Airyscan detection in multiphoton microscopy: super-resolution and improved signal-to-noise ratio beyond the confocal depth limit

The penetration depth of traditional confocal laser-scanning microscopy (LSM) systems is limited by light scattering. To avoid these limitations, multiphoton LSM uses a nonlinear fluorophore excitation process in combination with a non-descanned detection concept to greatly increase the penetration depth. However, in traditional multiphoton LSM, this increased depth necessitates a compromise on the achievable spatial resolution and signal-to-noise compared with that of confocal LSM. The novel Airyscan detection concept from ZEISS, used in combination with multiphoton excitation, overcomes these limitations and provides increased resolution and signal-to-noise with a 2–3× increase in penetration depth compared with that of traditional confocal LSM.

LSM has become a standard imaging technique for basic biomedical research, as it allows the interrogation of model systems with a high degree of contrast and specificity for measurements of morphology, molecular interaction and cellular interaction, and can yield functional and structural information with subcellular resolution. LSM has traditionally consisted of two major technologies, confocal and multiphoton microscopy, both of which provide researchers with optically sectioned images for increased contrast and resolution[1–3]. In 2014, Zeiss introduced Airyscan as a revolutionary new detector concept for confocal LSM with substantially and simultaneously increased resolution, signal-to-noise ratio (SNR) and speed (in Airyscan Fast Mode). However, the depth to which confocal microscopy can penetrate into a sample is limited because of the scattering of light by the sample and the prevalence of optical aberrations as a function of depth. Multiphoton LSM circumvents these depth-penetration limitations by utilizing the two-photon effect from a pulsed near-infrared laser for fluorophore excitation and non-descanned detector concepts to collect the emitted signal. The major tradeoffs for extended depth penetration in multiphoton microscopy are reduced resolution and SNR compared with that achieved in confocal imaging. However, combination of the Airyscan detection concept with multiphoton excitation leads to a 1.8× increase in resolution in all three spatial dimensions compared with that of traditional multiphoton LSM4. Further, a substantial increase in SNR beyond that of traditional multiphoton LSM is realized owing to the >10× increase in higher-frequency information provided by the Airyscan detector[4]. The achieved increases in resolution and SNR are realized with a 2–3× increase in depth penetration beyond that possible with confocal LSM for most scattering samples.

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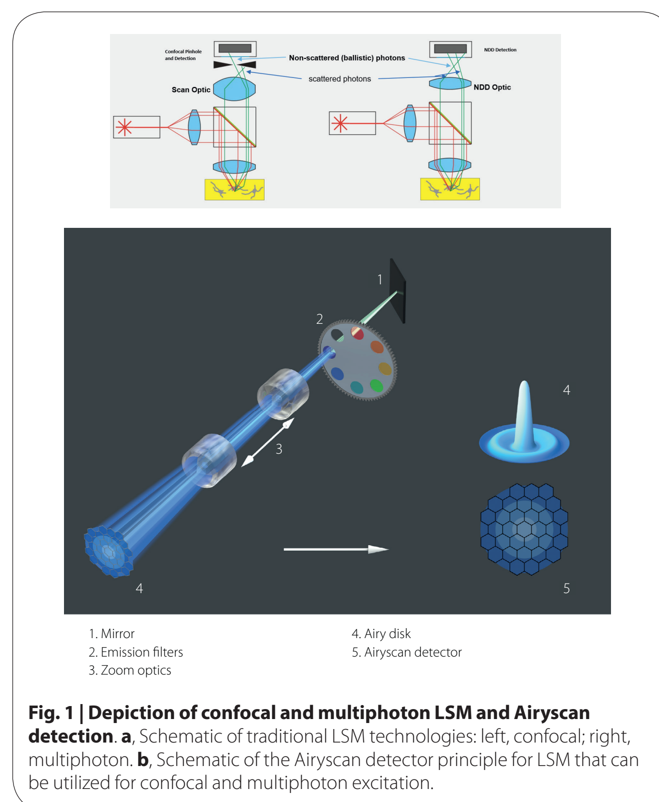


Fig. 1 | Depiction of confocal and multiphoton LSM and Airyscan detection. **a**, Schematic of traditional LSM technologies: left, confocal; right, multiphoton. **b**, Schematic of the Airyscan detector principle for LSM that can be utilized for confocal and multiphoton excitation.

