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Adherent cells avoid polarization gradients on periodically poled $LiTaO_3$ ferroelectrics

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Abstract

The response of fibroblast cells to periodically poled $LiTaO_3$ ferroelectric crystals has been studied. While fibroblast cells do not show morphological differences on the two polarization directions, they show a tendency to avoid the field gradients that occur between polarization domains of the ferroelectric. The response to the field gradients is fully established after one hour, a time at which fibroblasts form their first focal contacts. If suspension cells, with a lower tendency to establish strong surface contacts are used, no influence of the field gradients is observed.

Keywords: Fibroblast; Cell adhesion; Ferroelectrics; Permanent dipole; Polarization

Background

When a particle, cell or microorganism approaches a surface, electrostatic interactions are among the first forces encountered [1,2]. Especially for motile microorganisms, surface charge affects adhesion and extended Derjaguin, Landau, Verwey, Overbeek (DLVO) theory, in conjunction with electrostatic interactions, are used to describe observed experimental trends [1,3–6]. A series of studies by the Whitesides group show that an overall electrically neutral surface seems to be a prerequisite for an inert surface [7,8]. Electrostatic interactions may still be involved even for formally neutral surfaces, such as self assembled monolayers (SAM) of ethylene glycol, as theory suggests that hydroxyl ions can accumulate on top of the SAM and thus form a negatively charged interface [9]. Studies of electrostatic interactions between microorganisms or cells with surfaces in many cases rely on the presence of charged groups and differently charged surfaces are a result of a changed chemistry and it is desirable to experimentally access the impact and relevance of electrostatic contributions on adhesion phenomena. To restrict interaction studies to electrostatic contributions requires surfaces with constant surface energy and identical surface chemistry. Electrets with embedded charges in polytetrafluoroethylene are one option and recently used to correlate the polarity of the electret with the favorability of this substrate to be colonized by spores of the green algae *Ulva linza* [5]. Another class of surfaces capable retaining a stable oxide surface but permit local alterations polarization are periodically poled inorganic ferroelectric materials. One of the characteristic features of the ferroelectrics is the presence of electrically reversible polarization. In a properly oriented ferroelectric sample, for example (001)-cut $LiNbO_3$ crystal or (001)-grown $BaTiO_3$ thin film, the polarization can be aligned perpendicular to the surface in the positive or negative direction. In this case, the abrupt change in the normal component of the spontaneous polarization on a ferroelectric surface results in the appearance of a bound polarization charge, which in ambient conditions is compensated by accumulation of ionic species or dipole molecules and through redistribution of mobile carriers in the bulk [10]. This screening significantly affects the surface charge distribution and the surface potential and results in band bending [11]. Similarly, charged surface states can pin the surface Fermi level resulting in a change in both the surface charge, the surface potential, and change the molecular band offsets [12]. It has been shown recently that these electrically switchable properties of the ferroelectrics can be used to tailor surface reactivity. Several examples illustrating the effect of polarization on the photo-reduction rate of Ag^+ ions, the sticking

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coefficients and interaction energies of alcohol and water molecules can be found in literature [13–19], including the selective deposition of virus particles [20].

Regarding the effects of polarization on mammalian cells, several studies have shown that electrically active ceramics improve biological responses to artificial grafts in bones [21,22]. It has been suggested that these effects are linked to the adaptation of bone in response to mechanical loading, since polarization effects have been observed in hydroxyapatite. However, the mechanism by which surface polarization affects cell response is yet to be elucidated and it still remains to be investigated if the preferential adsorption of proteins and ions plays a role. Another limitation is the use of different individual surfaces with a specific polarization state where subtle differences in adhesion behavior are difficult to assess. With the patterned material used in this study, a side-by-side comparison becomes possible and we can study if strong field gradients at boundaries between the polarized areas influence early cell adhesion - a key event regulating several biological responses. It is important to bear in mind that due to the high concentration of ions in cell culture medium, the Debye length is in the order of 1 nm. This means that only those parts of the cell that are very close to the surface will be in any way affected by the gradient fields. The unique approach of using periodically poled substrate domains allows the cells to select locations of different polarizations and field gradients without being significantly perturbed by chemical differences.

