# Differentiation and Monitoring of Cells Using a Biochip for Regenerative Medicine*

## (Differentiation of Bone Marrow Stromal Cells and Myoblasts)

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A novel biochip is developed for culturing stem cells. Biochip is made of Polymer (PDMS), and cells can be loaded by gradient strains in one chip. They grow well on a hydrophilic membrane and differentiation is promoted by cyclic strains. In this paper, we propose the method for culturing and monitoring of stem cells such as bone marrow stromal cells (ST2 cells) and myoblasts (C2C12 cells), and the results of culture. First we analyzed strains on a membrane when an air hole is decompressed, and clarified their range. From experiment, bone marrow stromal cells grew well in a narrow range, and we quantified their ALP activity as a measure of differentiation. As myoblasts, the direction of their differentiation was perpendicular to a groove, that is, the same direction of uniaxial strains.

***Key Words:*** Medical Engineering, Biological Engineering, Micromachine, Muscle and Skeleton, Regenerative Medicine

## 1. Introduction

Regenerative Medicine is one of methods to recover human tissues damaged by such as sickness or injury. The research of Regenerative Medicine has been progressing rapidly, and there are several methods to promote regeneration by giving strains to tissue in vitro. The research of mechanotransduction, which investigates the relationship between mechanical stress and response from cells has been done for a long time. It is well known that bone cells and muscle cells respond to stress and for example it is known that it is effective for maintenance of bone structure and bone function to give constant and cyclic strains to bone tissue[1]. It is also known that dynamic stress is more effective for tissue than static stress[2]. The fact that the bone volume of an astronaut reduces during his stay in space and it recovers after his coming back to earth proves that the gravity of earth is important for the maintenance of bone tissue. In cellular level it is also known that mechanical stimulation to precursor cells of osteoblasts promotes the differentiation of them, but its mechanism is still unknown during the process of research so far. In addition, it is noted that the best condition of stress for cell differentiation depends on each person, so a tool for searching the best condition for differentiation is needed. Various apparatuses have been developed for the purpose of investigating the effect by stretching cells like osteoblasts, endothelial cells[3]-[5], smooth muscle cells[6],[7], fibroblasts[8],[9], fetal rat lung cells[10],[11], and many other cells[12]-[15], but the apparatus whose main purpose is searching for the best

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condition for cell differentiation has never been developed.

We developed a system in which cells can be given stress and strain in a small biochip, and made it possible to promote cell differentiation with less energy[16]-[18]. We also accomplished 4 different conditions of stress in one-time experiment, and quantified the level of differentiation in each condition by comparing them simultaneously.

In this paper, we propose a biochip for cell differentiation and a principle of cell stretching. We also report the results of experiments and evaluation of promoting cell differentiation using bone marrow stromal cells and myoblasts. In addition, we report the results of stress distribution using Finite Element Method (FEM) analysis.

## 2. Biochip for Cell Stretching

There are 2 kinds of systems that give stress to cells in vitro. One is the system that gives strains by mechanical force and the other is the one that gives strains by fluid force. As a system that gives mechanical strain to cells, Banes et al. developed a vacuum operated cell stretching system "Flexer Cell Strain Unit" in 1985[19]. Naruse et al. also developed a stretch chamber for stretching cells to uniaxial direction[20]. This is operated by using the characteristic of elastic Polydimethylsiloxane (PDMS), and is driven by motors but not by vacuum. Both of them stretches cells on a sheet of membrane, however, because only homogeneous strain can be accomplished in these systems with one-time experiment, it isn't easy to quantify the data after experiments. On the other hand, Wong et al. proposed a system in which cells can be given strains by fluid force[21]. In this case there is little possibility of disturbance from external environment, but the large size and complexity of whole system as well as the one of Owan et al.[22] may prevent us from observing objectives by a microscope easily. In addition, it is impossible to quantify the effect of various strains to cell differentiation at one-time experiment because cells are exposed to homogeneous strain in petri dishes or chambers.

