Detection and Quantification of Protein Biomarkers from Fewer than 10 Cells*

Saju Nettikadan‡§, Korinna Radke‡, James Johnson¶, Juntao Xu‡, Michael Lynch‡, Curtis Mosher‡, and Eric Henderson‡‖

The use of antibody microarrays continues to grow rapidly due to the recent advances in proteomics and automation and the opportunity this combination creates for high throughput multiplexed analysis of protein biomarkers. However, a primary limitation of this technology is the lack of PCR-like amplification methods for proteins. Therefore, to realize the full potential of array-based protein biomarker screening it is necessary to construct assays that can detect and quantify protein biomarkers with very high sensitivity, in the femtomolar range, and from limited sample quantities. We describe here the construction of ultramicroarrays, combining the advantages of microarraying including multiplexing capabilities, higher throughput, and cost savings with the ability to screen very small sample volumes. Antibody ultramicroarrays for the detection of interleukin-6 and prostate-specific antigen (PSA), a widely used biomarker for prostate cancer screening, were constructed. These ultramicroarrays were found to have a high specificity and sensitivity with detection levels using purified proteins in the attomole range. Using these ultramicroarrays, we were able to detect PSA secreted from 100 LNCaP cells in 3 h and from just four LNCaP cells in 24 h. Cellular PSA could also be detected from the lysate of an average of just six cells. This strategy should enable proteomic analysis of materials that are available in very limited quantities such as those collected by laser capture microdissection, neonatal biopsy microspecimens, and forensic samples. ***Molecular & Cellular Proteomics 5:895–901, 2006.***

The development of novel and improved proteomic technologies in recent years has resulted in major efforts toward the discovery of new protein biomarkers. This has resulted in a rapid increase in the list of potential clinically relevant biomarkers some of which have become useful in diagnostics, prognostics, and therapeutics (1).

It is unlikely that individual biomarkers will be sufficiently sensitive and reliable to serve as robust indicators of a disease state (2). Therefore, a concerted effort is ongoing to develop methods for discovering and quantifying expression profiles of ensembles of protein biomarkers that as a group provide robust diagnostic information. Many currently known biomarkers are found only in relatively low concentrations in very complex mixtures (3). Hence a successful strategy to detect these biomarkers has to provide reasonably high throughput as well as high sensitivity and the capacity for quantification.

Protein microarraying is a powerful strategy for multiplexed analysis of gene expression, development of diagnostic procedures, and discovery of new pharmacologically active agents (4, 5). Protein microarrays have distinct advantages over traditional macroscopic methods of using less sample volumes and increased number of analytes simultaneously detected. These advantages translate to reduced reagent costs, faster kinetics, higher throughput, and potentially improved sensitivity.

Typically creation of protein arrays involves deposition of suitable capture molecules, such as specific antibodies, in an array format on an appropriate substrate. Each spot of the array consists of capture antibodies against a single antigen. The antigens are captured specifically onto these domains and detected by a variety of techniques including direct labeling of the antigens and using a secondary antibody in a sandwich assay (4, 6). Several different signal generation schemes are used in antibody microarray applications including fluorescently labeled secondary/tertiary antibodies (7), staining of biotinylated antibodies with streptavidin conjugated with fluorescent or enzymatic tags (8, 9), rolling circle amplification (10), and resonance light scattering by gold-coated particles (11).

Contact printers and other arraying devices are used to deposit nanoliter quantities of proteins in spatial arrays with domain sizes typically ranging from 100 to 300 μm in diameter. Several studies have demonstrated the application of protein microarraying technology in studying the proteins from serum (12), cell culture (13), tissue (14), and culture medium (10).

Further miniaturization of the capture domains, with individual domains much smaller than conventional microarrays, offers many distinct advantages including higher array density, increased sensitivity by analyte harvesting, improved binding kinetics, smaller reaction volumes, potentially reduced reaction times, and reduced amounts of analyte and

From the ‡BioForce Nanosciences Inc., Ames, Iowa 50010, ‖Genetics, Development, and Cell Biology Department, Iowa State University, Ames, Iowa 50011, and ¶Department of Microbiology, Des Moines University, Des Moines, Iowa 50312

Received, October 27, 2005, and in revised form, February 15, 2006
Published, MCP Papers in Press, February 19, 2006, DOI 10.1074/mcp.M500350-MCP200

ligand reagents (15, 16). Ekins *et al.* (15) demonstrated that an assay that uses smaller capture domains and lower sample volumes is more sensitive than one that uses larger capture domains and larger sample volumes. This is particularly relevant in the context of non-amplifiable protein biomarkers that are available only in very small quantities (*e.g.* laser capture microdissected materials, neonatal samples, and forensics specimens). Importantly further miniaturization of surface-immobilized protein microarrays creates new opportunities for micro- and nanobiosensor development toward the goal of proteomic analysis at the single cell level.

