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Resolution in super-resolution microscopy — definition, trade-offs and perspectives

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Super-resolution microscopy (SRM) is gaining popularity in biosciences; however, claims about optical resolution are contested and often misleading. In this Viewpoint, experts share their views on resolution and common trade-offs, such as labelling and post-processing, aiming to clarify them for biologists and facilitate deeper understanding and best use of SRM.

How would you define resolution?

Kirti Prakash: There are two fundamental resolution metrics: the Abbe limit[1], wherein diameter $(d)_{x,y} = \lambda/(2\mathrm{NA})$ (in lateral direction; $d_z = 2n\lambda/\mathrm{NA}^2$ in axial direction), which provides a fundamental physical basis to resolution in terms of the vacuum wavelength of light (λ) and the numerical aperture (NA), and the Rayleigh criterion[2], wherein $d_{x,y} = 0.61\lambda/\mathrm{NA}$ and related full width at half maximum (FWHM), which are heuristic and estimate the closest distance between two fluorescent point sources, in which they can still be distinguished. The latter is frequently used to characterize the resolution of SRM methods, but it can be easily manipulated. Therefore, more rigorous measures need to be developed to define resolution for techniques that overcome the diffraction limit.

David Baddeley: Analysing resolution in the Fourier domain (that is, frequency bandwidth) is unambiguous and yields elegant explanations of the resolution improvements in structured illumination microscopy (SIM)[1], confocal, and multi-photon techniques. However, for stimulated emission depletion (STED)[2] and single-molecule localization microscopy (SMLM)[3] techniques, the frequency domain answer is simply 'theoretically unlimited' and the effective usable resolution is instead limited by noise or background. This ambiguity has led to the use of FWHM of the point spread function as a resolution proxy, although it is a poor measure for SMLM, wherein the density of detected localizations usually limits the practical resolution.

Reto Fiolka: In techniques such as SIM or image scanning microscopy (ISM; also known as confocal microscopy with photon reassignment, for example, Airyscan and iSIM), a clear cut-off frequency defines the highest possible resolution, which defines an upper limit for their resolving power. In techniques that have theoretically no resolution limit, such as STED, SMLM or minimal emission fluxes (MINFLUX), resolution might be defined by the highest spatial frequency that can be detected above the noise floor. To experimentally measure resolution, combining FWHM and Fourier ring correlation (FRC)[4] or image decorrelation analysis[5] on suitable samples (that is, beads that are more than twice smaller than the resolution limit, and sufficiently small-scaled biological structures) is preferable. Both methods have flaws (both involve somewhat arbitrary thresholds) and complementary strengths, so achieving agreement between both is a good sign.

Rainer Heintzmann: Most SRM techniques exploit prior knowledge of the sample and code the information transfer, often using the time dimension. Methods with a linear sample response that make no assumptions on the sample achieve improvement of up to twice as much resolution compared to the conventional diffraction limit whereas methods exploiting nonlinear sample response (for example, STED and nonlinear SIM) are conceptually unlimited in their ability to transmit spatial frequencies. Localization-based methods form a separate group as they explicitly exploit an object consisting of spatially discrete entities (that is, fluorescent probes); here, the resolution is better defined in terms of localization precision, which scales with the number of photons[3]. Notably, any optical method scales with the wavelength of light if only light is used to probe the sample.

Suliana Manley: One of the most useful definitions of resolution for SRM is based on decorrelation analysis[5], which can be used on single images and is agnostic of the microscopy method. As decorrelation is applied to images, it requires that data from SMLM be rendered as an image using a method that allows an unbiased estimate of resolution.

Aleksandra Radenovic: Resolution is not an absolute measure but an imaging modality performance indicator that must be contextualized with control experiments and a comprehensive understanding of all processes involved, from sample preparation to image post-processing.

Carlas Smith: Most resolution metrics provide a binary interpretation of resolution. Thus, it is more important to assess the spectral signal-to-noise ratio[6], which is a measure used to assess the quality of a signal to the level of noise in the frequency domain. Understanding the signal-to-noise ratio required to discern various structures provides much more valuable insight than a single number.

