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High resolution epifluorescence and TOF-SIMS chemical imaging comparisons of single DNA microarray spots

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Abstract

DNA microarray assay performance is commonly compromised by spot-spot probe and signal variations as well as heterogeneity within printed microspots. Accurate metrics for captured DNA target signal rely upon uniform spot distribution of both probe and target DNA to yield reliable hybridized signal. While often presumed, this is neither easily achieved nor often proven experimentally. High resolution imaging techniques were used to determine spot heterogeneity in identical DNA array microspots comprising varied ratios of unlabeled and dye-labeled DNA probes contact printed onto commercial arraying surfaces. Epifluorescence imaging data for individual array microspots were correlated with time-of-flight secondary ion mass spectrometry (TOF-SIMS) chemical state imaging of the same spots. Epifluorescence imaging intensity distinguished varying DNA density distributed both within a given spot and from spot-to-spot. TOF-SIMS chemical analysis confirmed these heterogeneous printed DNA distributions by tracking bound Cy3 dye-, DNA base, and phosphate- specific ion fragments often correlating to fluorescence patterns within identical spots. TOF-SIMS ion fragments originating from probe DNA and Cy3 dye are enriched in microspot centers, correlating with high fluorescence intensity regions. Both TOF-SIMS and epifluorescence supports Marangoni flow effects on spot drying, with high density DNA-Cy3 located in spot centers and non-homogeneous DNA distribution within printed spots. Microspot image dimensional analysis results for DNA droplet spreading show differing DNA densities across printed spots. The study directly supports different DNA probe chemical and spatial microenvironments within spots that yield spot-spot signal variations known to affect DNA target hybridization efficiencies and kinetics. These variations critically affect probe-target duplex formation and DNA array signal generation.

Keywords

microarray; chemical imaging; fluorescence; mass spectrometry; surface analysis; nucleic acid; hybridization

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INTRODUCTION

Despite years of development, microarray-based nucleic acid diagnostics continue to be plagued with numerous bioanalytical metric issues that block clinical translation. These include a recognized lack of direct correlation between absolute array spot fluorescent intensity – the common array signal read-out – and absolute target abundance in a sample, difficulties in sensitivity and reliability in complex biological samples, and both lab-to-lab and platform-to-platform assay repeatability issues.^{1, 2} Printed probe spot heterogeneity and non-uniform immobilized DNA density result from procedures common to microarray preparation.² Significantly, immobilized DNA probe density is critical for target DNA duplex formation, affecting the resulting assay signal generation in array-based formats.^{3–6} Control of printed DNA probe density is important to the reliability of this technology but very challenging to implement and assess.⁷

Both spot-to-spot and individual spot morphological heterogeneity arise from complex interactions between DNA probe micro-droplets during printing onto substrates of highly variable interfacial properties (e.g., droplet wetting, sorption, and immobilization kinetics), as well as temperature and humidity during array fabrication that affect droplet drying kinetics.^{8,9} The issues are a complex, interrelated set of parameters involving the intrinsic physical, chemical, kinetic and thermodynamic properties of DNA at surfaces under both probe and target exposure conditions. In typical DNA probe printing to prepare microarrays, nanoliter-scale droplets of DNA solution are dispensed under ambient, non-equilibrium surface printing conditions, leading to rapid droplet evaporation (~few seconds) on surfaces. Droplet evaporation rapidly increases droplet ionic strengths and DNA concentrations, forming distinct intra-spot drying features as a consequence of mass transport-limited mixing and non-equilibrium evaporation kinetics.¹⁰

Printed DNA spot drying is complicated due to the presence of complex mixtures of solutes in printed droplets and competing effects of simultaneous “coffee stain drying” and Marangoni flow effects within printed droplets.^{11–14} Previous theoretical and experimental reports show that droplet evaporation induces non-uniform cooling along the droplet surface.^{15, 16} During evaporation, both diffusion and three-dimensional convection deposits solutes and particles in droplet centers.¹⁷ The Marangoni effect is influenced by temperature-dependent solvent surface tension, viscosity and fluid dynamics of the sessile drop.^{18–20} Convective transport and circulation together yield local variations in droplet solute concentration, surface energies, contact angles and mass distributions during the drying process. Deposition of materials at drying spot centers is due to recirculatory flow driven by surface tension gradients produced by latent heat of solvent evaporation.^{20–22} This repeated event with increasing continuous evaporation leads to multiple concentric ring deposits of solute particles.^{17, 23, 24} This precludes uniform distributions of DNA during spreading and drying of printed droplets in microarray fabrication. DNA spot drying phenomena leading to spot heterogeneity in the presence of different concentrations of DNA and fraction of dye-labeled DNA can be explained from spot drying behavior in the presence of DNA-bound dye, surfactant and dissolved salts.²⁵ Addition of anionic surfactant (e.g., SDS) is known to initially facilitate uniform droplet spreading, stabilizing the evaporation and drastically altering the “coffee ring effect”. Surfactant-driven Marangoni flows arise when the local surfactant concentration at the pinned contact line increases due to the coffee ring effect thereby decreasing the local surface tension and inducing Marangoni flow towards the spot center.¹² The extent of circular Marangoni flow is influenced by variation of SDS concentration along the air-water interface.¹⁶ Also, the surfactant layers adsorbed at the liquid solid interface creates different phases, envisaged to limit the accessibility of the reactive sites for the DNA immobilization. The mode of evaporation can be influenced by the presence of salts, with the DNA probe deposition patterns following the salting trace.²⁵

