MIC Microscopy Workshop 2022
Mixed Zoom / In-person Seminars

- **In Person**

Please wear a mask, your neighbor may have a medical condition!
35 sqft = 6 ft away from anybody else
Please save questions for Q. breaks
Questions from in-person audience repeated *by* speaker for Zoom

- **Zoom**

Mute your microphone
Do not talk in your microphone, in-person audience cannot hear you
Questions in chat will be relayed *to* the speaker

Presentation PDFs from MIC staff available on demand
Session is being recorded

Microscopy Workshop, Spring 2022

Vincent Schram, Ph.D.

- **LIGHT MICROSCOPY 1: TRANSMISSION AND FLUORESCENCE**

  Monday May 9, B35 / GG607, 11 am - 1:30 pm

- **LIGHT MICROSCOPY 2: CONFOCAL, 2P AND LIGHT SHEET**

  Tuesday May 10, B35 / GG607, 11 am - 1:30 pm

- **LIGHT MICROSCOPY 3: SUPER-RESOLUTION IMAGING**

  Wednesday May 11, B35 / GG607, 11 am - 1:30 pm

- **IMAGE ANALYSIS WORKSHOP: IMAGEJ**

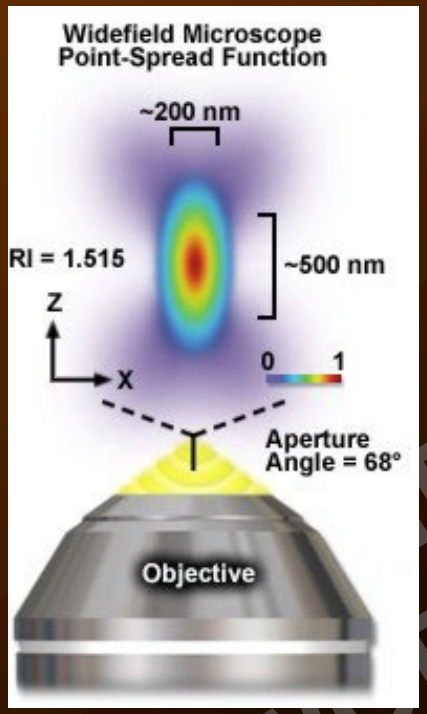
  Thursday May 12, B35 / GG607, 9:30 am - 12:30 pm / 1 pm - 4 pm

- **CONFOCAL MICROSCOPY HANDS-ON**

  Friday May 20, B35 / GD922, 9:30 am - 12:30 pm / 1 pm - 4 pm

SUPER-RESOLUTION IMAGING

- Sub-resolution particle imaged as Point Spread Function (PSF)



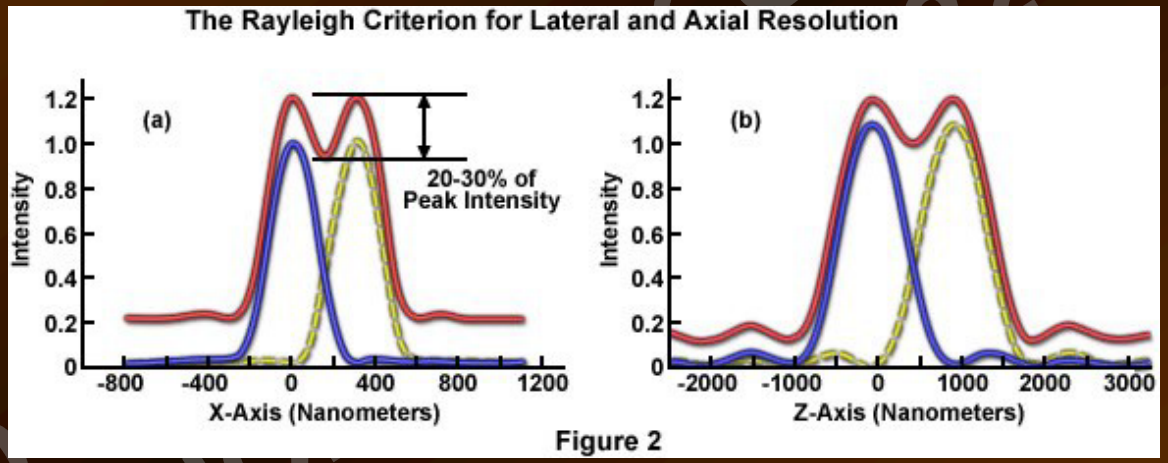


Figure 2

- Lateral resolution = 1.22 . λ / (2 . NA) or 0.2 μm
- Axial resolution = 2 . n . λ / $(NA)^2$ or 0.7 μm
- Gap between light and electron microscopy (10 – 200 nm)

→ ***TRICKS*** to increase resolution

BEAT THE RESOLUTION LIMIT...?

- Near-field imaging at glass / water interface
  → TOTAL INTERNAL REFLECTION FLUORESCENCE (TIRF)
- Undo optical distortions caused by the microscope:
  → DECONVOLUTION
- Fit PSF of individual molecules
  → LOCALIZATION MICROSCOPY: PALM, STORM...
- Spatial modulation of excitation beam
  → STRUCTURED ILLUMINATION, LATTICE SIM
- De-excite molecules at the edge of focal volume
  → STIMULATED EMISSION DEPLETION (STED)
- Close confocal pinhole to 0.2 AU and compensate light losses
  → Zeiss AIRYScan, Nikon SoRa

TIRF

Principle

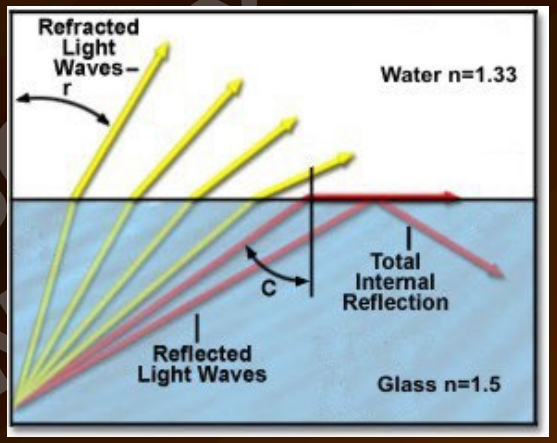
- Glass (n=1.5) / water (n=1.33) interface:

→ n1 . sin α1 = n2 . sin α2

Below 61° → Refraction

61° → Critical angle

Above 61° → Reflection

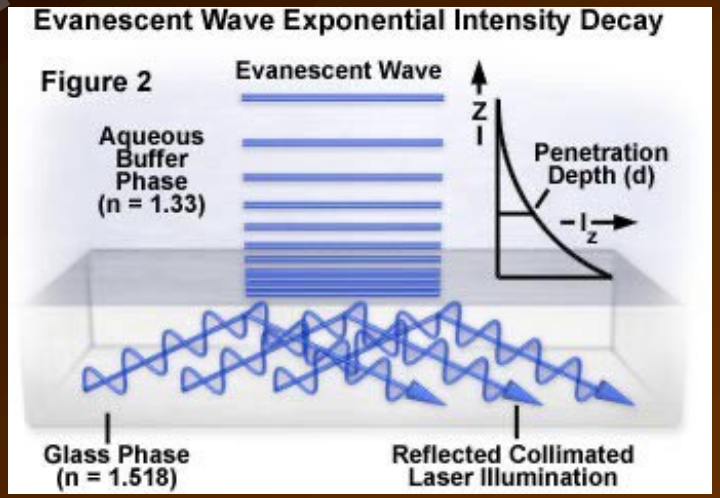


- Critical angle: Evanescent wave

Thin layer of light, < 200 nm

Exponential decay from interface

Thickness decays over 1-3 deg.



→ *Very localized excitation at glass/water interface*

→ *No signal from bulk solution*

→ *Axial resolution only, not xy*

→ ***Highly selective for membrane markers, no background***

TIRF - Objective

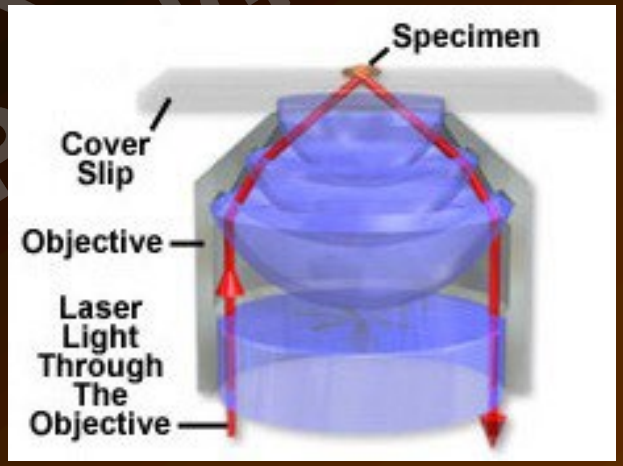
- All commercial TIRF microscopes are objective-based

- Minimum of 61˚ for glass / water TIRF:
  Numerical aperture = n . (sin α)

→ NA at least 1.33 (TIRF = oil only)

→ Oil objectives w/ high NA

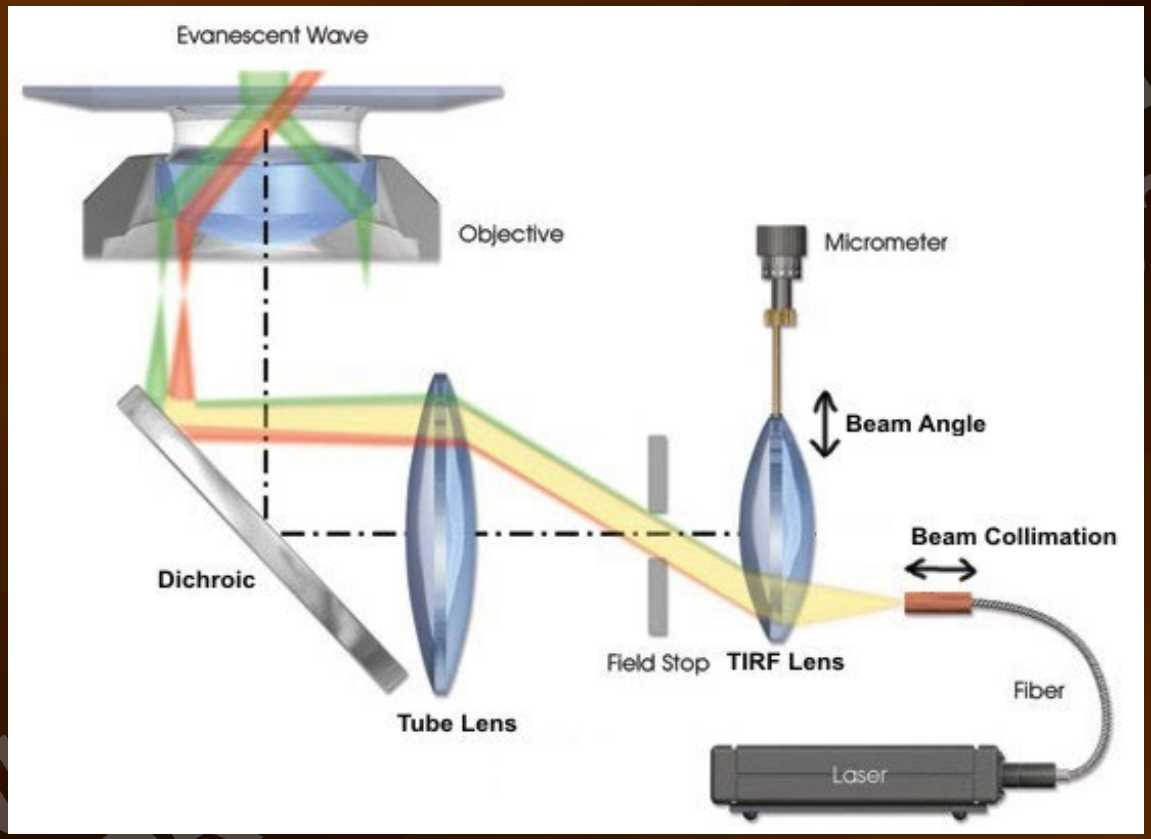
Olympus: APON 60xO 1.49 NA
Zeiss: Alpha Plan-Apo 63x 1.46NA
Nikon: CFI apo 100x 1.49 NA
Leica: HCX PL APO 100x/1.47



- Edge of FOV = poor chromatic correction
→ *Adjust TIRF angle and focus for each color*

- Edge of FOV → *precise collimation and alignment*

TIRF Launch



- Laser refocuses at objective back-focal plane → Full-field illumination
- Beam collimation → Distance between fiber tip and TIRF lens
- Beam angle → Fiber / TIRF lens coaxiality

Experimental Considerations

- Require high NA oil lens, high-quality #1.5 coverslip, hardware autofocus
- Glass / water interface → Strongly adherent cells, careful with coating!
- 200 nm from glass surface → membrane-bound events:

  Endo/exo-cytosis, membrane docking, aggregation state, lateral mobility
- To find TIRF angle:

  Increase angle until image disappears

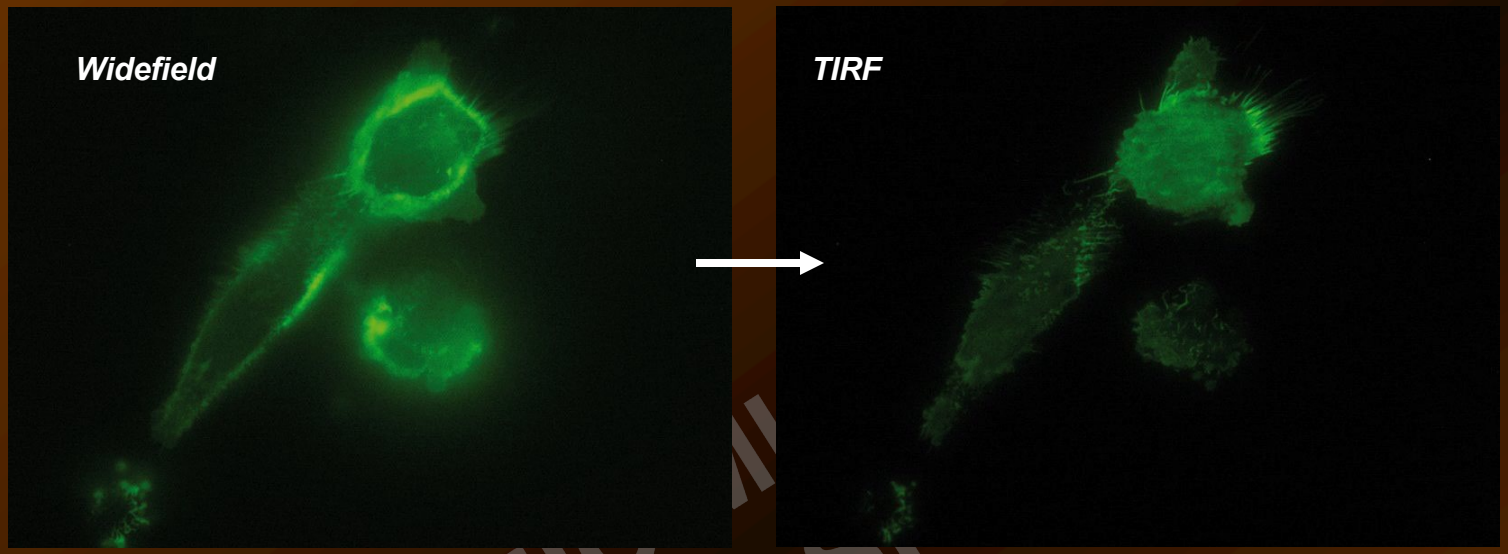
  Back up a little

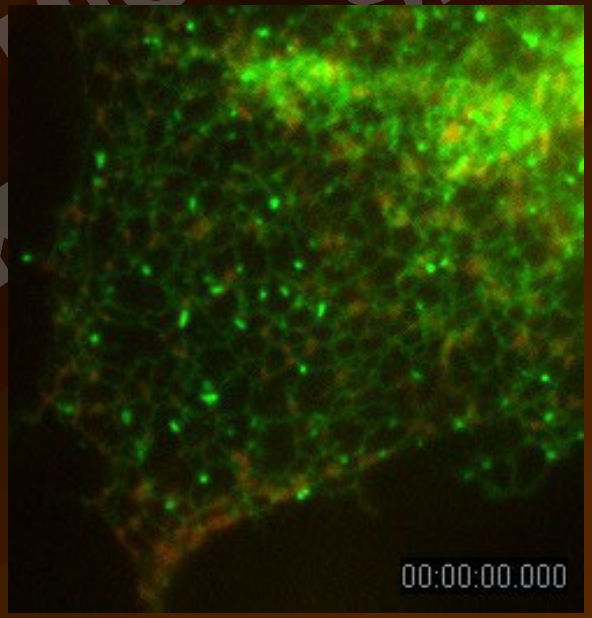
  Focus up and down: image should disappear, or put more angle

