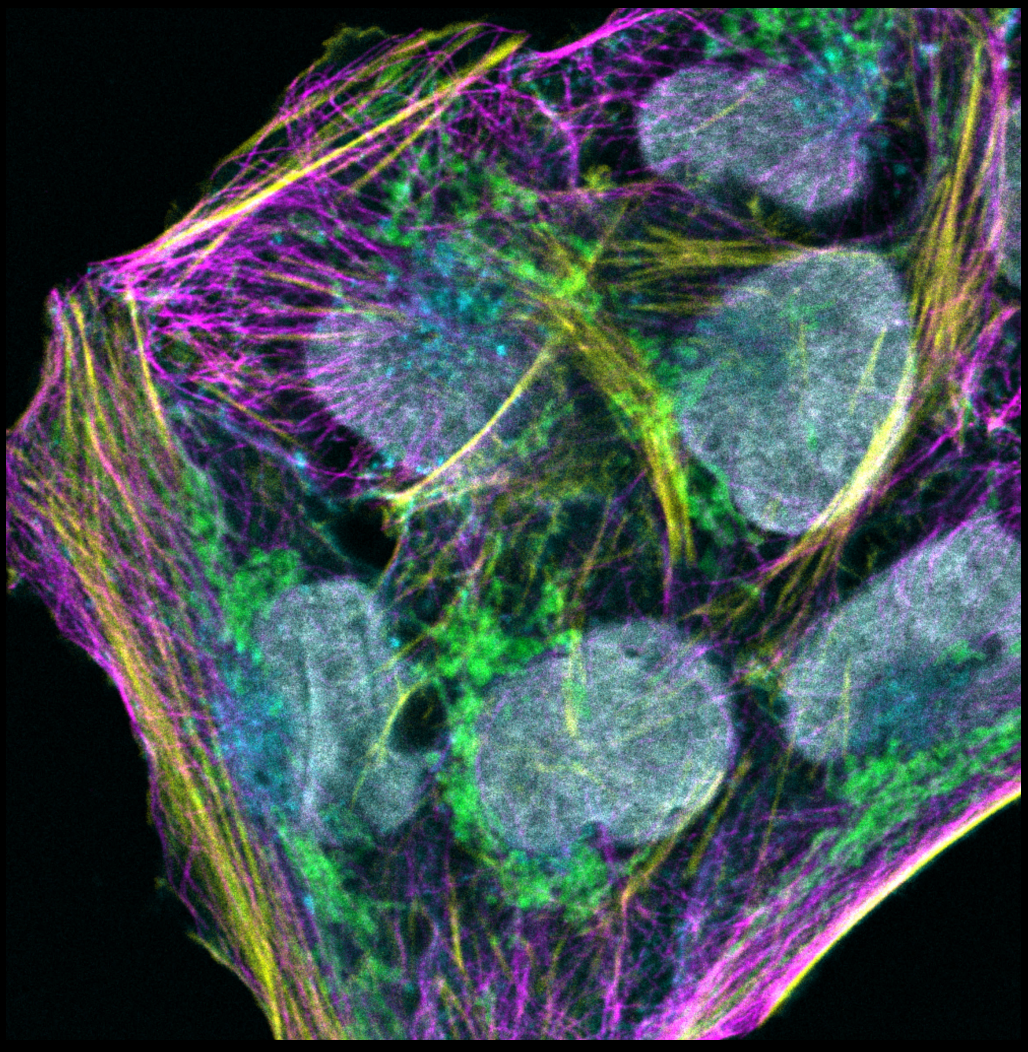
From Eye to Insight

Leica
MICROSYSTEMS

Application Note

A NEW WORLD OF CONFOCAL APPLICATIONS WITH THE NEXT GENERATION WHITE LIGHT LASERS



Introduction

As biological questions get more complex, there is an increasing need to study multiple events simultaneously in the same specimen. Consequently, more and more scientists find that they have to use multiple fluorescent labels to analyze their fixed samples or living cells with confocal microscopy. Some of the advantages of multicolor confocal microscopy are the ability to observe the spatial relationship and temporal dynamics of subcellular constituents, to monitor molecular interactions between differently labeled entities, and to understand the cellular make up of complex samples, such as cancer tissues.

Unfortunately, the visible light spectrum spans approximately a wavelength range between 400 and 700 nm. With each fluorophore and fluorescent protein requiring at least a 100 nm wavelength band, the visualization of multiple events or components with confocal microscopy is limited by the number of synthetic fluorophores or fluorescent proteins that can be simultaneously imaged without incurring bleed-through artifacts due to significant spectral overlap. Add to that technical constraints from the confocal system itself, where most confocal systems are limited by the number and specificity of available excitation lines, filters, and detectors.