Methods

Preparation of single poled and periodically poled $LiTaO_3$

Periodically poled $LiTaO_3$ of congruent composition with a domain pattern width of 22 μm were fabricated by depositing a photoresist mask on the + *c* sample face and applying a voltage of 10 kV through a fixture with an electrolyte solution [23,24]. After the poling process the photoresist was removed by chemical/mechanical polishing. The ferroelectric properties of the domain patterns were characterized using piezoresponse force microscopy (PFM) [25,26]. In Figure 1, dark stripes in the PFM phase image indicate domains with upward polarization and bright - with downward. A commercial atomic force microscope (Asylum MFP-3D) was used in this study. Domain visualization has been performed by applying a high-frequency modulating voltage (400–800 kHz, 1.0-1.5 V), using Pt-Ti-coated silicon (Mikromasch, CA, USA). The spontaneous polarization was ≈ 60 μC/cm^2, the intrinsic coercive field 1.7 kV/mm. All values for dielectric and piezoelectric constants can be found in the Additional file 1.

Protein adsorption assay

Protein adsorption assays were carried out following established protocols [27]. Alexa fluor 488 labeled fibrinogen was diluted in phosphate buffered solution (PBS) (both Invitrogen, Karlsruhe, Germany). Samples were immersed in 10 mL of filtered PBS for 1 min. Subsequently, 10 ml of 1 mg/mL fibrinogen solution was added and kept at room temperature for 30 min. Afterwards, the solution was continuously diluted with 500 mL deionized water and 500 mL Milli-Q water. The samples were dried and the protein layer was imaged by fluorescence microscopy using a Nikon TE-2000. The layer thickness was determined with X-ray photoelectron spectroscopy (XPS). The XPS device (Leybold-Heraeus MAX 200), equipped with a $K_{\alpha}Al$ (1486.6 eV) anode measured the attenuation of Ta 4f signal from which the protein thickness was calculated.

Cell culture and adhesion

REF52YFP

Cell adhesion on polarized $LiTaO_3$ was evaluated *in vitro* using rat embryonic fibroblast cells expressing YFP-paxillin (REF52YFP, kindly provided by B. Geiger, Weizmann Institute, Israel). In previous studies, we showed that these cells form robust focal adhesions, which are paxillin-rich contacts mediated by integrins [28]. The fibroblasts were cultured in a humidified incubator with

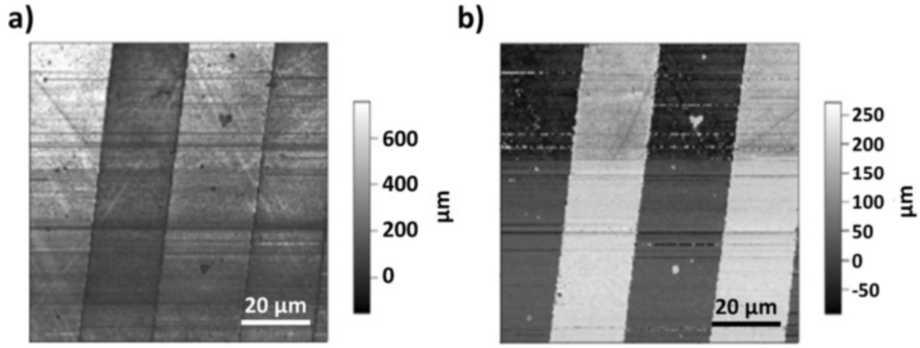

Figure 1 Piezoresponse force microscopy images of periodically poled $LiTaO_3$. (a) PFM amplitude image **(b)**: PFM phase image.

5% CO_2 at 37°C. The initial culture medium used was Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and 100 units/mL penicillin–streptomycin solution all purchased from Gibco (Invitrogen, Karlsruhe, Germany). The cells were harvested from tissue culture flasks by incubation with a 0.05% trypsin–EDTA solution for 5 min. Cells were then centrifuged and resuspended in complete media. Cells were seeded on the substrates at a density of 1×10^5 cells/mL.

KG-1a

Cell adhesion was also evaluated with the motile human leukemia cell line KG-1a. The KG-1a cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and 100 units/mL penicillin–streptomycin inside the same incubator. For adhesion experiments, KG-1a were directly taken from the culturing suspension and diluted with culture medium to 1×10^5 cells/mL.

