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Source: Zoological Science, 20(4) : 429-434

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.20.429

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ZOOLOGICAL SCIENCE **20**: 429–434 (2003)
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Growth Factor Array Fabrication Using a Color Ink Jet Printer

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ABSTRACT—We have developed a novel method for growth factor analysis using a commercial color ink jet printer to fabricate substrata patterned with growth factors. We prepared substrata with insulin printed in a simple pattern or containing multiple areas of varying quantities of printed insulin. When we cultured the mouse myoblast cell line, C2C12, on the insulin-patterned substrata, the cells were grown in the same pattern with the insulin-printed pattern. Cell culture with the latter substrata demonstrated that quantity control of insulin deposition by a color ink jet printer is possible. For further applications, we developed substrata with insulin-like growth factor-I (IGF-I) and basic fibroblast growth factor (bFGF) spotted in 16 different areas in varying combinations and concentrations (growth factor array). With this growth factor array, C2C12 cells were cultured, and the onset of muscle cell differentiation was monitored for the expression of the myogenic regulator myogenin. The ratio of cells expressing myogenin varied with the doses of IGF-I and bFGF in the sections, demonstrating a feasibility of growth factor array fabrication by a color ink jet printer. Since a printer manipulates several colors, this method can be easily applied to multivariate analyses of growth factors and attachment factors affecting cell growth and differentiation. This method may provide a powerful tool for cell biology and tissue engineering, especially for stem cell research in investigating unknown conditions for differentiation.

Keywords: myogenesis, growth factor, patterning, ink jet

INTRODUCTION

Human embryonic stem (ES) cells are essentially capable of differentiating into almost any cell type from the three embryonic germ layers. These cells can also be directed into various specific cell types when under the influence of specific molecules such as growth factors and secreted products of neighboring cells. On demand control of their developmental fate using such bioactive substances has now become an urgent need in regenerative medicine.

The extracellular environment *in vivo* is highly complicated but, certainly, is controlled or affected by the mixture of various growth factors, hormones, and other proteins associated with the extracellular matrices. In past years, it has been shown that no single growth factor is sufficient for deciding the differentiation fate of ES cells. Schuldiner *et al*. (2000) examined eight different growth factors for human ES cells and reported that none of these directed differentiation to only one cell type, but, rather, altered the relative proportions of a specific cell type. Specific conditions must exist which would allow stem cells to retain particular phenotypes *in vitro,* since this does occur *in vivo*. Consequently, if one could apply multiple growth factors in various conditions simultaneously to multipotent cells such as ES cells, it might be possible to specify conditions that would lead such cells to differentiate into certain cell types. However, in most published works, the effects of bioactive substances are examined singly, or doubly at most. In order to examine the effect of multiple bioactive substances in terms of their concentration and combination, enormous works will be required.

In the past decade, several novel approaches for studying growth factors have been examined. It has been reported that insulin and epidermal growth factor (EGF), which had been covalently immobilized onto culture substratum, sustained their mitogenic activity, and their activity was even higher than when in the soluble state (Chen *et al*., 1997; Ito *et al*., 1996; 1997a; 1997b). According to their reports, the enhancement of mitogenic activity was due to the inhibition of the internalization process of growth factors,

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resulting in continuous stimulation without down regulation of the growth factor receptors. In addition to insulin and EGF, nerve growth factor (NGF) and tumor necrosis factor (TNF) have also been reported to retain their activity in the immobilized state (Ito, 1998).

In recent years, ink jet technology has achieved such remarkable progress that it is possible to eject tiny droplets of ink (~10pl) with high precision. Among various applications of ink jet technology, there are several applications as a tool for biological experiments. Kelebe (1988) prepared a fibronectin-patterned substrate with an ink jet printer, which allowed cells to align along the printed pattern. Deposition of protein using an ink jet printer as a unique method for thin-layer immunoaffinity chromatography or simple protein microarrays has also been reported (Nilsson, 1995; Roda, 2000). Furthermore, fabrication of DNA microarrays using a color ink jet printer has attracted attention lately (Okamoto *et al*., 2000; Goldman and Gonzalez, 2000; Allain *et al*., 2001). In this study, we adopted the technique to immobilize growth factors on a substratum and applied an ink jet printer for use in analysis in terms of patterning, concentration control, and combination. In this work, we applied photoreactive growth factors so that no surface modifications of substrata are needed and easy control of growth factors were established. We synthesized photoreactive insulin, IGF-I, and bFGF, and these were printed onto substrata in different patterns, concentrations, and combinations. Here we report on this novel use of a color ink jet printer for growth factor analysis in cell-based research.

