PALM/STORM, BALM, STED
• Last class
  • 2-photon
  • Intro to PALM/STORM
  • Cyanine dyes/DRONPA

• This class
  • Finish localization super-res
  • BALM
  • STED
Localization microscopy

\[
\langle (\Delta x)^2 \rangle = \frac{s^2 + a^2/12}{N} + \frac{4\sqrt{\pi}s^3b^2}{aN^2}
\]

Figure 1
Practicalities in STORM

• To resolve small features, labeling has to be VERY dense

• The reconstructed image will represent a pixelated view of the actual sample

• To resolve features, must label AT LEAST at the Nyquist frequency

• With a resolution of 30 nm, must have probes every 15 nm

• Fluorophore size becomes an issue – antibodies are big

• If you’re using antibodies, have to ensure high efficiency and high coverage of labeling
Contrast ratio and spontaneous activation

- In active experiment, #Dark Cy5 >> #Active Cy5
- Noise can creep through spontaneous activation, and dark state fluorescence
- Contrast ratio measures fluorescence in bright state/dark state
- The red laser can drive a little bit of Cy5 photoactivation
- Between 1 and 5 in 10,000 fluorophores turn on spontaneously
- If sample is very densely packed, can override ability

\[
\text{Contrast} = \frac{F_{\text{br state}}}{F_{\text{dk state}}} \sim 1000
\]

1000 dark fluorophores = 1 bright
Can erode ability to detect and fit peaks

\[
\% \text{Active} = \frac{k_{\text{blink}}}{k_{\text{blink}} + k_{\text{off}}} \sim .0005
\]

\(k_{\text{off}}\) = rate of switching off with red light
\(k_{\text{blink}}\) = rate of reactivation by red light
STORM image display

• Concatenate all fitted points from collected images and display them on single picture

• Often use a Gaussian of calculated uncertainty width

• A 40µm x 40µm image displayed at 1 nm resolution would take ~4.5Gb of memory
STORM Equipment

- High NA objective (probably TIRF)
- Multicolor laser excitation (at least 2)
- High sensitivity camera
- Drift correction (if your sample moves by 20 nm, that’s now a lot in your image)
- Images take a long time to collect
- Autofocus (Quadrant photodiode with IR laser)
Multicolor STORM

• Two potential options
  • Single donor, multicolor acceptors
  • Many donors, single acceptor
3D STORM

- Use an cylindrical lens to purposely introduce astigmatism
- PSF changes as a function of depth
- Keep measurements with high ellipticity, and then assign a z-depth according to a/b ratio

Mitochondrial network with color encoding depth
STORM applications

- Cytoskeletal arrangement too small to be seen by widefield
- Axon specific rings, dendrites don’t show this pattern
- One of my favorite examples of new biology using super-resolution
STORM applications – telomere FISH
Live cell STORM

• Possible, but slow and hard
• Have to keep a thiol reagent (toxic) and blast cells with light
• Labels can obviously move, now, makes it harder to localize
• Time resolution on the order of seconds

Two color, 3D, live cell STORM
Clathrin and transferrin
PALM

• Photoswitchable proteins instead of Cy3-Cy5 pairs
• Dronpa (photoswitchable) and mEos (photoconvertible) are two most popular
• FPs are bigger and less bright
• Gain all the advantages of genetic labeling
Two color PALM

- Can use Dronpa and tdEos
- Dronpa significantly overlaps tdEOS pre-conversion
- First photoactivate (405 nm) and localize tdEos (561 nm)
- Photobleach all tdEos
- Then use 488 nm to localize and bleach Dronpa
- There also exists a photoswitchable mCherry
PALM vs STORM

- Same exact fitting protocol
- PALM uses FPs – easier to genetically attach, worse as fluorophores
- STORM uses photoswitching dyes, requires toxic thiol buffers
- Easier to do 3 colors in STORM
- Really, not much difference

Couldn’t find an image to represent differences, went with this cool picture
BALM - blinking assisted localization microscopy

• Rather than photoswitch, rely on endogenous blinking to isolate molecules
• Let laser and camera run continuously
• Take difference images between frames
• If dye blinks on, it will appear like a negative spot
• If dye blinks off, it will appear as positive spot
• Average together those frames to get single molecule spot
BALM

- Due to higher background, localization is not as good as PALM/STORM
- Still significant improvement over diffraction limited systems
- Easy to do multi-color BALM
Increasing fluorophore blinking

- Engineering fluorophores to undergo blinking with appropriate kinetics
  - Preparation of the blinking state
  - Recoding

  dSTORM (ex. Alexa647)
  - Thiols (MEA, ME, GSH)
  - Higher laser intensity

  Spontaneously blinking fluorophore
  - No additives
  - Lower laser intensity

- Too much blinking - > not enough signal for each fluorophore, too many on at one time
- Too little blinking -> Takes forever to build image

![Fluorophore Structures](image)

![Physiological pH (pH 7.4)](image)
BALM applications
STED – Stimulated Emission Depletion

• Another way to generate superresolution images
• 2014 Nobel prize – Stefan Hell
STED theory

• Consider a laser

• Electron is living in an excited state, doesn’t matter how it got there

• If a photon arrives with the energy equivalent to band gap, the electron de-excites, and 2 photons appear

$\Delta E \sim 2.5 \text{ eV}$
STED theory

- By sending in light at a given wavelength, we can control the color of the stimulated emission.
- Possible to design filters to let normal fluorescent light through, while excluding the stimulated photons.
STED theory

- The smallest spot we can excite is a diffraction limited spot (100s of nanometers)
- If we can selectively deplete some chunk of that region, we can limit the region of fluorophores that emit
- STED ideal is to make a donut around the excitation spot, limiting emission from a small region in the center. If you see fluorescent photons, you know where they came from to a much higher precision
STED imaging

- Develop image exactly like confocal
- Scan both excitation and STED laser around sample
- But you know below resolution limit where the fluorescence originated from
- Rebuild super-resolution image
- No need to post process images, unlike STORM/PALM
STED resolution

- Unlike STORM/PALM, resolution limit is dependent on intensity of depletion laser.
- Intensity must be above $I_s$, the threshold at which 50% fluorophores undergo stimulated emission (i.e. depleted fluorescence).
- STED pulse must be delayed in time to allow vibrational relaxing. Want to catch electrons in ground state of excited state, but before they decay.

\[ d = \frac{\lambda}{2n \sin \alpha \sqrt{1 + \frac{I}{I_s}}} \]

$d = \text{diffraction limit}$
$I_s = \text{Saturation intensity}$

$I_s$ fluorophore and wavelength dependent.
STED PSF is intensity and wavelength dependent

- More power = higher resolution
- Higher photobleaching and toxicity as well
- Diminishing returns in resolution, but increasing returns in cell death with increasing lasers

\[
d = \frac{\lambda}{2n \sin \alpha \sqrt{1 + \frac{I}{I_s}}}
\]

Full list at: http://nanobiophotonics.mpiibpc.mpg.de/old/dyes/
STED Practicalities

- STED depletion pulse is susceptible to changes in index of refraction
- Must use oil that exactly matches glass (type F)
- Must use coverslips that are 170 µm (+/- 3%). Normal coverslips can vary by as much as 20%. Have to order special
- Ultrafast pulsed lasers can be temporally separated to optimize depletion, but then they take longer to scan and cost more monies.
On to Matlab...