BMB/Bi/Ch 173 – Winter 2018

Homework Set 9.2 – Assigned 3-6-18, due 3-14-18 by 10:30 a.m (can turn in to my mailbox, which is directly adjacent to the north entrance of the Braun building)

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Office hours – Friday Mar 9 1:00pm - 3:00pm (in SFL 229) and Monday Mar 12 3:30pm - 5:00pm (in SFL 229), or by appointment

1. CryoPALM (50 points)

In 2014, the Jensen lab, led by Dr. Yi-Wei Chang, demonstrated the use of correlated cryogenic photoactivated localization microscopy and cryo-electron tomography to discover a new conformation of the type VI secretion system in *Myxococcus xanthus*.

Here's a link to the paper for your reference: https://www.nature.com/articles/nmeth.2961 You can do this problem WITHOUT reading the paper. You may want to reference this page http://zeiss-campus.magnet.fsu.edu/articles/superresolution/palm/introduction.html to supplement your understanding of PALM.

1.a. (4 points) Initial PALM experiments were done on fixed cells. What parameter made live cell PALM more difficult? Is this an issue in cryoPALM?

Temporal resolution. Taking PALM data usually requires minutes, and most fluorophores attached to things in living cells don't stay still that for that long due to diffusion. CryoPALM is frozen, so diffusion is not a problem.

1.b. (6 points) Name three factors that determine the localization precision you can achieve with a given fluorophore and a given microscope setup.

- Number of photons released before photobleaching
- Ability of objective lens to collect photons
- Density of fluorophore labeling

1.c. (2 points) Let's think about the cryoPALM setup Dr. Chang used in the paper. Why did Dr. Chang select long-working distance air objectives for the cryoPALM experiments? Oil immersion objective lenses have higher Numerical Apertures than air objectives.

The sample is held at liquid nitrogen temperatures, which is incompatible with oil because it will cause the oil to solidify. An air objective is compatible with low temperatures.

1.d. (5 points) In the paper, Dr. Chang used a 60X objective, but mentioned in the discussion that using a 100X objective would improve his localization precision. Calculate the theoretical point spread function (resolution) for a 60X long-working distance air objective (NA=0.7) and a 100X long-working distance air objective (NA=0.8) for 507 nm wavelength light.

Image Resolution, D=0.61∗λNA

Image Resolution, D=0.61∗507 nm0.7=442 nm

Image Resolution, D=0.61∗507 nm0.8=387 nm

1.e. (5 points) On average, extended type VI secretion system (T6SS) tubes are about 600 nm by 12 nm, while contracted tubes are about 275 nm by 15 nm. In this cell, there are also other tubes with similar diameters that aren't T6SS tubes as close as 200 nm from the T6SS tubes. Comment on the suitability of the 60X and 100X objectives for precisely localizing different types of tubes in a cell.

Contracted tubes are shorter than the PSF of both objectives, so it would be challenging to precisely locate them using this method. If tubes were 200 nm apart, neither objective could distinguish them.

1.f. (8 points) To improve the resolution of his fluorescence cryomicroscopy experiments, Dr. Chang used photoactivatable GFP to do cryoPALM. To calculate the localization precision of his cryoPALM experiments, Dr. Chang used the following equation:

$$(\sigma_{x,y})^2 = \left(\frac{s^2 + \left(\frac{a^2}{12}\right)}{N}\right) + \left(\frac{8\pi s^4 b^2}{a^2 N^2}\right)$$

N=number of photons per blink

a=pixel size (178 nm)

b=background noise (1)

s=standard deviation of the PSF (assume 2 pixels, so use 2*PSF from the previous answer)

Calculate the localization precision for N=200, 1000 for the 60X objective and the 100X objective discussed in the previous questions.

	60X objective	100X objective
N=200	126.6	100.4
N=1000	35.62	29.7

1.g. (5 points) A recent fluorescence cryomicroscopy paper investigated the use of small organic dyes in cryoPALM experiments (https://www.osapublishing.org/oe/fulltext.cfm?uri=oe-23-3-3770&id=311815). They report that small molecule dye Atto647N has an average yield of 3.8×10^4 photons at 294 K and 3.5×10^6 photons at 89 K with a laser setting of 300 W/cm^2. Dr.

