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The cell biologist's guide to super-resolution microscopy

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ABSTRACT

Fluorescence microscopy has become a ubiquitous method to observe the location of specific molecular components within cells. However, the resolution of light microscopy is limited by the laws of diffraction to a few hundred nanometers, blurring most cellular details. Over the last two decades, several techniques – grouped under the 'super-resolution microscopy' moniker – have been designed to bypass this limitation, revealing the cellular organization down to the nanoscale. The number and variety of these techniques have steadily increased, to the point that it has become difficult for cell biologists and seasoned microscopists alike to identify the specific technique best suited to their needs. Available techniques include image processing strategies that generate super-resolved images, optical imaging schemes that overcome the diffraction limit and sample manipulations that expand the size of the biological sample. In this Cell Science at a Glance article and the accompanying poster, we provide key pointers to help users navigate through the various super-resolution methods by briefly summarizing the principles behind each technique, highlighting both critical strengths and weaknesses, as well as providing example images.

KEY WORDS: Super-resolution microscopy, Live-cell imaging, Labeling, Sample preparation

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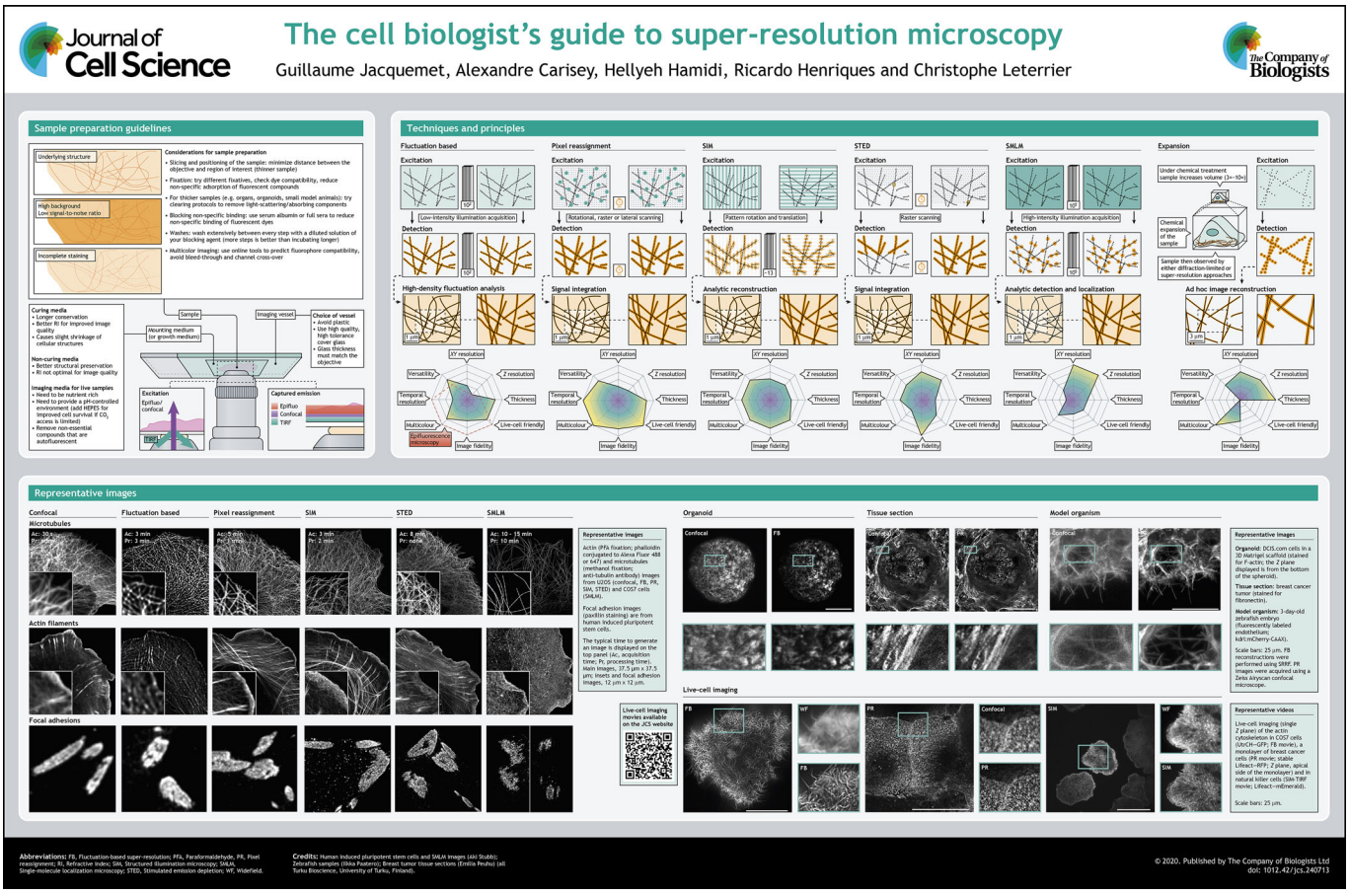
Introduction

Fluorescence microscopy allows biologists to selectively label and observe cellular components with high sensitivity in fixed and living samples (Combs and Shroff, 2017; Kremers et al., 2010; Masters, 2010), and is one of the leading technologies used to drive discoveries in life sciences. However, classical light



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microscopy is limited by the diffraction of light, which precludes the visualization of details below ∼200 nm (Vangindertael et al., 2018). Since the early 2000s, super-resolution microscopy (SRM) approaches have been devised that bypass this diffraction limit to resolve biological objects down to a few tens of nanometers (Sahl et al., 2017; Schermelleh et al., 2019; Sigal et al., 2018). A variety of techniques are now available as either commercial or open, do-it-yourself, instruments and software (Thorley et al., 2014), and cell biologists interested in applying SRM to their question of interest can feel lost among the number of options available. In particular, the different SRM techniques vary greatly in the resolution they attain, the constraints they place on sample type, and their general versatility and ease of use (Bachmann et al., 2015; Wegel et al., 2016). In this Cell Science at a Glance article and accompanying poster, we focus on SRM methods that we consider accessible to researchers, by being commercially available or that are easy to implement by non-experts. In Box 1, we highlight the general elements to be considered when preparing samples for SRM. We briefly explain the principles behind each SRM technique, then summarize its performance for relevant cellular imaging parameters using an indicative scoring system represented by radar plots on the poster (using a '1 to 5' scale of less adapted to more adapted; see 'Techniques and Principles' on poster). The advice we propose is based on our collective experience of applying various SRM techniques to different cell biological questions (Almada et al., 2019; Carisey et al., 2018; Gustafsson et al., 2016; Jacquemet et al., 2019; Stubb et al., 2019; Vassilopoulos et al., 2019). The aim of this Cell Science at a Glance article and the accompanying poster is to provide a short overview that will foster further reading or discussion with imaging facility specialists and help put these powerful techniques in the hands of diverse users.

Fluctuation-based super resolution microscopy

Principle

When excited with continuous light, the emitted light of every fluorophore randomly varies over time. This is due to transitions between the non-fluorescent states of the fluorophore (van de Linde and Sauer, 2014) or their interactions with the surrounding environment (Bagshaw and Cherny, 2006). After capturing these oscillations over a sequence of tens to hundreds of images, algorithms such as super-resolution optical fluctuation imaging (SOFI) (Dertinger et al., 2009) and super-resolution radial fluctuations (SRRF) (Gustafsson et al., 2016) use the temporal correlations in these oscillations to predict the presence and location of fluorophores at improved resolution (see 'Techniques and Principles' and 'Representative Images' on poster). The accuracy and resolution will considerably improve when samples are decorated with highly fluctuating probes, such as reversibly photoswitchable fluorescent proteins (Zhang et al., 2016) or organic dyes in a photoswitch-inducing buffer (van de Linde et al., 2011).

Versatility – 4/5

Fluctuation-based methods are purely analytical approaches that are compatible with most microscopes and do not depend on hardware modifications. They are thus easy to implement, and free software versions exist, but these require knowledge to properly tune the processing (Dedecker et al., 2012; Gustafsson et al., 2016). Moreover, the need to acquire a fast sequence of images (typically hundreds) can make multidimensional protocols complex.

