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Cell Culture in a Closed Nano-Space

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The minimum size of a closed nano-space in which cells can survive was determined using 4-nl nanowells. One or two cells could divide in the nanowell. Our results suggest that the cell division activity in the nano-space is determined by the conflict between intercellular effects and consumption of substrates.

[**Key words:** nano-space, chip culture, cell culture]

The development of microdevices has been greatly promoted by the progress of microfabrication techniques, and such devices have been widely developed for genomics, proteomics and drug discovery (1, 2). The use of microdevices can save enormously on experimental space and cost. Moreover, these devices have several advantages including the integration of multiple steps in complex analytical procedures and minimum consumption of rare reagents and samples. Therefore, microdevices have made it possible to culture a single cell within a micro- or nano-level space. The microcultivation of single cells can provide functional information of individual cells for drug screening and environmental monitoring. A fast and precise method for the positioning and microcultivation of single cells was developed using substrate ring electrodes by controlled suction through micro-suction holes (3). By combining multiphase laminar flow driven by pressure and micro-unit operations, subcellular processes could be analyzed in single living mammalian cells (4). A device for on-chip single cell microcultivation of bacteria was also constructed using a microchamber array with circulation of fresh medium (5). In fact, the use of laminar streams in a microfluidic channel can deliver fresh media for long-term cultivation. However, if long-term cell culture in a closed nano-space is realized, it will be possible to analyze more single cells easily and rapidly. Therefore, we examined human cell division in a closed nano-space.

The nanoculture chip was a simple configuration of a polydimethyl siloxane (PDMS) membrane sandwiched by glass slides. PDMS is an atoxic, soft silicone elastomer that is used for cell culture (6). A total of 150 nanowells were made from holes on the PDMS membrane (24×35 mm) using a UV laser beam. Nanowells of 4-nl volume (200×200 ×100 μm) were prepared every 1.5 mm on the membrane. The PDMS membrane and the glass slides were put into a collagen solution after ethanol and UV sterilization. HeLa epitheloid carcinoma cells were grown to 80% confluency on tissue culture polystyrene dishes in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 5% fetal bovine serum (FBS) in 5% CO_2 at 37°C. Cells at the logarithmic growth phase were harvested and cell concentration was adjusted to 8.0×10^5 from 4.0×10^5 cells/ml. After the PDMS membrane was put onto and adhered to the glass slide, 500 μl of the cell culture liquid was evenly dropped onto the membrane. Finally, the second glass slide was put onto the membrane with pressure using tweezers in order to remove the excess liquid. The nanowells thus became completely closed spaces due to surface tension. Immediately after establishment of the closed space, the number of cells per nanowell was counted under a TMS inverted microscope (Nikon, Tokyo).

We constantly found cells in approximately 85% of the 4-nl nanowells. For the remaining 15% of nanowells, two patterns were identified. One whereby no cells entered the

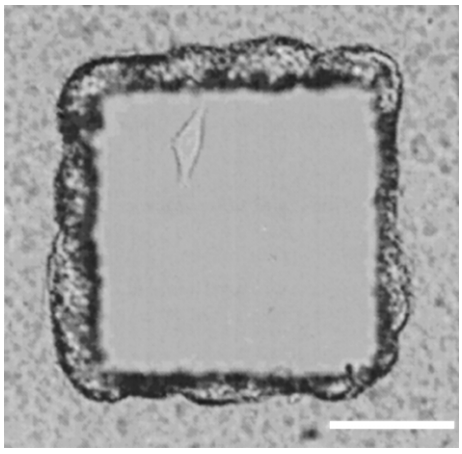

FIG. 1. A HeLa cell in a nanowell. A single HeLa cell in a 4-nl nanowell was observed after 12 h of incubation. The 4-nl nanowell was fabricated on a PDMS membrane. The scale bar shows 100 μm.

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TABLE 1. Ratios of cell division in the 4-nl nanowell

Cell division pattern	1	2	3	4	Average (SD)
1 cell to 2 cells	14.8% (7/47)	11.4% (4/35)	6.7% (3/45)	8.6% (3/35)	11.0% (4.1)
2 cells to 4 cells	4.5% (1/22)	4.8% (1/21)	4.3% (1/23)	5.0% (1/20)	4.7% (0.3)
3 cells to 6 cells	0.0% (0/11)	0.0% (0/11)	0.0% (0/8)	0.0% (0/11)	0.0% (0.0)

In parentheses, the dominator shows the number of nanowells that include one, two or three cells before cell division. The numerator shows the number of nanowells in which cells doubled after 24 h. Numbers 1 to 4 show independent experiments. SD represents a standard deviation.

nanowell and the other whereby air bubbles entered the nanowells at the establishment of the closed space. When we attempted to prepare a nanoculture chip with 1-nl and 2-nl nanowells, the number of nanowells occupied by cells markedly decreased and over 50% of nanowells displayed one of the two patterns outlined above. After counting the number of cells, the nanoculture chip was incubated at 37°C in the presence of 5% CO_2 using a CO_2 incubator (MCO-175; Sanyo, Osaka). If the HeLa cell activity remains in the nanowell, the cell can adhere to the glass slide as shown in Fig. 1. After 24 h of incubation, the number of cells per nanowell was checked again because HeLa cells have a doubling time of 23 h (7). We focused on the nanowells in which cells had doubled through cell division and designed four independent experiments, the results of which are shown in Table 1. Nanowells with cell numbers below the doubling value were excluded because we could not judge whether cells died before or during the cultivation. Approximately 7% to 15% of single cells divided in 4-nl nanowells, whereas Fig. 1 was approximately 4% to 5% for nanowells containing two cells. When the ratio of cell division for single cells is assumed to be 15%, the expected ratio for two cells is calculated to be 2.25%. The ratio of cell division for nanowells containing two cells was significantly higher than the expected ratio. This suggests that the environment in the nano-space was improved by intercellular interaction or cell–cell communication. However, when three cells were in the nanowells, all three could not divide in the 4-nl nanowells. These results suggest that the limit of the closed space volume in which a single cell can live for 24 h is around 2-nl. The cell-division activity in the nano-space is determined by the conflict between intercellular effects and consumption of substrates.

From the point of view of consumption of substrates, oxygen is considered to be the primary factor for determining cell survival in a closed space. The oxygen consumption rate of normal mammalian cells is approximately 1 mmol/g-dry-cell/h (8, 9). If the cell dry weight at a concentration of 10^6 cells/ml is 0.3 g/*l*, the specific oxygen consumption rate per cell is about 3×10^{-10} mmol/cell/h. Because the general oxygen content of water is 0.2 mmol/*l*, the time during which a single cell can live without the supply of oxygen in 4-nl of culture media is 2.7 h. Some single cells in nanowells survived longer than the expected time based on oxygen limitation. This longer survival time suggests that oxygen was supplied from the PDMS membrane because the gas permeates the PDMS membrane. In addition, it is probable that a shortage of glucose and glutamine in the medium is a plausible cause for suppression of cell division because mammalian cells utilize glucose and glutamine as primary energy sources (10). Moreover, accumulation of waste products and a pH change would adversely influence cell survival. The improvement of media, such as those containing more dissolved oxygen and being more pH stable, will make it possible to extend cell culture time and reduce the cell survival limit of the nano-space.

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