Chapter 10

Transfected Cell Microarrays for the Expression of Membrane-Displayed Single-Chain Antibodies

Baochuan Lin and James B. Delehanty

Abstract

Transfected cell microarrays, arrays of cells expressing defined cDNAs, are promising technologies that can enable the functional analysis of many proteins in parallel. This technique has been adapted for the comparative functional analysis of single-chain antibodies (scFvs) and to facilitate the screening and characterization of these antibodies for their use in diagnostic and therapeutic applications. In this method, membrane-targeting expression vectors encoding scFvs are mixed with transfection reagents and are deposited at high density onto a microscope slide. Adherent mammalian cells are subsequently added to the printed array. Upon attachment to the substrate, the cells take up the plasmid DNA and express the particular protein encoded at each location. The result is an array whose features are micrometer-sized clusters of cells expressing defined genes. This approach provides for the high-throughput functional analysis of many different proteins in parallel and can be considerably more informative and cost-effective relative to more traditional protein expression techniques.

Key words: Transfected cell microarray, single-chain antibody (scFv), reverses transfection, high-throughput functional analysis, immunoglobulin, fusion protein.

1. Introduction

The human genome project has fostered the development of high-throughput methodologies to obtain and analyze nucleic acid sequence information. DNA microarrays, collections of hundreds to thousands of nucleic acid probes immobilized on a solid support, have been a key technology to emerge from this effort. The spatial separation of the immobilized DNA probes allows for the interrogation of large numbers of targeted complementary nucleic acid sequences simultaneously. A variety of microarray formats has been developed for gene expression

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14. ABSTRACT
Transfected cell microarrays, arrays of cells expressing defined cDNAs, are promising technologies that can enable the functional analysis of many proteins in parallel. This technique has been adapted for the comparative functional analysis of single-chain antibodies (scFvs) and to facilitate the screening and characterization of these antibodies for their use in diagnostic and therapeutic applications. In this method membrane-targeting expression vectors encoding scFvs are mixed with transfection reagents and are deposited at high density onto a microscope slide. Adherent mammalian cells are subsequently added to the printed array. Upon attachment to the substrate, the cells take up the plasmid DNA and express the particular protein encoded at each location. The result is an array whose features are micrometer-sized clusters of cells expressing defined genes. This approach provides for the high-throughput functional analysis of many different proteins in parallel and can be considerably more informative and cost-effective relative to more traditional protein expression techniques.

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profiling, genetic polymorphism analysis, and microbe detection and diagnosis. Building on the principle of DNA microarrays, transfected cell microarrays, arrays of cells expressing defined cDNAs, have emerged as a methodology for analyzing gene function within the context of mammalian cells (1, 2). A transfected cell microarray is composed of "cDNAs" in the form of plasmids deposited at high density onto a microscope slide, where each spot can carry either one or a collection of different cDNAs and adherent mammalian cells that are subsequently added to the printed array. The cells internalize the plasmid DNA upon contact with the deposited DNAs on the spots and subsequently express the specific plasmid-encoded protein(s) at each location. The result is an array whose features are micrometer-sized clusters of cells expressing defined genes at a high-spatial density (1–3).

Single-chain antibodies (scFvs), also called single-chain variable fragments, are recombinant fusion proteins constructed by joining together the heavy- and light-chain variable regions of immunoglobulins with a flexible peptide linker. The scFv proteins (approximately one-sixth the molecular weight of the parent immunoglobulin) retain the specificity of the original immunoglobulins and, because the antigen-binding domain is expressed as a single peptide, it can be easily produced and genetically manipulated through recombinant DNA techniques. This has led to an increased interest in their use in diagnostic and therapeutic applications (4, 5). Concomitant with the development of using scFvs as diagnostics tools and therapeutic agents has emerged the need for a high-throughput means to assess the binding activity of scFvs and other recombinant fragments as they are displayed on the surface of mammalian cells. Transfected cell microarrays expressing membrane-displayed single-chain antibodies can address this need. In the method presented herein, a membrane-targeting expression vector, pDisplay (Invitrogen Life Technologies, Carlsbad, CA) was used to express scFvs as a fusion with a C-terminal myc-epitope tag and a PDGF receptor transmembrane domain (PDGFR-TM) (**Fig. 10.1a**). The plasmids encoding the scFvs were then mixed with a transfection reagent in a fixed ratio to obtain optimal transfection efficiency and they were subsequently printed onto microarray slides using a non-contact microarray printer. Adherent cells were then added to the printed arrays. After allowing sufficient time for expression of the scFvs, their expression on the cell surface was monitored. In this chapter, we have used well-characterized anti-fluorescein scFv 4-4-20 and its mutants to demonstrate the utility of the microarray format to determine the expression levels of the different clones and to characterize their differential binding affinities for the fluorescein ligand.

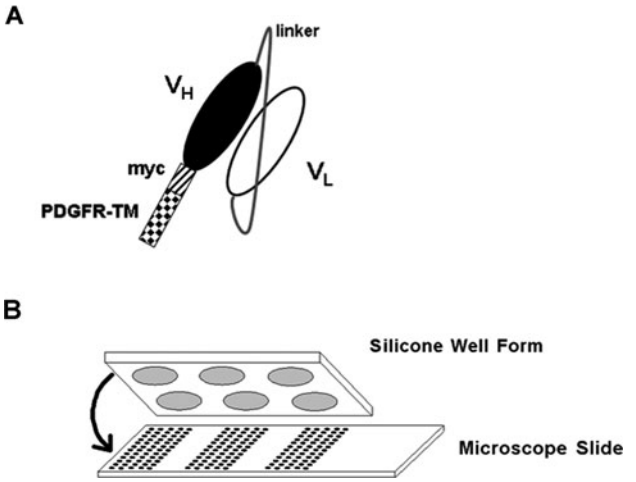

Fig. 10.1. 4-4-20 scFv and transfected cell array microarray format. (**a**) In the 4-4-20 scFv format, the light-chain variable (V_L) and heavy-chain variable (V_H) domains are joined by a 25-residue amino acid linker. The scFv is expressed as a fusion with a C-terminal myc-epitope tag for detection and a PDGF receptor transmembrane domain (PDGFR-TM). (**b**) In the transfected cell microarray, scFv plasmid DNA is mixed with transfection reagent and deposited in a gelatin/sucrose printing buffer onto the surface of a glass slide. A multiple-well silicon form is then aligned onto the printed array. The addition of cells to the array results in the transfection of cells in spatially discrete spots. Reproduced with permission from the ACS (3).