XY resolution – 2/5

The resolution improvement considerably depends on the capacity of the algorithm used to detect fluctuations in fluorophores. It is thus

Box 1. Sample preparation for optimized imaging

Experimental design

- The same pitfalls as in conventional microscopy apply to SRM and must be known to the experimentalist to avoid bias during image acquisition, analysis and data validation (Jost and Waters, 2019).
- For multicolor imaging, online tools should be used to predict the compatibility between the different fluorophores or fluorescent proteins (Lambert, 2019) and the microscope to be used.
- The speed of acquisition needs to be taken into consideration when choosing the most appropriate SRM technique. Slow acquisition strategies may lead to motion blur and/or artifacts during live imaging.
- Sample size should be computed ahead of the experiment using a pilot dataset.
- Consistency is key for preparation of fluorescent samples for SRM, but this does not eliminate the need for biological and technical replicates.

Sample labeling

- Fixation methods need to be carefully optimized as each fixative has specific advantages, drawbacks and dye compatibility. Live-cell imaging can be used to validate that no fixation artifacts have been introduced (Pereira et al., 2019).
- Reagents should be carefully validated, including antibodies and dyes.
- Aldehyde-based fixatives should be quenched using inert amine-containing molecules (glycine or sodium borohydride).
- The nonspecific adsorption of fluorescent compounds should be reduced by adding detergent and sera in the washing buffer. Ultimately, the ratio between signal of interest and background is more important than the overall brightness of the staining.
- Thick samples can be cleared to remove light-scattering and light-absorbing components of a tissue (Kolesova et al., 2016; Qi et al., 2019), allowing access to deeper structures.

Choice of vessel

- Glass coverslips should be used rather than plastic, as plastics introduce optical aberrations and are not compatible with immersion oils for long-term imaging.
- The thickness of the glass must match the expected value for the microscope used; 170 µm-thick cover glass (#1.5H) is often recommended. To ensure reliable performance, high quality, high tolerance cover glass should be chosen.
- Positioning of the sample must be designed to reduce the distance between the objective and the region of interest. This will help improve the resolution by reducing light dampening and distortion.
- Most techniques can accommodate a slide–cover-glass sandwich format, but some require access to the sample. In these cases, glass-bottom imaging dishes and multiwell slides are recommended.

Choice of the imaging medium

- For fixed samples, multiple mounting media are available, all designed to bring the refractive index (RI) of your sample close to the RI of the cover glass (RI=1.52).
- Curing mounting media allow for longer conservation and have an RI that is better for image quality, but lead to a slight shrinkage of cellular structures.
- Non-curing mounting media may be preferred due to convenience of use, and preservation of sample structure, despite the compromise in RI.
- For live-cell imaging, the priority of the medium is to ensure the survival of the biological sample.
- Imaging media need to be nutrient rich and provide a pH-controlled environment. For instance, adding 25 mM HEPES in imaging media may help to ensure cell survival when CO_2 access is non-optimal during imaging (Frigault et al., 2009).
- Consider removing any non-essential compounds from the medium that are autofluorescent (e.g. Phenol Red, flavins, nicotinamide adenine dinucleotide and lipofuscin) (Surre et al., 2018).

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difficult to predict the resolution that will be achieved prior to actual imaging, but an enhancement of two- to three-fold can typically be expected (Culley et al., 2018a).

Z resolution – 1/5
Resolution improvement in the *Z*-axis cannot yet be directly achieved by the algorithms, but specialized multiplane-imaging setups are being developed to tackle this limitation.

Thick-sample friendliness – 4/5
The resolution and quality of the data reconstructed will depend on the density of fluorophores captured in each image. As such, optical sectioning techniques, such as total internal reflection fluorescence (TIRF), confocal or light-sheet considerably improve the data generated and enable these methods to super-resolve samples with a thickness of tens of micrometers.

Live-cell friendliness – 4/5
Fluctuation-based SRM is one of the least phototoxic methods in existence; enhanced resolution can be achieved using an illumination intensity that is similar to conventional fluorescence imaging (mW/cm^2 magnitude). Imaging from minutes to hours without significant photobleaching or apparent light-induced cell stress has been demonstrated (Movie 1, Culley et al., 2018a), but slow speed can be an issue with moving samples (see below).

Image fidelity – 3/5
Intensity in the generated images weakly relates to the local stoichiometry of fluorophores. While images will represent the structure (with some degree of unwanted defects), care needs to be taken when employing further analysis routines that take pixel brightness into account (Culley et al., 2018b).

Multicolor – 4/5
Multicolor imaging is possible and straightforward since fluctuation imaging is compatible with most fluorophores.

Temporal resolution – 2/5
A temporal stream of a few hundred images needs to be collected to capture a single channel, generally taking one to five seconds.

Availability
Free implementations can be found for both SOFI and SRRF (Dedecker et al., 2012; Gustafsson et al., 2016), and SRRF has been implemented as onboard processing in some electron multiplying charge coupled device (EMCCD) cameras (Cooper et al., 2019).

Pixel reassignment super resolution microscopy
Principle
In this technique, single or multiple focal spots are used to scan the sample, as in confocal microscopy. However, the fluorescence signal is not captured by a single-point detector, such as a photomultiplier tube, but by an array detector (camera, concentric detector array or single-photon detector array) (Ströhl and Kaminski, 2016; Wu and Shroff, 2018). The signal detected in each element of the detector array is reassigned in space to achieve a smaller point-spread-function and thus higher resolution. After data processing, a 1.4-fold improvement in resolution can be achieved laterally with a minor axial improvement (Vangindertael et al., 2018). A number of variants exist for pixel reassignment, such as image scanning microscopy (ISM) (Müller and Enderlein, 2010), Zeiss Airyscan (Huff, 2015), rescan confocal microscopy (RCM) (Luca et al., 2013) and multifocal structured illumination microscopy (MSIM) (York et al., 2012). Pixel reassignment has been adapted to spinning disc and swept-field confocal microscopy (Azuma and Kei, 2015; Hayashi and Okada, 2015; York et al., 2013). There is an expectation that the majority of confocal systems will, in the future, integrate these concepts to improve resolution.

Versatility – 4/5
A lateral resolution improvement of 1.4-fold can be achieved purely through optics without the need for an additional analytical step. This means researchers can observe the resolution-enhanced sample in real-time. MSIM implementations (York et al., 2012), however, require the digital analysis of the images being collected.

XY resolution – 2/5
The 1.4-fold lateral-resolution improvement can be increased to two-fold through additional and careful deconvolution of the collected data.

Z resolution – 2/5
Minor (∼25%) axial resolution improvement has been demonstrated (York et al., 2013), and further improvement necessitates additional deconvolution of the collected data.

Thick-sample friendliness – 4/5
Reassignment systems are expected to generate images of similar fidelity to those collected in laser scanning confocal or spinning disc microscopy for thick samples.

Live-cell friendliness – 5/5
Pixel reassignment allows single-shot super-resolution imaging at low-illumination (Movie 2). Reassignment implies additional magnification, which requires the use of sensitive detection systems.

Image fidelity – 4/5
The pure-optical resolution enhancement does not generate additional image defects, such as those commonly occurring in super-resolution techniques that require data processing to generate a final image. However, the additional use of deconvolution can lead to image artefacts.

Multicolor – 5/5
The technique does not rely on specific fluorophores and can be used for multicolor imaging. It has the same multicolor capacity as laser scanning confocal or spinning disc microscopy.

Temporal resolution – 5/5
Owing to the mostly optical nature of the resolution improvement process, high-speed imaging is possible and expected to be similar to that of laser scanning confocal or spinning disc microscopes.

Availability
Commercial offerings include dedicated setups, and add-ons to a widefield or spinning disc microscope. Most commercial systems are relatively recent, so they are not yet widely available in imaging facilities.

Structured illumination microscopy
Principle
In the structured illumination microscopy (SIM) technique, the sample is illuminated using a patterned light. For each focus plane, multiple images are taken using a different pattern and are then

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combined by a computer algorithm to reconstruct a super-resolved image (Schermelleh et al., 2019). The most common patterns are parallel lines, but hexagonal or even random patterns can be used (Heintzmann and Huser, 2017). In the case of parallel lines, multiple shifted and rotated patterns are obtained using a grid or a spatial light modulator: 9 images for a 2D image (Gustafsson, 2000) and 15 images per plane for a 3D volume (Gustafsson et al., 2008).

