Chapter 1

General introduction: Goal and outline of this thesis

Geert van den Bogaart

1.1 Abstract

In this thesis, confocal microscopy is used to probe molecular diffusion in biological systems. The first section of this chapter introduces diffusion and its importance to biology. Subsequently, the confocal microscope and the various ways it can be employed to measure diffusion are introduced. The last section contains the outline of this thesis and briefly describes the achievements of this work.

1.2 Diffusion

Diffusion is the physical transport of matter by molecular motion. It was first observed in 1827 by the biologist Robert Brown, who noticed that pollen grains move through a droplet of water via an (by that time) unknown process, called Brownian motion. In 1904, the physicist William Sutherland proposed that Brownian motion is caused by molecular collisions \[^2\]; in this case the collisions of the pollen grains with the water molecules.

Since diffusion is a random process, it results in the net movement of particles down a concentration gradient. The concentration of particles \( C(r, t) \) in space \( r \)

\[^2\]This is often wrongly attributed to Albert Einstein, who independently reached the same conclusion in 1905; one year after Sutherland.
changes over time $t$ depending on its gradient, as is described by Fick’s second law of thermodynamics [3]:

$$\frac{\delta C(r, t)}{\delta t} = D \Delta C(r, t),$$

(1.1)

where $\Delta$ is the Laplace operator ($\frac{\delta^2}{\delta r^2}$) and $D$ is the diffusion coefficient, which is the speed the particle diffuses in m$^2$ s$^{-1}$. Since diffusion is a random process and particles move in both positive and negative directions, the mean net displacement (in free space) is close to 0, the mean square displacement, however, is linear in time with slope $D$ (Fig. 1.1).

![Figure 1.1: Diffusion.](image)

(a) Random walk of a particle (●) in two dimensions. The Brownian motion of the particle is caused by molecular collisions. (b) The mean square displacement $\langle \Delta x^2 \rangle$ is linear in time $t$, with the slope being the diffusion coefficient $D$.

The diffusion coefficient is related to the temperature $T$ and viscosity $\gamma$ of the medium as described by the Einstein-Stokes relationship:

$$D = \frac{k_B T}{6 \pi r_S \gamma},$$

(1.2)

where $k_B$ is Boltzmann’s constant and $r_S$ is the Stokes or hydrodynamic radius of the particle, defined as the radius of a hard sphere that diffuses with the same diffusion coefficient as the particle. For a spherical particle, $r_S$ is related to the (molecular) weight $M_W$ of the particle:

$$r_S = \sqrt[3]{\frac{3M_W}{4\pi N_A \rho}},$$

(1.3)

where $N_A$ is Avogadro’s constant and $\rho$ is the density of the particle. For proteins, a mean density of 1.35 g cm$^{-3}$ is often used [4].

Diffusion plays a role in essentially all processes in life, ranging from ecology (e.g. grain pollen movement), physiology (intestinal nutrient absorption), cell-biology (osmosis), to biochemistry (enzyme – substrate interactions). Still, diffusion in the often complex biological environment is poorly understood: How fast is diffusion in biological systems? How do inter-molecular interactions influence diffusion? How
does molecular crowding affect the diffusion? How do molecular dynamics, such as conformational changes, change the diffusion of (bio)molecules? In this thesis, diffusion-related problems in various biological systems are addressed, using laser-scanning confocal microscopy.

1.3 Confocal microscopy

When a screen with a pinhole is placed in the conjugate focal point of a lens, light emitted from the focal point is transmitted through the pinhole, whereas light from other positions is largely blocked by the screen (Fig. 1.2a). Confocal microscopy uses a pinhole conjugate to the focal point of the lens, thereby transmitting only information from the focal point. A laser is focussed to a diffraction limited spot of high intensity for excitation of the sample (Fig. 1.2b). The laser / pinhole combination results in a narrow region of excitation and only the fluorescence emitted from the focal point is being detected. Thus, a confocal microscope is in essence a fluorescence meter, where fluorescence can be measured in a very small open volume element (typically less than 1 fl $(10^{-15})$, Fig. 1.2c), called the detection or focal volume.

In laser-scanning confocal microscopy, often two mirrors mounted on motors, called the scanning-mirrors, enable to scan the focal volume through the sample in lateral directions $x$ and $y$ (perpendicular to the optical axis). The axial direction $z$ (parallel to the optical axis) can be scanned by changing the position of the focus in the sample, for instance by a piezo-translating stage mounted to the objective. A computer can now generate a three-dimensional image, pixel by pixel, corresponding to different positions of the focus and the scanning mirrors.

