

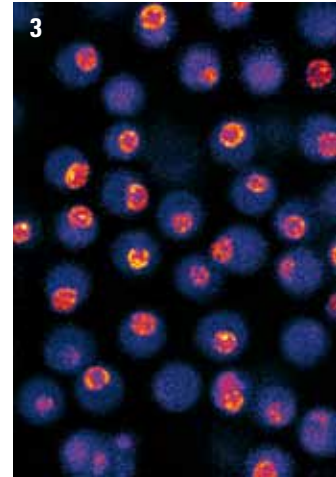
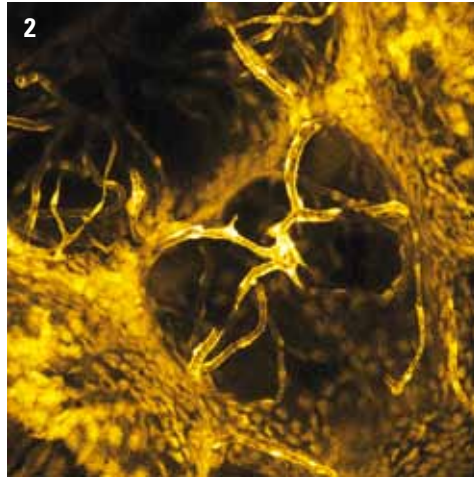
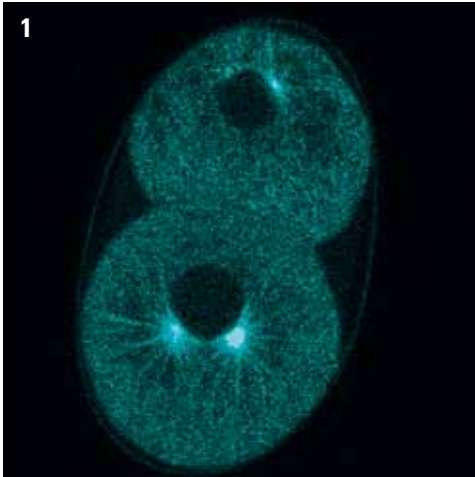


Leica HyD for Confocal Imaging

Hybrid detection technology for high fidelity

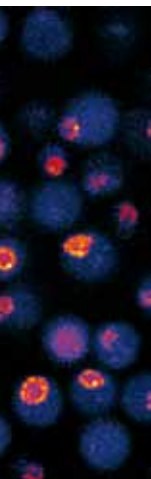
Living up to Life

Leica
MICROSYSTEMS



- Large dynamic range
- Improved cell viability
- High-speed imaging
- Single photon counting
- Open upgrade path





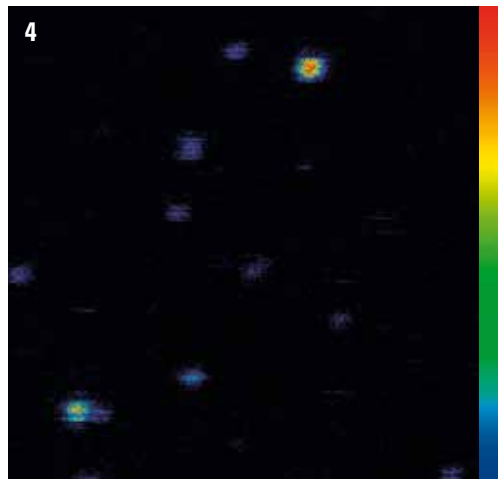
Biology's transformation into a data-driven, quantitative science is progressing. Demands on biological imaging are thus moving towards quantitative annotations of genes *in vivo*. The goal is to unravel the underlying functional interaction networks and to understand the spatio-temporal organization of live cells or living organisms. Hence, today's imaging instruments need to quantitatively reproduce the finest details with high fidelity.

Leica HyD

Your Road to Super-Sensitivity

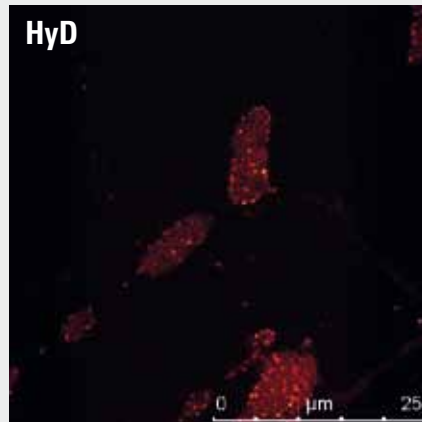
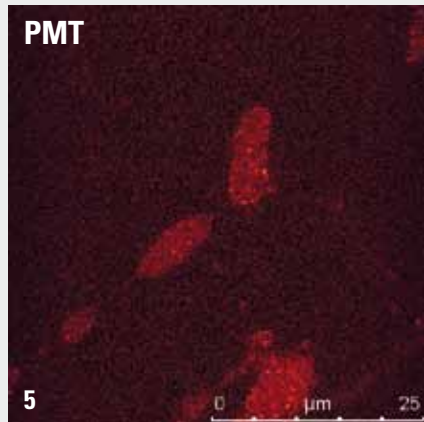
With its unparalleled contrast this detection system delivers publication-ready images out of the box – no post-processing necessary. Thus, all imaging tasks benefit from Leica HyD's low dark noise, superior sensitivity and large dynamic range. The latter is even further increased by single photon counting. Since the number of registered photons scales directly with the concentration of molecules under study, single photon counting represents the most attractive approach to image quantification. Thus, biochemical information becomes accessible through single photon counting and *in situ* spectroscopy.

With the Leica HyD series we present an integrated hybrid detector concept which can be freely combined with any new Leica TCS SP5 system as well as retrofit existing ones. Thanks to its high quantum efficiency (45 % at 500 nm typically), its low noise and large dynamic range the hybrid detector is the most versatile detector in our TCS SP5 confocal platform. Hybrid detectors along with Leica's filter-free beam-splitting concept and recycling loop-free spectral detection design make the TCS SP5 ideally suited for quantitative measurements and all-purpose imaging alike.



HyD Applications

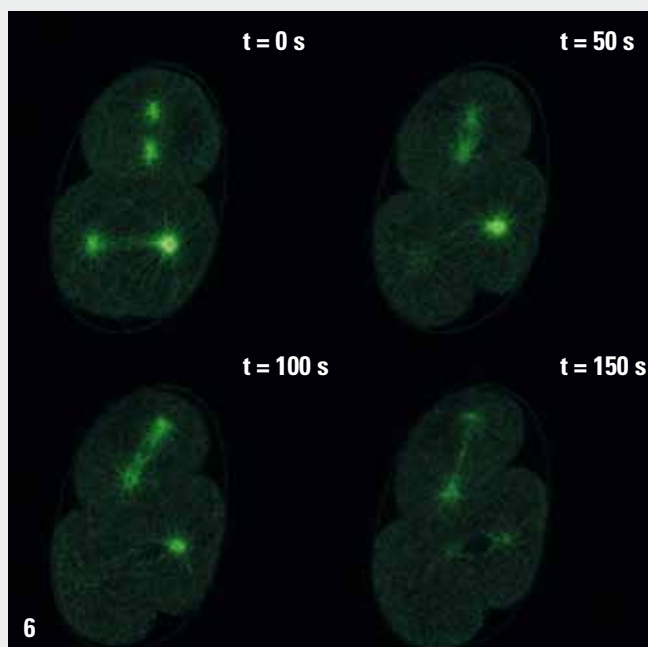
Low dark noise improves z-stacks of neuromuscular junctions



Critical live samples need to be imaged under low light conditions in high gain situations. Low noise can become the decisive advantage when it comes to recognizing weakly stained detail.

