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Quantitative analysis of cell proliferation and orientation on substrata with uniform parallel surface micro-grooves

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In order to quantify the effect of the substrata surface topography on cellular behaviour, planar and micro-textured silicon substrata were produced and made suitable for cell culture by radio frequency glow discharge treatment. These substrata possessed parallel surface grooves with a groove and ridge width of 2.0 (SiID02), 5.0 (SiID05) and 10 μm (SiID10). Groove depth was approximately 0.5 μm . Rat dermal fibroblasts (RDFs) were cultured on these substrata and a tissue culture polystyrene control surface for 1, 2, 3, 5 and 7 days. After incubation the cell proliferation was quantified with a Coulter Counter, and RDF size, shape and orientation with digital image analysis. Cell counts proved that neither the presence of the surface grooves nor the dimension of these grooves had an effect on the cell proliferation. However, RDFs on SiID02, and to a lesser extent on SiID05 substrata, were elongated and aligned parallel to the surface grooves. Orientation of the RDFs on SiID10 substrata proved to be almost comparable to the SiID00 substrata. Finally, it was observed that the cells on the micro-textured substrata were capable of spanning the surface grooves. © 1996 Elsevier Science Limited

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During the first part of this century, several investigators discovered that cellular behaviour is affected by the topographical morphology of the underlying surface. In 1912, Harrison¹ reported that substrata with a specific linear arrangement, as in the spider web, influence the direction of the movement as well as the form and arrangement of the cells. This was later confirmed by the studies of Loeb and Fleisher². They introduced the term stereotropism, which was described as the direction in which cells move, mainly governed by the contact with solids or very viscid bodies like fibres or fibrin. In 1945, Weiss³ called this cellular response to the topography of a substratum surface 'contact guidance', a term still in use today. Surprisingly, no further attention was paid to this guidance phenomenon until the early 1970s. It was Rovensky *et al.*^{4,5} and Maroudas^{6,7} who rediscovered that cells are able to react on the topography of a substratum surface. From this moment on, research on this subject has expanded, resulting in many publications, which were thoroughly reviewed recently by Singhvi *et al.*⁸.

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Most of the studies are focussed on the role of contact guidance in fundamental phenomena like embryogenesis and organogenesis. The possible effect of surface topography on the tissue response to implanted biomaterials has only been recognized for the last few years. Brunette *et al.*^{9,10}, for example, suggested the application of micro-grooved implant surfaces to prevent epithelial downgrowth around skin-penetrating devices. Campbell and von Recum¹¹ described the use of surface micro-patterns as a tool to reduce the inflammatory response at the implant-tissue interface. Although these studies have provided important information, the fundamental mechanism of, and optimal parameters for, cell control by guidance are still unknown. In addition, the reported results are often based on subjective, qualitative observations. To surpass this lack of knowledge it is evident that a systematic study of the influence of surface topography on the cellular behaviour is required. Therefore, the objective of our studies is to approach this guidance principle in a more orderly way.

In our first study¹² we reported on the effect of surface treatment on the wettability of surfaces and on

the growth behaviour of cells cultured on various surfaces *in vitro*. These experiments revealed that fibroblast proliferation on UV-treated surfaces was lower than on substrata treated with radio frequency glow discharge (RFGD) alone, or in combination with a UV treatment. The substrata that were used during these experiments also possessed parallel surface grooves. Scanning electron microscopic (SEM) examination of rat dermal fibroblasts (RDFs) on these microtextured surfaces suggested that parallel surface grooves of 2.0 and 5.0 μm were able to induce stronger cell orientation and alignment than grooves of 10.0 μm . Cellular orientation proved not to be affected by the various surface treatments. However, due to the number of substrata, only a qualitative conclusion regarding the cellular orientation could be formulated.

Based on the results of this first study¹², the purpose of this study was to test the hypothesis whether micro-geometrical surface patterns influence cellular behaviour only in terms of cell shape and orientation, or also alter the proliferation rate of the RDFs on these surfaces. In order to be able to quantify and test this hypothesis statistically, the experimental design of this study concerned a larger number of substrata than the first study to ensure good statistical power.

MATERIALS AND METHODS

The substrata

The experimental substrata were produced as described earlier by Schmidt and von Recum^{13,14}. Briefly, photolithography was used to manufacture smooth and textured silicon wafers. These textured wafers possessed parallel surface grooves with a groove width of 2.0 (SilD02), 5.0 (SilD05) or 10.0 μm (SilD10). All the grooves had a depth of 0.5 μm and were separated by a ridge, which had the same width as the groove. In order to obtain the final experimental substrata, these wafers or moulds were covered with polymethylsiloxane (silicon elastomer MDX 4-4210, Dow Corning). After polymerization the silicon rubber castings were removed from the wafers and cut into round discs with a diameter of 15 mm. These discs were then washed in a 10% Liquinox solution (Alconox Inc.), rinsed, ultrasonically cleaned for 30 min in a 1% Liquinox solution and given two 15 min ultrasonic rinses in distilled, deionized water. Subsequently, they were given a Soxhlet rinse for 12 h in distilled, deionized water. Finally, the substrata were air-dried and prepared for cell culture purposes by RFGD treatment (PDC-3XG, Harrick; Argon, 0.15 torr, 5 min). After RFGD treatment, the surface geometrical properties of the micro-textured substrata were characterized by SEM (SEM500, Philips) and atomic force microscopy (AFM; SP300, Polaron).

Cell proliferation assay and digital image analysis

RDFs were isolated from ventral skin grafts, taken from male Wistar rats, 40–43 days of age (100–120 g). After dissociation, these cells were incubated (37°C, 5%

CO₂–95% air) in α -MEM (minimal essential medium) with Earl's Salts and with L-glutamine (Gibco), supplemented with 15% (v/v) heat-treated fetal calf serum (Gibco), 2.5 $\mu\text{g ml}^{-1}$ amphotericin B (Gibco) and 50 $\mu\text{g ml}^{-1}$ gentamicin (Gibco). After approximately 3 days of culturing, the RDFs were rinsed with phosphate-buffered saline without magnesium and calcium (PBS Dulbecco; pH 7.2), supplemented with 5 $\mu\text{g ml}^{-1}$ amphotericin B and 100 $\mu\text{g ml}^{-1}$ gentamicin to remove non-attached cells. Subsequently, new culture medium was added and replaced every 2 days. Upon confluence, the RDFs were detached by trypsinization [0.25% (w/v) crude trypsin and 1 mM EDTA (ethylenediaminetetra-acetate) (pH 7.2)] and resuspended at a lower cell concentration in new culture flasks (Nunc) in fresh growth medium. After identifying the cells as fibroblasts by phase contrast morphology analysis as described by Freshney¹⁵, the fifth generation of these cells was used for all experiments.

Substrata with a smooth or micro-textured surface were placed in culture wells of 24-well plates (tissue culture polystyrene, Greiner). Subsequently, approximately 1.0×10^4 viable RDFs per ml, suspended in sterile growth medium, were added to each substratum. In addition, cell suspension was also added to wells without substrata to serve as a control (CTRL). The cultures were incubated for 1, 2, 3, 5 and 7 days (37°C, 5% CO₂–95% air) under static conditions. The growth medium was changed every 2 days. At the end of the various incubation periods, the cultures were rinsed with PBS Dulbecco to remove non-attached cells. The remaining RDFs on the substrata were detached by trypsinization and counted in triplicate with a Coulter Counter. After trypsinization the substrata were observed routinely with a phase contrast microscope (Leitz DMIL) to check whether all cells were removed. This experiment was performed on 10 occasions.

The effect of the surface microgeometry on the cellular morphology was quantified by digital image analysis (DIA). For DIA, the RDFs at six random evaluation areas (584.4 $\mu\text{m} \times 412.5 \mu\text{m}$) were photographed by phase contrast microscopy during the cell proliferation assay on days 1, 2, 3, 5 and 7. The evaluation areas were selected by dividing the substratum surface in 740 possible fields of observation of 584.4 $\mu\text{m} \times 412.5 \mu\text{m}$. Each field was given a number, which was entered in a randomization programme. Thus, five randomly selected evaluation areas and the field at the centre of each substratum were photographed. Registration of the coordinates of these areas assured that the same areas were observed and photographed during the entire period of incubation.

