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**Microenvironment and effect of energy depletion in the nucleus analyzed by mobility of multiple oligomeric EGFPs**

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## **ABSTRACT**

Four different tandem EGFPs were constructed to elucidate the nuclear microenvironment by quantifying its diffusional properties in both aqueous solution and the nuclei of living cells. Diffusion of tandem EGFP was dependent on the length of the protein as a rod-like molecule or molecular ruler in solution. On the other hand, we found two kinds of mobility, fast diffusional mobility and much slower diffusional mobility depending on cellular compartments in living cells. Diffusion in the cytoplasm and the nucleoplasm was mainly measured as fast diffusional mobility. In contrast, diffusion in the nucleolus was complex and mainly much slower diffusional mobility, although both the fast and the slow diffusional mobilities were dependent on the protein length. Interestingly, we found that diffusion in the nucleolus was clearly changed by energy depletion, even though the diffusion in the cytoplasm and the nucleoplasm was not changed. Our results suggest that the nucleolar microenvironment is sensitive to energy depletion and very different from the nucleoplasm.

## INTRODUCTION

The cell nucleus contains many proteins that form a multimolecular complex or a material such as chromatin and a nucleolus. Most of the proteins in the nucleus are concerned with molecular processing such as ribosome biogenesis, mRNA synthesis, transcription and molecular transportation to and from the nucleus. For these processes to be accomplished properly, proteins related to each process are expected to act dynamically and precisely in the nucleus. Consequently, the dynamics of various molecules such as RNAs and nuclear proteins in living cells have become a subject of major interest because mobilities of such molecules in the nucleus could provide important information about the molecular functions of the nucleus (1, 2, 3). On the other hand, such mobility of functional protein molecules in the nucleus might be mainly affected by the nuclear architecture and microenvironment (1, 4) as well as their function because the chromosomes and the nucleoli occupy a large portion of the nuclear space and changes depending on many factors such as gene expression, cell cycle progression, and other metabolic state of the cell. Therefore, for understanding the relation between functional proteins and nuclear microenvironment, it is helpful to analyze mobility of standard protein molecules with well-defined hydrodynamic properties as well as functional nuclear proteins (1, 5, 6) or labeled macromolecules (7).

In the last few years, many studies based on fluorescence microscopic techniques such as FRAP, single particle tracking (SPT), and fluorescence correlation spectroscopy (FCS) have been carried out for cell biology (8~15). The studies showed that a variety of small fluorescent probes such as BCECF (9), fluorescein-labeled macromolecules (dextran and Ficoll) from 3kD to 1000kD (13), and monomeric EGFP (14), move rapidly in the cytoplasm, whereas labeled linear dsDNA diffuse very slowly and has a size dependence of the diffusion constant (16). The key point of these studies is that the diffusion of small dextrans and Ficolls in the cytoplasm is only restricted mildly whereas that for large macromolecules can be greatly slowed.

On the other hand, a few studies of protein mobility in the cell nucleus have been carried out (10, 13, 14) with biologically inert protein, even though many studies have been carried out with nuclear proteins (1, 3, 6). A study based on FRAP and microinjection with diverse sizes of fluorescein-labeled dextrans (13) showed that diffusion in the nucleus was slowed approximately 4-fold compared with their diffusion in water. However, more variability in the measured data for the nucleus was found than for cytoplasm. Monomer GFP molecule showed much complex diffusion in nucleus than in cytoplasm (14). Recent studies of FRAP (1, 17) and FCS combined with FRAP experiment (18) using living cells have shown that various EGFP-fused nuclear proteins diffuse at different rates depending on their localization and function. Nuclear proteins could interact with target molecules or immobile structures such as chromatin, which slowed down the mobility of the proteins (5, 6, 19). An FCS experiment with monomeric EGFP showed that diffusion of EGFP, which is presumably inert to other proteins, was restricted depending on the position in the nucleus compared to diffusion in the cytoplasm (14). Furthermore, whether intranuclear mobility of many molecules result from passive diffusion or active transport is yet controversial (3, 20). The nuclear microenvironment, which may be one of the reasons, has yet not been clearly quantified under various physiological conditions.

FCS has been applied as a powerful technique for assessing biomolecular diffusion and interactions both in aqueous conditions and in living cells with single-molecule sensitivity (21~26). FCS detects fluorescence intensity fluctuations caused by Brownian motion of

fluorescent probe molecules in a tiny detection volume (~0.3 femtoliters) generated by confocal illumination. Through time correlation analysis of the fluorescence fluctuations, the diffusion coefficient, the molecular concentration, and the molecular interaction of probe molecules are accessible. Since FCS need only a very small detection volume and has high sensitivity, it will be also useful to measure diffusional mobility of proteins in very small regions of subnuclear microenvironments in living cells. Although FRAP is adequate for measuring the diffusion of fluorescent molecules and possible exchange in target organelles in the living cell (3, 7), the measurable minimal fluorescent intensity and diffusional speed range are limited to brighter and slower ranges than those for FCS. Therefore, we can anticipate that FCS will provide complementary information for faster movement at lower expression levels of various functional proteins in the nucleus.

EGFP is a powerful fluorescent bioprobe molecule with a well-known cylindrical structure (27~29). It has recently been used for various cell measurements in fluorescent imaging of cells as well as for analysis of molecular diffusion using FRAP and FCS. To develop a standard and reproducible method for diffusion analysis of proteins, we designed multiple oligomeric EGFPs with different molecular weights, which can be used as molecular rulers (MRs) for quantification of protein mobility in the nucleus. For this purpose, we constructed plasmids with different levels of oligomeric EGFP<sub>n</sub> (EGFP<sub>2</sub>~EGFP<sub>5</sub>, n=2 to 5) with molecular weights of 60, 90, 120 and 150 kD, respectively, tandemly linked by a random amino acid linker. Using multiple oligomeric EGFPs and FCS, we determined the diffusion of the proteins in the cytoplasm, nucleoplasm, and nucleoli of living HEK293, HeLa, and COS7 cells. For strict recognition of the two compartments in the nucleus, mRFP-fibrillarin and H2B-mRFP were used as red fluorescent markers for the nucleolus and the nucleoplasm, respectively.

In this study, FCS analysis by an one-component model showed that the diffusional mobility of EGFP<sub>n</sub> in aqueous solution was dependent on the length of EGFP<sub>n</sub> and was well consistent with the diffusion model of a rod-like structure. On the other hand, the diffusion of EGFP<sub>n</sub> in living cells analyzed by a two-component model showed that fast diffusional mobility in the cytoplasm and the nucleoplasm was consistent with the model of a rod-like molecule as shown in aqueous solution. The fast diffusion rates in the cytoplasm and the nucleoplasm were almost the same, and about 3.5-fold slower than in solution, regardless of the size of tandem EGFP<sub>n</sub> and cell type. Mobilities of tandem EGFP<sub>n</sub> found in the nucleoli of HeLa and COS7 cells were 5-fold and 7-fold slower than the fast diffusional mobility in the cytoplasm and the nucleoplasm, respectively. Moreover, the much slower diffusional mobility in the nucleolus was also dependent on the length of EGFP<sub>n</sub>, demonstrating tandem EGFP molecules was well-defined both in solution and in living cells. Interestingly, the slow diffusion in the nucleolus was related to the energy level of the living cell, because the slow diffusion of EGFP<sub>5</sub> in the nucleolus, but not in the cytoplasm and the nucleoplasm, was further slowed by ATP depletion.

## **MATERIALS AND METHODS**

### **Plasmid construction of tandem EGFP**

Plasmids expressing each tandem EGFP<sub>n</sub> were synthesized with the plasmid expressing EGFP-C1 (Clontech, Palo Alto, CA, USA). The EGFP-C1 was excised at the *Nde I* and the *Sma I* restriction sites and ligated between the *Nde I* and *Eco47 III* restriction sites of another EGFP-C1. The linker between EGFP<sub>n</sub> containing 25 random amino acid residues (SGLRSRAQASNSAVDGTAGPLPVAT) originated from the remaining bases of the multiple-cloning site. Plasmid constructs of H2B-mRFP and mRFP-fibrillarin were obtained as gifts from Drs. H. Kimura (Kyoto University, Kyoto, Japan) (30, 31) and T. Saiwaki (Osaka University, Osaka, Japan) (32), respectively. All plasmid constructs for transfection were purified using a plasmid DNA midiprep kit (QIAGEN).

### **Cell culture and expression of tandem EGFP<sub>n</sub> proteins**

For transient expression of tandem EGFP<sub>n</sub>, human embryonic kidney 293 (HEK293), HeLa, and COS7 cells were plated at confluence levels of 10-20% on LAB-TEK chambered coverslips with eight wells (Nalge Nunc Int., Naperville, IL, USA) for 12hrs or 24hrs before transfection. Cells were transfected with a EGFP<sub>n</sub> vector or cotransfected with a vector of EGFP<sub>n</sub> and H2B-mRFP or mRFP-fibrillarin, and grown in a 5% CO<sub>2</sub> humidified atmosphere at 37°C in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 10 mg/ml streptomycin. Transfection was carried out with FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany) or Effectene (QIAGEN, Hilden, Germany) as indicated by the manufacturer. The transfected cells were incubated for 24 hrs or 48 hrs and washed with Opti-MEM to remove phenol red dye in DMEM, and then the medium was replaced by Opti-MEM before LSM and FCS measurements. Energy depletion was performed by addition of 6mM 2-deoxyglucose (2-DG, Sigma) and 10mM sodium azide (NaN<sub>3</sub>, Sigma) to the culture medium (3, 20). LSM images were collected for the same cells before and after 2-DG and NaN<sub>3</sub> treatment.

### **Western immunoblotting**

The immunoblot analysis was performed according to the standard method. Cells expressing tandem EGFP<sub>n</sub> were grown on 10-cm culture plates for 48hrs after transfection, the BD Living Colors<sup>TM</sup> A.v. peptide antibody (BD Biosciences Clontech) was used as the primary antibody. Primary antibody-bound protein bands were detected with an alkaline phosphatase-conjugated secondary antibody (mouse anti-rabbit IgG, CHEMICON International) by BCIP/NBT dye solution (Sigma).

### **Cell homogenization**

After FCS measurements, the cultured cells on a LAB-TEK chambered coverslip were collected by centrifugation at 1500 rpm for 5min and then the pellets of cells were homogenized in 50 µl of buffer (10mM Hepes pH 7.9 containing 10mM NaCl, 3mM MgCl<sub>2</sub>, 1mM DTT, 0.4mM PMSF, and 0.1mM sodium orthovanadate). Each EGFP<sub>n</sub> protein solution was collected from the supernatant after centrifugation at 100,000 rpm for 20min and measured by FCS again.

### **Live cell imaging**

Fluorescence microscopy was performed using an LSM510 inverted confocal laser scanning

microscopy (LSM; Carl Zeiss, Jena, Germany). LSM observations were all performed at 25°C. EGFP<sub>n</sub> was excited at 488 nm of a CW Ar<sup>+</sup> laser through a water immersion objective lens (C-Apochromat, 40×, 1.2NA; Carl Zeiss) with emission detected above 505 nm for single scanning experiments using cells expressing EGFP<sub>n</sub>. Monomeric RFP-fibrillarlin or H2B-mRFP was imaged using a 543nm laser light and detection was above 560nm. The pinhole diameters for confocal imaging were adjusted to 70μm and 80μm for EGFP and mRFP, respectively. To avoid bleed-through effects in double-scanning experiments, EGFP and mRFP were scanned independently in a multitracking mode.

### FCS measurements and quantitative analysis

FCS measurements were all performed at 25°C on a ConfoCor 2 (Carl Zeiss) as described previously (24, 33). Excitation of EGFP was carried out at 488nm and 6.3mW by adjusting an acousto-optical tunable filter (AOTF) to 0.1%. Fluorescence autocorrelation functions (FAF;  $G(\tau)$ ), from which the average residence time ( $\tau_i$ ) and the absolute number of fluorescent proteins in the detection volume were obtained as follows;

$$G(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2} \quad (1)$$

where  $I(t+\tau)$  is the fluorescence intensity in single photon counting method obtained from the detection volume at delay time  $\tau$ . Brackets denote ensemble averages. The curve fitting for the multicomponent model is given by:

$$G(\tau) = 1 + \frac{1}{N} \sum_i y_i \left(1 + \frac{\tau}{\tau_i}\right)^{-1} \left(1 + \frac{\tau}{s^2 \tau_i}\right)^{-1/2} \quad (2)$$

where  $y_i$  and  $\tau_i$  are the fraction and diffusion time of component  $i$ , respectively.  $N$  is the number of fluorescent molecules in the detection volume defined by the beam waist  $w_0$  and the axial radius  $z_0$ ,  $s$  is the structure parameter representing the ratio of  $w_0$  and  $z_0$ . The detection volume made by  $w_0$  and  $z_0$  was approximated as a cylinder.

