# Chapter 2
# The White Confocal: Continuous Spectral Tuning in Excitation and Emission

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## 2.1 Fluorescence

In the middle of the nineteenth century, the scientific community became aware of a strange phenomenon. Under certain circumstances, one could detect color effects in otherwise homogenous or colorless solutions. First objects were solutions of chlorophyll and quinine. Solutions of chlorophyll are easily obtained from triturations of green plants. If focusing with a lens into such a solution, David Brewster (1781–1868) could detect a blood-red color at the lateral surface of the illumination cone. John William Herschel (1792–1872) realized a bluish shimmer at the glass–solution border in glass containers containing quinine solution when observed in the bright sun (Herschel 1845). Quinine is the working compound of extracts from the cinchona bark, a famous medicine for the prevention and healing of malaria. Quinine is also an ingredient in tonic water, the reason for tonic water to be promoted to the prime example specimen in demonstrations of fluorescence phenomenon to interested laymen. The famous researcher George Gabriel Stokes (1819–1903) repeated these experiments, especially with quinine solutions. He found out that the color of the illuminating light was changed when it interacted with the solution (Stokes 1852). If illuminated with short wavelength (blue), a longer wavelength (green-yellow) is returned. The change of that color, measured as displacement between excitation maximum and emission maximum, is therefore called "Stokes shift." Minerals were also known to yield such phenomena, which Stokes coined "Fluorescence," after the mineral Fluo-Spar and the word luminescence, which describes glowing effects in general.

As a matter of fact, fluorescence is not restricted to the visible range, but covers a wide range of the electromagnetic spectrum from Röntgen rays to far infrared. Nevertheless, microscopy basically uses the visible part with some minor excurses to the ultraviolet and near infrared.

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The most established description of the fluorescence process – that is the absorption of an energy-rich photon and the subsequent emission of a photon of lower energy – uses the term scheme that was introduced by Alexander Jablonski (1898–1980) (Jablonski 1935).

## 2.1.1 Fluorescent Specimen

As already mentioned, initially mainly organic compounds were objects that draw interest on research of fluorescence. Attempts to find out what could cause that behavior resulted in the discovery that certain chemical groups should be considered – in particular benzene, if linked to further groups. Such compounds were tagged "Luminophores" (Kauffmann 1900). For research with the microscope, August Köhler (1866–1948) already mentioned the "colorless dyes" (Köhler 1904). Indeed, more or less all living samples contain material that is fluorescent by nature, a phenomenon called "autofluorescence." Plant material is a very rich source for fluorochromes. The most famous and important is chlorophyll and a further wide variety of fluorescently active compounds that absorb light for the purpose of photosynthesis.

Besides substances that fluoresce without further modification, there are compounds in biological material that can be qualified for fluorescence by simple alterations. Some vitamins and hormones belong to that group.

Occasionally, it was found that some histological stains also show fluorescence, and that specific structures become visible. But it was clear that, for systematic research, completely different dyes needed to be applied. Such dyes were named "fluorochromes" (Haitinger 1934) and the process of staining is consequently called "fluorochromation". In histology, fluorochromation soon advanced to an extended science, because many fluorochromes show different emissions in different cell compartments. This effect is very well suited for "differentiation" of structures. To this group of dyes belong many compounds that are used until today – although occasionally in other applications – for example, fuchsin, rhodamine, fluoresceine, hematoxilin, eosin, acridine orange, and so on.

Differentiation by a single dye works, because emission of nearly any dye depends strongly on the molecular environment, that is, for example, the polarity, pH value, or molecules that bind to the dye. For modern fluorescence microscopy, this is both a blessing (molecular probes) and a curse (spectral shifts and quenching).

A revolution in fluorescence microscopy was triggered by the work of Coons et al. (1941) who introduced the immune-fluorescence staining (fluorescence immuno histochemistry resp. cytochemistry). The working principle of these stainings is based on the specific recognition of cellular structures, especially sugar residues and protein epitopes by antibodies. By appropriate methods, these structures can be decorated with antibodies inside cells or tissue sections. When the

antibody was decorated with chemically bound fluorochromes, then exactly and only those structures will light up under the microscope. By application of different dyes for various antibodies, a whole series of structures can be visualized simultaneously. By this approach, it was possible to specifically render the structure of many cell compartments, protein distributions, or cytoskeleton elements. By staining various cell states, e.g., during the cell cycle or during differentiation, this method allowed elucidation of the development and dynamic changes in these objects. Medical diagnosis also benefits from this technique.

One more revolution was the introduction of a similar procedure by Gall and Pardue (1969) for specific sequences of DNA or RNA, the fluorescence in situ hybridization (FISH). Here, a short polynucleotide chain is synthesized and chemically bound to fluorochromes. Subsequently, this marker is hybridized with the DNA that is abundant in the cell. Of course, this is possible only with locations on the DNA that are complementary to the artificially synthesized piece. Genetic research and medical care have introduced this method in large scale, meanwhile with various modifications and most diverse application possibilities.

Not only for structural information fluorescence methods were developed, but also dyes that allowed to detect various metabolites and inorganic ions in living cells. The best known case is detection of calcium via the $Ca^{2+}$-chelator FURA-2. The fluorescence parameters of this compound change significantly (both excitation and emission) upon binding of $Ca^{2+}$-ions. When applying a calibration curve to the data, the concentration of free $Ca^{2+}$ can be determined. These sorts of indicators are now available for a whole range of other ions and metabolites. The cumbersome and invasive method of injection of those dyes is meanwhile overcome by very ingeniously designed substances, so the living object is left unaltered in its native conditions.

