Relationships between Electric Impedance and Orientation of Biological Cells: Control by Micro-stripes Grooves In Vitro

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ABSTRACT

The relationships between the electric impedance and the orientation of biological cells have been studied *in vitro*. To control the orientation, parallel lines of micro rectangular grooves (1 μm depth, 3 μm width and interval) have been made between a pair of surface titanium electrodes on the scaffold of glass by the photolithography technique. C2C12 (mouse myoblast cell line) was cultured on the micro-patterned scaffold. The electric impedance (z) between electrodes was measured every 24 hours with the sinusoidal electric waves (1 kHz < frequency < 10 MHz; amplitude, ±0.05 V,). The orientation was evaluated by the orientation index (P) calculated by the mean value of the angle between the longitudinal direction of grooves and the major axis of each cell. The crossing index (Nn) was also calculated by the number of cells, which cross the straight-line connecting between electrodes. The experimental result shows that z relates to Nn, which relates to P. The present study shows that the measurement of the electric impedance is effective to estimate the orientation of cells perpendicular to the electric line of force.

Keywords: Biomedical Engineering, Electric Impedance, Micro-machining, C2C12 and Orientation.

1. INTRODUCTION

The biological tissue includes a lot of cells. The biological cell has the shell of the membrane. The membrane consists of the lipid-bilayer. The other part of the tissue is filled with the electrolyte solution. The electrolyte solution is conductive, although the lipid is not conductive. The membrane plays as a condenser when the electric current flows through the tissue [1].

The direct electric current may occur electrolysis in the biological tissue. The alternating electric current, on the other hand, can sense the distribution of the element of the lipid in the biological tissue [2]. The impedance depends on the number of cells. The electric property of the biological tissue also depends on the frequency of the alternating electric current.

The elongated cell has the orientation. The myoblast elongates on the scaffold of the cell culture *in vitro*. The orientation of myoblast can be controlled by the micro pattern on the surface of the scaffold plate [3–5]. Myoblasts differentiate into myotubes, and make muscles. The orientation of myotubes governs the function of muscles [6]. The orientation of myoblasts governs the orientation of myotubes.

The orientation of cells in the target area might relate to the electric impedance of the area, which might be applied to estimate the orientation of cells. In the present study, the impedance has been measured over several elongated cells between the surface electrodes, of which orientation is controlled on the scaffold surface with the several parallel lines of micro rectangular grooves.

2. METHODS

Stripe Pattern for Orientation of Cell

A scaffold of solid surface, which has both micro patterns and surface electrodes (Fig. 1), has been designed to measure electric signals of oriented cells. Several parallel lines of micro grooves have been made in the square area of 1 mm × 1 mm at the surface of the scaffold. The depth, the width, and the interval of the rectangular grooves are 1 μm, 3 μm, and 3 μm, respectively. Two types of arrangements of groove lines are designed at the micro ridges: parallel, and perpendicular to the electric line of force (Fig. 2).

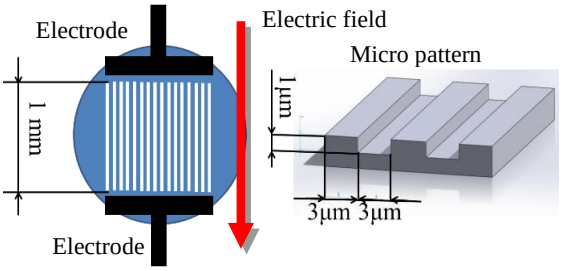

Fig. 1: Micro pattern (right) between electrodes (left).

