In vivo resolution of oligomers with fluorescence photobleaching recovery histograms

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Abstract Simple independent enzyme-catalyzed reactions distributed homogeneously throughout an aqueous environment cannot adequately explain the regulation of metabolic and other cellular processes in vivo. Such an unstructured system results in unacceptably slow substrate turnover rates and consumes inordinate amounts of cellular energy. Current approaches to resolving compartmentalization in living cells requires the partitioning of the molecular species in question such that its localization can be resolved with fluorescence microscopy. Standard imaging approaches will not resolve localization of protein activity for proteins that are ubiquitously distributed, but whose function requires a change in state of the protein. The small heat shock protein sHSP27 exists as both dimers and large multimers and is distributed homogeneously throughout the cytoplasm. A fusion of the green fluorescent protein variant S65T and sHSP27 is used to assess the ability of diffusion rate histograms to resolve compartmentalization of the 2 dominant oligomeric species of sHSP27. Diffusion rates were measured by multiphoton fluorescence photobleaching recovery. Under physiologic conditions, diffusion rate histograms resolved at least 2 diffusive transport rates within a living cell potentially corresponding to the large and small oligomers of sHSP27. Given that oligomerization is often a means of regulation, compartmentalization of different oligomer species could provide a means for efficient regulation and localization of sHsp27 activity.

INTRODUCTION

Proximal localization of enzymes involved in similar processes improves the likelihood of reaction intermediates reaching desired destinations (Pagliaro 2000). Furthermore, dynamic reorganization of cytoplasmic compartments provides an alternative means of regulating life processes through modulation of mass transfer limitations (Kaprelyants 1988). Cytoplasmic organization of proteins of different biochemical state, such as those that result in different oligomer sizes or binding affinities, could give rise to spatial domains within the cell of different apparent diffusive transport rates. We had hypothesized that resolution of these biochemical domains could be achieved through the generation of histograms of measured effective diffusion rates generated from a collection

of randomly sampled multiphoton fluorescence photobleaching recovery (MP-FPR) measurements taken throughout a population of cells. In addition, the generation of a diffusion rate histogram for a particular protein should facilitate the relative classification of an observed cytoplasmic diffusion rate with respect to the distribution of FPR measurements from the cell population. Visualization of cytoplasmic localization of different biochemical states might then be made through the classification of diffusion measurements performed along a spatial or temporal sampling grid within a cell.

A fusion of the green fluorescent protein variant S65T (eGFP) and the small heat shock protein sHSP27 is used to assess the ability of diffusion rate histograms to resolve compartmentalization of different oligomeric species. Existing as both a dimer and large multimer (up to 26 monomeric units; Lambert et al 1999), the oligomerization state of sHsp27 is regulated by the phosphorylation of 3 serine residues (Ser-15, Ser-78, Ser-82), whereby the number of sites phosphorylated determines its oligomeriza-

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tion equilibrium (Rogalla et al 1999). The large assortment of activities attributed to sHsp27, such as its ability to act as a chaperone (Haslbeck and Buchner 2002), ROS modulator (Arrigo 1999), and a barbed-end actin capping protein (Benndorf et al 1994), has given rise to the suggestion that these functions are regulated through the oligomeric state of sHsp27 (Garrido 2002). Depending on the need for a particular oligomeric state in a particular intracellular region, compartmentalization of these oligomers might be necessary, making sHsp27 an ideal protein to test our hypothesis.

Resolution of more than 1 diffusion rate through the regression of a multicomponent FPR model requires a signal-to-noise ratio (S/N) considerably better than what can be reasonably attained (Gordon et al 1995). Because of the approximate dependence of diffusivity on the cube root of the molecular mass of a species, monomers must associate into a 1000-unit (or greater) oligomer for the respective transport rates to be discerned. This requirement severely limits analysis of *in vivo* oligomerization through FPR rate regression analysis alone. As an alternative approach, we reasoned that spatial partitioning of oligomers would result in effective diffusion coefficient measurements corresponding to the dominant species within the microenvironment surrounding the measured focal volume. Consequently, a random sampling of effective diffusion coefficients throughout a population of cells might permit accurate diffusion rate estimates of each oligomer species through histogram analysis.

