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Fluorescence Correlation Spectroscopy (FCS) as a Tool for Single Molecule Detection

Diaa Atta, Aly Okasha, Osama Osman, Abdel Aziz Mahmoud, Walid El Hotaby, Zeinab Abdel Aziz and Ahmed Fakhry

Spectroscopy Department, National Research Centre, 12311 Dokki, Cairo, Egypt.

ABSTRACT

Single molecule measurements are considered among the most challenging points of research in recent trends of spectroscopy. Time resolved confocal microscope was presented as a successful tool for the molecular dynamics detection. Confocal volume is one from the most important parameters, which must be well defined before measuring using Fluorescence Correlation Spectroscopy (FCS) technique. The first step is to present how to calibrate it by two independent methods. The way to convert the time traces to correlation curve and how from the FCS curve were presented which in turn allow fast dynamics for the detection process. Moreover ,one can obtain some important dynamical information about the molecule like the hydrodynamic radius, the diffusion time and triplet state or dark state amplitude. Finally, some real measurements for biological materials like PGK protein as small molecule and ribosome as large molecule were presented. Moreover, an auto-correlation curve was presented for labeled nano disks as examples about how the molecule dynamics could be done by the FCS. Recently molecular modeling at different level of theory could be utilized as a conformational tool in the spectroscopic analyses of single molecule. In this paper some important aspects will be reviewed with important application examples in order to indicate the importance of the above mentioned spectroscopic tools of analyses.

Keywords: FCS-Single Molecule Detection, Protein dynamics,Confocal volume, Hydrodynamic radius, Diffusion time and Molecular modeling.

Introduction

Spectroscopic methods of analyses are the exact ways for the identification of substances. These methods of analyses are recommended for both atomic as well as molecular scale (Chang, 1971). Knowledge of the exact composition and structure of materials is an important aspect in many fields. Fourier transform infrared (FTIR) spectroscopy is widely used for qualitative identification of components (Pretzel,1998, Carbo,1996 and Michele, 1999). The technique is further modified to act as a powerful tool for elucidating molecular structures of many samples in different forms. One of the modified FTIR is the attenuated total reflectance- (ATR-) FTIR (Asensio *et al.,* 2009, Vahur *et al.,* 2010 and Vahur *et al.,* 2009), which is fast and requires little sample preparation. However, quantitative characterization needs further verification and confirmation which could be achieved by other techniques such as gas chromatography with mass spectrometry (GC/MS), Nuclear magnetic resonance (NMR) and Electron spin resonance (ESR) (Colombini *et al.,*2010). The identification and functional characterization of biogenic small molecules remains one of the most challenging tasks in chemical biology (Arthur *et al.,* 2010 and Nicholson and Lindon 2008), for instance, researcher interested in the use of vibrational spectroscopy for investigating biological applications. Because it is less frequently used, sum-frequency generation (SFG) is described at a greater length (Barth and Zscherp, 2002).The electromagnetic radiation in the infrared (IR) region of the spectra has oscillation frequencies that match the characteristic frequency of vibrational modes of matter, and therefore IR spectroscopies have been ubiquitously used as characterization techniques, such as a variation of the traditional transmission Fourier Transform Infrared spectroscopy (FTIR), developed by Greenler, (1966). Derivations of reflection–absorption IR technique were developed over the years, which include the internal total reflection–absorption FTIR spectroscopy (nowadays known as ATR — attenuated total reflection) (Fahrenfort, 1961). ATR is now among the most useful tools to characterize biological films supported by solid crystals (Binder, 2003 and Goormaghtigh *et al.,*1999) As a method to probe bio interfaces, IRRAS had its applicability largely expanded when it was adapted to Langmuir monolayers (Dluhy and Cornell 1985 and Mendelsohn *et al.,* 1995) .Spectroscopic studies on the single molecule level areone from the scientific challenges in the last decades. Many efforts have been exerted to develop spectroscopic techniques fordetectionon the single molecule level. One from the promising techniques especially in life science is the Fluorescence. Optical tweezers (Omar *et al.,* 2014 and Eriksson *et al.,*2010), magnetic traps, and single particle tracking, which are also potential techniques for diffusing molecules (Zander *et al.,* 2002 and Ha and Selvin, 2008). The spectroscopic analyses of single-molecule have gained plenty of attention in many areas of science such as life science (Ishijimaand Yanagida, 2001). There are so many important factors which could be gained from single molecule detection including kinetics of physical processes