2. Materials

2.1. Control Plasmid

1. pDsRed2-Nuc expression vector (which expresses the fluorescent protein DsRed2 within the nucleus of transfected mammalian cells by virtue of its fusion with a nuclear localization signal from SV40 virus) was obtained from BD Biosciences (Palo Alto, CA). The plasmid was stored at –20°C for up to 6 months or –80°C for several years.
2. LB kanamycin plates: 0.01% (w/v) tryptone, 0.005% (w/v) yeast extract, 0.005% (w/v) NaCl, 0.015% (w/v) agarose, and 50 μg/ml kanamycin. The plates were stored at 4°C for up to 3 months.
3. LB kanamycin broth: 0.01% (w/v) tryptone, 0.005% (w/v) yeast extract, 0.005% (w/v) NaCl, and 50 μg/ml kanamycin. The broth was stored at 4°C for up to 3 months.
4. QIAGEN Plasmid Midi kit (Qiagen) or PureYield Plasmid Midiprep System (Promega Corp.) was stored at room temperature for up to 12 months (*see* **Note 1**).

2.2. Cloning of Anti-fluorescein Single-Chain Antibodies

1. pDisplay vector was obtained from Invitrogen Life Technologies. The plasmid was stored at –20°C for up to 6 months or –80°C for several years.
2. *Pfu* Turbo DNA polymerase (2.5 U/μl) (Stratagene) and 10× *Pfu* DNA polymerase reaction buffer were stored at –20°C for up to 6 months (*see* **Note 2**).
3. 10 mM dNTPs (Invitrogen) were stored in 100 μl aliquots at –20°C for up to 6 months (*see* **Note 3**).
4. PCR primers obtained from Operon Biotechnologies Inc. were dissolved in TE buffer to a stock concentration of 100 μM and stored at –80°C for up to 1 year. Working solutions (1–10 μM) were prepared by diluting the stock into nuclease-free H_2O and stored at –20°C for up to 6 months (*see* **Note 3**).
5. PCR reaction buffer: 20 mM Tris–HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton® X-100, 0.1 mg/ml BSA, 200 μM of dNTPs, and 200 nM each of sense and antisense primers.
6. MasterPure™ DNA purification kit (Epicentre Biotechnologies, Madison, WI) was stored at room temperature for up to 12 months (*see* **Note 4**).
7. The restriction endonucleases, *AccI*, *EcoRI*, *SfiI*, and *SalI*, were obtained from New England BioLabs (NEB) and stored at –20°C for up to 6 months (*see* **Note 3**).
8. Calf intestinal alkaline phosphatase (10 U/μL, NEB) was stored at –20°C for up to 6 months (*see* **Note 5**).
9. T4 DNA ligase (1 U/μL) and 5× ligation buffer (Invitrogen) were stored at –20°C for up to 6 months (*see* **Note 6**).
10. Ligation reaction buffer contains 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 5% (w/v) polyethylene glycol-8000.
11. DH5α competent cells (Invitrogen) were stored in 50 μl aliquots at –80°C for up to 6 months (*see* **Note 3**).
12. LB kanamycin plates: 0.01% (w/v) tryptone, 0.005% (w/v) yeast extract, 0.005% (w/v) NaCl, 0.015% (w/v) agarose, and 50 μg/ml kanamycin. The plates were stored at 4°C for up to 3 months.
13. LB kanamycin broth: 0.01% (w/v) tryptone, 0.005% (w/v) yeast extract, 0.005% (w/v) NaCl, and 50 μg/ml kanamycin. The broth was stored at 4°C for up to 3 months.
14. LB ampicillin plates: 0.01% (w/v) tryptone, 0.005% (w/v) yeast extract, 0.005% (w/v) NaCl, 0.015% (w/v) agarose,

and 100 μg/ml ampicillin. The plates were stored at 4°C for up to 3 months.

15. LB ampicillin broth: 0.01% (w/v) tryptone, 0.005% (w/v) yeast extract, 0.005% (w/v) NaCl, 0 and 100 μg/ml ampicillin. The broth was stored at 4°C for up to 3 months.
16. QIAprep spin miniprep kit and QIAGEN Plasmid Midi kit (Qiagen) or PureYield Plasmid Midiprep System (Promega Corp.) were stored at room temperature for up to 12 months.
17. Glycerol solution: 65% glycerol, 0.1 M $MgSO_4$, and 25 mM Tris–HCl (pH 8.0). The glycerol solution was autoclaved and stored at room temperature for up to 12 months.