Cell adhesion

Prior to adhesion experiments the substrates were cleaned by immersion in 3 mmol NaOH containing 10% p.a. ethanol. After a 30 min immersion in a sonication bath, samples were rinsed with ethanol and water. The substrates were affixed to the bottom of a custom-made petri dish where a hole of 5 mm in diameter was cut into the center of the dish. The adhesion and spreading of cells was followed by time-lapse video microscopy (Nikon TE-2000) inside a custom build incubation chamber under standard culture conditions (37° C, 5% CO_2). Cells were imaged directly after seeding, at a time interval of 1 minute for the first two hours and then every 10 minutes up to 18 h of total incubation time with an automated microscope Nikon TE-2000. Time-lapse microscopy was carried out with a 10x Ph1 objective and static fluorescence imaging was performed with a 20x Ph1 objective (both Nikon, Tokyo, Japan). In the case of the fibroblast adhesion studies, several positions were observed simultaneously which was not possible for the weakly adherent leukemia cells because movement of the automated microscopy stage caused vibrations which caused immediate cell detachment. The fluorescence imaging of cell focal contacts was performed with a 20x phase contrast objective and a GFP/YFP filter.

Cell position analysis

Digital image analysis methods were applied to determine the preferential site for cells to adhere. Due to the unequal sizes of the two cell types and thus their relative size to the polarized domains of the substrate, the center of the nucleus was used as a measure of cell position on the substrate. The domains are visible in phase contrast microscopy as their optical properties change with polarization direction of the domains. The positions of cell nuclei were analyzed at different time points and related to the periodic pattern sequence A/B. The cell number along the periodicity was calculated by counting the number of cells within rectangular areas parallel to the stripes with a horizontal width of approximately 3.15 μm (5 px) across the full image. As the stripes had different widths, approximately 3.15 μm (5 px) relate to 1/7 of the width of the bright stripes (~35 px, 22 μm) and 1/8 of the dark stripes (~41 px, 25.8 μm).

Results and discussion

Protein adsorption on periodically poled $LiTaO_3$

A protein adsorption assay was performed on the flat backside of the periodically polarized surface to reveal if the polarization changes protein adsorption. Therefore fluorescence microscopy was used to identify local adsorption of Alexafluor 488 labeled fibrinogen on the surface (Figure 2). As it becomes obvious from panel b (GFP fluorescence channel), no preferential adsorption to either positive or negative polarization was found. The protein thickness as determined by XPS was 53 ± 2 Å throughout the entire sample surface.

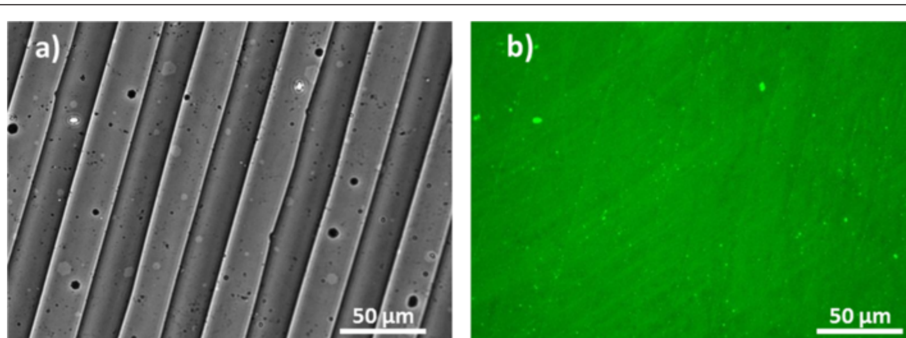

Figure 2 Light microscopy images of periodically polarized stripes after 30 min immersion into a 1 mg/mL solution of Alexafluor 488 labeled fibrinogen in PBS. (a) phase contrast micrographs **(b)** fluorescence micrographs using a GFP filter.

