Comparing the intracellular mobility of fluorescent proteins following *in vitro* expression or cell loading with streptolysin-O

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Abstract. The application of fluorescent proteins in live cells has greatly improved our ability to study molecular mobility, which both reflects molecular function in live cells and reveals the properties of the local environment. Although measuring molecular mobility with fluorescent fusion proteins is powerful and convenient, certain experiments still require exogenous macromolecules to be loaded into cells. Cell viability provides a rough gauge of cellular damage following membrane permeabilization, but it is unknown how permeabilization will affect intracellular mobility. We have used fluorescence correlation spectroscopy to measure the intracellular dynamics of the enhanced green fluorescent protein (EGFP) in living human embryonic kidney (HEK) cells under conditions where the EGFP is either expressed or loaded using streptolysin O (SLO) permeabilization to determine how permeabilization effects mobility. We found that purified EGFP loaded with SLO has the same mobility as the expressed EGFP, while the mobility of the expressed EGFP after SLO permeabilization treatment becomes slightly slower. Our results indicate that SLO permeabilization is often accompanied by the loss of cellular soluble proteins to the surrounding medium, which explains the apparent decrease in diffusion rates following treatment. These measurements are also relevant to the role of molecular crowding in the intracellular mobility of proteins.
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Keywords: molecular mobility; membrane permeabilization; streptolysin O; FCS; dual-color FCCS; anomalous diffusion; molecular crowding.

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1 Introduction

The study of the intracellular mobility of proteins and other molecules is attracting increasing attention from biophysical and life science researchers, since molecular mobility is in many cases related to molecular functions and interactions and also provides a useful tool for assaying the physical properties of the intracellular environment.[1] The use of fluorescent proteins as intracellular reporters has dramatically advanced our ability to study molecular dynamics and interactions in live cells. A variety of fluorescence imaging and spectroscopy techniques are now routinely applied to measure molecular mobility and dynamics in live cells, such as photobleaching methods [fluorescence recovery after photobleaching (FRAP), inverse fluorescence recovery after photobleaching (iFRAP), fluorescence loss in photobleaching (FLIP)], particle tracking, and fluorescence correlation spectroscopy (FCS).[2,3] Fluorescent proteins are particularly useful for studying protein dynamics and interactions in live cells, because the gene for fluorescent proteins can be fused to DNA for the protein of interest and conveniently transfected into cells using standard protocols. The protein of interest can then be monitored by fluorescence imaging and spectroscopy techniques without exogenous labeling as the cells manufacture the fusion protein with the fluorescent reporter. Among many available fluorescent proteins, the enhanced green fluorescent protein (EGFP) is one of the most commonly used in live cell applications to study intracellular protein dynamics due to its high molecular brightness and good photostability.[4–7]

Although expressing fluorescent fusion proteins is extremely useful and provides a versatile experimental tool, sometimes it is still advantageous or necessary to load external macromolecules into living cells. For example, some methods such as dual-color cross-correlation spectroscopy (FCCS), used to measure molecular interactions, require the use of multiple spectrally distinct fluorophores. Even though there are now many spectrally variant fluorescent proteins available, the choices are still limited for red emitters that are sufficiently bright and spectrally separated for dual-color fluctuation spectroscopy measurements. Therefore, red inorganic dyes are still often selected to pair with a green or blue fluo-

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rescent protein in dual-color fluctuation spectroscopy or even multicolor imaging applications.[8] In addition, specific biological applications may require the introduction of other exogenous molecules such as purified peptides, oligonucleotides, or drugs, since such molecules cannot be transfected and expressed by cells. For some proteins, the relatively large size of the fluorescent proteins may also inhibit molecular function of the fusion protein, so other fluorescence labeling methods may be preferable. Finally, in the case of mobility studies where the complex disordered intracellular environment may have size-dependent diffusion behaviors, it can be important to measure molecular mobility without the ~28 kD fluorescent fusion protein attached. So far it has been unclear to what extent the introduction of exogenous fluorescent reporters into living cells by membrane permeabilization will influence the observed molecular mobility through associated changes in the intracellular environment. A quantitative comparison of molecular mobility for proteins expressed in living cells versus those loaded by membrane permeabilization is the goal of these studies.

