Super-resolution Imaging of Amyloid Fibre with Localisation Microscopy

Final Report

CHS 802

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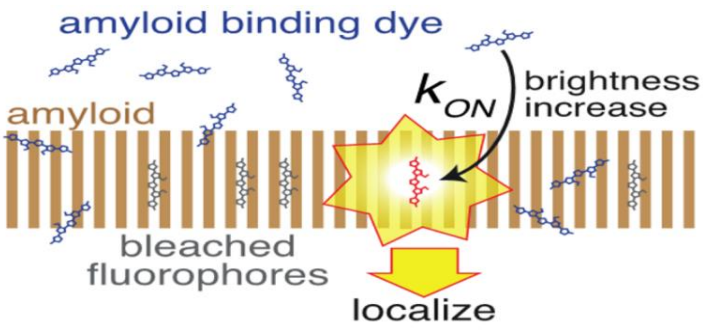
Abstract:

Amyloid fibre is well known for its cytotoxic nature and is responsible for various neuro-degenerative disease. This makes amyloid structure determination as a hot research field but it's equally challenging. What physiological condition exactly promotes soluble native protein to aggregate is still unknown. Additionally, native protein with same amino acid sequence aggregates to amyloid fibrils with different nature and toxicity, and discovery of non-toxic functional amyloid makes the situation more complex. Several well established method like XRD, Solution state NMR is not so useful in this regard due to insolubility and lack of crystal formation by amyloid fibrils. So, fluorescence based spectroscopy and microscopy in famous alternate for amyloid structure determination. Although with reasonable progress conventional optical microscopy is fundamentally bound with diffraction limited low resolution of ~250 nm which mask much structural and morphological information. Several super resolution imaging techniques with Sub-wevelength resolution is already establish like STED, SIM and Localisation microscopy techniques but have their own demerits when comes to imaging of biological sample. In this review we have demonstrated binding activated localisation microscopy (BALM) to achieve superresolved image of α-Synuclein amyloid fibres based on stochastic binding of Luminescent conjugated oligothiophens (LCO) probe pentamer-formyl thiophene acetic acid (p-FTAA) and subsequent photo bleaching. In contrast to conventional small molecule organic dyes like ThT or Nile Red LCOs shows amyloid morphology dependent spectral characteristics which makes BALM one of the best techniques in the field of amyloidosis drug designing.



Introduction:

Amyloids are insoluble extracellular protein aggregate fibril of β-sheet structure[1]. Originating from precursor amyloidogenic protein of completely different amino acid sequence and natively folded structure amyloid fibrils possess a common parallel (usually) cross β-sheet structure, where fibril axis propagates perpendicular to β-sheets. This infinite β-sheets forms long unbranched ribbon like protofibrils, three to five of which coils around a hollow axis to form the fibril structure Being the most stable structure in protein folding pathway amyloid is expected to form by all protein, when agitated with certain conditions like extreme pH, temperature change or mutation, whereas disordered proteins self-aggregate to amyloid fibre even in physiological condition and their extracellular deposit on cell membrane, brain tissue, synaptic gap causing several pathological condition, amyloidosis in general[2]. After establishing the pathogenic effect of amyloid fibre in

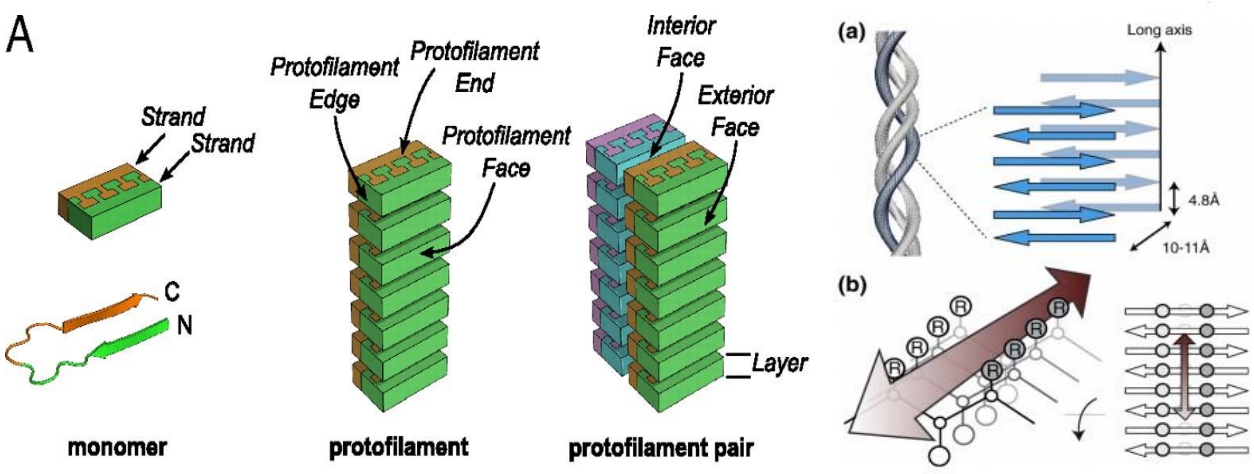


Figure 1: Cross β structure of amyloid fibre (a) amyloid protofibril (b) amino acid chain orients perpendicular to amyloid long axis[3,4]

