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# Non-contact production of oligonucleotide microarrays using the highly integrated TopSpot nanoliter dispenser

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For the first time we report on the production of oligonucleotide microarrays using a highly parallel and highly integrated, pressure driven TopSpot nanoliter dispenser. The system enables non-contact printing of different media like oligonucleotides, DNA or protein solutions. We optimized the printing buffer needed for oligonucleotides microarrays production with respect to two major aspects: microfluidical optimum for droplet dispensing and biochemical coupling efficiency on different commercially available microarray slides. Coefficient of variations (CVs) of generated spot diameters were measured to be smaller than 1% within one single dispensing nozzle and smaller than 1.5% within all 24 parallel nozzles of the printhead for all printing buffers used. No carry-over and no cross-talk was found, in extensive experiments with oligonucleotides. Optimized printing buffer compositions and concentrations for oligonucleotide microarrays were found, as well as optimized coupling protocols. Furthermore, buffers and protocols were adapted to a host of different microarray slides used. With this system, prime critical points of microarray production are solved, leading to high quality high throughput microarray fabrication.

## Introduction

Oligonucleotide microarray technology is established in many fields of application.[1] Three different techniques are commonly used for their production: On-chip synthesis as well as contact and non-contact spotting techniques.[2]

In on-chip synthesis, oligonucleotides are synthesized directly on the chip surface. Affymetrix (GeneChip®) uses UV-lithography adopted from semiconductor technologies to synthesize oligonucleotides on a surface. The photolithography process and solid-phase chemistry allow the fabrication of high density microarrays containing *e.g.* hundreds of thousands of different DNA fragments, but not all applications need such high density. High flexibility is in increasing demand, required for the production of different specialized microarrays (*e.g.* cancer, specific diseases, toxicity, apoptosis, pathway analysis) with a lower density of 20 to several hundreds of different probes. Batch sizes of significantly more than 100 slides at reasonable costs are asked for.

The most commonly used oligonucleotide microarray printing technology is contact based pin printing. Pins are dipped into a sample solution and brought in direct contact with the substrate. A small volume of fluid is thus transferred to the slide surface. Spot volumes range from 0.2 nL to 1.0 nL. Using standard solid pins, reloading must be done almost after every print. Micro-spotting-pins, which work like fountain pens, were developed to avoid this problem. They are able to aspirate liquid volumes between 0.2 μL and 1 μL. Even with this it is necessary to use many pins in parallel to have an efficient system. Thus it is not easy to obtain a set of printing pins, which are uniform in size, shape and height. Some instruments use up to 64 pins in parallel at 4.5 mm center-to-center spacing.[3] Another disadvantage of contact printing is the risk of deforming the pins during spotting, which can affect the reproducibility of spots. After the printing process of a probe, the pins have to be cleaned to avoid carry-over.

Non-contact printing techniques overcome the problems arising from the contact between printing tool and substrate. The fluid is "shot" onto the surface from a distance. A reservoir holds a larger volume of liquid so that there is no need to refill after every shot. Capillary forces or pumping mechanisms transport the liquid through channels from the reservoirs to the nozzles, where the droplet ejection takes place. Perkin Elmer and GeSiM supplied the piezoelectric tips,[4,5] and a glass capillary containing liquid is squeezed by a piezo-electric collar, which surrounds the capillary. This squeezing induces a drop to be formed out of the capillary. The fast response time of the piezoelectric crystal in combination with the acoustic droplet generation principle permits fast dispensing rates. The small deflection of the crystal generates drop volumes from sub-nanoliter to nanoliter. The main difficulties in using piezoelectric dispensing are: generation of air bubbles inside the dispenser, which reduce the reliability of the system, large sample volumes and difficulties in changing samples. To gain speed, parallel systems are built up.

This work is based on the TopSpot technology, a pneumatically driven fluid displacement technique. In contrast to other microarray spotting techniques the highly integrated TopSpot technology delivers the spotted probes at a very small pitch of 500 μm. This significantly reduces the effort which has to be spent for automation. TopSpot printheads can be loaded with reagent volumes up to 6 μL. So far, we have built printheads with up to 384 dispensing channels in parallel. This significantly increases throughput in microarray fabrication and allows one to fabricate microarray batch sizes in the order of 5000 slides without reloading the printhead.

