Increased intracellular crowding during hyperosmotic stress

Akira Kitamura[1*], Sho Oasa[2], Haruka Kawaguchi[1], Misato Osaka[1], Vladana Vukojević[2], Masataka Kinjo[1*]

1 Laboratory of Molecular Cell Dynamics, Faculty of Advanced Life Science, Hokkaido University, Sapporo, Japan

2 Department of Clinical Neuroscience (CNS), Center for Molecular Medicine (CMM), Karolinska Institutet, Stockholm 17176, Sweden

*To whom correspondence may be addressed. Email: akita@sci.hokudai.ac.jp and kinjo@sci.hokudai.ac.jp

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Abstract

Hyperosmotic stress activates in live cells numerous processes the net result of which is readily recognized at the cellular level through morphological changes. It also promotes intracellular protein/RNA aggregation and phase separation, observable through the formation of stress granules in the cytoplasm. However, the time course and extent of these changes remain largely uncharacterized. To investigate in live cells dynamic changes in intracellular macromolecular crowding (MMC) induced by hyperosmotic stress, we used Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Lifetime Imaging Microscopy (FLIM) to quantify changes in the local environment by measuring the diffusion and the fluorescence lifetime of the monomeric enhanced Green Fluorescent Protein (eGFP), respectively. In addition, Confocal Laser Scanning Microscopy (CLSM) was used to map the acceptor/donor emission ratio (Y/C ratio) in cells expressing the glycine-inserted mutant Förster Resonance Energy Transfer (FRET) sensor (GimRET) that is indicative of MMC. Real-time monitoring of eGFP fluorescence lifetime showed that a faster response to environmental changes due to MMC is observed than when measuring the Y/C ratio using the MMC-sensitive GimRET sensor. This suggests that eGFP molecular electronic states and/or collision frequency are affected by changes in the immediate surroundings due to MMC without requiring conformational changes as is the case for the GimRET sensor. Furthermore, slower eGFP mobility and a lower anomalous diffusion parameter (α) are measured in cells exposed to hyperosmotic stress, indicating higher intracellular viscosity due to increased MMC. Our findings reveal that changes in eGFP fluorescence lifetime and diffusion are early indicators of elevated intracellular MMC. These variables can therefore be used for quantitative characterization of MMC under various stress/disease conditions in live cells.

Introduction

Cells dynamically interact with their surroundings and changes in the extracellular environment are readily accompanied by adjustments of intracellular properties through the activation of numerous biochemical reaction pathways. For example, changes in solutes concentration outside the cell lead to altered movement of water across the plasma membrane, disrupting the intracellular osmolarity that is tightly regulated by balancing the intake and excretion of water and electrolytes[1, 2]. If an increase in solutes concentration is observed, hyperosmotic stress ensues, which, if not interfered with, can rapidly give rise to protein damage due to cellular water loss[3]. Cells activate various biochemical pathways in a non-steady-state manner to counteract the hyperosmotic stress[2]. Post exposure to a hyperosmotic environment, cells shrink due to dehydration, *i.e.,* due to the loss of water molecules from the cell to the surrounding. To diminish or shut down unwanted and unnecessary nascent polypeptide synthesis, aggregation of macromolecules leads to the formation of a dense network of interactions due to dehydration, strong enough to drive phase separation, resulting in the formation of stress granules (SGs) containing mRNA, RNA-binding proteins (RBPs) and ribosomes[4-6].

During hyperosmotic stress the cell volume changes to such an extent that intracellular macromolecular crowding (MMC) is considered to also change. To read out the MMC status at different locations in the cells, the Förster resonance energy transfer (FRET)-based sensor GimRET, a fusion protein between the cyan fluorescent protein (CFP) and the crowding-sensitive mutant of the yellow fluorescent protein (YFP) with an inserted glycine residue before Tyr145, was recently designed[7]. The aim of this work is twofold: to use GimRET to characterize MMC during hyperosmotic stress and to establish an alternative, fluorescence lifetime-based method for *in situ* monitoring of MMC changes during hyperosmotic stress in real-time.

Fluorescence lifetime (τ_{FL}), *i.e.,* the lifetime of a molecule in the excited singlet electronic state, is an intrinsic property of the fluorophore that does not depend on fluorophore concentration, excitation, and/or photobleaching, but rather depends on environmental factors, such as temperature, solvent polarity, and the presence of molecules with the capacity to quench fluorescence. It is therefore well suited to study environmental changes, such as MMC in live cells. τ_{FL} is defined as the mean time required for a population of molecules in the excited state to be reduced to 36.8 % of its original value (*i.e.* by a factor of Napier's constant, $e = 2.718$), by losing energy through fluorescence emission, which is a radiative process, and by various nonradiative processes, such as intersystem crossing to the triplet state, internal conversion, collisional, *i.e.* dynamic quenching, energy transfer, charge transfer, *etc.*[8, 9] As such, τ_{FL} is reciprocally proportional to the total decay rate, comprising contributions from the radiative and all non-radiative rate constants. Hence, τ_{FL} decreases when the rate constant of any of the non-radiative processes increases. τ_{FL} can be measured in either the frequency or the time domain. Here, time domain measurements were used[10, 11] to probe the local cellular environment[12]; FRET-donor and -acceptor ratiometric imaging using the GimRET sensor was

deployed to monitor the changes in MMC after cell exposure to hyperosmotic stress; and diffusion of eGFP was measured using fluorescence correlation spectroscopy (FCS)[6, 13, 14], to acquire information about local viscosity[15].

Results:

Fluorescence lifetime of eGFP rapidly decreased and slowly recovered in cells exposed to hyperosmotic stress

CLSM imaging revealed that eGFP transiently expressed in murine neuroblastoma Neuro-2a (N2a) cells is uniformly distributed inside the cells (Figure 1A, upper row). Following the exposure to hyperosmotic stress caused by 290 mM NaCl (Figure 1A, lower row), the morphology of the N2a cells markedly changed within some 20 s, as evident from the transmission bright field images in Figure 1A. As before, eGFP was uniformly localized in both, the cytoplasm and the cell nucleus, however, in a significant portion of N2a cells a pronounced eGFP fluorescence rim was observed at the boundary between the nucleus and the cytoplasm (Figure 1A, lower row and Supplemental Figure S1, lower row, white arrows). In addition, formation of SGs was observed in the cytoplasm, verified using the SG marker T cell intracellular antigen 1 tagged at the N-terminus with the Red Fluorescent Protein variant mCherry (mCherry-TIA1; Figure 1A).

