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Fluorescence Correlation Spectroscopy of λ-DNA

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Introduction

In this one-day practical course, you will learn key concepts in polymer physics related to the structure and transport of biopolymers. Double stranded DNA (ds-DNA) molecules of varying lengths will serve as a model system and will be investigated using optical microscopy and fluorescence correlation spectroscopy (FCS). You will prepare fluorescent DNA samples, operate a confocal microscope, and analyse microscopy images using the image analysis software ImageJ. FCS measurements will be performed and the measured time correlation functions will be fitted by theoretical models of molecular diffusion.

Background

Structural Properties of DNA

DNA was first isolated in 1869 by Friedrich Miescher in Tübingen, who identified a previously unknown substance in cell nuclei. Later, Phoebus Levene described the fundamental chemical components of DNA-deoxyribose sugar, phosphate groups, and the four nucleobases adenine (A), guanine (G), thymine (T), and cytosine (C). The correct three-dimensional structure was established in 1953 by James Watson and Francis Crick, based on X-ray diffraction data obtained by Rosalind Franklin and Raymond Gosling [Franklin and Gosling (1953); Watson and Crick (1953).

Structurally, DNA consists of two antiparallel strands forming a double helix. Each strand is a polymer composed of repeating nucleotide units (Fig. 1). A nucleotide comprises a nucleobase attached to a phosphate backbone. The nucleotides are covalently linked via phosphodiester bonds between the 3′-hydroxyl and 5′-hydroxyl groups, giving each strand a defined directionality (5′$\rightarrow$ 3′). The two strands associate through specific hydrogen bonding between complementary bases: adenine pairs with thymine via two hydrogen bonds, while guanine pairs with cytosine via three hydrogen bonds. This specificity is known as Watson-Crick base pairing and forms the basis of the helical DNA structure. The DNA helix has a characteristic geometry: one full turn corresponds to approximately 10.5 base pairs, with a base-pair spacing of about 0.34 nm. At a larger length scale we conceptualize DNA as a flexible rod-like molecule with a contour length $L_c = 0.34 N_{bp}$, where N_{bp} is the number of base pairs. λ-DNA specifically is the double-stranded genomic DNA of the bacteriophage λ, a virus that infects *Escherichia coli*. It has a length of 48502 base pairs, corresponding to a contour length of approximately $L_c = 16.5\,\mu$m. The length scale over which the orientation of a flexible rod in solution is lost, is the so-called persistence length. For DNA the persistence length is $L_p \approx 50$ nm. Hence at a microscopic length scale, ds-DNA adopts a random coil conformation. In this model, the behavior of the end-to-end distance $\langle \mathbf{R}_{ee}^2 \rangle \sim N$ is analogous to the mean-squared displacement of a random walk with N steps. (See Fig. 1 and Equation 5).

Polymer Mechanics of DNA

DNA is described as a semiflexible polymer ($L_c \approx L_p$), i.e. depending on its contour length, DNA can be described as a rigid rod ($L_c \ll L_p$), or a flexible polymer ($L_c \gg L_p$). The persistence length of an elastic rod

