Automated time-gated FLIM multi-well plate microscope for identifying interactions between endogenously labelled kinetochore proteins in live yeast cells based on FRET

Wenjun Guo[1,2], Frederik Görlitz[1], Sunil Kumar[1], Douglas Kelly[1], Sean Warren[1], Yuriy Alexandrov[1], Edwin Garcia[1], Ian Munro[1], Peter Thorpe[2,#], Chris Dunsby[1,#], Paul French[1,#]

[1]Photonics Group, Department of Physics, Imperial College London, UK; [2]Mitotic Control Lab, The Francis Crick Institute, London, UK; [#]Authors contributed equally to this work.

Förster Resonance Energy Transfer (FRET) enables the detection of interactions between fluorescently-labelled proteins in fixed or live cells and Fluorescence Lifetime Imaging (FLIM) of the donor fluorescence lifetime provides a robust a readout of FRET. Wide-field approaches provide rapid FLIM acquisition that can be applied to automated high content analysis. Recently we have developed an automated time-gated FLIM microscope that is controlled by a *μManager*-based open-source software *OpenFLIM-HCA* and can acquire FLIM images at a typical speed of ~10s per FOV in a multi-well plate format [1].

In this study, we have applied our *openFLIM-HCA* platform to query protein interactions in budding yeast kinetochores. To the best of our knowledge, this is the first report of time-gated FLIM applied to FP-labelled endogenous proteins in live cells, and here we present the strategies we have developed to address the associated challenges, in particular the low signals resulting from low copy number endogenous kinetochore proteins, which tightly cluster into one or two foci in each cell, the high cellular autofluorescence background, and the impact of photobleaching on both the signal and the background autofluorescence. We selected the CFP-variant mTurquoise2 as the FRET donor for its brightness, photostability, and monomeric intrinsic lifetime, with YFP as the acceptor. Optical sectioning was provided by a Nipkow spinning disk unit to reduce out-of-focus autofluorescence and a 60x water-immersion objective lens was used with an electronically controlled pump to maintain immersion water during automated imaging. Yeast cells were seeded in a 96 well plate in synthetic complete medium with additional adenine and FLIM was undertaken at room temperature. Each FOV was pre-photobleached for 15 s to reduce cellular autofluorescence, then time-gated images were acquired at 7 delays after excitation utilising a gated optical image intensifier (GOI) read out by a CCD camera with 3 s exposure time. Imaging each FOV took <60s including stage movement and autofocusing. 15 FOVs from each of 3 wells were acquired for each yeast strain to average over biological heterogeneity and noise in the measurement process. FLIM data were then processed with a MATLAB-based software to exclude bright dead cells, segment for kinetochore foci, and subtract the local cellular background from each kinetochore. Our open-source software tool, *FLIMfit* [2], was used to calculate lifetime parameters using global analysis. By comparing lifetimes from query strains with donor-only negative controls, we found that the protein interacting partners we identified, e.g. Spc24p and Spc25p in the NDC80 complex, agreed with previous reported data, validating our methodology. In the future, this combination of instrumentation and image analysis will be used to screen for interactions between endogenously labelled mitotic regulators and kinetochore proteins in live yeast cells, and should also be applicable to other live cell studies.

[1] Görlitz, F. *et al*. Open Source High Content Analysis Utilizing Automated Fluorescence Lifetime Imaging Microscopy. *J. Vis. Exp*. e55119–e55119 (2017).
[2] Warren, S. C. *et al*. Rapid global fitting of large fluorescence lifetime imaging microscopy datasets. *PLoS One* **8,** e70687 (2013).