Fluorescence microscopy & Single Molecule Localization Microscopy

Overview

In this module, we will study the application of fluorescence microscopy in biophysical research. We will see what advantages fluorescence microscopy has, the limit of resolution of optical microscopy and how single molecule localization microscopy can overcome this limit. For these purposes, you will image some biological specimens, understand how a fluorescence microscope works, build your own microscope and image the specimen with both regular and super-resolution fluorescence microscopy.

Introduction to fluorescence microscopy and single molecule localization microscopy

Absorption of light and subsequent re-emission (which happens nearly simultaneously, typically a few nanoseconds) by organic and or inorganic molecule is called fluorescence. Only a few molecules actually fluoresce, but those that do and can be placed at a position of interest, are extremely valuable. Watching where a labeled biomolecule is, or moves around, can be illuminating. When looking at the object through a microscope, fluorescence microscopy has become an essential technique in biological sciences due to its availability of high contrast and specificity.

The basic function of a fluorescence microscope is to irradiate the specimen with a specific band of wavelengths, and then to separate the much weaker emitted fluorescence from the excitation light (Fig. 1). In a properly configured microscope, only the emission light should reach the detector so that the resulting fluorescent structures of the specimen are visualized with high contrast against a dark (almost 0) background. The limits of detection are generally governed by the darkness of the background, and the brightness of the fluorescence. Under ideal conditions, it is possible to detect a single fluorescent molecule, provided that the background and detector noise are sufficiently low. (One real consideration, is that the excitation light, necessary to induce the fluorescence, is typically 10^5 -10^6 times brighter than the emission, so getting rid of this, is a very important.)

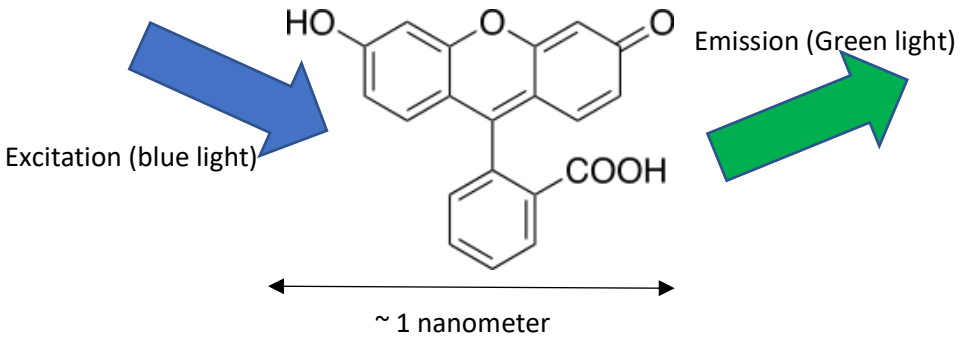


Figure 1. Absorption and fluorescent emission of a molecule. Excitation (blue) light is absorbed by the molecule and emitted (green) from the molecule.

Optical microscopy, i.e. where the wavelength of excitation or emission is roughly 400-750 nm, including fluorescence microscopy, has limitation of its resolution defined as $\lambda/2NA$, called the diffraction limit of light where λ is the wavelength of the light and NA is the numerical aperture --which is the range of angles over which the lens can collect the light-- of the detection objective of the

microscope. While the fluorescent object is only in nanometer in size or-so, it's emission (i.e., its fluorescence light) is ~ 250 nm. Why? Even though the light was emitted from a point source, at the image plane wavelets from different points of the wave front undergo interference, forming blurry Airy distribution of light which is ~λ/2.(See the interactive tutorial: http://micro.magnet.fsu.edu/primer/java/imageformation/airydiskformation/index.html and "What is resolution", which can be found on the web page of Physics 498EBP)

This limitation defines the minimal dimension of the structure one can distinguish in the specimen by using optical microscopy, which is about 250 nm for visible light.

