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Fast spatiotemporal correlation spectroscopy to determine protein lateral diffusion laws in live cell membranes

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Spatial distribution and dynamics of plasma-membrane proteins are thought to be modulated by lipid composition and by the underlying cytoskeleton, which forms transient barriers to diffusion. So far this idea was probed by single-particle tracking of membrane components in which gold particles or antibodies were used to individually monitor the molecules of interest. Unfortunately, the relatively large particles needed for single-particle tracking can in principle alter the very dynamics under study. Here, we use a method that makes it possible to investigate plasma-membrane proteins by means of small molecular labels, specifically single GFP constructs. First, fast imaging of the region of interest on the membrane is performed. For each time delay in the resulting stack of images the average spatial correlation function is calculated. We show that by fitting the series of correlation functions, the actual protein “diffusion law” can be obtained directly from imaging, in the form of a mean-square displacement vs. time-delay plot, with no need for interpretative models. This approach is tested with several simulated 2D diffusion conditions and in live Chinese hamster ovary cells with a GFP-tagged transmembrane transferrin receptor, a well-known benchmark of membrane-skeleton-dependent transiently confined diffusion. This approach does not require extraction of the individual trajectories and can be used also with dim and dense molecules. We argue that it represents a powerful tool for the determination of kinetic and thermodynamic parameters over very wide spatial and temporal scales.

fluorescence | protein dynamics | membrane heterogeneity | transient confinement | single molecule

The plasma-membrane “fluid mosaic” model was proposed in a seminal work by Singer and Nicholson in 1972 (1). Since then, an intense research effort has led to significant advancements; current models include the notion that membranes are crowded environments (2) with a complex topology and that they interact with the cytoskeleton and contain microdomains of different size and lipid/protein composition (3–5). Using electron tomography, the structure of the membrane skeleton on the cytoplasmic face of the plasma membrane was clarified (6). It was found that virtually all of the cytoplasmic surface is covered by the meshwork of the actin-based membrane skeleton, and that the latter is closely associated with the membrane (within 0.83 nm). Because transmembrane proteins protrude into the cytoplasm, their intracellular domains may collide with these actin filaments that can act as “fences,” inducing temporary confinement of the protein within mesh domains. Transmembrane proteins are assumed to hop between these domains whenever there is space between the membrane and the actin filament owing to membrane structural fluctuations and/or when the actin filament temporarily dissociates (for further details see ref. 7).

Overall, these phenomena are master regulators of specific molecular interactions involved, for instance, in cellular signaling (8, 9). A major challenge in the study of these dynamical processes/structures is the requirement of high temporal and spatial resolutions. In this context, high-speed single-particle tracking (SPT) techniques are particularly successful and yield information at the nanomerescale of membrane-component dynamics. Based on this approach, and mainly by the pioneering work by Kusumi and coworkers, the compartmentalization of the plasma membrane into submicrometer domains and the hop diffusion of many relevant molecules were investigated (for a review, see refs. 7 and 10). However, the SPT approach suffers from at least three main drawbacks: (i) a large number of trajectories must be recorded and analyzed to fit statistical criteria, i.e., the time required to gather and analyze SPT data is considerably high; (ii) experiments typically require production, purification, and labeling of a single molecule with a suitable marker particle (e.g., gold nanoparticle or semiconductor quantum dot); (iii) the latter typically is rather large on the molecular scale and can cause cross-linking of target molecules or induce steric hindrance effects that may affect the biological function under study and often hinder its application to intracellular molecules (colloidal-gold particles typically used have diameters of 20–40 nm). In this respect, it was shown that single-molecule measurements can be performed with small (organic) fluorescent tags (11) or fluorescent proteins (12), but at the expense of the observation times accessible. Here, fluorescence correlation spectroscopy (FCS) is a very attractive approach. In fact, because it preserves single-molecule sensitivity even in the presence of many molecules, it can easily afford good statistics in a limited amount of time, and works well with genetically encoded fluorescent proteins (FPs). Usually, however, FCS is used as a local measurement of the concentration and residency time of molecules present in the focal area. As a consequence its spatial resolution is intrinsically limited by diffraction (~250 nm). In addition, information on dynamics is obtained indirectly and is influenced by assumptions on the models used to describe the phenomena under study. Many efforts were made to extend the FCS principle to smaller spatial scales, such as those playing a role in cell-membrane organization and dynamics. For instance, it was shown that “FCS diffusion laws” can be recovered by performing fluctuation analysis at various spatial scales by variation of the laser focal area (13, 14). Based on these data, inferences can be drawn about the dynamical organization of cell-membrane components by extrapolation below the diffraction limit (15). By contrast, recent reports successfully exploited the ~30-nm observation

