Microarrays

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Generation of Live-Cell Microarrays by Means of DNA-Directed Immobilization of Specific Cell-Surface Ligands**

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Microarray technology has become one of the principal platform technologies for the high-throughput analysis of biological systems.[1] Starting with the evolution of DNA microarrays in the 1990s, the developments of peptide and protein microarrays to elucidate interaction partners, modification sites, and enzyme substrates,[2,3] this technology is nowadays moving towards the construction of microarrays of fixed-tissue samples[4] and live cells.[5] The latter are expected to help unravel complex cellular traits in both healthy and diseased states because cell microarrays provide the ability to molecularly delineate the characteristics of individual cells from complex mixtures. By immobilizing different cell-capture and analysis reagents on a solid support, mixtures of cells can be rapidly interrogated for their composition and phenotype, thus helping to identify and quantitate distinct cell types based on the expression of particular cell-surface molecules. Furthermore, this immobilization helps to analyze the response of different cell types to defined signals through the secretion of specific factors or other measurable cellular activities.[5]

To immobilize cell-capture moieties on suitable solid supports, proteins, peptides, or other ligands are usually attached to surfaces by using covalent coupling, chemisorption, or physisorption processes in combination with robotic spotting procedures.[1,5,6] Motivated by the extraordinary performance of the DNA-directed immobilization (DDI) of proteins and other molecular and colloidal components,[7] we herein describe that micropatterns of cell-surface ligands can be generated on DNA microarrays by the DDI method and that the resulting surfaces are suitable substrates for the growth of fibroblast cells (Figure 1). This approach should have significant advantages over conventional spotting procedures because it would enable the implementation of the power of DNA microarrays as decoding tools in combinatorial synthesis and screening of ligands[8] into cellular biology research. To experimentally investigate this hypothesis, we chose the well-studied recognition of RGD peptide ligands by integrin surface receptors of fibroblast cells as the model system.[9–11]

As depicted in Figure 1, covalent conjugates of streptavidin (STV) and single-stranded DNA (ssDNA), were used as molecular adaptors in the DNA-directed immobilization of biotinylated peptides. To this end, three different DNA–STV conjugates (**F1**, **F5**, **F10**, shown schematically in Figure 1) containing the 22-mer oligonucleotides **tF1**, **tF5**, and **tF10**, respectively, were prepared as previously described.[12,13] In separate reaction tubes, conjugates **F1** and **F5** were then coupled with one molar equivalent of the biotinylated peptide **bRGDF** (biotin-Gly_5-Arg-Gly-Asp-Phe-COOH),[10] and, as a control, **bG5** (biotin-Gly_5-COOH), thus leading to conjugates **F1–bRGDF** and **F5–bG5**, respectively. To allow for visualization of the immobilized conjugates by fluorescence analysis, five molar equivalents of biotinylated fluorescent dye Cy5 were added to the **F1** and **F5** conjugates subsequent to the coupling of the peptide. To estimate specificity of the proposed cell attachment, another control conjugate was prepared from **F10** by coupling of six molar equivalents of biotinylated Cy5 (conjugate **F10–Cy5**) and subsequent saturation of the remaining biotin binding sites with free D-biotin.[14] The conjugates were immobilized by hybridization on a DNA microarray containing three complementary capture oligonucleotides, **cF1, cF5**, **cF10**. The capture oligonucleotides were immobilized on commercially available H^P slides (Chimera Biotec, Dortmund) containing a poly(amidoamine) (PAMAM) dendrimer coating[15] modified with polyethylene glycol spacers and reactive epoxide end groups. Furthermore, the DNA array contained a fourth oligonucleotide (**cF9**) that is not complementary to any of the conjugates (Figure 2a) to allow for estimation of nonspecific hybridization and cell attachment. Figure 2b shows a typical fluorescence image of such an array, obtained after hybrid-

