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Fluorescence and single molecule analysis in cell biology

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ABSTRACT

An overview is presented which describes the development of fluorescence spectroscopy at the cellular level from its beginning as a quantitative tool to determine the content of cellular components to its present use. Analysis of individual biomolecules, their transport and kinetics within a single cell is now possible.

1. Introduction

The start of quantitative cell biology was initiated by Torbjörn Caspersson at Karolinska Institutet when he introduced UV microspectroscopy for analysis of the content of nucleic acids and proteins by measurement of their UV absorption. Caspersson's work started with his thesis [1] and provided crucial information on nucleic acid and protein synthesis in the nucleus and cytoplasm during the cell cycle [2]. As an important complement, fluorescence, providing highly sensitive and specific signals, was recognized and a systematic analysis was started with the construction of the first fluorescence microspectrograph [3]. This instrument allowed us to measure the turnover of nucleic acids (DNA and RNA) by complex formation with Acridine Orange in individual cells during the cell cycle. The sensitivity was high enough to detect double stranded nucleic acid (DNA) in single mitochondria, as well as in individual chromosomes [3].

1.1. Acridine Orange in nucleic acid analysis

Acridine Orange (AO), a strongly fluorescent basic acridine dye, interacts in different manners with negatively charged nucleic acid strands, depending on the tertiary structure of the nucleic acid chain. In double stranded nucleic acids, like DNA, the monomeric form of AO is preferred for interactions, mainly by the process of intercalation, while in single stranded DNA and/or RNA, a polymeric (aggregated form) form is observed. Both AO forms can be distinguished by their characteristic color (green for the DNA bound AO and red for the RNA bound AO). Based on this behavior, a strategy was developed to measure the synthesis of DNA in the nucleus and of RNA in the cytoplasm [3,4]. Similar behavior was found for acridine compounds, like quinacrine which later on became the prime tool for analysis of individual chromosomes by their quinacrine binding [5]. A particular finding was the observation that the banding capacity of AO and nucleic acid protein complexes could provide information on histone-modulated gene expression [6].

1.2. Fluorescence and chemical kinetics

In the summer of 1967, I met Manfred Eigen at the Nobel Symposium in Södergarn, on the island of Lidingö within the Stockholm archipelago. Our discussions resulted in an invitation to come to Göttingen to the Max Planck Institute for physical chemistry. Starting from my experience with Acridine/nucleic acid complexes, I introduced the analysis of fluorescence to chemical relaxation kinetics and built the first fluorescence temperature instrument which became a forerunner for most of the T jump machines built at this time. The papers on the fluorescence T jump [7] belong to this period, as well as those on the detailed kinetics of acridines interacting with nucleic acids of different base composition [8], and those on the conformational kinetics of fluorescently-labeled transfer RNA [9].

A remarkable development was the idea to use fluctuations of chemical systems instead of relaxation techniques to analyze the kinetics of a chemical reaction. Based on the fluctuation–dissipation theorem, thermal fluctuations, acting on individual particles (molecules) and their correlation will provide the same result as shifting the equilibrium state in a relaxation experiment. Together with Leo de Mayer and Klaus Gnädig we started in 1969 the first experiments to prove the validity of this idea and as example we used the system of acridine/DNA interactions [10]. Similar ideas where followed up by Elliott Elson and Watt Webb, and a first account of the fluctuations of a dye/nucleic acid equilibrium

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(ethidium bromide/DNA) was published in 1972 [11]. Within the following years the scene for a new tool in the analysis of molecular fluctuations [12–14] was set. It should, however, take another 15 years until fluorescence correlation spectroscopy (FCS) made its breakthrough.