Hari Shroff: The idea of the microscope system as a low-pass filter that attenuates spatial frequencies is attractive. In this picture, there is a rigorous 'resolution limit' in diffraction-limited microscopy, which is the Abbe cut-off — a maximum spatial frequency that the optics can transmit. Similarly, a natural notion of image contrast is spatial-frequency dependent (for example, contrast or modulation transfer function)[7].

Lothar Schermelleh: Resolution can be defined in various ways: do we refer to the theoretical resolution based on a specific system configuration, the measured resolution in perfect conditions using an ideal target (FWHM of beads, microtubules), or the effective achievable resolution within a biological sample? Also, do we measure the average resolution across the entire image or on selected 'cherry-picked' features? This confusion is often (mis)used to sell technology. A key objective for cell biologists is to determine the 3D shapes and distances of macromolecular structures in their

subcellular context, either in situ or in vivo. Thus, any resolution claim achieved in ideal conditions should be complemented by the 'effective resolution' of the sample image, ideally in *xy* and *z* directions.

How should biologists regard resolution claims and assess them in their data?

K.P.: Biologists should not be misled by specific resolution claims, particularly regarding SMLM techniques. Methods claiming a few nanometres to angstrom resolution typically refer to localization precision assessed on DNA origamis or similar well-defined structures with almost zero background[8]. A prudent test for the resolution power is the ability to resolve known biological test structures. For example, if the outer ring of the nuclear pore complex is resolved, the structural resolution is ≤100 nm; if the inner ring is resolved, it is ≤50 nm; and if the dimer structure of one of the eight subunits is resolved, then it is ≤12 nm. Owing to energy transfer, it will always be difficult to achieve a biological resolution of <10 nm.

D.B.: There are different scenarios in which biologists want to assess resolution. First, "Should I use/buy this?", that is, deciding whether a given technique can answer their question(s) and whether it is worth investing time and money in it. This issue is multidimensional, and a single (typically optimal) resolution metric is only marginally helpful. Second, "Is my system performing as it should?" For this assessment, FRC is a useful metric, as it is relatively easy to compute and good for detecting degradations within a modality caused by drift, vibration or changes in labelling. Last, "Can a claimed resolution improvement explain a new observation?" This question is also multi-factorial, especially when dealing with live-cell imaging, and the ideal answer would be to perform 'virtual microscopy' in silico with models of your biological system and microscopes. Failing that, robust controls and demonstrations of reproducibility are probably more valuable than a resolution metric when determining whether a new observation is real.

R.F.: For linear SIM and ISM techniques (and also for STED), small fluorescent nanosphere beads can serve as standards to measure resolution. However, beads are an ideal case (providing large signal-to-noise ratio with minimal impact of aberration) and as such demonstrate how close one can get to the theoretical resolution of a given technique. Some biological structures can test the resolution of SIM and ISM inside a cell, such as clathrin-coated pits that can serve as puncta and be analysed by FRC. SMLM and expansion microscopy may need different test samples. For expansion microscopy, stereotypical biological structures such as nuclear pores can serve as biological nano-rulers. As the resolution improves, researchers may use smaller structures, for which electron microscopy reconstructions exist.

R.H.: It is often difficult to validate resolution claims in typical biological samples. Standard measures such as FWHM or FRC can be misleading at best as they measure the repeatability of an experiment, but they do not necessarily inform how well biological features can be distinguished. Ideally, one would wish to use a range of standard samples differing in their (fractal) dimension. Examples include 30-nm (zero-dimensional) vesicles as an approximation to point-like objects, tubulin fibres as line-like (one-dimensional) samples, lamin-stained cell nuclei as two-dimensional samples, and DAPI-stained chromatin as an approximation to structured volume stains. The most convincing definition to biologically interested users is whether a particular feature known to exist in the sample is visible in the images before and after processing.

S.M. and H.S.: It is important to distinguish between 'resolution in theory' and 'resolution in practice'. Claims of resolution in proof-of-concept papers typically present a best-case result. They can be useful as a guide for which methods will be needed to resolve features at a desired scale. Achieving published resolutions often requires substantial sample and acquisition optimization. It is naive to use a single number (for example, the FWHM of a single bead) to infer the resolution in practice, and it is useful to combine complementary methods (Rayleigh, decorrelation, Fourier analysis and so on) and apply them in different imaging situations.

