A multicolor TIRF microscope

Setup using iChrome MLE for FRET and TIRF experiments

P. Meyer, M. Strackharn S. Baumann
Lehrstuhl für Angewandte Physik,
Ludwig-Maximilians-Universität, München

M. Lang
TOPTICA Photonics AG, Gräfelfing

Total internal reflection fluorescence microscopy (TIRFM) is one of the leading techniques to observe biological probes. It greatly decreases unwanted background and allows imaging thin optical sections. An evanescent field illuminates the sample surface and its near surrounding only in the order of 100 nm. A pillar of modern microscopy are single-molecule techniques that help to light up complex molecular behavior. Differently colored labels allow for even more detailed insights, especially in combination with FRET or superresolution methods.

We here present a multicolor TIRF microscope equipped with an iChrome MLE (TOPTICA Photonics) which permits to excite the sample with up to four different laser lines. We demonstrate the applicability of such an instrument to measure single-molecule dynamics and for research on whole cells.

Fig. 1: iChrome MLE – multi laser engine with up to four different colors.

Fig. 2: Custom-built TIRF microscope with EMCCD camera and iChrome MLE.

Experimental setup

The iChrome MLE (fig. 1) is coupled into a custom-build TIRF microscope (fig. 2,3). On a first stage the laser beam is expanded with a collimating lens and afterwards passes an aperture, which controls the width of the illuminated field. Then the laser is focused onto the back focal plane of the objective (Nikon Apochromat 100x NA1.49). Moving the stage in vertical direction shifts the distance between the

beam and the objective's optical axis. Moving the beam towards the periphery of the back focal plane changes the angle at which the rays emerge from the objective. Above a certain angle ("critical angle") total internal reflection occurs at the interface between the glass coverslip and the aqueous sample and an evanescent field extends into the sample. Since this field decays exponentially, it allows exciting the fluorophores within an approximately 100 nm thick section. The objective collects the fluorescence signal which is separated from the laser excitation with a "dichroic" mirror (Chroma zt405/488/561/640rpc). The fluorescence is subsequently focused with an achromatic tube lens on the input aperture of the Cairn Research OptoSplitIII (equipped with emission filters 525/36, 600/37, 676/29), which projects the three fluorescence channels on the Andor iXon EMCCD. The result is a false-color overlay of the three grayscale camera pictures (e.g. via the image processing software ImageJ on a PC).

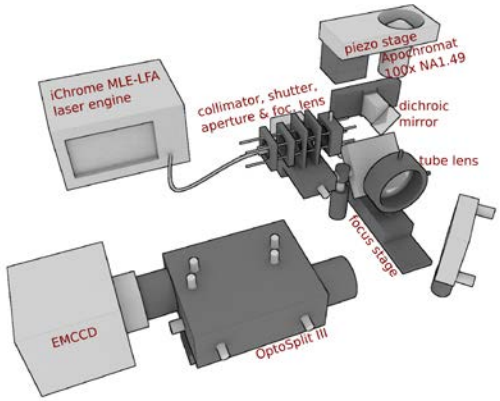

Fig. 3: Schematic drawing of the multicolor TIRF microscope.

FRET on Holliday junctions

As an example how multicolor experiments can help to gather information about molecule dynamics, we investigated Holliday junctions as described elsewhere[1]. A Holliday junction is an intermediate process in genetic recombination, where two homologue double helices join to exchange genetic information. The conformation at the time of separation determines the extent of exchanged segments.

We hybridized four DNA strands to form the typical quadruplex of a Holliday junction. Three of the DNA strands were labeled with a dye molecule each, Alexa488, ATTO565 and ATTO647N, respectively. To the fourth strand we attached biotin, which anchors the construct to the coverslip surface (fig. 4). Addition of 150 mM $MgCl_2$ induced pair wise stacking and flipping of the helices. As redox components we added 1 mM ascorbic acid and 1 mM methylviologen which both act as photostabilizers. To identify the fraction of completely assembled Holliday junctions we illuminated the sample with three lasers (488 nm, 561 nm, 640 nm) of the iChrome MLE simultaneously (see fig. 5, top). Thus we excited all three dyes and identified those spots where a strong emission signal occurred in all three channels. Then

