Invited Paper

# Nanoscale imaging of neurotoxic proteins

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## ABSTRACT

The misfolding and self-assembly of intrinsically disordered proteins into insoluble amyloid structures is central to many neurodegenerative diseases such as Alzheimer's and Parkinson's Diseases. Optical imaging of this self-assembly process *in vitro* and in cells is revolutionising our understanding of the molecular mechanisms behind these devastating diseases. In contrast to conventional biophysical methods, optical imaging, and in particular optical super-resolution imaging, permit the dynamic investigation of the molecular self-assembly process *in vitro* and in cells, at molecular level resolution. In this article, current state-of-the-art imaging methods are reviewed and discussed in the context of research into neurodegeneration.

## 1. INTRODUCTION

Proteins have an intrinsic propensity to self-assemble into aggregates. The process is initiated by the formation of an aggregation-nucleus from the native monomers; this is a small oligomeric species consisting of only a few monomer units, from which aggregation then proceeds *via* association of further monomers. Kinetically, the nucleus is a unit for which the rate of further monomer association is larger than that of dissociation. Such oligomers are called 'on-pathway' species, because their presence increases the propensity of the protein pool to aggregate. In contrast, oligomers that block the growth of aggregates are called 'off-pathway' species; these can dissociate, and act as a source for monomers[1]. Usually aggregation corresponds to the association of proteins, whose polypeptide chain is folded into a structural state that is non-functional; one says the protein is in a 'misfolded state'. Aggregation thus propagates the misfolded protein state and this phenomenon is at the heart of a number of so-called 'protein misfolding diseases'[2,3]. A key problem in misfolding diseases is that normal protein homeostasis is disrupted in the cell, as aggregation lowers the propensity of the protein pool to remain soluble. Small oligomeric species may furthermore elicit toxicity directly, through interference with vital metabolic processes in the cell. These problems are at the heart of major neurodegenerative diseases, such as Alzheimer's Disease (AD) and Parkinson's Disease (PD), in which usually harmless, soluble proteins self-assemble into highly regular, fibrillar aggregate structures called amyloids[4]. In AD and PD, amyloids are formed from intrinsically disordered proteins (IDPs), i.e. those that normally lack defined secondary and tertiary structures under physiological conditions. In response to changes in the molecular environment, or due to point mutations in their peptide sequence, IDPs partially fold and adopt metastable β-sheet conformations (see Figure 1). These partially folded states self-assemble into oligomers, which then gradually convert into stable, β-sheet rich conformations, thus forming aggregation nuclei, from which elongation into the fibrillar amyloid state then proceeds through consecutive monomer addition[5]. In both AD and PD, aggregation appears to be initiated in certain, well defined regions in the brain, before the gradual spreading of aggregated species takes place throughout the entire brain. In the final stages of the disease, a significant fraction of the brain mass of affected patients is replaced by insoluble solid plaques, which are predominantly composed of fibrillar amyloids of the proteins tau and amyloid β (Aβ) in Alzheimer's, and the protein α-synuclein in the case of Parkinson's Disease.

The environmental conditions that favour, or hamper, the self-assembly of proteins into amyloid fibrils; the propagation of misfolded species from cell to cell; the effect of point mutations on the aggregation process; the possible interaction with, and inhibition of, amyloid aggregation *via* small molecule drugs; the structural identification and physiological interaction of toxic species in brain cells; etc. – these are all questions at the heart of a better understanding of these devastating diseases, and the focus of much ongoing biophysical research. Optical techniques play a crucial role in this effort, because of their non-intrusive nature, their suitability to track the kinetics of the self-assembly process in time, and their high sensitivity and specificity in combination with fluorescent labelling techniques, which makes them suitable for applications in cells and even *in vivo*. In what follows we review techniques pioneered in our laboratory and by others, for studies of amyloid formation optically, with nanoscale resolution. We begin with indirect methods, which we term *multi-parameter imaging*, where molecular level information is conveyed *via* changes in spectral properties of

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reporter fluorophores, for example, changes in the fluorescence lifetime or the polarisation of emitted radiation. Bulk measurements are presented first, before their application is discussed in combination with microscopic imaging. We then review the application of single molecule super-resolution microscopy, which reveals morphological information of single aggregate clusters at the molecular scale.

