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Glass-Like Membrane Protein Diffusion in a Crowded Membrane

Ignacio L.B. Munguira^{1#}, Ignacio Casuso^{1#}, Hirohide Takahashi¹, Felix Rico¹, Atsushi Miyagi¹, Mohamed Chami² & Simon Scheuring^{1*}

¹U1006 INSERM, Université Aix-Marseille, Parc Scientifique et Technologique de Luminy, 163 avenue de Luminy, 13009 Marseille, France

²Center for Cellular Imaging and NanoAnalytics, Biozentrum, University of Basel, Mattenstrasse 26, CH-4058 Basel, Switzerland

[#]These authors have equally contributed to the work

*Correspondence should be addressed to S. Scheuring

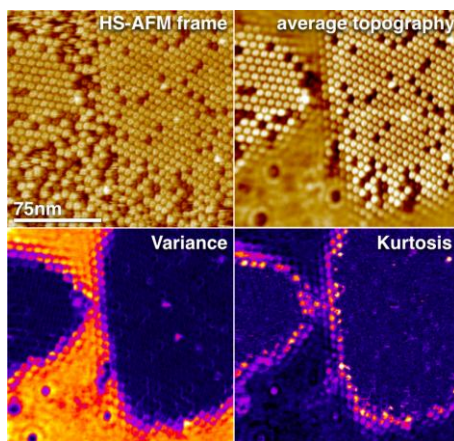
Email: simon.scheuring@inserm.fr

Tel.: ++33-4-91828777, Fax: ++33-4-91828701

Many functions of the plasma membrane depend critically on its structure and dynamics. Observation of anomalous diffusion *in vivo* and *in vitro* using fluorescence microscopy and single particle tracking has advanced our concept of the membrane from a homogeneous fluid bilayer with freely diffusing proteins to a highly organized crowded and clustered mosaic of lipids and proteins. Unfortunately, anomalous diffusion could not be related to local molecular details given the lack of direct and unlabeled molecular observation capabilities. Here, we use high-speed atomic force microscopy and a novel analysis methodology to analyze the pore forming protein lysenin in a highly crowded environment and document coexistence of several diffusion regimes within one membrane. We show the formation of local glassy phases, where proteins are trapped in neighbor-formed cages for time scales up to 10 seconds, which had not been previously experimentally reported for biological membranes. Furthermore, around solid-like patches and immobile molecules a slower glass phase is detected leading to protein trapping and creating a perimeter of decreased membrane diffusion.

Keywords: Anomalous Diffusion / Membrane Dynamics / Membrane Domains / High-Speed Atomic Force Microscopy / Single Molecule / Glass-Glass Transition

Graphical Table of Contents



1 Biological membranes are formed by lipids and proteins (and associated sugars). The
2 composition, relative concentration and density of lipids and proteins define their
3 aggregation and diffusion,¹ which in turn regulate membrane protein distribution and
4 function.^{2, 3} In the recent years, evidence has been accumulated that biological
5 membranes are complex and organized, featuring local aggregations of lipids and
6 proteins (rafts)^{1, 4} and protein species- and localization-dependent diffusion
7 properties,^{5, 6} which regulate the interaction of the membrane components and
8 modulate the biomolecular processes taking place in the membrane.

9 Diffusion can be Brownian, *i.e.* the mean-square displacement (MSD) scales linearly
10 with the time of observation. However, the observed diffusion trajectories of lipids and
11 proteins in biological membranes often deviate from Brownian behavior. Indeed, it is
12 observed that MSD of a membrane component has a nonlinear relationship with
13 observation time: these types of anomalous diffusions are termed superdiffusion and
14 subdiffusion when the object goes farther or stays closer to its initial position,
15 respectively, compared to a Brownian movement. Such intricate and diverse diffusion
16 properties at the cell membrane regulate biomolecular traffic and thus molecular
17 encounters and function. We are just at the beginning of understanding membrane
18 architecture and dynamics.⁷⁻⁹

19 Lysenin^{10, 11} is a 33kDa protein extracted from the coelomic fluid of the earthworm
20 *Eisenia fetida*. It belongs to the family of Pore-Forming Toxins (PFT), and its mechanism
21 of action is common to PFT and is described in six steps:¹² Secretion, binding to the
22 target membrane, two-dimensional diffusion, oligomerisation, assembly in hexagonal
23 close pack, and pre-pore to pore transformation.¹³ Besides Lysenin's affinity for
24 Sphingomyelin (SM), membrane-embedded cholesterol (Chol) has been shown to
25 facilitate the formation of Lysenin oligomers. Lysenin offers hence an excellent system
26 for studying fundamentals of membrane diffusion and aggregation allowing it to
27 interact with mixed SM/Chol bilayers at variable local density.¹⁴

28 The general approach to study membrane protein diffusion is fluorescence microscopy
29 combined with single molecule tracking. Fluorescence microscopy allows studying the
30 diffusion of fluorescence-tagged molecules with high temporal resolution and under
31 physiological conditions.^{6, 15, 16} Unfortunately, fluorescence microscopy comes with
32 some shortcomings: (i) Only tagged molecules can be studied and are visible as long as
33 they are not bleached, and (ii) the lateral and the spatial resolution are limited to
34 optical diffraction $\sim 200\text{nm}$ (distance between two objects that can be resolved in real
35 space at the same time) and to fitting of the point-spread function $\sim 20\text{nm}$ (typical
36 precision of localization). Furthermore, only one single molecule can be tracked –
37 keeping the environment non-fluorescent in the color of the observed tag is
38 prerequisite for the localization of the molecule of interest.