Effective diffusion coefficients were determined by MP-FPR analysis, in which transport coefficients are estimated by observing the rate of fluorescence recovery resulting from the influx of fluorophores into a volume rendered nonfluorescent by photobleaching (Axelrod et al 1976). Diffusion coefficients were then estimated through nonlinear regression to an appropriate transport model (Brown et al 1999). With the development of multiphoton microscopy, 3-dimensional diffusion coefficients can be determined with a spatial resolution of a few microns. Under physiological conditions, multiple MP-FPR measurements of diffusivity were made within a population of murine lung fibroblasts (L929) expressing eGFP-sHsp27. Histograms of several hundred independently measured diffusion coefficients were generated to identify subpopulations of protein diffusion rates within the cell population. These distributions were interpreted with respect to estimated diffusion coefficients of either large or small oligomers. The effects of heat shock were also investigated to examine the influence of hyperthermia on the structure of observed diffusion rate histograms.

MATERIALS AND METHODS

Cell culture

L929 cells (NCTC clone 929; L cell; derivative of Strain L) were grown in Dulbecco's Modified Eagles medium, with

iron-supplemented 10% bovine calf serum, and 250 mg/L geneticin. The molecular biology and generation of eGFP- and eGFP-sHSP27-expressing clones has been described previously (Borrelli et al 2002). Stock cultures were maintained as monolayers in T-25 flasks at 37.0°C in CO₂-enriched, humidified incubators. For imaging, cultures were grown as monolayers in 8-chamber coverslips (25 × 75 mm, Lab-Tek). Cells were maintained in the dark at all times when not being imaged to minimize potential phototoxic effects arising from eGFP expression. In prior studies, the eGFP-sHSP27 construct was demonstrated to behave in a manner completely consistent with native sHSP27 (Borelli et al 2002), suggesting minimum steric effects of the GFP on sHSP27 function.

Experimental apparatus

A mode-locked Ti:sapphire laser tuned to 840 nm (~200-femtosecond pulses at 80 MHz; 5W Verdi pump, MIRA 900F, Coherent Lasers) was used for MP-FPR. Laser intensity was modulated by a Pockels Cell (Conn Optics) driven with a square wave pulse generator. A 3× beam expander ensured overfilling of the back aperture of the microscope objective (63×, 1.4 N.A., oil immersion). The laser was scanned across the specimen with a MRC-600 scanhead (Biorad). A 650-nm short-pass dichroic mirror separated the fluorescence signal from the excitation beam. Fluorescence was measured with the photomultiplier tubes in the MRC-600.

The duration and intensity of photobleaching pulses were adjusted so that systematic error induced by fluorophore influx during bleaching was <3%. Pulse frequency was adjusted so that complete recovery of fluorescence occurred between successive pulses. To ensure minimal bleaching during fluorescence recovery monitoring, the monitoring laser intensity was attenuated so that the observed fluorescence signal did not decrease appreciably over time in the absence of bleach pulses. The focal volume size was estimated through nonlinear regression of MP-FPR analysis on a fluorophore solution of known diffusivity. A series of approximately 500 individual fluorescence recovery curves were averaged to minimize the effect of shot noise in the analysis (Fig 1). The MP-FPR model was fit with a Levenberg-Marquardt error minimization algorithm (nlinfit function of MATLAB[™]) to the averaged FPR data for estimation of diffusion coefficients. The absence of reversible photobleaching was confirmed by examining the effects of underfilling the back aperture of the objective lens on the fluorescence recovery curve. In addition, no fluorescence recovery was observed when photobleaching was performed on paraformaldehyde-fixed cells.

Fig 1. Typical MP-FPR analysis. (A) Fluorescence pulse train shows the progression of the recorded signal intensity; a.u., arbitrary fluorescence intensity units. (B) Successive pulses are averaged together to minimize the effect of shot noise. (C) The single-species MP-FPR model is fit to the normalized recovery curve (D) and residuals are examined to verify goodness of fit.

Effect of equilibria and oligomer size on D_{eff}

As derived by Brown et al (1999), the fluorescence recovery curve for a 2-photon excitation system is described by equation 1.

$$\frac{F(t)}{F_0} = \sum_{n=0}^{\infty} \left(\frac{1}{n!} \right) (-\beta)^n \times \left[\frac{1}{1 + n + (2nt/\tau_D)} \frac{1}{\sqrt{1 + n + (2nt/R\tau_D)}} \right] \quad (1)$$

$F(t)$ denotes fluorescence intensity as a function of time t . F_0 is the prebleach fluorescence intensity. The bleach depth parameter (β) is a function of the order of fluorophore excitation (n) and the laser intensity. The characteristic diffusion time of the fluorophore through the multiphoton focal volume is $\tau_D = w_r^2/8D$, where D is the diffusion coefficient. R is w_z^2/w_r^2 , where w_r and w_z are the e^{-2} radial and axial dimensions of the focal volume, respectively.

