SUPPORTING INFORMATION

Microscopic Viscosity of Neuronal Plasma Membranes Measured Using Fluorescent Molecular Rotors: Effects of Oxidative Stress and Neuroprotection

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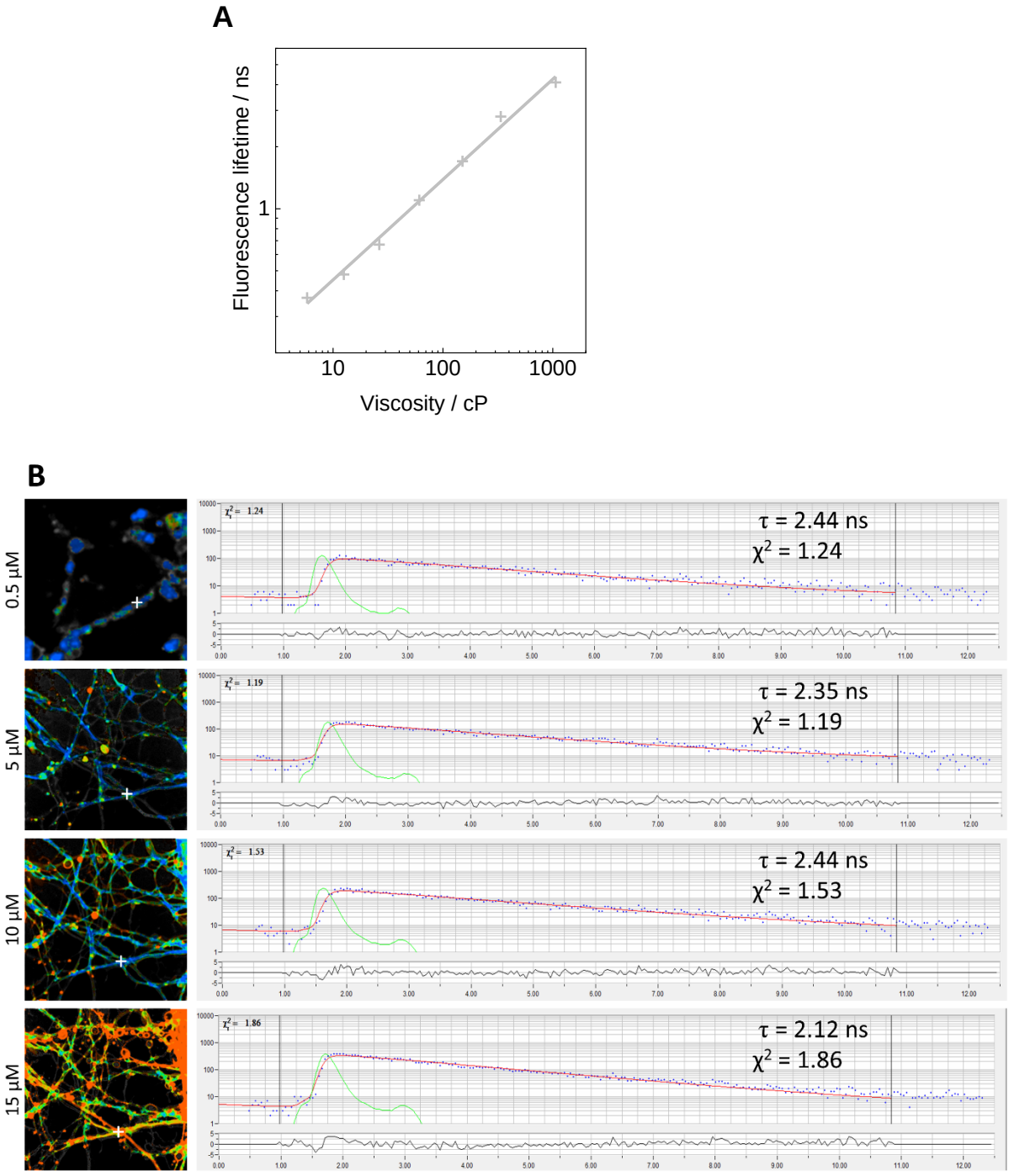

