Electronic Supplementary Material (ESI) for Lab on a Chip

Supplementary Section

Programmable and automated bead-based microfluidic devices for versatile DNA microarrays under isothermal conditions

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1. Fidelity of deoxyoligonucleotide hybridization in different buffer solutions suitable for isothermal DNA transfer. When using a pool of different oligonucleotides in hybridization experiments, the goal is to establish uniform conditions for all oligonucleotides that are stringent enough to guarantee specificity yet relaxed enough to allow formation of stable DNA hybrids at an acceptable rate. Different buffer solutions were tested for their capacity of making discrimination between perfectly matching DNA hybrids and those with various mismatches. All tested solutions are able to buffer an equal volume of 100 mM NaOH with a change of their pH values within 0.15 units. This allows the buffer solutions tested to be employed for repeated DNA hybridization transfer in microfluidics under isothermal conditions.

To investigate the fidelity of DNA/DNA hybridization in different buffer solutions, which allow DNA selection under isothermal conditions, a quenching system was used. One of the complementary strands was TAMRA (tetramethylrhodamine) labeled at the 3'-end (DNA20), while the other strands with 0 (DNA21), 5 (DNA22), 3 (DNA23), and 2 (DNA24) mismatches to DNA1 were labeled at the 5'-end with QSY-7 (**Fig. S1**). The hybridization reactions were carried out in different buffering solutions for 30 min at different temperatures. The quenching of the fluorescence signal was measured by a FluorMax-2 spectrofluorometer (Instruments S.A., Inc, Edison, New Jersey) with excitation at 546 nm and an emission signal was computed as the integrated signal from 560 to 600 nm. As a positive control, the fluorescence signal of TAMRA labeled DNA20 was measured without any complementary strand.

The ability of QSY-7 to quench the emission signal of TAMRA quantitatively was demonstrated with two perfectly matching strands as shown in **Fig. S1a**. Each type of hybridization experiments was performed 5 times, and the average results with the corresponding standard deviation are plotted in Fig. S1. First, the fluorescence signal of 1 μM 3′-end TAMRA-labeled DNA20 was measured and used as a control after incubation for 30 min at 25^{o}C in a 100 μl solution of a 500 mM Tris–acetate buffer, pH 8.3, and 50 mM NaCl. The next experiments were performed under the same conditions, but it was added 0.5 μM of 5′-end QSY-7-labeled DNA21 oligomer complementary to DNA20. This resulted, as expected, in 50% quenching of the fluorescence signal measured in the first set of experiments where no DNA21 was

present Fig. S1a. In addition, the same experiments were performed in the presence of equal concentrations of DNA20 and DNA21 (each 1 μM). The obtained data demonstrate that the quenching effect was by a factor of 41, which implies 97.50% quenching efficiency (**Fig. S1a**).

This quantitative quenching system was applied to investigate the fidelity of DNA hybridization in four different buffer solutions using DNA hybrids with 0 (DNA20/DNA12), 5 (DNA20/DNA22), 3 (DNA20/DNA23) or 2 (DNA20/DNA24) mismatches over a length of 21 nt (Fig. S1b). The free-energy gap between the perfect hybrids (DNA20/DNA21) and those with five mismatches (DNA20/DNA22) was sufficient (about 20 kcal/mol) to allow the DNA hybridization to reach equilibrium in 500 mM Tris-borate, pH 8.3, and 50 mM NaCl at 45^{o}C, in which no DNA hybrids with five mismatches were formed (i. e. no quenching - **Fig. S1b**). The free energy of DNA hybrids was computed by the nearest neighbor's method.

For perfectly matching strands, hybridization was 89%. The hybridization yield was obtained by calculating the percentage of quenching effect of faultlessly matching hybrids (DNA20/DNA21 in Fig. S1b) relative to the fluorescence signal of single-stranded TAMRA labeled oligomer (DNA20, **Fig. S1b**) under equal buffer conditions and temperature. A similar result was obtained for a Tris-acetate buffer solution, where the hybridization yield was higher than 94% (**Fig. S1b**). To convert the fluorescence signal into DNA20 concentration, a standard curve of integrated fluorescence signal from 560 to 600 nm of the 3′-end TAMRA-labeled DNA20 in different concentrations (3.0, 6.0, 12.5, 25.0, 50.0 and 100.0 pmol) was used. The curve obtained had a linear fit with a ±5% error margin defined by the Origin 6.0 software from Microcal Software, Inc., Northampton, MA (**Fig. S2**).