39 High-Speed Atomic Force Microscopy (HS-AFM)¹⁷ offers a novel view of membrane
40 protein architecture and dynamics.¹⁸ It contours proteins with about $\sim 1\text{nm}$ lateral and
41 $\sim 0.1\text{nm}$ vertical resolution in buffer at ambient temperature and pressure, and at
42 subsecond rate. Hence, HS-AFM does not only visualize the movement of unlabeled
43 proteins, but it images all molecules within the membrane. Given this novel and
44 unique feat, HS-AFM allows correlating diffusion to the molecular environment.

1

2 **RESULTS/ DISCUSSION**

3

4 Here, we use HS-AFM to determine the effect of local molecular crowding on the
5 diffusion behavior of lysenin on supported lipid bilayers (SLB) constituted of SM/Chol
6 1:1 (**Figure 1a, Supplementary Movie 1**). As previously reported,¹³ the molecules in
7 pre-pore and pore states can be distinguished based on their membrane protrusion
8 height. Both classes of molecules associate and diffuse next to each other as pre-pores
9 inserts into the first leaflet and pores into both leaflets of the membrane. The
10 resolution and signal-to-noise ratio in the HS-AFM movie allows almost each lysenin
11 ring to be clearly depicted over the entire movie (**Figure 1a, left 4 panels**). In a time
12 average over the entire movie (**Figure 1a, right panel**) areas of different morphology
13 and dynamics are readily distinguished: In certain areas proteins form stable lattices
14 and are well resolved after averaging. In contrast, in other areas, no structural features
15 are recognizable reporting high dynamics in these locations. In agreement, the
16 standard deviation (SD) map of the pixel height values over the entire movie duration
17 (**Figure 1b, left panel**) displayed low values (purple) in the hexagonally packed areas,
18 while the SD was high (yellow) in the diffusive areas. Interestingly, at the interface
19 between these domains, well-defined circular patterns were detected, corresponding
20 to lysenin localizations, where the SD-map displayed low to high SD values (**Figure 1b,**
21 **arrowheads**). This indicated that molecules at interfaces revealed association times of
22 varying duration from long (well-preserved topography and low SD) to short
23 (undefined topography and high SD). Some of the pores, about 0.5% of all molecules
24 got stuck by interactions with the mica support and served us as proof that the vast
25 majority of the molecules had motional freedom (**Figure 1b, dashed squares, Figure 1c,**
26 **Supplementary Movie 2**). Furthermore, these molecules mimic anchored protein-
27 pickets,¹⁹ and confirm experimentally that immobile molecules can strongly influence
28 the diffusion of its annular environment (**Figure 1c**): The immobile molecule creates
29 new borders that resemble structurally and dynamically the borders of the crystalline
30 domains.

31 We analyzed the local dynamics of the proteins, using an approach inspired by
32 Fluorescence Correlation Spectroscopy (FCS).²⁰ Since in an AFM measurement, the
33 pixel intensity is the molecular height, the pixel value is directly related to the presence
34 of a molecule under the tip. Hence, the time that a pixel keeps a certain value reports
35 directly about the residence time of a protein and a value change reports the diffusion
36 'under' that pixel. A section kymograph (**Figure 1d, along the dashed line in Figure 1a**)
37 illustrates the power of the approach: Pixels constituting the crystalline areas display
38 constant height values as a function of time (**Figure 1d, top**), while pixels that are part
39 of the diffusive areas presents rapid changes (**Figure 1d, bottom**). At the border pixels
40 are occupied with stable molecules for intermediate lag-times (seconds to minutes) in
41 alternation with rapid molecular redistribution (**Figure 1d, middle**).

42 In order to analyze the diffusion of molecules 'under' each pixel, we calculated
43 the difference of each pixel value (z_i) at time t with respect to a later moment $t+\tau$
44 ($\Delta z_i(t, \tau) = z_i(t + \tau) - z_i(t)$). As a result, histograms of the height changes $\Delta z_i(\tau)$ of

1 each pixel (i) and varying lag-time (τ) were obtained. Such histograms, also termed
 2 ‘van Hove distributions’, are commonly used in colloidal sciences to extract diffusion
 3 parameters.²¹ From each van Hove distribution on each pixel, the variance $V(\tau)$ is
 4 extracted, analogous to the MSD in single molecule tracking,²² following

$$V(\tau) = \frac{1}{n} \sum_1^n (\Delta z_i(\tau) - m_i(\tau))^2 \quad \text{eq. 1}$$

