Confocal Microscope Systems - A Comparison Of Technologies

by Nicolas George

Confocal microscopy offers a number of advantages when compared with traditional widefield optical microscopy. Confocal systems occlude out-of-focus light, vastly improving imaging contrast. By minimizing the haze of out-of-focus light and flare, they help the researcher view phenomena and structures not distinguishable under non-confocal conditions, particularly when using fluorescence imaging. In addition, confocal systems improve axial resolution and offer the potential to collect optical cross-sections from thick specimens.

Confocal imaging is accomplished by using a two-step process (Figure 1). First, excitation light that is focused on the specimen by the objective is initially passed through a small aperture, often a slit or pinhole. Alternatively, a very narrow beam of laser light can be introduced into the system via an optical fiber. By conditioning the excitation light this way, the amount of fluorescence not in focus can be controlled or minimized. Second, fluorescence emissions that originate from above or below the plane of focus are blocked by a second aperture or slit in front of the detector. The smaller this second opening, the higher the rejection rate of out-of-focus light and the thinner the optical section. These thin optical sections have greatly improved contrast and axial resolution, but they are obtained at the expense of overall specimen brightness.

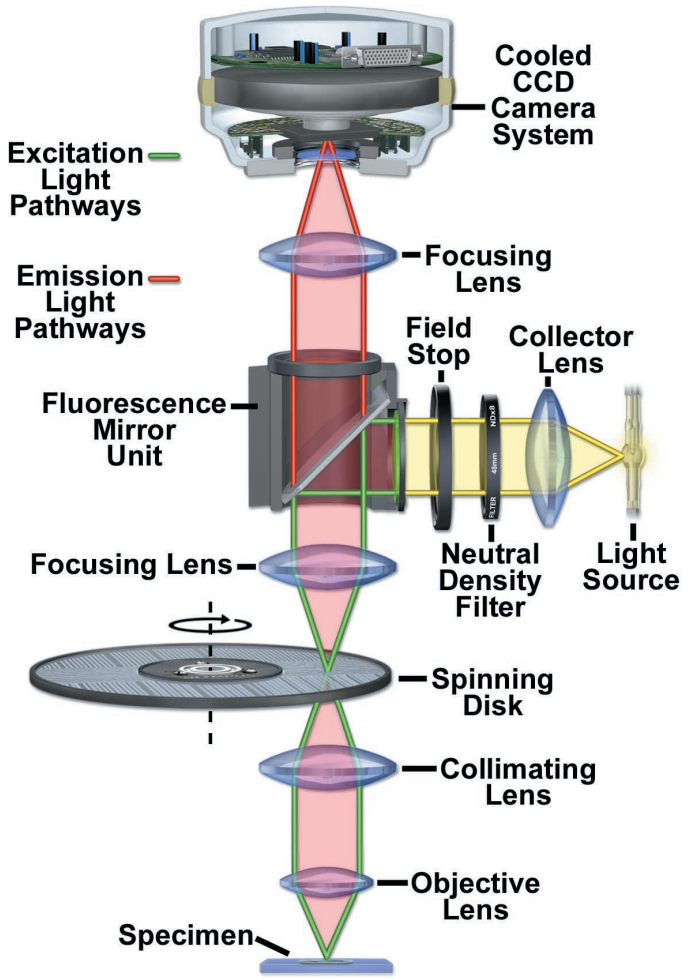

Figure 1: Confocal system layout, courtesy of Michael W. Davidson, Florida State University.

Laser scanning confocal systems

Two confocal techniques have been developed to deliver light to every point of a specimen within the focal plane. The first is the laser-based point scanning confocal system, where the excitation laser source is scanned across the specimen in a point-by-point raster pattern, so that, over time, a complete image of the focal plane is collected. The emitted light is then collected by the objective, passed through a pinhole aperture and detected by a photomultiplier tube (PMT). The resulting image is reconstructed and displayed by a computer. Often, multiple fluorescence probes are present in one specimen to highlight various structures or processes simultaneously. By compiling multiple optical sections of the specimen sequentially, the entire specimen can be reconstructed in three dimensions for analysis.

The disk provides great transmission while maintaining excellent confocality.

Laser-based point scanning confocal microscopes offer some extraordinary advantages. First, they allow extremely thin optical sectioning of specimens, permitting the viewing of structures and intracellular features never before seen — features deep within the specimen. Indeed, the use of a laser light source offers near-total control of where and how a very bright illumination source strikes the specimen. The point scanning confocal microscope is probably the most widely used type of confocal system in life science research. It offers the highest level of confocality and the ability to do very thin optical sections.

However, there are also several drawbacks to traditional laser-based confocal systems. These systems compromise detectable fluorescence in order to deliver their high degree of confocality. Because they use very small apertures and reject so much light, detectable emissions from the specimen must be very bright, and the objective used of the highest numerical aperture, or light gathering ability. This is why very intense laser light sources and very sensitive PMT detectors are popular in confocal systems with very small apertures. Lasers help address the low emissions problem, but the intensity of the laser light causes other serious repercussions — including both photobleaching in fluorescent probes, and phototoxicity in the specimen itself. In addition, the raster scan's point-by-point acquisition of the image is time consuming, making the system ineffective for recording short-time-period, live-cell events. For instance, scientists capturing a 1024 × 1024 high resolution image might obtain just one to three images per second, which may not be enough to capture a burst of light that lasts only a tenth of a second. Also, any specimen movement during the raster scan results in jagged edges in the image and poor definition of intracellular

details. Because of both the time it takes to capture an image and the damaging effects of so much light on living specimens, laser scanning confocal systems are typically used on fixed specimens. In addition, laser-based systems can be very expensive.

