

# Fluorescence microscopy & Single Molecule Localization Microscopy

Please read the overview and the introduction before the experiment starts and answer the “Pre-lab questions” at the end of the introduction

## 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.)

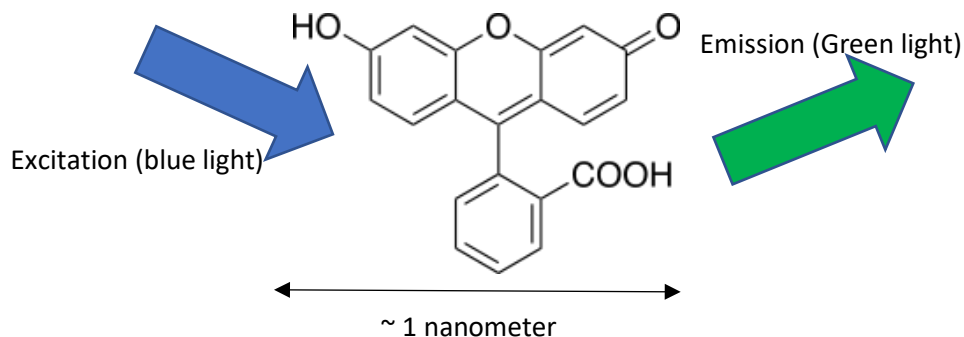


Figure 1. Absorption and fluorescent emission of a molecule. Excitation (blue) light is absorbed by the molecule and emitted (green) from the molecule.

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  $\lambda$  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  $\sim 250$  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  $\sim \lambda/2$ . (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.

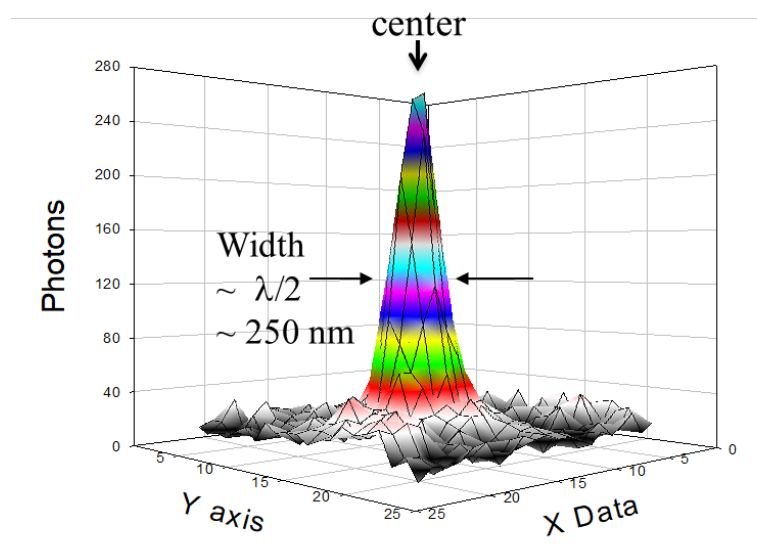


Figure 2. Principle of single molecule localization (FIONA). The figure shows a point spread function (PSF). The width of the PSF is blurred out ( $\sim 250$  nm) but one can estimate the center of the PSF much more accurately than the width.

How can we go from something whose emission is blurred out (to  $\sim 250$  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  $\sim$  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  $\sim s/2\sqrt{N} = \lambda/2NA\sqrt{N}$ . By collecting 5,000-10,000 photon counts per second, one can localize a fluorescent molecule to within a nanometer.

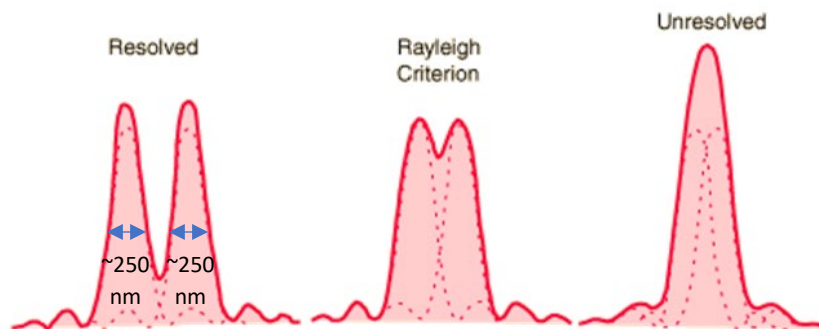


Figure 3. Two molecules located further than diffraction limit (left, resolved, shown at  $\sim 250$  nm) at about Rayleigh criterion (middle, diffraction limit) and closer (right, unresolved).

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?

