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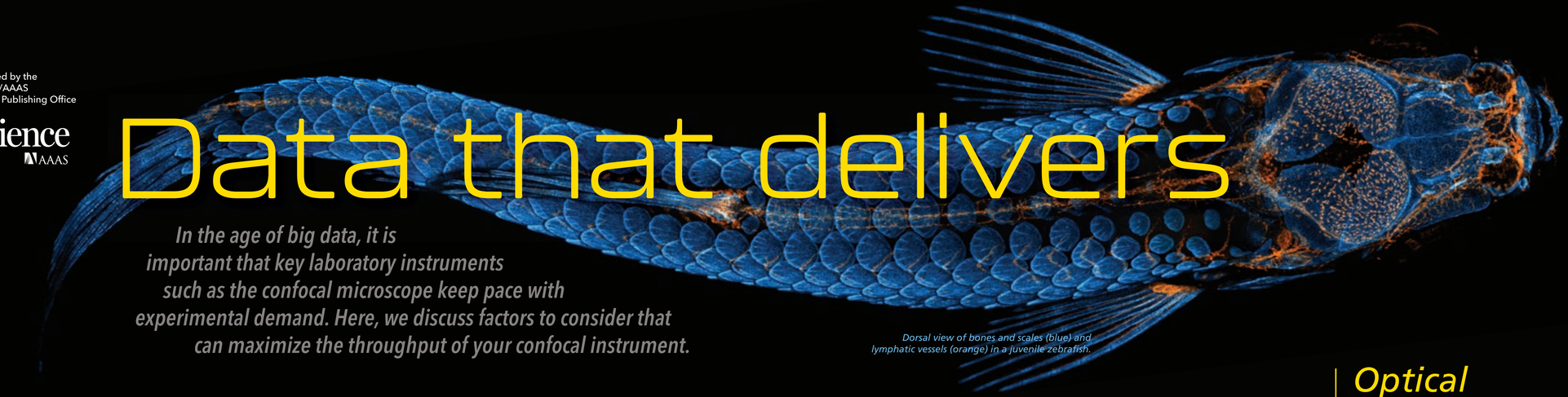
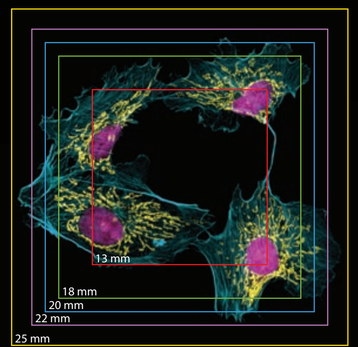
Data that delivers

In the age of big data, it is important that key laboratory instruments such as the confocal microscope keep pace with experimental demand. Here, we discuss factors to consider that can maximize the throughput of your confocal instrument.



Dorsal view of bones and scales (blue) and lymphatic vessels (orange) in a juvenile zebrafish.



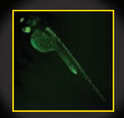
Actin (cyan), mitochondria (yellow), and nuclei (magenta).

Field of view (FOV)

A critical factor in confocal imaging throughput is the area of a single image field. A lower-magnification objective can image a larger field, but generally has lower resolving power, as measured by its ***numerical aperture (NA)***.

Optical systems have a magnification-independent, maximum field of view (FOV). Beyond this, the image quality rapidly degrades. The image to the left compares the FOV of different confocal instruments.

25-mm FOV



Danio rerio 2-day old embryo

Optimizing resolution across the FOV

To realize optimal performance at full resolution, it is important to achieve a pixel density that meets the ***Nyquist sampling criterion***. Sufficient sampling can be an issue with lower-magnification objectives. The FOV divided by the total magnification gives the usable field size (at the specimen plane). Once an objective is selected, the theoretical resolution limit can be estimated using its NA and a metric such as the ***Rayleigh criterion***.

The table shows how well a point-scanning system with a 25-mm FOV satisfies the Nyquist criterion with various low-magnification objectives (8,192 × 8,192-pixel resolution, 25-mm FOV, no scan zoom applied).

The NA determines the lens resolving power, calculated using the Rayleigh criterion (row 3). The back-projected virtual pixel size (last row) for the stated conditions should be less than or equal to the Nyquist sampling value in order to be within the range for imaging with the full resolving power (NA) provided by the lens.