The fluorescence recovery of a 2-species system is described by (Gordon et al 1995),

$$\frac{F_T(t)}{F_0} = x_F \frac{F_\alpha(t)}{F_{\alpha,0}} + (1 - x_F) \frac{F_\beta(t)}{F_{\beta,0}}, \quad (2)$$

where F_T , F_α , and F_β denote overall fluorescence of species α and β , respectively, and x_F is the fluorescence fraction of species α . $F_\alpha(t)/F_{\alpha,0}$ and $F_\beta(t)/F_{\beta,0}$ are as described in equation 1.

Given that associated oligomeric forms comprise more

fluorophores than the disassociated species, their contribution to a recovery curve is greater. As a result, x_F is not equivalent to mole fraction. Differences in fluorescence intensities can be taken into account with the relation

$$x_F = \frac{K_{\text{eq}}}{K_{\text{eq}} + \left(\frac{\text{MW}_\beta}{\text{MW}_\alpha} \right)}, \quad (3)$$

where MW denotes the molecular mass of the respective species and K_{eq} denotes the dissociation constant of species α . Equation 3 assumes no self-quenching (ie, fluorescence intensity is quantized on the basis of the number of fluorophores present within the oligomer [Iino et al 2001]).

The effects of oligomer equilibria and the number of monomeric units of the associated oligomer on the effective diffusivity estimated by equation 1 was investigated by generating theoretical recovery curves for 2 different oligomer species diffusing through a photobleached volume according to equation 2. The x_F was determined with equation 3 for specified mole fractions and number of monomeric units within the larger oligomer. The single-component model of equation 1 was then fit to the simulated 2-component recovery data to examine the effect on D_{eff} estimates made with a 1-component model for a "true" 2-component system.

Calculation of theoretical diffusion coefficients

Theoretical diffusion coefficients were calculated with the Stokes-Einstein equation as a rough estimate to determine whether resulting histogram peaks might correspond to specific oligomer species. Assuming that temperature and effective intracellular viscosity does not vary spatially and that the hydrodynamic radius of a diffusing species is directly proportional to the cube root of its molecular mass, equation 4 was used to estimate the molecular diffusion rate.

$$D_\beta = \left(\frac{\text{MW}_\alpha}{\text{MW}_\beta} \right)^{1/3} D_\alpha \quad (4)$$

RESULTS

Effect of equilibria and oligomer size on D_{eff}

Complete partitioning of associated and disassociated oligomers, in which one microenvironment significantly favors one species over another, would be ideal. However, such domains might not necessarily be ubiquitous and localized regions could occur in which various equilibria exist between the 2 oligomer species. The dependence of

Fig 2. Effect of oligomerization equilibria on effective diffusivity estimates. Values of D_{eff} were regressed to a theoretical recovery curve on the basis of the transport of 2 diffusing oligomer species of different size. Solid lines were generated with $D_{\text{max}} = 1 \times 10^{-7} \text{ cm}^2/\text{s}$ as the basis, whereas dashed lines were generated with an assumed $D_{\text{max}} = 1 \times 10^{-6} \text{ cm}^2/\text{s}$ (ie, effectively decreasing the molecular mass of the monomer by 3 orders of magnitude). The number of monomeric units in the associated oligomer ranged from a dimer (2-mer) to a decamer (10-mer) in these simulations. The dotted line denotes the dependence of D_{eff} on the equilibria of eGFP-sHsp27.

Fig 3. Multiphoton imaging of L929 cells expressing eGFP. In control cells, eGFP is distributed homogeneously throughout the cell. Images are single slices from an image stack filtered with the smooth3 function in the Matlab Image Processing Toolkit.

estimated D_{eff} values on oligomer equilibrium and size was therefore investigated. Theoretical fluorescence recovery curves of 2 diffusing species were generated from the 2-component diffusion model (equation 2), and estimates of D_{eff} were regressed with a single diffusing species model (equation 1). The effect of equilibria on estimates of D_{eff} was determined by plotting normalized 1-component model diffusivity estimates vs the fluorescence fraction of the small oligomer (ie, fast species) within the photobleached volume (Fig 2). The effect of the number of monomers in the associated oligomer was investigated by adjusting the ratio of diffusion coefficients between the large and small oligomers.

Fig 4. Diffusion histograms of eGFP in L929 cells. The distribution of eGFP was unimodal, with a mean of $31.2 \mu\text{m}^2/\text{s}$ and a standard deviation of $5.9 \mu\text{m}^2/\text{s}$. The unimodal distribution of eGFP suggests that eGFP-sHsp27 compartmentalization of transport rates is not likely due to variation in intracellular architecture.