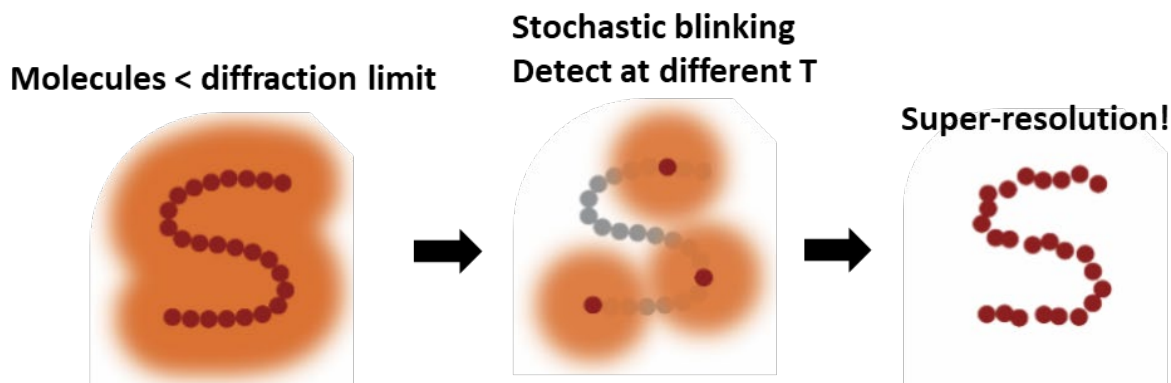


Figure 4. Principle of single molecule localization microscopy to get super-resolution. Here, the molecules within the diffraction limit of light are switching on stochastically, so that every molecule fluorescing can be localized at different time points. The collection of every localization results 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  $\sim 10$  nm localization accuracy. The collection of the localization of the molecules, finally show the structure of specimen with the resolution beyond the diffraction limit.

**Pre-lab questions (answer the questions and get them checked by TA before you start the experiment)**

1. What is fluorescence?
2. When two molecules are separated by 300 nm, what is the maximum wavelength to distinguish them?
3. A molecule is moving with 10 nm discrete step. To distinguish the step size of the molecule, how many photons do you need to collect for each step?

**1. Fluorescence vs. Bright field (Day 1 of week 1)**

First, you will image some specimens using fluorescence and bright field microscope to see the difference and advantage of them. You will use the setup built by the TA (and then you will build your own in next session!).

**Samples**

- Immobilized fluorescent beads: 40 nm, 100 nm, 200 nm, and 4  $\mu\text{m}$  diameter
- Fluorescently labeled cell (HeLa cell stained by anti- $\alpha$ tubulin-Alexa647)

**Image acquisition**

**0. When the laser is on, wear safety goggles.**

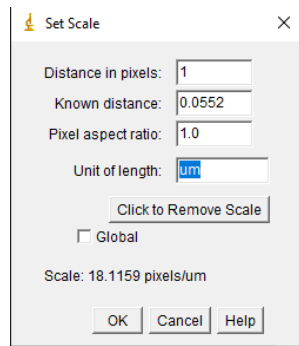
1. Turn on the computer and start FlyCapture software for the camera.
2. Put a drop of immersion oil on the objective lens.
3. Place the sample on the stage.
4. Using z-focusing stage, raise the objective so that the immersion oil touches the glass coverslip.
5. Cover the sample with black box.
6. Turn on the laser.
7. Using z-focusing stage, focus the sample to see clear image of the sample.
8. Take images of sample and save them with proper name.
9. For comparison, take bright field image of each sample as well.
  - a. For bright field image, turn off the laser and take off the block box from the sample.
  - b. Using flashlight of your smart phone, take images of the samples.
10. You will also take STORM image using TA setup. The detail instruction is in Session 3. TA will give on site instruction.
11. Each group member needs to take one set of images.
12. After all images are taken, wipe the objective lens with lens cleaning paper and methanol.


**Analysis**

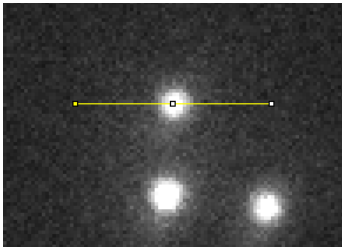
Use the images taken in the previous session to answer the lab report questions. Image J is a good tool for image analysis. For the analysis, refer to the instruction below.

**Instruction for measuring size**

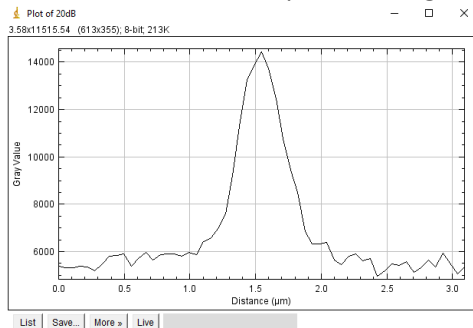
1. Start Image J and drag and drop your image file on Image J window to open the file for analysis.
2. First you need to set the scale (pixel size) of your image.
  - a. Go to "Analyze -> Set Scale"
  - b. Enter Distance in pixels: 1, Known distance: 0.0552, Pixel aspect ratio: 1.0 and Unit of length:  $\mu\text{m}$ .



- c. Click OK.
3. You can adjust brightness and contrast of the image by "Control+Shift+C"
4. Choose the object of which size (diameter, width or thickness) you want to measure.
5. Click  icon to select cross section of your target.
6. Click and draw a line across the object of your interest.



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

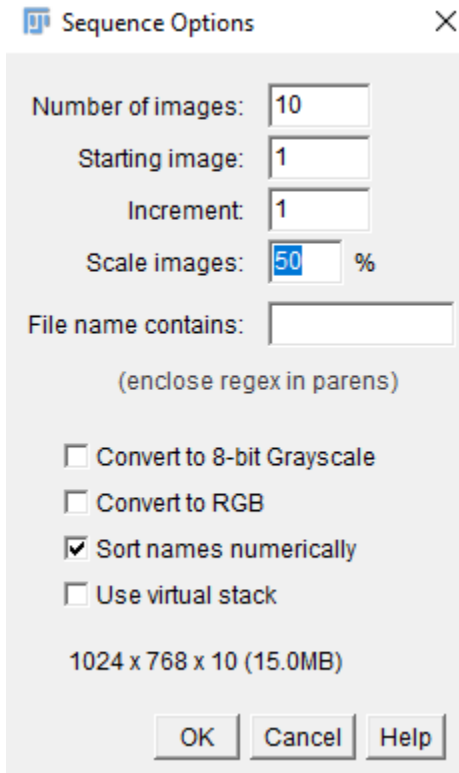


8. Click "List" to see the value.
9. For Gaussian fitting of the value copy the plot values. (Control+c)
10. Go to "Analyze -> Tools -> Curve fitting"
11. Paste the plot value and choose "Gaussian" for fitting curve.
12. Click "Fit" then Image J will show you the fitting parameters.