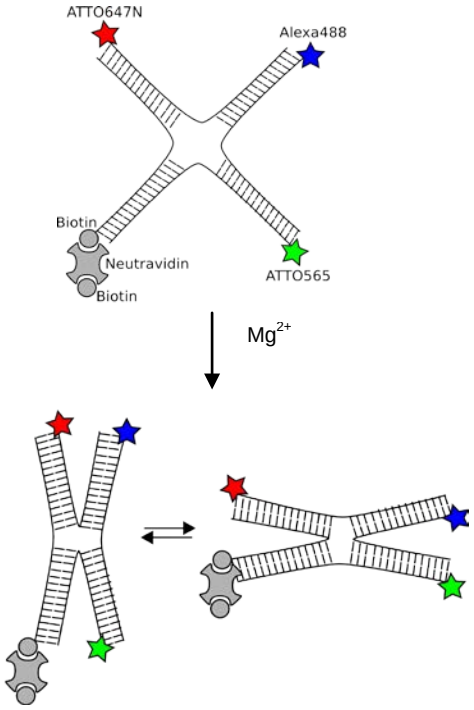

Fig. 4: Holliday junction of four DNA strands labelled with three different fluorophores and biotin. The junction can flip between two configurations where either Alexa488 and ATTO647N or Alexa488 and ATTO565 are close. The biotin binds the Holliday junctions to the surface of the glass slide.

we illuminated the sample with the 488 nm laser only and recorded the fluorescence signals in all three channels with the EMCCD at 20 Hz integration time (see fig. 5, bottom).

Acting as a FRET system, the excited Alexa488 (donor) transfers the energy either to the ATTO565 (acceptor) or the ATTO647N (acceptor). However, the transfer efficiency strongly depends on the distance between the dyes (~$1/r^6$). If the ATTO565 and Alexa488 dyes are in close proximity, the FRET efficiency between these two dyes is high and a strong fluorescence signal can be recorded from ATTO565. At the same time, the ATTO647N is furthest from the donor. In the second conformation, the situation is reversed: the ATTO565 is distant and the ATTO647N is next to the Alexa488. Thus FRET occurs between Alexa488 and ATTO647N. Compared to Alexa488 and ATTO565, Alexa488 and ATTO647N have less spectral overlap (fig. 6), so their FRET efficiency is lower. Nevertheless, the signal dynamics allow evaluating the molecular state of the Holliday junction in both conformations. An example for the time resolved emission of the three dye molecules is given in fig. 7.

Since the iChrome MLE furthermore allows fast switching between the laser lines (up to 20 MHz), an alternating laser excitation (ALEX)[2] scheme can be implemented. This technique permits a further and more detailed analysis of the FRET efficiencies.

	Excitation (nm)	Emission (nm)
ATTO647N	644 (red)	669
ATTO565	563 (green)	592
ALEXA488	495 (blue)	519

TIRF on stained cells

TIRF is well suited for the analysis of cellular processes near the plasma membrane. This technique minimizes the background noise, e.g. from the cytoplasm, and so achieves a higher contrast compared to other techniques.[3] Especially when multiple components are involved in the processes under investigation, a visualization of the individual compo-

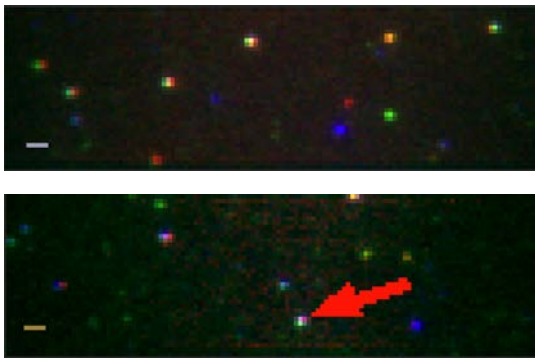

Fig. 5, top: Simultaneous excitation in blue, green and red of the immobilized and labelled DNA allows identifying the completely assembled complexes. Bottom: Excitation at 488 nm only: due to FRET the holiday junctions showed signals in all three color channels (scale bar: 1 µm).

