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Plasma Membrane Plasticity of *Xenopus laevis* Oocyte Imaged with Atomic Force Microscopy

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Key Words
Lipid bilayer • Membrane protein • Membrane fluidity • Molecular volume • *Xenopus laevis* oocyte

Abstract
Proteins are known to form functional clusters in plasma membranes. In order to identify individual proteins within clusters we developed a method to visualize by atomic force microscopy (AFM) the cytoplasmic surface of native plasma membrane, excised from *Xenopus laevis* oocyte and spread on poly-L-lysine coated glass. After removal of the vitelline membrane intact oocytes were brought in contact with coated glass and then rolled off. Inside-out oriented plasma membrane patches left at the glass surface were first identified with the lipid fluorescent marker FM1-43 and then scanned by AFM. Membrane patches exhibiting the typical phospholipid bilayer height of 5 nm showed multiple proteins, protruding from the inner surface of the membrane, with heights of 5 to 20 nm. Modelling plasma membrane proteins as spherical structures embedded in the lipid bilayer and protruding into the cytoplasm allowed an estimation of the respective molecular masses. Proteins ranged from 35 to 2,000 kDa with a peak value of 280 kDa. The most frequently found membrane protein structure (40/µm^2) had a total height of 10 nm and an estimated molecular mass of 280 kDa.
Membrane proteins were found firmly attached to the poly-L-lysine coated glass surface while the lipid bilayer was found highly mobile. We detected protein structures with distinguishable subunits of still unknown identity. Since *X. laevis* oocyte is a generally accepted expression system for foreign proteins, this method could turn out to be useful to structurally identify specific proteins in their native environment at the molecular level.

Introduction

In living cells, plasma membranes separate the cell interior from the extracellular space by using a lipid bilayer, 5 nm in thickness, for isolation and membrane proteins as selective barrier molecules for transmembrane signal transduction. Atomic force microscopy (AFM) is a method for visualizing native biological surfaces at macromolecular resolution under near-physiological conditions. Over the past years AFM was applied to biological membranes [1] artificial bilayers [2,3,4], 2D

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and 3D protein crystals [5], excised plasma membrane patches [6], isolated membranes [7], nuclear envelope [8] and also to living cells [9,10]. In molecular cell biology *X. laevis* oocyte serves as a well established expression system for membrane proteins. Although function of membrane proteins has been elegantly examined by electrophysiological and fluorescence microscopical techniques little is known about the specific distribution of the proteins within the plasma membrane. AFM visualizes native membranes under physiological conditions and, within the limits of resolution, allows imaging protein structures protuding from the membrane at nanometer range. A large proportion of proteins expected to be localized at the cell surface are transmembrane proteins that in part interact with peripheral membrane proteins [11]. This suggests that images from the inner plasma membrane surface must share common features with images from the outer surface [12]. We focused on the cytoplasmic side of oocyte membrane to avoid problems associated with the glycocalyx, a complex structure composed of highly branched sugars covering the cell surface. We developed a method to spread patches of oocyte plasma membrane "inside-out" on glass suitable for AFM scanning. Spreading a plasma membrane patch on a solid support is currently the method of choice for identifying individual protein structures at the cell surface since the softness of living cells prevents macromolecular resolution.

Materials and Methods

Oocyte preparation

The experiments were performed on *X. laevis* oocytes stage V, which were obtained as described [13] and stored in Barth medium (87 mM NaCl, 1 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 5 mM HEPES, pH 7.4 containing 100 IU/ml penicilline and 100 µg/ml streptomycine). The oocytes were mechanically defolliculated 1 h after collagenase treatment (1 mg/ml, type D collagenase, Boehringer, Mannheim) and then incubated for 2 h in Barth medium. Removal of the vitelline membrane was performed by hypertonic shrinkage in potassium aspartate buffer (10 min; 200 mM K-aspartate, 20 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.4) followed by mechanical stripping similar as previously reported [14]. The oocyte plasma membrane was stained with FM1-43 (0.1 µM in Barth medium; Molecular Probes, Eugene, Oregon, USA) for one minute and then rinsed with Barth medium twice.

Preparation of the glass coverslip

Glass coverslips (Cellocate®, Eppendorf, Hamburg, Germany) with a 55 µm grid were cleaned by 1 h treatment with concentrated H_2SO_4 / 30 % H_2O_2 (9:1), rinsed three times with double destilled H_2O and twice with acetone. 40 µl of an aqueous solution of poly-L-lysine (0.01 % w/v, Sigma, Deisenhofen, Germany) was applied to the clean side of each glass coverslip and removed 10 min later. Coated glass coverslips were then baked for one hour at 60°C.

Plasma membrane preparation

After removal of the vitelline membrane and staining with FM1-43 the respective oocyte was transiently attached to the coated glass for a minute and then removed. The plasma membrane patch remaining on the glass surface was rinsed with H_2O, dried on air and located by fluorescence microscopy. The glass coverslip contained a grid, which helped to locate an individual plasma membrane patch for AFM.

