Module 4: Superresolution

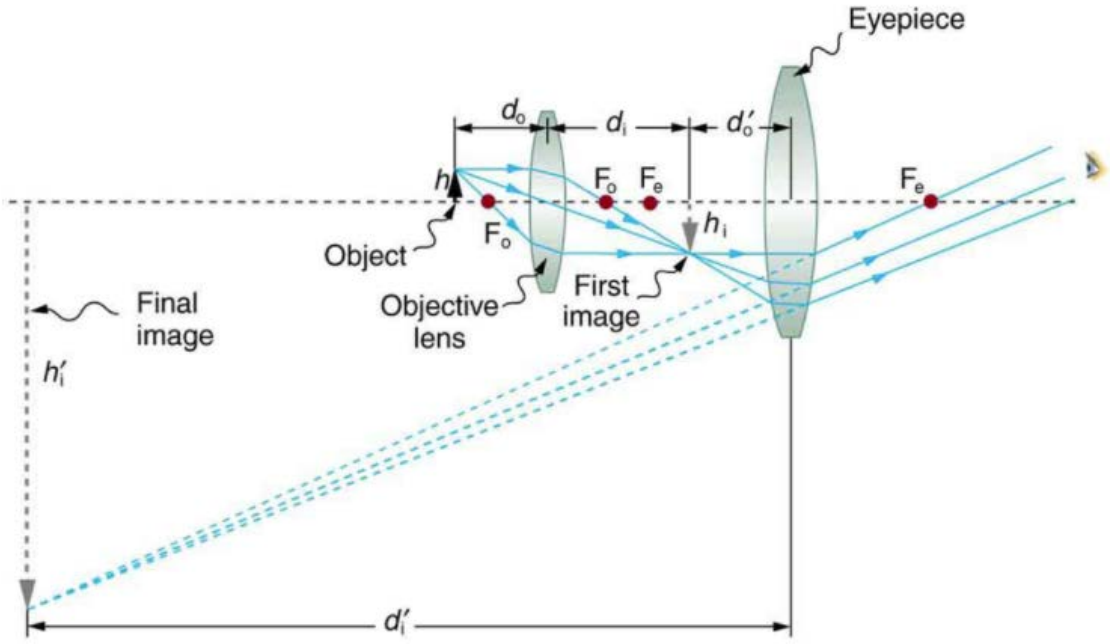
Problem Set

1. The Rayleigh criteria is given as: $\theta_{min} = 1.22\frac{\lambda}{D}$, where θ_{min} is the smallest resolvable angle, λ is the wavelength of light used and *D* is the diameter of the aperture or lens used. On the other hand, the Abbe limit is given as: $d = \frac{\lambda}{2\,n\sin(\alpha)} = \frac{\lambda}{2\,\mathrm{NA}}$, where *d* is the smallest resolvable angle, *n* is the refractive index of the medium between the object and the optical system, α is the biggest scattering angle (incident on the optical system) and NA is the numerical aperture. Since superresolution microscopy involves both, how do you relate them?

Ans. Both equations are in fact structurally similar. Rayleigh developed his criterion on diffraction of light on slits, whereas Abbe was working on microscopy. Therefore, you have a refraction index in one and not in the other. Rayleigh criteria is basically predicting an angular resolution for an object that is infinitely far away, the other a spatial resolution for an object that is close. However, you could bring the Rayleigh version close to the Abbe's. Rayleigh stated the following: $\theta_{min} \approx 1.22\lambda/d$, where θ_{min} represents the minimum angular radius of an Airy disk as seen from the center of the circular aperture, λ the wavelength of light and d the diameter of circular aperture. The factor 1.22 comes from the definition of Bessel function of 1st kind, the fact that 1st minima of the diffraction pattern appear at 1.22 units from the central zero. Now this is angular separation, and we have to bring it to spatial separation via $x_{min} = R\,sin(\theta_{min})$ and we get $x_{min} \approx 1.22\lambda R/d$, where R is distance between the slit and imaging screen. Now we can convert the R/d into the *sin*α term using *sin*α = (d/2)/R, and we get: $x_{min} \approx 0.61\frac{\lambda}{n\sin\alpha} \approx 0.61\frac{\lambda}{NA}$ and this is very close to Abbe's limit: $x_{min} = 0.5\frac{\lambda}{NA}$. So, all in all it is just how you define the minimum distance at which you can still separate two sources. Another way to look at it is to also realize that: The Rayleigh criterion states that in order for 2 closely placed PSF to

be resolved, the central maxima of one should lie exactly at the first minima of the second one. Since the Airy pattern is defined by the Bessel function, the minimum separation between the 2 patterns should be 1.22λ/2NA instead of just λ/2NA considering that the first minima will be at 1.22 times the unit from the central maxima.

2. Calculate the magnification of an object placed 6.20 mm from a compound microscope that has a 6.00 mm focal length objective and a 50.0 mm focal length eyepiece. The objective and eyepiece are separated by 23.0 cm.



Ans. The situation is similar to that shown in the figure above. To find the overall magnification, we must find the magnification of the objective, then the magnification of the eyepiece. This involves using the thin lens equation. The magnification of the objective lens is given as: $m_o = -\frac{d_i}{d_o}$, where d_o and d_i are the object and image distances, respectively, for the objective lens as labeled in the Figure. The object distance is given as: d_o = 6.20 mm, but the image distance, d_i, is not known. Isolating d_i, we have:

$\frac{1}{d_i} = \frac{1}{f_o} - \frac{1}{d_o}$, where f_o is the focal length of the objective lens. Substituting known values gives: $\frac{1}{d_i} = \frac{1}{6.00mm} - \frac{1}{6.20mm} = \frac{0.00538}{mm}$.

