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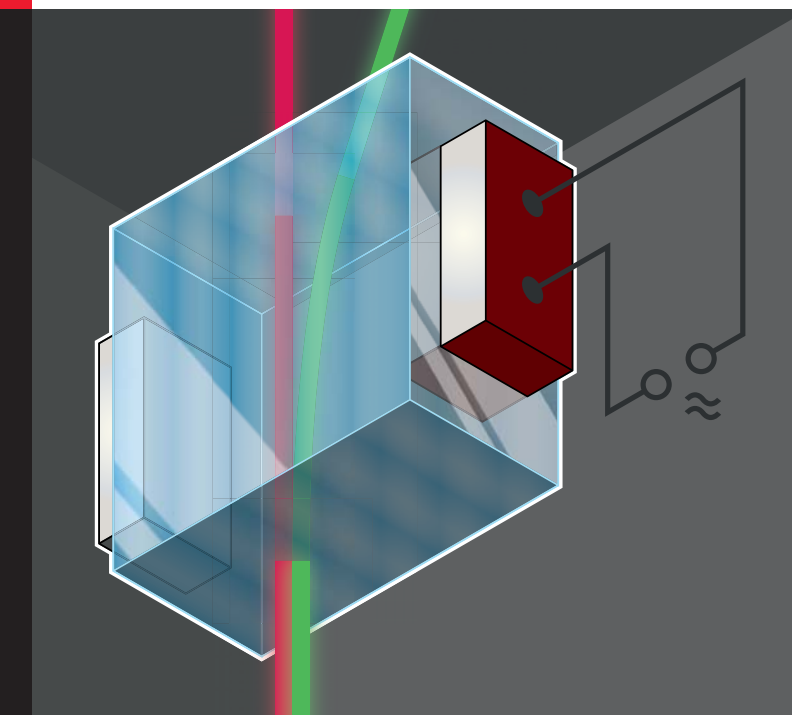
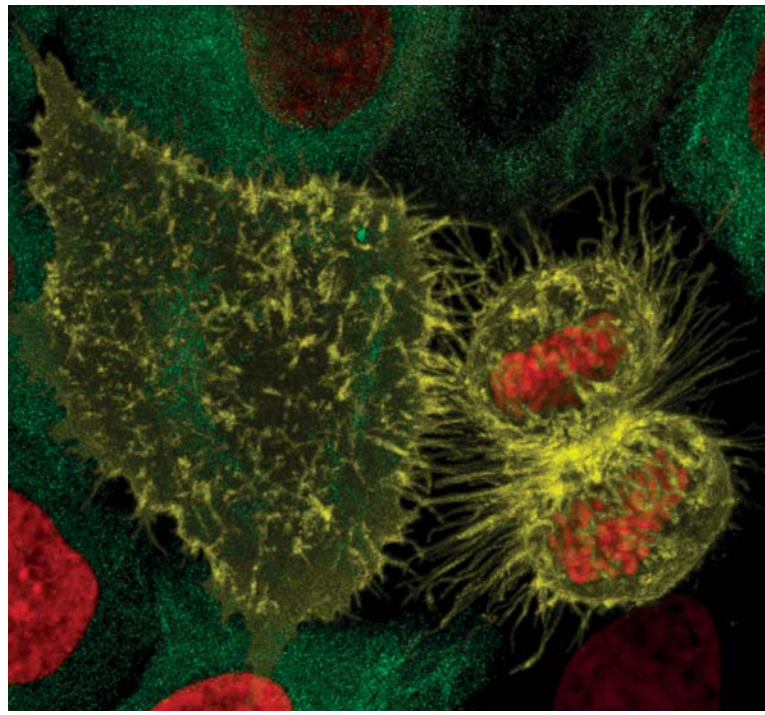
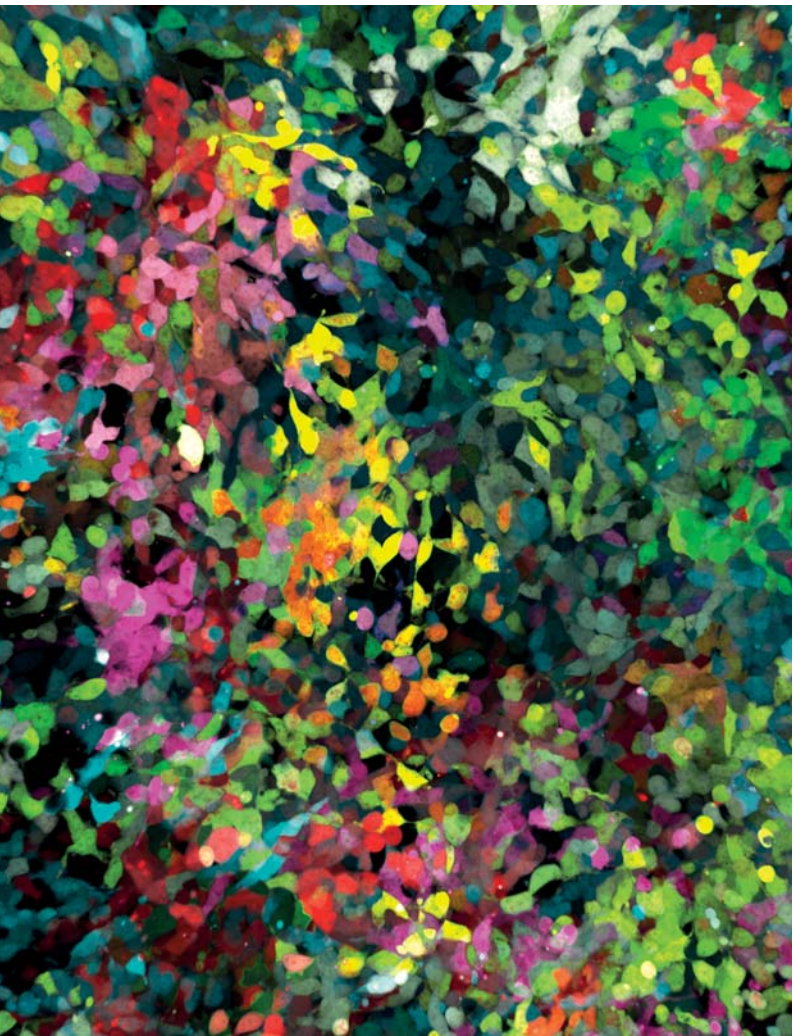






# AOBS® – The Most Versatile Beam Splitter

Acousto-Optical Beam Splitter for the TCS SP8 Confocal

- High transparency and narrow excitation bands increase cell viability and reduce photobleaching.
- The freely programmable AOBS perfectly adapts to challenging multicolor experiments, new laser lines, and the tunable White Light Laser of the TCS SP8 X.
- Provides fast line sequential imaging of living cells having spectrally close fluorophores.

# Discover the AOBS® – Changing Confocal Imaging

Fluorescence imaging is performed in an incident light configuration: the excitation light enters the specimen on the same side from which the emission is collected. This setup requires a device that separates excitation and emission light: a beam splitter. The AOBS is a uniquely flexible, efficient, and fast beam splitting device.

## FLEXIBILITY AND VERSATILITY SIMPLIFY MULTICOLOR IMAGING

The position of the band for excitation is tunable. Consequently, a single AOBS is programmable for any visible laser color – even for lines that are installed during laser upgrades or modifications of the laser configuration.

While the use of dichroic mirrors for more than three simultaneous excitations is complicated and inefficient, the AOBS easily operates up to eight tunable colors by design, even with narrow distances between excitation lines.

## GAIN MORE LIGHT WITH MAXIMUM SENSITIVITY

The reflection bands for excitation light through the AOBS are very narrow, leaving broad bands for the collection of the precious fluorescence photons. The result: maximum detection sensitivity. The efficient separation between excitation and emission is shown in the transmission curves (Fig. 1).

