論文の内容の要旨

Mobile microplate technology for multi-angle observation of adherent cells

(微小プレートによる接着性細胞の多角度観察)

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1. Introduction

Deformation of the plasma membrane plays an important role in the cellular response to external stimuli and in the spontaneous secretion of proteins. A real-time investigation of membrane deformation not only provides fundamental information about cell behaviors such as endocytosis and exocytosis, but also extends the applications of research field, including microbial infection. Although, conventionally, membrane deformation of two-dimensionally cultured cells has been observed under an optical microscope, unidirectional optical observation cannot capture the cross-sectional view of membrane deformation (Figure 1a). Multi-angle observation of the deformed membrane reveals its precise shape and provides information on membrane transportation, i.e. the trajectory and invasion angle of infectious microbes.

Many researchers have developed experimental systems to optimize the viewing angle toward multi-angle observation. Several previous studies monitored cellular events through the cell membrane by inclining the culture dishes or microscope lenses. Nevertheless, there are still technical limitations on inclination of the optical pathway because cell culture dishes and microscope components, such as lenses and stages, physically obstruct each other. Furthermore, it is difficult to adjust the microscopic focus on the targeted samples after light path optimization, which interferes with the high-resolution observation of the plasma membrane surface.

In this study, we propose two types of microfabricated magneto-active mobile microplates, microflaps and microdisks, to achieve angle-tunability in cell-culturing substrates for multi-angle observation, as shown in Figure 1b. Magnetically inclined microplates enable observation of the cells from multiple angles without physical microscopic obstruction or limited resolution. This system does not require an experimental setup in which the light path is inclined, and it is suitable for multi-angle observation of plasma membranes under the conventional microscope. Furthermore, the micro-sized inclination of the cell-laden microflaps facilitates easy adjustment of the microscopic focus, which enables cellular observation with high-magnification lens.

2. Design and fabrication

The microplates achieve angle-tunability in cell-culturing substrates for multi-angle observation. To make the microplates, we used three materials: a transparent biocompatible polymer, parylene; an alloy with high magnetic permeability, permalloy; alginate hydrogel as the sacrificial layers. The geometry of the microflap and microdisk is illustrated in Figure 2(a-b). The array of parylene structure with embedded permalloy pieces facilitates the batch manipulation of cell-laden mobile microplates under the control of a magnetic field, enabling observation of the cells from multiple angles without physical microscopic obstruction or limited resolution. The parylene surface and the glass substrate are coated with cell-adhesion protein and non-cell-adhesive polymer, respectively; thus cells are patterned only onto parylene surface.

Figure 2(c-i) shows the fabrication process of microplates. On a sacrificial alginate hydrogel layer, we deposited parylene, and evaporated Chromium and Permalloy layer to add a magnetic function to parylene microplates. Alginate hydrogel layer produces a distance between the glass substrate and parylene microplates for releasing from the substrate easily by dissolving it with little cytotoxicity. Then, to prevent the toxic damage to cells due to dissolution of permalloy, we coated permalloy with another parylene layer not to contact adherent cells directly. We patterned Al mask layer onto the parylene and etched it by O_2 plasma. Finally, we patterned fibronectin and 2-methacryloyloxyethyl phosphorylcholine polymer that inhibits protein adsorption and cell adhesion to make cells attached only to the microplates. By putting hollow magnets, we formed magnetic fields around microplates to immobilize, rotate and lift-up them. Figure 3(a-d) and 3(e-f) show optical micrograph and SEM images of the microplates, respectively. An array of parylene structure with embedded permalloy facilitates the batch inclination of cell-laden microplates under the control of a magnetic field. To manipulate microdisks magnetically, fabricated devices were set and suspended in Petri dishes, and neodymium magnets were put under the dishes to apply magnetic fields.

