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Single molecule detection by fluorescence and current blockade during translocation through a nanopore

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Single molecule detection by fluorescence and current blockade during translocation through a nanopore

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Abstract

A Zeiss Axiovert 200 microscope was modified for single molecule detection by fluorescence and current blockade as molecules translocate through a nanopore. Single molecule detection was achieved by fluorescence correlation spectroscopy. Detection with the nanopore incorporated will probably be achieved during 2005.

Keywords

Nanopore, single molecule detection, fluorescence correlation spectroscopy

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Single molecule detection by fluorescence and current blockade during translocation through a nanopore

Jonas Högström

Sammanfattning

Att kunna räkna stora biologiska molekyler när de åker genom ett väldigt litet hål, en nanopor, vore användbart inom biologin. I detta examensarbete beskrivs arbetet med att utforma en metod för att göra det. Molekylerna är elektriskt laddade, och kan därför drivas genom nanoporen av en elektrisk spänning som lagts över nanoporen, så att det går elektrisk ström genom den. När en stor molekyl åker genom nanoporen bromsas strömmen upp ett tag. Genom att mäta strömmen kan man detektera att en molekyl åker genom poren.

För att få en dubbel kontroll på att det var en molekyl som åkte genom nanoporen lyser en laserstråle på molekylen när den kommer ut på andra sidan. När molekylen träffas av lasern fluorescerar den, vilket betyder att den skickar ut ljus med en längre våglängd (annan färg) än lasern. Genom att sätta ett filter som bara släpper igenom den längre våglängden (och inte lasern) framför en ljusdetektor som mäter ljus där molekylen träffas av lasern kan man se när en molekyl åkte genom nanoporen.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala Universitet, januari 2005

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Introduction

The aim of this project is to detect single macromolecules both by fluorescence and by ionic current blockades as they go through a nanopore. The nanopore used in this study is a small hole, about 100 nm, in a silicone nitride (Si_3N_4) membrane supported on a 3 X 6 mm chip. The macromolecules to be detected will be fluorescently marked, and a laser beam will be focused to a very small volume (about 1 fL) on the exit side of the nanopore. As the molecules exit the nanopore, they enter the focused laser beam, and the fluorescence light is detected. An ionic current drives the fluorescently marked macromolecules through the pore. When a macromolecule occupies the pore, the ionic current is blocked and the current blockade is detected and measured. This way, the molecules are counted one by one as they go through the pore.

This instrument will provide a new method for analyzing single macromolecules, at very low concentrations. By measuring two different properties simultaneously – fluorescence and current blockade – the method makes it easier to tell the actual signal from noise. Another advantage with this method would be that the fluorescence signals should become consistent, i.e. the height and width of the peaks should be similar from time to time. This is because the molecules are always injected in the focused laser beam within the same microscopic volume. This is not the case in fluorescence correlation spectroscopy, which is based on random diffusion of molecules into varying portions of the illuminated volume.

Theory: Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) provides information by monitoring the fluctuations of the fluorescence from a small number of molecules in an open volume [1]. The open volume is typically about 1 fL [2]. This is very small compared to the larger closed volume of which it is a part (e.g. a droplet of water solution on a microscope objective), so that the closed volume can be considered to be infinitely large.

The small, open volume is defined by the overlap of the excited volume and the detected volume. A laser beam is focused to as small a spot as possible, which becomes the excitation volume. An image is projected from a plane in the large volume, and a light sensor with a small collecting area is placed in the plane of the projected image. The plane from which the image is projected and the small laser-illuminated spot are adjusted to overlap, and the light sensor is adjusted so that it is in the center of the projection of the laser-illuminated volume. When this is achieved, the laser illuminates the same volume as the light sensor detects. A filter is placed in front of the detector, to block any scattered laser light and allow only fluorescent emission through to the sensor.

The ideal case would be to have a clearly defined volume with even illumination and detection and no illumination or detection outside of that volume. In reality, however, the count rate from a fluorophore increases smoothly as it enters the open volume. For this reason an effective volume, which is considered to be ideal, can be calculated and used in further calculations [2][*]. For the purpose of this report, it is assumed that the ideal approximation of the open volume is correct.

When the large volume is at equilibrium, i.e. the flourophores are evenly distributed and the number of excited flourophores has reached its steady state, the fluorescence from the small, open volume will fluctuate around its equilibrium emission. These fluctuations are due to molecules diffusing in and out of the open volume and molecules transforming between fluorescent and nonfluorescent states. With more complex systems where molecules also react with each other or enter triplet states, other molecular events may also affect the fluctuations. By correlating the signal at time t with the signal at time $t + \tau$ and plotting the correlation as a function of τ, information about these events can be extracted. This is called the correlation function. When the time scales for the different types of events are different, they can be separated by looking at the correlation for different τ values [1], (appendix 1).

[*] [2] is only used for background information.

