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Strategies for increasing the throughput of super-resolution microscopies

Dora Mahecic[1], Ilaria Testa[2], Juliette Griffié[1] and Suliana Manley[1]

Fluorescence microscopy methods have been developed to circumvent the diffraction limit by exploiting nonlinearities in the interactions between light and fluorophores. Initially, these methods were up to orders of magnitude slower than standard microscopies. In recent years, a wide array of technological advances have increased the throughput of super-resolution microscopies, through parallelization, smart scanning or data processing, and sample expansion. Here, we review recent innovations for increased throughput, some applications that have benefitted from them, and how they could be improved in the future.

Addresses
[1] EPFL, Laboratory for Experimental Biophysics, Institute of Physics, Lausanne, 1015, Switzerland
[2] Department of Applied Physics and Science for Life Laboratory, KTH Royal Institute of Technology, 10044 Stockholm, Sweden

Corresponding author: Manley, Suliana (suliana.manley@epfl.ch)

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Introduction

Super-resolution fluorescence microscopies, or 'nanoscopies', offer methods that use light to resolve objects below the diffraction limit, which have transformed our understanding of resolution. In the process, they have enabled experiments that combine the advantages of fluorescence microscopy, such as specific and multicolor labelling of living or fixed samples with spatial information near the molecular scale. Because of their reliance on nonlinear photophysical transitions, early experiments were laborious and slow, creating a bottleneck for the application of super-resolution to fuel new scientific discoveries. Over the past few years, new technologies have emerged to address this limitation. In this brief review, we provide an overview of recent innovations that have increased the throughput of super-resolution fluorescence microscopies, as well as highlighting some applications that have benefitted from them.

There are a large number of methods that go beyond the diffraction limit, but many of them are based on a handful of fundamental mechanisms. One such mechanism exploits saturated transitions to a non-fluorescent state to reduce the emission volume of molecules illuminated by a focused excitation beam, as introduced by stimulated emission depletion (STED) [1] and reversible saturable or switchable optical fluorescence transitions (RESOLFT) [2,3] microscopies. These were initially implemented as point-scanning methods, where focused excitation light is scanned over the sample to recover spatial information. Scanned illumination presents intrinsically different obstacles to increased throughput compared with wide-field illumination, as used in single molecule localization microscopies (SMLM) such as photoactivated localization microscopy (PALM) [4], stochastic optical reconstruction microscopy (STORM) [5], or points accumulation in nanoscale topology (PAINT) [6]. The mechanism behind these methods is to use stochastic transitions of fluorophores between dark and fluorescent states, or stochastic binding, to isolate single molecules in time and space. The locations of isolated molecules can then be used to reconstruct super-resolved images. Finally, expansion microscopy (ExM) [7] uses physical expansion of a fixed and polymer-embedded sample, resulting in an improved capability to resolve structures using more rapid, conventional microscopes.

We do not focus here on another important mechanism for super-resolution, structured illumination used to generate interference patterns (SIM) [8] or pinholing of emission from focused excitation (Re-Scan, image scanning microscopy (ISM)) [9,10]. These methods have also benefited from increased acquisition speed [11], but have had relatively high throughput since their implementation, albeit with a typically smaller improvement in resolution (~2×).

There are unique challenges to increasing the imaging throughput of super-resolution microscopies, but some universal advantages come with collecting large datasets. The push into quantitative biology requires access to datasets large enough to capture (or average out) the variability of noisy biological processes. Super-resolution microscopy allows quantitative measurements of events at the molecular scale. Large datasets also lend the possibility

of different kinds of analysis, such as high-throughput screening [12], structural modeling, and particle reconstruction inspired by electron microscopy [13,14]. Another emerging direction is to use large super-resolution datasets to train neural networks, which then can further speed up data acquisition [15,16].

STED and RESOLFT fluorescence nanoscopy

As with any point-scanning microscope, STED and RESOLFT are faced with a tradeoff between the number of acquired pixels, or field of view (FOV) and recording speed. Increased data throughput in STED and RESOLFT fluorescence nanoscopies has primarily been the result of advances in two directions: 1) parallelized scanning, 2) increased scan rate.

Parallelization of STED and RESOLFT

The recent use of spatial light modulators and deformable mirrors to create, correct and align the STED and the excitation beam in an automated modality [17,18] allowed the imaging area to be increased, while facilitating the alignment procedure. The correction of chromatic aberrations in easySTED [19] implementations resulted in an increased scan area of more than $100 \times 100\,\mu m^2$ in ~30 min [20].

