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A Highly Parallel Nanoliter Dispenser for Microarray Fabrication

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Abstract. **We report about the correlation between satellite free droplet release and liquid viscosity in a highly parallel, pressure driven nanoliter dispenser. In extensive studies, we found that for liquids of different viscosities the duration of the pressure pulse is the predominant effect compared to pressure amplitude. This result is of essential importance when actuation parameters have to be adopted for different media like oligonucleotide, DNA or protein solutions as it is the case for the non-contact high throughput fabrication of microarrays (Ducree et al., 2000). Experiments with oligonucleotides as well as with different proteins showed ascertained carry-over and cross-contamination free printing of DNA and protein microarrays. With it a prime critical point of microarray production is solved, leading to high quality whilst high throughput microarray fabrication. For oligonucleotides printing, we found CVs to be better than 1% within one single dispensing channel and 1.5% within all 24 channels of a 24 channel printhead for each used printing buffer. By optimizing the protein printing buffer the CVs for protein printing were reduced to about 1% within all 24 channels. As a serious practical application test oligonucleotides microarrays were produced using our nanoliter dispenser system. With it a full DNA hybridization experiment was performed. Clear positive signals one hand and no signals in the negative controls on the other hand showed that our system is suited for microarray production.**

Key Words. **microfluidics, microarrays, droplet dispensing, highly parallel, oligonucleotides, proteins**

1. Introduction

High throughput screening methods are used in many domains of life sciences as well as in pharmaceutical and chemical sciences. Miniaturization, automation and parallelization allow to decrease costs of often expensive materials and leads to faster analyzing times (Schena, 2000).

The microarray technology has revolutionized the fields of biotechnology and life sciences. It has opened new perspectives for decoding the human genome, in DNA diagnostics, proteomics as well as in pharmacogenetics. The DNA microarray technology is established in many fields of applications (Nature Genetics Supplement, 1999). But as the biochemistry of proteins is orders of magnitude more complex than DNA biochemistry the protein microarray technology is in the majority of cases only in a laboratory stage (Mitchell, 2002). Furthermore, there are already efforts in binding complete cells to substrates.

The commonly most used technology to produce DNA microarrays is the contact-based pin printing technology. But pin printing has a limitation in speed and the risk of carry-over of arrayed spots. Another big problem of using pins is, especially in the field of protein microarrays, the variation of deposition properties onto glass slides, which is leading to inhomogeneous amounts of probes on the microarray. Also the influence of the metallic pin on protein structure remains unclear. The TopSpot technology overcomes these problems and enables contact free high throughput printing of microarrays (Gutmann et al., 2003).

The TopSpot technology is based on a micromachined printhead (Figure 1). The fabrication of this TopSpot printhead is described in Ducree et al. (2000). The printhead formats allow the simultaneous application of 24 or 96 different probes at a pitch of 500 μm in one step, respectively. The TopSpot printhead consists of three layers: pyrex glass, silicon and a second pyrex layer. The probes are contained in reservoirs drilled in the upper pyrex glass wafer and can be filled by standard liquid handling robots. The glass wafer is bonded to the micromachined silicon wafer by anodic bonding. The reverse side of the silicon wafer is bonded to a thin pyrex glass wafer with a square opening for the outlet nozzles. A recess in the upper pyrex (actuation chamber) opens the nozzles to the upper side of the printhead. Every reservoir is connected with one nozzle in the central area of the print head (nozzle array) via an etched microchannel system in the silicon wafer. Liquid is

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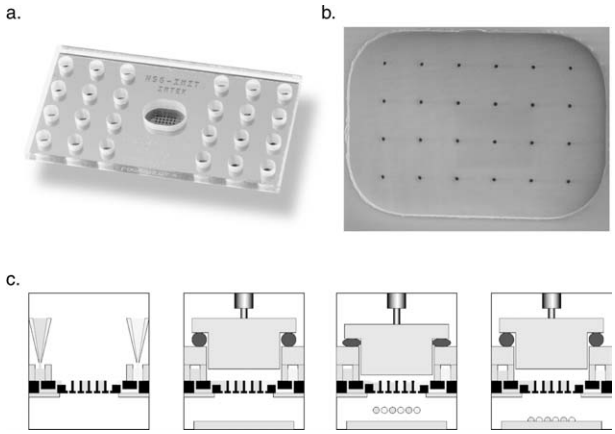


