Optics for Microscopy and Spectroscopy

Lecture Notes

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Contents

Preface

The following lecture notes have been developed for the Ph.D. course entitled *Optics for Microscopy and Spectroscopy*. These notes are meant to be an aid for the participants to study by summarizing the most relevant concepts explained during the course. These notes have not been carefully checked and may contain some errors. These notes cannot provide a complete and exhaustive explanation of optics, imaging, and microscopy. The interested reader should also consult the following books:

- Born, M., Wolf, E. *Principles of Optics* (7th ed.). Cambridge University Press.
- Goodman, J. *Introduction to Fourier Optics* (4th ed.). W. H. Freeman
- Boyd, R. *Nonlinear Optics*. (4th ed.). Academic Press
- Loudon, R. *The Quantum Theory of Light* (3rd ed.). Oxford University Press

Furthermore, much more in-depth information can be found in the vast scientific literature available in journals.

1 Elements of Optics

1.1 Geometrical optics

1.1.1 Rays and ray transfer matrices

In geometrical optics, the light is described by rays. These latter are 2-dimensional vectors

$$\boldsymbol{r} = \begin{pmatrix} x \\ \theta \end{pmatrix} \tag{1.1}$$

whose first element is the distance from the optical axis, and the second is the angle between the ray and the optical axis. The propagation of light through an optical element is calculated with ray transfer matrices (see table 1.1)

$$\boldsymbol{M} = \begin{pmatrix} A & B \\ C & D \end{pmatrix} \tag{1.2}$$

The new ray vector is calculated as the product between the matrix and the input vector

$$\boldsymbol{r}_1 = \boldsymbol{M} \cdot \boldsymbol{r}_0 = \begin{pmatrix} Ax + B\theta \\ Cx + D\theta \end{pmatrix} \tag{1.3}$$

The ray transfer matrix of a compound system is calculated as the product of the matrices of each component

$$\boldsymbol{M}_{\text{tot}} = \boldsymbol{M}_n \dots \boldsymbol{M}_2 \cdot \boldsymbol{M}_1 \tag{1.4}$$

Free space	Thin lens	Flat interface
$\begin{pmatrix} 1 & d \\ 0 & 1 \end{pmatrix}$	$\begin{pmatrix} 1 & 0 \\ -\frac{1}{f} & 1 \end{pmatrix}$	$\begin{pmatrix} 1 & 0 \\ 0 & \frac{n_1}{n_2} \end{pmatrix}$

Table 1.1: Ray transfer matrices of three common optical elements.

1.1.2 Scanning lens

A lens can be used to convert the angular displacement of a ray into a lateral displacement. Indeed, consider a ray originating from the optical axis with an angle θ. If its origin is distant f from a lens with focal length f, we find the following result

$$\begin{pmatrix} 1 & 0 \\ -\frac{1}{f} & 1 \end{pmatrix} \begin{pmatrix} 1 & f \\ 0 & 1 \end{pmatrix} \begin{pmatrix} 0 \\ \theta \end{pmatrix} = \begin{pmatrix} f\theta \\ 0 \end{pmatrix} \tag{1.5}$$

The exiting ray is laterally displaced by a quantity $f\theta$ and propagates with no angle.

1.2 Fourier optics

Maxwell's equations describe light propagation for all the components of the electric and magnetic fields

$$\begin{aligned} \boldsymbol{\nabla} \cdot \boldsymbol{E} &= \rho/\varepsilon & \boldsymbol{\nabla} \times \boldsymbol{E} &= -\frac{\partial \boldsymbol{B}}{\partial t} \\ \boldsymbol{\nabla} \cdot \boldsymbol{B} &= 0 & \boldsymbol{\nabla} \times \boldsymbol{B} &= \mu\varepsilon \frac{\partial \boldsymbol{E}}{\partial t} + \mu \boldsymbol{J} \end{aligned} \tag{1.6}$$

In a non-conducting medium ($\rho = 0$ and $\boldsymbol{J} = \boldsymbol{0}$), they can combined to generate the wave equations

$$\nabla^2 \boldsymbol{E} = \frac{n^2}{c^2} \frac{\partial^2 \boldsymbol{E}}{\partial t^2} \qquad \nabla^2 \boldsymbol{B} = \frac{n^2}{c^2} \frac{\partial^2 \boldsymbol{B}}{\partial t^2} \tag{1.7}$$

where $c = (\mu_0 \varepsilon_0)^{-\frac{1}{2}}$ is the speed of light in vacuum and n is the refractive index of the medium.

If the light is propagating through a homogeneous medium, the wave equations for each coordinate of the vectors are identical. Therefore, it is sufficient to solve only one scalar equation. In this case, the electromagnetic wave is effectively described by a scalar field that we identify with the symbol U. In the real world, no medium is perfectly homogeneous. Thus, a scalar description of light can be considered a good approximation only under certain conditions. The main assumption behind that theory is that the coupling between the differential equations is small, which is valid as long as the spatial inhomogeneities have a characteristic size much larger than the optical wavelength λ. Notably, different polarization components of light can still be described by the scalar theory as long as they can be treated independently.

1.2.1 Diffraction

If the scalar theory holds, we can consider a single wave equation for a scalar field U. The solution of such a differential equation is given by the *Huygens-Fresnel* integral. This latter describes how a field U_0 propagates into the U_z after a distance z

$$U_z(\boldsymbol{x}) = \frac{z}{i\lambda}\int_{\mathbb{R}^2} U_0(\boldsymbol{x}_0)\frac{\exp(ik\varrho)}{\varrho^2}\,\mathrm{d}\boldsymbol{x}_0 \tag{1.8}$$

where the position vector is $\boldsymbol{x} = (x, y)$ – the location on a plane orthogonal to the optical axis z – and ϱ is the distance between two planes, assumed to be much greater than λ, defined as

$$\varrho = \sqrt{(x-x_0)^2 + (y-y_0)^2 + z^2} = z\sqrt{1 + \left(\frac{x-x_0}{z}\right)^2 + \left(\frac{y-y_0}{z}\right)^2} \tag{1.9}$$

The diffraction integral can be simplified using the proper approximation. The choice for this latter depends on the value of

$$F = \frac{D^2}{z\lambda} \tag{1.10}$$

called the *Fresnel number*. In this definition, D is the linear size of field at the starting plane.

If $F \gtrsim 1$ the diffraction takes place in the *near-field* region and the propagation is better described by the Fresnel approximation. If $F \ll 1$ the diffraction takes place in the *far-field* region. In this case the Fresnel approximation still holds, but it can be further simplified with the Fraunhofer approximation.

1.2.1.1 Fresnel diffraction

Under the *paraxial approximation*, namely the assumption that diffraction angles are small with respect to the optical axis, we can expand ϱ in series

$$\varrho \sim z\left[1 + \frac{1}{2}\left(\frac{x-x_0}{z}\right)^2 + \frac{1}{2}\left(\frac{y-y_0}{z}\right)^2\right] \tag{1.11}$$

and replace it in equation 1.8. Keeping only the linear term for the denominator and up to the quadratic term for the argument of the exponential, we obtain

$$U_z(\boldsymbol{x}) = \frac{e^{ikz}}{i\lambda z}\int_{\mathbb{R}^2} U_0(\boldsymbol{x}_0)\exp\left[\frac{ik}{2z}(\boldsymbol{x}-\boldsymbol{x}_0)^2\right]\mathrm{d}\boldsymbol{x}_0 \tag{1.12}$$

This equation is known as the *Fresnel diffraction integral*, which can be seen as the convolution

$$U_z(\boldsymbol{x}) = \left[U_0(\boldsymbol{x}_0) * F_z(\boldsymbol{x}_0)\right](\boldsymbol{x}) \tag{1.13}$$

where F_z the Fresnel convolution kernel

$$F_z(x,y) = \frac{e^{ikz}}{i\lambda z} \exp\left[\frac{ik}{2z}\left(x^2+y^2\right)\right] \tag{1.14}$$

Interestingly, this kernel has a simple Fourier transform which greatly simplifies the analytical and numerical calculations of the propagation of light in free space

$$\hat{F}_z(\nu_x,\nu_y) = e^{ikz} \exp\left[-i\pi\lambda z\left(\nu_x^2+\nu_y^2\right)\right] \tag{1.15}$$

where $\boldsymbol{\nu} = (\nu_x, \nu_y)$ are the spatial frequencies.

1.2.1.2 Fraunhofer diffraction

At very large distances, the diffraction formula can be further simplified. Indeed, if we can neglect the quadratic terms of the argument of the exponential of equation 1.12, we obtain

$$U_z(\boldsymbol{x}) = \frac{e^{ikz}e^{\frac{ik}{2z}(x^2+y^2)}}{i\lambda z} \int_{\mathbb{R}^2} U_0(\boldsymbol{x}_0) \exp\left(i\frac{2\pi}{\lambda z}\boldsymbol{x}\cdot\boldsymbol{x}_0\right) \mathrm{d}\boldsymbol{x}_0 \tag{1.16}$$

This result is known as the *Fraunhofer diffraction integral*. Aside from negligible multiplicative factors, this integral is the Fourier transform of the field U_0 evaluated at spatial frequencies $\boldsymbol{\nu} = \left(\frac{x}{\lambda z}, \frac{y}{\lambda z}\right)$

$$U_z(\boldsymbol{x}) \propto \mathcal{F}\{U_0(\boldsymbol{x}_0)\}\left(\frac{\boldsymbol{x}}{\lambda z}\right) \tag{1.17}$$

1.2.2 Fourier transforming property of the lenses

The phase transformation applied by a lens to a field is

$$t_l(\boldsymbol{x}) = \exp\left(-\frac{ik}{2f}\boldsymbol{x}^2\right) \tag{1.18}$$

Notably, this transmission function has the expression of the Fresnel propagation kernel evaluated at $z = -f$, except for multiplicative constants

$$t_l(\boldsymbol{x}) \propto F_{-f}(\boldsymbol{x}) \tag{1.19}$$

Now, we calculate the effect of a lens with focal length f and a free-space propagation of the same length on a field U_0 originating from a distance z behind the lens. The result U_1 is calculated as follows

$$U_1 = [(U_0 * F_z)\cdot F_{-f}] * F_f \tag{1.20}$$

The above equation can be rewritten in frequency space by exploiting the convolution property of the Fourier transform

$$\hat{U}_1(\boldsymbol{\nu}') = \left(\left[\hat{U}_0(\boldsymbol{\nu}) \cdot \hat{F}_z(\boldsymbol{\nu})\right] * \hat{F}_{-f}(\boldsymbol{\nu})\right)(\boldsymbol{\nu}') \cdot \hat{F}_f(\boldsymbol{\nu}') = \\ = \int_{\mathbb{R}^2} \hat{U}_0(\boldsymbol{\nu}) \exp\left[-i\pi\lambda(z-f)\nu^2\right] \exp\left[-i2\pi\lambda f \boldsymbol{\nu}\cdot\boldsymbol{\nu}'\right] \mathrm{d}\boldsymbol{\nu} \tag{1.21}$$

Transforming back into real space, we obtain

$$U_1(\boldsymbol{x}) = \int_{\mathbb{R}^2} \hat{U}_0(\boldsymbol{\nu}) \exp\left[-i\pi\lambda(z-f)\nu^2\right] \underbrace{\int_{\mathbb{R}^2} \exp\left[-i2\pi\lambda f \boldsymbol{\nu}\cdot\boldsymbol{\nu}'\right] \exp\left[i2\pi\boldsymbol{x}\cdot\boldsymbol{\nu}'\right] \mathrm{d}\boldsymbol{\nu}'}_{\delta(\boldsymbol{x}-\boldsymbol{\nu}\lambda f)} \mathrm{d}\boldsymbol{\nu} = \\ = \hat{U}_0\left(\frac{\boldsymbol{x}}{\lambda f}\right) \exp\left[\frac{ik}{2f}\left(1-\frac{z}{f}\right)\boldsymbol{x}^2\right] \tag{1.22}$$

Interestingly, the field at the focal plane of the lens is the Fourier transform of the input field evaluated at spatial frequencies $\boldsymbol{\nu} = (\frac{x}{\lambda f}, \frac{y}{\lambda f})$, aside from a phase factor. when the distance between the input and the lens is matching the focal length, i.e. $z = f$, the phase factor equals to 1 and the result is exactly the Fourier transform. In other words, the field at the focal plane can be calculated as as Fraunhofer diffraction evaluated at $z = f$.

1.3 Vectorial Optics

1.3.1 Polarization

The solution of Maxwell's equations in vacuum (or in a homogeneous insulating material) can be written as a superposition of the following plane waves:

$$\boldsymbol{E}(z,t) = \begin{pmatrix} E_{0x}e^{i\phi_x} \\ E_{0y}e^{i\phi_y} \end{pmatrix} e^{i(kz-\omega t)} \tag{1.23}$$

where the amplitude term is described by a 2-vector called phasor. The polarization state of light depends on the relative amplitude and phase of the two components on the phasor.

1.3.1.1 Jones formalism

The normalized phasor is called *Jones vector*, which can be equivalently written in the linear or circular basis (see Table 1.2). Namely, a generic Jones vector $|P\rangle$ is

$$|P\rangle = c_H |H\rangle + c_V |V\rangle = \tag{1.24}$$
$$= c_R |R\rangle + c_L |L\rangle \tag{1.25}$$

where the coefficients c are complex numbers. Thus, the polarized electric field is

Linear basis		Diagonal basis		Circular basis	
$\|H\rangle$	$\begin{pmatrix}1\\0\end{pmatrix}$	$\|D\rangle$	$\begin{pmatrix}1\\1\end{pmatrix}$	$\|R\rangle$	$\frac{1}{\sqrt{2}}\begin{pmatrix}1\\-i\end{pmatrix}$
$\|V\rangle$	$\begin{pmatrix}0\\1\end{pmatrix}$	$\|A\rangle$	$\begin{pmatrix}1\\-1\end{pmatrix}$	$\|L\rangle$	$\frac{1}{\sqrt{2}}\begin{pmatrix}1\\i\end{pmatrix}$

Table 1.2: The most common bases used to represent a Jones vector with the corresponding Ket notation.

$$|E\rangle = E_0 e^{ikz-i\omega t}\,|P\rangle \tag{1.26}$$

Anisotropic optical elements can act either on the amplitude or the phase of a polarization state. Elements acting only on the amplitude are called *polarizers* and those acting only on the phase are called *phase retarders* or *wave plates*. Such elements are described by the 2×2 Jones matrices of Table 1.3. Notably, Jones Matrices are unitary and with determinant 1. In other words, they are members of the SU(2) group.

Linear Polarizer	Phase Retarder
$\begin{pmatrix}1 & 0\\0 & 0\end{pmatrix}$	$\begin{pmatrix}e^{ik\delta} & 0\\0 & 1\end{pmatrix}$

Table 1.3: Jones matrices of optical elements aligned horizontally.

