# ADVANCED FLUORESCENCE MICROSCOPY AS A TOOL TO CHARACTERIZE HETEROGENEITY AND TUNABILITY IN PROTEIN AGGREGATES

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Protein aggregates are characterized by high structural and morphological heterogeneity at the nano- and micro-scale and different stability that may translate into a variety of effects in biological systems. Multiple association mechanisms ranging from protein' conformational changes to liquid-liquid phase separation phenomena are at the basis of aggregates formation. For such a reason, methods of analysis with high spatio-temporal resolution and molecular scale sensitivity are highly demanded for the characterization of their structure.

In this scenario, fluorescence microscopy, arises as a perfect tool as it gives the possibility of obtaining quantitative spectroscopic measurement at diffraction limited spatial resolution. We here show how Fluorescence Lifetime Imaging Microscopy (FLIM) and Raster Image Correlation Spectroscopy (RICS) can be successfully applied for the investigation of protein aggregate structural heterogeneity and to monitor disassembly processes into sub-diffraction structures.

By means of FLIM measurements analyzed by a phasor approach, we developed a method to study structural heterogeneity in ensembles of protein aggregates. We correlated lifetime variations of Thioflavin T (ThT), a gold standard fluorescent dye for the analysis of amyloid structure, to the molecular architecture of the aggregates. This allows one to observe structural differences not only between supramolecular structures but also within the same aggregate. The same method was employed on aggregates of different nature and arising from different proteins, revealing common features. The analysis on ThT lifetime suggests that different types of protein-protein interactions are dominant in each of the self-assembly phenomena, which in turn regulate resulting aggregates stability. The disassembly of protein aggregates can be readily and dynamically monitored using RICS, facilitating the assessment of aggregates stability against harsh conditions. We show howuse of RICS presents several advantages with respect standard methods, which involve several steps and are largely time consuming as it allows to reveal, map and quantify the presence of small oligomeric species released by large aggregates and to simultaneously monitor the fate of micronscale and nanoscale aggregates.

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