Phys598BP Spring 2016 **University of Illinois at Urbana-Champaign**

STORM (STOCHASTIC OPTICAL RECONSTRUCTION MICROSCOPY)/ PALM (PHOTOACTIVATED LOCALIZATION MICROSCOPY)

Location: Loomis Lab. Rm. 363 (Selvin Lab)

Microscope: Nikon Eclipse Ti & a custom build setup based on Olympus IX-71

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Objective

This lab will provide the principles of super-resolution fluorescence imaging, sample preparation, the elements of a super-resolution microscope and the general algorithms of data analysis. Students will get hands-on training in obtaining images using our Nikon or custom setup, and will participate in basic data analysis. Due to the time limit, most of the sample preparation has been done, but the protocol is available upon request.

Outline

I. Background

A. Basic Concepts In Fluorescence Microscopy
B. Principles of PALM/STORM
C. Sample and Target Proteins
   a. Microtubule (MT)
   b. Glutamate Receptors (AMPA and NMDA) in the Central Nervous System
D. Instrumentation

II. Experimental Procedure

A. Experimental Procedure: STORM imaging of surface immobilized MTs (Week 1)
B. Experimental Procedure: 3D PALM/SPT imaging (Week 2)
C. Data Analysis
D. Reference and Online Resources

III. Lab report questions

I. Background

A. Basic Concepts In Fluorescence Microscopy

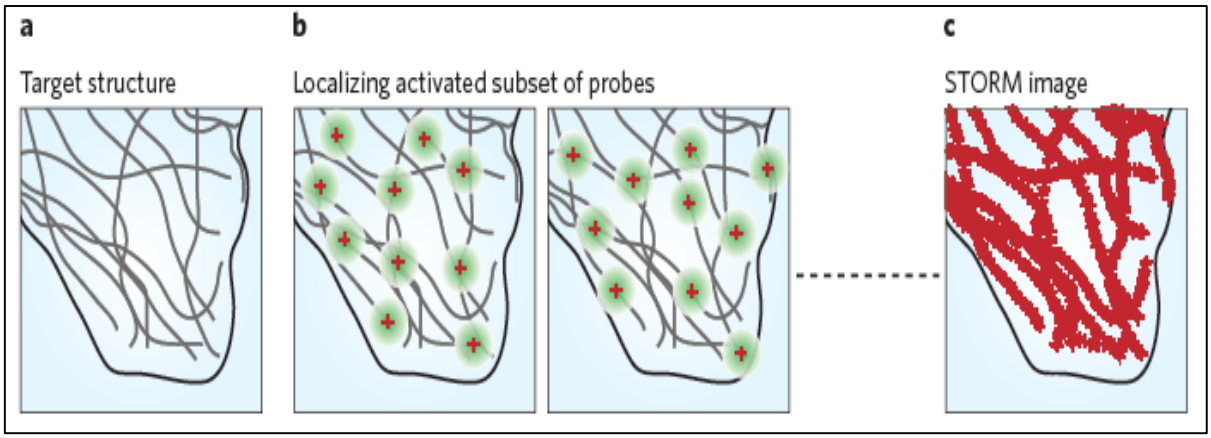
- Molecular specificity and *in vivo* imaging.

  Diffraction of the light limiting the resolution (Abbe's formula: $\mathrm{d} = \frac{\lambda}{2n \sin\theta}$

  where n is the refraction index in which the light travels and the θ is the angle of converging to a spot . The diffraction limit is about 300 nm, for red laser of wavelength around 600 nm.)