2.3. Site-Directed Mutagenesis

1. QuickChange® Site-Directed Mutagenesis Kit (Stratagene), containing *Pfu* Turbo DNA polymerase (2.5 U/μl), 10× reaction buffer, Dpn I digestion enzyme, oligonucleotide control primers, dNTP, and pUC18 control plasmid, was stored at –20°C for up to 6 months.
2. PCR primers (Operon Biotechnologies Inc.) were dissolved in TE buffer to a stock concentration of 100 μM and stored at –80°C for up to 1 year. Working solutions (1–10 μM) were prepared by diluting the stock into nuclease-free H_2O and stored at –20°C for up to 6 months (*see* **Note 3**).
3. XL1-Blue supercompetent cells (Stratagene), part of the QuickChange® Site-Directed Mutagenesis Kit, were stored in 50 μl aliquots at –80°C for up to 6 months (*see* **Note 3**).
4. NZY broth: 0.01% (w/v) NZ amine (casein hydrolysate), 0.005% (w/v) yeast extract, 0.005% (w/v) NaCl, 12.5 mM each of $MgCl_2$ and $MgSO_4$, and 0.4% (w/v) glucose. The broth can be stored at room temperature for up to 6 months.
5. QIAprep spin miniprep kit (Qiagen) was stored at room temperature for up to 12 months.
6. LB ampicillin plates: 0.01% (w/v) tryptone, 0.005% (w/v) yeast extract, 0.005% (w/v) NaCl, 0.015% (w/v) agarose, and 100 μg/ml ampicillin. The plates were stored at 4°C for up to 3 months.
7. LB ampicillin broth: 0.01% (w/v) tryptone, 0.005% (w/v) yeast extract, 0.005% (w/v) NaCl, 0 and 100 μg/ml ampicillin. The broth was stored at 4°C for up to 3 months.
8. QIAGEN Plasmid Midi kit (Qiagen) or PureYield Plasmid Midiprep System (Promega Corp.) was stored at room temperature for up to 12 months.

9. Glycerol solution: 65% glycerol, 0.1 M $MgSO_4$, and 25 mM Tris–HCl (pH 8.0). The solution was autoclaved and stored at room temperature for up to 12 months.

2.4. Sample Preparation and Microarray Fabrication

1. Effectene transfection reagent (Qiagen), a non-liposomal lipid reagent for DNA transfection (supplied with EC buffer and enhancer reagent), was stored at 4°C for up to 12 months.
2. 2× Transfection printing buffer: 0.2% gelatin and 0.2 M sucrose in MilliQ water. The solution was stored at 4°C for up to 6 months.
3. 0.2% Tween 20 (Sigma-Aldrich, St. Louis, MO) was stored in 100 μl aliquots at –20°C for up to 6 months.
4. GAPS II microscope slides (3-aminopropyl triethoxysilane slides) were obtained from Corning (Acton, MA) and stored dessicated at room temperature for up to 12 months.

2.5. Cell Culture and Microarray Transfection

1. Frozen stocks of human embryonic kidney (HEK 293T/17) cells (ATCC, Manassas, VA) at low-passage number (typically below four) were stored in liquid nitrogen.
2. Dulbecco's modified eagle's medium—high glucose (DMEM, high glucose) (Invitrogen) can be stored at 4°C for up to 6 months.
3. Fetal bovine serum (FBS, ATCC) was stored at –20°C for up to 12 months or –80°C for up to 24 months.
4. Complete growth media: DMEM, high glucose, 3.7 mM L-glutamine, 17 mM sodium bicarbonate, 1 mM sodium pyruvate, 1% antibiotic/antimycotic (v/v), 10% fetal bovine serum. The media can be stored at 4°C for up to 3 months.
5. Phosphate-buffered saline (PBS): 137 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4. The buffer was stored at room temperature for up to 6 months (*see* **Note 3**).
6. Trypsin solution: 0.2% trypsin/0.03 M EDTA in PBS.
7. Press-to-seal silicon adhesive well forms were obtained from Schleicher and Schuell (Keene, NH) and stored at room temperature.

2.6. Assays for Protein Expression

1. Fluorescein-5-isothiocyanate, isomer I (FITC)-conjugated anti-myc epitope polyclonal antibody (Novus Biologicals, Littleton, CO) was stored at 4°C for up to 6 months.
2. Phosphate-buffered saline (PBS): 137 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4. The buffer was stored at room temperature for up to 6 months (*see* **Note 3**).
3. Four percent paraformaldehyde in PBS (PFA/PBS, pH 7.4) was prepared by dissolving 4 g of paraformaldehyde in PBS

while stirring and heating. Once dissolved, the solution can be stored at 4°C for several weeks.

4. Vectashield mounting medium containing DAPI (4′,6-diamidino-2-phenylindole) (1.5 μg/ml) (Vector Laboratories, Burlingame, CA) was stored at 4°C in the dark for up to 3 months.

2.7. Assay for Fluorescein Binding Acitivity

1. FluoReporter FITC protein labeling kit (Molecular Probes) was stored at 4°C shielded from light for up to 6 months.
2. 1 M sodium bicarbonate buffer pH9: dissolve 0.84 g of $NaHCO_3$ in 9 ml deionized water. Adjust the pH to 9 with NaOH and add water to a final volume of 10 ml. The solution should be stored at 4°C and used within 1 week.
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4. The buffer was stored at room temperature for up to 6 months (*see* **Note 3**).
4. Four percent paraformaldehyde in PBS (PFA/PBS, pH 7.4) was prepared by dissolving 4 g of paraformaldehyde in PBS while stirring and heating. Once dissolved, the solution can be stored at 4°C for several weeks.
5. Vectashield mounting medium containing DAPI (4′,6-diamidino-2-phenylindole) (1.5 μg/ml) (Vector Laboratories, Burlingame, CA) was stored at 4°C in the dark for up to 3 months.

2.8. Image Collection and Data Analysis

1. Nikon E800 Eclipse fluorescence microscope equipped with fluorescein and rhodamine filters.
2. MCID Analysis image analysis software version 7 (Imaging Research Inc., Ontario, Canada).

2.9. Improvement of Cellular Adhesion on Microarrays

1. Lyophilized fibronectin was reconstituted to 1 mg/ml in cell-culture-grade PBS.
2. Working stock of fibronectin in PBS (25 μg/ml) was prepared by diluting the 1 mg/ml stock to 25 μg/ml. The solution was stored as 500-μl aliquots at –20°C for 3–6 months.

