Bringing Biology into Focus

In 1957, Marvin Minsky patented a "microscopy apparatus" that described the use of a pinhole to limit the light reaching the objective to only that which is in focus–making it the first description of a confocal system. We can be fairly certain that he could not have foreseen how this simple and elegant idea would be modified and refined into a technique that would revolutionize the field of microscopy. Some of this certainty comes from the fact that at the time his invention was patented, the technology was not yet even available to build it.

Confocality, which essentially describes the quality of light as being in the same focal plane, allows for the scanning of a sample to generate optical sections. Reconstruction of these individual images produces a sharp 2-D or 3-D final rendering, a boon for researchers seeking to visualize detail, especially in thick sections or even entire small organisms.

It took another few decades before a working version of Minsky's design was actually realized (unfortunately for Minsky, this was after the patent had already expired). Among other things, the field had to wait for the development of reliable and powerful lasers that could excite fluorophores at a variety of wavelengths, necessary for visualizing cells and subcellular structures. But it was not always smooth sailing for confocal microscopy. Image acquisition speed was in constant tension with detection sensitivity, and increases in either of these degraded resolution. These ongoing challenges can be represented by the so-called Eternal Triangle, with speed, resolution, and sensitivity at its apexes (see main poster). Generally speaking, pulling on one point of the triangle negatively impacts the others. So an increase in resolution invariably means slower image acquisition and/or decreased sensitivity.

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Researchers and microscope makers alike are constantly striving to push the boundaries of sensitivity, resolution, and speed. Their determination has produced a steady stream of advances in microscope technology, and researchers armed with these ever-improving tools and techniques are squeezing ever more and better data out of their samples. This poster, created by the *Science*/AAAS Custom Publishing Office and sponsored by ZEISS, leads the reader through the past, present, and future of confocal microscopy. Additionally, you can go online to find an interactive version of the poster with additional information and graphics (posters.sciencemag.org/confocal).

It is our hope that readers will find this poster both engaging and informative. In a field that is moving and growing so quickly, we are providing just a snapshot of where we are today and a glimpse at where we might be in the future. If the past is anything to go by, that future looks quite luminous.

Sean Sanders, PH.D., Commercial Editor, *Science*

Background: Confocal image of mouse brain cortex | Courtesy of Yi Zuo, Molecular, Cell and Developmental Biology (MCDB) department, University of California Santa Cruz

