Biophysical practical course R1b

Characterizing Products of the Polymerase Chain Reaction (PCR) by Fluorescence Correlation Spectroscopy (FCS)

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In this experiment we use polymerase chain reaction (PCR) to amplify DNA fragments of different lengths of a plasmid. Fluorescently labeled primers are used for this purpose. To check if the amplification of the DNA has worked we use gel electrophoresis. Amplification of the DNA is controlled by gel electrophoresis, before the various DNA fragments are further analysed by FCS.
The method of the PCR (Saiki et al. 1988) was developed by Kary B. Mullis in 1987. This procedure revolutionized genetic engineering. It enables to make millions of copies of a distinct nucleotide sequence *in vitro*. With this process called amplification even small amounts of DNA are easily accessible.

1 Polymerase Chain Reaction (PCR)

1.1 Field of Application

Usually, PCR is used for duplication of any gen fragment or for sequencing of a known fragment. By means of PCR it is also possible to prove if a distinct gene is present in an organism. PCR is also very important for the production of hybridization probes for example for DNA microarray technology. The main field of application however, is for medical diagnostics. Examples are the verification and quantification of viral and microbial gene sequences or the distinction of point mutations in otherwise identical DNA sequences. Furthermore, PCR is used for chromosome analysis, detection of chromosomal variations and in forensic analysis. Often only a little amount of DNA is detected on crime scenes, for example blood spots or strands of hairs, which can be amplified by PCR and analyzed in more detail afterwards.

1.2 Basic Principle

The process of the nucleic acid amplification by PCR is similar to the natural process of DNA replication (Figure 1). Based on a starter molecule a DNA polymerase synthesizes a new DNA strand on a single stranded nucleic acid matrix, the so called template DNA. Synthetic DNA oligonucleotides (called primers) are used as starter molecules at PCR. These primers hybridize to the DNA template. Beginning of the 3'-end of the template a thermostable polymerase synthesizes the new DNA double strand. By selection of a counter-rotating primer pair a specific DNA sequence between these two primers can be amplified (see Figure 1). The crucial principle of PCR is the cyclic repetition of the single reaction steps to achieve exponential amplification of the requested DNA sequence (Number of copies = $(2^n\text{-}2n)\cdot x$; n= number of cycles, 2n = products of 1^{st} and 2^{nd} cycle, x = copy number of primary DNA).
A cycle of the PCR starts with the thermal denaturation of the DNA template by melting the double stranded DNA at 95 °C. Thereby, single stranded DNA templates occur. Afterwards, the primers hybridize to the complementary sequences of the single stranded DNA at a temperature range between 37 °C to 65 °C. In this way the amplification of the sequence segment between the primers is initiated and is normally carried out at 72 °C. This step is performed by enzymes called polymerases. There are several polymerases like the thermostable polymerases of *Thermus aquaticus* (Taq), which do not own any correction mechanisms and produces more sequence errors than for example the Pfu-polymerase (*Pyrococcus furiosus*) which has a correction mechanism. The PCR experiments are done in thermocyclers. These units consists of a temperature-controlled reaction chamber, in which the PCR tubes are placed. An automated control regulates the cyclic temperature program. The temperature, the times for each reaction step and the numbers of cycles are individually programmable.

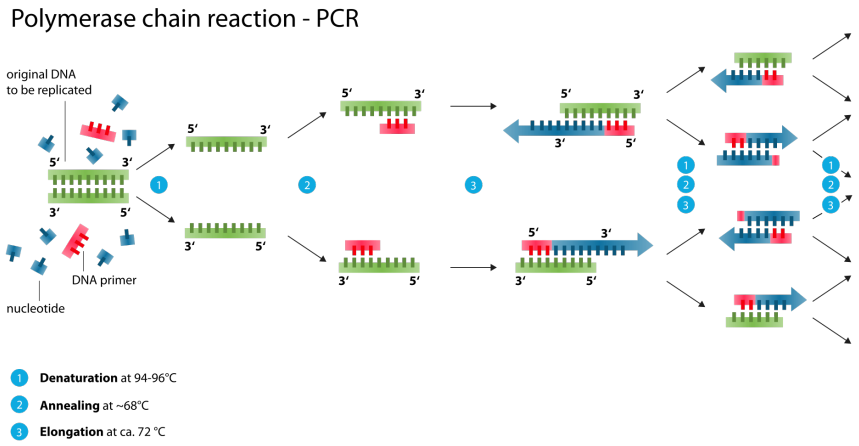

Figure 1: *Visualization of the Polymerase Chain Reaction, Source: Wikipedia*

Beside the right polymerase and the DNA template the selection of the primers is very important for the success of the PCR.
Important for primer selection:

