DEVELOPMENT OF A TWO PHOTON BURST INTEGRATED FLUORESCENCE LIFETIME SYSTEM FOR SINGLE-PAIR FRET TO ANALYSE INTERACTING PROTEIN POPULATIONS

Fluorescence detection of single molecules represents the ultimate in sensitivity for fluorescence-based assays in biology and medicine by eliminating ensemble-averaging.

We describe the development of a time-resolved burst integrated fluorescence lifetime (BiFL) methodology to observe single-molecule dynamics and measure fluorescence lifetime of these molecules as they diffuse through a femtoliter volume probed by two photon excitation.

ADVANTAGES OF SINGLE MOLECULE STUDIES

- Molecular reactions need not be synchronised
- Experiments can be performed on minute quantities
- Sub-populations in a mixed sample can be revealed
- Conformational changes can be studied
- Molecular temporal trajectories can be mapped

TWO PHOTON EXCITATION (TPE)

- Confined excitation of a sub-femtoliter probe volume
- Photobleaching is limited to zone of 2P excitation
- High background rejection providing efficient single molecule detection
- Three dimensional resolution by intensity-squared dependence of TPE
- Preliminary SM work was carried out on quantum dots (Q.Y=0.57) as fluorescent donors
- Alexa Fluor dyes acted as FRET acceptors
- Photostable and robust assay for characterising the single pair FRET experiments

EXPERIMENTAL TECHNIQUES

Burst Integrated Fluorescence Lifetime (BiFL)

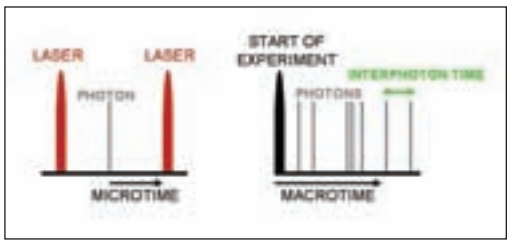

Figure 2. Schematic showing the temporal information obtained during single molecule data collection using Becker & Hickl TCSPC card single photon counter in First-In-First-Out (FIFO) mode.

To identify single molecules by their characteristic transit time, a real-time spectroscopic technique termed BiFL was used.

This technique monitored individual molecules diffusing through a focal volume and registered the macrotime and microtime simultaneously.

Microscopy Focus

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PROJECT AIM

The project aims to investigate FRET due to conformational changes on a single molecule level using biological models. Presented in this poster, is the study of intramolecular FRET of IgE complexes.

EXPERIMENTAL SET-UP

Microscope system: Time resolved system constructed around a Nikon ECLIPSE 90i upright microscope. The objective lens used is Nikon x60 1.49 NA (oil).

Laser system: DPSS pumped laser (Coherent Verdi) and femtosecond self-modelocked Titanium: Sapphire system (Coherent Mira).

Hardware: Becker & Hickl Time Correlated Single Photon Counting TCSPC board (SPC-830).

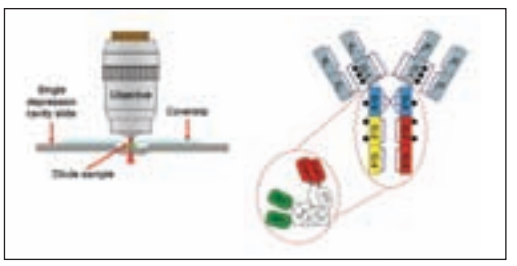

Figure 1. Illustration of experimental setup (left). Schematic showing the structure of human IgE antibody and the region of the chain where fluorescent proteins are attached (right).

EXPERIMENTS USING IgE CONSTRUCTS

- Nanomolar concentrations of freely diffusing IgE antibody chains labelled with GFP and mRFP at opposite terminals
- Control IgE-FC labelled with GFP only
- Samples were diluted in Phosphate Buffered Saline
- Instrument response of the system was acquired using 20mm colloidal Gold (Sigma-Aldrich Ltd)

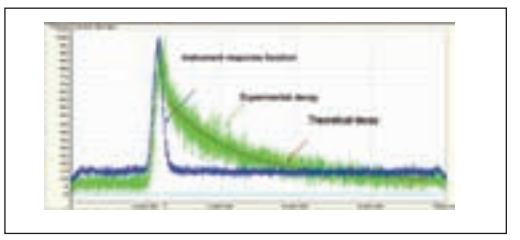

Figure 3. Fluorescence decay curve of a single burst of quantum dot transit convolved with the instrument response function (measured using colloidal Gold particles). Maximum Likelihood Estimation method was used to obtain the fluorescence lifetime of the quantum dots in the presence of varying concentrations of acceptors.

