Nanoprinting onto cells

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Growing cells on surfaces bearing nanotopography signals makes many changes in cell gene expression and downstream changes in phenotype but the mechanisms for this have, so far, been obscure. We consider the question of whether the topography directly nanoimprints onto the cell as a component of the signal transduction system. Evidence we present from SEM, TEM and fluorescence detection of the arrangements of cytoskeletal components is consistent with the possibility that cells are nanoimprinted by the substrate. The nanoprinting does not interfere with integrin-mediated adhesion processes and may perhaps work through them. Time-lapse video studies of cells moving from areas bearing nanotopography to flat areas and vice versa suggests that the nanoimprinting takes 1–6 h to appear on the cell and a similar time to disappear when the cell moves from a flat surface to a nanotopographic one and back. This nanoprinting of cells would appear to be a novel type of cell signalling.

**Keywords: nanoprinting; cell reactions to nanotopography; interface biological events;
persistence; nanotopography**

1. INTRODUCTION

Cells are known to be capable of mechanotransduction, which is the signalling into, out of and between cells by mechanical means (e.g. Wang *et al.* 1993; Knight *et al.* 2002). A wide variety of biological cells can react to growing on a nanostructured interface by changes in adhesion, cytoskeletal organization, gene expression and other cellular activities (Wojciak-Stothard *et al.* 1995; Dalby *et al.* 2002*a,b*, 2003*a,b*, 2004*a–c*; Riehle *et al.* 2002; Gadegaard *et al.* 2003; Arnold *et al.* 2004; Curtis *et al.* 2004). Some of these papers present evidence that mechanotransduction is involved. We have extended investigations on the mechanisms, by which cells can respond to a nanofeatured surface to the question of, whether they organize their surface structure in relation to those features. We consider, whether the cell can receive a direct imprint of the nanotopography of the substratum. The evidence on the reactions of cells to nanotopography and nanosurface chemistry suggests that this might happen (Dalby *et al.* 2003*a,b*, 2004*a–c*) but no direct evidence, for it appears to have been reported. Dalby and others have described many ordered and semi-ordered surface nanotopographies that induce considerable cytoskeletal reorganizations and alignments. Cells often align and adhere to nanometrically high-extended ridges (Curtis & Clark 1990) and that there is often an associated line of actin condensation very close to the edge (Wojciak-Stothard *et al.* 1996).

The simplest test for this idea is akin to the way in which the accuracy of printing is assessed—namely that the original pattern is transferred to the 'paper' as accurately as is required for easy reading. The original pattern is in the nanotopography and the replica is on

or in the cell. The techniques we have used include TEM, SEM and fluorescence microscopy. Fluorescence microscopy should reveal any pattern of proteins related to the original print surface, but it would be expected that these ordered proteins would probably, only be found close to the inside of the plasmalemma. We have also looked at evidence for the possible involvement of integrin signalling and the persistence of this type of mechanotransduction signalling.

2. EXPERIMENTAL SECTION

2.1. Nanofeatured surfaces

These were made by colloidal lithography or by electron-beam lithography (e-beam) followed by replication by embossing in to the polymers; polycaprolactone, polymethyl methacrylate and polycarbonate (figures 1 & 2). Details of the fabrication of such surfaces have been published by Gadegaard *et al.* (2003) and Hanarp *et al.* (2003).

2.2. Cell culture

Fibroblasts from skin (human, (h-tert BJ1) or endothelia (murine, LeII)) was grown on the nanofeatured substrata. Human platelets were extracted from whole blood, drawn with ethical permission by a qualified phlebotomist and with consent of the donors. The drawn blood was left for 40 min and the platelets were taken from the plasma rich supernatant. $1–2 \times 10^6$ cells were seeded per sample.

