| Novel FCS set up in TIFR Hyderabad bags international attention |

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In 2014, Kanchan Garai's lab set out to investigate the early stages of aggregation of amyloid proteins. These proteins are characteristic of diseases such as Alzheimer's disease and Type 2 diabetes. The researchers resorted to a technique called the Fluorescence correlation spectroscopy (FCS). FCS is a popular biophysical technique capable of characterizing single molecules even at very low concentrations. However, the researchers soon realized that conventional FCS was not suitable for studying protein aggregation in real time. It was only a matter of time until cuvettes were to make a fashionable comeback in the FCS setup.

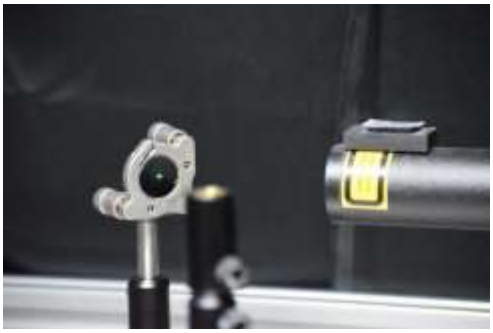
Fluorescence correlation spectroscopy was first conceptualized in 1974 by Douglas Madge, Eliot Elson and W. Webb. They studied the kinetics of a simple bi-molecular chemical reaction between DNA and ethidium bromide (EtBr). Intercalation of DNA with EtBr results in the formation of a fluorescent product. Upon excitation of this fluorescent complex with a laser, the DNA-EtBr complex emitted light of a longer wavelength, which was directed at a photocathode, generating a quantifiable electric current. The first experimental demonstration of fluorescence correlation spectroscopy was with a solution inside a thin walled cuvette. This method was aimed at studying 'fluctuations' in the medium. Now, what are these fluctuations? Consider the room, where you are currently located, to be the sample space. This space contains a mixture of gases. By FCS technique, one looks at portion of the sample space called the 'observable volume' and measures fluctuations. By observing the fluctuations, one can determine the rate of diffusion of the particles.

Though a remarkable study, this method did face challenges such as low sensitivity. To abrogate this hurdle, R. Rigler incorporated a confocal lens system in this set-up. With the advent of commercially available high numerical aperture objective lenses, FCS techniques can capture fluctuations in aqueous media with high sensitivity and resolution. However, usage of a high-powered objective lens would usually mean that you have smaller working distances. The samples could not be contained in thick walled cuvettes because the light could not be focussed beyond the wall of the cuvette. Hence, FCS techniques did away with cuvettes and resorted to confocal microscopes where the samples are placed on top of a thin coverslip.

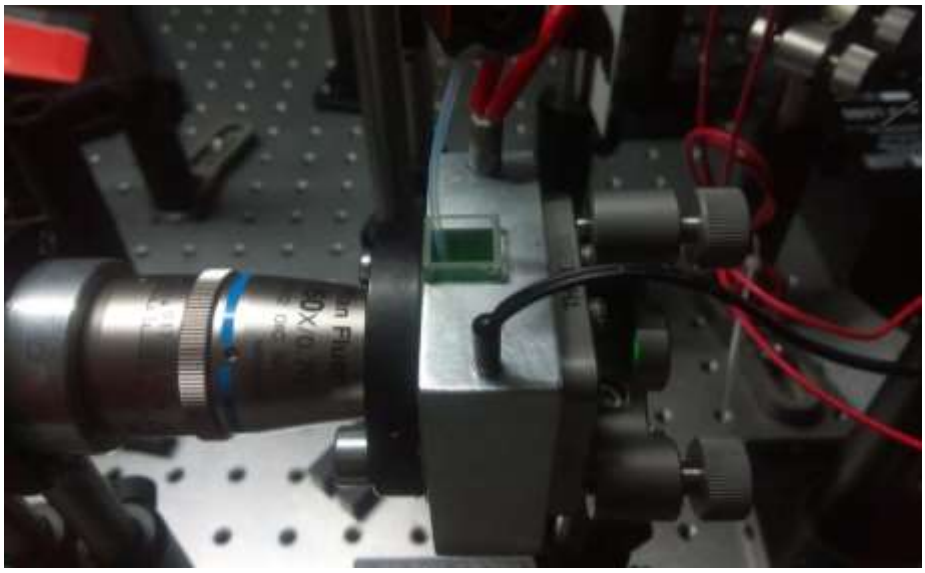
Protein aggregation is a slow process. Aggregation reactions sometimes go on for weeks, if not months. Many scientists speed up the aggregation of protein samples inside a cuvette by agitations such as heating or stirring. Microscope based FCS does not allow such agitations. Therefore, one needed to develop a set up where a confocal lens system could be used to collect data over extended periods of time, prompting the field to brainstorm regarding the incorporation of old fashioned cuvettes.

Researchers from Kanchan Garai's lab attempted the use of an Extra Long Working Distance (ELWD) objective lens with working distance of approximately 2 mm, as opposed to lower working distances of 0.3 mm of conventionally used objective lenses. This enabled them to focus inside the cuvette. The sensitivity of FCS depends on the square power of numerical aperture. However, the numerical aperture of ELWD objective is 0.7, which is about half of that of the conventionally used objective lenses. Additionally, another hurdle cropped up. ELWD lenses are not designed for single molecule

measurements. Hence, the correction for aberrations in these lenses is not quite extensive. The odds were stacked up against the ELWD lenses. Previous attempts of use of long working distance and low objectives in FCS yielded insufficient sensitivity.



The cuvette-FCS setup uses a green Helium-Neon laser (picture left). The laser is zoomed using a telescope and aligned precisely using several mirrors before being focused by the ELWD objective. When the laser beam excites the molecules, they emit fluorescence. This is detected by avalanche photodiodes (APD). APDs are very sensitive; it can detect even a single photon. Dr. Bankanidhi Sahoo, a postdoctoral fellow and Timir Baran Sil, a graduate student figured out that the quality and the precise alignment of cuvette are the two most critical factors for building a highly sensitive and high-resolution cuvette-FCS setup. Finally, they could create an observation volume ≈1.8 femtolitre and collect about 50,000 photons per second from one fluorophore molecule. The high resolution and sensitivity of cuvette-FCS makes it an attractive alternative of the microscope-based FCS.



FCS set up with the cuvette

This novel FCS setup offers numerous advantages over the conventional microscope-based FCS. It can perform measurements over a large range of temperature and in an array of solvents. Experiments, such as folding and unfolding of proteins, are very convenient in cuvette-FCS as compared to microscope-based FCS. Furthermore, cuvette-FCS can be tweaked to perform spatial scanning of the cuvette to allow detection of particles (including toxic substances) in very dilute solutions. Kanchan Garai's lab is already working on such developments.

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