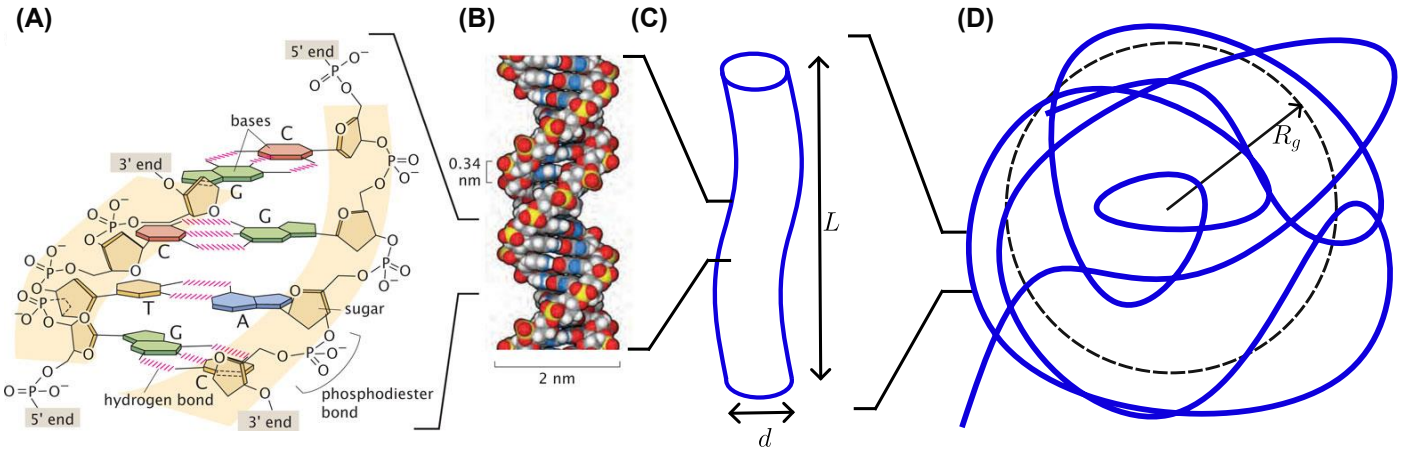

Figure 1 Schematic representation of ds-DNA at different length scales **(A)** At the molecular level ds-DNA is composed of a sugar-phosphate backbone linked to nitrogenous bases, denoted A, T, G and C. In Watson-Crick base pairing A and T are linked with two hydrogen bonds, G and C with three hydrogen bonds. **(B)** The two individual strands arrange in a double-helical structure [Adapted from Phillips *et al.* (2012)]. **(C)** At a length scale of 2-50nm DNA can be described as a rigid cylinder. **(D)** At a microscopic length scale the flexibility of the DNA double strand forms a random coil conformation with a extension length scale described by the radius of gyration, R_g.

parametrized by arc length s is defined by the decay length of the orientational correlation function

$$\langle \cos\theta(s) \rangle = e^{-\frac{s}{L_p}} \tag{1}$$

Here, $\theta(s)$ is the change of angle of a tangent vector at arc length s compared to the angle at $s = 0$. In the following we discuss the properties of the flexible polymer:

For a flexible polymer the mass density of monomers at position $\mathbf{R}$ is distributed according to a Gaussian distribution:

$$P(\mathbf{R}) = \left(\frac{3}{2\pi R_g^2}\right)^{\frac{3}{2}} \exp\left(-\frac{3\mathbf{R}^2}{2R_g^2}\right) \tag{2}$$

Here, the radius of gyration R_g gives a length scale. Specifically, R_g^2 is defined as the second moment of the mass distribution about the center of mass. The radius of gyration R_g will be a central observable in the experiments. It can be shown that for a semiflexible chain with no excluded volume (Worm-like chain model) R_g, $\mathbf{R}_{ee}$ and L_p relate as follows:

$$R_g^2 = \frac{1}{6}\langle \mathbf{R}_{ee}^2 \rangle = \frac{1}{3} L_p L_c \tag{3}$$

Hence, the radius of gyration scales as $R_g \sim N^{1/2}$ and thus we find the scaling exponent $\nu = 1/2$.
In real polymer systems, however, steric repulsion prevents monomers from overlapping, leading to a self-avoiding walk and effective swelling of the chain. As derived by Flory this leads to a different scaling as follows

$$R_g = b_K \left(\frac{V}{b_K^3}\right)^{1/5} N^{3/5} \tag{4}$$

where V is the excluded volume. The scaling exponent in 3D under good solvent conditions was later identified as $\nu = 0.588$ using renormalization group theory [De Gennes (1979); Rubinstein and Colby (2003)].