Corresponding Author: Diaa Atta, Spectroscopy Department, National Research Centre, 12311 Dokki, Cairo, Egypt.
E-mail: diaalolo2004@yahoo.com

and chemical reactions that are often hidden in ensemble measurements (Xieand Trautman, 1998). Among many techniques the total internal reflection fluorescence microscopy (TIRFM) has played an important role in this field (Daniel, 1989 and Ramseyand Van den Berg, 2001). It is stated earlier that, the observation and manipulation of single biomolecules allow their dynamic behaviors to be recorded to provide insights into its molecular structure as well as genetic structure (Shortreed *et al.,* 2000; Kang and Yeung 2002; Wirth *et al.,* 2003, Wayment and Harris 2006; Wazawa *et al.,* 2006 and Ma *et al.,*2000). Spectroscopic methods of analyses based on fluorescence continue to be a topic of much research work. It is well known in drug analyses, for example synchronous fluorescence spectroscopy (SFS) are based on measurement of the synchronous fluorescence intensity of the drug. Because of its sharp and narrow spectrum, it has superior advantages over conventional fluorescence spectroscopy, as it results in simple spectra, low interference and high selectivity (Walash *et al.,* 2011). In terms of sensitivity, the combination of SFS and derivative spectroscopy is more valuable than conventional direct spectrofluorimetry (El Din *et al.,* 2011 and Nevado *et al.,* 2000).Another application of fluorescence spectroscopy could be in imaging of biological molecules. For bio-imaging application, a fluorescent dye with far-red or NIR emission, large Stokes shift, and water-solubility is highly desired, because it can minimize auto-fluorescence background and increase penetration of excitation and emission light through tissues (Ntziachristos *et al.,* 2002).There are some aspects that must be put into consideration during the building up a single molecule fluorescence technique. The first is related to the detector which must be high sensitive and have low background .Moreover the whole optical set up must achieve the highest available signal to noise ratio, because we look for single emitters where in this case the signal will be weak compared to ensemble measurements. Second, the used fluorophores should be highly photostable (Fitter *et al.,* 2011), in other words have high quantum yields (in between 0.6 and 1) and high absorption coefficients (~ 10^4 and 10^5 $cm^{-1}M^{-1}$). Both increase the detection efficiency and thereby increase the signal to noise ratio. Third, about the excitation source it should be intense, monochromatic, and should deliver a well-collimated beam, Laser as excitation source typically provides these properties. Fourth, the optical elements must be of high quality, including high numerical aperture objectives, which give small detection volumes, open the measuring angle, and increase the lateral resolution. In addition, the developments of dichroic mirrors and filter coating reduce the background caused by scattering and by the light from unwanted sources. Over the past years laser induced fluorescence detection became a major technique in studying topics in chemistry, in biology, in medicine, and even in material science. In our study, we will take the protein molecules as an example of single molecule target. If we exclude the water molecule, the dominant molecules over the human body are the Proteins. In the cell, proteins carry out virtually all the chemical transformations. The protein molecule regarded as a natural polymer built up from a sequence of 20 different amino acids. By the act of the cell ribosome a polypeptide chain is formed by linking and sequencing the amino acids together, this sequence of the amino acid in the chain is called the primary structure of a protein. In most cases, during the polypeptide chain synthesis and elongation loops, the protein secondary structure elements (helices and/or beta sheets) are formed. The tertiary structure starts by the domain formation from the ready helices and beta sheets. A final protein complex may consist of several polypeptide chains, which is called quaternary structure. The final tertiary or quaternary structure is the key of the protein three-dimensional structure, which forms the protein functional regions or active sites. These regions or active sites are behind of the protein properties and characteristics (Clarence, 2009).

Materials and Methods

Chemicals: the proteins and chemicals (if it is not listed) are purchased from sigma Aldrich. Atto 655 was purchased from Atto Tec, Siegen, Germany and Alexa 488 was purchased from invetrogen.

Ribosomes labeling and characterization: the ribosomal protein L4 stained. Purification from the excess dye occurred using 25 cm self made chromatographic size exclusion column. The buffer exchanged with 50mM Na_2CO_3 buffer (PH 7.4).

