RGBV Fiber Optics for TIRF Microscopy and Biophotonics

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Many biophotonic methods, including fluorescence microscopy, require a broad spectrum of laser light for their full functionality. RGBV (red-green-blue-violet) fiber optics provide a self-contained, stable and compact system that can couple and collimate laser beams with wavelengths between 400 and 600 nm into a single polarization-maintaining combined beam.

Advanced fluorescence microscopy of the fundamental building blocks of life has revealed a level of detail which was impossible to imagine only a few years ago. Motor proteins, in particular, attract great interest because of their evolutionarily engineered structures and functions; they may be organized into complex structures (myofibrils or flagella) or, as in intracellular transport, act individually like autonomous beings. Consider, for example, the intracellular motor protein kinesin and its ability to transport remarkable biological loads, including cell organelles or vesicles, across the cell by simply walking along specially constructed microtubule pathways (colored red in Figure 1). Each step of the motor protein ‘foot’ is fuelled by the highly efficient consumption of chemical energy from a single molecule of ATP (adenosine triphosphate). Muscle contraction also relies upon a similar mechanism, only with apposed muscle fibers contracting in a coordinated fashion in opposite directions, with the fiber ‘feet’ all marching in step.

The study of molecular interactions by use of fluorescence relies upon specific molecules and sophisticated microscopy equipment. The attainment of signal specificity is 2-fold: a fluorophore must not only react selectively with particular chemical groups on different proteins but also emit light upon excitation by a defined wavelength of light. For the colocalization and relative movements of differentially labelled molecules to be observed, a single laser beam of combined wavelengths is coupled into a microscope in a highly precise orientation. Laser light, at defined wavelengths between 400–660 nm, is coupled using special apochromatic optics and a dichroic beam combiner into a common polarization-maintaining singlemode fiber, for delivery within the microscope at a precisely defined interface [1]. This is a feat almost unattainable with current free-beam optic systems, especially with their time-consuming requirement for multiple fine adjustments of the beam-deflecting apparatus.

The breakthrough in miniaturization, provided by the fiber coupling of laser beams, has enabled the production of easily set-up, robust and reliable systems. A number of other advantages ensue, including the ability to place the more sensitive measurement or imaging system at a distance from the light generation sources and, thereby, isolating it mechanically and thermally from their disturbing influences. A fiber-optic system is much less sensitive to vibration and variations in temperature and, being fully enclosed, enjoys a lower laser protection class and provides greater worker protection than an equivalent free-beam system. These miniature and modular systems are ideal for modernizing conventional set-ups and bring numerous benefits, including greater functionality, stability and ease of use.

TIRF microscopy of motor proteins

In addition to conventional fluorescence microscopy, Prof. Brenner and his research group at the Medical School of Hannover (Figure 2) use evanescent field microscopy, also known as TIRF (Total Internal Reflection Fluorescence) microscopy, to investigate the functions and movement of motor proteins such as kinesin, dynein and myosin [2–5]. For evanescent field or TIRF microscopy, an aqueous sample on a glass slide is illuminated from below at a low angle of incidence to obtain a total internal reflection at the optical density interface between the glass and the liquid (Figure 3). This generates a weakly penetrating evanescent electromagnetic field that decreases exponentially to a depth of 300 nm into the sample but is sufficient to excite the fluorophore-labelled motor proteins. The low quantum yield of the emitted light is at a longer wavelength than the corresponding specific exciting wavelength and this imposes severe design constraints on a polychromatic excitation and detection system.

The choice of fluorophores and corresponding lasers must be appropriate and must not overlap with the longer wavelengths of the weaker emission signals. The objective lens also has a high numerical aperture to capture these weak emissions and a sophisticated
series of notch filters prevents contamination from longer wavelength excitation and extraneous reflections. An ultrasensitive (electron-multiplying) EMCCD detector is used to capture and amplify the signal while maintaining quantum efficiency. The total internal reflection of the combined RGBV incident beam is designed to deliver the maximum excitation energy to the smallest sample volume for the induction of the evanescence. A precise geometry and also fine-positioning is required to achieve the critical incidence angle at the top of the glass slide and the bottom of the sample liquid. The immersion oil between the glass slide and the objective lens creates the correct distance and provides an optical density change for refracting the TIR beams. At its periphery, the objective is also a focussing lens for both the incident and reflected TIR beams. The specific excitation of the fluorophore-labelled molecules is limited to a very thin layer within the sample liquid, providing conditions ideally suited for the selective excitation of a variety of fluorophores using appropriate excitation wavelengths while minimizing the diffuse background fluorescence. Ideally, a collimated beam of polychromatic narrowband excitation wavelengths is required, with each individually controllable. Alternatively, a defined sequence of excitation pulses of different wavelengths can be used to stimulate specific fluorophores, with the added advantage that the pulsatile excitation also reduces the incidence of photobleaching. Each of the fluorescence signals is assignable to individual protein molecules, making each component directly visible and allowing their relative movements to be followed. This is particularly important when investigating the molecular structure and function of motor proteins and the effects of mutations, including those that can lead to disorders such as in Alzheimer’s disease.