The quenching system was used in two different hybridization solutions, a 500 mM Hepes buffer, pH 7.5, or a 500 mM Tris-acetate buffer, pH 8.3; both with 50 mM NaCl, 30% formamide) under the same DNA concentrations (1 μM) and temperature (45^{o}C).

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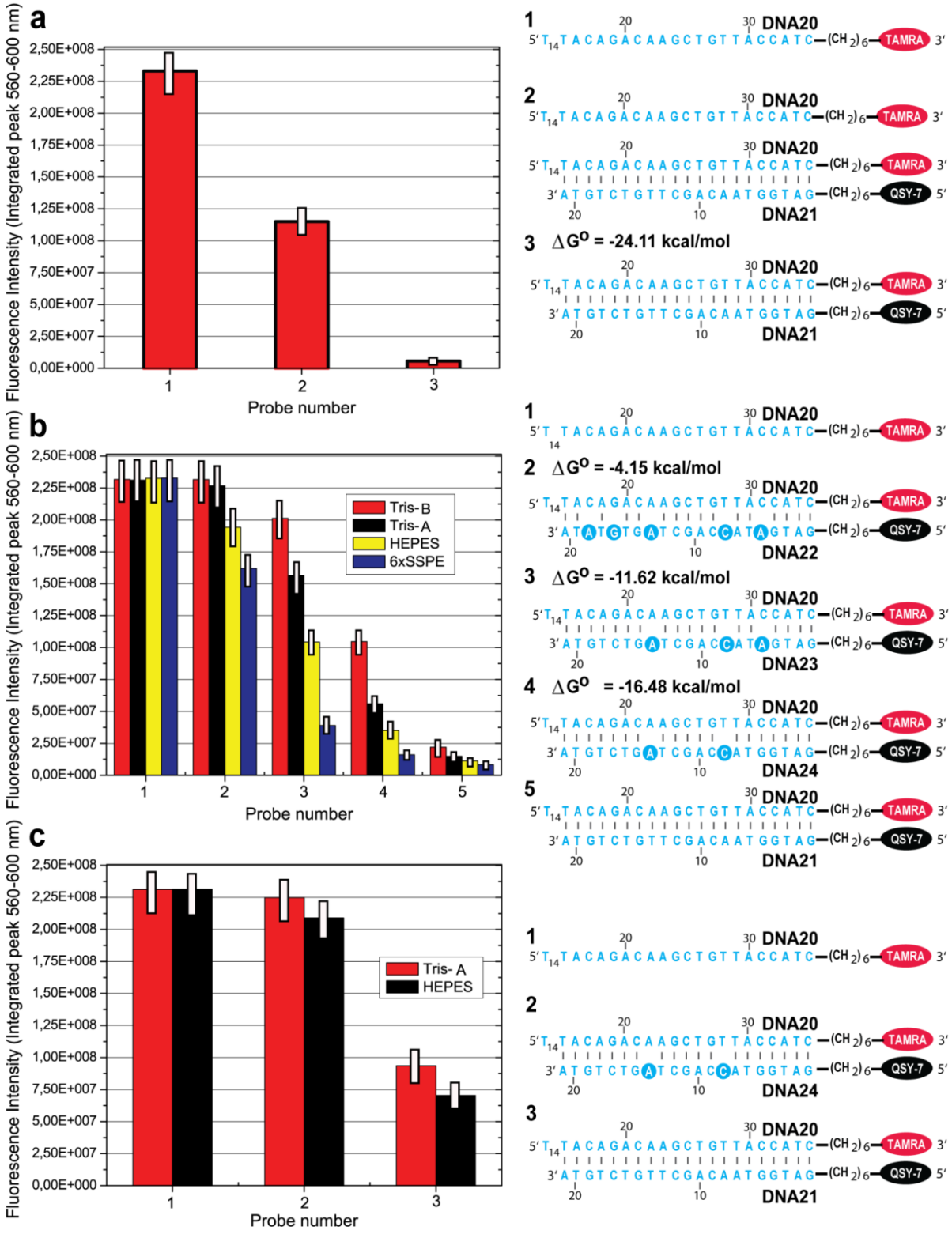

