International Workshop on
“Single Molecule Detection: Basics and Applications in Life Sciences”

Organized under the Auspices of the
Society for the Advancement of Medical, Biological and Environmental Technologies

Supported by **BiosQuant GmbH**

ㅅㄷ

Program

4 – 6 October 1995 at BiosQuant GmbH
Berlin-Adlershof (Germany)

List of Sponsors

(as of August 25, 1995)

The workshop is sponsored by

BASF AG

Becker & Hickl GmbH

BiosQuant GmbH

Edinburgh Instruments

Hamamatsu Photonics

Hoechst AG

L.O.T.

Melles Griot

Schering AG

Aims and purpose

Detection and spectroscopy of single molecules under biologically interesting conditions has gained considerable interest since the first report of single molecule detection (SMD) in a liquid 1976. The spectroscopic technique mostly involved in SMD is laser-induced fluorescence spectroscopy, mainly applied in two different experimental set-ups: detection within a focused laser beam and detection in a Near-Field Scanning Optical Microscope. Besides these spectroscopic techniques, other methods like Magnetic Resonance become also increasingly important for single molecule studies.

The possibility of detecting and even spectroscopically studying single molecules in solution offers far-reaching perspectives for the application of this technique in analytical chemistry and life sciences. One of the most striking challenges is the application of SMD for ultrafast DNA sequencing. But also the detection of minute amounts of substances and "direct" measurements of their concentration by counting single molecules in definite volumes seem to be possible. Another promising perspective is the monitoring of chemical and structural changes of molecules on a single molecule level, gaining new insight into complex processes which is impossible by bulk measurements.

The aim of the workshop is to review the results obtained so far and to exchange the experience of the groups working in the field. Special emphasis will be on the application of single molecule detection and spectroscopy to chemical and life sciences. We attempt

thereby the exchange of knowledge between the experts in SMD and interested scientists from chemical and life sciences.

Venue:

BiosQuant GmbH Berlin

Rudower Chausse 6a
Building 21.1
D-12489 Berlin

General Organization:

Dr. Jörg Enderlein

BiosQuant GmbH
Phone: +49-30-6392 4563
Fax +49-30-6392 4833
e-mail: joerg@bsq.fta-berlin.de

Registration Office:

BiosQuant GmbH Berlin, Wednesday, October 3 until Friday, October 6, 10 a.m. – 14 p.m.

Hotel addresses:

Radisson Plaza Hotel

Karl-Liebknecht-Str. 5
D-10178 Berlin
Tel.: 23828

Forum Hotel

Alexanderplatz
D-10178 Berlin
Tel.: 2389-0

Hotelschiff Victoria

Puschkinallee 16/17

D-12435 Berlin
Tel.: 27237-0

Hints for manuscript preparation

All contributions to the Workshop will be published in a special issue of the journal *Experimental Technique of Physics*. Invited papers are supposed to have a length not exceeding about 15 manuscript pages, contributed papers (oral and posters) a length not exceeding 8 pages. The manuscripts should be prepared according to the Instructions of the Journal enclosed.

Dead line for submission of the manuscripts will be at the beginning of the Workshop on October 4, 1995.

How to get to BiosQuant

from Airport/Railway Station Schönefeld	take any S-Bahn (city train) and travel two stations until Adlershof, then see plan on opposite page
from Railway Station Zoologischer Garten	take any S-Bahn in the following directions: Grünau (S8), Zeuthen (S6), Königs-Wusterhausen (S46), Flughafen Berlin-Schönefeld (S9/S45) and travel until Adlershof, then follow the directions on the next page
from Radisson Plaza Hotel	go to S-Bahn station Alexanderplatz (10 min away) and proceed as described in the case of Zoologischer Garten
from Forum Hotel	go to S-Bahn station Alexanderplatz (1 min away) and proceed as described in the case of Zoologischer Garten
from Hotelschiff Victoria	go to S-Bahn station Treptower Park (1 min away) and proceed as described in the case of Zoologischer Garten

Workshop Location

ㅅㄷ

Programme

(Subject to alterations)

