2 Integrated Barcode Chips for Rapid, Multiplexed Analysis of Proteins in Microliter Quantities of Blood

2.1 Introduction

Microfluidics has permitted the miniaturization of conventional techniques to enable high-throughput and low-cost measurements in basic research and clinical applications.[1,2] Systems for biomolecular assays[3,4] and bio-separations,[5,6] including the separation of circulating tumor cells or plasma from whole blood,[7-9] have been reported. We developed the integrated blood barcode chip (IBBC) to address the need for microchips that integrate on-chip plasma separations from microliter quantities of whole blood with rapid in situ measurements of multiple plasma proteins. The immunoassay region of the chip is a microscopic barcode, integrated into a microfluidics channel and customized for the detection of many proteins and/or for the quantification of a single or few proteins over a broad concentration range. We demonstrate versatility of this barcode immunoassay by detecting human chorionic gonadotropin (hCG) from human serum over a 10^5 concentration range and by stratifying 22 cancer patients via multiple measurements of a dozen blood protein biomarkers for each patient. We also use the IBBC to assay a blood protein biomarker panel from whole human blood, performing all key steps in the immunoassay within 10 minutes of blood collection by finger prick.

We first present an overview of the IBBC and then discuss control of assay sensitivity, extension of a single protein assay to an assay for a large panel of biomarkers and, finally,

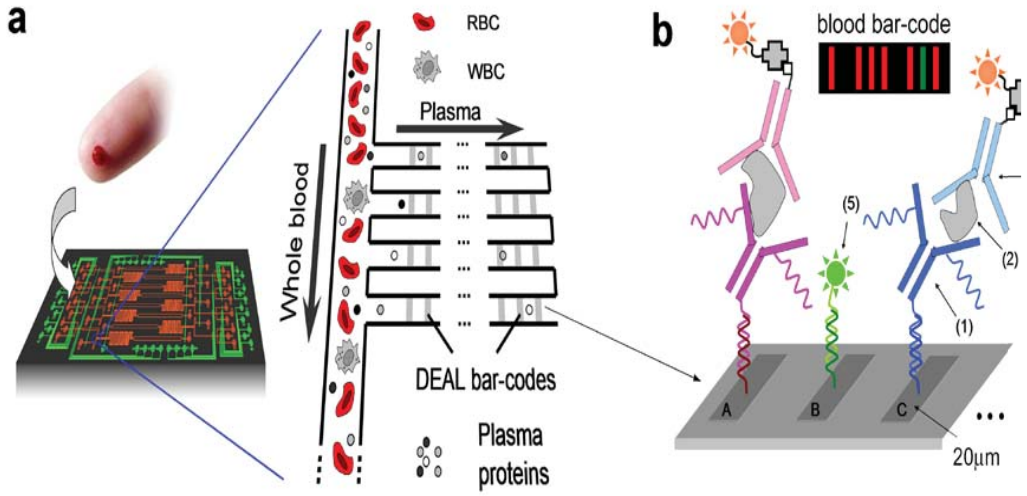

Figure 2.1 Design of an integrated blood barcode chip (IBBC). **(a)** Scheme depicting plasma separation from a finger prick of blood by harnessing the Zweifach-Fung effect. Multiple DNA-encoded antibody barcode arrays are patterned within the plasma-skimming channels for in situ protein measurements. **(b)** DEAL barcode arrays patterned in plasma channels for in situ protein measurement. A, B, C indicate different DNA codes. (1)–(5) denote DNA-antibody conjugate, plasma protein, biotin labeled detection antibody, streptavidin-Cy5 fluorescence probe and complementary DNA-Cy3 reference probe, respectively. The inset represents a barcode of protein biomarkers, which is read out using fluorescence detection. The green bar represents an alignment marker.

integration of plasma separation from whole blood, followed by the rapid measurement of a panel of protein biomarkers. **Figure 2.1** shows the design of an IBBC for blood separation and *in situ* protein measurement. We designed a polydimethylsiloxane (PDMS)-on-glass chip to perform 8–12 separate multiprotein assays sequentially or in parallel, starting from whole blood.

The Zweifach-Fung effect describes highly polarized blood cell flow at branch points of small blood vessels.[9-11] A component of the IBBC, redesigned from a previous report,[9] exploits this hydrodynamic effect by flowing blood through a low-flow-resistance primary channel with high-resistance, centimeter-long channels that branch off it at right angles (**Figure 2.1a**). As the resistance ratio is increased between the branches and the primary channel, a critical streamline moves closer to the primary channel wall adjoining the branch channels. Blood cells with a

radius larger than the distance between this critical streamline and the primary channel wall are directed away from the high-resistance channels, and ~15% of the plasma is skimmed into the high-resistance channels. The remaining whole blood is directed toward a waste outlet. The glass base of the plasma skimming channels is patterned with a dense barcode-like array of single-stranded DNA (ssDNA) oligomers before assembly of the microfluidics chip. A full barcode is repeated multiple times within a single plasma-skimming channel, and each barcode sequence constitutes a complete assay.

2.2 Experimental Methods

2.2.1 Micropatterning of Barcode Array

A PDMS mold containing 13–20 parallel microfluidic channels, with each channel conveying a different DNA oligomer as DEAL code, was fabricated by soft lithography. The PDMS mold was bonded to a polylysine-coated glass slide via thermal treatment at 80°C for 2 h. The polyamine surfaces permit significantly higher DNA loading than do more traditional aminated surfaces. DNA 'bars' of 2 μm in width have been successfully patterned using this technique. In the present study, a 20-μm channel width was chosen because the fluorescence microarray scanner we used has a resolution of 5 μm. Nevertheless, the current design already resulted in a DNA barcode array an order of magnitude denser than conventional microarrays fabricated by pin-spotting. The coding DNA solutions (A-M for the cancer serum test and AA-HH for the finger-prick blood test) prepared in 1X PBS were flowed into individual channels, and then allowed to evaporate completely. Finally, the PDMS was peeled off and the substrate with DNA barcode arrays was baked at 80°C for 2–4 h. The DNA solution concentration was ~100

μM in all experiments except in the hCG test, leading to a high loading of ~6 × 10^{13} molecules/cm^2 (assuming 50% was collected onto substrate).

2.2.2 Fabrication of IBBCs

The fabrication of PDMS devices for the IBBCs was accomplished through a two-layer soft lithography approach. The control layer was molded from a SU8 2010 negative photoresist (~20 μm in thickness) silicon master using a mixture of GE RTV 615 PDMS prepolymer part A and part B (5:1). The flow layer was fabricated by spin-casting the pre-polymer of GE RTV 615 PDMS part A and part B (20:1) onto a SPR 220 positive photoresist master at ~2,000 r.p.m. for 1 minute. The SPR 220 mold was ~17 μm in height after rounding by thermal treatment. The control layer PDMS chip was then carefully aligned and placed onto the flow layer, which was still situated on its silicon master, and an additional 60 minutes thermal treatment at 80°C was performed to enable bonding. Afterward, this two-layer PDMS chip was cut off the flow layer master and access holes were drilled. Finally, the two-layer PDMS chip was thermally bonded onto the barcode-patterned glass slide, yielding a completed integrated blood barcode chip (IBBC). In this chip, the DEAL barcode stripes are oriented perpendicular to the microfluidic assay channels. Typically, 8–12 identical units were integrated in a single chip with the dimensions of 2.5 cm × 7 cm.

2.2.3 Clinical Specimens of Cancer Patient Sera

The stored serum samples from 11 breast cancer patients (all female) and 11 prostate cancer patients (all male) were acquired from Asterand. Nineteen out of 22 patients were

European-American and the remaining three were Asian, Hispanic and African-American. The medical history is summarized in **Table 2.3**.

2.2.4 Collecting a Finger Prick of Blood

The human whole blood was collected according to the protocol approved by the institutional review board of the California Institute of Technology. Finger pricks were performed using BD microtainer contact-activated lancets. Blood was collected with SAFE-T-FILL capillary blood collection tubes (RAM Scientific), which we prefilled with 80 μL of 25 mM EDTA solution. A 10 μL volume of fresh human blood from a healthy volunteer was collected in an EDTA-coated capillary, dispensed into the tube, and rapidly mixed by inverting a few times. The spiked blood sample was prepared in a similar way except that 40 μL of 25 mM EDTA solution and 40 μL of recombinant solution were mixed and pre-added in the collection tube. Then 2 μL of 0.5 M EDTA was added to bring the total EDTA concentration up to 25 mM.

2.2.5 Execution of Blood Separation and Plasma Protein Measurement using IBBCs

The IBBCs were first blocked with the buffer solution for 30–60 minutes. The buffer solution prepared was 1% wt/vol bovine serum albumin fraction V (Sigma) in 150 mM 1X PBS without calcium/magnesium salts (Irvine Scientific). The fluid loading was conducted using a Tygon plastic tubing that is interfaced to the IBBC inlet with a 23 gauge metal pin. The Fluidigm solenoid unit was exploited to control the pressure (on/off) for both control valves and flow channels. A pressure of 8–10 p.s.i. was applied to actuate the valves, whereas the loading of fluid into assay channels was carried out with a lower pressure (0.5–3 p.s.i.) depending on the channel

flow resistance and the desired flow rate. Then DNA-antibody conjugates (~50–100 nM) were flowed through the plasma assay channels for ~30–45 minutes. This step transformed the DNA arrays into capture-antibody arrays. Unbound conjugates were washed off by flowing buffer solution through the channels. At this step, the IBBC was ready for the blood test. Two blood samples prepared as mentioned above were flowed into the IBBCs within 1 minute of collection. The IBBC quickly separated plasma from whole blood, and the plasma proteins of interest were captured in the assay zone where DEAL barcode arrays were located. This whole process from finger-prick to plasma protein capture took <10 minutes. In the cancer-patient serum experiment, the as-received serum samples were flowed into IBBCs without any pre-treatment (that is, no purification or dilution). Afterwards, a mixture of biotin-labeled detection antibodies (~50–100 nM) for the entire protein panel and the fluorescence Cy5-streptavidin conjugates (~100 nM) were flowed sequentially into IBBCs to complete the DEAL immunoassay. The unbound fluorescence probes were rinsed off by flowing the buffer solution for 10 minutes. At last, the PDMS chip was removed from the glass slide. The slide was immediately rinsed in ½X PBS solution and deionized water and then dried with a nitrogen gun. Finally, the DEAL barcode slide was scanned by a microarray scanner.

2.2.6 Quantitation and Statistics

All the barcode array slides used in quantification were scanned using an Axon GenePix 4000B two-color laser microarray scanner at the same instrument settings. For 635 nm and 532 nm excitation lasers, respectively, the following settings were used: Laser Power - 100% and 33%; Optical Gain - 800 and 700; Brightness/Contrast - 87 and 88. The output JPEG images were carefully skewed and resized to fit the standard barcode array mask design. Then, an image

processing software, NIH *ImageJ*, was used to produce intensity line profiles of barcodes in all assay channels. Finally, all the line profile data files were loaded into a home-developed program embedded as an Excel macro to generate a spreadsheet that lists the average intensities of all 13 bars in each of 20 barcodes. The means and standard deviations were computed using Microcal *Origin*. Non-supervised clustering of patients was performed using literature methods and algorithms.[12] To assess the significance of two patient (sub)groups, Student's t analysis was performed on selected proteins and all *p*-values were calculated at a significance level of 0.05, if not otherwise specified.

2.3 Results and Discussion

We used the DNA-encoded antibody library (DEAL) technique[13] (**Figure 2.5**) to detect proteins within the plasma-skimming channels. DEAL technology involves using DNA-directed immobilization of antibodies to convert a pre-patterned ssDNA barcode microarray into an antibody microarray, thereby providing a powerful means for spatial encoding.[14,15] The sequences of all ssDNA oligomer pairs used (labeled A/A'-M/M'), and their corresponding antibodies, are listed in **Tables 2.1** and **2.2**. To minimize cross-reactivity, these ssDNA molecules were designed *in silico* and then validated through a full orthogonality test (**Figure 2.6**). In that experiment, each of the complementary DNA molecules with Cy3 fluorescent label was added to a microwell containing a full primary ssDNA barcode array. The results showed only negligible cross-hybridization signals. In the DEAL assay, each capture antibody is tagged with approximately three copies of an ssDNA oligomer that is complementary to ssDNA oligomers that have been surface-patterned into a microscopic barcode within the immunoassay region of the chip. Flow-through of the DNA-antibody conjugates transforms the DNA

microarray into an antibody microarray for the subsequent surface-bound immunoassay. Because DNA patterns are robust to dehydration and can survive elevated temperatures (80–100°C), the DEAL approach circumvents the denaturation of antibodies often associated with typical microfluidics fabrication.

As only a few microliters of blood is normally sampled from a finger prick, on-chip plasma separation yields only a few hundred nanoliters of plasma. The ssDNA barcodes were patterned at a high density using microchannel-guided flow patterning (**Figure 2.7**) to measure a large panel of protein biomarkers from this small volume. We used a PDMS mold that was thermally bonded onto a polyamine-coated glass slide to pattern the entire ssDNA barcode. Polyaminated surfaces permit substantially higher DNA loading than do more traditional aminated surfaces[16] and provide for an accompanying increase in assay sensitivity (**Figures 2.8** and **2.9**). Different solutions, each containing a specific ssDNA oligomer, were flowed through different channels and evaporated through the gas-permeable PDMS stamp, resulting in individual stripes of DNA molecules. One complete set of stripes represents one barcode. All measurements used 20-μm-wide bars spaced at a 40-μm pitch. This array density represents an approximately tenfold increase over a standard spotted array (typical dimensions are 150-μm diameter spots at a 400-μm pitch), thus expanding the numbers of proteins that can be measured within a small volume. No alignment between the barcode array and the plasma channels (IBBC chip design presented in **Figure 2.11**) was required. All protein assays used one color fluorophore and were spatially identified using a reference marker that fluoresced at a different color.

