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Biophysics Internship

Single particle tracking at the air/water interface

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Importance of lateral diffusion

Lateral diffusion in the plasma membrane is of importance for a variety of biological processes. These include the transmission of signals with the aid of proteins and lipids, the formation and regeneration of membranes for cell-cell adhesion. From the lateral diffusion of membrane components, one can directly obtain information on the membrane architecture. First principle investigations were undertaken to gain knowledge about the validity of the Singer-Nicholson fluid mosaic model [1]. As the lipids adhere the membrane structure, the diffusion of lipids has been researched thoroughly [2],[3].

Of special importance for the insertion and distribution of membrane components is the fluidity of the membrane itself. The components are inserted locally via vesicle fusion and are then spread in the membrane by diffusion.

Diffusing molecules can also behave as amplifiers in the signal transduction of the cell. A very well researched example for protein diffusion in the context of signal transduction is the amplification of light through rhodopsin. The rhodopsin protein is mobile within the layer of the membrane. After activation by a photon it diffuses until it comes in contact with a G-protein, which it thereby activates. Then the rhodopsin, still activated, continues its movement, activating several G-proteins in its way and such acting as a signal amplifier in the light transduction path [4].

Diffusing lipids, especially phosphoinosites (PI), can also take part in signal transduction. The synthesis of PI is confined to small areas and triggered by an external chemical signal. The velocity of the diffusing PI (D=0.5 μm^2/s) determines the ability of the cell to locate the source of the signal. Thus the diffusion defines the spatial resolution of the signal perception of fibroblasts ($\pm 10\,\mu m$ for a lifetime of $40\,s$ for the PI [5]).

The lateral diffusion also affects the cell adhesion. At the first contact between membrane and substrate the area of adhesion contains a limited amount of receptors which bind to the substrate, getting immobilized in the process. As more receptors diffuse into the contact region they aggravate the adhesion force by binding to the substrate [6].

One result of the extensive research that has been done on the diffusion of membrane components, is the insight that diffusion can be used to gain insight into the physical structure of the membrane. As predicted by the Singer-Nicholson model a simple diffusion of membrane components, where proteins can move freely within the membrane, has been found [7],[8]. Nevertheless a series of experiments showed a variety of complex diffusion types of the membrane proteins [9],[10], which indicate the heterogeneous structure of the membrane.

Two dimensional diffusion

In homogeneous and continuous media the diffusion can be described by Fick's law,

$$\frac{dx}{dt} = D\,\Delta n \tag{1}$$

where $n(r,t)$ is the concentration of the solute at position $\boldsymbol{r}$ and time t and D is the diffusion coefficient. The

diffusion of a particle, e.g. a membrane component, can be expressed by the probability $P(\boldsymbol{r},t)$ to find the particle at the time t at position $\boldsymbol{r}$. This probability distribution is a solution of (1) with initial conditions $n(\boldsymbol{r},0)=\delta(\boldsymbol{r})$. For the two dimensional case, i.e. for a membrane, it is obtained:

$$P(\boldsymbol{r},t)=\frac{1}{4\pi Dt}\exp\left(\frac{-r^2}{4Dt}\right) \tag{2}$$

A useful quantity to describe the diffusion is the mean square displacement (MSD(t)).

$$MSD(t)=\langle \boldsymbol{r}^2\rangle=\int r^2 P(\boldsymbol{r},t)\,d\boldsymbol{r}=\int \frac{1}{4\pi Dt}\exp\left(\frac{-r^2}{4Dt}\right) \tag{3}$$
$$MSD(t)=4Dt$$

The MSD(t) is useful as it can be easily calculated from the trajectory of a diffusing particle $\boldsymbol{r}(t)$ and the diffusion coefficient D can be readily obtained from it.

Diffusive behaviour that follows (3) is called normal or Brownian diffusion. Brownian motion is the result of the collisions of the thermally fluctuating particles of the solvent with the particle under observation and leads to a diffusion coefficient which is given by [12],

$$D=\frac{k_B T}{\xi} \tag{4}$$

with the Boltzmann constant k_B, the absolute temperature T and the friction coefficient ξ This is the fundamental concept of diffusive transport.

Normal diffusion is very common (hence the name), as its stepsize distribution $P(\boldsymbol{r},t)$ is Gaussian. The central limit theorem (CLT) states that all distributions with a finite second moment become normally distributed in the long time limit [13].