Fig. S1. DNA/DNA hybridization analyses using a quenching system. (a) *Quenching efficiency of QSY-7 towards TAMRA. (1) Fluorescence intensity signal (the integrated peak from 560 to 600 nm) of 3'-end TAMRA labeled DNA20 oligomer in a concentration of 1 μM. (2) Fluorescence signal of 1 μM 3'-end TAMRA labeled DNA20 in the presence of 0.5 μM, 5'-end QSY-7 labeled, perfectly-matching complementary DNA21 oligomer. (3) Fluorescence*

*signal of 1 μM 3'-end TAMRA labeled DNA in the presence of 1 μM, 5'-end QSY-7 labeled, perfectly-matching complementary DNA21 oligomers. Both reactions were performed five times, and the average values are presented. The quenching efficiency was established to be about 97.5%. (**b)** Estimation of DNA hybridization yields in different buffer solutions by using of a quenching system DNA hybrid with 5 (2), 3 (3), 2 (4), and 0 (5) mismatches**.** Each assay is carried out in five repeats in four different buffer solutions at 45^{o}C and the average intensity signal of each assay is presented. The different buffer solutions used are: red, 500 mM Tris-borate buffer, pH 8.3, and 50 mM NaCl; black, 500 mM Tris – acetate buffer, pH 8.3, and 50 mM NaCl; yellow, 500 mM Hepes buffer, pH 7.6, and 50 mM NaCl, blue, 6xSSPE. Each DNA oligomer is in a concentration of 1 μM. (**c**) Estimation of hybridization yield of DNA hybrid with two mismatches over a length of 21 nt by means of a quenching system. The experiments are carried out five times each and the average intensity signal is plotted. Two different buffer solutions, both at 45^{o}C were used, denoted as follows: in red 500 mM Tris–acetate buffer, pH 8.3, 30% formamide and 50 mM NaCl, and in black: 500 mM: Hepes buffer, pH 7.6, 30% formamide and 50 mM NaCl each DNA strand in a concentration of 1 μM.*

As shown in **Fig. S1c**, there was no hybridization (no quenching) between the complementary strands with 2 mismatches (DNA20/DNA24) in the Tris-buffer and there was a quenching of about 30% for perfectly matching strands (DNA20/DNA21) under the same hybridization conditions. In the Hepes buffer, there were about 10% quenching of the DNA hybrid with 2 mismatches and a 40% quenching with the perfectly matching strands (**Fig. S1c**).

The experimental results obtained imply that all tested buffer solutions can be successfully employed for an isothermal DNA hybridization transfer. The Hepes buffer possesses a higher buffering capacity and ionic strength than that of Tris-acetate and Tris-borate buffers. The Hepes buffer leads to the highest DNA melting points as well. The choice of one of those buffers should mostly depend on the thermodynamic stability of the DNA hybrids formed and the temperature on which hybridization can be carried out. If we would make hybridization transfer of 15-22mer DNA strands at a room temperature the Tris-acetate buffer seems to be more appropriate than a Hepes buffer because of its lower ionic strength.

Note that the higher ionic strength of the hybridization buffer will require higher hybridization temperature. It is well known that DNA, and particularly RNA molecules

are not stable in a solution of NaOH at a high temperature. We use 50 mM NaOH for nucleic acid denaturation. To preserve the DNA/RNA molecules we wanted to do the hybridization/denaturation steps at as low temperature as possible. Therefore, we choose for our DNA the Tris-acetate buffer because of its lower ionic strength than that of Hepes buffer.

