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Enhancement of DNA micro-array analysis using a shear-driven micro-channel flow system

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Abstract

A very simple micro-channel flow system is used to investigate the potential gain in hybridization rate stemming from the induction of a convective flow past the surface of a DNA micro-array. Reporting on a series of preliminary experiments wherein a two-dimensional convective flow is created past the surface of a conventional micro-array slide, the analysis time could be brought down from overnight waiting (16 h) to some 10 to 30 min. The experiments open the road towards the development of novel, convection-driven hybridization systems yielding shorter analysis times, and/or lower detection limits.
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1. Introduction

In the past decade, DNA micro-array technology has gained wide use in analytical chemistry, with applications in important areas such as gene identification, gene mapping, DNA sequencing and clinical diagnostics [1,2]. Traditionally, DNA screening assays are effectuated by applying an aliquot of sample between two parallel plates, one carrying a high-density micro-array of different immobilized target DNA spots with known base sequence, while the other plate simply serves to seal off the system and to prevent evaporation of the sample liquid. In such systems, the transport of the sample molecules towards the target spots occurs in a passive mode, i.e., by pure molecular diffusion (cf. the diffusive path in Fig. 1a). As a result, micro-array analyses are very slow, and usually have to occur overnight. Another consequence of the slow diffusive transport is that micro-arrays are highly inefficient in terms of binding efficiency and detection limit. In general, twenty to forty million copies of the same sequence species have to be present in the sample to reach the typical detection limit of 20 000–50 000 hybridized strands per spot. This corresponds to a binding efficiency of less than 1%. Table 1 shows that this poor binding efficiency is due to the fact that, even after overnight incubation, a given target spot can typically only be reached by the probe DNA strands which are present within a distance of less than $\ell = 1$ mm from the target spot. The latter value is obtained from Einstein's law of diffusion:

$$\ell^2 = 2D_{\mathrm{mol}}t \quad (1)$$

using a typical value of $D_{\mathrm{mol}} = 10^{-11}$ m^2/s to

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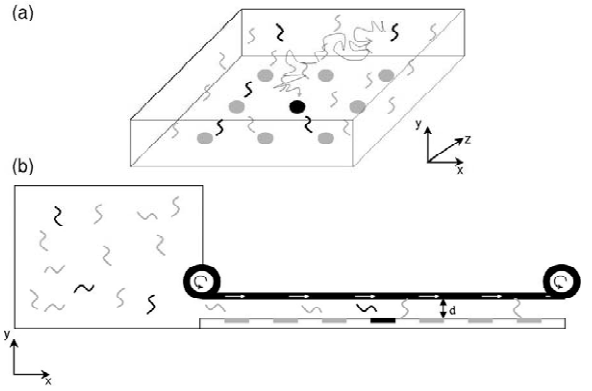

Fig. 1. (a) Schematic view of a conventional, diffusion-driven DNA micro-array, showing the diffusion path typically followed by a probe DNA molecule before reaching the target spot with its matching counterpart. (b) Schematic view of a shear-driven DNA micro-array analysis system.

represent the rate of diffusion of DNA strands in a typical micro-array analysis [3]. Comparing the area of a circular region with radius 1 mm to the total surface area of the chip, it can easily be calculated that each spot only ''sees'' less than 3 mm^2 out of the total 1875 mm^2 contacted with the DNA sample in a conventional, microscope slide-sized chip. This corresponds to a very poor maximal binding efficiency of less than 0.2%. Table 1 also shows that if one would try to bring this efficiency up to a level of 2% (requiring to sample the DNA from a region with radius 3 mm), a 6-day analysis would be required, while reaching a 20% binding efficiency would require a dramatically long 2-month analysis time.

To alleviate this diffusion limitation, several solutions have already been proposed: the application of electrically-induced flows [4] and hybridization in flow-through nanopores [5,6]. Both solutions however only consider the enhancement of the diffusion process in the direction perpendicular to the target spots, and do not solve the problem of the extremely slow lateral (i.e., in the x- and z-directions) transport. In the present contribution, we propose the use of shear-driven flows to generate a lateral convective transport across the micro-array surface.