For trypsin-digestion experiments plasma membrane was scanned in air and then floated with 0.05% trypsin in PBS-buffer. After 5 min of incubation, the liquid was removed, the sample washed twice with H_2O and dried at room temperature.

Atomic force microscopy

AFM was performed in contact mode using a Nanoscope III Multimode-AFM (Digital Instruments, Santa Babara, California, USA) with an E-type scanner (maximal scan area:15x15 µm). Glass coverslips (i.e. Cellocate®) were attached to stainless steel punches with double-sided adhesive tape and mounted in the commercially available fluid cell (Digital Instruments). V-shaped oxide sharpened cantilevers with spring constants of 0.06 N/m (Digital Instruments) were used for scanning in air. Images (512 x 512 pixels) were captured with scan sizes between 1 and 25 μm^2 at a scan rate of 12 Hz (12 scan lines/s). Images were processed using the Nanoscope III software (Digital Instruments). Particle counting and 3D presentation were performed with the software SPIP (Scanning probe image processor, Image Metrology, Lyngby, Denmark). This software allows analysis of particles located close to each other through the determination of „local minima" in height and therefore allows the definition of particle boundaries even from tightly packed structures. The precision of this process is affected by assignment of a threshold.

Molecular volume measurements of membrane proteins

In order to estimate the molecular mass (M_0) of individual membrane proteins we used a model published by Lärmer et al. [6] . The calculation is based on a simplified model imaging a membrane protein as a sphere embedded in the lipid bilayer. The volume of a single protein (V_{Prot}) was calculated using the sphere's volume equation ($V = 4/3\,\pi\, r^3$), with the protein radius, r, given by the half height of the protein. The molecular mass M_0 can then be calculated :

$$M_0 = \frac{N_A}{V_1 + d \cdot V_2} \cdot V_{Prot.}$$

In this equation N_A is the Avogadro constant ($6.022 \cdot 10^{23}$ mol^{-1}), V_1 is the partial specific volume of the protein (0.74 cm^3/g), V_2 is the specific volume of water (1 cm^3/g) and *d* is a factor describing the extent of hydration for air-dried proteins (0.4 mol H_2O / mol protein).