3. Experimental results and discussion of microflap

The inclination motion of the microflaps was tuned by a magnetic field applied perpendicularly to the glass substrate (Figure 4(a)). The magnetic response of the microflap was critically regulated by the hinge width, the hinge length and the permalloy volume (Figure 5(a-b)). Inclination angle of cell-laden microflaps was also precisely controlled by an applied magnetic field, enabling us to observe cell membrane boundaries from multiple angles while maintaining cell viability and morphology (Figure 6(a-b)). Since this system was equipped to the conventional microscopes, we were able to obtain the clear focused images of cell membrane boundaries with high magnification lens. To observe cell membrane boundary, fluorescent microparticles were attached to membrane surface of cells on the microflaps (Figure 6(c)). By applying a magnetic field, we were able to find an appropriate inclination angle and focus on the interface between the target cells and microparticles. Consequently, we obtained the images of the interface between the cells stained by Calcein-AM and fluorescent microparticles (Figure 6(d-e)). Continuous application of an optimized magnetic field held the cell-laden microflaps at a certain angle in the desired orientation and enabled us to observe the targeted microparticles from the desired angles.

To demonstrate the ability to observe cell membrane boundary, we traced microbial infection of the host cells (HFF) by a species of parasites, *Toxoplasma gondii* (*T. gondii*). We observed that *T. gondii* gradually approached the inclined HFF cells and exhibited two distinct forms to prepare for invasion: helical gliding and upright twirling (Figure 7(a-e)). In the transition from helical gliding to upright twirling, we found that they exhibited a bending motion. During the bending motion, the parasites paused, span their bodies, and moved while maintaining a concave shape; the radius of curvature of the concave shape is approximately 3 μm. Next, we traced the time-scale of parasite invasion into the host cells in real-time movies. The time-lapse images in Figure 7(f) indicate that after pause gliding for more than 1 min, it was less than 1 min to totally invade the host cells. The velocity of invasion was almost constant, and the invasion angle gradually increased from 10° up to 105° (Figure 7(g)). Compared with the conventional observation, the microflaps enabled us not only to determine the moments at which the invasion began and ended, but also to obtain detailed information about the trajectory and invasion angle. As a result, this method offers newly obtained information on three different modes of parasite invasion: upright twirling, helical rotation, and invasion.

4. Experimental results and discussion of microdisk

We fabricated the disk-shaped microplates, microdisks, with embedded rectangular shaped permalloy bars to manipulate single adherent cells. To demonstrate the single cell culture on the microdisks with embedded permalloy layer, we cultured four types of cells: HFF, PC12, HeLa and HepG2 cells. All types of cells attached to the fibronectin-coated surface of microdisks (Figure 8(a-d)). The long axis of permalloy bars were aligned to the direction of magnetic fields. Due to the lack of hinges, microdisks acquired higher degree-of-freedom than microflaps that is fixed to the glass substrates. The motion of manipulation is mainly composed of three parts: inclination, rotation, and translational motion (Figure 8(e-f)). Inclination can be achieved the magnetic field that is perpendicular to the glass substrates (Figure 4(b)). By making the magnetic field vertical to the microdisks, we could keep the microdisks inclined and observe the cell membrane boundaries of apical side. Then, by rotating magnetic field under the condition that the field is horizontal to the microdisks, the microdisks are forced to be rotated and aligned parallel to the magnetic field. By fixing the magnet location to keep applying magnetic field, the microdisks behave in translational motion. The combination of three magnetic motions enables us to freely manipulate, immobilize, and rearrange the targeted cells even under the microscopes.

By equipping the cell-laden microdisk with confocal microscopes, we were capable of obtaining scanned x-y images of intracellular structures. Since we can incline the cells on the microdisks in the desired orientation, this system enables higher-resolution cross-sectional imaging with single-molecule sensitivity at single image scanning than conventional methods of reconstruction and deconvolution (Figure 9(a-d)). In this system, we successfully observed the intracellular structure composed of immune-stained actin fibers and microtubules inside single adherent cells. Since the images can be taken at single scanning, it is possible to escape the following considerations: (i) long term scanning leads to photobleaching and phototoxicity; (ii) multiple focal planes lead to dominating depth-induced spherical aberration.