Spinning disk confocal aystems

The aforementioned limitations have led to another option for researchers — spinning disk confocal systems. These systems offer greater transmission, although they offer less confocality than laser-based systems. They are optimized for use by researchers for whom transmission, or speed, is an even higher priority than ultra-thin sectioning. The new spinning disk systems make possible far better speed, full frame imaging and higher transmission rates than laser scanning systems. With their larger pinhole or slit openings, however, they cannot deliver the same thinness of optical sectioning as their laser-based cousins. Researchers who prefer the spinning disk systems primarily include scientists whose work requires 4D (time-lapse) imaging of living cells.

There are two major types of spinning disk systems now available. The well-known Nipkow disk was first conceived by Paul Nipkow in 1884. Such disks can use either arc (white) sources of light, such as mercury or xenon, or they can use lasers. They use full-frame CCD cameras for image formation.

While Nipkow disk confocal systems offer good confocality and improved throughput, their tiny pinholes still do not allow as much light transmission as some experiments require. Furthermore, some systems have a "sandwich" of two disks; the microlenses that are placed at the pinholes to improve their light throughput makes these systems quite complex, both optically and mechanically. Finally, those disc systems that use lasers offer only limited excitation wavelengths. So until recently, researchers faced a trade-off that was less than optimal. Many have been seeking a spinning disk system that was optically optimized for both transmission and confocality, and used an arc light source — in short, a system that could provide better throughput than a Nipkow disk for time-lapse imaging, while still delivering excellent levels of confocality.

Recently, Olympus introduced the DSU disk scanning confocal microscope system (Figure 2). Proprietary, patent-pending disk technology delivers the optical sectioning capabilities of a Nipkow disk system but offers better light throughput. The DSU's disk is covered with a pattern of slits, rather than pinholes, providing greater transmission and making it markedly brighter while maintaining excellent confocality. Five easy-to-change disks of varying slit widths are available to suit different objective numerical apertures, magnifications and specimen thicknesses. Confocal images can be collected using objectives from 10× to 100×. Inserting the disk into the light path is a motorized operation, so the user can easily switch between confocal and widefield viewing via a computer. A full-spectrum mercury or xenon arc light source offers easy excitation wavelength selection via motorized filter turret, and the optical design delivers outstanding near-UV performance, even to 350 nm. The system offers full-frame CCD image capture at 15-to-20 frames per second with a streamlined single-disk, opto-mechanical design.

The DSU system can be combined with a motorized microscope for 3D confocal imaging. (Figure 3) The images can be deconvolved using any of the commonly-used deconvolution software packages available today.

The DSU confocal workstation is designed for the individual researcher, but can also be used in a multi-user facility to complement other microscopy methodologies. For instance, a researcher might grab time-lapse DSU images of a specimen to get a survey of events in the cell over time, and later tease out a specific region and acquire a laser-scanned confocal image using very thin sectioning for another perspective on the same cellular events.

For researchers who must balance the need for thin optical sectioning against the requirement for full-frame high-speed imaging, there are many choices in the field of confocal imaging. While laser scanning confocal systems offer unmatched image contrast, they are usually not optimized for acquiring high-resolution images of fast-changing cellular processes in action. For researchers who want maximum throughput and speed with minimal photobleaching and phototoxicity, the Olympus' DSU disk scanning confocal system offers an alternative to Nipkow disk systems and less flexible laser scanning confocal systems.

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Figure 2: The Olympus IX2-DSU confocal system offers a patent-pending spinning slit disk that delivers Nipkow-like optical sectioning with much higher transmission rates.

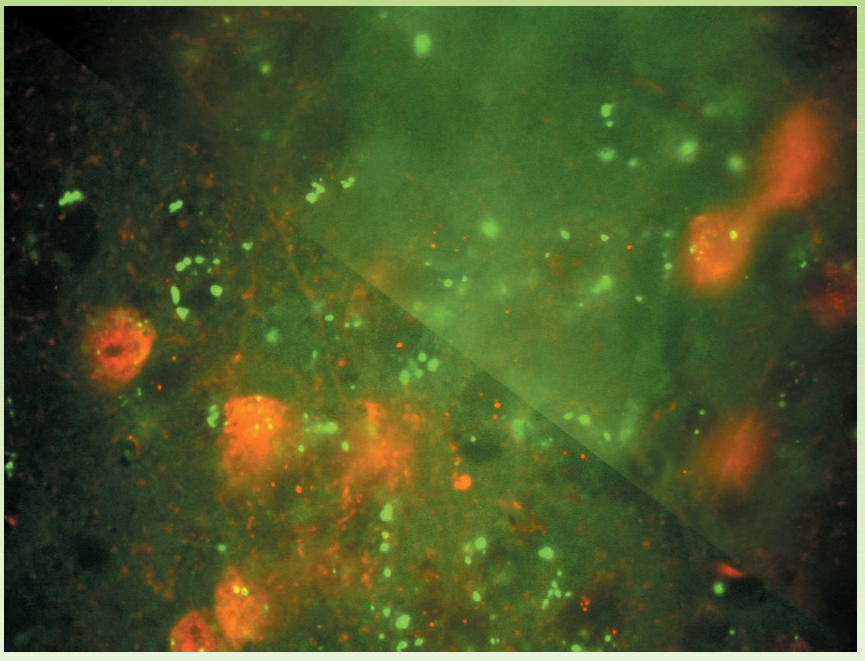

Figure 3: Comparison of widefield fluorescence and confocal images of a single specimen, a 50-micron section of mouse brain stained with GFP and Rhodamine and captured at 60X. Widefield fluorescence appears above the diagonal line; Olympus DSU confocal image using a PlanApo60X oil objective appears below the line. Specimen courtesy of Dr. Chris J. McBain, NICHD. Images courtesy of Olympus America Inc.