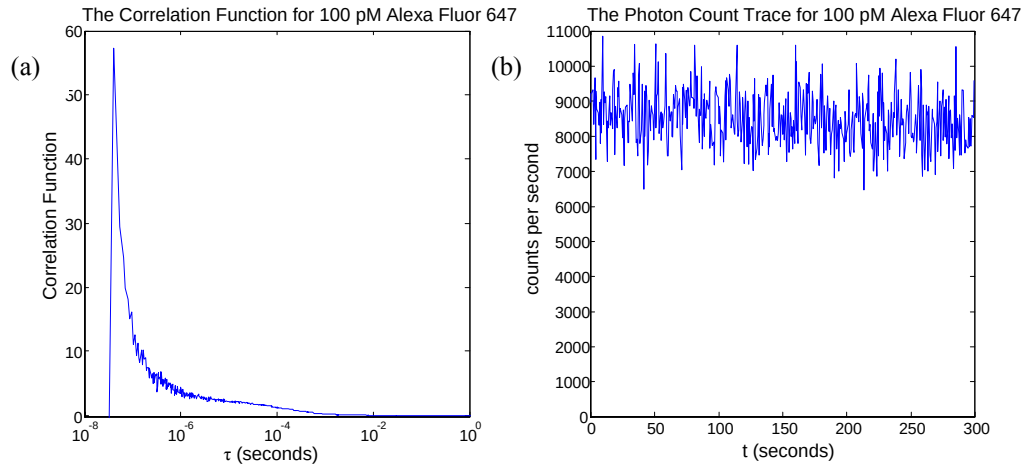

Fig. 1. The correlation function (a) describes the correlation of the photon count trace (b) at time t and t + τ. The plateau between $\tau = 10^{-4}$ s and $\tau = 10^{-6}$ s shows the correlation that is due to individual molecules diffusing in and out of the open volume. The average number of molecules in the open volume is about .5, the inverse of the height of the plateau.

If one follows the correlation function from right to left, the first increase above the base line (i.e. the longest τ at which a correlation between the signal at t and t + τ appears) should be due to the diffusion of individual molecules in and out of the small, open volume. The plateau that extends from this point toward smaller τ values yields information about the average number of molecules in the open volume [1]. The height of the plateau is inversely proportional to the average number of molecules in the open volume, with proportionality constant = 1. See fig. 1 for an example and appendix 1 for a more detailed mathematical description of the correlation function. Equation (11) in appendix 1 is the actual correlation function used throughout this report.

If the concentration of fluorophores is low enough, fluorophore molecules do not interact with each other, and the times between molecules' entering as well as exiting the open volume can be well approximated by Poisson distributions [8]. This makes it possible to perform statistical analysis of the correlation function to get the number of molecules in the open volume (appendix 1).

The experimental setup

Fig. 2. Overview of the experimental setup. See text for explanation.

The parenthesized numbers in the text refer to the numbers in fig. 2, 3 and 4. The setup is built on a Zeiss Axiovert 200 microscope, placed on an air-dampened table. The excitation light from the 633 nm or 488 nm laser (1) is routed to the rear right optical inlet of the microscope via a periscope (2) and a telescope (3). The periscope is used to adjust the beam so that it enters the microscope at the right spot and at the right angle. The telescope is used to compensate for some lenses in the microscope light inlet designed to direct light from of a lamp. With the telescope properly adjusted, the laser can be focused directly onto the surface of the pore chip. The power of the focused laser is about 150 µW for both lasers.

After entering the microscope, the laser beam passes through a filter that is designed to filter out other light than the laser light (4). It then is reflected from a dichroic mirror (4) at a 45° angle before being focused onto the pore. The fluorescence light from the focal point of the laser exits the same way the laser came in, but passes through the dichroic mirror and exits through an emission filter (4), which filters out laser excitation light. The dichroic mirror is designed to reflect as much of the laser light as possible, and let as much of the fluorescent light as possible through. The filter and mirror set XF45 from Omega Optics was used with the 633 nanometer laser, and XF25 was used with the 488 nm laser.

