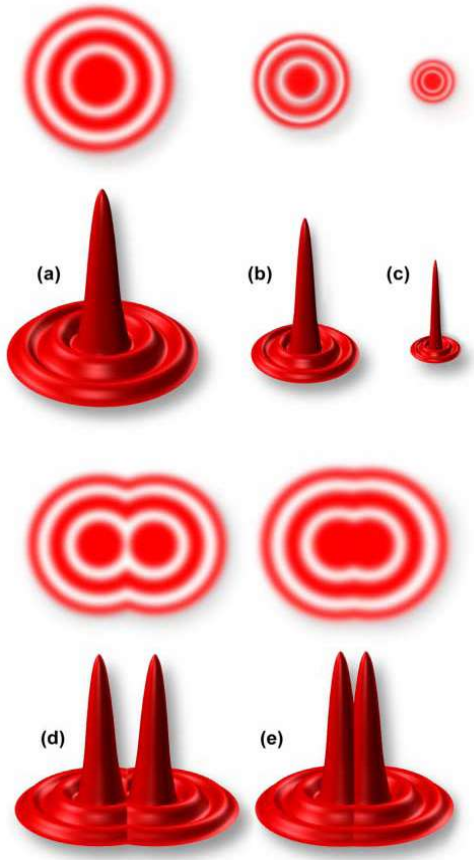
Lecture-13

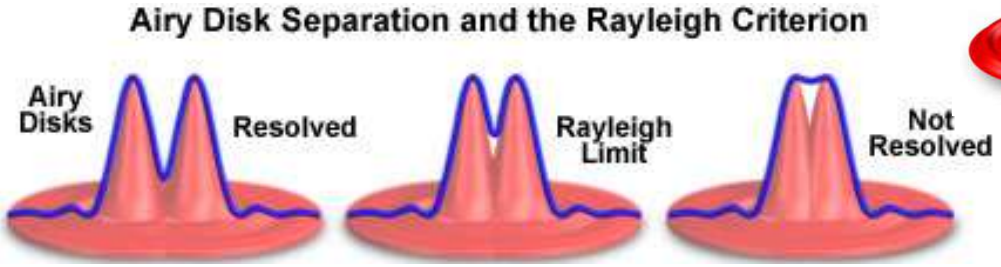
CSO202: Atoms, Photons & Molecules

Debabrata Goswami

MODULE 4 :

