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# Effect of parallel surface microgrooves and surface energy on cell growth

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To evaluate the effect of surface treatment and surface microtexture on cellular behavior, smooth and microtextured silicone substrata were produced. The microtextured substrata possessed parallel surface grooves with a width and spacing of 2.0 (SilD02), 5.0 (SilD05), and 10  $\mu\text{m}$  (SilD10). The groove depth was approximately 0.5  $\mu\text{m}$ . Subsequently, these substrata were either left untreated (NT) or treated by ultraviolet irradiation (UV), radiofrequency glow discharge treatment (RFGD), or both (UVRFGD). After characterization of the substrata, rat dermal fibroblasts (RDF) were cultured on the UV, RFGD, and UVRFGD treated surfaces for 1, 3, 5, and 7 days. Comparison between the NT and UV substrata revealed that UV treatment did not influence the contact angles and surface energies of surfaces with a similar surface topography. However, the

contact angles of the RFGD and UVRFGD substrata were significantly smaller than those of the UV and NT substrata. The dimension of the surface microevents did not influence the wettability characteristics. Cell culture experiments revealed that RDF cell growth on UV-treated surfaces was lower than on the RFGD and UVRFGD substrata. SEM examination demonstrated that the parallel surface grooves on the SilD02 and SilD05 substrata were able to induce stronger cell orientation and alignment than the events on SilD10 surfaces. By combining all of our findings, the most important conclusion was that physicochemical parameters such as wettability and surface free energy influence cell growth but play no measurable role in the shape and orientation of cells on microtextured surfaces. © 1995 John Wiley & Sons, Inc.

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## INTRODUCTION

All cell types that adhere to substrata reside in an environment with some form of topography. This topography may consist of other cells, extracellular matrix, other organisms, or artificial materials. The first observation of such a topographic reaction of cells dates from the beginning of this century.<sup>1</sup> Until the early 1970s, almost no further attention was paid to this phenomenon. Then, Rovinsky et al.<sup>2,3</sup> and Maroudas<sup>4,5</sup> rediscovered that cells are able to react on the topography of substratum surfaces. From that moment, research of this process flourished, resulting in a host of publications.<sup>6-17</sup> The underlying mechanism of this altered cellular behavior remains unknown. Several applicable theories are available, some of which assume that the geometric surface properties impose mechanical restrictions on the cytoskeletal components, which are involved in cell spreading and locomotion.<sup>6,17</sup>

Besides geometric properties, it is also recognized that physicochemical properties are able to influence cellular behavior. For example, it has been described that cellular adhesion tends to correlate with the surface free energy of the substratum material.<sup>18,24</sup> Surfaces with a low surface free energy are reported to be less adhesive than those with a high surface free energy.

Similar to the influence of surface topography, several mechanisms have been proposed to explain the influence of the wettability or surface free energy on cellular behavior. The most widely accepted theory is that these properties have a selective effect on the configuration or conformation of the proteins, which are deposited on the substratum surface.<sup>19,25</sup> These proteins play an important role in the cellular adhesion process. In this context, it has also been noted that the wettability of a substratum surface is primarily determined by the nature and packing of the outermost or exposed surface atoms in a solid. Therefore, it is independent of the chemical nature or arrangement of the underlying atoms and molecules.<sup>26</sup>

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Recognizing the potential effect of surface properties on cellular behavior, there are two other factors that need to be considered. First, it has been found that surface roughness or surface topography can have a disturbing effect on the wettability characteristics of a solid.<sup>27</sup> This may especially occur when a material has a uniform roughness or surface texture, but has been disputed by Schmidt and von Recum.<sup>38</sup> Second, in various experiments investigating the influence of the substratum surface topography on cellular behavior, several methods of surface treatments were used, such as ultraviolet irradiation<sup>28</sup> and radiofrequency glow discharge.<sup>39</sup> However, there is sufficient evidence that the applied surface treatment can modify the wettability properties and biologic performance of a material.<sup>29</sup>

Taking these factors into consideration, it is possible to suggest that the effect of surface topography on cellular behavior is caused not only by the surface pattern, but also by the altered wettability characteristics as a result of applied surface treatments. Therefore, the aim of this study was to evaluate the cellular growth rate and orientation of well-defined surfaces, which received a different surface treatment.

## MATERIAL AND METHODS

### Production of the substrata

The experimental substrata were produced as described by Schmidt and von Recum.<sup>30,31</sup> Briefly, photolithography was used to manufacture smooth and textured silicon wafers. These produced textured silicon wafers, which had parallel surface grooves with a 2, 5, or 10- $\mu\text{m}$  diameter. All of these grooves had a depth of 0.5  $\mu\text{m}$  and were uniformly distributed with spacing similar to the groove width. The configuration and dimensions of these surfaces are summarized in Table I and Figure 1. To obtain the final experimental substrata, these wafers or molds were covered with polydimethylsiloxane (silicone elastomer A-2186, Factor II). After polymerization, we removed the silicone rubber sheets by peeling them off the wafers.