Versatility – 4/5

SIM is generally easy to use, suitable for a wide variety of biological samples and is compatible with most fluorophores with the conditions that they are relatively resistant to photobleaching and non-blinking (Demmerle et al., 2017). SIM performances are affected by both the sample and the imaging conditions, and therefore dedicated training and optimizations are required to achieve good results.

XY and *Z* resolution – 3/5

Linear SIM typically doubles the spatial resolution in all three dimensions (Gustafsson et al., 2008). Non-linear SIM approaches that bypass this resolution use fluorophore saturation, but are not yet commercially available (Li et al., 2015; Rego et al., 2012).

Thick-sample friendliness – 3/5

The SIM design is often based on widefield microscopy. In this case, SIM performance can be strongly affected by the sample thickness, as well as by the presence of out of focus light. 'Grazing incidence' illumination (Guo et al., 2018) or TIRF (Kner et al., 2009; Li et al., 2015) can be used to image objects that are close to the coverslip with a better signal. In addition, lattice light-sheet (Chen et al., 2014) or slit-confocal (Schropp et al., 2017) arrangements can be combined with SIM to image deeper into cells.

Live-cell friendliness – 4/5

SIM is commonly used for live-cell imaging (Burnette et al., 2014; Carisey et al., 2018; Fiolka et al., 2012). Traditional 3D SIM often requires the acquisition of hundreds of images per time point (depending on the volume imaged) and can be phototoxic. Therefore, SIM-TIRF or grazing-incidence SIM are better suited for live-cell imaging (Movie 3). In addition, improvement in reconstruction algorithms are enabling the imaging of biological samples using low laser power over hour-long time lapses (Huang et al., 2018).

Image fidelity – 4/5

When using linear SIM, the fluorescence intensities in reconstructed images are not directly transposed from the intensities of the raw images (Heintzmann and Huser, 2017). The intensity of the reconstructed image typically correlates well with the brightness of the original structures, but SIM can attenuate constant signals and is thus not suited to image and quantify diffuse (cytoplasmic) staining.

Multicolor – 4/5

As SIM is compatible with most fluorophores, its setup can typically accommodate three to four different color channels (Jacquemet et al., 2019; Vietri et al., 2015). However, optimal resolution requires a precise tuning of the oil refractive index that can be slightly different for distinct channels (Demmerle et al., 2017).

Temporal resolution – 4/5

The temporal resolution of SIM widely depends on the setup used. Traditional 3D SIM requires the acquisition of hundreds of images per time point (depending on the volume imaged) and is relatively slow (several seconds per time point) (Fiolka et al., 2012). Other SIM implementations such as SIM-TIRF offer a faster acquisition speed of 11 Hz (Kner et al., 2009). Recent developments such as instant TIRF-SIM allow acquisitions as fast as 100 Hz, with the use of a sliding window over the varying patterns (Guo et al., 2018; Huang et al., 2018).

Availability

SIM microscopy requires a dedicated microscope and therefore can be expensive to implement. Multiple commercial systems are available.

Stimulated emission depletion microscopy

Principle

Stimulated emission depletion (STED) microscopy is based on laser scanning confocal microscopy where a second hollow beam (a donut-shaped STED beam) is overlaid on top of the excitation laser beam (Heine et al., 2017; Hell and Wichmann, 1994; Klar et al., 2000). In the zone of overlap between the two beams, the STED beam depletes the fluorophores before fluorescence takes place, thinning the emission area to a sub-diffraction sized spot. The donut-shaped STED beam is obtained using a vortex phase mask and the most recent systems are combining femtosecond lasers and detector time gating to improve the signal-to-noise ratio and significantly reduce the background (Hernández et al., 2015; Moffitt et al., 2011).

Versatility – 3/5

STED microscopy is very similar to, and has the same sample requirement as, classical confocal microscopy. STED does not need specific buffers, but special care should be taken during sample preparation (e.g. fixation) to ensure maximal preservation of cellular structures at the STED resolution (Blom and Widengren, 2017).

XY resolution – 4/5

In practice, in biological systems, the best resolution achieved are ∼40 nm in living tissue and cells (Bottanelli et al., 2016; Willig et al., 2006), and 20 nm in fixed samples (Göttfert et al., 2013), and a typical ∼60 nm resolution can be obtained in core facility settings. Recently, 1 nm resolution was achieved by using a hybrid microscopy technique combining STED and single-molecule localization microscopy (SMLM) (Balzarotti et al., 2017; Gwosch et al., 2020).

Z resolution – 4/5

In 3D STED, it is possible to also thin the emission spot along the *Z*-axis using a second phase mask, which provides up to a four-fold improvement in *Z* resolution when compared to point scanning confocal (Klar et al., 2000). For instance, one study achieved 90 nm in the *Z* dimension, while maintaining 35 nm in the *XY* dimensions (Osseforth et al., 2014).

Thick-sample friendliness – 2/5

Both the intensity and the geometry of the excitation and depletion beams are differently affected while traveling across the biological sample, leading to difficulties when imaging deep into tissues. This can be partially improved by using multiphoton excitation lasers, which allow for a deeper penetration imaging capability.

Live-cell friendliness – 3/5

Owing to the requirement for high-intensity illumination by the STED beam, this technique remains a challenge for live-cell imaging. From the various configurations available, depletion using a 775 nm laser line is the most live-cell friendly and has been shown to work for a panel of fluorescent proteins and probes

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(D'Este et al., 2015). Gentler approaches called RESOLFT, based on photoswitchable probes, allow resolution enhancement with a lower light dose (Grotjohann et al., 2011; Masullo et al., 2018).

Image fidelity – 5/5

STED microscopy does not require post-processing of the images, which strongly limits the risk for artifact generation. Image quality and signal-to-noise ratio can however be further improved by photon reassignment using computational deconvolution.

Multicolor – 2/5

The number of fluorophores compatible with STED remains limited and many commonly used dyes are irreversibly bleached by the high intensity STED beam. In addition, in multicolor experiments, special care must be taken when selecting fluorophores to ensure that no overlap exists between their excitation spectrum and the depletion laser to improve the resolution of the other channels. Sequential scanning can be used as a workaround but acquisitions are limited to a single time point and focal plane.

Temporal resolution – 3/5

STED microscopy allows the user to directly visualize the object of interest in super resolution without the need for offline computation or post-processing of multiple images. It is therefore suitable for the imaging of fast cellular events, such as organelle dynamics and protein trafficking (Bottanelli et al., 2016).

Availability

STED is commercially available from two companies as complete setups, with a range of costs that makes them more suited to shared facilities.

Single-molecule localization microscopy

Principle

During single-molecule localization microscopy (SMLM) experiments, the emission of individual fluorophores are recorded using a camera. The resulting images are composed of diffraction-limited spots (typically ∼200 nm in width) that are then fitted to precisely pinpoint the fluorophore position (typically within ∼10–15 nm) (Diezmann et al., 2017). In practice, tens of thousands of images of blinking fluorophores are acquired in rapid succession (Jimenez et al., 2019). A processing software is then used to fit the blinking events and create a super-resolved image (Baddeley and Bewersdorf, 2018). To detect single molecules from densely labeled samples, only a small fraction of the fluorophores can be emitting photons at any one time (Li and Vaughan, 2018). This can be achieved by using sparsely activated photoactivatable/photo-convertible fluorescent proteins in (fluorescence-) photo-activated localization microscopy [(F)PALM] (Betzig et al., 2006; Hess et al., 2006). Organic dyes can also be induced to blink using specific buffers in (direct) stochastic optical reconstruction microscopy [(d)STORM] (Heilemann et al., 2008; Rust et al., 2006) or ground-state depletion (GSD) microscopy (Fölling et al., 2008). Alternatively, blinking can be generated by the transient interaction between two short DNA sequences, one labeled and one unlabeled, in so-called DNA point-accumulation in nanoscale topography (DNA-PAINT) (Jungmann et al., 2014).

Versatility – 2/5

Owing to its high spatial resolution, SMLM requires specific care during sample preparation to ensure optimal ultrastructural preservation (Jimenez et al., 2019; Whelan and Bell, 2015). In addition, as single molecules are recorded, the density of labeling must be high enough to delineate the final structure of interest (Patterson et al., 2010).