Optical elements act differently depending on the orientation of their optical axis. The matrix J of a rotated optical element can be calculated as follows

$$J(\theta) = R(-\theta)JR(\theta) \tag{1.27}$$

where the rotation matrix is

$$R(\theta) = \begin{pmatrix}\cos\theta & -\sin\theta\\ \sin\theta & \cos\theta\end{pmatrix} \tag{1.28}$$

Two wave plates are of particular interest for their practical applications. The matrix of a half-wave plate can be obtained by assuming that the thickness of the retarder is $\delta = \lambda/2$. In this case, the phase difference of the two polarization components is π. Similarly, the matrix of a quarter wave-plate can be obtained assuming $\delta = \lambda/4$. The corresponding phase difference is $\pi/2$.

With some algebra, we can prove that a half-wave plate rotated by 45° rotates a linearly polarization state by 90°.

$$J_{\lambda/2}(45^\circ)\,|H\rangle = |V\rangle \qquad J_{\lambda/2}(45^\circ)\,|V\rangle = |H\rangle \tag{1.29}$$

Similarly, a quarter-wave plate rotated by 45° converts a linearly polarization state into a circularly polarization state.

$$J_{\lambda/4}(45^\circ)\,|H\rangle = |R\rangle \qquad J_{\lambda/4}(45^\circ)\,|V\rangle = |L\rangle \tag{1.30}$$

1.3.1.2 Stokes formalism

The Jones formalism is about the fields, while the Stokes formalism is about the intensity. A Stokes vector has four entries

$$\boldsymbol{s} = (s_0, s_1, s_2, s_3)^T \tag{1.31}$$

which are the Stokes parameters, calculated as

$$s_0 = |\langle E|E\rangle|^2 \tag{1.32}$$
$$s_1 = |\langle E|H\rangle|^2 - |\langle E|V\rangle|^2 \tag{1.33}$$
$$s_2 = |\langle E|D\rangle|^2 - |\langle E|A\rangle|^2 \tag{1.34}$$
$$s_3 = |\langle E|R\rangle|^2 - |\langle E|L\rangle|^2 \tag{1.35}$$

In other words, the first component describes the total intensity of light, while the other components describe the excess of linear, diagonal, and circular polarization, respectively. Importantly, the stokes parameters are not linearly independent. Namely, the following relation applies

$$s_0^2 = s_1^2 + s_2^2 + s_3^2 \tag{1.36}$$

If normalized with respect to s_0, the last three elements of the Stokes vector can be written as

$$s_1 = \cos(2\psi)\cos(2\chi) \tag{1.37}$$
$$s_2 = \sin(2\psi)\cos(2\chi) \tag{1.38}$$
$$s_3 = \sin(2\chi) \tag{1.39}$$

where ψ is the azimuthal angle and χ is the ellipticity angle. Namely, they parameterize in spherical coordinates the surface of a unit sphere, known as *Poincaré sphere*. A point on the surface of the Poincaré sphere uniquely defines a polarization state.

Optical elements can be described as matrices also using Stokes formalism. In this case, they become 4×4 matrices and are known as *Mueller* matrices M. They can be obtained from the corresponding Jones Matrices J using the following transformation

$$M = A(J \otimes J^*)A^{-1} \tag{1.40}$$

where $\otimes$ denotes the Kronecker product and

$$A = \begin{pmatrix} 1 & 0 & 0 & 1 \\ 1 & 0 & 0 & -1 \\ 0 & 1 & 1 & 0 \\ 0 & i & -i & 0 \end{pmatrix} \tag{1.41}$$

Two examples are shown in Table 1.4. Note that Mueller matrices have always real entries.

Linear Polarizer	Phase retarder
$\begin{pmatrix} 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \end{pmatrix}$	$\begin{pmatrix} 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & \cos k\delta & \sin k\delta \\ 0 & 0 & -\sin k\delta & \cos k\delta \end{pmatrix}$

Table 1.4: Mueller matrices of optical elements aligned horizontally.

Once again, we can find the matrix of a rotated component applying the following transformation

$$M(\theta) = R(-\theta)MR(\theta) \tag{1.42}$$

where, in this case, the rotation matrix is

$$R(\theta) = \begin{pmatrix} 1 & 0 & 0 & 0 \\ 0 & \cos(2\theta) & \sin(2\theta) & 0 \\ 0 & -\sin(2\theta) & \cos(2\theta) & 0 \\ 0 & 0 & 0 & 1 \end{pmatrix} \tag{1.43}$$

1.4 Quantum optics

The Hamiltonian of a harmonic oscillator is

$$\hat{\mathcal{H}} = \frac{\hat{p}^2}{2m} + \frac{m\omega^2\hat{q}^2}{2} \tag{1.44}$$

where $\hat{p}$ and $\hat{q}$ are, respectively, the momentum and position operators, which follow the canonical commutator relation

$$[\hat{q}, \hat{p}] = \hbar \tag{1.45}$$

They are conveniently rewritten into the following dimensionless operators

$$\hat{X} = \left(\frac{m\omega}{2\hbar}\right)^{1/2}\hat{q} \tag{1.46}$$

$$\hat{Y} = \left(\frac{1}{2m\hbar\omega}\right)^{1/2}\hat{p} \tag{1.47}$$

known as the *field quadrature operators*. The Hamiltonian can be rewritten using the quadrature operators as

$$\hat{\mathcal{H}} = \hbar\omega\left(\hat{X} + \hat{Y}\right) \tag{1.48}$$

The quadrature operators can be identified with the electric and magnetic part of an oscillating electromagnetic field. Equivalently, they can be seen as the in-phase and the in-quadrature part of the electric field. Namely, the operator representing the electric field with a specific polarization and wavelength is

$$\hat{E}(\boldsymbol{r}, t) = \left(\frac{2\hbar\omega}{\varepsilon_0 V}\right)^{1/2}\left(\hat{X}\cos(\omega t - \boldsymbol{k}\cdot\boldsymbol{r}) + \hat{Y}\sin(\omega t - \boldsymbol{k}\cdot\boldsymbol{r})\right) \tag{1.49}$$

We now define the following operators

$$\hat{a} = \hat{X} + i\hat{Y} \tag{1.50}$$

$$\hat{a}^\dagger = \hat{X} - i\hat{Y} \tag{1.51}$$

known, respectively, as the *destruction* and *creation* operators. Using those operators, the electric field can be rewritten as

$$\hat{E}(\boldsymbol{r}, t) = \left(\frac{\hbar\omega}{2\varepsilon_0 V}\right)^{1/2}\left(\hat{a}e^{-i(\omega t - \boldsymbol{k}\cdot\boldsymbol{r})} + \hat{a}^\dagger e^{i(\omega t - \boldsymbol{k}\cdot\boldsymbol{r})}\right) \tag{1.52}$$

Namely, the destruction and creation operators are proportional, respectively, to the positive and negative frequency part of the electric field. The Hamiltonian is reshaped as

$$\hat{\mathcal{H}} = \hbar\omega\left(\hat{N} + \frac{1}{2}\right) \tag{1.53}$$

where $\hat{N} = \hat{a}^\dagger\hat{a}$ is the number operator. The eigenstates $|n\rangle$ are called number states. The corresponding Eigenvalues are

$$E_n = \hbar\omega\left(n + \frac{1}{2}\right) \tag{1.54}$$

Namely, the energy levels are discrete with spacing $\hbar\omega$.

Each longitudinal mode and polarization mode of an electromagnetic field is described by the Hamiltonian 1.53 whose eigenstates are known as *photons*.

2 Image Formation Theory

2.1 Linear Space-Invariant Systems

A Linear Space-Invariant (LSI) system is a map

$$S : f \mapsto g \tag{2.1}$$

defined by the following properties

- **Linearity**: the output g is linear in the input f

$$g = S[f] \quad \Rightarrow \quad S[a \cdot f_1 + b \cdot f_2] = a \cdot S[f_1] + b \cdot S[f_2] = a \cdot g_1 + b \cdot g_2 \tag{2.2}$$

- **Shift-invariance**: the output of a shifted input is a shifted output

$$g(x) = S[f(x)] \quad \Rightarrow \quad g(x - x_0) = S[f(x - x_0)] \tag{2.3}$$

Any input function can be written as

$$f(x) = f(x) * \delta(x) = \int_{\mathbb{R}} f(\chi)\delta(x - \chi)\,\mathrm{d}\chi \tag{2.4}$$

Thus,

$$g(x) = \int_{\mathbb{R}} f(\chi)S[\delta(x - \chi)]\,\mathrm{d}\chi = \int_{\mathbb{R}} f(\chi)h(x - \chi)\,\mathrm{d}\chi = f(x) * h(x) \tag{2.5}$$

where $h(x)$ is the impulse response of the LSI system.

2.2 Geometrical Optics

With one or more lenses, it is possible to generate an image, namely to generate a rescaled copy of rays of light. In this section, we discuss imaging systems in terms of ray transfer matrices.

2.2.1 Single lens system

Consider a system composed of a single lens. Taking also into account the space before and after the lens, we find the following matrix

$$\begin{pmatrix} 1 & z_2 \\ 0 & 1 \end{pmatrix} \begin{pmatrix} 1 & 0 \\ -\frac{1}{f} & 1 \end{pmatrix} \begin{pmatrix} 1 & z_1 \\ 0 & 1 \end{pmatrix} = \begin{pmatrix} 1-\frac{z_2}{f} & z_1+z_2-\frac{z_1 z_2}{f} \\ -\frac{1}{f} & 1-\frac{z_1}{f} \end{pmatrix} \tag{2.6}$$

In order to have the formation of an image, the B element of the matrix has to be zero. Thus, the following condition has to be verified

$$\frac{1}{z_1}+\frac{1}{z_2}=\frac{1}{f} \tag{2.7}$$

Consequently, the A element equals $-\frac{z_2}{z_1}$ and can be interpreted as the magnification factor.

2.2.2 Two lenses system

Consider a system composed by two lenses separated by a distance d. The corresponding matrix is

$$\begin{aligned} &\begin{pmatrix} 1 & z_2 \\ 0 & 1 \end{pmatrix} \begin{pmatrix} 1 & 0 \\ -\frac{1}{f_2} & 1 \end{pmatrix} \begin{pmatrix} 1 & d \\ 0 & 1 \end{pmatrix} \begin{pmatrix} 1 & 0 \\ -\frac{1}{f_1} & 1 \end{pmatrix} \begin{pmatrix} 1 & z_1 \\ 0 & 1 \end{pmatrix} = \\ &= \frac{1}{f_1 f_2} \begin{pmatrix} f_1 f_2 + d z_2 - f_2 d - f_2 z_2 - f_1 z_2 & f_1 f_2 d + f_1 f_2 z_1 + f_1 f_2 z_2 - f_2 z_1 z_2 - f_1 z_1 z_2 + d z_1 z_2 - f_2 d z_1 - f_1 d z_2 \\ -f_1 - f_2 + d & f_2 f_1 + d z_1 - f_1 d - f_1 z_1 - f_2 z_1 \end{pmatrix} \end{aligned} \tag{2.8}$$

It is easy to verify that the B element is zero if $z_1 = f_1$ and $z_2 = f_2$. Moreover, if $d = f_1 + f_2$ the C element is also zero, meaning that the system is afocal. In this case, the lateral magnification is $A = -f_2/f_1$ and the angular magnification is $D = -f_1/f_2$.

2.3 Fourier Optics

We now consider a system composed by two lenses – with focal length f_1 and f_2 – separated by a distance d. We consider the starting field at a distance z_1 from the first lens and the output field at a distance z_2 from the second lens. The propagation is calculated as follows

$$U_1 = ((((U_0 * F_{z_1}) \cdot F_{-f_1}) * F_d) \cdot F_{-f_2}) * F_{z_2} \tag{2.9}$$

The Fourier transform of the above equation is

$$\hat{U}_1 = \Big(\Big(\Big(\Big(\hat{U}_0 \cdot \hat{F}_{z_1}\Big) * \hat{F}_{-f_1}\Big) \cdot \hat{F}_d\Big) * \hat{F}_{-f_2}\Big) \cdot \hat{F}_{z_2} \tag{2.10}$$

Explicitly, it is written as

$$\hat{U}_1(\boldsymbol{\nu}'') = \int_{\mathbb{R}^4} \hat{U}_0(\boldsymbol{\nu}) \exp\Big[-i\pi\lambda\Big(z_1\boldsymbol{\nu}^2 - f_1(\boldsymbol{\nu}' - \boldsymbol{\nu})^2 + d\boldsymbol{\nu}'^2 - f_2(\boldsymbol{\nu}'' - \boldsymbol{\nu}')^2 + z_2\boldsymbol{\nu}''^2\Big)\Big]\,\mathrm{d}\boldsymbol{\nu}\,\mathrm{d}\boldsymbol{\nu}' \tag{2.11}$$

Assuming $z_1 = f_1$ and $z_2 = f_2$, we have

$$\hat{U}_1(\boldsymbol{\nu}'') = \int_{\mathbb{R}^4} \hat{U}_0(\boldsymbol{\nu}) e^{-i\pi\lambda(d-f_1-f_2)\nu'^2} e^{-i2\pi\lambda f_1 \nu\nu'} e^{-i2\pi\lambda f_2 \nu'\nu''}\,\mathrm{d}\nu\,\mathrm{d}\nu' \tag{2.12}$$

Transforming back to the real space, we obtain

$$\begin{aligned} U_1(\boldsymbol{x}) &= \int_{\mathbb{R}^4} \hat{U}_0(\boldsymbol{\nu}) e^{-i\pi\lambda(d-f_1-f_2)\nu'^2} e^{-i2\pi\lambda f_1 \boldsymbol{\nu}\cdot\boldsymbol{\nu}'} \underbrace{\int_{\mathbb{R}^2} e^{-i2\pi\lambda f_2 \boldsymbol{\nu}'\cdot\boldsymbol{\nu}''} e^{i2\pi\boldsymbol{x}\cdot\boldsymbol{\nu}''}\,\mathrm{d}\boldsymbol{\nu}''}_{\delta(\boldsymbol{x}-\boldsymbol{\nu}'\lambda f_2)}\,\mathrm{d}\boldsymbol{\nu}\,\mathrm{d}\boldsymbol{\nu}' = \\ &= e^{-i\pi\lambda(d-f_1-f_2)\frac{r^2}{\lambda^2 f_2^2}} \int_{\mathbb{R}^2} \hat{U}_0(\boldsymbol{\nu}) e^{-i2\pi\boldsymbol{\nu}\cdot\boldsymbol{x} f_1/f_2}\,\mathrm{d}\boldsymbol{\nu} = \\ &= U_0\Big(-\frac{f_1}{f_2}\boldsymbol{x}\Big) e^{-i\pi\lambda(d-f_1-f_2)\frac{r^2}{\lambda^2 f_2^2}} \end{aligned} \tag{2.13}$$

This result shows that under imaging conditions (i.e. $z_1 = f_1$ and $z_2 = f_2$) the amplitude of the output field U_1 is a copy of the amplitude of the initial field U_0, but inverted and rescaled by the magnification factor $M = \frac{f_2}{f_1}$. This implies that the light intensity at the two planes is identical, thus at z_2 there is an *image* of the plane at z_1. If the distance between the two lenses is equal to $d = f_1 + f_2$, then the two fields are identical both in amplitude and in phase. In this case the two planes are said to be *optically conjugated*.

