

Antibody Microarray Analysis of Inflammatory Mediator Release by Human Leukemia T Cells and Human Non-Small Cell Lung Cancer Cells

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Abstract

In order to expedite the analysis of cytokine/chemokine expression levels, we have developed multiplexed, slide-based ExcelArray™ antibody microarrays. Each slide consists of 16 subarrays (wells) printed with 12 specific antibodies in triplicate and positive and negative control elements. This 16-well format allows for the analysis of 10 test samples using a six-point calibration curve. The microarray architecture is based on the "sandwich" enzyme-linked immunosorbent assay (ELISA), in which an analyte protein is "sandwiched" between an immobilized capture antibody and a biotinylated detection antibody. Analyte binding is detected using streptavidin-linked DyLight™ 649 dye for as a fluorescent detector. The Jurkat cell line was used as a model for human T cell leukemia and the A549 cell line was used as a model for human non-small cell lung carcinoma in our experiments. In order to evoke a cytokine/chemokine response, cells were stimulated with Tumor Necrosis Factor alpha (TNFα) (A549) or Phorbol-12-myristate-13-acetate (PMA, TPA) and Phytohemagglutinin (PHA) (Jurkat). Cell supernatants derived from both non-stimulated and stimulated cells were analyzed for expression of 41 unique analytes. Stimulated cells exhibited increased expression levels for many test analytes, including IL-8, TNFα, and MIP-1α, compared to the non-stimulated controls. Our experiments clearly demonstrate the utility of ExcelArray™ antibody microarray analysis for the profiling of cellular inflammatory mediator release.

Introduction

The microarray, or biochip, allows for the simultaneous screening of tens to tens of thousands of selected biological targets. The use of this tool in genotyping and gene expression experiments has matured over the past decade to the point where nucleic acid arrays are widely produced and utilized in numerous core facilities as well as smaller laboratories^{1,2}. Protein microarray technology is not as developed as nucleic acid microarray technology, but the potential uses of protein microarrays are equal to, if not greater than, those of the nucleic acid microarray. Examples of the utility of protein microarrays as screening tools for protein expression and protein interactions are abundant^{3,4}. We have constructed four human 12-plex cytokine/chemokine sandwich microarrays featuring many improvements over currently available alternatives (Table 1). These microarrays are printed on PATHplus™ microarray slides. The PATHplus™ surface allows for increased dynamic range, high signal-to-noise and excellent reproducibility compared with conventional derivatized glass and nitrocellulose pad slides. Thoroughly characterized antibody pairs are used as capture and detection reagents. Streptavidin-conjugated DyLight™ 649 is utilized as the detector molecule; superior photostability and fluorescence intensity are two attributes of this fluorescent molecule that make for a superior detector relative to other fluorophores. The general assay layout is illustrated in Figure 1.

Presented here is work in which the cytokine/chemokine response of Jurkat human T cell leukemia cells and A549 human non-small cell lung carcinoma cells were monitored using 2 distinct inflammation, a chemokine and an angiogenesis microarray. The contents of these microarrays are illustrated in Table 1. Cell culture supernatant samples were assayed on these microarrays, allowing for the simultaneous analysis of 41 different analytes. In an effort to further discern the role of several signaling proteins in the cytokine responses of TNFα stimulated A549 cells, NFκB, c-Jun and Akt expression levels were reduced through use of specific siRNA sequences. These "gene silencing" experiments illustrate the value of being able to monitor a multitude of targets simultaneously, as well as providing interesting insight into cytokine signaling pathways in the A549 cell line.

