Novel Uses of Microarrays in Detecting Gene Silencing

Elizabeth T. Jordan, Cathleen Karlak, Teresa Rubio, Luis Ugozzoli, and Jamie Wibbenmeyer.
Gene Expression Division, Bio-Rad Laboratories, Hercules, CA 94547 USA

BIO-RAD
Life Science Group
2000 Alfred Nobel Drive
Hercules, CA 94547 USA

Abstract

DNA microarrays allow many simultaneous parallel measurements, and transcriptional profiling using these arrays has provided scientists with a wealth of information. The same technology can be used to build protein arrays, which hold similar promise. We describe here an approach using protein arrays to screen cells for gene knockdown by β-actin siRNA and to examine changes in levels of the actin-binding proteins destrin and cofilin. Arrays were produced on the benchtop with a BioOdyssey™ Calligrapher™ miniarrayer. Antibodies against β-actin and phosphorylated cofilin (p-cofilin) were tested for specificity using western blots. In one experiment, arrays were processed to monitor the concentration of β-actin in cells, with a standard curve of purified human actin printed in the arrays. In another experiment on the same slide, changes in phosphorylation levels of cofilin were detected with an antibody specific for p-cofilin. We demonstrate that printed protein arrays can be screened using antibodies either singly or in pairs. Finally, the microarray results were validated using qPCR.

Introduction

Small interfering RNAs (siRNAs) can be used to degrade mRNA levels of a specific gene, thereby reducing the corresponding protein levels within cells. The use of siRNAs has many applications in functional genomics and proteomics, as well as in therapeutics, as researchers use this tool to identify proteins involved in specific pathways.

In a previous study, we silenced the β-actin gene in HeLa cells using siLentMer™ Dicer-substrate siRNA duplexes, and we confirmed the knockdown with a combination of 2-D gel analysis, western blotting, and qPCR (Liu et al. 2006). We also examined the expression levels of several actin-binding proteins, including cofilin, an actin depolymerization factor that binds to actin filaments and induces their cleavage (Hotulainen et al. 2005), and destrin, an actin depolymerization factor with a function similar to that of cofilin (Maciver and Hussey 2002, Ikeda et al. 2003). An increase in the level of p-cofilin was detected, whereas destrin levels appeared to be unchanged (Liu et al. 2006).

Here we demonstrate the use of reverse-phase protein microarrays as a screening method to identify cells that have been treated successfully with a β-actin siRNA. Microarrays were produced on the benchtop with the BioOdyssey™ Calligrapher™ miniarrayer to screen for β-actin, p-cofilin, and destrin. We validated the microarray results using qPCR. The arrays enabled higher throughput than traditional protein detection methods, and they allowed multiplexing.

Methods

A schematic of the gene silencing workflow is shown in Figure 1.

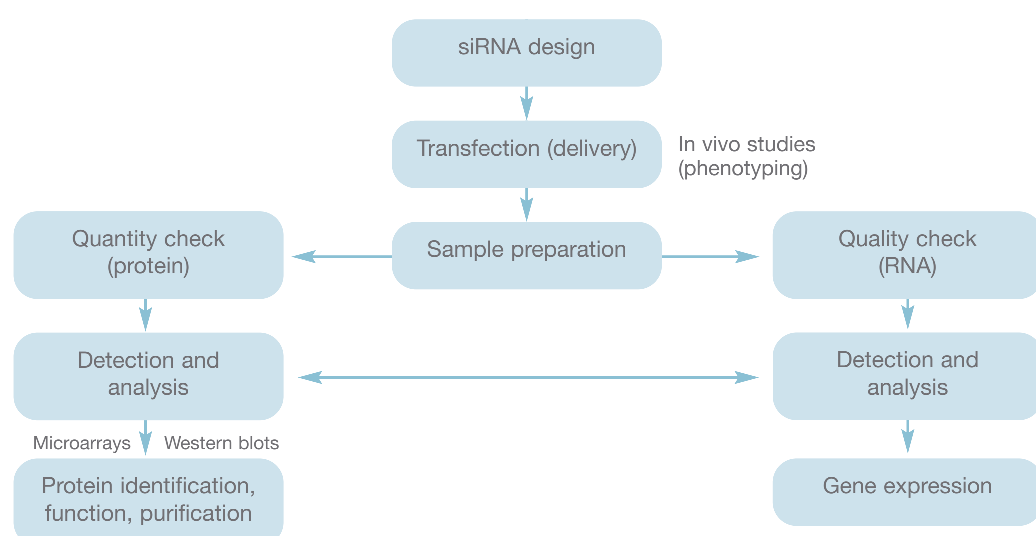

Fig. 1. Schematic of gene silencing workflow.