All FAFs in aqueous solutions were measured for 30s five times at 5s interval. In the case of intracellular measurement, FAFs were shortly measured for 15s one or three times and very low fluorescent cells under concentration of 20 molecules (<0.1M) per detection volume (0.3fl) were chosen for FCS measurement. Under these conditions, the effect of photobleaching on FCS analysis was minimized. The measurement position was chosen in the LSM image. Because the optical passes of LSM and FCS are not the same, the real position of FCS measurement was tuned to the position on LSM images with a coverglass coated by dried rhodamine 6G (Rh6G), following the protocol provided by the manufacturer (34). The real position of FCS measurement was also checked with bleaching of H2B-mRFP in living cells. Although there was no significant difference between the position of FCS measurements checked by a coverglass and living cells, misalignment under 1 micrometer was found. This range of misalignment may not be affect to analysis of diffusion in the region of nucleoplasm and nucleolus, diameters of which were of the order of 10μm and 2μm, respectively. The detection pinhole for FCS was fixed to a diameter of 70μm and emission was recorded through a 505-550nm bandpass filter for measurement of cells expressing EGFP<sub>n</sub> or through a 505-530nm bandpass filter for measurement of cells co-expressing EGFP<sub>n</sub> and mRFP tagged proteins for excluding any cross-talk signal from mRFP. In practice, there

was almost no cross-talk signal from mRFP using the three cell types expressing mRFP only. The fluorescence of cells expressing mRFP shows almost same background fluorescence signal under 15cps when a 505-550nm bandpass filter was used. All measured FAFs were fitted by the fit program installed on the ConfoCor 2 system using the model equation (2). FAFs in aqueous solutions were fitted by a one-component model ( $i=1$ ), and FAFs in cells by an one- or two-component model ( $i=1$  or  $2$ ) to consider free diffusion and restricted diffusion, respectively (see also the text). The pinhole adjustment of the FCS setup, structure parameter, and detection volume were calibrated everyday by FCS measurements of Rh6G solution with a concentration of  $10^{-7}$ M. Although the structure parameters determined by Rh6G after the pinhole adjustment were changed and ranged from 4 to 8 each day, FCS analysis was carried out with data sets with structure parameter ranging from 5 to 6, which are known to be a stable condition for FCS measurement. An average value of structure parameter was fixed for FCS analysis of all data carried out in a day under the same conditions. Diffusion time of component  $i$ ,  $\tau_i$ , is related to the translational diffusion constant  $D$  of component  $i$  by

$$\tau_i = \frac{w^2}{4D_i} \quad (3)$$

Diffusion of a spheroidal molecule is related to various physical parameters by the Stokes-Einstein equation as follows

$$D_i = \frac{k_B T}{6\pi\eta r_i} \quad (4)$$

where  $T$  is the absolute temperature,  $r_i$  is the hydrodynamic radius of the spheroidal molecule,  $\eta$  is the fluid-phase viscosity of the solvent, and  $k_B$  is the Boltzman constant. Because  $\tau_i$  is proportional to viscosity, the relative viscosity ( $\tau_{\text{cell}}/\tau_{\text{solution}}$ ) can be easily estimated. When the diffusion time of Rh6G is measured and the molecular weight of the sample molecule is known, the diffusion time of the sample molecule as a spherical shape can be simply calculated by the following equation (23).

$$\tau_{\text{sphere}} = \tau_{\text{Rh6G}} \left( \frac{MW_{\text{sphere}}}{MW_{\text{Rh6G}}} \right)^{1/3} \quad (5)$$

The diffusion time  $\tau$  is also related to the frictional coefficient of the diffusing molecules, which depends on the shapes of molecules undergoing diffusion in a solution of defined viscosity. The ratio of the frictional coefficient between spheroidal ( $f_0$ ) and ellipsoidal ( $f$ ) molecules and the relationship between the diffusion time and frictional coefficient are given by Perrin's equation (35, 36)

$$\frac{f}{f_0} = \frac{(p^2 - 1)^{1/2}}{p^{1/3} \ln(p + (p^2 - 1)^{1/2})} \quad (6)$$

$$\tau_{\text{ellips}} = \tau_{\text{sphere}} \frac{f}{f_0} \quad (7)$$

where  $f$  and  $f_0$  are frictional coefficients of ellipsoidal and spherical molecules, respectively,  $p$

is the axial ratio of the ellipsoidal molecule,  $\tau_{\text{ellips}}$  and  $\tau_{\text{sphere}}$  are the diffusion times of ellipsoidal and spherical molecules, respectively. Based on the known size of the EGFP molecule, 4nm in length and 3nm in diameter, and the average length of 25 amino acids, the predicted diffusion time of tandem EGFP<sub>n</sub> was calculated for spherical and ellipsoidal models using equations (5), (6), and (7). The lengths of amino acid linkers used for the calculation were 3.7nm for an  $\alpha$ -helix structure and 9.1nm for a simple linear structure of 25 amino acids. The diffusion constants of EGFP<sub>n</sub>s ( $D_{gfpn}$ ) in the solution and cells were calculated from the published diffusion constant of Rh6G,  $D_{\text{Rh6G}}$  ( $280 \mu\text{m}^2/\text{s}$ ) (37) and measured diffusion times of Rh6G ( $\tau_{\text{Rh6G}}$ ) and EGFP<sub>n</sub>s ( $\tau_{gfpn}$ ) as follows:

$$\frac{D_{gfpn}}{D_{\text{Rh6G}}} = \frac{\tau_{\text{Rh6G}}}{\tau_{gfpn}} \quad (8)$$

## RESULTS

### LSM observation

#### *Expressed oligomeric EGFP<sub>n</sub> localized in the nucleus*

To observe the distribution and localization of monomer EGFP and oligomeric EGFP<sub>n</sub> in HEK293, COS7, and HeLa cells, the cells were transiently transfected with DNA plasmids encoding EGFP<sub>n</sub> or cotransfected with plasmids encoding each EGFP<sub>n</sub> and H2B-mRFP. Cells expressing each oligomeric EGFP were observed at 24-48 h after transfection. Typical LSM images of HeLa cells expressing each EGFP<sub>n</sub> taken at 24 h after transfection are shown in Fig. 1. Monomer EGFP and EGFP<sub>2</sub> were uniformly distributed through the cytoplasm and nucleus in each cell except in the nucleolus (Fig. 1, A, F and B, G). In contrast, EGFP<sub>3</sub>, EGFP<sub>4</sub>, and EGFP<sub>5</sub> showed different distribution patterns in the cytoplasm and the nucleus. In the case of EGFP<sub>3</sub>, the fluorescent intensity of proteins in the cytoplasm was higher than that in the nucleus, although the difference was not significant (Fig. 1, C and H). For EGFP<sub>4</sub> and EGFP<sub>5</sub>, the fluorescent intensity in the nucleus was much weaker than that in the cytoplasm (Fig. 1, D, I and E, J). However, the fluorescence intensity of EGFP<sub>4</sub> and EGFP<sub>5</sub> in the nucleus was sufficient to be detected by LSM measurement (Fig. 1 I and J). The fluorescent intensities in the nucleus for EGFP<sub>2</sub>, EGFP<sub>3</sub>, EGFP<sub>4</sub>, and EGFP<sub>5</sub> at 48 h were increased compared with these at 24 h. For all oligomeric EGFP<sub>n</sub>, there was no speckled or aggregated distribution in the cytoplasm and the nucleoplasm and the fluorescence in the nucleoplasm except in the nucleolus had a uniform pattern (Fig. 1 F, G, H, I, and J). This uniform pattern of fluorescence in the nucleoplasm was confirmed by comparing the fluorescence of tandem EGFP with that of H2B-mRFP on HeLa or COS7 cells coexpressing EGFP<sub>3</sub> and H2B-mRFP or EGFP<sub>5</sub> and H2B-mRFP, respectively (supplementary Fig. S1), because it is known that H2B-GFP show heterogeneous fluorescent pattern in the nucleus depending on the density of chromatin (4, 31, 38). In the case of HEK293 and COS7 cells transfected with the tandem EGFP<sub>n</sub>, the difference of fluorescence intensity between the cytoplasm and nucleus was clearly discriminated from EGFP<sub>3</sub> regardless of the expression level, and the fluorescent intensity in the nucleus was decreased with the increase in size of tandem GFP (unpublished data).

LSM observations of HeLa cell indicated that tandem EGFP bigger than EGFP<sub>3</sub> (>90kD) had difficulty localizing in the nucleus. The localization of EGFP<sub>n</sub> in the nucleus was dependent on the size of the EGFP<sub>n</sub> molecule. Although all types of tandem GFP<sub>n</sub> could be localized in the nucleus, there was less tandem EGFP<sub>4</sub>, and EGFP<sub>5</sub> in the nucleus than monomeric EGFP, EGFP<sub>2</sub> and EGFP<sub>3</sub>. The small number of EGFP molecules in the nucleus (from 50 to 5 molecules in the detection volume of 0.3 fL) might be sufficient to be detected by FCS measurement even in very weak fluorescent cells. For the weak fluorescent intensity in the nucleus for EGFP<sub>3</sub>, EGFP<sub>4</sub>, and EGFP<sub>5</sub>, we did not need to select weakly expressing cells as explained in Materials and Methods, and could easily perform FCS measurement in the nucleus.

### FCS measurement in aqueous solution

#### *Tandem EGFP<sub>n</sub> diffuse in solution like a rod-like molecule*

For analysis of the diffusion properties of monomer EGFP and oligomeric EGFP<sub>n</sub> in aqueous solution, cells transfected with the EGFP<sub>n</sub> were homogenized and the proteins from the cell lysate were extracted and measured in aqueous solution. There was no drastic change or burst of average fluorescent intensity during FCS measurement resulting from

aggregated EGFP molecules or contaminants from the homogenized cell extracts during the measurement time of 60 s. The FAF of each tandem EGFP<sub>n</sub> was analyzed by a one-component model (equation (2),  $i=1$ ) and was well fitted. Figure 2 A shows typical FAFs of EGFP<sub>n</sub> obtained from aqueous solution. For comparison of the extents of diffusion speeds, the amplitude of  $G(\tau)$  ( $G(0)-1$ ) was normalized to unity. The autocorrelation functions of EGFP<sub>n</sub> shifted gradually to the right depending on the molecular weight of tandem EGFP<sub>n</sub> (Fig. 2 B). Diffusion times corresponding to the FAFs of EGFP<sub>1-5</sub> were  $86.8 \pm 3.9\mu\text{s}$ ,  $125.8 \pm 2.7\mu\text{s}$ ,  $147.4 \pm 4.8\mu\text{s}$ ,  $185.4 \pm 5.4\mu\text{s}$ , and  $200 \pm 8.5\mu\text{s}$ , respectively (Fig. 2 C). These results indicated that the diffusional mobility of EGFP<sub>n</sub> decreased with increasing molecular weight. That there was no degradation of monomeric and tandem EGFP<sub>n</sub> was also confirmed by the western blotting results (Fig. 2 B), which were well consistent with the expected molecular weight of each oligomeric EGFP<sub>n</sub>. Diffusion constants of monomeric and oligomeric EGFP<sub>n</sub> in solution are summarized in Fig. 2 D. The diffusion constant ( $76 \mu\text{m}^2\text{s}^{-1}$ ) of monomeric EGFP (263 a.a, 30kD) was similar to those ( $87 \mu\text{m}^2\text{s}^{-1}$ ) of previous studies (24, 39, 40) with recombinant GFP (238 a.a, 27kD) synthesized by bacterial expression.

Oligomeric EGFP<sub>n</sub> contains a linker of twenty-five random amino acids connecting monomer EGFP molecules. Consequently, oligomeric EGFP<sub>n</sub> can have different molecular shapes from spherical to linear. Because the linker can change  $p$ , the axial ratio of the protein molecule (equation (6)), the diffusional mobilities of tandem types of EGFP<sub>n</sub> from EGFP<sub>2</sub> to EGFP<sub>5</sub> may reflect the diffusional mobility of an ellipsoidal or rod-like molecule. For this case, diffusion times of oligomeric EGFP<sub>n</sub> from EGFP<sub>2</sub> to EGFP<sub>5</sub> could be much slower than those of the proteins in spherical shape. Figure 2 C shows a plot of the measured diffusion time (filled circles) of each EGFP and three plots of predicted diffusion times calculated by diffusion models for the spherical shape and two rod-like shapes with different  $p$  values (equation (5), (6), and (7)). Enhanced EGFP has a well-known cylindrical structure with a diameter of  $\sim 3\text{nm}$  and height of  $\sim 4\text{nm}$  (27). For simplification, monomer EGFP was assumed to be a spherical molecule and then the diffusion time of oligomeric EGFP<sub>n</sub> was calculated as a spherical molecule or rod-like molecule by equation (5). The measured diffusion time of monomer EGFP (30kD) agreed well with the calculated value obtained from equation (5) using the empirical diffusion time ( $21 \pm 2\mu\text{s}$ ) and the known molecular weight (0.479 kD) of Rh6G. The dashed line in Fig. 2 C plots the calculated diffusion time of oligomeric EGFP<sub>n</sub> with a spherical shape. The other two lines plot the predicted diffusion times of rod-like oligomeric EGFP<sub>n</sub> assuming that the amino acid linkers have an  $\alpha$ -helix (solid line) or a linear structure (dotted line) with lengths of  $\sim 3.7\text{nm}$  and  $\sim 9.1\text{nm}$ , respectively. With this simple assumption, EGFP<sub>1-5</sub> have longitudinal lengths of 4, 12, 20, 28, and 36 nm, respectively for an  $\alpha$ -helix linker and 4, 17, 30, 43, and 56 nm, respectively for a linear linker. As shown in Fig. 2 C, the measured diffusion times of oligomeric EGFP<sub>n</sub> (closed circles) are much longer than the calculated diffusion times of the EGFP<sub>n</sub> as a spherical molecule and well agreed with the rod-like model for the  $\alpha$ -helix linker, even though the diffusion time of EGFP<sub>5</sub> was slightly shorter than the calculated value. This indicated that diffusion of monomer and oligomeric EGFP<sub>n</sub> from EGFP<sub>2</sub> to EGFP<sub>5</sub> in solution reflected free diffusion of rod-like molecules and depended on the putative length of the oligomeric EGFP. Consequently, we concluded that monomeric and oligomeric EGFP<sub>n</sub> could be used as molecular rulers that change the diffusion time according to their own longitudinal length. This property of tandem EGFP<sub>n</sub> will be useful to analyze mobility of proteins in organelles, particularly in the subnuclear microenvironment.

### LSM and FCS measurement in cells

FCS measurements of oligomeric EGFP<sub>n</sub> *in vivo* were performed using three cell lines, HEK293, COS7, and HeLa. Cells expressing a comparatively low concentration of EGFP<sub>n</sub> under ~20 molecules (<0.1μM) per detection volume (0.3fl) were chosen because a dilute concentration of fluorescent molecules is adequate for FCS measurement. Even with this condition, there might be photobleaching effect on FCS measurements. Recently, a method combining FCS with photobleaching analysis was reported for studying intracellular binding and diffusion of functional proteins (41). This study suggested that the method is applicable to analyze mobility of monomer EGFP even in highly fluorescent cells. Nevertheless, it is noted that our study focused on the mobility of freely moving tandem EGFP<sub>n</sub> in the microenvironment contained the detection volume, but not that of immobile tandem EGFP<sub>n</sub>, which give rise to a photobleaching and make FCS analysis more complex. For excluding a possible photobleaching effect, we carefully selected cells with weak fluorescence or without photobleaching during FCS measurement.

All FCS measurements were performed after taking LSM images and multiple positions for FCS measurements in the cytoplasm excepting endoplasmic reticulum and plasma membrane and multiple positions in the nucleus were chosen in the LSM image of a cell. After FCS measurements, an LSM image was taken again to check whether measured positions of FCS were deviated from the LSM images. In weakly fluorescent cells, it was not easy to discriminate the nucleolus from the nucleoplasm, particularly, in cells expressing EGFP<sub>3</sub>, EGFP<sub>4</sub> and EGFP<sub>5</sub>, in which most of the proteins were located in the cytoplasm and only a few EGFP molecules were located in the nucleus. Figure 3 shows typical examples of LSM and FCS measurements for the three cell lines. LSM images for FCS measurement of a HEK cell expressed by EGFP<sub>1</sub>, a COS7 cell by EGFP<sub>4</sub>, and a HeLa cell by EGFP<sub>5</sub> are shown in Fig. 3 A, C, and E, respectively. On the weakly fluorescent HEK cell expressing EGFP (Fig. 3 A), the boundary between the cytoplasm and nucleus was not clear. On the other hand, the cells expressing EGFP<sub>4</sub>, and EGFP<sub>5</sub> (Fig. 3 C and E) show a clear contrast of the boundary resulting from the difference of fluorescence intensity between the cytoplasm and nucleus. The boundary between the cytoplasm and nucleus was not clear for weakly fluorescent cells expressing EGFP<sub>1</sub> and EGFP<sub>2</sub>, regardless of the cell type. However, the boundary was clearly visible with cells expressing EGFP<sub>3</sub>, EGFP<sub>4</sub> and EGFP<sub>5</sub>, depending on the size of tandem EGFP<sub>n</sub>, even though the fluorescence signals of the cells were weak. The clear boundary between the cytoplasm and nucleus for EGFP<sub>3</sub>, EGFP<sub>4</sub>, and EGFP<sub>5</sub> made it easy to discriminate the two.