several neurodegenerative desires including Parkinson's, Alzheimer, transmissible Prion diseases and many others cytotoxic nature[1] of amyloid fibre is well establish but how actually amyloid fibre rupture cell membrane and trigger cell death is not very clear. Way from soluble native protein to amyloid contains some charged colloidal oligomers state at certain point. Several discussions also claim those soluble oligomers as more toxic than mature amyloid fibre. But even oligomers are more toxic than amyloid they are likely to host oligomers to exhibit toxic effect. And in some amyloidosis, toxic role of amyloid is clearer from over deposit of amyloid in effected organs like α-Synuclein amyloid aggregate deposit in brain tissue in Parkinson's. Being protease resistant those amyloid deposits become irreversible and fatal.[5]

Besides several pathogenic amyloid discovery of non-pathogenic biologically functional amyloids[6] like melamine deposition, hormonal storage etc. makes the role of amyloid diverse and It's expected that complex physiological role of amyloid fibre must be originating from some structural consequence of amyloid fibre. But as already discussed structural diversity is very limited in amyloid fibrils compared to functional diversity and both pathologic and non-pathologic amyloid fibres posses same cross β structure. Along with that amyloid formed from same protein

in different agitation condition are found to possess different amount of cytotoxicity which added fog into the already invisible structure function relationship. For example when soluble insulin monomers are aggregated in a reducing environment form filament like cloudy amyloid fibre which are less toxic compared to febriler one formed in absence of such a reducing environment. Surprisingly despite of their difference in toxicity both filament and febrile form possesses the same cross β structure, exhibits same optical property while binds with small molecule which imparts a clear indication of structure-morphology relationship in toxicity of amyloid fibre. From this pathological advancement towards anti amyloidosis drugs design demands clear structural information of amyloid fibre including its morphological characterisation. Moreover to host or sink oligomers exhibiting increased toxicity, if amyloid really do so, surface charge mapping of amyloid fibre is essential considering charged colloidal nature of small oligomers.

In last few years several advancements are taken to realize the structure-morphology-toxicity relationship but challenges are faced due to insolubility of amyloid. Unlike protein structure determination, lack of proper crystal formation resists structural study of amyloid fibre using most conventional X-Ray diffraction crystallographic technique. Alternative conventional methods like liquid state NMR is also not possible due to insoluble solid fibril structure. Cyclic Dichorism (CD) study indicates common β-Sheet structure when performed with water suspension of amyloids formed from different proteins. Solid state NMR and electron microscopy although revels some structural insights, it remains dark when come to structure-toxicity relationship, moreover they are expensive and electron microscopy is destructive and not a very suitable method for biological sample imaging. In the regard of surface morphological information with high resolution AFM study is particularly helpful but alone can't provide inner structural information in very details. In last decade *Nelson et. al.* first performed direct X-Ray diffraction measurement of amyloid with a small 7 amino acid fragment (GNNQQNY) of East's prion protein which provides diffraction pattern at 4.7 Å and 10.4 Å which re-confirms the cross β structure of amyloid without revelling much structural details and structure-morphology-toxicity relationship[7].

Despite of their origin all amyloids are found to bind with certain small organic dye like ThT, Nile Red etc. and largely alter their optical property. So, fluorescence based optical spectroscopy and microscopy is one of the most used technique for amyloid characterization. Amyloid fibres are labelled with amyloid specific chromophore e.g. small molecule organic dyes, fluorescence protein e.g. GFP or specifically strained with chromophore labelled antibody in monomer level. This amyloid specific dyes like ThT, ThS, and Nile Red etc. specifically bind into the β-sheet channel of amyloid fibre and become highly fluorescent from mostly non-fluorescent unbound state due to restricted rotational freedom. There amyloid concentration dependent increase of intensity and shift of emission wavelength maxima on binding has made them excellent method for exhaustively studying aggregation-disaggregation kinetics, there role in diseases and also some steps towards drug designing has progressed in-vitro, but exploring molecular mechanism of intracellular interactions with conventional fluorescence microscopy suffers from diffraction limited low resolution of > 250 nm[8,9]. This also makes amyloid morphological analysis impossible. This method also suffers from the probable characteristic change of amyloid fibres during staining.

