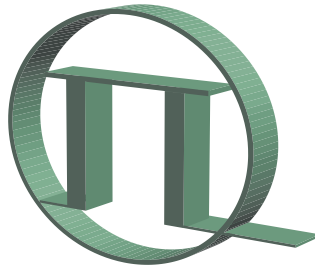


**5th International Workshop on
Single Molecule Detection
and Ultrasensitive Analysis
in Life Sciences**

Organized by
PicoQuant GmbH



PICOQUANT GmbH
Unternehmen für optoelektronische
Forschung und Entwicklung

Technical Program

29. September - 01. October 1998
at WISTA Campus
Berlin-Adlershof (Germany)

Aims and Purpose

The recent years have seen an ever increasing interest in the detection and spectroscopy of single molecules. Especially in genetic and biochemical screening and assaying, single molecule detection (SMD) under biologically native conditions became an important issue. The experimental method of choice in SMD is laser-induced fluorescence detection either on surfaces or SMD of freely moving molecules in liquids. In the first case, scanning techniques as Near-Field Scanning Microscopy or Confocal Scanning Microscopy, are the dominating techniques, although interesting results are emerging from the application of ultra-sensitive CCD camera systems. In the second case, the detection of single molecules in microdroplets, in flowing streams, and in (sub) picoliter detection volumes are the main approaches.

One of the most challenging topics in SMD is the application in fast DNA sequencing. There, the use of time-resolved techniques promises very compact systems capable of identifying the four nucleotides in a DNA-sequence according to their fluorescence lifetimes and spectra.

Already now, first applications have been reported from industry research groups concerning the use of SMD with picosecond diode laser systems. In the same context, two photon SMD has been reported using blue and green absorbing dyes as marker molecules. Similar techniques may prove very useful when incorporated into miniaturized systems for capillary electrophoresis, high density micro plate screening etc.

In the last couple of years, the annual Workshop on SMD, organized by PicoQuant in Berlin, has proven to be an excellent forum for the discussion of the topics mentioned above. The main goal is to give an overview of most recent results in the field and to stimulate new research and industrial applications. PicoQuant GmbH wants to encourage especially young scientists in SMD research. Therefore a special prize of 1500 DM is donated by the company for the "Students SMD Award 1999".

Venue

PicoQuant GmbH Berlin: Rudower Chaussee 29 (IGZ)
OWZ Building, Room 466
D-12489 Berlin

General Organization: Rainer Erdmann
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Registration office: WISTA Conference Center
Building 12.1
Einstein Kabinett
Wednesday 11.30 a.m. - 1.p.m.

Hotel address:
Transhotel Radickestr. 76
(across the street Adlergestell)
D-12489 Berlin
phone: +49-30-67095-0
fax: +49-30-67095-222

Hints for Manuscript Preparation

All contributions to the Workshop will be considered for publication (peer reviewed) in a special issue of the journal **Cytometry** (Wiley-Liss, they took over Bioimaging last year; Bioimaging has now it's own section edited by Prof. I. T. Young, Delft University).

Papers are supposed to have a length not exceeding 8 pages. The manuscripts should be prepared according to the Cytometry Instructions to Contributors, available during the workshop or at:

<http://www.interscience.wiley.com>

Dead-line for submission of the manuscripts will be **November 01, 1999**. Please inform the organizing committee if you plan to submit your paper.

How to get to PicoQuant GmbH

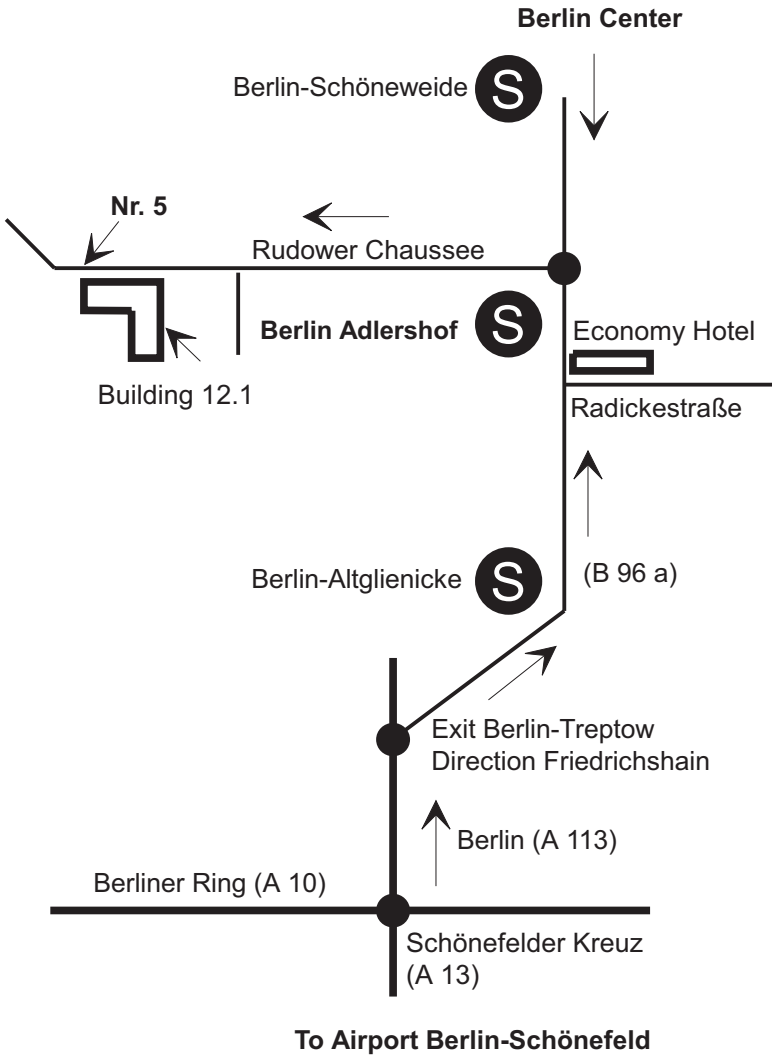
The workshop location is about 12 min walking distance from the S-Bahn station **Adlershof**. Please go through the railway bridge and follow the Rudower Chaussee on the left hand side until you see the workshop signs. Then enter the building 12.1 at the WISTA campus and follow the signs.

from Airport/Railway station Berlin-Schönefeld:
take S-Bahn **S9** or **S45**, go three stops to **Adlershof**.
Ticket: Short-distance ticket (valid 3 stations, 2.50 DM)

from Railway station Zoologischer Garten:
take the S-Bahn into one of the following directions:
S9 or **S45** to Flughafen Berlin-Schönefeld
S8 to Grünau
S6 to Zeuthen or
S46 to Königs-Wusterhausen
and go to **Adlershof**.
Ticket: Single ticket, fare area AB (valid 2 hours , 3.90DM)

from Airport Berlin-Tegel:
take the Express Bus number **X9** to the railway station Zoologischer Garten and follow the description given above.

Workshop Location



Program (subject to alterations)

Wednesday, 29 September 1999

11.30 - 13.00 Registration

13.15 - 13.40 R. Erdmann, Berlin

Opening Remarks and Presentation of "SMD Students Award"

13.45 - 14.20 N. van Hulst, Enschede, **(Invited Paper)**

Near-field Optical Photodynamic Studies of Single Molecules & Proteins

14.20 - 14.45 B. Schäfer, Jena

Relaxation of Individual DNA Molecules Described by a Harmonic Oscillator Model

14.45 - 15.10 F. van Veggel, Enschede

Functional Self-Assembled Monolayers of β -cyclodextrines on Gold: Single Host-Guest Interactions probed by AFM

15.10 - 15.35 B. Hecht, Zürich

Direct Measurement of the Longitudinal Electric Field Component in a Focus using Single Molecules

15.35 - 16.10 **COFFEE BREAK**

16.10 - 16.45 Th. Schmidt, Leiden **(Invited Paper)**

Single Molecule Microscopy on Biomembranes

16.45 - 17.10 G. Schütz, Linz

Diffusion of Single Lipid Molecules on Cell Membranes

17.10 - 17.35 C. Tietz, Chemnitz

Single Light Harvesting C(LHC-II) of Higher Plants

17.45 - 18.10 n.n.