The MSD(t) can also describe different types of motion. Of particular importance for diffusive motion in membranes is the anomalous diffusion and the confined diffusion [8]. for anomalous diffusion the MSD(t) is no longer in time, but follows a power law.

$$MSD(t)=4Dt^{\alpha} \tag{5}$$

In membrane literature the term anomalous diffusion and subdiffusion are used synonymously and describe diffusion with an exponent $\alpha<1$. Diffusion with $\alpha<1$ is called superdiffusive and can be observed if active transportation is present. One method to achieve subdiffusive motion is by binding of the particle to fixed or slower diffusing sites. If the waiting time distribution has a diverging second moment, the motion becomes subdiffusive [14].

As anomalous diffusion is only defined as a power law in the long time limit, an exact scaling is only possible for loarge sets of data.

For confined diffusion the MSD(t) is given by:

$$MSD(t)=\langle \boldsymbol{r}_c^2\rangle\cdot\left[1-\exp\left(\frac{-4Dt\cdot A}{\langle \boldsymbol{r}_c^2\rangle}\right)\right] \tag{6}$$

with $\langle \boldsymbol{r}_c^2\rangle$ being the area of the confinement and A is a geometric factor [8].

The velocity of the diffusion can be deduced from equation 4 and depends essentially on the friction

coefficient ξ. In three dimensions the friction coefficient of a sphere of radius a is given by $\xi = 6\pi\eta a$, η being the viscosity of the medium [12]. Unfortunately the parameter η is not well defined for two dimensional systems, as the corresponding equations of fluid dynamics do not have a (finite) solution. [15] is an example for literature that discusses a workaround to this dilemma.

A special method is to assume a three dimensional liquid that surrounds the membrane. For a cylindrical protein of radius a with height h the diffusion coefficient can by described by the Saffman-Delbrück-model [16],

$$D \approx \frac{k_B T}{4\pi\eta h} \ln\left(\frac{\eta h}{\eta' a}\right) \tag{7}$$

where η is the viscosity of the membrane which needs to be much larger than the viscosity of the surrounding medium η' [15]. For typical monolayer experiments $\eta \approx 100 \cdot \eta'$ so this assumption is valid.

A main difference to three dimensional diffusion is the fact that the D is far less sensitive to the particles size, logarithmic instead of linear. So the diffusion in a homogeneous layer only depends on the viscosity of the membrane, its thickness h and the temperature T, all which are almost constant in biological systems. Thus the control of diffusion in a cell must depend on different mechanisms.

The diffusion of lipids is even more complex. Single lipid molecules have the same size as as the molecules of the membrane so that the continuity solution of the Saffman-Delbrück model is not valid anymore. Diffusion under this boundary conditions is best described by the free volume model [17]. In this model the lipid moves by jumping into free space which appear due to fluctuations of the molecules in the membrane [25]. The diffusion coefficient therefore depends strongly on the packing density %rho of the lipids an the size of it [18].

$$D = C \cdot \exp\left(\frac{-\gamma a_0}{a - a_0}\right) \tag{8}$$

where a is the mean area per lipid and a_0 is the size of a single lipid, γ is a fitting parameter for the adjoining area, that the lipid needs to move into and C is a fittingparameter that depends on the activation energy which is needed to create the area $\gamma \cdot a_0$. The free volume model can be applied for molecules up to twice the size of a single lipid (approx $1\,nm^2$), above that the Saffman-Delbrück model has to be used [19].

Methods for measuring lateral diffusion

Several methods for observing lateral diffusion in membranes have been developed. The first experiments on lipid diffusion have focused on the time lipids of two different cells (or vesicles)need to evenly mix during cell fusion [21]. This method describes diffusion in the dependence on ensembles of molecules. This requires the usage of statistical models that usually assume homogeneous Brownian motion.

Typical lipid diffusion constants are about $1\,\mu m^2/s$ $(10^{-12} m^2/s)$ where as the ones of proteins are in the range of 10^{-4} to $1\,\mu m^2/s$. Thus methods have to be developed that work on timescales of picoseconds to hours, which is required for to cover the length scales from atoms to cells (Angstroms to μm). Neutron diffraction, EPR- and NMR- methods work on the length scale up to 100nm and up to times in the order of microseconds [20,22]. Measurements on a longer, biological more relevant time scale require the additional use of a probe as described

below. It should be mentioned that measurements on short timescales result in diffusion constants up to a factor of 100 smaller than at long timescales. It is assumed that this is a result of the hoping motion that lipids undergo in the free volume model, where the movement into the neighbouring free area is much faster than the creation of a new adjacent free area [23].

