Exercise 06

1 Super-resolution Microscopy

1.1 PALM - Localization precision

The localization precision can be decomposed into three contributions:

- Theoretically minimal localization precision
- Finite pixel size
- Background noise

All together give the localization limit as:

$$\sigma_{\mu_i} = \sqrt{\frac{s_i^2}{N} + \frac{a^2}{12N} + \frac{8\pi s_i^4 b^2}{a^2 N^2}}$$

s_i: width of the point spread function (PSF): $s_i \approx \frac{\lambda}{2NA}$.
N: number of photons
a: pixel size, by means of the size in the sample which gets mapped to one pixel on the camera chip
b: standart deviation of the background signal[1]

Solve questions (a-e) under the following assumptions: $NA = 1.44$, $a = 120\,\text{nm}$, $b = 4$

(a) Calculate the localization precision for *PA-GFP*, $\lambda_{\text{em}} = 517\,\text{nm}$, typical $N = 300$:

(i) Simple equation, not taking into account the pixel size and the background ($a = 0$, $b = 0$)

(ii) Taking finite pixel size into account

(iii) Also taking background noise into account

(iv) Under the given condition, Which factor contributes the most to the localization precision?

(b) Compare the localization precision of *PA-GFP* to that of the far-red dye *Alexa647* with $\lambda_{\text{em}} = 665\,\text{nm}$, $N = 6000$

(c) For the *PA-GFP*, how much would the photon number need to increase to compensate for localization precision loss due to the pixel size and background?

According tho the *Nyquist criterion* a certain number of samples are needed to resolve a structure. Which means a certain number of molecules. For a given spatial resolution (or spatial frequency) α and the dimensionality D the minimal required molecular density $\rho = \left(\frac{2}{\alpha}\right)^D$. As a **frame** we understand one cycle of PALM procedure, when under weak UV excitation small number of molecules is excited. By accumulating signals from randomly excited and further bleaching molecules through many cycles we get the final picture – see PALM principle.

(d) What is the maximal fluorophore density per frame in order to be able to properly localize them for the fluorophore *PA-GFP*?

(e) In order to prevent overlapping PSFs, imaging happens at an average fluorophore density of $1\,\mu\text{m}^{-2}$ per frame. How many frames are needed to be acquired minimally to reach a Nyquist resolution that is close to the localization precision found in (a.i)?

1.2 STED Microscopy

(a) Figure 1 represents a STED microscope. All the six necessary components of the STED microscope are numbered from **1** to **6**. Considering the principals of STED microscopy pick the correct element from the following list for each numbered component.

(i) Sample
(ii) Detector
(iii) Phase plate
(iv) Linear polarizer
(v) Lens
(vi) Long pass filter
(vii) Neutral density filter
(viii) Dichroic mirror

[1] The average background level does not matter, only the standard deviation.

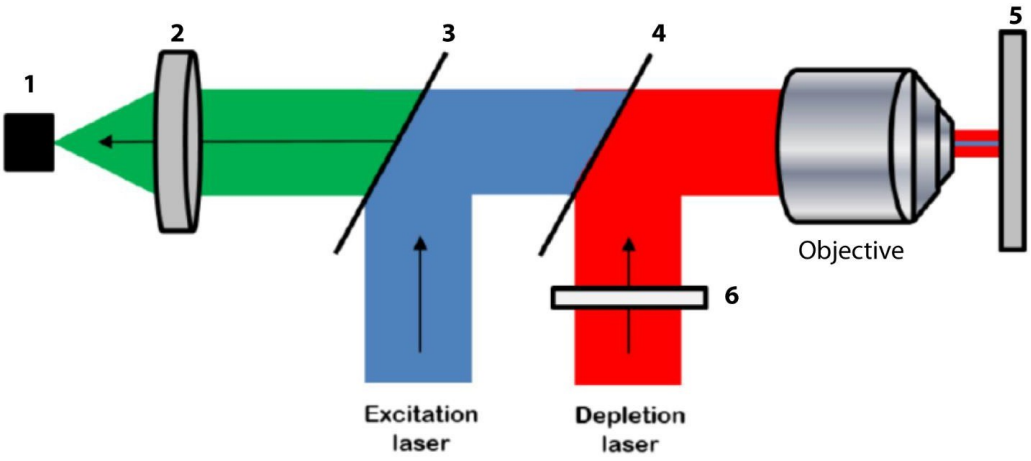

Figure 1: Schematic of a STED microscope setup.