2.3.1 Impulse response of an imaging system

We now consider the effect of the finite size of the lenses. The pupil function describes the limited aperture of a lens, and it is defined as follows

$$P(r) = \begin{cases} 1 & \text{if } r \leq R \\ 0 & \text{if } r > R \end{cases} \tag{2.14}$$

where $r = \sqrt{x^2 + y^2}$ and R is the radius of the lens. We now calculate the propagation of a point-like source $U_0(\boldsymbol{x}) = \delta(\boldsymbol{x})$ through a two-lenses imaging system. As shown before, the value of d has no effect on the intensity at the image plane. Therefore, we choose $d = 0$ for the sake of simplicity. The output field is

$$H = ((\delta * F_{z_1}) \cdot P \cdot F_{-f_1} \cdot F_{-f_2}) * F_{z_2} \tag{2.15}$$

Explicitly

$$H(\boldsymbol{x}) = \int_{\mathbb{R}^2} P(\boldsymbol{x}') \exp\left[\frac{ik}{2}\left(\frac{1}{nz_1} - \frac{1}{f_1} - \frac{1}{f_2} + \frac{1}{z_2}\right) r'^2\right] \exp\left[-\frac{ik}{z_2}\boldsymbol{x}\cdot\boldsymbol{x}'\right] \mathrm{d}\boldsymbol{x}' \tag{2.16}$$

Where we neglected pure multiplicative phase factors. The imaging condition implies

$$\frac{1}{nz_1} + \frac{1}{z_2} - \frac{1}{f_1} - \frac{1}{f_2} = 0 \tag{2.17}$$

Therefore, we impose $z_2 = f_2$ and $z_1 = f_1/n + z$. Using a McLaurin expansion, we get $\frac{1}{nz_1} \sim \frac{1}{f_1}\left(1 - n\frac{z}{f_1}\right)$. By substituting these values, we get

$$H(\boldsymbol{x}) = \int_{\mathbb{R}^2} P(r') \exp\left[-\frac{ik}{2}\frac{nzr'^2}{f_1^2}\right] \exp\left[-\frac{ik}{f_2}\boldsymbol{x}\cdot\boldsymbol{x}'\right] \mathrm{d}\boldsymbol{x}' \tag{2.18}$$

That is the Fourier transform of a circularly symmetric function. Therefore, we can rewrite the integral as a zero-order Hankel transform

$$H(r) = \int_0^R \exp\left(-\frac{ik}{2}\frac{nzr'^2}{f_1^2}\right) J_0\left(\frac{k}{f_2} r r'\right) r' \,\mathrm{d}r' \tag{2.19}$$

Changing the variable r' with $\rho = r'/R$ and defining the numerical aperture of the first lens as $\mathrm{NA} = nR/f_1$ we finally obtain

$$H(r, z) = \int_0^1 \exp\left(-\frac{ik}{2}\frac{\mathrm{NA}^2}{n}\rho^2 z\right) J_0\left(\frac{k\mathrm{NA}}{M}\rho r\right) \rho \,\mathrm{d}\rho \tag{2.20}$$

where we neglected pure multiplicative factors and used the definition of the magnification as $M = nf_2/f_1$.

2.3.2 Coherence of light

The finite temporal coherence of light can be described by random phase shifts of the electromagnetic wave

$$E_1(t) = \sum_{n=-\infty}^{+\infty} \exp\left(i\omega t + i\phi_n\right) \Pi\left(\frac{t}{T} - \frac{n}{2}\right) \tag{2.21}$$

Now consider the same electric field, time-shifted by τ

$$E_2(t-\tau) = \sum_{m=-\infty}^{+\infty} \exp\left(i\omega t - i\omega\tau + i\phi_m\right) \Pi\left(\frac{t-\tau}{T} - \frac{m}{2}\right) \tag{2.22}$$

The total intensity of the sum of the two fields is calculated as

$$|E_1 + E_2|^2 = |E_1|^2 + |E_2|^2 + E_1^* E_2 + E_1 E_2^* \tag{2.23}$$

The first two terms are proportional to the intensity of each field, the last two terms describe the interference between the two fields.

$$E_1^* E_2 = \exp(-i\omega\tau) \sum_{m,n} \exp(i\phi_n - i\phi_m) \Pi\left(\frac{t}{T} - \frac{n}{2}\right) \Pi\left(\frac{t-\tau}{T} - \frac{m}{2}\right) \tag{2.24}$$

given a fixed τ, the product of the two rectangular functions is either 0 or 1, depending on the value of $n - m$. We now consider only the couple (n, m) such as this product is equal to 1. Therefore,

$$\sum_{m,n} \exp(i\phi_n - i\phi_m) \approx \delta_{m,n} \tag{2.25}$$

Therefore the interference term is not zero only if $m = n$ and

$$\Pi\left(\frac{t}{T}\right) \Pi\left(\frac{t-\tau}{T}\right) > 0 \tag{2.26}$$

which implies $|\tau| < T$. Indeed, T is the coherence time and defines the maximum delay beyond which the interference term can be neglected.

The value τ defines the visibility of the interference. Indeed, the total signal collected in an ideally infinite amount of time from the interference terms is

$$\begin{aligned} &\int_{\mathbb{R}} \left[E_1^*(t) E_2(t-\tau) + E_1(t) E_2^*(t-\tau)\right] \mathrm{d}t = 2\cos(\omega\tau) \int_{\mathbb{R}} \Pi\left(\frac{t}{T}\right) \Pi\left(\frac{t-\tau}{T}\right) \mathrm{d}t = \\ &= (T - |\tau|) 2\cos(\omega\tau) \end{aligned} \tag{2.27}$$

Thus, the visibility of the interference $\cos(\omega t)$ decreases linearly with $|\tau|$. This linear behaviour is a consequence of this simplified model which uses rectangular coherence windows. A more realistic model would still predict a visibility monotonically decreasing with τ, but with a non-linear trend. For perfectly incoherent light $T \to 0$ and the interference signal can be seen as $\delta(\tau)$. The same reasoning which led to the results of this section can be applied to space to describe spatial coherence.

2.3.3 Incoherent imaging

The wide-field image formation is

$$\begin{aligned} i(\boldsymbol{x}) &= \int_0^T \left|\left[O(\boldsymbol{x}') * H(\boldsymbol{x}')\right](\boldsymbol{x})\right|^2 \mathrm{d}t = \\ &= T \int_{\mathbb{R}^6} O(\boldsymbol{x}') O^*(\boldsymbol{x}'') H(\boldsymbol{x} - \boldsymbol{x}') H^*(\boldsymbol{x} - \boldsymbol{x}'') \, \mathrm{d}\boldsymbol{x}' \, \mathrm{d}\boldsymbol{x}'' \end{aligned} \tag{2.28}$$

which can be seen as a spatial correlation function at delay $\boldsymbol{x}' - \boldsymbol{x}''$. For perfectly incoherent light, the correlation is

$$\int_{\mathbb{R}^6} E(\boldsymbol{x}')E^*(\boldsymbol{x}'')\,\mathrm{d}t = |E(\boldsymbol{x}')|^2\delta(\boldsymbol{x}' - \boldsymbol{x}'') \tag{2.29}$$

Therefore, the image formed with incoherent light is

$$i(\boldsymbol{x}) = T\int_{\mathbb{R}^3} |O(\boldsymbol{x}')|^2|H(\boldsymbol{x} - \boldsymbol{x}')|^2\,\mathrm{d}x' \propto [o * h](\boldsymbol{x}) \tag{2.30}$$

where

$$h(\boldsymbol{x}) = |H(\boldsymbol{x})|^2 \tag{2.31}$$

is the intensity Point Spred Function (PSF) and

$$o(\boldsymbol{x}) = |O(\boldsymbol{x})|^2 \tag{2.32}$$

is the distribution of light-emitters in the object plane.

2.3.4 Lateral and axial resolution

Using equation 2.20 with object plane coordinates, the intensity PSF is

$$h(r, z) = \left|\int_0^1 \exp\left(-\frac{ik}{2}\frac{\mathrm{NA}^2}{n}\rho^2 z\right) J_0(k\mathrm{NA}\rho r)\rho\,\mathrm{d}\rho\right|^2 \tag{2.33}$$

In perfect focus condition ($z = 0$), this equation becomes

$$h(r, 0) = \left|\int_0^1 J_0(k\mathrm{NA}r\rho)\rho\,\mathrm{d}\rho\right|^2 = \left|\int_0^{k\mathrm{NA}r} \frac{J_0(x)x}{(k\mathrm{NA}r)^2}\,\mathrm{d}x\right|^2 = \left|\frac{J_1(k\mathrm{NA}r)}{k\mathrm{NA}r}\right|^2 \tag{2.34}$$

where we used the property of Bessel functions $\frac{\mathrm{d}}{\mathrm{d}x}[J_\nu(x)x^\nu] = J_{\nu-1}(x)x^\nu$. The first zero of $J_1(x)$ is at $x_0 \approx 3.8317$. Solving the equation $k\mathrm{NA}r = x_0$ for r, we obtain the distance between the peak of the PSF and its first minimum

$$r_{\mathsf{min}} = 0.61\frac{\lambda}{\mathrm{NA}} \tag{2.35}$$

This is the minimum lateral distance resolvable by a standard imaging system, according to Rayleigh's criterion.

Along the optical axis ($r = 0$) the intensity profile is

$$\begin{aligned} h(0, z) &= \left|\int_0^1 \exp\left(-\frac{ik}{2}\frac{\mathrm{NA}^2}{n}\rho^2 z\right)\rho\,\mathrm{d}\rho\right|^2 = \left|\frac{n}{\mathrm{NA}^2 kz}\left[\exp\left(-\frac{ik}{2}\frac{\mathrm{NA}^2}{n}z\right) - 1\right]\right|^2 = \\ &= \left(\frac{2n}{kz\mathrm{NA}^2}\right)^2 \sin^2\left(\frac{kz\mathrm{NA}^2}{4n}\right) \end{aligned} \tag{2.36}$$

The first zero of the cardinal sine function $\frac{\sin(x)}{x}$ is at $x_0 = \pi$. Solving the equation $\frac{kz\mathrm{NA}^2}{4n} = x_0$ for z, we obtain the distance between the peak of the axial PSF and its first minimum

$$z_{\mathsf{min}} = \frac{2\lambda n}{\mathrm{NA}^2} \tag{2.37}$$

This is the minimum axial distance resolvable by a standard imaging system, according to Rayleigh's criterion.

2.4 Frequency analysis of imaging systems

A linear imaging system acts as a low-pass filter on the signal emitted from a sample. The spatial frequencies that can be collected are defined by the Optical Transfer Function (OTF), which is the Fourier Transform of the impulse response (PSF).

From equation 2.18, we see that in focus ($z = 0$) the field PSF is the Fourier transform of the pupil function. Thus, the OTF for coherent imaging is

$$\mathrm{OTF} = \mathcal{F}\{H(\boldsymbol{x})\} = P\left(\frac{\boldsymbol{x}'}{\lambda f}\right) \tag{2.38}$$

The OTF for incoherent imaging is

$$\mathrm{OTF} = \mathcal{F}\{h(\boldsymbol{x})\} = P\left(\frac{\boldsymbol{x}'}{\lambda f}\right) * P\left(-\frac{\boldsymbol{x}'}{\lambda f}\right) \tag{2.39}$$

If P is a rectangular function with length $2R$, then the incoherent OTF is a triangular function with cut-off frequency

$$\nu_o = \frac{2R}{\lambda f} = \frac{2\mathrm{NA}}{\lambda} \tag{2.40}$$

where $\mathrm{NA} = f/R$.

3

Deterministic super-resolution microscopy

3.1 Structured Illumination Microscopy

The illumination pattern is

$$e(\boldsymbol{x}) = 1 + \cos\left(\boldsymbol{K} \cdot \boldsymbol{x} + \phi\right) \tag{3.1}$$

the corresponding Fourier transform is

$$E(\boldsymbol{k}) = \delta(\boldsymbol{k}) + \frac{1}{2}\left[\delta(\boldsymbol{k} - \boldsymbol{K})e^{i\phi} + \delta(\boldsymbol{k} + \boldsymbol{K})e^{-i\phi}\right] \tag{3.2}$$

The image formation model is

$$i(\boldsymbol{x}|\boldsymbol{K}, \phi) = \left[o(\boldsymbol{x}) \cdot e(\boldsymbol{x}|\boldsymbol{K}, \phi)\right] * h(\boldsymbol{x}) \tag{3.3}$$

whose Fourier transform is

$$I(\boldsymbol{k}|\boldsymbol{K}, \phi) = \left[O(\boldsymbol{k}) + \frac{1}{2}O(\boldsymbol{k} - \boldsymbol{K})e^{i\phi} + \frac{1}{2}O(\boldsymbol{k} + \boldsymbol{K})e^{-i\phi}\right] \cdot H(\boldsymbol{k}) \tag{3.4}$$

We need to solve for $O(\boldsymbol{k})$, $O(\boldsymbol{k}-\boldsymbol{K})$, and $O(\boldsymbol{k}+\boldsymbol{K})$. Thus, we need (at least) three equations. We obtain these latter by changing the phase to $\phi \in \left\{0, \frac{2\pi}{3}, \frac{4\pi}{3}\right\}$. The solution is found by solving the linear system

$$\begin{pmatrix} I(\boldsymbol{k}|\phi_1) \\ I(\boldsymbol{k}|\phi_2) \\ I(\boldsymbol{k}|\phi_3) \end{pmatrix} = H(\boldsymbol{k}) \begin{pmatrix} 1 & \frac{e^{i\phi_1}}{2} & \frac{e^{-i\phi_1}}{2} \\ 1 & \frac{e^{i\phi_2}}{2} & \frac{e^{-i\phi_2}}{2} \\ 1 & \frac{e^{i\phi_3}}{2} & \frac{e^{-i\phi_3}}{2} \end{pmatrix} \begin{pmatrix} O(\boldsymbol{k}) \\ O(\boldsymbol{k} - \boldsymbol{K}) \\ O(\boldsymbol{k} + \boldsymbol{K}) \end{pmatrix} = \begin{pmatrix} 1 & \frac{e^{i\phi_1}}{2} & \frac{e^{-i\phi_1}}{2} \\ 1 & \frac{e^{i\phi_2}}{2} & \frac{e^{-i\phi_2}}{2} \\ 1 & \frac{e^{i\phi_3}}{2} & \frac{e^{-i\phi_3}}{2} \end{pmatrix} \begin{pmatrix} S_1(\boldsymbol{k}) \\ S_2(\boldsymbol{k}) \\ S_3(\boldsymbol{k}) \end{pmatrix} \tag{3.5}$$

The above system is solved for the vector of spectra $\boldsymbol{S}$ for different orientations $\boldsymbol{K}$. Indeed, to isotropically fill the Fourier space of the reconstructed object, the spatial frequency is chosen as

$$\boldsymbol{K} = (K\cos\theta, K\sin\theta) \tag{3.6}$$

where $\theta \in \left\{0, \frac{\pi}{3}, \frac{2\pi}{3}\right\}$ and K is chosen as the cut-off frequency of the illumination lens: $K = \frac{2\mathrm{NA}}{\lambda_{\mathrm{exc}}}$. The spectrum of the sample at each shift is then moved to the correct position $\boldsymbol{K}$ in the Fourier space, and the super-resolution image is generated by anti-transforming the sum of all the spectra

$$i_{\mathrm{SIM}}(\boldsymbol{x}) = \mathcal{F}^{-1}\left\{ A(\boldsymbol{k}) \frac{\sum_n \overline{H(\boldsymbol{k}+\boldsymbol{K}_n)} S_n(\boldsymbol{k}+\boldsymbol{K}_n)}{\sum_n |H(\boldsymbol{k}+\boldsymbol{K}_n)|^2 + \varepsilon} \right\} \tag{3.7}$$

where ε is a regularization parameter and $A(\boldsymbol{k})$ is an apodization function.