In order to solve these problems, we developed a small biochip in which we can give gradient strains with a single experiment, and made it simple for easy monitoring by a microscope. In this work we used PDMS as a material for the biochip. PDMS is biocompatible material with high accuracy of molding[23], and it has been used as substrates for cell culture and cell stretching by making use of its elasticity[20].

Figure 1 shows the schematic of cell loading. The grooves were fabricated by using convex and concave molds. A silicone tube is connected to the air hole surrounded by a membrane and grooves inside the biochip, and we can bend the membrane on the grooves by decompressing air through the tube as shown in Fig. 2 (b). As shown in Fig. 2 (c), cells on the membrane are given both tensile and compressive strains to uniaxial direction be-

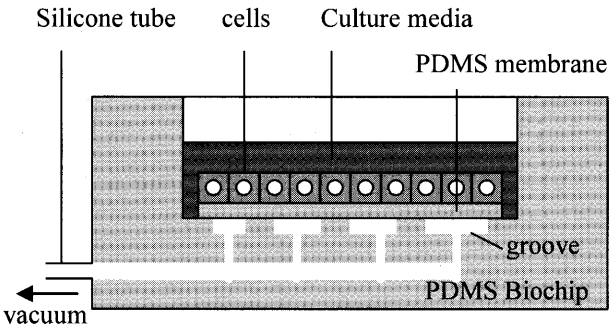



Fig. 1 Mechanism of cell-loading

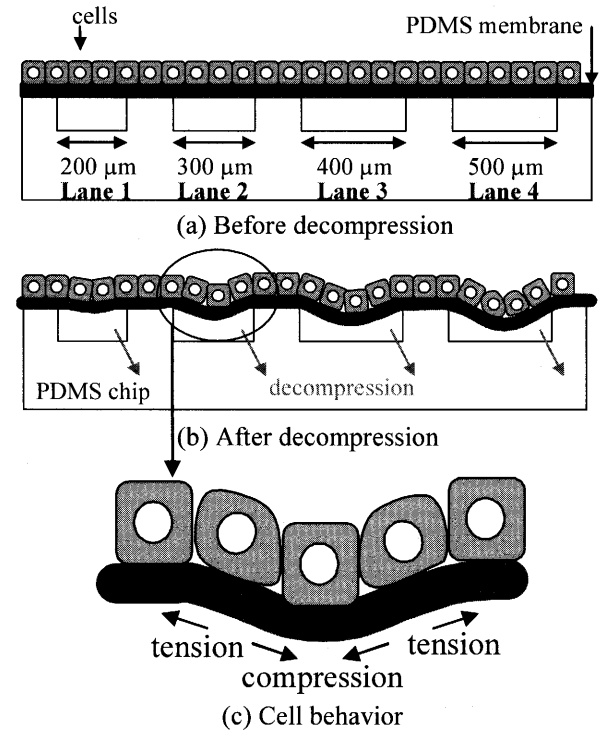
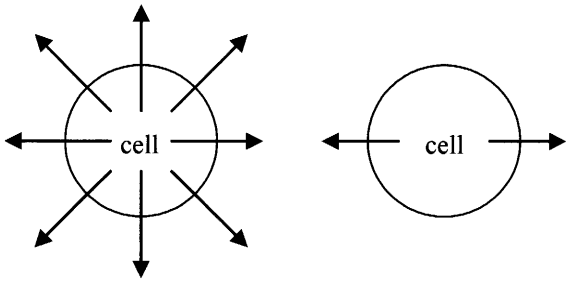