Table 1
Relation between time and mean diffusional distance of a 1000 bp single-stranded DNA ($D_{mol} \approx 10^{-11}$ m^2/s)

Time	Distance (mm)
2 h	0.38
8 h	0.76
16 h	1
6 days	3
2 months	10

Shear-driven flows have already been proposed as a solution for the pressure-drop limitation in on-chip liquid chromatography [7–9], and have the unique property that they can transport extremely thin fluid layers at very high velocities, without the aid of an electrical field and much faster than what is possible with a pumping system. The flow driving principle is very simple, and relies on the dragging action exerted by a moving surface on an adjacent fluid layer. The resulting velocity profile is linear ($u=0$ near the wall carrying the target spots, $u=u_{wall}$ near the moving wall), such that, due to the rapid diffusive radial equilibration, all liquid molecules travel at a mean velocity equaling one half of the moving wall velocity, independently of the channel thickness. In its most extreme limit, it could even be possible to use flow channels which are only a minimal number of times larger than the probe molecules, thereby nearly completely eliminating the time needed for radial diffusion (cf. y-direction in Fig. 1b), whereas the transport in the lateral direction (i.e., from spot to spot in the x-direction, see Fig. 1b) occurs by rapid convection (literally guiding the sample DNA strands from spot to spot).

In the present paper, the emphasis is on the potential increase of the hybridization rate which can be obtained by inducing a lateral convective flow past the chip surface. In a later stage, the further increase of the hybridization rate which can be expected from the use of thinner, and hence kinetically more advantageous channels will be investigated. The latter is however less important, because the diffusion limitation in conventional DNA chips is much more pronounced in the lateral direction (where the diffusion distances are of the order of centimeters) than in the radial direction (where the diffusion distances are only of the order of 50 μm). Furthermore, by decreasing the channel height, the amount of sample which is contacted with the target spots is reduced as well, unless more concentrated samples would be used in order to keep the total amount of DNA constant.

2. Operating principle and system design

Although the basic principle is quite straightforward, the inevitable evaporation of the sample liquid waiting to be dragged past the array surface requires an inventive design of the total system (cf. Fig. 1b). As we wanted to develop a system allowing the use of conventional microscope slides (still the substrate of choice in the majority of DNA screening laboratories and the sole format compatible with our laser scanning system), a channel lay-out as depicted in Fig. 2a has been used. With this lay-out, and using two independently movable linear displacement systems, each spot can be contacted with an amount of sample corresponding to a rectangular area with dimensions $a \times b$ (shaded cross-sectional area in Fig. 2b). In the x-direction, a relatively rapid, reciprocal movement with velocity u_x is induced, whereas in the z-direction a slow unidirectional flow is created ($u_z << u_x$). Noting that in a shear-driven flow system the liquid moves at one half of the moving wall velocity [8], the width (a) of the rectangular contact zone is simply equal to one half of the distance L_x over which the channel plate is moved in the x-direction:

$$a = \frac{L_x}{2} \tag{2}$$

In the z-direction the rectangular contact zone then extends over a distance (b), given by:

$$b = u_z t \tag{3}$$

wherein u_z is the fluid velocity in the z-direction and wherein t is the duration of the experiment. With the presently considered u_z velocity of $u_z = 40$ μm/min and with the employed reciprocal displacement distance of $L_x = 5$ mm, contact areas between 1 mm^2 (10-min experiment) and 6 mm^2 (60-min experiment) have been established (see Table 2). This is comparable to, and even larger than the contact area of about 3 mm^2 in an typical overnight experiment.