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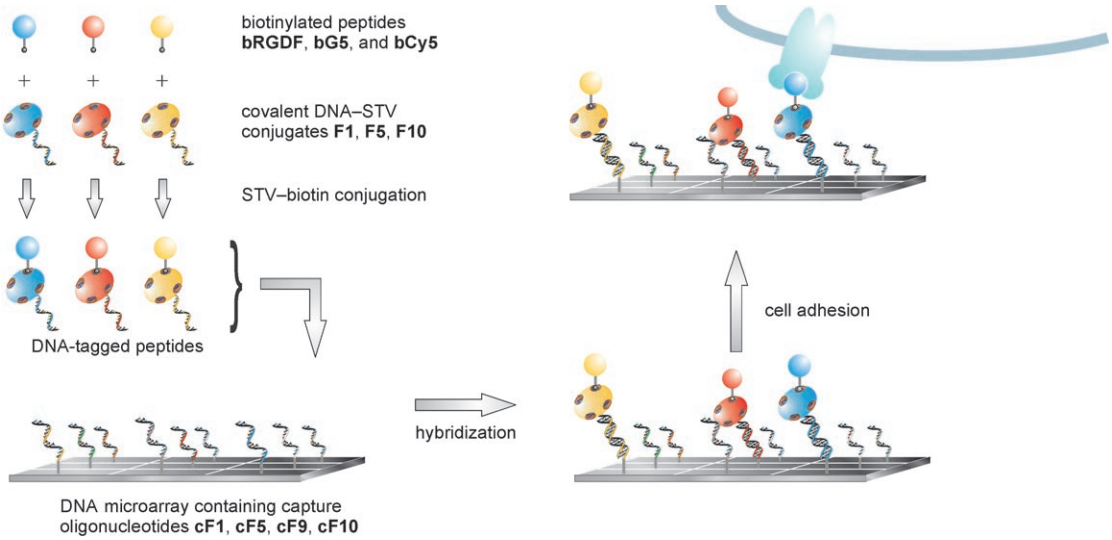

Figure 1. Schematic representation of the generation of live-cell arrays by DNA-directed immobilization of cell-specific ligands. The biotinylated ligands **bRGDF**, **bG5**, and **bCy5** are coupled to covalent conjugates of streptavidin and single-stranded DNA (DNA–STV **F1**, **F5**, and **F10**, respectively), hybridized with a DNA microarray containing complementary capture oligonucleotides, and the resulting array is then used for the adherence of NIH-3T3 fibroblasts.