  Short range of TIRF positions, higher angle = thinner layer
- Adjust focus & TIRF angle on every location…!
- Better to adjust TIRF angle / focus for every color

→ ***Suitable for single-molecule imaging***

TIRF Images





Green: PIS organelle & ER tubules
Red: STIM1 ER / mb junctions
Courtesy of T. Balla, NICHD

TIRF TAKE-HOME MESSAGE

- **Pros:**

Simple, cheap microscope addition
No dye restrictions
Extremely low photo-toxicity
Reference method for membrane attachment

- **Cons**

Require strong cell adhesion, coating may be a problem
Require careful microscope alignment
Color and location change require adjustments
Prone to interference stripes in image (Ilas rotating TIRF)

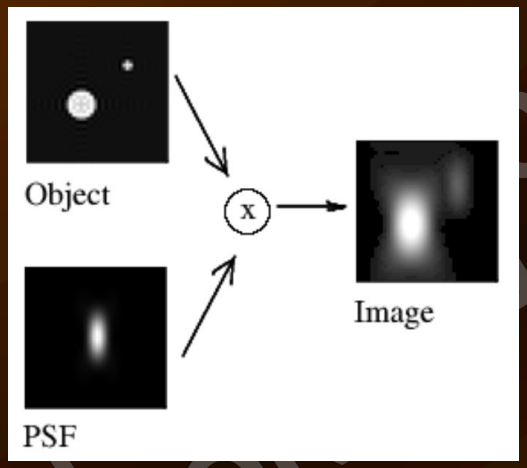
→ ***Reference method to document membrane attachment***

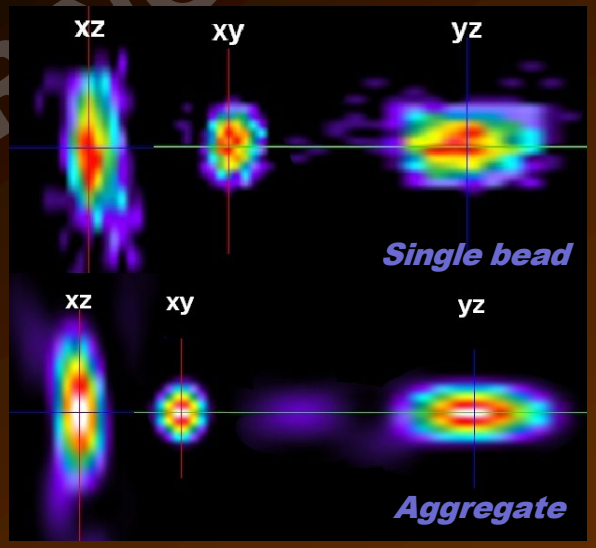
QUESTIONS

DECONVOLUTION

- **Acquire point scanner images at very tight spacing:**
  40 nm pixel size, 200 nm z increment (requires precise piezo z stage)

- **Feed image stack to deconvolution software (Hyugens / SVI)**
  → Iterative process that reverse optical distortion caused by the optics





- **Best results when measuring actual PSF from small beads**

  → *25% resolution improvement, higher signal, very slow*

LOCALIZATION MICROSCOPY

Fundamentals

Localization Imaging

Localization Principle

Sub-pixel localization, Localization precision, Localization sequence, Marker density

Localization Microscopy

Fluorescent proteins, PALM microscope, Images, Practical considerations, 3D localization, STORM

LOCALIZATION PRINCIPLE

Single molecule location can be established with 10-20 nm accuracy provided no other molecule present within 0.2 μm

- **Very low labeling density: single molecule imaging**
  - **Separate marker population in time:**

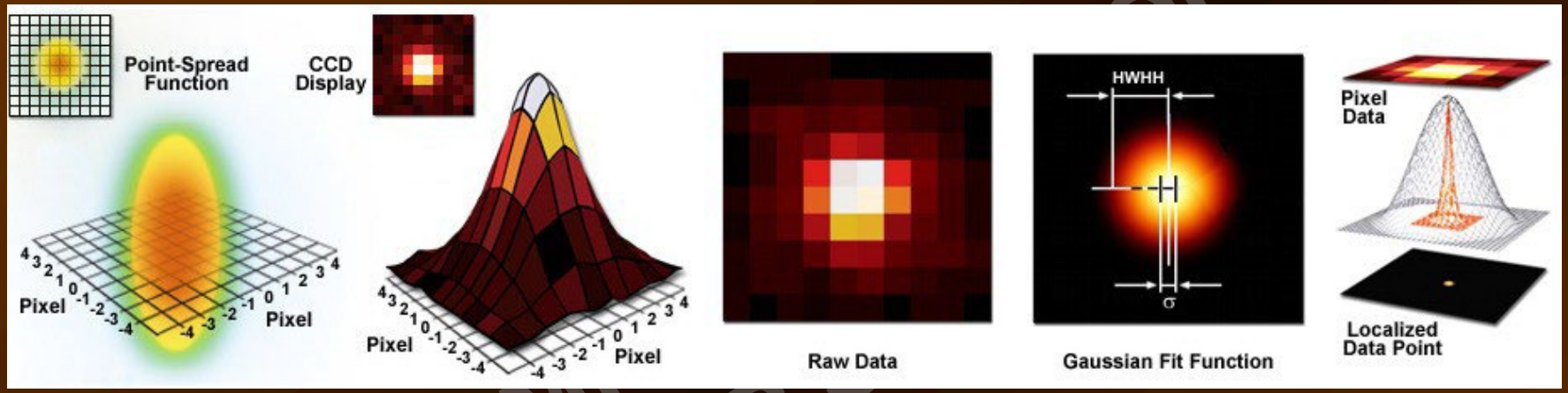
1) Make only a few scattered molecules visible
2) Record an image
3) Establish their position with nm resolution
4) Bleach molecules
5) Start again until all molecules imaged

Add each frame = nm-resolved image

→ 10x resolution improvement

→ Acquisition times in minutes

Sub-pixel Localization



- PSF of single molecule projects as an airy disk on CCD camera
- ***Intensity array fitted with 2D Gaussian***
- Top of fitted Gaussian = molecule location, ***sub-pixel accuracy***

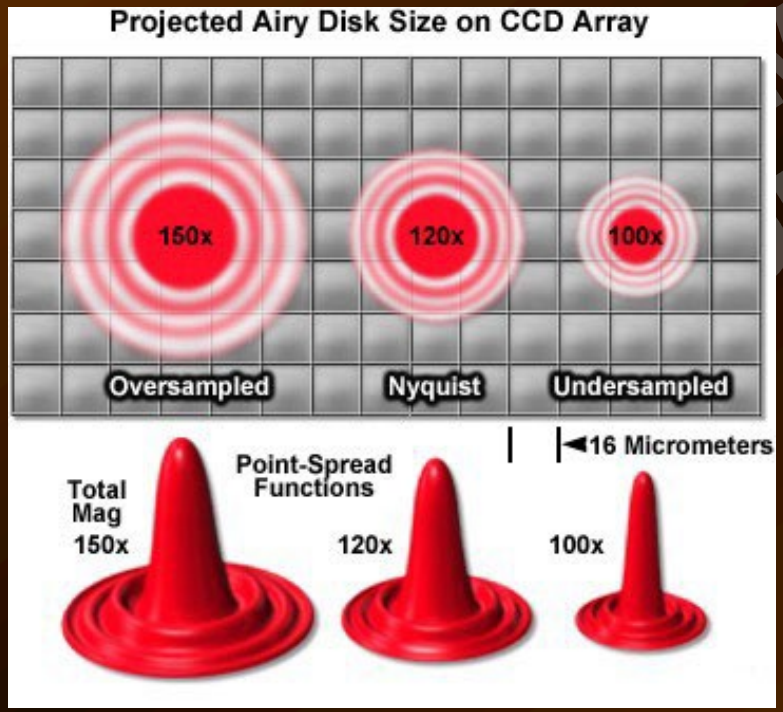
Localization Precision

- **Low noise: localization precision inversely proportional to photon count**

$$\sigma = \sqrt{\left(\frac{s^2}{N}\right) + \left(\frac{a^2/12}{N}\right) + \left(\frac{8\pi s^4 b^2}{a^2 N^2}\right)}$$

Photon noise — Detector pixel size — Background noise

σ = Localization precision (um)
S = Half-width at half-width (um)
N = Total number of photons
a = Pixel size on CCD (um2)
b = Standard deviation of background (photons)



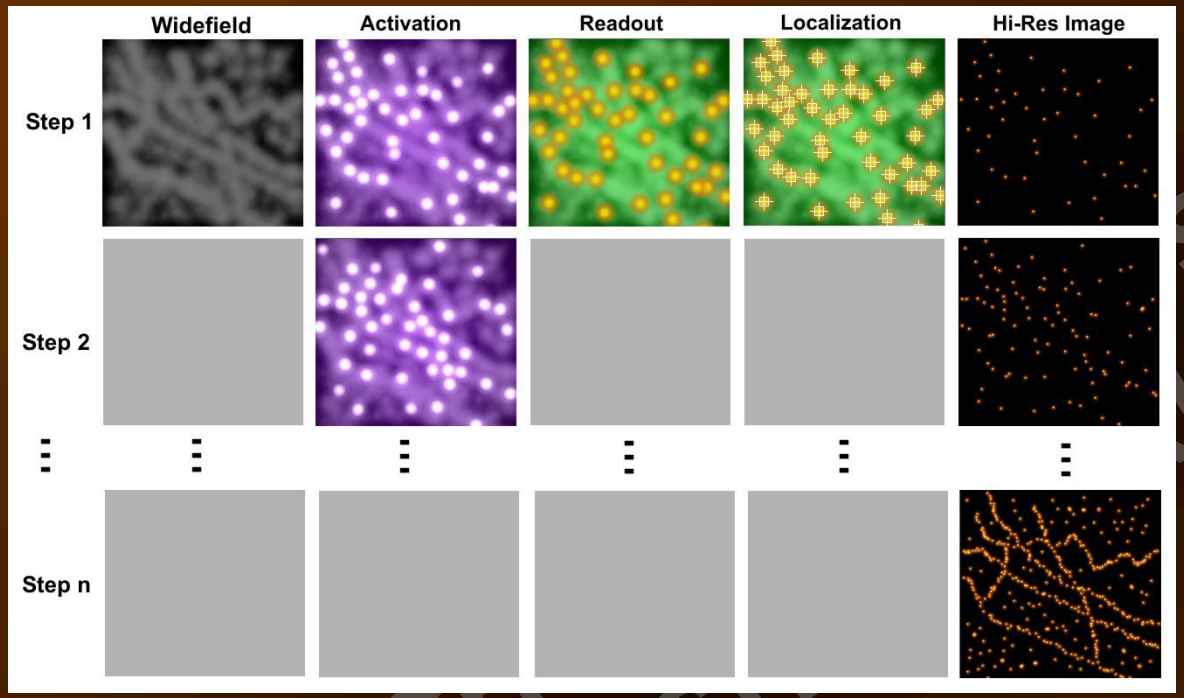
- **Magnification:**

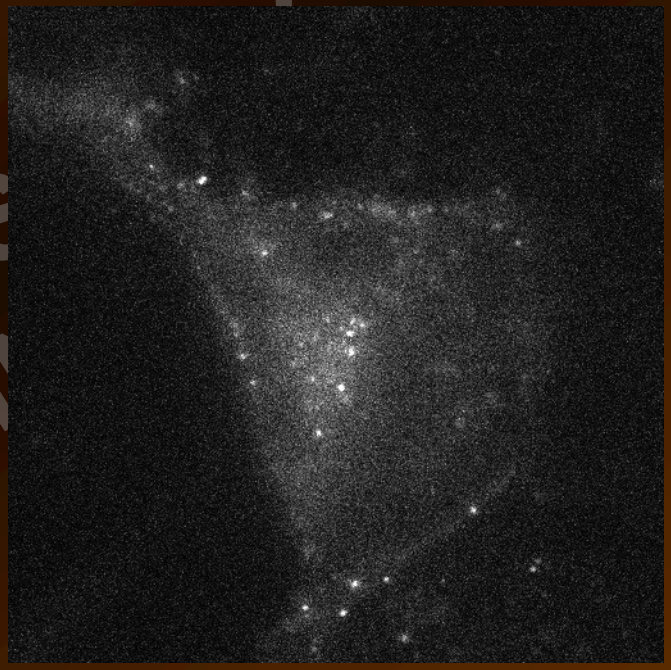
Signal / noise vs. resolution

Nyquist: pixel size = half-width

→Localization image = x,y coordinates + precision

Localization Sequence



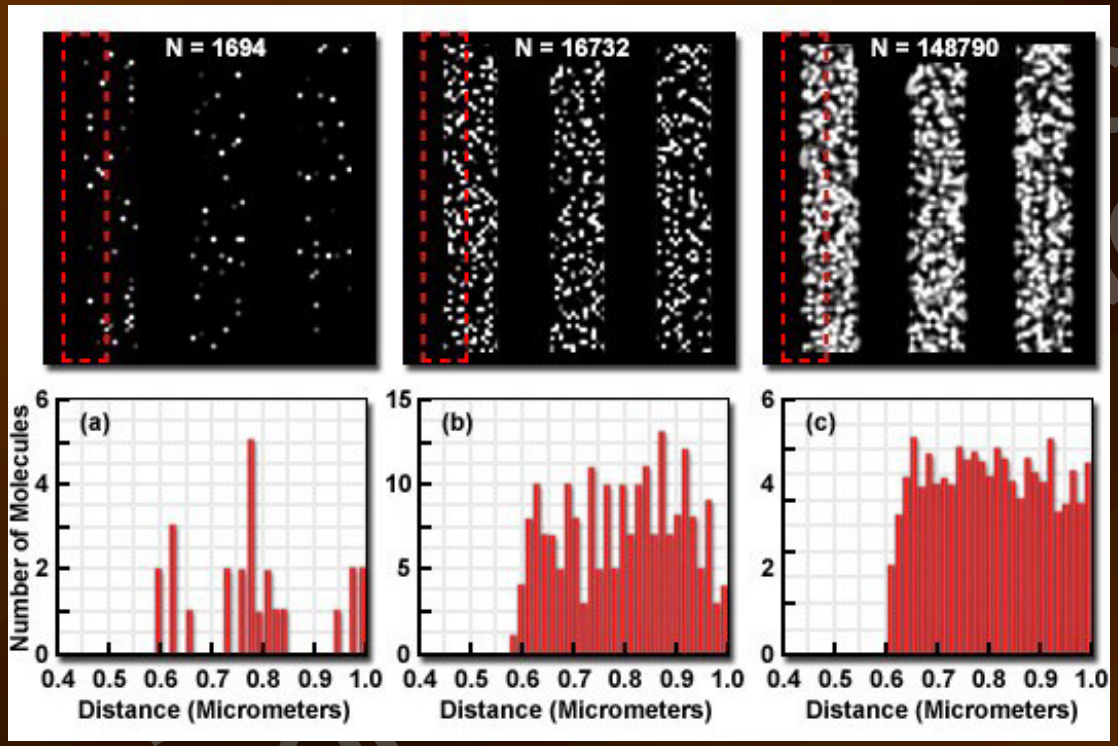


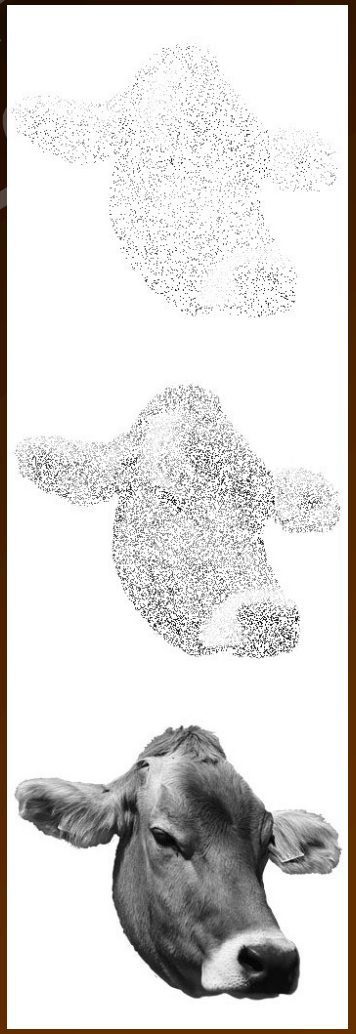
PA-mCherry / STIM1
Vincent Schram, Tamas Balla, NICHD

