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Citation	Biomedical Instrumentation Based on Micro- and Nanotechnology, 4265, 40-49 https://doi.org/10.1117/12.427973
Issue Date	2001-05-21
Doc URL	http://hdl.handle.net/2115/29929
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Type	proceedings
Note	Biomedical Instrumentation Based on Micro- and Nanotechnology, 24-25 January 2001, San Jose, USA
File Information	BIBM4265.pdf



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Culture of bone-marrow-derived cells in microfabricated pit arrays

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ABSTRACT

Tissue cells cannot survive without the attachment to extracellular matrix (ECM). Furthermore, the geometry of ECM is known to play an essential role in the regulation for these cells to proliferate and differentiate so that tissues with normal morphologies can be formed or maintained. We have introduced microfabricated surface structures coated with ECM protein collagen into cell culture studies to examine a possibility of three-dimensional patterning of ECM. In a preceding study, we created arrays of square or circular pits with side lengths or diameters from 25 to 600 μm and depth 10 μm as a simplest three-dimensional structure and tried to culture bone-marrow-derived cells there. The cells appeared to recognize the pits and to voluntarily enter into the pits particularly of 50 μm size. Furthermore, the cells showed an increased growth in the pits of sizes from 50 to 200 μm . We named the obtained effect of the pit-patterned ECM as "micropit effect". In the present study, we intended to more clearly show the micropit effect by newly creating arrays of square pits arranged with separations of 10 and 20 μm and introducing fluorescence microscopy with Acridine Orange staining. The latter allowed it to exactly count cells in the pits without detaching them from there. The micropit effect on the growth of cultured bone-marrow-derived cells of passage 2 (P2) was largest for 50- μm pits, when cells were counted on day 4 of culture, decreased with increase in pit size, and disappeared for 400- μm pits. The micropit effect for P4 cells weakened and the most effective size shifted to 100 or 200 μm .

Keywords: micropit array, extracellular matrix, cell culture, osteoblast

1. INTRODUCTION

Tissue cells are attached to one another and to extracellular matrix (ECM). Without this attachment, they undergo apoptosis, or programmed cell death.¹ This is also known as anchorage dependence. Furthermore, the geometry of ECM has been shown to play an essential role in the regulation for these cells to proliferate and differentiate so that tissues with normal morphologies can be formed or maintained. The signals of the attachment to ECM and also of the geometry are considered to be transmitted to the nucleus and genes via integrins and intracellular actin cytoskeleton.²

So far, studies concerning the geometry of ECM have been made particularly with osteogenesis; bone is constantly remodeled and it appears that osteoblasts recognize pits in bone matrix created by osteoclasts before proceeding to osteogenesis. Clinically, anomalous proliferation and differentiation of fibroblasts or osteoblasts cause heterotopic ossification when they enter into microcracks in other tissues such as ligaments. Allogeneic implantation of powders of demineralized collagenous bone matrix has been used to study the effect of geometry or size on the induction of bone.^{3,4} Coarse powders induced bone whereas fine powders did not. One of the present authors (Kuboki, Y.) and others^{5,6} have used porous hydroxyapatite particles and blocks with bone morphogenetic protein (BMP) and found that 300-400 μm pores were most effective in inducing attachment, differentiation and growth of osteoblasts and vascularization.

Singhvi et al.⁷ and Chen et al.⁸ introduced the photolithographic processes into in vitro cell culture studies to accurately define cell adhesion areas on a plane substrate and succeeded in controlling cell shape and survival. den Braber et al.⁹ used silicone microgrooved surfaces, which were replicas of microgrooved silicon surfaces, to study orientation of ECM protein deposition, fibroblast cytoskeleton, and attachment complex components on such textured surfaces.

We have further examined a possibility of three-dimensional patterning of ECM by coating microfabricated surface structures with ECM protein collagen. In preceding studies,^{10,11} we created arrays of square or circular pits with side lengths or diameters from 25 to 600 μm and depth 10 μm as a simplest three-dimensional structure and tried to culture bone-marrow-derived cells there. The distances between the pits were taken so that the area of a pit is equal to the surrounding surface area per pit. The cells appeared to recognize the pits and to voluntarily enter into the pits particularly of 50 μm size. Furthermore, the cells showed an increased growth in the pits of sizes from 50 to 200 μm . We named the obtained effect of the pit-patterned ECM as "micropit effect". In the present study, we intended to more clearly show the micropit effect by newly creating arrays of square pits arranged most closely, i.e., with separations of 10 and 20 μm , and introducing fluorescence microscopy with Acridine Orange staining.