Fig. 1. (a) Picture of a 24 nozzles printhead. (b) SEM picture of the 24 nozzles on the bottom side of the printhead. (c) Working principle of TopSpot. (1) Filling of the printhead reservoirs. (2) Placing a piston into the printhead. (3) Actuation by moving of the piston. (4) Retraction of the piston.

drawn to the nozzles simply by capillary forces. The actuation of the printhead is done by a piezostack actuator as depicted in Figure 1. The actuator drives a piston into the actuation chamber of the micromachined printhead. This generates a pressure pulse that affects all nozzles simultaneously. If the pressure pulse is high enough, it overcomes the capillary forces of the nozzles and the surface tension of the fluids and droplets are accelerated out of the nozzles. To achieve a homogenous ejection of droplets, the outer surface of the nozzle array is coated with hydrophobic silane.

One major advantage of the TopSpot technology over other nanoliter dispensing systems (e.g., inkjet printer) is that every nozzle can be supplied with another printing medium at the same time. In contrast to present proceedings (e.g., pin printer, piezo tips) contact-free high throughput production is possible. This eliminates problems arising from varying adhesion forces between probes, pins and substrate surfaces.

Printing microarrays requires very flexible printer systems. This publication reports on the evaluation of operating range and limits of a pressure driven droplet release and on the correlation between specific properties of printed media and corresponding printing conditions.

2. Experimental

2.1. Chemicals and materials

- 10 × PBS [phosphate buffered saline], Gibco BRL
- 20 × SSC [saline sodium citrate], Gibco BRL
- ArrayLink^{TM} buffer, Genescan Europe AG
- Boric acid, Sigma-Aldrich
- Sodiumcarbonate, Sigma-Aldrich
- Sodiumbicarbonate, Sigma-Aldrich
- DMSO [dimethylsulfoxid] [$(CH_3)_2SO$], Merck
- Glycerol 99% [$C_3H_8O_8$], ACROS ORGANICS
- Betaine monohydrate [$C_5H_{11}NO2xH_2O$], Fluka
- Nonidet P40 substitute, ionic surfactant, Fluka
- RBS N, non-ionic surfactant pH 7, Roth
- SDS (Sodium *n*-Dodecyl Sulfate), Roth
- Tween 20, Sigma-Aldrich
- Cy3-labeled oligonucleotide: (5′-5ac gta cgt acg tac gta cg-3′), BIG—Biotech GmbH, Germany
- Cy5-labeled oligonucleotide (20-caagaactatgcttcaag-cacatcaaccgttttttttttt-22), TIB MOLBIOL, Germany
- Biotin-labeled oligonucleotide (20-caagaactatgctt-caagcacatcaaccgttttttttttt-22), TIB MOLBIOL, Germany
- Random oligonucleotide (22-ttttttttttcgccatccacgtgtg-tacggatactagttaacccgtgctttggctccact), rnd oligo, TIB MOLBIOL, Germany
- Oligonucleotide 1: lambda493!277!43 (sense and biotin-labeled antisense), TIB MOLBIOL, Germany
- Oligonucleotide 2: phiX2253-2022F!40 (sense and biotin-labeled antisense), TIB MOLBIOL, Germany
- Oligonucleotide 3: lambda564!84!43 (sense and biotin-labeled antisense), TIB MOLBIOL, Germany
- Oligonucleotide 4: lambda784!555!41 (sense and biotin-labeled antisense), TIB MOLBIOL, Germany
- Oligonucleotide 5: phiX412-184F!40 (sense and biotin-labeled antisense), TIB MOLBIOL, Germany
- Streptavidin-Cy5, 1 mg/ml, Amersham pharmacia
- BSA (bovine serum albumine), fraction V, VWR
- Blocking Solution: 4 × SSC/0,2% Tween20/5% BSA
- Donkey anti Rabbit IgG-Cy5 (H + L), Dianova
- Donkey anti Mouse IgG-Cy5 (H + L), Dianova
- Donkey anti Goat IgG-Cy5 (H + L), Dianova
- ArrayLink^{TM} hyphob, Genescan Europe AG