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The authors declare no conflict of interest.

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area attained by stimulation-induced emission depletion (16) to directly measure the dynamics of membrane components at the nanoscale in living cells by fluctuation analysis (17–19). In alternative strategies introduced to extend FCS sensitivity to spatially heterogeneous phenomena, the focal area was duplicated (20), moved in space (21), or conjugated with fast acquisition cameras (22, 23). Also, Hebert et al. proposed a complete analysis of the full space–time correlation function by introducing the spatiotemporal image correlation spectroscopy (STICS) method (24). By measuring the position of the average spatiotemporal correlation function at each lag time, mean molecular displacements down to a few tens of nanometers were measured (24). Since its introduction, however, the STICS approach has been mainly used in conjunction with frame rates in the second time range. This in turn allowed accurate measurement of only slow protein fluxes (i.e., magnitudes of μm/min) such as those from retracting lamellar regions and protrusions for adherent cells, but neglected (fast) diffusive components (25, 26). Here, in an effort to overcome this limitation we propose a modified spatiotemporal image correlation spectroscopy approach that is suitable for determining diffusion laws of GFP-tagged integral membrane proteins in live cells with high temporal and spatial resolution and no need for the preliminary assumption of a specific interpretative model.

First, fast imaging of the region of interest on the membrane is performed; then, for each time delay in the resulting stack of images the average of the spatial correlation function is calculated by using the same mathematical basis of the STICS method. At this point, we shall show that by fitting the series of correlation functions, the actual protein diffusion law can be obtained directly from imaging, in the form of a mean-square displacement vs. time–delay plot (iMSD). Thanks to the iMSD vs. time plot, protein diffusion modes can be directly identified with no need for an interpretative model or assumptions about the spatial organization of the membrane. We shall first present simulated tests of this approach in the case of relevant 2D diffusion patterns (e.g., transient confinement and dynamic partitioning), and then actual experiments on live cells. In particular, we shall apply the present method to a GFP-tagged variant of the transmembrane transferrin receptor (TIR), a well-known benchmark of confined diffusion (27). In this latter case we shall show that our approach can successfully recover diffusion constants, confinement area, and confinement strength coefficients over many micrometers on the membrane plane, and their variation in response to drug treatments or temperature shifts. We believe that the present strategy may represent a useful asset for future research on membrane dynamic organization, particularly when the labeled particles under study are too dim or too dense to be individually tracked.

**Results**

**Extracting Protein Lateral Diffusion Laws from Imaging.** Fig. 1 schematically illustrates the presented method. First, fast imaging of a given membrane region of interest is performed for a suitable period (Fig. 1A). Then, recorded acquisitions corresponding to increasing time delays are correlated (i.e., each 2, 3, n repetitions). For each time delay, the average of the spatial correlation function is calculated (Fig. 1B). As thoroughly described in SI Text, if particles diffuse, the width of the correlation central peak increases with increasing time delay between frames (Fig. 1C, Upper). This peak can be fitted with a Gaussian function (Fig. 1C, Lower), whose variance \( \langle \sigma^2(t) \rangle \) (Eq. S4) represents the particle MSD directly extracted from imaging (labeled “iMSD” hereafter), with no need to extract and analyze individual single-particle traces. Thanks to the iMSD vs. time plot (Fig. 1D), protein diffusion laws can be directly identified with no need for an interpretative model or assumptions about the spatial organization of the membrane.

