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1 Welcome to St. Louis

On behalf of the organization committee of the "Second Carl Zeiss Symposium on Fluorescence Correlation Spectroscopy (FCS) and Related Methods", we wish you a pleasant stay and numerous productive discussions. Reviewing the program, we were impressed with the large number of promising contributions to this Workshop. The variety of subjects show that Fluorescence Correlation Spectroscopy is a well established research method, which has found its way into many diverse research areas.

The Second Carl Zeiss Workshop on Fluorescence Correlation Spectroscopy (FCS) and Related Methods continues the series of ConfoCor User Clubs organized by Carl Zeiss (1997 Leuven/Belgium, 1998 Jena/Germany, 50 participants each, and 2000 Jena/Germany, 100 participants).

The change in name reflects our intention to invite all scientists interested in FCS instrumentation, theory, applications and related techniques to participate in the meeting, regardless of whether they use a Carl Zeiss ConfoCor or not.

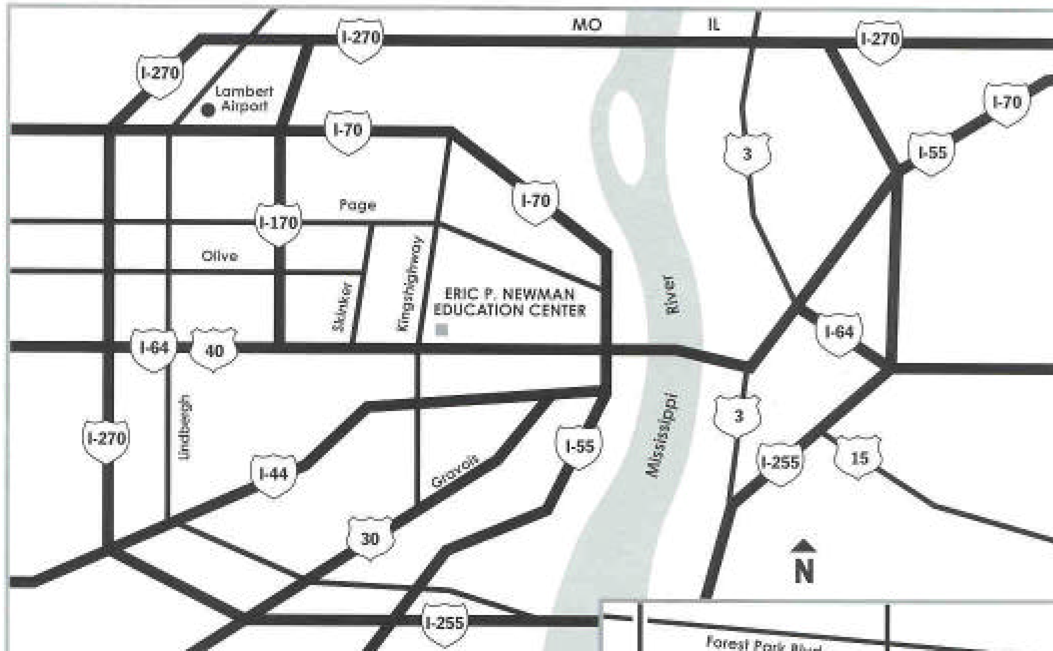
The Workshop begins Wednesday October 25th at 9:00am in the Eric P. Newman Education Center of Washington University. The participation of each of you ensures a successful, productive Workshop.

2 Location

Eric P. Newman Education Center
Washington University
320 S. Euclid
St. Louis, MO 63110
Phone: 314/747-6338
Fax: 314/286-2200

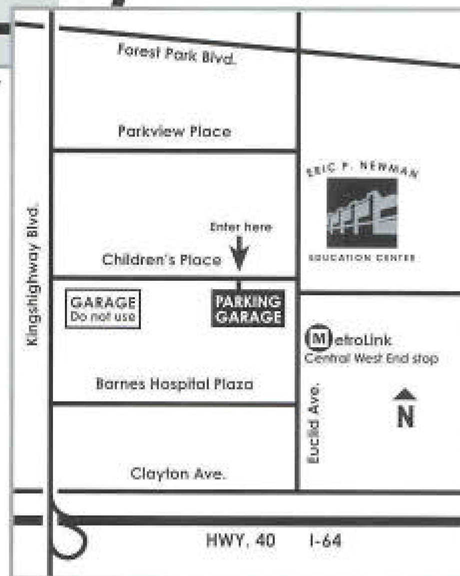
Please be aware that there are two University campuses - the Medical School and the Hilltop Campus. The Newman Center is in the Medical School Campus.

