Stable integration of isolated cell membrane patches in a nanomachined aperture

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We present a method for integrating an isolated cell membrane patch into a semiconductor device. The semiconductor is nanostructured for probing native cell membranes for scanning probe microscopy *in situ*. Apertures were etched into suspended silicon-nitride layers on a silicon substrate using standard optical lithography as well as electron-beam lithography in combination with reactive ion etching. Apertures of 1 μm diam were routinely fabricated and a reduction in size down to 50 nm was achieved. The stable integration of cell membranes was verified by confocal fluorescence microscopy *in situ*. © *2000 American Institute of Physics.* [S0003-6951(00)01834-9]

Proteins embedded in the lipid bilayer that forms cell membranes are responsible for a variety of elementary functions determining the behavior of the cell. These proteins enable transport of all kinds of molecules from the out- to the inside of the cell and vice versa, acting as carriers or simply as pores. They also act as receptors that activate processes inside the cell when a ligand binds to them on the outer surface.[1]

The application of scanning probe techniques (SPM), e.g., atomic-force microscopy (AFM) or scanning near-field optical microscopy (SNOM) can give detailed insight into the topology or even dynamics of proteins embedded into or attached to the membrane. For such an experimental approach it would be favorable to work with isolated, suspended membrane patches instead of whole cells. This approach ensures that only the topology of the membrane and related proteins is mapped. Membrane patches are routinely isolated by physiologists using the patch-clamp technique.[2,3] This method relies on forming a micron-sized contact with the cell membrane by means of an electrolyte-filled glass pipette. The open tip of the pipette is pressed against the membrane, defining an isolated patch. Due to the strong glass–membrane adhesion,[4] a mechanically stable seal is obtained. Withdrawing the pipette results in a membrane patch that spans across the tip of the pipette.

Scanning probe technique studies have been carried out on whole cells, investigating mechanosensitive ion channels[5] and voltage-dependent membrane displacements,[6] as well as on isolated membrane patches[7] with lateral resolution as low as 10 nm. Nevertheless, the application of scanning probe techniques on membrane patches in the tip of patch pipettes is technically rather demanding, due to the small tip geometry. Moreover, to access the membrane patch with SPMs, the pipette has to be inverted so that the tip points upwards. This requires a complicated setup, which can be circumvented with the biochip developed in this work.

The development of SNOM for liquid environments[8] has enabled high-resolution fluorescent imaging even from single molecules.[9] Also, the recent advances in using fluorescent resonance energy transfer (FRET) to monitor very accurately conformational changes of single molecules has led to an increasing number of studies.[10,11] Although FRET can be performed on single molecules, these studies measure the fluorescence signal of a large ensemble of proteins. To take full advantage of these powerful techniques, the probing of single proteins in a natural environment is desirable. This can be done using our device, where concomitant electrical recordings of conductance changes of proteins like ion channels are possible.

Our approach is based on a semiconductor probe with a suspended layer in which a submicron-sized aperture is etched. A cell membrane patch is suspended into this aperture by an integration procedure described below. The planar geometry of the arrangement is ideally suited for scanning probe experiments. Therefore, the presented chip device lends itself to probe single proteins and serves as a workbench for further detailed studies on these proteins.

A Si_3N_4 layer is suspended on a micron scale by etching a V-groove into the (100)-silicon substrate beneath. In order to build these suspended membranes we deposit a 120-nm-thick Si_3N_4 layer on both sides of a (100) low *n*-doped silicon substrate using low-pressure chemical-vapor deposition. Applying standard optical lithography and reactive ion etching (RIE), we define an etch mask on the backside of the samples. Subsequent anisotropic wet etching in a KOH solution results in a V-shaped groove, where the upper Si_3N_4 layer serves as an etch stop. Adjusting the size of the etch mask we build suspended Si_3N_4 layers with dimensions of a few tens of μm side length. Both optical lithography and low-energy electron-beam lithography are used to define an orifice which is transferred into the suspended layer by a RIE process. The lower inset in Fig. 1(a) shows apertures in such a suspended Si_3N_4 layer with sizes ranging from 500 down to 50 nm.

The integration of a cell membrane patch is achieved by

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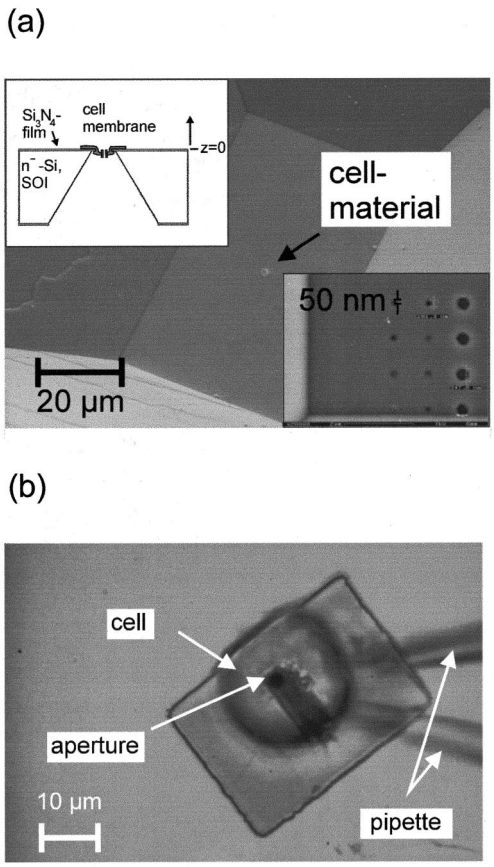