There are many ways to achieve cell permeabilization, and several methods are reviewed in reference.[9] Common methods include physical approaches to create membrane wounds such as electroporation, laser-induced microporation, loading by scraping, syringe loading, and chemical approaches to form pores on the membrane such as using lipsomes, bacterial toxins, or signal peptides.[9] Among the chemical methods, the use of streptolysin O (SLO), a bacterial toxin, has been demonstrated to achieve highly efficient and reversible permeabiliation as well as high cell viability.[10] Cell viability is an important indication of the integrity of the cellular functions after the permeabilization treatment, yet viability itself may not be enough to detect subtle differences that the treatment induces. For example, when molecular mobility is of interest, it is not clear *a priori* whether or not the permeabilization will significantly alter the intracellular environment in a way that modifies molecular mobility in the cells, and if so, to what extent. To make quantitative comparisons between mobility measurements with different fluorescence labeling methods, i.e., expression versus loading, it is essential to investigate the effects of the labeling method on the observed mobility. Thus, we measured and compared the mobility of the fluorescent protein EGFP in live human embryonic kidney (HEK) cells using three different conditions: (1) EGFP expression, (2) loading purified EGFP following SLO treatment, or (3) EGFP expression but measured following SLO treatment. These three conditions allowed a thorough evaluation to determine whether quantitative comparisons between mobility measurements made using the various approaches are directly comparable or not.

All measurement were made using FCS, which has become an important tool for measuring the mobility of proteins and other macromolecules in live cells and other complex media.[11–16] Some advantages of FCS include its high sensitivity, capability to measure dynamics at physiologically relevant low concentrations, and ability to make measurements with minimal cellular perturbation and low phototoxicity. FCS can be used to measure local concentration and molecular mobility as well as chemical or physical kinetic rates through the analysis of the autocorrelation function for the fluctuating fluorescence signal.[11–16] The diffusion of macromolecules in live cells is commonly observed to be anomalous, presumably due to the complexity of the intracellular environment in which mobility can be hindered by various factors such as molecular crowding, transient nonspecific binding interactions between diffusing macromolecules and cellular components, and interactions with other molecules or obstacles.[17–20] Therefore, the measured FCS data were fitted using an anomalous diffusion model,[18,21] and the results were used to evaluate how the SLO permeabilization treatment influenced the intracellular mobility of the cellular proteins.

2 Methods

2.1 *Cell Culture and Sample Preparation*

HEK 293 cells (ATCC, Manassas, Virginia) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, Virginia) supplemented with 10% feral bovin serum (Atlanta Biologicals, Atlanta, Georgia) and 100 U/mL penicilin and 100 μg/mL streptomycin (Mediatech, Herndon, Virginia). They were kept in a humidified incubator, Thermo Forma 370 (Thermo Electro Corporation, Marietta, Ohio) containing 5% CO_2 at 37°C. Cells were seeded in poly-D-lysine (0.1 mg/mL) (Fisher Scientific, Pittsburg, Pennsylvania) coated coverglass Lab-Tek II eight-well chambers (Naglenunc International, Rochester, New York) a day before transfection in a density that will grow into ~80% confluence on the day of transfection. Transfection was carried out using Lipofactamine 2000 (Invitrogen, Carlsbad, California) by following the manufacturer's protocol. The pEGFP-C1 mammalian transfection plasmid was used for EGFP expression. Experiments were done two days after transfection.

Purified EGFP diluted in nanopure water (18.2 mΩ/cm) was used to calibrate the laser excitation observation volume. For calibration purposes, the diffusion coefficient of EGFP in water was set to 78 $\mu m^2/s$ as reported.[22] The same eight-well chambers without coating were used for EGFP in solution. All FCS measurements, both *in vivo* and *in vitro*, were taken at room temperature. Data analysis was done with IgroPro (WaveMetrics, Lake Oswego, Oregon).