Simulations: Setting the Guidelines to Recognize Protein Diffusion Laws. To predict the output of the proposed analysis for membrane proteins dynamics, we performed four different sets of guided simulations for point particles in a 2D plane, exhibiting: (i) pure isotropic diffusion; (ii) confined diffusion due to a meshwork; (iii) partially confined diffusion; and (iv) dynamic partitioning into nanodomains (Fig. 2). These different diffusion modes show a clearly distinguishable behavior in the calculated iMSD plots. In simulation (i) the particles are diffusing isotropically with diffusivity “D” (Fig. 2A): as expected, the retrieved iMSD is linear in time with a slope that depends on the imposed diffusion coefficient (Fig. 2B). The plot in Fig. 2C shows the good agreement between the imposed diffusion coefficient \( D_{\text{micro}} \) and that derived from fitting iMSD to Eq. S11 (i.e., a stack of intensity images \( h(x, y, t) \) into (8) a stack of images representing the spatiotemporal evolution of correlation \( G(x, y, t) \)).

When particle dynamics is governed only by diffusion, the maximum of correlation remains in the origin (peak projection on Cartesian axis). It is possible to approximate \( G(x, y, t) \) with a Gaussian function whose variance corresponds to the particles average iMSD. (D) Plot of iMSD vs. time may be used to distinguish, for example, between free and confined diffusion.

Fig. 1. From STICS to iMSD to study protein diffusion on membranes. STICS operation converts (A) a stack of intensity images \( h(x, y, t) \) into (B) a stack of images representing the spatiotemporal evolution of correlation \( G(x, y, t) \).

(c) When particle dynamics is governed only by diffusion, the maximum of correlation remains in the origin (peak projection on Cartesian axis). It is possible to approximate \( G(x, y, t) \) with a Gaussian function whose variance corresponds to the particles average iMSD. (D) Plot of iMSD vs. time may be used to distinguish, for example, between free and confined diffusion.

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Fig. 2. iMSD analysis on simulated 2D diffusion. (A) Simulated condition: 2D isotropic diffusion, with diffusivity $D$. (B) iMSD is linear, with a higher slope for increasing $D$ values. (C) According between the theoretical $D$ value and that recovered from the analysis. (D) Simulated condition: 2D isotropic diffusion in a meshwork of impenetrable barriers (probability $P = 0$ to overcome the barrier). (E) iMSD plot starts linear and then reaches a plateau that identifies the confinement area and the corresponding linear size $L$. (F) Accordance between the theoretical $L$ value and that recovered from the analysis. (G) Simulated condition: 2D isotropic diffusion in a meshwork of penetrable barriers. Particles have probability $P > 0$ to overcome the barrier, thus generating a hop diffusion component. (H) iMSD plot starts linear (with a slope dependent on $D_{\text{trapping}}$) and then deviates toward a lower slope which depends on $P$. (I) Calculated $S_{\text{ord}}$ as a function of the imposed $P$. (J) Simulated condition: dynamic partitioning. Particles diffuse freely outside and inside the domain with diffusivity $D_{\text{in}}$ and $D_{\text{out}}$, respectively, and have probability $P_{\text{in}}$ and $P_{\text{out}}$ to enter or exit the domain, respectively. (K) Two characteristic iMSD traces in dynamic partitioning: diffusion (black) and trapping (gray). Diffusion shows linear iMSD, whereas trapping yields a constant iMSD. (L) Calculated $\tau_T$ as a function of the imposed $P_{\text{out}}$ (red dots) with respect to the trapping time directly calculated from molecular trajectories (black line).