1. Length of the primer: 20-30 nucleotides
2. GC-amount: 40-60 %
   Annealing temperature: 5 °C $< T_m$
   T_m is the melting temperature at which the examined DNA molecule is 50 % degenerated (DNA is single stranded).
   Melting temperature of the primer (approximation):
   $T_m = (A + T) \times$ 2°C + (G+C) $\times$ 4°C
3. No repeating motives
4. No distinct secondary structure
5. Primers are not permitted to be inter- and intramolecular complementary (otherwise: dimer formation of primers)
6. Sequence of 3‘-end: should contain GC not AT
   no repeats of 3 or more G or Cs at the end

One can easily approximate the concentration of the required DNA fragments. If $p_1^i, p_2^i, h_1^i, h_2^i$ are the concentrations of the desired products and of the half-side extended polymerization products (these are produced in every cycle at the original strand) after i PCR-cycles and o_1, o_2 are the concentrations of the

original strands, then one can postulate the following recursion relations:

$$\begin{aligned} h_1^i &= h_1^{i-1} + o_2 \\ h_2^i &= h_2^{i-1} + o_1 \\ p_1^i &= p_1^{i-1} + p_2^{i-1} + h_2^{i-1} \\ p_2^i &= p_1^{i-1} + p_2^{i-1} + h_1^{i-1} \end{aligned}$$

Here one assumes that all reactions – hybridisation and polymerisation – proceed ideally and that the primers are the dominant species in the solution. If, $o_1 = o_2 =: N_0$, then one can easily see that the number of the product strands after n rounds is given by

$$p_{1,2}^n = (2^{n-1} - 1) \cdot N_0 \tag{1}$$

and the number of the half-side extended products is just

$$h_{1,2}^n = n \cdot N_0. \tag{2}$$

These results can be strongly deviating, if one does not work stoichiometricaly and if one does not consider that the number of primers decays during the course of the reaction. In general, it can be observed that the number of the desired short reaction products that are flanked by the primer sequences increases exponentially and that after a few rounds these products are the dominant species in the sample.

1.3 Gel electrophoresis of nucleic acids

It is common practice to separate nucleic acids by agarose gels. The separation occurs in an electric field due to the fragment size. For huge DNA fragments agarose gels with low agarose concentration of 0.3-1.0 % agarose are used. For small fragments the separation is done with agarose concentrations of 1.0-2.0 % agarose. A little amount of each PCR sample ($5-10$ μl) together with a loading buffer is applied to one pocket of the gel. The loading buffer contains glycine to facilitate the sample loading and a dye to visualize the gel running. As a control and for size determination of the sample a standard with distinct size is applied to the gel as well. To make the DNA bands in the gel visible, the gel is incubated in SYBR Safe DNA Gel Stain, a DNA intercalating dye. Afterwards the bands are visible under UV-light.