The different types of lipids and proteins can be differentiated from other membrane components by attaching different markers to them [24].For fluorescence microscopy a fluorescent marker is attached to the molecule under observation and the diffusion of the dye is observed. The most widely used method is fluorescence recovery after photo bleaching (FRAP) [2],[3],[26],[27]. In this method a small fraction of the molecules (lipids or proteins) is labelled with a dye. In a small area (typically $1\,\mu m^2$) the dye molecules are bleached irreversible with an intensive light source (laser). As the molecules diffuse in and out of the area, the spot regains (part of) its fluorescence. The measured recovery curve, i.e. the increase of the light intensity, characterizes the the diffusive motion of the particles. The advantages of FRAP are its relatively easy set-up and the possibility to study diffusion on length scales from microseconds to hours. As a lot of parameters are needed for fitting a recovery curve, one FRAP measurement can be fitted to a sum of different fractions of different diffusion constants and under the assumption of anomalous and/or confined diffusion [7],[28].

Single Particle Tracking

To avoid this severe problem a technique has been devised that focuses not on molecule ensembles but on the trajectory of single molecules or particles, the so called Single Particle/Molecule Tracking (SPT,SMT) [8]. Molecules, like lipids or proteins, are too small to be observed directly and are thus labelled by a particle that is either visible due to fluorescence or diffraction of light [29],[30]. The movement of the label is assumed to be representative for the motion of the molecule itself. Comparing the size of the label to the molecule itself (30nm to 1nm for lipids and 5nm for proteins) this assumption seems to hold. The field of usage for SPT has not been completely exploited yet [31], but it results in diffusion constants that are in the order of the values obtained by ensemble methods, which use much smaller labels. For example gold colloids with a diameter of 30-100nm are used for dark field microscopy and fluorescent labelled polymer beads (67-150nm radius) and single fluorophore molecules are used in fluorescence microscopy. The size of single fluorophores is much closer to the size of proteins, but they usually bleach after approximately 20 steps.

The main advantage of SPT above FRAP is the possibility that the movement of the molecules can be characterized with a much higher spatial resolution, nanometres instead of microns. Thus fewer assumptions on the movement of the particles itself have to be made. In order to gain the maximum information from the trajectory the MSD(t) is calculated from

$$MSD(\Delta t)=\langle \boldsymbol{r}^2\rangle=\frac{1}{N}\cdot\sum_{j=1}^{N-n}\left[\boldsymbol{r}(j\,\delta t+n\,\delta t)-\boldsymbol{r}\,j\,\delta t\right]^2 \tag{9}$$

where $\boldsymbol{r}(t)$ denotes the the position of the particle at time t, N the total number of steps in the track, δt is the timestep between the individual steps and $n\cdot\delta t=\Delta t$. That means that the MSD is the average over all positions in the trajectory that are separated by equal times $n\,\delta t$. The MSD(t) can then be examined for normal, anomalous and confined diffusion.

One of the weaknesses has been the limited length of the trajectory which is usually in the order of several

hundred steps. This leads to a high statistical uncertainty and makes it very difficult to describe the scaling behaviour appropriately. The main reasons are the small observation area (half the cell surface), short lifetimes of the fluorophore and the limitation of early computers.

LANGMUIR MONOLAYERS

Water molecules at the air/water interface have a higher energy as molecules in the bulk due to the lower ability to form hydrogen bonds. The surface energy per unit area, the so called surface tension , is $73\,mN/m$ at $20\,°C$.

Surfactants, amphipilic molecules at the surface (tensides), reduce the free energy of the water molecules at the surface as polar parts form hydrogen bonds with the water molecules where as their non polar parts are directed towards the air. The surface tension $\Pi = \gamma - \gamma_0$ is the difference between the momentary surface tension γ and the the surface tension of the surfactant free surface γ_0. Π is indicative for the packing density of the monolayer and increases with increasing density.

By changing the surface pressure the monolayer undergoes phase transitions analogue to the three phases of a three dimensional gas. For many lipids there exist, at a certain pressure and temperature, a region at which different phases coexist, e.g. liquid condensed/liquid expanded (LC/LE). In this regime the different phases separate and the LC parts, which are almost incompressible, form domains in which the molecules are arranged in a hexagonal array, forming a two dimensional crystal. There exist different condensed phases that can be differentiated by the orientation of the lipid chains towards the surface (tilted condensed/ untilted condensed). If the pressure is high enough the monolayer collapses to a multilayer. The phase behaviour strongly depends on the chemical structure of the lipids, DMPC (di-myristoyl-phosphatidyl-choline)for example remains in the gaseous/liquid phase over the complete range of packing densities (at room temperature).