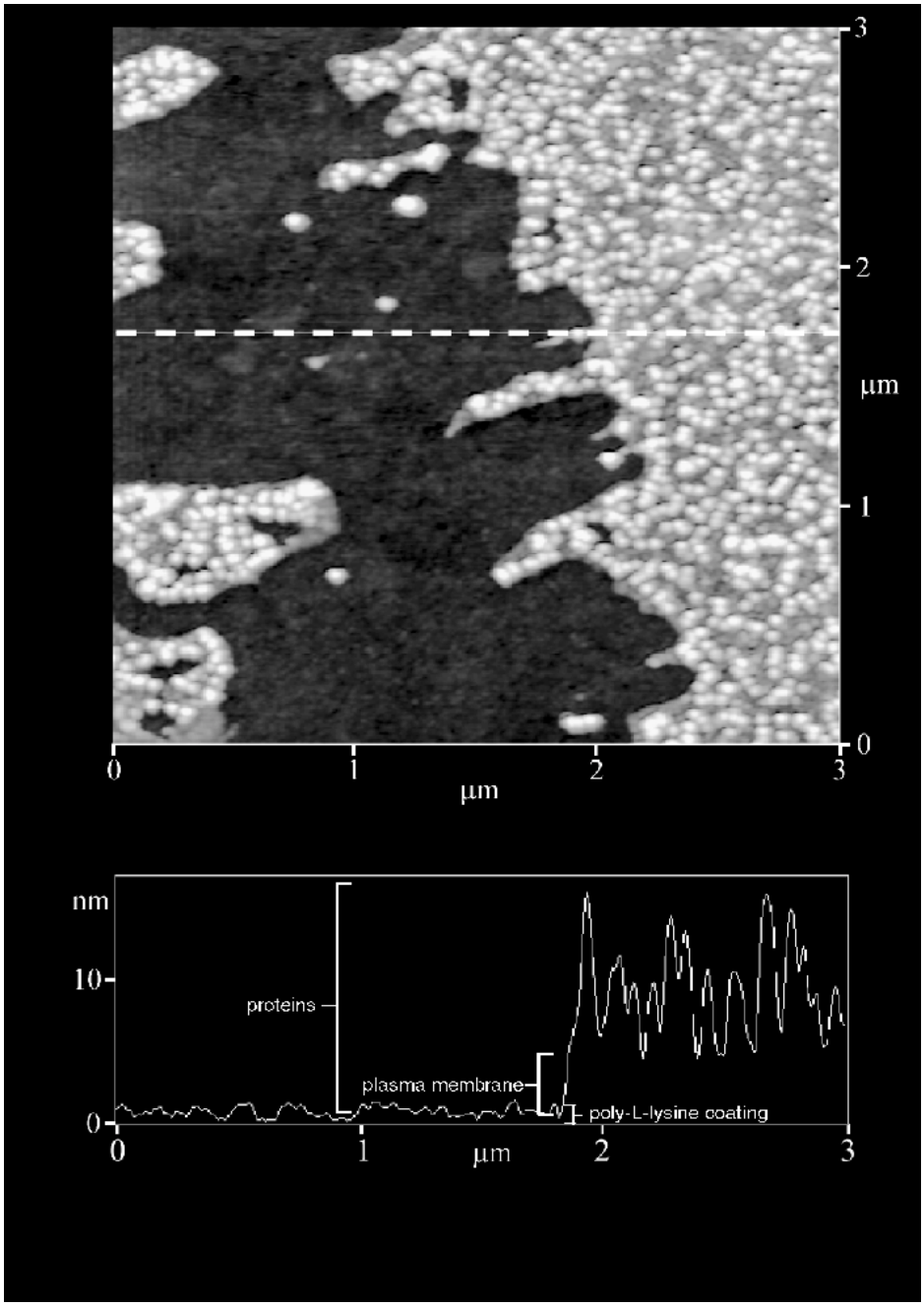

Fig. 1. View of a membrane fragment (cytosolic side) attached to poly-L-lysine coated glass. White spots covering the membrane are proteins protuding into the intracellular space. The broken line in the upper part corresponds to the profile line in the lower part. In the lower part of the figure the first height level is about 1.5 nm caused by poly-L-lysine coating. The second level corresponds to the 5 nm-height of the lipid bilayer. The third level (bracket) indicates the height of the proteins (up to 15 nm) protuding from the inner surface of the plasma membrane.

Results

Examination of cellocates by fluorescence microscopy revealed large patches of inside-out oriented plasma membrane, areas without membrane and small regions with relatively high structures (probably intracellular material; data not shown). For AFM experiments areas showing the outline of a membrane were chosen so that total height of plasma membrane and of protruding structures could be determined. Figure 1 shows the top view of a 9 μm^2 scan area containing plasma membrane fragments attached to the poly-L-lysine coated glass surface. White spots covering the membrane are proteins protuding into the intracellular space. Membrane fragmentation occurs frequently due to the preparation method we used. The broken line in the upper part of