Besides organic dyes, small fluorescent particles of semiconductor material have been developed (quantum dots) recently. These markers have a wide excitation spectrum and emit according to size and composition, in many different colors. Not yet sufficiently solved are biocompatibility and connection to antibodies, necessary preconditions for specific staining.

In the recent past all those concepts were radically excelled by a completely new method. A protein, which natively is abundant in jelly fish and other marine animals, expresses a natural fluorescence in the visible range: the green fluorescent protein (GFP). Initially, it was prepared from Aequatoria. Its triumphant success started when it became possible to clone the respective DNA into the genome of living cells (Chalfie et al. 1994). By appropriate genetic engineering, it enables the visualization not only of gene expression, but also of structural proteins in living and developing cells. Now living cells can be dyes without any external interference – and of course whole animals as well. The last sensational report was on a GFP-pig, expressing a greenish shimmering skin and green luminescent eyes. The application of this technique merely knows any boundaries. Meanwhile, a long list of proteins in all colors is available – with many secondary techniques to measure not only structural but also dynamic changes and processes in cells.

## 2.2   Fluorescence Microscopy

As mentioned above, A. Köhler was already investigating fluorescence for microscopy. The first commercially available fluorescence microscopy was produced and sold by Carl Reichert in Vienna. The systems at that time were basically ordinary microscopes with little variations that allowed connecting fluorescence devices. The microscope itself appears like a small appendage to the huge illumination apparatus, for example, coal or metal arc lamps with 10–30 A current drain.

To visualize fluorescence phenomena, it is first necessary to separate, using appropriate filters, an excitation band from the white light sources and guide it onto the sample for illumination. For this purpose, short-pass or band-pass filters are employed. Short-pass filters transmit light only below a specified wavelength; band-pass filters transmit only in a specified segment of the spectrum.

In the beginning, these instruments were dimensioned for illumination with near UV light or deep blue light, as fluorescence was more or less a synonym for UV illumination. Small and efficient sources for UV light were only available with the construction of compact high-pressure mercury arc lamps. These lamps also offer a high power density in the visible range and are evolved to a standard for the excitation of immunohistochemical stains.

To excite with UV light, filters made of black glass (Woods filter) were used. These filters have a good transmission between 300 and 420 nm. However, caution is advised as transmission increases at around 700 nm in these filters. This light is not visible, but can cause spurious effects in photographic exposures. By time, many color glasses were developed that were available in various thicknesses for many different purposes.

A revolution in filter technology was the invention of dielectric coatings that allowed depositing multiple layers on glass substrates that create all sorts of spectral bands. Such filters are still standard in today's fluorescence applications.

In general, microscopes come in two different types: transmitted light and incident light microscopes. Both types are suited for fluorescence, but the incident light version offers a significantly better separation of excitation and fluorescence light. The separation power is important, as the fluorescence intensity is usually at least 1,000 times less as compared to illumination intensity.

In case of incident light microscopes, the illumination light has to be guided into the objective lens by a reflecting mirror, while the emission is collected from the same side of the lens and has to pass that mirror for subsequent recording. The simplest case of the reflecting mirror is a "gray splitter", which reflects partially all colors equally and transmits complementarily. A 30/70 gray splitter, for example, has 30% reflectivity and 70% transmission. Consequently, the excitation energy and the fluorescence is partially lost, which is most severely a problem on the emission side and the reason not to use 50/50 splitters. Illumination is less precious as emission, so 70% are sacrificed in order to harvest 70% of the fluorescence. Even 05/95 splitters are sometimes used, if fluorescence is very weak or bleaches fast. With the introduction of dielectric coatings, mirrors became available that reflect

and transmit respectively different areas of the white spectrum. These "dichroitic splitting mirrors" were developed in large diversity and are used for a whole variety of dyes and dye combinations.

To remove residual excitation light completely from emission, a further filter is inserted between beam splitter and detector: the emission filter (barrier filter). Here, appropriate band-pass or long-pass filters are used.

In current instruments, excitation filter, beam splitter, and emission filter are combined in a "filter cube" that is easily exchanged. So, all the different regimes for illumination and detection can be switched quickly and simply. The extensive motorizations in modern research microscopes leave this concept dispensable and allow a wide flexibility by independent combination of the different plan-optical elements – without loss of time.
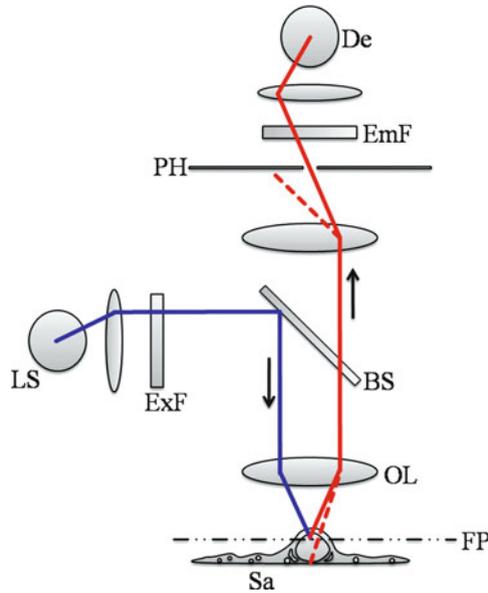
## 2.3   Confocal Fluorescence

A first application of a microscopic setup that allows to measure intensities in thicker samples without disturbance from other focal planes was published in 1951 (Naora 1951). Today, confocal microscopy is one of the most common tools in biological research (Diaspro 2002) for a graphical explanation of confocal microscopy (Fig. 2.1). Optical sectioning, as the fruit of a confocal setup, performs effectively only in incident light. By the currently rapid development of fluorescence techniques for biological research, also confocal microscopy mutated within 20 years from a dubious considered rare application to one of the most important daily routine methods. In confocal imaging, the illumination light has to be focused diffraction limited into the sample. For that reason, the light source has to be selected very carefully. Technical reasons leave lasers as the only suitable light source for confocal microscopes, as only they provide complete collimation at sufficient luminance.