2.3. Preparation for EM, AFM and fluorescence microscopies

For fluorescence microscopy, epitenon cells or human platelets were cultured for 24 h (epitenon) or 2 h

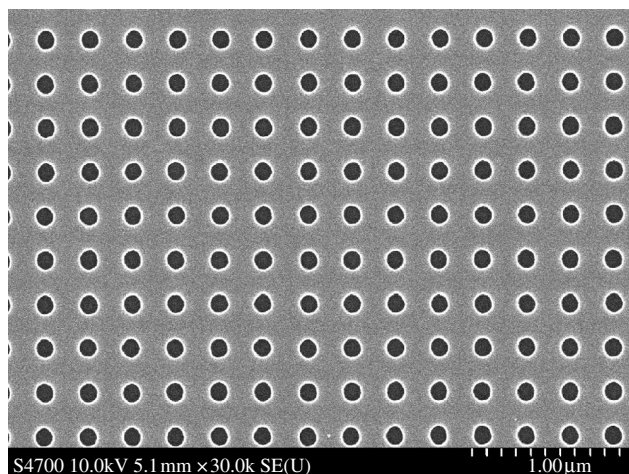**Author for correspondence (a.curtis@bio.gla.ac.uk).*

Figure 1. Nanopitted surface prepared by electron-beam lithography. It is an array of 120 nm diameter pits on a 300 nm centre-to-centre spacing.
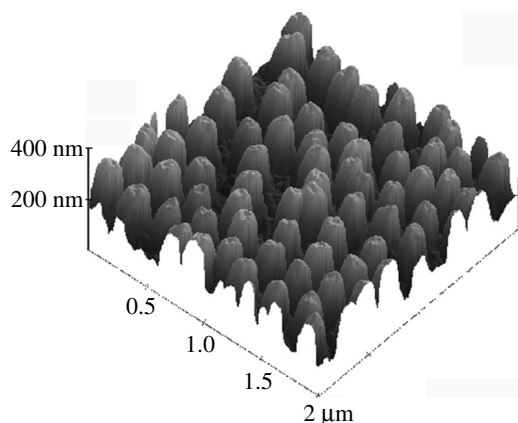

Figure 2. (right) Nanocolumns produced by colloidal lithography. It is an array of columns (fabricated by, and image) courtesy of Dr Duncan Sutherland, Chalmers University, Sweden. The columns are 160 nm high and have a diameter of 100 nm.

(platelets) on e-beam nanogrooves, fixed in 4% formaldehyde and stained with tetramethyl rhodamine–phalloidin for actin visualization. For SEM, h-tert cells were fixed in 1.5% gluteraldehyde after 4 days of culture on 160 nm high (100 nm diameter) nanocolumns fabricated by colloidal lithography figure 2 . Post fixation, they were osmium treated and dehydrated through alcohol and finally hexamethyl disilazane prior to viewing. For TEM, h-tert cells were fixed in 1.5% gluteraldehyde after 21 days of culture on 160 nm high (100 nm diameter) nanocolumns fabricated by colloidal lithography. Post fixation, they were osmium treated and dehydrated through an alcohol series prior to Araldite embedding and sectioning for TEM viewing. AFM images were acquired on a Nanoscope Dimension 3100 of SEM prepared samples.

2.4. Fast Fourier transforms of fluorescence images

These were carried out using the ImageJ program (available from http://rsb.info.nih.gov/ij/). The
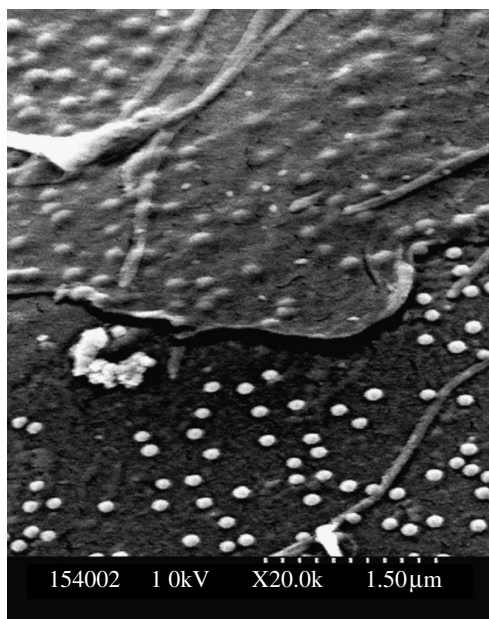