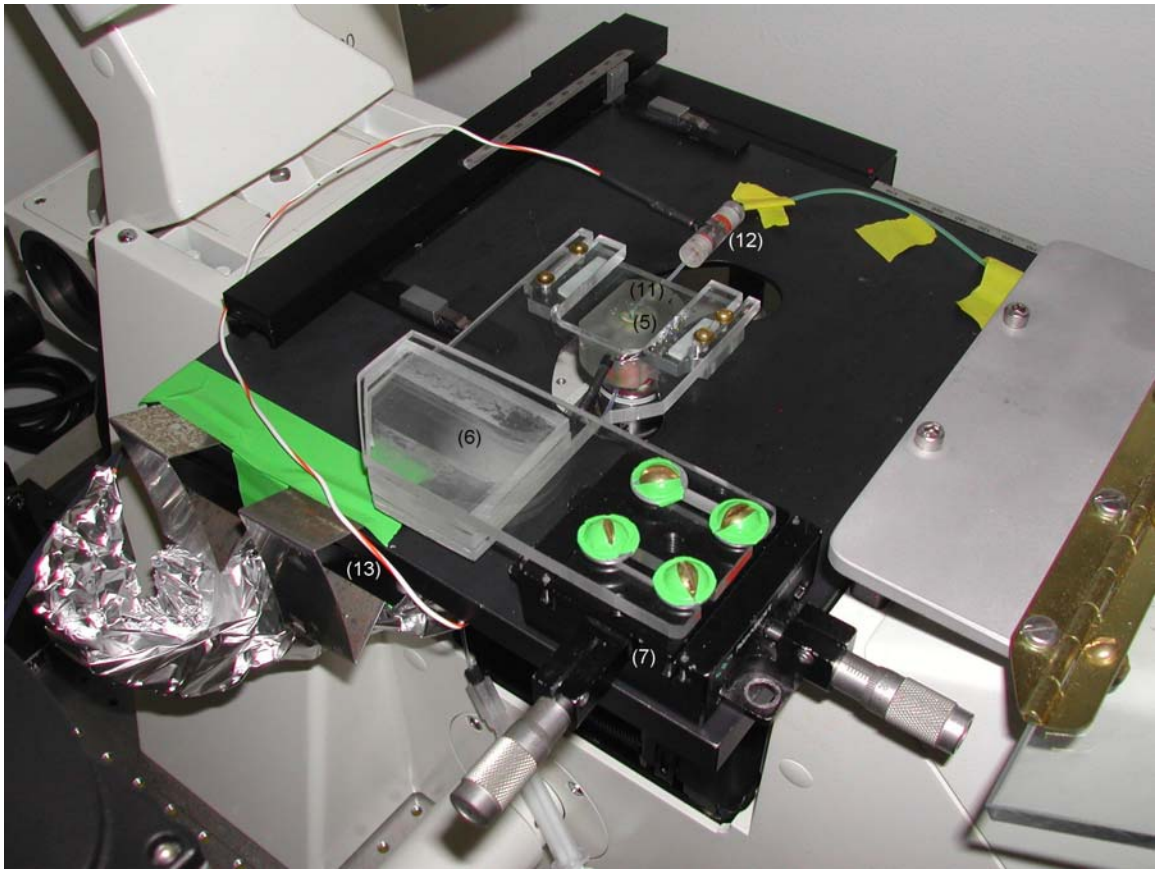

Fig. 3. The nanopore holder on the microscope. See text for explanation.

The Si_3N_4 pore chip with the 100 nm pore (5) is held in place by a plastic pore holder (6). Vacuum grease provides both adhesion and electrical insulation between the pore and the holder. The holder, in turn, is attached to a micromanipulator (7), which allows for the fine adjustments necessary to center the pore over the focused laser beam.

At the front left optical outlet of the microscope, there is a sliding mirror (8) for directing the light either to an optical fiber (9) that leads to an Avalanche Photon Detector, APD, or to a digital camera (10). The APD is used to detect photons under actual experiments, and the camera allows examination of the pore while focusing on the pore surface and centering the pore over the laser beam. The eyepieces cannot be used for viewing the focused laser beam on the pore surface, since it is too bright for the eye.

The pore holder also has a channel (11) for the electrolyte, which is used to carry a current through the pore. An electrode holder (12) is connected with plastic tubing to a syringe for loading in the one end, and to the electrolyte channel in the holder in the other end. A cable connects the electrode to the current generation and measurement probe (13), an Axon Instruments Axopatch 200B.

Fig. 4. The lower electrode on the objective. See text for explanation.

The lower electrode holder (14) is placed on top of the objective. It has a channel for the electrolyte, which passes by the electrode and then continues up to the objective, where the electrolyte comes into contact with the lower side of the pore. Plastic tubing leads into the lower side of this channel, which is connected to a syringe with electrolyte. The electrode is connected by a wire to the current generation and measurement probe (13).

In order to minimize electromagnetic interference, all radiating devices are placed as far away from the microscope as possible. Some initial efforts to shield it with aluminum foil have been done. The foil was wrapped around cables and larger sheets were placed around the setup of electrodes and the pore. These sheets were grounded.

Results and discussion

Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) with a reference dye was used to determine the effective open volume of the FCS setup. It was also performed to fine tune the detector before each actual experiment as well as to confirm that the laser and fluorescence detection were still aligned after each experiment.

Alexafluor 647 was used as the reference dye with the 633 nm HeNe laser. Before each experiment, the optical fiber was fine tuned by adjusting its position in the plane perpendicular to the light entering it. Initial experiments showed that maximizing the counts also maximized the height of the correlation function's first plateau above the baseline and minimized the τ value for which this plateau began, as seen in the correlation function viewed from right to left.

