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Supplementary Information

Molecular rotors report on changes of live cell plasma membrane viscosity upon interaction with beta-amyloid aggregates

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1. Materials and methods

1.1. Preparation of Aβ(1-42) peptides

Aβ(1-42) was purchased from rPeptide in vials containing 1mg of protein film, which was resuspended in 1,1,1,3,3,3-Hexafluoro-2-propanol (Sigma-Aldrich), aliquoted and dried under nitrogen flow into films containing 0.2 mg Aβ(1-42), which were stored at -20°C.

For each 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. Gel filtration using Zeba 5K MWCO spin-exchange columns was done in order to replace DMSO with 10 mM HEPES. Absorption spectra were recorded immediately to determine the concentration of Aβ(1-42), which was then diluted to 50 μM. To obtain Aβ(1-42) oligomers the solution was incubated for 2 hours at room temperature; to obtain fibrils, the solution was incubated for 7 days.

1.2. Cell culture, incubation of cells with Aβ, dye and H3 peptide

SH-SY5Y and HeLa cells (European Collection of Cell Cultures) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) with 10% Foetal Calf Serum. Cells were grown in T-25 flasks for 4-5 days to reach 80% confluency. For each experiment, cells were seeded into ibiTreat 8 well μ-slides (Ibidi) in 300 μL of culture media at 100,000 cells per well, and allowed to grow to confluence overnight prior to adding Aβ(1-42).

For each experiment, media in a well was replaced with 200 μl of Mg^{2+} and Ca^{2+} free HBSS (Sigma-Aldrich). 50 μL of the Aβ(1-42) HEPES solution was added to each well containing 200 μL of buffer, such that the final concentration of Aβ(1-42) in each well was 10 μM.

Prior to imaging, BODIPY **1** stock solution in DMSO was added to the well to a final concentration of 0.8 μM and mixed gently by pipetting in and out several times. Temperature in the wells was equilibrated and checked with a thermocouple.

The H3 peptide (sequence KELLTRELPSFLGKRT) was synthesized as a tetramer composed of four monomers coupled to a lysine backbone (Schafer-N, Denmark). Tetramerization was previously found to be necessary for the neuritogenic activity of S100A4.[1] For the experiments with H3 peptide, SH-SY5Y cells were incubated for 6 hours in culture media containing 20 μg/ml H3. Afterwards, Aβ(1-42) oligomers were added to the cells (apart from control wells), incubated for 2 hours, and membrane viscosity was analysed by BODIPY **1**.

1.3. Preparation of giant plasma membrane vesicles

Giant plasma membrane vesicles were prepared according to published protocol[2] using 2 mM NEM as vesiculating agent. HeLa cells cultured in DMEM w/ FCS in T-25 flasks were brought to 80% confluence and incubated with 3-4 ml of vesiculating buffer for 90 minutes before gently collecting the buffer with floating GPMVs.

1.4. Absorption and fluorescence spectra acquisition

Agilent 8453 UV-Vis spectrophotometer was used to acquire absorption spectra and FluoroMax4 spectrofluorimeter (Horiba) with a Xenon lamp as an excitation source was used to acquire fluorescence spectra. 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.

1.5. FLIM acquisition and analysis

Fluorescence lifetime imaging microscopy (FLIM) was performed via time-correlated single-photon counting (TCSPC) technique, using a confocal laser scanning microscope (Leica, SP5 II). 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 the excitation source, operated at 880 nm. 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 500 and 580 nm; short-pass filters of 700 and 715 nm were used. FLIM acquisition time was usually 180 seconds. Samples on chamber slides were mounted in the microscope chamber heated by a thermostat (Lauda GmbH, E200). SHG signal from urea crystals on a glass cover slide was used to obtain the instrument response function (IRF).