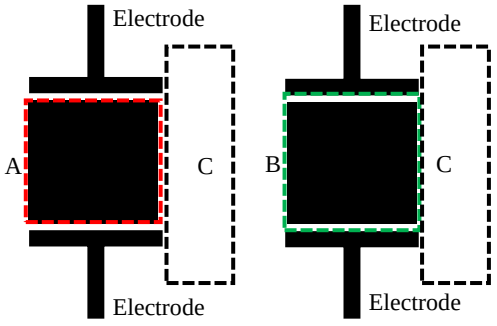

Fig. 2: Micro stripe pattern between electrodes: parallel (left), perpendicular (right): A, on micro pattern; B, between electrodes; C, control area.

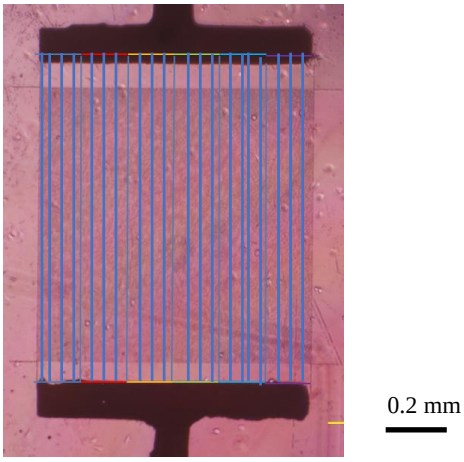

Fig. 3: Parallel straight lines connecting between T-shape of electrodes are drawn at regular intervals.

After plasma ashing, the positive photoresist material of OFPR-800 was coated with the spin coater. The pattern was drawn on the photoresist with a laser drawing system. The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3) for one minute. The glass disk was etched with the plasma gas using a reactive ion etching system (RIE-10NR). The pattern of the surface of the scaffold was confirmed by the laser microscope.

Electrode with Micro Pattern

After machining the micro-stripes grooves, titanium of 0.2 μm thickness was deposited on the surface of the glass in the electron beam vapor deposition apparatus. The positive photoresist material of OFPR-800 was coated with the spin coater. The photomask of the shape of the electrodes was mounted on the surface of OFPR-800, and the photoresist was exposed to the ultraviolet (UV) light through the mask in the mask aligner. The photoresist was developed with NMD-3. The titanium layer on the surface was etched with the plasma gas using RIE-10NR.

To insulate the residual area of the surface electrode, the titanium was covered with the coating of the negative photoresist material (SU-8). SU-8 was coated with the spin coater. The coating area was exposed to the UV light through the mask in the mask aligner. The photoresist was developed with SU-8 Developer.

The wall of culture dish is made of the donut-ring (10 mm inner diameter, 15 mm outer diameter, 10 mm height) of Polydimethylsiloxane (PDMS). The donut-ring of PDMS was adhered on the disk with the surface electrodes with the extra paste of PDMS. The lead wire was connected to the surface electrode with the crimp style terminals.

Cell Culture

C2C12 (Mouse myoblast cell line originated with cross-striated muscle of C3H mouse; passage between ninth) was used in the test. D-MEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (Fetal Bovine Serum) and 1% penicillin/ streptomycin was used for the medium. After the oxygen plasma ashing by FA-1, cells were seeded on the scaffold with the density of 6×10^3 cells/cm^2. Cells were cultured in the incubator at 310 K with 5% CO_2 for three days before the sub-confluent state. Cells were observed with an inverted phase-contrast microscope every 24 hours.

The number of cells crossing the electric line of force (*Ns*) was counted by the following method. On the microscopic image, parallel 25 straight lines connecting between T-shape of electrodes were drawn at regular intervals (Fig. 3). The contour of each cell was traced at "Image J" to count the position of each cell. The total number of cells (*Nc*), which makes crossing with the straight lines, was counted. The mean numbers (*Ns*) of cells, which makes crossing with the straight lines, was defined by Eq. (1).

$$Ns = Nc / k \tag{1}$$

Ns increases with numbers of cells in the area between electrodes (B in Fig. 2). The crossing index (*Nn*) of cells was calculated by Eq. (2), where *n* is the number of cells in the target area.