Wednesday, 4 October 1995

14.00 - 14.20	E. Klose, Berlin **Opening remarks**
14.20 - 14.55	S. Soper, Baton Rouge **Single Molecule Detection in the Near-Infrared: Monitoring Real-World Type Chemical Phenomena at the Ultimate Level of Sensitivity (Invited paper)**
14.55 - 15.30	P. Goodwin, Los Alamos **Progress Towards Single Molecule DNA Sequencing (Invited Paper)**
15.30 - 16.10	COFFEE BREAK
16.10 - 16.30	O. Greulich, Jena **Handling Individual DNA Molecules by Laser Microbeam and Optical Tweezers**
16.30 - 16.50	J. Enderlein, Berlin **Time-Correlated Single Photon Counting and Single Molecule Detection**
16.50 - 17.10	K.M. Ulmer, Cohasset

Single-Molecule DNA Sequencing with Native Nucleotides

18.00 RECEPTION

Thursday, 5 October 1995

9.30 - 10.05 C. von Borczyskowski, Chemnitz
Detection of Magnetic Resonance on Single Molecules (Invited paper)

10.05 - 10.25 Th. Basché, München
Dynamical Processes of Single Terrylene Molecules in a *p*-Terphenyl Crystal as Deduced from the Fluorescence Autocorrelation Function

10.25 - 11.00 COFFEE BREAK

11.00 - 11.20 K. Rebane, Riia
On the State and Prospectives of Single Molecule Detection and Spectroscopy

11.20 - 11.50 U.P. Wild, Zürich
Single Molecule Spectroscopy and Microscopy

11.50 - 12.10 J. Köhler, Leiden
Single Molecule Electron Paramagnetic Resonance

12.10 - 12.30 K. Kneipp, Cambridge (MA)

An Approach to Single Molecule Detection Using Surface-Enhanced Raman Scattering (SERS)

LUNCH

14.00 - 17.00 **POSTER SESSION AND PRODUCT PRESENTATION**

18.30 DINNER

Friday, 6 October 1995

9.30 - 10.05 P.W. Ambrose, Los Alamos
Near-Field Scanning Optical Microscopy with Single Molecule Sensitivity (Invited paper)

10.05 - 10.40 R. Rigler, Stockholm
Fluorescence Correlation Spectroscopy and Single Molecule Detection (Invited paper)

10.40 - 11.10 COFFEE BREAK

11.10 - 11.30 Th. Schmidt, Linz
Single Dye-Molecule Diffusion Watched in a Microscope

11.30 - 11.50 U. Kubitscheck, Münster
High Resolution Confocal Microscopy and Image Analysis

11.50 - 12.00 **Concluding remarks**

END OF THE WORKSHOP

Abstracts of Lectures

(in alphabetical order)

Near-Field Scanning Optical Microscopy with Single Molecule Sensitivity (Invited paper)

P. Ambrose

Los Alamos National Laboratory, Los Alamos NM, 87545, USA

We have developed a near field optical microscope with single fluorescent-molecule detection sensitivity. Our microscope is based on scanning a sample under a tapered fiber optic probe and has optical resolution between approximately 100 and 200 nm. We have used this system to demonstrate the detection of single molecules of Rhodamine-6G,[1] to observe the photobleaching of single molecules,[1] and to measure fluorescence lifetimes of single molecules.[2]

This work was supported by the US Department of Energy.

1. W.P. Ambrose, P.M. Goodwin, J.C. Martin, and R.A. Keller, "Single Molecule Detection and Photochemistry on a Surface Using Near-Field Optical Excitation," *Phys. Rev. Lett.* **72** (1994) 160-3.
2. W.P. Ambrose, P.M. Goodwin, J.C. Martin, and R.A. Keller, "Alterations of Single Molecule Fluorescence Lifetimes in Near-Field Optical Microscopy," *Science* **265** (1994) 364-7.