### FCS analysis in living cells

For all cells expressing EGFP<sub>1</sub> or tandem EGFP<sub>n</sub>, diffusive fluorescent regions in the cytoplasm and multiple positions in the nucleus were measured by FCS. Examples of FAFs of EGFP in HEK, EGFP<sub>4</sub> in COS7, and EGFP<sub>5</sub> in HeLa cells are shown in Fig. 3 B, D, and F, respectively. Cross hairs in the LSM images correspond to the FCS measurement points. In Fig. 3 B, position 1 of FCS measurement point was chosen for measuring cytoplasm, and positions 2 and 3 were presumed to be in the nucleus. In Fig. 3 D and F, position 1 of the cross hair corresponds to a point in the cytoplasm and positions 2 and 3 to random points in the nucleus. The amplitudes of all FAF ( $G(0)-1$ ) were normalized to unity for comparison of the shift of the curve. One of two FAFs obtained from the nucleus showed no or a small difference from that in the cytoplasm (curves 2 in Fig. 3. B, D, and F). Interestingly, other FAFs obtained from the nucleus largely shifted to the right, indicating much slower diffusional

mobility (curves 3 in Fig. 3 B, D and F). This slower diffusion was occasionally found in nuclei of all cells expressing monomeric and oligomeric EGFP<sub>n</sub> regardless of the cell type. This indicated that there were two types of diffusional mobility in the three compartments, the fast-diffusion-mobility (FAF curves 1 and 2 in Fig. 3) in the cytoplasm and the nucleus, and the slow-diffusion-mobility (FAF curves 3 in Fig. 3) in the nucleus (summarized in Table 1). Fluorescent intensity at the point of slower diffusion was weak compared to that at other places in the nucleus. However, we could not specify the precise position of the slow-diffusion-mobility in the cell nucleus because of the very weak fluorescence in the nucleus. Figure 3 G shows a plot superimposing normalized FAFs of EGFP<sub>n</sub> measured in the nucleus of HeLa cell excluding the slow-diffusion-mobility. The FAF of each oligomeric EGFP in the cell nucleus shifted to the right with the size of tandem EGFP<sub>n</sub>. This shift was well consistent with the result in aqueous solution (Fig. 2, A). This consistency suggests that the fast-diffusion-mobility of oligomeric EGFP<sub>n</sub> in the cell nucleus might follow the diffusion model of a rod-like molecule.

Analysis of FAF in cells was performed with a two-component model (equation (2),  $i=1$  and 2), a fast diffusing component (first component) and a slower diffusing component (second component), because almost FAF of each tandem EGFP<sub>n</sub> were cannot fitted by an one-component model, but best fitted by the two-component model. However, some FAFs were best fitted by an one-component model. In this case, we adopted the result of one-component analysis (supplementary Fig. S2). The first component was considered to be a freely diffusing component and the second component was assumed to be a slowly diffusing component (14, 24, 42). High density of the cellular solutes and some restricted mobility in a cellular microstructure may slow down free diffusion. With conditions of cells having a concentration under 20 EGFP<sub>n</sub> molecules ( $<0.1\mu\text{M}$ ) and a comparatively short measurement time under 30s, the influence of photobleaching on diffusion time, which gives rise to a very long diffusion time and an increase of the fraction ( $y_i$  value in equation (2)), could be minimized. Photobleaching effects were checked from the time trace of fluorescent intensity for all FCS data (supplementary Fig S3). Increasing the incubation time after transfection for a few days made the effect of photobleach on FCS measurement much stronger, because the promoter for protein expression is strong and not controlled one. In practice, photobleaching effect was very small for weakly fluorescent cells at early stage after expression of tandem GFP (supplementary Fig. S4). Background fluorescent signals under 2kcps and 10kcps were detected in medium and non-transfected HeLa, HEK, and COS7 cells (14). No significant correlation amplitudes were detected in the culture medium. In contrast, very weak correlations with very long diffusion times above  $10^5\mu\text{s}$  were sometimes detected in each cell types when FCS measurement was carried out over longer duration over 60 sec. This was derived from very slow and large fluctuation of fluorescence but not from photobleaching. To solve the background with very slow fluctuation, we adapted a shorter measurement time as described above. Considering each tandem EGFPs has much larger brightness per molecule than that of monomer EGFP (unpublished data) and the diffusion time of the proteins was an order of ms ranges, the short measurement time of FCS might be enough to obtain a reliable autocorrelation function.

### ***Two diffusional mobility in the nucleus:***

#### ***The fast-diffusion-mobility in the cytoplasm and the nucleus***

Figure 4 A shows a plot of the diffusion time of first component obtained from FAFs representing the fast-diffusion-mobility in the cytoplasm and in the nucleus of HeLa cells

(curves 1 and 2 in Fig. 3 B, D, and F). For these FAFs of the fast-diffusion-mobility, more than 90% of the fraction ( $\gamma_1$  in equation (2)) was defined as the first component, which represents free diffusion. These results were highly reproducible. As shown in Fig. 4 A (filled circles), the diffusion times of the fast-diffusion-mobility in the nucleus were gradually increased with the increase in the molecular size of tandem EGFP<sub>n</sub>. In the cytoplasm (Fig. 4 A open circles), the diffusion time of the first component for EGFP<sub>n</sub> also increased with size. No significant difference between the first components in the cytoplasm and nucleus was found. Average diffusion times of monomeric and oligomeric EGFP in HEK and COS7 cells also increased with increasing the size both in the cytoplasm and in the nucleus (Fig. 4 B and C). The ratio of the diffusion time of the first component in the cytoplasm and the nucleus of each cell type to that in aqueous solution ( $DT_{\text{cell}}/DT_{\text{sol.}}$ ), which indicates the ratio of viscosity (equation (3) and (4)), is shown in inserts in Fig. 4 A, B and C, respectively. Regardless of the cell type, the average ratios of viscosities in the cytoplasm and the nucleus were not significantly different and 3.5-fold higher than that in solution. Moreover, there was no dependency of the viscosity ratio on the size of oligomeric EGFP<sub>n</sub>. These results agreed with previous results obtained from microinjected fluorescent macromolecules and monomeric EGFP (7, 13, 42).

Using the result that the average viscosity in the cytoplasm and nucleus was 3.5-fold higher than that in solution, the expected diffusion times of tandem EGFP<sub>n</sub> in the cell were calculated. As shown in Fig. 2, the measured diffusion times of first components in living cells were also compared with three calculated diffusion times (Fig. 4 A, B, and C) assuming the shape of oligomeric EGFP to be spherical (dashed lines) or rod-like with the expected linker lengths of 4nm (solid lines) and 9nm (dotted lines). Dependency of the diffusion times on the size of oligomeric EGFP both in the cytoplasm and in the nucleus was consistent with that of a rod-like molecule rather than a spherical one (dashed lines). Based on the result that the diffusion properties of rod-like molecules of oligomeric EGFP in the cytoplasm and the nucleus are equivalent and consistent with the result in aqueous solution, the oligomeric EGFP<sub>n</sub> located in the nucleus was not truncated or degraded. Consequently, our results suggested that the diffusion of oligomeric EGFP<sub>n</sub> as a rod-like molecule was well conserved in the cellular circumstance in all of three cell lines.

Other diffusion times (second component) of the FAFs for the fast-diffusion-mobility (curves 1 and 2 in Fig. 3 B, D and F) were very slow, and ranged from  $10^4$  to  $10^5$   $\mu\text{sec}$  in the nucleus as well as in the cytoplasm. The range of these long diffusion times was very broad and so it is not clear that the diffusion time of the second component was also dependent on the size of tandem EGFP<sub>n</sub>. The fraction of the second component ( $\gamma_2$  in equation (2)) was very small (<10%), regardless of the size of tandem EGFP<sub>n</sub>. The slow drift of fluorescence could come from cell mobility or very large organelles such as vesicles in cytoplasm (24, 42) and such as a compact structure of chromatin in nucleus (4) during FCS measurement. Otherwise very weak photobleaching might be not completely excluded, even though data of photobleached samples were checked and excluded. However, a possibility of trapped diffusion in complex chromatin structures cannot be completely excluded. To analyze an effect of chromatin structures on the very slow diffusion time, we treated cells coexpressing tandem EGFP<sub>3</sub> and H2B-mRFP or EGFP<sub>5</sub> and H2B-mRFP, respectively, with Trichostatin A (TSA) (supplementary Fig. S5). It was previously reported that TSA inhibits histone deacetylation and so increases chromatin accessibility of relatively larger dextrans (4, 43). In LSM observation, no significant changes of fluorescent pattern for tandem EGFP<sub>3</sub> and EGFP<sub>5</sub> were found, although that of H2B-mRFP was significantly changed after TSA treatment

(supplementary Fig. S1 and S5). This result suggests that tandem EGFP<sub>n</sub> can freely and equally access to all regions of euchromatin and heterochromatin and so no effect of TSA treatment occurred. Moreover, there were no significant changes of diffusion time and fraction for the very slow component in the nucleoplasm after TSA treatment when FCS measurements on euchromatin (dilute H2B-mRFP fluorescent region) and heterochromatin (dense H2B-mRFP fluorescent region) were carried out (unpublished data). Because the fraction of the very slow component was very small (<10%) and the diffusion times were very broad with large standard deviation even before TSA treatment, it is likely that the effect of TSA treatment on mobility of tandem EGFP<sub>n</sub> in the nucleus cannot be detectable in our experimental system. Nevertheless, the result of LSM observation was consistent with the result of FCS measurement. Details and discussion of such very slow diffusion can be omitted in this paper because the fraction is small and we focus on well-defined diffusion property of tandem EGFP<sub>n</sub> as molecular ruler.

#### *The slow-diffusion-mobility in the nucleus*

On the other hand, the right-shifted FAFs for tandem EGFP<sub>n</sub>, which represent the slow diffusion-mobility, found in the nucleus (curves 3, dashed lines in Fig. 3 B, D, and F) showed a different range of diffusion times and a different fraction for the second component compared to those for the fast-diffusion-mobility (curves 1 and 2, solid and dotted lines in Fig. 3 B, D, and F). Obviously, although the diffusion times of the first component for the slow-diffusion-mobility in the nucleus were consistent with those for the fast-diffusion-mobility in the cytoplasm and in the nucleus (Table 1 B), the diffusion times of the second component for the slow-diffusion-mobility ranged from 800  $\mu$ s to 5,000 $\mu$ s, increasing with the size of tandem EGFP (e.g. curves 3 in Fig. 3, B, D, and F). Moreover, the fraction of the second component for the slow-diffusion-mobility varied from 20% to 100% depending on the cells, and even the measured position in the same nucleus. This observation was very reproducible, and was consistent among the three cell types. Obviously, our results indicated that the protein mobility in the nuclear microenvironment might be separated into two kinds of diffusing species (i.e. the first component of fast-diffusion-mobility and the second component of slow-diffusion-mobility). These two kinds of diffusing species had different ranges of diffusion time (or apparent viscosity) depending on the position inside the nucleus.

#### *The slow-diffusion-mobility of tandem EGFP<sub>n</sub> in the nucleolus*

Fluorescent intensity at the position of the slow-diffusion-mobility (i.e., the right-shifted FAFs) in the nucleus (positions 3 of A, C, and E of Fig. 3) was weak compared to other places inside the nucleus. In addition, the slow-diffusion-mobility was often found in the nucleolus in the cells expressing EGFP<sub>4</sub> and EGFP<sub>5</sub> with large and clear nucleoli. The density, the number and the morphology of the nucleolus changed according to the cell cycle as well as cell type and other cell conditions. Recently, the nucleolus has been detected by fluorescence microscopy in cell lines expressing fluorescent protein-tagged nucleolar proteins such as fibrillarin and B23 (32, 44). Fibrillarin is related to various steps of pre-rRNA processing and ribosome assembly and located in the dense fibrillar component (DFC) of the nucleolus during interphase (45). Using a nucleolar protein tagged with different fluorescent proteins will help in discriminating the nucleolar structures from nucleoplasm and tracing the changes of the nuclear structure during the cell cycle or depending on physiological cell conditions.

Figure 5 shows an LSM image and FCS measurement of a HeLa cell coexpressing

EGFP<sub>4</sub> and mRFP-fibrillarin. The strong red fluorescence in the nucleus (Fig. 5 A) indicates the nucleolus. A weak green fluorescence signal was also detected in the nucleoplasm (Fig. 5 B). This LSM observation for fibrillarin agreed with the previous results (12, 32). The shape, the size and the number of nucleoli were different from cell to cell. Using cotransfected HeLa cells, FCS measurement was carried out for positions of green fluorescent nucleoplasm and the red fluorescent nucleolus with a diameter of over 2 $\mu$ m in the x-y plane of the LSM image. FAF inside the nucleolus (Fig. 5 D, red line) shifted to the right compared to that in the nucleoplasm (Fig. 5 D, black line), which meant that the diffusion in the nucleolus was much slower than that in the nucleoplasm. The FAFs obtained from nucleolus fit well with the two-component model. Occasionally, some FAFs fit well even in the one-component model. The slow-diffusion-mobility in the nucleolus (Fig. 5 D; curve 1) was consisted with the first component of 700  $\mu$ s (40%) and the second component of 3900 $\mu$ s (60%). In contrast, the fast-diffusion-mobility in the nucleoplasm (Fig. 5 D; curve 2) was consisted with the first component of 900 $\mu$ s (94%) and the second component of 28000 $\mu$ s (6%). Diffusion times ( $1 \times 10^3 \sim 4 \times 10^3 \mu$ s) of second components for FAFs measured in the nucleolus (Fig. 5 E) were much shorter than those of second components measured in the nucleoplasm and the cytoplasm (ranging from  $10^4$  to  $10^5 \mu$ s). The time range (Fig. 5 E) was consistent with those obtained from B, D, and F of Fig. 3. In contrast, almost no such diffusional component in the range of  $1 \times 10^3 \sim 4 \times 10^3 \mu$ s was found in places other than the nucleolus. On the other hand, the diffusion time of the first component in the nucleolus was the same as those in the cytoplasm and the nucleoplasm. The fraction of the first component was decreased with the increased fraction of the second component. The diffusion times of second components in the nucleoli increased with the size of EGFP<sub>1</sub>, EGFP<sub>2</sub>, EGFP<sub>3</sub>, and EGFP<sub>4</sub>, even though there was little difference between EGFP<sub>4</sub> and EGFP<sub>5</sub> (closed circles in Fig. 5 E). The insert in Fig. 5 E shows the average ratio of the diffusion time of the second component in the nucleolus to the diffusion time of the first component in the nucleoplasm ( $DT_{NL}/DT_{NP}$ ). There was no dependency of the ratio on the size of EGFP<sub>n</sub> and average value of the ratio for all tandem EGFP<sub>n</sub> was about 5.2. The solid line in Fig. 5 E shows the calculated diffusion times of tandem EGFP<sub>n</sub> as a rod-like molecule with an  $\alpha$ -helix linker when the relative viscosity in the nucleolus is fixed by the average ratio of diffusion time (Fig. 5 E, insert). The measured diffusion times of tandem EGFP<sub>n</sub> were consistent with the calculated values. Our results indicated that the slow-diffusion-mobility in the nucleolus also reflected the diffusion of a rod-like molecule rather than a spherical molecule.