18.15 - 21.00 **RECEPTION**

Thursday, 30 September 1999

09.00 - 09.35 R. Rigler, Stockholm (**Invited paper**)
Non Markovian Processes in Enzyme Catalysis at the Single Molecule Level

09.35 - 10.00 C. Begon, Marseille
Spontaneous Emission Control and Detection of Biological Markers

10.00 - 10.25 M. Hattori, Tsukuba
Fluorescence Correlation Spectroscopy with Traveling Interference Fringe Excitation: Introduction of SMD Experimental Scheme

10.25 - 11.00 **COFFEE BREAK**

11.00 - 11.35 S. Soper, Baton Rouge (**Invited paper**)
Single Molecule Analysis in Genomics using Near-IR Fluorescence Detection and Microfluidic Devices

11.35 - 12.00 J. Enderlein, Regensburg
Tracking of Diffusing Molecules within Membranes

12.00 - 12.35 M. Sauer, Heidelberg
Single Molecule Detection and Identification in Submicrometer Channels: State of the Art and Future Prospects

12.35 - 13.00 M. Weber, Siegen
Triplet-state Lifetime of Single Molecules

13.00 - 14.00 **LUNCH**

14.15 - 17.30 **POSTER SESSION and PRODUCT PRESENTATION**

19.00 **DINNER**

Friday, 01. October 1999

09.00 - 09.35 N. Dovici, Edmonton (**Invited Paper**)
The Chemistry of Single Enzyme Molecules

09.35 - 10.00 M. Bennick, Enschede
Single DNA Molecule Approach to Homologous Recombination

10.00 - 10.25 V. Uhl, Jena
Microscopic Observation of Single Molecule Enzyme Kinetics in Femtoliter Droplets

10.25 - 11.00 **COFFEE BREAK**

11.00 - 11.35 M. Auer, Wien (**Invited Paper**)
Interfacing Novel Detection Technologies based on Single Molecule Fluorescence Spectroscopy with miniaturised High Throughput Screening in the Nanoliter Format.

11.35 - 12.00 A. Castro, Los Alamos
Detection of Specific Sequences in Individual Double-Stranded DNA Fragments

12.00 - 12.25 J. Schaffer, Göttingen
Multi-dimensional Site-specific Fluorescence Spectroscopy of Single DNA-Molecules in Solution

12.25 - 12.50 T. Ruckstuhl, Regensburg
Forbidden Light Detection from Single Molecules

12.50 - 13.50 **LUNCH**

14.00 - 14.35 C. Seidel, Göttingen (**Invited Paper**)
Applications of BIFL for Multi-dimensional Single Molecule Spectroscopy

14.35 - 15.00 P. Tinnefeld, Heidelberg
Characterization of Single Molecules by Time-resolved Fluorescence Scanning

15.00 - 15.25 M. Maus, Herverle
Intramolecular Excimer-like and Monomer Fluorescence of Multichromophoric Dendrimers Studied by Time- and Spectrally-Resolved Spectroscopy: From the Ensemble to the Single Molecule Behaviour

15.25 - 15.50 n.n.

15.50 - 16.00 S. Soper, Baton Rouge
Concluding Remarks

16.00 - 16.30 COFFEE BREAK

Abstracts

as received by the authors
(mostly in alphabetical order)

Papers not shown in the program will be presented
during the poster session.

The maximum size for a poster is 0,94 m width x 1,22 m height

Interfacing Novel Detection Technologies based on Single Molecule Fluorescence Spectroscopy with miniaturised High Throughput Screening in the Nanoliter Format.

Manfred Auer¹, Kurt A. Stoeckli², Werner Thumb², Karsten Gall³, Jörn Jungmann³, Stefan Jäger³, Leif Brand³, and Peet Kask³

¹ Novartis Forschungsinstitut, NFI, Vienna, Austria

² Novartis Pharma AG, Basel, Switzerland

³ EVOTEC Biosystems AG, Hamburg, Germany

Miniaturization is one of the key technology concepts of current high-throughput screening to meet the future needs of fast and cost-effective drug discovery. Confocal fluorescence techniques are amongst the most suitable methods enabling mechanism-based screening on miniaturized assay formats. In particular, fluorescence correlation spectroscopy (FCS) has already been applied to a high number of assays monitoring molecular interactions where either directly or indirectly a ~ ten-fold change in molecular weight takes place. In a powerful miniaturized HTS environment, however, it is necessary to exploit additional possible single molecule fluorescence detection possibilities. Of essential importance is to resolve populations of molecules based on their respective individual brightness. A new method, FIDA, (Fluorescence Intensity Distribution Analysis), allows the determination of the concentration of fluorescent particle species as function of their specific brightness. The analysis is based on fitting the measured photon count number distribution to a theoretical model. With the assumption that the sample contains a certain number of fluorescent species, the respective concentrations and specific brightness values can be estimated. The theoretically calculated photon count number distributions are sensitive to the spatial brightness function of the equipment, which is therefore carefully characterized before sample analysis. FIDA is an appropriate tool for resolving the molecular mechanism of complex biochemical interactions. Its power can be greatly enhanced by using two photon detectors for simultaneous monitoring different polarization components of fluorescence, or fluorescence of different color, either with a single or two excitation wavelengths. Anisotropy (2D-anisotropy FIDA), 2-color FIDA, burst coincidence, and cross correlation can be analyzed within a single experimental set-up. As a further essential step towards a comprehensive confocal screening set-up, fluorescence lifetime detection was integrated into the 1- and 2D-FCS and FIDA environment.

The lifetime of a suitable dye is the most sensitive parameter for investigating the molecular micro environment of the fluorophore. Due to this sensitivity and its independence of any optical alignment its performance may even surpass other confocal detection techniques. The strengths and weaknesses of new methods are always revealed with the practical robustness like in assay development for high-throughput screening. The status of practical applications of FCS, FIDA and fluorescence lifetime will be reviewed.

Fluorescence correlation spectroscopy: Lead discovery by miniaturized HTS. M. Auer, Keith J. Moore, F-J. Meyer-Almes, R. Guenther, A. J. Pope, K. A. Stoekli. *Drug Discovery Today*, 1998, 3, 457-465, Review.

Fluorescence Intensity Distribution Analysis (FIDA) and its Applications in Biomolecular Detection Technology Kask, P., Palo, K., Ullmann, D., Gall, K. *Proc. Natl. Acad. Sci.* in press,

Spontaneous Emission Control and Detection of Biological Markers

C. Begon, H. Rigneault

Laboratoire d'Optique des Surfaces et des Couches Minces (LOSCM)
Domaine Universitaire de St Jérôme, Marseille, France

Although a large number of high efficiency dyes have proved to be suitable for single molecule detection techniques [1], great potentialities are also offered by markers which allow reaction dynamics probing through the formation of donor-acceptor complexes and fluorescence resonance energy transfer (FRET). Nevertheless, those complexes remain delicate to handle and detect at the single molecule level especially if their quantum efficiency is not as high as current dyes.

Therefore, the problem of increasing the collection efficiency of the light emitted by these weak luminescent species is crucial, and can be treated by using mirrors or gratings to build micron-size resonant structures such as microcavities, that control and enhance the spontaneous emission in specific directions [3].

In this framework, two directions are investigated by our group:

- The first one consists in designing a grating structure which allows efficient spontaneous emission control in a direction normal to the grating plane. Promising results have been obtained using crossed grating [4] and will be presented at the conference both from theoretical and experimental aspects.
- The control of spontaneous emission is exploited for the detection of a few number of Cyanine-5 dye molecules, and of donor-acceptor complexes ([Sm³⁺]Cryptate-Cyanine or [Eu³⁺]Cryptate-Cyanine) by using a confocal optical set-up and the fluorescence correlation spectroscopy technique. The inherent difficulties together with the first results of this experiment will be presented and discussed.