We measured eGFP fluorescence lifetime ($\tau_{FL,eGFP}$) in live N2a cells under normal growth conditions (Figure 1B, left) and hyperosmotic stress (Figure 1B, right) using a home-built massively parallel FLIM system (mpFLIM) without laser scanning in which 16 × 16 confocal foci were generated using a Diffractive Optical Element (DOE) to split the incident laser beam into a 2-dimensional (2D) array of beams and a 2D Single Photon Avalanche Diodes (SPAD) array camera with matching geometry[11]. Subcellular positions where FLIM measurements were performed were identified using bright field and fluorescence imaging (Figure 1B). Under normal conditions, $\tau_{FL,eGFP}$ in the cytoplasm was $\tau_{FL,eGFP}^{cyt,\ norm}$ = (2.58 ± 0.02) ns (mean ± SD), whereas a slightly shorter fluorescence lifetime was measured in the cell nucleus, $\tau_{FL,eGFP}^{nuc,\ norm}$ = (2.55 ± 0.02) ns, (Figure 1C). Upon hyperosmotic stress, $\tau_{FL,eGFP}$ in both the cytoplasm and the nucleus markedly decreased, becoming significantly shorter, $\tau_{FL,eGFP}^{cyt,\ hyper}$ = (2.47 ± 0.04) ns and $\tau_{FL,eGFP}^{nuc,\ hyper}$ = (2.44 ± 0.03) ns (Figure 1C). Moreover, $\tau_{FL,eGFP}$ in N2a cells swiftly decreased just after addition of the hyperosmotic medium and gradually recovered thereafter (Figure 1D).

To confirm that the decrease of $\tau_{FL,eGFP}$ is due to MMC, control experiments were performed using eGFP in a series of solutions of bovine serum albumin (BSA), commonly used as a crowding agent in biochemical reactions[16], with concentrations of BSA varying from 0–250 mg/ml

(Supplemental Figure S2), or with a high NaCl concentration (Supplemental Figure S3). We observed that $\tau_{FL,eGFP}$ became shorter in a BSA-concentration-dependent manner (Supplemental Figure S2), which is indicative of dynamic quenching of eGFP fluorescence in BSA solutions of increasing concentration, *i.e.* increasing MMC. In contrast, a high NaCl concentration (290 mM) that is the same as in the extracellular medium for hyperosmotic stress, did not affect $\tau_{FL,eGFP}$ in solution (Supplemental Figure S3).

Y/C ratio of the MMC-sensitive GimRET sensor rapidly decreased and slowly recovered in cells exposed to hyperosmotic stress

To further assess whether MMC increases in cells during hyperosmotic stress, we monitored changes in MMC in live cells using the FRET-based MMC sensor GimRET[7]. In the GimRET sensor, quenching of YFP decreases FRET efficiency between CFP and YFP, resulting in a decrease in fluorescence intensity ratio between the YFP acceptor and the CFP donor (Y/C ratio) as MMC increases. In line with this, we have observed that the Y/C ratio of the GimRET sensor in live N2a cells abruptly decreased shortly after exposing the cells to the hyperosmotic medium, and then gradually recovered (Figure 2A & B). After incubation of the N2a cells in the hyperosmotic medium for 3 h, the Y/C ratio of GimRET was still lower than in the normal growth medium (Figure 2C). Cell shrinkage and morphology changes were also observed shortly after addition of the hyperosmotic medium, and remained visible throughout the experiment time as evident from the bright field image (Figure 2A, bottom row), showing cell volume recovery. Of note, the half recovery time of $\tau_{FL,eGFP}$ and Y/C ratio of GimRET after addition of stress medium was different, 9.7 s and 73.3 s, respectively (Figure 3).

eGFP diffusion and anomalous diffusion parameter (α) decrease in cells exposed to hyperosmotic stress indicating an increase in intracellular viscosity and heterogeneity

To elucidate how MMC induced by hyperosmotic stress affects intracellular viscosity, the diffusion of monomeric eGFP was measured using FCS. We have observed that eGFP diffusion time, that is the decay time of the autocorrelation curve, was longer both in the cytoplasm and in the nucleus when measured in N2a cells exposed to hyperosmotic stress compared to N2a cells under normal physiology (Figure 4A). This indicates that eGFP diffusion is slower in N2a cells exposed to hyperosmotic stress. By fitting the experimentally derived autocorrelation curves using the one-component anomalous diffusion model, eGFP diffusion coefficient and the anomalous diffusion parameter[17] were determined (Figure 4B & C). The diffusion coefficient of eGFP monomers in N2a cells during hyperosmotic stress was significantly lower than that under normal, non-stress conditions. In agreement with previous observations[15], differences between eGFP diffusion in the cytoplasm and the cell nucleus were not

observed. Diffusion analysis suggests that hyperosmotic stress induces an increase in intracellular viscosity *via* increased MMC. In N2a cells not exposed to hyperosmotic stress (control), the anomalous diffusion coefficient of eGFP in the cytoplasm and the cell nucleus were also not significantly different. Furthermore, the measured values of the anomalous diffusion parameter α were slightly less than 1.0, suggesting subdiffusion consistent with MMC[18]. In contrast, the anomalous diffusion coefficient of eGFP in N2a cells exposed to hyperosmotic stress significantly decreased compared to the non-stressed condition, as expected when MMC and spatial heterogeneity in the cells increase.

Discussion

Our data show that both, $\tau_{FL,eGFP}$ and Y/C ratio of the GimRET sensor, decreased swiftly after exposing the N2a cells to hyperosmotic stress, and then gradually recovered, but remained still low after 3 hours of incubation in a hyperosmotic medium (Figures 1 and 2). This observation is in line with transmitted light microscopy imaging, which showed cell volume shrinkage consistent with dehydration shortly after exposing N2a cells to the hyperosmotic medium. It also showed that the cell volume did not recover throughout the observation time – in line with what was previously observed for other neuronal cells[19, 20]. However, the half recovery time of eGFP fluorescence lifetime, $\tau_{FL,eGFP}$ and the Y/C ratio after stress medium addition were different, 9.7 s and 73.3 s, respectively, and eGFP lifetime changed faster than the GimRET Y/C ratio, reaching a minimum value a few seconds earlier (Figure 3). A possible explanation for this difference is that in GimRET conformational changes between the two linked fluorescent proteins affect FRET efficiency. The conformational rearrangement may be way slower than dynamic quenching of eGFP fluorescence, which may occur through a number of processes that increase radiation-less deactivation of eGFP, such as collisional quenching propelled by transiently increased concentration due to increased MMC and/or rapid reduction of cell volume.