Phase separation producing salt crystallization in evaporating droplets of DNA solutions containing salts occurs during drying.^{13, 24} Exceeding the local solubility limit of salts in evaporating droplets leads to rich morphologies of salt crystal and DNA.²⁴ Formation of radially varying salt concentration has a spacing between the concentric rings that depends on the DNA concentration and ion species concentration.²⁴ Marangoni inward flow of solution produces various salt patterns with deposition of the DNA-dye in concentric pattern in the spot.²⁶ The physicochemical property of the evaporating DNA solution is influenced by the dynamic change in DNA concentration, ionic strength and surfactant concentration influencing the final patterns.²⁵ Theoretical and experimental results conclude that at low concentrations of salt, the deposit more uniformly covers the entire surface beneath the droplet, whereas at higher concentration, salt deposits appear predominantly along the spot's outer rim.²⁴ At lower DNA concentration, DNA at the liquid-vapor interface is deposited at the droplet edge during initial evaporation stages and eventually, under developing Marangoni flow transitions, DNA aggregates at the spot centers.²⁷ Micro-droplets containing DNA, surfactant and dissolved salt undergo phase separation upon drying which is enhanced in the presence of cyanine dyes. Cyanine dyes are known to interact with dsDNA in aqueous media through various interactions, either by intercalation or minor groove binding between base pairs or dye-dye aggregation.^{28–30}

Heterogeneity of DNA immobilized probe and bound target in microarrays have been characterized with optical imaging, surface topography and many surface chemical analytical methods.³¹ Surface fluorescence mapping and imaging has been the preferred clinical and biomedical data quantification tool for decades.³² Fluorescence scanners, most frequently employed to quantify fluorescently labeled DNA patterns in arrays, are unable to quantify absolute amounts of probe or target DNA in spots, and are affected by numerous known surface issues that confound reliable absolute quantitative correlations of fluorescent signals. Hence, this method is almost always a relative quantitation method^{33–35} unable to report absolute density, biochemical or structural properties or states for bound DNA.^{36,37} Surface chemical methods such as X-ray photoelectron spectroscopy and TOF-SIMS provide information on nucleic acid elements, bonded structures, relative densities and coverage, and molecular fragments present in the top 2–10 nm of a surface – a dimension very appropriate to 20–40mer DNA probes and targets in many arrays.^{10, 38} Furthermore, TOF-SIMS surface chemical state imaging of DNA features has proven merit in providing both spatially and mass resolved chemical state information on printed surface heterogeneity, DNA spot and printed contaminant compositional variability, and chemical changes between probe and hybridized substrates.¹⁰ Importantly, processing of complex biomolecule TOF-SIMS data using multivariate methods such as principal component analysis (PCA) yields chemically informative images of different chemical species distributions within single printed spots at high sensitivity and sub-micrometer spatial resolution.^{10, 39–41} Salt presence affecting printed spot heterogeneity in carbohydrate microarrays has also been analyzed with TOF-SIMS.⁴² Spot morphology and signal heterogeneity have been claimed to result from incomplete target hybridization.⁴³ However, that DNA capture spot and signal variations might have significant common origins in heterogeneous probe immobilization motivated these studies of single spots and spot-spot variabilities to elucidate sources of assay variability and approaches to improve hybridization consistency. Published literature provides substantial evidence that spot printing steps produce irreversible, unalterable surface patterns that determine subsequent DNA interactions.^{10, 44–46}

In this study, we sought to evaluate if variations in amounts of fluorescent label affect DNA/DNA-dye mixing in spots, their printed spot morphology and fluorescence intensity (RFU). High resolution epifluorescence imaging of single DNA printed rinsed-blocked and dried spots demonstrating fluorescence heterogeneity typical for printed DNA probe spots was

compared to the identical spots analyzed using TOF-SIMS chemical state imaging.⁴⁶ This approach yields complementary molecular maps identifying specific chemical species in heterogeneous regions of a DNA spot. These new data provide a chemical basis for understanding DNA spot heterogeneity. Epifluorescence imaging of DNA spot morphologies, relative probe orientations and relative fluorescence unit (RFU) intensities exhibit intra- and inter- spot DNA density differences and heterogeneity. TOF-SIMS chemical imaging of identical spots provides unique chemical and spatial information on distributions of dye-labeled DNA in these printed spots. Together, these epifluorescence and TOF-SIMS techniques identify DNA spot variability from non-uniform distribution of DNA in the microspot due to spot drying effects: higher DNA probe densities at spot centers, regardless of varied DNA-dye concentrations and spotting volumes. This is proposed to influence subsequent target uptake kinetics and distributions in the same spot, impacting the assay's answer.^{44–46} Combining optical and chemical imaging techniques at micron resolution provides consistent and complimentary information regarding spot heterogeneity and its impact on array signal generation.