A typical example of a lipid that assumes all different phase states at room temperature is DMPE (di-myristoyl-phosphatidyl-ethanolamine). It has LC/LE coexistence region in which the monolayer is inhomogeneous. The phase transition is of first order. The shape of the domains is dendritic in the begining but relaxes to circular disks over time. The final size and shape is determined by a balance between the dipole-dipole repulsion of the individual lipids and the minimization of the length of the border between the two phases. The structure of the alkyl chain of the lipids can lead to shapes different from circles, e.g. DPPC (di-palmitoyl-phosphatidyl-choline).

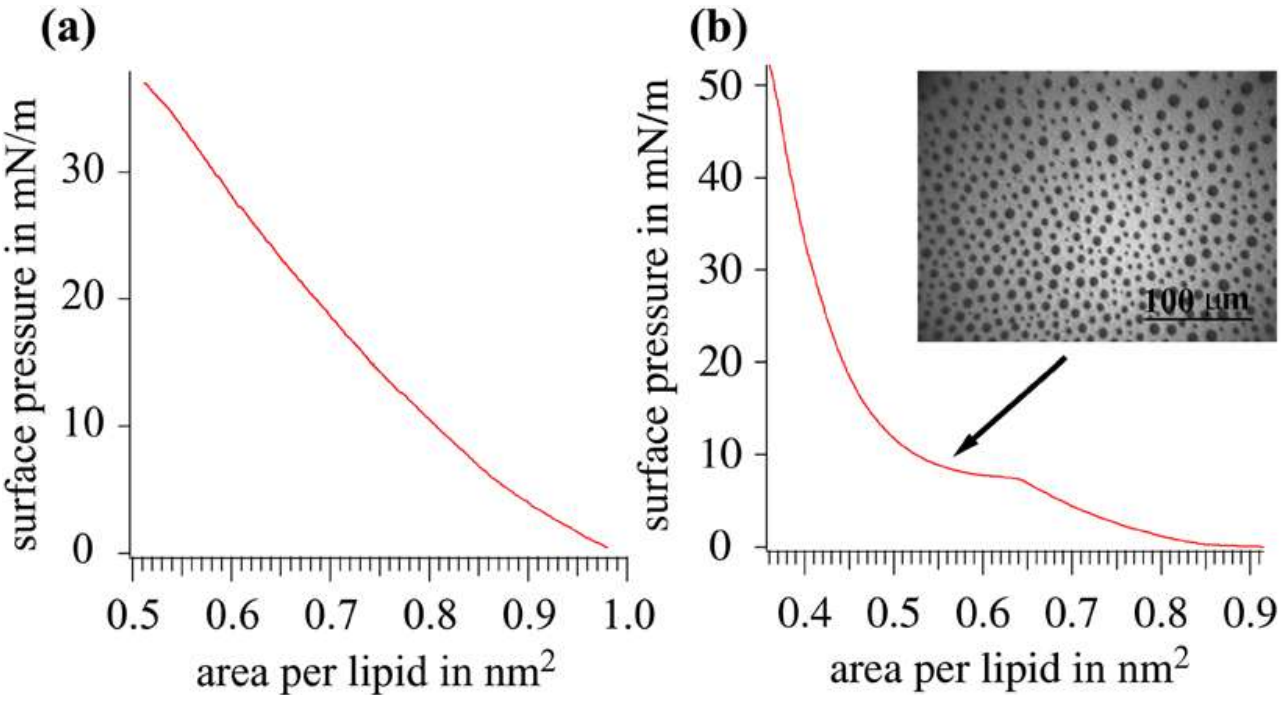

Fig. 1 ***Pressure-area-isotherm of Langmuir monolayer.***

(a) *DMPC isotherm* **(b)** *DMPE isotherm: the flat region is the liquid-condesed coexistence region.* **Inlay:** *flourescence image of the coexistence region, the dark areas are solid phase domains*

