

SUPUVIR Microscopy & Sensing Workshop, Cambridge, UK

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Single-molecule localization microscopy

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Research topics – Single molecule localisation microscopy.

See Laser Analytics Group website: <http://laser.ceb.cam.ac.uk/>

Aims of the practical

The aim of this practical is to introduce you to advanced optical imaging techniques. Specifically, this practical will focus on a super-resolution technique called single-molecule localization microscopy (SMLM). Super-resolution microscopy techniques are able to surpass the resolution barrier set by the diffraction of light. One of the main advantages of these imaging techniques is that the image contrast is based on fluorescence and therefore they feature a high molecular specificity and sensitivity, unlike other high-resolution techniques, such as electron microscopy.

In this practical, you will be introduced to SMLM and important related concepts such as fluorescence labelling, sample preparation for super-resolution imaging, image acquisition, detection sensitivity, and resolution.

Please read through this hand-out and address the questions **before** the practicals. Also, please read through the general review paper about super-resolution imaging from Huang *et al.* (Huang, Bates, & Zhuang, 2009).

<http://www.annualreviews.org/doi/abs/10.1146/annurev.biochem.77.061906.092014>

Further reading is also suggested (see page 5 for some additional key literature pieces).

Background

Optical fluorescence microscopy is a powerful research tool, particularly in biomedical research and cell biology. It can be used to study biological materials, even in live specimens under physiological conditions and many different properties of fluorescence (intensity, spectrum, polarization, lifetime etc.) can be probed, allowing the measurements of many local parameters within the sample, such as the local concentration of molecules of interest, local viscosity or temperature.

A disadvantage of conventional fluorescence microscopy is that features smaller than approximately half the wavelength of light cannot be resolved due to the diffraction limit as defined by Ernst Abbe in 1873:

$$\sigma = \frac{\lambda}{2 \times NA}$$

Where σ is the radius of image blur introduced by the diffraction of light (called the point spread function, or PSF), λ is the wavelength of light and NA is the numerical aperture of the imaging objective.

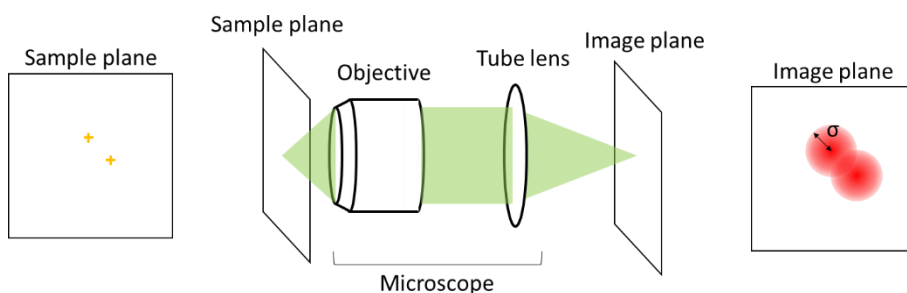


Figure 1: Diagram representing the resolution limit imposed by diffraction in an imaging system. The orange crosses represent point sources in the sample plane. They produce a blur in the image plane (camera). The blurred image produced in the image plane by a point source in the sample plane is called the point spread function (PSF).

In this practical, we will be using a 100x objective lens with numerical aperture (NA) = 1.49 to image the fluorescent emission of a dye called Alexa Fluor 647.

- 1) Using Abbe's equation for resolution and the fluorescence emission spectra of the dye, estimate the diffraction-limited resolution of our microscope. What is the collection solid angle of the fluorescence emitted from the sample fluorophores for this system? Discuss how resolution, working distance, spectral properties of the fluorophores and objective apertures are related to one another.