A successful strategy to increase the scan area without increasing the required measurement time is parallelization of the illumination and readout. Parallelization was achieved (Figure 1) by illuminating the specimen with multiple donut-shaped beams [21] or with the use of a lattice generated by superimposing two orthogonal standing waves, creating the equivalent of 2000–100 000 donuts [22–24]. As a result, a region 20 μm in diameter could be recorded with orders of magnitude fewer scanning steps than in conventional point-scanning STED. This implementation has the potential to reach reconstructed frame times of a millisecond, although the temporal resolution so far was limited by camera technology. Furthermore, the size of the FOV in parallelized STED was so far only limited by the available intensity of the STED laser. Since RESOLFT requires orders of magnitude less intensity for switching the molecules to the OFF state compared with stimulated emission, FOVs of 50–100 μm have been recorded with parallelized excitation [24–26].

Increased STED and RESOLFT scan rate

STED imaging throughput can be further increased by faster scanning and prolonged acquisition (Figure 1). The stimulated emission transition is extremely rapid; therefore, the measurement time mainly depends on the fluorescence photon emission lifetime, which is in the nanosecond range. In practice, enough fluorescence photons need to be collected to distinguish the signal from the shot noise. This results in dwell times of 0.1–100 μs, depending on the lifetime of the specific dye and the noise level.

The most commonly used and commercial STED nanoscopy implementations scan the beam across the specimen area. Beam scanning can be controlled with galvanometric mirrors, whose speed is limited by mechanical motion, or with faster resonant mirrors and electro-optical scanners, whose increased speeds have enabled recording of STED images in the millisecond time regime [27–29].

To prolong the recording of specimen regions, it is important to minimize photobleaching. For this, rapid line-scanning as well as an adaptive STED illumination scheme has been implemented [30,31]. In adaptive illumination an intensity-modulable STED beam is used to probe the specimen in a way that the exposure of the high intensities of the donut crest to the fluorophores is minimized. In MINFIELD the same result is obtained by confining small structures inside the donut light distribution. A second level of automation has been implemented in smart RESOLFT [32], where the scanning is accelerated and decelerated adapting to the specimen morphology. In this way dark pixels not containing any structure are rapidly probed while the RESOLFT imaging scheme is only performed in regions containing structures. This concept has the twofold benefit of speeding up recording and simultaneously reducing light exposure.

