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An improved macromolecular crowding sensor CRONOS for detection of crowding changes in membrane-less organelles under stressed conditions

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ABSTRACT

Membrane-less organelles (MLOs) formed by liquid-liquid phase separation (LLPS) play pivotal roles in biological processes. During LLPS, proteins and nucleotides are extremely condensed, resulting in changes in their conformation and biological functions. Disturbed LLPS homeostasis in MLOs is thought to associate with fatal diseases such as amyotrophic lateral sclerosis. Therefore, it is important to detect changes in the degree of crowding in MLOs. However, it has not been investigated well due to the lack of an appropriate method. To address this, we developed a genetically encoded macromolecular crowding sensor CRONOS (crowding sensor with mNeonGreen and mScarlet-I) that senses the degree of macromolecular crowding in MLOs using a fluorescence resonance energy transfer (FRET) system. CRONOS is a bright biosensor with a wide dynamic range and successfully detects changes in the macromolecular volume fraction in solution. By fusing to the scaffold protein of each MLO, we delivered CRONOS to MLO of interest and detected previously undescribed differences in the degree of crowding in each MLO. CRONOS also detected changes in the degree of macromolecular crowding in nucleolus induced by environmental stress or inhibition of transcription. These findings suggest that CRONOS can be a useful tool for the determination of molecular crowding and detection of pathological changes in MLOs in live cells.

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1. Introduction

Eukaryotic cells contain extremely high concentrations of proteins at 50–200 mg/ml and are filled with various nucleic acids and macromolecules in addition to proteins [1]. Such high concentrations of proteins in cells are crucial for efficient biochemical reactions [2]. In order for these molecules to coordinately maintain vital activities while avoiding disorderly mixing, it is efficient for biomolecules to form local compartments. The examples of classical compartments are intracellular membranous organelles such as mitochondria and endoplasmic reticulum partitioned by lipid membranes. The membranous organelles are suitable for sustaining constitutively specific biochemical reactions in an isolated environment. On the other hand, it has been reported that molecules that are not captured in the membrane organelles transiently undergo liquid-liquid phase separation (LLPS) and form membrane-less organelles (MLOs), which is preferable for transient biochemical reactions in response to various stimuli [3]. The biological roles of MLOs are highly diverse: nucleolus is the site for ribosome biogenesis and protein quality check under stress conditions [4,5]; stress granules control mRNA stability and

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protein translation under stress conditions [6]; nuclear speckles are the site for storage and modification of splicing factors [7]. MLOs consist of scaffold proteins specific for each MLO, their component proteins, and nucleic acids, and these proteins and nucleic acids undergo LLPS to form each MLO [8,9]. During the phase-separation process, the scaffolding proteins and nucleic acids are highly condensed in the droplet through biophysical forces such as electrostatic force, cation-pi interaction, and hydrophobic interaction derived from its component proteins and nucleic acids [10,11]. It is estimated that the concentration of specific proteins which undergo phase-separation can be up to more than hundreds-fold in the droplets compared with the concentration in the supernatant fraction [12]. Such a high density of specific species of proteins can alter the fashion of protein-protein interaction, reaction kinetics, protein conformation, and overall functions. In addition, disturbance of LLPS by pathological changes of the composing proteins result in impairment of LLPS homeostasis and malfunction of MLO, associating with fatal diseases including amyotrophic lateral sclerosis (ALS) [13,14]. Although MLOs play pivotal roles in a wide range of biological phenomena, there are many uncertain points to be clarified. For example, it remains unknown whether the degree of crowding varies in each type of MLO. Furthermore, it is unclear how the crowding of MLO is affected by biological processes, due to the lack of appropriate tools to measure them. The first attempt to directly measure macromolecular crowding in cells was achieved by the FRET-based biosensor with mCerluean-mCitrine (mCer-mCit) [15]. The sensor and its derivatives successfully detected the crowding change in cells induced by the osmotic shock and aging process [16–19]. To investigate macromolecular crowding especially in MLOs in live cells, we developed a FRET-based biosensor CRONOS (crowding sensor with mNeonGreen and mScarlet-I) by modifying the previously reported mCer-mCit sensor. CRONOS successfully detected the degree of macromolecular crowding of various MLO in live cells under normal and stressed conditions.