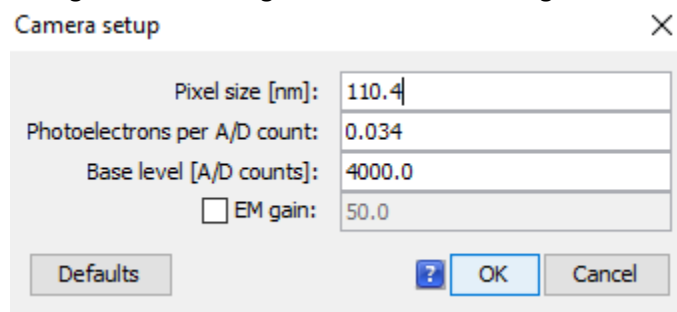
### Instruction for using ThunderSTORM for analysis

Here you will use Image J and Origin for the analysis.

1. Open an image file using Image J.
  - a. File -> Import -> Image sequence, go to the folder of your images, click the first file, and click open.
  - b. Put the parameters according to the image below. Scale images: 50% will 2x2 bin your image with averaging pixel value.



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.



Gain (dB)	Photoelectrons per A/D count (w/o binning)	Photoelectrons per A/D count (2x2 binning)
0	0.27	1.08
10	0.085	0.34
20	0.027	0.108
30	0.0085	0.034
40	0.0027	0.0108
48	0.0011	0.0044

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

Run analysis

Camera

Camera setup

Image filtering

Filter: Wavelet filter (B-Spline)

B-Spline order: 3

B-Spline scale: 2.0

Approximate localization of molecules

Method: Local maximum

Peak intensity threshold:  $2 \times \text{std}(\text{Wave.F1})$

Connectivity: ☒ 8-neighbourhood ☐ 4-neighbourhood

Sub-pixel localization of molecules

Method: PSF: Integrated Gaussian

Fitting radius [px]: 3

Fitting method: Weighted Least squares

Initial sigma [px]: 1.6

Multi-emitter fitting analysis: ☐ enable

Maximum of molecules per fitting region: 5

Model selection threshold (p-value):  $1.0 \times 10^{-6}$

☐ Same intensity for all molecules

☒ Limit intensity range [photons]: 500:2500

Visualisation of the results

Method: Averaged shifted histograms

Magnification: 5.0

Update frequency [frames]: 50

3D: ☐

Colorize z-stack: ☐

Z range (from:step:to) [nm]: -2000.0:100.0:2000.0

Lateral shifts: 2

Axial shifts: 2

Defaults Preview Ok Cancel

- 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).
- 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.

ThunderSTORM: results

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

Filter: Density filter Remove duplicates Merging Drift correction Z-stage offset

Filter:  Apply Restrict to ROI

Post-processing history: - Reset

☒ Preview Defaults Plot histogram Visualization Import Export

- Click the “plot histogram” button and then plot histograms for the parameters “intensity” (photon count per point per frame), “offset” (background photon count) and “uncertainty” (localization precision). Compare these parameters for different sets of data.

## 2. Building a simple fluorescence microscope (Day 2 of week 1 and week 2)

To achieve its function, a fluorescence microscope has number of optical components as illustrated in Figure 4. Figure 4 is a schematic diagram of a very simple epi-fluorescence microscope. In the first



session, you will build a simple fluorescence microscope which has capability of single molecule localization microscopy (SMLM) and understand the role of each optical component.

When the microscope is built, you will need to characterize your setup. Before you get the super-resolution, we want to know the diffraction limit of the system. By looking at the sample with the sub-diffraction limit size (40 or 100 nm fluorescent beads), you will be able to tell what the limit of the resolution of the system is. Then by using the technique called Fluorescence Imaging with One-Nanometer Accuracy (FIONA), you will learn that you can localize the position of the detected molecule with the accuracy that is higher than the diffraction limit.

For high quality of SMLM image, it is also important to have high signal-to-background ratio (SBR) or signal-to-noise ratio (SNR). Here, we will learn how the conditions and the parameters for the image acquisition affect the result of the molecular localization.