The fraction of injection is tunable. This allows the AOBS to be programmed to a 50/50 device, which is optimal for reflected light imaging. Reflected and flourescence imaging can be performed simultaneously, with the light from each mode detected by distinct channels.

## FAST, RELIABLE SWITCHING

Reprogramming the AOBS is a matter of a few microseconds. Even complicated illumination routines can be switched between scan lines without delay or pixel shift.

Also, complex region-of-interest scans can be defined. The AOBS minimizes crosstalk at high acquisition speeds by switching excitation lines in line-sequential multichannel scans.

## Transmission Curves for Different Beam Splitting Devices

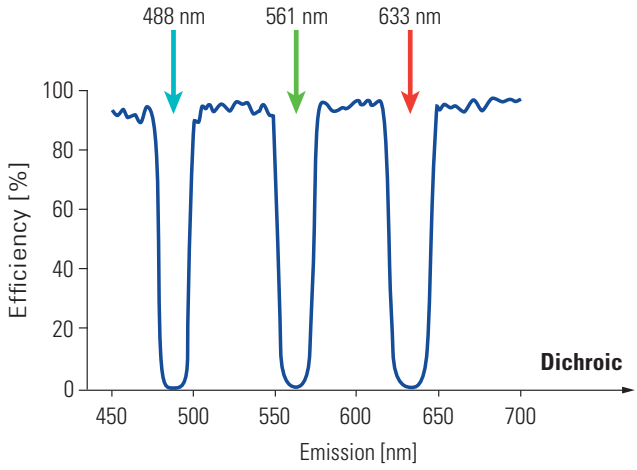



Fig. 1a: Dichroic. Non-flexible reflections on bands.

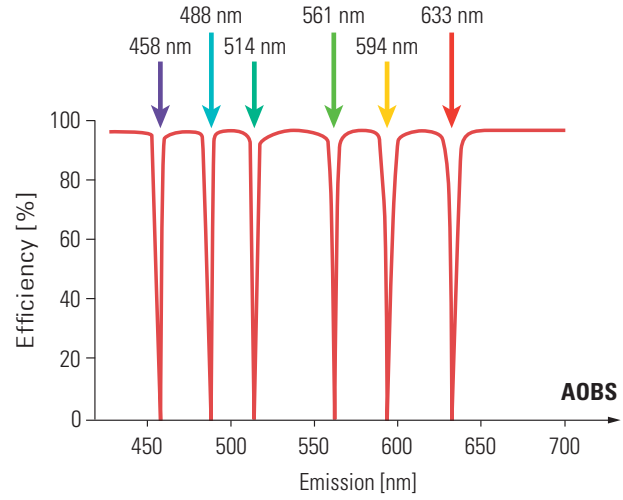



Fig. 1b: AOBS. All wavelength are fully flexible, adjusting to your experiment.

Comparing the transmission curves of a typical triple dichroic mirror (blue) and AOBS (red) for excitation with 488, 561, and 633 nm shows two major advantages of the AOBS:

- The AOBS reflection bands have steep edges and narrow bandwidth – allowing the collection of more emission light.
- The AOBS is more flexible and can accomodate up to eight reflection bands – facilitating the simultaneous imaging of multiple fluorophores.

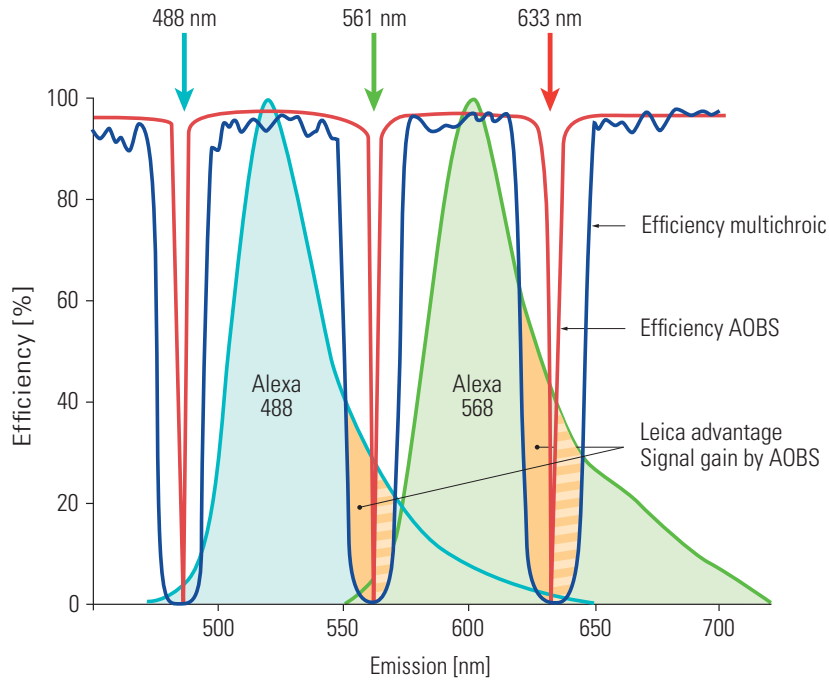



Fig. 1c: Overlay of emisson curves of Alexa 488 and Alexa 568 with transmission sof dichroic and AOBS.

Now we add the emission curves of Alexa 488 and Alexa 568 to an overlay of the transmission curves of a dichroic mirror and AOBS tuned to 488, 561, and 633 nm. The advantage of the AOBS becomes clear. Notice the increased detection efficiency for simultaneous excitation of Alexa 488 and Alexa 568 (shown in solid orange).

For sequential excitation with the two laser lines the spectral detection window can be increased so that the advantage of the AOBS becomes even larger (hatched orange area).

# A Comparison of Beam Splitting Methods

Classically, beam splitting is done by dichroic mirrors that reflect certain color bands and transmit between them. The reflecting part is used for excitation; the emission is collected through the transmitting band. An AOBS also separates excitation and emission light, but works in a completely different way as compared to a dichroic mirror. It is an active, programmable device that is uniquely flexible, efficient, and fast.

## SPLITTING MIRRORS HAVE FIXED SPECIFICATIONS

For different applications, the dichroic mirror needs to be mechanically exchanged to fit spectral requirements.

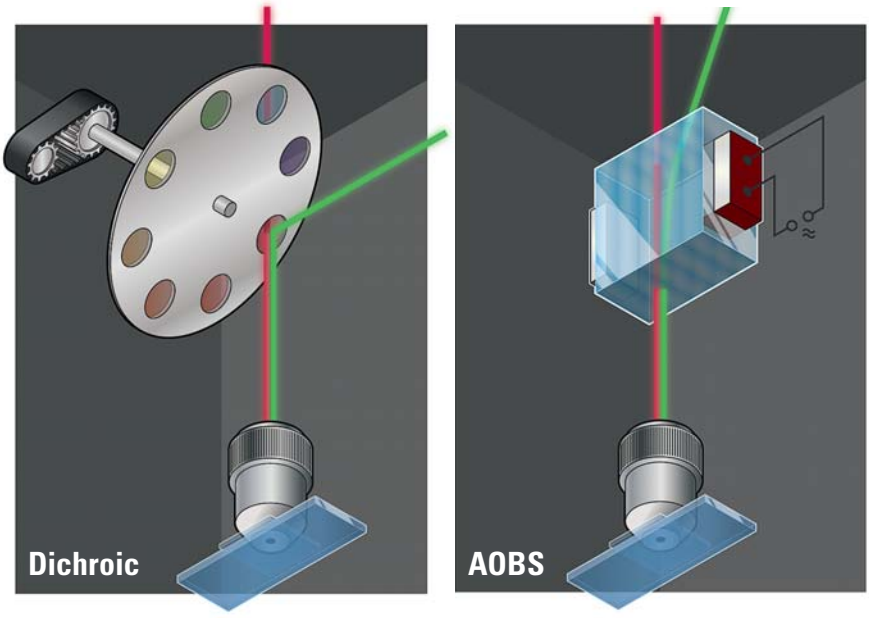
Changing mechanical parts takes time, is prone to misalignment (pixel shift), and requires a large set of mirror types in the repository.