Materials and Methods

Cell Culture

Jurkat human T cell leukemia cells and A549 human non-small cell lung carcinoma cells were obtained from ATCC (TIB-152 and CCL-185, respectively). These cells were cultured in ATCC media according to product recommendations. Cytokine release was stimulated by addition of the following reagents in fresh media: 75 nM PMA (Phorbol 12-myristate 13-acetate) and 5ug/ml PHA (Phytohemagglutinin) was applied to Jurkat cells at a final density of 5x10⁵ cells/ml or 25 ng/ml TNFα was applied to A549 cells as discussed below. Appropriate vehicle controls were performed for each cell treatment. Cell culture supernatants were collected at indicated times and either assayed immediately or stored short term at -20°C.

siRNA Transfection and Western Blot Analysis:

siRNA transfections were performed according to recommendations provided by Thermo Fisher Scientific (formerly Dharmacon, Inc.) with minor modifications. The day before transfection, A549 cells were plated at a density of 7x10⁵ per well in a 12 well plate. The cells were transfected with 100 nM either ON-TARGETplus siCONTROL Non-Targeting Pool (negative control), ON-TARGETplus NFκB siRNA, ON-TARGETplus c-jun siRNA, ON-TARGETplus AKT-1 siRNA or a combination of 50 nM each of ON-TARGETplus c-jun and ON-TARGETplus NFκB siRNA (as contained in SuperSignal™ siRNA/Antibody kits). A mock transfection (2 μl of transfection reagent, DharmaFect 1 only) was included as additional control. Forty-eight hours post-transfection, the media was replaced with fresh media (non-stimulated) or media containing 25 ng/ml TNFα (stimulated) and incubated for an additional 24 hrs. The cell culture supernatants were collected and assayed for cytokine/chemokine release.

After harvesting the supernatants, the A549 cells were washed once with cold PBS and lysed directly in wells using 1X SDS PAGE sample buffer. Equivalent volumes of cell lysates were separated on a 4-12% gradient polyacrylamide gel and transferred to nitrocellulose membrane. The membranes were blocked for 1 hr at room temperature and incubated overnight at 4°C with the indicated primary antibody. The blots were washed and incubated in secondary antibody for 30 min at room temperature. The blots were developed with SuperSignal™ West Dura and exposed to X-ray film. The X-ray films were scanned and the bands quantified using a Kodak Image Station 2000MM imaging system. Loading control densitometry values did not vary significantly, therefore specific target densitometry values were not normalized.

Microarray Protocol

Cell culture supernatants were assayed for cytokine/chemokine concentration using Thermo Fisher Scientific ExcelArray™ Inflammation I (prod # 81002), Inflammation II (#81003), Angiogenesis (#81005) and Chemotaxis (#81006) microarrays (Jurkat supernatants were not assayed on the Angiogenesis microarray). Assays were performed according to product instructions. Briefly, 100 μL standard (1000-12.3 pg/ml) or sample was applied to each well and incubated at room temperature for 2 hours. After sample incubation, the microarrays were washed with wash buffer. Pre-titrated biotinylated detector antibody was applied to the microarray and incubated at room temperature for 1 hour. Again, the microarray was washed and streptavidin-linked DyLight™ 649 was applied to the microarray and incubated for 45 minutes. The microarray was washed and dipped in a final rinse solution. Microarrays were imaged using an Alpha Innotech Alphascan™ microarray imager. Spot densitometry was performed using Arrayvision™ software.