The main advantage of confocal microscopy over regular widefield fluorescence microscopy is the higher $z$-resolution, because the confocal pinhole blocks out of focus light. This allows imaging of a thin section of the sample (diffraction limited resolution $\sim 0.5 \, \mu m$). In addition to having a higher $z$-resolution, the stray light suppression by the pinhole also results in a higher ($\sim 1.4$-fold) $x$- and $y$-resolution ($\sim 0.2 \, \mu m$) [5]. The exact size and shape of the focal volume are dependent on the wavelengths of the excitation and the emission light, the numerical aperture of the microscope objective, the size of the pinhole, and the aberrations of all optical components integrated in the system [6].

A laser-scanning confocal microscope can be used to measure diffusion in various ways, the most well-known being fluorescence correlation spectroscopy (FCS) and fluorescence recovery after photo-bleaching (FRAP). In this work, several of
Figure 1.2: The confocal microscope. (a) Principle of pinhole optics. The pinhole, located conjugate to the focal point \( f \) of the lens, transmits all light from the focal point (solid line) while out-of-focus light (dotted line) is largely blocked by the screen. (b) Scheme of a confocal microscope. The excitation light from the laser is focussed by the objective lens on the sample. The emission is separated from the excitation light by a beam splitter and focussed on the pinhole in front of the detectors. The combination of the laser and the pinhole results in a very small detection volume of less than 1 fl. The focal volume can be scanned in lateral directions \( x \) and \( y \) using two scanning-mirrors, and in axial direction \( z \) by changing the position of the focus in the sample. This allows to record a 3-dimensional image, pixel by pixel. (c) Plot of the focussed laser and the ‘egg-shaped’ focal volume. The projection in space is plotted where the measured fluorescence intensity dropped \( e^2 \)-times relative to the maximum intensity. Fluorescently labeled molecules (●) diffuse through the detection volume (dotted arrows), thereby resulting in fluctuations of the fluorescence intensity (d).
these techniques have been applied to study a number diffusion-related problems in biology.

1.4 FCS and FRAP

If the concentration of fluorophores in the sample is low ($< 1 \mu\text{M}$), the number of molecules in the focal volume of the microscope is limited ($< 100$). The events of fluorophores diffusing through the focal volume can now be observed as fluctuations in the fluorescence intensity (Fig. 1.2d). With FCS, the kinetics of the diffusion are obtained by temporally autocorrelating the fluorescent intensity $I(t)$ [7]:

$$G(\tau) = \frac{\langle I(t)I(t + \tau) \rangle}{\langle I(t) \rangle^2} - 1,$$

where $\langle$ and $\rangle$ indicate ensemble averaging. The autocorrelation curve $G(\tau)$ (Fig. 1.3a) is a measure of the probability that a molecule in the focal volume at $t = 0$ is still there at $t = \tau$. In addition to diffusion, other factors that cause the fluorescent intensity to fluctuate contribute to the autocorrelation curve, e.g. intersystem crossing (non-fluorescent transitions), photo-bleaching, detector noise, molecular conformational changes, and, in certain cases, chemical reactions. Photobleaching is the photochemical destruction of a fluorophore and intersystem crossing is the transition from the ‘light’ (singlet) to the ‘dark’ (triplet) state.

The diffusion coefficient can be obtained by fitting the fluorescence autocorrelation curve with a function assuming Brownian motion and approximating the focal volume as Gaussians [8]:

$$G(\tau) = \frac{1}{N} \frac{1}{1 + \frac{\tau_d}{\tau}},$$

where $N$ is the average number of particles present in the focal volume, and $\omega_{xy}$ and $z_0$ are the lateral (perpendicular to the optical axis) and axial (parallel) radii of the focal volume, respectively, defined as the point where the measured fluorescence drops $e^2$-times relative to the maximum. $\tau_d$ is the characteristic diffusion time through the focal volume and is related to the diffusion constant:

$$\tau_d = \frac{\omega_{xy}^2}{4D}.$$

In addition to the diffusion of free fluorophores in solution, the lateral diffusion of membrane-associated fluorophores can be measured (Fig. 1.3b). For this, the focal
volume is positioned on the membrane [9, 10] and the fluorescence autocorrelation
curve can be fitted with a model incorporating only lateral (2-dimensional) diffusion
[11]:

\[ G(\tau) = \frac{1}{N} \frac{1}{1 + \frac{\tau}{\tau_d}} . \]  

\[ (1.7) \]

Fluorescence cross-correlation spectroscopy (FCCS) uses two spectrally sepa-
rated fluorophores to detect molecular interactions [12]. For this, it is necessary
that the emission of both fluorophores can be individually detected in two spatially
overlapping detection volumes. The two fluorescent intensities are cross-correlated
to determine the level of coincidence:

\[ G_{cc}(\tau) = \frac{\langle I_1(t)I_2(t + \tau) \rangle}{\langle I_1(t) \rangle \langle I_2(t) \rangle} - 1 , \]  

\[ (1.8) \]

where \( I_1(t) \) and \( I_2(t) \) are the fluorescent intensities from the two detection channels.

We used FCCS to study the interactions of soluble proteins [13] and membrane
proteins [14].