CALIBRATION WITH RHODAMINE

- Determine optimal single molecule concentrations
- Determine laser power required to obtain enough photons
- At low concentrations or small excitation volumes, the probability of detecting more than one molecule in the excited state is negligible
- This separates signal (a burst of photons) of a single molecule from that of background

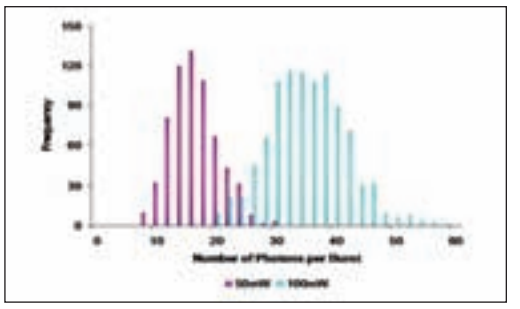

Figure 4. Photons emitted during fluorescence bursts of Rhodamine B ($1.09X10^{-9}$mol/L) transits. Increasing the laser power leads to the collection of sufficient number of photons for Bayesian analysis.

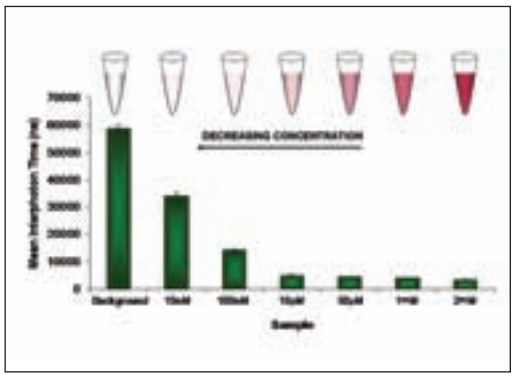

Figure 5: Interphoton time (Δt) is the time between the detection of 2 consecutive photons. Single molecule transits have much shorter Δt than background. The mean Δt for transit of Rhodamine B molecules is shown above.

ANALYSIS & RESULTS

Bayesian Analysis Method:

Bayesian analysis provides protocols for optimal extraction of quantitative information from 'noisy' data.

- Probability of a burst is calculated from posterior probability that the data conforms to the signal model rather than any background model
- Signal Model: $P(\Delta t|\omega) = (1- \omega 0)F(\Delta t|\omega 1)+ \omega 0/T$

Fraction of photons received from excitation event with arrival time distribution F(s| ω1) is 1-ω0, where ω0 is the fraction that represents background noise with uniform arrival time distribution of Fnoise(s)=1/T.

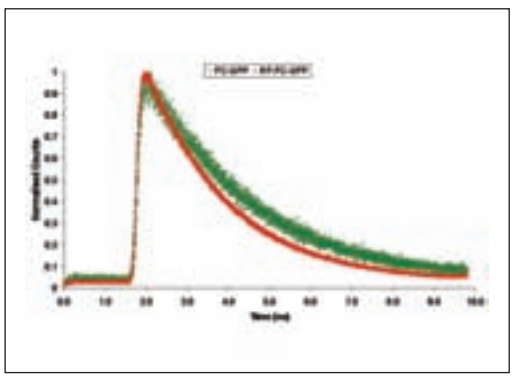

Figure 6. Decay curves of IgE FC complex with GFP as donor and GFP and mRFP as FRET acceptor. The ensemble fluorescence lifetime of GFP complex is 2.52 ns. In the presence of acceptors this decreases to 2.01 ns.