2.2 *SLO Treatment*

SLO (Sigma-Ardrich, St. Louis, Missouri) was diluted into phosphate buffered saline (PBS) containing 1% bovine serum albumin (Fisher Scientific, Pittsburg, Pennsylvania) to a concentration of 1 unit/μL. Cells were washed with PBS a few times before the SLO treatment. For the SLO loading of EGFP, 2 μM of purified EGFP in OPTI-MEM (Invitrogen, Carlsbad, California) with the final volume of 100 μL was added to each well of cells (surface area ~1 cm^2), then 2 μL of SLO was added onto the cell medium. Cells were kept in the incubator for SLO treatment for 10 minutes, and then 200 μL of regular growth medium was added to each well to stop the treatment. Cells were left in the incubator for at least 30 minutes to recover and then washed a few times with regular growth medium to get rid of the EGFP fluorophore in the culture medium before measurements. For the SLO treatment to cells expressing EGFP, an Alexa 633 labeled nuclear localization signal (NLS) peptide was used as an indicator of successful permeabilization treatment. The NLS peptide (**KR**TADGSEFES**PKKKRKV**E) was synthesized and conju-

gated with Alexa 633 maleimide (Invitrogen, Carlsbad, California), then purified with high-performance liquid chromatography (HPLC). For these experiments, 2 μM of Alexa 633-NLS (without purified EGFP) was diluted into the OPTI-MEM. Then the procedure described above for EGFP loading was followed.

2.3 *FCS and Imaging Instrumentation*

The two-photon excitation experimental setup is similar to what has been previously described.[23] An Olympus inverted microscope IX71 (Olympus, Melville, New York) with an Olympus 60x water immersion objective lens (numerical aperture=1.2) UPLSAPO60XW (Olympus, Melville, New York) was used. For FCS measurement, fluorescence is collected by an avalanche photodiode (APD) (EG&G, Vaudreuil, Canada), and in imaging mode, fluorescence is collected by a photomultiplier tube (PMT). A home-built beam scanning and imaging system was used for imaging, and a software-controlled motor stage ASI MS200 (Applied Scientific Instrumentation, Eugene, Oregon) was used to move the spots of interest in the cells to the laser focus. The wavelength used was 920 nm, and the average power of the 4x expanded laser beam at the sample was 2.8 mW for all the measurements. This wavelength efficiently excites both EGFP and Alexa 633. A dichroic mirror (575 DCXR) with red (645/75) and green (530/50) filters was used before the two PMTs to separate the EGFP and Alexa 633 emission for dual-channel imaging. For FCS, a dichroic mirror (625 DCXR) with red (666/68) and green (530/100) filters was used before two APDs. All filters and dichroic mirrors were from Chroma Technology (Rockingham, Vermont). The Alexa 633 and the filter sets were chosen such that there was no leakage of the red fluorescence signal into the green channel for FCS measurements.

3 Results and Discussion

We measured the intracellular mobility of EGFP expressed by HEK cells, the mobility of purified EGFP loaded into HEK cells using SLO permeabilization, and the mobility of the expressed EGFP after SLO treatment. Fluorescence imaging was performed before the FCS measurement to identify the cells of interest and to select the cellular regions for FCS measurements. Figure 1 shows sample images of cells expressing EGFP [1(a)], cells with successful EGFP loading by SLO permeabilization [1(b)], and cells expressing EGFP following SLO treatment [1(c) and 1(d)]. With the SLO treatment condition described in the methods section, about 50% of the cells were permeabilized, and over 90% of the permeabilized cells are viable after the treatment. A higher loading efficiency could be achieved if desired with a further optimized SLO dosage. For the experiment with loading of purified EGFP into HEK cells by SLO, it was straightforward to select the loaded cells because nonpermeabilized cells remain dark and were ignored for the FCS measurements [Fig. 1(b)]. For the case of cells expressing EGFP measured after SLO permeabilization treatment, it was not easy to distinguish successful and nonsuccessful permeabilization from the EGFP signal because there was no distinct difference between the two cases. To correctly identify permeabilized cells, we add Alexa 633-NLS, a red dye conjugated to a peptide coding a NLS, to the SLO treating medium. Since this peptide-dye

