Beginner's guide to producing super-resolved images on a widefield fluorescence microscope

Ilijana Vojnovic and **Ulrike Endesfelder** (Max Planck Institute for Terrestrial Microbiology and LOEWE Center for Synthetic Microbiology (SYNMIKRO), Germany)

The development of super-resolution microscopy techniques, which are able to achieve resolutions in the nanometre range and as such allow the visualization of subcellular structures and dynamics, has considerably expanded the possibilities of fluorescence microscopy in the life sciences. While a majority of these techniques require highly specialized hardware, *single-molecule localization microscopy* (SMLM) can be implemented on conventional widefield fluorescence microscopes. Here, we describe what technical upgrades are necessary and discuss some of the difficulties that can be encountered during sample preparation and imaging.

Visualizing life with fluorescence microscopy

Fluorescence microscopy sheds light on all areas of life. It visualizes the embryonic development of large multicellular organisms as well as the molecular organization within tiny microbes. The molecules of interest are labeled with fluorophores, which reveal their positions through their emitted fluorescence, producing colourful and contrast-rich images.

The development of super-resolution techniques has expanded the possibilities of fluorescence microscopy even further. These techniques are not restricted by the diffraction limit of light, and thus are able to attain resolutions of only a few nanometres. This has in many cases revealed unexpected, spectacular new biology. The demand for super-resolution setups, access to imaging facilities and collaborations with research groups operating such systems is, therefore, high.

Whilst some super-resolution techniques work with complex illumination patterns and require highly specialized hardware, the class of *single-molecule localization microscopy* (SMLM) techniques relies on special fluorophores that can be photoswitched between a non-emitting 'dark state' and a fluorescent 'on-state' to achieve sub-diffraction resolution. They are modulated in such a way that only few of them fluoresce at the same time. Fluorophores, therefore, appear as distinct fluorescence spots on the detector whose centres can be localized with high precision.

As a consequence, SMLM, in its simplest form, can be implemented on a widely available widefield fluorescence microscope. This makes it ideal for anyone wishing to explore the possibilities of super-resolution microscopy at a relatively low cost. In the following sections, we describe how you can equip your conventional widefield microscope for SMLM and what needs to be considered during sample preparation and imaging.

Preparing your microscope

The basic design of a widefield fluorescence microscope is depicted in Figure 1a. The excitation light, depicted in green, is reflected by a dichroic mirror onto the objective to illuminate the sample. The emitted fluorescence, depicted in orange, is collected by the same objective and now passes the dichroic mirror due to its Stokes shift to longer wavelengths. The fluorescence image is then recorded by a light-sensitive detector.

Unfortunately, most conventional widefield microscopes are not sensitive enough to detect the signal of single fluorophores, which is essential for SMLM. However, this can be remedied by upgrading three core components – the light sources, the objective and the detector.
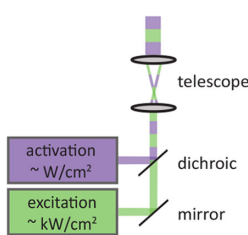
Conventional fluorescence microscopes typically employ gas discharge lamps or an array of *light-emitting*
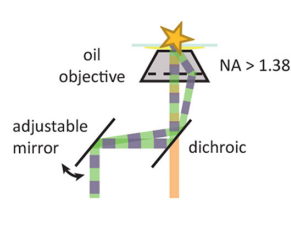
a) basic widefield microscope
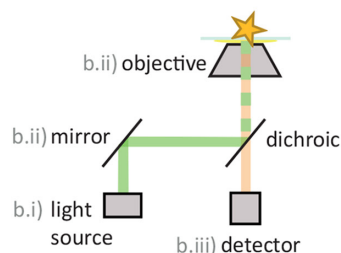
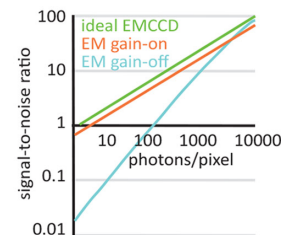### b) critical components of a SMLM setup

