Broadband Polarization-maintaining Fiber Optics
RGBV beam combiner enables TIRF microscopy of motor proteins
Anja Knigge, Ulrich Oechnser, Bernhard Brenner, and Tim Scholz

Modern fluorescence microscopy allows the fundamental building blocks of all life to be studied at a level of detail and accuracy that would have been inconceivable just a few years ago. One example is the movement of intracellular motor proteins responsible for muscle contraction or for the transport of biological cargo along microtubules within the cell. To study these systems using fluorescence microscopy, laser light over a wide range of wavelengths is required that has to be coupled precisely into the microscope. With free-beam optics, this can only be done to a limited extent and at considerable cost.

The effective solution is to couple the laser beam sources of the desired wavelengths into a single polarization-maintaining (PM) singlemode fiber using special apochromatic optics and dichroic multiplexing (Fig. 1) for the wavelength range 400 – 660 nm.

Using fiber optics to deliver the laser light has several advantages. The light sources and the measurement system are allowed to be physically separated, so that they are mechanically and thermally decoupled, preventing any mutual disturbance. The light guidance itself is totally enclosed resulting in a lower laser safety class, is insensitive to vibrations and exhibits long-term stability in terms of temperature fluctuations. In many cases, a measurement setup already equipped with laser beam sources can simply be upgraded to fiber optics, bringing a significant increase in stability and convenience without the need for costly and tedious adjustment of mechanically unstable, free-beam components.

TIRF microscopy of individual motor proteins

In conventional studies using large ensembles of molecules, many crucial functional parameters are not accessible or could be masked by ensemble averaging. Thus, to characterize motor protein function or pathological malfunction properly it is important to study motor proteins at the single molecule level. This is done in the group of Prof. Brenner at the Hannover Medical School (MHH) using total internal reflection fluorescence (TIRF) microscopy (also known as evanescent field microscopy). The technique allows the simultaneous detection of motor proteins like fluorophore-labelled kinesin, dynein or myosin along with fluorescently labelled ATP molecules or molecules associated with the cytoskeleton, such as the Alzheimer-related protein Tau at the single molecule level (Fig. 1). Fluorophores are molecules that emit light in response to a defined excitation. The TIRF microscopy utilizes the optical principle of total reflection which is described in detail in the box (p. 20).

RGBV fiber optic components for the TIRF analysis

For multicomponent experiments characteristic excitation wavelengths are needed for different individual fluorophores, so an ideal light source consists of several narrow-band excitation wavelengths that can be switched individually.

This can be realized by using different laser diode beam sources, each coupled into an individual PM fiber using laser beam couplers. If a low noise laser source is needed for the experiment, then fiber-coupled laser sources of the type 51nano are used. These are special low noise laser sources (< 0.1 % RMS, < 1 MHz) with a reduced coherence length and a low speckle contrast. Laser source qual-
ity (especially high pointing stability and good polarization characteristics) as well as the use of high quality polarization-maintaining fibers are essential (here with a polarization extinction ratio of more than 32 dB measured at 405 nm), as the efficiency of the following components are polarization sensitive.

**Broadband polarization-maintaining fibers with end caps**

For laser sources with larger powers and especially for smaller wavelengths, the PM fibers are equipped with end caps. End caps lead to an increase in the mode field diameter at the fiber endface which reduces the power density at the fiber-air interface significantly. This prevents damage to the fiber from e.g. scorching or photocontamination.

The different input wavelengths introduced through the PM fibers are combined using an RGBV Beam Combiner (Fig. 2), which means that they are first collimated using standard laser beam couplers (60SMS), combined using dichroic mirrors and the resultant beam is finally coupled into one collective single broadband polarization-maintaining fiber, designed for wavelengths 400 – 660 nm.

**RGBV beam combiner unit**

The transmission of the dichroic mirrors (here configured as long-pass filters) determines which wavelengths can be combined. Fig. 2 shows an optical scheme of the dichroic multiplexing using dichroic mirrors which have been adjusted to the nominal wavelengths (here e.g. 405 nm, 488 nm, 514 nm and 635 nm). The first dichroic reflects the green light and allows the red light to pass with high transmission. The following two dichroics reflect the blue and violet light respectively, while the combined wavelengths are transmitted with high efficiency. A precise adjustment of the dichroic mirrors is required for the particular spectral range and wavelength configuration to be combined. The polarization dependency determines that the polarization of the input laser beams must be clearly defined, which makes the use of PM fibers essential for a stable beam combination.

The combined laser light is then coupled into the PM fiber using a laser beam coupler equipped with an apochromatic lens. This lens was specially designed to minimize the chromatic focal shift for the wavelength range 400 – 660 nm as well as for spherical aberration, so that a highly efficient fiber-coupling can be achieved over the total wavelength range. The resulting combined laser light is then coupled into, for example, the fluorescence microscope.

The combiner unit is an example of a so called fiber port cluster. These are compact opto-mechanical units that split the radiation from one or multiple output polarization-maintaining (PM) fibers into one or multiple output polarization-maintaining fiber cables with high efficiency and variable splitting ratio. The beam delivery system consists of compact, modular opto-mechanic units. The modularity ensures that almost any desired system can be assembled and provides stable and compact, as well as sealed, beam delivery units.
Stable fiber coupling at short wavelengths

In the RGBV Laser Beam Combiner, the light is collimated and coupled into the final fiber using standard laser beam couplers. The laser beam coupler is a fundamental component of a fiber port cluster or in this case the beam combiner unit. The stability and efficiency of the total beam delivery is directly attributable to the stability of the laser beam coupler.