Figure S1 A) The fluorescence lifetime *vs.* viscosity calibration of BODIPY **1** in mixtures of methanol and glycerol of varying viscosities. **B**) Example FLIM maps and decays of neurons incubated with BODIPY **1** at 0.5, 5, 10 and 15 uM**.** The '+' sign indicates the position of the pixel of the fluorescence decay. The instrument response function is shown in green. We note that at concentrations below 0.5 uM the images are too faint for FLIM (on reasonable time scales), while above 10 uM a significant number of points appear where a bi-exponential model is required to fit the decays. This increase in χ^2 for higher incubation concentrations of BODIPY **1** is accompanied by the shortening of the detected lifetime. Based on our previous significant experience with BODIPY dyes[1,2] we assign these bi-exponential decays to dye aggregation.

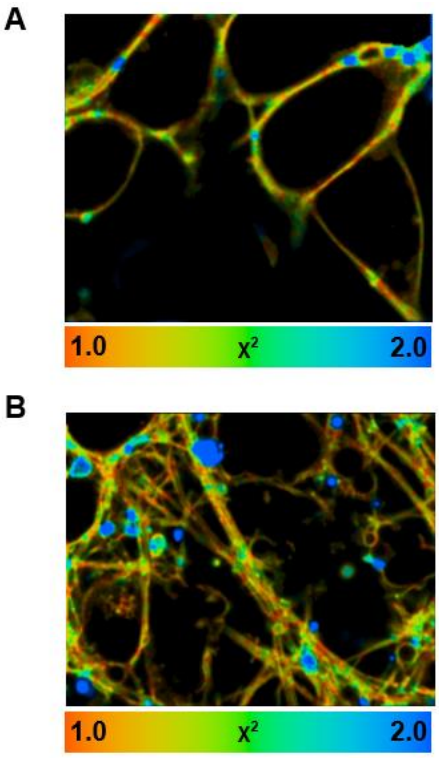

Figure S2 A) The χ^2 map of the FLIM image in Figure 3A (main text). **B)** The χ^2 map of the FLIM image in Figure 3B (main text). The values of χ^2 in the somata and neurites are between 1.0 – 1.4, confirming the correct use of mono-exponential fitting.

