Local Development of Stress Fibers in Endothelial Cells exposed to Nonuniform Strain of Substrate under Uniaxial Cyclic Stretching

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Abstract

We have developed a novel uniaxial cyclic stretching technique to apply a ventral nonuniform strain to cells. In this system cells are grown on a glass-embedded silicone substrate instead of the commonly used uniform substrates. This unique substrate has been developed to give a strain gradient of 0.2%/μm across each individual cell. Bovine aortic endothelial cells (BAECs) were cyclically stretched up to a maximum strain of 50% at 0.5 Hz for 30 min or 3 hours, focusing on the effect of the ventral strain gradient on local cell remodeling. After the experiments, BAECs were fixed and stained with rhodamine-phalloidin to observe actin filament structure. BAECs showed local development of stress fibers and localization of cell nuclei at regions exposed to higher strain. This result suggests that BAECs may sense ventral nonuniform strain and remodel cytoskeletal structure accordingly followed by the movement of cell nuclei.

1. Introduction

Endothelial cells (ECs) are subjected to mechanical stimuli *in vivo*, including fluid shear stress, cyclic stretching and so forth. It has widely been accepted that when subjected to uniaxial cyclic stretching, most cells align away from the direction of stretching. This has been demonstrated for endothelial cells [1-5], for smooth muscle cells [6-10], and for monocytes/macrophages [11]. So far, many experimental and numerical studies have attempted to understand the underlying mechanism of how cells change their shape and cytoskeletal structures in response to cyclic stretching. Wang *et al.* [12] developed microgrooved silicone surfaces to grow tendon fibroblasts and subjected them to cyclic stretching at 8% for 48 h. They showed that α-SMA protein expression levels of fibroblasts depend on cell orientation with respect to both the direction and the duration of stretching. Naruse *et al.* [13] have suggested that c-src activation in response to cyclic stretching of endothelial cells may be induced via SA channel activation and play an important role in the subsequent morphological changes. In contrast to these experimental findings, Yamada *et al.* [14] studied stress fiber orientation under cyclic stretching and validated a hypothesis that stress fibers align to minimize a mechanical stimulus in the fiber direction. Wang [15] has also used numerical methods to study the relationship between actin filament reorganization and substrate deformation, developing a mathematical model to consider ventral strain energy. The model predicted that actin filaments are formed in the direction where their ventral strain energies are minimally altered, which was later confirmed by experiments.

In our previous studies [16-19], exposure of bovine aortic endothelial cells to fluid shear stress resulted in elongation and orientation parallel to the direction of flow. In addition, development of stress fibers was observed more at the upstream regions of the cells. Furthermore, finite element analysis showed that intracellular stress was higher at the upstream regions. These results suggest a close correlation between local development of stress fibers and the intracellular stress state. In contrast, most studies of the cellular response to uniaxial cyclic stretching have used uniform substrates producing uniform strain through cells. Therefore, for better understanding of the cell remodeling response to uniaxial cyclic stretching, it is necessary to develop an experimental system with which nonuniform strain can be applied to each cell.

In this study, we have developed a novel experimental system, in which cells are grown on a glass-embedded silicone substrate instead of the commonly used uniform substrates [20]. This unique substrate can expose cells to a ventral nonuniform strain. Bovine aortic endothelial cells (BAECs) were exposed to uniaxial cyclic stretching on the substrate to evaluate the relationship between the ventral nonuniform strain and cell remodeling. In a separate study, the ventral nonuniform strain was quantitatively determined by finite element analysis.

2. Methods

2.1. Cell culture

BAECs were cultured in tissue culture flasks with Dulbecco's modified Eagle medium (DMEM, Gibco,

MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, KS, USA), penicillin and streptomycin (Gibco). Cultures were grown in a 37ºC humidified atmosphere of 95% air and 5% CO_2 gas. BAECs were confluent after 4-5 days and then passaged at a 1:4 split ratio using trypsin-EDTA. Fully confluent cell populations from the 4th to 10th generation were studied.

2.2. Fabrication of glass-embedded silicone substrate

A glass-embedded silicone substrate was fabricated to apply cells a ventral nonuniform strain to cells. Briefly, a silicone adulterant and a catalyst were mixed at 10:1 (v/v). A circular cover glass (70 μm in thickness, 15 mm in diameter) was then immersed in the mixture. The glass-embedded silicone substrate was baked at 80ºC for 3 h and attached to the bottom of a silicone chamber, as shown in Fig. 1. The chamber was sterilized and the surface of the substrate was coated with fibronectin. Prior to experiments, BAECs were seeded on the substrate and incubated until reaching confluence.

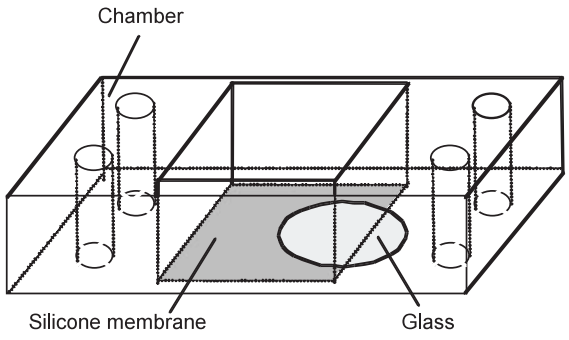


Fig. 1. Silicone chamber with a glass-embedded silicone substrate. [20]

2.3. Uniaxial cyclic stretching

For uniaxial cyclic stretching, the above chamber was mounted on a commercially available cyclic stretcher consisting of a stepping motor and a controller (Scholartec, Osaka, Japan), as shown in Fig. 2. The glass-embedded silicone substrate was cyclically stretched up to a maximum strain of 50% (strain on the non-glass region of the substrate) at a frequency of 0.5 Hz for 30 min and 3 h. For control experiments, commonly used uniform substrates were also used to apply a uniform strain of 20% (grip to grip strain) at 0.5 Hz for 3 h.