After completion of the cell culture experiments, these photographs were scanned digitally (400 dpi \times 400 dpi) and analysed with an Acorn R260 computer (RISC processor), the ArcImage 5 for the HAWK V12 software package (Foster Findlay Associates, UK) and additional self-programmed software. In short, the in-house written routines were used to trace all RDFs (approx. 50) in each digital phase contrast image and to prepare the resulting

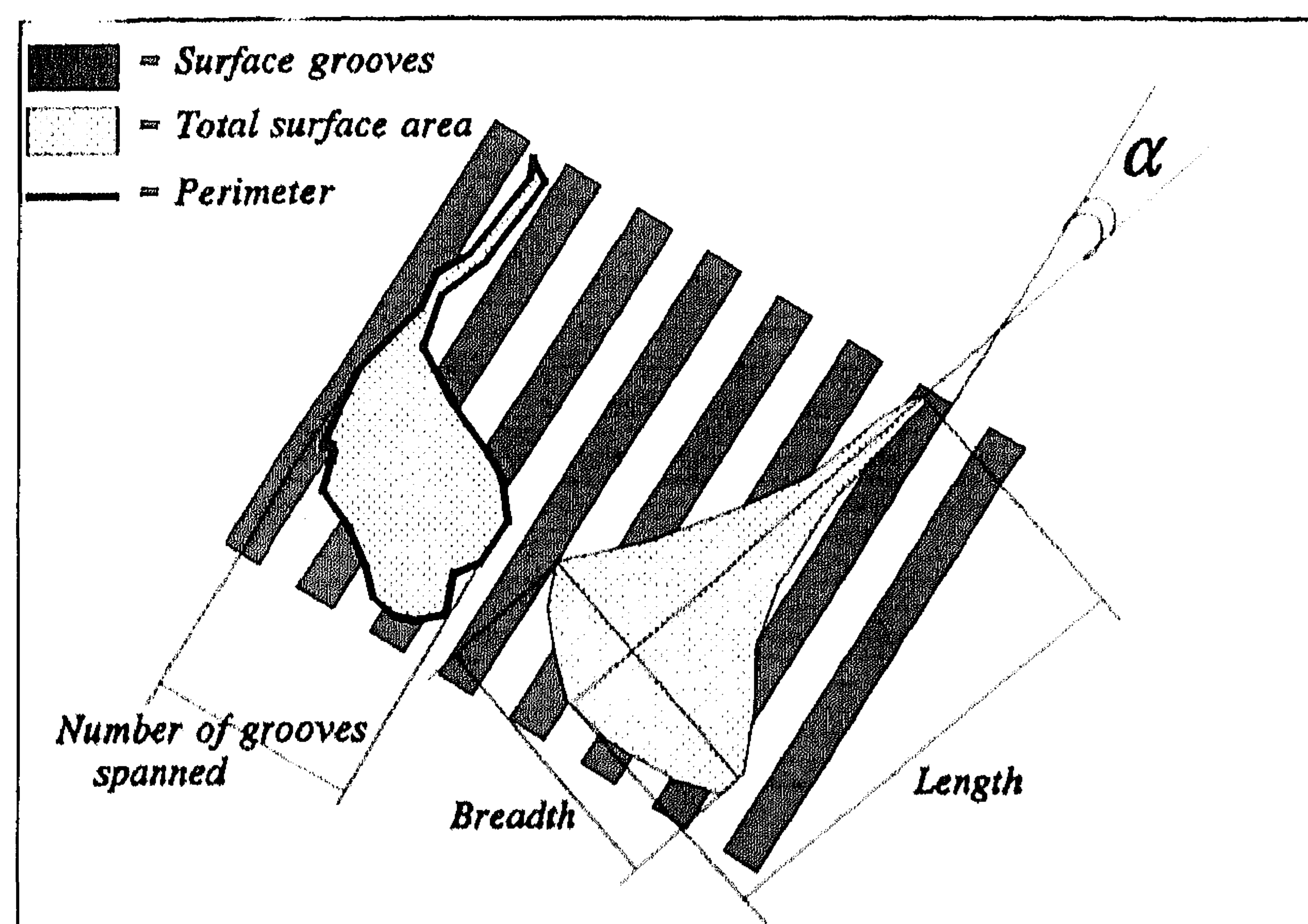


Figure 1 Digital image analysis parameters; α represents the angle of cellular orientation relative to the surface grooves.

image for image analysis with the ArcImage programme package. The ArcImage programme measured several cell parameters, i.e. the cellular surface area, cellular perimeter, cellular circularity, maximum cell length, cell breadth perpendicular to the maximum length and number of grooves spanned by a single cell. Furthermore, the angle of cellular orientation relative to the surface micro grooves was calculated. A diagram with the evaluated parameters, except circularity, is shown in *Figure 1*.

Circularity is defined as

$$\text{Circularity} = \frac{4\pi(\text{Area})}{(\text{Perimeter})^2}$$

and ranges between 0 and 1. In the theoretical situation that circularity equals 0, the cell has a perfect linear shape. However, if circularity is 1, the cell is shaped as a perfect circle.

After gathering the numerical DIA data, these parameters were analysed using univariate and general linear model procedures, including Scheffe's multi-comparison test.

RESULTS

Surface characterization

Surface inspection by SEM and AFM showed that the pattern of the parallel micro-grooves on the substratum surfaces had no defects or irregularities (*Figure 2*). However, AFM measurements did show a slight deviation between the dimensions of the micro-events on the silicon cast substrata and the designer values of the silicon moulds. These values can be found in *Table 1*.

Cell proliferation assay

Figure 3 shows the proliferation curves of the RDFs cultured on surfaces with several parallel surface groove configurations. Statistical evaluation of the proliferation data produced no evidence for a constant significant influence of the surface topography on the RDF proliferation rate. For example, on day 2, cell

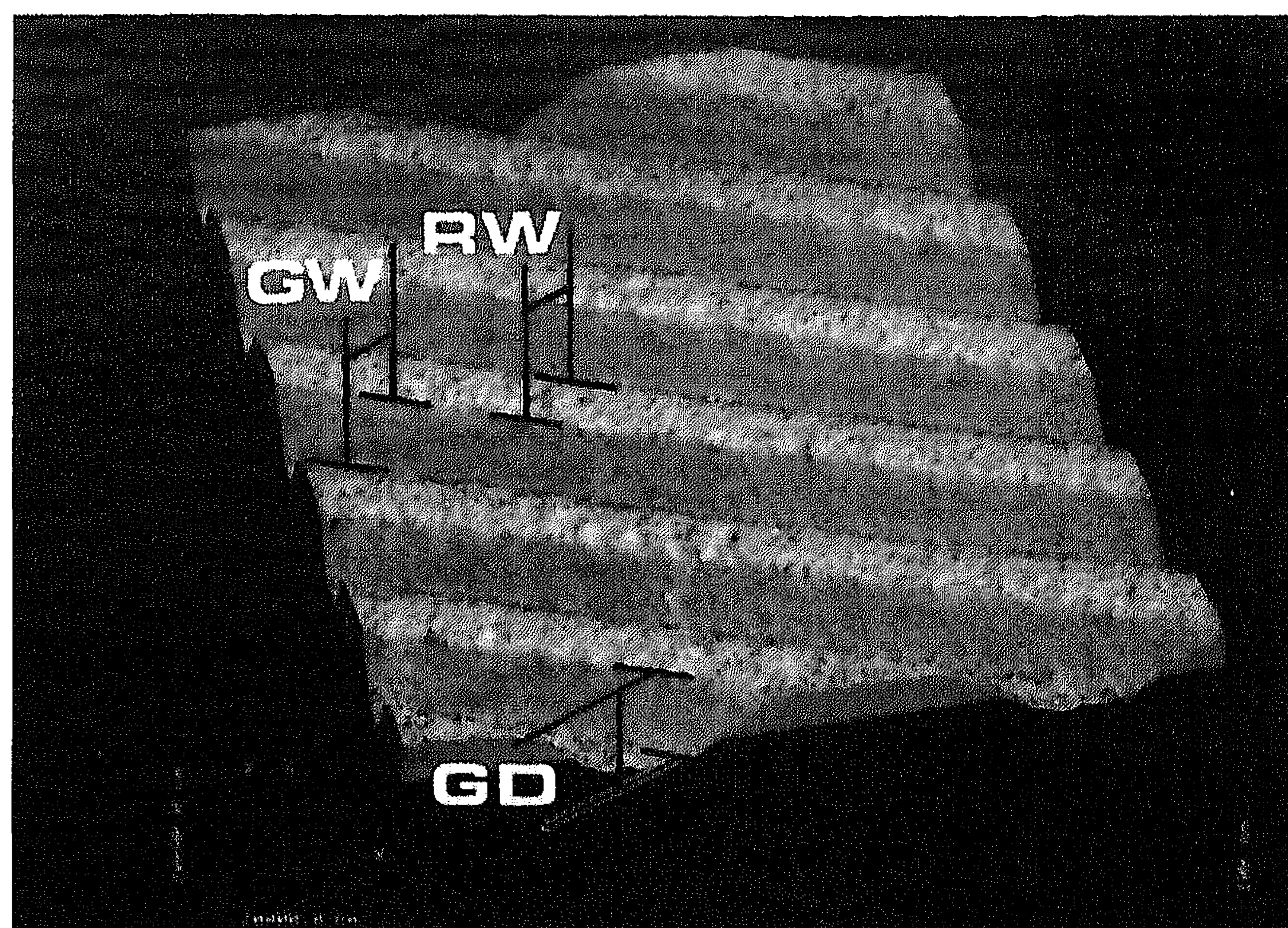


Figure 2 Three-dimensional representation of the results of the atomic force microscopy measurements on a SiID02 substratum. Different X- and Y-axis magnifications were used in this plot to clarify the conformation of the substratum surface. The codes in this image represent the ridge width (Rw), the groove width (Gw) and the groove depth (Gd).