## AOBS: A SINGLE, FLEXIBLE, AND RELIABLE OPTICAL ELEMENT

Because of its fast electronic programming, the AOBS does not rely on the previous selection of spectral specifications.

It is suitable for any set of laser lines and combinations of those in the visible range, and features maximum transmission between the excitation intervals.

**Acousto-Optical Beam Splitter**
This device replaces all conceivable dichroic and multichroic mirrors and wheel- or slider-based arrangements or combinations of these. The AOBS is a single programmable optical element for visible range laser scanning microscopy.



# Acousto-Optical Beam Splitting

## Technology in Detail

Beam splitting by the AOBS is based on acousto-optical diffraction in a $TeO_2$ crystal. These crystals are fully transparent from below 400 nm to more than 4 µm.

Applying a mechanical wave at radio-frequency results in periodic density variations within the crystal. The crystal density affects the refractive index. Thus, the acoustic wave creates a refractive index grid.

The grid constant is variable and depends on the frequency and amplitude of the applied wave, which makes it easily tunable.

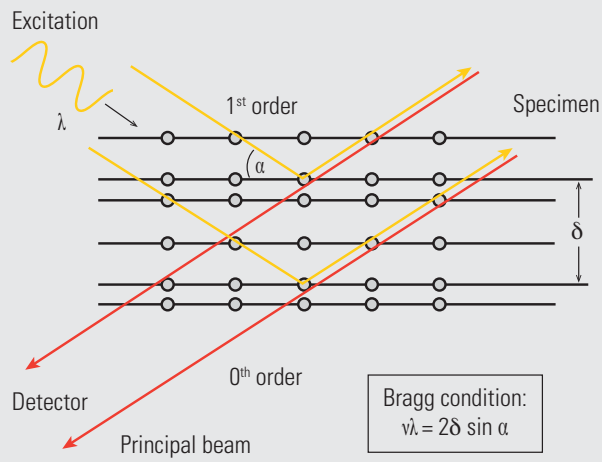
If excitation light of the desired color enters the crystal at an appropriate angle, the light will be acousto-optically refracted and will merge with the principal beam, which coincides with the optical axis of the microscope.

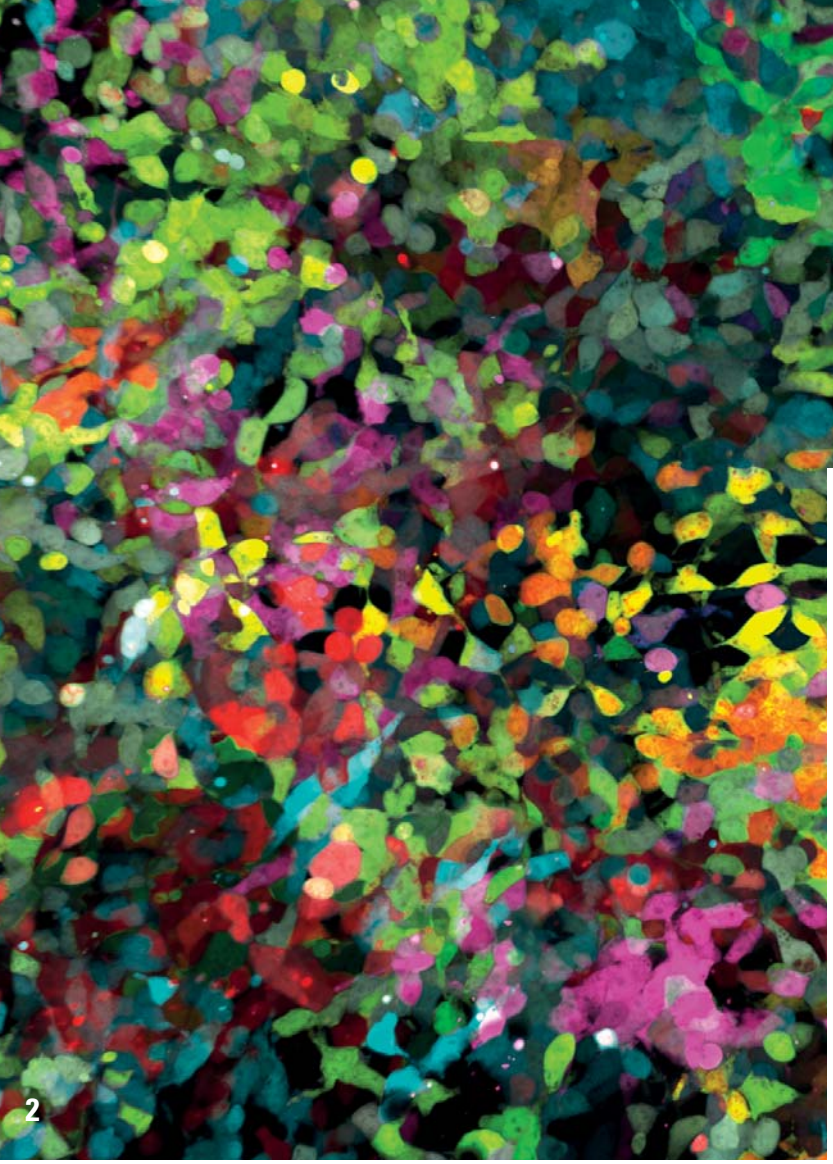
**Understanding Physics: The Principle of Acousto-Optics**

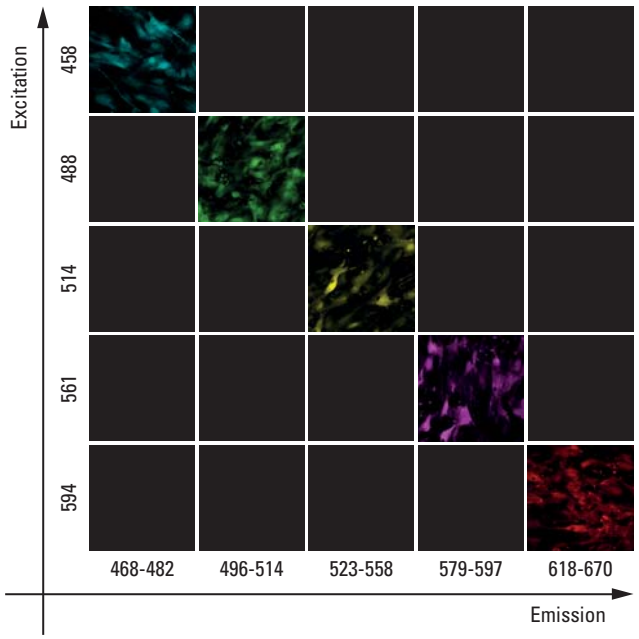
The incident angle, wavelength, and grid constant must fulfill the Bragg-condition. As the grid constant is tunable, any color can be merged with the optical axis.

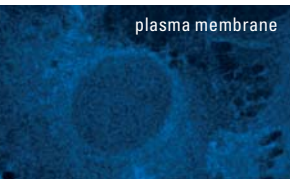
Furthermore, the refractive index grid of the AOBS can accommodate several wavelengths simultaneously for multicolor excitation. The design of the AOBS ensures that the first order injection of all visible wavelengths is coaxial.

