Chapter II

Review of Literature and Experimental Techniques

II. 1 Review of Literature

Luminescent quantum dots (QDs) are semiconductor fluorescent nanoparticles molecules on an order of 2-10 nm size made up of few hundred atoms. They are made up of II-VI, III-V and IV-VI group elements of the periodic table with a diameter on the order of the compound’s exciton bohr radius [1, 2]. It was Ryogo Kubo, a Japanese mathematical physicist, who first theoretically postulated the quantum dot effect during 1960s. Later on, scientists from Massachusetts Institute of Technology synthesized self assembled QDs in 1990s with metals belonging to second and sixth family.

QDs exhibit discrete conduction bands resulting in emission of light when an excited electron returns to the valence band. Another feature of quantum confinement effect in QDs is the energy band gap which is the distance between valence band and conduction band. The band gap in QDs is size dependent and is inversely related to size. In other words the band gap of the material increases as the size of the QD decreases. This size dependent property of band gap in QDs allows engineering the QD size to restrict emission frequencies. Thus, emission wavelength in QD shifts towards higher
wavelength as the size of the particle increases \cite{3, 4}. Another interesting property in QDs is their wide effective Stokes shifts resulting from broad absorption and narrow emission spectra \cite{5}.

QDs show unique optical and electronic properties as a result of strong confinement of excited electrons and corresponding holes called excitons in their structures \cite{6, 7}. QDs score over conventional fluorescent labels such as fluorescent dyes in terms of high photostability, high quantum yield, broad absorption spectra, narrow and stable emission spectra, color codes for multiplexed detection and size tunable fluorescent spectrum \cite{8}. The combination of all the above mentioned properties made researchers to think about QDs in qualitative and quantitative analysis \cite{9-11}. In this direction, QDs have been studied extensively and made use in targeted drug delivery system, cellular labeling, to detect site directed mutagenesis, molecular therapeutics \cite{12-14}, real time detection of electron tunneling \cite{15}, cancer cell imaging with QD-immunoconjugates \cite{16}, imaging \cite{17}, multicolor detection system based on internal reflection fluorescence microscopy \cite{18, 19}, Förster Resonance Energy Transfer (FRET) \cite{20, 21}.

II. 1.1 Development of Förster theory

Historically, Resonance Energy Transfer (RET) was referred to as “transfer by inductive resonance”. This descriptive term emphasizes that the electronic interaction promoting RET involves a coupling of transition moments of the donor and acceptor molecules via a columbic interaction. There is an important synergy between experiment and theory in studies of RET phenomena as well as in the use of RET as a spectroscopic tool \cite{22}. In recent years the complexity of the systems being studied has escalated, and
these systems have been investigated at a deeper level. This has necessitated the
development of more general theoretical approaches and has uncovered some new
principles underlying RET processes.

It was Theodor Förster who first outlined the quantum-mechanical behavior of
non-radiative resonance energy transfer between two molecules called FRET in the year
1946 and provided a strong theoretical framework for FRET [23]. FRET is a distance
dependent non-radiative energy transfer in a dipole-dipole interactions between
chromophores over long distances on an order of 10-100 Å. Here quantum of energy is
transferred from a donor chromophore to an acceptor chromophore in a close proximity
when absorption spectrum of acceptor overlaps with that of emission spectrum of the
donor molecule [24]. FRET being near field interaction, takes place in close proximity
and involves non-radiative dipole-dipole interaction between the electric fields of donor
and acceptor molecules [24]. During the process, most of the energy emitted in the form
of light called fluorescence comes from acceptor molecule. This may also result in
decrease of fluorescence from donor molecule called donor quenching that in turn
depends on efficiency of energy transfer or ability of acceptor molecule to absorb energy
non-radiatively [24]. Therefore, distance between donor-acceptor pair should be within
the range from 10 Å to 70 Å that may vary energy transfer efficiency [24]. Energy
transfer efficiency gets affected by donor quantum yield, loss of energy in the form of
non-radiative decay, dipolar orientation within the pair and spectral overlap between
donor and acceptor. However, FRET is being extensively used in biochemical interaction
studies such as interactions between proteins, protein folding, assaying enzyme activity.
It is also being used in DNA sequencing, genetic analysis by fluorescence in situ
hybridization (FISH), gene translocation studies, hybridization studies, application in molecular beacons, drug interaction studies, DNA-protein interaction and environmental monitoring along with other applications [22, 25]. FRET has been utilized for protein-protein association events [26], virus detection [27] and bacteria detection [28].