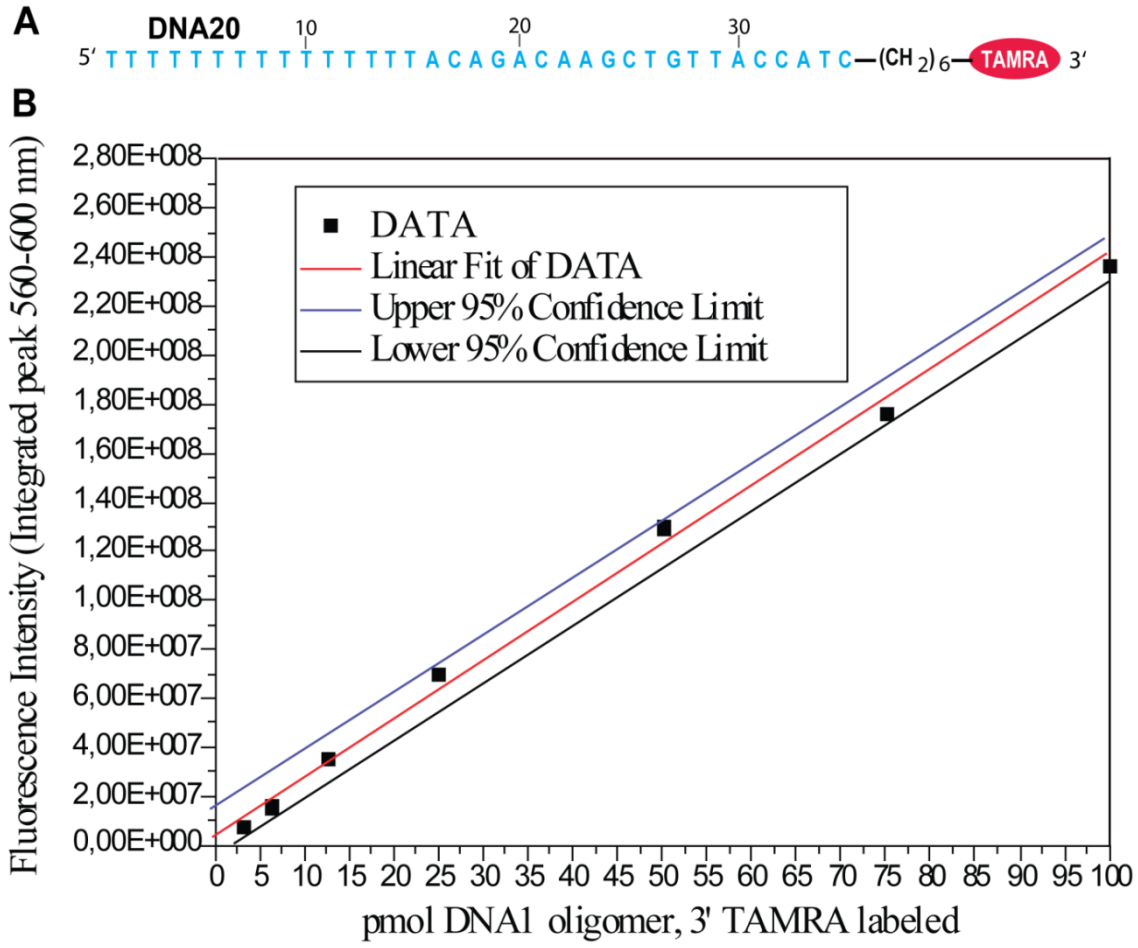

Fig. S2. A standard titration curve for quantification of 3'-end TAMRA labeled DNA. *(**a**) The fluorescence signal (integrated peak from 560 to 600 nm) of 3' TAMRA labeled DNA20. (**b**) The experiments were done in seven different concentrations (3.0 pmol; 6.0 pmol; 12.5 pmol; 25.0 pmol; 50.0 pmol; 75.0 pmol; 100.0 pmol) was measured and plotted the values being plotted in the dark squares. A linear fit was made, based on the measured data – the red line. The two green lines enclose a region of ± 5 % error rate (calculated by Origin Software). Each concentration was made three times and the average intensity signal is presented.*