We first illustrate aspects of the barcode assays via the measurement of a single biomarker, human chorionic gonadotropin (hCG), in undiluted human serum over a broad

concentration range. HCG is widely used for pregnancy testing and is a biomarker for gestational trophoblastic tumors and germ cell cancers of the ovaries and testes. For this assay, the barcode was customized by varying the DNA loading during the flow patterning step. The DNA barcode contained 13 regions (**Figure 2.2a**). There were two bars of oligomer B designed to detect the protein, tumor necrosis factor-alpha (TNF-α), as a negative control, one reference bar (oligomer M), one blank, and nine bars of oligomer A (designed for hCG detection and flow patterned at ssDNA concentrations that were varied from 200 μM to 2 μM). To perform the assay, we flowed a mixture of A'-anti-hCG and B'-anti-TNF-α through assay channels. Next, a series of standard hCG serum samples and two hCG samples of unknown concentration were flowed through separate assay channels. Biotinylated detection antibodies for hCG and TNF-α were then applied

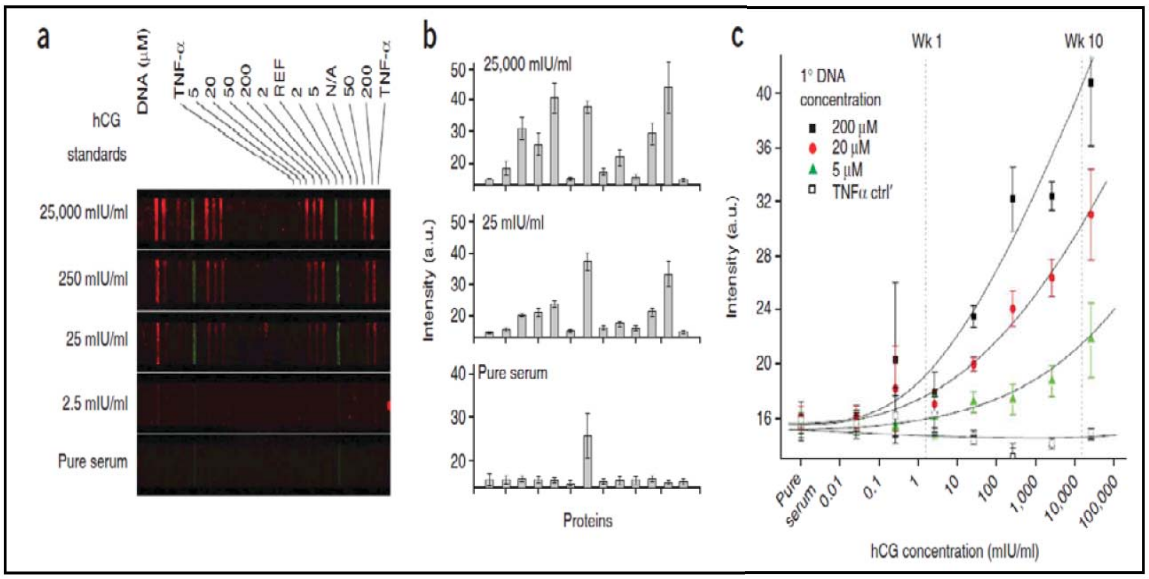

Figure 2.2 Measurement of human chorionic gonadotropin (hCG) in sera.
(a) Fluorescence images of DEAL barcodes showing the measurement of a series of standard serum samples spiked with hCG. The bars used to measure hCG were patterned with DNA strand A at different concentrations. TNF-α encoded by strand B was employed as a negative control. The green bars (strand M) serve as references. **(b)** Quantification of the full barcodes for three selected samples. **(c)** Mean values of fluorescence signals corresponding to three sets of bars with different DNA loadings. Broken lines indicate the typical physiological levels of hCG in sera after 1 or 10 weeks of pregnancy. Error bars, 1 s.d.

followed by a final developing step using fluorescent Cy5-labeled streptavidin (red) for all protein channels and Cy3-labeled M' oligomer (green) for the reference channels (**Figure 2.2a**). Quantifying the fluorescence intensity (**Figure 2.2b,c**) revealed a sensitivity (~1 mIU/ml) comparable to the enzyme-linked immunosorbent assay (ELISA) and a broad detected concentration range (~10^5). Using the microfluidics-entrained DEAL barcode in a blind test, we measured the hCG levels in the two unknown serum samples. Our measured levels, estimated at 6 and 400 mIU/ml for unknowns 1 and 2, are in good agreement with the values of 12 and 357 mIU/ml, respectively, obtained from an independent lab test (**Figure 2.12**). Even without quantification, the analyte concentrations can be estimated by eye through pattern recognition of the full barcode. The bar with the highest DNA-loading rendered the highest sensitivity, whereas the bar with the lowest DNA-loading was used to discriminate samples with high analyte concentrations. For example, the 25,000 mIU/ml and 250 mIU/ml hCG samples can be visually distinguished using stripes patterned with lower DNA concentrations, whereas the stripes loaded from 200 μM DNA solutions do not readily distinguish these samples. For circumstances in which accurate photon counting is not available, visual barcode inspection permits a rough estimation of the target quantity—a potential point-of-care application. When levels of hCG are tracked during pregnancy, concentrations in the blood increase from ~5 mIU/ml in the first week of pregnancy to ~2×10^5 mIU/ml 10 weeks after conception. The IBBC can cover such a broad physiological hCG range with reasonable accuracy.

To evaluate multiplexed measurements of a panel of 12 protein markers using the microfluidic DEAL barcode regions of the IBBCs, we quantified the cross-reactivity between the stripes within the DNA-encoded immunoassays. This test involved twelve human serum proteins, including: ten cytokines - interferon (IFN)-γ, TNF-α, interleukin (IL)-2, IL-1α, IL-1β,

transforming growth factor (TGF)-β1, IL-6, IL-10, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF); a chemokine - macrophage chemoattractant protein (MCP)-1; and the cancer biomarker, prostate-specific antigen (PSA). The results showed negligible cross-talk, with typical photon counts <2% compared to the correctly paired antigen-antibody complexes (**Figure 2.13**). We also assayed serial dilutions (from 5 nM to 1 pM) for these proteins on the DEAL barcode chip to establish a set of calibration curves for future estimates of protein concentration in sera (**Figure 2.14**). We fixed all the parameters associated with laser scanning and fluorescence quantification (e.g., power, gain, brightness and contrast) and performed quantitative analysis. Depending on the antibodies used, the estimated sensitivity varied from <1 pM for IL-1β and IL-12 to ~30 pM for TGF-β, and was comparable to the detection limits of ELISA based on the same antibody pairs. For example, according to the specifications of commercial kits (eBioscience), the detection limit for cytokines like TNF-α and IL-1β is ~8 pg/ml (~0.5 pM), which compares favorably with our observations. However, the statistical variation of the measured signals is relatively large compared to a commercial ELISA assay—a variation that is likely due to the fact that our chips are manufactured manually.

We assessed the utility of the DEAL barcodes for clinical blood samples by measuring the same 12 proteins from small amounts of stored serum collected from 22 cancer patients. These serum samples were thawed, and then assayed using two chips, each containing 12 separate assay units operated in parallel. In every unit, 20 full DEAL barcodes in each assay channel were used for statistical sampling. The proteins in this panel (**Figure 2.3a**) - the prostate cancer marker, PSA, and eleven proteins secreted by white blood cells - have been associated with tumor microenvironment formation, tumor progression, and tumor metastasis.[17-19] Thus, this panel provides information relevant to multiple aspects of cancer.

Figure 2.3b shows fluorescence images, each depicting four sets of randomly picked barcodes obtained from the 22 patient samples. The medical records for all patients are summarized in **Table 2.3**. B01–B11 denote 11 samples from breast cancer patients, whereas P01–P11 are from prostate cancer patients. Many proteins were successfully detected with high signal-to-noise ratios, and the barcode signatures are distinctive from patient to patient, excepting the assays on P05, P04, P10 and B10. These assays are from individuals who are heavy smokers (~11–20 cigarettes daily). Only one serum sample (P06) from a heavy smoker did not exhibit a high background. This high background may result from elevated blood content of the fluorescent protein carboxyhemoglobin, which has been shown to be relevant to the pathogenesis of lung diseases of smokers.[20] Although we have also measured high background in a number of stored serum samples, we have never measured a high background in assays from freshly collected blood, as described below. The results imply that, at least for stored samples, some pre-purification of the plasma or serum will be required to assay serum protein levels.

Barcode intensities were then quantified and the statistic mean value for each protein was computed (**Figures 2.15, 2.16, and 2.17**). The cancer marker PSA clearly distinguished between the breast cancer and the prostate cancer patients. The only exception was a false-positive result from B10 that had high nonspecific background. We independently validated our PSA measurements for all patient sera using standard ELISA. For eight of the prostate cancer patients, we compared our results with clinical ELISA measurements provided by the serum supplier. The results (**Figure 2.3c**) validated the applicability of the DEAL barcodes for assaying complex clinical samples. However, the statistical accuracy of the PSA barcode assay was not high, revealing only a modest linear correlation between ELISA and DEAL (**Figure 2.18**). Again, this is likely due to our manual chip manufacturing process. We are currently automating our barcode

fabrication, assay execution, and image quantification in an effort to bring statistical uncertainties to within 10–20%, close to the state of the art.

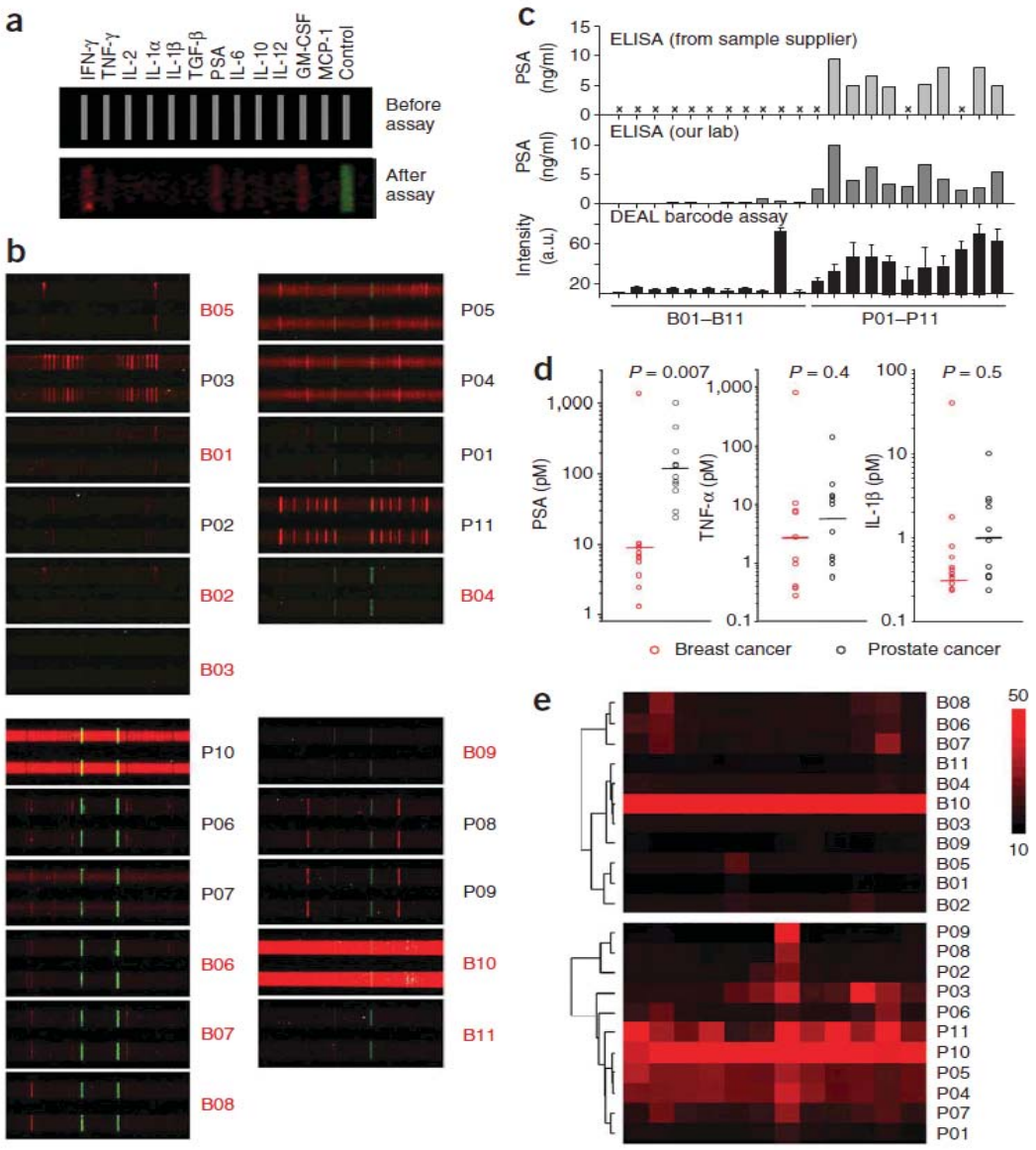

Figure 2.3 Multiplexed protein measurements of clinical patient sera. **(a)** Layout of the barcode array used in this study. Green denotes the reference (strand M). **(b)** Representative fluorescence images of barcodes used to measure the cancer marker PSA and 11 cytokines from 22 cancer patient serum samples. B01–B11, samples from breast cancer patients; P01–P11, samples from prostate cancer patients. The left and right columns represent measurements on different chips. **(c)** Validation of PSA DEAL barcode measurement using ELISA. x denotes PSA measurements were not provided by the serum supplier. Error bars, 1 s.d. **(d)** Distribution of estimated concentrations of PSA, TNF-α, and IL-1β in all serum samples. The horizontal bars mark the mean values. **(e)** Complete non-supervised clustering of breast and prostate cancer patients on the basis of protein patterns.

The cancer patient barcode data could be analyzed for absolute protein levels by comparing those data against the barcode quantification plots (**Figure 2.14**). Results for PSA, TNF-α and IL-1β are shown in **Figure 2.3d**. PSA concentrations range from 22 pM to 1 nM (or 0.7 to 33 ng/ml) with a log-scale mean of 117 pM (3.8 ng/ml) for prostate cancer patients. The estimated PSA concentrations for breast cancer patient sera have a mean of 9.1 pM. PSA readily differentiates between these two patient groups with good statistical accuracy (P = 0.0007). Nevertheless, the absolute PSA levels measured by either the standard ELISA or by the barcode assay are below those determined by the clinical ELISA—a likely result of sample degradation during storage (**Figure 2.3c**). As would be expected, neither TNF-α nor IL-1β allows prostate and breast cancer patients to be distinguished (P – 0.4 and 0.5, respectively, at a significance level of 0.2). Our estimates of absolute protein levels indicate that the protein concentration ranges assessed by the DEAL barcode assay are clinically relevant for patient diagnostics. For example, the serum level of cytokines such as interleukins and tumor necrosis factors can reach ~10–100 pg/ml in cancer patients,[21] ~500 pg/ml in rheumatoid arthritis patients, and 41 ng/ml[22] in septic shock.[23] These levels can all be captured using the barcode assay format.

We performed a complete non-supervised clustering (that is, using only the levels of assayed proteins without assigning any weight factors) of patients and generated a heat map (**Figure 2.3e**) to assess the potential of this technology for patient stratification. This analysis is only presented as a proof of principle. Nevertheless, the results are encouraging. For example, the measured profiles of breast cancer patients can be classified into three subsets—non-inflammatory, IL-1β positive and TNF-α/GMCSF positive ($P_{TNF\alpha}$ = 0.005, P_{GMCSF} = 0.04 for the latter two subsets). The prostate cancer patient data were classified into two major subsets based upon the inflammatory protein levels ($P_{TNF\alpha}$ = 0.016, P_{GMCSF} = 0.012). The multiplexed

measurement of cytokines[24] is relevant to cancer diagnostics and prognostics.[25,26] Our results demonstrate that IBBCs can be applied to the multi-parameter analysis of human health-relevant proteins in serum.

The ultimate goal behind developing the IBBC was to measure the levels of a large number of proteins in human blood within a few minutes of sampling that blood, to avoid the protein degradation that can occur when plasma is stored. In a typical 96-well plate immunoassay, the biological sample of interest is added, and the protein diffuses to the surface-bound antibody. Under adequate flow conditions, diffusion is no longer important, and the only parameter that limits the speed of the assay is the protein/antibody binding kinetics (the Langmuir isotherm),[27] thus allowing the immunoassay to be completed in just a few minutes.[28] Flow through our plasma skimming channels proceeds at velocities $>\sim 0.1$ mm sec^{-1} and can operate continuously and with near 100% efficiency unless the blood flow is clogged.