Neuromuscular junction in *Drosophila melanogaster* labeled with Bruchpilot::mStrawberry. The background of the PMT image is blurred by residual noise amplified by the maximum projection, while the HyD image is devoid of noise.

High speed makes *C. elegans* embryogenesis accessible



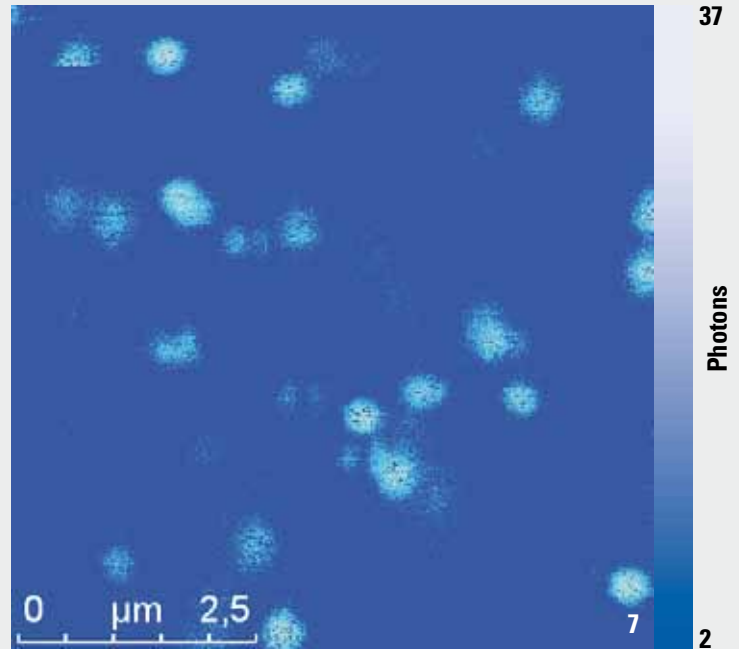
Developmental processes intrinsically involve the time dimension. In order to unravel the spatio-temporal formation of structure one needs to find the right balance between acquisition speed and clarity. Along with Leica's pioneering Tandem Scanner the HyD offers an unprecedented image quality. The tandem scanner's 8000 Hz line frequency comfortably leaves room for averaging or accumulation as needed, while retaining a large field-of-view.

Three- to four-cell stage of *Caenorhabditis elegans* labeled by EGFP-tubulin

High sensitivity for single molecules

Fixed single molecules represent the ultimate frontier to imaging. When measuring such weak signals close to a reflective surface, sensitivity, dark noise and the efficiency of the beam splitting system are stretched to their limits. Note the blinking (half moon shapes and horizontal lines) testifying the single molecule nature of the diffraction limited spots.

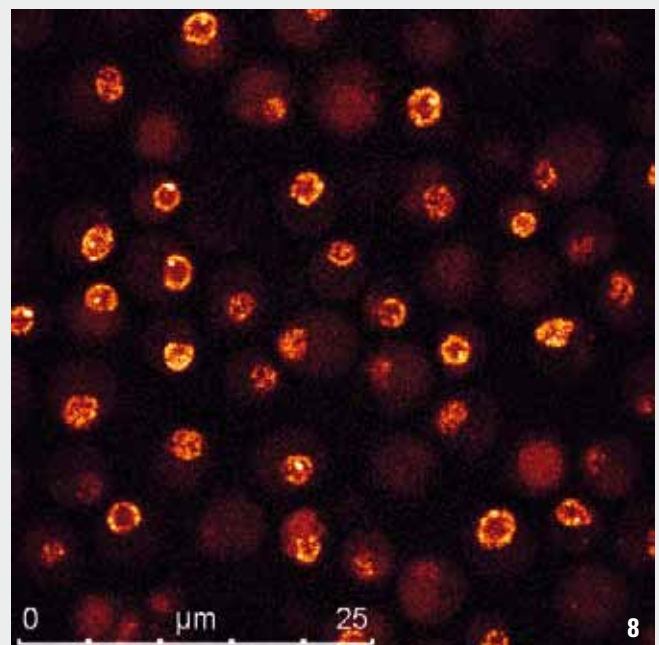
Polymer-embedded red fluorophores on a glass surface. They were imaged using a Leica TCS SP5 equipped with AOBs and white light laser.



Increased cell viability permits yeast imaging with a confocal point scanner

High sensitivity directly translates into reduction in light dosage delivered to the sample. Even delicate systems, such as yeast, are accessible to HyD detection – at full confocal resolution.

Live yeast cells double-labeled with EGFP at both the nuclear envelope and the telomere.

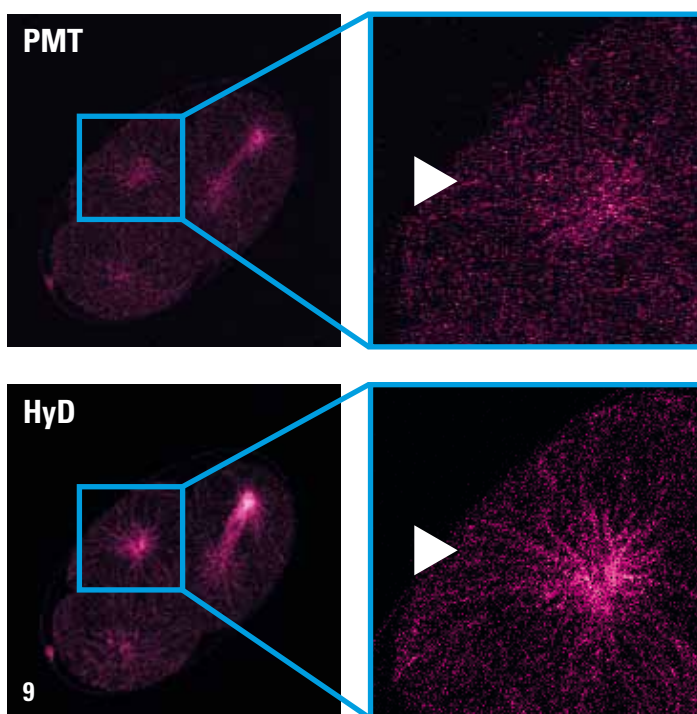


High Fidelity

- Signal efficiency for maximal information content
- Low dark noise for high fidelity
- Rendition of minute details
- Publication-ready images “out-of-the-box”