Figure 1. ExcelArray™ Assay Configuration

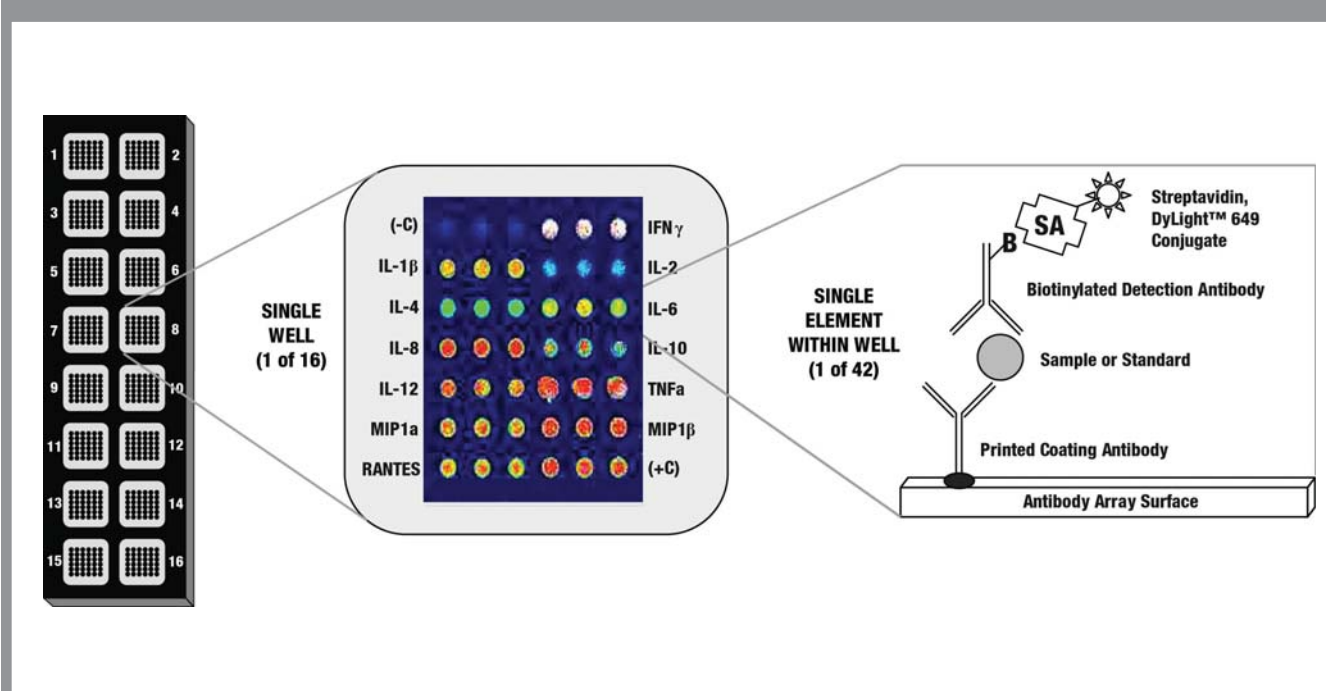


Table 1. ExcelArray™ Microarray Content

ExcelArray™ Inflammation I	ExcelArray™ Inflammation II	ExcelArray™ Angiogenesis	ExcelArray™ Chemotaxis
IFNγ	IL-1α	Angiogenin	Eotaxin
IL-1β	IL-3	EGF	I-309
IL-2	IL-5	FGFb	IP-10
IL-4	IL-7	GROα	MCP-3
IL-6	IL-13	HGF	MDC
IL-8	IL-15	IL-8	IL-8
IL-10	IL-17	HB-EGF	Fractalkine
IL-12p70	FasL	KGF	TARC
RANTES	G-CSF	PIGF	RANTES
MIP-1α	GM-CSF	VEGF	MIP-1α
MIP-1β	TNFβ	TIMP-1	MIP-1β
TNFα	MCP-1	TNFα	MCP-1

Table 2. Jurkat Cytokine/Chemokine Expression Levels

ExcelArray™ Inflammation I	ExcelArray™ Inflammation II	ExcelArray™ Chemotaxis
IFNγ	IL-1α	Eotaxin
IL-1β	IL-3, >1000pg/ml	I-309, >1000pg/ml
IL-2, >1000pg/ml	IL-5	IP-10, 93pg/ml
IL-4	IL-7	MCP-3
IL-6	IL-13, 400pg/ml	MDC, 95pg/ml
IL-8, >1000pg/ml	IL-15	IL-8
IL-10	IL-17	Fractalkine
IL-12p70	FasL, 40 pg/ml	TARC
RANTES	G-CSF, 105pg/ml	RANTES
MIP-1α, >1000pg/ml	GM-CSF, >1000pg/ml	MIP-1α
MIP-1β, 169pg/ml	TNFβ, 419pg/ml	MIP-1β
TNFα, 770pg/ml	MCP-1	MCP-1

Expression results obtained by ExcelArray™ analysis are tabulated for PHA/PMA stimulated Jurkat cell culture supernatants. Cytokines and chemokines that exhibited PHA/PMA responsiveness are highlighted in red with detected expression levels listed.