The highly parallel and highly integrated non-contact approach of this new technology accelerates the application of probes onto a substrate. It overcomes the formerly mentioned problems for conventional spotting techniques and enables high-throughput printing of low and medium density microarrays at reasonable costs.[6,7]

### The TopSpot principle

The TopSpot technology is based on a micromachined printhead, and the fabrication of this TopSpot printhead is described in ref. 6. Two printhead formats for the simultaneous application of 24 and 96 different probes in one step are commercially available.[7] A 384 channel printhead is already available for β-testers. The TopSpot printhead consists of three layers, Pyrex glass, silicon and another Pyrex layer (Fig. 1). The probes to be dispensed are contained in reservoirs in the upper pyrex glass and can be filled in by standard liquid handling robots. Each reservoir can be filled with up to 6 μL of probe, enough for printing thousands of microarrays without any

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washing and refilling steps. A recess in the upper Pyrex (actuation chamber) opens the nozzles to the upper side of the printhead. Every reservoir is connected to one nozzle *via* an etched microchannel system in the silicon wafer. Liquid is drawn to the nozzles by capillary forces. The actuation of the printhead is done by a piezostack actuator as depicted in Fig. 1. The actuator drives a piston into the actuation chamber. This generates a pressure pulse that affects all nozzles simultaneously. If the amplitude of the pressure pulse is high enough, it overcomes the capillary forces of the nozzles and the surface tension of the fluid, and droplets are ejected out of the nozzles. To achieve a homogenous ejection of the droplets, the outer surface of the nozzles is coated with hydrophobic silane.[8]

One of the major advantages of the TopSpot technology over other nanoliter dispensing systems (*e.g.* an inkjet printer) is the fact that every nozzle can be supplied with another printing medium. So in contrast to the present proceedings (*e.g.* pin printer, piezo tips) a contact-free high-throughput production is possible. The contact-free method eliminates problems arising from the varying adhesion forces between probes, pins and the substrate surface.

The TopSpot technology addresses the production of custom made low to mid density microarrays containing up to several thousands of different probes on a slide. This can be achieved by a modular concept enabling the user to operate several printheads filled with different printing media in one sequenced procedure. The number of simultaneously used printheads and therefore the number of employed modules is only dependent on the automation environment. The arrayer with lowest complexity (TopSpot Entry Arrayer) just contains one single printhead and allows one to spot 24, 96 or (for β-testers) 384 different probes on one slide. This type of performance can be achieved without any automation. A higher degree of automation is *e.g.* realized with the TopSpot Modular Arrayer (TopSpot/M) which can handle up to 5 printheads, simultaneously, printing up to 40 standard microarray substrates in one procedure. With a camera system and dedicated software, quality assurance is integrated. TopSpot/M enables flexible production of a small and medium series of microarrays with a throughput of 300 microarrays per hour each containing 480 different features when using printheads with 96 channels (for further information and contact details see: http://www.imtek.de/anwendungen). Companies using the TopSpot technology to produce custom made mid density microarrays are *e.g.* Picorapid Technologie GmbH in Bremen, Germany or Hoffmann-La Roche AG in Basel, Switzerland. In addition, customized machines containing up to 15 printheads (1440 different probes) have been realized so far.

Microarray production requires a very flexible printer technology. It is well known that different applications need specific adopted printing buffer systems, depending on printing media, coupling chemistry of used microarray slides and surface properties. This paper reports on the production of oligonucleotide microarrays using the highly parallel and highly integrated TopSpot non-contact nanoliter dispenser. Microfluidic aspects as well as biochemical aspects were investigated to optimize the microarray fabrication including the following steps: microarray printing, coupling of oligonucleotides to different microarray surfaces and oligonucleotide microarray usage.

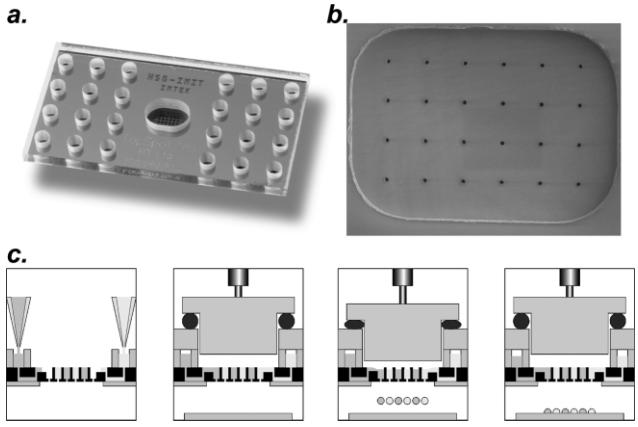


**Fig. 1** ***a.*** Picture of a printhead. ***b.*** SEM picture of the 24 nozzles on the bottom side of the printhead. ***c.*** Working principle of TopSpot. From left to right: i. filling of the printhead reservoirs; ii. placing a piston into the printhead; iii. actuation by moving of the piston; iv. retraction of the piston.