Still, the well-established gas lasers are the most popular ones – especially argon ion lasers and helium–neon lasers. The use of krypton and cadmium lasers has more or less vanished. The most important advantage of gas lasers is the emission of more than one line, and therefore, fewer units needed.

Modern developments are solid-state and diode lasers that offer a much higher degree of freedom in selection of the emission wavelength. Diode lasers are also used inside solid-state lasers as pump lasers. These lasers are quite compact and small, but have the disadvantage of emitting only a single line.

In the chapter on fluorescence specimens, it was shown that the huge varieties of dyes cover the whole spectrum of visible light. Thus, to achieve a differentiated representation of all fluorochromes, it is highly desirable to have the individual dye excited by arbitrarily selectable wavelengths. This is not possible with traditional lasers. Here, significant excitation gaps remain in the spectrum. To increase the density of lines, a large conglomeration of lasers is necessary, which needs complex and laborious coupling. These setups are of course prone to misalignments, are

**Fig. 2.1** Confocal fluorescence. *Left*: Light emitted from the light source LS passes the excitation filter ExF and is diffraction limited focused by the objective lens OL into the focal plane FP. *Right*: Emission from the focal plane (*solid line*) passes through objective lens and beam splitter BS and is focused to a spot in the intermediate image plane, where the pinhole PH is located. Focal emission can pass the pinhole and is filtered by an emission filter EmF before reaching the detector De. Emission from other planes than the focal plane (*broken line*) cannot pass the pinhole and thus is blocked from detection. The pinhole acts as spatial filter for the *z*-dimension and thus creates optical sections. Conventional lasers emit only one or a few narrow lines. Excitation filter, beam splitter, and emission filter are classically fixed-parameter devices, usually colored mirrors or glass filters. The white confocal concept uses spectrally tunable devices in all these places

quite expensive, and have high energy consumption. The efficiency is very low and therefore lost heat has to be removed by cooling devices, often accompanied by displeasing noise.

The most comfortable way to achieve optimal excitation would of course be a system that allows dialing the color by a simple knob or slider. So, for each dye and for any combination of dyes, the optimal line or set of lines would be available – a dream?

## 2.4   A Tunable Laser

Such a dream has become true. A classical laser emits only tiny narrow lines with a bandwidth of usually less than one nanometer. The basis for a tunable laser is a white laser. A laser that emits white light has a high energy density over a wide range of the

visible spectrum. In combination with an acousto-optical element permitting to select narrow bands of a few nanometer widths out of the white emission, a tunable laser was realized (Birk and Storz 2001).

Novel fiber technologies were the basic principle that led to the invention of white lasers (Knight et al. 1996). The main component of such an instrument is a fiber whose core is an ensemble of many symmetrically arrayed cavities. This fiber type is called "photonic crystal fiber." Initially, they were developed for telecommunication purposes, consequently for infrared light applications. If a short pulse of high energy is coupled into a photonic fiber, then at the glass–air interface many nonlinear photonic processes cause various recombinations of photons. The result is the conversion of the line spectrum into a wide band, therefore called a "super-continuum." The width of that band depends on the crystal structure and on the length of the fiber. By selecting appropriate structures, it was possible to extend the broadening into the visible range.

Initially, a seed laser is used to generate short pulses in the range of picoseconds at about 100 MHz repetition frequency in the infrared. These seed lasers are also fiber lasers. A series of diode lasers is fiber-coupled with the seed laser to amplify the intensity of the pulsed light to some 10 W of light (Fig. 2.2). These high energy pulses are then fed into the photonic crystal fiber that consequently emits about 1–2 W of visible light in the range of 450–700 nm. The power density arrives at 1 mW/nm visible, which is a good value to record high signal-to-noise images in confocal microscopy.

Although there are some rare exceptions that use the full white spectrum for illumination, the most typical application is fluorescence that requires selection of an appropriate spectral band for excitation, if a white light source is used. This is equivalent to classical widefield fluorescence microscopy using, for example,
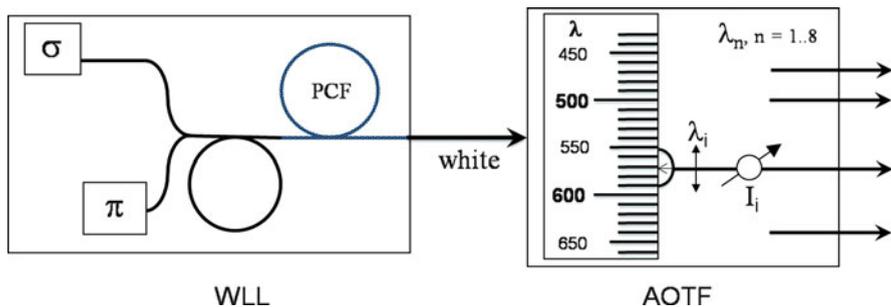