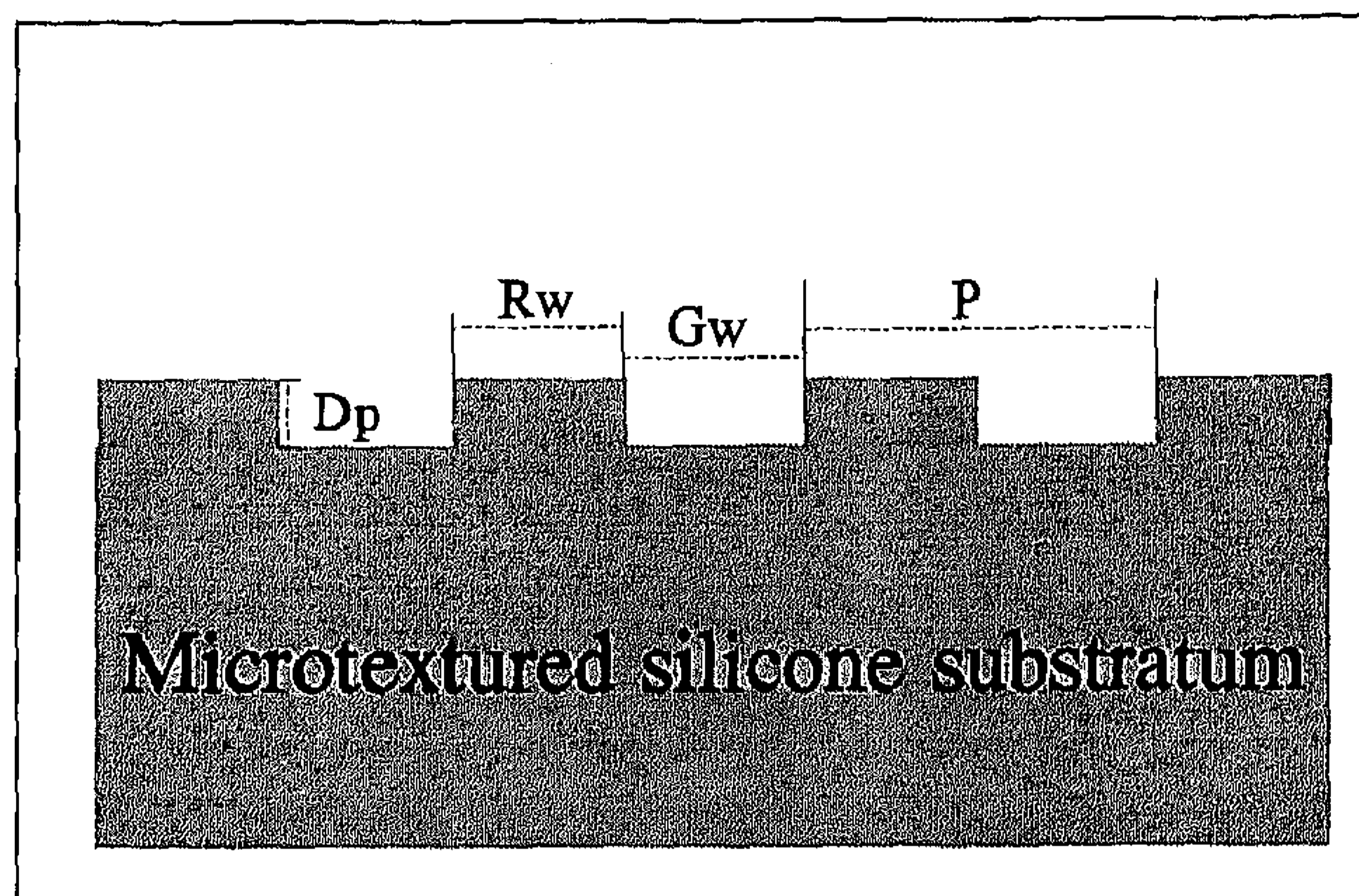


Figure 1. Cross-section through a microtextured substratum (not to scale); Dp, groove depth; Gw, groove width; Rw, ridge width; P, pitch.

### Surface treatment of the substrata

Prior to use, the microtextured silicone sheets were cut into 15-mm-diameter round discs. These experimental substrata were manually washed in 10% Liquinox solution (Alconox Inc.), rinsed, ultrasonically cleaned for 6 h in a 1% Liquinox solution, and given two 15-min ultrasonic rinses in distilled, deionized water. Subsequently, they were given a Soxhlet rinse for 24 h in distilled, deionized water to remove residue. Finally, the substrata were air-dried and randomly divided into four groups. These groups of substrata were either left untreated (NT) or treated by: 1) ultraviolet irradiation (UV; 254 nm, 8 h); 2) radiofrequency glow discharge (RFGD) treatment (PDC-3XG, Harrick; Argon, 0.15 mm Hg, 5 min); or 3) 8 h UV irradiation, followed by RFGD treatment (UVRFGD).

### Surface characterization of the substrata

After applying these treatments, the following methods were used to characterize the smooth and microtextured surfaces:

1. Scanning electron microscopy (SEM 500; Philips) and scanning probe microscopy (SPM, SP300; Polaron) for qualitative and quantitative inspection of the various surface textures.

TABLE I

Designer Values of Silicon Molds and Actual Values of the Microevents on Silicone Rubber Substratum Surface

Surface	Designer Values				Actual Values			
	Dp ( $\mu\text{m}$ )	Gw ( $\mu\text{m}$ )	Rw ( $\mu\text{m}$ )	P ( $\mu\text{m}$ )	Dp ( $\mu\text{m}$ )	Gw ( $\mu\text{m}$ )	Rw ( $\mu\text{m}$ )	P ( $\mu\text{m}$ )
SiD00	0.00				$\pm 0.02$			
SiD02	0.50	2.00	2.00	4.00	0.45	1.71	1.68	3.87
SiD05	0.50	5.00	5.00	10.00	0.45	4.65	4.98	9.49
SiD10	0.50	10.00	10.00	20.00	0.46	9.58	9.77	18.98

Dp, groove depth; Gw, groove width; Rw, ridge width; P, pitch.

2. Wettability measurements by using the Wilhelmy plate technique. The substrata for this particular analysis consisted of two square pieces of silicone rubber ( $15 \times 15$  mm) attached back to back, thus creating a substratum with two identical smooth or microtextured surfaces. A DCA 322/DACS (Cahn Instruments Inc.) was used to perform the wettability analysis in water and ethylene glycol, according to the two-liquid method.<sup>27</sup> The dip and retraction speed during contact angle measurements was  $2.5 \mu\text{m}/\text{second}$ . To exclude an effect of the groove orientation on the advancing and receding contact angles, the measurements were performed with three different substratum orientations (Fig. 2). Nine test pieces of each substratum were used. In addition to the measured contact angles, the surface tension of the various substrata was calculated (DCA Applications Software version 1.0; Cahn Instruments Inc.), according to the geometric mean method.<sup>27,32-34</sup>

## Cell culture

Fibroblasts (RDF) 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 at  $37^\circ\text{C}$  in sterile atmosphere of 5%  $\text{CO}_2$ –95% air in  $\alpha$ -MEM with Earl's salts and with L-glutamine (Gibco), supplemented with 15% (vol/vol) heat-treated fetal calf serum (Gibco),  $2.5 \mu\text{g}/\text{ml}$  amphotericin B (Gibco), and  $50 \mu\text{g}/\text{ml}$  gentamicin (Gibco). After approximately 3 days of culturing, the RDF were rinsed with phosphate-buffered saline without magnesium and calcium (PBS Dulbecco; pH 7.2), supplemented with  $5 \mu\text{g}/\text{ml}$  amphotericin B and  $100 \mu\text{g}/\text{ml}$  gentamicin to remove nonattached cells. Subsequently, the growth medium was replaced every 2 days by fresh growth medium. Upon confluence, the RDF were detached by trypsinization [0.25% (wt/vol)

crude trypsin and 1 mM EDTA (pH 7.2)] and resuspended at a lower cell concentration in new culture flasks (Nunc) in fresh growth medium. The cells were identified as fibroblasts by phase contrast morphology analysis as described by Freshney.<sup>35</sup> Fifth-generation cells were used in all experiments.

## Cell growth assay

Smooth and microtextured surface treated substrata were placed randomly in the wells of 24-well plates (Greiner). The orientation of the grooves was random, since the microgrooves are not macroscopically visible during this procedure. Subsequently, approximately  $1.0 \times 10^4$  viable RDF  $\text{ml}^{-1}$  suspended in sterile growth medium were added to each substratum. In addition, cell suspension was added to wells without substrata to serve as a control group (CTRL). The cultures were incubated for 1, 3, 5, and 7 days ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ –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 nonattached cells. The remaining RDF on the substrata were detached by trypsinization and counted using a Coulter Counter. After trypsinization the substrata were observed routinely with a phase contrast microscope to check whether all cells were removed. The results presented are based on the average of four experimental runs, which were counted in triplicate.

To demonstrate the effect of the surface microgeometry on the shape and orientation of the RDF, additional cultures of smooth and microtextured substrata were evaluated by SEM. After incubation the attached RDF were fixed and dehydrated by rinsing with 100% methanol for 5 min. Finally, the samples were air-dried, mounted on stubs, sputter-coated with gold, and investigated by SEM. This experiment was performed in triplicate.