Figure 1. Adapting a widefield microscope for SMLM imaging. In order to adapt an epifluorescence widefield microscope system as depicted in (**a**) for SMLM imaging, three components are necessary: illumination lasers (**b.i**), an immersion oil objective with high numeric aperture (**b.ii**) and a sensitive detector such as an EMCCD camera (**b.iii**). Together, these allow for single-molecule sensitivity.

diodes (LEDs) as light sources, thereby accommodating a wide range of fluorophores with diverse excitation spectra and allow for straightforward multicolour imaging. For SMLM imaging, these light sources need to be replaced by lasers. Unlike ensemble fluorescence microscopy, which records the joint signal of all fluorescent molecules, SMLM relies on the ability to detect individual fluorophores. This is only possible if a minimum of several hundred to a few thousands of photons per fluorophore reach the detector in a single imaging frame. As each emission of a photon requires the absorption of an excitation photon, fluorophores have to be excited as efficiently as possible to maximize the emitted fluorescence per time. Lasers provide the strong, coherent illumination to achieve this (Figure 1b.i).

Typically, small diode lasers or diode-pumped solid state lasers with wavelengths matching the absorption spectra of common SMLM fluorophores are used. By means of a telescope, the laser beams' diameters are adjusted in such a way that the illuminated area within the sample covers the field of view of the detector. In an efficient setup, lasers with powers of 100–500 mW are sufficient to achieve an illumination intensity of 0.5–2 kW/cm^2 that is needed for SMLM imaging. This requires that the expansion of the laser beams is not larger than necessary and that about 50% of the lasers' original power reaches the sample (this is typically only possible with open laser paths).

In addition to the excitation lasers, a so-called activation laser is needed to modulate the photoswitching fluorophores. Most fluorophores suitable for SMLM are photoswitched by near-UV laser illumination of ~400 nm wavelength. Typically, intensities of a few W/cm^2 of a 405-nm diode laser are sufficient (Figure 1b.i). Additionally, variants exist of many commonly used fluorescent proteins that can be photoswitched via so-called *primed photoconversion*, which uses a combination of 488 nm and near-infrared illumination. Primed photoconversion can be useful

to reduce phototoxicity and (in combination with a near-UV switchable fluorophore) for dual colour imaging. Another very convenient add-on is the fast mechanical shutters or an *acousto-optic tunable filter* (AOTF) to modulate the activation and excitation lasers for fast temporal illumination control.

The second crucial prerequisite for the detection of single fluorophores is an objective that collects as much fluorescence as possible from the sample (Figure 1b.ii). As the fluorescence emitted by a fluorophore is typically isotropic, an objective with a large light collection angle (also called aperture angle) is essential. However, light hitting the glass surface of the objective at a sharp angle will simply be reflected away if it comes from a less-dense medium. The space between cover slip and objective, therefore, needs to be filled with an immersion oil with a refractive index matching that of glass (about 1.51). This also prevents photon loss due to refraction at the boundary of cover slip and immersion medium. Aperture angle and the refractive index of the immersion liquid are, therefore, the key performance indicators of any objective. Together, they determine the so-called *numerical aperture* (NA) of an objective, which is defined as the product of the refractive index and the sinus of the half aperture angle. The higher the NA, the better the ability of the objective to collect light. For SMLM imaging, an objective with an NA of 1.45 or higher is typically necessary.

High NA objectives also come with a second advantage: they allow for objective-based *total internal reflection fluorescence* (TIRF) microscopy. The TIRF geometry creates an evanescent light field of only 100–200 nm width at the cover slip surface. It only excites molecules close to the surface, and as such drastically improves the signal-to-noise ratio when imaging small objects. An adjustable mirror can be installed in the optical path of the illumination lasers to easily switch between widefield and TIRF imaging modes (Figure 1b.ii).

The last critical component is a detector sensitive enough to detect the weak signal of single fluorophores. As a widefield microscopy technique, SMLM records images with a two-dimensional array detector, e.g., a *charge-coupled device* (CCD) sensor. CCD chips consist of an array of photodiodes that detect incident fluorescence photons by means of the photoelectric effect. To attain single-molecule sensitivity, CCDs are enhanced by electron multipliers (EMCCDs), which cause photoelectrons to trigger an avalanche of secondary electrons through impact ionization. The strength of this amplification can be adjusted through the EM gain of the detector, which can make even the signal of only a few photons detectable (Figure 1b.iii). Apart from EMCCD cameras, the new generation of *scientific Complementary Metal Oxide Semiconductor* (sCMOS) sensors are sensitive enough for SMLM imaging.