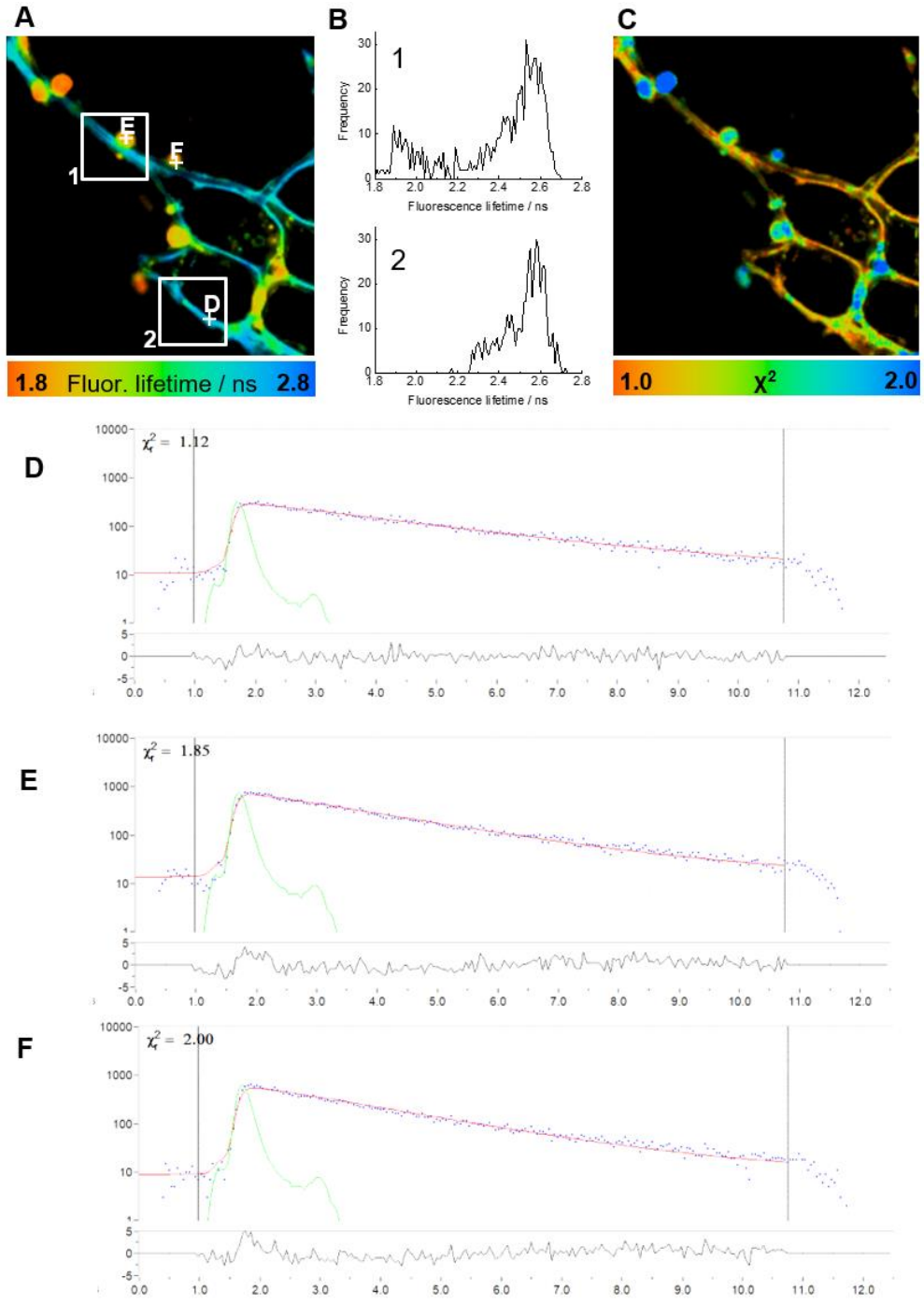

Figure S3 The fluorescence lifetime of BODIPY **1** in different regions of neuronal plasma membranes (mono-exponential fitting). **A)** The microscopic viscosity of the axon (1) is identical to the soma (2). **B)** The fluorescence lifetime distributions of the two regions show good overlap, apart from the low lifetime contribution of a spherical vesicle in region (1). **C)** The corresponding χ^2 map. Although χ^2 values are in the correct range in neurites and somata and confirm the correct use of mono-exponential fitting, the values are too high in the vesicles, requiring the use of bi-exponential fitting. **D)** The fluorescence decay in pixel 'D' in FLIM A) is overlaid with a mono-exponential fit (red) with a good $\chi^2 = 1.12$. **E)** The decay in pixel 'E' in FLIM A) is overlaid with a mono-exponential fit (red) with $\chi^2 = 1.85$, demonstrating the unsuitability of mono-exponential fitting. **F)** The decay in pixel 'F' in FLIM A) is overlaid with a mono-exponential fit (red) with $\chi^2 = 2.00$, demonstrating the unsuitability of mono-exponential fitting.

