The White Confocal – Spectral Gaps Closed

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This article summarizes the development and differences in design and functionality of confocal technology as far as spectral properties are concerned, from classical filter-based excitation and emission color selection to fully flexible spectral excitation and emission tuning. All three major components: light source with excitation color selection, beam splitting for incident illumination and detector emission filtering have been completely transformed.

Keywords confocal microscopy; white light laser; acousto-optical tunable filter; spectral detection; acousto-optical tunable beam splitter

1. Requirements for true confocal illumination

True confocal microscopy requires a complex system of illumination, beam splitting, detection, scan process performance, data processing, control software, image handling and much more besides. The light source itself must also fulfill a number of requirements to serve as a practicable illumination device for true confocal imaging.

1.1 Focusability of the illumination light

True confocal optical sectioning microscopes use simultaneous illumination and detection of a tiny spot. The smaller the spot, the better the sectioning efficiency. The tiniest spot that can be created by illumination (or sensation) with far-field optical designs in circular optical systems is the Airy diffraction pattern. In early systems, this boundary condition was fulfilled by focusing the light from an extended divergent light source onto a tiny aperture: the pinhole. The pinhole can then be regarded as the light source which is substantially smaller than the (magnified) image of the illumination spot. The efficiency of this design mainly depends on the focusability of the illumination light. Lasers – or similar sources – are usually easy to focus into a diffraction limited spot (Airy pattern), as the beam of the laser is typically cylindrical and parallel (~ not diverging). Good sources have focusing parameters that allow the excitation pinhole to be omitted completely.

1.2 Intensity of the light source

Secondly, the source must provide sufficient energy to create images in a reasonably short time at sufficient signal-to-noise ratio. Reasonably short is usually understood as at least one frame per second. Living material often requires some 10 frames per second to generate time-correlated images. Physiologists who want to follow metabolite changes or electrical alterations with fluorescent dyes ask for 100 frames per second, and in extreme cases 1000 frames per second. Nevertheless, the 1...10 frames per second range is sufficient in most applications. Concerning noisiness, a good image features signal-to-noise (SNR) of 10. Much higher values are not recognized by the eye (but might sometimes be meaningful for image processing). Lower ratios are accepted for physiological measurements, which usually bin a larger number of pixels for data evaluation. The important thing here is not to create a beautiful image, but to subject the sample to as little illumination as possible to avoid phototoxic effects. An SNR of even 0.1 is accepted for these applications. There are many more parameters governing the laser emission required to get decent images: the optical transmission efficiency of the excitation and emission path, the detector sensitivity, beam splitting efficiencies and so forth. As a rule of thumb, 1 mW of laser emission per excitation is more than sufficient for most imaging tasks. In everyday work, 0.1 mW will be appropriate. And with modern high-sensitive detectors, physiological data are usually recorded at 10 µW or even less.

1.3 Stability of the intensity

Noise of the light source will obviously cause noise in the images. Depending on the frequency range of the source noise, this will have different effects on the images recorded.

All light sources feature what is called photonic noise, a shot noise that is caused by the particle property of light. This noise scales with the square root of the number of particles in question. If we assume a laser emitting 0.4 mW of blue-green light at 500nm, the stream of particles would consist of ca 10^{15} photons per second. Even if we assume only 10% efficiency, 10^{14} photons per second would still reach the sample. The intrinsic noise is then controlled by the time we take to illuminate one single pixel. Assuming a typical frame size of 1000x1000 pixels at duty cycle 100% (usually, this is not realized) and an acquisition speed of 10 frames per second, the pixel dwell time would result in 10^{-7} seconds,

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i.e. 0.1 µs. Still, the number of photons would be 10^7, and the signal-to-noise ratio would exceed 1000 – far beyond detectable limits.

If, on the other hand, the source does not emit at constant intensity in the frequency range of the pixel time, then the intensity fluctuation will cause additional noise in the image. Noise at higher frequencies is averaged by the dwell time. 10% intensity fluctuations would be tolerable if the noise frequency were in the range of the pixel dwell time. If the fluctuations were slower, changes in intensity would be detectable, especially if the sample featured large homogeneous areas. If the fluctuations were slower than the line frequency, the image would show a wavy structure in the second (slower) scan direction, which is usually top to bottom. Very low changes in intensity would be recognized as source drifts and cause variations in long-term time lapse experiments introduced by the light source.

