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Controlling Microarray Spot Morphology with Polymer Lift-off Arrays

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Abstract

Biological arrays are hindered by the lack of uniformity in the deposition of biomaterials. Efforts aimed at improving this deposition have focused on altering the composition of the solution or the tool used to deposit the material. However, little attention has been paid to controlling material deposition by constraining the physical and chemical topography of the surface. Here we present the use of a hybrid hydrophilic/hydrophobic micropatterned surface to direct the deposition of spotted DNA on microarrays. These polymer "lift-off" arrays combine the hydrophobic surface properties of di-para-xylylene (Parylene) with photolithographically etched hydrophilic openings within the polymer. We show that the flow pattern of solutes on these substrates favors the concentration of dissolved material into the mesoscopic openings underlying the printed spot, resulting in significantly improved uniformity of deposition. Moreover, the micropatterned surface allows for increased replication of spotted materials. Finally, these polymer lift-off arrays display reduced array-to-array variation, improving the reproducibility of data acquisition. We envision that these novel substrates can be generalized to produce more uniform arrays of other patterned biomaterials.

Introduction

While microarrays have become ubiquitous in biological research, microarray data is widely known to be noisy and imprecise[1]. As the data obtained from DNA microarray-based experiments consists of measurements of thousands of individual genes, confidence in array data is critically dependent upon the printed spot morphology. However, the dynamics that underlie the drying of complex solutions (*e.g.* DNA contained within the printing solution) are not completely understood. As a result, variation in spot quality occurs both within and between arrays, affecting the reliability of data obtained from any given array and the ability to reproduce these measurements between arrays[2]. To compensate for the lack of spot uniformity, image analysis programs average pixel intensities across a given spot[3]. Also, a number of printing solutions have been introduced to control the drying rate of individual spots[4, 5]. Because these strategies have had only limited success, the development of novel technologies to produce more uniform spots would have a significant impact on the quality and precision of data from microarray experiments[6, 7].

There is limited understanding of the forces that drive drying patterns on microarrays. Prior studies examining the drying of simple, dilute aqueous solutions have shown that a droplet placed on a hydrophilic surface results in a pinned contact-line at the periphery of the area

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where the solution and surface meet[8]. As evaporation proceeds, the height of the droplet is reduced, but the contact area remains constant. This establishes a capillary flow at the surface which is driven towards the periphery to maintain the original radius of the spot. Dissolved solids are carried by this capillary flow to the periphery, producing the characteristic "coffee ring" pattern upon drying. Although microarray printing buffers are often more complex than the simple solution described in the "coffee ring" studies[8], we hypothesized that this capillary flow could explain the morphologically similar "donut" pattern of hybridization seen in many microarray experiments. As most microarrays are printed on hydrophilic, amine-functionalized surfaces, we theorized that evaporation of the spotted DNA droplets would drive dissolved nucleic acids to the periphery due to the pinned contact-line. Upon hybridization this would result in increased signal at the periphery of the spot and decreased signal in the center. Data from such "donut" shaped spots would rely on pixel averaging to compensate for the lack of uniformity of the original printed material.

To ameliorate the uneven DNA deposition problem and improve microarray reproducibility and reliability, we developed a hybrid substrate using photolithographically patterned surfaces. This substrate combines a hydrophobic polymeric surface with patterned openings that expose an amine-functionalized hydrophilic surface beneath the polymer. DNA spotted on the mixed surface would be subject to distinct capillary flow patterns during drying. In the simplest case, the portion of the droplet in contact with the hydrophobic surface would experience de-pinned contact-line drying, and an inward capillary flow[9]. Through evaporation, the spot would shrink until it covered only the hydrophilic micro-fabricated openings, resulting in increased uniformity of deposition due to the high DNA concentration within the openings.

Here we report on the fabrication and use of these polymer lift-off microarray substrates to address problems associated with microarray-based experiments. We show that the hybrid hydrophobic/hydrophilic nature of the arrays improves DNA deposition, spot uniformity, replication, and reproducibility between microarray experiments as compared with conventional substrates. We also show inward convective flow occurs on these hybrid substrates whereas the opposite behavior is observed on simple, hydrophilic surfaces. These polymer lift-off arrays represent a novel approach towards addressing the common "coffee ring" issue observed in drying of dilute analytes, and could be used to improve deposition of other types of arrays.

Experimental section

Lift-off microarray substrate fabrication

Polymer lift-off microarray substrates were fabricated on 1×3 inch, 1mm thick Pyrex glass slides (ESCO Products) as previously reported[10, 11]. Substrates were cleaned using piranha solution (1:3 H_2SO_4:H_2O_2), followed by a short oxygen plasma clean. Polyethylene glycol (1%, MW 200, Sigma-Aldrich) was spun on the substrates to reduce nonspecific binding. A thin (300nm) conformal coating of *di*-para-xylylene (Parylene, Specialty Coating Systems) was vapor deposited in a PDS-2010 Labcoater 2 Parylene deposition system (Specialty Coating Systems). Shipley 1827 positive photoresist was spun on the substrates to a thickness of 2.7μm and baked at 90°C. Photolithography was performed using an HTG System III-HR Contact Aligner. The exposed photoresist was developed in AZ300-MIF developer for 60 seconds, followed by a deionized water rinse and then dried under nitrogen. Exposed regions of the Parylene film were etched in a reactive oxygen ion plasma chamber, and residual photoresist was removed by washing successively with acetone and isopropanol. Substrates were functionalized for DNA adsorption with 3-aminopropyl-trimethoxysilane (APTMS, Sigma) in a Molecular Vapor Deposition-100 tool (Applied MicroStructures). Control slides were functionalized with APTMS in parallel.