XY resolution – 5/5

SMLM reconstructs images at 10 to 15 nm resolution. The final resolution achieved depends on the brightness of the fluorophores detected, their labeling density and the capacity to accurately detect individual fluorophores (Culley et al., 2018b). Importantly, at this scale, the size of the probe (antibody or fusion protein) can start to degrade the precision of the imaging (Magrassi et al., 2019).

Z resolution – 5/5

A common way of retrieving the *Z* coordinate of fluorophores is to deform the point-spread function into an ellipse using a cylindrical lens (Huang et al., 2008). This provides a typical *Z* localization precision of 20–30 nm (Diezmann et al., 2017).

Thick-sample friendliness – 2/5

SMLM is sensitive to light diffusion and spherical aberrations when imaging structures that are more than a few microns above the coverslip. More complex setups using light-sheet illumination schemes or adaptive optics allow to reach deeper in cells and tissue, but they are not yet broadly available (Liu et al., 2018).

Live-cell friendliness – 2/5

The time necessary to accumulate enough localizations and the high illumination intensities needed to visualize single molecules makes SMLM challenging to use on live samples (Tosheva et al., 2020; Wäldchen et al., 2015), although it has been performed (Huang et al., 2013; Jones et al., 2011).

Image fidelity – 2/5

The complexity of the required post-acquisition analysis (localization and image reconstruction) and the high precision attained by SMLM makes it prone to artifacts. Care must be taken to ensure proper quenching of fluorophores and to limit the density of their blinking.

Multicolor – 3/5

Multicolor remains a challenge for most SMLM strategies – photoactivatable and/or convertible proteins rapidly occupy all available channels in PALM (Shroff et al., 2007), and the photophysics of organic fluorophores makes it difficult to identify those that are spectrally distinct and have good blinking properties (Dempsey et al., 2011; Lehmann et al., 2015). However, DNA-PAINT allows for virtually unlimited sequential imaging of distinct targets, and is easily used for imaging of three to four colors (Jimenez et al., 2019; Jungmann et al., 2014).

Temporal resolution – 1/5

The necessity to acquire thousands of images for a single reconstruction is a strong impediment to fast acquisition in SMLM. Higher laser intensities and fast cameras can be used (Lin et al., 2015). Furthermore, the use of artificial intelligence shows promise in the ability to infer structure from a limited number of acquired images (Ouyang et al., 2018).

Availability

A regular epifluorescence microscope equipped with lasers is all that is needed to perform SMLM, with high-power lasers required

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for STORM; several free options exist to process and analyze SMLM data (Jimenez et al., 2019; van de Linde, 2019).

Expansion microscopy

Principle

Expansion microscopy (ExM) is a sample preparation technique that physically increases the size of the specimen (Chen et al., 2015). Multiple variations of the ExM protocol have been described, but they all share a common workflow. After fixation, the sample is embedded and cross-linked to a swellable gel that is then expanded using water (Wassie et al., 2019). The resulting enlarged specimen can then be imaged using classical microscopy techniques (Chen et al., 2015; Chozinski et al., 2016; Ku et al., 2016; Tillberg et al., 2016).

Versatility – 3/5

ExM has been successfully applied to a wide variety of samples, including cells (Chen et al., 2015), tissue sections (Zhao et al., 2017) and model organisms (*Drosophila* and zebrafish embryo) (Cahoon et al., 2017; Freifeld et al., 2017), as well as whole intact organs (Gao et al., 2019; Ku et al., 2016). ExM has also been used to observe RNA (Chen et al., 2016) and lipids (Karagiannis et al., 2019 preprint). Each new application of ExM needs careful and specific optimization.

XY and *Z* resolution – 4/5

The final 'resolution' of the image depends on the final size of the expended sample, as well as on the microscopy strategy used to acquire the images. ExM protocols typically lead to a 4.5-fold expansion in all dimensions (an expected resolution of ∼70 nm), but others attain a 10-fold expansion (an expected resolution of 25–30 nm) (Truckenbrodt et al., 2018). Samples can also be expanded sequentially (an expected resolution of ∼25 nm) (Chang et al., 2017). ExM is also compatible with other super-resolution modalities, including STED (Gao et al., 2018), SMLM (Shi et al., 2019 preprint) and SIM (Cahoon et al., 2017; Halpern et al., 2017).

Thick-sample friendliness – 5/5

ExM can be used on thick samples, such as small model organisms or whole mouse organs (Cahoon et al., 2017; Freifeld et al., 2017; Gao et al., 2019; Ku et al., 2016). However, it is important to note that post-expansion, samples will be at least 100-fold more voluminous and, therefore, they can be challenging to image at high magnification using classical microscopes. Light-sheet microscopy is especially well suited for imaging such samples (Gao et al., 2019).

Live-cell friendliness and temporal resolution – 0/5

ExM is only compatible with fixed samples.

Image fidelity – 3/5

ExM leads to an isotropic expansion and has been shown to preserve the structural integrity of the various cellular structures imaged. In addition, using the nuclear pore as a reporter, ExM was found to have a uniform accuracy in the range of 20 nm (Pesce et al., 2019). Nevertheless, one concern that remains is whether all the structures of interest within a sample expand at the same rate or to the same extent. For instance, in bacteria, the expansion efficacy varies across species (Lim et al., 2019). Moreover, the digestion procedure typically attenuates the fluorescence of organic or protein-based fluorophores, and the expansion itself dilutes the fluorescent signal spatially (a 4-fold expansion results in 64 times less fluorescence per volume unit), requiring sensitive microscopes for ExM sample imaging.

Multicolor – 5/5

ExM is compatible with standard dyes and fluorescent proteins and is therefore well adapted to multicolor experiments.

Availability

ExM sample preparation only requires commercially available reagents and is therefore readily available; it is also relatively inexpensive to implement (Asano et al., 2018).

Conclusions – going above and beyond

Regardless of the technology used to acquire the fluorescent signal, powerful signal post-processing methods, such as deconvolution or image restoration, can be used to increase the resolution of the final image. Various algorithms have been implemented directly in the acquisition software commercially available, while others, for instance SACD (Zhao et al., 2018 preprint), can be run independently of the pipeline. Another promising development is the use of artificial intelligence to improve the quality of the final image; here, large datasets of high-resolution images are used to train neural networks that can then be applied to low-resolution and/or noisy datasets datasets (Belthangady and Royer, 2019; Moen et al., 2019; von Chamier et al., 2019; von Chamier et al., 2020 preprint). The resulting image gains a dramatic resolution improvement thanks to the prior knowledge of the structure obtained from high-resolution images.

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Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.240713.supplemental

Cell science at a glance

A high-resolution version of the poster and individual poster panels are available for downloading at http://jcs.biologists.org/lookup/doi/10.1242/jcs.240713.supplemental

References

Almada, P., Pereira, P. M., Culley, S., Caillol, G., Boroni-Rueda, F., Dix, C. L., Charras, G., Baum, B., Laine, R. F., Leterrier, C. et al. (2019). Automating multimodal microscopy with NanoJ-Fluidics. *Nat. Commun.* **10**, 1-9. doi:10.1038/s41467-019-09231-9

Asano, S. M., Gao, R., Wassie, A. T., Tillberg, P. W., Chen, F. and Boyden, E. S. (2018). Expansion microscopy: protocols for imaging proteins and RNA in cells and tissues. *Curr. Protoc. Cell Biol.* **80**, e56. doi:10.1002/cpcb.56

Azuma, T. and Kei, T. (2015). Super-resolution spinning-disk confocal microscopy using optical photon reassignment. *Opt. Express* **23**, 15003. doi:10.1364/OE.23.015003

Bachmann, M., Fiederling, F. and Bastmeyer, M. (2015). Practical limitations of superresolution imaging due to conventional sample preparation revealed by a direct comparison of CLSM, SIM and dSTORM. *J. Microsc* **262**, 306-315. doi:10.1111/jmi.12365

Baddeley, D. and Bewersdorf, J. (2018). Biological insight from super-resolution microscopy: what we can learn from localization-based images. *Annu. Rev. Biochem.* **87**, 965-989. doi:10.1146/annurev-biochem-060815-014801

Bagshaw, C. R. and Cherny, D. (2006). Blinking fluorophores: what do they tell us about protein dynamics? *Biochem. Soc. Trans.* **34**, 979-982. doi:10.1042/BST0340979

