

A unified *in vitro* to *in vivo* fluorescence lifetime screening platform yields amyloid β aggregation inhibitors

Súil Collins,^{1,2,3} Liisa van Vliet,² Fabrice Gielen,² Matej Janeček,¹ Sara Wagner Valladolid,³ Chetan Poudel,³ Giuliana Fusco,¹ Alfonso De Simone,⁴ Claire Michel,³ Clemens F. Kaminski,³ David R Spring,^{1,*} Florian Hollfelder^{2,*} and Gabriele S Kaminski Schierle^{3,*}

¹Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, UK.

²Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge, CB2 1GA, UK

³Department of Chemical Engineering and Biotechnology, University of Cambridge, West Cambridge Site

Philippa Fawcett Drive, Cambridge, CB3 0AS, UK.

⁴Department of Life Sciences, Imperial College London, South Kensington Campus, London, SW7 2AZ, UK

Abstract

Inhibiting the aggregation of amyloid β (1-42) is a promising strategy for the development of disease-modifying Alzheimer's disease therapeutics. To date, however, no sufficiently efficacious inhibitors have been identified, despite the best efforts of >200 advanced drug development campaigns. This failure can be attributed to limitations in current compound screening and *in vivo* validation assays. Here, we report an *in vitro* to *in vivo* screening platform based on the use of a fluorescence lifetime aggregation sensor. The microfluidic "nanoFLIM" assay developed circumvents issues that plague conventional assays, such as lack of reproducibility, high cost and artefactual false read-outs. The fluorescence lifetime sensor can also dynamically monitor peptide aggregation in cellular and *Caenorhabditis elegans* disease models, providing directly comparable aggregation kinetics, which is not achievable by any other method. The power of this unified system for accelerating hit-to-lead strategies, lowering attrition rates and expediting *in vivo* screening, was demonstrated with a pilot screening campaign of 445 compounds, revealing a new inhibitor that can inhibit amyloid β self-assembly *in vitro* as well as in cellular and whole organism disease models.

Introduction

Aggregation of the peptide amyloid β (1-42) ($A\beta_{42}$) is a pathological hallmark of Alzheimer's disease (AD), a multifaceted neurodegenerative disorder for which no preventative measures or disease modifying therapies exist.^{1,2} The development of $A\beta_{42}$ aggregation inhibitors has long been recognised as a potential strategy for AD treatment,^{3,4,5,6} but to date only one inhibitory drug has been approved, subject to an unusual nine-year

post-approval trial.^{7, 8} Indeed, more than 200 unsuccessful attempts to develop medicines in clinical trials to treat and potentially prevent AD are on record,^{9, 10} suggesting that finding therapeutic molecules in this area is much harder than in other drug development campaigns. However, the number of drugs candidates in clinical trials at any time remains above 100.¹¹ Setbacks in the development of such clinical candidates stem from a variety of issues along the drug development pathway, including the types of assays historically used and the difficulty of integrating biophysical and cell-based studies.¹² The intrinsically disordered nature of the peptide and aggregate heterogeneity has limited structural studies on the peptide, thereby occluding structure-based design strategies.^{13, 14, 15} Available compound libraries often lack novelty and scaffold diversity,¹⁶ and current screening approaches are limited by reagent consumption, assay reproducibility and spectral inference from intrinsic properties of the test compounds. A major limitation in the development of AD therapeutics is the absence of methods to test the activity of hit compounds in cellular or whole organism models quickly and reliably: there are currently no high-throughput screening systems to directly monitor cellular A β ₄₂ aggregation in real time, or compare *in vitro* and cellular anti-aggregation activity.¹⁷ Current techniques rely on fixing cells to stain amyloid deposits or simple cell viability tests, neither of which are dynamic or revealing about the underlying aggregation processes. Filtering and prioritising *in vitro* hits for those that show the strongest *in vivo* activity is often too laborious to pursue extensive ‘hit-to-lead’ strategies, potentially resulting in the advancement of lead compounds whose sub-optimal *in vivo* activity only becomes apparent in late stage development.^{18, 19, 20}

Herein, we report an approach that addresses these issues by employing a unified assay format to cover *in vitro* to *in vivo* compound screening for A β ₄₂ aggregation inhibitory activity, in order to better identify and validate drug candidates, and to avoid wasting efforts on false positive results that have previously led to failed drug discovery campaigns. This comprehensive assay is based on the use of an amyloid aggregation fluorescence lifetime sensor, whereby A β ₄₂ aggregation is monitored by changes in the fluorescence lifetime of an attached fluorophore, which is significantly quenched upon self-assembly of the peptide.^{21, 22} Changes in the aggregation profile in the presence of a small molecule is indicative of a modulatory effect on A β ₄₂ self-assembly, be it to promote, delay or inhibit the process.

A medium-throughput microfluidic assay (dubbed the *nanoFLIM*) was designed to screen compound libraries using fluorescence lifetime imaging microscopy (*FLIM*), with large sample sizes (>100 assays per

experiment) and *nanolitre* volume requirements (18 nL per test). To demonstrate the potential of the system, a selection of novel chemical libraries – developed using diversity-oriented synthesis (DOS) or rationally targeted drug discovery strategies – was interrogated, yielding **MJ040**, a novel lead A β ₄₂ aggregation inhibitor. The fluorescence lifetime sensor protocol was also applied to dynamically screen compounds for A β ₄₂ anti-aggregation activity in SH-SY5Y neuroblastoma cells, where **MJ040** was again shown to display an inhibitory effect. The activity of a rationally designed prodrug version, **MJ040X**, was further validated by probing *in vivo* A β ₄₂ aggregation in the disease model *Caenorhabditis elegans*, in which treatment was seen to delay or completely inhibit the aggregation process.

In addition to an unrivalled economy in reagent consumption, the *nanoFLIM* platform provides the only unified direct comparison of A β ₄₂ aggregation propensity in the presence of small molecules *in vitro*, in live cells and in a whole organism disease model. The successful identification of **MJ040X** as a novel lead A β ₄₂ aggregation inhibitor, capable of preventing amyloid formation in all three relevant formats, suggests that promising candidates for the development of therapeutically active AD treatment readily result from this approach, thereby providing a route to drug development for a hitherto elusive challenge.

Results and discussion

Small molecule library screening at the nanoliter scale identifies novel inhibitory scaffold

To overcome problems that have plagued previous screening campaigns, namely assay variability, data quality and reagent consumption,^{12, 23} a novel workflow was designed in which A β ₄₂ aggregation was monitored within arrayed nanoliter droplets using fluorescence lifetime imaging.^{24, 25} The arrayed format enabled the simultaneous imaging of 110 droplets, each corresponding to a distinct experiment. **Figure 1a** shows an overview of the nanoFLIM assay platform, in which 110 traps sit along a serpentine channel in a microfluidic chip. The droplets (18 nL) lodged in these traps were generated and deposited using droplet-on-demand technology,^{26, 27} producing defined quantities of partially labelled monomeric peptide (845 pg per droplet) and the respective drug candidate molecule (360 pg per droplet) in a precisely known order (**Fig. 1a,b**). Once droplets were sequentially trapped in the device, reaction progress was imaged over several hours, to give characteristic aggregation profiles (**Fig. 1c**). 445 compounds, with 5-10 repeats per compound at a

single concentration, were screened to give start point and end point values, taking only ~70 hours for ~2400 experiments. The compounds originated from novel chemical libraries synthesised by diversity-oriented approaches or specifically designed to incorporate medically relevant features (**Supplementary Results, Supplementary Table 1**). A sample screen showing the fluorescence lifetime change of trapped A β ₄₂ droplets with 9 repeats of 9 compounds is shown in **Figure 2a**. Such screening efforts resulted in the identification of hit compound **MJ040** (**Fig 2b**), which originates from a cinchophen scaffold library and exerts a profound inhibitory effect on A β ₄₂ aggregation compared to other library members (**Fig. 2a**).²⁸ The ability of **MJ040** to inhibit A β ₄₂ aggregation was quantified by nanoFLIM measurement of A β ₄₂ aggregation kinetics at several concentrations of the compound, giving an IC₅₀ value of $3.8 \pm 0.8 \mu\text{M}$ (**Fig. 2c,d**). Notably, we multiplexed aggregation assays to overcome batch to batch variability even shown for recombinant A β ²⁹ by carrying out repeats. The high number of repeats in this assay made it possible to obtain reliable aggregation data immediately from one set of measurements, with incomparably low reagent consumption. The low micromolar range of the IC₅₀ value is similar to known *in vitro* inhibitory compounds, e.g. EGCG (IC₅₀ $6.4 \pm 0.7 \mu\text{M}$),³⁰ an inhibitor that has reached phase III clinical trials (NCT00951834) for its A β ₄₂ anti-aggregation activity. Two compounds, **MJ001** and **MJ042** (**Fig. 2a,b**), showed weaker, yet significant inhibitory activity and were carried forward together with **MJ040** for further validation.

104

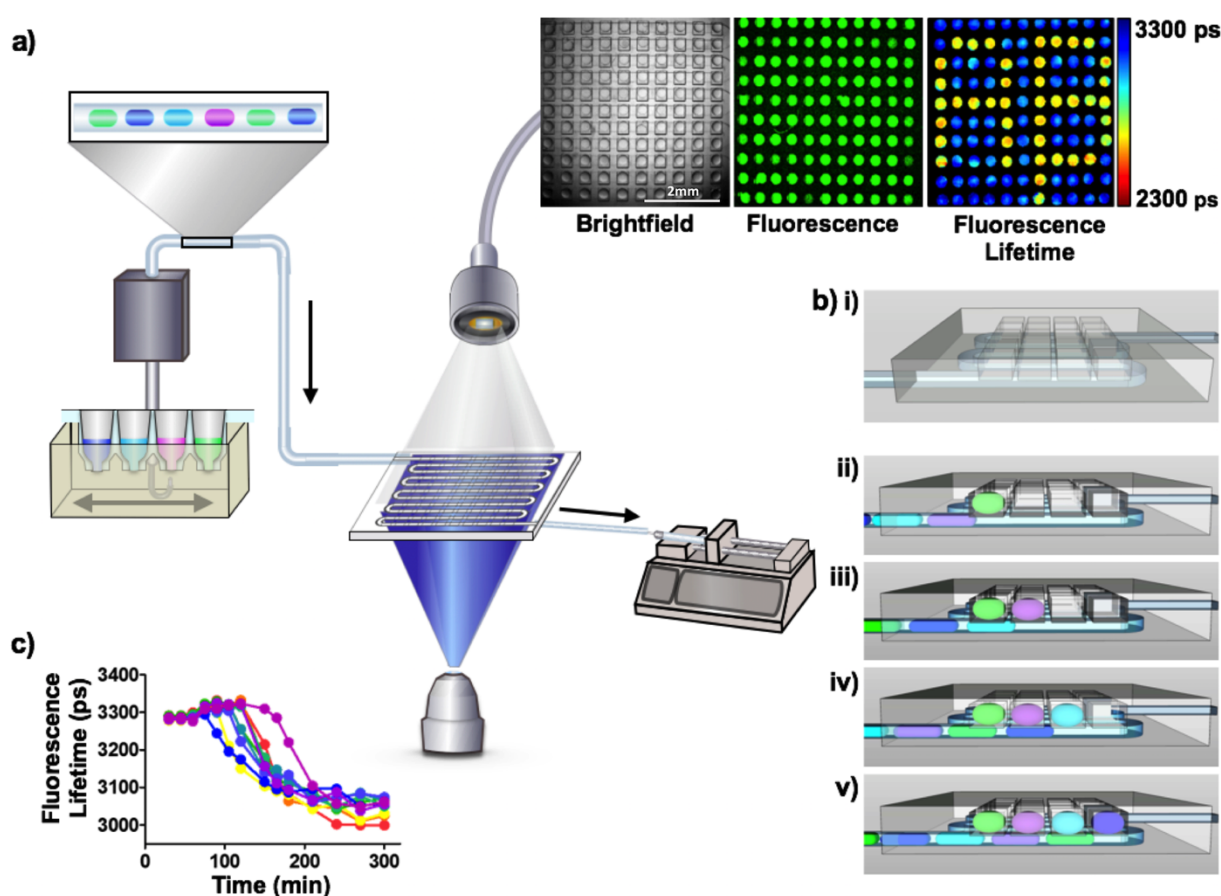


Figure 1. Overview of the nanoFLIM. a) Assay workflow. Using droplet-on-demand technology (Mitox Dropix, Dolomite²⁶) droplets containing monomeric peptide together with test compounds are formed and filled into a droplet array chip in a predefined order. The brightfield image insert shows the filled microfluidic device, in which 110 droplets, with 18 nL of aqueous solution per droplet, are trapped within square grids above a serpentine channel (Scale bar = 2 mm). Fluorescence lifetime imaging is used to monitor the aggregation kinetics of the peptide, which is partially labelled with a reporter dye, and the effect that different extrinsic factors or inhibitory compounds can have on the process. The fluorescence intensity of monomeric or aggregated peptide droplets are similar. However, the fluorescence lifetime sensor provides a quantitative measure of the aggregation state of the peptide, which are either monomeric (blue, lifetime ~3200 ps) or aggregated (yellow, lifetime ~2700 ps). The ability of the system to monitor the process of A β ₄₂ aggregation with different peptide labelling densities, under various pH, temperature, shearing conditions and with a variety of known inhibitory small molecules is shown in **Extended Data Figure 3, and Supplementary Figures 1-4.** b) *Schematic of microfluidic chip filling.* i) Empty device showing square grids aligned over a serpentine channel. ii) Droplets of a known sequence fill into the serpentine channel and travel until they reach an empty grid, where iii) the leading droplet floats upwards and is trapped in the first grid and held for several hours for imaging. iv) Subsequent droplets flow under previously trapped droplets until v) they reach an empty grid where they become trapped. c) A β ₄₂ aggregation profiles for 10 droplets formed from the same 10 μ L stock solution of peptide. A great discrepancy between the initiation of aggregation in the first droplet

and the last is observed, highlighting the need for many repeats when monitoring self-assembly of this highly aggregation prone peptide. 20 μ M A β ₄₂-488, 20% labelled.