AREA MAPS & DIRECTIONS



ST. LOUIS AREA MAP ▲

WASHINGTON UNIVERSITY MEDICAL CENTER MAP ►



From The Airport:

- I-70 east to I-170
- I-170 south to I-64, U.S. 40
- I-64, U.S. 40 east to Kingshighway
- Kingshighway north to Children's Place
- Children's Place east to parking, Euclid and the Eric P. Newman Education Center

From Downtown, Illinois and points east:

- I-64, U.S. 40 west to Kingshighway
- Kingshighway north to Children's Place
- Children's Place east to parking, Euclid and the Eric P. Newman Education Center

Via MetroLink:

- Detrain at the Central West End stop
- Ascend stairs to Euclid
- North (right) on Euclid to the Eric P. Newman Education Center

3 Hotels and Transportation

From the Airport:
Transportation to and from the hotels can be accomplished via taxi or Metrolink. Taxi service will cost approximately $25-30 each way. Tickets on the Metrolink are offered for $3 round trip anywhere on the system. The Metrolink can be caught at the eastern end of the main airport terminal.

To the Best Western Inn at the Park from the Airport:
Take the train east, (only one direction from the airport), to the Central West End Station. You will be only five blocks from the Hotel. (You also happen to be at the Convention Center where you will return for the conference.) If you have little or no luggage, you could walk to the hotel from this point. Turn right on Euclid and go five (5) blocks to Lindell Blvd. The hotel is immediately on your right at the corner of Euclid and Lindell Blvd. There is a lot of construction in this area. The hotel offers shuttle service between the train station and the hotel between 6:30am and 6:30pm. At the top of the platform at the street level you can call the hotel for pickup. It takes approximately 10-15 minutes for the shuttle to arrive. 800/373-7501 or 314/367-7500

To the Drury Inn at St. Louis Station from the Airport:
Take the train east, (only one direction from the airport), to Union Station. You must walk through the parking area to get to the hotel. It is on South 21st Street. The phone number is 314/231-3900.

Transportation to the Conference Center:
The Best Western provides shuttle service to the Conference Center. Due to the large number of participants staying at this location, there will be at least three bus runs between 7:45 and 8:30am. You must sign up on Tuesday evening for shuttle service at the front desk to reserve a specific time. Shuttles will depart promptly.

The Metrolink is the most direct route from the Drury Inn to the Conference Center. Two stops from the Union Station you go west and get off at Central West End Station. Head up the platform and the Conference Center is immediately to your left on Euclid Avenue.

4 Dinner at Bevo Mill Restaurant

On Wednesday evening there will be a cocktail reception and dinner at Bevo Mill Restaurant. Bevo Mill is an old converted brewery and a St. Louis Landmark. Buses will depart from the Convention Center at 6:00pm, and will return to both hotels and make a stop at the Convention Center for those local participants who opt to leave their cars at the Convention Center.

5 International Scientific Committee

Dr. Elliot Elson
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Prof. Rudolf Rigler
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6 Schedule

Wednesday October 25, 2000

Time	Authors	Title	Abstract (page)
		Session I: Basics Chair: E. Elson	
9:00-9:30	Elliot Elson	Introduction to the Symposium	11
9:30-10:00	Rudolf Rigler	"FCS and Single Molecule Analysis: New Tools in Bioscience"	
10:00-10:30	Hong Qian	Stochastic Theory and Statistical Analysis of FCS	23
10:30-11:00	Coffee Break		
11:00-11:30	Gregoire Bonnet	Unusual DNA Breathing Modes Unraveled by Fluorescence Correlation Spectroscopy	13
11:30-12:00	Alexander Sytnik	Structural Dynamics of Single Light-Harvesting Complexes LH2	25
12:00-1:00		**Lunch and Poster Viewing**	

Wednesday October 25, 2000

Time	Authors	Title	Abstract (page)
Session II: Studies on and in Cells and Membranes Chair: H. Qian			
1:00-1:30	Nils O. Petersen	Image Cross-Correlation Spectroscopy: Quantitative Measurements of Intermolecular Interactions for Receptors in Cells	21
1:30-2:00	Nancy Thompson	Total Internal Reflection Fluorescence Correlation Spectroscopy	26
2:00-2:30	Petra Schwille	Dual-Color Fluorescence Cross-Correlation and its Perspectives for Intracellular Applications	24
2:30-2:45	Coffee Break		
2:45-3:15	Aladdin Pramanik	The Image and Dynamics of Ligand-Receptor Interactions in Living Cells Monitored by ConfoCor 2	22
3:15-4:00	Masataka Kinjo	Fluorescence Correlation Spectroscopy as a Tool for Study of Microenvironment in Living Cells	18
4:00	**Molecular Biophysics Seminar**		
	Carlos Bustamante	"Following DNA Replication One Molecule at a Time"	
6:00	Bus picks up for dinner at Bevo Mill Buses will return at 10:00pm to respective drop off points		