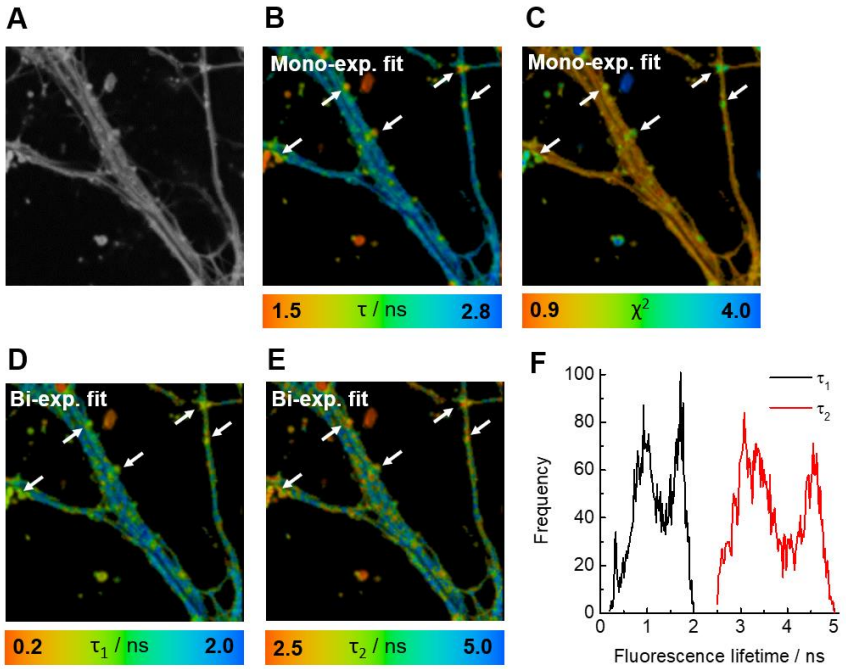

Figure S4 A) The fluorescence intensity image of BODIPY **1** in an axon with visible 'hot spots'. **B)** Mono-exponential fit of the FLIM, revealing the low fluorescence lifetime of 'hot spots' (marked by white arrows) relative to the axon. **C)** The high values of the χ^2 goodness-of-fit parameter demonstrate that a mono-exponential decay is not a suitable model for the behaviour of BODIPY **1** in 'hot spots' **D,E)** Here, the FLIM image was analysed using bi-exponential fitting required for a satisfactory goodness-of-fit in 'hot spots'. Shown are the lifetime maps of the two components τ_1 and τ_2. Both components exhibit lower lifetime in dendritic spines compared to the surrounding membrane. **F)** The bimodal distributions of τ_1 and τ_2 in the FLIM image analysed using bi-exponential fitting; the lower values originate from 'hot spots', higher values from the axon. τ_1 and τ_2 values at *ca.* 1 and 3 ns correspond to dendritic spines, while the higher values (nearly 2 ns and 4.5 ns) correspond to the 'bulk' of the axon. Of note, the multi-exponentiality observed in these 'hot spots' is potentially due to excessive dye accumulation and its aggregation as a result. It is well known that the aggregation of BODIPY dyes causes self-quenching of fluorescence and significant deviations of time resolved traces from the mono-exponential fit. In such case, the obtained lifetime values are no longer representative of the microviscosity.[1,2]