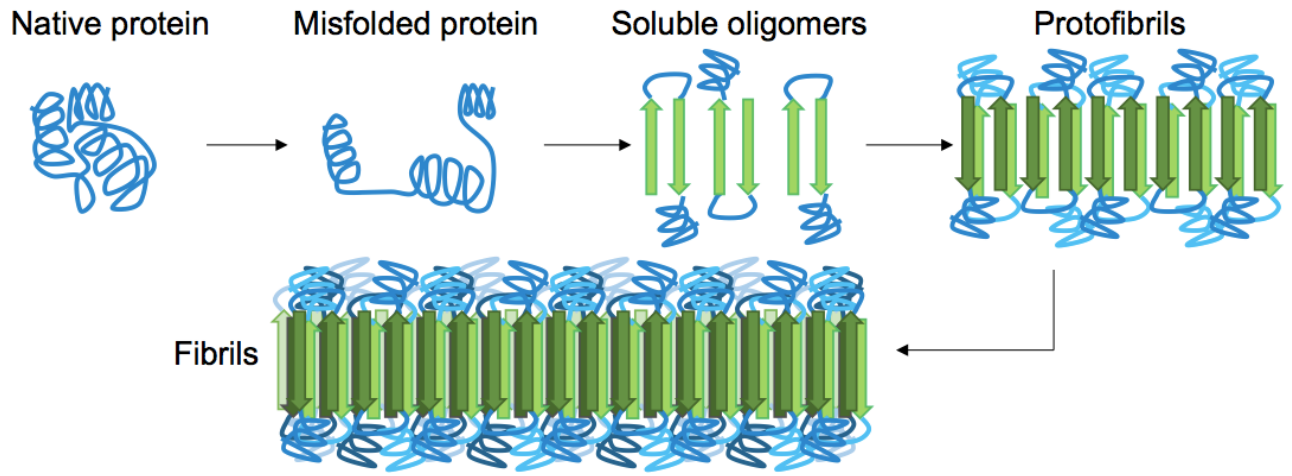


Figure 1: Simplified pathway towards amyloid fibril formation. In the misfolded state proteins have an increased propensity to oligomerise, *via* association of their metastable beta-sheet domains. These can convert into more stable beta sheet states and the ensuing oligomers act as the nuclei for the subsequent elongation reaction, that leads to the formation of so called protofibrils. The final amyloid fibril usually consists of a number or intertwined protofibrils.

## 2. BULK MEASUREMENTS TO STUDY FIBRIL GROWTH

Traditional optical techniques well suited for bulk measurements include the use of far UV circular dichroism (CD), and Fourier Transform Infrared spectroscopy (FTIR), which inform on the content of β-sheet to α-helical structure in unlabelled proteins[6,7]. Furthermore, small molecule dyes, such as thioflavin T (ThT), that bind with a high propensity to the β-sheet rich fibrillar state of amyloids can be used to track the aggregation state in bulk[8]. The rise in ThT fluorescence emission in mixtures containing aggregating peptides is a popular method to track the kinetics of amyloid formation *in vitro*[9]. Although the exact mechanisms by which ThT fluorescence is affected by fibril binding is still incompletely understood, it is thought that intercalation of the dye in the fibril grooves leads to a stabilisation of the molecule in a configuration, which enhances electron delocalisation and thus transition dipole moments.

An intriguing phenomenon recently discovered by us is that the formation of amyloid fibrils is accompanied by the adoption of a structure specific intrinsic fluorescence in the visible range, independent of the presence of aromatic residues in the polypeptide chain[10,11]. This provides for a new tool in the diagnostics of aggregation kinetics *in vitro* and has been used as a quantitative assay, for example, to study the elongation reactions of α-synuclein[12], see Figure 2. The study focused on the elongation of fibrillar template species in the presence of monomers of α-synuclein, and clearly exponential growth of fibrils is evident as a function of incubation time. Growth rates recede gradually, as the monomer concentration becomes depleted during fibril growth. The intrinsic fluorescence properties were found to be similar for many different amyloids tested, and strong enough to render fibrils visible under the microscope. The fluorescence was furthermore confirmed to be independent of the presence of aromatic side chains in the studied proteins and to occur in a wavelength range far removed spectrally from that of traditional intrinsic protein fluorescence (excitation near 400 nm, emission >450 nm emission range)[12].

