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reSOLUTION

Solving the Challenge of Fluorescence

Leica TCS SP5 X:
The First COMPLETELY
Tunable Confocal System

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Fluorescence 1833 to 2008+

In 1833, D. Brewster found a blood-red light in a solution of Chlorophyll at the edge of a focusing cone and in 1845, J. Herschel discovered a bluish shimmer when a quinine solution was observed in the bright sun. G. Stokes repeated those experiments and raised the first theory on the phenomenon and also coined the term *Fluorescence* (G. Stokes, 1852)[1]. At that time, the sun was the best light source for short wavelength, and the excitation filter was a piece of blue church glass. Emission could be separated in "transmitted fluorescence mode", as we would call it today, when the object was looked at through a glass of dark yellow wine – the barrier filter. The fluorescent dye they observed was quinine, the working ingredient in tonic water that prevents and cures malaria fever. Quinine has a broad absorption in the UV-blue range and emits green-yellow light. Commercial Tonic Water is therefore a very popular specimen to introduce beginners into the world of fluorescence effects.

Fluorescence was introduced as a new method in microscopy in the beginning of the 20th century, and the first commercially available fluorescence microscope was produced by Reichert in Vienna 1911. At that time, Fluorescence usually was done only with UV-illumination, using Cobalt-glass in front of bright arc-lamps as excitation source. The samples were stained materials, that empirically were found to fluoresce, and mineral samples. At this time, the most famous biological stain available was Feulgen-stain for DNA.

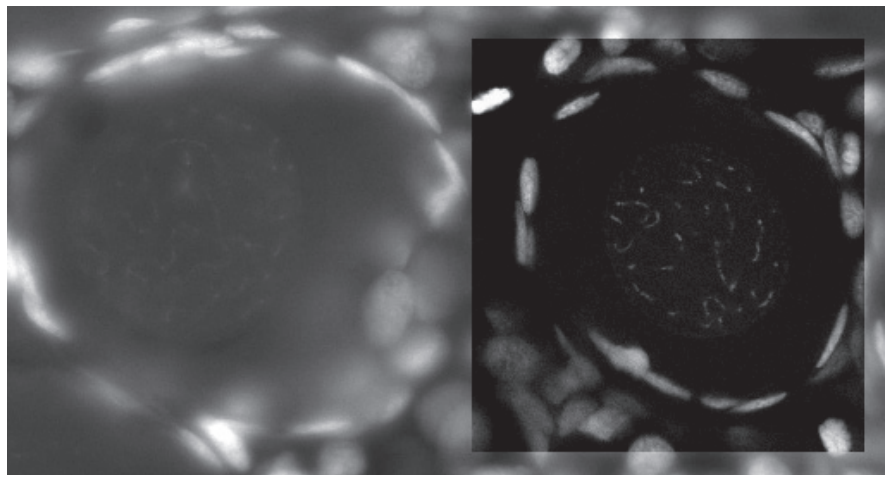


Fig 1: Feulgen stained mouse trophoblast. On the left is a fluorescence image taken with a widefield research microscope. On the right side, an optical section is recorded with confocal optics.

A new world of applications was discovered when A. Coons published methods using fluorescently labeled antibodies to visualize specifically single proteins in cells, what was then known as Immunohistochemistry (Immunocytochemistry) (Coons et al 1941)[2]. Further support for fluorescence microscopy was the introduction of Fluorescence *In Situ* Hybridisation of DNA (Gall & Pardue)[3]. These methods soon became the most useful tools in cell biology, and also stimulated the invention of even more dyes with various colors to simultaneously stain multiple structural features, in different colors, in the same cell or nucleus.

Biological research looks to understand the processes that make up life. Therefore, researchers always look for specific stains that work in living material, without interfering with the living organism. A very powerful method was the introduction of fluorescent proteins (D. Prasher)[4] that can be expressed as endogenic stains in living cells in various colors. Not only can genetic information on expression schemes of proteins be observed but also the proteins themselves can be fluorescent genetic constructs. More recently are the development of biological markers that follow metabolic or environmental changes in selected locations of the cell.

