TOWARDS HIGH-THROUGHPUT FLIM FOR PROTEIN-PROTEIN INTERACTION SCREENING OF LIVE CELLS AND TISSUE MICROARRAYS.

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ABSTRACT

Studying cellular protein-protein interactions *in situ* requires a technique such as fluorescence resonance energy transfer (FRET) which is sensitive on the nanometer scale. Observing FRET is significantly simplified if the fluorescence lifetime of the donor can be monitored. Results from live cells and tissue micro arrays are presented from an automated microscope incorporating time-domain TCSPC fluorescence lifetime imaging (FLIM). Novel hardware and software with a modular approach and scripting abilities allow us to work towards speed-optimized acquisition and ease of use to bring FLIM into the high-throughput regime.

Index Terms— FRET/FLIM, High-Content Screening, Tissue Micro Arrays, Protein-protein interactions

1. INTRODUCTION

In the current post-genomic era attention is drawn to the many thousands of proteins that enable and execute cell function. Many of these proteins will effect cancer progression and our motivation to forward the understanding of protein function comes from the potential for cancer therapies. The study of how and where these proteins interact to perform specific fundamental processes, requires the use of advanced microscopical imaging techniques. However, fluorescence microscopy with a resolution of a few hundred nanometers (the most highly resolved imaging technique for the study of live cells) is not sufficient to determine the interaction status of co-localized proteins. The *effective* resolution can be enhanced to a few nanometers through the detection of a transfer of energy between closely spaced donor and acceptor fluorophores, called Förster (or fluorescence) resonance energy transfer (FRET) [1,2]. FRET can be detected through intensity based methods, such as sensitized acceptor emission, but this method suffers from spectral bleedthrough (SBT) because

of the necessary spectral overlap of donor and acceptor precluding the exclusive excitation of the donor with detection of acceptor fluorescence, without some contribution from contaminating acceptor excitation and/or donor fluorescence [1]. Acceptor photobleaching avoids SBT but is unsuitable for time-lapse experiments with live cells [1]. Measuring the fluorescence lifetime of the donor fluorophore provides a more versatile means of FRET detection [3,4].

Fluorescence lifetime microscopy (FLIM) has been used for some time and systems are available commercially, however, to date, there has only been one example of an automated system that incorporates FLIM [5] and none that use time-domain FLIM via time-correlated single photon counting (TCSPC) [6]. The analysis of mixed populations of fluorophores, some experiencing FRET and some not, results in bi-exponential fluorescence decays making FLIM by single-frequency-domain methods troublesome.

With the ultimate goal of understanding protein-protein interactions in-vivo, studies must progress from fixed and live cells in-vitro and through to excised and sectioned tissue. Although work in all models is ongoing, we concentrate here on high-content and throughput live cell and sectioned tissue studies. The automated studies of tissue sections are aided by the use of tissue micro arrays (TMA's) [7].

In this paper we present techniques we have used to develop automated high-content microscopes. In particular, one that incorporates time-domain TCSPC FLIM and some experimental results gained through its use. Although automated microscopy systems are commercially available, the hardware they can control and the software that drives them is either proprietary or not sufficiently flexible to cope with our developments. We therefore develop in-house hardware as well as control and analysis software [8]. This includes novel software for global analysis of time-resolved data over multiple pixels and multiple images [9].

We present two experiments, biologically relevant to cancer research that demonstrate different aspects of the FLIM microscope. Firstly a live cell time-lapse experiment showing the time course of activation of a protein important for cell structuring (Cdc42) over several minutes at time intervals usually too short for TCSPC FLIM. Also shown is automated FLIM performed on a TMA showing ezrin-PKCα interaction which is implicated in breast cancer metastasis.

2. HARDWARE SYSTEM DESCRIPTION

The multi-photon time resolved microscope is based around a Nikon TE2000-E inverted microscope, Hamamatsu Orca camera, Becker & Hickl SPC830 TCSPC data acquisition card, Coherent Mira Titanium:Sapphire 76 MHz <150 fs pulsed laser, Märzhäuser Wetzlar motorized stage and in-house developed optical scanners and other components [8]; see Figure 1. We have taken an expandable and flexible approach with communication based on USB and the I^2C protocol.

