INTRODUCTION

Microarray technology, allowing the quantitative and simultaneous monitoring of the expression of thousands of genes, initialized a new era in gene expression studies (1). It provides a unique tool for the determination of gene expression at the level of messenger RNA (mRNA) and has become a widely utilized technology in laboratories around the world to perform gene expression profiling studies for clinical (2–6) and diagnostic applications (7–12) or to determine genetic variation through the detection of single nucleotide polymorphisms (SNPs) (13–15). Although the microarray technology has existed for more than a decade, there are a number of effects that remain ill-understood. One such example is the occurrence of doughnut patterns on the microarray scans. A doughnut or ring pattern can be defined as an intensity pattern occurring at the spot level in which the intensity signal is higher at the edges and lower at the center of the probe spot (Figure 1A). The intensity profile across a section of Figure 1B shows that the intensity between the center and the rims of the spot can differ more than 2-fold in intensity.

The presence of a ring-shaped intensity pattern makes the spots harder to analyze, because it leads to very high intraspot standard deviation values and some of the image analysis tools like GenePix® (Axon Instruments), ImaGene™ (Biodiscovery), QuantArray® (GSI Lumonics), and ScanAlyze do not contain algorithms that are able to detect and analyze spots with these artifacts in a correct manner. The spots are often flagged and left out of the analysis. It is therefore of great importance to get a better insight into the formation of ring-shaped hybridization patterns. Until now, two major causes have been proposed. There have been reports (16–18) claiming that doughnuts are created during the spotting process when contact is made between the spotting pins and the microarray surface. However, a good calibration of the microarray spotter can easily circumvent this type of doughnut formation. Another more likely explanation for the occurrence of these patterns is based on the so called coffee-stain effect (19). When a drop of liquid containing solutes dries on a solid surface, it leaves a dense, ring-like deposit over the entire perimeter. The ring formation can be explained as the consequence of a geometrical constraint of the droplet surface. As the free surface is constrained by the pinned contact line, the fluid is squeezed outwards to compensate for evaporative losses (19). Originally, the dissolved DNA material is homogeneously distributed over the entire droplet, but as the solvent evaporates, the DNA molecules flow outwards with the fluid flow. The DNA material is therefore accumulated on the edges of the spot. When spotting DNA microarrays, this diffusion limitation: a possible source for the occurrence of doughnut patterns on DNA microarrays

Kris Pappaert¹, Heidi Ottevaere¹, Hugo Thienpont¹, Paul Van Hummelen², and Gert Desmet¹

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Doughnut shaped hybridization patterns on DNA microarrays are mainly allocated to spotting or drying artifacts. The present study reports on results obtained from four different approaches that when combined generate a better view on the occurrence of these patterns. This study points out that doughnuts are not only formed during the spotting and drying process, but the hybridization process itself can be considered as an important cause. A combination of computer simulations, theoretical, optical, and experimental techniques shows how ring-shaped hybridization patterns occur when diffusion-limited conditions are present during the hybridization process. The theoretical assumptions as well as the simulations indicate that, for the basic geometry of a microarray hybridization experiment, a large amount of binding molecules reach the spot from the sides (and not from above the spot), leading to a preferential binding on the rims of the spot. These patterns seem to occur especially during hybridization with short oligonucleotides that have a very high binding probability and fast hybridization kinetics. Longer target DNA molecules lead to a more evenly distributed intensity signal. Furthermore, the diffusion-limited conditions also lead to pronounced hybridization intensity patterns on the scale of a whole spot block, where larger intensities are obtained on the edges of the block compared with the spots laying in the center of the block.