3.2 Confocal Microscopy

Consider the Intensity collected by a photodiode placed before a pinhole

$$\begin{aligned}
i(\boldsymbol{x}) &= \int_{\mathbb{R}^2} p(-\boldsymbol{x}') \cdot [o(\boldsymbol{x}'-\boldsymbol{x}) \cdot h_{\mathrm{exc}}(\boldsymbol{x}')] * h_{\mathrm{em}}(\boldsymbol{x}')\,\mathrm{d}\boldsymbol{x}' = \\
&= \int_{\mathbb{R}^4} p(-\boldsymbol{x}') o(\boldsymbol{x}''-\boldsymbol{x}) h_{\mathrm{exc}}(\boldsymbol{x}'') h_{\mathrm{em}}(\boldsymbol{x}'-\boldsymbol{x}'')\,\mathrm{d}\boldsymbol{x}'\,\mathrm{d}\boldsymbol{x}'' = \\
&= \int_{\mathbb{R}^2} o(\boldsymbol{x}''-\boldsymbol{x}) h_{\mathrm{exc}}(\boldsymbol{x}'') \int_{\mathbb{R}^2} p(-\boldsymbol{x}') h_{\mathrm{em}}(\boldsymbol{x}'-\boldsymbol{x}'')\,\mathrm{d}\boldsymbol{x}'\,\mathrm{d}\boldsymbol{x}'' = \\
&= \int_{\mathbb{R}^2} o(\boldsymbol{x}''-\boldsymbol{x}) h_{\mathrm{exc}}(\boldsymbol{x}'') [p(-\boldsymbol{x}'') * h_{\mathrm{em}}(-\boldsymbol{x}'')]\,\mathrm{d}\boldsymbol{x}'' = \\
&= o(\boldsymbol{x}) \star \{h_{\mathrm{exc}}(\boldsymbol{x}) \cdot [p(-\boldsymbol{x}) * h_{\mathrm{em}}(-\boldsymbol{x})]\} = \\
&= o(\boldsymbol{x}) * \{h_{\mathrm{exc}}(-\boldsymbol{x}) \cdot [p(\boldsymbol{x}) * h_{\mathrm{em}}(\boldsymbol{x})]\}
\end{aligned} \tag{3.8}$$

Namely, we proved that the confocal PSF is

$$h(\boldsymbol{x}) = h_{\mathrm{exc}}(-\boldsymbol{x}) \cdot [p(\boldsymbol{x}) * h_{\mathrm{em}}(\boldsymbol{x})] \tag{3.9}$$

We now consider two limit cases.

The first one is that of the infinitely large pinhole. Assuming normalized PSFs, we have $p(\boldsymbol{x}) * h_{\mathrm{em}}(\boldsymbol{x}) = 1$. Thus, the PSF of a laser-scanning system with an open pinhole is

$$h_{\mathrm{LSM}}(\boldsymbol{x}) = h_{\mathrm{exc}}(-\boldsymbol{x}) \tag{3.10}$$

The second case is that of the infinitely small pinhole. In this case, we assume $p(\boldsymbol{x}) = \delta(\boldsymbol{x})$ and thus $p(\boldsymbol{x}) * h_{\mathrm{em}}(\boldsymbol{x}) = h_{\mathrm{em}}(\boldsymbol{x})$. We found out that the PSF of a confocal laser scanning microscope is

$$h_{\mathrm{CLSM}}(\boldsymbol{x}) = h_{\mathrm{exc}}(-\boldsymbol{x}) \cdot h_{\mathrm{em}}(\boldsymbol{x}) \tag{3.11}$$

To quantify the enhancement in resolution due to CLSM we consider the simplified case of Gaussian PSFs

$$h_n(\boldsymbol{x}) = \frac{1}{2\pi\sigma_n^2} \exp\left(-\frac{\boldsymbol{x}^2}{2\sigma_n^2}\right) \tag{3.12}$$

where σ is the standard deviation. Thus, the resulting PSF is the product of two Gaussian functions. The result is a narrower Gaussian function with the following standard deviation

$$\sigma_{\mathrm{CLSM}} = \sqrt{\frac{\sigma_{\mathrm{em}}^2 \sigma_{\mathrm{exc}}^2}{\sigma_{\mathrm{em}}^2 + \sigma_{\mathrm{exc}}^2}} \tag{3.13}$$

Neglecting the Stokes-shift, we get that $\sigma_{\mathrm{ISM}} = \sigma_{\mathrm{LSM}}/\sqrt{2}$. However, this result requires a point-like pinhole, which would result in no signal reaching the detector. Thus, there is a trade off between resolution and signal-to-noise ratio in CLSM.

3.3 Image Scanning Microscopy

The scanned image generated by the detector element at position $\boldsymbol{x}_d$ can be written as

$$i(\boldsymbol{x}_s|\boldsymbol{x}_d) = o(\boldsymbol{x}_s) * h(\boldsymbol{x}_s|\boldsymbol{x}_d) \tag{3.14}$$

where $o(\boldsymbol{x}_s)$ is the specimen, here considered as a distribution of light emitters. The PSF is

$$\begin{aligned} h(\boldsymbol{x}_s|\boldsymbol{x}_d) &= h_{\mathrm{exc}}(-\boldsymbol{x}_s) \cdot [p(\boldsymbol{x}_s - \boldsymbol{x}_d) * h_{\mathrm{em}}(\boldsymbol{x}_s)] = \\ &= h_{\mathrm{exc}}(-\boldsymbol{x}_s) \cdot h_{\mathrm{det}}(\boldsymbol{x}_s - \boldsymbol{x}_d) \end{aligned} \tag{3.15}$$

In order to grasp the core idea behind image scanning microscopy (ISM), we first introduce its concept using a Gaussian approximation and later proceed with the general case.

3.3.1 Gaussian model

In this subsection, we simplify the model approximating the emission and excitation PSF with a Gaussian distribution with circular symmetry,

$$g(\boldsymbol{x}|\boldsymbol{\mu}, \sigma) = \frac{1}{2\pi\sigma^2} \exp\left[-\frac{(\boldsymbol{x} - \boldsymbol{\mu})^2}{2\sigma^2}\right] \tag{3.16}$$

where $\boldsymbol{\mu}$ is the mean and σ is the standard deviation. Thus, the resulting PSF is

$$h(\boldsymbol{x}_s|\boldsymbol{x}_d) = g(\boldsymbol{x}_s|\boldsymbol{0}, \sigma_{\mathrm{exc}}) \cdot g(\boldsymbol{x}_s|\boldsymbol{x}_d, \sigma_{\mathrm{det}}) \tag{3.17}$$

Notably, the product of two Gaussian function is another Gaussian function with the following properties:

$$\boldsymbol{\mu}(\boldsymbol{x}_d) = \frac{\boldsymbol{\mu}_{\mathrm{det}}\sigma^2_{\mathrm{exc}} + \boldsymbol{\mu}_{\mathrm{exc}}\sigma^2_{\mathrm{det}}}{\sigma^2_{\mathrm{det}} + \sigma^2_{\mathrm{exc}}} = \frac{\boldsymbol{x}_d\sigma^2_{\mathrm{exc}}}{\sigma^2_{\mathrm{det}} + \sigma^2_{\mathrm{exc}}} \qquad \sigma_{\mathrm{ISM}} = \sqrt{\frac{\sigma^2_{\mathrm{det}}\sigma^2_{\mathrm{exc}}}{\sigma^2_{\mathrm{det}} + \sigma^2_{\mathrm{exc}}}} \tag{3.18}$$

where $\boldsymbol{\mu}(\boldsymbol{x}_d)$ is known as *shift-vector*, and σ_{ISM} is independent of $\boldsymbol{x}_d$. The intensity of the resulting Gaussian function is also rescaled by the following rescaling factor

$$s(\boldsymbol{x}_d) = \frac{1}{2\pi\left(\sigma^2_{\mathrm{det}} + \sigma^2_{\mathrm{exc}}\right)} \exp\left[-\frac{\boldsymbol{x}_d^2}{2(\sigma^2_{\mathrm{det}} + \sigma^2_{\mathrm{exc}})}\right] \tag{3.19}$$

Thus, the resulting PSF can be written as

$$h(\boldsymbol{x}_s|\boldsymbol{x}_d) = s(\boldsymbol{x}_d) \cdot g[\boldsymbol{x}_s|\boldsymbol{\mu}(\boldsymbol{x}_d), \sigma] \tag{3.20}$$

Neglecting the Stokes-shift, we can assume $\sigma_{\mathrm{exc}} \sim \sigma_{\mathrm{det}}$ to obtain the well-known $\sqrt{2}$ gain in resolution. However, the images generated by each detector element are shifted with respect to the central element. Indeed, the scanned-image is

$$i(\boldsymbol{x}_s|\boldsymbol{x}_d) = o(\boldsymbol{x}_s) * h(\boldsymbol{x}_s|\boldsymbol{x}_d) = i[\boldsymbol{x}_s - \boldsymbol{\mu}(\boldsymbol{x}_d)] \tag{3.21}$$

If all the images are simply summed together, the resolution gain is lost, and the resulting image is equivalent to that generated by a traditional confocal microscope with an open pinhole (as large as the detector size). The pixel reassignment (PR) algorithm shifts back each image

$$i(\boldsymbol{x}_s|\boldsymbol{x}_d) \underset{\mathrm{PR}}{\longrightarrow} i[\boldsymbol{x}_s + \boldsymbol{\mu}(\boldsymbol{x}_d)|\boldsymbol{x}_d] = i(\boldsymbol{x}_s|\boldsymbol{0}) \cdot s(\boldsymbol{x}_d) \tag{3.22}$$

and constructs a new image summing the reassigned images

$$i_{\mathrm{ISM}}(\boldsymbol{x}_s) = i(\boldsymbol{x}_s|\boldsymbol{0}) \cdot \int_{\mathbb{R}^2} s(\boldsymbol{x}_d)\,\mathrm{d}\boldsymbol{x}_d \tag{3.23}$$

thus conserving the gain in resolution and enhancing the signal-to-noise ratio (SNR).

3.3.2 General model

Instead of using the theoretical values found using the Gaussian approximation, the shift vectors are found by the adaptive pixel reassignment (APR) algorithm. This latter first calculates the correlogram

$$R(\boldsymbol{x}_s|\boldsymbol{x}_d) = \mathcal{F}^{-1}\left\{\frac{\mathcal{F}\{i(\boldsymbol{x}_s|\boldsymbol{x}_d)\} \cdot \overline{\mathcal{F}\{i(\boldsymbol{x}_s|\boldsymbol{0})\}}}{\left|\mathcal{F}\{i(\boldsymbol{x}_s|\boldsymbol{x}_d)\} \cdot \overline{\mathcal{F}\{i(\boldsymbol{x}_s|\boldsymbol{0})\}}\right|}\right\} \tag{3.24}$$

and later finds the shift vectors as the position of maximum correlation

$$\mu(\boldsymbol{x}_d) = \arg\max_{\boldsymbol{x}_s}\{R(\boldsymbol{x}_s|\boldsymbol{x}_d)\} \tag{3.25}$$

and shifts each scanned image of the corresponding shift vector

$$i(\boldsymbol{x}_s|\boldsymbol{x}_d) \xrightarrow[\text{APR}]{} i[\boldsymbol{x}_s + \boldsymbol{\mu}(\boldsymbol{x}_d)|\boldsymbol{x}_d] \tag{3.26}$$

After reassignment, the scanned images are approximately identical and the final ISM image is calculated as the sum of reassigned images

$$i_{\text{ISM}}(\boldsymbol{x}_s) = \sum_{\boldsymbol{x}_d} i[\boldsymbol{x}_s + \boldsymbol{\mu}(\boldsymbol{x}_d)|\boldsymbol{x}_d] \tag{3.27}$$

The ISM image formation process can be seen equivalently from the perspective of the detector. In fact, the detector array can be considered as a small camera, capable of acquiring wide-field images that we call *micro-images*. The equation of these latter can be found as follows:

$$\begin{aligned}
i(\boldsymbol{x}_s|\boldsymbol{x}_d) &= o(\boldsymbol{x}_s) * [h_{\text{exc}}(-\boldsymbol{x}_s) \cdot h_{\text{det}}(\boldsymbol{x}_s - \boldsymbol{x}_d)] = \\
&= \int_{\mathbb{R}^2} o(\boldsymbol{x}_s - \boldsymbol{x}') \cdot h_{\text{exc}}(-\boldsymbol{x}') \cdot h_{\text{det}}(\boldsymbol{x}' - \boldsymbol{x}_d)\, \mathrm{d}\boldsymbol{x}' = \\
&= \int_{\mathbb{R}^4} o(\boldsymbol{x}_s - \boldsymbol{x}') \cdot h_{\text{exc}}(-\boldsymbol{x}') \cdot h_{\text{det}}(\boldsymbol{x}'')\delta(\boldsymbol{x}'' - \boldsymbol{x}' + \boldsymbol{x}_d)\, \mathrm{d}\boldsymbol{x}'\, \mathrm{d}\boldsymbol{x}'' = \\
&= \int_{\mathbb{R}^2} o(\boldsymbol{x}_s - \boldsymbol{x}'' - \boldsymbol{x}_d) \cdot h_{\text{exc}}(-\boldsymbol{x}'' - \boldsymbol{x}_d) h_{\text{det}}(\boldsymbol{x}'')\, \mathrm{d}\boldsymbol{x}'' = \\
&= [o(\boldsymbol{x}_s - \boldsymbol{x}_d) \cdot h_{\text{exc}}(-\boldsymbol{x}_d)] * h_{\text{det}}(-\boldsymbol{x}_d) = i(\boldsymbol{x}_d|\boldsymbol{x}_s)
\end{aligned} \tag{3.28}$$