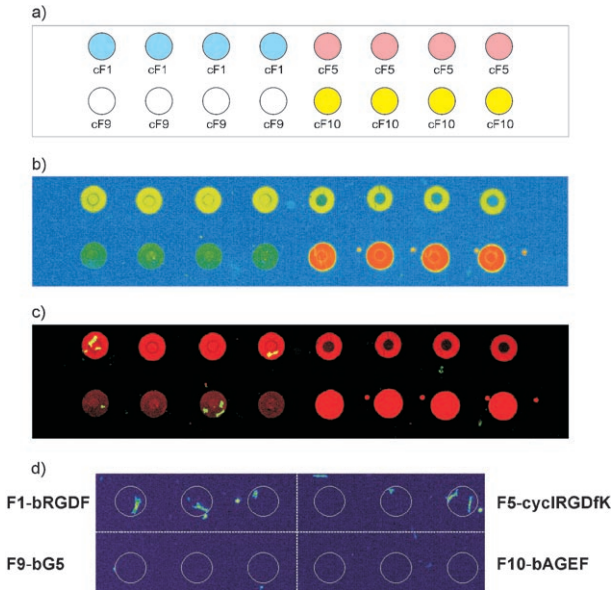

Figure 2. Typical microarrays obtained in the course of experiments described in Figure 1. a) Allocation of capture oligonucleotides **cF1**, **cF5**, **cF9**, **cF10** at the DNA microarray. b) Fluorescence image of the DNA microarray hybridized with a mixture of the DNA–STV conjugates **F1**–**bRGDF**, **F5**–**bG5**, **F10**–**Cy5**. Note that conjugates **F1**–**bRGDF** and **F5**–**bG5** contain less Cy5 labels and therefore yield lower fluorescent signals than **F10**–**Cy5**. The observed ringlike structures are due to the so-called doughnut effect, occurring during the drying of the spotted capture oligomers. The spot size of the shown arrays is 250 μm. c) Two-color overlay image of the array subsequent to adhesion of fibroblast cells. The grown cells are stained with the TRITC-conjugated phalloidin and are therefore visible as green objects. d) Reconfigured array containing four different peptide ligands after adhesion of fibroblast cells. For clarity, only the green fluorescence signals are shown.

ization of the labeled DNA–STV conjugates. Cy5 signals of high intensity were observed in the spots where complementary capture oligomers were bound (**cF1**, **cF5**, **cF10**, in Figure 2a), whereas in spots containing noncomplementary **cF9**, the low fluorescent signals corresponded to about 7% of the signal intensity observed for **F10**–**Cy5**, which was entirely labeled with biotinylated Cy5. This indicated that the various DNA–STV conjugates hybridized with their complements with high specificity, a result that is in full agreement with previous studies concerning the specific immobilization of these STV–DNA conjugates by the DDI method.[13,16]

The protein/peptide DNA array containing slides were then used as substrates for the adhesion of NIH-3T3 fibroblasts. To this end, cells at passage 5–25 were trypsinized and plated on the array at a density of 2×10^4 cells mL^{-1} in Dulbecco's modified eagle's medium (DMEM). After 12–24 h, cells were washed with buffer, fixed with formaldehyde and stained with tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin. The latter reagent is a high-affinity probe for F-actin prepared from the mushroom toxin phalloidin conjugated to fluorescent TRITC dye. Analysis of the slides with a microarray scanner allowed for the direct visualization of cell adhesion by taking advantage of the green (TRITC) and red (Cy5) detection channels. As shown in Figure 2c, cell adhesion preferentially occurred at spots containing either the **F1**–**bRGDF** or no DNA–protein conjugate (i.e., spots containing only capture oligomer **cF9**). In fact, counting the cells for a large number of spots ($n=384$) revealed a ratio of about 1:2:5:14 for the spots with **F10**–**Cy5**/**F5**–**bG5**/**F1**–**bRGDF**/**cF9**. Notably, the number of cells adhered to the **F10**–**Cy5** modified spots of the slide was similar or even smaller than in the background regions, which contained only the polyethylene glycol passivated areas.

The comparison of spots containing **F10**–**Cy5** or **cF9** indicated that the tendency of the NIH-3T3 fibroblasts to adhere to ssDNA-containing spots can be effectively suppressed by hybridization of the complementary DNA–STV conjugate (e.g. **F10**–**Cy5**) and thus the "capping" of the **cF9**

single-stranded capture oligomers. Quantitative assessment of the spots carrying different oligonucleotide–peptide conjugates revealed that the preference of the cells for spots containing the RGD motif as compared with those containing the **bG5** peptide in the majority of the cases varied between 10:1 and 10:4. Careful adjustment of the culture and washing procedures was identified as the main reason for the observed variation.[14]

To further demonstrate that our approach is suitable for the screening of diverse ligands, we took advantage of the modular chip design and reconfigured the above DNA capture array with different DNA–peptide conjugates (Figure 2d). To this end, a second control peptide, the nonapeptide biotin-Gly5-Ala-Gly-Glu-Phe-COOH denoted as **bAGEF** and containing two point mutations with respect to the RGDF motif, was coupled to the DNA–STV conjugate **F10** (**F10–bAGEF**). Also, a pentapeptide containing the cyclized RGDfK motif,[10] denoted as **b-cyclRGDfK** (see Figure S1 in the Supporting Information) was coupled with the DNA–STV conjugate **F5** (**F5–cyclRGDfK**). In addition, the conjugates **F9–bG5** and **F1–bRGDF** were also immobilized on the chip. Only very low numbers of cells were adhered on the **bAGEF**-containing spots, which is approximately similar in quantity to that observed for **bG5** spots. In contrast, the number of cells adhered to the **b-cyclRGDfK**-modified spots was comparable with that observed for the **bRGDF** spots (Figure 2d). These results clearly confirm that the effects observed for adhesion result from the RGD motif rather than from a variation of peptide lengths or DNA sequence.