1) **Fluorophores in dark state**
2) **Few random molecules photo-converted (UV light)**
3) **Molecules imaged**
4) **Localization established**
5) **Molecules bleached (does not affect dark-state fluorphores)**
6) **Repeat**

Marker Density

- Image definition increases with number of detected molecules:





→Localization imaging limited by labeling density

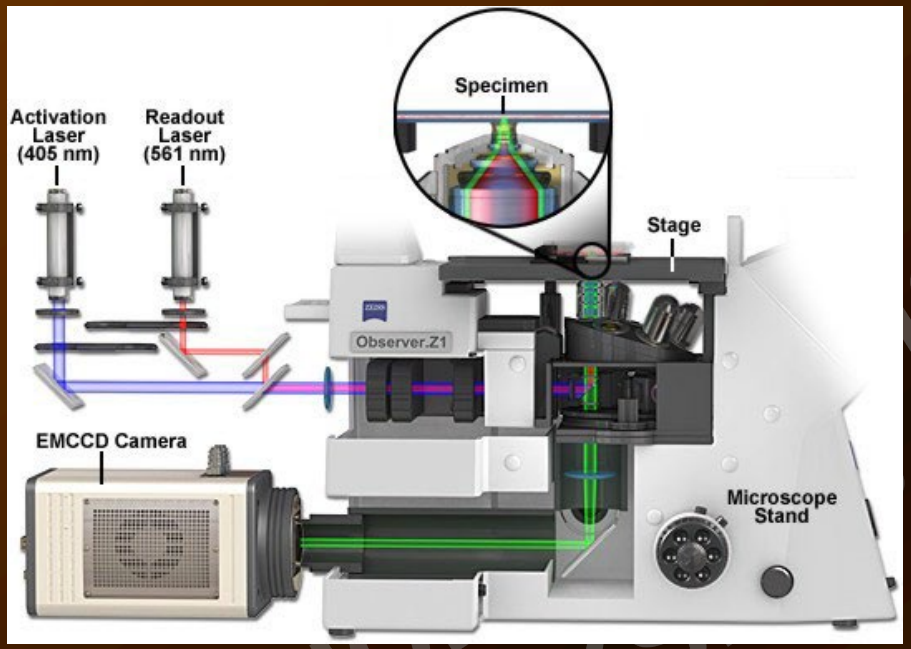
LOCALIZATION MICROSCOPY

Photo Activation Localization Microscopy (PALM) Fluorescent Proteins

- **Photo-activable: non-fluorescent FP until UV-irradiated**
- **Photo-convertible: fluorescence change upon UV**
- **Photo-switchable: reversible photo-conversion with UV**

Protein (Acronym)	Ex (nm)	Em (nm)	N Photons Emitted	Contrast Ratio	Quaternary Structure	Brightness (% of EGFP)
Photoactivatable Fluorescent Proteins						
PA-GFP (N)	400	515	70	NA	Monomer	8
PA-GFP (G)	504	517	300	100	Monomer	41
PA-mCherry (R)	564	595	ND	4000	Monomer	25
Photoconvertible Fluorescent Proteins						
tdEos (G)	506	516	ND	NA	Tandem Dimer	165
tdEos (R)	569	581	750	>4000	Tandem Dimer	59
mEos2 (G)	506	519	ND	NA	Monomer	140
mEos2 (R)	573	584	500	>2000	Monomer	90
Photoswitchable Fluorescent Proteins						
Dronpa	503	517	120	<1000	Monomer	240
Dronpa-3	487	514	ND	ND	Monomer	56

PALM Microscope

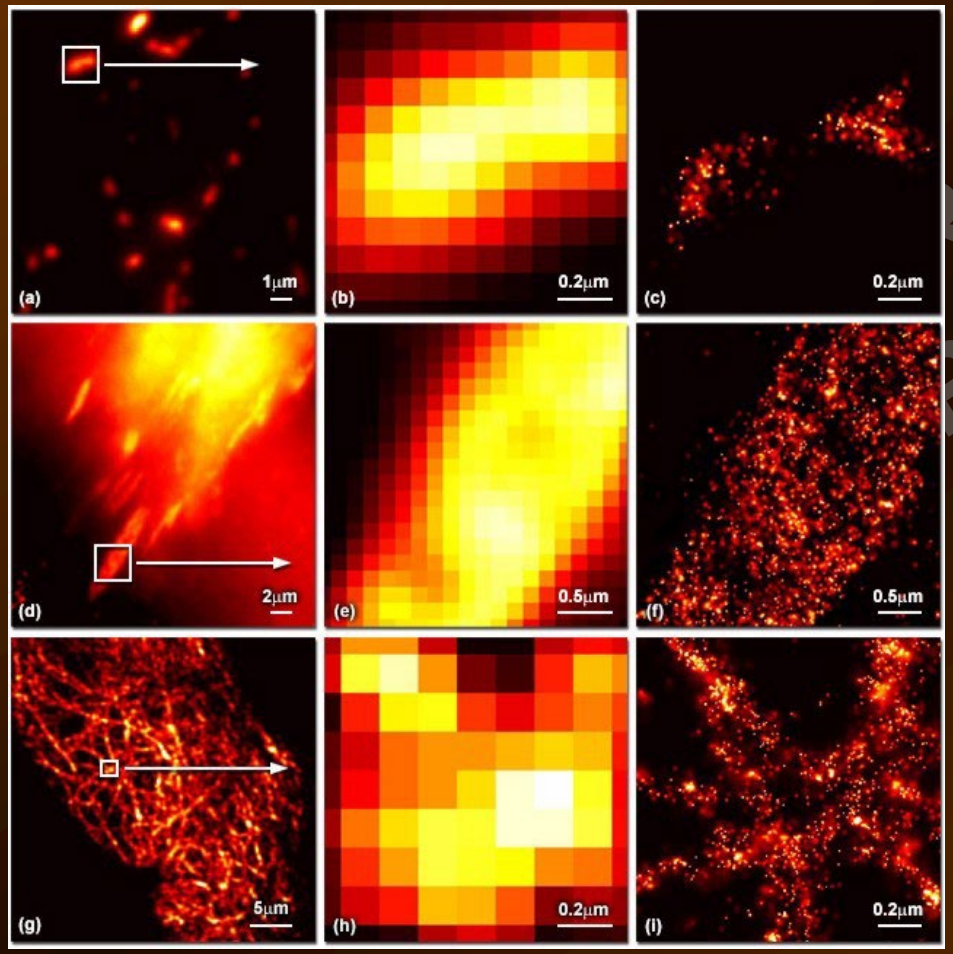


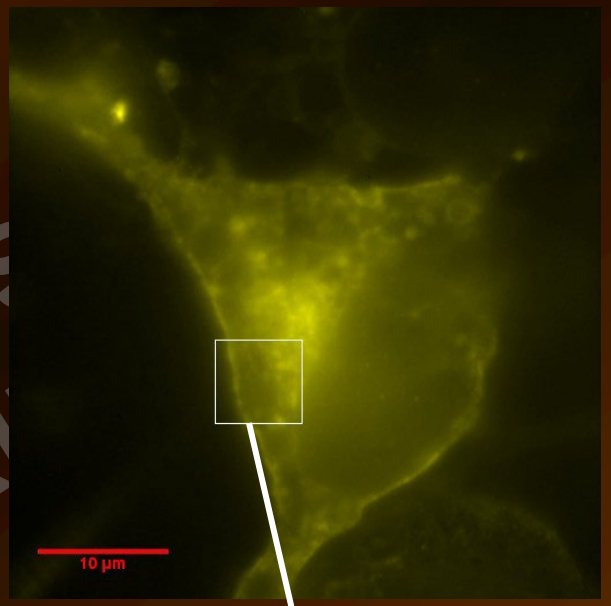
- Widefield fluorescence microscope
- 405 and 488 / 561 direct-coupled lasers or high-power lasers
- Additional focusing lens (power)
- High-sensitivity EM-CCD
- ***TIRF illumination*** (low background)

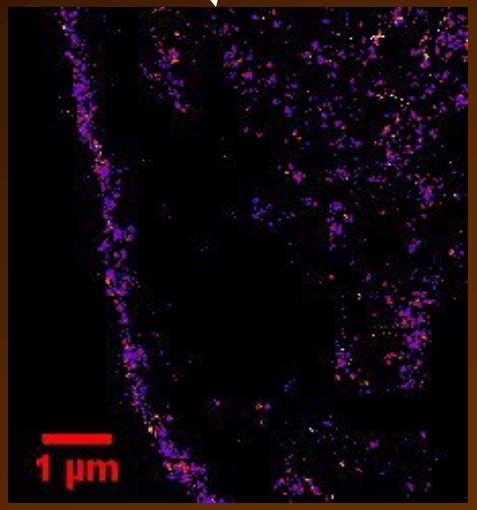
- Lowest mechanical drift:

→ Stable room environment, enclosure
→ Hardware autofocus
→ Stable specimen holder
→ xy drift compensation algorithm

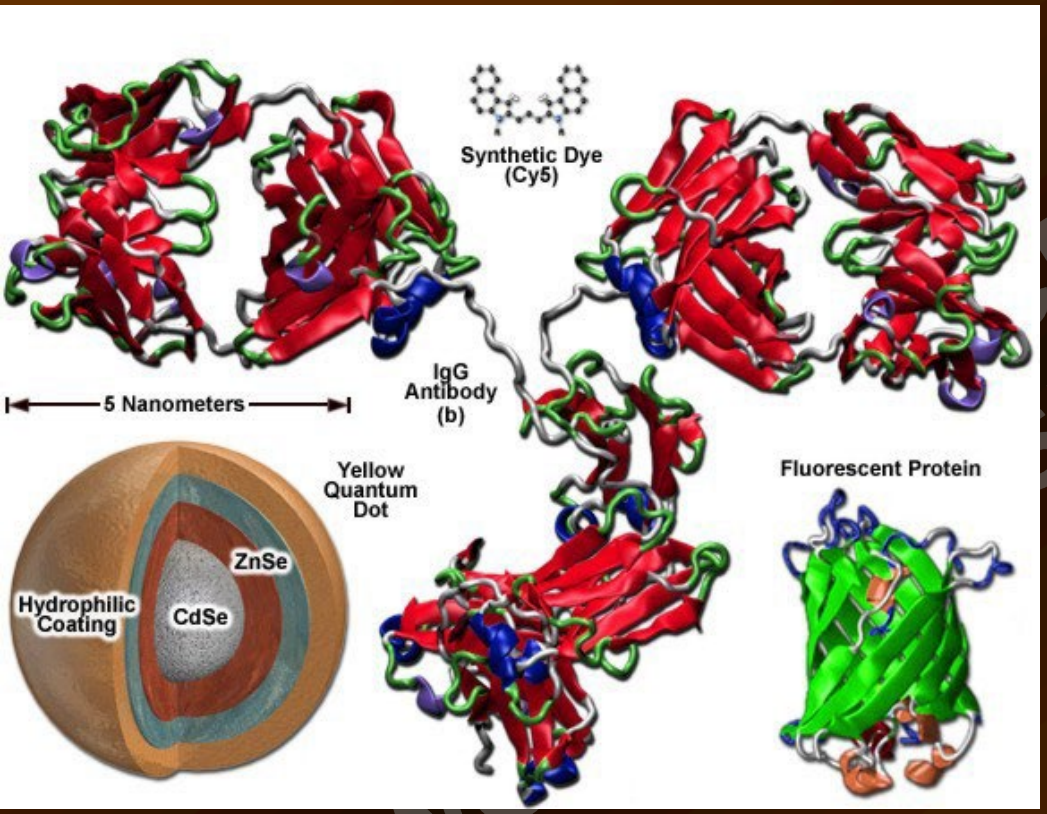
PALM Images







Practical Considerations



- 20 nm resolution imaging:
  → Fab-fragment / small marker

- Bright, photostable markers:
  PA-mCherry, mEOS2, dendra2...