MATERIALS AND METHODS

Materials

The sources of materials used in this work were as follows. Bovine insulin, human transferrin, 4-azidobenzoic acid N-hydroxysuccinimide ester, and Hoechst 33258 were from Sigma Chemical Co. (Missouri, USA), and N,N-Dimethylformamide (DMF) from Wako Pure Chemicals Ltd. (Osaka, Japan). IGF-I and bFGF were from Upstate Biotechnology, Inc. (New York, U.S.A.), and [^{125}I]IGF-I from Amersham Pharmacia Biotech (Buckinghamshire, UK). Anti-myogenin polyclonal antibody was purchased from Santa Cruz Biotechnology, and anti-rabbit IgG Alexa-Fluor 488 conjugate antibody from Molecular Probes (Oregon, USA). Millipore Centricon Centrifugal Filter Devices YM-3 were from Millipore (Massachusetts, USA). The myoblast cell line C2C12 was kindly provided by Dr. Yoichi Nabeshima.

Preparation of growth factor printed substrata

A phenyl azido group was introduced to growth factors according to the works by Ito et al. (1996) and Matsuda and Sugawara (1995). 4-azidobenzoic acid N-hydroxysuccinimide ester in DMF and insulin in phosphate buffered saline (PBS) were mixed and stirred in an ice bath for 72 h at 4°C. After stirring, the solution was ultrafiltrated by Millipore Filter, and the photoreactive insulin was retrieved. Photoreactive IGF-I, bFGF, and [^{125}I]IGF-I were also prepared by the same protocol.

The upper cover of an ink jet printer BJ F850 (Canon; Tokyo Japan) was removed and the printing head was washed carefully with distilled water. The polystyrene substratum or silicone film was placed about 1mm beneath the printing head. Photoreactive growth factors were injected into the printing head and printed onto the substratum. By changing the repeating times of the print, substrata printed with varying amounts of growth factor were obtained. To fabricate growth factor array, photo-reactive IGF-I and bFGF were printed onto the same substratum in different patterns respectively to form 16 different combinations of IGF-I and bFGF in single substratum. Then, each growth factor was covalently immobilized to the substratum by UV irradiation with a mercury lamp (Zeiss HBO 50: 50W) at the distance of 10cm for 15sec. After the irradiation, substrata were rinsed with PBS. At each stage of this process the printing fidelity and the immobilization efficiency were examined using [^{125}I]IGF-I and measuring the gamma ray intensity in the deposits. Pre-estimated gamma ray intensity of [^{125}I]IGF-I in the deposits was compared with the measured value.

Cell culture and growth factor analysis

C2C12 cells were grown in Dulbecco's modified eagle's medium (DMEM) containing 20% fetal bovine serum (FBS). For insulin-patterned substrata and quantity controlled substrata, cells were cultured at 10^4 cells/ml and, after 24hr, growth medium was replaced with DMEM containing 2% FBS for 48hr. To examine the effect of growth factors in soluble conditions, growth medium was changed to differentiation medium, DMEM containing 5 μg/ml human transferrin, 5 nM sodium selenite, and 1 mg/ml bovine serum albumin (BSA). For growth factor array analysis, 10 μg/ml insulin was added to differentiation medium.