2.2. Experimental setup

In our publication, we investigated printing with 24 channel printheads depicted in Figure 1. Parameters for diameter and pitch of the nozzles were 50 µm and 500 µm. Since the printhead is reused it is important to evaluate the washing procedure of the printhead to guarantee carry-over and cross-talk free usage of the printhead. Therefore, every second reservoir was filled with 1 µM Cy3-labeled oligonucleotides and the other reservoirs with pure printing buffer. The reagents have been printed onto microarray slides detected with a fluorescence reader (La Vision Biotech, Germany, sensitivity up to < 0.8 Cy3 molecules/µm^2). Without cross-talk they should look like a checker board. Afterwards the checkerboard-like filled printhead was washed in an ultrasonic bath with 5% v/v RBS N and

rinsed in excess of deionized water. Then the checkerboard-like filling was inversed, so every nozzle, which was filled with Cy3-labeled oligonucleotides before is now filled with buffer solution without fluorescence material. In the fluorescence reader, no leavings of former filling should be detectable, even at very high exposure times. The same tests were also done with a variety of proteins in different printing buffers, such as Cy5-labeled BSA or antibodies (each 200 µg/ml). For those experiments the washing procedure had to be adapted. It starts with a first rinsing step with 5% v/v RBS N, followed by an ultrasonic bath, a second rinsing in deionized water and a second ultrasonic bath with deionized water.

Surface tension and viscosity of printing media are the parameters with major impact on the droplet dispensing process. The main portion of printing medium is printing buffer. In the field of microarrays very different printing buffers are reported in publications. So, we investigated over 120 different buffers and additives in different concentrations and pH values covering a wide range of viscosities and surface tensions (Figure 5). In all cases, we determined optimal printing conditions for stable and satellite-free droplet ejection. Surface tensions of all used printing buffers were measured by a drop shape analysis system (DSA10 Mk2, Kruess, Germany) according to the ''pendant drop'' method. For correlation between printing media and needed printing conditions the piston movement of the printer device was recorded during actuation process using a laser-doppler-vibrometer (OFV 1102, Polytek, Germany). The actuation pressure pulse was calculated from piston movement and initial actuation chamber volume. These data enable us to analyze the correlation between amplitude and duration of pressure pulse on the one hand and quality of droplet release on the other hand.

A stroboscopic camera was used to evaluate droplet dispensing and droplet flight of different printing media towards microarray slides in a time resolved measurement after leaving the nozzle. Droplets form a regular spherical shape. This allows to measure the droplet volume during the flight via the analysis of the droplet diameter from the stroboscopic data. This droplet volume calculation was done automatically by the image processing system NeuroCheck® (NeuroCheck, Germany). Due to the limited resolution of the camera used and the arrangement of the nozzles on the printhead in several lines it is only possible to evaluate two but not all 24 nozzles at the same time by this stroboscopic method. In a similar configuration we were also able to evaluate the droplet impact onto a microarray slide.

To observe all 24 simultaneously printed droplets after printing on a slide in one step we used a fixed camera system, mounted on the TopSpot printer device. Each printed array was photographed automatically right after the print. The spot diameter was determined by geometric outline analysis of dispensed spots. In this configuration, NeuroCheck® was used for determination of spot diameter and horizontal deflection of the spots from their correct 500 µm spacing as well. Correct size and position of spots is essential for microarray usage. To achieve higher densities the spacing between the spots was lowered by printing between the spots of already printed arrays at 500 µm pitch, resulting in a 250 µm spacing of spots.

