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A DNA tetrahedral structure-mediated ultrasensitive fluorescent microarray platform for nucleic acid test

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ABSTRACT

Microarrays are key platforms for biomolecule detection owing to their parallelizability and amenability for high-throughput. However, conventional fluorescent microarrays still suffer from low specificity and sensitivity, therefore are unsuitable for detection of low abundance nucleic acids. In this study, we demonstrate a universal microarray platform with high specificity and ultra-sensitivity for fluorescent detection of DNA and micro RNA (miRNA), which employs a DNA tetrahedral structured probe (DTSP) together with a hybridization chain reaction (HCR) based signal amplification technique. By precisely modulating the number of base pairs on each tetrahedron side, we developed three different sized DTSPs: 17 base pairs, 26 base pairs, and 37 base pairs (DTSP-17, DTSP-26 and DTSP-37, respectively). A low detection limit of 10 aM was obtained by DTSP-26, notably much lower than detection limits of prior methods. Furthermore, our microarray platform can distinguish single base DNA mismatches and thus exhibits single nucleotide specificity. Lastly, our microarray platform can be implemented for miRNA detection, as we demonstrate in a mimic medium, demonstrating potential for its use in clinical diagnosis.

1. Introduction

In recent years, many techniques and methods have been reported for nucleic acid detection and testing, including nanopore sensors [1], nanomaterial based optical sensors [2–7], colorimetric sensors [8], northern blotting [9], quantitative polymerase chain reaction (qPCR) [10,11] and sequencing [12]. Microarray technologies are regarded as a promising method in life science research and clinical diagnostics, and are also widely used for nucleic acids detection [13–15] because of their ability to parallelize testing in a high-throughput manner. Different strategies have been developed for improving detection performance of microarray analysis. For example, Quantum dots (QDs) with high fluorescence quantum yields and long lifetimes have been introduced to increase signals [14]. Three-dimensional dextran-coated microarrays coated have also been designed to decrease background signals for the purpose of increasing sensitivity [16]. However, the sensitivity, selectivity, and reproducibility of microarrays remain insufficient for low abundant micro RNA (miRNA) detection [17]. Aforementioned limitations in microarray technologies are due to 1) the softness and easy to fall down of many microarray probes, which can lower the hybridization efficiency, and 2) high probe loading densities needed for the detection of low abundance analytes that often decrease the microarray assay selectivity. Hence, microarray technologies for low-abundance nucleic acid detection is an area requiring new strategies for

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programmable molecular recognition.

DNA nanotechnology has recently attracted much interest due to its nanoscale programmability and inherent biocompatibility [18–22]. DNA molecules can be self-assembled into DNA nanostructures [23] with various sizes, shapes, and geometries for applications in protein structure determination, medical diagnosis, biosensing, and drug delivery [24–36]. Among the many DNA nanostructure form factors is the Tetrahedron DNA nanostructures (TDNs), which was first designed by Goodman et al. [37] and initially used as a probe for biosensing by Pei et al. [28]. Many biosensing platforms have since been established for bioanalysis of DNA, miRNA, protein, and cells, based on DNA tetrahedra [31,38–40]. The advantages of the DNA tetrahedron for molecular detection lies in two aspects: first, the rigid tetrahedral scaffold can fix probes in a straightforward direction. Second, the controllable size can regulate the distance between probes to reduce hybridization steric hindrance [38,40–43]. By programming the size of the DNA tetrahedral structured probe, we can control its surface adsorption properties [44] and improve the effectiveness of probe recognition by optimizing ligand-probe binding. An exemplary study by Li et al. [40] demonstrated that DNA tetrahedron-based microarrays can achieve a 10 fM limit of detection (LOD) for a target miRNA, however, this LOD remains below the ~aM target sensitivity needed for detection of low abundance nucleic acids tests.

Separately, the hybridization chain reaction (HCR) involves the triggering of two stable DNA hairpins by an initiator to form long double helix strings through a cascade hybridization reaction. Labeled DNA can act as an amplifying tool for biodetection and bioimaging applications [16,31,39,45–47] whereby the HCR reaction proceeds to magnify the signal and can significantly increase the sensitivity of nucleotide detection. This amplification method is an enzyme-free reaction that can react at room temperature, and has been shown to achieve a 10 fM LOD [16] when incorporated into a microarray platform. HCR is an attractive platform for signal amplification because it is not an enzyme-coupled method, enabling its implementation in a broad range of pH, temperature, and buffer media conditions [48].