Labelling and imaging performance at single-molecule fidelity

The right hardware, however, is only part of the story. Due to the delicate nature of nanoscale imaging, special care needs to be taken during sample preparation and imaging. Single-molecule–sensitive microscopes provide single-molecule resolution. This means that factors that are hardly noticeable in conventional widefield images can have a large impact on the quality of an SMLM image.

One important aspect of fluorescence microscopy is the notion that one always only visualizes the fluorophores, not the molecules of interest themselves. As such, fluorescence microscopy techniques strongly depend on the fidelity of the chosen fluorescence label. Good labeling will have a high efficiency, i.e., a large part of the target molecules carry a label, and a high specificity, i.e., most other things do not.

In Figure 2a, we showcase a high-quality image of vimentin filaments in a mammalian HeLa cell at widefield (left), as well as SMLM resolution (right). In this specific example, the vimentin structures are labeled by a small, only a few nanometres, sized single-chain antibody, a so-called nanobody, which yields high labeling efficiency and specificity. The labels densely populate the filaments which are thoroughly stained. The nanobody carries an organic dye, Alexa Fluor 647, in a quantitative one-to-one labeling ratio, which is one of the best performing fluorophores in the SMLM field. It is a bright probe, its photoswitching is well-controlled and reliable, and its bleaching rate in SMLM buffers is low.

Using this SMLM data set, we simulate typical problems of labeling and imaging procedures.

In Figure 2b, we use only a fraction of our data set to visualize how low labeling efficiencies impact image quality. While the labeling efficiency is still acceptable for widefield resolution (Figure 2b.i), prominent gaps are visible in the vimentin structures in the SMLM image (Figure 2b.ii). In general, low labeling efficiencies can have many different causes: for affinity tags such as antibodies, their affinity could just not be sufficiently high or the targeted epitopes cannot be reliably reached (e.g., labels physically too big, epitopes partially buried in the structure). For transient expressions of genetic labels, the filaments simply could be a mixture of native and labeled proteins of interest. It is also possible for the sample preparation protocols to be too harsh, quenching fluorescence or destroying the epitopes of extrinsic labels.

The quality of SMLM images can also be impacted by false-positive signals caused by unspecific staining or autofluorescence. This is especially critical if the native abundance of the molecules of interest is low. We simulate unspecific, false-positive signals in the data set for our example by distributing random data points throughout the field of view (Figure 2c). Even though vimentin is a highly structural, filamentous protein, the interpretation of the image becomes ambiguous. It is difficult to evaluate if a registered signal results from specific staining, e.g., a pool of vimentin monomers, or from an unspecific, non–target-bound fluorophore (Figure 2c.ii). In general, autofluorescence can be mostly attributed to colourful metabolites or pigments within the specific organism, or to uptakes from the growth media. Unspecific staining originates from non–target-bound labels that were not washed out during sample preparation, e.g., sticking by charge. Genetic labels can be troublesome as well: overexpression can produce a large pool of non-physiological monomers which are not integrated into native structures, or tags can disturb the biological function of the protein of interest.