Fig 5. Multiphoton imaging of L929 cells expressing eGFP-sHsp27. Nuclear exclusion eGFP-sHsp27 is evident because the size of the oligomers prevent transport through the nuclear membrane. Images are single slices from an image stack filtered with the smooth3 function in the Matlab Image Processing Toolkit.

Estimated D_{eff} are always less than the arithmetic mean of the diffusivities for the large and small oligomers, indicating that estimates of D_{eff} favors the slower species. As well, this effect increases as the number of monomers in the associated oligomer increases. This is a result of the greater number of fluorophores within the larger oligomer, thereby increasing its relative signal contribution to the observed FPR recovery curve. To a lesser extent, increasing the monomer molecular mass also increased the influence of the larger oligomer on D_{eff} estimation. This effect is the result of a systematic deviation of the measured fluorescence recovery profile of the 2 diffusing species from the recovery for a single diffusing species given the independence of the fluorescence fraction, x_i , on the molecular mass of the monomer. With Figure 2, it should

be possible to estimate equilibria from D_{eff} measurements if the diffusion coefficients of the associated (D_{α}) and disassociated (D_{β}) oligomers and their relative sizes are known.

Partitioning of oligomer species permits estimation of equilibria within each compartment through transport measurements alone. By measuring D_{eff} at randomly selected locations, compartmentalization and equilibria can be resolved by analyzing the frequency of measured diffusion coefficients with the use of histograms. MP-FPR analysis of intracellular domains, consisting solely of either the large or the small oligomer, would produce histograms with peaks coinciding with D_{α} and D_{β} , whereas a histogram peak situated between these 2 values would indicate a state of equilibrium within compartments. Given that regressed values of D_{eff} favor the diffusivity of the larger oligomer at all concentrations, a histogram peak corresponding to the faster species will shift toward the transport rate of the slower species. Conversely, in the absence of compartmentalization, histograms of diffusion coefficients would not resolve the diffusivities of the 2 oligomer species, and a single histogram peak would result corresponding to the dominant equilibrium state within the cell.

eGFP histograms

Cells were cultured to 70% confluency and imaged with multiphoton laser scanning microscope (MPLSM) to resolve the distribution of eGFP in L929 cells in 3 dimensions. Image slices were collected every 0.18 μm from the base of the culture chamber. The resulting image stack was processed through the smooth3 function of Matlab. Expression of eGFP did not result in changes to cellular morphology, and fluorescence was generally distributed homogeneously throughout the nucleus and cytoplasm (Fig 3). L929 cells had an average thickness of $\sim 8 \mu\text{m}$ around the nuclei, with lamellipodia extending up to 30 μm from the nuclear envelope. A greater number of eGFP-excluded vacuoles were observed localized near the endoplasmic reticulum than the nucleus, but these vacuoles were smaller in diameter. The majority of the cell population was stationary over a period of 5 minutes, with the exception of a few cells that were morphologically active (eg, dividing or blebbing). No directed motion of vacuoles was observed during 3-dimensional imaging, verifying that minimal convective cytoplasmic transport occurred during the relatively brief MP-FPR measurements.

Effective diffusivity of eGFP (D_{eGFP}) was determined with 11-microsecond photobleaching pulses delivered every 10.3 milliseconds. This pulse sequence timing ensured negligible recovery during photobleaching and that 99% fluorescence recovery would be achieved before the

next bleaching pulse. The bleach depth parameter, β , ranged from 0.9 to 4.3, assuring compliance with assumed initial condition geometries of equation 1 (Brown et al 1999). Recovery curves (450) were averaged together to minimize the effect of shot noise and improve the statistical confidence of regressed parameters. The fit of equation 1 to the averaged recovery curve resulted in normally distributed residuals (Fig 1). One or 2 transport measurements were taken within each cell for a total of 168 independent estimates of D_{eGFP} . A Gaussian distribution was assumed and used to fit the resulting unimodal histogram to provide an estimate of the mean diffusivity (Fig 4). The average D_{eGFP} is 31.1 $\mu\text{m}^2/\text{s}$, with a standard deviation of 5.9 $\mu\text{m}^2/\text{s}$. The range of observed values is consistent with published values for GFP (Yokoe and Meyer 1996; Swaminathan et al 1997; Potma et al 2001). A histogram of diffusion coefficients measured in a fluorescein solution was used to examine whether the observed distribution for D_{eGFP} was solely the result of measurement uncertainty (Fig 4). Fluorescein FPR histograms are significantly narrower than those of eGFP within the intracellular environment, implying a heterogeneous distribution of cytostructural and macromolecular microenvironments within the cell.

eGFP-sHsp27 histograms

The fusion protein eGFP-sHsp27 exists as a hexadecamer (16-mer) and a hexamer (6-mer) as determined by analysis of cell lysates on linear glycerol gradients (Borrelli et al 2002). Lambert et al (1999) reported that hamster sHsp27 expressed in CCL39 and 3T3 cells resulted in associated and disassociated oligomers with 26 and 2 monomeric units, respectively (Lambert et al 1999). However, comparisons with wild-type sHsp27 oligomers within L929 cells revealed that the discrepancy in oligomer size could be attributed to cell line variation rather than the fusion of eGFP to sHsp27 (Borrelli et al 2002).