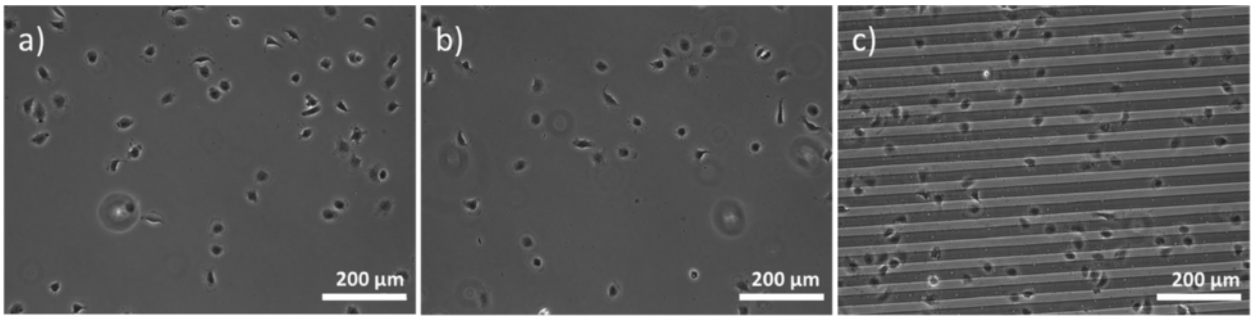

Figure 3 Optical micrographs of REF52 cells on $LiTaO_3$ surfaces with different polarization. Cells are equally well spread on **(a)** positive polarization, **(b)** negative polarization, and **(c)** periodically poled stripes (40 µm period).

Cell adhesion on periodically poled $LiTaO_3$

Fibroblasts were seeded on cleaned transparent $LiTaO_3$ substrates, pre-immersed for 30 min in supplemented culture medium, as noted, and the initial cell adhesion and cell spreading was characterized over 18 h. The adhesion experiments of REF52 fibroblasts were conducted on positive, negative and periodically poled $LiTaO_3$ surfaces at the same time under the same conditions. A correlation of the optical contrast with PFM measurements at the terminating lines allowed assignment of the bright areas (stripes) to 'down' domains and the dark background to 'up' domains. Images of fibroblast cells after 1 h of adhesion on positive, negative and periodically poled $LiTaO_3$ showed similar spreading on all substrates and polarization directions (Figure 3). Independent of their position on the substrate, cells started spreading after 10 min and were mostly spread after 1 h (Figure 4). This behavior is typical for moderately attractive artificial surfaces such as e.g. glass.

Cell orientation after 18 h is random and the stripes do not cause cell elongation, as it has been shown on microstructured patterns of PAA/PAH [16,29,30]. In order to determine if the organization of adhesive contacts at the cell periphery is altered by the polarized patterns, we imaged paxillin-rich contacts, known as focal contacts, in fibroblasts expressing YFP-labeled paxillin. The images in Figure 5 reveal a highly regular occurrence of focal contacts and surface polarization does not seem to influence the interaction of the cell with the surface.