3. Methods

The successful expression of membrane-displayed single-chain antibodies on cell-based microarrays depends primarily on two main factors. First, the nucleic acids encoding the single-chain antibodies must be cloned into an appropriate expression plasmid that directs their expression upon the surface of the cellular

membrane. Second, the expression plasmids must be deposited onto the microarray at high density with the optimal amount of transfection reagent to mediate efficient cellular internalization upon the addition of adherent cells to the array.

3.1. Control Plasmid—pDsRed2-Nuc Expression Vector

The plasmid pDsRed2-Nuc expression vector which expresses the fluorescent protein DsRed2 within the nucleus of transfected mammalian cells by virtue of its fusion with a nuclear localization signal from SV40 virus was used as control.

1. The plasmid was transformed into DH5α competent cells and grown overnight at 37°C on LB kanamcyin plates.
2. Single colony was selected from the LB kanamycin plate and grown overnight in 250 ml LB kanamycin broth.
3. Large-scale plasmid preparation was performed using the QIAGEN Plasmid Midi kit or PureYield Plasmid Midiprep System according to the manufacturer's protocols.
4. The plasmid was quantified by measuring the OD at 260/280 nm and was stored at –20°C for up to 12 months or at –80°C for several years (*see* **Note 1**).

3.2. Cloning of Anti-fluorescein Single-Chain Antibodies

A membrane-targeting vector (pDisplay) was used to express anti-fluorescein single-chain antibodies upon the surface of the cellular membrane.

1. The pDisplay vector was transformed into DH5α competent cells and grown overnight at 37°C on LB kanamycin plates.
2. Single colony was selected from the LB kanamycin plates and grown overnight in 250 ml LB kanamycin broth.
3. Large-scale plasmid preparation was performed using the QIAGEN Plasmid Midi kit or PureYield Plasmid Midiprep System according to the manufacturer's protocols.
4. The plasmid was quantified by measuring the OD at 260/280 nm and was stored at –20°C for up to 12 months or at –80°C for several years (*see* **Note 1**).
5. The coding region of the 4-4-20 anti-fluorescein antibody was amplified from the vector pRS316 prepro/MBP/4-4-20/Aga2 (a gift from Dr. Robert Siegel) using primers containing an *SfiI* restriction site (sense primer) and a *SalI* restriction site (antisense primer) (**Table 10.1**) (*see* **Note 7**).
6. The PCR reactions were performed in 50 μl volumes using 1 U of *PfuTurbo* DNA polymerase with 10 ng of pRS316 prepro/MBP/4-4-20/Aga2 template.
7. The amplification reaction was carried out in a Peltier Thermal Cycler-PTC225 with preliminary denaturation at 94°C for 3 min followed by 5 cycles of 94°C for 30 s, 44°C for 30 s, 72°C for 45 s, then followed by 35 cycles of 94°C for 30 s, 60°C for 45 s, and a final extension at 72°C for 10 min.

Table 10.1
Primers used for the cloning and site-directed mutagenesis of 4-4-20 scFv

Primer name	Sequence (5'→3')
scFV-SfiI	ATC GGC CCA GCC GGC CGA CGT CGT TAT GAC T
scFV-SalI	TTA GTC GAC TGA GGA GAC GGT GAC TGA GGT
FITC-stopmycBACK	GAA GAG GAT CTG TAA TAA AAT GCT GTG GGC
FITC-stopmycFOR	GCC CAC AGC ATT TTA TTA CAG ATC CTC TTC
HisL27dLysBACK	GTC AGA GCC TTG TAA AGA GTA ATG GAA ACA CCT ATT TAC
HisL27dLysFOR	GTA AAT AGG TGT TTC CAT TAC TCT TTA CAA GGC TCT GAC
HisL27dAlaBACK	GTC AGA GCC TTG TAG CCA GTA ATG GAA ACA CCT ATT TAC
HisL27dAlaFOR	GTA AAT AGG TGT TTC CAT TAC TGG CTA CAA GGC TCT GAC
TyrL32PheBACK	CAG TAA TGG AAA CAC CTT TTT ACG TTG GTA CCT G
TyrL32PheFOR	CAG GTA CCA ACG TAA AAA GGT GTT TCC ATT ACT G
ArgL34LysBACK	GGA AAC ACC TAT TTA AAA TGG TAC CTG CAG AAG CCA GG
ArgL34LysFOR	CCT GGC TTC TGC AGG TAC CAT TTT AAA TAG GTG TTT CC
ArgL34HisBACK	GGA AAC ACC TAT TTA CAT TGG TAC CTG CAG AAG CCA GG
ArgL34HisFOR	CCT GGC TTC TGC AGG TAC CAA TGT AAA TAG GTG TTT CC
ArgL34AlaBACK	GGA AAC ACC TAT TTA GCT TGG TAC CTG CAG AAG CCA GG
ArgL34AlaFOR	CCT GGC TTC TGC AGG TAC CAA GCT AAA TAG GTG TTT CC
TrpL96TyrBACK	CAA AGT ACA CAT GTT CCG TAT ACG TTC GGT GGA GGC
TrpL96TyrFOR	GCC TCC ACC GAA CGT ATA CGG AAC ATG TGT ACT TTG
TrpL96PheBACK	CAA AGT ACA CAT GTT CCG TTC ACG TTC GGT GGA GGC
TrpL96PheFOR	GCC TCC ACC GAA CGT GAA CGG AAC ATG TGT ACT TTG
TrpH33TyrBACK	CAC TTT TAG TGA CTA CTA TAT GAA CTG GGT CCG C
TrpH33TyrFOR	GCG GAC CCA GTT CAT ATA GTA GTC ACT AAA AGT G
TrpH33PheBACK	CAC TTT TAG TGA CTA CTT TAT GAA CTG GGT CCG C
TrpH33PheFOR	GCG GAC CCA GTT CAT AAA GTA GTC ACT AAA AGT G

