論文の内容の要旨

論文題目　Stretchable substrates for the measurement of cellular Mechanotransduction
（細胞の力学応答特性計測のための伸縮する細胞培養基板）

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1. Introduction

We propose a cell culture platform with embedded pneumatic chambers, specially designed to be able to demonstrate cellular mechanotransduction. Cellular mechanotransduction, the process whereby cells convert extracellular mechanical signals into intracellular biochemical signals, plays important roles in tissue development, like migration, growth, differentiation, apoptosis, and stem cell lineage switching, as well as in many disease process. In this reason, the cell assay in mechanically active environment is required to clarify pharmacological effect. In addition, the relationship is still elusive between very specific elements and global elements in a cell. Therefore, we propose pneumatic chamber embedded platform for applying global stress to single cell and local/very local stress to a cell within whole living cell (Fig. 1).

Various devices have been suggested for force application to cells. There are two general approaches to stretch cell for studying cellular mechanism. One is stretchable substrates provide global mechanical stress to a group of cells. It has a limit to obtain information of individual cell by averaging. In this reason, methods probing single cell have been developed. Optical and magnetic trap need particles to apply localized stress to a cell, which causes the limited inspection time due to phagocytosis. Glass pipette has been applied to stretch partial cell membrane. In this dissertation, we propose the pneumatic chamber embedded platform for stretching global, local, and very local region of cell membrane. The present platform can be designed to apply mechanical stress to local region of a single cell, in normal cellular environment of whole living cell, rather than contact portion locally fixed by glass pipette. Furthermore, the platform with embedded pneumatic chambers enables to apply mechanical stress without particles, which results in repeated force application over time. In addition, the stretchable substrates using the pneumatic chambers provide the automatically driven periodic force and the feasibility of integrating to micro chips, which have potentials to analyze diverse cell phenomena, such as rigidity and conductivity changes. In this dissertation, we integrate pneumatic chambers under the cell culture substrate, where the mechanical stress applied to the cells plated on the substrates. We measure the intracellular calcium ion concentration, $[Ca^{2+}]_i$, in response to global, local, and very local stress using the present stretchable substrates.

The stretchable substrate is composed of a thin and flexible PDMS layer and a glass slide. When the pressure is inputted to the pneumatic chamber under the substrate, the substrate is stretched, which results in generating the mechanical stress to the cells attached on the substrates (Fig. 1). The applied mechanical stress via integrins is tranmitted to actin filaments, which results in activation of mechanosensitive (MS) ion channels. Calcium ions enter into the cells through the active ion channels, and then, the intracellular calcium ion concentration increases (Fig. 2a). If we load calcium indicator into the cells on the stretchable substrate, the intracellular calcium ion concentration increases as the mechanical stress is supplied (Fig. 2b). Due to actin filaments, the calcium response can propagate to MS channels over long distance, as shown in Fig. 2c.

2. Theoretical Analysis and Design

In this dissertation, we employed micro-pneumatic actuators for stretching substrate, thereby applying tension and bending. Micro-pneumatic actuators enable to simulate mechanical stress only. Micro-pneumatic actuators also have a potential to measure diverse intracellular phenomena due to a transparent view. Furthermore, Micro-pneumatic actuators are fabricated by microfabrication, which provide easy-modification of changing the size of pneumatic chambers. We designed 3 sizes of pneumatic chambers for applying global, local, and very local stress. Pneumatic chambers having larger and smaller than a cell were applied for stretching global and local stress to cell membrane, respectively.

We tested 2 types of micro-pneumatic actuators composed of PDMS membrane-glass slide and PDMS membrane-PDMS slide. PDMS-glass micro-pneumatic actuators provide thin substrate, bearable to generated force, and high bond strength between layers, compared to PDMS-PDMS micro-pneumatic actuators. Therefore, we designed micro-pneumatic actuators composed of an elastic membrane, made by PDMS, and glass slide. The stretchable substrate for cell culture is the top surface of air chambers of the micro-pneumatic actuators. Connectors attach on the input port of the present devices. Through, the connector, we are able to apply pressure into the air chambers.