Table 1 Dimensions of the micro-events on the silicone rubber substratum surface*

Surface	Actual values			
	G_d (μm)	G_w (μm)	R_w (μm)	P (μm)
SiID00	± 0.02	—	—	—
SiID02	0.45	1.71	1.68	3.87
SiID05	0.45	4.65	4.98	9.49
SiID10	0.46	9.58	9.77	18.98

* G_d , groove depth; G_w , groove width; R_w , ridge width; P , pitch.

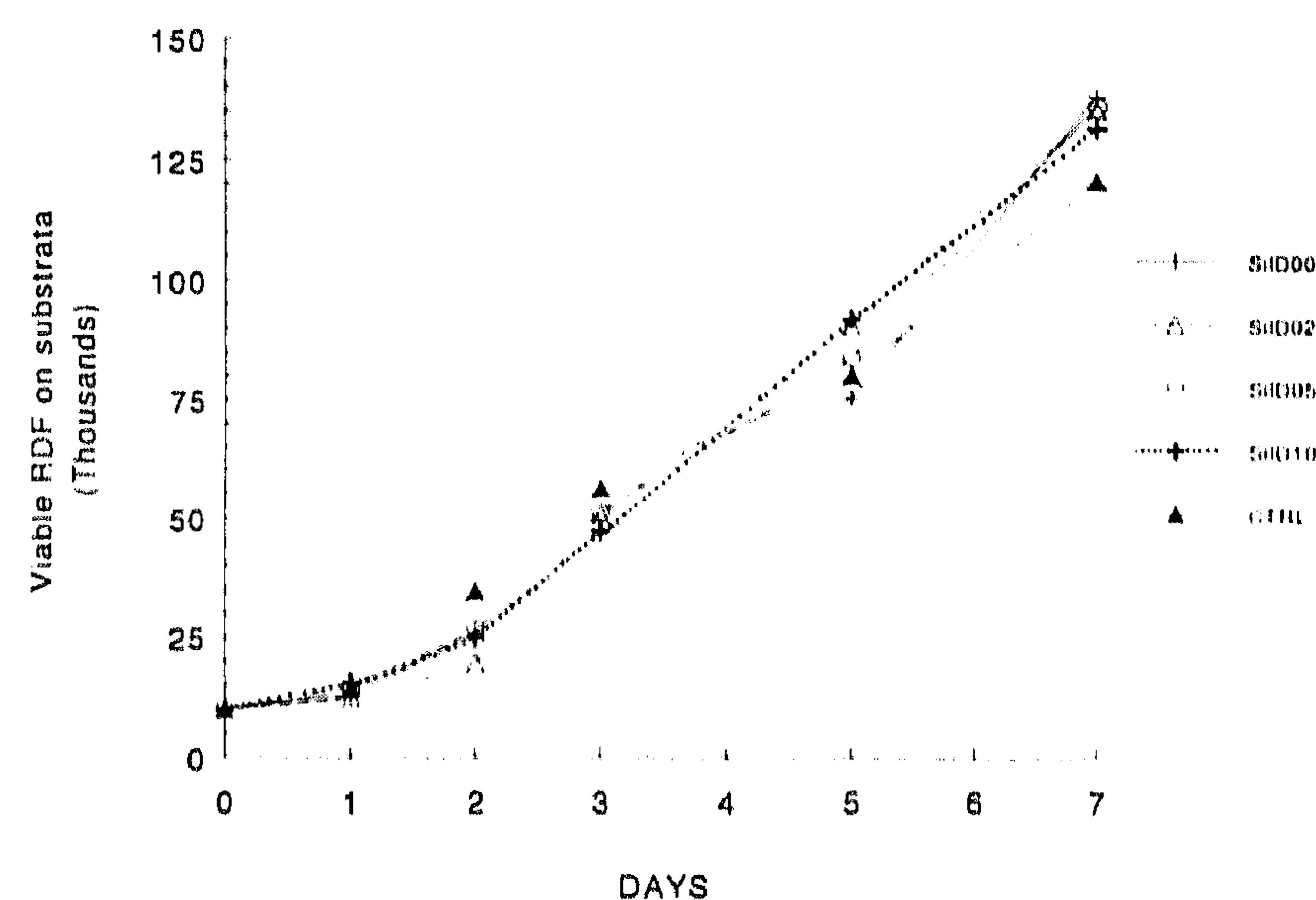


Figure 3 Proliferation of rat dermal fibroblasts on substrata with different groove configurations (average coefficient of variance = 14.76%), i.e. smooth silicon surfaces (SiID00), surfaces with 2 μm (SiID02), 5 μm (SiID05) and 10 μm grooves (SiID10). The proliferation data of the control group (CTRL) are also plotted (average coefficient of variance = 7.04%). Statistically significant differences between control and the experimental silicon substrata were only found on day 7 ($0.0001 \leq P \leq 0.0103$).

proliferation on SiID00 substrata was significantly higher than on SiID02 substrata ($P = 0.0001$), while on day 5 more RDFs were found on the SiID02 substrata than on the SiID00 surfaces ($P = 0.0020$).

Digital image analysis

Figures 4–7 show representative phase contrast images of the RDFs on the various surfaces after 3 days of incubation. On the smooth substrata, the RDFs are well spread and randomly orientated (Figure 4). In contrast, the cells on the 2- μm grooved substrata appear to align to the direction of the grooves (Figure 5). Most of these RDFs have a highly elongated spindle shape. RDFs on the SiLD05 and SiLD10 substrata show a more complicated picture (Figures 6 and 7). On both substrata, spindle shaped and flat, well spread RDFs can be seen. The cells on these surfaces are not aligned as strongly as the RDFs on the SiLD02 substrata.

DIA data confirmed this observed influence of the surface topography on the size, shape and orientation of the RDFs after the quantitative analysis of the measured cell parameters (Figure 1). RDFs were significantly smaller ($0.0002 \leq P \leq 0.0472$) on the SiLD02 than on the other surfaces up to day 5 (Figure 8). Evaluation of the RDF perimeter showed that the size of the cell perimeter was not significantly affected by the surface topography. However, RDFs were significantly more circular on the smooth