- Non-specific sparks (dust / dirt):
  → Etch coverslips (NH_3/H_2O_2)
  → Filter and UV-bleach buffers

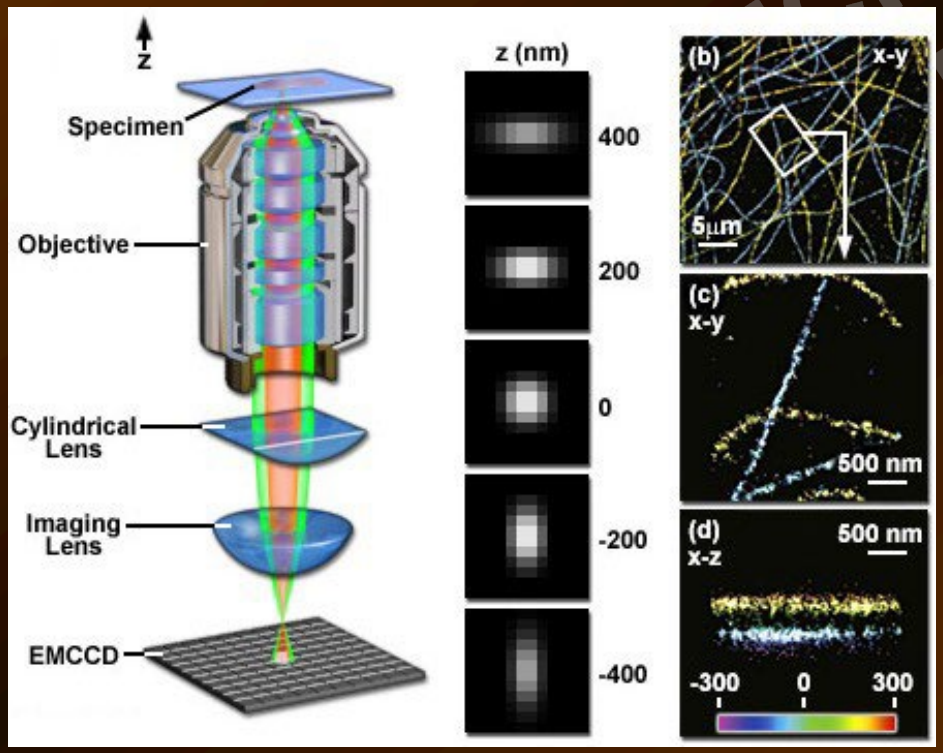
- Require perfect optics: high-quality #1.5 coverslips

3D Localization

- Cylindrical lens between specimen and EM-CCD

→ Horizontal / vertical distortion when above / below focal point

- Calibration curve ellipticity vs. z-position → 100 nm Z resolution



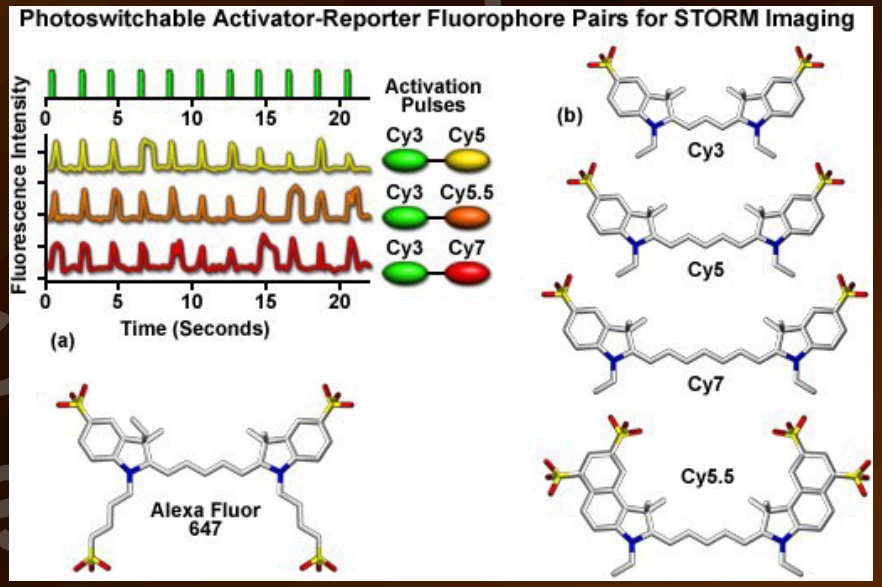
- Successfully applied to PALM and STORM

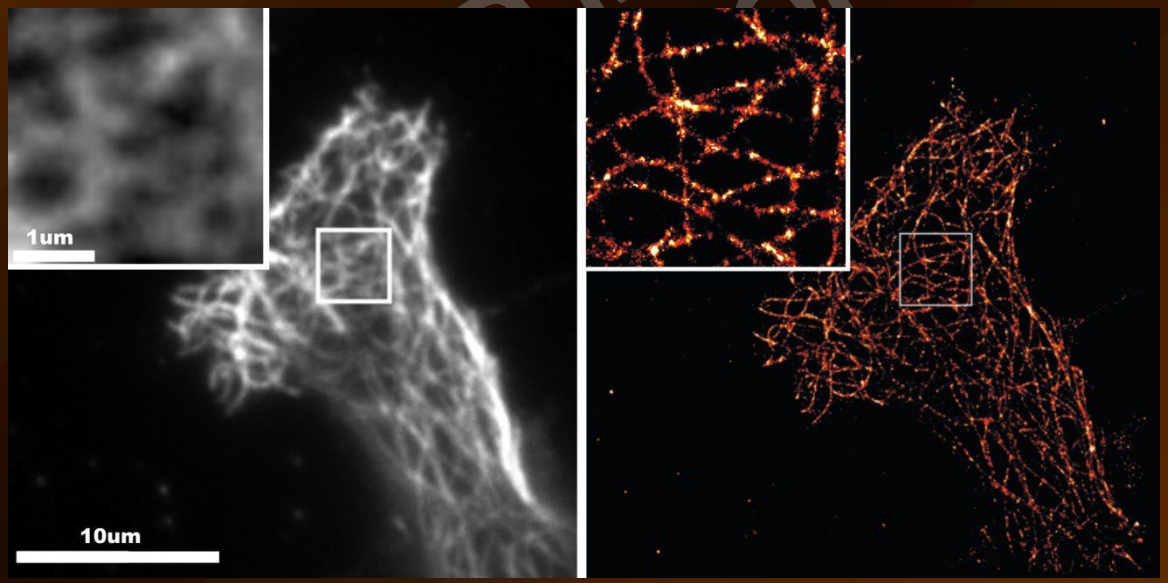
Stochastic Optical Reconstruction Microscopy (STORM)

- **STORM = PALM with chemical dyes**
- **Activator / photoswitcher pairs:**

1)All photoswitchers driven to dark state

2)Random molecules turned on by irradiating activator





- **d-STORM: single marker storm with O2 scavenging reagent**

Alexa 647 / Tubulin stain
Hammond, G. & T. Balla, NICHD

LOCALIZATION IMAGING TAKE-HOME MESSAGE

- **Pros**
  - ***Best resolution: 20 nm lateral, 100 nm axial***
  - **Low-cost equipment**
  - **Choice of methods and dyes**

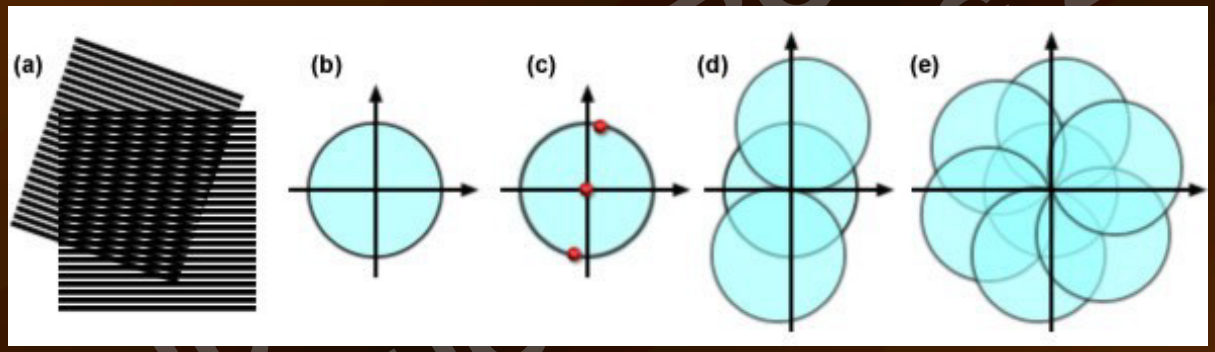
- **Cons**
  - **Slow, imaging time in minutes**
  - **Require special dyes**
  - **No live imaging, no tissue**
  - **Extensive post-processing**
  - **Require high mechanical stability**

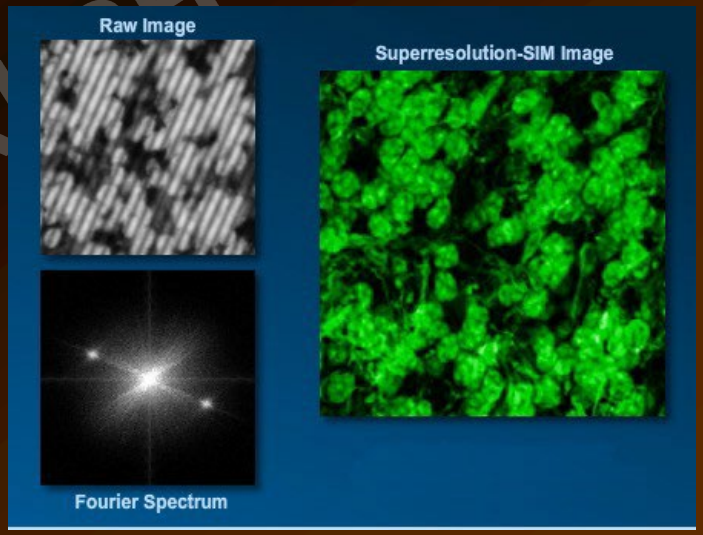
→ ***Powerful and wide-spread technique***

QUESTIONS

STRUCTURED ILLUMINATION MICROSCOPY (SIM)

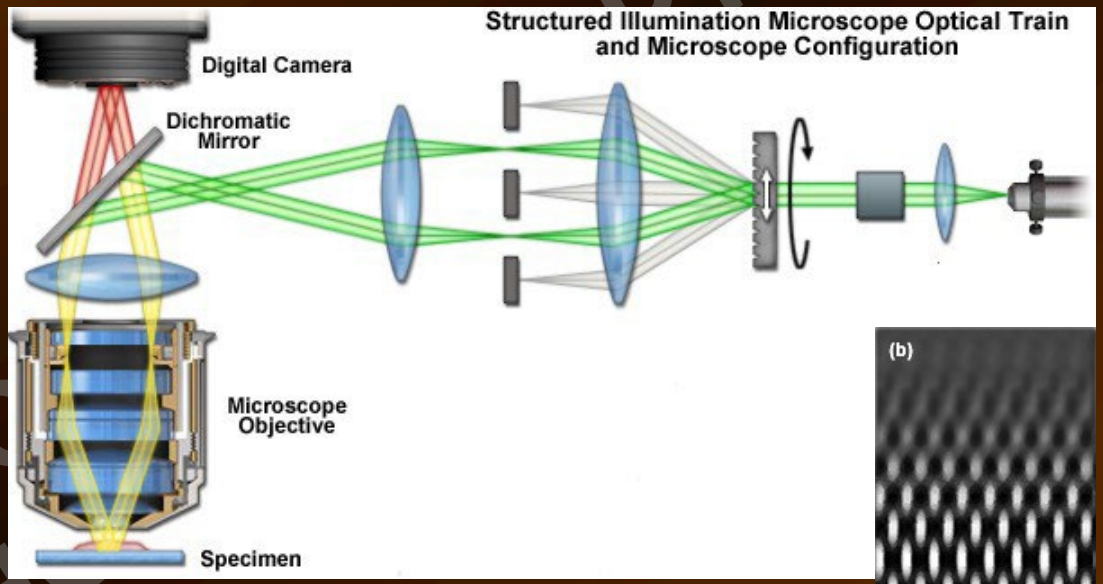
- **Full-field illumination with diffraction-limited interference pattern**
  - ➔ **Series of images at different phases (slight movement) and rotations**
  - ➔ **Fourier transform and image processing improve resolution (100 nm)**



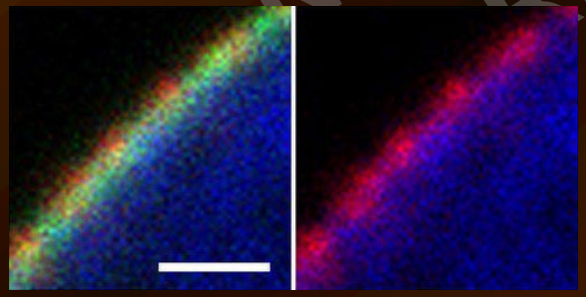


3D-SIM

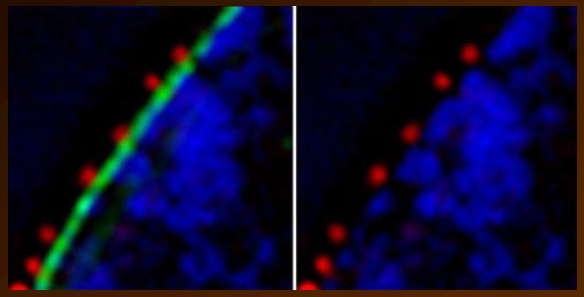
- Interference microscopy
- First order beams from polarizer and phase grating produce interference patterns
- 3D Bessel beam, 100 nm lateral, 300 nm axial periodicity



Confocal



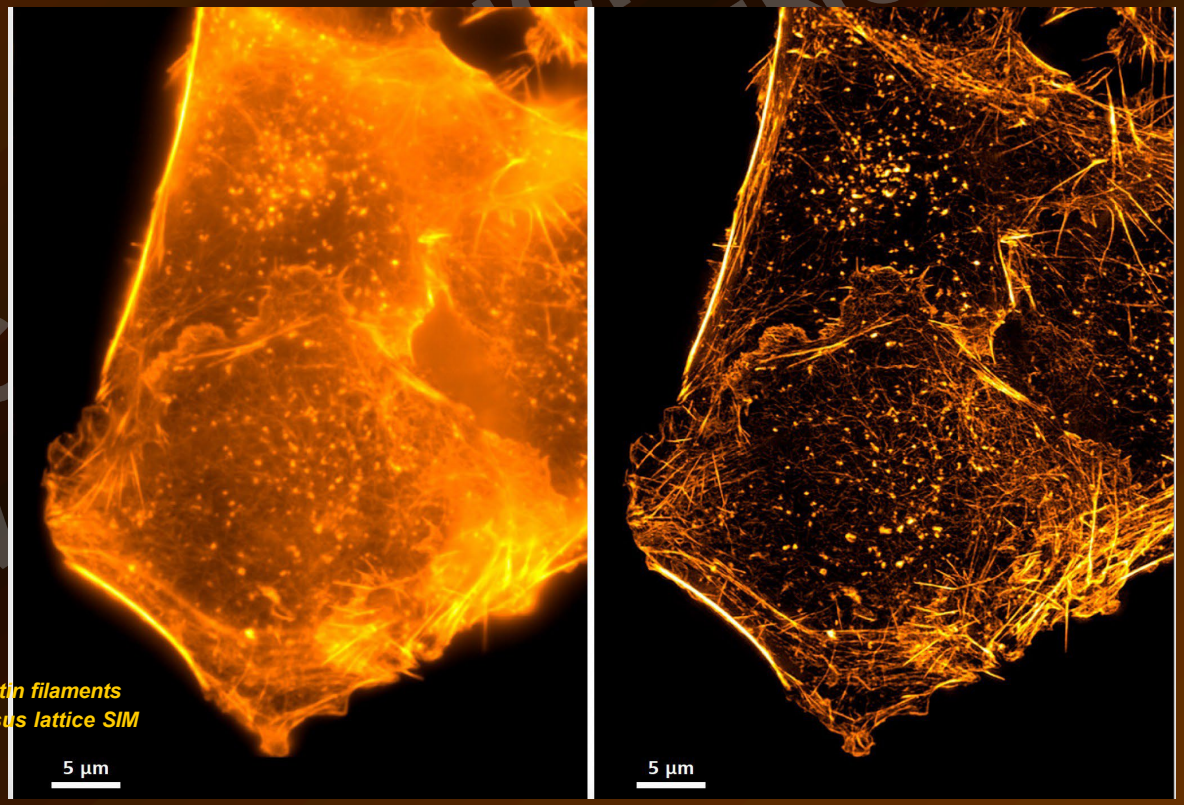
3D SIM



Red: α NPC (nuclear pores)
Green: α Lamin (nuclear envelope)
Blue: DAPI. Bar = 1 um
Schermelle & Sada, 2008

Zeiss Lattice SIM / Elyra 7

- **Developed by Eric Betzig at HHMI, patented by Zeiss**
  - **➔ Dot scanning instead of stripes**
  - **➔ 120 um after Fourier transform, “60 nm” after deconvolution (??)**
  - **➔ Very fast, bundled w/ PALM on Elyra 7**



Phalloidin / Actin filaments
Wide-field versus lattice SIM

STRUCTURED ILLUMINATION TAKE-HOME MESSAGE

- **Pros**
  - **No specimen or dye requirement**
  - **Compatible with live imaging**
  - **Significant resolution improvement (100 nm lateral, 300 nm axial)**

- **Cons**
  - **Require multiple exposures**
  - **Require extensive post-processing**
  - **Prone to artifacts**
  - ***Best resolution improvement for discrete structures (puncti, spines…)***
  - ***Zeiss lattice sheet =niche application***

