

## Colours Count: how the Challenge of Fluorescence was solved in Confocal Microscopy

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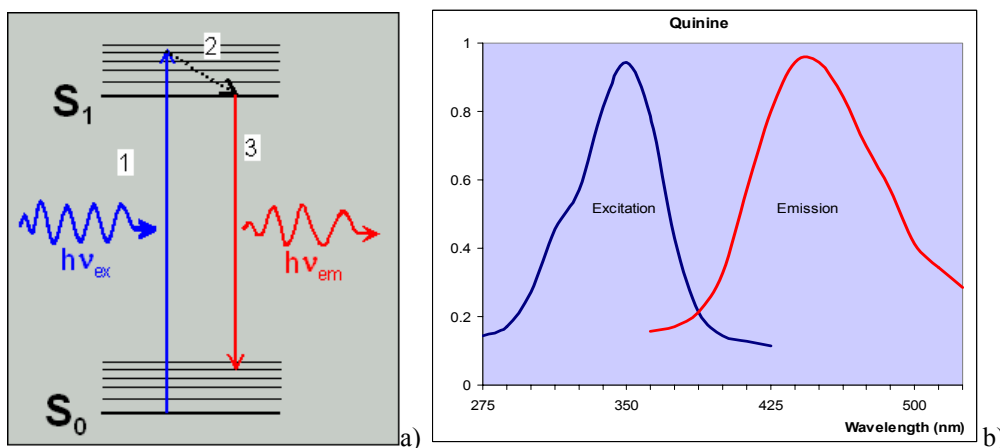
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Fluorescence requires four parties: the sample, proper illumination, efficient beam splitting and selective detection. As confocal microscopy is beneficial only in incident light mode, only reflected light and fluorescence is applicable. Biological research has a wide variety of fluorescent probes. Due to this coincidence, confocal fluorescence microscopy was booming in the last 20 years and there is by far no end in sight. With new technologies for increased resolution, for example stimulated emission depletion (STED), Fluorescence has again made it to the upper league and is in the business for the next era in modern biology. The most beneficial phenomenon in fluorescence is the fact that it comes in an infinite number of colours – which is at the same moment the most challenging feature. As a consequence, multiple colours are available simultaneously. But the price is a need for very elaborate illumination scheme, tricky beam splitting and efficient but selective detection of the various colours which are used to stain different structural elements in the sample. This article shows how ingenious inventions tackled the problems and found solutions for full spectral adaptation in illumination and detection – without loss of the precious fluorescence emission light.

**Keywords** fluorescence microscopy; confocal; multiparameter imaging; acousto optical; white laser; spectral confocal;

### 1. Fluorescence

The fluorescence phenomenon describes a sample's emission in a specific colour when illuminated by a different (shorter wavelength) colour. The first scientific report on fluorescence was given by F. W. Herschel in 1845 as some unusual effects he observed in a vessel of Quinine solution in bright sunlight [1]. It was G.G. Stokes who discovered the difference of excitation and emission in this phenomenon [2].



**Fig. 1** a) Jablonski Diagram for transition of electronic system upon illumination of the molecule by appropriate wavelength ( $h\nu_{ex}$ ). b) Spectral properties of a classical fluorochrome: excitation and emission of Quinine.

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Stokes was using very basic types of light filters: blue glass in a window for UV-excitation and yellow wine in a drinking glass as an emission filter. (Why he was lucky to have a church-window in combination with dense yellow wine handy is worth a separate investigation.) A more detailed report on the discovery of Quinine fluorescence is given in the introductory chapter of [3].

Fluorescence occurs when a photon is absorbed by a molecule (or an atom) and in consequence the electronic system of the molecule is transferred from the ground state  $S_0$  into the excited state  $S_1$ . From the excited state, the electron returns to the ground state under emission of a photon, which carries the energy away. This process is called fluorescence. There are other processes that involve higher excited states and triplet states and excited triplet states, but we can omit these processes in the context of what we discuss here. The electronic transitions are usually graphically displayed in Jablonski-diagrams, named in honour of A. Jablonski, a Polish scientist who accomplished many contributions to fluorescence both in theory and experiment. The Jablonski-diagram is read from left to right as the time-axis and indicated the energy level of the molecule on the y-axis. Usually, the time-axis is a mere hint on the sequence of events and should not be misunderstood as a scaled ruler. Due to thermal effects (excitation of oscillations), the various principal states have sub-states, called vibrational states. Absorption and emission occurs (with some rare exceptions) from the lowest vibrational states. Upon absorption or emission, the vibrational state is typically higher, but quickly relaxes into the lowest state.