Stochastic superresolution microscopic methods:

Therefore, advance morphological analysis requires non-traditional super-resolution microscopy or nanoscopy based techniques, which will also be essential to analysis amyloid-cell interaction in molecular level and will pave the way towards invention of new drugs for amyloidosis patients.

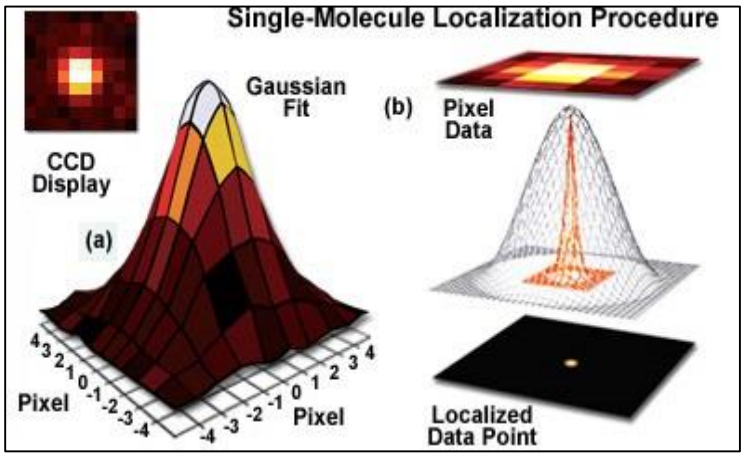


Figure 2: Methods of localisation microscopy (a) Display in CCD and Point-Spread function (b) Fitting of PSI with Gaussian and localisation in sub wavelength resolution [10]

These techniques are classified into two broad category. STED, GSD, RESOLFT and SSIM etc. involves sophisticated optics to point a LASER beam bellow diffraction point whereas in localisation microscopy point spread function (PSF) of a single emitter is fitted with Gaussian and from the maxima the molecule is precisely located, provided no other molecule is present in the diffraction limited spot. Such a condition is achieved in biological system with a low power LASER light when a small percentage of the fluorophores are stochastically activated in a circle, localised and then photo bleached. Repetition of this process reveals the reconstruction of the super-resolution image. With help of advance computation data acquisition speed can also be greatly increased with molecules of overlapping point spread function. Furthermore no requirement of specialized optics and use of conventional wide field setup make localization microscopy less expensive.

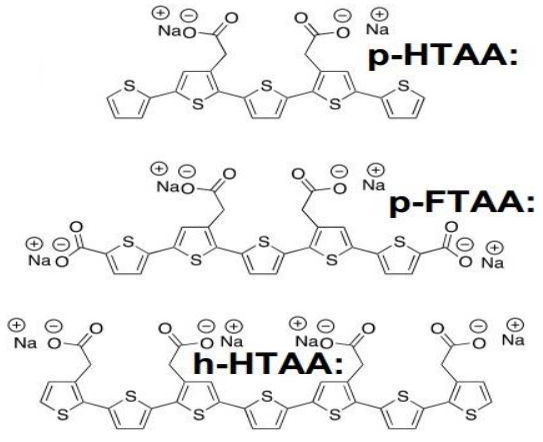


Figure 3: LCOs with different chain length[11]

Localisation microscopy has been recently used to study Alzheimer causing Aβ-fibrils, intrinsically disordered α-Synuclein aggregates etc. both in vitro and in vivo with a spatial resolution of ~20 nm. But this techniques requires incorporation of fluorescent dye into amyloid fibre, which suffers from several disadvantages. Bound dye molecule may significantly interfere with the characteristics, structure and function of the amyloid fibres. Background fluorescence from labelled monomer significantly reduces signal to noise ratio and makes it difficult to track amyloid growth over time. Alternatively, immunolabeling increases the ultimate size and changes the topology of amyloid fibres with incorporation of large antibodies ultimately results to significant decrease in resolution[11].