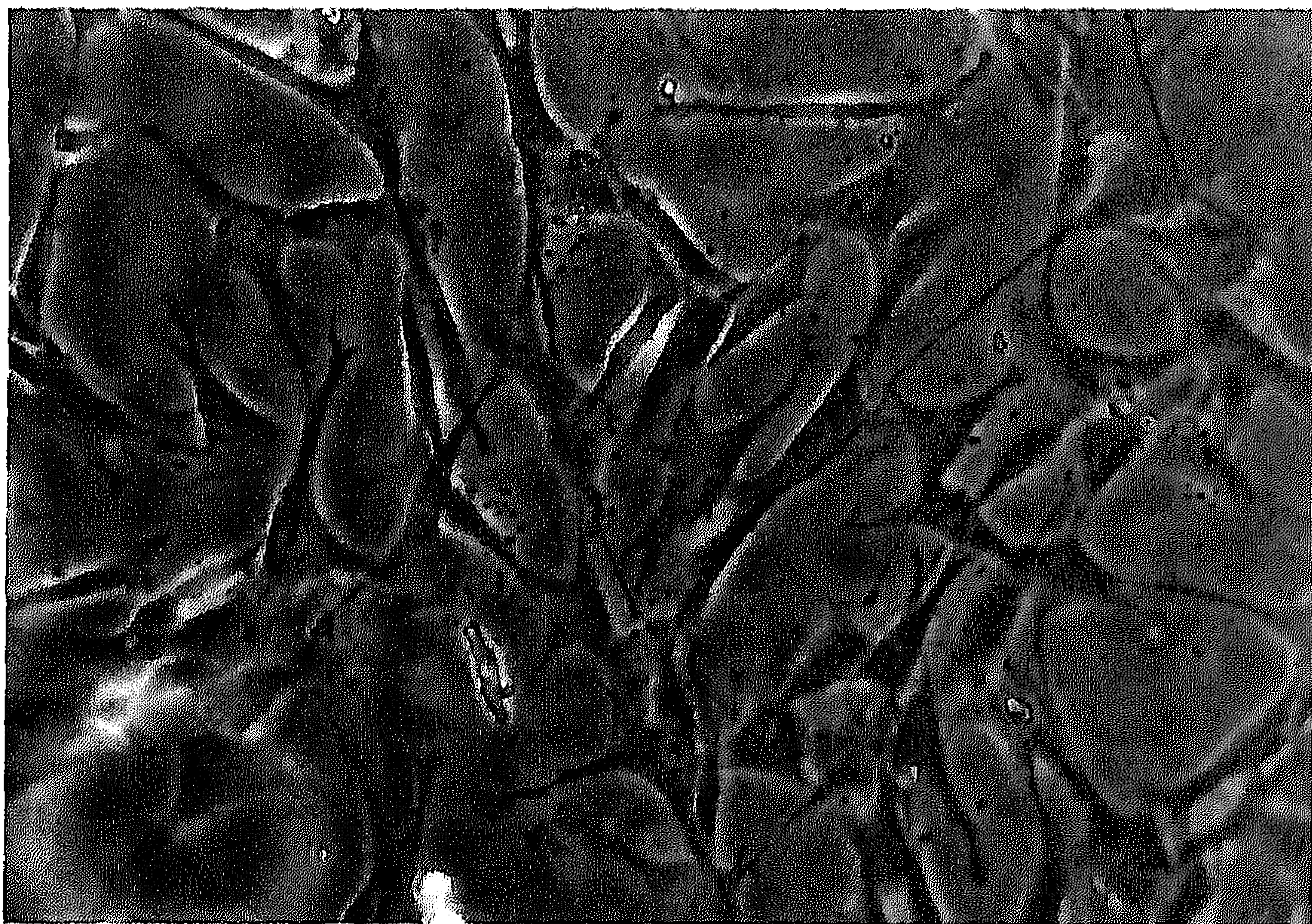


Figure 4 Phase contrast image of rat dermal fibroblasts on an SiLD00 substratum. The random cellular orientation is evident.

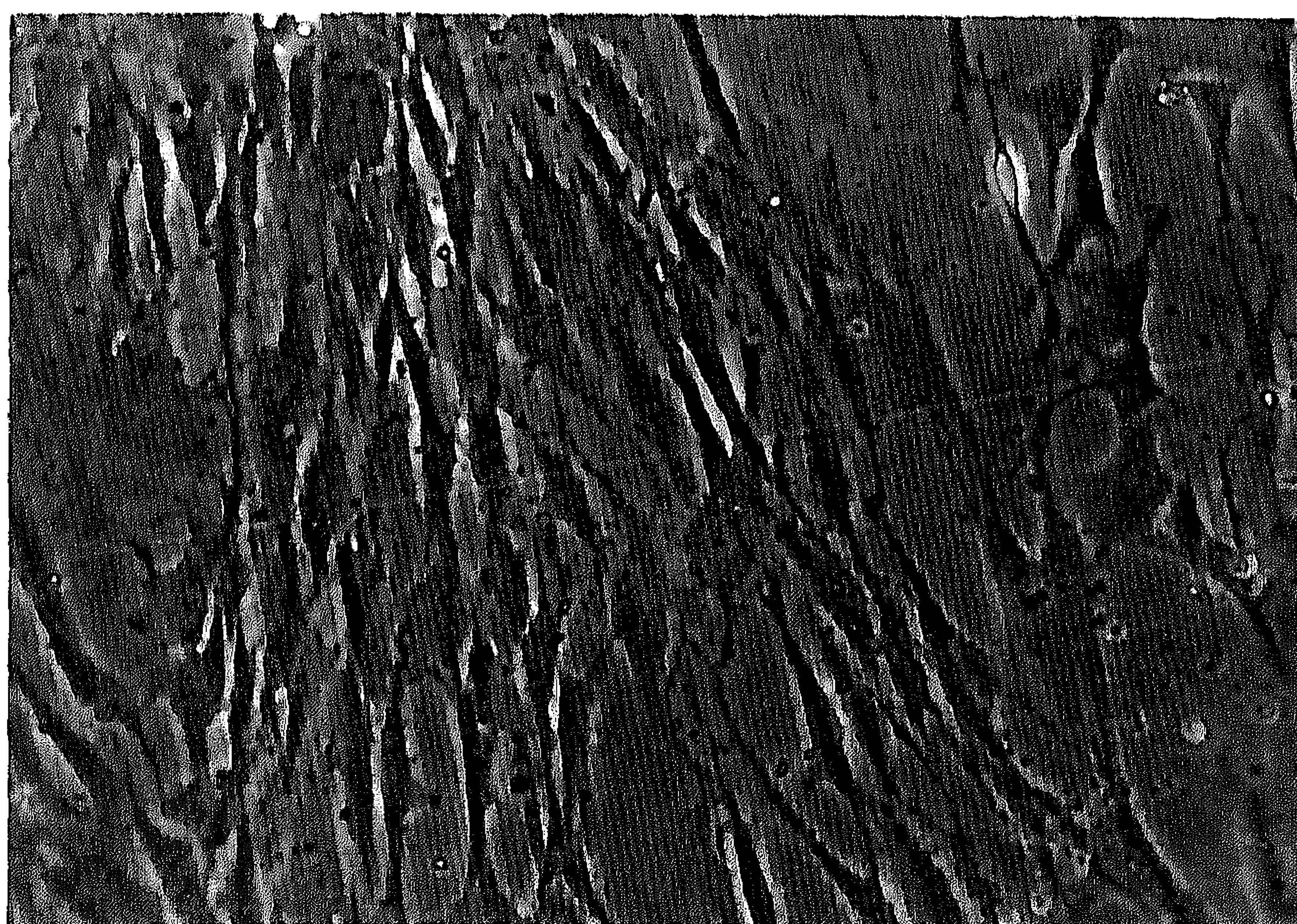


Figure 5 Phase contrast image of rat dermal fibroblasts on an SiLD02 surface. The cells are strongly aligned and elongated along the grooves.