Currently, these limitations can be overcome by carefully planning your sample preparation and use of fluorescent labels, sequential imaging, or with complex analytical approaches, such as using spectral unmixing algorithms that mathematically restore the signal from each fluorophore to its respective channel. However, most researchers decide to perform several experiments with a limited number of dyes each to ensure that they get successful results. But what if there was another way?

STELLARIS is the only confocal platform with an integrated next-generation White Light Laser (WLL) combined with Leica Microsystems' proprietary Acousto-Optical Beam Splitter (AOBS), spectral detection, and Power HyDs detector family. Such a combination enables tuning excitation and detection to perfectly match the spectral profile of the dyes in your sample, giving you the flexibility to perform multicolor experiments while obtaining clear, sharp images. Moreover, this is done automatically when you are setting up your experiment thanks to the ImageCompass smart user interface. There is now a simple way to do simultaneous imaging of multiple colors without having to resort to complex experimental procedures.

Spectral freedom to expand your research

Confocal microscopy is widely employed in cellular and molecular biology, where it is commonly used to study co-localization, co-expression, and other multiparameter analysis at the cellular and sub-cellular level. Some examples of these studies are DNA damage and repair, cellular signaling in immuno-oncology, tissue patterning in embryogenesis, protein trafficking and interactions, etc., the list is endless. However, the most basic confocal microscopes are unable to cleanly discriminate more than three fluorophores in a given sample, limiting their utility to gather results.

When it comes to choosing fluorescent probes for your multi-color experiments, you shouldn't have to compromise. Your confocal system should ensure that you get the maximum out of your sample. The STELLARIS confocal platform comes with a next-generation White Light Laser at its core to ensure you can combine exactly the right probes to answer your experimental questions.

Traditional lasers are generally monochromatic or emit just a few discrete laser lines at once which results in suboptimal excitation and can also cross-excite untargeted fluorophores in the sample. A White Light Laser is a single laser source that covers the full spectrum of visible light by producing a continuous spectral output. With the next-generation WLL in STELLARIS 5, that continuous spectral outcome allows you to choose any wavelength between 485 nm and 685 nm. With STELLARIS 8, the range goes from 440 nm to 790 nm. Even with an advanced setup used in a traditional confocal with individual laser lines, the wavelength range only extends up to 640nm. The freedom to choose excitation wavelengths would be a challenge for fixed-wavelength dichroic beam splitters typically found in confocal systems. Leica Microsystems' next-generation WLL is supported by the unique AOBS technology, which tunes automatically and allows you to select eight excitation lines from an almost infinite number of fluorophore combinations.

Our unique combination of high-efficiency pulsed WLL excitation, AOBS technology, and spectral detection provides the architecture essential for achieving complete wavelength freedom.

When using traditional confocal microscopy to image several different fluorescent labels in the same sample, sequential imaging of each color channel is often needed to avoid spectral bleed-through, which can degrade image quality. In the case of a kinetic experiment, that means you may miss rapid dynamic events due to the increased time it takes to acquire each time point. In addition, your sample remains on the stage for longer, making it more challenging to maintain cell health for the duration the experiment. The following image shows HeLa cells labeled with 5 different fluorophores to identify plasma membrane, nuclei, tubulin, actin and mitochondria. With STELLARIS, it was possible to collect all 5 channels in a single pass, rather than having to image the cells 5 times sequentially.

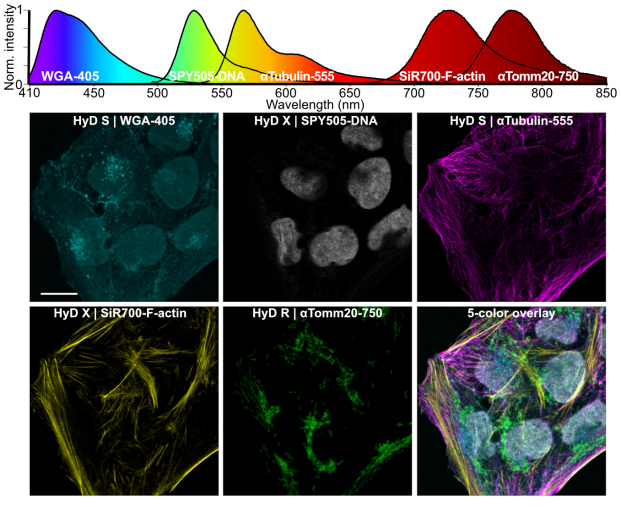


Figure 1: The Power HyD detector family enables flexible multicolor imaging across the visible to near-infrared range. Mammalian cells stained for five key cellular components. Emission spectra of the fluorophores used, and five-color image of cellular structures with detector types, fluorophores and targets indicated. Scale bar, 15 µm.