This relaxation causes the emitted photon to have maximally equal, typically less energy than the exciting photon. Consequently, the colour of the emitted light has longer wavelength, i.e. is shifted to the red. In honour of G.G. Stokes, the distance from excitation peak to emission peak is called “Stokes-shift”.

As a great variety of Fluorescence techniques have evolved to the most important and frequently used experimental tools in biomedical research, the amount of known, characterized and not-yet-characterized chemical fluorescent probes is enormous. In addition to those, silicon-based probes (quantum-dots) have been invented and made biocompatible. They are as well used for many different kinds of experiments where fluorescent probes are employed. As if this was not enough, a fully new chapter has been opened by the development of fluorescent proteins, which allow to link endogenous fluorescence to proteins, to metabolic states and to regulation parameters in cells and whole living animals, without the need for entering the probe from outside into the cells, tissues or organisms. Latest developments delivered photosensitive proteins that can be switched on and off by light, or feature a change of spectral properties upon illumination. New advanced probes and molecular tools currently grow faster than the applications of those can keep pace - thus, an exciting future is guaranteed.

All fluorescence techniques share a similar basic design-principle. The object has to be illuminated by an appropriate spectral band, the excitation light has to be separated from the emission, and the emission has to be clarified from residual excitation in order to detect dim emission against the (necessarily) very bright excitation light. According to the Stokes-shift, the emission band is at longer wavelengths as the excitation band.

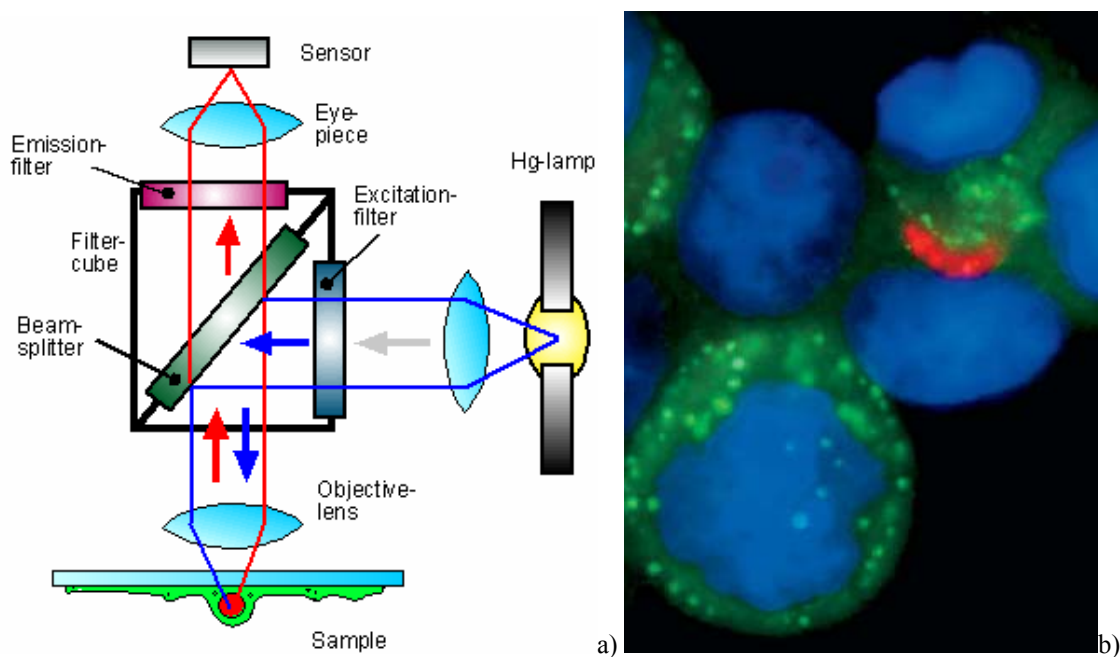
## 2. Fluorescence Microscopy

Fluorescence was not much considered for microscopy before the beginning of the 19<sup>th</sup> century. Fortunately, there is no fundamental difference between ordinary brightfield and fluorescence microscopes, a chance for those researchers who were interested to look at fluorescence in the microscope just some simple alterations to their instruments where necessary.