Requirements for Fluorescence in Microscopes

Fluorescence microscopy today is mainly performed by incident illumination. This improves the separation of the strong excitation light from the weakly emitted fluorescence. To accomplish this, an excitation filter is placed between an Hg-lamp and a beam splitting device. Beam splitters in common microscopes are dichroitic mirrors that reflect the short wavelength excitation light onto the specimen and transmit the longer wavelength of the emission. For observation, a barrier filter is placed in front of the detector, to remove as much as possible residual illumination. All these optical elements are typically mounted together in a filter block that is easily exchanged to allow observation of various stains and combinations of fluorochromes.

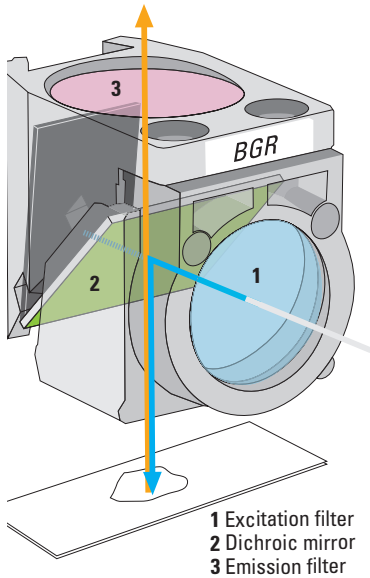


Fig 2: Filter cube for conventional fluorescence microscopes contains all filter optics needed for a given fluorochrome or fluorochrome combination.

A true confocal microscope focuses the illumination light to a single diffraction limited spot and moves that spot line by line over the specimen. The technical solution for diffraction limited high intensity illumination requires laser light, which is perfectly collimated. Consequently, current confocal laser scanning microscopes employ a series of lasers that (unfortunately) emit only at distinct lines.

Best cases are gas lasers, which emit a series (up to five) lines simultaneously. Nevertheless, to cope with the requirement for full spectral performance, a bulky setup of several combined lasers is necessary – still offering only the physically possible emission lines of lasers that restrict flexibility in tuning into the correct excitation wavelength for the many fluorescent probes.

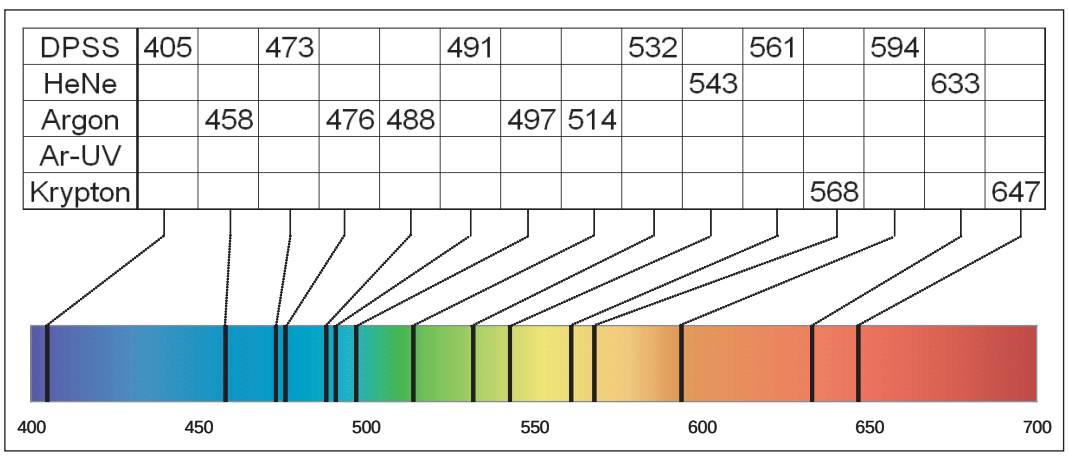


Fig 3: Some classical laser sources and their main emission lines.

(Note: not all lasers may be combined in one system due to restrictions with beam-combining optics or number of ports.)

A New Source: The White Light Laser

For flexible selection of the excitation wavelength, one would wish to have a laser that emits white light and allows selecting the excitation band similar to wide-field microscopy – or even a laser that emits narrow lines with the frequency tunable like a radio receiver. Until recently, the only technology that provided tunable emission (in the visible range) were dyes lasers – complicated instruments that suffered from dye-degradation and very high intensity fluctuations rendering this concept useless for imaging.

A new fiber technology was the breakthrough for the desired instrument: crystal photonic fibers, also called supercontinuum fibers. These fibers have a core that consists of an assembling of hollow tubes, usually arranged in a hexagonal pattern (crystal).

