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Doctoral thesis

Field model for lateral diffusion
of various transmembrane proteins
in living *Dictyostelium* cells

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February 2023

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Abbreviations

AIC: Akaike information criterion

CI: Confidence interval

DB: Development buffer

FITC: Fluorescein isothiocyanate

HMM: Hidden Markov model

MLE: Maximum likelihood estimation

MSD: Mean squared displacement

PDF: Probability density function

SD model: Saffman-Delbrück model

SE model: Stokes-Einstein-like model

TIRFM: Total internal reflection fluorescence microscope

TMR: Tetramethylrhodamine

Abstract

The lateral diffusion of transmembrane proteins on plasma membranes is a fundamental process for various cellular functions. Diffusion properties specific for individual protein species have been extensively studied, but the common features among protein species are poorly understood. Here I systematically studied the lateral diffusion of various transmembrane proteins in lower eukaryote *Dictyostelium discoideum* cells by using a Hidden Markov model for single-molecule trajectories. As common features, all membrane proteins varying from one to ten transmembrane regions adopted three free diffusion states with similar diffusion coefficients regardless of their structural variability. All protein species reduced their mobility similarly upon the inhibition of microtubules, actin cytoskeleton or myosin II. The relationship between protein size and diffusion coefficient was consistent with the Saffman-Delbrück model, meaning that membrane viscosity is a major determinant of lateral diffusion but protein size is not. These protein species-independent properties of multistate free diffusion were explained simply and quantitatively by free diffusion on three membrane regions with different viscosities, which is in sharp contrast to the complex diffusion behavior of transmembrane proteins in higher eukaryotes.

Chapter 1

Introduction

1.1 Membrane proteins diffuse across the cell membrane with complex structure.

Transmembrane proteins, together with lipids, are one of the major components of the cell membrane, which separates the inside and outside of the cell. There are various types of transmembrane proteins, including receptors, which bind to specific molecules and are responsible for signal transduction into and out of the cell, and channels, transporters, and pumps, which transport various molecules into and out of the cell. These functions are realized by the lateral diffusion of transmembrane proteins across the cell membrane. In recent years, many previous studies have reported that transmembrane proteins regulate their functions by interacting with specific molecules or localizing to specific regions, thereby altering their diffusion behavior (Hiroshima et al., 2018; Yanagawa et al., 2018; Kawakami et al., 2022). Therefore, it is essential to understand the diffusion dynamics of transmembrane proteins in order to advance our understanding of transmembrane proteins.

Diffusion kinetics are determined by the properties of the cell membrane surrounding the molecule and the properties of the molecule itself. The properties of the molecule itself include properties common to transmembrane proteins in general and type-specific properties, each of which affects diffusion kinetics. First, I will address the structures of the cell membrane. Various models have been proposed to understand the dynamics of membrane proteins on the cell membrane. The fluid mosaic model (Singer and Nicolson, 1972; Nicolson, 2014) was the first to propose a modern understanding of the cell membrane. In this model, the cell membrane is composed of a bilayer of phospholipid molecules, and protein molecules exist in a mosaic-like random mixture on the surface, inside, or penetrating up and down the bilayer. For membrane proteins, the lipid bilayer was thought to behave like a two-dimensional fluid that determined lateral diffusion. In a cell membrane such as this model, molecules diffuse very simply, as described below. However, since the proposal of this model, various membrane structures have been discovered and proposed that limit or inhibit lateral diffusion and make it more complex. Lipid rafts are membrane regions < 300 nm in size that are composed of specific lipids such as cholesterol and sphingolipids and proteins involved in signal transduction (Simons and Ikonen, 1997). It constitutes a less fluid region compared to other regions,

and molecules are thought to diffuse slower in this region (Fig. 1.1A). In addition, according to the picket-and-fence model, the actin skeleton just below the cell membrane constitutes a "fence"-like mesh structure that inhibits the diffusion of membrane protein molecules. In addition, various transmembrane protein molecules are present in the actin backbone as anchored "pickets" and similarly inhibit diffusion (Kusumi, 2003) (Fig. 1.1B). The plasma membrane contains a variety of factors that inhibit diffusion, and indeed, the diffusion of proteins and lipids in the plasma membrane is 5-50 times smaller than in artificially reconstituted membranes and liposomes (Kusumi, 2003).

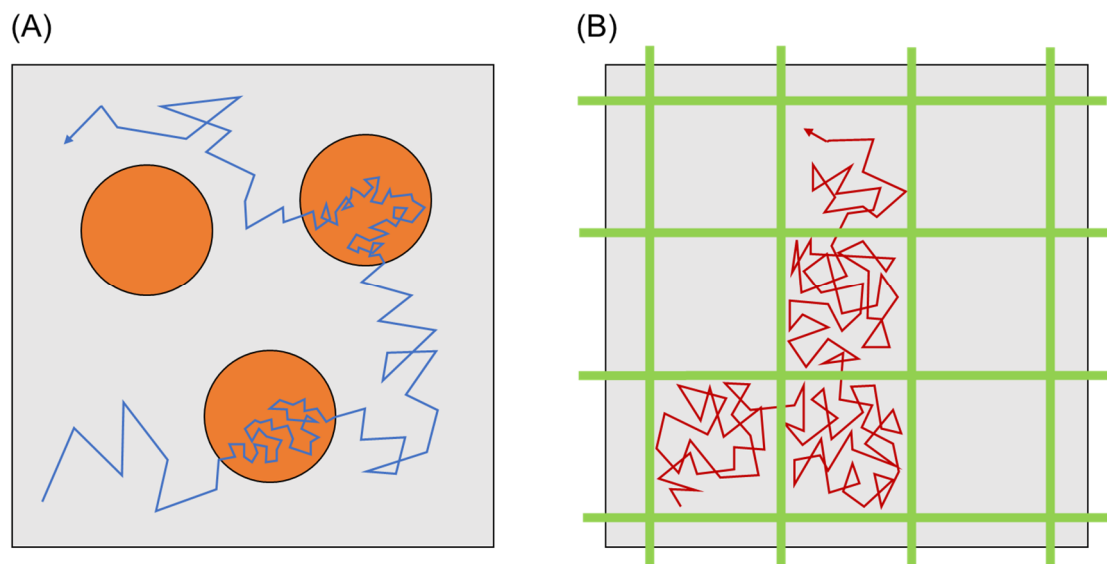


Fig. 1.1. Structure of the cell membrane that causes changes in molecular diffusion.

(A) Trajectory of a molecule diffusing on cell membrane with lipid rafts. The blue line represents the trajectory of molecular motion. The orange circular regions indicate lipid rafts. Molecules diffuse slowly in the lipid raft region. (B) Trajectory of a molecule diffusing on the membrane with a meshwork of actin cytoskeleton just below the cell membrane. The red line represents the trajectory of molecular motion. The green mesh structure represents the actin cytoskeleton just below the cell membrane. Molecules are restricted from diffusing by the fence of the actin cytoskeleton. This figure describes the cell membrane from the inside of the cell.

1.2 Membrane proteins diffuse across the cell membrane in various conformations.

Diffusion of transmembrane proteins is also thought to be affected by their own properties. For example, the structure (size) of the transmembrane protein molecule itself. The number of transmembrane regions varies with the type of transmembrane protein, and some transmembrane proteins form multimeric complexes, which vary in size on the membrane. There are previous studies that attempted to describe how much the size of the molecule itself affects the diffusion motion from the viewpoint of fluid dynamics. Basically, the question is to what extent the size (radius) of the molecule embedded in the membrane contributes to the diffusion coefficient of the molecule. The following models approximate the membrane-embedded part of the transmembrane protein as a cylinder of radius R and height h , and assume that it diffuses across the cell membrane with viscosity μ_m . The first model is the Saffman-Delbrück model (SD model) (Saffman and Delbrück, 1975). This model hypothesizes that the diffusion coefficient of molecules depends roughly inversely on the viscosity and thickness of the cell membrane, with little dependence on the size (radius) of the molecules embedded in the membrane. Another well-known model is the Stokes-Einstein-like model (Gambin et al., 2006) (SE model), which was proposed based on experimental verification of the SD model with artificial membranes. This model differs from the SD model in that it assumes that the diffusion coefficient of molecules depends inversely on the size (radius) of molecules embedded in the membrane in addition to the viscosity and thickness of the cell membrane (Fig. 1.2). In both models, the size of the extracellular or intracytoplasmic region of the molecule has little effect on diffusion because the viscosity of the extracellular or cytoplasmic region is negligibly small compared to the viscosity of the plasma membrane. Experiments have since been conducted on artificial membranes, and many results have been obtained that are consistent with the SD model (Ramadurai et al., 2009; Weiß et al., 2013). However, these models assume that the number of membrane protein molecules is sufficiently small to allow free diffusion without interaction with other membrane protein molecules. There are too many factors to take into account, and few studies have been done to test this in eukaryotic cells.

In addition to the size of the molecule, which is a general factor, there are many examples of diffusion being affected by interactions with the cell membrane in each type of molecule. For example, EGFR is known to diffuse slower after binding to EGF. This is thought to be due to increased friction with the cell membrane as EGFR forms multimeric complexes and the extracellular region collapses to adhere to the cell

membrane, thereby slowing it down. (Hiroshima et al., 2018). In complex membrane environments, the extent to which the structure of membrane proteins affects diffusion may vary greatly from species to species.

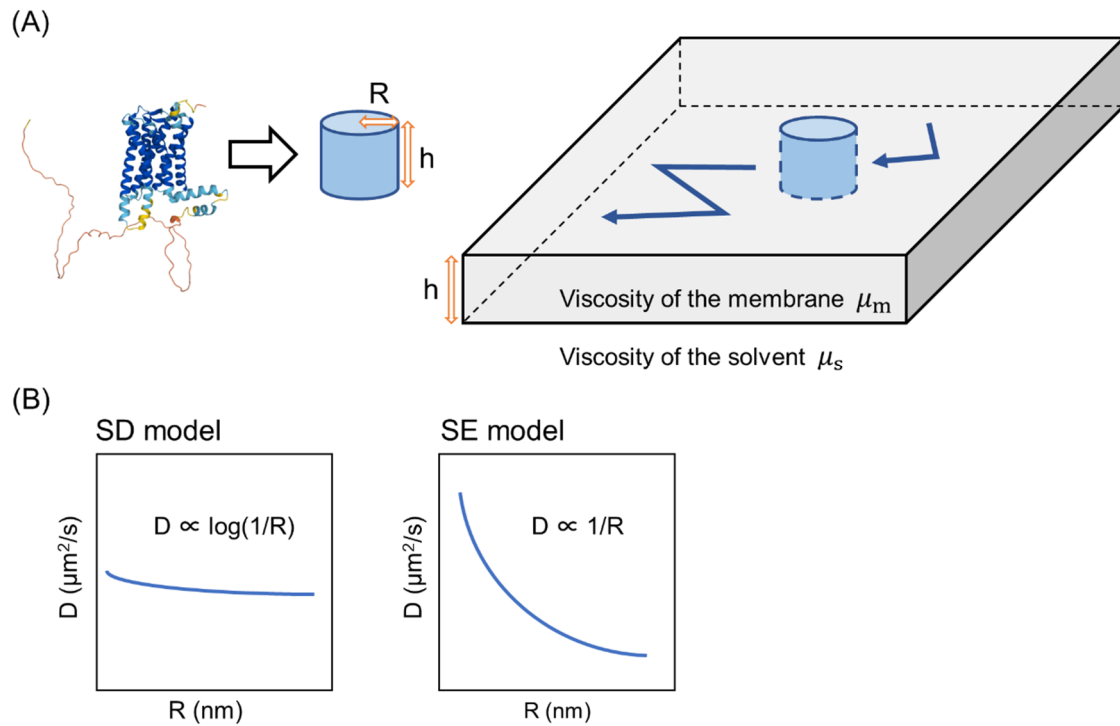


Fig. 1.2. Schematic of theoretical models of molecular diffusion.

(A) Diagram of the theoretical model. A transmembrane protein is considered as a cylinder of radius R and height h , and it is assumed to diffuse laterally through a membrane with viscosity μ_m . The viscosity of the solvent is represented by μ_s . (B) Comparison between the SD and the SE models. In the SD model, the diffusion coefficients of the molecules do not depend much on the radius, whereas in the SE model, the diffusion coefficients depend much more on the radius.

1.3 Diffusion on the cell membrane and analysis methods

In considering diffusion motion on cell membranes, since the SD and SE models are about membrane proteins in free diffusion in a single viscous two-dimensional fluid, they are missing two major elements: first, molecules diffusing on the membrane have different diffusion modes, and second, molecules diffusing on the membrane have multiple diffusion states with different velocity bands.

First, I will discuss diffusion modes. Molecules have three different diffusion modes on the cell membrane. The three modes are: free diffusion, in which no external force is exerted; superdiffusion, in which active transport by motor proteins, and so on, takes place; and subdiffusion, in which movement is restricted by the cytoskeleton and other factors. Compared to free diffusion, in which the direction of movement is randomly determined, superdiffusion moves more linearly, while subdiffusion moves to stay in one place (Fig. 1.3A). The main analytical method used to determine the diffusion pattern is the mean squared displacement (MSD) (Metzler and Klafter, 2000). The MSD is a measure of the deviation of a particle's position from a reference position over time. The magnitude of the slope of the MSD versus time interval Δt represents the magnitude of the diffusion coefficient for that molecule (Fig. 1.3B). Furthermore, the appearance of the increase in MSD indicates the diffusion mode of the molecule. When the MSD is proportional to $(\Delta t)^\alpha$, the molecule shows free diffusion when $\alpha = 1$, restricted diffusion when $\alpha < 1$, and superdiffusion when $\alpha > 1$ (Fig. 1.3C).

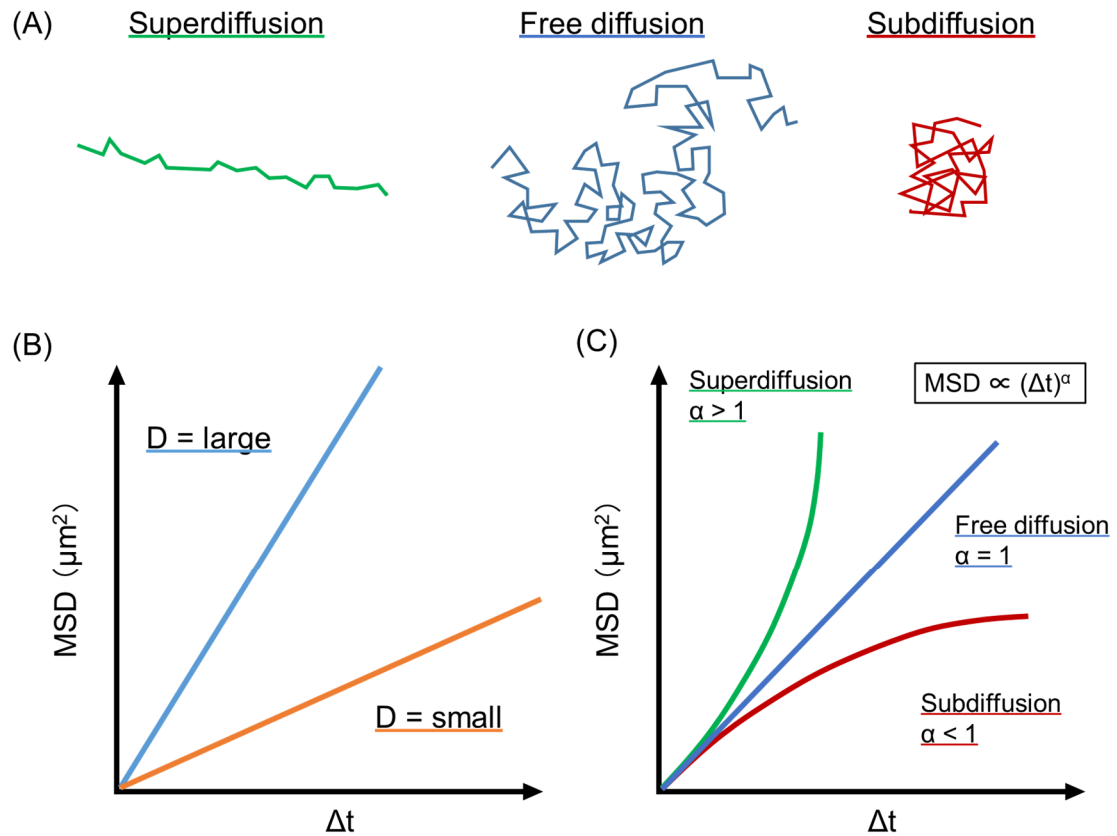


Fig. 1.3. Molecular diffusion modes and overview of the MSD analysis.

(A) Examples of a molecular trajectory for three diffusion mode. (B)(C) Examples of the MSD analysis (B) The larger the slope of the MSD increases relative to the time interval, the larger the diffusion coefficient of the molecule. (C) The shape of the increasing curve of MSD indicates the diffusion mode.

Second, I will discuss that molecules have multiple diffusion states. On the cell membrane, there are heterogeneously distributed regions of different viscosity, such as lipid rafts, and structures that limit motion, such as the cytoskeleton, which are very different from the two-dimensional fluid with a single viscosity assumed by the SD model and other models. In addition, membrane proteins themselves interact with a variety of molecules. In such an environment, membrane proteins are thought to have multiple diffusion states with different velocity bands and to transition from one diffusion state to another with time (Fig. 1.4A, B). Therefore, it is necessary to capture each diffusion state separately. The probability density function (PDF) analysis (Matsuoka et al., 2009) and the hidden Markov model (HMM) analysis (Das et al., 2009) are the analytical methods for capturing multiple diffusion states. The PDF analysis can estimate the diffusion coefficients and ratios of each of the multiple diffusion states based on the displacement of the molecule between frames. The HMM analysis can estimate the diffusion coefficient and ratio of each diffusion state, the ratio of transitions to other diffusion states, and which diffusion state the molecule is in at each time (Fig. 1.4C).

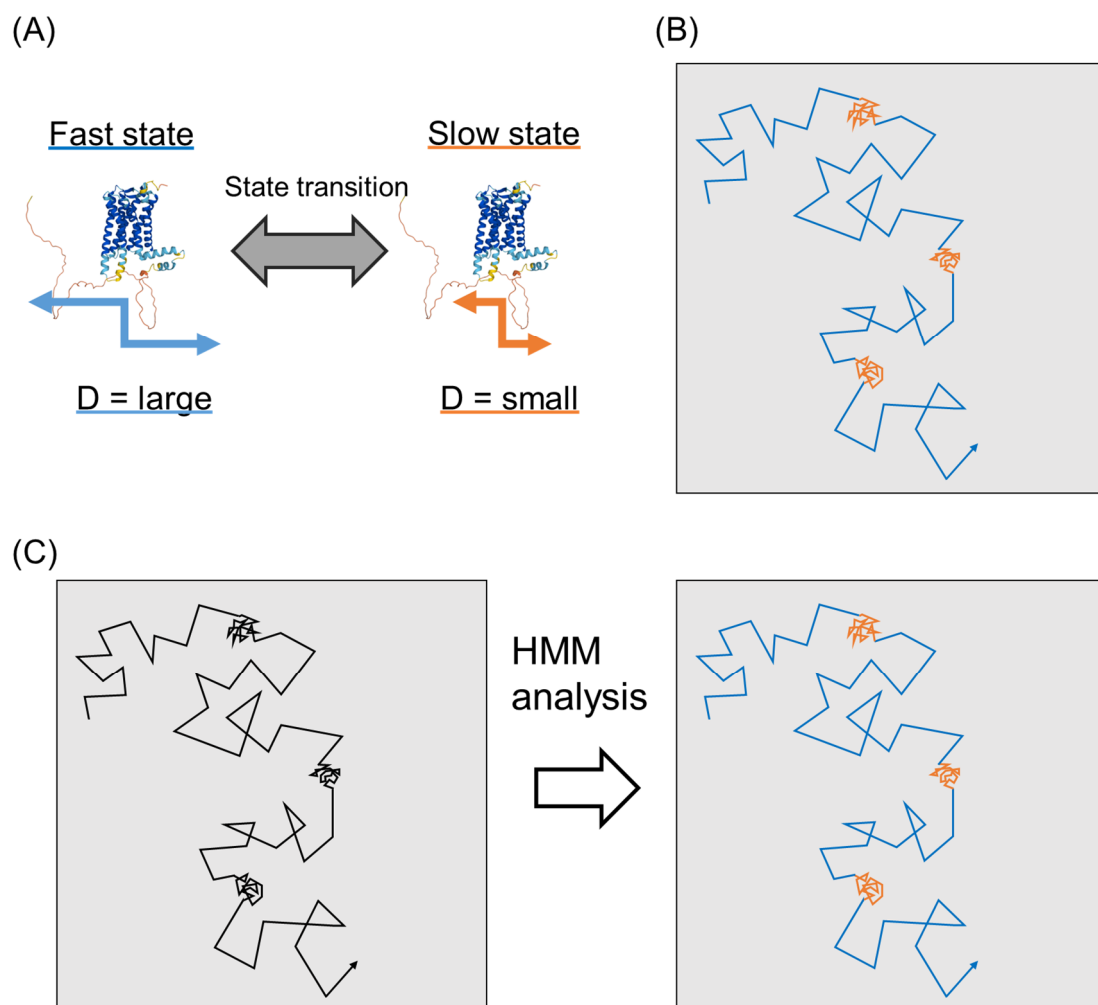


Fig. 1.4. Multiple diffusion states and the HMM analysis.

(A) Image of a molecule with two diffusion states. The molecule is in transition between a fast state with a high diffusion coefficient and a slow state with a low diffusion coefficient. (B) Example of the trajectory of a molecule in two diffusion states, showing the difference in diffusion between the fast state (blue line) and the slow state (orange line). (C) Example of state estimation by HMM analysis. The diffusion state at each time can be estimated from a trajectory whose diffusion state is unknown.

1.4 To investigate the factors that affect membrane protein diffusion in cell membranes

While there has been abundant research on diffusion properties specific for individual protein species, there has been little research on the common features in diffusion regardless of the protein species. Consequently, it is difficult to determine whether a given diffusional movement is caused by a protein species-specific feature or by the surrounding membrane environment. In order to develop a model widely applicable to various membrane proteins, it is important to dissect two parts: one derived from the intrinsic properties specific to the protein species and the other derived from the membrane environment surrounding the proteins. For this purpose, I analyzed systematically the lateral diffusion of many transmembrane proteins with structural variability in the same condition.

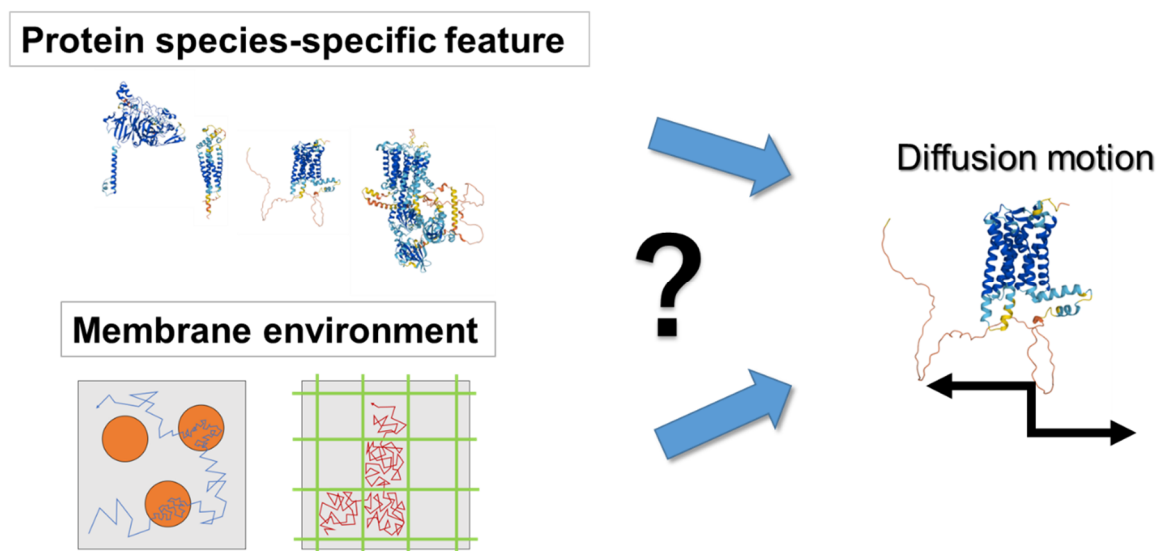


Fig. 1.5. Does the diffusion of a protein molecule depend on the protein species-specific feature or on the surrounding plasma membrane environment?

1.5 Results summary

First, to investigate the tendency of diffusion of membrane proteins, I measured the diffusion of 27 membrane proteins of *Dictyostelium discoideum* using single molecule imaging and performed the MSD analysis. The results showed that all membrane proteins exhibited free diffusion and their diffusion coefficients were relatively close. In addition, measurements and the MSD analysis were performed under conditions in which drugs were added that would affect the cytoskeleton. The results showed that the diffusion coefficients decreased regardless of the type of drug. On the other hand, almost all membrane proteins showed free diffusion.

Next, assuming that these membrane proteins have multiple diffusion states on the cell membrane, I estimated the number of diffusion states by using the probability density function (PDF) of displacement and the Akaike information criterion (AIC). I found that most membrane proteins have three diffusion states. I also performed the MSD analysis for each of the three diffusion states using the HMM. The results show that most of the molecular species exhibit free diffusion under all measurement conditions. The diffusion coefficients in each state were close to the analytical values regardless of the species. The diffusion coefficients for each state were better represented by the SD model, indicating that differences in membrane protein structure do not contribute significantly to diffusion.

The above measurements revealed that in *Dictyostelium*, membrane protein diffusion is highly dependent on the cell membrane. Based on this fact, I developed a phenomenological model of membrane protein diffusion, the membrane field model. In this model, the cell membrane has three regions corresponding to three diffusion states, and the diffusion state of a particle is determined by its position. Using this model to generate the diffusion motion of particles, I obtained analytical values that were consistent with the measured data.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Selection of candidates for single molecule measurements

I focused on the molecular species of membrane proteins with α -helix transmembrane regions in the plasma membrane of *Dictyostelium discoideum*. In order to include as many protein species as possible in the candidate list, UniProtKB was used for the selection (The UniProt Consortium, 2021). I selected molecular species registered in SwissProt, which were manually annotated and reviewed among the UniProtKB entries. There were 430 registered molecular species of membrane proteins with annotated sequences of the transmembrane region of the α -helix. Among them, 143 species with annotations for the extramembrane region (cytoplasmic, extracellular), which are likely to exist on the cell membrane, were selected as candidates for measurement. Of the 27 membrane proteins that exhibited stable expression on the plasma membrane of wild-type AX2 cells, no molecules had been reported to interact specifically with cytoskeleton molecules, such as microtubules or F-actin.

2.1.2 Cell culture and DNA constructs

Dictyostelium discoideum cells were used for all experiments. Ax2 was used as the wild-type parental strain (our in-house strain). Cells were statically cultured at 21°C in HL5 medium including glucose 15.4 g/L, yeast extract (Oxoid) 7.15 g/L, proteose peptone No.2 (BD) 14.3 g/L, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 1.28 g/L, KH_2PO_4 0.486 g/L, folic acid 200 mg/L, cyanocobalamin 0.06 mg/L, 6 ng/mL vitamin B12 supplemented with streptomycin sulfate 100 mg/L, and benzylpenicillin potassium 70 mg/L, as reported previously (Watts and Ashworth, 1970; Kamimura et al., 2016). Plasmids were generated by In-Fusion (Takara Bio Inc). The plasmid vector, pHK12-neo-C-terminal Halo, was digested using *Bgl*III. The genes encoding the membrane proteins were amplified by PCR with Phusion High Fidelity DNA Polymerase (NEB) from *Dictyostelium* genomic DNA. Primers were the first and last 20-30 nucleotides carrying an additional 15 nucleotides of the flanking vector sequences at the *Bgl*III digested site for the In-Fusion cloning. All primer sequences are listed in supplementary Table S1. The resultant plasmids allowed the expression of membrane proteins bound to HaloTag® (Promega) at the C-terminus. The expression plasmids were electroporated into *Dictyostelium* cells using the ECM 830 Square Wave Electroporation System (BTX) at the following setting: an effective voltage of 500 V, a pulse width of 100 μs , a pulse interval of 1.0 s, and pulse number of 15. A total of 10^7 cells were exponentially grown in a petri dish, then HL5 medium was removed, and the cells were washed with Development Buffer (DB; 5mM NaH_2PO_4 , 5 mM Na_2HPO_4 , 2 mM MgSO_4 , 0.2 mM CaCl_2) three times. Then the cells were collected from the dish in 1 mL of Electroporation Buffer (10 mM KH_2PO_4 , 50 mM Sucrose), and 400 μL was taken and mixed with 5 μg of plasmid DNA. The mixture was placed into a pre-chilled cuvette and allowed to stand for 5 minutes on ice. After electroporation, Cell/DNA mix was transferred to a new petri dish and mixed with 4 μL of Healing Buffer (100 mM CaCl_2 , 100 mM MgCl_2). After 15 min, 10 mL of HL5 buffer and 10 mL of HL5 medium were added to the electroporated cells. After 24 hours incubation, G418 (Nacalai tesque) was added to the medium at a final concentration of 10 $\mu\text{g}/\text{mL}$ to select transformed cells.

2.2 Methods (Measurement)

2.2.1 Phalloidin staining to observe actin cytoskeleton

After 1 h starvation in DB, the cells were incubated with 2.5 μ M Jasplakinolide (Abcam), 5 μ M latrunculin A (Sigma-Aldrich), 50 μ M nocodazole (Abcam), 100 μ M thiabendazole (Santa Cruz Biotechnology), 20 μ M benomyl (Riedel-de Haen) or 100 μ M blebbistatin (Sigma-Aldrich), in DB for 30 min. The drugs were removed, and the cells were fixed with 3.7% formaldehyde in DB for 30 min at 21 °C. After washing with DB, the cells were treated with 0.1% Triton X-100 in DB for 5 min and washed with PBS twice. The cells were stained with BODIPY-conjugated Phalloidin (Invitrogen) diluted 200 times in PBS for 30 min at 21 °C. After three washes with PBS, the cells were filled in PBS. The images were taken using an Olympus FV1000 confocal laser microscope system with an oil immersion objective lens (Fluor 60 $\times$ /1.49 NA).

2.2.2 Microtubules staining

Cells treated with the drugs were prepared for in the Phalloidin staining above. The drugs were removed, and the cells were fixed with methanol pre-chilled at -30 °C for 5 min. After two quick rinses with PBS, the cells were incubated in PBS for 15 min and subsequently in PBS containing 5 mg/mL BSA for 15 min at 21 °C. The cells were incubated with 1:400 anti- α -tubulin antibody labeled with FITC (Sigma-Aldrich, F2168) in PBS containing 5 mg/mL BSA at 4 °C overnight. After washing with PBS three times, the cells were filled in a 1:1 dilution of an antifade reagent using PBS (Nacalai tesque, 12745-74). The images were taken using the Olympus FV1000 confocal laser microscope system with an oil immersion objective lens.

2.2.3 Cell preparation for live single molecule imaging

For single molecule imaging of the transmembrane proteins in living cells, I used HaloTag®, which can be fluorescently labelled by Halo-ligands. A Halo-ligand, tetramethylrhodamine (TMR, Promega), was used for all measurements, as previously described (Miyanaga et al., 2009; Matsuoka et al., 2016). The cells were prepared as follows. About 5.0×10^6 cells carrying the membrane protein expression plasmids cultured in a 90-mm petri dish were transferred to a 35-mm petri dish. HL5 medium was removed,

and the cells were washed with DB three times followed by a one-hour incubation in 1 mL DB. To the dish, 5 μ L of 10 μ M HaloTag TMR ligand was added, and the mixture was incubated for 30 minutes. After the staining solution was removed, and the cells were washed with DB three times. The cells were transferred to a 96-well glass bottom plate (Greiner) and allowed to stand for 5 minutes. Drugs were added just before transferring to the 96-well plates. For the drug treatment, 2.5 μ M jasplakinolide, 5 μ M latrunculin A, 50 μ M nocodazole, 100 μ M thiabendazole, 20 μ M benomyl, or 100 μ M blebbistatin was added to the cells at final concentrations under the respective conditions (Clarke et al., 2002; Shu et al., 2005; Tang et al., 2008; Yumura et al., 2014; Sugiyama et al., 2015). The plate was then centrifuged at 1,500 rpm (500 g) for 2 minutes for the cells to adhere to the glass surface of the plate.

2.2.4 Single molecule imaging by TIRFM

Single molecule imaging was performed as described previously using an inverted fluorescence microscope (ECLIPSE Ti2-E, Nikon) (Miyanaga et al., 2009; Matsuoka et al., 2016). The objective lens was a 60 $\times$ lens (CFI Apochromat TIRF 60XC Oil, Nikon), and, together with a 1.5 $\times$ intermediate magnification unit, the magnification was 90 $\times$. A laser of wavelength 561 nm and an output power of 150 mW (OBIS 561-150 LS, Coherent) was used to excite the fluorescent ligands. Images were acquired using a CMOS camera (C13440-20CU, HAMAMATSU). Movies were captured at a size of 768 $\times$ 768 pixels for 100 frames at a rate of 30 frames/s. The pixel size was 72 nm. Images were taken of 5 cells for each drug condition of each membrane protein species twice on different days to obtain a total of 10 cell movies. From the single-molecule observation of 10 cells for each experimental condition, I obtained ~3000 single-molecule tracks, providing ~80000 displacements at 33-ms intervals for the diffusion analysis.

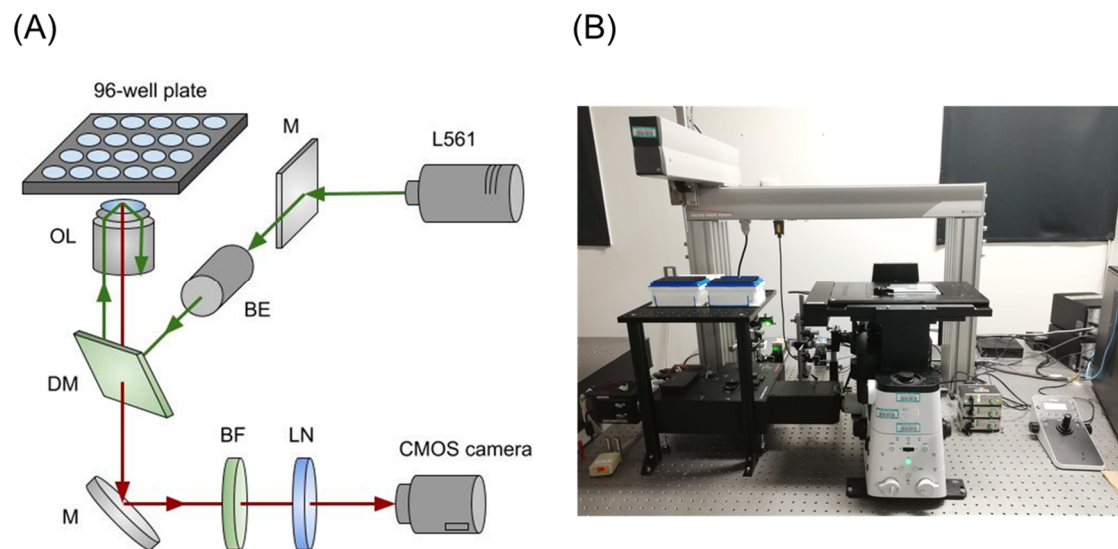


Fig. 2.1. Overview of the TIRFM.

(A) Schematic diagram of the TIRFM. L561 is a laser of wavelength 561 nm; M mirror, BE beam expander, DM dichroic mirror, OL objective lens, BF bandpass filter, LN lens.

(B) Photograph of the TIRFM.

2.3 Methods (Analysis)

2.3.1 Tracking of fluorescence signals.

Trajectories of the fluorescence signals from single molecules were automatically acquired from single-molecule imaging movies using the FIJI plugin TrackMate (version 5.0.1) (Tinevez et al., 2017). After manually selecting a cellular region for each movie, the LoG (Laplacian of Gaussian) detector was used for light spots detection, and the LAP (Linear Assignment Problem) tracker was used for frame-to-frame particle linking. Other parameters were set as follows:

DO_SUBPIXEL_LOCALIZATION = true

RADIUS = 3.0 pixels

THRESHOLD = 2.0

DO_MEDIAN_FILTERING = false

ALLOW_TRACK_SPLITTING = false

ALLOW_TRACK_MERGING = false

LINKING_MAX_DISTANCE = 6.0 pixels

GAP_CLOSING_MAX_DISTANCE = 30 pixels for ESCs, MES/60 pixels for NPCs

MAX_FRAME_GAP = 0 frames

TRACK_FILTER = TRACK_DURATION: 3.

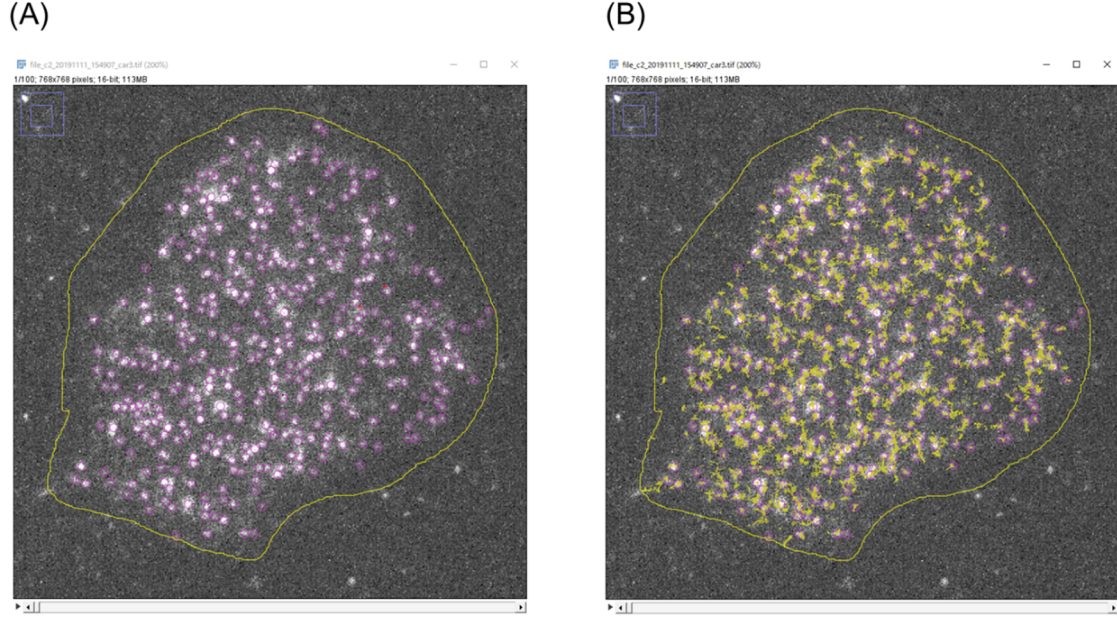


Fig. 2.2. Tracking of fluorescence signals using trackmate.

(A) Light spots detection using the LoG detector. Pink circles surround the detected light spots. (B) Frame-to-frame particle linking the LAP tracker. The yellow lines extending from the pink circles represent the trajectories of the light spots.

2.3.2 Mean squared displacement (MSD)

The diffusion coefficient, D , of the molecules was calculated using the mean squared displacement (MSD) (Miyanaga et al., 2009; Matsuoka et al., 2016). The MSD of molecular motion is calculated as follows,

$$\text{MSD}(n\Delta t) = \{[x_i(t + n\Delta t) - x_i(t)]^2 + [y_i(t + n\Delta t) - y_i(t)]^2\}_i \quad \text{Eq. (1)}$$

, where i is the number of the trajectory, and $x_i(t)$ and $y_i(t)$ are the x - and y -coordinates of the i -th trajectory at time point t , respectively. Δt is the time interval between frames (33 ms). n represents the frame number. MSD was calculated with n ranging from 1 to 15. $\{ \}_{i,m}$ means the average of i trajectories.

For two-dimensional free diffusion, the MSD is expressed using D_M and ϵ_M , which represent the diffusion coefficient and the localization error of the position of the molecule, respectively, as follows,

$$\text{MSD} = 4D_M\Delta t + 4\epsilon_M^2 \quad \text{Eq. (2)}$$

The MSD calculated from Eq. (1) was linearly approximated by the least-squares method, and D_M and ε_M were calculated from Eq. (2).

2.3.3 Probability density function (PDF) and Akaike information criterion (AIC)

The probability density function (PDF) of the displacement of the molecules and the Akaike information criterion (AIC) were used to estimate the number of diffusion states of the molecules (Akaike, 1974; Matsuoka et al., 2009; Matsuoka et al., 2016). The PDF of the displacement of freely diffusing molecules with multiple diffusion states is expressed by the following equation,

$$P_n(\Delta t, \Delta r) = \sum_{n=1}^N p_{\text{PDF}-n} \frac{\Delta r}{2D_{\text{PDF}-n}\Delta t} \exp\left(-\frac{\Delta r^2}{4D_{\text{PDF}-n}\Delta t}\right), \quad \sum_{n=1}^N p_{\text{PDF}-n} = 1. \quad \text{Eq. (3)}$$

, where Δr is the displacement between frames, Δt is the time interval between frames (33 ms), N is the number of diffusion states, p_n is the ratio of the diffusion state n , and D_n is the diffusion coefficient of the diffusion state n . The PDF does not include localization errors, because the errors vary depending on the diffusion state, and the errors for each diffusion state can be determined only after the trajectory has been separated into multiple states by the HMM analysis and then analyzed using the MSD, as described below (also see Table 3.4). Thus, $D_{\text{PDF}-n}$ is the apparent diffusion coefficient and used only to estimate the number of diffusion states.

To calculate AIC values, a maximum likelihood estimation (MLE) of the parameter θ is required for each model, and the log likelihood $L(\theta)$ can be described as follows:

$$L_n(\theta) = \sum_{m=1}^M \log P_n(\Delta r_m | \theta) \quad \text{Eq. (4)}$$

, where M is the total number of all Δr obtained from the single-molecule trajectory data. θ is estimated from the dataset of all Δr , assuming that the dataset is derived from the PDF with θ unknown. The values of θ are searched so as to return the maximum log-likelihood. From these MLE, I can obtain the apparent diffusion coefficient $D_{\text{PDF}-n}$ and the ratio $p_{\text{PDF}-n}$ as well as $L(\theta)$ for the four models ($N = 1$ to 4). The appropriate

number of diffusion states was determined by calculating the AIC_n from the calculated log likelihood (Akaike, 1974; Matsuoka et al., 2009; Matsuoka et al., 2016),

$$AIC_n = -2L_n(\theta) + 2k_n = -2 \sum_{m=1}^M \log P_n(\Delta r_m | \theta) + 2k_n \quad \text{Eq. (5)}$$

, where k_n denotes the number of parameters used for the model. The model with the number of states N that minimizes the value of the AIC is the optimal model. Because three states were optimal for any protein based on this analysis, the estimated apparent diffusion coefficients and the ratios were expressed as $D_{PDF-fast}$, $p_{PDF-fast}$, $D_{PDF-middle}$, $p_{PDF-middle}$ and $D_{PDF-slow}$, $p_{PDF-slow}$ for fast, middle and slow states, respectively, as summarized in Table 2.1.

