Leica MICROSYSTEMS

HyVolution—the smart path to confocal super-resolution

Super-resolution refers to any device or method that can resolve better than the classical Abbe limit. Apart from infinite super-resolution techniques such as STED (stimulated emission depletion) and SMLM (single-molecule localization methods) that can theoretically resolve to any detail, there are also methods for limited super-resolution. Here we present HyVolution by Leica, which merges optical super-resolution and computational super-resolution. The optical part is provided by confocal microscopy, and the computational part by deconvolution. Lateral resolution of 140 nm is demonstrated. HyVolution offers multiple fluorescence recording in truly simultaneous mode.

Super-resolution—perceived as resolution beyond the classical diffraction limit—is achievable by a number of methods and technologies[1]. We call methods that can theoretically attain infinitely high resolution "unlimited super-resolution." In general, such resolution can be achieved only by the use of switchable probes[2] or other nonlinear approaches. Techniques that exceed the classical margin but are still limited to a finite value we call "finite super-resolution." The latter will not generally exceed the classical limit by a factor of two.

Leica Microsystems provides two unlimited super-resolution methods. For scanning systems, STED shapes the emission volume through stimulated emission. For wide-field systems, GSDIM (ground state depletion microscopy followed by individual molecule return) is available as a localization technique. With the use of on-board tools, confocal laser-scanning microscopes can generate a smooth solution for finite super-resolution: HyVolution.

HyVolution is the combination of optical super-resolution by confocal microscopy at sub-Airy pinhole sizes with subsequent computational super-resolution by image deconvolution. Both techniques combined allow for resolution up to 140 nm, at prime signal-to-noise ratio and with truly simultaneous multicolor recording. HyVolution also includes fast and seamless data communication, an array of powerful graphics processing units (GPUs) with high-speed programming technology.

Classical diffraction-limited optical resolution in microscopes

Two references are cited in most treatises on microscope resolution. One of them is the Abbe limit[3], deduced from illuminating a sample that is regarded as a multitude of gratings and causes diffraction patterns that have to enter the entrance pupil of the microscope. The other is the Rayleigh criterion[4], which describes a distance between two points that generate diffraction patterns at the entrance pupil of the microscope. Similar to the Rayleigh criterion is the undulation criterion[5], which is numerically very close to the Abbe limit. A more practical definition of resolution is the full-width at half-maximum (FWHM) of a spot's diffraction pattern, as it is easily measured in actual images[6].

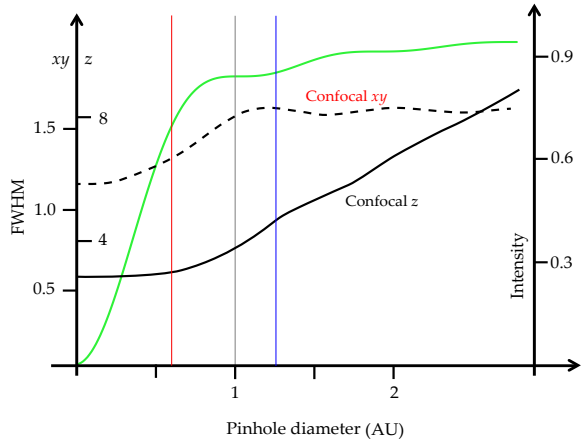

Figure 1 | Parameters for confocal super-resolution microscopy. The green line represents the focal intensity at the detector position as a function of the pinhole diameter in Airy units (AU). The intensity of the diffraction pattern is zero at 1 AU; therefore, the detected integral intensity is flat at that position. Increasing the size does not significantly increase the focal intensity but adds out-of-focus light only. The dashed line shows that the lateral resolution does not differ from classical diffraction-limited resolution for pinholes larger than 1 AU. The highest resolution achievable is about 28% better than the diffraction limited resolution. The solid black line shows the *z*-sectioning performance. When the pinhole is opened wider than 1 AU, the contribution from defocus positions increases linearly. Data for black curves obtained from ref. 8.