MATERIALS AND METHODS

Materials

High performance liquid chromatography (HPLC) purified (>90%) DNA oligomer sequences, with and without terminal Cy3 dye labeling, and also with and without reactive primary amine termini (Table S-1 Supporting Information), were purchased from TriLink Biotechnologies (San Diego, USA). Polymer-coated commercial microarray slides (Slide H, amine-reactive polymer-coated slides,⁴⁷ Schott Nexterion, Louisville, USA) were used as received. Unused slides were stored in original packaging under nitrogen at 4°C and used within one month of opening the package.

Microarray printing

A SpotBot®2 contact printer with Stealth SMP4 and SMP6 pins (Arrayit, Sunnyvale, USA) was used to print oligo-DNA probes onto commercial array surfaces. Microarrays (see layout shown in Figure S-1 and printing methodology in Supporting Information, similar to previous reporting¹⁰) were printed with multiple probe concentrations (10, 20 and 40 micromolar) and different molar ratios of Cy3 dye-labeled probe (Cy3-Oligo1-NH₂) to unlabeled probe (Oligo1-NH₂) (i.e., 0:1, 1:3, 3:1, and 1:0), yielding printed rows containing no Cy3-labeled probe (0:1) progressing to printed rows containing 100% Cy3-labeled probe (1:0). Non-specific polymer surface interactions (i.e., residual substrate primary amine chemical reactivity) were then blocked on DNA-printed slides using a step-wise protocol per manufacturer's recommendations (see Supporting Information). All spot analysis (TOF-SIMS, epifluorescence imaging) was performed on printed DNA microarrays that were rinsed-blocked and dried prior to analysis.

Fluorescence imaging of DNA microarrays

Probe-printed rinse-blocked and dried DNA microarrays were scanned for Cy3 (channel 535, GenePix 4100A scanner, version 6 software) before hybridization (after blocking), and prior to obtaining higher resolution fluorescent images with an epifluorescence microscope. For all scans, laser power was preset to 100%, brightness and contrast at 92%, PMT gain 400, the maximum pixel resolution is 5µm/pixel, adjustable from 5 to 100µm/pixel, and line average 3. Spots scanned before (data not shown) and after blocking have spot morphologies and patterns that are unchanged, indicating that spot heterogeneity occurs during droplet drying in the first step and remains for all steps thereafter.

Epifluorescence imaging of single DNA spots

High resolution fluorescent images of single DNA spots were acquired (Olympus IX81 epifluorescence microscope, Prior Scientific motorized XYZ environmental stage, motorized shutter/filter wheel, and Photometrics CoolSnap-ES scientific CCD camera in 36-bit color mode, controlled by Metamorph V6.2r6 software). Successive fluorescent images of individual DNA spots were captured under motorized XYZ stage control. Autofocus was set initially for the DNA spot containing the highest dye intensity (e.g., 40 μ M DNA, 100% Cy3) to establish maximum image contrast, and successively measured for all printed DNA spots in that array with the same optical parameters. Based on spot dye content, different microscope air objectives were used to maximize image information collection per spot (excitation filter 510–550 nm, dichroic long-pass filter from 510 nm, and emission band pass filter at 575–600 nm used for Cy3 image capture). All images are pseudo-colored and image contrast was adjusted using the auto-scale function in Image J. This processing can produce concentric rings of different colors with defined rings most often being artifactual, not real. Further description of DNA spot image analysis is provided in Supporting Information.

TOF-SIMS chemical imaging and analysis of DNA microarray spots

TOF-SIMS images were recorded on a TOF-SIMS V instrument (ION-TOF GmbH, Munster, Germany) using a Bi_3^+ cluster primary ion source. Each image has a resolution of 256×256 pixels, image sizes vary and are reported for each individual image. Positive and negative ion images were recorded using pulsed 25 keV, 0.18 pA primary ion beams in high current bunched mode. Both positive and negative ion images were taken of the same spot. Positive spectra were calibrated using CH_3^+ , C_2H_2^+ , C_4H_4^+ , C_7H_9^+ and negative spectra were calibrated using CH^- , C_2H_3^- , C_2H_3^- , CHO^- and $\text{C}_2\text{H}_3\text{O}_2^-$. A low energy electron beam was used for charge compensation. The mass resolution ($m/\Delta m$) was approx. 6000 for the m/z 26 peak in the positive spectra and 4000 for the m/z 25 peak in the negative spectra. TOF-SIMS image data were collected with primary ion dosing of 9.9×10^{11} ions/ cm^2 for images taken with 50 scans and 2×10^{12} ions/ cm^2 image taken with 100 scans (both doses are considered below the static limit).