DNA spot printing and hybridization

DNA was spotted using a custom-built microarrayer and 946 quill-pins (Telechem International). Ambient humidity was maintained between 55–60%. A positive control DNA, which has been shown to be an effective measure of hybridization sensitivity, was printed on all arrays[12]. To print the DNA we used a buffer containing 1× SSC/0.005% sarkosyl (subsequently referred to as printing buffer). Slides were post-processed using 1-methyl-2-pyrollidinone[13]. During the washing phase of the post-processing, the polymer was peeled away and discarded. Slides were briefly washed successively in three washes of 0.1× SSC, water, and 200 proof ethanol, and spun dry. Labeled target was generated using the indirect labeling method[14] and Trizol-purified total RNA from mouse liver. Slides were hybridized in a HS400 hybridization machine (Tecan), although conventional hybridization coverslips can also be used. Slides were pre-blocked in 3× SSC/0.1% SDS /1% BSA, and hybridized at 45° C in 3× SSC/0.1%SDS/50% formamide/20 mM Tris 8.0/0.1 mg/ml BSA/0.1 ug/ml salmon-sperm DNA for 18 hours. Unbound fluorescent target was removed by an initial wash in 6× SSC/0.005% Triton X-100 followed by three washes in 0.1× SSC/0.005% Triton X-100 and the slides dried under a stream of Argon gas. Slides were scanned using an Axon 4000B scanner (Molecular Devices).

Video recording and fluorescence microscopy

Hybridized slides were imaged with a LSM510 Meta laser scanning microscope (Zeiss), with 532 nm laser excitation line, and a Plan-Neofluar 20x/0.5NA objective. The drying profile of spotted droplets was observed with a camera set to record side views of the droplet. Videos were captured with a Panasonic DVD recorder and frames were extracted using VirtualDub MPEG software[15]. Droplets of different buffers containing fluorescent latex spheres (10^9 spheres/mL; Molecular Probes) were examined for flow patterns using an Olympus IX71 inverted microscope and a PLAPON 10x/0.4NA objective (NY/NJ Scientific). Videos were recorded as the spheres moved in the drying solution using a Cascade 512 cooled CCD camera (Roper Scientific/Biovision).

Spot morphology analysis

Fluorescence microscopy images were analyzed using a custom Matlab (The Mathworks) program based on Otsu's thresholding algorithm[16]. Once a threshold grayscale level was calculated, the raw image was separated into signal and background areas. A two pixel exclusion region between signal and background was generated to eliminate pixels that average signal and background values due to the image resolution. The program then calculated the mean, median, and standard deviation of pixel intensities for signal and background regions for every printed spot analyzed, and generated representative images for each processing step. For comparative measurement of uniformity and reproducibility we used the percentage standard deviation of the signal values obtained for the analyzed spots. The percentage standard deviation (PSD) was calculated as: PSD = (signal intensity standard deviation)/(mean signal fluorescence intensity)×100. Comparison of the PSD values was used to assess the uniformity of spot morphology via confocal fluorescence microscopy and microarray scanner images. The PSD was also used to compare reproducibility of spot morphology within and across arrays.

Results and Discussion

Generation of polymer substrates and application to conventional microarray technology

There are multiple microarray platforms currently in use. The most flexible and frequently employed design uses quill-pins to spot a variety of biological materials, including proteins, oligonucleotides, cDNAs, genomic DNA, and others[17]. Because the benefits of the proposed approach can be extended to these applications, we designed our hybrid substrates for general

use with conventional microarray equipment. We used glass as the base for the polymer lift-off arrays because glass is easily functionalized with silane chemistry and 1×3 inch slides can be mounted on most microarraying equipment. For the hydrophobic aspect of the polymer lift-off arrays, we chose Parylene, a polymer we have previously shown can be photolithographically patterned, is non-reactive, and compatible with a variety of biological molecules[10, 11]. We fabricated the polymer lift-off arrays as described in the Experimental Section and depicted in Figure 1. We confirmed that the Parylene surface was not amine-functionalized by contact angle measurements (data not shown). DNA was then spotted on lift-off arrays and on control, APTMS-coated slides. After printing, the Parylene was mechanically "lifted-off" from the polymer lift-off arrays, leaving behind the patterned, surface-bound DNA. Then, both sets of arrays were hybridized and processed in parallel using standard protocols[18].