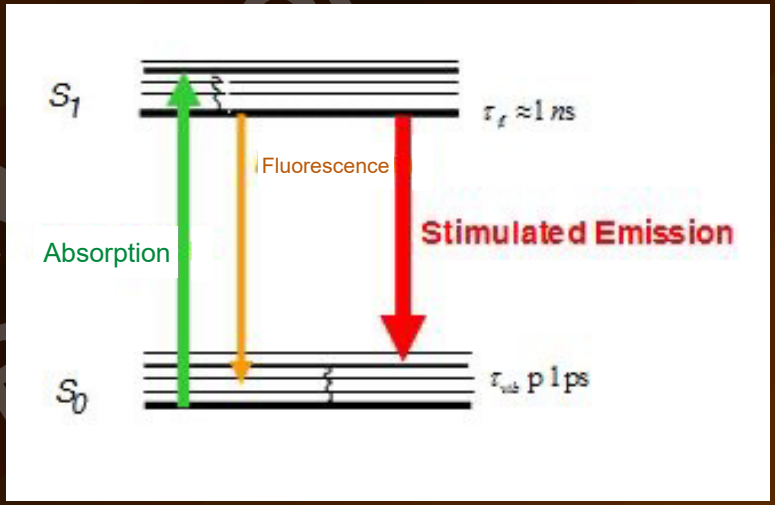
→ ***Uncertain future due to competing techniques…***

STIMULATED EMISSION DEPLETION

STED PRINCIPLE

- Once excited, fluorescent dye may be forced to re-emit by absorbing a photon of longer wavelength ("depletion beam")

- Difference in ground vibration state → "stimulated" photon is red-shifted compared to fluorescence



Fluorophore	Ex λ (nm)	STED λ (nm)	Resolution (lateral; nm)
Synthetic Dyes			
ATTO 425	440	532	70-80
ATTO 565	532	640	30-40
ATTO 663	635	750	40
Alexa Fluor 594	570	700	60
DyLight 594	570	700	60
RH 414	554	745	30
Fluorescent Proteins			
EGFP	490	575	70
Citrine	490	598	50
EYFP	490	598	70

- Not all dyes equally susceptible to stimulated emission

- Depletion lasers:
  - 592 nm (green dyes)
  - 660 nm (red dyes)
  - 775 nm (far-red dyes)

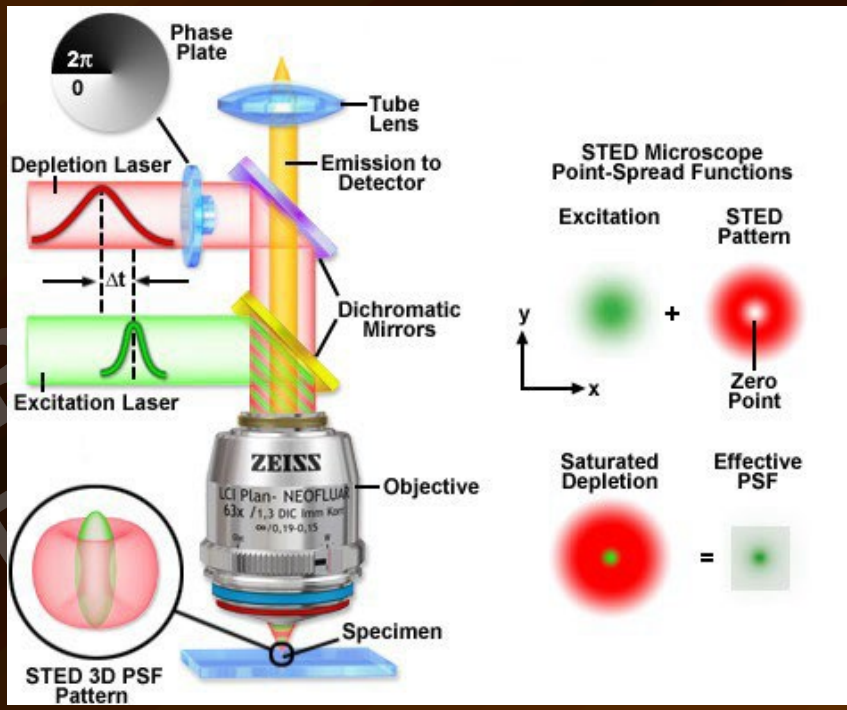
STED IMPLEMENTATION

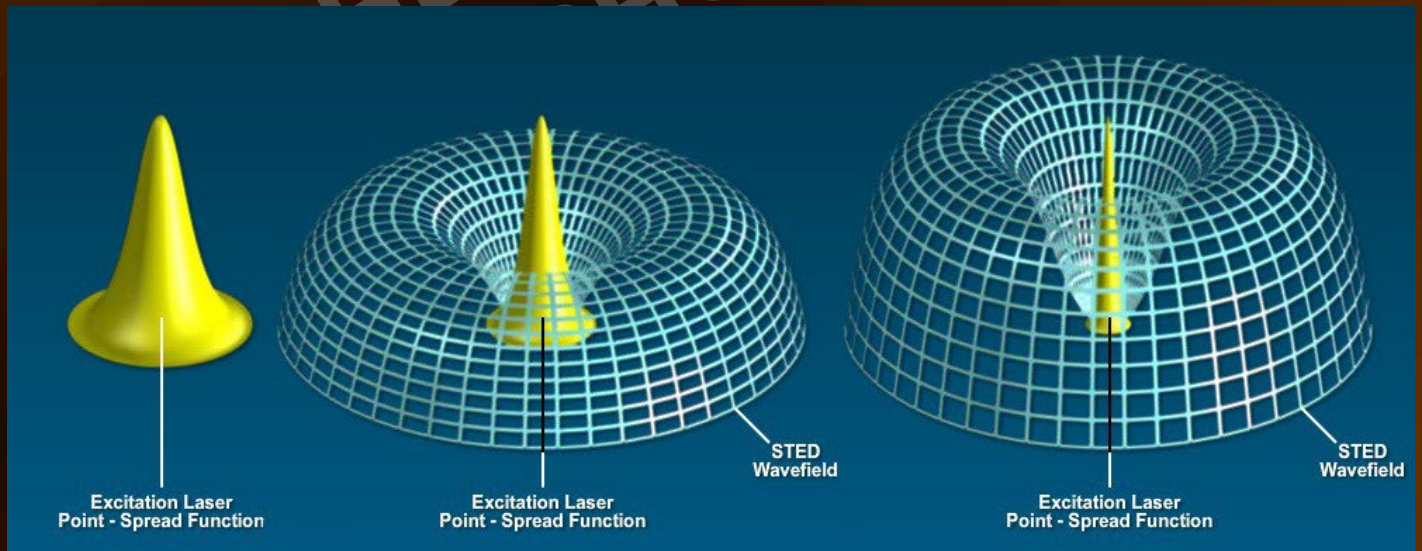
- Leica (well-established) / Abberior

- Confocal beam overlaid with doughnut-shaped depletion beam

→ Special depletion beam and fine overlay optics

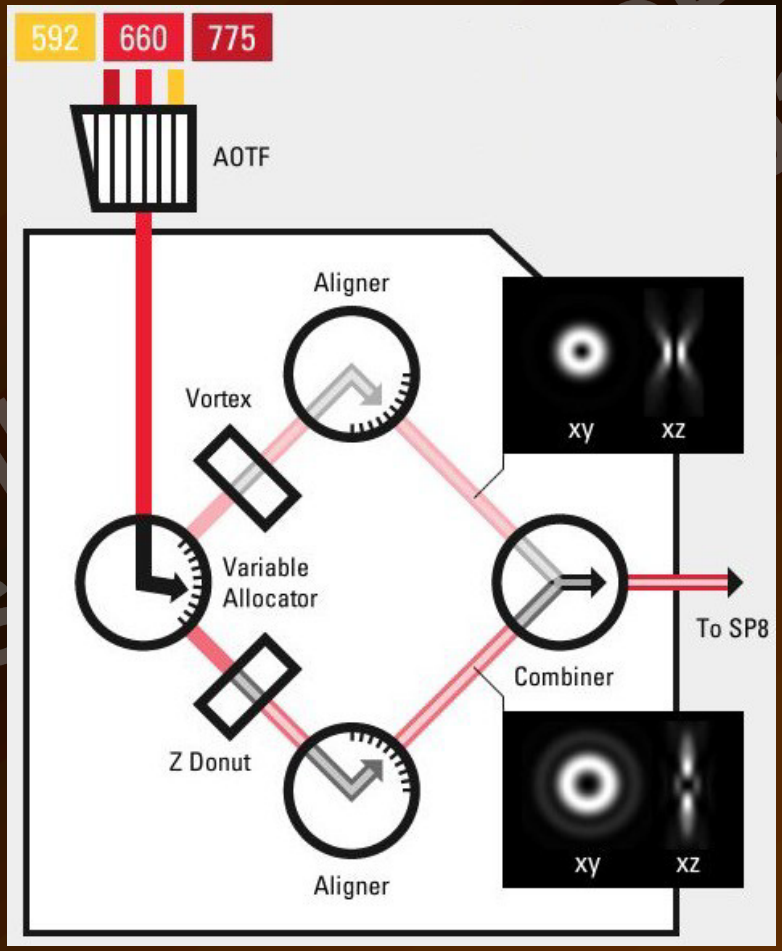
→ Higher depletion intensity shrinks effective PSF

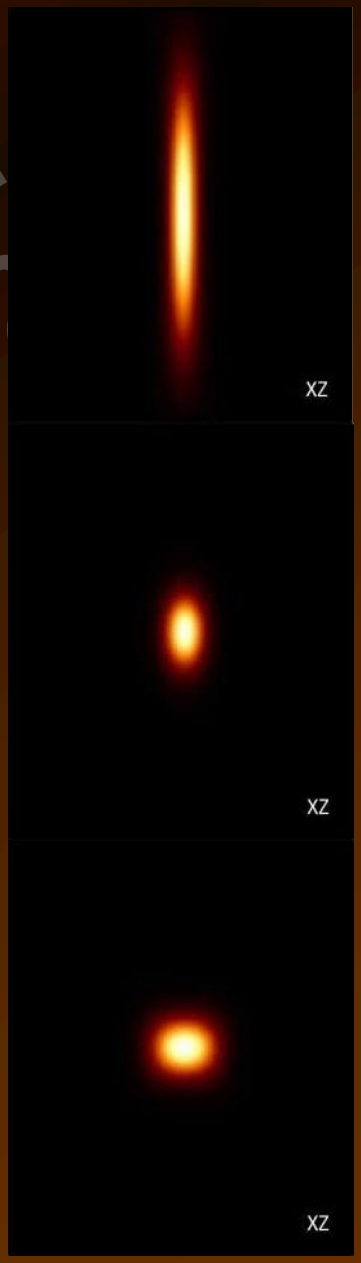




3D-STED

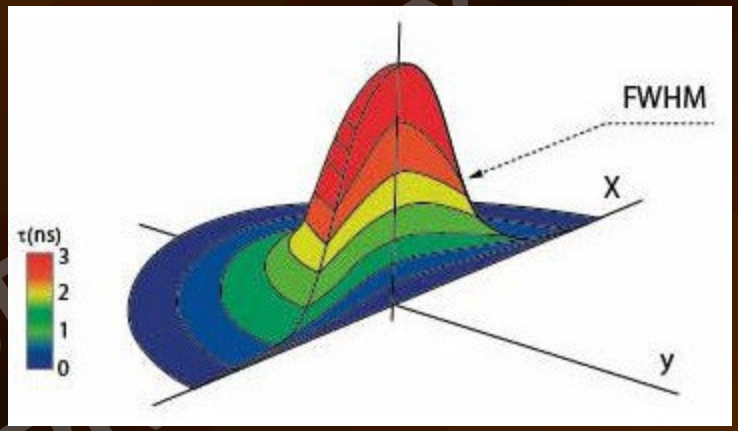
- Vertical STED variant where depletion laser is shaped in z
- Depletion power allocated between 100% lateral to 100% axial resolution enhancement

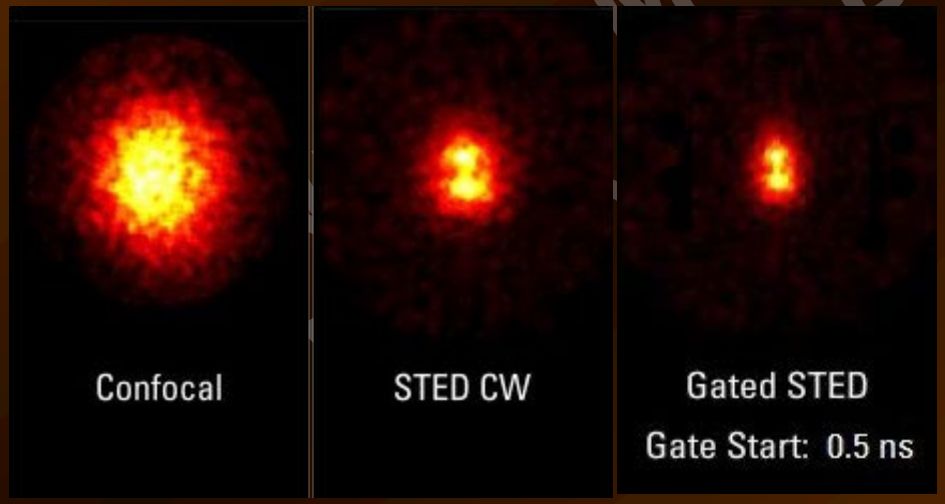




TIME-GATED STED

- White-light pulsed laser used for imaging
- Depletion laser reduces fluorophore lifetime



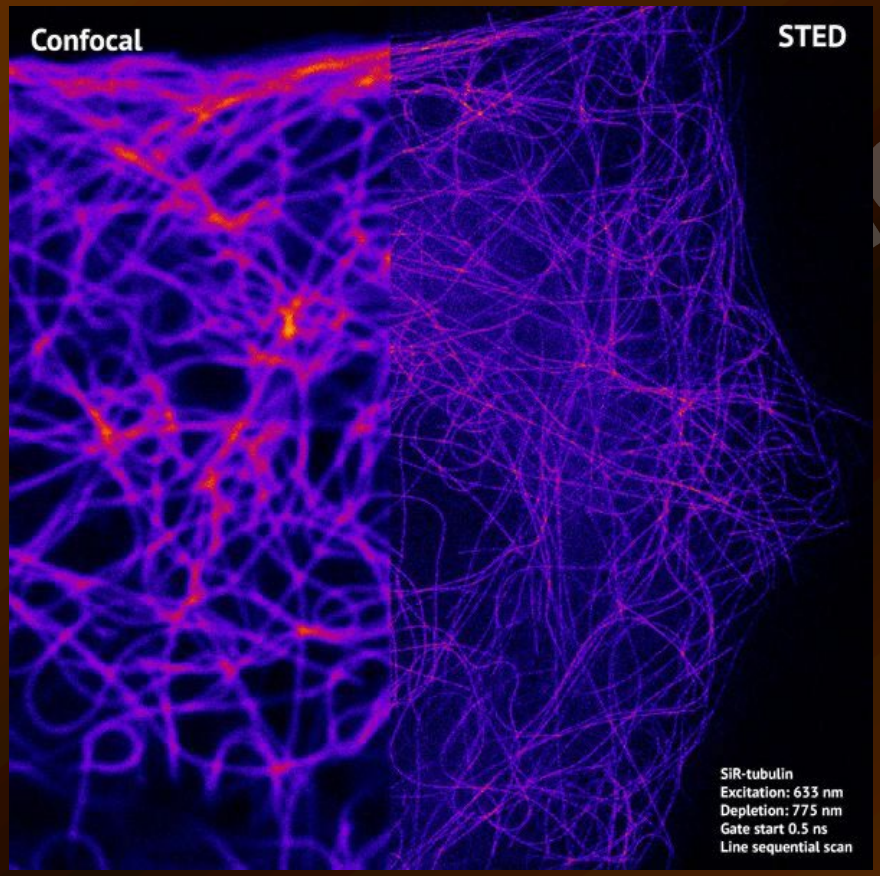


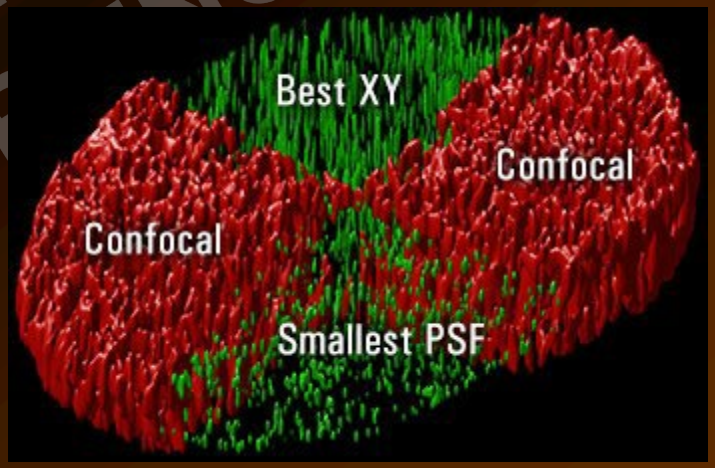
→Molecules exposed to the depletion beam can be filtered out by applying a time filter after each imaging pulse