Brownian Motion

Diffusion is a stochastic movement of particles arising from thermal fluctuations. It can be understood starting from a simple random walk model, where a particle performs several successive steps which are uncorrelated in their direction. After N steps, the average displacement is 0, but the mean square displacement (MSD) grows linearly over time:

$$\left\langle \mathbf{r}^2(t) \right\rangle = 2dDt \tag{5}$$

where d is the spatial dimension and D the diffusion coefficient. To relate D to thermal fluctuations, we describe the dynamics using the underlying Langevin equation

$$m\frac{\mathrm{d}\mathbf{v}}{\mathrm{dt}} = -\gamma\mathbf{v} + \xi(t) \tag{6}$$

where γ is the Stokes friction coefficient and $\xi(t)$ a random force with zero mean and delta-correlation ($\langle\xi(t)\xi(t')\rangle = 2\gamma kT\delta(t-t')$), representing thermal fluctuations. We find ourselves in the low Reynolds number regime, where inertia is dissipated immediately, hence $\gamma\mathbf{v} = \xi(t)$. The mean square displacement is then given by

$$\left\langle \mathbf{r}^2(t) \right\rangle = \frac{1}{\gamma^2}\int_0^t\int_0^t \langle\xi(t)\xi(t')\rangle \mathrm{d}t\mathrm{d}t' = \frac{2dkT}{\gamma}t \tag{7}$$

Comparing with equation 5 this gives

$$D = \frac{kT}{\gamma} \tag{8}$$

What is the hydrodynamic friction coefficient of a DNA molecule?

For a sphere of radius R under non-slip boundary conditions the friction coefficient is given by the Stokes formula

$$\gamma_{sphere} = 6\pi\eta R \tag{9}$$

where η is the viscosity of the surrounding medium. Equation 9 determines the so-called hydrodynamic radius R_H for any shape, as the size of a sphere with equivalent friction.
At the microscopic length scale DNA adopts a coil-like conformation. The friction of a coil can be described by the Stokes formula with an effective hydrodynamic radius:

$$R_H = \frac{3}{8}\sqrt{\pi}R_g \tag{10}$$

This formula was derived by Kirkwood and Riseman [Doi *et al.* (1988)] and states that the effective hydrodynamic radius is smaller than the characteristic size, R_g of the coiled DNA due to permeability (porosity) of the coil conformation compared to a rigid sphere.

Short strands of ds-DNA with $L_c \lesssim L_p$ can be approximated as a rigid cylinder (see Fig. 1). For a cylinder of length L and diameter d, no closed analytical expression for the Stokes friction γ exists, but the following expansion can be used [Tirado and De La Torre (1979)]:

$$D_{cyl} = \frac{kT}{\gamma_{cyl}} = \frac{kT}{3\pi\eta L}\left(\ln\frac{L}{d} + 0.312 + 0.565\frac{d}{L} - 0.1\left(\frac{d}{L}\right)^2 + \mathcal{O}\left(\frac{d}{L}\right)^3\right) \tag{11}$$