The modifications were very similar to those which G.G. Stokes used to look at his bottle of Quinine solution: add a filter for excitation and one for emission. The first commercially available systems [4] initially used huge carbon-arc lamps (30 Amps current!) for illumination and still required patience and serenity (“...observation was quite exhausting for the eyes, and it required a great deal of practice to discern nuances in fluorescence...” [5]). For illumination, Mercury high pressure lamps with sufficient UV and blue emission were employed soon; as fluorescence in the beginning was synonymous to ultra-violet illumination. The desired short-wavelength band was selected by glass filters containing Cobalt (smalt). This dense dark bluish glass unfortunately does transmit not only UV and blue, but also has a

second peak in the far red range. To avoid erroneous signal on the eye or the photographic paper, a cuvette with  $\text{CuSO}_4$  solution was inserted into the illumination path. This copper sulfate solution absorbed the red light and simultaneously worked as a heat-filter. Emission filter were inserted close to the eyepiece [6].

The fluorescence is typically 1.000-1.000.000 times weaker than the illumination intensity. Therefore, darkfield-illumination was introduced to get a black background. By incident light illumination, instead of transmitted light, the same effect can be obtained in a much more practical way. Current fluorescence microscopes use incident light. This technique requires a new part in the beam path: the beam splitter. The most simple beam splitter is a grey mirror at  $45^\circ$  which reflects the illumination light onto the sample and transmits the emitted light (or vice versa). Obviously, a setup with grey-splitters is not optimal: if a 50/50 splitter is employed, half of the illumination light is wasted – compensation is possible by larger light sources – the loss of half the emission is but a severe drawback. To get more fluorescence into the detection path, mirrors with different split-ratio are a (weak) workaround. If the light source is not limiting, a 90/10 splitter could at least collect most of the emitted light. Much more efficient in excitation and emission are dichroic (interference) mirrors. These devices have different properties in reflection and transmission; the according spectra depend on the multilayer coatings of the glass surfaces. In recent fluorescence microscopes, almost all optical filters are interference devices. The dichroic-technique was suggested and developed by J.S. Ploem [7].



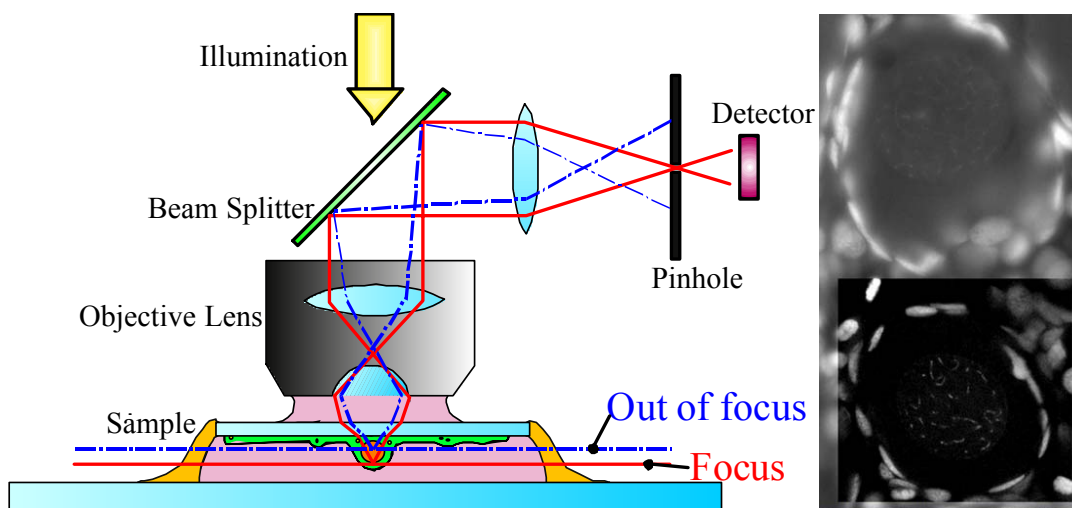
**Fig. 2** a) Elements of a fluorescence microscope: Lamp, excitation filter and beam splitter for illumination, beam splitter, emission filter and sensor for detection. b) Micrograph of threefold fluorescence stained sample: cancer cells stained with FITC, TRITC and Dapi. Courtesy of Hilary Russell, Belfast, Ireland.

