### **Glasslike Membrane Protein Diffusion in a Crowded Membrane**

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# **Supplementary Information**



Supplementary Figure S1) Electron microscopy (EM) analysis of lysenin. a) Negative-stain EM of individual lysenin combined with b) single particle analysis (gallery of 32 representative molecules). c) Cryo-EM of a lysenin2D-crystal andd) crystallographic analysis. Lysenin is a nonamer with pore diameter of ~35Å, inner ring density diameter of ~45Å, an outer density ring diameter of ~75Å, and full diameter of ~110Å.



**Supplementary Figure S2) Voronoi tessellation of lysenin distribution for the assessment of local protein density.a)** Delaunay triangulation (green lines) and Voronoi tessellation (blue lines) of the schematic lysenins (black disks) with determined center positions (black crosses). **b)** Left: HS-AFM movie frame (**Supplementary Movie 1**). Right: Voronoi tessellation of the HS-AFM same HS-AFM frame.



Supplementary Figure S3) Distribution of shape factors of the Voronoi cells as function of area fraction. This analysis detects structural heterogeneity at Voronoi cell size where dynamical heterogeneity was detected (see figure 3 in the main manuscript). The shape factor  $\xi$  is calculated as  $\xi = (C^2/4\pi A)$ , where C and Aare the Voronoi cell perimeter and area, respectively. As a guide: $\xi$ =1.0circle,  $\xi$ =1.10 hexagon,  $\xi$ =1.16 pentagon,  $\xi$ =1.27 square.The color scheme corresponds to the color schemes in the Variance and Kurtosis plots shown in Figure 3c) and d) in the main manuscript.



Supplementary Figure S4) Diffusion as a function of local density from automated particle tracking. a) Sequential HS-AFM movie frames (Supplementary Movie 3).
b) Variance and c) Kurtosis as function of lag-time and Voronoi cell area. Each point is the median of all molecules with the same characteristics. Molecules have been grouped according to the average Voronoi cell size at the location where the molecule was in the first frame.

#### Supplementary Movie 1:

#### HS-AFM observation of lysenindiffusion dynamics.

lysenin rings diffusive in fluid domains and form hexagonal packings in stable domains. HS-AFM movie of lyseninin a SM/Chol 1:1 SLB.

Movie parameters: full movie length 504832 ms; frame rate 493 ms; full image size 3650 Å; frame dimensions 195x188 pixels.

#### Supplementary Movie 2:

#### HS-AFM observation of lysenindiffusion dynamics.

Close-up view of lysenin diffusion in the fluid domain. A pore state lysenin gets stuck at t=168113 ms in the center of the frame, compare with figure 1c.

HS-AFM movie of lyseninin a SM/Chol 1:1 SLB.

Movie parameters: full movie length 504832 ms; frame rate 493 ms; full image size 900 Å; frame dimensions 50x50 pixels.

#### **Supplementary Movie 3:**

#### HS-AFM observation of lysenindiffusion dynamics and automated particle

**tracking.**Left: HS-AFM movie. Right: HS-AFM movie with overlay of automated particle tracking, compare with supplementary figure S3.

HS-AFM movie of lyseninin a SM/Chol 1:1 SLB.

Movie parameters: full movie length 504832 ms; frame rate 493 ms; full image size 3650 Å; frame dimensions 195x188 pixels.

#### Supplementary Movie 4:

#### Running SD maps( $\tau$ =10s).

Spatial cooperative motion and dynamic heterogeneity of molecules in the glassy fluid phase as reported by wave-like intensity variations of the SD over  $\tau$ =10s (20 subsequent movie frames).

Movie parameters: full movie length 497930 ms; frame rate 493 ms; full image size 3650 Å; frame dimensions 195x188 pixels.