5 where n is the number of frames of each pixel i , and m_i the pixel mean value. Using this
 6 approach, variance maps for varying τ are generated (**Figure 2a**). As expected, the
 7 variance increases with the τ in all the domains, though much less in the crystalline
 8 areas. Whatever the intensity of dynamics, as long as the behavior is Brownian then
 9 the van Hove distribution is Gaussian. In contrast, non-Brownian dynamics, as found for
 10 example in glasses, give rise to non-Gaussian van Hove distributions. This is because
 11 particles in glasses are constraint and transiently caged by the dense packing and
 12 interactions with neighboring particles, but undergo rare large displacements due to
 13 cage rearrangements. To characterize such complex dynamics the Kurtosis K of the
 14 distribution on each pixel is calculated, following

$$K(\tau) = \frac{\frac{1}{n} \sum_1^n (z_i(t + \tau) - z_i(t)) - m_i)^4}{\left(\frac{1}{n} \sum_1^n (z_i(t + \tau) - z_i(t)) - m_i\right)^2} \quad \text{eq. 2}$$

15 When $K=3$, then the distribution in the van Hove plot is Gaussian and the underlying
 16 motion is Brownian. Deviations from $K=3$ are associated with anomalous diffusion, and
 17 typically $K>3$ are signature of the cooperative behavior in glasses.²³

18 Plotting K on each pixel as a function of varying τ , areas of non-Brownian dynamics are
 19 highlighted (**Figure 2b**). Within the fluid areas, we find subregions, upper left corner,
 20 which display a Kurtosis ~ 3 at all τ , corresponding to free diffusion. However, most of
 21 the fluid domains revealed Kurtosis significantly >3 at short lag-times, and ~ 3 when
 22 analyzed over longer $\tau > 8s$, corresponding to the average trapping time of particles in
 23 this glass phase. Beyond this τ the glass behaves like a Brownian fluid. The
 24 interfaces between fluid and crystalline domains displayed high Kurtosis values >4 even
 25 over extended lag-times indicating that molecules are trapped for varying durations
 26 and eventually up to minutes. The crystalline domains despite their low dynamics
 27 displayed $K \sim 3$, and correspond therefore statistically to an area of Brownian diffusion.

28 To better understand the relationship between the structure and dynamics of these
 29 domains,²⁴ we undertook two types of analysis: First, for a more detailed
 30 comprehension of the crystalline domains we performed negative stain electron
 31 microscopy (EM) (**Supplementary Figure S1a**) combined with single particle analysis
 32 (**Supplementary Figure S1b**), and cryo-EM (**Supplementary Figure S1c**) combined with
 33 electron crystallography (**Supplementary Figure S1d**) of lysenin as individual molecules
 34 and in the crystalline arrangement, respectively. Both approaches depicted lysenin as a
 35 nonameric pore with two concentric density rings. The 9-fold symmetric molecule
 36 assembles with $p3$ -symmetry in the ‘hexagonal packing’ with $a=b=12nm$ and $\gamma=120^\circ$, in
 37 agreement with HS-AFM (**Figure 1a**). In such an arrangement one lysenin ring occupies a
 38 membrane area $A = 2 \cdot (\sqrt{3}/4 \cdot a \cdot b)$ of $125nm^2$ corresponding to an area fraction (ϕ)

1 of 0.91. Second, for a more detailed comprehension of the fluid domains, we
 2 performed Delaunay triangulation and Voronoi tessellation
 3 (**Supplementary Figure S2a**).²⁵ In the case of a 2D-lattice, each molecule has 6 nearest
 4 neighbors and the Voronoi cells are hexagons with 125nm^2 area. In the fluid domain,
 5 the average distance between molecules is $16.5\pm 3.5\text{nm}$, with about 5.5 nearest
 6 neighbors, and pores occupy areas up to 250nm^2 , an approximate area fraction of 0.45
 7 without short- or long-range order (**Supplementary Figure S2b**). Furthermore, the
 8 shape factor ξ of the Voronoi cells have been analyzed: Each cell is characterized by a
 9 shape factor $\xi = (C^2/4\pi A)$, where C is the perimeter and A the area of the Voronoi
 10 cell. In the range $\phi\approx 0.60-0.71$ a bimodal distribution of the shape factor is observed,
 11 meaning that molecules in the same density range have different number of
 12 neighbors, characteristic of phase transitions (**Supplementary Figure S3**).²⁶

13 Having in hand methodologies to characterize diffusion properties (**Figure 2**) and local
 14 protein density (**Supplementary Figure S2a**) ‘under’ each pixel, we had competence to
 15 determine how diffusion properties scale and change as function of local membrane
 16 structure. Plotting the variance of all pixels in the movie as a function of the Voronoi
 17 cell area that comprises each pixel and as a function of varying τ , a segregation of the
 18 data occurred (**Figure 3a**). A large number of data points gather at a Voronoi cell size of
 19 $\sim 125\text{nm}^2$ and small variance that remains unchanged with increasing lag-time; these
 20 pixels are part of the crystalline area. In contrast, pixels in areas of low protein density
 21 and Voronoi cell size $>200\text{nm}^2$ show high variance that further increases with τ . The
 22 Kurtosis of the crystalline area, Voronoi cell sizes of $\sim 125\text{nm}^2$, is stable at a value of ~ 3 ,
 23 Brownian rattling (**Figure 3b**). However, pixels that locate in the fluid areas with low
 24 protein density and Voronoi cell area $>200\text{nm}^2$ depict wide spread Kurtosis values with
 25 many significantly >3 at short τ .