Thursday October 26, 2000

Time	Authors	Title	Abstract (page)
Session III: Technology Chair: N.L. Thompson			
9:00-9:30	Sandra Turconi	Lifetime Measurements in a Confocal Volume	27
9:30-10:00	Samuel Hess	The Effects of Focal Volume Optics on Experimental Artifacts and Signal to Noise in Fluorescence Correlation Spectroscopy	17
10:00-10:30	Christian Eggeling	Do Confocal Techniques Shed New Light on FRET?	15
10:30-11:00	Coffee Break		
11:00-11:30	Peet Kask	A New Scope in Fluorescence Brightness Analysis: Multidimensional Fluorescence Intensity Distribution Analysis	
11:30-12:00	Daniel Larson	Fluorescence Correlation Spectroscopy in Heterogeneous Samples	19
12:00-2:00	**Lunch and Poster Viewing**		

Thursday October 26, 2000

Time	Authors	Title	Abstract (page)
Session IV: Applications Chair: N.O. Petersen			
2:00-2:30	Ulrich Haupts	Applications of FIDA to Different Assay Scenarios	
2:30-3:00	Edmund Matayoshi	Applications of FCS and Fluorescence Polarization to Protein-Ligand Interactions	20
3:30-4:00	Coffee Break		
4:00-4:30	Jan Bieschke	Sifting Amyloids: Highly Sensitive Detection and Characterization of Pathological Aggregates for the Diagnosis of Creutzfeldt-Jakob and Alzheimer's Disease	12
4:30-5:00	Zeno Foldes-Papp	Detecting and Characterizing Biological Molecules and Their Interactions on the Molecular Basis of Process by FCS	16

7 Poster Titles

No.	Authors	Title	Abstract (page)
1	N. Boyd University of Western Ontario London, Ontario, Canada	Using Imaging Correlation Spectroscopy to Analyse the Effect of Integrins on the Distribution of Adaptor Protein-2	
2	C. Eggeling, P. Kask, K. Palo, S. Jager, L. Brand, K. Gall EVOTEC Biosystems AG 22525 Hamburg, Germany	A New Scope in Fluorescence Brightness Analysis: Multidimensional Fluorescence Intensity Distribution Analysis	14
3	A. Kenworthy, J. Lippencott-Schwartz National Institutes of Health CBMB, NICHD, NIH Bethesda, MD, USA	Comparison of FCS and Confocal FRAP Measurements of GFP-tagged Proteins in Living Cells	
4	T. Laurence, D.S. Chemla, S. Weiss	Photon Arrival Interval Distributions (PAID): A New Way to Visualize and Measure Brightness and Correlations	

8 Abstracts

Introduction to the Symposium

Elliot Elson

Washington University School of Medicine, St. Louis, MO, USA

Fluorescence Correlation Spectroscopy (FCS) was originally developed as a method for measuring dynamic characteristics, e.g., diffusion and chemical reaction rates, of systems in equilibrium. This is accomplished by a statistical analysis of the time courses of spontaneous fluctuations of the number of fluorescent molecules in a defined open sample volume. More recently, methods have been developed to determine differences in spectroscopic properties and degrees of aggregation by analyzing the amplitudes of the fluctuations. That FCS focuses on the stochastic behavior of individual molecules brings it into a close relationship with the new and burgeoning field of single molecule studies. In practice, FCS is readily carried out as a single molecule measurement. Recent developments in experimental procedure have made FCS measurements both routine and robust. This has led to a further elaboration of basic concepts, development of measurement methods and a wide range of basic and technological applications. At this conference, we will discuss issues relating to each of these areas – concepts, methods and applications.

Sifting Amyloids: Highly Sensitive Detection and Characterization of Pathological Aggregates for the Diagnosis of Creutzfeldt-Jakob and Alzheimer's Diseases

Jan Bieschke, Armin Giese **, Hans Kretzschmar**, Manfred Eigen**

* Max-Planck-Institute for Biophysical Chemistry, Am Fassberg, 37077 Göttingen, Germany
** University of Munich, Dept. of Neuropathology, Marchionistr. 17, 81377 Munich, Germany

In Alzheimer's disease (AD) as well as in prion diseases, such as Creutzfeldt-Jakob-Disease (CJD), a structural transition of a protein fragment to a pathogenic isoform is at the center of the pathogenic process. Subsequently, the pathogenic proteins aggregate to form amyloid fibrils, which can accumulate in the nerve tissue in the form of neurotoxic plaques. A definite diagnosis of prion diseases relies on the detection of pathological prion protein (PrP^{Sc}). So far, no test for PrP^{Sc} in body fluids such as cerebrospinal fluid (CSF) has been available for this purpose. Based on a setup for confocal dual-color fluorescence correlation spectroscopy (FCS), a technique suitable for single molecule detection, we developed a novel, highly sensitive detection method for pathological aggregates. Aggregates were labeled by specific antibody probes tagged with fluorescent dyes, resulting in intensely fluorescent targets. The combination of a scanning setup with a two-color intensity analysis (*SIFT, scanning for intensely fluorescent targets*) allowed to detect fluorescent aggregates at femtomolar concentration.