INTRODUCTION

Microarray technology, allowing the quantitative and simultaneous monitoring of the expression of thousands of genes, initialized a new era in gene expression studies (1). It provides a unique tool for the determination of gene expression at the level of messenger RNA (mRNA) and has become a widely utilized technology in laboratories around the world to perform gene expression profiling studies for clinical (2–6) and diagnostic applications (7–12) or to determine genetic variation through the detection of single nucleotide polymorphisms (SNPs) (13–15). Although the microarray technology has existed for more than a decade, there are a number of effects that remain ill-understood. One such example is the occurrence of doughnut patterns on the microarray scans. A doughnut or ring pattern can be defined as an intensity pattern occurring at the spot level in which the intensity signal is higher at the edges and lower at the center of the probe spot (Figure 1A). The intensity profile across a section of Figure 1B shows that the intensity between the center and the rims of the spot can differ more than 2-fold in intensity.

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effect deposits more probe molecules on the edges of the spot and almost none in the center, resulting in an uneven signal distribution after hybridization.

In order to prevent doughnut patterns during the drying process, different technological adaptations have recently been proposed. A better control of the evaporation process [slower evaporation by using a dimethyl sulfoxide (DMSO) spotting solution, higher humidity in the spotting chamber] and the use of spotting substrates that are ultraphobic instead of the mildly hydrophobized glass slides have reduced the occurrence of doughnut patterns. Because the surface-pinned contact line is the major reason for the coffee-stain effect, a very hydrophobic surface with high contact angles can also be used to reduce the fixation of the contact line (20).

Although the number of ring-shaped patterns occurring during microarray analysis has been reduced by the technological adaptations cited above, these artifacts still keep arising in some cases (usually posthybridization). A wider investigation into the phenomenon of doughnuts on microarrays is hence necessary. The present study reports on results obtained from four different approaches that, when combined, show that doughnuts cannot only be formed during the spotting and drying process, but that the hybridization process itself can be considered as an important cause. In the present study, a combination of computer simulations, theoretical, optical, and experimental techniques is used to demonstrate that the occurrence of diffusion-limited conditions during the hybridization process can cause these hybridization patterns. With the term diffusion limitation, we refer to the situation in which the actual hybridization rate is slower than that expected on the basis of the intrinsic reaction kinetics.

MATERIALS AND METHODS

Simulations

In order to gain a better insight in the hybridization dynamics at the molecular level, random walk (RW) simulations were performed using a self-written C++ program, simulating the release and binding of a delimited number of target molecules in a three-dimensional (3-D) space of 2 × 2 mm with a height of 40 μm containing a circular spot area (200 μm diameter). At each time step, Δt, the targets had a chance to be displaced either in the x-, y-, and z-direction. At the edges of the simulated space, a total reflection condition was applied, and in the circular spot region, the target molecule had a probability χ to bind to the surface and a probability 1-χ to return to its previous location. The relation between the step length, Δy, and the jump time, Δt, on the one hand and the macroscopic diffusion coefficient, D_mol, on the other hand can be expressed using the well-established Einstein-Schmoluchowski relation (21).

\[ D_{\text{mol}} = \frac{\Delta y^2}{2\Delta t} \]  

[Eq. 1]

The simulations were conducted for typical values of \( D_{\text{mol}} = 2 \times 10^{-11} \text{ m}^2/\text{s} \) (22), \( \Delta y = 2 \mu \text{m} \), and \( \Delta t = 0.1 \text{ s} \). The corresponding rate coefficient (k) according to Equation 2 (23) is then 1.5 \( \times 10^6 \text{ m/s} \) for \( \chi = 1 \).

\[ k = \frac{3D_{\text{mol}}}{2\lambda} \chi \]  

[Eq. 2]

Different fluid layer heights, binding chances (χ), the mean molecular displacement length (λ), and diffusion rates (D_mol) were considered, covering a wide range of typical hybridization conditions. The simulations were performed for different values of D_mol needed and the adaptation of the Δy and Δt variables to keep the rate coefficient (k) constant.