## Material and methods

### Chemicals and materials

a. Buffers
- 10 × PBS buffer [1.54 M phosphate buffered saline], Gibco BRL
- 20 × SSC buffer [3.3 M sodium chloride sodium citrate], Gibco BRL
- Sodium phosphate buffer [1 M = 141.96 g $Na_2HPO_4$, 137.99 g $NaH_2PO_4{\cdot}H_2O$ in 2,000 mL $H_2O$ (deion.)
- Carbonate buffer [1 M = 10.6 g sodium carbonate, 8.4 g sodium bicarbonate in 200 mL $H_2O$ (deion.)]
- Borate buffer [1 M = 12.37 g boric acid, 4 g NaOH in 200 mL $H_2O$ (deion.)]
- Boric acid, Sigma-Aldrich
- Sodium carbonate, Sigma-Aldrich
- Sodium bicarbonate, Sigma-Aldrich
- DMSO [dimethylsulfoxide] [$(CH_3)_2SO$], Merck
- Glycerol 99% [$C_3H_8O_8$], Acros Organics
- Betaine monohydrate [$C_5H_{11}NO{\cdot}2H_2O$], Fluka

b. Surfactants
- Nonidet P40 substitute, ionic surfactant, Fluka
- RBS N, non-ionic surfactant pH 7, Roth
- SDS (sodium n-dodecyl sulfate), Roth
- Tween20, Sigma-Aldrich

c. Oligonucleotides
- Cy3-labeled oligonucleotide: (5′-5ac gta cgt acg tac gta cg-3′), BIG—Biotech GmbH, Germany
- Cy5-labeled oligonucleotide (20-caagaactatgcttcaagcacat-caaccgttttttttttt-22), TIB MOLBIOL, Germany
- Biotin-labeled oligonucleotide (20-caagaactatgcttcaagcacat-caaccgttttttttttt-22), TIB MOLBIOL, Germany
- Random oligonucleotide (22-ttttttttttcgccatccacgtgtgtacgga-tactagttaacccgtgctttggctccact), 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

d. Detection
- Streptavidin-cy5, 1 mg $mL^{-1}$, Amersham Pharmacia
- BSA (Bovine Serum Albumin), fraction V, VWR
- Blocking solution: 4 × SSC/0.2% Tween20/5% BSA

e. Microarray slides
- ArrayLink hyphob, epoxy-modified glass slides, Genescan Europe AG
- CMT-GAPS2, amino-modified glass slides, Corning
- QMT Epoxy, epoxy-modified glass slides, Quantifoil
- PMMA, plastic slides, Genescan Europe AG

f. Devices and software
- UV StrataLinker 2400, Stratagene, USA
- Bioanalyzer 4F/4S Fluorescence reader, La Vision Biotech, Germany (sensitivity up to $< 0.8$ Cy3 molecules $\mu m^{-2}$)

– Highspeed-Videostroboscope MOCON-RT, VISIT GmbH & Co. KG, Germany
– NeuroCheck®, NeuroCheck, Germany

## Experimental setup

Printing of oligonucleotide microarrays with 24 channel printheads was investigated. Parameters for diameter and pitch of the nozzles were 50 μm and 500 μm. As already mentioned, of prime interest for microarray production is the elimination of any possibility of sample cross-contamination. We made extensive cross-talk tests to verify the total isolation of all supply-channels, nozzles and dispensed droplets due to the highly parallel printhead approach. Every second reservoir was filled with 1 μM Cy3-labeled oligonucleotides and the other reservoirs with pure printing buffer. This checkerboard-like array was printed onto microarray slides and detected with the fluorescence reader. The printhead is reusable, so it is important to establish a proven washing procedure to guaranty carry-over free usage of the printhead. The checkerboard-like filled printhead used in the cross-talk test was washed for 5 min in an ultrasonic bath with 5% v/v RBS N and afterwards rinsed with 50 mL of deionized water. Then the checkerboard filling was inversed, so that every nozzle, which contained Cy3-labeled oligonucleotides in the previous step is now filled with buffer solution. In the fluorescence reader, no remains of former filling should be detectable.

Oligonucleotide microarray printing buffers were optimized to the microfluidic optimum of the TopSpot printing system, as well as to the biochemical requirements of coupling efficiency of oligonucleotides on different microarray slides.

For the microfluidic optimization the stroboscopic camera was used to record droplet dispensing, flight and impact of different printing media (Table 1). For the precise analysis of the recorded droplets the image processing system NeuroCheck® was used. A programmed measurement process was developed to measure the radius of dispensed spots on the flight for a correct evaluation of the spots. Thus the spot volume can be determined by geometric outline analysis of the dispensed spots.