II. 1. 2 Quantum dots (QDs) for FRET Applications

QDs have proved beyond doubt that they are promising in FRET based studies because of inherent quantum confinement effect, which dictates QDs to behave differently in contrast to their bulk counterparts.

In 1967, Stryer and Haugland showed this phenomenon could be used as a molecular ruler over a length scale of ~1-10 nm [29]. Since then large number of such studies has been performed. Andrey Rogach et al. have demonstrated FRET systems with semiconductor nanocrystals as at least one component and explained the exciting developments in this area involve controlling energy transfer on a nanometer scale where especially coupling with nanoparticles or dye molecules [30].

Mutlugan et al. [31] have reported efficient FRET between CdSe/ZnS nanocrystals and Rhodamine B for light harvesting in solution by utilizing the electrostatic interaction between them. They showed that the ZnS capped CdSe core-shell nanocrystals are sensitive to external factors such as pH and divalent cations and that it is advantageous to grow a shell of ZnS on the surface of CdSe cores as it has been found to protect the core from oxidation, significantly enhancing the PL efficiency and the quantum yield [13, 32-36].

Pons et al. have shown that fluorescent QDs are particularly suitable as energy donors for solution phase single particle FRET sensing; in particular they have explained
heterogeneity in QDs population. It allowed characterization of individual QD bioconjugate structures, which is an important parameter in sensor development based on QDs and FRET [37]. H. Lu et al. reported three fluorophore FRET systems, which enable the analysis of up to three mutually dependent energy transfer processes occurring between the fluorophores. Thus three fluorophore FRET systems are considered to possess a great potential for elucidating biomolecular structure and dynamics [38]. Most of the work published has been reviewed in ref. [39]. Clapp et al. [40] reported the potential of luminescent semiconductor quantum dots for development of hybrid inorganic-bio receptor sensing material. They demonstrated the use of luminescent CdSe/ZnS QDs as energy donors in FRET based assays with organic dyes as energy acceptors in QDs-dye labeled protein conjugates. In most cases, the energy transfer in QD based system is analyzed by using Förster theory assuming the donor and acceptors as points in the interaction space. Schrier et al. [41] theoretically demonstrated the shape dependence of resonance energy transfer between semiconductor nanocrystals. Since FRET physically originates from the weak electromagnetic coupling of two dipoles, one can imagine that the FRET limit can be circumvented by introducing additional dipoles and thus provides more coupling interactions.

Most recent work from our laboratory [42] reported the surface effects on QD based energy-transfer. The observed FRET efficiency from CdSe (core) and CdSe/ZnS (core/shell) QD donors to cresyl violet dye acceptor did not follow the linear dependence on the spectral overlap integral as expected from the Förster's theory. On the contrary CdSe/ZnS QDs show non-linear enhancement in fluorescence energy transfer efficiency on the spectral overlap as compared to CdSe core QDs, which could again be explained
by the involvement of QDs surface states as the time-resolved data reveal a still higher value of $A_3$ in decay component indicating that ZnS shell does not completely passivate the states present on the surface of core sample and consequently resulting in a non-linear dependence of energy transfer efficiency on spectral overlap. Specific applications, however, require multiple characteristics in a single system. For example, very small nanocrystals are desirable for in vivo imaging [43], yet multiplexing experiments require a range of sizes in order to achieve a range of fluorescence colors. Size also plays a role when nanocrystals must be incorporated into larger superstructures such as mesoporous materials in photovoltaics [44, 45]. One solution to the problem of dual requirements is to employ alloy nanocrystals. In recent times, alloyed nanocrystals have received great attention as their colour-tuning emission properties are controlled by changing their constituent stoichiometry without changing the particle size. Thus, by varying composition, we gain a second tool for altering physical and optical properties. Both size and composition may be tuned to select multiple desirable qualities simultaneously.