2. MATERIALS AND METHODS

a) Micropit arrays

Arrays of square pits of side lengths of 50, 100, 200, 400 μm , depth of 10 μm , and wall or separation widths of 10 and 20 μm were created in the (100) surface of a single-crystal silicon wafer, with one pit size and one wall width per chip area (12 x 12 mm), by dry-etching to 8 μm depth followed by wet-etching for the rest 2 μm . The chips are hereafter denoted as T(side length)-(wall width) such as T100-10. T is Japanese initial of a rice field; the present micropit array appears like it. Meanwhile, the previous chips are denoted as E(side length).

b) Coating with ECM collagen

Fully cleaned micropit array chips and plane chips of the same dimension were autoclaved in autoclavable capped plastic beakers. The chips were immersed in ice-cold 0.1 M acetic acid solution containing 0.1 % collagen (cell-matrix 1-A type) for 30 minutes and then rinsed with phosphate buffered saline (PBS; pH 7.0) and with a culture medium (Minimum Essential Medium Alpha Medium with 10 % fetal bovine serum (FBS); GIBCO BRL).

c) Cell preparations

Bone marrow stromal cells were taken from femurs of anesthetized Wistar rats (male, 6 weeks) and incubated in the culture medium in dishes using a CO₂ incubator (5 % CO₂, 37 C). When cells grew to become 80 % confluent, they were detached from the dishes by treatment with trypsin-EDTA (Sigma 1 x solution; 2 min) and suspended in the culture medium at a concentration of about 2.5×10^4 cells/ml. Cells thus prepared are P1 (passage 1). When P1 cells are cultured, they are denoted as P2. P3 cells were similarly prepared after three successive cell culture and harvest steps.

d) Cell culture in the micropit arrays

Three micropit array chips and one plane chip were placed in the same culture dish (diameter 36 mm). Two ml of the cell suspension was taken into each dish. After gently shaken, they were incubated for 4-7 days with refreshing the culture medium every other day.

e) Fluorescence microscopy and cell counting

On day 4 or day 7 of culture, 100 μl of Acridine Orange (Research Organics Inc., Ohio) solution was added to the medium (final concentration 1.8 μM) and kept for 5 min. Then, the medium was replaced with fresh one twice. Five randomly selected spots were observed by a fluorescent microscope (Nikon Optiphot) with B excitation and video pictures were printed using a video printer (Mitsubishi, CP710). Cells in each picture were counted by marking with a marker pen.

The present experimental procedures are illustrated in Fig. 1.

3. RESULTS

a) Bright field microscopy and fluorescent microscopy

Fig. 2 shows comparison of bright field microscopic observation and fluorescent one of the same spot in T50-10. As shown, cells were clearly observed by fluorescence with nucleus densely stained and cytoplasm palely stained. It is possible to count cells without missing.

b) Micropit effect for P2 cells on day 4

Figs. 3 through 6 show cells cultured in the micropit arrays of pit sizes 50, 100, 200, and 400 μm . Each figure contains pictures of 4 substrates placed in the same culture dish, including plane one as a control. The pictures are the