Gene Knockdown

Two actin-specific siRNAs (Act8 and Act9) and a nonsilencing scramble siRNA were used. HeLa cells (0.675 x 10^6) were seeded in 10 ml of medium on a 100 mm plate. 5 nM siRNA and 9 µl siLentFect™ lipid reagent were mixed and incubated for 20 min to form siRNA–lipid complexes. The complexes were then added to the cell cultures. Additional controls containing lipid only were also used. Biological replicates were prepared for each condition tested. After 24 hr incubation, the medium was exchanged with 10 ml of fresh medium to remove excess complexes.

Sample Preparation

At 48 hr posttransfection, medium was removed and cells were washed with PBS. Lysis buffer (1 ml 2% SDS, 50 mM Bis-Tris, pH 6.8, containing 0.1% β-mercaptoethanol, 0.1% Protease-Arrest (G Biosciences), and phosphatase inhibitor cocktail set II (Calbiochem)) was added to each plate. The extract was collected in tubes and sonicated for 1 min at 4°C. Insoluble material was removed by centrifugation for 1 min. Lysate was stored at –20°C until further analysis.

Total protein content of cell lysates was measured using an *RC DC*™ protein assay kit. RNA was isolated from cells using an Aurum™ total RNA kit, and the quantity was calculated using an Experion™ automated electrophoresis system. cDNA was synthesized using an iScript™ cDNA synthesis kit and RT-qPCR was performed in an iQ™5 real-time PCR detection system.

Western Blotting

Samples were diluted to 0.5 mg/ml in PBS, and 2 volumes of Laemmli buffer were added. After heating at 95°C, equal volumes of each sample were electrophoresed in Criterion™ gels. Gels were blotted to PVDF membrane in Towbin buffer. Blots were blocked with 5% nonfat dry milk (Bio-Rad) in PBST (0.1% Tween 20 in 1x PBS (all from Bio-Rad), then incubated with primary antibodies (1:1,500 in blocking buffer) for 1 hr at room temperature (RT). After three 10 min washes with PBST, blots were incubated with secondary antibodies (1:3,000 in blocking buffer) for 1 hr at RT, then washed again for 3 x 10 min with PBST. Mouse anti-β-actin and sheep anti-mouse IgG (whole molecule)-Cy3 were from Sigma. Rabbit anti-p-cofilin 1 was from Santa Cruz Biotechnology and goat anti-rabbit IgG (H+L) –Cy5 was from Abcam. Images were captured using a Molecular Imager® VersaDoc™ MP 4000 imaging system and Quantity One® software.

qPCR

Real-time qPCR was carried out in an iQ5 system using iQ™ SYBR® Green supermix and 300 nM β-actin-specific primers. Each 50 µl reaction contained one-tenth of a 1 µg cDNA synthesis reaction, and four replicates were run for each sample.

Microarray Printing

The BioOdyssey Calligrapher miniarrayer was used with BioOdyssey™ MCP™ 310S solid pins for printing arrays of protein samples onto 16-pad nitrocellulose-coated FAST slides (Whatman). Each sample was printed in triplicate, and PBS was printed between different samples to prevent carryover contamination. Identical samples were printed on 12 pads on the slide. A standard curve was prepared using human actin from platelets (Cytoskeleton, Inc.), which contain 85% β-actin and 15% α-actin.

Processing Protein Array Slides

Slides were air-dried and stored in a vacuum desiccator at RT until use. All incubation steps were carried out at RT with gentle agitation. Slides were rinsed briefly with PBST and blocked with 5% nonfat dry milk in PBST for 1 hr. Before use, insoluble material was removed by centrifugation to reduce background fluorescence. A FAST frame, which separates each grid on the slide into its own reaction chamber, was used for incubation with primary and secondary antibodies. All antibodies were diluted in blocking buffer as described under Western Blotting, and ~60–70 µl was applied per grid. All incubations with primary or secondary antibody were performed in triplicate for 1 hr, and were followed with three washes with PBST for 5 min each. Incubations with fluorophore-conjugated secondary antibodies were carried out in the dark. After the final PBST wash, slides were dried by centrifugation and scanned immediately with an Axon 4100a scanner at appropriate PMT gain settings. Arrays were analyzed with GenePix 6.0 software.