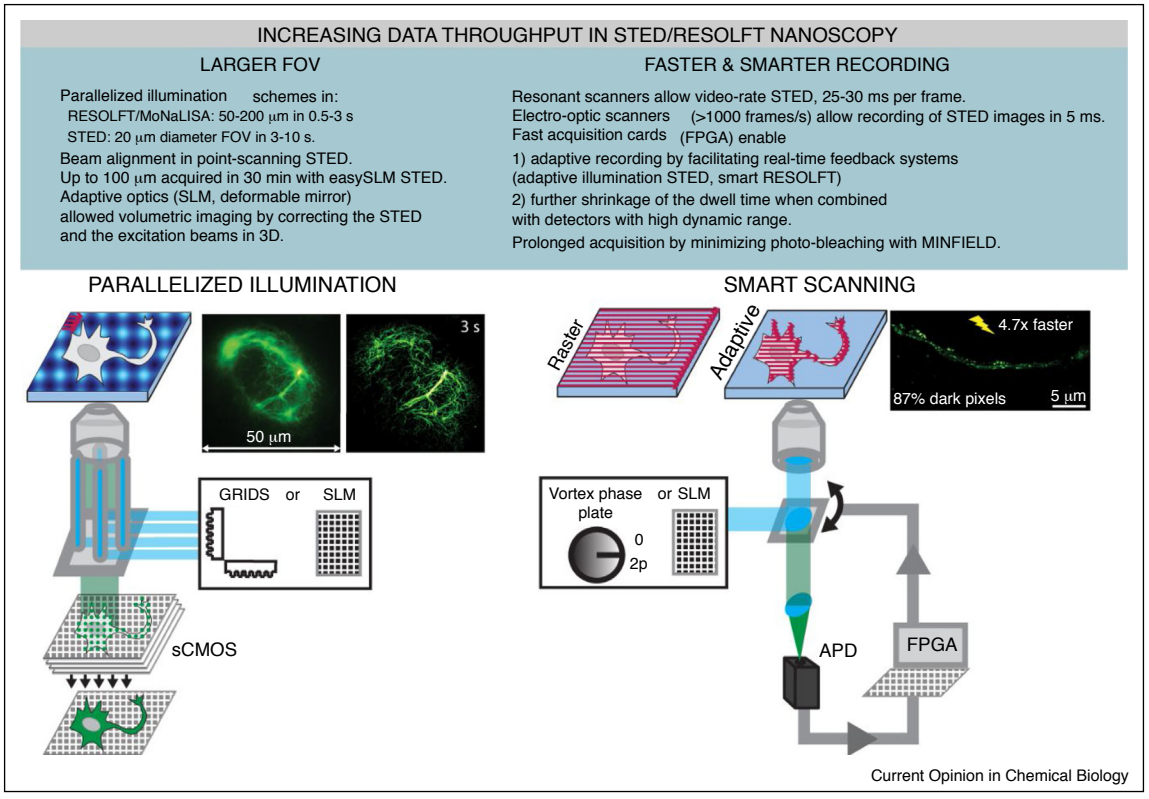
Single molecule localization microscopy

Single molecule localization microscopies use stochastic photoswitching (PALM, STORM) or stochastic binding of fluorescent probes (PAINT) to isolate single molecules in space and time. Subpixel localization of molecular positions is then an estimation problem, which has been solved using a wide variety of algorithms [33]. Such microscopies typically use wide-field illumination, to collect data simultaneously over a FOV. Limitations to speed are primarily due to the fluorophore on-state or binding lifetime, the requirement of single molecule isolation, and the need to collect enough localizations to fully sample the structure of interest [34]. Three main approaches have been used to improve the throughput of SMLM (Figure 2a): 1) automation of imaging over multiple FOVs, 2) enlargement or increase in speed of imaging each FOV, and 3) development of algorithms, either to manage overlapping signals from single molecules or to reconstruct images from undersampled localization data.

Automation for high-throughput SMLM

Achieving true single molecule isolation allows for the highest localization precision, but it requires careful choice of experimental parameters. This is because photoswitching rates depend on laser power, and as the measurement proceeds, the density of fluorophores available to be switched on decreases due to irreversible off-

Figure 1



High throughput in STED and RESOLFT nanoscopies.
The size of the FOV and the recording speed of current STED and RESOLFT microscopes depend on the illumination scheme (single point versus parallelized illumination), available technology (scanners, detectors, acquisition cards) and ultimately probe brightness and ON–OFF switching time.

switching [35]. Thus, creating an automated SMLM microscope implies laser control with real-time feedback, as has been implemented based on real-time localization [36,37], cumulative pixel on-time [38], or machine-learning-based molecule density estimation [39].

Each FOV typically takes tens of seconds to image, so using complementary modalities such as phase contrast or bright field microscopies to select sample regions of interest has also enabled increased throughput [37,38]. A microscope was recently engineered to image in multi-well plates, with laser automation, FOV selection, and real-time data processing, which allowed SMLM to be combined with high-content screening [12].

