Enhancing the Microarray Signal Detection of Single Nucleotide Polymorphisms (SNPs) by Using Homemade Immobilisation Buffers

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Our research is focused on improving the detection capabilities of single nucleotide polymorphisms (SNPs) with pathological significance within breast cancer 1, early onset gene (BRCA1). We employed the BRCA1 canonical probe sequence containing the common C allele and probes including the mismatches caused by C replacement with any of the other nucleotides (A, G or T). Two immobilization buffers with distinct pH and salt concentration were involved in the experimental and statistical analyses in order to assess the detection specificity and stability over time of the tethered probes in oligonucleotide microarrays. A high spreading of data points (standard deviation 0.16) was acquired from the fluorescent hybridization signal of the DNA probes diluted in Phosphate Buffered Saline (PBS), while the Disodium Phosphate (Na_2HPO_4) buffer was recommended by a standard deviation of 0.08 for further investigations. The high specificity of mismatch detection of DNA probes diluted in Na_2HPO_4 buffer was established by Student's t test, revealing the statistical reliability of the DNA-printed slides after 1, 4, 8 and 21 days.

Keywords: Microarray technology, immobilisation buffers, probe stability, SNP, BRCA1

Single nucleotide polymorphisms (SNPs) are genetic modifications which usually do not have noticeable influence on the life quality of individuals, but the development of various health conditions may occur when either a particular gene or the gene regulatory region is affected [1,2]. Therefore, these variations are used as biological markers for tracing the links between a gene and various predispositions or health conditions; moreover, such markers are often used to predict the response of a patient to certain drugs or to various environmental factors [3,4].

DNA microarray technology revolutionized the areas of genetics and molecular biology in the mid 90's by facilitating the simultaneous investigation of the expression levels for thousands of genes [5,6]. Later, this technique also proved useful for SNP genotyping and has had a key role in the identification of DNA variants which increase the predisposition to different disorders [1,7,8]. Nowadays, vendors such as Affymetrix and Illumina sell competing SNP arrays which provide increased coverage to examine genetic dissimilarity in human populations [9,10]. The advantages held by commercial platforms are the good SNP detection as a result of stream line production and highly standardized setup [11]. The disadvantages are represented by the high costs associated with creating customised arrays. This is why the homemade arrays are in the focus of many research groups due to the low cost, the possibility to select genes from any species and to work on any kind of support, and with any suitable type of functionalisation [12,13]. Our previous studies were mainly focused on assessing the discrimination efficiency between perfect-matched (PM) and mismatched sequences (MM) by developing 3D microarray platform on silicon, [14] but the issues in microarray related to poor reliability due to hybridisation signal intensity variation remained unexplored.

In general, one of the major and recurrent problems for any type of microarray is given by the large quantity of parameters which influence the results [15]. The printing of probes and their immobilisation, the blocking step and the hybridisation are factors which can be source of dissimilarity and they need to be optimised to reach stable experimental results. Thus, it is required to minimise the causes of variation step by step [16,17].

The optimum binding and maintenance of the probes throughout the entire printing/blocking/hybridisation/washing process, depends on three factors: the affinity of the modified single stranded DNA (ssDNA), the properties of the slide surface, and the ambient conditions, [18] which are necessary to control for attaining a reliable microarray assay [19-21]. Thus, it is essential to analyse the stability of tethered probes in the immobilisation buffer of choice, in order to avoid weak hybridisation results. Besides the reliable commercial immobilisation buffers, alternative homemade solutions are used to enhance the yield of probe attachment [22]. Moreover, the chemical stability of the slides sealed in their original foil pouch is guaranteed by the manufacturers, allowing their storage under dry conditions for an extended period of time. However, the studies regarding the stability over time of the printed slides which are subjected to several washing steps are scarce. Thus, Call et al. [23] investigated the unmodified and amine-modified probes' stability over time (0 - 16 h) printed on acid-washed or silanized surfaces, and found no significant differences in hybridisation signal attributable to soaking in 1x saline sodium citrate (SSC) for the aforementioned period of time. Gerdtsson et al. [24] have the first comprehensive storage stability evaluation of antibody arrays, up to 42 days prior to assay processing, reporting that the activity of the arrayed antibodies increased with storage time up to ten days. To the best of our knowledge, no thorough investigation of hybridisation specificity and sensitivity on slides preserved at 4°C after immobilisation and blocking steps has been reported.