Given the orthogonal advantages of DNA nanotechnology and HCR, herein we developed a platform combining both DNA tetrahedral structured-probes (DTSP) and HCR to produce a microarray platform with aM sensitivity. Firstly, we designed three differently sized DTSPs (DTSP-17, DTSP-26, DTSP-37). Secondly, we employed HCR to increase the sensitivity of our microarray platform by introducing coupling repeat units on the DTSP with fluorescent dyes. Our combined DSTP and HCR based microarray platform achieved a 10 aM LOD for a target low-abundance DNA, and enabled detection of a single-base mismatch. We lastly demonstrated that our microarray platform can be used for miRNA detection in simulated serum, suggesting our platform can be useful in biodetection and clinical assays for low-abundance nucleic acids.

2. Results and discussion

2.1. Design of the DTSP − HCR microarray platform

To address limitations in microarray sensitivity and specificity, herein we combine DSTP and HCR to maximize the performance of microarray technology. In our design, the DTSP contains one ssDNA extension as the detection probe at one vertex, and three amino groups at the remaining three vertices for tetrahedron immobilization via covalent coupling between amino groups on ssDNA and aldehyde groups to form imine bond on the glass surface (Fig. 1). With this approach, the DTSP anchored to the microarray glass surface with well-defined spacing of the probes in a consistently upright orientation, features which both contribute to higher analyte hybridization efficiency [38]. We designed HRC substrates to include an initiator that starts the reaction, and two subunits labeled with Cy3 (H_1 -Cy3 and H_2-Cy3). The initiator sequence was designed into a single stranded DNA hairpin named helper which includes the target complementary sequence, and a capture probe hybridized sequence.

As illustrated in Fig. 1, the DSTP was fixed on aldehyde-functionalized slides, leaving the fourth strand stretched out of the surface as the capture probe. In the presence of DNA or miRNA target, the hairpin structure of helper can be opened, and the initiator sequence and capture probe hybridization sequences become exposed. Once exposed, the HCR cascade reaction is triggered, and the polymerization process proceeds through exhaustion of H_1-Cy3 and H_2-Cy3, yielding fluorescent signal amplification that reads out the presence of the target DNA (Fig. 1).

2.2. Construction and optimization of our DTSP − HCR microarray platform

To evaluate our DTSP − HCR approach, we constructed a microarray platform with a 17 bp side length DSPT (DSTP-17) as the probe. As shown in Fig. 2a, by adding different concentrations of target DNA (DNA155) in a concentration range from 1 fM to 10 nM, we observed an exponential increase (see insert plot for exponential fitting) in the output of signal to noise ratio (SNR) of our microarray, indicating successful assembly of our DTSP − HCR microarray platform. We believe that at lower target concentration (< 10 pM), the signal is determined by how much target was added to trigger the cascade HCR mediated output. Even we have much excess of Cy3 labelled DNAs to generate signal, there is not much site they can bind since only a proportional probe was activated by the target. However, when the target concentration reaches nM, there will be a significant signal increase as shown in Fig. 2a, that is how the HCR strategy is designed. Meanwhile, comparing with the traditional sandwich assay, the HCR strategy showed more than 15 times higher of the SNR output (Fig. 2b). To further optimize our DSTP − HCR microarray platform, we investigated the impact of the following parameters: spotting solution, hybridization temperature, and annealing procedure. The spotting solution is important for microarray performance, as it is used to dissolve and immobilize the probe. An ideal spotting solution should form regular round spots for delineation and analyte quantification that is independent of the spot form factor and minimal autofluorescence. We demonstrated that compared to the Bio Capital Corp commercial spotting solution, DMSO provides a lower background signal and more homogenous microarray spot sizes (Fig. S2). Hence, DMSO was selected as the dissolving and spotting solution for DSTP deposition, and was also used as a negative control. Because temperature is a key factor that will influence hybridization, we tested two hybridization temperatures and observed stronger fluorescent signals at 37°C compared to 44°Cwith 1 pM DNA target in our experiment. Therefore, an annealing temperature of 37°C was chosen for all following experiments (Fig. S2). We also consider that hairpin strands may take undesired secondary structures leading to decreased hybridization efficiency. Thus, we tested our deposition reaction with and without an annealing procedure of pre-incubation of the hairpin strands at 95°C for 10 min. Our results show that fluorescent signals output with annealing step are around 2 times higher (Fig. S2) than those without, therefore we implemented this annealing step for all hybridization procedures.