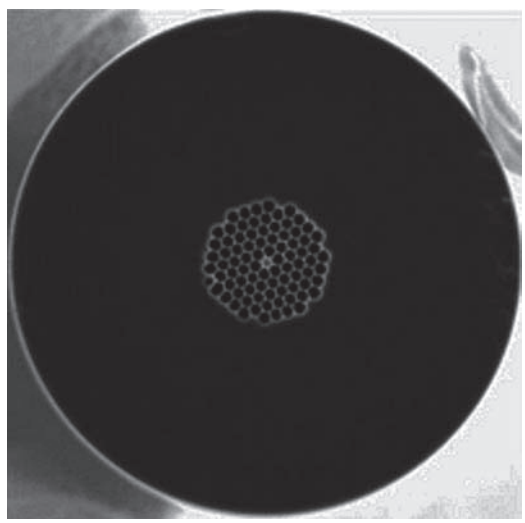


Fig 4: Cross section of a photonic crystal fiber. The diameter of the fiber is approx. 2 µm.

If intense light pulses of a conventional laser are delivered at the fiber-entrance, the originally narrow line is broadened to a wide emission band – a "white" emission. The efficiency in broadening depends on the fiber-pattern and on the mere length of the fiber. For imaging, the emission also needs to be bright enough to create a good and noise-free image in a reasonable time.

All these challenges have been solved[5]. The white laser used in the Leica TCS SP5 X consists of three fiber-based parts. First, a seed laser, generating a pulsed emission at 80 MHz in the infrared; second is a strong pump source, and third, the supercontinuum fiber, that emits the visible light. This arrangement produces a continuous spectrum from 470 nm to 670 nm.

The classical way to feed excitation light into the microscope would use a set of filters for the various spectral parts to select appropriate colors for the fluorochromes applied. A much better way is to employ an Acousto Optical Tunable Filter (AOTF) that is continuously tunable and offers intensity regulation at the same time. In addition, the excitation regime may be reprogrammed in a matter of microseconds. Consequently, the Leica TCS SP5 X laser is an integrated design of a white light emitting laser source with an appropriate acousto optical tunable filter. The control software offers sliders that can set to any wavelength in the emission range and have an additional handle to control the intensity of the line selected. Up to 8 lines may be active simultaneously.

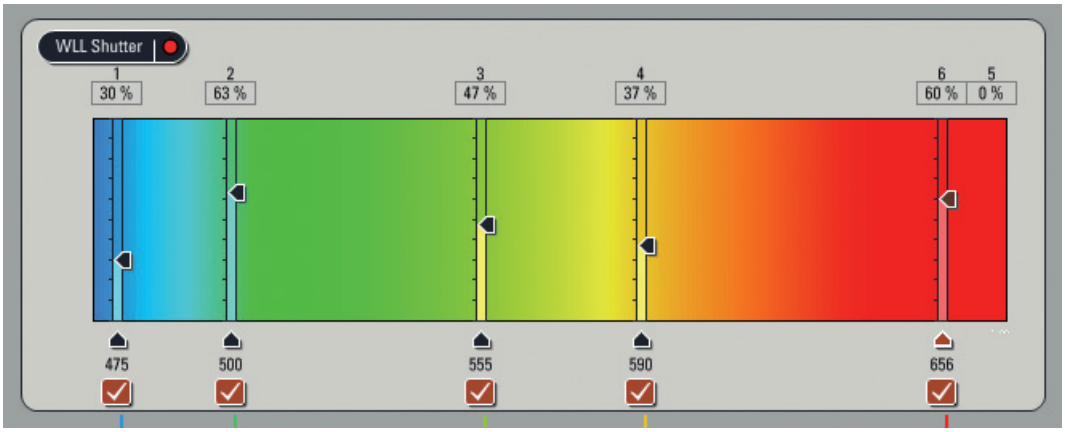


Fig 5: Control window for adjustment of wavelength and intensity for each peak used from the white emission. Up to 8 peaks could be used simultaneously.

How White Laser Fits So Nicely to AOBS and SP

To benefit from the tunability of the Leica WLL, it is also necessary to have the main beam splitting device following spectrally when excitation wavelengths are tuned. The perfect fit for this task is the active Acousto Optical Beam Splitter (AOBS®) that was introduced by Leica in 2002[6]. This device operates as a active programmable photon valve, which injects the dialed excitation light onto the sample and transmits the full emission (except for the nm size gap of the excitation line).