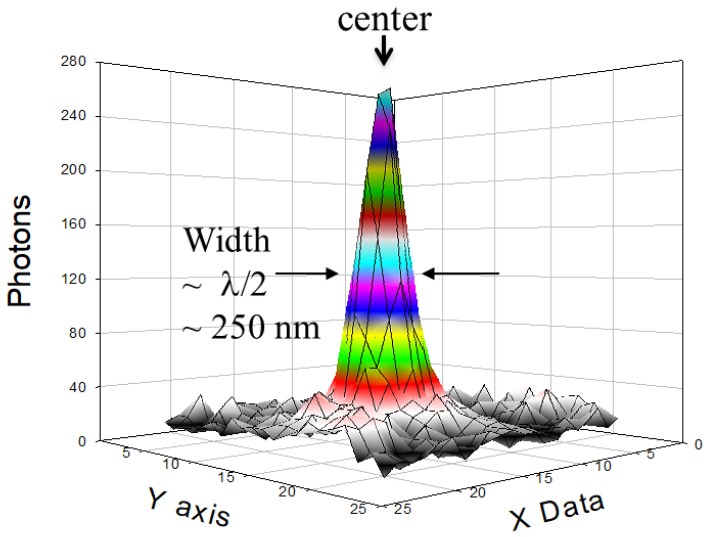


Figure 2. Principle of single molecule localization (FIONA). The figure shows a point spread function (PSF). The width of the PSF is blurred out (~250 nm) but one can estimate the center of the PSF much more accurately than the width.

How can we go from something whose emission is blurred out (to ~ 250 nm), to much smaller. If we know that there is a single molecule present, we can figure out where the center is with much better accuracy than the blurred image of the molecule.

FIONA (Fluorescence Imaging with One Nanometer Accuracy) is a powerful technique that can be used for making ~ nanometer accuracy localization of molecules. The premise behind FIONA is that one can measure the center of a point-spread function (PSF) much more accurately than the diffraction limit, provided enough photons can be collected. For FIONA, fluorescence from single dye molecules are recorded by a camera. The recorded images show bright spots which can be modeled as Gaussian functions (Figure 2). The accuracy of a measurement of the position (center) of the molecule is given by:

$$\sigma_i = \sqrt{\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{a\pi s_i^4 b^2}{a^2 N^2}}$$

where N is the number of photons collected, s is the width of the distribution in either the x or y direction, a is the pixel size of the camera (actual pixel size of the camera divided by the magnification of the system), and b is the standard deviation of the background. The first term under the square root describes the photon noise (shot noise), the second is the effect of the finite pixel size of the camera, and the third is the background noise. For a pixel size of approximately 100 nm, the photon noise term dominates therefore the accuracy of localization is ~ s/2√N = λ/2NA√N. By collecting 5,000-10,000 photon counts per second, one can localize a fluorescent molecule to within a nanometer.

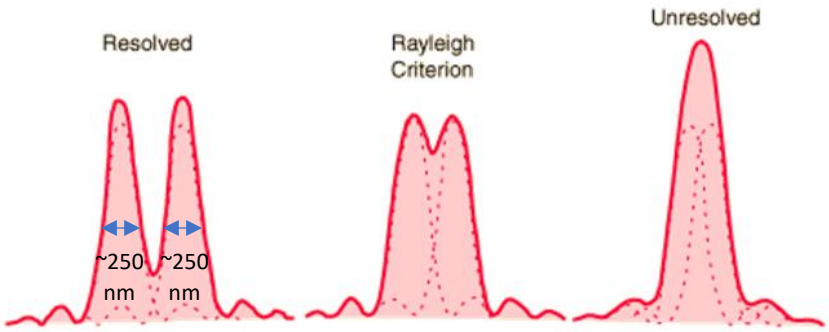


Figure 3. Two molecules located further than diffraction limit (left, resolved, shown at >~250 nm) at about Rayleigh criterion (middle, diffraction limit) and closer (right, unresolved).

The above depends on seeing a single molecule. What if we have two (or more molecules) within the diffraction limit? In Figure 3, you can see two molecules located at different distances from each other. When they are far enough (> diffraction limit) it is possible to distinguish two molecules. However, when they are closer than the diffraction limit (known as the "Rayleigh Criterion"), two molecules become unresolvable. How can we resolve multiple molecules located very close to each other?