In confocal microscopy, the optical-sectioning ability is created by placement of a field stop, the so-called pinhole, in a conjugate image plane in front of a detector along the fluorescence beam path. If the pinhole is sufficiently closed, out-of-focus light collected by the objective will be prevented from reaching the detector, thus creating an optically sectioned image. However, in the Airyscan detector from ZEISS, the traditional pinhole-and-detector design has been altered to offer greatly improved resolution and SNR. In the Airyscan detector, the physical confocal pinhole aperture and unitary detector assembly are replaced with a hexagonally packed detector array (Fig. 1). Just like the traditional confocal pinhole, the Airyscan detector is positioned in a conjugate focal plane relative to the excitation spot and uses a zoom optic arrangement to project a defined number of Airy unit orders onto the detector. By collecting the information of a pinhole-plane image in addition to a priori knowledge of the detection point spread function, the Airyscan detector increases both the spatial resolution and the SNR of all images, while maintaining the optical-sectioning ability of a traditional confocal microscope[3,5–7] (Fig. 1). The benefits of the Airyscan detection concept can also be extended to multiphoton excitation to gain substantial improvements in resolution and SNR (Fig. 2).

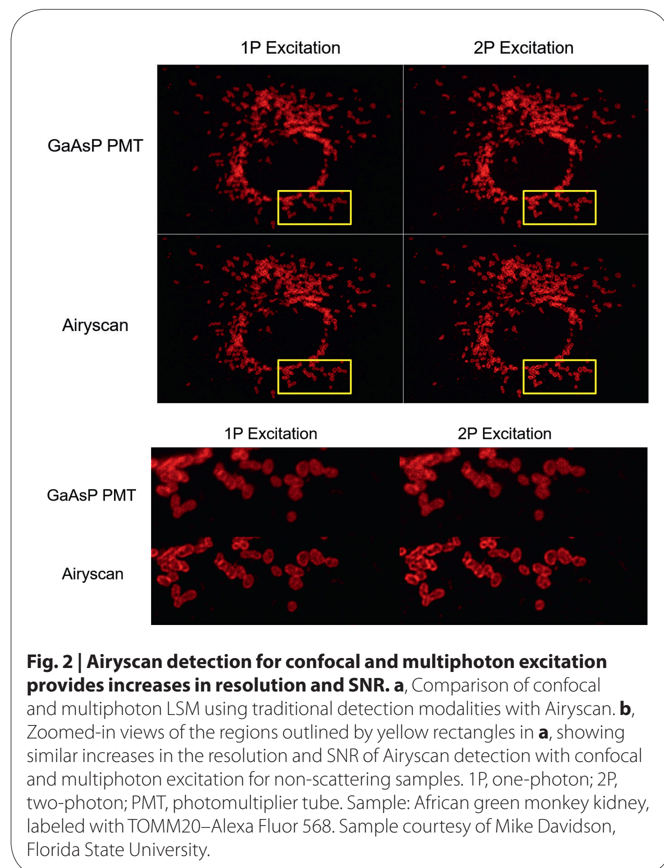
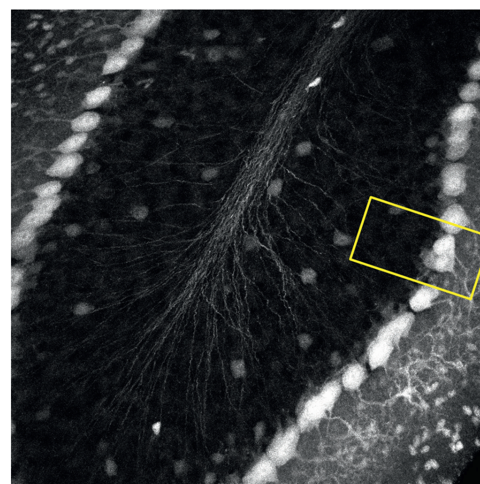


Fig. 2 | Airyscan detection for confocal and multiphoton excitation provides increases in resolution and SNR. a, Comparison of confocal and multiphoton LSM using traditional detection modalities with Airyscan. **b**, Zoomed-in views of the regions outlined by yellow rectangles in **a**, showing similar increases in the resolution and SNR of Airyscan detection with confocal and multiphoton excitation for non-scattering samples. 1P, one-photon; 2P, two-photon; PMT, photomultiplier tube. Sample: African green monkey kidney, labeled with TOMM20–Alexa Fluor 568. Sample courtesy of Mike Davidson, Florida State University.

Increase in depth penetration

Because of the limitations imposed by the scattering of light by the sample and the increased prevalence of optical aberrations as a function of depth, confocal LSM has a practical depth penetration limit of approximately <100 µm. As a response to this limitation, multiphoton LSM was developed for biological applications in 1990 as a way to increase the depth penetration and extend the application reach of LSM[2,3]. Like confocal LSM, multiphoton LSM profits from rastering of a diffraction-limited excitation point across a sample to create an image while also providing positional information about the excitation and detected fluorescence. In contrast, multiphoton LSM creates optically sectioned images by using a pulsed near-infrared laser to create a nonlinear excitation point spread function that is less prone to scattering from the sample[2]. Additionally, multiphoton LSM uses non-descanned detectors (NDDs) to maximize the percentage of emitted fluorescence photons collected from the scattering sample[3] (Fig. 1). For traditional multiphoton LSM, the light that reaches the NDD is a combination of both non-scattered and scattered fluorescence, and the proportion of scattered light increases with sample penetration depth8. Thus, as with image-scanning microscopy technologies, the Airyscan detection concept can be leveraged with multiphoton excitation to increase the imaging depth beyond that of traditional confocal LSM as long as the detected fluorescence has a high enough proportion of unscattered light[4,9–12]. If the non-scattered proportion is high enough, the resulting image resolution and SNR will be substantially increased, and the imaging depth will also be increased beyond what confocal LSM can achieve (~2–3×) (Figs. 2–4).



