A 1480/1064 nm dual wavelength photo-thermal etching system for non-contact three-dimensional microstructure generation into agar microculture chip

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Abstract

We have developed a new type of non-contact three-dimensional photo-thermal etching method for agar microculture chips exploiting the characteristics of two different wavelengths of infrared laser beams. We used two different wavelengths of infrared (1480 and 1064 nm) focused laser beams as a heat source to melt and remove a portion of 200 μm high agar gel layer on the 5 nm thick chromium-coated glass slide. As the 1480 nm infrared beam is absorbed by water, the agar gel on the light pathway is heated and melted. On the other hand, as the 1064 nm infrared beam is not absorbed by water and agar, the melting of the agar occurred just near the chromium thin layer that absorbs 1064 nm infrared light. Using this non-contact etching, we can easily make microstructures in agar-layer using infrared laser beam only within a few minutes; i.e. cell-culture holes are melted by 100 mW, 1480 nm laser and tunnels by 100 μm/s, 40 mW, 1064 nm laser, respectively. The size of holes and tunnels were also controlled by choosing the irradiation power and time of infrared lasers. Those results indicate that we can make and use microstructures for biological use without any expensive microfabrication facilities nor a series of complicated procedure and time.

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1. Introduction

Single-cell based cell assay becomes more and more important for drug screening and studies about genetic/epigenetic information in cells. In the field of genome studies, novel sequencing analysis methods like the multi capillary electrophoresis have led to dramatic progress. In contrast, effective approaches to studying cell-based epigenetic information are still being sought. One of the main interests of epigenetic studies is how the information is controlled and recorded. It is especially important to gain understanding of the effect of the number of cell groups and the topology of cell–cell interactions, which we call "community effect". For this purpose, a silicon wafer and a glass slide with holes and metal decorations using the latest micromachine technologies have been created and tested [1–4]. We have also developed an on-chip single-cell based cell cultivation system for studying epigenetic information in cells by using a microchamber array and optical tweezers [5–7]. Although these conventional microfabrication techniques provide structures with fine spatial resolution, it is still hard to change their shape during cell cultivation, which is usually unpredictable and is only defined during cultivation.

Creating small tunnels to connect two chambers without cells passing through them is essential in cell cultivation studies of cell–cell interactions such as the community effect. Thus a variety of materials and several well-known methods, including bonding, sacrificial layer techniques, and lamination have been used to create tunnel-shaped microstructures between two microchambers [8–10]. However, making these microstructures on a chip requires a number of steps, and it is impossible to fabricate them during cell cultivation; that is, we need to make all the structures on the chip before we use it. Thus we have developed a new single-cell cultivation method and a system using agar microstructures, based on 1064 nm photo-thermal etching.

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Using this method, though we can change the structure of the microchambers’ connection by making new tunnels using 1064 nm photo-thermal etching during cultivation, we still have to make microchambers by use of the cast molding before cultivation starts. In this paper, we have therefore proposed the improved photo-thermal etching method called ‘1480/1064 nm dual wavelength photo-thermal etching’. In this method, we exploit the characteristics of two different wavelengths of infrared laser beams, i.e., we used 1480 nm infrared beam to melt all the agar gel on the light pathway, and used the 1064 nm infrared beam for melting of the agar just near the chromium thin layer. This system gives us possible to create and use microstructures even when we do not have facilities for making micro casts like SU-8 microstructures. In other word, using this system, we can create desired shape of microstructures into the agar layer on the chip within 10–20 min in their cultivation room, not in the clean room. We believe it is innovative thing for popularization of on-chip technologies to cell biologists.

2. Apparatus design and experimental methods

2.1. A 1480/1064 nm dual wavelength photo-thermal etching system

Fig. 1 shows the schematic drawing of the 1480/1064 nm dual wavelength photo-thermal etching system. The system we have developed is basically the same as the one we previously reported [12], except for the addition of 1480 nm laser and the flexible-slide focusing lenses to control the focal points of infrared lasers. The system consists of the following two parts: a phase-contrast microscope (IX-70; with a phase-contrast objective lens, 40×, Olympus, Tokyo, Japan) with an automated X-Y stage (BIOS-201T, Sigma Koki, Hieda, Saitama, Japan), and the dual wavelength focused laser irradiation module with a 1064 nm Nd:YAG laser (maximum 1 W; Forte-1064, Laser Quantum, Emery Court, Vale Road, Stockport, Cheshire, UK) and a 1480 nm Raman fiber laser (maximum 1 W; PYL-1-10480-M, IPG Photonics, Oxford, MA, USA). For phase-contrast microscopy and μm scale photo-thermal etching, three different wavelengths (visible light for observation and 1480/1064 nm infrared lasers for spot heating) were used simultaneously to observe the positions of agar chip surface and to melt a portion of the agar in the heated area. Phase-contrast image was acquired by using a charge-coupled device (CCD) camera (CS230, Olympus). The dichroic mirrors and lenses in the system were chosen suitable for those three different wavelengths. The flexible-slide focusing lenses were added in the way of infrared laser beam to control the focal positions of the lasers for correcting their different focal lengths, which depend on their wavelengths.