For $L \gg d$ this can be simplified to

$$D_{cyl} = \frac{kT}{3\pi\eta L}\ln\frac{L}{d} \tag{12}$$

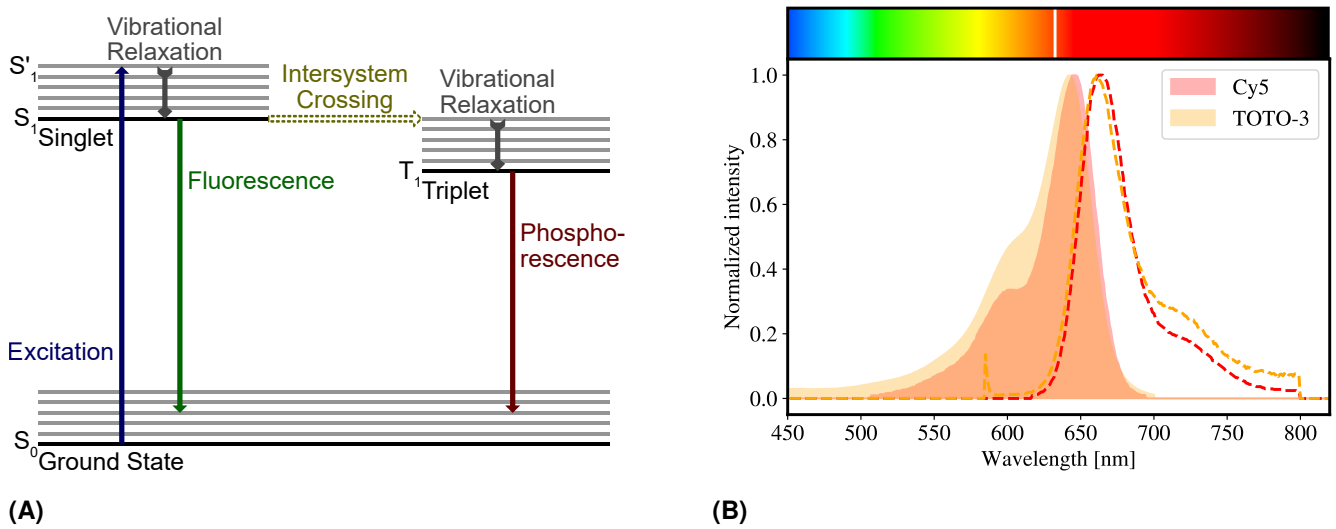

Figure 2 (A) Jablonski diagram of a generic photoluminescent dye. After absorbing a photon, the system is excited from ground state to a vibrational state from where it relaxes towards the excited singlet. Fluorescence occurs under the fast transition towards the ground state whereas phosphorescence involves an intersystem crossing to and a subsequent forbidden change in spin multiplicity resulting in a longer timescale. (B) Absorption (shaded) and emission (dashed) spectra of the FCS calibration dye Cy5 and intercalator dye TOTO-3. A laser line at 633 nm will be used.

Fluorescence

Luminescence is the emission of light that results from the excited electrons of a substance. In the specific case of fluorescence, these excited electrons are in singlet states (total spin $S = 0$). A common representation of the electron transitions involved is the so-called Jablonski diagram (Fig. 2A): Fluorescence occurs when an electron is raised from the ground state to an excited state by light absorption. From there, it first relaxes to the lowest-energy vibrational mode and, whilst emitting a red-shifted photon, falls back from there to a ground-state mode. Alternatively, it may also transition to the triplet state T_1 where $S = 1$.

From this state, a return to the singlet ground state is spin-forbidden by quantum mechanical selection rules, which is why its lifetime is considerably longer than that of an excited singlet state (Phosphorescence). Typical fluorescence lifetimes are in the 10 ns range, whilst triplet lifetimes are in the µs range. In the case of a dye, there are naturally several competing pathways via which it can return from the excited state to the ground state. Thus, a certain percentage of transitions always occur to the triplet state, and other non-radiative processes are also possible (rotation and vibration). The environment (de-excitation through collisions with solvent molecules) therefore also plays a role in fluorescence. An important parameter for characterizing dyes is the so-called "quantum yield" Q. If we denote the transition rate for non-radiative processes by k_{nr} and the rate at which photons are emitted k_r, Q is given by

$$Q = \frac{k_r}{k_r + k_{nr}} = \frac{\text{photons emitted}}{\text{photons absorbed}}, \tag{13}$$

so, the percentage of excitations that result in fluorescence [Lakowicz (2006)]. Since transitions are not only possible from specific wavelengths, excitation and emission properties are represented as spectra (Fig. 2B). Because emission occurs from lower energy singlet states, the emission spectrum is generally red-shifted (Stokes' shift). In this experiment, the DNA will be labeled with TOTO-3. This intercalating fluorophore inserts itself between DNA bases.