As an applied measurement method a typical microarray approach was used. Cy3-labeled oligonucleotides (1 µM) and Cy5-labeled antibodies (200 µg/ml) were printed and coupled to ArrayLink™ hyphob microarray slides. The unbound molecules on the slides were removed by washing the printed slides three times 10 minutes in a washing solution (2 × SSC 0.1% SDS). The intensity of signal integral of coupled molecules was determined. The fluorescence signal was used as a measure of dispensed volume.

Finally, as an applied experimental usage oligonucleotide microarrays were produced using the TopSpot technology. Eight different oligonucleotides were printed onto ArrayLink™ hyphob microarray slides. Two positive controls were printed to check whether the coupling onto the microarray slide and the coloration step was successful. As a negative control a random sequenced oligonucleotide was printed, which has no corresponding partner in the hybridization solution. As target molecules five sense oligonucleotides were printed. After printing the printed microarray slides were coupled and washed according to the UV-protocol of the microarray slide supplier (ArrayLink™ hyphob manual). A hybridization solution was prepared containing the five antisense oligonucleotides (25 nM) in hybridization buffer. The DNA was denatured for 5 minutes at 80 °C on a heating block, followed by 5 minutes on incubation on ice to improve DNA hybridization. The denatured hybridization solution was pipetted onto the microarray and incubated for 15 minutes at 60 °C. Afterwards the unbound molecules were removed by washing three times for 10 minutes in washing solution. For detection of the hybridization event in a specific spot the microarray was incubated for 30 minutes with a detection solution containing 5 µg/ml Streptavidin-Cy5 (the corresponding partner to the biotin label of the antisense oligonucleotides) and blocking solution (to minimize unspecific binding events). Once more the unbound molecules were removed by washing three times 10 minutes in washing solution. In consequence the binding of antisense oligonucleotides in a specific spot was detected and analyzed using a fluorescence reader.

3. Results and Discussion

Extensive experiments using labeled oligonucleotides and different labeled proteins showed the total isolation of the different microchannels, nozzles and dispensed droplets without any cross-talk even after thousands of printed arrays. For the re-usage of printheads the experiments showed that even after washing and re-using of printheads no carry-over of formerly printed media could be detected. For the purpose of protein printing the washing procedure of printheads was improved. With it we ensured reliably carry-over free printing of protein microarrays (Figure 2). The improvement was necessary due to the fact that compared to the simple poly-anion DNA the biochemistry of proteins are in orders of magnitude more complex and diverse. Many proteins have sticky properties and adhere easily to the hydrophobic coating outside the printhead nozzles. The improved washing procedure is more stringent, so it fulfills these requirements and cleans the printhead properly. The experiments ascertain carry-over free printing of DNA and protein-microarrays. In contrast to reloading pin printing techniques a prime critical point of microarray production is solved, leading to high quality whilst high throughput DNA and protein microarray fabrication.

Optimal droplet dispensing conditions are stable and satellite-free (Figure 3). In order to understand what happens, one has to look to the dynamics of the droplet formation process as depicted in Figure 4. The displacement of the actuating piston, the calculated pressure pulse and the stroboscopic images of the droplet release process are depicted. The oscillation of pressure pulse is due to oscillation of piezostack actuator around the wanted stroke (data not shown). The withdraw of

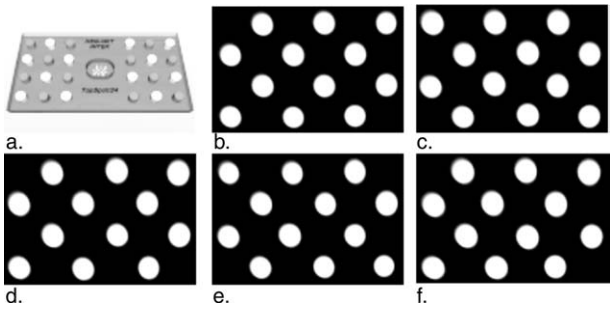


Fig. 2. *Carry-over test: The printhead was filled with Cy5-labeled BSA and buffer like a checker board. (a) The printed microarrays were evaluated in a fluorescence reader. (b) Afterwards the printheads were washed, refilled with the inversed checkerboard filling and printed. (c) The whole procedure was repeated five times (b to f). In the quantification of the 4 × 6 arrays only the spots with Cy5-BSA are visible, that means both no cross-contamination and carry-over occurs.*