Live cell work enabled by the thermostatically controlled enclosure that can maintain a 37 degree environment. Small animal in-vivo work is also supported within the enclosure.
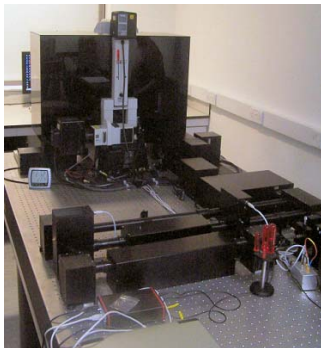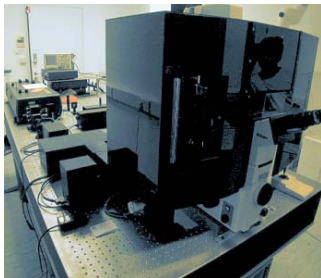

Figure 1, Pictures of the automated FLIM microscope used. The front view (top) shows the Nikon TE2000 microscope enclosed in an incubation chamber that maintains 37 degrees at the sample. The dual-processor control PC sits underneath the optical table. The Titanium:Sapphire laser can be seen at the far end of the table. The back view (bottom) shows black boxes that contain in-house developed optical systems, such as zoom telescopes and the optical mirror scanners, the laser beam traverses the black tubing that links components. The back of the microscope can be seen and the laser is just out of shot to the right.

3. AUTOMATION SOFTWARE

The automation software for this and other microscopes is an ongoing development as different systems can share software to perform similar tasks. As such, some past, current and future developments are discussed. The core software is programmed in the 'c' programming language under the Labwindows/CVI environment (National Instruments, UK). It brings together different hardware components under one software interface but also remains modular to aid testing and enable a different software 'package' to be put together of microscopes with different hardware components. Software 'signals' passed between modules makes this structure easier to maintain.

Importantly, in contrast to other groups, we have brought together the control of the optical scanners and detectors with the time-resolved TCSPC data acquisition provided by the SPC830 card. This 'Single Button Press' operation of many distinct and separate hardware components enables the full automation of this TCSPC FLIM microscope.

The lower level modules are tied together by higher level functions, such as: multiple-point time lapse; region scanning and mosaicing; and cell finding and revisiting. We have two approaches to these higher level functions. Firstly, the development of custom hardware components allow system functions that are optimized for speed and the software must be appropriately written for these. For example, the completion of a stage motion may trigger, through a hardware line, data acquisition, in much less time than the several software calls that would be required.

Secondly, experiments are always changing and the ease with which these higher level functions can be modified is important. Microscopes under development in our group incorporate a script interpreter based on the 'Python' programming language [10] that, for example, specifies the operations performed at each time point of a time lapse experiment. The problems that arise because of the conflict between speed of operation and ease of modification are being addressed with this approach.

4. IMAGE ANALYSIS

Data from the FLIM acquisition system consists of 3 dimensional data (2 spatial and 1 fast time dimension) and is saved in Imaging Cytometry Standard (ICS) files [11]. The stand alone analysis program allows the fitting of a multi-exponential curve to the fluorescence decay data in the fast time dimension, either on a pixel by pixel basis or globally over the whole image or multiple images. Figure 2 presents the time resolved analysis panel and shows how fits and data can be compared. Fitting is performed by iterative re-convolution with the instrument response function to minimize the Chi-square parameter, for which the Levenberg-Marquardt algorithm is used as described elsewhere [9].

Global analysis is highly suited to FRET experiments where fixed lifetimes can often be assumed for the non-interacting and interacting donors. The fractional contribution of the interacting species is allowed to vary locally, whereas the lifetimes can be both determined and fixed globally. The term 'globally' here can refer to over the spatial extents of the image or multiple images, and over the time of a time-lapse experiment. Importantly, our approach need not require any manual segmentation or selection of

control data from each image and is therefore appropriate for automated processing. Approximate timings to process an image of a single cell at a resolution of 0.5 microns per pixel were around 3 seconds for a global bi-exponential fit on a 2.4 GHz Intel Pentium 4 PC.