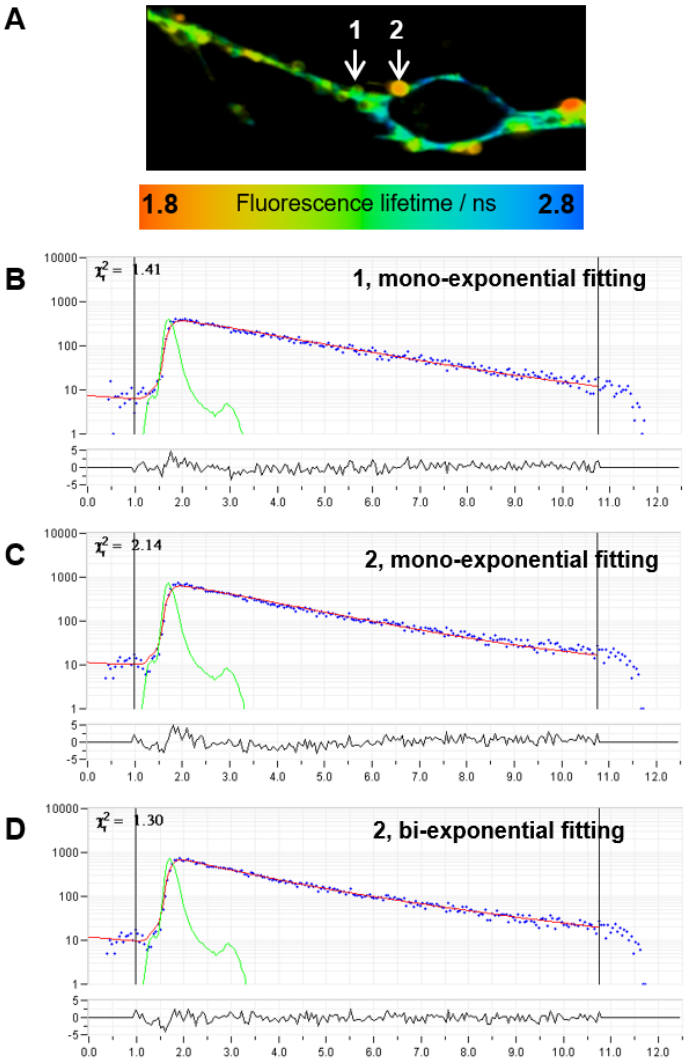

Figure S5 A) FLIM image of a neuron incubated with BODIPY **1**. The arrow 1 in FLIM A) shows the position of the pixel of the fluorescence decay in B), arrow 2 shows the position of the pixel of the fluorescence decay in C,D). **B)** The decay from pixel 1 in FLIM A) is overlaid with a mono-exponential fit (red) with χ^2 = 1.41. **C)** The decay in pixel 2 in FLIM A) is overlaid with a mono-exponential fit (red) with χ^2 = 2.14. **D)** The decay in pixel 2 of FLIM A) is overlaid with a bi-exponential fit (red) with χ^2 = 1.3. The instrument response functions are shown in green.

