Supplementary Material for:
Probing supramolecular protein assembly using covalently attached fluorescent molecular rotors

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Table of Contents

1) Additional spectroscopic and imaging data
   Figure S1 Molecular structure of DiSC2(3), sulfo-Cy3 and BODIPY-C10 2
   Figure S2 Absorption and emission spectra of sulfo-Cy3-HEWL and BODIPY-HEWL 2
   Figure S3 MALDI-ToF spectra of sulfo-Cy3-HEWL conjugates 3
   Figure S4 Phasor analysis of aggregation reactions monitored by sulfo-Cy3-HEWL 3
   Figure S5 Phasor analysis of sulfo-Cy3-NHS decays during the reaction with HEWL 4
   Figure S6 Mean fluorescence lifetime of sulfo-Cy3-HEWL in sucrose solutions 5
   Figure S7 Phasor analysis of HEWL aggregation monitored by freely added DiSC2(3) 6
   Figure S8 The disruption of blood clots after the application of TPA 6
   Figure S9 Phasors of sulfo-Cy3-fibrin conjugates in human and artificial clots 7
   Figure S10 Phasor analysis during assembly of various rotor-protein systems 8
   Figure S11 MALDI-ToF spectra of BODIPY-HEWL conjugates 9
   Figure S12 Monitoring lysozyme aggregation using BODIPY-HEWL 10
   Figure S13 Phasor clouds from FLIM images of BODIPY-HEWL during aggregation 11
   Figure S14 BODIPY-C10 in cells 12
   Figure S15 Electrospray mass spectrum of BODIPY-Aβ conjugates 13
   Figure S16 Electron microscopy images of Aβ aggregates 14
   Figure S17 Tyrosine fluorescence intensity during Aβ and BODIPY- Aβ aggregation 14
   Figure S18 Thioflavin T fluorescence intensity during Aβ aggregation 15
   Figure S19 Brightfield images of SH-SY5Y cells upon incubation with BODIPY-Aβ 15

2) Materials and methods

3) Synthesis and characterization of BODIPY-NHS
   Figure S20 1H NMR spectrum of 2 22
   Figure S21 13C NMR spectrum of 2 25
   Figure S22 1H NMR spectrum of BODIPY-COOH 26
   Figure S23 13C NMR spectrum of BODIPY-COOH 27
   Figure S24 ESI+ mass spectrum of BODIPY-COOH 28
   Figure S25 1H NMR spectrum of BODIPY-NHS 29
   Figure S26 13C NMR spectrum of BODIPY-NHS 30
   Figure S27 ESI+ mass spectrum of BODIPY-NHS 31

4) References

1
1 Additional spectroscopic and imaging data

**Figure S1.** Molecular structure of DiSC$_2$(3) (a), sulfo-Cy3 (b) and BODIPY-C10 (c). Note, in this study sulfo-Cy3-NHS was used with R= CH$_3$.

**Figure S2.** Absorption (dashed) and emission (solid) spectra of sulfo-Cy3-HEWL (orange) and BODIPY-HEWL (blue). The peak absorption and emission intensities were observed at 552/566 nm for sulfo-Cy3-HEWL and at 497/511 nm for BODIPY-HEWL. The absorption and emission spectra of sulfo-Cy3-HEWL and BODIPY-HEWL show close correspondence to the spectra of the unconjugated Cy3-based and BODIPY-based dyes.
Figure S3. MALDI-ToF spectra of sulfo-Cy3-HEWL conjugates. The peak at 14382.3 Da belongs to the unlabelled protein and the remaining two peaks belong to proteins labelled to a different degree, with the mass difference corresponding to the mass of the sulfo-Cy3 dye (598.74 Da). The presence of multiple peaks in addition to the unlabelled protein demonstrate that although a low pH was used to preferentially target the N-terminal amino group, some of the dye molecules additionally reacted with a different site on the protein.

Figure S4. Phasor analysis of two different aggregation reactions monitored by sulfo-Cy3-HEWL. The conjugation reactions to produce sulfo-Cy3-HEWL were performed on two different days, yet show a very similar trend.
Figure S5. Monitoring conjugation of sulfo-Cy3-NHS to HEWL. Phasor analysis of the fluorescence decays of sulfo-Cy3 acquired during the conjugation reaction. The arrow shows the direction of the trend. The reaction was monitored by fluorescence for 6 hours; it was apparent that after 2 hours the reaction was already near completion, as the 2 hours phasor (red) is close to the final 6 hours phasor (orange).
**Figure S6. Dependence of sulfo-Cy3-HEWL mean fluorescence lifetime on the viscosity of sucrose solutions at various temperatures.** The phasors of fluorescence decays recorded in aqueous sucrose solutions of various % sucrose (w/w) 30% (green), 50% (red), and 70% (blue) at a variable temperature between 0-60°C. The phasors of fluorescence decays recorded in sucrose solutions of various concentrations at a fixed temperature of 60°C is shown in black, with a fitted linear function at low viscosities only.