Western Blot Analysis of siRNA Treated A549 Lysates Using SuperSignal™ siRNA/Antibody Kits

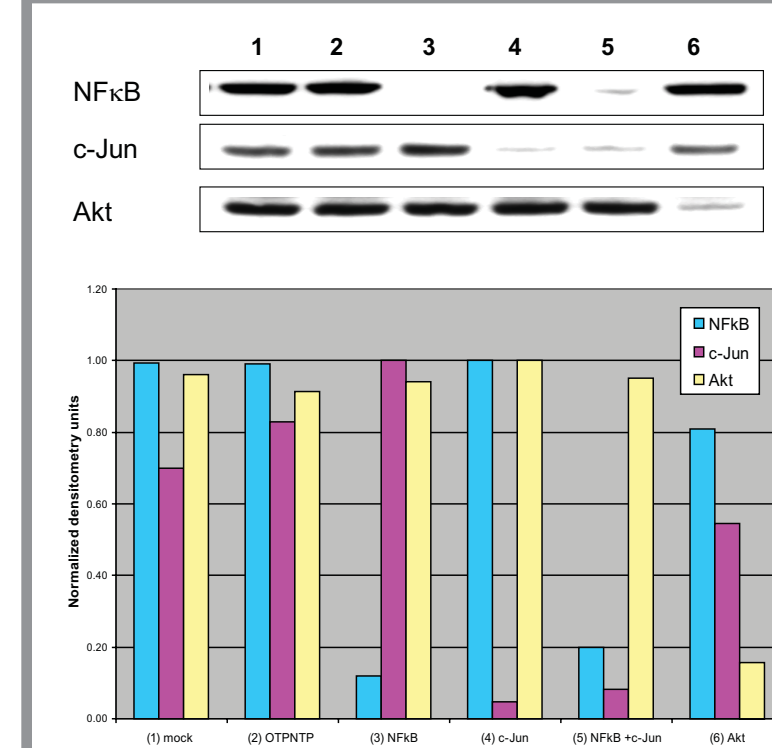


Figure 2. Total cell lysate was loaded on polyacrylamide gels, separated by SDS PAGE and transferred to blotting membranes for subsequent immunoblotting. NFκB, c-Jun and Akt antibodies were used to probe the membranes for expression levels of these specific targets. A significant variance in loading control (cyclophilin B) densitometry values were not observed, therefore, results were not normalized to the loading control. NFκB (blue), c-Jun (red) and Akt (yellow) normalized expression levels are graphically displayed for each cell treatment. Lane numbers correspond to cell lysates obtained from the following cell treatments: (1) mock transfection, (2) ON-TARGETplus siCONTROL Non-Targeting Pool (OTNTP), (3) ON-TARGETplus NFκB siRNA, (4) ON-TARGETplus c-Jun siRNA, (5) ON-TARGETplus NFκB + c-Jun siRNA and (6) ON-TARGETplus Akt siRNA.