Table 1 summarizes the diffusion constants of the fast-diffusion-mobility (the first component with a fraction more than 90%) found in the cytoplasm and the nucleoplasm, and the diffusion constants of the slow-diffusion-mobility (first and second components) found in the nucleolus. The average values were obtained from living cells only expressing monomer EGFP and tandem EGFP<sub>n</sub> without mRFP-fibrillarin. Diffusion constants of the fast-diffusion-mobility both in the nucleoplasm and in the cytoplasm decreased with the length of tandem EGFP<sub>n</sub> in HeLa, COS7, and HEK cells, even though the diffusion constants of EGFP<sub>4</sub> and EGFP<sub>5</sub> in the cytoplasm of HEK cells did not change. Diffusion constants of the first and the second components in the nucleoli of HeLa and COS7 cells also decreased with the length of EGFP<sub>n</sub>. There was little difference between diffusion constants in the cytoplasm and the nucleoplasm of HEK293 and HeLa cells. In contrast, diffusion constants in the cytoplasm of COS7 cells were slightly larger than those in the nucleoplasm. Base on these results, it was concluded that the diffusional motion of tandem EGFP<sub>n</sub> in the nucleus as well as in the cytoplasm and the nucleoplasm was well consistent with free diffusion of

rod-like molecules, regardless of the cell type. It is emphasized that the microenvironment of the nucleolus as well as the nucleoplasm and the cytoplasm could be quantitatively understood by diffusion analysis of the oligomeric EGFP<sub>n</sub> as molecular rulers (MR). Moreover, our results indicated that the microenvironment and apparent viscosity of the cytoplasm and the nucleoplasm were almost same, even though the constituents of the two compartments were very different.

Compared with those of the first component in the nucleoplasm, the fractions of the second components in the nucleoli were significantly changed from 20% to 100 % depending on the nucleolus, even in the same cell (unpublished data). Because the length of the z-axis (optical axis) of detection volume (<2μm) was six times longer than the diameter in the x-y plane (<0.2μm), FCS measurement of a nucleolus with a length in the z-axis shorter than 2μm might contain both the nucleoplasm and the nucleolus. This might affect the variability of the fraction. However, it is also presumed that the diffusion of oligomeric EGFP<sub>n</sub> in the nucleolus has more variability than that found in the cytoplasm and the nucleoplasm, indicating the dynamic change of the nucleolar microenvironment or the complexity of subnucleolar structures such as DFC, fibrillar centers, and the granular region (45, 46). More detailed study using two-color 3D imaging combined with FCS measurement is in progress for elucidating the large diffusion changes in the nucleolus according to a long time scale or the cell cycle of a single cell. Nevertheless, our results showed that the mobility of MR in the nucleolus was dependent on length of them, but was much slower than those in the cytoplasm and the nucleoplasm. Consequently, it was concluded that the diffusion of protein in the nucleus must be separated into two significant diffusing components, fast-diffusion-mobility in the nucleoplasm and slow-diffusion-mobility in the nucleolus.

#### *Nucleolar microenvironment is sensitive to energy depletion*

To examine effect of energy depletion on the mobility of oligomeric EGFP in the nuclear microenvironment, the culture medium containing HeLa cells expressing EGFP<sub>4</sub> or EGFP<sub>5</sub> was perfused with 2-DG and NaN<sub>3</sub> solution (3, 20) at 25 or 37°C. LSM and FCS measurements were carried out with HeLa cells expressing EGFP<sub>5</sub> or coexpressing EGFP<sub>5</sub> and mRFP-fibrillarin (or H2B-mRFP). For FCS measurement of cells transfected with EGFP<sub>5</sub>, HeLa cells with clear and large nucleoli (>4μm in diameter) were chosen despite the fluorescence signals of the cytoplasm being a little strong (for example, right upper cell in Fig. 3 E). FCS measurement was carried on the same position of single cells before and after the energy depletion. We confirmed the redistribution of H2B-mRFP and nuclear shrinkage through LSM images of cells coexpressing H2B-mRFP and EGFP<sub>5</sub> following ATP depletion at room temperature for 30 min (unpublished data). This result was consistent with a previous study (20). With cells expressing EGFP<sub>5</sub>, Fig. 6 A, B, and C show FAFs of EGFP<sub>5</sub> at the same positions in the cytoplasm, the nucleoplasm, and the nucleolus of a single HeLa cell, respectively, before (dashed black lines) and after the energy depletion (solid red lines). FAFs of EGFP<sub>5</sub> both in the cytoplasm and in the nucleoplasm were slightly shifted to the right by the energy depletion (Fig. 6 A and B). In contrast, the FAF of EGFP<sub>5</sub> in the nucleolus was significantly changed in the longer time range as shown in Fig. 6 C. The energy depletion induced a big tail on the FAF, which indicates that, a fraction with much slower mobility was newly produced. The diffusion time corresponding to the tail found in the nucleolus was 13-fold slower than that of the second component found before energy depletion, and the fraction of the new slower component was increased from 0 % up to ~32 % (Fig. 6 C).

Figures 6 D, E, and F show the average change of the diffusion time and the fraction in

each cellular compartment of five HeLa cells expressing EGFP<sub>5</sub>. Averaged diffusion times in the cytoplasm ( $0.8 \pm 0.04\text{ms}$  and  $0.85 \pm 0.04\text{ms}$ ) and the nucleoplasm ( $0.79 \pm 0.06\text{ms}$  and  $0.7 \pm 0.08\text{ms}$ ) before and after ATP depletion, respectively, were not changed (solid bars in D and E of Fig. 6). Instead, the fractions of first components were slightly decreased in the cytoplasm (~1%) and the nucleoplasm (~9%) (solid bars in insert of Fig. 6 D and E). On the other hand, diffusion times of the second component in the nucleolus were increased from  $4.6 \pm 0.8\text{ms}$  to  $22.4 \pm 6.7\text{ms}$  (open bars in Fig. 6 F), even though the fraction of the second component was decreased from 77% to 47% by the energy depletion (open bars in insert of Fig. 6 F). In addition to the change of the second component in the nucleolus, the diffusion time of the first component in the nucleolus were also increased from  $0.58 \pm 0.1\text{ms}$  to  $1.0 \pm 0.1\text{ms}$  (solid bars in Fig. 6 F). This indicated that the microenvironment inside the nucleolus, which was reflected by diffusion of EGFP<sub>5</sub> molecules, was more sensitive to energy depletion than those of the nucleoplasm and the cytoplasm.

## DISCUSSION

Tandemly linked EGFP<sub>n</sub> proteins were constructed for modeling rod-like molecules. The diffusion properties of the proteins were quantitatively dependent on their length. These series of standard proteins allowed us to analyze protein mobility in living cells. LSM observation of HeLa cells expressing monomer EGFP and four different kinds of tandem EGFP<sub>n</sub> showed that the proteins could be distributed to the cell nucleus regardless of their molecular weights. Monomeric EGFP, EGFP<sub>2</sub>, and EGFP<sub>3</sub> were easily distributed in the nucleus. In contrast, the fluorescent intensities in the nuclei of cells expressing EGFP<sub>4</sub> and EGFP<sub>5</sub> were lower than in the cytoplasm, even though they were also located in the cell nuclei. Although the tendency of fluorescence intensity was very much different for EGFP<sub>n</sub> in the nucleus, all tandem proteins were detected by LSM and could also be detected by FCS. Many studies have shown that the transport of inert molecules to the nucleus depends inversely on molecular size with an exclusion limit at about 5~10nm in diameter or 40~60kD in molecular weight (47, 48). These studies discussed only the exclusion limits of spherical molecules. Our results for tandem EGFP<sub>n</sub> with molecular weights of 60, 90, 120, and 150 kD showed that rod-like proteins could localize to the cell nucleus within 24hr after transfection depending on size, even though the mechanism for their transport to the nucleus was not clear.

Western blots of tandem proteins from cell lysates showed that the molecular weights of proteins synthesized in cells were well consistent with those expected from their numbers of amino acids. FCS measurement of monomer and tandem EGFP<sub>n</sub> in aqueous solution showed that their diffusion times also increased with molecular weight. Comparison of the measured diffusion time with the calculated diffusion time according to Perrin's equation (35, 36) indicated that the tandemly linked EGFP<sub>n</sub> behaved like rod-like molecules. The fact that diffusion times of tandem series of EGFP are proportional to their lengths in aqueous solution indicates that the proteins could be employed as molecular rulers (MR) in living cells.

Combining a well-defined MR with the high sensitivity of FCS measurement make possible analysis of protein mobility in living cells, in particular in the nucleus. In contrast to the cytoplasm, our results showed that there were two kinds of diffusional mobility in the nucleus, both of which also depended on the length of MR as shown in solution and cytoplasm. One was the fast-diffusion-mobility of tandem EGFP<sub>n</sub> found in the nucleoplasm as well as in the cytoplasm, in which the first component had a fraction above 90%, reflecting the free diffusion of the MR (represented by  $D$  of the first components in NP, Table 1 B). The other was the slow-diffusion-mobility (represented by  $D$  of the second components in NL, Table 1 B) observed in the nucleolus. The second component of the fast-diffusion-mobility in nucleoplasm showed a very slow diffusion ( $10^4\sim 10^5\mu\text{s}$ ) with fractions under 10%, indicating no length dependency and no significant change by TSA treatment. The first component of the slow-diffusion-mobility (represented by  $D$  of the first components in NL, Table 1 B) in nucleolus was almost equivalent to the first component of the fast-diffusion-mobility in nucleoplasm (represented by  $D$  of the first components in NP, Table 1 B). There was no significant change in the diffusion time and the fraction for the first component of the fast-diffusion-mobility and for the second component of the slow-diffusion-mobility by TSA treatment (unpublished data). The result of FCS before and after TSA treatment was well consistent with LSM observation using two-color imaging (supplementary Fig. S1 and S5). Previous studies (4, 38, 43) using LSM observation of labeled dextran with various sizes showed that a globular protein with molecular weight of 1MD (an apparent pore size of 14nm) might be no limitation in access to chromatin. Because molecular weights of tandem EGFP<sub>n</sub>

are much smaller than IMD, tandem EGFP<sub>n</sub> might be freely access to the two types of chromatin.

A study of FCS and monomer EGFP using both a two-component model and an anomalous subdiffusion model analysis (14) has shown that the diffusion of EGFP in the nucleus was much more complex than in the cytosol. The study described averaged diffusional mobility of EGFP in the entire nucleus but not in each compartments in the nucleus such as the nucleolus, and suggested that the ratio of diffusion mobilities in cells and in solution was not dependent on the two models used. The fast-diffusion-mobility of tandem GFP<sub>n</sub> in the cytoplasm and the nucleoplasm was dependent on length. The ratio of diffusion time in each compartment to that in solution showed that the apparent viscosities of the cytoplasm and nucleoplasm were identical. In addition, the apparent viscosity in the three cell lines (HeLa, COS7, and HEK293) was found to be about 3.5-fold higher than in aqueous solution. The viscosities in the cytoplasm and the nucleoplasm were well consistent with previous studies using FRAP (9, 13) and using FCS (14).

We investigated the protein mobility in the nucleolar microenvironment of living cells in detail. The size and shape of the nucleolus during each phase of the cell cycle are not constant. Moreover, it was not easy to discriminate between the nucleoplasm and nucleolus in the cells weakly expressing the monomer and tandem EGFP<sub>n</sub>. We marked the nucleolus with mRFP-tagged fibrillarin to distinguish it from the nucleoplasm. Our observations in the nucleolus (Fig. 5E and Table 1) indicated that mobility of the inert EGFP and tandem EGFP<sub>n</sub> in the nucleolus was also dependent on the length of the protein, but that the mobility was about 17-fold slower for HeLa and 24-fold slower for COS7 than in aqueous solution. Nevertheless, assuming a random walk model, the result suggested that it would take the tandem proteins just a few seconds to travel a distance of 4 $\mu$ m, roughly the diameter of a nucleolus. Rapid association or exchange of GFP-fibrillarin ( $0.046\mu\text{m}^2\text{s}^{-1}$ ) (12) and GFP-B23 ( $0.08\mu\text{m}^2\text{s}^{-1}$ ) (32) in the nucleolus was observed by FRAP. These results suggested that the nucleolus is not a static protein mass such as aggregates, and that proteins were dynamically exchanged between the nucleoplasm and the nucleolus. EGFP tagged fibrillarin was shown to have diffusion constants of  $0.53\mu\text{m}^2\text{s}^{-1}$  even in the nucleoplasm (12). On the other hand, diffusion of the MR in the nucleolus was much faster than for the nucleolar proteins (Table 1). For instance, the diffusion constants of tandem EGFP<sub>2</sub> were  $14.9 \pm 0.8$  and  $3.8 \pm 0.5\mu\text{m}^2\text{s}^{-1}$  in the nucleoplasm and the nucleolus of the HeLa cell, respectively, although the molecular weight and shape of EGFP<sub>2</sub> might be similar to EGFP-tagged fibrillarin (60kD). Our observations indicated that the architecture of nucleolus was not very tight and some proteins, at least GFP<sub>n</sub>, could be almost freely accessible inside of the compartment, because the mobility of GFP<sub>n</sub> was only slowed down about one-fifth compared with in the nucleoplasm and the cytoplasm. Consequently, our study of MR mobility in the nucleoplasm and the nucleolus might be very helpful to understand the variability of mobility of microinjected labeled macromolecules in the nucleus (3, 13) or the restricted mobility of monomeric EGFP (14) and various nuclear proteins (12, 20, 32, 41, 43). In those studies, the complex microenvironment inside of the nucleolus was not considered in detail, even though the mobilities of the nuclear proteins were measured in the nucleolus and the interactions with nucleoli were analyzed.