As expected, the micro-image is simply given by the wide-field image formation law, but applied to the illuminated object, i.e. the object multiplied by the excitation PSF. After APR, the reassigned micro-image is

$$i[\boldsymbol{x}_d|\boldsymbol{x}_s + \boldsymbol{\mu}(\boldsymbol{x}_d)] = \{o[\boldsymbol{x}_s - \boldsymbol{x}_d + \boldsymbol{\mu}(\boldsymbol{x}_d)] \cdot h_{\text{exc}}(-\boldsymbol{x}_d)\} * h_{\text{det}}(-\boldsymbol{x}_d) \tag{3.29}$$

For clarity, we now rewrite the new coordinates of the micro-images as

$$\boldsymbol{u} = \boldsymbol{x}_d - \boldsymbol{\mu}(\boldsymbol{x}_d) = \boldsymbol{x}_d \frac{\sigma_{\text{det}}^2}{\sigma_{\text{det}}^2 + \sigma_{\text{exc}}^2} \tag{3.30}$$

where we have used the theoretical value of the shift-vectors found using the Gaussian approximation for simplicity. In this new coordinate system, the post-APR micro-image is

$$i(\boldsymbol{u}|\boldsymbol{x}_s) = \left[o(\boldsymbol{x}_s - \boldsymbol{u}) \cdot h_{\text{exc}}\left(-\frac{\sigma_{\text{det}}^2 + \sigma_{\text{exc}}^2}{\sigma_{\text{det}}^2}\boldsymbol{u}\right)\right] * h_{\text{det}}\left(-\frac{\sigma_{\text{det}}^2 + \sigma_{\text{exc}}^2}{\sigma_{\text{det}}^2}\boldsymbol{u}\right) \tag{3.31}$$

In other words, the effect of APR is a digital shrinking of the excitation and detection PSFs with respect to the object. In detail, the standard deviations of the PSFs become

$$\sigma_{\text{exc}} \xrightarrow[\text{APR}]{} \sigma_{\text{exc}} \frac{\sigma_{\text{det}}^2}{\sigma_{\text{det}}^2 + \sigma_{\text{exc}}^2} \qquad \sigma_{\text{det}} \xrightarrow[\text{APR}]{} \sigma_{\text{det}} \frac{\sigma_{\text{det}}^2}{\sigma_{\text{det}}^2 + \sigma_{\text{exc}}^2} \tag{3.32}$$

which are always smaller than the original standard deviation.

3.4 Stimulated Emission Depletion Microscopy

The STED concept is about depleting the fluorescence (spontaneous emission) from the periphery of an excited spot by exploiting the phenomenon of stimulated emission.

The spontaneous emission rate is k_F. The stimulated emission rate is k_S and depends on the flux of STED photons and the corresponding cross-section. We define the saturation intensity I_{sat} as the STED intensity such that $k_S = k_F$. Thus,

$$k_S = k_F \zeta \qquad \zeta = I_{\text{STED}}/I_{\text{sat}} \tag{3.33}$$

where ζ is known as the saturation factor.

We now consider the rate equations of a fluorophore exposed with excitation and depletion light.

$$\frac{\partial N_1}{\partial t} = -k_F N_1 - k_S N_1 + k_S N_0^* \tag{3.34}$$

$$\frac{\partial N_0^*}{\partial t} = -k_V N_0^* - k_S N_0^* + k_S N_1 \tag{3.35}$$

We assume that at $t = 0$, we have $N_1(0) = 1$ and all other states unpopulated. We also assume a squared STED pulse with finite duration T_S. Lastly, we assume the vibrational state to have a vanishingly short lifetime ($k_V \to +\infty$). Therefore, $N_0^* = 0$ at each time, and we can neglect the last term of the first rate equation (anti-Stokes excitation). The rate equation of the excited state becomes

$$\frac{\partial N_1}{\partial t} = -k_F N_1 - k_S N_1 \tag{3.36}$$

and the solution is

$$N_1(t) = \begin{cases} \exp(-k_F t - k_S t) & 0 \le t < T_S \\ \exp(-k_S T_S)\exp(-k_F t) & t \ge T_S \end{cases} \tag{3.37}$$

We define the fluorescence probability per molecule as

$$F = \int N_1(t)\,\mathrm{d}t \tag{3.38}$$

For high STED intensities, we have that $k_S \gg k_F$, suggesting that it is useful to collect photons only after the STED pulse (gated-STED). The fluorescence probabilities with and without the STED beam are

$$F(\zeta) = \int_{T_s}^{+\infty} \exp(-k_S T_S)\exp(-k_F t)\,\mathrm{d}t = \frac{1}{k_F}\exp\left[-T_S k_F(1+\zeta)\right] \tag{3.39}$$

$$F(0) = \int_{T_s}^{+\infty} \exp(-k_F t)\,\mathrm{d}t = \frac{1}{k_F}\exp\left(-T_S k_F\right) \tag{3.40}$$

The ratio of the fluorescence probabilities gives the depletion function

$$\eta(\zeta) = \frac{F(\zeta)}{F(0)} = \exp\left(-k_F T_S \zeta\right) \tag{3.41}$$

The effective excitation PSF is given by

$$h_{\text{exc}}^{(\zeta)}(\boldsymbol{x}) = h_{\text{exc}}^{(0)}(\boldsymbol{x}) \cdot \eta[\zeta(\boldsymbol{x})] \tag{3.42}$$

In order to improve the lateral resolution, the fluorescence has to be suppressed only from the periphery of the excitation PSF. Thus, the intensity profile of the STED beam has to be annular shaped. For simplicity, we approximate the excitation PSF with a Gaussian function and the center of the STED beam with a parabolic function

$$k_F T_S \zeta(\boldsymbol{x}) \sim \frac{1}{2}\zeta \boldsymbol{x}^2 \tag{3.43}$$

Thus,

$$\begin{aligned} h_{\text{exc}}^{(0)}(\boldsymbol{x}) \cdot \eta[\zeta(\boldsymbol{x})] &= \exp\left(-\frac{\boldsymbol{x}^2}{2\sigma_{\text{exc}}^2}\right) \cdot \exp\left(-\frac{1}{2}\zeta \boldsymbol{x}^2\right) = \\ &= \exp\left[-\frac{1}{2}\left(\frac{1}{\sigma_{\text{exc}}^2} + \zeta\right)\boldsymbol{x}^2\right] \end{aligned} \tag{3.44}$$

Thus, the excitation standard deviation is reduced to

$$\sigma_{\text{exc}}(\zeta) = \frac{\sigma_{\text{exc}}}{\sqrt{1 + \zeta\sigma_{\text{exc}}^2}} \tag{3.45}$$

The complete PSF of a STED system is given by

$$h(\zeta)(\boldsymbol{x}) = h_{\text{exc}}^{(\zeta)}(-\boldsymbol{x}) \cdot h_{\text{det}}(\boldsymbol{x}) \tag{3.46}$$

Approximating also the detection PSF as a Gaussian, we have

$$\sigma_{\text{STED}} = \sqrt{\frac{\sigma_{\text{det}}^2 \sigma_{\text{exc}}^2}{\sigma_{\text{det}}^2(1 + \zeta\sigma_{\text{exc}}^2) + \sigma_{\text{exc}}^2}} \underset{\zeta \to +\infty}{\sim} \frac{\sigma_{\text{exc}}}{\sqrt{1 + \zeta\sigma_{\text{exc}}^2}} \tag{3.47}$$

4

Stochastic super-resolution microscopy

4.1 Super-resolution optical fluctuation imaging (SOFI)

Consider a widefield imaging system and a sample containing a single fluorescent molecule at position $\boldsymbol{r}_i$. The resulting image is

$$I(\boldsymbol{r}) = c \cdot h(\boldsymbol{r} - \boldsymbol{r}_i), \tag{4.1}$$

with $h(\boldsymbol{r})$ the PSF of the imaging system and c a constant taking into account the brightness of the molecule and the detector sensitivity. From now on, we assume for simplicity $c = 1$. A set of N different fluorophores at different positions thus gives

$$I(\boldsymbol{r}) = \sum_{i=1}^{N} h(\boldsymbol{r} - \boldsymbol{r}_i). \tag{4.2}$$

The core idea of SOFI comes from fluorescence fluctuation spectroscopy (see Chapter 6). If we assume that each emitter may **randomly and independently from each other** turn on and off, we get a time-dependent image:

$$I(\boldsymbol{r}, t) = \sum_{i=1}^{N} h(\boldsymbol{r} - \boldsymbol{r}_i) s_i(t), \tag{4.3}$$

with

$$s_i(t) = \begin{cases} 0, & \text{if the emitter is off at time } t \\ 1, & \text{if the emitter is active at time } t \end{cases} \tag{4.4}$$

Now, let us calculate

$$G^{(n)}(\boldsymbol{r}) = \langle I(\boldsymbol{r},t)^n \rangle \,, \tag{4.5}$$

where the brackets $\langle\rangle$ denote averaging over time. Then,

$$G^{(n)}(\boldsymbol{r}) = \left\langle \left[\sum_{i=1}^{N} h(\boldsymbol{r}-\boldsymbol{r}_i) s_i(t) \right]^n \right\rangle \tag{4.6}$$

$$= \sum_{i=1}^{N} h^n(\boldsymbol{r}-\boldsymbol{r}_i) \left\langle s_i^n(t) \right\rangle \tag{4.7}$$

$$= a \cdot \sum_{i=1}^{N} h^n(\boldsymbol{r}-\boldsymbol{r}_i), \tag{4.8}$$

with $a = \langle s_i(t) \rangle$ a constant.

Thus, the resulting image is the convolution of the sample with the n-th power of the PSF. In Fourier space, this results in an m-fold extension of the frequency support of the OTF of the imaging system. In addition, SOFI provides optical sectioning.

If we approximate the PSF as a 2D Gaussian,

$$h = A \exp\left(-\frac{x^2+y^2}{2\sigma^2} \right), \tag{4.9}$$

then raising h to the n-th power yields

$$h^n = A \exp\left(-\frac{x^2+y^2}{2\left(\frac{\sigma}{\sqrt{n}}\right)^2} \right), \tag{4.10}$$

indicating that the apparent resolution improvement only scales with $\sqrt{n}$.

Note that a more rigorous derivation of the SOFI principles can be found in literature, which uses the calculation of the n-th order cumulant image. For $n = 2$, this cumulant image $C_2(\boldsymbol{r},\tau)$ is

$$C_2(\boldsymbol{r},\tau) = \sum_{i=1}^{N} h^2(\boldsymbol{r}-\boldsymbol{r}_i) c_2(\tau), \tag{4.11}$$

with $c_2(\tau)$ the second-order temporal cumulant function of the emitters' fluorescence.

4.2 Camera-based single-molecule localization microscopy (SMLM)

Photo-activated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), and point accumulation for imaging in nanoscale topography (PAINT) are examples of camera-based SMLM.

The Cramér–Rao lower bound (CRLB) provides a lower limit to the variance of any unbiased estimator. For 1D SMLM, the CRLB is

$$\mathrm{Var}(\hat{x}) \geq -\left[\left\langle \frac{\partial^2}{\partial x^2} \ln p(l;x) \right\rangle\right]^{-1}, \tag{4.12}$$

with $p(l;x)$ the likelihood, i.e., the probability of observing l for an emitter at position x.

For a Gaussian shaped PSF, Eq. 4.12 gives the following localization uncertainty σ_{loc} (fundamentally limited by the emission PSF size and the number of photons N collected by the camera):

$$\sigma_{\mathrm{loc}} = \sqrt{\mathrm{Var}(\hat{x})} \geq \frac{\sigma_0}{\sqrt{N}}, \tag{4.13}$$

with σ_0 the standard deviation of the PSF (assumed to be Gaussian), typically around 100 nm. Assuming $N = 10^2 - 10^4$, this corresponds to σ_{loc} below 10 nm. However, the non-Gaussian shape of the PSF, background fluorescence, and the finite pixel size of the camera all worsen the localization uncertainty. A more complete expression, taking these factors into account is:

$$\sigma_{\mathrm{loc}} \geq \sqrt{\left(\frac{\sigma_0^2 + a^2/12}{N}\right)\left(\frac{16}{9} + \frac{8\pi\sigma_0^2 b^2}{a^2 N^2}\right)}, \tag{4.14}$$

with a the pixel size and b the background intensity.

Any localization algorithm without bias will have a localization uncertainty greater or equal than Eq. 4.14. The most common localization method is based on fitting of a 2D Gaussian.

The concept of SMLM can be extended to 3D. A common method is the use of a cylindrical lens in the detection path, that asymmetrically condenses light beams in either the x or y direction. This astigmatism-induced stretch is analyzed to find the z position.

An extension to conventional SMLM is resolution enhancement by sequential imaging (RESI), a technique in which the information from multiple localization events coming from the same sample position are merged. For K events, the resulting localization uncertainty is

$$\sigma_{\mathrm{RESI}} = \frac{\sigma_{\mathrm{SMLM}}}{\sqrt{K}}. \tag{4.15}$$

4.3 SMLM with minimum fluxes: MINFLUX

MINFLUX, meaning either localizing with MINimal emission FLUXes or Maximally INFormative LUminescence eXcitation probing, is a technique able to localize single molecules with a very

limited number of emitted fluorescence photons. Here, we consider 2D localization of an emitter at position $\boldsymbol{r}_e$, exposed to K different light intensities $\{I_0(\boldsymbol{r}), \ldots, I_{K-1}(\boldsymbol{r})\}$, yielding a collection of $\boldsymbol{n} = \{n_0, n_1, \ldots, n_{K-1}\}$ photons. Due to shot noise, each photon count number n_i follows Poissonian statistics with mean λ_i equal to

$$\lambda_i = \mathrm{c} I_i\left(\boldsymbol{r}_e\right), \tag{4.16}$$

with c a constant taking into account the absorption cross section and quantum yield of the fluorophore, as well as the collection efficiency of the system.