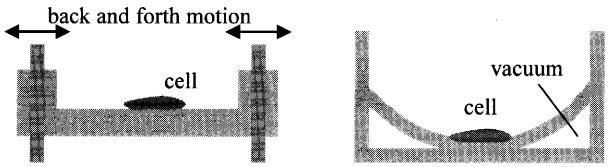

Fig. 2 Schematic of gradient strain

cause of bending. Though the method of using vacuum is the same as the one of Banes et al., 4 grooves with different width produce 4 different bending scale on each groove simultaneously with a single vacuum gage pressure in our case. This is why we can monitor differentiation under 4 different conditions of stress, can compare them simultaneously, and can search for the best condition of specific cell differentiation. We can also observe the effects of minute stress to cells thanks to the micro-scale grooves developed by microfabrication. Though membrane is bended on a circle-shape hole in the case of "Flexer Cell Strain Unit", we bend the membrane on rectangular grooves with high aspect ratio from top view, the form of stress given to a cell in our method differs from it as in Fig. 3. Basso et al. investigated about the way to load cells[24]. The point that we use PDMS as a membrane for stretching cells is the same as the one of Naruse et al., but how cells are given stress in our biochip also differs from




(a) Multiaxial stretch (b) Uniaxial stretch

Fig. 3 2 types of cell deformation




(a) Motor driven stretch (b) Vacuum driven stretch

Fig. 4 2 types of uniaxial stretch

theirs because the deformation of membrane differs as in Fig. 4.

You et al. described that the effects of osteoblasts' differentiation can be defined by single vertical strain[25], so it is important to develop a novel system where cells can be given vertical strain in order to make this point clear[24]. As cell stretching apparatus, our method differs from the motor driven method of Naruse et al. as shown in Fig. 4 and we can compare the effect of vertical strain to cells with Naruse's and Bane's ones. The summary of characteristics in our method is as follows.

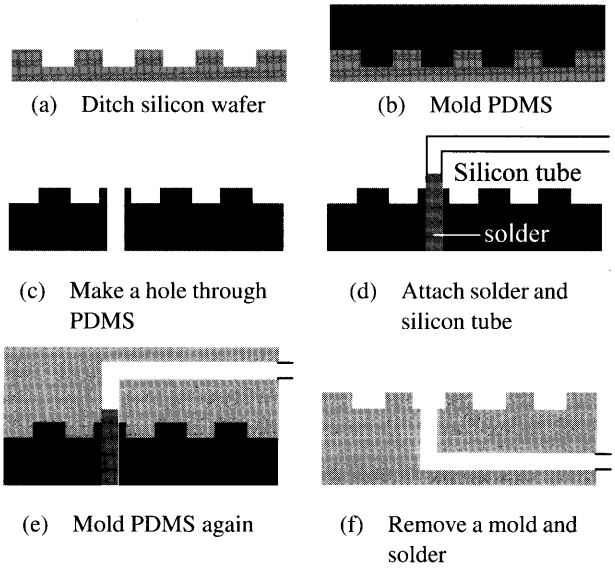
( 1 ) We stretch cells by vacuum operation.

( 2 ) Cells are given gradient strain that is compressive at the center of a groove and is tensile at the edge.

( 3 ) We can compare the activity of cell differentiation by using grooves with different width.

## 3. Design and Fabrication of a Biochip

Based on the schematic of Fig. 1, we fabricated a biochip according to the process of Fig. 5. There are 4 grooves with width of 200 μm, 300 μm, 400 μm, 500 μm and depth of 100 μm in the biochip (in the following sentence, we call these grooves as Lane 1, Lane 2, Lane 3, Lane 4 respectively), and a sheet of oxidized PDMS membrane is stuck on them. The interval between each groove is 1 mm. The space for injecting culture medium is 50 mm wide, 35 mm long and 15 mm high and there is a margin of 15 mm so that we can observe the whole area on grooves by an erecting microscope. We culture cells by filling culture media to the height of 5 mm. First we fabricated grooves on a silicon wafer as Fig. 5 (a) and print the pattern as Fig. 5 (b). After printing we made a hole through the convex mold of PDMS as Fig. 5 (c), then we penetrated solder in the hole and attach a silicone tube.