Exponential fitting was done in SPCI software (Becker-Hickl) using the nonlinear least squares method (NLLSM) and reconvolution algorithm. The χ^2 value and randomness of residuals were used to indicate the goodness of fit. Fluorescence decays were judged to be mono-exponential and followed the equation

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp\left(-t/\tau_i\right) \qquad (3)$$

where I is fluorescence intensity, t is time, and α_i are the amplitudes and τ_i the fluorescence lifetimes of the n exponentially decaying components (1 in this case). The threshold for each pixel was set to at least 100 fluorescence counts in the decay peak, pixels with fewer counts were not included in the analysis. The binning parameter was set to 1 or 2 in the case of images with lower intensity. Data was further processed with OriginPro 8.6.

1.6. Statistical analysis

Statistics were performed using IBM SPSS Statistics software by two-tailed non-paired *t*-test or one-way ANOVA with Tukey's HSD post-test to identify statistically significant groups. Unless indicated otherwise, results are expressed as mean ± SD, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

2. Supplementary figures

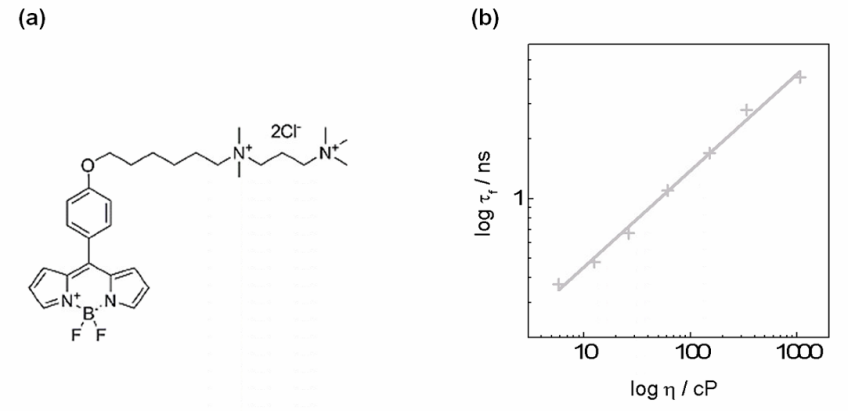

Fig. S1 Molecular structure and viscosity calibration of BODIPY 1. **(a)** Molecular structure of BODIPY **1**; (**b)** Viscosity calibration of the fluorescence lifetime of BODIPY **1** in methanol-glycerol mixtures of varying viscosities with a linear fit according to the equation $\log(\tau_f) = 0.49\log(\eta) - 0.83$.