2.3. Microarray detection sensitivity mediated by different sized-DTSPs

We hypothesize that the hybridization efficiency of the target DNA or RNA with the microarray probe is modulated by the probe density, which can be modulated by controlling the distance between adjacent probes [49]. By modulating the size of the DTSP, we can regulate the distance between probes to assess how DTSP size affects DTSP surface adsorption so as to maximize DNA or RNA analyte hybridization efficiency on the final assembled microarray.

To precisely control the distance between the probes, we designed three DTSPs with different sizes: DTSP-17, DTSP- 26 and DTSP- 37, for

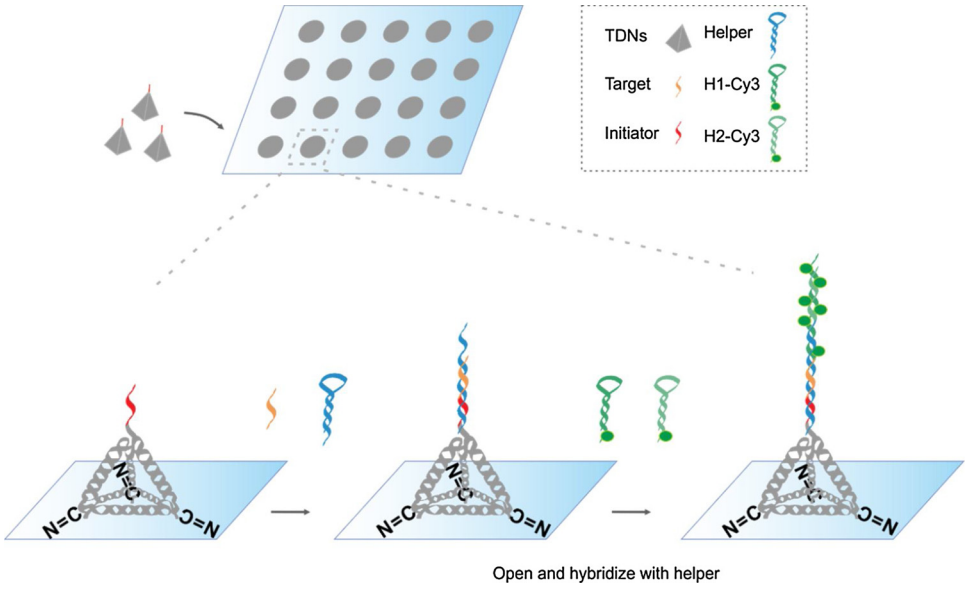

Fig. 1. Schematic illustration of hybrid DSTP – HCR microarray platform for low abundance DNA/miRNA detection. The DSTP is fixed on aldehyde-functionalized glass slides through imine bond, whereby the fourth upward-facing DSTP vertex contains an initiator strand (red). Upon addition of a helper (blue), helper hybridization to the initiator in the presence of the target DNA analyte (yellow) initiates a catalytic binding process with Cy3-labelled oligos (green) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

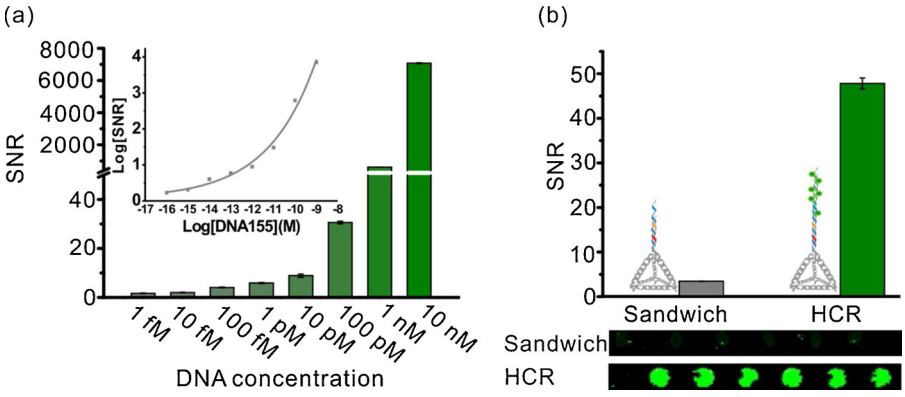

Fig. 2. DTSP – HCR detection calibration. (a) DTSP – HCR strategy shows strong signal output (SNR) amplification with corresponding increased DNA target concentration (from 1 fM to 10 nM). (b) Comparison between HCR and sandwich. The concentration of target is 0.1 nM. Inset: microscopic fluorescence images of corresponding HCR strategy and typical sandwich strategy. The error bars represented the SD of 18 spots from three independent measurements.