8. The PCR products were cleaned up with MasterPure™ DNA purification kit (*see* **Note 4**) and subsequently digested with *SalI* at 37°C for 7 h in 50 μl reaction volume. The *SalI*-digested PCR product was ethanol precipitated and digested with *SfiI* at 50°C overnight in a 50 μl reaction volume followed by ethanol precipitation and resuspension in 30 μl of nuclease-free water.
9. 5 μg of pDisplay vector was digested with *SalI* in 100 μl reaction volume at 37°C overnight. The *SalI*-digested vector was ethanol precipitated and digested with *SfiI* at 50°C overnight in 100 μl reaction volume. The *SfiI*- and *SalI*-digested vectors were incubated with 10 U of calf intestinal phosphatase overnight at 37°C. The dephosphorylated vector was cleaned with MasterPure™ DNA purification kit (*see* **Note 5**).

10. The digested, cleaned PCR products (7 μl) were ligated to 50 ng of pDisplay vector also digested with *SfiI* and *SalI* with 1U T4 DNA ligase in a 10 μl reaction volume containing 1x ligation buffer for 1 h at room temperature.
11. 1 μl of the ligation mixture was mixed with 50 μl DH5α competent cells and incubated on ice for 30 min. The cells were then subjected to heat-shock at 42°C for 45 s and then placed on ice for 2 min. An aliquot of SOC medium (0.45 ml) was added and the cells were incubated at 37°C for 1 h with shaking (225 rpm). A portion of the cells (200 μl) were spread onto LB ampicillin plates and incubated overnight at 37°C.
12. Five to ten colonies were selected from the LB plates and grown overnight in 5 ml LB ampicillin broth.
13. The plasmids were purified using the QIAprep spin miniprep kit following the manufacturer's protocol.
14. The purified plasmids were digested with *EcoRI* and *AccI* to confirm the presence of the 4-4-20 anti-fluorescein coding sequence and were subsequently sequenced for further confirmation.
15. Glycerol stocks of the clones were prepared by mixing 0.5 ml of the fresh overnight-culture with 0.5 ml of glycerol solution and snap-freezing the mixture in an ethanol-dry ice bath. Stocks could be stored at –80°C for years.

3.3. Site-Directed Mutagenesis

Site-directed mutagenesis of the 4-4-20 anti-fluorescein construct was performed using the QuikChange® Site-Directed Mutagenesis Kit. Complementary oligonucleotides containing the desired mutation were listed in **Table 10.1**.

1. Mutation-introducing PCR reactions were performed in 50 μl volumes with 2.5 U of *Pfu* Turbo DNA polymerase and 50 ng of pDisplay/4-4-20 anti-fluorescein plasmid as template.
2. The amplification reaction was carried out in a Peltier Thermal Cycler-PTC225 with preliminary denaturation at 94°C for 30 s followed by 18 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 6 min 10 s (*see* **Note 8**).
3. The PCR products were digested with 10 U of *Dpn* I at 37°C for 1 h.
4. An aliquot (1 μl) of the digested PCR products was incubated with 50 μl XL1-Blue super-competent cells on ice in a pre-chilled 14-ml BD Falcon polypropylene round-bottomed tube for 20 min. The cells were then heat-shocked at 42°C for 45 s. After incubating the heat-shocked cells on ice for 2 min, 0.5 ml of NZY medium (pre-warm to 42°C)

was added to the cells. The cells were incubated at 37°C for 1 h with shaking (225 rpm). 250 μl of the cells were spread onto LB ampicillin plates and incubated overnight at 37°C.

5. Two to three colonies were selected from the LB ampicillin plates and grown overnight in 5 ml LB ampicillin broth for plasmid miniprep.
6. The QIAprep spin miniprep kit was used to purify the plasmids from the colonies following the manufacturer's protocol (*see* **Note 1**).
7. The purified plasmids were digested with *EcoRI* and *AccI* to ensure that they contained insert of the appropriate size.
8. The presence of the desired mutation was confirmed by sequencing. Glycerol stocks of the clones were made by mixing 0.5 ml of the fresh overnight-culture with 0.5 ml of glycerol solution and snap-freezing in ethanol-dry ice bath followed by storage at –80°C for years.
9. Large-scale preparation of the plasmids (wild type and mutants) were performed using the QIAGEN Plasmid Midi kit or PureYield Plasmid Midiprep System according to the manufacturer's protocols and quantified by measuring OD at 260/280 nm. The plasmids were stored at –20°C for up to 6 months or –80°C for several years (*see* **Note 1**).

3.4. Sample Preparation and Microarray Fabrication

All microarrays were printed using a BioChip Arrayer I non-contact microarray instrument (Perkin-Elmer Life Sciences, Boston, MA) housed in an environmentally isolated chamber with a relative humidity of 35–45% at room temperature.

1. Plasmid DNA was prepared as a DNA-transfection reagent complex prior to printing. Samples (composed of DNA, EC buffer, Enhancer reagent, and Effectene transfection reagent) were prepared to a final volume of 25 μl in a 96-well medium-binding plate for printing. Plasmids encoding the wild-type pDisplay/4-4-20 anti-fluorescein scFv and its corresponding binding site mutants were diluted to 2.0 μg/ml with EC buffer in individual wells of the plate.
2. Enhancer reagent was added to each well at a ratio of 6:1 (μl reagent/μg plasmid) and incubated for 5 min at room temperature.
3. Effectene transfection reagent was then added to each well at the same 6:1 ratio (μl reagent/μg plasmid) and the mixtures were incubated at room temperature for 20 min.
4. An equal volume (25 μl) of 2× transfection printing buffer was added to the 25 μl DNA:transfection reagent mixture such that each well contained final concentrations of 0.1% gelatin and 0.1 M sucrose.