B. Principles of PALM/STORM

- FIONA (Fluorescence Imaging with One Nanometer Accuracy)
- Imaging small subset at a time
- Photoactivation mechanism & building an optical switch
  (X. Zhuang, "Nano-imaging with STORM", Nature Photonics 3, 365-367 (2009)

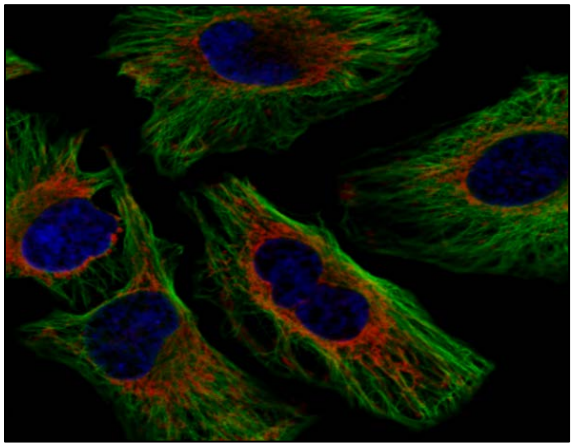


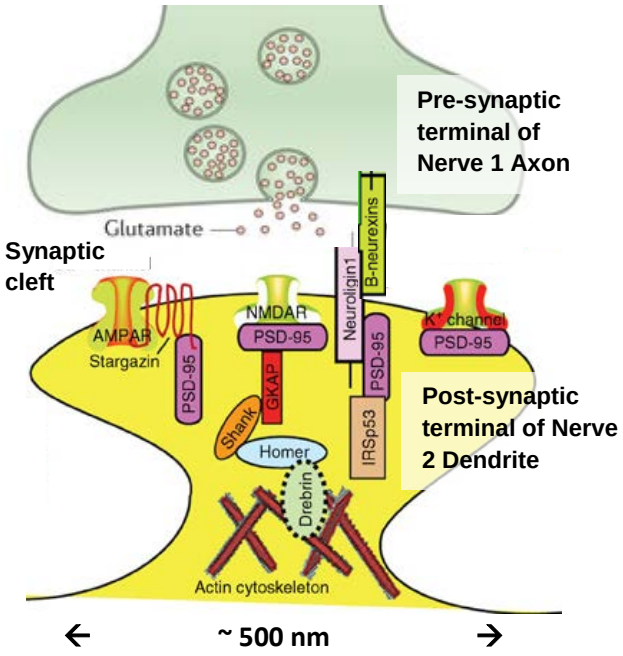
PALM and STORM were two localization-based super-resolution microscopy techniques discovered independently and around the same time. Both techniques rely on the same principle to achieve subdiffraction images: the excitation of a small subset of fluorescence emitters followed by the localization by using **F**luorescence **I**maging with **O**ne **NA**nometer resolution (FIONA), invented by Selvin and his colleagues in 2003. FIONA was originally invented to track organic fluorophores and quantum dots (QDs) with nanometer accuracy. In order to achieve the whole sample image, the procedure is repeated many times until sufficient number of molecules are detected to reconstruct the target sample structure. The main difference between PALM and STORM is the fluorophores used for the experiment and the mechanism of switching between the bright and dark states. PALM uses photo switchable/convertible fluorescent proteins (FPs), whereas STORM uses organic dyes as fluorescent probes for imaging.

C. Sample and Target Proteins

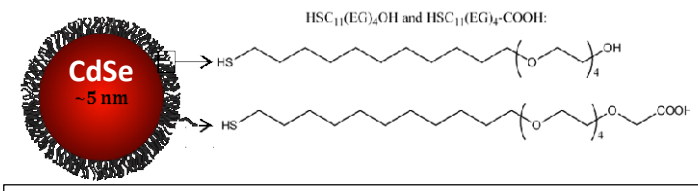
(1) **Microtubule (MT):** A key cytoskeleton in wide variety of cells composed of polymerization of α-tubulin/β-tubulin heterodimers. Each protein has a molecular weight of approximately 55 kDa. MTs are found throughout the cytoplasm of all eukaryotic cells and carry out a variety of functions, ranging from transport to structural support. MTs are about 25 nanometers in diameter. They form part of the cytoskeleton that gives structure and shape to a cell, and plays a key role in intracellular transport of cargo and organelles throughout the cytoplasm.