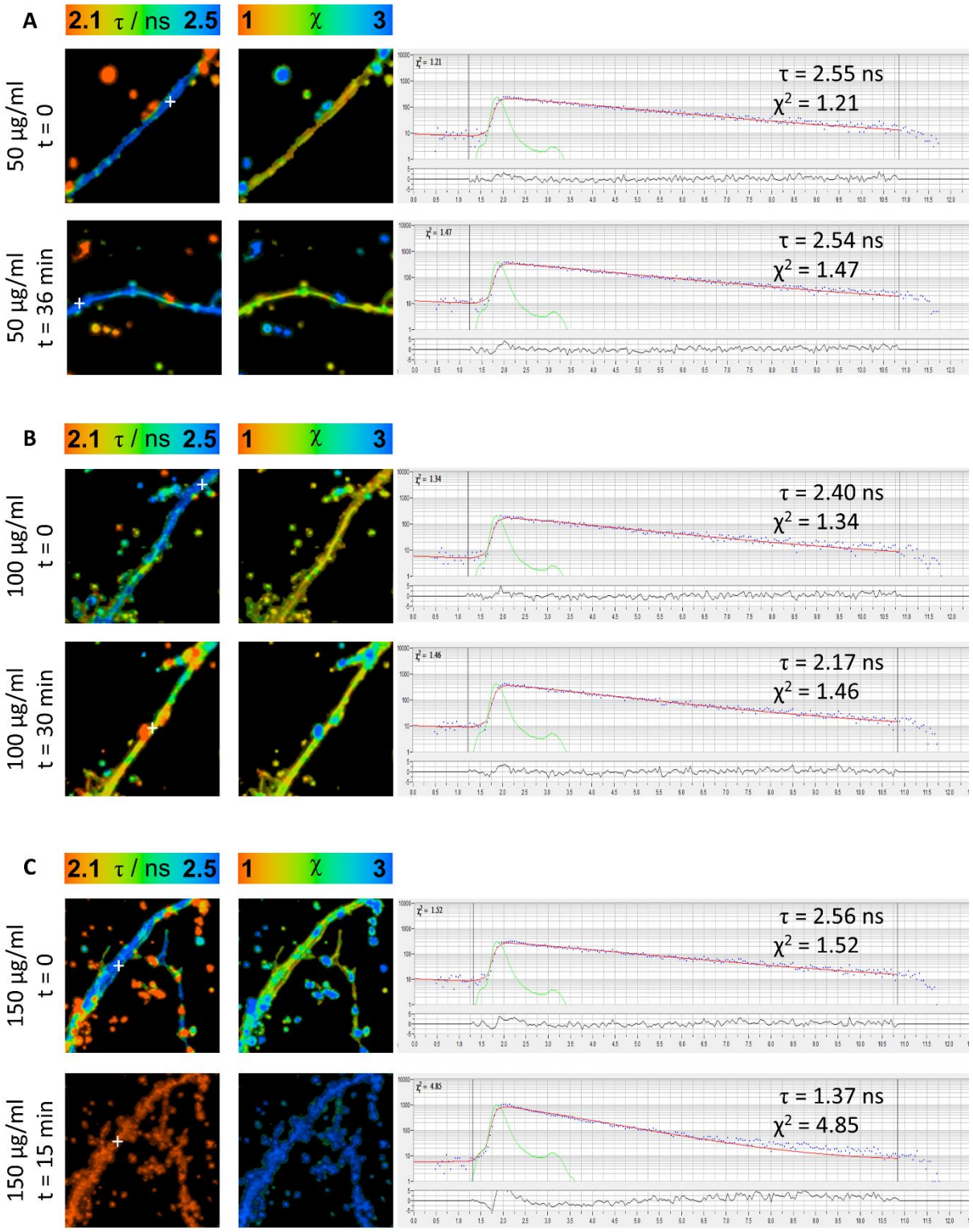

Figure S6 FLIM images, χ^2 maps, and sample decays following mono-exponential decay fitting of neurons incubated with BODIPY **1** and kainic acid at **A)** 50 µg/ml, **B)** 100 µg/ml and **C)** 150 µg/ml. The white '+' sign indicates the position of the pixel of the fluorescence decay. The instrument response function is shown in green.