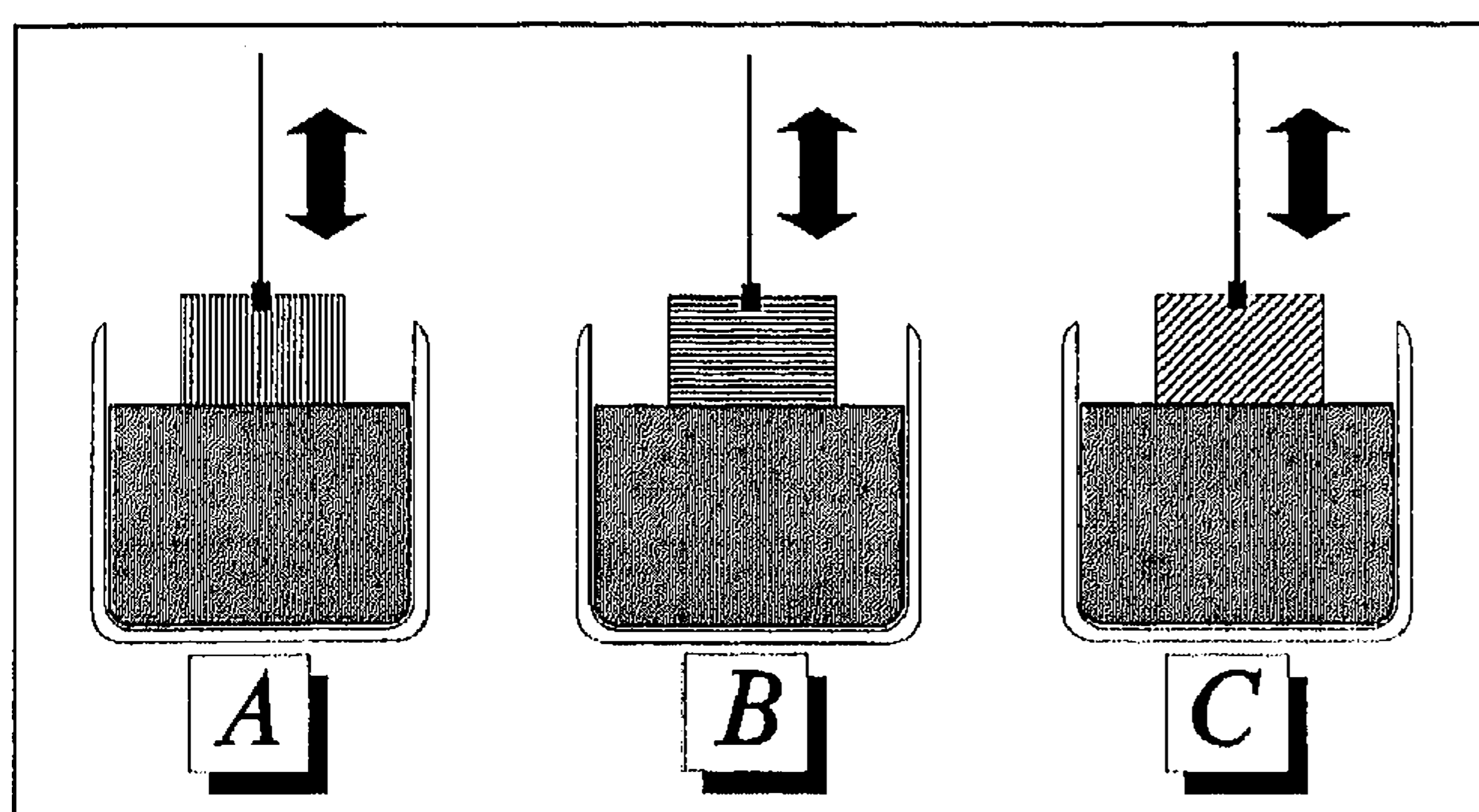


Figure 2. Illustration of the orientation of the microgrooves during wettability analysis.

## RESULTS

### Surface characterization

Scanning electron microscopic and SPM examination showed that none of the duplicated silicone surfaces had defects or irregularities in their surface pattern (Figs. 3 and 4). However, SPM measurements also showed a deviation between the values of the microevents on the silicone-cast substrata and the designer values of the silicon molds (Table I and Fig. 4).

The advancing and receding contact angle ( $\theta_{\text{ADV}}$  and  $\theta_{\text{REC}}$ ) of the various substrata were measured,

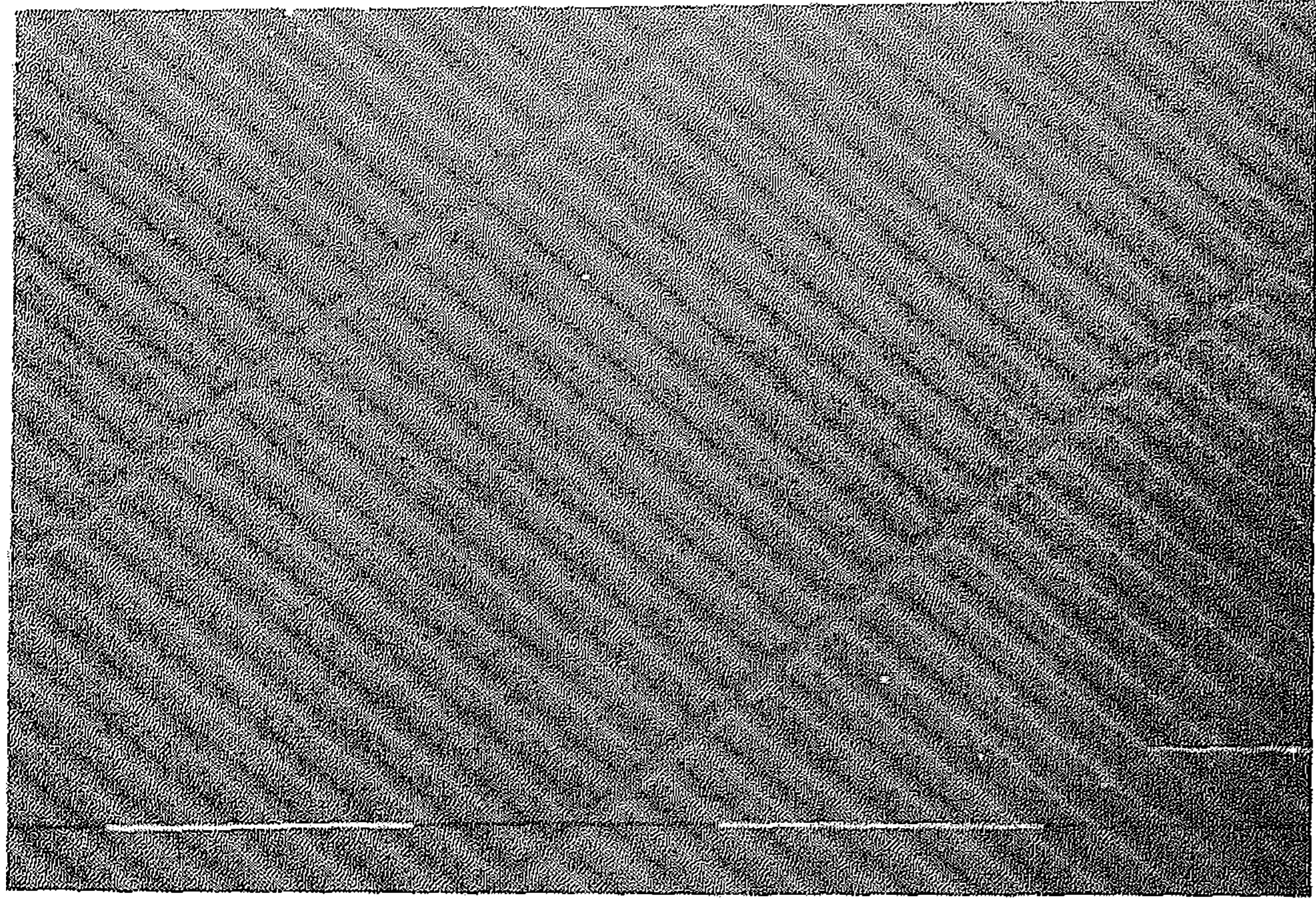


Figure 3. Scanning electron micrograph of the grooved surface of an SiID05 substratum (1 division = 100  $\mu\text{m}$ ).