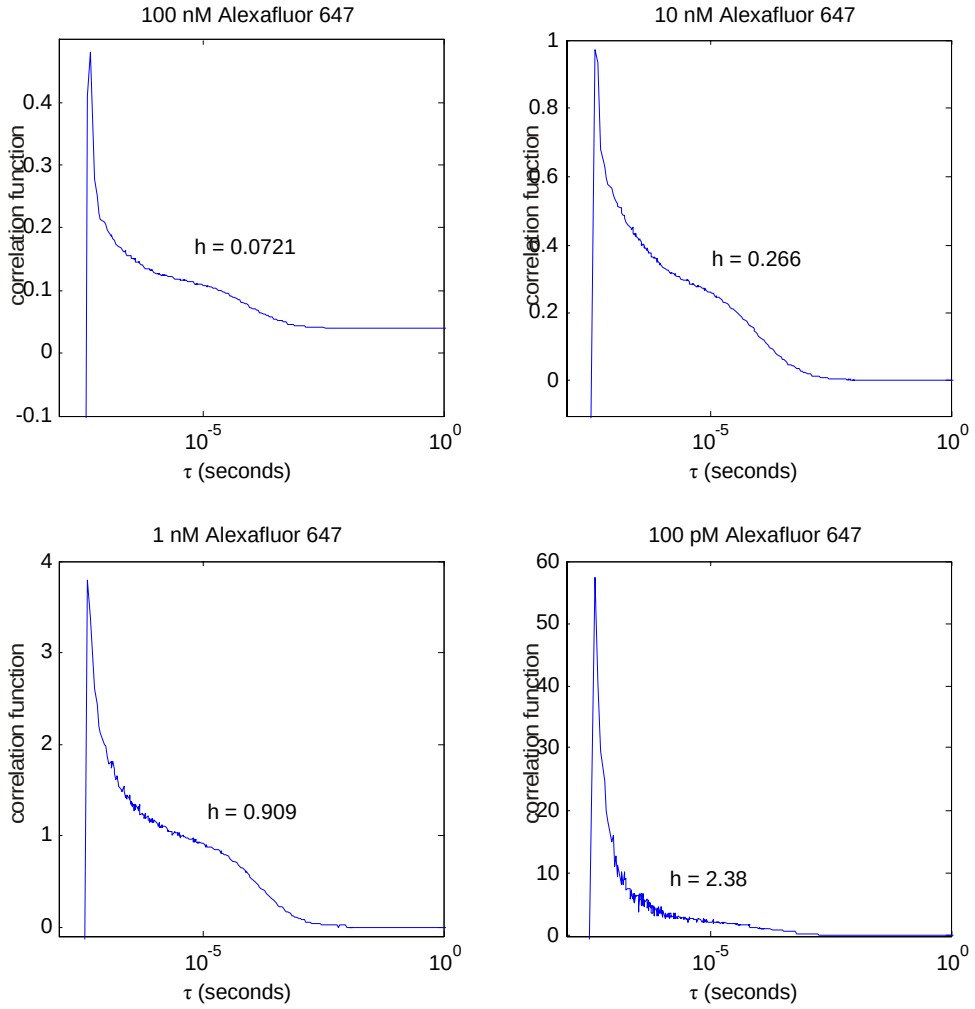

Fig. 5. The height of the plateau for τ values of 10^{-5} s is inversely proportional to the number of molecules in the open volume (with proportionality constant 1).

FCS experiments were carried out with four different concentrations of Alexafluor 647, to determine the open volume of the system. The graphs (fig. 5) show that the height of the correlation function at $\tau = 10^{-5}$ s appears to be a good representation of the height of the first plateau from the right. As discussed earlier, this height represents the correlation due to diffusion in and out of the open volume. In the top left plot, the level of the base line needs to be subtracted to get the correct height (appendix 1).

If the open volume v was constant for different dye concentrations, the number of molecules in the open volume n_v would be proportional to the concentration c. n_v is proportional to $1/h$, where h is the correlation function height. Thus, c would have been proportional to 1/h with a constant volume. This was, however, not the case. To test for a potential exponential relationship, the logarithms of c and $1/h$ were calculated and the latter plotted as a function of the former (fig. 6).

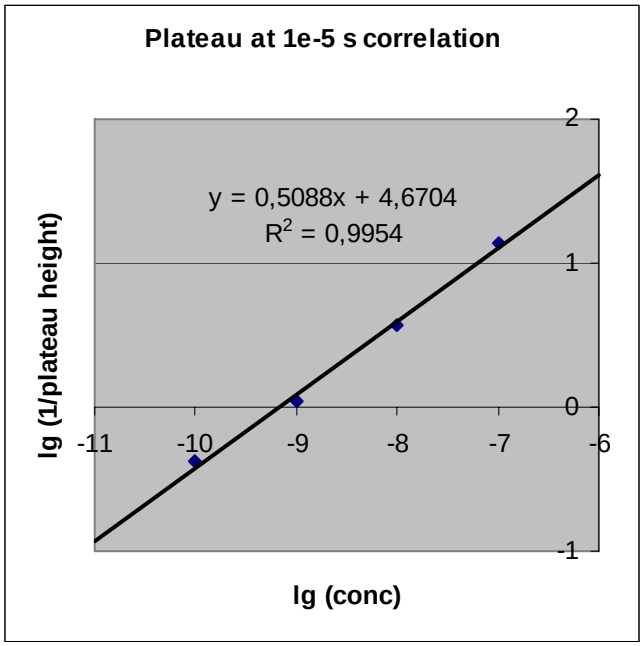

Fig. 6. The number of detected molecules increases as the square root of the increase in concentration. Thus, the open volume decreases as the molecule concentration increases.