For whole blood analysis, the microfluidic channels of IBBCs were precoated with bovine serum albumin blocking buffer. The DNA barcodes were transformed into antibody barcodes as described above, and blood samples were flowed into the device within 1 minute of fingerprick collection. The time from that finger prick to completion of blood flow through the device was ~9 minutes. We sampled both as-collected whole blood and protein-spiked blood from healthy volunteers. **Figure 2.4a** shows the effective separation of plasma in an IBBC. The few red blood cells that did enter the plasma channels (**Figure 2.4a**, right panel) did not affect the subsequent protein assay.

The plasma proteins detected in this whole-blood analysis experiment included a cancer marker (PSA), four cytokines, and three other functional proteins - complement C3, C-reactive protein (CRP), and plasminogen - involved in the complement system, inflammatory response,

fibrin degradation and liver toxicity (**Tables 2.1** and **2.2**). After exposure of the barcode assay region to the separated, flowing plasma for 8 minutes, the detection antibody solution and the fluorescence probes were added to complete the assay. All proteins in the spiked blood were detected (**Figure 2.4b,c**). Cytokines gave the strongest fluorescence signals due to the higher affinities of their cognate antibodies. The measurement of the unspiked fresh blood established a baseline for a healthy volunteer, in which IL-6, IL-10, C3, and plasminogen were detected. Using IBBCs for the separation and analysis of freshly collected blood consistently resulted in very clean DEAL barcodes, with little or no evidence of biofouling. We are planning a study to assess the importance of rapid measurements for obtaining accurate protein levels.

Our IBBC enables the rapid measurement of a panel of plasma proteins from a finger prick of whole blood. Integration of microfluidics and DNA-encoded antibody arrays enables reliable processing of blood and in situ measurement of plasma proteins within a time scale that is short enough to avoid most protein degradation processes that can occur in sampled blood. Use of the IBBC represents a minimally invasive, low-cost, and robust procedure, and potentially represents a realistic clinical diagnostic platform.

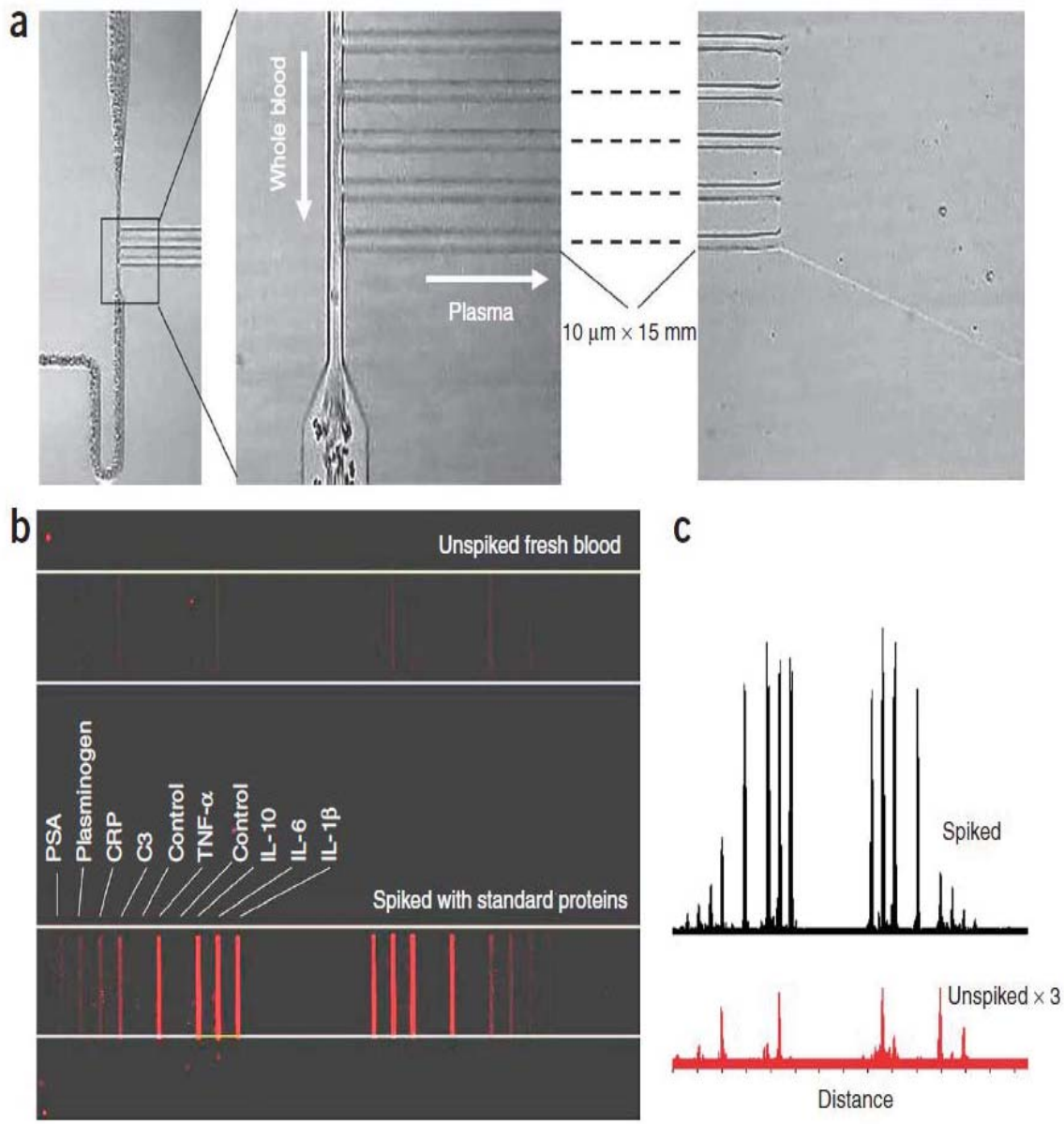

Figure 2.4 IBBC for the rapid measurement of a panel of serum biomarkers from a finger prick of whole blood. **(a)** Optical micrographs showing the effective separation of plasma from fresh whole blood. A few red blood cells occasionally seen downstream of the plasma channels did not affect the protein assay. **(b)** Fluorescence image of blood barcodes in two adjacent microchannels of an IBBC, on which both the unspiked and spiked fresh whole blood collected from a healthy volunteer were separately assayed. Eight plasma proteins are indicated. All bars, 20 µm wide. **(c)** Fluorescence line profiles of the barcodes for both unspiked and spiked whole blood samples. The distance corresponds to the full length shown in **b**.

2.4 References

1 Sia, S. K. & Whitesides, G. M. Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies. *Electrophoresis* **24**, 3563-3576 (2003).

2 Quake, S. R. & Scherer, A. From micro- to nanofabrication with soft materials. *Science* **290**, 1536-1540 (2000).

3 Huang, B. *et al.* Counting low-copy number proteins in a single cell. *Science* **315**, 81-84 (2007).

4 Ottesen, E. A., Hong, J. W., Quake, S. R. & Leadbetter, J. R. Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. *Science* **314**, 1464-1467 (2006).

5 Huang, L., Cox, E., Austin, R. & Sturm, J. Continuous particle separation through deterministic lateral displacement. *Science* **304**, 987 (2004).

6 Chou, C. F. *et al.* Sorting biomolecules with microdevices. *Electrophoresis* **21**, 81-90 (2000).

7 Toner, M. & Irimia, D. Blood-on-a-chip. *Annual Review of Biomedical Engineering* **7**, 77-103 (2005).

8 Nagrath, S. *et al.* Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* **450**, 1235-1239 (2007).

9 Yang, S., Ündar, A. & Zahn, J. A microfluidic device for continuous, real time blood plasma separation. *Lab on a Chip* **6**, 871-880 (2006).

10 Svanes, K. & Zweifach, B. W. Variations in small blood vessel hematocrits produced in hypothermic rates by micro-occlusion. *Microvascular Research* **1**, 210-220 (1968).

11 Fung, Y. C. Stochastic flow in capilary blood vessels. *Microvascular Research* **5**, 34-38 (1973).

12 Eisen, M., Spellman, P., Brown, P. & Botstein, D. Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences* **95**, 14863 (1998).

13 Bailey, R., Kwong, G., Radu, C., Witte, O. & Heath, J. DNA-encoded antibody libraries: a unified platform for multiplexed cell sorting and detection of genes and proteins. *J. Am. Chem. Soc* **129**, 1959-1967 (2007).

14 Boozer, C., Ladd, J., Chen, S. F. & Jiang, S. T. DNA-directed protein immobilization for simultaneous detection of multiple analytes by surface plasmon resonance biosensor. *Analytical Chemistry* **78**, 1515-1519 (2006).

15 Niemeyer, C. M. Functional devices from DNA and proteins. *Nano Today* **2**, 42-52 (2007).

16 Pirrung, M. C. How to make a DNA chip. *Angewandte Chemie-International Edition* **41**, 1277 (2002).

17 Coussens, L. & Werb, Z. Inflammation and cancer. *Nature* **420**, 860-867 (2002).

18 Lin, W. W. & Karin, M. A cytokine-mediated link between innate immunity, inflammation, and cancer. *Journal of Clinical Investigation* **117**, 1175-1183 (2007).

19 De Marzo, A. M. *et al.* Inflammation in prostate carcinogenesis. *Nature Reviews Cancer* **7**, 256-269 (2007).

20 Ashton, H. & Telford, R. Smoking and carboxhemoglobin. *Lancet* **2**, 857-858 (1973).

21 Chopra, V., Dinh, T. & Hannigan, E. Serum levels of interleukins, growth factors and angiogenin in patients with endometrial cancer. *Journal of Cancer Research and Clinical Oncology* **123**, 167-172 (1997).

22 Öncül, O., Top, C. & Çavu lu. Correlation of serum leptin levels with insulin sensitivity in patients with chronic hepatitis-C infection. *Diabetes Care* **25**, 937 (2002).

23 Pinsky, M. *et al.* Serum cytokine levels in human septic shock. Relation to multiple-system organ failure and mortality. *Chest* **103**, 565 (1993).

24 Schweitzer, B. *et al.* Multiplexed protein profiling on microarrays by rolling-circle amplification. *Nature Biotechnology* **20**, 359-365 (2002).

25 Lambeck, A. J. A. *et al.* Serum cytokine profiling as a diagnostic and prognostic tool in ovarian cancer: A potential role for interleukin 7. *Clinical Cancer Research* **13**, 2385-2391 (2007).

26 Gorelik, E. *et al.* Multiplexed immunobead-based cytokine profiling for early detection of ovarian cancer. *Cancer Epidemiology Biomarkers & Prevention* **14**, 981-987 (2005).

27 Heath, J. R. & Davis, M. E. Nanotechnology and cancer. *Annual Review of Medicine* **59**, 405 (2007).

28 Zimmermann, M., Delamarche, E., Wolf, M. & Hunziker, P. Modeling and optimization of high-sensitivity, low-volume microfluidic-based surface immunoassays. *Biomedical Microdevices* **7**, 99-110 (2005).

2.5 Appendix A: Supplementary Methods

2.5.1 DNA-Encoded Antibody Libraries (DEAL) Technique

The critical technique upon which this study is based is the DNA-encoded antibody library (DEAL) method.[1] When DEAL is utilized to measure proteins, it is used as follows (**Figure 2.5**). Capture antibodies (CAs) against the protein of interest are chemically labeled with single-stranded DNA (ssDNA) oligomers, yielding ssDNA-CA conjugates. The coupling reaction is accomplished using succinimidyl 4-formylbenzoate (SFB, Solulink) and succinimidyl 4-hydrazinonicotinate acetone hydrazone in *N*,*N*-dimethylformamide (DMF) (SANH, Solulink) as conjugation agents to link amine termini on DNA oligomers to the amine side-groups of proteins.[1] A size-exclusion column is used to purify the product by removing excess unreacted DNA molecules. Separately, the complementary ssDNA oligomers are deposited in a barcode pattern on a poly-L-lysine coated glass slide using microchannel-guided patterning (details described in **Figure 2.7**). At the beginning of a DEAL protein assay, incubation of ssDNA-CA conjugates with the complementary spatially-patterned ssDNA array assembles the CAs onto those specific sites through DNA hybridization. This step transforms the DNA microarray into an antibody microarray that is ready for a protein sandwich assay. Biological samples (i.e. plasma isolated from human whole blood) can be applied onto the CA microarray and antigens can be captured. Finally, detection antibodies and/or fluorescent read-out probes are introduced sequentially to complete the immuno-sandwich assay. DNA oligomer sequences are chosen with appropriate melting temperatures to optimize room-temperature hybridization to complementary strands while minimizing cross-hybridization (<5% in fluorescence signal).

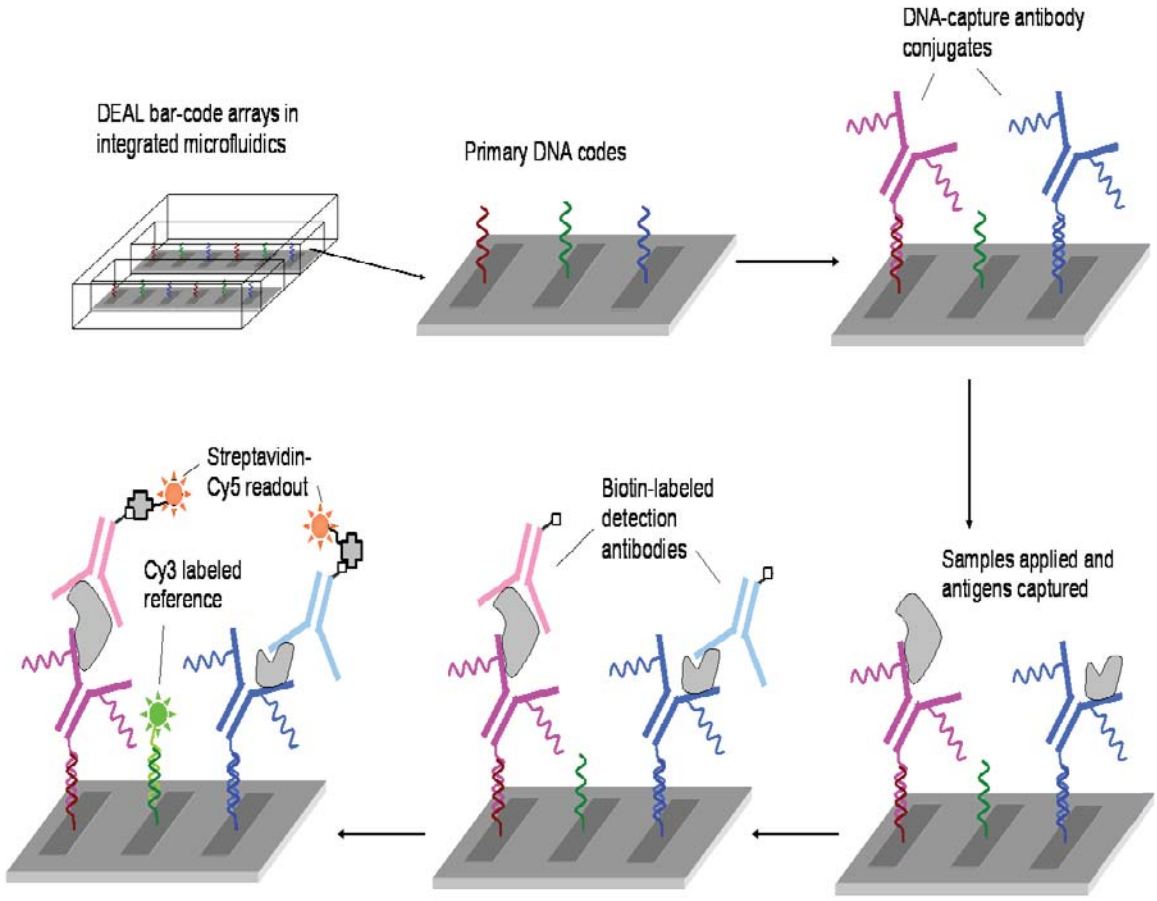

Figure 2.5 Schematic depiction of multi-parameter detection of proteins in integrated microfluidics using the DNA-Encoded Antibody Library (DEAL) technique.