The spatial distribution of eGFP-sHsp27 within L929 cells was investigated with MPLSM performed as above for eGFP (Fig 5). Nuclear exclusion of eGFP-sHsp27 and a homogeneous distribution of the fusion protein throughout the cytoplasm were observed. Decreases in fluorescence intensity near the endoplasmic reticulum are likely due to exclusion of eGFP-sHsp27 from membrane-bound compartments. Overall cellular morphology was identical to that of L929s expressing eGFP only.

MP-FPR measurements were performed in L929 cells at $\sim 70\%$ confluency. The resulting residuals did not suggest the presence of anomalous diffusion or multiple diffusing species. MP-FPR protocols of 11-microsecond photobleaching pulses every 23.2 milliseconds resulted in β values from 1.6 to 4.9. These parameter values all met the necessary conditions for accurate MP-FPR diffusion rate

Fig 6. Diffusion histograms of eGFP-sHsp27 in L929 cells (non-heat-shocked). The distribution of eGFP-sHsp27 resolved 2 major peaks and 1 minor peak with median values of 5.1, 7.1, and 8.1 $\mu\text{m}^2/\text{s}$ with standard deviations of 0.7, 0.3, and 1.68 $\mu\text{m}^2/\text{s}$, respectively. The histogram suggests the presence of compartmentalization of the oligomers.

measurements (Brown et al 1999). The intervals between photobleaching pulses were longer than those for eGFP experiments because of the slower diffusivity of eGFP-sHsp27. Better S/N was achieved with eGFP-sHSP27 because cells were somewhat brighter than L929 cells expressing eGFP only.

Multiple Gaussian functions were regressed to the resulting diffusion rate histograms to estimate the number of diffusion regimes observed within the cell population (Fig 6). This was achieved by incrementing the number of regressed Gaussian functions until the added parameters were no longer statistically justifiable. Three distributions were sufficient to model observed diffusion histograms (F -test: $F_{3,\text{Gauss}} = 12.9 > F_{\text{crit}} = 3.0$), where F_{crit} is the critical value for rejection of the null hypothesis (ie, the distribution was not Gaussian). This approach resulted in the resolution of 3 distinct maxima with a distribution similar to those estimated during glycerol fractionation (Borrelli et al 2002). The histogram, consisting of 232 independent diffusion rate estimates, resolved 2 major peaks centered about 5.5 and 7.1 $\mu\text{m}^2/\text{s}$, and a small minor peak at 8.1 $\mu\text{m}^2/\text{s}$, with standard deviations of 0.65, 0.32, and 1.68 $\mu\text{m}^2/\text{s}$, respectively. The width of the faster major peak ($D_{\text{eff}} = 7.1 \mu\text{m}^2/\text{s}$) is only slightly wider than the uncertainty attributed to each measurement as determined by MP-FPR analysis of fluorescein in glycerol solutions, suggesting that the distributions in Figure 6 are near the resolution limit of our system for discerning these peaks.

In response to thermal stress, a fraction of large oligomers of sHsp27 disassociate (Kato et al 1994). This dissociation should be reflected in a shift in the diffusion histograms. To investigate this, L929 cells were heat shocked at 43°C for 15 minutes, after which they were

Fig 7. Diffusion histograms of eGFP-sHsp27 in L929 cells (heat-shocked). The distribution of eGFP-sHsp27 resolved only 1 peak at 6.8 $\mu\text{m}^2/\text{s}$ with a standard deviation of 1.9 $\mu\text{m}^2/\text{s}$. Compartmentalization was not observed. The change in distribution suggests changes in the compartmentalization of oligomers.

allowed to recover at 37°C for 2 hours. This recovery time was allowed because cells were morphologically very active during the heat shock and early recovery phase, making FPR analysis difficult. On inspection, approximately 30% of the cells had fully retracted lamellipodia, indicative of heat stress, while the remaining population was in the process of recovering their original morphology. Nuclear exclusion of eGFP-sHSP27 was still observed, whereas bright aggregates of the protein appeared in the cytoplasm immediately after thermal stress. eGFP-sHsp27 was also observed to localize to the centromere after heat shock, which might be indicative of sHSP actin binding activity. In control experiments, no redistribution of eGFP alone was noted in heat-shocked cells.