SUPER-RESOLUTION: SUPER-RESOLVED FLUORESCENCE MICROSCOPY

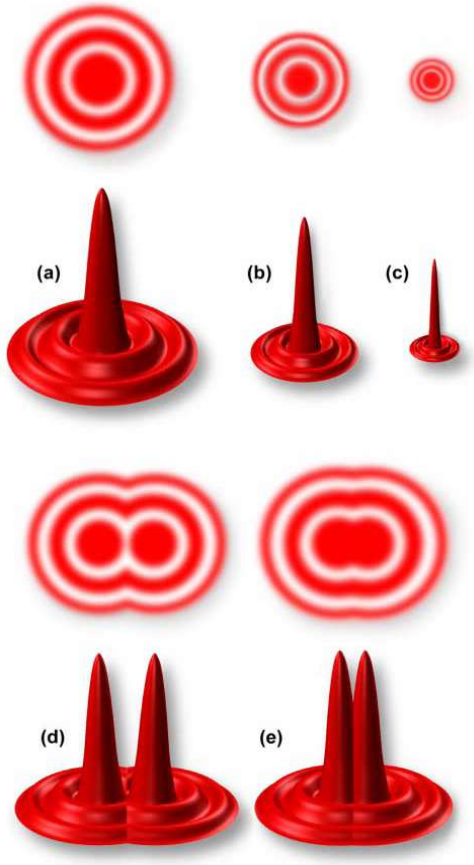




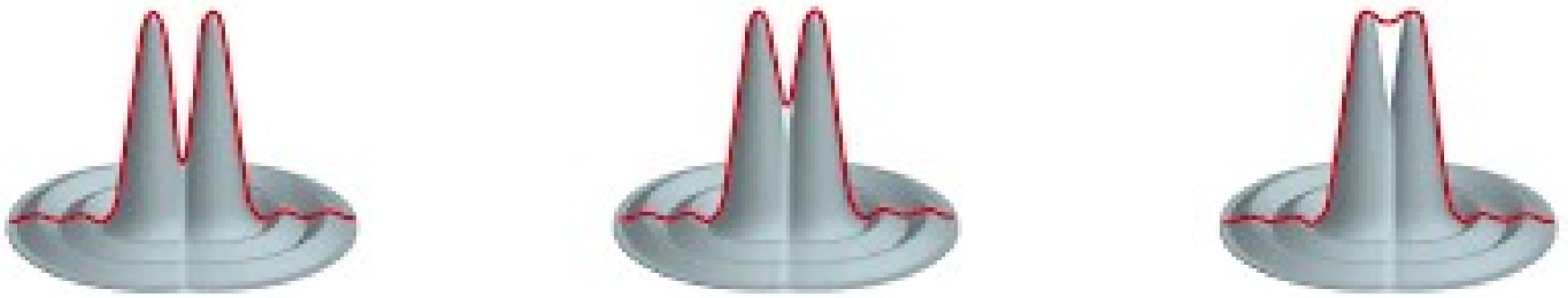
- The **Airy disk** (or **Airy disc**) and **Airy pattern** are descriptions of the best-focused spot of light that a perfect lens with a circular aperture can make, limited by the diffraction of light.
- The diffraction pattern resulting from a uniformly illuminated, circular aperture has a bright central region, known as the Airy disk, which together with the series of concentric rings around is called the Airy 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.



DEFINING RESOLUTION



RESOLVED **RAYLEIGH LIMIT** **NOT RESOLVED**

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.

Super-resolution microscopy is about enhancing the diffraction-limited resolution of a **microscope**

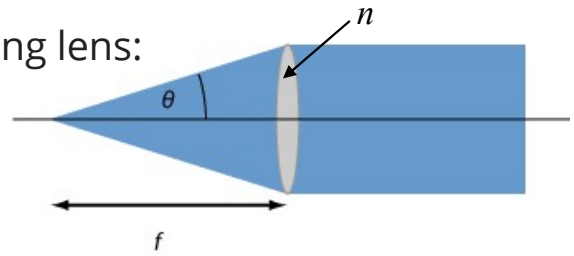
NUMERICAL APERTURE OF AN OPTICAL SYSTEM

The numerical aperture of an optical system is defined as the product of the **refractive index** of the beam from which the light input is received and the sine of the maximum ray angle against the axis, for which light can be transmitted through the system based on purely geometric considerations (*ray optics*):

$$NA = n \sin \theta_{max}$$

For the maximum incidence angle, the requirement is that the light can get through the whole system and not only through an entrance aperture.

Example:
A simple case of a collimating lens:



A collimating lens can theoretically accept light from a cone, the opening angle of which is limited by its size

The extreme rays that define the subtended angle (θ_{max}) are limited by the size of the lens, or in some cases somewhat less if there is a non-transparent holder. It is often not recommended to operate a lens or its full area, since there could be substantial spherical aberrations. The numerical aperture, however, is a completely geometrical measure, which is not considering such aspects. The numerical aperture of the lens is determined by its active diameter and its focal length.

MORE ON THE NUMERICAL APERTURE OF A LENS

A lens may not be designed for collimating light. It could, for example, be designed for imaging objects at a large distance. In that case, one will consider rays coming from that distant object, and the obtained numerical aperture will be correspondingly smaller – sometimes even much smaller. This shows that the numerical aperture depends on the location of some object plane determined by the designer according to the intended use.

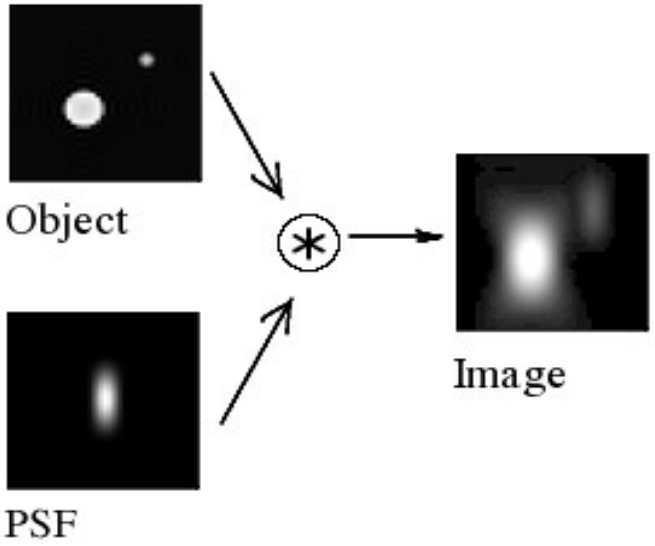
Some lenses are used for focusing collimated laser beams to small spots. The numerical aperture of such a lens depends on its aperture and focal length, just as for the collimation lens discussed above. The beam radius w_{lens} at the lens must be small enough to avoid truncation or excessive spherical aberrations. Typically, it will be of the order of half the aperture radius of the lens (or perhaps slight larger), and in that case ($w_{lens} = D / 4 = \mathrm{NA} \cdot f / 2$, with the beam divergence angle being only half the NA) the achievable **beam radius** at the focus w_f is