With the presently adopted approach, only part of the chip surface can be used. The total available surface is however large enough to accommodate some 1000 different target spots and this is already larger than the typical number of different target genes one is after in a typical DNA screening study. The current concept of shear-driven convective flow

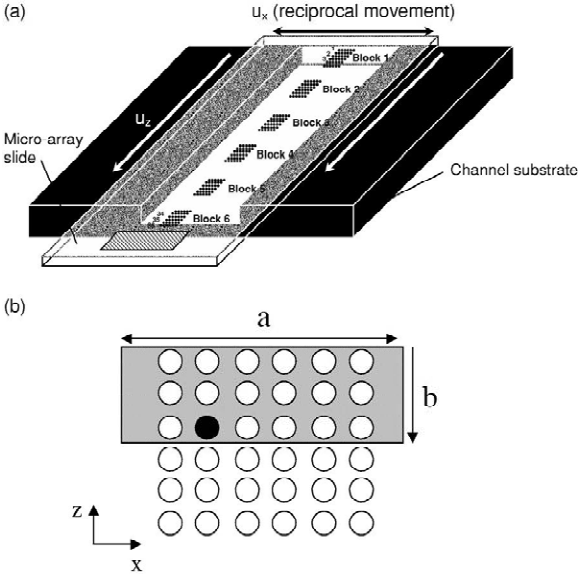

Fig. 2. (a) Birds eye view of the channel set-up and the microarray lay-out. (b) Detailed view of the sample liquid fraction (shaded area) brought into contact with a given target spot (black circle) in the two-dimensional shear-driven contacting mode.

generation can however also be employed in a variety of other designs wherein the number of target spots is much larger. One such design is based on the use of a circular channel etched into a round glass or silicon substrate, onto which a circular DNA microarray (spotted on a flat glass or plastic substrate) can be arranged. Considering for example a circular channel with a relatively narrow width of 2 mm and a radius of 5 cm (corresponding to the radius of the standard 10 in. wafer diameter; 1 in. = 2.54 cm), it can easily be calculated that such a system allows to accommodate some 10 000 target spots of diameter 200 μm (eight circles of approximately 1250 spots). During one rotation, each spot then sees about 1/8 of the total sample, a dramatic increase over the frac-

Table 2
Relation between contacted area and contact mode

Contact mode	Contacted area (mm^2)
Diffusion-driven (overnight)	3
Convection-driven (10 min)	1
Convection-driven (20 min)	2
Convection-driven (30 min)	3
Convection-driven (60 min)	6

tion of the sample reaching the spots during a conventional, diffusion-driven overnight hybridization assay (about 0.2%, see above).

3. Experimental

3.1. General procedure

All experiments were conducted using conventional micro-array procedures. To simplify the experiment, only six different target molecule types (cDNA fragments) were spotted. All different target strands [Nras (716 base pairs (bp)], PolA (330 bp), Rad52 (683 bp), Nia12E (914 bp), Nia12F (1000 bp) and Nia12G (1400 bp), were obtained by polymerase chain reaction (PCR) amplification and were arrayed on aminosilane coated slides (Takara, USA) using a commercial Generation III Array Spotter (Amersham Biosciences, Buckinghamshire, UK). The targets were all spotted at a concentration of 200 ng/μl. The spots (diameter 200 μm and spaced 50 μm apart) were grouped in six different blocks, regularly spaced across nearly the entire central region of the microscope slides (cf. Fig. 2a). Each block grouped six different rows, each consisting of six spots of the same target DNA (see also below, Fig. 6).

To investigate the occurrence of non-specific hybridization events, the probe DNA sample only contained matching DNA strands for three of the six different target types (Rad52, Nras and PolA). These probes were prepared by performing a nick translation with a labeled dCTP (Cy3) on a sample mixture containing the different DNA fragments. After purification, the probes were mixed with Huntsman hybridization buffer to a final total DNA concentration of 1 ng/μl. Prior to each experiment, the concentration of the DNA-samples and the amount of incorporated Cy3 molecules was measured with a NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). After heat denaturation of the probes, 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) to remove the non-specifically bonded DNA strands. The slides were subsequently rinsed with Milli-Q water and finally dried by centrifugation. The slides were then scanned at 532 nm using a commercial Generation III Scanner (Amersham Biosciences). Image analysis was done using Array Vision (Imaging Research, Canada). The hybridization intensities are finally represented in terms of the signal-to-noise (S/N) ratio, defined as the spot intensity minus the background intensity and divided by the standard deviation of the background intensity.