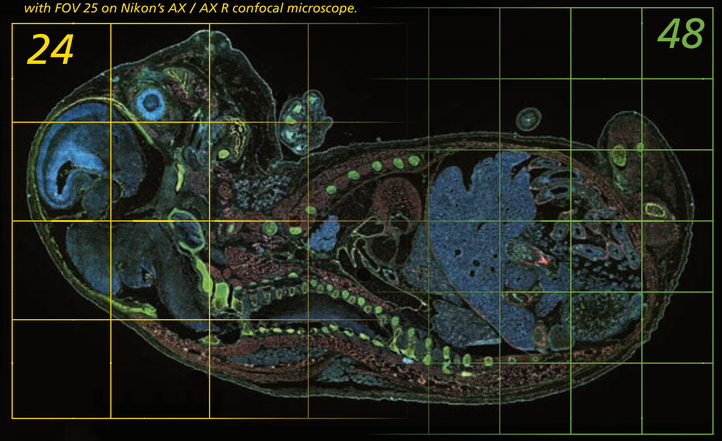
Objective Magnification (dry)	1X	2X	4X	10X	20X	40X
Numerical Aperture (NA)	0.04	0.1	0.2	0.45	0.75	0.95
Rayleigh Resolution Limit (XY, λ = 546 nm)	8.33 µm	3.33 µm	1.67 µm	0.74 µm	0.44 µm	0.35 µm
Nyquist Sampling (~2.3x, λ = 546 nm)	3.62 µm	1.45 µm	0.72 µm	0.32 µm	0.19 µm	0.15 µm
EXAMPLE: Minimum Effective Pixel Size (25-mm FOV)	2.16 µm	1.08 µm	0.54 µm	0.22 µm	0.11 µm	0.05 µm

Tiled acquisition

When the entire region of interest cannot be captured in a single image field, overlapping "tiled" images can be acquired and merged into a single file. The FOV determines the number of tiled images needed to capture the entire specimen. Decreasing the number of locations imaged can significantly shorten the overall acquisition time.

Entire region requires a total of about 24 frames with FOV 25 on Nikon's AX / AX R confocal microscope.

Entire region requires total of about 48 frames with conventional FOV 18.



Tiled multichannel fluorescence image of a rat embryo.

Scan speed

The image acquisition rate for ***spinning disk confocal*** systems is limited by the frame rate of the camera and the rotation speed of the disk. They scan many points in parallel to hasten image acquisition.

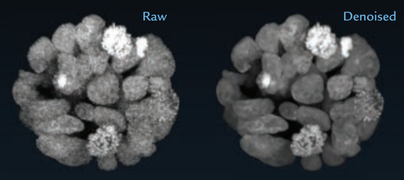
For ***point-scanning confocal*** systems, the laser-focus scan speed is limiting. These systems are inherently slower but can be accelerated using a resonant scanning mirror. Improvements in ***resonant scanners*** yield image quality approaching that of standard galvanometer (or galvano) scanners, where mirrors are driven using a sawtooth or triangular waveform, ensuring an equal dwell time at each point in the scan (but slower overall scan speed). Resonant scanners typically have lower pixel resolution than galvano scanners, requiring additional scan zoom, reducing the effective FOV but ensuring performance at full resolution.

Sensitivity

The signal collected from a specimen and the sensitivity of the detector impact the practical acquisition rate more than the maximum possible scan speed or camera frame rate do. Pairing a spinning disk system with a scientific complementary metal-oxide semiconductor (sCMOS) detector—the latest feature ***peak quantum efficiency (QE)*** values of ~95%—can achieve close to single-photon sensitivity. Point-scanning instruments rely on single-element detectors known as "photomultiplier tubes," which generally have a QE lower than that of the latest sCMOS cameras.

Camera images contain three principal noise sources: the camera readout, dark current, and shot noise. The ***signal-to-noise ratio (SNR)*** can be improved with post-acquisition analyses such as denoising and deconvolution. Nikon's unique Denoise.ai artificial intelligence software uses a pretrained neural network to rapidly denoise resonant-scanning confocal data, which is mostly affected by shot noise.

Deconvolution uses knowledge of the system's ***point-spread function*** to reassign out-of-focus light to its correct location. This can significantly improve SNR, even with confocal data where most out-of-focus light is eliminated by the pinhole aperture(s). Data for deconvolution can also be prefiltered using denoising analysis.



Raw versus denoised (using Nikon's Denoise.ai) resonant confocal image of Caco-2 cells stably expressing H2B-GFP, cultured in Matrigel.

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PUBLICATION: 19 November 2021

IMAGE CREDITS: Banner zebrafish: Courtesy of Daniel Castranova, Dr. Brant M. Weinstein, and Bakary Samasa (Eunice Kennedy Shriver National Institute of Child Health and Human Development); Sensitivity: Courtesy of Sally Cheung, Pelletier Lab, Department of Molecular Genetics, University of Toronto.