In a diagnostic model system, PrP^{Sc} aggregates were detected down to a concentration of 2pM PrP^{Sc}, corresponding to an aggregate concentration of approximately 2fM, which was more than one order of magnitude more sensitive than Western blot analysis. CJD specific aggregates could be detected specifically in presence of a 10^3-fold excess of AD-specific aggregates and vice versa.

The technique was applied to the detection of pathological aggregates in the spinal fluid of patients suffering from CJD and AD. PrP^{Sc}-specific signals could be detected in a number of CSF samples from CJD patients but not in control samples. When using AD-specific probes, aggregate signal was detected in the CSF of AD-patients.

By *SIFT*, pathological PrP was detected in CSF for the first time. The same technique provides the basis for a rapid and specific test for other disease-associated amyloid aggregates such as Alzheimer's disease. Furthermore, the method can be adapted to a number of disease specific aggregation processes and could provide a versatile tool for the differential diagnosis of a number of neurodegenerative diseases in an early stage.

Unusual DNA Breathing Modes Unraveled by Fluorescence Correlation Spectroscopy

Grégoire Bonnet*, Oleg Krichevsky**, Albert Libchaber*

*Center for Studies in Physics and Biology, The Rockefeller University, 1230 York Ave., New York, NY 10021

**Department of Physics, Ben Gurion University, Beer Sheva, 84105 Israel

The opening of basepairs of double-stranded DNA is a prerequisite for many biological DNA processing events, (replication, transcription, promoter recognition). The dynamics of basepair opening has been classically studied by NMR of the exchange of protons from imino groups with catalysts. However, this technique provides only a steady-state measurement of the lifetime of a closed basepair. We use FCS (fluorescence correlation spectroscopy) and fluorescence quenching to monitor the dynamics of the open basepair in dsDNA.

We find a typical lifetime of 100ìs for the open complex, two orders of magnitude smaller than the timescales measured by NMR. The inhomogeneity of timescales is unraveled as a stretched exponential (ß=0.5) for the correlation function. Our results do not comply to the standard nearest-neighbor model of DNA basepairing, and hint towards unusual structures of DNA, leading to enhanced breathing modes.

We will present our implementation of FCS, and emphasize practical considerations in the design of the samples to eliminate diffusion and photochemistry artifacts.

A new scope in fluorescence brightness analysis: Multidimensional fluorescence intensity distribution analysis

Peet Kask, Kaupo Palo, Stefan Jäger, Leif Brand, Christian Eggeling, and Karsten Gall

EVOTEC BioSystems AG, Schnackenburgallee 114, 22525 Hamburg

Fluorescence correlation spectroscopy (FCS) has proven to be a powerful technique for distinguishing single molecules due to their diffusion properties. It has found a convincing complement in the form of fluorescence intensity distribution analysis (FIDA; Kask et al., 1999; independently Chen et al., 1999) that resolves different species on the basis of their molecular brightness. This technique has recently been extended to two dimensions/detectors (2D-FIDA; Kask et al., 2000), opening the way to considerably broader applications. Polarization studies on the single molecule level are only one out of many possible employments. If used e.g. with spectrally tuned detectors, 2D-FIDA allows also the access to biochemical assays utilizing fluorescence resonance energy transfer (FRET) or, in combination with two-color excitation, coincidences of different molecules in time and space. Compared to a number of other methods, it is superior due to its ability to separate absolute concentrations of different species with extreme accuracy.

Another way to increase the read-out effectively by one dimension is the analysis of multiple fluorescence intensity distributions (FIMDA; Palo et al., 2000) obtained with a single detector. This novel technique combines the features of FCS and FIDA, i.e. it distinguishes fluorescent species on the basis of both, the specific molecular brightness and the translational diffusion time, and thus breaks the individual limits of FCS and FIDA.

The talk will describe the various techniques of analyzing fluorescence intensity distributions. The analysis will be demonstrated on exemplary HTS compatible biochemical assays for therapeutic targets.

Chen, Y., J.D. Müller, P.T. So, and E. Gratton. 1999. Biophys. J. **77**(7):553 - 567

Kask, P., K. Palo, D. Ullmann, and K. Gall. 1999. *Proc. Natl. Acad. Sci. USA.* **96**:13756 – 13761

Kask, P., K. Palo, N. Fay, L. Brand, Ü. Mets, D. Ullmann, J. Jungmann, J. Pschorr, and K. Gall. 2000. *Biophys. J.* **78**(4):1703 – 1713

Palo, K., Ü. Mets, S. Jäger, P. Kask, and K. Gall. 2000. *Biophys. J.* (submitted)

Do confocal techniques shed new light on FRET ?