Given that diffusion of non-reacting biomolecules is directly affected by the viscosity of the surrounding medium and that eGFP is not chemically reactive in cells, the significantly slower diffusion of monomeric eGFP is likely indicative of increased viscosity inside the cells primarily due to increased MMC [21], rather than due to changes in eGFP conformation, activity and/or quinary interactions [22] caused by volume reduction due to exposure to hyperosmotic stress.

Persistently high MMC in cells stimulates a direct apoptotic pathway[23]. For example, apoptosis signal-regulating kinase 3 (ASK3; also known as mitogen-activated protein kinase kinase kinase 15 [MAP3K15]) and its phosphorylation signaling play an important role in cell survival and osmotic stress response[24]. Since the MMC state in the cell affects kinase-substrate interaction[14], high MMC during hyperosmotic stress regulates phosphorylation signaling activity associated with hyperosmotic stress responses. Although a quantitative measurement system and stable fluorescent proteins against such intracellular environmental changes are indispensable to characterize such change, concentration

of several ions, and pH during dehydration easily change the photophysical property of fluorescent proteins, such as dynamic quenching, distorting the quantitative fluorescence measurement. An increase of intracellular MMC in *E.coli* during hyperosmotic stress was suggested using a FRET-based crowding sensor with a linker region that contains two α-helices and three random coils between a FRET pair: mCerulean3 and mCitrine, a chloride ion-insensitive YFP variant[25]; however, it has not been tested in mammalian cells. Furthermore, since we show that the recovery response of $\tau_{FL,eGFP}$ is faster than that of Y/C ratio of GimRET, these FRET-based crowding sensor may not show a rapid response due to the rate-determining step of structural change. We demonstrated that $\tau_{FL,eGFP}$ is sensitive to MMC change during hyperosmotic stress in mammalian cells, not affected by NaCl concentration and slight pH increase[26, 27]. Thus, FLIM using eGFP is a reliable method to measure MMC during hyperosmotic stress. Moreover, diffusion analysis by FCS is informative for determining intracellular viscosity. A somewhat shorter $\tau_{FL,eGFP}$ was observed in the nucleus than in the cytoplasm, while no difference was observed in diffusion state (Figures 1 and 4). We propose that MMC in the nucleus *via* chromatin condensation may be more susceptible to collisional quenching of eGFP. Therefore, a combination of both FLIM and FCS represents a more reliable method for determining MMC during hyperosmotic stress.

Accordingly, we demonstrate intracellular MMC and viscosity change during hyperosmotic stress using dynamic quenching of eGFP fluorescence. This strategy allows the quantitative analysis of various MMC states not only during hyperosmotic stress but also during other environmental changes in the cell.

Methods

Cell culture, transfection, and hyperosmotic medium

Murine neuroblastoma Neuro-2a (N2a) cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and maintained in DMEM (D5796, Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (12676029, Thermo Fisher Scientific, Waltham, MA), 100 U/mL penicillin G (Sigma-Aldrich) and 0.1 mg/ml streptomycin (Sigma-Aldrich) as previously described[28]. One day before transfection 2.0×10^5 N2a cells were transferred to a glass bottom dish (3910-035, IWAKI-AGC Technoglass, Shizuoka, Japan) for confocal imaging and FCS experiments or an 8-well chambered coverglass (155411, Thermo Fisher Scientific) for FLIM experiments. Plasmid DNAs were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol. After overnight incubation, the transfection medium was replaced with Opti-MEM I Reduced serum medium (Opti-MEM; 31985070, Thermo Fisher Scientific) for control experiments, which was thereafter replaced with Opti-MEM containing 290 mM NaCl for the hyperosmotic stress experiments.

Plasmids

To express the enhanced green fluorescent protein carrying the A206K monomeric mutation (eGFP), peGFP-C1 and pTKbX-eGFP plasmids were used for FLIM and FCS, respectively[28]. The plasmid for the crowding-sensitive glycine-inserted mutant of YFP (YFP1G) fused with CFP (GimRET) sensor is described elsewhere[7]. The DNA fragment encoding the T-cell-restricted intracellular antigen-1 (TIA-1) was digested from pFRT-TO-eGFP-TIA1 (#106094, Addgene) using Bsp1407I (TaKaRa, Shiga, Japan) and inserted into mCherry-C1 (mCherry-TIA1).

Multi-point fluorescence lifetime imaging microscopy (MP-FLIM)

The instrumental design and construction of the massively parallel imaging system without laser scanning is described previously[10, 11]. Briefly, simultaneous excitation of fluorescent molecules across the sample is achieved by passing a single pulsed laser beam through a DOE, which transforms it into a rectangular illumination matrix that consists of 16×16 foci. Fluorescent signal from 256 foci in the illumination matrix is detected by a matrix detector, single-photon avalanche photodiodes (SPAD) camera. Fluorescence and bright field images are captured by the 18.0 megapixel digital single-lens reflex camera Canon EOS 600D (Canon Inc., Tokyo, Japan) to identify GFP localization and visualize cell morphology. FLIM data acquisition and analysis was carried out using the MPD SPC3 software (http://www.micro-photon-devices.com/Software) and the homebuilt MP-FLIM software[10, 11]. Acquisition of a single FLIM curve lasted 3.7 s. $\tau_{FL,eGFP}$ was determined by fitting a single-exponential decay function to the experimental FLIM curves. To monitor the time course of FLIM changes, single FLIM curves were fitted and an average value from 9 spots in 5 cells is obtained for each time point (Figure 1D). To record data at steady-state conditions, that is before exposure to hyperosmotic stress and afterwards when a plateau value was reached, FLIM curves were acquired for 5 min and averaged to obtain low-noise FLIM curves at a single-pixel level and the average FLIM curve was fitted (Figure 1C).

