Measuring macromolecular crowding in cells through fluorescence anisotropy imaging with an AIE fluorogen

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METHODS

Materials. OptiMem, Dulbecco's modified Eagles Medium (DMEM), fetal bovine serum (FCS), glutamine, penicillin, streptomycin and ER Tracker Red were purchased from Thermo Fisher. Sorbitol and all other reagents used in this study was purchased from Sigma-Aldrich. TPE-Py-NCS was synthesized as described in Ref 1.

Cell culturing. Neuro 2A and HeLa cell-lines (from lab cultures originally obtained from ATCC) were used in this study and tested and cleared for mycoplasma. Neuro-2a cells were maintained in OptiMem (Life Technologies) supplemented with 10% FCS, 1 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 5% atmospheric CO_2. For microscopy experiments 9×10^4 Neuro-2a cells were plated on 8-well μ-slides (Ibidi).

HeLa cells were maintained in DMEM supplemented with 1 mM glutamine, 100 U/mL penicillin/streptomycin and 10% v/v FCS. Cells were cultured at 37 ºC in a humidified incubator with 5% atmospheric CO_2. For microscopy experiments 7×10^4 HeLa cells were plated on 8-well μ-slides (Ibidi).

Cell staining. TPE-Py-NCS was dissolved in DMSO as 1 or 2 mM stock solution. The stock solution was kept at 4 ºC in the dark. Plated cells were rinsed with PBS and then treated with freshly diluted TPE-Py-NCS (1-10 μM in PBS) for 30 min at 37 ºC. For live cell imaging, TPE-Py-NCS was removed and cells were rinsed with PBS prior to the image acquisition. For fixed cell imaging, after the removal of TPE-Py-NCS, the cells were fixed in solution with 4 % (w/v) paraformaldehyde for imaging. For co-staining experiment, ER-Tracker Red (ThermoFisher) was used according to manufacturer's instructions with 1μM staining concentration. Cells were fixed afterwards.

Cytotoxicity test. 7×10^4 HeLa cells were plated onto 12-well plates. The next day, cells were treated with TPE-Py-NCS as described above. Cells were detached in 400 μL PBS and assessed using the Countess Cell counter (Invitrogen) with Trypan blue for viability.

Cell lysate preparation and SDS-PAGE. The stained HeLa cells were lysed by repeated immersed in 300 μL native lysis buffer (20 mM Tris pH 8.0, 1% Triton X-100, 2 mM $MgCl_2$, 1 EDTA-free Complete protease inhibitor tablet (Roche)/10 mL, 150 μM NaCl, 20 u/mL benzonase (EMD Millipore)). Protein concentration was determined by bicinchoninic acid assay using bovine serum albumin as mass standard. The cell lysate was analyzed by 12% arylamide SDS-PAGE. TPE-Py-NCS fluorescence was recorded on unstained gels with a Gel Imaging system (Bio-rad). Protein ladder with fluorescence was also loaded for comparison.

Confocal imaging. After staining with TPE-Py-NCS, cells were fixed with 4% (v/v) paraformaldehyde in PBS for 15 min, with media replaced by PBS. Cells were imaged on the Leica SP5 Confocal microscope for TPE-Py-NCS (excitation: 405 nm, emission: 520–600nm) and ER Tracker Red (excitation: 587 nm, emission: 600–650 nm) using a 63× objective lens.

Fluorescence anisotropy microscope. The developed polarised microscope[2] is based on a modified commercial inverted frame system (Nikon, TE2000) using a high numerical aperture objective (Nikon, Apo 60x) (Fig. S5). The excitation source was a CW diode laser (Coherent Inc. 405 nm) coupled to the microscope by a polarisation maintaining single mode optical fibre (ThorLabs, PM-S405-XP). The polarisation of the excitation light was switched orthogonally by a liquid crystal half-wave variable retarder (ThorLabs, LCC1111-A and LCC25) placed immediately after the collimating lens. The fluorescence was collected by the same objective lens and passed through a polarisation splitter (Cairn Research, OptoSplit II), which facilitated the simultaneous collection of both emission polarisation images on the same EMCCD camera (Princeton In-struments, PhotonMAX 1024B). In this way, four images, corresponding to the four excitation and emission polarisation conditions I_{SS}; I_{SP}; I_{PP}; I_{PS} could be collected rapidly. From the intensity-based images (Fig. S6), fluorescence anisotropy map (Fig. S8), showing the extent of emission depolarisation due to processes occurring r (Eq. 1) could be generated by image manipulation at a pixel-by-pixel level using MatLab code. The G is the correction factor (0.98) to account for any polarisation bias of the detection system and B is background which subtract from each image using ImageJ (Fig S6).

