

Studying molecular machines by TIRF microscopy

by Dr Michael Schlierf and Prof. Stefan Diez

Fluorescence microscopy has become a powerful tool in cell biology. Developments over the past decade allow the efficient detection of individual fluorophores with the help of total-internal reflection fluorescence microscopy. Fluorophores can be attached to molecular machines, e.g. motor proteins or DNA interacting enzymes, and can be tracked with high resolution to study the chemo-mechanics of these enzymes and by extension yield insights into their cellular function.

Fluorescence microscopy

A prominent interface between physics and biology is the field of optical microscopy. Over the past centuries advances in light microscopy have often enabled a closer look on biological questions, while at the same time questions arising from biology have inspired technical or conceptual improvements for microscopy. This symbiosis has constantly been driven by the wish to better separate the desired optical signals from the surrounding background. One success story along these lines is fluorescence microscopy.

Fluorescence microscopy bears the intrinsic capability of signal-to-background improvement because the excitation light can be spectrally separated from the emission light. Cellular sections without a fluorophore appear black, while bright signals are observed for the fluorescently labelled sections. Specific cellular structures and proteins can be imaged, for instance with the help of immunofluorescence, where fluorescently labelled antibodies are allowed to bind the cellular structures of interest. This way, details, which had previously been hidden

became accessible. An additional advantage was then offered by the discovery of fluorescent proteins in combination with new methods in molecular biology that allowed the direct fusion of the fluorescent probes to the proteins of interest.

Thus, by now fluorescence microscopy has become one of the most powerful tools in cell biology and biochemistry. However, while the signal-to-background ratio of isolated structures is very good in fluorescence microscopy, there is the inherent problem that cells possess a three-dimensional structure. Consequently, fluorescence light components from both the in-focus imaging plane as well as from multiple out-of-focus planes contribute to the recorded images.

Total internal reflection fluorescence microscopy

One way to locally restrict the imaging plane to a thin layer is scanning confocal microscopy, nowadays one of the standard imaging techniques in cell biology. Confocal microscopy enables researchers to obtain not only two-dimensional images, but also to gain

information about the third dimension, due to the rejection of out-of-focus light by means of a pinhole in the optical path. However, scanning in the x-y-direction is time consuming and thus does not allow for the observation of processes, which exhibit fast dynamics.

Alternatively, widefield techniques like selective plane illumination microscopy (SPIM) [1] and total internal reflection fluorescence (TIRF) microscopy [2] have been developed that allow a higher time resolution by imaging a whole field-of-view on a camera chip. Both techniques restrict the excitation light to either a thin light sheet (in SPIM) or to a thin layer at the glass-buffer interface (in TIRF microscopy). SPIM, on one hand, allows changing the position of the illuminated plane in the z-direction. The height of that plane, however, is of several micrometers, thus limiting the z-resolution. TIRF, on the other hand, cannot be scanned in the z-direction but offers an ultra-thin illumination layer limited to the evanescent field of the excitation light at the glass-buffer boundary. The evanescent field arises when the excitation light



is totally reflected (i.e. when the angle of incidence is larger than the critical angle, which is set by the two indices of refraction). The intensity of the illuminating light then decays exponentially in the buffer solution and only fluorophores that are within a distance of about 50 – 200 nm from the glass-buffer interface are excited [3].

With respect to the actual experimental setups for the generation of evanescent fields, two distinct methods have evolved: (i) prism-type TIRF, where the laser light for the excitation of the fluorophores is coupled through a prism on top of a flow-chamber [Figure 1, top panel] and (ii) objective-type TIRF, where the laser light is coupled through the objective (Figure 1, center panel).

In both cases, the objective is used to collect the fluorescence light, which is subsequently recorded on a CCD or EMCCD camera [Figure 1, bottom panel]. Due to the localization of the evanescent field to the glass-buffer interface, TIRF microscopy has become a popular tool for *in vitro* experiments on cellular machines (like molecular motors or DNA interacting enzymes) immobilized on a glass surface. TIRF then allows the detection of single fluorophores attached to the individual proteins of interest.