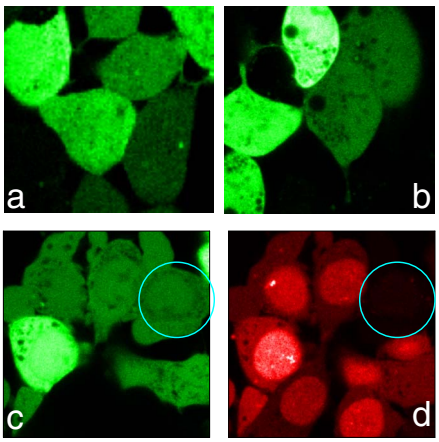

Fig. 1 Images of HEK cells (a) expressing EGFP or (b) loaded with EGFP by SLO treatment (b). (c) and (d) show cells expressing EGFP after SLO treatment and loading with Alexa 633-NLS. The green EGFP signal is shown in (c) and the red Alexa 633 signal is shown in (d). Most cells show Alexa 633-NLS intake, recognizable by the bright nuclear signal in (d), indicating successful SLO peameabilization, while the blue circles highlight an example of a cell with unsuccessful SLO permeabilization. (Color online only).

conjugate was not cell permeable, its intake to the cells would indicate successful pore generation upon SLO treatment. The EGFP signal had some spectral bleedthrough into the red imaging channel, so images could show a red channel signal even if the cell was not permeabilized. However, the NLS peptide directed these red labeled molecules to the cell nucleus where they were concentrated, providing a straightforward and simple marker for SLO permeabilization. In Figs. 1(c) and 1(d), cells show signal in both the green and red channels with a bright red cell nucleus, indicating successful permeabilization to the cells expressing EGFP. The two cells at the bottom of the image appearing in the red channel [Fig. 1(d)] but not appearing in the green channel [Fig. 1(c)] represent successful permeabilization of cells that were not expressing EGFP. One example of a cell expressing EGFP but with unsuccessful permeabilization is highlighted with a circle. Cells not expressing EGFP or not permeabilized were not included in the mobility measurement for the expressed EGFP with permeabilization treatment. We note that no Alexa 633 emission was detected in the EGFP detector channel, so this strategy for detecting successful SLO permeabilization did not distort FCS measurements of the intracellular EGFP mobility.

The imaging software allows computer mouse selection of points of interest in the imaged cells, and then a motorized stage moves the selected cellular points of interest to the focused laser beam for FCS measurements. For FCS experiments with each type of EGFP loading or expression, 10 to 15 cells are measured, and a total of 40 to 50 point measurements are taken. About half of the measurements are taken in the cytoplasm and half taken in the cell nucleus. For each point FCS measurement, four 30-second data runs were acquired. These four runs were then averaged and used to compute standard deviations.[24] Data were fit using an anomalous diffusion fitting function based on the published model,[18,21] with

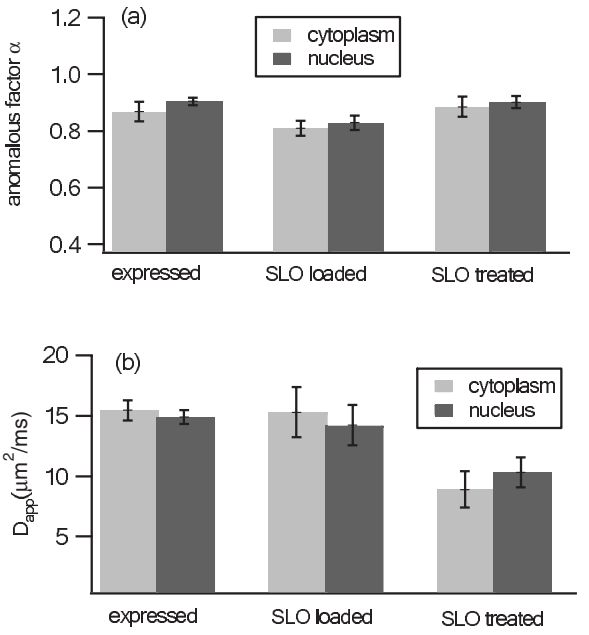

Fig. 2 (a) Anomalous factor α, and (b) apparent diffusion coefficient D_{app} for EGFP expressed, purified EGFP loaded into cells by SLO, and EGFP expressed in cells after SLO treatment. Error bars indicate one standard error.

