Lecture-11

CSO202: Atoms, Photons & Molecules

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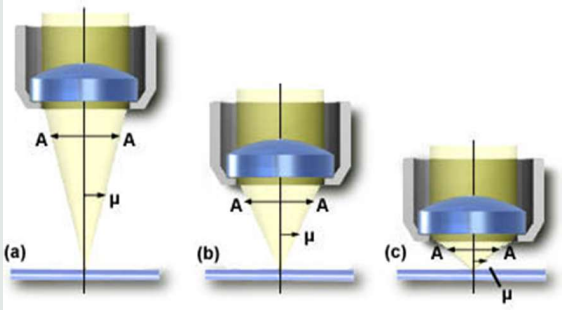
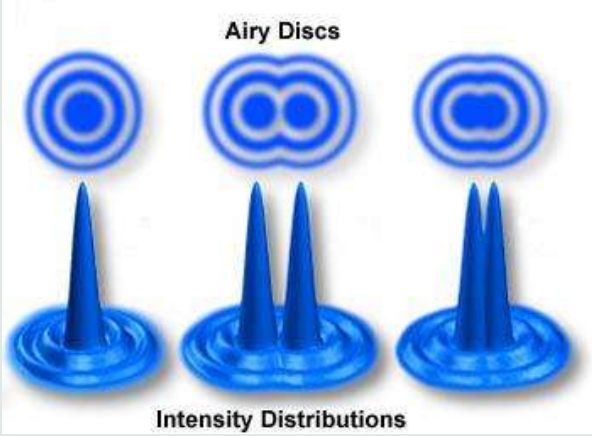
Resolution of Microscope

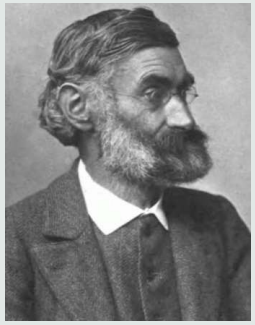


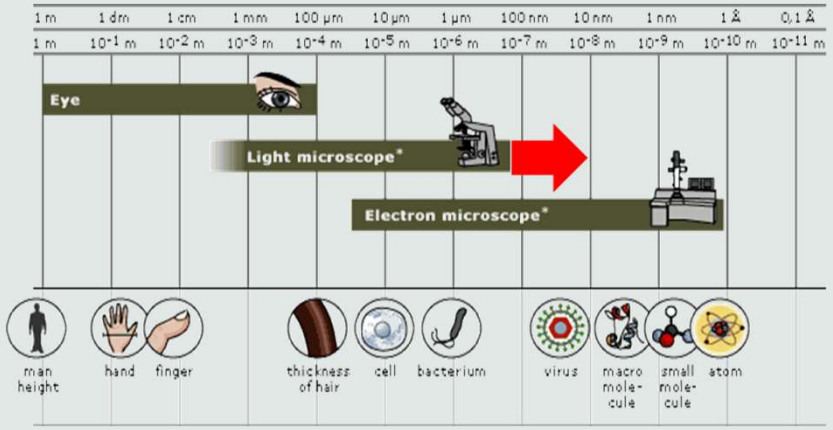


According to Geometrical optics: focal spot is a Point

Reality: it has Finite dimensions

Tightly focused beams form airy disc pattern



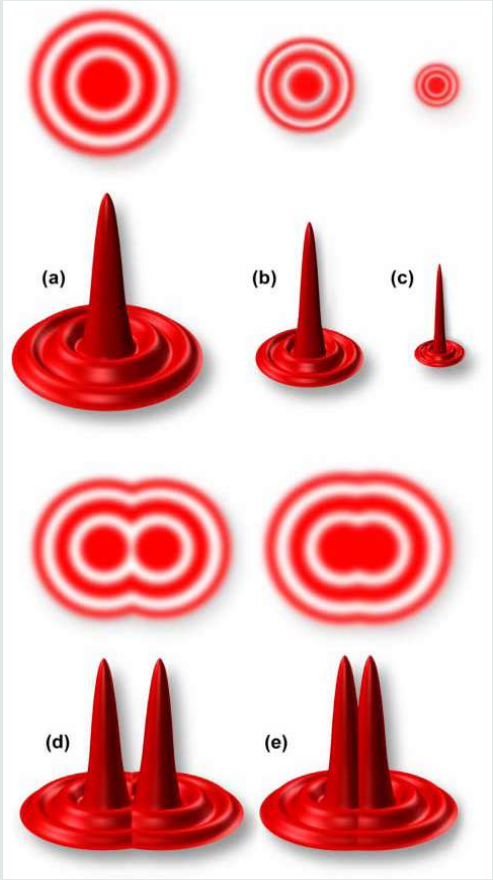


Airy Disks

➢The diffraction pattern resulting from a uniformly-illuminated circular aperture has a bright region in the center, known as the Airy disk which together with the series of concentric bright rings around is called the Airy pattern.

➢For small details in a specimen (rather than a grating), the objective projects the direct and diffracted light onto the image plane of the eyepiece diaphragm in the form of small, circular diffraction disks as Airy disks

➢High numerical aperture objectives capture more of the diffracted orders and produce smaller size disks than do low numerical aperture objectives.



Rayleigh Criterion

The Rayleigh criterion is the generally accepted criterion for the minimum resolvable detail - the imaging process is said to be diffraction-limited when the first diffraction minimum of the image of one source point coincides with the maximum of another.