Microarray Experiments

All hybridization experiments were conducted using conventional microarray procedures and made use of VIB_Mouse_5K_II chips (MicroArray Facility, Leuven, Belgium). All different spotted cDNA probe strands were obtained by PCR amplification and were arrayed on Amersham type VII star slides (GE Healthcare, Piscataway, NJ, USA) using a Generation III Array Spotter (GE Healthcare). The array elements were all spotted at a concentration of 100 ng/μL. The probe spots (diameter 100 μm and spaced 20–30 μm apart) were grouped in 265 different blocks of 12,632 spots. As target molecules, a complex mixture of mouse lung and testis total RNA was used (40 pmol Cy™3 and Cy5). The hybridization reaction was performed using the standard hybridization procedures described by Puskas et al. (24). The procedure consisted of pipeting a 30-μL sample and putting it on the microarray slide, which was then topped by a thin glass coverslip (2 × 5 cm) and sealed off with rubber glue. The slides were subsequently kept overnight (16 h) in an incubator at 42°C. The hybridization procedure using a universal target (23 base long target that hybridizes with all probe spots; 5’-TCCCAGTCACGACGTCGT-3’) was identical to the above described, except for the hybridization time (only 1 h).

Figure 1. Typical doughnut hybridization profiles. (A) Taken from a high density microarray slide hybridized at room temperature for 1 h with a Cy3-labeled universal primer (23 bases long target molecule), the image shows a typical doughnut pattern. The spots have a bright fluorescent intensity at the edges and dim centers appearing like a doughnut. (B) The intensity profile of the spots, taken along the white line, shows clearly how the rims of the spot are very bright compared with the center.
In another type of hybridization experiment, the microarray slides were entirely covered by more than 6000 identical probe spots (Figure 2). For these experiments, aminosilane-coated slides (Takara, Madison, WI, USA) were used, and the probes were spotted using a Lucidea Array Spotter (GE Healthcare). The spots (diameter 110 μm and spaced 50 μm apart) were grouped in an array of 4 × 8 blocks, regularly spaced across nearly the entire central region of the microscope slides (Figure 2). Each block consisted of 192 spots arrayed in 16 rows of 12 spots. The target sample used for the hybridizations contained matching strands prepared using a nick-translation technique (24). After purification, the targets were mixed with a hybridization buffer to a final total DNA concentration of 1 ng/μL and added on the microarray slide. However, instead of a coverslip, an etched substrate containing a microchamber with a specific controlled depth (15 μm) was used to cover the microarray slide. A hydraulic pressure system was used to keep both surfaces in intimate contact during the hybridization reaction. The whole system was incubated at a constant temperature of 42°C during the hybridization process. Excess target conditions were used for all microarray hybridization experiments.

Prior to each experiment, the concentration of the DNA samples and the amount of incorporated Cy3/ Cy5 molecules was measured with a NanoDrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). After heat denaturation of the targets, the hybridization was carried out at 42°C. After the hybridization, the slides were washed three times for 5 min in different sodium dodecyl sulfate (SDS)/sodium chloride-sodium citrate (SSC) solutions (respectively, 1× SSC/0.2% SDS, 0.1× SSC/0.2% SDS, and 0.1× SSC). The slides were subsequently rinsed with Milli-Q® water (Millipore, Billerica, MA, USA) and finally dried by centrifugation for 1 min at 1000 rpm in a Model CR412 centrifuge (VWR International, Leuven, Belgium). The slides were then scanned at 532 nm using a Generation III Scanner (GE Healthcare). Image analysis was done using ArrayVision™ (Imaging Research, GE Healthcare).

**WYKO Measurements**

In this work we have also used a WYKO NT2000 noncontact optical profiler (VEECO Instruments, Woodbury, NY, USA) to measure the surface profile of the different DNA probe spots. This surface profiler system uses two approaches to measure a wide range of surface heights. The phase-shifting interferometry (PSI) mode allows measuring fairly smooth and continuous surfaces (0.1 nm < heights < 160 nm), while the vertical-scanning interferometry (VSI) mode can measure rough surfaces and heights ranging between 160 nm and 2 μm. In the present study, the PSI mode was used, as the height differences were only a few nanometer large. The basic principle of this noncontact profiler is based on a Mirau interference microscope in which a white light beam passes through a beam splitter that reflects half of the incident beam to a reference surface and transmits the other half onto the sample. The light beams reflected from the sample and from the reference surface then recombine at the beam splitter to form interference fringes. The system measures the degree of the fringe modulation and the fringe contrast and determines the height differences from these measurements.