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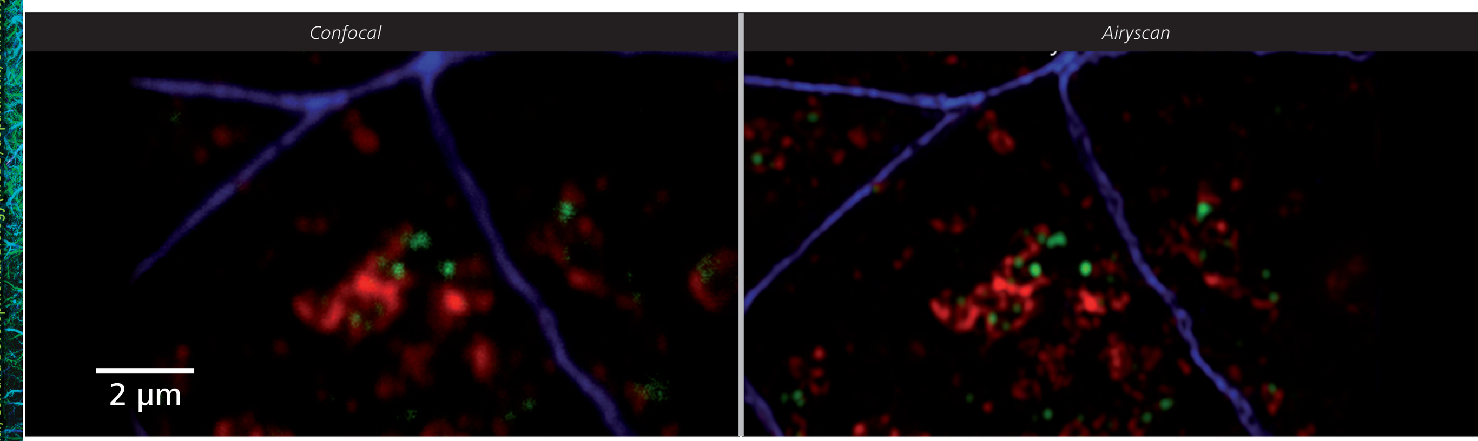
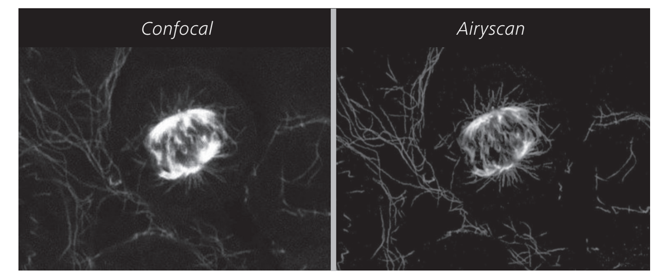
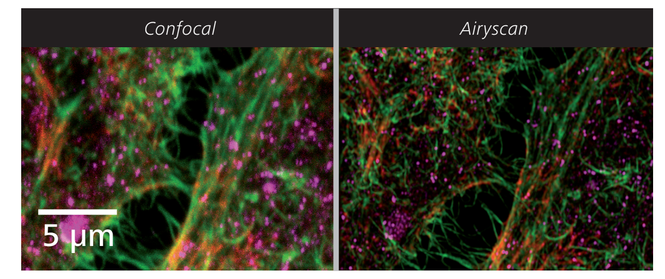
ZEISS LSM 880 with Airyscan at Work



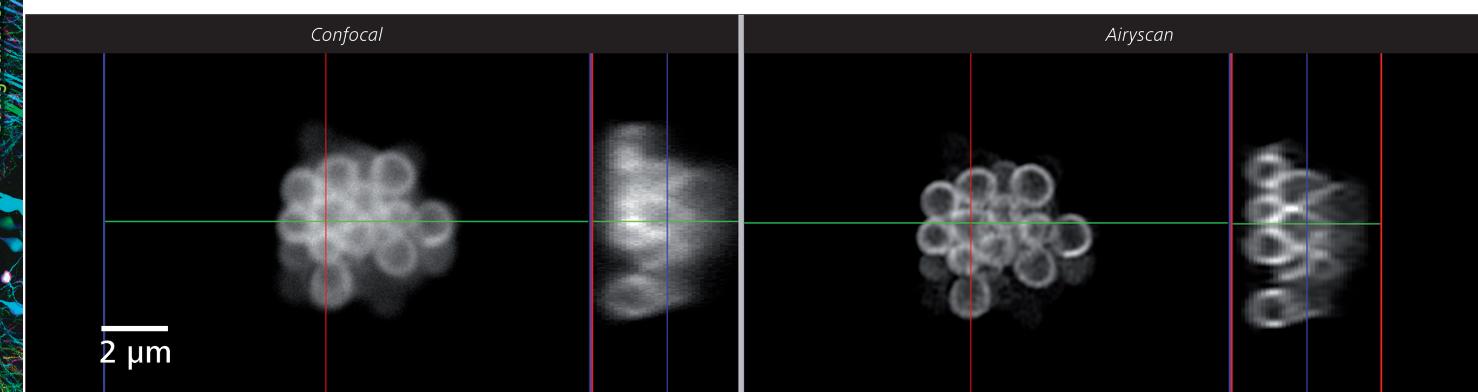
Human RPE cells, ZO1 (tight junction marker) in blue, photoreceptor outer segments stained with FITC in green, EEA1 (endosomal marker) in red. Courtesy of S. Almewadar, CRTD, TU Dresden, Germany



18 Fixed tumor cells, tubulin labelled with Alexa 555, Airyscan SR mode. Sample: courtesy of P. O`Toole and P. Pryor, University of York, UK



HeLa cells, Actin stained with Phalloidin-Alexa 546, AP3 with Alexa 488, DAPI. Courtesy of S. Traikov, BIOTEC, TU Dresden, Germany



Fluorescent 1µm ringbeads imaged at 488 nm.