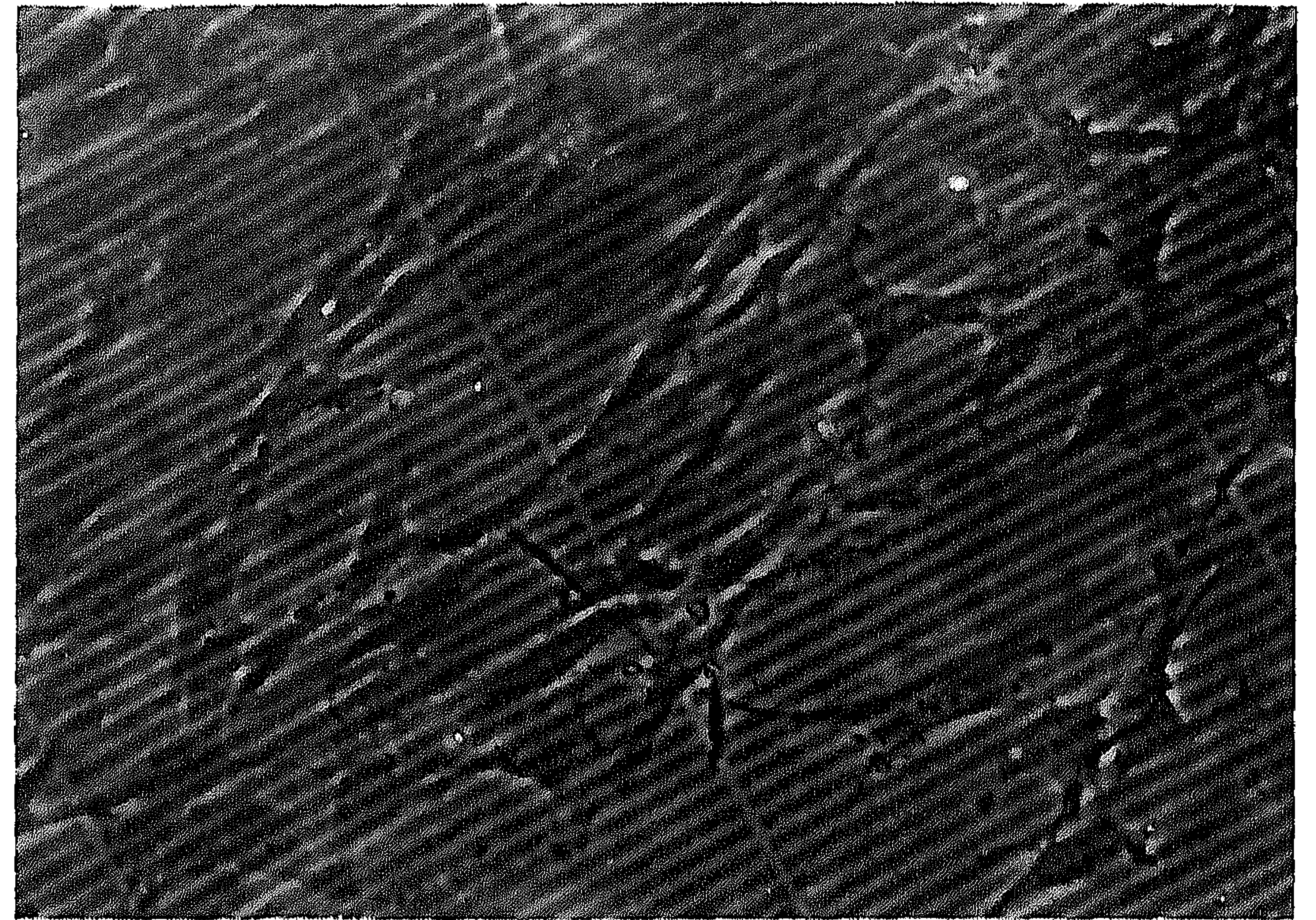


Figure 6 Phase contrast image of rat dermal fibroblasts on an SiLD05 substratum. The fibroblast orientation is not as clear as on the SiLD02 surfaces.

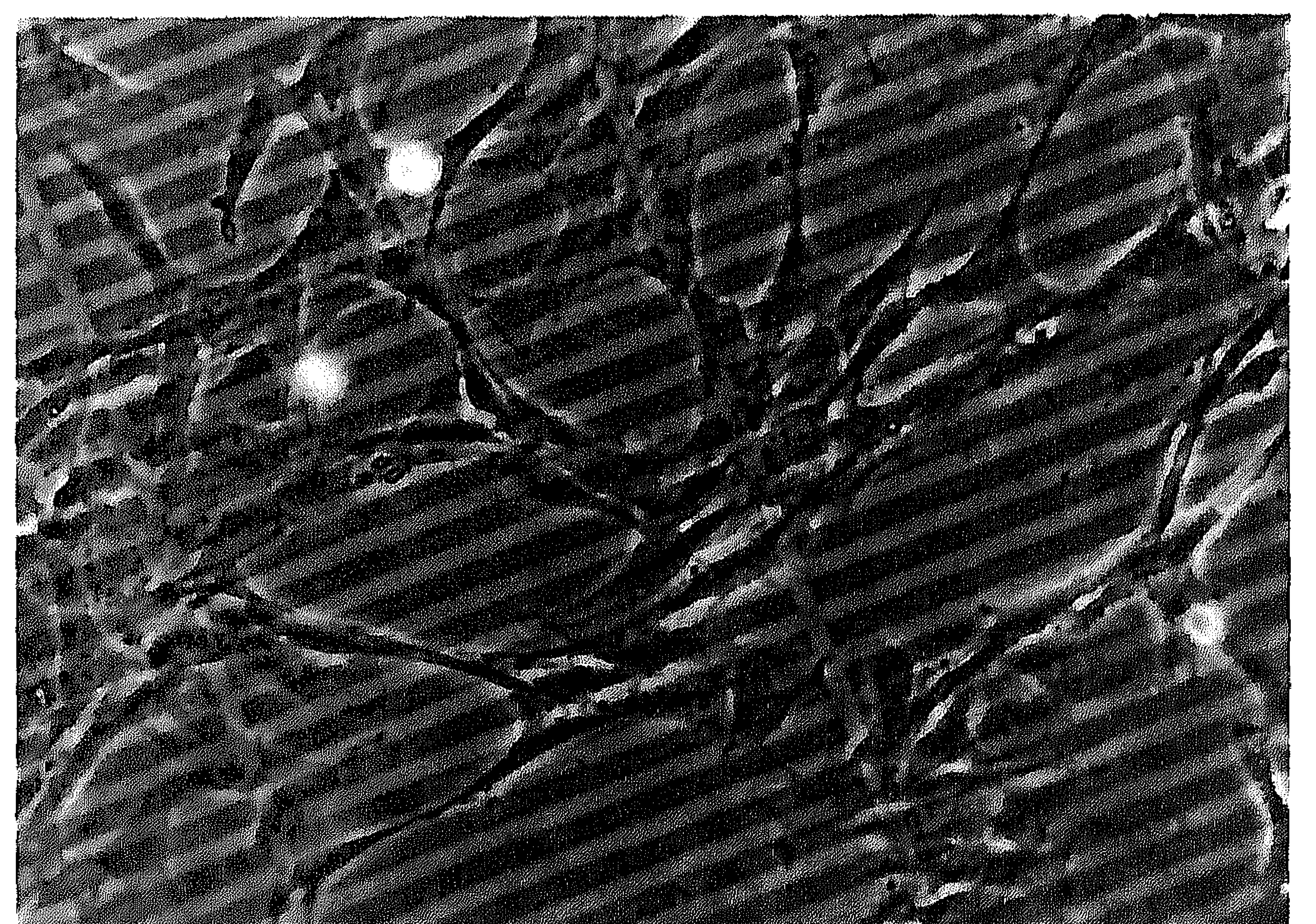


Figure 7 Phase contrast image of rat dermal fibroblasts on an SiLD10 surface. The orientation of the fibroblasts resembles that of the cells on the SiLD00 substrata.

substrata ($P_{\text{days } 3-7} = 0.001$) and CTRL surfaces ($P_{\text{days } 1-7} = 0.001$) than the cells on the textured substrata (Figure 9). In addition, RDFs on the grooved surfaces were significantly longer than the cells on the planar silicon substrata ($0.0001 \leq P_{\text{days } 2-7} \leq 0.0066$) and the CTRL surfaces ($0.0001 \leq P_{\text{days } 2-7} \leq 0.0349$). The breadth of the RDFs on the SiLD02 and SiLD05 substrata was significantly smaller ($0.0001 \leq P_{\text{days } 1-7} \leq 0.0184$) than the breadth of these cells on the SiLD00 and CTRL surfaces. The breadth of the RDFs on the SiLD02 surfaces proved to be the smallest, while no difference in breadth was observed between the RDFs on the SiLD10 substrata and the cells on the SiLD00 and CTRL surfaces.