3. Experimental Analysis

The stretchable substrates have developed by micro fabrication process (Fig. 3). Polyester film was employed for handling thin PDMS layer easily and separating the PDMS layer and mass (Fig.2a). From the fabricated devices (Fig. 4), we have characterized 1) the stress-strain distribution depending on the input pressure and 2) the intracellular calcium ion concentration in response to global, local, and very local stress. 1) The stress-strain of the present device was estimated from the maximum deflection of the substrate for the input pressure. The maximum deflections of the substrate for the input pressure were measured by a digital microscope (Keyence VK-500) and analyzer (Fig.5). 2) The intracellular calcium ion concentration has been measured using human foreskin fibroblasts (ATCC CRL-2097). For cell culture, the stretchable substrate was coated with extracellular matrix protein, fibronectin (Fig.6a). And then, cells were cultured on the stretchable substrate (Fig.6b). After 18h~24h from seeding fibroblasts on the present substrate, cells were loaded with OGB1, a calcium indicator, by incubation with OGB1-AM (10μM) for 10~30 min (Fig.6c). Figure 7 illustrates the experimental apparatus for imaging intracellular calcium.

4. Experimental Results

1) The stress-strain of the present device for stretching global membrane was estimated from the maximum deflection of the substrate for the input pressure. We obtained line profiles of the stretchable substrates, as shown in Fig.5a and b. The fabricated substrate shows strain over 10% for the input pressure of 34.6kPa~139kPa. Therefore, we captured calcium images for input pressure of 34.6kPa~139kPa (Fig. 5c). The present device for stretching local region of cell membrane was able to apply 10 pN~1000 nN, which is suitable to study cellular response depending on local stress (Fig. 6).

2) The intracellular calcium ion concentration has been measured using human foreskin fibroblasts. For characterizing stretch performance for global cell membrane, we the fluorescence images of $[Ca^{2+}]_i$ after supplying pressure of 34.6kPa~139kPa for 3s into the pneumatic chambers. Pseudo ratio of fluorescent intensity, $\Delta F/F_i$, increased in the stretched cells, showing the peak value of 40% after 1.7s from the force application (Fig. 7b). Otherwise, $\Delta F/F_i$, in the un-stretched cell showed no increase in intracellular calcium ion concentration over time. The peak value of pseudo ratio increases depending on stress (Fig. 8). It means that more calcium influx originated from the larger stress to cell membrane. We also verified that the mechanosensitive calcium response in calcium free solution and solution with gadolinium, the general inhibitors of MS channels. The increase in peak value could not be inspected in both cases (Fig. 9). We enable to conclude that the calcium influx is originated from extracellular calcium ions via MS channels.

For showing the potential of local and very local stress application, intracellular calcium concentration in the portions of a cell. For local stress by stretching the area of 491 μm^2, stretched portion of a cell responded faster than unstretched portions (Fig. 10). However, for very local stress by stretching the area of 20 μm^2, the portion placed over long distance from pressure-applied pneumatic chambers showed the faster mechanosensitive calcium response compared to stretched regions and controls (Fig. 11). From the results, pneumatic chambers having the diameter of 5 μm are needed to explore the link between specific and global elements. Therefore, we experimentally verified that the present stretchable substrates showed the feasible applications for studying cellular mechanotransduction in both subcellular and molecular levels.

5. Conclusions

In this dissertation, we designed, fabricated, and tested the stretchable substrate using micro-pneumatic actuators. The present device was satisfied the requirement for measuring mechanotransduction, 10% of strain, in the range of 34.6kPa~139kPa. The fabricated devices were able to measure the intracellular calcium concentration change responding to mechanical stress. We obtained over 10% of strain in the range of 34.6kPa~139kPa. The plated cells on the stretchable substrate showed increase in intracellular calcium ion concentration due to mechanical stress. The cells showed calcium response for repeated force application. However, they did not show the increase of intracellular calcium ion concentration maintained in solution with no calcium or gadolinium. For local stress by stretching the area of 491 μm^2, stretched portion of a cell responded faster than unstretched portions. However, for very local stress by stretching the area of 20 μm^2, the portion placed over long distance from pressure-applied pneumatic chambers showed the faster mechanosensitive calcium response compared to stretched regions and controls. On balance, we can conclude that the present stretchable substrate have a feasible potential application for analyzing cellular mechanotransduction for varying force application. Moreover, the air chamber size of stretchable substrate can be modulated by microfabrication. We also experimentally verified that the present stretchable substrate can not only globally but also specifically stimulate cell membrane.