As this device is controlled in the same way as the acousto optical tunable filter, both devices are coupled and work synchronously together without any need for the operator to select appropriate dichroics (and consequently never can select anything wrong). The AOBS offers both full adaptability to a tunable laser source and also represents the most photon-efficient way to separate excitation from emission.

Another device that needs to be tunable in order to make full use of the advantages of a tunable laser and tunable beam-splitter is the detector itself. Here, Leica introduced the SP® detector in 1997. This apparatus consists of a series of spectrophotometer-elements that are coupled by mirror sliders[7]. The emission is spread into a spectrum by a prism, and the mirror-sliders direct any fraction of the spectrum onto photomultipliers. The various emissions of the fluorochromes in the sample can be separated by steplessly tunable bands and up to five channels may be recorded simultaneously.

As the light passes only a single glass-element (the prism) and is directed by highly reflective mirrors (~99% reflectance) this apparatus is the most photon-efficient design to record multiparameter fluorescence in confocal microscopes. The fully tunable SP detector adapts perfectly to the selected excitations. As an additional benefit, the spectrophotometer design inherently offers the measurement of emission-spectra *in situ*.

Benefit No. 1: Tune in to the Excitation Optimum

If comparing excitation spectra and available emission lines of conventional lasers, the gap is obvious: most dyes cannot be excited at the position of their maximum cross section.

A common dye like Alexa 488, although it is designated as a "488 nm dye", has an excitation maximum at 500 nm, whereas the absorption at 488 nm is only 75%.

The white light laser allows users to steplessly tune the excitation, no matter what the name of the fluorochrome is suggesting. So you may excite the fluorochrome at its maximum cross section; for Alexa 546 this would be 561 nm.

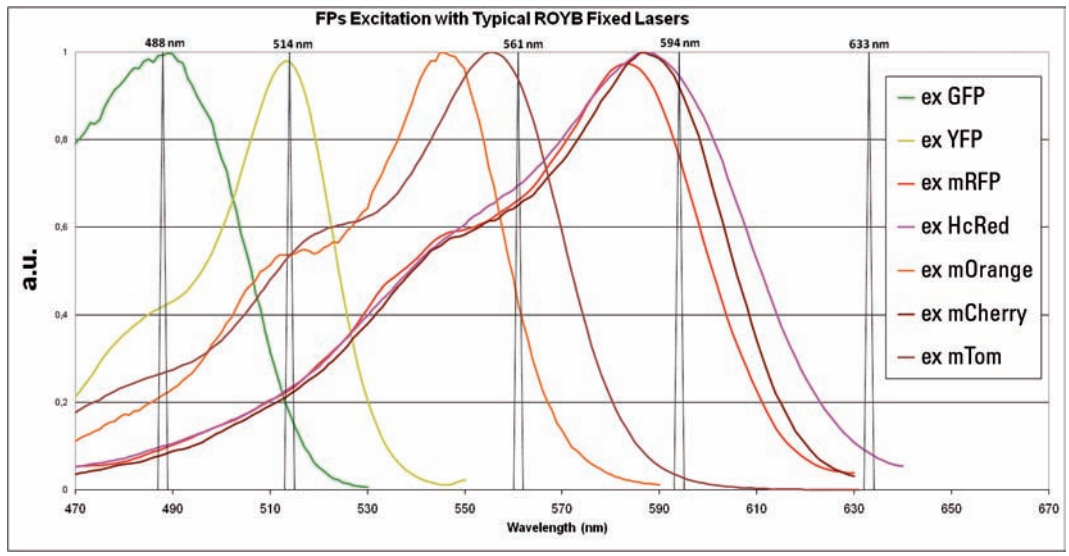


Fig 6: Excitation spectra of various fluorescent proteins and excitation lines of commonly used conventional lasers (ROYB-type). Obviously, most of the shown FPs can be excited optimally with the laser combination indicated. (Courtesy: K. Jalink, Netherlands Cancer Institute, Amsterdam)