A.R.: In SMLM, FRC resolution estimates are strongly dependent on multiple blinking corrections and grouping of localizations, whereas decorrelation analysis is not[5]; to paraphrase G. E. P. Box, all resolution estimates have their limitations, but some can still be quite useful.

The contributors

Kirti Prakash has degrees in computer science and biology. His expertise lies in single-molecule super-resolution microscopy, mathematical modelling and machine learning. He is dedicated to developing pioneering tools for advanced microscopy, with a research focus on epigenetics and chromatin structure.

David Baddeley is trained as a physicist and started working on super-resolution imaging problems in his doctorate. He has expertise in a range of methodologies including SMLM and a particular interest in the quantitative analysis of super-resolution data.

Christian Eggeling holds a degree in physics and, after gaining industry experience in single-molecule microscopy, joined the super-resolution microscopy group of Stefan Hell. He now leads an independent group advancing super-resolution microscopy and spectroscopy, focusing on molecular diffusion dynamics studies of cell membranes. He also manages a microscope facility.

Reto Fiolka, originally trained as a mechanical engineer in computational fluid dynamics, switched focus to optical microscopy during his doctorate. His laboratory is dedicated to developing new imaging techniques for biomedical research to be applied in 3D environments, ex vivo and in vivo, offering improved spatiotemporal resolution and multi-scale capabilities.

Rainer Heintzmann studied physics and computer science and develops super-resolution fluorescence microscopy methods such as linear and nonlinear structured illumination, pointillism and image inversion interferometry. He has a strong interest in computational optics and inverse problems such as deconvolution and in extracting multidimensional information from biological structures.

Suliana Manley studied physics and mathematics and became fascinated by complex biological systems. Her group studies the biophysical principles of organelle structure and dynamics and develops smart, automated and multi-modal microscopy methods. Their work is enriched by interplay between fundamental discovery and microscopy development.

Aleksandra Radenovic studied physics and is an expert in single-molecule biophysics and nanofluidics. Her research focuses on developing biosensors and optical imaging techniques for observing individual molecules and complexes, enhancing our understanding of their behaviour in various environments.

Carlas Smith studied aerospace engineering and applied physics. He now leads a group focusing on developing advanced computational microscopy techniques and the combination of opto-mechatronics and information processing algorithms, particularly for super-resolution imaging.

Hari Shroff has degrees in bioengineering and biophysics and entered the microscopy field as a postdoctoral researcher working on super-resolution imaging. He now leads a group focused on developing new optical and computational methods that offer the ability to interrogate biological structure and function across diverse spatiotemporal scales.

Lothar Schermelleh is a trained cell biologist who has specialized in the biological application of super-resolution structured illumination microscopy. His research focuses on studying chromatin organization and functional nuclear architecture. To this end, his group developed fluorescent labelling protocols and software tools for image data quality control.

C.S.: Biologists should be mindful of not equating resolution claims with absolute truth as they often involve nuances. For example, in MINFLUX, the purported close-to-infinite localization precision achieved through continuously decreasing scanning distance would require unrealistic experimental conditions (maximum modulation contrast, zero background). Furthermore, the iterative improvement in localization precision arises from integrating prior knowledge, thereby reducing the required number of photons for accurate localization. This means that the classical Cramer–Rao Lower Bound (CRLB) cannot be relied upon for accurate predictions. The Bayesian version of CRLB, known as van Trees inequality, emerges as a valuable measure for assessing the optimal achievable localization precision in various practical scenarios[9].

L.S.: Biologists should always treat resolution numbers with healthy skepticism as long as it is not clearly shown how the resolution was defined. For instance, an image created from a SMLM dataset claiming a resolution of 10 nm does not equate to, for example, an electron microscopy image of the same resolution because in SMLM, resolution numbers typically refer to localization precision and not to structural resolution. Applying the Nyquist rule, the structural resolution in SMLM is at least approximately twofold lower than the localization precision in case the labelling density matches the sampling criterion and even less if the sampling is lower. Also, biology is inherently three-dimensional; therefore, an increase in axial (z) resolution and optical sectioning capability may be equally important as an increase in lateral resolution.

What are the main trade-offs in microscopy?