→Effectively enhance xy and z resolution

STED Images

- Tubulin filaments: confocal vs STED





- Confocal vs STED (xy max) vs STED (z max)

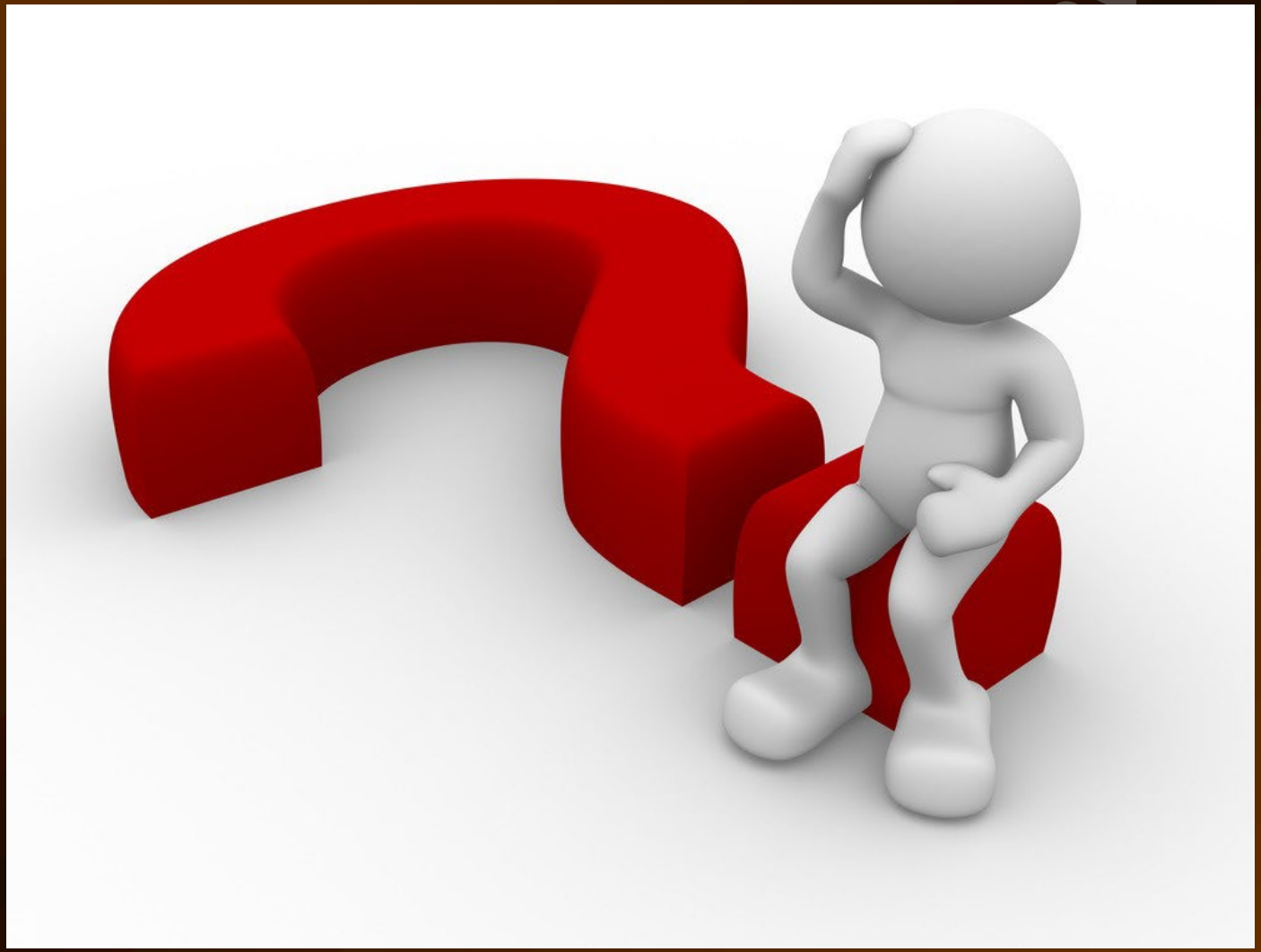
STED TAKE-HOME MESSAGE

- **Pros:**
  - 30 to 80 nm lateral resolution
  - Significant axial resolution enhancement
  - Widely available
  - Potential for high-speed imaging
  - Lower-cost add-ons (Abberior “Steadycon”)

- **Cons:**
  - Limited dye selection, especially endogenous
  - Leica mostly, complex and expensive hardware
  - High photo-damage, live imaging difficult
  - Depletion laser obliterates other channels
  - Very sensitive to choice of dye and sample quality
  - Require deconvolution for best results

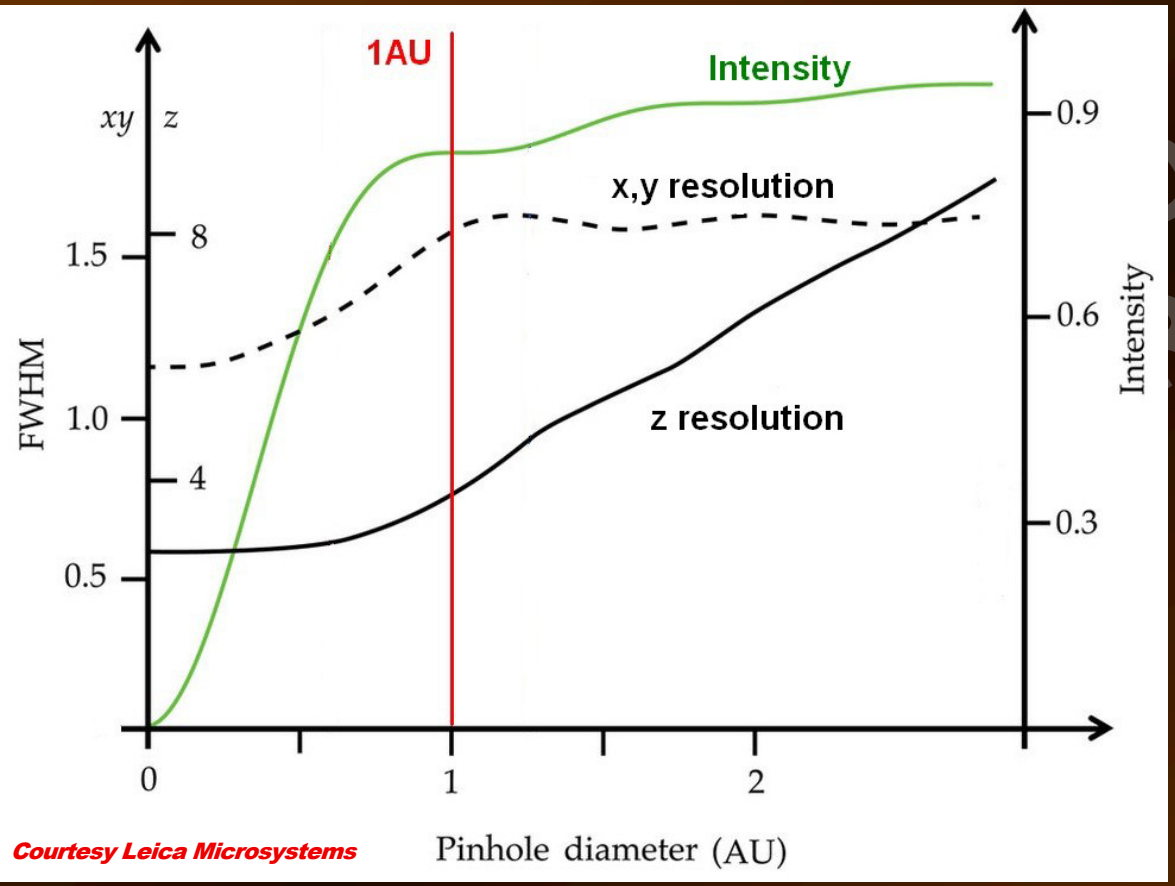
→ ***Important super-resolution technique from Leica***

QUESTIONS



ZEISS AIRY DETECTOR

Effect of pinhole size



Courtesy Leica Microsystems

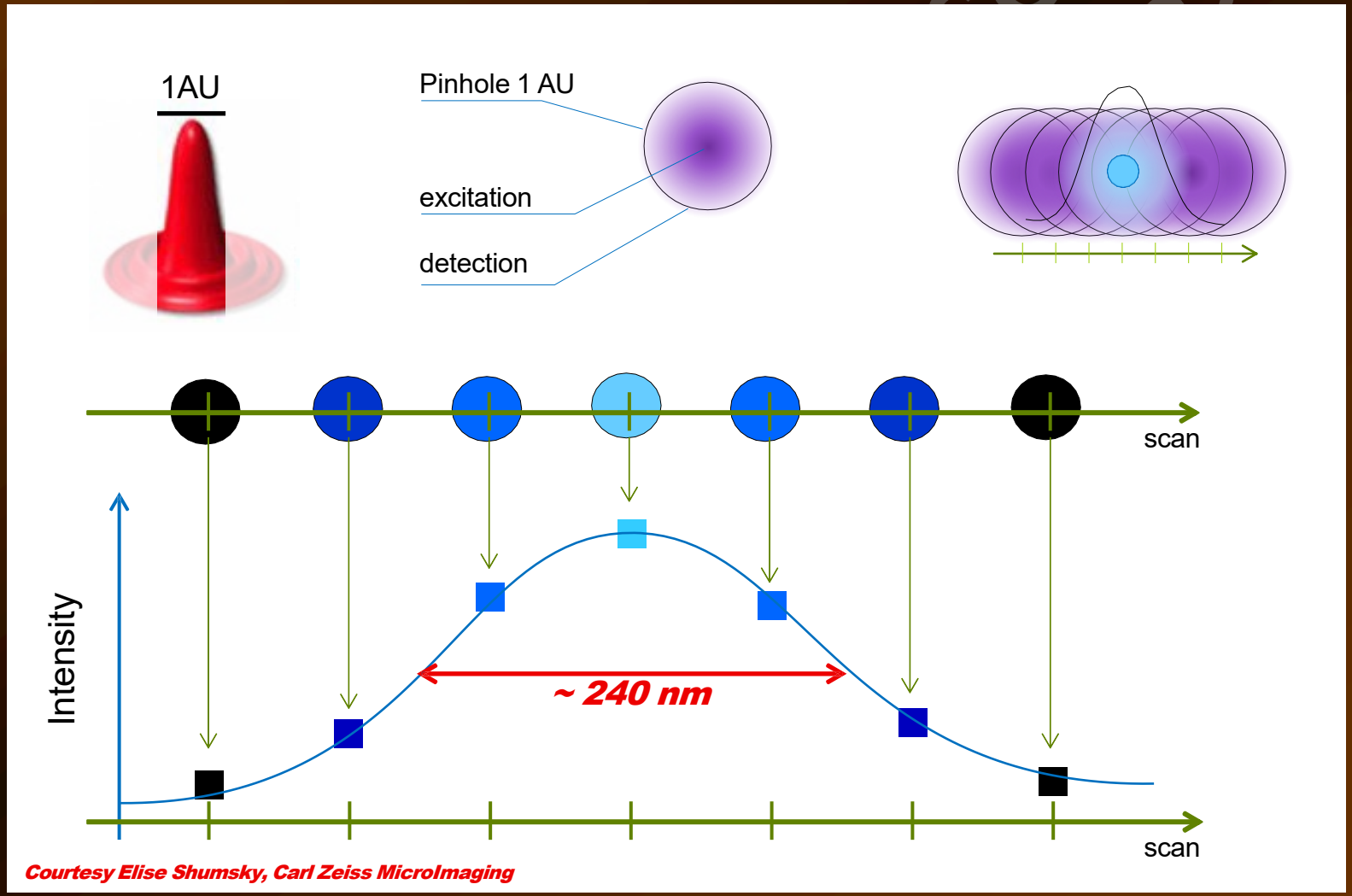
- **1 AU → Best compromise between sensitivity and resolution**
- **Above 1 AU:**
  x,y resolution constant
  z resolution down
  Increase in signal
- **Below 1 AU :**
  modest increase in x,y res. z resolution constant
  extreme loss of signal

→ ***Regular confocal at 0.7 AU: 20% better x,y res., 20% signal loss***

→ ***0.2 AU: 30% better x,y resolution, 90% signal loss***

Confocal pinhole at 1 AU

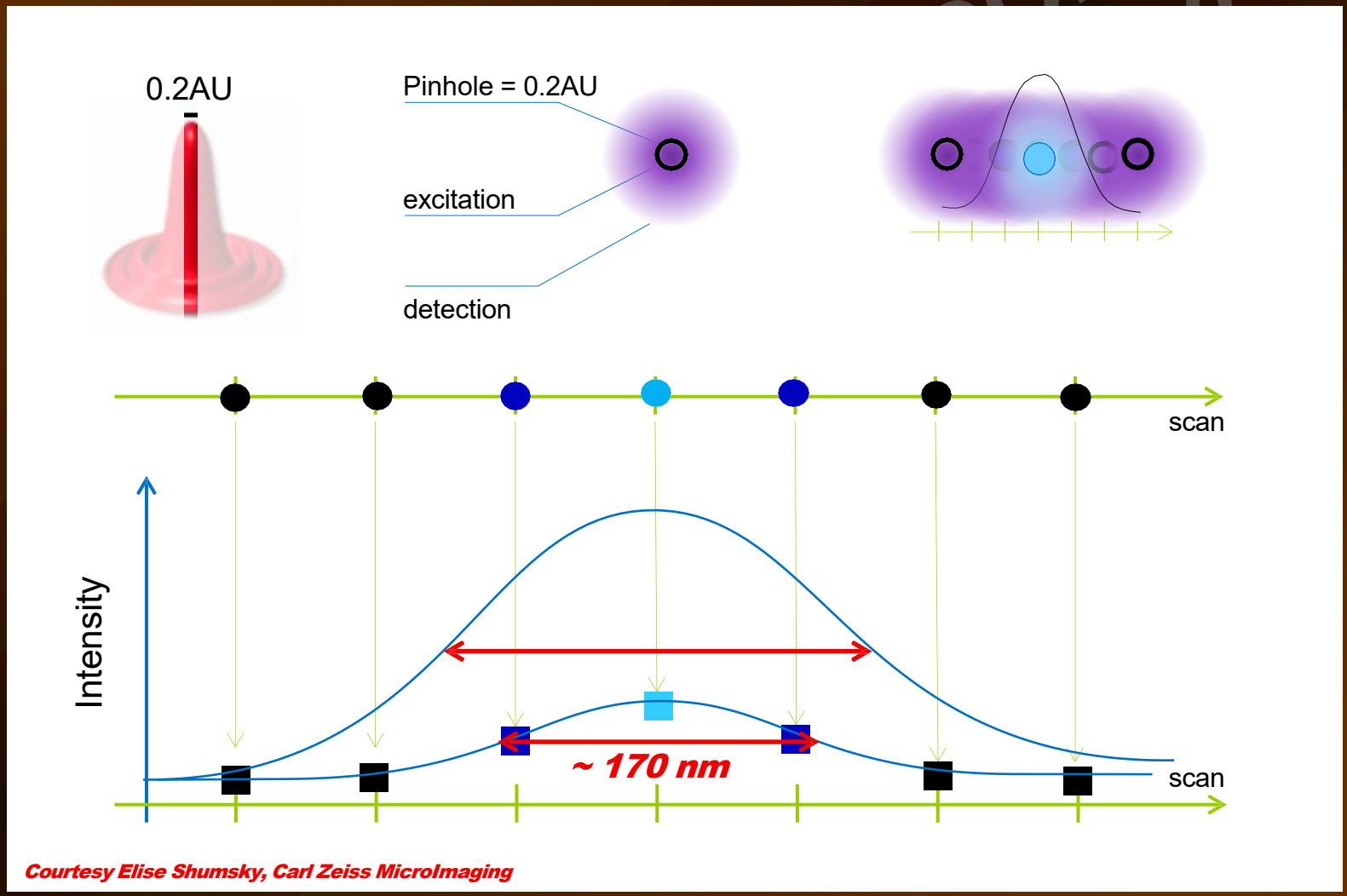
- **Confocal pinhole at 1 Airy Unit → Detector sees central peak of PSF**



