Ultramicroarrays for Biomarker Detection from Four Cells

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Abstract

The field of protein microarrays has enabled the rapid detection and identification of biomarkers. However the spot size limitation of protein microarray methods has limited the study of biomarkers to relatively large sample volumes. Here we describe the use of ultramicroarrays, defined as arrays with domain sizes in the two to twenty micrometer range, for protein biomarker detection. Detection of prostate specific antigen (PSA) from one microliter sample volumes was achieved. PSA capture was highly specific with sensitivity in the picogram per milliliter range (absolute sensitivity of femtograms). PSA was detected from two microliters of supernatant from as few as four LNCaP cells. Miniaturization of capture domains to these levels enables the analysis of extremely small volumes of samples that cannot be amplified or replaced such as those found in minimally invasive cell aspirates, neonatal samples and forensic specimens. This approach should be consistent with protein biomarker analysis from small populations, and even single cells acquired, for example, by laser capture microdissection (LCM).

BioForce Nanosciences has developed a dedicated instrument, the NanoArrayer™, for the construction of ultraminiaturized arrays of biomolecules on the sub-micron and micron scale. With droplet volumes in the attoliter to femtoliter range, these nanoarrays occupy a surface area thousands of times smaller than a standard microarray. The analyte volume required for analysis is drastically reduced, with typical assays consuming 1 µl or less. This spatial size is ideal because it allows for the development of novel applications such as single cell proteomic analysis while permitting acquisition of statistically meaningful data by a number of methods. Ultramicroarrays may be analyzed using conventional techniques such as

Figure 1. The NanoArrayer system used to create these ultraminiaturized biomarker arrays.

Methods

Arraying: 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. All capture antibodies were diluted 1:1 in protein spotting buffer and a minimum of 5 arrays were printed on each chip. Each experiment was repeated at least three times. Arraying was performed with a NanoArrayer (BioForce Nanosciences), which employs microfabricated surface patterning tools (SPTs) to actively transfer attoliter to femtoliter droplets of fluid to the surface. Spot diameter is user controlled, and in these experiments ranged from 5 µm to 8 µm. All surfaces were Prolinker SAM on 4mm x 4mm gold coated, alphanumeric indexed silicon chips (Sindex chips). Antibody arrays constructed in this fashion were incubated overnight at 70% humidity to facilitate binding, then blocked with ViriBlock (BioForce Nanosciences) prior to use.

Analysis: A Nikon TE 2000U inverted microscope with 40X oil objective and filter sets for Alexa 488 and Alexa 594 dyes was used for fluorescence imaging. A cooled CCD digital camera with 1.3mp resolution was used to collect images. Metamorph was used for image capture and analysis. Array Pro Analyzer 4.5 and Microsoft Excel were used for data analysis and the results were plotted using Sigma Plot.

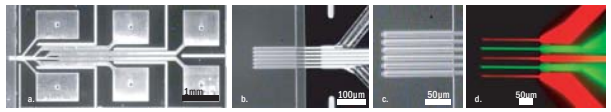

Figure 2.
(a-c) Optical images of 6 lever SPT showing reservoirs with microfluidic channels leading down each cantilever.
(d) Fluorescence image of 5 lever SPT loaded with two fluorescently labeled antibodies.

Methods

Secreted PSA Assays: Nine serial two-fold dilutions of cells were made in complete media. Dilutions were carried to an average of 4 cells/well. 3.8 µl were seeded in 10 replicate wells of a 1536 well plate (Belco) along with control media in wells and a like number of control non-PSA producing reptilian cells. Media and cells were incubated for 24 hours. Media 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 media was stored at –80°C until assayed. LNCaP cells prepared from 50% confluent T flasks were diluted so that each 38 µl plated into a well of a 96 well plate contained 100 cells at time zero. Media above the attached cells (3.8 µl) from 10 identical wells was collected at the indicated times and frozen at –80°C until assayed for PSA.