In this report we describe the use of protein "ultramicroarrays," defined as arrays with spots sizes in the 1–20-μm-diameter range and occupying $1/100$–$1/10{,}000$ of the surface area of a conventional (100-μm-diameter) microarray spot (17). Ultramicroarrays represents an optimal level of bioarray miniaturization because they contain a sufficient number of molecules to provide a statistically robust signal while also providing the benefits of miniaturization described above. Moreover ultramicroarrays permit analysis of protein profiles from extremely limited sample volumes. Finally ultramicroarrays can be readily visualized in a standard fluorescence system for readout and are also compatible with novel readout systems (*e.g.* atomic force microscopy).

We used prostate-specific antigen (PSA)[1] and interleukin-6 (IL-6) as a model system. Serum PSA measurement has become the most commonly used laboratory test for cancer (18). PSA is a 33-kDa androgen-regulated serine protease and member of the tissue kallikrein family of proteases (19). It is produced by epithelial cells in human prostate glands and secreted directly into the prostatic ductal system (20). PSA is a major protein in the seminal fluid with a concentration of 0.5–2.0 mg/ml (21). Pleiotropic cytokine, IL-6, is a 21–30-kDa glycoprotein that has been implicated in the androgen-independent activation of androgen receptor (22, 23). It has also been reported that the serum levels of IL-6 are elevated in patients with prostate cancer (24). The serum levels of IL-6 may also be a significant bioprognostic factor in prostate cancer (25).

Ultramicroarrays were constructed using capture antibodies against IL-6 and PSA on an amine-reactive surface. These ultramicroarrays were shown to capture their cognate antigen specifically and with high sensitivity. Each of the antigens could be detected in the picogram/milliliter range from purified proteins using only 1 μl of the sample. This translates to a detection sensitivity of femtogram quantities of proteins. Furthermore using these ultramicroarrays, we were able to detect PSA secreted from 100 LNCaP cells in 3 h and from just four LNCaP cells in 24 h. Cellular PSA could also be detected from the lysate of an average of just six cells.

[1] The abbreviations used are: PSA, prostate-specific antigen; IL-6, interleukin-6; SPT, surface patterning tool; a.u., arbitrary units.

EXPERIMENTAL PROCEDURES

Substrates—Silicon wafers patterned by reactive ion etching and diced into 4-mm squares (chips) were used as substrates. The chips were ultrasonically cleaned in water and absolute ethanol and sputter coated with 5 nm of chromium and 10 nm of gold using a dual gun, ion beam sputterer operating at 4 mA and 7 keV (South Bay Technologies). The gold-coated, patterned substrates were immediately immersed in a freshly prepared solution of prolinker B (Proteogen) (26) in chloroform and incubated for 1 h to form an amine-reactive surface. The surfaces were sequentially washed in chloroform and acetone, blown dry using a stream of dry nitrogen, and stored in a nitrogen atmosphere.

Antigens and Antibodies—Mouse anti-PSA and goat anti-PSA (Fitzgerald Industries) were used as the PSA capture antibody and detection antibody, respectively. Rat anti-IL-6 (R&D Systems) and biotinylated anti-IL-6 (eBioscience) were used as the IL-6 capture and detection antibodies, respectively. Purified PSA was purchased from Fitzgerald Industries, and recombinant IL-6 was purchased from R&D Systems. Texas Red-labeled donkey anti-goat IgG (Jackson Immunoresearch Laboratories, Inc.) and Alexa Fluor 594-labeled streptavidin (Molecular Probes) were used as the secondary antibodies to generate the fluorescence signal. Alexa 488-labeled rabbit anti-rat IgG (Molecular Probes) was used to tag the capture antibodies.

Construction of Arrays—Ultramicroarrays were constructed using an instrument we have developed called the NanoArrayer™ (27). The NanoArrayer system includes microfabricated "print cartridges" (surface patterning tools (SPTs™)) and uses precise motion and environmental control to transfer attoliter to femtoliter volumes of materials from a reservoir on the SPT onto a surface (28).

The protein to be deposited was mixed 1:1 in a spotting buffer (20% glycerol and 0.2 mg/ml *n*-octyl glucoside), and 1 μl of this mixture was placed onto a glass coverslip. Microfabricated SPTs were treated by exposure to ultraviolet light and ozone in a TipCleaner (BioForce Nanosciences, Inc.) for 30 min to render the device surface hydrophilic. The SPT and the coverslip containing the protein droplet were mounted into the NanoArrayer stage, and the distal end of the SPT cantilever was immersed into the protein droplet, thereby loading the SPT. The protein solution that spontaneously wicks into the hydrophilic channel in the SPT is sufficient for the construction of several hundred ultramicroscale domains. The SPT loaded with the protein was then brought in contact with the surface to be arrayed under constant force feedback and defined environmental conditions. A relative humidity of ~50% and temperature of 25 °C were used as the standard conditions for antibody depositions in this study. The dimensions of the deposited protein domains are determined by the geometry of the distal end of the SPT; the duration, angle, and force of contact of the SPT with the substrate; the chemical nature of the substrate; and the humidity levels at the deposition surface. In this report we optimized conditions for antibody deposition to create antibody domains of ~5–8 μm. The deposition time for each spot was less than 100 ms. This resulted in a 25-spot array being constructed in less than 25 s, including the translation time.