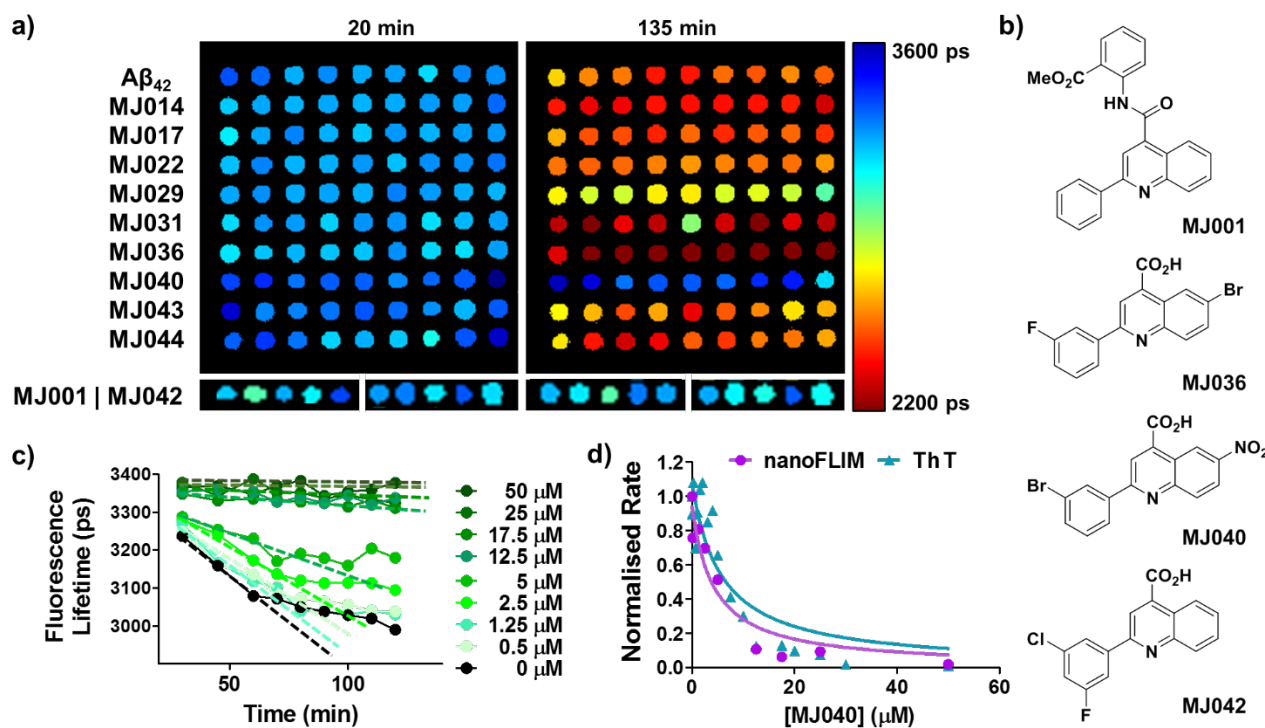


Figure 2: Screening of compound collections using the nanoFLIM identifies active inhibitory

compounds. a) Example of the nanoFLIM screening approach for hit identification. The raw FLIM images show rows of nine droplet replicates containing peptide and test compounds at start (20 min., left) and end (135 min, right) time points. The changes in fluorescence lifetime are monitored to give averaged aggregation profiles, as shown in panel c). Compounds capable of preventing the lifetime change associated with peptide aggregation, e.g. **MJ040** (row 8) and **MJ001** and **MJ042** (inserts) are selected for further study. 10 μ M A β ₄₂-488, 50% labelled, 10 μ M small molecule, droplet diameter = 200 μ m. (Details of the FLIM analysis and representative photon counts are shown in **Extended Data Figure 4**. The full cinchophen library and associated nanoFLIM data are shown in **Supplementary Figure S5 & S6**.) b) Structures of the hit compounds from the cinchophen library carried forward for further characterisation. c) A β ₄₂ aggregation profiles obtained by filling the device with a concentration gradient of **MJ040** (n = 9). d) IC₅₀ graphs using the initial rates obtained for a concentration gradient from the nanoFLIM and ThT fluorescence assays.³¹ nanoFLIM IC₅₀ = 4.3 $\pm$ 1.3 μ M, ThT fluorescence IC₅₀ = 5.8 $\pm$ 1.7 μ M. The lines are fits to the equation: $\frac{y}{y_{max}} = \frac{1}{1 + \frac{x}{IC_{50}}}$

Inhibitory activity of hit compounds validated by conventional Thioflavin T assays and structural analysis

Thus far, screening for A β ₄₂ aggregation inhibitors has mainly employed Thioflavin T (ThT) fluorescence assays, whereby a red shift and enhancement in the fluorescent signal of the ThT dye upon binding to structures rich in β -sheets is correlated with increasing protein aggregation.³² The anti-aggregation activity observed using the ThT fluorescence assay and the nanoFLIM was compared, and the data for four notable compounds are shown in **Figure 3**. Both the nanoFLIM (**Fig. 3a**) and ThT (**Fig. 3b**) assays indicated that **MJ040** exerts a strong inhibitory effect, with calculated IC₅₀ values of $4.3 \pm 1.3 \mu\text{M}$ and $5.8 \pm 1.7 \mu\text{M}$, respectively (**Fig. 2d**). The mode of interaction of MJ040 was investigated by a combination of NMR spectroscopy and molecular modelling, allowing us to locate the MJ040 binding site in a hydrophobic cleft near the C-terminus of monomeric A β ₄₂ (**Extended Data Figure 1**).

With compound **MJ036**, conflicting results were obtained in the two assay formats. In the fluorescence lifetime sensor measurements, no inhibitory activity was observed with **MJ036** (**Fig. 3a**), while the ThT fluorescence assay identified **MJ036** as a potent aggregation inhibitor (**Fig. 3b**). To resolve this apparent inconsistency, morphological analysis of the aggregation products by transmission electron microscopy (TEM) (**Fig. 3e**) and atomic force microscopy (AFM) (**Supplementary Fig. 7**) was performed. These orthogonal techniques rule out non-spectral and fluorescence interference, providing a method of assessing fibril formation independent of the presence of extrinsic dyes. Many aggregated species were observed following 7-day incubation with **MJ036** (**Fig. 3c-iii**), and only very few with **MJ040** (**Fig. 3c-iv**). This suggests that **MJ036** had been incorrectly assigned as a strong inhibitor by ThT fluorescence. Based on nanoFLIM it can be correctly discounted as a *false positive*. Dot blot assays, with the toxic oligomer-specific A11 antibody, were also used to investigate the formation of aggregated species A β ₄₂ in the presence of the compounds (**Fig. 3d**).³³ A11-sensitive species were not detected in the **MJ040** treated sample, suggesting that the small structures observed by the scanning microscopy techniques were innocuous aggregates. In contrast, A11-immunoreactive structures were detected in the **MJ036** treated sample. Thus, **MJ036** neither prevents the formation of A β ₄₂ fibrils nor potentially toxic oligomeric A β ₄₂ species, and was therefore incorrectly assigned as an inhibitor by ThT fluorescence analysis.

To further assess the inadequacy of the conventional ThT assay³⁴ for screening the cinchophen library, the fluorescence emission spectra of ThT with previously formed A β ₄₂ fibrils in the presence of the compounds was investigated (**Fig. 3c**). The addition of either **MJ036** or **MJ040** was shown to reduce the ThT emission signal (with 440 nm excitation, as typically used in the real time assay), indicating that the compounds interfere with the ThT assay readout as a result of their intrinsic fluorescence properties or competitive binding interactions with the peptide or ThT dye itself.³⁵ As such, the inhibitory activity of these compounds cannot be reliably assessed by means of a ThT fluorescence assay. A reduction in fluorescence intensity was not observed upon addition of **MJ036** and **MJ040** at 480 nm excitation, the wavelength used for fluorescence lifetime imaging (**Supplementary Figure 8**). The intrinsic spectroscopic properties of **MJ001** and **MJ042** are also believed to contribute to incorrect assignments in the ThT fluorescence assay, in this case *false negative* results. When added to the ThT- A β ₄₂ fibril sample, the emission at 488 nm (the emission wavelength used in the conventional ThT assay) is higher than that of the A β ₄₂ fibrils and dye alone, thereby potentially masking the compounds inhibitory activity against A β ₄₂ aggregation (**Fig. 3b,d**), which may explain why they will *not* be found in a screen based on ThT fluorescence. The inhibitory activity of **MJ001** and **MJ042** was validated using TEM, where in both cases only small aggregates were observed following compound treatment (**Fig. 3c-ii,-iv**). NanoFLIM screening, therefore, is less susceptible to misleading readouts that produce false positive and negative results than the conventional ThT assay when screening spectroscopically active small molecule libraries.