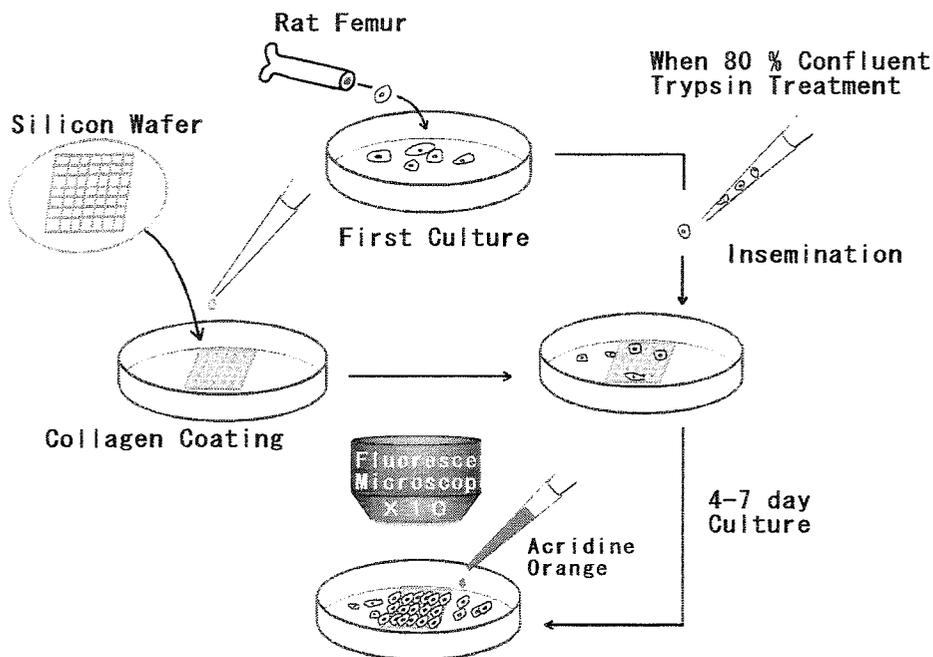


Fig. 1. Experimental Procedures.

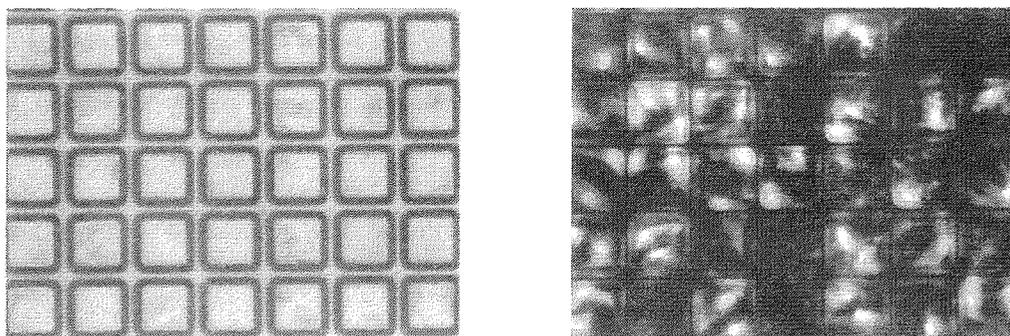


Fig. 2. Comparison of bright field microscopic observation and fluorescent one of the same spot in T50-10.

spots where maximum cell numbers were obtained among the randomly selected 5 spots. The numeral figures given to each picture are the total cell number in it. The cell number in T50-10 was twice larger than the control. The difference from control in cell number decreased with increase in pit size and disappeared for the 400 μm pit array. The cell numbers in T50-10 and T100-10 were larger than those in T50-20 and T100-20, respectively.

c) Micropit effect for P4 cells on day 4 and day 7

Figs. 7 through 10 show comparison for each pit size on day 4 and day 7. Similarly to the preceding figures, comparison is made between the spots where the cell number is maximum among the 5 spots in the substrates placed in the same dishes. Because culture could not be continued for the stained cells, those on day 4 and 7 are from different dishes. Micropit effect for P4 cells appeared to be weakened compared with that for P2 cells, and the pit size most effective for cell growth appeared to shift to 100 or 200 μm . Furthermore, contrary to P2 cells, the cell growth appeared to be larger in the pit arrays with 20 μm wall width than in those with 10 μm wall width.