Let N be the total number of photons collected, i.e.,

$$N = n_0 + \cdots + n_{K-1}. \tag{4.17}$$

Then probability of measuring a list of $\boldsymbol{n}$ photons is

$$P\left(\boldsymbol{n} \mid \{\lambda_i\}\right) = \prod_{i=0}^{K-1} \frac{e^{-\lambda_i} \lambda^{n_i}}{n_i!}. \tag{4.18}$$

However, this is the probability of observing $\boldsymbol{n}$ photons. We want to make the localization scheme independent of the apparent brightness of the emitters (c in Eq. 4.16). Therefore, we calculate $P(\boldsymbol{n})$ conditioned to N, i.e., $P(\boldsymbol{n}|N, \{\lambda_i\})$. Using Bayes' theorem, we have

$$P\left(\mathbf{n} \mid N, \{\lambda_i\}\right) = \frac{P\left(\mathbf{n} \mid \{\lambda_i\}\right)}{P\left(N \mid \{\lambda_i\}\right)} = \frac{\prod_{i=0}^{K-1} \frac{e^{-\lambda_i} \lambda^{n_i}}{n_i!}}{\frac{e^{-\lambda_{tot}} \lambda_{tot}^N}{N!}} = \frac{N! \prod_{i=0}^{K-1} \frac{e^{-\lambda_i} \lambda^{n_i}}{n_i!}}{e^{-\lambda_{tot}} \lambda_{tot}^N}, \tag{4.19}$$

with $\lambda_{\mathrm{tot}} = \sum \lambda_i$.

We can write $\lambda_i = N p_i$ with p_i the probability that if a photon is detected, it is detected under exposure i, i.e.

$$p_i\left(\boldsymbol{r}_e\right) = \frac{\lambda_i}{\sum_{j=0}^{K-1} \lambda_j} = \frac{I_i\left(\boldsymbol{r}_e\right)}{\sum_{j=0}^{K-1} I_j\left(\boldsymbol{r}_e\right)}. \tag{4.20}$$

Since $\sum_i p_i = 1$, we have

$$P\left(\mathbf{n} \mid N, \{p_i\}\right) = \frac{N! \prod_{i=0}^{K-1} \left(\frac{e^{-N p_i} N^{n_i} p_i^{n_i}}{n_i!} \right)}{e^{-N} N^N} = \frac{N!}{\prod_{i=0}^{K-1} n_i!} \prod_{i=0}^{K-1} p_i^{n_i}, \tag{4.21}$$

which is a multinomial distribution with p_i the event probabilities and N the number of trials. Note that $p_i = \frac{\lambda_i(\boldsymbol{r}_e)}{N} = p_i(\boldsymbol{r}_e)$ is a function of the emitter position. Thus, Eq. 4.21 gives for each potential emitter position $\boldsymbol{r}_e$ the probability to observe $\boldsymbol{n}$ photons, given a total number of N collected photons. If we assume no prior information, Eq. 4.21 is the likelihood function $\mathcal{L}$ for finding the emitter position at $\boldsymbol{r}_e$ given the $\boldsymbol{n}$ photon counts:

$$\mathcal{L}\left(\boldsymbol{r}_e \mid \boldsymbol{n}\right) = \frac{N!}{\prod_{i=0}^{K-1} n_i!} \prod_{i=1}^{K} p_i\left(\boldsymbol{r}_E\right)^{n_i}. \tag{4.22}$$

Eq. 4.22 can be used in practice to find the emitter position by finding arg max $\mathcal{L}$, i.e., the maximum likelihood estimation.

To find the localization uncertainty with which we can localize the emitter, we calculate the Fisher information matrix. For simplicity, we assume that a 1D scenario with an emitter that is illuminated with two displaced doughnut beams ($K = 2$), positioned at position $x = \pm L/2$. We further assume the emitter to be close to the doughnut minimum in which the illumination profile can be approximated as a quadratic function. Thus, we have:

$$\lambda_0 = c(x - L/2)^2, \tag{4.23}$$
$$\lambda_1 = c(x + L/2)^2 \tag{4.24}$$

Then, p_0 and p_1 are:

$$p_0 = \frac{\lambda_0}{\lambda_0 + \lambda_1}, \tag{4.25}$$
$$p_1 = \frac{\lambda_1}{\lambda_0 + \lambda_1} \tag{4.26}$$

The localization uncertainty σ_{CRB} is given by

$$\sigma_{\mathrm{CRB}} = \sqrt{\frac{1}{N\left(\frac{1}{p_0}\left(\frac{\partial p_0}{\partial x}\right)^2 + \frac{1}{p_1}\left(\frac{\partial p_1}{\partial x}\right)^2\right)}} \tag{4.27}$$

The derivative $\frac{\partial p_0}{\partial x}$ is

$$\frac{\partial p_0}{\partial x} = \frac{L(x^2 - L^2/4)}{2(x^2 + L^2/4)^2} \tag{4.28}$$

For an emitter at x = 0, we find for $\left(\frac{\partial p_0}{\partial x}\right)^2$

$$\left(\frac{\partial p_0}{\partial x}\right)^2 = \frac{8}{L^2} \tag{4.29}$$

And the same for $\left(\frac{\partial p_1}{\partial x}\right)^2$. Filling in Eq. 4.25, 4.26, and 4.29 in Eq. 4.27 yields

$$\sigma_{\mathrm{CRB}}(0) = \frac{L}{4\sqrt{N}}. \tag{4.30}$$

Unlike conventional SMLM, the uncertainty does not depend on the emission wavelength (nor the excitation wavelength) and scales linearly with L, an experimentally tunable parameters that can be chosen (almost) arbitrarily small. E.g. for $L = 50$ nm and $N = 100$ photons, a σ_{CRB} value of 1.25 nm is found. A similar linear dependence on L is found in 2D for four illumination patterns.

5

Non-linear microscopy

Note that throughout this chapter we assume that the medium is homogeneous and isotropic and as a consequence we can examine vectors and equations on a component-by-component basis.

A linear dielectric medium is characterized by a linear relation between the polarization density P and the electric field E:

$$P = \varepsilon_0 \chi E, \tag{5.1}$$

with ε_0 the dielectric permittivity of empty space ($= 8.854 \cdot 10^{-12} C^2/Nm^2$) and χ a dimensionless quantity called the susceptibility (which is a function of the frequency of the electric field in case of an oscillating field).

From Maxwell's equations (in one dimension), we can derive the inhomogeneous wave equation to describe the effect of the interaction between the electric field and the induced polarization P in the medium.

$$\frac{\partial^2 E}{\partial x^2} - \frac{1}{c^2}\frac{\partial^2 E}{\partial t^2} = \mu_0 \frac{\partial^2 P}{\partial t^2} \tag{5.2}$$

Combining Eq. 5.2 and 5.1, we have:

$$\frac{\partial^2 E}{\partial x^2} - \frac{1}{c^2}\frac{\partial^2 E}{\partial t^2} = \mu_0 \frac{\partial^2}{\partial t^2}(\varepsilon_0 \chi E) \tag{5.3}$$

Since $\frac{1}{c^2} = \varepsilon_0 \mu_0$:

$$\frac{\partial^2 E}{\partial x^2} - \frac{(1+\chi)}{c^2}\frac{\partial^2 E}{\partial t^2} = 0, \tag{5.4}$$

which is simply the homogeneous equation with $c \to c/\sqrt{1+\chi} = c/\sqrt{\epsilon/\epsilon_0} = c/n$, with n the (complex) refractive index.

5.1 Second Harmonic Generation microscopy

More generally, P is not proportional to E:

$$P = \varepsilon_0 \left[\chi^{(1)}E + \chi^{(2)}E^2 + \chi^{(3)}E^3 + \ldots\right] \tag{5.5}$$

Usually, $\chi^{(2)} > \chi^{(3)} > \chi^{(4)} > \chi^{(5)} \ldots$. The first term describes linear absorption, scattering and reflection of light, the second term describes second harmonic generation and sum and difference frequency generation and the third term describes two- and three-photon absorption, third harmonic generation and stimulated Raman processes and coherent anti-Stokes Raman scattering (CARS).

For strong enough electric fields, the nonlinear terms cannot be ignored. The wave equation then becomes:

$$\frac{\partial^2 E}{\partial x^2} - \frac{n^2}{c^2}\frac{\partial^2 E}{\partial t^2} = \varepsilon_0\mu_0\chi^{(2)}\frac{\partial^2}{\partial t^2}\left(E^2\right) + \varepsilon_0\mu_0\chi^{(3)}\frac{\partial^2}{\partial t^2}\left(E^3\right) + \ldots \tag{5.6}$$

Assume an oscillating electric field with angular frequency ω:

$$E(t) \propto E_0 \exp(i\omega t) + E_0^* \exp(-i\omega t). \tag{5.7}$$

Then,

$$E(t)^2 \propto E_0^2 \exp(2i\omega t) + 2\left|E_0\right|^2 + E_0^{*2} \exp(-2i\omega t) \tag{5.8}$$

We now have terms that vary at twice the original frequency, second harmonic generation. Since the amplitude of the second-harmonic light is proportional to E^2, also the second-harmonic intensity scales with the square of the intensity of the incident wave. Since the second harmonic emissions are added coherently, the intensity of the second-harmonic wave is also proportional to the square of the length of the interaction volume L.

Therefore, to maximize the SHG efficiency, the incident wave must have the larges possible power. This is typically accomplished by using pulsed lasers which may have a peak power of hundreds of kW (while keeping the average power low enough to limit the amount of photodamage) and focusing the beam with an objective lens.

The constant term in Eq. 5.8 corresponds to small steady contribution to the polarization density, which creates a potential difference across the nonlinear material when the light beam passes.

Energy ($\omega_1 + \omega_2 = \omega_3$) and momentum ($\vec{k}_1 + \vec{k}_2 = \vec{k}_3$) have to be conserved in the process of second harmonic generation. Conservation of energy shows that SHG generates a photon with twice the energy; "two red photons produce one blue photon". Conservation of momentum implies that

$$n(\omega)\frac{\omega}{c} + n(\omega)\frac{\omega}{c} = n(2\omega)\cdot\frac{2\omega}{c}. \tag{5.9}$$

Hence,

$$n(2\omega) = n(\omega) \tag{5.10}$$

This is usually not the case because of dispersion. However, birefringence can be used to satisfy the phase matching requirement. In birefringent materials, the speed at which the wave travels through the medium is different for different polarizations. By choosing the direction at which the waves enters the medium, the birefringence may exactly compensate for dispersion. For perfect phase matching, the SHG emission is 100% forward directed and co-propagates with the laser. This situation holds for SHG from uniaxial crystals (e.g., potassium dihydrogen phosphate (KDP) and β barium borate (BBO)) and from interfaces. The inherent randomness and dispersion in real biological tissues results in a distribution of nonzero Δk values. This imperfect phase matching gives rise to a corresponding distribution of forward and backward emitted components, and as a result SHG in tissues is best described as quasi coherent. Examples of biological materials that produce SHG are collagen and myosin and consequently can be easily imaged with label-free SHG microscopy.

From Eq. 5.5, it is clear that in systems that exhibit inversion symmetry, all even powers of χ disappear. Liquids, gases, amorphous solids (such as glass), and even many crystals display inversion symmetry, these materials cannot produce this type of nonlinear optical interactions. For third-order processes, there is no such condition.

Focusing a laser through an objective lens imposes the Gouy phase shift, which is a phase shift of the coherent light wave upon passing trough the focal point. The Gouy phase shift of the electric field traveling in the z-direction is given by

$$\zeta(z) = \arctan\left(\frac{z}{z_R}\right), \tag{5.11}$$

with

$$z_{\mathrm{R}} = \frac{\pi w_0^2}{\lambda} \tag{5.12}$$

the Rayleigh length.

All SHG scatterers inherit and preserve the phase of the illumination wave. Successive scatterers are therefore not in phase along the illumination wave vector but at a certain angle. To estimate this angle, consider two SHG scatterers separated a distance d apart along the z direction. Complete constructive interference of the SHG signal from both scatterers then occurs under the angle θ

$$\cos\theta \approx 1 - \frac{1}{z_R k_\omega}. \tag{5.13}$$

Hence, in order to collect most of the SHG signal, one can show that the NA of the condenser lens NA^C should be at least

$$\mathrm{NA}^C \approx \frac{2\mathrm{NA}^I}{\pi} \approx 0.64\mathrm{NA}^I, \tag{5.14}$$

with NA^I the NA of the objective lens.

Since SHG is most efficiently produced near the maximum intensity of the focused laser beam, SHG offers a better resolution than linear imaging modalities operating at the same wavelength. Alternatively, one can use a longer wavelength, which can penetrate more deeply into the sample and is less damaging. Moreover, the large wavelength difference between the laser beam and the SH signal allows easy filtering of the signal.

The design of an SHG microscope is very similar to a conventional laser-scanning confocal microscope. Typically, a femtosecond pulsed laser beam or relatively long wavelength (800-1100 nm) is focused by an objective lens onto the sample. A set of galvo scan mirrors scans the laser beam over the sample. Since most of the SHG signal is typically produced in "forward" mode, a condenser lens is used to collect the signal an focus is onto a large single-element detector, such as a PMT. A pinhole, and hence descanning, is not needed, since the SHG is intrinsically produced in a small focal volume. A sharp (5 or 10 nm wide) band-pass filter is installed in front of the detector to allow the SHG to pass. The forward SHG is emitted in a dual-lobed pattern, where the angle between these becomes larger at a higher NA. Thus, it is advantageous to use a condenser with somewhat higher NA than the excitation objective,

To penetrate deeply into the sample, what is needed is an objective with a long working distance (e.g., 3 mm for $\times$40, 0.8 NA), while keeping a reasonable NA (0.5–0.9, many higher-NA lenses have insufficient working distances) and optimized for transmission of the near-IR laser excitation.

Due to the coherence, the image formation process cannot be described by a convolution of the object with an intensity PSF. Instead, one has to work with the field H and the object O, and take the squared modulus after integration.

$$i\left(\boldsymbol{x}\right) = \left|\chi^{(2)}\right|^2 \left| \int H^2\left(\boldsymbol{x'} - \boldsymbol{x}\right) O(\boldsymbol{x'}) \mathrm{d}A \right|^2 \tag{5.15}$$

5.1.1 Non-linear effects

This section gives a more detailed description of non-linear effects.