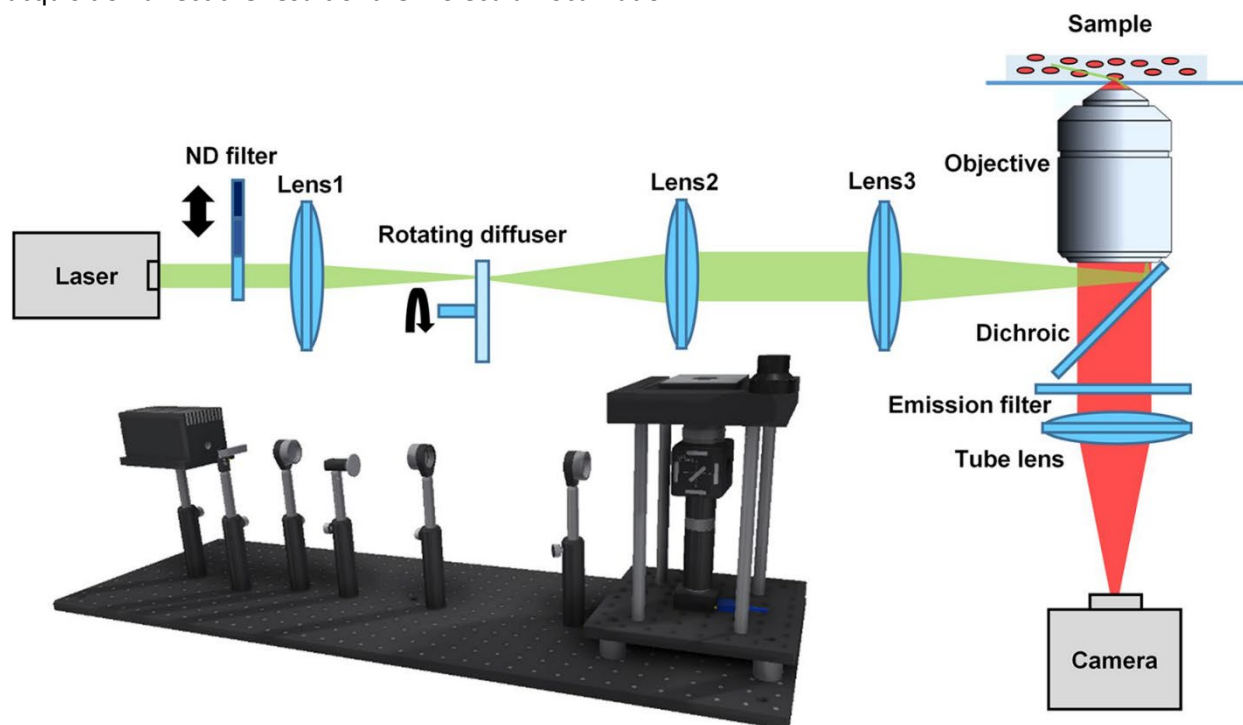


Figure 5. Schematic diagram for the simple SMLM setup.

### Questions before you start building!

Before building the setup, let's see the role of each optical component. You will need to answer these questions: What is the function and the role of each optical component in the microscopic system? (These questions will come again at the lab report question 3.)

- Major components
  - Laser:
  - Dichroic (dichromatic) filter (beam splitter):
  - Emission filter:
  - Objective lens:
  - Tube lens:
  - Lens 1&2 (Beam expander):
  - Lens 3 (as Kohler lens):

- Minor things  
ND filter:  
Diffuser:

### Building a simple epifluorescence microscope

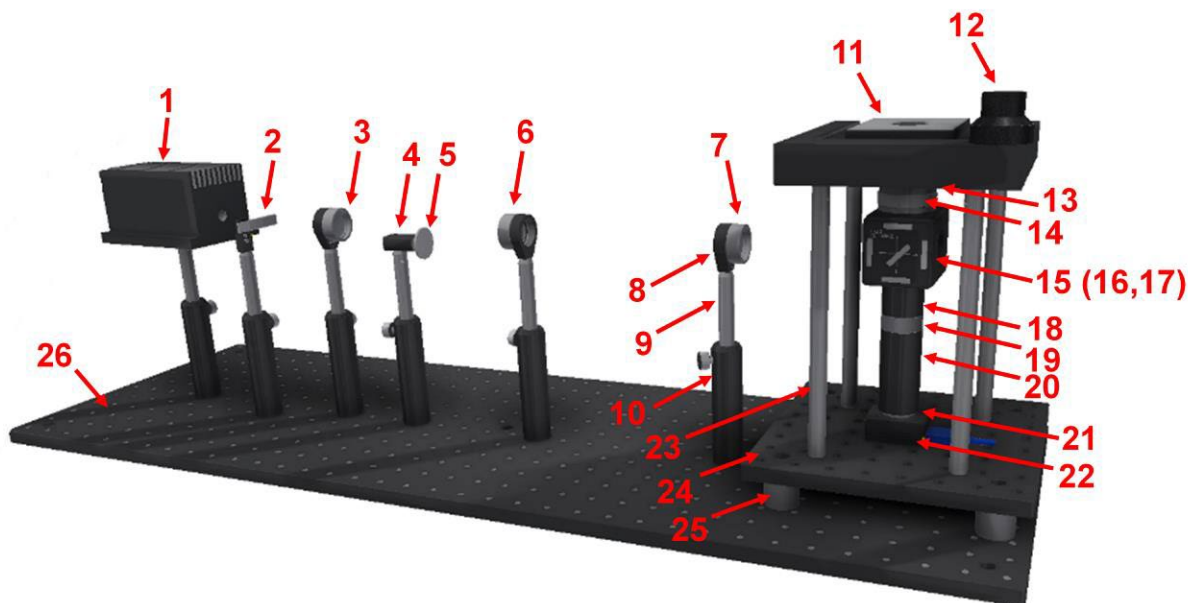


Figure 6. The 3D CAD schematic of the SMLM setup and the corresponding detailed list of components is shown in Table 1