(2) **Glutamate Receptors (AMPA and NMDA) in the Central Nervous System:** Glutamate is the main excitatory neurotransmitter in the human brain. An action potential in nerve 1 triggers a fusing of glutamate-containing vesicles to the membrane, releasing glutamate into a 20-40 nm neuronal synapse, where it diffuses across and then binds to AMPA and NMDA receptors placed on the post-synaptic membrane of nerve 2. These receptors are tethered to the membrane by a series of scaffolding proteins, PSD-95, Homer1, and SAP97, being among them. These receptors then allow calcium (and other positive ions) to enter, and results in an action potential in nerve 2. The number of AMPA receptors depends on its history, in that it will increase or decrease in a complex process known as plasticity. The amount of AMPA receptors is controlled by the number of NMDAR (which is also triggered by glycine, as well as glutamate) and does not seem to be a strong function of history. (Why this is, is the subject of another R01.) Nevertheless, the amount of intra- and extra-synaptic NMDAR and AMPAR varies tremendously during normal activity, contributing to learning and development, as well as development and during neurological diseases (ref). Understanding the steady-state and dynamics of AMPAR and NMDAR is critical. The glutamate receptors can be specifically labeled with small QDs (sQDs) to determine the precise location within the cell. Molecular level of the mechanism of neuroplasticity may be studied by combining 3D coordinates of synapses imaged by PALM and the nanometer-scale dynamic of glutamate receptor followed by FIONA in living cells over time under different stimulation conditions.

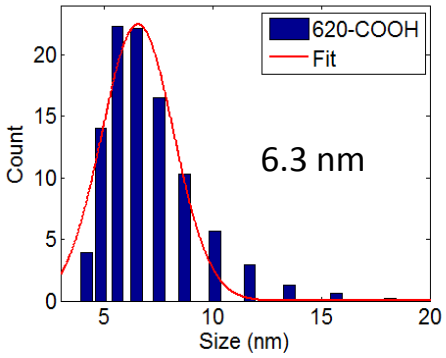




[Left] A confocal image of immunofluorescently labeled microtubules (green) and mitochondria (red); [Right] Schematic diagram of the pre- and post-synaptic termini with glutamate receptors.

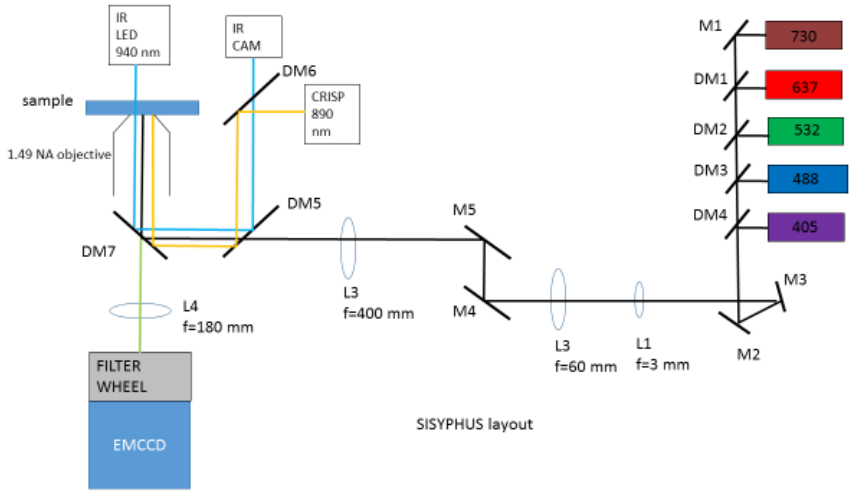


Structure of sQD. The CdSe core (d = 3-6 nm) with a thin ligand layer consisting of a SH group to bind tightly to the core, a hydrophobic 11-carbon linker to exclude water from quenching the fluorescence, then a short PEG to add water solubility. A small percentage ending in COOH is made for bio-attachment. The diameter is 6.3 nm for sQD emitting at 620 nm.