Among a variety of semiconductor materials, the binary type II-VI nanocrystals emitting visible light have been intensively investigated in recent years [46-52]. In the last two decades, research has been focused on the preparation of different color-emitting binary or core-shell nanocrystals with different particle sizes [4, 6]. However, of late tunable optical properties have been studied by changing the constituent stoichiometries in mixed ternary nanocrystals [53-57]. Zhong and co-workers reported ternary Zn$_x$Cd$_{1-x}$Se alloyed nanocrystals with luminescent properties comparable or even better than the best-reported binary CdSe based nanocrystals. A color-tunable emission of the alloyed
Zn$_x$Cd$_{1-x}$Se nanocrystals can be achieved by changing the particle composition besides the particle size [58].

Further, Sadhu et al. [59] taking an account of these alloyed quantum dots in FRET applications he reported that composition of quantum dots plays a significant role in energy transfer between QD donor and proximal dye acceptors, because the spectral overlap varies with changing composition without changing the particle size.

Because of these unique optical properties, nanoparticles are playing an important role in biosensor applications aiming at improving sensitivity and performance of biosensors [60-63]. As a result, applications of various nanomaterials such as quantum dots, gold nanoparticles, carbon nanotubes and magnetic nanoparticles in biosensor development are being extensively investigated.

Many studies successfully employing QDs as donors in FRET for developing chemical and biosensors have been reported [13, 17 27, 34, 61-63]. Such FRET based biosensor systems have been employed to detect maltose [5], cortisol [64] and some proteases [35]. In a FRET assembly reported by Medintz et al. [5] to detect maltose, QD was conjugated to *Escherichia coli* maltose-binding protein (MBP) that functioned as a sugar receptor. FRET was observed as β-cyclodextrin-QSY9 dark quencher binds to MBP saccharide binding site resulting in loss of QD fluorescence. In presence of maltose, QD regained its fluorescence as β- cyclodextrin-QSY9 gets displaced.

### II. 1.3 Quantum dots (QDs) in interaction studies

Serum albumins (SA) such as bovine serum albumin (BSA) and human serum albumin (HSA) are the major soluble protein constituents of the circulatory system; contribute to many important physiological functions like transport, buffer, nutrition etc.
Thus any kind of conformational change in the protein can alter the function of the protein. While the most important property of albumins is their ability to reversibly bind a large variety of exogenous compounds, including fatty acids, amino acids, drugs and pharmaceuticals [65–67].

Semiconductor nanoparticles (NPs) are receiving increasing research attention in all fields of science in the past few decades due to their attractive physicochemical properties. In addition, the size of NPs may be lower than that of the biological molecules and thus NPs can easily accumulate on the biomolecule surfaces and interact with them. Therefore understanding the mechanisms of interaction of nanoparticles with BSA is of prime importance.

Jiangong et al. [68] investigated the interaction between BSA and CdTe QDs with spectroscopic techniques. And he reported that fluorescence of BSA was quenched by CdTe QDs at pH 7.4. The interaction mechanism was also discussed and it was found that hydrophobic force and sulfhydryl group play an important role in the quenching process. Further results from Raman spectra showed some structural changes of BSA after interaction with CdTe QDs.

Ross et al. [69] reviewed the thermodynamic parameters characterizing self-association and ligand binding of proteins at 25°C. On the basis of expected signs of contributions to change in enthalpy and change in entropy of small molecules interactions, he concluded the contribution of forces in association process.

Recently Kothari et al. [70] investigated ZnO nanoparticles (with ~7.5 nm average size) quench the fluorophore of BSA by forming ground state complexes in solution, which however may get destabilized at higher temperatures. It is reported that
the calculated thermodynamic parameters suggest the binding occurs spontaneously involving hydrogen bond and van der Waals forces. Synchronous fluorescence spectra permitted us to establish that the microenvironment close to both the tyrosine and tryptophan residues of BSA is perturbed. In addition, the hydrophobicity of both residues increases in the presence of ZnO NPs. Finally, from the resonance light scattering and circular dichroism spectra they inferred on the formation of aggregates of BSA-ZnO NPs to induce slight conformational modification in BSA.

Jessy Mariam et al. [71] investigated interaction between silver nanoparticles (SNPs) and Bovine Serum Albumin (BSA) and reported that SNPs have a strong ability to quench the intrinsic fluorescence of BSA by both static and dynamic quenching mechanisms. They observed positive values of enthalpy and entropy change indicating that the interaction is mainly driven by hydrophobic forces. Synchronous fluorescence spectra indicate change in the microenvironment of tryptophan residues.