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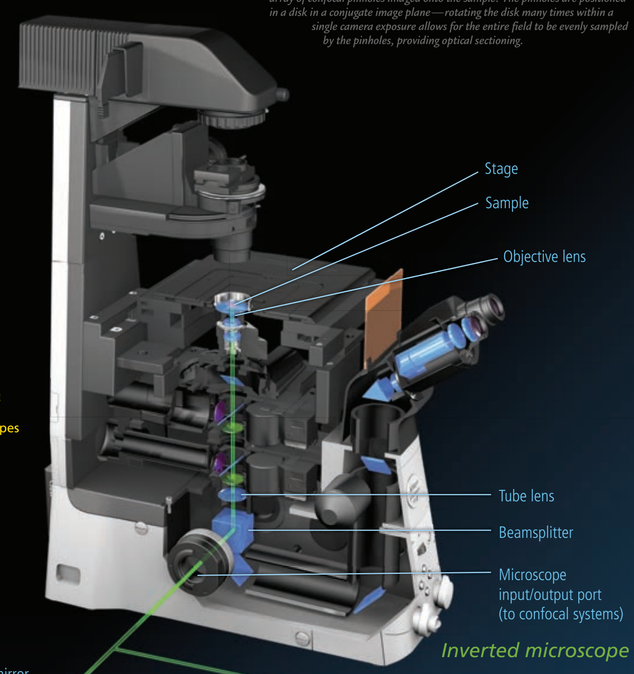
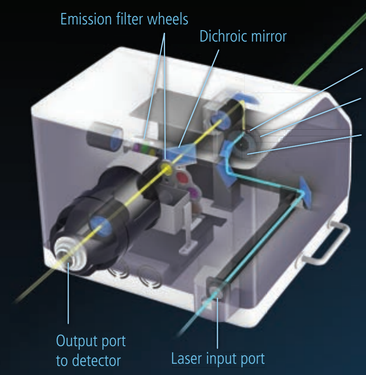
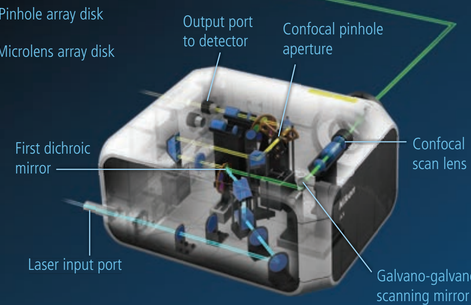
Optical path



Inverted microscope



Spinning disk confocal



Point-scanning confocal

Learn more about Nikon's range of confocal microscopes

GLOSSARY

Deconvolution: *the use of an iterative algorithm for reassigning out-of-focus light in a 3D dataset back to the correct plane/location to improve the signal-to-noise ratio (SNR) and available resolution.*

Numerical aperture (NA): *a measure of the resolving power of a lens, calculated using the formula NA = η sin(α), where η is the refractive index of the immersion medium and α is half the angular aperture of the lens.*

Nyquist sampling criterion: *requires that an analog signal (the image) be sampled at a rate of at least twice the highest frequency present (twice the resolution limit of the microscope). In practice, at least ~2.3X sampling is recommended.*

Peak quantum efficiency (QE): *QE is the percentage of incident photons that are successfully converted into photoelectrons by the detector. It is spectrally dependent, so manufacturers usually report the peak QE if citing a single value.*

Point-scanning confocal: *the confocal mode that scans the focus of a laser beam in the XY plane, imaging each point in the field. Emission light from each point is descanned and passes through a pinhole aperture in a conjugate image plane prior to reaching the detector. Out-of-focus light is mostly blocked by the pinhole, thus providing optical sectioning.*

Point-spread function (PSF): *the 3D diffraction pattern obtained by imaging an arbitrarily small (subresolution) object. The PSF can be thought of as a blurring function—its dimensions limit the obtainable resolution.*

Rayleigh criterion: *defines two objects as being optically resolved when the center of the diffraction pattern of one object overlaps with the first minimum of the other.*

Resonant scanners: *point-scanning confocal systems that use a resonant scanning mirror for the fast scan direction. Resonant scanning mirrors vibrate at a fixed, high-frequency rate. Galvano scanning mirrors are driven by an adjustable sawtooth signal, and scan speed is more limited by inertia.*

Signal-to-noise ratio (SNR): *the ratio of the detected signal to noise. Noise may originate either from quantum fluctuations in the number of emitted photons (which follows a Poisson distribution) or from the detector.*

Spinning disk confocal: *Instead of scanning a single point, these systems use an array of confocal pinholes imaged onto the sample. The pinholes are positioned in a disk in a conjugate image plane—rotating the disk many times within a single camera exposure allows for the entire field to be evenly sampled by the pinholes, providing optical sectioning.*

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