The diffraction limit is now being routinely surpassed by means of various "super-resolution microscopy" techniques. The immense potential of these techniques was acknowledged in September 2014 by the award of the Nobel Prize in Chemistry to three pioneers of these techniques: **W.E. Moerner**, **Eric Betzig** and **Stephan Hell**.

http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/



Three major implementations of super-resolution microscopy are available: stimulated emission depletion microscopy (STED) (Hell & Wichmann, 1994), structured illumination microscopy (SIM) (Gustafsson, 2000) and single-molecule localization microscopy (SMLM) (Betzig et al., 2006; Rust, Bates, & Zhuang, 2006). A good introduction to these techniques can be found in the paper by Huang *et al.* (Huang et al., 2009).

In this practical, however, we will only focus on SMLM (a.k.a. localization microscopy). In localization microscopy, the diffraction barrier is surpassed by imaging individual fluorescent dyes labelling a structure in the sample separately in time. Figures 2 and 3 illustrate this approach.

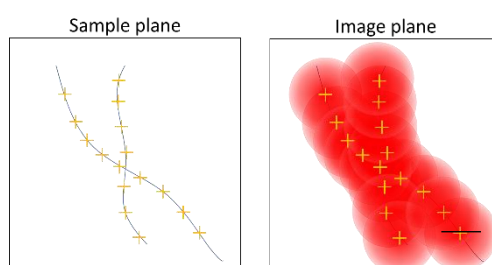


Figure 2: Diffraction limited image of a fluorescently labelled (yellow crosses) filamentous structure (blue lines). Imaging all fluorescent dyes at the same time leads to a diffraction-limited image. Each fluorescent dye leads to a blur in the image plane, the PSF, with radius equal to σ (shown by black line).

One method to achieve super-resolution via SMLM uses fluorescent dyes with photo switching properties (fluorescence emission can be switched off and on), such that the emission of individual dye molecules can be imaged sequentially.

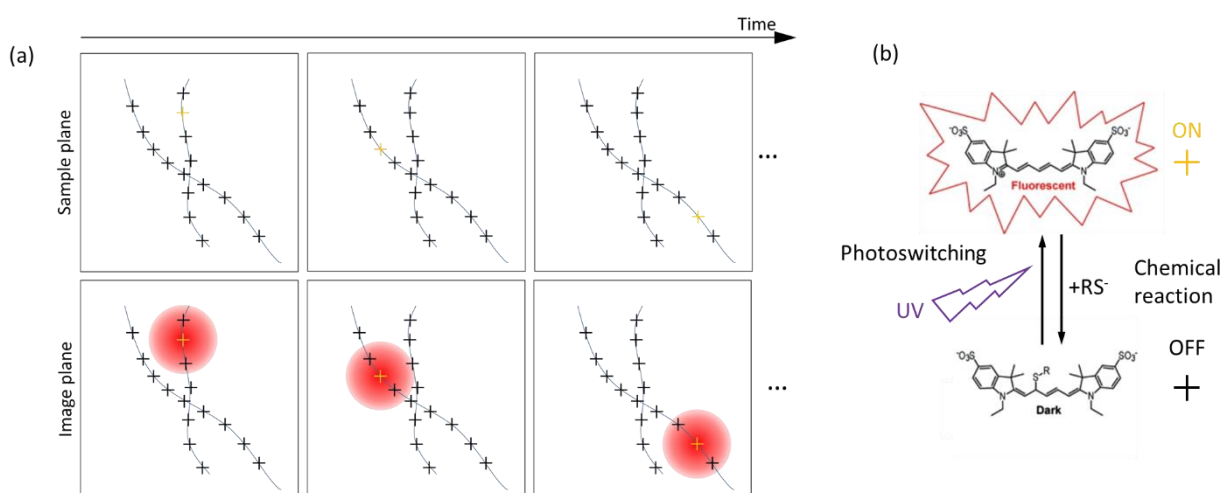


Figure 3: Principle of localization microscopy. (a) In each frame, the fluorescence of an individual fluorescent dye is imaged. All fluorescent dyes are switched off (black crosses) apart from one (yellow cross) in each frame of the sequential camera acquisition. (b) The switching on and off of the fluorescent dye is performed both chemically and optically. Adapted from Dempsey *et al.* (Dempsey et al., 2009).