Confocal pinhole at 0.2 AU

➔ **Improve lateral resolution by 30%**

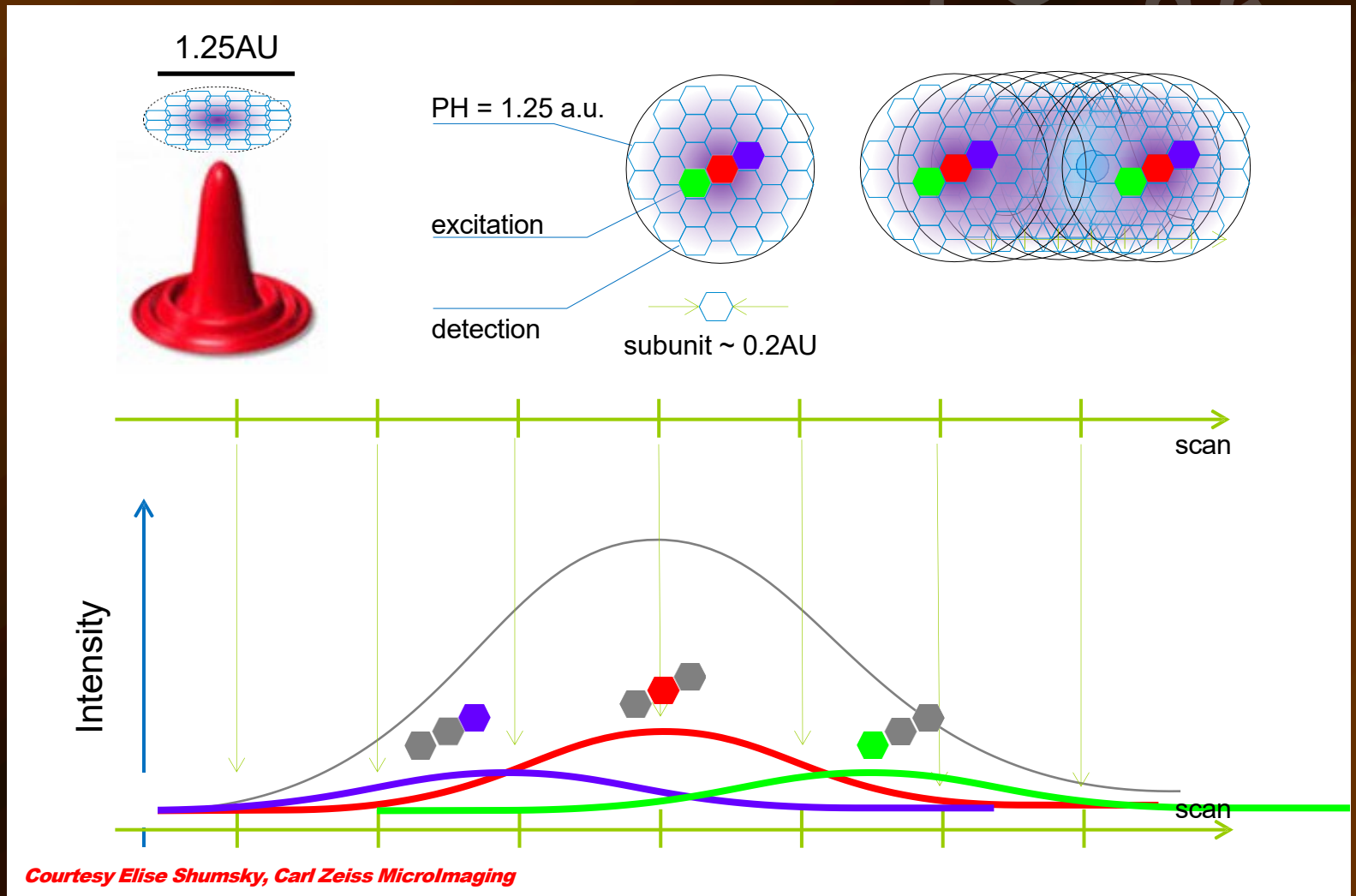
➔ ***90% light loss***



Courtesy Elise Shumsky, Carl Zeiss MicroImaging

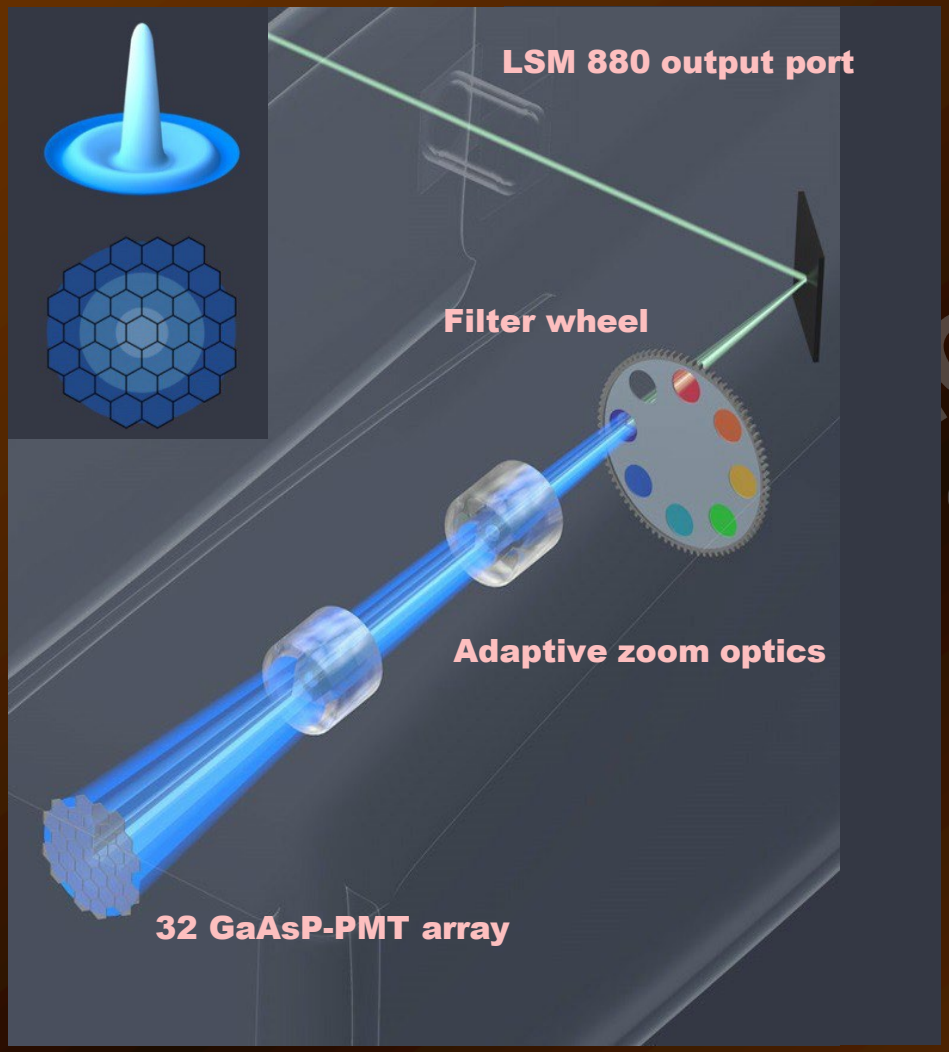
AIRY DETECTOR

- **Hexagonal array of 32 GASP detectors**
- **Each element = 0.2 AU, total array = 1.25 AU**
  - ➔ ***Image rebuilt by remapping each element to the PSF***

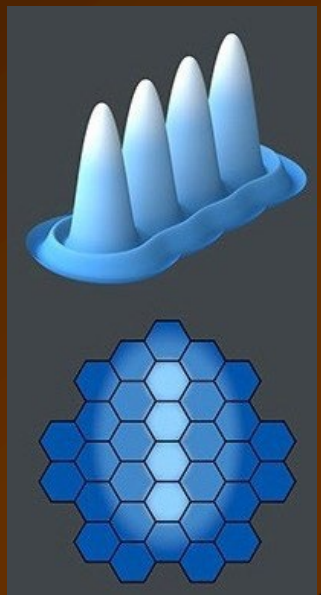


Courtesy Elise Shumsky, Carl Zeiss MicroImaging

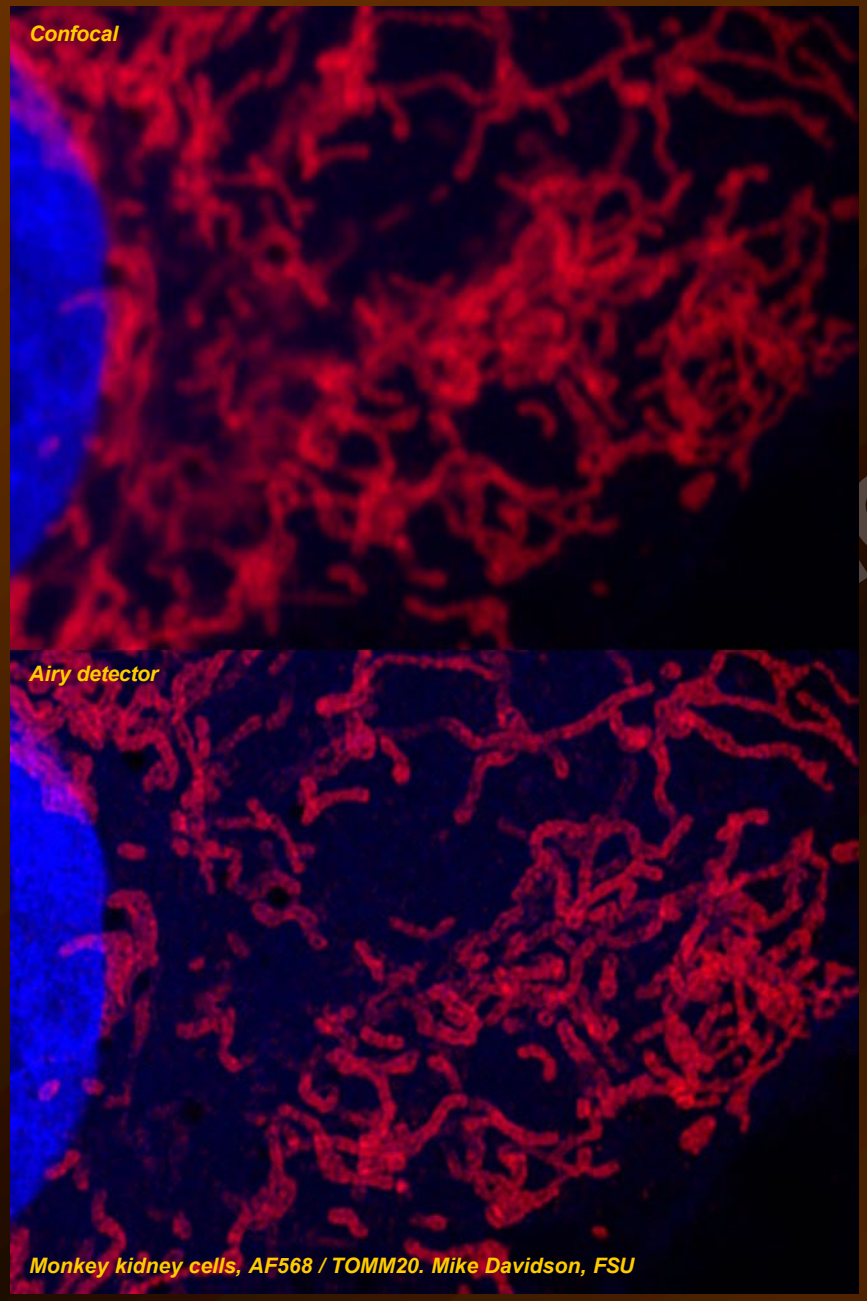
Implementation

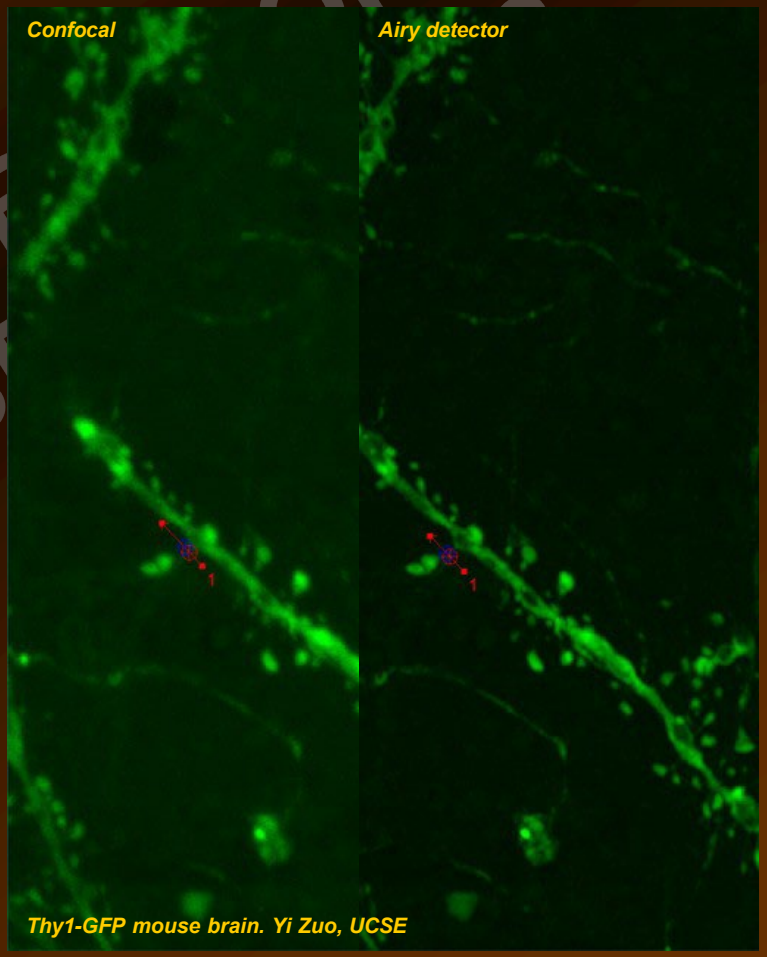


- Add-on to Zeiss 800/900 or 880/980 point scanner
- Adaptive zoom optics matched to each objective, require “Elyra” high-quality lenses (63x)
- 32-channel image “re-assigned” (PSF rebuilt), combined with deconvolution → 120 nm x,y, 400 nm in z
- “FAST” mode: laser elongated in y, re-assigned as 4 overlapping PSFs