An example of motor protein functionality that can be observed directly is the motor protein activity that releases the energy from ATP to drive the motor. By using ATP molecules labelled with the fluorophore CY3 (excitation at 550 nm, emission at 570 nm), it is possible to monitor the binding and quantum decay of ATP fluorescence in a series of images [3], as the high energy phosphate bond of ATP is cleaved to release energy and produce ADP (adenosine diphosphate). Similarly, the walking of kinesin molecules (Figure 1A) along the cytoskeleton pathways through the cell can be visualized. The sequence of TIRF images in Figure 1 highlight the movement of tetramethylrhodamine-labelled kinesin (shown in green, excitation at 532 nm) as it walks along a CY5-labelled microtubule (in red, 633 nm excitation) (Figure 1B) [2]. A single kinesin molecule can walk at a rate of 500-600 nm/s (Figure 1C), when it is loaded with cargo and sufficient ATP is available for each kinesin step.

**RGBV fiber optic components for TIRF implementations**

The particular wavelengths used by the Prof. Brenner team are provided by argon (476 nm, 488 nm, 514 nm) and HeNe (633 nm) lasers and converted into a single collimated beam with high efficiency by the RGBV combiner (Figure 4). The laser beams are coupled using polarization-maintaining singlemode fibers into the RGBV beam combiner, in which a dichroic mirror is utilized as a long-pass filter to launch the single collimated beam into a single polarization-maintaining output fiber. The multiple inputs and single output use large bandwidth apochromatic fiber collimators to preserve the integrity of polarization; an absolute requirement throughout the whole system for maintaining its high efficiency.
The combined output beam is modulated by a fiber-coupled acousto-optical tunable filter (AOTF) that selects the wavelengths at high frequencies (several hundred MHz), as appropriate for the fluorescence microscopy being performed. A simple aspherical lens is sufficient to correct for spherical aberration and, while an aspherical lens performs well for monochromatic applications, a strong chromatic aberration is obtained with polychromatic light and the relative focal points for several different wavelengths may be displaced by up to 400 microns (Figure 5A).

Lenses capable of correcting chromatic errors for two different wavelengths are termed doublets (Figure 5B). Although the superimposition of focal points is better, an efficient coupling over a wide spectral range is still not possible. A theoretically conceivable achromatic lens might achieve a maximum coupling efficiency for red light (660 nm) but would only attain 50% efficiency for light in the violet (405 nm) part of the spectrum.

Alternatively, an apochromatic lens can successfully correct chromatic aberration over more than two wavelengths. Specially developed apochromatic fiber-coupling optics can achieve highly efficient coupling across the entire 400–600 nm range (Figure 5C). Finally, the design of the optics must also provide a good spherical correction, as well as an efficient chromatic correction, for the best coupling efficiencies to be achieved.

Fiber-coupled laser sources

Wavelength combinations are possible for many laser beam sources, including diode lasers (DFB, DBR, etc.), frequency-doubled lasers, optically pumped semiconductor lasers (OPSL) or gas lasers (Figure 6). The newest laser sources are sold fiber-coupled but existing sources at an established test site can also be converted to fiber-coupling, as required.

Polarization-maintaining fibers

Polarization-maintaining singlemode fibers possess an approximately Gaussian mode field and are capable of coupling laser light, with its characteristic Gaussian beam profile, at high efficiency. Since the mode field diameter is of the order of 2–5 microns, a high degree of precision is required for truly efficient coupling. The two critical characteristics of a fiber that determine the precision of coupling are its designated numerical aperture, which determines the divergence angle of the radiation emanating from the fiber end, and the cut-off wavelength \( \lambda_0 \). Above the cut-off wavelength \( \lambda_0 \), the coupling of singlemode light in LP_{01} mode is assured, otherwise hopping between adjacent modes is manifest, while beyond about 1.3 \( \lambda_0 \) fiber bending losses impose.

Standard singlemode fibers produce a polarization state between linear and circular that is highly sensitive to physical displacement or bending of the fiber as well as any changes in ambient temperature. In contrast, a correctly coupled linearly polarized light beam is wavelength dependent, this results in chromatic aberration that can be severe in a polychromatic application. Traditionally, compound lenses of different glass compositions and characteristics are assembled in an attempt at providing a common focal point for several disparate wavelengths.

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laser beam within a polarization-maintaining singlemode fiber is fully protected by the rotationally symmetrical rigid supports within the fiber. Specialized broadband polarization-maintaining fibers are used when several wavelengths must be coupled into a single fiber.