followed by calculation of the surface free energy. The results are listed in Table II. The values were averaged over the three orientations as used for wettability analysis but were separately statistically tested. Statistical testing of these findings, using a Kruskal–Wallis test, showed that the orientation of the surface grooves had no measurable effect on the contact angles and surface free energies of equally treated substrata with an identical surface texture. The various topographic dimensions also did not influence the advancing and receding contact angles and surface energies. Furthermore, statistical testing revealed that UV treatment had no influence ( $P > .05$ ) on the contact angles and surface energies of sub-

TABLE II  
Average Water Contact Angles ( $\theta_{\text{ADV}}$  and  $\theta_{\text{REC}}$ ) and Surface Tension ( $\gamma_{\text{S}}$ ) of Smooth and Microtextured Substrata

Sample	$\theta_{\text{ADV}}$ (degrees)	$\theta_{\text{REC}}$ (degrees)	$\gamma_{\text{S}}$ (dyne/cm)
SiID00			
NT	111 (10.7)	68 (3.9)	24.9 (2.6)
UV	104 (2.4)	74 (0.3)	23.6 (2.9)
RFGD	17 (1.0)	17 (1.2)	125.8 (12.5)
UVRFGD	15 (1.5)	16 (1.7)	110.5 (14.5)
SiID02			
NT	96 (1.5)	66 (3.1)	14.9 (0.9)
UV	98 (0.8)	74 (0.3)	23.6 (2.9)
RFGD	17 (0.6)	15 (0.6)	133.1 (11.7)
UVRFGD	17 (0.7)	15 (0.8)	131.8 (10.3)
SiID05			
NT	100 (2.2)	66 (3.2)	17.7 (3.7)
UV	100 (1.5)	70 (2.0)	14.4 (1.4)
RFGD	29 (1.2)	18 (0.6)	111.4 (28.3)
UVRFGD	18 (1.2)	17 (1.2)	123.4 (4.1)
SiID10			
NT	90 (2.8)	67 (2.6)	16.6 (2.4)
UV	98 (2.0)	69 (3.1)	14.0 (0.3)
RFGD	23 (0.9)	23 (0.6)	127.5 (18.8)
UVRFGD	18 (0.7)	19 (0.4)	137.3 (12.4)

$\sigma_{n-1}$  is given between brackets ( $n = 9$ ).

strata with an identical surface topography. However, a significant difference was detected between identical textured substrata of the NT and RFGD groups ( $P = .0001$ ), the NT and UVRFGD groups ( $P = .0001$ ), the UV and RFGD groups ( $P = .0001$ ), and the UV and UVRFGD groups ( $P = .0001$ ).

### Cell growth assay

Figures 5–7 show the growth curves of the RDF cells on the various substrata. As indicated by these figures, the RDF cell growth on RFGD and UVRFGD-treated substrata was higher than on UV-treated substrata. Statistical evaluation of the data using a Kruskal–Wallis test confirmed this observation ( $P = .0001$ ). Statistical testing also revealed that the cell growth of the RDF of the CTRL group was significantly higher than that on the UV-treated substrata ( $.0001 \leq P \leq .005$ ). No significant difference in growth rate was found between RDF cultured on the other treated substrata and CTRL surfaces.

Statistical comparison of the growth data for each individual treatment group produced no evidence for a constant significant influence of the surface topography on the RDF growth rate. For example, in the RFGD group cell growth on SiID10 substrata was significantly higher than on SiID02 substrata on day 1 ( $P = .0376$ ), while on day 3 the reverse was found ( $P = .0002$ ). Furthermore, many nonsignificant differences

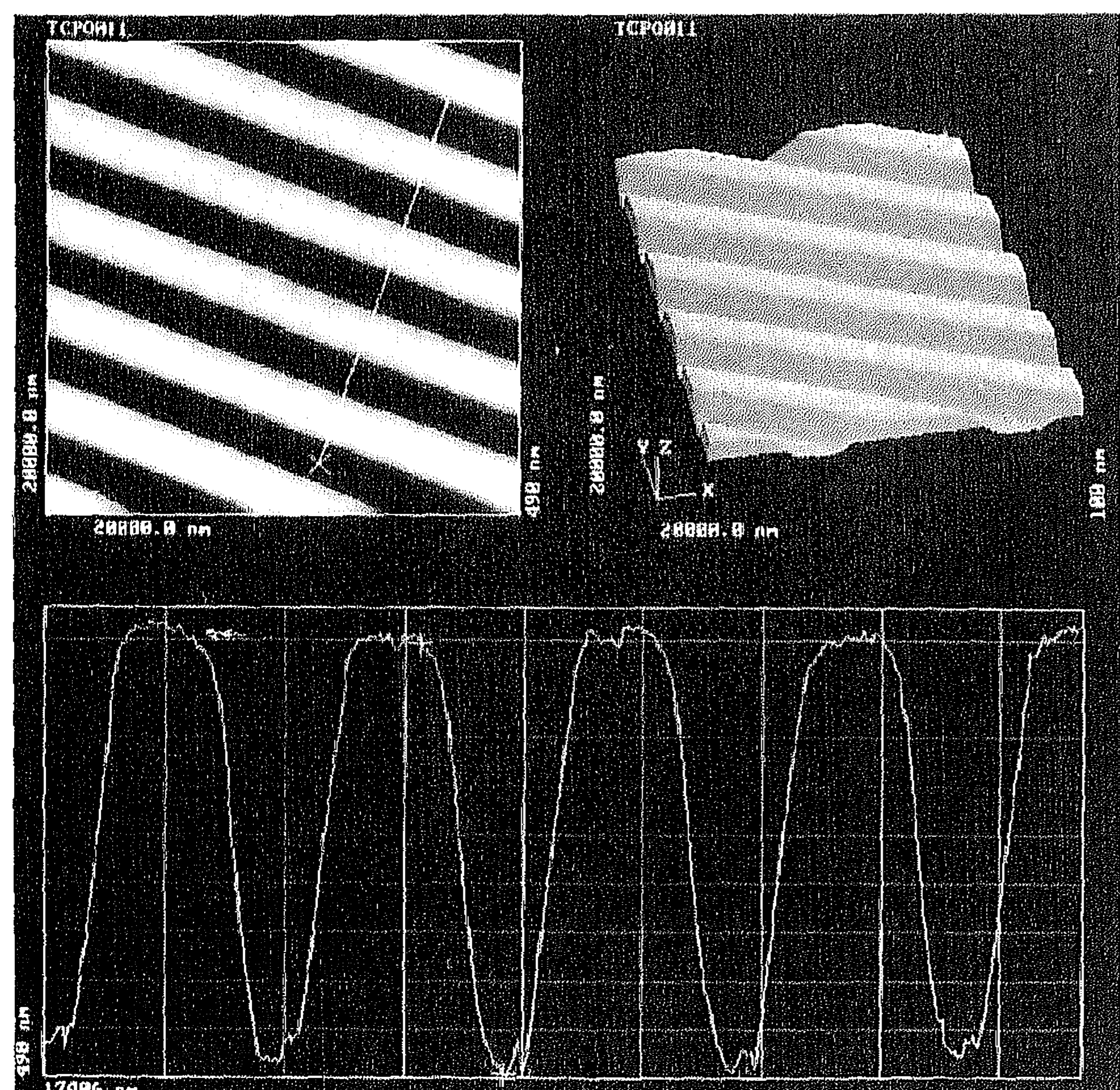
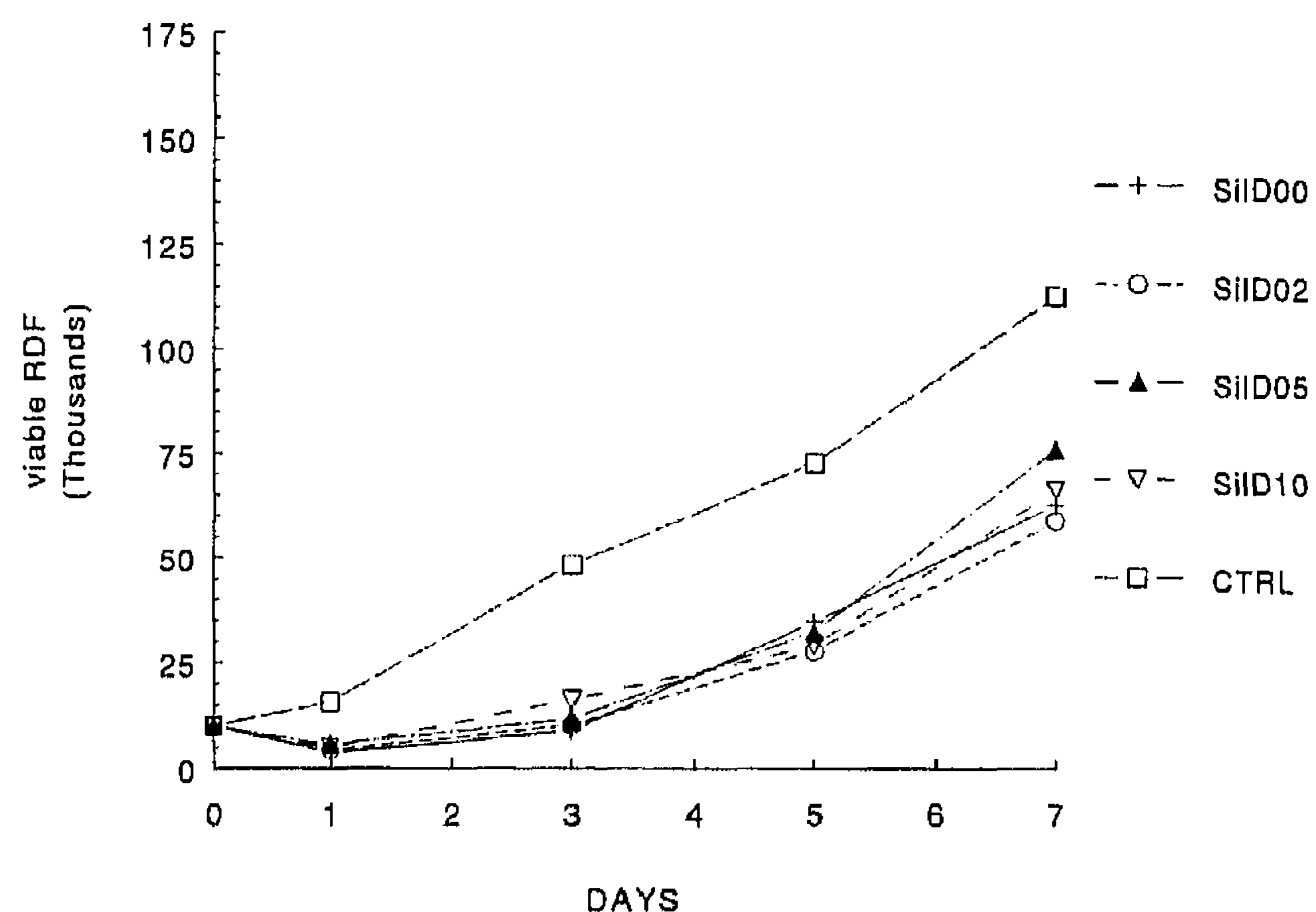