26 In analogy to typical MSD vs lag-time plots from single particle tracking, we plotted
 27 variance vs lag-time. Our approach has the advantage that proteins can be grouped
 28 together as a function of the local density in which they evolve. In general, the variance
 29 increases with lower local protein density (larger Voronoi cell). The slope of the variance
 30 as a function of τ is somewhat steeper at $240\text{nm}^2/\text{molecule}$ compared to areas at
 31 $125\text{nm}^2/\text{molecule}$ at short τ , flattening at longer τ . However, at intermediate protein
 32 density of $\sim 185\text{nm}^2/\text{molecule}$ the slope is increased, especially for longer τ , indicating
 33 particular diffusion of molecules at this density (**Figure 3c**, left). When plotting the
 34 variance vs Voronoi cell area for different τ , we observe a biphasic, maybe triphasic,
 35 behavior: Low variance for the crystalline areas with 125nm^2 to 165nm^2 per molecule,
 36 high variance for fluid areas with 200nm^2 to 250nm^2 per molecule, and a steep
 37 variance increase for intermediate molecular density regions with 165nm^2 to 200nm^2
 38 per molecule. Around Voronoi areas of 185nm^2 a weak variance plateau at short τ , is
 39 found (**Figure 3c**, right).

40 Plotting the Kurtosis as a function of lag-time for the different Voronoi cells
 41 revealed three different populations of Brownian and non-Brownian dynamics
 42 (**Figure 3d**, left): Densely packed molecules have Kurtosis ~ 3 , almost independent of τ .
 43 When the local protein density is lower than 165nm^2 per molecule, then the Kurtosis
 44 raises abruptly to a completely non-Brownian regime. A third population is found when
 45 the protein density loosens further with Voronoi cells of 200nm^2 and larger: In this

1 regime, the Kurtosis is significantly non-Brownian at $\tau < 10$ s, above this lag-time
 2 diffusion is Brownian. 10s seems to be the average trapping time of lysenin in the
 3 glassy-fluid phase. This striking triphasic behavior and its transitions are best visualized
 4 when plotting the Kurtosis as a function of Voronoi cell area (**Figure 3d**, right):
 5 Molecules evolving in a density regime of 165nm^2 to 200nm^2 membrane area (peaking
 6 at 185nm^2) display non-Brownian diffusion characteristics basically independent of the
 7 time span. These characteristics around $\phi \sim 0.61$ are in good agreement with theory and
 8 simulation of 2D-glasses.^{27, 28}

9 HS-AFM features the advantage to visualize (i) non-labeled molecules and (ii) not only
 10 single molecules but all molecules in the membrane. An approach inspired by FCS is
 11 used to detect dynamics ‘under’ each pixel with nanometer resolution, of particular
 12 importance in crowded systems. Qualitatively similar results were obtained
 13 from automated single particle tracking (**Supplementary Figure S4**). However
 14 considerable problems occur when tracking densely packed molecules, where the
 15 motion of the molecules is comparable to the inter-particle distance. Furthermore, a
 16 single local density cannot be attributed to a molecule trajectory as the molecule may
 17 diffuse through heterogeneous domains. In contrast, the pixel-by-pixel
 18 analysis approach used here (**Figure 3**) provides the possibility to correlate density and
 19 diffusion.

21 CONCLUSIONS

23 In summary, the membrane contains four phases with different diffusion dynamics: At
 24 ϕ ranging from 0.91 to 0.68 (solid phase), the proteins are essentially crystalline and
 25 rattle around their position with Brownian dynamics. At borders of the solid phase, the
 26 proteins evolve at ϕ between 0.68 and 0.56 (sliding glass) and are caged up to minutes.
 27 Morphologically, this glassy area resembles a ‘sliding puzzle’ where moving complexes
 28 occupy defined positions. At ϕ 0.56 to 0.45 (fluid glass) diffusion is fluid yet molecules
 29 are caged at shorter time periods < 10 seconds. Calculating SD maps running over 10s of
 30 movie acquisition revealed the presence of spatially correlated dynamics represented
 31 by SD fluctuation waves (**Supplementary Movie 4**). This glassy phase is characterized
 32 by lack of short-range order. At ϕ below 0.45 (liquid phase) proteins diffuse freely. Such
 33 a coexistence of several glasses has been described in colloidal systems.^{29, 30}

34 In a crowded mosaic biological membrane, molecules diffuse in an environment with
 35 $\phi \sim 0.5$ containing stable domains.^{1, 4} Furthermore, molecular heterogeneity favors the
 36 occurrence of glass dynamics.²⁴ Specialized membranes, e.g. photosynthetic
 37 membranes³¹ or retinal disk membranes^{32, 33} may be even more crowded and comply to
 38 cooperative rearrangements during their functional tasks.