As the demonstration of high resolution observation, we applied this microdisk handling technology to analyze the parasite invasion into host cells. Single host HFF cells on the microdisks were inclined at 90 degrees and observed under confocal microscopes, enabling us to obtain the cross-sectional images of parasite invasion scanned in x-y layer. Here, we found that during the parasite invasion process, cytoskeleton inside the host cells, especially actin fibers and microtubules, were dynamically moved and deformed. Figure 9(e-f) indicates that the parasites harness the actin fibers inside the host cells to migrate and invaginate the host cell membrane. This deformation is attributed to either active pulling up of actin fibers by *T. gondii* or passive deformation of host cell cytoskeleton. Figure 9(g-h) indicate that during the parasite invasion, they are encapsulated by microtubules derived from host cells. The founding obtained by integration of microdisks with confocal microscopy would broad the new scientific field and reveal the parasite invasion efficiency. This method can be an attractive platform for the manipulation of host cells, the observation of invading microbes and the analysis of microbe-host cell interaction.

5. Conclusion

This dissertation describes the mobile microplate technologies to manipulate adherent cells for multi-angle observation of cell membrane boundaries. We proposed two types of mobile microplate structure: hinged microplates, termed microflaps; microplates without hinges to increase the degree of freedom in location and motility, termed microdisks. In the applied magnetic field, cell-laden microplates can be inclined in the desired orientation and equipped on the conventional microscopies including bright-field and confocal microscopes, enabling us to observe the cells loaded onto the microplates from desired angles. By using the microflaps in the bright-field microscopes, we revealed that *T. gondii* exhibited a distinctive pattern of behaviors during invasion into host cells. By applying the microdisks to confocal observation, we obtained the single scanned image that indicates the dynamic deformation of host cell cytoskeleton during parasite invasion under the confocal microscopes. The new and detailed knowledge of parasite motion during infection cannot be accomplished without high angle-tunability and experimental versatility of the mobile microplate. Since infectious microbes, including parasites and bacteria, generally behave with motions in x-y, y-z and x-z plane, multi-angle observation on the microplates will be a promising method for real-time detailed analysis of the microbial motility under the conventional optical and confocal microscopes.

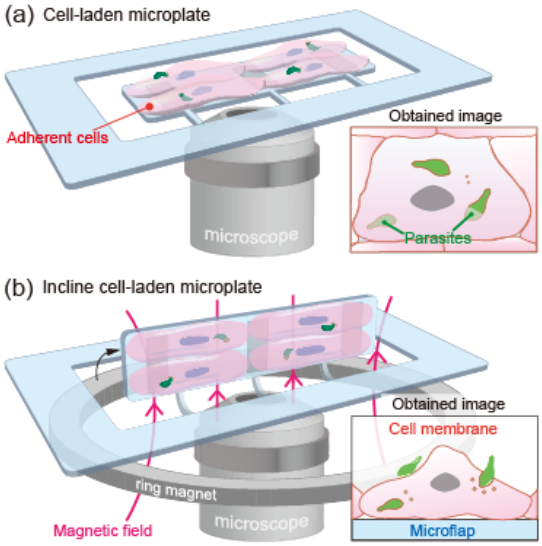

Figure 1. Inclination of adherent cells by microplartes. (a) Schematic image of observation of cell membrane surface of 2D-cultured cells. (b) Microflaps for inclining cells directly facilitate microscope observations of the cell plasma membrane from desired angles.