It is easy to imagine how the quantitative evaluation of SMLM data quickly becomes unfeasible for less-structured or low copy number targets if labeling efficiency and specificity are low. For example, a staining efficiency of 75% for a homotrimeric structure will, on average, result in only about 50% of labeled structures actually appearing as trimers – under otherwise ideal imaging conditions. In practice, the underlying structure would be further obscured by random noise and signals lost due to insufficient brightness, premature bleaching or improper photoswitching control. All in all, ensuring high labeling efficiency and specificity is essential in every SMLM project. It will often be necessary to modify or completely replace the chosen labeling strategy if, after some test runs, no satisfactory results are obtained. It is not uncommon to pursue two, three or even four different approaches before being successful.
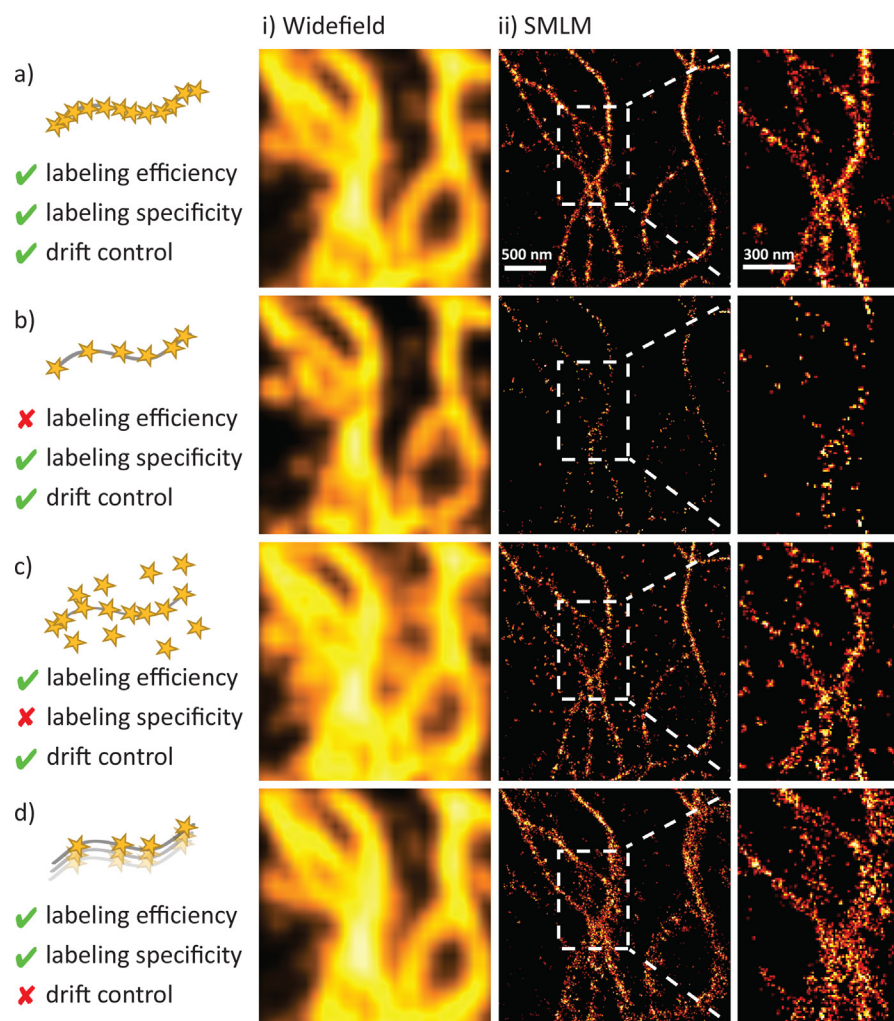
i) Widefield   ii) SMLM

a)

✔ labeling efficiency
✔ labeling specificity
✔ drift control

b)

✘ labeling efficiency
✔ labeling specificity
✔ drift control

c)

✔ labeling efficiency
✘ labeling specificity
✔ drift control

d)

✔ labeling efficiency
✔ labeling specificity
✘ drift control

Figure 2. Labeling and imaging performance at single-molecule fidelity. (**a**) SMLM image of vimentin structures in a HeLa cell. Cells were stained using nanobodies carrying the SMLM-suitable photoswitching fluorescent dye, Alexa Fluor 647. This original recording (taken from Virant et al., Nat Commun, 2018. 9(1)) illustrates a good practice example for SMLM imaging with high labeling efficiency and specificity and robust drift control. In (**b**)–(**d**), we used this data set to simulate typical problems in labeling and imaging performance. These include low labeling efficiency (**b**), unspecific signal (**c**) and drift during image acquisition (**d**). In all cases, shortcomings in fluorescence labeling and imaging performance are clearly visible in the SMLM images (**i**), and as such limit their quality. At the same time, they are hardly noticeable in the corresponding widefield fluorescence images (**ii**).