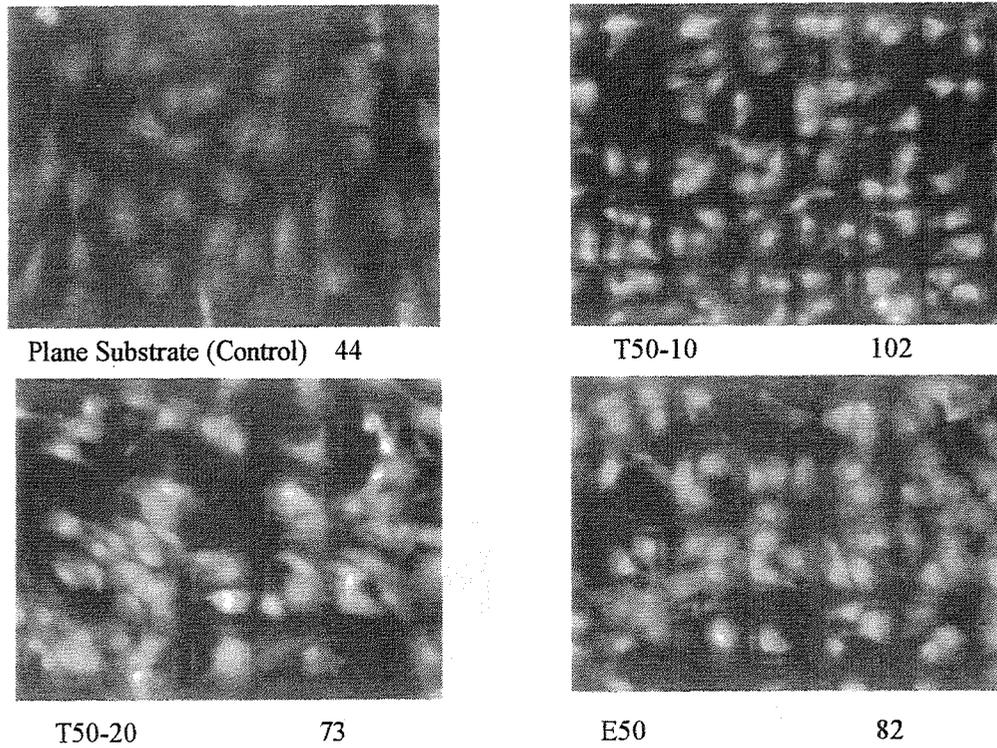


Fig. 3. Culture (day 4) of bone-marrow-derived cells (P2) in arrays of 50 μm square pits. The four substrates were in the same culture dish (the same for the rest figures).

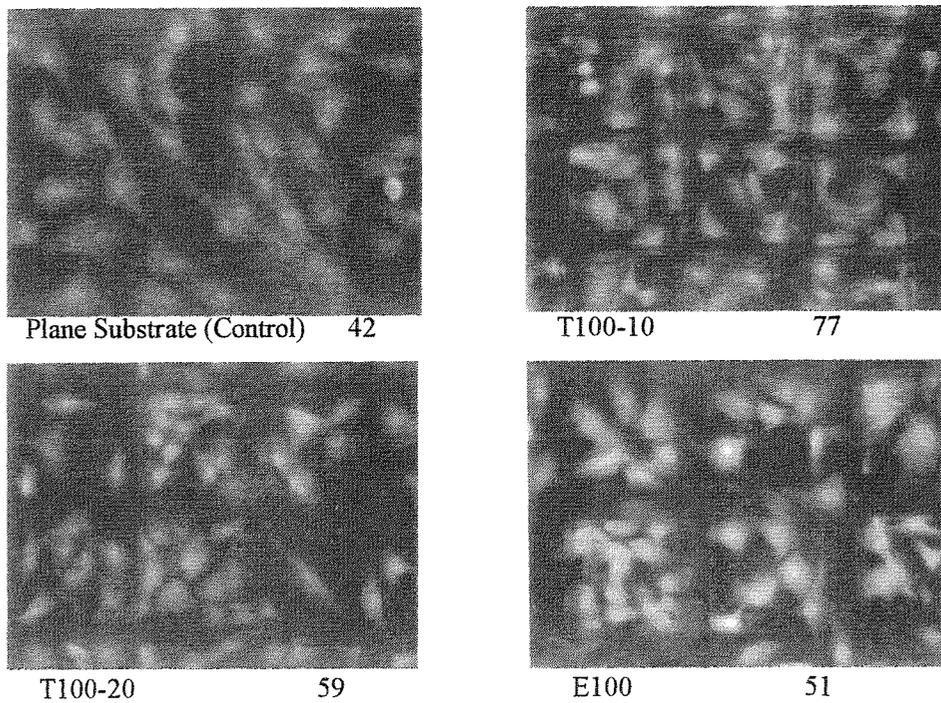


Fig 4. Culture (day 4) of bone-marrow derived cells (P2) in arrays of 100 μm square pits.



Plane Substrate (Control) 47



T200-10 58

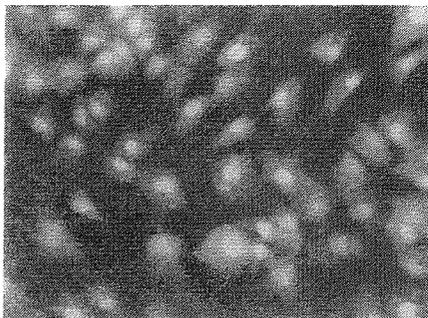


T200-20 53



E200 57

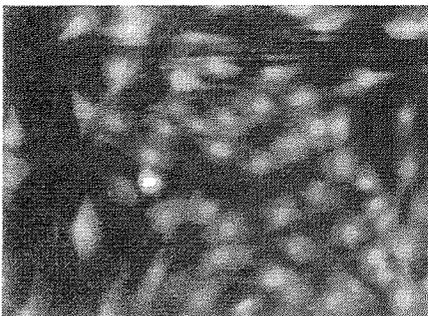
Fig. 5. Culture (day 4) of bone-marrow-derived cells (P2) in arrays of 200 μm square pits.