One of the benefits of fluorescence staining over other techniques is the colour of fluorochromes. With sufficiently narrow excitation- and emission bands at appropriate spectral ranges, the same sample can be stained with multiple markers simultaneously. Even if the bands are overlapping, the signals can be separated by applying simple linear mathematics. The filters and beam-splitters get of course quite complicated, as they need to show complex features of reflectance and transmittance. Increasing numbers of bands for simultaneous recording decreases the efficiency and increases the complexity of filter-blocks: the filters and mirrors are not tuneable!

To increase efficiency and separation-sharpness, a common solution is sequential recording (today with digital cameras) of the various fluorochromes from the same sample. Although the images are significantly improved, the complexity is not reduced. A battery of filter-blocks – in the better case on motorized devices – needs to be installed in the microscope and controlled appropriately. The advantage of simultaneous recording (which ensures time-correlation of observed dynamic processes) is impaired, as mechanical switching of filterblocks takes a fraction of a second to several seconds.

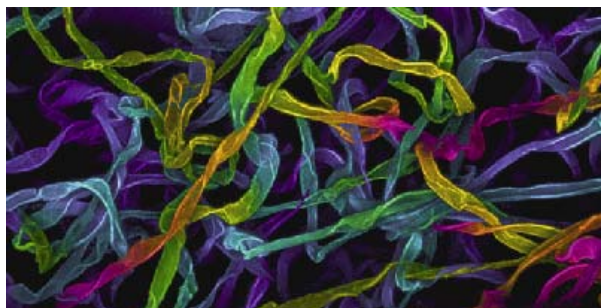
### 3. Confocal Microscopy

Confocal detection as a means to measure profiles and sections without disintegration of the sample was invented and described by M. Minsky [8] in 1957. Optical sectioning is performed by introduction of a small aperture in the detection beam path (pinhole) upon illumination in a single, diffraction-limited spot. For details on this comparably young but now very wide spread approach see [9, 10, 11]. The working principle is explained in Fig. 3. In essence, the confocal microscope can take optical sections from a sample, while the sample stays mechanically intact (e.g. whole animals, cell-layers in dishes or tissue sections). There are two application ranges: one area uses the sectioning performance to obtain three-dimensional stacks of images, from whose various types of renderings are calculated (see Fig. 4). This is the “structure” domain. The other part takes advantage of the spatially confined observation volume to measure time-depending changes in living material, i.e. four-dimensional kinetics. This is the “dynamics” domain. Of course, there are many intermediate applications and extensions to spectral, polarization and various other dimensions.



**Fig. 3** working principle of a confocal microscope. Light from the sample is only passing to the detector, if the origin is the focal plane. The result is an optical section. On the right side, a fluorescently stained mouse trophoblast (Feulgen) is shown. In the lower part, a square area was recorded confocally to remove any out-of-focus haze.

The single-point illumination and recording requires a sequential point-by-point image generation. This is accomplished by a pair of mirrors that perform the spot's displacement in x and y. The intensity is detected synchronously by a photon-multiplier tube and stored in a framestore for display or electronic archiving. As a consequence to this sequential data acquisition, recording of an image takes more time as compared to imaging by a camera. Many approaches try to compensate for this drawback, many of those introducing an array of spots allowing acquisition of multiple spots in parallel. Various kinds of scanning microscopes, single or multiple spots, two- and more photon excitation, resonant and non-resonant and many other variants are currently developed. There is currently a hot period of fast evolution in this technology-area [12].



**Fig. 4** Colours also can help to visualize three dimensions in a single image. Height-colour coded representation of a z-stack of optical sections taken from autofluorescing hairs on a leave of *vitis vinifera* (specimen: courtesy of K.-F. Krämer, Dielheim, Germany).

As far as the fluorescence part is concerned, there is no fundamental difference between a widefield and a confocal microscope. For that reason in early confocal systems the colours were treated by identical means as in ordinary fluorescence microscopy. The confocal requires diffraction limited illumination, which is best accomplished by lasers. Therefore, instruments for multiparameter fluorescence are equipped with a battery of lasers. To select the appropriate lasers for a given application, line selection filters are switched in the beam path. This is necessary for lasers emitting more than one line; all others may be switched by shutters. In addition, intensity is regulated by a series of grey-filters. To allow for various illumination schemes, a battery of beam splitters to separate illumination and emission is introduced, mounted on wheels or sliders. If the combination of laser lines for illumination is changed, the appropriate beam splitter has to be moved into the beam path. If the laser-device contains for example 8 different lines, all possible combinations of those would give  $2^8 = 256$  different splitters – on a very long slider. On the detection side, a set of barrier filters is required. For simultaneous recording of different colours, each detector needs a selection of barrier filters, again on wheels or sliders. The simultaneous detection also requires the different emission bands to be separated, which is done by secondary beam splitters. As requirements for these beam splitters depend again on the application, the splitters need to be exchangeable.