Christian Eggeling, Leif Brand, Stefan Jäger, and Karsten Gall

EVOTEC BioSystems AG, Schnackenburgallee 114, 22525 Hamburg, Germany

Fluorescence Resonance Energy Transfer (FRET) is a photophysical effect that can be used to reveal a lot of information not only on the internal structure of biomolecules but also on the interaction of different molecules. In recent times it has attracted much attention in the life sciences including its utilization in High Throughput Screening (HTS) for the identification of potential drug candidates. From the experimental perspective, this effect has been mostly used by recording the overall fluorescence intensity in a wavelength selective manner. Up to now, confocal techniques like Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Intensity Distribution Analysis (FIDA) play only a minor role in an industrial context. FCS and FIDA as well as their two-dimensional counterparts Fluorescence Crosscorrelation Spectroscopy (FxCS) and 2D-FIDA extract information on the single molecule level and are very attractive for HTS purposes since they are invariant to miniaturization, thus offering a great cost reduction potential. Here, we compare these techniques with respect to their individual capabilities and accuracy quantifying the FRET effect.

We studied an enzymatic reaction as a representative biological test system by determining the Proteinase K activity on a peptide substrate. Upon the addition of Proteinase K, the peptide which is labeled with a donor as well as with an acceptor dye is cleaved, resulting in a diminishing FRET signal. For analysis, the different methods, FCS, FIDA, FxCS, and 2D-FIDA together with the evaluation of the overall donor and acceptor fluorescence intensity are applied and the results are compared.

Detecting and characterizing biological molecules and their interactions on the molecular basis of processes by FCS

Zeno Földes-Papp and Rudolf Rigler
Department of Medical Biophysics, Karolinska Institute, S-17177 Stockholm, Sweden

In this lecture, we provide practical examples of molecular biological, biochemical and biophysical properties that can be analyzed by FCS. To illustrate the approaches, to assess the interactions between soluble fluorescent-tagged ligand/substrate and target, we examined a ribosome display system, a repertoire of diverse immunoglobuline specificities and a mutant enzyme generated by random mutagenesis of amino acids. The possibility to use FCS as a quantitation method might open up other applications beyond the scope so far; the biological libraries can be used for FCS-based selection technologies.

We developed a new concept for polymerase chain reactions applying two primers with different colors. This allowed the specific detection of the amplification product in the presence of large excess of primers and nucleotides without the need for separation steps. The two-color cross-correlation only resulted from fluorescence intensity fluctuations for which the specificity probes (primers) in two colors were incorporated into (or from the viewpoint of the FCS model 'bound to') the same target molecule. This is superior to the concept of the fluorogenic energy transfer probe in the TaqMan™ PCR detection strategy and to the concept of fluorogenic energy transfer hybridization probes in the LightCycler™ PCR detection strategy. Two-color cross-correlation FCS could specifically detect the target at concentrations below 10^{-18} M.

The idea to use single molecule spectroscopy for the detection of dye-tagged nucleotides following exonucleolytic degradation of high-density labeled DNA has been worked out for single molecule DNA sequencing. The results of complete, error-free labeling of test and native DNA sequences with dye-tagged nucleotides were obtained by a thermophilic, 'tailor-made' exonuclease-deficient DNA polymerase. The fluorophore-bearing nucleotide fully replaced the normal nucleotide in the reaction mixes. Alternatively, this can be achieved by filling-up reactions. In the new concept of high-density labeling of DNA developed by us, the quantitative and error-free incorporation of dye-tagged nucleotides was proven by two-color cross-correlation FCS in combination with the molecular biological technologies, which we first developed for this purpose. The recent developments of two-color cross-correlation FCS provided a unique sensitivity for the detection and analysis of molecular systems.

In summary, the studies presented in this lecture focus on spotlights of detecting and characterizing biological molecules and their interactions at the level of single molecules. FCS is still very much an evolving methodology and by no means a routine method.

THE EFFECTS OF FOCAL VOLUME OPTICS ON EXPERIMENTAL ARTIFACTS AND SIGNAL TO NOISE IN FLUORESCENCE CORRELATION SPECTROSCOPY

Samuel T. Hess, Petra Schwille, and Watt W. Webb

Fluorescence correlation spectroscopy (FCS)[i] is an increasingly powerful tool to investigate dynamic processes in biological systems on the submicrosecond to seconds timescales. The standard assumption of an ellipsoidal gaussian illumination and collection profile in three dimensions is invalid under many experimental conditions, and introduces artifacts into the measured autocorrelation. Numerical modeling of the point spread function for a high numerical aperture objective lens has been used to predict the diffusion time, autocorrelation function, collected fluorescence, observation volume, average number of molecules in that volume, and count rate per molecule (η), as a function of detector aperture and objective underfilling fraction. The simulation results are compared with FCS measurements. The signal-to-noise ratio, proportional to η, is maximized for a detector aperture radius of 4.5 optical units, or a ~50 µm diameter pinhole for a 40X 1.2 NA water immersion objective. Measurement artifacts are minimized by underfilling the objective back aperture and using a small confocal detector aperture, or by using two-photon excitation without a detector aperture. The artifacts manifest themselves in the correlation function as an extra exponential component with amplitude 0.05-0.30 which also shifts the fitted diffusion time, or might be represented as a second diffusing species. These artifacts of the optics are entirely unrelated to the real properties of the sample and may be deceptive to inexperienced users.