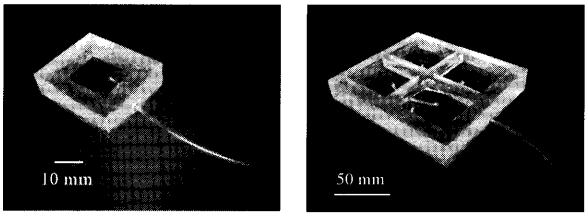



(a) Ditch silicon wafer (b) Mold PDMS

(c) Make a hole through PDMS (d) Attach solder and silicon tube

(e) Mold PDMS again (f) Remove a mold and solder

Fig. 5 Fabrication process of a biochip




(a) Single biochip (b) Integrated biochip

Fig. 6 Overview of a biochip

After that we put this stuff in a plastic case and poured pre-cured PDMS again, and finished making the biochip with micro-scale grooves by removing solder and plastic case after curing PDMS.

Figure 6 (a) shows the appearance of biochip we developed. Based on this concept, we can apply the way to develop a 4-coupled biochip (Fig. 6 (b)). In the 4-coupled chip we can culture cells by filling 4 different kinds of media in each area. By connecting tubes inside the chip, we can quantify the experimental data of cell culture under the same gage pressure and under different concentration of media. Therefore the biochip we propose can be applied easily and we can quantify the cell differentiation caused by gradient stress although this has never been done so far.

## 4. Cell Adhesiveness on the Membrane

At initial point of their proliferation, bone marrow stromal cells extend roots on the surface of PDMS membrane and fix their place. According to the result of Waters et al.[26], it is hard for cells to adhere on the original PDMS surface because of its hydrophobicity, but it becomes easier for them to adhere on oxidized and hydrophilic PDMS surface after $O_2$ plasma treatment. Based on this result, we made the surface of PDMS membrane hydrophilic by exposing of $O_2$ plasma ion (device name: Plasma Ion Bom-

barder, discharge current: 10 mA, discharge time: 3 min) right before the experiment of cell differentiation. Cells adhered well on the membrane due to the treatment of making PDMS hydrophilic. The appropriate way to keep its hydrophilic property is to save it in water[27].

## 5. Stress Analysis inside the Membrane

To calculate the stress that appears on the surface of membrane, we developed a cross-sectional model in which membrane and biochip are combined by ANSYS (software for finite element method) based on Fig. 2 (a) and (b), and analyzed the stress that appeared on the surface of the membrane under the condition of plane strain. Figure 7 shows the boundary condition of this model. We drew a virtual model that contains an air hole with the same size as the biochip for experiment, fixed its surroundings to $x$ and $y$ direction respectively, and gave negative gage pressure inside the air hole. As far as the binding condition between membrane and biochip, we assumed that they are unified completely. The Young's modulus of this model is the same value of PDMS (1.9 MPa). Figure 8 shows the configuration of mesh. We applied square mesh and made it relatively minute around the membrane for the purpose of yielding smooth distortion of the membrane. For each of 4 grooves with different width, we input gage pressure of −60 kPa that is the same condition of experimental cell culture, and calculated the amount of membrane distortion and stress on the membrane. The type of analysis is static-large-deformation analysis, and we applied non-linear-contact analysis for Lane 3 and Lane 4 because the membrane touched the bottom of grooves in these cases.