Plane Substrate (Control) 55



T400-10 47



T400-20 63



E400 57

Fig. 6. Culture (day 4) of bone-marrow-derived cells (P2) in arrays of 400 μm square pits.

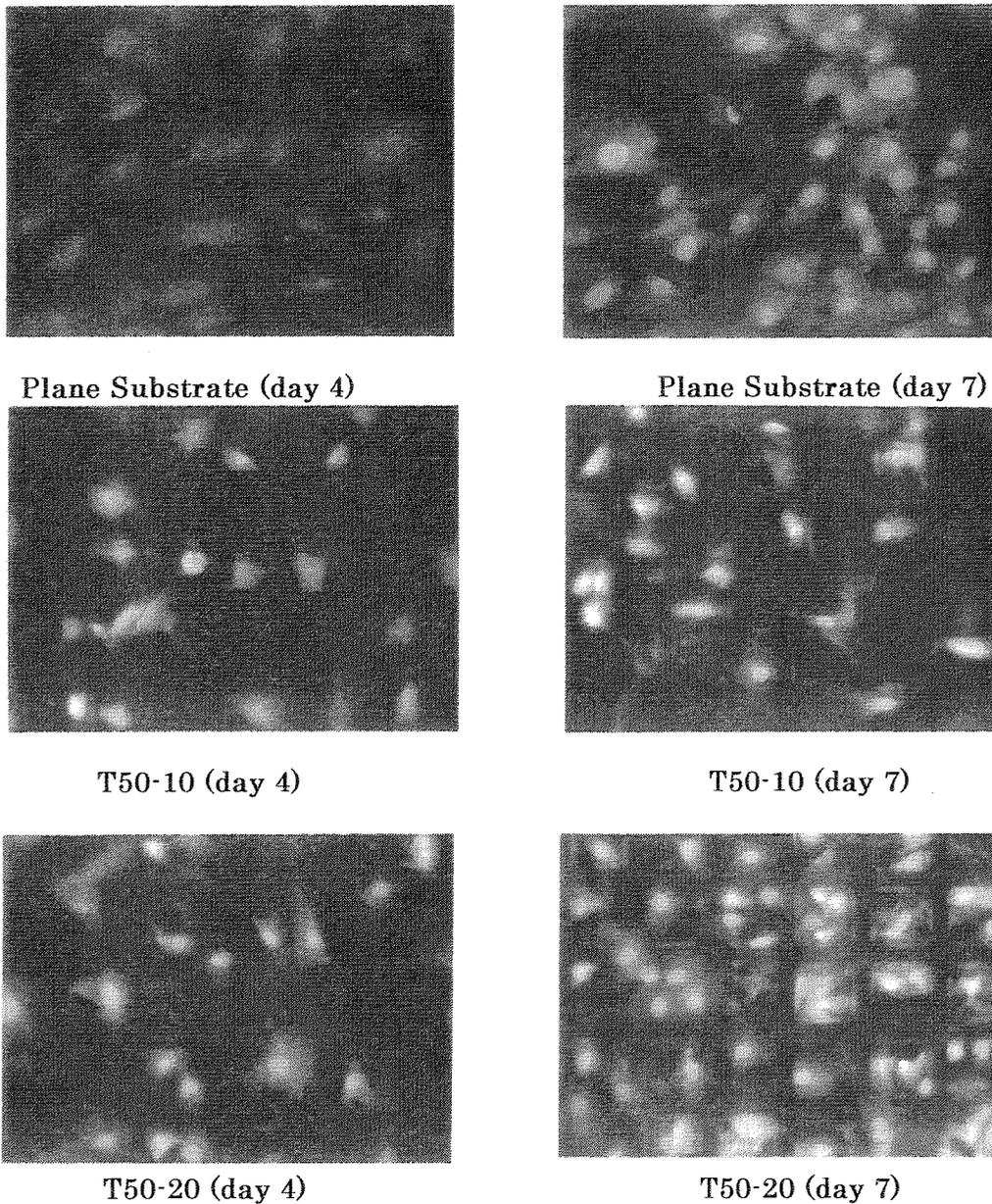
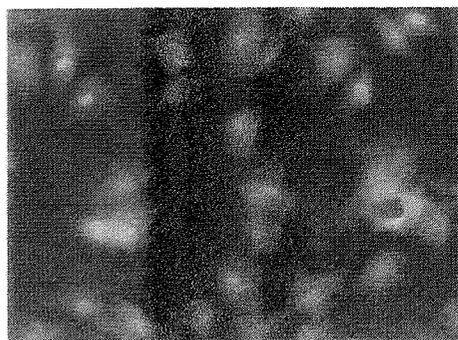


Fig. 7. Culture of bone-marrow derived cells (P4) in arrays of 50 μm square pits.