Standard Assay—The chips with the protein capture arrays were incubated overnight at 70% humidity at room temperature and then blocked with 1× ViriBlock solution (BioForce Nanosciences Inc.) at room temperature for 30 min on a rocker. The chips were rinsed in PBST (1× PBS, 0.2% Tween 80) and incubated with 1 μl of antigen (PSA or IL-6) at room temperature for 1 h in a humid environment. The chips were washed in PBST two times for 5 min each and incubated with the detection antibody (diluted 1:1,000 in PBST) for 1 h at room temperature. The chips were washed in PBST two times (5 min each) and tagged with Alexa Fluor 594-labeled streptavidin or Texas Red anti-goat IgG (diluted 1:1,000 in PBST) and Alexa 488 anti-rat IgG (diluted 1:1,000 in PBST). The surfaces were then washed three times in PBST and examined by fluorescence microscopy.

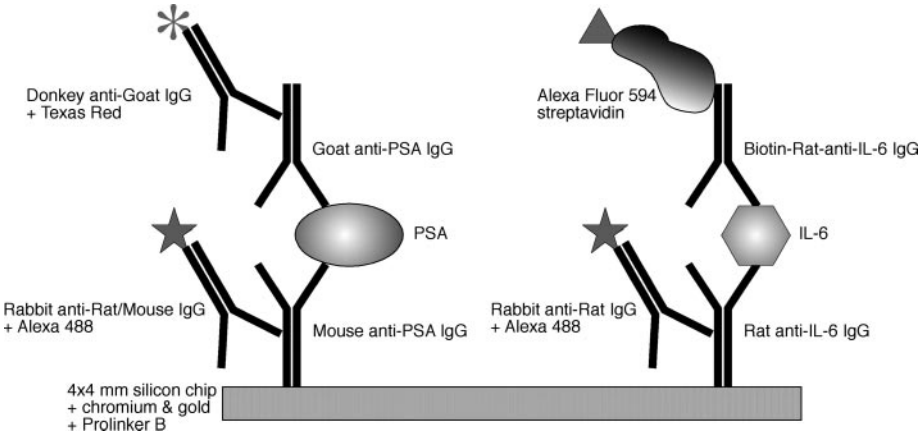

FIG. 1. **Illustration of the experimental system used in this study.** Mouse anti-PSA and goat anti-PSA were used as the PSA capture antibody and detection antibody, respectively. Rat anti-IL-6 and biotinylated anti-IL-6 were used as the IL-6 capture and detection antibodies, respectively. Texas Red-labeled donkey anti-goat and Alexa Fluor 594-labeled streptavidin were used as the secondary antibodies to generate the fluorescence signal. Alexa 488-labeled rabbit anti-rat IgG was used to tag the capture antibodies.

Data Acquisition—A Nikon TE 2000U inverted microscope equipped with a 40× oil objective and filter sets for Cy2™ (Alexa 488™) and Texas Red (Alexa 594™) dyes were used for fluorescence imaging. Images were captured using a cooled charge-couple device digital camera with 1.3-megapixel resolution.

Cell Culture—LNCaP cells (ATCC CRL 1740) were propagated in T-flasks containing complete medium (RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 90%; and fetal bovine serum, 10%). Flasks were incubated at 37 °C in an atmosphere of 95% air and 5% CO_2. Medium replacements were at 2-day intervals. At 70–80% confluency, cells were trypsinized using 0.25% trypsin, 0.53 mM EDTA, pH 7.3 (Sigma). Released cells were triturated using a 10-ml pipette and collected by centrifugation. Cells were counted using improved Neubauer chambers and seeded at $10–12 \times 10^3$ cells/cm^2 in 25-cm^2 T-flasks. When cells reached 50% confluency and existed mostly as individuals rather than clumps they were again released from the substratum using trypsin-EDTA and washed twice with complete medium and collected by centrifugation ($500 \times g$ for 3 min). Cells from one 25-cm^2 T-flask were suspended in 4 ml of complete medium, and aggregates were allowed to settle from the liquid column. The suspension containing mostly individual cells was collected, and cell numbers were again determined. The concentration of cells was adjusted with complete medium to 5.26×10^5 cells/ml (2,000 cells/3.8 μl).

Secreted PSA—Nine serial 2-fold dilutions of cells were made in complete medium. Dilutions were carried to an average of four cells/well. 3.8 μl were seeded in 10 replicate wells of a 1,536-well plate (Bellco) along with control medium in wells and a like number of control non-PSA-producing reptilian cells. Medium and cells were incubated for 24 h. Medium from each well for each cell type and serial dilution was collected (3 μl) and pooled to obtain a statistical average for the number of cells in each well. The pooled, collected medium was stored at −80 °C until assayed.