Recent LSM observation of human U2OS cells expressing yellow fluorescent protein tagged H2B and electron microscopic observation of ATP-depleted cells have shown that the chromatin structure changes with nuclear shrinkage under energy depletion, and suggest that movement of mRNA-protein complexes (mRNPs) is constrained by the structural changes in

the nucleus (20). It would be interesting to know whether the redistribution of the chromatin structure by energy depletion also affects other small proteins, and whether the nucleolar microenvironment is also changed by ATP depletion. To determine whether the diffusion of the longest EGFP<sub>5</sub> in the nucleolar microenvironment was affected by the cellular metabolism, we treated HeLa cells expressing EGFP<sub>5</sub> with metabolic inhibitors 2-DG and NaN<sub>3</sub>. Interestingly, our results showed that the diffusion of EGFP<sub>5</sub> in the nucleolus was slowed down by ATP depletion, but that in the cytoplasm and the nucleoplasm it was only slightly changed. The small change of EGFP<sub>5</sub> mobility (Fig. 6 B) in the nucleoplasm suggested that the microenvironment of nucleoplasm was not so changed. This result indicated that the mobility of proteins smaller than mRNP complex was not sensitive to the structural change in the nucleoplasm (20). Otherwise, the energy depletion would change large nuclear matrix structures (50~52), which affect the much larger molecular size of mRNP ( $r \sim 133\text{nm}$  as a circular mRNP with 2.8kb) rather than that of EGFP<sub>5</sub> (longitudinal length  $\sim 28\text{nm}$ ). Recent reports have indicated that nuclear diffusion can be limited by a mesoscale viscosity for particles that are larger than 100nm in diameter (53). In contrast, the change of EGFP<sub>5</sub> mobility in the nucleolus induced by ATP depletion suggested that the effect of energy depletion on the microenvironment of the nucleolus was bigger than that of the nucleoplasm and cytoplasm, even though the origin of the significant mobility change in the nucleolus was not clear. Nevertheless, our results clearly indicate that the microenvironment of the nucleolus is physiologically very different from that of the nucleoplasm. It is interesting to note that the two motor proteins, nuclear actin and myosin I are related to rDNA and are required for RNA polymerase I transcription (54). Such ATP-binding motor proteins can modify the nucleolar microenvironment.

In this study, we have demonstrated that combination of FCS and oligomeric EGFP<sub>n</sub> with different lengths is a novel method to elucidate the nuclear microenvironment of living cells. The microenvironment of the two compartments in the nucleus can be now differentiated and analyzed by using tandem MR, two-color imaging, and FCS. We found that MR EGFP<sub>n</sub>, which is presumably inert, could rapidly diffuse inside of the cell nucleolus as well as the nucleoplasm depending only on the length of the protein. Our experimental system can be applied to understand the mobility of other functional proteins in the nucleolus as well as in the cytoplasm and the nucleoplasm. More importantly, it is also suggested that the microenvironment of the nucleolus is very sensitive to pharmacological energy depletion compared to that of the cytoplasm and the nucleoplasm. Consequently, it is concluded that the physiological state of the nucleolar microenvironment can be understood through mobility analysis of tandem MR in living cells. Combining this method with other fluorescence microscopic methods such as time-lapse microscopy will allow complementary analysis of the nucleolar microenvironment of various cell types and single cells while varying the cell cycle or other physiological conditions such as cell stresses. Effects of GTP depletion or specific inhibitors such as actinomycin D, which primarily affects ribosome biogenesis in the nucleolus through the inhibition of RNA polymerase transcription, will be also important to understand the relations between the nucleolar microenvironment and physiological conditions in detail.

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### **Abbreviations used in this paper**

cps, count per second; Cyt, cytoplasm; 2-DG, 2-deoxyglucose; DT, diffusion time; EGFP<sub>n</sub>, tandemly linked oligomeric EGFP; FAF, fluorescence autocorrelation function; MR, molecular ruler; FCS, fluorescence correlation spectroscopy; FRAP, fluorescence recovery after photobleach; mRFP, monomeric red fluorescent protein; NP, nucleoplasm; NL, nucleolus; SPT, single particle tracking; TSA, Trichostatin A

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**Table 1** (C. Pack et al.)**A**

|   | <i>fast (fraction)</i> | <i>slow (fraction)</i> | <i>very slow (fraction)</i> |
|---|------------------------|------------------------|-----------------------------|
| <b>Fast-diffusion-mobility (Cyt and NP)</b> | ○<br>(>90%)            | -                      | ○<br>(<10%)                 |
| <b>Slow-diffusion-mobility (NL)</b>         | ○<br>(<80%)            | ○<br>(20%~100%)        | -                           |

**B**

| Cell line | Type of GFP      | Cyt ( <i>fast / first comp.</i> )      |              | NP ( <i>fast / first comp.</i> )       |              |
|-----------|------------------|--|--------------|--|--------------|
|           |                  | $D$ ( $\mu\text{m}^2\text{s}^{-1}$ )   | fraction (%) | $D$ ( $\mu\text{m}^2\text{s}^{-1}$ )   | fraction (%) |
| HeLa      | GFP <sub>1</sub> | 23.4 ± 2.5                             | 95           | 22.7 ± 2.3                             | 96           |
|           | GFP <sub>2</sub> | 16.4 ± 0.8                             | 92           | 14.9 ± 0.8                             | 93           |
|           | GFP <sub>3</sub> | 13.1 ± 1.4                             | 91           | 11.6 ± 1.7                             | 91           |
|           | GFP <sub>4</sub> | 9.0 ± 0.8                              | 93           | 8.6 ± 0.6                              | 90           |
|           | GFP <sub>5</sub> | 8.3 ± 1.1                              | 90           | 7.8 ± 1.2                              | 90           |
| COS7      | GFP <sub>1</sub> | 27.1 ± 3.0                             | 90           | 22.3 ± 1.3                             | 95           |
|           | GFP <sub>2</sub> | 15.8 ± 1.0                             | 93           | 14.9 ± 0.3                             | 92           |
|           | GFP <sub>3</sub> | 14.7 ± 0.6                             | 91           | 11.1 ± 0.8                             | 94           |
|           | GFP <sub>4</sub> | 10.8 ± 1.0                             | 92           | 9.2 ± 0.8                              | 91           |
|           | GFP <sub>5</sub> | 9.5 ± 1.0                              | 90           | 7.9 ± 0.4                              | 90           |
| HEK       | GFP <sub>1</sub> | 21.0 ± 1.6                             | 95           | 24.9 ± 2.1                             | 93           |
|           | GFP <sub>2</sub> | 17.4 ± 3.5                             | 92           | 16.4 ± 2.2                             | 95           |
|           | GFP <sub>3</sub> | 14.1 ± 1.4                             | 91           | 13.2 ± 0.9                             | 91           |
|           | GFP <sub>4</sub> | 10.3 ± 1.1                             | 90           | 10.5 ± 1.8                             | 90           |
|           | GFP <sub>5</sub> | 10.4 ± 0.5                             | 92           | 9.6 ± 0.4                              | 92           |
| Cell line | Type of GFP      | NL ( <i>fast / first comp.</i> )       |              | NL ( <i>slow / second comp.</i> )      |              |
|           |                  | $D^*$ ( $\mu\text{m}^2\text{s}^{-1}$ ) | fraction (%) | $D^*$ ( $\mu\text{m}^2\text{s}^{-1}$ ) | fraction (%) |
| HeLa      | GFP <sub>1</sub> | 24.2 ± 1.3                             | -            | 5.8 ± 1.6                              | -            |
|           | GFP <sub>2</sub> | 17.6 ± 1.2                             | -            | 3.8 ± 0.5                              | -            |
|           | GFP <sub>3</sub> | 13.0 ± 1.0                             | -            | 2.1 ± 0.3                              | -            |
|           | GFP <sub>4</sub> | 11.0 ± 0.7                             | -            | 1.9 ± 0.1                              | -            |
|           | GFP <sub>5</sub> | 8.9 ± 0.4                              | -            | 1.6 ± 0.4                              | -            |
| COS7      | GFP <sub>1</sub> | 22.8 ± 1.7                             | -            | 5.7 ± 1.6                              | -            |
|           | GFP <sub>2</sub> | 16.1 ± 2.9                             | -            | 3.7 ± 0.8                              | -            |
|           | GFP <sub>3</sub> | 13.3 ± 1.5                             | -            | 1.7 ± 0.2                              | -            |
|           | GFP <sub>4</sub> | 9.0 ± 0.3                              | -            | 1.3 ± 0.2                              | -            |
|           | GFP <sub>5</sub> | 9.1 ± 0.8                              | -            | 1.1 ± 0.2                              | -            |

**Table 1. Diffusion constants of monomeric EGFP and tandem EGFP<sub>n</sub> in the cytoplasm, the nucleoplasm and the nucleolus in three different living cells.** (A) Two kinds of mobility, fast-diffusion-mobility and slow-diffusion-mobility, were differentiated by 2-component analysis of  $G(\tau)$ . The fast-diffusion-mobility has “*fast*” and “*very slow*” components. In contrast, the slow-diffusion-mobility has “*fast*” and “*slow*” components. These three components have different ranges for the diffusion time and fraction. (B) The diffusion constants and fractions in cytoplasm (Cyt) and nucleoplasm (NP) correspond to the average diffusion constants and fractions for the first component of the fast-diffusion-mobility (“*fast*” in Table A). The diffusion constant and fractions of the second component (“*very slow*” in Table A) is not shown. The diffusion constant in the nucleolus (NL) corresponds to those for the first component (“*fast*” in Table A) and the second component (“*slow*” in Table A) of the slow-diffusion-mobility. The fraction in the NL was not shown because the values were very variable (*see also the text*). The values of  $D$  in NL indicated those having a fraction over 50%. Data were averaged over 15~20 cells for HeLa, COS7, and HEK293 (Mean  $\pm$  SEM of three independent experiments).

\* The average values of  $D$  in the nucleolus were obtained from cells only expressing monomer EGFP and tandem EGFP<sub>n</sub> without mRFP-fibrillarin. These values were consistent with the result of Fig.5 E obtained by coexpressed cells.

## Figure Legends

Figure 1. **Tandem EGFP located in the nucleus.** LSM images of HeLa cells expressing (A) monomer EGFP, (B) EGFP<sub>2</sub>, (C) EGFP<sub>3</sub>, (D) EGFP<sub>4</sub>, and (E) EGFP<sub>5</sub>, are shown. The images were taken at 24hrs and 48 hrs after transfection with EGFP and tandem EGFP<sub>n</sub>, respectively. Bars, 20μm. Figures from (F) and (J) show enlarged images of cells expressing EGFP<sub>n</sub> from EGFP<sub>1</sub> to EGFP<sub>5</sub>, respectively, showing their location on the nucleus. Bars, 10μm. The fluorescent intensity in the nucleus decreased with the increase in size of the oligomers. Tandem EGFPs, regardless of their size, were equally distributed in the nucleoplasm except the nucleolus (see also supplementary Fig. S1).

Figure 2. **FCS measurement of tandem EGFP<sub>n</sub> in aqueous solution.** (A) Immunoblots of EGFP and tandem EGFP<sub>n</sub> and (B) normalized FAFs of the proteins in aqueous solution are shown. Lysates from HEK293 cells expressing monomer EGFP and oligomer EGFP<sub>n</sub> were blotted. The amplitude of FAF,  $G(0)-1$ , was normalized to unity for comparison of the extent of diffusional speed. Diffusion times obtained by fitting the functions (B) with a one-component model (equation (2),  $i=1$ ) are plotted in (C). Error bar shows SD of three measurements. Dashed line shows diffusion times calculated by equation (5) using the molecular weight and the measured diffusion time of Rh6G assuming the oligomeric EGFP<sub>n</sub> are spherical. Solid and dotted lines show the calculated diffusion times assuming that the linker between EGFP forms a rigid  $\alpha$ -helix and a linear shape, respectively. (D) Diffusion constants for EGFP and tandem EGFP<sub>n</sub> in aqueous solution. Each diffusion constant was calculated from equation (8) using the known diffusion constant ( $280\mu\text{m}^2\text{s}^{-1}$ ) and the measured diffusion time ( $21 \pm 2\mu\text{s}$ ) of Rh6G. Data are averaged from five independent experiments. (Mean  $\pm$  SEM of five independent experiments).

Figure 3. **Two kinds of diffusional mobility in the nucleus.** For FCS analysis, very weakly fluorescent and non-photobleaching cells less than the molecular number of  $N = 20$  in the detection volume of FCS, which corresponds to a concentration under  $10^{-7}$  M, were selected at 24h after transfection. After recording LSM images of selected (A) HEK, (C) COS7, and (E) HeLa cells, FCS measurements were performed on multiple places in the cytoplasm and in the nucleus. Bars 5μm. For clarification, only three typical and normalized FAFs of EGFP<sub>1</sub>, EGFP<sub>4</sub>, and EGFP<sub>5</sub> in the cytoplasm (curves 1) and the nucleus (curves 2 and 3) in HEK, COS7, and HeLa cells are shown in (B), (D) and (F), respectively. The rightward shift of the FAF curve indicates the slow-diffusion-mobility. The nucleus has both fast diffusion (curves 2) and much slower diffusion (curves 3). The normalized FAFs of EGFP and tandem EGFP<sub>n</sub> in the nucleus of a HeLa cell with the fast-diffusion-mobility (curves 2) are summarized in (G). The normalized FAFs of the fast-diffusion-mobility were gradually shifted to the right according their molecular size. (Cyt, cytoplasm).

