**Title: - Insights into molecular mechanisms regulating the activity of multidomain proteins in living cells using FRET-FLIM**

## SUMMARY

Macromolecular association and dissociation are key events involved in the subcellular organisation below the limit of optical resolution. Foersters/Fluorescence Resonance Energy Transfer (FRET) in combination with Fluorescence Lifetime Imaging Microscopy (FLIM) is among the best quantitative methods to probe these events at the subcellular regime. In this work, FRET dynamics of Green Fluorescent Protein (GFP) based tandem constructs were investigated in living cells using a combination of FLIM and Fluorescence Lifetime Micro-Spectroscopy (FLMS) at picosecond time resolution and nanometer spectral resolution. Simultaneous detection and analysis of intensity decays of donor and acceptor probes as coupled excited state reactions identified the lifetimes participating in energy transfer. This method differentiated the involvement of multiple conformations of Cyan Fluorescent Protein (CFP) in energy transfer to Yellow Fluorescent Protein (YFP), by plotting pre-exponential factors of individual lifetimes along the wavelength resulting in the Decay Associated Spectra (DAS). A change in sign of pre-exponential factors from positive to negative at the acceptor emission maxima confirmed FRET in the multiexponential lifetime analysis. This approach discriminated the intramolecular energy transfer dynamics between the tandem constructs which differed in spacer lengths down to eight amino acids. The results allowed to obtain a kinetic model for FRET occurring from multi-exponential CFP to monoexponential YFP, which was a basis for interpreting results using the same fluorophores in the context of various biological applications like protein folding and conformational changes.

Lymphocyte specific protein tyrosine kinase (Lck) is among the first proteins to be recruited to the immunological synapse, implicating its importance in T cell signalling. Results from FRET-FLIM studies suggested that in resting T-lymphocytes Lck exists in equilibrium between closed (passive) and open (active) conformations. The structural prediction from the FRET-FLIM studies was coherent with the existing hypothesis for the structure of Src kinases. In stimulated T-lymphocytes, Lck indicated a temporary reversible change in its conformation from the closed to an open state. These transient changes were in correlation with the reported kinase activity of Lck, where an initial increase in kinase activity was observed during the early moments of formation of an immunological synapse, which returned to the basal level in 20 min.

Membrane-associated guanylate kinases (MAGUKs) are multidomain molecules pivotal in the architecture of various cell-adhesion interfaces. Synapse-associated protein 97/Human Discs Large (SAP97/hDlg) interacts with the SH3 domain of Lck using the proline-rich region at the N-terminus of the protein. The exon encoding this proline-rich region is subject to alternative splicing. The absence of Lck as well as the expression of the protein lacking its proline-rich region was observed to affect the localisation of SAP97/hDlg to T cell-bead interfaces or mock immunological synapses. The changes of intramolecular FRET in the conserved SH3-HOOK-GUK unit at the C-terminus of different MAGUKs (SAP97/hDlg and SAP90/PSD95) in response to elevated calcium levels were investigated. The observed changes were ascribed to the formation of parallel or anti-parallel dimers, creating a rigid molecular framework of cytoplasmic scaffolds.

Thus, with a combination of advanced microscopic methods, cell biology and molecular modelling, activity-dependent structural regulation and intramolecular association of multidomain proteins were studied during the initial moments of cell recognition events. The transient conformational changes and activity-dependent distribution of Lck and MAGUKs could be central in signal transduction machineries, efficiently distributing signals within the immunological synapse, and at the same time involved in preparing a dynamic molecular platform for assembling near-membrane scaffolding molecules.