Cellular PSA Assay: LNCaP cells prepared from 50% confluent T flasks were adjusted with complete media to 2,000 cells/50 µl and nine serial two-fold dilutions in complete media were prepared. Aliquots of 50 µl from each dilution were added to 500 µl microcentrifuge tubes (in triplicate) and the tubes were spun at 4000 rpm for 4 min in a fixed angle rotor of a microfuge. Media was carefully and totally removed from each tube using a blunt needle Hamilton syringe. The cells were then lysed by freeze-thaw method. The cellular debris was removed by centrifugation and the supernatant collected and assayed for PSA.

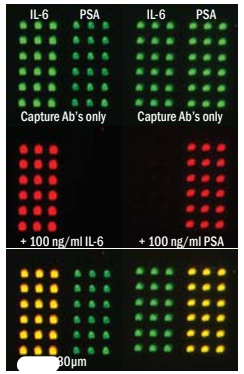

Figure 3.
Color combined images demonstrating specific capture of IL-6 or PSA.

Results

PSA Detection Sensitivity

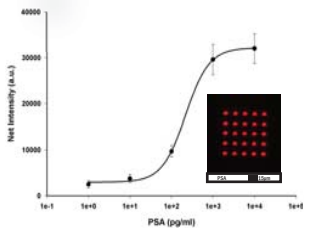

Figure 4. Ten-fold serial dilutions of PSA (1 µl) were assayed using ultramicroarrays of PSA capture antibodies. The fluorescent image was collected using the same camera settings for each of the PSA concentrations and the net intensity of the spots was obtained. The mean net intensity for each concentration from 3 different chips (125 spots/chip) was plotted. The limit of detection for this assay was 10 pg/ml using a 1 µl sample, for an absolute sensitivity at the zeptomole level.

PSA from LNCaP Lysate

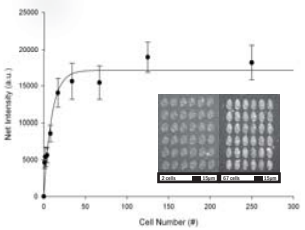

Figure 5. A serial dilution of LNCaP cells was lysed and the cellular PSA was assayed. The net fluorescent signal intensity was plotted as a function of the cell number. The inset shows the raw fluorescence data from 2 (left) and 67 (right) cells. The higher limit is the result of photosensor saturation under fixed conditions and does not represent the ultimate upper detection limit of the system.

Results

PSA Secretion - Culture Time

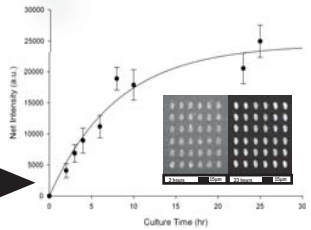

Figure 6. LNCaP cells were cultured in 1536 well plates with 100 cell/well for the indicated times. The supernatant (3 µl) was collected and assayed for PSA. The net fluorescence signal intensity was plotted as a function of time. The inset shows the raw fluorescent intensity at 2hr (left) and 23 hr (right).

PSA Secretion - Cell Count

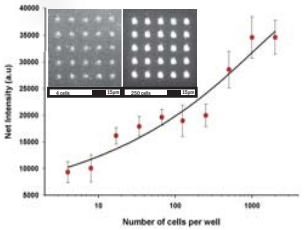

Figure 7. Two-fold serial dilutions of LNCaP cells were grown in 1536 well plates and the supernatant (3 µl) collected after 24 hours. The supernatant was assayed for PSA and the relationship between net fluorescence signal intensity and number of cells was plotted. Each point is the average of at least 3 trials. The inset shows the raw fluorescence data from 4 (left) and 250 (right) cells.

Conclusions

- The NanoArrayer is a useful tool for constructing ultraminiaturized protein biomarker assays
- Ultramicroarrays can be used to assay samples as small as 1 µl containing zeptomoles of target protein
- Ultramicroarrays can detect the PSA released by lysing as few as 2 LNCaP cells
- Ultramicroarrays can detect the PSA secreted by 100 LNCaP cells after as little as 2 hours in culture
- Ultramicroarrays can detect the PSA secreted by only 4 LNCaP cells after 24 hours in culture

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