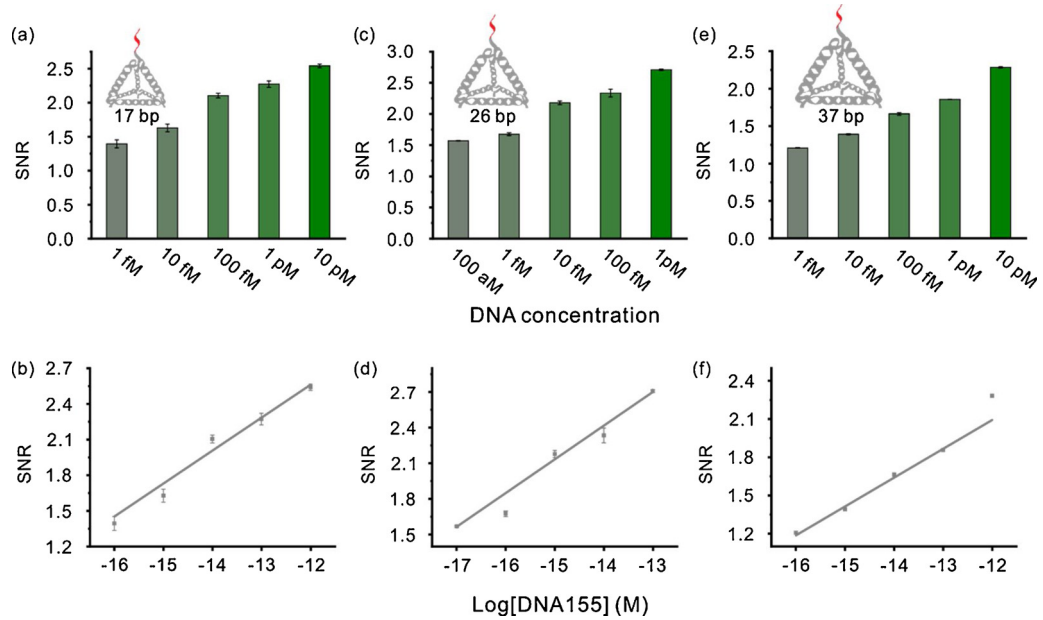

Fig. 3. Detection performance of different sized DTSPs on DNA detection. SNR value increased linearly with the target DNA concentration of DTSP-17 (a, b), DTSP-26 (c, d) and DTSP-37 (e, f). Error bars represented the SD of 18 spots from three independent measurements.

which each edge of the DTSP measured 17, 26 and 37 base pairs with corresponding theoretically calculated sizes of 5.8 nm, 8.8 nm and 12.6 nm, respectively. All DTSPs were designed with no undesired secondary structures and with a monomer probe yield of 41–50 %, as shown by native polyacrylamide gel (PAGE) analysis (Fig. S1). With these differently sized DTSPs, we next investigated the detection performance towards target DNA combined with the HCR strategy. As shown in Fig. 3, the observed SNR signals increased linearly with target DNA concentration in all DTSP sizes. However, each DTSP exhibited different dynamic ranges of target DNA detection: For DTSP-17 (Fig. 3a and b), the linear detection range was from 1 fM to 10 pM with a 1 fM limit of detection (LOD) and a regression equation of $y = 332.59338x + 7018.22952 \pm 374.98475$ ($R^2 = 0.95703$). For DTSP-26 (Fig. 3c and d), the linear detection range was from 10 aM to 100 fM with a 10 aM LOD. For DTSP-37 (Fig. 3e and f), the linear detection range was from 1 fM to 10 pM with a 1 fM LOD. These results support our hypothesis [38] that the density, orientation, and entanglement between DTSP probes can affect their ability to recognize a DNA target. Previous study by Lin [49] proposed that the hybridization thermodynamics/kinetics are dominated by the absolute number of probes instead of mass transport. Compared with DTSP-17, the hybridization efficiency was much higher in DTSP-26 since the distance between probes increases from 5.8 nm to 8.8 nm, which benefits the hybridization process. In contrast, when compared with DTSP-37, the number of probes decreased nearly 2-fold than that of the DTSP-26 (Table S2) [49], which in turn lowed its detection ability. Correspondingly, we find that DTSP-26 has the lowest LOD of 10 aM (Fig. 3d), which is a lower LOD than leading microarray assays [50–54], suggesting our approach is a valuable tool for detection of low-abundance DNA and RNA. For the remainder of our study, we focused on the use of DTSP-26 owing to its aM scale LOD.