We have recorded time resolved decays of sulfo-Cy3-HEWL in various sucrose/water solutions at variable temperatures and plotted them in the phasor space, Figure 2b (main text). In addition we have also performed exponential analysis of the individual decays and calculated the mean fluorescence lifetime, Figure S6. While the data overlap well in the phasor space (Figure 2b, main text), exponential fitting revealed a dependence of the position of the mean fluorescence lifetime on both the temperature and the viscosity of the mixture (Figure S6). Such behaviour is not unusual and was reported previously for molecular rotors.1

Thus, for calibration purposes, in order to remove the bias associated with temperature variations in the calibration set, we have recorded time resolved fluorescence decays of sulfo-Cy3-HEWL in sucrose/water mixtures at a fixed temperature of 60°C, the intended temperature of the aggregation reaction. The time resolved decays are shown in Figure 2a (main text) and the mean fluorescence lifetime calibration of the data is plotted in Figure S6 (black squares). From this dataset it is clear to see that an increase in viscosity causes a significant increase in the fluorescence lifetime of sulfo-Cy3-HEWL.
Figure S7. Phasor analysis of HEWL aggregation monitored by freely added DiSC\(_3\)(3) (blue) compared to sulfo-Cy3-HEWL (red). Only the centroids of phasor clouds are shown for clarity; the arrow shows the direction of the trend. The dynamic range of the conjugated dye is smaller than in the case of free dye.

Figure S8. The disruption of blood clots after the application of tissue plasminogen activator (TPA). Confocal fluorescence images of fibrinogen clots obtained at time 0, 1/2 h and 1h following the addition of the known clot-disrupting drug TPA (1.5 μg/ml) to the incubating solution. Excitation was at 1000 nm, detection was 600-700 nm. It is clear that the clot starts dissolving following the addition of the drug as monitored by the sulfo-Cy3 fluorescence. The fact that sulfo-Cy3 was covalently linked to fibrinogen was confirmed by the formation of a fluorescent fibrin mesh after blood clotting (a). Further evidence is provided by an experiment in which the fluorescent mesh containing Cy3-fibrin was disassembled due to the application a clot-dissolving drug, TPA. The effect can be clearly seen after ½-1 hour of the application of TPA (b and c, respectively).
Figure S9. Phasors corresponding to decays of sulfo-Cy3-fibrin conjugates in human blood clots compared to porcine plasma clots. In both cases, the phasor point recording after clotting lies anticlockwise from the phasor recorded before clotting, indicating a higher degree of crowding as expected.
Figure S10. Phasor analysis of lifetime evolution during aggregation in various *in vitro* rotor-protein systems studied in this work. Only the centroids of phasor clouds are shown for clarity; all trends proceed anticlockwise.

Lysozyme aggregation is monitored by BODIPY-HEWL (red) and sulfo-Cy3-HEWL (blue), Aβ(1-42) aggregation by BODIPY- Aβ(1-42) (green) and fibrin clotting by sulfo-Cy3-fibrinogen (orange). Clearly, the fluorescence lifetime sensitivity to microviscosity is very dependent on the specific rotor-protein system. Sulfo-Cy3-HEWL has the biggest dynamic range during the assembly of HEWL into fibrils, but the same rotor (sulfo-Cy3) in the sulfo-Cy3-fibrinogen system during clotting has the smallest dynamic range. This may be due to Cy3 rotation being largely hindered from the start, upon covalent attachment (as indicated by its initial high fluorescence lifetime); however, we believe that the rotor may not be ideally located on a suitable residue(s) on the protein, in order to strongly sense the crowding associated with fibrin mesh assembly.

All phasors are located within the universal circle, indicating that the rotors are experiencing an inhomogeneous environment, from the very start (i.e. conjugation) through to fibril formation. While the trends for sulfo-Cy3-HEWL (blue), BODIPY- Aβ(1-42) (green) and sulfo-Cy3-fibrinogen (orange) appear to proceed in a linear fashion in the phasor space (i.e. possibly involve the conversion between two species), the trend for BODIPY-HEWL (red) is clearly biphasic, see Figure S12 for more details.
Monitoring lysozyme aggregation using BODIPY-HEWL

We have tested whether it is possible to monitor protein aggregation with a hydrophobic BODIPY-based molecular rotor, previously used for a range of biological viscosity studies in lipid-based membranes.\textsuperscript{2–10} BODIPY-C10 (Figure S1c, Supplementary Material) displays a large dynamic range of lifetimes in the viscosity range between 20-5000 cP and is characterised by monoexponential time resolved fluorescence decays in homogeneous media,\textsuperscript{8,9} and was shown to measure viscosity in temperature-independent manner.\textsuperscript{1} However, the use of BODIPY-based rotors in water-based systems was precluded due to a poor solubility of the dye in an aqueous media.

We have synthesised BODIPY-NHS (Figure 1b, see Section 3 for detailed synthetic procedures and characterisation) and performed a conjugation to HEWL under conditions similar to sulfo-Cy3-NHS. From the MS data (Figure S1, Supplementary Material) it is apparent that each BODIPY-HEWL molecule was labelled with a single dye molecule, although, similarly to sulfo-Cy3-NHS, the labelling did not necessarily occur exclusively at an N-terminus position. The absorption and emission spectra of BODIPY-HEWL (Figure S2, Supplementary Material) show close correspondence to the spectra of the structurally similar unconjugated BODIPY-C10 with peak absorption and emission at 497 nm and 511 nm, respectively.