2.5.2 Serum Protein Biomarker Panels and Oligonucleotide Labels

The protein panels used in the cancer-patient serum experiment (panel 1) and finger-prick blood test (panel 2), the corresponding DNA codes, and their sequences are summarized in **Tables 2.1** and **2.2**. These DNA oligomers were synthesized by Integrated DNA Technologies (IDT), and purified by high pressure liquid chromatography (HPLC). The quality was confirmed by mass spectrometry.

Table 2.1 List of Proteins and Corresponding DNA Codes

DNA-code	Human Plasma Protein	Abbreviation
Panel (1)		
A/A'	Interferon-gamma	IFN-γ
B/B'	Tumor necrosis factor-alpha	TNF-α
C/C'	Interleukin-2	IL-2
D/D'	Interleukin-1 alpha	IL-1α
E/E'	Interleukin-1 beta	IL-1β
F/F'	Transforming growth factor beta	TGF-β
G/G'	Prostate specific antigen (total)	PSA
H/H'	Interleukin-6	IL-6
I/I'	Interleukin-10	IL-10
J/J'	Interleukin-12	IL-12
K/K'	Granulocyte-macrophage colony stimulating factor	GM-CSF
L/L'	Monocyte chemoattractant protein -1	MCP-1
M/M'	Blank control/reference	
Panel (2)		
AA/AA'	Interleukin-1 beta	IL-1β
BB/BB'	Interleukin-6	IL-6
CC/CC'	Interleukin-10	IL-10
DD/DD'	Tumor necrosis factor-alpha	TNF-α
EE/EE'	Complement Component 3	C3
FF/FF'	C-reactive protein	CRP
GG/GG'	Plasminogen	Plasminogen
HH/HH'	Prostate specific antigen (total)	PSA

Table 2.2 List of DNA Sequences used for Spatial Encoding of Antibodies

Sequence Name	Sequence	T_m °C (50mM NaCl)
A	5'-AAAAAAAAAAAAAATCCTGGAGCTAAGTCCGTA-3'	57.9
A'	5' NH3-AAAAAAAAAATACGGACTTAGCTCCAGGAT-3'	57.2
B	5'-AAAAAAAAAAAAAGCCTCATTGAATCATGCCTA -3'	57.4
B'	5' NH3-AAAAAAAAAATAGGCATGATTCAATGAGGC -3'	55.9
C	5'- AAAAAAAAAAAAAGCACTCGTCTACTATCGCTA -3'	57.6
C'	5' NH3-AAAAAAAAAATAGCGATAGTAGACGAGTGC -3'	56.2
D	5'-AAAAAAAAAAAAAATGGTCGAGATGTCAGAGTA -3'	56.5
D'	5' NH3-AAAAAAAAAATACTCTGACATCTCGACCAT-3'	55.7
E	5'-AAAAAAAAAAAAAATGTGAAGTGGCAGTATCTA -3'	55.7
E'	5' NH3-AAAAAAAAAATAGATACTGCCACTTCACAT -3'	54.7
F	5'-AAAAAAAAAAAAAATCAGGTAAGGTTCACGGTA -3'	56.9
F'	5' NH3-AAAAAAAAAATACCGTGAACCTTACCTGAT-3'	56.1
G	5'-AAAAAAAAAAGAGTAGCCTTCCCGAGCATT-3'	59.3
G'	5' NH3-AAAAAAAAAAAATGCTCGGGAAGGCTACTC-3'	58.6
H	5'-AAAAAAAAAAATTGACCAAACTGCGGTGCG-3'	59.9
H'	5' NH3-AAAAAAAAAACGCACCGCAGTTTGGTCAAT-3'	60.8
I	5'-AAAAAAAAAATGCCCTATTGTTGCGTCGGA-3'	60.1
I'	5' NH3-AAAAAAAAAATCCGACGCAACAATAGGGCA-3'	60.1
J	5'-AAAAAAAAAATCTTCTAGTTGTCGAGCAGG-3'	56.5
J'	5' NH3-AAAAAAAAAACCTGCTCGACAACTAGAAGA-3'	57.5
K	5'-AAAAAAAAAATAATCTAATTCTGGTCGCGG-3'	55.4
K'	5' NH3-AAAAAAAAAACCGCGACCAGAATTAGATTA-3'	56.3
L	5'-AAAAAAAAAAGTGATTAAGTCTGCTTCGGC-3'	57.2
L'	5' NH3-AAAAAAAAAAGCCGAAGCAGACTTAATCAC-3'	57.2
M	5'-AAAAAAAAAAGTCGAGGATTCTGAACCTGT-3'	57.6
M'	5' NH3-AAAAAAAAAAACAGGTTCAGAATCCTCGAC-3'	56.9
AA'	5' NH3-AAAAAAAAAAGTCACAGACTAGCCACGAAG-3'	58
BB	5'-AAAAAAAAAAGCGTGTGTGGACTCTCTCTA-3'	58.7
BB'	5' NH3-AAAAAAAAAATAGAGAGAGTCCACACACGC-3'	57.9
CC	5'-AAAAAAAAAATCTTCTAGTTGTCGAGCAGG-3'	56.5
CC'	5' NH3-AAAAAAAAAACCTGCTCGACAACTAGAAGA-3'	57.5
DD	5'-AAAAAAAAAAGATCGTATGGTCCGCTCTCA-3'	58.8

DD'	5' NH3-AAAAAAAAAATGAGAGCGGACCATACGATC-3'	58
EE	5'-AAAAAAAAAAGCACTAACTGGTCTGGGTCA-3'	59.2
EE'	5' NH3-AAAAAAAAAATGACCCAGACCAGTTAGTGC-3'	58.4
FF	5'-AAAAAAAAAATGCCCTATTGTTGCGTCGGA-3'	60.1
FF'	5' NH3-AAAAAAAAAATCCGACGCAACAATAGGGCA-3'	60.1
GG	5'-AAAAAAAAAACTCTGTGAACTGTCATCGGT-3'	57.8
GG'	5' NH3-AAAAAAAAAAACCGATGACAGTTCACAGAG-3'	57
HH	5'-AAAAAAAAAAGAGTAGCCTTCCCGAGCATT-3'	59.3
HH'	5' NH3-AAAAAAAAAAAATGCTCGGGAAGGCTACTC-3'	58.6

* All amine-terminated strands were linked to antibodies to form DNA-antibody conjugates using SFB/SANH coupling chemistry described by R. Bailey *et al.*[1] Codes AA-HH were used in the experiment examining fresh whole blood from a healthy volunteer. Codes A-M were used for the molecular analyses of cancer patient serum samples.

All matched antibody pairs and standard proteins (recombinants) were received from eBioscience except those described below. The antibody pairs for human C3 and CRP were received from Abcam. Their recombinants were from Sigma. The antibody pair and recombinant protein for human plasminogen were received from Molecular Innovations. The antibody pair for PSA was received from Biodesign. The PSA recombinant was from R&D Systems. The capture and detection antibodies for human hCG were received from Abcam and Chromoprobe, respectively. The antibody pair and the recombinant for human GM-CSF were both received from BD biosciences. All oligonucleotides were synthesized by Integrated DNA Technologies.

2.5.3 Cross-Reactivities of Oligonucleotide Labels

A full orthogonality analysis was performed to quantitate the cross-hybridization between the stripes within the DEAL barcode arrays. A 13-well PDMS slab was placed onto a barcode array chip consisting of thirteen distinct strands of coding ssDNA (A-M). In each well, a solution containing only one kind of complementary ssDNA from A'-M' (labeled with Cy3) was added,

and successful hybridization was visualized by fluorescence using a 532 nm laser excitation. The result (Figure 2.6) indicates negligible cross-hybridization across the entire panel of DNA codes used in our DEAL barcode assay.

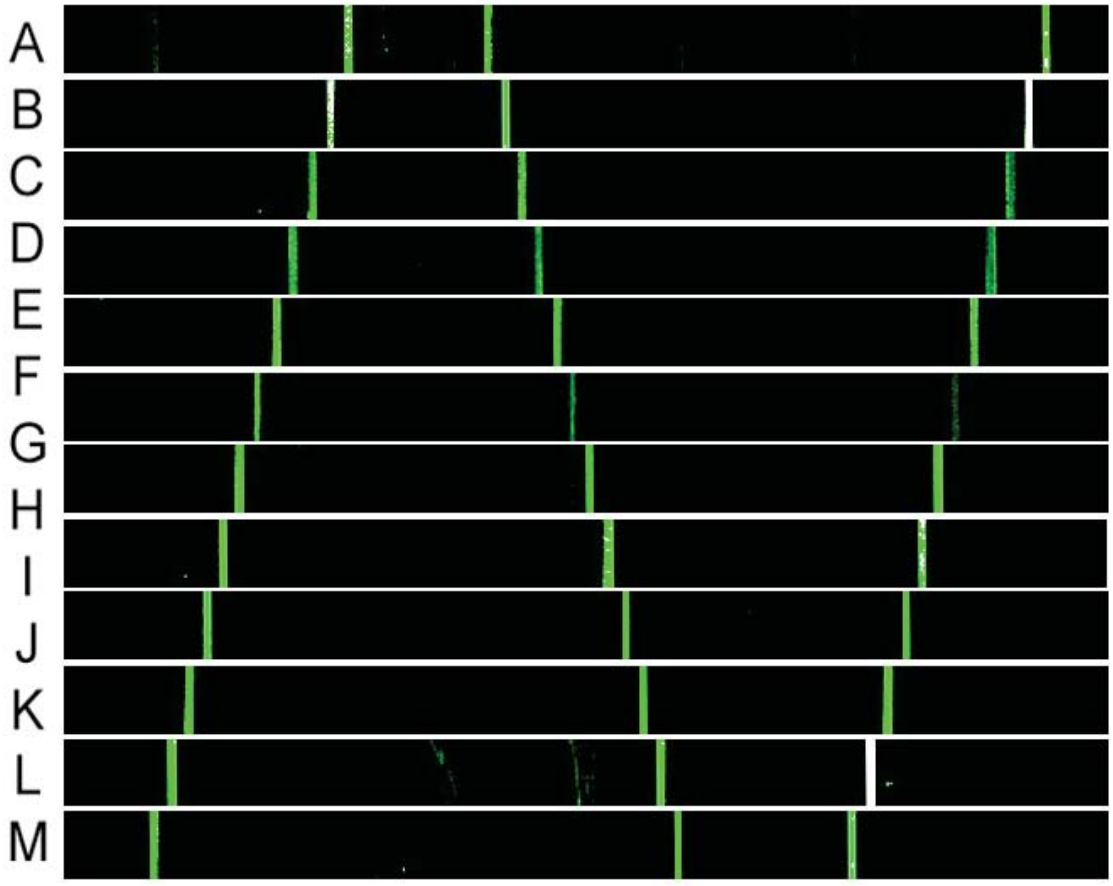

Figure 2.6 Cross-hybridization assay for all 13 DNA oligomer pairs that were used for encoding the registry of antibody barcode arrays.

2.5.4 Patterning of Barcode Arrays

Using the microchannel-guided flow-patterning approach (Figure 2.7), we fabricated DEAL barcode arrays that were ~10-fold denser than conventional microarrays. Microcontact printing can generate high density arrays of biomolecules with spot sizes of a few micrometers

(μms),[2,3] but extending stamping to large numbers of biomolecules is awkward because of the difficulty in aligning multiple stamps to produce a single microarray. Direct microfluidics-based pattering of proteins has been reported, but DNA flow-patterning with sufficient loading remains less successful compared to conventional spotting methods.[4,5] In the flow patterning process, a polydimethylsiloxane (PDMS) mold containing 13-20 parallel microfluidic channels, with each channel conveying a different biomolecule capture agent, was used. The number of channels could readily be expanded to include 100 or more different capture agents. Poly-amine coated glass surfaces permitted significantly higher DNA loading than do more traditional aminated surfaces, with a corresponding increase in assay sensitivity (**Figure 2.8**). DNA "bars" of 2 micrometers in width could be successfully patterned. In the present study, a 20-micrometer (μm) channel width was chosen because the fluorescence microarray scanner utilized has a resolution of 5 μm. The fabrication details are as follows:

Mold fabrication. The microfluidic-patterning chips were made by molding a PDMS elastomer from a master template, which was prepared using photolithography to create a photoresist pattern on a Si wafer. An alternative was to make a silicon "hard" master by transferring the photolithographically-defined pattern into the underlying silicon wafer using a deep reactive ion etching (DRIE) process.[6] The first method offers rapid prototyping, while the second method yields a robust and reusable mold, permitting higher throughput chip fabrication.