Last but not least, a robust drift correction is needed. Recording SMLM images takes time, which increases the chance for substantial sample drift accumulating over minutes of imaging (Figure 2d). Most microscope setups are built in a heavy, mechanically stable fashion which passively prevents large drift. Additionally, most commercial systems actively stabilize the focal plane to suppress axial drift, detecting shifts via the reflection of infrared lasers from the cover slip on quadrant photodiodes. However, these measures usually do not suffice to achieve nanometre precise drift control as is required for SMLM imaging. Thus, SMLM images are typically post-processed to remove residual drift, e.g., by means of trajectories of fiducial markers measured with the sample or by calculating spatiotemporal cross-correlations using the sample data itself. Most SMLM software supports such corrections.

To illustrate the severity of residual drift in SMLM imaging, we added an artificial linear shift of 0.02 nm per imaging frame to our data set. This small drift sums up to 200 nm of total drift over the 10,000 imaging frames – hard to spot in the widefield image (Figure 2d.i) but clearly visible in the SMLM image (Figure 2d.ii).

To gather some initial experience with SMLM imaging and to test the setup, it is a good idea to first image a well-known test sample. Cytoskeletal structures such as microtubules, actin or vimentin filaments are often used as visualized standards. For all of them, well-tested commercial antibodies – pre-labeled with Alexa Fluor 647 – are available. Microtubules have the additional advantage that they are hollow, have a well-defined diameter of 25 nm and do not form higher-order filaments bundling together several fibres. In high-quality SMLM images, a cross-section through a microtubule should, therefore, show two clear peaks separated by 25 nm plus two times the size of the label and can serve as a good control for successful staining and imaging.

Conclusion

We hope that this beginners' guide has been able to convince you that the first steps towards SMLM imaging are not too difficult. Requirements are a widefield setup equipped for single-molecule sensitivity and careful sample preparation and imaging routines. For further steps, e.g., towards quantitative or live cell SMLM imaging, please have a look at the (open access) overview articles listed below. Happy imaging. ■

Further reading

Introduction into the technical details of light microscopy
- Wegerhoff, R., Weidlich, O., Kässens, M. (2006) *Basics of Light Microscopy & Imaging*, GIT Verlag GmbH & Co. https://analyticalscience.wiley.com/do/10.1002/imaging.1654

Introduction into suitable fluorophores and their photoswitching. This review includes several overview tables.
- Turkowyd, B., Virant, D. and Endesfelder, U. (2016) From single molecules to life: microscopy at the nanoscale. *Anal. Bioanal. Chem.* **408**, 6885–6911 10.1007/s00216-016-9781-8

Strategies for multicolor labeling in SMLM imaging. This review includes an experimental design guideline and a troubleshooting table.
- Vojnovic, I., Winkelmeier, J. and Endesfelder, U. (2019) Visualizing the inner life of microbes: practices of multi-color single-molecule localization microscopy in microbiology. *Biochem. Soc. Trans.* **47**, 1041–1065. 10.1042/bst20180399

The example vimentin data set of Figure 2 is taken from this publication. It introduces a new nanobody label for SMLM imaging.
- Virant, D., Traenkle, B., Maier, J., et al. (2018) A peptide tag-specific nanobody enables high-quality labeling for dSTORM imaging. *Nat. Commun.* **9**, 930 10.1038/s41467-018-03191-2

Ilijana Vojnovic studied chemistry at the Goethe University in Frankfurt, Germany, and is now a PhD student at the Department for Systems and Synthetic Microbiology at the Max Planck Institute for Terrestrial Microbiology in Marburg, Germany. She applies super-resolution methods for structural studies in microbes, investigating multi-protein complexes. Email: ilijana.vojnovic@synmikro.mpi-marburg.mpg.de

Ulrike Endesfelder studied physics and has been a group leader at the Max Planck Institute for Terrestrial Microbiology in Marburg since 2014. She is a member of the German Young Academy. In summer 2020, she and her group will move to Carnegie Mellon University in Pittsburgh, USA, where she accepted a professorship position for Experimental Biophysics. Email: ulrike.endesfelder@synmikro.mpi-marburg.mpg.de