The photoswitching rate is typically regulated by a chemical reaction using a buffer containing an oxygen scavenger (such as catalase) and a reducing agent (typically mercapto-ethylamine or MEA) present in the imaging buffer (controlling the **off** rate). The photoswitching is induced by irradiance with high power laser light.

Once all fluorescent dyes in the sample have been imaged, the sequential acquisition ('movie') is stopped and the image data are analysed. The position of each individual fluorescent dye molecule can be estimated with sub-pixel accuracy from the intensity of its fluorescent image on the camera. This procedure is performed by image analysis algorithms which locate the PSFs (blurred image from a point-source) from individual emitters and fit a 2D Gaussian function to each PSF (Rees et al., 2012). Other methods involve transiently binding fluorescent probes, which can be excited with total internal reflection illumination (Jungmann et al., 2010).

Therefore, the data obtained from SMLM is a set of coordinates (x,y) for each fluorescent dye. This set of coordinates can be used to reconstruct a super-resolved image of the underlying fluorescently-labelled structure obtained from the sample (see Figure 4). A detailed review of the different steps of localization microscopy can be found in Deschout *et al.* (Deschout et al., 2014).

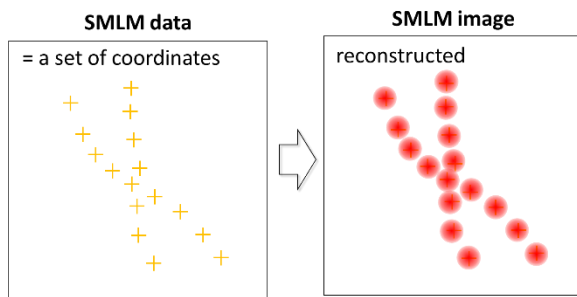


Figure 4: Reconstruction of the super-resolved image in SMLM. From the PSF fitting algorithm, a set of coordinates is obtained for each fluorescent dye. This can then be used to reconstruct a super-resolved image.

- 2) In the case of localization microscopy, what is the resolution related to? Think about the way the position of the fluorescent dye is obtained.
- 3) Intuitively, how do you think the reconstruction can be performed in order to obtain a fluorescent dye density map?
- 4) In localization microscopy, the pixel size of the reconstructed image can be chosen by the user. How would you decide what pixel size to use in the final image?

The precision of the determination of the centre of the PSF can be mathematically evaluated. It is called the localization precision and was first determined by Thompson et al. (Thompson, Larson, & Webb, 2002) and later corrected by Mortensen et al. (2010). It is given by the following equation:

$$\sigma_{loc}^2 = \frac{\sigma^2 + \frac{a^2}{12}}{N} + \frac{8\pi\sigma^4 b^2}{a^2 N^2}$$

Here σ_{loc} is the localization precision, σ is the diffraction limit given by Ernst Abbe's equation, a is the corresponding size of an individual camera pixel in the sample plane, N is the total number of photons

contained in the PSF and b is the standard deviation of the noise on the background level (in equivalent numbers of photons).

In this practical, we are using a 16-bit EMCCD camera with 512 x 512 pixels with a detector size of 8.2 mm x 8.2 mm.

- 5) *What is the physical camera pixel size?*
- 6) *What is the corresponding size of an individual camera pixel in the sample plane for our microscope?*
- 7) *Assuming there is no background noise, and assuming that on average a fluorescent dye gives out 500 photons in each PSF, estimate the localization precision.*

You can download a representative movie obtained from an SMLM acquisition on this link.

<https://drive.google.com/file/d/0ByllrVMresQWFdGTWJrMGJfUkk/view?usp=sharing>

The camera measures the intensity of the light in digital counts (ADC or Analogue to Digital Counts), the conversion factor of our camera is such that 1 photon gives on average about 16 ADC.