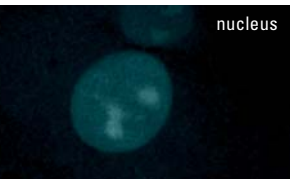
The emitted light is stokes-shifted, i.e. of different color, and so passes the crystal without being affected by the refractive index grid. The emission is then fed into the multichannel spectral detector of the TCS SP8.

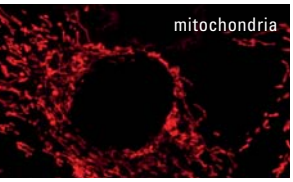


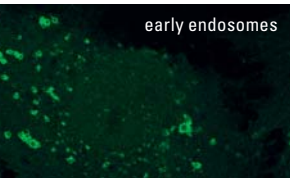


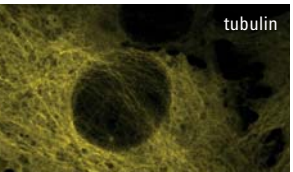

Excitation
458
488
514
561
594
468-482
496-514
523-558
579-597
618-670
Emission

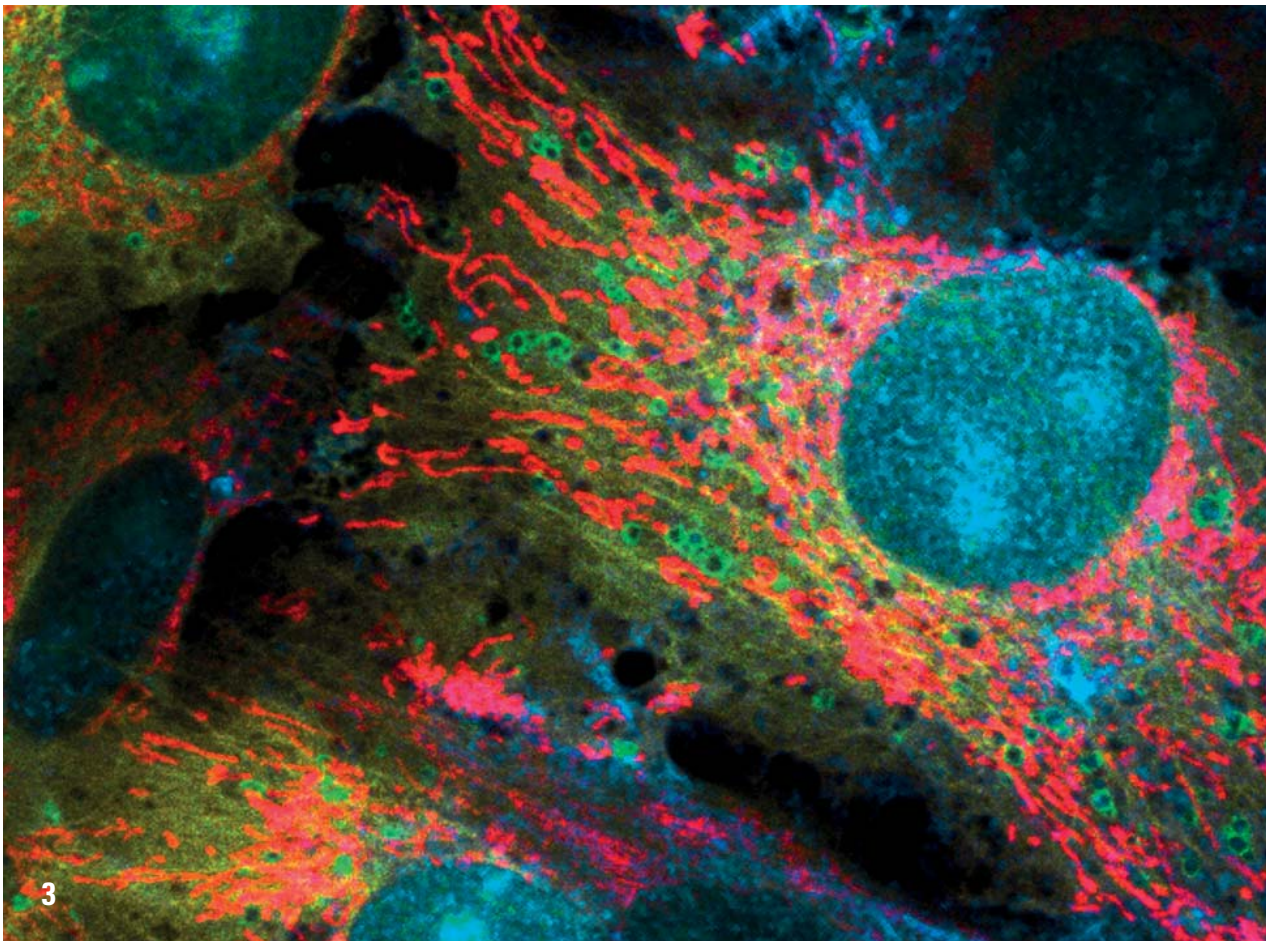

plasma membrane


nucleus


mitochondria


early endosomes


tubulin



# A Versatile Beam Splitter for all Confocal Imaging Applications

The AOBS is ideal for both single parameter fluorescence and sophisticated multichannel imaging – without changing dichroic mirrors. Upon selecting a set of colors for excitation, the AOBS will automatically be programmed to direct these lines onto the specimen and transmit the emission between them. No need to think about which mirror to use. The TCS SP8 with AOBS is perfect for core imaging facilities, where so many different fluorochromes need to be imaged each day. It largely reduces the training time needed for novice confocal users.

The spectral detector of the TCS SP8 comprises an array of up to five sensors that collect emissions of truly tunable bands with individual gain and offset. In combination with the AOBS, it allows simultaneous or fast line sequential imaging of many channels without the need to move optical parts or remove crosstalk after recording.

The AOBS is the ideal solution for advanced multichannel confocal microscopy, e.g. with many different fluorescent proteins, chemical dyes or combinations of those. The steep band-edge allows wider emission bands as compared with plan-optical mirrors and filters, therefore the AOBS offers maximum transmission efficiency. Excitation power can be reduced to decrease photobleaching and increase specimen viability.