5. The arrayer was programmed to deliver solutions to the surface of GAPS II microscope slides at 1.5 nl per spot at a spacing of 650 μm between spots (*see* **Note 9**).
6. The samples were deposited in horizontal rows across the surface of the slides for later alignment with the wells of a silicon well form (**Fig. 10.1b**).
7. A row of spots encoding the red fluorescent protein DsRed2 was deposited as an internal control to monitor the transfection efficiency.
8. The slides were stored at 4°C in a desiccator until ready to use (typically within 1–2 days).

3.5. Cell Culture and Microarray Transfection

1. HEK 293T/17 cells were cultured in complete growth medium and incubated at 37°C in a 5% CO_2 atmosphere tissue culture incubator.
2. For transfection, cells were trypsinized using trypsin solution. The trypsinized cells were pooled and adjusted to a final concentration of $\sim 2.5 \times 10^5$ cells/ml in complete growth media.
3. The printed slides were equilibrated to room temperature and a press-to-seal silicon well form was affixed to each slide such that the rows of arrayed spots were centered in the wells (**Fig. 10.1b**).
4. An aliquot (150 μl) of trypsinized cells was added to each well of the slide and the cells were allowed to attach to the printed spots. The cells were incubated on the arrays for 24–48 h to allow for plasmid internalization and protein expression.

3.6. Assay for Protein Expression

The expression levels of the cell membrane-displayed scFvs were assessed using immunocytochemistry using an anti-myc polyclonal antibody directed against the myc tag on the expressed scFv protein.

1. The anti-myc antibody was diluted to a final concentration of 5 μg/ml in complete growth medium and incubated on the cell arrays for 1 h.
2. Following the incubation period, the medium was removed followed by the removal of the silicon well form.
3. The slides were then rinsed with PBS that was pre-warmed to 37°C for 10 s and then fixed in 4% paraformaldehyde in PBS for 30 min at room temperature.
4. After fixing, the slides were rinsed with PBS at room temperature twice.
5. The slides were then mounted with two drops of Vectashield mounting medium containing DAPI and covered with a No.

1 coverslip. The edges of the coverslip were sealed with nail polish to prevent drying of the sample.

3.7. Assay for Fluorescein Binding Activity

The microarrays were used to characterize the relative binding affinities of the wild-type 4-4-20 scFv and its corresponding mutants. The native antibody binds fluorescein with an equilibrium dissociation constant of $2.0\pm0.2\times10^{-10}$ M (6) while the scFv form has an affinity that is within two- to threefold that of the native binding site. The mutation of W96 in the scFv light chain to phenylalanine ((L) W96F) causes a 170-fold decrease in the affinity of the scFv for fluorescein. The mutation of (L) R34 to either lysine ((L) R34K) or histidine ((L) R34H) decreases the affinity of the binding site by 415- and 750-fold, respectively (6–8). These binding data are summarized in **Table 10.2**.

1. Prepare fluorescein–BSA conjugate using the FluoReporter FITC protein labeling kit according to the manufacturer's instruction. Add 200 μl of BSA (0.5–1 mg/ml) to a reaction tube and then 20 μl of 1 M of sodium bicarbonate buffer to the tube. Warm DMSO and reaction dye to room temperature. Prepare 10 mg/ml reactive dye stock solution by adding 50 μl of DMSO and the reactive dye. Add 2.4–4.8 μl of reaction dye to the reaction tube while stirring, and stir the reaction at room temperature for 1 h shielding from light. Prepare the spin column by removing the top cap and the bottom closure and allow the column buffer to drain from the column by gravity. Place the spin column in a 2-ml collection tube and spin for 3 min at $1{,}100\times g$ using a swing bucket rotor. Discard the buffer and place the spin column back to the collection tube. Add the labeled proteins to the tube and allow the solution to absorb into the gel bed. Centrifuge the column for 5 min at $1{,}100\times g$ to collect the fluorescein–BSA conjugate (*see* **Note 10**).

Table 10.2
Binding constants of anti-fluorescein scFv 4-4-20 and mutants

Clone designation	K_a (M^{-1})[a] (soluble)	K_d (M)[b] (soluble)	K_d (M)[c] apparent (cell array)
Wild type	$4.9\pm0.5\times10^{9}$	$2.0\pm0.2\times10^{-10}$	$1.9\pm0.3\times10^{-9}$
(L) W96F	$2.9\pm1.3\times10^{7}$	$3.4\pm1.6\times10^{-8}$	$5.8\pm1.3\times10^{-8}$
(L) R34K	$1.2\pm0.2\times10^{7}$	$8.3\pm1.2\times10^{-8}$	$1.1\pm0.2\times10^{-7}$
(L) R34H	$6.6\pm1.8\times10^{6}$	$1.5\pm0.4\times10^{-7}$	$8.0\pm2.0\times10^{-7}$

[a] K_a values are reported in (6, 7).
[b] K_d values were determined from the identity $K_d = 1/K_a$.
[c] Apparent K_d values were determined from the microarray data shown in **Fig. 10.2**.

2. On a single microscope slide, replicate arrays of cells expressing the wild-type scFv 4-4-20 and the mutants (L) W96F, (L) R34H, and (L) R34K were constructed by depositing replicate spots of plasmid DNA and allowing the cells to express the scFvs for 24 h.
3. The binding activities of the cell-surface-expressed scFvs on the microarrays were assessed using a fluorescein–BSA conjugate. The conjugate consisted of BSA functionalized with 8–10 fluorescyl ligands. This configuration allowed for the binding of the fluorescyl ligand into the binding pocket of the scFv while leaving a sufficient number of fluorescyl ligands available to confirm binding via fluorescence.
4. The fluorescein–BSA conjugate was diluted to the effective working concentration in complete growth medium and incubated on the array for 1 h at 37°C.
5. After the 1-h incubation period, the culture media were removed and the silicon form was removed from the slide.
6. The slides were rinsed for 10 s with PBS that was prewarmed to 37°C and then fixed in 4% paraformaldehyde in PBS for 30 min at room temperature.
7. After fixation, the slides were rinsed twice with PBS.
8. The slides were then mounted with two drops of Vectashield mounting medium containing DAPI and covered with coverslips and sealed with nail polish.
9. The resulting fluorescence pattern obtained is shown in **Fig. 10.2a** and the binding curves determined from the microarrays are shown in **Fig. 10.2b**.
10. The relative rankings of the affinities of the scFvs as determined from the microarrays were in good agreement with the published values and were only slightly lower than those reported for the soluble versions of the antibodies (*see* **Table 10.2**).