LNCaP cells prepared from 50% confluent T-flasks as above were diluted so that each 3.8 μl plated into a well of a 96-well plate contained 100 cells at time 0. Medium above the attached cells (3 μl) from 10 identical wells was collected at the indicated times and frozen at −80 °C until assayed for PSA.

Cellular PSA—LNCaP cells prepared from 50% confluent T-flasks as above were adjusted with complete medium to 2,000 cells/50 μl, and nine serial 2-fold dilutions in complete medium were prepared. Aliquots of 50 μl from each dilution were added to 500-μl microcentrifuge tubes (in triplicate), and the tubes were spun at 4,000 rpm for 4 min in a fixed angle rotor of a microcentrifuge. Medium was carefully and totally removed from each tube using a blunt needle Hamilton syringe. The cells were then lysed by three cycles of freezing and thawing. The cellular debris were removed by centrifugation, and the supernatant was collected and assayed for PSA.

Statistical Calculations—Each experiment was repeated three times or more with at least five arrays for each attempt. Each data point was the statistical average of minimally 15 different data sets. The net fluorescence intensity (raw minus background intensity) of the arrayed spots was obtained using Array Pro Analyzer software (Media Cybernetics). Statistical calculations were carried out using Microsoft Excel, and the results were graphed using Sigma Plot (SPSS Inc.).

RESULTS

In this study, capture antibodies were deposited in ultramicrodomains and immobilized using an amine-reactive chemistry. The captured antigens were detected using standard sandwich assays and immunofluorescence microscopy. The ability to directly image, rather than scan, ultraminiaturized arrays makes data collection rapid and provides automatic internal control with respect to instrumentation variability. The experimental design is illustrated in Fig. 1.

Mouse anti-PSA ultramicroarrays were constructed adjacent to rat anti-IL-6 ultramicroarrays (both arrays were 3×6 with 15-μm pitch; spot size = 5–8-μm) on amine-reactive silicon surfaces. Alexa 488-conjugated anti-rat IgG, which is known to cross-react with mouse IgG, was used to evaluate the presence of the capture antibody on the chips (Fig. 2, *panels A* and *D*). The fluorescent images clearly demonstrate that the capture antibodies were bound to the chip only at expected locales. In one instance a chip so constructed was incubated with PSA, washed, and incubated with a goat anti-PSA detection antibody to complete the antibody-target-antibody sandwich. A Texas Red-conjugated anti-goat IgG was used to generate a fluorescence signal from the detection antibody and demonstrate that the arrayed capture antibody was functional and had captured PSA (Fig. 2, *panel B*). The fluorescence signals from both the Alexa 488 and Texas Red dyes were restricted to the antibody-arrayed domains, and there was a one-to-one correlation between the locations of the two dyes.

Molecular specificity and multiplexing capability of the ultramicroarrays used in this study were demonstrated by arraying capture antibodies against PSA and mouse IL-6 on the

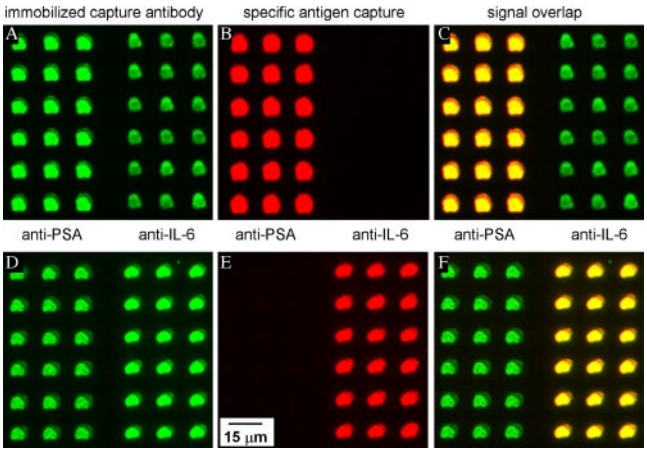

FIG. 2. **Ultramicroscale immunofluorescent antibody capture assay.** *Panels A* and *D* show the deposition domains containing capture antibodies for PSA and IL-6, visualized with an Alexa 488 (*green*)-coupled antibody. *Panels B* and *E* show specific capture of PSA and IL-6, visualized with an Alexa 594 (*red*)-coupled detection antibody. *Panels C* and *F* show the overlap of *panels A* and *B* and *panels D* and *E*, respectively, to illustrate the one-to-one correspondence between the capture antibody and the specific signal. *Scale bar*, 15 μm.