**RESULTS**

**Theoretical Work**

The basis for the assumption that diffusion-limited conditions can also be a cause of the formation of ring-shaped spots can easily be understood from the following simple calculation. When assuming that the target molecules are about 500 bases long and have a diffusion coefficient of about 10⁻¹¹ m²/s (22), it can be calculated from Einstein’s law of diffusion (Equation 1) that after an overnight waiting period,
the molecules will, on average, only have traveled a distance of 1 mm. For the case of an overnight experiment in which 30 μL hybridization solution is used to cover the microarray surface (2 × 5 cm) and a coverslip is used to cover the fluid (average fluid layer height is 30 μm), this means that for a spot with a diameter of 150 μm, only 0.56% of the targets that bind to the spot are sampled from the region right above the spot surface. Therefore, more then 99% of the matching targets come from the regions outside the spot. A target approaching the spot from the sides will have to move over the spot surface for more than 45 min before reaching the center of the spot. During that time period, it will be more likely for the target molecules to bind to the edges of the spot surface than it is to bind to the center (Figure 3). The limited diffusion of the target molecules is therefore a probable source for the occurrence of ring-shaped hybridization patterns. A similar effect has already been described by Sheehan and Withman (25), as they calculated the flux of analyte molecules toward different sensor regions as a function of time. As a microarray spot can be assumed to be a circular sensing region within a flat surface (26), the analytical solution for the steady-state flux to that circular sensing area, or sink, is similar to the Weber's disk problem, in which the flux toward the circular surface is given by

\[
 j(r) = \frac{2D_m N_v C_0}{\pi \sqrt{d - r}}
 \]

[Eq. 3]

where, \( N_v \) stands for the number of Avogadro and \( C_0 \) to the target concentration. As \( r \) is the distance from the center of the disk, the flux increases with the radius and is significantly enhanced at the edges (\( r = a \)). This enhancement at the edges of the sensing region can be assumed to be a possible explanation for the doughnut formation during the static hybridization process (25).

The hybridization process on the spot can be presumed to occur in two phases. In the first phase, it is essentially the target molecules present directly above the spot that are hybridizing. Once this region is depleted, the target molecules binding to the spot will come from the regions beside the spot. When there are enough target molecules above the spot surface to saturate the spot, the spot will be hybridized uniformly. In the case of very rapid binding kinetics and slow diffusion, molecules coming from outside the region above the spot immediately bind to the rim of the spot. This process continues until the rim is fully saturated and the target molecules start to conquer the adjacent more central ring, until this one is saturated as well, and the next ring zone is starting to become hybridized. Of course the above process is not perfect, and deviations (caused by the stochastic nature of the hybridization process) lead to a more gradually decreasing intensity profile of the inner edge of the doughnut region, while the outer edge is sharper due to the border between the spotted and nonspotted region.

**Simulation Results**

The above-described assumption can be validated using computer simulations. A 3-D RW program was used to simulate the hybridization reaction on a microarray spot. After a fixed time period, the simulation was stopped, and the hybridization pattern on the microarray spot was monitored. Simulations were performed for different fluid layer heights, binding chances (\( \chi \)), and diffusion rates.