K.P. and C.S.: For SMLM methods, the main trade-offs are emitter density, localization precision and sample quality. For instance, there is an interplay between emitter density and localization precision. At a high emitter density, the resolution is limited by the localization precision. Conversely, selecting signals that have photon count above a certain threshold to improve overall localization precision reduces the emitter density and the overall resolution.

D.B.: The trade-offs are hugely sample-dependent and application-dependent, but there is a classical triangle of time, resolution and dose. You can generally have good performance simultaneously in two of these parameters, at the expense of the third.

C.E. and R.F.: Microscopy faces various trade-offs such as labelling and molecular specificity, temporal and spatial resolution, volumetric coverage, signal-to-noise ratio and photobleaching or phototoxicity in (long-term) live-cell imaging, which are exacerbated when imaging deep-tissue samples or in more physiological conditions. To address these challenges, core facilities or similar settings that offer a range of SRM instrumentation and expertise can assist in finding the best solution for a specific application.

R.H.: Resolution, acquisition time and phototoxicity are the main trade-offs, and factors such as data size (resolution, time) or visibility (1/phototoxicity) are directly related to them. One would naively assume that these trade-offs are not very sample-dependent, but this is a misconception. Distinguishing complex 3D features packed within a larger volume such as the mammalian cell nucleus may require a very high signal-to-noise ratio (for example, ≫1,000 photons per voxel) whereas isolated, well-defined small features (for example, exocytotic vesicles) may be well 'resolved' with less than 10 photons.

H.S. and S.M.: The 'pyramid of frustration' is probably most relevant in live-cell imaging, with the vertices being spatial resolution, temporal resolution, signal-to-noise ratio and sample health. Depending on the application, other performance axes may also be of concern, such as imaging depth and bleaching.

L.S.: Imaging contrast and the available photon budget are the main constraining factors. In addition to the microscope hardware, sample preparation and acquisition settings have a huge role in tackling these constrains. The experimenter should aim for the best-possible labelling contrast (high specificity, low background) and high-as-possible dynamic range while keeping photobleaching and phototoxicity in check. Within the confines of the photon budget, a trade-off must be found between spatial resolution, temporal resolution, imaging volume and photodamage. Aiming for the highest spatial resolution inevitably comes at a price. Hence, it is important to determine what level of spatial resolution is needed to answer the research question before selecting the methodology and technique[10].

What are the benefits and risks of computational post-processing?

K.P. and C.S.: The utilization of artificial intelligence (AI) in biomedical research presents a considerable challenge owing to inherent biases in training data, for example, image regression tasks such as image denoising. AI-driven computational pipelines may inherit these biases, leading to skewed outcomes. Ensuring that researchers fairly train the data and openly share datasets and codes will be crucial for avoiding such outcomes. Additionally, assessing data quality and quantity is important for evaluating the performance and reliability of machine learning (ML) models. Improperly trained ML models might overfit the test data, resulting in poor generalization to new data. For instance, when using deep learning frameworks to convert widefield and confocal images into higher-resolution images, encounters of unseen image data during training can lead to intriguing artefacts, often misconstrued as novel findings.

D.B.: Post-processing methods can greatly improve the ability to segment and quantify noisy images; however, they can also introduce artefacts, 'hallucinate' false structures, or artificially remove image structures depending on biases present in their training sets. If based on a solid mathematical model of the imaging and noise processes, carefully executed deconvolution should be the preferred choice of post-processing techniques. Linear denoising filters such as blurring are also an acceptable option, albeit with some loss in resolution. ML/AI tools allow powerful analysis with comparatively little effort. However, they are typically black boxes, providing little insight into how a given result is obtained and having unpredictable failure modes. They perform best on classification-type problems when choosing between several known possible outputs, but they tend to generalize poorly beyond their training data or when data characteristics change. All post-processing techniques are best applied to enable better quantitative processing and/or segmentation of phenomena that have already been identified by examining the raw data, rather than for visualization or discovery. Anything that appears in the processed data but cannot be detected in the raw data should be treated with extreme suspicion.