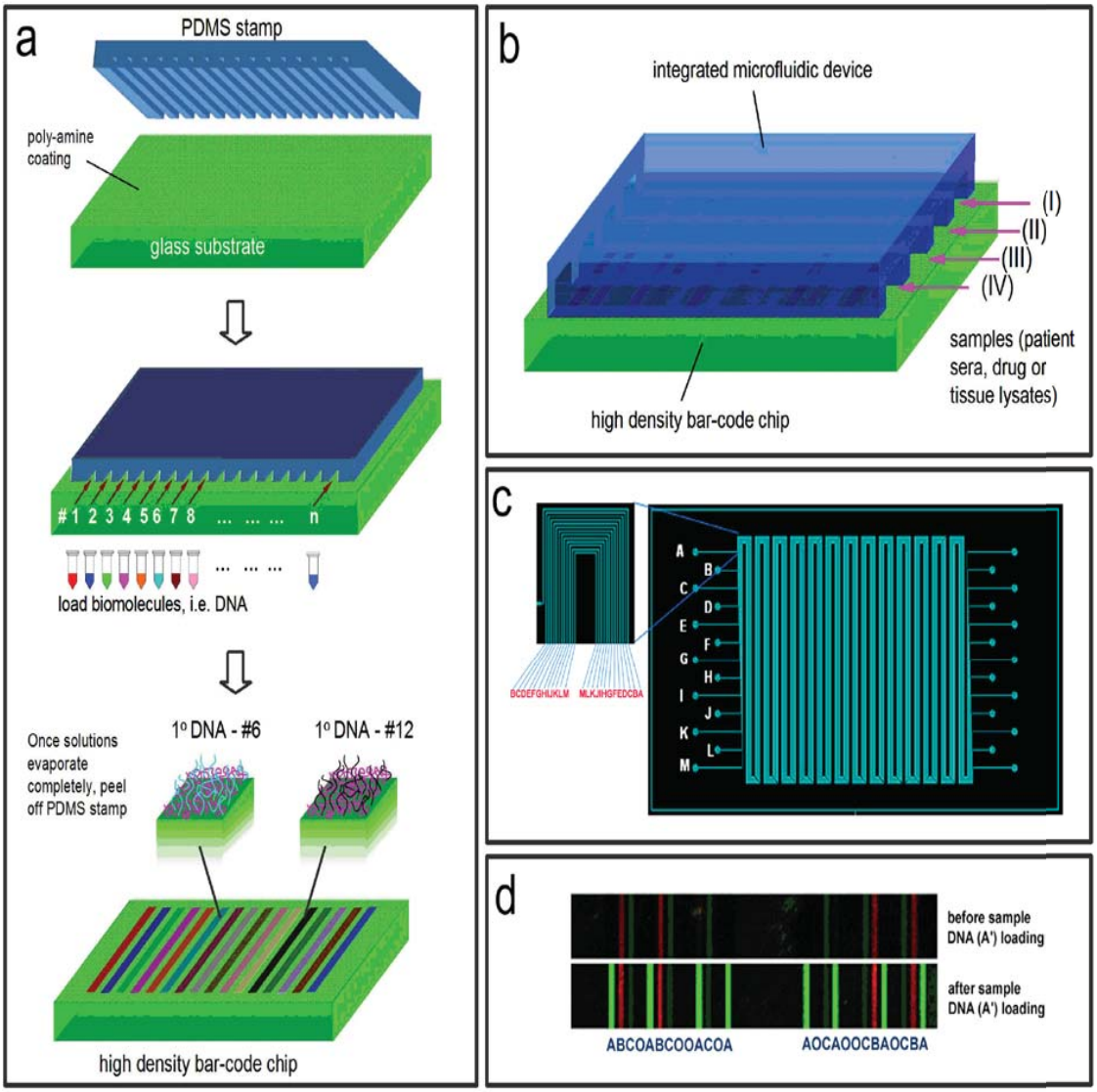

Figure 2.7 Microchannel-guided flow patterning of DEAL barcode arrays. **(a)** Depiction of the procedure. Each DNA bar is 20μm wide and spans the dimensions of the glass substrate. **(b)** Integration of a DEAL barcode-patterned glass slide with microfluidics for multiplexed protein assays. **(c)** Mask design of a 13-channel barcode. A-M denotes the flow channels for the different DNA molecules. **(d)** Validation of successful patterning of DNA molecules by specific hybridization of oligomer A to its fluorescent complementary strand A'. The primary strands B and C were pre-tagged with red and green dyes as references.

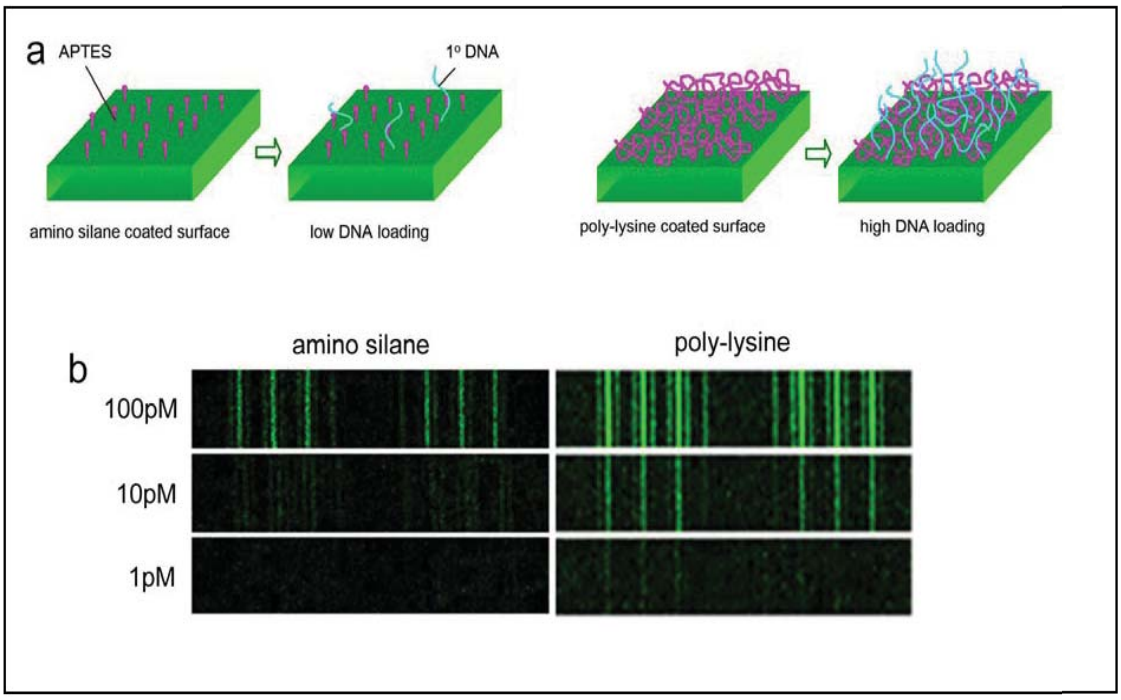

Figure 2.8 Effects of polylysine coating on DEAL assay. (a) Schematic illustration of polylysine coating for increased loading of DNA oligomer codes. **(b)** Fluorescence images showing a comparative study of the measurement of three human cytokines (IFN-γ, TNF-α, and IL-2) using substrates coated with amino-silane and polylysine, respectively.

PDMS patterning-chip fabrication. A polydimethylsiloxane (PDMS) elastomer slab was fabricated by casting the Sylgard® 184 wet PDMS (prepolymer:curing agent = 10:1 (w/w)) onto the molds described above followed by a curing step at 80°C for 50 minutes. This slab was peeled from the mold and was bonded onto a glass surface, which provided the base walls for the flow channels. Prior to bonding, the glass surface was pre-coated with the polyamine polymer, poly-L-lysine (Sigma-Aldrich), to increase DNA loading. The coating process is described elsewhere.[18] The number of microfluidic channels determines the size of the barcode array. In the present work, the PDMS chip, as shown in **Figure 2.7c**, contained 13 to 20 parallel microchannels designed to cover a large area (3cm × 2cm) of the glass slide with the DNA barcode microarray.

DEAL barcode patterning. Solutions, each containing a different primary DNA oligomer prepared in 1X PBS buffer, were flowed into each of the microfluidic channels. Then, the solution-filled chip was placed in a desiccator for several hours (or overnight) to allow solvent (water) to evaporate completely through the gas-permeable PDMS, leaving the DNA molecules behind. Last, the PDMS elastomer was removed from the glass slide, and the barcode-patterned DNA was fixed to the glass surface by thermal treatment at 80°C for 4 hours, or by UV cross-linking. Potassium phosphate crystals precipitate during solution evaporation, but are readily removed by rapidly dipping the slide in deionized water. The barcode-patterned DNA arrays demonstrated a marked improvement in sensitivity as compared to conventional pin-spotted microarrays (**Figure 2.9**). A side-by-side comparison study was performed by running DEAL assays on three cytokines under identical conditions. Using the microchannel-guided flow patterning method, a glass slide was patterned with DNA oligomers **A, B, C,** and a blank control **O** (20 μm-wide bars; 50-100 μM DNA solutions). The pin-spotted array, with a typical spot size of 150-200 μm, was printed at the Institute for Systems Biology using 100 μM oligomer concentrations. Six sets of spots were printed, corresponding to oligomers **A, B, C, D, E,** and **F**. Poly-L-lysine coated slides were used for both types of arrays.

Before the DEAL assay, the capture antibodies were conjugated to DNA oligomer codes as follows: **A'** to IFN-γ, **B'** to TNF-α, and **C'** to IL-2. Protein standards were diluted in 1% BSA/PBS solution at concentrations ranging from 1fM to 1nM. The incubation time for each step (blocking, conjugate hybridization, sample binding, detection-antibody binding, and fluorescent-molecule binding) was 30 minutes. The results (**Figure 2.9b**) reveal that the DEAL

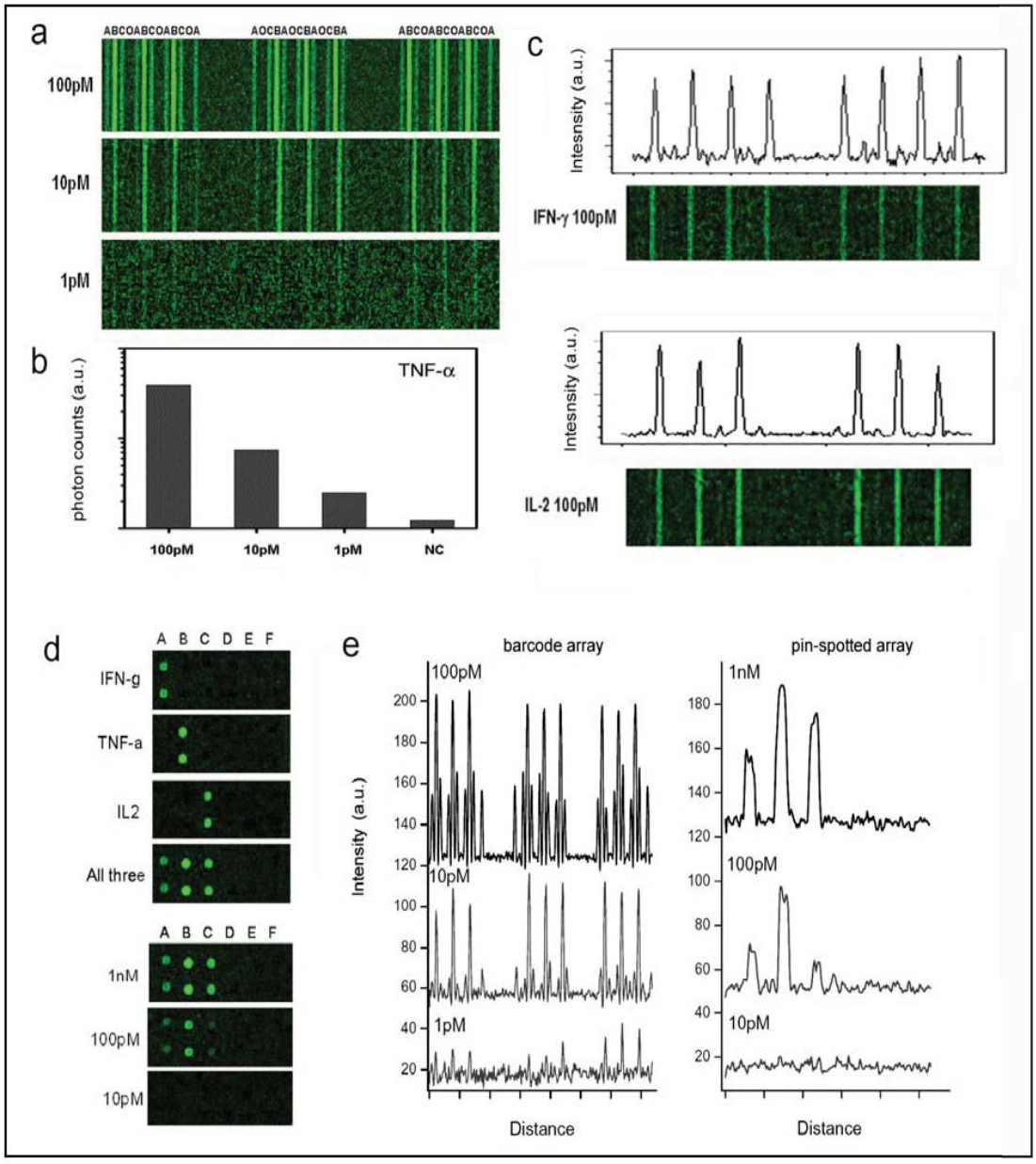

Figure 2.9 Increased sensitivity observed in immunoassays run on DEAL barcode arrays. **(a)** Concentration-dependent fluorescence signal for the detection of three human cytokines (A: IFN-γ, B: TNF-α, C: IL-2, O: negative control) using a DEAL barcode array. The bar width is 20 μm. **(b)** Quantitation of fluorescence intensity vs. TNF-α concentration. **(c)** Measurements of individual proteins, IFN-γ and IL-2, reveal no distinguishable cross-reactivity. **(d)** Comparison of the microfluidics flow-patterned DEAL microarrays with DEAL microarrays patterned using a conventional DNA pin-spotting method. The spot size is ~150-200 μm. **(e)** Fluorescence line profiles for the DEAL barcode array in **a** and the pin-spotted array in **d** at different protein concentrations. The curves were amplified in the y-coordinates for better visualization.

barcode array sensitivity is similar to the projected sensitivity limit of the commercial ELISA assay (~10 pg/mL, or 0.8 pM; eBioscience). Taking the example of the TNF-α assay, the detection sensitivity of the DEAL barcode array (better than 1 pM) is substantially improved over the 10-100 pM limit found for the microarrays spotted using conventional methods (**Figure 2.9d**). Therefore, the DEAL barcode array combines ELISA-like sensitivity with a high degree of multiplexing for protein measurements.

The difference in sensitivity between the barcode array and pin-spotted array platforms is likely attributable to the difference in feature size. The barcode array has a line-width of 20 μm, whereas the spot diameter in conventional arrays is more than 150 μm. These results are consistent with a recent report which demonstrated that DNA microarrays with smaller spot sizes could detect DNA with increased sensitivity.[7]

The ELISA-like sensitivity of the DEAL barcode assays is key for realizing the multiplexed measurement of human plasma proteins in blood. The human plasma proteome is comprised of three major classes of proteins – classical plasma proteins, tissue leakage proteins, and cell-cell signaling molecules (cytokines and chemokines). Cell-cell signaling molecules are biologically informative in a variety of physiological and pathological processes, i.e. tumor host immunity and inflammation. The concentration range of plasma proteins within the human plasma proteome spans 12 orders of magnitude, and the lowest end is approximately at the detection limit of mass spectrometry – a high-throughput protein profiling technique. The state-of-the art for clinical protein measurements is still the typically low-throughput ELISA assay. The high performance of the DEAL barcode chip, including its increased sensitivity, is a key to realizing highly multiplexed measurements of a panel of proteins from small quantities of clinical blood samples.