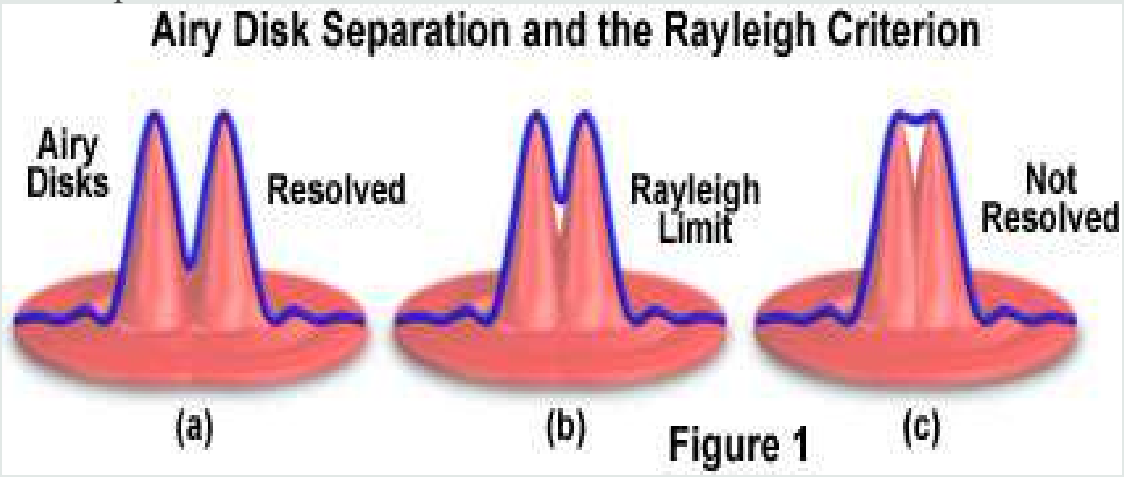


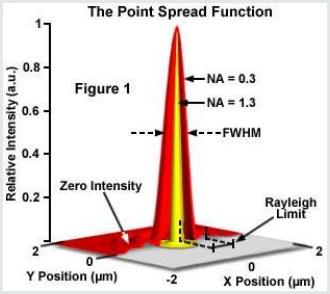
Figure 1

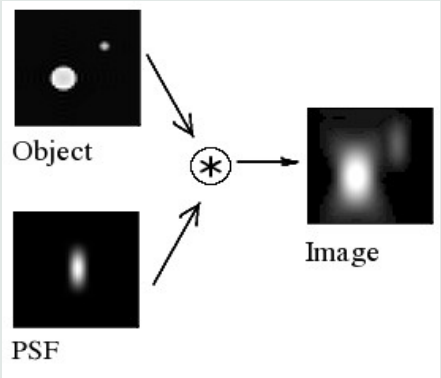
Minimum Separation between the centers of two Airy disks to be resolved is: $R = \frac{0.61\times\lambda}{N.A.} \approx \frac{\lambda}{2\times N.A.}$

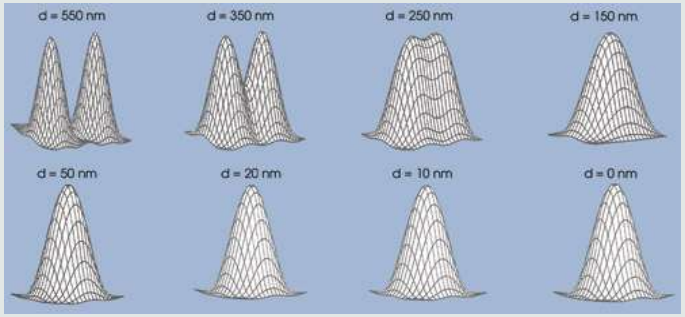
~ 200 nm for 400 nm light

Point Spread Function

Point **S**pread **F**unction (PSF)**:** describe the two-dimensional distribution of light in the telescope focal plane for astronomical point source**:** considered to be the fundamental unit of an image in theoretical models of image formation.







Rayleigh's resolution ~ 220 nm

Two objects residing within the Rayleigh resolution cannot be resolved

Way Out ?

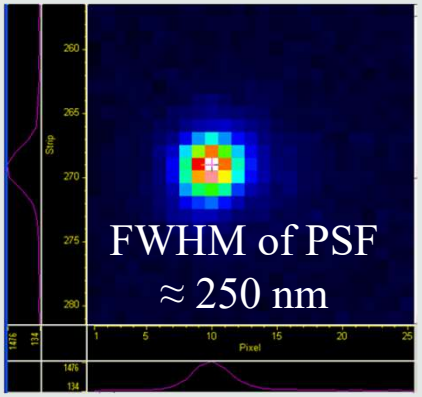
- Using the small probe once at a time: Single Molecule Spectroscopy; Photoactivated Localization Microscopy (PALM); Stochastic Optical Reconstruction Microscopy (STROM)

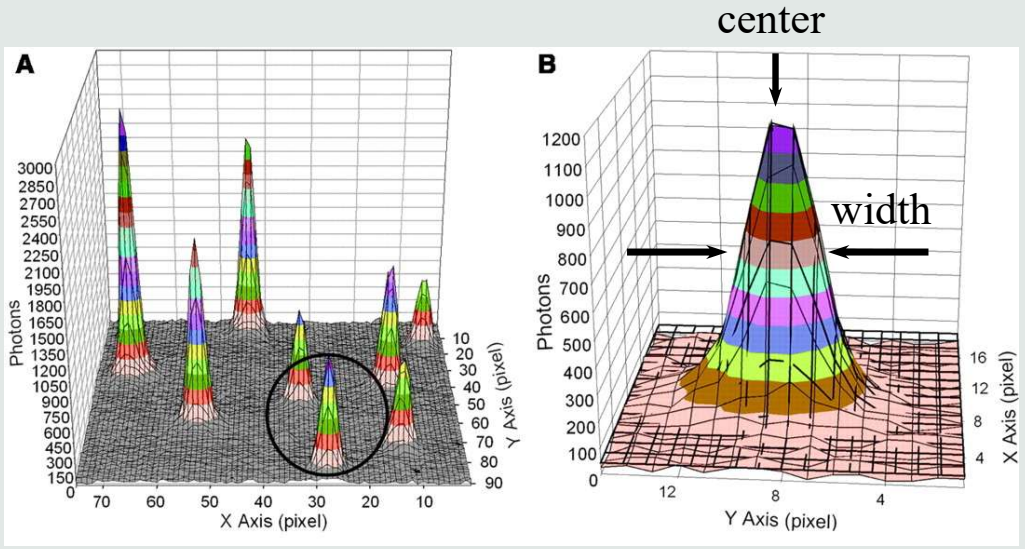
- Modifying the PSF using Structured light: Stimulated Emission Depletion Microscopy (STED)