Fluorescence Microscopy

Fluorescence microscopy allows for quantitative spatial and temporal visualization of fluorescent material at the microscopic level.

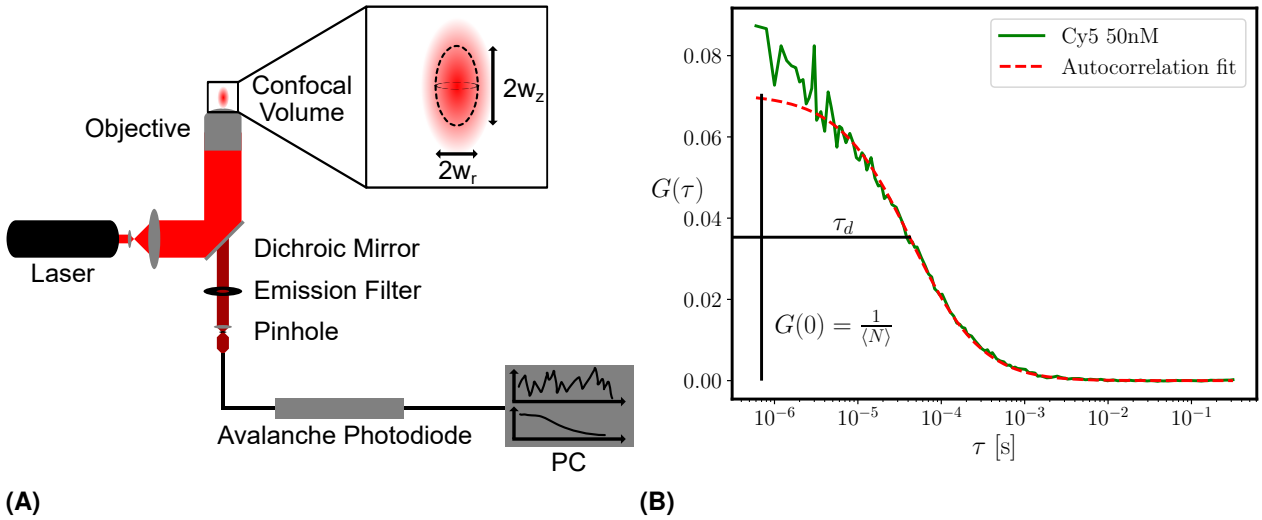

Figure 3 (A) Confocal microscopy setup for fluorescence correlation spectroscopy. The incoming laser beam is directed towards the sample through a dichroic mirror and objective. The fluorescent emission propagates back, through the emission filter and the pinhole which shapes the confocal volume. The intensity signal is then auto-correlated. The detection volume is characterized by radial waist w_r and axial waist w_z in z-direction. **(B)** Autocorrelation curve $G(\tau)$ of diffusing Cy5 fluorophors. The fit parameters diffusion time τ_D and $\langle N \rangle$ are defined at the inflection point and the onset value $G(0)$, respectively.

In a fluorescence microscope, excitation light from (commonly) a mercury lamp or laser is coupled into the optical path, typically via an optical fiber to ensure a stable and well-defined beam profile. The laser light is directed towards the sample by a dichroic mirror, which reflects the excitation wavelength while transmitting the longer-wavelength fluorescence emission. The objective lens focuses the excitation light into a small volume within the sample, typically positioned on the order of 200 µm above the coverslip. Fluorescence emitted by the sample is collected by the same objective and passes back through the dichroic mirror and into a camera. This technique will be used to image DNA coils and oligomers bound to a substrate on a glass surface.