II. 2. Experimental Techniques

This section presents the technical details of the related but yet diverse instruments contributing to this work: The most important instruments used in the present investigation for steady-state measurements are UV-vis absorption spectrophotometer, spectrofluorometer, refractometer and Fluorescence microscope. Time-resolved measurements were carried out by adopting time correlated single photon counting system. A concise description of these instruments is given in the following sections.

II. 2.1 Materials

Quantum dots (QDs) were acquired from several sources for the studies included in this thesis. Several varieties of Lumidots CdSe (core), CdSe/ZnS (core-shell) quantum
dots) and alloyed water soluble CdSeS/ZnS quantum dots produced from Sigma-Aldrich India Pvt. Ltd., were used as FRET donors and sulforhodamine B and Rhodamine dyes from lambda-physik, were used as acceptors for the work described in Chapter-III and Chapter-IV. Further, alloyed water soluble CdSeS/ZnS quantum dots were used for interaction studies and selected protein is Bovine serum albumin (BSA) (Sigma-Aldrich India Pvt. Ltd.) using various Spectroscopic techniques as described in Chapter V.

II. 2.2 Absorption spectrophotometry

We have recorded the absorption spectra using a UV-vis absorption spectrophotometer (Hitachi, model U-2800) in solution. The sample was dispersed in 10 mm Quartz cuvettes at room temperature (298K), the optical system of the instrument is shown in Fig. 2.1. It is a double beam ratio recording spectrophotometer, which features a half mirror to split in to reference and sample beams. The white light emitted from the source is fed to Seya-Namioka mount monochromator utilizing a concave diffraction grating (grating constant: 1/600 mm, a blaze wavelength: 250 nm and grating area: 20 mm × 25 mm) featuring high-energy efficiency and low stray light level, where it is transformed into a monochromatic beam.

The beam sent from the monochromator is reflected by the toroidal mirror (M3) and then separated into reference and sample beams by the half mirror (M4). The two beams after passing through the sample compartment are focused by lenses, onto the detector where they are converted into electric signals. The electrical signal converted from optical signal enters the LOG amplifier where it is LOG converted to provide absorbance data. This data is amplified, and is then converted in to digital signal to be
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Fig. 2.1: Optical layout of Hitachi model U-2800 UV-vis absorption spectrophotometer
processed as digital value. The processed results are displayed on the LCD and recorded onto the printer. U-2800 can also be controlled by the optional UV solution software through a Windows-based computer [72]. All the measurements were done using this computer controlled software (UV solutions 2.0). The concentration of the solutions was maintained in the range from $10^{-5}$ to $10^{-6}$ M.

II. 2.3 Fluorescence spectrophotometry

The fluorescence spectrophotometer (JY Horiba, model Fluoromax-4) was used for recording the fluorescence spectra of the selected probes in different solvents. In the steady-state measurements, the most commonly used continuous light source is a 150-W ozonefree xenon arc-lamp, giving output in the range of about 200-800 nm. Light from the lamp is collected by a diamond-turned elliptical mirror, and then focused on the entrance slit of the excitation monochromator. The lamp housing is separated from the excitation monochromator by a quartz window. This vents heat out of the instrument, and protects against the unlikely occurrence of lamp failure. Such lamps are generally useful because of their high intensity at wavelengths ranging upwards from 200 nm. The optical layout of the instrument shown in Fig.2.2 is equipped with Czerny-Turner monochromators for excitation and emission. The essential part of a monochromator is a reflection grating. A grating disperses the incident light by means of its vertical grooves. A spectrum is obtained by rotating the gratings, and recording the intensity values at each wavelength. The gratings in the FluoroMax-4 contain 1200 grooves mm$^{-1}$, and are blazed at 330 nm (excitation) and 500 nm (emission). Blazing is etching the grooves at a particular angle, to optimize the grating’s reflectivity in a particular spectral region in order to obtain accuracy better than 0.5 nm, and repeatability of 0.3 nm. The entrance and
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exit ports of each monochromator have continuously adjustable slits controlled by FluorEssence™. The width of the slits on the excitation monochromator determines the bandpass of light incident on the sample. The emission monochromator’s slits control the intensity of the fluorescence signal recorded by the signal detector. In addition, these monochromators use concave gratings, produced by holographic means, which further decrease stray light level.