As mentioned, the most effective limitations in fluorescence microscopes are emission losses due to filter characteristics and the lack of flexibility as glass-filters have fixed specifications. The confocal microscope – at least the true confocal single point scanning concept – offers but some fundamental differences in the optical setup that may be turned into very beneficial advantages for the fluorescence part, as will be described below.

#### 4. Line selection

The first significant difference was developed for the selection of laser lines. A laser beam has a very small diameter, typical in the range of less than 1mm. Beam diameters out of conventional lamps need to be in the range of several centimetres in order to give full and homogeneous illumination in the full field of view. Here, the small diameter allows introducing a new technology: acousto optical devices. These devices consist of a crystal, which is activated mechanically by a high frequency. When coloured light is then shone through the crystal, a very narrow band will be deflected at a certain angle, whereas all other colours pass through straight. The colour of the deflected portion is tuneable by the mechanical frequency fed in. In addition, the amount of light deflected is also adjustable by the amplitude of the stimulation frequency. In total, this “acousto optical tuneable filter (AOTF)” acts as a dimmer with tuneable colour. Fortunately, it is as well possible to impose various different mechanical waves simultaneously onto the crystal, with the effect of deflecting a series of narrow bands at the same angle.

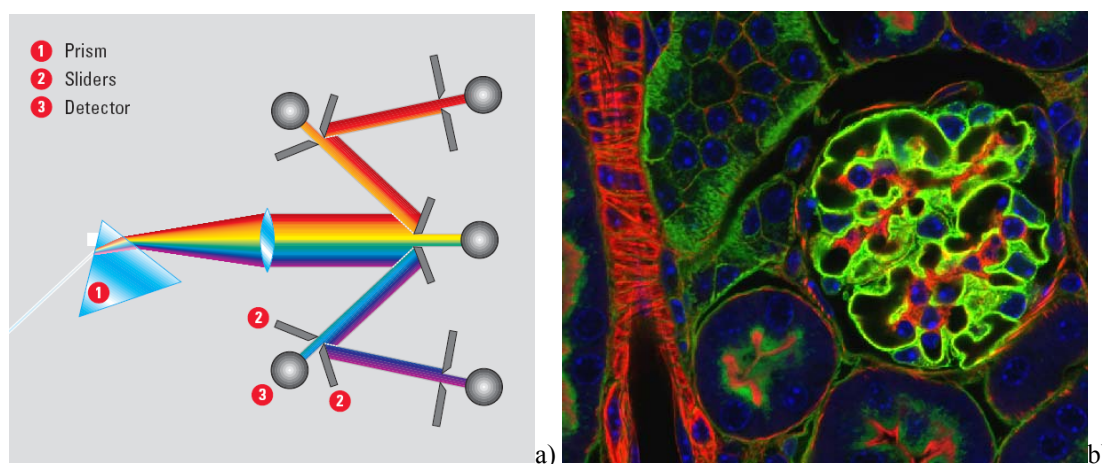
If the light shone on the crystal is the emission of a battery of lasers, and the deflected light is used as light source for a confocal microscope, one has a single device allowing for selection of any line and line-combination at individually steplessly tuneable intensity [13]. This type of illumination modulation has now become a standard for laser-based equipment.

The time required to change the illumination scheme with an AOTF is in the range of a microsecond, i.e. much faster than any mechanical device which may switch in the range of a second. This feature is

made use of in “region of interest illumination”. Here again, technical characteristics of true confocal microscopy - the scan-procedure – offers new concepts. In a controlled mode, the illumination is switched on only in predefined section of each line. The effect is illumination only in a fraction of the whole field against a dark (black) background. Most beneficial is this illumination pattern for dynamic measurements performed with FRAP and related experimental tools or by activation of photoswitchable proteins or dyes. Very advanced protocols allow illumination by different colours at different intensities in various regions in the field of view. These areas may be geometric (rectangles, circles, ellipses), manually painted irregular shapes or automatically segmented areas by application of image analysis routines (seeding strategies).