$$G(\tau) = G(0)\left[1 + \left(\frac{8D_{app}\tau}{\omega_0^2}\right)^{\alpha}\right]^{-1}\left[1 + \frac{1}{x^2}\left(\frac{8D_{app}\tau}{\omega_0^2}\right)^{\alpha}\right]^{\frac{-1}{2}},$$

where $G(0)$ is the amplitude of autocorrelation function, ω_0 is the radial beam waist, x is the structure factor that defines the axial-to-radial beam waist ratio, and α is the anomalous factor with $\alpha < 1$ indicating anomalous subdiffusion as observed in our measurements. We reported the measured mobility in terms of the time-dependent apparent diffusion coeffcient, $D_{app}(\tau_D)$, which specifies the average diffusion coefficient for anomalous diffusion on the measurement length-scale. The apparent diffusion coefficient also can be thought of as the diffusion coefficient that a freely diffusion molecule would have if it crossed the observation volume in the same time as the observed anomalous diffuser.[21,25]

We found that mobility measurements acquired from any single point within a cell were highly repeatable. For a collection of diffusion measurements from many points, we observe the expected biological variation in local mobility when analyzing measurements from different individual cells, but we found that the average diffusion coefficient and anomalous factor determined for a large number of point measurements were consistent from day to day. Within experimental error, the average diffusion parameters were consistently repeatable for the different labeling protocols, and the observed patterns and variations were statistically significant. Summaries of measured mobility for the cellular EGFP protein with all three different preparations are in Fig. 2 and Table 1.

3.1 *Mobility of Expressed EGFP*

The measured intracellular mobility of expressed EGFP is very similar in both the cytoplasm and the nucleus. The apparent diffusion coefficient at the measurement length-scale, as defined by the focused beam size, was $15.5 \pm 0.8\ \mu m^2/s$ and $14.9 \pm 0.6\ \mu m^2/s$ in the cytoplasm and cell nucleus, respectively. This is approximately five times smaller than the diffusion coefficient of EGFP in water ($78\ \mu m^2/s$) as reported,[22] although the anomalous diffusion in cells makes direct comparison of diffusion coefficients complicated and dependent on the time scale. The measured anomalous factor of $0.85 \sim 0.9$ is typical for hindered subdiffusion of proteins in the intracellular environment. Since EGFP is not known to interact specifically with other molecules in HEK cells, the reduced mobility and anomalous behavior of EGFP could be caused by a variety of factors including local viscosity, molecular crowding, or transient nonspecific interactions. The fact that inert EGFP molecules have essentially the same mobility in cytoplasm and the nucleus suggests that diffusion in the two compartments is very similar for the small 29 kD EGFP molecule. The slightly lower anomalous factor, i.e., more anomalous diffusion, in the cytoplasm may suggest that cytoplasm is more disordered than the cell nucleus. For convenience in this experiment, mobility for the expressed EGFP is measured two days after the transfection. Other experiments done in the laboratory (data not shown) have demonstrated that the mobility of expressed EGFP measured two days after the transfection is the same as the mobility measured in stable transfected HEK cells, indicating that the transfection does not alter the measured mobility.

Table 1 Measured values for the anomalous factor α and the apparent diffusion coefficient D_{app} with one standard error for expressed EGFP, purified EGFP loaded into cells by SLO, and expressed EGFP following SLO treatment.

EGFP *in vivo*	Anomalous factor α		$D_{app}(\mu m^2/s)$	
	cytoplasm	nucleus	cytoplasm	nucleus
Expressed	0.87±0.02	0.91±0.01	15.5±0.8	14.9±0.6
SLO loaded	0.81±0.03	0.82±0.03	15.3±2.1	14.2±1.6
SLO treated	0.89±0.04	0.90±0.02	8.9±1.5	10.3±1.3

3.2 *Mobility of Proteins upon SLO Permeabilization*

After measuring the intracellular mobility of EGFP using *in vitro* expression of the EGFP, we then examined the mobility of EGFP proteins upon SLO permeabilization. The measured mobility data for the loaded EGFP and the expressed EGFP with SLO treatment are shown in Fig. 2 and Table 1. The measured mobility of the loaded EGFP is $15.3 \pm 2.1\ \mu m^2/s$ and $14.2 \pm 1.6\ \mu m^2/s$ in the cytoplasm and cell nucleus, respectively. These results show that the apparent diffusion coefficient of the loaded EGFP is indistinguishable from that of the expressed EGFP in both cellular compartments. For the loaded EGFP, the anomalous factor is slightly lower, i.e., slightly more anomalous, than for the expressed EGFP, perhaps suggesting some subtle variation in the intracellular environment induced by permeabilization. SLO treated cells also show a slightly larger standard error than expressed cells. However, on the whole the highly similar mobility measure-

ments for EGFP expression and EGFP loading suggest that intracellular mobility measurements are not dramatically altered by exogenous loading, and that such results can indeed be compared quantitatively with measurements of expressed proteins. This finding will be useful in future investigations of intracellular dynamics that require some combination of expressed and loaded molecules.