Photoactivated Localization Microscopy (PALM)

Single Molecule Detection

If individual molecules can be used as a probe, it can give rise to a few nanometer (< 30 nm) resolution



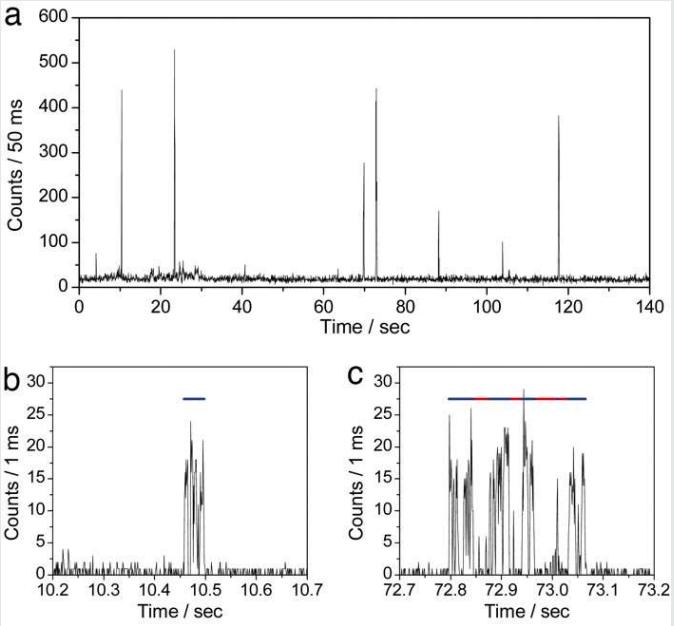


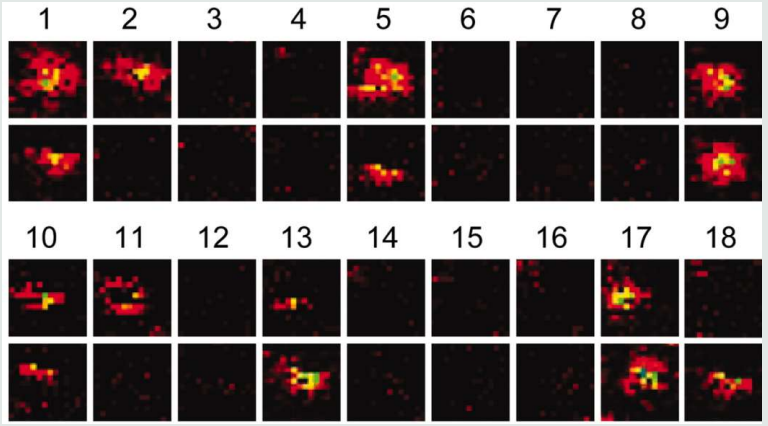
$$d_{PALM} = \frac{FWHM\ of\ the\ PSF}{\sqrt{Number\ of\ Photons\ collected\ (N)}}$$

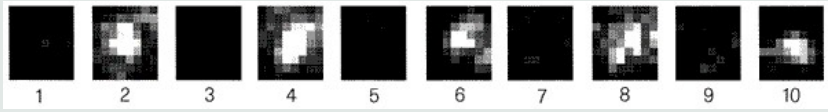
Typically N ~ 10^4 : Accuracy of center ~ ± 1.25 nm