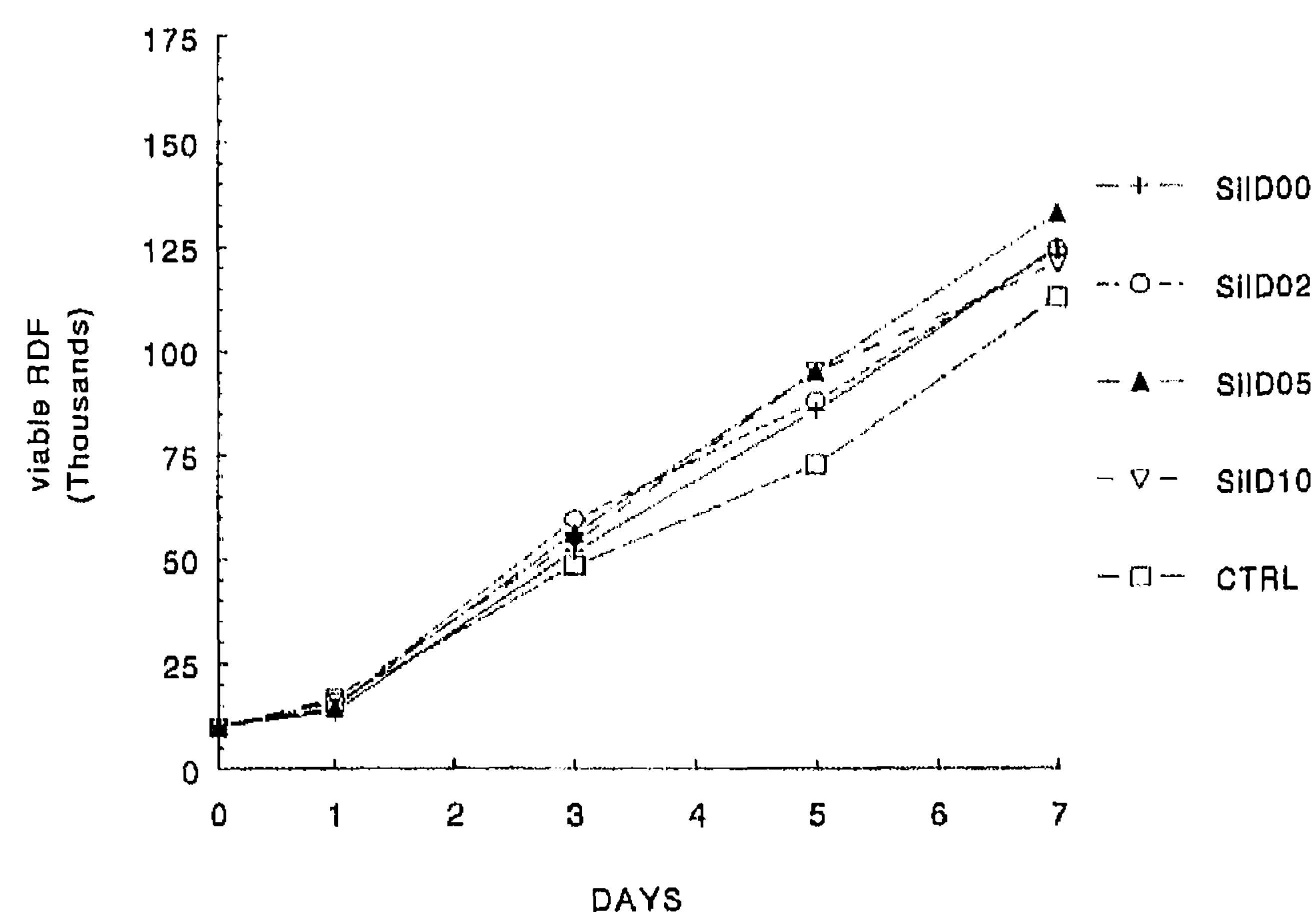
Figure 4. Results of SPM measurements on an SiID02 substratum. The two figures represent a two-dimensional and a three-dimensional height distribution plot. The graph shows the data points of the SPM measurements. X- and Y-axis have different magnifications.