Keep it simple: add one more fluorophore without altering your current sample preparation

Bleed-through and cross talk can be particularly cumbersome with live cell experiments. As there can be the need to conduct experiments with more than two or three fluorescent probes, designing an experiment becomes more complex, because the most often used fluorescent probes, often have overlapping emission spectra. For example, when using EGFP and EYFP the 488 nm laser line from traditional confocal will excite both fluorophores simultaneously. Thus, for experiments with these fluorescent probes, a different solution is required.

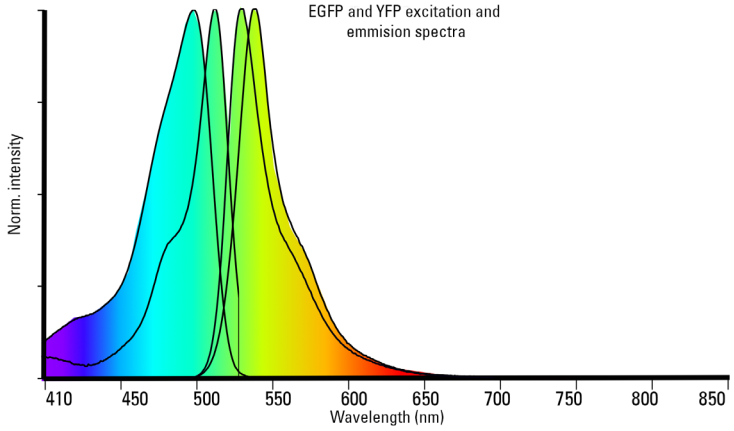
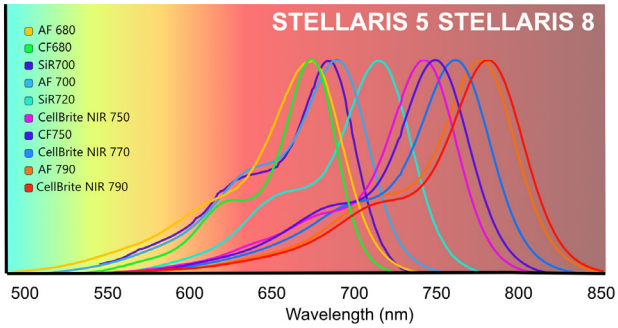


Figure 2: Exploiting the small shift in the excitation maximum of EGFP and EYFP. Now the signal can be separated using the WLL.

The next generation WLL configurations on STELLARIS 5 and STELLARIS 8 extend the range of accessible fluorophores into the near-infrared (NIR), giving you a more comprehensive spectral breadth. STELLARIS 5 has been optimized to produce a continuous spectral output between the wavelengths of 485 nm and 685 nm. This output provides you with an additional far red channel, so you can perform your usual experiment with commonly used fluorescent probes, such as Hoechst, GFP and mCherry, and study an additional event using dyes, like Alexa Fluor 750 orSiR700. STELLARIS 8 takes this flexibility even further with an extended WLL output ranging from 440 nm to 790 nm, enabling you to take advantage of dyes, such as CellBrite NIR 790.



An expanded WLL spectrum means that you now have more flexibility when designing your experiment, but without added complexity.

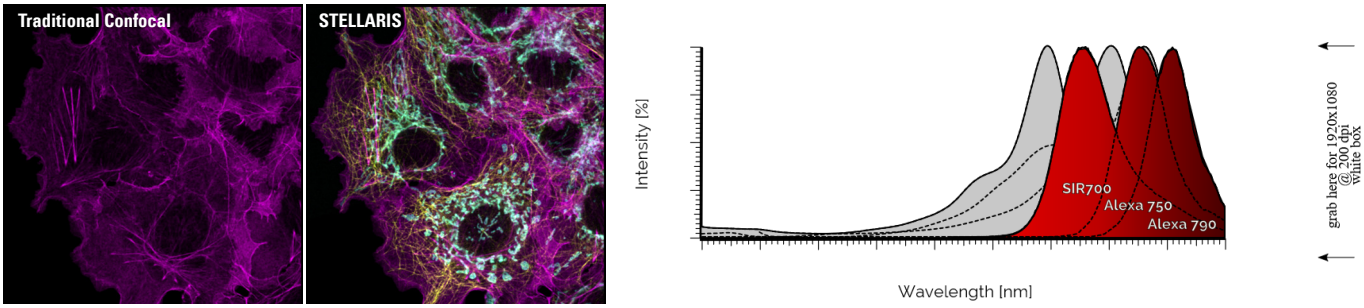


Figure 3: Images of Cos-7 cells, labeled with SiR-Actin (657 – 740nm detection range), AF750-Tom20 (760 – 790 nm), AF790-Tubulin (810 – 850nm). Sample courtesy of Jana Döhner, Urs Ziegler, University of Zürich. Left: Seen by conventional GaAsP detectors. Right: Seen by STELLARIS 8.