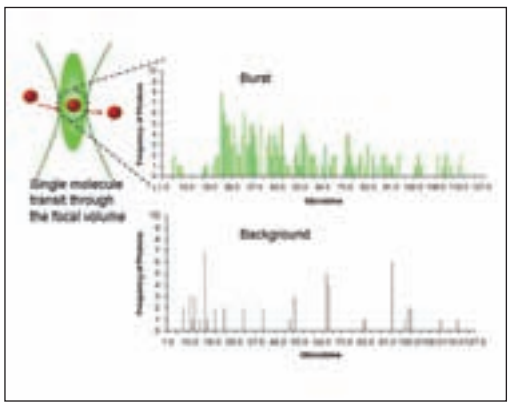

Figure 8. Decay IgE FC complex labelled with GFP obtained from photons from a single burst.

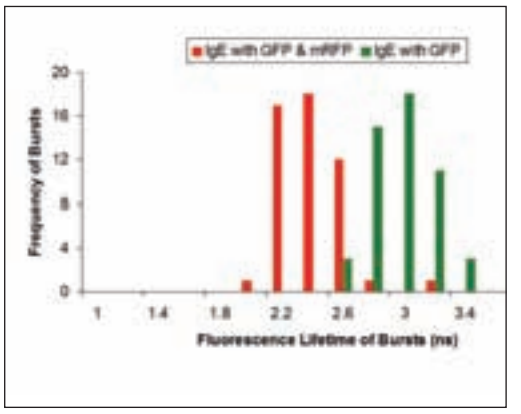

Figure 9. Histogram of fluorescence lifetime of all bursts of photons belonging to the control and dual labelled samples showing a change in lifetime.

We have presented the outcome of our experimental work employing single molecule spectroscopy to study intramolecular dynamics. Compared to a control antibody labelled with GFP alone, a decrease in fluorescence lifetime was found in dual labelled samples. This provided evidence of intra-molecular FRET. Future work will focus on identifying lifetimes of mixed population of molecules.

We gratefully acknowledge the financial support of the Medical Research Council. We would like to thank Professor A Coolen and Dr P Barber

Combining High Throughput, High-Resolution Polymorph Screening

The X'Pert PRO MPD X-ray powder diffraction (XRPD) system from **PANalytical** combines rapid data collection with unsurpassed data quality. This makes possible for the first time the use of a single analytical system for polymorph screening in combination with structural analysis.

Traditionally, screening instruments have been optimised for speed, providing only low-resolution data. As a result, the use of a second, high-resolution analysis technique has been common.

Now, sample data collected on Cimetidine (a histamine receptor antagonist), bovine insulin, and other complex organic materials, shows how the X'Pert PRO MPD takes high-resolution screening to a new level. The system offers high throughput, high-resolution screening that goes beyond pure polymorph identification to deliver semi-automatic crystallographic analysis.

The investigation of polymorphs - chemically identical substances in different crystallographic forms with possibly differing dissolution properties and varying bioavailability - is a key step in drug development. It aids the selection of a suitable solid form for development. In addition, because several polymorphs can have similar efficacy, it is essential that pharmaceutical companies discover and patent all polymorphs, hydrates and solvates of a drug.

XRPD is an accepted methodology for new product registrations and patent applications - an indexed XRPD pattern is frequently used to secure a patent.

Circle no. 157

Compact Personal Imaging with a Small Footprint

GRI are delighted to announce the launch of the red™ Personal Imaging System from Alpha Innotech. The red™ is a compact standalone system for gel documentation and fluorescent imaging. With a small footprint the red™ requires minimal bench space and houses a scientific grade camera that provides quality imaging along with motorised optics at an incredible price point. The red™ Personal Imaging System combines superior specifications such as a scientific-grade camera with motorised optics and a new touch panel interface into one dynamic package. The scientific grade camera offers high-resolution images while motorised optics allow the user to control settings through the touch screen. A customised graphics interface designed specifically for red™ makes image capture a breeze.

red™ is housed in a light-tight casing. The system features an integrated UV transilluminator and epi white LED lights. A three position filter wheel in addition to an optional white conversion screen makes red™ suitable for imaging a variety of UV fluorescent labelled gels in addition to white light applications. These features are designed for exciting and imaging a wide range of UV fluorescent labels as well as white light applications. The red™ Personal Imaging System and other Alpha Innotech products and are available immediately and exclusively from GRI.

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