1.4 Primer for detection of EGFP at the pEGFP-N1-vector (CLONTECH)

```
        1    tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata tggagttccg
       61    cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc cccgcccatt
      121    gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc attgacgtca
      181    atgggtggag tatttacggt aaactgccca cttggcagta catcaagtgt atcatatgcc
      241    aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgcccagta
      301    catgacctta tgggactttc ctacttggca gtacatctac gtattagtca tcgctattac
      361    catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg actcacgggg
      421    atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc aaaatcaacg
      481    ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg gtaggcgtgt
      541    acggtgggag gtctatataa gcagagctgg tttagtgaac cgtcagatcc gctagcgcta
      601    ccggactcag atctcgagct caagcttcga attctgcagt cgacggtacc gcgggcccgg
P1f   661    gatccaccgg tcgccaccat ggtgagcaag ggcgaggagc tgttcaccgg ggtggtgccc
P5r   721    atcctggtcg agctggacgg cgacgtaaac ggccacaagt tcagcgtgtc cggcgagggc
      781    gagggcgatg ccacctacgg caagctgacc ctgaagttca tctgcaccac cggcaagctg
      841    cccgtgccct ggcccaccct cgtgaccacc ctgacctacg gcgtgcagtg cttcagccgc
      901    taccccgacc acatgaagca gcacgacttc ttcaagtccg ccatgcccga aggctacgtc
P9r   961    caggagcgca ccatcttctt caaggacgac ggcaactaca agacccgcgc cgaggtgaag
     1021    ttcgagggcg acaccctggt gaaccgcatc gagctgaagg gcatcgactt caaggaggac
     1081    ggcaacatcc tggggcacaa gctggagtac aactacaaca gccacaacgt ctatatcatg
     1141    gccgacaagc agaagaacgg catcaaggtg aacttcaaga tccgccacaa catcgaggac
     1201    ggcagcgtgc agctcgccga ccactaccag cagaacaccc ccatcggcga cggccccgtg
     1261    ctgctgcccg acaaccacta cctgagcacc cagtccgccc tgagcaaaga ccccaacgag
     1321    aagcgcgatc acatggtcct gctggagttc gtgaccgccg ccgggatcac tctcggcatg
P13r  1381    gacgagctgt acaagtaaag cggccgcgac tctagatcat aatcagccat accacatttg
     1441    tagaggtttt acttgcttta aaaaacctcc cacacctccc cctgaacctg aaacataaaa
     1501    tgaatgcaat tgttgttgtt aacttgttta ttgcagctta taatggttac aaataaagca
     1561    atagcatcac aaatttcaca aataaagcat ttttttcact gcattctagt tgtggtttgt
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Sequence information:

P1EGFP (672-691f):	5’ CGC CAC CAT GGT GAG CAA GG 3’	Tm=(66°C) / 63.4°C
P5 EGFP (772-753r):	5´GGA CAC GCT GAA CTT GTG GC 3’	Tm= (64°C) / 61.4°C
P9 EGFP (971-952r):	5’ GTG CGC TCC TGG ACG TAG CC 3’	Tm= (68°C) / 65.5°C
P13 EGFP (1364-1383r):	5’ GTC CAT GCC GAG AGT GAT CC 3’	Tm= (64°C) / 61.4°C

All primers are labeled with the fluorophore Cy5 at the 5’-end.

Figure 2: *A part of the nucleotide sequence of the vector is shown. The forward primer is marked in green and the three reverse primers are marked in yellow. Additional information for each primer is given below.*

1.5 Procedure

For examination of the fragment length three PCR samples are prepared.

Primer Forward	**F Primer Reverse**	**Fragment (bp)**	T_m ($^\circ C$)
P1 (672-691f)	P5 (772-753r)	101	63.4 / 61.4
P1 (672-691f)	P9 (971-952r)	300	63.4 / 65.5
P1 (672-691f)	P13 (1364-1383r)	712	63.4 / 61.4

Therefore, the required components are thawed on ice and the samples are mixed as indicated in the table below. It is mandatory that all steps are done on ice.

Components	**Final concentration**	**μl for 50μl-preparation**
10× Buffer	1 ×	5
2mM dNTP-Mix	10 nmol	5
Primer F (10 pmol/μl)	50 pmol	5
Primer R (10 pmol/μl)	50 pmol	5
Template (pEGFP-N1-plasmid)	10 ng	1
Taq (5.000U/ml)	5 U	1
Water		Add to 50.0

The samples are put in the programmable thermocycler (PCR machine) and the following program has to be used:

1. step	Denaturation	95°C	5 min
Cycles (30)	Denaturation	95°C	30 s
	Annealing	65°C	30 s
	Elongation	68°C	60 s
Last step	Final elongation	68°C	5 min