Balzarotti, F., Eilers, Y., Gwosch, K. C., Gynnå, A. H., Westphal, V., Stefani, F. D., Elf, J. and Hell, S. W. (2017). Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes. *Sci. New York N Y* **355**, 606-612. doi:10.1126/science.aak9913

Belthangady, C. and Royer, L. A. (2019). Applications, promises, and pitfalls of deep learning for fluorescence image reconstruction. *Nat. Methods* **16**, 1215-1225. doi:10.1038/s41592-019-0458-z

Betzig, E., Patterson, G. H., Sougrat, R., Lindwasser, O. W., Olenych, S., Bonifacino, J. S., Davidson, M. W., Lippincott-Schwartz, J. and Hess, H. F. (2006). Imaging intracellular fluorescent proteins at nanometer resolution. *Science* **313**, 1642-1645. doi:10.1126/science.1127344

Blom, H. and Widengren, J. (2017). Stimulated emission depletion microscopy. *Chem. Rev.* **117**, 7377-7427. doi:10.1021/acs.chemrev.6b00653

Bottanelli, F., Kromann, E. B., Allgeyer, E. S., Erdmann, R. S., Baguley, S. W., Sirinakis, G., Schepartz, A., Baddeley, D., Toomre, D. K., Rothman, J. E. et al. (2016). Two-colour live-cell nanoscale imaging of intracellular targets. *Nat. Commun.* **7**, 10778. doi:10.1038/ncomms10778

Burnette, D. T., Shao, L., Ott, C., Pasapera, A. M., Fischer, R. S., Baird, M. A., Der Loughian, C., Delanoe-Ayari, H., Paszek, M. J., Davidson, M. W. et al. (2014). A contractile and counterbalancing adhesion system controls the 3D shape of crawling cells. *J. Cell Biol.* **205**, 83-96. doi:10.1083/jcb.201311104

Cahoon, C. K., Yu, Z., Wang, Y., Guo, F., Unruh, J. R., Slaughter, B. D. and Hawley, R. S. (2017). Superresolution expansion microscopy reveals the three-dimensional organization of the Drosophila synaptonemal complex. *Proc. Natl. Acad. Sci. USA* **114**, E6857-E6866. doi:10.1073/pnas.1705623114

Carisey, A. F., Mace, E. M., Saeed, M. B., Davis, D. M. and Orange, J. S. (2018). Nanoscale dynamism of actin enables secretory function in cytolytic cells. *Curr. Biol.* **28**, 489-502.e9. doi:10.1016/j.cub.2017.12.044

Chamier, Lv., Jukkala, J., Spahn, C., Lerche, M., Hernández-pérez, S., Mattila, P., Karinou, E., Holden, S., Solak, A.C., Krull, A. et al. (2020). ZeroCostDL4Mic: an open platform to simplify access and use of Deep-Learning in Microscopy. *bioRxiv* 2020.03.20.000133. doi:10.1101/2020.03.20.000133

Chang, J.-B., Chen, F., Yoon, Y.-G., Jung, E. E., Babcock, H., Kang, J. S., Asano, S., Suk, H.-J., Pak, N., Tillberg, P. W. et al. (2017). Iterative expansion microscopy. *Nat. Methods* **14**, 593-599. doi:10.1038/nmeth.4261

Chen, B.-C., Legant, W. R., Wang, K., Shao, L., Milkie, D. E., Davidson, M. W., Janetopoulos, C., Wu, X. S., Hammer, J. A., Liu, J. et al. (2014). Lattice light-sheet microscopy: Imaging molecules to embryos at high spatiotemporal resolution. *Science* **346**, 1257998. doi:10.1126/science.1257998

Chen, F., Tillberg, P. W. and Boyden, E. S. (2015). Expansion microscopy. *Science* **347**, 543-548. doi:10.1126/science.1260088

Chen, F., Wassie, A. T., Cote, A. J., Sinha, A., Alon, S., Asano, S., Daugharthy, E. R., Chang, J.-B., Marblestone, A., Church, G. M. et al. (2016). Nanoscale imaging of RNA with expansion microscopy. *Nat. Methods* **13**, 679-684. doi:10.1038/nmeth.3899

Chozinski, T. J., Halpern, A. R., Okawa, H., Kim, H.-J., Tremel, G. J., Wong, R. O. L. and Vaughan, J. C. (2016). Expansion microscopy with conventional antibodies and fluorescent proteins. *Nat. Methods* **13**, 485-488. doi:10.1038/nmeth.3833

Combs and Shroff (2017). Fluorescence microscopy: a concise guide to current imaging methods. *Curr. Protoc. Neurosci.* **79**, 2.1.1-2.1.25. doi:10.1002/cpns.29

Cooper, J., Browne, M., Gribben, H., Catney, M., Coates, C., Mullan, A., Wilde, G. and Henriques, R. (2019). Real time multi-modal super-resolution microscopy through Super-Resolution Radial Fluctuations (SRRF-Stream). In *Single Molecule Spectroscopy and Superresolution Imaging XII* (ed. Z. K. Gryczynski, I. Gregor, F. Koberling), p. 1088418. International Society for Optics and Photonics.

Culley, S., Tosheva, K. L., Pereira, P. M. and Henriques, R. (2018a). SRRF: Universal live-cell super-resolution microscopy. *Int. J. Biochem. Cell Biol.* **101**, 74-79. doi:10.1016/j.biocel.2018.05.014

Culley, S., Albrecht, D., Jacobs, C., Pereira, P. M., Leterrier, C., Mercer, J. and Henriques, R. (2018b). Quantitative mapping and minimization of super-resolution optical imaging artifacts. *Nat. Methods* **15**, 263-266. doi:10.1038/nmeth.4605

Dedecker, P., Duwé, S., Neely, R. K. and Zhang, J. (2012). Localizer: fast, accurate, open-source, and modular software package for superresolution microscopy. *J. Biomed. Opt.* **17**, 126008. doi:10.1117/1.JBO.17.12.126008

Demmerle, J., Innocent, C., North, A. J., Ball, G., Müller, M., Miron, E., Matsuda, A., Dobbie, I. M., Markaki, Y. and Schermelleh, L. (2017). Strategic and practical guidelines for successful structured illumination microscopy. *Nat. Protoc.* **12**, 988-1010. doi:10.1038/nprot.2017.019

Dempsey, G. T., Vaughan, J. C., Chen, K. H., Bates, M. and Zhuang, X. (2011). Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging. *Nat. Methods* **8**, 1027-1036. doi:10.1038/nmeth.1768

Dertinger, T., Colyer, R., Iyer, G., Weiss, S. and Enderlein, J. (2009). Fast, background-free, 3D super-resolution optical fluctuation imaging (SOFI). *Proc. Natl. Acad. Sci. USA* **106**, 22287-22292. doi:10.1073/pnas.0907866106

D'Este, E., Kamin, D., Göttfert, F., El-Hady, A. and Hell, S. W. (2015). STED nanoscopy reveals the ubiquity of subcortical cytoskeleton periodicity in living neurons. *Cell Reports* **10**, 1246-1251. doi:10.1016/j.celrep.2015.02.007

Diezmann, A. von, Shechtman, Y. and Moerner, W. E. (2017). Three-dimensional localization of single molecules for super-resolution imaging and single-particle tracking. *Chem. Rev.* **117**, 7244-7275. doi:10.1021/acs.chemrev.6b00629

Fiolka, R., Shao, L., Rego, E. H., Davidson, M. W. and Gustafsson, M. G. L. (2012). Time-lapse two-color 3D imaging of live cells with doubled resolution using structured illumination. *Proc. Natl. Acad. Sci. USA* **109**, 5311-5315. doi:10.1073/pnas.1119262109

Fölling, J., Bossi, M., Bock, H., Medda, R., Wurm, C. A., Hein, B., Jakobs, S., Eggeling, C. and Hell, S. W. (2008). Fluorescence nanoscopy by ground-state depletion and single-molecule return. *Nat. Methods* **5**, 943-945. doi:10.1038/nmeth.1257