FCS measurements: the time traces have been registered by a time resolved confocal microscope Micro Time 200 from PicoQuant around 20 micro liter of concentration around 500pM. The confocal volume was determined for the Atto655 free dye using its hydro-dynamical radius recorded by 2-FFCS. The fluorophores was excited by 640 nm for the Atto 655 and for Alexa 488 laser beam with wavelength 470 nm both are pulsed laser head with 20MHz repetition rate and power density around $0.1MW/Cm^2$ and reflected by 600dcr dichroic mirror for atto 655 and 490 dcr for Alexa 488 both are from Chroma. The emission collected with UPLANsApo 60x water immersed objective from Olympus then split by 50/50 beam splitter after passing through a 50 microns pinhole. The fluorescence was detected by avalanche photo diodes SPCM-AQ-14 from Perkin–Elmer after filtering using emission filters 690DF40 and 520DF40 for Atto 655 and alexa 488 from omega optical for every channel. The photons are counted by means of time correlated single photon counting card (pico-Harp 300) from pico Quant. The measurements are carried out at room temperature. To prevent the protein staking to the microscope glass slides we added 0.001% (w/v) Tween 20 to the buffer. The recorded values are averaged over 5 times repetitions for every measurement each last for 20 minutes duration with time pining 1ms. The data

are pre-collected using symphotime software from PicoQuant. The data plot has been performed by origin from OriginLap, self madematlab subroutines are used for more data analysis.

Results and discussions

One from the wide used and strong techniques investigating the dynamics and interactions (Lippincott-Schwartz and Snapp E, Kenworthy, 2001 and Mutze *et al.,* 2007) of ultra diluted samples down to the single molecule level is the FCS (Elson, 2011 and Ries and Schwille, 2012). The idea behind FCS is that the measuring and the correlation of the fluorescence intensity fluctuations is based on the fluctuation theory (Del Razo *et al.,* 2014) in other words it is based on the correlation of intensity time traces. It is not easy like freely diffusing samples but possible to apply FCS for immobilized molecules (Kim *et al.,* 2002 and Foldes-Papp *et al.,* 2001) as well. In this paper, we will concentrate on the FCS for diffusing molecules. The cornerstone in this case is to record the intensity fluctuation of the diffusing molecules through a very small confocal volume (in order of few femtoliters) (Dertinger *et al.,* 2007). Normally these fluctuationscome from the molecule conformation changes or from dynamical processes. The conformational changes make the fluorophores to act as reporters. The time range of the FCS sensitivity extends over time scale from picoseconds even smaller to hundreds of milliseconds.As mentioned before, the FCS is depending on the intensity fluctuation, which happens because of the diffusion of one or more molecules through the very tiny confocal volume. According to the sample concentration, one or two fluorophores will diffuse in and out of the confocal volume. In this case, one can compare intensity I (t) with a laterone I (t+ τ) to obtain the autocorrelation function G (τ). Since the intensity fluctuation follows the Poisson statistics (Foldes-Papp,2007), hence the autocorrelation function G (τ) is the time average of the product of the intensity I(t) at time (t), with the intensity I(t+ τ) at later time (t+ τ) (Enderleine J 2009 and Hess S. 2002),

$$G(t) = \langle I(t)I(t+\tau)\rangle$$
$$= \frac{1}{T}\int_0^T I(t)I(t+\tau)dt$$

Where T is the data accumulation time.

From the autocorrelation function one can get some physical parameters such as, the number of diffusing molecules (Schwille, 2001, Doose *et al.,* 2005 and Perevoshchikova *et al.,* 2010) in the confocal volume(N) from the reciprocal of the auto correlation amplitude (G (0)), where

N=1/G (0)

Other parameters could be extracted for example from the auto correlation curve and one can get the diffusion times (τ_D), as shown in figure 1

The corner stone in all the fluorescence correlation measurements is the accurate calculation of the detection volume (Shi *et al.,* 2010). From the diffusion time and the confocal volume geometrical parameters, one can get the diffusion coefficients (D) using the following equation (Meseth *et al.,* 1999 and Haeberlein, 2003).

$$\tau_D = \frac{\omega_o}{4D}$$

Where ω_o is the confocal volume semi minor. The autocorrelation function at any time τ could be directly related to the diffusion time τ_D and the detection volume parameters (semi major Z_o and semiminorω_o) as (Janmey and Schmidt 2006 and Singh *et al.,* 2012)

$$G(\tau) = \frac{1}{N}\left[1+\frac{\tau}{\tau_D}\right]^{-1}\left[1+\frac{\tau}{\tau_D}\left(\frac{\omega_o}{Z_o}\right)^2\right]^{-1/2}$$

According to the stock's Einstein equation one can get the hydrodynamic radius R of the diffusing molecule by relating the buffer viscosity η to the diffusion coefficient D and the absolute temperature T as (Fogolari *et al.,* 2012, Fang X. 2011 and Tsierkezos *et al.,* 2011)

$$D = \frac{TK_B}{6\pi R\eta}$$

Furthermore, we can obtain the molecule concentration (C) in the confocal volume using the following equation (Diaa, 2012)

$$C = \frac{N}{VN_A}$$

Where V is the confocal volume and N_A is the Avogadro's number.