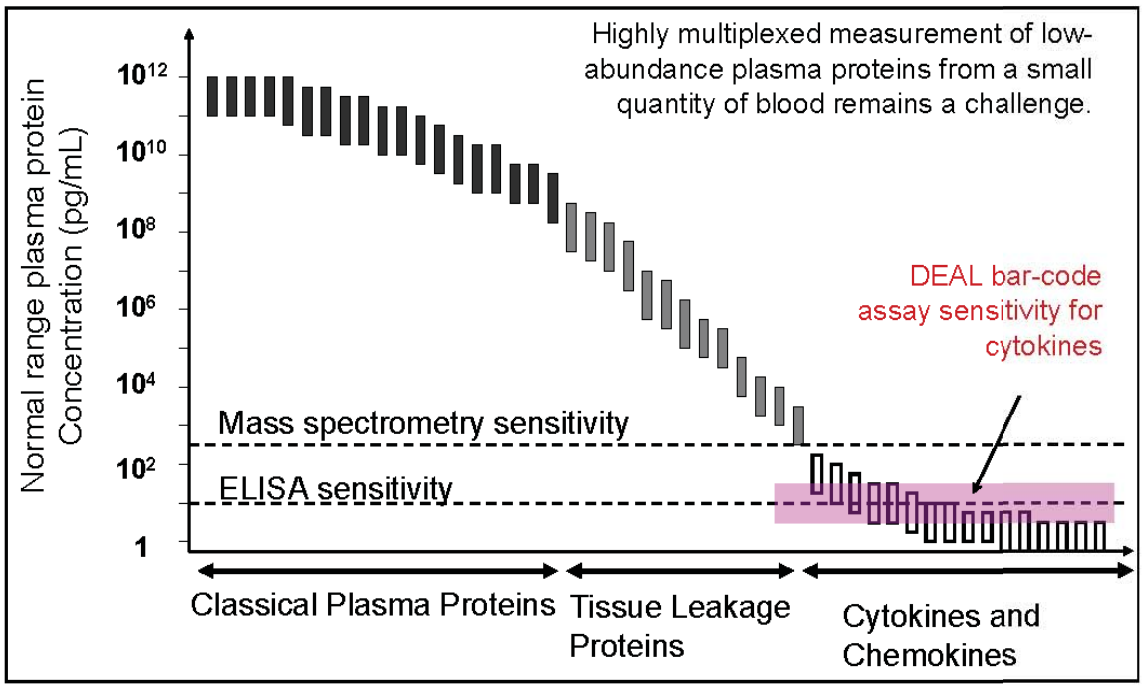

Figure 2.10 Schematic of human plasma proteome (refer to N.L. Anderson and N.G. Anderson, *Molecular & Cellular Proteomics* 11, 845, 2001). Our work demonstrates that the DEAL barcode assay has a markedly increased sensitivity, comparable to ELISA, leading to the feasibility of multiplexed detection of plasma proteins, including low-abundance cell-cell signaling molecules, e.g. cytokines and chemokines, from a small quantity of sample.

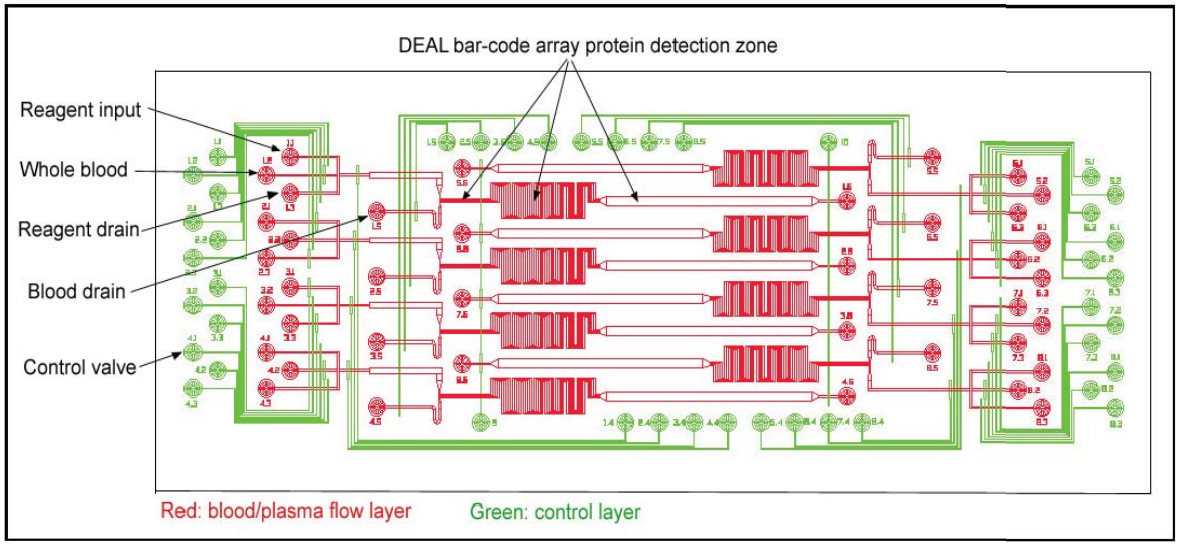

Figure 2.11 AutoCAD design of an IBBC. Underneath the PDMS microfluidic chip is a large-scale DNA barcode array. Flow layer in red; control layer in green.

2.5.5 Fabrication of IBBCs

The fabrication of the IBBCs was accomplished through a two-layer soft lithography approach.[8,9] A representative chip design is shown in **Figure 2.11**. The silicon master for the control layer (red) was fabricated by exposing a spin-coated SU8 2010 negative photoresist film (~20-μm thickness). Prior to molding, the master was silanized in a trimethylchlorosilane (TMCS) vapor box for 20 minutes. A mixture of GE RTV 615 PDMS prepolymer part A and part B (5:1) was prepared, homogenized, and then applied onto the control layer master. After degassing for 15 minutes, the PDMS was cured at 80°C for 50 minutes. The solidified PDMS chips were then cut and peeled off the master, and access holes were drilled with a 23-gauge stainless-steel hole punch.

The flow-layer master (blue) was fabricated using SPR 220 positive photoresist. After exposure and development, the photoresist pattern was baked at 120°C in a convection oven to round the flow channels. The resultant flow layer was typically 15-20 μm in thickness. Silanization using TMCS was performed right before applying the fluid PDMS prepolymer. Next, a mixture of GE RTV 615 PDMS part A and part B (20:1) was prepared, homogenized, degassed, and then spun onto the flow layer master at 2000-3000 rpm for 1 minute. It was cured at 80°C for 30 minutes, at which point the PDMS control layer was carefully aligned and placed onto the flow layer. Finally, an additional 60-minute thermal treatment at 80°C was performed to bond the two PDMS layers together. The bilayer chip was then peeled off of the flow-layer master and access holes were drilled.

The last assembly step was to bond the PDMS chip to the DEAL barcode slide via thermal treatment at 80°C for 4 hours, yielding a completed integrated blood barcode chip (IBBC). In this chip, the DEAL barcode stripes are orientated perpendicular to the microfluidic

assay channels. The IBBC features a microfluidic biological fluid-handling module, specifically a whole blood separation unit, and a DEAL barcode array for highly multiplexed protein measurements. In a typical design, 8-12 identical blood separation and detection units were integrated within a single 2.5 cm x 7 cm chip.

2.5.6 Execution of Blood Separation and Multi-Parameter Protein Assay using IBBCs

The compatibility of the DEAL technique with integrated microfluidics yielded rapid blood separations and reliable measurements of a panel of proteins. The experimental procedure is detailed below.

a. *Blocking:* Prior to use of the IBBC, all microfluidic channels were blocked with the assay buffer solution (1% w/v BSA/PBS solution prepared by adding 98% pure Bovine Serum Albumin, Fraction V (Sigma) to 150 mM 1X PBS without calcium/magnesium salts (Irvine Scientific) for 30-60 minutes.
b. *DEAL formation (introducing conjugates):* A solution containing all the DNA-antibody conjugates was flowed through the assay channels of the IBBCs for ~30-45 minutes, thus transforming the DNA barcode microarray into an antibody microarray, enabling the subsequent surface-bound immunoassay. The unbound conjugates were removed by flowing the assay buffer solution for 10 minutes. The DEAL-conjugate solution was prepared by mixing all synthesized conjugates in 1% BSA/PBS with a final concentration of 5 μg/mL. The DNA coding oligomers were pre-tested for orthogonality to ensure that cross-hybridization between non-complementary oligomer strands yielded a fluorescence intensity that did not exceed 5% of the complementary pair signal intensity.

c. *Collecting a finger-prick of blood:* Finger pricks were carried out using BD Microtainer Contact-Activated Lancets (purple lancet – for low volume, single blood drop). Blood was collected with SAFE-T-FILL capillary blood collection tubes (RAM Scientific), which were prefilled with a 25 mM EDTA solution as discussed below. Two samples were prepared from the drop of whole blood:

   i. Unspiked Blood Samples: The blood collection tube was pre-filled with 80 μL of 25 mM EDTA solution, and then 10 μL of fresh human blood was collected in the EDTA-coated capillary, dispensed into the tube and rapidly mixed by inverting a few times.

   ii. Spiked Blood Samples: The blood collection tube was pre-filled with 40 μL of 25 mM EDTA solution. Forty microliters of recombinant protein solution, containing all the protein standards, was added. Then, 2 μL of 0.5 M EDTA was added to bring the total EDTA concentration up to 25 mM. Finally, 10 μL of fresh human blood was collected in an EDTA-coated capillary, added to the tube and quickly mixed by inverting a few times. The final concentrations for all protein standards were ~10 nM. However, the quality of these "standards" and the affinity of capture antibodies vary substantially. The purpose of spiking in protein standards was to contrast the signal at high protein concentrations with that of as-collected fresh whole blood.

d. *Blood sample assay:* These two blood samples were flowed into the IBBCs within 1 minute of collection. The plasma was quickly separated from blood cells within the chip, and the proteins of interest were captured in the downstream assay zone containing the DEAL barcode arrays. The entire process from finger prick to the completion of plasma

protein capture was very rapid (<10 minutes). Owing to the reduced diffusion barrier in a flowing microfluidic environment, the sample assay was executed within *ten minutes*. With regards to the cancer patient serum tests, the as-received serum samples (Asterand) were flowed into IBBCs without further treatment.

e. *Applying detection antibodies:* A mixture of biotin-labeled detection antibodies was flowed into the microfluidic devices for ~30 minutes to complete the DEAL assay. The detection-antibody solution contained biotinylated detection antibodies at ~5 μM prepared in 1% BSA/PBS. Afterwards, unbound detection antibodies in the IBBCs were removed by flowing the assay buffer for 10 minutes.

f. *Fluorescence probes:* For the cancer serum experiments, Cy5 fluorescent dye-labeled streptavidin and the reference, Cy3-labeled complementary ssDNA (DNA code M/M'), were mixed together and were then flowed into the IBBCs for 30 minutes. Finally, the assay buffer was flowed for 10 minutes to remove unbound Steptavidin-Cy5.

g. *Rinse:* The PDMS blood chip device was removed from the DNA-patterned glass slide. The slide was immediately dipped 6 times each in the following solutions in order: 1% BSA/PBS solution, 1X PBS solution, ½X PBS solution, deionized Millipore H_2O. The slide was rinsed for a few seconds under a Millipore H_2O stream, and then dried with a nitrogen gun.

h. *Optical readout:* The slide was scanned by an Axon Instruments GenePix Scanner. The finest resolution (5 μm) was selected. Two color channels (the green Cy3 channel and the red Cy5 channel) were turned on to collect fluorescence signals.

2.5.7 Consideration of Microfluidic Environment for Rapid Immunoassay

In a microfluidic environment, at sufficiently high flow rates, diffusion is not limiting, and the rate at which a biological assay can be completed is determined by the kinetic parameters that describe the capture of the biomolecule by the surface-bound capture agent.[10] Under chemical equilibrium, the relative amount of biomolecule that is complexed to the surface-bound capture agent is given by:

Keq = [biomolecule-CA complex] / [biomolecule in solution][Surface Bound CA]

where *Keq* is the equilibrium constant. For a given concentration of biomolecule, the surface-bound assay sensitivity depends upon several factors, including:

- The equilibrium constant, *Keq*: a large *Keq* corresponds to a large amount of the biomolecule-CA complex.
- The concentration of surface-bound CA: we find that the sensitivity limits of the assay directly correlate with the concentration of surface-bound CA.[1] During microchannel-guided flow-patterning of the DEAL barcode arrays, the glass surface was modified by treatment with poly-L-lysine (a poly-amine), yielding a three-dimensional matrix for DNA adsorption and markedly increasing the amount of DNA loading. Our DNA-loading density is estimated to be 6×10^{13} molecules/cm^2, an order of magnitude higher than typical loading densities on amino-silane coated glass slides.[11] As a result, the protein detection sensitivity was improved by an order of magnitude, and the dynamic range was increased to 4 orders of magnitude, as compared with 2-3 orders of magnitude for the small-molecule amine (i.e. amino-propyl-triethoxyl silane, APTES) functionalized glass surface. The comparative study is shown in **Figure 2.8**.

- Feature size: smaller feature sizes can lead to increased sensitivities.[7] The feature sizes (20 μm-wide stripes) of DEAL barcode chips are substantially smaller than are generated using more traditional spotting methods (150-μm diameter spots).

2.6 Appendix B: Supplementary Data

2.6.1 Blind Test of Serum Samples Containing Unknown hCG Concentrations

Two serum samples containing unknown concentrations of hCG were measured in a blind test using a DEAL barcode assay. These two samples were introduced alongside eight standards of hCG-spiked serum on the same DEAL barcode chip. Fluorescent images were acquired at the same laser settings and quantified from 20 sets of barcodes. Using the resulting standard curve (at 200-μM DNA loading), we estimated the hCG concentration. The results are shown in **Figure 2.12** (inset table), and compared to the test results from an outside independent laboratory (Labcorp; the test was requested by the Nanotechnology Characterization Laboratory at the National Cancer Institute). The DEAL barcode assay result is in reasonable agreement with the Labcorp results.

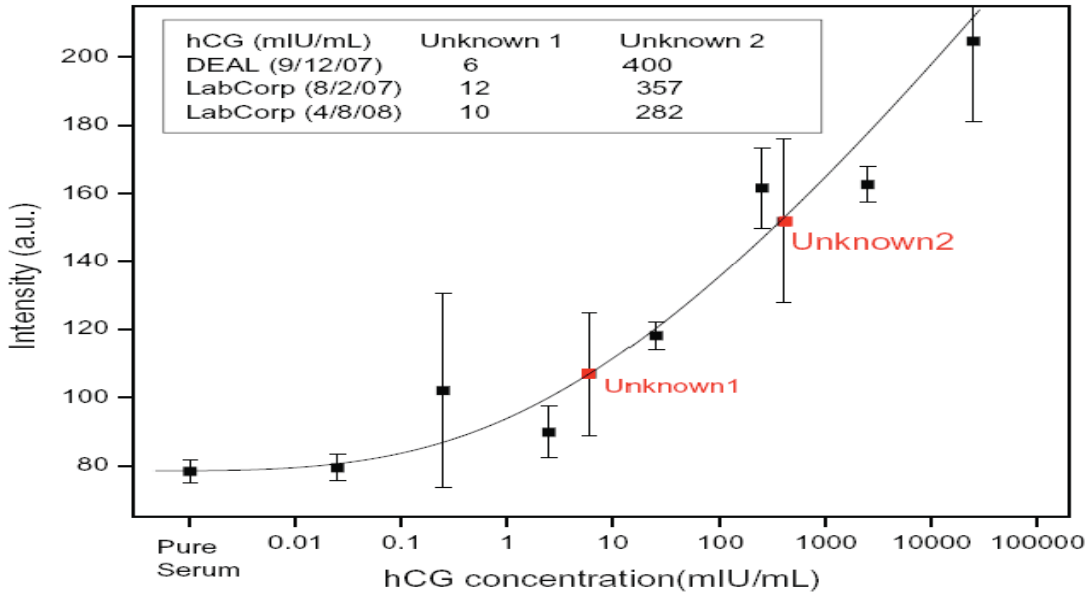

Figure 2.12 Blind test of hCG-containing "unknown" samples. The black squares correspond to the 200 μM DNA loading shown in **Figure 2.2**, and the red squares show the statistical means of two unknowns. The insets show representative images of barcode assay results for two unknowns, plus a table showing the estimated concentrations measured using the DEAL barcode assay and by an independent laboratory (Labcorp).