Superior signal-to-noise

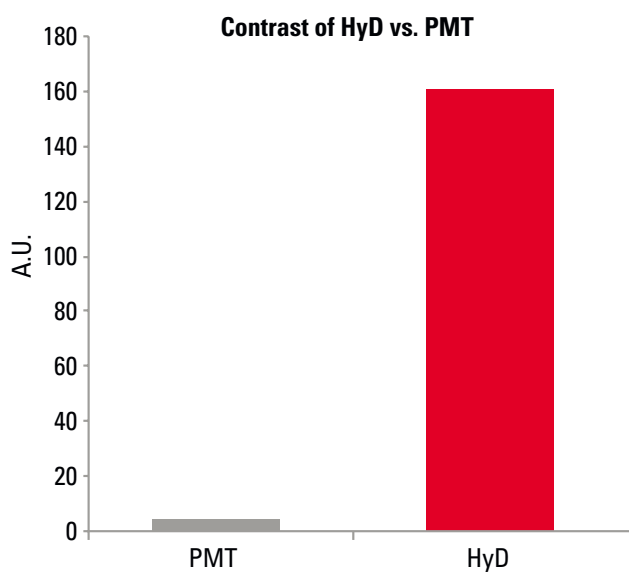
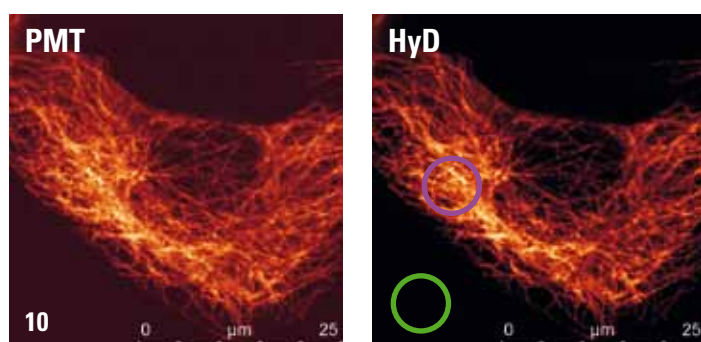
For maximal signal efficiency photons can be accumulated resulting in an increased signal-to-noise ratio of the image. To this end a detector with a low dark noise is imperative; otherwise noise will accumulate in the background of the image. Low dark noise helps to render finest detail from any sample – even tricky ones, such as highly scattering tissue slices.



C. elegans embryos labeled with EGFP-tubulin imaged by either PMT (top row) or HyD (bottom row) using identical instrument parameters. The insets (right column) reveal how the HyD captures the delicate microtubules (arrow) radiating from the aster.

Exquisite contrast

Dark noise also contributes to the overall background of an image. By reducing it the image contrast improves automatically. Thus, the information content is increased and the images are publication-ready right away without any need for image processing.



In this example tubulin was imaged at < 0.2 % of the Argon laser's 488 nm total power with 16 times signal accumulation. The gap-less detection band was 500 – 620 nm and the PMT's voltage was set to 800 V (typical user scenario), the HyD's gain to 100 %. These settings shed light on the contribution of dark noise to the overall image for PMT and HyD, respectively. A tremendous amount of detail was captured in this way with strongly improved contrast. The contrast ratio was plotted as the ratio of mean intensity in the darkest region (green circle) and the brightest region (purple circle).

Dynamic Range is Key

Four ways to maximize dynamic range

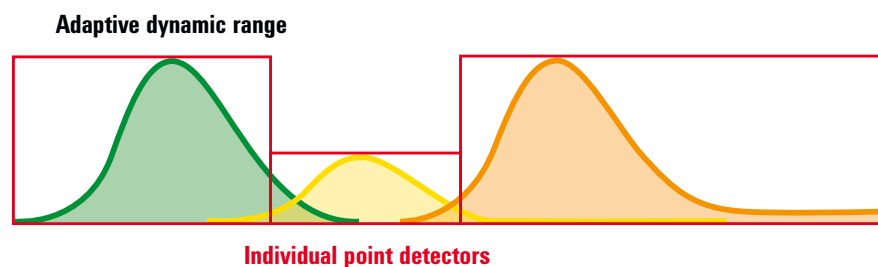
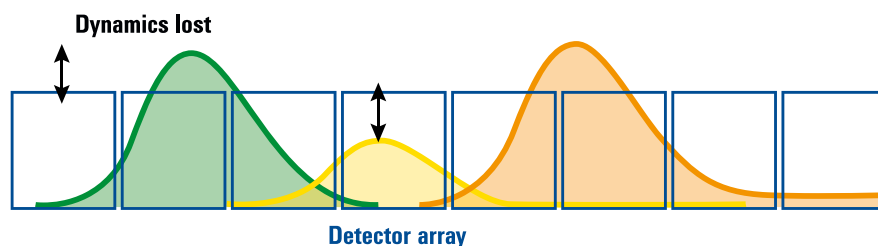
- Combine PMTs with HyD in one system
- Use 12-bit or 16-bit dynamic resolution
- BrightR mode for large dynamic range
- Use photon counting

Many very sensitive detection systems, such as avalanche photon diodes or classical GaAsP photomultipliers or arrays thereof suffer from their low dynamic range, meaning they cannot convert high light intensities into signals. This renders them a special purpose detection system for very dedicated applications only.

Have the confocal system adapt to your sample – not vice versa

For optimal imaging of arbitrary samples with varying brightness, the system must offer the broadest dynamic range possible. Unlike array-based spectral detection designs, the Leica SP detector allows a customized balance between highest sensitivity and highest dynamic range. Discrete detectors allow individual gain settings for each detector, rather than being forced to use the same gain for all array elements. Combining up to five channels comprised of PMTs and HyDs each with an adjustable gain the overall dynamic range of the confocal system is maximized. This effectively obviates the need to record time-consuming exposure series and hence reduces the photon dosage delivered to the sample. Moreover, the workflow remains straightforward, because no additional software tools are needed to create images with a large dynamic range.

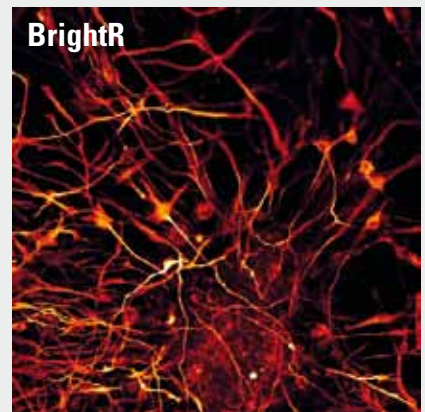
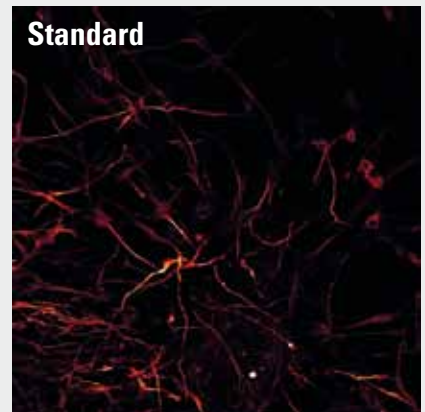
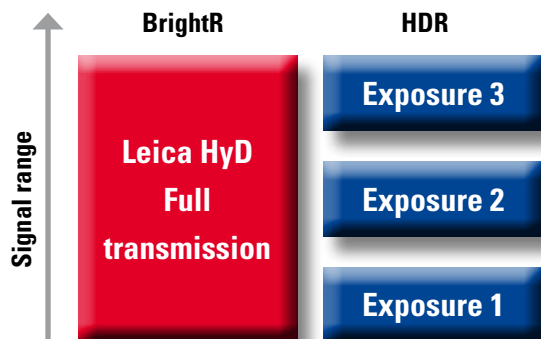
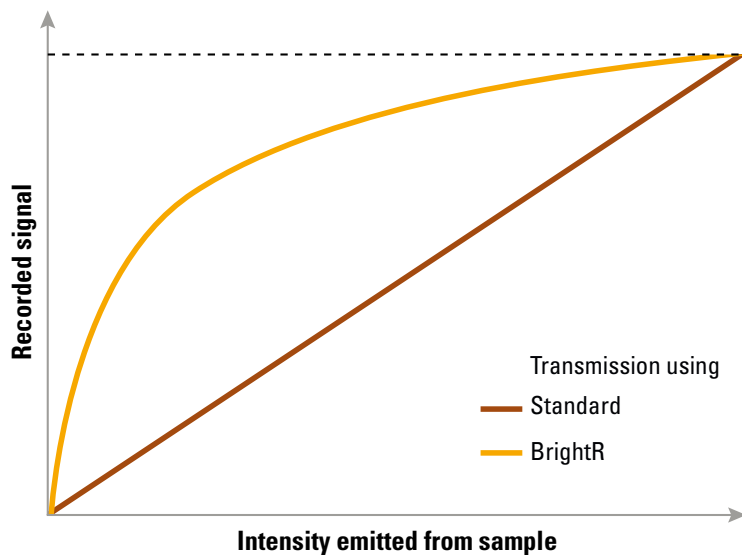
Why put your fluorescence spectra behind bars?