[1] R.A. Keller et al., Appl. Spectrosc. 50, A12 (1996)

[2] S. Weiss, Science 283, 1676-1683 (1999)

[3] H. Rigneault, S. Robert, C. Begon, B. Jacquier, P. Moretti, Phys. Rev. A 55, 1497-1502 (1997)

[4] H. Rigneault, F. Lemarchand, A. Sentenac, H. Giovannini, Optics Letters 24 (3), 148-150 (1999)

Single DNA Molecule Approach to Homologous Recombination

M.L. Bennink, O.D. Schärer, R. Kanaar, B.G. de Groot and J. Greve

Applied Physics / Biophysical Techniques, University of Twente,
Enschede, The Netherlands

A single λ -phage DNA molecule is held stretched by two polystyrene beads. A micropipette is used to hold the first bead. The second bead is held using optical tweezers. Moreover these tweezers are used to determine the longitudinal tension in the DNA molecule. Flowing in RecA protein molecules and ATP enabled us to explore the binding kinetics of this protein to double-stranded DNA. The binding rate appeared to be force-dependent. In nature the RecA protein plays a central role in the process of homologous recombination by mediating homologous pairing and strand-exchange reactions. In this capacity it binds a double-stranded and a single-stranded DNA molecule and furthermore it aligns homologous sequences within these two molecules. We conducted experiments in which we added RecA coated single-stranded pieces (5 kbp) which sequence is identical to part of the λ -phage double-stranded DNA. Monitoring the length of the complex as a function of the longitudinal tension, reveals detailed information on the kinetics of the homologous recombination process. Moreover it is possible to determine the mechanical properties of the triple-stranded intermediate structure.

Four-dimensional Site-specific Fluorescence Spectroscopy of Single DNA-Molecules in Solution

S. Berger, E. Schweinberger, J. Schaffer, C. Eggeling, A. Volkmer, Fries, J. Widengren, G. Striker, C. A. M. Seidel.

Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany

Four-channel-detection for simultaneous registration of fluorescence intensity, lifetime τ , anisotropy r and spectral range of single molecules as a new application of the real-time spectroscopic technique BIFL (Burst Integrated Fluorescence Lifetime) is introduced [1,2].

This extension of BIFL is carried out by inducing fluorescence by pulsed laser excitation and using a confocal epi-illuminated microscope with polarizing and dichroic beam-splitters. The multi-dimensional parameters are obtained after sliding burst analysis of the registered bursts where each burst reflects a single molecule in the focus.

The extended technique is applied to study the conformational dynamics of DNA-oligonucleotides in aqueous solutions (10^{-12} M) which are single- or double-stranded and singly or doubly labeled with fluorescent dyes. Usually, Rhodamine 6G is used for single-labeled oligonucleotides, but for double-labeled strands investigating fluorescence resonance energy transfer (FRET) donor-acceptor dye-pairs (i.e. Rhodamine Green-Cy5) are employed.

As results for single-labeled oligonucleotides, two major conformational states (stable in a ms-range) of characteristic properties are obtained. Site-specific analysis of lifetimes and intensities of these conformational states indicates different photophysical parameters and additional faster dynamics, which is a direct proof for further branching of the energy landscape.

These investigations allow a better understanding of dye-nucleobase interactions and DNA-dynamics. Using four-dimensional BIFL, perspectives for monitoring biomolecular dynamics by FRET studies on single molecule level will be discussed.

- [1] Fries, J. R., Brand, L., Eggeling, C., Köllner, M., Seidel, C. A. M., J. Phys. Chem. A. 102, 6601-6613 (1998).
- [2] Schaffer, J., Volkmer, A., Eggeling, C., Subramaniam, V., Striker, G., Seidel, C. A. M., J. Phys. Chem. A. 103, 331-336 (1999).
- [3] Eggeling, C., Fries, J. R., Brand, L., Günther, R., Seidel, C. A. M., Proc. Natl. Acad. Sci. USA. 95, 1556-1561 (1998).

Conformational Changes of H⁺-ATPase upon Nucleotide Binding detected by Fluorescence Correlation Spectroscopy

Michael Börsch

Institut für Physikalische Chemie, Universität Freiburg, Germany

abstract n.a. at time of printing

Detection of Specific Sequences in Individual Double-Stranded DNA Fragments

Alonso Castro and Lorraine Paz-Matos

Biophysics Group, Los Alamos National Laboratory , U.S.A.

We describe a new method for the direct detection of specific sequences in double-stranded DNA at the single-molecule level of sensitivity. This method allows analysis of genomic samples without the need for a denaturing step. In addition, it can be combined with other single-molecule detection techniques that allow sizing DNA fragments by measuring their individual electrophoretic velocities (Castro and Shera, *Anal. Chem.* 67, 3181 (1995)). The present technique is based on a two-color, single fluorescent molecule detection technique. The basis of our approach is to monitor for the presence of a specific nucleic acid sequence of bacterial, human, plant or other origin. The detection scheme involves the use of a peptide-based nucleic acid probe which binds to a complementary sequence in a double stranded DNA target. When the probe is added to the sample under analysis, and if the target is present, hybridization occurs. In addition, an intercalating dye is added to the sample, which provides a signature for the passage of each fragment through the detection volume. Simultaneous detection of the intercalator fluorescence signal and the labeled probe signifies the presence of the target molecule. When there is no target present, the free probe will emit a signal that is not coincident in time with that of the intercalator.

A Comparative Study of Single Molecule Fluorescence Decays by the Least-Squares and Maximum-Likelihood Estimation Methods

M. Cotlet, M. Maus, J. Hofkens, T. Gensch, F.C. DeSchryver

Dept. Photochemistry and Spectroscopy, Katholieke Universiteit Leuven, Belgium

J. Schaffer, C.A.M. Seidel

MPI für Biophysikalische Chemie, Göttingen, Germany

Single Molecule (SM) fluorescence decays of hexaphenyl-peryleneimide fluorophores in polyvinylbutyral films are comparatively analyzed by the Least-Squares (LS) and Maximum-Likelihood Estimation (MLE) Methods. Both methods lead to comparably good fit quality parameters for a monoexponential excited state decay model and each of them yield an average decay time which is slightly larger than that derived from the corresponding bulk measurement (4.1 ns). A linear correlation between the decay times analyzed by LS and MLE, respectively, is obtained, even though the MLE results in larger values. The influence of excitation polarization, emission count rate per second and the number of counts per decay is investigated taking into account the time trajectories of the SM fluorescence. The goodness of both analysis methods is evaluated and photophysical effects are discussed.

The Chemistry of Single Enzyme Molecules

Norman J. Dovichi, Robert Polakowski, Douglas B. Craig,
Jerome Wong, and Edgar Arriaga,

Chemistry Department, University of Alberta, Edmonton,
Alberta T6G 2G2

Single molecules of alkaline phosphatase are captured in a capillary filled with a fluorogenic substrate. During incubation, each enzyme molecule creates a pool of fluorescent product. After incubation, the product is

swept through a high sensitivity laser-induced fluorescence detector. Replicate incubations are used to construct a kinetic curve for a single molecule. While the activity of a single molecule is constant and independent of incubation time, the activity of different molecules is heterogeneous. We measure the activation energy for the reaction catalyzed by a single enzyme molecule. The activation energy is also heterogeneous for different molecules, but the average activation energy is identical to the activity of a bulk solution. This result confirms the first postulate of statistical mechanics: a thermodynamic property of a single molecule, averaged over time, is identical to the value determined from an ensemble of molecules. Last, we present evidence that heterogeneity in the enzyme properties is a result of post-translational modification to the enzyme.

Tracking of Diffusing Molecules within Membranes

Jörg Enderlein

Institute of Analytical Chemistry, Chemo- and Biosensors
University of Regensburg, Germany

A new method is proposed for tracking fluorescing single molecules diffusing within a two-dimensional membrane. It is based on a confocal microscopy set-up with a constantly rotating laser focus, which follows the position of the molecule. The optimization and efficiency of the method are theoretically studied for a broad range of experimentally realistic conditions. The proposed method allows for a long-time observation of diffusing molecules while permitting the application of fast spectroscopic techniques such as fluorescence decay time or fluorescence anisotropy measurements.