It appears that the open volume was inversely proportional to the square root of the concentration of the dye. Hence, when the concentration increases, the open volume decreases. This could be in part due to less quenching at lower concentrations, as well as a less obstructed light path. However, the size of the open volume is not likely to be of any greater importance when incorporating a nanopore, since there would be almost no fluorescent molecules on the exiting side (where the laser is focused), and all molecules would be injected to the same place in the open volume.

Detecting single particles

When using concentrations low enough for the open volume to be occupied by less than one molecule on average, the actual entering and exiting of individual molecules

should be visible in the trace of the signal. Experiments done with low concentrations of Alexafluor 647 and fluorescent microspheres indeed showed that this is the case (fig. 7).

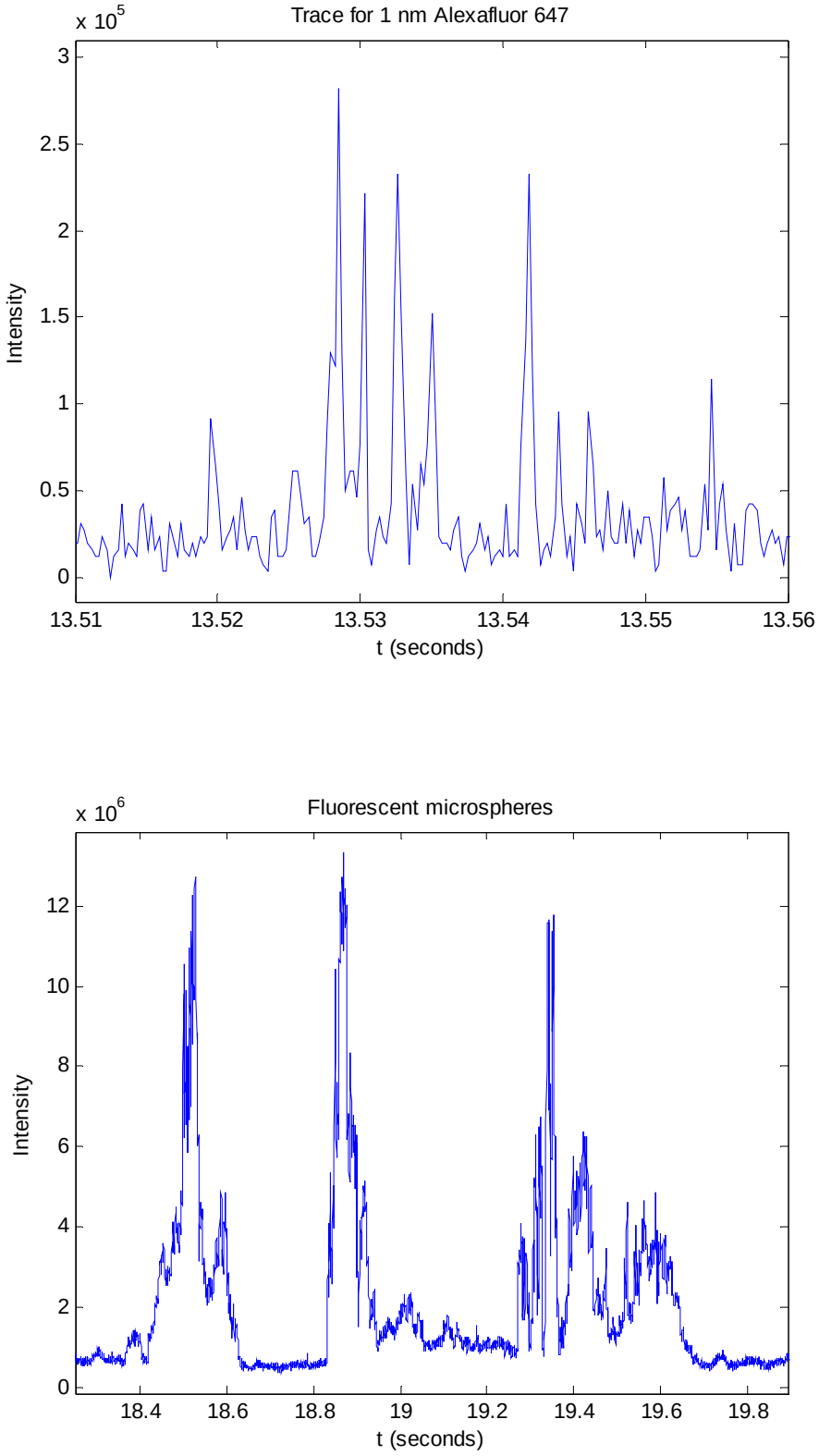

Fig. 7. Very low concentrations of dye molecules (Alexa Fluor 647) and microspheres allows for single molecules/particles to be detected as they are in the open volume.