- 8) *Use the open-source image analysis software Fiji (<http://fiji.sc/Fiji>) to obtain a rough estimate of σ , b and N . (Hint: simply draw line profiles through the fluorescence patterns from individual molecules and measure the standard deviation of the resulting intensity distribution).*
- 9) *What is the average localization precision achievable with this image?*

Further reading prior to the practical (required reading underlined)

[1] Art and artefacts in single-molecule localization microscopy: beyond attractive images. **Endesfelder U. et al. (2014)** – Nature Methods

<http://www.nature.com/nmeth/journal/v11/n3/full/nmeth.2852.html>

[2] Superresolution imaging using single-molecule localization. **Patterson G. et al. (2010)** - Annual Review of Physical Chemistry

<http://www.ncbi.nlm.nih.gov/pubmed/20055680>

[3] Photons in - numbers out: Perspectives in quantitative fluorescence microscopy for in situ protein counting –**Grussmayer et al. 2018** – BioArxiv.

<https://arxiv.org/pdf/1809.02464.pdf>

[4] **VanDeLinde et al. (2011)** - this paper describes the fundamental processes governing direct STORM (dSTORM) which is the technique we primarily use, and how it compares to other single molecule localisation techniques.

[5] **Sauer and Heilemann (2017)** - comprehensive review on Single molecule localisation studies in eukaryotes. This paper is really long so I wouldn't recommend necessarily going through the full thing, but the intro chapter of the review is very well written and gives a wide overview of the kind of research that led to the development of single molecule localisation techniques.

[6] **Rust et al (2006)** - seminal paper on STORM, the first version of the technique we use in the lab. This paper is definitely required reading, especially the supplementary information.

- [7] **Chao, J., Sally Ward, E., & Ober, R. J. (2016).** Fisher information theory for parameter estimation in single molecule microscopy: tutorial. *Journal of the Optical Society of America A*, 33(7), B36. <https://doi.org/10.1364/JOSAA.33.000B36>
- [8] **Jungmann, R., Avendano, M. S., Woehrstein, J. B., Dai, M., Shih, W. M., & Yin, P. (2014).** Multiplexed 3D cellular super-resolution imaging with DNA-PAINT and Exchange-PAINT. *Nature Methods*, 11(3), 313–318. <https://doi.org/10.1038/nmeth.2835>
- [9] **Dertinger, T., Colyer, R., Iyer, G., Weiss, S., & Enderlein, J. (2009).** Fast, background-free 3D super-resolution optical fluctuation imaging (SOFI). *Proceedings of the National Academy of Sciences of the United States of America*, 106(52), 22287–92. <https://doi.org/10.1073/pnas.0907866106>

Outline of the practical

In this practical, you will use localisation microscopy to obtain a super-resolution image of a pre-prepared specimen, and then estimate the resolution improvement that has been obtained, in comparison with a conventional fluorescence microscopy image.