The limited resolution of the used camera and the arrangement of the nozzles on the printhead does not allow the evaluation of all 24 nozzles at the same time. To observe all 24 droplets in one step we used a fixed camera system, mounted on the TopSpot printer device. Each printed 24 spot array was photographed automatically directly after the print. Additionally NeuroCheck® was not only used for determination of spot diameter but also for evaluation of horizontal deflection of the spots from their correct 500 μm spacing. To achieve higher densities the spacing between the spots was lowered by interlacing prints, resulting in a 250 μm spacing of spots.

The limits of the TopSpot printhead with respect to viscosity and surface tension of printing media were tested with different concentrations and lengths of oligonucleotides and varying printing buffer solutions. Regarding biochemical aspects of microarray production we optimized the concentration and pH of different printing buffer solutions according to the coupling protocol and the microarray slide (Fig. 2). For the optimization of coupling we used commercially available microarray slides and printed 20 μM Cy5-labeled oligonucleotides in different printing buffer solutions. The printing concentration of 20 μM is a commonly used concentration in the field of oligonucleotide microarrays production and was kept constant during this evaluation. The optimized buffers were used to print microarrays with 1 μM oligonucleotides and the optima were compared to the results of 20 μM oligonucleotides.

The following coupling protocols for the immobilization of oligonucleotide to microarray slide surfaces were performed, optimized and compared to each other:

a. Water bath protocol: 2 h incubation in a humid box at 50 °C (humidity approx. 100%)

b. Oven protocol: 30 min incubation in an oven at 80 °C (humidity not set)

c. UV coupling protocol: 600 mJ in a humid box in the UV-StrataLinker (humidity approx. 60%)

Each protocol was followed by a slide washing procedure to remove unbound oligonucleotides: 2 × 5 min in 2 × SSC 0.1% SDS, 2 × 5 min in $H_2O_{deion}$ and drying with $N_2$. To avoid smearing of spots during the washing procedure we established a dip-washing device (Fig. 3) for reproducible washing of microarrays after the coupling protocol. Main criteria of the evaluation were the amount and reproducibility of fluorescence signal on different microarray slides with the different coupling protocols.

**Table 1** Concentrations and pH values of printing buffers used for the optimization of droplet dispensing and biochemical coupling efficiency on different microarray slides

| a | | | | | |
|---|---|---|---|---|---|
| **Printing buffer** | **pH range** | | | | |
| Sodium phosphate (0.1 M–0.5 M) | 6.0 | 6.8 | 7.4 | 8.0 | |
| PBS (0.1 M–0.5 M) | 6.0 | 6.8 | 7.4 | 7.8 | |
| SSC (0.1 M–0.5 M) | 6.0 | 6.5 | 7.0 | 7.6 | |
| Borat (0.1 M–0.5 M) | 7.7 | 8.0 | 8.5 | 9.0 | |
| Borat (0.1 M–0.5 M) | 9.2 | 9.6 | 10.0 | 10.4 | |
| Sodium carbonate (0.1 M–0.5 M) | 9.2 | 9.6 | 10.0 | 10.4 | |
| **b** | | | | | |
| × M betaine 0.5 × −3.0 × SSC (pH 7) | 1.5 | 1.0 | 0.5 | 0.4 | 0.3 |
| × % DMSO | 50 | 40 | 30 | 20 | |
| × % DMSO | 15 | 10 | 5 | 2.5 | |

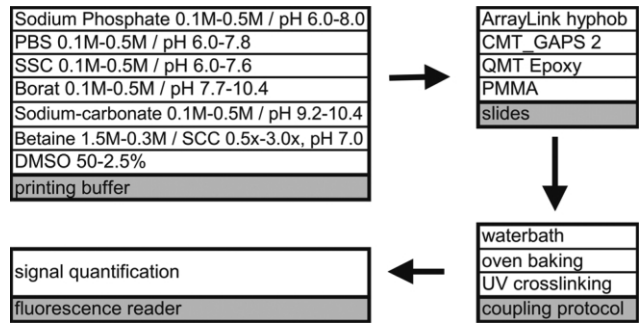


**Fig. 2** Scheme of experimental setup: fluorescence labeled oligonucleotides were solved in seven printing buffers in different concentrations and pH values were printed on four different slide types. The slides were processed according to three different coupling protocols and the coupling quantified in a fluorescence reader.