[i] D. Magde, E. Elson, and W.W. Webb, "Thermodynamic Fluctuations in a Reacting System- Measurement by Fluorescence Correlation Spectroscopy," Phys. Rev. Lett. **29** (11) 705-708 (1972).

Fluorescence Correlation Spectroscopy as a Tool for Study of Microenvironment in Living Cells

Masataka Kinjo, Naoto Yoshida, Takuya Muto and Kenta Saito
Laboratory of Supramolecular Biophysics, Research Institute for Electronic Science, Hokkaido University, Sapporo 060-0812, Japan

Fluorescence correlation spectroscopy (FCS) is a unique method to determine the diffusional properties and the concentrations of fluorescent molecules in aqueous solutions with high spatio-temporal resolution. Using FCS measurement, we have tried to analyze the dynamic behavior of fluorescent molecules in living cells.

The diffusion coefficients (D) of calcein (MW655) rhodamine-labeled latex beads (14 and 35nm in diameter) ingested in endosomes in cultured bovine aortic endothelial cell were measured. The average values of the obtained D were calculated to be 50-60% of those in water, suggesting that the physical microenvironment of the aqueous phase of endosome is very similar to water and/or culture medium.

On the other hand, the moblility of transiently expressed GFP (MW 27000) in cytoplasm, and fluorescent-labeled tetra peptide (Rho-S-S-G-A) injected in squid giant axon were much slower than those in water. This suggests a part of the mobility of macromolecule is restricted in the cytosol and the environment of cytoplasm is not similar to endosome.

The dynamics of macromolecule in living cells are affected not only by the viscosity but also by other physical properties of its circumstance such as pH and hydrophobicity. FCS can analyze these parameters by the measurement of fluorescence intensity per molecule and also number of molecules.

Therefore, it is expected that more detailed microenvironment of internal space of cell and organelles can be clarified by FCS.

Fluorescence Correlation Spectroscopy in Heterogeneous Samples

Daniel R. Larson[1], Watt W. Webb[2]

[1] Biophysics, Cornell University
[2] Applied and Engineering Physics, Cornell University

Fluorescence correlation spectroscopy has been utilized effectively for studies of non-equilibrium processes in homogenous samples. The measurement of diffusion coefficients of fluorescent species in solution is an example of a common application. However, it is also desirable to extend fluorescence correlation spectroscopy (FCS) to heterogeneous samples such as living cells. FCS has been done in living cells, but the implementation has been hampered by a lack of precise image information and a limited understanding of the factors that affect correlation measurements on heterogeneous samples. We have designed an instrument that combines multiphoton imaging and multiphoton FCS. This microscope is designed for 'point-and-click' FCS: the user can visualize the fluorescent sample using laser scanning multiphoton microscopy and then select a region of interest to perform FCS. Multiphoton excitation is a critical part of this integrated instrument. Intrinsically defined three-dimensional focal volumes ensure that one can perform correlation measurements on the same region that is visualized with laser scanning microscopy. The microscope is based on a modified Bio-Rad MRC 600 scan box, a Hamamatsu GaAsP photon counting PMT, and an ALV correlator card. We have characterized the stability of this instrument and compared correlation measurements taken with this microscope to those taken in a traditional manner. The resolution of this microscope is ultimately limited by jitter in the location of a parked laser beam and long-term mechanical drift. Several preliminary applications will be discussed.

Applications of FCS and Fluorescence Polarization to Protein-Ligand Interactions

Edmund Matayoshi[1], Kerry Swift[1], and Sergey Tetin[2]

From the [1]Department of Structural Biology, Drug Discovery, Pharmaceutical Products Division, and the [2]Therapy Monitoring Area, Abbott Diagnostics Division
Abbott Laboratories, Abbott Park, IL 60064

Abstract

FCS and Fluorescence Polarization (FP) provide researchers with biophysical tools of very general applicability to studies on protein-ligand associations in homogeneous solution. While FP is a mature methodology that has been used for a variety of biological problems for over 30 years, FCS is currently evolving and commercial instrumentation has become available only recently. In this talk FCS and FP will be compared by presenting examples from our laboratories which span both ligand size (from small molecules of MW ~ 1 kDa, to large protein ligands) and affinity (picomolar to micromolar). The discussion will show that while there clearly is overlap in the types of problems amenable to FCS and FP, the information each provides is unique and highly complementary, and it will be desirable to apply them in tandem whenever feasible.

Image Correlation Spectroscopy: Quantitative Measurements of Intermolecular Interactions for Receptors in Cells

Nils O. Petersen
University of Western Ontario, London, Ontario, Canada

Image Correlation Spectroscopy provides a convenient and quantitative measurement of receptor cluster density and the degree of aggregation. Image Cross-Correlation Spectroscopy provides the additional ability to measure the density of clusters with more than one receptor as in co-localization experiments. When performed carefully, it is possible to measure the fraction of clusters of one receptor that is associated with the other and vice versa.