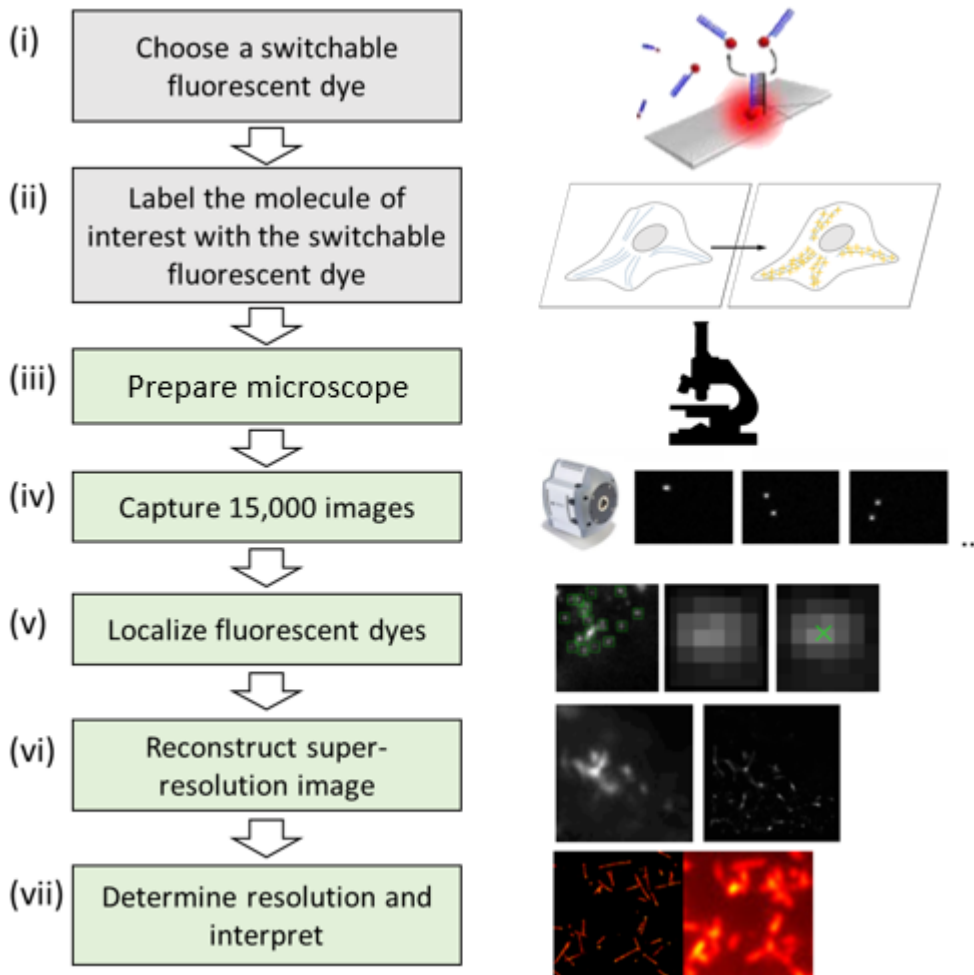


Figure 5: Outline of the practical. A suitable microscopy specimen has been pre-prepared, so this section of the experiment is greyed out (the fluorescent labelling step can take a few hours or more, depending on the protocol). You will capture super-resolution microscopy data, analyse it, and determine image resolution.

(i) and (ii) - Sample preparation. The first two steps of the procedure were performed beforehand. DNA origami scaffolds (Schmied et al., 2014) called “nanorulers” will be used here as ground-truth samples. The nanorulers are short oligonucleotide strands, some of which are “docked” to the coverslip and others which float freely in solution above the coverslip and have an AlexaFluor647 molecule attached to one of their ends. The photowitching behaviour is based on transient binding of the floating “imager” strand to the stationary “docking” strand. Figure 6 illustrates how a DNA origami nanoruler may look like. These nanorulers are attached to the bottom of cover slips by biotin/streptavidin binding.

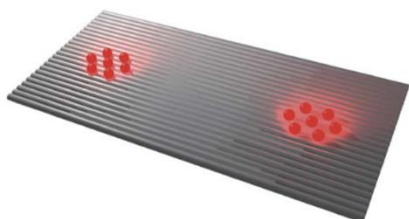


Figure 6: Schematic of the DNA origami nanoruler with two marks formed of 7 binding sites for Alexa Fluor 647. The distance between the two marks can be accurately determined by the structure of the DNA origami base plate. Reproduced from (Schmied et al., 2014).

(iii) - Preparation of microscope. The preparation of the microscope is essential as it allows the discrimination of background fluorescence and in-focus signal of blinking molecules.

10) Prepare the microscope by focusing onto the DNA origami and adjusting TIR illumination.

(iv) - Setup of the localisation microscope and acquisition.