Real Time Light-driven Dynamics of the Fluorescence Emission in Individual Copies of the Green Fluorescent Protein

Maria F. Garcia-Parajo, Ine G.M.J. Segers-Nolten, Joost A. Veerman, Jan Greve and Niek F. van Hulst

Applied Optics Group, Dept. of Applied Physics & MESA+ Research Institute, University of Twente, Enschede, The Netherlands

The use of the green fluorescence protein (GFP) as individual marker for applications in molecular biology requires detailed understanding of its photophysical and photodynamical properties. We have applied real time single molecule fluorescence detection to study the light driven dynamics on the fluorescence emission of the S65T mutant of the GFP [1]. We have combined confocal microscopy and near-field scanning optical microscopy (NSOM) to obtain information on the photodynamics of GFP. Both techniques allow to record real time fluorescence trajectories of individual molecules at different excitation conditions (time resolution of 0.1 ms). In addition, NSOM provides a higher spatial resolution and correlated topographic information. Our experiments show that the excitation intensity has a dramatic effect on the GFP "blinking", with a reduction of the fraction of molecules being in the "on" state as a function of increasing excitation power. Correspondingly, we find that the on times become shorter at high intensity. We will present most recent results and a preliminary model that fits quite accurately with our observations. In addition, we have found an optimal excitation power in which the GFP will be preferentially in the on state. This result has implications for the use of GFP as marker in the study of biological processes at the single molecular level.

[1] Garcia-Parajo MF, Veerman JA, Segers-Nolten GMJ, de Grooth BG, Greve J, van Hulst NF, Visualizing individual green fluorescent proteins with a near field optical microscope, Cytometry 36, 239-246 (1999)

Dynamics of GFP-labeled Proteins in Cells

Greg S. Harms¹, Gerhard J. Schütz², Heike Kahr², Nikolai M. Soldatov³, Christoph Romanin², and Thomas Schmidt¹

¹ Departments of Biophysics, University of Leiden, The Netherlands

² Departments of Biophysics, University of Linz, Austria,

³ Department of Pharmacology, Georgetown University, Washington, DC, USA.

Strategies to detect small ensembles of membrane-proteins linked with the green-fluorescent protein (EGFP) as a fluorescence tag which have been expressed in cells will be presented. The use of selected cell-lines that have reduced autofluorescence permitted to unquestionably identify low concentrations of EGFP by comparison of the signals obtained with those for single EGFP's in solution. The diffusion of these EGFP-labeled proteins was characterized in their native environment. As predicted, the EGFP-labeled membrane-proteins exhibit characteristically slower mobility than that observed for EGFP-labeled proteins in solution. It is anticipated that the observed mobility reflects the diffusion and conformational motions of the membrane-proteins.

Fluorescence Correlation Spectroscopy with Traveling Interference Fringe Excitation: Introduction of SMD Experimental Scheme

Mineyuki Hattori and Hideaki Shimizu

Supermolecular Science Division, Electrotechnical Laboratory, Tsukuba, Japan

A new improved fluorescence correlation spectroscopy apparatus for determining motional states of particles, in which the scheme of fluorescence correlation spectroscopy with traveling interference fringe excitation (FCSTFE) is applied, is presented. In this method, the modulated fluorescence signal from particles excited by a moving interference fringe is detected, and cosine and sine Fourier coefficients at the frequency of the traveling fringe are recorded. The autocorrelation function of the fluorescence intensity consists of terms which are characterized by the size of the excitation region and the spacing of the fringe. In this method the modulated fluorescence signal from particles excited by interference fringe moving at constant velocity is detected and cosine, sine Fourier coefficients at the frequency of the traveling fringe are recorded. Autocorrelation functions of the Fourier coefficients express the motion of the fluorescent particles. The decrease of the fringe spacing gives the shorter characteristic time of the fluctuation and the increase of radius of the excitation region gives the more fluorescent photons. The autocorrelation functions include information on interactions of particles in correlated Brownian motion, and such information on correlations among several particles may not be extracted effectively in FCS experiments with circular-spot excitation. The FCSTFE for each pixel detected by the planar imaging device is applicable in an advanced version of imaging fluorescence correlation spectroscopy. Microscopic mapping of the parameter of dynamics and reactions in the biological systems is expected.

Direct Measurement of the Longitudinal Electric Field Component in a Focus Using Single Molecules

B. Hecht, B. Sick and U.P. Wild

Physical Chemistry Laboratory, Swiss Federal Institute of Technology, ETH-Z, Zürich, Switzerland

Single fluorescent molecules, due to their small size, can map electromagnetic field components with molecular-scale spatial resolution [1]. It has been shown recently that at room temperature single molecules of terrylene doped into a crystal of p-terphenyl are extremely photo-stable [2] and thus are ideally suited as local probes. Scanning confocal optical microscopy was used to image single molecules of terrylene in crystal flakes of p-terphenyl. The orientation of terrylene molecules in such a crystal is nearly perpendicular to the prominent (a,b) crystal plane [3] which is perpendicular to the beam axis. Due to this fact the terrylene molecules map the longitudinal component of the electric field in the excitation focus. We find that for circular polarized excitation the spatial distribution of the longitudinal electric field component has the shape of a doughnut. The results agree nicely with predictions by Richards and Wolf [4]. Our findings may be useful for the direct determination of single molecule orientations in a scanning confocal optical microscope.

[1] E. Betzig and R.J. Chichester, *Science* 262 (1993) 1422.

[2] L. Fleury, B. Sick, G. Zumofen, B. Hecht, and U.P. Wild, *Mol. Phys.* 95 (1998) 1333.

[3] S. Kummer et al., *J. Chem. Phys.* 107 (1997) 7673.

[4] B. Richards and E. Wolf, *Proc. Roy. Soc. A* 253 (1959) 358

Non-classical photon statistics in single-molecule fluorescence at room temperature

Jean-Manuel Segura, Ludovic Fleury, Gert Zumofen, Bert Hecht, and Urs P. Wild

Physical Chemistry Laboratory, Swiss Federal Institute of Technology, ETH-Z, Zürich, Switzerland

The fluorescence of single terrylene molecules in a crystalline host was investigated at room temperature by scanning confocal optical microscopy with cw excitation. The arrival times of individual fluorescence photons were recorded in a dead-time-free Hanbury Brown/Twiss arrangement. In such a configuration the statistics of fluorescence photon arrival times can be studied with nanosecond time resolution. The data are analyzed in terms of inter-photon time distributions, second order correlation functions, and the variance of the photon number probability distribution. Antibunching at short times, crossing over to bunching behavior for longer times is observed, associated with sub- and super-Poissonian statistics, respectively. The results are in agreement with a rate-equation analysis of the molecular level populations from which photophysical parameters of the chromophores can be extracted.

Real Time Evaluation of a Hydrolysis Reaction of Single DNA Molecules by Fluorescence Video Microscopy

Yoshiaki Tachi-iri, Mitsuru Ishikawa, Ken-ichi Hirano, and Hiroki Sano

Joint Research Center for Atom Technology (JRCAT), Angstrom Technology, Partnership (ATP), 1-1-4 Higashi, Tsukuba, Japan

We developed a novel method for evaluating the hydrolysis kinetics of exonuclease III (exo III) molecules, which hydrolyse phosphorester bonds in DNA, in situ and in real time based on observing a single DNA molecule by fluorescence video microscopy. When a single DNA molecule was immobilized at one end on a coverslip and labeled with a fluorescent bead at the other end, the motion of the bead was limited within a circle area determined by the length of DNA. We evaluated the radius of a circle area of a fluorescence image formed by the trajectory of the bead. When exo III was added the radius decreased at the rate of 150 nt/min on average in the presence of both Mg^{2+} and single-strand binding proteins (SSB), which roll single-stranded DNA around themselves. However, a decrease in the radius was not observed without Mg^{2+} , which is a cofactor of the exo III activity, even when exo III and SSB existed together. These observations conclusively indicated that the decrease in the radius was due to hydrolysis of DNA by exo III. We thus evaluated the hydrolysis reaction rate of single DNA molecules by exo III from the decrease in the radius.

Single LHCII: High Resolution Spectroscopy and Polarisation Study at Cryogenic Temperature.