**Fig. 2.2** White light laser and spectral tuning. *WLL*: The white light laser is a compound fiber-based collimated light source that consists of a seed laser $\sigma$, a high-power pump laser $\pi$, and the photonic crystal fiber PCF. The emission is a continuous spectrum with close to white characteristics in the visible range. *AOTF*: The acousto-optical tunable filter allows to simultaneously pick (currently up to eight) independent narrow bands (bandlets) from the white spectrum. For each bandlet the center wavelength $\lambda_i$ is steplessly tunable, and also is the intensity ($I_i$). This device allows creating any illumination pattern in the wavelength–intensity space that might be required for fluorescence and is especially suited for confocal microscopy

a mercury high-pressure lamp and appropriate filters. Also, for selection of bands from the white laser, filters could be a solution. A much more efficient, flexible, and elegant solution is the employment of an acousto-optical device for the purpose. An acousto-optical crystal is capable of deflecting out of a white beam one or a series of narrow bands of only a few nanometers bandwidth. The crystal is excited mechanically and the wave grid effectively acts as a diffractive element that causes specific wavelength to be deflected. The deflected light exits the crystal in an angle (first order) to the principal beam (zeroth order). The deflected wavelength is controlled by the mechanical excitation frequency, and the intensity by the amplitude of the mechanical excitation. Casually speaking, the crystal works as a "photonic track switch". These devices are therefore called "acousto-optical tunable filters" (AOTFs).

By applying appropriate measures when designing the crystal and the electronic control, the light at the crystal's exit is collinear to the microscope's optical axis and thus suitable for illumination in the microscope. AOTFs are used in confocal microscopy already since 1992 to select laser lines and control their intensity. In comparison to plan-optical solutions (with filter glasses), an AOTF offers even more advantages. Many excitations of the crystal can be superimposed interference-free, so that a number of laser lines are simultaneously selectable. Current instruments use typically eight channels. A further advantage is the control of the amplitude that influences the intensity of the deflected light. In consequence, the device is a "dimmer" for many colors at the same time – each color's intensity can be controlled independently. And the whole regime of deflected lines can be reprogrammed within a matter of microseconds. The fast switch of illumination regimes is a necessary precondition for some applications to work at all, such as the illumination of hand-selected regions of interest or sequential illumination by different colors of single lines, which build up the image. Such methods help to reduce crosstalk or are used to excite ratio dyes in a timely correlated manner.

The combination of a white emitting laser and an acousto-optical tunable filter is consequently the ideal light source for confocal fluorescence microscopy (Fig. 2.2). Up to eight lines may be selected continuously in color and in intensity, where the switch time of illumination regimes is in the range of the duration of a single picture element.

## 2.5   Tunable Beam Splitting

The next step in incident light fluorescence microscopy is coupling the excitation light into the incident light beam path to illuminate the sample. Even though the classical solution by gray splitter or dichroic mirrors will work in connection with a tunable laser, these concepts invalidate the benefits of the new invention.

The simplest solution is of course a gray splitter that would also allow tuning the excitation wavelength continuously without the need of changing the splitting mirror. The major disadvantage, though, is the significant loss of both the applied

laser energy and the emitted fluorescence light, which is highly undesirable. For that reason, a gray splitter solution would be a little attractive makeshift.

The implementation of dichroitic splitting mirrors is also not ideal, as already mentioned above. They work more efficiently, but are very inflexible as many splitters would be necessary to interchange. For a reasonably dense covering over 250 nm, at least ten different splitting mirrors would be necessary, mounted on a wheel or slider. This is mechanically fragile, slow, and expensive. Not to mention that for the typical case, that is multiple staining, multiple splitters for a huge number of combinations need to be available as well. In order to serve for the mentioned eight lines, allowing excitation only at distances of 36 nm (compared to a continuous, i.e., stepless tuning!), $2^8$ different splitters, i.e., 256 different elements, would be necessary to be mounted and operated – not really a serious suggestion.

Here again, by employment of acousto-optical elements, a potent and very elegant solution was found: the acousto-optical beam splitter (AOBS) (Birk et al. 2002). Basically, this is an AOTF in reverse operation, which, however, requires the resolution of a couple of sophisticated technical problems.

If the acousto-optical crystal is tuned for a selected wavelength, then upon illumination with white light, this wavelength will be deflected into the first order. All other colors will pass the crystal straight and leave at the zeroth order. As the light pass is symmetrical, it is as well possible to guide light of a very narrow band into the first order, which will exit coaxially at the original entrance of the crystal. If adjusted correctly, this light could be used to irradiate the sample. The emitted fluorescence will never have the same wavelength as the excitation, rather always be shifted to the red. On its way back through the crystal, the emission is therefore not deflected into first order and can be collected completely by the detector. As the first-order bands in acousto-optical devices are always very narrow (typically around 1–2 nm), the excitation is very efficient and the losses for the precious fluorescence emission are very low. This is especially beneficial when compared to multiple-band dichroitic mirror splitter systems.