When pores are created following membrane peameabilization, cellular contents can leak into the surrounding medium through the pores. We performed an additional control measurement to identify how the mobility changes when EGFP is expressed but cells are still treated with SLO (no exogenous EGFP protein loaded). We observed that after cells expressing EGFP underwent SLO treatment, the measured mobility of EGFP became significantly slower while the anomalous factor remained the same as was measured without SLO treatment. The apparent diffusion coefficient went down to 8.9 ± 1.5 and 10.3 ± 1.3 $\mu m^2/s$ in the cytoplasm and cell nucleus, respectively. This somewhat surprising result, which at first appears to contradict the results shown above, can be explained after considering that the average fluorescence intensity of the expressed EGFP dropped significantly following the SLO treatment, with an average intensity decrease to around 40% of the original signal and individual cells with intensity decreases ranging from 10% to 80%. This variation presumably depends on the number of membrane pores induced in any given cell during the SLO treatment. The cells without successful permeabilization showed only minimal or no decrease in fluorescence intensity, and in all cases less than a 5% decrease was observed. The reduced intensity from SLO treated cells thus indicates that EGFP molecules are leaking from the cell into the surrounding media. The reduced diffusion coefficients following protein leaking seems to indicate that the most mobile EGFP molecules escaped from the cell, while a small subpopulation of EGFP molecules were for unknown reasons either trapped or confined in environments where they diffused more slowly. To further test this hypothesis, we examined how the measured diffusion coefficient of EGFP after SLO treatment correlated with the extent of membrane permeabilization, which was estimated by measuring the Alexa 633-NLS signal levels. We assumed brighter red signals indicated more permeabilized membranes and more protein leakage, which would thus predict slower mobility as well. As shown in Fig. 3, this prediction was indeed observed in the cell cytoplasm, with higher Alexa 633 signals correlaed with reduced cytoplasmic mobility of the expressed EGFP. This is consistent with our hypothesis that the reduced mobility of EDFP after SLO treatment was indeed due to the leaking of more mobile EGFP through the SLO pores to the surrounding medium, resulting in an enhanced population of the less-mobile EGFP molecules.

By combining the intracellular mobility results for all three types of EGFP preparations, the scenario became clear. First, there existed both the main relatively fast-moving EGFP population and a small population of less-mobile EGFP molecules in the cells expressing EGFP. We do not detect the slower-moving population in cells expressing EGFP or loaded with exogenous EGFP, presumably because it was only a small fraction of the total protein in the cell and the diffusion coefficients were not sufficiently distinct to resolve two diffusing components. When pores are formed on a cell membrane through SLO permeabilization treatment, proteins up to ~100 kD can leak through the pores, which can be as big as 35 nm in diameter.[10] Cells treated with SLO without adding additional EGFP then undergo enhancement of the slow-moving population, since the faster-moving population leaks out of the cell more efficiently. This finding is also consistent with the dramatically reduced average EGFP signal in these cells. The observation that EGFP diffusion rates were the same for the expressed and loaded EGFP seems to suggest that the intracellular environment was not drastically different following SLO treatment, at least as seen by a ~30 kD diffuser. However, we cannot rule out the possibility that some of the slow-diffusing population following SLO treatment was indeed related to some intracellular changes induced by the SLO permeabilization.

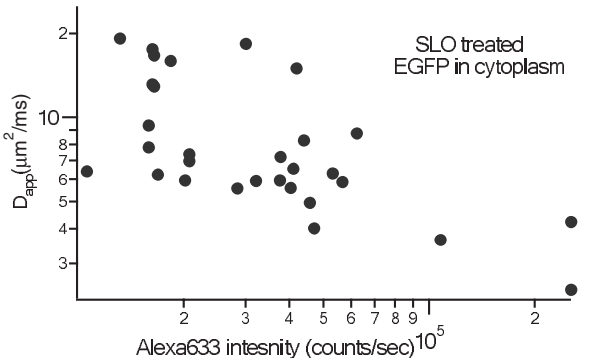

Fig. 3 Apparent diffusion coefficient of the EGFP expressed in HEK cells after SLO treatment varied with the fluorescene intensity of loaded Alexa 633-NLS. The measured diffusion rate descreases with increasing Alexa 633-NLS intake. This observation is consistent with our explanation that the reduction in measured mobility is due to the loss of mobile EGFP from the cell to the surrounding media since increasing Alexa 633-NLS signal also indicates more permeabilization of the membrane.