F. Jelezko¹, Tietz¹, A. Schubert², U. Gerken¹, J. Schuster¹,
J. Wrachtrup¹

¹ Institute of Physics, University of Technology Chemnitz, Germany

² Institute of Biology, Humboldt University Berlin, Germany

The permanent interest to pigment-protein complexes is caused by their role in the harvesting of light energy in nature. Photophysical study of light-harvesting complexes (LHC) on single molecule level provide the possibility to avoid ensemble averaging and to obtain new information about energy transfer and protein dynamics. We report on spectroscopic investigations of single LHC complexes from higher plants (LHCII) isolated in polymer matrix. Low temperature fluorescence emission and fluorescence excitation spectra show narrow ($<2\text{cm}^{-1}$) zero-phonon lines (ZPL) corresponding to the pure electronic transition of chlorophyll molecules. Spectral jumps of ZPL due to the protein dynamics at low temperature are observed. Polarization study at low temperature shows that for most LHCII there is an energy transfer among different chlorophyll molecules.

Fluorescence Correlation Analysis of Bioactive Protein in Single Living Cell

Masataka Kinjo

Laboratory of Supramolecular Biophysics, Research Institute for Electronic Science, Hokkaido University, Sapporo, Japan

Fluorescence correlation spectroscopy (FCS) is a sensitive analytical method to detect changing number of fluorescent molecules in homogeneous solution. FCS also analyse translational diffusion coefficient of the molecules in aqueous condition, however, its sensitivity is rather low since the translational mobility depends on the radius of spherical molecule such as protein. In order to detect the protein's behavior in living cell, we must focus not only the changing number of fluorescent molecules and diffusion properties but also fluorescent intensity per protein complex. To study protein-protein interaction at the single molecules level in a cell, plasmid gene of GFP fusion protein was transfected and expressed in cell at native condition, and the fluorescent emission was detected by optical microscope with FCS setup. We report the successful application of FCS to analysis a hormone sensitive protein bound with GFP. The changing number, diffusion time and fluorescent intensity of the protein complex in cytosol were monitored by using FCS, and these parameters were affected by stimulus from out of cell membrane. In vitro transcription/translation system of GFP was also carried out under FCS monitoring and compared with in vivo system, live cell.

New Intelligent Probes for Nucleic Acid Detection on the Single-Molecule Level

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We have developed new fluorescently labeled oligonucleotide probes which can report the presence of specific nucleic acids in homogenous solutions. As in case of the well known molecular beacons (Tyagi and Kramer 1996) our probe oligonucleotides are hairpin shaped molecules with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid. Hence, such oligonucleotide probes are ideally suited for homogenous assays, i.e. only specifically bound probe molecules contribute to the measured signal. Our oligonucleotide probes consist of a loop which is a sequence complementary to the target sequence and a stem which is formed by annealing of a an arm containing several cytosine residues and an complementary arm (guanosine residues). The fluorescent dye (rhodamine or oxazine derivative) is attached at the 5'-end of the cytosine arm via a C6-aminolinker. Upon close contact to the complementary guanosine containing arm the rhodamine or oxazine dye is quenched through an electron transfer mechanism. Since the used rhodamine and oxazine derivatives recognize their microenvironment, i.e. DNA bases in close contact, we call our oligonucleotide probes "Intelligent Probes". In contrast to the commonly used molecular beacons only one coupling reaction is necessary for the synthesis of our intelligent probes.

We demonstrate highly sensitive detection of target sequences in solution on the single-molecule level. Using intelligent probes in combination with time-resolved fluorescence microscopy target sequence concentrations down to 10^{-12} M can be detected in a homogeneous assays within seconds.

Tyagi S. and Kramer F. R. (1996) Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnology* 14, 303-308.

Intramolecular Excimer-like and Monomer Fluorescence of Multichromophoric Dendrimers Studied by Time- and Spectrally-Resolved Spectroscopy: From the Ensemble to the Single Molecule Behaviour.

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Time- and spectrally resolved spectroscopy, such as single photon counting fluorescence and transient absorption, reveal a substantial contribution of intramolecular excimer-like emission to the fluorescence of isolated peryleneimide chromophores in bulk second (G2) and third (G3) generation multichromophoric dendrimers. The absence of the red-shifted emission in a corresponding monomer model (G0) confirms the assignment to excimer-like fluorescence in the dendrimers. This ensemble behaviour is studied on the single molecule level, e.g., by the simultaneous measurement of fluorescence decay times, spectra and time trajectories.

While the monomer model G0 exhibits only structured spectra and monoexponential fluorescence decays, the G2 and G3 dendrimers clearly show the expected jumps between structured and structureless fluorescence spectra as well as non-single exponential decays even in certain intensity levels of the time trajectories binned with 10 ms. In addition, the effect of excitation polarization and wavelength is investigated.

The results are discussed in terms of intramolecular monomer-excimer-like emission, energy transfer processes and orientation dynamics taking place on different (femtosecond to the second) timescales, respectively.

Two-Dimensional Time-Resolved Fluorescence Detection of Individual Probe Molecules on Surfaces

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We present the first 2-dimensional time-resolved fluorescence detection system which is based on pulsed laser excitation using a short-pulse diode laser in combination with a gated CCD-camera system for time-correlated single-photon counting (TCSPC). In order to homogeneously excite a surface area of about $1\text{-}2 \cdot 10^4 \mu\text{m}^2$ the beam of a pulsed diode laser emitting at 640 nm was focused into the microscope objective. The average excitation power at the sample was 20 W/cm^2 . Fluorescence signal of the sample is collected by the same microscope objective and imaged onto a micro channel plate (MCP). The amplified signal is imaged by a cooled CCD camera. The MCP is triggered with a repetition rate of 55.8 MHz from the pulse generator of the diode laser. After each trigger signal the MCP is activated for 200-1000 ps. Within this period, generated electrons are accelerated by a voltage of 900V, thus creating an avalanche of secondary electrons which produce a highly intensified signal on a scintillation-plate. To efficiently suppress scattered laser light a time-gated detection method is used, i.e. the MCP is activated after the optical pulse by a delay line. By varying the delay and activation period of the MCP the fluorescence decay is obtained in each pixel. We present first 2-dimensional time-resolved fluorescence images obtained from immobilized single dye molecules, labeled oligonucleotides, labeled protein molecules and cells.

Multidimensional Photon Counting and Sequential Analysis for Rapid Identification of Single Molecules in Ambient Conditions

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A new method to identify single molecules rapidly and with a high efficiency based on simple probability considerations is proposed. The method utilises spectrally resolved time-correlated photon counting and sequential statistical analysis. Four dyes were selected varying both in fluorescence lifetime and spectra. However, no model function is required for the analysis. After each detected photon the probability that this photon originated from a molecule of one of the four species is computed. The joint probabilities of having one of the four molecules in the detection volume thus evolves as the number of detected photons increases. Monte-Carlo simulations show that in this framework less than 200 photons are sufficient to distinguish between Rhodamine 6G, DiI, Sulphorhodamine B, and DBATT with an accuracy higher than 99.99%.

Non Markovian Processes in Enzyme Catalysis at the Single Molecule Level.

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We have developed a model to study the substrate-product turnover of single enzyme molecules using surface bound single horse radish peroxidase (HRP) molecules (1). The analysis of the observed fluctuations in the generation of products gives evidence for the existence of a large distribution of conformational substates in HRP of which only few are involved in the catalysis. We have further analysed the existence of Markovian and non Markovian processes pointing to the existence of a time dependent memory landscape which may have significance in enzyme catalysis.

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Forbidden Light Detection from Single Molecules

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The detection of fluorescent dyes bound at a water-glass interface is a major task in fluorescence spectroscopy. When detecting surface bound molecules, it is important to take into account their emission characteristics. The angular distribution of the fluorescence emission of a molecule at a refractive index discontinuity differs significantly from that of a molecule within a homogeneous medium. For a molecule adsorbed from water onto glass, about one third of its total emission into the glass consists of the so-called forbidden light which is emitted above the critical angle of total internal reflection. Here, we present a new concept for ultrasensitive detection of surface generated fluorescence and demonstrate its capacity by detecting single molecules. It is especially efficient in collecting the forbidden light modes of the fluorescence emission. Such a detection method leads to an increase in the light collection efficiency compared to that of a conventional high aperture microscope objective. Moreover, by detecting the forbidden light only, the detection volume in solution can be reduced to as little as 10^{-17} liters.