1.3. FCS and single molecule detection

The start for a new area of identification of molecular fluctuations was set by the introduction of the confocal volume element determined by the optical diffraction limit of a focused laser beam and the detection of the radiation (fluorescence) emitted by a collection of particles [15–17]. In our experiments, we could show that single dye molecules could be observed with a background which was orders of magnitude (1000-fold) smaller than the signal [18–20] (Fig. 1A). The high sensitivity and almost noise-free detection was reached by avalanche photo diodes (APD) originally developed by RCA (now Perkin Elmer).

The detection of single molecules in a confocal volume element allowed us to establish their image. Together with Johannes Dapprich, a student of Manfred Eigen, we constructed a single molecule imager based on confocal APD detection combined with a scanning stage, and were able to create the image of Rhodamin-labeled DNA strands [21] (Fig. 1B). This was the start of single molecule imaging which now is performed in many laboratories. Different modifications are used with the confocal volume element

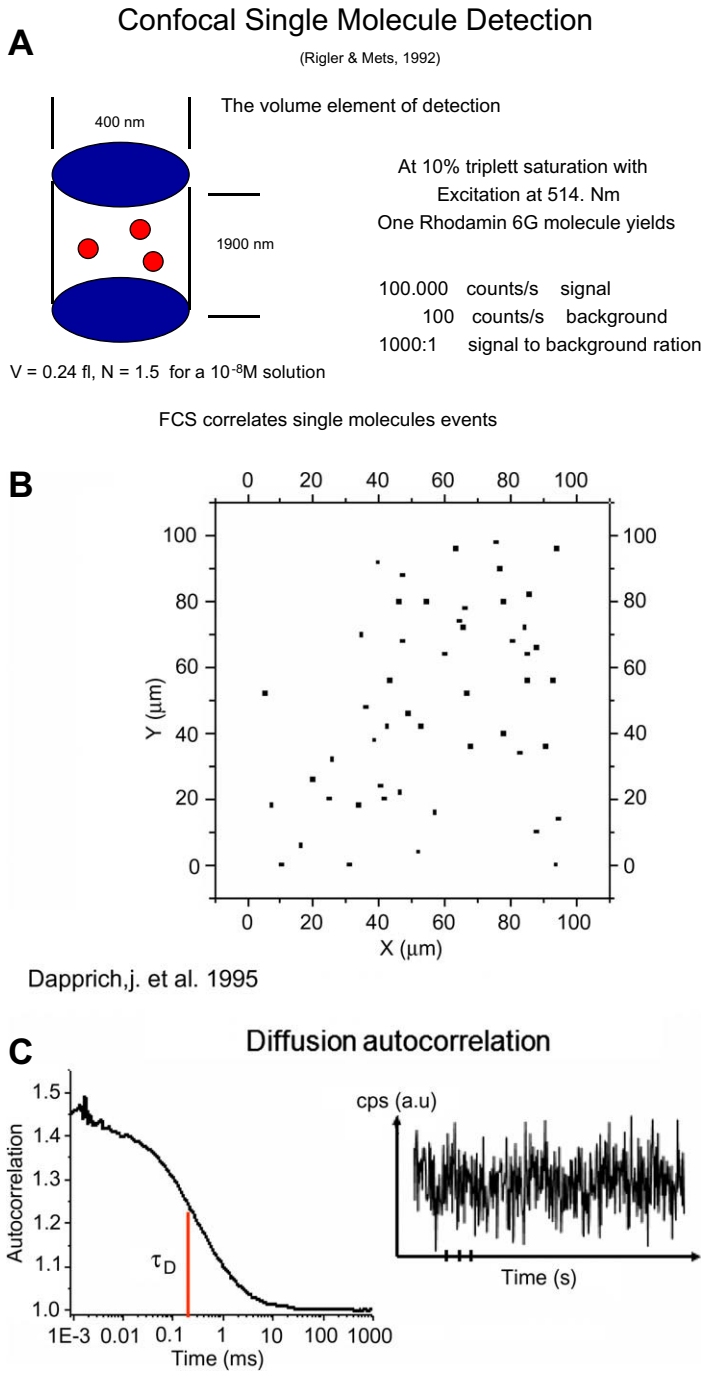

Fig. 1. Single molecule detection in a confocal volume element of size 0, 24 fL (A). Image of single DNA fragments (217 bp single stranded M13) visualized by a Rhodamine-labeled complementary sequence (B). Diffusional correlation function with autocorrelation amplitude equal to $1/N$ and t_D (diffusion time) equivalent to $\omega^2/4D$ (C).

verified by pixels of a multipixel detector or monomode glass fibers [10].