2.3.4 Hidden Markov model (HMM)

Single-molecule trajectories were analyzed using the HMM, as reported previously (Hiroshima et al., 2018; Yanagawa et al., 2018). The number of states of the HMM used in the analysis was 3. The HMM has three parameters: the initial probabilities $p_{HMM0-fast}$, $p_{HMM0-middle}$, $p_{HMM0-slow}$, which represent the probability of which diffuse state is in the first frame (Fig. 2.1A); the transition probability matrix A where each element can be represented by $A_{fast \rightarrow fast}$, $A_{fast \rightarrow middle}$, $A_{fast \rightarrow slow}$, $A_{middle \rightarrow fast}$, $A_{middle \rightarrow middle}$, $A_{middle \rightarrow slow}$, $A_{slow \rightarrow fast}$, $A_{slow \rightarrow middle}$, $A_{slow \rightarrow slow}$, which represents which state each state will transition to in the next frame (Fig. 2.1B); and the observed symbol probability distributions of B_{fast} , B_{middle} , and B_{slow} , which generates a displacement Δr from each state that cannot be known directly from the measurements (Fig. 2.1C). Given that the molecules are freely diffusing in each state, B_{state} can be expressed as follows,

$$B_{state}(\Delta t, \Delta r) = \frac{\Delta r}{2D_{HMM-state}\Delta t} \exp\left(-\frac{\Delta r^2}{4D_{HMM-state}\Delta t}\right) \quad \text{Eq. (6)}$$

, where the state is fast, middle or slow. Given that the only parameter of the distribution B is the diffusion coefficient, $D_{HMM-state}$ was used as the model parameter. The initial values of the initial probabilities ($p_{HMM0-fast}$, $p_{HMM0-middle}$, $p_{HMM0-slow}$) and the transition probability matrix A were determined randomly. The initial value of $D_{HMM-state}$ was

determined by dividing $\Delta r^2/4\Delta t$ of each dataset into three clusters using the k-means method, and the central value of the cluster was used. The Baum-Welch algorithm was used for the model parameter estimation (Baum and Petrie, (1966); Baum and Eagon, (1967); Baum and Sell, (1968); Baum et al., (1970); Baum, (1972)). The Viterbi algorithm was used to estimate which state the molecule is in at each time (Viterbi, 1967). From this estimation, three mobility states were assigned along the trajectories. The obtained trajectories for each state were used to calculate the MSD,

$$\text{MSD}_{S\text{-state}} = 4D_{S\text{-state}}\Delta t + 4\varepsilon_{S\text{-state}}^2 \quad \text{Eq. (7)}$$

, where $D_{S\text{-state}}$ and $\varepsilon_{S\text{-state}}$ are the diffusion coefficient and localization error for each diffusion state, respectively; the diffusion coefficients are represented as $D_{S\text{-fast}}$, $D_{S\text{-middle}}$ and $D_{S\text{-slow}}$ for each state (Table 2.1, Table 3.4). To obtain the steady state ratios of each state, the probabilities of each state at each time determined by the HMM were averaged for all trajectories and represented as $p_{S\text{-fast}}$, $p_{S\text{-middle}}$, and $p_{S\text{-slow}}$ (Table 2.1, Table 3.4).

To calculate the lifetimes of each state, the trajectories of each state were extracted by the Viterbi algorithm, as described above. The time duration of individual trajectories was collected and used to obtain the histogram in a cumulative form with $t = 0$ as the time when the state started. Typically, the statistical distribution of the time duration exhibited an exponential-like decay curve. The state lifetime, τ , was calculated from the equation $\tau = 1/\lambda$ by fitting the histogram with the exponential function $N = N_0 e^{-\lambda t}$ using the least-squares method and the obtained λ .

For the HMM, the localization error was not included in Eq. (6). Thus, $D_{\text{HMM-state}}$ is the apparent diffusion coefficient for each state. After being assigned into the three diffusion states along the trajectories by the HMM, the obtained trajectories for each state were used to calculate the MSD using Eq. (7), from which I obtained $D_{S\text{-state}}$ with the corresponding $\varepsilon_{S\text{-state}}$ for each state. Considering that the fluorescent spots of single molecules are obtained by an accumulation of fluorescence over a period of 33 ms, it is reasonable that the error depends on the diffusion state. In fact, the localization errors differed depending on the different diffusion states, in which the fast diffusion state showed relatively large errors (~90 nm), while slow diffusion showed conversely small errors (~10 nm), as shown in Tables S3 and S5. When the localization error for each state was included as an unknown parameter in Eq (6), the parameter estimation by the HMM did not work well.

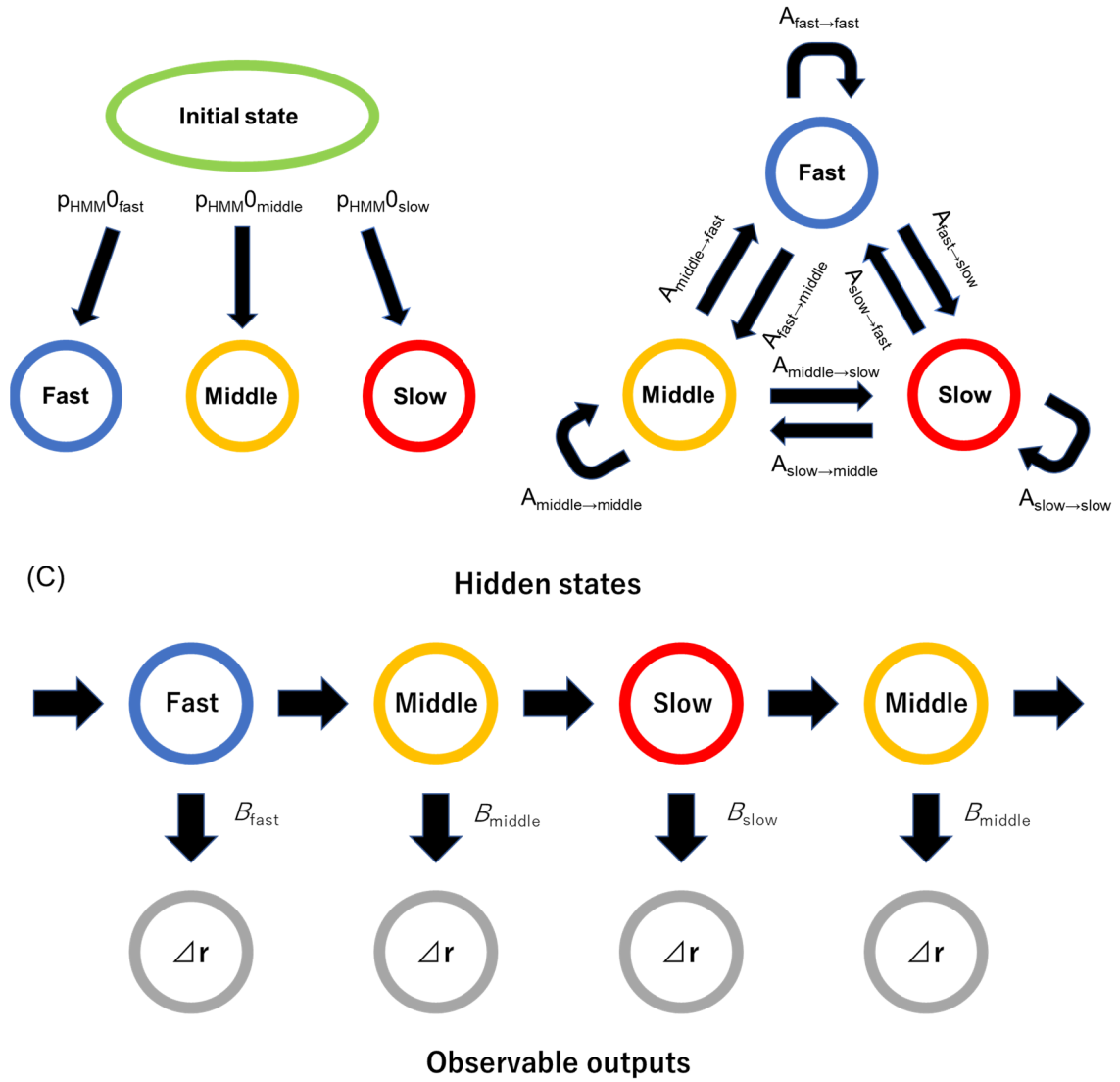


Fig. 2.1. Conceptual Diagram of Hidden Markov Model.

- (A) The initial diffusion state is determined according to the initial state probabilities p_{HMM0} .
- (B) The diffusion state transitions each frame according to the state transition probabilities A .
- (C) Displacement Δr is output according to output probabilities B from the unobservable diffusion state for each frame.

2.3.5 Bootstrap analysis for parameter estimation and AIC-based model selection

To calculate confidence intervals (CI) for the parameters estimated by the diffusion analysis, the bootstrap method was used (Efron, 1979). For the MSD and HMM analyses, 10 datasets were created by random sampling with overlap allowed from the original datasets of the single-molecule trajectories. The estimated parameters were obtained from the 10 datasets.

To calculate the AIC value, 10 datasets of displacement Δr were created by random sampling with overlap from the original Δr datasets. The diffusion parameters were estimated by MLE using Eq. (4), and log-likelihoods were obtained using Eq. (5) for the 10 datasets. From the calculated log likelihoods, AIC values were obtained (Fig. S6). The relative likelihoods for the model with three versus two components were determined by calculating $\exp((AIC_3 - AIC_2)/2)$.

For the diffusion analysis of the particle trajectory data obtained from the numerical simulation, 10 sets of 5000 trajectories from 100 frames were generated for each condition. The datasets were analyzed in the same manner as for the experimental data.

2.3.5 Comparison with theoretical models

The Saffman-Delbrück model (SD model) is expressed using the following equation (Saffman and Delbrück, 1975),

$$D_{SD} = \frac{k_B T}{4\pi\mu_m h} \left(\ln \frac{\mu_m h}{\mu_s R} - \gamma \right) \quad \text{Eq. (9)}$$

The Stokes-Einstein-like model (SE-like model) is expressed using the following equation (Gambin et al., 2006),

$$D_{SE} = \frac{k_B T \lambda}{4\pi\mu_m h R} \quad \text{Eq. (10)}$$

, where k_B is Boltzmann's constant ($k_B = 1.38 \times 10^{-23} \text{ m}^2 \text{ kg} / \text{s}^2 \text{ K}$), T is the absolute temperature, μ_m is the viscosity of the cell membrane, μ_s is the viscosity of the solvent surrounding the cell membrane, h is the thickness of the cell membrane, R is the radius of the membrane protein molecule embedded in the cell membrane, γ is Euler's constant

($\gamma \approx 0.5772$), and λ is the characteristic length. T was set to $T = 294$ K based on the room temperature at the measurement, μ_s was set to 9.6×10^{-4} Pa·s, and h was set to 3.8×10^{-9} m. The radius, R , of membrane protein molecules with the same transmembrane number was assumed to be the same. The radii of DD3-3 for single-transmembrane molecules and car1 for seven-transmembrane molecules were used as representatives. R of the membrane protein molecules was estimated using PyMol with available 3D models, which were predicted using AlphaFold (Jumper et al., 2021; Varadi et al., 2021). If the region embedded in the cell membrane was oval, the average of the long and short radii was used. The fitting of each model to the measured data was performed using the least-squares method with μ_m as a variable.

2.3.6 Parameters used in the diffusion analyses.

Since each of the MSD, PDF, and HMM analyses uses parameters with similar meanings, they are displayed together in Table

Analysis	Parameter	Unit	Equation No.	Meaning
MSD	D_M	$\mu\text{m}^2/\text{s}$	(2)	Diffusion coefficient obtained from MSD of the entire trajectory data
	ϵ_M	nm		Localization error obtained from MSD of the entire trajectory data
PDF	$D_{\text{PDF-state}}$	$\mu\text{m}^2/\text{s}$	(3)	Diffusion coefficient for each diffusion state estimated by MLE
	$p_{\text{PDF-state}}$	%		Ratio of each diffusion state estimated by MLE
HMM	$D_{\text{HMM-state}}$	$\mu\text{m}^2/\text{s}$	(6)	Diffusion coefficient for each diffusion state estimated by HMM
	p_{HMM}^0	%		Initial probability of each diffusion state for each trajectory estimated by HMM
	$p_{\text{S-state}}$	%		Steady state ratio for each diffusion state obtained by averaging the probability of diffusion state at each time for each trajectory estimated by HMM
MSD after state estimation by HMM	$D_{\text{S-state}}$	$\mu\text{m}^2/\text{s}$	(7)	Diffusion coefficient obtained from MSD of each diffusion state estimated by HMM
	$\epsilon_{\text{S-state}}$	nm		Localization error obtained from MSD of each diffusion state estimated by HMM

Table 2.1. List of notations for diffusion parameters.

2.4 Methods (Simulation)

2.4.1 Field generation and particle simulation of diffusional motion

The trajectory data of all molecular species were analyzed together for each drug condition, and a field model was developed to reproduce the results of the analysis. The whole field was set as a square with a size of $10\ \mu\text{m} \times 10\ \mu\text{m}$, which is equivalent to the size of one *Dictyostelium discoideum* cell. The field is divided into three regions. The analysis of the experimental data using the HMM concluded that the transition between fast and slow states was rare, and thus fast and slow regions do not adjoin each other. I randomly placed clumps of fast and slow squares in the middle field so that they were spaced apart from each other. By changing four parameters that include the size of the fast and slow clumps and their number (occupied % area), I prepared various fields.

The following procedure was used to generate the trajectories of particles in the field.

Step 1: Randomly select the position of the particle in the field.

Step 2: Check the state of the position where the particle is located.

Step 3: Generate movement of the particle with distance Δr from the distribution of the diffusion coefficient that matches the state (Eq. (8)). The values of the diffusion coefficient $D_{S\text{-}state}$ of each state, which were experimentally obtained, were used.

Step 4: Select the direction of movement randomly.

Step 5: Return to step 2 after the movement.

After generating the trajectory of the particle, the error of the position corresponding to the state ($\epsilon_{S\text{-}state}$) was added to the position of the particle in each frame.

For particles leaving the field, I made the particles move to the position where they were reflected at the boundary of the field. However, the trajectory was broken at the reflected frame, and a new trajectory started as if another molecule had flowed in from outside.

The positions of individual particles were initially chosen randomly. During the simulation of the diffusion, the particles gradually accumulated to the slow squares due to the difference in diffusion coefficients in each region and then reached a steady state after a sufficient amount of time had passed. In this simulation, the trajectory of 100 frames from the beginning of the motion was deleted for the analysis of the particle diffusion. Three thousand trajectories with 100 frames were generated for the diffusion analysis under each condition. The particle trajectories obtained by the simulation were

analyzed in the same way as the single-molecule trajectories obtained by the experiments.

2.4.2 Size and shapes of clump in the field model

Fields were prepared with various sizes of fast or slow clumps, and the obtained particle trajectories were analyzed by the HMM. The lifetimes of each state were calculated from the datasets of each state as described above. Because the lifetimes of fast and slow states under control condition were about 0.046 ± 0.000 and 0.468 ± 0.008 s (mean $\pm$ 95% CI), respectively (Table 3.8), the clump sizes selected for the field model covered a range of lifetimes (Fig. 3.2.3A).

A square clump was used for simplicity and ease of repeatability of the model calculation, but squares are thermodynamically unlike membrane microdomains. We therefore examined the effects of changing the shape of the clumps from squares to circles. Fields with one square or circular clump in the middle region were prepared, and single-particle trajectories were generated in the fields under control condition. The square size was set as 50×50 and 250×250 nm² for fast and slow clumps, respectively. The change in clump shape had no obvious effect on the particle mobility if the area was kept the same (Fig. 3.2.8). The squares in the field model shown in Fig. 3.2.2 can be replaced by circles, which more closely reflects the thermodynamics of lipid microdomains.

2.4.3 Reproduction of tracking interruptions

From the HMM analysis, I estimated the ratio of each state in each frame. The results show that the proportion of faster states is high in the first frame, but as the frame progresses, the proportion of these states decreases rapidly to reach a steady state. This is due to interruption of the single molecule tracking. Molecules with fast diffusion motion are more likely to lose their tracking. When analyzing the trajectories, the fast state tends to be exposed at the start point by the interruption. The time when the molecules appear is set to zero, and the start points of the trajectories are aligned. As a result, the ratio of faster states becomes higher at the first frame of the trajectories. In order to reproduce this phenomenon in the simulation, I included the interruption of the particle trajectories generated by the simulation. When the tracking of the particles is not interrupted, I set F, M, and S to denote the numbers of trajectories that take the fast, middle and slow states at the start point of the trajectories, respectively. When an interruption does occur, a trajectory is divided into two parts: the front part with the start point counted by the

numbers F , M , and S , and the back part with the new start point. f , m , and s , respectively, denoted the number of trajectories that take the fast, middle and slow states at the start of the trajectories with the newly created start point. The initial probability of each state ($p_{HMM0_{fast}}$, $p_{HMM0_{middle}}$, $p_{HMM0_{slow}}$) and the steady state probability of each state (p_{S-fast} , $p_{S-middle}$, p_{S-slow}) can be described as follows,

$$\begin{aligned} p_{HMM0_{fast}} &\cong \frac{F + f}{F + M + S + f + m + s} , \\ p_{HMM0_{middle}} &\cong \frac{M + m}{F + M + S + f + m + s} , \\ p_{HMM0_{slow}} &\cong \frac{S + s}{F + M + S + f + m + s} , \end{aligned} \quad \text{Eq. (10)}$$

and

$$\begin{aligned} p_{S-fast} &\cong \frac{F}{F + M + S} , \\ p_{S-middle} &\cong \frac{M}{F + M + S} , \\ p_{S-slow} &\cong \frac{S}{F + M + S} . \end{aligned} \quad \text{Eq. (11)}$$

From the HMM of the experimental data, I can obtain the initial probability $p_{HMM0_{state}}$ and the steady state probability $p_{S-state}$ of each state experimentally. From the HHM of the trajectory data simulated on a field without the interruption, I can obtain F , M and S for the various fields. By evaluating F , M and S using Eq. (11), I can select the field that reproduces the steady state ratio (p_{S-fast} , $p_{S-middle}$, p_{S-slow}) of each mobility state. Then, the cutting points of the simulated trajectories were selected randomly along the trajectories, and I obtained f , m , and s for the interrupted trajectories. Because the initial probability of the slow state, $p_{HMM0_{slow}}$, was relatively small in the experimental data, I assumed that the interruption at the slow state is rare and set $s = 0$. Using Eq. (10), I determined the number of cutting points that can reproduce the initial probabilities $p_{HMM0_{fast}}$ and $p_{HMM0_{middle}}$.

2.4.4 Moran's I

To see the dependency of the particle mobility on the spatial arrangements of the fields, I

generated various fields with extreme spatial patterns. As shown in Fig. 3.2.7 A, two fields, Field 1 and Field 2, are set as follows.

Field 1 (Fig. 3.2.7 A left): a field of 28×28 squares (784 squares in total) with only slow squares of 250 nm equally spaced in a middle field of 10 μm ;

Field 2 (Fig. 3.2.7 A right): a field of 56×56 squares (3136 squares in total) with only fast squares of 75 nm equally spaced in a middle field of 10 μm .

The first step is to select 320 squares from the slow squares in Field 1 and erase the other slow squares. The next step is to select 900 fast squares from Field 2 that do not overlap with the selected slow squares and place the fast squares in the same locations in Field 1 to create the field. Using this procedure, I can prepare various fields with the same parameters such as % area occupancy and sizes of the slow and fast regions.

In order to evaluate the degree of bias in the arrangement of the fields, I used Moran's I (Moran, 1950). This index takes values from -1 and 1. The spatial distribution is dispersive or repulsive in the case of negative values, but it is gathering or attractive in the case of positive values. The random distribution gives a value of 0. The definition of Moran's I is

$$I = \frac{N}{\sum_i \sum_j w_{i,j}} \frac{\sum_i \sum_j w_{i,j} (x_i - \bar{x})(x_j - \bar{x})}{\sum_i (x_i - \bar{x})^2} \quad \text{Eq. (12)}$$

Using this equation, I examined the arrangements of the slow squares. In this case, N is the number of slow squares shown in Field 1, giving $N = 784$. i and j represent the spatial points of the field, such that $i, j = 1, 2, \dots, 784$. x_i is the attribute value of point i . The attribute value of the points where the slow square exists was set to 1, while that of the other points was set to 0. $\bar{x}$ is the mean of the attribute values of all points. $w_{i,j}$ is an element of the matrix of spatial weights between points i and j . The matrix of spatial weights is the four nearest neighbors. This means that, except in the periphery, the points above, below, left and right of a point are considered adjacent to each other. For example, the matrix of spatial weights for 3×3 squares (9 squares in total) can be described as follow,

$$\begin{pmatrix} 0 & 1 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 1 & 0 & 1 & 0 & 1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 & 1 & 0 & 1 & 0 & 0 \\ 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 \\ 0 & 0 & 1 & 0 & 1 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 1 & 0 & 1 \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 & 1 & 0 \end{pmatrix}$$

As shown in Fig. Fig. 3.2.6 B, the five patterns of fields were evaluated by Moran's I: scattered, random, two squares in close proximity, four squares in close proximity, and gathering. After setting the patterns of the slow squares, fast squares were distributed randomly. The obtained fields were used for the simulation of particle diffusion in Fig. Fig. 3.2.6 C.

2.4.5 Particle simulation of diffusion motion using HMM.

The following procedure was used to generate the trajectories of particles using HMM.

- Step 1: Determine the diffusion state for the first frame according to the initial state probabilities p_{HMM0} .
- Step 2: Transition the diffusion states according to the transition probability matrix A to create a sequence of diffusion states for the duration of the trajectory.
- Step 3: Generate the travel distance Δr using the diffusion coefficients $D_{S-state}$ according to the diffusion states in each frame.
- Step 4: Randomly determine the direction of movement in each frame.
- Step 5: Determine the position at each time based on the distance Δr and the direction of movement at each time with the starting point as the origin (0,0)

In section 3.2.6, after generating the trajectory of the particle, the error of the position corresponding to the state ($\epsilon_{S-state}$) was added to the position of the particle in each frame.

If not explicitly stated, the trajectory dataset follows the methods of Section 2.4.2 to cut trajectories and reproduce trajectory interruptions.

Since the initial probability of each diffusion state p_{HMM0} obtained from the measurement data deviates from the steady state probability p_s as shown in Sections 2.4.2 and 3.2.3, the trajectory generated by the above procedure shows state changes affected

by artifacts as shown in fig. 3.2.5(A). In order to eliminate the influence of this artifact, the first 100 frames of the generated trajectory were not used for analysis, and the data from after 100 frames were used for analysis. For example, if a trajectory of 100 frames in length is generated for analysis, 200 frames are generated and the first 100 frames are not used. In Section 327, trajectories are generated using HMMs without state transitions, but if there are no state transitions from the first frame, the HMM parameter estimation cannot be correctly evaluated because the ratio of states does not change from initial probability to steady state. Therefore, I set the HMM to have state transitions during the first 100 frames that are not used for analysis, and no state transitions after 100 frames have elapsed.

Chapter 3

Results

3.1 Results (measurement & analysis)

3.1.1 Membrane protein selection and preparation of cells for measurement

I used the lower eukaryote *Dictyostelium discoideum* as a model, since these cells have been established for the single molecule imaging of membrane proteins (Ueda et al., 2001; Matsuoka et al., 2006; Miyanaga et al., 2009; Matsuoka et al., 2016). I selected 143 transmembrane proteins of *D. discoideum* that are annotated as having α -helix transmembrane regions embedded in the plasma membrane and also as having a presumed function when preparing the tagged proteins with a HaloTag at the C-terminus (Table 3.1) (Los et al., 2008; Matsuoka et al., 2016). When introduced into *D. discoideum* wild type AX2 cells, only 27 proteins exhibited stable expression on the plasma membrane, and the corresponding stable transformants were obtained for the subsequent diffusion analysis. The proteins have 1-10 transmembrane regions as estimated by UniProtKB (Table 3.1) (The UniProt Consortium, 2021).

Gene	UniplotKB accession number	Number of transmembrane α -helix	Length	Mass (Da)	Vector	Protein tag	Forward primer's sequence	Reverse primer's sequence
DD3-3	Q58A42	1	1851	69,498	pHK12 (neo)	HaloTag2	ATGAGATTTTTATCAAGTTTAATTATTT	TTAAACTAATCTCTTGAGACCTGGTACTTTTGAAC
psiM	Q54P69	1	2718	79,232	pHK12 (neo)	HaloTag2	ATGAAAAAATAAACAAATAAAATTT	TTATGTTGGTGAGTTATAGAGTGGAATTTACCTCC
tgrO1	Q55G18	1	887	99,445	pHK12 (neo)	HaloTag7	ATGGAGAAAAATTACTAATAATAGTT	TTATTGTTTCATTAATCTTTCTCTTTTGATGA
tgrC1	P42523	1	889	97,443	pHK12 (neo)	HaloTag7	ATGGAAAAGAAAATAATATTACTGATT	TTAAAATTTTTTACCTATAAAATTTTTTCACACG
comC	Q55AP8	1	1501	162,794	pHK12 (neo)	HaloTag7	ATGATTAATAATATTTATTTTATTT	TTATGATTGTGATTACCACCATCACTACCACT
tspE	Q54F08	4	778	26,527	pHK12 (neo)	HaloTag2	ATGACATTTGTGGATAATTTGAATTC	TTAATCTTGTAATAAATGATTTTGATTTTAC
car2	P34907	7	1228	43,301	pHK12 (neo)	HaloTag2	ATGACTATTATGTCAGATATTATCGCAC	TTAAATCATATCATTTTTATTATTATAT
car1	P13773	7	1322	44,312	pHK12 (neo)	HaloTag2	ATGGGTCTTTTAGATGGAAATCCAGCC	TCAATTATTTCCCTTGACCATTTGTTGAAG
fscD	Q75JP9	7	1506	50,663	pHK12 (neo)	HaloTag2	ATGTTTATTATCCTAAATTTTTAATATC	TTAATTTTTGAATCTACTCCTGATAAC
car4	Q9TX43	7	1427	51,467	pHK12 (neo)	HaloTag2	ATGAAAGTATTACAAGAGATTAATCTTAC	TCAAACCTTATTATCTTTTTTTCTC
fscE	Q559M5	7	1535	50,531	pHK12 (neo)	HaloTag2	ATGATATCTCATATTAATAATTTATAAA	TTATGGATCATGTTTAGAATCATCAATAC
fscF	Q1ZXB0	7	1694	52,173	pHK12 (neo)	HaloTag2	ATGATTTTTAATAATTTAAAAATC	TTAAATTCATCTACACTATTTAATGAAG
car3	P35352	7	1638	56,161	pHK12 (neo)	HaloTag2	ATGGAAATTTAAATACAACAAGTACGGC	TTAAACATTTGAATCTTTTTTTTGTAC
fscA	Q54H37	7	1940	63,275	pHK12 (neo)	HaloTag2	ATGAAATTTAATTTAAATTAATTTAATA	TTAATTATTTTCATCATTATTGCTATTATT
fslG	Q54J71	7	1806	64,284	pHK12 (neo)	HaloTag2	ATGAAATCAATAATATTTATACTTTTTT	TTAAATTTTTCCAGTTTTATTTCATTTTG
fslQ	Q1ZXE4	7	2019	65,439	pHK12 (neo)	HaloTag2	ATGAAAAATTCATTTTAATAAATATTTA	TTATGGTTGTTGATTTATTTCAGTAGAC
fslF	Q54J77	7	1857	66,711	pHK12 (neo)	HaloTag2	ATGAAAAATTTAATAATTTTATTATTTT	CTAATTAATTTAATTGATTCTAATTC AAC
fslJ	Q556J4	7	2538	68,945	pHK12 (neo)	HaloTag2	ATGGTCTCAAATAAAAAATCTTTTACC	TTATAGAGCATCAGAAATTTGAATCTAATTC
fslE	Q54J78	7	2225	69,006	pHK12 (neo)	HaloTag2	ATGGAAATGATTAGAATTTTTTAATTTAT	TTATTTTTTTCAAGTTCAATAGATTTAC
fslN	Q55CY2	7	2094	69,796	pHK12 (neo)	HaloTag2	ATGAAATGAAATGAAATTCCTTTAATT	TTAAGCTTGAGATTGTTTATCATCATTATT
fslL	Q54PF8	7	2177	70,185	pHK12 (neo)	HaloTag2	ATGATTACAAATAAATCAAAGTACTATTTT	TTAAGATTTTGAAACTTCGATATCTTTTAA
fslH	Q556C6	7	2183	69,274	pHK12 (neo)	HaloTag2	ATGAATCTGAATTTTTATAACTTAATTTT	TTAAAGATCATCATCATCAGATTATAATT
fslM	Q556N7	7	2183	70,199	pHK12 (neo)	HaloTag2	ATGAAATCAATATTTATTATTATTTTATT	TTATTTTATATTAACAGAACCAATTTTC
grlB	Q86HH3	7	2344	84,118	pHK12 (neo)	HaloTag2	ATGAAAAATTTAATTTCAATTATTCTTTTA	TTAAAGGTTATTAGAATCAATTTCAACTTC
grlO	Q55AP3	7	2699	92,649	pHK12 (neo)	HaloTag2	ATGAAAAAAGTGTTTTTTTGATTTAATT	TCAAGTATCTGAAATAATTTCAATTTTC
slc44a2	Q54I48	9	2096	71,809	pHK12 (neo)	HaloTag2	ATGAGTTCAGAGGATTTACAGGATCACC	TTAAACAACATTTGAACGACCTTTTCC
patB	P54679	10	1058	117,268	pHK12 (neo)	HaloTag7	ATGGATAATAATCAAATTCAAAAAAT	TTAAACTTTATTATCGGTAACAACAGATTATG

Table 3.1. List of all transmembrane proteins analyzed and all primers used in this study.

3.1.2 Single Molecule Imaging and Tracking

After the tag was stained by a fluorescent Halo-ligand conjugated with tetramethylrhodamine (TMR), non-polarized vegetative cells were observed under objective-type total internal reflection fluorescence microscopy (TIRFM) for 100 frames at 30 frames/s. Individual membrane proteins exhibited lateral diffusion on the basal membrane of the cells (Fig. 3.1.1A). For each experimental condition, single molecule observations of 10 cells and single molecule tracking with TrackMate (Tinevez et al., 2017) resulted in approximately 3,000 single molecule tracks, providing approximately 8,0000 displacements at 33 ms intervals for diffusion analysis. A histogram of trajectory lengths is shown in Fig. 3.2. The mean and deviation of the track durations were 23.9 ± 28.9 frames (Fig. 3.1.2A). Durations more than 1 s constitute more than 70% of the total data (Fig. 3.1.2B).

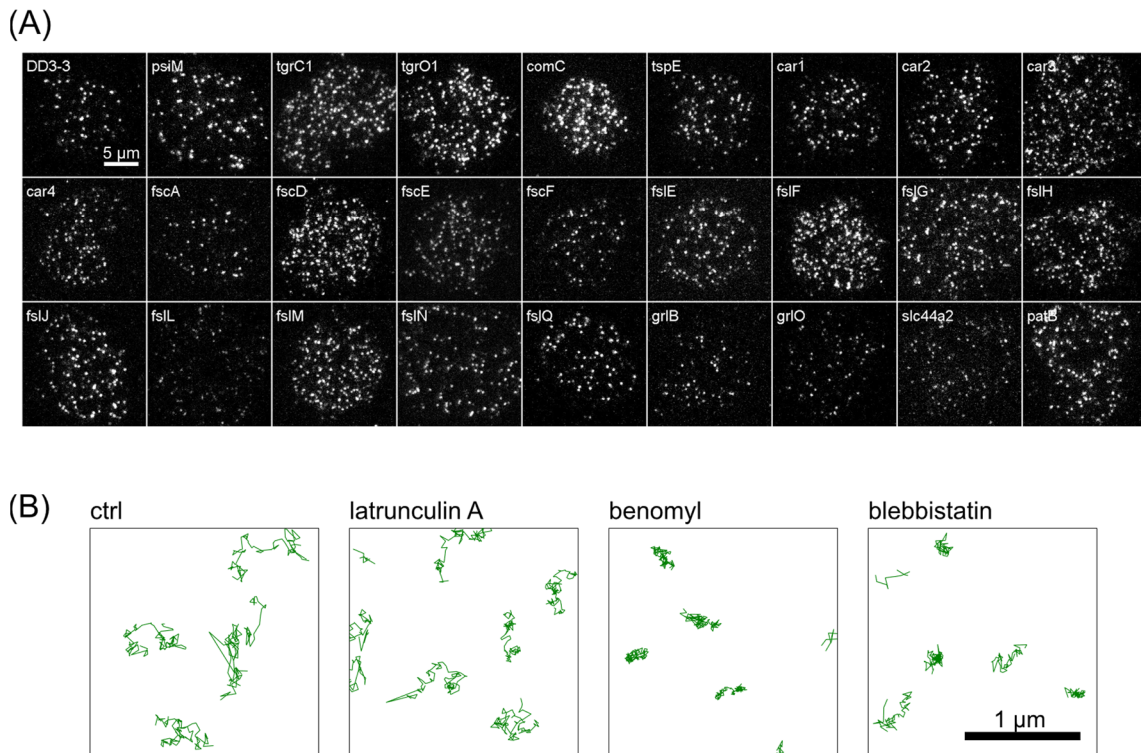


Fig. 3.1.1. Single molecule imaging of transmembrane proteins.

(A) Single molecule images of 27 kinds of transmembrane proteins in *D. discoideum* cells. Scale bar, 5 μm . (B) Representative trajectories of the lateral diffusion of one membrane protein (fslN) in each drug condition. Scale bar, 1 μm .

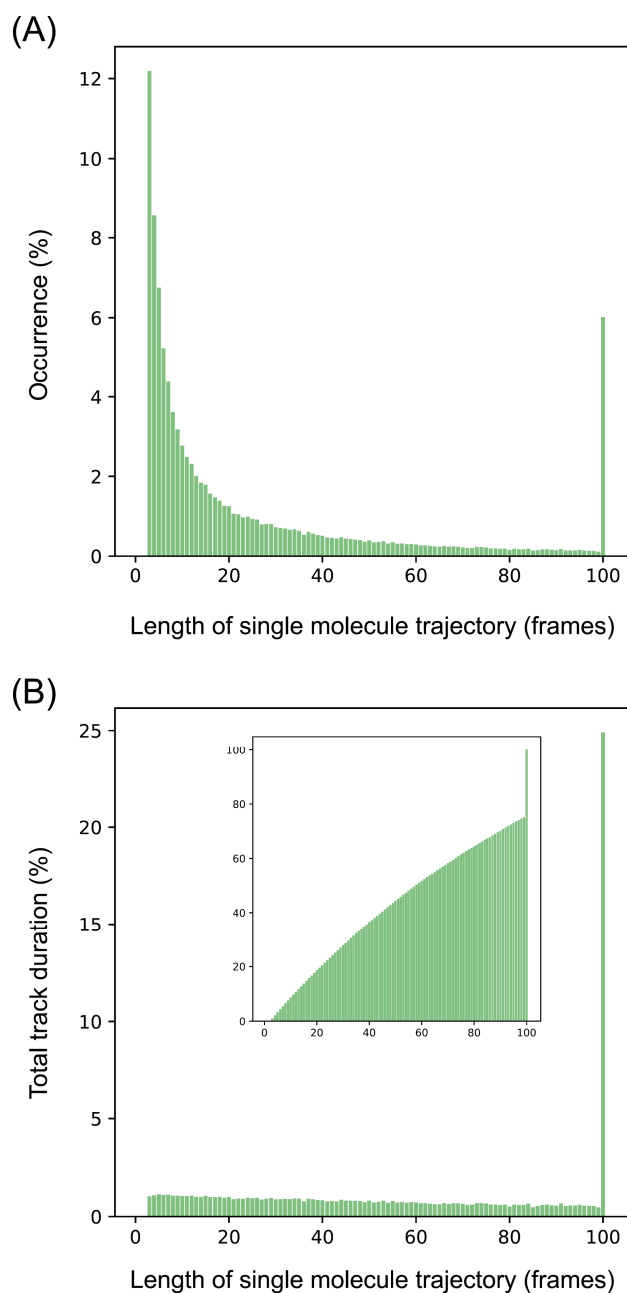


Fig. 3.1.2. Distribution of trajectory length obtained by single-molecule tracking.

(A) The occurrence of each length in the single-molecule trajectories obtained experimentally for the 27 membrane proteins. Interruption in the single-molecule tracking resulted in trajectories shorter than 100 frames. The mean and deviation of the tracking were 23.9 ± 28.9 frames (mean $\pm$ SD). (B) Percentage of each trajectory length. **Inset**, Culminated percentage of each trajectory length. Durations longer than 1 second (30 frames) constitute more than 70%.

3.1.3 All transmembrane proteins undergo free diffusion.

To characterize the diffusion modes of individual protein species, the single-molecule trajectories were obtained, and analyzed by calculating the mean square displacement (MSD) (Fig. 3.1.1B, Fig. 3.1.4). The MSDs were linear with time for all species, indicating that they all underwent free diffusion. The diffusion coefficients were different for each molecular species and ranged from 0.019 to 0.033 $\mu\text{m}^2/\text{s}$, consistent with those reported previously (Miyanaga et al., 2018). Regardless of the structural variability within the 1 to 10 transmembrane regions and 27 to 163 kDa molecular weight (Table 3.1), the obtained diffusion coefficients for the membrane proteins were not so different from each other. Because molecular size is a critical factor for the diffusion coefficients in the Stokes-Einstein-like model (Gambin et al., 2006), this model cannot explain the similar diffusion coefficients among protein species. The average diffusion coefficient for all 27 molecular species was $D = 0.024 \pm 0.004$ (SD) $\mu\text{m}^2/\text{s}$, which is about one-tenth that measured for the free diffusion of various membrane proteins in mammalian cells (Mashanov et al., 2021), showing an apparently higher viscosity of the membrane of *Dictyostelium* cells than of mammalian cells. In addition, the observation that all molecular species underwent simple diffusion is a characteristic feature in this lower eukaryote that distinguishes it from the diverse diffusion modes in mammalian cells.

3.1.4 All membrane proteins showed free diffusion even under drug conditions.

The possible involvement of the cytoskeleton in the mobility of membrane proteins was examined using inhibitors for actin and microtubules, because the cytoskeleton influences lateral diffusion in mammalian cells (Kusumi et al., 2005; Kusumi et al., 2014). I used latrunculin A and jasplakinolide to inhibit actin polymerization and depolymerization, respectively, and used benomyl, nocodazole, and thiabendazole to disrupt microtubules (de Keijzer et al., 2011; Miyanaga et al., 2018). The inhibition of the cytoskeleton was confirmed by phalloidin staining for actin and immunofluorescence staining for microtubules (Fig. 3.1.3). All molecular species underwent free diffusion when any drug was applied (Fig. 3.1.4). The MSDs of all molecular species were close to each other in each drug condition, showing a consistent trend of the drug-induced changes in diffusion regardless of the molecular species. All molecular species showed reduced diffusion for all four cytoskeleton inhibitors except for jasplakinolide (Fig. 3.1.5, Table 3.2). In addition, I investigated whether myosin II

affect the diffusion by using the inhibitor, blebbistatin. All molecular species underwent slower free diffusion with the drug than without (Fig. 3.1.5, Table 3.2). Thus, the cytoskeleton contributes to the lateral diffusion of all membrane proteins commonly in *Dictyostelium* cells, probably via some factors that act in common with various protein species rather than intrinsic factors specific to the species. Moreover, the reduced diffusion suggests that cytoskeletal fences do not play a major role in determining diffusion properties in *Dictyostelium* cells.

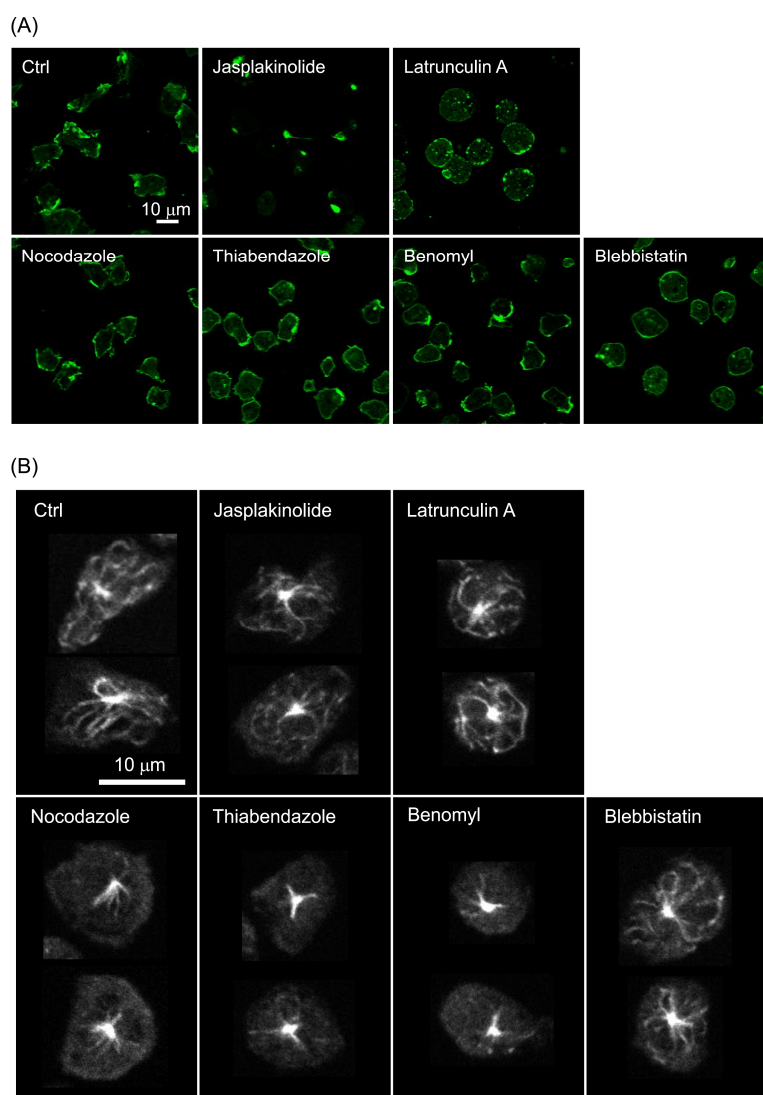


Fig. 3.1.3. Drug effects on F-actin and microtubules in *Dictyostelium* cells.

(A) Effects of six drugs (jasplakinolide, latrunculin A, nocodazole, thiabendazole, benomyl and blebbistatin) on F-actin. The cells were stained with BODIPY-conjugated Phalloidin. Latrunculin A-treated cells exhibited punctuated staining of F-actin on the cell contour. Jasplakinolide-treated cells exhibited aggregates of F-actin, showing severe defects of the actin organization. There were no severe defects of F-actin observed in the nocodazole-, thiabendazole-, benomyl- and blebbistatin-treated cells. Scale bar, 10 μ m. (B) Effects of the six drugs on microtubules. The microtubules were immuno-stained with 1:400 anti- α -tubulin antibody labeled with FITC (Sigma-Aldrich, F2168). The nocodazole-, thiabendazole- and benomyl-treated cells exhibited severe defects in microtubules, in which the microtubules remained only around the centrosome. There were no severe defects of microtubules observed in the jasplakinolide-, latrunculin A- and blebbistatin-treated cells. Scale bar, 10 μ m.