Single Molecule Detection and Identification in Submicrometer Channels: State of the Art and Future Prospects

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During the last few years several new very promising detection methods have been developed to monitor the fluorescence characteristics of individual biomolecules in solution and on surfaces. However, the background due to impurities always sets detection limits, especially in biological samples containing buffer, enzymes and other macromolecular compounds. Therefore we decided to use short pulse diode lasers emitting in the red region for the fluorescence excitation of single chromophores in biological relevant fluids and surroundings. In addition, the use of pulsed excitation in combination with time-resolved detection enables a better discrimination between the fluorescence signal of probe molecules and impurities or scattered light. Furthermore, the fluorescence kinetics of single probe molecules can be used as a sensor for microenvironmental changes.

Using pulsed excitation with diode lasers in combination with mechanical manipulation by functionalized etched optical fibers individual molecules can be handled, detected and identified. For efficient detection of each analyte molecule given in a present sample electrophoresis in submicrometer microchannels is applied. Hence, all molecules passing the detection area are counted and identified with high efficiency. Identification of the labeled analyte molecules is done by time-resolved fluorescence detection (TCSPC) in combination with an efficient maximum likelihood estimator (MLE). In combination with activated optical fibers individual molecules can be transferred into the microchannel and analyzed. This technique can be applied for various important applications, i.g. for single molecule DNA sequencing. State of the art and future prospects of this technique will be discussed.

Another possible application of single molecule detection techniques is biodiagnostics, i.e. the early-stage diagnosis of a viral or bacterial infection. Here, we are always confronted with the problem of discrimination between free and bound probe molecule in a relatively large excess of free labeled probe molecules. For discrimination of bound and free probe molecules independent on the change in molecular weight either single molecule electrophoresis or so-called "intelligent" probes can be applied

As an example, through small changes in the overall charge of peptides a precise control of the electrophoretic mobility in microchannels can be achieved. Hence, individual bound and free labeled peptide molecules can be separated, even in a 1000fold excess of free labeled peptide molecules.

First results on the development of intelligent probes which directly display information of their microsurrounding, i.e. exhibit a strong increase in fluorescence intensity upon specific binding, will be presented. These probes are ideally suited for in vivo measurements in cells and other biological interesting solvents, such as undiluted serum samples.

Relaxation of Individual DNA Molecules Described by a Harmonic Oscillator Model

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Under typical physical-chemical conditions as they are required in many experiments DNA molecules show a tendency to coil up. However, it is possible to adjust the experimental conditions in a way that DNA molecules can be stretched to their full extension by a buffer flow. For such experiments, one end of a SYBRGreen labelled DNA molecule is bound to a microsphere held by optical tweezers when the molecule is stretched by hydrodynamic flow. The DNA molecule relaxes to a minimal length when the buffer flow stops. The structural transition of the molecule in pure water from the elongated form to a coiled globular form has a time constant of 1 to 2 seconds. An exponential law has been proposed by Perkins et al. to describe the time course of such a relaxation. In this work, a harmonic oscillator model is used to fit the experimental data, which allows the calculation of the friction coefficient and the spring constant. Relevant forces obtained from this model are in the order of 10^{-18} N, far below the pN forces found by the groups of Block, Bustamante and Chu for overstretching the molecule. The interaction of minor groove binders with DNA is changing the flexibility by local stiffening of the macromolecule. The specific binder netropsin and the non-specific one SN6113 have been employed to show their effect on the calculated parameters. The model of the entropic spring is used to verify the results of the harmonic oscillator model. For this, the model of the entropy force responsible for the relaxation of the DNA molecule is estimated.

Multi-dimensional Site-specific Fluorescence Spectroscopy of Single DNA-Molecules in Solution

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Multichannel-detection for simultaneous registration of all information on single-molecule fluorescence (intensity F , lifetime τ , anisotropy r and spectral range) is introduced as a new application of the real-time spectroscopic technique BIFL (Burst Integrated Fluorescence Lifetime) [1,2]. This extension of BIFL is carried out by pulsed laser excitation and using a confocal epi-illuminated microscope with polarizing and dichroic beam-splitters. The time traces of multi-dimensional parameters [3] are obtained after sliding burst analysis of the registered bursts where each burst reflects a single molecule in the focus.

The concept of data analysis is discussed. Furthermore three-dimensional measurements of F , τ and r are presented to study the conformational dynamics of DNA-oligonucleotides in aqueous solutions (10^{-12} M) which are single- or double-stranded and labeled with Rhodamine 6G. Two major conformational states (stable in a ms-range) with characteristic properties were found. Specific analysis of lifetimes and intensities of these conformational states indicates different photophysical parameters, charge-transfer dynamics and additional faster conformational dynamics. This is a direct proof for further branching of the energy landscape, which allows for a better understanding of dye-nucleobase interactions and DNA-dynamics.

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Single Molecule Microscopy on Biomembranes

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Due to technological advances and scientific understanding, optical experiments at the level of individual molecules became possible in various laboratories. Employing wide-field video-enhanced fluorescence microscopy we have been able to detect individual fluorescence labeled molecules on artificial as well as native biomembranes. The wide-field approach, in contrast to scanning approaches, permits the observation of dynamical processes, like diffusion, rotation, and association in real-time. A spatial accuracy of about 40 nm was achieved. It will be shown by means of typical examples how single molecule techniques could become a valuable tool for the study of structural properties of biological membranes with the prospect to observe association dynamics in the millisecond time range.

Single Molecule Diffusion in Thin Wetting Films

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The microscopic dynamics of wetting is of high technological and scientific interest.

In particular the formation of thin liquid layers from a precursor state to a complete wetting of a surface is of fundamental interest. In order to analyze the dynamics of this precursor layer diffusion of single molecules in thin wetting films of glycerol and ethylene glycole has been studied by fluorescence correlation spectroscopy and fluorescence burst analysis. It is found that the diffusion in thin liquid films is slowed down compared to bulk liquids. For further understanding an exact analysis of the diffusion data and computer simulations of diffusing molecules have been performed. CCD imaging of individual freely diffusing molecules in liquids reveals that the origin of reduced diffusion constants in thin liquid films has to be attributed to attachment and detachment of molecules on the surface while diffusing.

Diffusion of Single Lipid Molecules on Cell Membranes

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The lateral heterogeneity in the distribution of lipids and proteins within the plasma-membrane of a biological cell is mediated by dynamical arrangements of the constituents in combination with immobilization to confined areas. While protein mobility in cells on submicron length scales is widely studied using Single Particle Tracking experiments, the diffusional behavior of lipids is largely unknown. We present here studies on the lateral mobility of individual lipid molecules within the membrane of biological cells in vivo on submicron length scales using Single Molecule Optical Microscopy. Clear deviations from free Brownian motion are observed and used to determine the lateral dimension and the surface density of the confinement areas.

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Applications of BIFL for Multi-dimensional Single Molecule Spectroscopy

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Multichannel-detection for simultaneous registration of all information on a signal burst (intensity, lifetime τ , anisotropy r and spectral range) is a versatile tool for spectroscopy single-molecules/particles. These applications of BIFL (Burst Integrated Fluorescence Lifetime) [1,2] are carried out by pulsed laser excitation and using a confocal epi-illuminated microscope with polarizing and dichroic beam-splitters. The time traces of multi-dimensional parameters [3] are obtained after sliding burst analysis of the registered bursts where each burst reflects a single molecule in the focus. Hence this method gives direct access to time trajectories. Applications of BIFL for analytics and conformational dynamics of fluorescent labeled molecules are discussed.

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Two-photon Fluorescence Excitation in Detection of Biomolecules

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Two-photon fluorescence excitation has been found as a very powerful method for enhancing the sensitivity and resolution in far field light microscopy. Two-photon fluorescence excitation also provides a substantially background free detection on single molecule level. It allows direct monitoring of formation of labeled biomolecule complexes in solution. Because the emission of two photon excitation is a quadratic process with respect to illumination intensity, only the fluorescence that is formed in the clearly restricted 3-dimensional vicinity of the focal point is excited. We have developed an assay concept that is able to distinguish optically between the signal emitted from a microparticle in the focal point of the laser beam, and the signal emitted from the surrounding free labeled reagent. Moreover, the free labels outside the focal volume do not contribute any significant signal. This means that the assay is separation free. The method based on two-photon fluorescence excitation makes possible fast single step and separation free immunoassays, for example, for whole blood samples. Since the method allows a separation free assay in very small volumes, the method is very useful for high throughput screening assays. Consequently we believe that two-photon fluorescence excitation will make an remarkable impact as a research tool and a routine method in many fields of analysis.