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Bringing Biology into Focus

THE PAST, PRESENT, AND FUTURE OF CONFOCAL MICROSCOPY

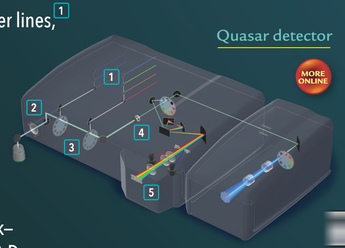
The Technology of Confocal Laser Scanning Microscopy (CLSM)

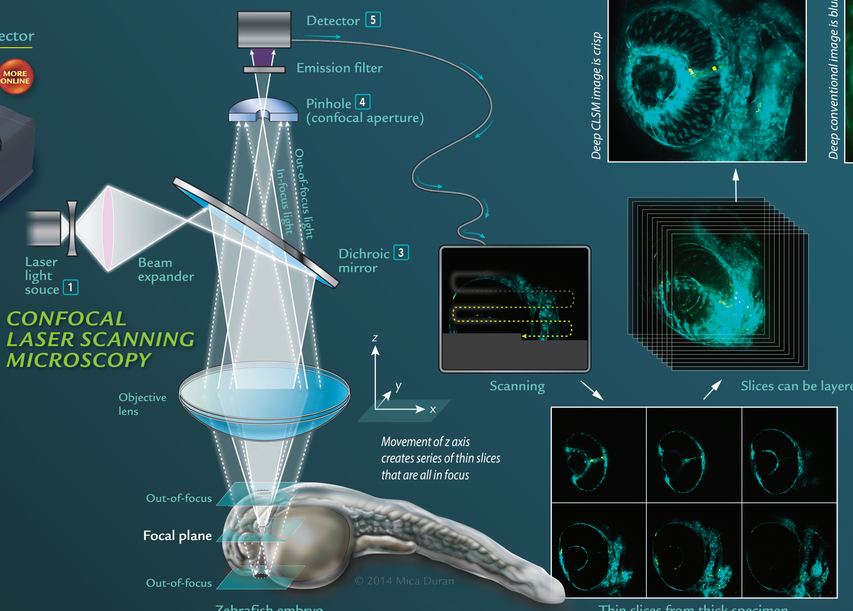
A point-scanning confocal system features multiple laser lines,[1] galvanometric scanning mirrors,[2] beamsplitters, and dichroic elements[3] to guide excitation and emission light on their respective paths.

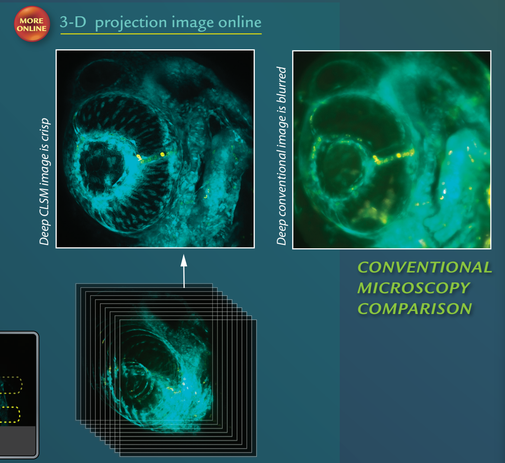
A pinhole[4] in the detection beam path blocks light from above or below the desired focal plane, producing an image of a thin optical section. By moving the focal plane up and down, researchers can obtain multiple virtual slices of a tissue–called a *z*-stack–which can be analyzed frame by frame or rendered in 3-D.

The pinhole aperture size is configurable: by making the pinhole larger or smaller, spatial resolution and section thickness can be increased or decreased. But the microscope is still constrained by the diffraction limit of light; however small the pinhole, lateral resolution cannot surpass ~200 nm.