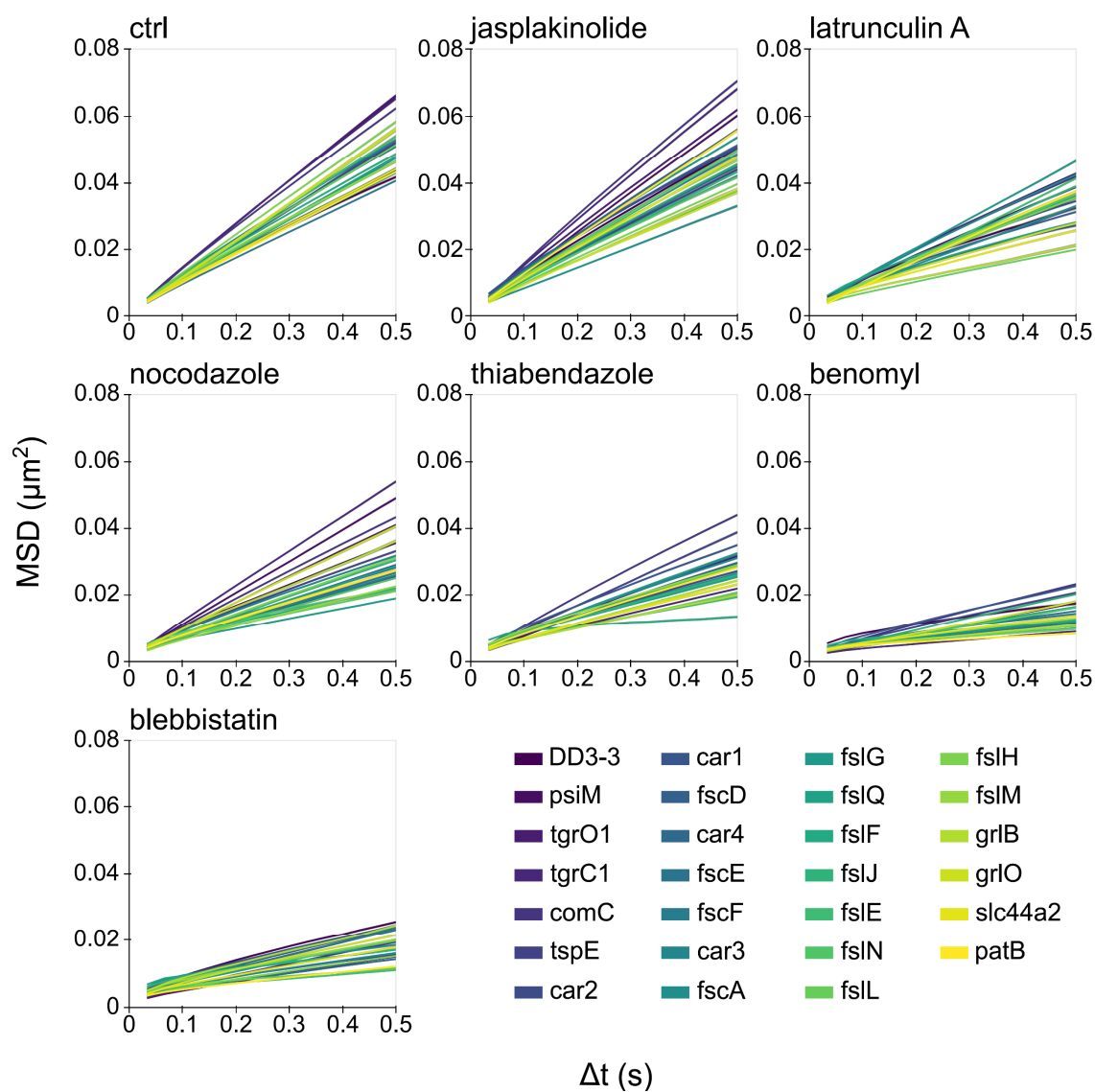


Fig. 3.1.4. Free diffusion of transmembrane proteins under drug treatment.

MSD of 27 kinds of membrane proteins in the control and seven drug conditions are shown in different colors.

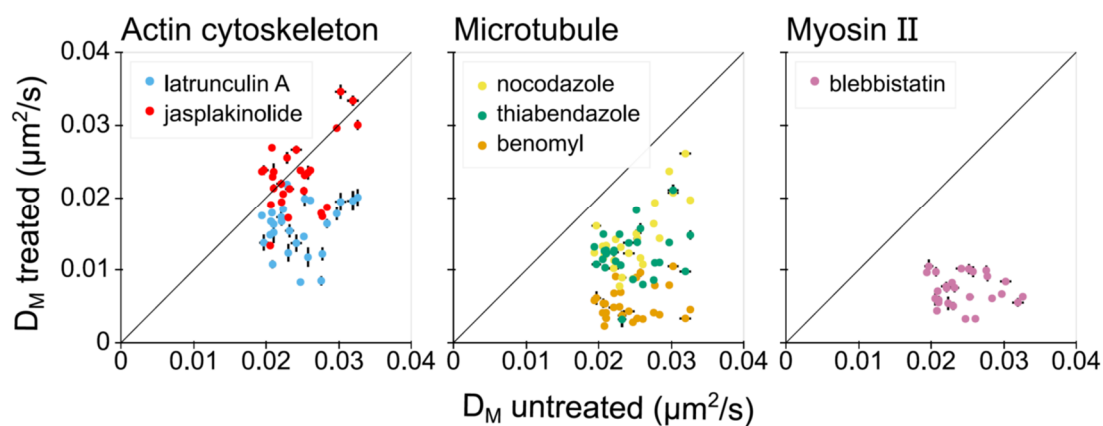


Fig. 3.1.5. All molecular species underwent slower free diffusion with the drug than without.

Scatter plots of diffusion coefficients (D_M , mean $\pm$ 95% CI) estimated from the MSDs. Diagonal lines indicate no effect on D_M . (n = 10 bootstrap datasets created from trajectory data of 10 cells).

Gene	Ctrl		Jasplakinolide		Latrunculin A		Nocodazole		Thiabendazole		Benomyl		Blebbistatin	
	D_M ($\mu\text{m}^2/\text{s}$)	ϵ_M (nm)	D_M ($\mu\text{m}^2/\text{s}$)	ϵ_M (nm)	D_M ($\mu\text{m}^2/\text{s}$)	ϵ_M (nm)	D_M ($\mu\text{m}^2/\text{s}$)	ϵ_M (nm)	D_M ($\mu\text{m}^2/\text{s}$)	ϵ_M (nm)	D_M ($\mu\text{m}^2/\text{s}$)	ϵ_M (nm)	D_M ($\mu\text{m}^2/\text{s}$)	ϵ_M (nm)
DD3-3	0.020±0.001	27±1	0.024±0.000	29±1	0.014±0.001	38±1	0.016±0.000	31±0	0.011±0.000	32±1	0.006±0.000	39±1	0.010±0.000	36±1
psiM	0.030±0.000	17±1	0.030±0.000	19±1	0.018±0.000	31±1	0.024±0.000	20±1	0.014±0.000	23±1	0.008±0.000	22±0	0.007±0.000	23±0
tgrO1	0.033±0.000	19±1	0.030±0.000	24±1	0.020±0.000	30±1	0.020±0.000	22±1	0.015±0.000	25±0	0.005±0.000	26±0	0.006±0.000	26±0
tgrC1	0.032±0.000	22±0	0.033±0.001	22±1	0.020±0.000	32±1	0.026±0.000	21±1	0.010±0.000	26±0	0.003±0.000	25±0	0.006±0.000	30±0
comC	0.030±0.000	24±1	0.035±0.001	23±1	0.019±0.000	31±0	0.021±0.000	24±1	0.021±0.000	25±1	0.010±0.000	26±0	0.008±0.000	26±0
tspE	0.025±0.000	21±1	0.021±0.001	25±1	0.015±0.000	30±0	0.015±0.000	30±1	0.018±0.001	24±1	0.009±0.000	28±1	0.010±0.000	31±1
car2	0.021±0.000	25±1	0.024±0.000	33±0	0.015±0.000	36±1	0.013±0.000	25±0	0.012±0.000	28±0	0.003±0.000	33±0	0.006±0.000	33±0
car1	0.026±0.000	18±1	0.023±0.000	35±1	0.012±0.000	35±1	0.012±0.000	25±1	0.016±0.000	32±0	0.010±0.000	31±1	0.010±0.000	36±1
fscD	0.024±0.000	28±1	0.027±0.000	28±0	0.014±0.000	34±1	0.012±0.000	28±0	0.014±0.000	26±0	0.004±0.000	31±1	0.010±0.000	28±1
car4	0.021±0.000	23±1	0.021±0.000	26±1	0.016±0.000	29±1	0.013±0.000	27±1	0.013±0.000	29±1	0.004±0.000	29±0	0.006±0.000	29±0
fscE	0.019±0.000	22±1	0.024±0.000	27±0	0.018±0.000	26±1	0.012±0.000	23±0	0.013±0.000	28±0	0.006±0.000	31±1	0.010±0.000	31±1
fscF	0.021±0.001	24±1	0.019±0.000	32±1	0.017±0.000	28±1	0.012±0.000	27±1	0.015±0.000	26±1	0.005±0.000	32±1	0.010±0.000	34±0
car3	0.025±0.000	26±0	0.023±0.000	27±0	0.020±0.000	30±0	0.014±0.000	30±0	0.014±0.000	31±0	0.003±0.000	33±1	0.006±0.000	31±0
fscA	0.021±0.001	23±1	0.013±0.000	30±1	0.015±0.000	29±1	0.013±0.000	24±0	0.011±0.000	31±1	0.004±0.000	32±1	0.006±0.000	41±1
fslG	0.023±0.000	19±1	0.026±0.000	29±1	0.022±0.000	27±1	0.008±0.000	30±0	0.015±0.000	27±0	0.005±0.000	28±1	0.008±0.000	31±0
fslQ	0.023±0.000	21±1	0.017±0.001	27±1	0.012±0.000	33±1	0.013±0.000	26±0	0.011±0.000	33±0	0.007±0.000	27±0	0.005±0.000	42±0
fslF	0.023±0.000	26±0	0.021±0.000	28±1	0.015±0.001	37±1	0.009±0.000	33±0	0.003±0.000	44±1	0.004±0.000	36±1	0.008±0.000	35±0
fslJ	0.022±0.000	23±1	0.021±0.000	25±1	0.018±0.000	25±0	0.014±0.000	25±0	0.011±0.000	32±0	0.009±0.000	24±1	0.005±0.000	41±0
fslE	0.026±0.000	22±1	0.024±0.001	25±1	0.020±0.000	22±0	0.011±0.000	31±1	0.008±0.000	31±1	0.003±0.000	30±1	0.003±0.000	34±0
fslN	0.022±0.000	24±1	0.019±0.000	29±1	0.017±0.000	29±1	0.011±0.000	26±0	0.013±0.000	30±1	0.007±0.000	26±0	0.008±0.000	35±0
fslL	0.025±0.000	24±1	0.024±0.000	24±1	0.008±0.000	30±0	0.009±0.000	31±0	0.009±0.000	29±0	0.003±0.000	33±0	0.003±0.000	36±0
fslH	0.028±0.000	21±1	0.019±0.000	25±0	0.016±0.000	28±1	0.014±0.000	26±1	0.011±0.000	26±0	0.004±0.000	29±0	0.006±0.000	28±0
fslM	0.022±0.000	26±1	0.022±0.000	29±1	0.017±0.000	28±1	0.010±0.000	25±0	0.012±0.000	33±0	0.005±0.000	30±0	0.008±0.000	35±0
grlB	0.028±0.001	12±2	0.018±0.001	25±2	0.009±0.000	33±1	0.016±0.001	28±2	0.009±0.000	28±1	0.004±0.000	27±1	0.010±0.001	34±1
grlO	0.028±0.001	13±2	0.017±0.000	25±1	0.012±0.001	32±1	0.019±0.000	23±1	0.011±0.000	25±0	0.008±0.000	24±0	0.009±0.000	30±0
slc44a2	0.021±0.001	25±1	0.023±0.001	23±1	0.011±0.000	34±1	0.012±0.001	27±1	0.010±0.000	27±1	0.005±0.000	29±0	0.007±0.000	31±1
patB	0.021±0.000	21±1	0.027±0.000	23±1	0.018±0.000	23±0	0.012±0.000	28±0	0.013±0.000	29±0	0.002±0.000	31±0	0.004±0.000	29±0

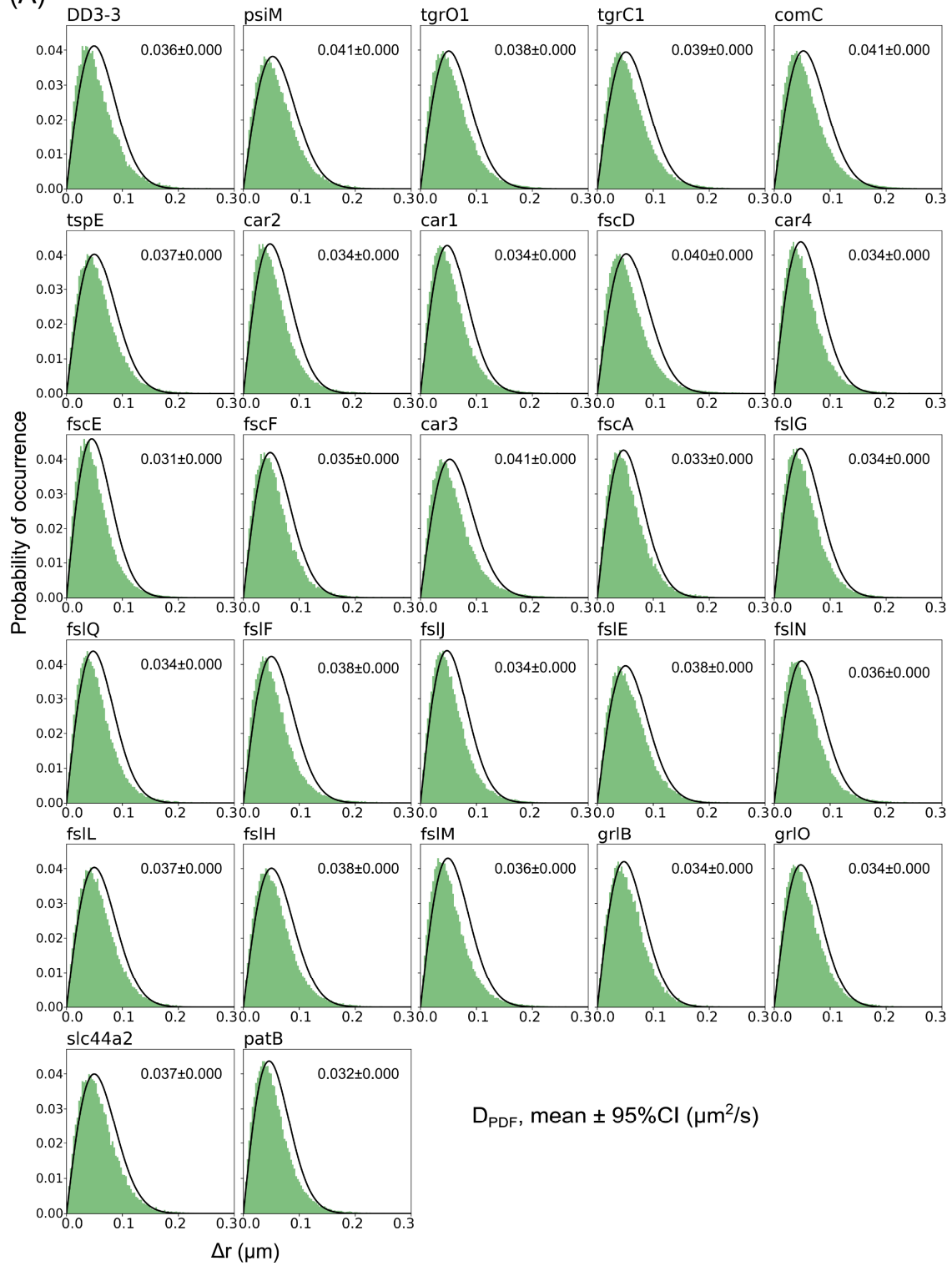
Table 3.2. List of diffusion coefficients determined from the MSD analysis.

For each experimental condition, 10 cells were observed under TIRFM and recorded for 100 frames at a rate of 30 frames/s, from which ~3000 single-molecule tracks with ~80000 displacements at 33ms intervals were obtained for the diffusion analysis. The estimated values are shown as means±95% CI. To calculate CI, the bootstrap method was used (see Methods). ϵ_M is the localization error of diffusing fluorescent spots and determined using Eq. (2).

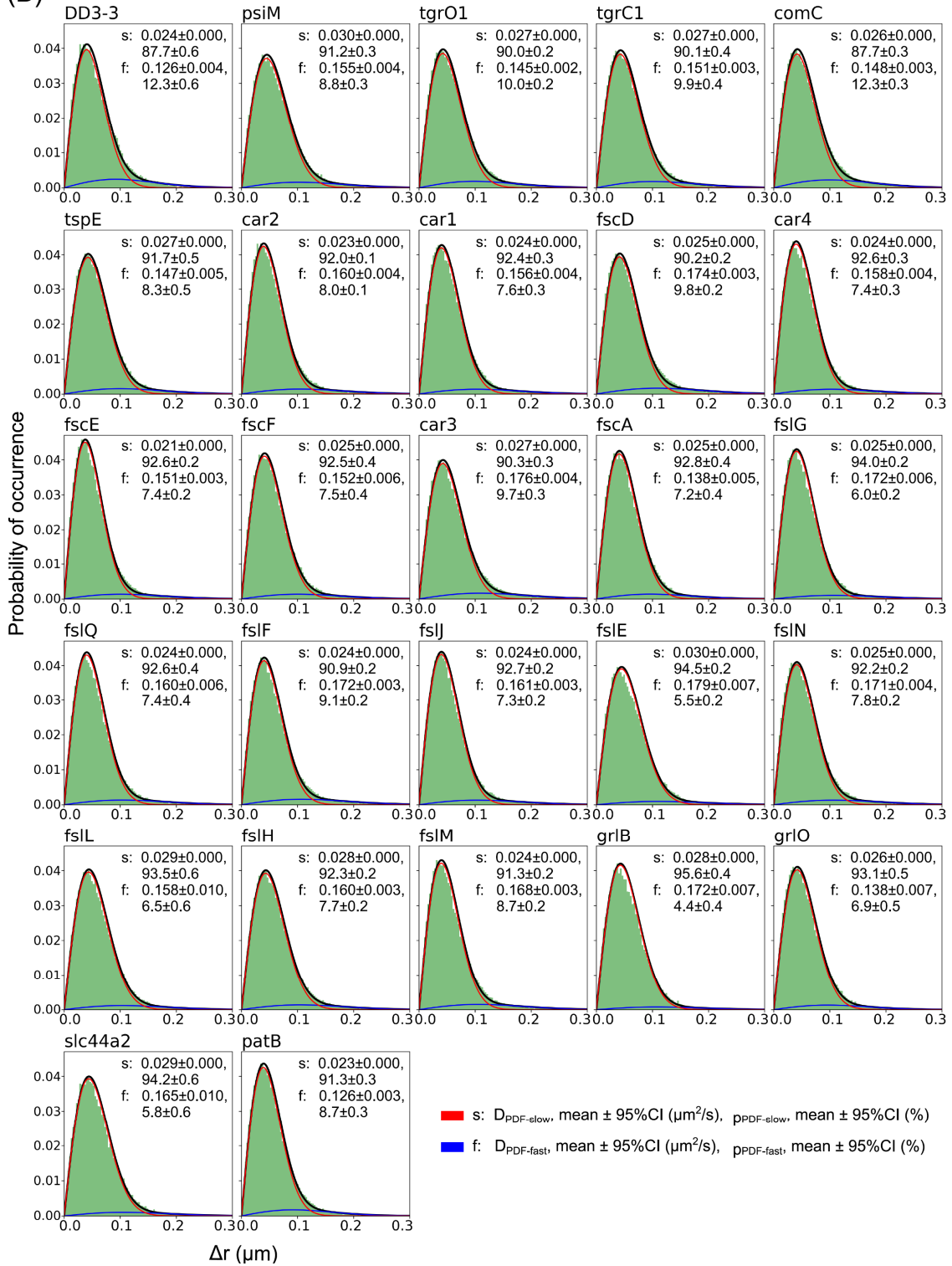
3.1.5 Almost all types of membrane proteins had three diffusion states.

Individual molecules observed under TIRFM exhibited a transition in lateral mobility regardless of any molecular species, as observed previously with transmembrane proteins such as EGFR and GPCRs (Low-Nam et al., 2011; Hiroshima et al., 2018; Yanagawa et al., 2018; Clarke and Martin-Fernandez, 2019). To further characterize the diffusion properties, I analyzed the displacement distribution using a mixed Gaussian function (Matsuoka et al., 2009; Matsuoka et al., 2016). A one-state model of free diffusion was not consistent with the experimental data, and the model selection using the Akaike Information Criterion (AIC) (Akaike, 1974), which can estimate the number of diffusion states, found that three diffusion states were optimal for any protein species (Fig. 3.1.6).

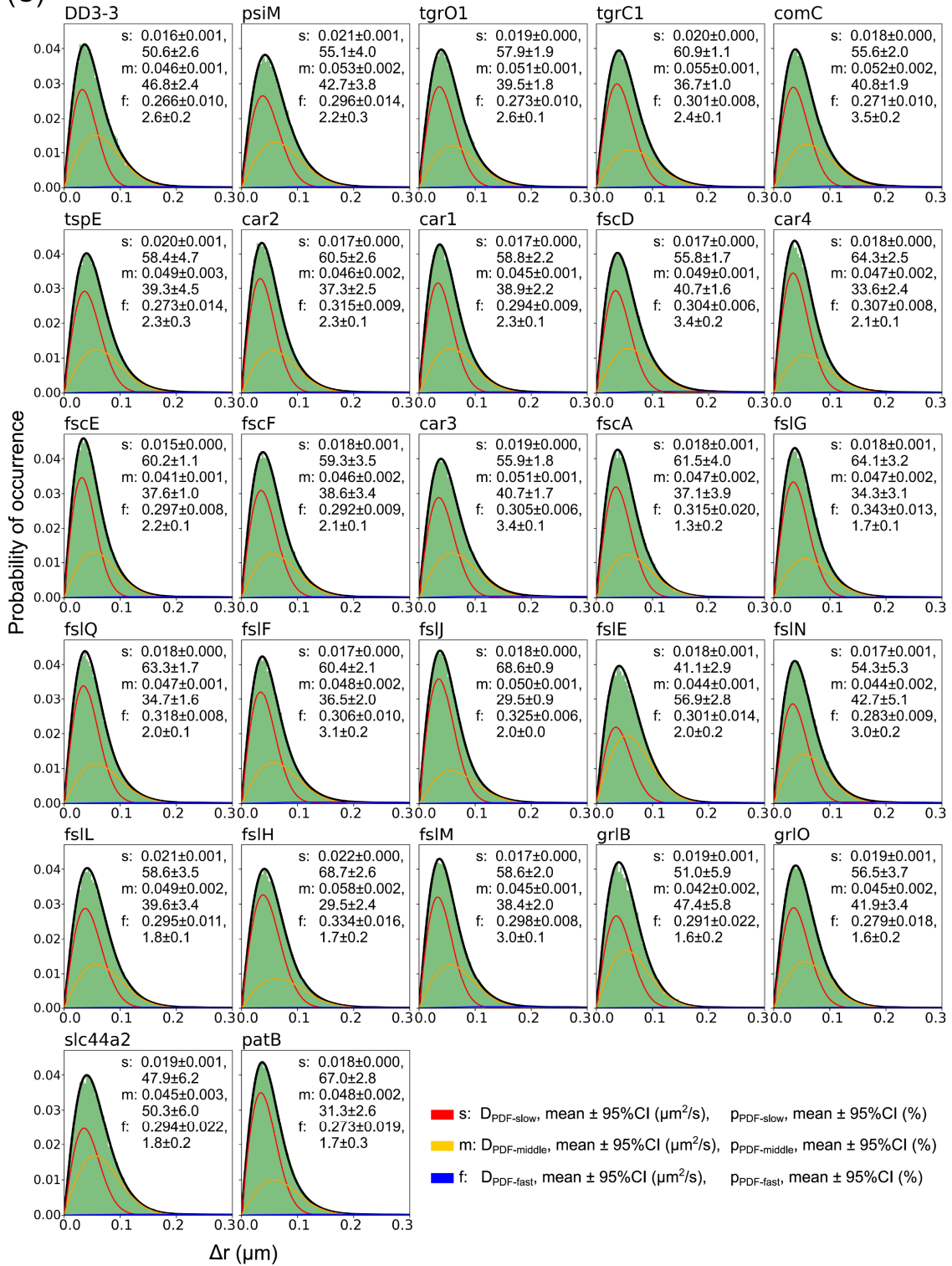
(A)



(B)



(C)



(D)

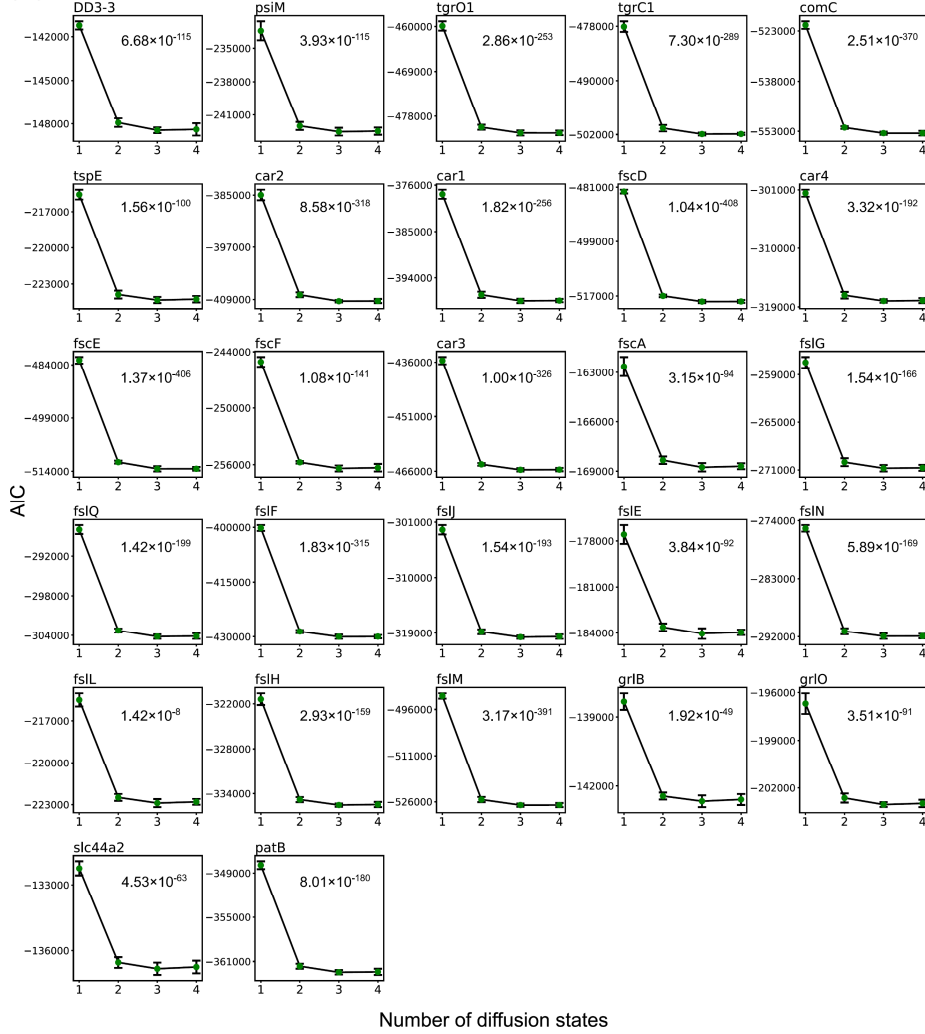


Fig. 3.1.6. Histogram of displacements (Δr) at 33-ms intervals of 27 different transmembrane proteins and AIC values for the 27 transmembrane proteins.

Histograms of the displacements (green) are overlaid with the PDF of the single diffusion state (A), the two diffusion states (B) and the three diffusion states (C) (Eq. (3)) (black). The colored lines indicate the PDFs corresponding to each diffuse state: fast (blue), middle (yellow), slow (red). $D_{PDF-fast}$, $p_{PDF-fast}$, $D_{PDF-middle}$, $p_{PDF-muddle}$, $D_{PDF-slow}$ and $p_{PDF-slow}$ were obtained using the MLE from the Δr datasets. The numbers in each figure are the diffusion coefficient $D_{PDF-state}$ (top) and ratio $p_{PDF-state}$ (bottom) of the fast (f), middle (m) and slow (s) states (mean $\pm$ 95% CI). The bin size for the histograms is 3 nm. (n = 10 bootstrap datasets created from trajectory data of 10 cells) (D) The AIC values (mean $\pm$ 95% CI) were calculated using Eq. (5) for $N = 1$ to 4, where N is the number of diffusion states. The relative likelihood of the three-component model relative to the two-component model, $\exp((AIC_3 - AIC_2)/2)$, was calculated and is shown in each figure.

Gene	State	Ctrl		Jasplakinolide		Latrunculin A		Nocodazole		Thiabendazole		Benomyl		Blebbistatin	
		D _{PDF} (μm ² /s)	P _{PDF} (%)	D _{PDF} (μm ² /s)	P _{PDF} (%)	D _{PDF} (μm ² /s)	P _{PDF} (%)	D _{PDF} (μm ² /s)	P _{PDF} (%)	D _{PDF} (μm ² /s)	P _{PDF} (%)	D _{PDF} (μm ² /s)	P _{PDF} (%)	D _{PDF} (μm ² /s)	P _{PDF} (%)
DD3-3	fast	0.266±0.010	2.6±0.2	0.314±0.011	3.3±0.2	0.379±0.012	4.9±0.2	0.332±0.012	3.6±0.2	0.302±0.008	3.7±0.2	0.373±0.009	5.4±0.2	0.354±0.010	4.2±0.1
	middle	0.046±0.001	46.8±2.4	0.051±0.002	41.5±3.2	0.045±0.003	36.3±3.3	0.045±0.001	41.3±2.5	0.046±0.002	35.7±2.2	0.037±0.001	38.5±2.4	0.041±0.001	37.1±2.2
	slow	0.016±0.001	50.6±2.6	0.019±0.001	55.2±3.4	0.017±0.001	58.7±3.4	0.017±0.000	55.2±2.6	0.015±0.000	60.7±2.3	0.012±0.000	56.1±2.5	0.014±0.000	58.7±2.3
psiM	fast	0.296±0.014	2.2±0.3	0.272±0.011	2.1±0.1	0.296±0.009	3.9±0.1	0.288±0.014	2.4±0.2	0.256±0.007	2.6±0.1	0.246±0.010	2.5±0.1	0.243±0.007	2.7±0.1
	middle	0.053±0.002	42.7±3.8	0.046±0.001	45.1±1.9	0.042±0.001	40.0±1.8	0.040±0.001	45.7±2.7	0.036±0.001	34.0±0.9	0.028±0.001	34.8±0.9	0.029±0.001	30.4±0.7
	slow	0.021±0.001	55.1±4.0	0.017±0.000	52.8±2.0	0.014±0.000	56.1±1.9	0.014±0.001	51.9±2.8	0.011±0.000	63.4±1.0	0.008±0.000	62.7±0.9	0.008±0.000	66.9±0.7
tgrO1	fast	0.273±0.010	2.6±0.1	0.274±0.004	3.7±0.1	0.283±0.005	3.7±0.1	0.274±0.009	2.6±0.2	0.282±0.006	2.8±0.1	0.247±0.006	3.0±0.1	0.237±0.006	3.3±0.1
	middle	0.051±0.001	39.5±1.8	0.051±0.001	39.4±1.1	0.045±0.001	38.1±1.1	0.042±0.001	35.6±1.4	0.038±0.001	34.7±0.9	0.028±0.001	31.8±0.9	0.030±0.001	28.9±0.7
	slow	0.019±0.000	57.9±1.9	0.018±0.000	56.9±1.1	0.014±0.000	58.2±1.2	0.014±0.000	61.7±1.6	0.011±0.000	62.5±0.9	0.008±0.000	65.2±1.0	0.008±0.000	67.8±0.8
tgrC1	fast	0.301±0.008	2.4±0.1	0.280±0.007	3.4±0.1	0.296±0.009	3.6±0.1	0.285±0.012	2.5±0.2	0.260±0.007	3.2±0.1	0.271±0.007	2.7±0.1	0.259±0.005	4.0±0.1
	middle	0.055±0.001	36.7±1.0	0.050±0.000	49.4±0.8	0.049±0.001	36.7±1.1	0.049±0.001	35.3±1.8	0.035±0.001	30.4±0.7	0.026±0.000	30.4±0.8	0.033±0.001	30.7±0.7
	slow	0.020±0.000	60.9±1.1	0.016±0.000	47.2±0.8	0.016±0.000	59.7±1.2	0.017±0.000	62.2±1.9	0.010±0.000	66.4±0.7	0.006±0.000	66.9±0.8	0.008±0.000	65.4±0.8
comC	fast	0.271±0.010	3.5±0.2	0.265±0.008	3.8±0.2	0.280±0.008	4.1±0.1	0.283±0.008	2.6±0.1	0.279±0.007	3.0±0.1	0.253±0.008	3.0±0.1	0.259±0.007	3.1±0.1
	middle	0.052±0.002	40.8±1.9	0.051±0.002	46.1±2.8	0.043±0.001	43.4±1.0	0.044±0.001	36.3±1.4	0.044±0.001	34.9±1.1	0.034±0.001	33.9±1.4	0.034±0.001	30.4±0.8
	slow	0.018±0.000	55.6±2.0	0.018±0.001	50.1±3.0	0.014±0.000	52.5±1.1	0.015±0.000	61.1±1.5	0.014±0.000	62.0±1.1	0.010±0.000	63.1±1.5	0.009±0.000	66.5±0.9
tspE	fast	0.273±0.014	2.3±0.3	0.353±0.015	1.9±0.1	0.289±0.008	3.3±0.1	0.284±0.006	3.5±0.1	0.346±0.012	2.2±0.1	0.309±0.014	1.5±0.1	0.292±0.010	4.1±0.1
	middle	0.049±0.003	39.3±1.5	0.042±0.001	46.7±2.4	0.048±0.001	34.6±1.6	0.048±0.001	34.8±1.2	0.038±0.001	48.8±2.4	0.038±0.001	42.1±2.4	0.039±0.001	36.8±0.8
	slow	0.020±0.001	58.4±4.7	0.014±0.001	51.4±2.4	0.018±0.000	62.1±1.6	0.016±0.000	61.7±1.3	0.013±0.000	49.0±2.5	0.013±0.000	56.4±2.4	0.011±0.000	59.1±0.9
car2	fast	0.315±0.009	2.3±0.1	0.293±0.007	5.0±0.3	0.291±0.007	4.4±0.1	0.306±0.007	2.5±0.1	0.276±0.011	3.4±0.2	0.301±0.007	3.7±0.1	0.282±0.005	4.3±0.1
	middle	0.046±0.002	37.3±2.5	0.053±0.001	41.8±1.5	0.045±0.001	41.4±1.7	0.039±0.001	32.7±1.1	0.041±0.001	34.5±1.4	0.038±0.001	35.1±1.0	0.037±0.001	36.0±1.3
	slow	0.017±0.000	60.5±2.6	0.017±0.000	53.2±1.7	0.015±0.000	54.2±1.8	0.012±0.000	64.8±1.2	0.012±0.000	62.1±1.6	0.010±0.000	61.2±1.0	0.010±0.000	59.6±1.4
car1	fast	0.294±0.009	2.3±0.1	0.358±0.005	5.8±0.1	0.331±0.008	3.4±0.1	0.272±0.008	2.5±0.1	0.317±0.005	4.7±0.1	0.300±0.010	4.2±0.2	0.326±0.006	5.7±0.2
	middle	0.045±0.001	38.9±2.2	0.051±0.001	42.1±1.1	0.043±0.002	38.5±2.5	0.036±0.001	31.7±1.0	0.044±0.001	36.4±0.7	0.036±0.002	37.4±2.1	0.042±0.001	36.1±1.2
	slow	0.017±0.000	58.8±2.2	0.017±0.000	52.1±1.1	0.014±0.000	58.1±2.6	0.011±0.000	65.8±1.1	0.013±0.000	58.9±0.8	0.010±0.000	58.4±2.3	0.011±0.000	58.1±1.3
fscD	fast	0.304±0.006	3.4±0.2	0.291±0.009	3.9±0.2	0.309±0.006	4.0±0.1	0.284±0.009	3.6±0.1	0.292±0.012	2.6±0.1	0.304±0.009	3.4±0.1	0.297±0.009	3.1±0.1
	middle	0.049±0.001	40.7±1.6	0.050±0.002	48.5±2.4	0.043±0.001	40.1±1.7	0.037±0.001	36.3±0.8	0.041±0.001	33.2±1.4	0.037±0.001	29.5±0.8	0.037±0.001	34.0±1.8
	slow	0.017±0.000	55.8±1.7	0.017±0.001	47.6±2.5	0.015±0.000	55.9±1.7	0.012±0.000	60.1±0.9	0.013±0.000	64.2±1.5	0.009±0.000	67.0±0.8	0.011±0.000	62.9±1.9
car4	fast	0.307±0.008	2.1±0.1	0.273±0.010	3.6±0.2	0.293±0.007	3.1±0.2	0.281±0.009	3.0±0.2	0.299±0.008	3.7±0.2	0.273±0.010	3.2±0.2	0.306±0.008	3.2±0.1
	middle	0.047±0.002	33.6±2.4	0.040±0.001	51.5±1.7	0.043±0.002	38.5±2.5	0.038±0.002	35.7±3.0	0.040±0.001	35.7±0.9	0.031±0.001	38.5±2.4	0.034±0.001	32.4±1.2
	slow	0.018±0.000	64.3±2.5	0.013±0.000	44.9±1.9	0.016±0.000	58.4±2.6	0.013±0.000	61.3±3.1	0.012±0.000	60.6±1.0	0.009±0.000	58.3±2.5	0.010±0.000	64.4±1.3
fscE	fast	0.297±0.008	2.2±0.1	0.314±0.008	3.0±0.1	0.307±0.006	2.6±0.1	0.275±0.007	2.5±0.1	0.312±0.009	2.9±0.2	0.301±0.009	3.1±0.1	0.292±0.009	3.9±0.2
	middle	0.041±0.001	37.6±1.0	0.048±0.002	47.2±3.1	0.046±0.001	31.2±2.0	0.033±0.001	33.7±1.9	0.047±0.002	30.1±1.8	0.041±0.001	31.6±1.1	0.039±0.001	40.6±1.6
	slow	0.015±0.000	60.2±1.1	0.019±0.001	49.8±3.2	0.016±0.000	66.2±2.0	0.012±0.000	63.8±1.9	0.015±0.000	67.0±1.9	0.013±0.000	65.3±1.1	0.013±0.000	55.6±1.7
fscF	fast	0.292±0.009	2.1±0.1	0.281±0.007	4.0±0.2	0.297±0.012	3.0±0.2	0.293±0.007	2.6±0.1	0.299±0.015	2.7±0.2	0.283±0.013	3.7±0.2	0.305±0.006	4.1±0.2
	middle	0.046±0.002	38.6±3.4	0.054±0.002	44.3±2.0	0.049±0.002	32.6±2.3	0.039±0.001	35.2±1.7	0.040±0.002	40.1±2.6	0.038±0.001	31.7±1.6	0.043±0.001	41.2±1.4
	slow	0.018±0.001	59.3±3.5	0.020±0.000	51.7±2.2	0.018±0.000	64.4±2.5	0.013±0.000	62.1±1.7	0.013±0.000	57.3±2.7	0.011±0.000	64.7±1.8	0.013±0.000	54.7±1.5
car3	fast	0.305±0.006	3.4±0.1	0.318±0.012	3.1±0.2	0.279±0.004	3.7±0.1	0.291±0.008	4.0±0.2	0.291±0.010	4.7±0.2	0.295±0.006	4.4±0.2	0.272±0.014	4.0±0.3
	middle	0.051±0.001	40.7±1.7	0.051±0.002	37.5±2.7	0.047±0.001	38.8±1.2	0.043±0.001	33.7±1.7	0.044±0.001	35.4±1.2	0.034±0.001	33.8±1.4	0.038±0.002	31.4±1.2
	slow	0.019±0.000	55.9±1.8	0.017±0.001	59.5±2.9	0.016±0.000	57.6±1.3	0.014±0.000	62.3±1.9	0.013±0.000	59.9±1.4	0.009±0.000	61.8±1.6	0.010±0.000	64.6±1.4
fscA	fast	0.315±0.020	1.3±0.2	0.280±0.018	2.8±0.3	0.336±0.011	3.0±0.1	0.270±0.011	2.4±0.2	0.281±0.008	4.1±0.2	0.296±0.013	2.9±0.2	0.375±0.004	6.0±0.1
	middle	0.047±0.002	37.1±3.9	0.045±0.003	32.9±3.2	0.044±0.002	37.0±2.6	0.036±0.001	42.2±1.6	0.042±0.001	40.5±1.6	0.041±0.002	36.2±1.8	0.048±0.002	31.3±1.3
	slow	0.018±0.001	61.5±4.0	0.015±0.001	64.3±3.5	0.016±0.000	59.9±2.7	0.012±0.000	55.4±1.8	0.013±0.000	55.4±1.7	0.012±0.000	60.9±1.9	0.013±0.000	62.7±1.4

Table 3.3. (1/2) List of ratios and diffusion coefficients for each diffusion state determined by PDF analysis