Comparative Subarray Images

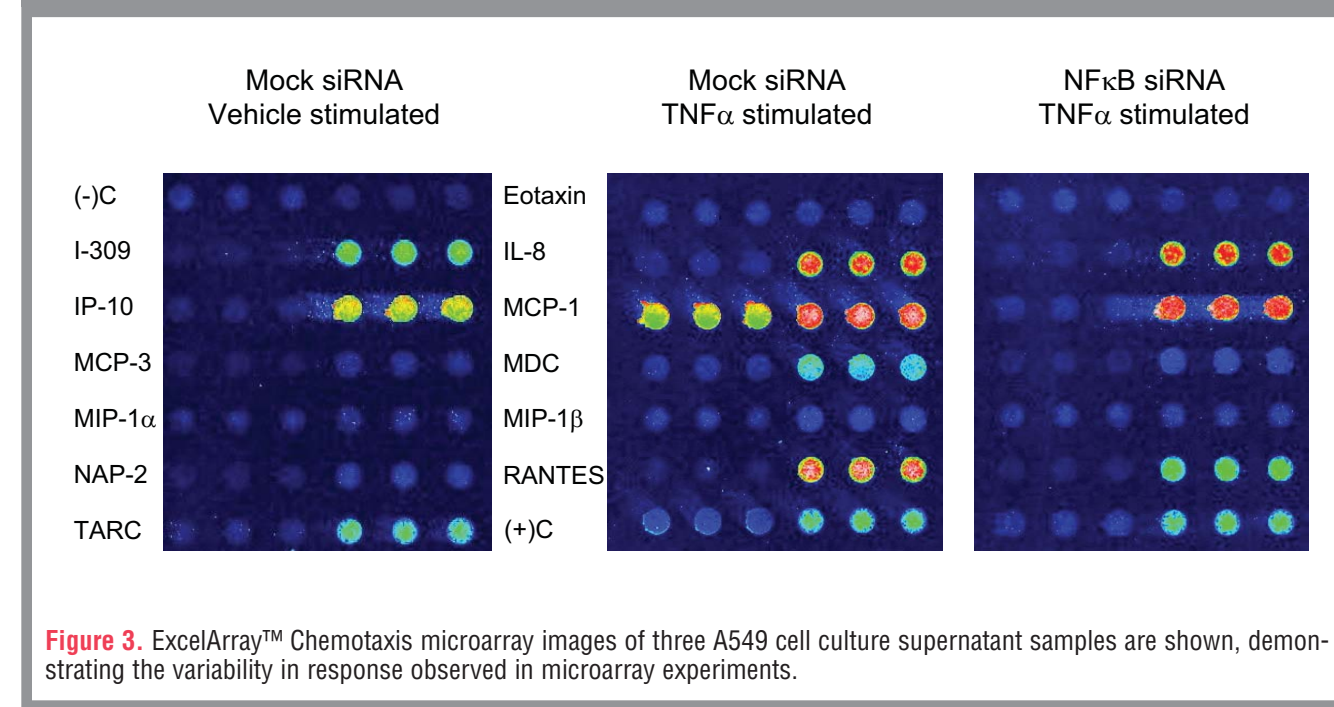


Figure 3. ExcelArray™ Chemotaxis microarray images of three A549 cell culture supernatant samples are shown, demonstrating the variability in response observed in microarray experiments.