2.4. Fluorescence staining

After experiments, fluorescence staining of F-actin filaments was performed as follows. Briefly, BAECs were fixed with 10 % formaldehyde for 5 min and were then stained with 150 nM concentration of rhodamine-phalloidin for 20 min. Fluorescence images were observed using an inverted microscope (IX-70 、 Olympus, Tokyo, Japan) equipped with a confocal laser scanning microscope (CSU10, Yokogawa, Tokyo, Japan) and then transferred to a personal computer through a digital CCD camera (ORKA-ER, Hamamatsu Photonics, Hamamatsu, Japan) controlled by a frame glabber in AQUACOSMOS (U7501, Hamamatsu Photonics, Hamamatsu, Japan).

2.5. Morphological analysis

Morphological analysis was performed on BAECs to evaluate parameters including the angle of cell orientation and the shape index [21]. The cell outline was manually extracted by tracing outsides of peripheral thick actin filaments in fluorescent images in the public domain NIH Image program version 1.62

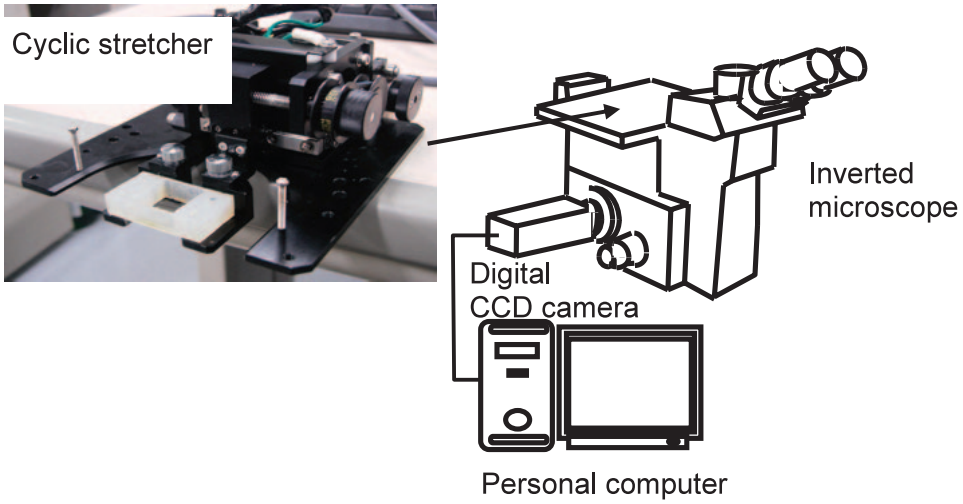


Fig. 2. Experimental setup consisting of a uniaxial cyclic stretcher, inverted microscope and imaging system. [20]

(National Institute of Health, USA). This produced cell area and cell perimeter. In addition, an equivalent ellipse for the cell outline, with an equal area and moment of inertia to the corresponding cell shape was also determined. The angle of cell orientation is defined as the deviation of the major axis of the equivalent ellipse from the direction of flow as shown in Fig. 3. For control cells, the horizontal direction from left to right in the images was defined as 0°. The shape index is defined as follows:

$$\text{Shape index} = 4\pi A / P^2 \qquad (1)$$

where A is the cell area, and P the cell perimeter. The shape index is defined as 1.0 for a circle and approaches zero for highly elongated shape.

Statistical comparisons were made using the unpaired Student's t-test and unpaired Welch's t-test for equal variance and unequal variance, respectively. A value of $p < 0.05$ was considered significant in all analyses. Statistical data were shown in terms of the mean±standard deviation (mean±SD).

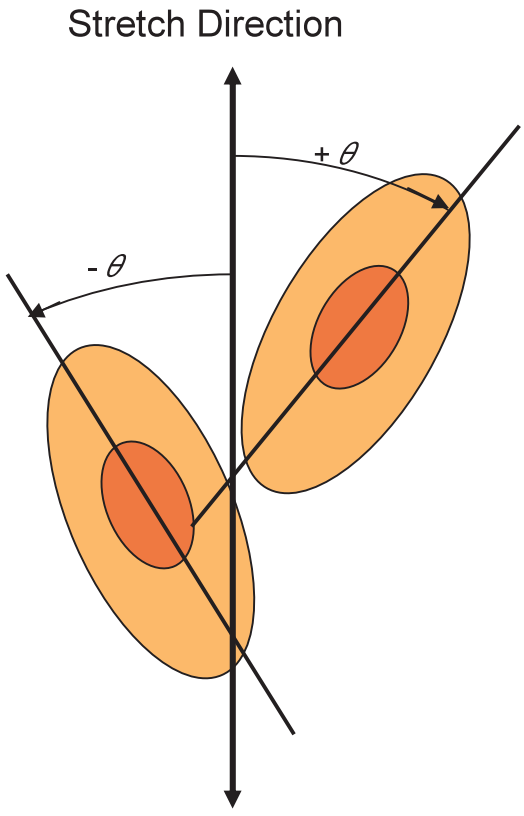


Fig. 3. Definition of angle of cell orientation. [22]

2.6. Measurement of nonuniform strain and FE analysis

In a separate study, 0.2 μm-diameter fluorescent beads were sprayed on the glass-embedded silicone substrate as a marker to quantitatively determine nonuniform strain. Fluorescence images including the border between silicone and glass-embedded regions were obtained before and after stretching, as shown in red and green in Fig. 4(a) and (b), respectively. A superimposed image in Fig. 4(c) shows excellent co-localization of the fluorescent beads before and after stretching with the exception of the region close to the glass-silicone border, indicating no displacement of the most fluorescent beads on the glass-embedded silicone substrate. In contrast, the fluorescent beads on silicone substrate were found to move in the direction of stretching, away from the border region.