The radiation from the xenon arc-lamp is converged at the entrance slit of the excitation monochromator through the lens. Only the light dispersed by the excitation concave grating enters the exit slit. The excitation beam from the exit slit is reflected by the concave mirror to the beam splitter which splits the light emerging from the excitation monochromator. A beam splitter, placed in the excitation light path, consists of a thin piece of clear quartz, which reflects about 8% of the incident light. This amount is generally adequate for a reference channel, which does not use a monochromator. One of the two-excitation beams directed to the monitor detector for its measurement, and the other beam passing through the beam splitter is converged to the sample cell through the lens and is used to excite the sample in the cuvette. The fluorescence emitted by the sample is normally collected at right angle to the excitation beam and is restricted into the entrance slit of the emission monochromator through the lenses. The fluorescence dispersed by the emission concave grating passes through the exit slit and is converged onto the photo multiplier through concave mirror for intensity measurement [73-75].

II. 2.4 Synchronous fluorescence scan spectroscopy

Synchronous Fluorescence Scan (SFS) spectroscopy is one of the most recent and powerful techniques for multi-component analysis of QDs mixture solutions without
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resorting to tedious separation procedures and is extremely useful for routine analysis. In conventional fluorescence spectroscopy, two types of spectra are generally measured. When a sample is excited at a fixed wavelength ($\lambda_{ex}$), an emission spectrum is produced by recording the emission intensity as a function of the emission wavelength ($\lambda_{em}$). An excitation spectrum may be obtained when $\lambda_{ex}$ is scanned while the observation is conducted at a fixed $\lambda_{em}$. The broad nature and spectral overlap of conventional fluorescence spectra can be overcome, and enhanced selectivity can be obtained using synchronous fluorescence spectroscopy (SFS). In SFS, the $\lambda_{ex}$ and $\lambda_{em}$ are scanned simultaneously. Depending on the scan rate three basic types of SFS technique are possible [76]. Constant-wavelength SFS is very simple technique as the scan rate is constant for both monochromators and, therefore, a constant wavelength interval, $\Delta \lambda$, is kept between $\lambda_{em}$ and $\lambda_{ex}$. Second technique is known as the variable-angle SFS. The excitation and emission wavelengths may be varied simultaneously but at different rates. The third technique, constant-energy SFS, has not been used much. SFS is often considered as a convenient technique for the analysis of multi-component samples without resorting to tedious separation procedures [77-81].

The sharpness and narrowness of the peak of a SFS spectrum, compared to that of conventional spectrum, may be explained with reference to a simplified Jablonski diagram (Fig. 2.3). A molecule can be excited in the whole absorption band starting from wavelengths $A_1$, $A_2$, . . . . . . $A_9$ and could give fluorescence at wavelengths $F_1$, $F_2$, . . . . . . $F_9$. Generally, the fluorescence emission spectrum of a fluorophore remains unchanged, irrespective of the excitation wavelength, except for a variation in the fluorescence
Fig. 2.3: Jablonski Diagram provides explanation of synchronous fluorescence scan
intensity, which depends on the probability of the electronic transition of the molecule. To get a fluorescence emission spectrum, the molecule is generally excited at its absorption maximum (A5) and fluorescence is collected in all the emission wavelengths, i.e., F₁, F₂, F₃, . . . . . . F₉.

A fluorescence excitation spectrum is obtained by exciting the molecule at all possible excitation wavelengths, e.g., A₁, A₂, . . . . . . A₉, and collecting the fluorescence only at the emission maximum (F₅). But, in the case of SFS, a particular wavelength interval is chosen, so that a signal is observed only when Δλ matches the interval between an absorption band and an emission band. Therefore, initially, e.g., taking Δλ = A₅ ~ F₅, we will not see any fluorescence until the excitation monochromator is at A₅ and fluorescence wavelength is at F₅. In the next moment, the molecule will be excited at A₆, A₇ . . . . . . A₉ and corresponding fluorescence will be recorded at F₆, F₇ . . . . . . . F₉, respectively. This process continues till a full spectrum is recorded. As the emission intensity is a function of the excitation wavelength (which is related to the probability/population of transition), and Δλ defines the matching of absorption and emission band, we obtain a sharper peak in SFS compared to a conventional spectrum [82]. The combination of synchronous and derivative fluorometry enhances minor spectral features and allows more reliable identification of chemical species [80-82].