BrightR – brightness reinforcement for highly dynamic samples

Some biological samples accumulate far more fluorescent labels in specific structures, while others do not pick them up as much. Likewise, the physical size of labeled structures can vary greatly. Both result in a highly dynamic distribution of light intensity. Such samples are intrinsically difficult to record, because either the bright parts of the image get overexposed or the dim parts underexposed.

BrightR represents a Leica innovation to address this imaging task. It amplifies dim structures more than bright ones. Hence an image with an extended dynamic range ensues, capturing both very bright structures and intricate detail in the same image.



BrightR

- Retains dynamic information in bright structures
- Makes dim structures accessible
- Transmits a very large dynamic range in one shot
- Makes exposure series and post-processing – usually employed by high dynamic range approaches – obsolete

One Detector – From Single Photon to Whole Organism

Standard imaging and photon counting for

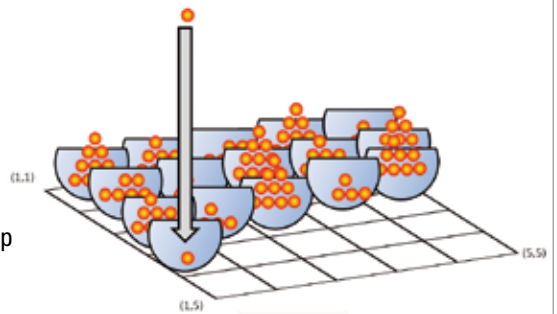
- Extended image dynamics
- Biochemical information
- Ratio imaging
- FRET
- Image correlation

Photon counting for quantitative imaging

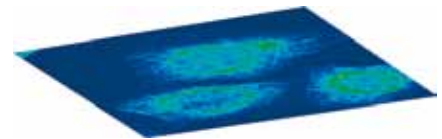
Photon counting not only enhances the dynamic range of the imaging system, but also opens the door to a new level of quantitative imaging. The former is achieved by preventing the dynamic overflow of single pixels or regions. Quantitative imaging benefits from photon counting as one can obtain direct insights into photophysical processes and apparent concentrations of labeled molecules. The light emitted by a specific fluorophore is directly proportional to its concentration in the sample. This is why photon counting is so attractive for quantitative measurements, such as ratio imaging, FRET and image correlation.

The Photon Counting Principle

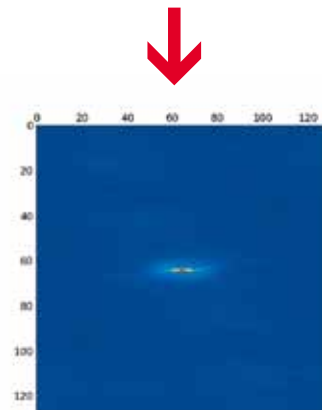
Single photon counting during acquisition – filling up pixels with photons



Color coded image scaled in photon counts

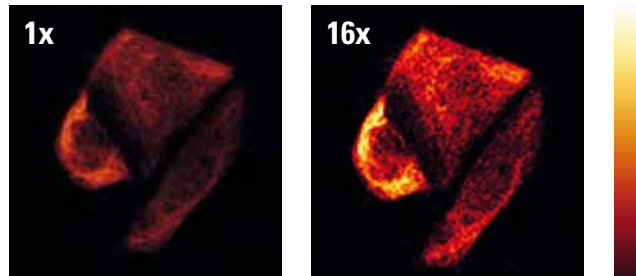


Statistical analysis

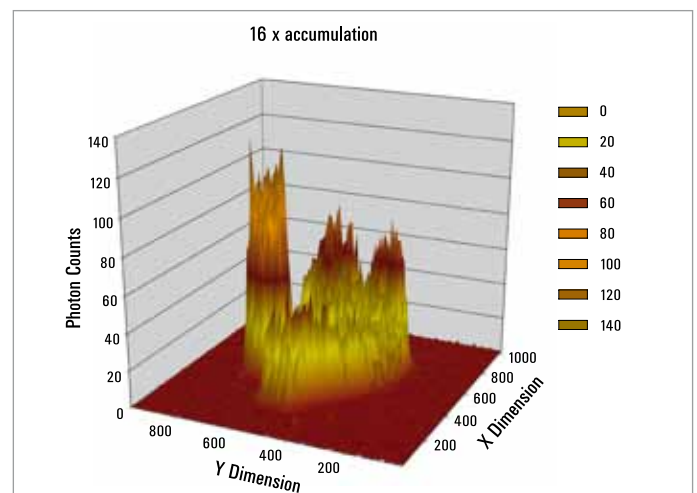
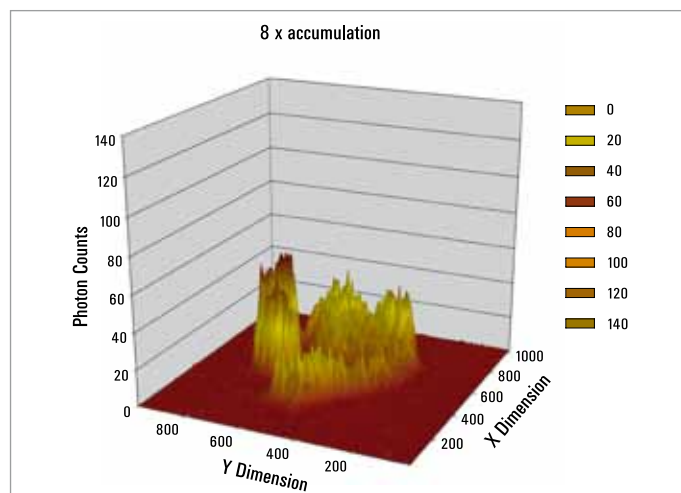
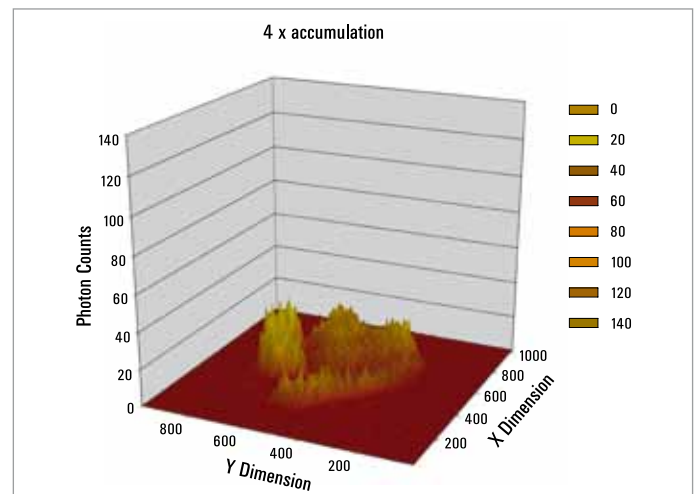
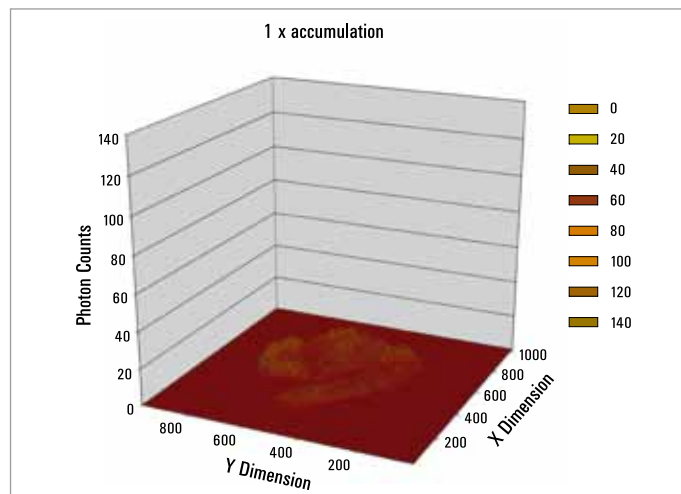