Closer analysis of the cells by fluorescence microscopy revealed significant differences in the morphology of the adhered cells (Figure 3). In spots containing **F1–bRGDF** or **cF9**, the fibroblasts showed the normal spindle or pyramidal morphology with a well-defined cytoskeleton visible through the stress fibres within the cytoplasm (Figure 3a,b). Moreover, extended periods of growth of the cells adhered to spots containing **F1–bRGDF** showed that the entire spot is populated after about 40 h (see Figure S7 in the Supporting Information).[17] This indicates that the cells are capable of propagation. Thus, they can be cultured on the microarray and they behave similar to classical tissue culture. The observed preference of the NIH-3T3 fibroblasts to adhere to the ssDNA-containing spots was surprising and the reason for this phenomenon is presently unclear.[18] It is known that the cell surface carries a net negative charge and, thus, one should expect Coulomb repulsion to occur between the DNA polyanion and the cell. Therefore, possible explanations include electrostatic attraction owing to the shielding of the DNA's negative charges by increased concentrations of divalent cations or positively charged membrane proteins, or the existence of receptor moieties at the cell's surface. A detailed analysis of this phenomenon by using immunostaining and live-cell microscopy is under way.

In contrast with the above-described morphologies, cells adhered to either the spots containing **F5–bG5**, **F10–Cy5**, **F10–bAGEF**, or the glass surface (Figure 3c,d,e and Figure S6 in the Supporting Information, respectively), revealed a round morphology, and showed increased outgrowth of

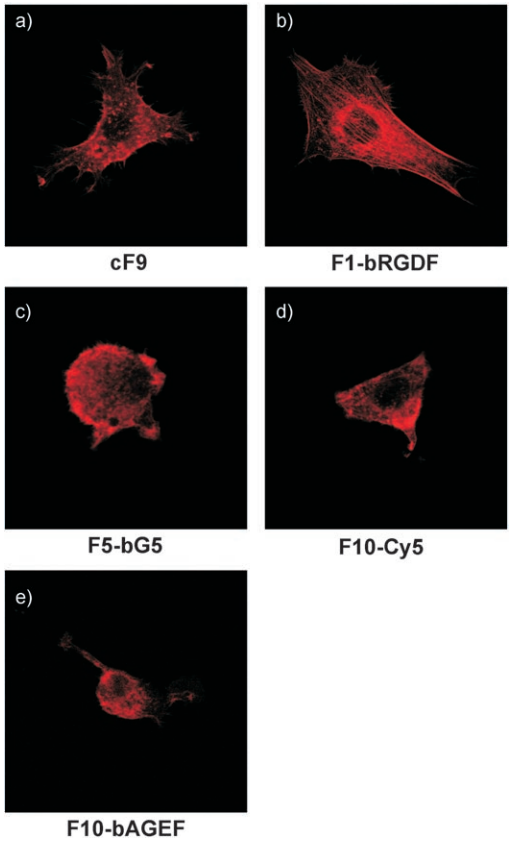

Figure 3. Representative fluorescence microscopy images (64-fold magnification) of cells grown on individual spots of the array shown in Figure 1 and 2. e) Representative image of a cell on spots with **F10–bAGEF** conjugate shown in Figure 2d.

filopodia, the radial thin extensions of the cell that are needed for attachment at substrate surfaces. This morphology is typical for cell growth on substrates lacking suitable attachment points that are needed for the development of a normal phenotype. Because integrin-mediated cell spreading and focal-adhesion formation trigger survival and proliferation of anchorage dependent cells, the absence of attachment causes the formation of round cell shapes and often apoptosis.[10] Therefore, our observation of round-shaped cells adhered to surface sites containing **F10–Cy5**, **F5–bG5**, **F10–bAGEF**, or the surrounding passivated glass surface are in agreement with the expectation that the fibroblasts can not form proper focal contacts with these surfaces. In contrast, the regular cell morphology observed on spots containing either the RGD motif and, surprisingly, ssDNA suggest that the NIH-3T3 cell line studied herein is indeed capable of forming the focal contacts necessary to develop the normal phenotype.