Results

Western blots were used to demonstrate the specificity of the antibodies used in microarray studies (Figure 2). These data also confirmed effective knockdown of β-actin expression and an associated increase in the level of p-cofilin. The actin level in Act9-treated samples was less than 20% of levels in the control samples (scramble siRNA and no treatment); while the level of p-cofilin was at least 2-fold higher in actin knockdown samples than in controls.

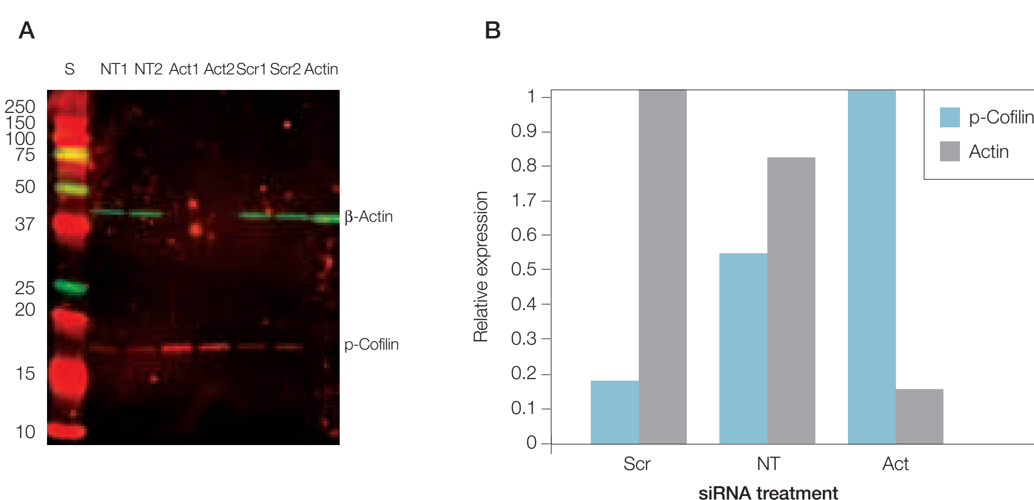

Fig. 2. Validation of antibodies by western blot analysis. A, Western blots labeled with antibodies against β-actin (Cy3) and p-cofilin (Cy5). Individual images were combined using the multi-channel viewer in Quantity One software. **S**, protein standards; **NT1** and **NT2**, biological replicates of untreated controls; **Act1** and **Act2**, biological replicates of cells treated with Act9 siRNA; **Scr1** and **Scr2**, biological replicates of cells treated with scramble siRNA. **Actin**, mixture of β-actin and α-actin used in standard curve. **B**, Relative expression levels of β-actin and p-cofilin in cells treated with Act9 siRNA (**Act**), scramble siRNA (**Scr**), or medium alone (**NT**). Fluorescence intensity was measured using Quantity One software. To compare across treatments, the actin level in Scr samples and the p-cofilin level in Act samples were set to 1.

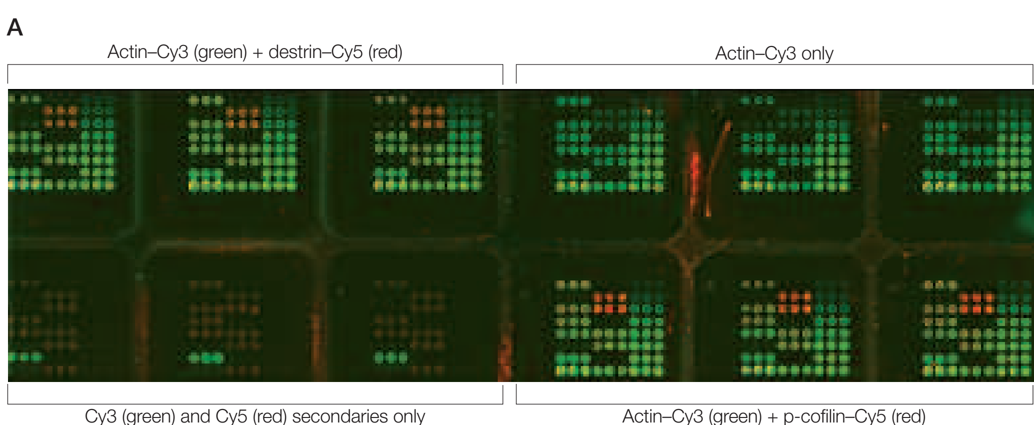

Fig. 3. Detection of proteins on microarrays. A, identical samples printed on 12 pads of a single slide. Grids were labeled in triplicate with primary and secondary antibodies as labeled. **B**, enlarged grids (rightmost upper and lower grids from A) showing placement of printed lysates and proteins. **White**, two biological replicates of lysates from cells treated with Act9 siRNA, spotted in triplicate; **orange**, biological replicates of lysates treated with scramble siRNA; **green**, biological replicates of untreated lysates; **red**, triplicate positive control spots (primary antibody); **yellow**, PBS spots; **blue**, actin standard curve (highest concentration, 382.5 µg/µl, is in lower left corner of grid).