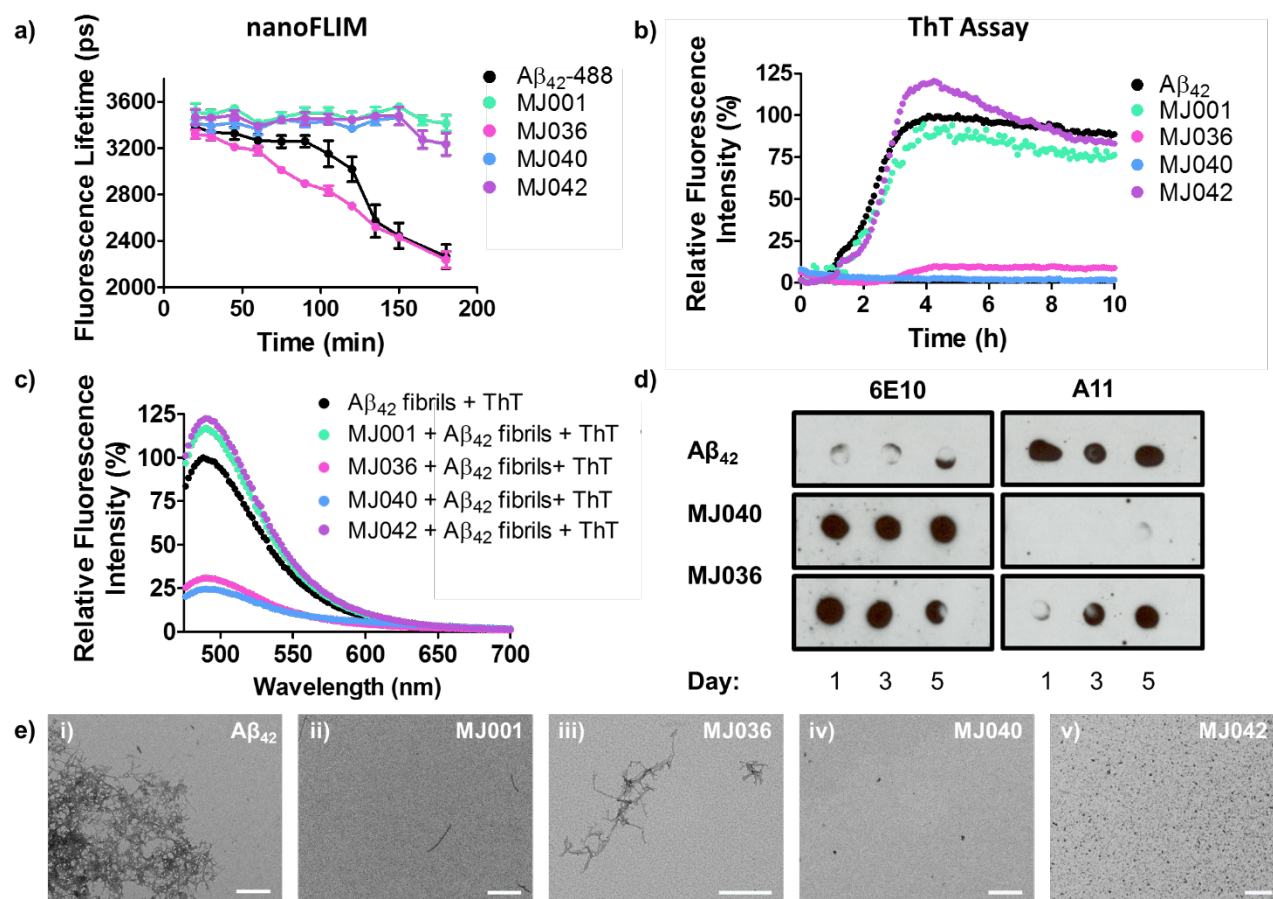


Figure 3: Comparison of the inhibitory activity detected using the nanoFLIM, ThT fluorescence assays, A11 dot blots and TEM imaging. **a)** NanoFLIM Aβ₄₂ aggregation profiles in the presence of select compounds. **MJ001**, **MJ040** and **MJ042** exert an inhibitory effect, but aggregation is seen to proceed in the presence of **MJ036**. 10 μM Aβ₄₂-488, 50% labelled, 50 μM compound. **b)** Aβ₄₂ aggregation curves with the addition of the select compounds, as monitored by ThT fluorescence. The curve for Aβ₄₂ (black) represents the time course of Aβ₄₂ aggregation in the absence of inhibitors, the plateau of which is taken to represent fibril mass concentration and is set as 100%.^{7, 30} Compounds **MJ040** and **MJ036** show a strong inhibitory effect. **MJ001** does not appear to perturb the aggregation process and the profile of **MJ042** is overly fluorescent relative to that of Aβ₄₂ alone. The relative ThT fluorescence data for the full cinchophen library is shown in **Supplementary Figure 9**. 10 μM Aβ₄₂, 20 μM ThT, 50 μM compound, ex = 440 nm, em = 488 nm. **c)** Emission spectrum of pre-formed Aβ₄₂ fibrils and ThT samples in the presence of select compounds (ex = 440 nm), expressed relative to that of fibrillary Aβ₄₂ and ThT alone (peak set 100%). **MJ040** and **MJ036** significantly quench the ThT fluorescence in the presence of fibrils. **MJ001** and **MJ042** are intrinsically fluorescent at the emission wavelength used to monitor aggregation in the ThT fluorescence assay (488 nm), which may mask the inhibitory activity of the compounds in the ThT fluorescence aggregation screen. The relative emission data for the full cinchophen library in the presence of preformed fibrils at 440 nm excitation is shown in **Supplementary Figure 10**, and **Supplementary Figure 11** shows the absorbance spectra for the compounds relative to Aβ₄₂ fibrils with **d)** Dot blot time course investigating the formation of potentially toxic species, using oligomer-specific antibody A11.³³ The 6E10 control antibody, which detects all Aβ₄₂ species,

shows a positive result for all samples. Oligomeric structures were present following 1, 3 and 5 days of incubation in the absence of any compounds or in the presence of **MJ036**. No A11 sensitive species were detected following incubation with **MJ040**. **e)** TEM images of the A β ₄₂ species formed following 7-day incubation with the compounds of interest. **i)** A β ₄₂ control; **ii)** **MJ001**; **iii)** **MJ036**; **iv)** **MJ040**; **v)** **MJ042**. In all cases nanoFLIM screening correctly predicted if the compound had a modulatory effect on the aggregation process. The ThT assay incorrectly identified one false positive (**MJ036**) and two false negatives (**MJ001** and **MJ042**). Scale bar = 500 nm.

Prodrug synthesis generates a cell active inhibitory lead compound.

To investigate the ability of lead **MJ040** to inhibit A β ₄₂-induced cell death, MTT cell vitality tests were performed. SH-SY5Y cells were employed, as this human-derived neuroblastoma cell line displays many biochemical and functional features of human neurons and has consequently seen widespread use as a human neuronal cell model in cytotoxicity assays.^{33, 36, 37} Monomeric A β ₄₂ and increasing concentrations of **MJ040** were added to cells and incubated for 48 h. Addition of A β ₄₂ resulted in a pronounced reduction in cell vitality (~33%, measured as a function of impaired cellular metabolic activity), and the addition of **MJ040** to the extracellular medium was not capable of significantly rescuing the cells (**Fig. 4a**). In a bid to improve cellular activity, the carboxylic acid of the compounds was masked as a methyl ester, to generate the prodrug **MJ040X** (**Fig. 4b, Supplementary Synthetic Procedures**). This compound displayed poor inhibitory activity *in vitro* (**Supplementary Fig. 12**), suggesting the carboxylate is necessary for interaction with the peptide. In the cell-based assays, however, the compound resulted in a significant increase in cell vitality (**Fig. 4a**). The rescuing effect suggests that the lack of activity observed for the original compound **MJ040** is caused, at least in part, by poor cellular uptake as a consequence of the anionic group. The rationally designed prodrug **MJ040X** successfully permeates into the cells, where the ester is then hydrolysed to generate the free active drug, which inhibits the aggregation process in the cellular environment.

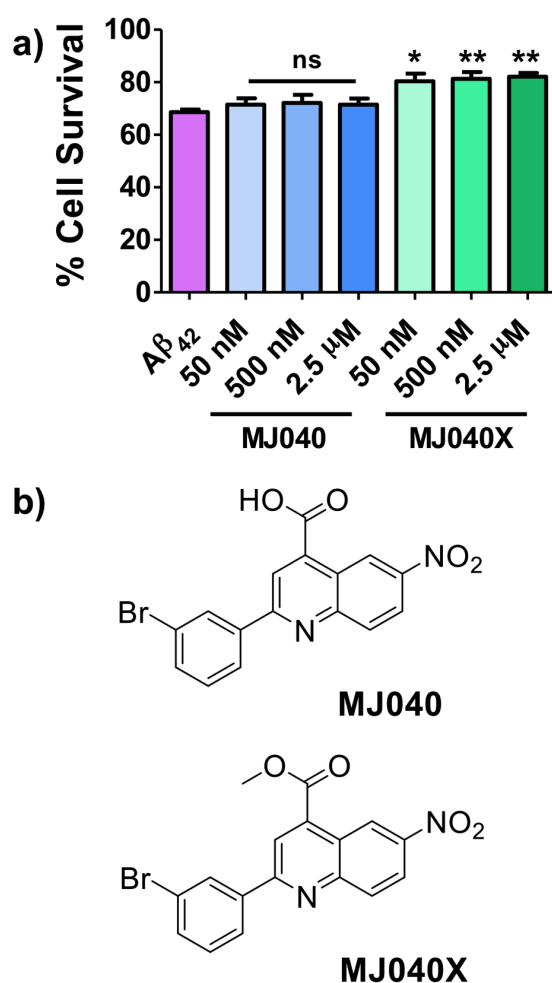


Figure 4. Methyl ester prodrug MJ040X rescues neuronal cells from Aβ₄₂-induced cellular stress. a) Cell vitality tests monitoring the rescuing effect of **MJ040** and **MJ040X** from monomeric Aβ₄₂-induced toxicity. Aβ₄₂ monomers (500 nM) with or without the presence of small molecules were added to SH-SY5Y cells. Following 48 h incubation, the cell vitality was assessed using a MTT cell vitality assay. Anionic **MJ040** was unable to rescue Aβ₄₂ induced cellular stress, but prodrug **MJ040X** could significantly increase cell vitality. The vitality of untreated cells was set as 100%. Error bars represent SEM, n = 4, statistical analysis performed by one-way ANOVA with Dunnett's multiple comparison post-test; *p<0.05; **p<0.01. The rescuing effect of **MJ040** and **MJ040X** on preformed Aβ aggregate-induced cell stress is shown in **Supplementary Figure 13**, and the rescuing effect of **MJ001** and **MJ042** in **Supplementary Figure 14b**) Structure of original hit **MJ040** and methyl ester prodrug **MJ040X**.

Development of a live cell fluorescence lifetime sensor assay confirms cellular A β ₄₂ anti-aggregation activity of hit compound

There is a severe shortage of methods to monitor A β ₄₂ self-assembly in cellular models in real time. Amyloid aggregates in fixed cells can be imaged with immunological staining, which suffers cross reactivity issues with the amyloid precursor protein and its derivatives,^{38, 39} or with the use of amyloid sensitive dyes such as ThT and Congo Red. These effectively stain fixed aggregates, but are generally unsuitable in dynamic live cell studies, as they cannot detect small aggregates low in β sheet content and can induce a mild inhibitory effect on the aggregation process.⁴⁰ Several studies have monitored the uptake of labelled A β species, and tracked their movement throughout the cell.^{41, 42} Whilst useful in terms of localisation studies, these cannot report on the underlying aggregation processes and what aggregated species exist at particular time points, as the peptide is uniformly labelled and will exhibit similar fluorescence intensity in a monomeric or aggregated state. Invasive protocols are therefore required to quantify the formation of aggregates. Current cellular methods rely almost exclusively on cell vitality tests. Although these provide a means to quickly elucidate if a compound or the specific A β species it generates are toxic to cells, such work does not provide insight into how the compounds are actually acting within the cellular environment, as they do not inform on how the peptide functions within the cells or its aggregation state, and instead only highlight damage to the membrane or dysfunction of the metabolic system.^{43, 44}

In order to remedy these shortcomings, we considered that the fluorescence lifetime sensor could be used to follow the uptake and any subsequent aggregation of A β ₄₂ in the presence of hit compounds in SH-SH5Y cells, allowing a direct comparison of A β ₄₂ aggregation propensity *in vitro* and in live cells. The transport of partially labelled A β ₄₂-488 (250 nM) from the extracellular medium into the SH-SY5Y cells was monitored and a drop in the fluorescence lifetime of the attached fluorophore, indicative of peptide aggregation, observed after 12 h (**Fig. 5a**).²⁵ To test if the system could be used for small molecule inhibitor screening, the previously reported inhibitor EGCG was employed.⁴⁵ It was found that addition of EGCG to the extracellular medium at the same time as A β ₄₂ addition had little inhibitory effect on intracellular peptide aggregation, with both conditions reaching the same fluorescence lifetime value (**Supplementary Fig. 15**). However, pre-incubating the cells in a drug solution for one hour prior to the addition of the peptide, was shown to significantly inhibit the aggregation in the cells, as measured at 12, 24 and 48 h (**Fig. 5a**). The measurable change in the fluorescence lifetime observed here is directly comparable to that monitored in the *in vitro* format, permitting

a comparative analysis of compound activity in both formats, which is not possible by any other method. This protocol was validated by testing a range of other known inhibitory small molecules, which were each seen to inhibit the aggregation to different degrees (**Supplementary Fig. 16**). Treatment with **MJ040** reduced the extent of A β_{42} aggregation relative to that of peptide alone, and the modified **MJ040X** provided an even stronger inhibitory effect (**Fig. 5b,d**). This supports the working idea that **MJ040** displays limited permeability as a result of its anionic centre, and that masking this functionality confers more desirable pharmacokinetic properties. In order to show that the aggregation inhibition did not occur during the pre-incubation of the drug with A β_{42} prior to their uptake into cells but indeed intracellularly, we also tested **MJ040X** using a HEK293 cell line overexpressing mCherry-A β_{42} intracellularly.⁴⁶ As shown in the **Extended Data Figure 2**, **MJ040X** was capable of significantly reducing mCherry-A β_{42} aggregation as detected by FLIM analysis.

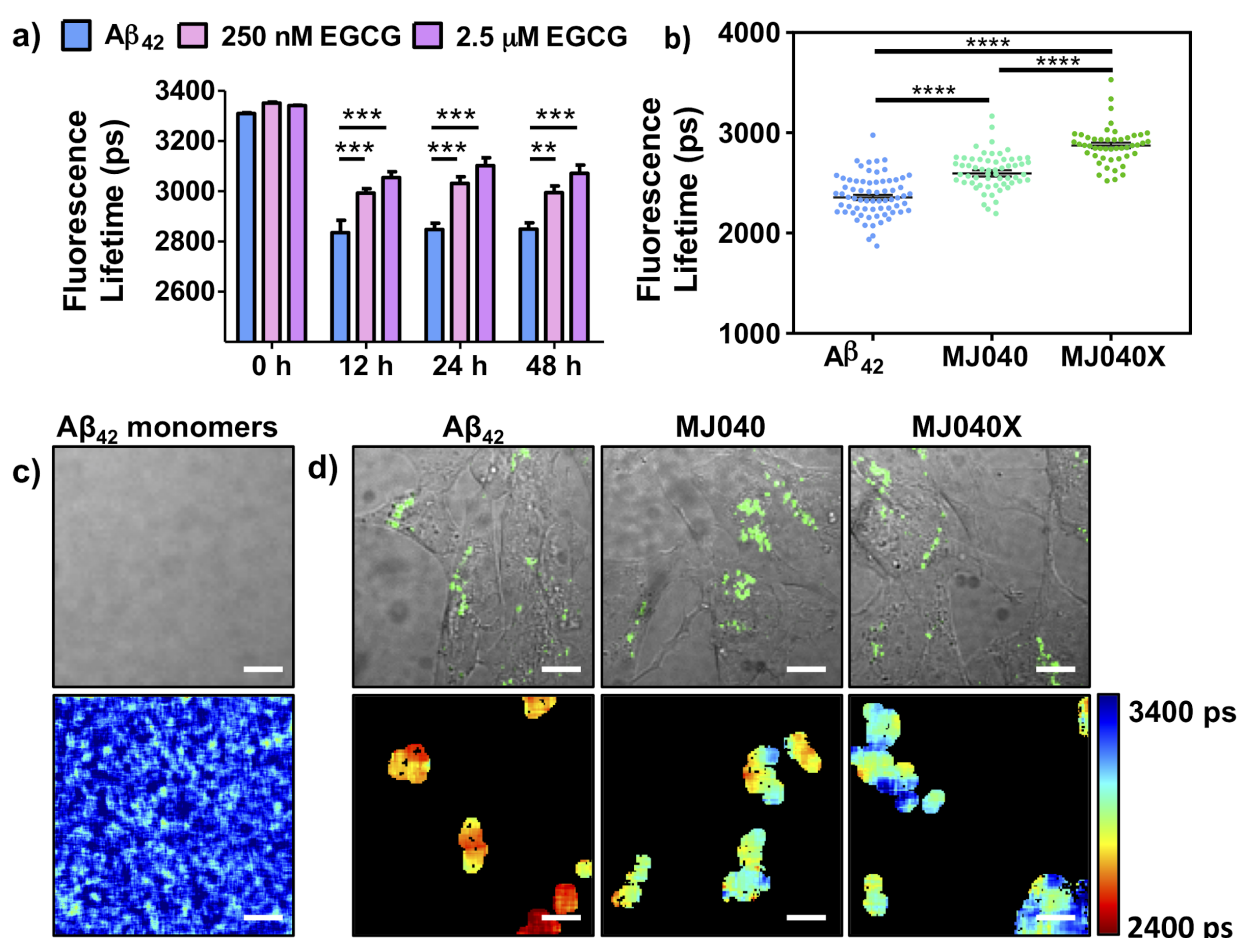


Figure 5: The change in fluorescence lifetime of labelled A β_{42} reports on its aggregation in live cells, which can be inhibited with the use of small molecules. a) Bar diagram displaying the mean fluorescence