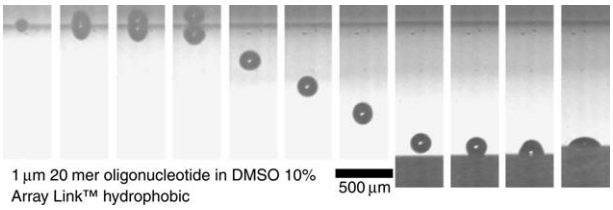


Fig. 3. *The printing process from the nozzle of the printhead down to the microarray slide. The droplet dispensing process is highly stable and reproducible with all tested printing media.*

piston generates a negative pressure pulse and facilitate thereby the droplet release. Generally, droplet release took place in the first minimum of pressure pulse (Figure 4). We correlated optimal droplet dispensing conditions of a specific printing solution to the liquid properties of used buffer. So we found that droplet release of higher viscous fluids takes longer. This can be explained with the higher flow resistance of higher viscous fluids. The response of fluid takes more time and the pressure pulse has to be adapted. In contrast it seems to be not necessary to charge higher pressure pulse for higher viscous fluids. The highest stable printable viscosity and surface tension were 30% v/v glycerol-water and 0.5% v/v Nonidet P40-buffer solution, respectively.

With the used automated image processing system, we measured 250 volumes recorded per printing buffer by the stroboscopic camera for droplets during flight (Figure 3). Based on this we found the droplet diameter at optimal conditions to be dependent on viscosity and surface tension as well. For oligonucleotides as printing media the reproducibility of droplet diameter within one single channel was in general better than 1% independent from used printing buffer (Figure 5). The droplet diameter and CVs of protein media (per single nozzle) were measured by the mounted camera system on the TopSpot printer device. They were slightly higher (Figure 6), but can be explained by tiny inhomogeneities of the used microarray substrate surface and by differing solubility of proteins in buffer solutions. But with optimal printing conditions for both media CVs below 1% were achieved.

Evaluation of recorded arrays on the slides showed CVs between all 24 different printing channels of a printhead to be better than 1.5% and a spot position deflection below 10 µm from the regular 500 µm grid on the slide. Higher spot densities were printed successfully by printing between the spots of printed arrays, so that even with a 500 µm nozzle spacing in a printhead a 250 µm spacing of spots was achieved (Figure 7). One main reason for droplet deflection and increased CVs for all 24 nozzles is the hydrophobic coating of the nozzle surface. Tiny differences in the homogeneity have significant influence on the droplet release process.