2.4. Discrimination ability of our DTSP – HCR microarray platform for low abundance nucleic acid biomarkers

Nucleic acid biomarkers including DNA and RNA are important for diagnosing and monitoring various diseases and the efficacy of their therapeutics [55–58]. However, due to the low abundance and high sequence homology of DNA and RNA biomarkers, detection of nucleic acids and in particular miRNA biomarkers in complex systems remains a challenge.

To test the specificity of our DTSP – HCR microarray platform for detection of low abundance nucleic acid biomarkers, we first tested the detection of DNA targets (DNA 155) to validate our method. DTSP-26 was employed to compare the microarray signal of single-base mismatched DNA sequences, triple-base mismatched DNA sequences, and a perfectly matched DNA sequence. As shown in Fig. 4a, the signals observed from all of the mismatched sequences were less than 10 % of the signal observed from the perfectly hybridized sequence. Therefore, we can distinguish single-base mismatches from target DNA sequences using our microarray method. We contributed this to the high specificity of the design, as we can see in Fig. S3, without the DNA target, the HCR amplification strategy did not work. We next chose three microRNA pancreatic cancer biomarkers [59]: miRNA 155, miRNA 210 and miRNA 196a to evaluate the miRNA detection ability of our microarray platform. We assayed all miRNAs at a 10 fM concentration, chosen to reflect miRNA concentrations in human serum. Comparing to control group with no target, we observed a 3.6, 5.1, and 5.7 fold fluorescence increment by introducing of miRNA 155, miRNA 196a and miRNA 210 targets respectively (Fig. 4b).

Finally, we assayed different miRNAs in a mimic media system (containing 10 % fetal calf serum) to further verify the discrimination ability of our DTSP – HCR microarray in a complex system. Our results show only the matching miRNA targets induce a significant signal compared to mismatched miRNA sequences, and our microarray can easily distinguish different miRNAs even in a mimic system (Fig. 4c), Taken together, our results suggest our microarray system, which combines DTSP and HCR technologies, holds great promise for further clinical study.

3. Conclusions

We present an ultrasensitive microarray platform mediated by programmable DNA nanotechnology, employing DNA tetrahedral structure as probes together with HCR signal amplification technology. Compared to conventional sandwich assays that are implemented to detect DNA and RNA targets, our DTSP – HCR strategy exhibited over 10-fold increased fluorescence signals towards target polynucleotides, which enabled a lower ~aM limit of detection than previously reported for microarray technology. We demonstrated that differently sized DTSP probes demonstrate a linear response to their target nucleotides, suggesting our platform is generalizable for various microarray applications in which DTSP spacing and microarray dynamic range may be desirable tunable parameters. Among targets tested herein, DTSP-26 demonstrated the highest fluorescent signals and the lowest limit of detection of 10 aM, which suggests our approach holds particular promise for low abundance miRNA detection. Moreover, our microarray platform can distinguish single-base mismatches and remains functional for miRNA detection in human serum and FBS-based media. Based on the above results, this ultrasensitive microarray technology could become quite a promising detection and quantification method in clinical diagnosis.

4. Materials and methods

4.1. Sources of materials

All chemicals were purchased from Sangon Biotech (Shanghai, China) Co., Ltd. unless otherwise stated. Aldehyde-functionalized slides were purchased from Capital Bio Corp. (Beijing, China). Dimethyl sulfoxide(DMSO) was purchased from Sigma-Aldrich (St. Louis, Mo). Fetal calf serum was purchased from Invitrogen (Shanghai, China). All oligonucleotides were synthesized and purified by Sangon Biotech Co. Ltd (Shanghai, China), and the sequences are listed in Table S1. The mature miRNAs were synthesized and purified by Invitrogen (Shanghai, China).