FIG. 1. (a) SEM micrograph of a V-groove in (100) silicon with a suspended Si_3N_4 layer on top. In the central aperture cell material is incorporated. The upper inset depicts the arrangement of the semiconductor–cell hybrid. The lower inset shows a series of holes in a suspended membrane with dimensions down to 50 nm. (b) Photograph of the probe with the cell positioned on top of the aperture.

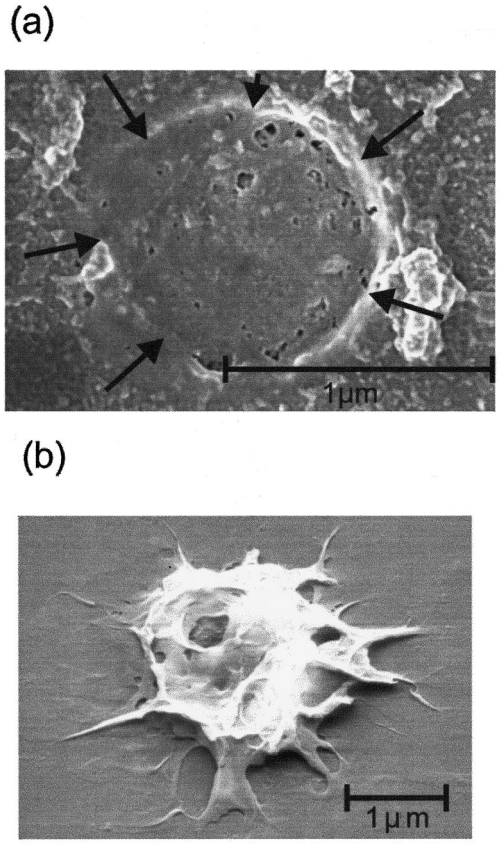

FIG. 2. (a) Top-view of the aperture with incorporated cell material. The arrows indicate the circumference of the opening. (b) Close-up of the cell membrane taken from the backside protruding from the opening.

positioning a single cell on top of the aperture, which is surrounded with standard Ringer's electrolyte solution (270 mOsm) from both sides. Cultured embryonic cells from rat striatum or C6-glioma cells are acutely dissociated applying standard trypsin treatment and trituration. The inset in Fig. 1(a) schematically shows the arrangement of the semiconductor/cell membrane hybrid. In order to carry out electrical measurements, the ensemble can be connected to an amplifier via $Ag/AgCl_2$ electrodes in the electrolyte.

A glass suction pipette is used to move an isolated cell onto the aperture, as shown in Fig. 1(b). By applying negative pressure from below, the cell membrane is partially sucked into the opening. In order to obtain a cell-free patch, the glass pipette is used to remove the cell body, leaving an excised membrane patch in the aperture. In Fig. 2, this excised patch is shown from both sides of the device. After fixation of the sample in 1% glutaraldehyde solution, dehydration in a graded alcohol series, and drying in a critical point drier, micrographs are taken with a low-voltage scanning electron microscope (SEM) with a resolution of about 1 nm. Figure 2(a) shows a top view of the aperture. Cellular material (presumably cytoskeletal elements) are seen to fill the entire lumen. Figure 2(b) shows a close-up view of the cell membrane that has been dragged into the opening. Fixation, dehydration, and drying are responsible for the somewhat distorted surface structure of the cell membrane.

Furthermore, we apply confocal laser scanning fluorescence microscopy (Biorad MRC 1024, United Kingdom) to image the hybrid in the ionic solution, i.e., in a situation where the membrane proteins and their functions are intact. In order to visualize the membrane, we incubate isolated cells with a solution containing the fluorescent marker bisoxanol (Molecular Probes) prior to integrating the membrane into the probe. The fluorophore is excited by blue light (488 nm) emitted from an Ar-ion laser. In Fig. 3(a) a scanning micrograph of the membrane–semiconductor hybrid taken with a confocal fluorescence microscope is shown. On the suspended Si_3N_4 layer, fluorescent cell debris is found in the environment of the aperture. A structure of a more regular, round shape can be discerned near the center of the image. It represents a fluorescent cellular membrane incorporated in the aperture. As shown in Fig. 3(b), using a *z*-scan series, this structure can be clearly distinguished from the surrounding debris. The graph shows a plot of the fluorescence intensity as a function of the distance of the confocal plane from the probe surface. Thus, successive optical sections parallel to the probe surface ranging from about -4 to 4 μm with zero set at the Si_3N_4-membrane level are taken. The three curves correspond to the normalized fluorescence light intensity emitted from the clean Si_3N_4 film, the debris on top of the film, and the membrane in the aperture, respectively. Obviously, the fluorescence of the fractured cell material is emitted starting from a *z* position larger than that of the Si_3N_4 film. In contrast, the fluorescence of the incorporated membrane displays a *z* range on the same level or even lower than