Airy Detector images





AIRY DETECTOR TAKE-HOME MESSAGE

- **Pros**
  - **Significant improvement: 120 nm lateral, 400 nm axial**
  - **Low-cost, add-on to Zeiss point scanner**
  - ***Widespread availability***
  - ***No dye nor specimen restriction***
  - **Compatible with live imaging**
  - **Airy detector very sensitive at AU = 1**
  - **Four-fold "high speed" FAST option**

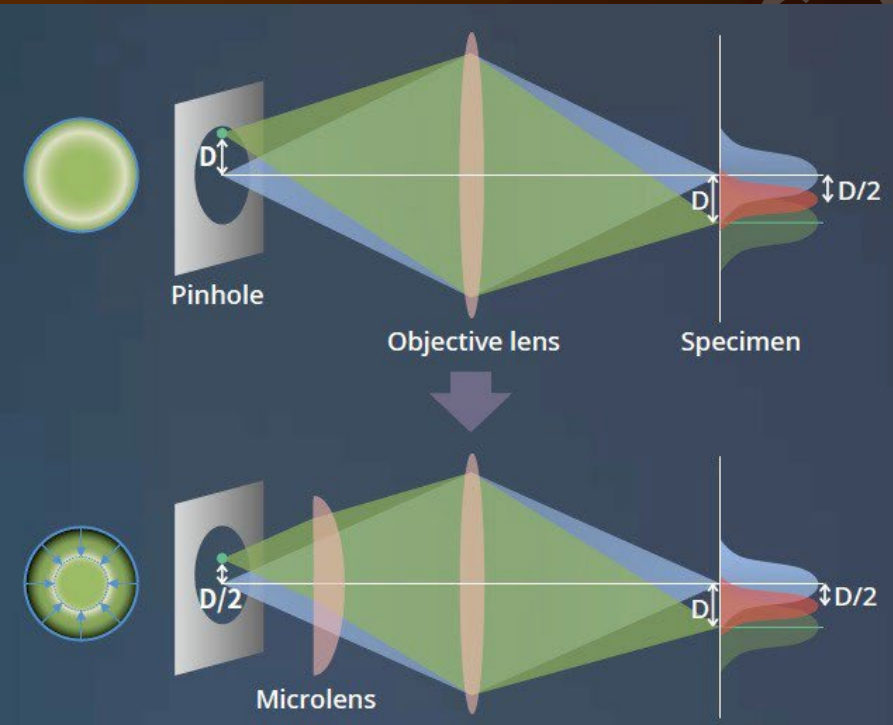
- **Cons**
  - **Oversampling (deconvolution)= higher phototoxicity**
  - **Slow acquisition, stability issues with tilling**
  - **"FAST" option no substitute for spinning disk**

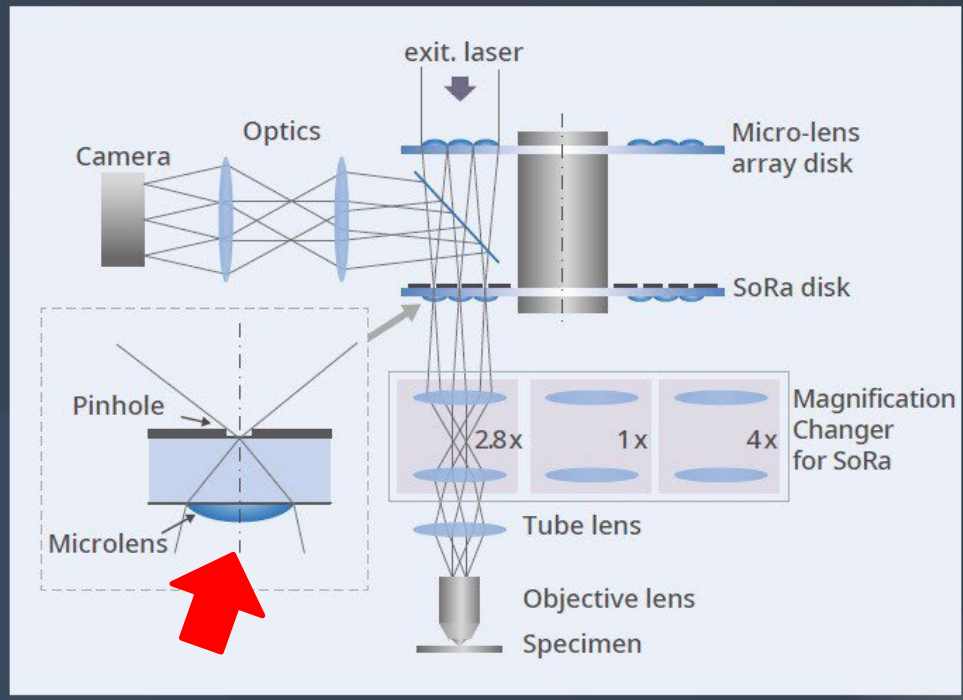
→ ***Modest but easy resolution improvement***

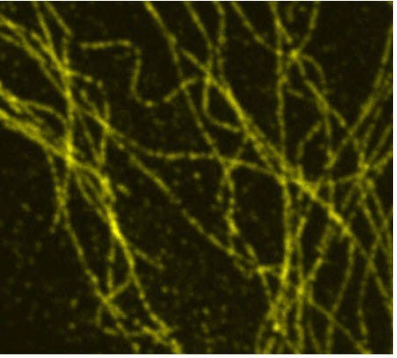
Super-resolution Spinning Disk

Principle

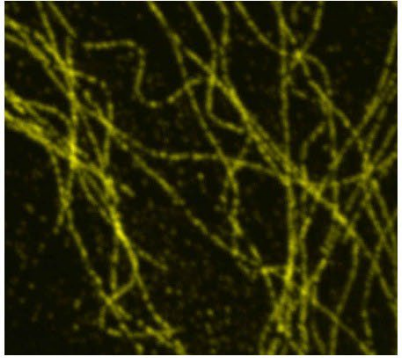
- "SoRa" system from Yokogawa
- Spinning disk with smaller pinholes + additional collection lenses to avoid light losses (50% improvement)
- Additional deconvolution → 120 nm in xy, 400 nm in z
- Magnification changer matched to objective



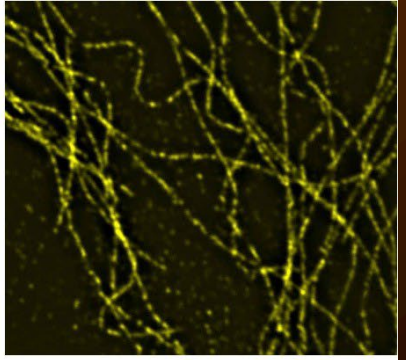




SD 50µm

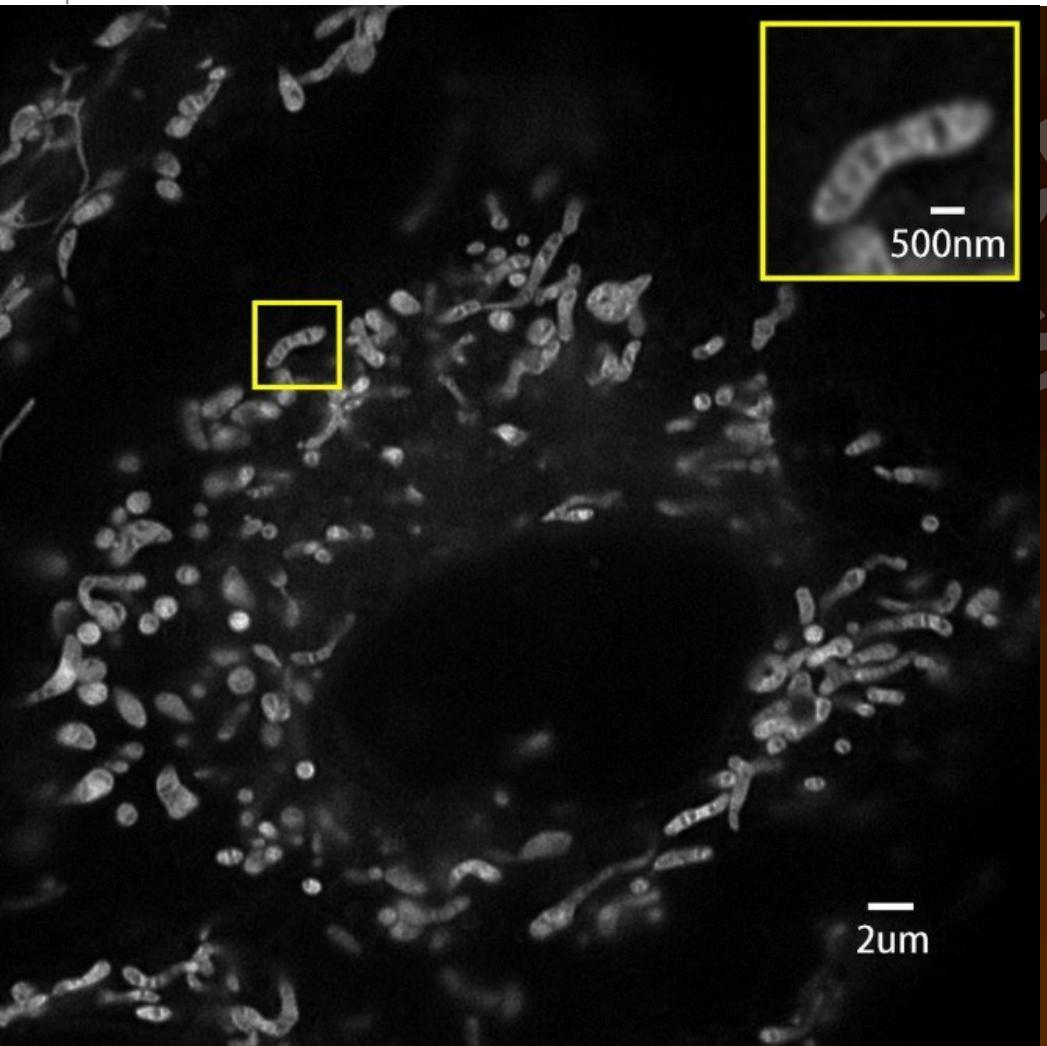


SoRa



SoRa DCV

SoRa images



Movie at:
https://bcove.video/2O63eAf

SoRa SPINNING DISK TAKE-HOME MESSAGE

- **Pros**
  - **Significant improvement: 120 nm lateral, 400 nm axial**
  - ***No dye nor specimen restriction***
  - **Spinning disk = suitable for live imaging**
  - ***High speed compared to the Airy***
  - ***Suitable for high-volume imaging***

- **Cons**
  - **Full-field illumination = prone to lateral bleed-through**
  - **Laser exposure on high side**

→ ***Attractive option for high-speed 120 nm***

ABBERIOR MINIFLUX

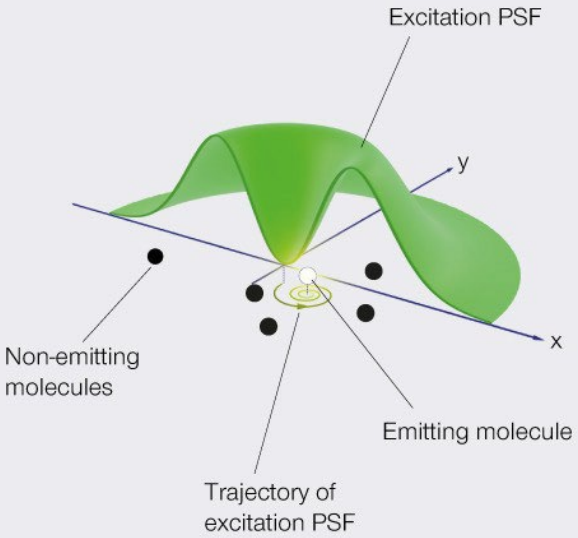
- **Combination of STED and localization microscopy**

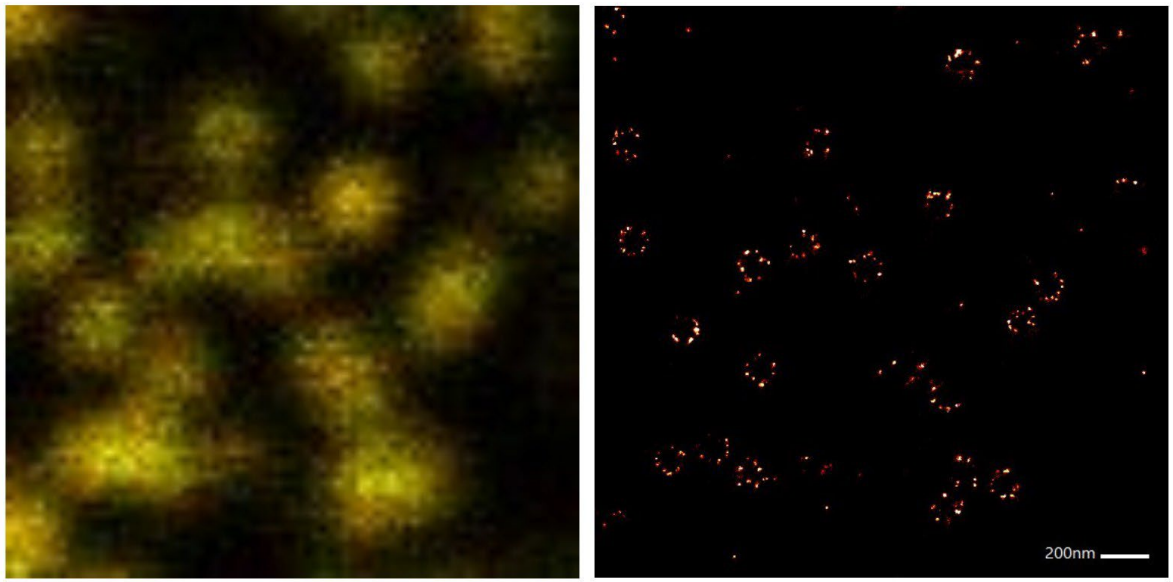
→ Emiter activated one at a time

→ Donut-shaped excitation beam raster scan FOV

→ As soon as signal detected, zero in on molecule by putting it in the center (lowest emission)

→ 3D variant, sample xyz active stabilizer





Comparative images of Nup96 complexes labelled with Alexa 647. Left: standard confocal mode, right: 2D Minflux mode.

MINIFLUX TAKE-HOME MESSAGE

- **Pros**
  - ***True molecular resolution: 1-3 nm in xyz***
  - **Suitable for fast molecular dynamics in vivo (SPT)**

- **Cons**
  - **Slower scan times (localization)**
  - **Labelling density requirements**
  - **Limited selection of dyes**
  - ***Extraordinarily expensive ($2M)***

→ ***Bleeding edge, still in development***

SUPER-RESOLUTION MICROSCOPY

Membrane only

- **TIRF:** 200 nm z, live OK, low photo-damage, very fast

170 nm

- **Deconvolution:** 170 nm xy, 550 nm z, slow, cheap

120 nm

- **SIM:** 100 nm xy, 300 z axial, live OK
- **Lattice SIM:** faster SIM option
- **AIRY:** 120 nm xy, 400 nm z, live OK, slow
- **SoRa:** 120 nm xy, 400 nm z, very fast, excellent for live and tilling

50 nm

- **STED:** 50-100 nm xy, 200 z, live difficult, special dyes, expensive hardware

20 nm

- **Localization:** 20 nm xy, 100 nm z, special dyes, fixed only, no tissue, very slow

3 nm

- **Miniflux:** 1-3 nm xyz, few dyes, rapid molecular dynamics, complex, enormously costly

→ ***Methods under active development...***

QUESTIONS