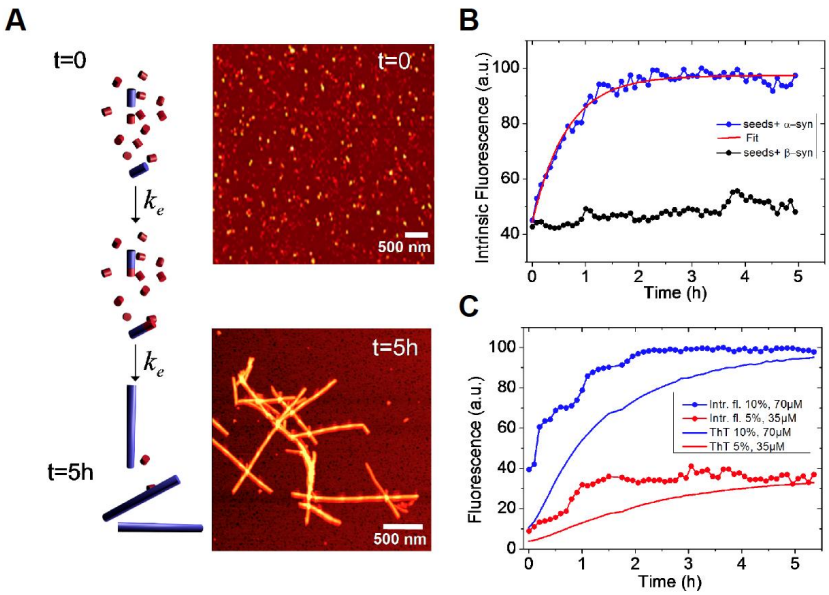


Figure 2: Intrinsic fluorescence informs on amyloid growth kinetics without the requirement for any extrinsic fluorescence labels. A, left: Short, fibrillar seeds of α-synuclein, shown in blue, serve as the template from which fibril elongation proceeds through addition of monomers, shown in red. The top panel to the right shows AFM images of short fibril seeds at the start of the reaction, at t=0. Below is an image of the same sample at t=5 h. Clearly, the elongation of the seeds into long amyloid fibrils has taken place. B: Rise in intrinsic fluorescence from the amyloid fibrils in time. The blue points correspond to the elongation of α-synuclein seeds in the presence of α-synuclein monomer. The black points correspond to the addition of β-synuclein monomers to the α-synclein seed fibrils, and confirm that the two species cannot 'cross-seed' aggregation. The fluorescence measurements were performed with a diode laser emitting near 405 nm and signals were detected around 450 nm. Figure reprinted (adapted) with permission from[12]. Copyright 2013 Wiley .

Whilst a useful tool for kinetic studies of purified protein *in vitro*, the intrinsic fluorescence is too unspecific and weak to be useful in more complex assays, for example for high resolution microscopy techniques or for studies in cells or organisms. Specificity and sensitivity can be conferred *via* fluorescent labels that are covalently linked to the protein of interest. Labelling of primary amine groups is possible via succinimidylesters, but control over the binding site can be difficult, causing possible interference with the amyloid self-assembly process[13]. Site specific labeling is possible *via* maleimide dyes, which react only with cysteine residues. Specific cysteine residues can be replaced or introduced through site selective mutagenesis; this allows the covalent and specific labeling of domains, that lie outside of the amyloid specific misfolding region, thus minimizing the risk of steric interference with the aggregation process[14]. For super-resolution microscopy in particular, this permits the introduction of highly efficient organic dyes, optimally suited to the imaging task at hand.

In all cases, the propensity of the protein to aggregate with comparable behavior to the wild type protein, and to form fibrils featuring similar morphologies, needs to be carefully checked using biophysical assays, such as NMR, AFM and scanning electron microscopy. This is particularly true for the use of fusion proteins as labels, whose size can exceed that of labeled peptide. Nevertheless, the use of variants of green fluorescent protein, GFP, has been demonstrated successfully for a range of amyloid peptides, yielding similar aggregation kinetics and fibril morphology as the wild type protein[15].