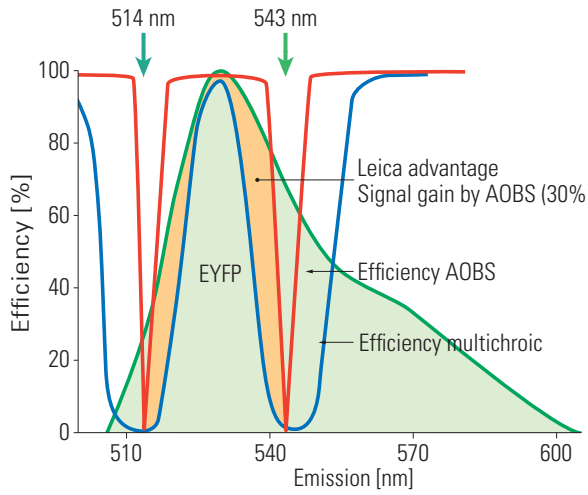
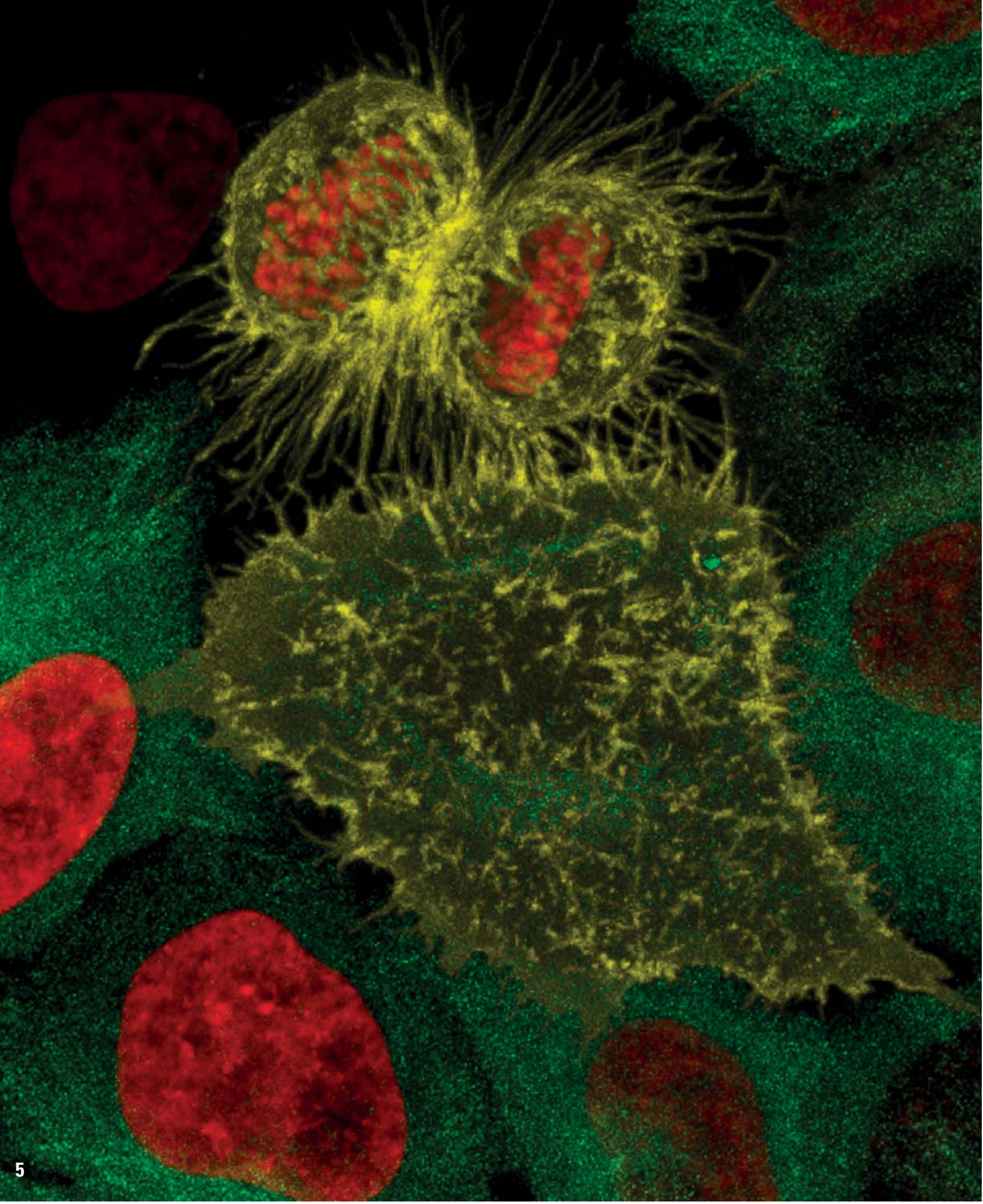



**Fig 4**: Gain of efficiency: double excitation at 514 and 543 nm.
Green: Emission of EYFP, Red: transmission of AOBS (2 nm width), blue: typical transmission of dichroic (20 nm width).
Orange area: 30 % efficiency increase by AOBS.

**Fig. 2**: NIH3T3 cells transduced with five individual fluorescent protein (FP) vectors.
FPs: Cerulean, EGFP, Venus, tdTomato and mCherry. Each FP was visible only in the cells transduced with the corresponding vector. AOBS fast line sequential scan with five excitations and five emission bands. No unmixing.
Image courtesy of Daniela Malide, NIH Bethesda, MD USA

**Fig. 3**: PAE cell line stably expressing five fluorescent subcellular markers (FPs) with ex and em: EBFP2-Nuc (nucleus) 405/430-450, mTFP1-FYVE (early endosomes) 458/485-510, mCitrine-Tub (tubulin) 514/525-545, mCherry (mitochondria) 543/585-620, mPlum-PLC-PH (plasma membrane) 633/640-800.
Image courtesy of Philipp Berger, Paul Scherrer Inst. Villingen, CH



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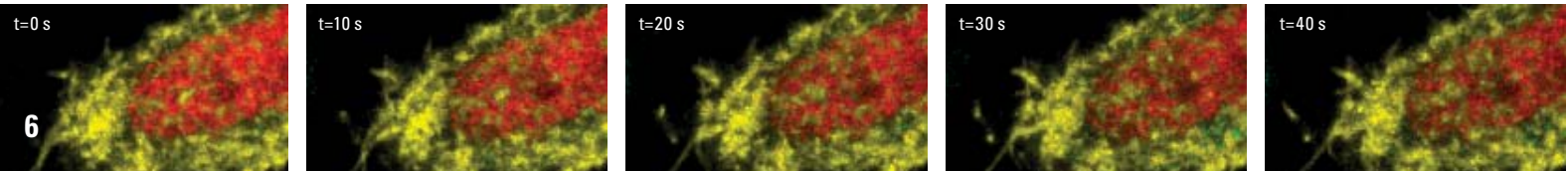


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# Live Cell Imaging with Spectrally Close Fluorophores

With dichroic beam splitters, fluorophores with a large spectral separation between excitation peaks must be selected for multiple staining of biological specimens. Otherwise, there is insufficient space for emission collection. Additionally, multichroic mirrors do not allow for closely adjacent excitation lines. This limitation is removed with the AOBS.

## CHOOSE SIMULTANEOUS OR SEQUENTIAL MODE

The AOBS offers excitation with laser lines that are spectrally close and in various imaging modes.

› In simultaneous mode, with simultaneous emission collection of all channels.
› In simultaneous mode, with mathematical separation by unmixing.
› In sequential mode, switching the excitation fast between lines or in a more relaxed way between frames.

## FAST SWITCHING FOR LIVE CELL IMAGING

The AOBS offers sufficiently short switching times (< 10 µs) even for living specimens, whereas sequential scanning only makes sense in line mode; otherwise the spatial correlation is lost.

› Fast switching of the illumination routine for line sequential scanning in living specimens.

## FAST SPECTRAL SEPARATION OF GFP AND YFP

The efficiency of the AOBS plus the free tunability of the detection bands in the SP detector open the door to imaging dye combinations that were previously not separable without post processing.

GFP and YFP, for example, require sequential scanning with different excitation lines that are comparably close together. No existing dichroic mirror can accomplish this task, the AOBS is the only solution.

With the AOBS one can separately excite with 488 nm and 515 nm, and the illumination can be changed between lines.

› The AOBS allows spectral separation of two close excitation lines.

Fig. 5: Separation of GFP and YFP in living cells. HeLa cells expressing three different fluorescent proteins: GFP-tubulin (green) Ex 476 nm, Em 485-509 nm, YFP-GPI-filipodia (yellow) Ex 514 nm, Em 517-556 nm, mCherry-H2B-nucleus (red) Ex 561 nm, Em 571-671 nm. Three channels, simultaneously recorded.

Fig. 6: 4D live cell imaging with GFP, YFP, and mCherry. Per time point, a stack of images is recorded and visualized as a maximum projection. Movement of the filipodia becomes visible in the YFP channel, here shown in yellow. GFP is simultaneously recorded and mCherry (nucleus) as reference. Sample courtesy of J. Bulkescher, EMBL Heidelberg, Germany. Simultaneous recording. No unmixing.

# Detect Fluorescence and Reflection Simultaneously

The AOBS can easily convert to a 50/50 splitter for a desired wavelength for optimal reflected light contrast. Simultaneously, other laser lines can still be used for fluorescence excitation and corresponding emission.