$D_{\text{in}} = D_{\text{out}}/5$, and eventually exit the domain with probability $P_{\text{out}} < 1$ (Fig. 2F). Overall, such a system needs two Gaussian components for a satisfactory fitting (Fig. S1). This reflects the distinct molecular behavior of (i) isotropic diffusion outside of the nanodomains and (ii) trapping within the nanodomains. The former obviously produces a linear iMSD trace with a slope proportional to $D_{\text{out}}$, whereas the latter yields a constant iMSD trace (Fig. 2K). The constant iMSD, together with the concomitant temporal decrease in the amplitude of the correlation function (Fig. S2), is proof of molecular trapping within the nanodomains. In this case, fitting the amplitude of the correlation allows measurement of the average trapping time ($\tau_T$) within the nanodomains, according to Eq. S8. Fig. 2L shows the values of the recovered $\tau_T$ as a function of the probability to exit the domain (red dots) with respect to the actual trapping times directly calculated from particle trajectories (black line). As expected, the recovered $\tau_T$ decreases with increasing $P_{\text{out}}$. Note that as soon as the trapping time becomes comparable with the characteristic diffusion time on the PSF scale (around 10 ms in Fig. 2L), the contribution of the latter cannot be neglected and leads to a deviation of the calculated $\tau_T$ from its expected value, i.e., the “diffusive” and “trapping” components cannot be properly separated (gray area in Fig. 2L; for further details see SI Text). All together, these simulations set the guidelines for the identification of the different modes of diffusion based on the experimental iMSD trend. As shown in Fig. S3 for 2D free diffusion, the iMSD trace calculated from the variance coincides with the iMSD calculated from the temporal correlation by means of Eq. S5. Thus, fitting the amplitude could represent an independent strategy to measure particle diffusivity. These considerations do not apply when the average number of particles ($N$, Eq. S5) varies as a function of time (e.g., in the case of photobleaching). The iMSD calculation is feasible under the typical experimental conditions met with FP-tagged molecules and live cells (i.e., high particle density, low signal-to-background ratios), as quantitatively analyzed through simulations (Fig. S4).

Simulations: $\sigma_{\text{iMSD}}$ and Particle Size. Thus far, we have presented iMSD traces as obtained after subtraction of the $\sigma_{\text{iMSD}}^2(z = 0)$ value, hereafter indicated as $\sigma_{\text{MSD}}^2$ extracted by fitting iMSD with the proper model (SI Text). However, this parameter can provide quantitative information about the size of the particle under study. To demonstrate this property, we performed a set of simulations in which particles of increasing sizes ($R$) freely diffuse in a box as shown in Fig. 3A, changing the particle size does not affect the iMSD overall shape, but impacts on the intercept value. As expected, $\sigma_{\text{iMSD}}^2$ increases for increasing $R$ values. The difference between $\sigma_{\text{iMSD}}^2$ and the independently measured PSF value (in the present example 300 nm) yields the actual particle size. Fig. 3B shows the recovery of particle size in a rather broad range spanning from a few tens to several hundred nanometers. Note that in a wide-field acquisition, the calculated spatial correlation $G(\xi, \chi, 0)$ contains a “broadening” effect compared with the extrapolated $\sigma_{\text{PSF}}^2$ value due to the extent of particle diffusion during exposure time (Fig. S5). This observation implies that fast-diffusing particles will appear larger in $G(\xi, \chi, 0)$ if the time of frame acquisition is longer than the time during which a particle will diffuse a significant distance on the PSF scale.