1.4. Molecular mobility and diffusion

Molecular mobility due to Brownian motion is conveniently analyzed by measurement of the diffusion of molecules over the confocal area ω^2. A typical signature is the correlation function of diffusion which is obtained by measurement of the positional time correlation function (Fig. 1C). From the half width of the correlation function, the diffusions constant D can be obtained and from the amplitude the inverse number of molecules ($1/N$) giving rise to the fluctuation [19].

The development of FCS instruments (microscopes) by Carl Zeiss, Jena (Confocor1, Confocor 2 and Confocor 3) as well as by Leica, Olympus and Hamamatsu has provided exquisite and reliable tools for FCS experiments.

Details relating to triplet kinetics, translational and rotational diffusion, chemical kinetics, excited states kinetics, molecular folding, electron transfer and other modes have been analyzed in the following decennium and have been presented in a comprehensive collection [22]. The further development up to now can be found in the proceedings of the Nobel Symposium on Single Molecule Spectroscopy [23].

An important development was the protocol of fluorescence cross correlation [24,25] which enables the study of time-linked molecular processes. A typical situation where FXS was successfully used is the detection of expressed genes by hybridization with gene probes carrying different colors [26,27] (Fig. 2). Analysis of the specific expression of the GAPDH gene interacting with two complementary gene probes labeled with Rhodamin 110 (green fluorescence) and with Bodipy 630 (red fluorescence) has recently been observed in the cytoplasm of HEK 239 cells (Westerlund and Norstedt, personal communication). From the spatial cross