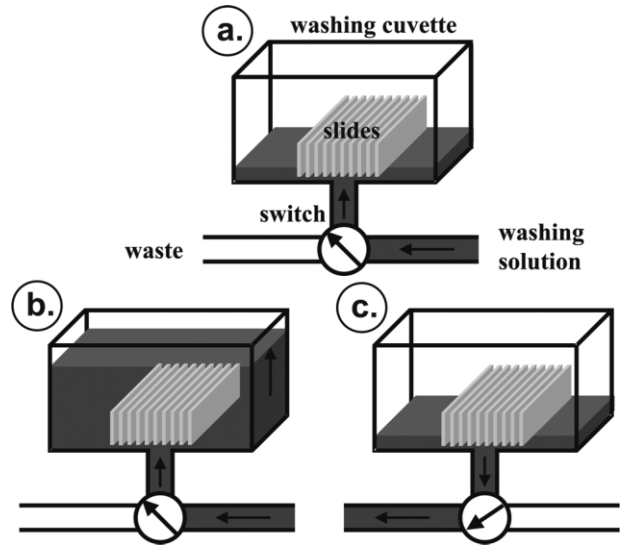


**Fig. 3** Scheme of the dip-washing device: ***a.*** slides are placed into a washing cuvette, washing solution flows in from the bottom with a constant low flow rate, ***b.*** slides are incubated in the washing solution for 5 min, followed by ***c.*** draining of used washing solution.

Finally, oligonucleotide microarrays were produced using the TopSpot technology. We used the optimized printing buffer for fabrication of oligonucleotide microarrays. With the performed antisense hybridization experiments we verified the acquired optima in a oligonucleotide microarray approach. Eight different oligonucleotides were printed in triplicates on ArrayLink™ hyphob microarray slides and coupled with the UV protocol followed by dip-washing of the slides. As target molecules five sense oligonucleotides were printed (oligonucleotides 1–5). Additionally, two positive controls were printed to ensure that the coupling (Cy5-labeled oligonucleotide) and the coloration step (biotin labeled oligonucleotide) on the microarray slide were successful. As a negative control a random oligonucleotide was printed, which had no corresponding partner in the hybridization solution. The hybridization mix was prepared containing the five biotin labeled antisense oligonucleotides (25 nM) in hybridization buffer. The DNA was denatured for 5 min at 80 °C on a heating block, followed by 5 min incubation on ice to improve subsequent DNA hybridization. 65 μL of the hybridization mix were pipetted onto the microarray and incubated for 15 min at 60 °C. Afterwards the slides were washed two times in 2 × SSC 0.1% SDS at 40 °C for 10 min, quickly rinsed in 0.5 × SSC and dried with $N_2$. For detection of the hybridization event in a specific spot the microarray was incubated for 30 min with 20 μL of coloration solution containing 5 μg $mL^{-1}$ Streptavidin-Cy5 and blocking solution to minimize unspecific binding events. Finally the slides were washed 3 × 5 min in 4 × SSC 0.2% Tween20, quickly rinsed in $H_2O_{deion}$ and dried with $N_2$. The binding of antisense oligonucleotides in a specific spot was detected and analyzed using the fluorescence reader.

## Results and discussion

Extensive experiments using the printhead for printing checkerboard arrays of labeled oligonucleotides and buffer solution showed the total isolation of the different microchannels, nozzles and dispensed droplets. No cross-talk was detectable between the 500 μm spaced nozzles even after thousands of printed arrays (Fig. 4). For the re-usage of printheads the experiments showed that after washing and re-using of printheads no carry-over of formerly printed media was detectable. The experiments ascertain carry-over and cross-talk free printing of oligonucleotide microarrays and in contrast to reloading pin printing techniques a prime critical point of microarray production is solved, leading to high quality and high-throughput microarray fabrication.

With the automated image processing system used we measured 250 droplet volumes per printing buffer on the flight (Fig. 5). Based on this we found that the diameter of dispensed droplets is highly dependent on characteristics of printing fluid, mainly viscosity and surface tension. In all dilution series the volume increased with increasing viscosity and at the same time with lower surface tension (Fig. 5). Higher viscosity leads to higher friction between the fluid layers so that more fluid gets pushed out of the fluid meniscus. Assisted by the surface tension of the printing fluid, a small filament of liquid is formed right before the droplet tears off. Lower surface tension delays the formation of a droplet resulting in a higher droplet volume. Using 1 μM oligonucleotides in different printing buffer solutions the reproducibility of droplet diameter within one single nozzle was better than 1%, independent of printing buffer used (Fig. 6). A comparison of different single nozzle records of all 24 different nozzles showed a CV better than 1.5%.

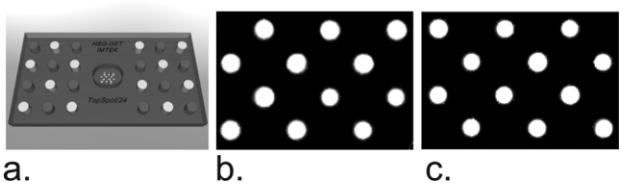


**Fig. 4** Carry-over test: ***a.*** The printhead was filled with Cy3-labeled oligonucleotides and printing buffer like a checkerboard. ***b.*** The printed microarrays were evaluated in a fluorescence reader. ***c.*** Afterwards the printheads were washed, refilled with the inversed checkerboard filling and printed. Only the spots with Cy3-oligonucleotides are visible, that means neither cross-contamination nor carry-over occurs.