$$r = \frac{(I_{SS}-B)-G.(I_{SP}-B)}{(I_{SS}-B)+2.G.(I_{SP}-B)} \qquad (1)$$

Scanning confocal time-resolved fluorescence imaging measurement. The pulsed excitation (440 nm) source was the frequency-doubled output of a mode-locked and cavity-

dumped titanium sapphire laser (Coherent Mira 900f/APE PulseSwitch, ~120 fs, ~5.4 MHz) pumped by ~4.5W from a DPSS laser (Coherent Verdi V10). This excitation source was delivered to the confocal scanning inverted microscope (Olympus FV300) via a single-mode optical fibre (S405 ThorLab) and focused with a 60x objective lens (Olympus PLANAPO water). Confocally isolated emission was acquired by an SPC-150 single photoncounting unit and controlled using the SPCM TCSPC operating software. The fluorescence decay data were analyzed using "SPC Image" FLIM data analysis software (Becker & Hickl GmbH).

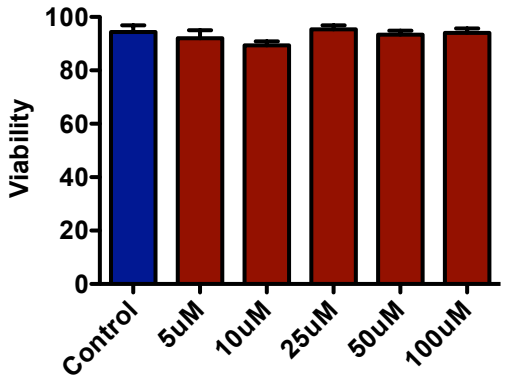

Fig. S1 Cytotoxicity test of TPE-Py-NCS in HeLa cells. Three replicates with mean are shown for cells treated with the dye for 30 min.

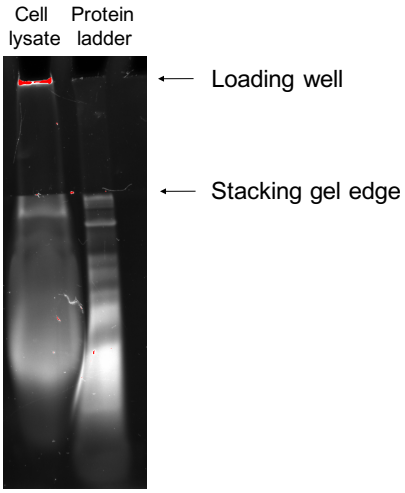

Fig. S2 In-gel fluorescence of TPE-Py-NCS stained HeLa cell lysates by SDS-PAGE (left lane). Protein ladder was shown for comparison.

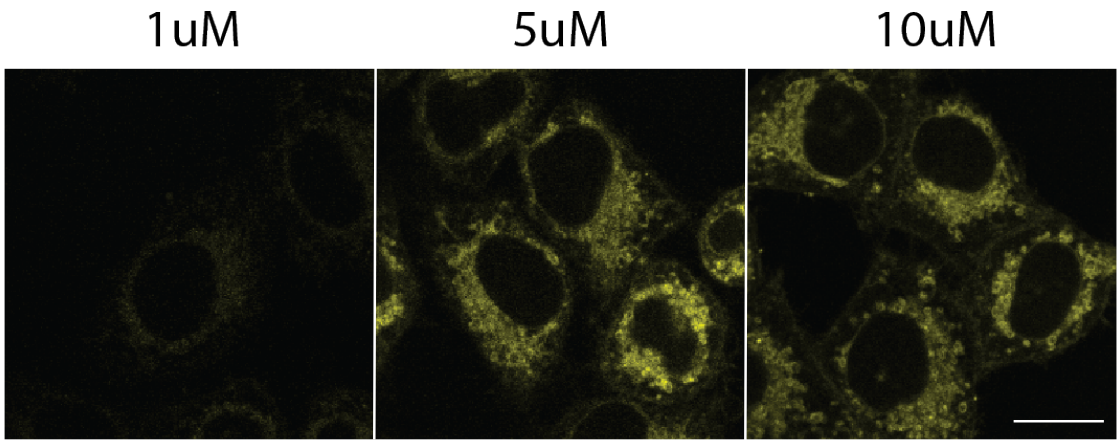

Fig. S3 Confocal image of HeLa cells stained with TPE-Py-NCS at different concentrations. Scale bar: 20 μm.