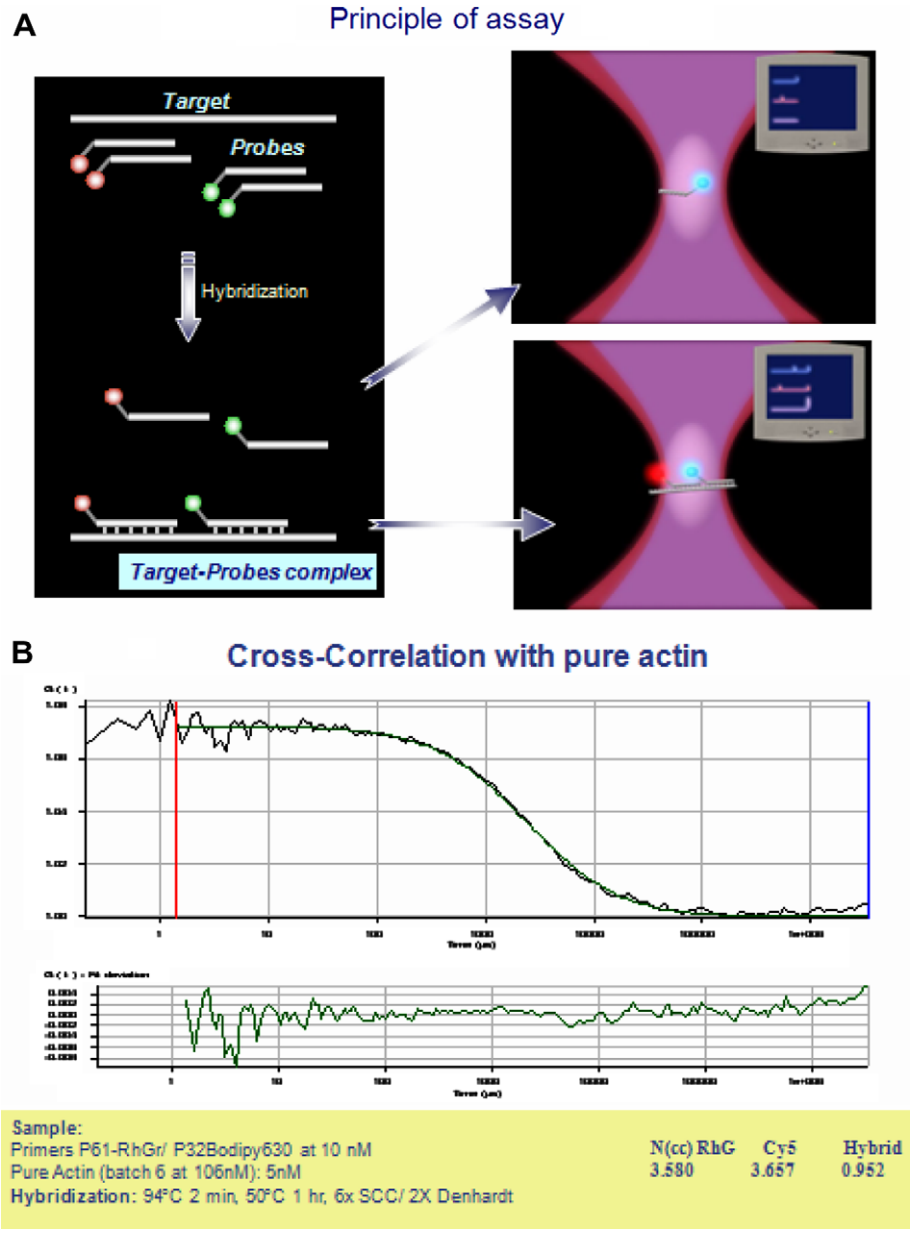

Fig. 2. Principle of the detection of gene expression by analysis of the interaction between the expressed gene sequence and the complimentary interaction of two complementary gene probes labeled with Rhodamine Green and Bodipy 630 (A) fluorescence cross correlation curve of the Rhodamine Green and Bodipy 630 gene probes linked to the expressed gene sequence (actin) (B) [26,27].

correlation curve the position of the expressed GAPDH genes in the cytoplasm can be identified.

1.5. Single molecule enzymology

A field which created high interest in recent years was the detection of molecular fluctuations in single enzyme molecules as observed by Xie et al. in cholesterol oxidase [28] and by Edman et al. in horse radish perodidase [29]. These results have triggered fluctuation analysis in a variety of other enzymes, such as lipases [30,31] and β-galactosidase [32].

Recent results, obtained in a total internal reflection (TIRF) set-up for FCS analysis built by Hassler [33,34] (Fig. 3A) shows the recording of individual substrate-product fluctuations in the catalysis of leukorhodamine (Fig. 3B and C). The enzyme turnover comprises silent and active periods of the catalytic events (Fig. 3D). Detailed theoretical models related to this behavior have been presented by Edman and Rigler [29], Lerch et al. [35] and by Klafter and associates [31].

1.6. FCS and single molecule detection in individual cells

The introduction of single molecule analysis and FCS into the domain of a single cell was a step near at hand. However it needed the introduction of APD detectors, characterized by negligible dark count to reach the background which is necessary for single