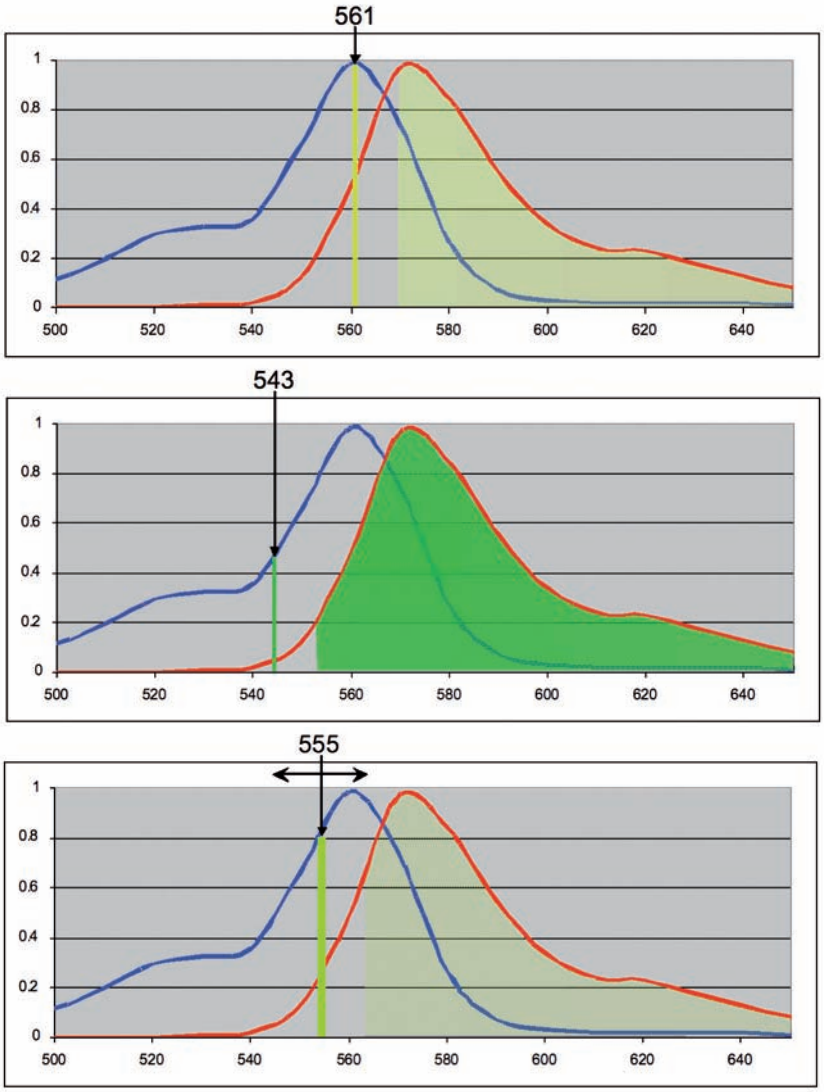


Fig 7: Top: excitation at the peak of absorption (here with 561 nm from a DPSS laser) is restricting the band for emission collection. Therefore, it might be beneficial to use a line that excites on the blue slope. Bottom: for Alexa 546, this could be the HeNe 543 nm line. Unfortunately, this line is very weak and not efficient for a cross section of only 50%.

Fig 8: Tunable excitation and ample power density allows to optimize the excitation wavelength and the collection band for emission.

Nevertheless, this is not necessarily the best position of excitation. In order to avoid excitation light to enter the detector, a certain "security-distance" has to be kept between excitation and the blue edge of the emission band that is collected. If the stokes shift is rather low, then the residual window for emission collection might cut off a significant part of the available photons, which is not desirable. So it sometimes does indeed make sense to excite the dye somewhat off the peak in the blue range (Alexa 488 case) and compensate for the lesser absorption by increasing the laser intensity. Then you can collect the full emission spectrum.

With the Leica tunable laser source you may also find a more efficient excitation for Alexa 546, and get better images at e.g. 555 nm. Just try out and evaluate the best settings during a few scans online!

The combined operation of tunable excitation and tunable emission can help to find the best setting for excitation and emission: a software tool is available, that acquires images at incrementing excitation wavelength (excitation scan) and also adjusts automatically the blue cut-off of the emission band, for example always 10 nm off the excitation to avoid reflected light to enter the detector.

Furthermore, the environment in which the sample is surrounded will sometimes shift excitation. In some cases, specific dyes have been developed to exploit this phenomenon (pH or Ca^{+2} dyes). Here, with the tunable laser you can adapt to these alterations and ensure optimal excitation under any circumstances.

Benefit No. 2: Reduce Crosstalk Easily

A common challenge with multiparameter fluorescence is that excitation even by a narrow line will excite not only the targeted dye, but also other dyes in the sample. In some cases, this might be beneficial, as you can excite more than one dye by the same wavelength. In most cases this is not wanted as the separation of various channels suffers from cross-excitation making some applications, like FRET experiments, difficult to work with.