Each microarray detection experiment was run in triplicate to ensure good reproducibility (Figure 3). While there was some variability between biological replicate samples, the β-actin level in the Act9-treated cells was about 20% of that in untreated and scramble siRNA controls (Figure 4A), like in western blots (Figure 2). Actin knockdown resulted in a >2-fold increase in p-cofilin, whereas the level of destrin remained basically unchanged (Figure 4B).

Dilutions of purified human actin were printed on each of the microarrays and labeled with antibodies in singleplex or multiplex. The resulting standard curves were well correlated (Figure 5). Actin in the siRNA knockdown samples was below the limit of detection, so quantitation of β-actin in knockdown samples was not possible. Spotting more material may result in measurable amounts.

qPCR results indicated that siRNA treatment reduced mRNA levels to about 5% of the level in untreated samples (Figure 5), confirming the effectiveness of β-actin knockdown.

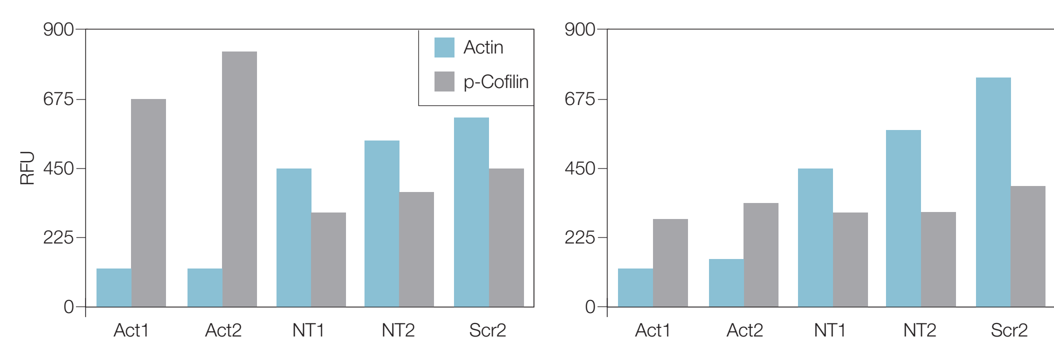

Fig. 4. Relative expression of β-actin, p-cofilin, and destrin. Expression levels varied with treatment, confirming earlier findings. **A**, β-actin was knocked down and phosphorylation of cofilin increased when cells were treated with actin-specific siRNA (**Act1** and **Act2**), but not when cells were treated with scramble (**Scr2**) or not treated (**NT1** and **NT2**). **B**, destrin levels were basically unaffected by siRNA treatment. **RFU**, relative fluorescence units.

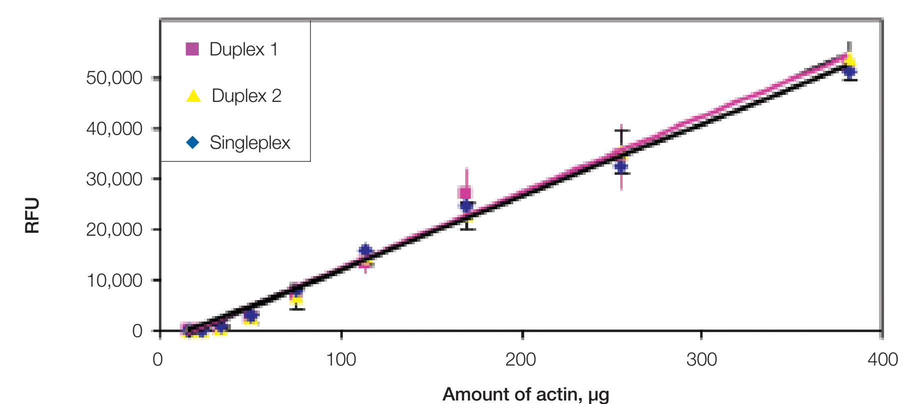

Fig. 5. Standard curve of human β-actin in singleplex and multiplex experiments. The β-actin standard curve was similar regardless of whether arrays were incubated with anti-β-actin antibody alone (**singleplex**); $y = 143.05x - 2,721.3$, $r^2 = 0.992$, with anti-actin plus anti-p-cofilin antibody (**duplex 1**); $y = 149.88x - 3,295.7$, $r^2 = 0.989$, or with anti-actin plus anti-destrin antibody (**duplex 2**); $y = 151.37x - 369$, $r^2 = 0.997$. **RFU**, relative fluorescence units.