Gene	State	Ctrl		Jasplakinolide		Latrunculin A		Nocodazole		Thiabendazole		Benomyl		Blebbistatin	
		$D_{PDF} (\mu m^2/s)$	$P_{PDF} (\%)$	$D_{PDF} (\mu m^2/s)$	$P_{PDF} (\%)$	$D_{PDF} (\mu m^2/s)$	$P_{PDF} (\%)$	$D_{PDF} (\mu m^2/s)$	$P_{PDF} (\%)$	$D_{PDF} (\mu m^2/s)$	$P_{PDF} (\%)$	$D_{PDF} (\mu m^2/s)$	$P_{PDF} (\%)$	$D_{PDF} (\mu m^2/s)$	$P_{PDF} (\%)$
fslG	fast	0.343±0.013	1.7±0.1	0.315±0.008	3.4±0.1	0.298±0.007	3.7±0.1	0.301±0.008	3.2±0.2	0.268±0.009	3.0±0.1	0.275±0.009	3.3±0.2	0.297±0.009	3.7±0.1
	middle	0.047±0.002	34.3±3.1	0.052±0.001	43.6±1.9	0.046±0.001	41.8±2.2	0.040±0.001	34.4±1.7	0.044±0.001	35.3±1.6	0.030±0.001	35.1±1.0	0.039±0.001	35.2±1.0
	slow	0.018±0.001	64.1±3.2	0.019±0.000	53.0±2.0	0.018±0.000	54.5±2.3	0.013±0.000	62.4±1.9	0.015±0.000	61.7±1.6	0.008±0.000	61.6±1.1	0.012±0.000	61.1±1.1
fslQ	fast	0.318±0.008	2.0±0.1	0.279±0.007	3.5±0.2	0.341±0.011	2.9±0.1	0.265±0.011	2.8±0.2	0.332±0.008	4.6±0.2	0.284±0.006	2.8±0.1	0.345±0.006	6.4±0.1
	middle	0.047±0.001	34.7±1.6	0.043±0.002	45.6±3.5	0.046±0.001	34.7±1.8	0.040±0.001	33.4±1.2	0.045±0.001	30.0±1.3	0.035±0.001	30.4±1.0	0.057±0.001	35.2±0.7
	slow	0.018±0.000	63.3±1.7	0.015±0.001	50.9±3.6	0.015±0.000	62.5±1.8	0.013±0.000	63.8±1.3	0.013±0.000	65.4±1.3	0.010±0.000	66.9±1.0	0.015±0.000	58.4±0.8
fslF	fast	0.306±0.010	3.1±0.2	0.286±0.011	4.1±0.4	0.304±0.009	5.5±0.2	0.318±0.011	4.0±0.2	0.386±0.013	6.6±0.2	0.337±0.010	4.3±0.2	0.307±0.009	4.4±0.2
	middle	0.048±0.002	36.5±2.0	0.050±0.003	40.3±3.8	0.051±0.001	41.8±1.8	0.044±0.002	34.8±2.6	0.046±0.001	34.3±1.2	0.045±0.001	30.4±1.1	0.046±0.001	35.9±1.4
	slow	0.017±0.000	60.4±2.1	0.017±0.001	55.6±4.1	0.018±0.000	52.7±1.9	0.014±0.000	61.2±2.8	0.012±0.000	59.1±1.3	0.012±0.000	65.3±1.2	0.014±0.000	59.7±1.4
fslJ	fast	0.325±0.006	2.0±0.0	0.297±0.013	2.6±0.2	0.293±0.009	2.0±0.2	0.290±0.012	2.5±0.1	0.294±0.007	4.1±0.1	0.320±0.010	1.9±0.1	0.325±0.007	5.3±0.2
	middle	0.050±0.001	29.5±0.9	0.047±0.002	39.6±2.3	0.049±0.001	28.7±1.0	0.038±0.001	34.8±1.8	0.040±0.001	40.6±1.2	0.038±0.002	27.5±1.5	0.051±0.001	37.2±1.2
	slow	0.018±0.000	68.6±0.9	0.017±0.000	57.8±2.4	0.016±0.000	68.6±1.1	0.012±0.000	62.6±1.9	0.013±0.000	55.3±1.2	0.011±0.000	70.6±1.5	0.016±0.000	57.5±1.3
fslE	fast	0.301±0.014	2.0±0.2	0.316±0.016	2.1±0.1	0.295±0.013	2.0±0.2	0.374±0.014	4.1±0.3	0.299±0.009	3.5±0.1	0.344±0.014	2.5±0.1	0.290±0.010	4.1±0.3
	middle	0.044±0.001	56.9±2.8	0.052±0.001	36.7±1.8	0.045±0.002	33.5±3.4	0.037±0.002	48.0±3.2	0.042±0.001	31.4±1.8	0.035±0.001	34.7±2.8	0.040±0.002	33.2±2.2
	slow	0.018±0.001	41.1±2.9	0.021±0.000	61.2±1.8	0.018±0.001	64.4±3.5	0.014±0.001	48.0±3.5	0.013±0.000	65.1±1.9	0.011±0.000	62.8±2.8	0.012±0.000	62.8±2.4
fslN	fast	0.283±0.009	3.0±0.2	0.301±0.011	3.5±0.2	0.304±0.009	3.6±0.2	0.310±0.011	2.6±0.1	0.291±0.018	3.5±0.3	0.303±0.008	2.6±0.1	0.324±0.009	3.9±0.2
	middle	0.044±0.002	42.7±5.1	0.049±0.001	39.4±1.7	0.042±0.001	40.9±1.9	0.038±0.001	35.6±1.3	0.044±0.002	37.4±2.6	0.034±0.001	31.4±1.2	0.046±0.002	35.3±2.3
	slow	0.017±0.001	54.3±5.3	0.017±0.000	57.1±1.9	0.014±0.000	55.5±2.1	0.011±0.000	61.8±1.4	0.015±0.000	59.2±2.6	0.010±0.000	66.0±1.3	0.015±0.000	60.7±2.4
fslL	fast	0.295±0.011	1.8±0.1	0.313±0.018	2.5±0.3	0.275±0.008	3.4±0.2	0.308±0.012	3.6±0.2	0.277±0.012	3.3±0.3	0.303±0.018	3.3±0.3	0.288±0.010	5.0±0.3
	middle	0.049±0.002	39.6±3.4	0.051±0.003	38.0±4.1	0.041±0.001	32.7±1.2	0.041±0.002	37.0±2.7	0.040±0.001	35.9±1.5	0.039±0.002	35.5±2.7	0.042±0.001	36.3±1.1
	slow	0.021±0.001	58.6±3.5	0.020±0.001	59.5±4.4	0.013±0.000	63.9±1.3	0.013±0.000	59.4±2.9	0.013±0.000	60.7±1.6	0.011±0.000	61.2±3.0	0.012±0.000	58.7±1.2
fslH	fast	0.334±0.016	1.7±0.2	0.280±0.008	2.9±0.1	0.332±0.008	2.4±0.1	0.278±0.013	3.0±0.2	0.273±0.008	2.7±0.1	0.283±0.008	3.2±0.1	0.265±0.007	3.4±0.1
	middle	0.058±0.002	29.5±2.4	0.043±0.001	37.1±1.4	0.049±0.002	29.1±1.6	0.041±0.001	31.9±1.6	0.037±0.001	32.2±1.5	0.032±0.001	31.0±1.3	0.034±0.001	32.2±1.2
	slow	0.022±0.000	68.7±2.6	0.015±0.000	60.0±1.5	0.017±0.000	68.5±1.6	0.014±0.000	65.1±1.7	0.012±0.000	65.1±1.6	0.009±0.000	65.8±1.4	0.009±0.000	64.4±1.3
fslM	fast	0.298±0.008	3.0±0.1	0.295±0.009	3.7±0.2	0.298±0.013	3.1±0.2	0.274±0.006	2.7±0.1	0.309±0.006	4.0±0.1	0.308±0.007	3.3±0.1	0.309±0.005	4.1±0.1
	middle	0.045±0.001	38.4±2.0	0.049±0.001	43.6±1.4	0.047±0.002	31.6±1.5	0.034±0.001	29.1±0.6	0.049±0.001	36.0±1.3	0.039±0.001	28.1±1.1	0.045±0.001	35.1±1.6
	slow	0.017±0.000	58.6±2.0	0.017±0.000	52.7±1.5	0.016±0.000	65.3±1.6	0.011±0.000	68.2±0.7	0.016±0.000	60.0±1.4	0.011±0.000	68.6±1.1	0.015±0.000	60.8±1.6
grlB	fast	0.291±0.022	1.6±0.2	0.460±0.024	1.4±0.1	0.369±0.026	3.0±0.4	0.430±0.010	2.1±0.1	0.338±0.030	2.2±0.3	0.337±0.029	1.8±0.2	0.384±0.013	3.6±0.1
	middle	0.042±0.002	47.4±5.8	0.043±0.002	31.5±5.0	0.043±0.003	26.9±5.3	0.043±0.002	34.6±4.3	0.042±0.006	19.9±6.7	0.039±0.002	21.9±1.5	0.039±0.002	34.2±4.4
	slow	0.019±0.001	51.0±5.9	0.020±0.001	67.1±5.0	0.016±0.001	70.1±5.6	0.020±0.001	63.3±4.3	0.016±0.001	78.0±6.9	0.012±0.000	76.3±1.6	0.016±0.001	62.2±4.5
grlO	fast	0.279±0.018	1.6±0.2	0.325±0.019	1.9±0.2	0.359±0.016	2.7±0.1	0.308±0.017	2.0±0.2	0.314±0.016	2.0±0.1	0.285±0.020	2.0±0.2	0.311±0.011	2.7±0.1
	middle	0.045±0.002	41.9±3.4	0.040±0.002	38.6±3.6	0.039±0.001	41.6±3.3	0.035±0.002	53.2±5.2	0.034±0.002	34.7±3.4	0.040±0.001	24.8±1.1	0.042±0.002	26.8±2.1
	slow	0.019±0.001	56.5±3.7	0.016±0.001	59.5±3.7	0.015±0.000	55.7±3.3	0.015±0.001	44.9±5.3	0.013±0.000	63.3±3.4	0.013±0.000	73.2±1.2	0.013±0.000	70.4±2.1
slc44a2	fast	0.294±0.022	1.8±0.2	0.300±0.022	1.5±0.1	0.307±0.014	3.8±0.3	0.252±0.016	2.5±0.2	0.341±0.016	1.7±0.1	0.310±0.016	2.5±0.2	0.278±0.011	3.3±0.2
	middle	0.045±0.003	50.3±6.0	0.048±0.003	43.3±4.1	0.049±0.002	36.3±2.4	0.041±0.001	41.3±2.4	0.037±0.001	41.0±2.7	0.040±0.002	27.3±2.3	0.040±0.001	34.1±2.2
	slow	0.019±0.001	47.9±6.2	0.020±0.001	55.2±4.3	0.016±0.001	59.9±2.6	0.015±0.000	56.2±2.5	0.013±0.001	57.2±2.8	0.012±0.000	70.1±2.5	0.013±0.000	62.6±2.3
patB	fast	0.273±0.019	1.7±0.3	0.284±0.007	3.0±0.2	0.261±0.010	2.2±0.1	0.263±0.014	3.5±0.2	0.293±0.011	3.4±0.2	0.253±0.007	4.1±0.1	0.278±0.012	3.0±0.3
	middle	0.048±0.002	31.3±2.6	0.050±0.001	40.7±1.7	0.043±0.001	28.8±1.7	0.040±0.001	33.3±1.4	0.043±0.001	34.2±0.8	0.037±0.001	28.9±0.9	0.038±0.002	28.5±1.5
	slow	0.018±0.000	67.0±2.8	0.018±0.000	56.3±1.8	0.016±0.000	68.9±1.8	0.013±0.000	63.2±1.5	0.013±0.000	62.5±0.9	0.010±0.000	67.1±0.9	0.011±0.000	68.5±1.7

Table 3.3. (2/2) List of ratios and diffusion coefficients for each diffusion state determined by PDF analysis.

3.1.6 All transmembrane proteins undergo multistate free diffusion with similar diffusion coefficients.

Then I analyzed the single molecule trajectories by classifying them into three states (fast, middle, and slow) using a hidden Markov model (HMM), as reported previously (Hiroshima et al., 2018; Yanagawa et al., 2018). This analysis can assign three states along the trajectories, from which the transition probability and the proportion of each state can be determined (Fig. 3.1.7). By calculating the MSDs from the displacement for each state, I examined the diffusion modes for each state of all molecular species in the presence and absence of the inhibitors. I found that all membrane proteins observed exhibited simple free diffusion in each state under control and inhibitor-treated conditions (Fig. 3.1.8), which shows a marked difference from the multi-modal mobility observed in mammalian cells (Hiroshima et al., 2018; Yanagawa et al., 2018). Reduced mobility was observed in the middle- and slow-mobile states with any inhibitor except for jasplakinolide, and was also the case in the fast state (Fig. 3.1.9A, Table 3.4). The middle- and slow-mobile states ranged from 42.8 - 69.4% and 24.6 - 54.7% for various protein species, respectively, while the fast-mobile state (2.1 - 7.2%) was a minor subpopulation in control cells. The ratio of each state was relatively maintained with any inhibitor (Fig. 3.1.9B, Table 3.4). Thus, three diffusion states were a common feature for all protein species. This observation can be explained by simple diffusion on a heterogeneous membrane structure shared among transmembrane proteins and the drug-dependent modulation.

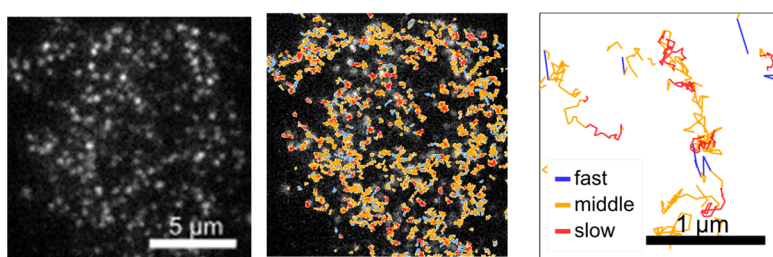


Fig. 3.1.7. State estimation using hidden Markov models.

Representative single molecule images of the membrane protein car4 (left). Overlaid trajectories categorized into three different states, fast (blue), middle (yellow), slow (red), by HMM (middle). Scale bar, 5 μm. An enlarged view of the superimposed trajectories (right). Scale bar, 1 μm.

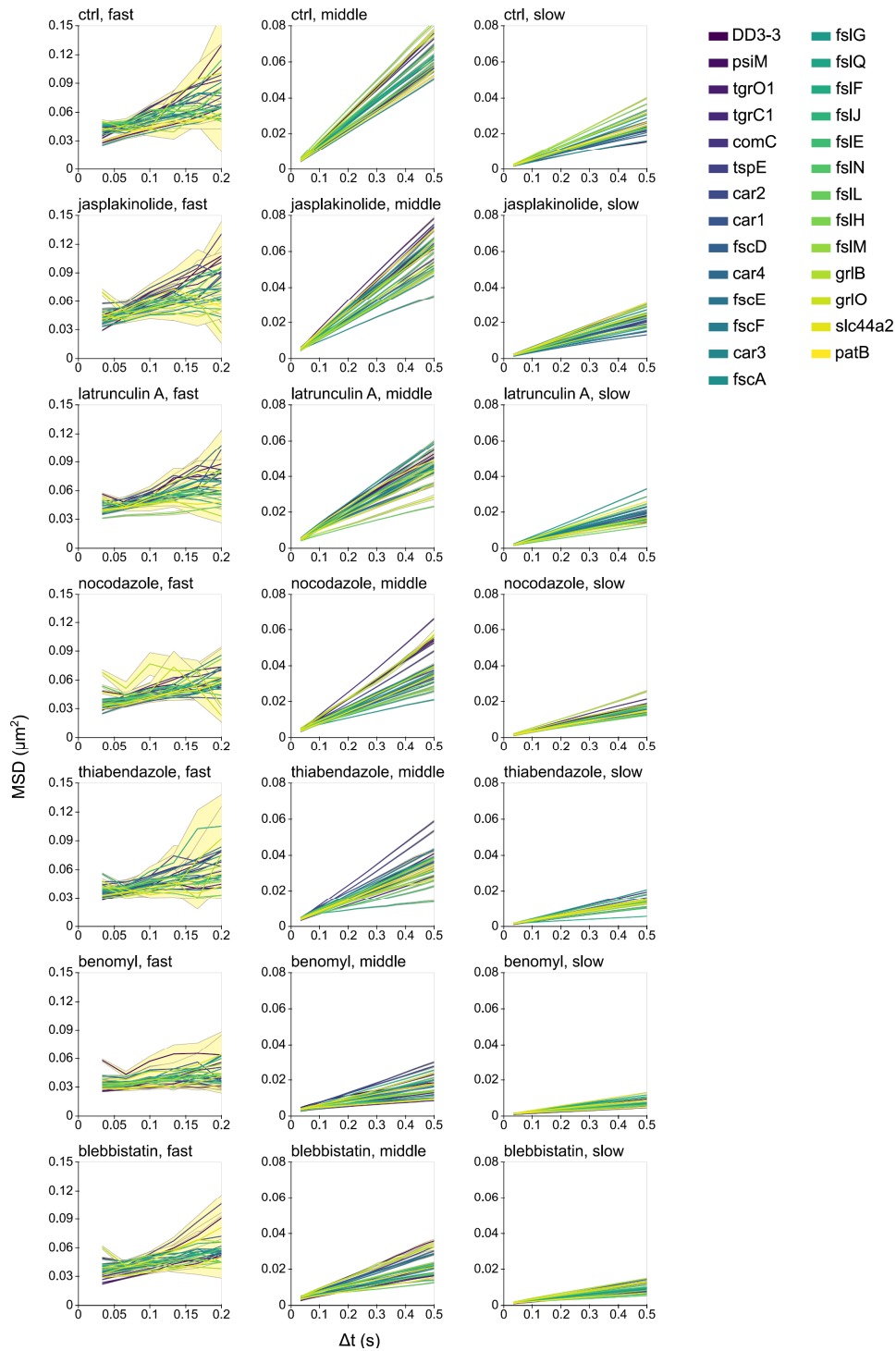


Fig. 3.1.8. MSDs of three diffusion states under drug-treated conditions.

MSD of fast, middle and slow states. The mobilities of the 27 transmembrane proteins were observed under the control and seven drug conditions. Using the HMM, the mobilities were separated into the three states, from which the MSD was calculated. Error bars represent SE. The data of the 27 transmembrane proteins are represented in different colors.

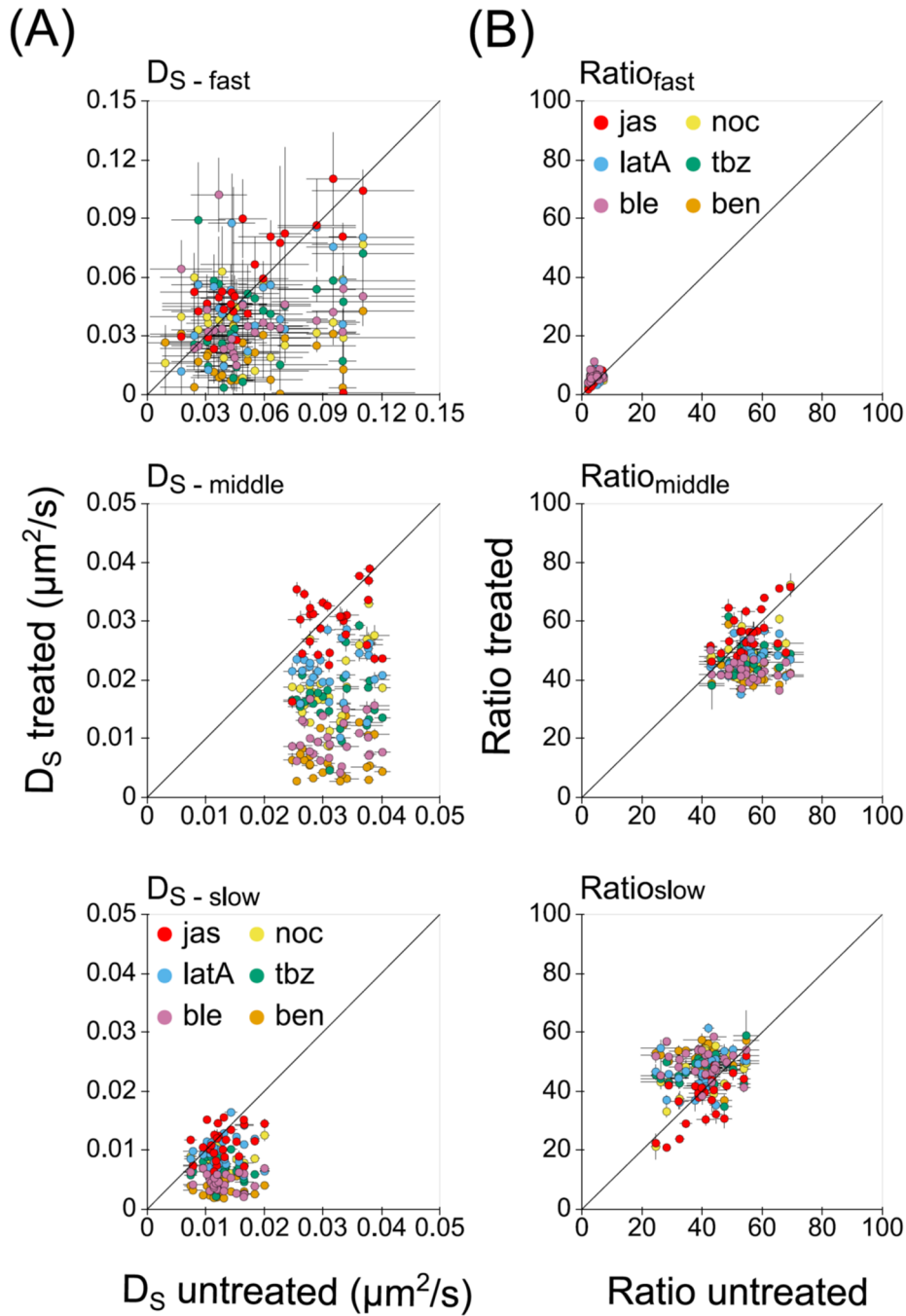


Fig. 3.1.9. Lateral diffusion of membrane proteins is defined by three different states.

(A, B) Scatter plots of the diffusion coefficients (A) and ratios (B) for each state (top, middle, bottom) under drug-treated conditions. Diagonal lines indicate no effect on the diffusion coefficients (D_S , $\text{mean} \pm 95\%$ CI). ($n = 10$ bootstrap datasets created from trajectory data of 10 cells)

		Ctrl			Jasplakinolide			Latrunculin A			Nocodazole			Thiabendazole			Benomyl			Blebbistatin		
Gene	State	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ε_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ε_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ε_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ε_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ε_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ε_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ε_s (nm)
DD3-3	fast	0.049±0.012	7.2±0.6	74±4	0.090±0.020	5.7±0.4	83±7	0.046±0.023	6.2±0.4	103±6	0.009±0.007	4.9±0.3	108±3	0.007±0.008	6.3±0.4	99±3	0.027±0.023	6.2±0.3	108±6	0.046±0.022	5.7±0.3	97±7
	middle	0.028±0.001	60.6±2.7	12±3	0.031±0.001	57.7±2.3	18±3	0.020±0.001	49.5±3.2	29±3	0.027±0.001	50.6±1.6	11±5	0.017±0.001	48.6±1.9	24±2	0.007±0.001	40.1±1.8	36±1	0.017±0.001	46.1±1.8	26±2
	slow	0.007±0.001	32.2±3.2	18±1	0.012±0.001	36.6±2.7	14±1	0.009±0.001	44.4±3.5	17±2	0.009±0.000	44.6±1.8	14±1	0.006±0.000	45.2±2.0	17±1	0.004±0.000	53.7±1.9	17±1	0.006±0.000	48.2±2.1	16±0
psiM	fast	0.111±0.026	5.9±0.4	64±12	0.104±0.011	6.0±0.4	63±3	0.080±0.025	6.5±0.4	81±9	0.077±0.013	6.5±0.6	70±5	0.072±0.012	5.7±0.2	64±5	0.043±0.008	4.8±0.4	69±2	0.050±0.013	6.1±0.3	62±3
	middle	0.038±0.001	69.4±1.3	8±2	0.034±0.001	71.6±1.0	6±2	0.025±0.001	46.9±2.2	23±3	0.027±0.001	72.3±3.9	3±3	0.019±0.000	48.8±1.5	15±2	0.011±0.000	42.3±1.4	21±1	0.010±0.000	42.0±1.9	17±1
	slow	0.013±0.001	24.6±1.5	13±2	0.010±0.000	22.4±1.4	10±2	0.011±0.001	46.6±2.5	9±1	0.006±0.001	21.2±4.4	13±1	0.007±0.000	45.5±1.7	10±1	0.005±0.000	52.9±1.7	11±0	0.004±0.000	51.9±2.1	12±0
tgrO1	fast	0.087±0.020	5.9±0.5	80±6	0.087±0.023	5.6±0.2	92±7	0.085±0.012	5.9±0.4	81±4	0.032±0.009	5.0±0.3	90±3	0.054±0.006	6.0±0.2	78±2	0.025±0.003	5.9±0.3	73±1	0.038±0.005	6.8±0.3	68±2
	middle	0.038±0.001	65.2±2.1	10±3	0.039±0.001	52.4±1.0	17±3	0.026±0.001	48.3±1.0	27±2	0.027±0.001	51.9±1.3	9±3	0.020±0.001	51.2±1.0	14±2	0.005±0.000	43.9±0.5	23±0	0.007±0.000	41.5±0.9	23±0
	slow	0.012±0.001	28.9±2.6	10±2	0.015±0.000	42.0±1.1	8±1	0.010±0.000	45.8±1.2	14±0	0.009±0.000	43.1±1.6	12±2	0.006±0.000	42.8±1.2	12±0	0.003±0.000	50.2±0.7	12±0	0.003±0.000	51.7±1.1	12±0
tgrC1	fast	0.100±0.007	6.1±0.3	76±4	0.081±0.007	8.0±0.3	85±4	0.036±0.008	7.3±0.5	92±4	0.059±0.007	6.2±0.5	84±4	0.017±0.007	5.9±0.1	87±6	0.004±0.006	4.9±0.2	84±3	0.032±0.007	6.7±0.2	83±4
	middle	0.038±0.001	65.7±1.2	9±2	0.037±0.001	71.1±0.7	11±2	0.024±0.000	55.7±1.2	26±1	0.033±0.001	60.7±1.6	13±3	0.013±0.001	44.3±1.1	20±2	0.003±0.000	38.3±1.1	26±1	0.007±0.001	36.4±0.8	28±1
	slow	0.012±0.001	28.2±1.3	11±3	0.007±0.001	20.9±0.9	15±1	0.010±0.001	37.0±1.6	12±1	0.011±0.001	33.1±2.0	11±2	0.005±0.000	49.9±1.2	12±1	0.002±0.000	56.8±1.2	12±0	0.003±0.000	56.9±0.9	13±0
comC	fast	0.095±0.014	6.8±0.3	81±6	0.110±0.024	8.3±0.4	76±11	0.076±0.010	7.1±0.5	82±2	0.037±0.006	5.8±0.5	88±3	0.058±0.014	5.5±0.2	86±5	0.031±0.005	5.7±0.4	80±3	0.042±0.006	6.3±0.3	73±2
	middle	0.036±0.001	60.7±1.4	12±3	0.038±0.001	67.9±1.2	7±2	0.025±0.001	56.7±1.8	23±3	0.026±0.001	56.8±2.2	15±2	0.029±0.001	51.8±1.6	5±3	0.013±0.000	45.8±1.1	23±1	0.011±0.000	42.9±1.0	23±1
	slow	0.011±0.001	32.5±1.5	13±1	0.010±0.001	23.8±1.4	14±1	0.007±0.001	36.2±2.3	17±0	0.010±0.001	37.4±2.6	10±2	0.010±0.000	42.7±1.8	11±1	0.006±0.000	48.5±1.3	12±1	0.005±0.000	50.8±1.2	12±0
tspE	fast	0.037±0.014	3.8±0.3	97±4	0.050±0.011	4.8±0.5	87±6	0.044±0.015	5.2±0.5	92±6	0.038±0.003	7.0±0.3	86±3	0.057±0.024	3.8±0.4	93±7	0.009±0.011	4.2±0.7	88±7	0.102±0.019	7.4±0.6	62±7
	middle	0.034±0.002	48.8±2.3	15±4	0.028±0.001	64.5±2.9	12±3	0.021±0.001	41.3±1.9	28±2	0.023±0.001	50.4±1.2	15±2	0.026±0.002	61.5±2.7	2±3	0.014±0.001	58.9±1.5	22±1	0.015±0.001	41.6±1.7	23±2
	slow	0.017±0.001	47.4±2.5	4±2	0.007±0.001	30.7±3.2	15±1	0.011±0.000	53.6±2.3	15±1	0.007±0.000	42.6±1.5	16±0	0.007±0.001	34.8±3.0	14±1	0.003±0.000	36.9±2.0	18±1	0.006±0.000	51.0±2.3	14±1
car2	fast	0.063±0.016	4.4±0.4	87±6	0.081±0.008	7.7±0.3	91±3	0.056±0.020	6.1±0.1	98±6	0.019±0.007	4.3±0.2	96±3	0.041±0.008	7.1±0.3	80±3	0.008±0.005	7.0±0.4	89±2	0.035±0.010	7.0±0.3	86±2
	middle	0.028±0.001	54.6±3.1	17±4	0.031±0.001	52.7±0.6	20±1	0.021±0.001	50.3±1.0	33±2	0.018±0.000	42.9±1.4	24±1	0.016±0.001	52.4±1.0	19±1	0.003±0.000	44.7±1.2	31±1	0.007±0.000	43.4±1.3	29±1
	slow	0.011±0.001	40.9±3.5	13±1	0.010±0.000	39.6±0.8	16±1	0.009±0.000	43.6±1.0	14±1	0.008±0.000	52.8±1.6	11±1	0.005±0.000	40.5±1.2	14±1	0.002±0.000	48.3±1.5	16±0	0.003±0.000	49.5±1.5	16±0
car1	fast	0.071±0.013	4.0±0.3	88±5	0.082±0.044	7.1±0.3	103±13	0.034±0.017	5.2±0.3	98±6	0.025±0.003	5.4±0.3	82±2	0.045±0.008	6.4±0.2	97±3	0.029±0.012	5.9±0.4	94±5	0.046±0.011	8.1±0.2	93±4
	middle	0.034±0.001	52.8±2.5	11±5	0.030±0.001	49.1±1.1	30±4	0.016±0.002	49.1±1.3	33±3	0.016±0.000	49.6±1.0	17±1	0.020±0.001	46.2±1.3	27±2	0.012±0.001	45.3±1.5	26±1	0.013±0.001	45.5±1.1	30±2
	slow	0.013±0.001	43.3±2.7	10±2	0.012±0.001	43.8±1.3	14±1	0.008±0.000	45.7±1.4	16±1	0.007±0.000	45.0±1.1	11±1	0.007±0.000	47.4±1.4	16±1	0.005±0.000	48.7±1.8	14±1	0.006±0.000	46.4±1.2	13±1
fscD	fast	0.052±0.016	5.7±0.2	96±5	0.041±0.010	7.1±0.3	96±4	0.022±0.013	5.3±0.3	107±3	0.021±0.007	5.7±0.3	90±6	0.052±0.014	5.3±0.3	81±5	0.018±0.010	6.1±0.2	88±3	0.035±0.009	5.1±0.2	89±3
	middle	0.030±0.001	59.7±1.8	20±1	0.033±0.001	64.0±1.1	6±3	0.020±0.001	47.9±1.6	30±1	0.017±0.000	46.0±2.0	22±1	0.017±0.001	48.6±1.6	23±2	0.004±0.000	40.1±1.1	29±1	0.014±0.001	42.1±1.3	24±2
	slow	0.010±0.001	34.6±2.0	15±1	0.011±0.001	29.0±1.2	12±3	0.009±0.000	46.8±1.8	14±1	0.007±0.000	48.3±2.3	14±1	0.008±0.000	46.2±1.9	10±1	0.002±0.000	53.8±1.1	15±0	0.007±0.000	52.8±1.4	12±1
car4	fast	0.043±0.012	4.2±0.2	90±3	0.052±0.009	6.3±0.3	86±3	0.088±0.025	5.7±0.3	72±10	0.031±0.013	4.4±0.3	91±4	0.031±0.007	5.8±0.2	93±7	0.008±0.005	6.2±0.6	84±4	0.029±0.006	5.8±0.3	87±3
	middle	0.028±0.001	54.6±2.9	15±2	0.027±0.001	63.3±1.7	13±2	0.022±0.001	52.7±1.9	17±2	0.019±0.001	41.2±2.0	24±4	0.017±0.001	45.3±1.1	25±2	0.006±0.000	51.0±3.4	25±2	0.008±0.000	41.9±1.3	25±1
	slow	0.011±0.001	41.2±3.1	12±1	0.006±0.001	30.4±2.0	15±1	0.010±0.000	41.6±2.2	13±1	0.008±0.000	54.4±2.2	14±1	0.007±0.000	48.9±1.2	13±1	0.002±0.000	42.8±3.9	16±0	0.004±0.000	52.3±1.5	14±0
fscE	fast	0.034±0.010	3.9±0.2	92±4	0.024±0.008	3.9±0.2	111±2	0.055±0.016	4.3±0.2	89±4	0.024±0.008	3.9±0.2	91±3	0.058±0.008	4.8±0.3	87±3	0.012±0.006	5.3±0.4	92±4	0.033±0.010	5.7±0.2	91±3
	middle	0.027±0.001	51.6±2.2	14±1	0.035±0.001	48.0±1.3	16±3	0.023±0.001	44.9±1.6	26±2	0.016±0.000	40.7±1.2	24±1	0.016±0.001	42.7±1.6	30±2	0.006±0.000	42.9±1.6	31±1	0.013±0.001	45.4±0.8	26±2
	slow	0.010±0.000	44.5±2.4	12±1	0.015±0.001	48.1±1.4	12±2	0.011±0.000	50.8±1.8	11±1	0.009±0.000	55.4±1.2	10±1	0.009±0.000	52.5±1.8	13±1	0.004±0.000	51.8±2.0	16±0	0.006±0.000	48.8±0.9	15±0
fscF	fast	0.038±0.015	3.6±0.3	92±7	0.053±0.007	6.3±0.4	93±3	0.041±0.012	5.7±0.2	86±9	0.063±0.023	7.0±0.7	64±20	0.026±0.008	4.8±0.6	91±4	0.010±0.006	5.0±0.6	91±4	0.034±0.012	7.7±0.4	90±6
	middle	0.027±0.001	56.3±2.5	20±2	0.024±0.001	52.5±1.8	29±2	0.022±0.001	49.1±1.7	24±1	0.015±0.000	52.6±4.3	23±2	0.021±0.001	50.4±2.5	19±3	0.008±0.000	39.9±1.2	27±1	0.013±0.000	53.9±1.1	27±1
	slow	0.011±0.001	40.0±2.6	13±2	0.011±0.001	41.2±2.1	16±1	0.011±0.001	45.2±1.9	12±1	0.007±0.001	40.4±4.9	12±1	0.009±0.001	44.8±2.9	11±2	0.004±0.000	54.1±1.4	16±0	0.005±0.000	38.4±1.4	17±0
car3	fast	0.059±0.013	5.4±0.5	92±5	0.059±0.008	5.8±0.3	92±4	0.055±0.013	5.9±0.2	88±5	0.058±0.008	6.0±0.3	84±2	0.043±0.004	7.9±0.4	87±2	0.031±0.022	7.2±0.4	84±7	0.037±0.011	6.5±0.1	83±4
	middle	0.034±0.002	55.7±3.5	13±3	0.031±0.001	56.3±1.2	13±4	0.029±0.001	51.5±1.1	17±1	0.019±0.000	47.3±1.3	25±1	0.019±0.001	50.3±1.9	22±3	0.003±0.000	43.6±1.7	29±1	0.009±0.000	39.2±0.8	28±1
	slow	0.011±0.001	38.9±0.4	14±2	0.010±0.001	37.9±1.3	15±1	0.010±0.000	42.6±1.3	13±1	0.008±0.000	46.7±1.6	14±1	0.006±0.001	41.9±2.2	15±1	0.002±0.000	49.1±2.0	15±0	0.004±0.000	54.3±0.9	15±0
fscA	fast	0.044±0.009	5.9±1.0	72±7	0.043±0.063	5.4±0.5	84															

Gene	State	Ctrl			Jasplakinolide			Latrunculin A			Nocodazole			Thiabendazole			Benomyl			Blebbistatin		
		D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ϵ_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ϵ_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ϵ_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ϵ_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ϵ_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ϵ_s (nm)	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ϵ_s (nm)
fslG	fast	0.055±0.028	2.8±0.2	95±9	0.067±0.014	5.1±0.2	98±5	0.035±0.009	5.2±0.2	99±3	0.012±0.016	5.1±0.3	96±6	0.049±0.021	5.0±0.3	86±7	0.022±0.007	5.6±0.4	83±5	0.032±0.006	6.3±0.3	88±3
	middle	0.031±0.001	49.0±2.8	19±3	0.033±0.001	53.1±1.6	25±3	0.029±0.001	45.3±2.4	25±3	0.009±0.000	42.3±1.3	31±1	0.018±0.001	45.9±1.2	28±2	0.006±0.000	43.2±1.6	25±1	0.010±0.001	43.9±1.0	28±1
	slow	0.014±0.001	48.2±2.9	5±2	0.014±0.001	41.8±1.7	12±2	0.016±0.000	49.5±2.6	6±2	0.006±0.000	52.7±1.5	14±1	0.010±0.000	49.1±1.2	9±2	0.003±0.000	51.2±1.9	13±0	0.005±0.000	49.8±1.2	15±1
fslQ	fast	0.031±0.011	4.1±0.2	96±5	0.029±0.018	6.6±0.7	88±6	0.013±0.015	4.6±0.2	104±4	0.037±0.007	5.4±0.4	81±3	0.015±0.004	6.9±0.3	98±3	0.021±0.005	4.7±0.2	86±2	0.032±0.008	11.3±0.4	95±6
	middle	0.031±0.001	52.7±1.5	11±3	0.023±0.001	56.4±3.3	20±3	0.016±0.001	45.0±1.1	32±1	0.017±0.001	43.6±1.4	23±2	0.015±0.001	43.8±1.4	25±2	0.009±0.000	40.1±0.7	27±1	0.007±0.001	45.7±1.2	35±1
	slow	0.013±0.000	43.2±1.6	8±2	0.009±0.001	37.0±3.9	12±1	0.009±0.000	50.4±1.2	16±1	0.009±0.000	51.0±1.7	11±1	0.007±0.000	49.3±1.4	13±1	0.005±0.000	55.2±0.8	13±0	0.004±0.000	43.0±1.3	17±1
fslF	fast	0.026±0.014	4.9±0.2	103±5	0.043±0.009	7.6±0.5	90±5	0.056±0.007	8.8±0.3	89±2	0.033±0.009	6.6±0.4	93±2	0.089±0.029	8.4±0.2	86±11	0.017±0.009	7.0±0.3	98±3	0.025±0.006	6.9±0.4	95±2
	middle	0.031±0.001	50.6±1.9	21±3	0.025±0.001	60.2±2.7	20±2	0.020±0.001	55.9±1.1	27±1	0.011±0.001	43.9±1.6	30±1	0.005±0.000	41.5±1.3	36±1	0.005±0.000	41.0±1.2	33±1	0.009±0.001	45.9±1.7	34±2
	slow	0.012±0.000	44.6±2.0	12±1	0.008±0.001	32.2±3.2	15±1	0.007±0.000	35.3±1.3	18±1	0.006±0.000	49.5±1.8	16±0	0.002±0.000	50.0±1.4	18±0	0.003±0.000	52.0±1.4	17±0	0.004±0.000	47.2±2.0	18±0
fslJ	fast	0.031±0.023	3.3±0.2	105±6	0.047±0.015	4.4±0.3	94±5	0.045±0.008	5.2±0.3	88±3	0.040±0.008	4.9±0.5	85±4	0.027±0.008	6.5±0.2	93±3	0.020±0.006	4.6±0.4	84±2	0.043±0.006	8.6±0.3	92±7
	middle	0.030±0.001	42.8±2.1	26±3	0.029±0.001	51.5±1.5	15±2	0.023±0.001	44.4±1.9	25±2	0.019±0.001	47.5±2.5	17±2	0.014±0.000	51.1±0.9	26±1	0.012±0.001	46.4±2.1	22±1	0.006±0.001	50.1±1.4	35±1
	slow	0.015±0.000	53.9±2.2	9±3	0.011±0.001	44.2±1.6	13±2	0.012±0.000	50.4±2.2	6±2	0.009±0.001	47.6±2.9	10±1	0.007±0.000	42.5±0.1	15±1	0.006±0.000	49.0±2.4	12±1	0.003±0.000	41.3±1.6	20±0
fslE	fast	0.068±0.024	2.9±0.3	92±7	0.078±0.039	4.1±0.3	87±16	0.039±0.010	3.7±0.3	90±4	-0.004±0.012	4.7±0.3	113±6	0.015±0.007	5.6±0.4	93±6	0.001±0.011	4.3±0.2	97±3	0.034±0.015	6.3±0.2	89±7
	middle	0.033±0.001	58.4±2.9	16±4	0.031±0.001	56.6±1.7	15±4	0.027±0.001	47.0±2.9	16±3	0.014±0.001	46.0±4.2	31±4	0.010±0.001	43.4±1.2	29±1	0.004±0.000	43.9±2.1	30±1	0.005±0.001	39.8±1.8	32±1
	slow	0.017±0.002	36.7±3.1	4±3	0.014±0.000	39.3±1.7	8±2	0.014±0.001	49.3±3.2	7±2	0.008±0.001	49.3±4.3	15±1	0.005±0.000	51.0±1.4	16±0	0.003±0.000	51.7±2.1	16±1	0.003±0.000	53.9±1.9	18±0
fslN	fast	0.046±0.006	5.1±0.3	87±3	0.028±0.015	6.0±0.3	98±5	0.039±0.007	6.4±0.6	89±3	0.045±0.014	5.0±0.3	85±3	0.014±0.011	5.1±0.3	100±5	0.008±0.006	4.8±0.2	88±2	0.015±0.010	6.3±0.4	100±4
	middle	0.029±0.001	57.2±3.4	13±3	0.024±0.001	52.2±1.3	23±2	0.022±0.001	56.6±3.1	20±2	0.017±0.001	47.3±1.1	19±2	0.018±0.001	45.3±2.2	26±3	0.009±0.000	44.2±1.6	23±1	0.009±0.000	46.9±1.0	32±1
	slow	0.012±0.001	37.7±3.6	7±2	0.012±0.000	41.7±1.4	7±2	0.008±0.001	36.9±3.6	15±1	0.007±0.000	47.7±1.4	12±1	0.009±0.000	49.5±2.2	13±1	0.005±0.000	51.0±1.8	12±0	0.005±0.000	46.8±1.3	18±0
fslL	fast	0.039±0.009	2.8±0.2	103±3	0.044±0.011	3.8±0.3	100±4	0.015±0.004	6.5±0.3	86±2	0.019±0.008	5.8±0.4	95±4	0.004±0.008	6.7±0.6	89±5	0.015±0.015	6.1±0.5	87±4	0.023±0.005	8.6±0.5	87±3
	middle	0.033±0.002	53.0±2.4	21±3	0.031±0.002	47.5±2.1	26±4	0.010±0.000	44.7±0.7	28±1	0.013±0.001	46.4±1.4	27±1	0.012±0.000	50.9±0.3	24±2	0.003±0.000	45.6±1.3	32±1	0.004±0.000	43.7±1.5	32±1
	slow	0.017±0.000	44.1±2.6	5±3	0.015±0.000	48.7±2.4	11±2	0.006±0.000	48.9±0.9	15±0	0.006±0.000	47.8±1.7	15±1	0.005±0.001	42.4±3.5	14±1	0.003±0.000	48.3±1.7	17±0	0.002±0.000	47.7±2.0	18±1
fslH	fast	0.024±0.017	3.5±0.5	109±6	0.053±0.008	4.9±0.3	89±4	0.030±0.017	4.0±0.3	99±4	0.060±0.012	5.2±0.3	77±7	0.026±0.006	5.3±0.3	86±3	0.004±0.004	6.0±0.2	94±2	0.024±0.007	6.3±0.2	81±4
	middle	0.040±0.001	46.3±3.0	20±6	0.024±0.001	49.0±2.0	21±2	0.021±0.001	41.9±1.6	33±1	0.019±0.001	47.8±1.9	22±2	0.014±0.000	47.4±1.6	24±1	0.004±0.000	45.1±0.8	25±0	0.008±0.000	41.6±0.7	26±1
	slow	0.018±0.001	50.3±3.4	4±2	0.012±0.000	46.2±2.3	8±1	0.012±0.000	54.1±1.8	13±1	0.009±0.000	47.0±2.1	12±1	0.006±0.000	47.4±1.9	13±1	0.003±0.000	48.8±0.9	13±0	0.004±0.000	52.1±0.8	13±0
fslM	fast	0.045±0.014	5.0±0.3	94±5	0.050±0.011	5.6±0.4	96±6	0.030±0.008	5.8±0.2	93±3	0.030±0.006	4.4±0.1	85±3	0.034±0.010	7.0±0.3	93±3	0.028±0.026	6.0±0.4	84±6	0.019±0.004	6.7±0.3	94±2
	middle	0.028±0.001	53.9±2.5	21±2	0.032±0.001	48.9±2.1	15±3	0.023±0.000	53.3±1.6	22±2	0.013±0.000	41.8±0.8	24±1	0.018±0.001	52.0±1.4	23±2	0.006±0.000	39.1±1.3	29±1	0.010±0.001	47.5±1.2	31±1
	slow	0.012±0.001	41.1±2.8	11±2	0.013±0.001	45.5±2.4	14±1	0.008±0.000	40.9±1.6	15±1	0.007±0.000	53.8±0.8	11±1	0.006±0.000	41.0±1.6	17±1	0.004±0.000	54.9±1.6	15±0	0.005±0.000	45.8±1.5	17±1
grlB	fast	0.009±0.017	2.1±0.2	104±5	-0.012±0.012	1.8±0.2	124±5	-0.008±0.015	4.3±0.3	110±3	0.016±0.011	2.5±0.2	123±5	-0.019±0.009	3.0±0.5	109±6	0.027±0.009	3.9±0.4	82±5	-0.031±0.016	4.1±0.3	120±5
	middle	0.038±0.003	43.2±4.1	17±4	0.026±0.003	46.2±4.2	23±5	0.013±0.001	45.4±1.4	27±3	0.027±0.002	48.0±5.0	12±6	0.012±0.001	38.1±8.0	30±4	0.005±0.001	38.8±3.8	30±2	0.015±0.001	41.8±1.9	31±3
	slow	0.020±0.001	54.7±4.2	10±3	0.015±0.001	52.0±4.3	8±3	0.006±0.000	50.3±1.5	17±1	0.013±0.001	49.5±5.1	11±2	0.007±0.001	58.9±8.4	17±1	0.004±0.000	57.2±3.9	15±1	0.007±0.000	54.0±1.8	18±0
grlO	fast	0.101±0.037	2.9±0.4	70±16	0.001±0.015	3.1±0.3	105±6	0.058±0.014	3.5±0.1	96±7	0.029±0.040	2.7±0.4	102±11	0.048±0.018	3.1±0.3	80±8	0.013±0.005	4.5±0.3	80±3	0.054±0.007	4.4±0.3	88±3
	middle	0.039±0.003	53.2±3.9	15±5	0.024±0.001	56.5±3.2	18±3	0.020±0.001	49.6±2.3	25±2	0.028±0.002	58.2±5.2	7±3	0.015±0.001	49.2±2.2	23±1	0.011±0.001	41.9±3.0	21±2	0.016±0.001	37.0±0.7	26±2
	slow	0.016±0.001	43.9±4.2	8±2	0.009±0.001	40.4±3.5	15±1	0.008±0.000	46.8±2.4	16±1	0.009±0.001	39.1±5.5	18±1	0.007±0.000	47.7±2.3	12±1	0.006±0.000	53.5±3.2	13±1	0.006±0.000	58.5±0.9	16±0
slc44a2	fast	0.018±0.016	3.1±0.4	98±8	0.030±0.011	2.8±0.6	98±7	0.012±0.008	6.1±0.5	100±3	0.040±0.011	4.5±0.3	82±5	-0.012±0.016	3.0±0.3	103±8	0.031±0.023	4.6±0.6	89±14	0.064±0.015	5.6±0.5	76±5
	middle	0.026±0.002	57.0±5.7	23±4	0.030±0.001	56.1±3.7	23±2	0.016±0.001	47.8±2.7	30±2	0.019±0.001	49.1±1.8	23±2	0.015±0.001	46.8±2.3	25±2	0.007±0.000	38.0±1.8	29±2	0.009±0.000	40.5±2.8	31±2
	slow	0.012±0.001	39.9±6.1	14±1	0.012±0.002	41.1±4.2	17±1	0.007±0.001	46.0±2.9	19±1	0.007±0.001	46.4±1.8	17±1	0.007±0.000	50.1±2.5	15±1	0.004±0.000	57.4±2.2	16±1	0.006±0.000	53.9±3.3	16±1
patB	fast	0.043±0.011	5.0±0.4	81±5	0.046±0.011	5.0±0.4	101±4	0.054±0.013	3.4±0.2	87±4	0.040±0.007	6.8±0.3	78±4	0.031±0.004	6.8±0.3	85±2	0.007±0.003	7.1±0.2	83±2	0.025±0.006	6.2±0.3	80±2
	slow	0.026±0.001	52.9±1.7	16±2	0.035±0.001	49.4±2.2	19±3	0.024±0.001	35.1±1.5	32±4	0.016±0.001	47.7±0.8	20±2	0.017±0.000	48.4±1.2	22±2	0.003±0.000	36.8±1.4	30±1	0.006±0.000	41.2±1.6	27±1
	fast	0.013±0.000	42.1±1.9	9±1	0.016±0.001	45.6±2.5	4±2	0.013±0.000	61.4±1.6	11±1	0.007±0.000	45.5±1.1	12±1	0.006±0.000	44.8±1.4	15±1	0.002±0.000	56.1±1.5	17±0	0.003±0.000	52.6±1.9	15±0

Table 3.4. (2/2) Diffusion coefficients D_s estimated from HMM and MSD analysis of the 27 membrane proteins under drug-treated conditions.