A stable and reproducible droplet ejection was mainly achieved by improvements in the homogeneity of the nozzle surface coating.[9] For example, one of the main reasons for the 0.5% difference between dispensed volume for all 24 nozzles is the hydrophobic coating of the nozzle surface. Tiny differences in the homogeneity of the hydrophobic layer have significant influence on the droplet release process. In that case, during the droplet ejection process deficient coated areas around a nozzle get coated with printing solution, leading to a bigger tear-off edge and as a consequence to larger droplet volumes. Of minor importance are variations in the manufacturing of the nozzles due to the very high precision of the etching process used.

Of importance for stable and reproducible droplet ejection are the improvements in the printhead cleaning procedure, especially the nozzle surface. A clean nozzle surface avoids deflection of the droplets during the ejection process and leads to high reproducibility. The optimized protocol is described in the experimental setup. Additionally, filtering of the printing buffer solutions using a 0.2 μm filter avoids clogging of the printhead nozzles. Controlled, stable environmental conditions during printing (40–60% relative humidity) also reduce salt out effects in the printhead.

The pictures of printed arrays, made by the mounted camera, verified the results of the stroboscopic camera. The oligonucleotide spots showed CVs of droplet diameter below 1.5% per printed array (intra-array CV). Comparison of all printed spots per array with all spots of the subsequently printed arrays showed CVs below 1.6% (inter-array CV), including the errors of environmental influences and especially microarray slide inhomogeneities. Since hundreds of spots were evaluated this also showed the high reproducibility of the whole printing process.

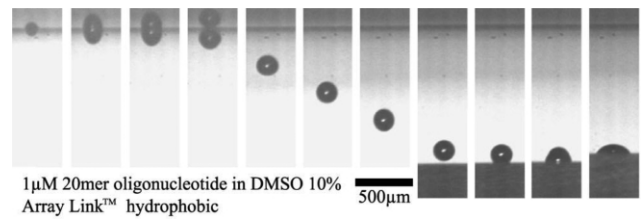


**Fig. 5** The printing process from the nozzle of the printhead down to the microarray slide. The droplet dispensing process is highly stable and reproducible with all tested printing media.

| 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 | 3xSSC xM 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 | 3xSSC 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. 6** Table of printing buffer solutions used and the corresponding CVs of droplet diameters on the flight using optimal printing conditions. CVs with respect to 250 droplet ejections.

Also, the highly correct positional accuracy of microarrays spotted with the TopSpot printing device could be shown. The spot positions of a sequence of printed arrays were measured. The distance between nozzles and slide surface was 800 μm. The deflection of spots was less than 10 μm from the regular 500 μm grid. Higher spot densities on the microarray slides were generated successfully by printing between the spots of a printed array, so that even with a 500 μm nozzle spacing of the printhead a 250 μm spacing of spots was achieved (Fig. 7).

In the experiments it was possible to print reproducibly print solutions with up to 1% (v/v) of surfactant solution (NP40 substitute). This represents a surface tension of approximately 26 mN $m^{-1}$. Lower surface tension led to leakage of fluid from the nozzles, wetting of nozzle and with it less reproducible or completely missing droplet dispensing. Experiments with high viscous fluids showed that up to 30% (v/v) glycerol/water mixture (viscosity = 1.9 mPa s) was printable. Higher concentrations led to no droplet ejection because of the high fluidic resistance of these high viscous solutions.

Reconsidering the measured droplet diameter (Fig. 6) of different printing media, the volume of dispensed droplets is highly dependent on properties of printing media, mainly viscosity and surface tension. To achieve satellite-free droplet dispensing of all printing media the duration and amplitude of pressure pulse was adjusted to the specific printing media. Using the droplet diameter of different printing media used the calculated volume of dispensed droplets ranged from 1.0 nL (for deionized water) to 1.5 nL (for 50% v/v DMSO). In all dilution series of printing media the droplet volume increased with increasing viscosity and lower surface tension. Nevertheless, if the printing buffer is fixed the droplet volume is well defined and homogenous over all nozzles. For microarray production usually a uniform printing buffer is used to solve the different oligonucleotides, thus TopSpot can be regarded as a robust and reliable printing technology.