Diffusion histograms of heat-shocked eGFP-sHsp27 were generated with protocols identical to those used for non-heat-shocked cells. As with the previous MP-FPR measurements, residuals did not suggest the presence of anomalous diffusion. After heat shock, multiple Gaussian functions were again regressed to the measured diffusion rate histograms. The resulting distributions were well fit by a single Gaussian function. Thus, heat shock resulted in a shift from a 3-peak distribution to a single-peak distribution with a median value of 6.8 $\mu\text{m}^2/\text{s}$ (Fig 7).

Diffusion rate maps

Given that FPR analysis of a population of cells exhibiting either an associated or disassociated species exclusively would also produce multiple peaks on a histogram, an F -test was performed to determine whether the observed variance between cells could account for the observed population variance. On investigating the variance between 6 cells, for a total of 99 estimates of diffusivity, it was determined that intercellular variance could not ac-

count for the distributions observed in the histograms (ANOVA analysis; $F_{\text{crit}} \gg F_{\text{cal}}$ at $\alpha = 0.05$), where F_{cal} is the calculated ratio of the intracellular to the intercellular measurement variance (ie, the observed distribution is not due to intracellular variation). Consequently, the observed bimodal distribution is likely the result of a heterogeneous spatial distribution of intracellular compartments of different transport rates, possibly arising from compartments consisting of associated or disassociated species.

Fluorescence loss in photobleaching (FLIP) was also performed to determine whether oligomer compartmentalization was the result of membrane-bound enclosures. Physical partitioning by compartments can be resolved through continuous photobleaching of a selected volume within a cell while observing the resulting decrease in fluorescence in regions distal to the bleach volume. (Cole et al 1996; Nehls et al 2000). FLIP analysis within eGFP-sHsp27-expressing cells (data not shown) resulted in a gradual decline of fluorescence throughout the cell, suggesting that compartmentalization of eGFP-sHsp27 was not maintained by membrane-enclosed domains.

The multimode diffusion histograms of non-heat-shocked cells suggest the presence of intracellular partitioning of oligomers. To investigate this effect, diffusion measurements were taken along a predefined sampling grid within a cell to resolve compartmentalization (Fig 8). Values between measured points were interpolated so that a continuous map of diffusivity could be visualized. Through MP-FPR automation, the focal volume was rapidly repositioned to minimize the effects of temporal variation during map generation and to ensure the submicron accuracy of focal volume repositioning. Diffusion maps were then superimposed over an image of the sampled cell so that variation in measured D_{eff} might provide insights into the compartmentalization of oligomers.

A temporal pooled variance was estimated over 5 minutes to determine whether apparent compartmentalization changed rapidly with respect to the time required to generate a diffusion map. This analysis provided a measure of the likelihood that an intracellular environment consisting of a particular oligomer species, changed to the other species before completion of map data acquisition. MP-FPR measurements were taken at fixed locations for 5 minutes, whereupon the resulting temporal variance was estimated. To reduce the effects of cellular damage, the number of measurements at a particular location was kept to a minimum and a total of 18 independent measurements of temporal variance were pooled to improve accuracy. The resulting pooled temporal variance was $8.53 \times 10^{-17} \text{ cm}^4/\text{s}^2$ ($\nu = 72$). The probability of an oligomeric state changing before completion of map data acquisition was estimated by the extent to which the area of the temporal variance distribution ($\bar{X} = D_{\text{eff}}$ of initial

oligomeric state, $s^2 = \text{temporal variance}$) overlapped the distribution corresponding to the other oligomeric state ($\bar{X} = D_{\text{eff}}$ of final oligomeric state, $s^2 = \text{temporal variance}$). Because of a broader distribution of the slower peak, the probability of the disassociated state changing to an associated state (20%) was determined to have a greater likelihood than an associated oligomer undergoing disassociation (10%) during map generation.

DISCUSSION

GFP has proven to be a versatile noninvasive fluorescent marker for proteins and is largely unaffected by the range of conditions present within the intracellular environment (Tsien 1998). In addition to studies of protein expression and localization, GFP has also been used to investigate the rheologic properties of the cytoplasm (Yokoe and Meyer 1996; Swaminathan et al 1997; Dayel et al 1999; Potma et al 2001). Given the low presence of ionizable or hydrophobic groups on its surface (Ormo et al 1996; Yang et al 1996), GFP binds minimally to intracellular constituents, making it an effective inert spherical tracer (Luby-Phelps 2000). Reported estimates of cytoplasmic GFP diffusion coefficients range from 17 to 46 $\mu\text{m}^2/\text{s}$ (Yokoe and Meyer 1996; Swaminathan et al 1997; Potma et al 2001). This distribution of values has been attributed to variation in cytoplasmic architecture or cell volume (Luby-Phelps et al 1986; Seksek et al 1997; Potma et al 2001), which in turn varies between cell lines. As a result, to investigate the diffusivity of a specific GFP fusion protein, it was necessary to determine the diffusion coefficient of GFP within the cell line used to evaluate the effect of cellular architecture on observed transport rates.