As is common for DNA micro-arrays, the employed slides could only be scanned once. This implies that, in all the presented time series, each different experimental point relates to a different experiment, performed with a different slide and with freshly prepared sample, started at time $t=0$ and stopped after the desired analysis time.

3.2. Fluorescence intensity calibration experiment

To investigate the relation between the amount of hybridized Cy3 labels and the resulting fluorescence intensity the following calibration experiment was performed. First, a batch of PCR amplified and purified Rad52 was labeled with Cy3 using a nick translation reaction. From the prepared batch, two sevenfold dilution series were made to obtain a broad concentration range. A 0.8-nl volume of each dilution was then spotted on a micro-array slide and the fluorescence intensities were measured.

3.3. Regular overnight hybridization experiments

All regular (i.e., diffusion-driven) overnight experiments were conducted according to the standard procedure of the MicroArray Facility Laboratory. The procedure simply consists of pipetting 30 μl of the sample and putting it on the micro-array slides, which were then topped by a thin glass cover-slip (2×5 cm wide). The slides were subsequently kept overnight (16 h) in an incubator at 42 °C.

3.4. Shear-driven flow hybridization experiments

The shear-driven flow experiments were carried out in wide micro-channels (length = 5 cm and width = 1 cm), etched in flatly polished borosilicate glasses (Radiometer Nederland, The Netherlands) by

hydrofluoric acid (HF)-etching in a 10 or 50% HF solution (Fluka, Buchs, Switzerland). After the etching, the channel depth was measured using a Talystep apparatus (Rank Taylor Hobson, UK). The channel substrates were subsequently coated hydrophobically: 2 h in a 1 *M* KOH solution (E. Merck, Darmstadt, Germany), 1 h in a 0.03 *M* HCl solution (Fluka) and overnight in a methanol solution (Fluka) with [3-(heptafluoro-isopropoxy)propyl]-trichlorosilane, 97% (Aldrich, Steinheim, Germany). All experiments were effectuated with an automated translation stage (M-531.5iM-Intellistage; PI, Karlsruhe, Germany), controlled with Macro editor software for M-511.5i IntelliStages (PI). For a schematic view of the set-up, the reader is referred to Fig. 2a. In practice, the channel substrate was attached to the translation stage using a self-built holder, while the micro-array slide was held in place using a stationary holding frame. Intimate contact between the channel substrate and the micro-array slide was maintained by putting a relatively large mass of about 200 g on top of the micro-array slide. As the two channel parts are completely detached from each other, the micro-array slide can simply be picked up from the channel substrate after the experiment. All experiments presented in Figs. 4 and 5 were conducted with a u_z velocity of 40 μm/min and a u_x velocity of 0.05 mm/s.

4. Results and discussion

All experiments were conducted in a concentration range wherein a nearly linear relation ($R^2 = 0.96$) between the amount hybridized (DNA concentrations varying from 6 to 0.06 ng/μl) and the measured Cy3 fluorescence intensity exists.

To investigate the influence of the flow velocity on the hybridization rate, a broad range of different flow velocities has been investigated. The results are given in Fig. 3. As can clearly be noted, there is a relatively broad range of flow velocities up to 1 to 2 mm/s wherein the hybridization intensity remains large. At flow velocities larger than 2 mm/s, the contact time τ during which the individual DNA molecules dwell past a given spot apparently becomes too short to have a sufficiently large binding