## FREELY COMBINE FLUORESCENCE WITH REFLECTION

The AOBS is not just a programmable chromatic splitter. It can be used as a 50/50 beam splitter as well. You can choose which wavelength you want to use for the reflected imaging mode.

In reflection mode, the AOBS will perform as a 50/50 splitting device (this ratio is the most efficient for reflected light imaging). Simultaneously, up to seven additional lines can be used for fluorescence excitation.

## FAST SWITCHING BETWEEN REFLECTION AND FLUORESCENCE

Any excitation color can be employed for reflected light. And you may even wish to collect different colors in reflection mode, for example in a sequential scan, where fluorescence and reflection are collected from the very same illumination color.

## STUDY OF CANCER CELL INVASION BY REFLECTION AND FLUORESCENCE*

An interesting application example for combining reflection and fluorescence imaging is the study of cell movement through tissue. It is accompanied by lysis and removal of collagen fibers – visualized by reflection imaging – to make way for the invading cells.

* Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion, Wolf K, Wu YI, Liu Y, Geiger J, Tam E, Overall C, Stack MS, Friedl P, Nature Cell Biology 2007.

**Structures that give good contrast in reflected light without staining:**

- collagen fibers and similar structures in connective tissue
- lipid conglomerations
- cytoskeleton components

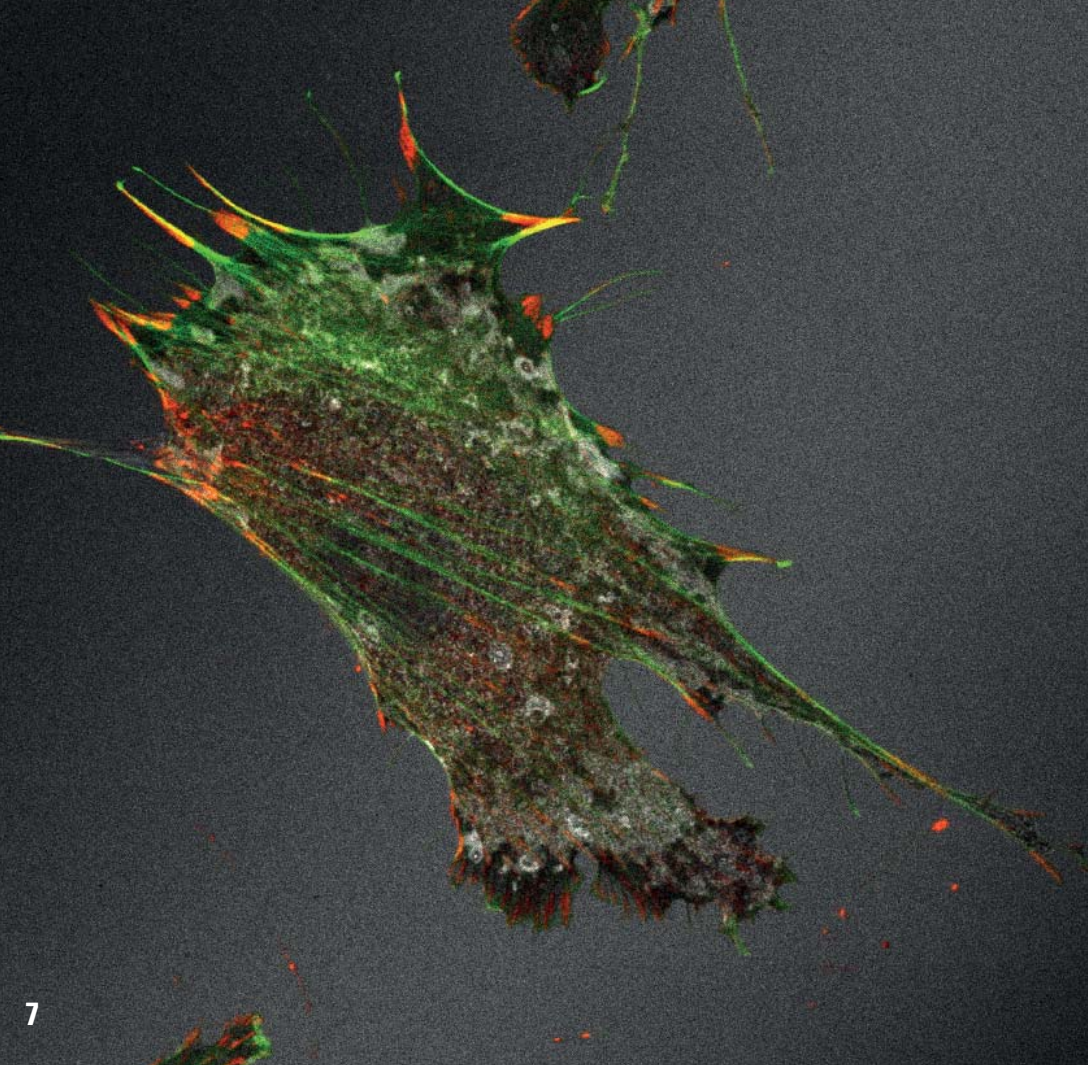



Fig. 7: NIH3T3 fibroblasts. Actin Alexa 488, Ex 488 nm, Em 500-550 nm (green). Reflection of focal contacts, III 476 nm Det 467-492 nm (gray). Paxilin Cy3, Ex 561 nm, Em 570-627 nm (red). Image courtesy of M. Bastmeyer, KIT Karlsruhe, Germany.