lifetime values of partially labelled A β ₄₂ after 12, 24 and 48 h when incubated in the presence of 250 nM or 2.5 μ M of EGCG, a known active aggregation inhibitor. Plot shows mean fluorescence lifetime + SEM, statistical analysis performed by two-way ANOVA with Bonferroni post-test, n = 18-30 (see Supplementary Table S2). b) Dot plot displaying the mean fluorescence lifetime values of A β ₄₂-488 obtained following 48 h treatment with either **MJ040** or **MJ040X** (2.5 μ M). The prodrug **MJ040X** shows a greater effect on reducing peptide aggregation. The plot shows mean fluorescence lifetime + SEM, statistical analysis was performed by one-way ANOVA with Tukey's multiple comparisons post-test, based on three biological repeat experiments in which 48-60 cells were analysed (see Supplementary Table S3). c) The brightfield/fluorescence and fluorescence lifetime image of 50% labelled A β ₄₂-488 monomers (250 nM) in cell culture medium. Scale bar = 10 μ m d) Brightfield/fluorescence (above) and corresponding fluorescence lifetime image (below) of SH-SY5Y cells following 48 h incubation with A β ₄₂-488 and hit compounds. A β ₄₂-488 (250 nM, 50% labelled) with or without the presence of **MJ040** (2.5 μ M) or **MJ040X** (2.5 μ M) was added to the extracellular medium of the cells. The cells to receive **MJ040** or **MJ040X** treatment were pre-incubated with the compound (2.5 μ M) for 1 h prior to A β ₄₂-488 addition. Following 48 h incubation, the cells were washed with medium and the fluorescence lifetime of the internalised A β ₄₂-488 analysed. Scale bar = 20 μ m. A typical time trace and exponential decay fits for the data can be found in **Supplementary Figure S17**.

Hit compound MJ040X inhibits A β ₄₂ aggregation in whole organism disease model

Whole organism studies using a *C. elegans* disease model were carried out to demonstrate the ability of our unified fluorescence lifetime sensor assay to report on aggregation in matched *in vitro* and *in vivo* formats. Worm models have been extensively employed for studying the aggregation of proteins associated with neurodegenerative disease.^{47, 48, 49} Here, a Pmyo3::GFP::A β ₄₂ construct was used (**Supplementary Figure 18**). In this model system, the myo-3 promoter (Pmyo-3) drives expression of GFP-A β ₄₂. The muscle myosin gene *myo-3* is turned on in post-mitotic embryonic body-wall muscle and, as such, fluorescently labelled A β ₄₂ is easily identified along the periphery of the worms. Similar to the fluorophore previously employed, GFP can act as reporter to inform on the aggregation state of attached amyloidogenic peptides, with the extent of fluorophore quenching indicating the degree of peptide self-assembly.⁵⁰ The fluorescence lifetime of GFP was monitored throughout the life span of adult worms (~15 days), which were either treated with **MJ040X** from the first day of adulthood or directly from the earliest larval stage after synchronisation. A measurable decrease in fluorescence lifetime, indicative of A β ₄₂ aggregation, was observed in accordance with ageing, with a statistically significant reduction observed at day 12 (**Fig. 6a,b,d**). It was found that treatment with the

MJ040X from adulthood delayed this process. A statistically significant difference in fluorescence lifetime between the treated and untreated control was observed at day 12 of adulthood, but by day 15 peptide aggregation was evident in the treated worms also (**Fig. 6d**), suggesting a narrow, but significant therapeutic window. Treatment from larval stage, however, prevented $A\beta_{42}$ aggregation until day 15, the last day of measurement for adult worms (**Fig. 6e**).

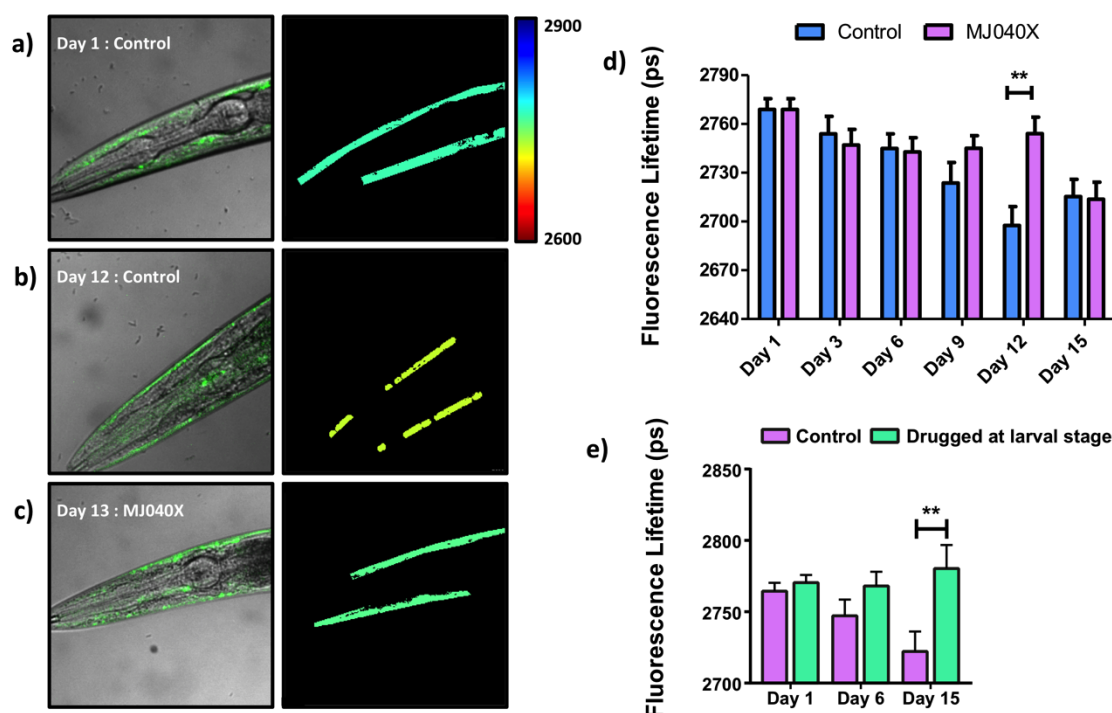


Figure 6: The change in fluorescence lifetime of GFP- $A\beta_{42}$ in disease model *C. elegans* reports on its aggregation in ageing adult worms, and can be used to probe the *in vivo* activity of small molecule inhibitors. Confocal and corresponding fluorescence lifetime images of **a)** day 1 control **b)** day 12 control and **c)** day 12 **MJ040X**-treated worms, expressing GFP- $A\beta_{42}$ in the body muscles. *C. elegans* were grown on OP50 *E. coli* seeded plates, with 0.7 μ M **MJ040X**. The worms were changed to new drugged plates on day 8 of adulthood. Scale bar = 20 μ m. **d)** Bar diagram displaying the mean fluorescence lifetime values at each time point measured, following the initiation of drug treatment at day 1 of adulthood. A delay in $A\beta_{42}$ aggregation with **MJ040X** treatment was observed in two biological repeats with 8-10 worms analysed for each. Data were analysed by a two-way Anova and a Bonferroni post-test (see Supplementary Table S4). **e)** Bar diagram displaying a comparison of the mean fluorescence lifetime values observed when the *C. elegans* were treated from day 1 of the larval stage. Drug treatment from the larval stage prevents aggregation until day 15, based on two biological repeats with 8-18 worms analysed per repeat. All data are reported as mean fluorescence lifetime + SEM and the statistical analysis was performed using a one-way ANOVA with Sidak's multiple comparison test (see Supplementary Table S5). A typical time trace and exponential decay fits for the data can be found in Supplementary Figure 17.

342

343 **Implications and Conclusions**

344 Recent failures in clinical trials suggest that current aggregation screening strategies are limited in their ability
 345 to provide therapeutically viable hit compounds.^{12, 19, 51} Limitations stem from issues in reproducibility
 346 (partially due to peptide quality and solubility in a heterogeneous assay),⁵² fluorescence interference caused
 347 by intrinsic properties of the test compounds,³⁵ and inability to efficiently validate and prioritise hit
 348 compounds *in vivo*. There is currently no single method that permits protein aggregation and its inhibition as
 349 measured *in vitro* to be directly correlated to processes in a cellular environment in a mammalian cell or in
 350 small organisms. After years of failing to find a suitable therapeutic drug against AD, researchers are still
 351 using historically established assays, such as MTT, Thioflavin S staining or fluorescence intensity based
 352 aggregate counting to validate their *in vitro* or *in silico* hits.^{53, 54, 55} The unified fluorescence lifetime screening
 353 system described in this work overcomes this historical burden and bridges the gap that intrinsically exists
 354 between *in vitro* and *in vivo* approaches, permitting results from a medium-throughput microfluidic screen to
 355 be directly compared to physiologically relevant cellular and whole organism analysis.

356 The availability of the nanoFLIM provides a unique opportunity to directly follow the aggregation of A β ₄₂
 357 with large screening libraries, with great sensitivity and temporal resolution, undeterred by limitations that
 358 restrict conventional screening approaches. The microfluidic system is advantageous in many respects: (i)
 359 *Sample size and reagent consumption*. The nanoFLIM allows for up to 110 replicates per screen, providing
 360 an unparalleled sample size and circumventing the issues of irreproducibility that persistently complicate *in*
 361 *vitro* biophysical A β ₄₂ experiments,^{12, 23, 56} even with recombinant material.²⁹ The minimised volume (18 nL
 362 per droplet vs 100 μ L per 96-well plate experiment) necessitates only 219 μ g of A β ₄₂ to screen all 445
 363 compounds – with 10 replicates - in nanoFLIM. This is 50-fold less than the 10.7 mg of A β ₄₂ in a ThT
 364 experiment performed in 24 96-well plates (see Supplementary Information for details of the comparisons).
 365 The miniaturisation and automation reduces time spent performing laborious peptide preparation protocols
 366 and makes screening precious novel libraries more feasible. (ii) *Costs*. A 1115-fold reduction in the peptide
 367 costs per single experiment is achieved with the nanoFLIM, calculated as 0.087 p per droplet using this assay
 368 and £0.97 per well in a 96 well plate format. (iii) *Avoidance of artefacts*. The correct assignment of the
 369 inhibitory activity of four spectroscopically active compounds, as validated by TEM imaging analysis,

suggests that the fluorescence lifetime sensor is less susceptible to the detection of false positive and negative results than the ThT fluorescence assay. This conventional assay is more prone to bias as a result of intrinsic properties of the screening libraries,³⁵ which generally arises from fluorescence interference caused by spectroscopic properties of the test compounds or through competition with the ThT binding site or interaction with the ThT itself.^{35, 57, 58} Removal of these limitations with the nanoFLIM assay increases the true hit rate, and its impact is demonstrated by a screen of DOS- and medically-focused compound collections, in which the nanoFLIM permitted the detection of inhibitory activity that was *not* picked up by the conventional ThT assay.