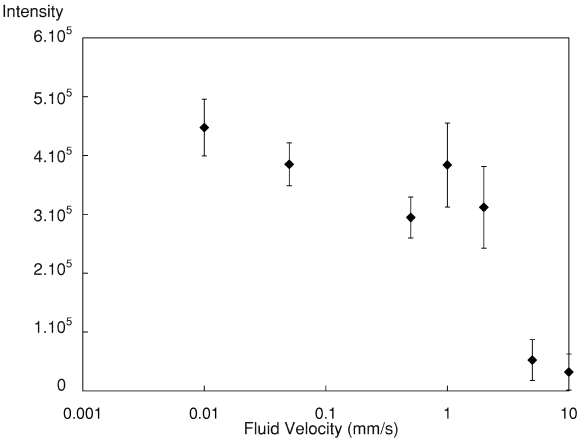

Fig. 3. Variation of the recorded hybridization intensity of Rad52 as a function of the flow velocity. Channel depth = 30 μm. Displacement in the *x*-direction only. The total contact time between the fluid and the target spots was equal to 10 min for all the presented experiments.

probability. This contact or dwell time τ is simply given by:

$$\tau = d_{\text{spot}}/u_x \quad (4)$$

wherein d_{spot} is the spot diameter and u_x the fluid velocity in the *x*-direction. Ideally, this dwell time should be selected such that it is equal to the time needed for hybridizing all the matching DNA strands present in the entire fluid layer segment topping a given spot. A more detailed analysis of the relation between the flow velocity and the hybridization rate will be presented in a future report.

Fig. 4 shows the obtained evolution of the *S*/*N* ratio of the hybridized spots as a function of the analysis time for a number of typical experimental conditions. For convenience, only the data for the Rad52 targets are shown, but it should be noted that fully similar plots are obtained for the Nras and PolA target. For the PolA probe, the shear-driven data in fact consistently compared more favorable to the regular overnight than the Rad52 data shown in Fig. 4. As can be noted, the hybridization pattern obtained with the shear-driven contacting mode is quite uniform and comparable to the spatial uniformity of the regular overnight experiments. Spatial variations of the hybridization intensity are quite common for DNA hybridization assays [10], and are often blamed on the non-flatness of the microscope slides and the

