Fluorescence Lifetime Imaging of Protein Aggregation to Understand the Etiology of Neurodegenerative Diseases

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Abstract: Time-domain fluorescence lifetime imaging microscopy is presented for the detection of alpha-synuclein aggregation in neurons and for determining spread, thereby facilitating understanding of the development and progression of Parkinson's disease.

Neurodegenerative diseases involve the progressive dysfunction and eventual loss of neurons in the brain. While the mechanism for many of these diseases is unclear, protein aggregation has been identified as a shared characteristic [1]. This is evidenced through the formation of highly organized, amyloid-like fibrillar protein deposits having a β-sheet structure. Aggregation evolves from monomers to fibrils with intermediate oligomers of varying assembly states. It is currently unclear which of these aggregated species imparts neurotoxicity, and determining aggregate type and level in neurons has been challenging. We demonstrate the detection of different aggregation states for alpha-synuclein (aSyn), a neuronal protein that forms aggregates in the brains of patients with Parkinson's disease (PD), using a joint fluorescence (Förster) resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM) framework.

Amyloid-like protein fibrils, owing to their β-sheet structure, develop intrinsic absorption and fluorescence in the visible spectral region [2, 3]. The absorption spectrum for fibrils formed by proteins such as aSyn overlaps with the emission spectrum for yellow fluorescent protein (YFP), thus enabling a FRET interaction in which conjugated YFP acts as the donor and the growing aSyn aggregate acts as the acceptor [4]. This results in a concomitant decrease in the fluorescence lifetime of the YFP label as aggregation proceeds, thus providing an optical reporter of the aggregation state. This sensing strategy is depicted in Figure 1(a), where intermediate oligomers are categorized based on the detected fluorescence lifetime of the YFP label.

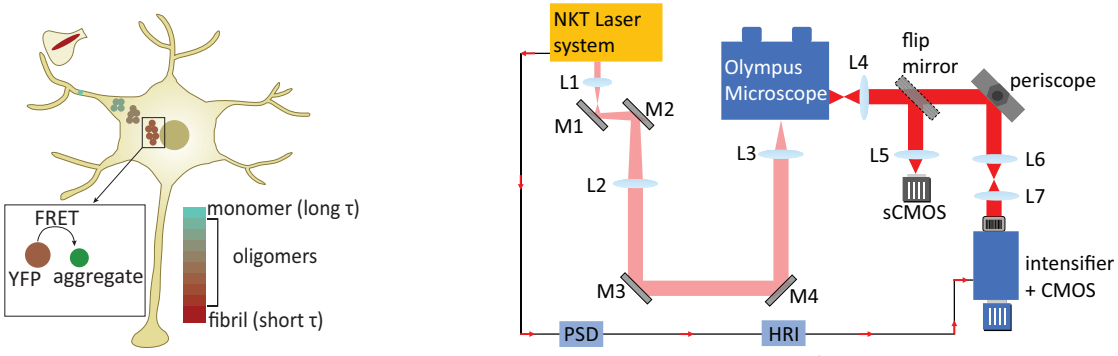
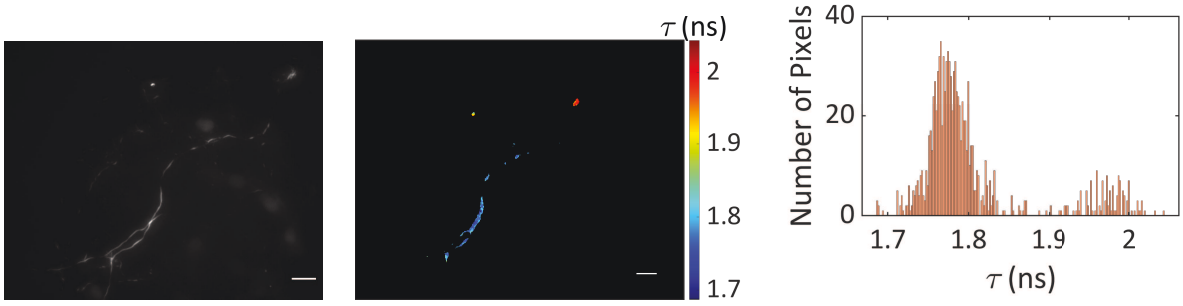