Fluorescence correlation spectroscopy (FCS)

To measure eGFP diffusion under normal physiology, FCS measurements were performed in N2a cells transiently expressing eGFP that were grown in the Opti-MEM medium (control) in a glass bottom dish. To measure eGFP diffusion under hyperosmotic stress, the Opti-MEM cell culture medium was replaced with the hyperosmotic stress medium, Opti-MEM with 290 mM NaCl. FCS measurements were performed using an LSM510 META ConfoCor3 system (Carl Zeiss, Jena, Germany) equipped with a C-Apochromat 40×/1.2NA W Korr. UV-VIS-IR water immersion objective, a multi-line Argon laser and Avalanche Photodiode (APD) detectors. eGFP was excited using the 488 nm laser line of the Argon laser. To remove the detector-derived afterpulsing noise, fluorescence was split using a half mirror (HF50/50), and detected, after passing through a 505-540 nm band-pass filter (BP505-540),

using two APD detectors. Auto- and cross-correlation functions (ACFs and CCFs, respectively) were calculated for different lag times (τ) and plotted as a function of lag-time to derive corresponding auto- and cross-correlation curves. The cross-correlation curves were further analyzed by fitting a theoretical function for three-dimensional anomalous diffusion of one-component with blinking due only to an equilibrium between dark and bright states (Eq. 1) using the ZEN2012 (Carl Zeiss) software:

$$G(\tau) = 1 + \frac{1}{N}\left[\frac{T}{1-T}\exp\left(-\frac{\tau}{\tau_{\mathrm{Relax}}}\right)\right]\left[\frac{1}{\left(1+\left(\frac{\tau}{\tau_{\mathrm{D}}}\right)^{\alpha}\right)^{-1}\left(1+s^{-2}\left(\frac{\tau}{\tau_{\mathrm{D}}}\right)^{\alpha}\right)^{-\frac{1}{2}}}\right] \quad (1)$$

In Eq.1, $G(\tau)$ is the cross-correlation function; τ_{D} is the diffusion time of eGFP; α is the anomalous diffusion parameter[29]; N is the average number of eGFP molecules in the effective detection volume; s is the structure parameter representing the ratio of the radial (w_0) and the axial (z_0) $1/e^2$ radii of the effective volume; T is the exponential relaxation fraction; and τ_{Relax} is the relaxation time of eGFP blinking dynamics. In calibration experiments, the diffusion time (τ_{Rh6G}) and structure parameter (s) were determined using 0.1 μM Rhodamine 6G (Rh6G) as a standard. The diffusion coefficient of eGFP was calculated using equation 2.

$$D = \frac{\tau_{\mathrm{D}}}{\tau_{\mathrm{Rh6G}}} D_{\mathrm{Rh6G}} \quad (2)$$

where D is the diffusion coefficient of eGFP; D_{Rh6G} is the diffusion coefficient of Rh6G (414 $\mu m^2/s$); τ_{D} and τ_{Rh6G} are the diffusion times of eGFP and Rh6G, respectively.

Confocal fluorescence microscopy

Confocal images were acquired using a laser scanning microscope system LSM 510 META (Carl Zeiss) equipped with a C-Apochromat 40×/1.2NA W Korr. UV-VIS-IR water immersion objective, diode laser 405 nm, multi-line Argon and yellow He-Ne 594 nm laser, and photomultiplier tube (PMT) detectors. eGFP and mCherry were excited at 488 nm and 594 nm, respectively. Excitation and emission were separated using the main dichroic beam splitter HFT488/594. To spectrally separate eGFP and mCherry fluorescence, the secondary dichroic mirror NFT545 was used to split fluorescence emission at 545 nm. eGFP fluorescence was detected after passing through a 505-550 nm band-pass filter (BP505-550), and mCherry fluorescence was detected after passing through a 615 nm long pass filter (LP615). The pinhole was set to 71 μm for eGFP and 86 μm for mCherry. GimRET fluorescence was excited at 405 nm. Excitation and emission light were separated using the main dichroic beam splitter HFT405/514/594. Fluorescence was detected using a spectrometric multi-channel detector; the

range 430-473 nm was collected for CFP and 558-655 nm for YFP. Pinhole was set to 300 μm. Acquired images were processed and analyzed using Fiji-ImageJ 1.52n. The Y/C ratio images were constructed by dividing in each pixel the background-subtracted fluorescence intensity of YFP with the background-subtracted fluorescence intensity of CFP and smoothed using the Gaussian-blur function in ImageJ.

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Author contribution

AK and MK designed the experiments and prepared the first draft of the manuscript. AK performed and analyzed confocal imaging using eGFP, GimRET, and mCherry-TIA1, in addition to preparation of all plasmids. HK and MO performed the FCS experiments and analyzed the data together with AK and MK. SO and HK performed the mpFLIM experiments and analyzed the data under the supervision of VV. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Figure legends

Figure 1. Hyperosmotic stress-induced changes in macromolecular crowding (MMC) followed by phase separation observed through the formation of stress granules (SGs) and environmental changes reflected by variations in eGFP fluorescence lifetime

(A) Fluorescence and transmitted light images of live N2a cells transiently expressing eGFP monomers and TIA1-mCherry when grown under normal conditions (upper row) and upon exposure to hyperosmotic stress (lower row). The images were acquired using Confocal Laser Scanning Microscopy. Scale bar: 10 μm. The white arrows point to stress granules (SGs). **(B)** Fluorescence images of eGFP-expressing N2a cells acquired using the home-built microscope for massively parallel fluorescence lifetime imaging (mpFLIM) superimposed on corresponding brightfield images. Scale bar: 10 μm. Bright green spots indicate individual excitation foci where FLIM measurements were simultaneously performed. **(C)** Box and whisker plots showing eGFP fluorescence lifetime in the cytoplasm and the nucleus of N2a cells under normal conditions (light grey) and during hyperosmotic stress (dark grey). The numbers on the box graph show mean ± SD. The number of independent spots in which eGFP fluorescence lifetime was measured were: 87 in the cytoplasm and 37 in the nuclei of 8 cells under normal physiology, and 84 spots in the cytoplasm and 70 in the nuclei of 15 cells exposed to hyperosmotic stress. Statistical significance was tested by Student's *t* test: ***$p < 0.001$. **(D)** Time course of changes in eGFP fluorescence lifetime in live N2a cells before and after exposure to hyperosmotic stress, recorded using the mpFLIM system. The hyperosmotic stress medium was added at 0 s. Dots and bars show mean ± SD (the number of analyzed spots from 5 cells, n = 9).