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In this paper, we investigated the immobilisation buffer as a source of experimental variation, analysing the stability over time of the tethered probes subsequently to the blocking and washing steps, a parameter which has not been methodically evaluated so far. Considering the statistical power enhancement when a higher number of technical replicates are employed [25], the effects of the mismatches in terms of specificity was investigated in *BRCA1* canonical probe sequence (containing the common C allele) having perfect complementarity with the target molecule, whereas the other three variants of *BRCA1* probe sequences were designed to have the C nucleotide replaced with another nucleotide (C > A, C > G and C > T). The oligonucleotide sequences corresponding to *BRCA1* gene were chosen because any modifications, such as single nucleotide polymorphisms, increase the lifetime risk for the development of both breast and ovarian cancer [26,27] as revealed by SNP arrays [28].

We demonstrate herein that using 200 mM Na_2HPO_4, *p*H 8.5 as immobilisation buffer increases the efficiency of hybridisation between the complementary target oligonucleotide and *BRCA1* canonical probe sequence (which has a free aliphatic amine modification at one end), enhancing the microarray sensitivity. Furthermore, the average quality of the spot morphology and the detection specificity were not substantially affected by the storage of the printed slides for 21 days. The study implied a high number of technical replicates which increased the statistical nucleotide change discrimination for SNP detection.

Experimental part

Materials and reagents

The Superaldehyde 3 Premium Microarray Substrates were purchased from ArrayIt Corporation (Sunnyvale, USA).

The probes were provided by Biomers.net (Germany).

Phosphate buffered saline (PBS), sodium phosphate dibasic (Na_2HPO_4), sodium dodecyl sulfate (SDS), bovine serum albumin, fraction V (BSA), sodium citrate, sodium chloride (NaCl), Ficoll 400, Polyvinylpyrrolidone (PVP) were supplied by Sigma-Aldrich.

Nuclease-free water and coverslips were purchased from Roth (Germany). Herring sperm DNA was obtained from Promega (USA). Adhesive films for microtiter plates were purchased from EXCEL Scientific (USA). Sterile microtiter plates were acquired from BRAND (Germany).

Microarray experiment

For this study, we employed oligonucleotide probes and target sequences which correspond to *BRCA1* gene, as presented in table 1.

The design of the probes and target oligonucleotides was performed starting from the *BRCA1* gene sequence (indexed in NCBI) [29], involving a C6 amino-link modification at 5' end with three types of single nucleotide mismatch in the same locus. The mismatched sequences were designed in accordance with the SNPs reported for the pathogenic allele, available in The Single Nucleotide Polymorphism Database (dbSNP) of nucleotide Sequence Variation [30,31]. If found in homozygous form (two copies), the Rs28897696 polymorphism enhances the risk of developing breast or ovarian cancer [32].

The complementary target sequence had a Cy3 dye attached to the 5' end, required for the fluorescent detection of hybridisation.

Two different immobilisation buffers were used to prepare the probes for the microarray experiments: (i) 10 mM phosphate buffered saline (PBS), *p*H of 7.4; and (ii) 200 mM Na_2HPO_4, *p*H of 8.5, respectively. and the spotting was conducted at a concentration of 50 µM with an Omni Grid Micro Contact printer (Genomic Solutions). During the experiment, we controlled the duration and the contact force of the pins with Ø 200 nm tips, while the plotting parameters were maintained at previously established values [33]. The covalent binding of the probes to the surface was attained by overnight incubation of the glass slides at room temperature and constant 80% humidity in the printer chamber. The unbound probes were removed from the slides by washing the slides for three times successively in each of the following solutions: (i) 2x SSC/ 0.1% w/v SDS; (ii) 1x SSC; (iii) deionized water (DIW). The blocking of unreacted sites was performed by immersing the microarray platforms for 1 h, at 42 °C and 450 rpm in a preheated solution (42°C) of 1% w/v BSA in 5x SSC and 0.1% w/v SDS.