Freifeld, L., Odstrcil, I., Förster, D., Ramirez, A., Gagnon, J. A., Randlett, O., Costa, E. K., Asano, S., Celiker, O. T., Gao, R. et al. (2017). Expansion microscopy of zebrafish for neuroscience and developmental biology studies. *Proc. Natl. Acad. Sci. USA* **114**, E10799-E10808. doi:10.1073/pnas.1706281114

Frigault, M. M., Lacoste, J., Swift, J. L. and Brown, C. M. (2009). Live-cell microscopy – tips and tools. *J. Cell Sci.* **122**, 753-767. doi:10.1242/jcs.033837

Gao, M., Maraspini, R., Beutel, O., Zehtabian, A., Eickholt, B., Honigmann, A. and Ewers, H. (2018). Expansion stimulated emission depletion microscopy (ExSTED). *Acs Nano* **12**, 4178-4185. doi:10.1021/acsnano.8b00776

Gao, R., Asano, S. M., Upadhyayula, S., Pisarev, I., Milkie, D. E., Liu, T.-L., Singh, V., Graves, A., Huynh, G. H., Zhao, Y. et al. (2019). Cortical column and whole-brain imaging with molecular contrast and nanoscale resolution. *Science* **363**, eaau8302. doi:10.1126/science.aau8302

Göttfert, F., Wurm, C. A., Mueller, V., Berning, S., Cordes, V. C., Honigmann, A. and Hell, S. W. (2013). Coaligned dual-channel STED nanoscopy and molecular diffusion analysis at 20 nm resolution. *Biophys. J.* **105**, L01-L03. doi:10.1016/j.bpj.2013.05.029

Grotjohann, T., Testa, I., Leutenegger, M., Bock, H., Urban, N. T., Lavoie-Cardinal, F., Willig, K. I., Eggeling, C., Jakobs, S. and Hell, S. W. (2011). Diffraction-unlimited all-optical imaging and writing with a photochromic GFP. *Nature* **478**, 204-208. doi:10.1038/nature10497

Guo, Y., Li, D., Zhang, S., Yang, Y., Liu, J.-J., Wang, X., Liu, C., Milkie, D. E., Regan, P. Moore, et al. (2018). Visualizing intracellular organelle and cytoskeletal interactions at nanoscale resolution on millisecond timescales. *Cell* **175**, 1430-1442.e17. doi:10.1016/j.cell.2018.09.057

Gustafsson, M. G. L. (2000). Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. SHORT COMMUNICATION. *J. Microsc* **198**, 82-87. doi:10.1046/j.1365-2818.2000.00710.x

Gustafsson, M. G. L., Shao, L., Carlton, P. M., Wang, C. J. R., Golubovskaya, I. N., Cande, W. Z., Agard, D. A. and Sedat, J. W. (2008). Three-dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination. *Biophys. J.* **94**, 4957-4970. doi:10.1529/biophysj.107.120345

Gustafsson, N., Culley, S., Ashdown, G., Owen, D. M., Pereira, P. M. and Henriques, R. (2016). Fast live-cell conventional fluorophore nanoscopy with ImageJ through super-resolution radial fluctuations. *Nat. Commun.* **7**, 12471. doi:10.1038/ncomms12471

Gwosch, K. C., Pape, J. K., Balzarotti, F., Hoess, P., Ellenberg, J., Ries, J. and Hell, S. W. (2020). MINFLUX nanoscopy delivers 3D multicolor nanometer resolution in cells. *Nat. Methods* **17**, 217-224. doi:10.1038/s41592-019-0688-0

Halpern, A. R., Alas, G. C. M., Chozinski, T. J., Paredez, A. R. and Vaughan, J. C. (2017). Hybrid structured illumination expansion microscopy reveals microbial cytoskeleton organization. *Acs Nano* **11**, 12677-12686. doi:10.1021/acsnano.7b07200

Hayashi, S. and Okada, Y. (2015). Ultrafast superresolution fluorescence imaging with spinning disk confocal microscope optics. *Mol. Biol. Cell* **26**, 1743-1751. doi:10.1091/mbc.E14-08-1287

Heilemann, M., van de Linde, S., Schüttpelz, M., Kasper, R., Seefeldt, B., Mukherjee, A., Tinnefeld, P. and Sauer, M. (2008). Subdiffraction-resolution fluorescence imaging with conventional fluorescent probes. *Angewandte Chemie Int. Ed. Engl.* **47**, 6172-6176. doi:10.1002/anie.200802376

Heine, J., Reuss, M., Harke, B., D'Este, E., Sahl, S. J. and Hell, S. W. (2017). Adaptive-illumination STED nanoscopy. *Proc. Natl. Acad. Sci. USA* **114**, 9797-9802. doi:10.1073/pnas.1708304114

Heintzmann, R. and Huser, T. (2017). Super-resolution structured illumination microscopy. *Chem. Rev.* **117**, 13890-13908. doi:10.1021/acs.chemrev.7b00218

Hell, S. W. and Wichmann, J. (1994). Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Opt. Lett.* **19**, 780. doi:10.1364/OL.19.000780