II. 2.5 Refractive index measurements

Refractive index (n) of probe solutions for sodium-D line was measured using the thermostatically controlled Abbe’s refractometer (Atago 3T, Tokyo, Japan). Precision of the instrument was ±0.0001 units. This refractometer is fitted with hollow prism casings through which water is circulated. The temperature of the prism casings is observed with
digital display (±0.1°C). The instrument has two prisms placed one above the other in front of the telescope. With the insertion of a drop of the test liquid using a hypodermic syringe, the incident ray forms a line of demarcation between light and dark portions of the field. This is viewed in the telescope, which moves with the scale. The instrument directly provides the value of $n$.

The refractive index measurements have been carried out at 25°C by circulating water from a thermostat. In order to obtain precise data, the refractometer was calibrated frequently using a glass piece of known refractive index provided along with the instrument [83]. It was also ensured by measuring the refractive index of the pure water twice. A built-in sodium-D lamp was used as a source of light and an average of four trials was considered in all the calculations.

II. 2.6 Time-resolved fluorescence decays

Steady-state measurements cannot always resolve the individual components, which may contribute to overall fluorescence. Measurements of the fluorescence decay time are able to do so. Two techniques used to resolve fluorescent components are: Time Correlated Single Photon Counting (TCSPC), and phase modulation techniques [84]. The TCSPC method based on the repetitive precisely timed registration of single photons of a fluorescence signal has been employed in the current study.

II. 2.6.1 Time Domain spectrometry using Time Correlated Single Photon Counting (TCSPC) technique

Fluorescence lifetime of the fluorophores is measured using various methods among which, the time correlated single photon counting (TCSPC) is rated as the best.
TCSPC is a digital technique, which counts the photons that are time correlated with the excitation pulse. The schematic of the experimental setup is given in Fig. 2.4 A.

II. 2.6.1.1 Principle of operation

The fluorescence decay of each fluorophore obeys first order kinetics, since fluorescence decay is a unimolecular process. This allows the resolution of different fluorescence components. TCSPC involves an elaborate instrument to measure the time between an excitation light pulse, generated by a pulsed light source such as a flash lamp or laser, and the arrival of a fluorescent photon at a microchannel plate-photomultiplier tube. The time-to-amplitude converter (TAC) is the device that achieves the time correlation between excitation and emission events. Upon receipt of a start signal, and after a certain fixed delay, a timing capacitor is charged linearly from a constant current source. The charging is discontinued upon the acceptance of a stop pulse and an output pulse is generated with amplitude derived from the final charge in the capacitor. Therefore, the output pulse height is proportional to the time difference between start and stop pulses. If no stop pulse is received after a certain amount of time, charging is automatically discontinued and the capacitor is reset [84]. The measured times are digitized by the electronics and then output to a computer-controlled multichannel analyzer. This accumulates the photon counts in data channels assigned to different time points, typically 2048 at 10 ps/channel each. The resulting histogram illustrates the fluorescence decay of a sample. Each point on the decay profile is obtained from a single pulse. The excitation intensity is attenuated with a neutral density filter, so that only one fluorescence photon is detected per 100 excitation pulses. At this rate, approximately 8000 pulses are detected in 1 second for an 82 MHz excitation source. To optimize the
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Fig. 2.4 A: Schematic of Picosecond Laser and TCSPC setup
signal to noise ratio, 10000 counts are collected in a typical curve. An intensity-time profile of the laser pulse, also known as the instrument response function (IRF), can be generated using the Stokes Raman scattering or Raleigh scattering of pure water at an appropriate wavelength [85].