The hybridisation step was carried out using a solution containing the labelled complementary sequences diluted to 10 µM in a buffer preheated to 60°C, which consisted of 2x Denhardt's solution, 10x SSC and 200 µg/mL herring sperm DNA. The hybridisation solution was dispersed on the surface by encasing each biochip with a coverslip and the target evaporation was prevented by incubating the glass slides in a humid chamber for 3 h at 42 °C. Prior to scanning, the same washing conditions mentioned after the immobilisation step were employed to remove the unbound target sequences from the microarrays.

Investigation methods

The hybridized DNA was detected with a laser scanning confocal fluorescence system (GeneTAC UC4 Microarray Scanner, Genomic Solutions). The microarray slides were scanned at pixel size of 5 µm and with the Cy3 (532 nm)

BRCA1		
Perfect Matched Sequence (PM)		5'-*C6-NH_2*-CTAGGAATTGCGGGAGGAAAATGGG-3'
Sequences with 1 mismatch (MM)	C>A	5'- *C6-NH_2*-CTAGGAATTG**A**GGGAGGAAAATGGG-3'
	C>G	5'- *C6-NH_2*-CTAGGAATTG**G**GGGAGGAAAATGGG-3'
	C>T	5'- *C6-NH_2*-CTAGGAATTG**T**GGGAGGAAAATGGG-3'
Complementary sequence (C)		5'-Cy3-CCCATTTTCCTCCCGCAATTCCTAG-3'

Table 1
PROBE AND TARGET SEQUENCES

excitation laser, adjusting the photomultiplier (PMT) gain and the background cut-off to obtain the accurate quantification of the microarray assay. The raw images were imported into GenePix® Pro 7 Software for spot detection and quantification of the intensity of hybridisation signal. None of the obtained data points were labelled as *bad* at this stage; they were eliminated afterwards from the graphical and statistical analysis of the mismatches.

Statistical analysis

All experiments include 320 technical replicates/probe type and, unless otherwise stated, values in graphs represent average signal intensities with an applied background correction (accomplished by subtracting the local background intensity from the average signal intensity) and pre-processed using $\log_{10}$ transformation [34]. The values situated at 2σ (standard deviation) from the mean were discarded as outliers from the graphical and statistical analysis of the mismatches.

Student's *t* test with Welch's correction, available in GraphPad Prism 5 (Graph Prism Software Inc., La Jolla, CA) [35] was used for the statistical analysis of the hybridisation data. The significance level $p < 0.05$ is considered representative for these tests and it can be found in our tables as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results and discussions

Image analysis of spot morphologies

Figure 1 illustrates the hybridisation results obtained for *BRCA1* oligonucleotides, consisting of a collage of correctly hybridized spots (fig. 1A) and different errors which may occur during a normal microarray experiment (figs. 1B-F), respectively. The spots presented in figures 1A-E were obtained using 200 mM Na_2HPO_4, *p*H 8.5 as immobilisation buffer, whereas for the spots depicted in figure 1F 10 mM PBS, *p*H 7.4 was used as immobilisation buffer.

The experimental accuracy and the biases issued from the overall hybridisation conditions, improper washing or microarray fabrication are evaluated by fluorescent scanning. Figure 1A illustrates conform hybridized spots, having the same size and a perfectly circular shape, which is well discerned from the low and uniform background intensity. For DNA microarrays printed on glass slides, much of the variation might appear during the deposition of DNA oligonucleotides onto the solid support [36,37]. In Figure 1B, some of the spots' centres appeared darker, an indication of a poor hybridisation process, which may be the result of uneven distribution of the probe solution, after microarray printing. This effect is often noticed during contact printing, being caused by either the high evaporation rate of the solution and/or by the migration of the solution towards the periphery of the feature. Nevertheless, the former reason is unlikely, since the deposition and the incubation processes were carried at a humidity of 80%.

The irregular spot shapes observed in figure 1C resulted from the scratches on the surface, coming from the way the pins pressed on the surface and affecting the local hybridisation.

In figure 1D, the hybridisation solution was not uniformly distributed and thus, an air bubble was formed between the two slides, causing a local increase in the background signal and a decrease of the spots' intensities.