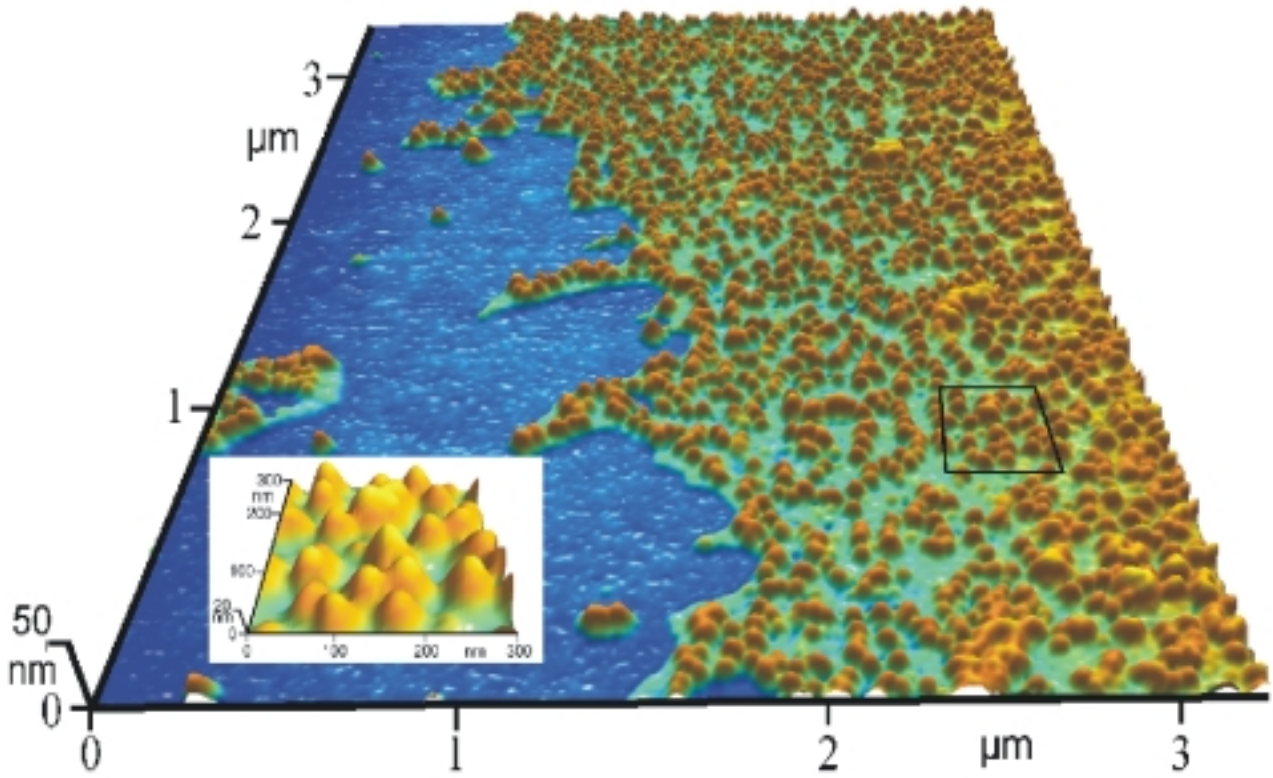

Fig. 2. 3D color-coded view of figure 1. Poly-L-lysine coated glass is shown in ”blue”, the lipid bilayer membrane is shown in ”turquoise” and the membrane proteins are shown in ”brown”. The insert shows a detail from the right part of this image (marked with a black rectangle) at higher magnification.

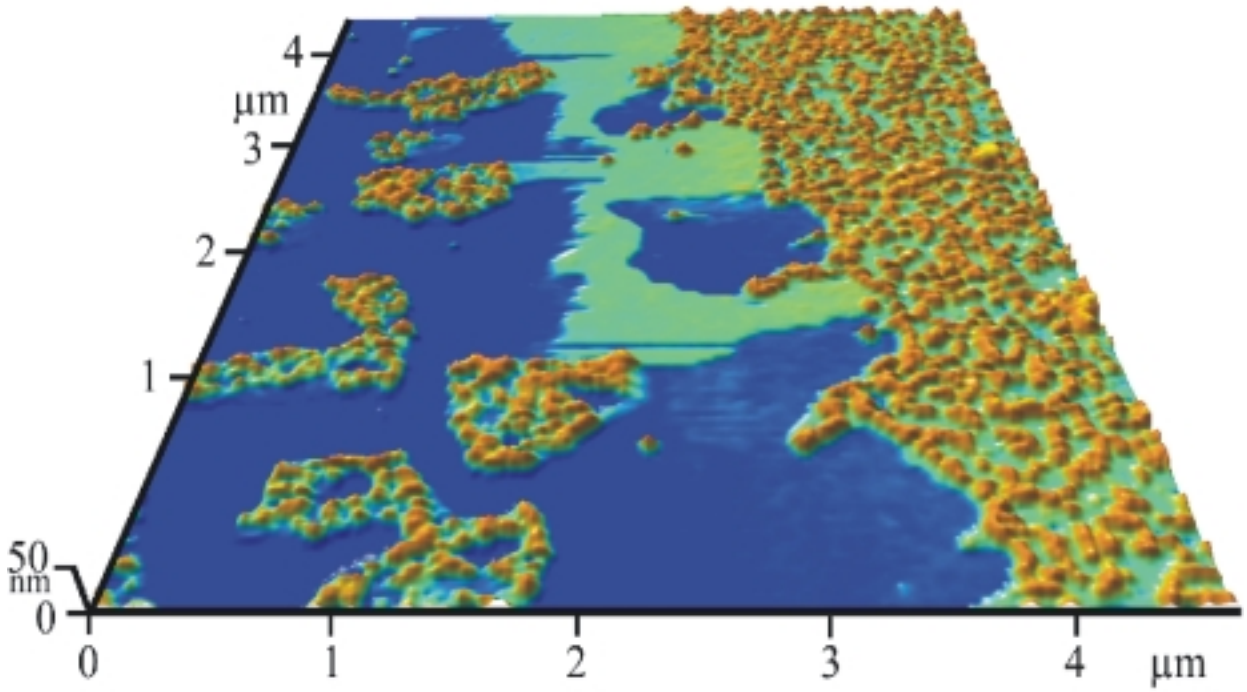

Fig. 3. 3D-view of the same membrane sample shown in Fig. 1 and 2. It was obtained actually before obtaining the image displayed in figure 2. It shows a large area of lipid membrane (”turquoise”) without any protuding proteins. Poly-L-lysine coated glass is shown in ”blue”, the lipid bilayer membrane is shown in ”turquoise” and the membrane proteins are shown in ”brown”.