A powerful attribute of the nanoFLIM is the unique fluorescence lifetime readout, which can also be applied for measurements in live cells and *C. elegans* disease models, thereby provided a means to probe amyloid aggregation and the inhibitory effect of added small molecules in multiple formats along the drug development pipeline. The aggregation of A β ₄₂ *in vivo* is considerably more complex than reductionist *in vitro* experiments and remains poorly understood, leading to conflicting interpretations and precluding experimental elaboration of lead compounds that work in an organismal context.²⁰ This use of the fluorescence lifetime sensor demonstrates how hit compounds identified in microfluidic droplet screening can quickly and easily be assessed in live cells and disease model whole organisms. Previously, there has been no other single technique that could be used to measure amyloid aggregation in these distinct relevant formats, and this capability gives an inimitable opportunity to reduce attrition rates by bridging *in vitro* and *in vivo* studies. Furthermore, there is great potential to start the compound screening process in the living systems, where the cooperative effects of chaperones, internal surfaces, molecular crowding and other intracellular factors are better taken into consideration. As such, there would be a reduced chance of missing active compounds that do not function in the simplified *in vitro* format, or favouring the detection of non-toxic compounds or those capable of penetrating biological membranes. This system is amenable to a high-throughput scale up through the use of a 96 well plate format of the *in vivo* systems, which could also be easily automated, further speeding up the screening process.

To illustrate the potential of this system, a pilot screening campaign was performed with 445 compounds from medically-relevant chemical libraries, yielding a total hit rate of 13% (>30% A β ₄₂ aggregation inhibition). The lead inhibitor identified, **MJ040**, and rationally designed prodrug **MJ040X**, were shown to

exert strong inhibitory effects *in vitro*, in live cells and in disease model *C. elegans*, emphasising that biologically active inhibitors can be identified through this comprehensive assay platform. We also believe that it could be easily adapted to screen for aggregation inhibitors of other amyloidogenic proteins, including functional bacterial amyloids, thereby holding potential for the identification of hit compounds for the treatment of a range of amyloid disorders and bacterial pathogenesis.⁵⁹

The breadth of the fluorescence lifetime screening platform and the potential high-throughput afforded by its use, will expedite the rate at which hits are identified, validated *in vivo* and prioritised for future hit development strategies, with the attrition rate in moving through these stages minimised by the unified analysis. The efficiency afforded by this approach has already yielded a lead in **MJ040X**, but the high hit rates observed here suggest further campaigns with existing industrial compound libraries are likely to identify further modulators of the aggregation of A β ₄₂, providing new opportunities to overcome the current unsatisfactory situation, where no Alzheimer's disease treatments exist.

References

1. Querfurth HW, LaFerla FM. Alzheimer's disease. *N Engl J Med* 2010, **362**(4): 329-344.
2. Alzheimer's Disease Facts and Figures, Vol. 13. . In: Association As, editor. *Alzheimers Dement.*; 2017. pp. 325-373.
3. Citron M. Alzheimer's disease: strategies for disease modification. *Nat Rev Drug Discov* 2010, **9**(5): 387-398.
4. Necula M, Kaye R, Milton S, Glabe CG. Small molecule inhibitors of aggregation indicate that amyloid beta oligomerization and fibrillization pathways are independent and distinct. *J Biol Chem* 2007, **282**(14): 10311-10324.
5. Saunders JC, Young LM, Mahood RA, Jackson MP, Revell CH, Foster RJ, *et al.* An *in vivo* platform for identifying inhibitors of protein aggregation. *Nat Chem Biol* 2016, **12**(2): 94-101.
6. Joshi P, Chia S, Habchi J, Knowles TP, Dobson CM, Vendruscolo M. A Fragment-Based Method of Creating Small-Molecule Libraries to Target the Aggregation of Intrinsically Disordered Proteins. *ACS Comb Sci* 2016, **18**(3): 144-153.
7. Mullard A. Landmark Alzheimer's Drug Approval Confounds Research Community. *Nature* 2021, **594**: 309-310.
8. Walsh S, Merrick R, Milne R, Brayne C. Aducanumab for Alzheimer's disease? *BMJ* 2021, **374**: n1682.
9. Patterson C. *The World Alzheimer Report 2018*: London, 2018.

- 431 10. Plascentia-Villa G, Perry G. Status and future directions of clinical trials in Alzheimer's disease. In:
432 Söderbom G, Esterline R, Oscarsson J, Mattson MP (eds). *Metabolic and Bioenergetic Drivers of*
433 *Neurodegenerative Disease: Neurodegenerative Disease Research and Commonalities with*
434 *Metabolic Diseases*. Academic Press, 2020, pp 3-50.
- 435 11. Cummings J, Lee G, Ritter A, Sabbagh M, Zhong K. Alzheimer's disease drug development pipeline:
436 2020. *Alzheimers Dement (NY)* 2020, **6**(1): e12050.
- 437 12. Doig AJ, Del Castillo-Frias MP, Berthoumieu O, Tarus B, Nasica-Labouze J, Sterpone F, *et al.* Why
438 Is Research on Amyloid-beta Failing to Give New Drugs for Alzheimer's Disease? *ACS Chem*
439 *Neurosci* 2017, **8**(7): 1435-1437.
- 440 13. Metallo SJ. Intrinsically disordered proteins are potential drug targets. *Curr Opin Chem Biol* 2010,
441 **14**(4): 481-488.
- 442 14. Cheng Y, LeGall T, Oldfield CJ, Mueller JP, Van YY, Romero P, *et al.* Rational drug design via
443 intrinsically disordered protein. *Trends Biotechnol* 2006, **24**(10): 435-442.
- 444 15. Kotler SA, Walsh P, Brender JR, Ramamoorthy A. Differences between amyloid-beta aggregation in
445 solution and on the membrane: insights into elucidation of the mechanistic details of Alzheimer's
446 disease. *Chem Soc Rev* 2014, **43**(19): 6692-6700.
- 447 16. Galloway WR, Isidro-Llobet A, Spring DR. Diversity-oriented synthesis as a tool for the discovery
448 of novel biologically active small molecules. *Nat Commun* 2010, **1**: 80.
- 449 17. Shineman DW, Basi GS, Bizon JL, Colton CA, Greenberg BD, Hollister BA, *et al.* Accelerating drug
450 discovery for Alzheimer's disease: best practices for preclinical animal studies. *Alzheimers Res Ther*
451 2011, **3**(5): 28.
- 452 18. Ringman JM, Frautschy SA, Teng E, Begum AN, Bardens J, Beigi M, *et al.* Oral curcumin for
453 Alzheimer's disease: tolerability and efficacy in a 24-week randomized, double blind, placebo-
454 controlled study. *Alzheimers Res Ther* 2012, **4**(5): 43.
- 455 19. Salloway S, Sperling R, Keren R, Porsteinsson AP, van Dyck CH, Tariot PN, *et al.* A phase 2
456 randomized trial of ELND005, scyllo-inositol, in mild to moderate Alzheimer disease. *Neurology*
457 2011, **77**(13): 1253-1262.
- 458 20. Karran E, Hardy J. A critique of the drug discovery and phase 3 clinical programs targeting the
459 amyloid hypothesis for Alzheimer disease. *Ann Neurol* 2014, **76**(2): 185-205.
- 460 21. Chen W, Young LJ, Lu M, Zaccane A, Strohl F, Yu N, *et al.* Fluorescence Self-Quenching from
461 Reporter Dyes Informs on the Structural Properties of Amyloid Clusters Formed in Vitro and in Cells.
462 *Nano Lett* 2017, **17**(1): 143-149.
- 463 22. Kaminski Schierle GS, Bertoncini CW, Chan FTS, van der Goot AT, Schwedler S, Skepper J, *et al.*
464 A FRET sensor for non-invasive imaging of amyloid formation in vivo. *Chemphyschem* 2011, **12**(3):
465 673-680.
- 466 23. Giehm L, Otzen DE. Strategies to increase the reproducibility of protein fibrillization in plate reader
467 assays. *Anal Biochem* 2010, **400**(2): 270-281.
- 468 24. Chan FT, Kaminski Schierle GS, Kumita JR, Bertoncini CW, Dobson CM, Kaminski CF. Protein
469 amyloids develop an intrinsic fluorescence signature during aggregation. *Analyst* 2013, **138**(7): 2156-
470 2162.
- 471 25. Esbjorner EK, Chan F, Rees E, Erdelyi M, Luheshi LM, Bertoncini CW, *et al.* Direct observations of
472 amyloid beta self-assembly in live cells provide insights into differences in the kinetics of Abeta(1-
473 40) and Abeta(1-42) aggregation. *Chem Biol* 2014, **21**(6): 732-742.
- 474 26. Gielen F, van Vliet L, Koprowski BT, Devenish SR, Fischlechner M, Edel JB, *et al.* A fully
475 unsupervised compartment-on-demand platform for precise nanoliter assays of time-dependent
476 steady-state enzyme kinetics and inhibition. *Anal Chem* 2013, **85**(9): 4761-4769.

- 477 27. Buryska T, Vasina M, Gielen F, Vanacek P, van Vliet L, Jezek J, *et al.* Controlled Oil/Water
478 Partitioning of Hydrophobic Substrates Extending the Bioanalytical Applications of Droplet-Based
479 Microfluidics. *Anal Chem* 2019, **91**(15): 10008-10015.
- 480 28. Janecek M, Rossmann M, Sharma P, Emery A, Huggins DJ, Stockwell SR, *et al.* Allosteric
481 modulation of AURKA kinase activity by a small-molecule inhibitor of its protein-protein interaction
482 with TPX2. *Sci Rep* 2016, **6**: 28528.
- 483 29. Stephens AD, Lu M, Fernandez-Villegas A, Kaminski Schierle GS. Fast Purification of Recombinant
484 Monomeric Amyloid-beta from E. coli and Amyloid-beta-mCherry Aggregates from Mammalian
485 Cells. *ACS Chem Neurosci* 2020.
- 486 30. Sum TH, Sum TJ, Galloway WR, Collins S, Twigg DG, Hollfelder F, *et al.* Combinatorial Synthesis
487 of Structurally Diverse Triazole-Bridged Flavonoid Dimers and Trimers. *Molecules* 2016, **21**(9):
488 1230.
- 489 31. Sato M, Murakami K, Uno M, Nakagawa Y, Katayama S, Akagi K, *et al.* Site-specific inhibitory
490 mechanism for amyloid beta42 aggregation by catechol-type flavonoids targeting the Lys residues. *J*
491 *Biol Chem* 2013, **288**(32): 23212-23224.
- 492 32. LeVine H, 3rd. Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides:
493 detection of amyloid aggregation in solution. *Protein Sci* 1993, **2**(3): 404-410.
- 494 33. Kaye R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, *et al.* Common structure of
495 soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 2003, **300**(5618):
496 486-489.
- 497 34. Thompson AJ, Herling TW, Kubankova M, Vysniauskas A, Knowles TP, Kuimova MK. Molecular
498 Rotors Provide Insights into Microscopic Structural Changes During Protein Aggregation. *J Phys*
499 *Chem B* 2015, **119**(32): 10170-10179.
- 500 35. Hudson SA, Ecroyd H, Kee TW, Carver JA. The thioflavin T fluorescence assay for amyloid fibril
501 detection can be biased by the presence of exogenous compounds. *FEBS J* 2009, **276**(20): 5960-5972.
- 502 36. Agholme L, Lindstrom T, Kagedal K, Marcusson J, Hallbeck M. An in vitro model for neuroscience:
503 differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of
504 mature neurons. *J Alzheimers Dis* 2010, **20**(4): 1069-1082.
- 505 37. Bieschke J, Herbst M, Wiglenda T, Friedrich RP, Boeddrich A, Schiele F, *et al.* Small-molecule
506 conversion of toxic oligomers to nontoxic beta-sheet-rich amyloid fibrils. *Nat Chem Biol* 2011, **8**(1):
507 93-101.
- 508 38. Winton MJ, Lee EB, Sun E, Wong MM, Leight S, Zhang B, *et al.* Intraneuronal APP, not free Abeta
509 peptides in 3xTg-AD mice: implications for tau versus Abeta-mediated Alzheimer
510 neurodegeneration. *J Neurosci* 2011, **31**(21): 7691-7699.
- 511 39. Gouras GK, Tampellini D, Takahashi RH, Capetillo-Zarate E. Intraneuronal beta-amyloid
512 accumulation and synapse pathology in Alzheimer's disease. *Acta Neuropathol* 2010, **119**(5): 523-
513 541.
- 514 40. Frid P, Anisimov SV, Popovic N. Congo red and protein aggregation in neurodegenerative diseases.
515 *Brain Res Rev* 2007, **53**(1): 135-160.
- 516 41. Garai K, Frieden C. Quantitative analysis of the time course of Abeta oligomerization and subsequent
517 growth steps using tetramethylrhodamine-labeled Abeta. *Proc Natl Acad Sci U S A* 2013, **110**(9):
518 3321-3326.
- 519 42. Johnson RD, Schauerte JA, Wisser KC, Gafni A, Steel DG. Direct observation of single amyloid-
520 beta(1-40) oligomers on live cells: binding and growth at physiological concentrations. *PLoS One*
521 2011, **6**(8): e23970.
- 522 43. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation
523 and cytotoxicity assays. *J Immunol Methods* 1983, **65**(1-2): 55-63.