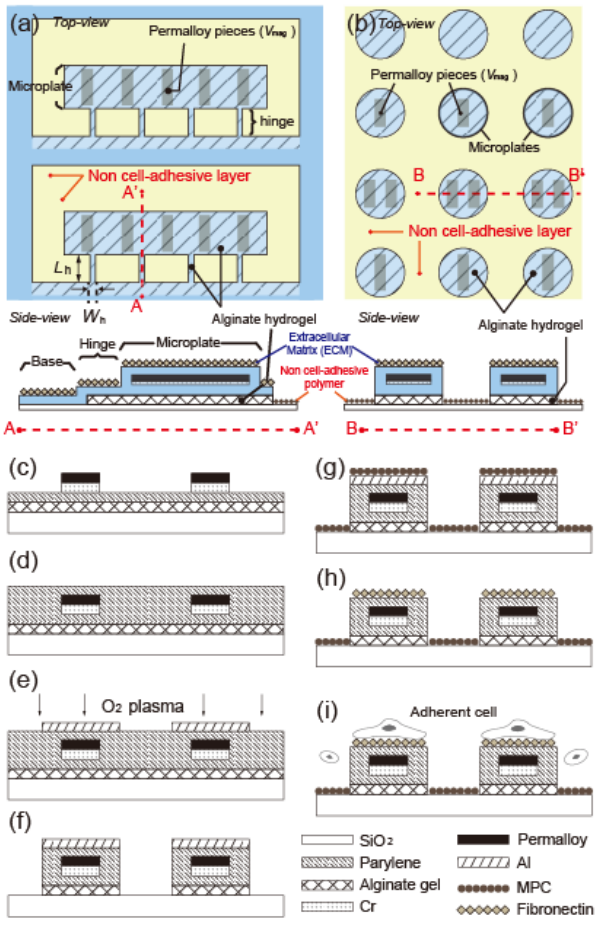

Figure 2. Geometry and fabrication process of mobile substrate for culturing adherent cells. Both of microflaps (a) and microdisks (b) are composed of three parts: parylene structure with embedded permalloy pieces, and sacrificial layer made from alginate hydrogel underneath a glass substrate. (c-i) Fabrication process of microdisks for culturing single adherent cells. Microdisk is made of parylene and a magneto-active material, permalloy as well as microflap. Alginate hydrogel are coated underneath the parylene layer.

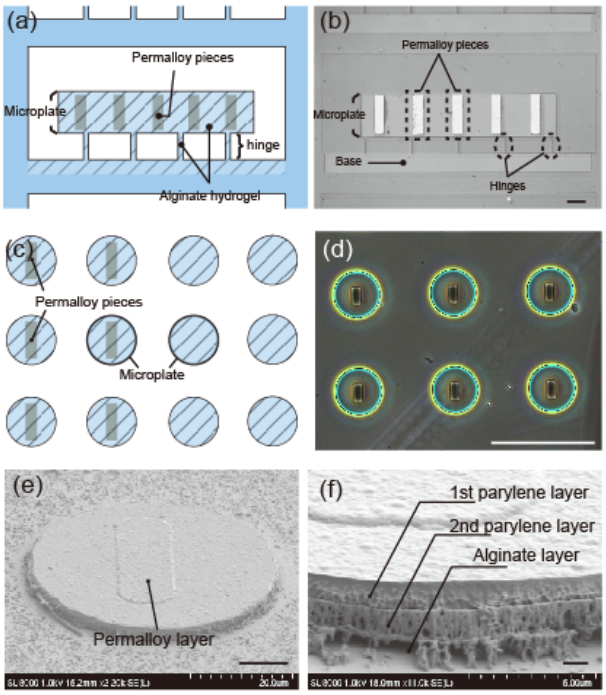

Figure 3. Fabricated parylene microflap and microdisk with embedded permalloy layer. (a) Schematic illustration of microflaps with hinges. (b) Phase-contrast images of fabricated microflaps. (c) Schematic illustration of microdisk. (d) Phase-contrast images of fabricated microdisks. (e-f) SEM images of microdisks with embedded permalloy layer and alginate hydrogel layer.

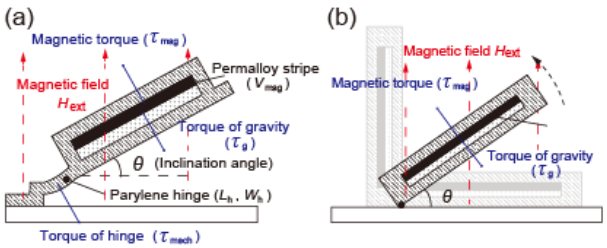

Figure 4. Hinge deformation of microflaps. (a) A schematic illustration of deformed microflaps. We can define three torques: the magnetic one, the one of gravity, and torque of hinges. (b) A schematic illustration of inclined microplates.