5 μl of each PCR sample is mixed with 1 μl 6× loading buffer and applied to one pocket of a 1 % agarose gel. 6 μl of two DNA standards (1 kb ladder and 100 bp ladder of NEB) are applied to other pockets. The gel is connected to a power supply unit for around 1 hour at ca. 80 V. Afterwards, the gel is stained for about 30 minutes with SYBR Safe DNA Gel Stain and photographed under UV-light. The remaining 45 μl of the PCR samples are purified using the purification kit of QIA-quick and the concentration of the PCR fragments is measured spectroscopicaly using the NanoDrop.

1.6 Analysis of PCR

Mark the photo of the gel showing the PCR fragments (sample names corresponding of the lanes, fragment sizes of the DNA standards). Generate a calibration graph using the DNA standards (Figure 3). Plot the migration distance against the logarithm of the DNA length of each fragment (in bp) on semi

logarithmic paper. Determine the length of the fragments on the basis of your calibration curve and explain why these lengths do not correspond exactly with the mentioned length.

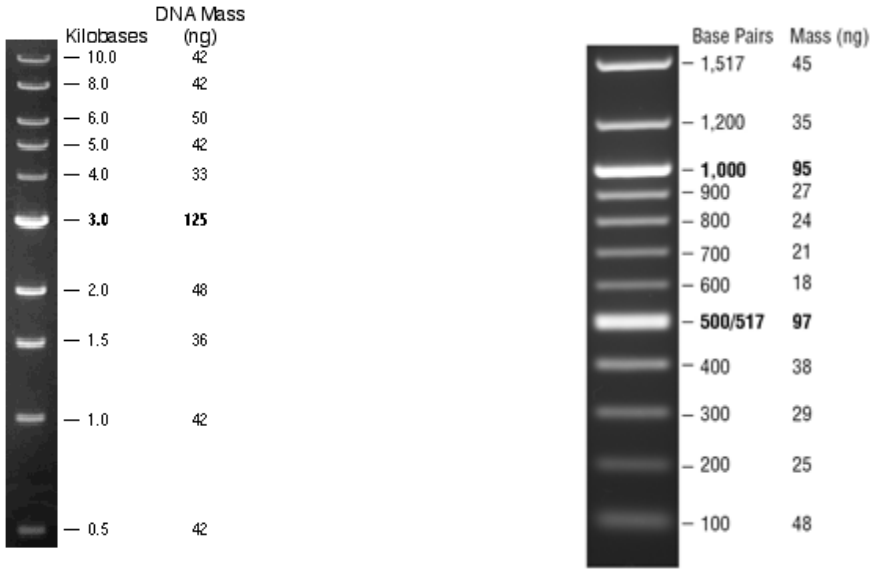

Figure 3:
left: 1 kb DNA ladder, source: www.neb.com/products/n3232-1-kb-dna-ladder, right: 100 bp DNA ladder, source: www.neb.com/products/n3231-100-bp-dna-ladder

2 Diffusion of DNA

In the following, we will discuss how one can determine the diffusion constant of a fluorescently labeled object with the help of fluorescence correlation spectroscopy. In this experiment this approach should be validated by using PCR products of different length. What is the expected diffusion behavior of these DNA molecules?
For spherical objects with radius r the known Stokes-Einstein-relation holds true

$$D = \frac{k_B T}{6\pi\eta(T)r}, \tag{3}$$

where $k_B T$ is the thermal energy and η(T) the viscosity of the surrounding medium at temperature T. The DNA fragments used in this experiment have lengths of 100, 300 and 700 base pairs and cannot be considered as spheres at all. The distance of two base pairs of B-shaped DNA is 0.34 nm, so the DNA

fragments have a contour length of approx. 35 nm, 100 nm und 240 nm. A multitude of biophysical studies showed that DNA behaves mechanically like a "semiflexible polymer" with a so-called persistence length of about 50 nm. At this length scale one can consider DNA to be a stiff cylinder. Therefore, we will compare the experimentally determined diffusion constants with the ones for a cylinder with diameter $d = 2$ nm and length $L = 35$ nm, 100 nm und 240 nm. There exists no exact description for the diffusion constant of a cylinder, but the following approximation is valid:

$$D = \frac{k_B T}{3\pi\eta L} \times \left\{ \ln L/d + 0.312 + 0.565 d/L - 0.100 (d/L)^2 \right\} \tag{4}$$

For $L \gg d$ the first term inside the brackets is determinant and one can also use the simplified equation (see Figure 4):

$$D = \frac{k_B T}{3\pi\eta L} \times \ln L/d \tag{5}$$

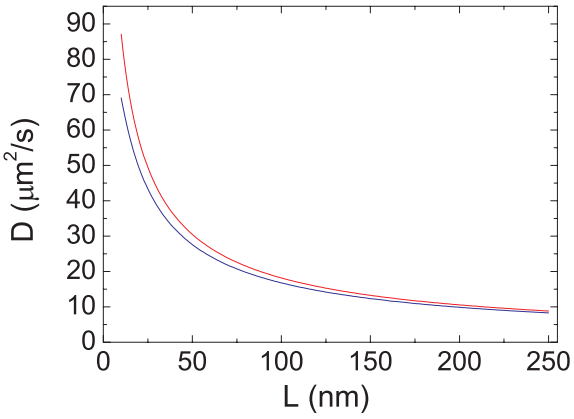

Figure 4: *Comparison of both approximations of the diffusion constant of a cylinder with length L with diameter $d = 2$ nm. The upper curves comes from the more precise expression used in Equation 4.*

3 Fluorescence

Luminescence is the emission of light that originates from excited electrons of a substance. In the special case of fluorescence these excited electrons are in the singlet-sate (total spin is zero). A typical illustration of the involved electron transitions is the so called Jablonski-diagram (Figure 5).

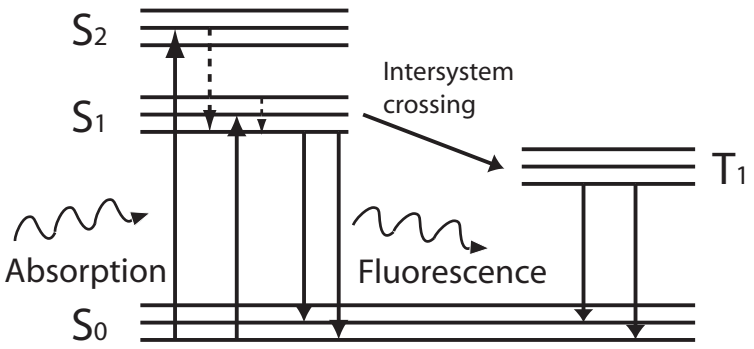

Figure 5: *Simplified Jablonski-diagram. Originating from a singlet-ground state (S_0), electrons are excited to higher orbitals (S_1, S_2) which are energetically separated into vibrational and rotational modes.*