\textbf{Figure S11. MALDI-ToF spectrum of BODIPY-HEWL conjugates.} The peak at 14501 Da belongs to the unlabelled protein, the peak at 14854.9 Da belongs to single-labelled protein, and the mass difference equals to the mass of the BODIPY dye (353.16 Da).
**Figure S12. Monitoring lysozyme aggregation using BODIPY-HEWL.**

**a)** Fluorescence decays of BODIPY-HEWL in 75% glycerol-water solution at 10-60°C giving the viscosity range 0.9-107.2 cP; **b)** The evolution of centroids of phasor clouds measured during aggregation of HEWL (red); the arrow shows the direction of the trend. Phasor analysis of the free BODIPY-C10 decays recorded in methanol-glycerol solutions between 50-900 cP (grey) is also shown. The phasor of the BODIPY-C10 decay measured at 77K in the glassy matrix of ethanol-methanol (η=2x1014 cP; τ = 6 ns) is shown in green.

The viscosity sensitivity of the BODIPY-HEWL conjugates was confirmed by measuring the time resolved fluorescence decays in water/glycerol mixtures of varied viscosities (Figure S12a). It is clear to see that increasing viscosity resulted in increasing fluorescence lifetime of BODIPY-HEWL, indicating that the viscosity sensitivity of BODIPY was retained even after conjugation.

Lysozyme aggregation was induced in the same manner as for Cy3-HEWL and fluorescence decays traces of BODIPY-HEWL were recorded during the course of aggregation, until fibril formation was apparent and there was no further change in the time resolved traces recorded from the mixture. As expected, the mean fluorescence lifetime increased throughout aggregation, proving that BODIPY conjugates are effective for monitoring the aggregation process (Figure 5b, main text).

The time resolved decays recorded for free BODIPY-C10 in methanol/glycerol mixtures of different viscosities can be fitted with a monoexponential function and therefore fall onto the universal circle in phasor space (Figure 11b, grey). By contrast, the BODIPY-HEWL conjugate shows a substantial deviation from monoexponentiality and, in addition, the dynamic range of lifetimes is smaller for the conjugate than for the free dye, likely due to an increased rotation barrier caused by conjugation. While the more complex shape of decays can cause difficulties in analysis, it does not preclude the monitoring of the aggregation using BODIPY-HEWL.

To ascertain that the high fluorescence lifetime observed for BODIPY-HEWL during aggregation is not due to an artefact we have measured the time resolved fluorescence decay of BODIPY-C10 in the glassy matrix of ethanol-methanol at 77K with a known dynamic viscosity of η=2x1014 cP, where we expect the rotor to be completely immobile. This decay can be fitted with a monoexponential function and is characterised by a lifetime of 6 ns, which lies anticlockwise from the extrapolated point C’ of BODIPY-HEWL aggregation. Thus we confirm that BODIPY is not completely immobilised in the final fibrillar state of HEWL, since it displays lower lifetime than in a solid matrix at 77K and thus it is in principle capable of sensing even higher microscopic viscosity than that achieved during the aggregation of HEWL.
Figure S13. The evolution of phasor clouds from FLIM of BODIPY-HEWL obtained during HEWL aggregation. a) The phasor clouds obtained during the first stage of the aggregation A to B; b) the phasor clouds obtained during the second stage of aggregation B to C; c) only centroids of the decays obtained during the aggregation of HEWL are shown for clarity.

Two stages can be distinguished in the evolution of phasors during aggregation, which become apparent from the direct analysis of phasor clouds (Figure S13). In the first minutes after seeding the phasor clouds proceed along a line (A to B), which can be interpreted as a transition between two states of different microviscosity (or crowding) experienced by BODIPY (with viscosities characterised by points A’ and B’ on the universal circle). After approximately 6 minutes, the trend changes and the phasor clouds start proceeding along the second line (B to C), which again in the phasor space indicates an interconversion between two species.

The transition towards a third state characterised by the viscosity value corresponding to C’ can be explained by the appearance of a new species of aggregate. If extrapolated towards the universal circle (C’), it is apparent that the trend is evolving towards a region of very high fluorescence lifetime, which, nevertheless, is less than the maximum achievable lifetime of BODIPY rotor recorded in a solid glass matrix at 77K, 6 ns (point D, Figure S13 c).
Figure S14. BODIPY-C10 in live cells. Confocal fluorescence image (114 × 114 μm) obtained following 488 nm excitation, 550 (±25) nm fluorescence detection from SK-OV-3 cells incubated with 1 μM solution of BODIPY-C10 for 10 minutes. The dye does not directly interact with the plasma membrane of the cells and shows efficient endocytosis, even at early incubation times (0-10 min).

Figure S15. Electrospray mass spectrum of BODIPY-Αβ conjugates. (a) High-mass spectrum. The peak at 4514 Da belongs to the unlabelled Αβ(1-42) and the peak at 4865 Da belongs to single-labelled protein, the mass difference corresponds to the mass of BODIPY without the NHS moiety (M₀ = 354.16); (b) Low-mass spectrum. The peak at 470 Da demonstrates the presence of unreacted BODIPY-NHS even after two-fold gel filtration.
Figure S16. Transmission electron microscopy (TEM) images of 10 μM (a) unlabelled Aβ(1-42) during the course of aggregation and (b) BODIPY- Aβ(1-42) during the course of aggregation at time 0, 1, 2, 3, 4 and 24 hours after the start of aggregation. Scale bars = 200 nm.
Unlabelled Aβ(1-42) was allowed to aggregate at conditions identical to fluorescently labelled BODIPY- Aβ(1-42) mixture. According to the TEM images, the ‘time 0’ Aβ(1-42) samples are composed of small spherical species (a), which is also the case for the labelled BODIPY- Aβ(1-42) sample (b).