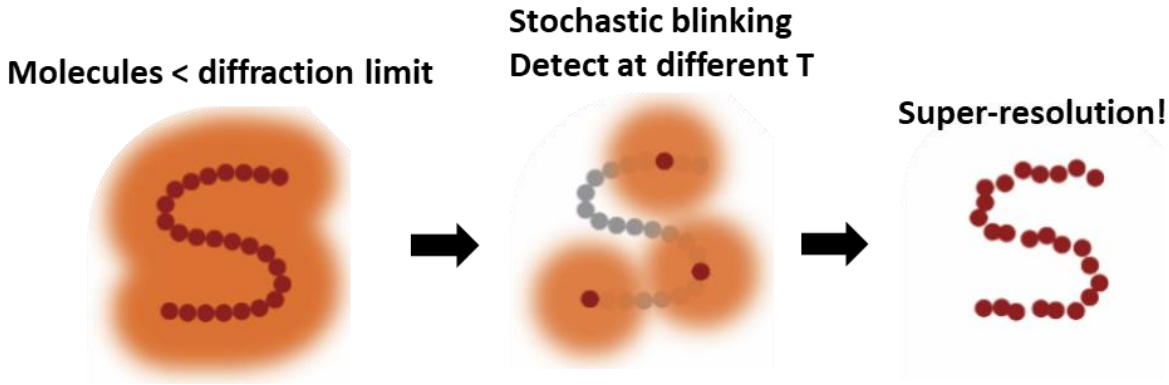


Figure 4. Principle of single molecule localization microscopy to get super-resolution. Here, the molecules within the diffraction limit of light are switching on stochastically, so that every molecule fluorescing can be localized at different time points. The collection of every localization results in the super-resolution image of the structure.

In Figure 4, multiple fluorescent molecules are located within the diffraction limit. The image of the molecules is shown as a blurred image and it is not possible to visualize the structure beyond the limit (left). However, using photochemical properties of the molecules we can make the molecules blink so that only a few molecules far from each other can be detected at a time. By detecting the molecules randomly blinking at different time points, one can localize each molecule with higher localization accuracy using FIONA (middle). By repeating this over time until all the molecules in the specimen are detected, one can get the position information of every fluorescent molecule of the sample with ~10 nm localization accuracy. The collection of the localization of the molecules, finally show the structure of specimen with the resolution beyond the diffraction limit.

1. Fluorescence vs. Brightfield & Diffraction limit of light

Data: images in "Fluorescence vs. Brightfield" folder.

- Brightfield and fluorescence images of fluorescent beads with 4 different sizes
- Brightfield and fluorescence image of a cell

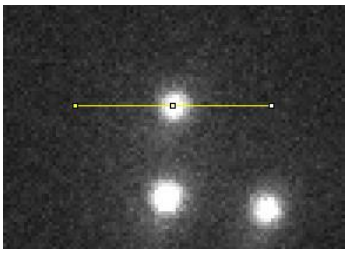
Instruction for analysis of images using Image J

Use the images to answer the lab report questions. Image J is a good tool for image analysis. For the analysis, refer to the instruction below.

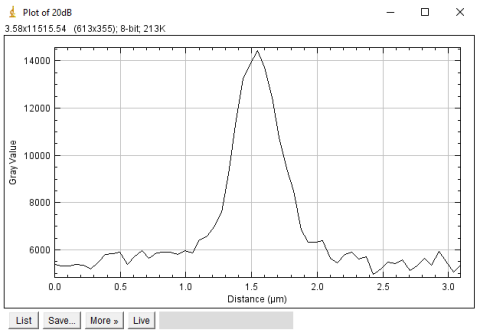
First you need to download and install ImageJ (https://imagej.nih.gov/ij/download.html). Then download and install a plugin called "ThunderSTORM." (https://github.com/zitmen/thunderstorm/wiki/Downloads) the instruction for installation can be found here (https://github.com/zitmen/thunderstorm/wiki/Installation)

Instruction for measuring size of objects

1. Start Image J and drag and drop your image file on Image J window to open the file for analysis.
2. You can adjust brightness and contrast of the image by "Control+Shift+C"
3. Choose the object of which size (diameter, width or thickness) you want to measure.
4. Click  icon to select cross section of your target.
5. Click and draw a line across the object of your interest.