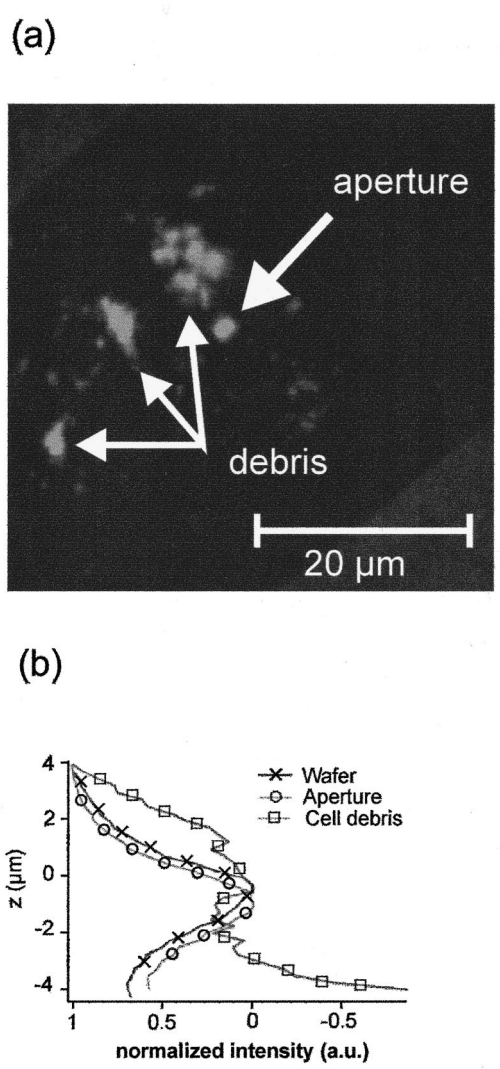

FIG. 3. (a) Fluorescence scanning micrograph taken with a confocal microscope. The cell membrane is labeled with a fluorescent marker (thick arrow). Some cell debris is also visible (thin arrows). (b) *z* series taken from the fluorescence of the cell membrane, the Si_3N_4 layer, and the debris on top of it.

the reference Si_3N_4 film. The increase of fluorescence intensity of the debris in the negative *z* range is related to the backscattering of excitation light from the Si_3N_4 layer acting as a birefringent mirror. Since the integrated cell membrane is freely suspended, this effect is not seen in the aperture.

The morphological findings described here show that it is possible to stably integrate an isolated patch of native cell membrane into a nanomachined device with a submicron-scale aperture, in a configuration which is suitable for scanning probe investigations. When scanning the suspended cell membrane patch with AFM/SNOM cantilevers, the question of membrane stability arises. As known from membrane patches in glass pipettes, electrical and mechanical stress leads to electroporation and membrane breakdown.[12] For natural as well as for artificial lipid membranes, the elastic area expansion moduli K vary from 50 to nearly 2000 dyn/cm and the tensile strengths τ range from 2 to 30 dyn/cm.[13] The maximum pressure a circular membrane patch with radius r can withstand is given by $P=2\tau/r$, therefore, minimizing the aperture size improves the ruggedness of the device. This very stable arrangement increases the possible resolution for SPM, because a higher scanning force can be applied without breaking the membrane.

As known from experiments with artificial lipid membranes, small perturbations can lead to the breakdown of a membrane. Therefore, a stabilized system is also very desirable for long-term measurements. Ultimatively, the miniaturization of the membrane patch will enable the fabrication of devices that are to some extent shock and vibration resistant. This is one of the key issues for building any kind of sensor based on ion channels in lipid membranes, which is a goal pursued by different groups.[14–16] The use of an ion channel as a molecular sensor for highly sensitive devices is very compelling and the presented structure is a first step towards an integrated hybrid biosensor.

In conclusion, we have shown a cell membrane patch integrated into a nanostructured semiconductor device verified by confocal fluorescence microscopy and SEM micrographs. With the presented chip it is now possible to attach native cell membranes to nanostructured probes. The geometry of this device can be freely chosen, also allowing the integration of additional features on-chip, e.g., gate electrodes. Furthermore, measurements of ionic currents through ion channels in the membrane patch may be possible, if sufficient electrical insulation of the silicon with respect to the surrounding electrolyte can be achieved. This would allow a detailed study of structure/function relations of the channel proteins and opens the way for device applications based on ion channels.

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