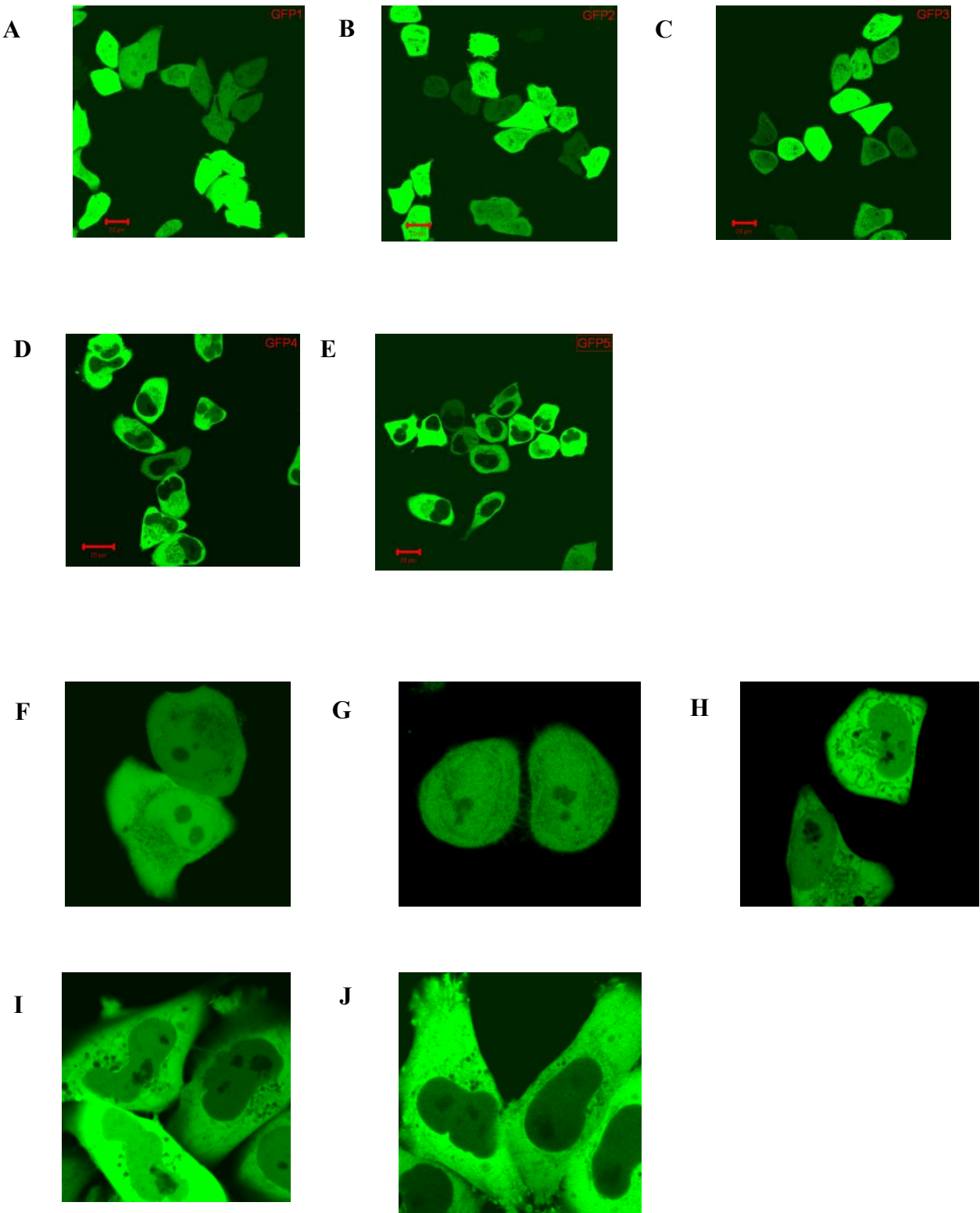
**Figure 4. Fast diffusional mobility of tandem EGFP<sub>n</sub> is dependent on the molecular length.** FAFs were fitted with a two-component model using equation (2),  $i=2$  for analyzing the diffusion times and the fractions of components 1 and 2. For the FAFs of the fast-diffusion-mobility in Fig. 3 G, the fraction of the first component was more than 90% for EGFP and tandem EGFP<sub>n</sub> in the cytoplasm and the nucleus. The diffusion times of the first components in HeLa cells (A), HEK cells (B), and COS7 cells (C) are plotted. The plots of measured diffusion times shown in (A), (B), and (C) show the average values of 5 cells for each protein. The error bars represent SD. The diffusion times of the first components were increased according to the molecular weight. As shown in Fig. 2, the calculated diffusion times for a spherical and a rod-like molecule were plotted for comparison. Dashed line shows diffusion times calculated by equation (5) using the molecular weight and the measured diffusion time of Rh6G assuming the oligomeric EGFP<sub>n</sub> are spherical. Solid and dotted lines show the calculated diffusion times assuming that the linker between EGFP forms a rigid  $\alpha$ -helix and a linear shape, respectively. Inserts show the ratio of diffusion times of the first component in the cytoplasm and the nucleus to that in aqueous solution.

**Figure 5. Slow diffusional mobility of oligomeric EGFP found in the nucleolus is much slower than that in the nucleoplasm.** For clear discrimination of nucleoplasm from nucleolus in cells weakly expressing EGFP and tandem EGFP<sub>n</sub>, a nucleolar protein of mRFP-fibrillarin was coexpressed. (A) LSM images of HeLa cells coexpressing mRFP-fibrillarin and (B) tetrameric EGFP, and (C) a merged image are shown. Bars, 5 $\mu$ m. FCS measurement was carried at 24hr after cotransfection of genes encoding mRFP-fibrillarin and EGFP<sub>n</sub>. Nucleoli with a diameter of over 2 $\mu$ m were selected for FCS measurement. FCS measurements were performed on multiple places in the nucleoplasm and the nucleolus. For clarification, (D) two normalized FAFs ( $G(0)-1=1$ ) of EGFP<sub>4</sub> measured on two points in the nucleoplasm (black curve) and the nucleolus (solid curve) of a single cell are shown. The normalized FAF indicated that the diffusion in the two positions was clearly different. The error bars represent SD for three measurements of a single cell. (E) The diffusion times of the second components (closed circles) for EGFP and tandem EGFP<sub>n</sub> in the nucleoli in HeLa cells are plotted. The insert shows the ratio of diffusion time in the nucleolus to that in the nucleoplasm ( $DT_{NL}/DT_{NP}$ ). Solid line shows the calculated diffusion time using the rod-like model (with an  $\alpha$ -helix linker) assuming that the apparent viscosity of the nucleolus is 5.2-fold higher than the nucleoplasm (insert). The plots of measured diffusion times shown in (E) represent the average values of 6 cells for each protein. The error bars represent SEM. (NP, nucleoplasm; NL, nucleolus).

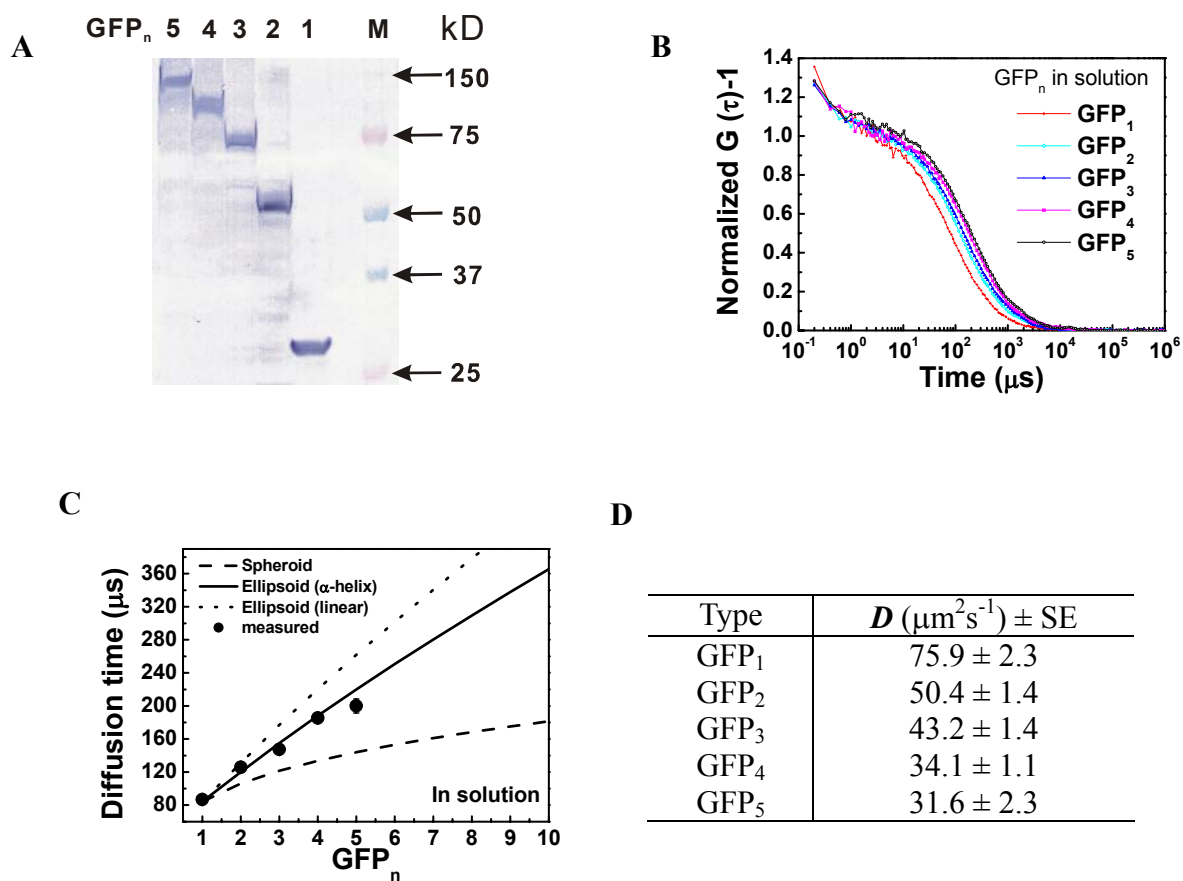
**Figure 6. MR mobility in the nucleolus is changed by the energy depletion.** HeLa cells were transiently transfected with EGFP<sub>5</sub>, or cotransfected with EGFP<sub>5</sub> and H2B-mRFP. The FAF of EGFP<sub>5</sub> in single HeLa cells is shown before (dashed black lines) and 30min after addition (solid red lines) of 2-deoxyglucose (6mM) and sodium-azide (10mM) to culture medium at room temperature. The redistribution of H2B-mRFP on the cells coexpressed with EGFP<sub>5</sub> was confirmed by LSM before and after the treatment. The changes of the normalized FAF on (A) cytoplasm, (B) nucleoplasm, and (C) nucleolus of a single cell induced

by energy depletion are shown. The amplitude of FAF,  $G(0)-1$ , was normalized to unity. The average diffusion times and the average fractions (insert) of the first (closed bars) and the second (open bars) components for EGFP<sub>5</sub> in the cytoplasm, the nucleoplasm, and the nucleolus before and after ATP depletion are shown in (D), (E), and (F), respectively. The error bars are the measured SEM (n=8 cells).

**Figure 1** (C. Pack et al.)



**Figure 2** (C. Pack et al.)



**Figure 3** (C. Pack et al.)

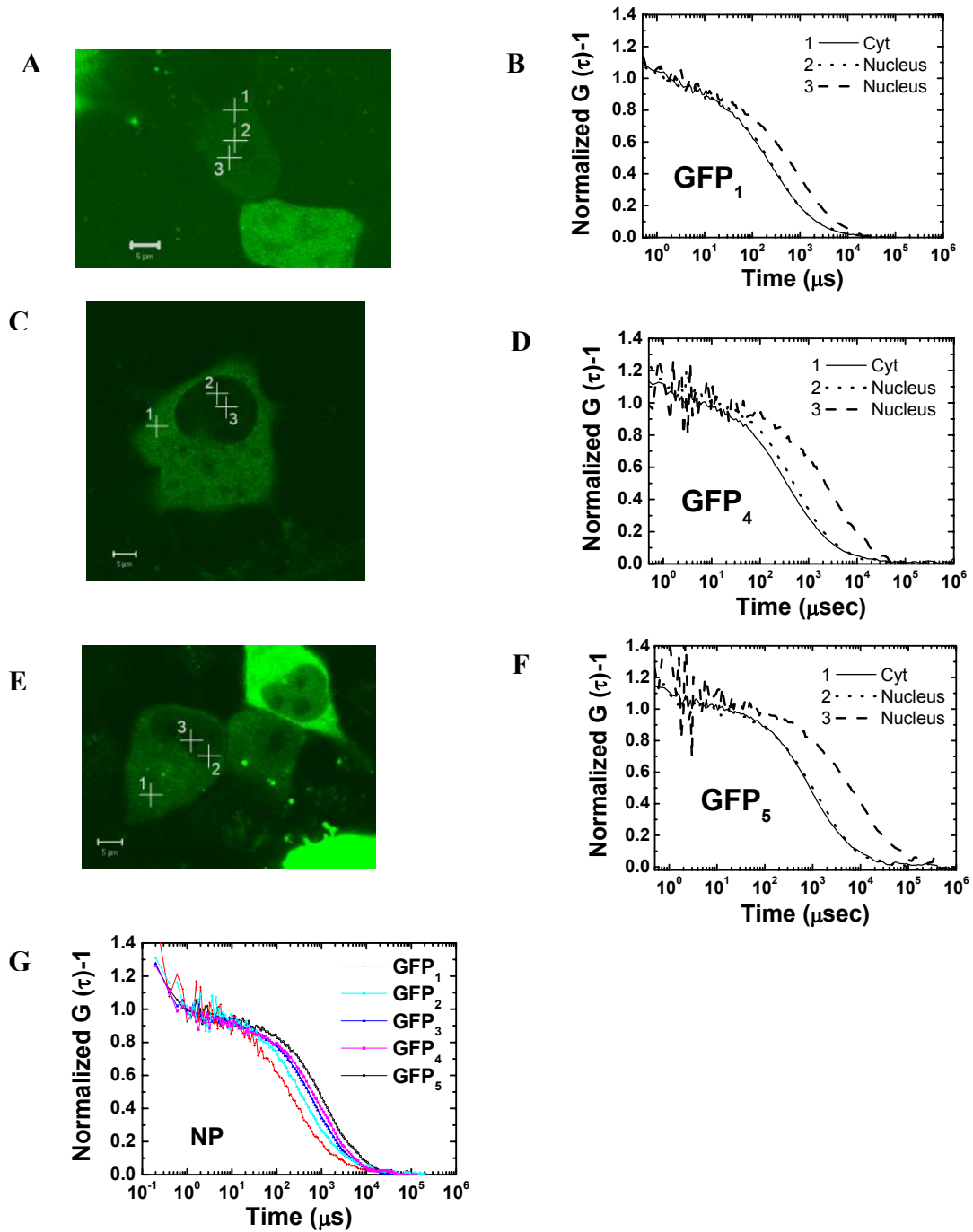
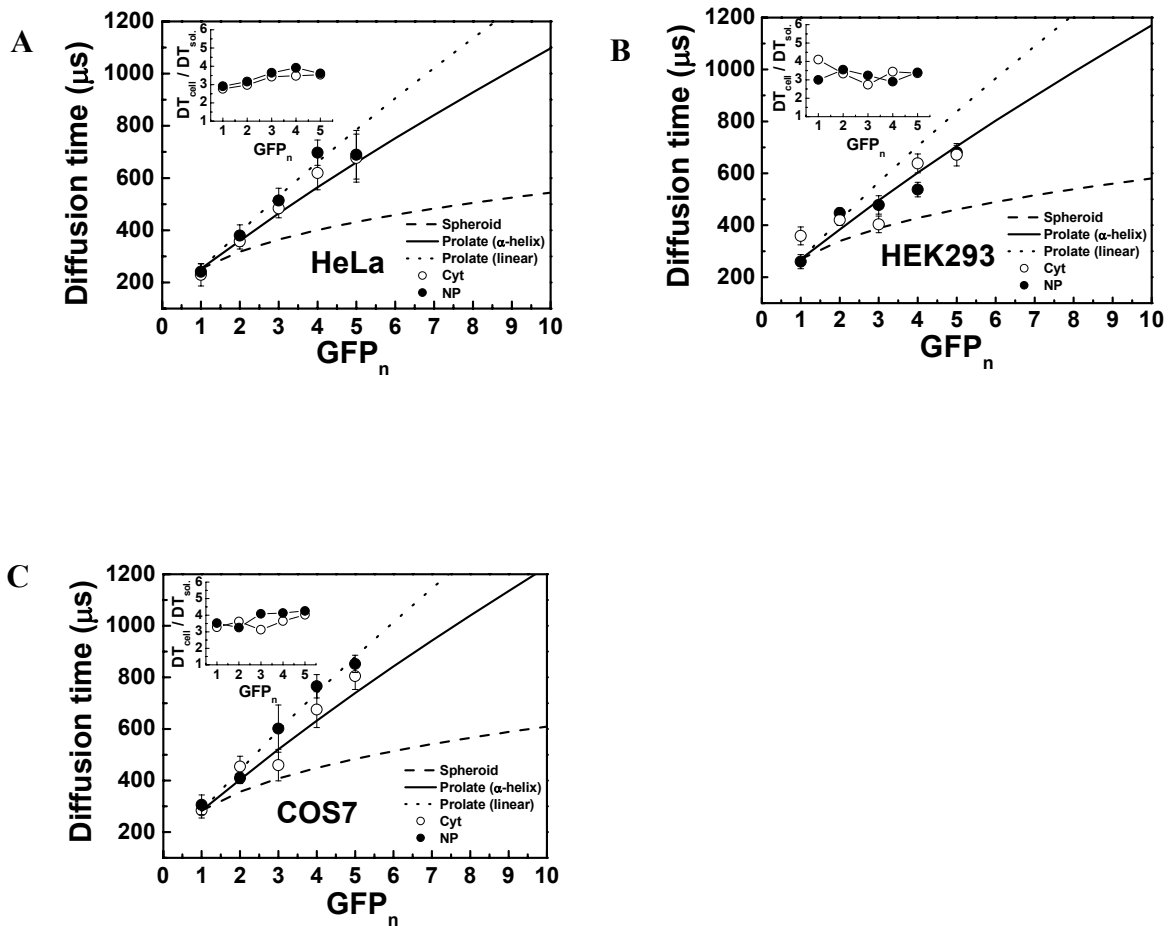
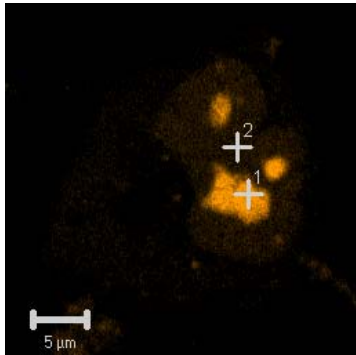