To quantify the spatial distribution of strain, finite element analysis was performed using ANSYS 6.1 commercial code (SAS IP, PA, USA) to identify the position of the beads before and after stretching. The finite element model was generated from the rectangular region in Fig. 4(c) on the basis of the position of the fluorescent beads, as shown in Fig. 5(a). The finite element domain was descritized by the two-dimensional 4-node solid element (135 elements). The Young's modulus was set to be 100 kPa and the Poisson's ratio 0.49. The analytical result showed that the substrate strain was increased monotonically with the distance from the border and reached 50% strain at 300 μm from the border, as shown in Fig. 5(b). The strain gradient was 0.2%/μm, which was considered to be sufficient to expose a single cell to local strain differences.

Finite element analysis for the glass-embedded silicone substrate used in the experiment was done for a model shown in Fig. 6. As a result, strain distribution along the direction of stretch was obtained as shown in Fig. 7. From this, it is clear that sharp strain gradient generates around the interface between glass and silicone membrane.

3. Results

Fluorescence images of actin filaments and the angle of cell orientation of BAECs before and after stretching at 20% at 0.5 Hz for 3 h on uniform substrates are shown in Fig. 8. Before stretching, BAECs have thick actin bundles, called dense peripheral bands, at cell peripheries with a relatively rounded shape. The angle of cell orientation for unstretched cells varies widely distributed from -90° to 90°. In contrast, after exposure to stretching, BAECs show marked elongation and orientation almost perpendicular to the direction of stretching. Thick stress fibers are formed in the central portion of the cells and aligned with the cell major axis. The angle of cell orientation was equally divided between angles approaching -90° or 90°. Furthermore, the shape index significantly decreased from control (0.82±0.08) to stretched BAECs (0.72±0.12, $p < 0.01$).

Figure 9 shows fluorescence images of actin filaments in BAECs in the border region of nonuniform substrates after stretching at 50% for 3 h. On the glass-embedded region of the silicone substrate, BAECs showed neither elongation nor orientation in response to tensile strain.

