Fluorescence Interferometry of Neuronal Cell Adhesion on Microstructured Silicon

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We measured the separation of cell membranes from a surface of silica with nanometer precision taking advantage of the fluorescence of an organic dye in the standing modes of light above silicon. For neural cells from rat brain we found about 105 nm on a surface coated with laminin and about 60 nm with fibronectin. No plaques of close adhesion were seen within a lateral resolution of 400 nm. The wide homogeneous cleft raises the question about the nature of the attractive and repulsive forces in cell adhesion. [S0031-9007(98)07867-3]

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Migration and outgrowth of living cells are investigated generally on solid substrates coated with proteins of the extracellular matrix [1–3]. Little is known about the microscopic structure of the cell adhesion which is the basis for any description on a molecular level. We measured the distance of the membrane of neuronal cells from a silica surface coated with laminin and fibronectin, taking advantage of the fluorescence of a membrane-bound dye in the standing light waves above a microstructured silicon chip. The method is insensitive to ill known optical parameters of the cell, yielding a systematic error of about 1 nm. We found a surprisingly wide extracellular cleft of 105 and 60 nm on laminin and fibronectin, respectively, which was surprisingly homogeneous within 5 nm without visible plaques of close adhesion.

Standing waves of the electromagnetic field form in front of a reflecting interface. The fluorescence intensity of a dye molecule depends on the distance from the interface due to a modulation of excitation and emission [4–7]. We consider a cell grown across microscopic steps of silicon dioxide (height $\Delta d_{\mathrm{ox}} = 20$ nm, width 2.5 μm) on silicon (Fig. 1) with a distance d_{cleft} between cell membrane and chip. The membrane is placed by the steps at different positions in the standing waves. If it is stained with a dye, we expect a pattern of fluorescence intensity in register with the steps. The intensity as a function of the thickness of oxide d_{ox} depends on the distance d_{cleft}. Vice versa d_{cleft} can be obtained by fitting experimental data by a multireflection optical theory [8,9]. This method of fluorescence interference contrast (FLIC) microscopy was tested in model systems such as Langmuir-Blodgett films [8] and erythrocyte membranes [9]. It relies on homogeneous staining, illumination, and sensitivity of detection.

Chip.—Four inch silicon wafers (100 surface) were oxidized at 1000 °C in wet oxygen up to 320 nm [10]. A profile with sixteen levels of oxide (Fig. 2, inset) was fabricated. Photolithography, etching with ammonium fluoride, and cleaning with Caro solution (96% sulfuric acid, 30% hydrogenperoxide, 3:1) were repeated with four alignments of a mask with 5 μm stripes. The quadratic steps gave rise to homogeneous Newton-type interference colors in white light (Fig. 2, inset). After applying a hydrophobic coating (2% dimethyldichlorosilane in toluol for 15 min) their height was measured by ellipsometry on larger reference squares with an accuracy of 0.3 nm [9].

Cells.—The chips were sterilized in UV light. Laminin (Sigma, Heidelberg, F-2020) or fibronectin (Sigma, F-2006) was adsorbed from 10 μg/ml solutions in phosphate buffered saline (pH 7.0) for 12 h. (After drying, the adsorbed laminin was optically equivalent to 3–4 nm silicon dioxide.) Polylysine (MW 10000, Sigma) was adsorbed to hydrophilic chips [9]. Neural cells were dissociated from the hippocampi of Wistar rats (Thomae, Biberach) at 18 days gestation considering rules of animal welfare. They were preplated in DMEM/glutamax I (Gibco, Eggenstein, 61965-026) with 10% fetal bovine serum (Gibco, 10270-031) and 1% penicillin/streptomycin (Gibco, 15146-114) to remove glia [11,12]. They were resuspended in L15/glutamax I (Gibco, 31415-029) with 5% serum (1000 cells/ml), applied to the chips and—after