Importantly, the spatial resolution in conventional fluorescence microscopy is diffraction-limited. According to the Rayleigh criterion, two point emitters can only be resolved if their separation exceeds

$$d = \frac{1.22\lambda}{2 \cdot NA} \tag{14}$$

where $NA = n \sin\theta$ is the numerical aperture of the objective, n the refractive index of the medium, and θ the half opening angle of the objective. The point spread function (PSF) of a single emitter is described by the Airy diffraction pattern resulting from Fraunhofer diffraction at a circular aperture. Consequently, an extended fluorescence distribution, such as that of λ-DNA, is observed as the convolution of the underlying fluorophore distribution with the PSF. [Herman (2020)]

In case of a *confocal microscope*, a pinhole placed in a conjugate image plane blocks out-of-focus light (blur), thereby defining a small detection volume and providing axial resolution (Fig. 3A). The remaining signal is detected with a sensitive detector, such as an avalanche photodiode, which allows for the measurement of low light intensities with high temporal resolution. Confocal microscopes generally employ lasers for excitation. The focal intensity distribution can be described by a Gaußian ellipsoid

$$\Omega(x, y, z) = \Omega_0 \exp\left(-2\left(\frac{x^2 + y^2}{w_r^2} + \frac{z^2}{w_z^2}\right)\right) \tag{15}$$

where w_r and w_z are radial and axial waist respectively. This defines the structure parameter $S = \frac{w_z}{w_r}$. Typical values for S range between 2 and 10, resembling a prolate ellipsoid. The effective confocal volume is the

equivalent uniform volume that would produce the same fluorescence signal as the actual Gaussian-shaped detection profile. It is given by

$$V_{eff} = \frac{\left(\int \Omega \, \mathrm{d}V\right)^2}{\int \Omega^2 \, \mathrm{d}V} = \pi^{\frac{3}{2}} w_r^2 w_z \tag{16}$$

A confocal microscope setup will be used to perform FCS.

Fluorescence Correlation Spectroscopy

FCS measures fluctuations in fluorescence intensity from a small confocal detection volume ($V_{eff} \sim 1$ fL $= 1\,\mu\mathrm{m}^3$). At nanomolar concentrations, only a few fluorescent particles are present in the focal volume (e.g. for 10 nM: $\langle N \rangle = cV = 10\,\mathrm{nM} \cdot 1\,\mathrm{fL} = 10 \cdot 10^{-9} \cdot 1.6022 \cdot 10^{23} \cdot 10^{-15} \approx 1.6$). By diffusing in and out of this volume, they generate measurable intensity fluctuations $I(t)$. In FCS, these fluctuations are quantified by temporally autocorrelating the recorded intensity signal in a hardware correlator card. This measure of self-similarity between $I(t)$ and $I(t+\tau)$ is formulated as a convolution:

$$G(\tau) := \frac{\langle I(t) I(t+\tau) \rangle}{\langle I(t) \rangle^2} \tag{17}$$

For a single species diffusing under Brownian motion, the ACF is computed analytically as (for a detailed derivation see Schwille and Haustein (2000)):

$$G(\tau) = G(0) \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{S^2 \tau_D}\right)^{-1/2} \tag{18}$$

where the lateral diffusion time τ_D, also known as the average dwell time in the confocal volume, is obtained via equation 5:

$$\tau_D = \frac{w_r^2}{4D} \tag{19}$$

For a prolate confocal volume, it can be interpreted as the time after which correlations have decayed to approximately half of $G(0)$ (see Fig. 3B).

$G(0)$ is evaluated according to Equation 17

$$G(0) = \frac{\mathrm{Var}(I)}{\langle I \rangle^2} = \frac{\langle N \rangle}{\langle N \rangle^2} = \frac{1}{\langle N \rangle} \tag{20}$$

where $\langle N \rangle$ denotes the average particle number in the confocal volume. The relation $\mathrm{Var}(I) = \langle \Delta I^2 \rangle = \langle I \rangle$ holds since particles entering the confocal volume follow a Poisson process (continuity in time and independence of events), where the variance equals the mean.

Finally, time scales below lateral diffusion can also be seen in the ACF, such as triplet states, rotational diffusion or antibunching. They are not captured in Equation 18.