Maximal Dynamic Resolution by Photon Counting

Photon counting allows as much information to be accumulated as needed for any statistical analysis.

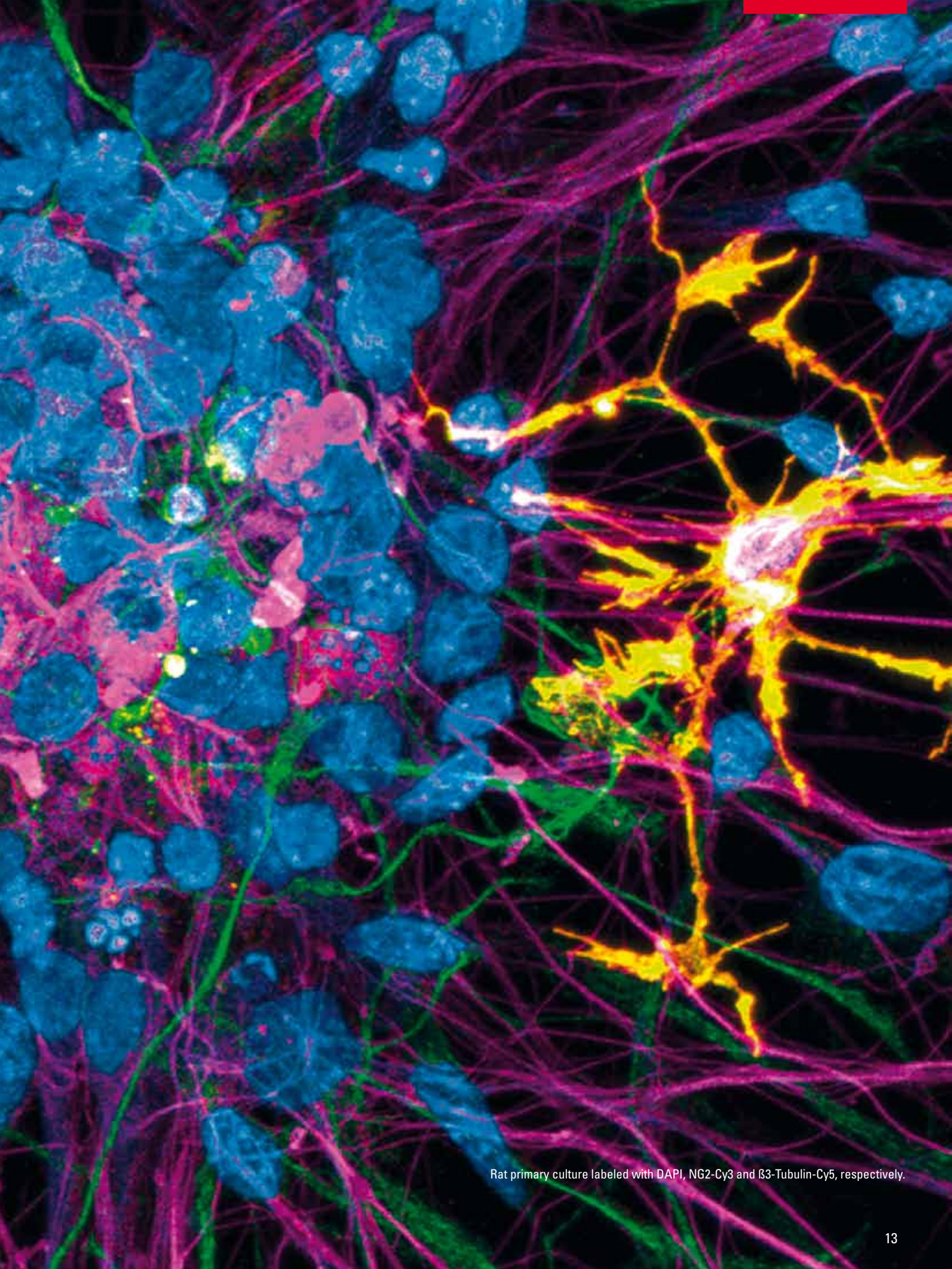


In photon counting each pixel behaves like a bucket which can be filled with photons. The longer one counts, the more photons are collected. The higher bit-depth modes available, 12-bit and 16-bit, represent very large buckets: In 12-bit mode, one can fill 4096, in 16-bit 65356 photons into one pixel. Thus, an enormous dynamic range with very low statistical per-pixel variance is available. The photon numbers are displayed via a look-up-table (LUT) on the screen. In this case the colors have a physical equivalent, photons.



Extended Z-volume Stacks

One of the reasons for using a confocal instrument is its z-sectioning capabilities. Hence, the most widely used functionality is extended focus reproduction by maximum projection along the z-axis – even if the stack consists of 100 z-slices per channel as demonstrated by the primary culture from rat brain displayed here. The HyD's low dark noise renders z-stacks with great contrast and high fidelity. Further image processing becomes obsolete.



Rat primary culture labeled with DAPI, NG2-Cy3 and β 3-Tubulin-Cy5, respectively.