II. 2.6.1.2 Experimental setup

A schematic of the experimental setup of fluorescence lifetime (1. IBH, UK, Model 5000U. 2. ISS Chronous BH USA) is shown in Fig.2.4 (A and B). The second harmonic output from the Tsunami mode locked picosecond laser was used as the excitation source. The mode locked 375 nm laser pulses are focused on the sample and the fluorescence photons from the sample were collected at right angles to the excitation beam. These emitted photons were detected by MCP-PMT (R3809U, Hamamatsu) after passing through the emission monochromator. The output of the MCP-PMT was fed to a discriminator whose output serves as a stop signal for the TAC. The start signal for TAC is derived from a high-speed red sensitive silicon photo detector (DET 210, Thor Labs Inc.). The fundamental output (750 nm) from the Tsunami mode locked picosecond laser was focused on the photodiode. The photodiode signal is converted to a TTL signal by a pulse converter (model TB-01, IBH). The output TTL signal from the pulse converter is used as a start pulse for the TAC. The TAC output is fed to MCA card (Oxford Corporation, UK) and the data collection was carried out by a software (Data station 2000) provided by IBH. Repetitive laser pulsing and emitted photon collection produces a histogram of counts against voltage (time). This histogram represents the fluorescence decay of the sample under study. For recording the lamp profile, a scatterer is placed in
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Fig. 2.4 B: Chronos BH instrument top view
place of the sample and the above procedure was repeated. The response time of this instrument is ~50 ps.

Fig. 2.4 B represents the top view of Chronos BH fluorescence lifetime instruments. The fluorescence signal sent to photomultiplier tubes (PMTs) (model-H5773) by Hamamatsu PMC-100. Chronos is designed on a T-format geometry: one excitation channel and two identical emission channels positioned on the same axis and at 90 degrees with respect to the optical axis of the excitation channel.

The excitation channel houses an automatic shutter, a beam-splitter, a filter holder, and an automatic polarizer holder. An additional automatic filterwheel can be positioned in the excitation channel. The light beam enters into the excitation channel from the back aperture and travels down the excitation channel all the way to the sample. A fraction of the excitation light beam diverted by the beam splitter enters into the reference channel that has a filter holder and an automatic shutter. A PMT detects the light from the reference channel and the signal is used for correction of excitation spectra and for correction when acquiring fluorescence on a long time period.

Each emission channel is equipped with an automated polarizer holder, filter holder, and an automatic shutter. The connections at the ends of the two emission channels are M28-threaded coupling rings, which accommodate the ISS housings for photomultiplier tubes or the monochromators. Computer controlled filterwheels can be placed in the emission channels.

The polarizers holders are controlled by stepper motors which make polarization measurements fully under computer control. A polarizer is permanently attached to each motorized holder; that eliminates the need to insert or remove any polarizer from its
holder. The light detectors are mounted on the emission channels. The signals from the light detectors are diverted to the acquisition card inserted into the computer or to the processing electronics. The instrument response function was measured using starch in water as a scatterer, and multi-exponential curve fitting was done with the Vinci Analysis software. The response time of this instrument is about 45 ps.

II. 2.6.1.3 Analysis model used in Vinci

Decay times model

In a multi-component environment containing $i^{th}$ fluorescent molecules, the fluorescence is described by the relationship:

$$I(\lambda,t) = I_0 \sum_i \alpha_i(\lambda_i) \exp \{-t/\tau_i\}$$  \hspace{1cm} (2.1)

where the coefficients $\alpha_i(\lambda_i)$, called the pre-exponential factors and the decay times $\tau_i$ characterize the fluorescence decay of the $i^{th}$ component of the mixture. The pre-exponential coefficients are related to the fractional contributions $f_i$, that is the fraction of the total fluorescence emitted by the $i^{th}$-component of the mixture.

$$f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i}$$  \hspace{1cm} (2.2)

The Vinci Multidimensional Fluorescence Spectroscopy Analysis software determines the pre-exponential coefficients $\alpha_i$, the fractional contributions $f_i$ and the decay times $\tau_i$ of up to four components.

II. 2.6.1.4 Picosecond laser source

The diode laser pumped continuous wave neodymium yttrium vanadate (NdYVO$_4$) laser (Millennia V, Spectra Physics, USA) was used to pump the Ti:sapphire
rod in the mode locked picosecond laser (Tsunami, Spectra Physics). The diode laser contains two laser diode bars each having 19 diode elements. These diodes have an output at 809 nm with a power of 13 W, which are fiber coupled to pump an NdYVO\(_4\) laser that produces an output at 1064 nm. The frequency doublet using lithium triborate (LiB\(_2\)O\(_3\)) crystal produce 5 W of green light at 532 nm. This NdYVO\(_4\) laser now pumps the original Ti: sapphire laser, which is tunable over a wavelength range 720 to 840 nm.