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Although they use different assumptions, the results of all three approaches are quite comparable, and as a reference, it is a good convention to use the Abbe limit, $d_A = \lambda/(2 \times NA)$, where NA denotes the numerical aperture and λ is the wavelength.

Confocal super-resolution

As mentioned, the first step of HyVolution is the optical performance of confocal microscopes in the regime, where lateral resolution is improved. This aspect is explained below.

The huge impact of confocal microscopy on biomedical research and routine is mainly due to the fact that confocal microscopes transmit only focal signal and thus create optical sections[7]—if operated properly. Proper operation requires the pinhole to be closed enough for extrafocal light to be rejected at the pinhole in the detection path. Sectioning performance and lateral resolution[8] are shown in **Figure 1**. Also shown is the measurable focal intensity as a function of the pinhole size, calculated by integration of the radial intensity distribution of a point object diffracted at a circular aperture (Airy pattern). These dependencies reveal three important facts:

1. The lateral resolution is independent of the pinhole diameter if the diameter exceeds the value of λ/NA (= 1 AU). For smaller pinholes, the resolution increases by up to some 28% at pinhole zero. At a diameter of about 0.6 AU, the resolution has already improved by 20%.
2. The sectioning performance (thin optical sectioning) approaches a limit at pinhole zero. At a pinhole of 0.6 the diffraction-limited value is more or less achieved. At the usually suggested pinhole size of 1 AU, the section has already broadened by 30%. If the pinhole is opened further (e.g., to 1.25 AU), the performance drops by 70%.
3. The focal intensity reaches 84% of the total amount of light if the pinhole diameter is set to 1 AU. At 0.6 AU the intensity drops by some 12%. If the pinhole is opened to 1.25 AU, the intensity increases by only 1%.

These considerations show that there is good reason to use a pinhole size below 1 AU, with a good compromise offered at 0.6 AU. The lateral resolution is increased significantly, whereas the *z*-sectioning performance is approaching the best value. The intensity is impaired by only 1/8 of the total. It is also obvious that high transmission of the confocal microscope in conjunction with a highly sensitive sensor at very low noise and background will compensate several times over for the minor concession of omitting the pattern margins.

It is also evident that increasing the diameter above 1 AU significantly impairs the *z*-sectioning performance—the very feature that occasioned all the outlay for a confocal microscope. The gain in focal intensity is obviously nearly none, as the diffraction pattern has a zero at 1 AU, and the following increase is much less compared to the central peak. If one were to open the pinhole in order to get "brighter images," all the brightness would be out-of-focus light, whereas the initial aim was to prevent such light from reaching the sensor by introducing a confocal beam path.

Super-resolution by deconvolution

The second step of HyVolution is the extraction of resolved information from the collected data by image restoration[9], usually referred to as deconvolution. This is yet another technique that allows an improvement in resolution with a proper and efficient confocal setup. Deconvolution is more than simply a mathematical operation that averages noise or tries to enhance features in a given image. True image restoration uses *a priori* information about how the microscope modifies a point signal. The transformation of a point into a diffraction pattern is described by the point-spread function.

Point-spread functions can be calculated via computation of the three-dimensional diffraction pattern on the basis of the optical parameters, NA and λ. Alternatively, one can measure the point-spread function by recording a three-dimensional image stack of a tiny object (usually fluorochromized latex beads) that is significantly smaller than the classical resolution limit.

True deconvolution has two beneficial effects on the image quality:

1. Improvement of resolution. The separability of close point objects can be augmented by a factor of ca. 2 in the lateral and ca. 4 in the axial direction[10].
2. Improvement of signal-to-noise ratio (S/N). The noise components are effectively reduced by the deconvolution process.

As is the case with any analog or digital signal processing, a good low-noise image is a desirable starting condition. The microscope should be designed to collect as many photons as possible from the fluorescing volume and show very little dark signal. To perform the calculations speedily and view the results immediately after data acquisition, modern computers are equipped with additional arrays of GPUs, driven by specialized software.