For the first 2h both samples (a) and (b) are mainly comprised of oligomeric species, although a very small number of short fibrils are already present at the 1 hr time point.

The first Aβ(1-42) fibrils seem to appear slightly later than the labelled BODIPY-Aβ(1-42) fibrils. However, this is likely due to the fact that BODIPY-Aβ(1-42), has already started aggregating during a conjugation reaction, which was allowed to run for 1 hour.

After ca 4 hours the formation of first long fibrils (>1 µm) can be clearly seen in both unlabelled and labelled Aβ(1-42) samples.

In both unlabelled Aβ(1-42) (a) and labelled BODIPY-Aβ(1-42) (b), fibril cluster formation is apparent at the 24hr time point, with similar fibril morphology.

The morphology of the fibrils and kinetics of aggregation appear to be very similar for unlabelled Aβ(1-42) and labelled BODIPY-Aβ(1-42) samples, which gives us confidence that the labelling did not disrupt the secondary structure and aggregation properties of Aβ(1-42).

![Figure S17](image_url)

**Figure S17.** The evolution of the fluorescence intensity of tyrosine measured at 303 nm over the period of 48 hours during the aggregation of wild type Aβ(1-42) (black) and BODIPY-Aβ(1-42) (red). The intensity decrease trend is very similar for both unlabelled and BODIPY-labelled Aβ(1-42) over the course of 48 hours. As Aβ(1-42) aggregates, the tyrosine residue at position 10 becomes buried. The change in tyrosine environment can be followed using fluorescence at 303 nm.13
**Figure S18.** (a) The increase of Thioflavin T fluorescence intensity measured at 480 nm over the course of Aβ(1-42) aggregation; (b) the fluorescence lifetime of BODIPY-Aβ(1-42) during aggregation.

**Figure S19.** Evidence of toxic effects of BODIPY-Aβ(1-42) aggregates on SH-SYSY cells. The brightfield images were taken at 35 minutes (a), 5 hours (b), and 22 hours (c) upon incubation with 10 µM Aβ(1-42). At 5 hours there is evidence of cells starting to round up and by 22 hours cell death is widespread. Scale bars = 24 µm.
2. Materials and methods

Conjugation of NHS esters to lysozyme

Hen egg white lysozyme (HEWL, 14307 Da, 129 amino acids, product code L6786) and all solvents (spectroscopic grade) were purchased from Sigma-Aldrich; BODIPY-NHS was synthesised by adaptation of literature procedures as detailed in the Supplementary Material; sulfo-Cy3-NHS was kindly donated by Cyandye.com (Moscow, Russia).

For the conjugation reactions with sulfo-Cy3-NHS and BODIPY-NHS, HEWL was dissolved at 1 mg/mL (70 μM) in 10 mM phosphate buffer at pH 6.4. Stock solutions of sulfo-Cy3-NHS and BODIPY-NHS were prepared by dissolving the dyes in anhydrous DMSO to a concentration of 68 mM and 19 mM, respectively. Small aliquots of dye stock solutions were added to the protein solution to obtain 350 μM of the dye. The final solution consisting of a 5:1 molar ratio of NHS ester to protein was heated at 30°C for 16 hours with constant stirring to enable conjugation. Following the conjugation reaction, the solution was centrifuged for 2 minutes at 10000 rpm; the supernatant was recovered and the pellet containing HEWL aggregates was discarded. Excess free dye was removed by 7 MWCO Zeba spin desalting columns (Thermo Scientific) pre-equilibrated with a 50 mM HCl, 100 mM NaCl solution.

Absorption spectra were measured to determine the ratio of labelled to unlabelled protein. In the case of sulfo-Cy3 conjugates, the ratio of labelled to total protein obtained from absorption spectrometry was 0.36; the solution contained 5.6 μM sulfo-Cy3-HEWL and 15.5 μM total protein. As for BODIPY conjugates, the labelling efficiency was lower; the solution contained 3.6 μM BODIPY-HEWL and 14.4 μM total protein.

The mixtures were further analysed by MALDI-Tof mass spectrometry (Waters Corporation, Micromass MALDI micro MX) to confirm the presence of conjugates. Analyte samples were mixed 1:1 with a 10 mg/mL sinapinic acid matrix solution (50/50, acetonitrile/0.1% TFA) and deposited on to a MALDI target pre-coated with a thin layer of sinapinic acid. The spectrometer was operating in positive ion, linear-mode, and 100 laser shots per spot were combined. The results of MALDI-Tof mass spectrometry are discussed in the relevant sections below.