6. Hit "k" to measure line profile. Image J will show the following plot.



7. Click "List" to see the value.
8. For Gaussian fitting of the value copy the plot values. (Control+c)
9. Go to "Analyze -> Tools -> Curve fitting"
10. Paste the plot value and choose "Gaussian" for fitting curve.
11. Click "Fit" then Image J will show you the fitting parameters.

2. How does SMLM achieve "super-resolution?"

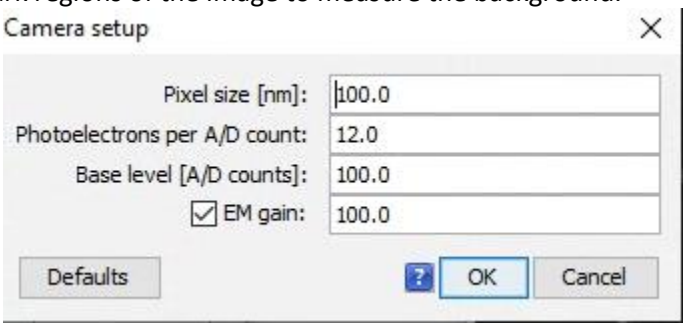
In the second session, you will image a biological specimen, mammalian cell whose actin filament (one of cytoskeletons in cells) is fluorescently labeled, with super-resolution. In the previous sessions, you learned the diffraction limit, and how to overcome the limit in terms of localization of single molecules separated more than the diffraction limit. Here, in addition to the localization, you will learn how to get super-resolution using stochastic photo-switching of fluorophores.

Data: images in "SMLM" folder.

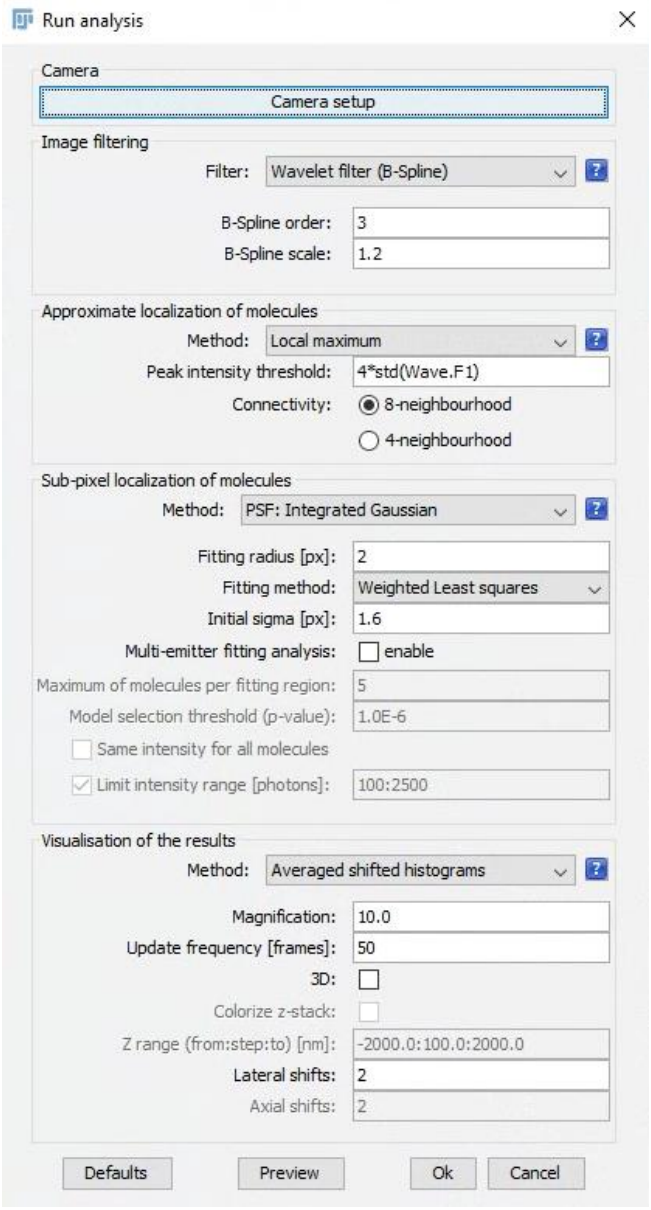
- Regular resolution image of microtubule (regular.tif)
- Sequence of SMLM image for analysis (sequence.tif, 27,529 frames)