2. Materials and methods

2.1. Crowding sensor design

The fluorescent protein-based FRET sensor was designed based on the previously reported molecular crowding sensor with a FRET pair of mCerulean-mCitrine (mCer-mCit) [15]. The sensor consists of mCer as a FRET donor and mCit as a FRET acceptor, sandwiching a linker consisting of α-helix forming sequences. We sought for a better FRET-pairs for the sensor and found that the mNeonGreen-mScarlet-I (mNG-mScaI) pair would work better [20]. CRONOS (crowding sensor with mNeonGreen and mScarlet-I) consists of mNG as a FRET donor and mScaI as a FRET acceptor, sandwiching the same linker which is used in the mCer-mCit sensor. The cDNA encoding CRONOS was synthesized and subcloned into pET28a vector containing His_6 tag (Genscript, NJ, USA).

2.2. Purification of recombinant CRONOS

E. coli BL21 strain was transformed with pET28a-CRONOS and plated on an LB-agar plate containing ampicillin, incubated overnight. A single colony was picked up and after culturing overnight at 37 °C, the expression of recombinant CRONOS was induced by incubation with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 30 °C. The recombinant CRONOS was purified with His 60 Ni Gravity Column Purification Kit (Clontech) following a manufacturer's protocol. After elution by 300 mM imidazole, the recombinant CRONOS was dialyzed with phosphate-buffered saline (PBS) using Slide-A-Lyzer dialysis cassette (ThemoFisher) for 4 h. The purified CRONOS was then frozen until use.

2.3. In vitro experiments

Recombinant CRONOS was challenged with crowding macromolecules, polyethylene glycol (PEG) of various molecular weights, Ficoll PM400 and D-glucose dissolved in PBS at different concentrations (5–40% weight/volume) as indicated. CRONOS was added to the solution at the final 2–3 μg/ml otherwise mentioned. After mixing the solution and sensor, the FRET efficiency was assessed by RF-6000 fluorescence spectrophotometer (Shimadzu, Kyoto, Japan). The donor mNG was excited at 506 nm and the emission spectra were measured. To calculate the FRET ratio of CRONOS, the fluorescence intensity of 588 nm was divided by the fluorescence intensity of 522 nm.

2.4. Cellular experiments

For cytosolic expression in mammalian cells, cDNA encoding CRONOS was subcloned into pcDNA3.1 (+) vector. For targeting the sensor to each MLO, we cloned cDNA of each MLO marker protein by PCR with HeLa cell cDNA, and the CRONOS was fused to N-terminus of human nucleophosmin 1 (NPM1) (nucleolus) or N-terminus of human TIA1 (stress granule). HeLa cells were cultured in Dulbecco-modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, maintained in a standard cell culture condition. For imaging, HeLa cells, plated on a chambered cover glass (Matsunami) or a chambered slide glass (Matsunami), were transiently transfected with pcDNA3.1-CRONOS derivatives using Lipofectamine 2000 reagent (ThermoFisher). The cells were challenged with heat shock at 42 °C for 3 h, or 2 μg/ml of Actinomycin D treatment for 3 h. The cells were imaged with LSM710 laser confocal microscopy using a 63 × water immersion objective lens (Zeiss) approximately 24 h after transfection. CRONOS was imaged by exciting the donor mNG at 488 nm and collecting two simultaneous images of the donor mNG (520–560 nm) and the acceptor mScaI (590LP) at the same gain settings. Image ratio calculation was performed with Fiji software (www.imagej.net).

2.5. Statistics

All data are represented as mean ± standard deviation. Statistical analysis was performed with SPSS software 26 (IBM).

3. Results

3.1. Design of CRONOS and its characterization

There are several types of macromolecular crowding sensors using the FRET systems such as fluorescent protein-based sensors and nucleic acid-based sensors [15,17,21,22]. Because we intended to investigate the degree of crowding in MLOs, we needed to control the delivery of the sensor to specific MLO of interest. For this end, the protein-based sensor has the advantage to add organelle-targeting signal sequences. The previously reported mCer-mCit crowding sensor consists of N-terminal mCer (cyan fluorescent protein) and C-terminal mCit (yellow fluorescent protein) sandwiching two α-helical peptides with a hinge which works as a conformationally flexible linker, and the sensor could detect cellular response to osmotic stress induced by sorbitol in live cells [15]. The physiological changes of macromolecular crowding in MLOs might be more subtle than the changes induced by chemical macromolecular crowders and thus we sought for a better FRET pair that confers a wider dynamic range to the sensor. From the literature search, we chose the FRET pair with best performance [20] consisting of mNG (green fluorescent protein: ex. 506 nm/em. 517 nm) [23] as a donor protein and mScaI (red fluorescent protein:

ex. 569 nm/em. 593 nm) [24] as an acceptor protein and named the sensor CRONOS (crowding sensor with mNeonGreen and mScarlet-I) (Fig. 1A). Both mNG and mScaI work as a monomer with minimal self-association [23,24]. When we challenged these sensors to a macromolecular crowding reagent, 20%-PEG8000, CRONOS successfully detected the macromolecular crowding (Fig. 1B). To deny the possibility that the change was caused by self-associated intermolecular FRET between multiple sensor molecules due to high concentration, we titrated the concentration of CRONOS from 10 nM to 200 nM, and we confirmed the FRET ratio was not changed by the concentration of the sensor (Fig. 1C). We also examined whether the expression levels of CRONOS affect the FRET ratio in live cells. We expressed CRONOS in HeLa cells, and measured the fluorescence intensity of mNG and FRET signals and we confirmed that the FRET ratio was not significantly affected by the expression level (Fig. 1D).

3.2. CRONOS detects changes in macromolecular crowding by various crowding reagents

Next, we performed a detailed characterization of CRONOS. To avoid a leak of excitation light, we used fluorescence intensity at 522 nm as mNG signal hereafter. Titration of CRONOS with PEG300 or PEG8000 increased FRET ratio (1.57-fold increase in 40% solution of PEG300, 9.0-fold increase in 40% solution of PEG8000, respectively) but D-glucose did not increase FRET ratio either (Fig. 2A, B, 2C, and 2D). The original mCer-mCit sensor showed 2.86-fold increase in 40% solution of PEG8000, indicating that CRONOS have a wide dynamic range to measure macromolecular concentration (Fig. 2D). We also measured the change of FRET ratio by Ficoll, and CRONOS responded with the incremental concentration of Ficoll (Fig. 2E). We next examined the effect of volume exclusion effect of different size of PEG. When the molecular weight of PEG increased, the volume exclusion effect also gets greater, suggesting that the bending of the sensor efficiently occurs (Fig. 2F). When we mixed the CRONOS with 10% of PEG with different molecular weights, the FRET ratio was increased according to the molecular weight of PEG (Fig. 2G and H).

3.3. CRONOS detects differences in macromolecular crowding levels in MLOs

Next, we tested whether CRONOS can detect the macromolecular crowding in live cells, especially in MLOs. To this end, we made three CRONOS constructs, CRONOS (without targeting sequence), CRONOS-NPM1 (nucleolus-targeted), and CRONOS-TIA1 (stress granule-targeted). In previous studies, both of the protein-based crowding sensor and nucleic acid-based sensor indicate that cytosolic macromolecular density is higher than that of the nucleus [21,22]. When we overexpressed CRONOS without targeting sequence in HeLa cells, it diffusely located to cytosol and nucleus (Fig. 3A). The FRET ratio of mScaI/mNG showed that nuclear crowding was lower than that of cytosol as reported previously (Fig. 3A). On the other hand, CRONOS-NPM1 mainly distributed to the nucleolus. Overexpression of TIA1 is reported to induce the formation of stress granules, and indeed CRONOS-TIA1 formed stress granule-like dots in the cytosol with the highest FRET ratio among organelles measured (Fig. 3A and B). These data showed that macromolecular crowding varies among MLOs.

We further examined if CRONOS can sense the physiological change of crowding in MLOs. The nucleolus is one of the most important and largest MLOs in cells and the nucleolar architecture dynamically changes in response to various stress conditions [25]. When the cells are exposed to extensive heat shock, the nucleolus