Module	Item #	Item	Description
Light Source	1	Laser module	Wave length: $638 \pm 3$ nm, Maximal power: 500 mW, Output stability: 5%, Beam size: 5 mm, Divergence: $<1.5$ mrad, $M^2$ : $<1.6$ , TTL/Analog power control
	2	ND filter	Rectangular Step ND Filter, 10 mm wide, OD: 0.3 - 2.0. You need to use sliding holder to assemble it on the post.
	3	Lens	$f=100$ mm, $\varnothing 1$ " Achromatic Doublet, SM1-Threaded Mount, ARC: 400-700 nm
	4	DC motor	6V 250mA Brushed DC Motor, No-load speed: $9100 \pm 1800$ rpm It will be held by the clamp holder
	5	Engineered diffuser	Round 1 inch, Divergence angle: $5.48^\circ$ It is glued on the motor shaft.
	6	Lens	$f=100$ mm, $\varnothing 1$ " Achromatic Doublet, SM1-Threaded Mount, ARC: 400-700 nm
	7	Lens	$f=200$ mm, $\varnothing 1$ " Achromatic Doublet, SM1-Threaded Mount, ARC: 400-700 nm
	8	Lens Mount	Lens Mount with Retaining Ring for $\varnothing 1$ " Optics, 8-32 Tap

	9	Optical Post	Ø1/2" Optical Post, SS, 8-32 Setscrew, 1/4"-20 Tap, L = 6"
	10	Post Holder	Ø1/2" Post Holder, Spring-Loaded Hex-Locking Thumbscrew, L = 4"
Microscope	11	Sample holder	Self-designed sample holder with aperture of 20 mm
	12	XY translation stage	X-Y Travel Distances: 75 x 56mm, Active Area: 37.6mm in diameter
	13	Objective lens	100X (Oil) Infinity-corrected Plan Fluor Objective Lens, NA = 1.3, tube length: 160 mm
	14	Z-focusing stage	SM1 Zoom Housing for Ø1" Optics, Non-Rotating, 4.1 mm Travel
	15	Filter cube	Kinematic Fluorescence Filter Cube, 30 mm Cage Compatible, 1/4"-20 Tapped Holes
	16	Dichroic mirror	660 nm edge BrightLine single-edge dichroic beam splitter
	17	Emission filter	emission filter for Alexa-647
	18	Spacer	SM1 Lens Tube Spacer, 1" Long
	19	Tube lens	f=100 mm, Ø1" Achromatic Doublet, SM1-Threaded Mount, ARC: 400-700 nm
	20	Spacer	SM1 Lens Tube, 2.50" Thread Depth, One Retaining Ring Included
	21	Thread adapter	Adapter with External C-Mount Threads and Internal SM1 Threads
	22	Camera	Sony IMX265 sensor, 2048 x 1536 3.45 µm pixels, 71% QE, 2.89e- read noise, USB3.0
	23	Optical post	Ø1/2" Optical Post, SS, 8-32 Setscrew, 1/4"-20 Tap, L = 8"
	24	Microscope base	Aluminum Breadboard 8" x 8" x 1/2", 1/4"-20 Taps
	25	Vibration isolating feet	Ø27.0 mm Sorbothane Feet, Internal 1/4"-20 Mounting Thread, 4 Pieces
Optical Table	26	Breadboard	Aluminum Breadboard 12" x 36" x 1/2", 1/4"-20 Taps
Miscellaneous	-	Alignment target	Fluorescent target with RMS thread
	-	Thread adapter	Thread adapter for the objective lens (RMS to SM1)
	-	Fluorescent card	

The goal of this session is to “align the laser” for the microscope system. For the proper “alignment”, there are two specific checkpoints for this session.

- The laser should be “collimated” after the beam expander.
  - o What is “collimation”? and how would you check whether the beam is collimated?
- The profile of the beam should be uniform (you can see the profile after the beam expander by using fluorescence detection card.
- The laser should be focused at the back focal plane of the objective lens.

**Before you start:**

- **You should wear safety goggles when the laser is ON. Even if you are using low-power laser, focused laser can damage your retina or skin.**
- You should work with the high-power laser ONLY IN THE PRESENCE OF TA. This is for your safety.
- You should wear gloves to prevent the contamination of optical elements: lenses, objective lens, filters. Fingerprints or oil from skins can damage or degrade the performance of the elements.

**General workflow for this session:**

1. Place the breadboard on the flat and stable place.
2. Set the heights of the parts (posts) as the same as the height of the aperture of the filter cube.
3. Place and assemble the parts accordingly to get the beam “collimated” after the beam expander.
4. By using diffuser, make the beam profile uniform.
5. Using Kohler lens (condenser lens) focus the beam at the center of the back focal plane of the objective lens (using fluorescence target).
6. Assemble the objective and start imaging test samples.
  - Samples: fluorescent beads with diffraction-limited size (40 nm, 100 nm or 200 nm given by TAs)
7. The points to check:
  - 1) With bead samples check whether you can see diffraction limited PSF and whether it's symmetric.
  - 2) Homogeneous illumination intensity over wide enough area ( $\sim \frac{1}{4}$  of the whole camera area).

### **3. How does SMLM achieve “super-resolution?” (week 3)**

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.

**STORM image of cytoskeleton (microtubule or actin)****Preparation of the specimen (HeLa cells labeled by anti-tubulin-Alexa 647 (for microtubule) or phalloidin-Alexa 647 (for actin)) - done by TAs.**

1. Coat a glass bottom dish with poly L lysine (PLL) for 30 min
2. Wash the dish with PBS or MBG water and put 1/20 dilution of 200 nm gold particle and incubate for 3 hrs.
3. After the incubation, wash with PBS or MBG water. For better cell adhesion, coat PLL for another 30 min before the seeding.
4. Seed the cell on the dish. In general, 1 ml of cell culture from the 25 cm<sup>2</sup> culture flask is good. Culture until cells settled on the surface.
5. When cells are ready, take out the culture media and wash with PBS for 1 time. Fix the cell with 4% paraformaldehyde (PFA) for 10 min.
6. Wash with PBS for 2 times, add 0.5% Triton-X and incubate for 10 min.
7. Wash with PBS for 2 times and incubate the cell in 5% BSA in PBS for 1 hr at 37C, or overnight at 4C for blocking non-specific binding.