Recently, Luminescent conjugated polyethylene or Luminescent conjugated oligothiophens (LCOs) has demonstrated to preferentially strain and distinguish between amyloid fibres[11]. Asluend A. el al. have well utilised Pentameric Thiophene Derivatives including pentamer-formyl thiophene acetic acid (p-FTAA) as fluorescence specific probe due to their very high fluorescence intensity increase on specific binding with amyloid fibre. Figure 4 shows thousand fold higher intensity of p-FTAA emission spectra on binding with Aβ mature amyloid fibre compared to freshly dissolved peptide. This results to a significant reduction of background noise level leading to high signal to noise ratio. Along with that LCO has special advantage of amyloid morphology dependent optical property whereas small molecule organic dyes have almost same response for amyloid of same protein infect their optical property is pretty similar for any amyloid until the concentration is same. For example emission spectra of both fibril and filament form of Insulin amyloid is same when probed with small molecule organic dye like ThT, Nile Red etc but they are differentiable when probed with LCOs (will be discussed latter in details). So, for a structure-morphology-toxicity relationship LCOs can be considered as better amyloid specific dye compared to conventional small molecule organic dye like ThT, Nile Red etc.

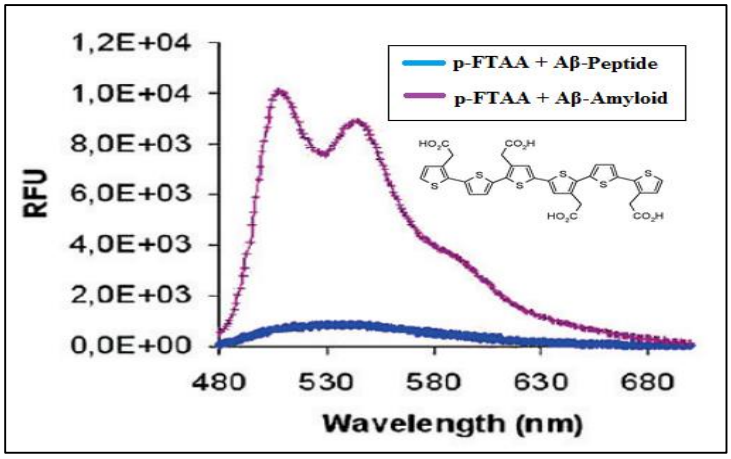


Figure 4: Fluorescence emission spectra of pentamer-formyl thiophene acetic acid (p-FTAA) with freshly dissolved Aβ peptide (blue) and that in presence of Aβ (1-42) amyloid fibre of ~10 µM concentration (magenta) showing large increase of fluorescence intensity[12]

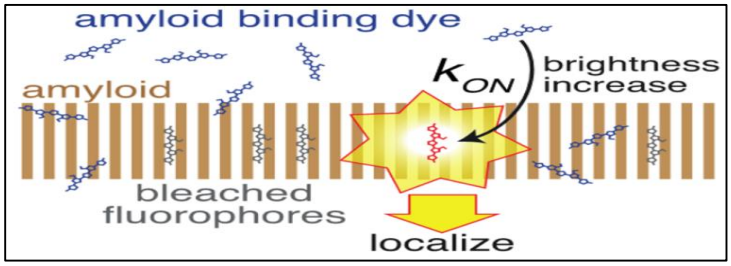


Figure 5: Stochastic binding of dye results a strong fluorescence enhancement[9]

Finally, to avoid this pre-binding interference, here Binding Activated localization microscopy (BALM) has been used in conjugation with p-FTAA to achieve superresolved image of very high resolution ~14nm. Here, fluorescent probe molecule is used in nanomolar concentration with amyloid fibre in buffer solution leading to stochastic binding to amyloid fibre and activation. On binding to amyloid fibre specific dyes become highly fluorescent and precisely localized before photo bleaching. New dye molecule rebind to the empty site of the amyloid fibre and repetition of this process ultimately uses to reconstruction of the entire image, unless all binding sites are filled with photo bleached dye molecule. As no prelabelled monomer present in solution noise label is significantly reduced. This method have advantage of no activity or morphology change of amyloid fibre on binding with dye molecule or due to immunolabelling. Moreover, in this method

density of localisation is easily controlled by altering the concentration of dye molecule which provides flexibility in acquisition speed and resolution as requirement.

Inefficiency of Amyloid Imaging with Conventional Microscopy:

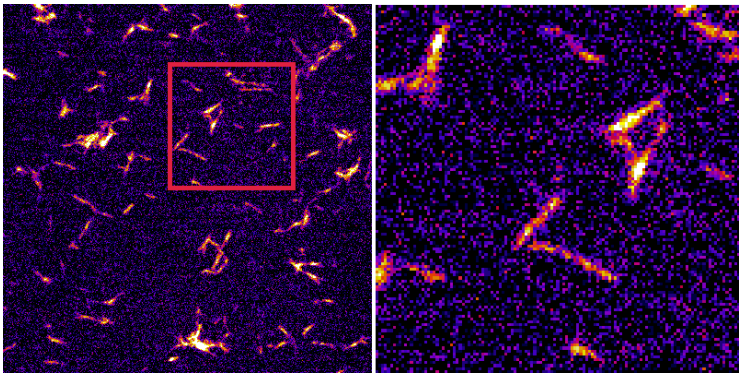


Figure 6: Diffraction limited image of amyloid fibre strained with ThT. Zoomed view of selected area in right side. (Data from our Lab)