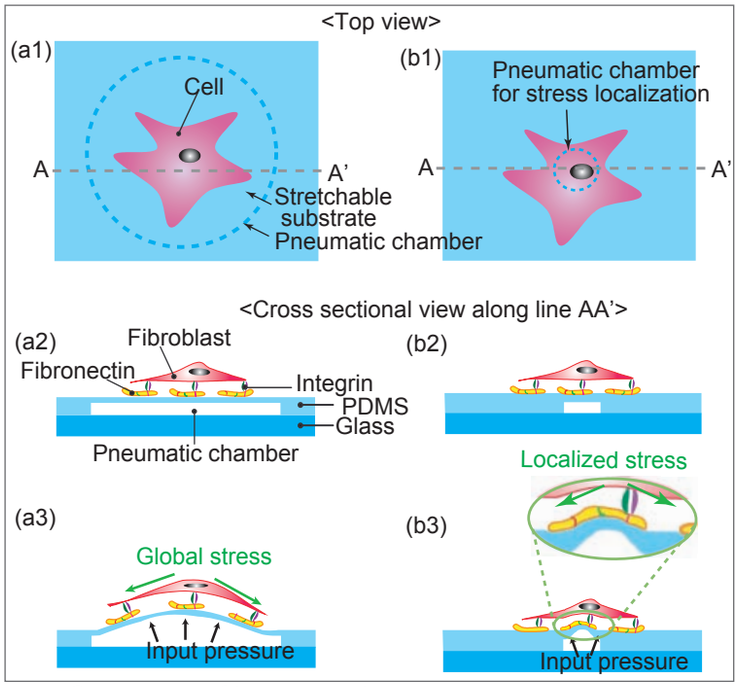


Fig. 1 Comparison of the pneumatic chamber embedded substrates: (a) The micro strestchable substrate for stretching whole region of cell membrane having larger dimater than a cell (a1). Stretchable substrate is top surface of a pneumatic chamber. PDMS is coated by fibronetin, extracellular matrix protein, to attach fibroblasts on PDMS substrate (a2). Stress is applied to a cell depending on pressure application to the pneumatic chamber (a3); (b) The miro stretchable substrate for stretching local region of cell membrane having smaller diameter than a cell (b1). The portion of cell on the pneumatic chamber is stretched depending on input pressure (b2 and b3).

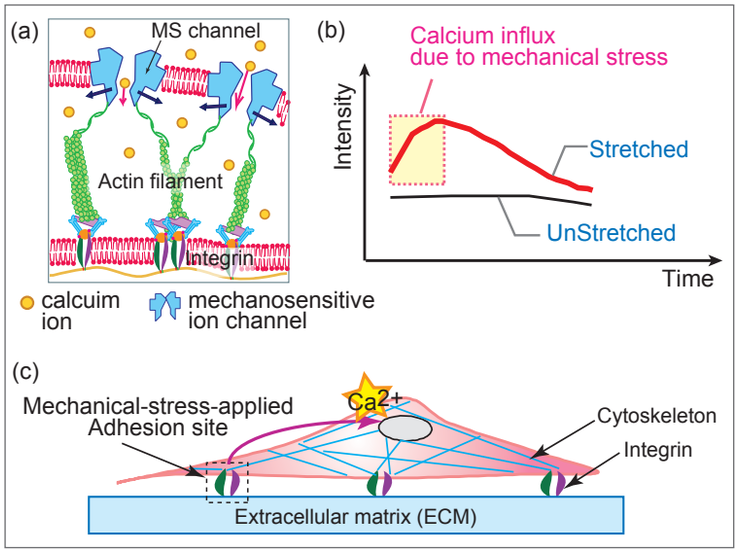


Fig.2 Mechanosensitive calcium reponse: (a) Intracellular calcium increase due to the activation of mechanosensitive (MS) channel. Mechanical stress is input through integrin, transmitted by actin filament, and then permits MS channel open; (b) Fluorescence intensity increase in a stretched cell responding to mechanical stress; (c) Cytoskeleton as a transducer for propagating mechanical stress over long distance.