8. After blocking, incubate with 1/200 diluted anti tubulin-Alexa647 or 0.8 U/100 ul phalloidin-Alexa 647 in blocking buffer for 30 min.
9. Wash the cell with PBS for 2 times.
10. Prepare the imaging buffer. Students will test 3 different imaging buffer conditions.

Imaging buffer:

	Oxygen scavenger	Thiol
1	-	-
2	+	-
3	+	+

Oxygen scavenger: Gloxy (1 mg/ml glucose oxidase, 0.15 mg/ml catalase, 5 mg/ml glucose)

Thiol: 10-20 mM MEA.

In PBS

11. Add the imaging buffer to the sample and Image the cell immediately. Reducing condition of imaging buffer can destabilize the structure in the specimen, therefore in order to image fine structure, the sample should be imaged within a few hours.

### Image acquisition

1. Wear the safety goggles always.
2. Put a drop of immersion oil on the objective lens.
3. Place the sample on the stage.
4. Connect the camera to the computer through USB connection.
5. Start the operation software (Point Grey Fly capture).
6. Focus to the sample by rotating z focusing stage.
7. By moving the sample stage find appropriate region of interest (ROI).
  - a. The ROI should include well-labeled cells and a few gold particles attached on the glass surface for the drift correction.

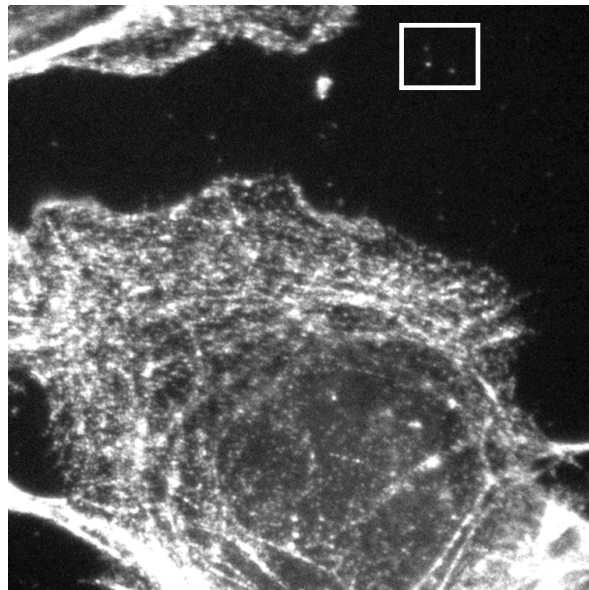


Figure 7. Example of a good ROI. Attached gold particles are highlighted by the white square.

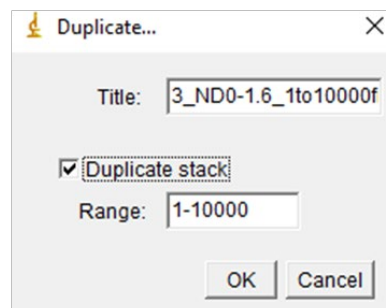
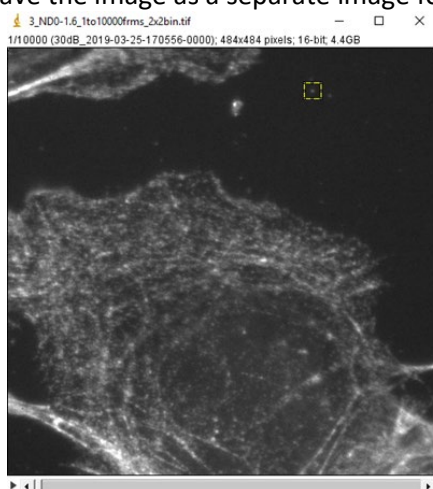
- b. Since cells have 3-dimensional structure, the structure of actin filament varies between cells. Focus on the gold particle for the drift correction and find a nice structure of actin filaments.

8. Set the parameters for imaging. Here is one example for the image acquisition. **You may vary the parameters for better Signal-to-Background Ratio (SBR). Make sure the pixel value is not saturated.**
  - a. Frame rate: 25 fps
  - b. Exposure time: 40 ms
  - c. Gain: between 10-48 dB depending on the signal
9. You will see quick photobleaching of the dye as rapid decrease of the signal. In a few seconds you will be able to see random blinking of single Alexa 647 molecules.
10. Once you observed the blinking of the molecules, start recording images. 5,000-10,000 frames will be enough number.

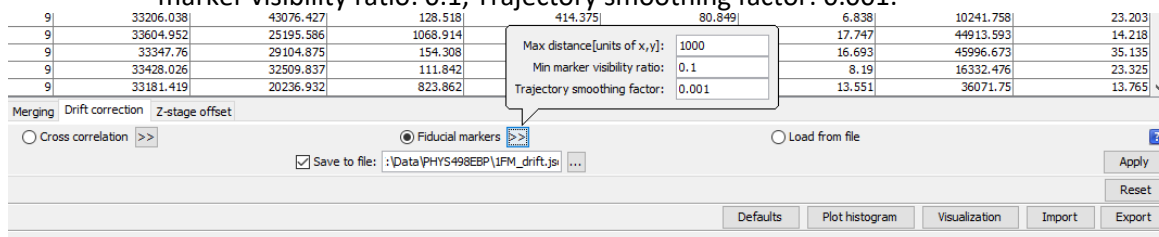
#### Session 4: Analysis of STORM image

Here you will use an Image J plugin, “ThunderSTORM” for the analysis of STORM image. Refer to step 2 for opening files and setting up the camera.