If the application requires better intensity stability than offered by the technology employed, images need to be corrected for the fluctuations. This could be done by monitoring the source intensity and subsequent computerized correction of the image data. A second option would be a stabilized light source, e.g. by feeding back the intensity measurement into an acousto-optical device that would automatically alter the excitation energy and thus keep the output into the microscope at a constant level, even at high frequencies in the MHz range.

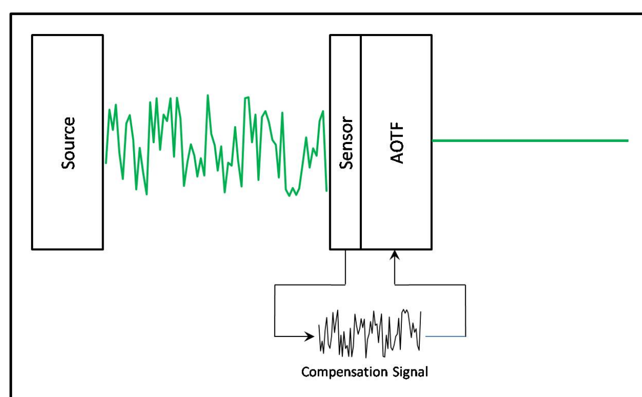

Fig. 1 Feedforward loop to stabilize the intensity delivered into a confocal microscope. The AOTF, an acousto-optical device that is usually employed to control the excitation energy, is used to keep the output power of the light source at a constant level.

1.4 Trans or Epi?

A fluorescence molecule is excited by absorption of a photon that has the appropriate wavelength. Also, the polarization direction must subcoincide with the transition moment of the excitable electron system. The emission is parallel to the transition moment, but under standard conditions, the fast rotation of the molecules depolarizes the emission completely (dedicated applications measure the dynamics of depolarization, though). As a consequence, the emission is non-coherent and covers the full 4-pi sphere homogeneously. Concerning the fluorescence yield, it makes no difference which direction the fluorescence is measured in, and therefore this will not differentiate trans (forward) fluorescence from epi (backward) fluorescence. However, the epi technique has become a standard, for good reason. The fluorescence intensity is – very roughly speaking – some 100,000 times dimmer as compared to the excitation light applied. In a trans-fluorescence system, it would be necessary to filter this immense background away. It is possible, but not easy. The background for epi-fluorescence is just the light that is reflected in the sample – which is typically 1000 times less (very broadly speaking). Separation of excitation and emission light is consequently much easier when using an epi technique, although it indeed complicates the beam path.

2. Multiparameter fluorescence

Fluorescence microscopy has become the most important tool in biomedical research, as fluorescence can be assigned to individual structures. Some fluorescence dyes have intrinsic affinities to certain cellular components. DAPI for example, will interact with DNA and RNA. Consequently, DNA and RNA will light up in the cell, but the rest remains jet black. By the way, DAPI fluorescence when bound to DNA has a different color compared to RNA fluorescence. It is an intrinsic multiparameter stain. The most specific binding in cellular terms is exerted by antibodies. If antibodies are chemically connected ("decorated") with fluorescent molecules, the resulting emission pattern shows very specifically only those molecules that are recognized by the antibody in use. This technique allows the identification of the locations of thousands of proteins and sugars in any organic tissue. The latest staining method employs fluorescence caused by proteins that are expressed by the living cell itself. This method enables in-situ measurements of dynamic changes and transport phenomena in an intact organism.

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Besides the structural information and its dynamic changes, other parameters are explored by fluorescence methods: ion and metabolite concentration and their dynamic change, and even electrical potential measurements are accessible with potential-sensitive fluorescent dyes.

2.1 Multiparameter recording patterns

The intrinsic double stain with DAPI for DNA vs. RNA is a rare exception. Usually, each target substance has to be stained by a separate dye. In case of immunohistochemistry, this requires a set of antibodies decorated with the same number of distinguishable fluorochromes to be introduced into the specimen. For fluorescent protein samples, a set of differently emitting fluorescent proteins must be genetically engineered as chimera together with the protein under research. Also, many kinds of composed techniques are used: pure reflected light combined with immunochemistry; or immunochemistry labels with fluorescent proteins; or structural probes with kinetic probes, e.g. to monitor Ca^{2+} concentration changes and so forth.