Some fluorescent microspheres of unknown size and concentration were available in the lab, but even so they served their purpose. After sonicating and diluting them to a very low concentration, the trace showed large bursts in the signal approximately once a second. These bursts were about 0.1 s wide, which means that it took one sphere about a tenth of a second to diffuse through the open volume. Of course this time varies, depending on the sphere's path. Passing trough the perimeter of the open volume would produce a smaller and narrower burst than passing through the center of the same.

With the microspheres, aggregation is a possibility. Therefore, the large peaks for the fluorescent microspheres in fig. 7 could in fact be due to aggregates. Whether this is the case or not is of lesser importance, since the instrument is sensitive enough to detect individual dye molecules (fig 7).

These two experiments suggest strongly that individual particles are detected, both single molecules and large spheres with many fluorescent molecules on them.

Noise from the nanopore

The next step, after detecting single molecules with FCS, was to incorporate the 100 nm Si_3N_4 nanopore in the setup. Because focusing the laser beam on the exiting side of the nanopore chip also means focusing it on the chip itself, the intensity of the reflected light could be high enough to cause significant noise, even though a filter was installed. The pore somehow fluorescing could also be a source of noise. It should be mentioned that the pore is much smaller than the area to which the laser can be focused, and hence aiming in the center of the pore to avoid the scattered light noise does not work.

After aligning the system, the pore was placed on top and brought into focus. In order to check that the laser was indeed focused on the pore, the camera connected to the microscope was employed. At this point, the emission filter that blocks the laser from reaching the detector (and hence the camera when it was used) was not installed. As mentioned, the reason for using the camera instead of the oculars is that the laser is too intense to safely be viewed by the eye.

After reinstalling the emission filter, the APD detector was turned on and a measurement was made. Considerable noise was present, and detection of single Alexafluor 647 molecules would not have been possible. Installing a second emission filter decreased the noise to an acceptable level. FCS with Alexafluor 647 was then possible, even though the signal was slightly lower with double filters installed.

To see if the noise was due to any special characteristics of the nanopore chip, a piece of glass was mounted in place of the nanopore chip and another measurement was made. The results were very similar to those obtained with the nanopore chip. Hence, the noise was probably due to reflected light, and not to special characteristics of the Si_3N_4 nanopore. This is good, since reflected excitation light is easier to filter out than fluorescence light with a possible overlap with the fluorescence from the molecule to be detected.

Evaluation of macromolecules

After evaluating liposomes and DNA, ribosomes were chosen as a target to fluorescently mark and use with the pore. To-Pro-3 from Molecular Probes was evaluated as a marker molecule. However, it did not work very well. The dye probably was lost from the ribosomes after they were stained and separated from any remaining free dye. This caused too high noise levels for satisfactory single molecule detection.

A problem with ribosomes is that they contain RNA. This adds the risk of getting RNase contamination and ribosome breakdown. Therefore, liposomes with encapsulated dye seem to be the test molecules of choice. There are several different kinds of liposomes available, and any dye can be encapsulated. Alexafluor 647 will be the first dye to be encapsulated, since it has good fluorescent properties.

Switching to the 488 nm diode laser widens the choices of dyes significantly. This will be very helpful, since finding a dye that will stain the molecule to be detected without leaking is crucial for this method to work once other molecules than liposomes are to be detected.

Ongoing work

To allow broader choice of dyes, a blue Protera Novalux 488 nm laser, generating 5 mW of power, was installed, together with an XF 25 filter set from Omega Optics. After aligning the laser and detector, a good correlation function could not be achieved. It was noisy and showed practically no correlation for any τ value. Several readjustments were made for weeks, and the red 688 nm HeNe laser was temporarily reinstalled to ensure that it could still be aligned, but the 488 nm laser still presented problems.

The noise level with a nonfluorescent sample was as low as with the red laser, and the count rates with the reference dye were even slightly higher. Note that the reference dye for the red laser was Alexa 647, and that fluorescein was used with the blue laser. The reference concentration was the same, 10^{-7} M.

Peter Goodwin at the Los Alamos National Laboratory [4] suggested that the problem with aligning the system with the blue laser might be due to the laser having different modes, i.e. the laser light is in fact not completely collimated. If that is the case, it can easily be fixed with a pinhole lens and a telescope [4], but time has not allowed for that yet.

Work on this project will continue in February, 2005. I am still optimistic about being able to reach the goal of efficient single molecule detection. The next big issues to be addressed are getting the 488 nm laser collimated, and isolating the setup from electromagnetic radiation.

After collimating the 488 nm laser, evaluation of many new dyes will be possible, as well as single molecule detection as they exit the nanopore.

Acknowledgements

First of all, I want to thank Professor David W. Deamer at UCSC for supervising this project, giving me feedback on the report and letting me work in his splendid group. I also want to thank everybody in the Deamer 'Origin of Life' and 'Nanopore' groups for their support and ideas. A special thanks for the help on aligning the laser for FCS to Peter Goodwin, without whose help this project would not have gone at all as far as it did. To Reay Dick for the help with constructing electrodes and setting up the software for the current measurements. To Breton Veach for helping me to start things up during the first weeks.