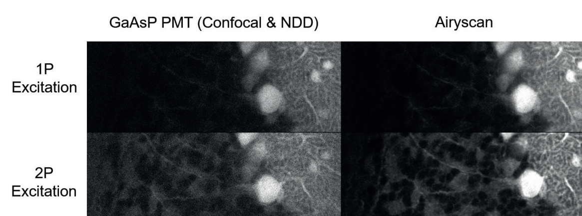


Fig. 3 | Airyscan detection with multiphoton excitation provides improved resolution and signal-to-noise beyond that of traditional confocal and multiphoton LSM. **a**, A *Drosophila* brain section with endogenous GFP in motor neurons imaged 300 µm from a sample surface excited at 900 nm. **b**, Zoomed-in views of the region outlined by the yellow rectangle in **a**, showing increased resolution and SNR of Airyscan detection with confocal and multiphoton excitation.

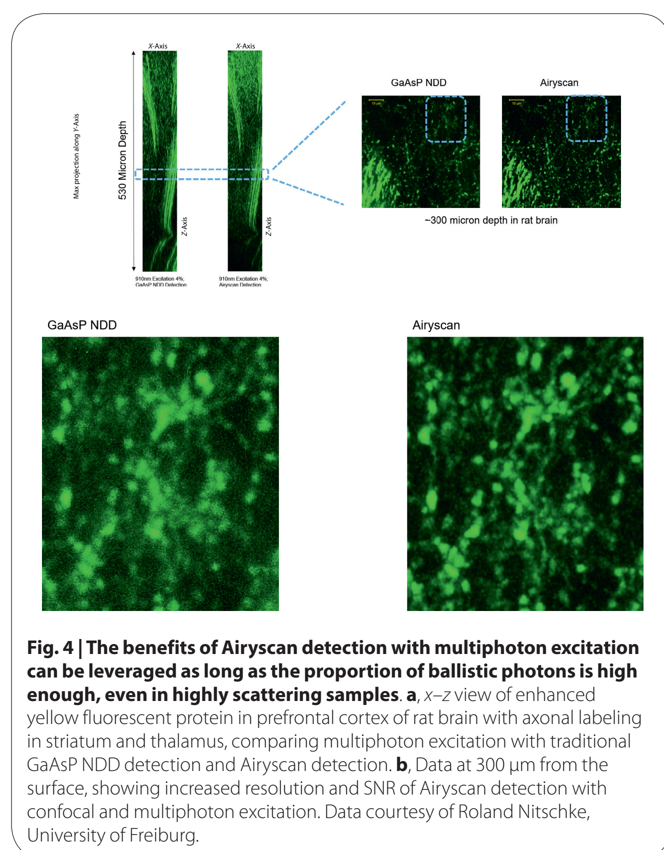


Fig. 4 | The benefits of Airyscan detection with multiphoton excitation can be leveraged as long as the proportion of ballistic photons is high enough, even in highly scattering samples. **a**, *x*–*z* view of enhanced yellow fluorescent protein in prefrontal cortex of rat brain with axonal labeling in striatum and thalamus, comparing multiphoton excitation with traditional GaAsP NDD detection and Airyscan detection. **b**, Data at 300 µm from the surface, showing increased resolution and SNR of Airyscan detection with confocal and multiphoton excitation. Data courtesy of Roland Nitschke, University of Freiburg.

Summary

Multiphoton LSM, like confocal LSM, creates an image by scanning an excitation laser and detecting the fluorescence at each position. The combination of the scan-position information with the additional positional information provided by the Airyscan detector can lead to substantial improvements in resolution and SNR. Further, as long as the proportion of non-scattered signal is high enough, Airyscan detection combined with multiphoton excitation allows for increased image contrast with high-spatial-frequency information not available in traditional multiphoton systems, at depths beyond what can be achieved with confocal LSM. Ultimately, Airyscan detection with multiphoton excitation delivers a simultaneous 1.8× increase in resolution and substantial increase in SNR compared with that obtained with traditional multiphoton LSM. Further, this improved performance is realized at depths 2–3× beyond what typical confocal LSM provides for most scattering samples.

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