## 3. FUNCTIONAL IMAGING OF AMYLOIDS GROWTH KINETICS

Fusion protein technology permits the imaging directly at the live cell-, or even organism-, level[16,17]. This opens a whole range of possibilities for functional imaging techniques, so called multi-parameter imaging techniques that report on molecular self-assembly directly in aggregation models of patho-biological relevance. The use of homoFRET, for

example, reports on aggregation *via* an increasing loss of fluorescence *polarisation* in growing aggregates, upon excitation with polarised laser light[18]. It was possible with this technique, to image with high sensitivity the formation of aggregates in an α-synuclein-YFP model of aggregation, using a confocal microscopy set-up with polarisation resolved fluorescence detection[15]. The fluorescence *lifetime* is also a sensitive reporter on the local environment of a fluorophore[19]. For example, it was shown that the fluorescence lifetime from appropriate fluorophore labels covalently attached to environmentally responsive polymers informs on the *folding* kinetics of the latter in live cells[20],[21]. We recently discovered that *aggregation* into β-sheet rich amyloid fibrils is similarly accompanied by dramatic changes in the fluorescence lifetime. However, here the mechanism differs, and the phenomenon appears to be caused by energy transfer to the emerging energy levels in fibrillar aggregates in a FRET like process; as a result, the fluorescence lifetime of appropriately selected reporter fluorophores decreases upon fibril formation and this can be used as an *in vivo* tool to track the aggregation kinetics with microscopy[22]. We have used this technique to report, for the first time, on the aggregation of α-synuclein in a live *C. elegans* model of protein aggregation, see figure 3, and were able to correlate the appearance of aggregate species with the emergence of a toxic phenotype[23]. Similarly, in a recent study we have used this technique to track the structural 'fate' of amyloid-beta protein ingested in neuronal cell lines and found that the compartmentalisation of Aβ into small acidic vesicles, induces aggregation of the protein, a finding that is significant in context of the disease, providing potential targets for therapeutic strategies[24].