Using the HMM, single-molecule trajectories were separated into three states, from which the MSD was calculated, and D_s was obtained for each state. The estimated values are shown as means±95% CI. To calculate CI, the bootstrap method was used (see Methods). ϵ_s is the localization error and determined using Eq. (7).

Gene	State	Ctrl					Jasplakinolide					Latrunculin A				
		D_{HMM} ($\mu m^2/s$)	P_{HMM0} (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)	D_{HMM} ($\mu m^2/s$)	P_{HMM0} (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)	D_{HMM} ($\mu m^2/s$)	P_{HMM0} (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)
DD3-3	fast	0.157±0.009	28.1±2.3	66.6±1.5	2.8±0.2	0.0±0.0	0.235±0.012	16.6±2.0	55.0±2.1	3.7±0.1	0.0±0.1	0.327±0.013	12.5±0.8	54.7±1.9	5.2±0.4	0.0±0.1
	middle	0.034±0.001	66.3±1.9	33.4±1.6	94.2±0.5	3.9±0.5	0.039±0.001	73.4±1.9	43.3±1.8	92.4±0.8	4.2±0.5	0.036±0.001	73.1±2.9	44.8±2.5	87.6±1.0	4.7±0.4
	slow	0.014±0.001	5.6±1.1	0.0±0.2	3.0±0.5	96.1±0.5	0.018±0.001	9.9±1.6	1.7±0.7	3.9±0.7	95.8±0.5	0.016±0.001	14.4±3.5	0.5±1.7	7.3±0.6	95.3±0.4
psiM	fast	0.179±0.008	26.1±1.7	63.8±1.6	2.1±0.1	0.1±0.1	0.159±0.007	25.5±1.8	64.0±1.4	2.1±0.1	0.4±0.1	0.215±0.009	24.4±1.7	68.6±1.9	3.2±0.3	0.1±0.0
	middle	0.038±0.001	67.6±1.9	35.1±1.5	95.6±0.4	5.0±0.6	0.032±0.001	66.3±1.6	34.8±1.6	96.2±0.3	4.3±0.7	0.034±0.001	65.8±1.2	31.4±2.1	91.7±0.8	3.4±0.2
	slow	0.017±0.000	6.3±0.8	1.1±0.5	2.3±0.4	94.9±0.6	0.013±0.000	8.2±0.6	1.2±0.5	1.7±0.3	95.2±0.6	0.013±0.000	9.8±1.0	0.0±0.5	5.1±0.6	96.5±0.2
tgrO1	fast	0.183±0.009	18.3±1.3	51.6±1.6	3.6±0.1	0.4±0.1	0.221±0.006	15.9±0.9	46.8±1.3	5.0±0.2	0.1±0.1	0.218±0.010	17.2±1.4	51.0±2.0	5.1±0.2	0.2±0.1
	middle	0.035±0.001	73.1±0.9	45.4±1.5	92.7±0.6	7.0±0.5	0.040±0.001	72.2±1.2	49.3±1.5	86.7±0.5	8.8±0.6	0.035±0.001	71.0±0.9	47.5±1.9	86.3±0.4	7.2±0.2
	slow	0.016±0.001	8.6±0.8	2.9±0.9	3.7±0.7	92.6±0.5	0.017±0.000	11.9±0.9	3.9±1.1	8.4±0.5	91.1±0.6	0.014±0.000	11.7±0.7	1.5±0.7	8.6±0.3	92.7±0.2
tgrC1	fast	0.187±0.006	19.9±0.8	56.4±1.4	3.3±0.1	0.0±0.1	0.180±0.005	22.5±0.8	53.5±1.4	4.4±0.3	0.4±0.1	0.201±0.008	23.1±1.7	58.0±1.5	4.6±0.1	0.0±0.0
	middle	0.036±0.001	75.5±1.3	42.9±1.4	91.6±0.5	9.7±0.7	0.035±0.000	71.9±0.9	44.6±1.5	94.0±0.2	4.3±0.4	0.033±0.001	70.9±1.9	42.0±1.5	90.0±0.5	6.1±0.4
	slow	0.015±0.000	4.6±0.6	0.7±0.5	5.0±0.5	90.3±0.6	0.012±0.000	5.5±0.2	1.9±0.3	1.6±0.2	95.3±0.3	0.013±0.000	5.9±0.8	0.0±0.0	5.5±0.5	93.9±0.4
comC	fast	0.197±0.005	17.5±1.0	49.8±1.5	4.9±0.2	0.3±0.2	0.180±0.004	23.0±1.4	51.4±1.4	4.9±0.2	0.6±0.2	0.204±0.010	22.9±1.8	53.9±2.4	4.9±0.2	0.0±0.1
	middle	0.037±0.001	76.1±1.1	45.8±1.8	88.6±0.7	10.4±0.5	0.036±0.000	70.7±1.4	46.3±1.2	92.6±0.3	5.5±0.5	0.032±0.001	70.9±1.8	46.1±2.5	88.6±0.8	8.2±0.5
	slow	0.015±0.000	6.4±0.8	4.5±0.8	6.5±0.6	89.3±0.4	0.014±0.000	6.3±0.7	2.3±0.4	2.5±0.2	93.9±0.4	0.012±0.000	6.2±0.9	0.0±0.2	6.5±0.6	91.8±0.4
tspE	fast	0.211±0.006	12.5±1.2	48.8±2.1	3.4±0.3	0.0±0.1	0.204±0.019	19.3±1.9	60.4±2.0	2.1±0.2	0.1±0.1	0.226±0.008	19.0±1.7	66.6±2.4	3.2±0.2	0.0±0.0
	middle	0.041±0.001	75.1±1.2	51.2±4.3	89.7±0.9	5.1±0.4	0.032±0.001	72.6±2.3	38.2±2.2	94.9±0.6	4.7±0.4	0.040±0.001	67.5±0.8	31.9±2.5	89.2±0.9	4.0±0.2
	slow	0.019±0.000	12.1±1.4	0.0±3.1	6.9±0.8	94.9±0.3	0.012±0.001	8.1±1.3	1.4±0.8	3.0±0.7	95.2±0.4	0.018±0.000	13.5±1.3	1.5±0.9	7.6±0.7	96.0±0.2
car2	fast	0.215±0.012	17.6±1.5	55.6±2.0	2.8±0.1	0.1±0.0	0.234±0.004	19.9±0.8	53.6±0.6	6.0±0.1	0.0±0.0	0.242±0.005	17.8±0.8	50.4±1.7	5.0±0.2	0.0±0.0
	middle	0.034±0.001	73.9±1.3	44.4±1.9	90.8±0.9	6.5±0.4	0.041±0.000	69.8±0.5	45.7±0.8	86.3±0.3	8.3±0.2	0.037±0.001	76.2±1.0	49.6±2.1	86.8±0.7	6.4±0.2
	slow	0.015±0.001	8.5±1.3	0.0±0.5	6.4±0.9	93.4±0.4	0.016±0.000	10.3±0.6	0.7±0.5	7.7±0.3	91.6±0.2	0.014±0.000	6.0±0.6	0.0±0.8	8.2±0.5	93.6±0.2
car1	fast	0.216±0.008	14.2±1.3	49.6±1.2	3.1±0.2	0.1±0.1	0.316±0.009	18.7±0.9	48.2±1.3	6.5±0.2	0.0±0.0	0.257±0.010	14.5±1.0	54.4±1.6	4.0±0.2	0.0±0.0
	middle	0.035±0.001	73.4±1.4	49.3±1.5	89.5±0.8	7.2±0.5	0.044±0.001	70.6±1.3	51.6±1.4	83.0±0.9	9.0±0.3	0.035±0.001	80.0±1.2	45.5±1.8	87.7±0.5	5.4±0.4
	slow	0.016±0.000	12.4±1.1	1.1±1.0	7.4±0.8	92.7±0.5	0.016±0.000	10.7±1.0	0.2±0.5	10.5±0.8	91.0±0.3	0.013±0.000	5.5±1.2	0.0±0.4	8.3±0.5	94.6±0.4
fscD	fast	0.231±0.009	16.3±0.6	48.9±1.3	4.2±0.2	0.0±0.1	0.213±0.006	20.1±1.0	57.0±1.1	3.8±0.2	0.4±0.1	0.263±0.008	14.9±1.0	49.5±1.4	4.7±0.2	0.1±0.1
	middle	0.037±0.001	75.6±0.7	48.5±1.8	88.7±0.8	10.3±0.4	0.038±0.001	72.9±0.8	43.0±1.1	92.4±0.3	5.8±0.3	0.037±0.001	74.7±1.2	49.7±1.5	87.0±0.8	5.9±0.3
	slow	0.014±0.000	8.1±0.8	2.6±1.2	7.0±0.7	89.7±0.3	0.015±0.000	7.0±0.7	0.0±0.1	3.7±0.2	93.9±0.3	0.014±0.000	10.3±1.1	0.8±1.0	8.3±0.7	94.0±0.4
car4	fast	0.209±0.009	13.2±1.1	56.8±1.9	2.9±0.2	0.0±0.0	0.197±0.007	21.3±1.6	57.7±1.5	3.4±0.2	0.0±0.0	0.207±0.010	17.1±0.9	57.8±2.3	3.9±0.2	0.0±0.0
	middle	0.034±0.001	77.2±1.2	43.0±1.9	92.1±0.9	4.9±0.4	0.032±0.001	73.8±1.1	42.1±1.5	91.5±0.6	8.6±0.4	0.033±0.001	73.9±1.6	41.6±2.2	90.4±0.6	5.2±0.3
	slow	0.016±0.001	9.5±1.0	0.2±0.3	5.0±0.8	95.1±0.4	0.011±0.001	4.9±0.8	0.2±0.3	5.1±0.5	91.4±0.4	0.014±0.000	9.0±1.9	0.6±0.5	5.7±0.5	94.8±0.2
fscE	fast	0.212±0.009	13.0±1.2	53.0±1.3	3.0±0.1	0.0±0.0	0.270±0.010	12.3±0.8	51.9±0.8	3.3±0.1	0.0±0.0	0.228±0.006	14.3±0.8	55.6±1.1	3.6±0.1	0.0±0.0
	middle	0.032±0.001	76.8±1.0	47.0±1.3	89.5±0.8	6.8±0.3	0.044±0.001	75.8±1.3	45.9±1.9	88.1±0.5	6.3±0.4	0.034±0.001	74.8±1.3	44.4±1.4	87.2±0.6	6.0±0.3
	slow	0.014±0.000	10.1±0.9	0.0±0.3	7.5±0.7	93.2±0.3	0.020±0.000	11.9±1.7	2.1±1.7	8.7±0.5	93.7±0.4	0.015±0.000	10.9±1.5	0.0±0.6	9.2±0.5	94.0±0.3
fscF	fast	0.214±0.012	11.1±1.6	54.9±2.2	2.4±0.1	0.1±0.1	0.223±0.007	16.4±1.4	50.9±1.2	4.2±0.2	0.1±0.0	0.200±0.007	18.4±1.1	67.0±0.9	3.2±0.1	0.0±0.0
	middle	0.036±0.001	81.4±2.0	42.2±2.3	91.2±0.9	6.8±0.7	0.045±0.001	71.4±1.9	41.1±1.6	89.2±0.6	6.3±0.4	0.035±0.001	71.4±1.7	33.0±0.9	90.9±0.6	4.5±0.5
	slow	0.016±0.000	7.5±0.8	2.9±1.3	6.4±0.9	93.1±0.7	0.019±0.000	12.2±1.8	0.0±0.1	6.6±0.6	93.6±0.4	0.016±0.000	10.1±1.4	0.0±0.1	5.8±0.6	95.5±0.5
car3	fast	0.236±0.014	14.5±1.7	50.8±1.4	4.2±0.1	0.0±0.0	0.224±0.008	18.2±1.0	53.6±0.8	4.1±0.2	0.0±0.0	0.217±0.005	15.9±1.2	56.4±0.7	4.2±0.1	0.3±0.1
	middle	0.040±0.002	77.2±1.0	45.4±1.4	87.2±1.4	10.2±0.5	0.036±0.001	73.9±1.1	46.3±0.9	89.3±0.5	7.8±0.3	0.036±0.001	73.8±0.8	42.0±1.1	89.4±0.3	5.8±0.2
	slow	0.017±0.001	8.3±1.5	3.7±1.6	8.6±1.3	89.8±0.4	0.014±0.000	7.9±0.9	0.1±0.3	6.6±0.4	92.2±0.3	0.015±0.000	10.4±0.9	1.6±0.9	6.5±0.3	93.9±0.2
fscA	fast	0.145±0.018	24.9±1.0	68.4±2.9	1.8±0.2	0.0±0.0	0.193±0.013	22.8±2.2	68.5±1.4	2.3±0.2	0.2±0.1	0.272±0.015	14.0±1.1	58.0±1.5	3.7±0.3	0.0±0.0
	middle	0.031±0.002	69.7±2.8	31.6±2.8	95.4±1.2	5.6±0.5	0.032±0.002	65.1±1.5	30.7±1.9	90.6±2.0	6.2±0.8	0.038±0.002	68.3±1.7	41.4±1.3	86.1±1.0	5.9±0.5
	slow	0.014±0.001	5.4±1.5	0.0±0.2	2.8±1.2	94.4±0.5	0.013±0.000	12.1±1.7	0.8±0.9	7.2±1.9	93.5±0.8	0.016±0.000	17.7±2.2	0.7±1.3	10.2±0.8	94.1±0.5

Table 3.5. (1/5) List of estimated hidden Markov model parameters.

Gene	State	Ctrl					Jasplakinolide					Latrunculin A				
		$D_{HMM} (\mu m^2/s)$	$P_{HMM}^0 (\%)$	$A_{fast \rightarrow state} (\%)$	$A_{middle \rightarrow state} (\%)$	$A_{slow \rightarrow state} (\%)$	$D_{HMM} (\mu m^2/s)$	$P_{HMM}^0 (\%)$	$A_{fast \rightarrow state} (\%)$	$A_{middle \rightarrow state} (\%)$	$A_{slow \rightarrow state} (\%)$	$D_{HMM} (\mu m^2/s)$	$P_{HMM}^0 (\%)$	$A_{fast \rightarrow state} (\%)$	$A_{middle \rightarrow state} (\%)$	$A_{slow \rightarrow state} (\%)$
fslG	fast	0.255±0.017	10.1±0.9	50.3±2.3	2.4±0.1	0.0±0.0	0.253±0.007	14.4±0.6	52.9±1.6	3.9±0.2	0.0±0.1	0.244±0.009	17.3±0.8	59.3±1.7	3.7±0.1	0.0±0.1
	middle	0.037±0.001	77.5±1.7	45.2±3.2	90.8±0.6	5.2±0.5	0.043±0.001	76.2±1.2	46.1±1.5	87.0±0.9	9.2±0.6	0.040±0.001	70.8±1.0	39.4±2.0	89.0±0.8	4.5±0.3
	slow	0.017±0.001	12.4±1.9	4.6±2.2	6.8±0.6	94.8±0.5	0.018±0.000	9.3±1.3	1.0±0.7	9.1±0.8	90.8±0.6	0.019±0.000	11.8±1.3	1.3±0.8	7.3±0.7	95.5±0.3
fslQ	fast	0.215±0.010	14.3±1.1	58.0±1.9	2.7±0.1	0.0±0.0	0.194±0.009	29.4±2.0	64.5±2.3	2.8±0.1	0.0±0.0	0.257±0.011	13.9±1.0	49.6±2.2	4.5±0.2	0.0±0.1
	middle	0.035±0.001	71.5±1.3	41.5±1.7	91.8±0.6	5.4±0.5	0.034±0.001	62.6±1.5	35.5±2.2	92.4±0.7	5.6±0.4	0.036±0.001	77.1±1.6	50.4±2.1	86.6±0.8	5.3±0.2
	slow	0.016±0.000	14.2±0.7	0.5±0.8	5.5±0.6	94.6±0.5	0.014±0.001	7.9±1.3	0.0±0.5	4.7±0.6	94.4±0.4	0.013±0.000	9.0±1.2	0.0±0.5	8.9±0.6	94.7±0.2
fslF	fast	0.239±0.007	13.8±0.8	49.2±1.4	4.3±0.1	0.1±0.1	0.206±0.010	22.6±1.2	59.3±2.4	4.2±0.2	0.1±0.2	0.233±0.007	23.0±1.3	61.6±1.3	5.0±0.2	0.0±0.0
	middle	0.037±0.001	73.9±1.3	48.0±1.4	86.3±0.9	8.7±0.4	0.035±0.001	70.8±1.4	40.7±2.4	90.5±1.1	7.7±0.5	0.039±0.001	71.0±1.6	38.4±1.3	88.5±0.7	7.3±0.6
	slow	0.016±0.000	12.3±1.2	2.8±1.3	9.4±0.9	91.2±0.4	0.014±0.001	6.6±0.7	0.0±0.4	5.3±1.0	92.2±0.5	0.015±0.000	6.0±0.9	0.0±0.0	6.5±0.6	92.7±0.6
fslJ	fast	0.243±0.014	11.6±0.9	51.0±1.7	3.2±0.1	0.0±0.0	0.225±0.007	13.8±0.9	57.4±1.1	3.1±0.2	0.0±0.0	0.207±0.008	18.7±1.1	60.7±1.5	3.8±0.2	0.1±0.0
	middle	0.038±0.001	74.0±1.6	47.2±1.8	87.7±1.0	5.4±0.3	0.038±0.001	74.9±1.8	42.6±1.1	90.7±0.7	5.4±0.6	0.034±0.001	67.2±0.9	39.3±1.3	88.8±0.6	4.9±0.2
	slow	0.017±0.000	14.4±1.9	1.8±0.8	9.1±1.0	94.6±0.3	0.016±0.000	11.3±1.3	0.0±0.0	6.2±0.7	94.6±0.6	0.015±0.000	14.1±1.4	0.0±0.4	7.4±0.5	95.1±0.2
fslE	fast	0.241±0.007	11.2±0.7	51.0±1.5	2.0±0.2	0.0±0.0	0.223±0.012	11.5±0.8	51.1±1.3	2.9±0.2	0.3±0.1	0.210±0.011	15.6±1.3	63.2±2.3	2.2±0.2	0.0±0.0
	middle	0.040±0.001	80.0±1.8	49.0±1.7	93.0±0.7	5.4±0.7	0.039±0.001	79.8±2.6	47.9±2.4	91.7±1.0	5.4±0.9	0.035±0.001	72.9±1.5	36.6±2.9	91.8±0.9	3.9±0.4
	slow	0.020±0.001	8.8±2.0	0.0±0.2	5.0±0.7	94.6±0.7	0.018±0.000	8.8±1.9	1.0±1.3	5.4±0.9	94.3±0.8	0.017±0.001	11.5±1.8	0.3±0.9	5.9±0.9	96.1±0.4
fslN	fast	0.208±0.007	18.0±1.5	62.4±1.6	2.7±0.2	0.0±0.1	0.222±0.007	16.9±1.1	58.0±1.2	4.1±0.2	0.1±0.1	0.214±0.008	19.0±1.6	61.0±1.9	3.7±0.3	0.0±0.1
	middle	0.035±0.001	73.4±1.4	36.2±1.9	92.2±0.9	6.0±0.5	0.038±0.001	70.5±1.4	41.0±1.5	91.7±0.5	3.7±0.3	0.031±0.001	75.0±0.9	39.0±1.9	91.5±0.9	5.2±0.3
	slow	0.016±0.001	8.6±1.7	1.4±0.7	5.1±0.8	94.0±0.6	0.016±0.000	12.6±1.1	1.0±0.5	4.2±0.3	96.1±0.2	0.012±0.001	6.0±0.9	0.0±0.0	4.8±0.7	94.8±0.3
fslL	fast	0.234±0.010	8.8±0.6	47.0±2.0	2.4±0.1	0.0±0.0	0.249±0.015	10.0±1.0	52.9±2.0	3.4±0.2	0.0±0.1	0.189±0.005	23.2±0.9	68.0±1.2	3.8±0.2	0.0±0.0
	middle	0.041±0.001	81.7±1.2	48.8±3.5	90.4±0.9	6.4±0.6	0.042±0.001	75.3±1.3	47.1±2.4	88.5±0.8	5.9±0.2	0.029±0.001	63.6±1.3	32.0±1.2	90.6±0.2	3.9±0.2
	slow	0.020±0.000	9.5±1.3	4.2±2.0	7.1±1.0	93.6±0.6	0.019±0.000	14.7±1.9	0.0±1.5	8.0±0.6	94.1±0.2	0.012±0.000	13.2±0.9	0.0±0.0	5.6±0.2	96.1±0.2
fslH	fast	0.235±0.020	10.9±1.4	46.6±3.0	3.5±0.1	0.0±0.0	0.209±0.007	17.2±0.9	54.4±1.2	3.8±0.1	0.0±0.0	0.247±0.010	12.6±1.1	53.3±3.1	3.8±0.2	0.1±0.1
	middle	0.042±0.002	77.0±1.7	53.4±4.4	85.0±1.0	8.2±0.6	0.033±0.001	69.9±1.1	45.4±1.3	89.8±0.7	5.2±0.2	0.037±0.001	75.5±2.4	44.6±3.3	87.6±0.8	4.4±0.3
	slow	0.020±0.000	12.1±2.2	0.0±2.0	11.5±1.1	91.8±0.6	0.014±0.000	12.8±1.4	0.3±0.4	6.4±0.7	94.8±0.2	0.016±0.000	11.9±2.2	2.1±1.0	8.6±0.6	95.5±0.3
fslM	fast	0.223±0.007	15.4±0.9	50.3±1.7	4.0±0.1	0.0±0.0	0.234±0.009	18.6±1.1	65.4±1.7	3.1±0.1	0.1±0.1	0.209±0.007	17.7±0.8	57.0±1.6	4.0±0.1	0.0±0.0
	middle	0.035±0.001	75.7±1.1	49.3±1.7	88.4±0.9	7.7±0.3	0.042±0.001	71.0±1.2	32.1±1.7	88.8±0.6	6.8±0.6	0.032±0.001	76.0±0.9	43.0±1.6	89.1±0.5	6.7±0.3
	slow	0.015±0.000	8.9±1.6	0.4±0.5	7.5±0.8	92.3±0.3	0.018±0.000	10.4±1.6	2.5±0.9	8.1±0.5	93.1±0.6	0.013±0.000	6.3±0.8	0.0±0.2	6.8±0.4	93.3±0.4
grlB	fast	0.249±0.016	7.9±1.7	51.0±1.9	2.1±0.3	0.0±0.0	0.394±0.024	4.9±0.8	47.2±4.7	1.6±0.1	0.0±0.0	0.298±0.009	6.3±1.0	57.9±2.2	4.0±0.4	0.1±0.1
	middle	0.040±0.002	77.6±4.2	46.7±3.2	89.9±1.8	4.5±0.8	0.036±0.001	77.8±5.7	52.8±4.7	89.3±1.9	4.2±1.7	0.031±0.001	82.3±2.4	42.1±2.2	87.6±1.5	3.5±0.8
	slow	0.021±0.001	14.5±3.7	2.3±2.1	8.0±1.7	95.5±0.8	0.019±0.001	17.3±5.5	0.0±0.0	9.2±1.8	95.8±1.7	0.014±0.000	11.3±2.1	0.0±0.0	8.4±1.4	96.5±0.8
grlO	fast	0.204±0.014	13.6±1.4	53.5±2.9	1.8±0.2	0.1±0.1	0.245±0.019	8.7±1.4	49.6±3.0	2.5±0.1	0.0±0.0	0.302±0.018	8.4±1.1	57.4±2.7	2.8±0.2	0.0±0.0
	middle	0.038±0.001	72.5±2.3	46.5±2.9	93.8±0.7	3.8±0.5	0.032±0.001	81.6±2.0	50.4±2.8	92.5±1.1	4.9±0.5	0.034±0.001	79.9±2.3	42.4±2.9	91.3±1.1	3.8±0.6
	slow	0.018±0.001	13.8±2.9	0.0±0.0	4.3±0.7	96.1±0.5	0.015±0.001	9.7±1.7	0.0±0.7	5.0±1.0	95.1±0.5	0.014±0.000	11.6±1.9	0.2±0.9	6.0±1.0	96.2±0.6
slc44a2	fast	0.219±0.026	8.4±2.3	59.5±3.8	2.0±0.3	0.0±0.1	0.214±0.026	10.0±2.4	59.1±3.6	1.7±0.2	0.0±0.0	0.233±0.014	20.7±1.0	63.8±2.5	3.5±0.2	0.0±0.0
	middle	0.040±0.002	80.1±3.5	40.5±3.8	91.4±1.6	7.3±0.4	0.041±0.002	77.2±1.7	40.9±3.5	91.5±0.8	7.5±0.7	0.038±0.001	70.8±1.8	36.2±2.6	89.1±0.8	5.0±0.4
	slow	0.019±0.001	11.5±4.7	0.0±0.0	6.6±1.3	92.7±0.4	0.019±0.001	12.8±2.2	0.0±1.8	6.8±0.9	92.5±0.7	0.015±0.001	8.5±1.6	0.0±0.2	7.4±0.7	95.0±0.4
patB	fast	0.160±0.009	19.3±1.5	58.9±2.2	3.2±0.2	0.0±0.0	0.218±0.011	17.1±1.9	49.7±1.3	4.3±0.2	0.0±0.0	0.206±0.008	11.7±0.9	56.3±1.5	3.8±0.2	0.0±0.0
	middle	0.032±0.001	69.1±1.9	40.5±2.3	92.9±0.4	3.7±0.4	0.040±0.001	72.8±1.7	49.0±1.4	87.2±1.1	7.4±0.5	0.035±0.001	68.0±1.2	40.7±1.9	84.7±1.4	5.3±0.3
	slow	0.016±0.000	11.6±1.3	0.6±0.8	4.0±0.5	96.3±0.4	0.018±0.000	10.2±0.9	1.3±0.8	8.5±1.0	92.6±0.5	0.016±0.000	20.3±1.2	3.0±0.8	11.5±1.2	94.7±0.3

Table 3.5. (2/5) List of estimated hidden Markov model parameters.

Gene	State	Nocodazole					Thiabendazole					Benomyl				
		D_{HMM} ($\mu m^2/s$)	$P_{HMM}0$ (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)	D_{HMM} ($\mu m^2/s$)	$P_{HMM}0$ (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)	D_{HMM} ($\mu m^2/s$)	$P_{HMM}0$ (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)
DD3-3	fast	0.273±0.012	13.6±0.7	59.0±1.4	3.4±0.1	0.0±0.1	0.221±0.010	17.4±1.7	61.4±2.4	4.3±0.2	0.1±0.0	0.341±0.013	15.9±1.7	49.7±1.6	6.8±0.4	0.0±0.1
	middle	0.038±0.001	76.0±1.2	41.0±1.4	88.9±0.5	6.3±0.3	0.034±0.001	68.4±1.6	38.6±2.4	90.8±0.6	3.8±0.3	0.034±0.001	63.1±1.7	50.1±2.0	85.6±0.8	4.1±0.7
	slow	0.016±0.000	10.3±1.6	0.0±0.1	7.7±0.6	93.6±0.3	0.013±0.000	14.2±1.3	0.0±0.0	4.9±0.4	96.2±0.3	0.012±0.000	21.0±2.0	0.2±0.7	7.6±0.6	95.9±0.6
psiM	fast	0.164±0.012	26.8±2.1	65.3±1.5	2.3±0.2	0.2±0.1	0.160±0.004	23.4±1.4	61.0±1.3	3.7±0.1	0.1±0.0	0.160±0.009	21.3±2.1	68.2±1.3	3.0±0.1	0.0±0.0
	middle	0.027±0.001	67.8±1.3	34.1±1.5	95.6±0.6	6.0±0.3	0.025±0.001	62.9±1.5	38.2±1.2	91.9±0.3	3.9±0.3	0.022±0.001	61.1±1.7	31.8±1.2	92.7±0.3	2.8±0.2
	slow	0.009±0.001	5.4±1.3	0.6±0.3	2.1±0.6	93.8±0.3	0.010±0.000	13.7±0.9	0.8±0.5	4.4±0.3	96.0±0.3	0.008±0.000	17.5±1.1	0.0±0.2	4.3±0.3	97.1±0.2
tgrO1	fast	0.192±0.009	17.7±1.1	52.0±1.8	3.9±0.1	0.0±0.0	0.179±0.005	20.7±1.1	56.6±1.3	4.3±0.1	0.1±0.0	0.159±0.004	24.1±0.7	67.1±1.0	3.8±0.1	0.0±0.0
	middle	0.030±0.001	72.7±0.6	48.0±2.0	88.9±0.3	7.0±0.4	0.026±0.001	66.0±1.1	43.4±1.2	91.4±0.4	4.1±0.2	0.020±0.000	56.6±0.7	32.9±1.0	92.1±0.2	3.2±0.1
	slow	0.012±0.000	9.6±1.3	0.0±0.7	7.2±0.3	93.0±0.4	0.009±0.000	13.3±0.8	0.0±0.3	4.3±0.3	95.8±0.2	0.007±0.000	19.4±0.7	0.0±0.1	4.1±0.2	96.8±0.1
tgrC1	fast	0.178±0.009	20.6±1.6	56.5±1.9	3.6±0.1	0.2±0.1	0.181±0.005	20.3±0.8	57.9±1.1	4.9±0.2	0.0±0.0	0.179±0.006	23.4±1.4	63.7±1.3	3.8±0.2	0.1±0.0
	middle	0.032±0.001	74.2±1.4	42.0±1.7	91.3±0.5	7.3±0.3	0.024±0.000	64.9±0.9	40.7±1.4	88.8±0.4	4.7±0.2	0.019±0.001	54.0±1.6	36.3±1.3	90.2±0.4	3.6±0.2
	slow	0.014±0.000	5.2±0.8	1.5±0.5	5.1±0.5	92.5±0.3	0.008±0.000	14.8±0.8	1.3±0.7	6.3±0.3	95.3±0.2	0.006±0.000	22.6±0.9	0.0±0.3	6.0±0.4	96.4±0.2
comC	fast	0.182±0.012	20.9±1.7	56.5±1.7	3.7±0.1	0.1±0.1	0.200±0.005	16.4±1.1	52.7±1.5	4.2±0.2	0.4±0.1	0.173±0.009	20.0±1.6	60.5±1.0	4.2±0.3	0.0±0.1
	middle	0.030±0.001	70.9±1.4	42.6±1.3	91.3±0.5	6.1±0.3	0.032±0.001	72.6±1.3	43.3±1.9	89.2±0.8	6.5±0.5	0.025±0.001	65.0±1.5	39.5±0.9	89.4±0.5	5.0±0.3
	slow	0.012±0.000	8.2±1.3	1.0±0.6	5.0±0.5	93.8±0.3	0.013±0.000	11.0±0.8	4.0±1.0	6.6±0.7	93.1±0.4	0.009±0.000	15.0±0.7	0.0±0.1	6.4±0.4	95.0±0.3
tspE	fast	0.194±0.007	24.8±1.3	64.1±1.6	3.8±0.2	0.1±0.1	0.247±0.018	15.7±1.6	56.5±2.6	2.0±0.1	0.0±0.0	0.169±0.020	16.0±1.9	65.2±2.2	1.9±0.3	0.0±0.0
	middle	0.034±0.001	64.9±1.1	35.9±1.5	91.2±0.5	4.3±0.4	0.032±0.001	75.4±0.8	41.5±2.2	94.4±0.4	5.0±0.5	0.029±0.001	75.1±1.9	34.5±2.4	95.0±0.3	3.6±0.4
	slow	0.014±0.000	10.3±0.8	0.0±0.3	5.0±0.5	95.6±0.4	0.012±0.001	8.9±1.2	2.1±0.8	3.9±0.4	95.0±0.5	0.011±0.000	8.9±1.1	0.4±0.7	3.1±0.3	96.3±0.4
car2	fast	0.218±0.010	16.0±0.9	56.4±1.5	3.7±0.2	0.0±0.0	0.180±0.006	23.6±0.8	60.2±1.0	4.6±0.1	0.0±0.0	0.202±0.008	23.3±1.6	67.7±0.9	4.2±0.2	0.0±0.0
	middle	0.030±0.001	71.8±1.2	43.6±1.5	88.6±0.5	4.7±0.2	0.027±0.000	65.9±0.8	39.8±0.9	91.5±0.3	3.9±0.2	0.028±0.001	62.6±1.2	32.3±0.8	90.2±0.6	4.1±0.3
	slow	0.012±0.000	12.2±1.2	0.0±0.1	7.6±0.3	95.3±0.2	0.010±0.000	10.6±0.8	0.0±0.1	3.9±0.3	96.1±0.2	0.009±0.000	14.1±0.8	0.0±0.2	5.6±0.6	95.8±0.3
car1	fast	0.170±0.004	20.1±0.9	60.1±1.0	3.6±0.1	0.1±0.0	0.265±0.006	17.2±0.6	49.8±0.8	6.2±0.2	0.0±0.0	0.241±0.010	17.0±1.2	55.5±2.1	5.4±0.2	0.0±0.1
	middle	0.024±0.000	68.3±1.0	39.6±1.0	92.1±0.2	3.6±0.2	0.035±0.001	72.6±0.6	50.2±1.0	82.4±0.8	8.8±0.4	0.029±0.001	69.7±1.0	44.5±2.1	87.4±0.6	5.4±0.3
	slow	0.010±0.000	11.6±0.8	0.3±0.4	4.3±0.2	96.3±0.2	0.012±0.000	10.2±0.6	0.0±0.6	11.5±0.7	91.2±0.4	0.010±0.000	13.3±1.4	0.0±0.2	7.2±0.5	94.6±0.3
fscD	fast	0.211±0.009	18.1±1.3	61.5±1.9	4.2±0.2	0.0±0.0	0.194±0.009	18.4±0.9	56.4±2.0	4.0±0.2	0.0±0.0	0.210±0.009	19.5±1.0	61.9±1.5	5.3±0.3	0.0±0.0
	middle	0.028±0.001	71.9±0.8	38.5±1.9	88.2±0.7	5.6±0.3	0.029±0.001	67.3±1.0	43.6±1.6	89.9±0.5	5.1±0.4	0.025±0.001	65.7±1.5	38.1±1.4	88.4±0.6	3.6±0.2
	slow	0.011±0.000	10.0±1.3	0.0±0.0	7.6±0.6	94.4±0.3	0.011±0.000	14.3±1.3	0.0±0.8	6.0±0.3	94.9±0.4	0.008±0.000	14.8±1.0	0.0±0.1	6.3±0.3	96.4±0.2
car4	fast	0.221±0.011	13.8±1.1	61.0±1.2	3.8±0.3	0.0±0.0	0.228±0.007	19.5±1.0	58.1±1.0	4.5±0.1	0.0±0.0	0.178±0.013	24.3±2.4	67.9±1.9	3.2±0.2	0.0±0.0
	middle	0.033±0.001	71.6±1.2	39.0±1.2	87.1±1.0	5.3±0.3	0.031±0.001	68.1±0.8	41.9±1.0	87.4±0.4	5.8±0.2	0.023±0.001	63.7±2.6	32.1±1.9	92.3±1.1	4.5±0.3
	slow	0.013±0.000	14.7±1.4	0.0±0.1	9.1±0.9	94.7±0.3	0.012±0.000	12.3±0.8	0.0±0.2	8.0±0.4	94.1±0.2	0.008±0.000	11.9±1.3	0.0±0.2	4.5±1.0	95.5±0.3
fscE	fast	0.208±0.010	13.3±0.8	56.8±1.6	3.7±0.2	0.0±0.0	0.232±0.010	16.1±0.9	59.6±0.9	3.9±0.2	0.0±0.0	0.218±0.013	16.8±1.7	61.5±2.2	4.2±0.2	0.0±0.0
	middle	0.027±0.001	71.1±1.0	43.2±1.6	87.8±0.5	4.9±0.3	0.035±0.001	69.7±1.0	40.3±1.4	87.7±0.6	5.2±0.3	0.030±0.001	70.2±1.7	38.5±2.2	88.5±0.5	4.6±0.2
	slow	0.011±0.000	15.7±1.3	0.0±0.1	8.5±0.4	95.1±0.3	0.014±0.000	14.2±1.4	0.1±0.8	8.4±0.6	94.8±0.3	0.011±0.000	12.9±1.5	0.0±0.0	7.2±0.5	95.4±0.2
fscF	fast	0.162±0.011	29.4±2.9	76.4±1.6	2.2±0.2	0.0±0.0	0.210±0.017	16.8±1.7	60.7±2.2	3.1±0.1	0.0±0.0	0.200±0.013	20.1±1.6	63.9±2.5	4.5±0.4	0.0±0.0
	middle	0.026±0.002	61.3±2.2	23.6±1.6	93.2±1.1	4.7±0.4	0.031±0.001	73.9±1.3	39.1±2.1	91.7±0.6	4.2±0.4	0.029±0.001	69.4±1.6	35.1±2.0	88.2±0.7	3.7±0.3
	slow	0.011±0.001	9.3±1.1	0.0±0.3	4.6±1.0	95.3±0.4	0.012±0.000	9.3±1.5	0.2±0.4	5.2±0.6	95.8±0.4	0.010±0.000	10.6±1.5	1.0±1.0	7.2±0.5	96.3±0.3
car3	fast	0.228±0.008	17.3±1.0	56.6±1.4	4.9±0.2	0.1±0.1	0.215±0.008	26.2±2.2	63.3±1.1	4.7±0.2	0.1±0.1	0.212±0.010	26.6±1.3	65.1±1.9	4.9±0.3	0.0±0.0
	middle	0.032±0.001	70.6±0.8	42.7±1.5	87.4±0.5	6.2±0.3	0.031±0.001	69.0±2.3	36.6±1.0	87.0±1.2	7.9±0.2	0.025±0.001	58.9±0.9	34.9±1.9	89.0±0.7	4.5±0.4
	slow	0.013±0.000	12.1±0.9	0.7±0.5	7.7±0.5	93.8±0.3	0.011±0.000	4.7±0.4	0.1±0.4	8.3±1.0	92.1±0.2	0.008±0.000	14.5±1.4	0.0±0.1	6.1±0.6	95.5±0.4
fscA	fast	0.184±0.017	19.2±2.1	66.5±1.8	2.2±0.1	0.0±0.0	0.205±0.010	25.0±1.2	70.2±1.9	3.3±0.2	0.0±0.0	0.192±0.021	21.6±2.3	69.3±2.4	3.7±0.4	0.0±0.0
	middle	0.029±0.001	69.4±2.2	33.4±1.7	93.6±0.5	3.9±0.3	0.033±0.001	64.2±1.2	29.8±1.9	91.1±0.7	4.7±0.4	0.032±0.002	67.3±1.6	30.7±2.4	90.5±0.5	3.3±0.3
	slow	0.011±0.000	11.3±1.1	0.0±0.2	4.2±0.5	96.1±0.3	0.012±0.000	10.8±0.9	0.0±0.1	5.6±0.6	95.3±0.4	0.011±0.000	11.2±1.6	0.0±0.0	5.7±0.5	96.7±0.3

Table 3.5. (3/5) List of estimated hidden Markov model parameters.