The response of the material to an electric field is described through the density of dipoles, also known as the polarization P. We write the dependency on the electric field as the following power series

$$P(t) = \varepsilon_0 \sum_{n=1}^{+\infty} \chi^{(n)} E^n(t) = \underbrace{\varepsilon_0 \chi^{(1)} E(t)}_{P^{(1)}} + \underbrace{\varepsilon_0 \sum_{n=2}^{+\infty} \chi^{(n)} E^n(t)}_{P^{(NL)}} \tag{5.16}$$

The first term describes the linear response, while higher terms describe the non-linear response. Note that second-order nonlinear optical interactions can occur only in noncentrosymmetric crystals. Namely, only materials that do not display inversion symmetry. Instead, third-order nonlinear optical interactions can occur for both centrosymmetric and non-centrosymmetric media. Higher

order interactions are typically neglected, being extremely inefficient. For simplicity, we now consider only the second-order non-linearity

$$P^{(2)}(t) = \varepsilon_0 \chi^{(2)} E^2(t) \tag{5.17}$$

We now assume an electric field as follows

$$E(t) = E_1 e^{-i\omega_1 t} + E_2 e^{-i\omega_2 t} + \text{c.c.} \tag{5.18}$$

Typically, the two fields are called *pump* and *idler*. The beam generated by the non-linear process is called *signal*. The second-order polarization is then

$$P^{(2)}(t) = \epsilon_0 \chi^{(2)} \left[E_1^2 e^{-2i\omega_1 t} + E_2^2 e^{-2i\omega_2 t}\right] + 2\epsilon_0 \chi^{(2)} \left[E_1 E_2 e^{-i(\omega_1+\omega_2)t}\right] + \tag{5.19}$$

$$+ 2\epsilon_0 \chi^{(2)} \left[E_1 E_2^* e^{-i(\omega_1-\omega_2)t}\right] + \epsilon_0 \chi^{(2)} \left[E_1 E_1^* + E_2 E_2^*\right] + \text{c.c.} \tag{5.20}$$

The following terms describe the second harmonic generation

$$P\left(\pm 2\omega_1\right) = \epsilon_0 \chi^{(2)} E_1^2 \tag{5.21}$$

$$P\left(\pm 2\omega_2\right) = \epsilon_0 \chi^{(2)} E_2^2 \tag{5.22}$$

The following terms describe the sum frequency generation

$$P\left(+\omega_1 + \omega_2\right) = 2\epsilon_0 \chi^{(2)} E_1 E_2 \tag{5.23}$$

$$P\left(-\omega_1 - \omega_2\right) = 2\epsilon_0 \chi^{(2)} E_1^* E_2^* \tag{5.24}$$

The following terms describe the difference frequency generation

$$P(\omega_1 - \omega_2) = 2\epsilon_0 \chi^{(2)} E_1 E_2^* \tag{5.25}$$

$$P(\omega_2 - \omega_1) = 2\epsilon_0 \chi^{(2)} E_1^* E_2 \tag{5.26}$$

The following terms describe the optical rectification

$$P(0) = 2\epsilon_0 \chi^{(2)} \left(E_1 E_1^* + E_2 E_2^*\right) \tag{5.27}$$

If the non-linear terms are not neglectable, the wave equation becomes

$$\nabla^2 E - \frac{n^2}{c^2} \frac{\partial^2 E}{\partial t^2} = \frac{1}{\epsilon_0 c^2} \frac{\partial^2 P^{(NL)}}{\partial t^2} \tag{5.28}$$

Namely, the non-linear polarization acts as a source. Solving the non-linear wave-equation shows that the generated fields have non-neglectable intensity only if the phase matching conditions are respected

$$\Delta \boldsymbol{k} = \boldsymbol{k}_1 + \boldsymbol{k}_2 - \boldsymbol{k}_3 = \boldsymbol{0} \tag{5.29}$$

$$\Delta \omega = \omega_1 + \omega_2 - \omega_3 = 0 \tag{5.30}$$

The first condition describes the *conservation of momentum*, while the second one describes the *conservation of energy*. Using the relation $ck = \omega$ and assuming collinear beams, we can rewrite the first condition as

$$\frac{n_1\omega_1}{c} + \frac{n_2\omega_2}{c} = \frac{n_3\omega_3}{c} \tag{5.31}$$

However, such condition cannot be achieved, because $n(\omega)$ is typically a monotonically increasing function of ω. This limitation is practically circumvented by using birefringent materials. By aligning the polarization of the field with the highest frequency to the crystal axis showing the smallest refractive index, it is possible to achieve anisotropic phase-matching.

5.2 Two-photon fluorescence microscopy

Two-photon fluorescence may look very similar to SHG, since two low-energy photons are 'converted' into one higher-energy photon, but the two processes are quite different. SHG is a second-order scattering process, while two-photon fluorescence is a third-order process that involves actual absorption to an excited state. (In SHG, the destruction and creation of the photons involves virtual transitions in which no energy is absorbed by the specimen. These virtual energy levels are not energy eigenstates of the atom.)

The absorption cross-section σ for two-photon absorption processes is:

$$\sigma = \sigma^{(2)} I, \tag{5.32}$$

where $\sigma^{(2)}$ is the quantity describing the strength of the two-photon absorption process. Under certain assumptions (e.g., the molecular transition rate must be small enough not to alter the population of molecules in the ground state available for excitation), the two-photon absorption cross-section can be expressed as follows:

$$\sigma^{(2)} = \frac{4\pi^2\hbar\omega^2 \operatorname{Im}\chi^{(3)}}{n^2c^2}. \tag{5.33}$$

The transition rate of an absorption process is given by

$$R = \sigma I/\hbar\omega. \tag{5.34}$$

Hence, we find

$$R = \frac{\sigma^{(2)} I^2}{\hbar\omega}. \tag{5.35}$$

Thus, two-photon absorption scales with the square of the excitation intensity. Two-photon absorption was first described by Maria Goeppert-Mayer in 1931. The molecular two-photon absorption cross-section is usually quoted in the units of Goeppert-Mayer (1 GM $= 10^{-50}$ cm^4 s photon^{-1}).

Not that two-photon absorption can also happen with two photons of different energies. This is called non-degenerate two-photon absorption or two-color two-photon excitation.

Compared to single-photon excitation microscopy, two-photon excitation microscopy has several advantages: fewer photointeractions enabling long term imaging of living samples (and photo-damage by UV excitation can be avoided), imaging of thick specimens up to a depth of about 1 mm, simultaneous excitation of different fluorescent molecules reducing 3D colocalization errors.

Similar to SHG, two-photon fluorescence is only produced in the small volume of a focused laser beam.

$$V \approx \frac{33n\lambda^3}{\pi^3(\mathrm{NA})^4}. \tag{5.36}$$

For typical values (NA $= 1.3$, $\lambda = 500$ nm, n $= 1.33$), V is around 0.06 fL.

The two-photon fluorescence intensity collected in a two-photon microscope with objective NA NA, equipped with a laser with power P and wavelength λ is

$$I_f \propto \sigma^{(2)} P^2 \left(\frac{\mathrm{NA}^2}{\lambda}\right)^2. \tag{5.37}$$

The imaging process in a two-photon fluorescence microscope can be described similarly as for one-photon:

$$i(\boldsymbol{x}) \propto [o * h_2](\boldsymbol{x}), \tag{5.38}$$

with o the distribution of emitters in the object plane and h_2 the two-photon PSF.

$$h_2(u,v) = \left| 2\int_0^1 J_o(v\rho)\exp\left(-iu\rho^2/2\right)\rho d\rho \right|^4, \tag{5.39}$$

with $u = k(NA)^2 z$ and $v = k(NA)r$.

Two-photon fluorescence is typically detected in non-descanned detection in backward mode, but also the combination with descanned detection is possible, for example to combine it with ISM.

6 Fluorescence spectroscopy

6.1 Fluorescence Correlation Spectroscopy

The three-dimensional diffusion equation for particles undergoing Brownian motion is:

$$\frac{\partial \rho(\mathbf{r},t)}{\partial t} = D\nabla^2 \rho(\mathbf{r},t), \tag{6.1}$$

with ρ the particle density at position $\mathbf{r}$ at time t and D the diffusion coefficient. For simplicity, we continue with one spatial dimension:

$$\frac{\partial \rho(x,t)}{\partial t} = D\frac{\partial^2 \rho(x,t)}{\partial x^2}. \tag{6.2}$$

If we assume a single particle at $x_0 = 0$ at $t_0 = 0$, the probability density of finding it at position x at time t is:

$$\rho(x,t) = \frac{1}{\sqrt{4\pi D t}} \exp\left(-\frac{x^2}{4Dt}\right) \tag{6.3}$$

One can prove that the mean squared displacement (MSD) $\langle x^2(t)\rangle$ of this particle is

$$\left\langle x^2(t)\right\rangle = \int x^2(t)\rho(x,t)dx = 2Dt. \tag{6.4}$$

The diffusion coefficient gives information on the dynamics in the sample, and therefore also on the size of the particle via the Stokes-Einstein equation:

$$D = \frac{k_\mathrm{B}T}{6\pi\eta r}, \tag{6.5}$$

with k_{B} the Boltzmann constant, η the dynamic viscosity and r the particle radius.

Measuring the MSD for individual particles is often infeasible (too high concentration, too fast for camera detection, etc.). Fluorescence correlation spectroscopy (FCS) provides a solution.

Consider a confocal setup measuring the time-dependent fluorescence intensity $F(t)$ in a sample of randomly moving fluorescent particles. Then,

$$F(t) = \alpha \int W(\mathbf{r})\rho(\mathbf{r}, t)dV, \tag{6.6}$$

with α a constant describing the quantum yield and detector sensitivity, $W(\mathbf{r})$ the observation volume, and $dV = dxdydz$.

The autocorrelation of this signal is

$$G(\tau) = \frac{\langle \delta F(t)\delta F(t+\tau)\rangle}{\langle F(t)\rangle^2}, \tag{6.7}$$

with

$$\delta F(t) = F(t) - \langle F(t)\rangle. \tag{6.8}$$

Alternatively, combining Eq. 6.7 and 6.8 and making use of $\langle F(t)\rangle = \langle F(t+\tau)\rangle$, G can also be calculated as:

$$G(\tau) = \frac{\langle F(t)F(t+\tau)\rangle}{\langle F(t)\rangle^2} - 1. \tag{6.9}$$

For $\tau = 0$, we have

$$G(0) = \frac{\langle \delta F(t)\delta F(t)\rangle}{\langle F(t)\rangle^2} = \frac{\langle (\delta F(t))^2\rangle}{\langle F(t)\rangle^2}. \tag{6.10}$$

The number of emitted/detected photons follows a Poisson distribution. Therefore, the numerator, which describes the variance in F, is equal to $\langle F\rangle$. Thus,

$$G(0) \propto \frac{\text{Variance}}{\langle N\rangle^2} \propto \frac{1}{\langle N\rangle} \tag{6.11}$$

If we assume that the change in intensity is solely caused by the translational movement of the particles, and if we assume a $3D$ Gaussian focal volume,

$$W(\vec{r}) = I_0 e^{-2\left(x^2+y^2\right)/\omega_0^2} e^{-2z^2/z_0^2}, \tag{6.12}$$

then the autocorrelation can be analytically calculated.

$$\delta F(t) = \alpha \int_V W(\vec{r})\delta\rho(\vec{r}, t)dV \tag{6.13}$$

Thus,

$$G(\tau) = \frac{\iint_{VV'} W(\vec{r}) W\left(\vec{r}'\right) \left\langle \delta\rho(\vec{r},0)\delta\rho\left(\vec{r}',\tau\right)\right\rangle dV dV'}{\left(\int_V \delta\rho(\vec{r},0) W(\vec{r}) dV\right)^2}, \tag{6.14}$$

with

$$\left\langle \delta\rho(\vec{r},0)\delta\rho\left(\vec{r}',\tau\right)\right\rangle = \langle\rho\rangle \frac{1}{(4\pi D\tau)^{3/2}} e^{\left(\vec{r}-\vec{r}'\right)^2/4D\tau}. \tag{6.15}$$

Plugging Eq. 6.15 into Eq. 6.14 gives the final result:

$$G(\tau) = \frac{1}{\langle N\rangle}\left(1+\frac{4D\tau}{\omega_0^2}\right)^{-1}\left(1+\frac{4D\tau}{\omega_z^2}\right)^{-1/2}. \tag{6.16}$$

Here, $\langle N\rangle = \langle C\rangle V_{eff}$ is the average number of particles in the so-called effective focal volume. Note that different definitions of V_{eff} are used. Here, we take $V_{eff} = \omega_z\omega_0^2\pi^{3/2}$.

If we assume that the volume is strongly elongated along the z direction, i.e., $\omega_z \gg \omega_0$, then for a τ value of $\tau_D = \omega_0^2/(4D)$, we find that

$$G(\tau_D) = \frac{1}{2\langle N\rangle} = \frac{1}{2}G(0). \tag{6.17}$$

Thus, τ_D corresponds to the time shift for which the autocorrelation has dropped to half the "starting value". This value is called the diffusion time and is related to how long the particles stay on average in the focal volume.

From τ_D or, equivalently, D, one can calculate the size of the particle with the Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi\eta r}, \tag{6.18}$$

with k_B the Boltzmann constant, T the temperature, η the dynamic viscosity, and r the radius.

Note that, although in theory, FCS allows seeing the formation of dimers from monomers, this is experimentally difficult. Indeed, for a doubling in volume, only a factor $2^{1/3}$ (26 %) increase in the radius and in τ_D, is found.

Many variations on FCS exist: cross-correlation FCS, two-color FCS, cross, spatial and spatiotemporal image correlation spectroscopy, Fluorescence Fluctuation Spectroscopy, etc. Many other analytical or empirical fit models exist: models for two-photon FCS, second harmonic generation "F"CS, free diffusion of two (or more) components, anomalous diffusion, directional transport (flow), rotational diffusion, bleaching correction, detector afterpulsing, etc. can be found in the literature.

6.2 Fluorescence Lifetime

The fluorescence lifetime is a measure of how long a fluorophore remains in an excited state before returning to its ground state by emitting a photon. The fluorescence lifetime can provide

information about the environment surrounding the fluorophore, such as temperature, pH, polarity or viscosity. In addition, in combination with fluorescence resonance energy transfer, the fluorescence lifetime can give information on the distance between two molecules, which can be used for example to distinguish between the status (open/closed) of ion channels.

Fluorescence lifetime measurements can also be used to distinguish between different fluorophores that emit light at the same wavelength but have different fluorescence lifetimes.

Several methods exist for measuring fluorescence lifetimes but, especially in the combination with microscopy, time-correlated single-photon counting (TCSPC) is one of the most popular.

In TCSPC, a pulsed laser is used and the time between a laser pulse and the arrival of a photon is measured. The histogram of the arrival times is then analyzed to get the lifetime, e.g. by curve fitting or phasor analysis.

6.2.1 Curve fitting

Curve fitting is a widely used method for fluorescence lifetime analysis. Note that the measured fluorescence signal is a convolution of the intrinsic fluorescence decay $F(t)$ with the instrument impulse response function (IRF) $h(t)$, which deviates from an ideal Dirac-delta function due to instrument electronics and other delay components:

$$d(t) = F(t) * h(t). \tag{6.19}$$

The IRF can be measured with a sample that has (close to) no lifetime, such as SHG or quenched fluorescence. The IRF can then be used to deconvolve $d(t)$ and obtain $F(t)$.