In conclusion, we herein demonstrated that DNA microarray technology can be adopted to the generation of live cell arrays. By using DNA microarrays for the controlled immobilization of combinatorial libraries of cell-specific ligands, positionally encoded cell arrays can be generated that contain individual spots colonized by small populations of live cells which are affected by the presence of the immobilized ligands. Given the unprecedented affinity of cells to adhere to DNA surfaces, the employment of DNA arrays might also open up

new possibilities for the design of cell arrays by taking advantage of self-assembled DNA nanoarchitecture to implement additional functionality, such as switchable release and mechanical properties to the surfaces.[19,20] We therefore anticipate that the approach described herein should be useful not only for fundamental research in molecular cell biology but also, for instance, as a screening platform for applications in the area of drug development.

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[1] J. Sobek, K. Bartscherer, A. Jacob, J. D. Hoheisel, P. Angenendt, *Comb. Chem. High Throughput Screening* **2006**, *9*, 365 – 380.
[2] D. H. Min, M. Mrksich, *Curr. Opin. Chem. Biol.* **2004**, *8*, 554 – 558.
[3] K. Y. Tomizaki, K. Usui, H. Mihara, *ChemBioChem* **2005**, *6*, 782 – 799.
[4] T. Braunschweig, J. Y. Chung, S. M. Hewitt, *Expert Rev. Proteomics* **2005**, *2*, 325 – 336.
[5] D. S. Chen, M. M. Davis, *Curr. Opin. Chem. Biol.* **2006**, *10*, 28 – 34.
[6] Orthogonal chemical coupling strategies, which allow for directional and oriented immobilization of small molecules and whole proteins, are essential for the generation of arrays in which the immobilized probes retain their undisturbed biological functionality. For examples, see: M. N. Yousaf, M. Mrksich, *J. Am. Chem. Soc.* **1999**, *121*, 4286 – 4287; C. D. Hodneland, Y.-S. Lee, D.-H. Min, M. Mrksich, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5048 – 5052; M. Köhn, R. Wacker, C. Peters, H. Schröder, L. Soulere, R. Breinbauer, C. M. Niemeyer, H. Waldmann, *Angew. Chem.* **2003**, *115*, 6010 – 6014; *Angew. Chem. Int. Ed.* **2003**, *42*, 5830 – 5834; ; A. Watzke, M. Köhn, M. Gutierrez-Rodriguez, R. Wacker, H. Schröder, R. Breinbauer, J. Kuhlmann, K. Alexandrov, C. M. Niemeyer, R. S. Goody, H. Waldmann, *Angew. Chem.* **2006**, *118*, 1436 – 1440; *Angew. Chem. Int. Ed.* **2006**, *45*, 1408 – 1412; ; A. Dantas de Araújoa, J. M. Palomoa, J. Cramer, M. Köhn, H. Schröder, R. Wacker, C. M. Niemeyer, K. Alexandrov, H. Waldmann, *Angew. Chem.* **2006**, *118*, 302 – 307; *Angew. Chem. Int. Ed.* **2006**, *45*, 296 – 301; ; A. Watzke, M. Gutierrez-Rodriguez, M. Köhn, R. Wacker, H. Schröder, R. Breinbauer, J. Kuhlmann, K. Alexandrov, C. M. Niemeyer, R. S. Goody, H. Waldmann, *Bioorg. Med. Chem.* **2006**, *14*, 6288 – 6306, and references therein.
[7] C. M. Niemeyer, *Int. J. Environ. Anal. Chem.* **2005**, *85*, 639 – 643.
[8] M. Lovrinovic, C. M. Niemeyer, *Angew. Chem.* **2005**, *117*, 3241 – 3246; *Angew. Chem. Int. Ed.* **2005**, *44*, 3179 – 3183; .