For recording fluorescence, both excitation and emission require some spectral considerations. The excitation light should fit the absorption spectrum of the fluorochrome. Usually, one would try to hit the absorption maximum, although this is not always the best choice. Widefield systems that employ white sources, e.g. mercury lamps, use band filters to specify an appropriate excitation band. Classical lasers are intrinsically monochromatic, or emit a sequence of lines from which a single line is extracted either by glass filters or acousto-optical filters. The most suitable solution, a white light laser source, is discussed below. The emission is also filtered in order to remove residual excitation light and to avoid contamination of the desired signal by other fluorescence that might be abundant in the specimen.

If the sample contains a set of different fluorochromes that is to be recorded (autogenic fluorescence is often an unwanted signal that has to be removed), there are two strategies to acquire the data. Solution 1: the dyes are excited and recorded individually, and each image is stored before the next color. At the end, all images could be overlaid in different colors to show the respective occurrence in the sample. This scheme is called “sequential scanning”. Solution 2: all dyes are excited simultaneously and the emission bands are collected simultaneously in separate channels. The channels could be displayed as individual images in black and white (split screen display) or as a colored overlay, immediately during image acquisition. This scheme is called “simultaneous scanning”. Simultaneous recording is quicker than sequential recording, as the sample only has to be scanned once. This is essential for live sample research, where temporal correlation of various signals is necessary, and where movements of the targeted structures may cause false colocalization results.

2.2 Spectral challenges in multiply stained samples

The benefit of sequential scanning is that it gives a better chance of recording spectrally clean signals. If two emissions are collected simultaneously by selection of different emission bands, there is always a distinct possibility that some fluorescence of the wrong dye will contribute to the signal in the selected dye. This phenomenon is called “crosstalk” and is one of the most discussed sources of error in multiparameter fluorescence. If the dyes are recorded one after the other, applying different excitations and emission bands in each recording, the chance of crosstalk is reduced. In confocal microscopes, sequences of different recording patterns are usually applied to sequentially scanned image frames, or sometimes to whole three-dimensional image stacks. If patterns can be switched fast enough, it is possible to record a set of single lines with the different patterns before incrementing the y-direction in order to create a two-dimensional image. This is called “line sequential” and has the benefits of immediate creation of the composed image and the least movement of artifacts in living samples. In cases where the number of channels is more than two, individual recording patterns may also record a number of fluorochromes simultaneously, such that a compromise is possible between speed and crosstalk.

In simultaneous recording, crosstalk reduction is a demanding task. Both the excitation and the emission have to be set spectrally in optimal positions. And obviously, a system should provide maximum flexibility for controlling these parameters. We will show in this article that a combination of a tunable white light laser source and a spectral detector featuring tunable bands is the superior solution for meeting this challenge.

3. Classical lasers for confocal microscopy

As mentioned above, lasers are the gold-standard illumination sources for confocal microscopy. Since the introduction of confocal microscopy in research labs between 1980 and 1990, laser technology has of course made significant improvements. There is a huge choice of lasers, each with particular benefits and drawbacks. These will not be discussed here in detail. There are some general features of classical lasers that should be discussed briefly.

3.1 Features that make classical lasers a good source for confocal illumination

Lasers have been used for confocal microscopy for their excellent focusability. The core of confocal imaging is the creation of a diffraction-limited spot. The extremely low divergence of a laser beam makes it the preferred light source.

Although other light sources could be implemented, lasers have been used from the very beginning. And, as lasers are also employed in many other technologies that have changed our everyday life, they are now available in many versions and at reasonable prices - although reasonability is different for the various applications.

The second parameter that makes lasers a good source for confocal is their intensity. Although there are lasers that deliver enough energy to weld steel (and even to ignite a fission plasma), the energy delivered by a small laser still has a high intensity. The focused intensity is defined as the intensity per unit area. If a laser line emits 20mW, this intensity is typically delivered in a beam of roughly $1mm^2$, resulting in a focused intensity of 2 W/cm^2, which is by far sufficient to damage the retina. When focused to an area of $1\mu m^2$, which corresponds to a medium sized microscopic focus, the focused intensity is 2 MW/cm^2. Such high intensities are sufficient to easily bleach most of the fluorochromes used in biomedical research. Sometimes bleaching is intended, e.g. for FRAP experiments. Consequently, lasers for confocal microscopes emit in the range of a few milliwatts. For normal imaging, the intensity has to be attenuated to $10^{-1}...10^{-3}$ of the nominal value. Today, attenuation is usually performed by acousto-optical filters that allow stepless and quick alterations over a broad dynamic range.