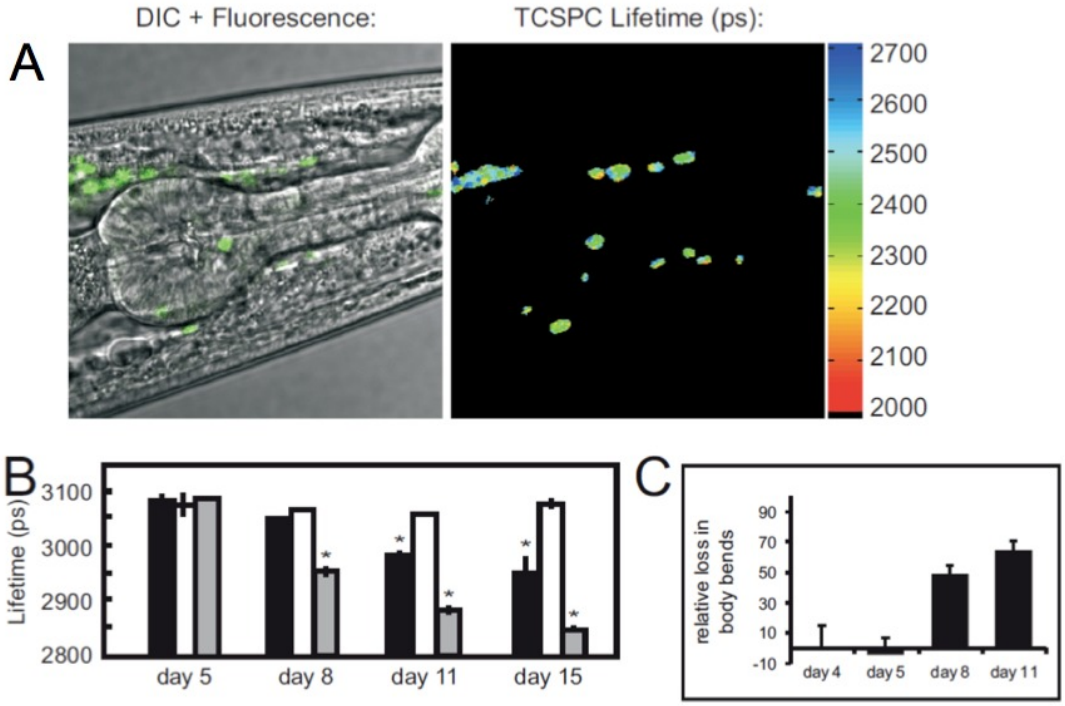


Figure 3: Imaging the aggregation state of α-synuclein in a live *C. elegans* model of α-synuclein aggregation. A: Example images of protein expressed in neuronal cells in the live worms. The panel on the left shows a bright field image of the nematode, on which the fluorescence image of the labelled amyloid is superimposed in green. The right panel shows the corresponding fluorescence lifetimes determined by time correlated single photon counting (TCSPC) confocal microscopy. B: Fluorescence lifetime as a function of age for live worm expressing different protein constructs. Black bars: α-synuclein-YFP; white bars: YFP controls; grey bars: Q40-YFP worms. Clearly a monotonic decrease in the lifetime is observed with age for the worms expressing α-synuclein-YFP, indicating increasing progress of aggregation. For the strongly aggregating Q40-YFP the corresponding drop in lifetime is even more rapid than for α-synuclein. C: Increasing aggregation of α-synculein leads to an emerging toxic phenotype: From day 8, worms exhibit increasing loss of movement, concurrent with the appearance of small toxic oligomers as verified by TCSPC imaging. Figure reprinted (adapted) with permission from[23], copyright 2011 Wiley.

## 4. OPTICAL SUPERRESOLUTION IMAGING OF AMYLOID FORMATION

Whilst multi-parameter optical microscopy offers powerful glimpses into the kinetics of aggregation, its optical resolution is limited by diffraction, and it is thus not capable of offering morphological information on the formed species, or to verify the co-localisation with subcellular targets, for example vesicles or mitochondria, with good enough spatial resolution. The advent of optical super-resolution microscopy techniques has led to a paradigm shift in this field: Using the technique of direct Stochastic Optical Reconstruction Microscopy, *d*STORM[25], we were able to image *in vitro* and in cells the morphology of Aβ fibrils with a resolution better than 20 nm[26]. The technique thus affords a spatial resolution approaching that of AFM or SEM techniques, whilst offering the advantages of specificity and sensitivity that come with all-optical techniques. In Figure 4 we show how *d*STORM permits the tracking of fibril growth at the single molecule level, offering completely new information on the self-assembly process, which is not accessible from bulk measurements[27]. The images correspond to the 'seeded growth' assay of α-synuclein discussed in Section 2, for which bulk fluorescence data are shown in Figure 2.