The simulations showed that for target molecules with high binding chances (large \( \chi \)-values) and for thin fluid layers, doughnut patterns are more likely to be formed (Figure 4). This can be explained by the fact that

**Figure 4. Simulations of the formation of doughnut patterns using a three-dimensional (3-D) random walk simulation.** A decrease of the average number of attempts needed for a correct binding to occur (larger \( \chi \)-values), such that faster binding kinetics leads to a more pronounced doughnut effect. Simulation parameters: spot diameter, 200 μm; chamber height, 40 μm.
for target molecules that only need a limited amount of attempts before the hybridization can occur ($\chi = 1$ and $\chi = 0.1$), the binding occurs at a very high rate. As most target molecules approach the spot from the sides, they will bind directly near the rims of the spot, hence, leading to a ring-shaped hybridization pattern. A reduction of the fluid height tends to have the same effect, as it implies that a relatively higher amount of target strands need to come from the regions aside the spot before the final equilibrium can be established. This will eventually lead to higher signal intensity on the edges of the spot. On the other hand, the hybridization pattern is more uniform for cases where $\chi$ is low ($\chi = 0.01$ and $\chi = 0.001$), as can be noticed from Figure 4. In these cases, more attempts are needed before a successful hybridization occurs, and the spots are more evenly covered with targets. For systems where the fluid layer is large, relatively more binding target molecules will come from the region directly above the spot, hence leading to a more even distribution. The diffusion coefficients that were originally applied (ranging from $10^{-10}$ to $10^{-11}$ m$^2$/s) did not seem to have a major influence on the occurrence of doughnut shaped hybridization patterns. It was only when the simulations were performed for unrealistically high molecular diffusion coefficients ranging from $10^{-7}$ to $10^{-8}$ m$^2$/s, that the ring-shaped hybridization patterns for the case of $\chi = 0.1$ disappeared. Therefore, it can be assumed that the recently developed automated hybridization systems (27–31), in which the target mixture is continuously mixed during the hybridization process, hence enhancing the mobility of the target molecules, will lead to a significant lower amount of doughnut shaped hybridization patterns.

The doughnut shapes obtained with these simulations (Figure 4) might look different from the ones obtained experimentally (Figures 1 and 5A). The simulations show a much thinner doughnut region compared with the doughnuts obtained in the experiments. This is due to the higher resolution of the simulations ($1 \times 1 \mu$m), where the experimental microarray scanner resolution is only $10 \times 10 \mu$m. Furthermore, the simulations do not take into account the halo effect created by the fluorescent dyes that makes the doughnut region larger than it actually is.

### Experimental Results

The results of the conducted simulations could also be supported experimentally by comparing the hybridization of small DNA molecules to that of large DNA molecules. For the hybridization with small DNA molecules, very short fluorescently labeled DNA targets are used (only 20 to 30 bases long). These targets are typically used to check for possible errors and dirt on the microarray substrates after spotting. The short sequence is designed to match all the spots on the microarray, and because of its reduced length, it hybridizes very fast. An average hybridization experiment conducted under a coverslip with this type of targets only takes 1 h, suggesting very fast kinetics. As can be noted from the zoomed images presented in Figure 5, the occurrence of doughnut patterns in these systems is much higher than in a hybridization system using a complex target mixture consisting of RNA molecules that are much longer (>500 bases) and where the kinetics are much slower. The ring-shaped patterns were consistent over the entire array, and several similar experiments showed identical results. Given that the large target strands have a smaller diffusion coefficient, it can be assumed that the slow binding kinetics have a dominant effect over the slow diffusion process, because on the sole basis of the slower diffusion process, one would have expected that the longer strands could have a higher tendency to form doughnut patterns than the short DNA strands.

The diffusion limitation effect is not only noticed at the spot level. When overlooking an entire spot block, there are also effects that can be attributed to diffusion limitation. When performing hybridization experiments on microarrays that contain blocks of $12 \times 16$ identical probe spots, it can mostly be noticed that the spots positioned at the edges of the blocks have a significantly higher intensity signal compared with the center spots (Figure 2). Figure 2, B and C, show the vertical and horizontal readouts of the average spot intensity. The effect is extremely visible in the horizontal readout but lesser in the vertical readout as the space between the spot blocks is smaller in this direction. This effect was especially noticed when the hybridization occurred in small microchambers (15–30 $\mu$m). There are two possible reasons to explain this effect. The first reason is that the observed effect could be a spotting artifact. Normal microarray experiments showed that the intensity signal increases when spots are positioned more to the right bottom of a block. However, hybrid-