3.2 Classical lasers have drawbacks

Lasers usually only emit at one single color. The bandwidth of the emission is a fraction of a nanometer, which is why the band is called “laser line”. Some lasers can emit a series of lines, e.g. argon gas lasers, which offer some 5 different emissions in the blue range. Nevertheless, to excite a set of fluorescent dyes in the visible range it is necessary to employ a set of different laser instruments. For that reason, current confocal systems based on classical laser technology are composed of 3 to 5 (or even 10) different lasers in the visible range to cover the required spectral band. Still, these lasers always emit only at their nominal laser line(s). There is no way to tune the wavelength if necessary, to optimize the excitation and minimize crosstalk. It is important to keep in mind that even a few nanometers can affect efficiency and cleanliness when crosstalk is a problem (which it always is). Tunable visible lasers are technically possible: the dye lasers. Unfortunately, these dye lasers have a very unstable emission intensity and are consequently very noisy, they are prohibitively cumbersome to operate and can still emit only a single line at a time.

The multi-laser systems require all lasers to be combined in a way to simultaneously excite fluorochromes in the sample. Therefore, the laser beams have to be combined with semi-transparent mirrors. A set of lasers requires a cascade of beam-combining mirrors that are prone to misalignment over time and create a bulky system which is referred to as “laser battery”. Without question, this solution has given good results and has been the workhorse for the last 20 years of confocal microscopy. For that reason, they are still available and most probably will not vanish entirely in the very near future. The main drawback of these laser batteries is the lack of stepless tunability. Recently, a new technology has enabled the ideal light source for confocal imaging to be designed:

4. White Light Laser source – the ideal laser for confocal

The ideal solution for fluorescence excitation in true confocal microscopes has a high intensity and a very broad spectral range. Such devices became available with the development of nonlinear light fibers. These fibers create a broad spectrum (“supercontinuum”) and still provide the high spatial coherence required for focusing.

4.1 The photonic crystal fiber

Very high energy densities in light beams cause a number of nonlinear effects which alter the properties of the light significantly. Even in plain air, such effects are observable. When feeding an intense laser pulse e.g. through a piece of glass, the nonlinear effects increase significantly(1). More alterations are visible when using an optical fiber. The most efficient in that respect is a fiber that has a microstructure. These fibers are called photonic crystal fibers. They feature a regular pattern in their core, usually hollow pipes arranged in honeycomb patterns.

In these media, a monochromatic input pulse will undergo a number of nonlinear modifications. Headwords are self-phase modulation, soliton fission, Raman scattering and four-wave mixing. The most striking and useful result is a broad spectrum at the output upon a monochromatic input. The broadening and spectral distribution depends on the photonic crystal structure, the fiber length and the input pulse wavelength, duration and intensity.

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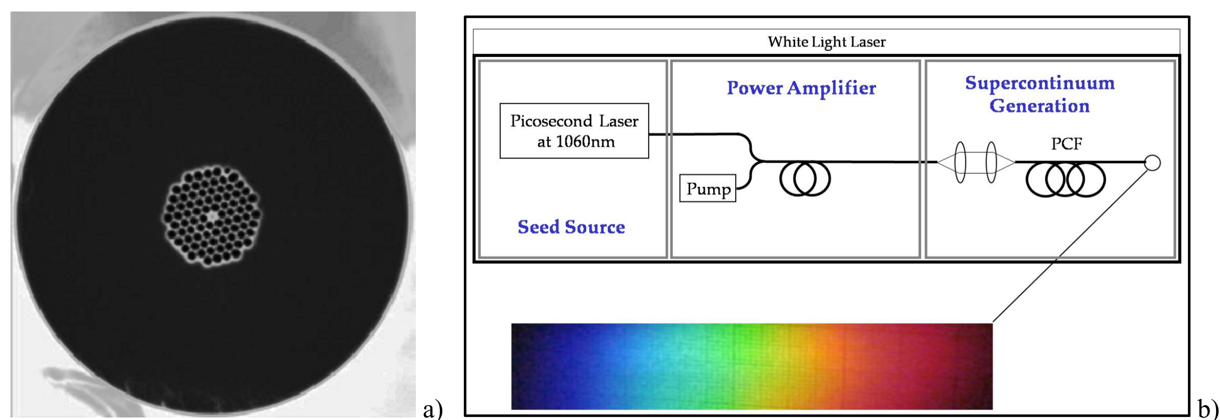

Fig. 2 a) Cross section of a photonic crystal fiber. The structure causes many nonlinear interactions. A high intensity monochromatic pulse is spread into a spectrum that can easily span an octave. b) Arrangement of seed-fiber laser, pump-fiber laser and photonic crystal fiber in a supercontinuum generating white light laser.