[9] K. E. Gottschalk, H. Kessler, *Angew. Chem.* **2002**, *114*, 3919 – 3927; *Angew. Chem. Int. Ed.* **2002**, *41*, 3767 – 3774.
[10] U. Hersel, C. Dahmen, H. Kessler, *Biomaterials* **2003**, *24*, 4385 – 4415.
[11] The integrin-based attachment of fibroblast cells to micro- and nanostructured surfaces has previously been studied by using RGD and related peptide motifs bound through chemisorption to gold surfaces. For example, see B. T. Houseman, M. Mrksich, *Biomaterials* **2001**, *22*, 943 – 955; M. Arnold, E. A. Cavalcanti-Adam, R. Glass, J. Blummel, W. Eck, M. Kantlehner, H. Kessler, J. P. Spatz, *ChemPhysChem* **2004**, *5*, 383 – 388; E. A. Cavalcanti-Adam, A. Micoulet, J. Blummel, J. Auernheimer, H. Kessler, J. P. Spatz, *Eur. J. Cell Biol.* **2006**, *85*, 219 – 224). For the effects of nanotopography roughness and substrate effects of cellular function, see: M. J. Dalby, M. O. Riehle, D. S. Sutherland, H. Agheli, A. S. Curtis, *Eur. J. Cell Biol.* **2004**, *83*, 159 – 169, and references therein. The deposition of fibronectins by dip-pen nanolithography has been reported: K. B. Lee, S. J. Park, C. A. Mirkin, J. C. Smith, M. Mrksich, *Science* **2002**, *295*, 1702 – 1705.
[12] R. Wacker, C. M. Niemeyer in *Current Protocols in Nucleic Acid Chemistry*, *Vol. Supplement 21* (Ed.: D. E. Bergstrom), Wiley, New York, **2005**, pp. 12.17.11 – 12.17.15.
[13] U. Feldkamp, R. Wacker, W. Banzhaf, C. M. Niemeyer, *ChemPhysChem* **2004**, *5*, 367 – 372.
[14] Oligonucleotide sequences, detailed protocols for preparation of the DNA array, synthesis of biotinylated peptides, preparation of the DNA–STV conjugates, and cell culture as well as additional microscopic images of array adhered cells are available in the Supporting Information.
[15] R. Benters, C. M. Niemeyer, D. Wöhrle, *ChemBioChem* **2001**, *2*, 686 – 694.
[16] R. Wacker, C. M. Niemeyer, *ChemBioChem* **2004**, *5*, 453 – 459.
[17] We observed that the fibroblast cells, in principle, can be cultured on all the various peptide–DNA surfaces investigated in our study. We assume that this propagation occurs owing to favorable cell–cell interactions that overrule the influence of cell–surface interaction. However, we observed marked differences in growth rates, such that cells grow with normal characteristics on peptide spots, whereas those adhered to the glass surface showed strongly decreased growth. The different growth rates are the reason that first the spots and only subsequently the surrounding areas are colonized with cells after about 40 h and 80 h of cultivation, respectively (see Figure S8 in the Supporting Information).
[18] Initial results from a study with HeLa cells (ATCC Nr. CCL-2) revealed that this cell line also preferentially adheres to the DNA-/peptide-modified spots instead of the passivated background area of the glass slide. In particular, spots containing only the DNA capture oligomers are colonized effectively (see Figure S9 in the Supporting Information).
[19] N. C. Seeman, *Nature* **2003**, *421*, 427 – 431.
[20] U. Feldkamp, C. M. Niemeyer, *Angew. Chem.* **2006**, *118*, 1888 – 1910; *Angew. Chem. Int. Ed.* **2006**, *45*, 1856 – 1876.