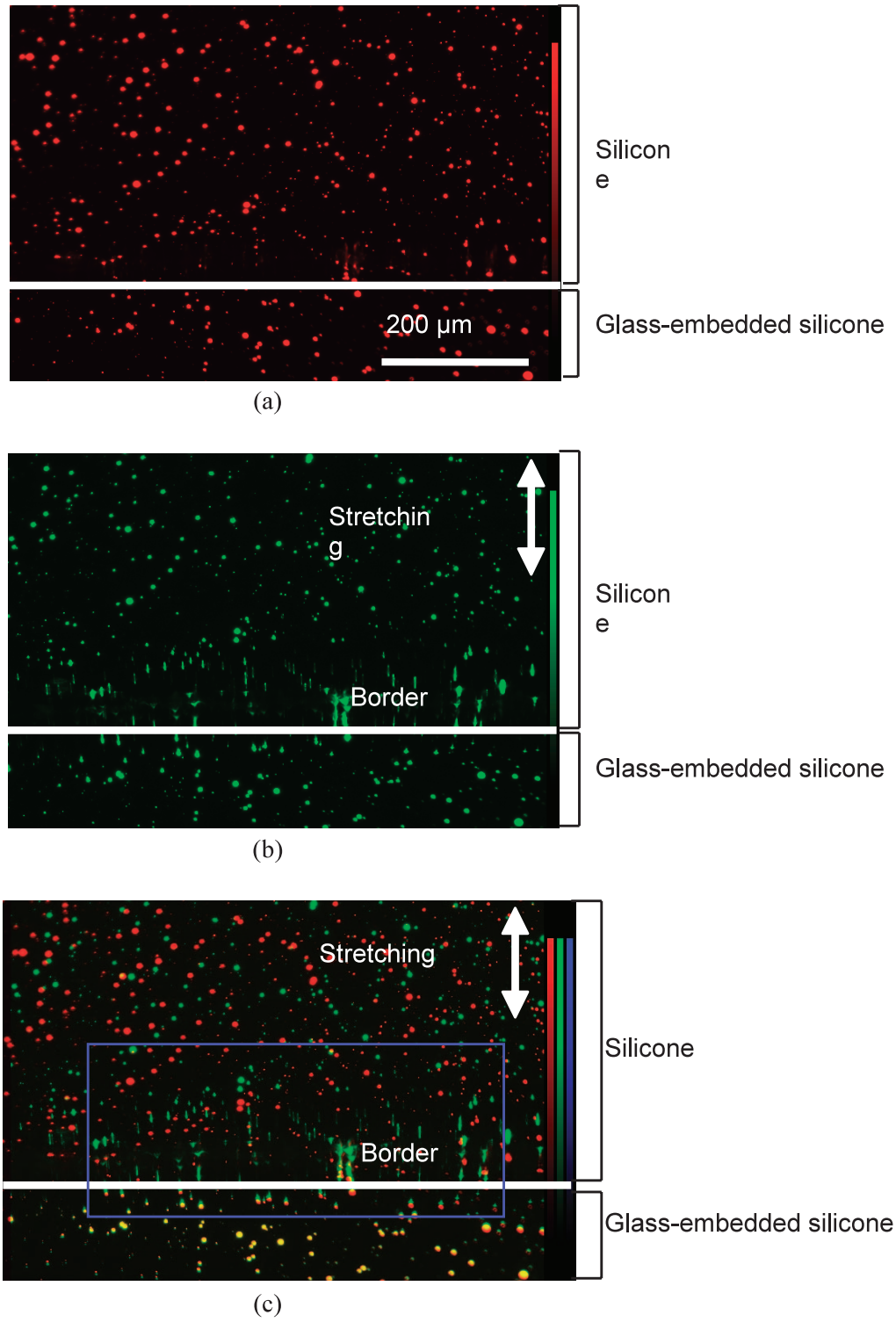


Fig. 4. Fluorescence images of 0.2 μm diameter fluorescent beads sprayed on the silicone substrate. Images obtained (a) before and (b) after stretching are superimposed in (c). [20]

In contrast, on the pure silicone substrate (non-glass region), BAECs aligned perpendicular to the direction of stretching. Fluorescence images of actin filaments and the angle of cell orientation of BAECs from this region are shown in Fig. 10 for 30 min and 3 h stretching at 50% strain, 0.5 Hz. Note that these fluorescence images were obtained on the pure silicone substrate far from the border (50% strain region). After 30 min (Fig. 10(a)), some BAECs start o reorient, as suggested by the distribution of the angle of cell orientation. After 3 h (Fig. 10(b)), most BAECs had finished their elongation and orientation with concomitant development of stress fibers, in a manner similar to that recorded for uniform substrates. In particular, the angle of cell orientation was

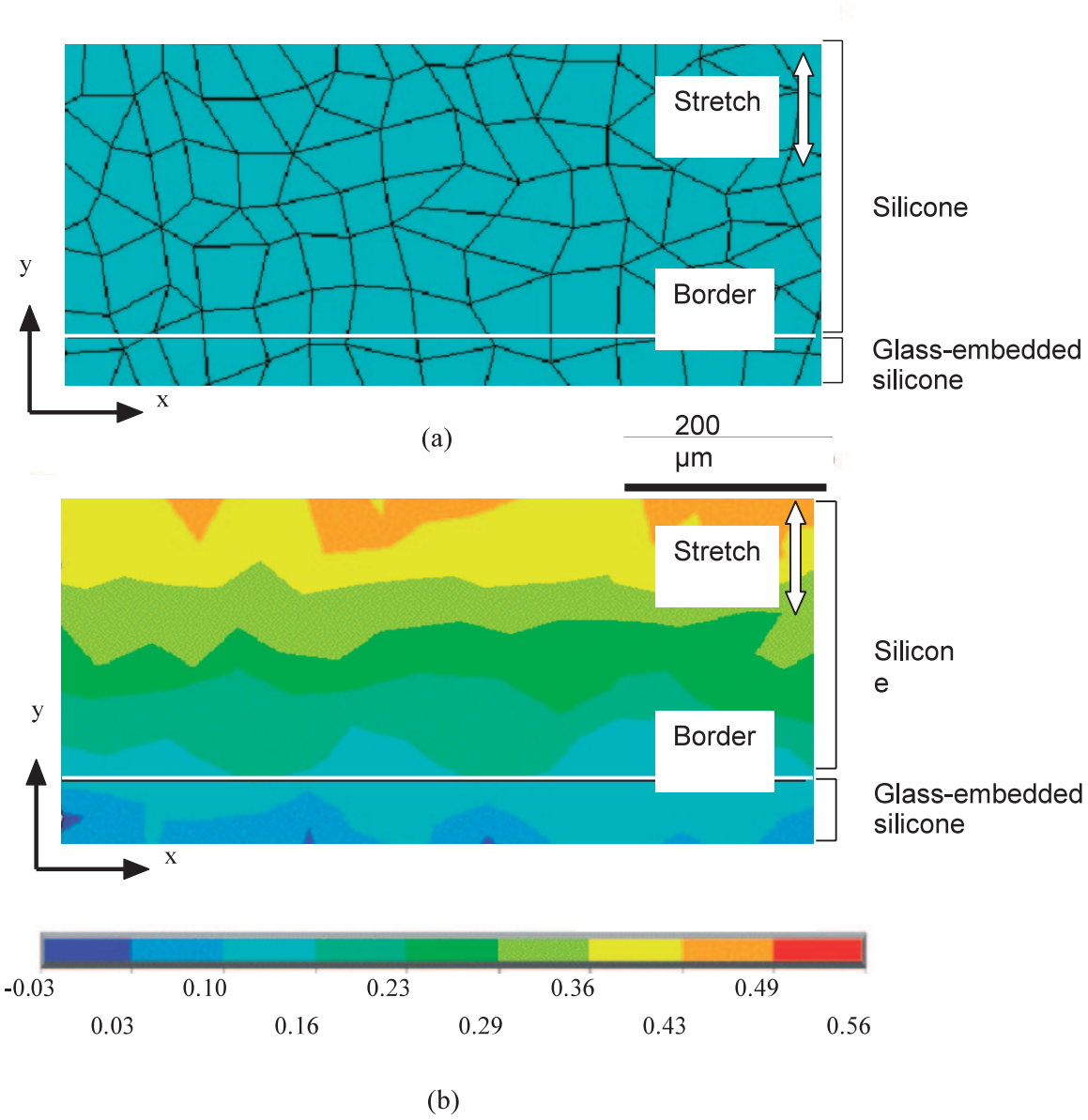


Fig. 5. Finite element analysis to quantify nonuniform strain on the silicone substrate. (a) finite element model and (b) calculated nonuniform strain distributions. [20]

largely around ±90°. In addition, the shape index significantly decreased from 30 min (0.73±0.12, p <0.01) to 3 h (0.56±0.10, p < 0.05).