4. Seed and pump lasers

High intensity laser pulses are available e.g. from Ti:S lasers. These lasers provide IR pulsed light in the range of watts and with repetition rates of about 80MHz. The pulse duration is in the fs or ps range. Unfortunately, these sources are very complex, large and expensive. A more appropriate solution employs fiber lasers to generate precisely pulsed high energy IR pulses. These lasers are typically composed of a seed source (which acts like a precise clock, but at low energy) and a pump laser as an amplifier (which amplifies the clock to some Watts average power).
Seed lasers usually contain a diode laser as a pump, which can be controlled very efficiently and precisely. This diode pumps an active fiber which is doped with rare earth elements, usually erbium or ytterbium. These seed lasers provide precisely clocked IR pulses at e.g. 1060nm with high excellent beam properties.

To arrive at the required high intensities for supercontinuum generation, the seed pulses are amplified in a fiber-pump laser. This device couples a set of high power laser diodes with the seed pulse in a spliced fiber arrangement. The output power of the amplifier may easily reach some watts and has the same pulse characteristics and beam profile as the seed laser.

4.1Brightness and broadness

The high power pulses fed through the photonic crystal fiber create high power pulses at the pcf output. Instead of a monochromatic IR pulse, the spectrum is broadened over a couple of hundreds of nanometers (depending on various parameters, as indicated above)(2). Therefore, the emission is called a white spectrum. For confocal applications(3), the range between 450nm and 650nm is normally used as a source for visible excitation. Despite the broad spectrum, the beam still has a very high spatial coherence which is essential for focusing the light into a diffraction-limited spot. This perfect combination is sometimes referred to as “broad as a lamp and bright as a laser”.

The supercontinuum generation in the photonic crystal fiber is not a process of light amplification by stimulated emission of radiation, which sometimes causes discussions on whether a white laser is a laser at all. One might agree or disagree, nevertheless: the source contains a laser (the seed-pump fiber laser) which is modified by a filter (the pcf). Thus, the whole arrangement is as much a laser as an ordinary argon gas laser combined with a glass filter for line selection. And what ultimately counts is the fact that it is the perfect source for confocal imaging.

5. Spectral tunable multiline laser: WLL and AOTF

White sources have been around for microscopy from the very beginning. A very good source, with high intensity, low divergence and the full spectrum from radio waves to x-rays, is sunlight. Unfortunately, the intensity is very unstable at a given point on earth, and the angle of incidence is continuously changing. The classical sources for fluorescent illumination have been arc lamps, including mercury vapor for better blue and ultraviolet yield.

5.1 White sources need excitation filters

All these white sources require excitation filters that select an appropriate band of the spectrum to fit the absorption of the fluorochrome. During the development of fluorescence applications, a great number of optical color filters has been designed and used. For excitation, a short pass filter is the most common filter, as the excitation photon energy is higher than that of the emission photons. Consequently, a “bluer” light is required for illumination. To simultaneously excite a set of fluorochromes and collect the emission between the excitation bands, multiband filters have been developed.

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Originally, the filters were based on colored compounds with which the glass matrix was doped, the prototype being “Woods glass”, a filter glass that transmits only in the UV and near IR but blocks the visible spectrum. It was originally developed for secret communication during World War I. The UV transparency makes it a good filter for UV fluorescence excitation, which was the most common excitation in the beginning (fluorescence illumination is sometimes still referred to as UV illumination). Modern fluorescence filters are based on interference phenomena that occur in complicated layers with which a glass matrix is coated. The spectral properties are freely designable over a wide range and they are ideal for multiband applications.

Nevertheless, even these dichroitic multiband filters are fixed and if different spectral responses are needed, a set of filters must be implemented and used in sequence. Although tunable devices have been built with dielectric coated filters, these are not suitable for optical instruments, as they tune by changing the angle of incidence, causing various directions of the reflected light and alterations in the beam position of the transmitted light.

5.2 A tunable optical filter: AOTF

For sufficiently small beam diameters (such as laser emissions), a better solution is available: the acousto-optical tunable filter (AOTF). These devices use an optical crystal (typically TeO_2), which is transparent by nearly 100 per cent (0th order: straight pass). If the crystal is mechanically excited by a standing wave, then a tiny fraction of the white spectrum is directed to a different angle (1st order). This “bandlet” has a width of a couple of nanometers depending on the color. The color that is deflected to the 1st order depends on the frequency of the mechanical wave. As the wave is steplessly tunable, one can easily control which color of the white spectrum exits at 1st order – which in turn is fed into the microscope for extremely specific excitation of any fluorochrome. It is very obvious that such a device is much more flexible than a fixed filter system. Consequently, all recent confocal microscopes implement an AOTF to select lines for actual excitation out of the multiple lines from the laser battery, which offers some 3 to 8 lines.