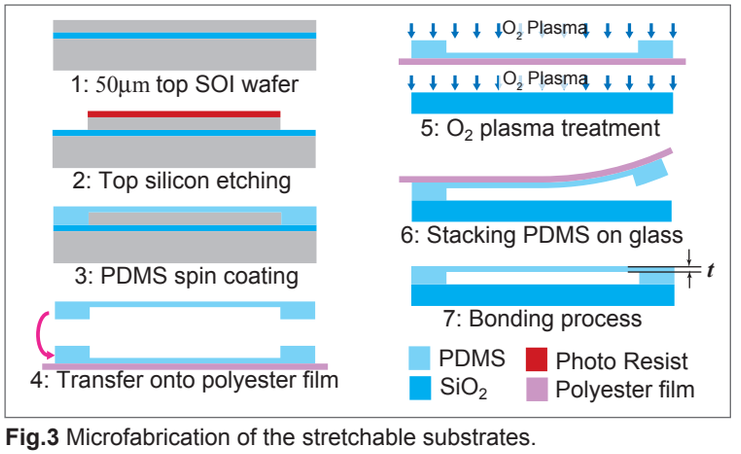
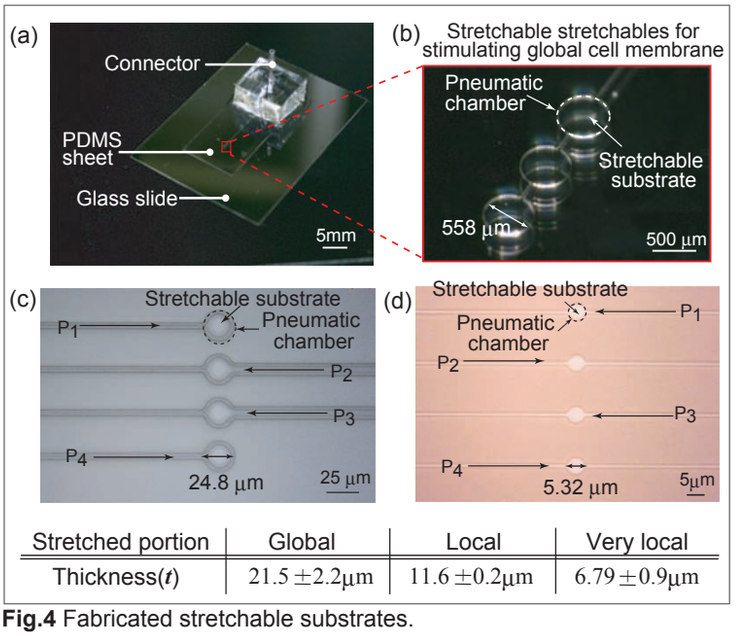
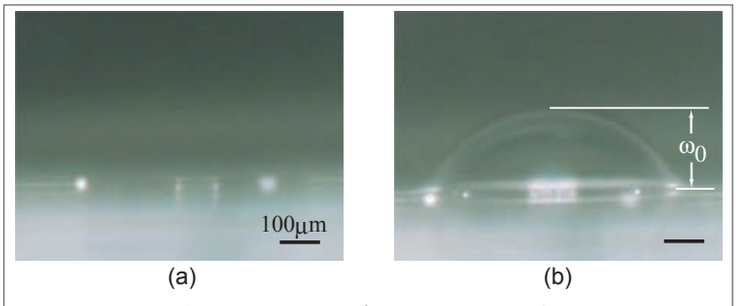


Fig.3 Microfabrication of the stretchable substrates.



Stretched portion	Global	Local	Very local
Thickness(t)	21.5 ±2.2μm	11.6 ±0.2μm	6.79 ±0.9μm

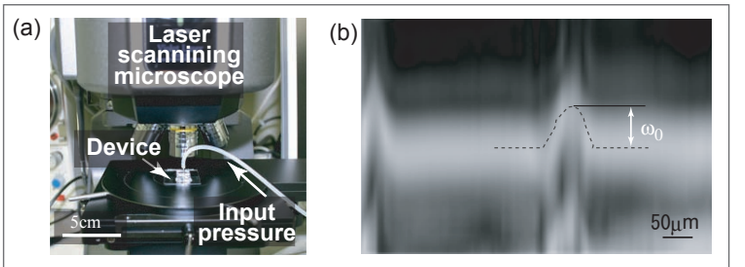
Fig.4 Fabricated stretchable substrates.