Next, we extensively observed fluorescence images of BAECs in the nonuniform strain region close to the border (0 - 300 μm from the border) after 30 min or 3 h stretching. Prominent features of the remodeling are shown in Fig. 11. Interestingly, stress fibers were locally developed perpendicular to the direction of stretching, particularly near the cell edge where higher strain was observed, as shown in Fig. 11(a) and (c). Well developed stress fibers in the perpendicular to the direction of stretching in a whole area of cell are also observed in Fig. 11(b) and (d). In addition, cell nuclei were found to exist not in the cell center but near the cell edge (Fig. 11(b), (c) and (d)). These results indicate that BAECs may sense ventral nonuniform strain and remodel actin filament distributions according to the gradient of strain followed by the movement of cell nuclei.

4. Discussion

For uniform substrates, BAECs show marked elongation and orientation together with development of stress fibers perpendicular to the direction of stretching in response to 20% stretching. In this study, BAECs were found to nearly complete this reorientation within 3 h. This result is in excellent agreement with previous studies. For example, Wang *et al.* [4] have shown that under cyclic stretching, human aortic endothelial cells

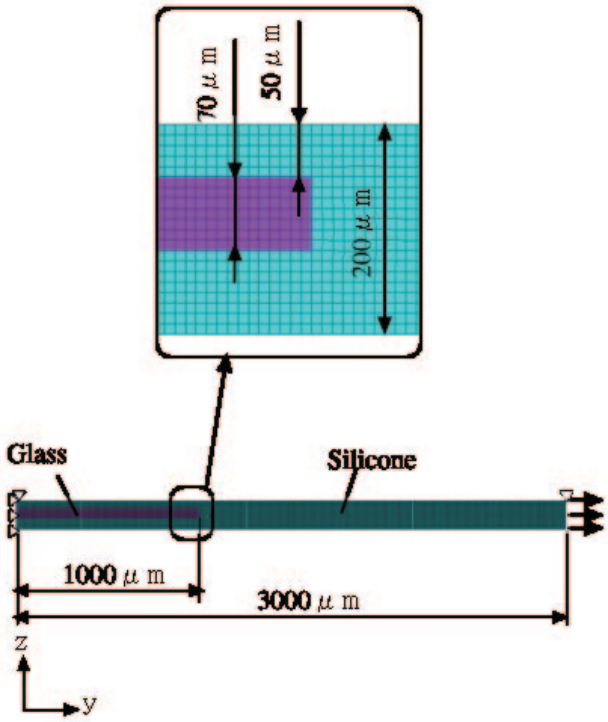


Fig. 6. Finite element model for glass-embedded silicone substrate used in the experiment. [22]

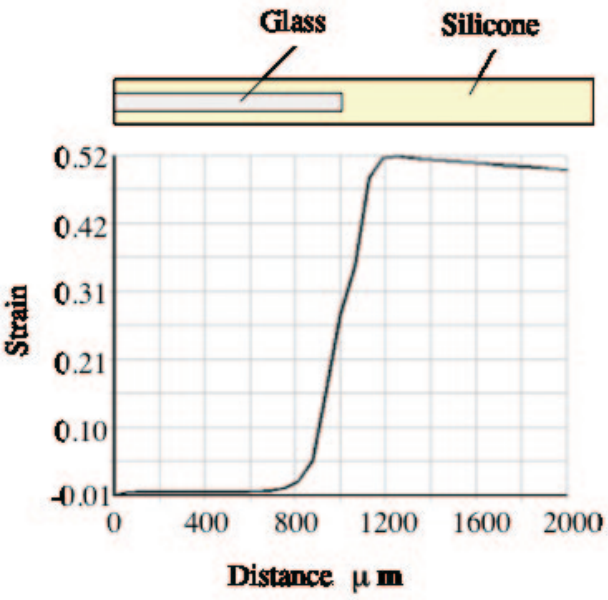


Fig. 7. Strain distribution around interface between glass and silicone membrane. [22]

reorient specifically to the direction with minimal substrate deformation and that the rate and extent of cell reorientation depend predominantly on the magnitude of stretching. Moreover, stress fibers developed in the direction of minimal substrate deformation. Similarly, Takemasa *et al.* [3] showed that stress fibers in endothelial cells align oblique to the stretching direction when subjected to an amplitude of 50% for 30 min, and that the definitive angle of orientation decreases, with respect to the stretching direction, with an increase in stretching amplitude. These studies indicate that stress fibers sense the magnitude of stretching, which is consistent with our results. These results strongly suggest that cell remodeling is undertaken, with the aim of reducing intracellular mechanical stress. However, the detailed underlying mechanism of this cell remodeling is still unclear, therefore we have decided to focus on a single cell under cyclic stretching.