Characteristics of the Single Molecule Fluorescence

Single molecule fluorescence shows blinking phenomena

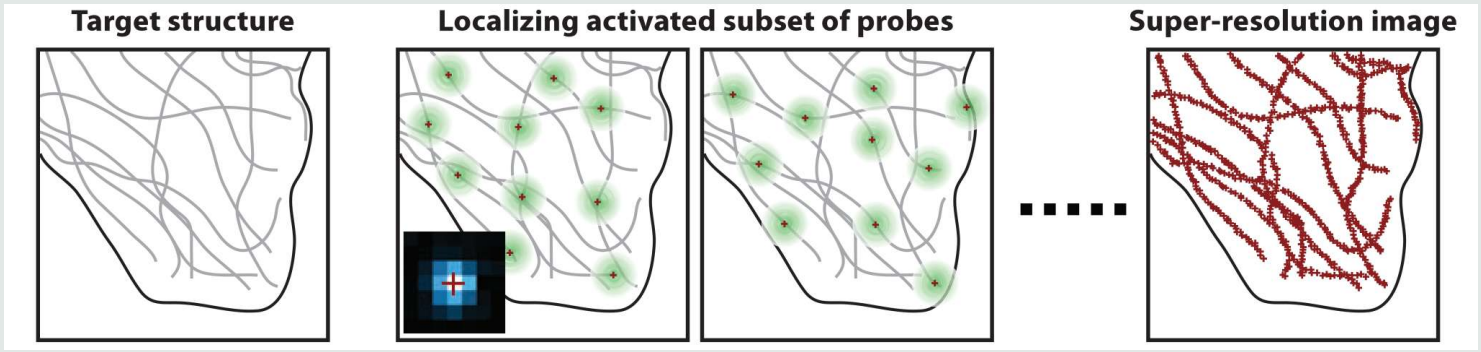


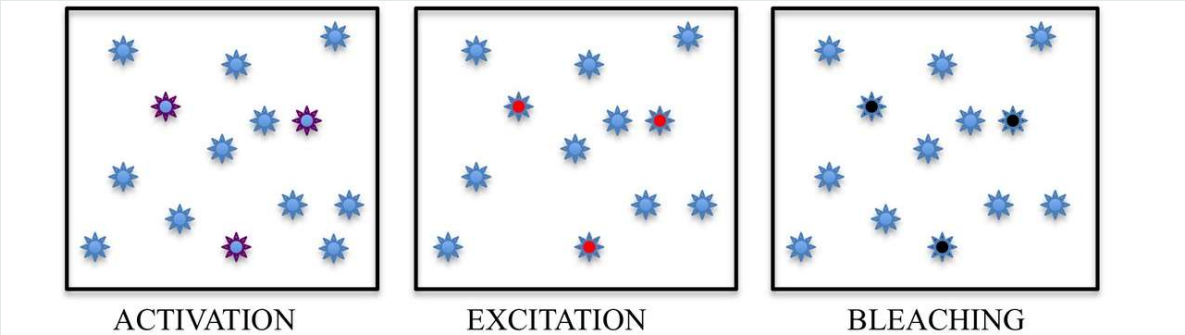


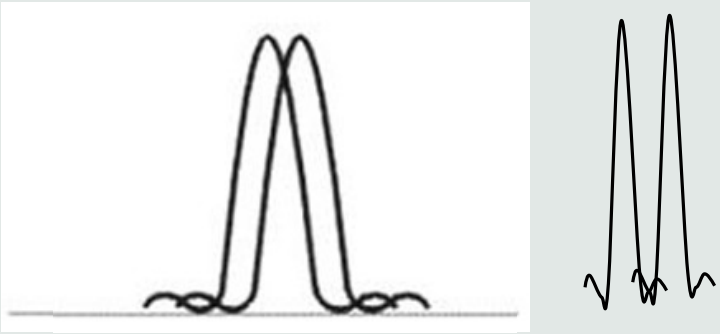


Exploiting the blinking process, in principle, fluorescence coming from a single fluorophore can be collected to give rise to the super resolved microscopy

