

PD-PAM

Use of *in-vitro* cell-models for assessing alpha-synuclein aggregate morphology and other polymorphic amyloid variants using amyloid specific fluorescent ligands

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Aims, research questions and working hypothesis:

The Parkinson's Disease (PD) is one of the well-known neurodegenerative disorders affecting majorly the ageing society. PD involves dysregulated molecular alterations in proteins that are crucial for the proper functioning of our brain. At the molecular level, in PD, the alpha-synuclein (α syn) protein undergoes extensive structural rearrangement into amyloid fibrils, to form inclusions in the form of Lewy bodies in PD as well as α syn deposits in other synucleinopathies. The clinical presentation of PD patients is highly heterogenous at the early disease stages. Notably, α syn protein deposits are known to form distinct structural morphologies which may be attributed to variable clinical features observed at the early disease phase in the gut or in the brain. In our project it is anticipated that the cellular milieu in which the α syn protein resides and forms aggregates, also impact the structural morphology of α syn amyloid formation. In the project, we are investigating the effect of α syn fibrillation in various cell models (from the brain and kidney). The currently available protein aggregate detection methods pose a caveat in identifying different polymorphic amyloid variants. In our research project, we aim to use amyloid specific fluorescent ligands, known as luminescent conjugated oligothiophene (LCOs) to assess the poly-morphology of α syn in different cell-models. Our project aims to combine new molecular research tools with strategies to examine the biological markers and molecular mechanisms behind this disease.

Means/methods implemented by the consortium:

First, the fluorescence spectral and decay time properties of the LCOs used are investigated in detail in various solvents. Next, the *in-vitro* binding of LCOs to the PFFs are evaluated by assessing the emission profile of the LCOs by varying their concentration and keeping the concentration of PFFs fixed. Hereby, the binding affinity can be analyzed to get an estimation of the concentration of LCO needed for staining the aggregates in cells. We have initially used recombinant α syn pre-formed fibrils (PFFs) derived from human or mouse gene sequence.

Second, the aggregate morphology is investigated in cell models, where we started with HEK293 (but also SH-SY5Y and astrocytes are on the project plan). This was achieved by transfecting the cells with either wildtype (WT- α syn-HEK293) or A53T-mutated α syn (A53T- α syn-HEK293) to induce endogenous expression of monomeric α syn in cells. The α syn expression was quantified using FACS (Fluorescence assisted cell sorting) and western blotting using conventional antibody labelling strategies.

The α syn-transfected cells are then exposed to various PFFs to induce aggregation. Subsequently, the transfected cells, exposed to PFFs, are stained with LCO and imaged using confocal laser scanning microscopy. The binding of LCO to the aggregates in the cellular milieu are examined by spectral imaging in microscopy. In addition, the conformational based fluorescence lifetime measurements of the LCO binding to the aggregate are evaluated using Fluorescence Lifetime Imaging Microscopy (FLIM).

What are the outcomes of the project?

1. Spectral emission profiles and binding curve of LCO, h-FTAA to the PFFs showing h-FTAA effectively binds to PFFs with a binding constant < 200nM concentration