This presentation will describe the experimental approach, the image analysis approach and the tools for interpretation. Selected examples of interactions of receptors among themselves and with coated pits will be used to illustrate the power and limitations of the approach.

The Image and Dynamics of Ligand-Receptor Interactions in Living Cells Monitored by ConfoCor 2

Aladdin Pramanik and Rudolf Rigler, Dept. of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 77 Stockholm, Sweden

Fluorescence correlation spectroscopy (FCS) allows detection of the interaction of ligands with binding sites of receptors on the molecular level in their native environment on cell surfaces with single-molecule detection sensitivity. The fact that with FCS it has been possible to discover new receptors for the proinsulin C-peptide (Rigler et al. (1999) PNAS 96:13318-13323) and the neuroactive natural product kavain (Boonen et al. (2000) Planta Med. 66:7-10), and a new subtype receptor for the neuropeptide galanin (Pramanik et al. (1999) Biomed. Chromatogr. 13:119-120), makes an important point that FCS permits the identification of receptors or target molecules which were not possible before to detect by other conventional binding techniques, specially by isotope labeling. Very recently, we have for the first time been able to make images of ligand-receptor interaction and to measure its Brownian motion at the same spot in living cells using ConfoCor 2. These images confirm our discovery of C-peptide (CP) receptor in the cell membrane since FCS measurement at the membrane part of an image shows a similar diffusion time for CP-receptor complex as obtained earlier by ConfoCor 1. The binding of rhodamine-labeled CP (Rh-CP) to receptors presents a localization of colour in the cell membrane. The localization of colour disappears upon addition of non-labeled C-peptide, confirming that Rh-CP binds specifically to a receptor. Both the images and correlation curves for the Rh-CP binding on cell membranes will be presented and discussed.

Stochastic Theory and Statistical Analysis of FCS

Hong Qian, Department of Applied Mathematics, University of Washington, Seattle, WA, USA

The theoretical foundation of FCS is the stochastic dynamics of single molecules in aqueous solution. We show how the conventional theory of FCS is related to the transport properties and chemical kinetics of single molecules. Even though statistical analyses based time-correlation function and waiting time distributions are powerful methods for analyzing FCS data without explicit molecular models, in general, a model-dependent statistical analysis is necessary for more complex kinetics of biological macromolecules. We argue stochastic thinking is essential for interpreting experimental data obtained by FCS.

DUAL-COLOR FLUORESCENCE CROSS-CORRELATION AND ITS PERSPECTIVES FOR INTRACELLULAR APPLICATIONS

Petra Schwille, AG Experimentelle Biophysik, Max-Planck-Institut für biophysikalische Chemie, am Fassberg 11, 37077 Göttingen

Confocal fluorescence correlation spectroscopy, as a time-averaging fluctuation analysis combining maximum sensitivity with high statistical confidence, has proved to be a very versatile and powerful tool for detection and temporal investigation of biomolecules at ultralow concentrations on surfaces, in solutions and in living cells. To probe the interaction of different molecular species for a detailed understanding of biologically relevant mechanisms such as protein-protein interactions or enzyme kinetics, cross-correlation studies on dual- or multiple fluorophore assays with spectrally distinct excitation and emission properties are particularly promising because of their considerably increased detection specificity. In respect to intracellular applications of confocal FCS, two-photon excitation of fluorescent probes has recently been shown to be a particularly attractive alternative since many problems encountered in spectroscopic measurements on living tissue such as light scattering, autofluorescence and photodamage can be reduced. It is demonstrated that two-photon excitation combined with dual-color FCS can be the key to significantly simplify simultaneous investigations of multiple fluorescent species on a single molecule scale. Two-photon excitation allows to access common fluorophores of largely distinct emission by the same excitation wavelength, because differences in selection rules and vibronic coupling can induce considerable shifts between the one-photon and two-photon excitation spectra. The concept of dual-color, two-photon fluorescence cross-correlation (TPCC) analysis is introduced and experimentally demonstrated with an established assay probing the selective cleavage of dual labeled DNA substrates by restriction endonuclease *Eco*RI. The experimental performance that can be obtained is found to be at least comparable to respective one-photon setups employing two different excitation lines, in terms of detection specificity. In this respect, the dual-color variant offers great promises for simplified measurement schemes of intracellular cross-correlation applications.