Leica Tandem Scanner with

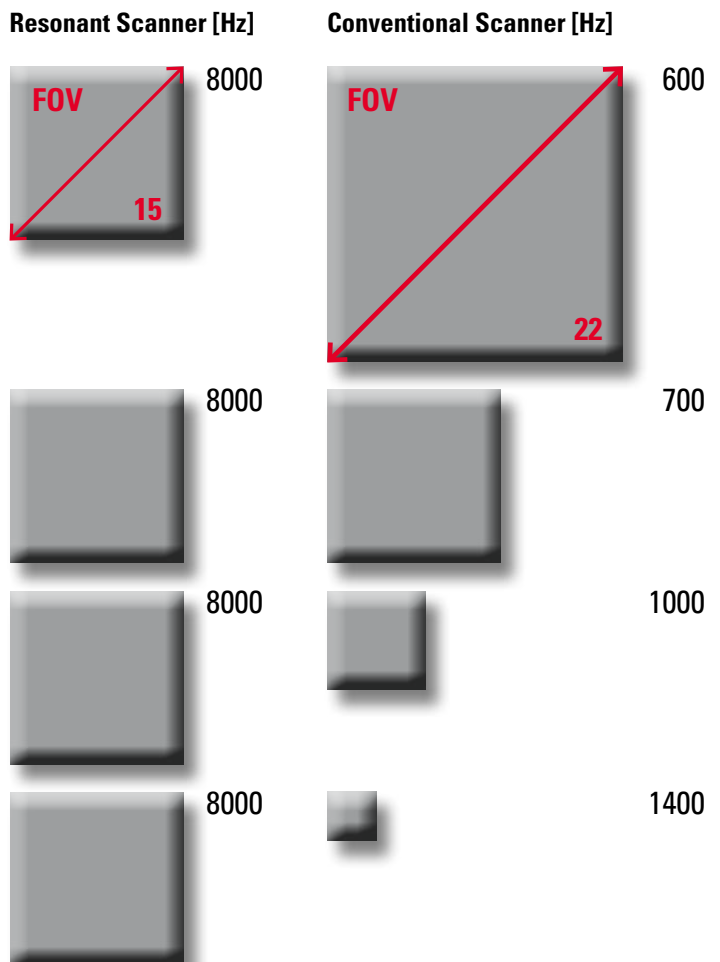
- Widest field of view available in any confocal point scanning system
- Optically correct images free of geometric distortions
- Higher time resolution to observe rapid biological processes
- Enhanced molecular brightness due to short pixel times (triplet suppression)

See more, rapidly

Leica Microsystems is the pioneer of rapid point scanning technology and sports a unique frame rate of 29 fps at scan format 512x512 with the widest field of view (FOV) available. The FOV using the standard scanner is 22 mm and using the tandem scanner up to 15 mm (e.g. the image width is up to 1 mm wide using a 10x lens and 16000 Hz line frequency or 1.5 mm using a 10x lens and 2800 Hz). This FOV goes along with parallax free imaging thanks to Leica's three-mirror scanner design, thus producing optically correct images free of geometric distortions.

Next to allowing greater insight into dynamic biological processes it has been shown that low pixel times avoid dark states and enhance the molecular brightness of dyes. Combine now the power of rapid point scanning with low noise hybrid detection

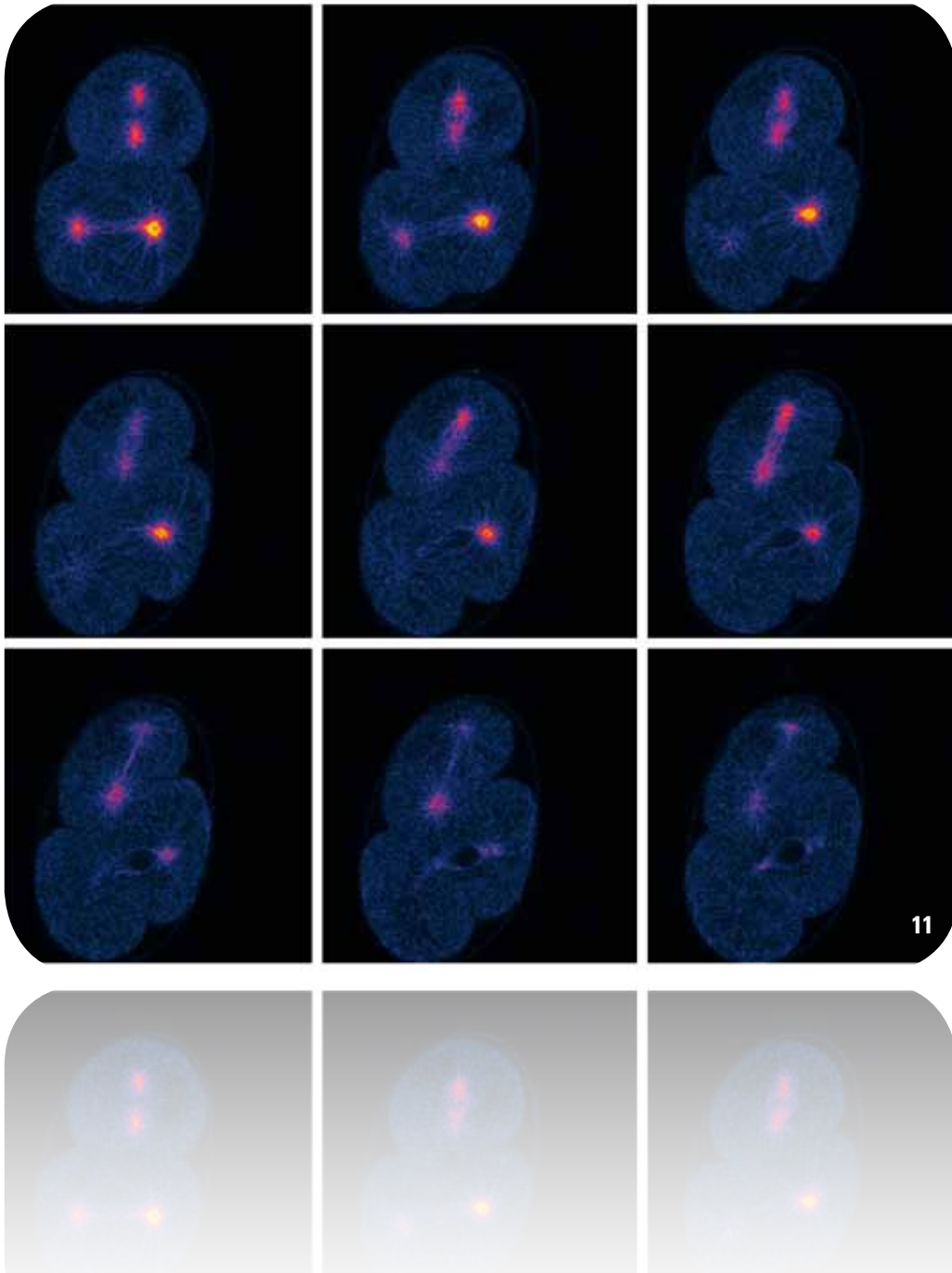
The resonant scanner has a constant FOV of 15, thus very large samples are accessible to high-speed imaging. At lowest zoom with a 10x objective lens, for example, the image width measures about 1 mm.



th HyD: Fastest Super-Sensitive Point Scanner

HyD and Tandem Scanner – an ideal combination

With pixel dwell times in the sub-microsecond range a standard PMT only captures very few photons per pixel, resulting in visible shot noise. The HyD's very good dark noise behavior combined with its higher quantum efficiency make it ideally suited for high speed imaging with the resonant scanner. The Leica TCS SP5 with HyD and Tandem Scanner defines the benchmark for the combination of high speed and image quality.



Choose As Much Super-Se

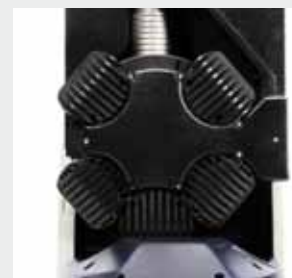
- Designed with upgradeability in mind
- Parallel detection of up to four multi-spectral super-sensitive channels
- Retrofit existing systems

Open upgrade path

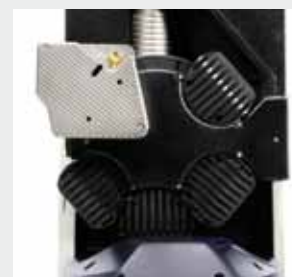
Rapidly changing needs in your lab go along with biology's dynamic nature. In acknowledgement of that the HyD concept was designed with upgradeability in mind. You can equip up to four HyD modules right away for parallel detection of up to four multi-spectral super-sensitive channels. Rather than offering this as a monolithic closed system you can, however, also start with one HyD and increase this number later as the lab's requirements grow. It is even possible to retrofit existing Leica TCS SP5 systems. Invest in tomorrow's technology today.