R.F.: With the exception of STED and expansion microscopy, all SRM techniques rely on some form of data post-processing to arrive

at their final images. Additional denoising and deconvolution can, in principle, benefit any imaging technique. Further post-processing steps might be necessary to analyse image data (tracking, segmentation and statistical methods). Some post-processing steps can now be undertaken with AI/ML, thereby generating new possibilities and challenges. Generally, laboratories that develop computational pipelines should do so with mathematical rigour and validate their algorithms. Practically, it is important to consider how well non-computational laboratories can apply these methods and understand the risks of potential artefacts.

R.H.: The human mind is evolutionarily not optimized for inspecting intensity–volume data or even very noisy two-dimensional images. Therefore, computational post-processing can sometimes seemingly achieve miracles in perceiving the underlying sample structure, often by implementing prior knowledge. AI-based post-processing has recently pushed the field of SRM as it can implicitly incorporate more specific prior information, which is particularly powerful with time-series data. If post-processing reveals any new feature, it should be validated by methods that require fewer prior assumptions.

S.M.: As with any measurement method, fluorescence microscopy has benefits and risks. For example, it requires labelling of target molecules and exposure of the sample to light, which can both interfere with biological function. Computational post-processing should be treated with the same caution as any other method, with independent validation of discoveries, careful benchmarking against existing methods, design of appropriate experimental controls, and transparency about failure modes.

H.S.: The benefits and risks of computational post-processing depend on the method. For example, if one assumes or can characterize a point spread function and noise model, carefully applied deconvolution can improve effective contrast or resolution. ML denoising allows the reduction of laser illumination and delivers plausible reconstructions over a much longer imaging acquisition. In all cases, it is worth carefully stating or verifying the assumptions of the method and remembering that all such methods, at best, estimate (or predict) some feature of the image. Similar to any other tool, for achieving the most robust conclusions, it is best to validate the data using complementary methods.

L.S.: Computational imaging and AI-based analysis are currently revolutionizing the field of SRM. Smart acquisition automation will reduce activation barriers and help to democratize advanced imaging. AI will enable the extraction of vastly more information with higher quality and increased throughput. However, there is also a danger for the average biologist dealing with a 'black box' of taking results for granted, and that reliance on the power of AI leads to lowering the standards for sample preparation and doing proper controls. Thus, it will be important to better understand the potential biases and limitations of AI tools and learn how to best utilize them.

What are the main effects of labelling on super-resolution microscopy?

K.P. and C.S.: An often-overlooked aspect in SMLM is the significance of sampling and the necessity for acquiring a minimum number of signals to decode the underlying structure. Test samples with known structures and minimal background (for example, DNA origami or microtubules) facilitate the selection of signals with the highest photon counts to improve localization precision at the cost of structure sampling requirements. The consecutive measurement of intensities, particularly with unstable emitting dyes, tends to influence both the sampling process and the density of localizations. Thus, blind samples (without prior knowledge of the structure) are needed for accurate calibration and resolution claims. Aiming for single-nanometre resolution when the smallest tag itself measures around 3 nm seems biologically pointless. Considering the resulting uncertainty in the relative fluorophore position, the claimed resolution (or localization precision) can only be attributed to the fluorophore, not the protein target. Although particle averaging can compensate for limited or incomplete labelling, it carries a risk of losing biological heterogeneity.

D.B.: For most biological applications, labelling density is more important than linker length. Short linkers can have adverse effects (particularly in SMLM, but also in any microscope using laser excitation) as dyes can no longer freely rotate and probe dipole moments need to be considered. Even in the case of standard dual-antibody immunofluorescence detection, one needs to operate at resolutions of below 20 nm before the distance between epitope and fluorophore (~12 nm) becomes relevant. Conversely, label density has a huge effect on effective resolution, both in SMLM and in non-switching methodologies. Innovations in genetic tagging and direct labelling have great potential for addressing these issues: reduced steric hindrance and better access enabled by these techniques will probably be more important for image quality than the label offset distance.

C.E.: In conventional methods such as confocal microscopy, label size has never had a big effect, and unspecific binding often had a lower influence. However, when reaching spatial scales in the range of the label size, these issues lead to significant biases because minute unspecific background becomes visible and distances or co-localization (which is no longer a valid expression as nearby objects can never fully co-localize) are influenced by label size. Therefore, one of the biggest current challenges in recently developed super-resolution microscopy techniques such as MINFLUX and DNA-PAINT SMLM is the quest for small labels and their interrelation on small spatial scales.