Quantitative DIA also demonstrated that the angle of cellular orientation (α) relative to the surface grooves (Figure 1) was the smallest on the SiLD02 surfaces during the first 5 days of incubation (Figure 10). This angle proved to be larger with the cells cultured on the SiLD05 substrata, while the largest angle of orientation was found on the SiLD10 surfaces. After 7 days of incubation the angles were comparable for the RDFs on the SiLD02, SiLD05 and SiLD10 substrata. In Figure 11, the range of the measured angle of cellular

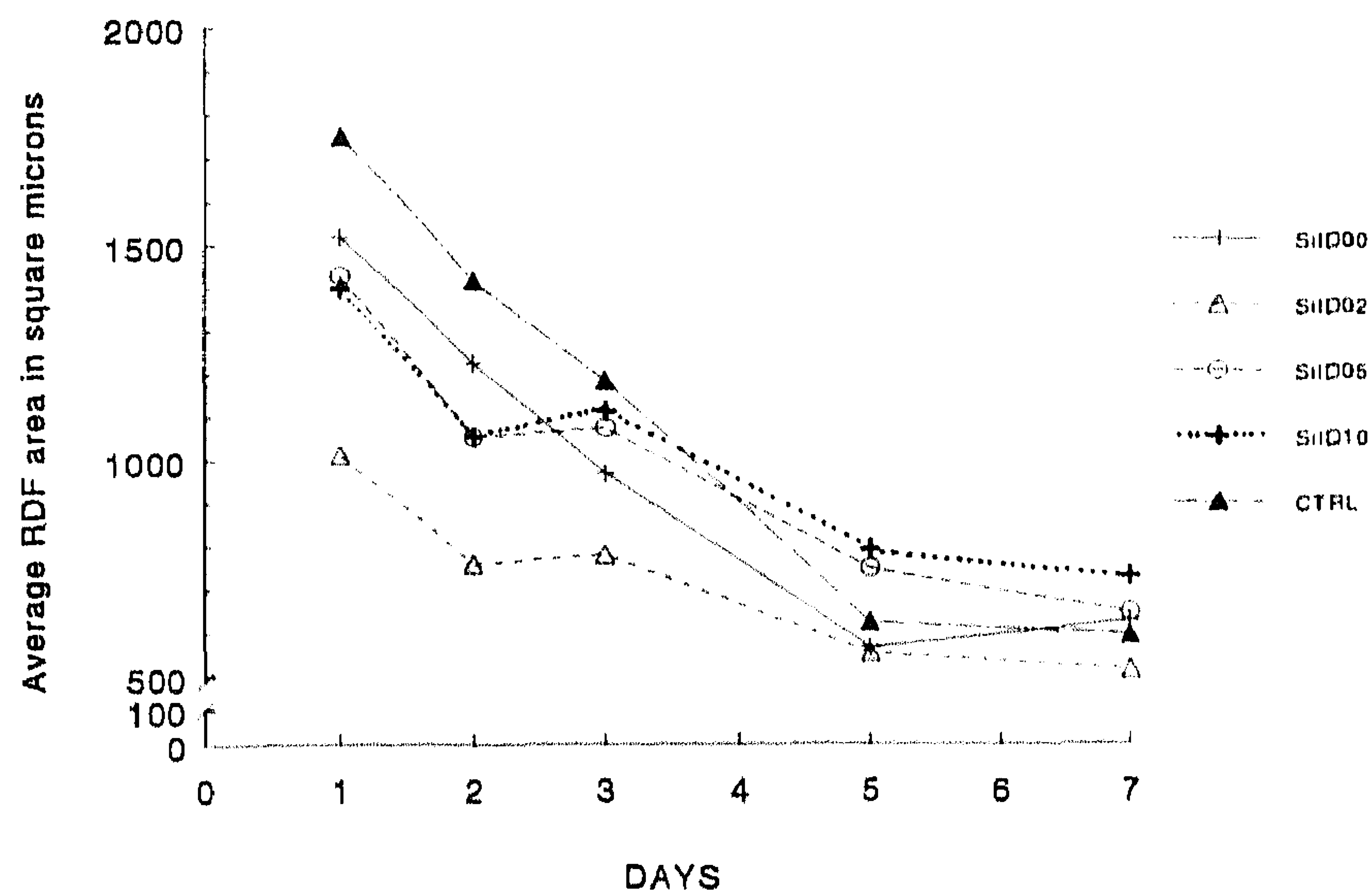


Figure 8 Average rat dermal fibroblast surface area on the various surfaces in μm^2 (average coefficient of variance = 17.85%). The surface area of the cells on the SiD02 substrata is the smallest.

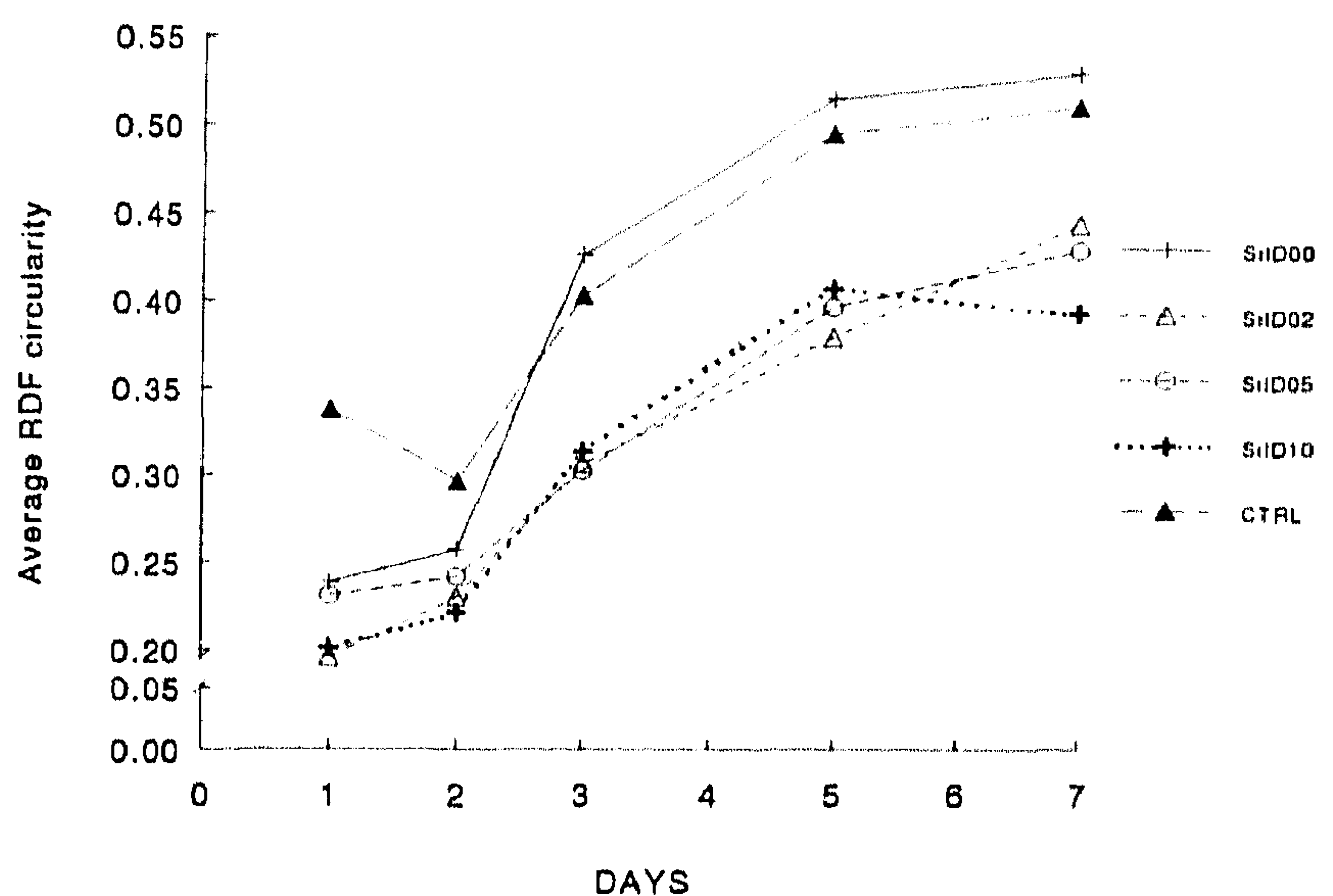


Figure 9 Average circularity of the rat dermal fibroblasts on the various surfaces (average coefficient of variance = 4.60%). The cells on the SiD00 and control surfaces are rounder than the fibroblasts on the textured substrata.