Figure 2. Hyperosmotic stress-induced changes in intracellular macromolecular crowding (MMC) monitored using the using the MMC-sensitive GimRET sensor.

(A) Fluorescence time-lapse images before (- 2 s) and after the addition of 290 mM NaCl to N2a cells expressing the MMC-sensitive sensor GimRET. Time of NaCl addition was at 0 s. The Y/C ratio was determined by dividing the background-corrected fluorescence intensity in the YFP channel by the background-corrected fluorescence intensity in the CFP channel and smoothed using the Gaussian blur function. The pseudo color scale is shown in the right bottom corner. Scale bar: 20 μm. **(B)** Corresponding changes in the Y/C ratio over time. Dots and bars indicate mean ± SD (the number of analyzed cells, n = 18). **(C)** Box and whisker plot showing Y/C ratio values measured in live N2a cells expressing the GimRET sensor after 3 hours incubation in hyperosmotic or control medium (the number of analyzed cells, n = 53). Statistical significance was tested by Student's *t* test: ***$p < 0.001$.

Figure 3. eGFP fluorescence lifetime is an earlier marker of hyperosmotic stress-induced macromolecular crowding (MMC) than the Y/C ratio of the MMC-sensitive GimRET probe

Normalized recovery curves showing changes in eGFP fluorescence lifetime (pink) and Y/C ratio of GimRET (green) caused by hyperosmotic stress-induced MMC. The normalized curves are derived from the data shown in Figure 1D and Figure 2B. The thin solid lines are smoothed using adjacent averaging of two data points The dashed lines show an exponential recovery model fitted to all data points.

Figure 4. Hyperosmotic stress-induced changes in intracellular macromolecular crowding (MMC) slow down eGFP diffusion in live N2a cells and increase intracellular heterogeneity

(A) Autocorrelation curves normalized to the same amplitude, $G_n(\tau) = 1$ at $\tau = 10$ μs, reflecting differences in eGFP diffusion in live N2a cells before (Stress-) and after (Stress+) exposure to hyperosmotic stress, recorded in the cytoplasm (Cyt, solid line) and the nucleus (Nuc, dashed line). **(B)** Diffusion coefficient values determined by non-linear fitting of autocorrelation curves using Eq. 1 derived for anomalous three-dimensional diffusion of one-component with blinking due only to an equilibrium between dark and bright states. Bars and the numbers above them indicate mean ± SD (the number of analyzed cells, n = 10). (C) Box-and-whisker plot showing variations in the anomalous diffusion parameter (α) obtained by fitting Eq. 1 to autocorrelation curves shown in (B). The box indicates 25th and 75th percentile. The whisker indicates SD. The bold line shows mean value. The numbers above the bars indicate mean ± SD (the number of analyzed cells, n = 10).

References

1. Gleeson, D.; Corasanti, J. G.; Boyer, J. L., Effects of osmotic stresses on isolated rat hepatocytes. II. Modulation of intracellular pH. *The American journal of physiology* **1990,** *258* (2 Pt 1), G299-307.

2. Schliess, F.; Reinehr, R.; Haussinger, D., Osmosensing and signaling in the regulation of mammalian cell function. *The FEBS journal* **2007,** *274* (22), 5799-803.

3. Burkewitz, K.; Choe, K.; Strange, K., Hypertonic stress induces rapid and widespread protein damage in C. elegans. *American journal of physiology. Cell physiology* **2011,** *301* (3), C566-76.

4. Anderson, P.; Kedersha, N., RNA granules. *The Journal of cell biology* **2006,** *172* (6), 803-8.

5. Protter, D. S. W.; Parker, R., Principles and Properties of Stress Granules. *Trends in cell biology* **2016,** *26* (9), 668-679.

6. Kitamura, A.; Nagata, K.; Kinjo, M., Conformational analysis of misfolded protein aggregation by FRET and live-cell imaging techniques. *International journal of molecular sciences* **2015,** *16* (3), 6076-92.

7. Morikawa, T. J.; Fujita, H.; Kitamura, A.; Horio, T.; Yamamoto, J.; Kinjo, M.; Sasaki, A.; Machiyama, H.; Yoshizawa, K.; Ichimura, T.; Imada, K.; Nagai, T.; Watanabe, T. M., Dependence of fluorescent protein brightness on protein concentration in solution and enhancement of it. *Sci Rep* **2016,** *6*, 22342.

8. Lakowicz, J. R., *Principles of fluorescence spectroscopy*. 3rd ed.; Springer: New York, 2006; p xxvi, 954 p.

9. Chang, C. W.; Sud, D.; Mycek, M. A., Fluorescence lifetime imaging microscopy. *Methods in cell biology* **2007,** *81*, 495-524.

10. Vitali, M.; Bronzi, D.; Krmpot, A. J.; Nikolic, S. N.; Schmitt, F. J.; Junghans, C.; Tisa, S.; Friedrich, T.; Vukojevic, V.; Terenius, L.; Zappa, F.; Rigler, R., A Single-Photon Avalanche Camera for Fluorescence Lifetime Imaging Microscopy and Correlation Spectroscopy. *Ieee J Sel Top Quant* **2014,** *20* (6).

11. Oasa, S.; Krmpot, A. J.; Nikolic, S. N.; Clayton, A. H. A.; Tsigelny, I. F.; Changeux, J. P.; Terenius, L.; Rigler, R.; Vukojevic, V., Dynamic Cellular Cartography: Mapping the Local Determinants of Oligodendrocyte Transcription Factor 2 (OLIG2) Function in Live Cells Using Massively Parallel Fluorescence Correlation Spectroscopy Integrated with Fluorescence Lifetime Imaging Microscopy (mpFCS/FLIM). *Analytical chemistry* **2021,** *93* (35), 12011-12021.