Dynamical Processes of Single Terrylene Molecules in a *p*-Terphenyl Crystal as Deduced from the Fluorescence Autocorrelation Function

S. Kummer, R. Kettner, C. Bräuchle, Th. Basché
Institut für Physikalische Chemie, Universität München, D-80333 München, FRG

By recording the fluorescence intensity autocorrelation function from the photon stream emitted by a single molecule in a low temperature solid, one can investigate the intramolecular photophysical dynamics of the molecule as well as the dynamics caused by its interaction with the environment simultaneously over more than ten orders of magnitude in time. In this contribution we report on particular results obtained with single molecules of terrylene doped into a *p*-terphenyl host crystal. At short times the correlation function shows a strong anticorrelation which is termed photon-antibunching and which can be qualitatively explained by the simple fact that a single quantum system cannot emit two photons at the same time. Under strong excitation conditions the correlation function displays coherent Rabi oscillations in the nanosecond time regime the damping of which can be employed to determine energy and phase relaxation times. Finally, the decay of the correlation function due to intersystem crossing into and out of the triplet state (timescale: μs - ms) permits the measurement of population and depopulation rates of the triplet state.

Detection of Magnetic Resonance on Single Molecules (Invited paper)

C. von Borczyskowski
Institut für Physik, Technische Universität Chemnitz-Zwickau, D-09126 Chemnitz, FRG

Spectroscopy of single molecules in organic solids is a sensitive tool to study both static and dynamic parameters of chromophores. In combination with (optical) detection of magnetic resonance this new technique opens the wide range of in chemistry and biology well known techniques for extremely low concentrated chromophores in solids, thin films and on surfaces. Recent results and perspectives will be reported.

Time-Correlated Single Photon Counting and Single Molecule Detection

J. Enderlein, R. Erdmann, E. Klose, R. Krahl, U. Ortmann
BiosQuant GmbH, D-12489 Berlin, FRG

The technique of single photon counting is ideally suited for ultrasensitive fluorescence detection. Thus, it is widely used in Single Molecule Detection experiments by counting the photons emanating from the laser-excited molecule's fluorescence. Because of the very low signal-to-noise ratio, it is desirable to extract from the detected photons as much as possible information. Here, time-correlated single photon counting (TCSPC) adds an additional informational dimension, facilitating the detection of single molecule events on the background of a strong noise signal, which has a different time-characteristics. But not only noise discrimination can be achieved by exploiting the time information contained in the measured photon counts, it is also feasible to distinguish different molecules by their fluorescence decay.

The paper studies the current status and the perspectives of TCSPC in Single Molecule Detection experiments.

Progress Towards Single Molecule DNA Sequencing (Invited Paper)

P. Goodwin
Los Alamos National Laboratory, Los Alamos NM, 87545, USA

We describe progress towards sequencing DNA at the single molecule level. Our technique involves: incorporation of fluorescently tagged nucleotides into a target sequence, anchoring the labelled DNA strand into a flowing stream, progressive exonuclease cleavage of the DNA strand, and efficient detection and discrimination of the single tagged nucleotides.

Handling Individual DNA Molecules by Laser Microbeam and Optical Tweezers

C. Hoyer, S. Monajembashi, N. Endlich*, K.O. Greulich
Inst. Mol. Biotech., Dept.. of Single Molecule Techniques, D-7708 Jena, FRG
* Phys. Chem. Inst. Univ. Heidelberg, D-69120 Heidelberg, FRG

The probably most prominent molecular individual is the DNA molecule. The prospect to gain analytical access to individual DNA molecules is one of the driving forces for the development of single molecule detection techniques. Individual DNA molecules have to be handled, for example transferred into the analytical apparatus, before they can be analyzed. One approach is to couple the DNA molecule to a microbead and to move the latter with optical tweezers, basically an infrared laser focused into a microscope. Since enzyme reactions are required for several types of DNA analysis, for example in attempts to sequence single molecules, the environment for these operations has to be close to physiological. Under such conditions DNA tends to condense and precautions are required to prevent this process. In addition, for some purposes it is helpful to cut the molecule in order to obtain pieces of optimal length. Experiments are reported which represent a first step towards a complete handling of individual DNA molecules by laser light.