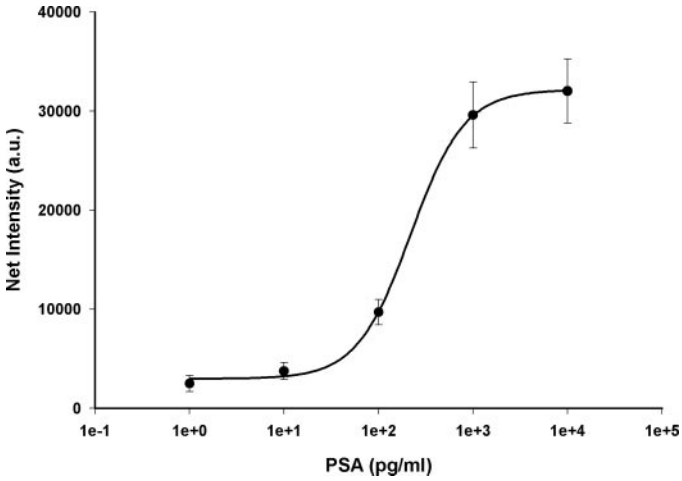

FIG. 3. **PSA detection sensitivity.** 10-fold serial dilutions of PSA were captured using chips with ultramicroarrays of PSA capture antibodies. The fluorescent image was collected using the same camera settings for each of the PSA concentrations, and the net intensity (raw minus background) of the spots was obtained. The mean net intensity for each concentration from three different chips (125 spots/chip) was plotted. The higher limit is the result of photosensor saturation under fixed conditions and does not represent the ultimate upper detection limit of the system.

same chip. As described above, the arrayed capture antibodies were labeled with Alexa 488-conjugated anti-rat antibody to demonstrate that they were immobilized at the expected locations. The multiplexed arrays were incubated with either purified PSA or purified recombinant IL-6 followed by completion of the antibody sandwich and fluorescence microscopy. Comparing the fluorescence data presented in Fig. 2, *panels B* and *D*, illustrates that only the arrayed PSA and IL-6 antibodies captured the cognate proteins with virtually no cross-talk within the limits of detection. Again there was a one-to-one correspondence between the location of the capture antibody and the IL-6-specific fluorescence signal (Fig. 2, *panels C* and *F*).

To evaluate the detection sensitivity of anti-PSA ultramicroarrays multiple PSA capture chips were constructed with at least five arrays on each chip. These chips were incubated with 1 μl each of 10-fold serial dilutions of PSA. At least three chips were incubated with each concentration of PSA. The net fluorescence intensity (raw minus background intensity) of the arrayed spots was obtained. Statistical calculations were carried out, and the results were plotted. Each data point on the graph was the statistical average of at least 15 different data sets. The results presented in Fig. 3 indicate that anti-PSA ultramicroarrays are capable of detecting PSA at a level of ~10 pg/ml from only 1 μl of the sample corresponding to sensitivity at the attomole level.

To evaluate anti-PSA ultramicroarrays in a biological system we used the well characterized cell culture model for prostate cancer, LNCaP, which constitutively produces PSA. Ten replicate 2-fold serial dilutions, starting at 2,000 cells/well, were incubated for 24 h. The supernatants from each serial dilution were pooled and stored at −80 °C. A small volume of the pooled medium (3 μl) was mixed with an equal volume of PBST buffer and incubated on PSA capture chips at room temperature for 1 h in a humid environment. The chips were treated and evaluated as described under "Experimental Procedures." Each analysis was repeated at least three times for each medium pool. The data presented (Fig. 4, *inset*) compare the fluorescence intensities of the signal obtained from the supernatant of 250 cells and that from four cells. The net intensity over the range of dilutions used is plotted in Fig. 4. These results show that PSA could be readily detected from the supernatant of LNCaP cells. More importantly, a reliable signal was obtainable from the PSA secreted by just four cells in 24 h.

To further evaluate the anti-PSA ultramicroarrays in a biological system, LNCaP cells were grown in a 1,536-well plate with 100 cells in each well. The cells were incubated for varying periods of time, and the supernatant was collected at time points starting at 1 h to 24 h postincubation. The supernatant was collected from at least five wells at each time point, pooled, and mixed with an equal volume of PBST buffer. A small volume of the pooled medium (3 μl) was mixed with an equal volume of PBST buffer and incubated on PSA capture chips at room temperature for 1 h in a humid environment. The chips were treated and evaluated as described under "Experimental Procedures." The net intensity of the fluorescence signal over the indicated course of time is presented in Fig. 5. The data presented in Fig. 5, *inset*, compare the fluorescence intensity of the signal obtained from the secreted PSA by 100 LNCaP cells in 2 h and that in 23 h.

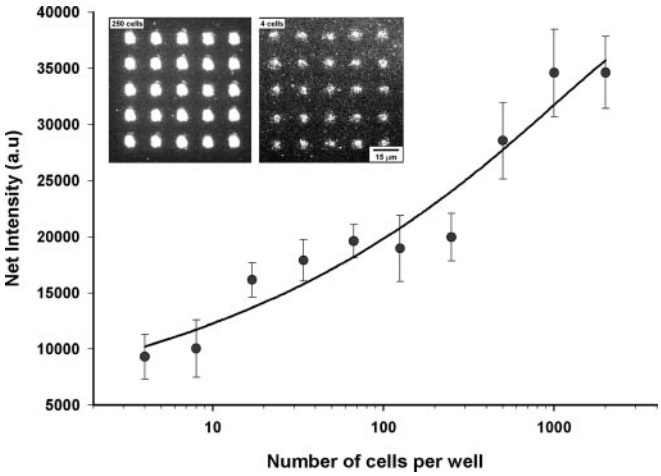

FIG. 4. **PSA detection from a model cell culture system.** 2-fold serial dilutions of LNCaP cells were grown in 1,536-well plates, and the supernatant was collected after 24 h. The supernatant was assayed for PSA, and the relationship between net fluorescence signal intensity and number of cells was plotted. Each point is the average of at least three trials. The *inset* shows the raw fluorescence data from 250 (*left*) and four (*right*) cells.