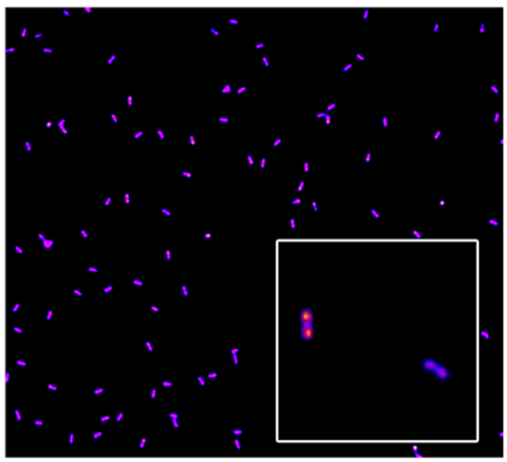

Figure 2 | Perfectly resolved DNA origamis (Gattaquant DNA Technologies) by Leica SP8 HyVolution. The space between the two markers in these origamis is 140 nm. The inset shows two origamis at higher magnification.

Performance requirements and components

As mentioned above, the combination of small pinhole diameters and deconvolution algorithms will benefit from a superior performance of the confocal device (which is desirable anyway). HyVolution,

the Leica TCS SP8 Confocal Microscope equipped with the SVI Huygens deconvolution suite, communication software and GPU arrays, meets these expectations to the user's utmost satisfaction and offers the following benefits:

1. Most transparent beam path. Acousto-optical beam splitting in combination with multiband spectrometric detection has very few air-glass interfaces, as there are no planar filters or lossy gratings involved. As a consequence, the beam path provides maximum transmission from the sample to the sensor.
2. Efficient and low-noise hybrid detectors (HyD). The perfect sensor for imaging featuring extreme low dark signal, very low peak noise and short pulse width. It still offers a high dynamic range for any biomedical fluorescence application. HyDs use a GaAsP photocathode for maximum quantum efficiency and have no multiplicative noise as caused by dynodes in conventional photomultiplier tubes. They are ideally applied with photon-counting equipment.
3. Well-regarded and professional deconvolution software Huygens Suite by SVI. This software provides true image restoration by applying algorithms using a point-spread function to reconstruct the original emitter.
4. Enhanced communication between acquisition software and deconvolution software to ensure seamless data exchange and image presentation.
5. Implementation of high-speed GPU arrays driven with CUDA software for top-end mathematical processing even of large data sets.

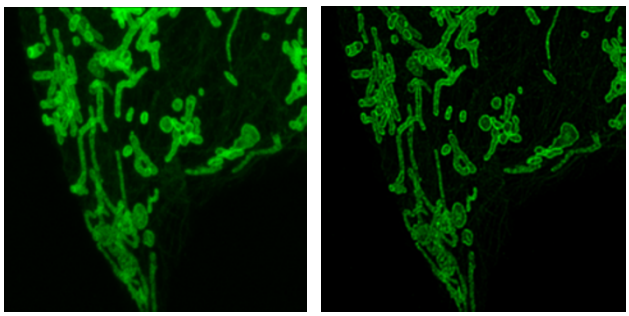

Figure 3 | Resolution improvement in xy. Images show mitochondrial membranes labeled with TOM20-GFP. Sample courtesy of Urs Ziegler, ZMB, University of Zurich, Switzerland. Left, confocal imaging; right, HyVolution.

HyVolution and benefits

The Leica TCS SP8 HyVolution offers superior imaging with super-resolution performance down to less than 140 nm at very low noise levels (**Figs. 2** and **3**). Images can be recorded with multiple channels in parallel (true simultaneous scanning—not just line-sequential). Super-resolution and high-S/N imaging is available for both classical and resonant scanning, offering large fields of view and very fast time sampling. All combinations of lasers and fluorochromes are compatible with the HyVolution technique. The HyVolution super-resolution approach does not introduce additional optical elements, so it does not compromise the efficiency of the confocal microscope.

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