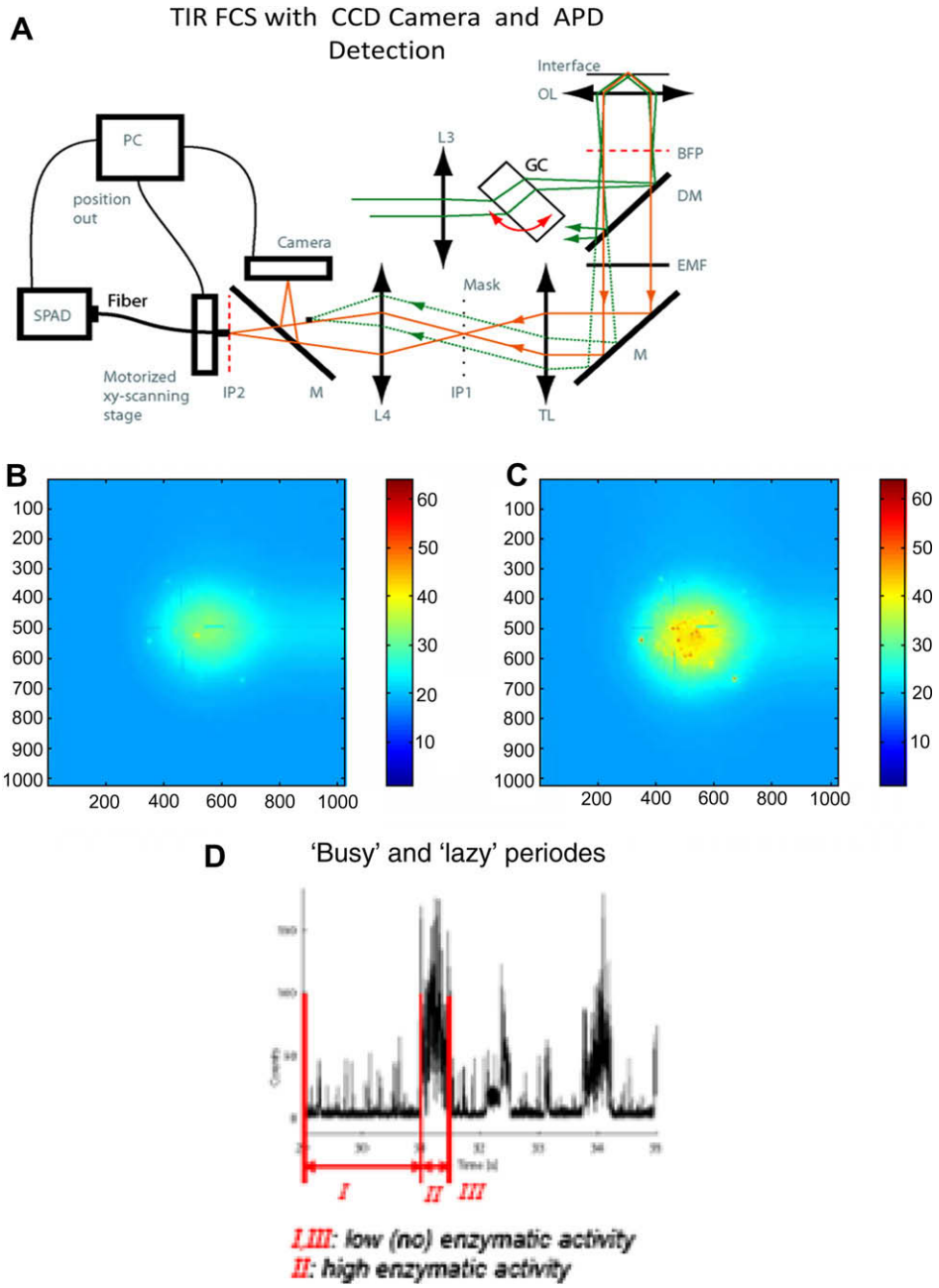

Fig. 3. TIRF setup with inverse epi-illumination for FCS analysis (A). The excitation beam is focused in the back focal plane of the objective and totally reflected at the glass surface carrying the sample. The evanescent component on the other side of the glass surface is exciting the sample. The emission is collected by a high NA objective and focused in the glass fiber cable serving as confocal pinhole. For fast overall detection of fluorescent spots generated by the enzyme catalysis, a CCD camera is used. For single molecule detection and FCS the confocal fiber is moved to the position indicated by the CCD camera with a motorized x–y stage. Turnover of horseradish peroxidase in the absence (B) and presence of cosubstrate (H_2O_2) is shown (C). Busy and lazy periods of horse radish peroxidase exist. In the busy periods, stretched exponential kinetics are observed, while in the lazy periods single exponential kinetics are found (D) [33,34].