Tracking molecular motors with nanometer precision

A prototypic example for imaging single molecules at work are cytoskeletal motor proteins, enzymes that convert the chemical energy

released during ATP hydrolysis directly into mechanical work. For example, the processive movement of individual kinesin-1 motor molecules along surface-immobilized, fluorescently labelled microtubules can be visualized by labelling the motor proteins with green fluorescent proteins (GFP) or quantum dots (Qdots) [Figure 2A]. The evanescent field of the TIRF illumination excites the respective fluorescent probes allowing to resolve the linear motion of individual motor molecules [Figure 2B]. With the help of computerized tracking algorithms [4,5] the “walked distance” of individual motors can be followed with nanometer precision [Figure 2C]. Step-finding algorithms (resulting in the blue lines in Figure 2C) can then yield information about the motor’s step size [Fig. 2D] and thus provide insight into the chemo-mechanical function of these motor proteins.

Visualizing enzyme activity with sub-nanometer resolution

Enzymes like kinesin or myosin are unusual enzymes in respect of their large conformational changes on the order of tens of nanometers during their active motion along their tracks. Most other enzymes fulfill tasks that either do not go along with any conformational changes or where the involved conformational changes are on a nanometer to sub-nanometer scale.

An ideal tool to probe the dynamics of enzymes on the sub-nanometer scale is Förster Resonance Energy Transfer (FRET) in combination with TIRF microscopy [6]. FRET experiments usually require two different fluorophores, a donor fluorophore and an acceptor fluorophore, with Cy3/Cy5 or CFP/YFP being typical choices. After attaching the fluorophore pairs to specified

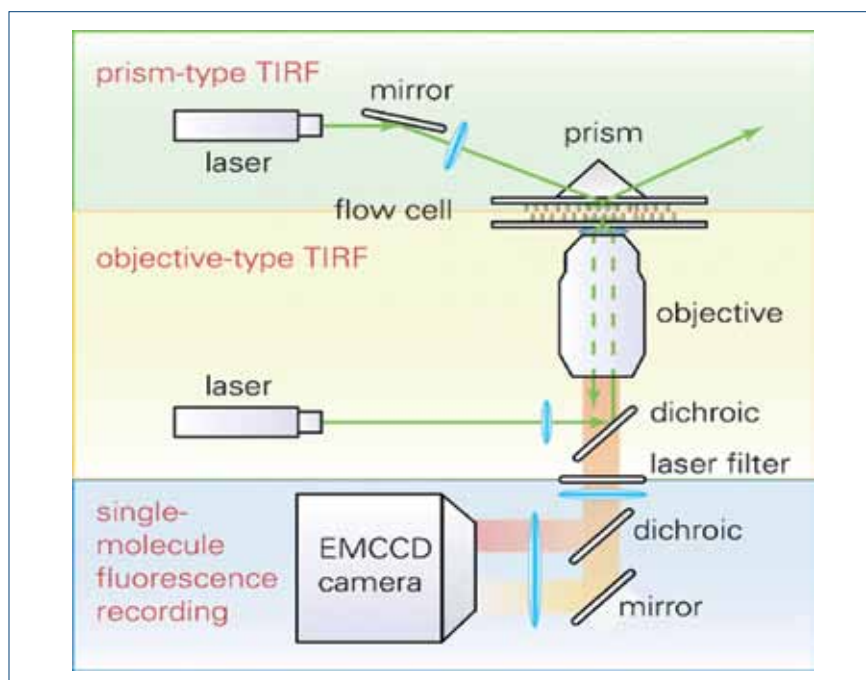


Figure 1. Schematic illustration of two typical TIRF microscopy geometries. Fluorophores can be excited through the prism-type geometry (top panel) or objective-type geometry (center panel). The fluorescence signal is collected through the objective, and spectrally separated from the excitation light. Further spectral separation of two fluorescence signals can be achieved in the case of simultaneous imaging with an additional dichroic mirror in front of the EMCCD camera, typically used for FRET microscopy (bottom panel).

locations in enzyme, the donor can transfer its energy non-radiatively to the acceptor with an efficiency that is strongly distance-dependent on a length scale from 2 to 8 nm [7] [Figure 3A].