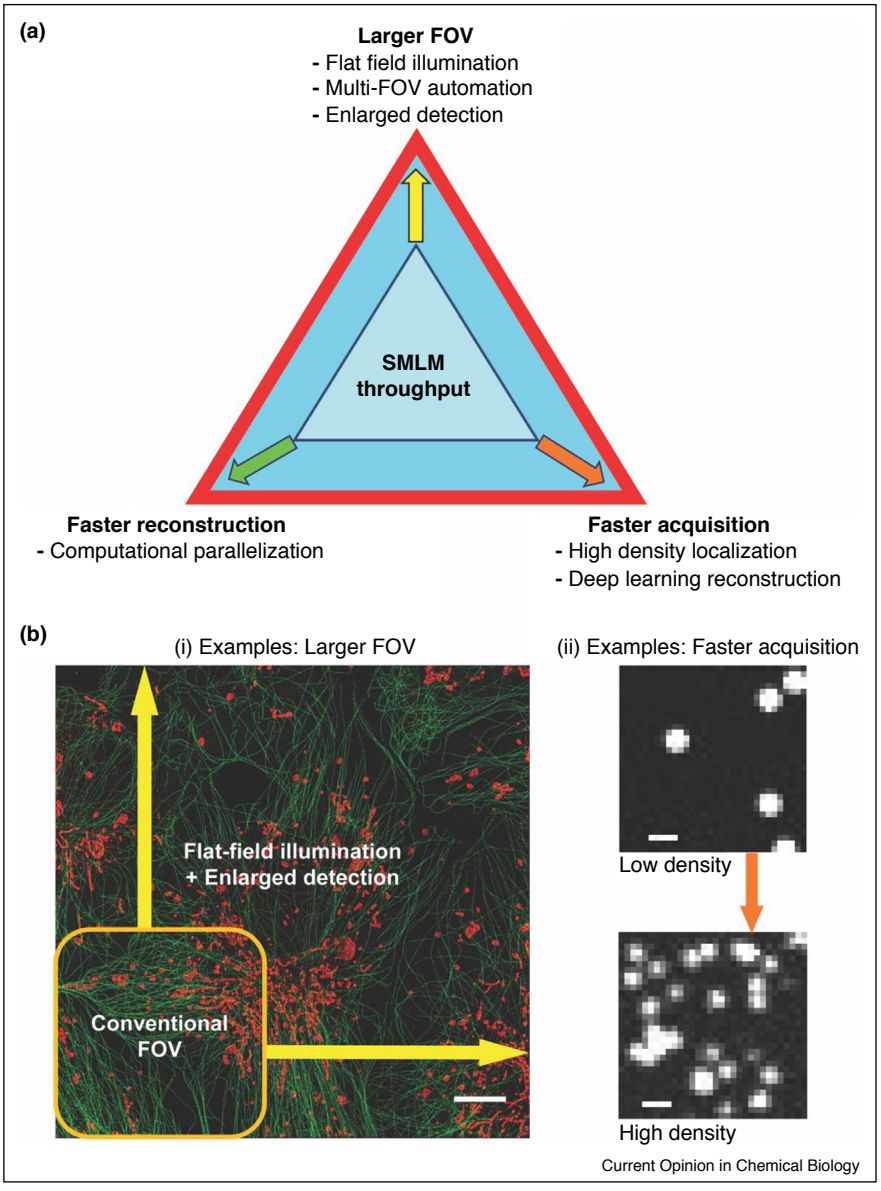
Increased size and acquisition speed of SMLM images

Wide-field microscopies are inherently parallelized, since multiple pixels are interrogated simultaneously. Thus, straightforward ways to increase throughput include increasing the size of illumination and collection areas, or increasing the frame rate of the detector and the photoswitching rate. On the collection side, the adoption of scientific CMOS cameras with increased array size and frame rate allowed for video-rate data collection, once pixel-dependent noise was corrected [40]. On the illumination side, flat-field illuminators increase the FOV, while ensuring uniform photoswitching (Figure 2b) [41–43]. The resulting improved data quality and throughput have allowed ideas from electron microscopy to be extended to super-resolution microscopy, such as 3D particle reconstruction, to reveal the organization of protein complexes with protein identity and in multiple colors [44,45]. Methods for producing large and uniform evanescent fields with waveguides [46,47] or diffractive beam-shaping elements [48] have brought the same advantages to imaging objects near the coverslip.

New algorithms for high-density and high-speed single molecule data processing

Another way of increasing the speed of acquiring data on a single FOV is to relax the requirement of single molecule isolation (Figure 2b). In its most recent edition, the localization microscopy software challenge included high-density 3D datasets for benchmarking [49]. As an alternative, methods to analyze fluctuations instead of localization maps have met with some success [50–52]. Analysis of high-density data increases throughput by reducing the number of raw images required to

Figure 2



High throughput single molecule localization microscopy.
(a) SMLM throughput has been increased by technological improvements along three axes. **(b)** (i) Example of larger FOV, accomplished by a combination of flat-field illumination and an enlarged detection area. (ii) Example of faster acquisition, accomplished by increasing the density of emitting fluorophores (simulated emitters). Scale bar: b. (i) 10 mm, (ii) 5 pixels.

reconstruct an image. Another promising development approaches this problem from a different direction, by using deep learning to reconstruct SMLM-like images using orders of magnitude fewer localizations [15].