In figure 1E, the spots appear more doughnut-shaped, even though the spotting solution consisted of 200 mM Na_2HPO_4, *p*H around 8.5. A similar result was reported by Pappaert et al. [38], indicating that in the case of rapid binding kinetics, a large amount of target molecules reach the spot from the sides, not from above, leading to a preferential binding on the perimeter of the spot. Thereafter, the unhybridized target molecules diffuse towards the centre of the spot and bind to the probes.

In figure 1F, the poor results are given by the use of PBS as spotting solution which has physiological *p*H and low salt concentration (10 mM); in this case, a volcano-shaped spot was generated. Here, the reason of weak hybridisation signal might be related to the negative charging of DNA which causes an electrostatic repulsion between the surface and the single stranded DNA probe. The negative charge can be shielded using a spotting buffer with higher salt concentration. The DNA probe used had at one end a

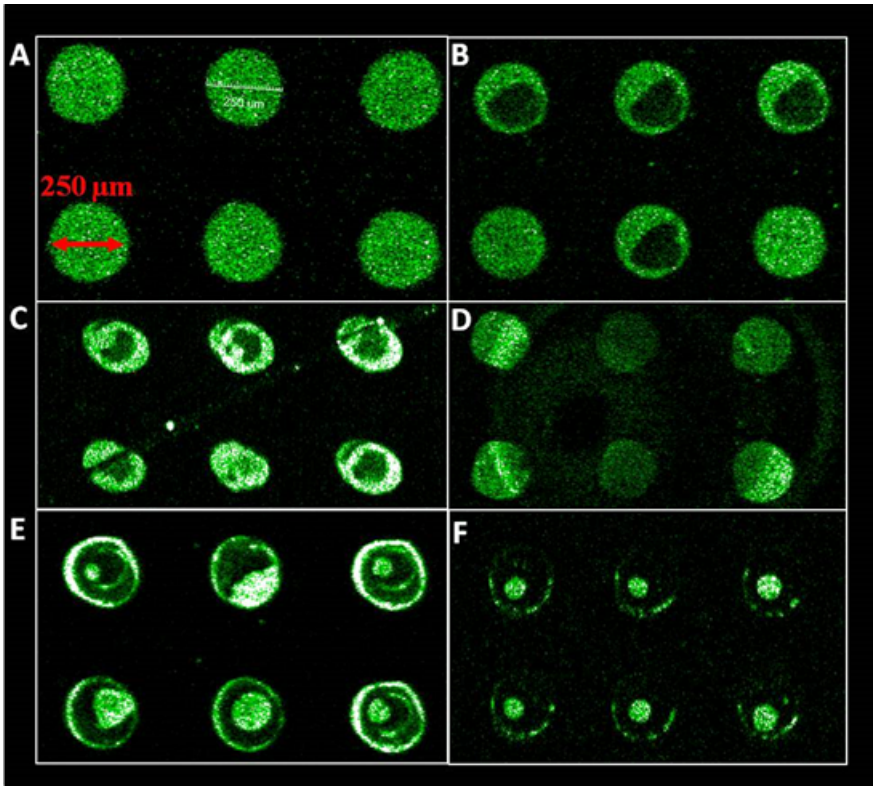

Fig.1. Images of various morphologies of the spots, with a measured diameter of 250 µm. (A) uniformly hybridized spot; (B) doughnutshaped features; (C) scratched biochip and irregular shapes; (D) patchy distribution of the hybridisation solution; (E) irregular hybridisation causing fried-egg effect; (F) "volcano" shaped spot. Slides were scanned using GeneTac UC4.

free aliphatic amine modification, moderately basic. Nevertheless, in the case of aliphatic amines, the concentration of the free base form below *p*H 8.0 is very low, a higher *p*H (8.5-9.5) being optimal for oligonucleotide conjugation.

Graphical comparison of the hybridisation efficiency for the spots prepared in PBS versus Na_2HPO_4 buffer

The choice of spotting buffer represents a key factor for obtaining high quality microarray data with low experimental errors. Consequently, it is important to determine the optimal *p*H and salt concentration for the buffer, in order to identify the best spot morphology. Two spotting buffers were taken into consideration, i.e. 10 mM PBS, *p*H 7.4 and 200 mM Na_2HPO_4, *p*H 8.5. The immobilisation experiments were performed in Petri dishes, at controlled humidity of 80%. After the overnight incubation, the slides were blocked and washed as detailed in *Microarray experiment* section.