1. Follow the step 1-3 in the session 2 to open the image. Use 50% (2x2 binning).
2. Crop an ROI with 1 gold particle and duplicate (Ctrl+Shift+D) the ROI (check Duplicate stack). You can save the image as a separate image for the drift correction step (step 5).

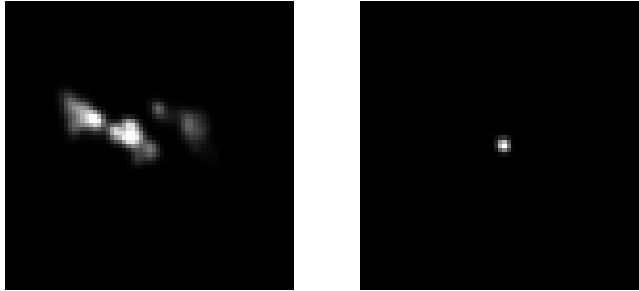


3. Go back to the original STORM image. Run analysis with the image.
4. After analysis is done, export the sheet in your data folder. It will be saved as \*\*.csv format.
5. Drift correction
  - 1) Open the image file of the gold particle you chose in step 2.
  - 2) Run ThunderSTORM analysis with the image.
  - 3) Export the result as “filename\_Drift.csv”.
  - 4) Go to “Drift correction” tab.
  - 5) Choose “Fiducial markers”. Use the following parameters: Max distance: 1000, Min marker visibility ratio: 0.1, Trajectory smoothing factor: 0.001.

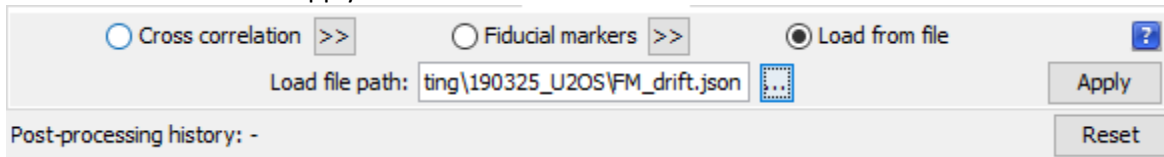


- 6) Check “Save to file” and save the file in your folder.

- 7) Click “Apply”, then the software will correct the stage drift and save the drift information in the folder you choose. You will see the effect of the drift correction in the visualization window. Following image shows the position of the gold particle before (left) and after (right) the drift correction. The file will be saved in \*\*.json format.



- 8) Close the current result window, and import your original result (STORM image).  
9) Go to “Drift correction” tab, choose “Load from file”, select the file you saved in step 7, then click “Apply”.



- 10) Drift correction will be done in the image.
6. 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) & (100 < intensity) & (intensity < 10000).”
7. The resulting image will be shown, for example, as below.

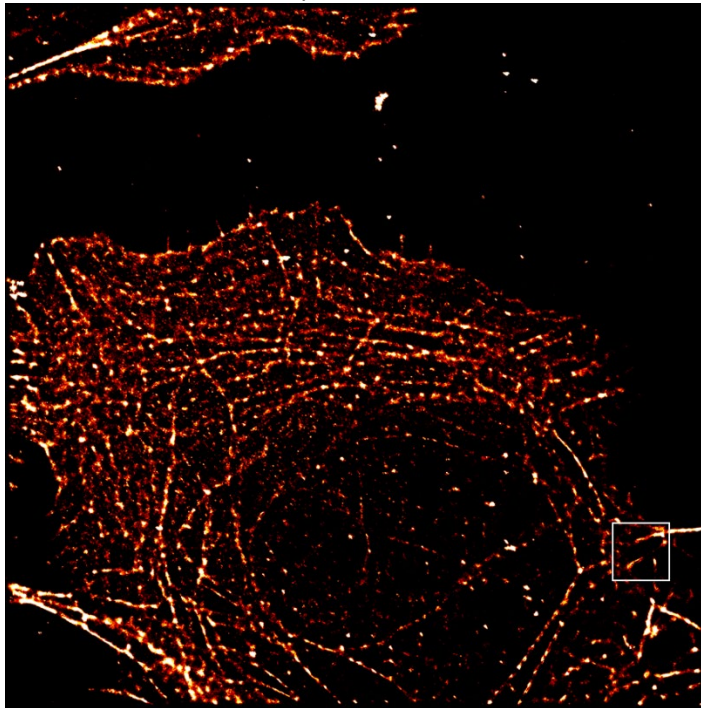


Figure 8. STORM image of microtubule. Highlighted area with the white box is used in Figure 9.



8. You will be able to show images of regular resolution image and STORM image of the same area. An example is below. You can use the images for measuring the width of a filament for lab report questions.