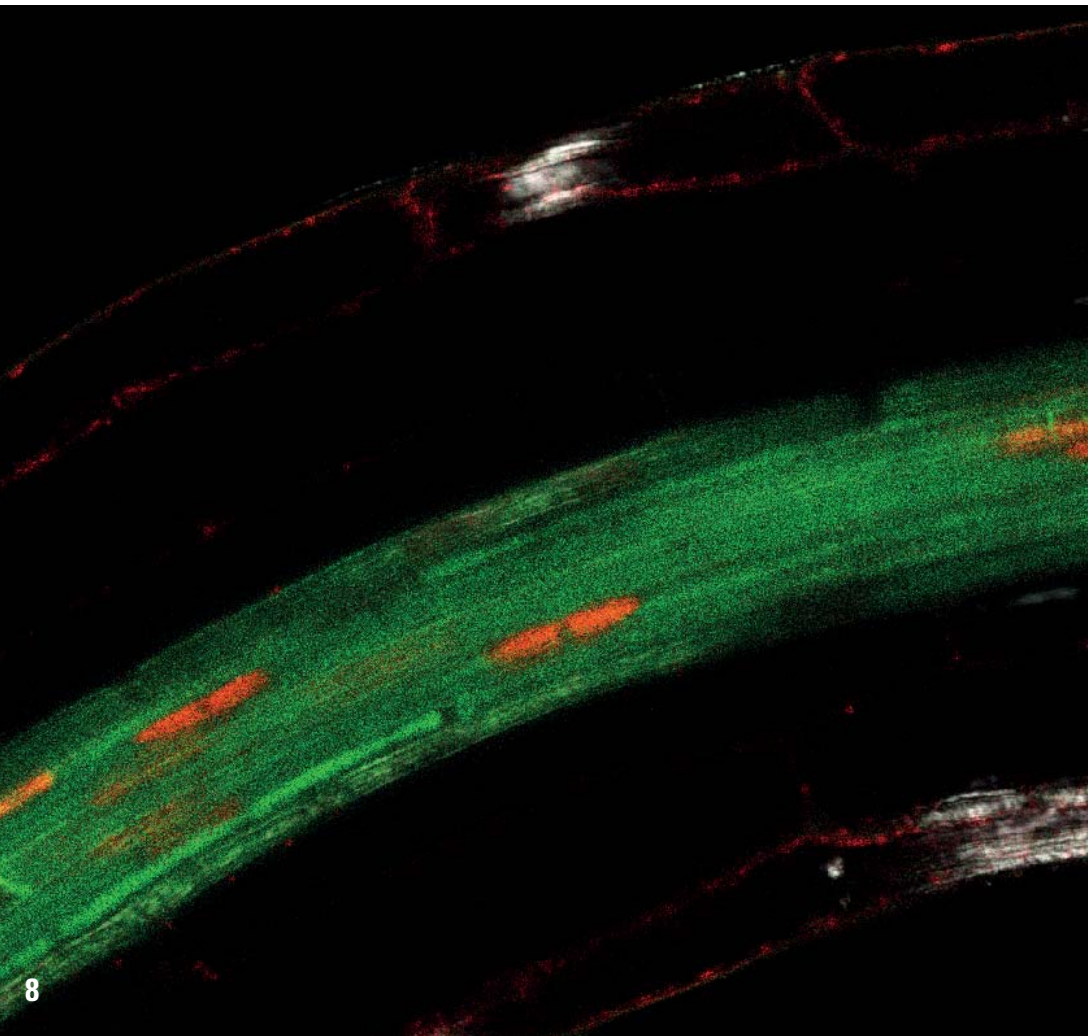



Fig. 8: Arabidopsis thaliana, root tissue. PINI-GFP (membrane, green), REV-2xYFP (nucleus, red), reflection (gray).
Image courtesy of M. Heisler, EMBL Heidelberg, Germany.


**Background Information:**
**White Light**

With supercontinuum fiber lasers, a light source is available that covers a wide spectral range and fulfills the requirements for confocal microscopy. A truly white source emits photons in a spectral range, e.g. visible light, without gaps.

Light composed of three laser lines, e.g. red, yellow, and blue, is perceived by the human eye as "white" as well, but is far from covering a spectral range without gaps. Such devices should not be called "white sources".

# A Freely Tunable Beam Splitter for the White Light Laser

The White Light Laser (WLL) used in the TCS SP8 X combines a white light source that covers the spectral range from 470 nm to 670 nm and an acousto-optical tunable device for the selection of single lines. Up to eight lines can be selected from the spectrum, each line being freely tunable in both color and intensity.

## TUNABLE EXCITATION REQUIRES FREELY TUNABLE BEAM SPLITTING

The WLL provides any color combination within the visible range. Illumination routines are switched within a few microseconds.

The WLL is tunable in 1 nm steps, resulting in 200 different colors, plus the permutations that you can create with one to eight colors simultaneously. The number of required mirror designs would dramatically exceed all limits.

The AOBS, which is also freely tunable to up to eight illumination lines, perfectly matches the beam splitter requirements for the White Light Laser.

## FAST AND EASY AUTOMATIC SETUP OF THE BEAM SPLITTER

The above-mentioned acousto-optical color selector (which in essence is an AOTF) is easily co-controlled with the AOBS, the sister component in such a setup. All you need to do is select the colors for excitation from a continuous spectrum.

The setup is automatically recognized by the AOBS control, setting the appropriate beam splitting characteristics. If any selected colors from the source are changed, the AOBS instantly follows.

## TRUE SPECTRAL DETECTION WITH THE TCS SP8

The continuously tunable illumination and splitting is efficiently complemented by the continuously tunable detection of Leica's SP detector.

It can collect the transmission bands between the excitation by the tunable spectral photometer bands – at utmost transmission efficiency and in five true dye emission channels simultaneously.

To separate the channel information, mathematical cleaning is just an option you can add on demand.

Fig. 9: Primary culture of rat cortical neurons imaged with White Light Laser. Excitation and detection settings were adjusted to optimal image contrast.

Application Example:

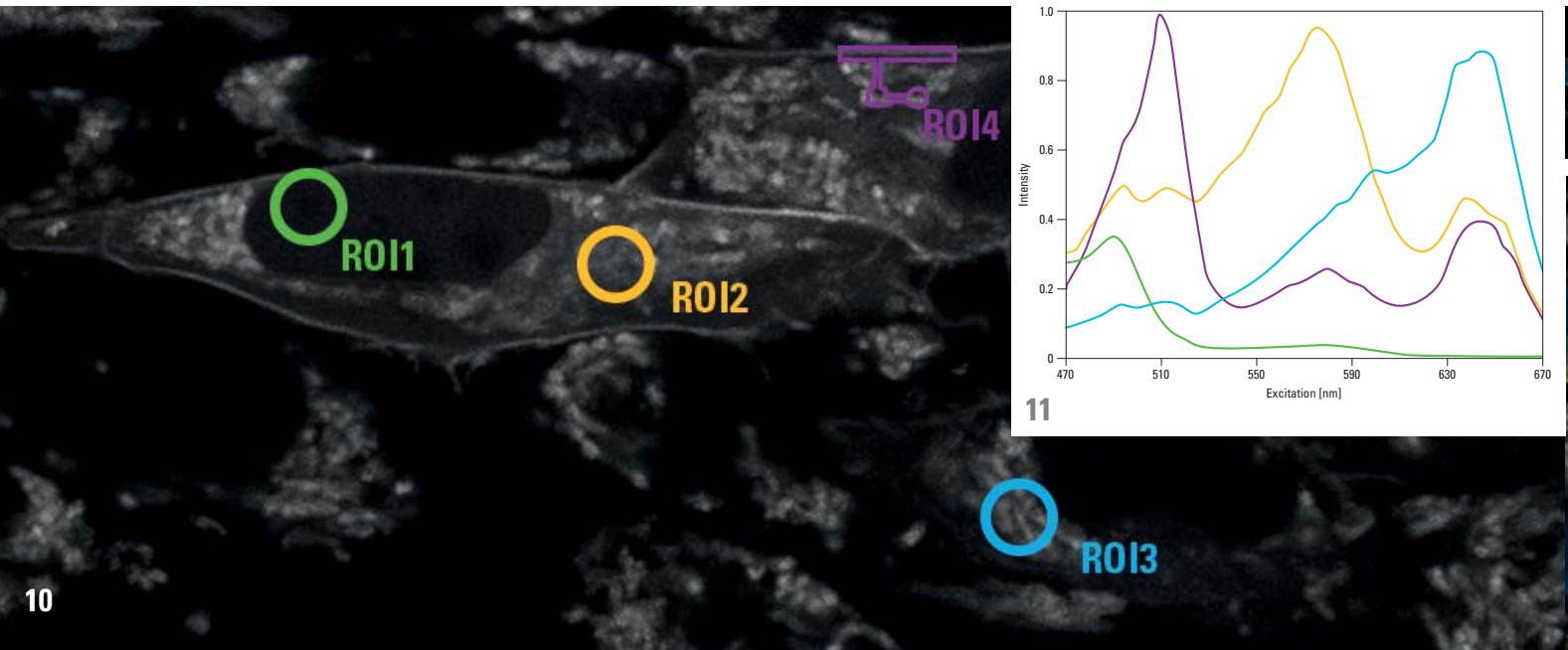
# White Light Laser combined with AOBS

The following example covers most of the applications and benefits achieved by combining WLL and AOBS. Any of the steps might be applied individually; the sequence shown here explains the multiple operation possibilities.

**1.** To identify the best excitation colors for the four visible stains (DAPI is excited with a separate 405 nm laser), an excitation spectrum is recorded. Here, the emission of the WLL is automatically incremented, by steps (adjustable) with 1 nm accuracy. The AOBS will automatically follow this incrementation. The detector collects all fluorescence emitted on the red side of the excitation by automatically incrementing the blue edge with the illumination color.