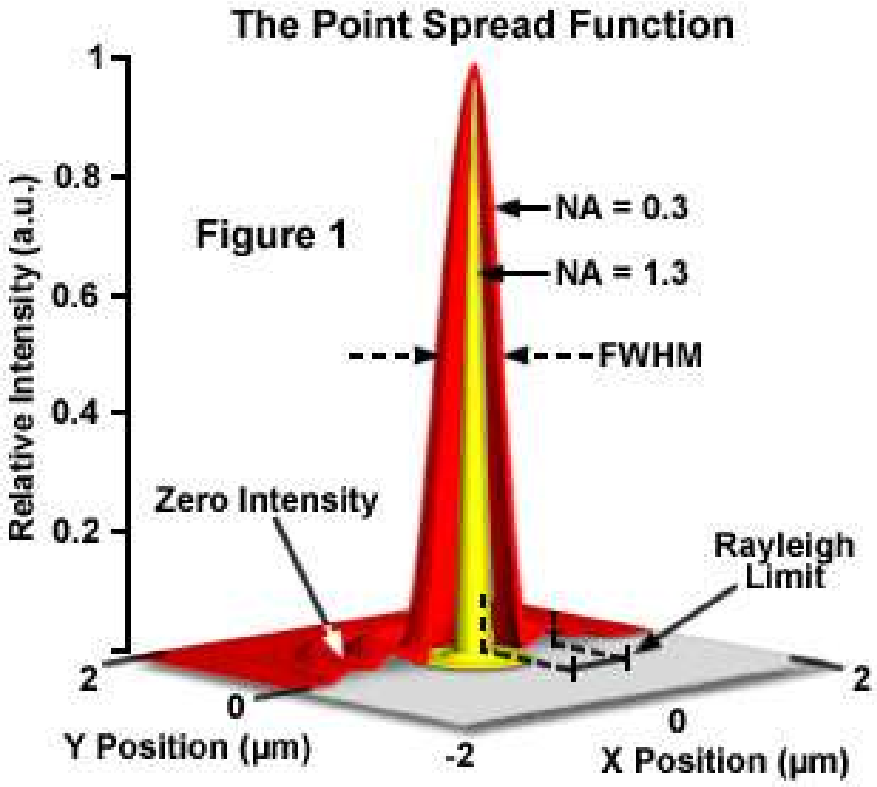
$$w_f = \frac{\lambda f}{\pi w_0} = \frac{4 \lambda f}{\pi D} \approx \frac{2 \lambda}{\pi \, \mathrm{NA}}$$

where D is the aperture diameter, f the focal length and λ the optical wavelength. Note that the calculation is based on the **paraxial approximation** and therefore not accurate for cases with very high NA. A somewhat smaller spot size may be possible with correspondingly larger input beam radius.