RESULTS AND DISCUSSION

Printing-dependent DNA spot heterogeneity

High resolution epifluorescence images of individual printed rinsed-blocked and dried DNA microarray spots exhibit highly variable, non-uniform intra-spot distributions of fluorescence intensity, reflecting heterogeneous distribution of dye-labeled DNA. Figure 1A schematically represents DNA spot heterogeneity observed by epifluorescence imaging; depicting higher Cy3-labelled DNA density at the spot center compared to the periphery at both high and low DNA-Cy3 concentrations (see Figure 1 cartoon depicting high intensity regions in spot center). Actual pseudo-colored (Image-J) single spot epifluorescence intensity images in Figure 1B show dry DNA films for two printed diameters, each with high and low DNA-dye:DNA probe ratios at differing total DNA print concentrations. All images under all print conditions exhibit higher DNA dye densities in spot centers shown by the bright pseudo-colored regions, irrespective of DNA concentration or Cy3 dye-labeled DNA probe ratio. Pseudo-coloring of dried DNA showed in radial (circular) contours within each spot (i.e., ring-like deposits each of variable intensities) however, this was not observed in gray scale DNA spot images. Both the gray scale and pseudo-colored images showed high intensity at the spot center with profile plots (Image J) indicating a dominant Marangoni inward flow during spreading and drying of DNA spots (see Figure S-2).^{12, 48} High Cy3 dye-labeled DNA intensities consistently seen in Figure 2 at spot centers support dominant Marangoni effects²⁵ during DNA spot drying on NHS slides.⁴⁷ Regardless of the printing method and deposited volume, heterogeneous dried spots result.^{25, 27, 49–51} Intra-spot DNA

high intensity center-type heterogeneity was consistently observed for both dried spot diameters of ~100 and ~200 microns (Figure 1 and 2), as shown in epifluorescence images for DNA spots (probe only) with different mixed probe DNA-Cy3:DNA ratios and different total printed DNA concentrations.

Epifluorescence imaging to assess DNA spot heterogeneity and spot-spot variations

In addition to non-uniform probe distributions within single spots, spot-spot variations in DNA probe and target duplex densities are major sources of experimental variability in microarray-based analyses.^{52, 53} To enhance spot quality and reproducibility, surfactants are often used in probe immobilization buffers: nonionic Tween 20 and zwitterionic sarcosine surfactants were added to 300mM sodium phosphate print buffer here, and anionic surfactant, sodium dodecyl sulfate (SDS), was added to rinse buffer used after blocking. These have notable influence on droplet/drying physicochemical results (i.e., droplet wetting and evaporation rates) to produce well-controlled round, consistent diameter spots.²⁵ Figure 2 expands the examples of DNA heterogeneity shown in Figure 1 with epifluorescence images of printed DNA spots for a wide DNA spot compositional matrix involving different total DNA concentrations and highly varying DNA:DNA-Cy3 ratios. Drying phenomena seen in Figure 1 are consistently observed. Therefore, the Marangoni thermal stress must also dominate droplet drying across this broad DNA compositional matrix, causing spot heterogeneity. Maintaining 60% relative humidity during printing and drying did not alter this observed inter-spot or intra-spot variability, as reported earlier.⁵⁴ Use of surfactants such as Tween and SDS commonly employed in various array fabrication steps (also here) creates Marangoni stress⁴⁸ conditions that otherwise are not generally favored in aqueous systems.^{16, 25, 27, 48}

Spot-spot variation for DNA microspots using identical printing conditions for 5-replicate sets during a single print run shows spot heterogeneities also observed for same-concentration DNA-dye printed spots using surface pixel intensity plots (Figure S-3, Supporting Information), consistent with the two-dimensional intensity plots shown in Figures 1 and 2. As both total printed DNA concentration and DNA-Cy3 content increase, spot-spot heterogeneity also increases in fluorescence intensity. Spots printed with higher DNA concentrations exhibit higher DNA fluorescence fractional distribution in spot centers. Also, increasing DNA concentration leads to comparative increases in printed dried spot diameters, contributed from initial droplet spreading. A steady increase in spot diameter is observed with increasing DNA concentration. Increasing DNA-dye labeling produced increasing spot diameters as seen in TOF-SIMS, epifluorescence and fluorescence scanner images comparing 0% Cy3-labeled DNA spots with 25%, 75% and 100% DNA-Cy content spots (see Figure 3, Table S-2 and Figure S-12). Despite much evidence for DNA aggregation upon drying, there is little evidence for non-ideal mixing (i.e., DNA-Cy3 phase separation) of unlabeled DNA.