Single Molecule Electron Paramagnetic Resonance

J. Köhler, A.C.J. Brouwer, E.J.J. Groenen, J. Schmidt
Centre of study of excited states of molecules, Huyghens Laboratorium, Leiden University, NL-2300 RA Leiden, The Netherlands

Magnetic-resonance techniques add a new dimension to the field of single-molecule spectroscopy. It has been found possible to observe triplet sublevel transitions of a single molecular spin. This will be shown for a single crystal of *p*-terphenyl ($C_{18}H_{14}$) doped with pentacene ($C_{22}H_{14}$) where the difference in the mean residence time of pentacene in the triplet sublevels allows the optical detection of the magnetic-resonance transition as a decrease in fluorescence. It will be shown that we have been able to select optically individual pentacene molecules which contain a single ^{13}C nucleus in a specific position of the molecule. The ^{13}C nucleus (I=½) manifests itself by its contribution to the hyperfine interaction which leads to a significant broadening of the magnetic-resonance line allowing the assignment of the position of the ^{13}C substitution.

Recently we have observed the magnetic resonance of a single molecule in an external magnetic field. We have used samples of pentacene-d_{14} in *p*-terphenyl-d_{14} which results in considerably narrower magnetic-resonance lines. In an external magnetic field the magnetic-resonance line of a molecule which contains a ^{13}C nucleus shows a splitting which is caused by the hyperfine interaction of a single molecular (electronic) spin with a single nuclear spin.

An Approach to Single Molecule Detection Using Surface-Enhanced Raman Scattering (SERS)

K. Kneipp*, Y. Wang, H. Kneipp**, R.R. Dasari, M.S. Feld

George R. Harrison Spectroscopy Laboratory, Massachusetts Institute of Technology, Cambridge, MA 0213, USA

* present address: Optical Institute, Technical University Berlin, D-10623 Berlin, FRG

** Spektrum Laser-Entwicklungs- und Vertriebs-GmbH, D-13597 Berlin, FRG

We have measured surface-enhanced Raman scattering (SERS) spectra on dye molecules at concentrations as low as 10^{-15} - 10^{-16} M in colloidal silver solution activated by NaCl ions. Experiments conducted with small scattering volumes show that less than 1000 molecules are sufficient to give rise to a SERS spectrum with reasonable signal to noise ratio. The experiments show that SERS detection of a single dye molecule is possible with some simple improvements in experimental conditions.

Our experimental results show that for selected dyes, effective SERS cross sections are on the same order of magnitude (10^{-17} - 10^{-16} cm^2/molecule) as the product of absorption coefficient and fluorescence quantum yield. However, SERS provides greater sensitivity to molecular structure compared to fluorescence, permitting discrimination of different molecules. In addition, more scattering photons per unit time occurring under saturation conditions and higher photostability of adsorbed molecules can favour SERS over fluorescence for single molecule detection.

Identification and Localisation of Single Nuclear Pore Complexes by High Resolution Confocal Microscopy and Image Analysis

U. Kubitscheck, P. Wedekind, O. Zeidler, R. Peters
Inst. für Medizinische Physik und Biophysik, Westfälische Wilhelms-Universität Münster, D-48149 Münster, FRG

The nuclear pore complex (NPC), a large protein complex spanning the nuclear envelope of eukaryotic cells, mediate the selective transport of macromolecules between nucleus and cytoplasm. We used high resolution confocal laser scanning microscopy and special image analysis methods to identify and localise single NPCs in semi-intact cells. The plasma membrane of 3T3 cells was permeabilized with digitonin, and the NPC labelled using the monoclonal antibody mAb 414 and a fluorescent secondary antibody. Stacks of optical sections of individual nuclei were generated by confocal imaging at high resolution. Single NPCs could be detected as bright diffraction limited spots. Centers of these spots were determined by a fit to a 3D Gaussian, employing in case of overlapping intensity distributions up to six Gaussians simultaneously. This yielded an accuracy of < 1 pixel, i.e. < 40 nm in c- and y-, and < 200 nm in z-direction. From these data 3D-maps were created revealing the distribution of NPCs in the nuclear surface.

The nearest neighbour distribution and pair correlation functions of the NPCs were calculated and compared with computer simulations of various particle distributions. Analysis of the distribution functions indicated that the minimum center-to-center distance between individual NPC were significantly larger (140-200 nm) than expected on the basis of the NPC diameter (125 nm) as determined by electron microscopy.

