Abstract to the Dissertation of:

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INVESTIGATING PROTEIN-PROTEIN INTERACTIONS BY FRET-FLIM IN LIVING CELLS: INSIGHTS INTO INTERACTIONS MEDIATED BY THE PRESYNAPTIC PROTEIN BASSOON

SUMMARY

Förster's Resonance Energy Transfer (FRET) combined with Fluorescence Lifetime Imaging Microscopy (FLIM) is becoming a powerful tool to map protein-protein interactions in living specimens. In contrast to the in vitro methods which are useful to assess the potential of proteins for physical interactions, a combination of these techniques provides high spatial (nanometer) and temporal resolution (picosecond) in monitoring interactions within protein complexes in their natural environment. A completely new microscopic system including non-scanning detectors based on Time and Space Correlated Single Photon Counting was constructed for the interaction studies. In time domain FLIM, the plots of contributions of the different lifetimes of the donor and acceptor fluorophores as a function of wavelength, (Decay Associated Spectra/DAS), were used to study the presence of FRET.

The developmental shift of chloride ions in neurons is important in both neurobiological as well as biophysical aspects. It is an essential feature to maintain equilibrium between excitation and inhibition in the developing brain. Also, the biophysical properties of the fluorescent probes are significantly affected due to this shift. A ratiometric chloride sensor Clomeleon, consisting of CFP (Cyan Fluorescent Protein) and a chloride sensitive variant of YFP (Yellow Fluorescent Protein) called Topaz, was used for studying the changes in intracellular chloride concentrations during neuronal development. A multi-wavelength approach with simultaneous detection and analysis of donor and acceptor probes was employed, with which a discrimination of energy transfer from other excited state reactions was possible along development of living hippocampal neurons. Clomeleon was used as an optical indicator for monitoring the intracellular chloride concentrations in living cells by steady-state and time-resolved spectroscopy. A direct correlation of FRET in Clomeleon with the overall development of individual neurons as well as at different subcellular compartments was also possible. The results helped to arrive at a kinetic model for the mechanism when competitive quenching effects as well as energy transfer simultaneously occur in the same molecule.

A principal candidate for investigation was the presynaptic protein Bassoon, a major scaffolding component of the CAZ (Cytomatrix at the Active Zone). Currently it is believed that prefabricated complexes comprising of presynaptic scaffolding proteins including Bassoon are assembled at the trans-Golgi network and are transported via dense-core vesicles to the presynapses. Using immunofluorescence techniques we could show that a central fragment of Bassoon, which contains essential signals for the recruitment of Bassoon to the presynapse, can simultaneously recruit CtBP1 and CAST to the same molecular complexes. Time-lapse studies indicated a co-transport of these proteins along neuronal axons and growth cones to possible synapses. In order to address a Bassoon-dependent recruitment of CtBP1 to different subcellular compartments, the studies were extended to observe a direct physical association of these proteins using a combination of FRET and FLIM in COS-7 cells and in the trans-Golgi network and synapses of living hippocampal neurons. The fluorescence properties of the donor probe were observed to be significantly affected depending on the property of the protein component to which it was fused and the environment in which it was expressed. Even in presence of high challenges posed for FRET-FLIM studies due to the

varying intracellular ionic concentrations in neurons, our modified approaches in combination with immunocytochemistry made it possible to address the questions of interest.

Thus a deep investigation to address the changes in intracellular chloride along neuronal development and their photophysical effects on fluorescence, as well as interactions of proteins involved in synaptogenesis was done, which included the construction of a highly complex microscopic system as well as characterisation of the biophysical and biochemical properties of the proteins involved by microscopic, spectroscopic, biochemical, molecular biological and computational techniques.