12. Pliss, A.; Levchenko, S. M.; Liu, L.; Peng, X.; Ohulchanskyy, T. Y.; Roy, I.; Kuzmin, A. N.; Qu, J.; Prasad, P. N., Cycles of protein condensation and discharge in nuclear

organelles studied by fluorescence lifetime imaging. *Nature communications* **2019,** *10* (1), 455.
13. Kitamura, A.; Inada, N.; Kubota, H.; Matsumoto, G.; Kinjo, M.; Morimoto, R. I.; Nagata, K., Dysregulation of the proteasome increases the toxicity of ALS-linked mutant SOD1. *Genes to cells : devoted to molecular & cellular mechanisms* **2014,** *19* (3), 209-24.
14. Sadaie, W.; Harada, Y.; Matsuda, M.; Aoki, K., Quantitative in vivo fluorescence cross-correlation analyses highlight the importance of competitive effects in the regulation of protein-protein interactions. *Molecular and cellular biology* **2014,** *34* (17), 3272-90.
15. Pack, C.; Saito, K.; Tamura, M.; Kinjo, M., Microenvironment and effect of energy depletion in the nucleus analyzed by mobility of multiple oligomeric EGFPs. *Biophysical journal* **2006,** *91* (10), 3921-36.
16. Kuznetsova, I. M.; Turoverov, K. K.; Uversky, V. N., What macromolecular crowding can do to a protein. *International journal of molecular sciences* **2014,** *15* (12), 23090-140.
17. Saxton, M. J., A biological interpretation of transient anomalous subdiffusion. I. Qualitative model. *Biophysical journal* **2007,** *92* (4), 1178-91.
18. Woringer, M.; Izeddin, I.; Favard, C.; Berry, H., Anomalous Subdiffusion in Living Cells: Bridging the Gap Between Experiments and Realistic Models Through Collaborative Challenges. *Front Phys-Lausanne* **2020,** *8*.
19. Mackert, B. M.; Staub, F.; Peters, J.; Baethmann, A.; Kempski, O., Anoxia in vitro does not induce neuronal swelling or death. *Journal of the neurological sciences* **1996,** *139* (1), 39-47.
20. Wilson, C. S.; Mongin, A. A., Cell Volume Control in Healthy Brain and Neuropathologies. *Curr Top Membr* **2018,** *81*, 385-455.
21. Rashid, R.; Chee, S. M.; Raghunath, M.; Wohland, T., Macromolecular crowding gives rise to microviscosity, anomalous diffusion and accelerated actin polymerization. *Physical biology* **2015,** *12* (3), 034001.
22. Gnutt, D.; Timr, S.; Ahlers, J.; Konig, B.; Manderfeld, E.; Heyden, M.; Sterpone, F.; Ebbinghaus, S., Stability Effect of Quinary Interactions Reversed by Single Point Mutations. *Journal of the American Chemical Society* **2019,** *141* (11), 4660-4669.
23. Rana, P. S.; Kurokawa, M.; Model, M. A., Evidence for macromolecular crowding as a direct apoptotic stimulus. *Journal of cell science* **2020,** *133* (9).
24. Watanabe, K.; Umeda, T.; Niwa, K.; Naguro, I.; Ichijo, H., A PP6-ASK3 Module Coordinates the Bidirectional Cell Volume Regulation under Osmotic Stress. *Cell reports* **2018,** *22* (11), 2809-2817.
25. Liu, B.; Hasrat, Z.; Poolman, B.; Boersma, A. J., Decreased Effective Macromolecular Crowding in Escherichia coli Adapted to Hyperosmotic Stress. *Journal of bacteriology* **2019,** *201* (10).
26. Nakabayashi, T.; Wang, H. P.; Kinjo, M.; Ohta, N., Application of fluorescence lifetime imaging of enhanced green fluorescent protein to intracellular pH measurements. *Photochemical &*

photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology **2008,** *7* (6), 668-70.

27. Suhling, K.; Siegel, J.; Phillips, D.; French, P. M.; Leveque-Fort, S.; Webb, S. E.; Davis, D. M., Imaging the environment of green fluorescent protein. *Biophysical journal* **2002,** *83* (6), 3589-95.

28. Kitamura, A.; Nakayama, Y.; Shibasaki, A.; Taki, A.; Yuno, S.; Takeda, K.; Yahara, M.; Tanabe, N.; Kinjo, M., Interaction of RNA with a C-terminal fragment of the amyotrophic lateral sclerosis-associated TDP43 reduces cytotoxicity. *Scientific reports* **2016,** *6*, 19230.

29. Banks, D. S.; Fradin, C., Anomalous diffusion of proteins due to molecular crowding. *Biophysical journal* **2005,** *89* (5), 2960-71.