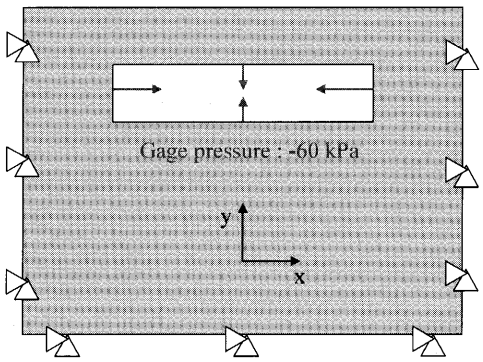



Fig. 7 Boundary condition of model

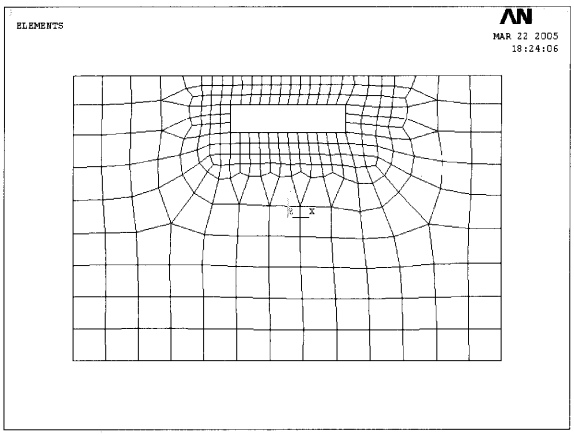
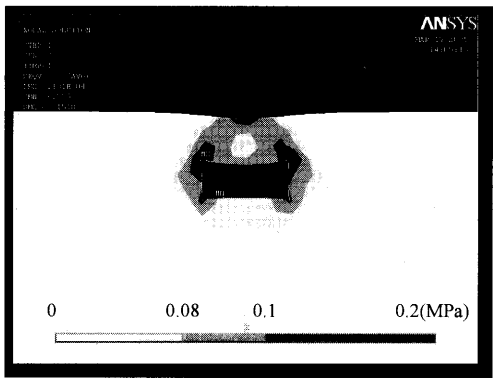



Fig. 8 Finite element mesh (Lane 3)

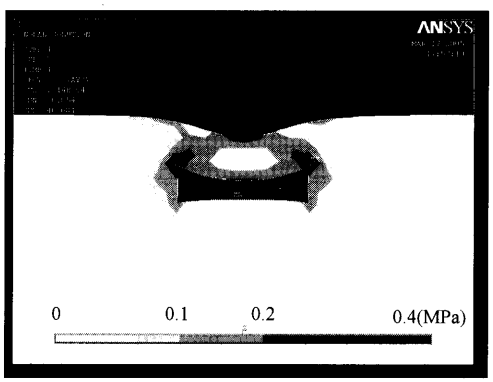
As a result of calculation, we got a distribution chart of equivalent stress shown in Fig. 9. In the result of Lane 3 and Lane 4 membrane touches the bottom of groove, and there is little stress on the membrane in the case of Lane 4 due to the contact. Figure 10 shows the range of stress that appears in the model for each Lane. This indicates that compressive and tensile stress in the order of 0.1 MPa appears on the membrane, and this affects bone marrow stromal cells that spread their roots inside the membrane surface.

## 6. Experiment of Cell Differentiation Using Bone Marrow Stromal Cells and Myoblasts

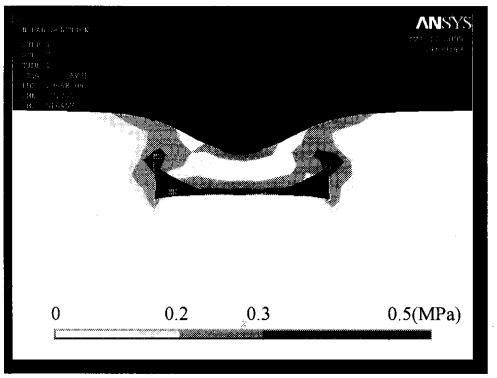
We seeded bone marrow stromal cells (ST2) and myoblasts (C2C12) separately on the biochip, and performed 7-day experiment of cell differentiation. Figure 11 shows the schedule of cell culture. We stretched cells to uniaxial direction from day 5 to day 7 on the condition that gage pressure inside the air hole is −60 kPa. After cell stretching, we stained osteoblasts as shown in Fig. 12 for the purpose of investigating ALP (Alkaline Phosphatase) activity, which is a marker of ST2 cells' differentiation. These 4 pictures qualitatively indicate that cell differentiation was more active on narrower grooves. To investigate this result quantitatively, we performed image processing for these 4 pictures and quantified the effect of cell loading for each width of groove as shown in Table 1. As in Fig. 13 we processed the image including the lane, extract the ALP activated area with white color, and calculated the ratio of pixels between white area and whole area on the lane. Therefore magnitude of number in each lane is proportional to ALP activity in Table 1. In areas of non-loaded, Lane 1, Lane 2, Lane 3, Lane 4, ALP activity varies as Lane 1 > Lane 2 > Lane 3 > Lane 4 = non loading (standardization in the same area between loaded and non-loaded area). Therefore cell differentiation was relatively active on narrow lane and there was little differentiation on Lane 4. This result corresponds to the result of finite element analysis (Fig. 9) that there was little stress on the membrane stuck on Lane 4. The environment with local and moderate stress is appropriate for the differentiation of bone marrow stromal cells. We also counted the cell number in the area of Lane 1, Lane 2, Lane 3, Lane 4, Non-Lane, Petri dish after experiment, and confirmed that each environment makes little difference as far as cell proliferation (Fig. 14). There seems to be no negative effect in PDMS chip because there is no difference of cell proliferation compared with Petri dish (polystyrene). By