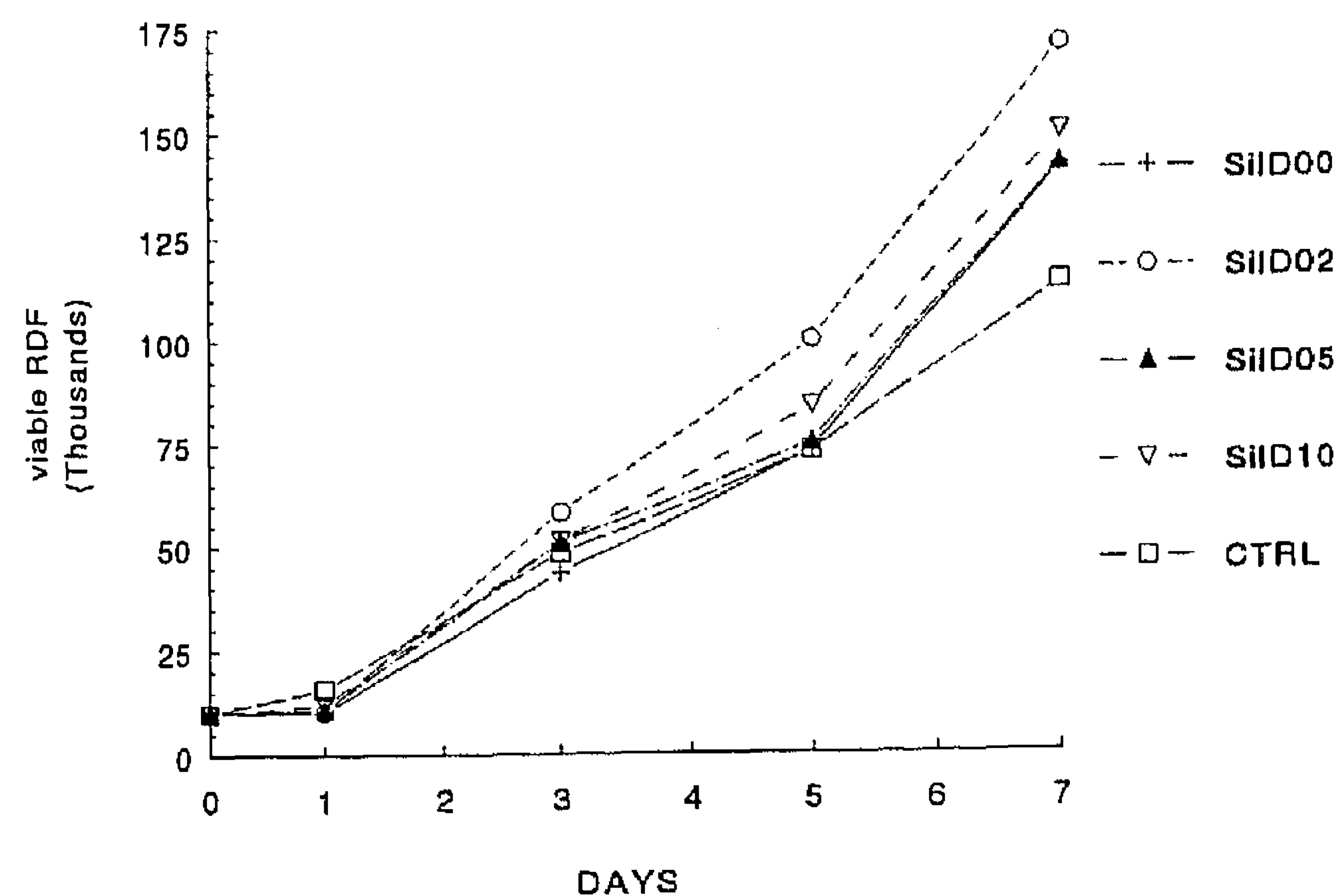
**Figure 5.** Growth of RDF on substrata of the UV group ( $CV_{\text{average}} = 24.4\%$ ). The growth data of the control group (CTRL) are also plotted ( $CV_{\text{average}} = 4.3\%$ ). Statistically significant differences were found between CTRL and UV-treated surfaces ( $0.0001 \leq P \leq .0005$ ).

in cell growth were found. These findings were consistent for all treatment groups.

In contrast with these growth rate findings, SEM evaluation revealed a clear influence of the surface topography of the substrata on the shape and orientation of the cells. This influence was independent of the surface treatment used. Scanning electron micrographs of cells cultured on the various patterned surfaces are shown in Figures 8–11. These micrographs show that cells grown on the SiID00 substrata were randomly spread and oriented. Although no quantitative procedures were performed, it is clear that cells on the SiID02 and SiID05 substrata were aligned parallel to the surface grooves. Furthermore, despite their oriented shape, some of these cells also possessed protrusions which extended over several



**Figure 6.** Growth of RDF on substrata of the RFGD group ( $CV_{\text{average}} = 11.7\%$ ). The growth data of the control group (CTRL) are also plotted ( $CV_{\text{average}} = 6.3\%$ ). No statistically significant differences were found between CTRL and RFGD-treated surfaces.



**Figure 7.** Growth of RDF on substrata of the UVRFGD group ( $CV_{\text{average}} = 13.84\%$ ). The growth data of the control group (CTRL) are also plotted ( $CV_{\text{average}} = 5.4\%$ ). No statistically significant differences were found between CTRL and UVRFGD-treated surfaces.

grooves and ridges. RDF, growing on SiID10 substrata, differed in two ways from cells cultured on the other surfaces. First, these cells were elongated, but their body was not aligned parallel to the surface pattern. Second, these RDF were not totally randomly oriented like the cells observed on the smooth SiID00 substrata. These findings proved to be comparable for all incubation periods.

## DISCUSSION AND CONCLUSIONS

Scanning probe microscopy measurements showed a deviation between the designer values of the silicon mold and the actual values of the microevents on the silicone substratum surface. These dimensional changes were probably caused by polymerization shrinkage, due to the minimal amount of filler that is added to the polymer.<sup>26</sup> However, it has to be noted that only the dimensions of the molds were determined. Therefore, it cannot be completely excluded that the dimensions of the textured wafers deviated from the original designer values.