The most important processes are indicated in the diagram. Normal fluorescence occurs if an electron is lifted form the ground state to an excited state by absorption of light. First, it relaxes from there to the energetically lowest vibrational mode and then drops from there back to the ground state by emitting a red-shifted photon. Alternatively, a transition to the triplet state T_1 can occur. From that state a transition to the singlet ground state is forbidden. Therefore, its life time is much more prolonged compared to the one of an excited singlet state. Typical fluorescence life times are in the order of 10 ns, while triplet life times are in the range of milliseconds to seconds. A dye can return from the excited state to the ground state over several pathways. Due to this, there is always a certain percentage for the transition to the triplet, but also other non-radiant processes are possible. The surroundings play an important role for fluorescence (depletion via collision with solvent molecules). An important parameter for the characterization of dyes is the so-called "quantum yield" Q. If one defines the transition rate for non-radiant processes with k_{nr} and the rate for the radiation of photons with Γ, the following is valid

$$Q = \frac{\Gamma}{\Gamma + k_{nr}}, \tag{6}$$

which is the percentage of excitation that leads to a fluorescence signal.

In our experiment we use the fluorescent dye "Cy5" (Cyanine 5) for labeling of the PCR products (Figure 6). It has an absorption maximum at 649 nm and an emission maximum at 670 nm (slight variations, depending on the solvent). Its quantum yield is larger than 0.28 (data from Amersham Biosciences), its molar extinction coefficient is $\epsilon = 250'000\ \mathrm{M}^{-1}\mathrm{cm}^{-1}$. The molecular weight of the pure dye is approx. 470 *Dalton* and its diffusion constant is about 250 $\mu\mathrm{m}^2/s$.

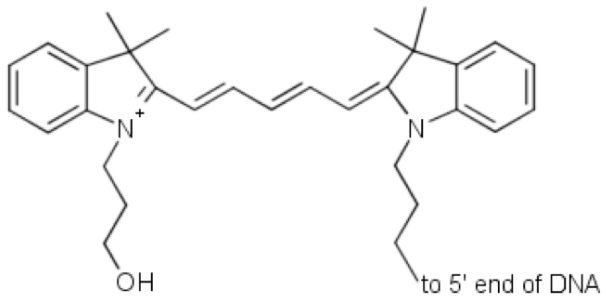

Figure 6: *Like most fluorescent dyes, Cy5 contains an extended π- electron system. The aromatic rings at the sides are attached via π-conjugated bridges. The OH-group at the alkane chain at the bottom left makes the molecule more soluble in water. The dye is bound to the 5'OH-Gruppe of the DNA via the linker on the right hand side.*

4 Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy is an elegant method to determine relevant physical parameters like the diffusion constant of biomolecular systems, by analysing the fluctuations of a fluorescence signal. The central measurement parameter of FCS is the temporal autocorrelation function $\bar{G}(\tau)$ of the fluorescence signal $F(t)$ of a fluorescent sample. $G(\tau)$ is defined by

$$\tilde{G}(\tau) := \frac{\langle F(t+\tau)F(t)\rangle}{\langle F(t)\rangle^2} \tag{7}$$

Using

$$F(t) = \langle F\rangle + \delta F(t)$$

one obtains

$$\tilde{G}(\tau) = 1 + \frac{\langle \delta F(t+\tau)\delta F(t)\rangle}{\langle F(t)\rangle^2} =: 1 + G(\tau) \tag{8}$$

At $\tau = 0$:

$$G(\tau = 0) = \frac{varF}{\langle F\rangle^2} \tag{9}$$

The fluorescence signal of a highly diluted sample is recorded by a confocal microscope set up. In the process, only fluorescent molecules are excited that are located inside the confocal volume ($V \approx 1\mu m^3$). Using a concentration of about $c = \ 10\ nM = \ 10\ nmol/l$ only approximately

$$N = c \cdot V = 10 \times 10^{-9} \times \mathrm{N_A} \times 10^3 \mathrm{m}^{-3} \times 10^{-18} \mathrm{m}^3 \approx 6$$

fluorescent molecules are contributing to the signal (N_A is the Avogadro constant). In this regime the fluctuations are strong enough and also contain physical relavant information. For our experiment, the observation of the autocorrelation function of a simple, diffusing fluorescent object is sufficient. More

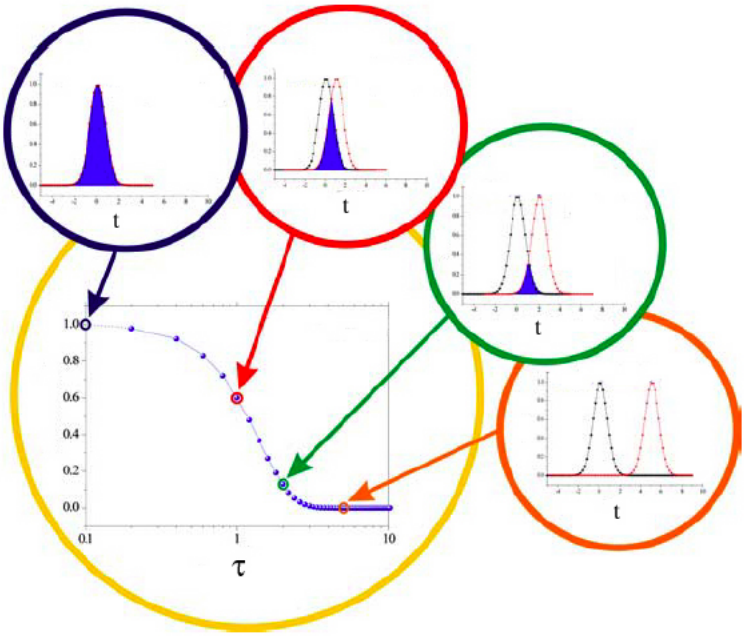

Figure 7: *Illustration of the autocorrelation function, Source: P.Schwille and E.Haustein*