molecule analysis. Based on the confocal Laser scan microscope with the Confocor 3 system a new instrument was developed in cooperation between the Karolinska Institutet and Carl Zeiss Jena [36]. This made image acquisition with low background noise possible, and the detection of single molecules within the cytoplasm or nucleus had become a reality (Fig. 4).

The very high sensitivity of the APD imaging enabled a very low expression level of GFP constructs and allowed to identify e.g. the transcription factor–DNA complex which otherwise would have been impossible to detect behind a green fluorescent screen of free transcription factor in the nuclear region of the salivary gland cell (Fig. 4A).

A heat shock protocol was developed which allowed to increase the concentration of the nuclear transcription factor as a function of time such that the complex formation within the nucleus could be followed (Fig. 4B and C) In this way a typical titration curve within the cellular domain could be produced and specific and unspecific interaction be measured [38,39].

The development of single molecule detection in individual cells as well as their dynamic components is just at the beginning. It is evident that molecular details behind gene regulation, the expression of gene products at the nuclear and the cytoplasmic level will be available as well as new insights into the molecular level of cellular processes [37–39].

We believe that the journey from Caspersson's UV spectroscopy which opened first insights into the quantitative behavior of nucleic acid and protein turnover in individual cells has been implemented and substituted by the tools of modern fluorescence spectroscopy where the detection of individual photons and their time dependence provides not only the image of molecular details but also the functional intertwining of molecular processes.

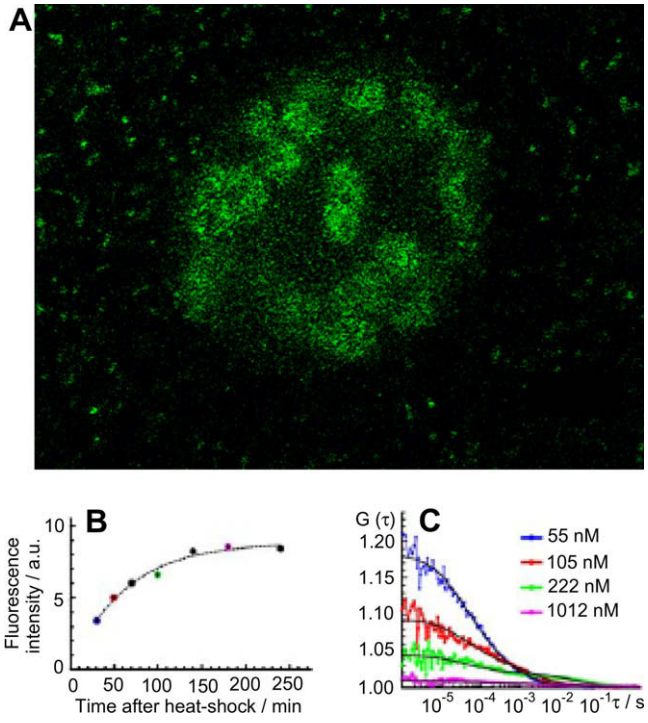

Fig. 4. Synthesis of GFP-labeled transcription factor in the cytoplasmic reticulum and presentation of the transcription factor bound to the chromosomal DNA in the salivary gland of the Drosophila fly (A). From the concentration of the transcription factor at different times after heat shock the binding constant of specific and unspecific interaction between transcription factor and chromosomal DNA can be determined [38,39]. Time dependent production of GFP marked transcription factor after heat shock measured by FCS (B). Corresponding FCS curves (C) [3].

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