Of course, there are various protocols to enhance separation, and it is good practice to select dyes that separate well. It is then a good idea to start with the first step: appropriate combination of excitation lines. Here again, the tunable wavelength of the white light laser source is an easy cure for cross-excitation. The longer-wavelength excitation can be moved out of the absorption of the blue dye. Here, an interactive optimization for reduced cross-excitation and efficient emission-collection is easily done by dialing the excitation and adapting the emission-band – online by a few scans only.

If separation is still not optimized, the TCS SP5 also offers sequential scan: that is recording a line with the first excitation and subsequently the same line with the next excitation for the next dye – and so on. Furthermore, the SP-detector as a tunable device can be specified to collect sufficiently narrow bands for minimal crosstalk on the emission side. If the above procedures still do not provide optimal separation, linear unmixing for dye separation – emission or excitation – is also implemented.

FRET Optimized

A commonly used method to obtain information on the distance of two proteins is called Förster Resonance Energy Transfer (FRET). If a fluorochrome, A, has an overlap of its excitation spectrum with the emission spectrum of a fluorochrome, D, then there is a probability, that the absorbed energy from D will pass directly without radiation to A. This allows A to emit a photon according to the emission properties of A. In FRET experiments, D is called the Donor and A is called the Acceptor.

Förster Resonance Energy transfer occurs if the fluorophores distance is in the range of a few nanometers. If one can detect emission of A upon excitation of D, one can conclude that the distance is very close. By applying some calibrations, it is even possible in some cases, to roughly estimate the distance. If the fluorochromes are bound to proteins, one can estimate the spatial distances of the proteins in question.

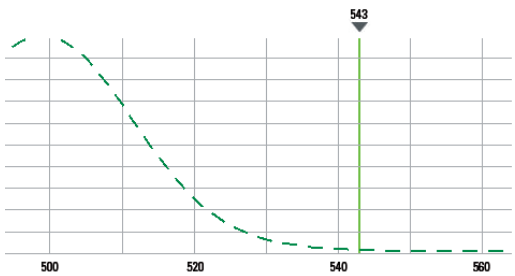
In fixed samples, the method *FRET AB*, that is FRET by Acceptor-Bleaching, is frequently applied. Here, the emission of the donor is measured, followed by bleaching of the acceptor molecules by sufficient high laser irradiation, and then the donor is measured again. If there was FRET before the bleaching, the donor molecule will be brighter in the second measurement, as there is no energy transferred to the acceptor and thus more fluorescence will be emitted.

As an example, a FRET AB experiment with the FRET-pair Alexa 488 and Alexa 568 is described. This is only one out of a long list of possible FRET pairs, but commonly used. The bleaching of the acceptor is performed by the 543 nm HeNe laser line, if there is no better choice available. According to officially published data, the absorption of the donor is sufficiently low (< 2%), to not interfere with the acceptor-bleaching (fig. 9). The experiment was done as mentioned, and as a result, no increase of the donor was detectable,

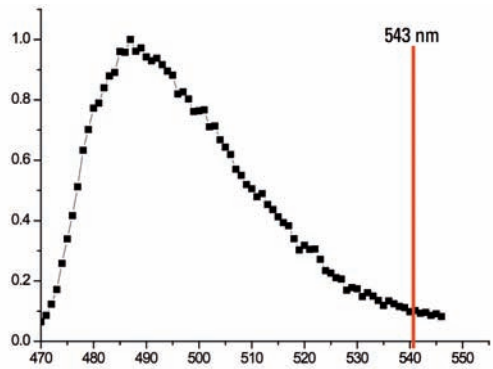
so one would conclude that the proteins are not "colocalized", and at least 10 nm apart. When measuring the excitation spectrum of the donor *in situ* (by white laser), it turned out, that the absorption at 543 nm is indeed much higher, about 11%.

When the same experiment was done with laser light tuned to 580 nm for the acceptor, the donor emission increased significantly by approx. one third – corresponding to 33% FRET-efficiency (fig. 10). 580 nm is sufficiently away from donor excitation, so the donor is not bleached during acceptor-bleaching and will emit more fluorescent light after the FRET-partner is removed. The lack of increase in the previous experiment was due to bleaching of donor by the laser light applied for acceptor-bleaching.