Confocal microscopy demonstrates increased uniformity of deposition on lift-off arrays

An advantage of using the lift-off arrays is that the patterning mask can be designed to produce openings of various sizes in the polymer. For our application, the openings had to be small enough so that drying was largely governed by the hydrophobic surface while the capillary flow within the hydrophilic openings was minimized. However, the diameter required to achieve this effect could not be predicted *a priori*. Spot diameters of DNA printed on standard glass slides typically range from 80–150μm; we generated polymer lift-off arrays with 10–40μm diameter openings. An unlabeled, positive control DNA[12] was then printed on these substrates and hybridized with Cy3-labeled target made from total mouse liver RNA. Confocal microscopy was used to assess the uniformity of individual spots. It was observed that for openings with diameters larger than 20μm (data not shown), DNA dried in a ring-like pattern similar to that of DNA printed on control substrates (Figs. 2a, 2e). For 20μm openings, the relative thickness of the ring increased, and more of the opening was filled with the deposited DNA (Figs. 2b and 2f). For 10μm openings, the coffee-ring pattern was significantly reduced, producing more uniform DNA distribution within the opening (Figs. 2c, 2g). While even smaller openings would further improve deposition, most commercial microarray scanners have a maximum resolution of 5μm/pixel. Thus, insufficient pixel information would be obtained with smaller diameters. Because one of our goals is to make the polymer lift-off arrays compatible with current microarray technology, we maintained the opening diameter at 10μm for the experiments described below.

To quantitatively assess the improvement in uniformity of deposition, the variation of the fluorescence intensity across each printed spot was calculated. Discrimination between signal and background pixel populations was performed with a Matlab program based on Otsu's thresholding algorithm[16] as described in the Experimental Section. After the signal and background pixels were identified, the mean and standard deviation from each population was calculated. The percentage standard deviation (PSD) was used as a comparative measure of the uniformity of deposition because the standard deviation is a measure of the spread in fluorescence intensity values across each spot. By calculating the fraction of the mean signal intensity represented by the standard deviation, we ensured that the observed variations did not stem from differences in the amount of adsorbed material. Figure 2d shows that the PSD calculated from confocal data for spots printed on control slides is larger than that of spots generated by lift-off arrays with 10μm openings. The visual and quantitative data obtained from the experiments show that DNA printed and hybridized on polymer lift-off arrays is distributed more uniformly than on control slides.

Replicate sub-spots yield highly uniform microarray data

A benefit of using 10μm wide openings within the polymer is that multiple signals (sub-spots) are obtained per printed spot. Each one of these highly uniform replicate sub-spots provides

precise data, which represents an obvious advantage over conventional arrays where a single spot is often printed per gene due to spatial constraints. However, because of the relatively low resolution of commercial scanners, each sub-spot on our polymer lift-off array only generates a small fraction of the pixel information of the average, 100μm spot. We reasoned that using a commercial scanner would represent a stringent test of the utility of these sub-spots, as even small variations in the polymer lift-off array data would have a significant effect on the standard deviation. It would also determine whether existing scanners could be used to analyze data from the lift-off arrays.

After hybridization and scanning on an Axon scanner (set to 5μm/pixel resolution), each of the 9–12 sub-spots generated per printed spot were taken as within-array replicates. The mean and standard deviation of the fluorescence intensity of these replicates was calculated with the algorithm described above. In the simplest case, we can compare the PSD for the population of 9–12 sub-spots generated per printed spot on the polymer lift-off array against the single printed spot present on the control slide. However, to gain more insight into the general behavior of these sub-spots in providing replicate information, the control DNA was spotted four times on each substrate. Each of the four sets of sub-spots could then be compared against the corresponding spots printed on the standard slide. We repeated this experiment three times, and analyzed a total of 12 spots and 12 sub-spot sets. The PSD obtained from the sets of sub-spots generated by each printed spot on lift-off arrays was compared with the PSD for the identical spots on control slides (Fig. 3). Variation was greatly reduced for all sub-spot sets as compared to the spots printed on control slides. Thus, despite the use of a commercial scanner and the concomitant decrease in pixel information associated with the sub-spot measurements, the data obtained from the sub-spots was still significantly more precise than that obtained from spots printed on control slides. This is true whether a single spot is printed on each array or if the same DNA is printed multiple times on each array. In the first instance, the sub-spot data for any given spot (*e.g.* “spot 1”) is more uniform than that obtained from a spot printed on a control slide. In the latter case, the sub-spot data across spots (e.g. “spots 1–4”) is less variable than that obtained from a control slide.