Gene	State	Nocodazole					Thiabendazole					Benomyl				
		D_{HMM} ($\mu\text{m}^2/\text{s}$)	P_{HMM}^0 (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)	D_{HMM} ($\mu\text{m}^2/\text{s}$)	P_{HMM}^0 (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)	D_{HMM} ($\mu\text{m}^2/\text{s}$)	P_{HMM}^0 (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)
fslG	fast	0.228±0.007	19.2±1.1	61.9±1.1	3.7±0.1	0.0±0.0	0.202±0.010	14.8±1.2	55.9±2.2	4.2±0.3	0.0±0.0	0.193±0.010	20.9±1.1	63.0±2.3	4.0±0.2	0.1±0.0
	middle	0.032±0.001	65.7±1.2	38.1±1.0	89.9±0.4	3.8±0.2	0.034±0.001	70.7±1.6	43.2±2.7	88.2±0.5	5.6±0.4	0.023±0.001	63.9±1.3	35.7±2.0	90.4±0.5	3.9±0.2
	slow	0.012±0.000	15.1±1.0	0.0±0.3	6.4±0.3	96.2±0.2	0.014±0.000	14.6±1.0	0.9±0.7	7.6±0.3	94.4±0.4	0.008±0.000	15.2±1.3	1.3±0.9	5.7±0.4	96.0±0.2
fslQ	fast	0.183±0.009	20.2±1.4	62.5±2.4	3.8±0.2	0.0±0.0	0.257±0.011	17.5±1.2	57.8±1.4	6.1±0.3	0.0±0.0	0.204±0.006	18.5±1.0	62.3±1.0	3.7±0.2	0.0±0.0
	middle	0.029±0.001	64.2±1.1	37.5±2.4	90.4±0.6	3.8±0.2	0.032±0.001	68.5±1.6	42.2±1.4	86.3±0.9	4.9±0.3	0.026±0.000	64.3±1.2	37.7±1.0	90.8±0.3	3.0±0.1
	slow	0.012±0.000	15.6±0.7	0.0±0.4	5.8±0.5	96.2±0.2	0.012±0.000	14.0±1.4	0.0±0.0	7.7±0.6	95.1±0.3	0.010±0.000	17.2±1.0	0.0±0.0	5.4±0.2	97.0±0.1
fslF	fast	0.235±0.012	20.1±1.4	64.4±1.9	4.6±0.3	0.0±0.0	0.329±0.009	18.8±0.8	51.6±1.5	8.9±0.4	0.0±0.1	0.249±0.007	22.6±0.9	61.7±1.5	5.5±0.3	0.1±0.1
	middle	0.033±0.001	69.1±1.4	35.3±1.8	87.2±0.8	5.4±0.4	0.036±0.001	66.6±1.0	47.2±1.6	81.0±0.9	6.3±0.3	0.032±0.001	64.0±1.1	38.3±1.7	88.2±0.5	3.7±0.2
	slow	0.013±0.000	10.7±1.3	0.4±0.3	8.2±0.6	94.6±0.4	0.011±0.000	14.6±1.1	1.1±0.4	10.1±0.6	93.7±0.3	0.011±0.000	13.4±1.0	0.0±1.2	6.3±0.4	96.2±0.2
fslJ	fast	0.196±0.013	18.8±1.9	57.3±2.1	3.6±0.2	0.0±0.0	0.223±0.009	22.9±1.0	60.1±1.2	4.1±0.2	0.0±0.0	0.185±0.010	20.2±2.2	63.2±1.6	2.9±0.2	0.1±0.0
	middle	0.028±0.001	68.4±0.8	42.7±2.1	91.2±0.5	3.9±0.3	0.031±0.001	67.5±1.0	39.9±1.2	90.8±0.4	4.5±0.2	0.024±0.001	67.2±2.3	36.8±1.8	92.7±0.4	3.0±0.2
	slow	0.011±0.000	12.8±1.7	0.0±0.2	5.2±0.4	96.1±0.3	0.012±0.000	9.5±0.9	0.0±0.0	5.1±0.3	95.5±0.2	0.010±0.000	12.6±1.2	0.0±0.2	4.4±0.4	96.9±0.2
fslE	fast	0.334±0.018	9.4±0.6	53.8±4.2	4.3±0.6	0.0±0.0	0.224±0.010	17.4±1.5	60.9±1.8	4.4±0.4	0.0±0.0	0.236±0.011	14.9±1.4	67.6±2.1	2.8±0.3	0.0±0.0
	middle	0.036±0.002	78.7±5.6	46.2±4.2	81.9±1.7	8.3±1.1	0.031±0.001	70.6±1.9	38.3±1.9	88.2±0.6	4.7±0.2	0.028±0.001	73.0±3.0	31.8±2.4	90.3±0.7	4.5±0.7
	slow	0.015±0.001	11.9±5.6	0.0±0.1	13.8±1.2	91.7±1.1	0.012±0.000	12.1±1.1	0.8±0.5	7.4±0.6	95.3±0.2	0.010±0.000	12.1±2.3	0.5±0.6	6.9±0.6	95.5±0.7
fslN	fast	0.208±0.011	20.4±0.8	63.8±2.1	2.9±0.1	0.0±0.0	0.233±0.010	16.1±1.4	60.1±1.7	3.8±0.3	0.0±0.1	0.205±0.008	22.1±1.5	64.2±2.0	3.0±0.2	0.0±0.0
	middle	0.028±0.001	66.4±1.1	36.2±2.1	92.0±0.4	3.8±0.3	0.036±0.001	72.3±1.2	39.2±1.7	88.4±1.1	5.2±0.3	0.025±0.001	62.9±1.1	35.8±2.0	92.6±0.3	2.9±0.3
	slow	0.010±0.000	13.2±0.8	0.0±0.5	5.1±0.4	96.2±0.3	0.015±0.000	11.3±1.4	0.7±0.4	7.9±0.8	94.8±0.3	0.009±0.000	15.0±1.5	0.0±0.0	4.5±0.2	97.1±0.2
fslL	fast	0.228±0.012	21.8±0.8	63.5±1.4	3.7±0.3	0.0±0.0	0.182±0.014	22.4±1.4	68.6±2.4	3.4±0.2	0.0±0.1	0.207±0.012	24.1±2.0	65.9±2.3	3.6±0.3	0.0±0.0
	middle	0.033±0.001	66.6±1.1	36.5±1.6	91.6±0.4	3.3±0.2	0.029±0.002	69.3±1.1	31.4±2.4	91.4±1.0	4.7±0.3	0.029±0.001	63.3±2.1	34.1±2.3	91.5±0.4	3.5±0.4
	slow	0.012±0.000	11.6±0.9	0.0±0.4	4.7±0.4	96.7±0.2	0.011±0.001	8.3±1.5	0.0±0.1	5.3±0.9	95.2±0.3	0.010±0.000	12.6±1.4	0.1±0.2	4.9±0.3	96.4±0.4
fslH	fast	0.203±0.009	17.0±0.9	56.3±1.3	4.0±0.1	0.1±0.1	0.185±0.009	17.3±0.8	59.4±1.5	4.0±0.1	0.0±0.0	0.186±0.005	21.6±1.3	67.2±0.9	3.8±0.2	0.0±0.0
	middle	0.029±0.001	71.7±1.3	43.0±1.5	90.0±0.6	4.5±0.2	0.026±0.001	71.3±0.7	40.6±1.4	89.8±0.5	4.9±0.2	0.021±0.000	62.5±1.5	32.8±0.9	91.5±0.4	3.6±0.2
	slow	0.012±0.000	11.3±1.2	0.8±0.4	6.0±0.5	95.4±0.2	0.011±0.000	11.4±0.8	0.0±0.1	6.2±0.4	95.1±0.2	0.008±0.000	15.9±0.5	0.0±0.1	4.6±0.3	96.4±0.2
fslM	fast	0.203±0.005	16.0±0.5	56.1±0.9	4.0±0.1	0.0±0.0	0.224±0.006	21.5±1.2	57.2±1.8	4.9±0.2	0.0±0.0	0.210±0.010	25.6±1.0	65.3±1.3	4.2±0.1	0.0±0.0
	middle	0.025±0.000	69.1±1.0	43.9±0.9	89.2±0.4	4.0±0.1	0.035±0.001	69.7±1.5	42.8±1.8	89.5±0.7	5.3±0.3	0.027±0.001	58.3±1.0	34.7±1.3	90.0±0.6	3.2±0.2
	slow	0.010±0.000	15.0±0.9	0.0±0.0	6.7±0.3	96.0±0.1	0.013±0.000	8.8±1.3	0.0±0.0	5.6±0.5	94.7±0.3	0.009±0.000	16.1±0.8	0.0±0.1	5.8±0.6	96.8±0.2
grlB	fast	0.388±0.029	5.3±0.5	54.9±3.4	1.9±0.3	0.1±0.1	0.274±0.028	7.2±1.6	51.0±6.1	3.5±0.6	0.0±0.1	0.202±0.019	15.5±2.9	73.8±3.5	2.0±0.5	0.0±0.0
	middle	0.036±0.002	81.2±7.4	45.1±4.0	87.0±1.7	5.8±1.1	0.029±0.002	73.9±8.0	48.9±5.9	88.2±1.6	2.8±1.4	0.025±0.001	74.0±2.5	26.2±3.5	90.1±1.5	3.5±0.6
	slow	0.018±0.001	13.4±7.4	0.0±1.7	11.1±1.5	94.1±1.1	0.014±0.001	18.9±8.7	0.0±2.2	8.3±1.5	97.2±1.4	0.010±0.000	10.5±2.1	0.0±0.0	8.0±1.1	96.5±0.6
grlO	fast	0.252±0.025	8.0±1.5	49.7±3.7	2.1±0.2	0.0±0.0	0.237±0.018	8.2±1.1	50.7±2.6	2.9±0.2	0.0±0.0	0.173±0.009	22.1±2.4	70.6±1.0	2.2±0.3	0.0±0.0
	middle	0.032±0.001	78.4±2.4	50.3±3.7	93.7±1.0	4.6±0.8	0.027±0.001	84.1±1.4	49.1±3.0	90.6±0.6	4.6±0.4	0.026±0.001	64.8±2.2	29.4±1.0	92.9±0.7	2.7±0.1
	slow	0.015±0.001	13.6±2.6	0.0±0.0	4.2±0.9	95.4±0.8	0.012±0.000	7.7±1.4	0.2±0.9	6.5±0.5	95.4±0.4	0.011±0.000	13.2±2.1	0.0±0.7	4.8±0.5	97.3±0.1
slc44a2	fast	0.182±0.009	15.8±1.6	65.3±1.9	2.6±0.2	0.0±0.1	0.240±0.025	13.0±1.7	62.0±3.7	1.9±0.1	0.0±0.0	0.212±0.015	16.9±2.8	61.3±3.2	4.0±0.4	0.0±0.0
	middle	0.035±0.001	71.2±2.3	34.7±2.0	91.0±0.6	5.2±0.6	0.032±0.001	75.9±1.7	36.3±3.4	92.8±0.9	3.4±0.6	0.029±0.001	70.6±2.5	38.7±3.2	89.6±0.8	2.7±0.2
	slow	0.014±0.000	13.0±1.6	0.0±0.4	6.4±0.7	94.8±0.6	0.013±0.000	11.1±1.9	1.7±1.0	5.3±0.8	96.6±0.6	0.011±0.000	12.6±1.3	0.0±0.9	6.4±0.7	97.2±0.2
patB	fast	0.177±0.005	25.5±1.1	62.9±1.5	4.4±0.2	0.0±0.0	0.194±0.006	21.9±1.2	63.3±1.1	4.4±0.2	0.0±0.0	0.180±0.005	29.7±1.6	72.1±2.0	4.3±0.4	0.0±0.0
	middle	0.028±0.000	63.2±1.0	37.1±1.5	91.0±0.3	3.7±0.4	0.029±0.001	66.3±0.9	36.7±1.2	90.0±0.6	4.8±0.3	0.027±0.001	52.4±2.1	27.9±2.1	89.9±0.8	3.1±0.2
	slow	0.011±0.000	11.3±0.8	0.0±0.1	4.6±0.4	96.3±0.4	0.011±0.000	11.8±0.6	0.0±0.0	5.6±0.4	95.2±0.3	0.010±0.000	17.9±1.1	0.0±0.4	5.8±0.5	96.9±0.2

Table 3.5. (4/5) List of estimated hidden Markov model parameters.

Gene	State	Blebbistatin				
		D_{HMM} ($\mu\text{m}^2/\text{s}$)	P_{HMM}^0 (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)
DD3-3	fast	0.292±0.009	13.1±0.8	56.7±1.4	4.6±0.2	0.1±0.1
	middle	0.034±0.001	72.2±2.2	40.5±1.6	87.5±0.9	5.1±0.5
	slow	0.014±0.000	14.7±2.4	2.8±1.3	7.9±0.9	94.8±0.5
psiM	fast	0.143±0.005	33.0±1.4	73.5±1.0	2.7±0.1	0.1±0.0
	middle	0.019±0.001	50.4±1.3	26.2±0.8	93.6±0.3	2.4±0.2
	slow	0.007±0.000	16.5±1.3	0.3±0.4	3.7±0.2	97.5±0.1
tgrO1	fast	0.151±0.004	28.1±0.8	68.2±1.0	4.4±0.1	0.0±0.0
	middle	0.020±0.000	55.5±0.6	31.8±1.0	90.2±0.3	3.6±0.1
	slow	0.007±0.000	16.4±0.7	0.0±0.0	5.3±0.2	96.4±0.1
tgrC1	fast	0.186±0.007	27.7±0.9	64.9±1.2	5.3±0.3	0.0±0.0
	middle	0.025±0.001	54.8±0.9	35.1±1.1	88.5±0.4	3.2±0.2
	slow	0.008±0.000	17.5±0.5	0.0±0.3	6.2±0.3	96.8±0.2
comC	fast	0.166±0.006	26.8±1.0	68.3±1.0	3.7±0.2	0.0±0.0
	middle	0.023±0.001	57.9±1.3	31.7±1.0	91.3±0.4	3.4±0.2
	slow	0.008±0.000	15.3±0.8	0.0±0.0	5.0±0.3	96.6±0.2
tspE	fast	0.200±0.010	30.0±2.5	69.2±2.2	4.0±0.3	0.0±0.0
	middle	0.030±0.001	59.2±2.2	30.5±2.1	90.3±0.8	3.3±0.2
	slow	0.010±0.000	10.8±0.9	0.4±0.3	5.7±0.6	96.6±0.2
car2	fast	0.209±0.007	24.1±1.6	64.3±1.1	4.9±0.2	0.0±0.0
	middle	0.028±0.001	63.4±1.5	35.6±1.1	88.2±0.5	4.9±0.2
	slow	0.010±0.000	12.5±1.4	0.0±0.2	6.9±0.3	95.1±0.2
car1	fast	0.261±0.007	23.8±1.0	63.4±1.2	5.8±0.1	0.0±0.0
	middle	0.032±0.000	67.9±1.0	36.6±1.2	87.1±0.4	5.1±0.3
	slow	0.010±0.000	8.2±0.5	0.0±0.1	7.1±0.4	94.9±0.3
fscD	fast	0.217±0.012	20.7±1.5	62.8±1.4	3.6±0.2	0.0±0.0
	middle	0.029±0.001	65.8±1.4	37.2±1.5	90.2±0.6	3.7±0.2
	slow	0.011±0.000	13.4±1.0	0.0±0.4	6.2±0.4	96.3±0.2
car4	fast	0.208±0.011	26.1±1.5	64.1±1.7	3.8±0.1	0.0±0.0
	middle	0.025±0.001	61.1±1.6	35.9±1.7	91.0±0.4	3.1±0.2
	slow	0.009±0.000	12.8±0.6	0.0±0.0	5.2±0.3	96.9±0.2
fscE	fast	0.229±0.008	18.7±1.1	56.8±0.9	4.7±0.2	0.0±0.0
	middle	0.032±0.001	68.6±1.1	43.1±1.0	88.9±0.3	4.5±0.2
	slow	0.013±0.000	12.7±1.1	0.0±0.4	6.5±0.2	95.5±0.2
fscF	fast	0.207±0.008	28.6±1.6	63.5±1.4	4.0±0.2	0.0±0.1
	middle	0.031±0.001	63.7±1.5	36.3±1.4	91.4±0.4	5.0±0.2
	slow	0.011±0.000	7.7±0.9	0.2±0.3	4.6±0.4	95.0±0.3
car3	fast	0.205±0.005	25.7±0.5	65.9±1.4	4.3±0.2	0.1±0.1
	middle	0.029±0.001	57.9±1.0	33.2±1.8	87.4±0.2	4.8±0.2
	slow	0.010±0.000	16.4±0.8	0.9±0.6	8.3±0.2	95.1±0.2
fscA	fast	0.294±0.010	23.2±1.2	57.9±1.2	7.0±0.3	0.0±0.0
	middle	0.032±0.001	67.9±1.4	42.1±1.1	87.0±0.8	4.1±0.3
	slow	0.012±0.000	8.9±1.0	0.0±0.2	6.0±0.5	95.9±0.3

Gene	State	Blebbistatin				
		D_{HMM} ($\mu\text{m}^2/\text{s}$)	P_{HMM}^0 (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)
fslG	fast	0.213±0.007	21.2±1.0	65.0±1.1	4.3±0.2	0.0±0.0
	middle	0.030±0.001	64.4±1.4	35.0±1.0	89.6±0.5	4.3±0.2
	slow	0.011±0.000	14.4±0.6	0.0±0.4	6.2±0.5	95.7±0.2
fslQ	fast	0.245±0.005	37.8±0.9	73.3±0.7	4.7±0.2	0.0±0.0
	middle	0.039±0.001	53.7±1.2	26.3±0.8	90.0±0.5	4.2±0.3
	slow	0.013±0.000	8.5±0.7	0.4±0.3	5.3±0.4	95.8±0.3
fslF	fast	0.235±0.010	21.1±1.0	59.2±1.3	5.3±0.2	0.0±0.0
	middle	0.035±0.001	67.8±1.0	40.1±1.2	87.5±0.4	5.3±0.4
	slow	0.013±0.000	11.1±1.1	0.7±0.7	7.2±0.4	94.7±0.3
fslJ	fast	0.244±0.008	23.3±1.0	63.5±1.2	5.5±0.2	0.0±0.0
	middle	0.037±0.001	68.3±1.0	36.5±1.2	88.4±0.5	5.5±0.3
	slow	0.013±0.000	8.5±0.8	0.0±0.0	6.2±0.4	94.5±0.3
fslE	fast	0.220±0.008	25.4±1.1	66.0±1.7	4.2±0.3	0.0±0.0
	middle	0.031±0.001	63.6±1.2	34.0±1.7	90.1±0.7	2.9±0.2
	slow	0.011±0.000	10.9±1.1	0.0±0.1	5.7±0.4	97.1±0.2
fslN	fast	0.245±0.011	20.3±1.6	59.6±1.8	4.6±0.1	0.0±0.0
	middle	0.035±0.001	70.6±2.1	40.4±1.8	88.5±0.7	5.0±0.3
	slow	0.013±0.000	9.2±0.7	0.0±0.1	6.9±0.6	95.0±0.3
fslL	fast	0.206±0.010	28.8±2.1	69.5±1.9	4.8±0.3	0.1±0.0
	middle	0.031±0.002	61.9±1.7	30.5±1.9	89.6±0.7	3.7±0.3
	slow	0.011±0.000	9.4±0.9	0.0±0.0	5.7±0.5	96.3±0.2
fslH	fast	0.179±0.005	25.6±0.9	66.0±1.2	4.2±0.2	0.0±0.0
	middle	0.024±0.000	56.7±1.1	34.0±1.2	91.2±0.4	3.0±0.2
	slow	0.008±0.000	17.7±0.6	0.0±0.1	4.6±0.3	97.0±0.2
fslM	fast	0.231±0.010	22.0±1.5	61.6±1.3	4.5±0.2	0.0±0.0
	middle	0.034±0.001	69.3±1.6	37.8±1.3	90.0±0.6	4.1±0.3
	slow	0.013±0.000	8.8±1.0	0.6±0.6	5.5±0.4	95.9±0.3
griB	fast	0.350±0.012	8.3±0.9	50.0±2.4	4.6±0.6	0.0±0.0
	middle	0.034±0.001	75.3±3.8	50.0±2.6	84.6±1.3	4.9±0.9
	slow	0.015±0.000	16.5±3.3	0.0±0.7	10.8±1.1	95.1±0.9
griO	fast	0.230±0.009	15.5±1.1	63.1±2.0	3.8±0.3	0.0±0.0
	middle	0.031±0.001	67.0±1.3	36.9±2.0	88.6±0.6	3.4±0.3
	slow	0.013±0.000	17.5±1.2	0.0±0.3	7.5±0.5	96.6±0.3
slc44a2	fast	0.200±0.014	25.1±2.1	67.6±1.9	3.2±0.2	0.0±0.0
	middle	0.032±0.002	61.7±1.7	32.3±1.9	91.2±0.9	2.8±0.3
	slow	0.013±0.000	13.2±2.0	0.0±0.4	5.6±0.7	97.2±0.3
patB	fast	0.179±0.006	27.2±1.7	66.5±0.8	4.0±0.1	0.0±0.0
	middle	0.026±0.001	57.9±1.6	33.5±0.8	90.1±0.6	3.7±0.2
	slow	0.010±0.000	14.9±0.8	0.0±0.3	5.9±0.5	96.3±0.2

Table 3.5. (5/5) List of estimated hidden Markov model parameters.

The estimated values are shown as means±95% CI. To calculate CI, the bootstrap method was used (see Methods).

3.1.7 Membrane protein size does not significantly affect lateral diffusion.

Two general physical models have been proposed to explain the lateral diffusion of membrane proteins (Saffman and Delbrück, 1975; Gambin et al., 2006). In the Saffman-Delbrück model (SD model), the diffusion coefficient is inversely proportional to the membrane viscosity, μ_m , and the log of the radius, R , of the protein embedded in the membrane (Eq. 9), meaning that protein size is not a major determinant for lateral diffusion. In the Stokes-Einstein-like model (SE model), the diffusion coefficient is inversely proportional to μ_m and R (Eq. 10).

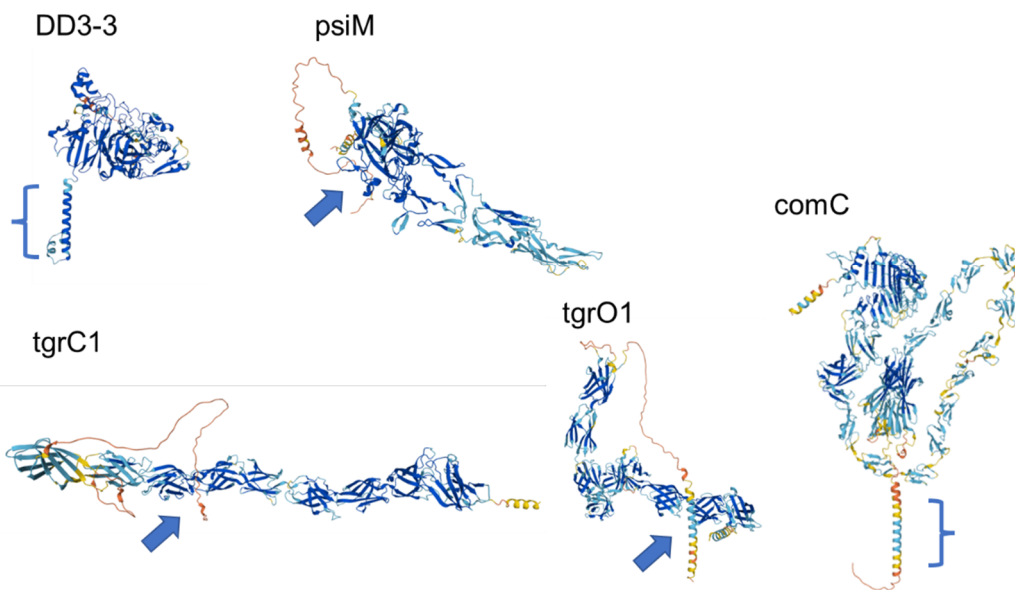
The structure of each molecule was predicted using AlphaFold to compare the models and measurements (Jumper et al., 2021; Varadi et al., 2021) (Fig. 3.1.10). Although some molecules were less accurately predicted in regions outside the plasma membrane, the overall structures were consistent for each protein family. For the single-transmembrane proteins psiM and tgrC1, the predicted transmembrane regions in the database were not well predicted. The nine-transmembrane slc44a2 was predicted to have the N- and C-termini on the same side, despite the odd number of transmembrane regions. The radii of the molecules were measured from the predicted structures using PyMol. This estimation did not take into account the oligomerization of the molecules and treats them as monomers.

Figure 3.1.11 shows that the middle- and slow-mobile states were more consistent with the SD model than the SE model, meaning that the radius diversity ranging from 1 to 10 transmembrane regions has no significant contribution to the diffusion of these two states. Since all molecular species adopted the middle- or slow-mobile states for more than 92% of trajectories under all conditions measured, the lateral mobility of membrane proteins is determined primarily by differences in membrane viscosity and not the intrinsic structural variability of the proteins. The viscosity of the membrane, μ_m , was estimated by fitting to the SD models: 1.5×10^2 and 3.1×10^2 Pa·s for the middle- and slow-mobile states, respectively. The estimated μ_m of the fast state (5.1×10 Pa·s) was also larger than estimated previously in mammalian cells (~ 1.0 Pa·s) (Kashirina et al., 2020), meaning that viscosity is a major determinant also for mobility of the fast-mobile state. These results are consistent with three states reflecting free diffusion in three membrane fields with different viscosities.

The predicted molecular structures show that the molecules have regions of various sizes outside the plasma membrane: the single-transmembrane molecules all have very large structures in the extracellular region relative to the transmembrane region; the seven-transmembrane molecules have large extracellular structures in the fsl and grl

families compared to the car family, which has a small extracellular region. The ten-transmembrane molecule patB has a large structure in the cytoplasmic region. Although detailed comparisons are not possible in the present study because the extramembrane region cannot be approximated by a simple shape, the size of the extramembrane region generally has little effect on molecular diffusion, given the results of previous analyses in which diffusion coefficients did not vary greatly among the species.

Number of transmembrane: 1

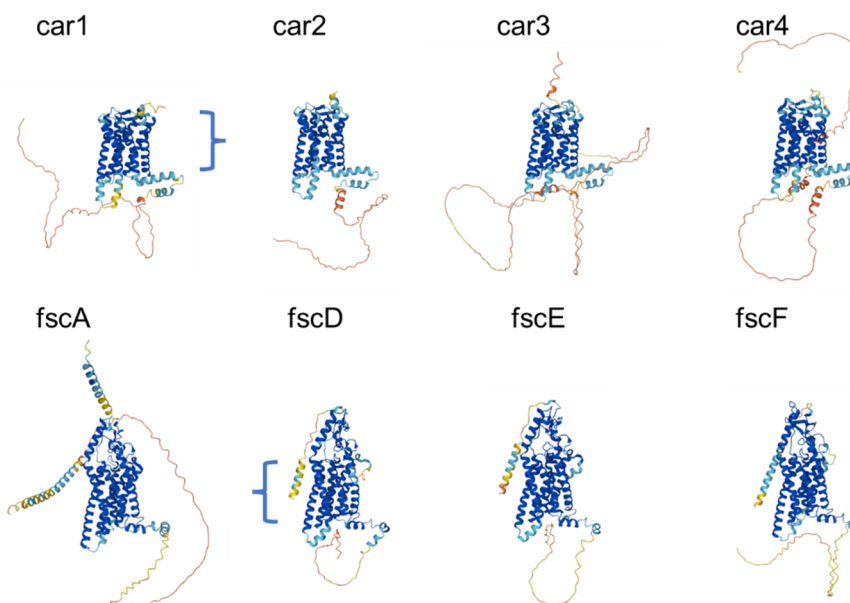


Number of transmembrane: 4

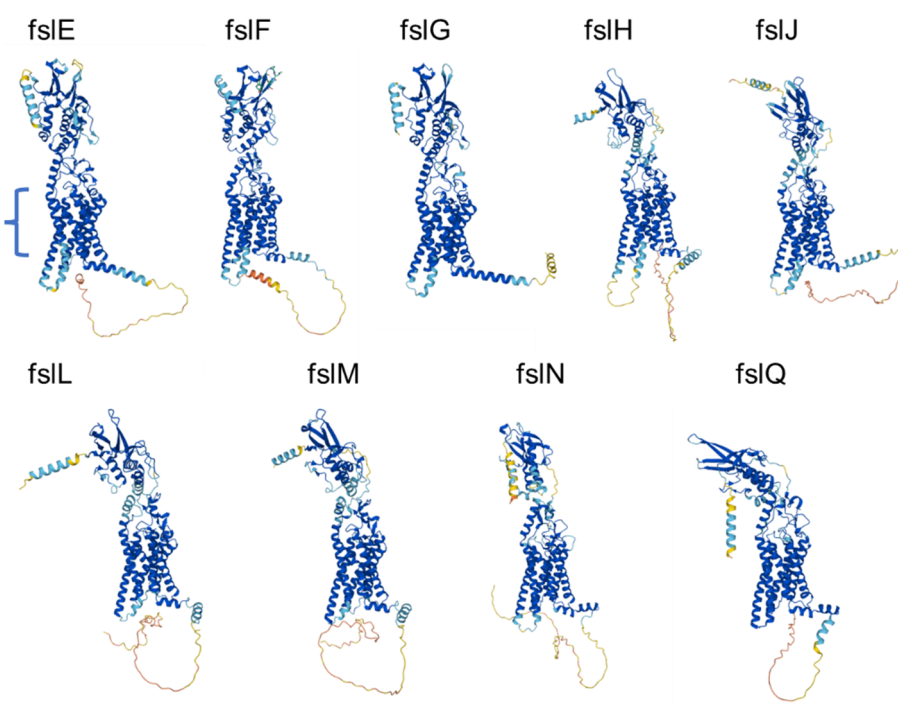
tspE



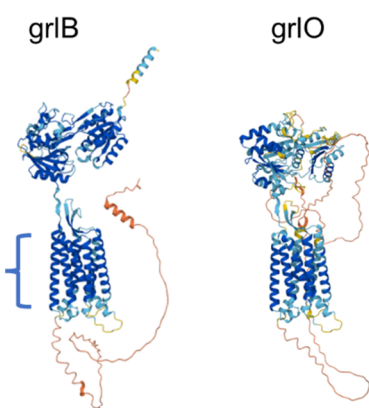
Number of transmembrane: 7



Number of transmembrane: 7

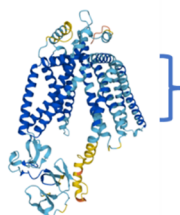


Number of transmembrane: 7



Number of transmembrane: 9

slc44a2



Number of transmembrane: 10

patB

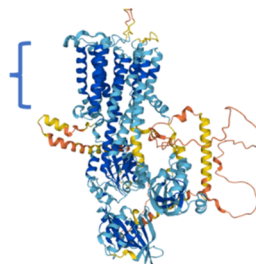


Fig. 3.1.10. Structures of the transmembrane proteins predicted using AlphaFold.

3D structures predicted using the AlphaFold of the 27 transmembrane proteins used in the measurements. The transmembrane regions of the molecules are positioned vertically, with the extracellular regions on top and the intracytoplasmic regions on the bottom. The regions indicated by arrows or brackets represent transmembrane regions. The color of each part of the molecules represents the confidence level of the prediction; the bluer the color, the higher the confidence level, and the redder the lower the confidence level. For psiM and tgrC1, the transmembrane regions registered in the database are not correctly predicted.

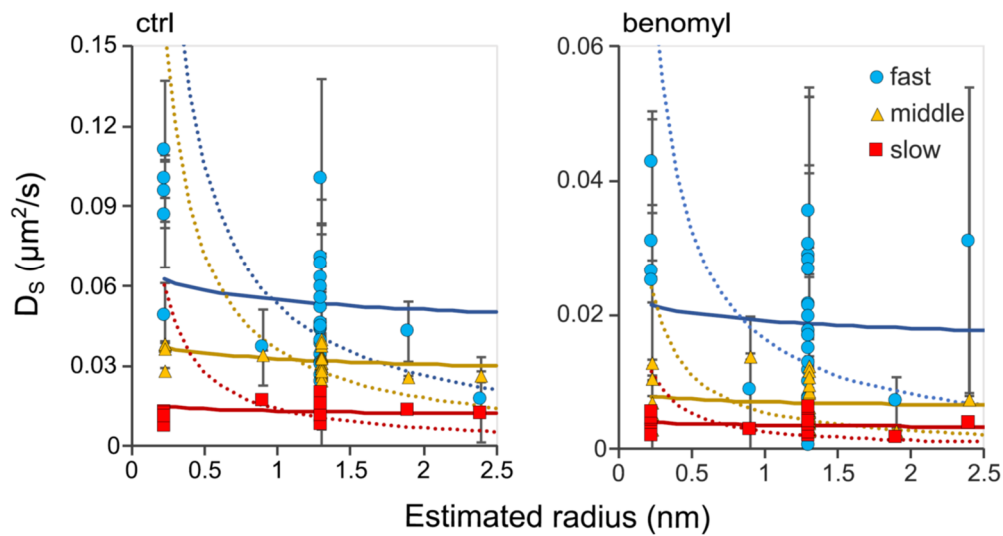


Fig. 3.1.11 Lateral diffusion of membrane proteins is defined by three different states.

Diffusion coefficients (D_s , mean $\pm$ 95% CI) for the fast (blue), middle (yellow), and slow states (red) of the 27 membrane proteins are plotted over the estimated radii of the proteins. The solid and dotted lines show theoretical states derived from the Saffman-Delbrück model and Stokes-Einstein-like model, respectively.

3.1.8 Each of the three diffusion states has a unique lifetime.

An HMM analysis of single molecule trajectories can provide time duration data for each state, from which the lifetimes of each state can be obtained (Fig. 3.1.12 ctrl). Because the lifetimes reflect the escape from the corresponding diffusion area, a larger area results in longer lifetimes. For each of the 27 protein species, the middle- and slow-mobile states exhibited longer lifetimes than those of the fast-mobile state, although there were species-dependent variations (Fig. 3.1.13A, Table 3.6). Benomyl-treated cells exhibited shorter, intermediate and longer lifetimes for the fast-, middle- and slow-mobile states, respectively, which is similar with the untreated control (Fig. 3.1.12 benomyl and Fig. 3.1.13B, Table 3.6). The same tendency was also observed under all other drug-treated conditions (Fig. 3.1.12, Table 3.6). The average lifetimes and diffusion coefficients of the three states independent of the protein species were determined from summing the trajectory data of all proteins to surmise the overall tendency (Fig. 3.1.14A-D; Table 3.8). The average lifetimes for each of the 27 proteins was also determined to examine protein-specific variations (Fig. 3.1.14E, F). Scatter plots of the averaged lifetimes and diffusion coefficients clearly indicate three discrete states with relatively constant lifetimes and drug-dependent mobility (Fig. 3.1.14G). These results imply that three diffusion fields are maintained at their average sizes but with changes in their viscosities by the drug treatment. The HMM analysis also revealed almost no transition between the fast- and slow-mobile states, suggesting physical separation of the membrane fields corresponding to these two states (Fig. 3.2.4, Table 3.9).

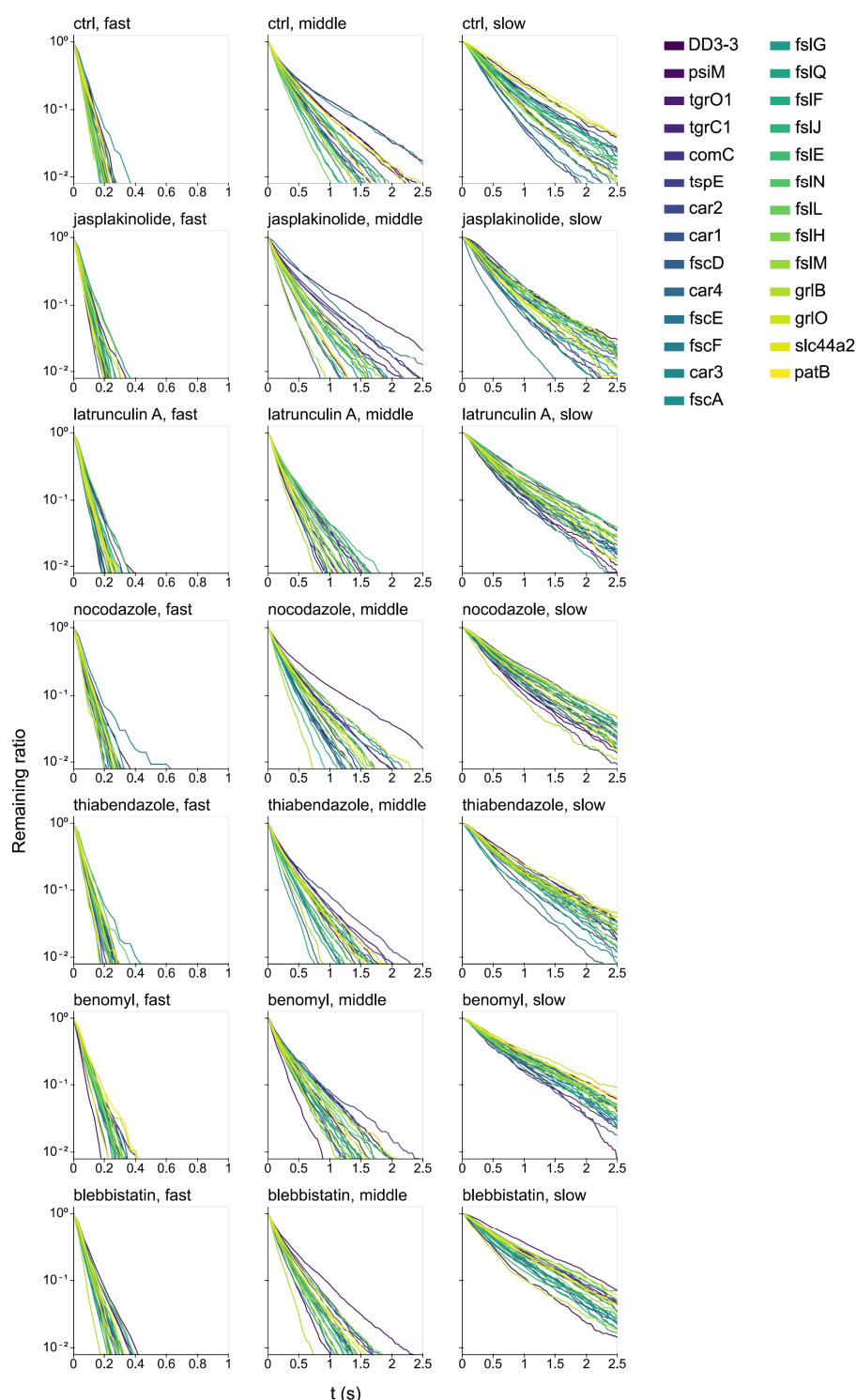


Fig. 3.1.12. Lifetimes of three diffusion states under drug-treated conditions. Lifetimes of the fast, middle and slow states. The mobilities of the 27 transmembrane proteins were observed under the control and seven drug conditions. Using the HMM, the mobilities were separated into three states, from which the lifetimes were calculated. The data of the 27 transmembrane proteins are represented in different colors.

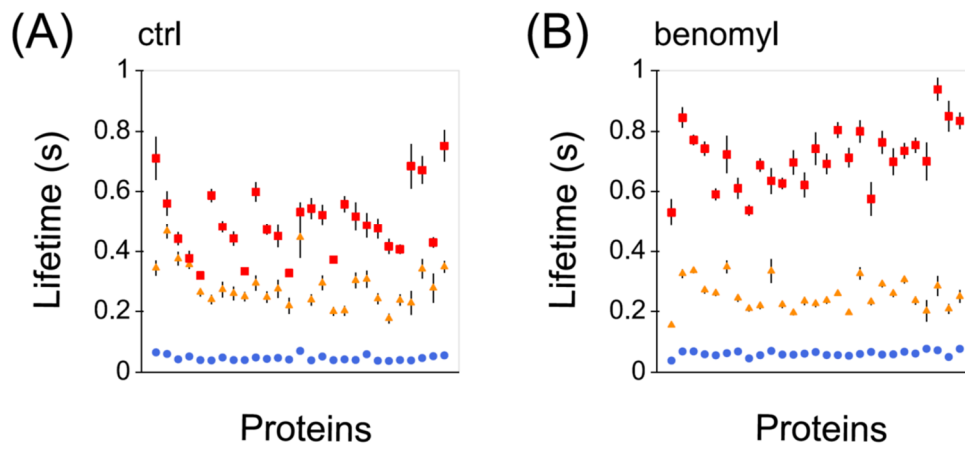


Fig. 3.1.13 Each of the three diffusion states has a unique lifetime.