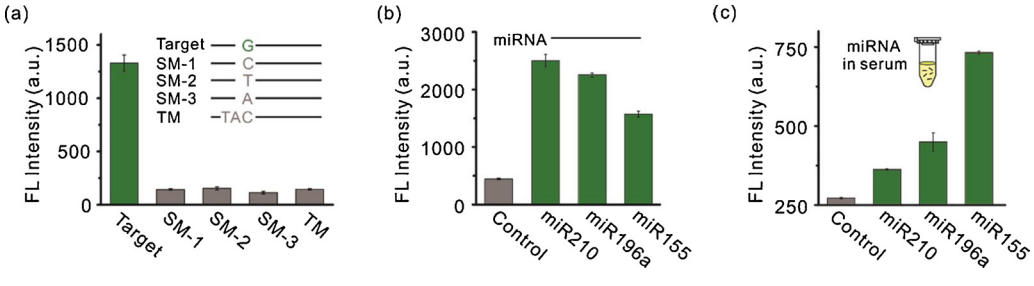

Fig. 4. Specificity evaluation of DTSP-26 towards DNA targets and their mismatches. (a) Fluorescence intensity in the presence of 1 pM DNA of different sequences. (b) Fluorescence response of microarray upon addition of 10 fM of different miRNA targets. (c) Fluorescence intensity of 10 nM miRNA 210, miRNA 196a or miRNA 155 respectively with helper for miRNA 155 in 10 % FBS. The error bars represented SD of 18 spots from three independent measurements.

4.2. Formation of DNA tetrahedron

Different sizes of DTSPs were formed based on modified protocols [32]. Tetra-A and three amino-modified single strands (tetra-B, tetra-C and tetra-D) were added in an equimolar mixture in TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH 8.0), then assembled through annealing at 95℃ for 10 min and followed by cooling to 4℃ in 30 s. The raw self-assembled DSTP was further characterized by 6% native polyacrylamide gel electrophoresis (PAGE).

4.3. DNA spotting

The fabrication of all microarrays was based on the universal spotting method unless otherwise stated. DTSPs in a spotting buffer (DMSO) were printed onto aldehyde-functionalized glass slides using a PersonalArrayer 16 programmed by matching software PersonalArrayer. The average spot diameter was 100 μm, and the distance between neighboring spots was 500 μm. The concentration of immobilized DSTP was 10 μM. The arrayed slides were incubated overnight at 37℃ in a constant temperature incubator. The slides were immersed in an aldehyde blocking solution (4 mg/mL BSA in PBS buffer with 0.2 % SDS) at room temperature for 1 h to passivate unreacted aldehyde groups. After being washed with PBS buffer, the slides were stored in a drying cupboard for further use.

4.4. DNA detection

DNA detection was carried out using both a DTSP-sandwich gap strategy and a DTSP−HCR gap strategy, respectively. Both detection principles are schematically demonstrated in Fig. 1. Before hybridization, the helper for DNA155, H_1-Cy3 or H_2-Cy3 was heated separately to 95℃ for 5 min, and then cooled down to room temperature over 15 min. The mixture containing DNA155 at variable concentrations (10 aM, 100 aM, 1 fM, 10 fM, 100 fM, 1 pM and 10 pM) and helper for DNA155 (2 nM), H_1-Cy3 (1 nM) and H_2-Cy3 (1 nM) were added to the rectangular fence grids adhere to the chip and allowed to adhere to the slides by incubation at 37℃ for 2 h. We repeated the same procedure on other targets with their own corresponding helper. After washing with washing solution A for 2 min, washing solution B for 5 min, and washing solution C for 5 min, fluorescent signals were collected with a Genepix 4100A microarray scanner and analyzed with the accompanying commercial software (Genepix Pro 6.1).The composition of washing solutions is listed in Table S3. The excitation wavelength was 532 nm (Cy3) and fluorescence signals in the range of 575 ± 17.5 nm were collected. To assess ability to discriminate signals between DNA mismatched targets, different DNA sequences with one or three mismatched bases were used instead of the completely complementary sequence.

4.5. microRNA detection

All buffers in the microRNA assays were prepared with DEPC water (DNase and RNase-free). For microRNA detection, the mixture containing miR155 (1 pM), miR210 (1 pM) and miR196a (1 pM) with their own designed helper, H_1-Cy3 and H_2-Cy3 were added to the slide and subject to the same procedure as for DNA detection. To mimic real samples and further demonstrate the detection ability of our platform, miR155, miR210 and miR196a were first mixed into 10 % FBS solution, then added to the platform using the same procedure as for DNA targets described above.

Author contributions

X.Q.M. supervised the whole project and guided writing the manuscript. H.Z. and X.X.L. carried out the experiments, analyzed the data and wrote the manuscript. Y.Z, M.L and L.H.W verified the analytical methods and supervised the findings of this work. X.L.L helped with the spotting experiments. All other authors discussed the results and contributed to the final manuscript.

Notes

The authors have declared that no competing interest exists.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2020.128538.

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