For soluble growth factor analysis, glass cover slips pre-treated with collagen were placed in each well of a 24-well tissue culture plate, and C2C12 cells were cultured at 2×10^4 cells/well. After 24 hr of culturing, growth medium was replaced with differentiation medium supplemented with 16 different combinations of IGF-I and bFGF (0, 2, 8, or 20 ng/ml, respectively). After 24 hr, cells were fixed with methanol and immunostained with anti-myogenin antibody then further stained with anti-rabbit IgG-Alexa Fluor 488 conjugate. Nuclei were also stained with Hoechst 33258. For growth factor array analysis, cells were inoculated with 50 cells/mm^2 in growth medium, and after 48hr of culturing, medium was changed to differentiation medium and cultured for further 24 hr, then cells were fixed and stained in the same manner mentioned above.

RESULTS

Evaluation of growth factor immobilization rate

To examine the fidelity of growth factor printing and the efficiency of immobilization, photoreactive [^{125}I]IGF-I was first synthesized. The [^{125}I]IGF-I we used in this experiment contained BSA whose quantity was more than 1000 times greater than [^{125}I]IGF-I. Since BSA is capable of being immobilized on the substrata in the same method as the growth factors, we regarded the amount of BSA as the total protein to be immobilized (Matsuda, 1995). Therefore, the approximation formula for the immobilization efficiency was calculated using the values of the BSA. The gamma ray intensity measured from the printed [^{125}I]IGF-I and the estimated value based on the quantity deposited matched very closely (Fig. 1). After the immobilization process, the substrata were rinsed with PBS. Again, gamma ray intensity was measured and compared with the value before rinsing. The result indicated that some [^{125}I] IGF-I was washed away during the rinsing process. The immobilization efficiency was dose-dependent and an approximation formula was obtained ($y=-0.0005x+0.8857$).

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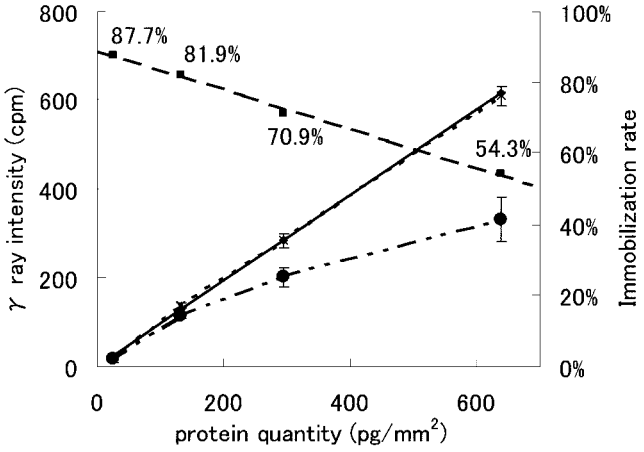


Fig. 1. Fidelity of growth factor printing by an ink jet printer and an immobilization rate of [^{125}I] IGF-I. Gamma ray intensity of the substratum was measured immediately after the printing of [^{125}I] IGF-I (×) and after UV irradiation and PBS rinsing (●). Pre-estimated gamma ray intensity printed is also shown (◆). From this result, an approximation formula for immobilization rate of [^{125}I] IGF-I was obtained: y=–0.0005x+0.8857 (■).

Patterning and concentration control of photo-reactive insulin

Photoreactive insulin was printed and immobilized on a silicone film in simple patterns. When C2C12 cells were cultured on the insulin-patterned substrata, cell growth was largely enhanced in the insulin-printed area (Fig. 2). Then, we investigated the validity of the quantity control of depositing growth factors by the ink jet printer. Substrata consisting of multiple areas which varied with the number of printing times used were obtained, and C2C12 cells were cultured on these in low serum medium (1% horse serum in DMEM) for 48hr. Although little mitogenic activity was observed in the areas with no insulin printed, a marked effect on the cell growth was observed in the insulin-printed areas in a dose-dependent manner (Fig. 3A & B).