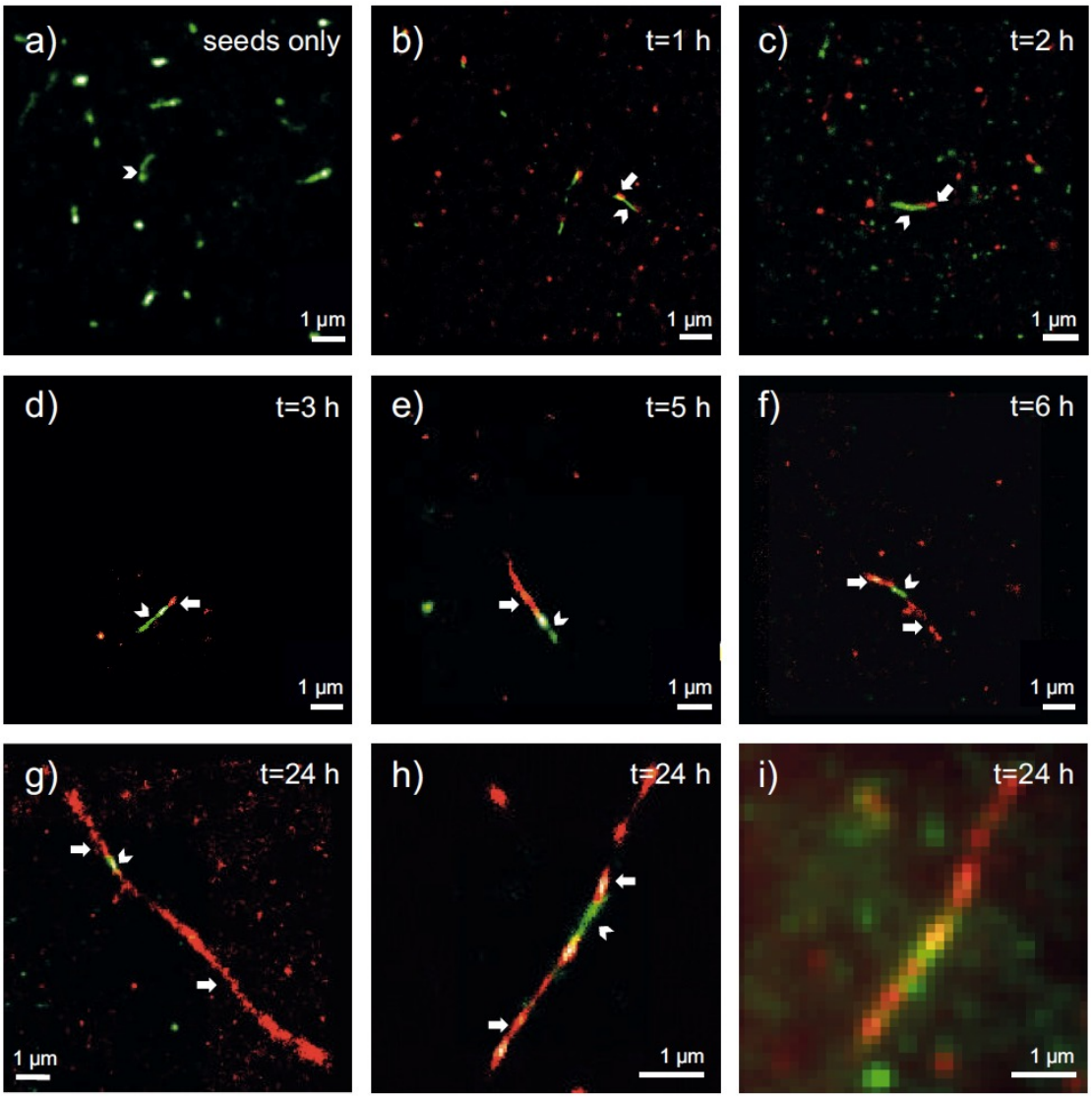


Figure 4: α-synuclein elongation assay performed with *d*STORM super-resolution microscopy. The assay corresponds to the same conditions as for the bulk measurements shown in Figure 2. Small fibril seed species, shown in green, are incubated in solution containing monomeric α-synuclein, shown in red. α-synuclein seeds were covalently labelled with Alexa Fluor® 568 and monomer with Alexa Fluor® 647 dyes, respectively. The images show the time-sequenced growth of individual α-synuclein fibrils. Clearly, growth takes place from both ends of the seed fibril, extending to several micrometers in length after 24 hours. The last image shows a conventional fluorescence microscopy image, blurred by optical diffraction. Adapted with permission from[27], copyright 2014 American Chemical Society.

Clearly, growth is seen for individual molecular species. Several new insights were obtained from this study that could not have been verified otherwise: First, fibril growth clearly proceeds from both ends of the fibril seeds, a hypothesis, that had been the subject of considerable scientific debate for lack of conclusive evidence. Secondly, we were able to show that the growth rates of individual fibrils varied by orders of magnitude; whilst some fibrils grew hardly at all, others extended over disproportional length over the same aggregation period[27]. The experiment dismissed both random diffusion or the so called 'stop and go' models of monomer addition, and instead points to the likely existence of several 'misfolding states', for which aggregation can proceed, but which feature individually differing growth rates. It is possible then with such techniques to verify whether certain mutants of amyloids 'cross-seed' other species, e.g. whether certain toxic species can 'force' healthy species to co-aggregate etc. The latter is a variant of the 'prion like propagation' model of amyloid spreading in disease.