We invert this to find d_i : $d_i = 186$ mm

Substituting this into the expression for m_o gives: $m_o = -\frac{d_i}{d_o} = -\frac{186mm}{6.20mm} = -30.0.$

Now we must find the magnification of the eyepiece, which is given by:

$$m_e = -\frac{d_i'}{d_o'},$$

where d'_i and d'_o are the image and object distances for the eyepiece (see Figure). The object distance is the distance of the first image from the eyepiece. Since the first image is 186 mm to the right of the objective and the eyepiece is 230 mm to the right of the objective, the object distance is $d'_o = 230mm - 186mm = 44.0mm$. This places the first image closer to the eyepiece than its focal length, so that the eyepiece will form a case 2 image as shown in the figure. We still need to find the location of the final image d'_i in order to find the magnification. This is done as before to obtain a value for $1/d'_i$:

$$\frac{1}{d_i'} = \frac{1}{f_e} - \frac{1}{d_o'} = \frac{1}{50.0mm} - \frac{1}{44.0mm} = -\frac{0.00273}{mm}.$$

Inverting gives: $d_i' = -\frac{mm}{0.00273} = -367mm.$

The eyepiece's magnification is thus: $m_e = -\frac{d_i'}{d_o'} = -\frac{-367mm}{44.0mm} = 8.33.$

So, the overall magnification is: $m = m_o m_e = (-30.0)(8.33) = -250.$

3. Consider the STED principle from one of your reading materials.

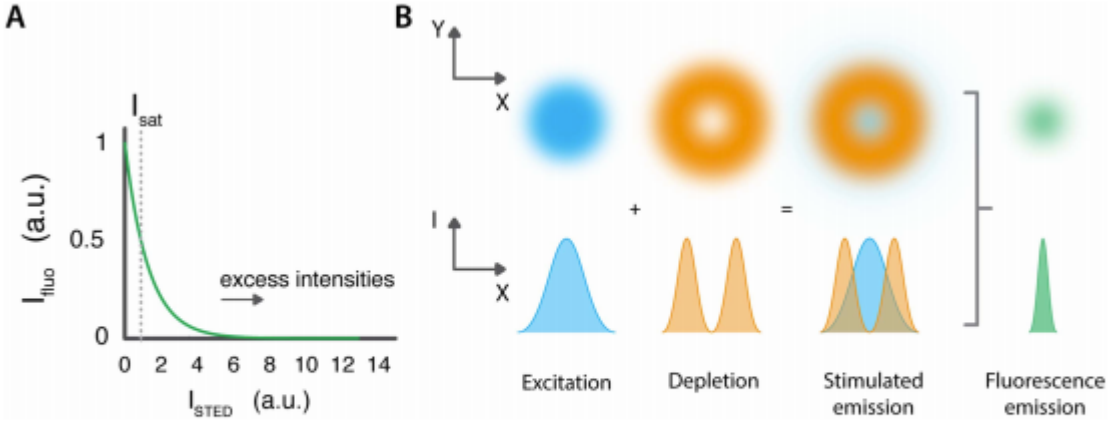


Figure 35. The STED principle. (A) The ability of a fluorophore to emit fluorescence decreases nearly exponentially with the intensity of the beam de-exciting the fluorophore by stimulated emission. I_{sat} is defined as the intensity at which the fluorescence signal is reduced by 50%. (B) The confocal excitation beam is approximately Gaussian in shape while the depletion beam is donut shaped, i.e. it features a central region of (near) zero intensity. After stimulated emission of fluorophores in the donut region, spontaneous emission from the center most molecules can be recorded. Modified from Göttfert *et al* [70].

In STED, the achievable resolution is strongly tied to the efficiency of the stimulated emission process, which in turn is directly proportional to the depletion beam intensity. Moreover, high intensity beam results in a steeper intensity gradient between the central zero and donut crest, reducing the diameter of the zero-intensity region from which fluorescence is ultimately collected. STED resolution and its relation to the depletion intensity can elegantly be expressed as a modified version of Abbe's formula: $$d = \frac{\lambda}{2 \cdot NA \cdot \sqrt{1 + \frac{I}{I_{sat}}}}$$

where, I is the applied STED intensity and I_{sat} the saturation intensity of the fluorophore, with d a measure for lateral resolution. Based on this expression, comment on the resolution of a STEAD microscope.

Ans. Based on the expression: $d = \frac{\lambda}{2 \cdot NA \cdot \sqrt{1 + \frac{I}{I_{sat}}}}$, it possible to state that given a

sufficiently powerful depletion laser, the resolution would practically be unlimited. While it is true that in some very particular cases, extremely high resolutions, (PSF FWHM < 6 nm) could be attained when imaging highly stable nitrogen vacancy defects in diamond samples, in general, however, values in the 30 to 80 nm range are more common. This is because, at increased intensities, photobleaching becomes a significant issue for most emitters. This is why, perhaps more so than in any other super-resolution modality, the illumination conditions and characteristics of the excitation and depletion sources become critical determinants of the attainable resolution. They are perhaps as important as the choice of fluorophore. In STED, the depletion source ideally saturates stimulated emission, strongly suppressing population of the electronic excited state. This would prevent processes competing with fluorescence emission from occurring at all and thus ultimately reduce photobleaching. Unfortunately, higher state transitions and triplet state interconversions are typically red shifted, increasing the risk of re-excitation by the depletion laser. Even if the probability of these transitions is low, i.e. if their excitation cross section is low, they are almost impossible to avoid at the high laser intensities used in a typical STED experiment. Moreover, due to the long lifetime of the first triplet state, there is a significant risk of ‘pile up’ or saturation of this state, further increasing the risk of triplet-triplet transitions. So, in order to mitigate the risk of higher state excitation, the depletion pulse is typically delayed relative to the excitation.