If simultaneously a set of different mechanical frequencies are applied to the crystal, a set of different colors will exit at 1st order. This allows the combination of any of the various lines available from the different lasers for simultaneous excitation. Furthermore, the amplitude of the mechanical wave controls the fraction of the desired color reaching the microscope. Thus, the device is also perfect for controlling the excitation intensity.

5.3 The excitation light source that is tunable in color and intensity

The AOTF allows any combination of given laser lines to be selected from a laser battery, as the color for excitation is tunable. This directly offers a tunable color over the full spectrum if the laser is emitting white light. Indeed, the perfect excitation source for confocal microscopy is the combination of a supercontinuum generator with an acousto-optical tunable filter. This device is capable of providing any color in the visible range. The color is steplessly tunable. The tuning is very fast – a matter of microseconds. That compares to a significant fraction of a second for classical filter wheels or sliders. And on top of that, one can request a set of excitation colors simultaneously. Currently, typical setups allow the control of eight different colors, each tunable in wavelength and in intensity. For optimized excitation, there is now nothing left to be desired in terms of fluorescence yield and cross-talk reduction.

6. Threading tunable excitation in an incident light beam path

If fluorescence is recorded in trans-mode, a tunable excitation does not cause significant design challenges. In epi-mode, the situation is different. For to create an incident light beam path, a device must be inserted that passes the excitation light onto the sample, but at the same time carries the emission onto the detector from the same direction.

6.1 Fixed beam splitters

The simplest device which performs such a task is a semitransparent mirror. Such mirrors are available in various specifications. If a splitter is 50/50, half of the excitation and half of the emission will be lost. As excitation light is usually easy to increase, higher split ratios such as 80/20 have been used. They will waste 80 per cent of the excitation and 20 per cent of the emission. This is not a good compromise. Better solutions with fixed mirror devices use dichroitic beam splitters. Similar to dielectric color filters, they consist of complicated coatings applied on a glass matrix. Simple dichroitic mirrors will reflect e.g. short wavelengths and transmit long wavelengths. The split wavelength is adjustable by design. As in this case both the transmission and reflection part are used, tuning by changing the incident angle is obsolete. Like pass filters for excitation and emission, there is a wide variety of dichroic beam splitters for various split frequencies and also for different angles of incidence (which is fixed in a given optical setup).

To excite a set of fluorochromes with an appropriate set of colors, the beam splitter must work for this set of colors and the subsequent set of emissions. Although the grey-splitter solution will work for any color combination, it is not very suitable due to the prohibitively high losses. Another possibility is provided by sophisticated dielectric coatings which are used in multiband beam splitters. These splitters offer a series of reflection bands for excitation and are

transparent in between to collect the emission. Meanwhile, many types of these multi-dye dichroitic beam splitters are available: for two, three or four colors, for different angles of incidence and in many variations for the uncountable set of dye combinations. Still, they are not tunable, but need to be mechanically exchanged for each set of fluorescent dyes.

6.2 Tunable beam splitter

The ingenious tunable white laser would not be usable with all its benefits without a device to seamlessly carry the dialed colors from the laser into the sample. Such a device has now finally been invented: the acousto-optical beam splitter AOBS(4). In comparison to traditional beam splitters, this device is not a mirror that somehow splits the spectrum into fixed parts. It is a completely different approach for threading excitation light into the incident beam path of a fluorescence measuring instrument. The basis is again an acousto-optical crystal, like in the AOTF. As explained above, the beam path of a small bandlet is different if the crystal is tuned by applying a mechanical frequency. This principle can be used in reversed mode: if the crystal is tuned to the color for excitation, the excitation light is fed into the 1st order direction. The crystal will consequently bend that color and coalign it to the optical axis of the microscope. The light will travel through the excitation part and prime fluorescence in the sample. The fluorescence emission light is of a different color, due to the Stokes shift in the fluorescence process. Consequently, it will pass straight through the crystal, as it does not meet the diffraction conditions of the mechanical frequency to which the crystal is tuned. The emission is then easily detected in the ordinary emission beam path of the microscope.