Advantages of curve fitting for fluorescence lifetime analysis include:

- Accurate determination of fluorescence lifetime: curve fitting allows for accurate determination of fluorescence lifetime by fitting a mathematical model to the measured fluorescence decay curve (if the SNR is high).
- Detection of multiple fluorescence lifetimes, which can be indicative of multiple fluorophores or different conformational states of the same fluorophore.
- Easy to implement

Disadvantages are:

- Sensitivity to noise: curve fitting is sensitive to noise; small fluctuations in the data can result in significant errors in the fitted parameters.
- Model dependence: curve fitting relies on a mathematical model to describe the fluorescence decay curve, and the accuracy of the fitted parameters depends on the validity of the model.
- Limited information: Curve fitting only provides information about the fluorescence lifetime and other parameters that are explicitly included in the model. It does not provide information about the underlying molecular mechanisms or interactions.
- Computationally intensive, especially when fitting complex models or large datasets, which can be a limitation when working with limited computational resources

6.2.2 The phasor analysis

Exponential decays are conveniently analyzed in Fourier space. We consider the case of a single exponential

$$d(t) = \begin{cases} 0 & t < 0 \\ d_0 \exp\left(-t/\tau\right) & t \geq 0 \end{cases} \tag{6.20}$$

with fluorescence lifetime τ. The origin of the temporal reference frame represents the excitation event triggered by the laser pulse.
The Fourier transform of the normalized signal is

$$\frac{\mathcal{F}\{d(t)\}(\omega)}{\int_0^{+\infty} d(t)\,\mathrm{d}t} = \frac{1}{1+i\omega\tau} = \underbrace{\frac{1}{1+(\omega\tau)^2}}_{g(\omega)} - i\underbrace{\frac{\omega\tau}{1+(\omega\tau)^2}}_{s(\omega)} \tag{6.21}$$

where we defined $g(\omega)$ and $s(\omega)$ respectively as the real and imaginary part of the Fourier transform of the decay. Notably, these quantities are related by the following equation

$$\left[g(\omega) - 1/2\right]^2 + s^2(\omega) = 1/4 \tag{6.22}$$

The vector (g, s) is known as phasor and lies on the semicircle of the complex plane described by the above equation, commonly named the *universal circle*. This fact implies that the phasors of single exponential decays are bound to lie on the universal circle. Multi-exponential decays are linear combination of single exponential decays and their corresponding phasors lie within the universal circle.

From equation 6.21 it is possible to calculate the lifetime the estimate in two ways. By defining

$$\tan\left[\phi(\omega)\right] = \frac{s(\omega)}{g(\omega)} \tag{6.23}$$

$$m^2(\omega) = s^2(\omega) + g^2(\omega) \tag{6.24}$$

we have that

$$\tau_\phi = \frac{1}{\omega}\tan\left[\phi(\omega)\right] \tag{6.25}$$

$$\tau_m = \frac{1}{\omega}\sqrt{\frac{1}{m^2(\omega)} - 1} \tag{6.26}$$

Note that – for single exponential decays – the two estimates of the lifetimes are identical and do not depend on the frequency ω.

Sampled data are inherently discrete. Thus, we need to generalize our analysis writing the phasor coordinates as the real and imaginary part of the discrete Fourier transform (DFT) of the sampled signal

$$g(h) = \frac{1}{I}\sum_{p=0}^{n_p-1} d(p)\cos(2\pi hp/n_p), \tag{6.27}$$

$$s(h) = \frac{1}{I}\sum_{p=0}^{n_p-1} d(p)\sin(2\pi hp/n_p), \tag{6.28}$$

where $I = \sum_p F(p)$ and n_p is the number of data points. Identifying $\omega = 2\pi h f_{\mathrm{exc}}$, we have the following numerical estimates of the fluorescence lifetime

$$\tau_\phi = \frac{1}{2\pi h f_{exc}}\tan\left[\phi(h)\right] \tag{6.29}$$

$$\tau_m = \frac{1}{2\pi h f_{exc}}\sqrt{\frac{1}{m^2(h)} - 1}. \tag{6.30}$$

Importantly, phasors can be calculated at any discrete frequency h. However, low frequencies carry most of the signal. As such, a typical choice is $h = 1$, commonly referred to as the first harmonic.

Advantages of the phasor analysis include:

- Simple graphical representation and interpretation. Note that the phasor of a sum of two components is on a line connecting the phasors of the two individual components.
- phasor analysis is a model-independent method, meaning that it does not rely on a specific mathematical model to describe the fluorescence decay curve.
- High-throughput: phasor analysis can be performed rapidly and can be easily implemented in automated data analysis pipelines, making it suitable for high-throughput data analysis.

Disadvantage are:

- Poor accuracy for low SNR data.
- Susceptible to error from instrument response

7 Image Analysis

7.1 Fourier Ring Correlation

The Fourier Ring Correlation (FRC) analysis calculates the Discrete Fourier Transform (DFT) of two images i_1 and i_2, identical but acquired in two separate moments. Thus, under the hypothesis of uncorrelated noise, only the signal contents of the two images correlate. Therefore, a correlation function in frequency space drops to zero above a certain spatial frequency. The inverse of this latter is the resolution of the imaging setup at the experimental conditions used to acquired the images.

In order to reduce the inherent spectral leakage, we apply to the images a 2D Hann window defined as follows

$$W(n_x, n_y) = \frac{1}{4}\left[1 - \cos\left(\frac{2\pi n_x}{N_x - 1}\right)\right]\left[1 - \cos\left(\frac{2\pi n_y}{N_y - 1}\right)\right] \tag{7.1}$$

where $n_{x,y}$ and $N_{x,y}$ are, respectively, the pixel index and the pixel size of the image. The FRC is defined as the normalized cross-correlation function between $I_1 = \mathrm{DFT}(i_1)$ and $I_2 = \mathrm{DFT}(i_2)$. It is calculated as

$$\mathrm{FRC}\,(q) = \frac{\sum\limits_{k_x,k_y \in q} I_1\,(k_x, k_y)\, I_2^*\,(k_x, k_y)}{\sqrt{\sum\limits_{k_x,k_y \in q} |I_1\,(k_x, k_y)|^2 \sum\limits_{k_x,k_y \in q} |I_2\,(k_x, k_y)|^2}} \tag{7.2}$$

where k_x and k_y are, respectively, the horizontal and vertical spatial frequencies, and $q = \sqrt{k_x^2 + k_y^2}$ is the radial spatial frequency. The resulting curves are denoised using a Locally Weighted Scatterplot Smoothing (LOWESS) algorithm. Importantly, if the SNR of the images

is low, correlations from the camera detector may appear at high frequencies. Therefore, we subtract from the FRC curves an offset calculated as the mean of FRC samples at frequencies above Abbe's resolution limit.

In order to measure the effective resolution of the microscope, we calculate the following variable-threshold function

$$T(q) = \frac{\sigma}{\sqrt{N_q/2}} \tag{7.3}$$

where N_q is the number of pixels contained in the ring of radius q. In this work, we used the so-called 3-σ threshold criterion by setting $\sigma = 3$. The intersection between the threshold and FRC curves identifies the spatial frequency q_t. Below this latter, correlations between the two images emerge from the random noise correlations. Thus, we can interpret q_t as the highest spatial periodicity with enough contrast to be distinguishable from noise fluctuations. The optical resolution of the images is $1/q_t$.

7.2 Image deconvolution

The Bayes theorem states

$$P(o \mid i) = \frac{P(i \mid o)P(o)}{P(i)} \tag{7.4}$$

where

- $P(o)$ is the *prior* probability, i.e. the probability of the hypothesis i before the data o are observed.
- $P(i)$ is the *marginal likelihood*, i.e. the probability model for the data i.
- $P(i \mid o)$ is the *likelihood*, i.e. the conditional probability of measuring i, having fixed an hypothesis o.
- $P(o \mid i)$ is the *posterior* probability, i.e. the conditional probability of the probability o, having measured i.

It is possible to estimate $\hat{o}$ as the mode of the *posterior* probability by maximizing it. If there is no knowledge on the prior distribution, it is possible to assume it as uniform. In that case, maximizing the *posterior* is the same as maximizing the *likelihood*

$$\hat{o} = \arg\max_o P(i \mid o) \tag{7.5}$$

In imaging problems, i is the image and o is the object. Those quantities are related by the law

$$i = o * h + \epsilon \tag{7.6}$$

where h is the point spread function of the imaging device and ϵ is the noise.

7.2.1 Wiener Filter

Under the assumption of Gaussian noise, the likelihood – seen as a function of the object o – is

$$P(i \mid o) = \prod_x \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{|i-(o*h)|^2}{2\sigma^2}} \tag{7.7}$$

finding its maximum is equivalent to finding the minimum of the log-likelihood, which is defined as

$$\mathcal{L}(o) = -\ln\left[P(i \mid o)\right] = \int \ln\left(\sqrt{2\pi}\sigma\right) \mathrm{d}x + \frac{1}{2\sigma^2}\int |i-(o*h)|^2 \,\mathrm{d}x \tag{7.8}$$

This problem is equivalent to minimizing the following loss function

$$\ell(o) = \underbrace{\int |i - o*h|^2 \,\mathrm{d}x}_{\ell_1} + \lambda \underbrace{\int |o|^2 \,\mathrm{d}x}_{\ell_2} \tag{7.9}$$

where we added a regularization term.

Since we want to minimize $\ell(o)$, we need to impose its functional derivative to be equal to zero. This latter, is defined from the following relation:

$$\lim_{\rho\to 0} \frac{\ell(o+\rho s) - \ell(o)}{\rho} = \left\langle \frac{\partial \ell}{\partial o}, s \right\rangle \tag{7.10}$$

where ρ is a constant, s is an arbitrary function, and $\langle\cdot,\cdot\rangle$ is the L^2 inner product.

We start calculating the derivative of $\ell_1(o)$. We have that

$$\begin{aligned} \ell_1(o+\rho s) &= \int |i - o*h - \rho s*h|^2 \,\mathrm{d}x \sim \\ &\sim \int |i-o*h|^2 \,\mathrm{d}x + 2\rho \int (s*h)(o*h-i)\,\mathrm{d}x \end{aligned} \tag{7.11}$$

Therefore, the difference quotient is

$$\begin{aligned} \lim_{\rho\to 0} \frac{\ell_1(o+\rho s) - \ell_1(o)}{\rho} &= 2\int (s*h)(o*h-i)\,\mathrm{d}x = \\ &= 2\langle o*h, s*h\rangle - 2\langle i, s*h\rangle = 2\langle h^* * (o*h), s\rangle - 2\langle h^* * i, s\rangle = \\ &= \langle 2h^* * [(o*h)-i], s\rangle = \left\langle \frac{\partial \ell_1}{\partial o}, s \right\rangle \end{aligned} \tag{7.12}$$

where h^* is the adjoint function of h, defined as $h^*(x) = h(-x)$. By comparison, we find that the derivative of ℓ_1 is

$$\frac{\partial \ell_1}{\partial o} = 2h^* * [(o*h) - i] \tag{7.13}$$

Repeating the same calculations for the regularization term, we find

$$\ell_2(o+\rho s) = \int |o+\rho s|^2 \,\mathrm{d}x \sim \int |o|^2 \,\mathrm{d}x + 2\int os \,\mathrm{d}x \tag{7.14}$$

$$\lim_{\rho\to 0} \frac{\ell_2(o+\rho s) - \ell_2(o)}{\rho} = 2\int os \,\mathrm{d}x = \left\langle \frac{\partial \ell_2}{\partial o}, s \right\rangle \tag{7.15}$$

Therefore, the derivative of ℓ_2 is

$$\frac{\partial \ell_2}{\partial o} = 2o \tag{7.16}$$

In order to find the minimum, we now want to impose the complete derivative to be equal to zero

$$\frac{\partial \ell}{\partial o} = \frac{\partial \ell_1}{\partial o} + \lambda \frac{\partial \ell_2}{\partial o} = 0 \tag{7.17}$$

Therefore

$$h^* * [(o * h) - i] + \lambda o = 0 \tag{7.18}$$

Calculating the Fourier Transform of the above equation, we have

$$H^* O H + \lambda O = H^* I \tag{7.19}$$

where the capital letters represent the Fourier transformed functions and H^* is the complex transpose of H. In Fourier space this equation has a simple solution, which in real space is

$$\hat{o} = \mathcal{F}^{-1}\left\{ \frac{H^* I}{|H|^2 + \lambda} \right\} \tag{7.20}$$

7.2.2 Richardson-Lucy

Under the assumption of Poissonian noise, the likelihood is

$$P(i \mid o) = \prod_x \frac{(h * o)^i e^{-(h*o)}}{i!} \tag{7.21}$$

The corresponding log-likelihood is

$$\mathcal{L}(o) = -\ln\left[P(i \mid o)\right] = \int \left[(h * o) - i \cdot \ln(h * o) + \ln(i!)\right] \mathrm{d}x \tag{7.22}$$

and the functional to be minimized is

$$\ell(o) = \int \left[h * o - i \cdot \ln(h * o)\right] \mathrm{d}x + \lambda \int |o|^2 \,\mathrm{d}x \tag{7.23}$$

where we added a regularization term.

In order to find the derivative, we calculate

$$\ell_1(o+\rho s) = \int \left(h*o + \rho s * h - i \cdot \ln\left[(h*o)\left(1 + \frac{\rho s * h}{o*h}\right)\right]\right) \mathrm{d}x \sim \tag{7.24}$$

$$\sim \int \left[h*o - i\cdot\ln(h*o) + (\rho s * h)\left(1 - \frac{i}{o*h}\right)\right] \mathrm{d}x \tag{7.25}$$

Therefore,

$$\lim_{\rho\to 0} \frac{\ell_1(o+\rho s) - \ell_2(o)}{\rho} = \int (s*h)\left(1 - \frac{i}{o*h}\right)\mathrm{d}x = \langle s*h, 1\rangle - \left\langle s*h, \frac{i}{o*h}\right\rangle = \tag{7.26}$$

$$= \langle s, h^* * 1\rangle - \left\langle s, h^* * \frac{i}{o*h}\right\rangle = \left\langle h^* * \left(1 - \frac{i}{o*h}\right), s\right\rangle = \tag{7.27}$$

$$= \left\langle \frac{\partial \ell_1}{\partial o}, s\right\rangle \tag{7.28}$$

So, the derivative is

$$\frac{\partial \ell_1}{\partial o} = h^* * \left(1 - \frac{i}{o*h}\right) \tag{7.29}$$

Therefore,

$$\frac{\partial \ell}{\partial o} = h^* * \left(1 - \frac{i}{o*h}\right) + 2\lambda o = 0 \tag{7.30}$$

If h is normalized, we have that $h^* * 1 = \int h\,\mathrm{d}x = 1$. Therefore, the above equation is satisfied when

$$\left[h^* * \left(\frac{i}{o*h}\right)\right]\frac{1}{1+2\lambda o} = 1 \tag{7.31}$$

This equation implies an iterative algorithm. Assuming that at convergence $\frac{o_{k+1}}{o_k} \to 1$, we can build the following multiplicative gradient-descent iteration rule:

$$o_{k+1} = \left[h^* * \left(\frac{i}{o_k * h}\right)\right]\frac{o_k}{1+2\lambda o_k} \tag{7.32}$$