$$Nn = Ns / \sqrt{(n)} \tag{2}$$

Electric Measurement

The sinusoidal electric waves (frequency, 1 kHz < *f* < 10 MHz; amplitude, v_2 = ±0.05 V) were generated with an electric stimulator. The stimulator was connected to the electrodes, and the sinusoidal electric waves were applied to the medium of cells. An electric resistance (*R*) of 10 kΩ is serially inserted between the electrode and the stimulator. The electric voltages (v_1, v_2) were monitored by an oscilloscope during measurement of impedance of the medium. The values v_1 and v_2 are the voltages between the terminals of the resistance, and of the electric stimulator, respectively. The impedance of the specimen (*z*) is calculated by the voltage (*v*) between electrodes divided by electric current (*i*) (Eq. (3)).

$$z = v / i \tag{3}$$

The voltage (*v*) is calculated by the difference between v_2 and v_1. The electric current (*i*) is calculated v_1 divided by *R*. The phase difference (α) between *v* and *i* is counted on the display of oscilloscope. One period corresponds to 360 degrees of the phase.

Orientation Index

The orientation index (*P*) was defined by the Eq. (4).

$$P = \frac{1}{n}\sum_{k=1}^{n}\left(1 - \frac{\theta_k}{90}\right) \tag{4}$$

In Eq. (4), *n* is he number of cells in the target area, and θ is the angle (0 degree < θ < 90 degree) between the longitudinal direction of the approximated ellipsoid of each cell and the longitudinal direction of the scaffold stripe pattern of the micro grooves (Fig. 4). *P* becomes 1, when every cell aligns along the longitudinal direction of the stripe pattern (θ_k = 0). *P* becomes 0.5, when the alignment of each cell has the uniform distribution to every direction.

3. RESULTS

The mean values of *P* at the area of A (on micro pattern), B (between electrodes), and C (control) (Fig.2) were 0.78, 0.75,

and 0.51, respectively.

Fig. 5 exemplifies cells cultured on striped-micro pattern between surface electrodes for 24 hours. The microscopic images show that cells make orientation along the striped micro pattern: parallel (Fig. 5a), and perpendicular (Fig. 5b) to the electric line of force between electrodes.

The number of cells increased day by day during cell culture. The impedance increases with the number of cells between electrodes (n) (Fig. 6).

Fig. 7 exemplifies the relationship between the impedance and the frequency. The impedance shows the minimum value at the frequency between 0.01 MHz and 0.1 MHz. At the frequency lower than 0.1 MHz, α is larger at the orientation perpendicular to the electric line of force than at the orientation parallel to the electric line of force (Fig. 8). The difference of α between at the perpendicular orientation and at the parallel orientation, however, is smaller at the higher frequency than 1 MHz. The phase difference α does not depend on *Ns*.

Fig. 9 shows the orientation index (P). The mean values of the orientation indexes were 0.5 during cell culture for 48 hours on the scaffold surface with no pattern, which correspond to the random alignment of cells. The value of P is higher at the area A than at the area B, which correspond to the regular arrangement of cells on the pattern. The high value of P on the stripe pattern decreases with time owing to interaction between cells at the higher density of cells.

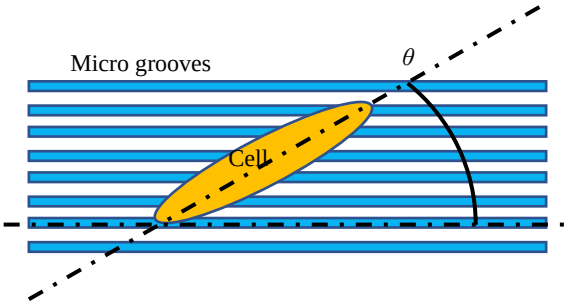

Fig. 4: Angle (θ) between longitudinal direction of each cell and stripe pattern of grooves.