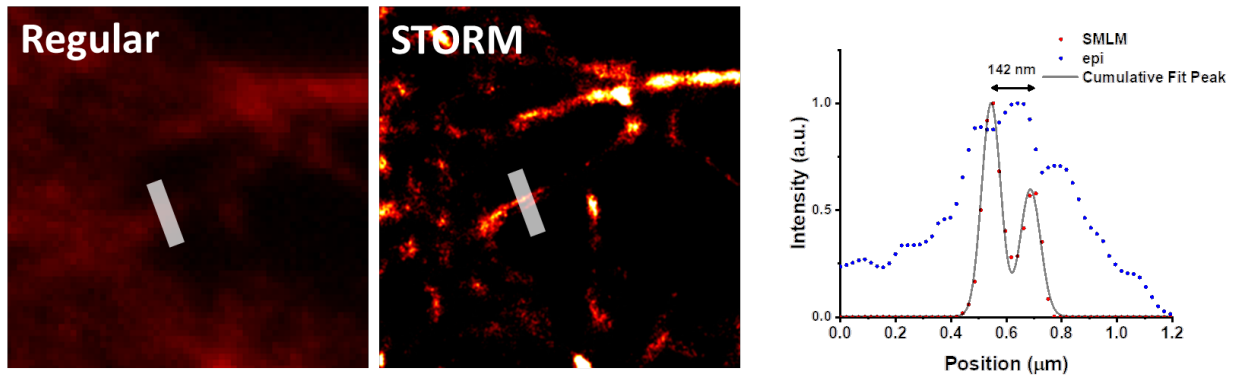
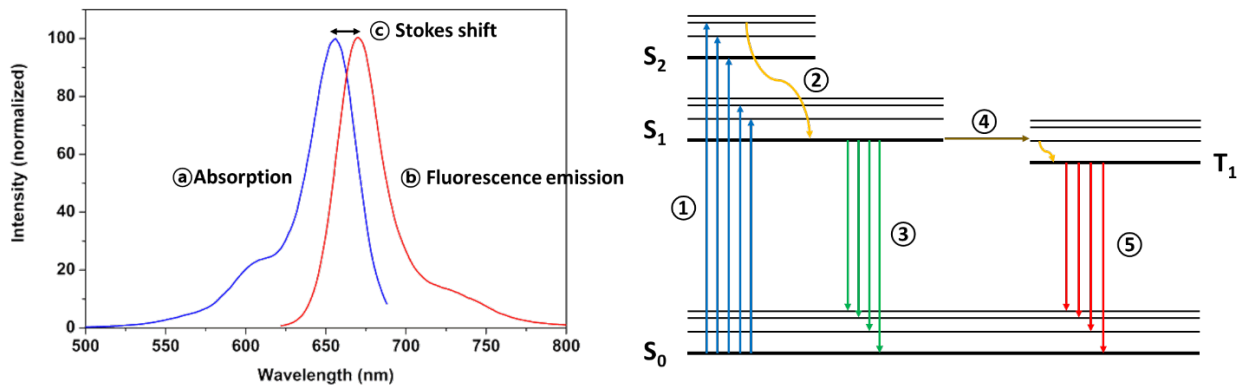


Figure 9. Images of microtubule in the white box in Figure 8. Regular (diffraction limited, left) vs. STORM image (middle). The plot on the right side is the intensity profile along the white line in the figures.

### 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.



- Connect relevant or corresponding process in Jablonski diagram to ① absorption, ② fluorescence emission, and ③ Stokes shift.
  - What's the difference between ③ and ⑤?
  - In the spectrum plot, why is emission spectrum a (almost) mirror image of absorption spectrum?
- 2) Questions about the microscope setup. See Figure 5 for the questions.
- What is the function and the role of following optical components in the microscopic system?
    - Major (essential) components
      - Laser:
      - Dichroic (dichromatic) filter (beam splitter):
      - Emission filter:
      - Objective lens:



- v. Tube lens:
- vi. Lens 1&2 (Beam expander):
- vii. Lens 3 (as Kohler lens):
- Minor components
  - i. ND filter:
  - ii. Diffuser:
- b. You are using  $f=100$  mm lens for Lens 1 & 2. If the Lens 1 is changed to  $f = 50$  mm lens, how does it effect on the system? What if Lens 2 is changed to  $f = 50$  mm lens? (The location of lens will be matched with its focal length.)
- c. What is the role of tube lens? If you use  $f = 200$  mm lens for the tube lens instead of  $f = 100$  mm, how does it effect on the image (pixel size of the camera)?
- 3) Let's compare bright field (transmission) microscopy and fluorescence microscopy.
  - a. In both the bead and the cell image, what structure can you see? Is the structure visible both in brightfield images and fluorescence images? If not, why?
  - b. Based on the result, what is the advantage of fluorescence microscopy over transmission microscopy? Explain with the concept of signal-to-noise ratio (SNR).
- 4) 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?
- 5) 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?
- 6) Use the analyzed results of the bead images. Pick one of the beads in the fluorescence image. What is the position (e.g. x-y coordinate) of the bead? How accurately can you define its position?
- 7) You tested different imaging buffer conditions (+/- oxygen scavenger, +/- thiol). How are the images different in different conditions? Based on that, what are the roles of oxygen scavenger and thiol? How do they help to achieve super-resolution image?
- 8) How do you define resolution in STORM imaging? What is the resolution of your image? What is the localization accuracy of the fluorophore detected in the image? Answer this with the STORM image you have taken in the experiment and the analysis results (e.g. the width of the microtubule filament in the image).
- 9) For SMLM (Single Molecule Localization Microscopy or STORM), what is the advantage of using fluorescence technique?

## Resources

### Simple SMLM setup

- H. Ma, R. Fu, J. Xu, Y. Liu, "A simple and cost-effective setup for super-resolution localization microscopy", Scientific Reports 7, 1542 (2017)

### 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)