Several factors that contribute to intracellular diffusion must be considered to identify sources of diffusive variability in the cytoplasm. Given that the viscosity of intracellular water does not differ significantly from that of pure water (Luby-Phelps 2000), the decrease in protein mobility is thought to primarily originate from mechanical obstructions arising from the actin cytoskeleton and other intracellular constituents (Swaminathan et al 1997). These effects have been investigated through cytoskeletal disruption and osmotic perturbation (Potma et al 2001). The cytoskeleton contributes ~50% to the overall reduction of protein mobility, whereas other mechanical barriers account for the remainder. As a result, the distribution of eGFP diffusion values within L929 cells is largely indicative of varying distributions of cytoskeleton and other intracellular constituents.

Histograms of eGFP diffusivity yielded a unimodal distribution with median value 31.1 $\mu\text{m}^2/\text{s}$, which is comparable to literature values. Potma et al (2001) analyzed the spatial variation of eGFP diffusivity within polarized *Dictyostelium* and found diffusivities ranging from 29 to

Fig 8. Intracellular diffusion map of EGFP-sHsp27 in an L929 cell. A grid sampling protocol (right inset) was used to generate a diffusion map. The map reveals localizations of fast and slow rates of transport. Independent MP-FPR measurements (49) were used to generate the map.

$33 \mu\text{m}^2/\text{s}$. eGFP diffusivity within L929 cells ranged from 15 to $47 \mu\text{m}^2/\text{s}$. The greater number of diffusion estimates in this investigation, and the relative precision of MP-FPR, results in the ability to resolve relatively low-frequency diffusion rates. Taken together, the broad eGFP diffusion rate histogram suggests the presence of environments in which cytostructural and intracellular constituents have a minimal effect on protein mobility, whereas other micro-environments exert a high degree of impedance.

The absence of multiple peaks within a diffusion rate histogram does not necessarily imply an absence of compartmentalization, but rather a homogeneous distribution of intracellular states relative to the multiphoton focal volume. Given that diffusion rate histograms are the summation of the distributions of compartmentalized species, distribution variances for each species must be sufficiently small to resolve their respective medians. Factors such as statistical uncertainty of MP-FPR measurements, variation in cytoplasmic architecture, degrees of monomer interaction, and effects that contribute to equilibria, will all have an effect on the overall structure of the histogram. In addition, compartmentalized regions smaller than the diffraction-limited multiphoton excitation volume, or multiple compartments at different equilibrium, would also result in the summation of multiple distributions to a histogram with a single resolved peak. Although a large number of possible mechanisms might inhibit the resolution of multiple peaks, the resolution of multi-peaked distributions is nevertheless indicative of spatial domains of protein mobility and thereby makes histogram analysis a useful approach to investigating intracellular organization.

Diffusion rate histograms have resolved at least 2 different diffusion rate populations of sHSP27 within living cells expressing eGFP-sHSP27. The discrimination of multiple diffusion rate peaks within histograms, and their relative position with respect to theoretical values, suggest that the MP-FPR measurements were sampling focal volumes in which either oligomeric form of sHSP27 was dominant. Given that oligomerization is often a means of regulation, compartmentalization of different oligomer species could provide a means for efficient allocation of sHsp27 activity. For example, the disassociated form of sHsp27 is involved in the enhancement of actin stability (Benndorf et al 1994; Lavoie et al 1995; Geum et al 2002) and inhibition of Daxx-mediated apoptosis (Charette et al 2000). The associated forms of sHsp27 exhibit chaperone activity in vitro (Ehrnsperger et al 1997) and inhibit apoptosis by interfering with cytochrome *c*-induced caspase activation (Bruey et al 2000) and decreasing levels of intracellular free radicals (Rogalla et al 1999). With these diverse roles, lack of compartmentalization would result in the homogeneous distribution of these activities throughout the cell.