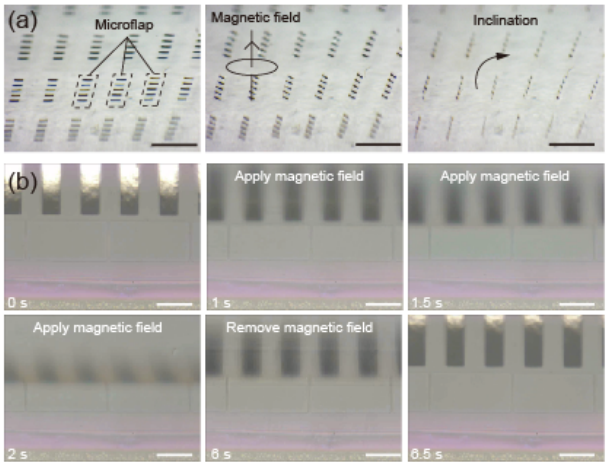

Figure 5. Inclination of microflaps after applying the magnetic fields. (a) Sequential bright field images of an array of microflaps in a batch inclination. (b) Phase contrast images of hinge function as a beam.

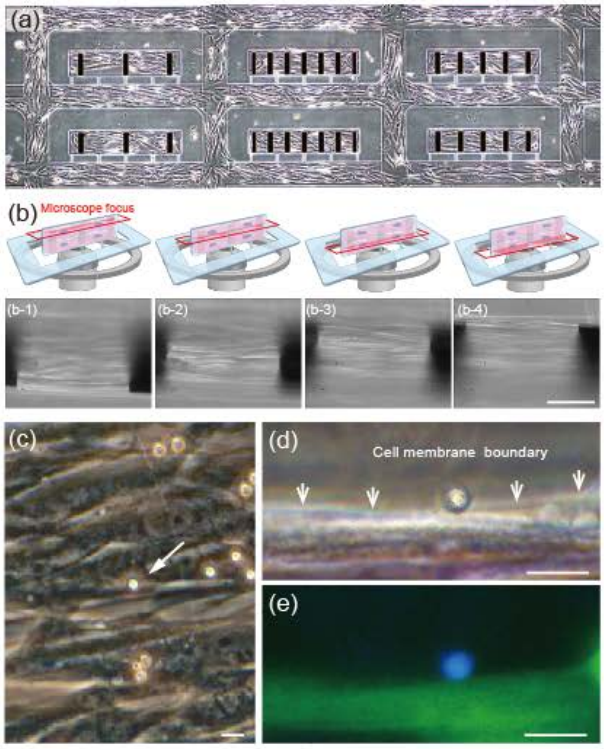

Figure 6. Inclination of cell-laden microflaps. (a) Cells were cultured on the surface of microflaps. (b) Cell membrane of the cells inclined on the microflaps were observed undert the microscopes. (c-d) The interface of microparticles and cell membrane was detected.

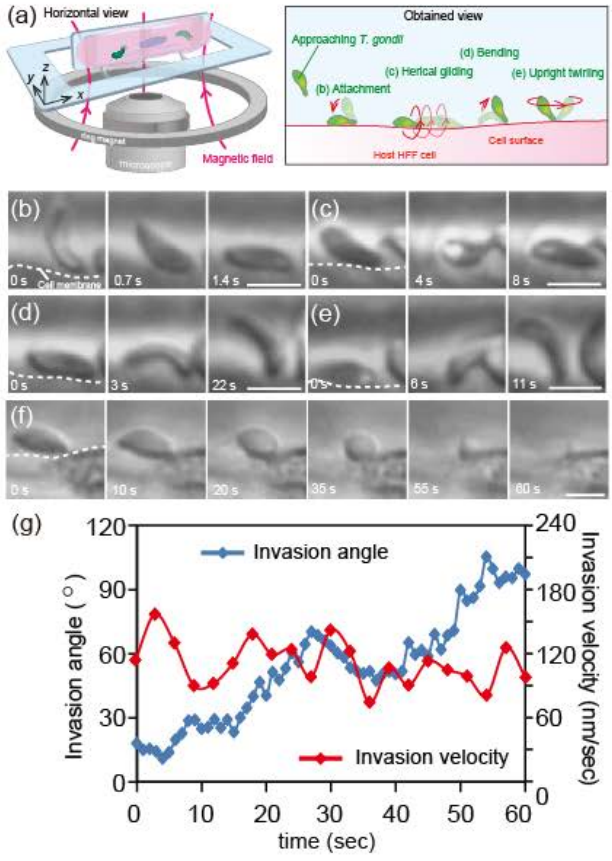

Figure 7.(a) Observation of parasite behavior on their host cells inclined by the microflaps. (b-f) Time-lapsed images of parasite attachment, gliding, bending and invasion. (c) Angle and velocity of T. gondii during the invasion into HFF cells.