In addition to FCS, another well-established method to measure diffusion with
a confocal microscope is FRAP (Fig. 1.4) [14, 15, 16]. With FRAP, a small region
of the sample is irreversibly photo-bleached with a short, intense light pulse. Using
an attenuated probe-beam, the diffusion of unbleached fluorophores into the photo-
bleached area is subsequently measured. The speed of this recovery is inversely
related to the diffusion coefficient of the fluorophore. Alternatively to an attenuated

\[ \text{Figure 1.3: Fluorescence correlation spectroscopy. (a) Autocorrelation curve } G(\tau) \text{ of free Alexa fluor 633 diffusing in water. The inset shows the fluorescent intensity fluctuations. (b) FCS to measure the lateral diffusion (dotted arrow) of membrane-associated fluorophores (\textbullet{}). The focal volume is positioned on the membrane.} \]
probe-beam, the recovery can be monitored by taking a time-series of images (chapter 7, reviewed in [16]). Generally, FRAP is employed at higher concentrations of fluorophore than FCS.

Figure 1.4: Fluorescence recovery after photo-bleaching. At $t_0$, fluorophores located in a limited spot in the sample are photo-bleached with a focussed laser beam at high intensity. Subsequently, the recovery of the fluorescence due to influx of unbleached fluorophores is monitored, using an attenuated probe-beam. The graph shows a typical recovery curve of a FRAP experiment. The half time of recovery $t_{1/2}$ is inversely related to the diffusion constant $D$. Note that the level of recovery is not 100%, due to for instance immobile or a limited pool of fluorophores. Image adapted from [16].

In this thesis, both FCS and FRAP were applied to measure diffusion in various biological systems. In addition, several new techniques have been developed to study problems that cannot be readily addressed with FCS or FRAP. The next section provides a brief overview of the new techniques and findings of each chapter.

1.5 Outline of this thesis

Part I of this thesis presents dual color fluorescence burst analysis (DCFBA); a newly developed technique that was employed to study diffusion of fluorophores through membrane pore proteins. For this, liposomes were fluorescently labeled and leakage-marker molecules were encapsulated that were labeled with a second,
spectrally separated fluorophore. The fluorescence bursts that originate from the liposomes diffusing through the focal volume of a confocal microscope coincide with those from the encapsulated leakage-marker molecules. The internal concentration in the liposome of leakage-marker molecules can be calculated from the fluorescence bursts, with single liposome resolution. In addition to the diffusion through membrane-pores, DCFBA was used to quantitatively measure the binding of proteins to membrane proteins. Chapter 2 introduces the technique and discusses its applications in biology.

In chapter 3 [17], DCFBA was employed to study the effective pore-size of the mechano-sensitive channel MscL from *Escherichia coli*. MscL is an osmolyte pore protein that functions as a safety valve for the cell and opens upon a decrease in the external osmolyte concentration, hence preventing cell lysis [18]. It was found that molecules with a diameter smaller than about 2 – 3 nm can freely permeate through MscL, whereas larger cannot.

Chapter 4 [19] presents the mechanism of pore-formation of the antimicrobial peptide melittin, again using DCFBA. Melittin is a lytic peptide that kills cells by auto-inserting in their membranes and forming pores [20]. It was found that melittin causes leakage via two different mechanisms: For phospholipid membranes composed of neutral, bilayer-forming lipids, the pore-size depended on the melittin concentration. For phospholipid membranes containing anionic and / or non-bilayer forming lipids, melittin induced a-specific leakage accompanied by fusion or aggregation.

Part II of this thesis presents a number of techniques other than DCFBA that were used to measure diffusion in various biological systems.

In chapter 5 [21] FCS was applied to study the effects of different sugars on the lateral mobility of lipids. It was found that the disaccharide sucrose slows diffusion more than all other tested sugars, including trehalose. Using molecular dynamics (MD) simulations, we show that this is the result of increased hydrogen bonding between sucrose and the lipid head-groups, compared to trehalose.

Chapter 6 [22] presents a pulsed version of FRAP, pulsed-FRAP. With conventional FRAP it is technically very difficult to measure diffusion in bacterial cells because of their small size (∼ 2 µm for *E. coli*), which is close to the diffraction limit of the visible light. Pulsed-FRAP allows to measure diffusion in bacteria, since the cell size and geometry of the bacterial cells are taken into account. Pulsed-FRAP was used to study intracellular protein diffusion in *E. coli* as a function of osmotic stress.

In chapter 7, FRAP was employed to measure nucleocytoplasmic transport through the nuclear pore complexes (NPC) in living yeast cells. Here, a quantitative version
of selective-FRAP was developed, that is based on partial photo-bleaching of fluorophores present in the nucleus and monitoring of the subsequent influx of intact GFP from the cytoplasm. This chapter also presents a new mechanism for regulation of protein synthesis in the mother and emerging daughter cell of budding yeast.