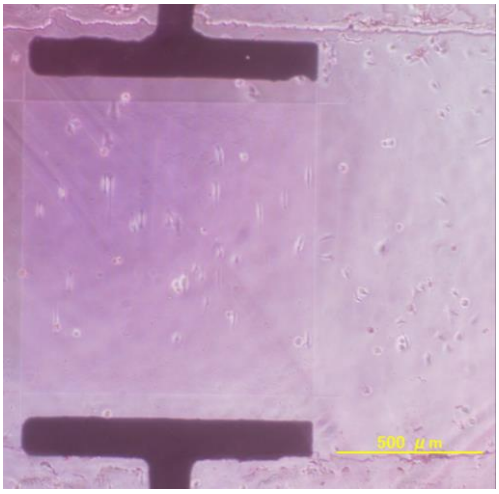

Fig. 5a: Cells on micro stripe pattern (parallel) between electrodes.

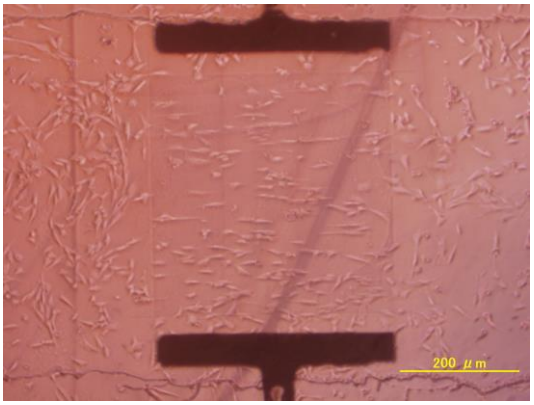

Fig. 5b: Cells on micro pattern (perpendicular) between electrodes.

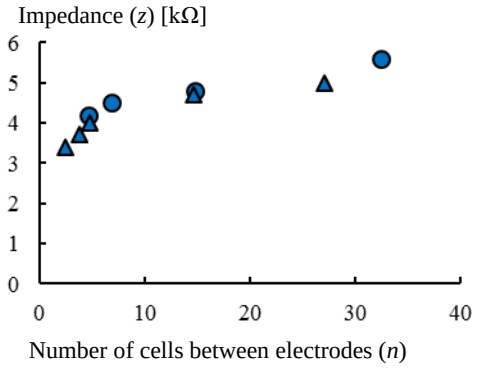

Fig. 6: Impedance z related to number of cells between electrodes (n) at 10 kHz: perpendicular (circle), and parallel (triangle).

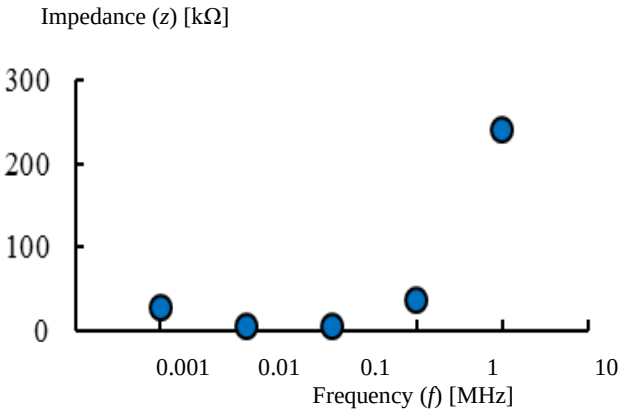

Fig. 7: Impedance z related to frequency f.

Fig. 10 shows relationships between impedance (z) and the crossing index (*Nn*). At the frequency of 1 kHz (Fig. 10a), the values of impedance are split each other between parallel (circle in Fig. 10a) and perpendicular (triangle in Fig. 10a) orientation of cells. The impedance at 1 kHz tends to increase with the number of cells between electrodes (with *Nn*). At the frequency of 10 MHz, on the other hand, data were normalized between parallel and perpendicular orientations of cells, and the impedance tends to decrease with the crossing index (*Nn*).