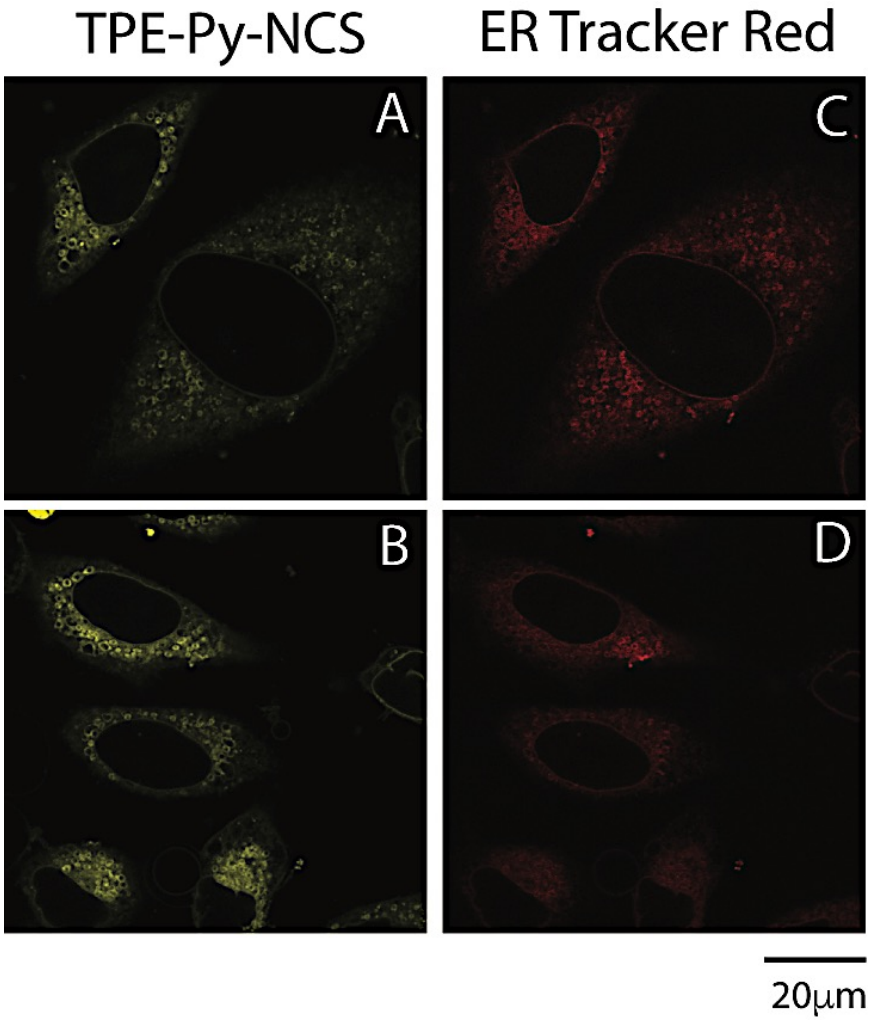

Fig. S4 Fluorescence co-localization imaging of HeLa cells stained with 10 μM TPE-Py-NCS and 1 μM ER Tracker Red. (A,B) Image from TPE-Py-NCS (excitation: 405 nm, emission: 520–600nm). (C,D) Image from ER Tracker Red (excitation: 587 nm, emission: 600–650 nm).