Reproducibility of data from polymer lift-off is improved over standard arrays

Given the inherent noise in microarray data, technical replicates are often performed using the same RNA source hybridized to two arrays so that the observed measurements can be averaged across experiments. If each gene is printed once on an array, obtaining two measurements for each gene using the same RNA stock can help reduce the effects of variation in printing quality or hybridization conditions. Although we have shown that the multiple sub-spots produced on any given lift-off array can improve the precision of the acquired data from that particular slide, we next assessed if these arrays could improve data acquisition across experiments and arrays at various levels of signal intensity. We printed positive control DNA at successive, two-fold serial dilutions (100 to 0.78 ng/μl) with four replicate spots per dilution. This has been shown to be a means of normalizing data between arrays and enable comparisons of hybridization efficiency between experiments[12]. Comparisons both within and between arrays were performed by comparing measurements from the various spots. All arrays (conventional and lift-off) were processed in parallel and hybridized with an aliquot from a common stock of labeled target. The conditions were designed to be as identical as possible to produce highly similar technical replicates. After hybridization and scanning, we determined sub-spot intensity data across all arrays and dilutions in 3 independent experiments (12 slides). Sub-spot information was averaged for each dilution printed in each slide. Comparison of reproducibility across arrays was determined by averaging pixel intensity across all like arrays and calculating the standard deviation. Significantly lower standard deviation was seen in technical replicates of lift-off arrays than in control arrays (Fig. 4), arguing for better inter-array reproducibility. Thus, despite performing 12 technical replicates in a manner designed to generate data with

highly reduced standard deviations on conventional slides, lift-off arrays still produced more uniform data. While 12 replicates are unlikely to ever be performed in routine experiments, the use of lift-off arrays will improve the quality of data obtained even with just two replicates.

Lift-off polymer arrays display different flow patterns as compared with standard substrates

To determine if the increased uniformity of deposition and reproducibility reflect alterations in flow patterns of spotted droplets on our hybrid surface, droplets of printing buffer containing fluorescent latex spheres were spotted on patterned and control surfaces. This approach has been used previously to follow capillary flow in drying droplets of simple solutions[8, 19]. By tracking fluorescent sphere movement, we observed that flow in droplets deposited on control substrates was consistently directed towards the periphery of the droplet (Fig. 5a). However, flow in droplets deposited on lift-off substrates was directed towards the center of the droplet (Fig. 5b). These findings suggest that the capillary flow on lift-off substrates promotes concentration of the material at the center of the droplet while the flow on control surfaces promotes concentration at the periphery. This behavior helps to explain the observation of the reduction of the coffee-ring pattern and the enhanced uniformity in the lift-off substrates.

Despite the inward flow, we found that the drying profile of a droplet of printing buffer on the patterned slides differed from that of a solution of phosphate-buffered saline (PBS). As predicted by the model proposed by Deegan and colleagues[8], drying of the aqueous droplet (*i.e.* PBS) was not constrained by a pinned contact-line (Fig. 5d). Nevertheless, the droplet containing printing buffer showed a pinned contact-line (Fig. 5c). Thus, the observed flow behavior cannot be uniquely associated with the presence of a pinned contact-line as in the proposed model. On the other hand, the drying profiles of both PBS and printing buffer on control surfaces were the same (Figs. 5e, 5f). The observed differences in the drying profiles can be attributed to the presence of a small percentage of detergent in the printing buffer which effectively disrupts the polar interactions of the solution with the polymer substrate. It has previously been shown that the coffee-ring pattern can be suppressed by the Marangoni effect which generates additional vortices in the drying solution[20, 21]. The interactions of a complex fluid (*i.e.* printing buffer) with a mixed hydrophilic/hydrophobic surface require further modeling to help explain the observed effects. This is beyond the scope of the present paper, which aims to demonstrate the use of lift-off arrays to improve spot morphology in DNA microarrays.

Conclusions

We have shown that the polymer lift-off substrates can be easily adapted to conventional microarray technology. The increase in uniformity, replication, and reproducibility demonstrate the advantages of polymer lift-off over conventional arrays. We anticipate that these results can be applied to other biomolecule arrays[22] (*e.g.* protein, antibody, RNA) and platforms, and should be particularly valuable in creation of matrix-assisted laser desorption/ionization targets. We have shown that these benefits stem directly from the constrained deposition of DNA into targeted openings by the tailored surface properties. The use of these substrates could be extended to experiments which require controlled drying of biological materials, with potential applications such as concentration of dilute analytes[23] or particle self assembly[24].