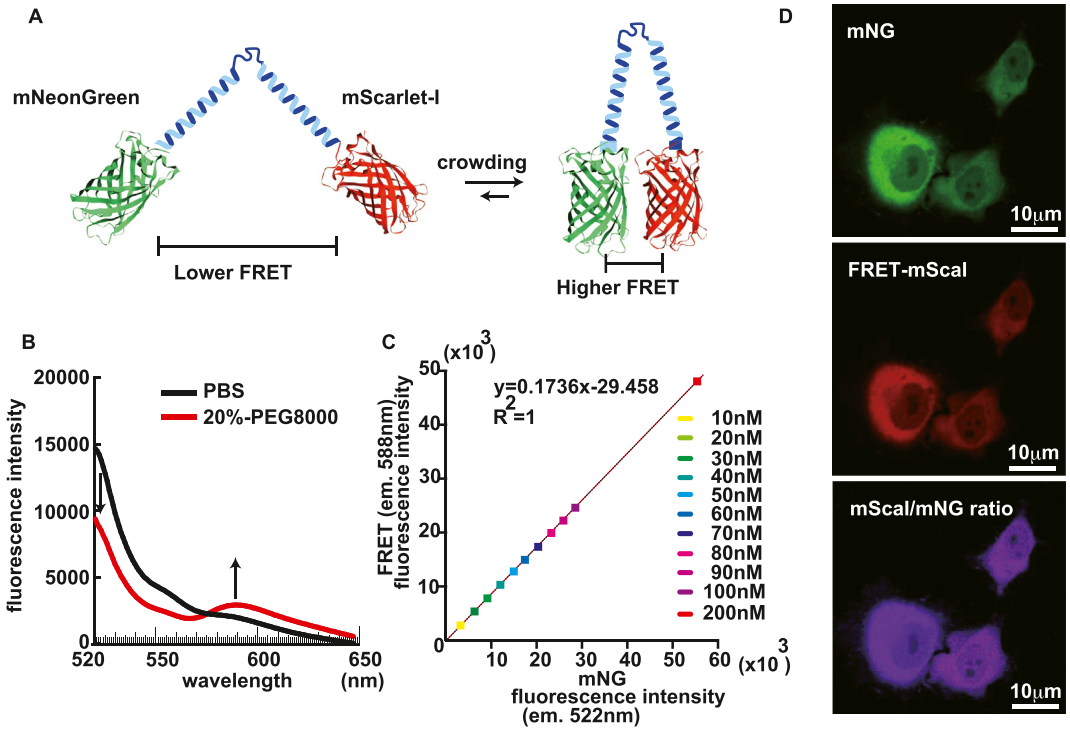

Fig. 1. Characterization of a bright, protein-based macromolecular crowding sensor CRONOS. (A) The scheme shows the design and concept of the CRONOS FRET system. The FRET donor and acceptor are connected via a conformationally flexible linker composed of two α-helical peptides with a hinge. Upon macromolecular crowding, the sensor confirmation gets more condensed, resulting in higher FRET efficiency. The template structure of the fluorescent protein was obtained from GFP (4EN1) on Protein Data Bank (PDB). (B) Fluorescence emission spectra of CRONOS in PBS or in 20% PEG8000-PBS revealed that macromolecular crowding by PEG increased FRET efficiency of CRONOS. (C) The effect of protein concentration on the FRET ratio. (D) Confocal images of CRONOS expressed in HeLa cells.