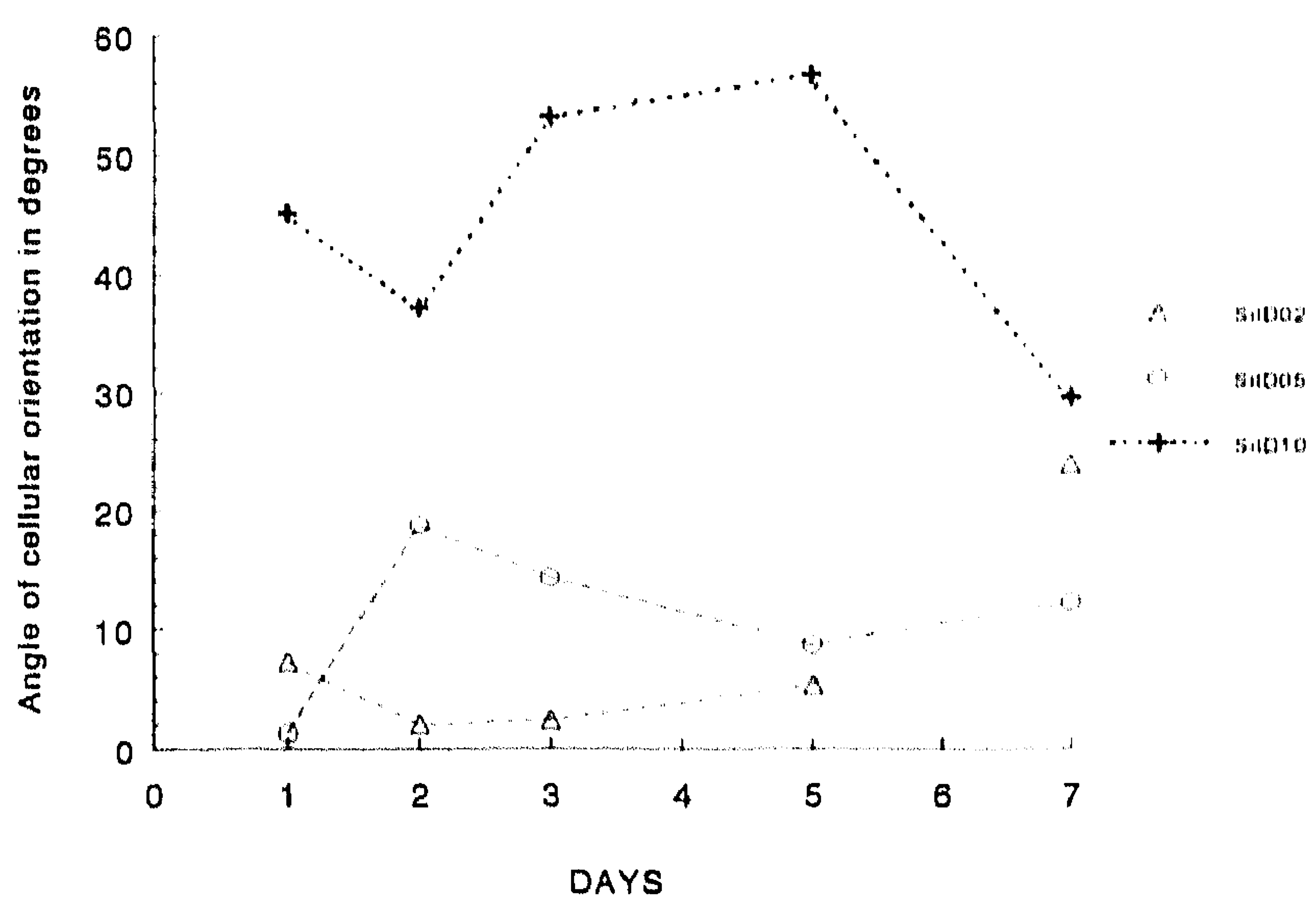


Figure 10 Average angle of rat dermal fibroblast orientation relative to the surface grooves. Especially the fibroblasts on the SiD02 substrata ($1.67 \leq \text{s.d.} \leq 4.237$), but also the cells on the SiD05 surfaces ($3.02 \leq \text{s.d.} \leq 6.28$) are orientated along the surface grooves. The cells on the SiD10 substrata ($3.76 \leq \text{s.d.} \leq 13.74$) are randomly aligned considering the average angle of orientation of 45° .

orientation relative to a virtual X-axis is plotted. This graph shows that the range of this angle is the smallest with the RDFs on the SiD02 substrata, while it is the largest with the cells cultured on the SiD10 surfaces.

Finally, the phase contrast images (Figures 4–7) also show that the RDFs were able to span the grooves on the textured surfaces. Apparently, the dimension of the grooves did not influence the ability of the RDFs to span these micro-events. For example, on day 1 the RDFs on the SiD02, SiD05 and SiD10 substrata spanned an average of 7.10, 3.85 and 2.1 grooves, respectively. Further evaluation of these data showed that the number of grooves that were spanned decreased after more days of incubation. In order to compare this decrease of the cells on the various textured surfaces, the grooves spanned on day 1 were defined as 100%. The number of grooves spanned on the following days were calculated as a fraction of this percentage (Figure 12). Although the dimension of the grooves on the various surfaces was different, the decrease of the number of spanned grooves did not differ significantly.

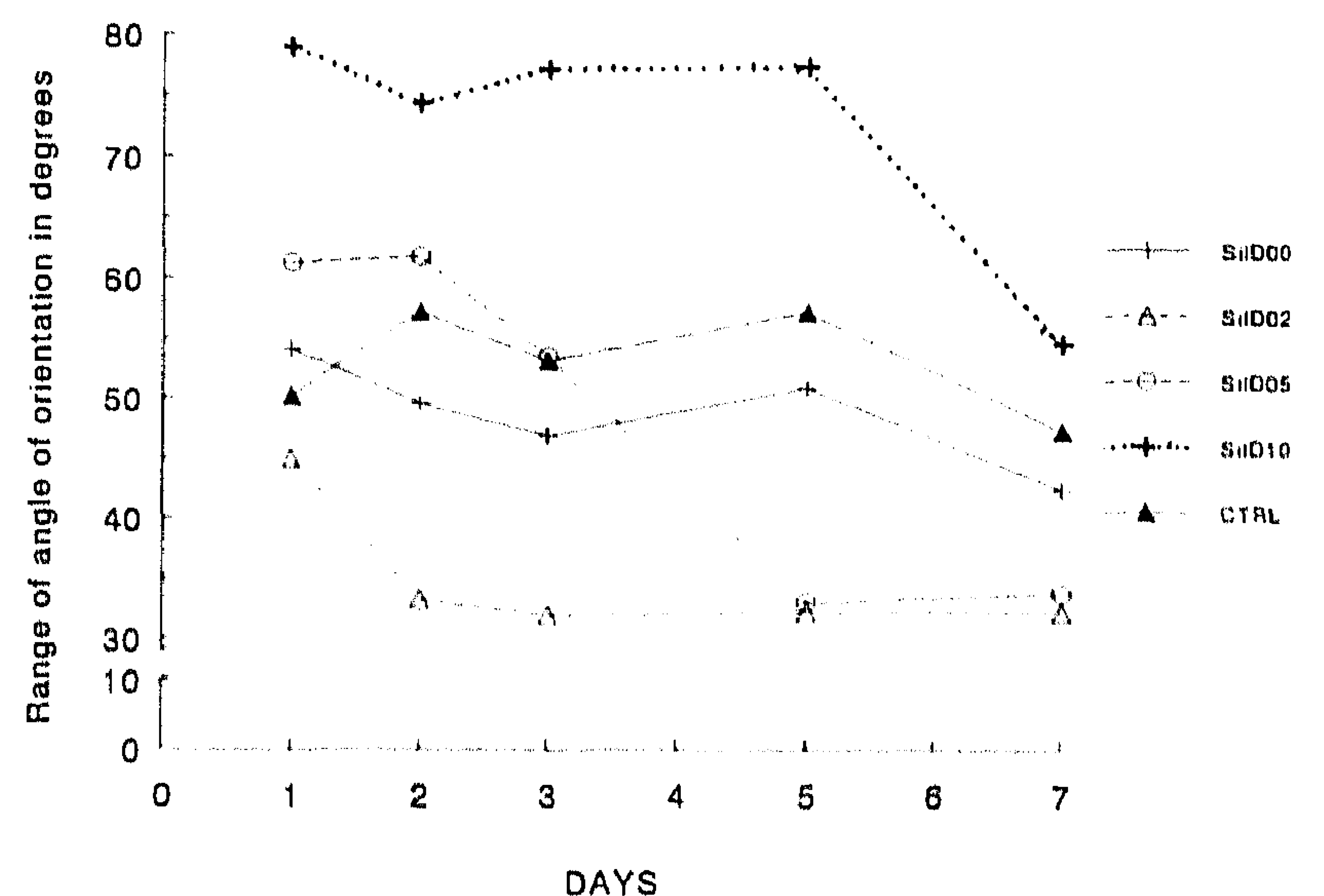


Figure 11 The range of the angle of rat dermal fibroblast orientation. As a result of contact guidance, the range of cellular orientation is much smaller among cells on the SiD02 substrata than on the SiD10 surfaces.