TNFα Stimulated A549 Expression Levels of IL-8, IP-10, MIP-1β and RANTES

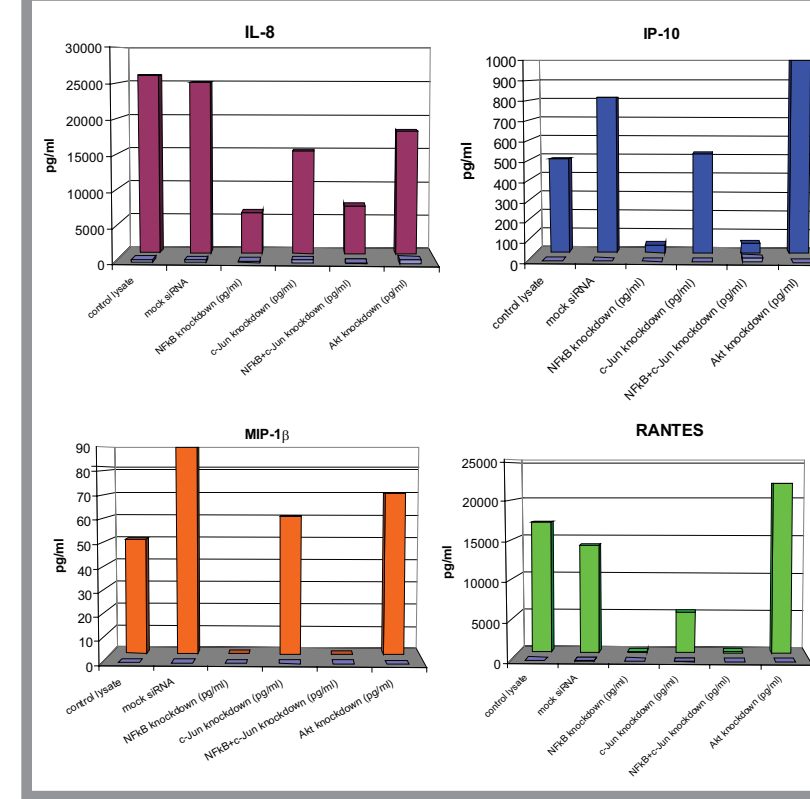


Figure 4. Results obtained for several protein microarray targets are graphically displayed. These graphs demonstrate the variability of response to gene silencing of various proteins. Responses range from partial knockdown in protein expression level (IL-8; NFκB and c-Jun lanes), to total knockdown in protein expression level (IL-8; NFκB and c-Jun lanes), to an increase in expression level (RANTES; Akt lane). For each graph, supernatants from TNFα stimulated supernatants are shown in color and vehicle stimulated controls are shown in gray.

Table 3. A549 Cytokine/Chemokine Expression Levels

target	condition	control lysate	mock siRNA	NFκB knockdown	c-Jun knockdown	NFκB+c-Jun knockdown	Akt knockdown
IL-8	unstim	360	330	220	430	90	530
	stim	26000	25000	6000	15000	7050	18000
IP-10	unstim	>10	>10	>10	>10	20	>10
	stim	490	810	40	520	85	1000
MCP-1	unstim	820	1250	810	1000	790	1000
	stim	18000	21000	2390	13000	4280	14000
MDC	unstim	>10	>10	>10	20	>10	20
	stim	250	300	40	300	120	620
MIP-1β	unstim	>10	>10	>10	>10	>10	>10
	stim	50	90	>10	60	>10	70
RANTES	unstim	20	50	>10	30	>10	50
	stim	17000	14000	110	5300	150	22000
TARC	unstim	>10	20	>10	30	20	20
	stim	130	130	60	90	70	540
G-CSF	unstim	>10	>10	>10	>10	>10	>10
	stim	120	100	>10	70	>10	180
GROα	unstim	>250	530	>250	>250	>250	600
	stim	38000	37000	6500	30000	10000	31000
IL-6	unstim	>10	>10	>10	>10	>10	>10
	stim	80	120	>10	90	>10	110
TIMP-1	unstim	33000	43000	44000	35000	25000	33000
	stim	54000	56000	47000	46000	47000	28000
Angiogenin	unstim	12000	14000	16000	17000	10000	8000
	stim	10000	12000	10000	11000	13000	8400

Expression results obtained by ExcelArray™ analysis are tabulated for tested A549 cell culture supernatants. All microarray analyses for which a variation in expression level was noted after TNFα stimulation are shown. For each row color codes correspond to a variance from vehicle control and mock transfection controls. Yellow is no change, green is an increase in protein levels and red corresponds to a decrease in detected protein level.