When plated on nonuniform substrates, we found that BAECs had nearly completed their reorientation after 3 h stretching at 50%, but not at 30 min. It has been reported that formation of stress fibers at a particular angle is observed only at 20 min in response to 50% stretching [3]. Taken together, it is speculated that the actin cytoskeleton is a prerequisite for cellular reorientation, as cells lacking this do not orient. We also observed fluorescence images of BAECs in the nonuniform strain border region after 30 min or 3 h of stretching. We found that stress fibers were locally developed perpendicular to the direction of stretching, particularly in the higher strain region. Similarly, Takemasa *et al.* [3] reported that the formation rate of stress fibers significantly depends on the strain magnitude. This study, therefore, indicates that the strain-dependent formation of stress fibers may exist at the single cell level. In addition, some nuclei were localized near the cell edge. The existence of direct linkages between actin filaments and the nucleus has previously been demonstrated by Maniotis *et al.* using micromanipulation methods to pull directly on integrins [23]. In addition, Takayama *et al.* [24] reported that disruption of actin filaments in fibroblasts might induce movement of cell nuclei. Taken together, these suggest that the process of BAECs remodeling in response to nonuniform strain may involve local development of stress fibers according to ventral nonuniform strain, followed by movement of cell nuclei induced by reorganization of stress fibers.

In vivo, vascular endothelial cells are subjected to not only cyclic circumferential strain but also wall shear stress. In the light of this, Zhao *et al.* [25] have exposed aortic endothelial cells simultaneously to circumferential stretch and wall shear stress. They found that those two mechanical loadings synergistically induced morphological changes in endothelial cells, particularly highlighting the role of circumferential strain in modulation of sensitivity of cells towards shear stress. Therefore, it is necessary to study this synergistic effect for better understandings of cell remodeling.

In summary, we observed BAECs remodeling after uniaxial cyclic stretching using nonuniform substrates and showed that BAECs may sense ventral nonuniform strain fields and locally remodel their shape and microstructure according to the strain gradient. The mechanical connections between stress fibers and nuclei may play an important role in BAECs remodeling.

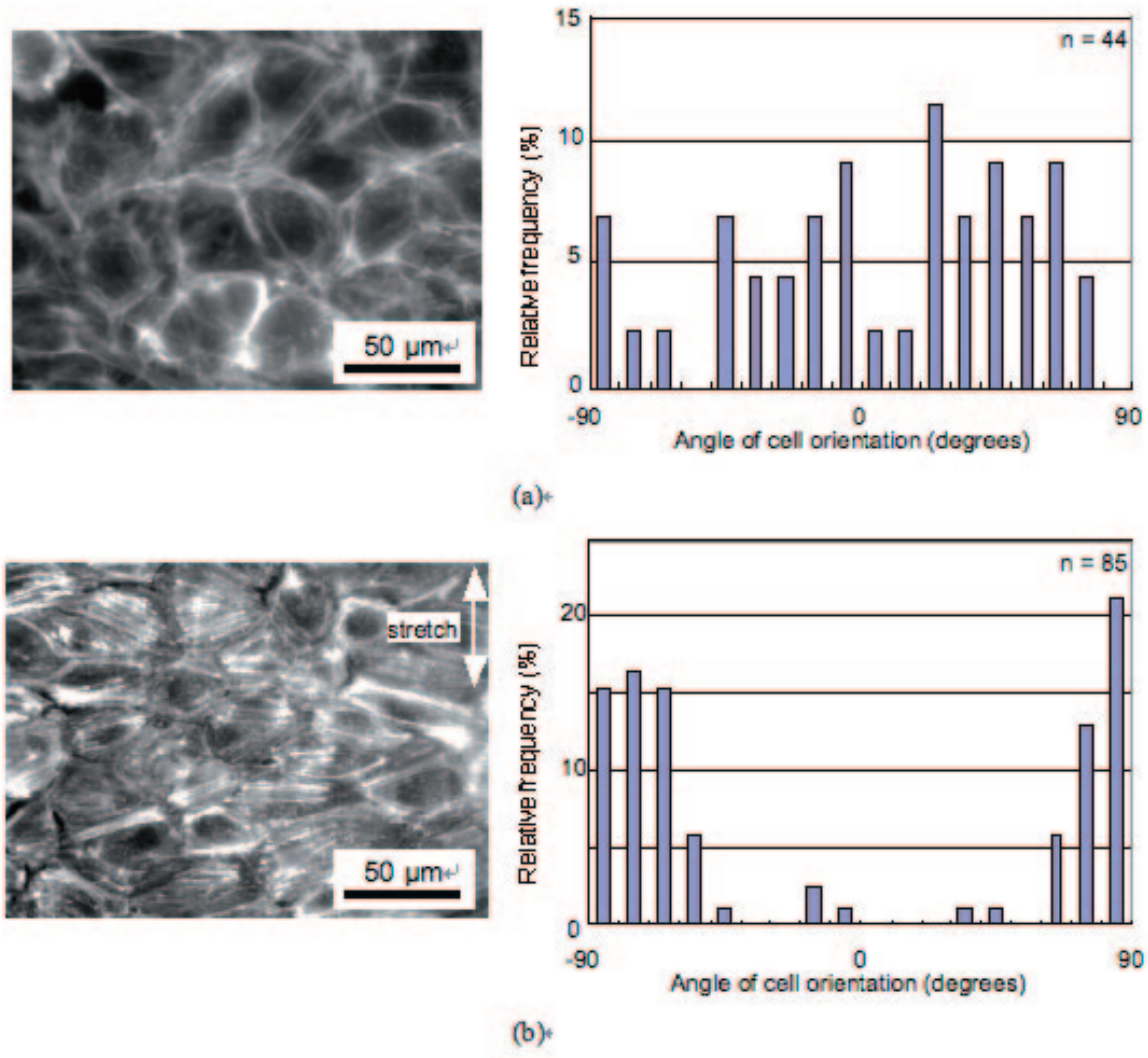


Fig. 8. The angle of cell orientation and fluorescence images of rhodamine-labelled actin filaments for uniform substrates (a) before and (b) after stretching at 20% at 0.5 Hz for 3h. [20]