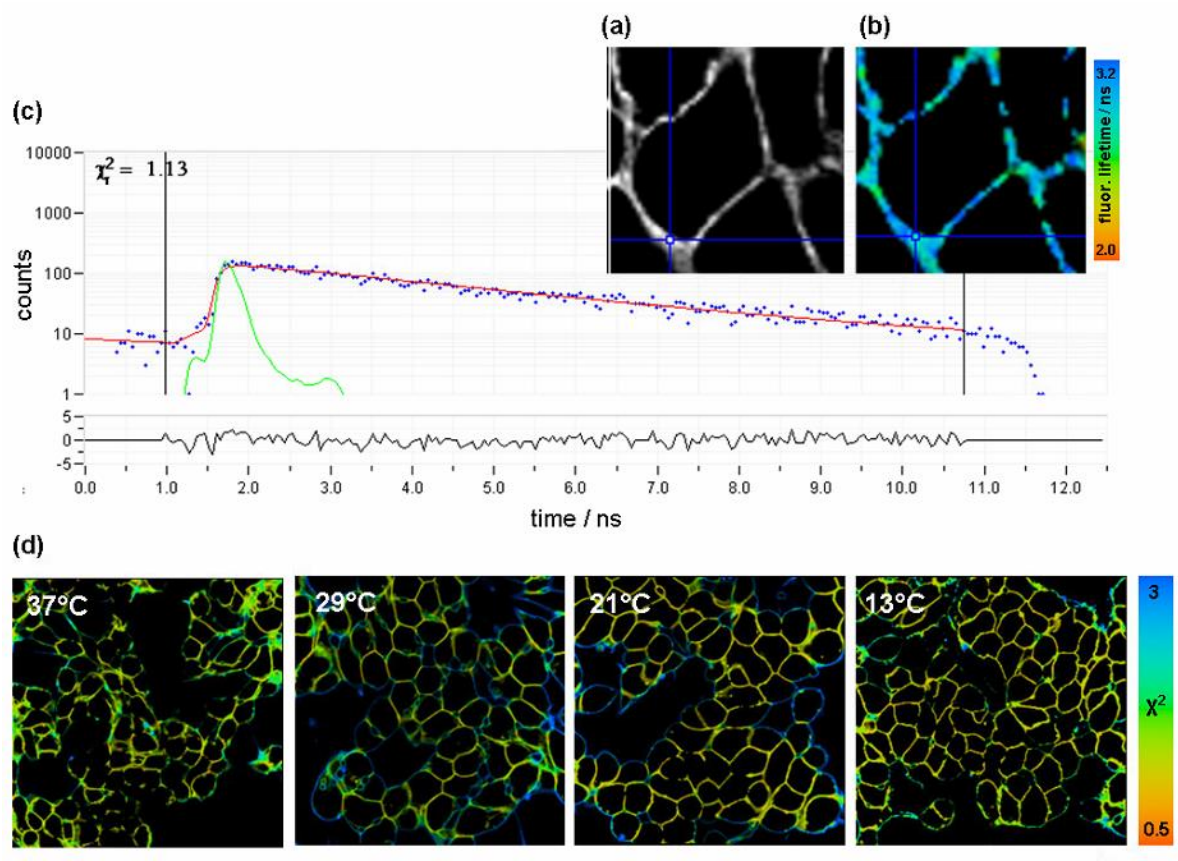

Fig. S2 Mono-exponential fitting of a FLIM image of BODIPY 1 in the cellular plasma membrane.

(a) A fluorescence intensity image of BODIPY **1** in live cell plasma membranes, acquired using two-photon excitation at 880 nm and emission between 500 - 580 nm. **(b)** The corresponding FLIM image. The fluorescence decay in each pixel was fitted using a mono-exponential decay model. The colour coding corresponds to fluorescence lifetime values extracted from the fits. **(c)** The time resolved fluorescence decay (blue) belongs to the pixel where the cursor is positioned on the FLIM image (b). The decay in each pixel was fitted with a mono-exponential function (red) using nonlinear least squares method (NLLSM) and reconvolution. The threshold for each pixel was set to at least 100 fluorescence counts in the decay peak, pixels with fewer counts were not included in the analysis. The instrument response function (green) was recorded using a coverslip with urea crystals. The goodness of fit is demonstrated by the residuals underneath the decay curve (black) and the chi-squared value above it. **(d)** Images of the "goodness of fit" statistical parameter X^2, which determines how well the fitted mono-exponential model fits the raw data (fluorescence decays) in each pixel. The X^2 images correspond to FLIM images in Fig. 1 (main text) of B++ in SH-SY5Y cell plasma membranes at different temperatures.

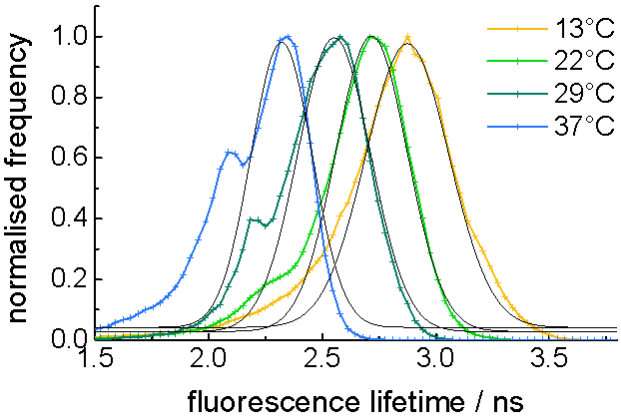

Fig. S3 Fluorescence lifetime of BODIPY 1 in plasma membranes at different temperatures. Gaussian fits of the distributions of fluorescence lifetime from FLIM images of BODIPY **1** in the plasma membranes of SH-SY5Y cells at four different temperatures. Each distribution was averaged out of 12 different FLIM images and normalised.