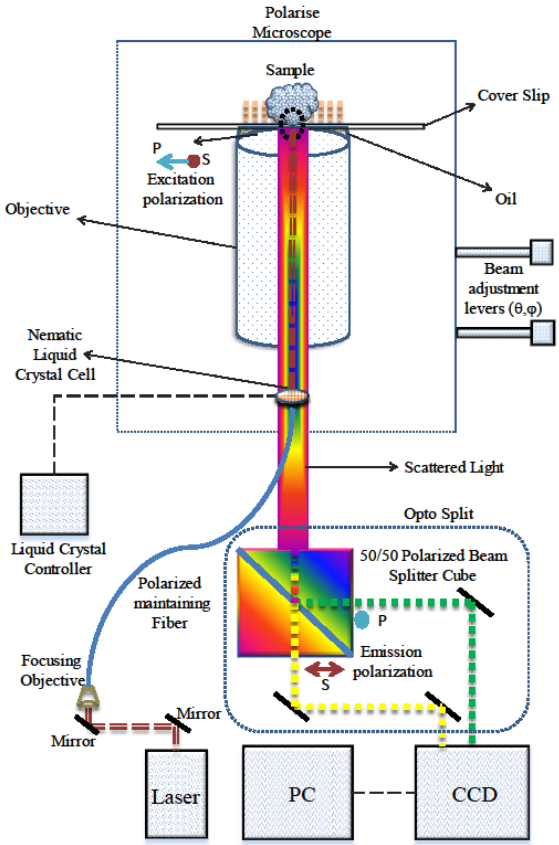

Fig. S5 Schematic diagram of the anisotropy imaging using developed polarised microscope.

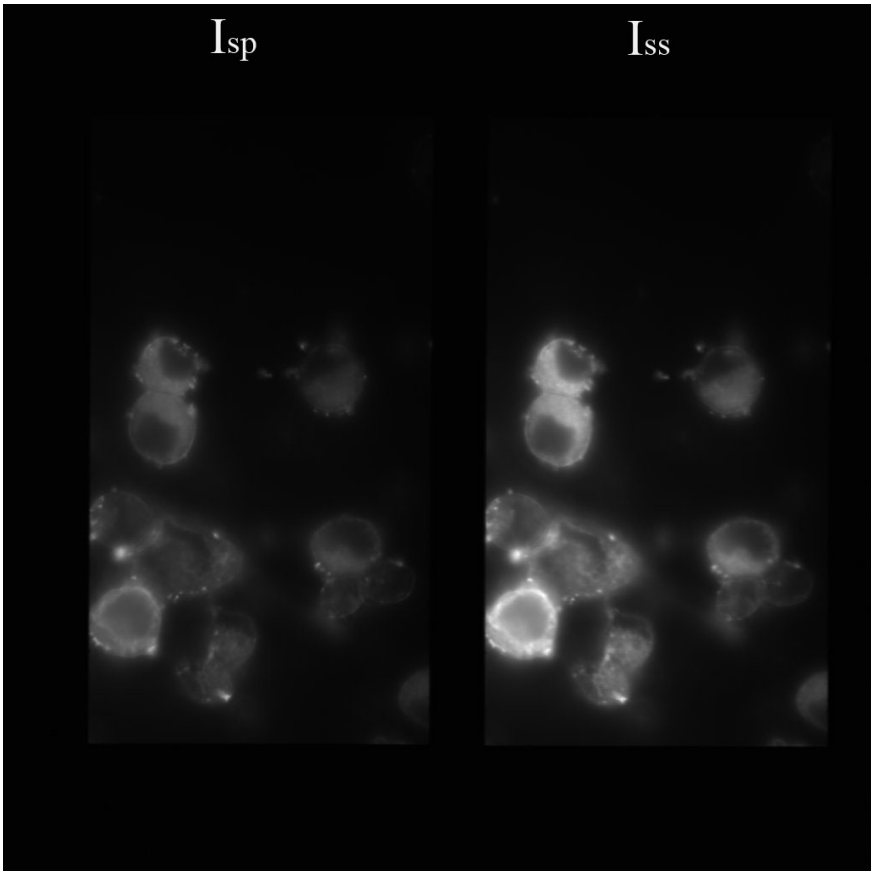

Fig. S6 The original intensity image (*cf.* Fig.3A) divided into the separate polarisations via beam splitter and acquired by CCD camera.