Figure 3. SEM of fibroblast lamella on nanocolumns produced by colloidal lithography. The cell lamella is shown at the top of the micrograph and the shapes of the columns are clearly visible in the lamella.
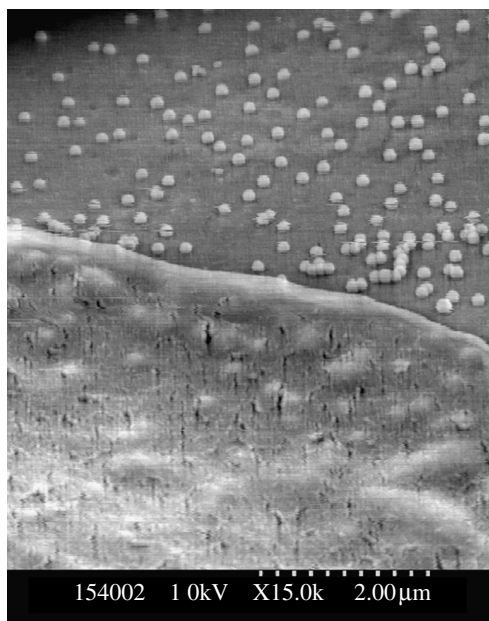

Figure 4. SEM of fibroblast lamellae on nanocolumns produced by colloidal lithography. The cell lamella is shown at the bottom of the micrograph and the shapes of the columns are clearly visible in the lamella.

images used were obtained from fluorescent-antibody or rhodamine–phalloidin stained cells.

2.5. Use of anti-integrin antibody

An anti-integrin alpha 1 antibody from a mouse myeloma fusion line with immunized mouse splenocytes SE8O9 was bought from Upstate Inc (Lake Placid, NY, USA; Lot no. 24929). The reactivity of this antibody with the cells was tested using an immunocytochemical test.
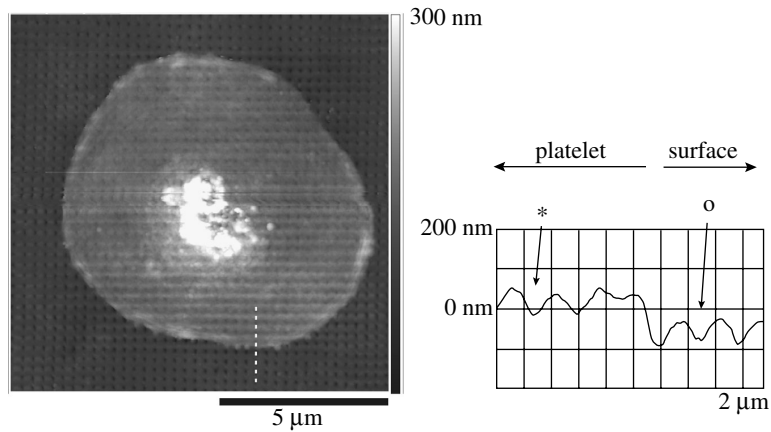
Figure 5. AFM micrograph of a human platelet on the same nanopitted surface as in figure 1. The underlying topography is clearly shown through the thin (80 nm) platelet membrane. This is confirmed by the cross-section where the pits clearly are seen in both the surfaces (o) and the platelet membrane. (These results demonstrate that the cell has the appropriate mechanical properties for nanoimprinting.)
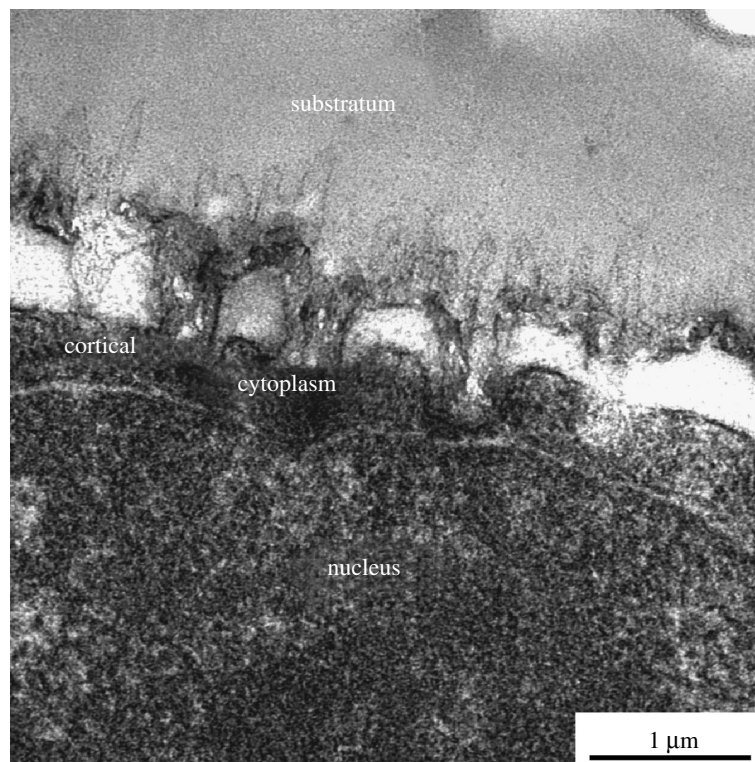