39 HS-AFM allows correlating structure with diffusion behavior, and glassy diffusion is only
 40 detectable when both movement and environment are simultaneously assessed.
 41 Therefore, biologists may have missed glass-like diffusion in crowded membranes³⁴
 42 due to the technical limitation of only tracking single molecules. Given the
 43 crowdedness of cellular membranes, we hypothesize that glassy dynamics might be a
 44 frequent feature of membrane protein regulation *in vivo*.

1

2 **METHODS/EXPERIMENTAL**

3

4 ***Protein purification***

5 A cDNA fragment, coding for Lysenin (GenBank: BAA21518.1, GenScript, USA) and
6 cloned into pET28a vector at BamHI and Hind III, and this vector transformed into
7 BL21(DE3) strain (New England BioLabs France, Evry, France). The transformed cells
8 were inoculated into 1 liter of LB medium containing 100 µg/ml kanamycin sulfate, and
9 incubated at 37 °C while shaking at 200 rpm until the OD₆₀₀ value reached 0.6. For
10 induction of Lysenin expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was
11 added at final concentration of 0.5 mM, and cells shaken overnight at 20°C and
12 200 rpm. The bacteria were collected by centrifugation at 2000g for 10 minutes, and
13 disrupted with 3 probe sonicator intervals of 15 seconds sonication and 30 seconds ice
14 cooling each. The resulting suspension was shaken at 4°C for 30 minutes in Triton X-
15 100 at 0.1 % and RNase/DNase at 10 µg/ml concentrations. The mixture was
16 centrifuged at 10⁴g for 30 minutes. The supernatant (volume: 9 ml) was collected and
17 mixed with 1 ml of metal chelating resin, chelating sepharose Fast flow (GE Healthcare
18 France), composed of chelating cobalt in 100 mM NaCl, Hepes-NaOH, pH 7.5. Lysenin
19 binding was performed by 1 hour incubation at 4°C with gentle shaking, and the
20 resulting resin centrifuged at 100g for 1 minute to discard the supernatant. The resin
21 was washed with fresh 14 ml of 100 mM NaCl, 100 mM imidazole-HCl, Hepes-NaOH,
22 pH 7.5 by 3 centrifuge/washing cycles. Lysenin was eluted in 1 ml of 100 mM NaCl,
23 250 mM imidazole-HCl, Hepes-NaOH, pH 7.5. To eliminate the imidazole, the eluate
24 was dialyzed against 1 liter of 100 mM NaCl, Hepes-NaOH, pH 7.5. The resulting protein
25 sample was directly used for the HS-AFM experiment.

26

27 ***Sample preparation for High-speed atomic force microscopy (HS-AFM) observation***

28 Egg Sphingomyelin (SM) and Cholesterol (chol) (Avanti Polar Lipids, Alabama, USA) were
29 used to form giant unilamellar vesicles (GUVs) at a molar ratio SM:Chol 1:1 through
30 electroswelling.³⁵ Of each lipid 10 µl at 3 mM dissolved in chloroform:methanol 3:1
31 were deposited in two glass plates coated with indium tin oxide with 70-100 Ω
32 resistivity (Sigma-Aldrich) and placed 60 minutes in the desiccator for complete solvent
33 evaporation. A U-shaped rubber piece of ~1 mm thickness was sandwiched between
34 the two indium tin oxide coated slides. The so-formed chamber was filled with
35 ~400 µl of 200 mM sucrose solution and exposed to 1.5 V sinusoidal 10 Hz AC current
36 for 3 hours followed by squared 5 Hz AC current for 15 minutes, at 55°C. GUVs were
37 harvested from the chamber. To form the supported lipid bilayers (SLBs) for HS-AFM,
38 1 µl of GUV solution was placed on a 1.5 mm-diameter freshly cleaved mica disk
39 covered with 1 µl of phosphate buffer saline (PBS) and incubated for 30 minutes. To
40 remove lipid that was not firmly attached the SLB was intensely rinsed with PBS. Once
41 the bilayer was formed, 1 µl of purified lysenin was incubated for 15 minutes. Excess of
42 protein was again rinsed with PBS.

43

44 ***High-speed atomic force microscopy (HS-AFM)***

1 HS-AFM movies were acquired with an Ando-type setup¹⁷ equipped with a super
 2 luminescent diode (emission wavelength: 750 nm; EXS 7505-B001, Exalos, Schlieren,
 3 Switzerland) and a digital high-speed lock-in Amplifier (Hinstra, Transcommerz,
 4 Budapest, Hungary).³⁶ 8 μ m-long cantilevers with spring constant $k = 0.15 \text{ Nm}^{-1}$,
 5 resonance frequency $f_{(r)} = 500\text{-}700 \text{ kHz}$ and quality factor $Q \approx 1.5$ in liquid (USC-1.2,
 6 NanoWorld, Neuchâtel, Switzerland), featuring an electron beam deposition (EBD) tip,
 7 were used. For high-resolution imaging the tip was sharpened by helium plasma
 8 etching using a plasma cleaner (Diener electronic, Ebhausen, Germany), resulting in a
 9 final tip radius tip of about 2 nm, as judged from analysis of the indentation inside the
 10 Lysenin rings. Amplitude modulation was used for imaging with free amplitude of
 11 $\sim 1.2 \text{ nm}$ and operating set point amplitude of $\sim 0.9 \text{ nm}$. Under these conditions we
 12 estimate the applied force following $F = (k_c/Q_c) * (A_0(1 - A_s/A_0) + h_0 \sin(\theta/2))$ where A_0 is
 13 the free amplitude, A_s is the setpoint amplitude, h_0 is the step height of the sample,
 14 and θ is the phase delay of the feedback. Under our imaging conditions $F = 44 \text{ pN}$.³⁷ All
 15 experiments were performed at room temperature and in physiological buffer.