On the State and Prospectives of Single Molecule Detection and Spectroscopy

K. Rebane
Inst. of Physics, Lab. of Laser Spectroscopy, Estonian Academy of Sciences, EE-2400 Tartu, Estonia

Short review and comparison of existing methods of single molecule detection and spectroscopy (ion trapping, near field fluorescence, correlation spectroscopy, single impurity molecule high resolution spectroscopy), their applications and potentials in chemical and life sciences are given. Prospectives for fast DNA sequencing are discussed. As a decisive feature the possibility of utilization of coherent phenomena is analyzed.

Single impurity molecule high resolution spectroscopy based on low temperature zero-phonon lines is considered in more detail and compared to the high resolution persistent spectral hole burning. The latter is an efficient tool for large scale fully parallel fast optical data storage and processing based on the utilization of coherent phenomena via various versions of holography. The error corrective capability is also gained.

In the case of single molecule imbedded in condensed matter the existing methods do not provide the coherent processes to be used and, if so, exclude this very promising opportunities of fast large scale storage and processing.

Fluorescence Correlation Spectroscopy and Single Molecule Detection (Invited paper)

R. Rigler
Karolinska Institutet, Dept. of Medical Biophysics, S-17177 Stockholm, Sweden

With the introduction of confocally limited volume elements of sizes down to 0.2 fl single organic dye molecules can be detected in aqueous solutions with signal to background ratios of > 1000:1 in time intervals below 10^{-4} s. From the correlation of single molecule events molecular properties and interactions can be evaluated. Developments for trapping and selecting single molecules as well as applications in chemistry and biology will be discussed.

Single Dye-Molecule Diffusion Watched in a Microscope

Th. Schmidt, G.J. Schütz, W. Baumgartner, H.J. Gruber, H. Schindler
Inst. of Biophysics, University of Linz, A-4040 Linz, Austria

The ultimate goal of sensitive detection schemes are observations on the single molecule level. This came into reach by the invention of scanning probe microscopy which has brought a wealth of new insights since. Optical methods allowed for detection of single atoms and single fluorophores in liquids, crystals, and glasses by confocal fluorescence-microscopy with the prospect to high-resolution spectroscopy. We show that conventional, high-sensitive, fluorescence-microscopy makes it possible to detect single, mobile fluorescence-molecules in a biological model-membrane. Single molecules were detected with a signal-to-noise ratio of 29 when viewed for 5 ms. This fact and reliable detection allowed to determine their position down to 40 nm and watch their diffusion in the membrane with 35 ms time-resolution. The application of this method to study mobile systems on surfaces of living cells and as method for microscopy with “stochiometric resolution” is discussed.

Single Molecule Detection in the Near-Infrared: Monitoring Real-World Type Chemical Phenomena at the Ultimate Level of Sensitivity (Invited paper)

S. Soper

Louisiana State University, Baton Rouge, LA 70803-1804, USA

Many laboratories have recently demonstrated the ability to detect single molecules in solution using fluorescence. The commonality in many of these experiments is the use of chromophores which possess absorption and emission properties in the visible region of the electromagnetic spectrum. The difficulty with this approach is that the detection efficiency is limited by the presence of fluorescence interferences (impurities), which cannot typically be discriminated against using spectral or time-filtering methods. We have initiated research on using near-infrared (NIR) chromophores in single molecule experiments in order to minimize the problem associated with these interferents, since few molecular species possess electronic transitions in the NIR. The single molecule detection apparatus consists of a passively mode-locked Ti:sapphire laser as the excitation source and a single photon avalanche diode with time-filtering to reduce background arising from solvent scattering. Our presentation will focus on the photophysical characteristics of the NIR dyes, single molecule detection in the NIR, determining thermodynamic and photophysical properties of NIR chromophores in heterogeneous environments (surfactant solutions) using single molecule monitoring and finally, ultrasensitive detection in the microcolumn separation techniques, such as capillary zone electrophoresis.

Single-Molecule DNA Sequencing with Native Nucleotides

K.M. Ulmer, Y. Yu, P.M. Mitsis, N.A. Nicklaus, M.J. Fulwyler
SEQ. Ltd., Cohasset, MA 02025, USA

Schemes for single-molecule DNA sequencing were proposed as early as 1970. The approach is conceptually quite simple. A single molecule of DNA is isolated in an extended conformation in a laminar flow stream, and an exonuclease cleaves single nucleotides from the end of the DNA. The resulting individual nucleotides are then detected and identified down stream in their original sequence. Two independent groups began serious efforts to develop such a sequencing method in the late 1980's.