The average lifetimes obtained by fitting the decay curves to exponentials (see Methods) ($\text{mean} \pm 95\% \text{ CI}$). (A) Ctrl condition, (B) Benomyl-treated condition. ($n = 10$ bootstrap datasets created from trajectory data of 10 cells)

Gene	State	Ctrl (s)	Jasplakinolide (s)	Latrunculin A (s)	Nocodazole (s)	Thiabendazole (s)	Benomyl (s)	Blebbistatin (s)
DD3-3	fast	0.066±0.003	0.046±0.003	0.043±0.002	0.052±0.002	0.058±0.004	0.039±0.003	0.049±0.002
	middle	0.345±0.025	0.274±0.019	0.155±0.009	0.206±0.008	0.239±0.013	0.156±0.007	0.174±0.009
	slow	0.710±0.071	0.575±0.055	0.380±0.020	0.447±0.024	0.606±0.031	0.531±0.044	0.435±0.028
psiM	fast	0.061±0.003	0.063±0.003	0.066±0.004	0.064±0.003	0.058±0.003	0.069±0.004	0.078±0.002
	middle	0.466±0.024	0.539±0.015	0.257±0.020	0.484±0.034	0.312±0.012	0.326±0.014	0.365±0.013
	slow	0.560±0.039	0.594±0.034	0.692±0.023	0.508±0.013	0.710±0.029	0.844±0.034	0.938±0.039
tgrO1	fast	0.043±0.002	0.037±0.002	0.043±0.002	0.044±0.003	0.050±0.002	0.069±0.002	0.071±0.003
	middle	0.375±0.022	0.221±0.008	0.201±0.007	0.256±0.006	0.299±0.010	0.336±0.008	0.281±0.007
	slow	0.441±0.022	0.373±0.018	0.427±0.009	0.461±0.022	0.618±0.019	0.770±0.017	0.747±0.016
tgrC1	fast	0.052±0.002	0.044±0.002	0.049±0.003	0.050±0.003	0.053±0.002	0.060±0.003	0.062±0.003
	middle	0.355±0.014	0.386±0.006	0.255±0.012	0.313±0.016	0.248±0.008	0.272±0.012	0.226±0.009
	slow	0.376±0.025	0.546±0.025	0.491±0.021	0.431±0.015	0.610±0.021	0.741±0.023	0.779±0.028
comC	fast	0.040±0.002	0.042±0.002	0.045±0.003	0.049±0.003	0.045±0.003	0.056±0.001	0.070±0.002
	middle	0.264±0.014	0.350±0.010	0.244±0.014	0.303±0.018	0.251±0.015	0.262±0.009	0.297±0.013
	slow	0.320±0.011	0.495±0.022	0.389±0.015	0.510±0.016	0.464±0.024	0.591±0.020	0.754±0.027
tspE	fast	0.039±0.003	0.056±0.003	0.063±0.005	0.062±0.003	0.048±0.004	0.063±0.004	0.064±0.004
	middle	0.240±0.016	0.357±0.030	0.193±0.014	0.266±0.014	0.338±0.025	0.348±0.020	0.218±0.014
	slow	0.587±0.021	0.550±0.035	0.638±0.026	0.636±0.047	0.536±0.028	0.722±0.061	0.716±0.027
car2	fast	0.049±0.003	0.044±0.001	0.039±0.002	0.048±0.002	0.056±0.002	0.069±0.003	0.061±0.003
	middle	0.273±0.025	0.205±0.005	0.177±0.008	0.220±0.010	0.301±0.011	0.245±0.011	0.215±0.009
	slow	0.482±0.019	0.373±0.009	0.436±0.017	0.615±0.011	0.683±0.025	0.611±0.035	0.587±0.022
car1	fast	0.041±0.002	0.038±0.001	0.044±0.002	0.055±0.002	0.039±0.001	0.046±0.003	0.056±0.002
	middle	0.261±0.022	0.151±0.008	0.171±0.006	0.301±0.008	0.154±0.007	0.211±0.009	0.191±0.006
	slow	0.442±0.023	0.343±0.011	0.439±0.024	0.735±0.025	0.359±0.012	0.538±0.018	0.531±0.028
fscD	fast	0.040±0.002	0.049±0.002	0.038±0.002	0.057±0.004	0.049±0.003	0.057±0.002	0.057±0.003
	middle	0.250±0.016	0.302±0.010	0.177±0.010	0.214±0.014	0.255±0.009	0.220±0.011	0.236±0.013
	slow	0.334±0.011	0.468±0.018	0.457±0.022	0.547±0.014	0.576±0.026	0.687±0.021	0.699±0.022
car4	fast	0.049±0.003	0.051±0.002	0.051±0.003	0.056±0.002	0.050±0.002	0.071±0.004	0.058±0.002
	middle	0.294±0.025	0.307±0.017	0.248±0.013	0.196±0.014	0.210±0.007	0.334±0.039	0.256±0.010
	slow	0.599±0.031	0.376±0.017	0.541±0.017	0.554±0.023	0.519±0.012	0.635±0.043	0.777±0.032
fscE	fast	0.044±0.002	0.045±0.002	0.049±0.002	0.049±0.003	0.053±0.003	0.059±0.005	0.049±0.001
	middle	0.248±0.018	0.203±0.008	0.196±0.010	0.207±0.008	0.204±0.012	0.224±0.012	0.225±0.006
	slow	0.472±0.018	0.475±0.018	0.511±0.017	0.602±0.024	0.556±0.027	0.627±0.018	0.615±0.017
fscF	fast	0.048±0.003	0.052±0.002	0.068±0.002	0.078±0.005	0.056±0.004	0.058±0.006	0.057±0.003
	middle	0.276±0.029	0.225±0.011	0.261±0.014	0.327±0.051	0.270±0.018	0.197±0.009	0.265±0.011
	slow	0.450±0.037	0.459±0.020	0.617±0.036	0.605±0.037	0.636±0.035	0.696±0.041	0.562±0.024
car3	fast	0.042±0.002	0.044±0.002	0.051±0.001	0.051±0.003	0.059±0.002	0.062±0.004	0.062±0.002
	middle	0.220±0.026	0.249±0.009	0.243±0.007	0.209±0.008	0.205±0.017	0.235±0.014	0.202±0.003
	slow	0.328±0.012	0.404±0.015	0.501±0.013	0.488±0.022	0.404±0.009	0.621±0.041	0.601±0.014
fscA	fast	0.071±0.005	0.070±0.004	0.049±0.003	0.065±0.004	0.072±0.006	0.067±0.005	0.048±0.002
	middle	0.445±0.067	0.265±0.051	0.167±0.014	0.343±0.028	0.254±0.014	0.227±0.011	0.170±0.009
	slow	0.532±0.031	0.507±0.059	0.483±0.033	0.672±0.033	0.591±0.032	0.742±0.053	0.629±0.028

Table 3.6. (1/2) Lifetimes of three diffusion states of the 27 membrane proteins under drug-treated conditions.

Gene	State	Ctrl (s)	Jasplakinolide (s)	Latrunculin A (s)	Nocodazole (s)	Thiabendazole (s)	Benomyl (s)	Blebbistatin (s)
fslG	fast	0.040±0.003	0.045±0.002	0.053±0.002	0.056±0.002	0.048±0.004	0.057±0.005	0.062±0.002
	middle	0.239±0.018	0.205±0.012	0.202±0.013	0.231±0.008	0.215±0.007	0.238±0.012	0.234±0.010
	slow	0.544±0.035	0.363±0.019	0.576±0.019	0.655±0.015	0.548±0.028	0.691±0.033	0.611±0.021
fslQ	fast	0.052±0.003	0.058±0.004	0.039±0.003	0.059±0.005	0.050±0.002	0.057±0.002	0.074±0.002
	middle	0.295±0.024	0.301±0.027	0.176±0.009	0.256±0.014	0.182±0.010	0.260±0.006	0.217±0.009
	slow	0.522±0.034	0.507±0.027	0.499±0.017	0.687±0.027	0.517±0.026	0.803±0.026	0.596±0.024
fslF	fast	0.040±0.002	0.054±0.004	0.055±0.003	0.060±0.004	0.040±0.002	0.055±0.002	0.052±0.002
	middle	0.201±0.015	0.269±0.025	0.208±0.008	0.197±0.011	0.124±0.006	0.197±0.008	0.198±0.008
	slow	0.372±0.011	0.407±0.023	0.390±0.023	0.535±0.027	0.414±0.021	0.712±0.032	0.533±0.023
fslJ	fast	0.043±0.003	0.051±0.002	0.054±0.003	0.051±0.004	0.053±0.002	0.061±0.002	0.058±0.002
	middle	0.203±0.016	0.254±0.015	0.226±0.013	0.279±0.017	0.262±0.008	0.326±0.020	0.214±0.009
	slow	0.558±0.027	0.534±0.041	0.577±0.015	0.680±0.018	0.585±0.019	0.799±0.037	0.479±0.020
fslE	fast	0.041±0.002	0.045±0.002	0.060±0.005	0.040±0.004	0.055±0.003	0.067±0.007	0.060±0.003
	middle	0.303±0.025	0.276±0.017	0.271±0.028	0.161±0.009	0.216±0.009	0.233±0.011	0.211±0.013
	slow	0.517±0.047	0.513±0.042	0.703±0.041	0.497±0.026	0.613±0.029	0.576±0.055	0.839±0.042
fslN	fast	0.060±0.003	0.051±0.002	0.055±0.003	0.059±0.004	0.054±0.003	0.059±0.003	0.052±0.003
	middle	0.307±0.029	0.252±0.013	0.263±0.020	0.269±0.012	0.203±0.021	0.292±0.011	0.206±0.012
	slow	0.487±0.041	0.598±0.027	0.537±0.024	0.634±0.025	0.532±0.024	0.762±0.037	0.585±0.023
fslL	fast	0.038±0.003	0.043±0.003	0.070±0.003	0.057±0.002	0.071±0.006	0.060±0.005	0.068±0.005
	middle	0.243±0.018	0.208±0.014	0.263±0.007	0.259±0.011	0.266±0.028	0.261±0.012	0.216±0.016
	slow	0.476±0.034	0.505±0.014	0.683±0.037	0.712±0.025	0.570±0.032	0.698±0.044	0.680±0.027
fslH	fast	0.038±0.004	0.046±0.002	0.042±0.004	0.050±0.002	0.055±0.002	0.068±0.002	0.065±0.003
	middle	0.178±0.016	0.254±0.016	0.179±0.007	0.252±0.014	0.259±0.014	0.306±0.012	0.274±0.007
	slow	0.416±0.024	0.557±0.015	0.603±0.017	0.640±0.018	0.588±0.014	0.734±0.026	0.795±0.020
fslM	fast	0.041±0.003	0.064±0.004	0.048±0.002	0.050±0.001	0.050±0.003	0.062±0.003	0.057±0.002
	middle	0.238±0.018	0.224±0.014	0.241±0.012	0.241±0.008	0.239±0.013	0.237±0.013	0.231±0.010
	slow	0.407±0.015	0.453±0.030	0.469±0.018	0.684±0.020	0.547±0.023	0.753±0.025	0.668±0.038
grlB	fast	0.039±0.002	0.036±0.003	0.046±0.002	0.043±0.004	0.041±0.010	0.078±0.008	0.038±0.003
	middle	0.229±0.039	0.145±0.013	0.140±0.009	0.138±0.017	0.159±0.030	0.203±0.035	0.131±0.009
	slow	0.684±0.073	0.436±0.070	0.467±0.038	0.355±0.019	0.599±0.094	0.700±0.062	0.415±0.027
grlO	fast	0.047±0.004	0.040±0.003	0.044±0.004	0.040±0.006	0.041±0.004	0.073±0.003	0.055±0.003
	middle	0.341±0.032	0.305±0.027	0.200±0.013	0.331±0.039	0.252±0.012	0.285±0.033	0.190±0.007
	slow	0.671±0.046	0.551±0.046	0.562±0.051	0.559±0.045	0.634±0.033	0.938±0.037	0.730±0.034
slc44a2	fast	0.053±0.006	0.054±0.007	0.056±0.005	0.063±0.005	0.055±0.006	0.051±0.005	0.065±0.004
	middle	0.278±0.048	0.275±0.026	0.192±0.013	0.270±0.015	0.266±0.023	0.210±0.016	0.231±0.023
	slow	0.429±0.016	0.436±0.039	0.507±0.025	0.568±0.047	0.719±0.080	0.849±0.051	0.833±0.047
patB	fast	0.056±0.004	0.039±0.002	0.050±0.003	0.060±0.003	0.062±0.002	0.077±0.004	0.067±0.002
	middle	0.348±0.020	0.221±0.021	0.175±0.016	0.286±0.008	0.262±0.012	0.250±0.021	0.264±0.018
	slow	0.751±0.052	0.446±0.025	0.607±0.024	0.721±0.045	0.587±0.023	0.833±0.027	0.730±0.019

Table 3.6. (2/2) Lifetimes of three diffusion states of the 27 membrane proteins under drug-treated conditions.

Using the HMM, single-molecule trajectories were separated into three states, and the lifetime of each was calculated. The estimated values are shown as means±95% CI. To calculate CI, the bootstrap method was used (see Methods).

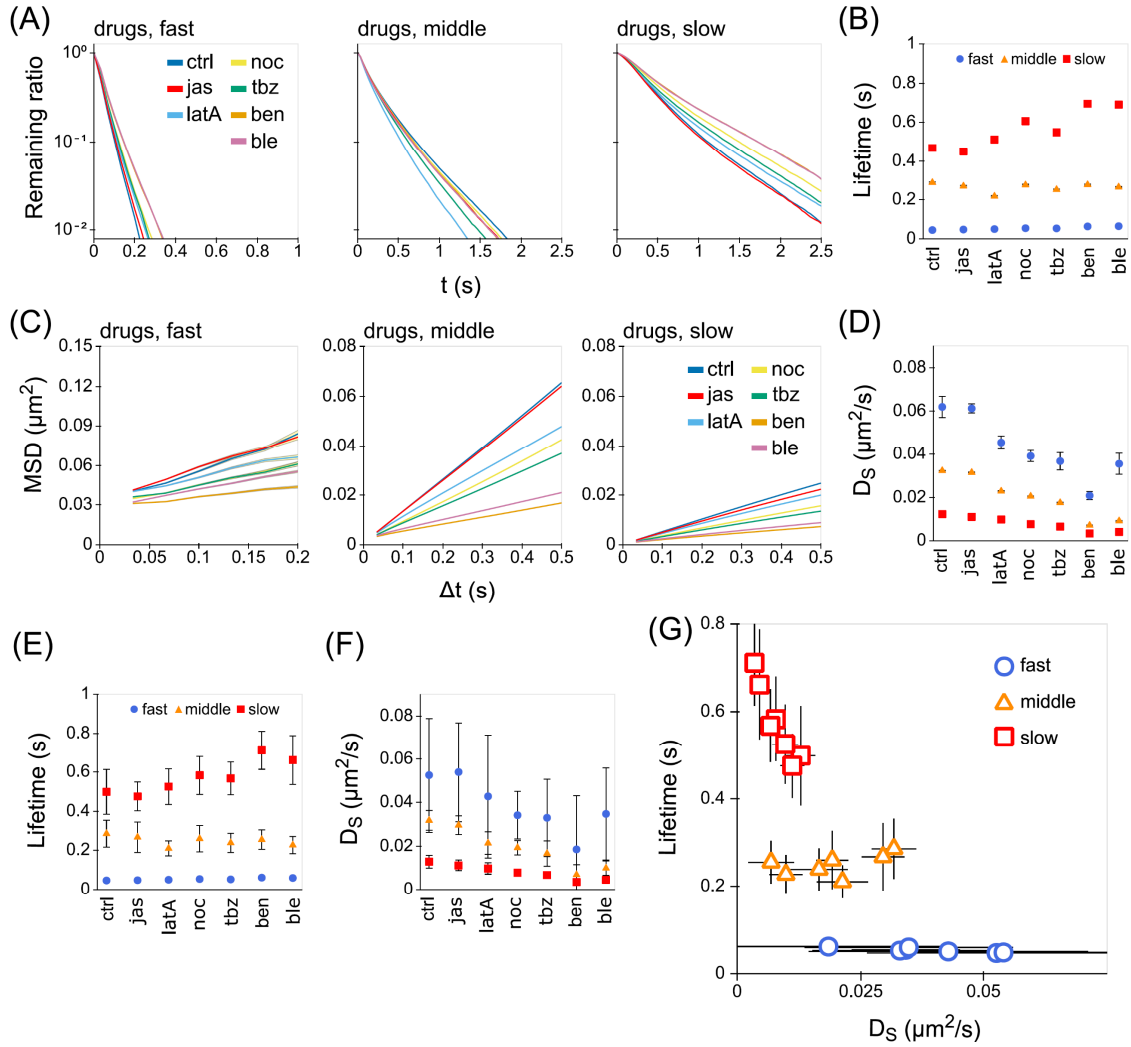


Fig. 3.1.14. Each of the three diffusion states has a unique lifetime.

(A-D) The lifetimes (A), average lifetimes (mean $\pm$ 95% CI) (B), MSD (C) and D_s (mean $\pm$ 95% CI) (D) were obtained from summing the data of the 27 proteins under control and drug-treated conditions, shown in different colors. (n = 10 bootstrap datasets created from trajectory data of 10 cells)

(E, F) The average lifetimes (E) and D_s (F) for the 27 proteins (mean $\pm$ SD).

(G) The relationship between D_s and lifetimes as a scatter plot (mean $\pm$ SD).

3.1.9 Comparison of HMM analysis results in 2 and 3 states

The estimated ratio of fast states by HMM analysis was very low for all types of molecules (2.1-7.2% under the ctrl condition), with approximately 95% of the remaining states being occupied by middle and slow states. To demonstrate that the fast state in the three-state model was not redundant for describing the measurement data, I performed HMM analysis using a two-state model. The results showed that the estimated diffusion parameters for car1 in the two-state model were different from those in the three-state model (Table 3.7). However, the fast-mobile state detected in the two-state model was a minor component with a short lifetime and large localization errors, which was similar to the fast-mobile state detected in the three-state model. Furthermore, the PDF analysis detected the fast component for all 27 protein species (Fig. 3.1.6B), and the AIC results (Fig. 3.1.6D) supported the necessity of including the fast state to accurately describe the data.

Number of diffusion states	State	D_s ($\mu\text{m}^2/\text{s}$)	P_s (%)	ϵ_s (nm)	Lifetime (s)
2	fast	0.041 ± 0.006	11.2 ± 0.5	87 ± 5	0.068 ± 0.002
	slow	0.022 ± 0.000	88.8 ± 0.5	7 ± 2	0.573 ± 0.010
3	fast	0.071 ± 0.013	4.0 ± 0.3	88 ± 5	0.041 ± 0.002
	middle	0.034 ± 0.001	52.8 ± 2.5	11 ± 5	0.261 ± 0.022
	slow	0.013 ± 0.001	43.3 ± 2.7	10 ± 2	0.442 ± 0.023

Table. 3.7. Comparison of HMM analysis results between 2-state and 3-state models

Results of HMM analysis under ctrl condition for car1. The estimated values are shown as means $\pm$ 95% CI. To calculate CI, the bootstrap method was used (see Methods).

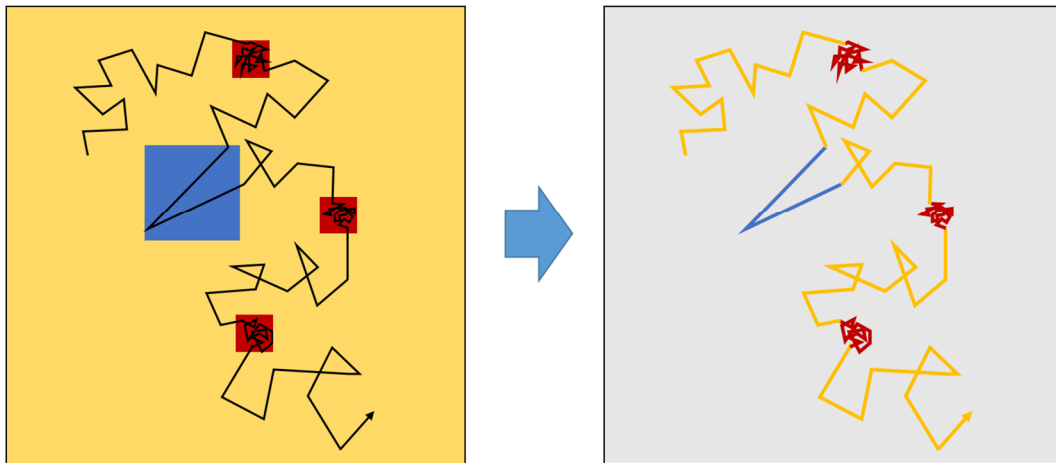
3.2 Results (simulation)

3.2.1 Field model for multistate lateral diffusion of various transmembrane proteins

Based on the analysis that the diffusion of transmembrane proteins is determined by the cell membrane rather than by the type of protein, I hypothesized that the three diffusion states consist of three regions of the cell membrane with different viscosities (Fig. 3.2.1A). I devised a novel model in which diffusion is determined by the cell membrane alone, the cell membrane field model, and verified whether it can reproduce diffusion as in the measured data. The membrane field consists of three regions corresponding to fast, middle and slow simple diffusion, in which fast- and slow-mobile squares are placed randomly on the basement of the middle-mobile region spread out all over (Fig. 3.2.1B, Fig. 3.2.2). Since there were few transitions between fast and slow states from the HMM analysis, fast and slow regions were arranged so that they are not adjacent to each other (Fig. 3.2.4). Each particle undergoes simple diffusion in each region with the corresponding diffusion coefficients that were experimentally determined (Fig. 3.1.14C, D; Table 3.8). Four parameters are used to create the field model: the sizes of the fast and slow regions and the area ratios of these regions to the entire field. The sizes of the fast and slow regions were determined by the simulation of the particle diffusion on the field. Because larger sizes result in longer lifetimes, the region sizes were set based on the lifetimes of the fast- and slow-mobile states obtained experimentally (Fig. 3.2.3A). For example, the region sizes are estimated to be 50 and 250 nm for the fast- and slow-mobile states in control cells, respectively (Table 3.7). Then I searched for the occupied % area of the fast and slow regions that made the MSD and HMM results from the particle trajectories match the measured values. It is important to note that the ratio of each diffusion state does not equal the occupied % area of each region. I found that when particles are diffused over a field, they tend to cluster in slower regions. For a completely random arrangement, the ratio of each diffusion state and the occupied % area of each region will match, but the arrangement of the particles in the steady state on the field will not match because of the bias toward the slower regions. Therefore, I searched for a value range for the occupied % area such that the ratio of each diffusion state and the ratio of particles in each region would match. Furthermore, the steady placement of particles on the field and the breakdown of molecule tracking were implemented, which are described in section 3.2.2 and 3.2.3. As a result, for each drug condition, I found a field that reproduces the experimental measurements of the lateral diffusion (Fig. 3.2.2, Table 3.7).

The field model I propose here was consistent with the data of the MSD (Fig. 3.2.3D, Table 3.8), lifetimes (Fig. 3.2.3E, Table 3.8), and HMM (Fig. 3.2.4, Table 3.9).

(A)



(B)

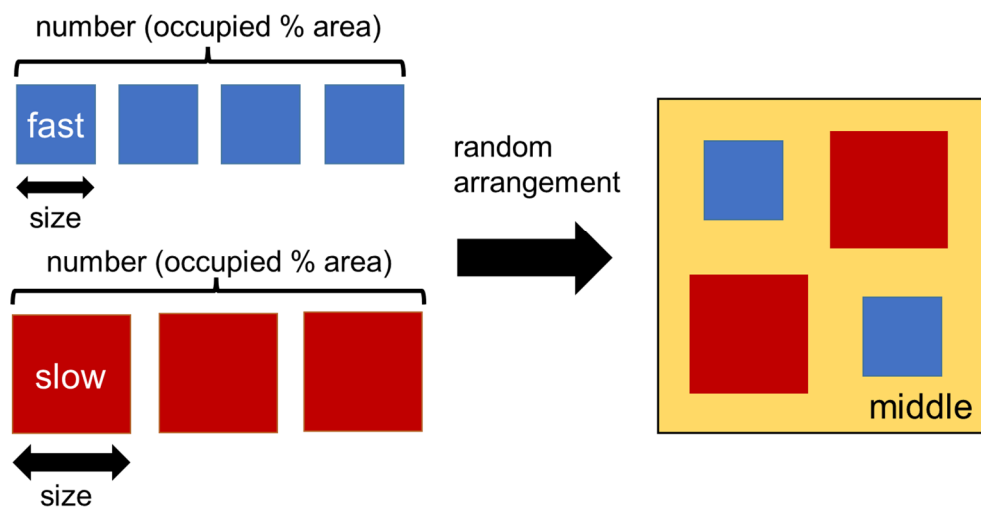


Fig. 3.2.1. Schematic of the cell membrane field model

(A) Assumptions of the field model. Molecular diffusion through the cell membrane, which consists of three regions of different viscosity (left), creates trajectories consisting of three diffusion states (right). (B) Method of creating a field model. The field is created by randomly arranging squares of fast and slow regions in a field consisting entirely of middle regions. The field consists of four parameters: the sizes of the fast and slow regions and their ratios of the total area.

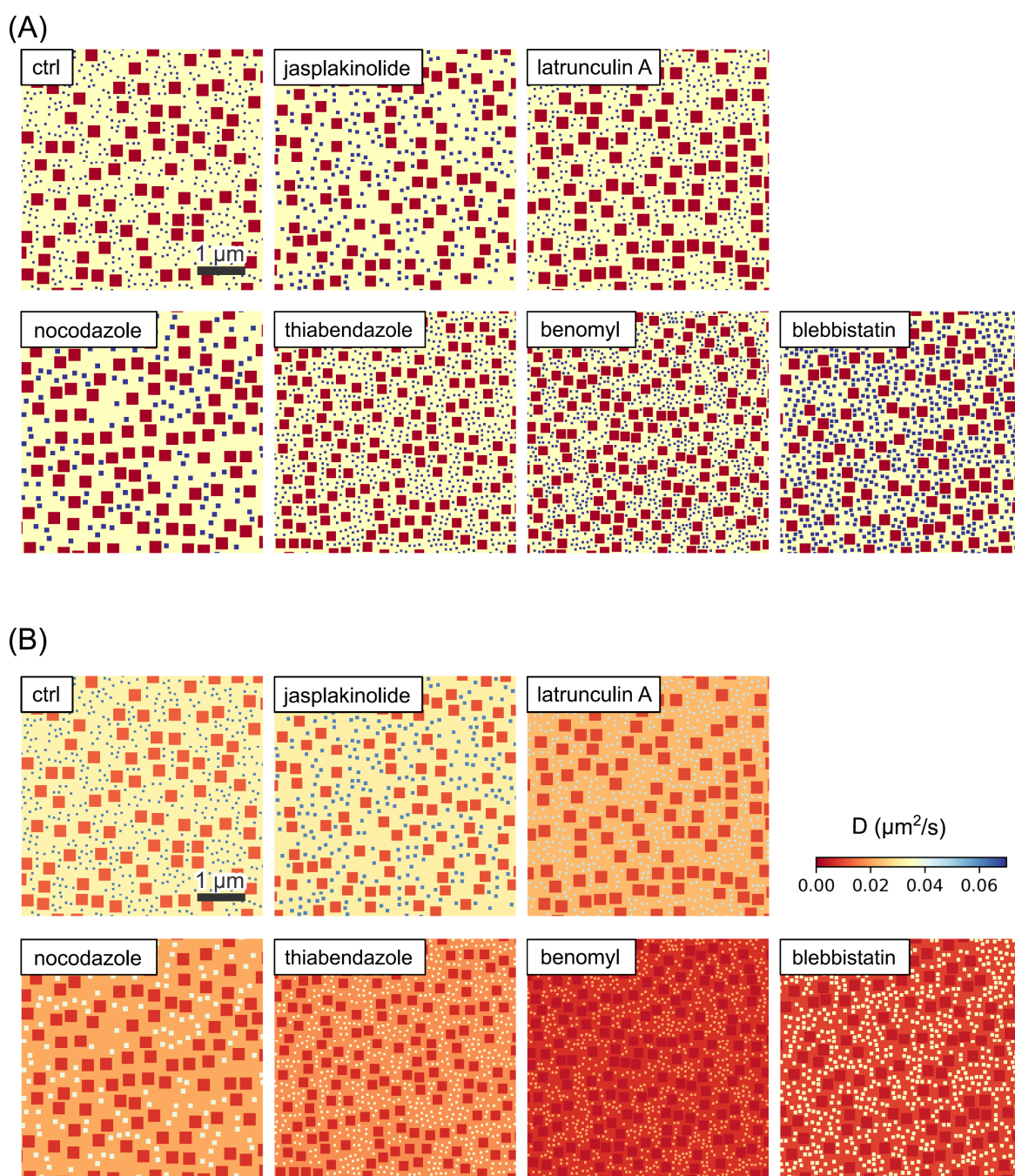


Fig. 3.2.2. Field models with three distinct regions corresponding to three diffusion states.

(A) Field models for the lateral diffusion of the transmembrane proteins under control and drug-treated conditions. The fast, middle, and slow states are represented by the blue, yellow, and red areas, respectively. (B) The fields are redisplayed as heat maps of the magnitudes of the diffusion coefficients for each region. The size of the fields is $5 \mu\text{m} \times 5 \mu\text{m}$. Scale bar, $1 \mu\text{m}$.

Drug	Size of a fast state squares (nm)	Size of a slow state squares (nm)	Ratio of the fast state area (%)	Ratio of the slow state area (%)	D_{S-fast} ($\mu m^2/s$)	$D_{S-middle}$ ($\mu m^2/s$)	D_{S-slow} ($\mu m^2/s$)	ϵ_{S-fast} (nm)	$\epsilon_{S-middle}$ (nm)	ϵ_{S-slow} (nm)
ctrl	50	250	5	20	0.062	0.032	0.012	88	12	10
jasplakinolide	75	225	6	19	0.061	0.031	0.011	92	15	13
latrunculin A	50	250	6	26	0.046	0.023	0.010	91	24	13
nocodazole	100	250	5	25	0.039	0.021	0.008	86	16	12
thiabendazole	50	200	8	23	0.037	0.018	0.007	87	21	13
benomyl	50	200	10	27	0.021	0.007	0.003	83	25	14
blebbistatin	75	225	16	24	0.036	0.009	0.004	83	26	14

Table 3.7. Parameters in each drug condition for the field model-based simulation.

Particle trajectories obtained by the simulation were analyzed in the same manner as the experimentally-obtained single-molecule trajectories.

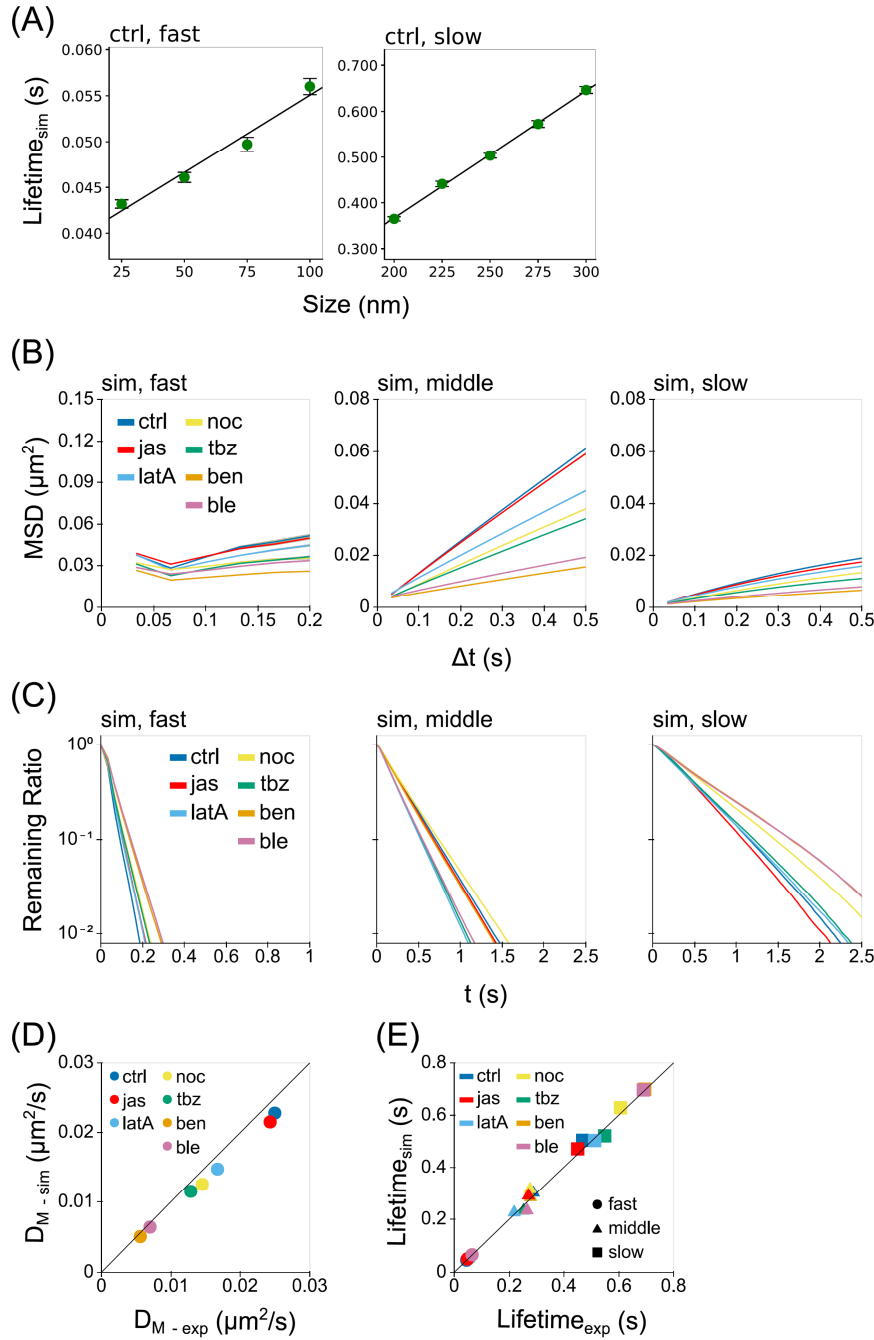


Fig. 3.2.3 A field model of the plasma membrane can recapitulate heterogeneous mobility of many membrane proteins observed experimentally.

(A) Relationship between field sizes and lifetimes in field model under control conditions. Mean $\pm$ 95% CI. (n = 10 datasets consisting of 5000 trajectories of 100 frames) (B and C) Reproduced mobility of particles in the field models represented as the MSD (B) and lifetimes (C) in different colors for the indicated conditions. (D and E) Comparison of the reproduced mobility with the experimental data for diffusion coefficients (D) and lifetimes (E).



Fig. 3.2.4 Hidden Markov models from experimental and simulated data.

Schematic diagrams of the HMMs and parameters of the models obtained from simulations and experiments in the control and six drug conditions. The values shown next to the arrows represent the state transition probabilities of the models. $D_{HMM-state}$ and $p_{HMM0-state}$ represent the diffusion coefficients and the initial probabilities of the hidden Markov models corresponding to the three states, respectively. The particle trajectories obtained by the simulation were analyzed in the same manner as the experimentally-obtained single-molecule trajectories by the HMM analysis. Mean $\pm$ 95% CI. (n = 10 datasets consisting of 5000 trajectories of 100 frames)

Drug	State	Experiment						Simulation					
		D_S ($\mu\text{m}^2/\text{s}$)	p_S (%)	ε_S (nm)	Lifetime (s)	D_M ($\mu\text{m}^2/\text{s}$)	ε_M (nm)	D_S ($\mu\text{m}^2/\text{s}$)	p_S (%)	ε_S (nm)	Lifetime (s)	D_M ($\mu\text{m}^2/\text{s}$)	ε_M (nm)
ctrl	fast	0.062±0.004	4.7±0.0	88±1	0.046±0.000			0.043±0.005	5.6±0.1	68±3	0.046±0.001		
	middle	0.032±0.000	57.0±0.5	12±0	0.289±0.005	0.025±0.000	23±0	0.030±0.000	58.8±0.2	19±0	0.301±0.004	0.023±0.000	26±0
	slow	0.012±0.000	38.3±0.5	10±0	0.468±0.008			0.009±0.000	35.6±0.3	20±0	0.503±0.005		
jasplakinolide	fast	0.061±0.002	6.0±0.1	92±1	0.048±0.001			0.035±0.004	5.7±0.1	75±2	0.051±0.001		
	middle	0.031±0.000	57.4±0.3	15±0	0.271±0.004	0.025±0.000	23±0	0.029±0.000	58.6±0.4	20±1	0.291±0.006	0.022±0.000	28±0
	slow	0.011±0.000	36.6±0.4	13±0	0.450±0.006			0.008±0.000	35.7±0.4	21±0	0.470±0.005		
latrunculin A	fast	0.046±0.002	5.5±0.1	91±0	0.051±0.001			0.033±0.003	5.9±0.1	69±2	0.051±0.000		
	middle	0.023±0.000	49.1±0.5	24±0	0.219±0.005	0.017±0.000	29±0	0.021±0.000	51.4±0.2	28±0	0.226±0.003	0.015±0.000	31±0
	slow	0.010±0.000	45.3±0.5	13±0	0.513±0.006			0.007±0.000	42.7±0.3	20±0	0.502±0.005		
nocodazole	fast	0.039±0.002	5.4±0.1	86±0	0.055±0.001			0.016±0.003	3.3±0.1	76±2	0.054±0.002		
	middle	0.021±0.000	50.5±0.4	16±0	0.277±0.005	0.015±0.000	30±0	0.018±0.000	53.2±0.3	21±1	0.315±0.004	0.013±0.000	24±0
	slow	0.008±0.000	44.1±0.4	12±0	0.607±0.005			0.006±0.000	43.5±0.3	17±0	0.628±0.007		
thiabendazole	fast	0.037±0.003	6.1±0.1	87±1	0.054±0.001			0.025±0.003	7.7±0.1	65±2	0.054±0.000		
	middle	0.018±0.000	50.4±0.3	21±0	0.253±0.004	0.013±0.000	27±0	0.016±0.000	50.2±0.2	24±0	0.233±0.002	0.012±0.000	29±0
	slow	0.007±0.000	43.5±0.4	13±0	0.549±0.005			0.005±0.000	42.1±0.2	18±0	0.521±0.004		
benomyl	fast	0.021±0.001	6.0±0.1	83±1	0.064±0.001			0.012±0.000	7.5±0.1	65±0	0.065±0.001		
	middle	0.007±0.000	45.4±0.3	25±0	0.278±0.005	0.006±0.000	29±0	0.006±0.000	47.1±0.2	27±0	0.285±0.002	0.005±0.000	28±0
	slow	0.003±0.000	48.6±0.4	14±0	0.695±0.009			0.003±0.000	45.4±0.2	16±0	0.700±0.008		
blebbistatin	fast	0.036±0.004	7.4±0.1	83±2	0.065±0.001			0.019±0.001	9.6±0.1	70±1	0.067±0.000		
	middle	0.009±0.000	47.2±0.2	26±0	0.266±0.003	0.007±0.000	32±0	0.008±0.000	44.9±0.3	29±0	0.234±0.002	0.006±0.000	32±0
	slow	0.004±0.000	45.5±0.3	14±0	0.690±0.006			0.004±0.000	45.5±0.3	17±0	0.697±0.006		

Table 3.8. Characteristic values of multiple diffusion states estimated from experimental data and field model-based simulations.

D_M and D_S are the diffusion coefficients obtained by the MSD and HMM using Eq. (2) and Eq. (6)-(7), respectively. ε_M and ε_S are the localization errors of diffusing fluorescent spots determined using Eq. (2) and Eq. (7), respectively.

Particle trajectory data obtained by the numerical simulation of the field model were analyzed in the same way as the experimental data to obtain the diffusion coefficient $D_{S-state}$, the lifetimes of each state, and the diffusion coefficient D_M .

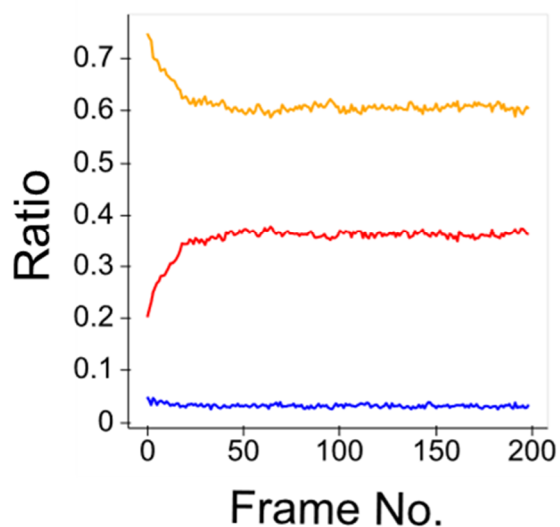
Drug	State	Experiment					Simulation				
		D_{HMM} ($\mu m^2/s$)	P_{HMM}^0 (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)	D_{HMM} ($\mu m^2/s$)	P_{HMM}^0 (%)	$A_{fast \rightarrow state}$ (%)	$A_{middle \rightarrow state}$ (%)	$A_{slow \rightarrow state}$ (%)
ctrl	fast	0.209±0.001	15.5±0.2	53.4±0.2	3.2±0.0	0.1±0.0	0.189±0.001	17.1±0.3	51.2±0.4	3.8±0.1	0.0±0.0
	middle	0.036±0.000	75.2±0.2	45.2±0.6	91.1±0.1	6.6±0.1	0.036±0.000	73.8±0.4	46.9±0.7	91.4±0.1	5.8±0.1
	slow	0.016±0.000	9.3±0.2	1.4±0.6	5.6±0.1	93.4±0.1	0.016±0.000	9.0±0.3	1.9±0.6	4.8±0.1	94.2±0.1
jasplakinolide	fast	0.219±0.002	18.3±0.3	55.8±0.6	3.8±0.0	0.0±0.0	0.206±0.002	19.7±0.2	54.6±0.3	3.4±0.0	0.0±0.0
	middle	0.038±0.000	72.7±0.2	43.4±0.6	90.8±0.1	6.6±0.1	0.037±0.000	71.4±0.2	45.0±0.5	91.3±0.2	6.3±0.1
	slow	0.015±0.000	8.9±0.2	0.8±0.2	5.4±0.1	93.4±0.1	0.016±0.000	9.0±0.2	0.4±0.3	5.4±0.2	93.7±0.1
latrunculin A	fast	0.225±0.002	17.5±0.2	58.3±0.5	3.9±0.1	0.0±0.0	0.193±0.001	17.1±0.2	55.9±0.3	4.1±0.1	0.0±0.0
	middle	0.035±0.000	72.6±0.3	41.6±0.5	89.1±0.3	5.5±0.1	0.040±0.000	73.0±0.4	43.5±0.5	89.4±0.1	5.1±0.1
	slow	0.014±0.000	9.8±0.3	0.1±0.1	7.0±0.2	94.5±0.1	0.015±0.000	9.9±0.2	0.6±0.2	6.5±0.1	94.9±0.1
nocodazole	fast	0.198±0.001	19.3±0.2	60.6±0.2	3.5±0.0	0.0±0.0	0.185±0.001	19.1±0.1	57.6±1.0	1.5±0.1	0.0±0.0
	middle	0.029±0.000	69.7±0.2	39.1±0.3	91.4±0.1	4.5±0.1	0.028±0.000	70.1±0.3	42.4±1.0	93.3±0.1	4.2±0.1
	slow	0.012±0.000	11.0±0.2	0.3±0.2	5.1±0.1	95.5±0.1	0.012±0.000	10.8±0.3	0.0±0.0	5.2±0.1	95.8±0.1
thiabendazole	fast	0.208±0.002	20.1±0.1	59.8±0.4	4.1±0.0	0.0±0.0	0.173±0.001	21.9±0.3	57.9±0.2	5.4±0.1	0.0±0.0
	middle	0.029±0.000	69.4±0.3	40.1±0.5	90.1±0.1	5.2±0.0	0.030±0.000	66.4±0.4	41.8±0.4	88.4±0.1	5.4±0.1
	slow	0.011±0.000	10.5±0.2	0.1±0.1	5.7±0.1	94.8±0.0	0.012±0.000	11.7±0.3	0.3±0.2	6.3±0.0	94.6±0.1
benomyl	fast	0.190±0.003	22.7±0.2	66.2±0.4	3.8±0.0	0.0±0.0	0.155±0.001	23.7±0.4	63.5±0.2	4.9±0.0	0.0±0.0
	middle	0.024±0.000	63.5±0.2	33.8±0.4	91.3±0.1	3.7±0.1	0.026±0.000	60.3±0.4	35.3±0.3	90.5±0.0	3.8±0.1
	slow	0.008±0.000	13.8±0.2	0.0±0.0	5.0±0.1	96.3±0.1	0.009±0.000	16.0±0.2	1.2±0.2	4.6±0.1	96.2±0.1
blebbistatin	fast	0.200±0.002	27.2±0.3	67.1±0.2	4.1±0.0	0.0±0.0	0.171±0.001	29.4±0.3	64.8±0.2	6.0±0.1	0.0±0.0
	middle	0.027±0.000	61.7±0.3	32.9±0.2	91.3±0.1	3.6±0.0	0.030±0.000	57.5±0.5	33.3±0.2	89.3±0.1	3.5±0.0
	slow	0.009±0.000	11.1±0.1	0.0±0.0	4.6±0.1	96.3±0.0	0.010±0.000	13.1±0.3	1.9±0.2	4.7±0.1	96.5±0.0

Table 3.9. Estimated HMM parameters in experimental and simulated data.