complex situations include the presence of serveral differently labelled species, the existence of "internal" fluctuations and association or dissociation processes. For mathematical considerations the confocal volume (focus of the laser beam) is approximated by using a Gaussian intensity distribution:

$$I(x,y,z) = I_0 \exp\left(-\frac{2(x^2+y^2)}{w_{xy}^2} - \frac{2z^2}{w_z^2}\right). \tag{10}$$

Here z is chosen as the beam direction of the laser beam. w_{xy} is approximately the radius of the intensity profile inside the focus, w_z the extension in beam direction. The diffusion equation

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

is also valid for fluctuations of the concentration $\delta c(\mathbf{r},t) = c(\mathbf{r},t) - \bar{c}$, so

$$\frac{\partial}{\partial t}\delta c(\mathbf{r},t) = D\nabla^2 \delta c(\mathbf{r},t) \tag{11}$$

An important assumption about the fluctuations $\delta c(\mathbf{r},t)$ in highly diluted samples is

$$\langle \delta c(\mathbf{r},t)\delta c(\mathbf{r}',t)\rangle = \bar{c}\delta(\mathbf{r}-\mathbf{r}'), \tag{12}$$

this means that fluctuations of the concentrations at the same time, but at different locations are statistically independent from each other. In case of Poisson statistics the prefactor is equal to the average concentration. The Fourier transformation of the fluctuations gives:

$$\tilde{c}(\mathbf{q},t) = \int d^3\mathbf{r}\ e^{i\mathbf{q}\cdot\mathbf{r}}\ \delta c(\mathbf{r},t), \tag{13}$$

with the back transformation

$$\delta c(\mathbf{r},t) = \frac{1}{(2\pi)^3}\int d^3\mathbf{q}\ e^{-i\mathbf{q}\cdot\mathbf{r}}\ \tilde{c}(\mathbf{q},t) \tag{14}$$

In Fourier space the diffusion equation turns into

$$\frac{\partial}{\partial t}\ \tilde{c}(\mathbf{q},t) = -q^2 D\ \tilde{c}(\mathbf{q},t), \tag{15}$$

thus, one solution is given by

$$\tilde{c}(\mathbf{q},t) = \tilde{c}(\mathbf{q},0)e^{-q^2 Dt}. \tag{16}$$

The fluctuations of the concentration of the fluorescent species δc lead to fluctuations of the fluorescence δF, characterized by:

$$\delta F(t) = \gamma\epsilon Q\int d^3\mathbf{r}\ I(\mathbf{r})\delta c(\mathbf{r},t) \tag{17}$$

Here ϵ is the extinction of the sample, Q the quantum yield of the dye, and γ takes the efficency of the detection by the set-up into account. Taken together, these parameters describe how many photons are collected effectively inside the confocal volume. Inserted into the formula for the correlation function, one obtains:

$$G_{diff}(\tau) = \frac{\gamma^2\epsilon^2 Q^2}{\langle F\rangle^2}\int\int d^3\mathbf{r}\ d^3\mathbf{r}'\ I(\mathbf{r})I(\mathbf{r}')\langle\delta c(\mathbf{r},t+\tau)\delta c(\mathbf{r}',t)\rangle \tag{18}$$

First, let's have a look at the correlation function of the fluctuations of the concentration:

$$\begin{aligned}
&\langle\delta c(\mathbf{r},t+\tau)\delta c(\mathbf{r}',t)\rangle && (19)\\
&\quad= \frac{1}{(2\pi)^3}\int d^3\mathbf{q}\ e^{-i\mathbf{q}\cdot\mathbf{r}}\langle c(\mathbf{q},t+\tau)\delta c(\mathbf{r}',t)\rangle && (20)\\
&\quad= \frac{1}{(2\pi)^3}\int d^3\mathbf{q}\ e^{-i\mathbf{q}\cdot\mathbf{r}}e^{-q^2 D\tau}\langle c(\mathbf{q},t)\delta c(\mathbf{r}',t)\rangle && (21)\\
&\quad= \frac{1}{(2\pi)^3}\int d^3\mathbf{q}\ \int d^3\mathbf{r}''\ e^{i\mathbf{q}\cdot(\mathbf{r}''-\mathbf{r})}e^{-q^2 D\tau}\langle\delta c(\mathbf{r}'',t)\delta c(\mathbf{r}',t)\rangle && (22)\\
&\quad= \frac{\bar{c}}{(2\pi)^3}\int d^3\mathbf{q}\ e^{i\mathbf{q}\cdot(\mathbf{r}'-\mathbf{r})}e^{-q^2 D\tau} && (23)
\end{aligned}$$

If one inserts this result into Equation 18, one obtains

$$G_{diff}(\tau) = \frac{\bar{c}\gamma^2\epsilon^2 Q^2}{(2\pi)^3\langle F\rangle^2}\int d^3\mathbf{r}\int d^3\mathbf{r}'\int d^3\mathbf{q}\; e^{i\mathbf{q}\cdot(\mathbf{r}'-\mathbf{r})}e^{-q^2D\tau}I(\mathbf{r})I(\mathbf{r}'). \quad (24)$$