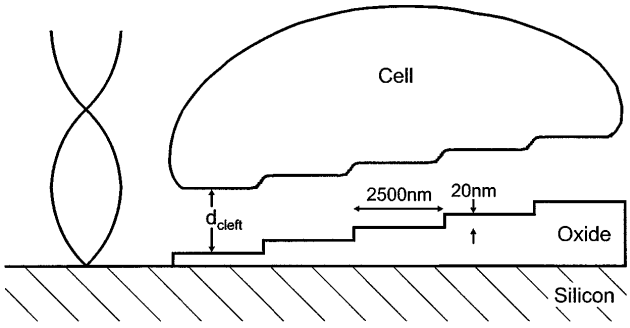

FIG. 1. Fluorescence interferometry of cell adhesion. The electromagnetic field forms standing modes above silicon. A cell grows across microscopic steps of silicon dioxide. Each step has a height of 20 nm and a width of 2.5 μm. The vertical scale of the wave, of the steps, and of the contact region is blown up by a factor of 20. The cell membrane is stained with a fluorescent dye. The fluorescence depends on the height of the steps d_{ox} and the width d_{cleft} of the extracellular cleft. The set of experimental intensities is fitted by an optical theory with d_{cleft} as a free parameter.

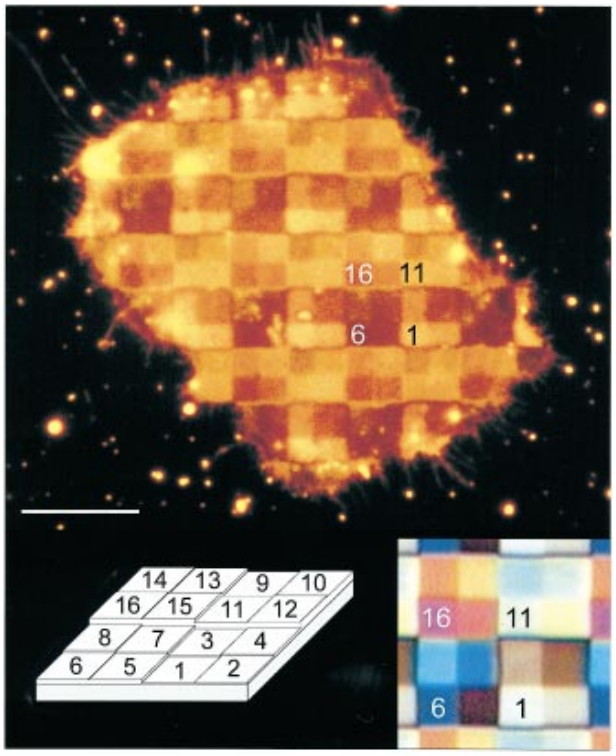

FIG. 2(color). Fluorescence micrograph of an astrocyte on silicon with quadratic steps (2.5 μm $\times$ 2.5 μm) of oxide coated with laminin. The membrane was stained with the dye S27/DiIC$_{18}$. The checkerboard pattern originates from the lower membrane. Bar: 10 μm. Left inset: Unit cell of the chip. The height (enhanced $\times 2$) of the 16 steps of oxide increases from $d_{ox} = 20$ nm to $d_{ox} = 320$ nm in the order of the numbers. Right inset: Micrograph of a unit cell in white light with Newton-type interference colors.

settling for 2 h—cultivated in a 1:1 mixture of both media for 3 days at 37 °C and 10% CO_2.

Fluorescence.—We prepared an aqueous dispersion of the amphiphilic cyanine dye S27/DiIC$_{18}$ [13] by adding 10 μl of a 2.5 mM ethanolic solution to 30 ml of TRIS buffered saline (pH 7.4). The cell membranes were stained by substituting the culture medium for 5 min and repeating the procedure once. For the measurements we kept the cells in DMEM/glutamax I buffered with 7 mg/ml HEPES. Fluorescence pictures were taken through a water immersion objective (100×, numerical aperture 1.0, lateral resolution 400 nm) (Zeiss Axioskop) at an illumination of 546 nm (mercury lamp) and a detection between 580–640 nm choosing appropriate filter combinations with a CCD camera (effective pixel size 90 nm $\times$ 90 nm, illumination time 40–320 ms).

Results.—A micrograph of a stained astrocyte on laminin is shown in Fig. 2. The checkerboard pattern of fluorescence corresponds to the 2.5 μm $\times$ 2.5 μm steps of oxide. CCD pictures of the astrocyte and of neurons (soma and growth cone) on laminin and of an astrocyte on fibronectin are shown in Fig. 3. Inhomogeneities within the squares are small compared with the contrast between squares differing in height by 20 nm. Some bright spots are due to microcrystals of the dye. We assign the checkerboard pattern to the fluorescence of the lower membrane: Some microcrystals of the dye were deposited on top of the cells; when these came into focus the checkerboard pattern disappeared. A possible undulation of the upper membrane cannot give rise to a modulation of fluorescence because the higher orders of standing modes are smoothed due to the large aperture and to defocusing [9]. Only in the periphery, the upper membrane gives rise to fringes [Figs. 3(c) and 3(d)] where it crosses the first maxima of standing modes [9].