Hernández, I. C., Buttafava, M., Boso, G., Diaspro, A., Tosi, A. and Vicidomini, G. (2015). Gated STED microscopy with time-gated single-photon avalanche diode. *Biomed. Opt. Express* **6**, 2258. doi:10.1364/BOE.6.002258
Hess, S. T., Girirajan, T. P. K. and Mason, M. D. (2006). Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophys. J.* **91**, 4258-4272. doi:10.1529/biophysj.106.091116
Huang, B., Wang, W., Bates, M. and Zhuang, X. (2008). Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. *Sci. New York N Y* **319**, 810-813. doi:10.1126/science.1153529
Huang, F., Hartwich, T. M. P., Rivera-Molina, F. E., Lin, Y., Duim, W. C., Long, J. J., Uchil, P. D., Myers, J. R., Baird, M. A., Mothes, W. et al. (2013). Video-rate nanoscopy using sCMOS camera-specific single-molecule localization algorithms. *Nat. Methods* **10**, 653-658. doi:10.1038/nmeth.2488
Huang, X., Fan, J., Li, L., Liu, H., Wu, R., Wu, Y., Wei, L., Mao, H., Lal, A., Xi, P. et al. (2018). Fast, long-term, super-resolution imaging with Hessian structured illumination microscopy. *Nat. Biotechnol.* **36**, 451-459. doi:10.1038/nbt.4115
Huff, J. (2015). The Airyscan detector from ZEISS: confocal imaging with improved signal-to-noise ratio and super-resolution. *Nat. Methods* **12**, i-ii. doi:10.1038/nmeth.f.388
Jacquemet, G., Stubb, A., Saup, R., Miihkinen, M., Kremneva, E., Hamidi, H. and Ivaska, J. (2019). Filopodome mapping identifies p130Cas as a mechanosensitive regulator of filopodia stability. *Curr. Biol. Cb* **29**, 202-216.e7. doi:10.1016/j.cub.2018.11.053
Jimenez, A., Friedl, K. and Leterrier, C. (2019). About samples, giving examples: optimized single molecule localization microscopy. *Methods* **174**, 100-114. doi:10.1016/j.ymeth.2019.05.008
Jones, S. A., Shim, S.-H., He, J. and Zhuang, X. (2011). Fast, three-dimensional super-resolution imaging of live cells. *Nat. Methods* **8**, 499-508. doi:10.1038/nmeth.1605
Jost, A. P.-T. and Waters, J. C. (2019). Designing a rigorous microscopy experiment: validating methods and avoiding bias. *J. Cell Biol.* **218**, 1452-1466. doi:10.1083/jcb.201812109
Jungmann, R., Avendaño, M. S., Woehrstein, J. B., Dai, M., Shih, W. M. and Yin, P. (2014). Multiplexed 3D cellular super-resolution imaging with DNA-PAINT and Exchange-PAINT. *Nat. Methods* **11**, 313-318. doi:10.1038/nmeth.2835
Karagiannis, E. D., Kang, J. S., Shin, T. W., Emenari, A., Asano, S., Lin, L., Costa, E. K., Consortium, I. G. C., Marblestone, A. H., Kasthuri, N. et al. (2019). Expansion microscopy of lipid membranes. *Biorxiv* 829903. doi:10.1101/829903
Klar, T. A., Jakobs, S., Dyba, M., Egner, A. and Hell, S. W. (2000). Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission. *Proc. Natl. Acad. Sci. USA* **97**, 8206-8210. doi:10.1073/pnas.97.15.8206
Kner, P., Chhun, B. B., Griffis, E. R., Winoto, L. and Gustafsson, M. G. L. (2009). Super-resolution video microscopy of live cells by structured illumination. *Nat. Methods* **6**, 339-342. doi:10.1038/nmeth.1324
Kolesova, H., Capek, M., Radochova, B., Janacek, J. and Sedmera, D. (2016). Visualization of GFP mouse embryos and embryonic hearts using various tissue clearing methods and 3D imaging modalities. In European Microscopy Congress 2016: Proceedings, pp. 256-257. American Cancer Society.
Kremers, G.-J., Gilbert, S. G., Cranfill, P. J., Davidson, M. W. and Piston, D. W. (2010). Fluorescent proteins at a glance. *J. Cell Sci.* **124**, 157-160. doi:10.1242/jcs.072744
Ku, T., Swaney, J., Park, J.-Y., Albanese, A., Murray, E., Cho, J. H., Park, Y.-G., Mangena, V., Chen, J. and Chung, K. (2016). Multiplexed and scalable super-resolution imaging of three-dimensional protein localization in size-adjustable tissues. *Nat. Biotechnol.* **34**, 973-981. doi:10.1038/nbt.3641
Lambert, T. J. (2019). FPbase: a community-editable fluorescent protein database. *Nat. Methods* **16**, 277-278. doi:10.1038/s41592-019-0352-8
Lehmann, M., Lichtner, G., Klenz, H. and Schmoranzer, J. (2015). Novel organic dyes for multicolor localization-based super-resolution microscopy. *J. Biophotonics* **9**, 161-170. doi:10.1002/jbio.201500119
Li, H. and Vaughan, J. C. (2018). Switchable fluorophores for single-molecule localization microscopy. *Chem. Rev.* **118**, 9412-9454. doi:10.1021/acs.chemrev.7b00767
Li, D., Shao, L., Chen, B.-C., Zhang, X., Zhang, M., Moses, B., Milkie, D. E., Beach, J. R., Hammer, J. A., Pasham, M. et al. (2015). Extended-resolution structured illumination imaging of endocytic and cytoskeletal dynamics. *Science* **349**, aab3500-aab3500. doi:10.1126/science.aab3500
Lim, Y., Shiver, A. L., Khariton, M., Lane, K. M., Ng, K. M., Bray, S. R., Qin, J., Huang, K. C. and Wang, B. (2019). Mechanically resolved imaging of bacteria using expansion microscopy. *PLoS Biol.* **17**, e3000268. doi:10.1371/journal.pbio.3000268
Lin, Y., Long, J. J., Huang, F., Duim, W. C., Kirschbaum, S., Zhang, Y., Schroeder, L. K., Rebane, A. A., Velasco, M. G. M., Virrueta, A. et al. (2015). Quantifying and optimizing single-molecule switching nanoscopy at high speeds. *PLoS ONE* **10**, e0128135. doi:10.1371/journal.pone.0128135
Liu, W., Toussaint, K. C., Okoro, C., Zhu, D., Chen, Y., Kuang, C. and Liu, X. (2018). Breaking the axial diffraction limit: a guide to axial super-resolution fluorescence microscopy. *Laser Photonics Rev.* **12**, 1700333. doi:10.1002/lpor.201700333
Luca, G. M. R. D., Breedijk, R. M. P., Brandt, R. A. J., Zeelenberg, C. H. C., de Jong, B. E., Timmermans, W., Azar, L. N., Hoebe, R. A., Stallinga, S. and Erik, M. M. et al. 2013). Re-scan confocal microscopy: scanning twice for better resolution. *Biomed. Opt. Express* **4**, 2644-2656. doi:10.1364/BOE.4.002644
Magrassi, R., Scalisi, S. and Cella Zanacchi, F. (2019). Single-molecule localization to study cytoskeletal structures, membrane complexes, and mechanosensors. *Biophys. Rev.* **11**, 745-756. doi:10.1007/s12551-019-00595-2
Masters, B. R. (2010). The development of fluorescence microscopy. In: *Encyclopedia of Life Sciences* (ELS). John Wiley & Sons, Ltd, Chichester. doi:10.1002/9780470015902.a0022093
Masullo, L. A., Bodén, A., Pennacchietti, F., Coceano, G., Ratz, M. and Testa, I. (2018). Enhanced photon collection enables four dimensional fluorescence nanoscopy of living systems. *Nat. Commun.* **9**, 1-9. doi:10.1038/s41467-018-05799-w
Moen, E., Bannon, D., Kudo, T., Graf, W., Covert, M. and Van Valen, D. (2019). Deep learning for cellular image analysis. *Nat. Methods* **16**, 1233-1246. doi:10.1038/s41592-019-0403-1
Moffitt, J. R., Osseforth, C. and Michaelis, J. (2011). Time-gating improves the spatial resolution of STED microscopy. *Opt. Express* **19**, 4242-4254. doi:10.1364/OE.19.004242
Müller, C. B. and Enderlein, J. (2010). Image scanning microscopy. *Phys. Rev. Lett.* **104**, 198101. doi:10.1103/PhysRevLett.104.198101
Osseforth, C., Moffitt, J. R., Schermelleh, L. and Michaelis, J. (2014). Simultaneous dual-color 3D STED microscopy. *Opt. Express* **22**, 7028. doi:10.1364/OE.22.007028
Ouyang, W., Aristov, A., Lelek, M., Hao, X. and Zimmer, C. (2018). Deep learning massively accelerates super-resolution localization microscopy. *Nat. Biotechnol.* **36**, 460. doi:10.1038/nbt.4106
Patterson, G., Davidson, M., Manley, S. and Lippincott-Schwartz, J. (2010). Superresolution imaging using single-molecule localization. *Annu. Rev. Phys. Chem.* **61**, 345-367. doi:10.1146/annurev.physchem.012809.103444
Pereira, P. M., Albrecht, D., Culley, S., Jacobs, C., Marsh, M., Mercer, J. and Henriques, R. (2019). Fix your membrane receptor imaging: actin cytoskeleton and CD4 membrane organization disruption by chemical fixation. *Front. Immunol.* **10**, 675. doi:10.3389/fimmu.2019.00675
Pesce, L., Cozzolino, M., Lanzanò, L., Diaspro, A. and Bianchini, P. (2019). Measuring expansion from macro- to nanoscale using NPC as intrinsic reporter. *J. Biophotonics* **12**, e201900018. doi:10.1002/jbio.201900018
Qi, Y., Yu, T., Xu, J., Wan, P., Ma, Y., Zhu, J., Li, Y., Gong, H., Luo, Q. and Zhu, D. (2019). FDISCO: Advanced solvent-based clearing method for imaging whole organs. *Sci. Adv.* **5**, eaau8355. doi:10.1126/sciadv.aau8355
Rego, E. H., Shao, L., Macklin, J. J., Winoto, L., Johansson, G. A., Kamps-Hughes, N., Davidson, M. W. and Gustafsson, M. G. L. (2012). Nonlinear structured-illumination microscopy with a photoswitchable protein reveals cellular structures at 50-nm resolution. *Proc. Natl. Acad. Sci. USA* **109**, E135-E143. doi:10.1073/pnas.1107547108
Rust, M. J., Bates, M. and Zhuang, X. (2006). Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* **3**, 793-796. doi:10.1038/nmeth929
Sahl, S. J., Hell, S. W. and Jakobs, S. (2017). Fluorescence nanoscopy in cell biology. *Nat. Rev. Mol. Cell Biol.* **18**, 685-701. doi:10.1038/nrm.2017.71
Schermelleh, L., Ferrand, A., Huser, T., Eggeling, C., Sauer, M., Biehlmaier, O. and Drummen, G. P. C. (2019). Super-resolution microscopy demystified. *Nat. Cell Biol.* **21**, 72-84. doi:10.1038/s41556-018-0251-8
Schropp, M., Seebacher, C. and Uhl, R. (2017). XL-SIM: extending superresolution into deeper layers. *Photonics* **4**, 33. doi:10.3390/photonics4020033
Shi, X., Li, Q., Dai, Z., Tran, A. A., Feng, S., Ramirez, A. D., Lin, Z., Wang, X., Chow, T. T., Seiple, I. B. et al. (2019). Label-retention expansion microscopy. *Biorxiv* 687954. doi:10.1101/687954
Shroff, H., Galbraith, C. G., Galbraith, J. A., White, H., Gillette, J., Olenych, S., Davidson, M. W. and Betzig, E. (2007). Dual-color superresolution imaging of genetically expressed probes within individual adhesion complexes. *Proc. Natl. Acad. Sci. USA* **104**, 20308-20313. doi:10.1073/pnas.0710517105
Sigal, Y. M., Zhou, R. and Zhuang, X. (2018). Visualizing and discovering cellular structures with super-resolution microscopy. *Science* **361**, 880-887. doi:10.1126/science.aau1044
Ströhl, F. and Kaminski, C. F. (2016). Frontiers in structured illumination microscopy. *Optica* **3**, 667. doi:10.1364/OPTICA.3.000667
Stubb, A., Guzmán, C., Närvä, E., Aaron, J., Chew, T.-L., Saari, M., Miihkinen, M., Jacquemet, G. and Ivaska, J. (2019). Superresolution architecture of cornerstone focal adhesions in human pluripotent stem cells. *Nat. Commun.* **10**, 1-15. doi:10.1038/s41467-019-12611-w
Surre, J., Saint-Ruf, C., Collin, V., Orenga, S., Ramjeet, M. and Matic, I. (2018). Strong increase in the autofluorescence of cells signals struggle for survival. *Sci. Rep.* **8**, 12088. doi:10.1038/s41598-018-30623-2
Thorley, J. A., Pike, J., Rappoport, J. Z., Cornea, A. and Conn, P. M. (2014). Super-resolution microscopy: a comparison of commercially available options. In *Fluorescence Microscopy: Super-Resolution and Other Novel Techniques*, (ed. A.