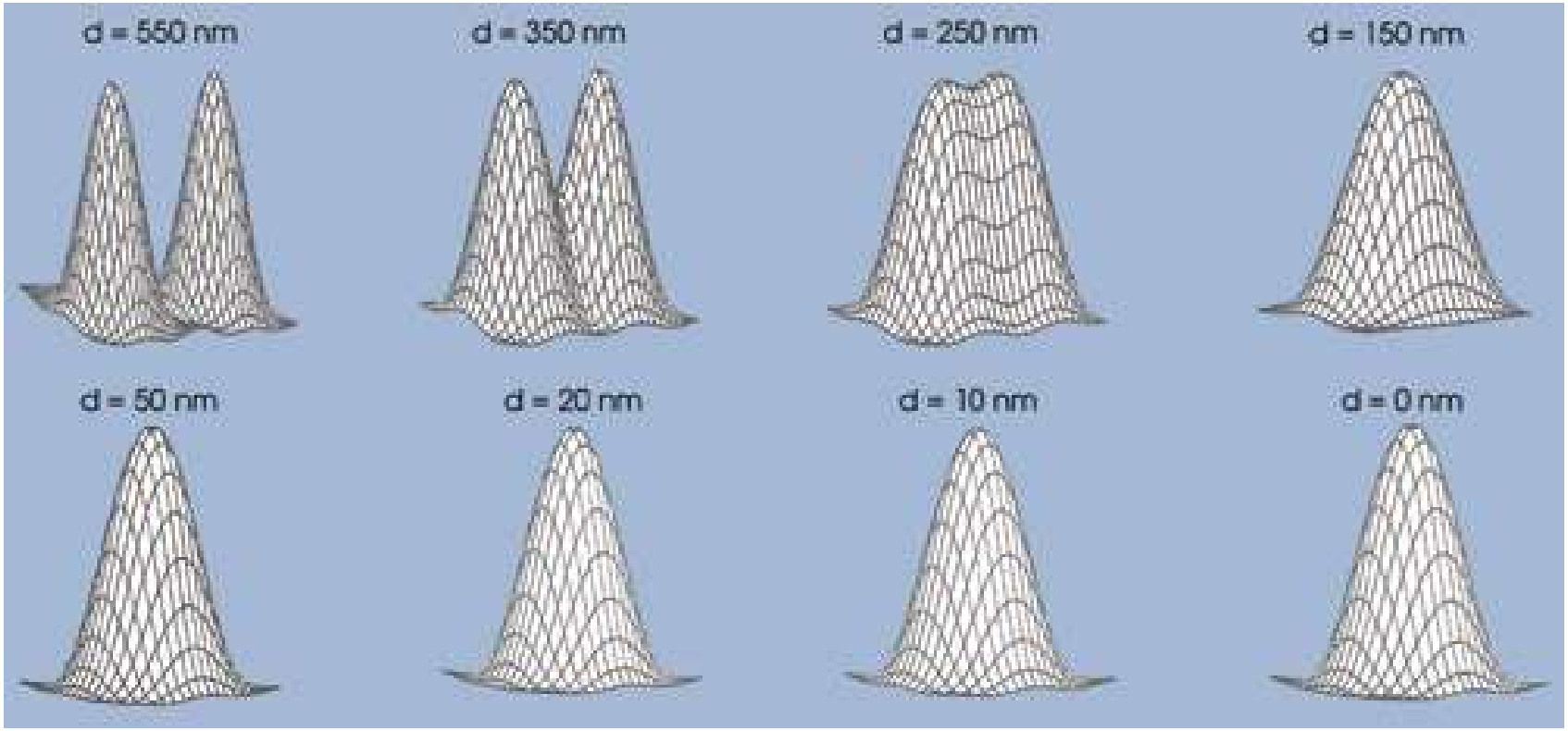
POINT SPREAD FUNCTION

- **P**oint **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.





TWO OBJECTS RESIDING WITHIN THE RAYLEIGH RESOLUTION CANNOT BE RESOLVED



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

Rayleigh's resolution ~ 200 nm for 400 nm light

Super-resolved Fluorescence Microscopy

The 2014 Nobel Prize in Chemistry was awarded to **Stefan W Hell**, **Eric Betzig** and **William E Moerner** for their contributions concerning the the development of superresolution microscopy.

Their research has been of great importance for the advancement of the new field of **Superresolution Microscopy** and has provided a much more detailed understanding of the fundamental processes in chemistry and biology.

IMPACT : BREAKS THE LAW THAT IS MORE THAN 140 YEARS OLD & ETCHED IN STONE !

Memorial to Ernst Karl Abbe, who, in 1873, approximated the diffraction limit of a microscope as, $d = \frac{\lambda}{2n\,sin}$, where d is the resolvable feature size, λ is the wavelength of light, n is the index of refraction of the medium being imaged in, and θ (depicted as α in the inscription) is the half-angle subtended by the optical objective lens (representing the numerical aperture).

Ernst Abbe memorial stone at the Friedrich Schiller University of Jena, built 1977

HOW WAS THIS DIFFRACTION LIMIT LIMITATION FINALLY SURPASSED?

THE WAY OUT…

Two approaches to surpass Abbe's magic limit (chronologically):

1. Modifying the Point Spread Function (PSF) using Structured light
   - STimulated Emission Depletion (STED) microscopy: S. Hell
2. Using a small probe once at a time
   - PhotoActivated Localization Microscopy (PALM): E. Betzig & W.E. Moerner

Interesting Note: W. E. Moerner – first to detect a single fluorescent molecule—1989

(1997) Eight years later Moerner took the next step towards single-molecule microscopy, building on the previously Nobel Prize-awarded discovery of *the green fluorescent protein (GFP)*. Roger Tsien (Nobel 2008)

Nature, (1997) Molecular-sized lamps turning on and off

Also Note, the different contexts for the use of the term "Single Molecule". Here, it is an 'on an average' measure, while in Module 1, it was truly 'single molecule'.

THE PRIZE

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

The STED microscope collects light from a multitude of small volumes to create a large whole. In contrast, the second principle rewarded, *single-molecule microscopy*, entails the superposition of several images. Eric Betzig and W. E. Moerner (who always has been called by his initials, W. E.) have independently of each other contributed different fundamental insights in its development. The foundation was laid when W. E. Moerner succeeded in detecting a single small fluorescent molecule.

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

Further methods developed afterwards, for example:
Stochastic Optical Reconstruction Microscopy (STROM)
(not recognized in the prize)

The Principle of STED Microscopy (Theoretical Proposal, Hell 1994)