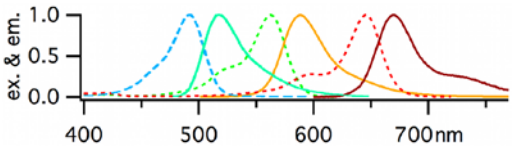

Fig. 6: Excitation (dashed) and emission (solid) spectra of Alexa488, ATTO565 and ATTO647N (from left to right).

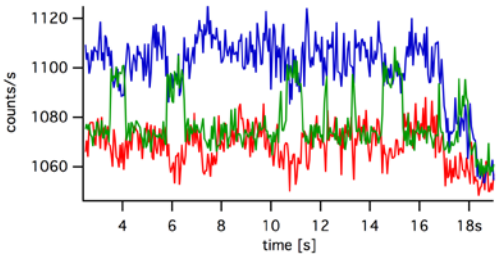

Fig. 7: An individual Holliday junction was excited with the 488 nm laser only. The fluorescence time traces were recorded on all three detection channels (blue: Alexa488, red: ATTO647N, green: ATTO565). Switching between the two helix conformations causes different FRET signals (after 18 s the donor bleaches).

nents on different color channels is required. Fig. 8 shows multicolored stained HEK-293 cells (human embryonic kidney) in the microscope.

	Excitation (nm)	Emission (nm)
YFP	514 (blue)	527
Phalloidin TRITC	543 (green)	572
TO-PRO-3 iodide	642 (red)	661

We performed fixation with formaldehyde and permeabilized the cells with Triton X-100. The actin filaments were stained with tetramethylrhodamine modified phalloidin conjugate (Sigma-Aldrich), which is excited by the 561 nm laser. We used TO-PRO-3 iodide (Invitrogen) as a counterstain for the nucleus (excitation at 640 nm). And as a third color the HEK-293 cells were co-expressing YFP (488 nm excitation) with prestin in the lateral plasma membrane.

Images from cells on a coverslip surface were taken with the Andor iXon EMCCD and post-processed for brightness and color in ImageJ. The well distinguishable color contributions of nucleus, actin and prestin allow mapping the distribution of these components (fig. 8).

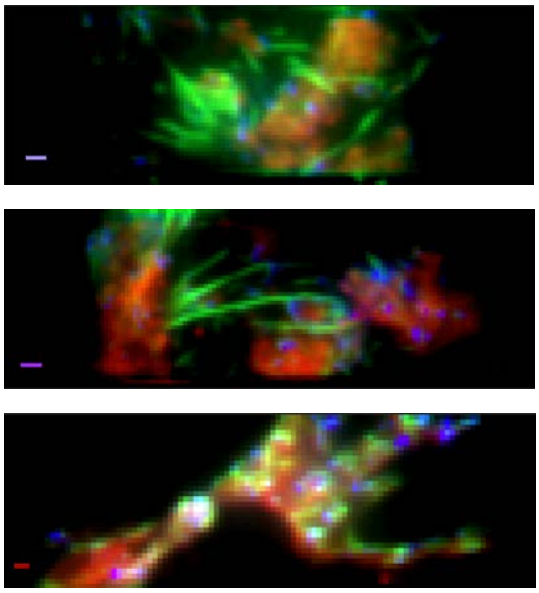

Fig. 8: Stained HEK-293 cells in TIRF microscopy. The multicolor excitation allows distinguishing actin (green), prestin (blue) and the nucleus (red) in the farther background (scale bar: 1 µm).

Advantages of iChrome MLE for TIRF/FRET

- COOL^{AC} technology: no manual realignment
- Compact system with fiber delivery
- Easy integration due to flexible interface
- Hands-off operation
- High fiber coupled output powers
- Fast switching between laser lines

[1] B. Person, I. H. Stein, C. Steinhauer, J. Vogelsang, P. Tinnefeld, ChemPhysChem 2009, 10, 1455-14

[2] Kapanidis, A. N.; Lee, N. K.; Laurence, T. A.; Doose, S.; Margeat, E.; Weiss, S. Proc. Natl. Acad. Sci. U.S.A. 2004, 101, 8936

[3] A. L. Mattheyses, S. M. Simon, J. Z. Rappoport, Journal of Cell Science 2010, 123, 3621-3628