Sensitivity As You Need



5 PMTs



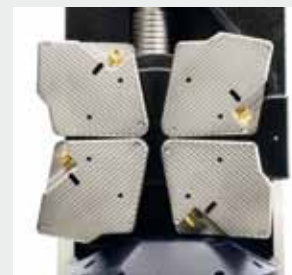
4 PMTs, 1 HyD



3 PMTs, 2 HyDs



2 PMTs, 3 HyDs



1 PMT, 4 HyDs

Different configurations of the spectral scan head allow for a customized balance between super-sensitivity and dynamic headroom.

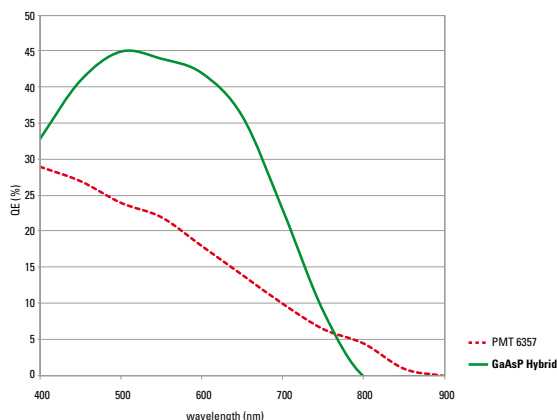
Pushing Sensitivity to a New HyD



“In summary we can say that hybrid detectors are an excellent alternative to PMTs.”

Dr. Dirk-Peter Herten,
Single-Molecule Spectroscopy, Cellnetworks
Cluster & Physical Chemistry,
University of Heidelberg

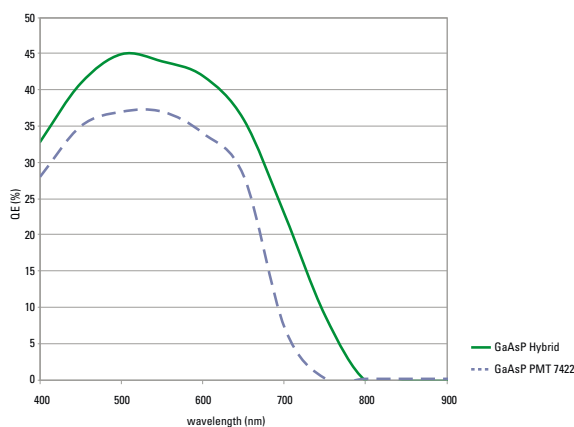
HyD's quantum efficiency is superior to standard PMTs ...



Quantum efficiency is a measure for a detector's capability to translate photons into usable electrical signals. With a quantum efficiency of typically 45% at 500 nm the hybrid detector is about two to three times more sensitive than a standard photomultiplier (PMT).

This new level of sensitivity will give scientists the edge in low-light applications where traditional PMT-based confocals would fail, such as imaging of yeast or *C. elegans*.

... and even to GaAsP PMTs

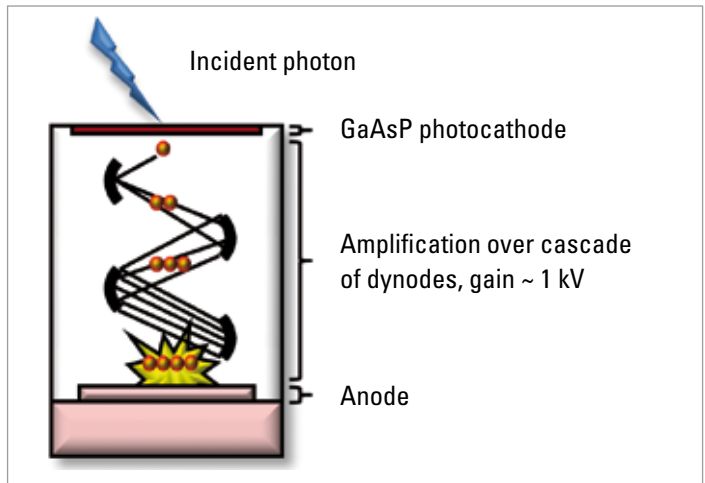


Traditional PMTs using a GaAsP photocathode (Gallium-Arsenide-Phosphide) are susceptible to damage by overexposure. Leica's HyD design avoids this shortcoming, making the HyDs both highly sensitive and versatile with a large dynamic range. In terms of quantum efficiency the HyD even supercedes standard GaAsP PMTs. This well-rounded detector delivers outstanding sensitivity along with durability.

Hybrid Detector Technology – the Best of Both Worlds

Traditional PMT with GaAsP photocathode

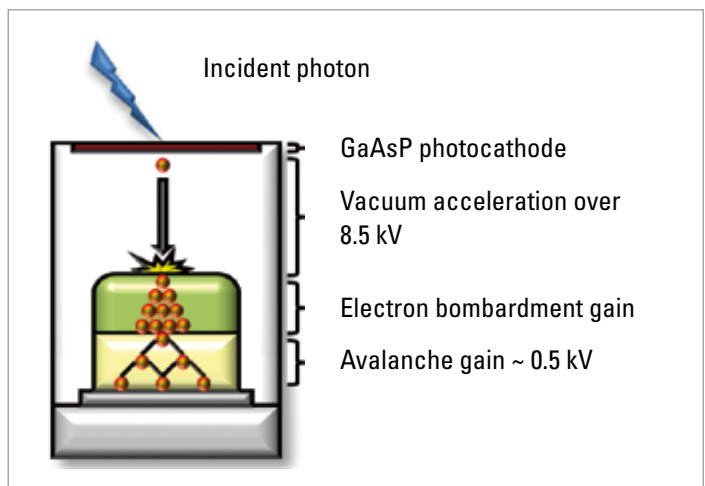
A traditional GaAsP detector is a photomultiplier tube (PMT) with a photocathode made of a Gallium-Arsenide-Phosphide (GaAsP) alloy. It amplifies secondary electrons via a cascade of dynodes, each of which is increasingly positively charged. Amplification is achieved at each dynode stage. The more dynodes the more amplification. Unfortunately, each amplification step also amplifies noise. Also, GaAsP is very efficient in translating light into electrons. The result may be strong currents or cations formed in the path of the electrons. Both may lead to ageing or destruction of the detector.



Hybrid detector

The hybrid detector employs a unique working principle. It combines functional elements used in PMTs and APDs. This results in large dynamic range and super-sensitivity combined with low dark noise.

The photoelectrons created at the GaAsP photocathode are accelerated in a strong electrical field, passing an electron bombardment and an avalanche element. Thus, further gain is realized. The detectors are more durable than traditional GaAsP PMTs, have less noise and higher sensitivity. The high voltage of the first stage prevents electrons from flying back into the photocathode, which would damage the detector. Hybrid detectors are much more versatile because they can amplify much stronger signals and are hence usable for every day samples as well as dim ones.