The slide spotted with the deposed oligonucleotides diluted in PBS was scanned at a PMT gain of 41% and a background cut-off of 75% because of the noise redundant signal. Secondly, the slide with the probes diluted in Na_2HPO_4 was scanned at a PMT gain of 43% and a background cut-off of 60% because it was less noisy. Thereafter, the variation which comes from the deposition of the oligonucleotides diluted in PBS spotting buffer in comparison with the results coming from the probes diluted in Na_2HPO_4 buffer was graphically analysed (fig. 2). The images of the hybridisation results are presented in figure S1 and figure S2(a) (Electronic Supplementary Material).

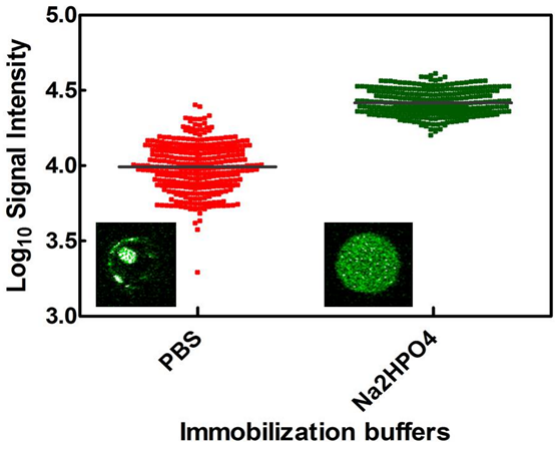

Fig. 2. Assessment of target attachment efficiency to the surface-tethered DNA probes diluted in two different spotting solutions (320 replicates per slide)

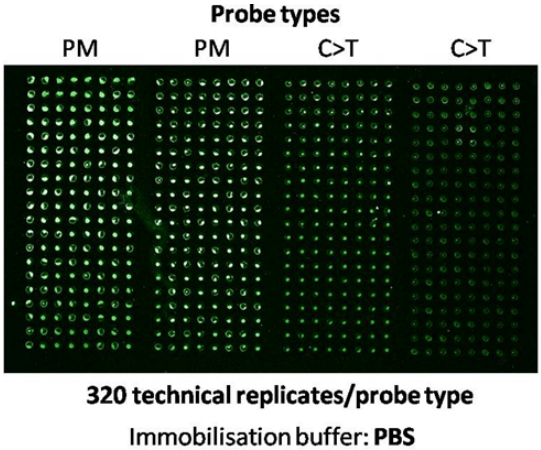

Fig. S1: Fluorescent hybridisation signals of the arrays printed with probes prepared in PBS immobilisation buffer

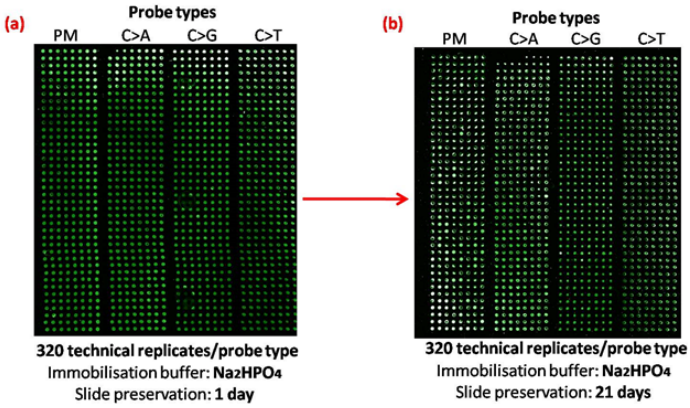

Fig. S2: Visualisation of the immobilisation stability by hybridising the slides after: (a) 1 day of slide storage; (b) 21 days of slide storage of arrays printed with probes prepared in Na_2HPO_4 immobilisation buffer

The hybridisation results clearly demonstrate that the degree of variability is highly dependent on the nature of the spotting solution. A very high spreading of results was observed for the slides with DNA probes diluted in PBS, corresponding to a standard deviation of 0.16 whereas, a significant improvement was achieved when the Na_2HPO_4 buffer was used, resulting a two-fold smaller standard deviation (0.08). It is noteworthy that the spots resulted for Na_2HPO_4 buffer present excellent morphologies and, also, higher signal intensity is obtained. This improvement is an effect of the uniform distribution of the DNA probes due to the higher salt concentration and basic *p*H, which contributed to a reproducible hybridisation signal within an array of many technical replicates.