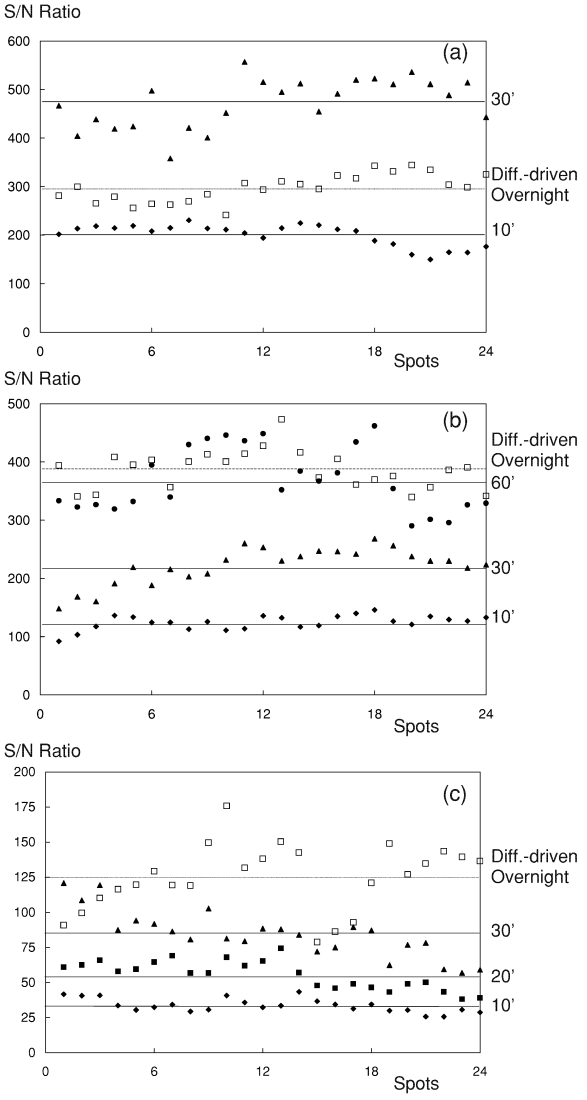

Fig. 4. Spatial variation of the hybridization intensity (S/N ratio) of Rad52 as a function of the spot number (referring to the position on the slide, see Fig. 2) for different values of the analysis time ($'$ = min) in the shear-driven contact mode (full symbols). The regular overnight experiment (duration = 16 h, open symbols) was always performed with sample taken from the same batch as used in the longest shear-driven experiment. (a) Time series A: total DNA concentration = 1 ng/μl; channel depth = 16.0 μm. (b) Time series B: total DNA concentration = 1 ng/μl; channel depth = 5.5 μm. (c) Time series C: total DNA concentration = 0.2 ng/μl; channel depth = 5.5 μm. The full horizontal lines represent the spatially averaged S/N values.

inevitable differences in DNA concentration of the individually spotted target spots. Given that each set of experimental points (= each row of symbols) stems from a different experiment, conducted with a freshly spotted slide and freshly prepared sample, and given that the difference in hybridization intensity correlates very well with the analysis time (see below, Fig. 5), it can be concluded that our prototype set-up operates in a robust and reproducible way.

The intensities of the shear-driven experiments and the regular overnight experiments cannot be compared directly, due to the different amounts of

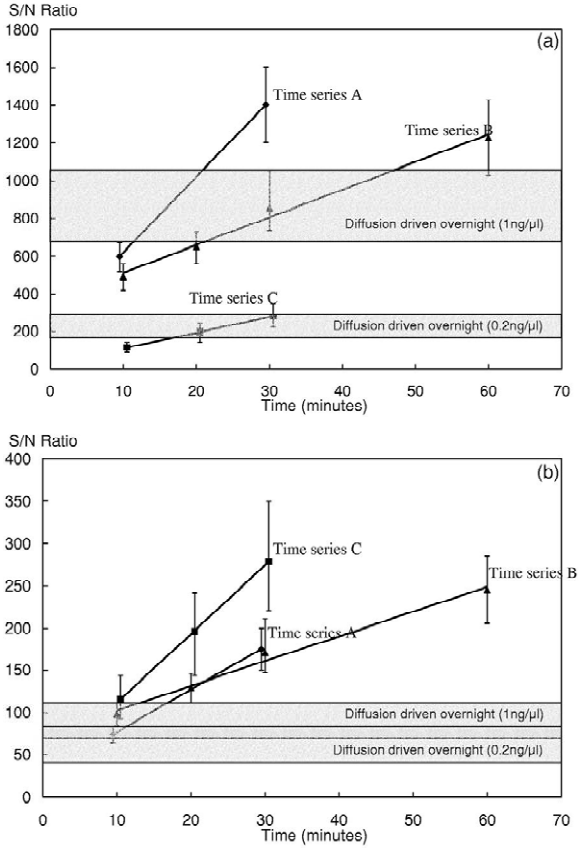

Fig. 5. (a) Evolution of the spatially averaged total S/N ratios (summed over the three different probe strands: Rad52, Nras and PolA) with the analysis time for the time series A, B and C represented in Fig. 4. The shaded areas represent the range of average S/N ratios obtained for the conducted regular overnight experiments. (b) Same data as above, but now corrected (see text) for the different amounts of DNA with which the micro-arrays were contacted.

sample which are brought into contact with the array. In the shear-driven experiments, the fluid layer height (a direct measure for the amount of DNA brought into contact with the target spots) is determined by the channel depth, respectively, $d = 16.0$ μm in Fig. 4a and 5.5 μm in Fig. 4b and c. In the regular overnight experiments, the fluid height is controlled by the total applied sample volume, which was 30 μl for the total effective slide area of 5.5× 2.25 cm (size of the employed cover slip). This corresponds to a liquid layer height of about 25 μm, which is, respectively, about 1.5- and four-times larger than the depth of the micro-channels used for the shear-driven experiments. This implies that in the regular overnight experiments the array is contacted with proportionally more sample than in the shear-driven micro-channel experiments. The influence of the channel layer height can for example also be noted by comparing the S/N ratios obtained in the 16 μm channel (Fig. 4a) and in the 5 μm channel (Fig. 4b): for the 30-min experiments, the spots in the 5 μm channel reach an average S/N ratio of about 220 (Fig. 4b), whereas in the 16 μm channel an average S/N value of about 480 is obtained in the same time (Fig. 4a). The influence of the DNA concentration was verified by lowering the DNA concentration by a factor of 5 (Fig. 4c).