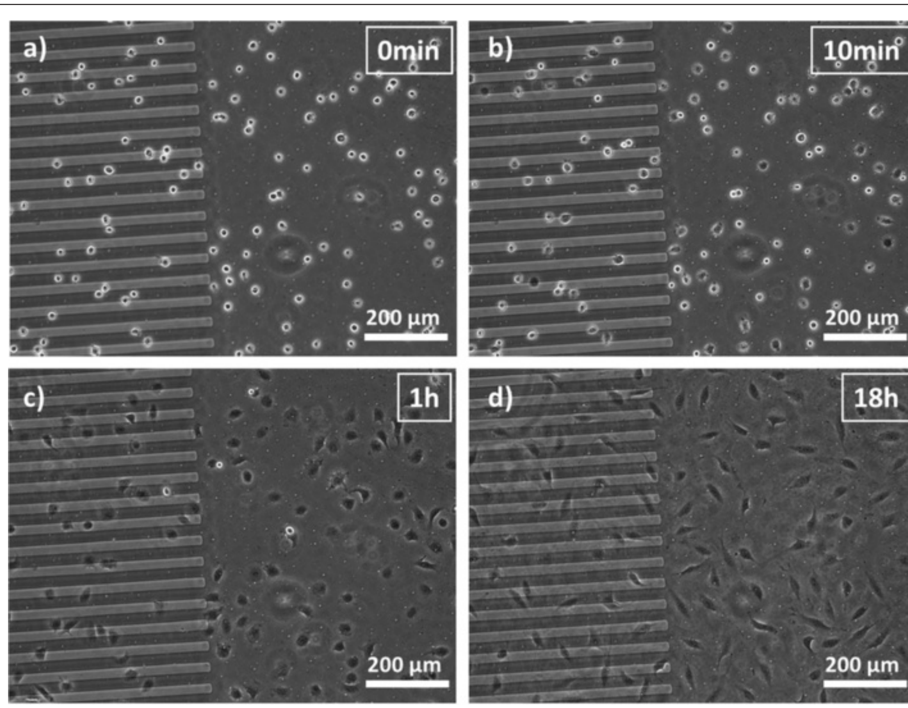

Figure 4 Light microscopy images of the adhesion process of REF52 cells on patterned ferroelectric $LiTaO_3$. (a) 0 min **(b)** 10 min **(c)** 1 h and **(d)** 18 h after seeding.

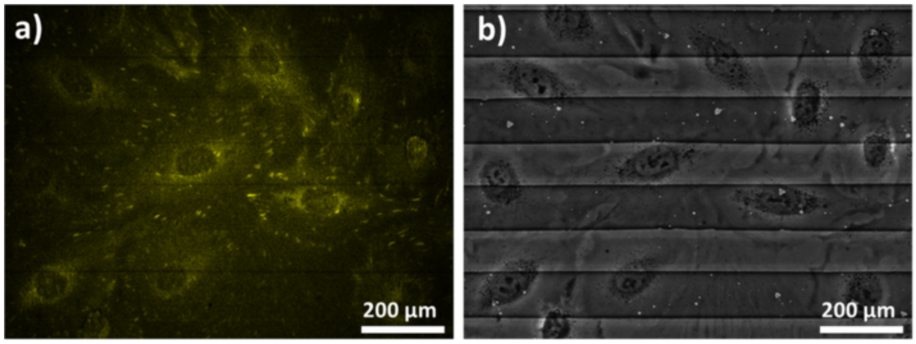

Figure 5 REF52YFP after 18 h of seeding on periodically poled $LiTaO_3$. Cell focal contacts imaged by fluorescence microscopy of cells YFP stained paxillin **(a)** and the phase contrast image **(b)**. The cells did not show either oriented anisotropy in shape or preferential positioning of focal contacts with respect to the surface polarization domains.

Cell response to polarization of periodically poled $LiTaO_3$

As no evidence was found for a polarization domain dependence of the cell spreading kinetics and focal adhesion, the settlement sites with respect to the position of the polarization domain boundaries were analyzed in greater detail . The positions of the center of the nucleus of ~ 550 cells were determined (see methods). The positions were related to the unit cell of the periodic pattern and then the total numbers of marked positions with respect to the position on the bright/dark areas were summed up. The result for different adhesion times is shown in Figure 6. During initial adhesion of the round fibroblasts the nucleus center showed a random distribution on the period of the polarized stripes. During cell spreading the probability to find a nucleus between the two stripes was diminished and more cells were found in the middle of the stripes. This observation indicated that spread fibroblast cells avoided positioning their nucleus on the border between the polarization domains or stripes, i.e. the ferroelectric domain boundaries were avoided. Analyzing all cells between 1 h and 11 h in Figure 7 shows that nearly twice as many nuclei centers were found in the middle of the polarization domains (~ 40) compared to the number of nuclei positions at the border of the poled domains (~ 20). The slight preference of the cells for the up domains (dark background) against the down domains (bright stripes) is too close to the error bars to draw meaningful conclusions at this stage.

In order to visualize the kinetics of cell reorientation with time, single time points, especially in the initial cell adhesion period were analyzed. To do so, we analyzed the number of nuclei per area at the border of the domain N_B (9.4 µm; 15 px width of the bins), in the middle of the downwards-poled ferroelectric domain stripes N_D (6.3 µm; 10 px width of the bins), and in the middle of the up domains (dark background) N_U (9.4 µm; 15 px width). As the width of the bright lines was smaller, the width of the area analyzed had to be slightly smaller than that on the dark lines, thus restricting analysis to regions of minimum effect coming from the border region. To account for the slightly different total areas analyzed, the number of cells per area was used to calculate the cell distribution ratio R of cells on the stripes and cells at the border between the domains as:

$$R = \frac{N_D + N_U}{2 \cdot N_B}$$

In Figure 8, the ratio obtained is plotted against the cell adhesion time. The figure shows that fibroblasts require ≈ 10 minutes to responded to the patterned polarized surface and establish the fully visible response within one hour after cell seeding. This is about the time fibroblasts needed to attach, spread and form first focal