Chang observed an average of 206 photons per PA-GFP blinking event with a laser setting of 0.2-0.4 mW/cm^2. Let's say a small molecule dye called Yeah507 exists that is as bright as Atto647N and has an emission wavelength of 507 nm. Calculate the localization precision Yeah507 for 60X and 100X as in the previous questions, assuming N=3.5x10^6 photons.

For the 60x objective:

((8*π*884^4)/(178^2*3500000^2)+((884^2+(178^2)/12)/(3500000)))^(1/2) = 0.47

For the 100x objective:

((8*π*773^4)/(178^2*3500000^2)+((773^2+(178^2)/12)/(3500000)))^(1/2)= 0.41

1.h. (2.5 points) Dr. Chang had to add a cryoprotectant to his *M. xanthus* cells before freezing to do cryoPALM. Without the cryoprotectant, he observed crystalline ice, which ruined the potential of the cryotomograms he wanted to take after doing cryoPALM. What part of the cryoPALM protocol caused the formation of crystalline ice?

Laser power heated up the ice and caused a phase transition from vitreous ice to crystalline ice

1.i. (2.5 points) Typical room temperature PALM setups use a laser power of 10-80 mW/cm^2 to photoactivate PA-GFP (http://www.pnas.org/content/102/27/9511). Dr. Chang used a laser power of 0.2 to 0.4 mW/cm^2 to photoactivate PA-GFP. Why did Dr. Chang select a relatively low power to do cryoPALM?

Higher laser intensities cause vitreous ice to transition to crystalline ice, which would be bad for the subsequent tomography experiments.

Higher laser intensities could also induce radiation damage that would be bad for the subsequent tomography experiments.

From the paper: "These ranges of laser intensities were chosen to avoid ice crystallization on the frozen sample but still strong enough to excite bright signals and bleach before the stage drifted notably."

1.j. (5 points) If you repeated Dr. Chang's experiment with Yeah507 excited by 300 W/cm^2 laser settings, your cryoCLEM experiment would fail. Why would it fail? What parameter would you have to adjust for a successful cryoCLEM experiment and what tradeoff would there be in terms of resolution. *Hint: I'm looking for broad statements, not a derivation*

This laser power is too high, so it would cause crystalline ice to form.You would have to reduce the laser intensity, which would probably result in fewer photons unless you elongated the exposure time by a lot.

1.k. (5 points) Stimulated emission depletion (STED) microscopy experiments have achieved higher resolution than many PALM experiments. Should Dr. Jensen's lab focus on cryoSTED instead of cryoPALM? Briefly explain why or why not.

STED bathes the sample in radiation. The laser power would be too high and would cause crystalline ice to form.

2. Future methods in structural biology (25 points)

The following two reviews provide a nice overview of the concepts, advantages, and limitations of micro-electron diffraction (MicroED) and serial femtosecond crystallography (SFX) with x-ray free electron lasers:

MicroED: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5656569/

XFELs: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4839532/

Use these reviews as a reference to complete the following table:

	X-ray crystallography	SFX	MicroED
Phasing methods	Anomalous dispersion Isomorphous replacement Molecular replacement Direct Methods	Anomalous dispersion (difficult) Isomorphous replacement Molecular replacement Direct methods	Isomorphous replacement Molecular replacement Direct methods Direct imaging (hypothetical)
Sample type			
Specific advantages			
Technical limitations			

	X-ray crystallography	SFX	MicroED
Phasing methods	Anomalous dispersion Isomorphous replacement Molecular replacement Direct Methods	Anomalous dispersion (difficult) Isomorphous replacement Molecular replacement Direct methods	Isomorphous replacement Molecular replacement Direct methods Direct imaging (hypothetical)
Sample type	Relatively large X-ray crystal (no smaller than 10 µm in any dimension)	Small crystals (low µm to nm dimensions) frozen on an EM grid	Small crystals (low µm to nm dimensions) in a liquid stream

Specific advantages		Smaller crystals, EMs could end up being much more accessible	Smaller crystals, allows detection of dynamic changes in the protein
Technical limitations	Requires synchrotron and large crystals	May be more difficult to perform de novo phasing	Requires access to very expensive high-energy free electron laser, difficult to coordinate the beam and the liquid stream of crystals