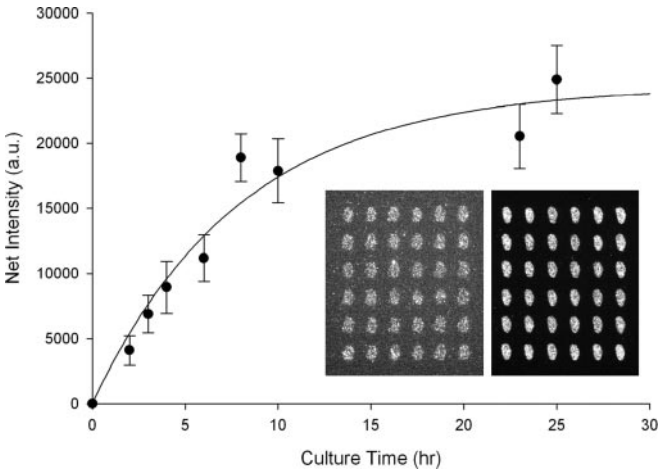

FIG. 5. **PSA detection from LNCaP cell as a function of time.** LNCaP cells were cultured in 1,536-well plates with 100 cells/well for the indicated times. The supernatant was collected and assayed for PSA. The net fluorescence signal intensity was plotted as a function of time. The *inset* shows the raw fluorescence intensity at 2 h (*left*) and 23 h (*right*).

These results show that PSA secreted by 100 cells could be detected in the growth medium in less than 3 h.

In an effort to detect and quantify the cellular PSA levels, LNCaP cells were harvested and diluted such that there were 2,000 cells/50 μl. Nine 2-fold serial dilutions of the cells were carried out and collected by centrifugation. Extra precautions were taken to ensure that all of the secreted PSA was removed by sequential washing of the cells before lysis. The cells were lysed, and the supernatant was used to assay for the presence of PSA. The data presented in Fig. 6, *inset*, compare the fluorescence intensity of the signal obtained

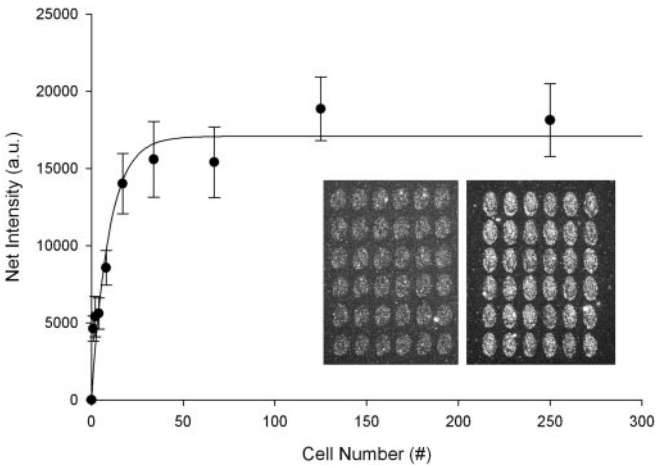

FIG. 6. **Detection of cellular PSA.** A serial dilution of LNCaP cells was lysed, and the cellular PSA was assayed. The net fluorescence signal intensity was plotted as a function of the cell number. The *inset* shows the raw fluorescence data from two (*left*) and 67 (*right*) cells.

from the cellular PSA from just two cells to that from 67 cells. The intensity of the fluorescence signal over the number of cells is presented in Fig. 6. The results demonstrate that the cellular PSA from just six cells can be easily detected using ultramicroarrays and a standard sandwich fluorescence assay. This level of sensitivity is unprecedented in the microarray capture assays with fluorescence readout.

DISCUSSION

This study demonstrates the utility of ultramicroscale protein capture arrays for the detection and quantification of protein biomarkers. Ultramicroarrays, *i.e.* arrays with domains less than 20 μm in diameter, were constructed using instrumentation and methodologies developed and previously described by our research group (17, 28). This report presents the first study demonstrating the significant advantages of using ultramicroarrays in protein detection. Haab *et al.* (4) arrayed 115 distinct antibodies and demonstrated that they could detect antigens with a sensitivity of 1 ng/ml. A similar study showed that cancer antigens could be detected at a concentration of 5 ng/ml (13). Detection of epidermal growth factor receptor and Her2 in a microarray format was achieved at 1 ng/ml levels using a sandwich assay and was increased to 0.1 ng/ml levels by direct labeling methods (7). Standard sandwich immunofluorescence assay was used to detect 75 different cytokines at a concentration of 1 ng/ml in a microarray format. Because this level of sensitivity was not sufficient to detect physiologically relevant levels of cytokines, Schweitzer *et al.* (10) used rolling circle amplification to increase the sensitivity to the order of 1–100 pg/ml. We have demonstrated that PSA and IL-6 can be detected in the range of 10 pg/ml, levels previously unattainable by other arraying technologies using sandwich assays. We show that protein ultramicroarrays can be multiplexed and that they are capable of meaningful detection of a cancer protein biomarker, PSA, from the

supernatant of just four LNCaP cells.

