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Assessing resolution in super-resolution imaging

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ABSTRACT

Resolution is a central concept in all imaging fields, and particularly in optical microscopy, but it can be easily misinterpreted. The mathematical definition of optical resolution was codified by Abbe, and practically defined by the Rayleigh Criterion in the late 19th century. The limit of conventional resolution was also achieved in this period, and it was thought that fundamental constraints of physics prevented further increases in resolution. With the recent development of a range of super-resolution techniques, it is necessary to revisit the concept of optical resolution. Fundamental differences in super-resolution modalities mean that resolution is not a directly transferrable metric between techniques. This article considers the issues in resolution raised by these new technologies, and presents approaches for comparing resolution between different super-resolution methods.

1. Introduction

Microscopic images at the theoretical limit of optical resolution have been available for well over 100 years. Recently, the diffraction limit has been overcome by a number of new optical imaging techniques, collectively known as super-resolution microscopy [1,2]. Two critical questions pose themselves to scientists interested in applying these new technologies. Firstly, what is the actual resolution of the resulting image? Secondly, is the increased effort and expense of super-resolution, as compared to conventional microscopy, worth the increase in resolution? The recent introduction of a range of commercial super-resolution instruments means that resolution has once again become a battleground between different microscope technologies and rival companies.

In this paper we classify super-resolution microscopy into three broad classes and assess the achievable resolution in each. As different super-resolution techniques produce images that are amenable to different methods of resolution measurement, we consider these different measurement methods, to which super-resolution microscopy methods they can reasonably be applied, and address some possible artifacts in their application. We also provide metrics for a realistic expectation of achievable resolution using relatively standard techniques and commercially available instruments (Table 1). In all cases these resolutions have been surpassed, but measurements from standard biological samples, with commercially available equipment, are a useful comparison for assessing the relative merits of different super-resolution techniques.

It should be noted that resolution is a multifaceted concept, and that measuring it is not as trivial as it may appear. To meaningfully compare different techniques it is essential to specify the exact definition of the resolution one is measuring. Further, resolution is only one of many contributors to the utility of a given microscopy technique. There are also a number of external factors such as sample labeling and signal-to-noise ratio that can affect image quality While these do not change the system's resolution, such factors significantly affect the overall image quality, even if the resolution is still extremely high.

2. Material and methods

2.1. Sample preparation

Drosophila macrophages were prepared from third instar larvae as previously described [3]. Cells were prepared for imaging with an immunofluorescence protocol modified for macrophage microtubules (Wegel et al., manuscript in preparation), with monoclonal mouse anti α-tubulin (Sigma–Aldrich T6199, clone DM1A) primary antibody, and polyclonal donkey anti mouse AlexaFluor 488 (Life Technologies A11015) secondary antibody. Samples were embedded in Prolong Gold (Life Technologies P36930) and cured for 16–20 h before sealing.

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Table 1
FWHM of microtubules, generated from images in Fig. 1 and other similar images to get representative averages. Reported as mean ± standard deviation. $n = 11$ for 3D-SIM; $n = 33$ for gSTED; $n = 11$ for dSTORM.

Technique	FWHM
3D-SIM	108 ± 5 nm
gSTED	63 ± 20 nm
dSTORM	42 ± 4 nm

2.2. Microscopy

2.2.1. 3-Dimensional Structured Illumination Microscopy (3D-SIM), widefield, and widefield-deconvolution data

3D-SIM data was generated on a DeltaVision OMX V3 Blaze system (GE Healthcare) equipped with a 63× 1.42 N.A. PlanApo oil immersion objective (Olympus), a 488 nm diode laser, and Edge 5.5 sCMOS cameras (PCO). Image stacks of ~5 µm were acquired with a *z*-distance of 125 nm and with 15 raw images per plane (five phases, three angles). Raw datasets were computationally reconstructed with a channel-specific measured optical transfer function (OTF) and a Wiener filter set to 0.002 using the softWoRx 6.1 software package (GE Healthcare). Negative intensity values of the 32-bit "raw" reconstructed data that do not carry any structural information were clipped using the "Threshold & 16-bit conversion" utility of the *SIMcheck* plugin suite for ImageJ (Ball et al., manuscript submitted). Pseudo-widefield and widefield-deconvolution data were generated with softWoRx 6.1 software by averaging all phase-shifted images from all angles for each *z*-position of the raw SIM dataset and by subsequently applying an iterative 3D deconvolution with softWoRx 6.1.

2.2.2. gated STimulated Emission Depletion (gSTED) data

gSTED data was generated on a SP8 gated STED system (Leica), equipped with a HCX PlanApo 100× 1.40 N.A. oil immersion objective (Leica), and HyD detectors (Leica). Probes were excited with a pulsed 488 nm laser and depleted with a 592 nm STED laser. Images were acquired with Leica LAS AF software's TCS SP8 module.