These time resolved image analysis functions are built into a processing application, called TRI2, which aims to be flexible and easy to use with a multi-image user interface and many built in functions such as spectral linear un-mixing, colocalisation and blob counting with the CHARM algorithm [12].
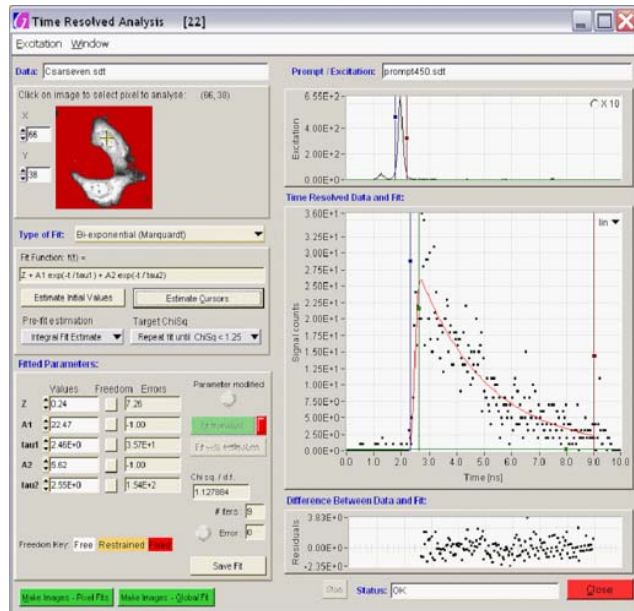

Figure 2, The time-resolved analysis software runs independently to the microscope in a stand alone application. This transient fitting panel shows some of the options available for fitting multi-exponential models to the fluorescence decay signals, and to make parametric images of lifetimes in a global or pixel-by-pixel manner.

5. CDC42 ACTIVATION TIME COURSE IN LIVE CELLS

The first biological example, relevant to cancer metastasis, involves imaging live cells in the study of the important cell structuring and motility protein Cdc42. The Rho family of small GTPase proteins (Cdc42, Rac and Rho) integrate changes in the extra or intracellular environment, e.g. the actin cytoskeleton, and the production of filopodia [13,14,15]. The human epithelial carcinoma cell line A431 was stimulated with epidermal growth factor (EGF) and Cdc42 activity monitored using the FRET probe Raichu-Cdc42 [16] which was adapted to make it suitable for two-photon FLIM. Cells were imaged at 1 frame approximately every 15 s.

This experiment demonstrates the systems ability to acquire automated FLIM time-lapse data with a 10 second interval. Applying global analysis to all the images in the time series enables the estimation of bi-exponential

parameters from extremely low photon count data showing a time course of interacting fraction as the EGF acts to increase Cdc42 activity (Figure 4).
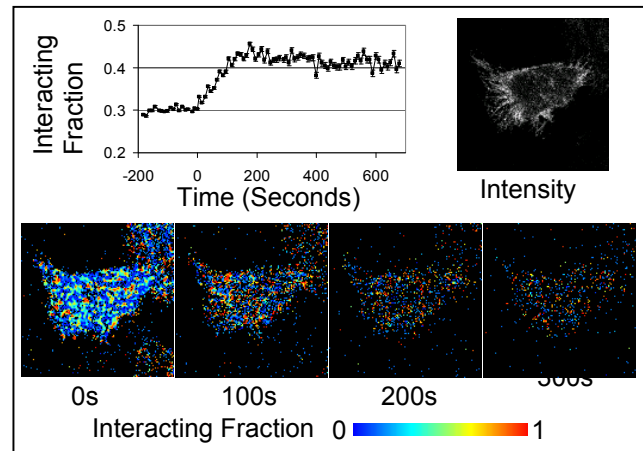

Figure 4, Monitoring Cdc42 activity before and after the addition of EGF (at time zero) using the FRET probe Raichu-Cdc42 in a low-photon-count time-lapse experiment with a short image interval. Performing a multi-image global bi-exponential fit produces robust lifetime estimates. It also reveals an interacting FRET efficiency of 0.57 and a time course of interacting fraction showing a significant before-after effect and importantly maintains both the spatial and temporal image resolution. The error bars represent the standard error in the average interacting fraction from each image. Representative images demonstrate the trend. Data was binned with a Gaussian kernel with sigma equal to 6 pixels.

6. EZRIN-PKCα INTERACTION ON A TISSUE MICRO ARRAY

The study of a cytoskeletal/membrane protein, ezrin, and the signaling protein PKCα may be significant for understanding metastasis of breast cancer [17]. This experiment confirms the ability perform automated scanning of an array of 84 tissue samples an acquire data from different modalities (FLIM and wide-field fluorescence) in a reasonable time scale.