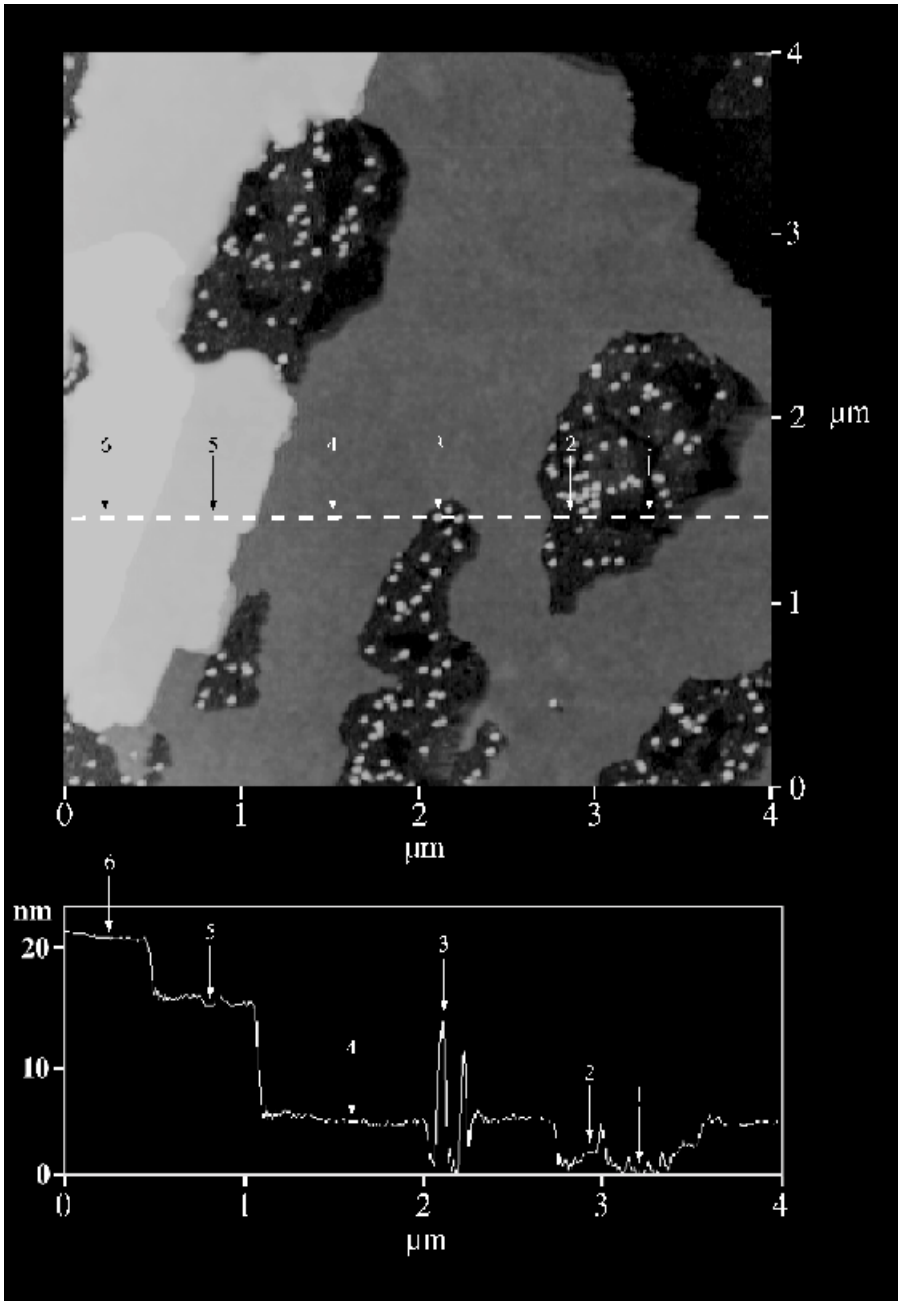

Fig. 4. Lipid membrane (arrow 4) attached to the glass support (arrow 1) but lacking proteins is shown. It further shows membrane proteins (arrow 3) firmly attached to poly-L-lysine coating (arrow 2) but lacking lipid bilayer. In the left part, multiple planes of bilayers in a lamellar arrangement are visible. The first plateau is a membrane of 5 nm in height (arrow 4), while the second plateau (arrow 5) represents two lipid bilayers (10 nm in height) on top of the former one (total height measured from the glass support = 15 nm). A fourth phospholipid bilayer (5 nm in height; total height in reference to glass surface = 20 nm) is visible in the image on far left (arrow 6).

figure 1 corresponds to the profile line in the lower part. Basically three different height levels can be determined: The first level is a height value of about 1.5 nm caused by poly-L-lysine coating. The second level corresponds to the 5 nm-height of the lipid bilayer. The third level indicates the height of the proteins (up to 15 nm) protuding from the inner surface of the plasma membrane. The same image is shown in figure 2 as a 3D color-coded view of the three different levels. Poly-L-lysine coated glass is shown in ”blue”, the lipid bilayer membrane is shown in ”turquoise” and the membrane proteins are shown in ”brown”. The insert shows a detail from the right part of this image (marked with a black rectangle) with higher magnification. The proteins appear with different heights and shapes. Some proteins are located so close to each other that they overlap or merge into one structure.