Diffraction limited image of α-synuclein **(Figure 6** left panel) was obtained with a hand build wide field epifluorescence setup while strained with ThT. The sample was excited with 405 nm laser and emission was collected from 420-500 nm region. Owing to have a diffraction limited resolution of ~280 nm Figure **6** showing how much data is be skipped due to low resolution. In right panel although well separated single fibrils are clearly visible hardly any structural or morphological feature is observable, whereas fibrils close to each others are not resolvable separately. Similar blur image with different amyloid fibril was also obtained with several groups with resolution of ~250nm. And as this data clearly can't fill the goal of structure-morphology-toxicity relationship unless aided by additional technique. So a direct visualisation of amyloid morphology, by fluorescence based microscopy, super resolved imaging is the way out.

Super-Resolved Imaging of α-Synuclein with Binding Activated Localisation Microscopy (BALM)

BALM image of α-Synuclein amyloid with sub-wavelength resolution is shown in **Figure 7**. Compared to diffraction limited image in **Figure 7a**, BALM image of same area in figure **Figure 7b** has much more higher resolution. In **Figure 7c,d** selected area is magnified showing much details of structural information. Fibrils with different length and diameter are clearly observable which indicates an overall heterogeneity in amyloid fibrils. From lower region of **7b** individual single fibrils are clearly differentiable.

To determine the resolution of BALM in α-Synuclein amyloid imaging intensity profile across a thin filament is plotted in **figure 7e**, with a measured FWHM (Full Width Half Maxima) value of 14 nm. Considering induced blurring due to finite thickness of α-Synuclein amyloid fibrils, their specific morphology and curvature, the actual resolution is supposed to be higher. Even if with 14 nm spatial resolution two fibre of 50 nm apart should be clearly resolvable. Intensity profile of an apparently looking single fibre in **figure 7f** poses two maxima separated by 47 nm, clearly resolving two amyloid fibre close into ~50 nm. In localisation microscopy resolution can be further increased by rejecting localisations with higher error, with decreased number of total localisation and hence demands more labelling density. In covalent monomer labelling an excess of non-labelled monomer is required to maintain the amyloid structure and morphology, which further reduces the labelling efficiency. Immunolabelling also gives moderate labelling efficiencyIn