TMA sections containing 84 1.5-mm diameter tissues cores were co-stained with two fluorophore-labelled immunoglobulins: anti-2H3 labelled with Cy2 which recognises the protein ezrin and anti-T(P)250 labelled with Cy3, which recognises activated PKCα. When in close proximity, Cy2 will transfer energy to Cy3 via FRET. In a series of automated data acquisition runs, a region near the centre of each core was identified and stored by the operator. Each region was then imaged in an automated fashion: firstly, autofocus was performed by a software algorithm based on the Fibonacci series in widefield fluorescence mode; the region was imaged using 3 different fluorescence cubes (G-2A for Cy3, FITC for Cy2 and UV-2A for autofluorescence); lastly, a time-resolved FLIM image of the Cy2 channel was acquired.

Figure 5 presents a set of images from one particular region were FRET is thought to be occurring as indicated by a reduction in the donor fluorescence lifetime. The

widefield images are to be used for manual grading as well as automated image segmentation of membrane, cytoplasm and nucleus for compartmentalised analysis (unpublished). The wider project aims are to correlate this interaction with clinical diagnosis and patient outcome.
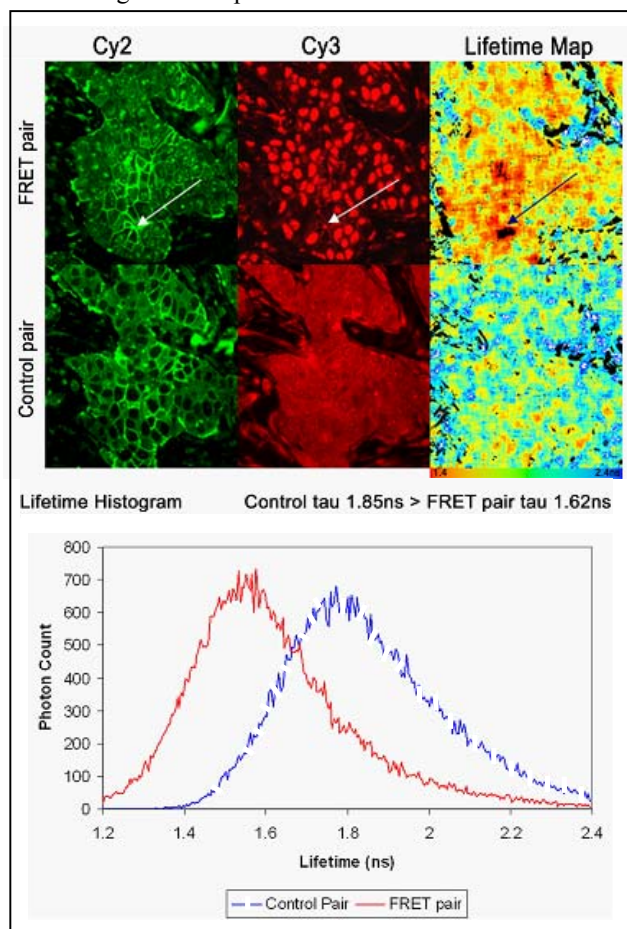

Figure 5, Images of a tissue section from a TMA of 84 tissue cores showing Cy2 labelled ezrin and Cy3 labelled PKCα as well as the lifetime map of Cy2 from time-resolved imaging. FRET is indicated by a reduction in the Cy2 (FRET donor) lifetime at a cell membrane (Upper panels and arrow). The lifetime histogram demonstrates an overall reduction in the Cy2 lifetime in the FRET sample compared to the control.

7. SUMMARY AND FUTURE WORK

An automated microscopy system that incorporates time-domain TCSPC FLIM and associated software for control and analysis is described. Two demonstrations have been presented. Firstly, live-cell time lapse imaging with short imaging intervals, leading to low-photon count data which was analyzed for FRET with a multi-image global analysis algorithm. Secondly, FRET was also analyzed in the tissue sections of a tissue micro array in an automated manner.

Future work will be aimed at bringing FLIM into the high-throughput domain with optimized acquisition procedures (fast photon binning) and fitting of low-photon count data (Bayesian data analysis). In the future, results will be imported into a lab-wide database.

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