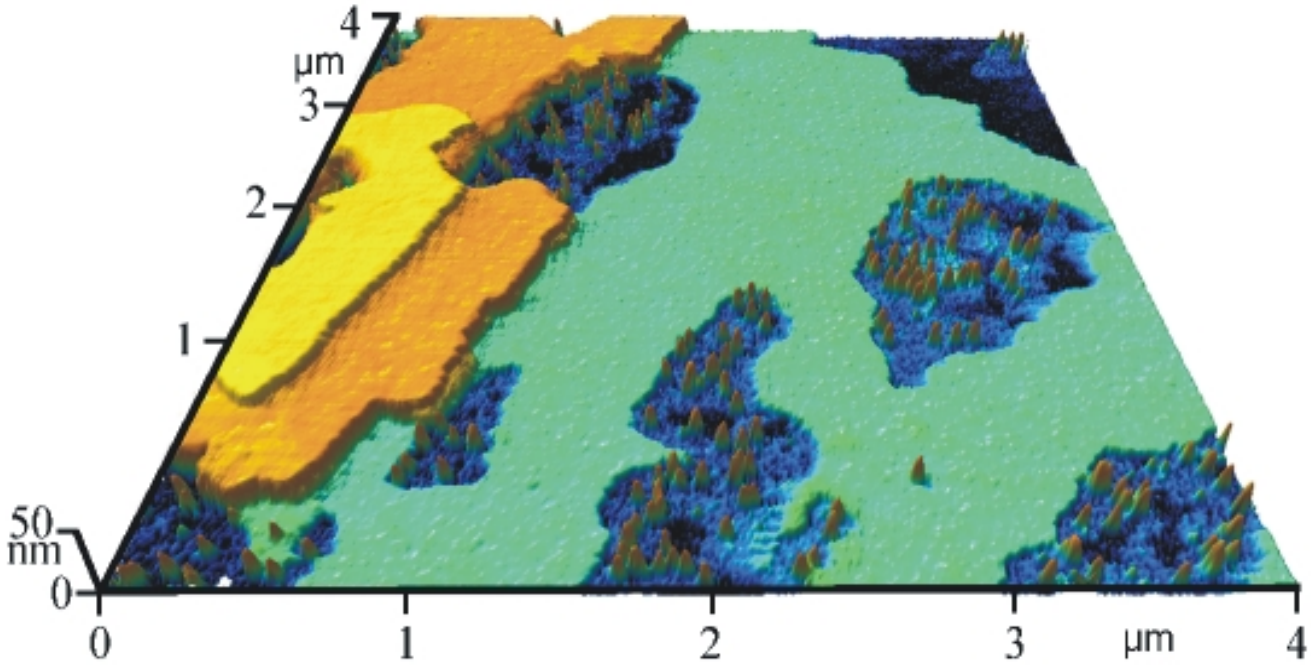

Fig. 5. Color-coded three-dimensional image of the membrane pattern explained in figure 4. „Black“ corresponds to glass surface and „blue“ to poly-L-lysine coating. „Brown“ particles are plasma membrane proteins, „turquoise“ indicates the first lipid bilayer, „orange“ the next two lipid bilayers and finally „bright yellow“ corresponds to the fourth lipid bilayer on top.