Detecting Single TIR Diffusing on Live Cell Membranes. Here, we choose the TIR as a paradigmatic example of transmembrane protein confined by the cytoskeleton meshwork (27) (Fig. 4A). In particular, we used a GFP-tagged variant of the TIR protein transiently transfected into live Chinese hamster ovary (CHO-K1) cells (Fig. 4B, Left). Typically, a membrane region of ~50 $\mu$m$^2$ was selected (red box and Fig. 4B, Right) and imaged repeatedly at ~100 frames per second (fps). STICS analysis shows that molecules disperse in a symmetric fashion due to diffusion only, thus broadening the correlation Gaussian (and the related Gaussian fit) in every direction (Fig. 4C). Our total acquisition time ($\sim$60 s, Fig. 4D) is long enough to accurately remove the immobile fraction contribution, as described in SI Text and Fig. 5A. The resulting...
iMSD vs. delay time plot is presented in Fig. 4E (red curve). Based on these data, it is straightforward to conclude that GFP-TfR experiences transient confinement within the analyzed membrane region. On the other hand, in keeping with the theoretical model, the dynamic partitioning into nanodomains experienced by glycosylphosphatidylinositol (GPI)-anchored GFP, a well-known raft marker, can be qualitatively distinguished from transient confinement by our method, because it requires a two-component model for a satisfactory fitting (details in Fig. S7 and SI Text). By fitting the experimental TfR iMSD curve to Eq. S13, all relevant parameters can be calculated. From the data set we obtain: GFP-TfR diffusion within the mesh domains $D_{\text{micro}} = 0.15 \, \text{μm}^2 \cdot \text{s}^{-1}$ (also Fig. 4E, Inset), long-range “hop” diffusion $D_{\text{macro}} = 0.03 \, \text{μm}^2 \cdot \text{s}^{-1}$, and confinement length $L = 400 \, \text{nm}$. Cumulative results are reported in Table 1, and show excellent agreement with SPT data obtained on a timescale similar to that used here (28). Note that this analysis is correct as far as the region sampled is much larger than the average displacement explored by the molecule in the chosen time window. Accordingly, here we analyze GFP-TfR displacement within a 1-s time window, during which the protein explores an average of less than 1 μm in linear length ($D = 0.2 \, \text{μm}^2 \cdot \text{s}^{-1}$), compared with a region linear size of 6.4 μm. As described by $P(\xi, \chi, \tau)$ (Eq. S3), less than 0.3% of TfR molecules will travel up to a distance of 2 μm from their initial position in 1 s. To verify the effect of cytoskeleton integrity on TfR dynamics, we treated cells with Table 1. Cumulative results on GFP-TfR dynamics in live cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$\sigma_0^2$, $\text{μm}^2$</th>
<th>$D_{\text{micro}}$, $\text{μm}^2 \cdot \text{s}^{-1}$</th>
<th>$D_{\text{macro}}$, $\text{μm}^2 \cdot \text{s}^{-1}$</th>
<th>$L$, nm</th>
<th>$S_{\text{conf}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-TfR, 10 ms</td>
<td>0.098 ± 0.004*</td>
<td>0.20 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>520 ± 30</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>GFP-TfR+Lat-B, 10 ms</td>
<td>0.12 ± 0.03*</td>
<td>0.21 ± 0.04</td>
<td>0.07 ± 0.02</td>
<td>ND†</td>
<td>3 ± 1†</td>
</tr>
<tr>
<td>GFP-TfR, 125 μs</td>
<td>0.055 ± 0.012‡</td>
<td>0.8 ± 0.2</td>
<td>0.13 ± 0.06</td>
<td>130 ± 15</td>
<td>6 ± 4</td>
</tr>
</tbody>
</table>

*Parameters are expressed as mean ± SD ($n = 10$ cells each group).

**PSF in these experiments is 0.075 μm².

†Linear size of confinement cannot be properly calculated if the iMSD plot becomes almost linear, as in the case of Lat-B–treated cells.

‡$P < 0.05$ (0.026) Mann–Whitney test against TfR.

§PSF in these experiments is 0.055 μm².
Latrunculin-B (Lat-B), a well-known actin-perturbing agent (Fig. 4F). In these conditions, the iMSD exhibits a dramatic shift toward a linear trend, thus indicating that the work-induced confinement is almost completely eliminated (green curve in Fig. 4E). Average results are collected in Table 1. As a further characterization, we analyzed the temperature dependence of GFP-TfR dynamics. iMSD curves for the two limit temperatures considered are reported in Fig. 5A. The long-range iMSD slope increases with temperature, confirming that hop-controlled TIR diffusion ($D_{\text{macro}}$) is favored at higher temperatures. This is expected considering that diffusion in the presence of rigid barriers (i.e., the cytoskeleton architecture) is an energy-activated process. Indeed, one can expect that the temperature variation of the measured long-range diffusivity ($D_{\text{macro}}$) is related to the activation energy necessary for membrane-skeleton remodeling. Fig. 5B shows that by linearly fitting $D_{\text{macro}}$ data in an Arrhenius plot, we estimated an activation energy $1.4 \pm 0.5$ eV, a value comparable to that previously determined for thermally activated cytoskeleton rearrangements (29).