44. Kepp O, Galluzzi L, Lipinski M, Yuan J, Kroemer G. Cell death assays for drug discovery. *Nat Rev Drug Discov* 2011, **10**(3): 221-237.
45. Ehrnhoefer DE, Bieschke J, Boeddrich A, Herbst M, Masino L, Lurz R, *et al.* EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. *Nat Struct Mol Biol* 2008, **15**(6): 558-566.
46. Lu M, Williamson N, Mishra A, Michel CH, Kaminski CF, Tunnacliffe A, *et al.* Structural progression of amyloid-beta Arctic mutant aggregation in cells revealed by multiparametric imaging. *J Biol Chem* 2019, **294**(5): 1478-1487.
4. David DC, Ollikainen N, Trinidad JC, Cary MP, Burlingame AL, Kenyon C. Widespread protein aggregation as an inherent part of aging in *C. elegans*. *PLoS Biol* 2010, **8**(8): e1000450.
48. Link CD. *C. elegans* models of age-associated neurodegenerative diseases: lessons from transgenic worm models of Alzheimer's disease. *Exp Gerontol* 2006, **41**(10): 1007-1013.
49. Alexander AG, Marfil V, Li C. Use of *Caenorhabditis elegans* as a model to study Alzheimer's disease and other neurodegenerative diseases. *Front Genet* 2014, **5**: 279.
50. Murakami T, Yang SP, Xie L, Kawano T, Fu D, Mukai A, *et al.* ALS mutations in FUS cause neuronal dysfunction and death in *Caenorhabditis elegans* by a dominant gain-of-function mechanism. *Hum Mol Genet* 2012, **21**(1): 1-9.
51. Toyn J. What lessons can be learned from failed Alzheimer's disease trials? *Expert Rev Clin Pharmacol* 2015, **8**(3): 267-269.
52. Hellstrand E, Boland B, Walsh DM, Linse S. Amyloid beta-protein aggregation produces highly reproducible kinetic data and occurs by a two-phase process. *ACS Chem Neurosci* 2010, **1**(1): 13-18.
53. Pasioka A, Panek D, Szalaj N, Espargaro A, Wieckowska A, Malawska B, *et al.* Dual Inhibitors of Amyloid-beta and Tau Aggregation with Amyloid-beta Disaggregating Properties: Extended In Cellulo, In Silico, and Kinetic Studies of Multifunctional Anti-Alzheimer's Agents. *ACS Chem Neurosci* 2021, **12**(11): 2057-2068.
54. Griner SL, Seidler P, Bowler J, Murray KA, Yang TP, Sahay S, *et al.* Structure-based inhibitors of amyloid beta core suggest a common interface with tau. *Elife* 2019, **8**.
55. Robinson M, Lou J, Mehrazma B, Rauk A, Beazely M, Leonenko Z. Pseudopeptide Amyloid Aggregation Inhibitors: In Silico, Single Molecule and Cell Viability Studies. *Int J Mol Sci* 2021, **22**(3).
56. Doig AJ, Derreumaux P. Inhibition of protein aggregation and amyloid formation by small molecules. *Curr Opin Struct Biol* 2015, **30**: 50-56.
57. Noormagi A, Primar K, Tougu V, Palumaa P. Interference of low-molecular substances with the thioflavin-T fluorescence assay of amyloid fibrils. *J Pept Sci* 2012, **18**(1): 59-64.
58. Pinotsi D, Buell AK, Dobson CM, Kaminski Schierle GS, Kaminski CF. A label-free, quantitative assay of amyloid fibril growth based on intrinsic fluorescence. *Chembiochem* 2013, **14**(7): 846-850.
59. Knowles TP, Vendruscolo M, Dobson CM. The amyloid state and its association with protein misfolding diseases. *Nat Rev Mol Cell Biol* 2014, **15**(6): 384-396.

Methods

Reagents. Synthetic A β ₄₂ (>95%, Eurogentec) and A β ₄₂ Hilyte™ Fluor 488 (>95%, labelled at the N-terminus) were purchased from Anaspec as lyophilised powder. ThioflavinT was purchased from AbCam. The compound screening libraries were obtained from the Spring Research group (Department of Chemistry, University of Cambridge). All other chemicals, unless otherwise stated, were purchased from Sigma Aldrich.

Peptide Preparation. The peptide was prepared as previously described.³⁰ Briefly, lyophilised A β ₄₂ (1 mg) was dissolved in ice cold trifluoroacetic acid (200 μ L), sonicated at 0 °C for 60 s, then lyophilised overnight. Ice cold 1,1,1,3,3,3-hexafluoro-2-propanol (1 mL) was added, sonicated at 0 °C for 60 s and aliquoted into 20 μ L portions. The samples were lyophilised overnight and were stored at –80 °C until use. The concentration of the aliquots was determined using amino acid mass spectrometry analysis. The required concentration of unlabelled A β ₄₂ was prepared by dissolving the solution in dimethyl sulfoxide (DMSO) (5% of total solvent volume), then adding sodium phosphate buffer (NaPi, 50 mM, pH 7.4). Prior to use, the solution was sonicated at 0 °C for 3 min, centrifuged at 13,400 rpm at 0 °C for 30 min to remove preformed aggregates. Lyophilised A β ₄₂ Hilyte™ Fluor 488 peptide (0.1 mg) was dissolved in 1% NH₄OH (200 μ L) and sonicated for 60 s at 0 °C. The sample was aliquoted into 5 μ L units, snap frozen in liquid N₂, then stored at –80 °C. Before use, the sample was thawed on ice and NaPi buffer was added to bring the solution to the required concentration.

For studies with partially labelled peptide, each peptide was prepared as above then mixed at the appropriate ratios before each set of experiments. This was aliquoted into small units, then snap frozen and stored at –80 °C until use.

Microfluidic Device Fabrication. The silicon master mould was fabricated by MicroLiquid (Gipuzkoa, Spain) using a two layer soft lithographic technique, as previously described.^{60, 61} The depth of the first layer with a serpentine channel was 175 μ m, while the depth of the square traps were 250 μ m. PDMS replicates of these devices were bonded to a thin glass coverslip (thickness 130 μ m) using oxygen plasma. The devices were silanized by pipetting a fresh solution of 2% Trichloro(1H,1H,2H,2H-perfluorooctyl)silane in HFE-

7500 (3M) into the chips. Subsequently, PTFE tubing (diameter: 200 μ m) was manually inserted in designed entrance channels and glued in place by curing PDMS over. This ensured air-tight connection as well as preserving the order of the droplets as they transited from tubing to chip. One tubing was then connected to a syringe pump (Chemyx Fusion 200) operating in withdrawal mode, while the other tubing was inserted and clamped into the stainless steel hook of a Mitos Dropix. A gas-tight glass syringe (100 μ L) was used to fill the device with HFE-7500 oil containing 1% Pico-Surf surfactant (Dolomite Microfluidics) and the chip was inspected to confirm the absence of air bubbles. Next, 10 μ L of each compound was pipetted into the loading strip of the Dropix. The droplet sequence was programmed to obtain 18 nL droplets with 36 nL oil spacing between each drop. Typical flow rate for producing the droplets was 2 μ L/min. Before reaching the device, the droplets were slowed down to 1 μ L/min and the filling process was monitored with a bright-field camera of an inverted microscope (Olympus, IX71). After completion of the filling process, the flow was stopped, unless specified in shearing experiments.

Spectroscopic Assays. Final concentrations of 20 μ M ThT with 10 μ M unlabelled peptide in sodium phosphate (NaPi, 50 mM) were used for all samples. For screening the chemical libraries, 50 μ M of the test compound was added. The assay samples (total volume 100 μ L) were mixed in a black non-binding 96-well plate (Greiner Bio-One, Switzerland). The plate was sealed (Nunc™, polyolefin acrylate film) and loaded into the fluorescence plate reader (Tecan, Switzerland) at 37 °C. Fluorescence kinetics were measured at 5 min reading intervals, with 15 sec shaking before each read. The excitation and emission wavelengths were 440 nm and 480 nm, respectively.

Fluorescence lifetime imaging microscopy. Fluorescence lifetime imaging was performed on a custom-built confocal microscope (FV300, Olympus) as previously described²¹ that utilises a time-correlated single photon counting (TCSPC) module (SPC-830, Becker & Hickl GmbH) and is shown in the Extended Data Figure 3 and 4, Supplementary Figure S17). Briefly, a supercontinuum source (SC390, Fianium) operating at a 40 MHz repetition rate was used for excitation. The excitation light was filtered using an acousto-optic tunable filter (AOTF_{NC}-400.650, QuantaTech) centered at 480 nm to excite GFP and AF488 or at 585nm to excite

mCherry. Fluorescence emission from the sample passed through a band-pass filter (FF01-525/39-25 or FF01-624/40-25, Semrock) before reaching the detector (PMC-100, Becker & Hickl GmbH). The photon detection rate for each pixel was kept below 1% of the laser repetition rate in order to avoid photon pile-up. Air objectives (PlanApo 2x and 40x, Olympus) were used for imaging the microfluidic chip and *C. elegans*, respectively. An oil objective (PlanApo 60x BFP1 C2, Olympus) was used to image cells. All FLIM data were analysed using either commercial software SPCImage (Becker & Hickl GmbH) or open-source FLIM-fit software.⁶² A biexponential fit was used for nanoFLIM and for FLIM data from AF488 containing cells. A single exponential fit was used for FLIM data from *C. elegans* data and from mCherry containing cells (Extended Data Figure 2). The phasor plot analysis⁶² feature in SPCImage was used to validate the use of a biexponential fit on certain FLIM data (Extended Data Figure 4).

AFM. A freshly cleaved mica surface was prepared by sequential treatment with potassium hydroxide and 0.1% poly-lysine solution. After the specified time of incubation (20 μ M A β ₄₂, 100 μ M compound), samples were transferred directly to the slides and were allowed to dry for 30mins. Samples were then rinsed with Milli-Q water and dried in the air. AFM images were acquired on a commercial system (Bioscope RESOLVE, Bruker) and Nanoscope software (Bruker). The instrument was operated in tapping mode in air.

Transmission Electron Microscopy (TEM). Samples were stained with 2 % (w/v) uranyl acetate. Images were obtained at various magnifications using a Tecnai G2 80-200kv transmission electron microscope and captured with a bottom mounted digital camera (AMT).

Dot blot. 20 μ M A β ₄₂ and 100 μ M compound were incubated at 37 °C. At specified time points, 10 μ L aliquots were removed and stored at -20 °C until use. 5 μ L samples were spotted onto a nitrocellulose membrane (Amersham Hybond ECL, GE Healthcare Life Sciences) and were allowed to dry for 1 h. The membranes were blocked with 5% non-fat milk in tris-buffered saline (TBS), then washed with TBS. The membranes were treated with the primary antibodies A11 (Invitrogen) and 6E10 (Invitrogen) for 12 h. After washing (0.01% Tween20 in TBS), anti-rabbit horseradish peroxidase (HRP) conjugated antibodies

(ThermoScientific) were added. After 2 h, the membranes were washed (0.01% Tween20 in TBS). An enhanced chemiluminescent (ECL, Thermo Scientific Pierce™) substrate was added and the samples exposed to X-ray film, as per manufacturer instructions. Positive A11 stained dots are indicative of the presence of oligomeric species, but not A β monomer or fibrils. 6E10 stained dots are indicative of any A β species, irrespective of the conformation.

SH-SY5Y cell culture. Human neuroblastoma cells (Sigma-Aldrich, Gillingham, UK) were grown in a serum-containing medium (SCM) consisting of 15% FBS, 1% non-essential amino acids (Sigma), 1% L-glutamine (Life Technologies, UK) and 1:1 minimal essential medium (Sigma) and nutrient mixture F-12 Ham (Sigma). For cell viability assays and uptake experiments the cells were cultured using a serum free medium (SFM), in which the FBS was replaced with 2% B27 complement (Life Technologies).

mCherry HEK cell culture. Flp-In™ T-REx™ 293 cell line (Invitrogen), with a stably integrated FRT site and a TetR repressor, served to create new stable cell lines expressing mCherry-A β 42(WT), i.e. pcDNA5/FRT/TO-mCherry-A β (WT) under the Flp In™ expression vector.^{46, 63} All cell lines were grown in cell culture medium containing: Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS; 10%; Thermo Fisher Scientific), antibiotic-antimycotic (1%; Thermo Fisher Scientific), and glutaMAX (1%; Thermo Fisher Scientific). Cells were incubated in T75 flasks at 37 °C, in a 5% CO₂ atmosphere, and passaged approximately every 3–4 days to reach 80–90% confluency. Cell lines were regularly tested for mycoplasma contamination with the MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza, Walkersville).

Cell Vitality Assay. Monomeric peptide experiments: SCM containing the test compounds were added to the SH-SY5Y and were incubated for 1 h. The media was removed and the cells were rinsed with minimum essential medium (MEM). A β 42 monomers (500 nM) with or without the presence of test compound in SFM were added. Following 48h incubation the cytotoxicity was assessed using an MTT cell viability assay (measuring cellular metabolic activity as an indicator of cell vitality), according to the manufacturer's instructions (Invitrogen). Briefly, medium was aspirated from the cells and replaced with 100 μ L of fresh

674 medium and 10 μ L of 12 mM MTT. After incubation for 4 h at 37 °C, 85 μ L of the medium was removed and
675 50 μ L of DMSO added. The samples were mixed thoroughly, incubated overnight at 37 °C for 10 min, then
676 the absorbance was recorded at 540 nm.

677 Pre-aggregated peptide experiments: A β ₄₂ (10 μ M) was pre-incubated for 24 h with or without the test
678 compounds. The sample were diluted with SFM (500 nM A β ₄₂) and added to SH-SY5Y cells. After 48 h
679 treatment, cell vitality was evaluated using an MTT assay as previously described.