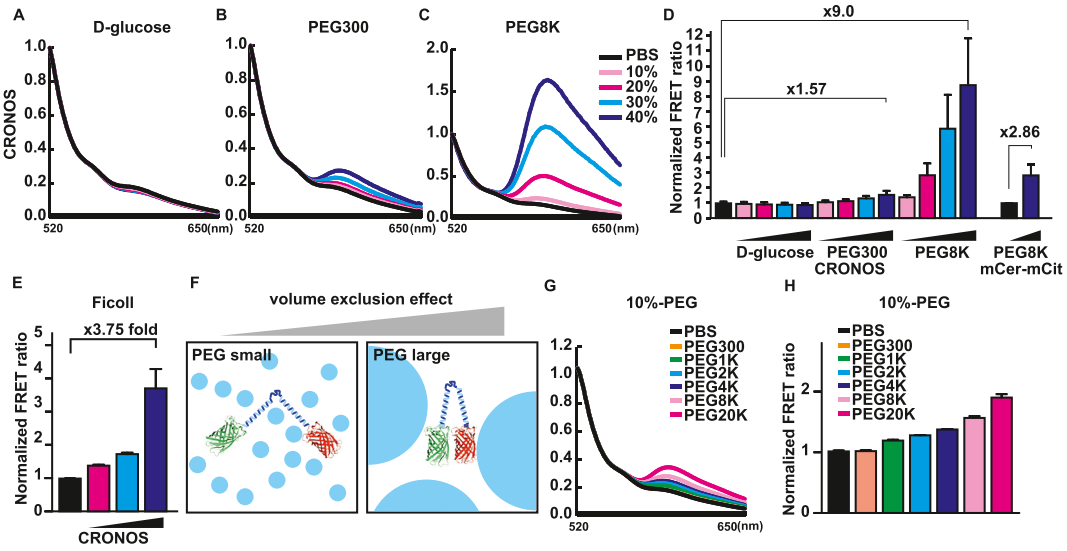

Fig. 2. CRONOS detects macromolecular crowding changes in solutions. (A) (B) (C) Fluorescence emission spectra of the CRONOS upon titration with 10%-, 20%-, 30%- or 40% of D-glucose (A), PEG300 (B), or PEG8000 (C) were shown. The fluorescence emission spectra were normalized to the donor fluorescence. (D) The FRET ratio of CRONOS exposed to different concentrations of D-glucose, PEG300, or PEG8000. (E) The FRET ratio of the CRONOS upon titration with 5%-, 10%-, 20% of Ficoll. (F) Schemes show the volume exclusion effects by different sizes of PEG molecules. (G) (H) Fluorescence emission spectra and FRET ratio of CRONOS exposed to 10% PEG with different molecular weights. The error bars indicate ±S.D. obtained from three independent measurements.