The integrals of $\mathbf{r}$ and $\mathbf{r}'$ are in each case Fourier transformations of the intensity profile. For a Gaussian profile this is given by:

$$I_0\int d^3\mathbf{r}\; e^{i\mathbf{q}\cdot\mathbf{r}}\exp\left(-\frac{2(x^2+y^2)}{w_{xy}^2}-\frac{2z^2}{w_z^2}\right) \quad (25)$$

$$= I_0\left(\frac{\pi}{2}\right)^{3/2} w_{xy}^2 w_z \exp\left(-\frac{w_{xy}^2(q_x^2+q_y^2)}{8}-\frac{w_z^2 q_z^2}{8}\right) \quad (26)$$

Inserted above, one obtains:

$$G_{diff}(\tau) = \frac{\bar{c}\gamma^2\epsilon^2 Q^2 I_0^2 w_{xy}^4 w_z^2}{64\langle F\rangle^2} \quad (27)$$

$$\times \int d^3\mathbf{q}\; \exp\left(-\frac{(w_{xy}^2+4D\tau)(q_x^2+q_y^2)}{4}-\frac{(w_z^2+4D\tau)q_z^2}{4}\right). \quad (28)$$

To simplify this formula, we determine the average fluorescence signal:

$$\langle F\rangle = \gamma\epsilon Q\bar{c}\int d^3\mathbf{r}\; I(\mathbf{r}) = \gamma\epsilon Q\bar{c}I_0\;\frac{\pi w_{xy}^2}{2}\;\sqrt{\frac{\pi w_z^2}{2}}, \quad (29)$$

where we once more took advantage of the Gaussian shape of the intensity profile. After determination of the integral, Equation 28 turns with this into

$$G_{diff}(\tau) = \frac{1}{(2\pi)^3\bar{c}}\;\frac{4\pi}{w_{xy}^2+4D\tau}\;\sqrt{\frac{4\pi}{w_z^2+4D\tau}} \quad (30)$$

$$= \frac{1}{\pi^{3/2}w_{xy}^2 w_z\bar{c}}\;\frac{1}{1+4D\tau/w_{xy}^2}\;\sqrt{\frac{1}{1+4D\tau/w_z^2}} \quad (31)$$

Now we introduce some important abbrevations to simplify the notation.
The effective sample volume is defined by $V = \pi^{3/2}w_{xy}^2 w_z$, with that $\bar{N} = V\bar{c}$ is the average number of particles inside this volume. The time $\tau_D := w_{xy}^2/4D$ is the diffusion time perpendicular to the beam direction. According to that $\tau_D' := w_z^2/4D$ is the diffusion time along the beam direction. Finally, the aspect ratio of the longitudinal to the lateral extension of the focus $w := w_z/w_{xy}$ is introduced. w is also called the structure parameter. The correlation function of a one-component fluorescent sample is given by

$$G_{diff}(\tau) = \frac{1}{\bar{N}}\;\left(1+\frac{\tau}{\tau_D}\right)^{-1}\;\left(1+\frac{\tau}{w^2\tau_D}\right)^{-1/2} \quad (32)$$

First, in the experiment, S is determined with the help of a sample with a known diffusion time. The experimentally determined correlation curve is fitted with the above mentioned equation using $\bar{N}$ and τ_D as fitting parameters.

5 Set up

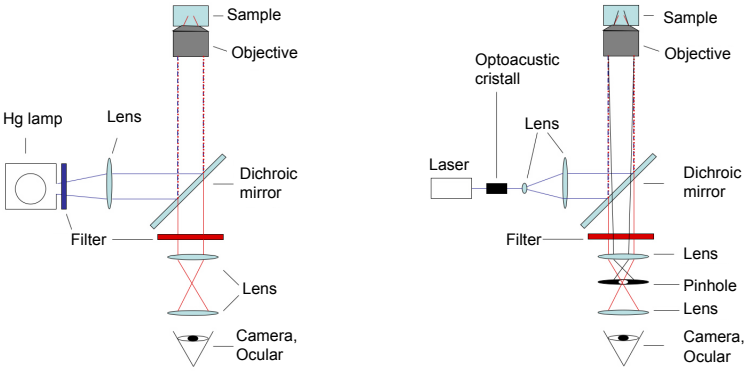

Figure 8: *Conventional fluorescence microscope and confocal FCS-set up*

The experiments will be performed using a confocal FCS-Set up from Zeiss (ConfoCor2). A sketch of the set up is shown in Figure 8. Laser light of the required wavelength is fed by a glass fibre. The light is reflected by a dichroic mirror and is focused through the objective about 200 μm above the bottom of the Nunc-Lab chambers. Set ups that work without laser light use a mercury vapor lamp. The light of the lamp is filtered and passes a pinhole before it reaches the mirror. Inside the confocal volume, the fluorophores are excited and they emit light of a certain wavelength. The light is collected by the objective and directed towards the dichroic mirror, which can be passed by the emitted light. The light then passes suited filters that absorb or reflect unwanted radiation. A pinhole selects the light of the focal plane before the remaining parts of the light hit an avalanche photodiode. The intensity of the signal is recorded during a certain time with a temporal resolution of nanoseconds.

6 Performance of the FCS experiment

6.1 Calibration