Use of non-ionic/zwitterionic surfactants can produce two phases of DNA segregation:²⁵ one enriched in DNA and another in surfactant, promoting formation of DNA precipitates with increasing electrolyte and surfactant concentration with evaporation.^{26, 55, 56} Furthermore, supersaturated salt conditions promote DNA phase separation,^{24, 25} at very low concentrations of both DNA and surfactant, and is further enhanced by electrolytes.^{10, 56–58} Similar aggregation is predominant at higher DNA concentrations (i.e., micromolar to molar)²⁸ and with dye-labeled DNA oligomers⁵⁹. It is likely that such concentrations are achieved in droplets seconds after printing due to water evaporation with rapid drying. Furthermore, cyanine dyes analogous to Cy3 are known to readily form molecular aggregates in aqueous media.²⁹ Cyanine dye aggregation is also reported in Langmuir-Blodgett monolayers of amphiphilic nucleic acids during evaporation rates controlled by mass transfer across the aqueous-film-air interface.⁶⁰ In cyanine dye-labeled

single-strand probe DNA, dye–dye interactions and dye–nucleotide⁴⁴ aggregation with increasing ionic strength, and surfactant concentrations reduce probe stability and quench fluorescence.^{59,67,70} Singly-labeled fluorescent DNA probes exhibit dye–DNA interactions as studied by fluorescence anisotropy and lifetime assays.⁵⁹ As DNA–Cy3 content increases in print solutions up to 100% DNA mass fraction, DNA aggregation is much more predominant, increasing dye quenching and reducing fluorescence intensity (shown in later section). Hence, DNA–dye aggregates predominately at spot centers,^{13, 48, 61} and fewer DNA–Cy3 probes deposit at droplet edges, altering DNA chain interactions and density as the droplet evaporates.^{27, 50, 61} (Figure S-4, Supporting Information schematically summarizes DNA droplet drying phenomenon in the presence of salt and surfactant).

TOF-SIMS chemical imaging of DNA-dye concentration-dependent printed spot heterogeneity

Further DNA-specific chemical evidence for printed spot heterogeneity comes from TOF-SIMS chemical imaging of single 100-micron diameter DNA printed spots containing various total DNA print and fractional DNA–Cy3 probe concentrations. Individual DNA spot chemical images obtained with TOF-SIMS for different mass fragments characteristic of different spot components are shown in Figure 3A. Selected chemical fragment images of individual printed DNA spots are displayed for: (Figure 3A, row 1) DNA ribose chemistry [$m/z=81$, $C_5H_5O^+$], (Figure 3A, row 2) all DNA-derived positive ions identified from TOF-SIMS spectra of deoxynucleoside controls [cytosine m/z 112 ($C_4H_6N_3O^+$); adenine 119 ($C_5H_3N_4^+$) and 136 ($C_5H_6N_5^+$); guanine m/z 135 ($C_5H_3N_4O^+$), 152 ($C_5H_6N_5O^+$), and 174 ($C_7H_4N_5O^+$)]; (Figure 3A, row 3) all positive ions associated with Cy3 [m/z 130 ($C_9H_8N^+$), 144 ($C_{10}H_{10}N^+$), 167 ($C_{10}H_{10}N^+$), and 180 ($C_{10}H_{10}N^+$)]; (Figure 3A, row 4) nucleotide phosphate fragment m/z 63 (PO_2^-), and (Figure 3A, row 5) all DNA negative ions [thymine m/z 125 ($C_5H_5N_2O_2^-$); cytosine m/z 110 ($C_4H_4N_3O^-$); adenine m/z 134 ($C_5H_4N_5$); guanine m/z 133 ($C_5H_3N_5^-$), 150 ($C_5H_4N_5O^-$)].⁴⁰ It should be noted that TOF-SIMS image pixel intensities indicate relative differences in amounts of ions within that image, but are not necessarily comparable between images. Collectively, all single-spot chemical images also show heterogeneity across the entire spot area, consistent with that seen in epifluorescence images of DNA spots (Figures 1 and 2).

In Figure 3, TOF-SIMS chemical fragment heterogeneity in Figure 3A is compared to epifluorescence images of the identical DNA spots in Figure 3B, as well as with the standard fluorescence scanner fluorescence pixel maps of the identical spots in Figure 3C. Because TOF-SIMS samples a $\sim 15\text{--}20\text{\AA}$ depth into the surface while epifluorescence emits from much deeper zones (micron depths) within spots, there are some differences in the two different spot images and morphologies. However, regardless of differences in depth of analysis and pixel resolution, textures in images providing chemical information on spot heterogeneity shown in Figures 3A (TOF-SIMS) correlate well with pixel intensity heterogeneities for identical spots seen in Figure 3B (high resolution epifluorescence images). Similarly, TOF-SIMS and epifluorescence images show similar spot heterogeneity with increased spot diameter (i.e., 200-micron diameter, increased spotting volume) as shown in Figure S-5 (Supporting Information) for 40 μM DNA spotting concentrations containing increasing amounts of Cy3 dye-labeled DNA