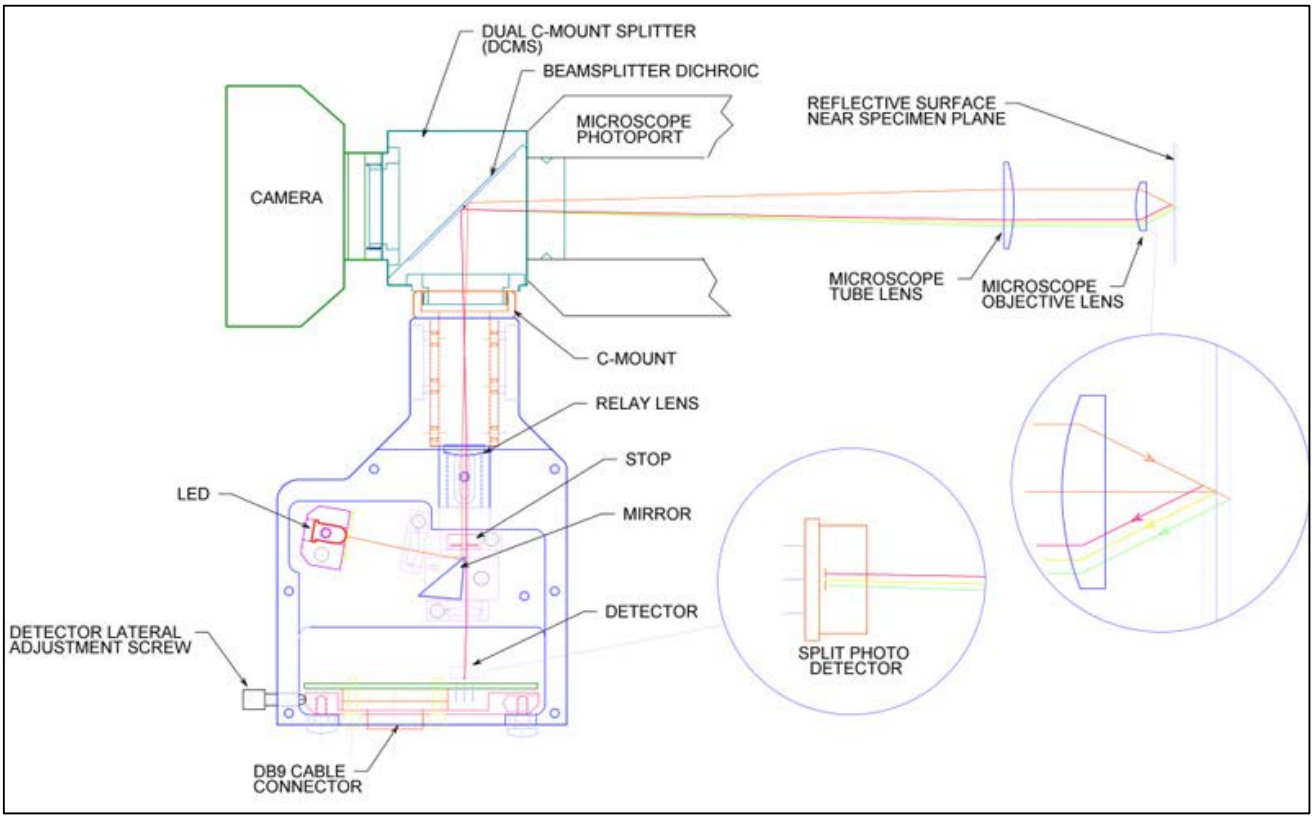


D. Instrumentation

(B. Huang, et al., Science 319, 810-813 (2008)), the right most plot is for our setup



STORM microscope



ASI CRISP (Continuous Reflection Interface Sampling and Positioning System)

II. Experimental Procedure

A. Experimental Procedure: STORM imaging of surface immobilized MTs (Week 1)

Note: At this point, the microscope should have been on for at least 30 min to minimize the drift. Use 100x objective (oil, NA = 1.49) and 1.5x additional magnification setting. For 2D STORM, make sure that the cylindrical lens is NOT inserted.

1) Sample: Obtain Cy3B labeled MT chemically fixed in an aminosilanized channel with beads.