2.6.2 Protein Cross-Reactivities

We assessed the level of cross-reactivity of each antigen with DEAL stripes that are not specific to that antigen. DNA-encoded capture antibodies and biotinylated detection antibodies for all 12 antigens were used as usual, but a distinct antigen (10 nM) was added to each assay lane. Cy5-Streptavidin (red-fluorescence tag) was added to visualize the extent of analyte capture. The reference marks (DNA strand M) were visualized in all lanes with fluorescent green Cy3-M' DNA molecules. The 12 proteins showed a negligible extent of cross-reactivity (**Figure 2.13**), with typical photon counts under 2% compared to the correctly paired antigen-antibody complexes. Even at this low level, most of these cross-talk signals were found to be due to degraded recombinants (protein standards) and did not appear reproducibly once a new recombinant was used. This minimal cross-talk was also validated in pin-spotted microarrays using the same set of primary DNA codes. The negligible cross-talk in our DEAL assays is largely attributable to our significant efforts to screen for orthogonal DNA pairs (**Tables 2.1** and **2.2**). A non-fully orthogonal DNA pair leads to cross-hybridization, and resultant cross-reactivity in the DEAL protein assay.[1]

2.6.3 Dilution Curves for all Proteins used in the DEAL Barcode Assay

We performed assays on serial dilutions of all 12 proteins on the DEAL barcode chip. Because each device allows a maximum of 12 parallel assays to be executed, we chose 6 lanes for cross-talk validation, leaving 6 lanes for dynamic range studies. As a result, we combined 2 proteins in each assay lane (**Figure 2.14**). On the same chip, we assayed all proteins over the concentration range of 1 nM – 1 pM (except PSA and TGF-β: 5 nM to 5 pM), and quantified the fluorescence signal vs. concentration for all 12 antigens (**Figure 2.14b**). All assay lanes were

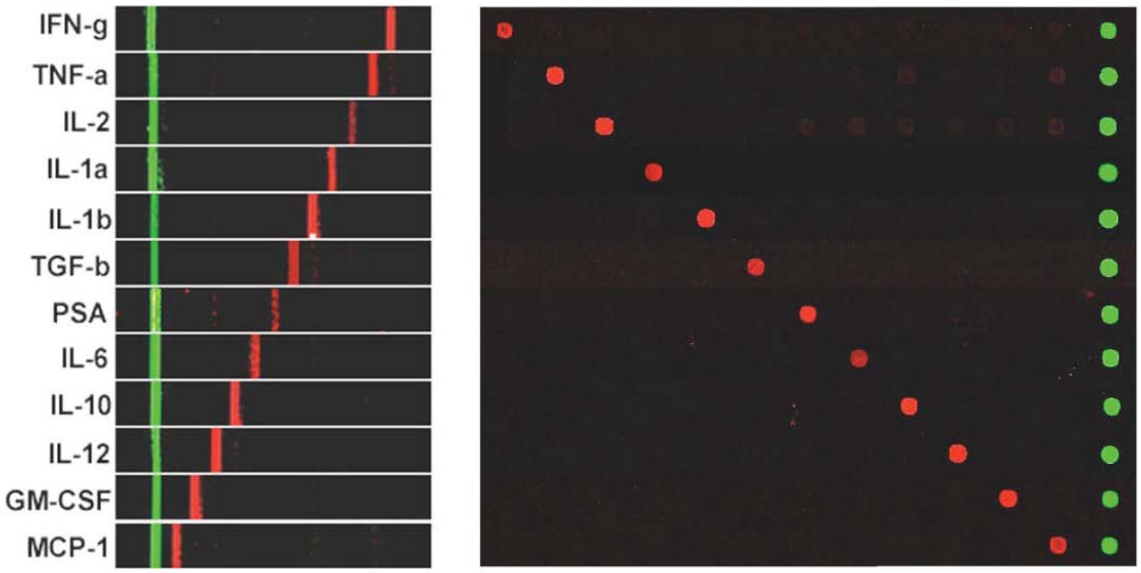

Figure 2.13 A cross-reactivity assay for the entire biomarker panel of 12 proteins. Both barcode (left panel) and pin-spotted (right panel) microarray formats are shown. The green bars represent the reference stripe/spot – M. Each protein can be readily identified by its distance from the reference. The designation of proteins in the barcode is the same as in **Figures 2.3 and 2.4**.

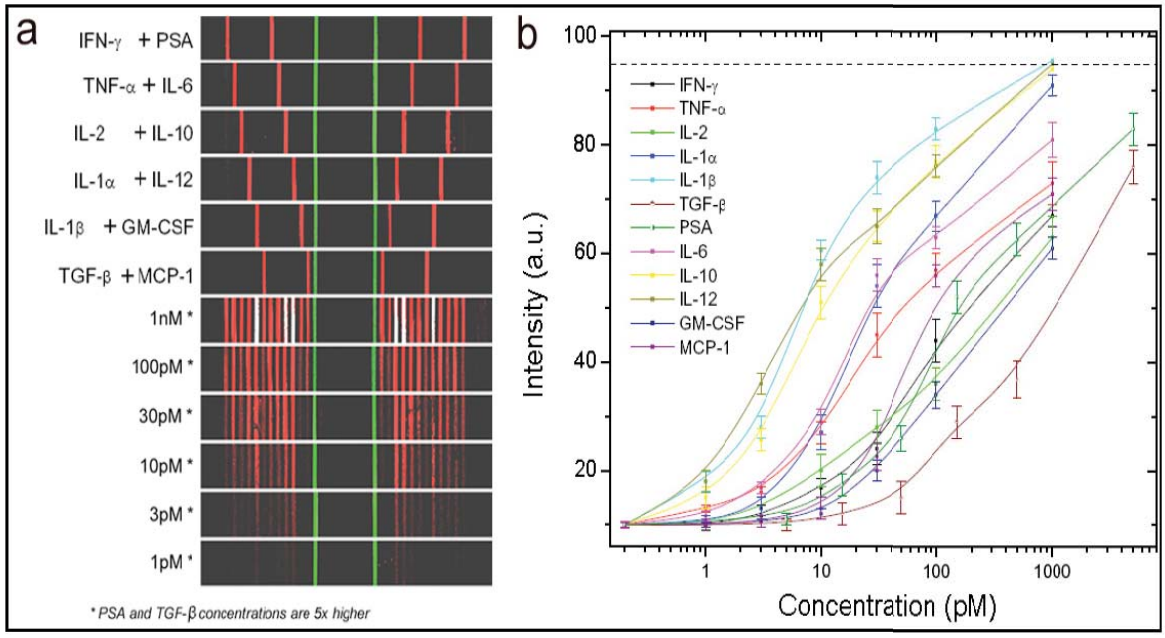

Figure 2.14 Dilution curves for the 12 proteins measured using DEAL-based barcodes entrained within microfluidic channels. (a) Barcode images from one device showing minimal crosstalk, and a series of standard antigens ranging from 1 nM to 1 pM for all 12 proteins (* the concentrations of PSA and TGF-β are 5x higher). **(b)** Quantitation of fluorescence intensity vs. concentration for all 12 proteins. Error bars: 1 s.d.

imaged using the GenePix scanner (with the same scanning parameters as described in the Experimental Methods section). Apparently, the estimated sensitivity varies substantially depending upon the antibodies being used, from ~0.3 pM (e.g. IL-1β and IL-12) to 30 pM (TGF-β). The TGF-β antibody pair has a relatively lower binding affinity and a poorer detection limit in ELISA (~70 pg/mL, compared to 5-10 pg/mL for most other cytokines, according to the specifications sheet). Predictably, this gave rise to a poorer performance in the DEAL assay. Although these curves reflect the ability of the microfluidics-patterned DEAL assays to assess specific antigens over broad concentration ranges, the statistical variation is relatively large compared to a commercial ELISA assay.

2.6.4 Standardized Quantification of the Patient Serum DEAL Barcode Data

Barcode signal quantitation was performed through a standardized process designed to minimize arbitrary bias in the image analysis. First, the fluorescence from the barcodes was visualized under fixed conditions, using the Axon GenePix 4000B two-color laser microarray scanner with identical instrument settings. For 635 nm and 532 nm excitation lasers, respectively, the following settings were used: Laser Power - 100% and 33%; Optical Gain - 800 and 700; Brightness/Contrast - 87 and 88. Next, the resulting JPEG image was exported from *GenePix 6.0* and was resized to match the standard barcode-array mask-design image. NIH *ImageJ* was employed to generate an intensity line profile of each assay channel and subchannel.

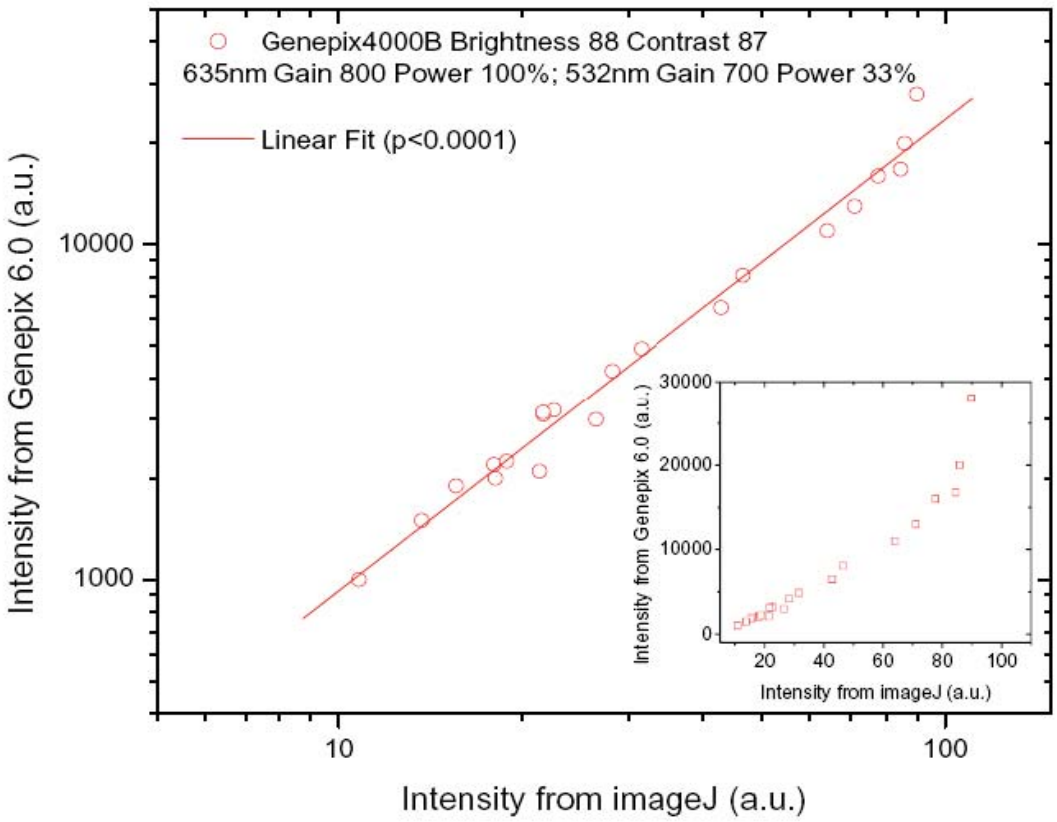

Figure 2.15 Comparison of fluorescence intensities quantitated using the *GenePix 6.0* and NIH *ImageJ* programs. The graph shows good linear correlation up to 85 a.u. (per pixel), where the *ImageJ* quantitation begins to saturate.

Each data point in the line profile was averaged from 40 pixels (200 μm) along the vertical direction. Fourth, all the line profile data files were loaded into a custom-written Excel macro that generated a spreadsheet tabulating the average intensities of all 13 bars (1 bar = 5 × 40 pixels, or 25 × 200 μm area) in each of 20 barcodes. The statistical analysis generated mean values and standard deviations (in Microcal Origin).

To demonstrate the feasibility of using the NIH *ImageJ* program, we performed a calibration by comparing the photocounts from the GenePix scan and the brightness values quantitated from *ImageJ* (**Figure 2.15**). A linear correlation ($p<0.0001$) exists if the intensity from *ImageJ* is no higher than 90. The scale of brightness in red-green-blue (RGB) mode is 0-255, so each color has a brightness maximum at 85. Since a common baseline (~10) is

superimposed onto the red fluorescence signal, the actual maximum is at ~90-100. The calibration data validates the use of *ImageJ* for quantitating the DEAL barcode data. We are currently pursuing a fully automated process for feature recognition, signal quantitation, and concentration extraction.

The mean values of measured protein levels for every patient were exported into a matrix for non-supervised clustering of patients. This was performed using the software, *Cluster 3.0,* and the heat map was generated using the software, Java *Treeview*. To assess the statistical significance between two patient (sub)groups, the Student's t analysis was performed on selected proteins and all *p*-values were calculated at a significance level of 0.05 if not otherwise specified. This standardized quantitation process diminished any possible biases in the manual quantitation process, but was unable to identify and exclude interfering speckles and noises in the images. A dust particle atop a barcode can give rise to an extremely bright scattering signal, thus causing large errors in quantitating such a barcode. In addition, several samples such as P04, P05, P10, and B10 exhibited significant bio-fouling and introduced a large non-specific background into quantitation. These issues remain to be resolved in further development of the DEAL barcode assay.

Quantitation and statistics of the barcodes for all patient sera are shown in **Figures 2.16** and **2.17**. The data for all proteins in these plots were shown in the same order as indicated in **Figure 2.3a**.