Significantly, neither of these higher resolution DNA spot heterogeneities is duplicated in the lower resolution fluorescent scanner images of the same spot shown in Figure 3C (pixel resolution with fluorescence scanner imaging is $\sim 6\times 6$ pixels, TOF-SIMS 256×256 pixels and epifluorescence 572×512 pixels (see Figure S-6)). TOF-SIMS images of characteristic DNA and Cy3 dye fragments consistently exhibit Marangoni drying influences, producing higher density of both DNA and Cy3 dye chemical fragments in spot centers and reduced outward radial image intensity, comparable to high resolution epifluorescence images

(Figure 3B). Lower resolution scanner images cannot capture this radially heterogeneous pixel information, and instead exhibit homogeneous spot intensities. Clearly, spot details important to DNA densities, amounts and distributions as a function of spot radius are consistent using higher resolution imaging methods on single spots, but lost in lower resolution fluorescence scanner image (Figure 3C).

Significantly for most microarray signal analysis, imaging technology used in Figure 3C represents the most commonly applied analytical method for commercial and clinical DNA arraying signal assays (i.e., fluorescence scanners). Importantly, integrated fluorescent intensity within each spot becomes the assay “answer” for gene presence and abundance in such assays. However, details obtained from high resolution techniques for intra-spot DNA spot heterogeneity and poor spot-spot reproducibility provide clear bases for sub-optimal assay quality obtained using low resolution scanner formats that cannot account for such variabilities or discern spot heterogeneity. Inconsistencies between these 3 different analytical imaging methods (on identical DNA spots) distinguish between the higher resolution of TOF-SIMS chemical imaging (Figure 3A), epifluorescence spot images (Figure 3B), and lower resolution spot fluorescence scanning.

More homogeneous fluorescence distribution, regardless of intensity differences, reflects DNA signal emanating from thicker spots from multiple depths. However, in most cases, spot morphology and density variations from epifluorescence imaging are comparable to TOF-SIMS chemical species imaging. Specifically, DNA-Cy3 dye fluorescence and relevant chemical fragment intensities are greatest at spot centers, decreasing outwardly with radius to spot edges. TOF-SIMS images provide direct evidence for Cy3 dye fragment distributions in single spots based on chemical fragment ($m/z=130$, $C_9H_8N^+$) enhancement at spot centers. Additionally, images based on positive ion fragments derived from DNA ribose sugars ($m/z=81$, $C_5H_5O^+$), negative ion phosphate peak (PO_2^-) - known DNA-derived positive and negative ion fragments - are consistently co-located with fluorescent high intensity regions for DNA-Cy3 images. TOF-SIMS chemical species imaging corroborates epifluorescence images of DNA distribution for both intra- and inter-spot.

High resolution epifluorescence and TOF-SIMS image comparisons of individual DNA microspots

Further single-spot analysis was used to compare prominent image features in spots, leveraging high resolution imaging capabilities from both epifluorescence and TOF-SIMS against lower resolution scanner images. This provided more insight into sources of DNA printed spot morphological and density heterogeneity by direct comparisons of chemically and optically distinct spatial features within respective images. Identical spot comparisons in TOF-SIMS and epifluorescence must correlate and align image and slide orientation. Comparisons of printed probe DNA spots using high-resolution epifluorescence, TOF-SIMS image and fluorescence scanner images are provided in Figure S-7 (Supporting Information).

Influence of DNA-Cy3 spotting concentrations on spot fluorescence intensity

Plots of DNA-Cy3 probe fluorescent intensity with varying DNA-Cy3 and DNA-Cy3:total DNA probe print concentrations do not exhibit a linear correspondence of increasing spot RFU with commensurate increases in Cy3-dye label fraction. Figure S-8 (Supporting Information) shows that integrated spot RFU signals exhibit non-linear intensity responses to increasing DNA-Cy3 additions. DNA-Cy3 dye content within each spot should increase the spot's relative fluorescence intensity (RFU) linearly and proportionally as long as dye-dye quenching or Cy3 quantum yield perturbations are negligible. Since the fluorescence intensity RFU value is the assay “answer”, spot morphology and spot fluorescent reporting

fidelity is crucial to accurately determine experimental spot-to-spot variation and experimental assay “answer” validity.⁶² Possible Cy3 aggregation, dimerization, DNA-Cy3^{59, 63, 64} and dye-substrate surface interactions that reduce fluorescent intensity for printed ssDNA are offered as possible explanations for this non-linearity observed. Lateral non-radiative energy transfer (self-quenching) is known between cyanine dyes (i.e., Cy3 and Cy5) at higher surface densities and bulk concentrations.⁶⁵ Quenching due to dye-dye and dye-surface interactions is predominant at short separation distances,^{66–70} favored at higher DNA labeling densities and supported by lower-than-expected increases in spot integrated RFUs as DNA-Cy3 fractions increase from 25 to 75 to 100%. Figure S-8 shows integrated spot fluorescence intensities from epifluorescence images of varying DNA-Cy3 concentration not correlating with actual DNA probe concentrations. This presents a difficult validation issue for use of this format as an industry-standard and clinically applied assay wherein low resolution fluorescence scanners are expected to produce integrated spot RFU intensity as the assay “answer”.