For initialization of the instrument, a dye solution is put into one well of the microwell-plate. First, the position of the microwell-plate above the objective is determined using the menu "Carrier". The position of the confocal volume will be set to 200 μm above the bottom of the well. By choosing "Count rate" in the menu "Optimize" one can see the count rate of the fluroescence intensity. This value is supposed to be between 10 and 100 kHz. To determine the radius of the confocal volume ω_{xy} in Equation 23, the set-up will be calibrated using a fluorescent molecule with a known diffusion constant. For this purpose we will use the dye Alexa Fluor 633 (LifeTechnologies) with a diffusion constant

of 280 $\mu m^2/s$. Alexa Fluor 633 has an excitation maximum at 633 nm and an emission maximum at 647 nm. A HeNe-Laser with 633 nm is used for excitation. In Figure 9, left the raw data of a fluorescence signal are shown. The correlation curve $\tilde{G}(\tau)$, resulting from Equation 8, is shown in Figure 9, right. The fluorescence signal will be recorded for 10×20 seconds. The corresponding autocorrelation functions $\tilde{G}(\tau)$ will be fitted using Equations 8 and 32 with the free parameters τ_D and N. The characteristic diffusion time τ_D, the structure parameter S and the average of the number of particles N inside the confocal volume are determined. Using the relation $\tau_D = {\omega_{xy}}^2/4D$ one can determine the current radius of the confocal volume ω_{xy}. The effective focal volume V_{eff} can be calculated using $V_{eff} = \pi^{3/2}{\omega_{xy}}^2\omega z$.

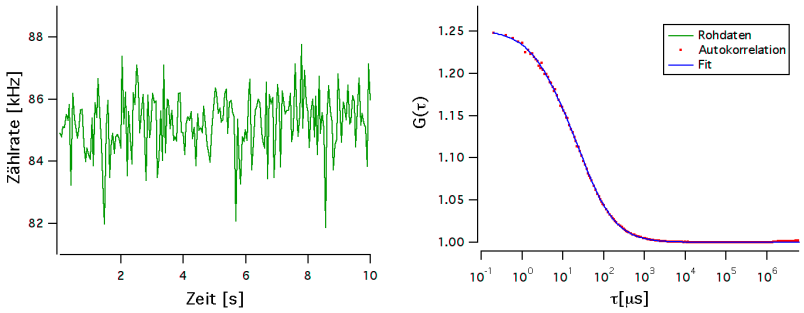

Figure 9: *Calibration of the FCS set-up using Alexa Fluor 633. left: raw data of the fluorescent signal of Alexa Fluor 633. right: autocorrelation function of the fluorecence signal fitted by Equations 8 and 32.*

6.2 Measurement

After calibration the actual measurement can be started. The sample will be diluted to a concentration of about 10 nM using a suited buffer and will be put in one well of the microwell-plate. The measurement will be performed using the original software form Zeiss. The measurements will be started from the menu "Optimize" and can be aborted there, if necessary. The laser power should not be changed because the shape of the confocal volume will be changed and one would have to calibrate the instrument again. It is easier to change the concentration of the sample than recalibrating the instrument. Normally one measurement needs 10×20 seconds.

6.3 Analysis of the Measurements

In the upper part of the window the raw data and the corresponding autocorrelation fuction can be seen. In the lower part one can see the results of the single measurements. In the last row the averages of the single measurements

are shown. In the right part of the window one can chose different functions of the analysis software. At the beginning, the program is set to "Correlation". Using the "Fit"-mode, one can define the fitting parameters. The structure parameter should be fixed to the value determined by the calibration measurement. On trial one can examine the effect of the triplet decay. After changing one parameter, all values need to be calculated again. This can be done by pressing the "Apply to all"-button. In the "Correlation" mode one can select one or a number of measurements and can print a summary of the data using "Preview". Please take care that "Selection" is chosen, otherwise all single measurements will be printed. Export the raw data of the average correlation curves to be able to reproduce the fitting in your report. In order to do this you have to click on "Export", rename the file to ".txt" and save it. One can import this file into a program like gnuplot, Igor or Origin and analyze and fit the data. The determined diffusion times should be converted into diffusion constants or the physical dimensions of the diffusing objects by the relations given in chapter 4. The results should be compared with the theoretical values in a diagram. For analysis the viscosity of water at room temperature can be used as an approximation.