Acknowledgments

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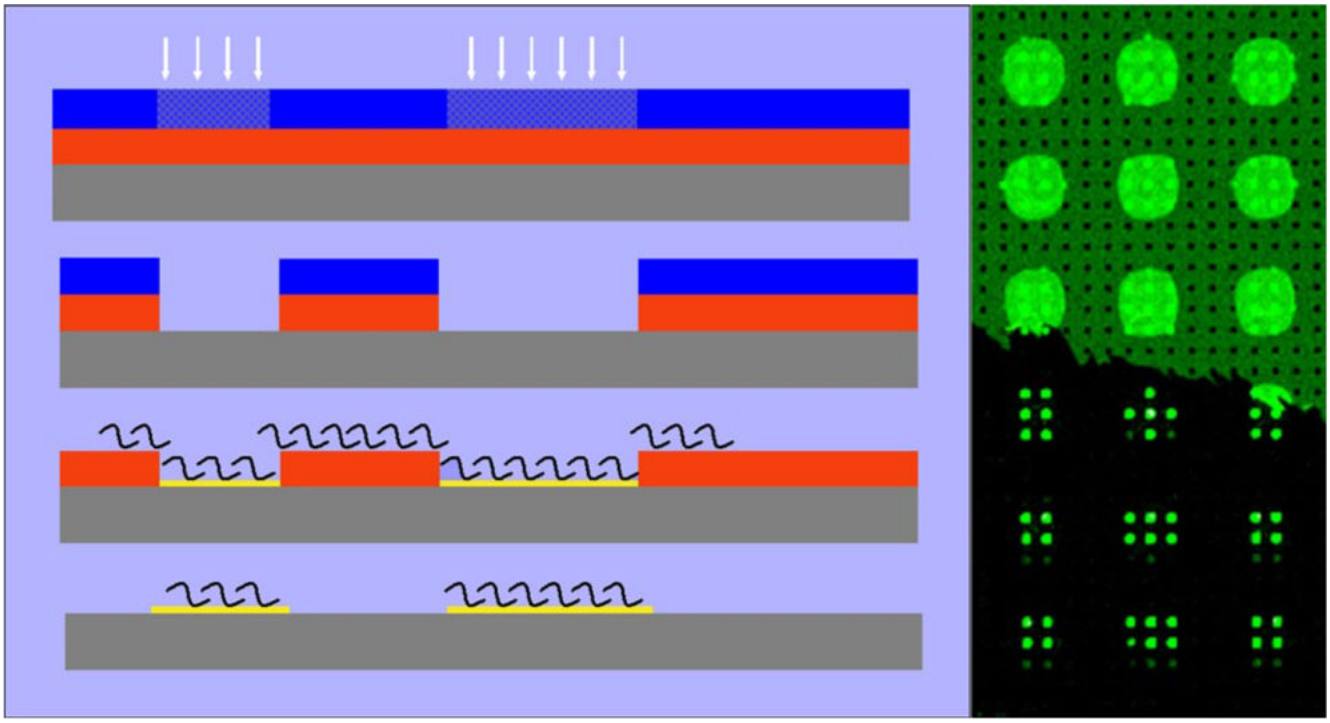

Figure 1.

Left: Fabrication of polymer lift-off arrays. A glass substrate (gray) is coated with Parylene (red). Photoresist (blue) is spun on and UV-exposed (white arrows). Exposed resist (cross-hatched area) is removed and the exposed Parylene is etched through. APTMS (yellow) is applied to the exposed glass surface, and DNA is spotted on the surface (black curves). Parylene is then peeled away, leaving behind only the patterned material (bottom). Right: 10μm opening polymer lift-off array where Parylene has been partially removed (bottom) after spot printing and drying. Note each printed spot covers multiple openings in the polymer. Each opening covered by a printed spot is considered a sub-spot for analysis purposes.