DNA spot morphology and droplet spreading comparisons during printing/drying

Spot drying effects on both DNA-Cy3 distribution in printed spots and spot morphologies were evaluated by assessing printed spot dimensions and distinct spot drying features (e.g., DNA distribution in different DNA drying regions). Spot dimensions, areas, diameters, and high fluorescent intensity from DNA spreading/drying were measured for epifluorescence images of printed DNA spots. Figure S-9 shows gross printed spot dimensions measured from both epifluorescence and processed TOF-SIMS images of identical printed DNA spots. Image scaling measurements confirming DNA spot diameter and central high intensity DNA-Cy3 regions in epifluorescence imaging are obtained by converting pixels to microns (Figure S-10, Supporting Information). Epifluorescence imaging provides higher resolution spot data compared to TOF-SIMS images, showing clearly high intensity central region of DNA-Cy3 distribution in the spot. Similarly, dimension of high intensity region of DNA spot images of ~200 micron dried 40 μ M DNA spot with 100% DNA-Cy3 dye were prominent and measurable for Cy3 dye distribution within the spot, and were comparable using both techniques (Figure S-11, Supporting Information).

Uniform DNA spot morphologies and fluorescence intensities are important for accuracy, repeatability and reproducibility of the DNA array assay RFU “answer,” especially using common lower resolution scanner fluorescence image pixel integration. Both integrated spot intensity and relative spot size are important for reliable RFU analysis of DNA array spots.⁷¹ As shown in Figures S-9 and Figure S-11, spot sizes obtained from contact printing could be precisely determined from diameter measurements using both high-resolution imaging techniques. While low-resolution fluorescence scanner images of DNA spot size are uniform without notable bleeding/spreading of either DNA or printer buffer, this imaging method cannot consider spot non-uniformity and resulting changes in spot signal intensities as observed with high resolution techniques. TOF-SIMS validates printed spot size by showing that all relevant DNA chemical signatures correlate to the same spot dimension as the same DNA spot's epifluorescence image signature. While this correlation confirms the accuracy of high-resolution epifluorescence to capture spot Cy3 fluorescent heterogeneity and signal accurately, lower-resolution scanners, while accurately determining actual spot size (Table S-2 Supporting Information), cannot provide sufficient resolution to resolve spot fluorescence heterogeneity. Hence, integrated spot RFU signal intensities, independent of instrumentation differences, will necessarily be unique for each method. This is supported by line scan analyses for corresponding spots (data not shown).

Epifluorescence and TOF-SIMS images also demonstrate increasing spot diameters and spread areas with increasing amounts of DNA and fractions of DNA-Cy3 for the 100-micron spot regimes (constant drop volume, Figure S-12A, Supporting Information). Figure S-12B

compares both 100- and 200- micron spot diameters for 40 μ M total printed probe DNA with increasing DNA-Cy3 content (different drop volumes). Both TOF-SIMS and epifluorescence imaging are closely comparable in demonstrating increased dried spot diameter with increasing DNA-Cy3 content. Common buffers such as PBS and SSC used for DNA spot printing lead to size and morphological inhomogeneities.²⁵ However, droplet evaporation induces DNA phase separation with increasing ionic strength, surfactant, and Cy3 dye-DNA concentrations, influencing spot drying patterns and DNA immobilized densities.^{72–75} This is important since high density DNA probe immobilization and DNA-DNA crowding influence DNA target surface capture (hybridization), hindering duplex formation at surfaces by both steric and coulombic blockade repulsive barriers to incoming DNA targets.^{3, 6, 72, 73} This means that DNA target binding efficiency, dependent on local probe density,⁷⁶ will also be heterogeneous given wide variations in both intra- and inter-spot probe density shown to result from Cy3 dye-labeled DNA altered interactions with surfaces during printing and drying⁷⁷ and evaporatively induced DNA phase separation.²⁷ Notably, high amounts of probe dye labeling used here to monitor spot optical properties are not typical of most arraying experiments and are shown to lead to greater phase segregation and spot size alterations.⁵⁹ Additionally, DNA exhibits only modest drying-induced non-specific surface binding upon printing and clearly reacts covalently in presence of NHS chemistry. Spots lacking DNA-specific chemical coupling or buffer-only spots can be imaged from residual DNA non-specific binding in the former case and background autofluorescence generated from buffer spot wetting/drying in both cases.⁴⁴ Whether DNA surface coupling chemistry proceeds after print buffer evaporation is not known, however it seems likely that residual amounts of DNA in printed spots areas (after evaporation) are primarily a non-specific drying-induced physisorbed aggregation of non-coupled DNA mixed with surface-coupled DNA, leaving substantial amounts of non-coupled but drying-stabilized DNA on the surface despite rinsing.⁷⁸ This aggregated disorganized multilayer DNA film comprises the microspot that undergo target capture, with a higher DNA density (high DNA concentration, substantially greater spot thickness⁷⁸) at its center due to DNA transport under Marangoni drying.