Fig. 11 shows relationships between *Nn* and *P* in the area between electrodes (B in Fig. 2). At the perpendicular orientation of cells (Fig. 11b), *Nn* tends to increase with *P*. At the parallel orientation of cells (Fig. 11a), on the other hand, relationship between *Nn* and *P* is not clear.

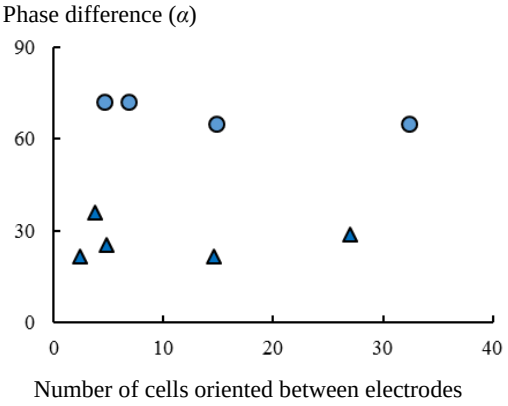

Fig. 8: Phase difference (α) related to number of cells oriented between electrodes (*Ns*) at 1 kHz: perpendicular (circle), and parallel (triangle).

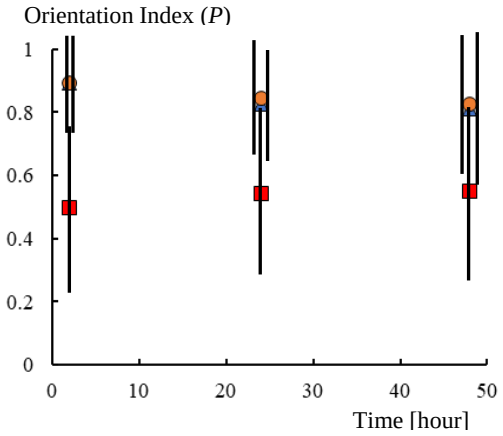

Fig. 9a: Orientation index (*P*) vs. culture time (mean ± standard deviation (bar), 67 < *n* < 415) at parallel pattern: circle (A), triangle (B), square (C): *n*, number of samples.

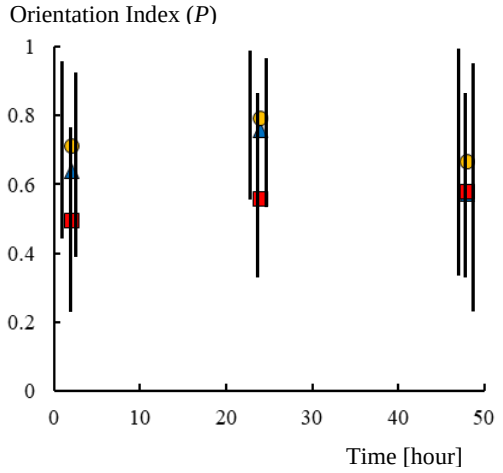

Fig. 9b: Orientation index vs. culture time (mean ± standard deviation (bar), 13 < *n* < 148) at perpendicular pattern: circle (pattern), triangle (between electrode), square (no pattern).

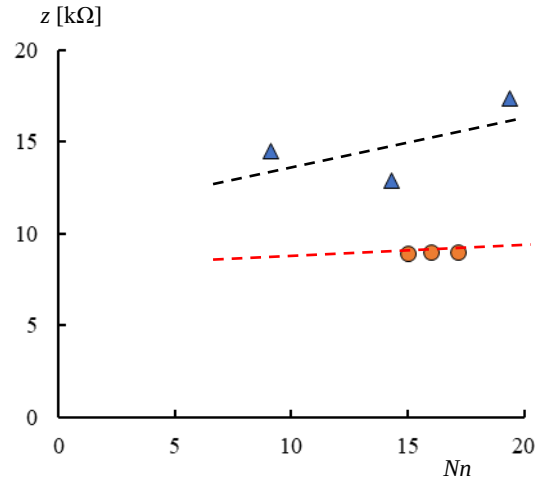

Fig. 10a: *z* (1 kHz) vs. *Nn*: circle (r = 0.84), parallel; triangle (r = 0.63), perpendicular: dotted line (regression line): r, correlation coefficient.