Growth factor analysis using growth factor arrays

Growth factor arrays consisting of 16 areas of various combinations of IGF-I and bFGF were fabricated. The amount of growth factors used was calculated according to the approximation formula obtained from the experiments with [^{125}I]IGF-I. IGF-I was deposited at 0, 21, 64, 149 pg/mm^2 and bFGF at 0, 41, 79, 175 pg/mm^2. C2C12 cells were inoculated on the growth factor array, followed by 48 hr culturing in growth medium, and then culturing in differentiation medium for further 24 hr. In order to compare the effect of growth factors immobilized on the array with the effect of those in a soluble state, C2C12 myoblasts were also cultured under 16 different conditions corresponding to combinations of four different concentrations (0, 2, 8, 20 ng/ml) of IGF-I and bFGF. For both experiments, the onset of myogenin expression was examined. When C2C12 cells were cultured on the growth factor arrays, the myogenin expression ratio varied among the different combinations of IGF-I and

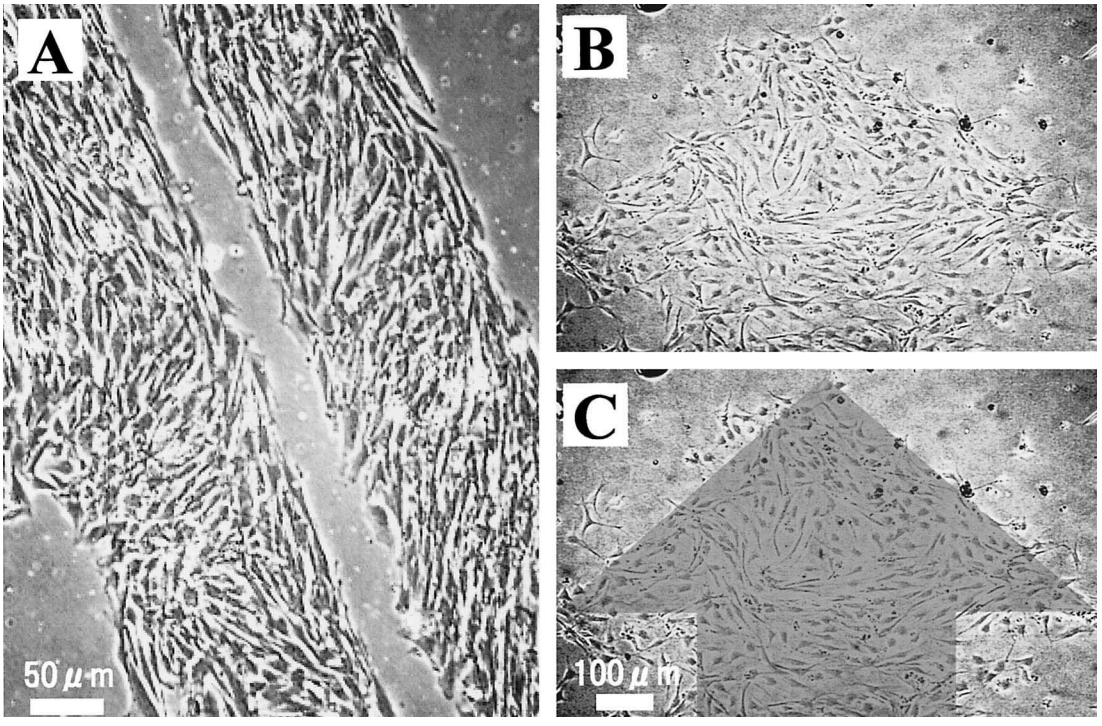


Fig. 2. Phase contrast images of C2C12 cells cultured on insulin-printed silicone films. Cells were grown in the aligned insulin-printed area (A). In the insulin printed area (arrow shape), the number of cells significantly increased (B). To indicate the insulin printed area in B, arrow shape image was superposed on the original image (C). B and C are same magnification. Bar, 50 μm (A), 100 μm (B and C).