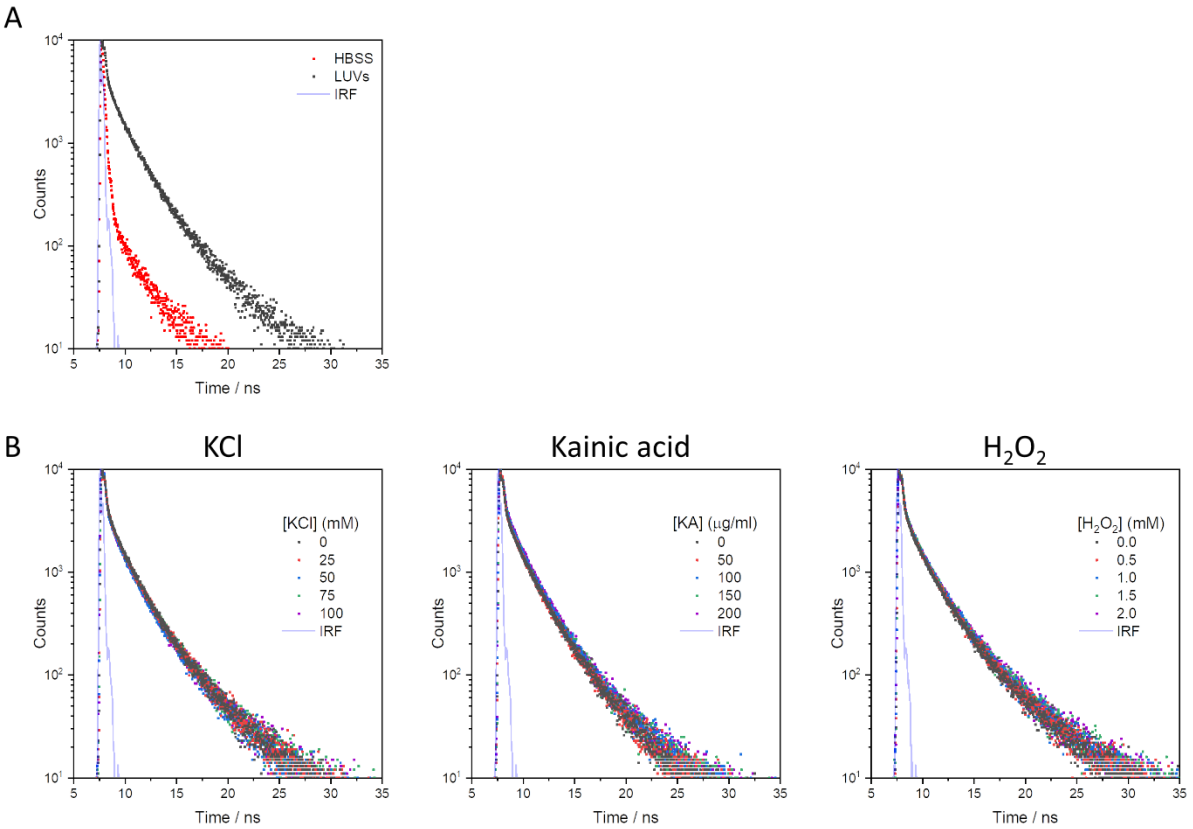

Figure S7 Fluorescence lifetime decays of BODIPY **1** in **A)** aqueous buffer HBSS, and **B)** LUV model lipid bilayers (composed of unsaturated lipid DOPC) during treatment with KCl (0, 25, 50, 75 and 100 mM), KA (0, 50, 100, 150 and 200 μg/ml) and H_2O_2 (0.0, 0.5, 1.0, 1.5 and 2.0 mM). λ_{ex} = 404 nm, λ_{em} = 515 nm (± 16 nm), 420 and 495 nm long pass cut off filter were placed between the sample and detector.