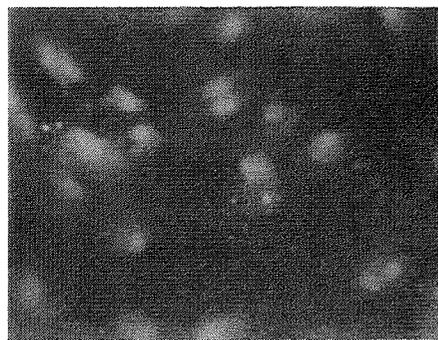
4. DISCUSSION

In the present study, we could show more clearly the micropit effect particularly for P2 cells by using the most closely arranged micropit arrays and fluorescent microscopy. In the preceding studies,^{10,11} we designed the micropit array so that the pit area and the rest surface area are equal with each other. It can be shown by a simple calculation that this condition is attained for square pits by taking 0.4 x pit side length for the separation distance between adjacent pits. Therefore, E50 is equal to T50-20. As shown in Fig. 3, similar cell numbers were obtained for the two micropit arrays.

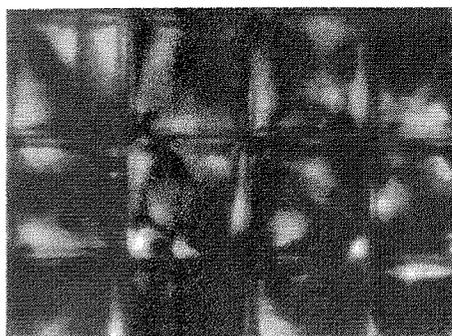
In the preceding reports,^{10,11} we described that 90 % of cells were observed to exist in the pits of 50 μm size and that the cell number became maximum for 50 or 100 μm pits when intact cells were counted microscopically and for 200 μm pits when cells detached from the substrates were counted. The present results appear to agree with the



Plane Substrate (day 4)



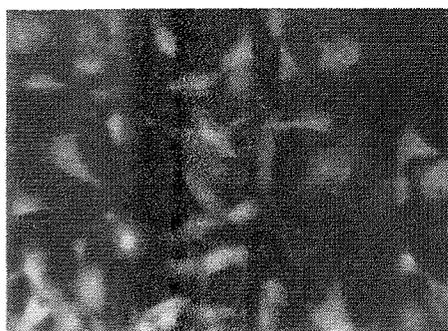
Plane Substrate (day 7)



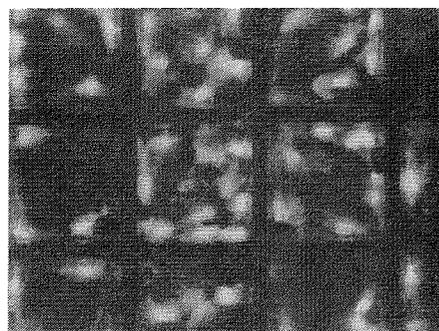
T100-10 (day 4)



T100-10 (day 7)



T100-20 (day 4)



T100-20 (day 7)

Fig. 8. Culture of bone-marrow derived cells (P4) in arrays of 100 μm square pits.

preceding ones as a whole. The growth maximum of P2 cells was obtained for 50 μm pits in the present study. It is supposed that, in the previous studies, a considerable number of cells were uncounted in the bright field microscopic preceding ones as a whole. The growth maximum of P2 cells was obtained for 50 μm pits in the present study. It is supposed that, in the previous studies, a considerable number of cells were uncounted in the bright field microscopic counting particularly for 50 and 25 μm pits and also that a considerable number of cells remained particularly in 100, 50 or 25 μm pits against the detaching treatment. Then the discrepancy between the two counting methods in the previous studies and the difference with the most effective pore size between the present and previous studies could be explained. The merit of use of fluorescent microscopy becomes clearer from those previous problems.