Figure 6. (above) TEM of a fibroblast (bottom) cultured on nanocolumns (top). The nanocolumns can clearly be seen making indents on the bottom of the cell; cell is at the bottom of the picture. The proximity of the nucleus to the nanostructure should be noted.

Thirty-three or 66 µg of the antibody was added to a 5 ml culture of 50 000 cells and the cells allowed to settle for 2 h being observed at 60 and 120 min. The cells were then fixed and stained to determine the proportion adhering to plain or nanopatterned polycarbonate.

2.6. Time-lapse video recording

Time-lapse videos were made of the areas of transition between flat surfaces and topography. This was done using 10× phase contrast objectives and a time-lapse video recorder. Videos were shot for approximately 24 h duration at 1/80th normal speed. This was done to discover, how long it took a cell transiting from one type of substratum to another or vice versa to change morphology after making the transit. Films were analysed visually for the time for cells at the point of crossing from one topography to another, till transition and morphology change were complete or morphology change had not taken place in a 12 h period.

3. RESULTS AND DISCUSSION

Comparison of the substratum nanopattern and the SEMs of thin parts of cells showed a good correspondence between the patterns in both cell and substratum
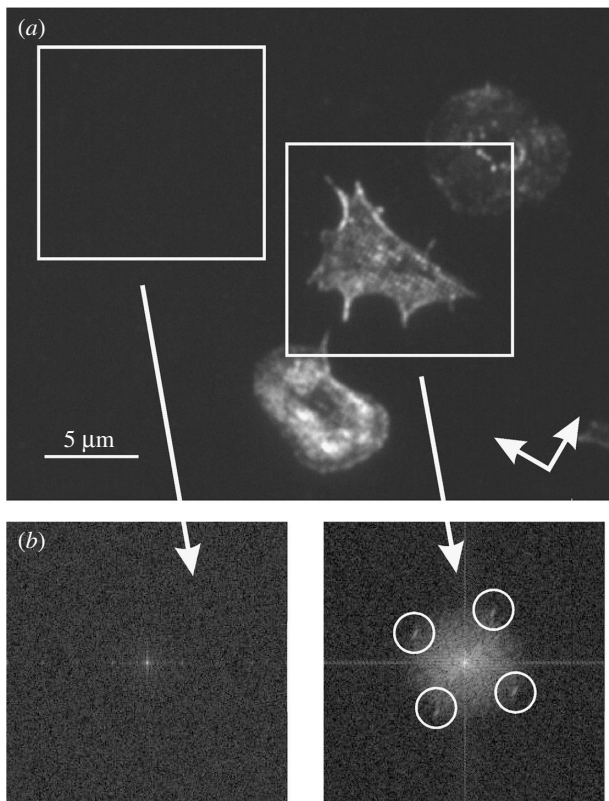
Figure 7. (*a*) Actin labelled platelets on a nanopitted surface. The surface has a square array of 120 nm diameter pits on a 300 nm pitch with the orientation indicated by the arrows. (*b*) Corresponding FFT of the left-hand side image. The condensation of the actin cytoskeleton reacts to the underlying nanotopography. The position of the four peaks (white circles) corresponds to the 300 nm periodicity of the underlying nanotopography. The corresponding FFT of the flat surface image shows no characteristic features related to the underlying nanotopography.

figures 3 and 4. Both these images show parts of the substratum.

Similar evidence for printing can be seen in an AFM image of the surface of a blood platelet, figure 5.

TEM examination of sections of cells growing on nanotopography shows that there is a very close apposition of the cells to the substratum though the possibility of artefacts of preparation cannot be discounted, see figure 6.