**2.** The result is a sequence of images that shows the maximum fluorescence detectable as a function of excitation wavelength, i.e. each pixel contains an excitation spectrum. From that data, the excitation spectra of the regions of interest (ROI) are extracted, which are associated with the different fluorochromes (Fig. 10), either in selected frames from the series, or from a maximum projection. The spectra from these regions are displayed graphically (Fig. 11).

**3.** These *in situ* excitation spectra show the actual excitation properties of the dyes in the specimen. Most dyes show shifts in their spectral behavior, depending on the molecular micro-environment. Thus, the spectra allow the optimal excitation color to be found for the fluorochromes. In combination with the emission spectra, the excitation is also optimized in order to minimize crosstalk from the very beginning. The TCS SP8 X's software also offers an automatic excitation-emission scan: “lambda square imaging”.

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4. When the system is set up to record images with the optimal excitation colors, the AOBS is automatically programmed to inject the desired colors into the specimen and transmit the emission bands between them. The positions of excitation can still be manually fine-tuned, if desired, and the AOBS will always automatically set the correct spectral bands.

5. Then, the SP detector is set to select only the emission bands. Here again, it is possible to control the red and blue edge of each band online in order to optimize signal separation while observing the resulting image on the screen. All operations are instant, i.e. without delays for mechanical or other system adjustments. The outcome is immediately revealed on the display.

6. Once the optimal imaging parameters are worked out, they can be stored and retrieved for later use on the same or similar specimens. Personal imaging parameters are collected in a library, together with the factory-supplied library.

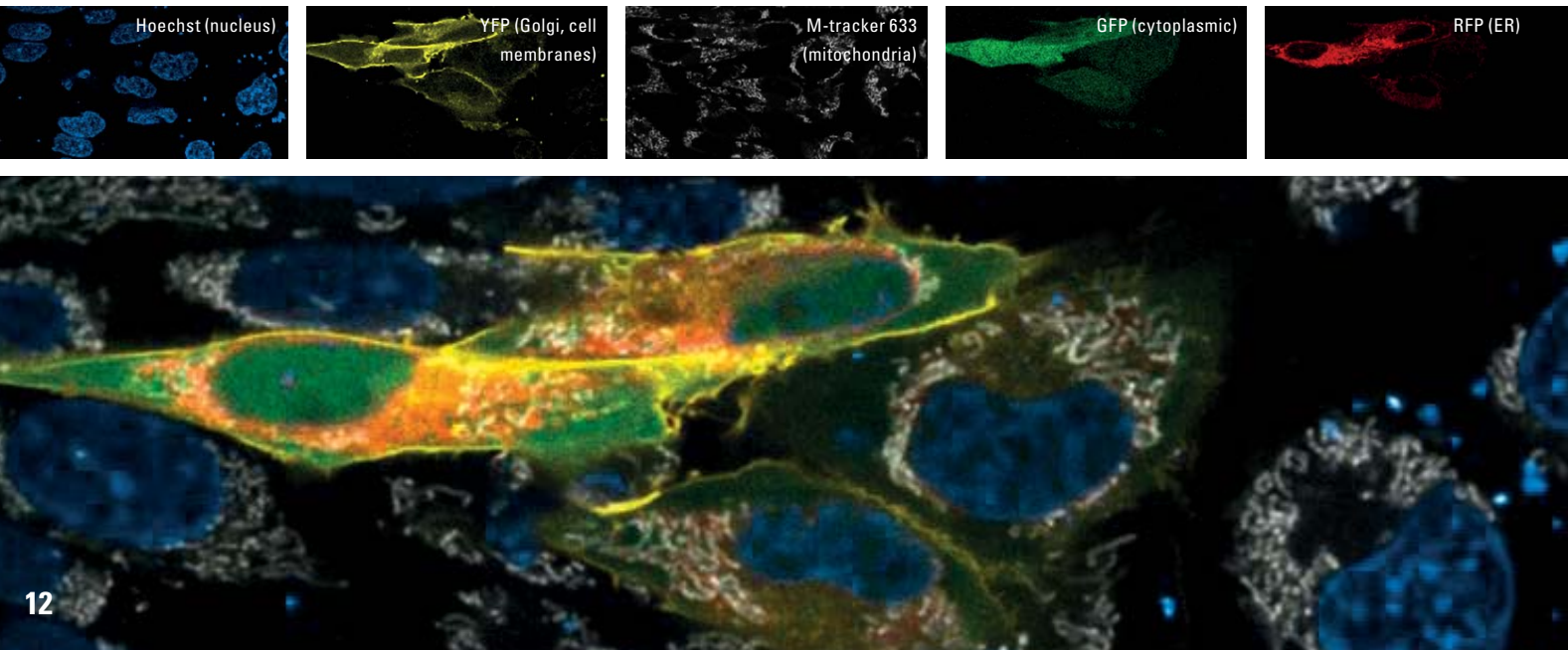
A practical implementation of a white light source, i.e. a freely tunable excitation color, is only possible with a freely tunable beam splitting device, the AOBS.

Fig. 10: Definition of regions of interest for analysis of an excitation spectrum.

Fig. 11 (inset): Excitation spectra in the regions of interest, as indicated in the image above.

Fig. 12: Five-channel confocal imaging with *insitu* optimized excitation and emission. The five signals are shown individually in the upper row. Overlay of all five channels below.

Quintuply stained CHO cells. Sample courtesy of J. Lippincott-Schwartz, NIH, Bethesda, MD (USA).



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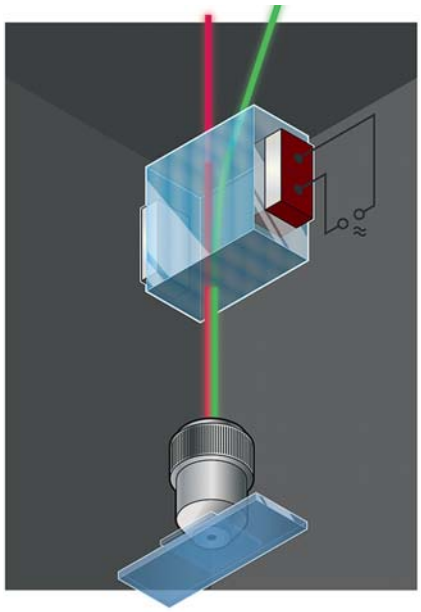

## What is the AOBS?

Leica's acousto-optical beam splitter (AOBS) replaces all collections of mirror-based beam splitters (gray splitters, dichroic splitters, and multichroic splitters) for visible light illumination in confocal microscopy.

- Highly efficient for maximum sensitivity – get more light from the specimen to the detector.
- Freely tunable for the most versatile multicolor imaging – no more hassle with filters or combinations thereof.
- Quick reprogramming for efficient multicolor live cell – adopt to your specimen's excitation without the limitations of ridgid reflection bands.

## When to use the AOBS?

- The AOBS is the perfect choice for all imaging applications as it is the most flexible, easy-to-use and efficient beam splitter. It is fully future-proof because it easily adapts to any new visible laser line.
- Sites that use different fluorochromes, or will often use new fluorochromes with classically stained samples. Imaging core facilities and dye development laboratories must have the AOBS.
- Experimental situations that are not supported by mirror-type beam splitters; if the fluorochromes' emissions are very close, or if, e.g. reflected light imaging is to be combined with fluorescence imaging. In these cases, the AOBS is the solution of choice.
- Using tunable excitation sources (WLL) combined with an AOBS is the only viable solution. This combination, linked with a freely tunable multiband SP detector is ideal – you can have it now!
- Ultimately, if you want to research with the latest and most advanced technology, there is no other choice; the TCS SP8 X "white confocal" with White Light Laser and AOBS leaves nothing to be desired.

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