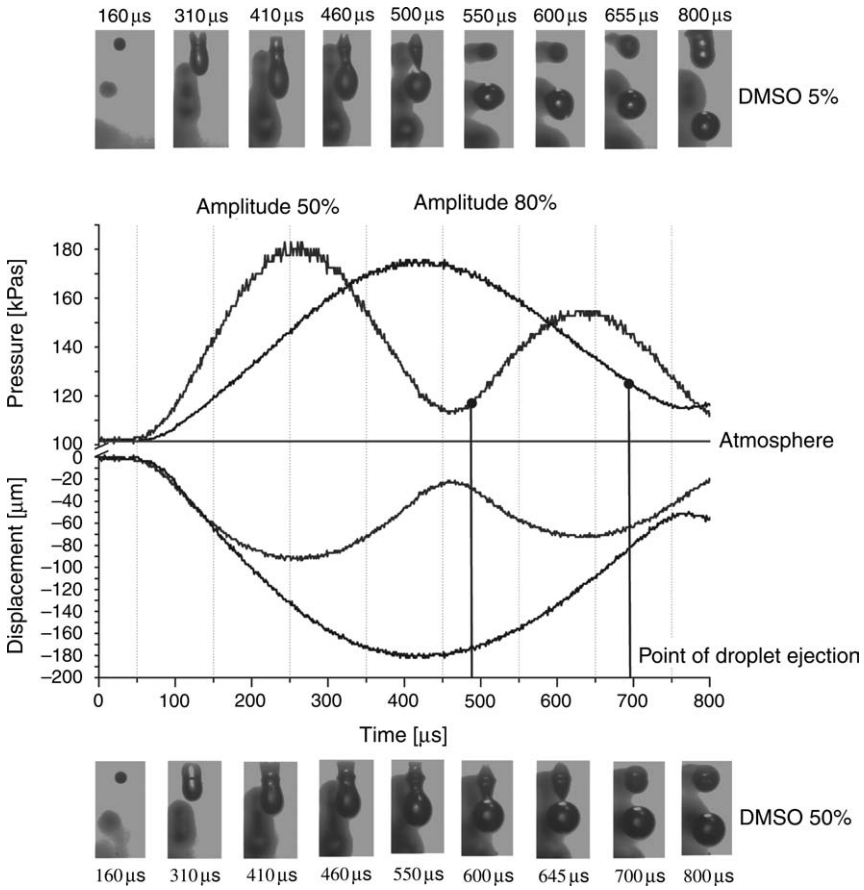


Fig. 4. *On the top and the bottom of the graph the droplet ejection of two different concentrations of DMSO is shown (gray lines: DMSO 5%, dark lines: DMSO 50%). In the graph the displacement of the piston (lower curves) and the calculated pressure pulse (upper curves) are plotted against the time. The points of droplet release are indicated.*

Buffer	Conc.	Diameter of dispensed droplets in µm	CV %	Buffer	Conc.	Diameter of dispensed droplets in µm	CV %
PBS in M	0.0375	128.88	0.32	Glycerol in Vol. %	2.5	130.08	0.08
	0.075	128.88	0.22		5	131.98	0.33
	0.15	131.84	0.33		10	139.42	0.32
	0.225	134.18	0.29		15	141.32	0.55
	0.3	135.74	0.13		20	143.3	0.33
	0.45	135.32	0.17		30	142.58	0.30
SSC in M	0.075	127.9	0.20	3×SSC *x*M betaine	0.2	129.22	0.22
	0.15	128.14	0.29		0.3	127.64	0.26
	0.3	128.8	0.19		0.4	127.8	0.22
	0.45	129.44	0.16		0.5	128.84	0.14
	0.6	131.40	0.24		1.0	134.28	0.33
	0.9	132.10	0.24		1.5	139.68	0.22
DMSO in Vol. %	5	129.14	0.19	3×SSC *x*% Nonidet	0.0005	131.58	0.22
	10	128.22	0.10		0.001	136.02	0.10
	20	132.52	0.24		0.01	137.32	0.48
	30	136.74	0.29		0.05	135.54	0.32
	40	141.64	0.27		0.1	133.40	0.39
	50	143.58	0.25		0.5	122.80	0.81

Fig. 5. *Table of used printing buffer solutions for oligonucleotides printing and the corresponding droplet diameters with CVs using optimal printing conditions. Droplet diameters and* CVs *out of 250 droplet ejections using a stroboscopic camera.*

During the droplet ejection process less coated areas, get wetted by printing solution, leading to bigger tear-off edge diameter and in consequence to higher droplet diameter and deflection of droplets. Of minor importance should be variations in the manufacturing of the nozzles, due to the very precise etching process used. The experiments showed also that the volume of dispensed droplets is highly depending on the characteristics of the printing fluid, mainly viscosity and surface tension. In all dilution series the volume increased with increasing viscosity, but also with lower surface tension (Figure 8). The higher viscosity is leading to higher friction between the molecular fluid layers so that more fluid is pushed out of the fluid meniscus. A small filament of liquid is formed right before the droplet tears off caused by the surface tension of the printing fluid. Lower surface tension delays the formation of a droplet, leading to higher droplet volume. If a defined liquid is used, the