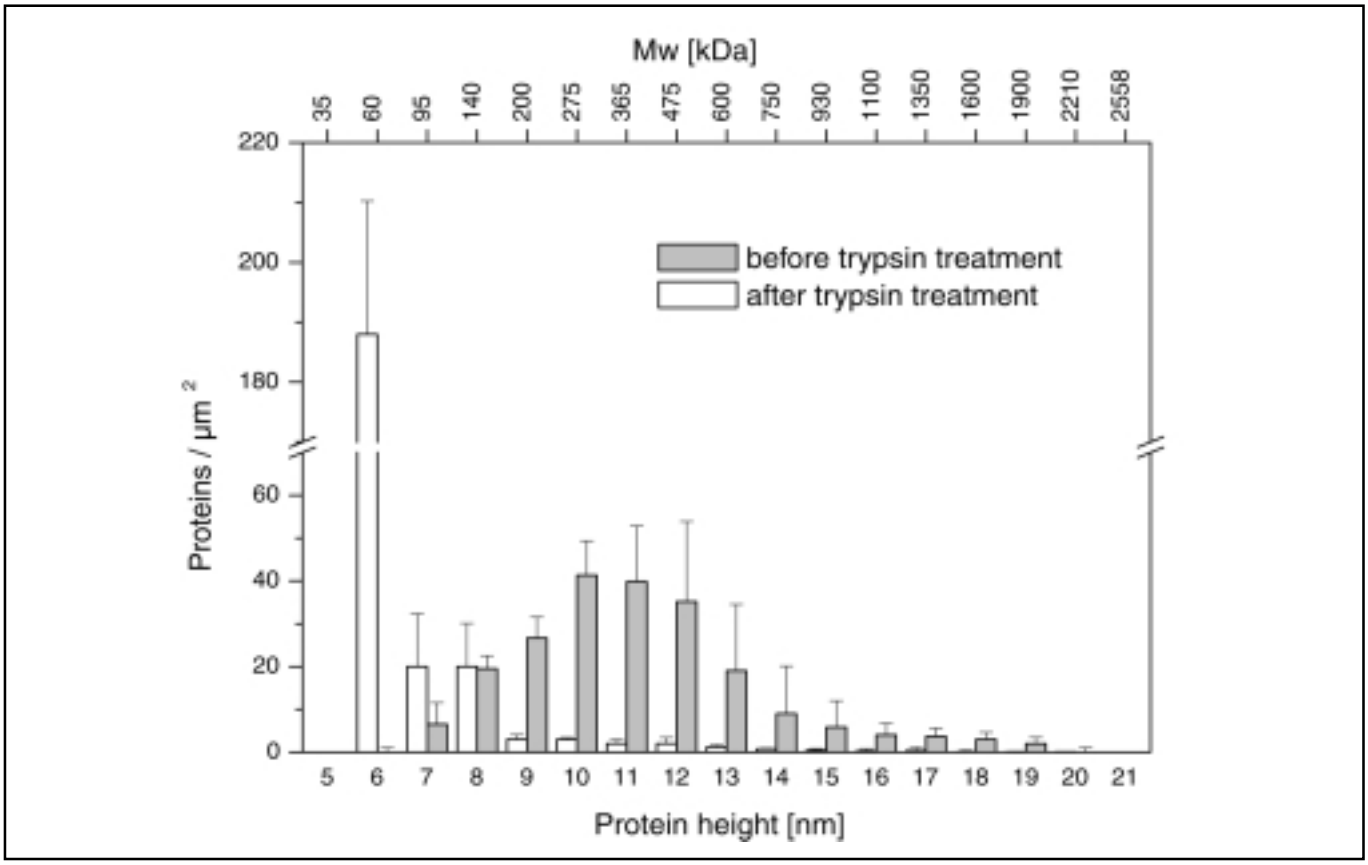

Fig. 6. Histogram of the plasma membrane protein distribution (intracellular side) in relation to measured protein heights and calculated molecular weights before and after incubation with trypsin. Molecular weights were calculated from the respective volume measurements (for details see me-thods).

Figure 3 is another 3D-view of the same membrane sample. It was obtained actually before imaging figure 2. It still shows a large area of lipid membrane (turquoise) without any protuding proteins. Repetitive scanning removes the phospholipid bilayer which lacks proteins whereas lipid bilayer that contains proteins, resists multiple scanning (compare figures 2 and 3).

Figure 4 shows a complex scenario indicating high plasticity of plasma membranes. The image shows lipid membrane (arrow 4) attached to the glass support (arrow 1) but lacking proteins. It further shows membrane proteins (arrow 3) firmly attached to poly-L-lysine coating (arrow 2) but lacking lipid bilayer. Such a scenario is obviously caused by incomplete poly-L-lysine coating. Membrane proteins need coating for attachment to glass whereas lipid membrane component do not. In figure 5, on left, multiple planes of bilayers in a lamellar arrangement are visible. The first plateau is a membrane of 5 nm in height (arrow 4), while the second plateau (arrow 5) represents two lipid bilayers (10 nm in height) on top of the former one (total height measured from the glass support = 15 nm). A fourth phospholipid bilayer (5 nm in height; total height in reference to glass surface = 20 nm) is visible in the image on far left (arrow 6).

Figure 5 shows the color-coded three-dimensional image of the membrane pattern explained in figure 4. „Black“ corresponds to glass surface and „blue“ to poly-L-lysine coating. „Brown“ particles are plasma membrane proteins, „turquoise“ indicates the first lipid bilayer, „orange“ the next two lipid bilayers and finally „bright yellow“ corresponds to the fourth lipid bilayer on top. The ripple pattern in this image may be the result of tangential stress exerted by the AFM tip on the deformable lipid bilayer. In order to test whether protusions emerging from the lipid membrane towards the cytoplasmic space were indeed proteins we incubated membranes with the enzyme trypsin. About 5 min after incubation with 0.05 % trypsin at room temperature protuding structures are dramatically decreased in height. Profiles taken before and after trypsin treatment clearly indicate that protusions are indeed proteins sensitive to trypsin digestion (data not shown). Figure 6 shows a histogram of plasma membrane protein distribution in relation to measured protein heights and calculated molecular weights. Measurements were exclusively taken from the intracellular aspect of the plasma membrane. Molecular volumes were estimated from protein heights measured by AFM. Molecular weights were then calculated from the respective volume measurements (for details see methods). Before trypsin treatment we found a wide range of different molecular weights with peak values up to 500 kDa. After trypsin treatment we observed a dramatic shift to the left with a sharp peak appearing at 60 kDa.