16

17 ***High-speed atomic force microscopy (HS-AFM) image treatment***

18 Image treatment was limited to the correction of a first-order XY plane fit and XY drift
 19 correction of the HS-AFM movie.³⁸

20

21 ***High-speed atomic force microscopy (HS-AFM) data analysis***

22 The HS-AFM movie is considered a four dimensional matrix with lateral dimensions X
 23 and Y, height dimension Z, and a time t . Time is subdivided in time-intervals τ , the
 24 shortest τ is the time passing between the acquisitions of two subsequent frames.
 25 From this matrix, the height changes Δz as a function of varying lag-time τ , was
 26 calculated by subtraction, following $\Delta z_i(t, \tau) = z_i(t + \tau) - z_i(t)$ on each pixel. So-
 27 called van Hove plots, *i.e.*, the histogram distribution of the height variations was
 28 calculated for each pixel. Following, the shape of the van Hove plots for each τ was
 29 analysed according two parameters: Variance V and Kurtosis K . While the variance
 30 informs about the width of the van Hove plots, hence about the intensity of motion,
 31 the Kurtosis reports about the non-Gaussianity, hence about non-Brownian behaviour.
 32 Each of these steps is performed for every lag-time τ and for every pixel. This data
 33 treatment resulted in variance and Kurtosis maps as shown in figure 2. Variance and
 34 Kurtosis were calculated using pre-built functions in Matlab (Matlab, Mathworks,
 35 Natick, USA).

36 To determine the local density, Voronoi tessellation was calculated from the
 37 localization of all particles. In order to determine the localization of all molecules in all
 38 frames, cross-correlation searches between a 360-fold symmetrized Lysenin ring
 39 (artificial reference) and each movie frame was performed. This resulted in cross-
 40 correlation maps of each frame that featured about 700 cross-correlation peaks each.
 41 Peak searches allowed the localization of about 740000 molecules in the movie (about
 42 700 in each frame). A lab-developed package^{18, 38} integrated in ImageJ was used for the
 43 cross-correlation analysis and trajectory extraction. Using the particle localizations,
 44 Voronoi tessellation was calculated using a pre-built function in Matlab (Matlab,
 45 Mathworks, Natick, USA).

1 Combining, the two above-described analysis allowed correlating variance and Kurtosis
 2 with local protein density and evaluating how diffusion properties scale as a function
 3 of membrane structure.

4 The area fraction ϕ was calculated taking in to account that the area occupied by a
 5 Lysenin ring is $A_{(mol)} = \pi d^2/4 = 113nm^2$ (where d is the diameter of the lysenin ring,
 6 *i.e.* center-to-center distance in the crystal packing), and the unit cell area of the
 7 hexagonal close packing is $A_{(unit\ cell)} = 2 \left(\frac{\sqrt{3}}{4} ab \right) = 124.7nm^2$, resulting in

8 $\phi=0.906899$. For our analysis ϕ is the ratio between A (mol) and the unit cell or Voronoi
 9 cell in the real HS-AFM movie, in which it is located.

10

11 ***Sample preparation for transmission electron microscopy (TEM) observation***

12 Adsorption and oligomerization of Lysenin on asphingomyelin-containing lipid
 13 monolayer were performed in custom-designed Teflon wells of 4mm in diameter and
 14 1mm in depth. 0.5 μ l of lipid solution (Sphingomyelin/Phosphatidylcholin 1:4, Avanti
 15 Polar Lipids, Alabama, USA) at 0.1mg/ml in chloroform were spread on 15 μ l of Lysenin
 16 at 50 μ g/ml and incubated for 1 hour at room temperature to reconstitute oligomers or
 17 overnight to form 2D-crystal patches. The interfacial surface formed by the lipid
 18 monolayer and the adsorbed protein was transferred to carbon-coated grids and
 19 analyzed by transmission electron microscopy.

20

21 ***Negative stain transmission electron microscopy (TEM) and image processing of*** 22 ***single Lysenin oligomers***

23 For imaging of negatively stained samples, the grid was washed with three droplets of
 24 pure water and subsequently negatively stained with 2% (w/v) uranyl-acetate. The
 25 prepared grids were imaged using a Philips CM10 TEM (FEI Company, Eindhoven, the
 26 Netherlands) operated at 80kV. The images were recorded by the 2k x 2k side-
 27 mounted Veleta CCD camera (Olympus, Germany) at magnification of 130 000 x. Under
 28 these conditions the pixel size at the sample level is 3.7 Å.