Input pressure, P_{in} (kPa)	Max. deflection, ω_0 (μm)	Max. stress, σ_{max} (MPa)	Min. stress, σ_{min} (MPa)
16.8±4.6	84.2±2.5	0.0566	0.0430
34.6±0.8	128± 2	0.126	0.0971
53.1±0.3	151± 2	0.190	0.147
69.3±0.0	176 ±2	0.218	0.157
87.1±0.0	198± 2	0.231	0.167
104 ±0.3	213 ±3	0.242	0.176
121 ±0.0	231 ±3	0.261	0.189
139 ±1.0	239 ±2	0.271	0.196

(c)

Fig. 5 Measurement of maximum deflection for input pressure: (a) Line profile of the stretchable substrat for 0kPa; (b) Line profile for 139kPa. ω_0 indicates maximum deflection; (c) Estimated stress from the measured max. deflections for input pressure of 16.8kPa~139kPa.



(c)

Diameter (μm)	Max. deflection, ω_0 (μm)	Max. stress (kPa)
49.7 ±0.6	3.08±0.31	272
44.6 ±0.3	2.43±0.06	243
39.8 ±0.3	2.05±0.03	215
34.7 ±0.3	1.54±0.10	187
29.6 ±0.1	1.07±0.06	158
24.8 ±0.6	0.77±0.05	131
19.6 ±0.3	0.50±0.09	102
14.5 ±0.2	0.27±0.03	74.0
9.83 ±0.2	0.17±0.04	49.3

Fig. 6 Characterization of stress for the input pressure: (a) Experimental apparatus for measuring maximum deflection; (b) Captured image of the cross section of platform with embedded pneumatic chambers; (c) Estimated stress from the measured maximum deflection for varying diameter. Input pressure of 139 kPa is applied to pneumatic chambers.

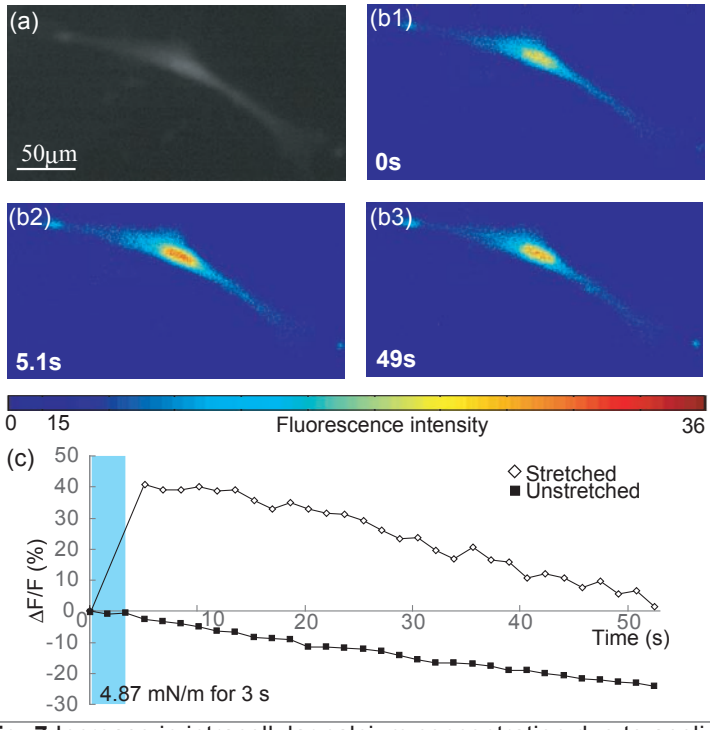


Fig. 7 Increase in intracellular calcium concentration due to applied stress using the micro stretchable substrate: (a) Intracellular calcium detected by OGB1-AM imaging and (b) colored images of the cell in (a). Stress was applied after (b1) was captured for 3 s. Color bar indicates fluorescence intensity. Fluorescence intensity indicates rapid increase after the stress was applied and then decrease, as shown in (b2) and (b3); (c) Increase in intracelluar calcium concentration in a stretched cell unlike in an unstretched cell.