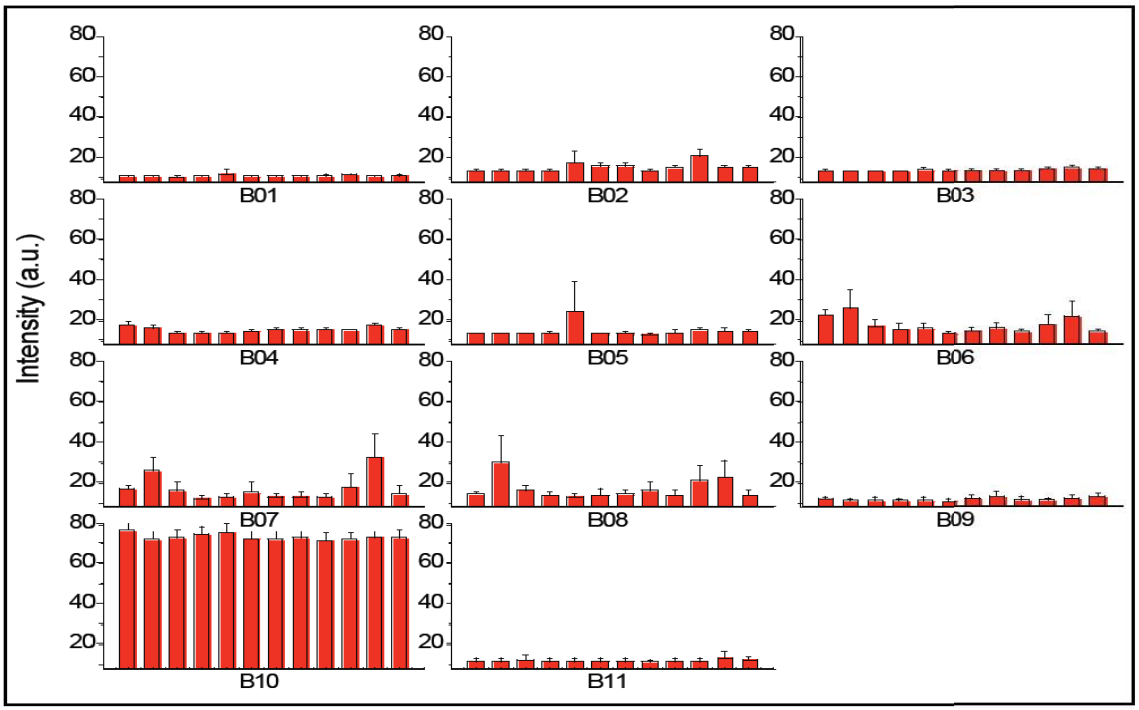

Figure 2.16 Quantitation of the fluorescence intensities from measurements of 11 breast cancer patients (B01-B11). The proteins are shown in the same order as described in **Figure 2.3a**.

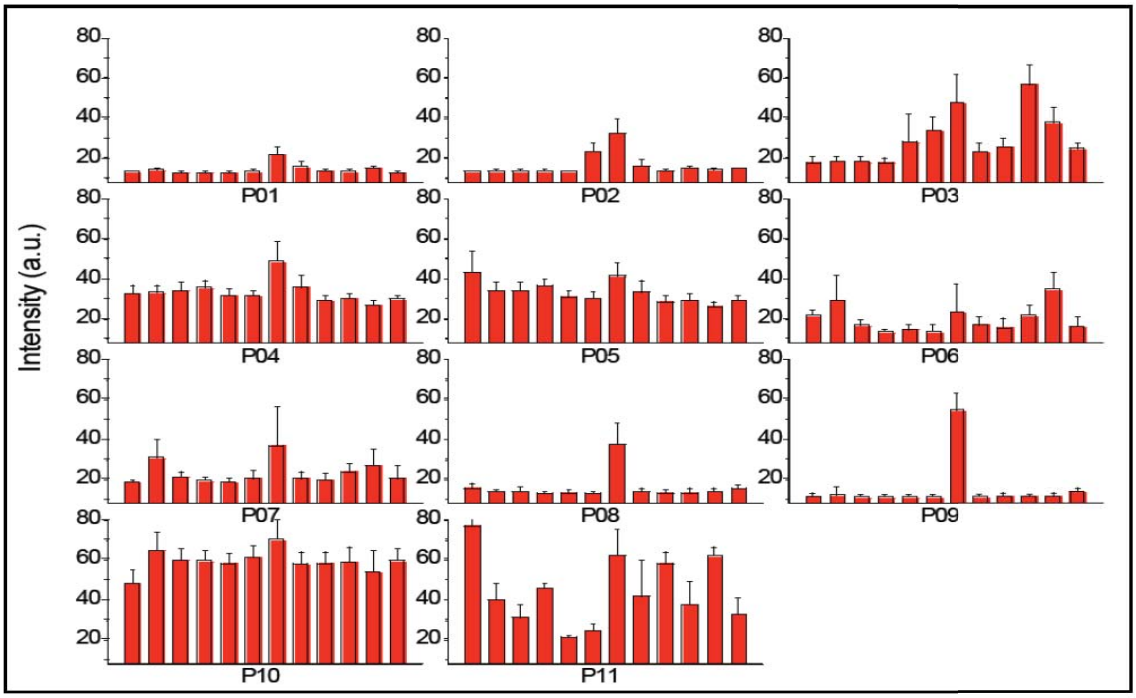

Figure 2.17 Quantitation of the fluorescence intensities from measurements of 11 prostate cancer patients (P01-P11). The proteins are shown in the same order as described in **Figure 2.3a**.

2.6.5 ELISA Validation of DEAL Barcode Assay

Given our limited serum sample quantities, and the amount of material required for carrying out standard protein measurements, we could only perform strategic cross-validations of the DEAL barcode measurements. PSA was the single protein that readily discriminated between breast and prostate cancer patients, and, in addition, clinical measurements of PSA levels for most of the prostate cancer patients were available through the serum vendor (Asterand). Thus, for all 22 patient serum samples, we performed enzyme-linked immunosorbant sandwich assays (ELISAs) to independently assess the PSA levels.

A comparative study of PSA levels measured by ELISA and by the DEAL barcode assays is shown in **Figure 2.3c**. One set of ELISA data was collected in our lab using the standard 96-well plate format. A second set of ELISA data, for 8 of the prostate cancer patient serum samples, was measured in commercial (clinical) labs. The DEAL barcode data is taken from **Figure 2.3b**. For our own ELISA measurements (**Figure 2.18a**), the first row of wells was loaded with PSA standards at serial dilutions. In all, 22 serum samples and a negative control (buffer) were measured. All three data sets are presented in **Figure 2.3c**, and are in good agreement with one another. The DEAL barcode assay detects the presence of PSA with 100% accuracy, but is less accurate relative to the ELISA tests in terms of quantitating small changes in concentration. This may be due, in part, to slight variations both in our manual chip assembly procedures and in our manual assaying procedures. It may also be that the higher sensitivity and concentration range of the DEAL assays is accompanied by a reduction in accuracy. We are in the process of fully automating both our microfluidics barcode patterning method and our assay

procedure. Those advances will allow us to more fully assess the trade-offs between the sensitivity and accuracy of the DEAL barcode arrays versus traditional ELISA formats.

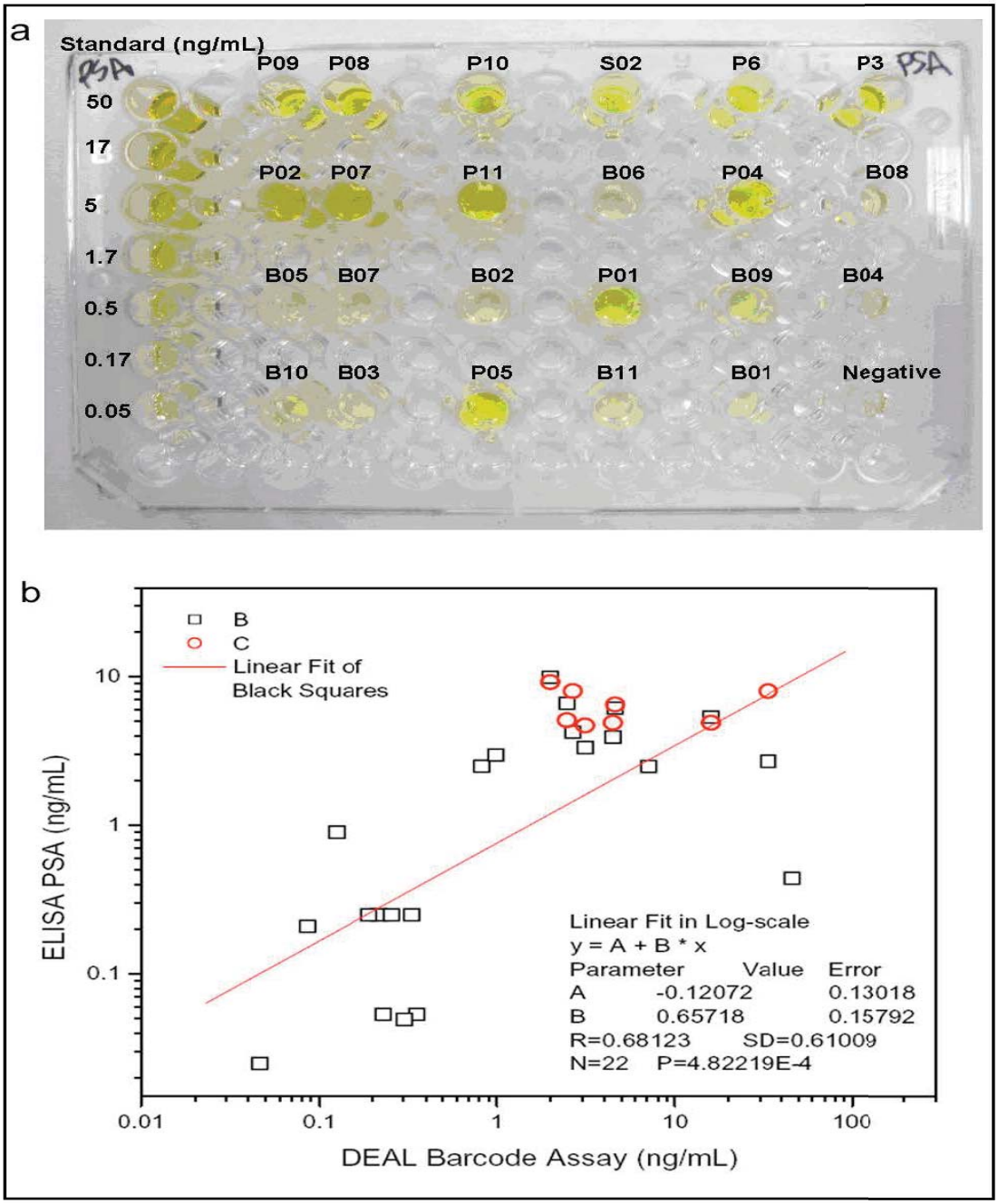

Figure 2.18 Validation of PSA detection using ELISA. (a) ELISA assays performed on PSA standards and 23 as-received serum samples. **(b)** Correlation between our ELISA and DEAL barcode assays. All DEAL tests were done in multiplex. Levels for most of the prostate cancer patients were available through the serum vendor (Asterand). Thus, for all 22 patient serum samples, we performed enzyme-linked immunosorbent sandwich assays (ELISA) to independently assess the PSA levels.

In comparison with the clinical ELISA results measured on fresh sera, our own ELISA measurements are generally lower in intensity. This likely reflects the influence of long-duration storage (>1year) and freeze/thaw cycles (>1 cycle). The correlation between the ELISA and DEAL barcode assays is analyzed using literature methods.[12] The R-value is comparable to literature measurements that compare multiplexed assays against ELISA standards.[13] The good agreement between standard ELISA and the DEAL barcode method with respect to PSA measurements provides validation of the IBBC for measuring proteins from human sera.

2.6.6 Cancer Patients: Medically-Relevant Information

Full medical records were provided for all serum samples acquired from Asterand. Selected information is listed in **Table 2.3**. Sample IDs were excluded for privacy protection.

Table 2.3 Cancer Patients: Selected Information

PATIENT	CANCER	GENDER/AGE	RACE	UICC STAGE	GLEASON SCORE	OTHERS
B01	Breast	Female/62	Caucasian	T2N0M0		wine 200mL/day
B02	Breast	Female/79	Caucasian	T4N2M0		
B03	Breast	Female/71	Caucasian	T1cNXM0		1-2 drinks/day
B04	Breast	Female/72	Caucasian	T2NXM0		hypertension
B05	Breast	Female/89	Caucasian	T3N0MX		arthritis
B06	Breast	Female/56	Asian	T1NXM0		
B07	Breast	Female/54	Caucasian	T2N2M0		hypertension, obesity
B08	Breast	Female/55	Caucasian	T2NXM0		1-5 cigs/day, wine 200mL/day
B09	Breast	Female/83	Caucasian	T4N0M0		coronary artery disease, cerebral atherosclerosis
B10	Breast	Female/63	Hispanic	T3N2MX		6-10cigs/day, hyperthyroid, hypertension, osteoarthritis
B11	Breast	Female/63	Caucasian	T1NXM0		arterial hypertension
P01	Prostate	Male / 51	Caucasian	T2cNXM0	4+3=7	
P02	Prostate	Male / 64	Caucasian	T3bN0MX	3+4=7	
P03	Prostate	Male / 47	Caucasian	T2cN0M0	3+3=6	hypertension
P04	Prostate	Male / 55	Caucasian	T2bN0M0	3+3=6	11-20 cigs/day
P05	Prostate	Male / 73	Caucasian	T3aNXMX	4+4=8	hypertension,11-20 cigs/day
P06	Prostate	Male/64	Caucasian	T3N0M0		chronic bronchitis, 11-20cigs/day
P07	Prostate	Male/60	Caucasian	T3aN0M0	3+4=7	gastroesophageal reflux
P08	Prostate	Male/72	African Am.	T2aNXMX	3+3=6	1-5cigs/day
P09	Prostate	Male/78	Caucasian	T3aN1MX	4+3=7	hypertension, atrial fibrillation
P10	Prostate	Male/66	Caucasian	T2aN0MX	3+3=6	hypertension, 11-20 cigs/day
P11	Prostate	Male / 47	Caucasian	T2cN0M0	3+3=6	hypertension
S01	Unknown					
S02	Unknown					

2.7 Additional References

1 Bailey, R., Kwong, G., Radu, C., Witte, O. & Heath, J. DNA-encoded antibody libraries: a unified platform for multiplexed cell sorting and detection of genes and proteins. *J. Am. Chem. Soc* **129**, 1959-1967 (2007).

2 Michel, B. *et al.* Printing meets lithography: Soft approaches to high-resolution patterning. *Chimia* **56**, 527-542 (2002).

3 Lange, S. A., Benes, V., Kern, D. P., Horber, J. K. H. & Bernard, A. Microcontact printing of DNA molecules. *Analytical Chemistry* **76**, 1641-1647 (2004).

4 Delamarche, E., Bernard, A., Schmid, H., Michel, B. & Biebuyck, H. Patterned delivery of immunoglobulins to surfaces using microfluidic networks. *Science* **276**, 779-781 (1997).

5 Bernard, A., Michel, B. & Delamarche, E. Micromosaic immunoassays. *Analytical Chemistry* **73**, 8-12 (2001).

6 Thuillier, G. & Malek, C. Development of a low cost hybrid Si/PDMS multi-layered pneumatic microvalve. *Microsystem Technologies* **12**, 180-185 (2005).

7 Dandy, D., Wu, P. & Grainger, D. Array feature size influences nucleic acid surface capture in DNA microarrays. *Proceedings of the National Academy of Sciences* **104**, 8223 (2007).

8 Thorsen, T., Maerkl, S. & Quake, S. Microfluidic large-scale integration. *Science* **298**, 580 (2002).

9 Hong, J. & Quake, S. Integrated nanoliter systems. *Nature Biotechnology* **21**, 1179-1183 (2003).

10 Heath, J. R. & Davis, M. E. Nanotechnology and cancer. *Annual Review of Medicine* **59**, 405 (2007).

11 Pirrung, M. C. How to make a DNA chip. *Angewandte Chemie-International Edition* **41**, 1277 (2002).

12 Fredriksson, S. *et al.* Multiplexed protein detection by proximity ligation for cancer biomarker validation. *Nature Methods* **4**, 327-329 (2007).

13 Schweitzer, B. *et al.* Multiplexed protein profiling on microarrays by rolling-circle amplification. *Nature Biotechnology* **20**, 359-365 (2002).