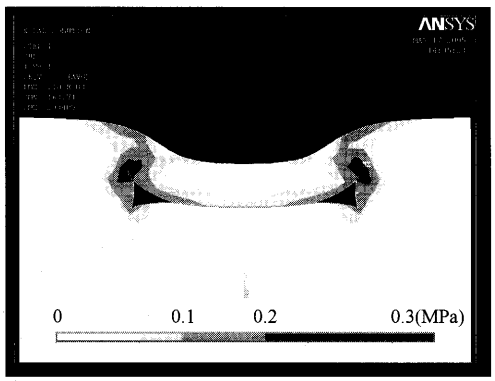

(a) Lane 1




(b) Lane 2




(c) Lane 3




(d) Lane 4

Fig. 9 Equivalent stress distribution in 4 areas

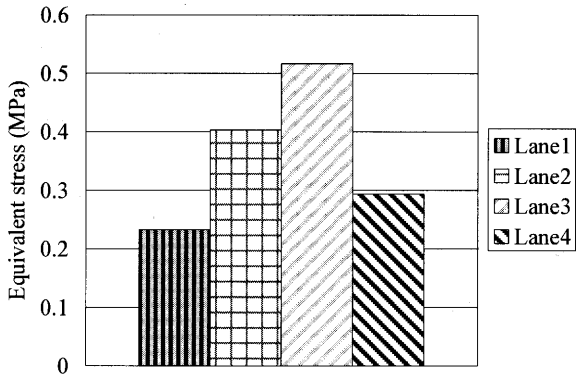



Fig. 10 Range of stress in 4 areas

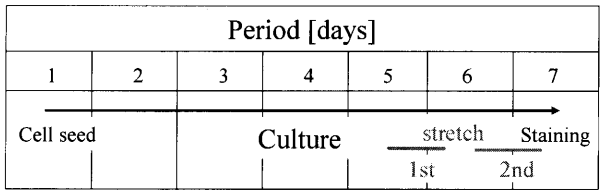
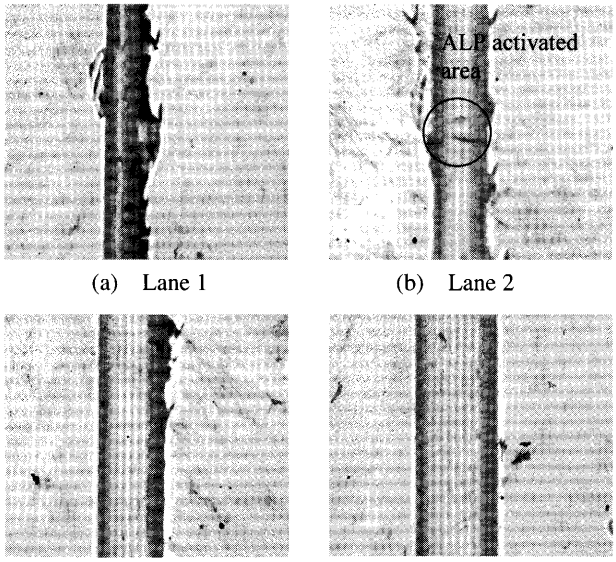