Fig. 5 shows the total S/N ratios (summed over the three different probes: PolA, Nras and Rad52) of the time series shown in Fig. 4. Despite of the fact that each data point refers to a different experiment (DNA micro-analysis are reputed to have a poor run-to-run reproducibility), all different time series display a clear linear relation between the amount of hybridized DNA and the analysis time. This shows that in the shear-driven contacting mode, the hybridization intensity increases in a linearly proportional way with the amount of DNA brought into contact with the target spots.

The different intensity levels in Fig. 5a correspond qualitatively to the differences in channel depth (time series A versus time series B) and to the differences in employed DNA concentration (time series B versus time series C). To correct for the fact that in the $d = 16$ μm channel (time series A) the target spots are contacted with about three-times more DNA than in the $d = 5$ μm channel (time series B and C), the S/N ratios of time series A have been divided by this factor and are represented as such in Fig. 5b. In a similar manner, the S/N ratios of the overnight diffusion-driven experiments have been divided by a factor of 4.4 to account for the fact that the fluid layer height during these experiments was about 25 μm (see above). In addition, we have also divided the experiments conducted at a concentration of 1 ng/μl by a factor of 5, in order to compare them with the 0.2 ng/μl experiments on the basis of equal amounts of contacted DNA (time series C). The results of these corrections are plotted in Fig. 5b. It is interesting to note that the shaded areas representing the overnight experiments performed at different concentrations nicely overlap after the correction. From Fig. 5b, it can be concluded that, when comparing the diffusion and the convection-driven contact mode on the basis of equal amounts of contacted DNA, the overnight level is already reached after about 10 min, and that the overnight hybridization level can be surpassed by about a factor of 3 in some 30 to 60 min time.

Apart from yielding large hybridization rates, it is also an absolute prerequisite that the increased hybridization rates are not accompanied by an increase of the number of non-specific bindings, i.e., the measured intensity signal should only come from the hybridization of exactly matching DNA strands and not from non-specifically bounded DNA. Fig. 6 clearly shows that this is not the case, even not under the strongest hybridization conditions (30 min data from time series A). The small residual signal for the Nia12E probe was also present in the regular overnights, and is hence not caused by the intensified hybridization mode.

5. Conclusions

As could readily be anticipated, the generation of a shear-driven convective flow past the surface of a conventional DNA micro-array allows to drastically enhance the speed of analysis. For the presently considered channel thickness, the analysis time needed to obtain a certain hybridization level decreased from overnight waiting for the traditional diffusion-driven assay down to 30 min for a convection-driven assay, without leading to any non-specific hybridization events and false positive measure-