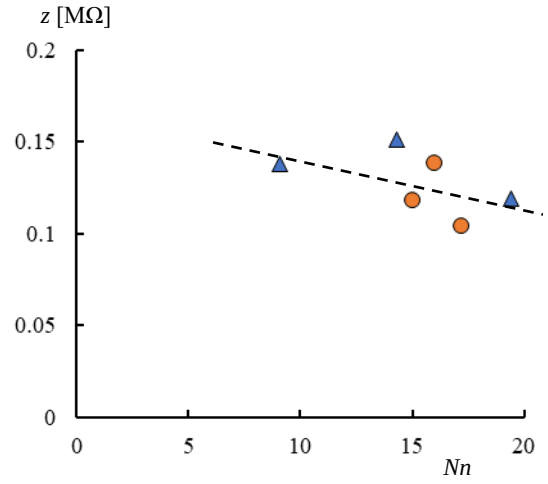

Fig. 10b: *z* (10 MHz) vs. *Nn*: circle, parallel; triangle, perpendicular: dotted line (regression line), r = −0.51.

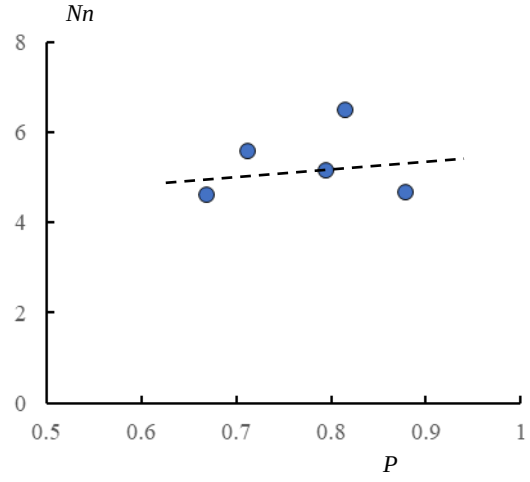

Fig. 11a: *Nn* vs. *P* (16 < *n* < 50) parallel: dotted line (regression line), r =0.14.

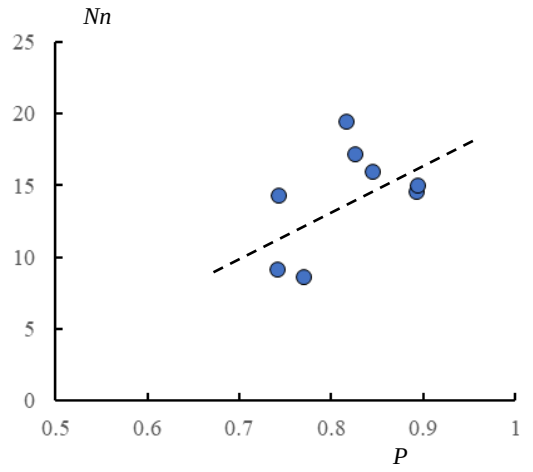

Fig. 11b: *Nn* vs. *P* (59 < *n* < 256): perpendicular: dotted line (regression line), *r* = 0.51.

4. DISCUSSION

In previous studies, the electric impedance was used to monitor cell behaviors [7–10]: cell adhesion [11, 12], spreading [13], shape change [14], proliferation [15], differentiation [16], cell growth [17], cytotoxicity [17], metastatic cells [18], activity of cell [19, 20]. Some of them are using evaporated gold electrodes [21], microelectrode array [6, 22], or vibrating electrode [23]. The sensitivity depends on the morphology of the surface electrodes [24]. The lipid-bilayer at the membrane of the cell governs the frequency response to the alternating electric current [1, 2]. The optical impedance is also applied to measure the confluent cell culture [25]. The orientation of cell depends both on the micro pattern [26] and on the electric field [27]. The orientation of floating cells is measured by the optical technique [28, 29].