Buffer	Conc. in M	Diameter of droplets on the substrate	CV %	Buffer	Conc. in M	Diameter of droplets on the substrate	CV %
PBS	0.05	239.74	0.53	Array Link	0.05	220.44	0.37
	0.1	241.00	0.42		0.1	210.66	0.51
	0.2	242.90	0.66		0.2	210.78	0.63
	0.3	244.76	0.42		0.3	213.76	0.66
	0.4	244.86	0.81		0.4	219.54	0.67
	0.5	245.80	0.34		0.5	219.90	0.48
SSC	0.05	252.22	0.52	Borate	0.05	208.44	0.67
	0.1	255.96	0.62		0.1	209.32	0.47
	0.2	254.66	0.68		0.2	209.64	0.53
	0.3	257.70	0.49		0.3	212.10	0.64
	0.4	256.16	0.47		0.4	213.88	0.81
	0.5	261.70	0.84		0.5	218.32	0.81
Sodium-carbonate	0.05	225.84	0.28				
	0.1	216.70	0.30				
	0.2	218.62	0.31				
	0.3	218.90	0.59				
	0.4	219.40	0.17				
	0.5	220.72	0.43				

Fig. 6. *Table of used printing buffer solutions for BSA printing and the corresponding droplet diameters on a microarray substrate with CVs using optimal printing conditions. Droplet diameters and CVs out of 25 pictures of printed arrays using the mounted camera system of the TopSpot printer device.*

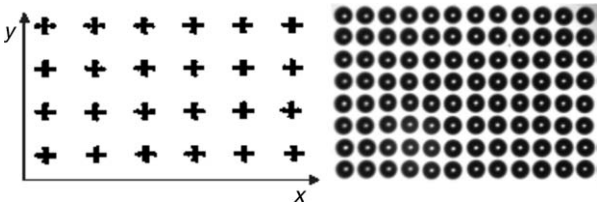


Fig. 7. *Overlaying of the xy-coordinates of 16 printed arrays measured by NeuroCheck®. Each cross indicates the spot center and the cross width the spot diameter. The deflection of all spots is less than 10 µm from regular 500 µm grid. On the right a picture of a 250 µm spot spacing by interlacing printing is shown.*

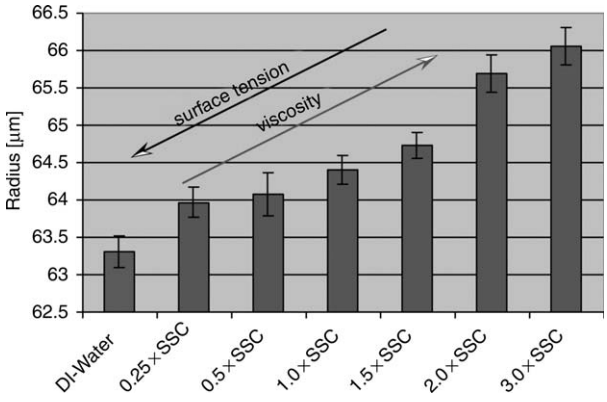


Fig. 8. *Droplet size depending on viscosity and surface tension: The arrows indicate the growing viscosity and surface tension of different SSC buffer concentrations on droplet size. (Data of the other tested buffers are not shown.)*