With the very good microfluidical printing performance of this printhead we were able to optimize also the biochemical aspects of oligonucleotide microarray production. Cy5-labeled oligonucleotides were used as printing media to optimize the printing buffer concentration and pH with respect to the used coupling protocol and microarray slide type (Fig. 8). Compared to 20 μM, using 1 μM Cy5-labeled oligonucleotide led to similar printing buffer concentration and pH value optima. However, in contrast to 20 μM oligonucleotide spots, the 1 μM spots tend to form a very inhomogeneous fluorescence signal with no or low fluorescence signal in the centre of the spot and high signal at the outline of the spot, the well-known and unwanted donut effect. Even with constant environmental conditions, these drying effects were very dominant and constrained proper evaluation of the fluorescence signal.

During the manual slide washing procedure, smearing of spots occurred very often. The smearing always happened in the opposite direction to the slide dipping into the washing cuvette. Therefore, we assumed that this is an effect of too fast and uncontrollable manual dipping of slides into the washing solution. With the reported dip-washing device from Fig. 3 we were able to avoid reliable smearing of spots during the washing procedure (Fig. 9). The problem was solved by continuously wetting the slides very slowly and steadily, so that the unbound molecules of the spots become solved immediately in the washing solution. No overlap or smearing of the unbound molecules around the spots occurs. In this way, we have been able to wash microarrays after the coupling protocol more reliably and therefore obtain more reproducible results.

In extensive experiments the three most commonly used coupling protocols were compared to determine the one best suited for oligonucleotide microarray printing, using the highly parallel TopSpot dispensing system. Independent of printing buffer used the highest Cy5 signals were measured using the UV cross-linking protocol. As a consequence the best Cy5 signals of a printing buffer constitution (concentration, pH) was found on microarray slides processed using the UV protocol. Each printing buffer has an optimal constitution, when looking at the Cy5 signal intensity of the printed array, but there were big differences between the tested microarray slide types (Fig. 8). For the determination of the overall best suited printing buffer the signal to noise ratio was not only taken into account but also stable and reproducible results are at least of same importance. The formation of donut spots arising from the printing buffer has to be eliminated to achieve homogenous and therefore reproducible spot results. Taking this into account we

| optimal printbuffer | ArrayLink hyphob | CMT-GAPS 2 | QMT Epoxy | PMMA |
|---|---|---|---|---|
| Sodium Phosphate | 0.5M pH 7.4 | 0.5M pH 8.0 | 0.5M pH 7.4 | 0.5M pH 7.4 |
| PBS | 0.5M pH no influence | 0.1M pH no influence | 0.5M pH no influence | 0.5M pH no influence |
| SSC | 0.5M pH 6.5 | 0.1M pH 6.5 | 0.5M pH 6.5 | 0.5M pH 6.5 |
| Borat | 0.1M pH 9.2 | 0.1M pH 7.7 | 0.2M pH 10.0 | forming donuts |
| Sodium-carbonate | 0.5M pH no influence | 0.3M pH 9.2 | 0.5M pH 10.0 | 0.4M pH 10.0 |
| Betaine / SSC | 0.3M 1.5x | 1.5M 0.5x | 0.2M 1.5x | 1.5M 3.0x |

**Fig. 8** Overview of the optimum conditions for oligonucleotide (20 μM) coupling on the different slide types tested. Indicated are the printing buffers which led to the highest fluorescence signal per slide with the UV cross-linking protocol. Absolute highest signal to noise ratios were observed with 0.5 M sodium phosphate buffer pH 8, for all slide types used.

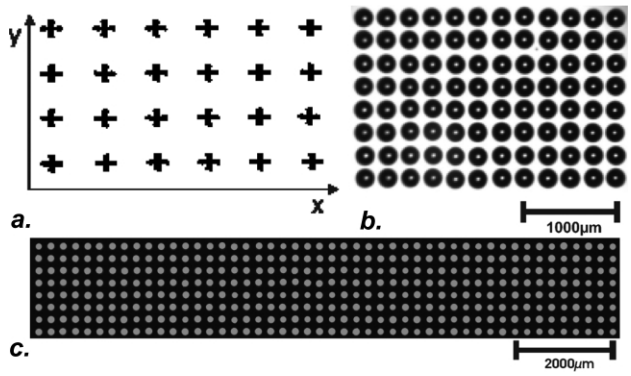


**Fig. 7** ***a.*** Overlaying of the *xy*-coordinates of 16 printed arrays measured by NeuroCheck®. Each cross indicates the spot centre. The cross width indicates the spot diameter. The deflection of all spots is less than 10 μm from a regular 500 μm grid. ***b.*** Camera picture of a 250 μm spot spacing by interlacing printing using four times a 24 nozzle printhead. ***c.*** Fluorescence reader image of 250 μm spot spacing by interlacing printing using four times a 96 nozzle printhead.