Application example:

In this section, biological and non-biological samples will be shownas real FCS measurements on the single molecule level. The measurements show that the FCS technique is able to detect the diffusion of single nano-disk; figure 2 shows the diffusion of single silver nano-disk as an example of non-biological samples. From the figure, it is clear the FCS diffusion curve and the diffusion parameters are clearly shown.

Curr. Sci. Int., 3(3): 199-207, 2014

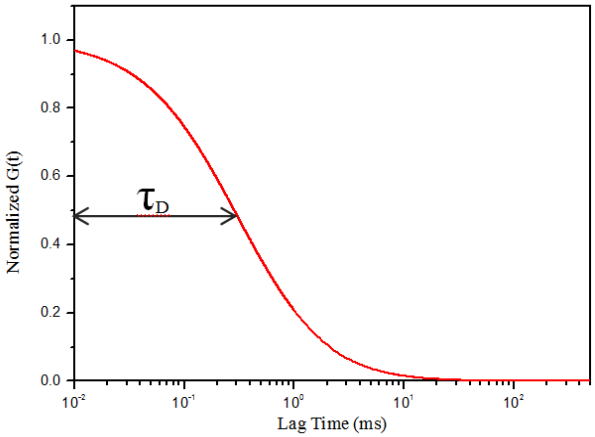

Fig. 1: Example of anauto correlation curve.

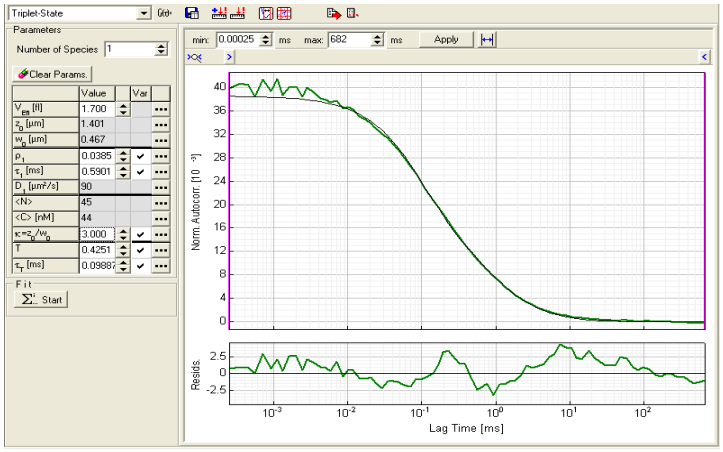

Fig. 2: The diffusion of single silver nano-disk.

It was tested with numerous inorganic dyes like following the diffusion of free Alexa 488 shown in figure 3.

Investigation of protein folding for a nascent chain is differing from the refolded protein (Katranidis *et al.,* 2009). One from the methods is to study the rotational diffusion of the ribosome and the nacent chain by time resolved fluorescence anisotropy decay (Kempe *et al.,* 2014).In the previous studies only labled ribosome diffusion was measured by FCS. The diffusion of Alexa 633 labeled ribosomes was followed as shown in figure 4. In this figure we can notice second component, which referred to larger particle that diffuses in the detection volume. This larger particle might be an aggregated ribosomes, in this case we have to apply fit the auto correlation curve (green line) with such appropriate model as shown in the figure (black line).

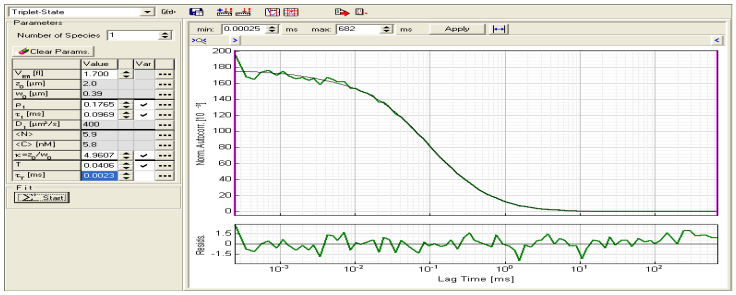

Fig. 3: The diffusion of single Alexa 488 molecule.