2.2.3. direct Stochastic Optical Reconstruction Microscopy (dSTORM) data

dSTORM data was generated with a modified DeltaVision OMX V2 system (GE Healthcare) equipped with a 100× PlanApo 1.40 N.A. oil immersion objective (Olympus), a 488 nm diode laser, Evolve 512 Delta EMCCD cameras (Photometrics), and a customized light path with approx. 30 kW/cm^2 laser intensity. 4000 images were acquired in widefield illumination with an integration time of 50 ms. Localizations were produced with *fastSPDM*, a maximum-likelihood-based algorithm with a sliding window for background subtraction [4], and adapted to the hardware configuration of the microscope. Super-resolution images were generated from the position data based on nearest-neighbor distances in order to account for label density effects limiting the resolution [5].

2.3. Analysis

Fourier spectra were generated using the FFT function in the Fiji implementation of ImageJ [6,7]. The power spectrum, the amplitude squared of the complex FFT, is log scaled in order to enable visualization of the high central peak and the low intensities near the noise floor. The FFT images were then normalized and radially averaged around the center to generate FFT line profiles using the *Radial_Profile* ImageJ plugin (http://rsb.info.nih.gov/ij/plugins/radial-profile.html). Overlaid concentric rings indicate the equivalent spatial resolution as distance from the center point. These radial profiles of the frequency distribution clearly show how structures at different sizes are represented in the image, thus allowing assessment of the image resolution.

Full Width Half Maximum (FWHM) of microtubules were measured in ImageJ by generating line profiles of single microtubules integrated over a width of 165 nm in all image types and fitting these profiles with a Gaussian distribution. The FWHM was calculated as $2\sqrt{(2\ln 2)}\sigma$, where σ is the Gaussian width parameter.

3. Measuring resolution

There is a dichotomy between the theory and practice of resolution in optical microscopy. The theoretical approach is almost universally based upon the Rayleigh Criterion [8], whereby the resolution is defined as the distance at which the first trough in the Airy disk of one point object falls exactly on the peak of another. This is calculable from Airy's original theory [9] and works out as the classic resolution limit of $1.22\lambda/2$ NA, where λ is the wavelength and NA is the system's numerical aperture (for epi-fluorescence, in transmission the denominator becomes (NA_{cond} + NA_{obj}), where NA_{cond} is the condenser NA and NA_{obj} the objective NA). This is equivalent to a contrast limit of about 25%, *i.e.* there is an ~25% dip in intensity between the two peaks and the trough between them. In practice, the resolution is usually measured as the Full Width Half Maximum (FWHM) of a point object, such as a sub-resolution fluorescent bead. To improve the sensitivity of these measurements, the single peak is then usually fitted with a Gaussian distribution (see Section 2.3).

Another resolution definition, which may be thought to be more representative of achievable resolution in a system, is the Sparrow Criterion [10]. Briefly, this is similar to the Rayleigh Criterion, but says that objects are distinguishable until there is no dip between the peaks. This has two major drawbacks over the Rayleigh criterion: (1) it is very signal-to-noise dependent, and (2) it has no easy comparison to a readily measured value in real images, such as the FWHM for the Rayleigh Criterion.

The theoretical and practical approaches are fundamentally different; however in practice the results are similar, as the FWHM is similar to the distance between the peak and the first trough of a real Airy disk. Critically, this depends upon the shape of the Point Spread Function (PSF). A Gaussian is generally a good approximation of the point spread function of a microscope objective, so equating these different measurements is reasonable. It should be noted that this is an approximate equality, and so extra care should be taken in extreme circumstances such as super-resolution imaging.

The underlying reason for the difference between the theoretical and practical approaches is that the theoretical approach aims to precisely calculate the achievable resolution of a system under ideal conditions, whereas the practical approach aims to be simple and robust. If one, *a priori*, knows that there are two point sources, then measuring their separation, and hence calculating the system's resolution is purely limited by Signal-to-Noise Ratio (SNR). The Rayleigh Criterion is a realistic rule of thumb, giving an approximate measure of the smallest object resolvable in a well-calibrated optical microscope. However, with additional information, such as the fact that there is a single point object in a given region, significantly more accurate results can be obtained. This is the fundamental technique behind Single Molecule Localization Microscopy (SMLM) based techniques (see Section 3.2.3.), and the reason why "system resolution", "localization precision", and "image resolution" are not identical.

Traditionally, approximating the resolution of a conventional microscope with the FWHM of a Gaussian fit to a PSF (the image of a single sub-resolution point source) is a good approximation