Figure 4 (C. Pack et al.)

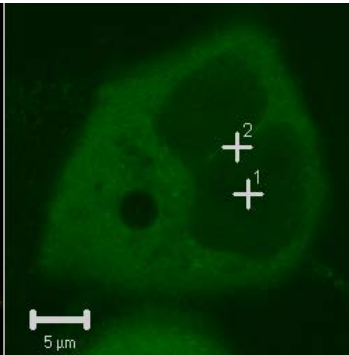


**Figure 5** (C. Pack et al.)

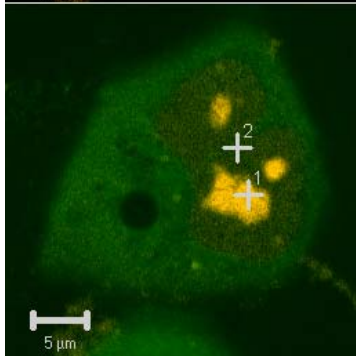
**A**



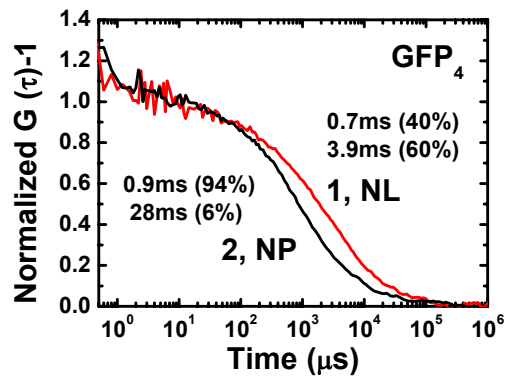
**B**



**C**



**D**



**E**

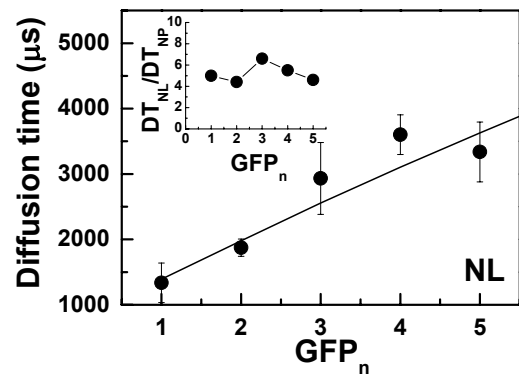
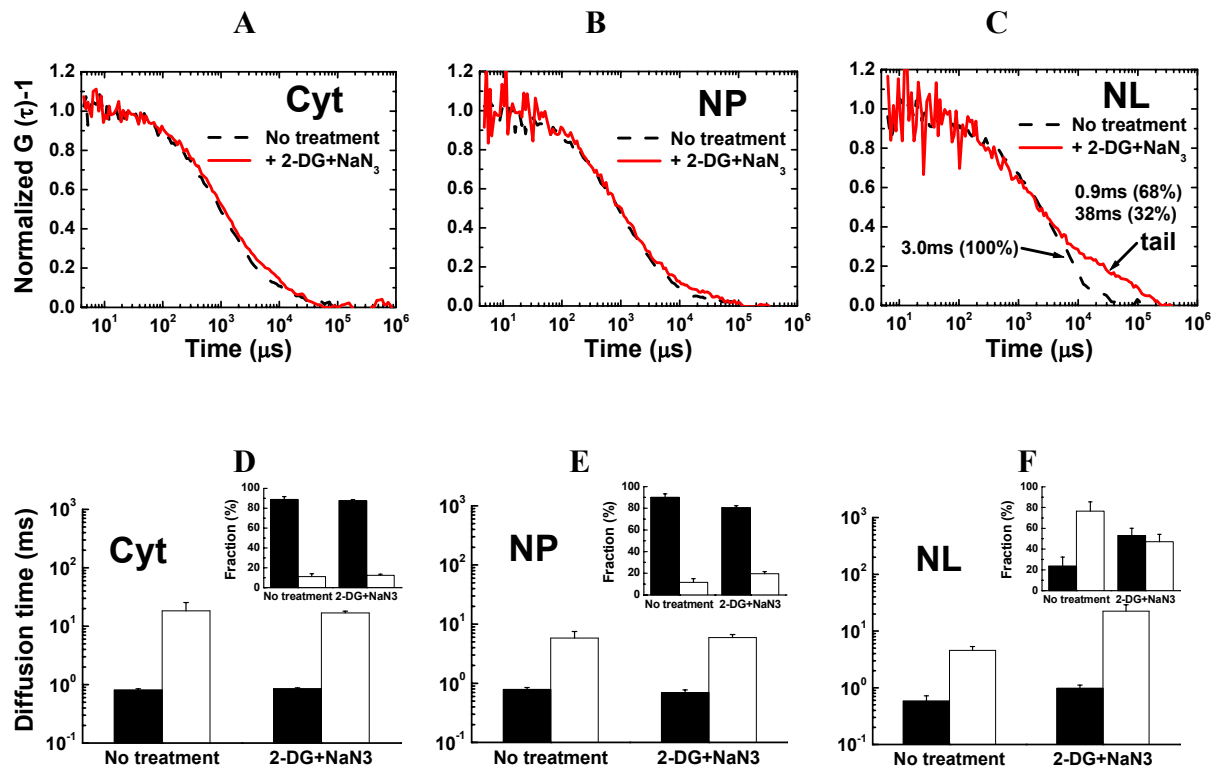


Figure 6 (C. Pack et al.)



## Supplementary Figures

Fig. S1. **Distribution of tandem EGFP in the nucleus.** LSM images of COS7 cells coexpressing (A) EGFP<sub>3</sub> and H2B-mRFP, (B) EGFP<sub>5</sub> and H2B-mRFP, respectively. The images were taken at 48 hrs after cotransfection with the plasmids of H2B-mRFP and tandem EGFP<sub>n</sub>. Bars, 10 $\mu$ m. The arrow lines indicate the direction of the corresponding intensity profiles that are shown in (C) and (D), respectively. The fluorescent signal of tandem EGFP is in green and the signal of H2B-mRFP is in red. H2B-mRFP was heterogeneously distributed in the nucleoplasm. In contrast, EGFP<sub>3</sub> (C) and EGFP<sub>5</sub> (D) located in the nucleoplasm were equally distributed

Fig. S2. **Fluorescence correlation function of tandem EGFP<sub>5</sub> in the nucleus.** Three FAFs (thin solid line) of EGFP<sub>5</sub> measured at three positions (see Fig. S4) in the nucleus of a COS7 cell and fitted functions (thick solid line) were shown. FAFs were measured for 15s three times at one position. There was no significant decrease of fluorescent intensity (Fig. S4) after FCS measurement. FAFs at position 1 (A) and 2 (B) were not fitted well by an one-component model, but by a two-component model. In contrast, FAF at position 3 (C) was well fitted by an one-component model. The diffusion times and the fractions were inserted on each FAF panels. The measured positions of FCS correspond to positions of 1, 2, and 3 in Fig. S4, respectively.

Fig. S3. **Time trace of fluorescence intensity on COS7 cells expressing EGFP<sub>5</sub>.** Three types of cell with high (A), medium (B), and low (C) fluorescence intensity were sequentially traced as a function of measurement time for checking the photobleaching effect by immobilized fraction. Cell expressing EGFP<sub>5</sub> 24hr or 48 hr after transfection were used. Inserts in (A), (B), (C) shows the FAFs separately obtained with measurement time of each 100sec. The first half of FAF was indicated in black curve and the latter half of FAF in red one.

A: Cells with high fluorescence often showed rapid photobleaching at the initial part of time trace by immobilized fraction and it affected significantly on slower diffusion time. DTs and fractions of first half part of measurement were 530  $\mu$ s with 91% and 210 ms with 9% (black). Instead, DTs and fractions of latter half were 510  $\mu$ s with 94% and 22 ms with 6% (red).

B: The relative small photobleaching of freely mobile GFP<sub>n</sub> was sometime observed, however this weak photobleaching may effect very small part of FAFs at long time correlation time. DTs and fractions of first half part of measurement were 690  $\mu$ s with 90% and 13 ms with 10% (black). Instead, DTs and fractions of latter half were 710  $\mu$ s with 93% and 18 ms with 7% (red).

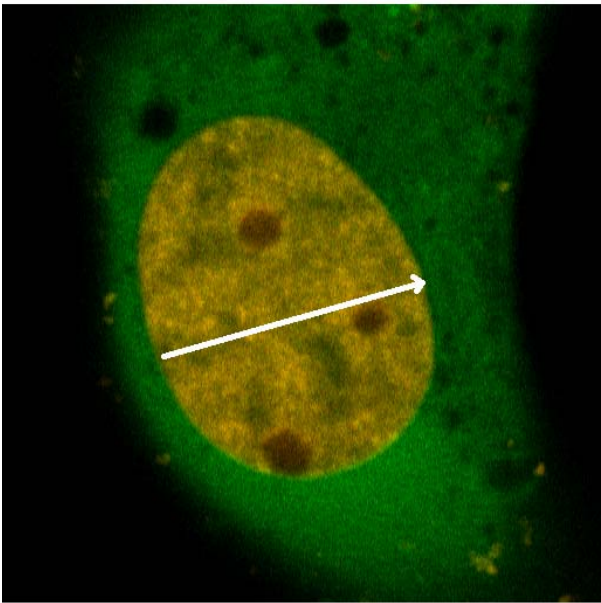
C: Some cells with very low fluorescence (< Number of molecules = 10) showed almost nothing of photobleaching. DTs and fractions of first half part of measurement were 540  $\mu$ s with 91% and 10 ms with 9% (black). Instead, DTs and fractions of latter half were 690  $\mu$ s with 93% and 24 ms with 7% (red).

**Fig. S4. Selection of weak fluorescent cells expressing tandem EGFPs.** LSM image of COS7 cell expressing EGFP<sub>5</sub> before (A) and after (B) FCS measurement (Fig. S2) in the nucleus are shown. The images were taken at 24 hrs after transfection. Bars, 10 $\mu$ m. Careful selection of cells with weak fluorescence in the nucleus allows analysis of diffusional mobility excluding a strong photobleaching effect.

**Fig. S5. Effect of Trichostatin A on the distribution of tandem EGFP.** LSM images of COS7 cells after 4hrs of TSA treatment with 200ng/ml (Sigma) (43) are shown. (A) and (B) indicate COS7 cells coexpressing EGFP<sub>3</sub> with H2B-mRFP, and EGFP<sub>5</sub> with H2B-mRFP, respectively. The cells were incubated in the culture medium before TSA treatment during 48 hrs after cotransfection with the plasmids of H2B-mRFP and tandem EGFP<sub>n</sub>. The arrow lines indicate the direction of the corresponding intensity profiles that are shown under the images. The fluorescent signal of tandem EGFP<sub>n</sub> is in green and the signal of H2B-mRFP is in red. Inhibition of histone deacetylation by Trichostatin A made fluorescence of H2B-mRFP much flattened compared to that of H2B-mRFP before TSA treatment (Fig. S1). In contrast, there was no significant change of fluorescence pattern for tandem EGFP<sub>3</sub> (C) and EGFP<sub>5</sub> (D) regardless of TSA treatment. Bars, 10 $\mu$ m.

Supplementary Figures  
Fig. S1 (C. Pack et al.)