One of the advantages of using ultramicroarrays is the high density of the arrays, and thereby the large numbers of different capture domains, that can be constructed in a small surface area. This enables the direct analysis of very small sample volumes without having to dilute the sample and thereby losing sensitivity. The 1–6-μl volumes used in this study were a vast volumetric excess in comparison to the spatial requirements of ultramicroarrays, which are compatible with submicroliter volumes. Another advantage of ultramicroscale capture domains is a reduction in the depletion of analyte from the samples; this translates to enhanced reaction kinetics.

The apparent limited dynamic range (Figs. 3, 5, and 6) is due to saturation of the camera system used in this study. A fixed exposure time was used for all samples to permit direct comparison of fluorescence data. This resulted in charge-coupled device saturation at high PSA concentrations. Use of shorter exposure times or a more sophisticated light capturing system would mitigate this apparent saturation and reveal the true dynamic range of the system. A key feature of ultramicroarrays is that the molecular population of antibodies is on the order of several hundred thousand molecules in a 5-μm-diameter spot. This was calculated based on the dimension of an IgG to be 14.5 $\times$ 8.5 $\times$ 4 nm (29, 30) and an optimal packing of IgG molecules. Therefore a 5-μm spot will create the opportunity for at least 3–4 logs of dynamic range while retaining the advantages of ultraminiaturization. This consideration is critical when evaluating single molecule approaches to diagnostic testing.

Protein capture ultramicroarrays such as those demonstrated here enable the detection and analysis of protein biomarkers from extremely small samples. This capability provides a new pathway to the realization of opportunities in many areas including protein biomarker analysis of laser capture microdissected, neonatal, and forensics microspecimens. Protein capture ultramicroarrays are, in fact, a significant advance toward the detection of protein biomarkers from single cells.

Acknowledgment—We thank Asrun Kristmundsdottir for the critical reading of this manuscript.

* This work was supported by NCI, National Institutes of Health Grant R43 CA105659-01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: BioForce Nanosciences Inc., 1615 Golden Aspen Dr., Suite 101, Ames, IA 50010. Tel.: 515-233-8333 (ext. 107); Fax: 515-233-8337; E-mail: snettikadan@bioforcenano.com.