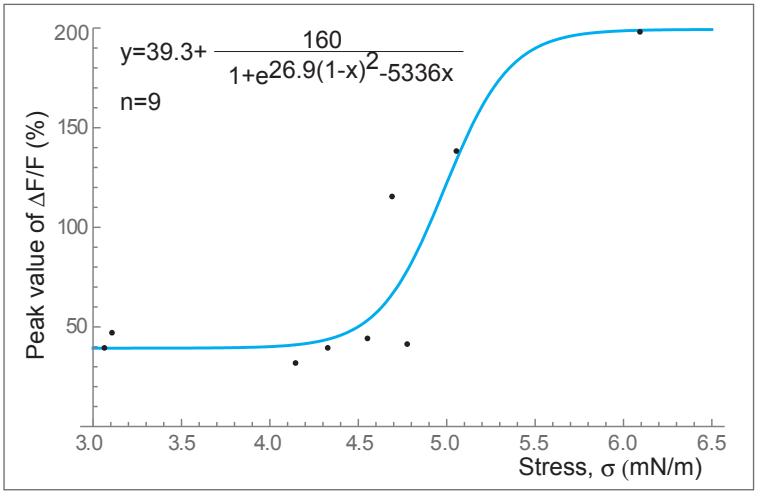


Fig. 8 Mechanosensitive calcium response as a function of the degree of mechanical stress.

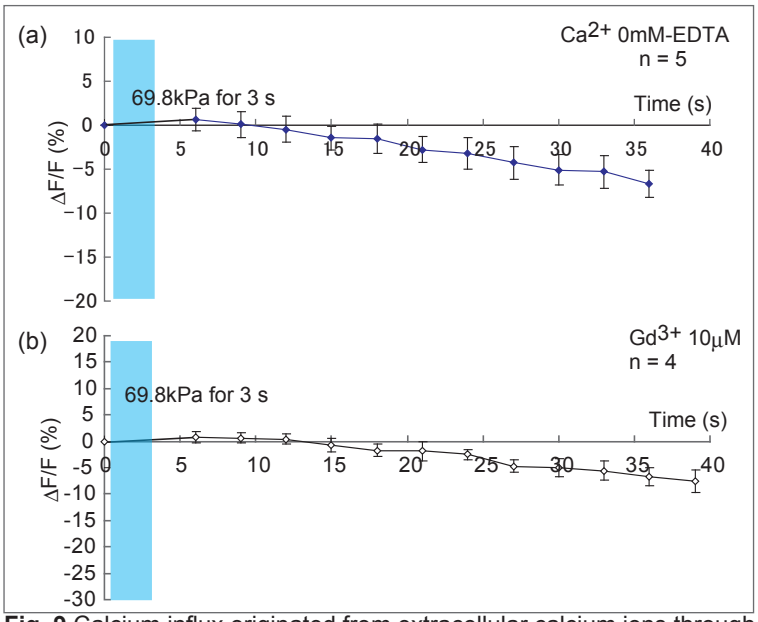


Fig. 9 Calcium influx originated from extracellular calcium ions through MS channels: (a) No mechanosensitive calcium response in solution without calcium; (b) No mechanosensitive calcium response if MS channels were inhibeted by gadolinium.