Fig. 1. (a) General FRET-FLIM framework for identifying protein (aSyn) oligomers and fibrils during aggregation based on the recorded fluorescence lifetime (τ) of the YFP label. (b) Schematic of the time-domain FLIM system (top view, not drawn to scale), where the excitation and emission optical paths are depicted in light and dark red, respectively. L: lens, M: mirror, PSD: picosecond delay unit, and HRI: high-rate image intensifier.

To perform time-domain FLIM experiments, a time-gated, wide-field FLIM system was constructed using a modified Olympus iX73 inverted microscope (Fig. 1(b)). The excitation source is a 20 MHz pulsed supercontinuum fiber laser coupled with a VARIA tunable filter, resulting in an output spectral range of 400-800 nm (NKT Photonics). The system is equipped with two image-capturing devices, a sCMOS camera (Photometrics PRIME) capable of up to 100 fps collection rates for intensity-based imaging, and a high-rate image-intensified (PicoStar HR12, LaVision) CMOS (Imager-M-Lite, LaVision) integrated system for fluorescence lifetime recording. A picosecond delay unit (PSD) is responsible for introducing delays between the intensifier gate and exciting laser pulse. The YFP label was excited at 488 nm and the fluorescence intensity decay was sampled with an intensifier

gate width of 200 ps in 250 ps increments. The fluorescence emission was collected through a bandpass filter with a transmission range of 510-550 nm (3-dB). Fluorescence lifetime images were then computed based on the recorded fluorescence intensity data.

We measured the fluorescence lifetime of mutant A53T aSyn-YFP overexpressed in primary rat midbrain neurons. These neurons were treated with aSyn pre-formed fibrils (PFFs) in order to induce the aggregation of intracellular aSyn. To investigate the formation of amyloid-like fibrils, neurons were fixed with 4% (w/v) paraformaldehyde containing 1% v/v Triton-X 100 7 days following aSyn PFF administration. Triton X-100 detergent was used to remove soluble protein so that only mature, insoluble fibrils remained in the sample. PFF administration successfully induced aggregation in these neurons, as evidenced by the distinct high intensity structures in the collected intensity image in Fig. 2(a). We then investigated these fibrillar species via fluorescence lifetime measurements. The lifetime map in Fig. 2(b) highlights two distinct fibrillar species from the soma and axons of two different neurons. The associated lifetime histogram presented in Fig. 2(c) shows that the average lifetime is 1.77 ns for the axonal region (blue), whereas it is 1.98 ns for the somatic region (red). This difference is influenced by variation in the size of the insoluble aggregates and possibly the local environment. We characterized monomeric aSyn by measuring the lifetime of YFP conjugated to aSyn monomers in solution, finding a value of 3.24 ns. As expected, this is significantly longer than the lifetimes obtained for the aSyn aggregate regions shown in Fig. 2(c). These results demonstrate the capability of FLIM to detect different populations of fibrils from distinct neuronal regions and lay the foundation for identifying different stages of protein aggregation in neurons in relation to PD pathology.



(a) (b) (c)

Fig. 2. Fluorescence lifetime measurements of aSyn-YFP expressed in primary midbrain neurons treated with aSyn PFFs and fixative supplemented with Triton X-100 detergent. (a) Intensity image. (b) Processed lifetime image after 50% intensity thresholding. (c) Lifetime histogram. Scale bar: 22 μm.

Coupled with a super-resolution strategy, FLIM could enable live-cell determination of protein aggregation in relation to functional aspects of the lysosome, such as endocytic escape of aggregates, hence giving further insight into aggregate spread. Importantly, using a deep-tissue *in vivo* FRET model [5] for sensing aSyn aggregation, in conjunction with precise localization [6], could enable future animal model PD studies.

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