Probe stability over time

The stability of the tethered probes by hybridising the slides was evaluated after 1 day, 4 days, 8 days and 21 days of slides' storage at 4°C, subsequently to the blocking and washing steps. For this study, only perfect-matched probes were taken into consideration. Because of the different scanning parameters between slides, we finally applied an across-slide correction by subtracting the means from the $\log_{10}$ normalised values, creating a scatter-plot (fig. 3).

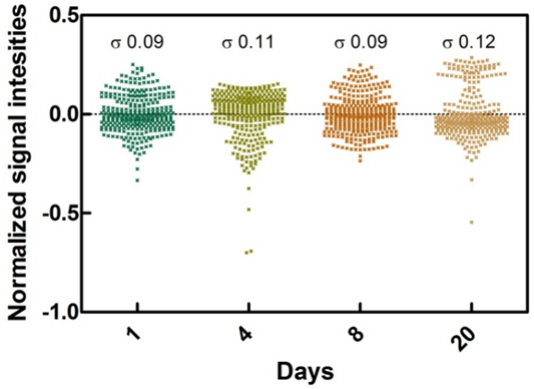

Fig.3. Assessment of the stability over time of surface-attached DNA probes (320 replicates per slide)

The scatter plot correlated with the standard deviations from each set revealed that the average quality of the spots was not substantially affected by the storage over the given time.

Statistical investigation of the mismatch detection capabilities

The mismatch detection capabilities by hybridising the slide with the probes diluted in Na_2HPO_4 one day after the

		Working parameters	
		This work immobilisation buffer: Na_2HPO_4 technical replicates: 320	[14] immobilisation buffer: PBS technical replicates: 4
PROBES	PM vs. C>A	ns 0.2714	ns 0.9073
	PM vs. C>G	*** <0.0001	** 0.0040
	PM vs. C>T	*** <0.0001	* 0.0483
	C>A vs. C>G	*** <0.0001	** 0.0015
	C>A vs. C>T	*** <0.0001	* 0.0136
	C>G vs. C>T	** 0.0015	*** 0.0003
		p-values	

Table 2
STUDENT'S *T*-TEST RESULTS FOR 50 µM PROBE SEQUENCES DILUTED IN NA_2HPO_4 HYBRIDIZED WITH 10 µM TARGET MOLECULES AFTER ONE DAY OF INCUBATION

Table 3
STUDENT'S *T*-TEST RESULTS FOR 50 µM PROBE SEQUENCES DILUTED IN NA_2HPO_4 HYBRIDIZED WITH 10 µM TARGET MOLECULES AFTER TWENTY DAYS OF SPOTTED PLATFORM PRESERVATION

PMvs. C>A	PM vs. C>G	PM vs. C>T	C>A vs. C>G	C>A vs. C>T	C>G vs. C>T
P values					
0.1828 ns	<0.0001 ***	<0.0001 ***	<0.0001 ***	<0.0001 ***	<0.0001 ***

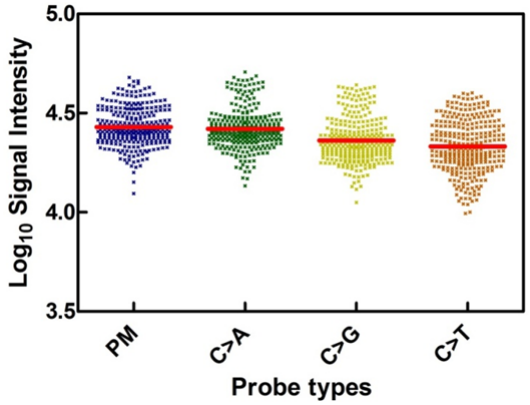

Fig.4. Assessment of the signal intensity trends between the PM probe and the mismatches represented by C > A probe, C > G probe and C > T probe (320 replicates per each probe type)

washing and blocking steps were evaluated. For this study, the perfect-matched (PM) probes were taken into consideration in comparison to the mismatched probes. The scatter-plot obtained in GraphPad Prism 5 programme is presented in figure 4.