The Los Alamos group observed that the quantum yields of fluorescence for native nucleotides under physiological conditions were 10^{-3} - 10^{-4}, and therefore decided to focus on the detection and discrimination of highly-fluorescent dyes which could be covalently coupled to the individual nucleotides to function as reporter groups. Although this greatly simplifies the detection problem, it poses serious technical challenges for the enzymatic incorporation and removal of such dye-tagged nucleotides into DNA.

For over 30 years, however, it has been known that the quantum yields of fluorescence of native nucleotides are increased by orders of magnitude if measured in a highly-cohesive, hydrophilic glass matrix at cryogenic temperatures. Our approach to single-molecule sequencing has therefore focused on the low-temperature, solid-state detection and discrimination of native nucleotides. Such an approach eliminates all of the difficulties with dye-tagging.

Single Molecule Spectroscopy and Microscopy

U.P. Wild

Physical Chemistry Laboratory, ETH-Zentrum, CH-8092 Zürich, Switzerland

Optical detection and spectroscopy of single molecules has been achieved in solids at very low temperature. Until now only a few host-guest combinations have fulfilled the stringent requirements for high-resolution single-molecule spectra. A large absorption cross section has to be pared with high fluorescence quantum yield, high photochemical stability, as well as the absence of any significant shelving in a bottle-neck state. The photophysical properties of the single fluorescent molecule are strongly influenced by its 'nano-environment'. Here we report the properties of a new system: terrylene in the *Shpol'skii* matrix hexadecane. In this system a peak emission rate of more than 10^7 photons/s is achieved. It is expected that such a *Shpol'skii* system is a prototype of a new class of materials which allow single-molecule spectra to be easily recorded.

Abstracts of Posters

(in alphabetical order)

Flow Analysis by Means of Fluorescence Correlation Spectroscopy

M. Brinkmeier
Max-Planck-Institut für Biophysikalische Chemie, D-37077 Göttingen, FRG

A method has been developed to measure the flow properties of fluorescent molecules in solution. The principal measuring system is based on Fluorescence Correlation Spectroscopy (FCS). Due to the high detection yield of the set-up, even the transient of single molecules can be detected.

Microstructures have been manufactured in which the molecules pass through defined channels. This opens up the possibility to do analysis of molecules in defined surroundings and also in vivo.

Scanning Single Molecules

J. Dapprich, Ü. Mets, R. Rigler
Karolinska Institutet, Dept. of Medical Biophysics, S-17177 Stockholm, Sweden

Single fluorescent molecules such as rhodamine 6G and tetramethylrhodamine have been imaged in a gel matrix using confocal detection.

Molecules of biological interest like viral DNA or DNA-primers for hybridization purposes were labelled with tetramethylrhodamine either by incorporating fluorescent nucleotides (Rho-dUTP) into a complementary strand (M13 viral DNA) or by attaching the dye to the 5'-end of a primer sequence.

A scanning device of sub-micrometer spatial resolution was used to obtain images of the molecules dispersed at different concentrations in an agarose matrix. The detection is performed with a confocal set-up that allows further investigation of the scanned samples by fluorescence correlation spectroscopy. Our results indicate the feasibility of the method to perform large number screening of rare processes on two- or three-dimensional samples.

We thank O. Kristensen for technical assistance, N. Walter for supplying a number of probes and the Alexander von Humboldt-foundation for financial support of this research.

Path Integral Approach to the Autocorrelation Function in Single Molecule Detection Experiments

J. Enderlein
BiosQuant GmbH, D-12489 Berlin, FRG

The autocorrelation function of the fluorescence photon flux in single molecule detection experiments is easy to be measured and contains a wealth of information about the molecule's photophysics. But for extracting this information from measured data, one needs a theoretical model, which connects the molecule's parameters like the diffusion constant, absorption cross section, fluorescence quantum yield, intersystem crossing rate, triplet state lifetime and photodestruction constant to the form of the autocorrelation function. For larger sample of molecules in the detection volume, and for low photodestruction rates, there are well known formulas for the fluorescence autocorrelation function, which are widely used in Fluorescence Correlation Spectroscopy. But in the case of single molecule detection, one tries to extract as many photons from the single molecule as possible, and in that case, the photodestruction of the molecule becomes a strong factor influencing the auto-correlation pattern. The correct evaluation of the autocorrelation for such cases is very complicated, and in the present paper an approach basing on path integral techniques, well knwon from quantum field theories, is applied.