The average number of cells insemated is $6/(300 \mu\text{m} \times 400 \mu\text{m})$, where $300 \mu\text{m} \times 400 \mu\text{m}$ is the picture size. The cell number increased by 6-8 times on the plane substrate and by about 15 times in the array of 50 μm pits by culture of P2 cells for 4 days. The time required for cells to be doubled is estimated to be about 32 h on the plane substrate

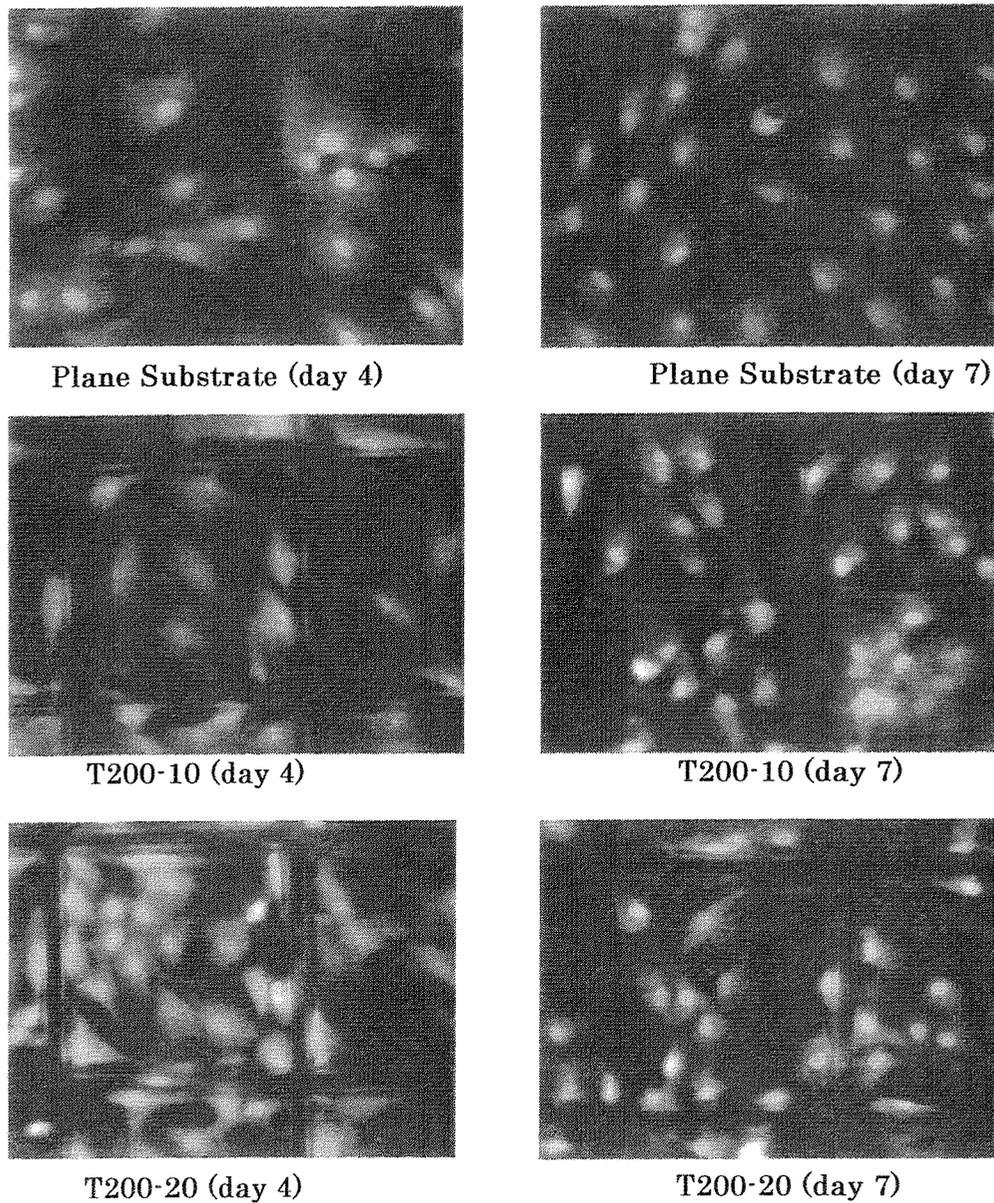


Fig. 9. Culture of bone-marrow derived cells (P4) in arrays of 200 μm square pits.

and about 24 h in the micropit array. The cell number inseminated corresponds to one cell per 5-6 pits for the 50 μm pit array. Therefore, cells proliferated actively climbing over the walls between the pits. The present results indicate that the P2 cells climbed over the walls of 10 μm width better or faster than those of 20 μm width, while P4 cells climbed over the walls of 20 μm width better or faster. Considerable differences are supposed to exist in the mechanical or motile properties between P2 and P4 cells.