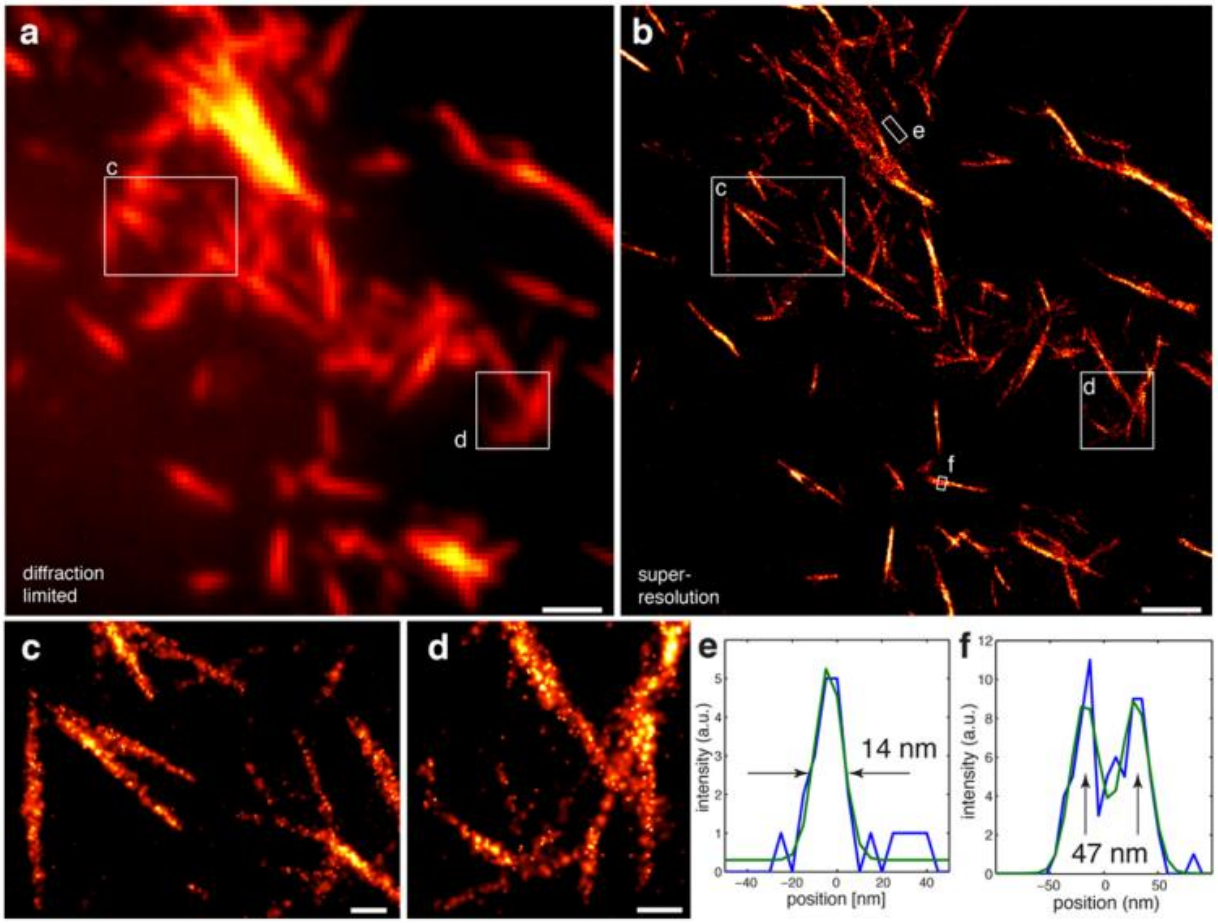


Figure 7: BALM imaging of α-synuclein fibrils. (a) Diffraction limited image and (b) superresolution image of α-synuclein fibrils obtained with BALM using the LCO p-FTAA. (c,d) Magnifications of (b) (positions as indicated in (b)). (e) profile of a thin structure (blue) and fit to Gaussian (green) to determine the resolution of this approach. The apparent thickness of the structure is 14 nm (fwhm). (f) Profile across two neighboring fibrils with a distance of 47 nm that can be distinguished. Scale bars 1 μm (a,b) and 200 nm (c,d). Regions of interest used to construct the profiles (e,f) are marked in (b)[9]

BALM binding efficiency can be easily tuned with optimized probes concentration such that resulting higher localisation efficiency without harming structural information. In this study authors had achieved about 2000 photons per localisation.

Along with obtaining α-Synuclein amyloid fibril image of <20 nm resolution BALM require no pre-labelling with either large fluorescent protein like GFP or inclusion of dye molecule in monomer level, so expected alternation in amyloid structure and morphology due to interaction with GFP or antibody is absent. Background level remains significantly low due to no labelled monomer in solution. As dye binds to amyloid only after cross β structure formation change in amyloid structure due to dye binding is unexpected.

So, BALM provides a direct method of visualisation of amyloid fibrils. This expects it possible for dynamic measurement of fibril growth with time. Initially the seed fibrils could be exposed to

dye until all the binding sites are filled with photo bleached bye molecule. Then this seeds can be used in presence of monomer to measure the growth dynamically. Monomer concentration is to be adjusted to obtain localisation from dye binding to newly fibre in proper time scale. Reconstruction of this localisation with time will generate movie showing growth of amyloid fibril with time.

Future possibilities:

In comparison to small molecule organic dyes like ThT or Nile red LCOs shows amyloid morphology dependent spectral behaviour and paves the way for BALM study to establish structure-morphology-toxicity relationship. **Figure 8** shows excitation and emission screening of fibril and filament form of same insulin amyloid having different toxicity. Physiological fibril form of insulin amyloid is found to possess much more toxicity compared to filament form formed in a reduced environment. When probed with small organic dye like ThT or Nile Red there is merely any difference in the spectroscopic behaviour. In bottom panel of **figure 8** both pHTAA and pFTAA have very low emission intensity in absence of amyloid when excited above 400 nm. Emission intensity largely increased in presence of 50 µM insulin amyloid in both fibril and filament form in top and middle row respectively.