Fig. 1

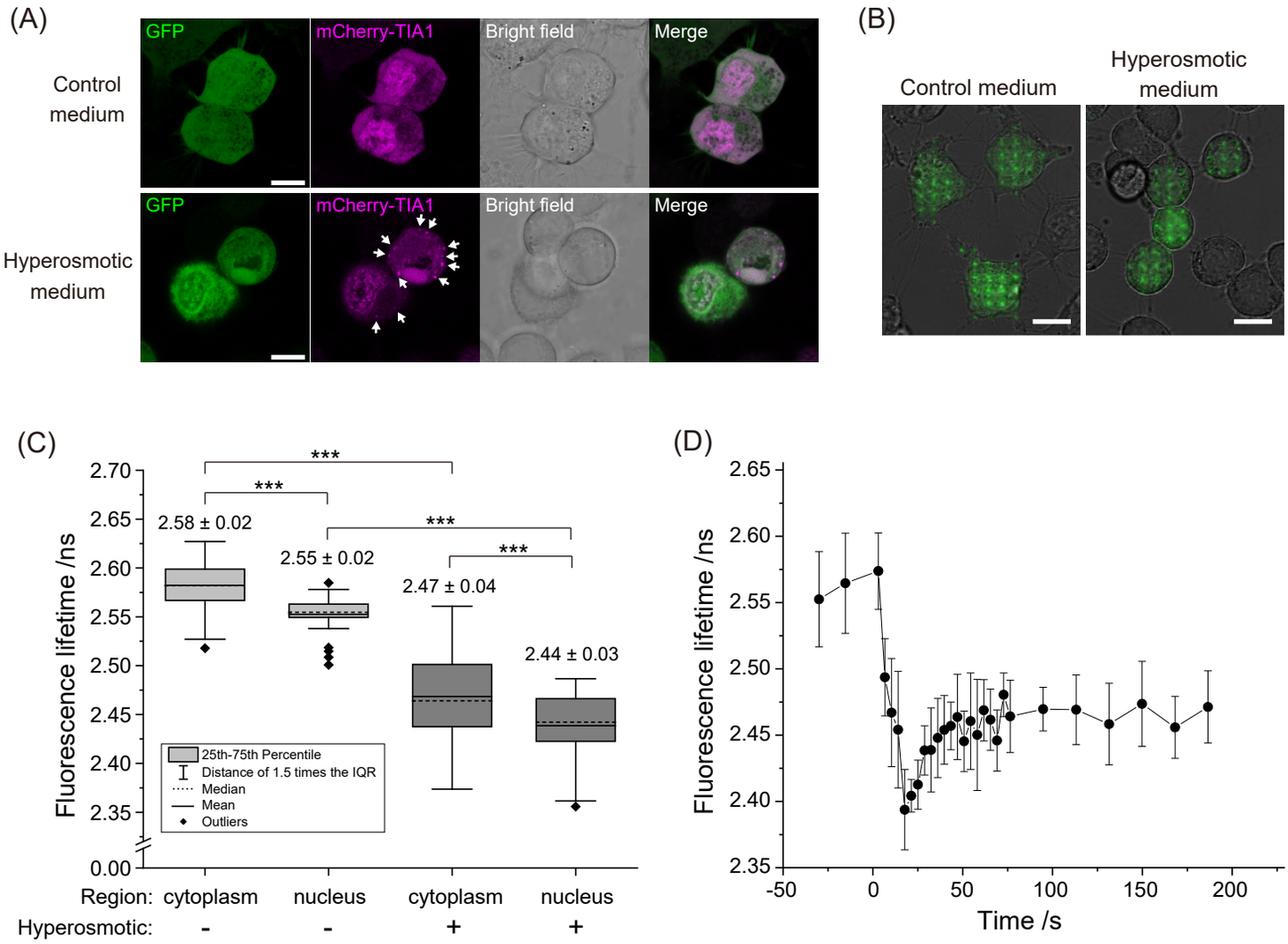

(A)
Control medium
Hyperosmotic medium
GFP
mCherry-TIA1
Bright field
Merge
(B)
Control medium
Hyperosmotic medium
(C)
Fluorescence lifetime /ns
2.58 ± 0.02
2.55 ± 0.02
2.47 ± 0.04
2.44 ± 0.03
***
25th-75th Percentile
Distance of 1.5 times the IQR
Median
Mean
Outliers
Region: cytoplasm nucleus cytoplasm nucleus
Hyperosmotic: - - + +
(D)
Fluorescence lifetime /ns
Time /s

Fig. 2

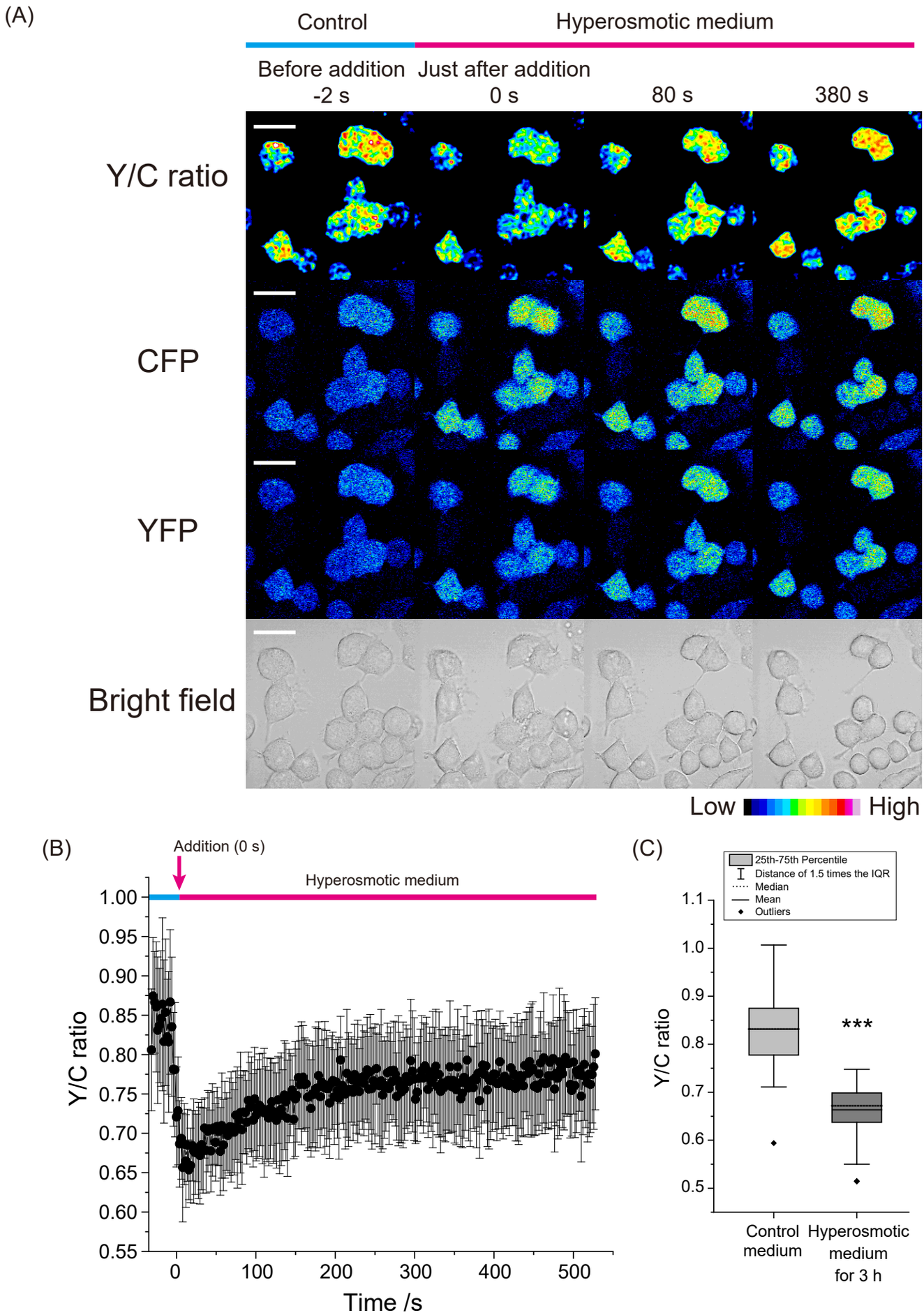

(A)
Control
Hyperosmotic medium
Before addition
Just after addition
-2 s
0 s
80 s
380 s
Y/C ratio
CFP
YFP
Bright field
Low
High
(B)
Addition (0 s)
Hyperosmotic medium
Y/C ratio
Time /s
(C)
25th-75th Percentile
Distance of 1.5 times the IQR
Median
Mean
Outliers
Y/C ratio
***
Control medium
Hyperosmotic medium for 3 h