On the squares marked in Figs. 3(a)–3(d), we fitted histograms of fluorescence intensity with Gaussians. Average and standard deviation were plotted in Figs. 3(e) and 3(f) versus the thickness of oxide. For laminin the intensity was highest on the thinnest oxide. This indicated a wide extracellular cleft, as the standing modes have a node near silicon. For comparison Fig. 3(f) shows the results of an erythrocyte on polylysine with a minimal intensity on the thinnest oxide due to a narrow cleft [9].

Theory.—We used an optical model with five layers: bulk silicon, silicon dioxide ($d_{ox} = 20$–320 nm), extracellular cleft (d_{cleft}), cell membrane ($d_{mem} = 4$ nm), and cytoplasm. Silicon was described by a refractive index and an attenuation factor [14]. Silicon dioxide, extracellular medium, cell membrane, and cytoplasm were characterized by refractive indices $n_{ox} = 1.460$, $n_{cleft} = 1.333$, $n_{mem} = 1.450$, and $n_{cyt} = 1.333$ [9]. The dye molecule was placed near the outer surface of the membrane with the transition moments of excitation and emission parallel to the surface [15]. The aperture was 47.3° for excitation at 546 nm and 48.6° for emission at 580–640 nm (numerical apertures 0.985 and 1.0) [9]. The observed photons per unit time J_{fl} depend on the probabilities P_{ex} and P_{em} of excitation under stationary illumination and of emission into the detector according to Eq. (1) with a scaling factor a and a background b.

$$J_{fl}(d_{ox}) = aP_{ex}(d_{ox}, d_{cleft})P_{em}(d_{ox}, d_{cleft}) + b\,. \qquad (1)$$

The functions $P_{ex}(d_{ox}, d_{cleft})$ and $P_{em}(d_{ox}, d_{cleft})$ are given by a multireflection finite aperture theory for self-interference of exciting light and of emitted light [8,9]. All directions, polarizations, and wavelengths of illumination and detection are taken into account as well as the orientation and the absorption and fluorescence spectra of the dye. Changes of lifetime by radiative and nonradiative effects [4–6] can be neglected for large distances of dye and silicon (Lambacher & Fromherz, unpublished).

Distance.—We fitted the data of Figs. 3(e) and 3(f) according to Eq. (1) and obtained an average distance $\overline{d}_{cleft}$ and its statistical error. On laminin we measured 109 $\pm$ 1.4 nm for an astrocyte, 104 $\pm$ 1.1 nm for a neuronal soma, and 104 $\pm$ 2.0 nm for a growth cone. For an astrocyte on fibronectin we obtained 65 $\pm$ 2.1 nm. From the error bars of the data points and the slopes of the theoretical relation we estimated a local variation