Single Molecule Analysis in Genomics using Near-IR Fluorescence Detection and Microfluidic Devices

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The processing of single molecules has been well documented in genomics as a strategy to improve throughput and decrease reagent consumption due to scaling of the assay to the nano-liter regime. In this presentation, we will discuss three applications where single molecule analysis will be demonstrated, namely amplifying single copy DNAs using PCR, single assay detection of hybridization events on poly-lysine and polymethyl methacrylate (PMMA) derivatized slides and the high speed processing of restriction fragments for determining rare polymorphisms (mutations) in genomic DNA. The tools used for each of these applications are the ultra-sensitive near-IR fluorescence detection and micro-fabricated devices for handling sub-microliter sample volumes. The common approach for preparing sequencing templates from hydrodynamically sheared yeast artificial chromosome (YAC) clones is to insert the clones into M13 vectors and amplify them in *E. coli* cells, which requires extensive sample manipulation and centrifugation steps. To alleviate these cumbersome steps, we are developing a method to amplify single YAC clones using PCR, since it is conducive to automation. Factors that influence the amplification in PCR will be discussed, such as the polymerase enzyme, hot start conditions and template size. The PCR products are analyzed using capillary gel electrophoresis with near-IR, laser-induced fluorescence detection. DNA micro-arrays have recently been documented as an attractive method for high throughput screening of DNAs for mutations and also expression profiling. The major technical challenge in this approach is the fact that the measurement is performed on a mono-layer coverage of the appropriately-labeled DNAs deposited on solid supports. We have built a sensitive near-IR fluorescence scanner (confocal imager with epi-illumination) to read hybridization events from DNA micro-arrays. The scanner uses time-gated detection to minimize back scattered photons generated from the array surface, allowing the monitoring of single molecules on PMMA or poly-lysine coated slides. Our final application involves the use of restriction fragment length polymorphism analysis to screen single DNA molecules for mutations. The device for making this measurement consists of a microfabricated structure with channels containing surface immobilized restriction enzymes and an ultrasensitive near-IR fluorescence detector for analyzing the restriction products generated.

Single Light Harvesting C(LHC-II) of Higher Plants

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The light harvesting chlorophyll complex (LHC-II) of higher plants is the major light absorbing pigment protein complex in nature. Here microscopy and spectroscopy of single LHC-II embedded in a PVA matrix at variable temperature are presented. Fluorescence excitation and emission spectra at low temperature ($T=2\text{K}$) show narrow lines ($\Delta v_{\text{ZPL}} < 2\text{cm}^{-1}$) and a wide range of spectral diffusion due to protein dynamics. It is shown that spectral jumps take place between distinct spectral sites which we interpret as a direct manifestation of conformational substates in protein dynamics. The range of spectral diffusion ($\Delta v_{\text{diff}} \sim 100\text{cm}^{-1}$) is comparable to the line width of bulk spectra. A further physiologically interesting question is whether the excitation is distributed over all monomers in the trimeric subunit or whether it is located on one subunit. Using temperature dependent polarization microscopy it becomes clear that at high temperature the excitation is distributed over all subunits. At low temperature part of the complexes show excitation localized on a single chlorophyll molecule.

Characterization of Single Molecules by Time-resolved Fluorescence Scanning

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During the last years, several methods for the detection of individual fluorescently labeled molecules have been developed. Especially, for the study of inhomogenous complex systems single-molecule spectroscopy can provide information that is difficult to obtain from ensemble measurements. Due to the limited observation time of individual freely diffusing molecules in solution fluorescence scanning systems were introduced to monitor the dynamical behavior of immobilized probe molecules. Here, fluctuations in fluorescence intensity were used to describe different microenvironments or conformational changes of individual molecules. We further extended the number of obtainable parameters by developing a scanning system for time-resolved fluorescence detection. The system consists essentially of a confocal fluorescence microscope and a x,y-microscope scanning stage. Fluorescence of probe molecules was excited using a short-pulse diode laser emitting at 640 nm with a repetition rate of 50 MHz. Fluorescence decays of different samples were detected at each pixel by an avalanche photodiode in combination with a PC plug-in card for time-correlated single-photon counting (TCSPC). Synchronization of the alignment and measurement process were developed to allow data acquisition without dead times.

We present time-resolved measurements of individual dye molecules, labeled protein molecules and labeled oligonucleotide molecules of different sequence immobilized on amino silanized and/or streptavidin coated glass surfaces. In order to investigate the dynamical fluorescence behavior, i.e. dark state formation and quenching due to different interaction geometries between the dye and biomolecule, a single molecule search program was developed. Furthermore, we show time-resolved fluorescence scanning of labeled probe molecules in living cells. Our data clearly indicate, that the additional parameter fluorescence decay time provides detailed information about the microenvironment of individual probe molecules. Since time-resolved fluorescence scanning (TRFS) is independent of the excitation/detection efficiency and the transition moment of the molecule direct information about the system is obtained that is difficult to obtain from intensity measurements.

Microscopic Observation of Single Molecule Enzyme Kinetics in Femtoliter Droplets

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The fusion of femtoliter droplets allows measuring the kinetics of reactions in liquid environment that are catalyzed by single or few enzyme molecules. Under normal experimental conditions, the measurement of such reactions is impossible because of diffusion of the reacting agents. In femtoliter droplets however, the diffusion is limited to the very small volume of the droplet. Up to nine droplets of enzyme and substrate solution are placed on a microscope cover slide and fused with a glass needle under microscopic observation. Subsequently, the microscope is switched to fluorescence microscopy and the increasing fluorescence of the reaction product is recorded. The autofluorescence of the reaction product is used to monitor the reaction rate. From the volume of the droplets of enzyme solution and from a statistical approach the number of enzyme molecules that catalyzed the reaction is estimated. Typical numbers are between 10 and 100. Two types of enzyme catalyzed reactions are studied: the conversion of NAD^+ to NADH and the conversion of resazurin to resorufin in conjunction with NAD^+/NADH . Catalytic effects of the buffer solution itself, which are too small to be observed in bulk experiments, are discussed.

Near-field Optical Photodynamic Studies of Single Molecules & Proteins

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Recent near-field optical photodynamic studies of single molecules are presented: inter-system-crossing dynamics, photo-induced switching of green fluorescent protein mutants and dendritic macromolecules. We apply near-field optical single molecule detection because of its distinct advantages [1,2]: nanometric localization accuracy, high orientation sensitivity, direct correlation with topography and possibility of local manipulation. Our high brightness aperture probes allow saturation of the molecular transition (up to 10 kW/cm^2), count rate $> \text{MHz}$ and real time resolution down to microseconds. Real-time single molecular singlet-triplet jumping is observed. It is found that both triplet state lifetime and intersystem crossing yield vary in time due to the temporal and spatial heterogeneity. The range of the variation during long observation times shows a strong similarity with the distribution obtained during short observation times of many individual molecules dispersed in space: ergodicity [3]. The rich photodynamics (intensity fluctuations, blinking, long dark states) of the S65T-GFP mutant [4] was monitored in time over a wide dynamic time scale from 10^{-4} to 10^3 seconds. Photo-induced switching to a dark state is observed. We have indications for the existence of an intermediate state. The lifetime of the non-fluorescent state is typically in the order of a second. Finally dendritic macromolecules are under study. Co-localization of the fluorescent core and surrounding dendritic shell allows discrimination of different dendritic assemblies from free fluorescent cores. Intra-molecular rotation of the fluorescent sub-unit is observed.