**The Impact of Sampling Rate on the iMSD: A Complete Picture of TIR Dynamics.** As demonstrated above, an iMSD intercept value higher than the instrumental waist can be associated with a nonnegligible particle size. The average $\sigma^2_t$ for GFP-TIR in physiological conditions is $0.098 \mu m^2$, a value significantly higher than the waist ($0.075 \mu m^2$) (Table 1): this may indicate the presence of large protein aggregates. Note, however, that an apparent “particle size effect” can also result from slow sampling rates if the protein undergoes transient confinement (13). Indeed, as shown for GFP-TIR in Fig. 6A, imaging a transiently confined protein (iMSD represented by light-red curve) using a frame rate lower than the confinement time results in a linear iMSD (red dots) with an apparently higher intercept (black line). To understand if the experimentally determined $\sigma^2_t$ for GFP-TIR does correspond to real protein aggregates, we explored a faster timescale. In the case of large aggregates, $\sigma^2_t$ will remain unchanged at a faster timescale. On the contrary, in the case of isolated proteins, the iMSD at a shorter timescale will be recalculated at this shorter timescale and $\sigma^2_t$ should match the PSF. Thus, we performed an $\sim 100 \times$ faster acquisition by line scanning with a confocal microscope: Fig. 6B shows a representative iMSD trace obtained in physiological conditions. Notably, we are able to reconstruct the TIR diffusion law at a short timescale (125 μs–10 ms): we obtained a transient confinement with a linear size of 140 nm ($D_{\text{macro}} = 0.7 \mu m^2/s$ and $D_{\text{macro}} = 0.2 \mu m^2/s$; cumulative results are in Table 1), far below the diffraction limit. Remarkably, the obtained confinement parameters are in perfect agreement with SPT measurements conducted at this timescale (28). Also, the newly obtained $\sigma^2_t$ value matches the instrumental PSF (Table 1), thus excluding the presence of large diffusing protein aggregates. Note that by plotting the line-scan data up to longer acquisition times ($\tau = 0.5$ s), the two characteristic time regimes of TIR transient confinement can be concomitantly probed in the same experiment (Fig. S8).