In the case of sulfo-Cy3-NHS conjugation to HEWL fluorescence lifetime imaging (FLIM) was used to monitor the aggregation process as described below (in ‘Time resolved measurements’ section).
Protein aggregation

A fresh solution of HEWL was prepared at 4 mg/mL with 50 mM HCl, 100 mM NaCl. The monomer solution was passed through a syringe driven filter of 0.22 μm pore size. A fraction of sulfo-Cy3-HEWL conjugates prepared as described above was added to the fresh solution of unlabelled proteins. Aggregation was triggered by seeding with 1 % v/v sonicated preformed fibrils. The seeds were prepared by incubating a solution of 60 mg/mL HEWL in 50 mM HCl, 200 mM NaCl at 65°C for 16 hours and subsequent sonication to obtain short seed fibrils. The pH of the mixture was less than 2 for all experiments. Fluorescence lifetime imaging (FLIM) was used to monitor the aggregation process as described below (in ‘Time resolved measurements’ section).

Viscosity calibration of rotor-HEWL conjugates

Sucrose-water mixtures of 40-75% w/w sucrose at variable temperature were used for producing a lifetime calibration set for sulfo-Cy3-HEWL as a function of viscosity; values of viscosity for different sucrose solutions were taken from literature. In the case of BODIPY-HEWL conjugates, water-glycerol mixtures were used for calibration and viscosity values of these mixtures were calculated using the published empirical formula. Time resolved fluorescence measurements was used to produce lifetime/viscosity calibrations of these mixtures as described below (in ‘Time resolved measurements’ section).

Blood clotting

For whole blood clot experiments, fresh venous blood was acquired by venepuncture from a consenting adult volunteer. 125 µl of anticoagulant was added per millilitre of blood and the aliquots were stored at 4°C until required, within 48h.

For plasma clot experiments, fresh frozen porcine plasma was prepared. Whole blood was collected from the aorta of a female Large White x Landrace pig and anticoagulated with 125µL anticoagulant per millilitre blood. The blood was aliquotted in 50 mL centrifuge tubes and immediately spun at 4,400rpm in a 15cm radius centrifuge for 20 minutes at room temperature. The supernatant was collected and stored at -80°C for up to three years.

Calcium chloride, thrombin from bovine plasma and fibrinogen from bovine plasma (75% clottable) were from Sigma Aldrich; Human Factor XIII was from Enzyme Research Laboratories (Swansea, UK). CPDA-1 anticoagulant was obtained from Fenwall Inc. (Lake Zurich, IL, USA). TNE buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4) was prepared according to Collet et al.
In order to prepare sulfo-Cy3-fibrinogen conjugates, fibrinogen was dissolved at 8.8 mg/mL in 10 mM phosphate buffer at pH 8.1 and kept at 37°C; 5 μL of sulfo-Cy3-NHS stock solution in anhydrous DMSO (68 mM) was added to 0.845 mL of the fibrinogen solution to create a 20:1 molar excess of sulfo-Cy3-NHS to fibrinogen (final sulfo-Cy3-NHS concentration 0.4 mM). The mixture was heated at 30°C for 15 hours with constant magnetic stirring. Next the solution was centrifuged for 2 minutes at 10000 rpm; the supernatant was recovered and the pellet containing precipitate was discarded. Excess free dye was removed by 5 mL 7 MWCO Zeba spin desalting columns (Thermo Scientific).

Samples were prepared by aliquoting 400 μL of whole blood or porcine plasma into an 8-well chamber slide (LabTekII Chamber Coverglass) and mixing in 40 μL of the Cy3-fibrinogen conjugate solution. Clotting was achieved by adding 40 μmol of calcium chloride with a solution of thrombin in TNE buffer, for a final concentration of 0.61 NIH units / mL.

**Beta amyloid aggregation in live cells**

SH-SY5Y cells obtained from ECACC (European Collection of Cell Cultures) were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) which was supplemented with 10% Foetal Calf Serum. Cells were grown in T25 flasks for 4-5 days before being passaged after reaching 80% confluency; cells were not used before passage 19. For fluorescence imaging cells were plated on ibiTreat 8 well μ-slides (Ibidi) in 300 μL of culture media at a plating density of 40,000 cells per well and allowed to grow to confluence for 24 hours prior to adding Aβ1-42.

Aβ(1-42) was purchased from rPeptide in vials containing 1mg of protein film, which was resuspended in HFIP (Sigma-Aldrich), aliquoted and dried under nitrogen flow into films containing 0.2 mg Aβ(1-42), which were stored at -20°C. For an aggregation experiment, one 0.2 mg Aβ(1-42) film was resuspended in HFIP, sonicated for 5 minutes at room temperature, vortexed for 1 minute and solvent evaporated under nitrogen flow. The resulting film was resuspended in 200 μL of anhydrous DMSO, vortexed and stirred for 1 minute.

For the conjugation reaction, BODIPY-NHS stock solution was prepared in anhydrous DMSO. Under constant stirring, 20 μL of 10 mM bicarbonate buffer (pH 8.3) was added to the Aβ(1-42) solution in DMSO and 5 μL of the stock solution of BODIPY-NHS was added to provide a dye:protein ratio of 1:2. The mixture was covered with foil and stirred for 1 hour. Two rounds of gel filtration were performed on of BODIPY-Aβ(1-42) conjugates after the conjugation reaction was completed. Gel filtration in DMSO was done in order to remove free dye from the mixture, followed immediately by buffer exchange of DMSO to 10 mM HEPES in a second gel
filtration column. Absorption spectra were recorded immediately to determine the concentration of Aβ(1-42), which was then diluted to 50 μM. 50 μL of this solution was added to wells containing SH-SY5Y cells in 200 μL of phenol-red free media, such that the final concentration of Aβ(1-42) in each well was 10 μM.