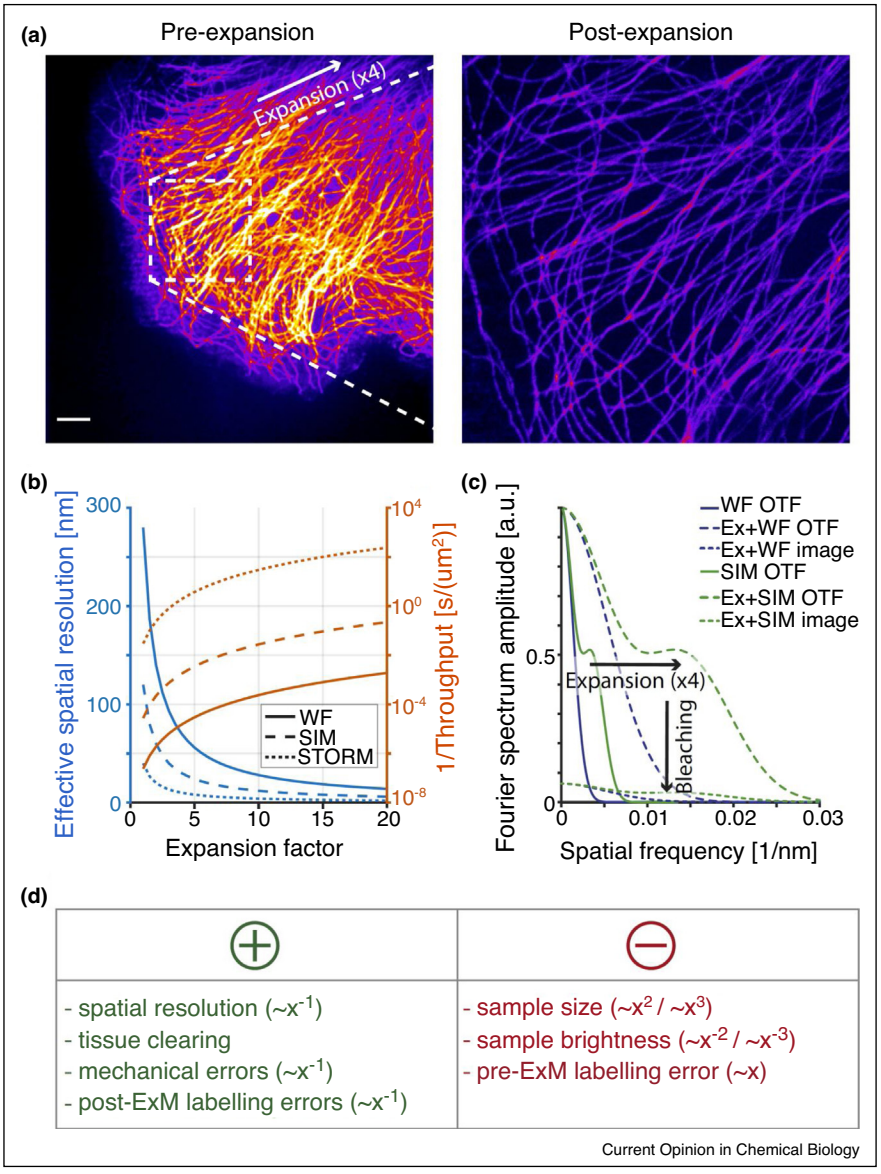
Expansion microscopy

Expansion microscopy (ExM) does not use optical tricks to overcome the diffraction limit, but instead physically magnifies the sample, bringing diffraction-limited features into the range observable by standard microscopy [7]. Most variants provide a linear expansion factor of ~4 along each dimension, resulting in an effective lateral resolution of ~70 nm on a conventional microscope. Once expanded, samples can be imaged with either conventional or super-resolution microscopies.

ExM samples imaged with rapid conventional microscopies

Sample expansion increases the distance between features of interest, but it simultaneously increases the overall sample size. Therefore, sample expansion shrinks voxels along each dimension, reducing the effective FOV

Figure 3



High-throughput super-resolution microscopy combined with sample expansion.
(a) Individual FOV showing Cos7 cells stained with αtubulin-Alexa-594 pre-expansion (simulated from expanded sample, left) and post-expansion (right). Image brightness not to scale. **(b)** Effect of expansion on the effective spatial resolution and throughput for widefield imaging (WF, full line), structured illumination microscopy (SIM, dashed line) and STORM (dotted line) imaging. **(c)** Effect of expansion on the effective microscope optical transfer function (OTF) and image spatial frequency components due to changes in sample. **(d)** Table listing main advantages (+) and disadvantages (−) of sample expansion and how they scale with the expansion factor (×). Scale bar: (a) 2.5 μm.

size and hence throughput (Figure 3a). ExM still has strong potential for high-throughput super-resolution imaging, because it benefits from faster acquisition speeds of diffraction-limited microscopes (Figure 3b). This creates a niche for fast techniques such as widefield epifluorescence microscopy, spinning disk confocal microscopy (SDCM) and light sheet microscopy (LSM), which offer effective subdiffraction resolution when combined with ExM. For example, SDCM was used to image expanded brain tissue up to 100 μm in depth [7], and LSM has been successfully combined with ExM [53]. More specialized microscopes, such as the lattice light sheet microscope have been combined with ExM, generating super-resolved images of an entire *Drosophila* brain, at orders of magnitude faster acquisition rates than comparable super-resolution techniques [54].