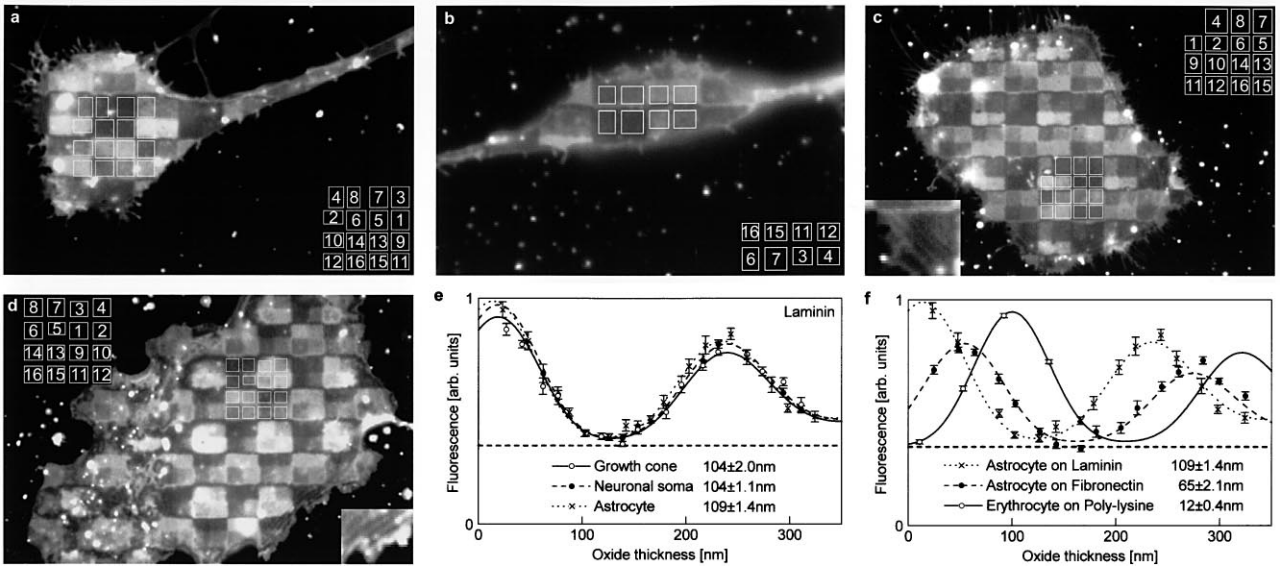

FIG. 3. Fluorescence micrographs of stained neuronal cells. Size of the squares 2.5 μm $\times$ 2.5 μm. (a) Neuronal growth cone on laminin. (b) Neuronal soma on laminin. (c) Astrocyte on laminin. The inset shows interference fringes near the periphery due to the upper membrane. (d) Astrocyte on fibronectin with similar inset. (e) Intensity (average $\pm$ sigma) on marked areas of growth cone, soma, and astrocyte on laminin versus thickness d_{ox} of oxide fitted with the width d_{cleft} of the extracellular cleft. (f) Intensity versus d_{ox} of astrocyte on fibronectin (fitted with $d_{\mathrm{cleft}} = 65$ nm) and on laminin. For comparison the data are shown for an erythrocyte ghost on polylysine.

$|\delta d_{\mathrm{cleft}}| < 5$ nm in all systems. The distances were reproducible for several cultures on various chips as shown in Fig. 4, with a population average of 105 nm on laminin and 60 nm on fibronectin.

Roughness.—We studied the adhesion of a neuron on polylysine in order to check the sensitivity of the method for structural roughness. Figure 5(a) shows that the checkerboard pattern of fluorescence was disturbed drastically. The intensities of small homologous areas are plotted in Fig. 5(b). From a fit we obtained a distance of 35 nm. With respect to that reference, the bright patches on oxide No. 1 and dark patches on oxide No. 3 indicate an upward bulging of the membrane. We reconstructed a complete profile of d_{cleft} from the pattern of fluorescence [area No. 3 in Fig. 5(a)] using Eq. (1) as shown in Fig. 5(c). For comparison a profile of a neuron on laminin [area No. 3 in Fig. 3(b)] is shown in Fig. 5(d). Here the adhesion is smooth within 4 nm. We never observed patches of close adhesion on laminin and fibronectin within the lateral resolution of 400 nm.

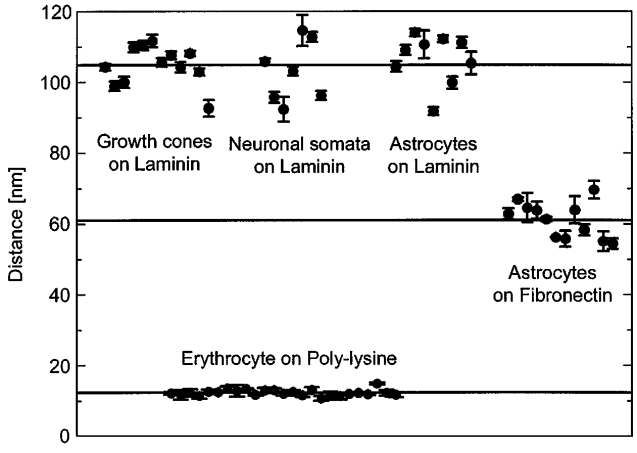

FIG. 4. Width d_{cleft} of the extracellular cleft (average and statistical errors) on laminin (growth cones, neuronal somata, and astrocytes) and on fibronectin (astrocytes). The average distance was 105 ± 6 nm on laminin and 62 ± 5 nm on fibronectin. The average distance of erythrocyte membranes on polylysine was 12 ± 1 nm.