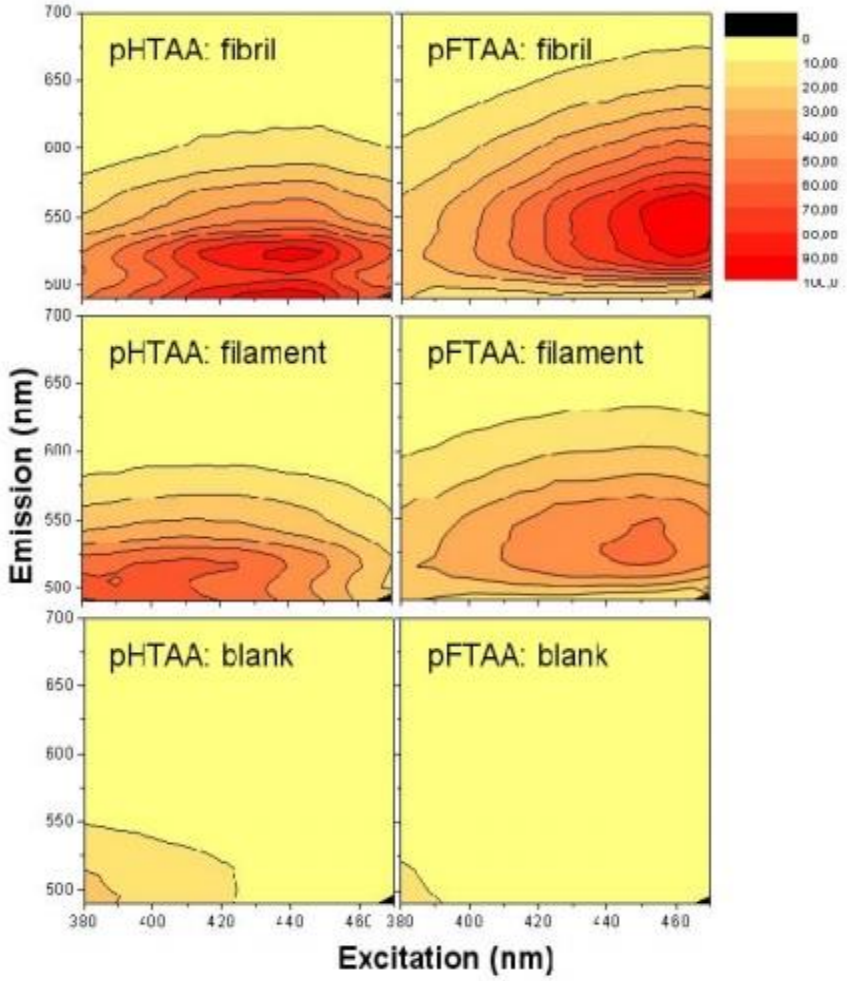


Figure 8: Excitation and emission spectra screening of LCOs pHTAA and pFTAA with fibril and filament form of insulin amyloid.[12]

Surprisingly, in contrast to small molecule organic dye, the excitation-emission screening profile of fibril and filament form of same insulin fibrils are looking different while probed with LCOs. Both excitation and emission spectra is blue shifted with filament form compared to fibril form. From this point the potential ability of LCOs to differentiate between different form of amyloid fibril is expected. Further experiments are performed to determine surface zeta potential and percentage of hydrophobic region present. It's observed that although in acidic pH charge density

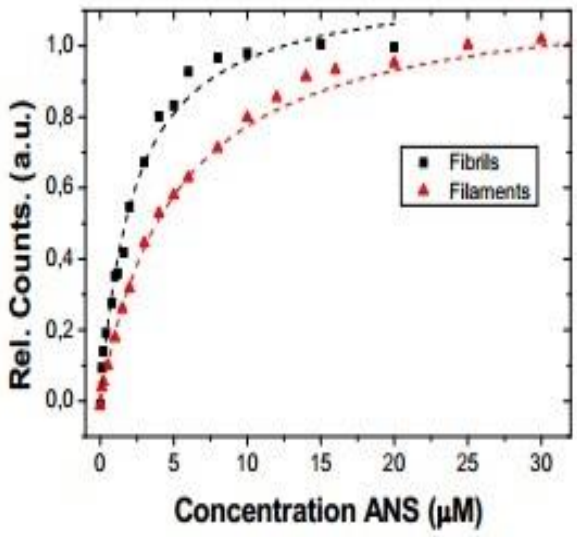


Figure 9: Hydrophobicity difference between two forms of insulin: Relative counts vs ANS concentration plot[12]

of fibril and filament form varies slightly, in neutral pH they are roughly same whereas the trend in excitation-emission scanning is unaltered. Whereas fibrils are found to be more hydrophobic compared to filaments, when fluorescence intensity in plotted against different ANS concentration (**figure 9**). ANS, being hydrophobic dye, bounds to hydrophobic region of amyloid specifically, and gives a qualitative measure of hydrophobic region present on amyloid fibres in terms of fluorescence intensity