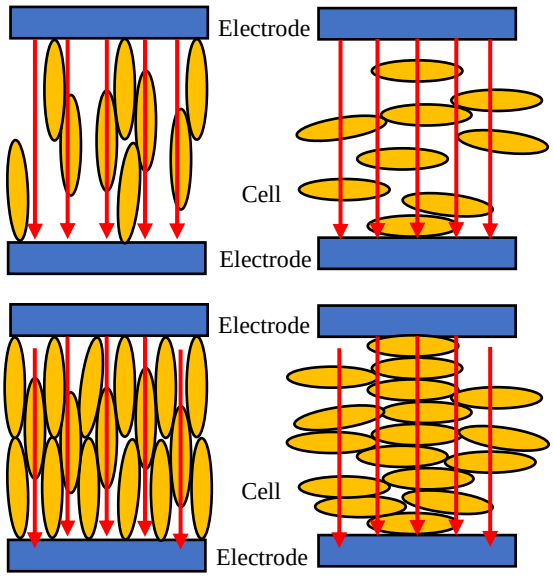

Fig. 12: Orientation of cells and impedance: parallel (left), and perpendicular (right); number of cells increase from upper to lower; arrow (electric field).

The effect of the membrane on the electric impedance (*z*) depends on the number of cells (*Ns*) at the crossing electric line of force (Fig. 12). *Ns* relates to orientation, and density of cells. *Ns* of the perpendicular orientation of cells is larger than *Ns* of the parallel orientation of cells (Fig. 12). The electric impedance might increase with *Ns*, especially at the higher frequency of the alternating electric current. The orientation is represented by the orientation index (*P*). The value of *P* is the highest in the pattern area (A in Fig. 2), where cells make alignment along the micro-stripes grooves. *Ns* related to the orientation of cells. In the confluent state, *Ns* of the elongated cells is higher at the orientation perpendicular to the electric line of force. At the lower frequency, the impedance increases with the density of cells, which is proportional to the crossing index (*Nn*) (Fig. 10a). The lower frequency (< 0.1 MHz) of electric current is effective to monitor the orientation of cells by the phase difference θ related to the membrane. *Ns* is sensitive to the orientation of cells, when the electric line of force is perpendicular to the orientation of cells (Fig. 12). *Ns*, which depends on *n*, is normalized to *Nn*. The crossing index (*Nn*) has proportional relationship to the orientation index (*P*) of cells in the perpendicular direction. The electrodes had better be placed to apply the electric field in the perpendicular direction to the orientation of cells for detection. Several cells show elongated shape on the scaffold. The orientation of cells in the tissue is related to the property of the tissue. The micro-topography of the surface of the scaffold is available to control the orientation of cell [4, 5, 8, 24]. To minimize the effect of the topography of the surface of the scaffold on the impedance of the substance between electrodes, the depth of the microgroove is set within 1 μm in the present study [3].

5. CONCLUSION

The relationships have been studied between the electric impedance (*z*) and the orientation of cells *in vitro*. The orientation was evaluated by the orientation index (*P*) calculated by the mean value of the angle between the longitudinal direction of micro-strips grooves and the major axis of each cell. The crossing index (*Nn*) was also calculated by the number of cells, which cross the straight-line connecting between electrodes. The experimental result shows that *z* relates to *Nn*, which relates to *P*. The phase difference between voltage and current is larger at the arrangement of myoblasts oriented in perpendicular position than that in parallel position. The present study shows that the measurement of the electric impedance is effective to estimate the orientation of cells perpendicular to the electric line of force.

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