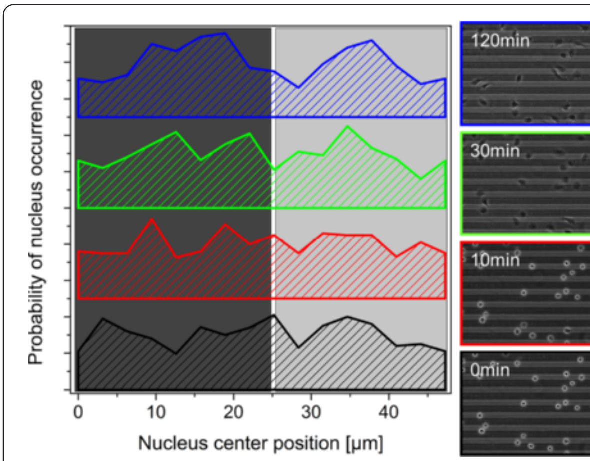

Figure 6 Analysis of the position of the cell nucleus on the polarized stripes at different times after seeding. During initial adhesion, the probability to find cell nuclei on a certain position is equal across the surface. With increasing spreading, the probability of the nucleus center to be found on the borders between the stripes decreased.

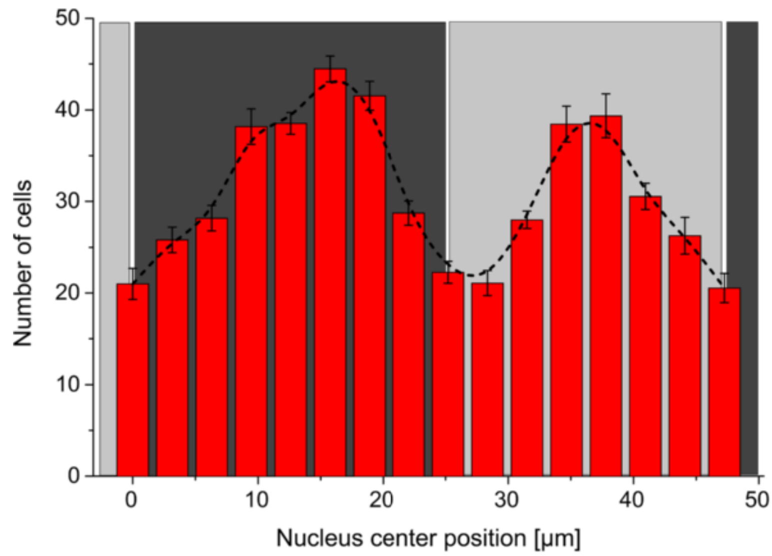

Figure 7 Summed time points of pixel analysis for spread fibroblasts between 1 h and 18 h of cell adhesion. The bars are the mean of four sample position. Error bars indicate the SD. The minima are clearly connected to the borders between two stripes with opposite polarization.

contacts [31]. Albeit being too close to the error bars to be of solid relevance, we note that between 1 h and 3 h, the ratio of cell localization between the center of a ferroelectric domain and the domain boundaries seems higher than several hours later.

From this finding, one might speculate that the formation of focal adhesions and the cell response to the strong field gradients of a domain boundary are related. It may well be that sufficient surface contact needs to be established to allow a cell to actively respond, and this is inhibited in the presence of a strong field gradient, but not by the surface polarization alone, but until the cell is