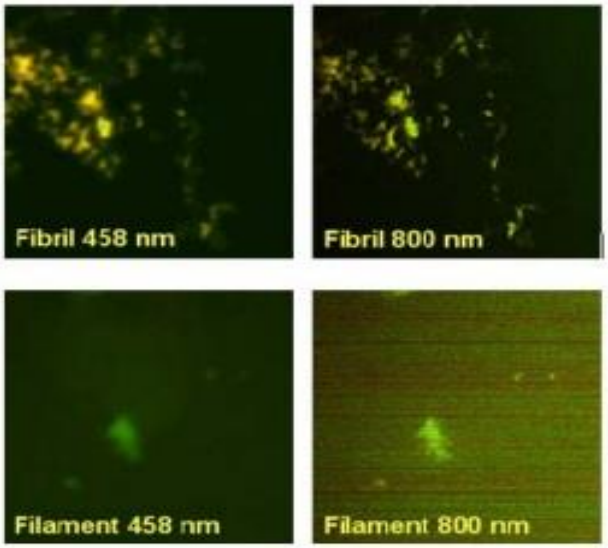


Figure 10: Flurescence imaging of insulin amyloid with h-HTAA. Right column is with two photon excitation[12]

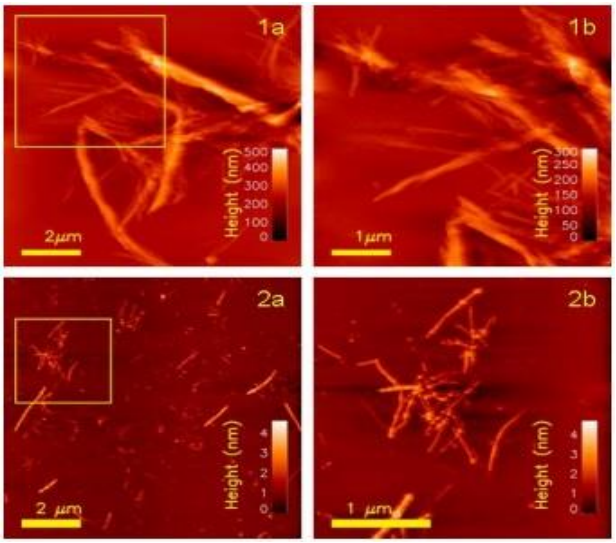


Figure 11: AFM image of Insulin amyloid (top) Fibril (bottom) Filament. Right Column is magnified view of selected portion[12]

during titration. This observation again favours for different morphology of two different.

Diffraction limited image of fibril and filament form of insulin fibre is shown in **figure 10**. In right panel resolution is quite higher with two photon imaging. This confirms structural difference between fibril and filament form of insulin amyloid but due to diffraction limited resolution morphology of individual fibril is not clear.

Although in AFM image of fibril and filament form of amyloid fibril (**figure 11**) there morphological difference is clear a direct visualisation is not possible without a super resolution imaging technique. This resists further possibility of dynamic measurements as AFM image can't

be acquired in a solution phase. Thus advancements toward amyloidosis drug design requiring measurement in physiological mimicking environment BALM will be very helpful technique.

Summery

Preceding discussion well establishes the necessity of superresolution microscopy or nanoscopy in regards to understand structure-morphology-toxicity relationship of amyloid fibre and concludes BALM as one of the most demanded superresolution imaging technique for studying amyloid fibres. In addition this requires no use of specialised optics and can be performed with conventional wide field setup which makes it operationally more flexible and cost worthy. Primary advantages includes no prelabeling with either fluorescent dye or fusion with large fluorescent protein like GFP or immunolabelling with antibody and thus allowing to study amyloid fibre in a less altered morphology. As LCOs binds to amyloid only after cross β sheet structure formation it's expected to have very less impact on the amyloid structure. Since the LCO probes binds to amyloid fibre only during measurement, the background noise level due to labelled monomer is significantly reduced. Optimization of labelling density and hence further improved resolution is also possible by tuning LCOs concentration. LCOs further facilitates in differentiating different morphologies of amyloid fibre. Synthesis of possible verities of LCOs with similar amyloid specific enhanced emission intensity make it experimentally more flexible. In contrast to sub wavelength imaging by AFM in solid state, BALM is performed in physiologically similar buffer like environment and perhaps will be extended to study the invivo interactions of amyloid fibres with organelles and relating amyloid toxicity with such interactions. With BALM based amyloid-organelles interaction study it will be much cheaper to design drugs against fatal neurodegenerative diseases and explore there effect invivo at a molecular level. As real time dynamic measurement is possible it's expected BALM will very soon cross the border of amyloid study and pave the way of drug characterisation in general.

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