Cornea and P. M. Conn), pp. 199–212. Academic Press. doi:10.1016/B978-0-12-409513-7.00014-2

Tillberg, P. W., Chen, F., Piatkevich, K. D., Zhao, Y., Yu, C.-C. J., English, B. P., Gao, L., Martorell, A., Suk, H.-J., Yoshida, F. et al. (2016). Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies. *Nat. Biotechnol.* **34**, 987-992. doi:10.1038/nbt.3625

Tosheva, K. L., Yuan, Y., Pereira, P. M., Culley, S. and Henriques, R. (2020). Between life and death: strategies to reduce phototoxicity in super-resolution microscopy. *J. Phys. D: Appl. Phys.* **53**, 163001. doi:10.1088/1361-6463/ab6b95

Truckenbrodt, S., Maidorn, M., Crzan, D., Wildhagen, H., Kabatas, S. and Rizzoli, S. O. (2018). X10 expansion microscopy enables 25-nm resolution on conventional microscopes. *EMBO Rep.* **19**, e45836. doi:10.15252/embr.201845836

van de Linde S. (2019). Single-molecule localization microscopy analysis with ImageJ. *J. Phys. D Appl. Phys.* **52**, 203002. doi:10.1088/1361-6463/ab092f

van de Linde, S. and Sauer, M. (2014). How to switch a fluorophore: from undesired blinking to controlled photoswitching. *Chem. Soc. Rev.* **43**, 1076-1087. doi:10.1039/C3CS60195A

van de Linde, S., Löschberger, A., Klein, T., Heidbreder, M., Wolter, S., Heilemann, M. and Sauer, M. (2011). Direct stochastic optical reconstruction microscopy with standard fluorescent probes. *Nat. Protoc.* **6**, 991-1009. doi:10.1038/nprot.2011.336

Vangindertael, J., Camacho, R., Sempels, W., Mizuno, H., Dedecker, P. and Janssen, K. P. F. (2018). An introduction to optical super-resolution microscopy for the adventurous biologist. *Methods Appl. Fluores* **6**, 022003. doi:10.1088/2050-6120/aaae0c

Vassilopoulos, S., Gibaud, S., Jimenez, A., Caillol, G. and Leterrier, C. (2019). Ultrastructure of the axonal periodic scaffold reveals a braid-like organization of actin rings. *Nat. Commun.* **10**, 1-13. doi:10.1038/s41467-019-13835-6

Vietri, M., Schink, K. O., Campsteijn, C., Wegner, C. S., Schultz, S. W., Christ, L., Thoresen, S. B., Brech, A., Raiborg, C. and Stenmark, H. (2015). Spastin and ESCRT-III coordinate mitotic spindle disassembly and nuclear envelope sealing. *Nature* **522**, 231-235. doi:10.1038/nature14408

von Chamier, L., Laine, R. F. and Henriques, R. (2019). Artificial intelligence for microscopy: what you should know. *Biochem. Soc. Trans.* **47**, 1029-1040. doi:10.1042/BST20180391

von Chamier, L., Jukkala, J., Spahn, C., Lerche, M., Hernández-pérez, S., Mattila, P., Karinou, E., Holden, S., Solak, A.C., Krull, A. et al. (2020). ZeroCostDL4Mic: an open platform to simplify access and use of Deep-Learning in Microscopy. *bioRxiv* 2020.03.20.000133. doi:10.1101/2020.03.20.000133

Wäldchen, S., Lehmann, J., Klein, T., van de Linde, S. and Sauer, M. (2015). Light-induced cell damage in live-cell super-resolution microscopy. *Sci. Rep.* **5**, 15348. doi:10.1038/srep15348

Wassie, A. T., Zhao, Y. and Boyden, E. S. (2019). Expansion microscopy: principles and uses in biological research. *Nat. Methods* **16**, 33-41. doi:10.1038/s41592-018-0219-4

Wegel, E., Göhler, A., Lagerholm, B. C., Wainman, A., Uphoff, S., Kaufmann, R. and Dobbie, I. M. (2016). Imaging cellular structures in super-resolution with SIM, STED and localisation microscopy: a practical comparison. *Sci. Rep.* **6**, 27290. doi:10.1038/srep27290

Whelan, D. R. and Bell, T. D. M. (2015). Image artifacts in single molecule localization microscopy: why optimization of sample preparation protocols matters. *Sci. Rep.* **5**, 7924. doi:10.1038/srep07924

Willig, K. I., Rizzoli, S. O., Westphal, V., Jahn, R. and Hell, S. W. (2006). STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis. *Nature* **440**, 935-939. doi:10.1038/nature04592

Wu, Y. and Shroff, H. (2018). Faster, sharper, and deeper: structured illumination microscopy for biological imaging. *Nat. Methods* **15**, 1011-1019. doi:10.1038/s41592-018-0211-z

York, A. G., Parekh, S. H., Nogare, D. D., Fischer, R. S., Temprine, K., Mione, M., Chitnis, A. B., Combs, C. A. and Shroff, H. (2012). Resolution doubling in live, multicellular organisms via multifocal structured illumination microscopy. *Nat. Methods* **9**, 749-754. doi:10.1038/nmeth.2025

York, A. G., Chandris, P., Nogare, D. D., Head, J., Wawrzusin, P., Fischer, R. S., Chitnis, A. and Shroff, H. (2013). Instant super-resolution imaging in live cells and embryos via analog image processing. *Nat. Methods* **10**, 1122-1126. doi:10.1038/nmeth.2687

Zhang, X., Zhang, M., Li, D., He, W., Peng, J., Betzig, E. and Xu, P. (2016). Highly photostable, reversibly photoswitchable fluorescent protein with high contrast ratio for live-cell superresolution microscopy. *Proc. Natl. Acad. Sci. USA* **113**, 10364-10369. doi:10.1073/pnas.1611038113

Zhao, Y., Bucur, O., Irshad, H., Chen, F., Weins, A., Stancu, A. L., Oh, E.-Y., DiStasio, M., Torous, V., Glass, B. et al. (2017). Nanoscale imaging of clinical specimens using pathology-optimized expansion microscopy. *Nat. Biotechnol.* **35**, 757-764. doi:10.1038/nbt.3892

Zhao, W., Liu, J., Kong, C., Zhao, Y., Guo, C., Liu, C., Ding, X., Ding, X., Tan, J. and Li, H. (2018). Faster super-resolution imaging with auto-correlation two-step deconvolution. arXiv 1809.07410. https://arxiv.org/abs/1809.07410v4