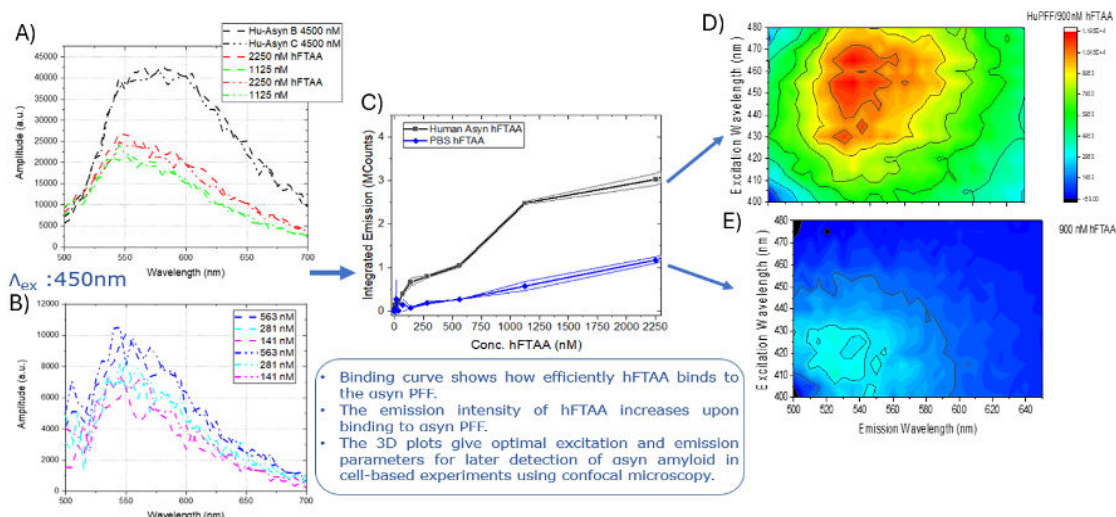


Figure 1: Emission profile, binding curve and 3D spectral plot showing varying concentrations of h-FTAA binding to human PFFs, fixed at 1µM, with the h-FTAA being excited at 450nm. Emission profile showing increase in fluorescence intensity of h-FTAA at **A)** higher (1.1µM to 4.5µM) loading and at **B)** lower conc. (0.1µM to 0.6µM) upon binding to PFFs, fixed at 1µM conc. **C)** The emission profiles of h-FTAA are integrated and plotted against the increasing conc. of h-FTAA, to obtain the binding curve. The black curve shows the binding of h-FTAA to PFFs, and concomitant increase of fluorescence intensity as opposed to the blue curve, which is the integrated emission curve of h-FTAA alone in PBS. The 3D excitation, emission scan gives an insight about the precise excitation and emission parameters to be used when looking for h-FTAA-stained regions in cell-based experiments.

2. Spectral imaging and FLIM of h-FTAA binding to asyn aggregate morphology in A53T-asyn and WT-asyn HEK 293 cells showing distinct emission and lifetime profiles

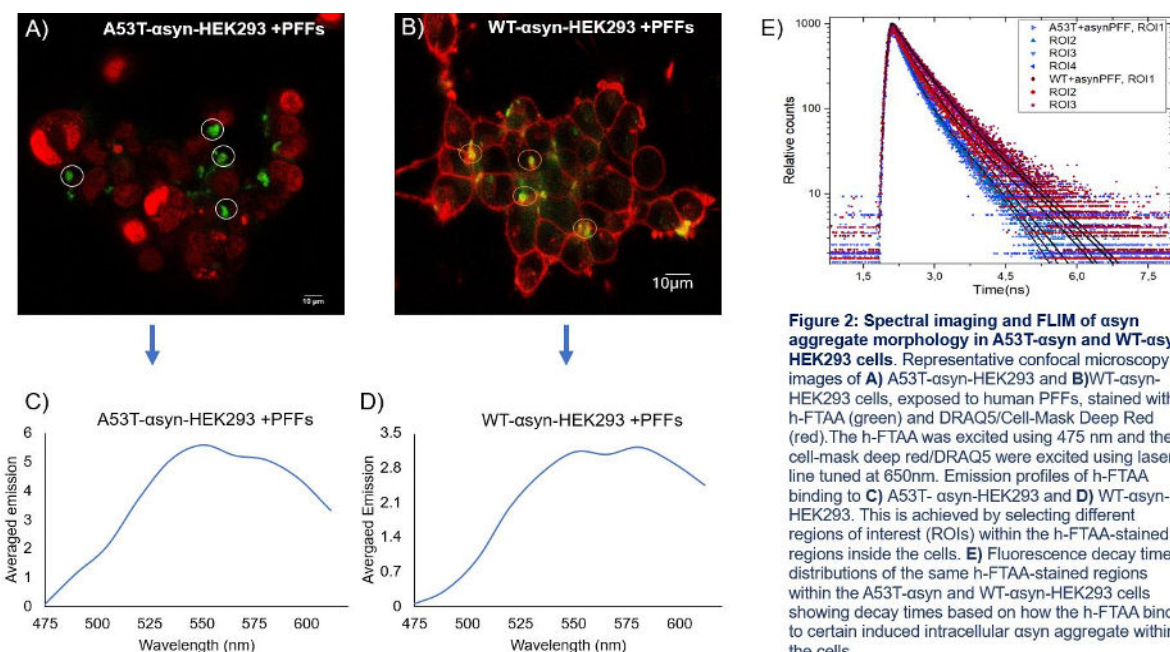


Figure 2: Spectral imaging and FLIM of asyn aggregate morphology in A53T-asyn and WT-asyn HEK293 cells. Representative confocal microscopy images of **A)** A53T-asyn-HEK293 and **B)** WT-asyn-HEK293 cells, exposed to human PFFs, stained with h-FTAA (green) and DRAQ5/Cell-Mask Deep Red (red). The h-FTAA was excited using 475 nm and the cell-mask deep red/DRAQ5 were excited using laser line tuned at 650nm. Emission profiles of h-FTAA binding to **C)** A53T-asyn-HEK293 and **D)** WT-asyn-HEK293. This is achieved by selecting different regions of interest (ROIs) within the h-FTAA-stained regions inside the cells. **E)** Fluorescence decay time distributions of the same h-FTAA-stained regions within the A53T-asyn and WT-asyn-HEK293 cells showing decay times based on how the h-FTAA binds to certain induced intracellular asyn aggregate within the cells.

Significance and impact of the work on the field

The detection and binding of LCOs to distinguish between distinct morphotypes in cell-culture may allow identification of subtype-specific conformations of asyn in the future. This could enable subtyping of PD, based on the structural variations observed between distinct asyn conformers and potentially create a therapeutic window for early intervention in the early disease phase.

Next steps and future challenges

This approach will be extended to investigate the amyloid seeding capacity onto our cell models by various asyn strains, both *in-vitro* and from animal models. A challenge will be the further development of relevant and targeted molecular assays focused on measuring the spectrum of PD pathophysiology. This also emphasizes the importance of cell models as an interdisciplinary research platform for bridging this gap.