Instruction for using ThunderSTORM for analysis

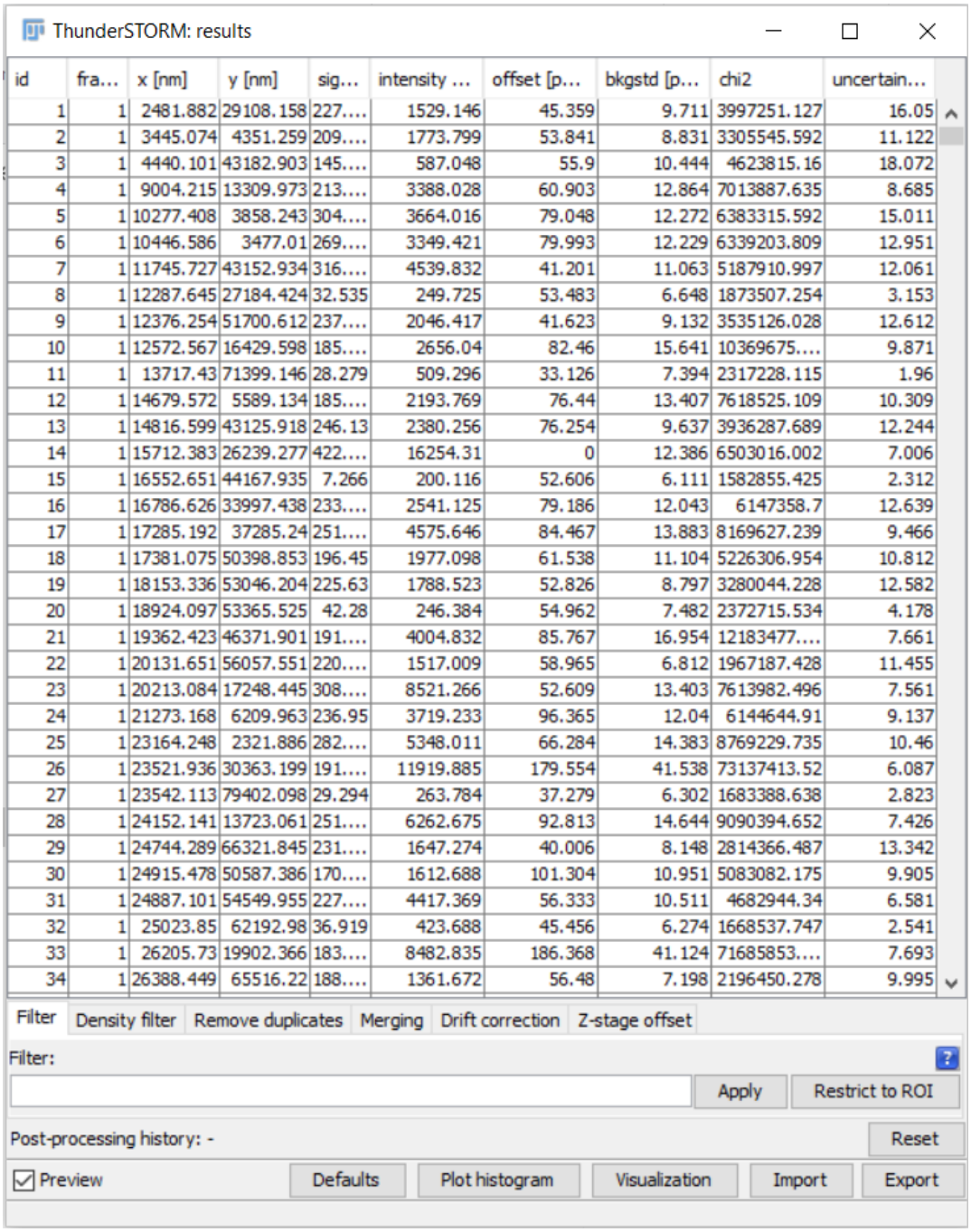
1. Start Image J and drag and drop your image file on Image J window to open the file for analysis.
2. Plugins -> ThunderSTORM -> Camera setup. Enter the parameters according to the picture below. Photoelectrons per A/D count will vary depending on the gain value you use (Refer to the table). Base level [A/D counts] value will depend on the background count of your image. Move the cursor to dark regions of the image to measure the background.