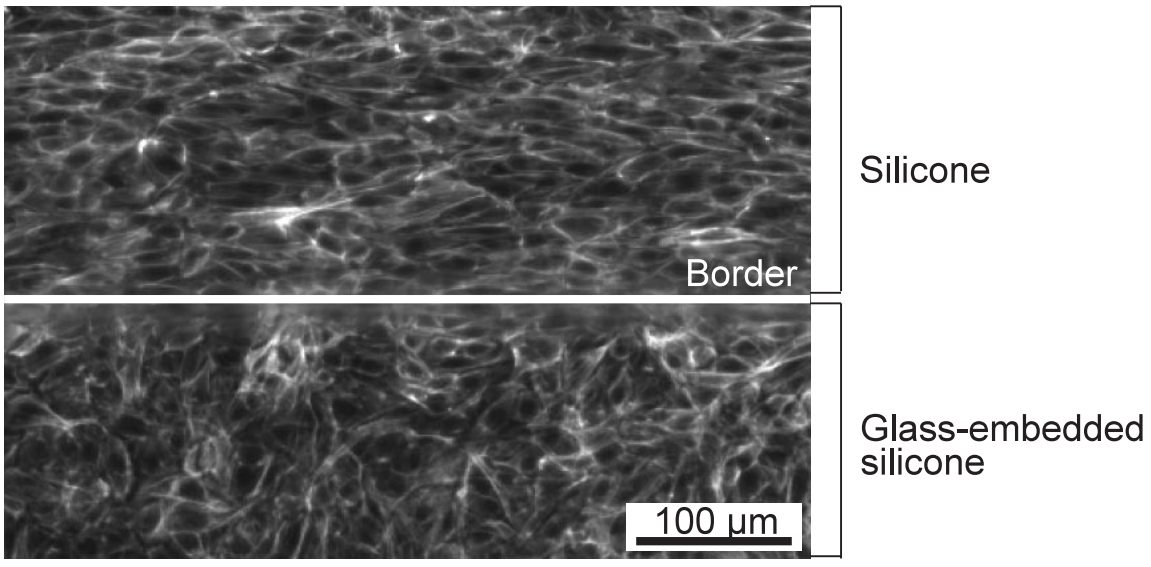
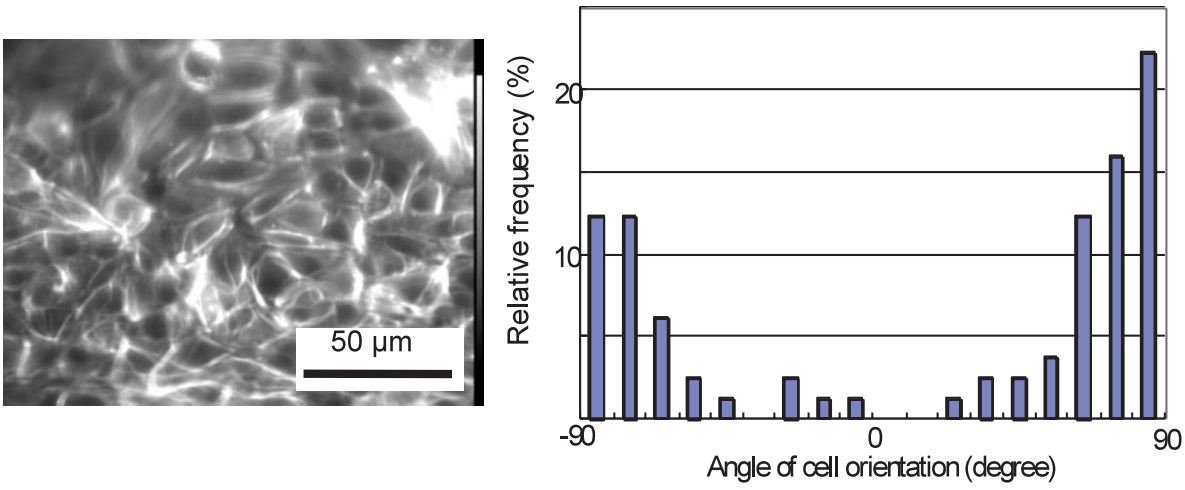
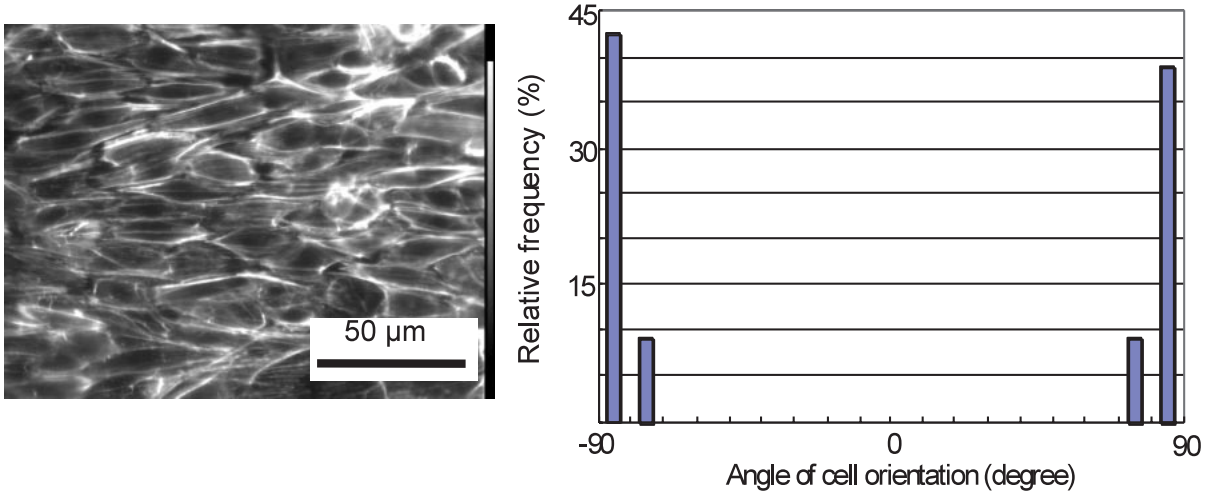