Our measurements confirm that BODIPY **1** is capable of measuring the degree of lipid organisation in the plasma membrane of live SH-SY5Y cells. Our data suggest that the relationship between temperature (T, °C) and viscosity (η, cP) of SH-SY5Y plasma membranes follows a linear trend (Fig. 1d, main text), which we fitted using the following equation:

$$\boldsymbol{\eta = -6.85T + 544}$$

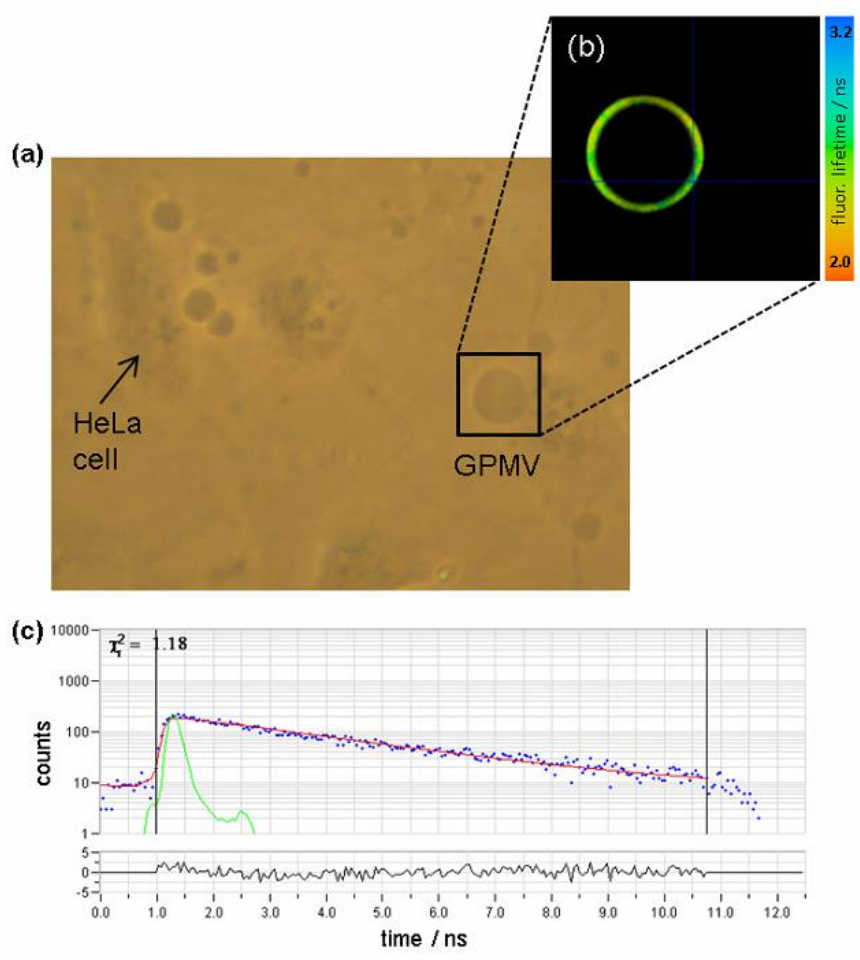

Fig. S4 Preparation and FLIM of HeLa-derived GPMVs. (a) HeLa cells with visible GPMVs upon incubation with the vesiculating agent. **(b)** Fluorescence lifetime map of BODIPY **1** staining a GPMV membrane; the lifetime decays were fitted to a mono-exponential decay model using the nonlinear least squares method (NLLSM) and reconvolution method. **(c)** Fluorescence decay (blue) originating from a single pixel of the FLIM image, the coordinates of which are shown by the blue cursor in (b). A mono-exponential model was chosen (red). The instrument response function (green) was recorded using a coverslip with urea crystals. The goodness of fit is demonstrated by the residuals underneath the decay curve (black) and the chi-squared value above it. In the case of the decay shown in (c), the X^2 value was 1.18, meaning that the fitted mono-exponential (red) is a good model for representing the raw fluorescence decay data (blue).