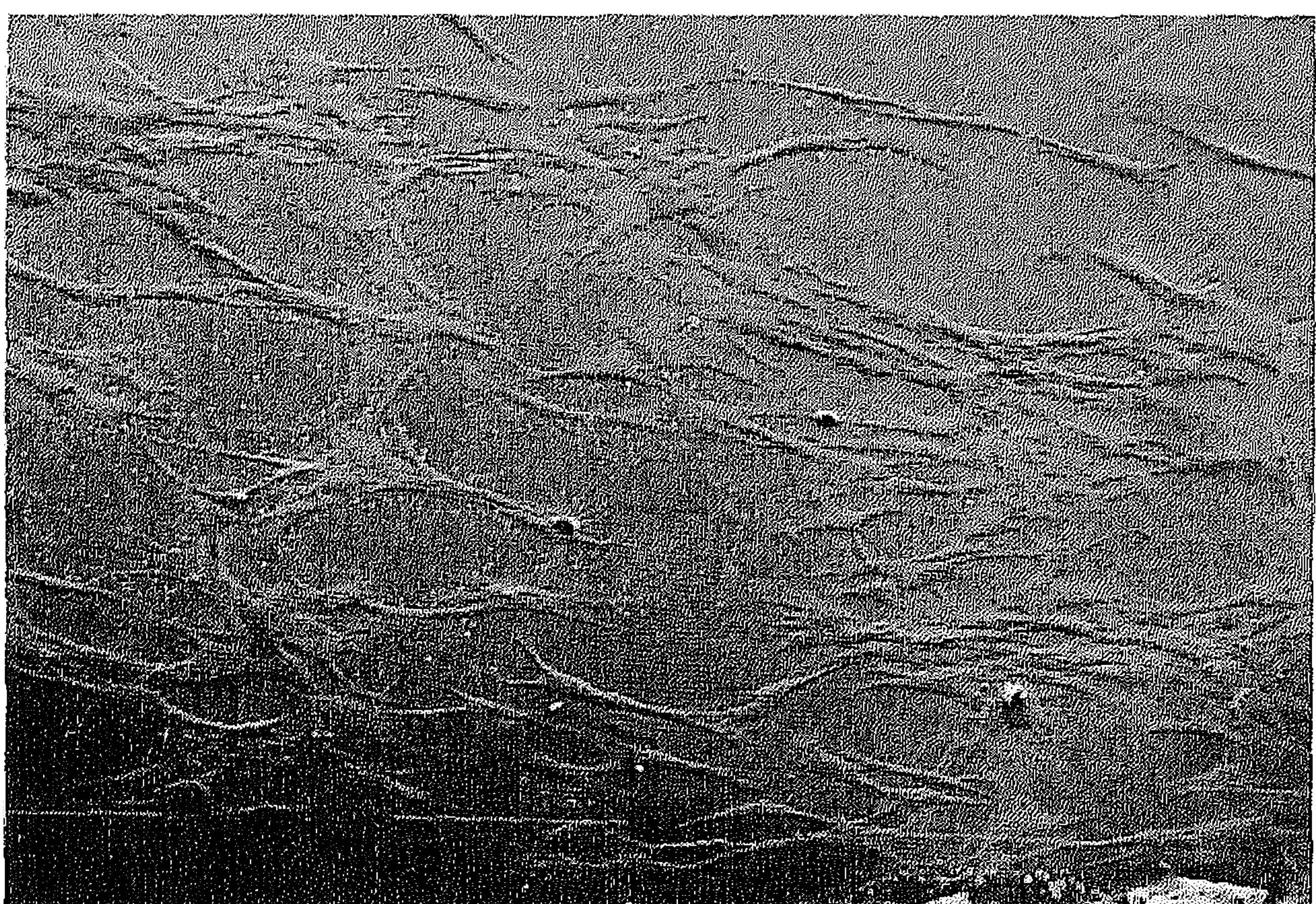
During the cell culture experiments the NT group was excluded, because the growth rate could be very seriously affected by possible microbiologic contamination. The effect of such contamination on the growth rate would introduce an additional variable, which would obscure the relation between surface treatment and cell growth. Application of conventional sterilization methods such as sterilization by heat, gas, or  $\gamma$ -irradiation can have negative effects or cause damage to silicone rubber substrata and the growth behavior of cells cultured on these substrata.<sup>19,36</sup> Therefore, UV irradiation was chosen as an additional surface treatment. This choice was



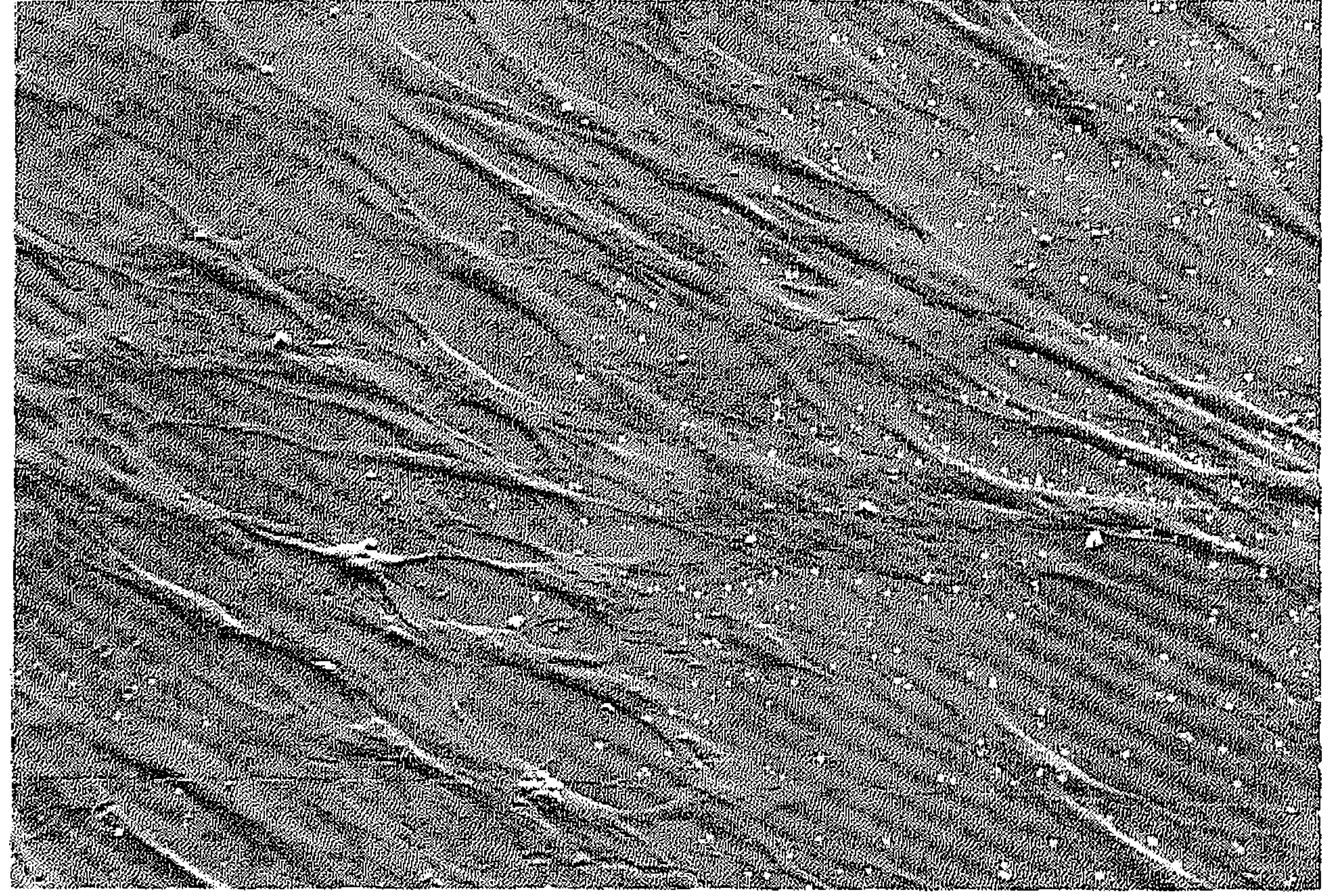
**Figure 8.** Scanning electron micrograph of RDF on an SilD00 surface after an incubation period of 3 days (1 division = 100  $\mu\text{m}$ ). Note the random orientation.

guided by the fact that UV treatment is commonly used for the sterilization of cell culture specimens. Furthermore, as demonstrated by our contact angle measurements, the wettability properties of the NT and UV substrata are similar.

The experimental data in Table II show that substrata of the same treatment group had the same contact angles and surface energy despite their different surface grooves. The contact angles and surface free energies were only increased after RFGD treatment. These results also demonstrated that RFGD treatment increased the wettability of UV-treated substrata to the same level as did RFGD alone. Therefore, a correlation between wettability and surface topography or roughness was not demonstrated. Although this observation is not in agreement with some earlier studies,<sup>37</sup> it corroborates the findings of Schmidt and von Recum,<sup>38</sup> who reported that square 2, 5, 8, and 10- $\mu\text{m}$  events on silicone surfaces did not increase the



**Figure 9.** Scanning electron micrograph of RDF on an SilD02 surface after an incubation period of 3 days (1 division = 100  $\mu\text{m}$ ). The RDF are aligned parallel to the surface grooves.