Discussion

Plasma membranes are composed of proteins and fatty-acid-based lipids, held together mainly by non-covalent interactions. This array of proteins, sterols, and phospholipids is organized into a liquid crystal, a structure that lends itself to rapid cell growth. The phospholipid molecules diffuse readily in the plane of the bilayer, sliding over the face of the membrane as their loose chemical bonds permit this [15]. Many of the membrane proteins also have this freedom of movement, while others are fixed in the membrane by interaction with the cytoskeleton. Surprisingly, we did not find cytoskeletal structures attached to the intracellular surface of the plasma membrane. We assume that binding between cytoskeletal structures and membrane proteins was disrupted during the process of membrane excision leaving behind the membrane proteins embedded in the lipid bilayer firmly attached to the coated glass surface. Nevertheless we cannot exclude the possibility that membrane proteins firmly bound to cytoskeletal structures were pulled off the lipid bilayer leaving behind membrane lacking cytoskeletal bound proteins.

Most frequently we found plasma membrane protein structures with about 10 nm for protein height (cytoplasmic domains: 5 nm in height). The proteins differ from each other in height and shape. Some are hardly detectable, others are up to 20 nm in height. Since plasma membrane height is 5 nm, we cannot detect proteins smaller than 5 nm, i.e. membrane-embedded proteins. The apparent shape of the proteins is found usually conical most likely due to the fact that lateral dimensions of the base of individual proteins are overestimated as a result of AFM-tip geometry [6]. Some proteins apparently exhibit „shoulders“, while others stand so close to each other that they overlap (insert of Fig. 2). From the calculated molecular weights we assume that single proteins assemble into multimeres or clusters. Currently we have no direct arguments whether the protein accumulations imaged are homo- or heteromultimers. This important issue has to be addressed in further experiments designed to identify specific proteins in the plasma membrane.

Usually membrane fragmentation occured in our experiments. This was most likely due to an imbalance of the forces between poly-L-lysine coating, proteins and lipid components. There are some „rules“ that in general can be drawn from the images: First, membrane proteins need poly-L-lysine coating for firm attachment on glass. Second, the lipid bilayer is stable only when protein density of the membrane is high. The pure lipid component of plasma membrane on poly-L-lysine coating usually does not resist scanning. Third, poly-L-lysine coating facilitates protein attachment. These phenomenons explain at least in part the inhomogeneous distribution of lipid membrane and membrane proteins found in this study. Another reason why membrane proteins seem neccessary for stabilizing the lipid bilayer could be the hydrophobic interaction between phospholipids and transmembrane domains of the proteins. Finally, high protein density shields the lipid bilayer from applying lateral scanning forces caused by the AFM-tip. We assume that the affinity of the phospholipids to uncoated glass is higher than the lipid affinity to poly-L-lysine. This apparently results in a flow of the lipid bilayer away from proteins (attached to poly-L-lysine) towards naked glass. The hydrophobic interactions between phospholipids and proteins are usually strong enough to hold the lipids back. However, when membrane proteins occur at low density in the lipid membrane, hydrophobic interactions are too rare and thus lipids flow off.

Derived from our observations we postulate a minimum of four different forces that should be considered when plasma membrane is attached to glass. One force is the (possibly electrostatic) attraction force between membrane proteins and poly-L-lysine (F1). A second force is the attraction force between the hydrophobic portions of the membrane proteins and the lipid bilayer (F2). An attraction force of similar magnitude is assumed to occur between the phosholipid bilayer and the naked glass surface (F3). Finally, a repelling rather than an attraction force is assumed to exist between lipid bilayer and poly-L-lysine (–F4). Besides these four forces (F1 to F4) the density and intrinsic nature of the membrane proteins (size, glycosylation, electrical charges, etc) in the lipid bilayer determine whether a native plasma membrane can be successfully spread on glass or not.

Modelling plasma membrane proteins as spherical structures protruding from the lipid bilayer allowed an estimation of their possible molecular weights [6]. Proteins ranged from 35 to 2,000 kDa with a peak value of 280 kDa. The most frequently found protein structure (40 proteins per μm^2) had a total height of 10 nm (including the intramembrane domain), an apparent lateral dimension of the cytoplasmic domain of about 40 nm and an estimated molecular weight of 280 kDa.

In conclusion, this sample preparation could serve as a suitable method for imaging the inner surface of oocyte plasma membrane and determining number and distribution of membrane proteins. It could be useful in identifying specific proteins in native membranes with “immuno-AFM” [16,17]. Using the oocyte as an expression system for foreign proteins in combination with specific antibodies it should be feasable to identify individual members of a protein family forming a functional cluster in the plasma membrane as previously proposed [18].

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