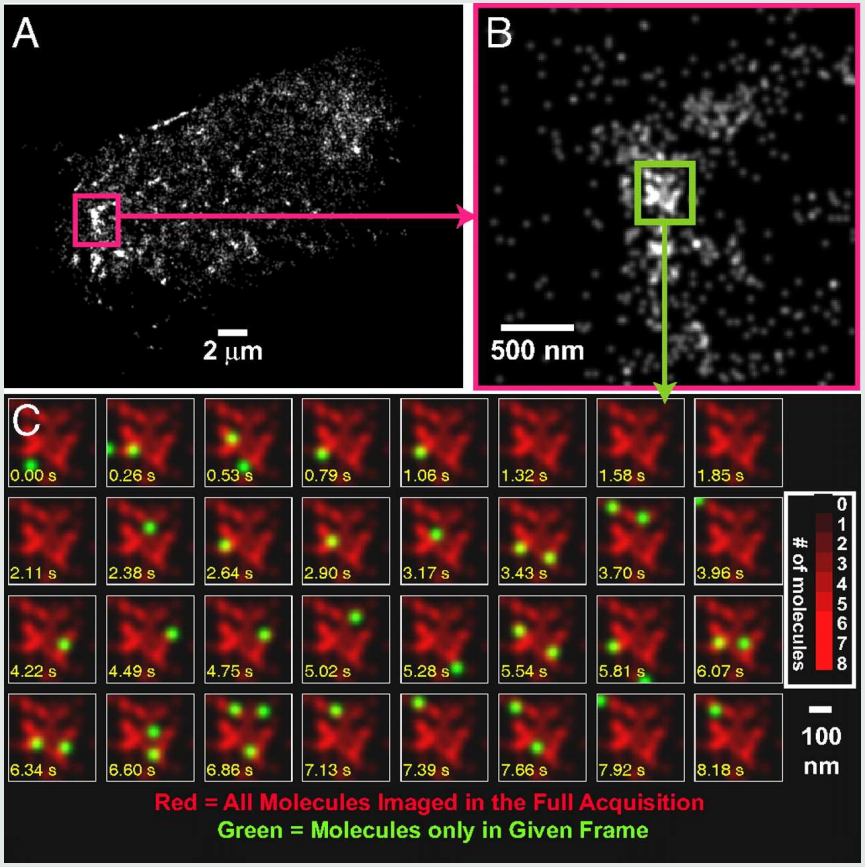
Mechanism of PALM



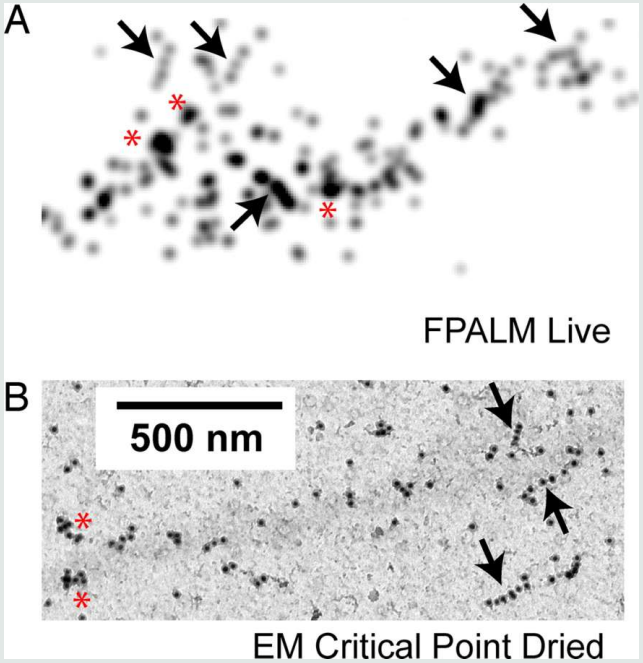




Mechanism of PALM



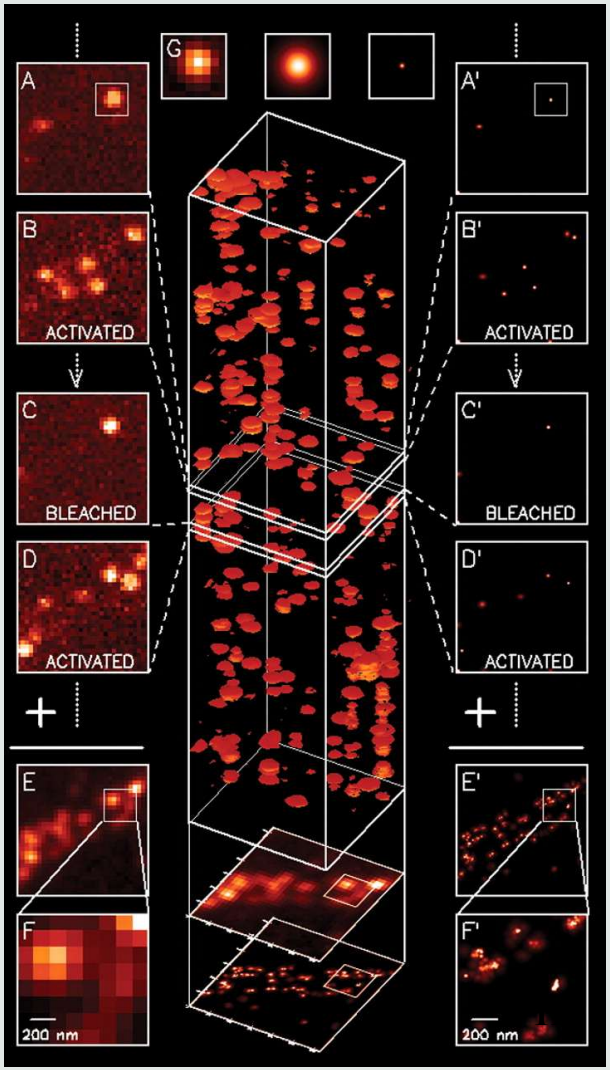
Time dependence of positions of localized HA molecules within an HA cluster in a live fibroblast at room temperature.



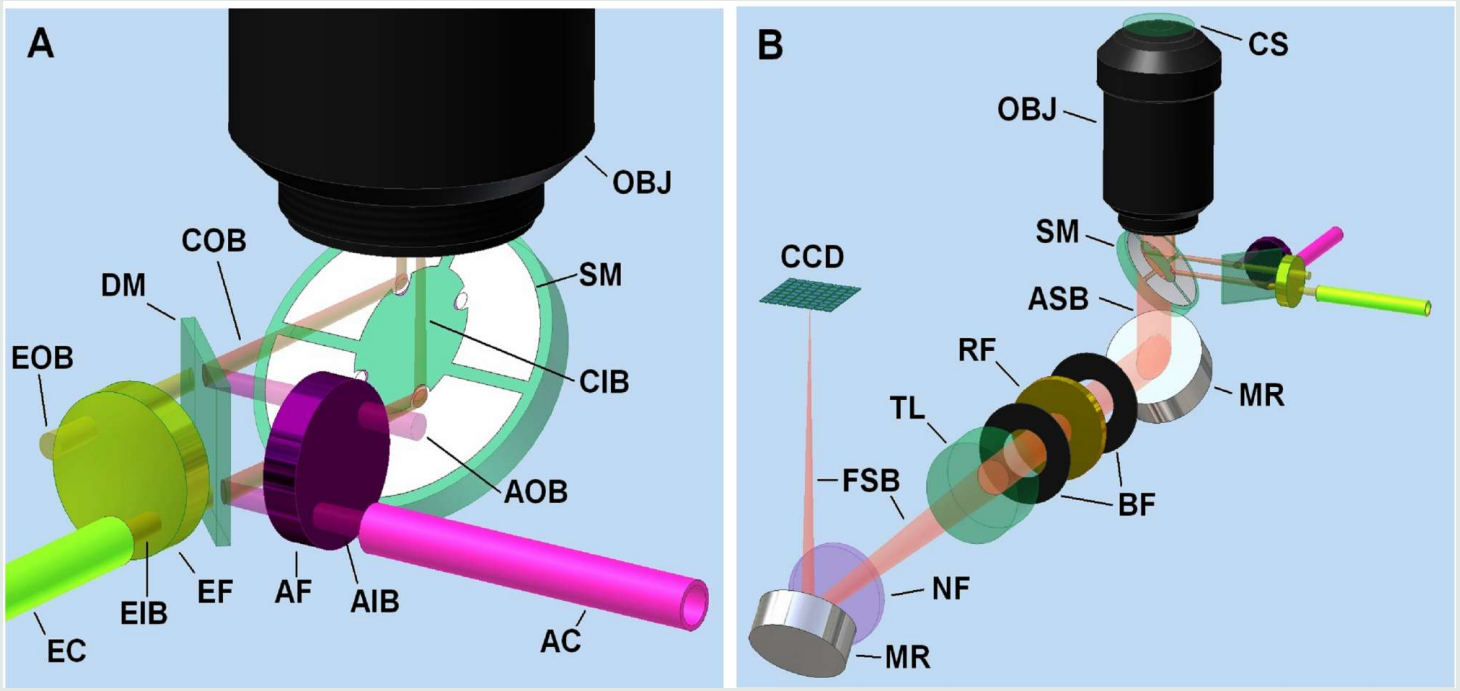
(A) FPALM image of a sub region of the coverslip-proximal plasma membrane of a live fibroblast, with molecular positions plotted in grey. (B) Transmission electron micrograph. Red asterisks: compact clusters and black arrows: elongated cluster.