We are further studying the proliferation of cells in the micropit arrays for other cell species with an expectation of considerable differences between different species.

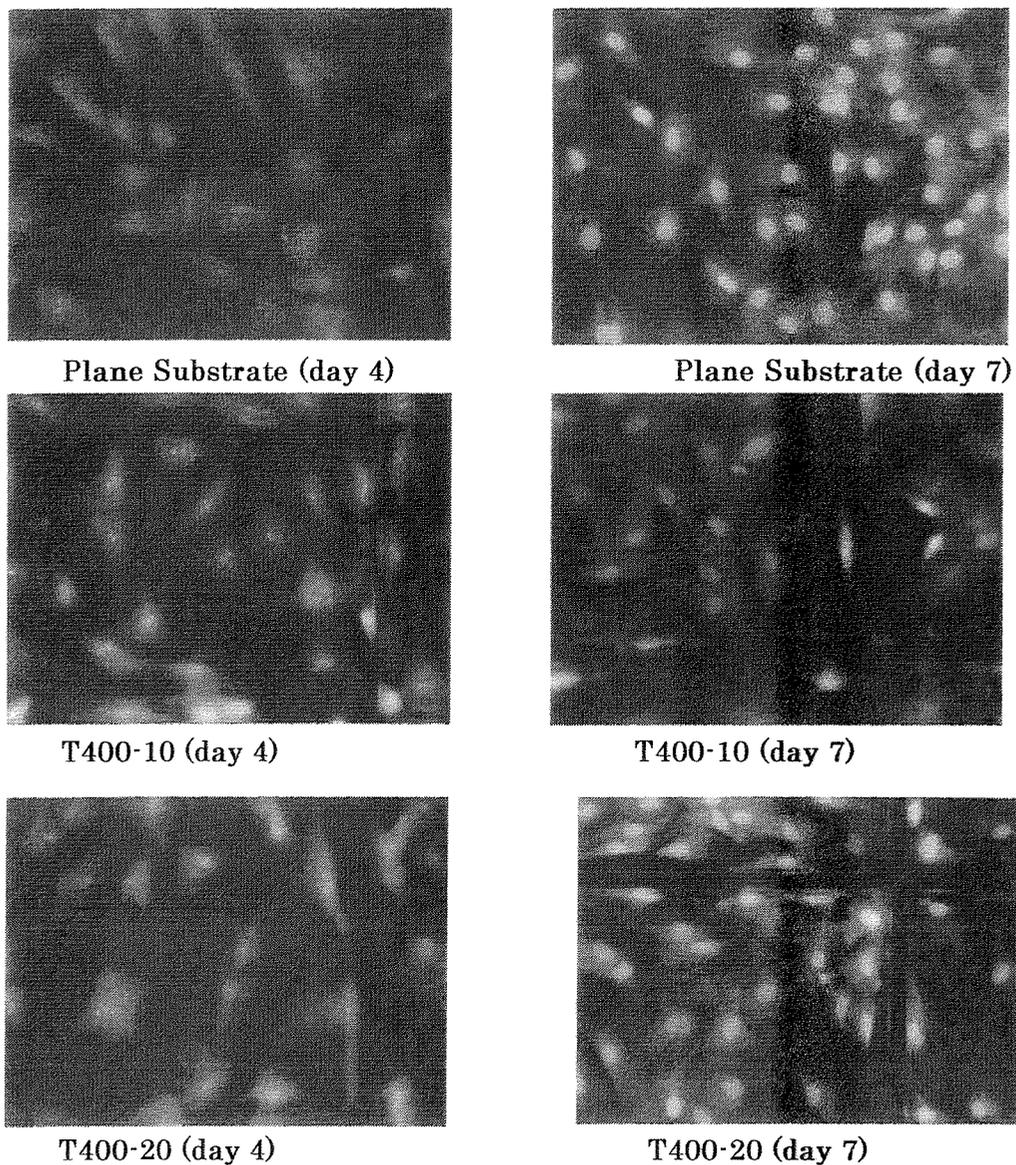


Fig. 10. Culture of bone-marrow derived cells (P4) in arrays of 400 μm square pits.

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