With the white laser source it was possible to find the root cause for the problem by recording an excitation spectrum and to cure the problem by tuning to the appropriate laser wavelength.

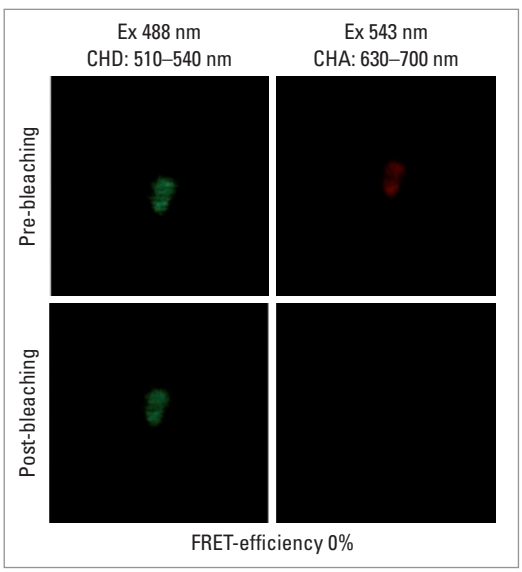


Invitrogen Spectra Viewer: Abs_{543} = 2%



Donor *In-situ* Excitation Spectrum
Excitation at 543 = 10%

Fig 9: Excitation spectra of Alexa 488. Left: data from Invitrogen Spectra Viewer. Right: in-situ measured excitation, data taken with white laser excitation scan. (Courtesy: V. Caorsi, Genoa)



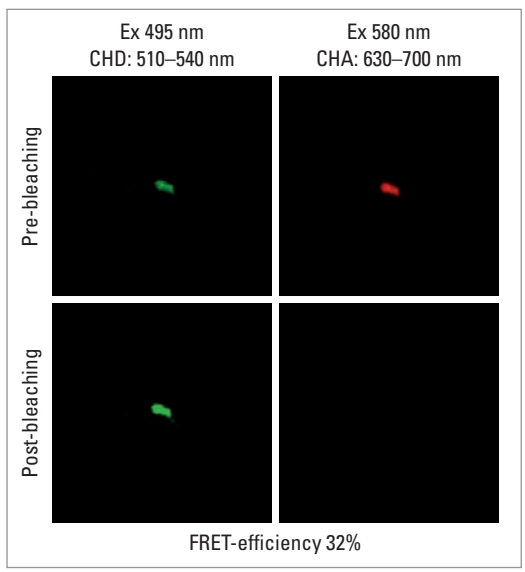


Fig 10: FRET AB measurements. FRET efficiency is measured by increase of donor emission after photobleaching of acceptor. In the left example with conventional lasers, donor emission is not increasing (green images in the left row). The right side shows a strong increase in donor fluorescence using a WLL tuned excitation for acceptor. (Courtesy: V. Caorsi, A. Diaspro, Genoa)

Benefit No. 3: Measure Excitation Properties *In Situ*

The white laser source in combination with an AOTF as fast programmable line-selector provides very straight forward a means to generate excitation spectra from dyes *in situ*. The software allows for automatic incrementing the excitation wavelength while the scanner takes images at each λ-position. There are two options to collect the emission. The standard operation will keep a preset emission band; the result is a direct measure for excitation-dependence of the dye as a function of wavelength. A second operation mode will move the blue edge of the emission band synchronously with the excitation in order to always record the maximally available fluorescence. This is especially valuable for screening new or unknown dyes to find out the best excitation-emission settings.

Evaluation of excitation spectra is easily possible by just drawing regions of interest into the recorded images. The software will provide graphs of fluorescence for all regions. As the measurement is done in the sample directly, the results are much more reliable as compared to published data. You may also measure series of spectra with varying conditions of the solvent to find out about spectral changes, e.g. at various pH-levels.

Fluorescent Proteins Excitation Spectra *In Situ*

A set of new fluorescent proteins was examined by excitation scans. The data may be used to optimize excitation and reduce crosstalk when recording images with those FPs.

This information is also needed when planning FRET pairs. And in general, changes in fluorescence parameters in living cells and under controlled conditions can be studied.