680

681 **Cellular fluorescence lifetime sensor experiments.** SH-SH5Y cells (30,000 cells/well) were plated in Lab-
682 Tek II chambered coverglass plates (NUNC™, Thermo Fisher Scientific, Cramlington, UK) in SCM, and
683 were incubated for 24 h. The media was removed and fresh SCM containing the test compound of interest, or
684 vehicle control, was added. The cells were allowed to incubate at 37 °C for 1 h. The media was removed and
685 SFM containing 50% labelled 250nM A β ₄₂-488 and the test compound was added to the cells, which were
686 then incubated for 24 or 48 h. mCherry-A β ₄₂(WT) cells (20,000 cells/well) were plated in Lab-Tek II plates
687 (Nunc™, Thermo Fisher Scientific) containing cell culture medium supplemented with tetracycline (1
688 μ g/mL), to induce construct expression. The compound of interest (M040X) or vehicle control (DMSO) was
689 added. MJ040X was first dissolved in DMSO to make a stock solution (20 mM), and then further diluted
690 with NaPi buffer (50 mM, pH 7.4) to make a working solution (1 mM), which was then added to cell culture
691 medium to obtain the required final concentration (2.5 μ M). Samples were incubated for 48 h (at 37 °C, 5%
692 CO₂) before analysis. The cells were rinsed 3 times with MEM then imaged in a chamber at 37 °C and 5%
693 CO₂ on the microscope stage. 8-10 images were taken per condition, with 3-10 cells per image, depending
694 on magnification. The FLIM data was analysed using the FLIMfit software, using image-wise analysis on
695 segmented regions.⁶⁴

696 **Worm model experiments.** *C. elegans* (Pmyo3:: GFP::A β ₄₂; expressing A β ₄₂ in its muscle myosin; Fig.
697 S21) were cultivated on a diet of OP50 *E. coli* and were maintained at 20 °C on nematode growth medium
698 (NGM) according to well described protocols.⁶⁵ Compound treatment was achieved by adding solutions of
699 the compound or vehicle control (700 nM, 0.05% DMSO) to the bacterially-lawned agar plates and allowing
700 the solvent to dry off before adding the worms. Worms treated from adulthood were transferred to drugged

5-fluoro-2'-deoxyuridine (FUDR, 75 μ M) containing NGM plates 2.5 days post synchronization (NaOH 0.25 M, NaOCl 0.8%, 6 min). Worms treated from larvae were transferred to drugged NGM plates directly after synchronization, then transferred to drugged FUDR plates after 2.5 day. All plates were seeded with concentrated OP50 *E. coli*. Adults were transferred to freshly drugged FUDR NGM plates after 8 days. Imaging was carried out at day 3,6,9,12 and 15. Two repeats of each time point were taken, with 8-10 worms imaged per condition for day 1 adult treated worms. Two repeats of each time point, with 8-18 worms imaged per condition for worms treated from larvae.

Statistical Analysis. Data were analysed using GraphPad Prism software, version 5. All comparisons were accompanied by one-way or two-way analysis of variance (ANOVA), with a Dunnett's, Sidak or Bonferroni post-test respectively utilised to analyse variance between each set of treatments (see Supplementary Tables S2-4). P-values are annotated * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and **** for $p < 0.0001$.

Nuclear Magnetic Resonance. To probe the binding properties of MJ040 with A β 42, we used ^1H - ^{15}N HSQC experiments in solution NMR spectroscopy. Recombinant ^{15}N -labelled A β 42 peptides with ammonium acetate counterions were obtained from AlexoTech (Umea, Sweden) and handled on ice at all times. The peptide powder was solubilized in 10 mM NaOH at concentrations of 5 mg/mL and stored at -80°C until required. To prepare NMR samples, A β 42 stock solutions were diluted into sodium phosphate buffer (50 mM Na 2HPO_4 / Na H_2PO_4) to give a final peptide concentration of 100 μ M and a pH of 7.5. NMR experiments were carried out at 5°C on an NMR spectrometer operating at the ^1H frequency of 700 MHz and equipped with triple resonance HCN cryo-probe. The ^1H - ^{15}N HSQC experiments were recorded using a data matrix consisting of 2048 (t_2 , ^1H) $\times$ 140 (t_1 , ^{15}N) complex points. Assignments of the resonances in ^1H - ^{15}N -HSQC spectra of A β 42 were derived from our previous study.⁶⁶

Modelling. We utilised a set of structures of the A β 42 peptide selected from clusters of conformations within an ensemble generated using molecular dynamics simulations. Using these clusters, we identified the binding 'hot spots' for MJ040 on the monomeric form of the A β 42 peptide following our published protocol⁶⁷ based

on the application of the FRED program.⁶⁸ In order to inform the selection of bound states from the docked structures, we utilised the experimental observations providing the probability of contacts between MJ040 and Abeta42, as derived from the NMR measurements. The results showed that transient hydrophobic clefts are formed in the C-terminal region of the peptide, providing hot-spots for the binding of the hydrophobic parts of MJ040, with hydrophilic groups of the molecule pointing toward the solvent.

Data Availability. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References

60. Qin D, Xia Y, Whitesides GM. Soft lithography for micro- and nanoscale patterning. *Nat Protoc* 2010, **5**(3): 491-502.
61. Xia Y, Whitesides GM. Soft Lithography. *Angew Chem Int Ed Engl* 1998, **37**(5): 550-575.
62. Ranjit S, Malacrida L, Jameson DM, Gratton E. Fit-free analysis of fluorescence lifetime imaging data using the phasor approach. *Nat Protoc* 2018, **13**(9): 1979-2004.
63. Wu H, Zhang F, Williamson N, Jian J, Zhang L, Liang Z, *et al.* Effects of secondary metabolite extract from *Phomopsis occulta* on beta-amyloid aggregation. *PLoS One* 2014, **9**(10): e109438.
64. Warren SC, Margineanu A, Alibhai D, Kelly DJ, Talbot C, Alexandrov Y, *et al.* Rapid global fitting of large fluorescence lifetime imaging microscopy datasets. *PLoS One* 2013, **8**(8): e70687.
65. Stiernagle T. Maintenance of *C. elegans*. *WormBook* 2006: 1-11.
66. Newby FN, De Simone A, Yagi-Utsumi M, Salvatella X, Dobson CM, Vendruscolo M. Structure-Free Validation of Residual Dipolar Coupling and Paramagnetic Relaxation Enhancement Measurements of Disordered Proteins. *Biochemistry* 2015, **54**(46): 6876-6886.
67. Zhu M, De Simone A, Schenk D, Toth G, Dobson CM, Vendruscolo M. Identification of small-molecule binding pockets in the soluble monomeric form of the Abeta42 peptide. *J Chem Phys* 2013, **139**(3): 035101.
68. McGann M. FRED pose prediction and virtual screening accuracy. *J Chem Inf Model* 2011, **51**(3): 578-596.
69. Fusco G, Sanz-Hernandez M, Ruggeri FS, Vendruscolo M, Dobson CM, De Simone A. Molecular determinants of the interaction of EGCG with ordered and disordered proteins. *Biopolymers* 2018, **109**(10): e23117.

763 Acknowledgements

764 The authors would like to acknowledge Dr Kaveh Ashrafi and Jason Liu for generating the *C. elegans* strain,
765 Anna Lena Ellermann for help with preparation of figures, Dr Tessa Sinnige for help with *C. elegans* cultures,
766 and thank Dr Della David and Prof. Michele Vendruscolo for helpful discussions. S.C. acknowledges a
767 studentship from the Biotechnology and Biological Sciences Research Council (BBSRC) and support from
768 the Cambridge Trust. FH holds H2020 ERC Advanced (695669) and PoC (665631) awards. G.S.K.S.
769 acknowledges funding from the Wellcome Trust (065807/Z/01/Z) (203249/Z/16/Z), the UK Medical
770 Research Council (MRC) (MR/K02292X/1), Alzheimer Research UK (ARUK) (ARUK-PG013-14), the
771 Michael J Fox Foundation (16238) and Infinitus China Ltd.

772

773 Author contributions

774 S.C., F.G., L.v.V., D.R.S., F.H. and G.S.K.S. designed research; S.C., F.G., M.J., L.v.V., S.W.-V., C.P., G.F.
775 and A.D.S. performed research; S.C., L.v.V., F.G., C.M., D.D, C.F.K., D.R.S, F. H. and G.S.K.S analysed
776 data; and S.C., L.v.V., D.R.S, F.H. and G.S.K.S wrote the paper with comments and input from all authors.

777

778 Competing financial interests

779 The authors declare no conflicts of interest.

780

781 Additional information

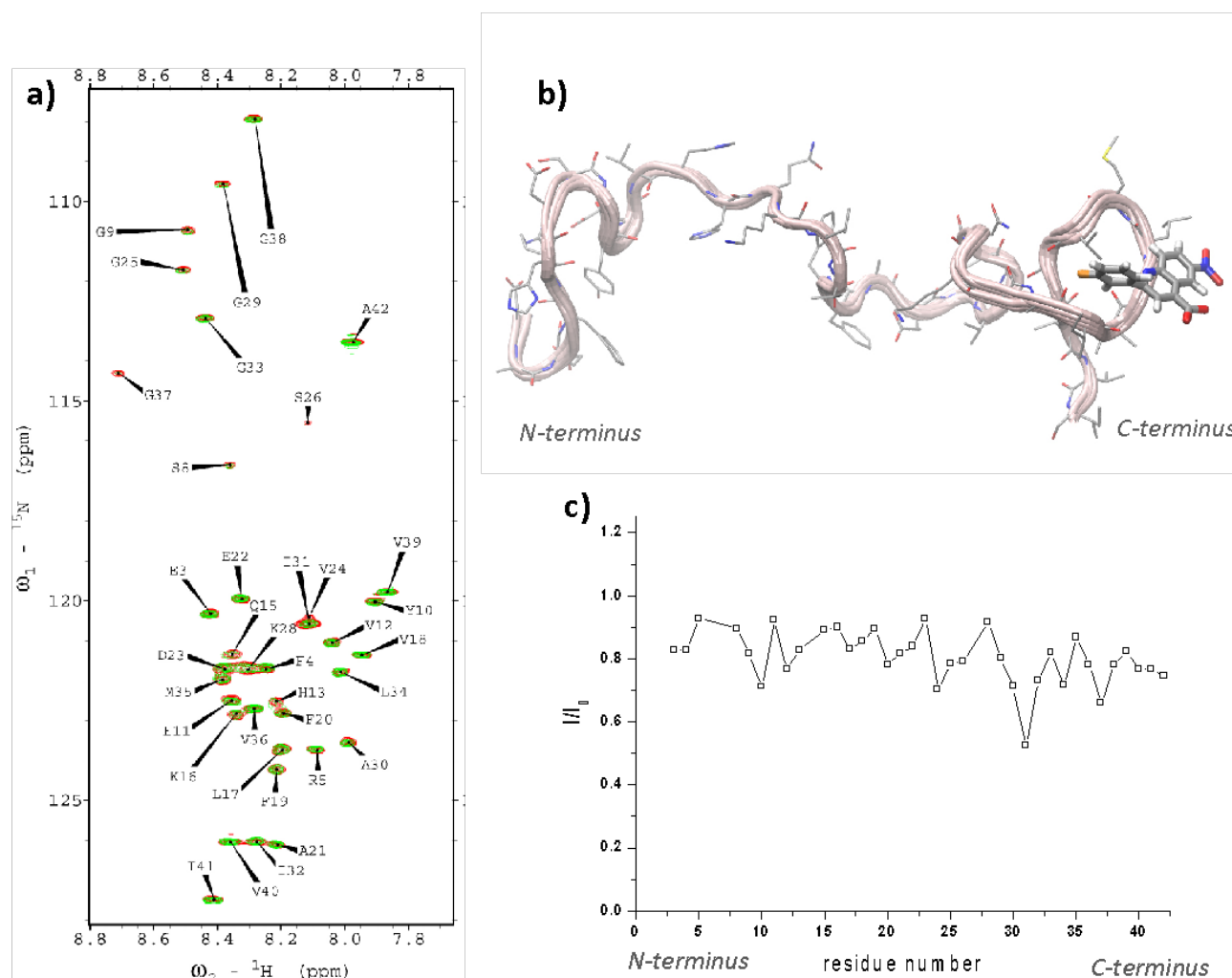
782 **Supplementary information** The online version contains supplementary material available at
783 <https://doi.org/...> and supplementary videos 1-3 showing details of device operation.

784 **Correspondence and requests for materials** should be addressed to Florian Hollfelder, Gabriele S Kaminski
785 Schierle or David Spring.