Although the compartmentalization of eGFP-sHsp27 oligomers provides a potential explanation for the observation of multiple histogram peaks, alternative explanations clearly could account for this distribution. For example, high concentrations of solvated macromolecules could force a substantial portion of cytoplasmic water into an ordered state (Clegg 1984), effectively reducing solvent mobility to 1 or 2 dimensions. The distribution corresponding to the faster peak would then relate to the transport of eGFP-sHsp27 along the reduced dimension, rather than the smaller oligomer species. However, it is likely that constrained dimensionality would have been observed during FLIP because the continual depletion of fluorophores in either the reduced-mobility region or the bulk water phase would have resulted in fluorescence intensity differences between the 2 transport regimes.

Multiple diffusion rate histogram peaks can also arise from localized intracellular regions of reversible binding to cytoplasmic constituents, thereby reducing the rate of fluorescence recovery and resulting in the slower diffusion rate peak (Kruhlak et al 2000; Phair and Misteli 2000). The faster peak in turn, would represent eGFP-sHsp27, for which mobility is not constrained. Extensive in vitro evidence suggests sHsp27 acts as a chaperone (Ehrnsperger et al 1997; Lindner et al 2000) and as a barbed-end F-actin capping protein (Benndorf et al 1994; Huot et al 1996), both of which require transient adsorption/desorption. Upon heat shock, the collapse of actin filaments and denaturation of thermolabile proteins would have increased the number of available binding sites, causing an increase in the peak corresponding to the slower species.

The binding of large eGFP-sHsp27 oligomers to denatured proteins should result in an increase of its apparent hydrodynamic radius. Following heat shock, the histogram should therefore shift toward slower diffusion coefficients resulting from the binding of eGFP-sHsp27. Although this was not observed, it is not possible to discount eGFP-sHsp27 chaperone activity from influencing our results. The inability of the diffusion rate histogram to resolve compartmentalization after heating indicates equilibrium within any given focal volume that does not significantly favor either oligomer species. Under these conditions, a fraction of the large oligomers can be bound to denatured proteins while smaller oligomers remain unbound, increasing the observed effective diffusion coefficient.

Although the resolution of a single peak on heat shock is indicative of an absence of oligomer partitioning, compartment size might have been reduced to less than the resolution limit of the focal volume. Resolution could have also been compromised through the formation of more transient compartments, existing for less than the observation time of a MP-FPR measurement. This would result in a temporally averaged diffusion rate estimate of each transient oligomeric state. The reduction in the number of peaks resolved in the eGFP-sHSP27 histogram after heating could also be indicative of the presence of multiple equilibria within the sampled focal volume. The presence of additional peaks arising from multiple equilibria would result in a broad single-peaked distribution, given that the widths of the peaks in Figure 6 were close to the 95% confidence interval of a MP-FPR measurement.

Diffusion maps generated with our approach confound temporal and spatial effects. However, the method provides a good illustration of the spatial heterogeneity of transport rates within the cytoplasmic environment. Faster acquisition of map data is required to minimize the influence of temporal variation on the generated diffusion map. However, the use of alternative techniques for measuring intracellular transport rates might not necessarily resolve this issue. The partially bleached femtoliter volume in MP-FPR results in a rapid recovery of fluorescence to its prebleach intensity, allowing for rapid estimation of 3-dimensional diffusion coefficients. In comparison, confocal FPR measurements performed in the cytoplasm require 5 to 10 seconds for full recovery (Verkman 2002). Other techniques such as fluorescence correlation spectroscopy could require observation times of up to 20 minutes (Hess and Webb 2002). As a result, the relatively rapid collection of MP-FPR measurements makes it ideal for the generation of diffusion rate maps. Significantly, the small photobleached volume in MP-FPR also limits bleached fluorophore concentrations within a cell.

The ratios of diffusion coefficients of the 2 primary peaks in Figure 6 ($D_{fast}:D_{slow}$) was 1.40. Substituting the

molecular masses of the 2 oligomers into equation 4 yields a theoretical diffusion rate ratio for these 2 oligomers of 1.39. The close agreement of theoretical and measured values strongly suggests that the 2 primary peaks are the result of the near exclusive partitioning of an associated or disassociated oligomer in a particular focal volume. Our results suggest that intracellular targeting of specific oligomeric states could be used as a means of regulating sHsp27 function. Changes in the diffusion histogram in response to heat shock are also indicative of a physiological response, further supporting the interpretation that the observed distributions in Figure 6 are due to the physical partitioning of oligomers. Furthermore, histograms also provided a means of interpreting diffusion maps by assigning specific transport rate measurements to observable clusters within the cell population. Given that the fluorescence signals generated from associated and disassociated oligomers are nearly identical, the generation of diffusion maps might allow one to resolve compartmentalization otherwise undetectable with standard fluorescence microscopy.

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