3. After setting up the camera, go to Plugins -> ThunderSTORM -> Run Analysis/ and enter the values according to the picture.



4. Click “Preview”. You should be able to see red dots overlapping on the beads. If there are more or less red dots, go back to the camera setup and adjust the base level accordingly (increase if there are more red dots than the beads, otherwise decrease).
5. After making sure that the preview looks fine, click “Ok” button next to the “preview” button. You will get a screen similar to the one below.



id	fra...	x [nm]	y [nm]	sig...	intensity ...	offset [p...	bkgstd [p...	chi2	uncertain...
1	1	2481.882	29108.158	227....	1529.146	45.359	9.711	3997251.127	16.05
2	1	3445.074	4351.259	209....	1773.799	53.841	8.831	3305545.592	11.122
3	1	4440.101	43182.903	145....	587.048	55.9	10.444	4623815.16	18.072
4	1	9004.215	13309.973	213....	3388.028	60.903	12.864	7013887.635	8.685
5	1	10277.408	3858.243	304....	3664.016	79.048	12.272	6383315.592	15.011
6	1	10446.586	3477.01	269....	3349.421	79.993	12.229	6339203.809	12.951
7	1	11745.727	43152.934	316....	4539.832	41.201	11.063	5187910.997	12.061
8	1	12287.645	27184.424	32.535	249.725	53.483	6.648	1873507.254	3.153
9	1	12376.254	51700.612	237....	2046.417	41.623	9.132	3535126.028	12.612
10	1	12572.567	16429.598	185....	2656.04	82.46	15.641	10369675....	9.871
11	1	13717.43	71399.146	28.279	509.296	33.126	7.394	2317228.115	1.96
12	1	14679.572	5589.134	185....	2193.769	76.44	13.407	7618525.109	10.309
13	1	14816.599	43125.918	246.13	2380.256	76.254	9.637	3936287.689	12.244
14	1	15712.383	26239.277	422....	16254.31	0	12.386	6503016.002	7.006
15	1	16552.651	44167.935	7.266	200.116	52.606	6.111	1582855.425	2.312
16	1	16786.626	33997.438	233....	2541.125	79.186	12.043	6147358.7	12.639
17	1	17285.192	37285.24	251....	4575.646	84.467	13.883	8169627.239	9.466
18	1	17381.075	50398.853	196.45	1977.098	61.538	11.104	5226306.954	10.812
19	1	18153.336	53046.204	225.63	1788.523	52.826	8.797	3280044.228	12.582
20	1	18924.097	53365.525	42.28	246.384	54.962	7.482	2372715.534	4.178
21	1	19362.423	46371.901	191....	4004.832	85.767	16.954	12183477....	7.661
22	1	20131.651	56057.551	220....	1517.009	58.965	6.812	1967187.428	11.455
23	1	20213.084	17248.445	308....	8521.266	52.609	13.403	7613982.496	7.561
24	1	21273.168	6209.963	236.95	3719.233	96.365	12.04	6144644.91	9.137
25	1	23164.248	2321.886	282....	5348.011	66.284	14.383	8769229.735	10.46
26	1	23521.936	30363.199	191....	11919.885	179.554	41.538	73137413.52	6.087
27	1	23542.113	79402.098	29.294	263.784	37.279	6.302	1683388.638	2.823
28	1	24152.141	13723.061	251....	6262.675	92.813	14.644	9090394.652	7.426
29	1	24744.289	66321.845	231....	1647.274	40.006	8.148	2814366.487	13.342
30	1	24915.478	50587.386	170....	1612.688	101.304	10.951	5083082.175	9.905
31	1	24887.101	54549.955	227....	4417.369	56.333	10.511	4682944.34	6.581
32	1	25023.85	62192.98	36.919	423.688	45.456	6.274	1668537.747	2.541
33	1	26205.73	19902.366	183....	8482.835	186.368	41.124	71685853....	7.693
34	1	26388.449	65516.22	188....	1361.672	56.48	7.198	2196450.278	9.995