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**Figure 5.** Comparison of the scanned images after a 1-h hybridization on Mouse 5K microarray slides (Clontech, Mountain View, CA, USA) with (A) a 23-base-long fluorescent oligonucleotide and (B) an overnight hybridization with a total RNA sample containing 500-base-long target strands. Both images are identical regions on the microarray slide and contain identical spots (from the same spotting batch), with only the applied target sample differing in length. All hybridizations were performed according to the techniques described in the Materials and Methods section. To check whether this difference is due to the size of the target molecules and not to the hybridization time, overnight hybridizations with the M13 targets gave identical results as the 1-h hybridizations. On the other hand, 1-h hybridizations with the complex target mixtures resulted in very low intensity values that cannot be interpreted.
ization experiments using the Lucidea Automated Slide Processor (ASP; GE Healthcare), a system where the hybridization sample is continuously pumped around (and wherein no diffusion limitation is present), showed that this spotting effect was only minor and not to the extent seen in the experiments where the sample fluid was kept stationary in a thin fluid layer. The cause of the effect observed in Figure 2 can therefore mainly be attributed to the fact that the spots near the edges of a block can sample targets from a larger region compared with the spots in the center. Since all spots are identical, they all compete for the same pool of target molecules. The spots positioned in the middle of a block will have more competition from the eight neighboring spots compared with the spots on the edges of the block, surrounded by only three or five neighboring spots (the competition is only present at one side of the spot). This difference in competition results in an enhancement of the hybridization signal of the spots positioned at the edges.

Optical Results

The occurrence of doughnut patterns can also be visualized using a WYKO NT2000 noncontact optical profiler. This optical technique was used to visualize microarray spots before and after hybridization (Figure 6). Prior to hybridization, the spots have a regular even shape, and the DNA probe molecules are homogeneously covering the spot. After hybridization, there is a clear border present at the edge of the spot. The hybridization of the slide has clearly changed the coverage of the microarray spot from a normal coverage to a ring shape. This shows that the target molecules preferentially bind at the edges of the spot, hence, creating the doughnut effect. Since this pattern has emerged during the hybridization process, the coffee-stain effect mentioned in the introduction can be excluded as the cause of the observed ring-shaped pattern.

**DISCUSSION**

The combination of the theoretical analysis, simulations, experimental, and optical work conducted in the present study shows that diffusion limitation plays an important role in the creation of ring-shaped patterns during the hybridization process. Even if the spotting conditions and substrates are ideal, and if the coffee-stain effect is avoided during the drying of the deposited probe spots, the occurrence of diffusion-limited hybridization conditions increases the risk of doughnut formation. The patterns occur especially for small target molecules like oligonucleotides that have very fast hybridization kinetics. The theoretical work as well as the simulations show that most of the binding target molecules come from the regions beside the probe spot surface and will preferentially bind to the rims of the spot when the hybridization kinetics are high. This shows that the faster the binding kinetics of the target-probe complex, the higher the risk for a more dense coverage of the edges of the microarray spot. The experimental work confirms these results, as they show that the occurrence of doughnuts is much more pronounced for hybridizations with small targets. Finally, the optical measurements show that the ring-shaped patterns are created during the hybridization reaction.

One solution in preventing ring-shaped patterns is applying some form of active mixing of the hybridization fluid, as this will give each target molecule the opportunity to bind at every location on the spot surface, hence bypassing the diffusion limitation. Reasoning even further on this diffusion limitation inducing the doughnut formation, it can also be inferred that the effect will be more pronounced with large spot diameters.
and will disappear for very small spot diameters (radius on the order of the width of the current doughnut bands).

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address correspondence to Kris Pappaert, Department of Chemical Engineering, Transport Modeling & (Bio)analytical Separation Science Group (TMA$), Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium. e-mail: kpappaert@vub.ac.be

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