29 Image processing was achieved with EMAN2 software package.³⁹ The images were CTF
 30 (contrast transfer function) corrected and the particles were semi-automatically
 31 selected. The 'e2refine2d' program was used to classify the particles, and produce
 32 reference-free class averages. The most populated class represented the top view.

33

34 ***Cryo Transmission electron microscopy (cryo-TEM) and image processing of 2-crystal*** 35 ***patches of Lysenin***

36 For cryo-TEM, the grid was blotted with Whatman filter paper and vitrified through
 37 plunging it into liquid nitrogen cooled liquid ethane using a vitrobot (FEI company,
 38 Netherlands). Frozen grids were transferred into a Philips CM200-FEG electron
 39 microscope using a Gatan 626 cryo-holder. Electron micrographs were recorded at an
 40 accelerating voltage of 200kV and a nominal magnification of 50 000 x, using a low-
 41 dose system (10 e⁻/Å²) and keeping the sample at liquid nitrogen temperatures.

1 Defocus values were around $-2.5\ \mu\text{m}$. Micrographs were recorded on a 4K x 4K CMOS
2 camera (TVIPS, Germany). The pixel size at the sample level is $2.1\ \text{\AA}$. The 2D-crystal
3 images were selected based on the presence of diffraction patterns with well-defined
4 spots and further treated using the 2dx software.⁴⁰

5

6 **ASSOCIATED CONTENTS**

7

8 **Supporting Information**

9 The Supporting Information is available free of charge on the ACS Publications
10 website at DOI:

11

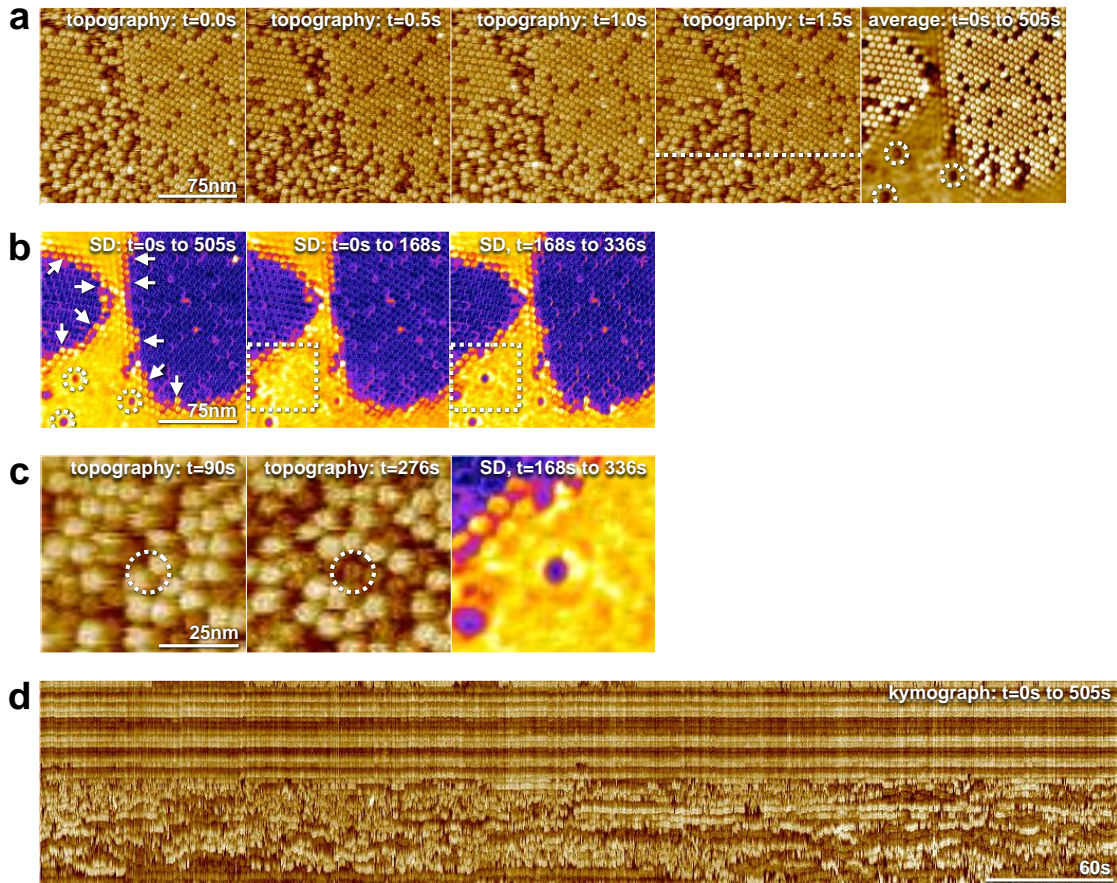
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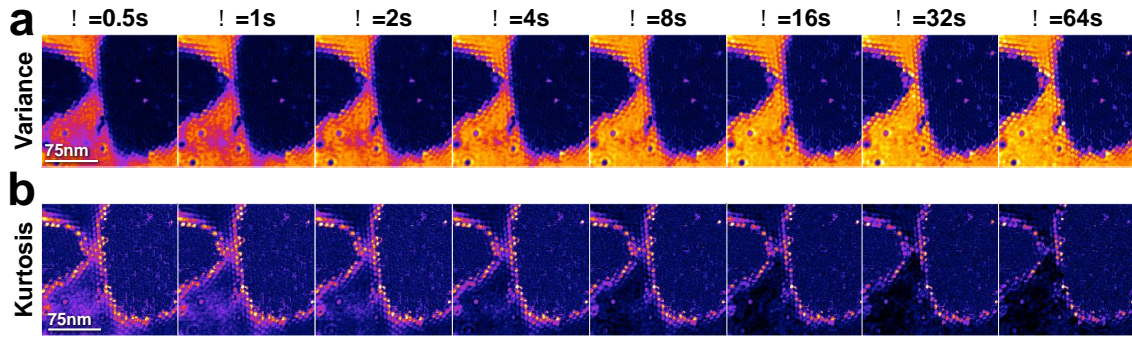
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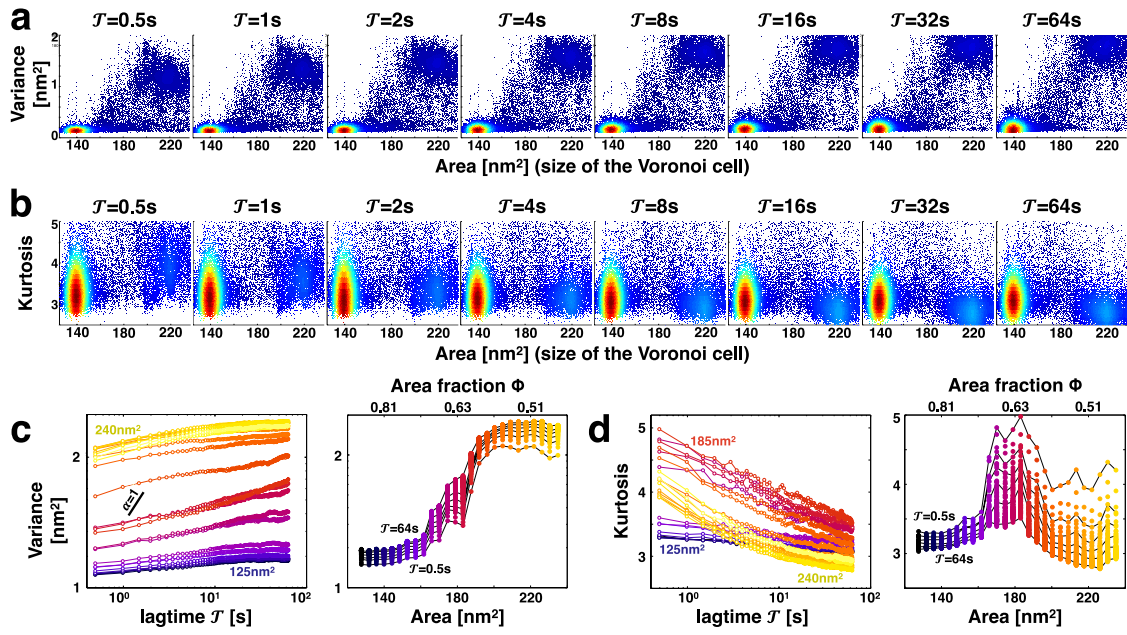