Single-molecule FRET experiments are currently used to study protein and nucleic acid structure, protein-protein interactions, protein-nucleic acid interactions, protein folding and enzyme kinetics. In order to obtain information on individual enzymes, typically a substrate or the enzyme of interest are immobilized on a passivated glass surface (e.g. a DNA helicase opening a double-stranded DNA, Figure 3B, [8]). Donor and acceptor fluorescence can be spatially separated with the help of a dichroic mirror and thus be recorded simultaneously on an EMCCD camera (as depicted in Figure 1). Subsequent post-experimental video analysis allows direct access to individual FRET fluorophore pairs and the kinetic information of a single enzyme. Figure 3C depicts the fluorescence intensities of donor and acceptor over time (green and red, respectively). These quantities can be converted to a FRET efficiency as function of time. Further analysis allows to either obtain structural information (e.g. relative distances between donor and acceptor fluorophores) or kinetic information (e.g. how long it takes the enzyme to perform a certain task such as the unwinding of an 18bp long double-stranded DNA by a helicase) [Figure 3C]. Thus, single-molecule FRET experiments can give direct access to various enzyme activities, e.g. burst and pause phases, and, by extension, teach us about the efficiency of nature's molecular machines.

Future developments

Single-molecule TIRF microscopy has become a popular tool to study

a wide range of molecular machines that are involved in various cellular tasks, including transport processes and cell division as well as DNA replication, recombination and repair. Present developments further explore the ability to characterize molecular machines for nanotechnological developments, e.g. transport processes on the nanometer scale, controlled assembly of nanostructures or even detection devices based on molecular motors [9].

However, to obtain a more sophisticated picture of the functionality, diversity and chemo-mechanical mechanism of these enzymes, further technological developments are necessary. We are still limited

in temporal resolution from several points of view:

(i) Currently available EMCCD cameras with single fluorophore sensitivity provide a time resolution on the order of 10 ms. However, enzymes often function on the microseconds time scale.

(ii) Currently available organic fluorophores (1-2 nm in diameter) exhibit a finite photon flux, which limits the spatio-temporal precision. Qdots provide a possibility to overcome these limitations, however, Qdots are significantly larger (about 20-30 nm in diameter). Recently, the use of scattered light (e.g. from gold nanoparticles) has been suggested as an alternative to using