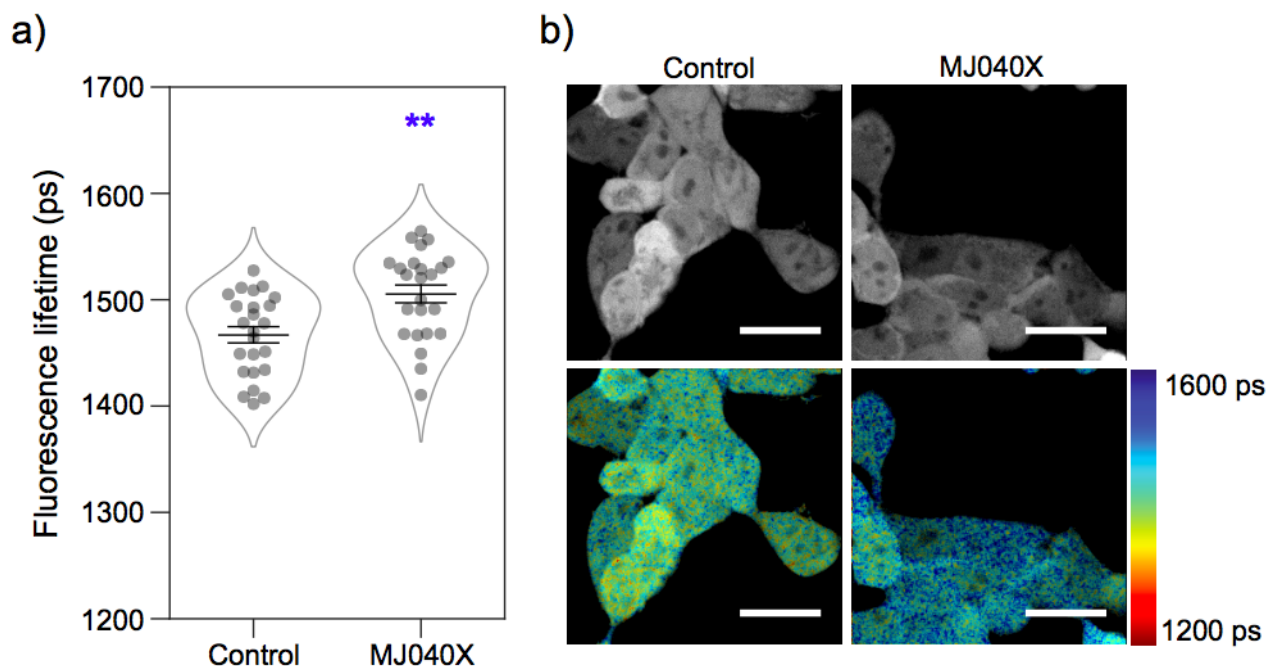
786 **Peer review information** *Nature Chemistry* thanks the anonymous reviewers for their contribution to the
787 peer review of this work.

788 **Reprints and permissions information** are available at www.nature.com/reprints.

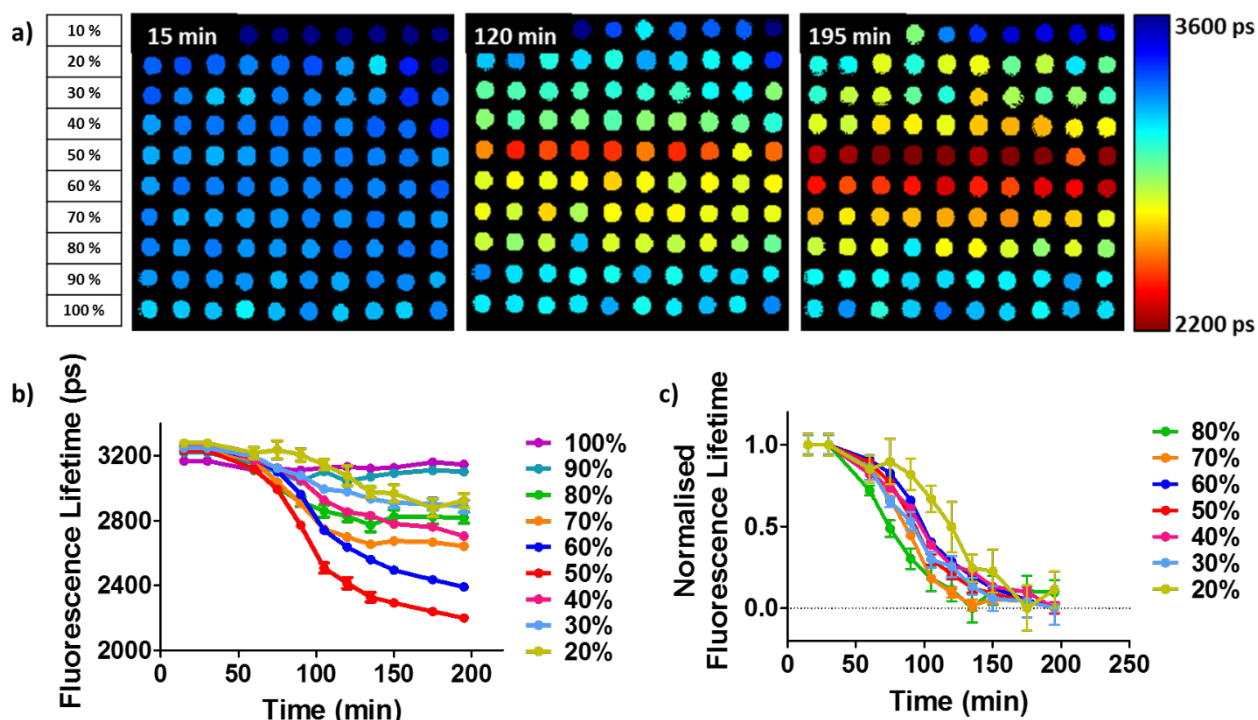
789



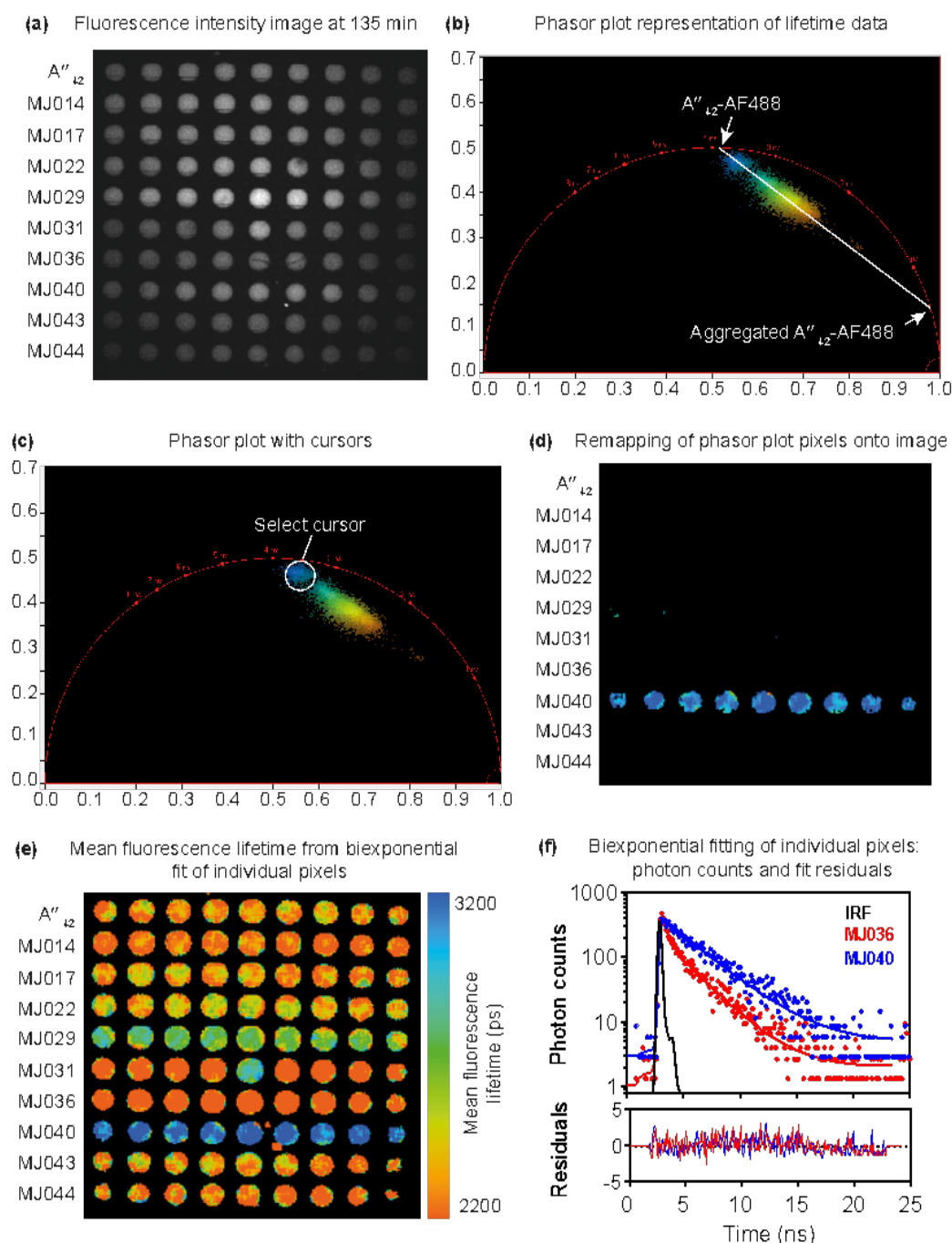
Extended Data Figure 1: NMR analysis and modelling reveal the binding site of MJ040 in the C-terminal of monomeric A β_{42} . a) ^1H - ^{15}N -HSQC spectrum of A β_{42} (100 μM) measured (50 mM phosphate buffer, pH 7.5, 2% DMSO) in the presence (green) or absence (red) of MJ040X (500 μM). Both experimental conditions included 2% DMSO. The spectra were measured at 278K using an NMR spectrometer operating at the ^1H frequency of 700 MHz. No significant chemical shift changes were observed upon addition of MJ040X to A β_{42} . However, the interaction with the molecule induced *broadening* of the resonances, with strongest effects in I/I_0 found in the C-terminal region (specifically in residues E31 and G37). Assignments of the resonances in ^1H - ^{15}N -HSQC spectra of A β_{42} were derived from a previous study.⁶⁹ b) Modelling of the interaction between MJ040X and A β_{42} conformations using an approach to identify the binding modes of small molecules with this protein⁶⁵. The programme FRED⁶⁸ was used to perform the docking of the molecule in these hot spots. c) Variation of signal intensity over the sequence of A β_{42} in the presence of MJ040X (intensity of the green resonances (with MJ040X)/intensity of the background A β_{42} resonance in a).



Extended Data Figure 2: MJ040X treatment increases fluorescence lifetime of mCherry-A β ₄₂ HEK cells. Intensity-averaged fluorescence lifetimes of mCherry-A β ₄₂ cells treated with DMSO (control) or MJ040X (2.5 μ M) for 48 h. The intensity-averaged fluorescence lifetimes are 1467 ± 8 ps and 1505 ± 8 ps, for the control and MJ040X-treated cells, respectively, confirming that MJ040X does inhibit the aggregation of A β ₄₂ inside the cell. The diagram displays mean fluorescence lifetime $\pm$ SEM. Statistical analysis was performed using an unpaired t-test, 3 biological repeats ** = $p < 0.01$. Scale bar = 20 μ m.



Extended Data Figure 3: Effect of labelling density on rate of peptide aggregation and change in fluorescence lifetime. **a)** Labelling density gradient in microfluidic chip. Each row contains 10 droplets of increasing labelled A β_{42} (10 μ M). **b)** Aggregation profiles of A β_{42} at different labelling densities. 50% labelled shows the largest dynamic range. **c)** Normalised aggregation profiles at different labelling densities. Aggregation curves from 90% and 100% labelled A β_{42} are omitted due to negligible aggregation. Plots show mean $\pm$ SEM, n = 10.



825

826 **Extended Data Figure 4: Detailed analysis of compound collection screening with a phasor plot and**
827 **biexponential fit.** (a) Raw fluorescence intensity image of nine droplet replicates containing peptide and test
828 compounds at the 135-minute time point. (b) Phasor plot analysis of the lifetime data from the entire
829 nanoFLIM image. This is an unbiased analysis approach, which does not rely on a fitting model of exponential
830 decay data. Native unaggregated $A\beta_{42}$ tagged with AF488 lies in the universal circle near 4 ns and fully
831 aggregated $A\beta_{42}$ -AF488 has a very short (quenched) fluorescence lifetime close to 1 ns. All droplets contain
832 a mixture of these two species in different proportions. (c) The phasor plot shows a bimodal distribution in
833 which a small subgroup of points (selected with the cursor) are closer to the native, unaggregated $A\beta_{42}$ species
834 than the rest of the points. (d) Points selected by the cursor in (c) with the least amount of aggregated $A\beta_{42}$
835 correspond to the droplets containing MJ040, showing the effectiveness of the compound in inhibiting

aggregation. (e) The phasor plot reveals that decay curves in image (a) are biexponential as they lie within the universal circle between two pure single exponential species. Standard biexponential fitting to decay curves are performed to extract quantitative lifetime parameters. The mean fluorescence lifetime is high for droplets containing MJ040, which shows that A β ₄₂ aggregation is inhibited in these droplets. (f) Typical fluorescence decay curves (with a comparison between MJ036 and MJ040), biexponential fits to the raw data, and the fit residuals for individual pixels of the FLIM image. The instrument response function (IRF) for the setup is also plotted.