In a recent study led by Michel et al, we use both multi-parameter and super-resolution imaging to verify how the protein tau propagates in a cell model of tau aggregation[28]. In the study, a short fragment of the human form of the protein tau, K18Tau, covalently labelled with a synthetic fluorophore, was used as a monomeric solution to incubate neuron-like cells (see Figure 5). Confocal TCPSC microscopy reveals that the protein is readily taken up by cells, and is compartmentalised in endocytotic vesicles, small acidic compartments that quite apparently enhance the aggregation rate of the ingested protein: The fluorescence lifetime of the reporter fluorophore drops dramatically upon ingestion into the cells indicating a strong degree of tau aggregation is taking place. This led to the proposal that acidic conditions favour tau aggregation, which was verified *in vitro*, again via multi-parametric imaging (see panels B and C in Figure 5). Indeed this finding suggests that uptake of tau via endocytosis is potentially a detrimental process: acidification and crowding upon ingestion into transport vesicles appear to provide conditions by which tau can aggregate. The last panel in Figure 5, panel D, shows a two-colour super-resolution image of fibrillar species found in the extracellular medium, after extended periods of K18Tau incubation of the cells. Intriguingly, the cell expunges fibrillar aggregates that contain both the ingested K18Tau, shown in red, and endogenous wild type tau, already present in the cell, shown in green. The image proves beyond doubt, that the extracellular K18Tau which aggregates on ingestion into neuron like cells, acts as a template, which 'seeds' the co-aggregation of endogenous, 'healthy' Tau. This has major pathological implications, as the model suggests that ingested Tau could potentially become toxic and lead to the formation of aggregation nuclei, from which co-aggregation of healthy protein species can proceed and propagate from cell to cell. The study furthermore suggests that the mere presence of tau in the extracellular space, for example following a brain trauma that causes local cell death and thus release of tau, can have potentially devastating effects: subsequent ingestion by adjacent, healthy cells triggers its aggregation and the consecutive recruitment and co-aggregation of endogenous Tau, starting a vicious circle, which mimics the disease cascade that may go on in humans. More generally, the study complements our other study on Aβ uptake and aggregation[24] in suggesting that vesicular compartmentalisation and acidification may be quite a general principle by which amyloid aggregation may proceed in cells.