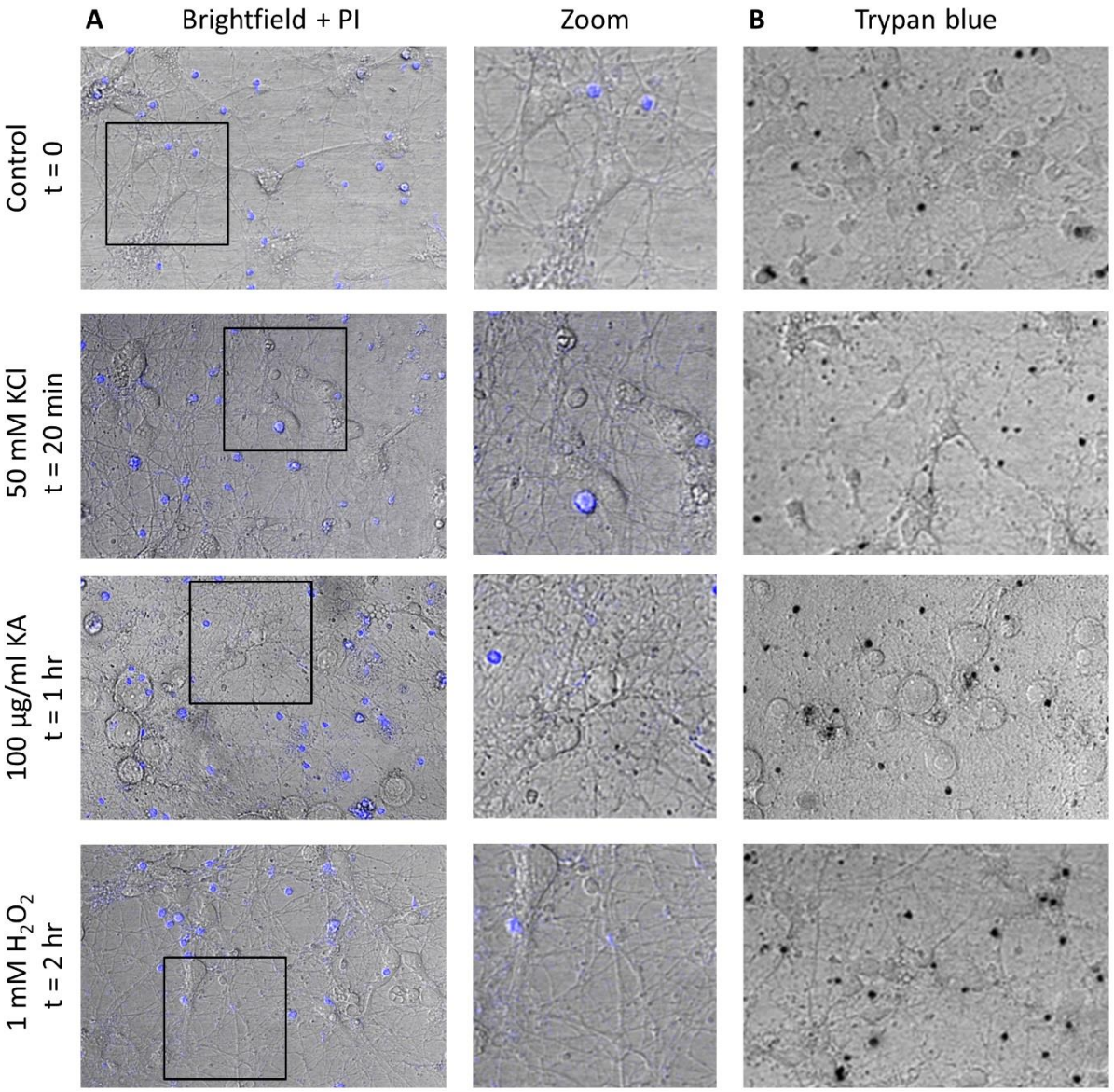

Figure S8 Propidium iodide (PI) and trypan blue assays to test cell viability after KCl (50 mM, 20 min), KA (100 µg/ml, 1 hr) and H_2O_2 (1 mM, 2 hr) treatment in HBSS. **A)** Left|: Brightfield and PI (blue) merge images after staining with PI (12.5 µg/ml, 2 min, λ_{ex} = 458 nm, λ_{em} = 600-800 nm). Right: Enlarged area marked in the left panel by black squares. **B)** Brightfield images from a different area of the same cultures after staining with trypan blue (1:1 dilution, 2 min). Images taken at 20x magnification.

References

(1) Wu, Y.; Štefl, M.; Olzyńska, A.; Hof, M.; Yahioglu, G.; Yip, P.; Casey, D. R.; Ces, O.; Humpolíčková, J.; Kuimova, M. K. Molecular Rheometry: Direct Determination of Viscosity in Lo and Ld Lipid Phases via Fluorescence Lifetime Imaging. *Phys. Chem. Chem. Phys.* **2013**, *15* (36), 14986.

(2) Mika, J. T.; Thompson, A. J.; Dent M. R.; Brooks N. J.; Michiels J.; Hofkens J.; Kuimova M.K. Measuring the Viscosity of the Escherichia coli Plasma Membrane Using Molecular Rotors. *Biophys. J.* **2015**, 111 (7), 1528-1540.