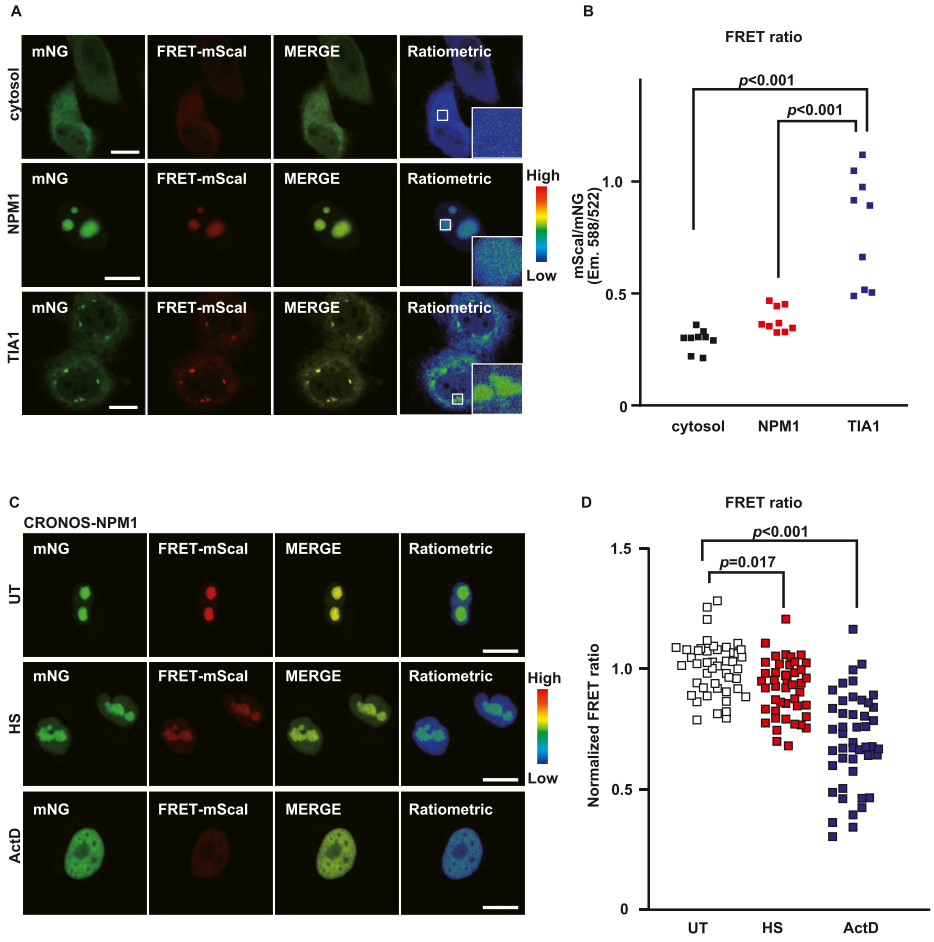

Fig. 3. CRONOS sensor detects the changes of macromolecular crowding in different membrane-less organelles. (A) (B) HeLa cells overexpressing CRONOS without tag (cytosol), CRONOS-NPM1 (nucleolus), or CRONOS-TIA1 (stress granule) were imaged by confocal microscopy. The cytosol, nucleolus, and stress granules were imaged with the same setting, enabling the comparison of their crowding. Whiter arrows showed nucleoli. The white squared areas were zoomed. (C) Confocal images HeLa cells expressing CRONOS-NPM1 under untreated condition (UT) (upper panels), heat shock (HS) (middle panels), or treatment with Actinomycin D (ActD) (lower panels). Heat shock was done by incubation at 42 °C for 3 h. Ratiometric images were pseudo-colored. (D) FRET ratio of CRONOS-NPM1. N = 45 cells/each condition. The scale bar shows 10 μm.

reportedly gets condensed, confirmed by transmission electron microscopy [25]. On the other hand, treatment with actinomycin D, an anti-cancer compound that inhibits RNA polymerase activity, causes dispersion of NPM1 from the nucleolus to nucleoplasm [5]. To examine the effects of environmental or chemical stress on nucleolus, the CRONOS-NPM1 sensor was expressed in HeLa cells, and the cells were exposed to heat shock (42 °C for 3 h) or actinomycin D (2 μg/ml for 3 h) (Fig. 3C). The treatment with actinomycin D caused nucleolar disruption and the release of CRONOS-NPM1 from the nucleolus to nucleoplasm with a little remnant in the nucleolus. The FRET ratio from the sensor in nucleolus revealed that the degree of crowding was unexpectedly reduced by heat shock (Fig. 3D). The effect of heat shock on the localization of CRONOS-NPM1 was negligible, and the effect of temperature on the FRET ratio of recombinant CRONOS was also little (data not shown), suggesting that attenuation of rRNA synthesis by heat shock may explain the lower density of the nucleolus [26]. The actinomycin D treatment strongly reduced the macromolecular crowding in nucleolus, also supporting the idea that repression of rRNA synthesis decreases nucleolar density. These results clearly indicate that CRONOS can detect changes in macromolecular crowding in the nucleolus.

4. Discussion

The emerging importance of LLPS and MLOs in biology provokes strong demand for the technique to measure the biochemical features in the phase-separated droplets. In this study, we optimized the FRET pair of the protein-based sensor, and successfully measured the macromolecular density of MLOs in live cells.

First, we sought for optimal FRET pair for creating a new crowding sensor with a wider working range, enabling easier detection. The previous study reported the superior performance of mNG-mScaI pair for FRET [20], and substitution of mCer to mNG as a FRET donor and mCit to mScaI as a FRET acceptor allowed us to create CRONOS, a novel crowding sensor. Because mNG is one of the brightest green fluorescent proteins [23] and mScaI is one of the brightest red fluorescent proteins [24], CRONOS is also very bright, enabling direct measurement of fluorescence in live cells with various modalities.

The advantage of a protein-based sensor over nucleic acid-based one is its controllable delivery to a specific region of interest by adding appropriate targeting signals [27]. When we fused the sensor to NPM1 or TIA1, we successfully delivered the sensor to different MLOs, nucleolus and stress granules. Although both nucleolus and stress granules are formed via LLPS, their ingredients are different and their crowding is also supposed to be different. Our study here revealed the previously undescribed difference of crowding among different MLOs: stress granules marked by TIA1 have a much dense environment inside compared with nucleolus marked by NPM1.

Finally, we successfully detected changes of crowding in nucleolus under stressed conditions such as heat shock or inhibition of transcription by CRONOS-NPM1. Because nucleolus is the site for the synthesis of ribosomal RNA, it was not surprising that suppression of transcription by heat shock or ActD treatment resulted in the less-crowded nucleolus. In conclusion, we present here a novel crowding sensor CRONOS which allowed us to measure macromolecular crowding in MLOs in live cells. Further investigation of crowding in MLOs under various conditions and disease-related status will provide a novel insight into the pathophysiology of diseases caused by malfunction of MLOs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.10.055.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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