Fig. 9. Fluorescence images of rhodamine-labelled actin filaments of BAECs after stretching at 50% strain, 0.5 Hz for 3 h in the region close to the border for nonuniform substrates. [20]



(a)



(b)

Fig. 10. The angle of cell orientation and fluorescence images of rhodamine-labelled actin filaments for nonuniform substrates after (a) 30 min and (b) 3 h stretching at 50% strain, 0.5 Hz. [20]

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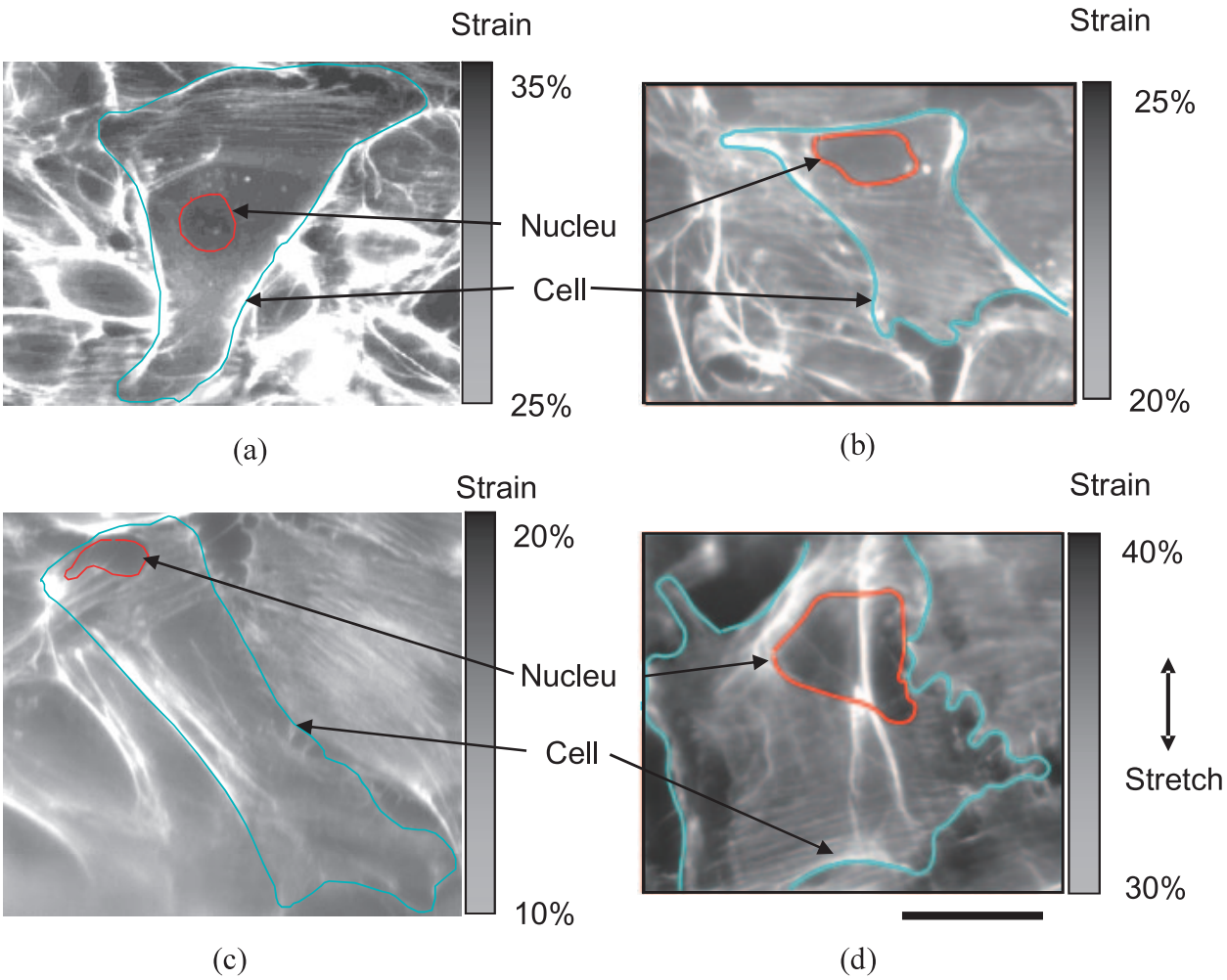

Fig. 11. Prominent features of BAECs remodeling under nonuniform strain. Stretch direction is vertical. (a), (b) after 30 min, and (c), (d) after 3 h. Bar = 25 μm [20, 22]

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