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Appendix 1

Mathematics of the auto correlation function

The auto correlation function is defined as:

$$G(\tau) = \frac{1}{T} \cdot \int_0^{T-\tau} I(t) \cdot I(t+\tau) \cdot dt \qquad (1)$$

The mean over time is denoted by brackets; $\langle a \rangle$ means the time average of a. τ is considered small enough compared to T for the approximation T- $\tau \approx T$ to be accurate.

$$G(\tau) = \langle I(t) \cdot I(t+\tau) \rangle \qquad (2)$$

$\delta I(t)$ is defined as the fluctuation from the average.

$$I(t) = \langle I \rangle + \delta I(t) \qquad (3)$$

$$G(\tau) = \langle (\langle I \rangle + \delta I(t)) \cdot (\langle I \rangle \delta I(t+\tau)) \rangle \qquad (4)$$

$$G(\tau) = \langle \langle I \rangle^2 + \delta I(t) \cdot \delta I(t+\tau) + \langle I \rangle \cdot \delta I(t) + \langle I \rangle \cdot \delta I(t+\tau) \rangle \qquad (5)$$

$$G(\tau) = \langle I \rangle^2 + \langle \delta I(t) \cdot \delta I(t+\tau) \rangle + \langle \langle I \rangle \cdot \delta I(t) \rangle + \langle \langle I \rangle \cdot \delta I(t+\tau) \rangle \qquad (6)$$

The time average of the fluctuations from the same average must be equal to zero.

$$\langle \langle I \rangle \cdot \delta I(t) \rangle = 0 \quad , \quad \langle \langle I \rangle \cdot \delta I(t+\tau) \rangle = 0 \qquad (7, 8)$$

$$G(\tau) = \langle I \rangle^2 + \langle \delta I(t) \cdot \delta I(t+\tau) \rangle \qquad (9)$$

Normalization with $\langle I \rangle^2$.

$$G(\tau)_{norm} = 1 + \frac{\langle \delta I(t) \cdot \delta I(t+\tau) \rangle}{\langle I \rangle^2} \qquad (10)$$

Subtract 1 to make statistical analyses easier.

$$g(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t+\tau) \rangle}{\langle I \rangle^2} \qquad (11)$$

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Since the fluctuations depend on the entering and exiting of an open volume by individual molecules that are considered independent, it depends on events that are Poisson distributed [8]. This allows for statistical analyses of the auto correlation function.

The number of molecules in the open volume, *n*, fluctuates around its average, $\langle n \rangle$. The signal strength, *I*, is proportional to *n*, with proportionality constant α:

$$I = \alpha \cdot n \tag{12}$$

The variance for a Poisson distribution is the same as its expectation value [8], which in this case is $\langle n \rangle$. Let τ go towards zero in (11):

$$\lim_{\tau \to 0} \left(\frac{\langle \delta I(t) \cdot \delta I(t+\tau) \rangle}{\langle I \rangle^2} \right) = \frac{\langle \delta I(t)^2 \rangle}{\langle I \rangle^2} \tag{13}$$

(12) + (13)

$$\frac{\langle \delta I(t)^2 \rangle}{\langle I \rangle^2} = \frac{\alpha^2}{\alpha^2} \cdot \frac{\langle \delta n(t)^2 \rangle}{\langle n \rangle^2} \tag{14}$$

$\langle \delta n(t)^2 \rangle$ is the variance of *n*, and thus its expectation value, $\langle n \rangle$ [8].

$$\langle \delta n(t)^2 \rangle = \langle n \rangle \tag{15}$$

(14) + (15)

$$\frac{\langle \delta I(t)^2 \rangle}{\langle I \rangle^2} = \frac{1}{\langle n \rangle} \tag{16}$$

(11) + (13) + (16)

$$\lim_{\tau \to 0} \big(g(\tau) \big) = \frac{1}{\langle n \rangle} \tag{17}$$

When plotting the correlation function for a signal that is only dependent on how many molecules there are in the open volume, $g(\tau)$ has approximately the same value for all values of τ that are significantly smaller than the time scale of the molecules' diffusion in and out of the volume. Therefore, the value of $g(\tau = 0)$ can be obtained by looking at a different $g(\tau)$ for a small enough τ value.

When additional events with different time scales affect the signal, for example molecules entering and exiting triplet states, one can look at the plateau for the event of interest. Any events with faster time scales than the interesting event will cause

plateaus only for τ values that are even smaller than the time scale for those events. When this happens, one simply looks at the plateau for τ values that are smaller than the time scale of the interesting event, but larger than the time scale of the other event. Events with a longer time scale than the interesting event will case plateaus that begin for larger τ values than the plateau of the interesting event. In this case, one simply considers the height of the plateau for τ values that are larger than the time scale of the interesting events as the base line. In other words, the height of this plateau is subtracted from the correlation function. In these ways, events with different time scales can be separated.