Literature

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Kettling U., Koltermann A., Schwille P. and Eigen M. (1998) Real time enzyme kinetics of restriction endonuclease EcoRI monitored by dual-color fluorescence cross-correlation spectroscopy, Proc. Natl. Acad. Sci. USA. 95 (4) 14116-14120

Schwille P., Haupts U., Maiti S., and Webb W.W. (1999) Molecular dynamics in living cells observed by fluorescence correlation spectroscopy with one- and two-photon excitation, Biophys. J. 77:2251-2265

Heinze K.G., Koltermann A. and Schwille P. (2000) Simultaneous Two-Photon Excitation of Distinct Labels For Dual-Color Fluorescence Cross-Correlation Analysis, Proc. Natl. Acad. Sci. USA, in press

Structural Dynamics of Single Light-Harvesting Complexes LH2

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Single molecule methods are well suited to study features of conformational dynamics that are not readily obtained in bulk measurements. We have employed scanning confocal microscopy to investigate structural dynamics of single light-harvesting complexes LH2 from purple bacteria Rhodopseudomonas acidophila. The structure of this photosynthetic assembly is notable for its high symmetry arrangement of the nine dipeptides that form cylindrical scaffold holding two rings of bacteriochlorophylls. Based on the crystal structure, the LH2 complex must be electronically cylindrical. Single LH2 assemblies were immobilized on mica surfaces and examined by measuring their fluorescence spectra and anisotropic properties. The average fluorescence spectra of free and mica-immobilized LH2 complexes were identical confirming the structural integrity of surface-bound assemblies. The spectral peak position and the width of single LH2s were fluctuating on the time scale of seconds. When single LH2 assemblies were examined by detecting their fluorescence after polarized light excitation, it was found that they are generally not cylindrically symmetric. Surface-bound LH2 complexes were distorting under equilibrium conditions into electronically elliptical structures with lifetimes of milliseconds to seconds. We are relating this dynamic electronic behavior of single LH2 complexes to the mobile dissociation-association at the interface between two dipeptides. It would be challenging to observe these effects in the bulk, where they are submerged by the incoherent dynamics of many LH2s.

TOTAL INTERNAL REFLECTION FLUORESCENCE CORRELATION SPECTROSCOPY

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Total internal reflection with fluorescence correlation spectroscopy (TIR-FCS) is a method for characterizing the dynamic behavior and absolute concentrations of fluorescent molecules near the interface of solution and a planar substrate. A general theoretical form for the TIR-FCS autocorrelation function when both surface association/dissociation kinetics and diffusion through the evanescent wave contribute to the fluorescence fluctuations has been derived. The autocorrelation function is predicted to depend, in general, on the kinetic association and dissociation rate constants, the surface site density, the concentration of fluorescent molecules in solution, the solution diffusion coefficient, and the depth of the evanescent field. Approximate expressions applicable to samples in which either surface association/dissociation or diffusion through the evanescent field is dominant have also been derived. The theoretical results have been compared with experimental TIR-FCS data for the diffusion of fluorescently labeled IgG through evanescent fields adjacent to substrate-supported planar membranes as well as for the specific interaction of fluorescently labeled IgG with Fc receptors that have been purified and reconstituted into substrate-supported planar membranes.

Application of Confocal Fluorescence Lifetime Detection in HTS Assays

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Fluorescence has established itself as the detection technique of choice for ultra-HTS due to its high sensitivity, signal information content and amenability to miniaturisation[1]. However, fluorescence measurements can be prone to artifacts due to background interactions (e.g. autofluorescence, light scatter or inner-filter effects) arising from the test biological matrix or test compounds. Consequently, several approaches have been developed to address this problem, such as confocal detection methods, multi-photon excitation and time-resolved measurements. Within routine HTS applications, time-resolution was so far only been implemented in time-gated measurements of long-lived lanthanide fluorescence signals (e.g. in time-resolved energy-transfer [TRET]). In this case the separation of the analyte signal (τ ~msec.) from background is straightforwardly achieved by appropriately gating the emission measurement. The fact that the lifetime of the fluorescent label can change upon interaction (e.g. in TRET the donor lifetime is considerably shortened) has not, so far, been used as an independent detection parameter. Moreover, lifetime-resolved measurements of standard "prompt" dyes (e.g. fluorescein, rhodamine, Cy dyes etc.) are currently not possible using standard HTS-compatible instruments.

The development of methods to identify fluorescence tracer lifetimes in uHTS could be extremely important. One major reason behind this is that lifetime decays have finite dimensions, whereas simple fluorescence intensity (FLINT) is a function of a whole range of factors (concentration, volume, optical set up etc.). Accordingly, FLINT-based assays require large signal changes (>5-fold) in order to produce robust HTS runs and are therefore restricted to certain applications (e.g. enzyme substrates). Robust lifetime measurements may open up many new types of application (e.g. using environmentally sensitive probes).

In this paper, we describe the first application of lifetime as a readout parameter in HTS. Confocal Lifetime Analysis (cFLA, Evotec Biosystems) was applied to both model and real biological assay systems. Experiments were designed to test the amplitude of the lifetime change, data quality at short measuring times and robustness of the readout in presence of library compounds. These results will be discussed in context of other fluorescence methods, both confocal and macroscopic, presently applied in HTS.

References:

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