The graphical analysis shows that the averages between PM and C > A hybridized oligonucleotides were not significantly different. On the other hand, the average signal intensities for C > G and C > T probes appear lower than PM and C > A hybridized probe types. The results of Student's *t* tests with Welch's correction which complete the graphical analysis are presented in table 2.

The results from the statistical analysis (table 2) show a good distinction of the different mismatch types. Qualitatively, by corroborating these results with previous findings, the results are statistically assessable. However, quantitatively, a larger amount of technical replicates allow a higher accuracy of detection.

Hence, the hybridisation with the C > G and C > T probes displays signal intensities significantly lower in contrast with the PM probes (*** <0.0001), showing also an increased statistical power comparative to the prior results (** 0.0040 – PM vs. C > G; * 0.0483 PM vs. C > T). The same case is for the differences between the mismatches, which are also statistically assessable, with an improved statistical power (*** < 0.0001 C > A vs. C > G; *** < 0.0001 C > A vs. C > T; *** < 0.0001 C > G vs. C > T).

Instead, the signal intensities of PM and C > A probes have close values and the differences between the two categories are not statistically significant (ns 0.2714), the same observation being reported in the prior research (ns 0.9073). Thus, this type of mismatch does not affect the DNA duplex formation and stability [39], the purine-purine base pair fitting perfectly into the B-form standard DNA duplex [40,41].

Statistical investigation of the mismatch detection reliability after 21 days of printed slide preservation

To further investigate stability over time of DNA spots printed using Na_2HPO_4 solution, the slides were stored for different periods before the hybridisation reaction. Subsequently, the mismatch detection capability was evaluated by hybridising the selected slide with the probes diluted in Na_2HPO_4 twenty days after the washing and blocking steps. The perfect-matched probes were compared to the mismatched ones, and the resulting scatter-plot is presented in figure 5. The visual study of the immobilisation stability by hybridising the slides after 1 day and 21 days of incubation is presented in figure S2 (Electronic Supplementary Material).

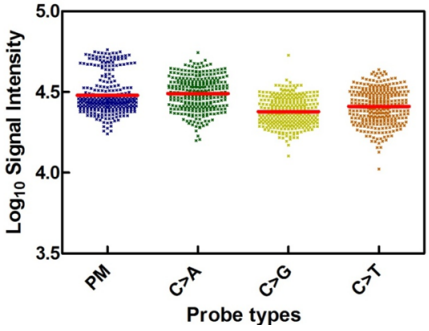

Fig.5. Assessment of the signal intensity stability over time and trends between the PM probe and the mismatches represented by C > A probe, C > G probe and C > T probe (320 replicates per each probe type)

The graphical representation shows that the averages between PM and C > A hybridized oligonucleotides are not significantly different. On the other hand, the bar corresponding to the average signal intensities for C > G

and C > T appear lower than PM and C > A hybridized probe types. The graphical analysis is completed by the Student's t tests with Welch's correction which are presented in table 3.

The Student's *t* tests confirm the hybridisation signal intensities are statistically different between PM and C > G, PM and C > T, as well as between the different probe types. Thus, the statistical analysis reveals that the reliability of the mismatch analysis is not affected by the 21 days preservation of the DNA-printed slides at 4 ℃.

Conclusions

The laser-assisted visualization of the microarrays correlated with the data analysis disclosed superior morphology and higher hybridisation signal intensities when using the 200 mM Na_2HPO_4, *p*H 8.5 immobilization buffer, in the detriment of 10 mM PBS, *p*H 7.4. The higher pH buffer is optimal for amine-modified oligonucleotide conjugation.

The stability over time of the tethered probes was subsequently established for 1 day, 4 days, 8 days and 21 days of incubation, proving the quality of the spot morphology by the scatter plot correlated with the standard deviations of each set, with constant mismatch detection trends. Thus, we determined that using homemade immobilisation buffer is a reliable solution for a reproducible hybridisation and improved signal detection.

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