Assume that you need to image cytoskeletons of cells, specifically, the microtubules which have an average diameter of $d = 25\,\text{nm}$. The cytoskeleton is labelled with a specific fluorophore well suited for STED microscopy with fluorescence lifetime of $\tau = 10\,\text{ns}$ and absorption cross section $\sigma = 1 \times 10^{-16}\,\text{cm}^2$. You have an excitation laser that emits at $\lambda_{\text{exc}} = 650\,\text{nm}$ and laser for stimulated emission depletion that emits at $\lambda_{\text{dep}} = 750\,\text{nm}$ at your disposal. In addition, the imaging setup has an objective with $NA = 0.8$.

(b) Calculate the saturation intensity of the fluorophore $\frac{\text{W}}{\text{cm}^2}$?

(c) What is the required laser power (in W) to resolve two next by lying microtubules ?

(d) What are the advantages of pulsed laser system in STED microscopy setup?

(e) What are the advantages of continuous wave laser systems in STED microscopy optical setup

2 Multi-photon Microscopy

2.1 Two photon microscopy with *Atto647N*

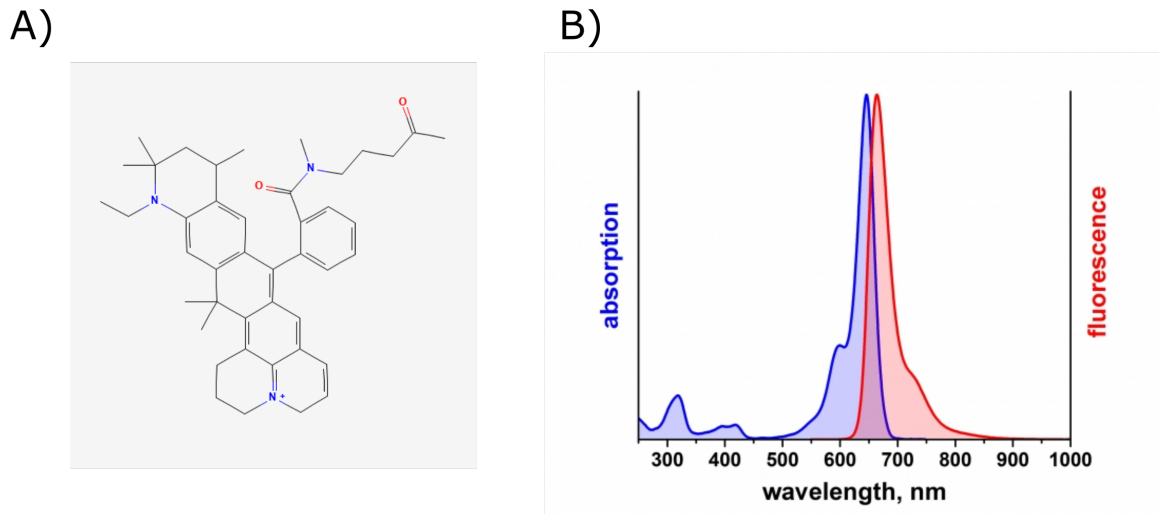

Figure 2: A) Chemical structure of Atto647N. B) Excitation (blue) and emission (red) spectrum.

In your lab you have fluorophore *Atto647N* as well as the following pulsed lasers to perform fluorescent imaging:

- 800 nm Ti:Sapphire
- 1064 nm Nd:YAG
- 1250 nm Cr:F
- 1330 nm Telecom

(a) It can be shown that absorption cross section of the molecules is equal to absorption coefficient $\sigma_{\text{abs}} \sim \epsilon_{\text{abs}}$. Calculate the absorption cross-section of the *Atto647N* molecule in units of Å, when $\epsilon_{\text{abs}} = 1.5 \times 10^5\,\text{M}^{-1}\,\text{cm}^{-1}$.