The somewhat surprising result that the mobility of the expressed EGFP without treatment had similar mobility to the loaded EGFP is relevent to the discussion of the role of molecular crowding in intracellular diffusion rates.[1,26,27] In particular, as discussed above, the SLO treatment resulted in a significant loss of soluble protein from the cells to the surrounding media. While we do not have good quantitative indicators of crowding for these measurements, it seems reasonable to assume that with the loss of soluble proteins the intracellular space should become less crowded and molecules should diffuse more rapidly. That we did not observe an increase in diffusion rates following SLO treatment seems to indicate that molecular crowding was not the major determinant of the observed intracellular mobility. More systematic studies of this effect for different size proteins and with some direct measure of the intracellular crowding would be required to confirm this conclusion.

4 Conclusion

We have shown that expressed and loaded EGFP have essentially the same measured intracellular mobility, and therefore, cell loading may be used as an experimental tool even when molecular mobility measurements are of interest. One caveat based on the above discussion is that if one is interested in the

mobility of an expressed protein following SLO treatment, caution is needed in interpreting the data because the remaining molecular populations following treatment may be enhanced for less-mobile fractions. This need for caution is further confirmed by the observation that the measured apparent diffusion coefficient for the expressed EGFP with SLO treatment varies more from cell to cell as indicated by the larger standard error for this set of measurements (Fig. 2 and Table 1). Again, this is probably due to the variation in the number of membrane pores created by SLO treatment and the variation in the recovery process, with the degree of selection for less-mobile fractions influenced by the number of pores. Thus, if one is interested in the mobility of an expressed protein following SLO introduction of an additional species, careful control measurements will be required to find conditions where the mobility of the expressed protein is not dramatically altered.

In the context of fluorescence fluctuation spectroscopy, one of the main motivations for exogenous loading of fluorescent molecules is to use dual-color FCCS to measure molecular interactions within living cells. Our findings suggest that SLO loading may indeed provide an effective approach to introduce a red labeled molecule to complement a green EGFP fusion protein for FCCS measurements. Caution will be required in interpreting such measurements to account for possible changes in mobility and the possible selective enhancement to the population of less-mobile molecules, which could have some influence on the extent of molecular interactions. Thus, FCCS measurements with exogenous loading of red fluorescent interaction partners will require careful analysis of the variations in mobility and concentration of the EGFP fusion protein and selection of loading conditions that produce minimal variation in these parameters.