2) Reagents to obtain

a) Sodium borohydride (NaBH4, powder)
b) MilliQ H_2O
c) 10x PBS
d) Glucose (20%)
e) 10x MEA (1 M)
f) 100x gloxy

3) Exercise 1: Chemical reduction of MTs

a) Put a drop of oil on the objective and place the sample chamber on the slide holder. Slowly bring the objective close to the coverslip and focus it.
b) Acquire 3-5 images of MTs from different ROIs using low laser intensity (~10%).
c) Prepare 1 mg/ml $NABH_4$ stock solution in PBS.
d) Dilute $NABH_4$ to 0.1 mg/ml using PBS.
e) Add diluted NaBH4 solution into the channel and start the timer.
f) Acquire 3-5 images of MTs from different ROIs every 5 min for 20 min to observe the fluorescence intensity change of MTs over time.
g) Thoroughly wash with 1x PBS.

4) Imaging buffer preparation.

a) For 50 µl imaging buffer, mix 5 µL 10x PBS + 5 µL 10x MEA + 12.5 µL 20% glucose + 27 µL H2O + 0.5 µL gloxy (Add gloxy last)
b) Add the imaging buffer into the channel and check with microscope whether the fluorophores are bleached less.

5) Exercise 2: Parameter Optimization

In a real experiment, various imaging parameters (*e.g.*, excitation laser intensity, exposure time, reducing agent concentration, labeling density, activation laser power) are optimized to achieve the best STORM image. Here, test different activation laser power (405 nm) to determine an optimal condition. Acquire short movies files (1000 frames, 50 ms exposure each) at a same imaging field with 3-4 different activation laser power. Repeat this at total of 3 different regions of interest (ROIs).

6) Exercise 3: STORM imaging

a) Exchange the buffer with a fresh imaging buffer.
b) Acquire 4 x 4,000 frames of STORM images (total of 16,000 frames) with a drift correction movie. Adjust the activation laser power between each movie accordingly.

B. Experimental Procedure: 3D PALM/SPT imaging (Week 2)

Note: At this point, the microscope should have been on for at least 30 min to minimize the drift. Use 100x objective (oil, NA = 1.49) and 1.0x magnification setting. For 3D STORM, make sure to insert the cylindrical lens.

1) **Sample:** Fixed and embedded neuron sample with sQD labeled on AMPAR and mGeos-Homer1

2) **SPT measurement**
   a) Using the 488 nm excitation, find a cell that is expressing mGeos-Homer1.
   b) Switch to 561 nm excitation and visually check that most of sQD signals are in a close proximity to Homer1 clusters.
   c) Acquire sQD movie (1000 frames, 50 ms exposure time, 561 nm excitation) with a drift correction movie (IR camera). (One movie for each student.)

3) **PALM measurement**
   As soon as the SPT measurement is completed, acquire PALM movie (1000 frames, 50 ms exposure time, 50 cycles of 20 frames of 561 nm excitation plus 2 frames of 405 nm excitation) with a drift correction movie (IR camera). (One movie for each student.)

C. **Data Analysis**

See additional handout for data analysis.

D. Reference and Online Resources

1) PALM/STORM principles
   a) B. Huang, M. Bates, X. Zhuang, "Super-resolution Fluorescence Microscopy", Annual Review Biochemistry 78, 993-1016 (2009)
   b) B. Huang, W. Wang, M. Bates, X. Zhuang, "Three-dimensional Super-resolution Imaging by Stochastic Optical Reconstruction Microscopy", Science 319, 810-813 (2008)
   c) 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
   d) Proteins at Nanometer Resolution”, Science 313, 1642-1645 (2006)
   e) S. T. Hess, T. P. K. Girirajan, M. D. Mason, “Ultra-High Resolution Imaging by Fluorescence Photoactivation Localization Microscopy” Biophysical Journal 91, 4258 (2006)
   f) J. C Vaughan, S. Jia, X. Zhuang, “Ultrabright photoactivatable fluorophores created by reductive caging” Nature Methods 9, 1181–1184 (2012)
2) STORM/PALM analysis software
   a) quickPALM: http://www.nature.com/nmeth/journal/v7/n5/full/nmeth0510-339.html
   b) a-livePALM: http://pubs.acs.org/doi/abs/10.1021/nn4009388
3) Removing free dyes after labeling antibodies
   a) http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4006051B.pdf
4) Fluorescence SpectraViewer (excitation and emission spectra of different fluorophores)
   a) http://www.lifetechnologies.com/us/en/home/life-science/cell- analysis/labeling-chemistry/fluorescence-spectraviewer.html
5) Z-axis drift correction
   a) http://www.asiimaging.com/index.php/products/focus-tracking-stabilization/crisp-autofocus-system/
6) Also see reference sites from Zeiss, Nikon and Olympus.