**Laser Beam Couplers**

A sufficiently high-quality laser beam coupler with apochromatic optics is absolutely critical when the laser beam to be coupled is a combination of numerous overlapping wavelengths (Figure 7). The informed choice of beam coupler provides a diffraction-limited laser spot perfectly matched to the mode field of the fiber and should any fine-tuning be required then this can be performed using the integrated tilt adjustment. Efficient coupling also requires the perfect alignment of the polarization axes of the laser beam, the laser beam coupler and the polarization-maintaining fiber at all beam transfer points within a system. The coupling efficiency attains a maximum when there is an exact correspondence between the convergence of the focussed beam and the angle of divergence of the fiber, which is specified by NA of the fiber, and also between the diameters of the laser spot and the mode field at the fiber end. For laser beam sources of high quality (M² <1.05), well-coupled systems can achieve coupling efficiencies of up to 85%, with the minimized losses predominantly a consequence of residual and essentially unavoidable Fresnel reflection (~8%) at the numerous input and output ports that are used by the coupled beam.

**RGBV Beam Combiners**

The RGBV laser beam combiner is the critical node in the system, where all of the individual laser beams are received, collimated using dichroic mirrors, and combined into a single beam for output into a single fiber, with full retention and perfect alignment of the individual characteristics of the incoming beams (see scheme in Figure 8). The collimation of the incoming beams is also attained using laser beam couplers. When combining numerous wavelengths (e.g. from several argon lines) and coupling them into a single output fiber then apochromatic laser beam couplers must be used. Figure 8 depicts a RGBV beam combiner used to bundle the red, green, blue and violet beams from 4 different laser sources into a combined and fully aligned “white” light output.

Careful choice of the transmission spectra of the long-pass filters determines which wavelengths are combined at each of the dichroic combination mirrors. In this example, the nominal wavelengths of 405 nm, 460/488 nm, 532 nm and 630 nm have been selected, although the transmitted wavelengths can vary from the nominal when a sufficiently broad input wavelength is used or desired.

Red light is transmitted and green reflected by the first dichroic mirror with high efficiency. The subsequent mirrors reflect the blue and violet light, respectively, whereas the already combined wavelengths are transmitted without significant loss. Particular configurations require a careful choice of dichroic components appropriate to the particular wavelength combination used. The polarization dependence of the dichroic beam combiner also imposes constraints on the polarization alignment of the polarization-maintaining fibers.
A modular RGBV laser beam combiner provides the opto-mechanical stability that is normally unattainable in conventional, overly complex, free-beam systems. Full miniaturization and the readily exchanged components allow customized applications with exotic choices of wavelength to be contemplated for the first time. The experimental setup used by Prof. Brenner and his team incorporates two laser sources and a total of four fully superimposed wavelengths, dichroically combined into a single broadband fiber output.

Fiber Collimators

The beam emanating from the end of a fiber can be divergent and is transformed into a collimated beam by using a fiber collimator. The collimator lens produces a beam with a Gaussian beam profile, whose diameter is determined by the numerical aperture of the fiber and the focal length of the collimation objective. To ensure collimation without vignetting or diffraction, the numerical aperture of the fiber collimator must be greater than the numerical aperture of the fiber. For applications requiring a wide spectral range then the usual apochromatic requirement also applies to the collimation objective.

AOTF (acousto-optical tunable filter)

Many applications require that the different wavelengths are often switched, precisely and at high frequency. In fluorescence spectroscopy, for example, this is essential to avoid bleaching of the fluorophores.

AOTFs are tunable acousto-optical filters that enable fast switching times between a number of wavelength channels, with each addressed and modulated precisely and individually. The modulation is performed by the diffraction of an acoustic wave which passes through an anisotropic material. The amplitude and frequency of the acoustic wave can be manipulated to provide the switching and modulation of the different wavelengths, either individually or in selected combinations. Fiber-coupled AOTFs exhibit the expected improvement in mechanical stability and laser safety that elude conventional free-beam systems.

Faraday Isolator

A frequency-stabilized laser beam emanating from a laser diode can reduce the lifetime of the diode significantly when scatter and back-reflection of the laser beam into the diode is not prevented. The frequency stability and intrinsic noise of the diode are also detrimentally affected. An effective preventative measure is the use of a Faraday isolator as an optical diode (Figure 9). While light is permitted in the beam direction with low absorption loss (<0.5 dB), any light in the reverse direction is very effectively blocked (>30 dB) and the diode protected.

Conclusions

The coupling of laser beam sources into polarization-maintaining singlemode fibers has provided a quantum leap in efficiency that has permitted the novel and diverse application of lasers in both science and industry. Laser beams coupled using fiber optics and a beam combiner have provided tunable and switchable laser beams of numerous defined wavelengths to be superimposed for precise illumination of individual molecules using fluorescence microscopy. Highly sensitive measurement systems, such as are required for monitoring motor protein movement and activity, can be physically separated from the beam-producing sources, isolating them from heat and vibration. The tedious, time-consuming and tricky adjustment of conventional free-beam deflector systems has been consigned to history. The higher technical standards necessitated by the optico-mechanical components of fiber-optic systems, their compactness, robustness and modularity, have opened up previously unimaginable opportunities in scientific and commercial microscopy and biophotonics.

References:


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