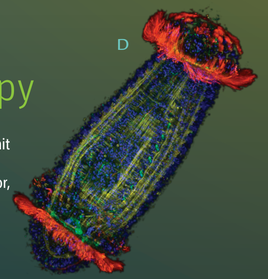
The instrument's detectors are also configurable. In the simplest optical configuration, emission light of the desired wavelength is directed through filters to one or more detectors. Live-cell and multicolor imaging applications require faster, more flexible detection systems capable of sampling much of the visual spectrum at once, such as the 34-channel Quasar detector[5] diagrammed above. MORE ONLINE







A Brief History of Confocal Microscopy

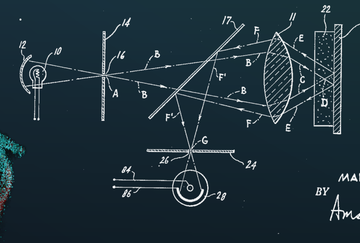


Given uniform excitation, as realized in widefield microscopy, all fluorescent molecules in a sample emit light simultaneously. Both in- and out-of-focus light emanating from these molecules reaches the detector, the latter causing blurring of the image. By contrast, confocal microscopy illuminates the sample point by point and blocks out-of-focus light using a pinhole positioned in focus with (confocal to) the focal plane, yielding a clearer picture.

Harvard University researcher Marvin Minsky first advanced this idea in 1957. His design imaged a sample point by point using an intense light source, assembling the data into a composite image.

In the following decades, multipoint (spinning disk) illumination strategies, developed in the 1960s, were succeeded by advances in laser excitation, new optical elements, and powerful computers. By 1987, the first confocal laser scanning microscope (CLSM)–a laser-based extension of Minsky's original idea–was commercially available.

Early designs held the light source steady and moved the sample on the stage. Today's CLSMs reverse that, using moving mirrors to scan the laser over the sample–a faster and more robust approach, especially for biological samples.



Marvin Minsky, "Microscopy Apparatus" U.S. Patent 3 013 467, December 19, 1961.
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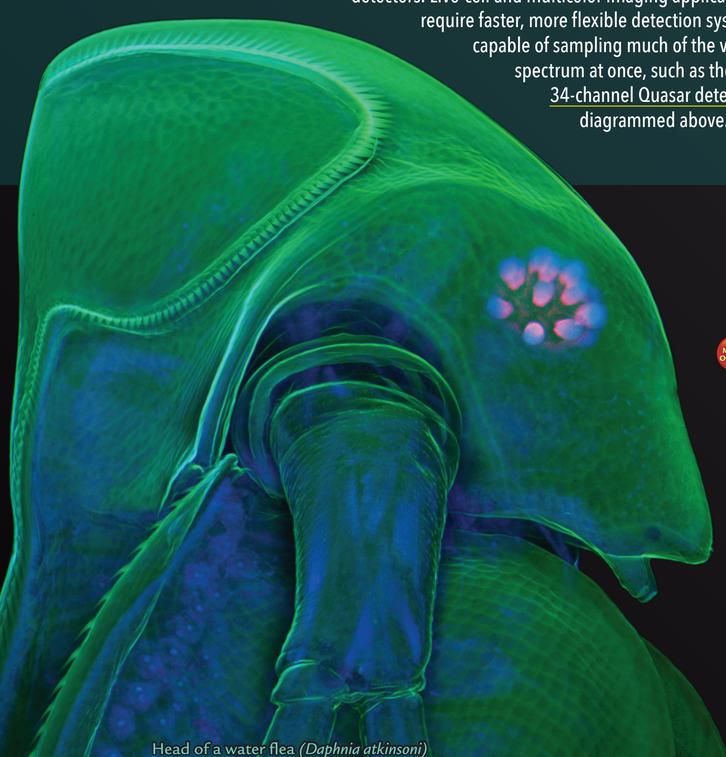


Head of a water flea *(Daphnia atkinsoni)*
Courtesy of Jan Michels, Institute of Zoology, Kiel University, Germany

Going Live

MORE ONLINE

The discovery and development of fluorescent proteins has greatly facilitated live-cell imaging and broadened the color palette for today's microscopists. The use of multiple markers has become standard, often with overlapping spectra.