## 5. Emission tuning

The next challenge was the emission- (barrier-) filters in combination with the above described secondary dichroics. Besides the fact, that a solution with batteries of barrier filters for each channel and a set of secondary dichroics for separation is very bulky but still restricted to given filter-specifications, these arrangements are comparably inefficient in transmission. This is particularly true for simultaneous recording of many (more than 2) fluorescence channels. The concept of recording only data through a single spot at a time again opens the way for new paradigms – that are indeed very well known and introduced elsewhere since long time ago [14]. Instead of barrier filters, one can use dispersive elements and record the intensity in a fraction of the spectrum by separating the interesting band by means of a slit. The slit is mechanically adjustable to any position in the spectrum and allows continuously tuning the width of that band. This device is called a spectrophotometer and immediately fits in the detection part of a single beam confocal microscope. The obvious benefit is tunability to any width and centre frequency without the need of bulky batteries of (non-tuneable) filters. As known, spectrophotometers also allow recording of spectra, in this case the emission spectra of the fluorochromes in the sample.



**Fig. 5** a) schematic graph of the multiband spectral detector for confocal microscopes. Emission light enters the prism (1) from left; the dispersed light is led to the first spectrophotometer device where the inner part is transmitted to the first detector. Shorter or longer wavelengths are reflected to the next detector (3), where again reflecting barriers (2) create further spectral bands. b) optical section in three fluorescence channels of mouse kidney (glomerulus, tubes and blood vessels).

So far, so good. The challenge here is the request for simultaneous recording of multiple channels at a time. The first and still the best answer was a multiband spectral photometer [15]. In this device, the barriers which make the slit defining the spectral band that passes onto the detector, are composed of mirrors. Each of the two barriers directs the light which is reflected to a further device of the same properties. The result is a cascade of spectrophotometers which are individually tuneable and can select



any band of the spectrum emitted from the sample in any combination. Here, the efficiency in comparison to barrier-filter and secondary-dichroic solutions is even more pronounced, as there are no secondary dichroics needed. Losses at the barrier-mirrors are negligible.

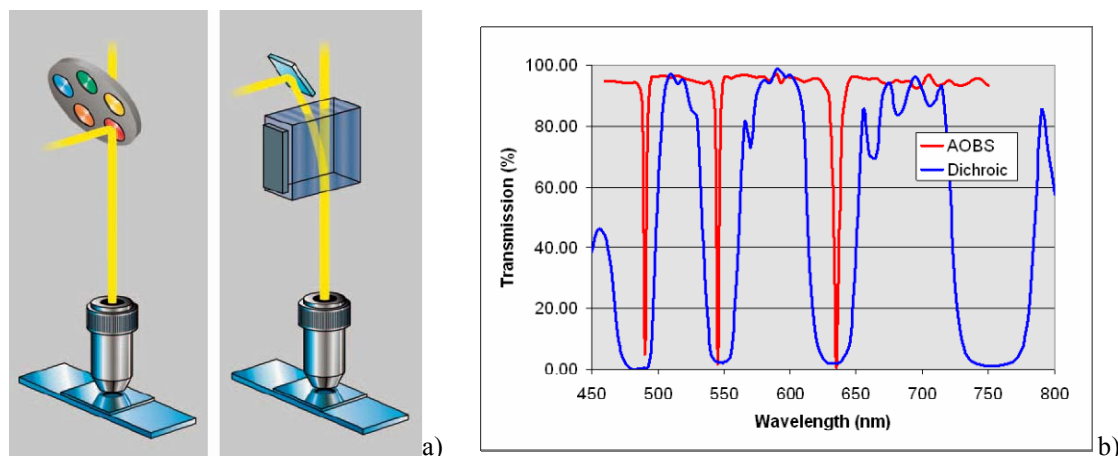
Alternative solutions try to increase the number of simultaneously recorded channels by introduction of detector arrays [16], which have their benefits and drawbacks. There are also other attempts to use diffraction grids as dispersive elements in order to get better spectral resolution. The prism has the advantage to transmit fluorescence at ca. 95%, whereas grids – whatever technique is used – typically transmit only ca. 50% unpolarized light over a sufficiently broad spectrum.