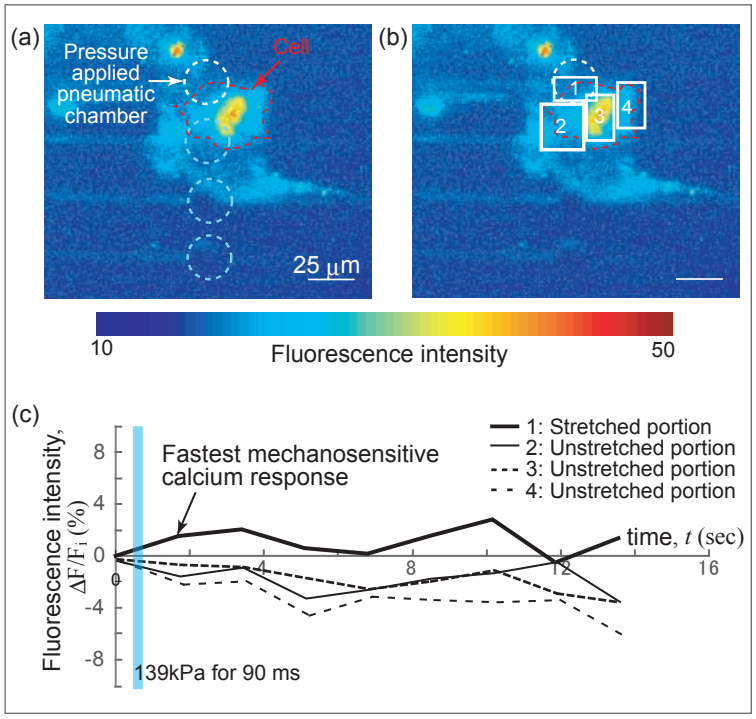


Fig. 10 The measurement of intracellular calcium ion concentration increase responding to local stress on the pneumatic chamber embedded platform: (a) Flourescence images of intacellular Ca^{2+} on pneumatic chambers. Red dotted line indicates a cell. White and blue dotted line indicates pneumatic chamber with input presure and no input pressure, respectively; (b) Portions of cell in (a). Portion 1 applied mechanical stress; (c) Pseudo ratio of fluorescence, $\Delta F/F_i$, of each portion of the cell in (b). Stretched portion shows the faster increase in mechanosesntive calcium response compared to unstretched portions.

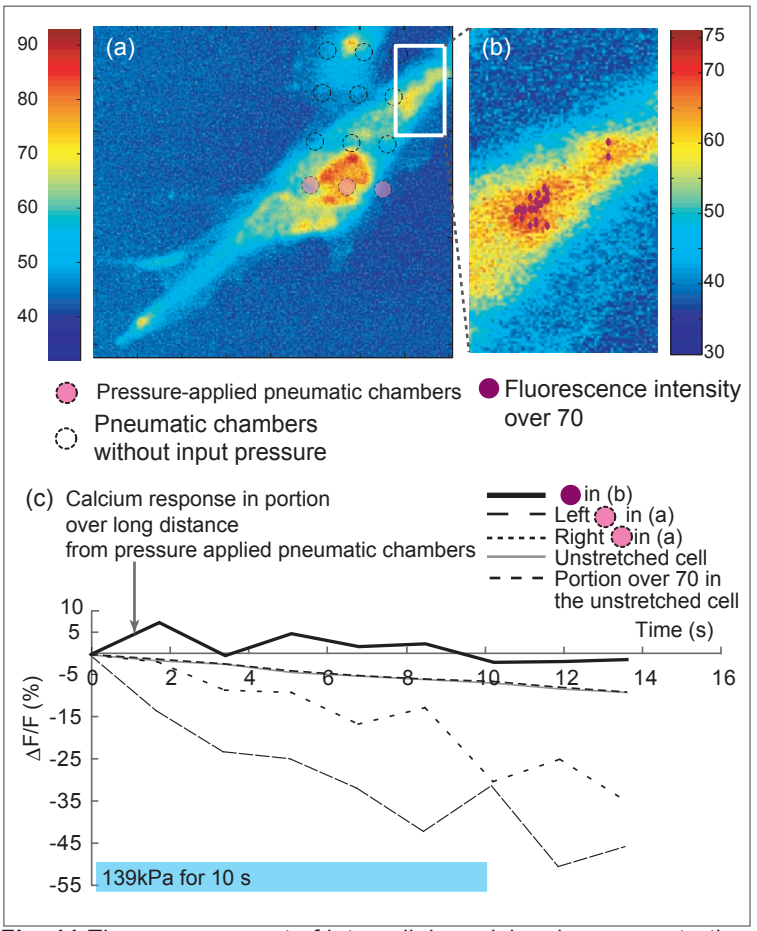


Fig. 11 The measurement of intracellular calcium ion concentration increase responding to very local stress on the pneumatic chamber embedded platform: (a) Flourescence images of intacellular Ca^{2+} on pneumatic chambers. Pink circles with dotted line indicates pressure applied pneumatic chambers. White and blue dotted line indicates pneumatic chamber; (b) Enlarged view of portion enclosed in white box in (a). Purple dots indicate the portion whose fluorescence intesity is over 70; (c) Pseudo ratio of fluorescence, $\Delta F/F_i$, of each portion of the cell in (b) and controls. The portion which places over long distance from pressure applied pneumatic chambers shows calcium increase, otherwise potions applied pressure are not.