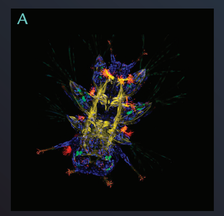
MORE ONLINE

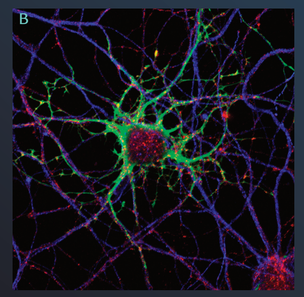
Linear unmixing strategies can resolve overlapping colors by collecting a dataset representing a single optical section collected at different wavelengths, known as a lambda stack. By analyzing that stack pixel by pixel, the system can deconvolve the signal into distinct channels. Acquiring these multidimensional images over long time periods and large sample regions has driven the need for increased acquisition speed, as well as increased data-processing and data-handling capabilities.

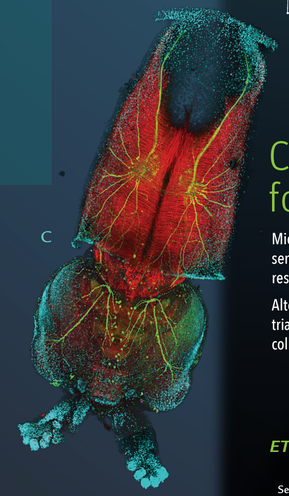
Fluorescent proteins have broadened the color palette for today's microscopists.

Combining fluorescent molecules with novel imaging strategies reveals molecular dynamics. Photoactivatable fluorophores (which are dark until activated by a light pulse) and photoconvertible/photoswitchable fluorescent proteins (whose emission color can be altered with light) can highlight local sub-populations of molecules of interest in order to follow their dynamics. FRAP (fluorescence recovery after photobleaching) uses intense illumination to photobleach a defined sample region to measure how quickly new fluorophores migrate into or out of it, while FCS (fluorescence correlation spectroscopy) monitors molecules diffusing in and out of an illuminated region. RICS (raster image correlation spectroscopy) details the motion of molecules as they move across the scanned region (and between *z* frames) during image acquisition.

MORE ONLINE For more information, go online to: posters.sciencemag.org/confocal







Left to right:
(A) *Platynereis* larva.
(B) Primary neuronal cultures stained for *Bornavirus* antigens and tetanus toxin.
(C) *Loligo pealei* embryo.
(D) *Capitella* larva.

Confocal Microscopy for the 21st Century

Microscopy systems represent a balance between acquisition speed, resolution, and sensitivity, the so-called *eternal triangle*. Basically, acquiring an image faster means resolution and sensitivity inevitably suffer.

Alternative technologies enable researchers to "stretch" the corners of the eternal triangle, pictured below. Resonance scanning provides speed at the expense of collected signal. Superresolution strategies such as stimulated emission depletion microscopy (STED) circumvent the diffraction limit by effectively shrinking the fluorescent region using two overlapping lasers, sacrificing both imaging speed and sensitivity. Widefield superresolution techniques like PALM and STORM boost resolution at the expense of speed by localizing molecules using iterative imaging and bleaching strategies.

A new approach is Airyscanning, which images the diffraction-limited illumination point (Airy disk) with an area detector, rather than a single-point detector. In Airyscanning, emission light is not blocked by a pinhole, thereby enhancing sensitivity, speed, and resolution.

COMPARISON OF IMAGING TECHNOLOGIES

	CLSM	Airyscan	Spinning Disk	Resonance Scan	SR-SIM	STED	PALM/ STORM
Signal-to-noise Ratio							
Avoiding Bleaching							
Speed							
Resolution							
Penetration Depth							
Spectral							

Microscopy Image Author Credits: (A, D) Nathan Kenny, Kathryn McClelland, Sophie Miller, Marine Biological Laboratory, Woods Hole, MA, USA. (B) Caroline Charlier and Daniel Dunia, Inserm U1043, CPTP, Toulouse, France. (C) Hooi Lynn Kee, Marine Biological Laboratory, Woods Hole, MA, USA.

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