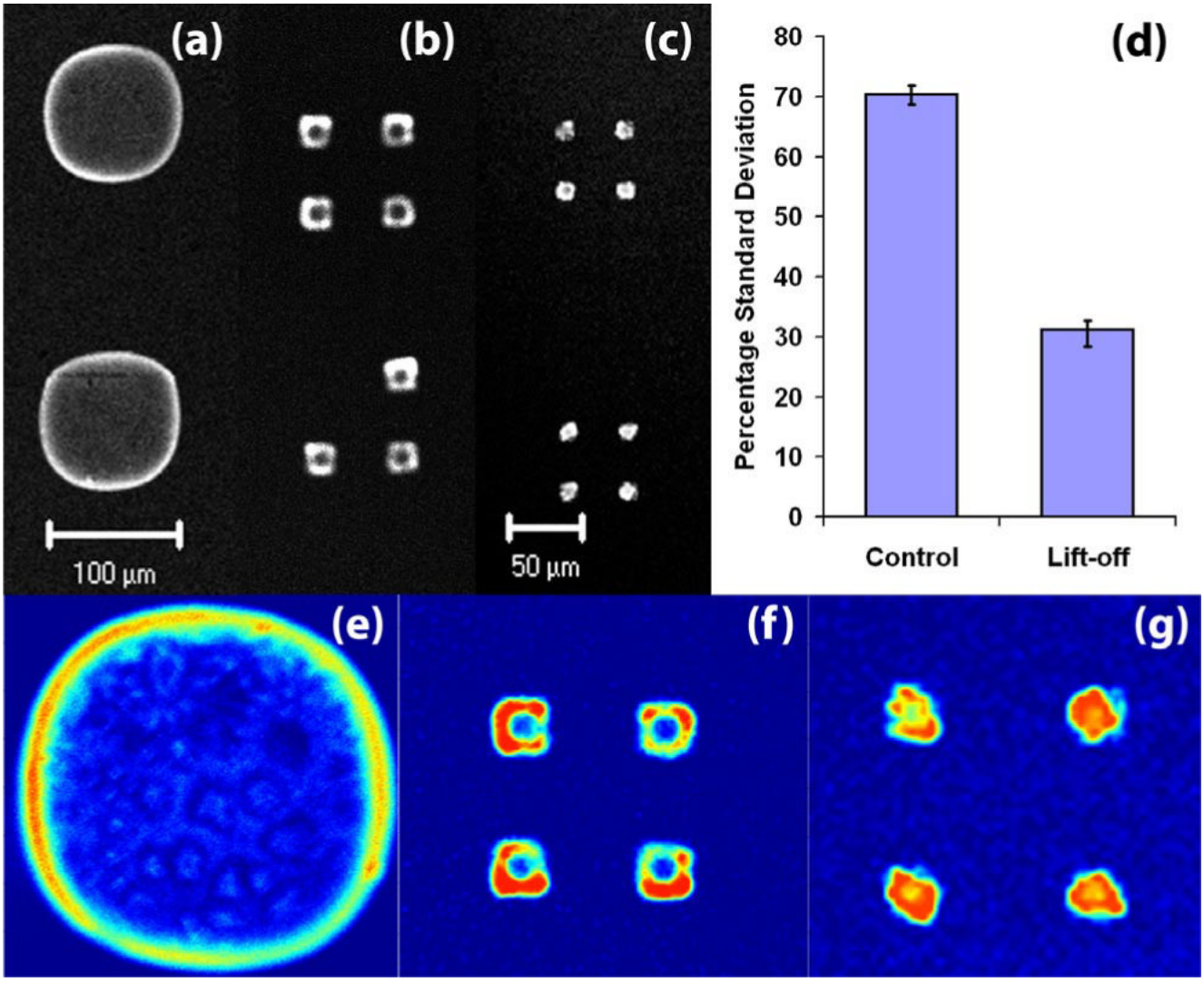

Figure 2.

Confocal imaging shows the improvement of spot uniformity after hybridization obtained with polymer lift-off arrays. (a)–(c) Raw images from control and lift-off arrays. (a) Spot printed on amine functionalized surface shows uneven coffee-ring pattern. (b)–(c) Lift off polymer arrays with 20 and 10µm openings. The relative thickness of the coffee-ring inside the opening increases as the diameter decreases, yielding the most uniform deposition at 10µm. Scale is the same for both micropatterned arrays. (d) Comparison of the percentage standard deviation of the mean fluorescence intensity for control vs. 10µm opening lift-off arrays. (e)–(g) Pseudo-color of intensity of arrays in (a)–(c) for uniformity comparison.