Results

Initial experiments consisted of treating Jurkat cells with PHA and PMA, as outlined in Materials and Methods. In these experiments, the Inflammation I, Inflammation II and Chemotaxis microarrays were utilized (Table 1). The expression levels of FasL, G-CSF, GM-CSF, I-309, IL-2, IL-3, IL-8, IL-13, IP-10, MDC, MIP-1α, MIP-1β, TNFα and TNFβ were increased in stimulated cells, as compared to vehicle stimulated controls (Table 2). The signaling mechanisms involved in these cytokine responses were further studied in siRNA experiments. Efforts to silence NFκB expression in the Jurkat cells using lipid mediated transfection were unsuccessful. Jurkat cells are a non-adherent cell line and are not efficiently transfected using lipid based reagents. To this end, the experimental model was switched to A549 non-small cell lung carcinoma cells, an adherent line. A549 cells were stimulated with TNFα and assayed on each of the four microarrays. TNFα stimulation resulted in an increased release of 12 of the 41 tested cytokines/chemokines, including IL-8, MCP-1, RANTES and GROα. A549 cells were treated with siRNA designed to reduce NFκB, c-Jun and Akt expression levels. Target silencing was confirmed by Western blot, shown in Figure 2. Significant reduction in the protein expression levels were observed for each of the targets (NFκB, c-Jun and Akt). Reduction in NFκB, c-Jun and Akt protein levels resulted in significant alterations in detected levels of several cytokines and chemokines in cell culture supernatant taken from TNFα stimulated A549 cells. Results are graphically demonstrated in Figure 4 for IL-8, IP-10, MIP-1β and RANTES. The effect of NFκB, c-Jun and Akt knockdown on the expression of the 12 TNFα responsive cytokine and chemokine targets is summarized in Table 3.

Conclusions

- PHA and PMA stimulation of Jurkat cells results in increased expression levels of 14 cytokines and chemokines (31 targets analyzed)
- TNFα stimulation of A549 cells results in increased expression of 12 cytokines and chemokines (41 targets analyzed)
- NFκB, c-Jun and Akt gene silencing (using SuperSignal™ siRNA/Antibody kits) in A549 cells results in decreased protein expression of these target proteins and a modified response to TNFα stimulation.
- ExcelArray™ antibody arrays allowed for the simultaneous screening of 41 human cytokines and chemokines, resulting in time and sample savings over traditional ELISA methods.

References

- [1] F.S. Collins, Microarrays and macrosequences, *Nature Genet.* 21 (1999) pp. 2.
- [2] E.S. Lander, Array of hope, *Nature Genet.* 21(1999) pp. 3-4.
- [3] E. Southern, K. Mir, M. Shechepinov, Molecular interactions on arrays, *Nature Genet.* 21 (1999) pp. 5-9.
- [4] L.G. Mendoza, P. McQuarry, A. Mongan, R. Gangadharan, S. Brignac and M. Eggers, High-throughput microarray based enzyme-linked immunosorbent assay, *Biotechniques* 27 (1999) pp. 778-788.
- [5] R.P. Ekins, F.W. Chu, Microarrays: their origins and applications, *Trends Biotechnol.* 17 (1999) pp. 217-18.
- [6] A. Lueking, M. Horn, H. Eickhoff, K. Bussow, H. Lehrach, G. Walker, Protein microarrays for gene expression and antibody screening, *Anal. Biochem.* 270 (1999) pp. 103-111.
- [7] B.B. Haab, M.J. Dunham, P.O. Brown, Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions, *Genome Biol.* 2(2) (2001) research 0004.1-0004.13.
- [8] R. Wiese, Y. Belosludtsev, T. Powdrell, P. Thompson, M. Hogan, M. Simultaneous multianalyte ELISA performed on a microarray platform, *Clin. Chem.* 47 (2001) pp. 1451-1457.
- [9] S. Tam, R. Wiese, S. Lee, J. Gilmore, K.D. Kumble, Simultaneous analysis of eight human Th1/Th2 cytokines using microarrays, *J. Immunol. Methods* 261(1-2) (2002) pp. 157-65.