The most striking benefits of this method in combination with a tunable laser are of course the fact that the reflection peaks (the very narrow bands that allow light to pass from first order to the entrance of the crystal) can be controlled directly and without any detour, together with the selection of the excitation peak (Fig. 2.3). As soon as a certain frequency is applied to the AOTF in order to provide a selected excitation, the AOBS crystal that is coupled electronically to the AOTF crystal will be reprogrammed synchronously without any interactive control needed by the operator. This enables the continuous tuning of the excitation, prevents losses due to improper beam splitters, avoids losses in time due to mechanical switch of splitting mirrors, and at the same time represents a highly transmissive optical component that needs to be capable of simultaneous injection of various tunable excitations and transmission of the consequent fluorescence emissions.

And still there is one more merit: the operator does not need to follow all this reasoning. "By accident we used the wrong beam splitter" does not happen anymore.
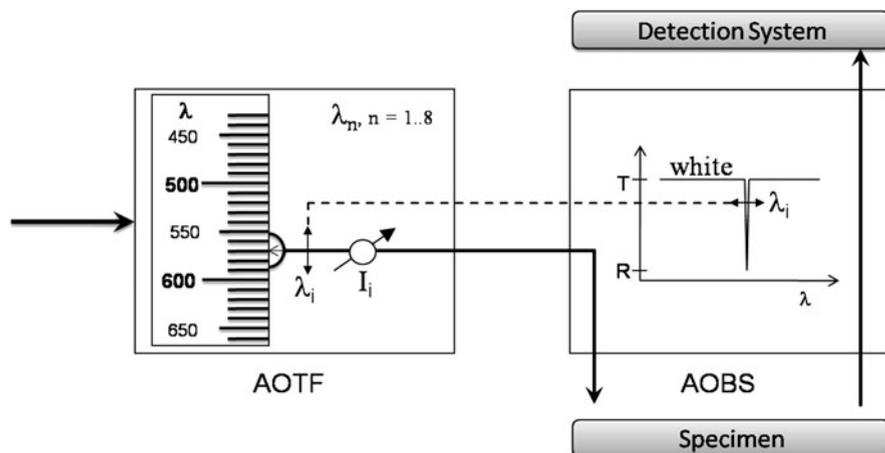
**Fig. 2.3** White beam splitting by acousto-optical beam splitter (AOBS). The color- and intensity-shaped excitation light (multiple bandlets) is guided by the AOBS into the specimen. From there, emission light is transmitted into the detection system. The transmission is white, with the exception of the narrow bandlets that are used for excitation. Both devices, AOTF and AOBS, are controlled synchronously in parallel (*dotted line*) when the wavelength of the illumination pattern is modified. No further interaction ("selection of dichroic") is necessary. The white transmission is better than 95% and allows immediate recording of emission spectra that are not chromatically modified by beam splitters

## 2.6   Tunable Spectral Detectors

To record fluorescence flexibly as offered by a tunable laser and a tunable beam splitter, it is also desirable to have emission filters tunable. Basically, tunable band filters already exist for a long time: spectral analysis of light started when Joseph von Fraunhofer (1787–1826) invented the spectrometer in 1814. By spreading the light spectrally by means of a dispersive element (here: a prism) and collecting only the desired band by blocking the unwanted parts by barriers, the task can be done easily. This is the concept of all modern spectrophotometers. The challenge was to allow the recording of multiple bands simultaneously and at the same time reduce losses as much as possible. This task was solved by an ingenious combination of comparably simple devices. A multiband detector allows the simultaneous recording of multiple parts of the spectrum, and the bands are adjustable individually and steplessly (Engelhardt 1997).

For signal reasons, a prism is chosen over a grid, since a grid has many disadvantages in terms of photon inefficiency as they create many orders and show severe polarization effects. The slit is made from two independently movable barriers, very similar to a conventional spectrophotometer. In a commercial spectrometer, a lot of effort is made to avoid any reflections from the barriers, as this may cause stray light and consequently distort the measurements. In a multiband detector, the edges of the barriers are designed as highly reflective mirrors.

The initially unwanted parts of the spectrum are reflected and directed to successive detectors that are designed with similar barriers. Repeated combination of this setup allows to record a series of bands simultaneously (Fig. 2.4). The losses at the mirrors are very low (<1%) and the slit itself has no absorption at all, of course.

This configuration constitutes a detection system, where many bands can be recorded simultaneously and the cut-off edges of the bands are individually and steplessly tunable. This is the ideal detector for multiple fluorescence samples. And it is the natural extension to a laser with multiple, tunable emission peaks.

As a side effect, the multiband detector also allows to record emission spectra, as it anyway consists of a series of spectrophotometers. The original intension was to record multiple bands simultaneously at negligible losses

## 2.7 Optimal Excitation

The abundance of many different dyes applied in biological research for fluorescence microscopy does make a flexible solution for exciting these dyes indispensable. Thus, the most important benefit of a tunable laser is obvious: setting the excitation wavelength to the most optimal position – in the simplest case to the excitation maximum of the dye.

Commercially available dyes are often named by excitation wavelength. Nevertheless, this name does not necessarily indicate the excitation maximum, but probably the best classical laser line that should be used to excite that dyes. The routinely used dye "Alexa488," for example, was baptized according to the most often used line emitted by the argon ion plasma, that is 488 nm. The excitation maximum of Alexa488 is around 500 nm; at 488 nm, the absorption dropped to only 80%.