Mechanism of PALM

The principle behind PALM. A sparse subset of PA-FP molecules that are attached to proteins of interest and then fixed within a cell are activated (A and B) with a brief laser pulse at λ_{act} = 405 mm and then imaged at λ_{exc} = 561 mm until most are bleached (C). This process is repeated many times (C and D) until the population of inactivated, unbleached molecules is depleted. Summing the molecular images across all frames results in a diffraction-limited image (E and F). However, if the location of each molecule is first determined by fitting the expected molecular image given by the PSF of the microscope [(G), centre] to the actual molecular image [(G), left], the molecule can be plotted [(G), right] as a Gaussian that has a standard deviation equal to the uncertainty $\sigma_{x,y}$ in the fitted position. Repeating with all molecules across all frames (A′ through D′) and summing the results yields a super resolution image (E′ and F′) in which resolution is dictated by the uncertainties σx,y as well as by the density of localized molecules..

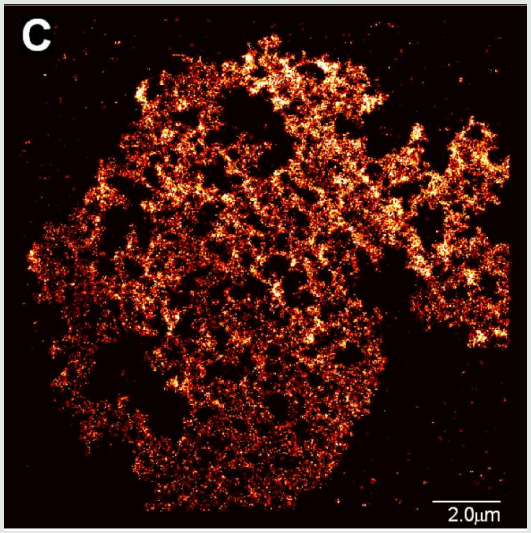


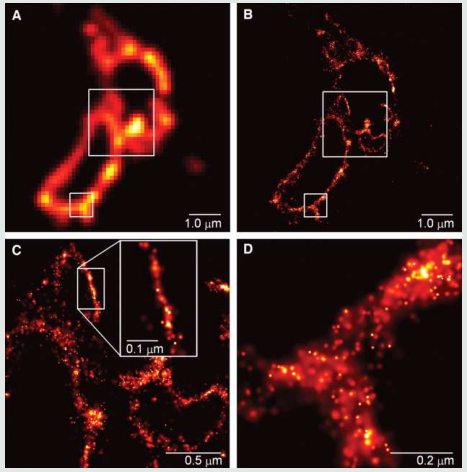
Experimental Setup

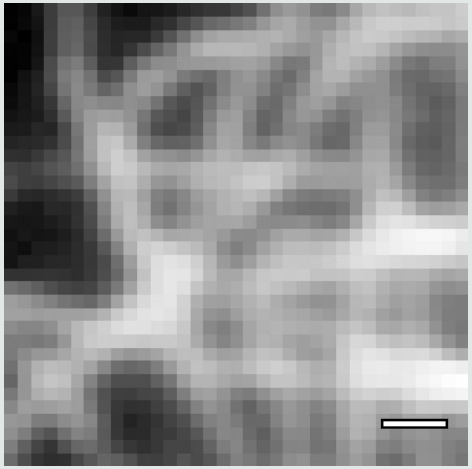


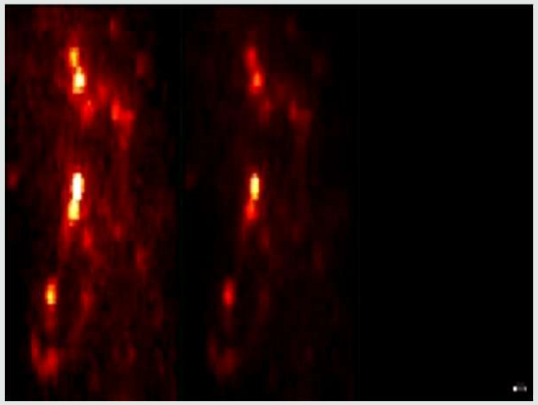
EIB: Excitation Input Beam; AIB: Activation Input Beam;
CIB: Combined Input Beam; ASB: Signal Beam