## 6. Beam splitting

Having removed all filters and splitters from the detection unit, there is still the main or primary dichroic which has to separate excitation from emission light. Also here, classical devices have restrictions in efficiency and are not tuneable. Is there an alternative solution for this central element of fluorescence microscopes?

The solution again is provided by acousto optics and restricted to small beam diameters – that rules out widefield application [17]. The basic idea is, to use the above described AOTF device in a reversed manner. The white transmission must serve for the fluorescence emission; otherwise the efficiency is dramatically reduced. But the excitation is done by lasers featuring very narrow spectral bands, a property that allowed acousto optical devices to be employed in the first place. So how to put these requirements and features together to solve the problem?

When the crystal is tuned to a certain frequency, the deflection path is open for a narrow band in the colour spectrum, whereas all other colours pass through straight. Consequently, the emission must go straight through the crystal. Now we must tune the frequency appropriately to the laser line, which we want to use for fluorescence excitation. Then we can inject the excitation light from the laser at the deflection angle into the beam and the laser light will exit the crystal coaxially and finally reach the sample to be absorbed by fluorochromes. The emitted light is anything but the same wavelength as the excitation and therefore will pass on its way back undeflected through the crystal [18].



**Fig. 6** a) Beam splitting devices. Traditional dichroic mirrors (left) and single-crystal electronically controlled acousto optical valve (AOBS, right). b) Transmission spectrum of a conventional dichroic beam splitter for three bands (blue) compared to transmission spectrum of AOBS, tuned to three lines (red). Total transmission equals the area under the curve and shows the better performance of AOBS.

This device has several significant benefits over a classical glass beam splitter: The transmission for emission light is much better (see Fig 6 b). Better transmission is always identical to better signal to noise, i.e. crisper images. Or it allows reducing excitation intensity for constant S/N in order to reduce

bleaching and phototoxicity. As the future is research at the living creatures, reduction of illumination intensity is a must.

The device is fully electronically programmable. There are no mechanically moving devices. The switching time is in the range of a microsecond. And the proper frequencies may be controlled by the system automatically when the operator selects a set of laser lines for excitation.

Other alternatives to classical beam splitters have been introduced. For line-scanning designs only, the “Achromate” uses the Fourier-transform of a line-shaped excitation structure, which is again a line (although at 90° rotation). Placing a reflecting line in the back focal plane, one can illuminate a line in the sample and still collect from the rest of the aperture the fluorescence. Therefore, that device does not need any adaptation to different illumination schemes; though it is restricted to line-scanning [19].

## 7. Continuous Excitation

So far, both the detection module and the beam-splitting have been designed as a white device, i.e. fully continuously tuneable (in the visible light range). The classical lasers have still narrow-band emissions – the main weakness as compared to conventional light sources in standard fluorescence microscopy. Most lasers have only one single fixed emission, which is especially true for the more modern diode-lasers.

Some of the (less modern) gas lasers emit a series of lines. The Argon-plasma for example, emits at a usable intensity two lines in the UV and 5 lines between 457nm and 514nm. The researcher is thus forced to select out of the given lines those, which fit best his application, which in turn is ruled by the dyes chosen. This might include suboptimal excitation (not at the dye’s peak absorption) or unnecessary and unwanted cross-excitation of other dyes abundant in the sample. The dream of colour microscopists therefore is a laser emitting over a broad range of the spectrum.

New developments in fibre technology achieved this goal [20, 21]. The heart of these devices is a photonic crystal fibre (PCF) which is fed with high energy pulses of infrared and emit the desired broad visible spectrum. The conversion occurs by nonlinear optical effects in the core of these fibres which is made up of a pattern of hollow tubes forming a microstructure inside the fibre. These white light laser sources have been successfully explored for confocal imaging [22, 23, 24].