3.2.2 Particles take a non-random, steady-state arrangement on the field.

The initial position in the field for diffusing particles was randomly determined. The change in the ratio of states in each frame of the generated trajectory showed that the ratio of fast and middle states decreased and the ratio of slow states increased with time from the first frame, and that each ratio reached a steady state after 50 frames (Fig. 3.2.5 A). A comparison of snapshots of the placement of particles in frame 1, which was a completely random arrangement, with ones in frame 99, where particles were free diffusing and time had elapsed, showed that particles accumulated in slower regions over time due to differences in diffusion coefficients in each region (Fig. 3.2.5 B). The rapid ratio change from the first frame was found to be caused by a change from a random arrangement to a biased, steady-state position. In order to obtain trajectories in which the ratio of states did not change significantly due to particle diffusion, it was decided to diffuse particles from random initial positions and use the data after 100 frames had elapsed.

(A)



(B)

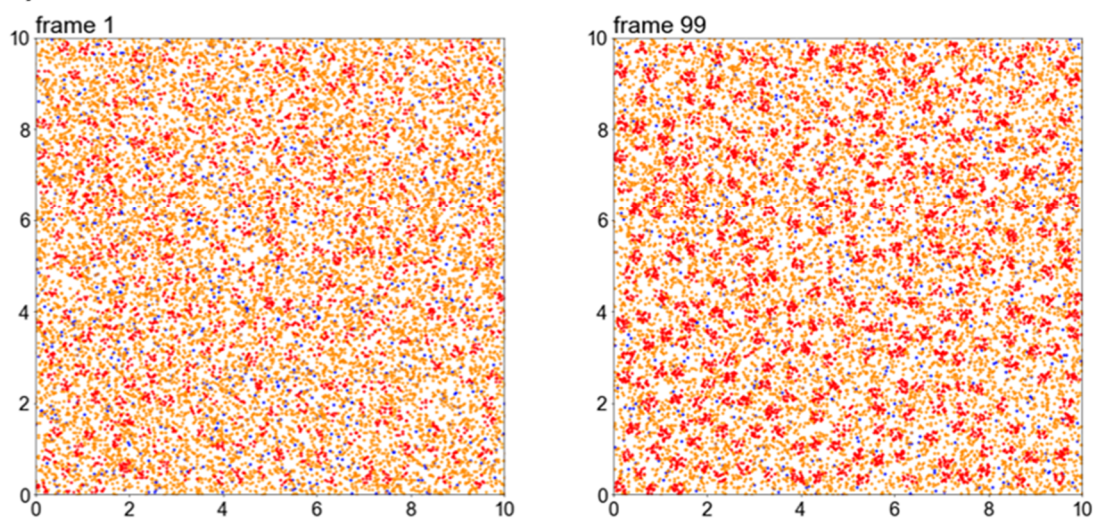


Fig. 3.2.5 Change over time of particle arrangements in the simulation.

(A) Ratio change of each diffusion state in each frame. The fast, middle, and slow states are represented by the blue, yellow, and red lines, respectively. (B) Snapshots of particle arrangements. The left shows the particle arrangements at frame 1, and the right shows the particle arrangements at frame 99. The fast, middle, and slow states are represented by the blue, yellow, and red, respectively.

3.2.3 HMM analysis on measured data contains artifacts in the initial probabilities.

Comparing the initial probability $p_{\text{HMM}0}$ and the state probability p_s at all times obtained from the HMM analysis, there was a tendency for $p_{\text{HMM}0}$ to have a larger ratio of faster states under all measurement conditions (Table 3.4, Table 3.5). The change in the ratio of the states from the first frame of each trajectory was examined. The ratio of the fast and middle states decreased and the ratio of the slow state increased with time from the first frame until a steady state was reached at over 20 frames (Fig. 3.2.6 A). I assumed that this ratio change was due to a rapid change in the cell membrane environment caused by laser irradiation. This assumption turned out to be incorrect, since state estimation after removing the first few dozen frames from the trajectory data showed exactly the same change in the ratio of each diffusion state. Next, I assumed that the cause of this phenomenon is that the tracking of the molecules is broken (Fig. 3.2.6 B). If the faster a molecule moves, the more it breaks tracking, then tracking should be more likely to break when the molecule is in the fast state. If a trajectory is interrupted in the fast state, a new trajectory with the fast state at the top is generated. $p_{\text{HMM}0}$ is calculated for the top of the trajectories, and as a result, the ratio of the fast state is estimated to be high. Under the above assumptions, the frequency of breaks in the measured trajectories is estimated using $p_{\text{HMM}0}$ and p_s , and when the generated trajectories are cut at that frequency, $p_{\text{HMM}0}$ can be made close to the measured value with almost no change in the other analysis values (Table 3.8, Table 3.9).

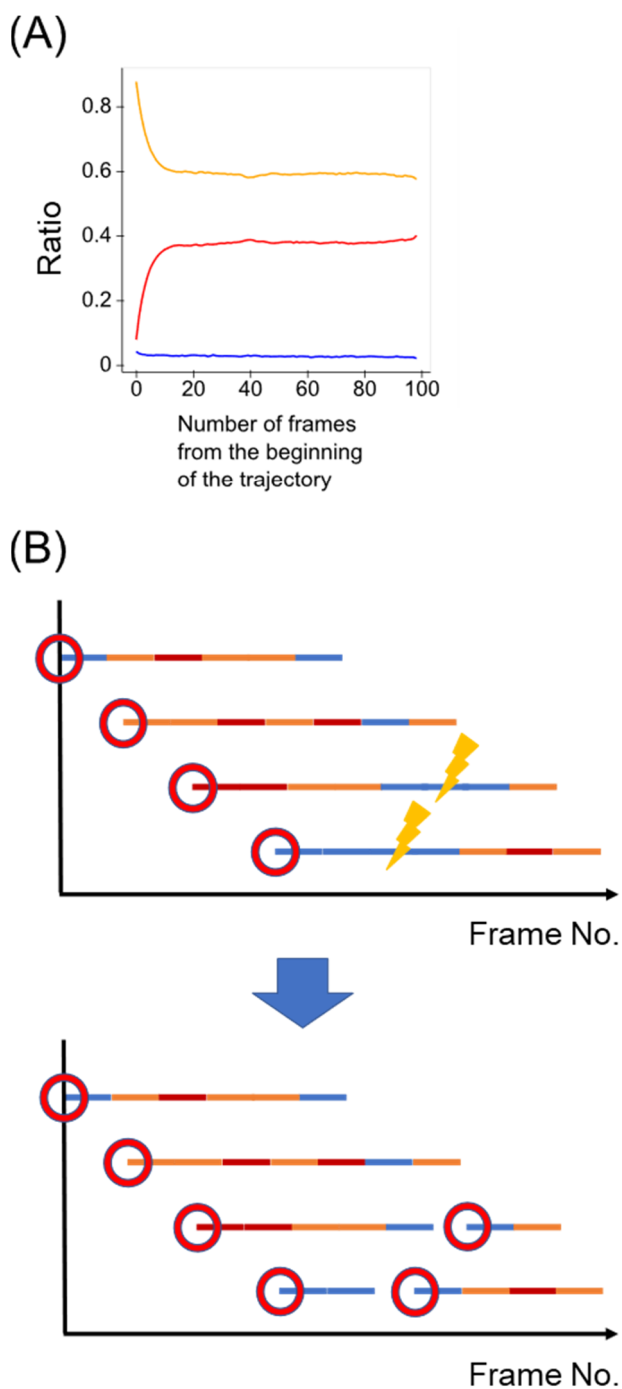


Fig. 3.2.6 Change over time of diffusion state in measured data.

(A) Ratio change of each diffusion state from the first frame of each trajectory. The fast, middle, and slow states are represented by the blue, yellow, and red lines, respectively.

(B) Schematic diagram of trajectory interruption phenomenon. Tracking of a trajectory is tend to be broken when the molecule is in a fast state, and the tip of the new trajectory created by the break in the trajectory is in a fast state.

3.2.4 Membrane fields are to some extent robust to bias in area arrangement.

The fields have been created by randomly arranging fast and slow regions according to four parameters: fast and slow size and area ratio. Because of the random arrangement, it is possible that the analytical values of trajectories obtained from some fields may deviate significantly from the analytical values of trajectories obtained from other fields. In order to estimate the bias of the region arrangement and its effect, I created artificially extremely biased fields while maintaining the four parameters. Moran's I, a measure of spatial autocorrelation, was used to evaluate the bias of the arrangement (Eq. (12)) (Moran, 1950). Moran's I takes values in the range $-1 \leq I \leq +1$, with $I = +1$ when the arrangement is most biased and $I = -1$ when it is most dispersed. Five fields were created, ranging from the most dispersed to the most biased arrangement possible to varying degrees of cohesion of the slow mass (Fig. 3.2.7 B) The trajectories were generated using each of the five fields and the overall diffusion coefficient D was calculated and compared (Fig. 3.2.7 C). When the arrangement of fast and slow squares is not extremely biased, the numerical simulation reproduced robustly the diffusion characteristics of the experimental results. This result suggests that even if the fields were randomly generated and the cohesion were increased, most arrangements would produce similar analytical values.

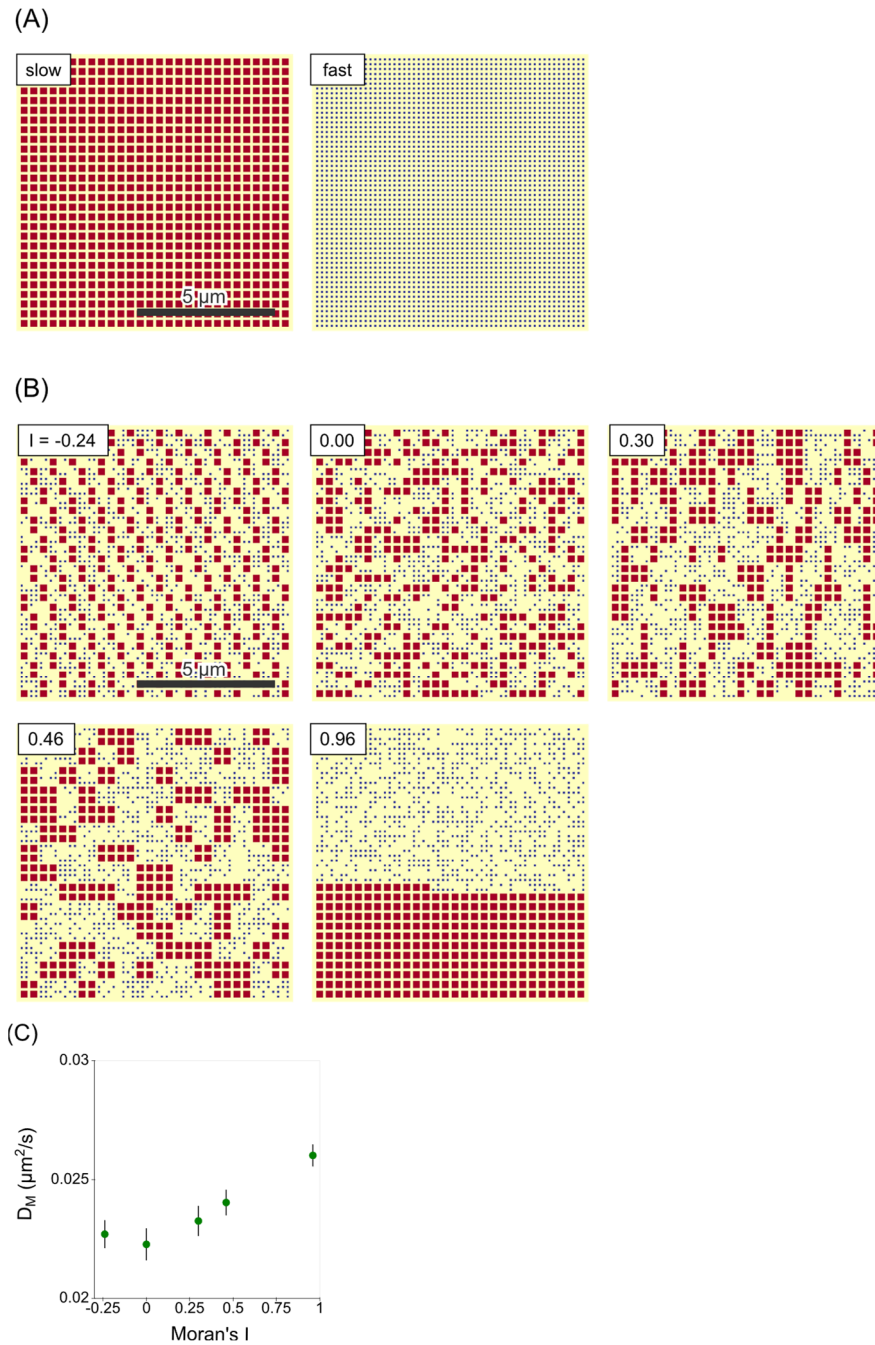


Fig. 3.2.7 Evaluation of arrangements of Field by Moran's I.

(A) Field models with only slow or fast regions used to create field models with different Moran's I. (left) Field 1, (right) Field 2. Scale bar, 5 μm . (B) Field models with different Moran's I. The five patterns of the fields are scattered, random, two squares in close proximity, four squares in close proximity, and gathering. The respective Moran's I values are shown. Scale bar, 5 μm . (C) Relationship between the spatial autocorrelation of the field arrangement defined by Moran's I and diffusion coefficient. Mean $\pm$ 95% CI. (n = 10 data sets consisting of 5000 trajectories of 100 frames)

3.2.5 Verification of the effect of clump shape on diffusion

In the field model, the fast and slow regions were set to be square in order to make arrangement simpler. However, it is assumed that no matter what the physical reality of the fast and slow regions is, it is almost impossible to have a square region on the cell membrane. From a hydrodynamic point of view, a region such as a lipid microdomain is considered to be circular on the cell membrane. Therefore, I replaced the square clumps in the field with circular ones to verify the effect of the clump shape. Using the parameters of the field model in the ctrl condition, I created a field such that the middle region had only one square or circular fast clump with the same area, and generated single molecule trajectories (Fig. 3.2.8A). In the same way, a field with only one slow clump was also created and single molecule trajectories were generated (Fig. 3.2.8B). Analysis of the obtained trajectories using HMM showed that there was little difference between squares and circles in both cases for fast and slow clumps, indicating that squares and circles are interchangeable with little effect of region shape for the clump size used in this study.

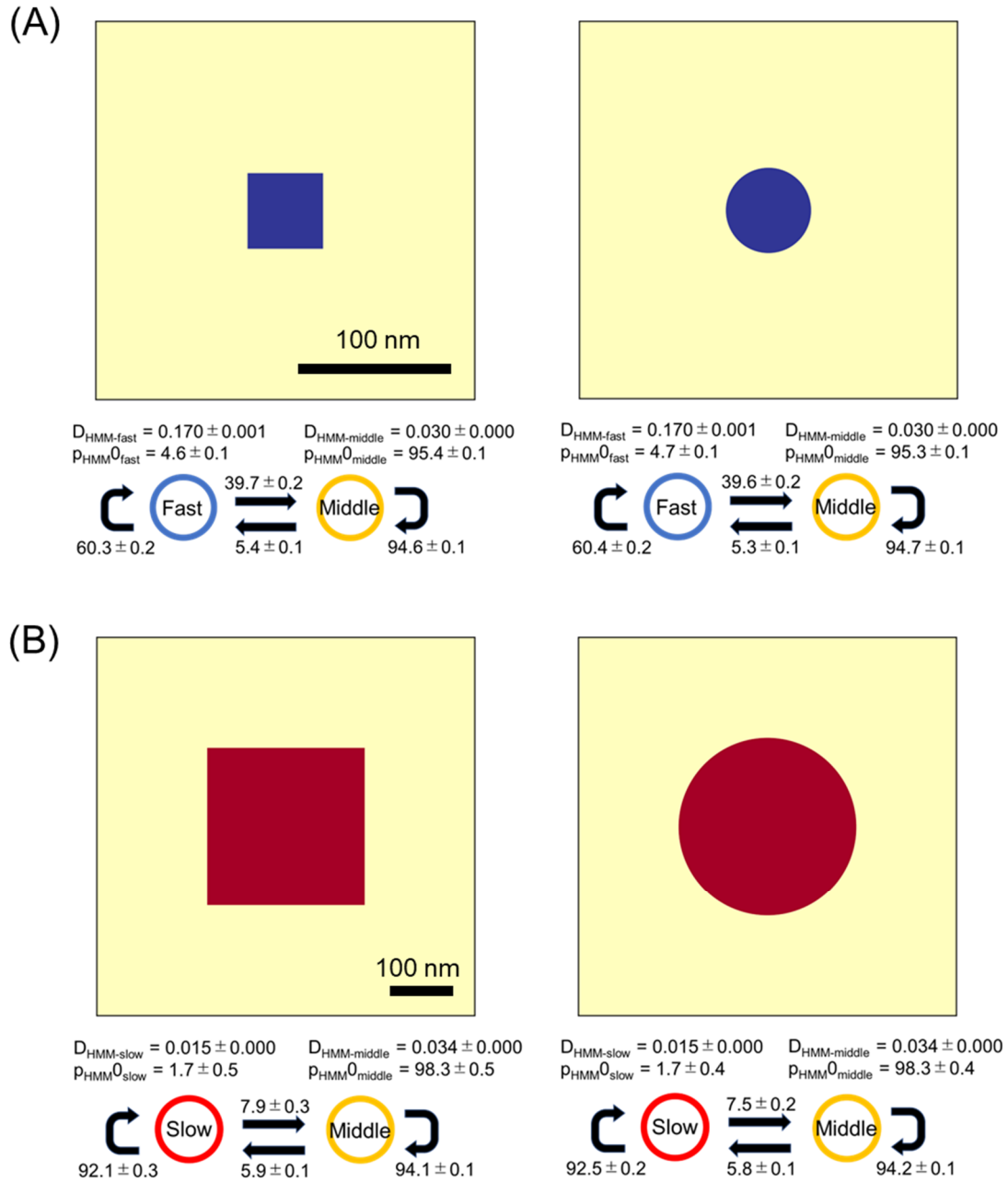


Fig. 3.2.8. Effects of clump shape on mobility properties.

Fields with one fast clump (A) and one slow clump (B) in the middle region under control condition. To examine the effects of the clump shape on the particle mobility, square and circular clumps of the same size were prepared (The size of one side of the fast and slow square clumps is 50 nm and 250 nm, respectively). Below each field is the result of the HMM analysis of the generated trajectories. Scale bar, 100 nm. Mean $\pm$ 95% CI. (n = 10 data sets consisting of 5000 trajectories of 100 frames)

3.2.6 Verification that the HMM can correctly estimate the measurement localization error.

In order to see whether the HMM can estimate the diffusion state along a trajectory for which the localization error of the fluorescent spots depends on the diffusion state, the particle trajectories generated by the numerical simulation were analyzed using the HMM. The particle trajectories were generated using the HMM with $D_{S-state}$ for each state with initial probabilities p_{HMM0} and a transition probability matrix A obtained from data for all protein species under control condition (Fig. 3.2.9A). A localization error was added to the coordinates of each particle in each frame based on $\epsilon_{S-state}$. The values of $\epsilon_{S-state}$ were 10, 12, and 88 nm for the slow, middle and fast states, respectively (Table 3.8). The obtained trajectories with the localization errors were analyzed by the HMM and MSD using Eq. (6) and Eq. (7). I confirmed that the HMM can estimate $D_{S-state}$ with the corresponding $\epsilon_{S-state}$ (Fig. 3.2.9B, C).

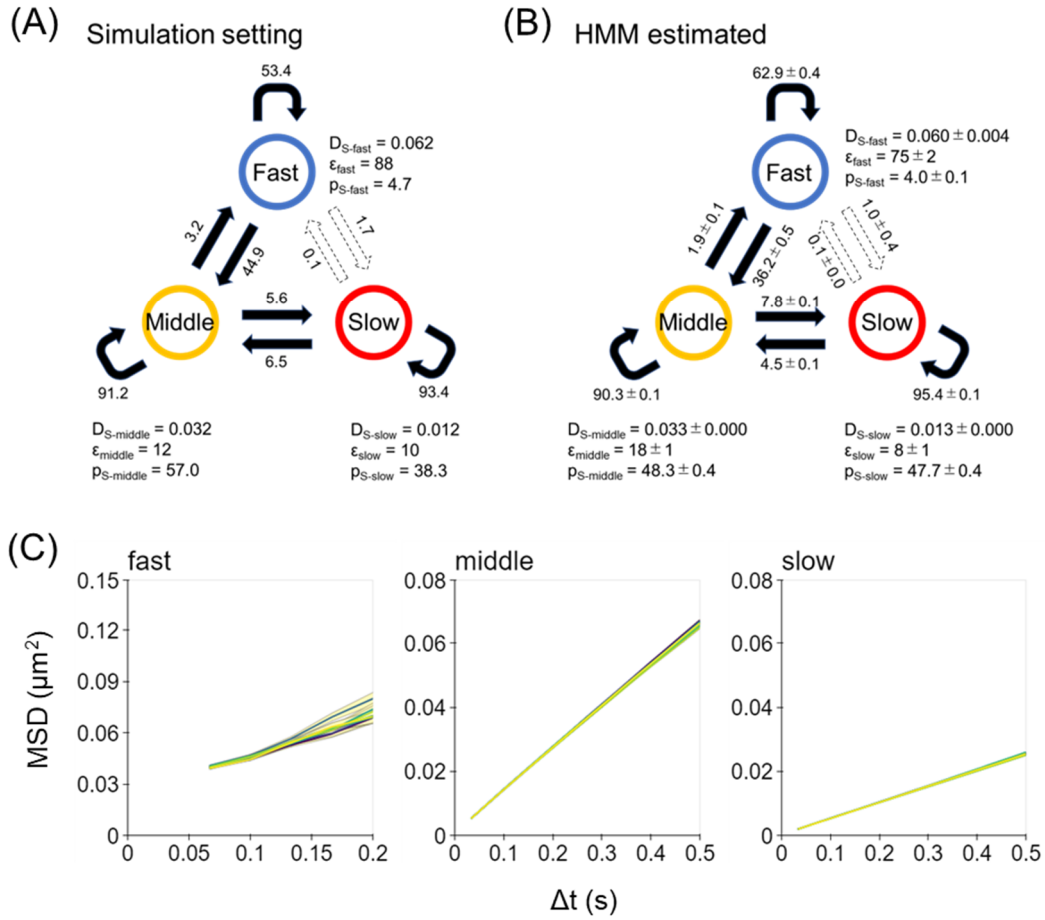


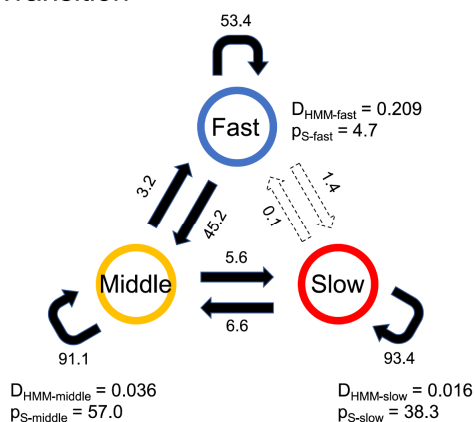
Fig. 3.2.9. Effects of localization errors of single-molecule trajectories on diffusion properties by the HMM.

(A) Particle diffusion with three states for the simulation. (B) Parameters estimated by analyzing the simulation trajectories using the HMM. (C) MSDs of fast, middle, and slow states estimated using the HMM. Mean ± 95% CI. (n = 10 data sets consisting of 5000 trajectories of 100 frames)

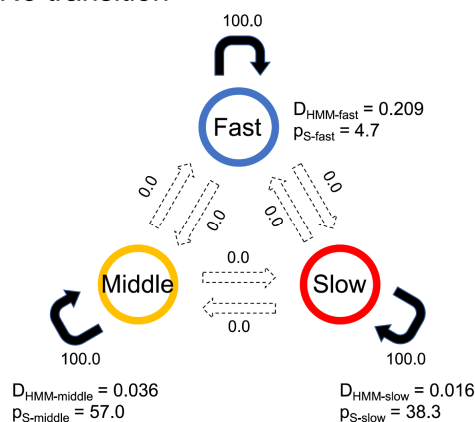
3.2.7 Verification of whether the HMM can detect the presence or absence of state transitions.

HMM analysis of the measured data showed that no transition between the fast and slow states was observed for any type of molecule under any drug condition (Fig 3.2.4, Table 3.5, Table 3.9). I verified whether the HMM can detect the state transition of diffusion, using trajectory data sets generated by simulations with the HMM. Trajectory data sets with and without state transitions were generated using the parameters obtained from the measurements under the ctrl condition (Fig 3.2.10A). HMM analysis of each of the generated trajectory data sets showed that the presence or absence of state transitions could be detected correctly, and the estimated parameters were almost identical except for the state transition probabilities (Fig 3.2.10B).

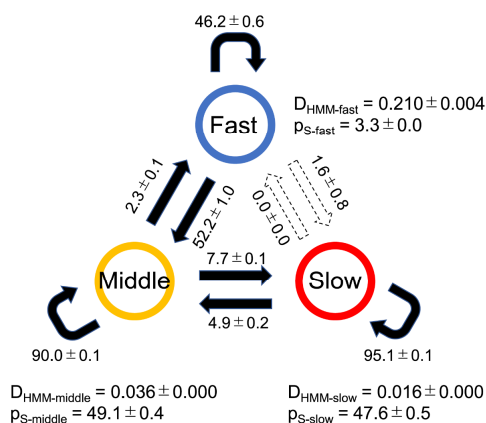
(A) Simulation setting
Transition



No transition



(B) HMM estimated
Transition



No transition

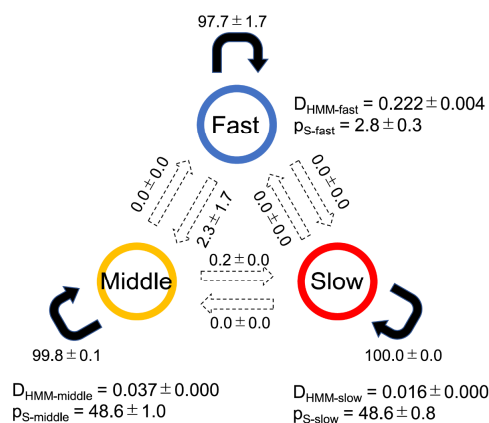


Fig. 3.2.10. Detection and estimation of state transitions by the HMM.

(A) Parameters to generate particle trajectories by numerical simulations using the HMM. The parameter values are the same as those obtained from the experimental data of the single-molecule trajectories for all protein species combined under the control condition (see Fig. 3.2.4, Ctrl Exp.). The trajectories were generated by numerical simulation with (left) and without (right) state transitions. (B) Estimated mobility parameters by the HMM from the trajectories obtained by the simulation with (left) and without (right) state transitions. Mean $\pm$ 95% CI. (n = 10 data sets consisting of 5000 trajectories of 100 frames)

3.2.8 Verification of the effect of trajectory interruptions on the estimation of diffusion properties using HMMs

The trajectories obtained from the measurement were only taken for a number of frames shorter than 100 frames of the measurement time due to the interruption (Fig. 3.1.2). I verified whether the shortened trajectories affected the parameter estimation of the HMM and the state estimation at each frame, using trajectory data sets generated by simulations with the HMM. I generated a trajectory dataset with a duration of 100 frames for all trajectories using the parameters obtained from the measurements under the ctrl condition and a dataset with a shorter trajectory length by cutting this trajectory dataset based on the parameters of the ctrl condition (Fig. 3.2.11A). The average length of trajectories in the dataset with shorter trajectories is 21.0 ± 20.1 frames (mean $\pm$ SD), and the histogram of trajectory lengths also shows that there are many trajectories shorter than 100 frames (Fig. 3.2.11C). The parameters obtained from HMM analysis of both trajectory datasets were compared to the parameters of the HMM used to generate them, and the results of parameter estimation for both datasets were almost identical, except for the difference in the initial probability of each state discussed in Section 3.2.3 (Fig. 3.2.11B). In addition, when comparing the accuracy of the state estimation at each frame, there was little difference between the two datasets with an accuracy of about 80% for both (Fig. 3.2.11D). The length of the trajectory obtained in this measurement is considered to have little effect on the HMM analysis.

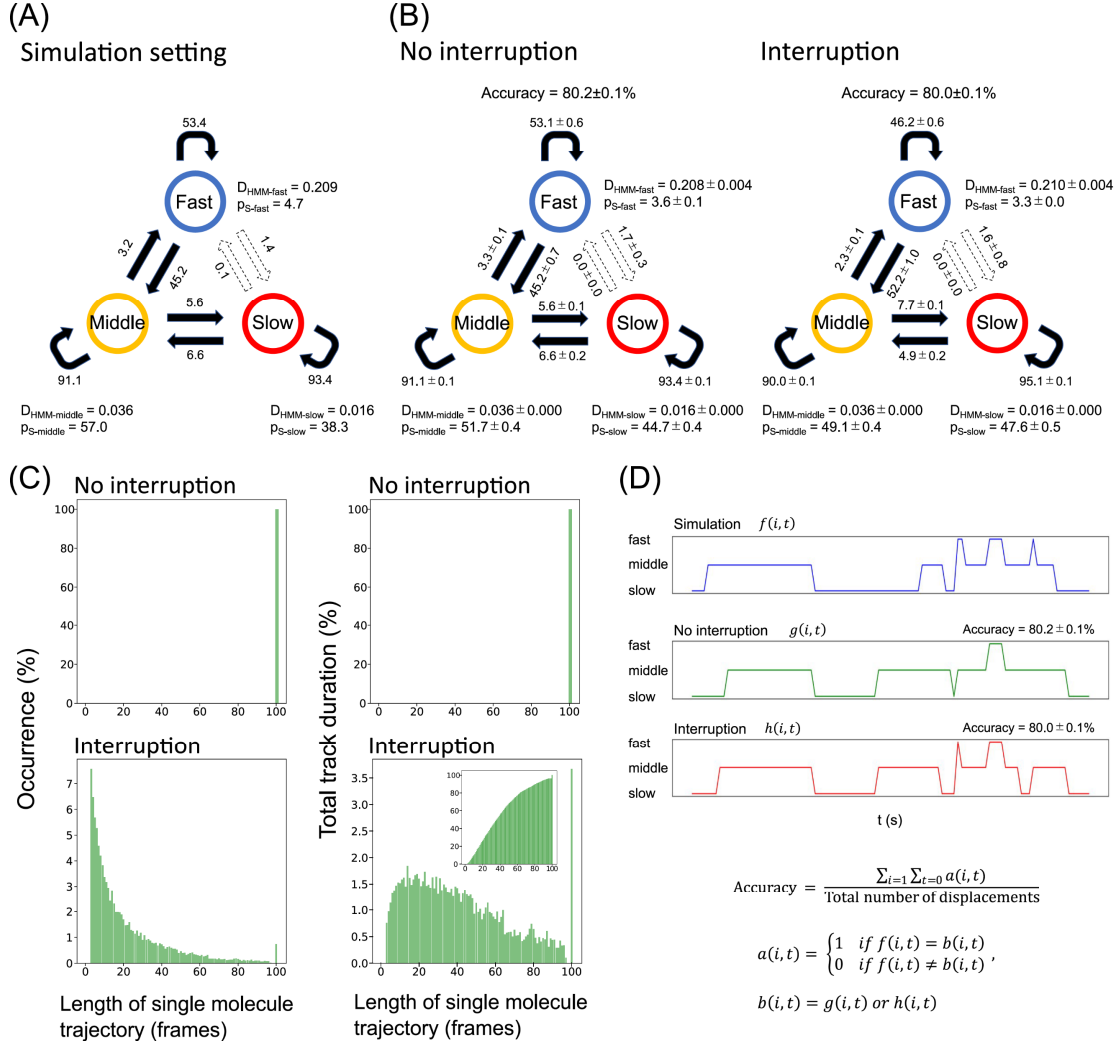


Fig. 3.2.11. Effects of trajectory interruption on the estimation of diffusion properties using the HMM. (A) Particle diffusion with three states for the numerical simulation based on the HMM. (B) The estimated parameters with and without trajectory interruption. Mean $\pm$ 95% CI. (C) The occurrence of each particle trajectory length generated by the numerical simulation. The track duration was 21.0 $\pm$ 20.1 frames (mean $\pm$ SD). **Inset**, Culminated percentage of trajectory lengths. (D) Definition of the coincidence between the simulated diffusion state and the estimated diffusion state. To evaluate the degree of coincidence between the numerically generated trajectory and HMM, I defined accuracy as follows. The state of the i -th trajectory at frame t of the generated trajectory is denoted as $f(i, t)$, the state estimated by the HMM of the full-length trajectory is denoted as $g(i, t)$, and the state estimated by the HMM of the interrupted trajectory is denoted as $h(i, t)$. Using $a(i, t)$, the accuracy rate can be expressed as the “accuracy”. Mean $\pm$ 95% CI. ($n = 10$ data sets consisting of 5000 trajectories of 100 frames)

Chapter 4

Discussion

General properties of the diffusion motion of transmembrane proteins of *Dictyostelium*.

The 27 transmembrane proteins used in this study varied in the number of transmembrane sites and in the structures predicted using AlphaFold. For example, all of the single-transmembrane molecules had very large conformations in the extracellular region relative to the transmembrane region. Also, these 27 proteins are likely to have diverse structures on the cell membrane, although it is not known whether they form multimeric complexes. In addition, some proteins, such as tgrC1 and car1, are known to be expressed at different times and have completely different functions (Wang and Shaulsky, 2015; Sun et al., 1990).

The results of the measurements of these diverse membrane proteins indicate that their diffusion kinetics are similar for all types. Most membrane proteins had three diffusion states, and the analyzed values were close. The diffusion coefficients were uniformly lower under the drug conditions except for jasplakinolide. Under the drug conditions, membrane proteins had almost all three diffusion states, and the analytical values were close to each other. Comparison with theoretical models of diffusion on lipid bilayers showed that the radius of membrane proteins embedded in the membrane did not significantly affect the diffusion motions. The above facts indicate that the average behavior of membrane proteins' diffusion motions on the cell membrane of *Dictyostelium* is determined by the cell membrane. By measuring various types of membrane proteins, I was able to estimate the factors that contribute to the diffusion behavior, which had not been captured in previous studies.

Only a simple membrane structure model could reproduce the average behavior of diffusion.

Even though measurements have revealed that the cell membrane is a major factor in determining diffusion motion, it was not possible to create a model that reproduced diffusion using only the cell membrane. This is because analyses such as MSD and HMM, which are commonly used to analyze diffusion, only use information on the displacement

Δr between frames and cannot obtain information on the distribution and structure of the membrane. In addition, the density of bright spots could not be increased sufficiently due to the nature of single molecule imaging, so it was not possible to estimate the membrane structure directly from the trajectory position. In this study, I was able to create a model of the cell membrane by assuming that there are three membrane regions corresponding to three diffusion states and that the duration of the state corresponds to the size of the membrane region. The simple nature of diffusion in *Dictyostelium*, such as the free diffusion of all types of molecules under all drug conditions and the absence of transitions between the fast and slow states, was essential for model construction. It was found that the field model could reproduce the average diffusion motion of membrane proteins obtained from the measurements by simply diffusing particles through three regions of different viscosity. Furthermore, I was able to estimate the membrane structure that produces diffusion under each drug condition.

Type-specific effects of membrane proteins on diffusion

By creating a field model, I was able to reproduce the average behavior of the 27 types of molecules measured. However, the differences in diffusion coefficients and the ratio of states for each type that deviate from the mean may arise from features specific to the species of protein. In this analysis, I was not able to find any diffusion features that lead to deviations from the mean, nor was I able to find any common features among the 27 species of molecules that were outliers. I would like to make detailed measurements of individual protein species in the future so that I can more clearly distinguish between the species-specific effects and the effects of the surrounding membrane environment on diffusion.

Diffusion is slowed by inhibition of the cytoskeleton.

The cytoskeleton, especially actin filaments, has been considered in previous studies (Kusumi, 2003) to inhibit the diffusion kinetics of membrane proteins. However, in this study, I found that drugs that reduce the cytoskeleton reduced the diffusion coefficients of membrane proteins, which is contrary to my initial assumption. The field models show that the diffusion coefficient is reduced across the entire plasma membrane. Of the 27 membrane proteins, no molecules are reported to interact directly with cytoskeleton molecules such as microtubules or F-actin. As for the possible roles of the cytoskeleton

in the diffusion of membrane proteins, one might speculate three possibilities. One is a fence against the diffusion of membrane protein. Another is vesicular transport to the membrane, which would modulate the composition of lipids and other molecules in the membrane. Lastly, force generation by the cytoskeleton might affect the plasma membrane mechanically. Currently, we have no evidence to distinguish these possibilities, but the reduced diffusion by inhibition of the cytoskeleton suggests that cytoskeletal fences do not play a major role in the diffusion properties of membrane proteins in *Dictyostelium* cells, which is in sharp contrast to mammalian cells.

The entities of the three areas of the field model

The three diffusion states are for convenience, and it is not clear what physical state each diffusion state corresponds to, nor is it clear if they are the same for each drug condition. However, the sizes of the fast and slow regions did not vary significantly in the fields created. It is possible that each region corresponds to approximately the same region. Moreover, the size of the slow region is close to the size of the lipid rafts in mammalian cells in the previous study (<200 nm) (Pike, 2006), suggesting that they are related.

All molecular species underwent free diffusion.

It was also surprising that most membrane proteins showed free diffusion in all diffusion states under all drug conditions. In mammalian cells, many membrane proteins exhibit subdiffusion (Hiroshima et al., 2018; Yanagawa et al., 2018), which is thought to be due to cytoskeletal fences and structural changes in membrane proteins that act to restrict membrane protein diffusion. This suggests that the cell membrane of *Dictyostelium* has a relatively simple structure, although it is capable of generating multiple states of diffusion.

Non-uniform distribution of particles on the field

It is important to note that in a field where all molecules exhibit free diffusion, a bias in the distribution of molecular positions is created at steady state (Fig. 3.2.5B). It suggests that the uneven distribution of lipids of different viscosities on the membrane can generate localization of membrane protein molecules without any mechanism to control movement in the membrane structure, such as a fence in the cytoskeleton that

restricts movement, or in the membrane protein molecules themselves. This is consistent with the original concept of the lipid raft hypothesis (Simons and Ikonen, 1997; Simons and Toomre, 2000). Lateral diffusion at the plasma membrane of *Dictyostelium* may be the simplest evolutionary implementation of this concept.

Discrepancy between initial state probabilities $p_{\text{HMM}0}$ and steady state probabilities p_s in HMM.

In constructing the field model, it became clear that there is a large discrepancy between the initial state probabilities and the steady state probabilities obtained from the state transition probabilities in the hidden Markov model (Section 3.2.3). Few previous studies have found such a discrepancy to be problematic. In general, initial state probabilities and steady state probabilities are often treated as if there is no discrepancy but interpreting data without considering the existence of a discrepancy can lead to serious misunderstandings. In this study, I assumed trajectory interruptions as a factor that could create deviations, and by implementing this assumption, I was able to successfully reproduce the experimental data. However, experimental verification has not yet been conducted, and the factors that cause discrepancies have not yet been determined. I intend to address this issue in order to improve the reliability of our measurement and analysis methods.

Toward more direct estimation or observation of membrane structure.

Although the field model estimated the membrane structure without using the positional information of the molecules, it is important to investigate whether such a membrane structure exists in order to validate the model. It would be difficult to stain and visualize each region due to spatial resolution factors. It is also difficult to obtain spatial information directly from single molecule imaging, since a single cell is about $10\ \mu\text{m}^2$ in size and has only about 300 light spots, which is not dense enough. To obtain information on membrane structure, it is essential to measure light spots at a higher density. For example, the InferenceMAP attempts to visualize the membrane structure by obtaining the positional information of a large number of light spots using super-resolution technology (Beheiry et al., 2015). I would like to address this issue by introducing a new measurement system.

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Publication List

1. Takebayashi, K., Kamimura, Y., & Ueda, M. (2023).

Field model for multistate lateral diffusion of various transmembrane proteins observed in living *Dictyostelium* cells.

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List of conference presentation

1. 竹林和俊、上村陽一郎、上田昌宏

「膜タンパク質側方拡散運動の細胞膜フィールドモデル」，日本細胞性粘菌学会第12回例会，2022年10月8~9日開催，オンライン開催，（口頭発表）

2. 竹林和俊、上村陽一郎、上田昌宏

“Field model for multistate lateral diffusion of various transmembrane proteins in living *Dictyostelium* cells”，日本生物物理学会第60回年会，函館アリーナ・函館市民会館，2022年9月28~30日開催，（ポスター発表）

3. 竹林和俊、上村陽一郎、上田昌宏

「膜タンパク質分子の拡散運動の細胞膜フィールドモデルの構築」，日本細胞性粘菌学会第11回例会，2021年10月23日開催，オンライン開催，（口頭発表）

4. 竹林和俊，宮永之寛，上田昌宏

「生細胞の細胞膜上での膜タンパク質の拡散の網羅的解析」，研究会「理論と実験」2017，広島大学東広島キャンパス 理学研究科 E 棟，2017年10月6~7日開催，（ポスター発表）

5. 竹林和俊，宮永之寛，上田昌宏

「生細胞での膜タンパク質の拡散運動の網羅的解析」，日本生物物理学会第55回年会，熊本大学黒髪北地区，2017年9月19~21日開催，（ポスター発表）

6. 竹林和俊，宮永之寛，上田昌宏

「細胞性粘菌の生きた細胞での膜タンパク質の拡散の網羅的解析」，日本生物物理学会第54回年会，つくば国際会議場，2016年11月25~27日，（ポスター発表）

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