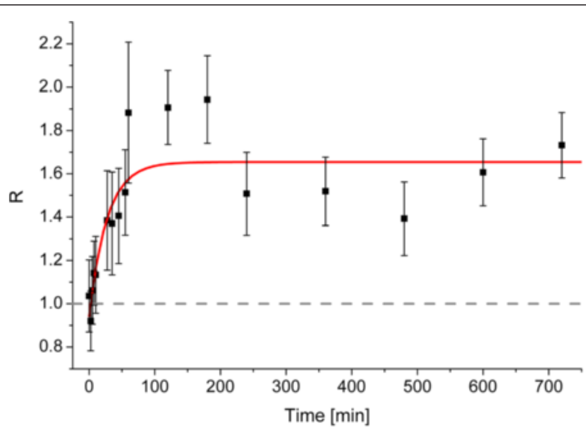

Figure 8 Kinetics of cell nucleus repositioning characterized by the cell distribution ration R. A ratio R of one (gray dotted line) indicates a random distribution of nuclei and no preference of a specific site. Values different from one indicate that the middle of the stripes is preferred and cells avoid the borders. An exponential fit curve depicts the cell response behavior and response seems fully established after one hour.

localized, a more random distribution of delocalized cells is a significant perturbation to the cell distribution. This observation could be explained by the Debye length of only ≈ 1 nm, which means that a cell approaching of loosely resting in the vicinity of the surface might not yet be able to sense differences in polarization. However, a thorough contact with the surface brings the cells much closer to the surface and thus the ferroelectric fields. Thus we complemented our investigation by using the highly motile, non-spreading leukemic cell line KG-1a and determined its response to periodically poled $LiTaO_3$ substrates. KG-1a cells were seeded onto the periodically poled $LiTaO_3$ substrate preimmersed in culture medium. After seeding, the cells were imaged by time-lapse microscopy for 2 h under physiological conditions inside the incubation microscope. During experiment, the presence of a slight convection within the petri dish caused the cells to move at ~ 2 µm/min. This flow phenomenon is quite common and can only be avoided in a controlled microfluidic environment similar to the one we described recently [32]. Unfortunately, due to their small size, the substrates could not be placed into the microfluidic shear force assay which was capable to keep the weakly adherent leukemia cells under controlled flow [33]. The analysis revealed a more uniform distribution of cells with respect to the lateral position of the polarized lines, indicating that KG-1a cells respond to a lesser extent, compared to spreading fibroblast cells (Figure 9). Probably the short Debye screening length in the cell culture media was again not sufficiently overcome by weakly adherent cells.

Summarizing, adhesion and spreading of cells were investigated on poled and structured $LiTaO_3$ surfaces and it was found that fibroblasts spread equally on homogeneously up

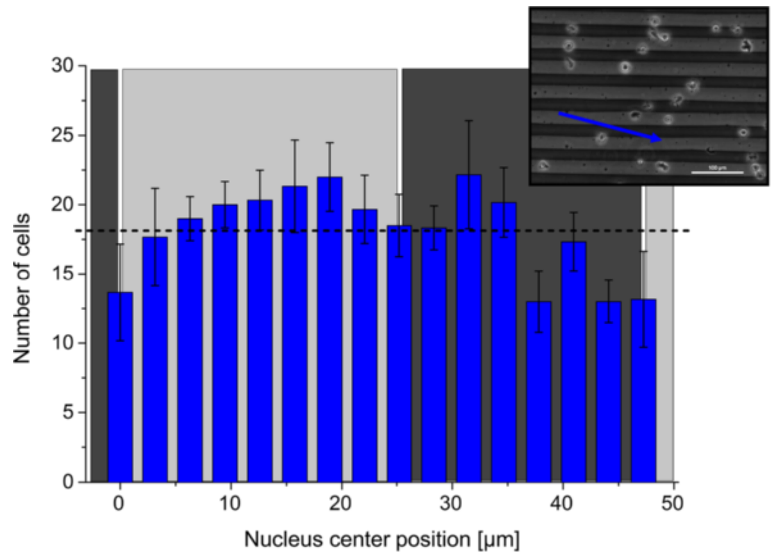

Figure 9 KG-1a leukemic cells on periodically poled $LiTaO_3$. Cell position pixel analysis reveals no preferential adhesion site of KG-1a with respect to polarization. Insert: Microscopy image of KG-1a during movement on $LiTaO_3$. Blue arrow indicates the liquid flow direction caused by convection.