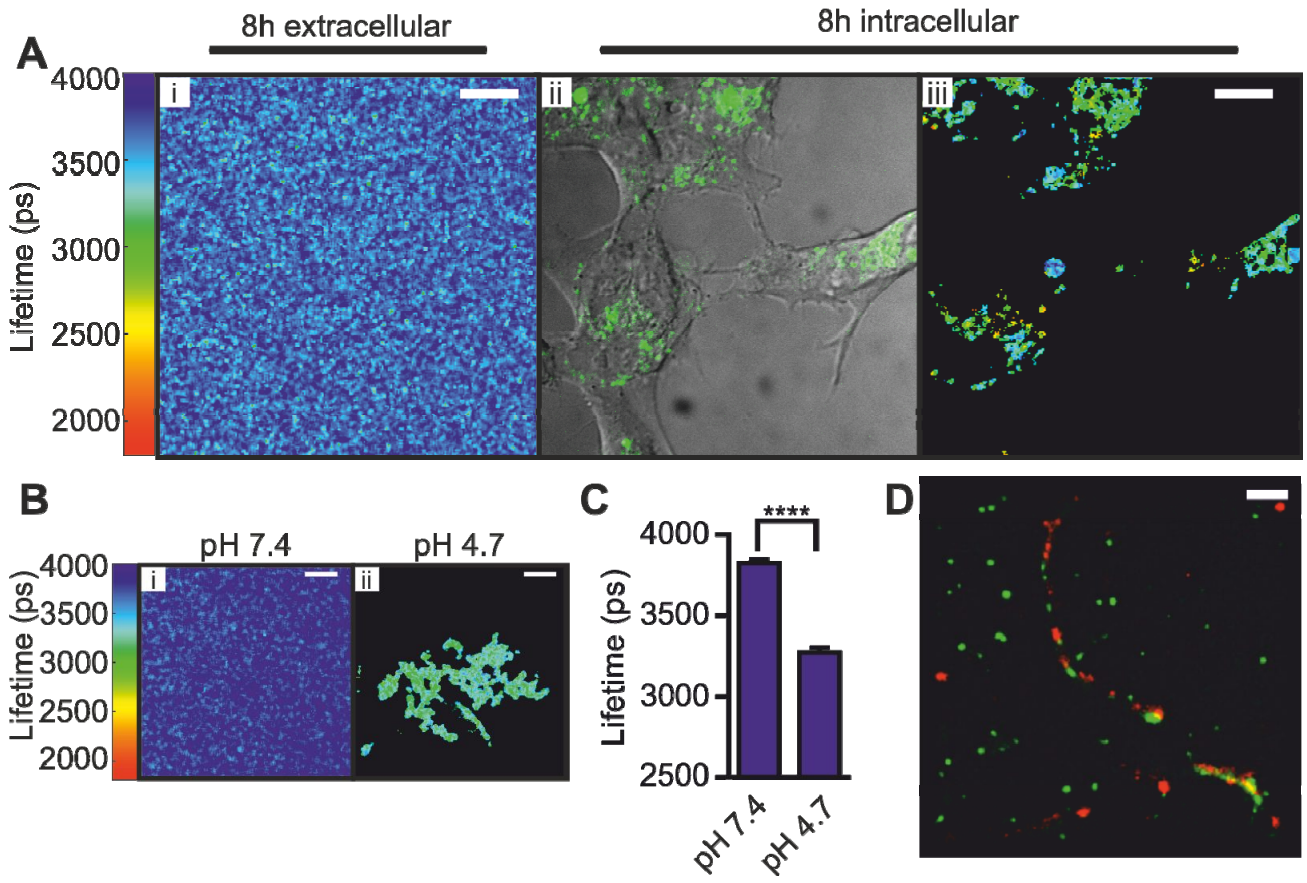


Figure 5: Uptake of monomeric Tau protein into neuron-like cells causes its aggregation and subsequent recruitment of endogenous Tau, normally present in the cell. A, i: Lifetime image of K18tau, covalently labelled with Alexa Fluor® 488 fluorophore, which does not aggregate in the extracellular space. A, ii: Confocal fluorescence intensity image displaying the distribution of K18Tau inside the cell upon ingestion. Independent experiments confirm compartmentalisation in acidic, endocytotic vesicles (not shown). A, iii: TCSPC -fluorescence lifetime images of the ingested protein suggests that significant aggregation has taken place upon protein ingestion. Compared to the incubating solution, shown in A i, the vesicular species show significantly reduced lifetimes, A iii, down by ca. 500 ps. B: *in vitro* confirmation by TCSPC to show that low pH leads to aggregation of monomeric tau. Left: K18Tau at pH 7.4, Right: Tau aggregate forming at pH 4.7. C: Average fluorescent lifetimes of monomers and aggregates shown in B. D: Incubation of cells with K18Tau leads to the release of fibrillar aggregates, containing both endogenous, intracellular tau, green, co-aggregating with the extracellular K18Tau, which was ingested by the cell, shown in red. The 2-colour *d*STORM super-resolution experiment confirms a prion-like propagation of misfolded tau species in this cell model, that may be highly relevant to the human form of Alzheimer's disease. This figure was originally published in[28], © the American Society for Biochemistry and Molecular Biology, reproduced here with permission.

## 5. CONCLUSIONS

This short review has put in focus recent developments in optical imaging that permit us to gain information, at the nanoscale, of the self-assembly process of neurotoxic protein species. Functional imaging techniques, so-called multi-parameter imaging techniques, use spectroscopic signature changes to inform on the protein aggregation process. Optical super-resolution techniques on the other hand, reveal greater spatial detail than has been hitherto possible. In combination these techniques are greatly empowering new ways to research in neurodegenerative diseases, and to probe their molecular origins. A myriad of unresolved questions are now open to 'illumination' by these tools, raising hopes to find a cure for these devastating illnesses.

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