3.8. Image Collection and Data Analysis

1. Individual images of transfected cell spots were collected using a Nikon E800 Eclipse fluorescence microscope equipped with fluorescein and rhodamine filters.
2. Within each array area, control spots were included to determine the transfection efficiency within each array as described elsewhere (9). Briefly, within each control spot, the number of transfected cells was determined by totaling the number of cells expressing the red fluorescent protein, DsRed2, within the nucleus. The total number of cells was determined by counting the number of DAPI-stained nuclei. The transfection efficiency was determined as the number of transfected cells (red) divided by the number of total cells (blue).

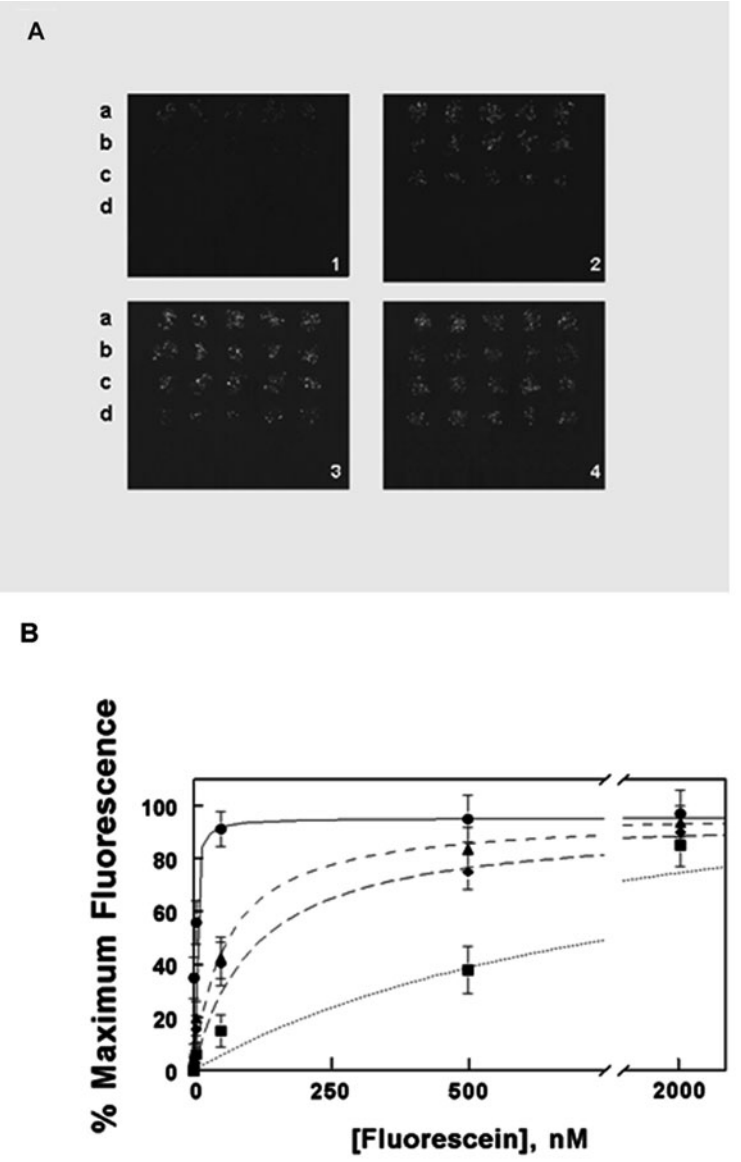

Fig. 10.2. Determination of cell-surface displayed scFv affinity using transfected cell microarray. (**a**) Replicate spots of plasmid DNA encoding the (*a*) wild-type scFv 4-4-20 and the mutants (*b*) (L) W96F, (*c*) (L) R34K, and (*d*) (L) R34H were deposited as described in the text. After the addition of cells and a 24-h expression period, the arrays were incubated with a fluorescein–BSA conjugate at the following concentrations: (*1*) 0.5, (*2*) 50, (*3*) 500, and (*4*) 2,000 nM. (**b**) Dose–response curves for the binding interaction of expressed 4-4-20 scFv with fluorescein–BSA. Each data point is the mean±SEM of 10 replicate spots taken from slides prepared in duplicate. A one-site ligand-binding equation was used to determine the best line through the points, and the results are plotted as percent of maximum fluorescence. Symbols represent the following: (●) wild-type, (▲) (L) W96F, (♦) (L) R34K, and (■) (L) R34H. Reproduced with permission from the ACS (3).

3. For quantification of the relative expression level of membrane-displayed scFvs, images of cell clusters consisting of 80–100 cells were collected and the fluorescence intensities of each image were determined using MCID Analysis image analysis software version 7.
4. The fluorescence signal for each image was determined as the product of the average pixel intensity times the total number of pixels within the image minus the fluorescence intensity of the background.
5. For the dissociation constants, the fluorescence intensity values were determined at each fluorescein concentration and transformed into a corresponding value of percent maximum fluorescence relative to the fluorescence value corresponding to a saturating concentration of fluorescein.
6. The percent maximum fluorescence was plotted versus the fluorescein concentration, and the dissociation constant was determined from the following equation:

$$\%\ \text{maximum fluorescence} = X/(X + K_{\mathrm{d}})$$

where X represents the fluorescein concentration and K_{d} represents the apparent dissociation constant.