Combining enhanced optical resolution with ExM

Although the benefit of ExM is most apparent with diffraction-limited methods, combining ExM with

existing fast super-resolution techniques further improves resolution. For instance, ExM has been combined with SIM to achieve ~30 nm resolution close to the coverslip [55,56], at >20× the speed of SMLM techniques. However, more powerful super-resolution techniques struggle to adapt to ExM samples, mainly due to the increased sampling requirements, and decreased labelling density and brightness of expanded samples. STED has been combined with ExM, achieving 10–20 nm resolution close to the coverslip [57,58], but since it requires scanning the excitation over the imaging area, expansion significantly increases the imaging time for the whole expanded volume. SMLM techniques such as PALM [59] and STORM [60] have been combined with ExM, but at drastically lower expected throughput (Figure 3b).

Beyond higher resolution, ExM samples can sometimes yield higher image quality [61,62]. This could be a consequence of several factors. The preparation of expanded samples is similar to optical clearing, since the expanded sample is composed almost entirely of water, eliminating aberrations and scattering associated with deep imaging. While the brightness of the expanded sample is significantly decreased as fluorophores are diluted during expansion [59,60,63], (Figure 3c), this can be addressed by as multi-epitope labelling [57], use of quantum dots [58], dual labelling strategies [55,58] or *in situ* signal amplification [53,65]. Post-expansion labelling may also improve labelling efficiency due to reduced steric hindrance after expansion [53,59] and labelling precision since the size of the probe is divided by the expansion factor [66]. Variants of the ExM protocol enabled highly multiplexed labelling of protein targets [67], or combined protein labelling with highly multiplexed imaging of RNA [53]. These experiments demonstrate the versatility of ExM for use in biological discovery.

Conclusions and future developments

As the field of super-resolution microscopy has matured, application-oriented technology development has become increasingly important. High-throughput is just one aspect of this, but it is an important one in an age of open data and machine learning. In microscopies in general, the ability to collect large datasets is becoming recognized as a way to improve reproducibility, data quality, and deepen our understanding of biological systems. However, there is room for improvement with each of the highlighted approaches.

In the case of STED or RESOLFT microscopies, high dynamic range and efficient detectors [68], like SPAD arrays and hybrid detectors, will be key to achieving the minimal theoretically possible dwell times for rapid STED imaging. At the state of art, the fastest STED/RESOLFT imaging (5–30 ms per frame) was recorded in small FOVs of a few micrometers with rapid and adaptive scanning. However, we expect that the future development of faster and gated cameras or larger SPAD arrays will allow the same recording speed in 10–100 times larger FOVs thanks to the parallelization of the illumination.

Localization microscopies were initially the slowest of super-resolution methods, but improvements in detectors, large, uniform illumination, and algorithms to make use of fewer raw images have brought acquisitions into the subsecond range. These improvements, combined with automation, make SMLM competitive with other nanoscopies when considering data throughput. It will be important to spread these technologies by generalizing solutions to make them truly transferrable. Furthermore, algorithms that treat high-density or sparse datasets to reconstruct super-resolved images can be prone to artifacts, and quality assessment tools are needed for non-expert users.

Overall, ExM enables super-resolution imaging of fixed samples at the speeds of faster, more modest resolution, and widely available microscopes, providing interesting advantages. Labeling post-expansion eliminates amplification of the labelling error during the expansion process and improves probe density and brightness [59,63,66]; this could be further improved by use of smaller and brighter labels such has nanobodies. We expect that better control of sample expansion will allow users to choose between tradeoffs (Figure 3d) [65,67,69,70]. Finally, standard oil objectives — preferred for super-resolution imaging, have small working distances and introduce spherical aberrations when used with expanded samples composed mostly of water. Development of higher refractive index immersion media compatible with expansion microscopy [58] would be of interest.

Beyond increased throughput, we expect that super-resolution microscopies will become more powerful through smarter control and real-time adaptation. The developments highlighted here set the stage for future exciting developments in intelligent microscopy.

Conflict of interest statement

Nothing declared.

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