(b) How does the absorption cross-section relates to the molecule size (shown in Figure 2)? Consider that a C-C bond length in the picture in a benzene ring is $\sim 1.4\,$Å.

(c) Which laser from those presented in your lab is the most appropriate to perform 2-photon fluorescent imaging of *Atto647N*? Explain why using absorption/emission graph.

In a two-photon excitation process, the fluorophore molecule is consecutively excited by 2 photons, passing from a ground state (0) through a short-lived transition state (i), to an excited state (1). Effectively, two-photon absorption is a second-order process, consisting of two consecutive first-order processes. First, the molecule in it's ground state (0) is excited by an incident photon. We can designate this transition 0i and the absorption cross section is $\sigma_{0\text{i}}$. This excites the molecule to a highly unstable transition state (i) with a typical lifetime τ_i. τ_i can be derived from Heisenberg uncertainty principle, which relates energy and time:

$$\Delta E \cdot \Delta t \geq \hbar, \text{ where } \Delta E = \frac{hc}{\lambda_{\text{abs}}} \text{ and } \hbar = \frac{h}{2\pi}$$

In the second step, while the molecule is in the transition state, it is excited by another incident photon to reach the excited state (1). The absorption cross-section here is σ_{i1}. Absorption cross-sections $\sigma_{0\text{i}}$ and σ_{i1} are cross-sections of first order processes and can be assumed equal to σ_{abs} of single-photon absorption. The overall absorption cross.section of the two-photon process can be found as:

$$\sigma_{2\text{ph}} \approx \sigma_{0\text{i}} \cdot \sigma_{\text{i1}} \cdot \tau_i$$

(d) Calculate two-photon absorption cross-section of *Atto647N* with the laser that you chose in question (c). Assume that at the chosen source wavelength σ_{abs} is reduced by 60 % compared to the maximum value that you found in question (a).

To perform two-photon excitation of fluorophores, high-power pulsed lasers are used. The efficiency of the two-photon excitation process can be described in terms of the number of two-photon absorption events per laser pulse. This number N_{abs} can be estimated as follows:

$$\begin{aligned} N_{\text{abs}} &= \Phi_{\text{pulse}} \cdot \sigma_{0\text{i}} \cdot \tau_i \cdot \Phi_{\text{pulse}} \cdot \sigma_{\text{i1}} \cdot \tau_{\text{pulse}} \\ &= \Phi_{\text{pulse}}^2 \cdot \sigma_{2\text{ph}} \cdot \tau_{\text{pulse}} \end{aligned}$$

Where Φ_{pulse} is the photon flux of excitation:

$$\Phi_{\text{pulse}} \approx \underbrace{\frac{\lambda_{\text{exc}}}{hc}}_{1/h\nu} \cdot \underbrace{\frac{P_{\text{exc}} T_{\text{optics}}}{\tau_{\text{pulse}} f_{\text{repetition}}}}_{\text{peak pulse power}} \cdot \underbrace{\frac{1}{\pi r_{\text{Airy}}^2}}_{\text{Airy disk}}$$

(e) Consider a fluorophore with $\sigma_{2\text{ph}} = 6.63 \times 10^{-56}\,\text{m}^4\text{s}$. Assume that only one molecule is excited per laser pulse. What average laser power P_{exc} is required for one excitation per pulse with the following pulsed laser excitation parameters?

- Pulse duration $\tau_{\text{pulse}} = 100\,\text{fs}$
- Repetition rate $f_{\text{repetition}} = 80\,\text{MHz}$
- Wavelength $\lambda_{\text{exc}} = 1250\,\text{nm}$
- Refractive index $n_{\text{H}_2\text{O}} = 1.33$
- Numerical aperture $NA = 1.2$
- Transmission $T_{\text{optics}} = 50\,\%$