High-Speed Electronics for Fast Detection of Time-Resolved Fluorescence Detection in Single Molecule Experiments

R. Erdmann, J. Enderlein, W Becker*, M. Wahl
BiosQuant GmbH, D-12489 Berlin, FRG
* Becker & Hickl GmbH, D-10829 Berlin, FRG

In Single Molecule Detection by Laser Induced Fluorescence, a main problem is the low signal to noise ratio due to scattering of the exciting laser light. There are several approaches to circumvent this problem, among with is the application of time-resolved measurement techniques. Due to the short-time decay of the scattered light intensity with respect to the molecule's fluorescence, it is possible to discriminate effectively between noise and signal. Moreover, for higher overall count numbers per molecule it is feasible to use the time-information of the time-resolved fluorescence measurment to distinguish single molecules with different fluorescence decay times. This will be of special interest for applications like fast DNA-sequencing, where a distinction between different bases solely by the decay times of the attached fluorescence labels can be imagined.

An important prerequisite for the experimental application of these

ideas is a high-speed electronics capable of processing time-resolved single photon counts at very high count rates. When a single molecule is traversing the focused laser beam, count rates of more than 1 Mcps and traversing times of less than 1 ms are common. Thus, the system should be able to record a single time-resolved fluorescence curves with with a count rate of more than 1 Mcps and a repetition rate of 1 kHz, corresponding to a maximum of 10^3 molecules per second passing through the laser beam.

In the present paper, a high speed electronics is presented, capable of contineously processing and recording fluorescence decay curves with a rate of more than 10^3 curves per second. The maximal time-resolution of the fluorescence decay measurement is about 30 ps, the maximum allowed count rate is about 5 Mcps. Thus, the system is ideally suited for fast TCSPC-measurements in any kind of fluorescence detection flow experiment.

Single Molecule Detection by Near-IR Laser-Induced Fluorescence in a Capillary: Rubidium Metal Vapor Filter Studies

R. Guenard

Dept. of Chemistry, University of Florida, Gainsville, FL 32611-7200, USA

With the proliferation of single molecule detection (SMD) by laser-induced fluorescence (LIF) and the wide spread use of microbore capillary separation techniques, such as capillary electrophoresis and microbore liquid chromatography, a marriage between the two is a natural progression. Specular scattering of the laser light from a capillary is very intense which makes the detection of single molecules in a column a challenge. To surpass this problem, post column single molecule detection by LIF has been done using hydrodynamic focusing[1] and levitated microdroplets.[2] It has been recently shown in our laboratory that on-column SMD is possible b using a novel atomic spectral filter to attenuate the laser scatter.[3] A rubidium metal vapor filter (MVF) is placed between the capillary and the single photon avalanche photodiode detector. By resonantly tuning a cw Ti:sapphire laser to the absorption maxima of the rubidium vapor within the filter, the laser light was attenuated over eight orders of magnitude while still allowing the passage of the molecular fluorescence photons. In order to evaluate and improve the performance of the MVF, studies on the spectral composition of background light transmitted through the filter during SMD were done. In this manner, the spectral components of the background could be elucidated with the goal of improving the performance of the MVF for SMD. Parameters such as MVF temperature and buffer gas pressure as well as laser power were investigated. It was found that the performance of the filter could be improved by simply optimizing the physical parameters of the MVF. The material presented will include an introduction to MVFs and the results from theoretical and experimental studies on the performance of the rubidium metal vapor filter used for SMD.