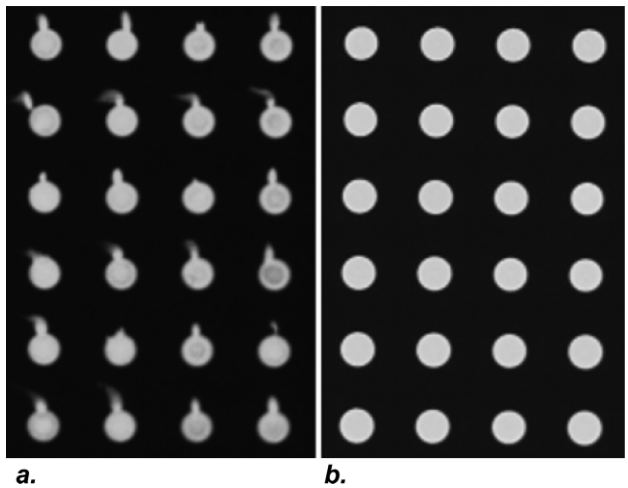


**Fig. 9** ***a.*** Smearing of spots during the slide washing procedure by manually dipping. ***b.*** No smearing of spots by microarrays processed with the dip-washing device.

found 0.5 M sodium phosphate at pH 8 to be the best suited printing buffer for oligonucleotide printing for all microarray slide types used.

Optimized sodium phosphate buffer was used to verify the data of the coupling optimization in an hybridization experiment with anti-sense oligonucleotides of the printed microarray spots. The experiments showed the same results as in the coupling optimization experiments (Fig. 10). For gene expression experiments it will be necessary to study the influence of UV cross-linking on the hybridization efficiency of cDNA molecules.

## Conclusion

We have optimized buffers and protocols for the TopSpot printing system when used for oligonucleotide microarray production. In extensive experiments with oligonucleotides, no carry-over and no cross-talk was detectable, enabling high quality production of microarrays. The printing buffer was optimized with respect to the two major aspects of microarray production: microfluidical optimum for droplet dispensing and biochemical coupling efficiency on different commercially available microarray slides. CVs of generated spot diameters were below 1% within one single dispensing nozzle and below 1.5% within all 24 different nozzles of used printhead for all printing buffers used. Optimized printing buffer concentrations and coupling protocols for oligonucleotide microarrays were found, adapted to the different commercial microarray slides commonly in use. Independently of printing buffer used, the UV cross-linking protocol led to the highest signal to noise ratios. The dip-washing device reliably prevented the smearing of spots during the slide washing procedure and led to high quality spots for microarray analysis in the fluorescence reader.

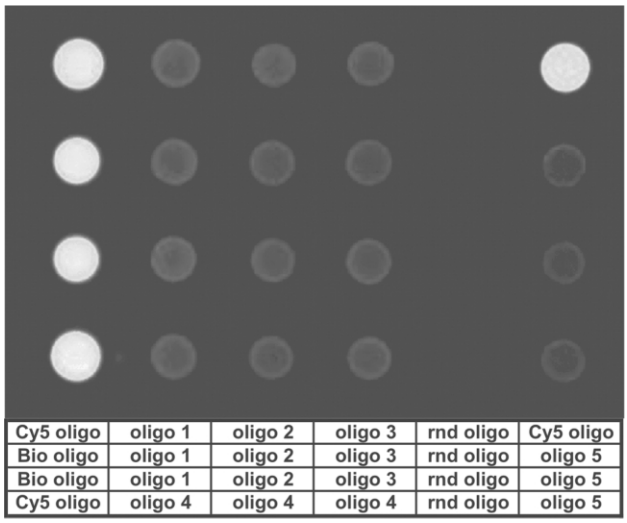


**Fig. 10** Antisense hybridisation experiment: Eight different oligonucleotides were printed in the shown arrangement: Cy5 and biotin labeled oligonucleotides as experimental positive controls and a random oligonucleotide as negative control; five different sense-oligonucleotides, which were detected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

## Outlook

Future work will extend on these results towards other biological molecules of higher complexity and molecular weight such as proteins and even cells. Higher viscosities and the varied biochemical properties will influence the microarray production. The hydrophobic coating of the nozzle area has to be improved to achieve a stable and reproducible droplet ejection of printing media with very sticky properties. Printheads with different nozzle diameters will be fabricated to achieve lower droplet volumes, *e.g.* for printing on non-standard microarray slides, especially very hydrophilic substrates, without mixing of spots. In the presented system and based on one liquid, the droplet volume is not modifiable through pressure modification. In contrast to the presented pressure driven system, ejection of variable droplet volumes will be possible in the future with the further development of the TopSpot system using a direct displacement actuation principle described in ref. 10. Evaluation of this direct displacement system with oligonucleotides and other biological molecules will be peformed in the future.

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