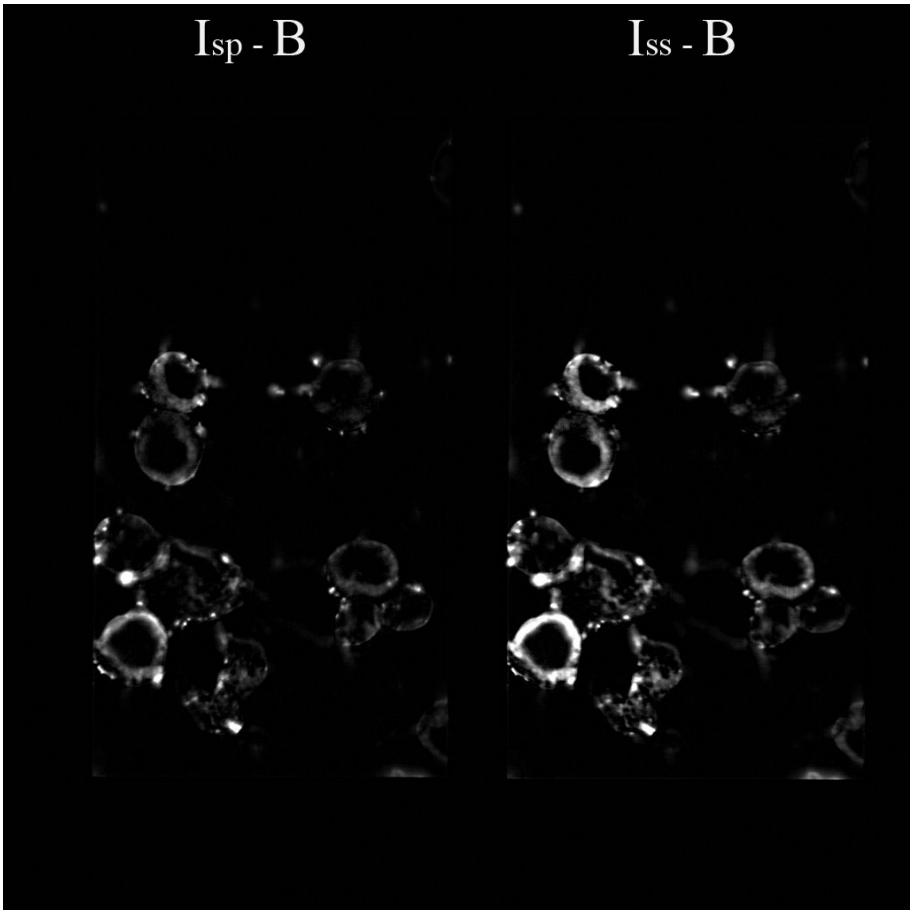

Fig. S6 The subtracted background (using ImagJ) from original intensity image (Fig. S5) which is divided into the separate polarisations via beam splitter and acquired by CCD camera.

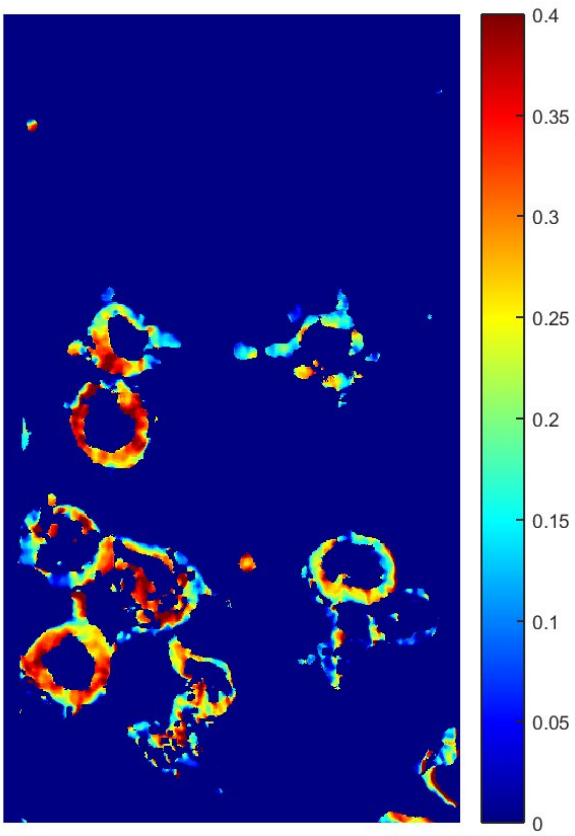

Fig. S7 The anisotropy map (*cf.* Fig.3A) which is calculated using Equation 1 via MatLab code from Fig. S6 left and right side.

References

1. W. Zhang; R. T. K. Kwok, Y. Chen, S. Chen, E. Zhao, C. Y. Y. Yu, J. W. Y. Lam, Q. Zheng and B. Z. Tang, *Chem. Commun.* **2015**, *51*, 9022.
2. H. Soleimaninejad, Y. Hong and T. A. Smith in *SPIE Nanoscience+ Engineering.* 99230M-99230M-99239 (International Society for Optics and Photonics).