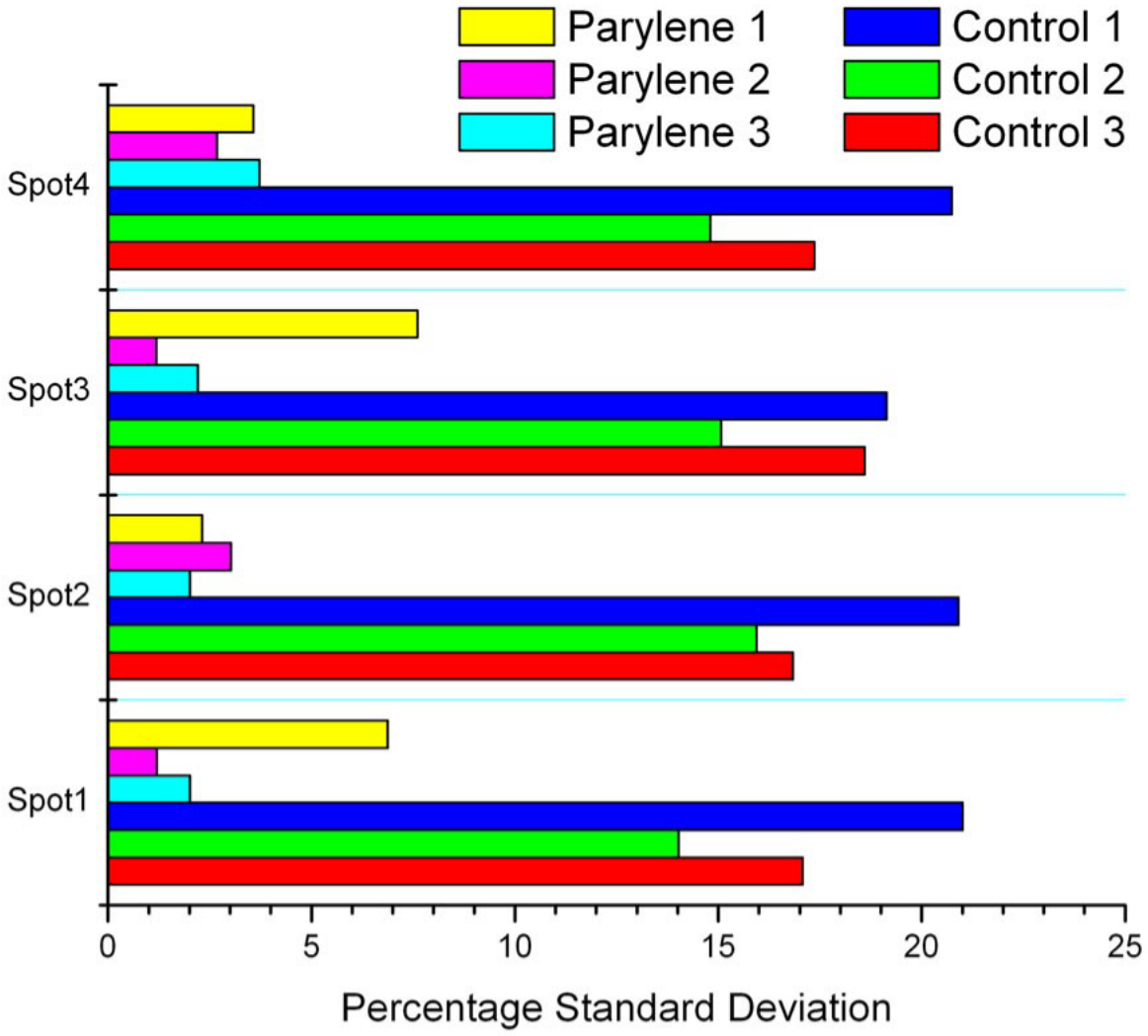

Figure 3.
Percentage standard deviation from sub-spot sets generated by individually printed spots is lower than that from conventional array spotted data. The percentage standard deviation of pixel intensities across each spot is shown for four replicate spots printed on lift-off versus control substrates (3 slide replicates).

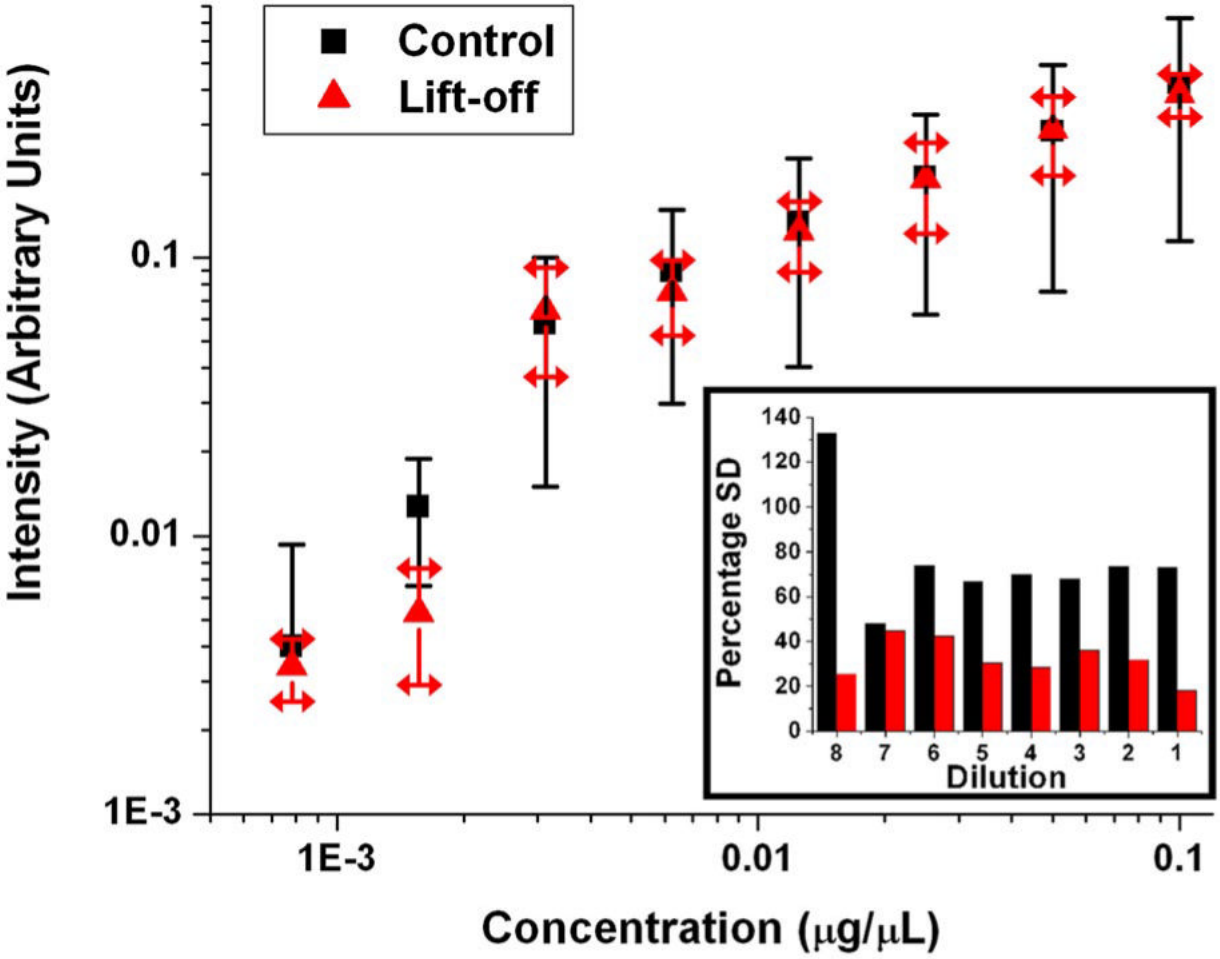

Figure 4.
Standard deviation associated with measurements of pixel intensities across arrays for successive dilutions. Inset: Standard deviation for individual dilutions represented as a percentage of the mean intensity.