3.9. Improvement of Cellular Adhesion on Microarrays

1. One common technical hurdle often experienced with cellular microarrays is the loss of cells due to poor adhesion to the microscope slide surface. While the extent of this problem varies with the particular cell line being used, we demonstrate here how the incorporation of an appropriate cell adhesion molecule during array construction can significantly improve the adhesion of cells to the microarray.
2. Passive adsorption of the cell adhesion molecule, fibronectin, onto the surface of GAPS II slides prior to the plasmid DNA deposition and its inclusion in the gelatin/sucrose printing buffer dramatically improves the adhesion of HEK 293T/17 cells to GAPSII slides while having no deleterious effect on transfection efficiency.
3. GAPSII slides were coated with fibronectin overnight at 37°C, washed with distilled water, and dried under a stream of nitrogen. 9×9 microarrays of plasmid DNA encoding the red fluorescent protein DsRed2-Nuc were printed in gelatin/sucrose printing buffer prior to the addition of HEK 293T/17 cells. In comparison to slides without fibronectin coating, slides coated with fibronectin exhibited superior retention of cells after processing of the slides (**Fig. 10.3**).
4. The addition of fibronectin to the printing buffer, while improving cellular adhesion, has no deleterious effect on the transfection efficiency (**Fig. 10.4**).

4. Notes

1. Other plasmid isolation kits can also be used for this step.
2. Other high-fidelity DNA polymerase can also be used to generate PCR products. Good laboratory practice (e.g., use

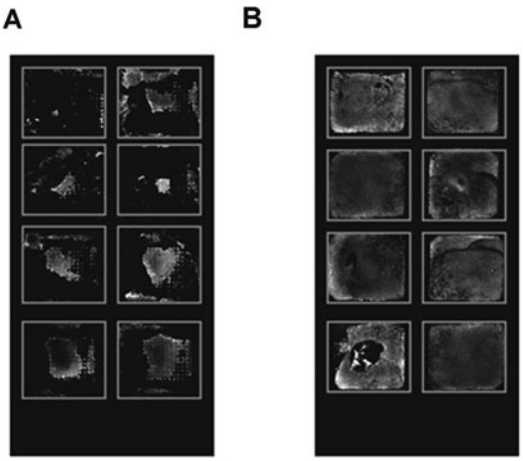

Fig. 10.3. Fibronectin improves cellular adhesion of HEK 293T/17 cells on well-based reverse transfection microarrays. GAPSII were used as supplied (**a**) or were coated with 25 μg/ml fibronectin prior to printing of microarrays (**b**). Subsequently, eight separate 9×9 microarrays were printed onto the surface. After deposition and culture of cells, the arrays were processed and stained with DAPI to visualize all cells retained within the wells. Slides coated with fibronectin exhibited superior retention of cells after processing of the slides.

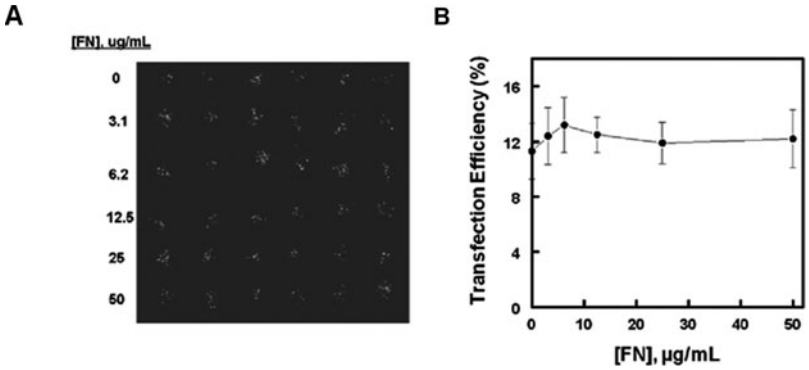

Fig. 10.4. Fibronectin-improved cell adhesion on two microarray printing platforms. Microarrays for reverse transfection were printed using a non-contact printer (BioChip Arrayer). 6×6 microarrays in which each spot contained 30 pg of a plasmid encoding a nuclear-localizing red fluorescent protein (DsRed2-nuc) were printed onto the surface of GAPSII slides coated with fibronectin. Fibronectin (FN) was also included in the printing buffer at the concentrations indicated. HEK 293T/17 cells were added and cultured for 24 h prior to fixing and staining with DAPI. The quantification of the transfection efficiency as a function of fibronectin concentration is shown.

of filtered pipette tips, gloves, and a UV-irradiated PCR hood) is necessary to prevent contamination issue in the PCR reactions.

3. Competent cells, dNTPs, PCR primers, restriction endonucleases, and PBS from other commercial suppliers can also be used.
4. Other PCR purification kits can also be used for cleaning up PCR products.
5. Other phosphatases (e.g., antarctic phosphatase) can also be used. Some alkaline phosphatases can be heat-inactivated without the need to purify the dephosphorylated vector.
6. T4 DNA ligase is unstable on ice for long periods of time. It is best to keep the enzyme at –20°C until ready for use and return it to –20°C immediately after use. Ligation buffer contains ATP and DTT which can be degraded by repeat freezing and thawing and will reduce the ligation efficiency. It is best to store the ligation buffer in small, single-use aliquots.
7. When designing oligonucleotide primers with linker sequences, it is necessary to consider using cloning sites on the vector that will generate the right orientation of the coding sequence.
8. The extension time is based on the size of the plasmid used according to the manufacturer's recommendation.
9. For all printing operations, a rinse step was performed between each sample aspiration/dispense cycle. The tips were rinsed with 0.2% Tween-20 liberally in a sonicating bath followed by a final rinse with distilled water.
10. The conjugate can be prepared in advance and stored at 4°C in the dark for up to 3 months.

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