**Transmission electron microscopy**

The solution of 10 μM Aβ(1-42) was prepared as described above and 4 μl aliquots were placed on Formvar/carbon coated 400 mesh copper grids (Agar Scientific) at the progressive time points to monitor aggregation. The Aβ(1-42) was allowed to adsorb onto the grid for two minutes before being blotted dry. The grid was then washed with 4 μl milliQ-filtered water and again blotted dry before being negatively stained with 4 μl of 2% w/v uranyl acetate for two minutes. The uranyl acetate was blotted and then left to air dry before being imaged using a JEM-1400Plus 120 kV transmission electron microscope (JEOL, USA). All images were acquired with a GATAN One View camera.

**Absorption and fluorescence spectra**

Absorption spectra were measured using an Agilent 8453 UV-Vis spectrophotometer. Fluorescence spectra were measured using a FluoroMax4 spectrofluorimeter (Horiba Scientific) with a Xenon lamp as an excitation source. Spectra were corrected for wavelength-dependent efficiency of the light source and sensitivity of the detector. Quartz cuvettes with 1 cm path length were used in all measurements.

For the measurement of Thioflavin T fluorescence intensity, 10 μM Aβ(1-42) was allowed to assemble at 37°C in a cuvette inside the spectrometer (Horiba Scientific), fluorescence was excited at 430 nm and emission was collected at 480 nm, with both slits set to 5 nm.

For the measurement of tyrosine fluorescence intensity, 40 μM Aβ(1-42) and BODIPY- Aβ(1-42) were allowed to assemble at 37°C and an aliquot placed in a fluorescence micro-cuvette with 1 cm path length at each time point. Fluorescence emission was collected in a Varian Cary Eclipse Fluorescence Spectrophotometer, with an excitation wavelength of 280 nm and emission wavelength of 303 nm; excitation and emission slits were set at 5 nm and 10 nm, respectively. The sample compartment was kept at room temperature. Three measurements were averaged for each time point.

**Time-resolved measurements**
Time resolved fluorescence decays were collected using time-correlated single-photon counting (TCSPC) technique. A mode-locked femtosecond Ti:Sapphire laser (Coherent, Chameleon Vision II) tunable over the 680-1080 nm range (140 fs pulse duration, 80 MHz) was used as an excitation source. The excitation light was frequency doubled using a second harmonic generation (SHG) crystal (Harmonic, Coherent). The detection system consisted of a DCC-100 detector control module (Becker-Hickl), PMC-100-1 photomultiplier tube (Hamamatsu), Omni-λ 150 monochromator (LOT-Quantum Design), and a qpod cuvette holder heated by a TC 125 Peltier thermostat (Quantum Northwest). For the calibration measurements of sulfo-Cy3-HEWL, the excitation wavelength was 480 nm, the detection wavelength 580 nm and a 550 nm longpass filter was used. In the case of BODIPY-HEWL conjugates, fluorescence was excited using 470 nm wavelength, detected at 560 nm and a 550 nm longpass filter was used. Data acquisition was performed using a single-photon counting card (Becker-Hickl, SPC-830). The instrument response function (IRF) was recorded using a scattering solution (Ludox colloidal silica, Sigma-Aldrich).

Fluorescence lifetime imaging microscopy (FLIM) was performed using a confocal laser scanning microscope (Leica, SPS II) with the Ti:Sapphire laser in two-photon excitation mode operated at 960 nm. Short-pass filters of 700 or 715 nm were used. A PMC-100-1 photomultiplier tube (Hamamatsu) and a SPC-830 single-photon counting card (Becker-Hickl) were used for data acquisition. Fluorescence was collected between 525 and 700 nm. The decay acquisition time was between 30-60 seconds. Samples were placed in chamber slides (LabTekII Chamber Coverglass) mounted in a microscope chamber heated by a circulating thermostat (Lauda GmbH, E200). The IRF was obtained by measuring SHG signal from urea crystals on a glass cover slide.

**Data analysis and representation**

Multi-exponential fitting was done in SPCI software (Becker-Hickl) using the nonlinear least squares method and reconvolution algorithm for finding the best fit. Goodness of fit was judged by the $\chi^2$ value and randomness of residuals. Decay models of fluorescence for all conjugated dyes were judged to be bi-exponential and followed the equation

$$ I(t) = \sum_{i=1}^{n} \alpha_i \exp\left(-\frac{t}{\tau_i}\right) $$

(1)
where $I$ is fluorescence intensity, $t$ is time, and $\alpha_i$ are the amplitudes and $\tau_i$ the fluorescence lifetimes of the $n$ exponentially decaying components. Data was further processed with OriginPro 8.6.

Mean fluorescence lifetime was calculated according to the equation

$$\tau_{avg} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i}$$  \hspace{1cm} (2)

Phasor analysis was chosen for data representation. This method does not require nonlinear multiexponential fitting and can be performed without prior knowledge of the number of components in the decay; it is highly suitable for large data sets and the visualisation of complex processes.