6. Each column indicates following information.
   • id: (optional) column to identify each localization.
   • frame: frame number.
   • x [nm] and y [nm]: x and y coordinates of the fitted localizations. (0,0) is top left as conventional in ImageJ.
   • sigma [nm]: standard deviation of the gaussian fitted on the peak.
   • intensity [photon]: integrated photons number under the peak (value used to calculate the uncertainty in the Webb/Mortensen formulas).
   • offset [photon]: baseline of the peak (background absolute value).

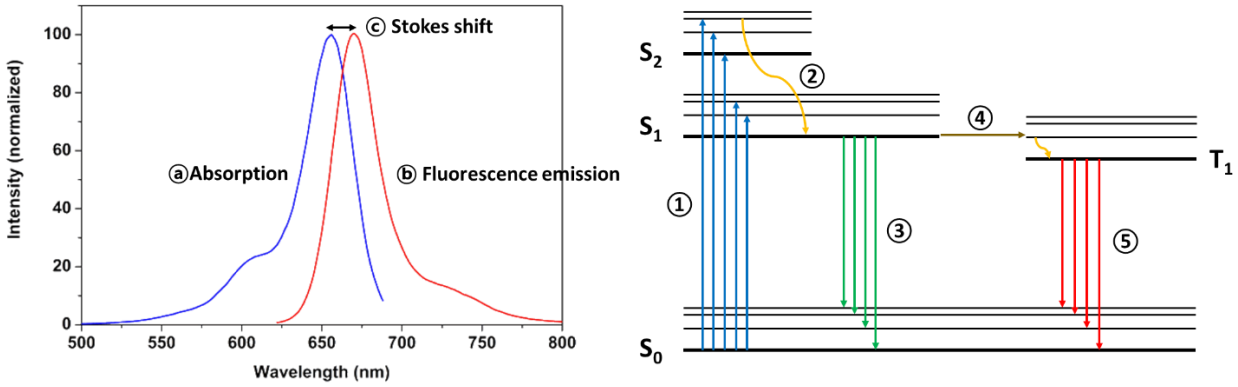
• bkgstd [photon]: standard deviation of the background (used to calculate the uncertainty in the Webb/Mortensen formulas).
• uncertainty [nm]: the lateral localization accuracy.

7. Optional: Since there are some artifact (noise) of the image, it is recommended to filter further. In "Filter" tab of the ThunderSTORM results window, use "offset > 1 & offset < 200".

Figure 5. Images of microtubule in the white box in Figure 8. Regular (diffraction limited, left) vs. STORM image (middle). The plot on the right side is the intensity profile along the white line in the figures.

Lab Report Questions

1) Fluorescence: Figure on the left side is the absorption and fluorescence emission spectra of Alexa647 the fluorescent dye we used for the experiment, right figure is called Jablonski diagram which shows possible electronic transitions by photo-excitation.



a. Connect relevant or corresponding process in Jablonski diagram to ⓐ absorption, ⓑ fluorescence emission, and ⓒ Stokes shift.
b. What's the difference between ③ and ⑤?
c. In the spectrum plot, why is emission spectrum a (almost) mirror image of absorption spectrum?