Actin organization related to the underlying topography is shown in figures 7 and 8 and a fast Fourier transform (FFT) of one of these images is also shown. It shows a periodicity of actin dots corresponding to the spacing of the nanofeatures at 300 nm. Figure 7 was acquired using an high NA, high-magnification system so that it is possible to see a distinct organization of the actin cytoskeleton corresponding to the underlying substratum. The FFT image (figure 7*b*) was obtained from image data in all three cells shown in figure 7*a*.

3.1. Involvement of integrins

The proportion of cells adhering to nanotopography and to flat polycarbonate in the presence and absence of antibody is shown in table 1. Statistical analysis of the results (see table 1) shows that both topography and
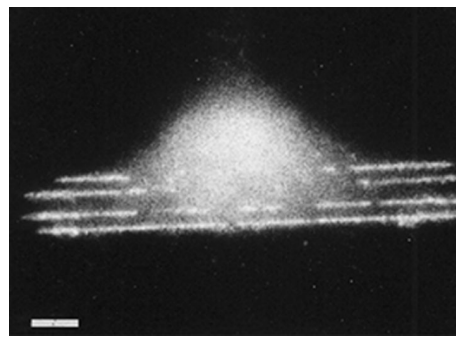

Figure 8. Previously published image (from Wojciak-Stothard *et al*. 1996) of a macrophage growing on 100 nm deep 5 μm wide grooves fabricated in silica. Fluorescent rhodamine–phalloidin stained. Note lines of actin corresponding to the grooves. Scale bar 5 μm.

the presence of antibody depress cell adhesion significantly ($p < 0.01$ in all comparisons in table 1.

If there were 100% cell attachment the mean count would be 71 cells per area. All *t*-test results shown significant at $p < 1\%$.

The term nanoprinting implies that there will be a degree of persistence in the printed surface. Obviously, this persistence is unlikely to be maintained when the cell is separated from any surface by enzymic or other dissociation. But we would expect that the actin cytoskeleton might take some time to reorganize itself when a cell moves from one substratum to another. Persistence in direction of locomotion (Dunn 1983) can be explained as a persistence of cytoskeletal organization in one mode that takes time to be replaced by another. So, we examined, what happens as a cell moves over a nanoprinted surface or from a nanofeatured surface to a flat one or vice versa.

Results are presented in table 2 for two different types of substrata. The 'correct' morphology on the flat substratum is the fibroblast-like flattened and spread morphology. On the nanotopography, there are three possible morphologies, non-adherent, rounded-up but adherent and the extended hour-glass-type cells, see Gallagher *et al*. (2003) who first observed this. We measured the times for cells to adopt a morphology appropriate to their substratum after moving to that substratum type. Persistences up to a mean of 2 h were observed.

The transition in morphology, on the low adhesion substratum is likely to be more rapid than the reverse transition to the flattened morphology on the flat substratum, since, usually the cell will round-up while the reverse transition requires the cell to actively spread on the substratum. This is indeed observed. A further prediction is that cells will tend to move from the nanotopographic surface to the flat one since, they are more adhesive on the latter.

3.2. Discussion

Previous results published by Dalby *et al*. (2002*a,b*, 2004*a–c*) and show that several cell types react to growth on nanotopographical surfaces by changes in gene expression, motility, cytoskeletal organization, tyrosine kinase activity and other features in manner

Table 1. Mean counts (10 repetitions) for a counting area 1/696 of total dish area.

antibody added (μg)	mean number of cells on nanotopography	mean number of cells on flat surface	comparison nanotopog/ flat *t*-test value	comparison Ab added/no Ab added *t*-test values
0	10.5 ($n=14$)	31.8 ($n=10$)	7.9	NA
33	4.4 ($n=10$)	14.4 ($n=6$)	9.1	4.0/4.3[a]
66	4.7 ($n=10$)	12.2 ($n=10$)	4.1	3.9/6.1[b]

[a] Comparison for nanotopographic surfaces.
[b] Comparison for flat surfaces.

suggesting that there is some type of transduction of the signal that the surface has topographic features. The type of reaction is related both to the shape, spacing and actual dimensions of the features (Curtis *et al.* 2001, 2004; Dalby 2004*a–c*, Dalby *et al.* 2005) so that it is clear that several different types of signal can be discriminated. Little has been reported on the actual mechanism(s) involved.

The observations, where the cells were examined by SEM or AFM (figures 3–5) do not provide evidence that the internal organelles and mechanisms of the cells were changed on nanotopography, but they do show that the cells can take the imprint of the substratum. This implies that the cells have the surface mechanical properties appropriate to accept the imprint. This is conceptually, exactly equivalent to the printing or embossing of a surface.