Fig. 11 Schedule of experiment




(a) Lane 1 (b) Lane 2

(c) Lane 3 (d) Lane 4

Fig. 12 Differentiated ST2 cells

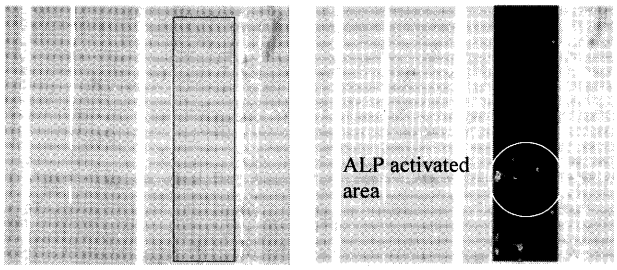
Table 1 ALP activity ratio in 4 areas

| Stress | ALP activity | | | |
|---|---|---|---|---|
| | Lane No. [μm] | | | |
| | 1[200] | 2[300] | 3[400] | 4[500] |
| Loading | 4.53±2.65 | 3.83±1.46 | 1.46±0.28 | 1.01±0.25 |
| No Loading* | 1 | 1 | 1 | 1 |

Note : mean of 3 experimental results±SD

this chip we can achieve safe cell culture at the same level of Petri dish and can promote cell differentiation by giving stress to them.

In the same way we gave strains to myoblasts (C2C12) and performed immunostaining to extract MHC (Myosin Heavy Chain) after differentiation. Figure 15 (a)




(a) Before processing (b) After processing

Fig. 13 Image processing of ST2 cells

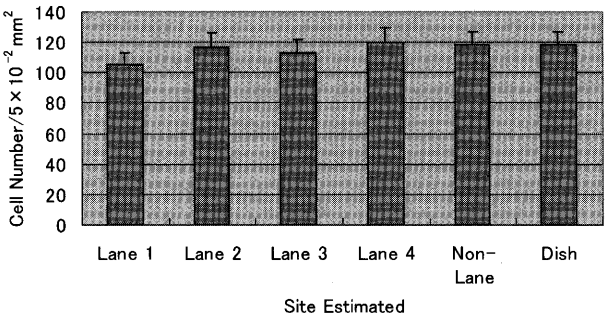
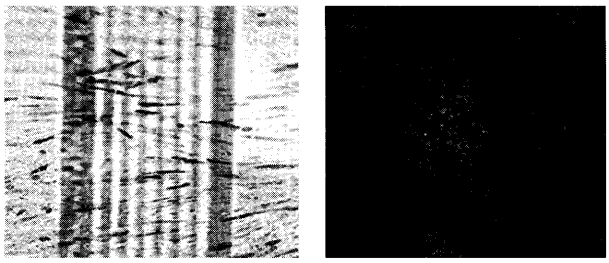



Fig. 14 Comparison of cell number in various areas



(a) Direction of differentiation (b) DAPI staining

Fig. 15 Direction of differentiation

shows that there is possibility that myoblasts differentiate perpendicularly to the lane. From this result we can expect that their differentiation occurs to the uniaxial direction of strain. Figure 15 (b) is DAPI stained myoblasts (Cells exist in white area). This biochip is also useful as a tool to discover cells those differentiations respond to the direction of strain such as myoblasts.

## 7. Conclusion

Differentiation of cells is followed by regeneration of tissue and plays an important role in Regenerative Medicine. In this paper we designed and developed a cell stretching system using a biochip as a method of promoting cell differentiation, and performed experiments of cell differentiation using bone marrow stromal cells and myoblasts. We also calculated the stress on the membrane where cells proliferate by finite element method. As experimental result, we succeeded to give gradient stress to cells in a biochip unlike experiments so far, confirmed that differentiation of bone marrow stromal cells was relatively active on narrow groove, and found the possibility that the direction of differentiation is perpendicular to lane as myoblasts. In the future, this biochip will serve as a helpful tool for searching the appropriate condition of cell differentiation.

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