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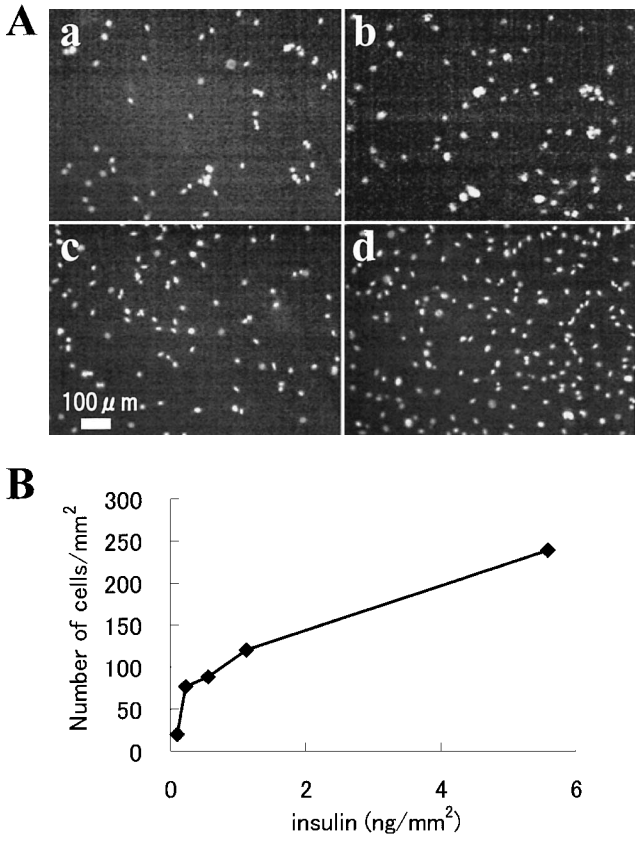


Fig. 3. Effect of quantity-controlled insulin on C2C12 growth. More cells were observed in areas with higher amounts of printed insulin (A). The amount of insulin immobilized on the substrata were 0.22 ng/mm^2 (a), 0.56 ng/mm^2 (b), 1.1 ng/mm^2 (c), and 5.6 ng/mm^2 (d). The number of the cells increased in an insulin dose-dependent manner (B).

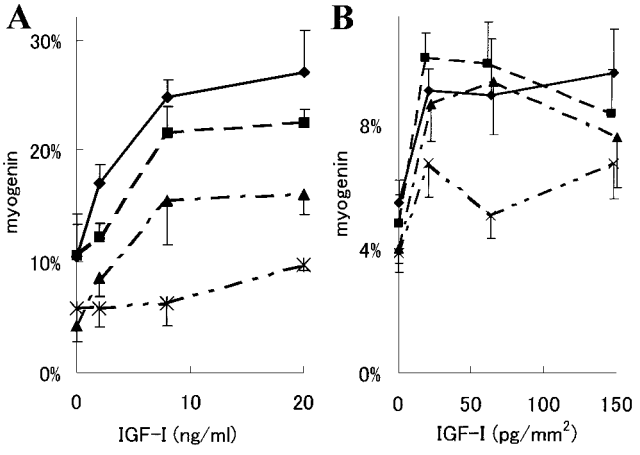


Fig. 4. Effect of IGF-I and bFGF on myogenin expression. (A): C2C12 cells were treated with soluble IGF-I (0, 2, 8, 20 ng/ml) in combination with soluble bFGF (0 (◆), 2(■), 8(▲), 20(×) ng/ml). (B): C2C12 cells were cultured on growth factor arrays containing 16 sections consisting of IGF-I (0, 21, 64, 149 pg/mm^2) and bFGF (0 (◆), 41 (■), 79 (▲), 175 (×) pg/mm^2).

bFGF (Fig. 4B). However, the myogenin expression pattern was different from that of the soluble growth factors. For soluble growth factors, the effect of IGF-I was remarkably high at 8 ng/ml, and approximately 20 ng/ml of IGF-I appeared to be close to saturation (Fig. 4A). On the growth factor arrays, the ratio of the myogenin expression increased with 21 pg/mm^2 of IGF-I, though the higher quantity of IGF-I barely increased its stimulation. In contrast, the suppressive effect of bFGF was very clear for soluble bFGF. However, the effect of the bFGF immobilized on the growth factor array seemed to be weaker. Only the concentration of 175 pg/mm^2 bFGF lowered the myogenin expression ratio.