Like in an AOTF, the AOBS can carry a set of excitation bandlets at the same time. Thus, the device can work as a tunable multiband beam splitter, with up to eight excitation bandlets (current technical standard) and very high transparency between the excitation bandlets. In addition, it does not need additional attention by the operator: if the desired set of excitation colors is dialed by the AOTF, the electronic control system will automatically tune the AOBS to meet the desired excitations. This is possible without any complicated translation, as the electronic devices are identical.

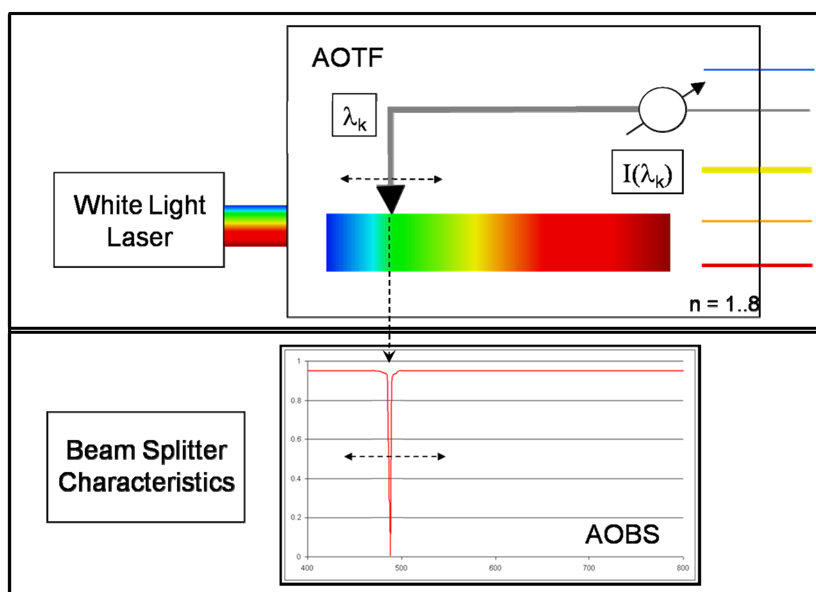

Fig. 3 Acousto-optical tunable beam splitter (AOBS). The concept allows seamless control of a set of tunable excitation bandlets to be fed into the incident beam path and lets the emission pass through at high efficiency.

7. Tunable spectral detection in combination with tunable excitation

Once the excitation is tunable by means of a stepless tunable white laser and an equally stepless tunable acousto-optical beam splitter, the obvious demand is for a tunable detector. Indeed, this tunable detector was available before the tunable exciter. The feature of tunable emission bands was introduced with a spectral multiband detector(5). This device is based on a prism (for high transmission efficiency) and a set of cascaded spectrophotometer slits. The slit barriers consist of mirrors that direct the fraction of the spectrum that is not required for the actual detector into different angles to successive detection devices that also have mirrors as barriers which allow operation of further detectors. This concept allows any band to be picked from the white spectrum spanned by the prism in each of the detectors. A set of 5 tunable detectors is currently the standard. The full spectrum can be distributed in any 5-fold fragmentation conceivable. To improve color separation, the bands can be restricted to smaller fractions, leaving out parts of the spectrum if wanted.

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Other concepts - employing linear arrays of detectors – are also available. But they are not tunable, as the bandwidth is ruled by the physical properties of the detection elements. They are also restricted to use linear dispersion elements, usually gratings. These gratings are much less efficient than prisms and consequently the whole instrument is less sensitive. Their benefit is simultaneous recording of more than 5 channels at a time, but with fixed band properties. The unavoidable severe crosstalk between the fixed bands requires mathematical separation of the signal by unmix protocols.

The prism-based tunable multiband detectors do not require unmixing, as all bands are individually tunable both in center wavelength and in bandwidth. Unmixing is here just an additional option to clear residual spillover. The tunable multiband spectral detector also offers recording of emission spectra by acquiring data with incremented center frequencies (moving windows), very much like in any other spectrophotometer. The increment and the bandwidth are freely controllable – which is not possible in array devices.

Most important is the tunability of the bands in conjunction with a tunable light source. Each time the excitation is changed, the emission bands can be adjusted precisely and specifically to the new excitation scheme, which might use one single or a whole set of different excitation colors.