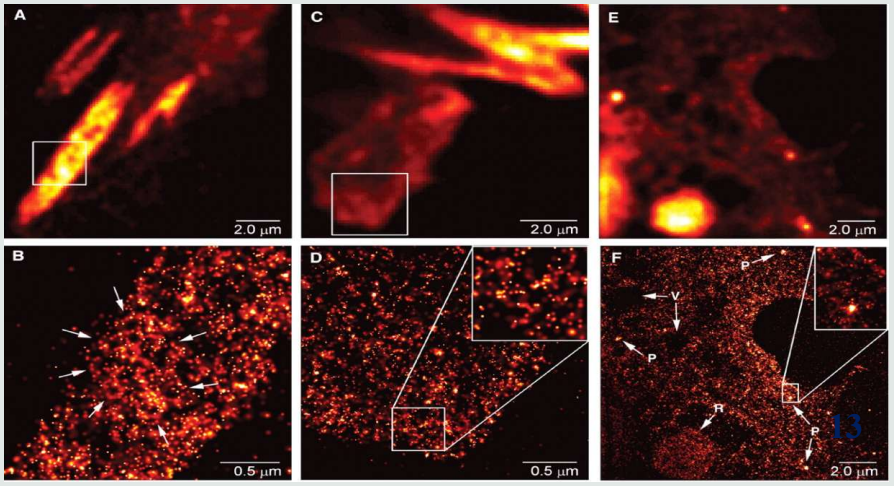
PALM Images


C
2.0μm

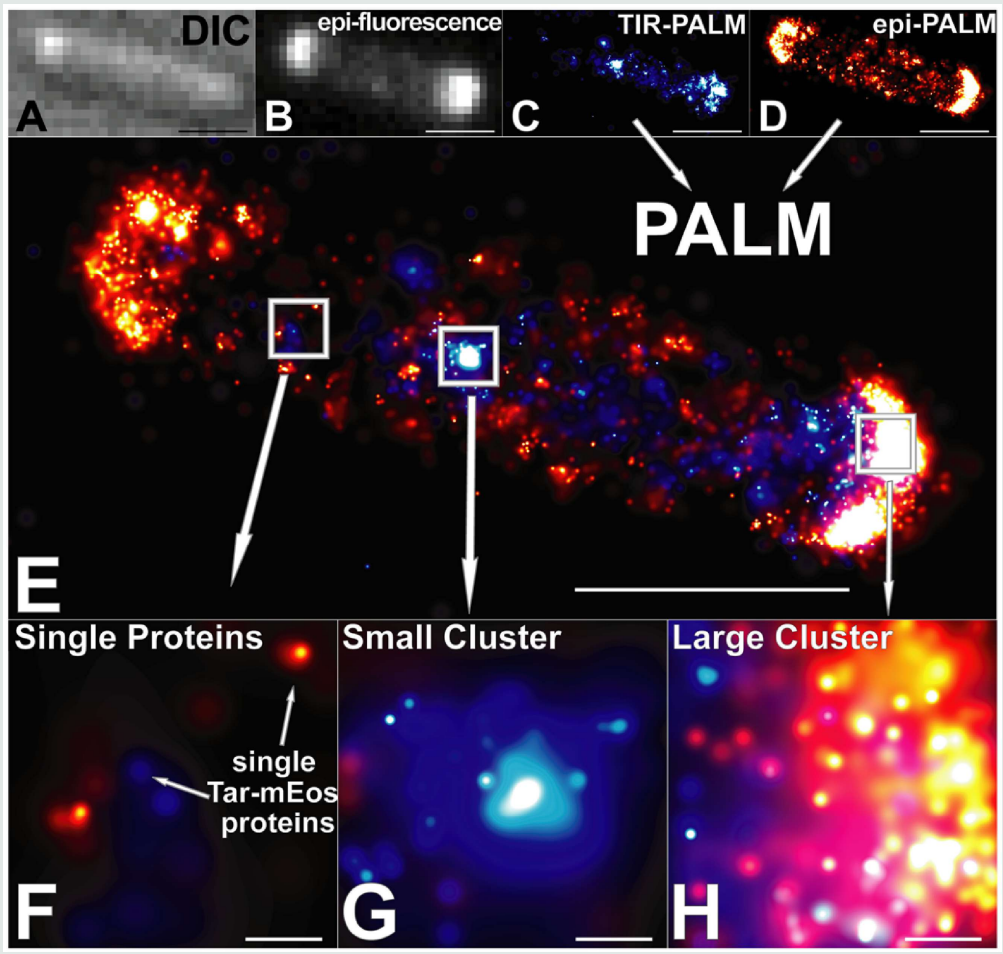

A
1.0 μm
B
1.0 μm
C
0.1 μm
0.5 μm
D
0.2 μm

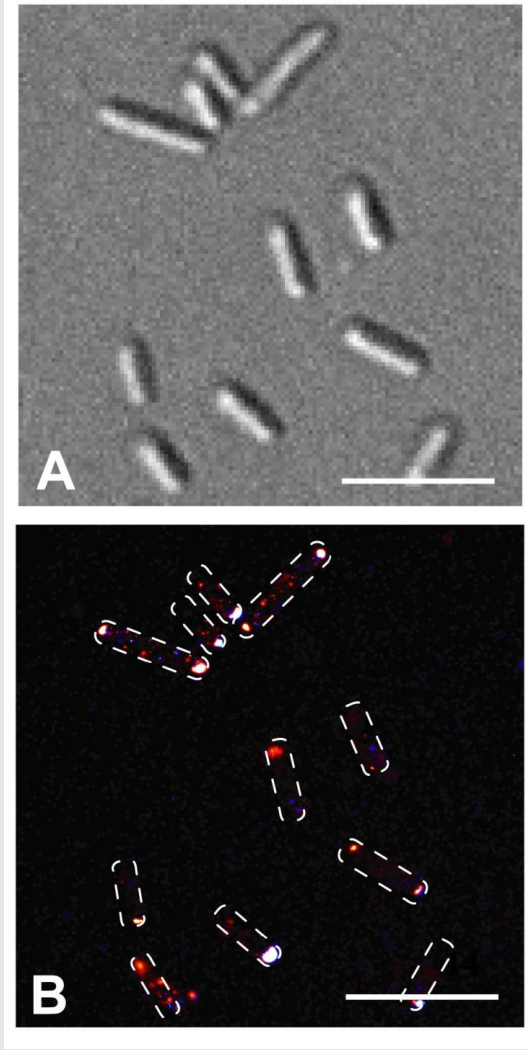




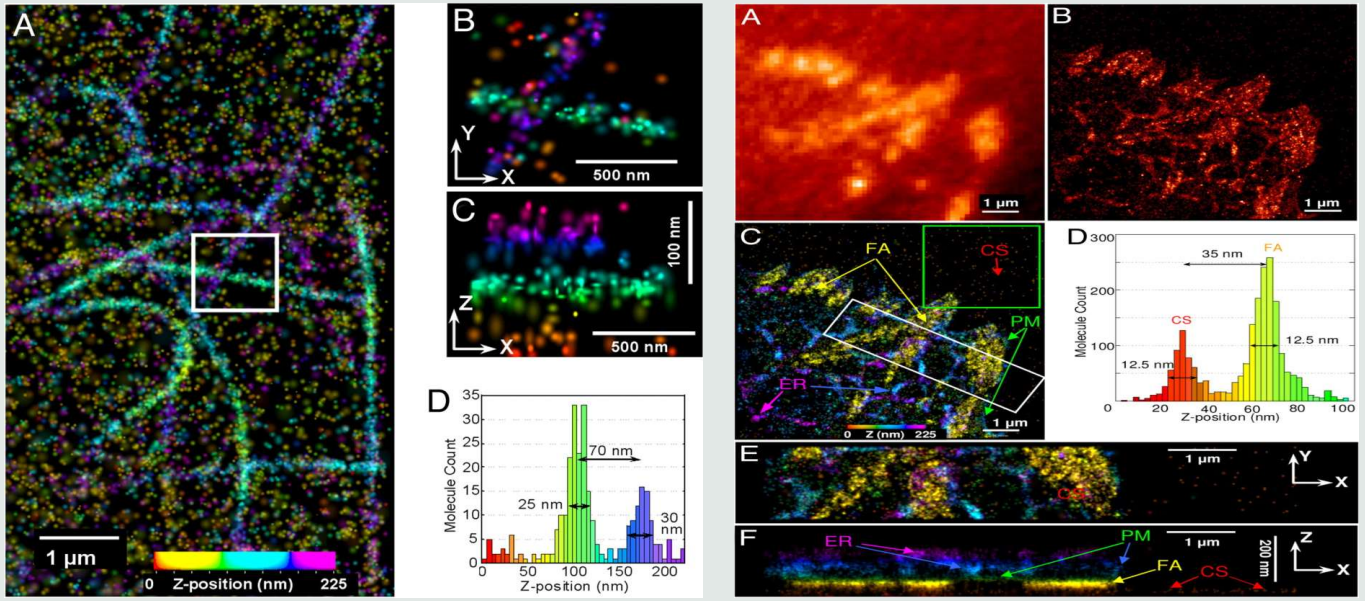

A
2.0 μm
C
2.0 μm
E
2.0 μm
B
0.5 μm
D
0.5 μm
F
V
P
P
R
P
2.0 μm

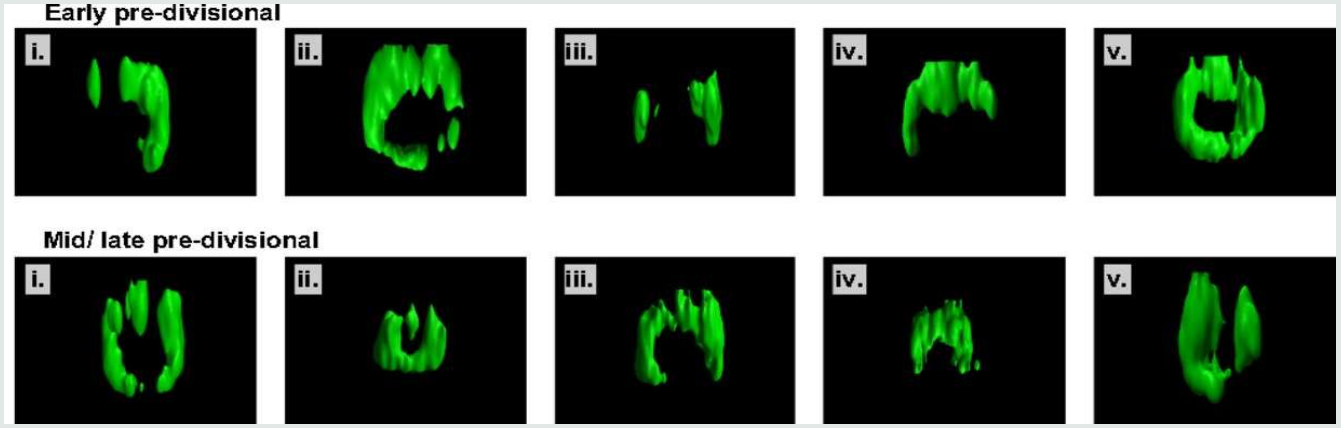
PALM Images


DIC
epi-fluorescence
TIR-PALM
epi-PALM
A
B
C
D
PALM
E
Single Proteins
Small Cluster
Large Cluster
single
Tar-mEos
proteins
F
G
H


A
B

3D PALM Images





PALM Summary

❖Using Photo activated dye to get nano-meter spatial resolution.

❖Using TIRF or Near TIRF angle illumination to reduce background to increase detection efficiency.

❖Product: Zeiss, Nikon, Leica

❖Advantage

➢Can get very high spatial resolution (~10-20nm) in 2D.

➢Can get high Z resolution (~60nm)

➢System will be cheaper than other high resolution product – half or less

❖Disadvantage:

➢Long Exposure to get image (15-30min)

➢Computation is required – not direct imaging.

✓ *Different Analysis software can output different result.*