R.F.: For many live-cell imaging applications, labelling methods that minimally perturb the underlying biology (that is, endogenous expression level) are paramount. Probe dimensions and achievable labelling density become crucial for SMLM and MINFLUX as they approach molecular scales, which until recently has been considered the ultimate resolution limit for these techniques. Sequential DNA-barcoding localization precision below the label size has been achieved[11]. This advancement, however, raises the question of whether the label can freely wiggle around or would steric hindrance bias the results. Nevertheless, the push for ever-smaller labels will be crucial for super-resolution in the realm of ultrastructure and tracking of protein dynamics.

R.H.: As microscopy resolution has reached the molecular level, images will look more and more like pointillistic renderings. Ultimately the world consists of molecules, and we cannot expect to see a line as the rendering of a filament at molecular resolution. Yet it is often not quite clearly stated what exactly a rendered SMLM image represents, which leads to some confusion in its interpretation. The higher the resolution becomes, the more important it is for the visual and quantitative interpretations to not miss molecular entities.

S.M.: All probes introduce a linkage error, which is based on the size of the probe and on how it is targeted. The smallest effective

linkage errors arise from the incorporation of directly labelled unnatural amino acids, and from expansion microscopy followed by labelling. In the latter case, the effective linkage error is reduced by the sample expansion factor, which can be interesting for super-resolution microscopy of fixed samples.

A.R.: Ideally, fluorescent dyes should be able to rotate freely, which averages out their dipole orientations. However, this rotation may be restricted at low temperatures or when using very short dye linkers, resulting in a fixed orientation of the dye molecules. Several methods and detection strategies, for example, using polarization-sensitive excitation, are being developed that account for and minimize localization biases. Future developments in SMLM will be closely connected with advancements in cryo-electron microscopy (cryo-EM). Specifically, correlative cryo-SMLM-EM may become a key area of focus.

L.S.: Progress in SRM is intricately linked with the development of new and improved fluorescence dyes[12], labelling tools (for example, nanobodies and aptamers)[13–15], and methodologies that enable multiplexed SRM (for example, exchange-PAINT). Ever-increasing physical resolution and the drive towards live-cell imaging push emphasis on labelling density, linker size and orientation, target accessibility, and biological compatibility, in addition to the general photophysical properties of fluorophores.

Where do you see super-resolution microscopy developing into in the coming years?

K.P.: In the upcoming 5–10 years, there may be a pivot towards sequential correlative super-resolution microscopy, aiming to image the same structure with different methods of comparable resolution and under identical imaging conditions. This shift could facilitate the independent validation of structural details. A considerable focus is also anticipated on quality control, ground truth and artefact correction algorithms, which are expected to become integral components of standard image-processing workflows to elevate image fidelity and precision. In terms of new method development, super-resolution histology, which is a combination of 3D high-resolution imaging and sequencing, might be of great interest.

A.R., S.M. and C.S.: The emergence of adaptive microscopy and event-driven microscopy is particularly exciting. New correlative and in operando microscopy techniques will enable us to design experiments suitable for studying complex systems at cellular, organ and organism levels. Now is the perfect time to dive into this exciting and transformative research.

C.E., R.F., R.H. and C.S.: It would be exciting if ultrastructural details and dynamics of molecular complexes could be imaged in living cells. Combined with correlative cryo-EM, X-ray crystallography, NMR studies, or structure reconstructions of those complexes, it could open up exciting opportunities.

H.S.: The greatest progress will probably be made in development of probes and targeting strategies rather than in optical methods. Adapting many of the existing super-resolution methods to living systems and the integration of AI tools are promising directions.

L.S.: Through the widespread implementation of computational imaging, SRM will become more user-friendly, more robust, more automated and more quantitative, thereby enabling the harvest of a much greater wealth of information in more economical ways. We will see optical microscopy (with or without physical expansion) increasingly competing with electron microscopy for application in structural biology. Key to this development will be the co-development of labelling methods that allow dense-enough coverage of biomolecules to benefit from the spatial resolution that is already achievable, for example, with MINFLUX and related techniques. Furthermore, the wider implementation of adaptive optics using programmable mirrors will overcome current limitations caused by optical aberrations that are hampering many live-tissue and deep-tissue SRM applications.

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