2.2. Principle of 1480/1064 nm dual wavelength photo-thermal etching

In this system, we used a new type of non-contact three-dimensional photo-thermal etching method for agar-microetching exploiting the characteristics of two different wavelengths of infrared laser beams (1480 and 1064 nm). As the 1480 nm infrared beam has the absorbance to water and agar gel, the agar gel on the light pathway was heated and melted all. On the other hand, as the 1064 nm infrared beam does not have the absorbance to water and agar, the melting of the agar occurred just near the chromium thin layer, which absorbs 1064 nm infrared laser beam.

![Diagram](image-url)
Fig. 2. A schematic drawing illustrating photo-thermal etching. First, a 1064 nm infrared laser beam was focused on the chromium layer on the glass slide (a); next, the focused beam was moved parallel to the chip surface and a portion of agar at the spot-heated point melted and diffused into water through agar mesh (b); finally, after the heated spot had been moved, a tunnel was created at the bottom of the agar layer (c). On the other hand, a 1480 nm infrared laser beam was focused on the agar glass slide (d); next, the focused beam was moved parallel to the chip surface and a portion of agar in the light pathway melted and diffused into water (e); finally, after the heated spot had been moved, a hole was created on the glass slide (f).

Using this non-contact etching, we could easily make microstructures like holes and tunnels only within a few minutes. As shown in Fig. 2, the melting of agar by laser occurred as follows: when a 1064 nm infrared laser beam was focused on the chromium layer on the glass slide, the agar at the focal point near the chromium layer started to melt (a); next, when the focused beam was moved parallel to the chip surface, a portion of agar at the spot-heated point melted and diffused into water through agar mesh (b); finally, after the heated spot had been moved, a tunnel was created at the bottom of the agar layer (c). On the other hand, when a 1480 nm infrared laser beam was focused on the agar glass slide, the agar on the light pathway started to melt (d); next, when the focused beam was moved parallel to the chip surface, a portion of agar in the light pathway melted and diffused into water (e); finally, after the heated spot had been moved, a hole was created on the glass slide (f).

2.3. Agar microchambers and photo-thermal etching

The agar chip is a microcultivation chip for cells covered with 200 µm thick low-melting-point agar on the 5 nm chromium-coated glass slide. Although, in our previous report [12], we made a series of 50 µm square holes molded by using a 50 µm thick cube array cast of thick photoresist, SU-8, microstructure, in this report we made holes using non-contact photo-thermal etching using 1480 nm focused laser of the system, which we have described above.

The following process was used for the agar chip creation. First, a 200 µm thick agar layer was made on the 5 nm chromium-coated glass slide with spreading sol state 2% (w/v) agar (ISC BioExpress, GenePure LowMelt: melting temperature 65 °C). Next, agar-decorated chip was placed in a refrigerator at 4°C until it was hardened into gel. The microstructures were next fabricated using the dual-wavelength non-contact photo-thermal etching in the system described.
above. After the position of the heating spot is checked by optical microscope, 1480/1064 nm laser was irradiated on the chip. Then the melting was confirmed by the microscope and the heating was continued until the size of sport reached to the desired one, or the heating position was shifted to make a desired shape.

3. Experimental results and discussion

3.1. Photo-thermal etching

As explained above, photo-thermal etching is an area-specific melting of agar microchambers by spot heating using a focused laser beam and a thin layer made of a light-absorbing material such as chromium. Although agar does not absorb light with a 1064 nm wavelength, it does with a 1480 nm wavelength. Thus we could melt a portion of agar on the thin chromium layer at a spot-heated by the 1064 nm laser beam and could also melt whole agar on the light pathway by the 1480 nm laser beam. One example of the process of creating microstructure in an agar chip is shown in Fig. 3: first, we focused a 1480 nm infrared laser beam on the agar chip to create two holes having 50–100 μm in diameter and 200 μm deep (b). Next, we focused a 1064 nm infrared laser beam on the chromium layer of the chip at the position of one of the holes, and moved it in the direction to the other hole. Finally, a portion of the bottom of the agar layer melted, so that a tunnel was created and two adjacent holes were connected (Figs. 3(c) and (d)). As shown in Fig. 3(d), the cross-sectional view of microstructure was checked by the confocal microscopy (Fluoview FV300, Olympus) and found that the holes, created by 100 mW, 1480 nm focused laser, were successfully opened and the tunnel, created by 40 mW, 1064 nm focused laser, was also opened just on the chromium layer in the 200 μm thick agar layer.