1. E.B. Shera, N.K. Seitzinger, L.M. Davis, R.A. Keller, S.A. Soper, *Chem Phys Lett* **174**, (1990), 553-7.
2. W.B. Whitten, J.M. Arnold, J.M. Ramsey, B.V. Bronk, *Anal Chem* **63**, (1991), 1027-31.
3. Y.H. Lee, R.G. Maus, B.W. Smith, J.D. Winefordner, *Anal Chem* **66**, (1994), 4142-9.

Steps towards Two-Dimensional Single Molecule Detection in Solution

M. Köllner, J. McCaskill
Inst. für Molekulare Biotechnologie, D-07745 Jena, FRG

We have reproduced single molecule detection in solution in diffraction limited volumes with confocal laser-induced fluorescence. Detection in larger than diffraction limited volume elements will be possible with the help of two-dimensional single molecule detection. This is a prerequisite in evolutionary biotechnology for screening larger samples in shorter times. To achieve this goal, a special purpose detector has been designed for temporally and spatially resolved single photon counting with high count rate. This detector is set up to allow rejection of stray light by temporal discrimination when working with a mode locked laser as excitation source. It is based on recent developments of detectors for elementary particles in high-energy physics.

Circuit for Efficient Storing the Fluorescence Lifetime Data of Single Molecules in Solution

Ü. Mets, R. Rigler
Karolinska Institute, Dept. of Medical Biophysics, S-17177
Stockholm, Sweden

Some of the potential applications of single molecule detection, e.g. fats DNA sequencing,[1] require fast “on-line” identification of single molecules. The fluorescent molecules can be identified either by the emission wavelength or by the lifetime of the excited state[2] or both. The present methods of storing the lifetime data of single molecules are relatively inefficient, requiring either recording of the timing information in both the milli-/microsecond scale and the nanosecond time scale for all detected photons,[3-4] or use random discontinuous measuring intervals resulting in many undetected events.[5]

The problem in selecting the nanosecond timing information for permanent storage arises from the fact that at the moment for detection of the single molecule event, a considerable fraction of the event has already passed.

We propose a circuit which can be used for on-line selection of nanosecond timing data for permanent storage. The circuit will ensure that only the information concerning the single molecule events will be stored, thereby reducing the requirements for memory capacity, but no events will pass undetected, as it is the case with discontinuous sampling. Together with a digital averager the circuit could work as an on-line lifetime identifier.

1. J.H. Jett, R.A. Keller, J.C. Martin, B.L. Marrone, R.K. Moyzis, R.L. Ratliff, N.K. Seitzinger, E.B. Shera, C.C. Stewart, *J Biomolec Struct and Dyn* **7**, (1991), 301-9.
2. J. Tellinghuisen, P.M. Goodwin, W.P. Ambrose, J.C. Martin, R.A. Keller, *Anal Chem* **66**, (1994), 64-72.
3. S.A. Soper, L.M. Davis, E.B. Shera, *J Opt Soc Am* **B 9**, (1992), 1761-9.
4. C.W. Wilkerson, P.M. Goodwin, W.P. Ambrose, J.C. Martin, R.A. Keller, *Appl Phys Lett* **62**, (1993), 2030-2.
5. L. Edman, Ü. Mets, R. Rigler, submitted to *Science*

Design and Application of a Fluorescence Near-Field Microscope

J. Wrachtrup, A. Gruber, M. Vogel, A. Dräbenstedt, C. von Borczyskowski

TU Chemnitz, Inst. of Physics, D-09107 Chemnitz, FRG

In the past years single molecule spectroscopy has experienced an unpreceded blossoming. Part of the work has been carried out at low temperature on highly diluted individual chromophores in solid matrices via high resolution laser spectroscopy. Due to the possible long observation periods and high spectral resolution a number of interesting experiments such as magnetic resonance on single molecules can be performed. However, only a small number of systems may be investigated via this technique. An approach of much wider interest is to study individual molecules with a scanning near-field optical microscope (SNOM). Here even experiments on single molecules at room temperature can be performed. In our contribution we will discuss a novel design for a fluorescence SNOM based on an alternative detection scheme of the shear force detection without optical readout which is of special importance for fluorescence studies. Furthermore the set-up has the advantage of minimal adjustments which allows easy and versatile application. Recordings of the topography of the test samples as well as fluorescence studies on thin solid films will be presented.

Notes