In this technique, fluorescence decays collected at a single frequency are Fourier transformed and their imaginary parts are plotted against the real parts in a so-called phasor plot.\textsuperscript{12} The real ($g$) and imaginary ($s$) components are calculated as follows:

$$g(\omega) = \frac{\int_0^\infty I(t) \cos(\omega t) dt}{\int_0^\infty I(t) dt}$$  \hspace{1cm} (3)$$

$$s(\omega) = \frac{\int_0^\infty I(t) \sin(\omega t) dt}{\int_0^\infty I(t) dt}$$  \hspace{1cm} (4)

where $\omega$ is the angular repetition frequency ($2\pi \times 80$ MHz), $t$ is time and $I(t)$ is the measured fluorescence decay. The resulting vectors ($g$, $s$) are called phasors. Phasors of monoexponential decays lie on the “universal circle”, a semi-circle centred at [0.5, 0], while phasors of multi-exponential decays lie on the inside of the semi-circle. Phasors corresponding to lifetime $\tau \rightarrow 0$ would be found at [1, 0]; with increasing lifetime the phasors move anticlockwise along the universal circle.

Phasor analysis was performed using an in-house MATLAB R2012a code. In our measurements, all phasors were corrected using an instrument response function.
3. Synthesis and characterization of BODIPY-NHS

3.1 General Materials and Methods

The manipulation of all air and/or water sensitive compounds was carried out using standard inert atmosphere techniques. All chemicals were used as received from commercial sources without further purification. Anhydrous solvents were used as received from commercial sources. Analytical thin layer chromatography (TLC) was carried out on Merck® aluminium backed silica gel 60 GF254 plates and visualization when required was achieved using UV light or I₂. Flash column chromatography was performed on silica gel 60 GF254 using a positive pressure of nitrogen with the indicated solvent system. Where mixtures of solvents were used, ratios are reported by volume.

Nuclear magnetic resonance spectra were recorded on 400 MHz spectrometers at ambient probe temperature. Chemical shifts for ¹H NMR spectra are recorded in parts per million from tetramethylsilane with the solvent resonance as the internal standard (chloroform: δ = 7.27 ppm). ¹³C NMR spectra were recorded with complete proton decoupling. Chemical shifts are reported in parts per million from tetramethylsilane with the solvent resonance as the internal standard (¹³CDCl₃: 77.0 ppm). ¹⁹F NMR spectra were recorded with complete proton decoupling. Chemical shifts are reported in parts per million referenced to the standard hexafluorobenzene: −164.9 ppm. Mass spectra were carried out using ElectroSpray Ionization (ESI), and only molecular ions are reported.

3.2 Synthetic Procedures

Scheme 1. Synthesis of BODIPY-NHS: (i) tert-butyl(chloro)diphenylsilane, DMAP, Et₃N, THF, 0°C to rt, 24h, yield: 60%; (ii) neat pyrrole, TFA, rt, 1h, yield: 90%; (iii) DDQ, CH₂Cl₂, rt, 1 h and then BF₃·(OEt)₂, Et₃N, CH₂Cl₂, rt, overnight, yield: 22%; (iv) N-hydroxysuccinimide, DCC, DMAP, THF, 0°C to rt, 16h, yield: 55%.

The synthesis of BODIPY-NHS started from the 4-[(4-formylphenoxy)butanoic acid 1. It is worth noting that the direct transformation of compound 1 into rotor BODIPY-NHS was
hampered by the lack of solubility of derivative 1 in pyrrole. This problem was overcome by converting 1 into the corresponding silyl protected ester 2 in 60% yield. Therefore, BODIPY-COOH was obtained via condensation of compound 2 with an excess of pyrrole to give the corresponding dipyrromethane, which was further reacted with DDQ and followed by treatment with BF₃·OEt₂. The silyl protecting group was removed during the work-up treatment. After purification by column chromatography, BODIPY-COOH was isolated in 22% yield from aldehyde 2. Finally, BODIPY-NHS was obtained in 55% yield by coupling of the carboxylic acid group of BODIPY-COOH with N-hydroxysuccinimide in the presence of DCC.

**Compound 2.** tert-Butyl(chloro)diphenylsilane (3.6 g, 12.9 mmol) was added to a mixture of 4-(4-formylphenoxy)butanoic acid 1 (1.8 g, 8.6 mmol), DMAP (0.1 g, 0.8 mmol) and Et₃N (1.8 mL, 12.9 mmol) under N₂ atmosphere in dry THF (30 mL) at 0 °C. The reaction mixture was stirred at room temperature for 24 h, then diluted with CH₂Cl₂ (100 mL). The organic solution was washed with an aqueous solution of NaHCO₃ (2 x 100 mL, 0.5M), H₂O (100 mL), and dried over anhydrous MgSO₄ and filtered. The solvent was evaporated under vacuum and the residue was purified by flash chromatography (3:2 CH₂Cl₂:petroleum ether), Rf 0.4, to give 3 as a colorless oil. Yield: 2.3 g (60%).