A second commonly used dye, Alexa546, actually does absorb maximally at 561 nm. There is a good laser line from solid-state lasers at 561 nm. However, this does not imply that this would also be the best color for excitation. As a matter of fact, a tunable laser easily enables the dialing of the excitation according to the excitation maximum, but one has to keep in mind that between excitation and emission a certain distance has to be maintained in order not to distort the measurement. For this reason, in many cases it is recommendable to excite at shorter wavelengths than maximum, and especially if the Stoke's Shift is comparably short and the emission spectrum consequently has severe overlap with the excitation spectrum. In those cases one can still collect the larger part of the emission by shifting the excitation to the blue and moving the edge of the emission band accordingly. This procedure is usually not a disadvantage, as the laser is rarely used at full power (Fig. 2.5).

Thus, to compensate for the lesser absorption, one may just increase the laser intensity to reach a better signal-to-noise ratio in the recorded data. A combination of a tunable laser, an electronically coupled tunable beam splitter, and a tunable band detector – this optimal setting of excitation is found very conveniently by
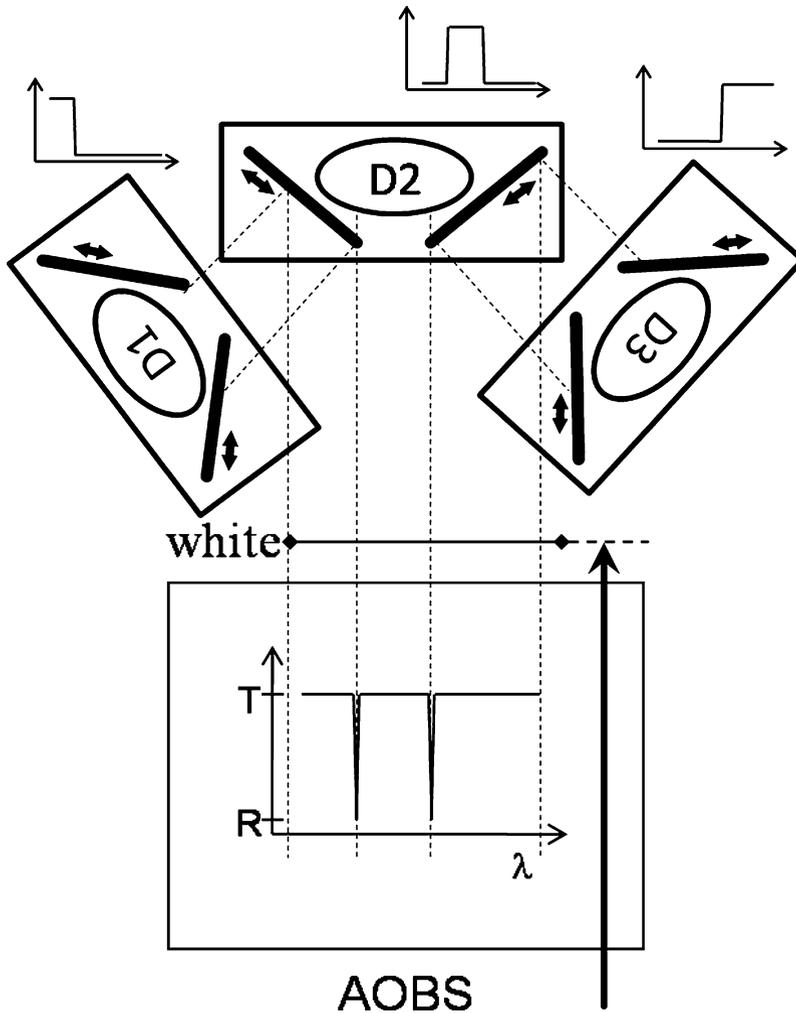
**Fig. 2.4** Spectral multiband detection. The emission from the specimen is passed by the AOBS to a detection system that is made of a sequence of classical spectrophotometer devices. The emission light is dispersed by a prism (not shown) and directed to a detector D2. In front of this detector, a spectral band is selected by two movable barriers that only pass the desired band. Parts of the spectrum with shorter wavelength are reflected to the next detector D1, as the barriers are made of high-reflecting mirrors. The detector D1 is also equipped with an equivalent band selection device. The same is true for that fraction of the spectrum that has longer wavelength than required for D2. This way of cascading any required number of band selector devices allows distributing the spectrum in any possible fractions to different detectors. This is an immediate tool for simultaneous recording of multiparameter fluorescence at lowest possible losses and maximum flexibility. The photometer slits have no chromatic properties, as compared to filters
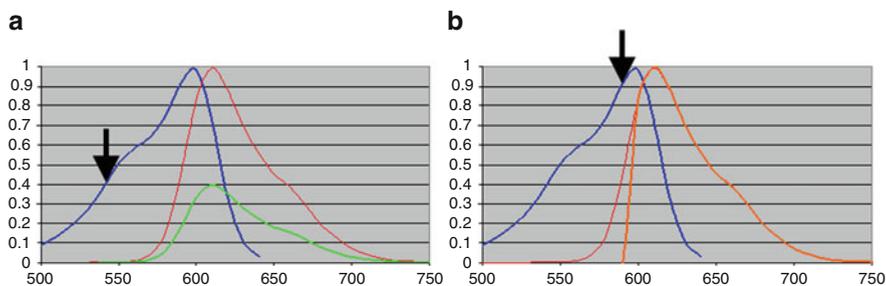
**Fig. 2.5** Optimal fluorescence excitation by tunable devices. (**a**) A theoretical fluorescence dye with excitation spectrum (*blue*) and emission spectrum (*red*) is excited with a fixed wavelength laser (*arrow*). The intensity of the emission equals the area under the green curve, if a white detection system is used (collection of all emission from about the excitation wavelength to the *red border* – here: 750 nm). (**b**) Excitation tune to an optimal wavelength, where the dye is well excited and the band for recording emission is not cutting away any significant part of the emission spectrum. Recorded emission intensity is ca 2.5 times as compared to example (**a**)

recording a few test images. You need just to vary the parameters during scanning until you find the best result, interactively within a matter of seconds. Of course, the system also is capable of incrementing the parameters automatically and record a series of images. And you may wish to select the desired parameters from the image stack afterwards.