**A**



**B**

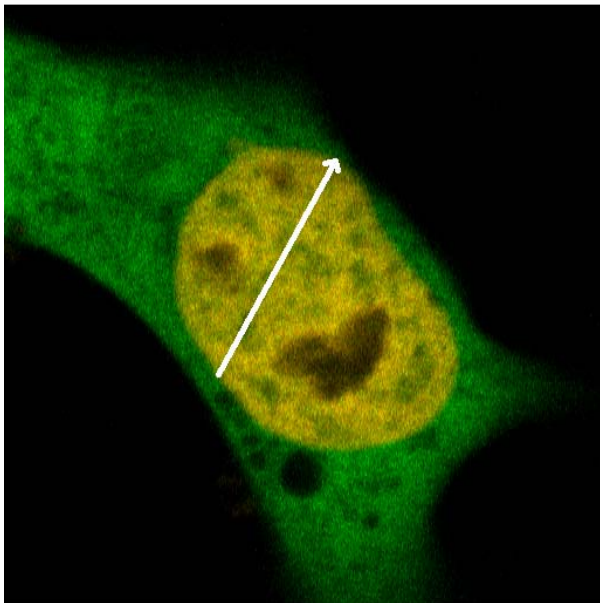
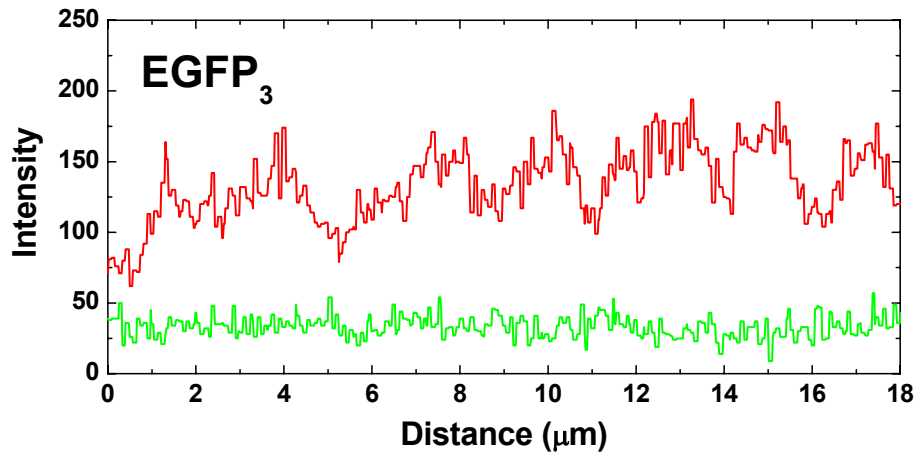


Fig. S1 (C. Pack et al.)

C



D

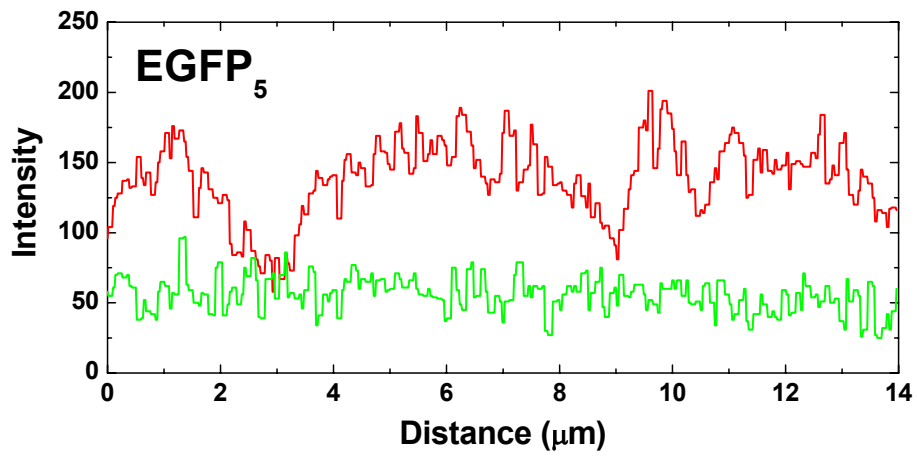
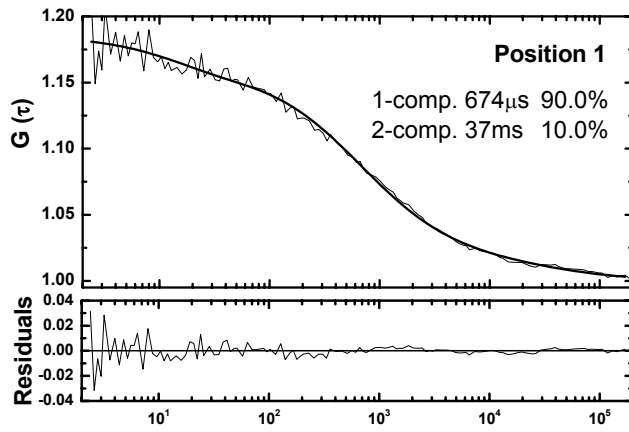
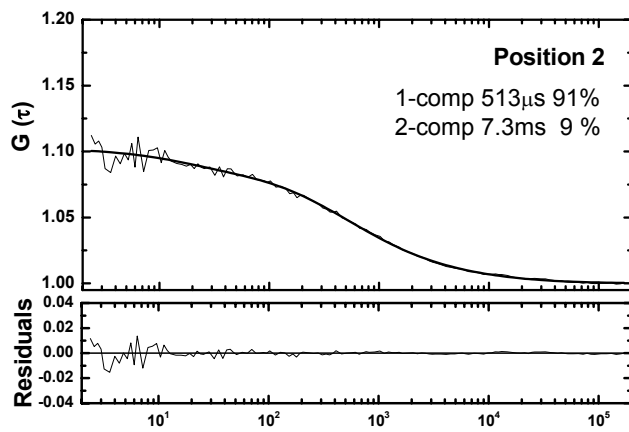


Fig. S2 (C. Pack et al.)

**A**



**B**



**C**

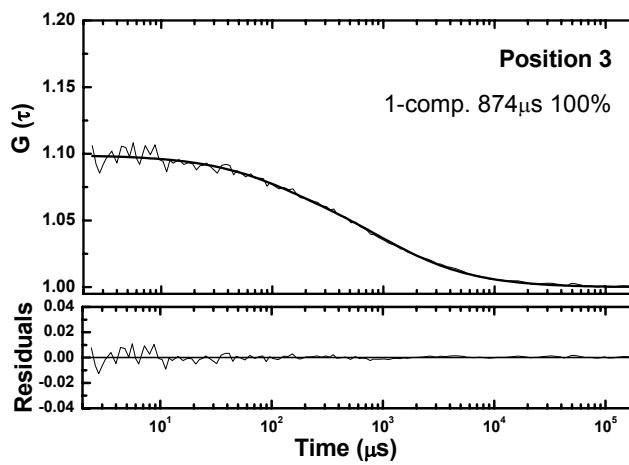
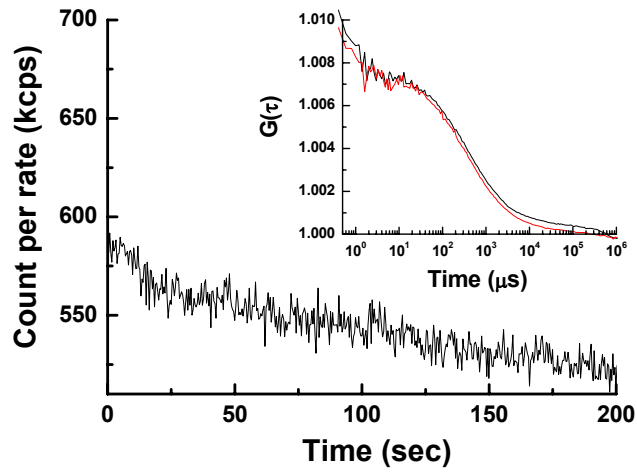
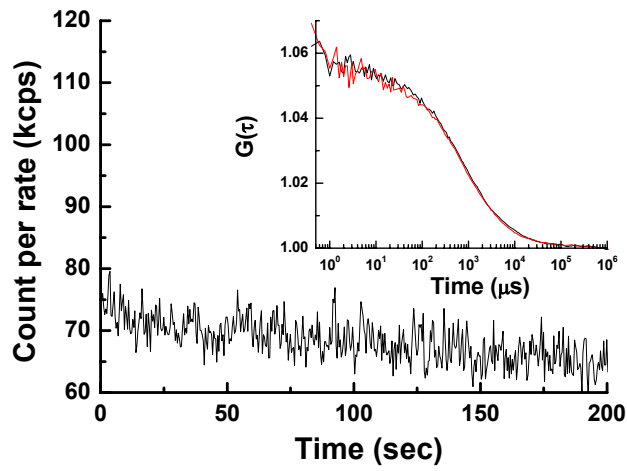


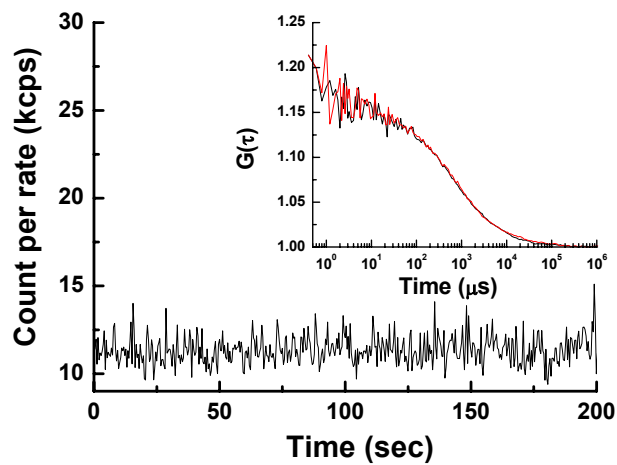
Fig. S3 (C. Pack et al.)



A



B



C

Fig. S4 (C. Pack et al.)

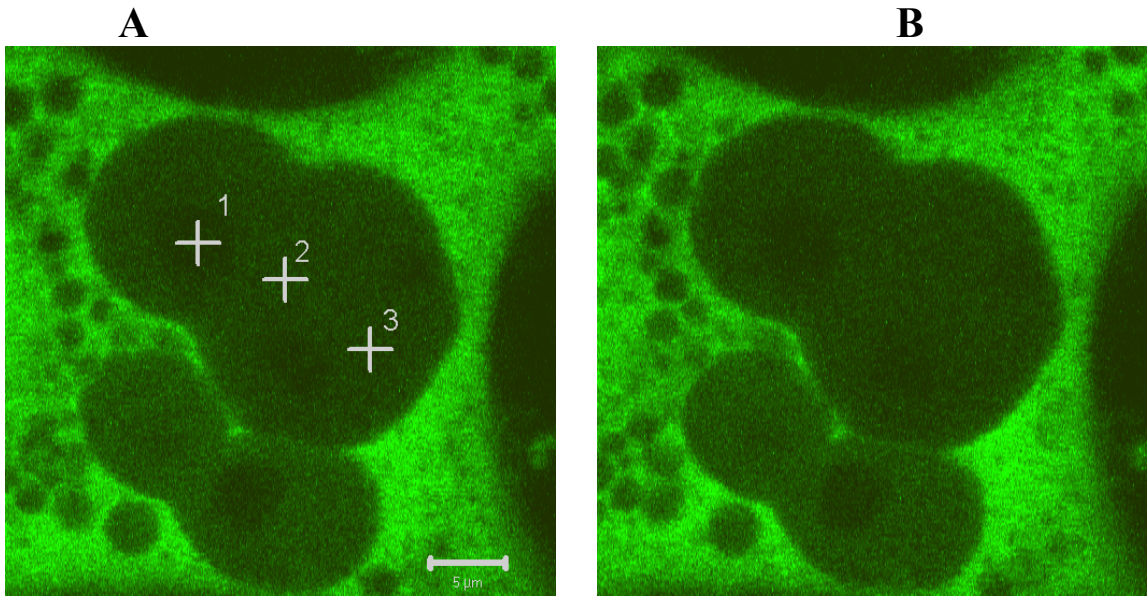
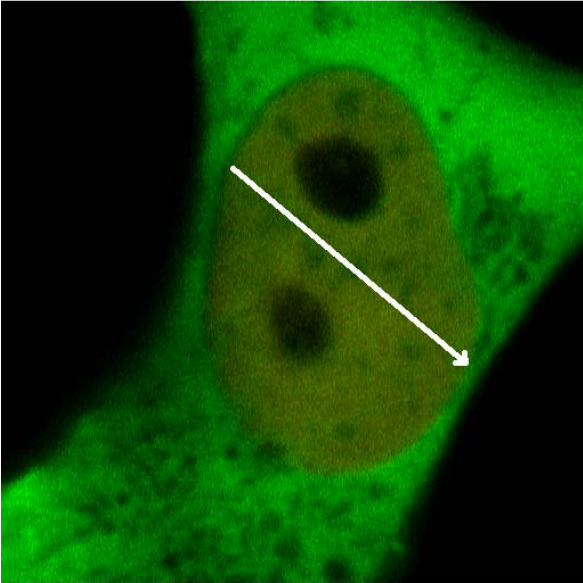


Fig. S5 (C. Pack et al.)

**A**



**B**

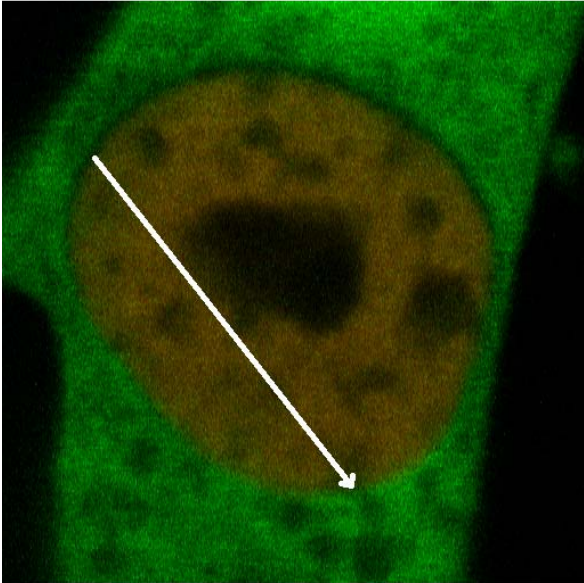
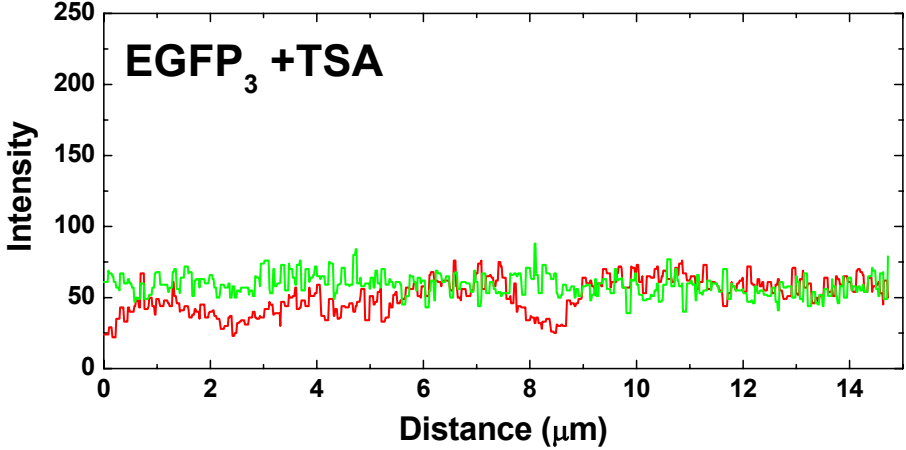


Fig. S5 (C. Pack et al.)

C



D