**BODIPY-COOH.** A solution of 2 (2.2 g, 4.9 mmol) in freshly distilled pyrrole (10 mL, 230 mmmol) was degassed by bubbling with N₂ for 30 minutes before the addition of TFA (0.05 mL, 0.6 mmol). The mixture was stirred for 1 hour at room temperature, diluted with CH₂Cl₂ (100 mL) and then washed consecutively with H₂O (100 mL), NaHCO₃ (100 mL, 0.5M) and H₂O (100 mL). The organic extracts were dried over anhydrous MgSO₄ and filtered and evaporated. The excess pyrrole was removed using high vacuum to give the dipyrromethane as a dark viscous oil. The crude dipyrromethane was purified by flash chromatography (2:1 CH₂Cl₂:petroleum ether) to give a green viscous oil. Yield: 2.5 g (90%). The corresponding dipyrromethane (1.2 g, 2.1 mmol) was dissolved in dry CH₂Cl₂ (100 mL) and DDQ (1.5 g, 6.6 mmol) was added under N₂ atmosphere. The reaction mixture was stirred at room temperature shielded from light for 1 h. Then, Et₃N (3 mL, 21.3 mmol) was added, followed immediately by the addition of BF₃·(OEt)₂ (3 mL, 24.4 mmol) and the reaction mixture was stirred at room temperature overnight.
organic solution was washed with H₂O (100 mL), aqueous HCl (100 mL, 1 M), aqueous NaHCO₃ (100 mL, 0.5 M) and finally H₂O (100 mL), and then dried over anhydrous MgSO₄, filtered and evaporated to give a black viscous oil which was purified by column chromatography (40:1 CH₂Cl₂:methanol), Rf 0.2, to give **BODIPY-COOH** as a red-orange sticky solid. Yield: 171 mg (22%).

**1H NMR** (400 MHz, CDCl₃) δH 7.93 (br s, 2H), 7.54 (d, J = 8.5 Hz, 2H), 7.04 (d, J = 8.5 Hz, 2H), 6.97 (d, J = 4.0 Hz, 2H), 6.55 (dd, J = 4.0, 1.4 Hz, 2H), 4.14 (t, J = 6.5 Hz, 2H), 2.66 (t, J = 6.5 Hz, 2H), 2.21 (qn, J = 6.5 Hz, 2H); **13C NMR** (100 MHz, CDCl₃) δC 177.75, 161.22, 143.46, 134.83, 132.43, 131.33, 126.47, 118.29, 114.49, 66.78, 30.18, 24.24; **19F NMR** (377.5 MHz, CDCl₃) δF −148.24; **MS (ESI)** m/z 351.1327 (C₁₉H₁₇BFNO₃, [M−F]+, requires 351.1316);**BODIPY-NHS**. Dicyclohexylcarbodiimide (76 mg, 0.37 mmol) was added to a solution of **BODIPY-COOH** (137 mg, 0.37 mmol), N-hydroxysuccinimide (43 mg, 0.37 mmol) and DMAP (4.5 mg, 0.037 mmol) under N₂ atmosphere in dry THF (10 mL) at 0 °C and the mixture was stirred for 1 h. The ice-bath was removed and stirring was continued at room temperature overnight. The dicyclohexylurea was removed by filtration, and the filtrate was diluted with CH₂Cl₂ (100 mL), which was washed with an aqueous solution of HCl (100 ml, 0.05 M), brine (100 ml) and dried over anhydrous MgSO₄. The solvent was removed by rotary evaporation and the crude was purified by flash chromatography (2 CH₂Cl₂ / 1 petrol ether), Rf 0.35. Fractions were evaporated to dryness to give **BODIPY-NHS** as an orange solid. Yield: 95 mg (55%).

**1H NMR** (400 MHz, CDCl₃) δH 7.93 (br s, 2H), 7.55 (d, J = 8.5 Hz, 2H), 7.06 (d, J = 8.5 Hz, 2H), 6.98 (d, J = 4.0 Hz, 2H), 6.55 (dd, J = 4.0, 1.4 Hz, 2H), 4.19 (t, J = 6.5 Hz, 2H), 2.91 (t, J = 6.5 Hz, 2H), 2.87 (br s, 4H), 2.31 (qn, J = 6.5 Hz, 2H); **13C NMR** (100 MHz, CDCl₃) δC 169.06, 168.22, 161.09, 147.34, 143.43, 134.81, 132.41, 131.36, 126.56, 118.27, 114.55, 66.18, 27.70, 25.58, 24.31; **19F NMR** (377.5 MHz, CDCl₃) δF −148.28; **MS (ESI)** m/z 448.1474 (C₂₃H₂₀BFN₃O₅, [M−F]+, requires 448.1480).
Figure 20. $^1$H NMR spectrum of 2 (400 MHz, CDCl$_3$).
Figure 21. $^{13}$C NMR spectrum of 2 (100 MHz, CDCl$_3$).
Figure 22. $^1$H NMR spectrum of BODIPY-COOH (400 MHz, CDCl$_3$).
Figure 23. $^{13}$C NMR spectrum of BODIPY-COOH (100 MHz, CDCl$_3$).
Figure 24. ESI+ mass spectrum of BODIPY-COOH.
Figure 25. $^1$H NMR spectrum of BODIPY-NHS (400 MHz, CDCl$_3$).
Figure 26. $^{13}$C NMR spectrum of BODIPY-NHS (100 MHz, CDCl$_3$).
Figure 27. ESI+ mass spectrum of BODIPY-NHS.
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