CONCLUSIONS

Analysis of DNA printed spot heterogeneity and spot-to-spot variation for printed dye-labeled DNA microspots often shows higher DNA density at the centers of printed DNA microspots than spot edges. High-resolution epifluorescence optical imaging of DNA-Cy3 and TOF-SIMS chemical imaging of different DNA or Cy3 chemical fragment ion species within spots together provide complementary information for assessing dried DNA density and distributions on coated glass commercial microarray substrates. High-density DNA regions in spot centers obtained from epifluorescence imaging compare very well to those obtained with TOF-SIMS chemical state imaging. Routine, conventional fluorescence scanner images provide information for integrated spot pixel intensity, shape and morphology, but lack all details for intra-spot DNA heterogeneity and intra-spot structural issues known to affect target capture and duplex hybridization kinetics critical to this assay's answer development and diagnostic reliability. Comparative spot-spot assessment with multiple complementary analytical techniques consistently detected DNA spot-spot variability, heterogeneity within individual DNA spots and DNA density distributions.

Dried DNA spot non-uniformities obtained using contact printing techniques are attributed to array fabrication process parameters including non-equilibrium drying under influences of buffers, humidity and evaporation during microarray fabrication. Marangoni drying under these evaporative conditions produce chemical and physical interactions between DNA and non-volatile solutes in the spot, yielding DNA aggregation and surface deposition within the original spotted, wetted footprint. Direct side-by-side comparisons of dried DNA spot

dimensions, DNA density distributions, lateral heterogeneities and trends in variations in spot DNA densities with varying DNA print concentrations are consistent in asserting these influences on dried spot morphologies. Non-uniform DNA probe distributions produce non-linear changes in integrated spot fluorescence intensities with increasing DNA-Cy3 printed content. Significantly, probe spot heterogeneities also affect DNA target capture efficiencies and reliabilities, and ultimately the entire microarray assay signal generation. Hence, variations in DNA target capture duplexes both within spots and spot-to-spot have origins in probe print drying artifacts. Improved assay consistency and “answer” reliability and reproducibility therefore should focus on probe printing controls and consistent surface, buffer and drying properties in order to limit stochastic variability. Study results justify necessary improvements in methods and quality controls for DNA surface immobilization processes that influence analyte (i.e., DNA target) capture and that affect microarray assay signal reliability, reproducibility and clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Spot heterogeneity in printed DNA microspots with varied DNA print concentrations and volumes. A) Schematics of spot fluorescence high intensity with DNA Cy3-dye content at spot center for 2 Cy3- concentrations (dotted line indicates fluorescence intensity variation with dye-labelled DNA present across printed spot radius. B) Comparison of single spot epifluorescence images showing spot anisotropy with different contents of Cy3-labeled DNA probe with unlabelled DNA in ~100 micron (left images are $125\mu\text{m} \times 125\mu\text{m}$) and ~200 micron diameter (right images are $200\mu\text{m} \times 200\mu\text{m}$) spots (total DNA print concentrations of $40\mu\text{M}$ and $10\mu\text{M}$ for top and bottom rows, respectively). Note: All images are pseudo-colored with contrast adjusted using the auto-scale function in Image J.

Figure 2.

Epifluorescence images of printed DNA spots comparing two side-by-side dried spots each of identical DNA concentration and DNA-Cy3 content for 10, 20 and 40 μM . DNA print concentrations (rows across) and increasing 25–100% Cy3-DNA ratios (columns down). Images are 125 μm ×125 μm . All images are pseudo-colored with intensity contrast adjusted using the auto-scale function in Image J to depict highest intensity of DNA-Cy3 dye at the spot's center.

Figure 3.

Comparison of TOF-SIMS, epifluorescence and fluorescence scanner images of identical ~100 micron DNA spots (each vertical column) of varying total DNA concentration (major triplicate columns) and fractional DNA-Cy3 dye label content (each vertical column). A) TOF-SIMS chemical images of different DNA chemical fragments: 10 μ M and 20 μ M DNA spot images were 150 μ m $\times$ 150 μ m and the 40 μ M spots were 200 μ m $\times$ 200 μ m. B) epifluorescence images of DNA-Cy3, images are 125 μ m $\times$ 125 μ m and C) conventional fluorescence scanner images lacking spot features seen in (A) and (B). DNA spots with 0% DNA-Cy3 in (B) are only visible with increasing exposure time in epifluorescence imaging. Corresponding fluorescence scanner image of the same 0% DNA-Cy3 spot (C) is only very faint with 100% laser intensity. Note: All images in (B) are pseudo-colored with intensity contrast adjusted using the auto-scale function in Image J.