REFERENCES

1. Ludwig, J. A., and Weinstein, J. N. (2005) Biomarkers in cancer staging, prognosis and treatment selection. *Nat. Rev. Cancer* **5,** 845–856
2. Mikolajczyk, S. D., Song, Y., Wong, J. R., Matson, R. S., and Rittenhouse, H. G. (2004) Are multiple markers the future of prostate cancer diagnostics? *Clin. Biochem.* **37,** 519–528
3. Liotta, L. A., Espina, V., Mehta, A. I., Calvert, V., Rosenblatt, K., Geho, D., Munson, P. J., Young, L., Wulfkuhle, J., and Petricoin, E. F., III (2003) Protein microarrays: meeting analytical challenges for clinical applications. *Cancer Cell* **3,** 317–325
4. Haab, B. B., Dunham, M. J., and Brown, P. O. (2001) Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. *Genome Biol.* **2,** RESEARCH0004
5. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R. A., Gerstein, M., and Snyder, M. (2001) Global analysis of protein activities using proteome chips. *Science* **293,** 2101–2105
6. Nielsen, U. B., and Geierstanger, B. H. (2004) Multiplexed sandwich assays in microarray format. *J. Immunol. Methods* **290,** 107–120
7. Nielsen, U. B., Cardone, M. H., Sinskey, A. J., MacBeath, G., and Sorger, P. K. (2003) Profiling receptor tyrosine kinase activation by using Ab microarrays. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 9330–9335
8. Wang, C. C., Huang, R. P., Sommer, M., Lisoukov, H., Huang, R., Lin, Y., Miller, T., and Burke, J. (2002) Array-based multiplexed screening and quantitation of human cytokines and chemokines. *J. Proteome Res.* **1,** 337–343
9. Wiese, R., Belosludtsev, Y., Powdrill, T., Thompson, P., and Hogan, M. (2001) Simultaneous multianalyte ELISA performed on a microarray platform. *Clin. Chem.* **47,** 1451–1457
10. Schweitzer, B., Roberts, S., Grimwade, B., Shao, W., Wang, M., Fu, Q., Shu, Q., Laroche, I., Zhou, Z., Tchernev, V. T., Christiansen, J., Velleca, M., and Kingsmore, S. F. (2002) Multiplexed protein profiling on microarrays by rolling-circle amplification. *Nat. Biotechnol.* **20,** 359–365
11. Yguerabide, J., and Yguerabide, E. (1998) Light-scattering submicroscopic particles as highly fluorescent analogs and their use as tracer labels in clinical and biological applications. *Anal. Biochem.* **262,** 157–176
12. Miller, J. C., Zhou, H., Kwekel, J., Cavallo, R., Burke, J., Butler, E. B., Teh, B. S., and Haab, B. B. (2003) Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. *Proteomics* **3,** 56–63
13. Sreekumar, A., Nyati, M. K, Varambally, S., Barrette, T. R., Ghosh, D., Lawrence, T. S., and Chinnaiyan, A. M. (2001) Profiling of cancer cells using protein microarrays: discovery of novel radiation-regulated proteins. *Cancer Res.* **61,** 7585–7593
14. Knezevic, V., Leethanakul, C., Bichsel, V. E., Worth, J. M., Prabhu, V. V., Gutkind, J. S., Liotta, L. A., Munson, P. J., Petricoin, E. F., III, and Krizman, D. B. (2001) Proteomic profiling of the cancer microenvironment by antibody arrays. *Proteomics* **1,** 1271–1278
15. Ekins, R. P., Berger, H., Chu, F. W., Finckh, T., and Krause, F. (1998) Minuaturised immunoarrays: ultrasensitive multianalyte immunoassays on a chip. *Nanobiology* **4,** 197–220
16. Silzel, J. W., Cercek, B., Dodson, C., Tsay, T., and Obremski, R. J. (1998) Mass-sensing, multianalyte microarray immunoassay with imaging detection. *Clin. Chem.* **44,** 2036–2043
17. Lynch, M., Mosher, C., Huff, J., Nettikadan, S., Johnson, J., and Henderson E. (2004) Functional protein nanoarrays for biomarker profiling. *Proteomics* **4,** 1695–1702
18. Hernandez, J., and Thompson, I. M. (2004) Prostate-specific antigen: a review of the validation of the most commonly used cancer biomarker. *Cancer* **101,** 894–904
19. Yousef, G. M., and Diamandis, E. P. (2001) The new human tissue kallikrein gene family: structure, function, and association to disease. *Endocr. Rev.* **22,** 184–204
20. Balk, S. P., Ko, Y. J, and Bubley, G. J. (2003) Biology of prostate-specific antigen. *J. Clin. Oncol.* **21,** 383–391
21. Lovgren, J., Valtonan-Andre, C., Marsal, K., Lilja, H., and Lundwall, A. (1999) Measurement of prostate-specific antigen and human glandular kallikrein 2 in different body fluids. *J. Androl.* **20,** 348–355
22. Grossmann, M. E., Huang, H., and Tindall, D. J. (2001) Androgen receptor signaling in androgen-refractory prostate cancer. *J. Natl. Cancer Inst.* **93,** 1687–1697
23. Chung, T. D., Yu, J. J, Spiotto, M. T., Bartkowski, M., and Simons, J. W. (1999) Characterization of the role of IL-6 in the progression of prostate cancer. *Prostate* **38,** 199–207

24. Adler, H. L., McCurdy, M. A., Kattan, M. W., Timme, T. L., Scardino, P. T., and Thompson, T. C. (1999) Elevated levels of circulating interleukin-6 and transforming growth factor-β1 in patients with metastatic prostatic carcinoma. *J. Urol.* **161,** 182–187
25. Nakashima, J., Tachibana, M., Horiguchi, Y., Oya, M., Ohigashi, T., Asakura, H., and Murai, M. (2000) Serum interleukin 6 as a prognostic factor in patients with prostate cancer. *Clin. Cancer Res.* **6,** 2702–2706
26. Lee, Y., Lee, E. K., Cho, Y. W., Matsui, T., Kang, I. C., Kim, T. S., and Han, M. H. (2003) ProteoChip: a highly sensitive protein microarray prepared by a novel method of protein immobilization for application of protein-protein interaction studies. *Proteomics* **3,** 2289–2304
27. Henderson, E., Mosher, C., and Lynch, M. P. (June 3, 2003) Method and apparatus for solid state molecular analysis, BioForce Nanosciences Inc., U. S. Patent 6,573,369
28. Xu, J., Lynch, M., Huff, J. L., Mosher, C., Vengasandra, S., Ding, G., and Henderson, E. (2004) Microfabricated quill-type surface patterning tools for the creation of biological micro/nano arrays. *Biomed. Microdevices* **6,** 117–123
29. Silverton, E. W., Navia, M. A., and Davies, D. R. (1977) Three-dimensional structure of an intact human immunoglobulin. *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5140–5144
30. Wadu-Mesthrige, K., Amro, N. A., Garno, J. C., Xu, S., and Liu, G. (2001) Fabrication of nanometer-sized protein patterns using atomic force microscopy and selective immobilization. *Biophys. J.* **80,** 1891–1899