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Functional Self-Assembled Monolayers of β -cyclodextrines on Gold: Single Host-Guest Interactions probed by AFM

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Organic molecules with functional groups for the selective recognition of neutral molecules and cations from aqueous solutions have been self-assembled on gold surfaces. Characterization of these highly ordered monolayers has been done by XPS, SIMS, grazing-angle FT-IR, contact angle measurements, cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS). Cyclodextrines have been derivatized for the self-assembly on gold in highly ordered layers. Cyclodextrines are well-known for their binding properties in aqueous solution of small organic compounds (e.g. adamantane and ferrocene derivatives). Some layers have also been studied by AFM showing hexagonal.

The binding of 1,8-ANS and 1,6-TNS by these monolayers has been studied by impedance spectroscopy, showing Langmuir-type binding isotherms with binding free energies much higher than in aqueous solutions.

In order to study the host-guest interactions at the molecular level, an AFM tip was coated with a thin layer of gold on which a self-assembled monolayer of 2-mercaptoethanol and 6-mercaptohexylferrocene (99 : 1) was applied. AFM studies showed that the pull-off curves are accompanied by some fine structure, i.e. before the critical pull-off force is reached, a number of smaller pull-offs were measured. These smaller pull-offs are quantized to $56(10 \text{ pN})$ and multiples thereof and are ascribed to single β -cyclodextrine ferrocene interactions.

The addition of 1,8-ANS blocks the β -cyclodextrine cavities and hence reduces the number of the quantized pull-offs almost completely. Removal of 1,8-ANS by washing restores the quantized pull-offs.

Time-varying Triplet State Lifetimes of Single Molecules

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It is found that triplet state lifetimes and intersystem crossing yields of individual molecules embedded in a polymer host at room temperature are not constant in time. The range over which the triplet lifetime of a single molecule varies during long observation times shows a strong similarity with the distribution of lifetime values obtained during short observation times of many individual molecules dispersed in space. The similarity is an elegant manifestation of the ergodic principle of statistical physics [1].

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Near-field Scanning Optical Microscopy of Single Fluorescent Dendritic Molecules

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Individual dendritic molecules [1] adsorbed on glass containing a single fluorescent Rhodamine B core have been observed with Near-field Scanning Optical Microscopy (NSOM) [2]; height and fluorescence images were obtained simultaneously. The dendritic assemblies can be discriminated from free fluorescent cores on the basis of accurate simultaneous localization of both, the fluorescent core and the surrounding dendritic shell. There are no significant differences between the photo-physical properties of the free and dendritic fluorophores. The full three-dimensional orientation of each individual fluorescent core can be resolved and millisecond time resolution accuracy has been achieved. Most dendritic structures exhibited rotational motion of the fluorescent core on a millisecond to second timescale, revealing intramolecular conformational dynamics [3].

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Single Molecule Detection and Spectroscopy of Single Perylene Diimide Dendrimers

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In recent years it became possible to conduct single molecule detection and spectroscopy, allowing investigation of the detailed dynamics of individual fluorescent molecules. Using a special class of molecules, dendrimers, one can control properties of interest of a molecule on a well-defined way. Two generations of dendrimers with a fluorescent core consisting of four polyphenylene branches attached to a perylene diimide core and a model compound were synthesized. Samples were prepared by spincoating a solution containing 10^{-10} M dendrimer and Zeonex (a polymer) on a glass coverslip. Detection and spectroscopy were conducted using a plate scanning confocal microscope and a scanning near-field optical microscope. These instruments combined with a high efficiency photon detection system, an avalanche photo diode, allow us to investigate fluorescence intensity changes, excited-state lifetimes, and spectral changes of the single dendrimers. Using two avalanche photodiodes in the detection path we can also detect polarized emission. Using these techniques, we are able to study the behavior of the different generations in the polymer matrix and the effect of increasing size of the branches on the photophysical properties. The latter can give insight in photophysical properties otherwise lost in the averaging of bulk experiments and information about polymer blending on a nanometer scale.

Time-resolved Fluorescence Detection and Identification of Single Molecules Using a Novel Versatile PC Plug-in Card for Time-correlated Single Photon Counting

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Lifetime analysis of laser induced fluorescence by means of Time-Correlated Single Photon Counting (TCSPC) provides a powerful additional discrimination feature to distinguish molecules of interest from background or other species.

We have developed a compact and easy-to-use TCSPC system on a single PC board based on a new solid state design. The circuit allows measurement rates up to 3MHz and provides a time resolution down to 30ps. The detector input has a programmable Constant Fraction Discriminator (CFD). These features qualify the board for use with all common single photon detectors such as Photomultiplier Tubes and Single Photon Avalanche Photodiodes. The time resolution is well matched to these detectors and an overall Instrument Response Function (IRF) down to 200ps FWHM can be achieved with inexpensive PMTs and fast diode lasers. The board is fully software reconfigurable to operate in different modes. Controlled by software, by a timer circuit or an external signal it is able to switch between 32 measurement curves for on-line histogramming. A sophisticated real-time memory switching technique is used to prevent dead times in multi channel mode $F(t, T)$ (continuously recording curves to hard disk in real time). Similarly a continuous Time-Tagged Time-Resolved (TTTR) measurement mode permits the recording of all photon events with a real-time tag to allow for single molecule photon burst detection and subsequent offline data analysis with unlimited flexibility, e.g. for burst detection and selective histogramming. Finally even time gated MCS mode is possible.

Demonstrating the capabilities of the system we have studied single molecule bursts of rhodamine 6G labeled oligonucleotides of different sequence in solution. The sequence of the oligonucleotide exhibits strong influence on the single molecule fluorescence kinetics. Furthermore, using TTTR-mode and surface immobilized labeled oligonucleotides we followed the dynamics in fluorescence kinetics and dark gap formation.

Triplet-state Lifetime of Single Molecules

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We have investigated the fluorescence of single molecules with a confocal laser-scanning-microscope. By means of a closed loop scanning table we were able to observe individual molecules for a long period of time. The excitation intensity can be varied within microseconds by an acousto-optical modulator. The dependence of the fluorescence intensity on the excitation intensity was examined. We have found a theoretically based saturation in the fluorescence intensity. The time-resolution of the detectors allowed us to examine dark states of the molecules. The statistical analysis of the data showed that most of the dark states can be attributed to the triplet state. The results show that the triplet lifetime of a single rhodamine 6G molecule depends on its individual environment. The triplet lifetime of molecules at the glass-air-interface is clearly below the lifetime of molecules covered by a polymer film.

Direct Observation of Interactions of Single Fluorescent Nucleotide Analogue Molecules with DNA Polymerase I

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The interaction of a fluorescent nucleotide analogue, 2'-(or-3')-O-(2, 4, 6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP), with the Klenow fragment of DNA polymerase I (Pol I) was investigated at a single-molecule level. The fluorescence intensity of a single TNP-ATP molecule increased when it was bound to an enzyme molecule. A light -induced binding process between TNP-ATP and the Klenow fragment of Pol I was observed in the single-molecule fluorescence imaging measurement.

Single-Molecule Separation, Detection and Identification in Capillaries

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One point of interest in analytical chemistry is to answer, whether there is a specific species of molecules present in a given sample. In this context capillary electrophoresis (CE) as well as high performance liquid chromatography (HPLC) have been established as standard methods for separation and detection of different analyte molecules in solution. Furthermore single-molecule detection (SMD) is on the threshold to become a routine technique to detect individual fluorophores with an outstanding sensitivity. Therefore it offers itself to combine the technique of SMD with CE or HPLC.

In the present work a confocal set-up is presented which allows to perform electrophoretical separation of different dye molecules in capillaries with a sensitivity high enough to detect and characterize individual molecules. The set-up consists essentially of a confocal microscope. For excitation of the fluorophores a commercial diode laser at an emission wavelength of ca. 635 nm and a repetition rate of 64 MHz is used. Fluorescence was detected by an avalanche photo diode and registered by a PC-adapter for time-correlated single-photon counting.

Call for Papers 2000

We would like to invite all participants of this meeting and all other interested scientists to the

6th International Workshop on Single Molecule Detection and Ultrasensitive Analysis

which will be held in Berlin in late September 2000. The final date will be announced towards the end of this year on our web page

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We would like to invite people to submit their papers as soon as possible. From our experience organizing the previous meetings we recommend to do all correspondence by e-mail. Please send abstracts in RTF format or plain ASCII text.

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