## 8. The White Confocal

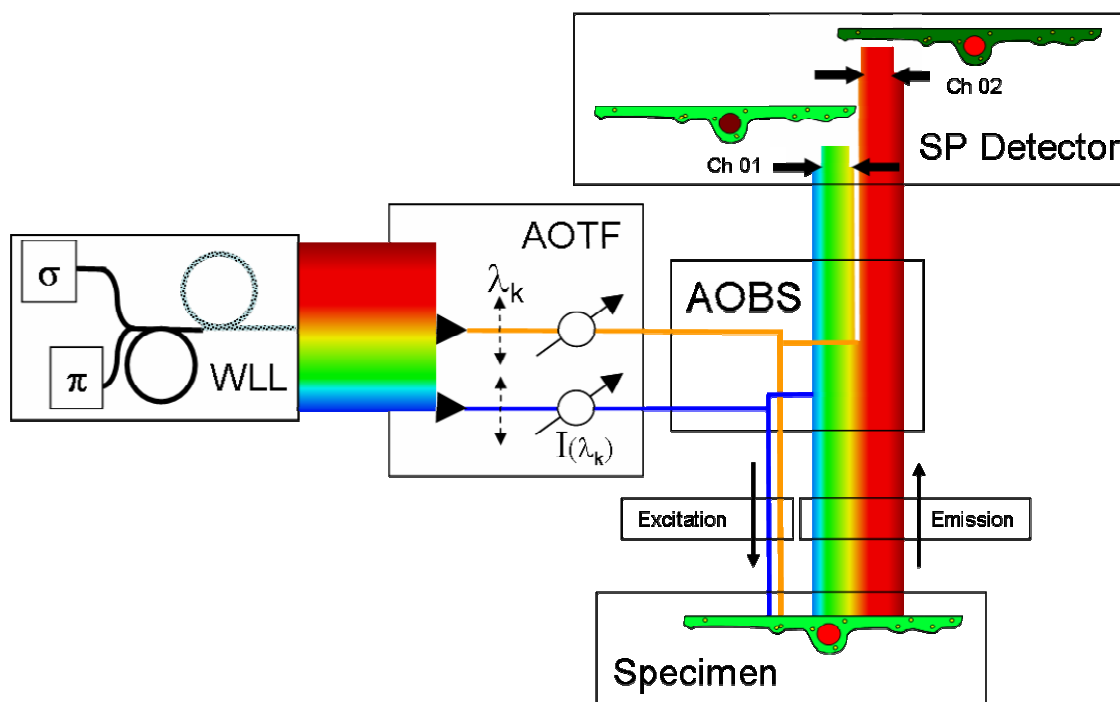
Of course, the white laser is not sufficient for illumination, as the sunlight was not sufficient for Stokes’ discoveries: we need the church window. An ideal window would be one that is tuneable to any colour present in the laser’s spectrum. And this window is already available: the acousto optical tuneable filter. The AOTF deflects a narrow band from the full spectrum shone onto the crystal. This band is only a few nanometers broad and therefore may be referred to as a line with sufficient reason – although a bold one. The wavelength is continuously adjustable and the intensity is continuously variable. And one can select as many lines as needed (there are some technical restrictions, and currently the limit is set to eight). The combination of a white laser with the AOTF control is the ideal source for confocal fluorescence illumination. Such a light source excites optimally any newly designed fluorochromes and fluorescent proteins or combinations of those.

The next element in the beam path is the beam splitter. Of course, a fixed parameter beam splitter would destroy the benefits we gained from the tuneable laser source. But the solution is already invented: it is the fast tuneable multiline light switch AOBS. If this device is electronically directly coupled to the wavelength-settings for the laser, the operator comfortably has nothing to adjust or even to ponder about. And in addition it is most efficient in transmitting the precious fluorescence emission.

When the light finally reaches the detection module, we again need a device that can benefit from the flexibility and adapt appropriately to the selected illumination scheme. The spectral detector provides the required features with tuneable bands fitting into the emission segment between two excitation lines. It also effectively suppresses the excitation light by leaving out small tuneable fractions at the positions of the lines. If designed with a glass-prism for dispersion, the transmission will fulfil the high-efficiency requirements for crisp images and sample preservation.



All spectral measurement techniques and methods which so far have been developed on the detection side may now be exploited in a similar way on the illumination side, e.g. linear unmixing or FRET. On top of that, fluorochromes can be characterized by excitation-emission correlation matrices [25].



**Fig. 7** The white confocal. A white source (WLL) allows to select a set of (currently 8) different lines from the spectrum by AOTF. These lines are guided onto the Specimen for excitation of fluorescence. The emission passes nearly unaltered the acousto optical beam splitter (AOBS) and reaches the spectral (SP) detector where multiple (currently 5) channels of freely tuneable emission bands are simultaneously recorded.

Starting from 1911 with a first “Luminescence Microscope”, less than a hundred years later both the fluorescence probes and the instrumentation have indeed undergone a paradigm shift [26].

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