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References

1. K. Luby-Phelps, "Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area," *Int. Rev. Cytol.* **192** (Microcompartmentation and Phase Separation in Cytoplasm), 189–221 (2000).
2. C. Chamberlain, and K. M. Hahn, "Watching proteins in the wild: fluorescence methods to study protein dynamics in living cells," *Traffic (Oxford, U. K.)* **1**(10), 755–762 (2000).
3. J. Lippincott-Schwartz, E. Snapp, and A. Kenworthy, "Studying protein dynamics in living cells," *Nat. Rev. Mol. Cell Biol.* **2**(6), 444–456 (2001).
4. V. Tozzini, V. Pellegrini, and F. Beltram, "Green fluorescent proteins and their applications to cell biology and bioelectronics," in *CRC Handbook of Organic Photochemistry and Photobiology*, edited by W. M. Horsphool and F. Lenei, CRC Press, Washington, D.C., pp. 139/1–139/23 (2004).
5. J. C. March, G. Rao, and W. E. Bentley, "Biotechnological applications of green fluorescent protein," *Appl. Microbiol. Biotechnol.* **62**(4), 303–315 (2003).
6. R. Y. Tsien, "The green fluorescent protein," *Annu. Rev. Biochem.* **67**, 509–544 (1998).
7. M. Zimmer, "Green fluorescent protein (GFP): applications, structure, and related photophysical behavior," *Chem. Rev. (Washington, D.C.)* **102**(3), 759–781 (2002).
8. S. A. Kim, K. G. Heinze, M. N. Waxham, and P. Schwille, "Intracellular calmodulin availability accessed with two-photon cross-correlation," *Proc. Natl. Acad. Sci. U.S.A.* **101**(1), 105–110 (2004).
9. J. L. Lauer and G. B. Fields, "In vitro incorporation of synthetic peptides into cells," *Methods Enzymol.* **289** (Solid-Phase Peptide Synthesis), 564–571 (1997).
10. I. Walev, S. C. Bhakdi, F. Hofmann, N. Djonder, A. Valeva, K. Aktories, and S. Bhakdi, "Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O," *Proc. Natl. Acad. Sci. U.S.A.* **98**(6), 3185–3190 (2001).
11. D. Magde, E. Elson, and W. W. Webb, "Thermodynamic fluctations in a reacting system. Measurement by fluorescence correlation spectroscopy," *Phys. Rev. Lett.* **29**(11), 705–708 (1972).
12. J. D. Muller, Y. Chen, and E. Gratton, "Fluorescence correlation spectroscopy," *Methods Enzymol.* **361**, 69–92 (2003).
13. R. Rigler and E. L. Elson, Eds., *Fluorescence Correlation Spectroscopy*, Springer-Verlag, Berlin (2001).
14. K. M. Berland, "Fluorescence correlation spectroscopy: a new tool for quantification of molecular interactions," *Methods Mol. Biol.* **261** (Protein-Protein Interactions), 383–397 (2004).
15. P. Schwille, "Fluorescence correlation spectroscopy: perspectives for in vivo characterization of single-molecule dynamics," in *Visions of the Cell Nucleus*, P. Hemmerich and S. Diekmann, Eds., American Scientific Publishers, Stevenson Ranch, Califonia, pp. 248–267 (2005).
16. W. W. Webb, "Fluorescence correlation spectroscopy: Inception, biophysical experimentations, and prospectus," *Appl. Opt.* **40**(24), 3969–3983 (2001).
17. S. Havlin and D. Ben-Avraham, "Diffusion in disordered media," *Adv. Phys.* **51**(1), 187–292 (2002).
18. P. Schwille, J. Korlach, and W. W. Webb, "Fluorescence correlation spectroscopy with single-molecule sensitivity on cell and model membranes," *Cytometry* **36**(3), 176–182 (1999).
19. M. J. Saxton, "Anomalous diffusion due to binding: a Monte Carlo study," *Biophys. J.* **70**(3), 1250–1262 (1996).
20. M. J. Saxton, "Anomalous diffusion due to obstacles: a Monte Carlo study," *Biophys. J.* **66**(2, Pt. 1), 394–401 (1994).
21. D. S. Banks C. Fradin, "Anomalous diffusion of proteins due to molecular crowding," *Biophys. J.* **89**(5), 2960–2971 (2005).
22. Y. Chen, J. D. Muller, Q. Q. Ruan, and E. Gratton, "Molecular brightness characterization of EGFP in vivo by fluorescence fluctuation spectroscopy," *Biophys. J.* **82**(1), 133–144 (2002).
23. A. Nagy, J. Wu, and K. M. Berland, "Observation volumes and gamma-factors in two-photon fluorescence fluctuation spectroscopy," *Biophys. J.* **89**(3), 2077–2090 (2005).
24. T. Wohland, R. Rigler, and H. Vogel, "The standard deviation in fluorescence correlation spectroscopy," *Biophys. J.* **80**(6), 2987–2999 (2001).
25. T. J. Feder, I. Brust-Mascher, J. P. Slattery, B. Baird, and W. W. Webb, "Constrained diffusion or immobile fraction on cell surfaces: A new interpretation," *Biophys. J.* **70**(6), 2767–2773 (1996).
26. R. J. Ellis, "Macromolecular crowding: obvious but under appreciated," *Trends Biochem. Sci.* **26**(10), 597–604 (2001).
27. A. S. Verkman, "Solute and macromolecule diffusion in cellular aqueous compartments," *Trends Biochem. Sci.* **27**(1), 27–33 (2002).