5 Fluorescence spectroscopy

5.1 Fluorescence Correlation Spectroscopy

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

$$\frac{\partial \rho(\mathbf{r},t)}{\partial t} = D\nabla^2 \rho(\mathbf{r},t), \tag{5.1}$$

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:

$$\frac{\partial \rho(x,t)}{\partial t} = D\frac{\partial^2 \rho(x,t)}{\partial x^2}. \tag{5.2}$$

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:

$$\rho(x,t) = \frac{1}{\sqrt{4\pi D t}} \exp\left(-\frac{x^2}{4Dt}\right) \tag{5.3}$$

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

$$\left\langle x^2(t)\right\rangle = \int x^2(t)\rho(x,t)dx = 2Dt. \tag{5.4}$$

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:

$$D = \frac{k_\mathrm{B}T}{6\pi\eta r}, \tag{5.5}$$

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,

$$F(t) = \alpha \int W(\mathbf{r})\rho(\mathbf{r},t)dV, \tag{5.6}$$

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

$$G(\tau) = \frac{\langle \delta F(t)\delta F(t+\tau)\rangle}{\langle F(t)\rangle^2}, \tag{5.7}$$

with

$$\delta F(t) = F(t) - \langle F(t)\rangle. \tag{5.8}$$

Alternatively, combining Eq. 5.7 and 5.8 and making use of $\langle F(t)\rangle = \langle F(t+\tau)\rangle$, G can also be calculated as:

$$G(\tau) = \frac{\langle F(t)F(t+\tau)\rangle}{\langle F(t)\rangle^2} - 1. \tag{5.9}$$

For $\tau = 0$, we have

$$G(0) = \frac{\langle \delta F(t)\delta F(t)\rangle}{\langle F(t)\rangle^2} = \frac{\langle (\delta F(t))^2\rangle}{\langle F(t)\rangle^2}. \tag{5.10}$$

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,

$$G(0) \propto \frac{\text{Variance}}{\langle N\rangle^2} \propto \frac{1}{\langle N\rangle} \tag{5.11}$$

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,

$$W(\vec{r}) = I_0 e^{-2\left(x^2+y^2\right)/\omega_0^2} e^{-2z^2/z_0^2}, \tag{5.12}$$

then the autocorrelation can be analytically calculated.

$$\delta F(t) = \alpha \int_V W(\vec{r})\delta\rho(\vec{r},t)dV \tag{5.13}$$

Thus,

$$G(\tau) = \frac{\iint_{VV'} W(\vec{r})W\left(\vec{r}'\right)\left\langle \delta\rho(\vec{r},0)\delta\rho\left(\vec{r}',\tau\right)\right\rangle dVdV'}{\left(\int_V \delta\rho(\vec{r},0)W(\vec{r})dV\right)^2}, \tag{5.14}$$

with

$$\left\langle \delta\rho(\vec{r},0)\delta\rho\left(\vec{r}',\tau\right)\right\rangle = \langle\rho\rangle\frac{1}{(4\pi D\tau)^{3/2}}e^{\left(\vec{r}-\vec{r}'\right)^2/4D\tau}. \tag{5.15}$$

Plugging Eq. 5.15 into Eq. 5.14 gives the final result:

$$G(\tau) = \frac{1}{\langle N\rangle}\left(1+\frac{4D\tau}{\omega_0^2}\right)^{-1}\left(1+\frac{4D\tau}{\omega_z^2}\right)^{-1/2}. \tag{5.16}$$

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

$$G(\tau_D) = \frac{1}{2\langle N\rangle} = \frac{1}{2}G(0). \tag{5.17}$$

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:

$$D = \frac{k_B T}{6\pi\eta r}, \tag{5.18}$$

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.

5.2 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 (note that also other methods, such as .

5.2.1 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:

$$d(t) = F(t) * h(t). \tag{5.19}$$

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

5.2.2 The phasor analysis

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

$$d(t) = d_0 \exp\left(-t/\tau\right) \tag{5.20}$$

with fluorescence lifetime τ. The Fourier transform of the normalized signal is

$$\frac{\mathcal{F}\{d(t)\}\left(\omega\right)}{\int_0^{+\infty} d(t)\,\mathrm{d}t} = \frac{1}{1+i\omega\tau} = \underbrace{\frac{1}{1+(\omega\tau)^2}}_{g(\omega)} - i\,\underbrace{\frac{\omega\tau}{1+(\omega\tau)^2}}_{s(\omega)} \tag{5.21}$$

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

$$\left[g(\omega) - 1/2\right]^2 + s^2(\omega) = 1/4 \tag{5.22}$$

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 5.21 it is possible to calculate the lifetime the estimate in two ways. By defining

$$\tan\left[\phi(\omega)\right] = \frac{s(\omega)}{g(\omega)} \tag{5.23}$$

$$m^2(\omega) = s^2(\omega) + g^2(\omega) \tag{5.24}$$

we have that

$$\tau_\phi = \frac{1}{\omega}\tan\left[\phi(\omega)\right] \tag{5.25}$$

$$\tau_m = \frac{1}{\omega}\sqrt{\frac{1}{m^2(\omega)} - 1} \tag{5.26}$$

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

$$g(h) = \frac{1}{I} \sum_{p=0}^{n_p-1} d(p) \cos(2\pi hp/n_p), \tag{5.27}$$

$$s(h) = \frac{1}{I} \sum_{p=0}^{n_p-1} d(p) \sin(2\pi hp/n_p), \tag{5.28}$$

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

$$\tau_\phi = \frac{1}{2\pi h f_{exc}} \tan\left[\phi(h)\right] \tag{5.29}$$

$$\tau_m = \frac{1}{2\pi h f_{exc}} \sqrt{\frac{1}{m^2(h)} - 1}. \tag{5.30}$$

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