## 2.8 Optimal Excitation for Multiple Stainings

A tool for stepless tuning of excitation wavelength is of even more importance for samples that are multiply stained. Here, the "cross-talking" between channels is a severe problem, when two or more fluorescence channels are recorded simultaneously. On the excitation side, a laser line meant to excite a shorter wavelength dye often will also excite the long-wavelength dye, although at a lower cross-section. This causes a better signal collected in the emission band for the long-wavelength dye, but at the same time the emission from that same dye may be increased in the short-wavelength channel, if the emission spectrum is wide enough. This of course will corrupt the separation of the two signals. This effect is called "bleed though" or "cross-talking." To tackle that problem, the short-wavelength excitation may be tuned further into the blue range – by a tunable laser. The excitation for the blue dye will be reduced, too, but usually not to the same degree as the excitation for the red dye. How those ratios change is, of course, strongly dependent on the dyes used.

   As the laser available is usually more than sufficient to excite the dye at maximum absorption, the reduced blue signal may be corrected for by increasing the short-wavelength excitation intensity. A laser that is steplessly tunable both in wavelength and intensity is the ideal source for these optimizations.

Similarly, it may occur that the long-wavelength excitation also excites the blue dye, causing a wrong blue signal in the red channel (without the benefit of a better signal in the blue channel, as emission is always of longer wavelength than excitation). For correction, the excitation wavelength must be tuned further red. This case is more critical, as it implies as well the need to narrow the collection band for the red signal.

With a tunable laser, it is very easy to balance the signals for multiple dyes and, at the same time, to reduce the unwanted signal in channels of complementary dyes. And in combination with a spectral band detector, it is possible to adapt the collected emission bands in order to increase signal to noise and further reduce crosstalk. A method that employs tunable laser lines would be rather laborious without an automatic synchronous tracing of the beam split parameters and the collection edges of the emission bands. With respect to the selection of appropriate filters and beam splitters, in many cases it would be just impossible.

When excitation wavelengths are optimized, the precision of separation may be enhanced further by the known procedures – if any are necessary. Here, fit in the already mentioned adaptation of the emission bands, the method of sequential scanning of the single channels, and – if nothing else will help – mathematical treatment by unmixing algorithms. The best method is of course: use simple to separate dyes – if any possible.

## 2.9  Förster Resonance Energy Transfer Problems

These days, fluorescence microscopy knows many new methods that well exceed the mere recording of images for rendering morphologic structures. An example that benefits particularly from tunable excitation wavelengths is the Förster resonance energy transfer (FRET) method. In FRET pairs of fluorochromes, the shorter wavelength dye is called the donor (D) and the longer wavelength dye is called the acceptor (A). In order for FRET to occur, the emission of D needs to spectrally overlap with the excitation of A. Then, the energy from D may be transferred to A without involving a photon (radiationless transfer). As radiationless transfer occurs only if the molecules are spatially very close (a few nanometers), the occurrence of FRET gives some evidence for the spatial colocalization of the fluorophores. If the fluorophores are bound to biological structure elements, e.g., proteins, then this conclusion also holds for the biological molecules that are investigated.

Detection of FRET is possible by two different methods: FRET-AB (for "FRET acceptor bleaching") and FRET-SE (for "FRET-sensitized emission"). FRET-SE tries to measure directly the acceptor's emission that was caused by donor excitation. Under normal (non-FRET) conditions, only the donor would emit photons (EmD) when excited with appropriate wavelength (ExD). If radiationless transition

occurs, then a second color is emitted, the acceptor emission (EmA) upon the single excitation by ExD. Separation of both emission channels is not trivial, as one has to quantitatively measure the comparably weak FRET signal against the strong donor emission. A separation that is just pleasing the eye is not sufficient. As in living objects the fluorochromized molecules move comparably fast, it is essential to record both emission channels simultaneously. As excitation is done by one single wavelength, there is a good chance to find emission of donor not only in the short-wavelength band for EmD, but also in the emission channel for EmA. The latter should only render the FRET signal. Due to the strong donor emission, this spill into the FRET channel causes significant disturbance in measurement accuracy of the faint FRET signal. The errors due to overlap of emissions of both channels may be corrected for by adaptation of the emission-band characteristics and by mathematical correction. Normally, for these kinds of experiments, a series of control measurements have to be recorded in order to capture all the parameters that cause crosstalk. These parameters are then applied to the data to get a significant measure for the radiationless transfer. Under good conditions, even the distance of the "fretting" molecules might be estimated.

A further source of errors that can have an impact on the precision of the FRET measurement is direct excitation of the acceptor molecule by the donor excitation wavelength. This of course causes directly a "bogus FRET signal," as there is no way to discern whether the emission from the acceptor was caused by direct excitation or as consequence of a FRET event. Here, the tunable laser offers the solution: the excitation wavelength may be selected from the bluer range of the spectrum until the effect is as small as possible or vanishes entirely. This way, by fine-tuning excitation, distinctly improved qualitative data may be obtained.