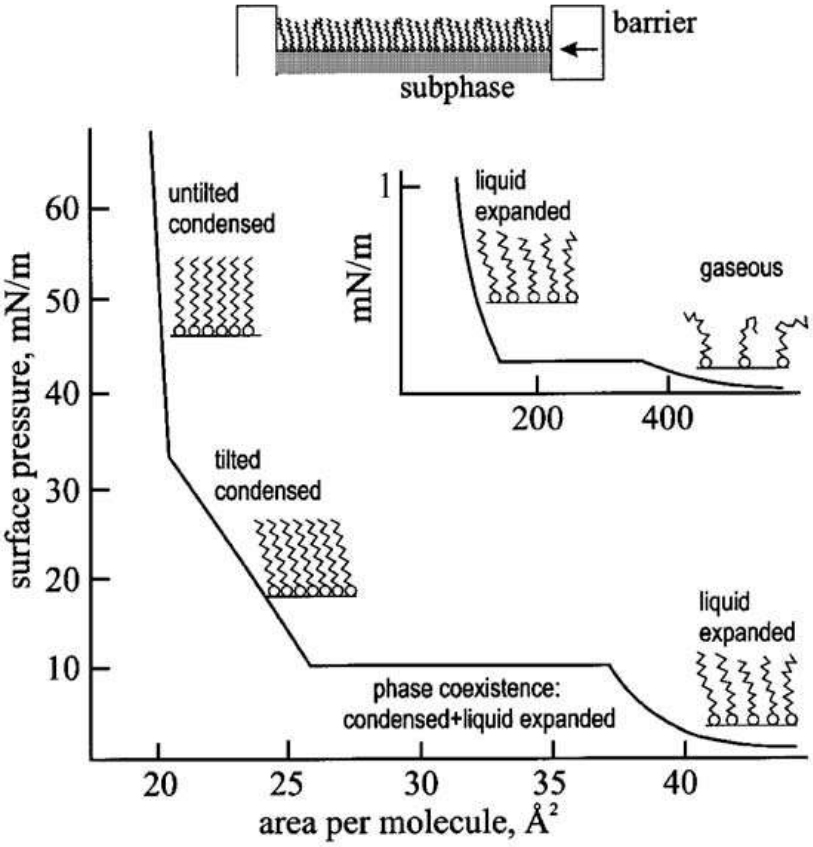

Fig 2 ***Schematic isotherm of a Langmuir monolayer.***

upper part: *a monolayer being compressed by a barrier* .**lower part***: transition form liquid to condensed phase with coexistence region. In the liquid expanded phase the molecules have long range oreder despite mutual interactions. The liquid condensed phase is an ordered hexagonal grid of lipids.* **inlay:** *transition from gaseous to liquid phase*

Tasks

a) Record the isotherm of DMPE on PBS at pH 7.5, room temperature

b) Record the motion of polystyrene-beads on PBS, in a fluorescent labelled DMPE monolayer at 5 different pressures. (the track length should be about 5000 frames or longer).

c) Record the motion of polystyrene-beads on PBS, in a fluorescent labelled DMPC monolayer at the same pressures as in b).

d) Analyse and discuss the corresponding trajectories and the MSD(t).

<u>Note</u>: The tracking of the particle takes several hours, and should be done over night. As the calculation of the MSD(t) needs an equivalent amount of time and some prior work, you will need to come to the lab one more time for about 30min-1h. Please arrange an appointment for one of the days following your experiment.

Commands for the single particle tracking routine

recording of the movie

open the ImDTD Writer Demo:	**Icon Desktop: ImDTD Writer Demo2.vi**
run the program:	**Operate -> Run** or **Ctrl+R**
insert file name:	**Configuration File path**
adjust film length:	**# of images** (preset 25.000 frames=16min)
start record:	**Acquire**
stop record:	**automatically after the adjusted time** or **F10**
replay the movie:	**ImDTD Reader Demo.vi**

Analysis of the movie

start IDL

Create tracks

read in frame ## of the movie:	**a=read_dtd(frame=##)**
output window:	**window, xs=768,ys=576**
display frame:	**tv,a**
highpass filter:	**b=bpass(a,1,13)**
display filtered image:	**tvscl,b**
locate beads:	**f=feature(b,13,masscut=?????,masstop=?????)**
massscut: lower intenstity bound	
masstop: upper intensity bound	
display found beads:	**fo=fover2d_pal(a,f,/cir,rad=10)**
run the tracking routine:	**autotrack_pal,masscut=????,masstop=????,/nodelete**

Calculation of the MSD(t)

A fixed particle is needed in order to eliminate the drift of the monolayer. the fixed particle should be close to the one you are looking at and should stay visible long enough. It might help to consider different fixed particles for different tracks, but you should keep in mind that the calculations take a long time.

read in file name:	**file=dialog_pickfile()**
read in the DTD information	**info=dtdinfo()**
number of the fixed bead:	**fixed=##**
calculation of the MSD(t):	**fullanalysis_pal,file,fixed,info**

Analysis of the Data

import

tracks diff from ## MSD C.txt	t,MSD0,errMSD0,MSD1,errMSD1,...
and **tracks diff from ## C.txt**	x0,y0,frame0,x1,y1,frame1,x2,y2,frame2,....

into Origin, Igor Pro etc.

create a weighted linear fit of the MSD(t) to obtain D

create a linear fit of the log-log plot of the MSD(t) to determine possible subdiffusion.

Due to their statistical significance only the first 10-30% of the tracks should be used!

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