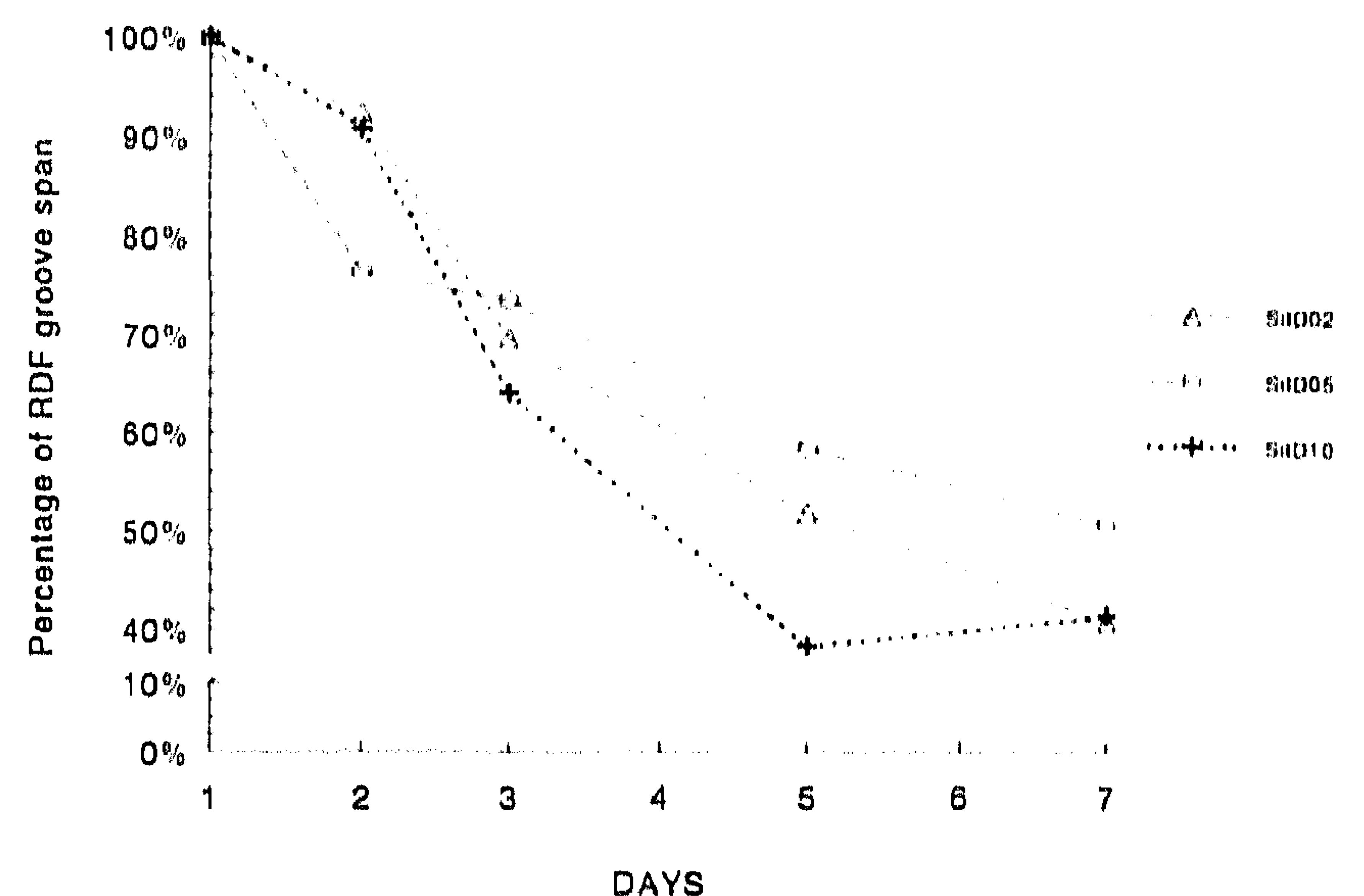


Figure 12 Percentage of rat dermal fibroblast groove span. The total number of grooves spanned on day 1 is defined as 100%. The decrease of the number of grooves spanned by the cells is not significantly influenced by the dimension of the grooves.

DISCUSSION AND CONCLUSIONS

On the basis of these results it can be concluded that the dimensions of the parallel surface grooves, as used in our experiments, did not result in a higher RDF proliferation rate. This observation is in contrast with the findings of Green *et al.*¹⁶ and Ricci *et al.*¹⁷. For example, Green *et al.*¹⁶ reported that especially abdomen fibroblasts (CCD-969sk) cultured on surfaces with 2.0 and 5.0 μm square pillars showed increased proliferation rates. Ricci *et al.*¹⁷ evaluated the *in vitro* growth of rat tendon fibroblasts and rat bone marrow colonies on unidirectional (grooved) surface micro-geometries. They found that the overall colony growth rate was changed and concluded that surface micro-geometry could be used to control the growth rate at implant surfaces. However, this study by Ricci *et al.* also showed that the response to surface topography is dependent on cell type, which could take account of the results found in our present and other studies^{12, 18–20}, which find no correlation between micro-textured surfaces and cell proliferation.

With respect to our proliferation results, it has to be mentioned that RFGD resulted in an optimal cell culture surface, since results proved to be comparable with tissue culture polystyrene. Nevertheless, it is still possible that, for instance, the amount and composition of secreted proteins is different between cells cultured on smooth and textured surfaces, especially since DIA data showed a marked influence of the surface grooves on the shape, size and orientation of the RDFs. In addition, stronger contact guidance was observed on the SilD02 and SilD05 substrata than on the SilD10 surfaces. This becomes even more evident when the alignment criteria that Clark *et al.*²¹ suggested are applied on the data plotted in Figure 10. These investigators defined a population of cells as highly aligned when the long axis of these cells makes an angle of $< 10^\circ$ with the direction of the grooves. Review of the data in Figure 10 shows that the cells on the SilD02 substrata, and occasionally on the SilD05 surfaces, have an orientation which lies between 0° and 10° . Therefore, these cells have to be considered as highly aligned. These observations support the findings of other studies^{12, 16, 18, 19, 22}, which conclude that surface features in the range of 1.0–5.0 μm have a high capacity of inducing cell guidance. Furthermore, it has to be noted that these findings were based on the result of a semi-automatic analysis procedure, which eliminates possible bias that could be present with an optical method as used by Clark *et al.*²¹. At this point it is also appropriate to mention that the incubation period in our study ranged from 1 to 7 days, which is longer than in other studies. The significance of this prolonged incubation has been proven by the fact that all the data show that, after 7 days, the influence of the micro-textured surfaces on cellular behaviour decreases. This reduction might be caused by the formation of cell-cell contacts²³. Consequently, it can be supposed that the observed guidance phenomenon is an initial response of cells to certain micro-textured surfaces, which is gradually lost after prolonged incubation. Still, it should also be noted that the process of wound

healing is a multi-factorial process in which many cell types and activation mechanisms play a role. This makes it difficult to apply the results of *in vitro* studies to *in vivo* studies. Therefore, the possible consequence of our finding for the final clinical use of surface micro-geometry in the design of implants can only be questioned and has to be investigated in *in vivo* studies.

Comparison of our results with other studies^{4, 7, 8, 17, 21, 22, 24–26} demonstrates that in most studies substrata were used with grooves of at least 1.0 μm deep and not 0.45 μm . Despite this difference, a similar influence on cellular alignment was found. This proves that the behaviour of RDFs can already be influenced by very shallow grooves. Unfortunately, no comparable numerical data are available from the other studies. This makes it impossible to investigate the existence of quantitative differences of the effect of the groove depth on the cellular interactions.

Our study confirmed the influence of surface micro-geometry on fibroblast behaviour. The mechanisms of this phenomenon, however, still remain unknown. As hypothesized by Meyle *et al.*²⁵, it is possible that the strong induction of contact guidance by 2.0, and to a lesser extent by 5.0 μm grooves, indicates the need of cells for mechanical stabilization against interfacial motion and improved initial cell adhesion purely by mechanical interlocking. Another explanation could be that the orientation and alignment of cells on micro-textured surfaces are a part of the cellular efforts to reach a biomechanical equilibrium with the net sum of forces minimized^{27, 28}. It is possible that the anisotropic geometry of the grooves and ridges establishes stresses and shear-free planes that influence the direction of microtubule growth²⁶ in order to create a force economic situation. Although economic force management is a common matter in nature, it does not explain the differences in susceptibility to topographical guidance that are found between different cell types²¹. This might be caused by functional differences between the cells in an *in vivo* situation, which can result in a difference in cytoskeleton organization²¹. This hypothesis needs to be thoroughly investigated in view of the recent findings, which suggest that the altered cell-substratum interactions can be based on the resemblance of these surfaces with the topography of fibrillar extracellular matrix²³.

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