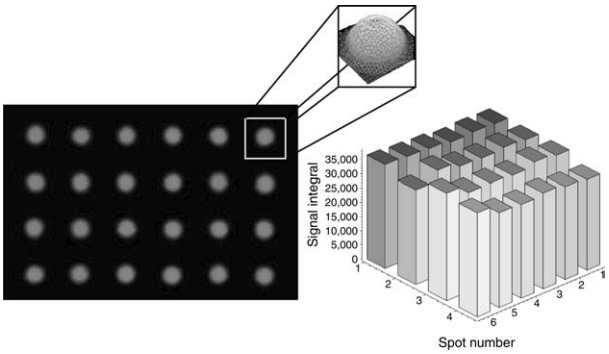
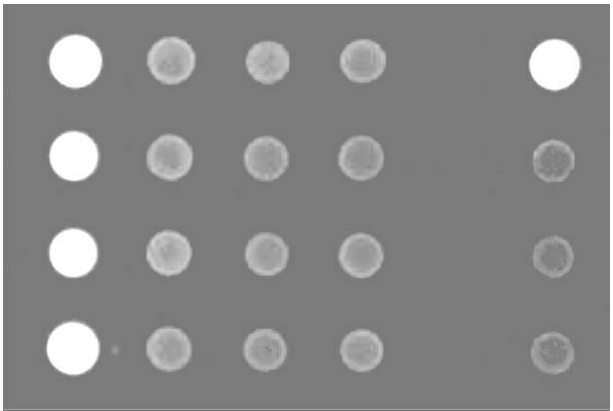


Fig. 9. *1 µM Cy3-labeled 20 mer oligonucleotides in 30% v/v DMSO on a microarray slide. The left side shows the fluorescence analysis of printed spots with a CV of 2.1% over all spots. On the top the distribution of fluorescence signal within one spot is shown in detail, demonstrating the high homogeneity of the printed spots.*

droplet volume for this type of liquid is fixed. It cannot be varied through pressure modification of the actuation without leaving the ideal printing parameters. Nevertheless, a tuning of droplets within a wide range is possible by a modified actuation system which is discussed in Steinert (2003) and which is not within the focus of the experiments described here.

Microarray experiments, including printing, coupling and washing, have peen performed with Cy3-labeled oligonucleotides. Fluorescence-based quantification



Cy5 oligo	oligo 1	oligo 2	oligo 3	rnd oligo	Cy5 oligo
Bio oligo	oligo 1	oligo 2	oligo 3	rnd oligo	oligo 5
Bio oligo	oligo 1	oligo 2	oligo 3	rnd oligo	oligo 5
Cy5 oligo	oligo 4	oligo 4	oligo 4	rnd oligo	oligo 5

Fig. 10. *Antisense hybridization experiment: Eight different oligonucleotides were printed in the shown arrangement. Cy5 and biotin labeled oligonucleotides as experimental positive controls. A random oligonucleotide as negative control. Five different sense-oligonucleotides, which were detected with their corresponding antisense-oligonucleotide in the hybridization solution. Clear positive signals in the control spots and sense-oligonucleotide spots on the one hand and no signals in the negative control spots were showing the utilizability of the TopSpot dispenser system for microarray production.*

showed CVs of signal integral to be better than 4% within one array depending on used printing buffer (Figure 9). The results include coupling and washing efficiency of microarrays as well as the measurement errors due to the fluorescence method. With it the results of the comparison of all 24 nozzles were verified by a typical microarray approach and enabled the system for high quality microarray production.

To show the qualification of the TopSpot dispenser in a real application, oligonucleotide microarrays were produced. With the microarrays DNA hybridization experiments were performed and the results analyzed in a fluorescence reader (Figure 10). The results of the microarray hybridization experiments showed clear signals in the positive control (Cy5 and biotin labeled oligonucleotides) spots and the oligonucleotide spots (sense-oligonucleotides 1 to 5). But, very important for microarray experiments, no signals in the negative control spots (random oligonucleotide) were detectable. So successful usage of TopSpot printed oligonucleotide microarrays was shown.

In the presented experiments, it was not possible to vary viscosity and surface tension independently. To assure which parameter has the predominant effect a computer simulation of the printing process will be established in future work. Further work will be the evaluation of higher complex molecules.

Acknowledgments

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