Curr. Sci. Int., 3(3): 199-207, 2014

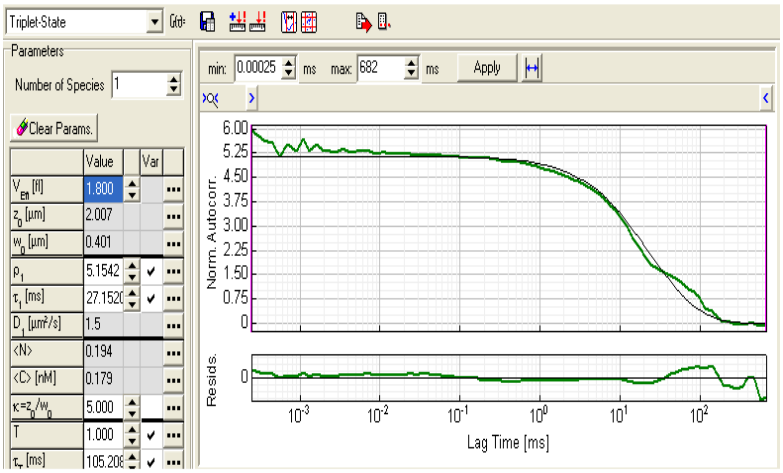

Fig. 4: The diffusion of single Ribosome labeled with Alexa633.

Exploring molecules using molecular modeling:

Molecular modeling depends on simulating given structures numerically, based in full or in part on the fundamental laws of physics (James, 1997). These classes of computational work compute the energy of a particular molecular structure, geometry optimization and compute the vibrational frequencies as well as many other important parameters (Hehreand Radom 1986). It covers the research interest of many scientists and engineers specially whom involved with studying molecular phenomena (Zsaboand Neil 1996). Molecular modeling specially those depending on electronic structure method are the most powerful tools in computational modeling. DFT among electronic structure method has an error, which is systematic, and can be overcome by an empirical scale factor, which makes the calculated results in a good agreement with experimental results (Foresman and Frisch 1996 and Ibrahim and Elhaes, 2013).These methods of calculations could compute single molecules in different phases and/or interfaces. It could be used successfully to describe some biological and pharmaceutical functionality of molecules even in its single state. Molecular modeling at higher level of theory together with quantitative structure activity relationship (QSAR) were utilized to describe the structural and electronic properties of new fullerene derivatives and their possible application as HIV-1 protease inhibitors (Ibrahim *et al.,* 2010a,b, and Ibrahim *et al.,* 2012).Molecular modeling together with FTIR was utilized to understand the constitution of natural protein. The contribution of each amino acid in the structure of gelatin was described by means of density functional theory DFT calculations then confirmed with the deconvlution of the experimental FTIR spectra of gelatin (Ibrahim *et al.,* 2011). The structure of protein could be also described not only for understanding protein but also to describe its interactions. Molecular modeling atsemiemperical level was utilized to understand the mechanism of interaction between chromium and native hide protein. The interaction is supposed to be within three molecules (amino acids) within the native protein (Nashy *et al.,* 2012). This paves the way toward utilization different level of theory to understand the behavior of single molecules even if they are part of other matrix. The model could isolate the molecule and describe its interaction as it behaves alone without its surrounding matrix. The interaction between amino acids and polysaccharides was described with molecular modeling (Ibrahim *et al.,* 2012) the models describe the active sites whereas the polysaccharide could interact with amino acid through it. Some molecules were designed for certain biological jobs then its ability as inhibitor could be tested with molecular modeling. QSAR was used in order to test novel peptidomimetic NS3 protease inhibitors(Ibrahim *et al.,* 2013a). Chitosan as an example for polysccarides was simulated in nano scale in order to describe its possible interaction with αB-crystalline protein. The process is described in order to overcome certain defects in this important protein (Gawad and Ibrahim, 2013). The effect of pollutants with divalent heavy metals upon protein structure was simulated and studied with molecular modelling with some help of FTIR spectroscopic technique.

Based upon the above considerations and computational work one can describe the applications of molecular modelling as a technique that could describe the mechanism of certain interaction and/or staring certain experimental phenomena in case the experimental techniques are limited or unavailable. It is very important technique to describe biological molecules even if these molecules are in single state.

Conclusion:

Spectroscopic tools of analyses offer reliable techniques for identifying matter in both atomic as well as molecular scale. Special challenges are arising for scientists dealing with single molecule measurements. FCS is now regarded as one from the most powerful single molecule techniques especially those needing high time resolution. Many methods could be applied to carry out information from the FCS time traces like FRET, PET even time decay anisotropy (Rosenkranz, 2009). More details could be extracted by measuring simultaneously one FRET or the PET measurement with the time decay anisotropy. It could be concluded that molecular modeling are techniques that fulfill the applications of spectroscopic techniques specially those dealing with single molecule measurements. This class of computational work is essential to describe and/or initiate experimental phenomena. It could be described as techniques supporting the availability of the well known spectroscopic tools.

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