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 2 **Figure 1) Lysenin dynamics is location dependent.** **a)** Four left panels: HS-AFM movie frames
 3 (**Supplementary Movie 1**) of Lysenin in a sphingomyelin/cholesterol (1:1) bilayer. Right panel: Time-
 4 averaged frame displaying the positional stability and the high mobility of proteins in the solid and fluid
 5 domains (full false color scale: 10nm). **b)** Left: standard deviation (SD) map of the pixel height values
 6 (t=0-505s). Lattice borders with well-defined molecular positions of varying SD (arrows). SD maps (t=0-
 7 168s, center) and (t=168-336s, right): At t=168s a location (dashed squares) drastically changed
 8 dynamics (full false color scale: $0.3 < SD < 1.6$ nm). **c)** Individual frames t=90s and t=276s of the molecular
 9 organization corresponding to the outlines in **b)**, see also supplementary movie 2. Right: SD map (t=168-
 10 336s) of this membrane region displaying the annular alteration of diffusion dynamics around the stuck
 11 molecule. **d)** Kymograph (of the white dashed line in **a)**. Stable (top), highly mobile (bottom), and
 12 molecules switching between stability and high mobility (middle) are visible during the entire movie.
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Figure 2) Detection of areas of non-Brownian dynamics. a) Variance(V) of the distribution of height changes (false color scale: $0 < V < 2 \text{ nm}^2$). **b)** Kurtosis(K) (non-Gaussianity) of the distribution of the height changes (false color scale: $2.5 < K < 5.0$).



1
 2 **Figure 3) Diffusion as function of local density.** a) Distribution of Variance and b) Kurtosis for all pixels as
 3 a function of Voronoi cell area and lag time (color indicates the concentration of data points in the plot
 4 area from blue (low abundance) to red (high abundance)). c) Variance and d) Kurtosis as function of lag
 5 time and Voronoi cell area: Each point is the median of all pixels with the same characteristics.