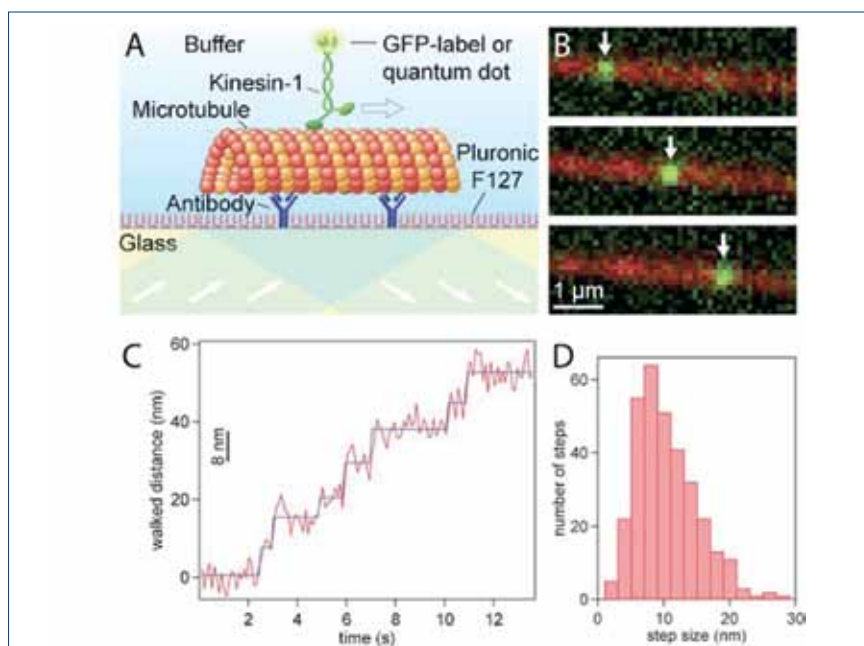


Figure 2. Imaging single kinesin-1 molecules moving along microtubules. (A) Schematic drawing of the assay. Microtubules are bound to a passivated glass surface with anti-tubulin antibodies. Single, GFP- or Qdot-labelled kinesin-1 motors are imaged using the evanescent field generated by a totally internally reflected laser beam. (B) Three frames of a stream showing a single kinesin-1 molecule (indicated with arrows) moving along a microtubule. (C) Walked distance of a tracked Qdot projected onto its average path plotted against time. Steps identified by a step-finding algorithm are overlaid. (D) Histogram of step sizes identified by the step-finding algorithm (data from 12 molecules). (modified from [11])



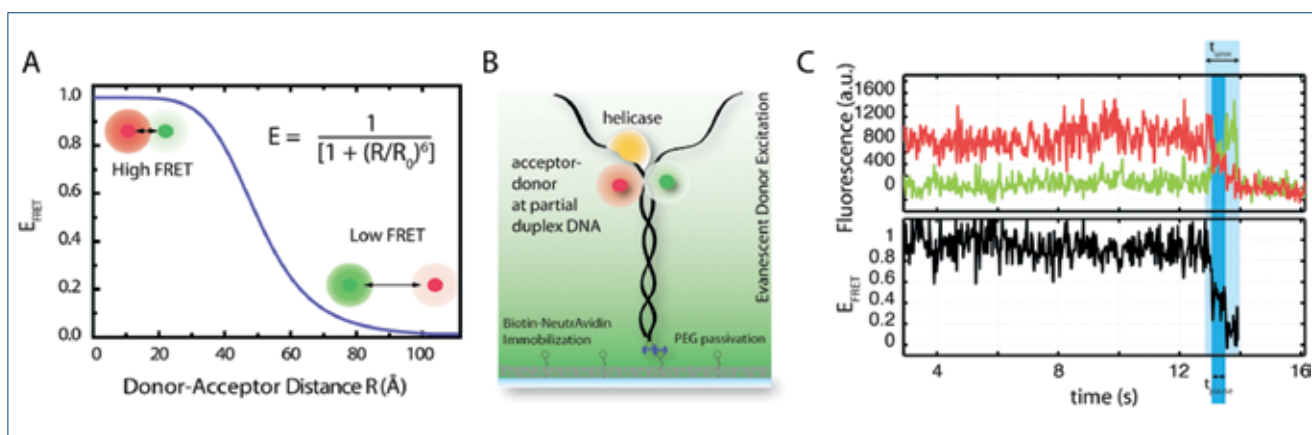


Figure 3. FRET efficiency as measure for distance changes on the nanometer scale. (A) If the donor (green) is in close proximity to the acceptor (red) the efficiency of energy transfer is close to 1, while if donor and acceptor are separated more than 10nm, the efficiency is close to 0. (B) Immobilized partial double-stranded-DNA with specifically attached donor and acceptor fluorophores and a helicase that will separate the double-stranded DNA into single-stranded DNA. (C) Typical single-molecule recording of a helicase unwinding double-stranded DNA. The donor (green) and acceptor (red) fluorescence signal can be converted to a FRET efficiency. During the unwinding process (highlighted in light blue), pausing times can be directly observed for this helicase (dark blue).

fluorescence light. When attached, for example, to molecular motor proteins, these gold nanoparticles provide for an unlimited photon flux characterized by the absence of photobleaching, blinking, and signal saturation. Consequently, not only a higher localization precision but also an increased time resolution can be achieved.

(iii) Another challenge for future single-molecule fluorescence microscopy will be the expansion of the observables. Both, single-molecule nanometer tracking as well as single-molecule FRET are limited to one observable at a time. This intrinsic limitation has recently been overcome by the combination of several high-resolution single-molecule techniques, e.g. optical tweezers and single-molecule FRET (reviewed in [10]). Ultimately, in order to understand molecular machines in their natural context, single-molecule experiments should be performed in an environment mimicking the cellular conditions or in cells directly.

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