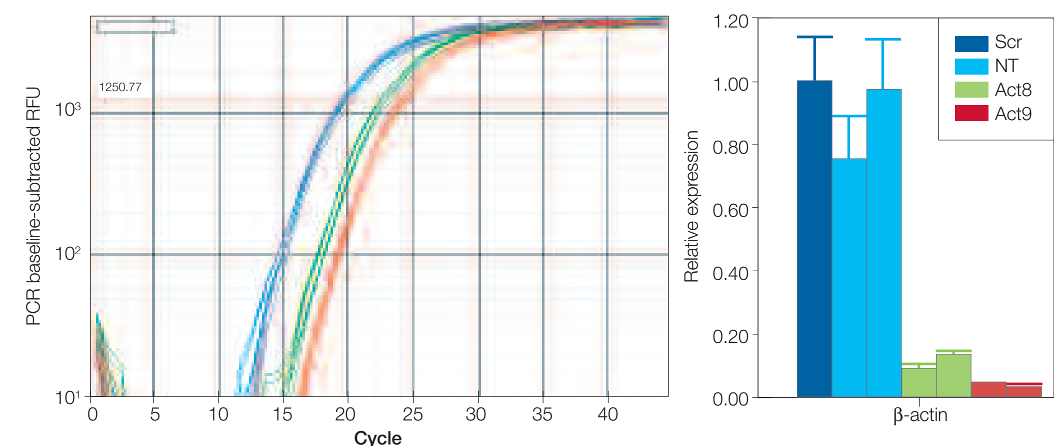

Fig. 6. Quantitation of gene silencing by qPCR. A, treatment of HeLa cells with the actin-specific siRNA Act9 (**red**) resulted in the greatest knockdown of β-actin cDNA compared to treatment with another actin-specific siRNA (Act8, **green**), scramble siRNA (**dark blue**), and no treatment (**light blue**). **B**, relative levels of β-actin cDNA from siRNA-treated vs. control cells. Act9 siRNA was used in the other experiments presented here.

Conclusions

We have demonstrated that reverse-phase protein arrays can be used as a screening tool to examine cell lysates for the effects of gene silencing. Additionally, we were able to use two primary antibodies in combination. Our results confirm previous findings of an increase in p-cofilin (Liu et al. 2006).

Using the BioOdyssey Calligrapher miniarrayer with FAST slides offers the following advantages:

- More than 72 (400 µm) samples, including replicates, controls, and standard curves, can be printed on each pad
- Identical grids can be printed on each of the 16 pads on a slide, for screening multiple antigens and/or replicating experiments
- Antigen up- or downregulation can be assessed qualitatively, and multiplex screening can be performed on a single grid, saving time and sample
- Limited quantitative data may be derived using the printed standard curve by repeatedly spotting dilute samples — a feature of the Calligrapher

References

Liu N et al., Cell Motil Cytoskeleton 64, 110–120 (2006)

Hotulainen P et al., Mol Biol Cell 16, 649–664 (2005)

Ikeda S et al., Human Mol Genet 12, 1029–1037 (2003)

Maciver SK and Hussey PJ, Genome Biol 3, reviews 3007 (2002)

Cy is a trademark of GE Healthcare. FAST is a trademark of S&S Biosciences, Inc. Tween is a trademark of ICI Americas Inc. SYBR is a trademark of Molecular Probes, Inc. Bio-Rad Laboratories, Inc. is licensed by Molecular Probes, Inc. to sell reagents containing SYBR Green I for use in real-time PCR, for research purposes only.

Bio-Rad's real-time thermal cyclers are licensed real-time thermal cyclers under Applera's United States Patent No. 6,814,934 B1 for use in research and for all other fields except the fields of human diagnostics and veterinary diagnostics.

LabChip and the LabChip logo are trademarks of Caliper Life Sciences, Inc. Bio-Rad Laboratories, Inc. is licensed by Caliper Life Sciences, Inc. to sell products using the LabChip technology for research use only.

06-0838 0107