DISCUSSION

Growth factor printing and immobilization

Lately, the ink jet printer has become so sophisticated that the picture printed is now as fine as a film photo, which indicates that the positional control and the quantity control of ink ejection are very precise. However, it was necessary to confirm the fidelity and reproducibility of growth factor ejection, because the printing conditions are very different from the normal uses of an ink jet printer. In order to monitor the growth factor deposition, we synthesized [^{125}I]IGF-I and checked the printing quantity and quality. The growth factor was found to be ejected with high accuracy, although the immobilization efficiency decreased as the quantity of growth factor increased. According to previously published work, growth factors immobilized with a phenyl-azido arm may form multi-layers on the substrata (Ito *et al*., 1996; 1997). This result suggests that when the quantity of the printed growth factors increases, these growth factor may form multi-layers, preventing a direct reaction with the substratum, and some of those might be washed away by rinsing with PBS.

Patterning and concentration control of insulin

Photoreactive insulin was printed on silicone film in several patterns. After 48hr of culturing in low serum conditions (1% horse serum: HS), the cell density became remarkably high in the insulin-printed area. We have also performed the same experiment with substrata printed with BSA instead of insulin. Although there were some cells on the BSA printed area, little effect on cell growth was observed (data not shown). Therefore, the promotion of growth on the insulin-printed area is due to not only the enhancement of cell attachment but also the original mitogenic effect of insulin, which suggests that the insulin retains its activity even in the immobilized state. This result matches with the work of Ito *et al*. (1996). In the substratum that contained multiple areas with different amounts of insulin immobilized, cells were cultured in DMEM containing low HS (1%). After 48hr of culture, the density of the cells was greater in the area with a high amount of printed insulin, while little increase in cell number was observed in the low insulin printed area. As the amount of insulin printed increased, the density of the cells

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increased, which suggests that the quantity control of growth factor deposition by the color ink jet printer was successful.

Growth factor array analysis

To study the feasibility of using growth factor arrays, we fabricated arrays consisting of IGF-I and bFGF. We first confirmed the effect of soluble IGF-I and bFGF for myogenic differentiation. Several laboratories have shown that soluble IGF-I, at concentrations up to 20 ng/ml, promotes myogenic differentiation in a dose-dependent manner (Magri *et al*., 1981; Ewton and Florini, 1981; Milansinic, 1996). On the other hand, it has also been reported that bFGF inhibits expression of the myogenic transcription factor MyoD (Florini, 1989, 1991; Vaidya, 1989). In the present work, bFGF inhibited the myogenin expression induced by IGF-I in a dose-dependent manner. Interestingly, soluble bFGF did not completely inhibit the myogenin expression. Approximately 5% of the cells remained myogenin-positive. According to the previous work, cultured muscle cells synthesize substantial amounts of IGF-I, which may cause the basal myogenin expression (Tollefsen *et al*., 1989).

Compared to that of soluble growth factors, the effect of IGF-I immobilized on the growth factor array was lower. There are two possibilities for this discrepancy. The first possibility is a weakened activity of photo-reactive IGF-I from introducing the azido-phenyl group. In this method, the azido-phenyl group is supposed to be introduced to the amino groups of the N-terminus or to lysine residues. Since IGF-I contains 3 lysine residues, this chemical modification may cause conformational changes that can interfere with its interaction with the IGF-I receptor. The same problem might have occurred in bFGF as well, because bFGF also has a large number of lysine residues (14 residues). The other possibility is an excess of IGF-I. A previous work reported that excess amounts of soluble IGF-I (360 ng/ml) nearly abolish its promotive effect on myogenic differentiation (Florini, 1986). Therefore, the lowest quantity of IGF-I in this growth factor array (21 pg/mm^2) might have already exceeded optimum concentrations. As for bFGF, it elicited an inhibitory effect at its highest concentration in the growth factor array (175 pg/mm^2). However, the inhibitory effect of bFGF seemed to be rather ambiguous. Similar to the case of IGF-I, conformational changes of bFGF, due to the introduction of the azido-phenyl groups, might have affected its activity, or the amount of IGF-I might have been too excessive for bFGF to suppress its effect.