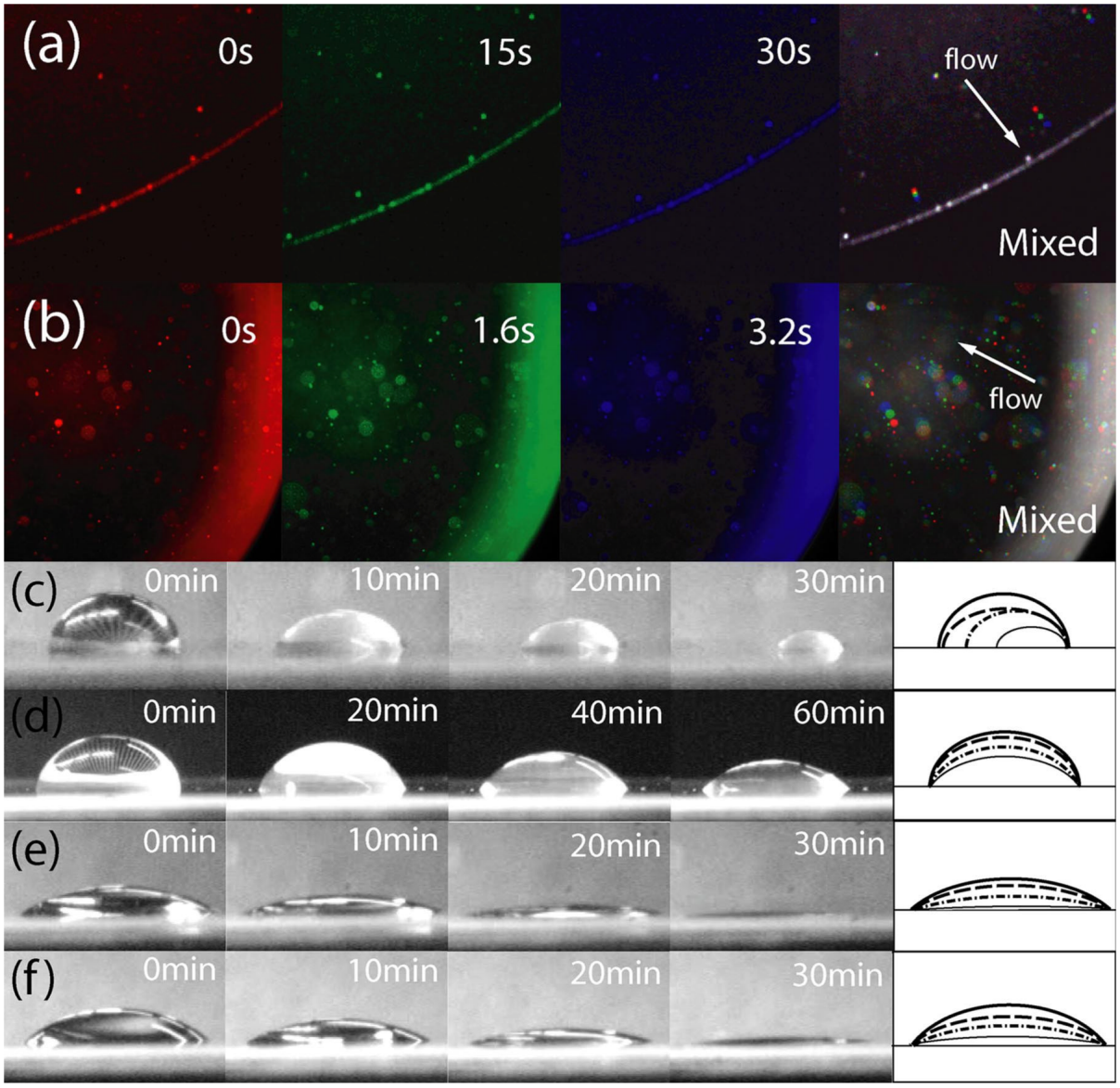

Figure 5.

The drying behavior of the printing buffer is different on lift-off substrates than on control slides. Panels a–b: Capillary flow behavior near the substrate surface for amine functionalized control slides (a), and lift-off substrates (b). Fluorescent beads were used as markers for the flow behavior. Different color images are snapshots in time extracted from recorded videos. Panels c–f: Drying droplet profile for an aqueous solution (phosphate buffered saline) and printing buffer on lift-off and control substrates. Individual images show snapshots of the recorded video as the droplet dried. Final image on each panel shows the droplet profile for each of the previous shots. Treatments: (c) aqueous solution on Parylene, (d) printing buffer on Parylene, (e) aqueous solution on control slide, and (f) printing buffer on control slide.