Simplifying complex experiments: setting up a multicolor experiment with ImageCompass

The more colors you want to image, the more complex the experimental setup. That is true for traditional confocal systems. With the STELLARIS user interface, ImageCompass, setting up a complex experiment is done in an intuitive manner thanks to its drag and drop fluorophore menu.

Let's think for a moment about an experiment that requires 5 fluorescent labels. With STELLARIS, all you need to know to set up the experiment is which labels are used for your experiment. Select the labels from the list and drag them onto a detection channel. ImageCompass automatically adjusts the excitation and detection parameters of the channels to your fluorophore combination, so that you obtain optimal results. This way, you are done with the setup in just a few seconds. Furthermore, ImageCompass gives information on cross excitation and cross talk for the combination of fluorophores imaged, giving instantaneous feedback on the quality of the spectral separation.

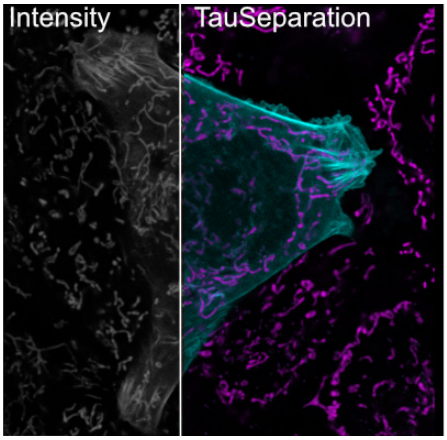
With this significant reduction in complexity, you can now design your multicolor experiments without having to worry about anything other than your sample preparation. Watch the video to find out more about ImageCompass: https://www.leica-microsystems.com/products/confocal-microscopes/p/stellaris-8/media/

Explore a new dimension: White Light Lasers enable lifetime imaging

What if you have no other option than to perform two imaging experiments, because your fluorescent probes have overlapping emission spectra? Using traditional confocal microscopy, it would be extremely challenging or impossible to differentiate them in the image. However, these probes could be separated using their fluorescence lifetimes which provide a form of contrast that is independent from fluorescence intensity.

A key advantage of the pulsed WLL technology is that it enables photon arrival time measurements. These measurements are fundamentally important for the imaging performed with TauSense, the powerful new set of lifetime-based imaging tools at the core of STELLARIS. Using the TauSense tools, you can assign your fluorophores to two different channels based on their different lifetimes and, therefore, they can be visualized as two separate images coming from the photons collected in the same spectral detection window.

Fluorescence-lifetime-based information delivered by TauSense adds a powerful new dimension to your research, enabling you to increase the number of fluorescent probes which can be resolved in a single sample[1].



Using fluorescence-lifetime-derived information, TauSense opens up many possibilities for multiplexing, expanding the number of simultaneous detection channels that can be used in each experiment.

Figure 4: TauSeparation of mammalian cells expressing LifeAct-GFP (manufactured by ibidi GmbH) and labeled with MitoTracker Green. Scale bars, 10 µm.

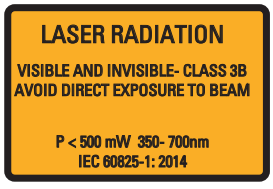
EXPAND YOUR POWER WITH STELLARIS

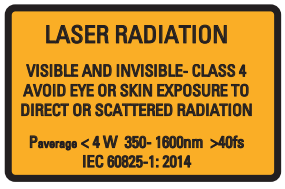
With traditional confocal systems, designing an experiment for optimal results requires the thoughtful selection of fluorescent labels, sequential modalities, the spectral detection band, and control specimen correction factors. With this scenario, situations often arise where the choice of experimental parameters is limited and the use of fluorescent probes which are well separated spectrally is not feasible.

STELLARIS has been designed to accommodate multilabel experiments without complexity, giving you the power to see more. With the compact, easy to maintain next-generation WLL, you can now add additional fluorophores, increasing the quantity of results you get from your experiment, without having to perform difficult offline techniques to separate your colors. With STELLARIS you can benefit from the freedom to choose excitation wavelengths that allow you to use your preferred combination of fluorophores for demanding experiments.

References

[1] Roberti, M.J., Lopez, L.O., Ossato, G., Steinmetz, I., Haas, P., Hecht, F. and Alvarez, L.A.J. (2020) TauSense: a fluorescence lifetime-based tool set for everyday imaging. Nat. Methods,.





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