Ito *et al*. (2001) reported that the effect of epidermal growth factor (EGF) on PC12 cells depends on its state, soluble or insoluble. According to their reports, for PC12 cells, soluble EGF shows a mitogenic effect, while immobilized EGF stimulates differentiation. The authors assumed that since immobilized EGF could not be internalized by the cells the duration of MAPK activation was elongated, resulting in a similar effect to that of nerve growth factor (NGF) in soluble state, which activates MAPK longer. As a result, the effect of immobilized EGF became NGF-like, promoting nervous differentiation. Taking this possibility into account, in our results, the effect of IGF-I and bFGF might have changed when they were immobilized. The different effect of immobilized IGF-I and bFGF from those in soluble state in the present work, suggests an urgent need for seeking alter-

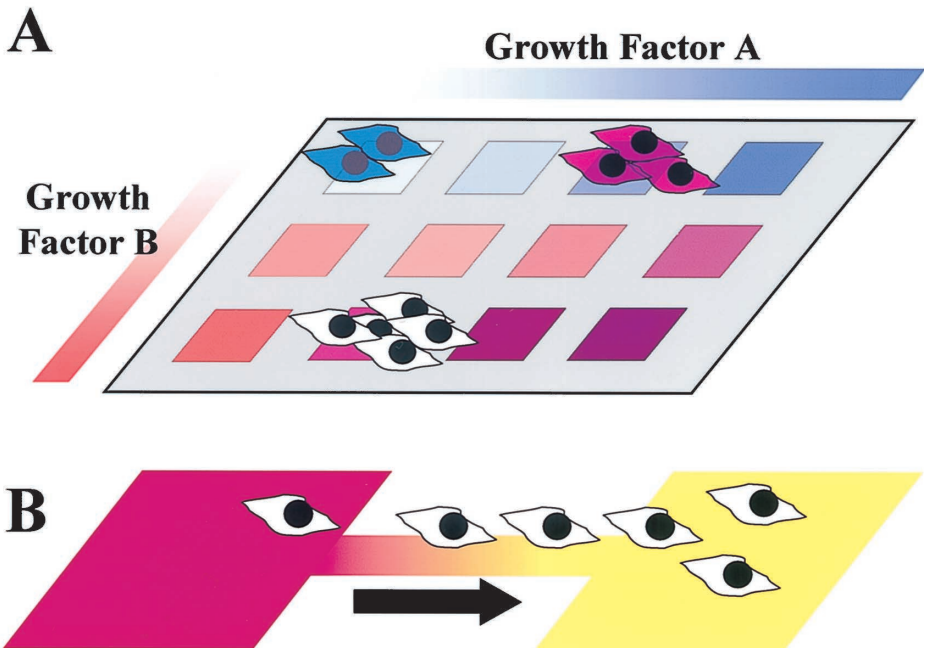


Fig. 5. Expected application models of color ink jet printer for growth factor analyses. (A), a simple growth factor array consisted of two different growth factors, which we fabricated in this work. (B), a model of chemotactic analysis by gradient pass between two different growth factors. In this figure, color gradation represents the concentration of each growth factor.

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native immobilization technique of growth factors such as introducing a chemical arm in different functional groups or inserting a longer arm.

In most previous works, growth factors were immobilized and patterned by a photolithograph technique. On the other hand, the ink jet printer can overcome the limitations of the photolithograph method such as quantity control, on-demand patterning, and the analysis with multiple samples. The advantages of using a growth factor array and its fabrication by a color ink jet printer are unquestionable. There are many possible applications, such as arrays for checking differences in growth factor sensitivity between individuals (Fig. 5A). By applying its flexibility to fabricate the desired pattern, it may be used for chemotaxis analysis (Fig5 B). In addition, by combining immobilized growth factor with soluble factors or extracellular matrices, we may produce a very complicated environment *in vitro*. This method will provide a powerful tool for tissue engineering and regenerative medicine, especially for determining the differentiation conditions necessary for ES cells.

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