III. **Lab Report Questions** (50 pts)

1.) How do you define a resolution in STORM imaging? If you are given a STORM setup, how would you measure the resolution? Answer this with the concept of FWHM (Full-width-half-maximum). (4 pts)

2.) Usually one pixel on our EMCCD is corresponding to 100 nm, but STORM imaging claims 20 nm lateral resolution. How can the resolution be better than the size of one pixel sensing? (4 pts)

3.) Within the data acquisition, we ensure that blinking spots don't overlap with each other, by ensuring that spots appear sufficiently sparse to each other. What is your estimate of the minimal distance between two spots (appearing in a single frame) to be distinguishable by the software? (Assume the excitation laser is 647 nm, EMCCD pixel one pixel is corresponding to 100 nm, each peak will be fitted with 19 X 19 ($pixels^2$) box) (4 pts)

4.) Exercise 1: In exercise 1, Cy3B-labeled MTs were incubated in $NaBH_4$ solution and the intensity change was observed over time. Show the progression with images and plot the average intensity over the course of $NaBH_4$ incubation. Briefly explain the chemical mechanism behind your observation. (5 pts)
Reference: *"J. Vaughan, S. Jia, X. Zhuang, "Ultra-bright Photoactivatable Fluorophores Created by Reductive Caging", Nature Methods 9, 1181-1184 (2012)"*

5.) In Exercise 2, the activation laser (405 nm) power was systematically changed to observe its effect. Plot number of molecules/imaging area, number of photons and localization precision as a function of activation laser power. How did the laser power influence each of these parameters? Explain why each of the parameter changed. (5 pts)

6.) In Exercise 3, STORM imaging of MT was done. (10 pts)
   a. Analyze the data and plot drift uncorrected and corrected STORM images. Explain how the drift correction was done and why this is necessary. What is the limitation of this method?
   b. Sample 5-10 cross sections of MT from the conventional and super-resolution images to determine the average cross sectional diameters. Plot a sample cross section from each type of images. Report the threshold values used to reconstruct the image. Do not forget to add the scale bar.
   c. Resolution improvement. What is the localization precision from your fitting? Compared to the theoretical resolution limit, how many fold improvement did you achieve? How many fold improvement of the resolution did you achieve from your cross section comparison between the conventional and the super-resolution image?

7.) Discuss how each of the following parameters/chemicals influences the photophysical properties of the dye (*e.g.*, on time, off time, number of switching cycles and photons) and ultimately the final STORM image. (10 pts*)*

a. Reducing agent concentration (*e.g.*, BME, MEA)
b. Oxygen scavenging system
c. Excitation laser power (in MT experiment, 561 nm)
d. Exposure time

References:

- Heilemann, M., et al., "Super-Resolution Imaging with Small Organic Fluorophores", *Angew. Chem. Int. Ed.*, 2009, 48, 6903-6908. doi:10.1002/anie.200902073
- "Subdiffraction-Resolution Fluorescence Imaging with Conventional Fluorescent Probes"
- "Superresolution Microscopy on the Basis of Engineered Dark States"
  "A Reducing and Oxidizing System Minimizes Photobleaching andBlinking of Fluorescent Dyes"

8.) In one of the experiments we directed labeled the microtubules with the organic dye, what do you expect in terms of difference when measuring the cross section of the microtubule compared to antibody labeling? (4 pts)

9.) In the neuron experiment, do AMPA receptors appear to be colocalized with the PSD-95 molecules? Could this experiment be done in live cells as well? What are the advantages/disadvantages of PALM over STORM. (4 pts)