The observations on the actin and vinculin distributions in the cell (figures 7 and 8) show that there is a internal response of the cell to the topography organized on the print master.

This, then leads onto to further speculation about the speed with which, this reaction takes place and its persistence, if the cell moves to a different substratum. Early work suggests that the plasmalemma may have rheopectic properties, namely that it hardens on being subject to shear (Curtis 1961). Obviously, the type of test for persistence we present here, suggests that it is possible that one of the effects of exposure to a given surface structure persists but it does not directly demonstrate persistence of the nanoprinting itself. We hope to submit further evidence of this for publication in a later paper.

If the plasmalemma is imprinted, this would provide a very simple explanation of the effects of ordered nanotopography on cytoskeletal organization since, the inwards projecting features would provide an ordered array of anchor points for the actin cytoskeleton.

At certain relationships of the aspect ratio, of the inwards facing prints of substratum pillars, actin polymers would be limited in length, by falling in the 'shadow' of an inward facing pillar. One possibility that must be considered is that, all the cells have done is to endocytose the particles. Cursory examination of the images we present, might suggest that the cells have endocytosed the nanocolumns from the surface but Dalby *et al.* (2004*a–c*) show that cells cannot exert large enough forces to detach them.

The data we present, shows that growing cells (of several different types) on surface bearing nanotopography leads to an imprint of the topography not only

Table 2. Time (in min) to respond to new type of substratum.

transition	topographic pattern	number of observations	time (min)	
			mean	(s.d.)
flat to topography	orthogonal	6	108	75
flat to topography	hexagonal	3	44.4	23
topography to flat	orthogonal	6	129	97
topography to flat	hexagonal	10	44.4	21

on the surface of the cell but also one which is sufficiently penetrative to be seen from the other side of the cell, when the cell is relatively thin and which can be detected in interior cell components, such as the cytoskeleton and perhaps, even on the nucleus (figure 6). These novel results suggest that this nanoprinting may be an important mechanism in cell interaction and signalling. It raises the possibility that this is also an explanation of the results of raised adhesion on discontinuities and diminished adhesion on orthogonal arrays of pits (Curtis *et al.* 2001, 2004).

While the experiment on inhibition of cell attachment with an anti-integrin alpha 1 antibody shows that integrin-mediated adhesion can be involved in the adhesion of these cells to the polycarbonate, an experiment similar to that of Arnold *et al.* (2004), comparison with the effects of nanotopography table 1, shows that there is a additive effect of the topography which reduces adhesion to a still lower value.

The general relevance of our findings is that we have demonstrated the existence of an unsuspected mechanism by which a nanotopographic signal can be imparted to a cell. This mechanism might, well be part of the mechanotransduction system seen in several cells, especially the chondrocyte (Knight *et al.* 2002)

Though, we have obtained our evidence from the reactions of cells to nanotopography it should be borne in mind that intercellular material such as collagen fibres, biominerals and many implanted materials present nanotopography to the cells around them.

ADDENDUM

We do not think that it is possible to resolve the periodic pattern of the substrate optically. However, by using fluorescence microscopy it is possible to detect features much smaller than the diffraction limit ($d_0 = \Lambda/2$ NA), in fact, single molecules as well as their spacing. In our case, we can also estimate the diffraction limit and thus demonstrate that we have the required power to resolve the periodic actin condensation. The image was acquired with a $100\times$ oil immersion objective (NA$=1.4$) and the actin was Rhodamine labelled ($\Lambda_{em} > 590$ nm) which yields 210 nm. The pixel resolution of the images is approximately 60 nm pixel^{-1}. It is also well known that it is possible to determine the position of an object with sub-pixel resolution by finding its centre of mass. During the FFT transformation, the latter is amplified and is thus very sensitive to the periodicity of the observed actin condensation.

The figure below is a clear demonstration of the ability to resolve the 300 nm periodic actin condensation and that it, in fact, originates from the actin cytoskeleton. An FFT transformation of the cell gives a clear twofold symmetry with peaks at 300 nm red circles. However, there is no symmetry in the FFT image of the background. Thus indicating no specific order is resolved. That includes the periodic pattern of the substrate.

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