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	989172.766	2	494586.383	7.236	.001
Within Groups	4237722.577	62	68350.364		
Total	5226895.343	64			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: VAR00002

Tukey HSD

(I) VAR00007	(J) VAR00007	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-336.22486*	88.38252	.001	-548.4541	-123.9957
	3.00	-196.92644	83.14806	.054	-396.5863	2.7335
2.00	1.00	336.22486*	88.38252	.001	123.9957	548.4541
	3.00	139.29842	74.91120	.159	-40.5827	319.1795
3.00	1.00	196.92644	83.14806	.054	-2.7335	396.5863
	2.00	-139.29842	74.91120	.159	-319.1795	40.5827

*. The mean difference is significant at the 0.05 level.

Fig. S5 One-way ANOVA of BODIPY 1 fluorescence lifetime in different membranes. The means of fluorescence lifetime of BODIPY **1** in three different membrane systems were analysed: HeLa GPMVs (variable group 1), HeLa plasma membranes (variable group 2) and SH-SY5Y plasma membranes (variable group 3). There was a statistically significant difference between groups on the p = 0.01 level, a Tukey post-hoc test revealed that there was a significant difference between the mean fluorescence lifetime of HeLa GPMVs and HeLa cells ($p < 0.001$). The difference between SH-SY5Y cells and HeLa cells was not significant ($p = 0.159$).

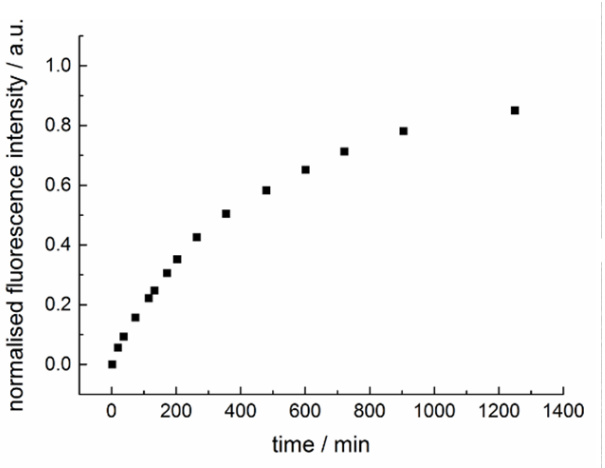

Fig. S6 The aggregation of 50 µM Aβ(1-42) monitored by a Thioflavin T. The ThT fluorescence intensity increase reflects the formation of fibrillar Aβ(1-42) aggregates. Normalised to the saturation value of fluorescence intensity.

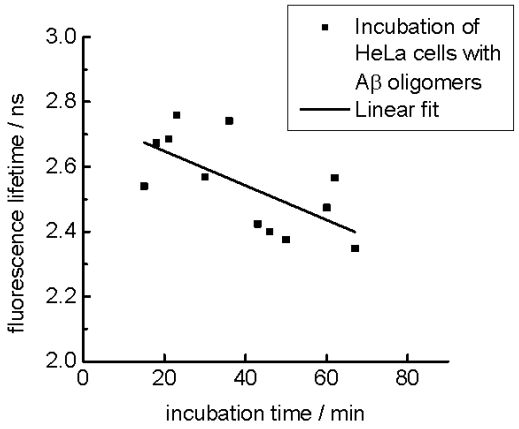

Fig. S7 Time course of BODIPY 1 fluorescence lifetime during incubation with Aβ(1-42) oligomers. The data points were overlaid with a linear fit to demonstrate the gradual decrease of BODIPY **1** fluorescence lifetime over 1 hour of incubation of HeLa cells with 10 µM Aβ(1-42) oligomers.

3. References

1 V. Novitskaya et al., *J. Biol. Chem.*, 2000, **275**, 41278–41286.

2 E. Sezgin et al., *Nat. Protoc.*, 2012, **7**, 1042–1051.