The regenerative mode locked pulses of the laser are obtained by Kerr effect and the pulse width of the mode locked Tsunami laser is < 2 ps operating at 82 MHz. The pulse width is measured using an autocorrelator (model 409-08, Spectra Physics), which employs second harmonic generation with background free configuration technique. The measured pulse is displayed on a high impedance oscilloscope (Scientific, 300 MHz) for real time viewing. The pulse picker (model 3980, Spectra Physics) selects the pulses at a rate of 4 MHz from the 82 MHz trains of pulses from Tsunami laser.

A flexible harmonic generator (FHG) (Spectra Physics) is used to generate the second and third harmonic laser outputs. In this unit second harmonic generation is accomplished by focusing the laser from the pulse selector into LBO crystal. In order to generate third harmonic signal, both the fundamental and second harmonic beams should overlap perfectly with time collinear and space in \(\beta\)-barium borate (BBO) crystal. With the standard optics, Tsunami generates 750 nm pulses as a fundamental output and the second harmonic output from the FHG at 375 nm is used as an excitation source for the samples. The fluorescence photons emitted from the sample are detected at right angles to
the excitation beam, by a high gain Micro Channel Plate Photomultiplier Tube (R3809U MCP-PMT, Hamamatsu).

II. 2.6.1.5 NanoLED source

NanoLED is a novel light source system that uses multiple light emitting diode (LED) and laser diode (LD) devices to generate nanosecond and picosecond optical pulses over a wide spectral range. A typical LED source of HORIBA Jobin Yvon is shown in Fig.2.5. LED-based sources generate nanosecond pulses in the UV and visible, while LD-based sources generate picosecond or nanosecond pulses in the UV (375nm), violet, blue, red and NIR. NanoLEDs are ideal sources for fluorescence lifetime and biomedical applications. They are compact, easy to use and are extremely reliable. The experienced spectroscopists appreciate the quality of optical pulses produced by the NanoLED system. The NanoLED system consists of a NanoLED controller (stand alone module or FluoroHub card) module and a range of interchangeable NanoLED sources, each designed for use over a specific wavelength range. LED-based sources generate nanosecond pulses, while Laser diode based sources generate either picosecond or nanosecond pulses depending on the version ("H" suffix indicates high intensity version with nanosecond pulse). Each NanoLED light source contains adjustable light collection optics and a bayonet mounting flange to make incorporation into any optical system easy. NanoLED has been developed for replacement of mode-locked lasers and flash lamps in low cost or portable applications. High intensity LD versions are now available for those who require the most energy per pulse, which typically produce pulses of 0.9ns duration.
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II. 2.6.1.6 Fluorescence decay analysis

The measured fluorescence decay is the convolution of true fluorescence decay, excitation function and the instrument response function (IRF). The fluorescence kinetic parameters (lifetime, amplitude, etc.) are obtained by deconvoluting the excitation and the IRF from the measured fluorescence decay. The data analysis was accomplished by programming software known as DAS-6 provided by IBH. DAS program acts as a user-friendly interface for the creation of decay associated spectra. This is based on the reconvolution technique using iterative nonlinear least square methods. The reconvolution is preceded by a series of iteration until a chi-square ($\chi^2$) value is reduced. The quality of fit is normally identified by the reduced $\chi^2$, weighted residual and the autocorrelation function of the residual. The reduced $\chi^2$ values for all the accepted fits were close to unity and the weighted residuals were randomly distributed among the data channels.
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II. 3 References


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[23] T. Förster, Naturwissenschaften. 6 (1946) 166
II. Review of Literature and Experimental Techniques


II. Review of Literature and Experimental Techniques

[72] Instrument manual, Cat. No. UV-Visible absorption spectrophotometer (U-2800), Hitachi company Ltd., Tokyo, Japan
[73] Operational manual, Part number 810005 version B, Fluoromax-4 Spectrofluorometer, Horiba Jobin Yvon, USA
[74] FluorEssence Software Version 3.5, Part Number 810000, Horiba Jobin Yvon, USA
II. Review of Literature and Experimental Techniques