Experimental Procedure and Analysis

We prepare 2 TOTO-stained, biotinylated DNA samples in buffer (TE + 10 nM NaCl):

1. Short DNA (50 bp, "DNA Oligo")
2. λ-DNA (~48 kbp)

20 bp/dye gives sufficient staining.

Surface Immobilization Assay and Fluorescence Microscopy

A streptavidin-coated Ibidi slide is first prepared.

- Decide on the right DNA dilution into appropriate buffer to achieve coverage of the slide while keeping overlap minimal. Apply it to the slide, where it is incubated to allow binding via the streptavidin–biotin interaction.
- Estimate the incubation time as the time required for DNA to reach a binding site, i.e. the substrate, under diffusion.

After incubation, the slide is gently washed to remove unbound DNA molecules. The immobilized DNA is then imaged using fluorescence microscopy under consistent illumination conditions, with appropriate correction for background signal and photobleaching. Note down the specifications of the objective (NA) to later quantify the PSF. Finally, we induce flow within the Ibidi slide to qualitatively observe the extension behavior of the DNA molecules.

- Analyze the fluorescence microscopy images using Fiji/ImageJ to identify individual DNA molecules and extract their intensity profiles.
- Perform Gaußian fits on the molecules to determine their radius of gyration and total intensities. You can use a plugin like ThunderSTORM.
- Compare the total fluorescence intensities for DNA of different lengths.
- From the NA of the objective, calculate the resolution limit and compare the FWHM of the PSF with the spatial intensity distribution of the DNA oligo.
- Assuming an ideal chain and Equation 3, how much does L_p deviate from the literature value?
- Discuss, how the intercalator might change the mechanic properties of DNA (specifically L_c and L_p).
- Finally, investigate the effect of induced flow in the channel on DNA conformation. Observe how the molecules elongate under flow and relate this behavior to the laminar flow profile in the channel.

Fluorescence Correlation Spectroscopy (FCS)

Calibration

Before measuring DNA samples, the device is initialized in consultation with a supervisor. To determine the structure parameter S and the focal radius w_r in Equation 19, the setup is calibrated using a fluorescent molecule with a known concentration and diffusion constant [Buschmann *et al.* (2009)]. For this purpose, the Cy5 dye (Amersham Bioscience) is used, which has a diffusion constant of 250 $\mu m^2/s$. We excite the dye with the 633 nm line of a HeNe laser. After recording 5×60 s measurements at 1% laser power, we extract τ_D and $G(0)$ from the fitted autocorrelation curves. Laser power must not be changed after calibration.

Measurement

Once the instrument has been calibrated, actual measurements can begin. The 2 DNA samples are each diluted to approximately 0.5 nM in buffer and each placed into one of the six chambers of the sample holder. The measurement and analysis is carried out almost entirely within the microscope software. To start the measurement, select the "AnalyzeFCS" menu. First, select the position of the holder above the objective in the "Carrier" menu. The focus is set 200 μm above the glass slide. Selecting the "Laser" button in this menu window displays the detector count rate. This should be between 10 and 100 kHz. The laser power must not be adjusted, as this alters the profile of the focal volume and the instrument needs to be re-calibrated. It is easier to adjust the concentration of the sample. In the "Method" menu, the measurement parameters can be checked. From the "Optimize" menu, measurements can be started and, if necessary, canceled. 5×60 s of measurement are recorded.

- Fit the ACFs in the FCS software. Infer the structure parameter from the calibration and fit the values τ_D and $\langle N \rangle$. Does the concentration value gained from the ACF match your dilution concentration?
- Calculate the diffusion coefficients from the fitted diffusion times.
- Compare the models discussed in the manual (cylinder and coil) with your data.

- Consider potential systematic errors in fluorescence correlation spectroscopy measurements and how they may affect the extracted parameters. Discuss how the effective size of the confocal volume is affected by the size of the diffusing species [Wu *et al.* (2008)].
- How do static and dynamic properties of DNA compare? How does Equation 10 fit your data?

Literature

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