Fig. 3

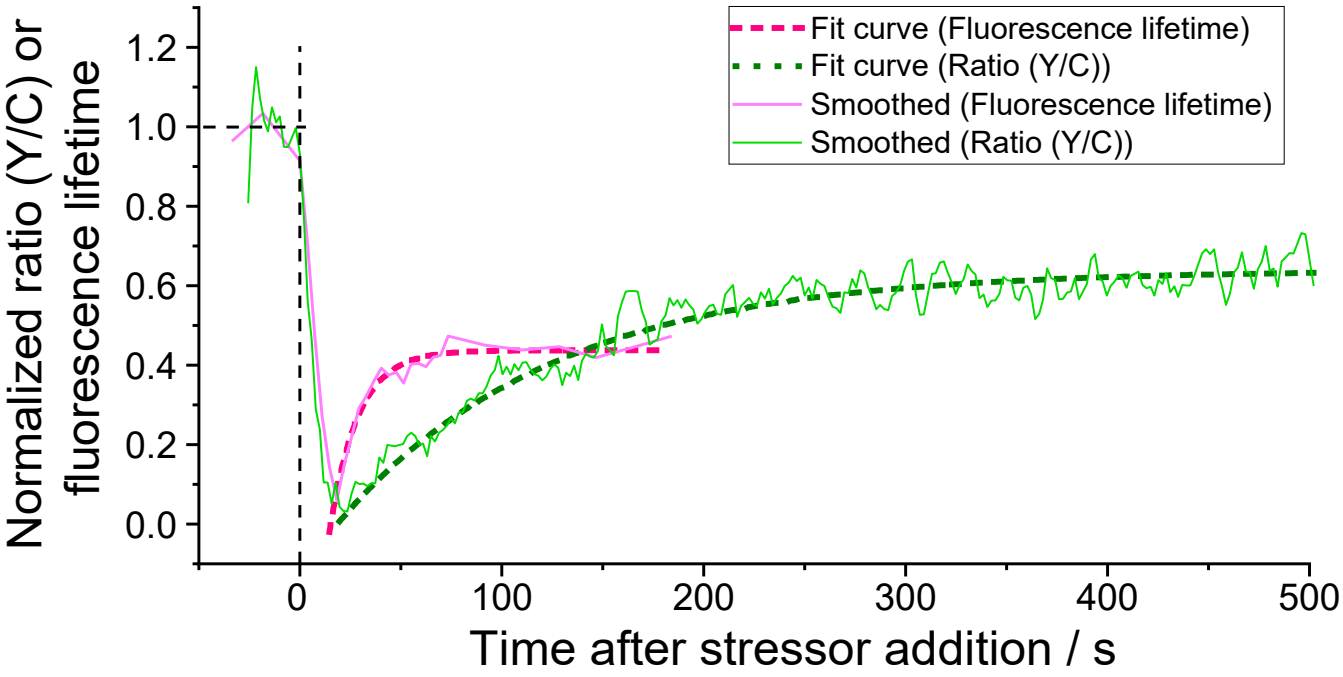
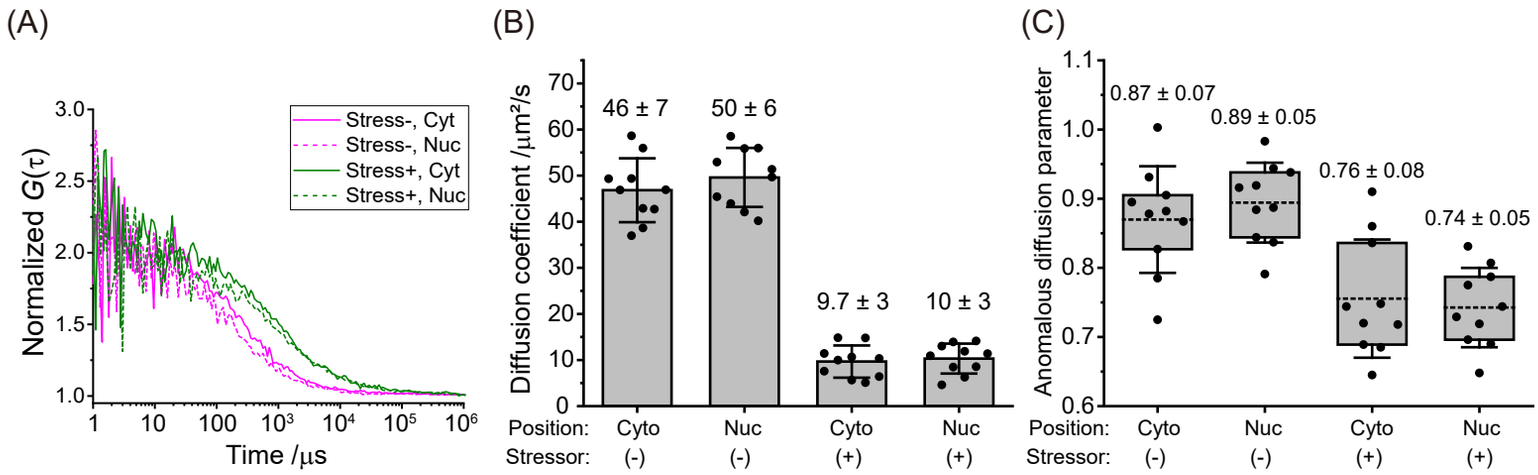

Fit curve (Fluorescence lifetime)
Fit curve (Ratio (Y/C))
Smoothed (Fluorescence lifetime)
Smoothed (Ratio (Y/C))
Normalized ratio (Y/C) or fluorescence lifetime
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
Time after stressor addition / s

Fig. 4


(A)
Normalized G(τ)
Time /μs
Stress-, Cyt
Stress-, Nuc
Stress+, Cyt
Stress+, Nuc
(B)
Diffusion coefficient /μm²/s
46 ± 7
50 ± 6
9.7 ± 3
10 ± 3
Position: Cyto Nuc Cyto Nuc
Stressor: (-) (-) (+) (+)
(C)
Anomalous diffusion parameter
0.87 ± 0.07
0.89 ± 0.05
0.76 ± 0.08
0.74 ± 0.05
Position: Cyto Nuc Cyto Nuc
Stressor: (-) (-) (+) (+)