Exercise 05 - Solutions

1 Fluorescence microscopy

1.1 Wide-field Fluorescence Microscopy

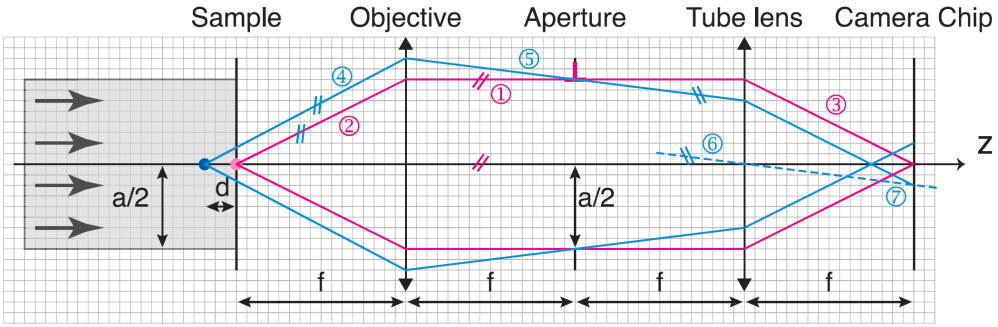

Figure 1: Schematic of the wide-filed optical system with the emerging rays traced for the fluorescent molecules (one in focus and one out-of focus).

(a) Figure 1 shows the rays for the two fluorophore configurations.

(b) The numerical aperture NA in air ($n = 1$) is given by $NA = \sin(\alpha)$, where $\tan(\alpha)$ is given by the ratio $\frac{a}{2f}$. Hence:

$$\begin{aligned} NA &= \sin(\alpha) \\ &= \frac{1}{\sqrt{4\left(\frac{f}{a}\right)^2 + 1}} = 0.6 \end{aligned}$$

(c) Since intensity is by definition the amount of power coming through the unit area, then to calculate power we find:

$$P_{\text{ex}} = I_{\text{ill}} \cdot \pi r_p^2 = \frac{P_{\text{ill}}}{\pi(a/2)^2} \cdot \pi r_p^2 = P_{\text{ill}} \frac{4r_p^2}{a^2},$$

where we took into account that the area of incident beam was equal to $\pi(a/2)^2$ (according to the picture).

(d) The percentage of light collected by the microscope (ECF) is the ratio of the spherical sector intercepted by the objective lens to the surface of such a sphere:

$$\text{ECF} = \frac{A_{\text{collect}}}{A_{\text{tot}}} = \frac{2\pi r^2 (1 - cos(\alpha))}{4\pi r^2} = 0.5 - \frac{f}{2\sqrt{f^2 + \left(\frac{a}{4}\right)^2}} = 10\,\%$$

(e) It is important to realise that the rays outgoing form the displaced point will intersect the optical axis behind the tube lens at a distance d before the detector and with an angle that is the same as the one of the outgoing rays at the point source. (Use ABCD formalism to check analytically!). Hence the image of the displaced fluorophore will be a disc of radius r_{im}.

$$r_{\text{im}} = d\tan(\alpha) = d\frac{a}{2f} = 750\,\text{nm}$$

(f) The ECF is the same as in (d) since the maximum angular opening is still defined by the aperture size (check the marginal rays).

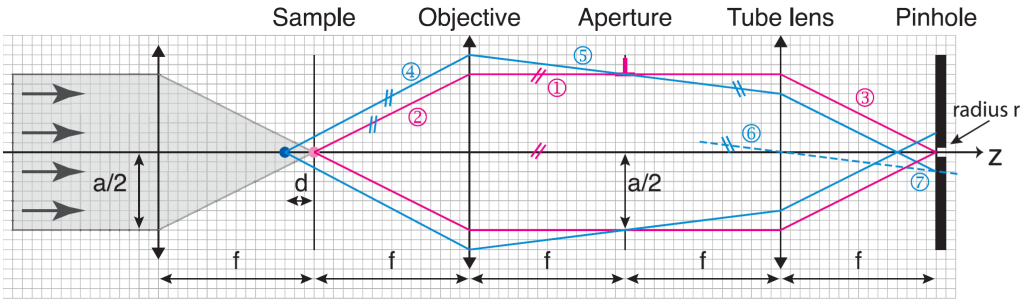

Figure 2: Schematic of the confocal optical system with the emerging rays traced for the fluorescent molecules (one in focus and one out-of focus).

1.2 Confocal Fluorescence Microscopy

(a) Figure 2 shows the rays for the two fluorophore configurations. Notice that the out-of focus marginal ray does **not** pass through the pinhole.

(b) The excitation power for the two fluorophores is:
in focus: All light is exciting the fluorophore, hence: $P_{\text{exc}} = P_{\text{ill}} = 1\,\text{W}$
out-of focus: Only a fraction of the illumination is exciting the fluorophore:

$$\frac{P_{\text{exc}}}{P_{\text{ill}}} = \frac{r_p^2 \pi}{\left(\frac{a^*}{2}\right)^2 \pi}$$

The quantity a^* can be found by considering similar triangles. We find the relation:

$$\frac{a^*}{2d} = \frac{a}{2f} \Rightarrow a^* = \frac{a \cdot d}{f}$$

Therefore the excitation power for the out-of focus fluorophore is:

$$P_{\text{exc}} = P_{\text{ill}} \frac{r_p^2 \pi}{\left(\frac{a^*}{2}\right)^2 \pi} = P_{\text{ill}} \frac{4 r_p^2 f^2}{a^2 d^2} = 0.7\,\text{mW}$$

(c) The detected light power of the two fluorophores is:
in focus: All light is exciting the fluorophore, hence: $P_{\text{det}} = \text{ECF} \cdot P_{\text{exc}} = \text{ECF} \cdot P_{\text{ill}} = 0.1\,\text{W}$ Where ECF is the *Efficiency Collection Function* calculated as $\text{ECF} = \frac{1}{2}(1 - \cos(\alpha))$
out-of focus:

$$P_{\text{det}} = \text{ECF} \cdot P_{\text{exc}} \cdot \frac{\text{Surface of det}}{\text{Image size}} = \text{ECF} \cdot P_{\text{ill}} \cdot \frac{4 r_p^2 f^2}{a^2 d^2} \cdot \frac{4 r^2 \pi f^2}{a^2 d^2 \pi} = 7.9\,\mu\text{W}$$

Where we used the image size from the calculation in exercise 1(e) and used a pinhole with radius $r = 250\,\text{nm}$ which is in the typical range of 0.25 AU to 1 AU for visible light.

(d) Compared to the wide-field microscope where for both points all light is collected and detected on the camera, in confocal microscopy the amount of light is limited. Because there are two foci involved (one in the sample plane and one in the detector plane), it is called *con-focal*. This is the underlying reason why the confocal microscope has very good optical sectioning properties. In this exercise choosing an appropriate pinhole, the fluorophore in focus is more than 10000 times brighter than the fluorophore out of focus.