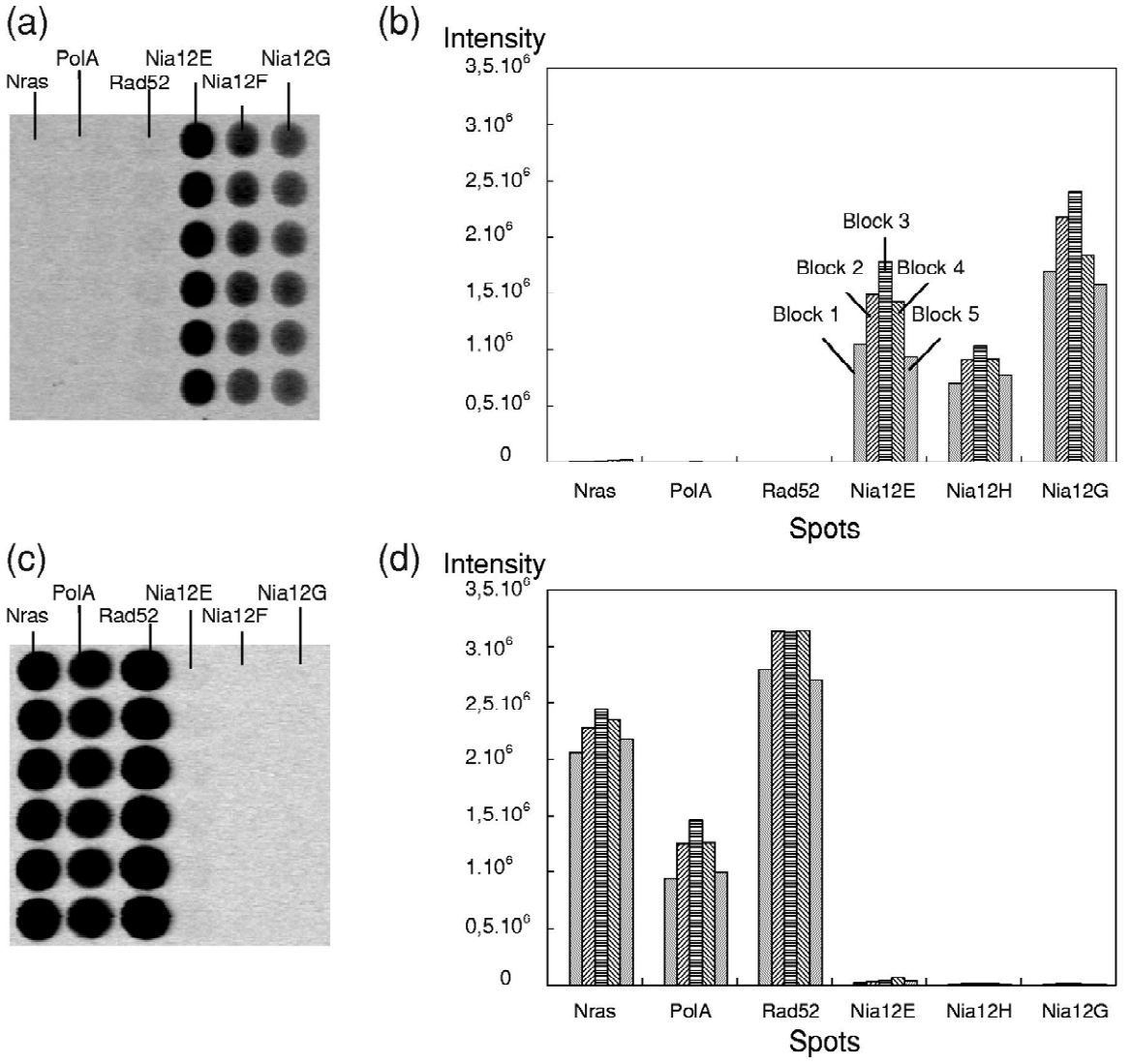

Fig. 6. (a, b) Fluorescence intensity image of given target spot block (block 1) and scanner intensity read out of all blocks after 2 h diffusion-driven hybridization with an M13 primer solution (only expected to hybridize specifically to the Nia12E, Nia12F and Nia12G spots) and (c, d) the same information for the 30-min shear-driven hybridization experiment (data point with largest S/N ratio in Fig. 5) using the normal probe mixture (only expected to hybridize specifically to the Rad52, Nras and PolA spots).

ments. Comparing the data on the basis of equal contacted DNA amount, the overnight level is even already reached after about 10 min. Over the considered time span of 1 h, the shear-driven contact mode allows one to increase the hybridization intensity in a linearly proportional way with the analysis time, i.e., with the amount of DNA contacted with the spots. This suggests that it should be possible to achieve much lower detection limits, eventually capturing each matching probe strand present in the mixture.

Future research efforts will involve a more complete investigation of the optimal displacement velocities, an investigation of the influence of the channel height, and the design of channel and micro-

array lay-outs allowing to accommodate more target spots than what is possible with the current channel system.

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