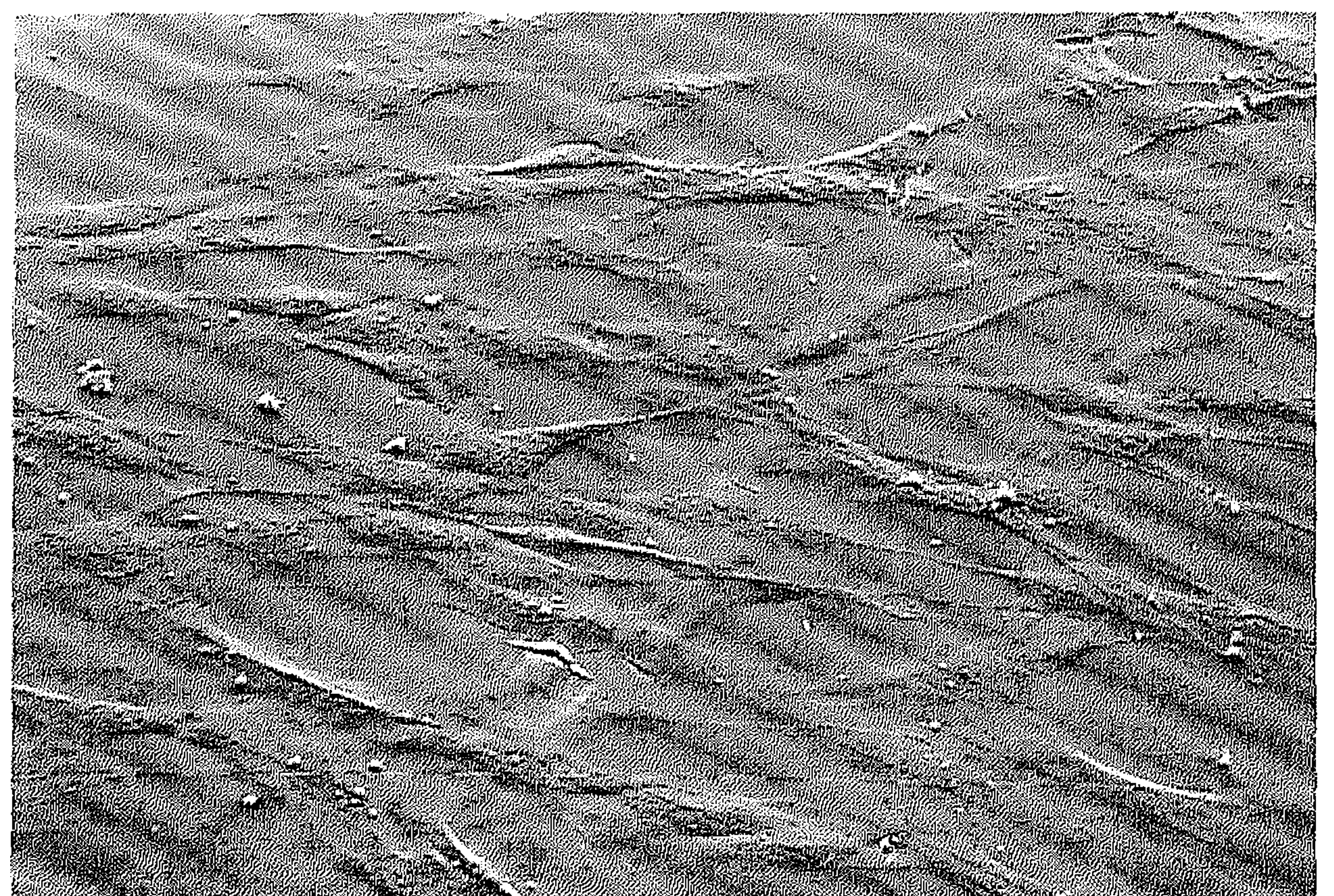


**Figure 10.** Scanning electron micrograph of RDF on an SilD05 surface after an incubation period of 3 days (1 division = 100  $\mu\text{m}$ ). The RDF are aligned parallel to the surface grooves.

critical surface tension and energy of these surfaces compared with smooth silicone substrata.

Our study showed that the growth rate of the RDF on UV-treated substrata was lower than the growth rate of these cells on the substrata of the RFGD, UVRFGD, and CTRL groups. Differences among these groups were not detected. We found no clear evidence that within a single treatment group the dimension of the microfeatures on the substratum surface facilitated a higher growth rate.

Furthermore, the SEM micrographs demonstrated a marked influence of the various surface structures on the orientation of RDF. These results confirm the findings of other investigators,<sup>3,4,6-17</sup> who also observed contact guidance of cells cultured on microtextured surfaces. However, contrary to the substrata used in our study, their substrata did not possess 0.45- $\mu\text{m}$ -deep grooves, but grooves of at least 1  $\mu\text{m}$



**Figure 11.** Scanning electron micrograph of RDF on an SilD10 surface after an incubation period of 3 days (1 division = 100  $\mu\text{m}$ ). Orientation of the RDF becomes more random compared to RDF cultured on SilD02 and SilD05 substrata.

depth. It was not surprising to find that some RDF cells were able to span several grooves and ridges on all our microtextured surfaces, since this had already been observed by other investigators.<sup>39</sup> In addition, our results showed that SiLD02 and SiLD05 substrata were able to induce a stronger contact guidance than SiLD10 substrata. The random orientation of the RDF on SiLD00 substrata proved that no contact guidance was evident on these substrata. These last two observations support the studies of Meyle et al.<sup>16</sup> and Schmidt and von Recum,<sup>31</sup> who concluded that surface features especially in the range of 1–5  $\mu\text{m}$  promote cellular conformation.

Finally, a comment has to be made about the SEM fixation and dehydration method used. The authors realize that the use of methanol is not a widely accepted means of fixating and dehydrating cells, which can cause a great loss of delicate cell structures.<sup>40</sup> Nevertheless, this method was chosen since other accepted methods (such as critical point-drying, freeze-drying, and dehydration with tetramethylsilane) cause severe damage to cells cultured on silicone rubber and make it impossible to gather information about cell orientation. This damage probably occurs because the substrata consist of polydimethylsiloxane. During critical point-drying, high pressure compresses the silicone rubber, thus causing cell damage or detachment of the RDF. Freeze-drying results in a rapid drop in temperature, which acts as a fixative. However, during this process silicone rubber acts as an insulator, retaining heat and permitting crystals to form which destroy the cell. Dehydration with tetramethylsilane, on the other hand, causes the silicone rubber substrata to swell. Consequently, the cells that attached to the silicone rubber are exposed to forces which deform, and ultimately detach or damage the cells.

By combining all our findings, the most important conclusion that can be drawn is that physicochemical parameters such as wettability and surface free energy play no measurable role in the shape and orientation of cells on microtextured surfaces. Apparently, the cells are forced into place by the surface texture. For example, as already mentioned earlier by Meyle et al.,<sup>15</sup> it can be hypothesized that the strong induction of contact guidance by 2- and 5- $\mu\text{m}$  grooves indicates the need of cells for mechanical stabilization against interfacial movement. However, it cannot be excluded that this orientation phenomenon is caused by the efforts of the cell to reach a biomechanical equilibrium with the net sum of forces minimized.<sup>39,41–43</sup>

Finally, in light of earlier reports,<sup>28,31</sup> no effect of surface features on the fibroblast growth rate could be undeniably proven in this study. This growth rate is, however, significantly changed by the applied surface treatment method.

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