(+) and down (–) poled substrates. Also, uniform spreading over up and down domain stripes was observed on periodically poled substrates. Random distribution of focal contacts over the domain stripes was confirmed by fluorescence microscopy of long term incubated YFP-paxillin REF52 fibroblasts. These results indicate that cell attachment and spreading were not affected by the polarization of the substrate. In contrast, analysis of the positions of the nuclei in the time lapse images revealed that during spreading fibroblasts preferably placed their nucleus in the middle of periodically poled domain stripes. This is the position on the surfaces where the lowest field gradients are present. In turn, the field gradient on the border of polarity domains is highest and these positions are less attractive. Kinetic analysis revealed that the positioning of the cell nucleus took place on the same timescale as the formation of cell focal contacts (30 min to 2 h). In the case of weakly adherent suspension cells KG-1a, the response was much weaker compared to fibroblasts. At present we can only speculate that the KG-1a response might be weaker due the larger distance of the nucleus to the surface for a suspension cell compared to an adherent fibroblast. Based on the short Debye length in the order of one nanometer, the weaker field strength could cause a weaker response. However, also the specific function of a particular cell type in the body might be connected with its ability to sense fields.

The fibrinogen adhesion data suggests that polarization-dependent surface chemistry can be excluded in the selective adhesion of these cells and the effects visible are due to the ferroelectric field present. Interestingly, isolated proteins [34], as some amino acids like cysteines [24], albeit in very low concentrations, have been seen to exhibit a preference for polar surfaces rather than domain walls. Adsorption from low concentration BSA solutions is higher on positively poled surfaces than on negatively poled ones [35]. In comparison, our experimental conditions contained very high concentration of proteins and the sticky fibrinogen leads to a rapid formation of a proteinaceous overlayer that was found to be independent of the polarization. While the adsorption of molecules on polarized surfaces seems to be quite complex, the general notion that molecular interaction with the surfaces might depend on the fields present is supported by the observation that in the region of the ferroelectric domain boundaries the photo-reduction rate of $AgNO_3$, $HAuCl_4$, Pt $(NO_3)_2$ and other compunds is increased [23,36–39]. In addition it was found that nanowires of pure Ag, Au and Pt were grown along the predefined 180^0 ferroelectric domain walls on c-cut congruent $LiNbO_3$ and lead zirconate titanate $[Pb(Zr_xTi_{1-x})O_3]$ single crystals. Thus, field gradients affect adsorption, chemical reactions, and crystal growth.

Less was so far known on interaction of cells with patterned, polarized substrates. Polarization-specific adhesion of cells does seem to occur under isothermal conditions and with minimal optical excitation, but cannot be attributed to the effect of pyroelectric or photoinduced charge [17]. Our fibroblast adhesion experiments show that both, up and down poled domains, are equally preferred and the ferroelectric domain boundaries at which the strongest field gradients are present are avoided. However, no obvious morphological differences were seen in the middle of stripes of different polarization. This observation is in agreement with recent studies of Baxter et al. who found no long-term effect of polarization direction of non-structured hydroxyapatite-barium titanate ceramics on the morphology of adherent Saos-2 cells [40]. In comparison to non-poled ones, Tarafder et al., however, observed fewer cell-cell attachments with the extension of filopodia on positively poled surfaces. These morphological differences went along with a delayed initial proliferation [41], irrespective of the sign of the surface charge. Research on osteoblast-like cells in some cases supports the preference of negatively charged surfaces [42], albeit in other cases contradicts this trend [43]. To the best of our knowledge our study is the first on cell adhesion on microstructured, polarized surfaces and shows that the differences between surfaces with different polarization direction seems to be dominated by the strong influence of the high field gradients present at the domain boundaries. Especially the shift of the position of the nucleus of the cells is a so far an unknown observation. At present we can only speculate that electric signals that are connected with ion transport might be involved.

Conclusion

Summarizing, a detailed investigation on the adhesion of cells on structured, polarized ferroelectric samples was presented and we could for the first time show that cells actively reorient the position of their nucleus and avoid positions of high field gradients.

Additional file

Additional file 1: Characterization of periodically poled $LiTaO_3$.

Competing interests

The first authors and all co-authors confirm that there are no potential competing interest to disclose.

Authors' contributions

KK, AG, PD prepared and characterized the samples. CC, ECA, MH, MG and AR designed, performed and analyzed the biological experiments. All authors contributed to writing the article. All authors read and approved the final manuscript.

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