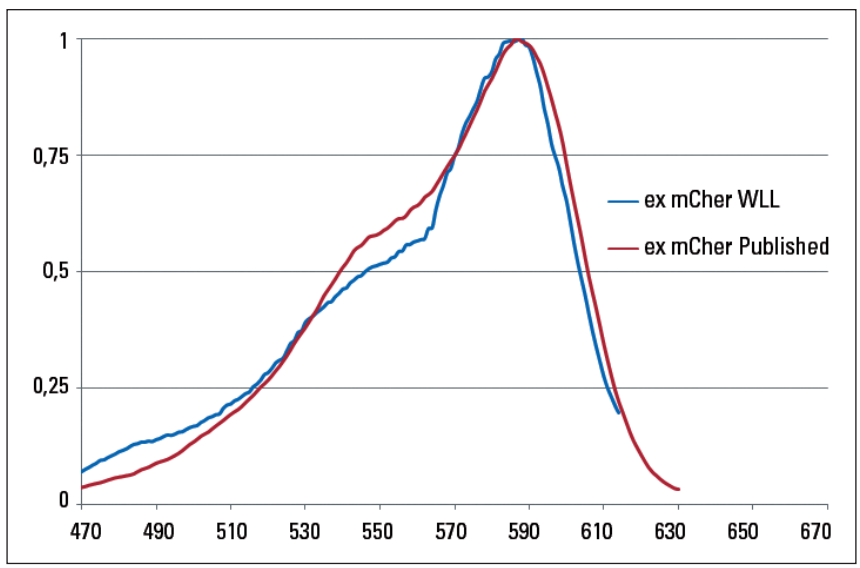


Fig 11: Comparison of published data for excitation of mCherry and excitation scan with WLL. (Courtesy: K. Jalink, Netherlands Cancer Institute, Amsterdam)

λ^2-Maps: More Parameters for More Information

In systems with many fluorochromes – either artificially multilabelled samples or in plant material with complex mixtures of chromophores, the identification of the various molecular species by just the emission spectra is sometimes a problem. In those cases, a second parameter is very beneficial for enhanced separation. The white light laser as a source for excitation scans in combination with the SP-detector for emission scans provides this feature: by recording emission scans at a sequence of excitation wavelength, the single-dimensional information of an emission spectrum can be spread out in two dimensions as excitation-emission map. Here, dyes that are indistinguishable by the emissions might be separable by their excitation parameters. Thus, the Leica TCS SP5 X offers new tools for new research and new insights.

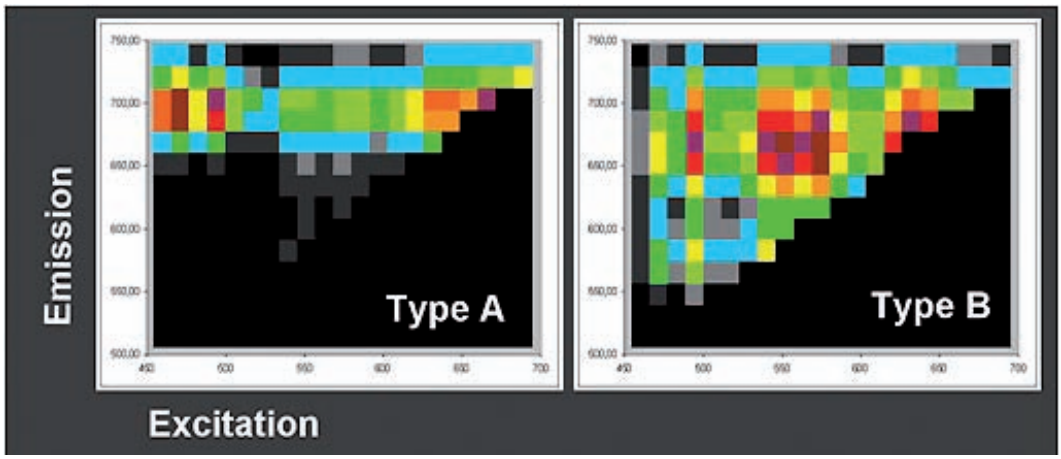


Fig 12: Excitation – Emission maps of fluorescence from biofilms. Two different species of Cyanobacteria are presented and show variant patterns of fluorescence[8].

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Cover image:
Autofluorescent image of chitin from *Coccus sp.* Color-coded z-series projection acquired on a Leica TCS SP5 X confocal system. Excitation 670 nm and detection range from 685-840 nm. (Courtesy: K. Jalink, Netherlands Cancer Institute, Amsterdam)

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