When coupling back into the polarization-maintaining fibers, the laser beam couplers produce a diffraction-limited spot that matches the mode field diameter and the numerical aperture of the fiber. It is only when this condition is met that fiber coupling with high coupling efficiencies of up to 80 % are achieved. Typical mode field diameters range from 3 µm (405 nm, NA 0.12) to 5 µm (780 nm, NA 0.12). The required pointing stability of the laser beam coupler can be visualized with an example: For a focal length of 5 mm, an angular misalignment of the coupler of a mere 0.2 mrad (0.01°) would result in a lateral displacement between the laser spot and the mode field of the fiber of 1 µm. A displacement only of 0.4 µm at λ = 400 nm and NA 0.12 is enough to decrease coupling efficiency by as much as 10 %. Thus, for high coupling efficiencies and long-term stability, a sub-micron precision and pointing stability of the coupling optics is required, especially in the ultra-violet range.

Tests have shown that the standard laser beam couplers (made from nickel silver) have a power stability during temperature cycling (15 – 35 °C) with a typical maximum deviation of only 3 %, in a test setup with a focal length of 4.5 mm at 405 nm [1].

Standard fiber port clusters made up with modular aluminum cluster units and standard nickel silver laser beam couplers, have already been used e.g. in zero-G experiments and are already very stable and resilient against outside influences.

The RGB beam combiner unit or any fiber port cluster can be further enhanced by using titanium components.

Titanium has the highest strength-to-weight ratio of any metal and a low coefficient of expansion. It is corrosion resistant and also amagnetic, so that titanium components can be used in environments that require very defined magnetic fields.

Functional analysis of motor proteins

Using the RGBV setup in combination with TIRF microscopy, individual motor proteins can be characterized. For localization of individual molecules, including their movement over time (Fig. 4), fluorophores are attached to the proteins. The emitted fluorescence signal, which is shifted towards longer wavelengths, is collected by the same objective lens used to generate the evanescent field (see box). For an experimental arrangement comprised of several different molecules of interest, each component has to be labelled with a distinct fluorophore (Fig. 4a). Therefore, several individually switchable, characteristic excitation wavelengths of tunable intensities are needed to excite different individual fluorophores in a defined sequence, one at a time. This challenge of exciting and detecting individual fluorescently labelled molecules leads to the problem of photo-bleaching, since bleaching of a fluorophore causes an immediate loss of signal. Photo-bleaching can be reduced by a biochemical enzymatic approach to deplete reactive oxygen from the sample solution. To further reduce bleaching pulsed excitation can be used. Pulsed excitation has been reported to reduce photo-bleaching as well as to increase fluorophore survival and fluorescence yield [2]. Direct modulation of the laser diode sources or using switchable elements such as AOTFs or AOMs allows generation of fast excitation pulses of different wavelengths, which permits interrupted relaxation of fluorophores from vulnerable excited states to their ground states.

The TIRFM technique offers an experimental way to directly visualize individual kinesin molecules moving along their microtubule tracks [3, 4]. Current
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models of Alzheimer disease regard increased levels of the microtubule-associated protein Tau as possible obstacles for microtubule-dependent motor proteins, such as kinesin and dynein, thus impairing long-haul axonal transport in neurons. To test this hypothesis, it was necessary to detect both motor proteins and Tau molecules on microtubules simultaneously. Fig. 4b shows a time sequence of TIRF images in a so-called kymograph – a time-vs.-position plot. In a kymograph, stationary molecules are represented by vertical lines, while moving molecules deviate to the left or right. Following the experimental scheme shown in Fig. 4a, green fluorescent protein (GFP) labelled kinesin molecules (shown in blue, excitation at 488 nm) moved from left to right along a Cy5-labelled microtubule (shown in red, excitation at 633nm) in the presence of the tetramethylrhodamine-(TMR-) labelled microtubule-associated protein Tau (shown in green, excitation at 514 nm). Over a period of several seconds, directed movement of multiple individual kinesin molecules could be observed, whereas the green Tau molecules did not behave as proposed by current models and did not remain stationary bound (Fig. 4b). Tau protein dilution to the low picomolar range and the tracking of individual Tau molecules by TIRF microscopy led to the discovery of Tau diffusion along microtubules [5, 6].

To study not only the mechanical action of motor proteins but the biochemical enzymatic ATPase activity at the single molecule level one can make use of fluorescently labelled ATP molecules. Due to the dramatically reduced background fluorescence in the TIRFM arrangement it is possible to detect individual fluorophores attached to ATP molecules. Such labelled ATP molecules only become visible as fluorescence signals when they get bound by surface-immobilized myosin molecules (Fig. 4d, e). Following the course of ATP fluorescence at different myosin molecules (Fig. 4f) allows studying characteristic differences among or changes in the kinetic behaviour of individual myosin molecules even in mixed populations of pathologically altered and wild-type myosin or mixtures of different motor isoforms [7, 8].

Summary

The coupling of laser light into PM fibers provides an opportunity for the efficient use of laser applications in both research and industry. After beam combination, the resultant single beam can be used as a light source for fluorescence microscopy, by creating a defined interface between the light source and the microscope. Exceptionally sensitive measurement systems (e.g., when observing individual motor proteins) benefit from the physical separation of beam combiner and measurement system (e.g., fluorescent microscope), while also avoiding costly, time-consuming and tedious beam adjustment. Optical and mechanical components that meet the highest technical standards enable the creation of modular and compact fiber optic systems with remarkable long-term stability.

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References

[1] Physics Best, April 2013, Wiley-VCH