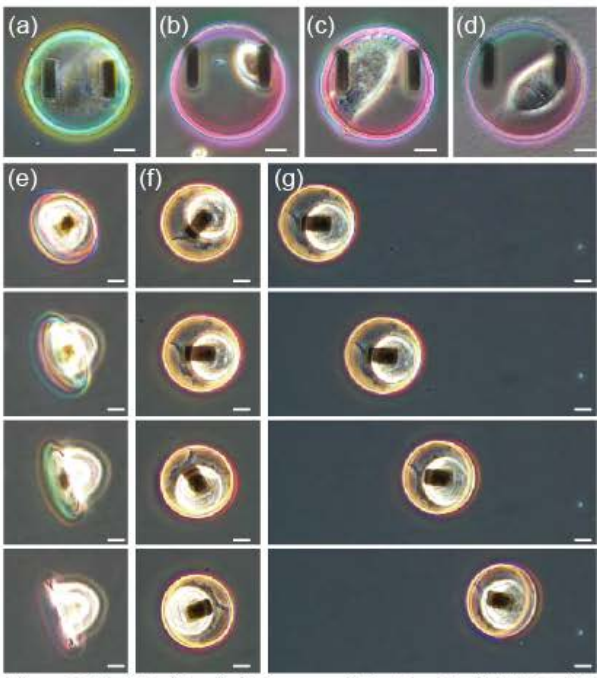

Figure 8. (a-d) Bright-field images of single cells ((a) HFF, (b) PC12, (c) HeLa and (d) HepG2 cells) on the microdisks at 12 h after cell incubation. (e-g) Magnetic manipulation of cell-laden microdisks: inclination, rotation and translational motion.

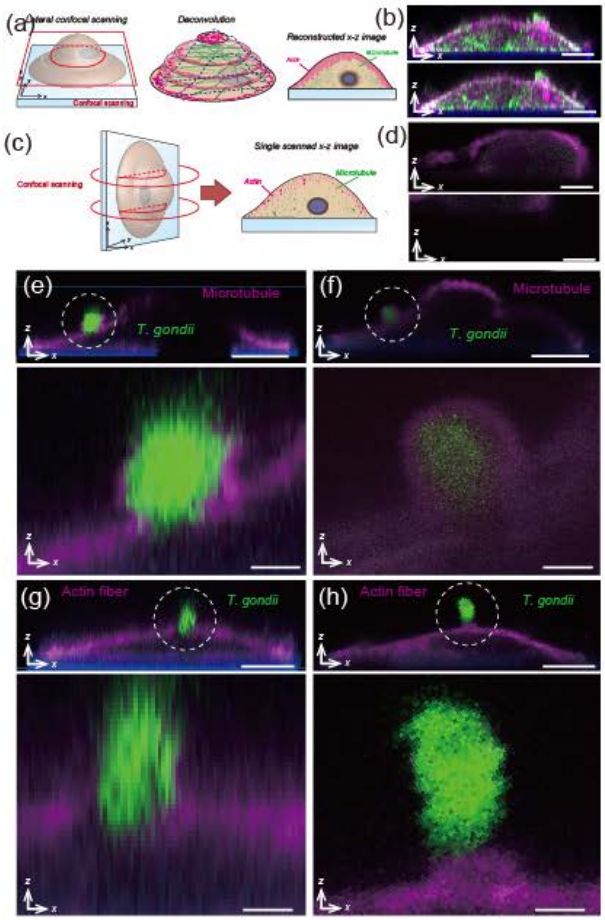

Figure 9. Confocal observation of stained cells. (a-b) Reconstruction of cross-sectional intracellular images. (c-d) Single scanned images of the cross-sectional intracellular structure. (e) Reconstruction of artificial stacked images of intracellular structures. (f) Single scanning of inclined host cells. (g) Reconstruction of artificial stacked images of intracellular structures. (h) Single scanning of inclined host cells.