In FRET-AB, which is preferentially appropriate for fixed samples, a quantifiable donor image is recorded. Then, the acceptor molecules are destroyed by application of a high dose of light with a wavelength at high absorption of the acceptor ("bleaching"). If prior to bleaching FRET has occurred, then radiationless transfer is now interrupted and the donor emission should increase. Therefore, a second donor image is recorded and the intensity is compared to the first recording prior to bleaching. As was shown by Valentina Caorsi (Bianchini et al. 2010), a serious problem is caused by the fact that during bleaching of the acceptor, also donor molecules may be destroyed if donor absorption is too strong at the bleaching wavelength. Then, the donor fluorescence is reduced again after bleaching , and the increase caused by FRET is compensated for or at least reduced. The absorption properties in a real sample differ usually significantly from data published in literature, where in most cases spectra of the dye in solvent are published. The tunable laser offers here a means to measure the real absorption properties (spectrum) of the donor and to compare that with data from the literature. And in addition, it offers as well a solution to the problem: the wavelength for bleaching may be moved as far as necessary into the red part of the spectrum, until the donor

will not absorb and the donor molecules will not be bleached and sensible FRET data can be obtained.

## 2.10    Excitation Spectra In Situ

The tunability of the white laser consequentially offers a possibility to automatically increment the excitation wavelength and record data at each color. The result is a stack of data in the excitation wavelength dimension. By selection of regions of interests in the image plane, the excitation spectra of various structures can be displayed graphically and the spectral data are available for further evaluation as well. As already mentioned, the optical properties of fluorochromes strongly depend on the local environment that the molecule is sensing. This fact was the precondition to use fluorescent dyes as pH indicators, polarity sensors, or potential-sensitive probes. The modern bioindicators are as well based on this interaction of the fluorochromes and the local environment – where the environment could as well be a second fluorochrome. Here, in many cases the sensing mechanism involves a molecule that binds to the probed metabolite. Binding then causes a conformational change or cleavage, rendering the attached fluorochromes to a different environment, or changing the distance to a second fluorochrome (FRET-based indicators).

As there are a tremendous number of modern fluorescence-based indicators, especially the continuously newly developed mutants of fluorescent proteins, the measurement of the spectral properties inside the sample became indispensable.

Excitation spectra also help to separate dyes by unmixing methods based on excitation. That is of particular interest with dyes that have strongly overlapping emissions or emissions that are more or less identical. As a consequence, a new method is available: spectral excitation unmixing.

## 2.11    $\lambda^2$-Maps

With the freedom to record spectra by varying both emission and excitation, a new and additional analytical method is available: two-dimensional maps that correlate excitation wavelength and emission wavelength ($\lambda^2$-maps). For this purpose, emission spectra are recorded in a series of excitation wavelengths. With systems that provide automatic incrementation of excitation color and the corresponding emission bands, this becomes an easy task. The obtained data are displayed as intensity distributions in the spectral landscape.

$\lambda^2$-mapping is significantly beneficial when samples contain multiple stains (i.e., more than approximately four) or are composed of a complex mixture of fluorescent molecules. In complex samples, such as autofluorescing biofilms or eightfold-stained samples, this type of analysis is applied with great success. (Borlinghaus et al. 2006; Bianchini et al. 2010).

## 2.12   Fluorescence Lifetime Imaging

As mentioned above, the white laser is pumped by an IR laser that is pulsed at around 100 MHz. Consequently, also the white light is pulsed at the same frequency. The pulse width is in the range of some 20 ps. These parameters make this white light source an ideal tool for measurement of fluorescence lifetime. Classical fluorescence lifetime imaging (FLIM) is performed by detecting the delay time between a light pulse that excites the fluorochromes and the arrival of the first photon at the sensor. As this delay is distributed statistically, measurements have to be repeated many times (at the same position) and the data are plotted in a time histogram. The typical decay time $\tau$ is then extracted from these data by standard curve-fitting algorithms. A scanning microscope allows doing this calculation at each picture element. The resulting image consequently does not show intensities as values, but fluorescence decay times and is hence called a $\tau$-map. With the tunable laser that is described above, it is not only possible to select the wavelength for optimal excitation, but also to record and evaluate sequences of $\tau$-maps against excitation wavelength. This yields an entirely new quality of information: the correlation of fluorescence lifetime and excitation color, which was not accessible by now.

By means of a spectral detector it is also possible to correlate fluorescence lifetime and emission color (a method called "spectral FLIM"). In a combination of all these methods, the excitation tuning provides a third dimension when measuring fluorescence lifetimes. And it also opens up the possibility of correlating excitation and emission wavelength by fluorescence lifetime ($\lambda^2\tau$-maps).

## 2.13   Unlimited Spectral Performance

As shown in this article, the use of a tunable visible laser that allows multiple lines simultaneously and fast switching ($\mu$s) is a crucial improvement for confocal fluorescence microscopy. This is particularly true, when the corresponding parameters for beam splitting and emission band selection are continuously adaptable to the selected excitation (Borlinghaus 2007). Then, such an instrument is a completely new tool allowing new insights by new methods and applications that have not been available so far. This is true for structural imaging, for measurements of dynamic processes in living material, and for the new fluorescence applications in biomedical research, like FLIM.

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