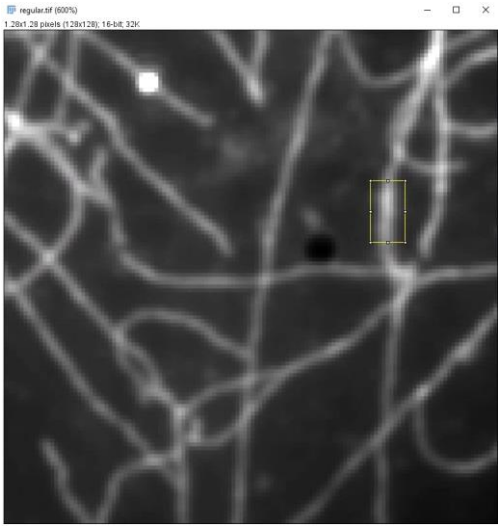
2) Fluorescence vs. Brightfield microscopy

a. In the images of beads, in which image can you see the shape of beads? Is the bead visible in both brightfield and fluorescence image?
b. In the image of cell, what can you see? Is what you see visible in which image?
c. Based on the result, what is the advantage of fluorescence microscopy over transmission microscopy? Explain with the concept of signal-to-noise ratio (SNR).

3) **Diffraction limit of light:** In the images of the fluorescent beads, what is the measured size of the beads? What is the actual size of them? If they are different, why? Take a few beads in the image and measure their Full-width-half-maximum (FWHM) of Gaussian fit. Also, measure the thickness of microtubule filaments in the fluorescence image of the cell in the same way. How think is it? Is the value the same as known thickness of the microtubule?

4) From question 4, how are the results related to the optical resolution of the system? How do you define a resolution of the system? How is it related to the diffraction limit of light?

5) **Single molecule localization microscopy:** In the highlighted area in the regular resolution image ("regular.tif") how many microtubule filaments can you see?



6) In the reconstructed image of SMLM sequence, how many filaments can you see in the same area? If you have multiple filaments, what is the separation between them?
7) What is the thickness of microtubule filaments in the images? Compare the thickness measured in the regular image and the SMLM image. If they are different, why?
8) How can SMLM image the structure smaller than the diffraction limit of light, how can it resolve multiple filaments that locate closer than the diffraction limit of light? How can it break the diffraction limit?

Resources

FIONA principles

- R. E. Thompson, D. R. Larson, W. W. Webb, “Precise Nanometer Localization Analysis for Individual Fluorescent Probes”, Biophysical Journal 82, 2775-2783 (2002)
- A. Yildiz, J. N Forkey, S. A. McKinney, T. Ha, Y. E. Goldman, P. R. Selvin, “Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization”, Science 300, 2061-2065 (2003)

PALM/STORM principles

- B. Huang, M. Bates, X. Zhuang, "Super-resolution Fluorescence Microscopy", Annual Review Biochemistry 78, 993-1016 (2009)
- B. Huang, W. Wang, M. Bates, X. Zhuang, "Three-dimensional Super-resolution Imaging by Stochastic Optical Reconstruction Microscopy", Science 319, 810-813 (2008)
- E. Betzig, G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, J. S. Bonifacino, M. W. Davidson, J. Lippincott-Schwartz, H. F. Hess “Imaging Intracellular Fluorescent Proteins at Nanometer Resolution”, Science 313, 1642-1645 (2006)
- S. T. Hess, T. P. K. Girirajan, M. D. Mason, “Ultra-High Resolution Imaging by Fluorescence Photoactivation Localization Microscopy” Biophysical Journal 91, 4258 (2006)
- J. C Vaughan, S. Jia, X. Zhuang, “Ultrabright photoactivatable fluorophores created by reductive caging” Nature Methods 9, 1181–1184 (2012)