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Appendix 2

Computer routines for data extracting and plotting

Matlab from The MathWorks Inc. was used to extract data from the correlation software and to do some plots. Since this is merely a way to overcome the correlation software's weaknesses in exporting data, the code is appended as a general reference only, and is not referred to specifically in the text when used. The extraction files are specifically designed to work on the computer where they were used, with the correlation data files being placed in a specific folder structure.

get_trace.m, extracts the detailed trace from the detailed trace file

```
function [dtrace namedate] = get_trace(file, date) %returns a matrix
%with the trace function when called with file name and date of
%creation

% Author: Jonas Högström
% Uppsala University: Molecular Biotechnology Engineering
% University of California Santa Cruz: Chemistry, Prof. David W.
% Deamer's group

deltat= 262.144e-6; %the sample time from the correlator

pat(1:77) = 'C:\Documents and Settings\Peter\Skrivbord\Jonas
exjobb\Mikroskoprummet\Jonas\';
pat(78:83) = date;
addpath(pat); %%%opens the specified path to look for the file
dtrace(:,2)=importdata(file); %read the file and return in trace
for i=1:1:size(dtrace,1)
    dtrace(i,1)=deltat*(i-1);
end
namedate{1}= date;
namedate{2}= file;

plot(dtrace(:,1),dtrace(:,2)/deltat);
title([namedate{1},' - ',namedate{2}]);
xlabel('seconds');
ylabel('trace');
```

get_corr.m, extracts the correlation function from the correlation function file

```
function [corr namedate] = get_corr(file, date) %returns a matrix
%with the correlation function when called with file name and date of
%creation

% Author: Jonas Högström
% Uppsala University: Molecular Biotechnology Engineering
% University of California Santa Cruz: Chemistry, Prof. David W.
% Deamer's group

m = 16; %number of header lines to skip before reading data
p = 1088; %no of rows to read to obtain correlation function data
xmin = 1e-8;
xmax = 1;
pat(1:77) = 'C:\Documents and Settings\Peter\Skrivbord\Jonas
exjobb\Mikroskoprummet\Jonas\';
pat(78:83) = date;
addpath(pat); %%%opens the specified path to look for the file

fid = fopen(file); %opens the specified file

C = textscan(fid,'%f','headerLines',m); %reads away m header lines,
%then reads data

for i=1:p %put the shuffled data in an ordered matrix and subtract 1
from y to get the true correlation function
    x(i)=C{1}(2*i-1);
    y(i)=C{1}(2*i)-1;
end

fclose(fid); %close the file
rmpath(pat); %remove the path so that it is not accidently looked in
%later
corr = [x',y']; %return the correlation function
namedate{1}=date;
namedate{2}=file; %%return the info about the file name and date

semilogx(corr(:,1),corr(:,2)); %plot the correlation function
title([namedate{1},' - ',namedate{2}]);
xlabel('seconds');
ylabel('correlation function');
V=axis;
axis([xmin,xmax,V(3:4)]); %change the x axle range
```

get_corr_trace.m, extracts the correlation function and the rough trace from the correlation function file

```
function [corr trace namedate] = get_corr_trace(file, date) %returns
%a matrix with the correlation function when called with file name
%and date of creation

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% Deamer's group

m = 16; %number of header lines to skip before reading correlation
%data
```

```
p = 1088; %no of rows to read to obtain correlation function data
q = 1093; %no of header lines to skip before reading trace data

pucko = 1024;
deltat = 262.144e-6;

xmin = 1e-8;
xmax = 1;
ymin = -.1;
pat(1:77) = 'C:\Documents and Settings\Peter\Skrivbord\Jonas
exjobb\Mikroskoprummet\Jonas\';
pat(78:83) = date;
addpath(pat); %%%opens the specified path to look for the file

fid = fopen(file); %opens the specified file

C = textscan(fid,'%f','headerLines',m); %reads away m header lines,
%then reads correlation data
D = textscan(fid,'%f','headerLines',q); %reads away q header lines,
%then reads trace data

for i=1:p %put the shuffled data in an ordered matrix and subtract 1
from y to get the true correlation function
    x(i)=C{1}(2*i-1); % -1 here is to get the right index
    y(i)=C{1}(2*i)-1; % -1 here is to get the true correlation
%function
end

for i=1:size(D{1},1)/3 %put the shuffled data in an ordered matrix
    trace(i,1) = D{1}(3*i-2);
    trace(i,2) = D{1}(3*i-1);
    trace(i,3) = D{1}(3*i);
end

fclose(fid); %close the file
rmpath(pat); %remove the path so that it is not accidently looked in
%later
corr = [x',y']; %return the correlation function
namedate{1}=date;
namedate{2}=file; %%return the info about the file name and date

subplot(1,2,1);
semilogx(corr(:,1),corr(:,2)); %plot the correlation function
title([namedate{1},' - ',namedate{2}]);
xlabel('seconds');
ylabel('correlation function');
V=axis;
axis([xmin,xmax,ymin,V(4)]); %change the x axle range



subplot(1,2,2);
plot(trace(:,1),trace(:,2)/pucko/deltat); %plot the trace
title([namedate{1},' - ',namedate{2}]);
xlabel('seconds');
ylabel('counts per second');
```

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