11) Record key details of the hardware setup of the localisation microscope and sample in order to be able to reproduce the experiment.

12) Are the current settings of the fluorescence microscope optimal for the excitation and detection of the fluorescent dye used here? You can use <http://searchlight.semrock.com/>

Place the microscope specimen on the oil objective lens of the microscope. You can focus on the sample using the eyepiece and the white light illumination or via the camera and the laser illumination.

Cover the specimen before you begin laser illumination. See Safe use of laser section below.

Use the Labview control software on the microscope PC to set up suitable illumination conditions (laser power) and camera settings to capture a conventional fluorescence image of an area of the specimen.

Set up suitable conditions to collect single-molecule localization image data. In practice, one would consult relevant publications such as van de Linde *et al.* (van de Linde et al., 2011) to determine suitable conditions for localisation microscopy. Capture a suitable set of data for the area of the specimen for which you previously measured a conventional fluorescence image.

13) What is the expected file size (in Bytes) of the movie (TIFF stack) acquired?

(v) and (vi) - Process SMLM data to obtain a super-resolved image. For that, a number of localization microscopy software packages are available such as “rainSTORM” (Rees et al., 2012), “rapidSTORM” (Wolter et al., 2012), and thunderSTORM (Ovesny et al. 2014). Here, we will be using rapidSTORM. The software first performs a rough spot detection (find the PSF in the image), determines the local signal-to-noise ratio (SNR) in the neighbourhood of the detected spot, filters out spots with low SNR, fits the remaining spots with a 2D Gaussian, and outputs the coordinates of each PSF (along with the uncertainty of the fit and the number of photons in each spot). These coordinates can then be used to reconstruct a super-resolved image.

14) Start the rapidSTORM software installed on the computer and enter the appropriate value in PSF size. What will setting a minimum SNR effectively do to the image?

(vi) - Analysis and interpretation. An empirical measure of resolution is to take a cross section through the image of a feature with known dimensions – say a 1-dimensional specimen such as filament (microtubule), in which the true sample is simply a straight line. This is typically done using image analysis software such as Fiji (Fiji is just ImageJ).

A more systematic approach to calculating the resolution attainable with single molecule localisation methods is to calculate the Fourier Ring Correlation (FRC) of the data, which involves separating the localisation data set into two halves, generating two independent super-resolution reconstructions of the same structure, and then computing the correlation of their frequency spectra in Fourier space to determine the highest spatial frequency supported by the image. There are Fiji plugins and Matlab scripts available to perform this calculation.

15) Estimate the average number of photons N which corresponds to the empirical resolution you estimated. Compare this with the number of photons of signal captured by the camera from the individual molecule images in practice.

Safe use of lasers in this practical

Laser light poses a potential blinding hazard. This practical must be done under supervision of an experienced user of the localisation microscope, and the following key considerations must be followed.

1. The lasers used in this microscope are visible lasers of **Class 3B**. Class 3B lasers pose an eye hazard only if the beam is viewed directly. Hence, it should not be possible to directly view the laser beam (or to view a mirror used to steer the beam) when the laser is being operated at anything above a low power setting (1 mW is a suitable low power setting, since this is a low power setting that does not pose an eye hazard).
2. Laser beams should be fully enclosed during normal use, to prevent any chance of direct exposure to laser light.
 - a. The light source enclosure should be closed during the actual measurement.
 - b. The microscopy specimens should be covered with a light-proof black box before the illumination shutter is opened.
3. **Laser beams should be set to a low power (less than 5 mW) before any part of the laser beam path is uncovered for any reason** (for example, for alignment).
4. In this practical, students should not perform any laser beam alignment, except for minor adjustments of illumination angle using the "TIRF mirror."
5. The TIRF mirror adjusts the laser beam illumination between a normal epifluorescence mode (in which the laser beam emerges vertically upwards from the objective lens) and an inclined illumination mode (in which the beam emerges from the objective lens at an oblique angle that potentially could direct the beam towards users in the room with the system). If the sample is enclosed by a box then minor adjustments of this mode may be made with the illumination above 5 mW power – this is typically done to improve image contrast. Adjustment of the TIRF mirror with the sample uncovered must only be done by with the laser illumination at low power, i.e. less than 5 mW, and a piece of paper should be used to monitor beam position. Once the experiment is complete, the beam must be returned to the vertical (epifluorescence) alignment, which allows the beam alignment to be safely located by the next user.