Filter-free Super-Sensitivity

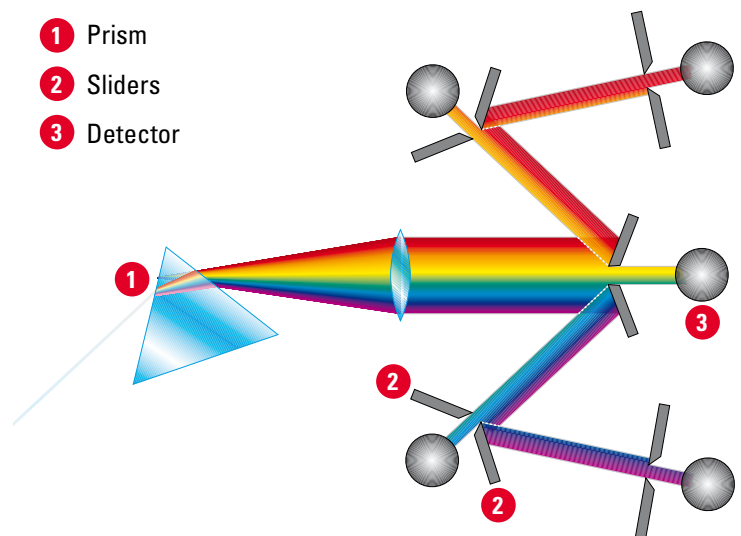
- True confocal point scanner for maximal axial resolution
- Gapless spectral emission bands for spectrometry
- Prism-based spectral dispersion maximizes photon efficiency
- Beam parking

The distinguished Leica TCS SP5 provides high photon efficiency and gap-less spectral fluorescence detection, both of which are ideal prerequisites for quantitative measurements.

Spectral multiband SP detector

The HyD SP modules seamlessly integrate into Leica's distinguished spectral detection design, the SP detector module. This design offers simultaneous detection of gapless variable emission bands. The SP detector resembles a multiband spectrophotometer, based on a prism and mirror sliders. The patented design using a prism represents the most efficient dispersion concept without the need to recycle photons or lose intensity with a grating-based design.

The Spectral Detection Module



Choice: Leica AOBS and SP Detection

Your choice: Dichroic beam splitter or AOBS

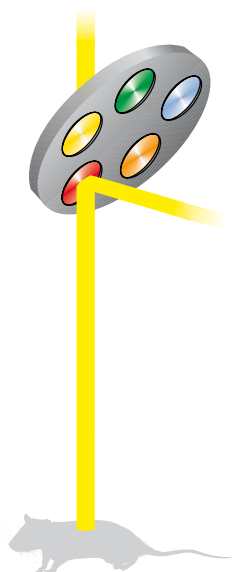
A critical element of incident light fluorescence microscopy is the beam splitter as it has a large impact on the system's light efficiency and flexibility. Leica offers both dichroic beam splitters and the AOBS (Acousto-Optical Beam Splitter).

The dichroic beam splitting system is your entry to super-sensitivity. The reflection suppression and transmission of the dichroic beam splitters have been improved for best results with your sensitive detection system.

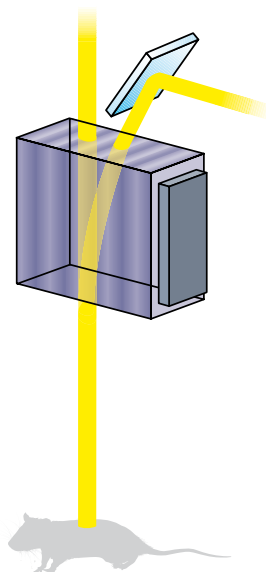
Cost efficient and widely applied dichroic beam splitters represent the standard option in terms of transmission and filter cut-off. Leica Microsystems has defined the benchmark in confocal imaging with the introduction of the patented AOBS. This optical device is a programmable deflection crystal, which very specifically directs narrow excitation lines to the sample while passing the full emission onto the detection module. The high transparency and neutral transmission characteristics of the AOBS allow non-distorted emission spectra to be used in such applications as multi-spectral FRET analysis. Instead of considering possible filter combinations you can focus on your work while your system adapts to it.

- Super-sensitivity with dichroic beam splitter or AOBS
- Full transmission beam splitting
- Faithful reproduction of excitation and emission spectra
- Multi-spectral FRET analysis

Beam Splitter



AOBS



Acknowledgements:

We gratefully acknowledge the following scientists for providing images, data, and valuable support to figures:

1, 6, 9, 11

Caenorhabditis elegans embryo labeled by EGFP-tubulin.

Courtesy of Prof. Pierre Gönczy, École Polytechnique Fédérale Lausanne (EPFL), Lausanne, Switzerland

#2

Danio rerio brain vascularization labeled with Fli1a::eGFP.

Courtesy of Anko de Graaf, Utrecht University, Utrecht, Netherlands (sample originally created by Bas Ponsioen)

#3

Labeled telomeres and nuclear envelope in yeast cell nuclei (see also legend to figure 8).

Courtesy of Prof. Susan M. Gasser, Friedrich Miescher, Institute for Biomedical Research, Basel, Switzerland

#4

Immobilized Atto 647N molecules on glass substrate.

Courtesy of Anton Kurz and Dr. Dirk-Peter Herten, Junior Research Group Single Molecule Spectroscopy, BioQuant Institute, University of Heidelberg, Germany

#5

Drosophila melanogaster neuromuscular junction labeled with BRP-short::mStrawberry.

Courtesy of Prof. Stefan J. Sigrist, Institut f. Biologie/Genetik, Freie Universität Berlin, Berlin, Germany

#7

Polymer-embedded red fluorophores.

Courtesy of Dr. Christian Hellriegel, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain

#8

Courtesy of Prof. Susan M. Gasser, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland



Abbreviations

AOBS – Acousto-Optical Beam Splitter
APD – Avalanche Photo Diode
BrightR – Brightness Reinforcement
EGFP – Enhanced Green Fluorescent Protein
FOV – Field Of View
GaAsP – Gallium-Arsenide-Phosphide
FRET – Fluorescence Resonant Energy Transfer
HyD – Hybrid Detector
PMT – Photomultiplier



Leica Design by Christophe Apothélos

“With the user, for the user”

Leica Microsystems

Leica Microsystems operates globally in four divisions, where we rank with the market leaders.

• Life Science Division

The Leica Microsystems Life Science Division supports the imaging needs of the scientific community with advanced innovation and technical expertise for the visualization, measurement, and analysis of microstructures. Our strong focus on understanding scientific applications puts Leica Microsystems' customers at the leading edge of science.

• Industry Division

The Leica Microsystems Industry Division's focus is to support customers' pursuit of the highest quality end result. Leica Microsystems provide the best and most innovative imaging systems to see, measure, and analyze the microstructures in routine and research industrial applications, materials science, quality control, forensic science investigation, and educational applications.

• Biosystems Division

The Leica Microsystems Biosystems Division brings histopathology labs and researchers the highest-quality, most comprehensive product range. From patient to pathologist, the range includes the ideal product for each histology step and high-productivity workflow solutions for the entire lab. With complete histology systems featuring innovative automation and Novocastra™ reagents, Leica Microsystems creates better patient care through rapid turnaround, diagnostic confidence, and close customer collaboration.

• Medical Division

The Leica Microsystems Medical Division's focus is to partner with and support surgeons and their care of patients with the highest-quality, most innovative surgical microscope technology today and into the future.

The statement by Ernst Leitz in 1907, “with the user, for the user,” describes the fruitful collaboration with end users and driving force of innovation at Leica Microsystems. We have developed five brand values to live up to this tradition: Pioneering, High-end Quality, Team Spirit, Dedication to Science, and Continuous Improvement. For us, living up to these values means: **Living up to Life.**

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