**Discussion**

Single-particle tracking allows the direct monitoring of single/few protein(s) motion and reveals details of membrane molecule dynamics that were not accessible to bulk observation techniques owing to ensemble averaging (30–32). In the case of transmembrane proteins, typical SPT trajectories display random motion within finite-sized domains, with infrequent transitions to neighboring compartments (“hop diffusion”). Estimates for confinement-domain size, protein residency time, and diffusion coefficient can be derived by calculating the MSD of the protein throughout its trajectory. However, this approach does suffer from the peculiar drawbacks of the SPT technique (introductory paragraphs). Alternative data-analysis tools adapted from fluctuation-correlation methods were able to extend the SPT approach to larger molecule densities (33). Along this reasoning, it is widely accepted that FCS methods hold great promise to complement SPT-based approaches (10, 34). To date, however, there are no reports on their successful application to the study of biological membranes. To fill this gap, here we propose an image-correlation analysis method for the study of protein dynamics confined in live-cell membranes. The present method has the same mathematical basis of conventional STICS (24). However, by pushing the temporal resolution down to $10^{-3}$ s, we gain access to the time window characteristic membrane-molecule diffusion. By analyzing the full space–time correlation function, we can monitor the temporal evolution of many single fluorescently labeled membrane proteins at the same time. In this approach we do not extract and track each molecule, but we probe population behavior by studying all molecules in a given region. In the case of random diffusion of the proteins and absence of fluxes/anisotropies (that we verified to be the case for the receptor investigated), the spatiotemporal correlation peak remains centered at the origin whereas it decays in amplitude and spreads out uniformly in space as time increases. By Gaussian fitting of the central peak of the correlation function at each time delay we can reconstruct the average iMSD vs. time plot, which in turn provides the actual diffusion law of the analyzed molecule. Compared with the FCS diffusion law recovered by the spot-size variation approach (13, 14, 17), the iMSD vs. time plot can be easily reconstructed on wide spatial and temporal scales by a single acquisition, yielding a quantitative description of the temporal evolution of the average molecular positions with nanometer accuracy and with no need for interpretative models. Here, we apply such a fluctuation analysis to study the regulation of protein diffusion taking place in the micro- to millisecond time range on micrometer-sized membrane regions in live cells. By using a GFP-tagged variant of the TIR,
we probed the regulation of protein diffusion imparted by the cytoskeleton meshwork. Notably, we obtain values for diffusion coefficient, confinement area, and confinement strength (and their dependence on specific drugs) that agree with those calculated for the same molecule in high-speed SPT measurements (27). The latter complete diffusion law of a fluorescently labeled protein on the plasma membrane is reconstructed over a 4-order-of-magnitude timescale by a fluctuation-correlation technique. It is noteworthy that, unlike SPT, this correlation-based method works well with high molecular densities, low signal-to-background ratios, and high counting-noise levels. This combination of properties opens the way to “single-molecule” experiments with GFP-tagged proteins in the presence of many labeled molecules diffusing at the same time and within the PSF. Furthermore, these same data provide an estimate of the size of the diffusing species simply by extrapolation of the iMSD intercept value (27). This latter information is not achievable in the single-molecule regime, where the spatial correlation of trajectories of neighboring molecules cannot be measured (only isolated molecules are tracked). The present iMSD approach was also used to investigate the temperature dependence of TIR diffusivity. Notably, our observations nicely mirror the temperature-dependent cytoskeleton rearrangements observed by Snyuer et al. (29). In fact, the energy barrier imposed by membrane skeleton on TIR long-range diffusion could be estimated by linear interpolation of the Arrhenius plot of $D_{macro}$. We obtained $1.4 \pm 0.5$ eV, a value consistent with the previously reported activation energy associated with cytoskeleton remodeling dynamics (~1 eV; ref. 29). Furthermore, our observation on the temperature dependence of confinement size is in line with reports by Snyuer et al., who demonstrated by SPT that cytoskeleton filaments explore larger displacements with increasing temperatures. In the same context, Nohe et al. reported an opposite trend (decrease of confinement area with increasing temperatures) for glycosylphatidylinositol (GPI)-anchored proteins (35). This observation is somewhat complementary to what is shown here because, contrary to TIR, GPI-anchored proteins are enriched in the lipid-raft phase of the membrane, which is in turn stabilized at low temperatures (10, 34). Such a quantitative view of the thermodynamic landscape underlying protein diffusion on membranes would not be easily accessible by SPT techniques, where only a limited number of (isolated) molecules can be followed for each selected membrane region. Overall, we believe that the iMSD analysis method represents a powerful tool to analyze large data sets and easily determine a number of average kinetic and thermodynamic parameters on wide spatial and temporal scales, thus fully complementing high-speed single-molecule techniques in the investigation of membrane dynamics at the nanoscale.

Materials and Methods

Further details of materials and methods are included in SI Text. CHO-K1 were grown in DMEM-F12 medium supplemented with 10% (vol/vol) FBS and maintained at 37 °C and 5% CO$_2$ (vol/vol). Cells were transiently transfected with GFP-TfR plasmid (Origenes), GFP-GPI, or actin-GFP (Clontech) using Lipofectamine (Invitrogen) according to the manufacturer’s protocol. For actin filament disassembly cells were treated with 1 μM Lat-B for 15 min in serum-free medium (Sigma–Aldrich).

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References

1. Singer SJ, Nicolson GL (1972) The fluid mosaic model of the structure of cell mem-