References

- Betzig, E., Patterson, G. H., Sougrat, R., Lindwasser, O. W., Olenych, S., Bonifacino, J. S., ... Hess, H. F. (2006). Imaging intracellular fluorescent proteins at nanometer resolution. *Science (New York, N.Y.)*, 313(5793), 1642–5. doi:10.1126/science.1127344
- Dempsey, G. T., Bates, M., Kowtoniuk, W. E., Liu, D. R., Tsien, R. Y., & Zhuang, X. (2009). Photoswitching mechanism of cyanine dyes. *Journal of the American Chemical Society*, 131(51), 18192–3. doi:10.1021/ja904588g
- Deschout, H., Cella Zanacchi, F., Mlodzianoski, M., Diaspro, A., Bewersdorf, J., Hess, S. T., & Braeckmans, K. (2014). Precisely and accurately localizing single emitters in fluorescence microscopy. *Nature Methods*, 11(3), 253–66. doi:10.1038/nmeth.2843
- Gustafsson, M. G. L. (2000). Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *Journal of Microscopy*, 198(2), 82–87. doi:10.1046/j.1365-2818.2000.00710.x
- Hell, S. W., & Wichmann, J. (1994). Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Optics Letters*, 19(11), 780–2. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/19844443>
- Huang, B., Bates, M., & Zhuang, X. (2009). Super-resolution fluorescence microscopy. *Annual Review of Biochemistry*, 78, 993–1016. doi:10.1146/annurev.biochem.77.061906.092014
- Rees, E. J., Erdelyi, M., Pinotsi, D., Knight, A., Metcalf, D., & Kaminski, C. F. (2012). Blind assessment of localisation microscope image resolution. *Optical Nanoscopy*, 1(1), 12. doi:10.1186/2192-2853-1-12
- Rust, M. J., Bates, M., & Zhuang, X. (2006). Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nature Methods*, 3(10), 793–5. doi:10.1038/nmeth929
- Thompson, R. E., Larson, D. R., & Webb, W. W. (2002). Precise nanometer localization analysis for individual fluorescent probes. *Biophysical Journal*, 82(5), 2775–83. doi:10.1016/S0006-3495(02)75618-X
- Jungmann, Ralf, et al. "Single-molecule kinetics and super-resolution microscopy by fluorescence imaging of transient binding on DNA origami." *Nano letters* 10.11 (2010): 4756-4761.
- Jürgen J Schmied, Mario Raab, Carsten Forthmann, Enrico Pibiri, Bettina Wünsch, Thorben Dammeyer & Philip Tinnefeld (2014). DNA origami-based standards for quantitative fluorescence microscopy. *Nature protocols*, 9, 1367–1391 (2014) doi:10.1038/nprot.2014.079
- Van de Linde, S., Löschberger, A., Klein, T., Heidbreder, M., Wolter, S., Heilemann, M., & Sauer, M. (2011). Direct stochastic optical reconstruction microscopy with standard fluorescent probes. *Nature Protocols*, 6(7), 991–1009. doi:10.1038/nprot.2011.336
- Wolter, S., Löschberger, A., Holm, T., Aufmkolk, S., Dabauvalle, M.-C., van de Linde, S., & Sauer, M. (2012). rapidSTORM: accurate, fast open-source software for localization microscopy. *Nature Methods*, 9(11), 1040–1. doi:10.1038/nmeth.2224