Accuracy.—The careful analysis of FLIC microscopy shows that the maximal systematic error of d_{cleft} is 1 nm—independent of the value of d_{cleft} [9]. The main contribution originates in the aperture: A change of 0.5° affects d_{cleft} by 0.5 nm. Of course, a deviation of the refractive index in the cleft from $n_{\mathrm{cleft}} = 1.333$ would lead to a proportional change of d_{cleft}. The results are insensitive to the optical properties of the cell, because the interference is dominated by the interface of silicon and silicon dioxide. A change of the refractive index of the cytoplasm from 1.33 to 1.4 would affect d_{cleft} by 0.5 nm. Intracellular structures play no role, as the cell membrane is labeled specifically by the fluorescent probe. A 5% variation of membrane thickness or of dye orientation has no significant effect. The oxide spacers provide an internal calibration for each cell, such that no absolute measurement of intensity and no difficult adjustment is required.

Cell adhesion has been observed *in situ* by interference reflection microscopy [16,17] and by total internal reflection fluorescence microscopy [18]. There a measurement of d_{cleft} is disturbed by light reflection from intracellular surfaces [19,20] and by background

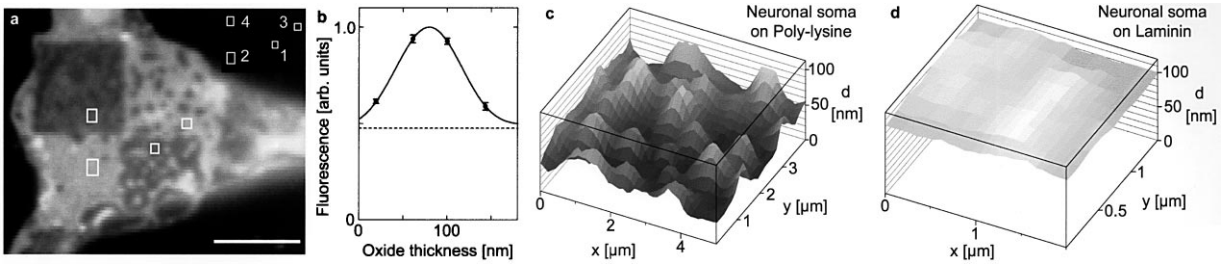

FIG. 5. Profile of adhesion. (a) Fluorescence micrograph of neuronal soma (stained with $S27/DiIC_{18}$) on polylysine. The size of a step was 5 μm $\times$ 5 μm, the thickness of four oxides was $d_{ox} = 20$, 60, 100, and 140 nm. Bar: 5 μm. (b) Intensity of fluorescence (average $\pm$ sigma) on the marked areas versus thickness d_{ox} of oxide fitted with a distance $d_{cleft} = 35 \pm 1$ nm. (c) Profile of the distance $d_{cleft}(x, y)$ on area No. 3. (d) Profile of the distance $d_{cleft}(x, y)$ for a neuronal soma on laminin [area No. 3 of Fig. 3(b)].

fluorescence, respectively [21,22]. Color and brightness in interference reflection microscopy indicated a large separation of neuronal growth cones from a substrate coated with laminin and a somewhat smaller separation for fibronectin [23,24]. Fluorescence interferometry provides a precise measurement of these separations. We did not find patches of close contact on laminin and fibronectin that correlate with point contacts as defined by local binding of antibodies to integrins [25]. It may be noted that the dark patches seen in interference reflection microscopy, which are interpreted usually as regions of close adhesion, may be related to reflection at the cytoplasmatic surface of the membrane which is thickened locally by protein complexes [16,17,19,20].

The length of laminin and fibronectin molecules is about 120 nm [26]. The membrane-bound integrin receptors point out from the membrane by about 15 nm [27,28]. Thus, the attraction of cell and substrate can be mediated over a large distance by laminin and fibronectin without deformation of the membrane. The question remains what repulsive force keeps the membrane at a large and well defined distance. We exclude major effects of a steric force [29,30] of neuronal membranes, because we never saw fluctuations of fluorescence as in the case of lipid bilayers (Zeck and Fromherz, unpublished). Is there a steric force of dangling laminin and fibronectin molecules which are not attached to the membrane in a binding equilibrium [31]?

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