3.2. The dependence on the 1480 nm irradiated-laser power of hole width

In the previous report [12], we reported that the melting of agar in the chip by 1064 nm infrared laser in detail. When the power was weaker than 3 mW, etching did not occur (Fig. 10 in [12]). When the power was stronger than 50 mW, etching was also difficult because of the bubble generation caused by boiling of water. When the irradiated-laser power was between 3 and 50 mW, the channel width could be controlled linearly, and it ranged from 2 to 50 μm. The channel width could be estimated as:

$$W (\mu m) = 0.96I (mW), \quad (1)$$

where $W$ is the width of the channel, and $I$ the laser power. The above linear relationship indicates that the photo-thermal etching occurs only as a result of direct heating by a spot of light of an area where the balance between heating and cooling was higher than that of the melting point. In contrast to the width data, the height of the tunnel was not proportional to the laser power and seemed to have a limit of 20 μm (Fig. 10(c) in [12]). Thus the cross-sectional shape of tunnel changed from a circle to an ellipse according to the laser power increased.

![Fig. 4. The dependence of the width of thermally etched holes on the power of the 30 s irradiated 1480 nm laser. Upper micrographs show the phase-contrast images of holes from 40 to 120 mW, middle is the same images using confocal microscopy, lower micrographs show the heights of the photo-thermally etched holes.](image)
In contrast to the 1064 nm infrared focused laser, the 1480 nm infrared laser can melt the agar in light pathway all. Fig. 4 shows the dependence of the width of photo-thermally etched holes on the power of the 30 s irradiated 1480 nm laser. Upper and middle micrographs in Fig. 4 show the phase-contrast and confocal top-view images of holes with laser power from 40 to 120 mW, and the lower micrographs show the cross-sectional views of the photo-thermally etched holes, respectively. In this example, the irradiation time of laser was 30 s, each. Although the holes could be observed by the phase-contrast microscopy, we could not observe them by the confocal images at less than 93 mW. This is because 30 s irradiation at 1480 nm was not enough to open the holes at the top surface, which is essential to introduce fluorescent dye into the holes for confocal imaging. Fig. 5 shows the dependence of the hole width on the laser power measured by the optical micrographs (see Fig. 4). The results indicated that the power dependency of the hole width in 1480 nm laser showed the linear relationship as was the case for 1064 nm laser:

\[ W(\mu m) = I - 20 \text{ (mW)} \] (2)

3.3. The time-dependency of melting spot size

Fig. 6 shows the micrographs showing the spot created by photo-thermal etching with 120 mW, 1480 nm infrared laser. Fig. 7 summarizes the time course of the change in the size of the melting spot. As shown in the graph, the melting spot sizes became larger linearly according to the time progress, which is the contrast to the 1064 nm laser, which becomes to the equilibrium size within 20 s after laser irradiation started. It should be noted that we should strictly control the irradiation time for fabrication the desired shape in agar chip.

3.4. Three-dimensional structure of the agar-microchamber chip

We examined the three-dimensional structure of the agar microchambers and tunnels by using a confocal microscopy system. The vacant space in agar layer in the chip was col-
Fig. 7. Time course of the change in melting spot size.

Fig. 8. Phase-contrast micrographs and their confocal cross-sectional micrographs of the agar microchamber array with holes created by 1480 nm laser and tunnels by 1064 nm photo-thermal etching. The irradiated 1064 nm laser powers are as shown in figures, and the tracing speed of 1064 nm spot was 100 μm/s.

ored by filling the microchambers with a fluorescence dye solution as shown in Figs. 3 and 4. Left micrographs in Fig. 8 are the top-view of the agar microchambers connected by small channels. The cross-sectional views are shown in right of Fig. 8, in which we can easily see a narrow tunnel connecting cylindrical holes in a thick agar layer just on the chromium layer. These cross-sectional micrographs prove that we can successfully create the cell cultivation holes and narrow tunnels in the agar layer by the dual wavelength photo-thermal etching.

3.5. Controlling the direction of synaptic connection of hippocampal cells using photo-thermal etching method

After creating the agar microstructures into the agar substrate, we cultured hippocampal cells in the chip. We first made an agar microchambers with 1480 nm photo-thermal etching, and made tunnels between adjacent chambers, one end of which was connected to chambers but the other side was not connected, with 1064 nm photo-thermal etching (Fig. 9(a)). After axons were elongated enough into the tunnels, we next melted a portion of agar substrate at the closed ends of the tunnels to connect two adjacent microchambers (see black arrows in Fig. 9(b)). A day after connecting the
4. Conclusion

We developed a 1480/1064 nm dual wavelength photothermal etching system that can be used in combination with photo-thermal etching to flexibly change the shape of agar microchambers simply and quickly without microfabrication facilities. It also enables creating tunnels in a thick agar layer with a thin light-absorbing layer at the bottom. This system must be valuable for the biological researchers, who do not have microfabrication facilities in the clean room, because they can make whole microstructures on the agar chip only with this improved system without any special facilities for microfabrication. Thus this system can potentially be used for the next stage of single-cell cultivation and measurement in laboratories of biological/medical fields.

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References


Biographies

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