Not only is the emission window tunable for recording of emission spectra, but with the tunable laser source described above, excitation spectra also become a standard measurement in confocal microscopy. In conjunction with the tunable beam splitter and the tunable detector, both types of spectra are subject to fully automated spectral recordings. Last but not least, both types can be combined to yield two-dimensional maps of fluorescence intensities as a function of excitation and emission wavelength. These lambda-square maps(6) allow detailed analysis of fluorescence landscapes, which is especially beneficial in samples with multiple stains that are unknown (e.g. natural fluorescence potpourris in plant material or biofilms).

8. Lifetime measurements

As a rule, fluorescence measurements are understood as measurements of intensity of emitted light. The emission intensity from a given sample in a given area depends on excitation intensity, excitation wavelength, all sorts of losses in the instrument, and on the specified spectral band for collecting emission in the detection device. In the sample, determining parameters are the concentration of the fluorochrome, the type and cross section of the fluorochrome, often microenvironmental conditions, the layer (depth) currently being measured and absorption effects of the dye and of other components in the sample.

8.1 Fluorescence lifetime

A different parameter that is characteristic for a given dye is the fluorescence lifetime. The fluorescence lifetime specifies the mean time the fluorochrome lingers in the excited state after excitation before it leaves this state to produce a fluorescence emission photon. Instead of returning to the ground state, the excited molecule may also assume any other path, for example to drop into a long-lived triplet state, which is a common alternative. It can also give away the energy to a nearby molecular partner without emitting any radiation. This is then called Förster resonance energy transfer, which has become an important phenomenon that is used in modern biomedical fluorescence measurements. One more parameter that effects the fluorescence lifetime is the abundance of photons that fit to cause stimulated emission for the fluorochrome in question.

The fluorescence lifetime obviously depends on the quantum properties of the molecule and on the microenvironmental conditions (including photons). It does not depend on all the other parameters mentioned in addition for intensity measurements.

Therefore, measurement of fluorescence lifetime is becoming more and more popular, as it is less prone to sample and instrument conditions (although the instrument is critical), it additionally allows identification of fluorochrome species by a second parameter and it can be used to measure microenvironmental changes, on which the working principle of FRET biosensors is based.

8.2 Lifetime measurement technique

To actually measure fluorescence lifetime, different methods are in use. One employs an oscillating excitation intensity and extracts the fluorescence lifetime from the amplitude and phase modulation in the emission signal. A second method excites the fluorescence by a light pulse and measures the emission in a series of gates.

The third and most precise method is the measurement of a single photon after a pulsed excitation. This requires the measurement of many events from which a decay curve is derived, which in turn can be analyzed by fit procedures to extract their fundamental decay components – which correspond to fluorescence lifetimes of the different fluorescent species in the sample. To perform time-correlated single photon counting (TCSPC), a pulsed source is needed, where the pulse is short enough not to interfere with the typical fluorescence lifetime, and the pulse distance is not too long as compared to the lifetime decay. Typical laser pulses are in the regime of femto- or a few picoseconds. This is short enough, as typical fluorescence lifetimes range somewhere between 0.1 and 10 nanoseconds. As mentioned, to create a

significant decay curve, many events have to be measured. Therefore, the pulse interval should not allow too much "idle" time, i.e. should be ca. 2-3 times the decay time. Many lasers pulse at 80 MHz, corresponding to 12.5 nanoseconds. That fits many fluorescence dyes. To measure longer decays, the pulse rate can be reduced by a "pulse picker", a device that passes only every second, third … pulse.

8.3 White light laser and fluorescence lifetime

Fortunately, the white light supercontinuum laser is intrinsically a pulsed light source. The repetition rate is usually 80MHz, and pulse pickers are readily available. Thus, fluorescence lifetime measurements can generally be performed with such a light source(7). The great advantage over all other solutions is the tunability of the laser wavelength. It is therefore immediately possible to generate and analyze excitation spectra not only of the intensity, but also of the fluorescence lifetime(8). And in analogy to intensity measurements, it is also possible to create two-dimensional maps of fluorescence lifetime as a function of excitation and emission wavelength. This enables even the most complicated multiply labeled samples and their possibly equally complex autogenous fluorescence to be characterized and analyzed. It also allows for the first time the systematic characterization of fluorescence dyes with their lifetime spectrum, both in controlled media and in complex microenvironments.

9. Gated detection with tunable excitation and emission

One last thing: all pulsed lasers offer the opportunity to only collect fluorescence emission during the dark time between the pulses. All you need to do is to stop the detection system to record data during the pulse. This has become possible with fast gatable hybrid detectors (HyDs). The benefit is that there is no need to worry about significant or spurious interference of the fluorescence signal with reflected light of whatever color.

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