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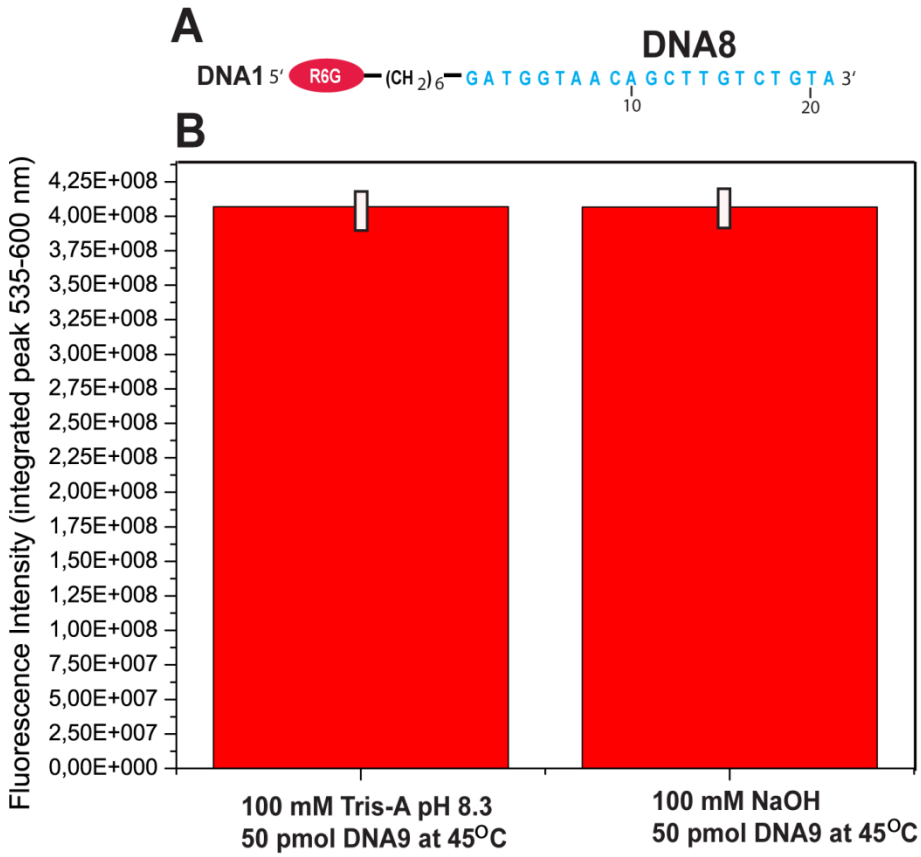

Fig. S3. Testing stability of the fluorescence signal of rhodamine 6G in 100 mM NaOH. *(**a**) 50 pmol 5'-end R6G-labeled DNA8 was incubated for two hours at 45°C in two different solutions. (**b**) The first solution was a 100 mM Tris-acetate buffer, pH 8.3, and the second solution was 100 mM NaOH. The experiments were repeated five times as the average values are plotted. There is no difference in the fluorescence signal of R6G between these two solutions. Thus the 100 mM NaOH does not influence the fluorescence signal of R6G under these conditions.*

Amino-modified (5'-end) deoxyoligonucleotides at a concentration of 10 µM were immobilized to 5 mg 15 µm polystyrene carboxyl-coated polystyrene beads in the presence of 50 mM EDAC (1-ethyl-3-(3-dimethylaminoethyl) carbodiimide), 100 mM MES (2-(N-morpholino) ethanesulfonic acid) buffer, pH 6.1, and 100 mM NaCl in a final volume of 200 µl at a room temperature under shaking. The immobilization reaction results in the formation of a peptide bond between the 5'-end amino-group of the modified DNA oligomer and the carboxyl-group on the bead surface. To create a negative control for non-specific DNA attachment, the same reaction was performed without EDAC and to 30 mg beads instead of 5 mg beads (**Fig.S3**).

2. Testing the mixing efficiency in cascade microchambers. To estimate the mixing efficiency in the second chamber, a 100 mM NaOH solution was delivered at a flow rate of 0.005 µl/min through inlets 2 and 3. A 500 mM Tris-acetate buffering solution, containing 50 mM NaCl and 1 µM R6G was delivered simultaneously through inlet number 1 at a flow rate of 0.01 µl/min (**Fig. S4**). Fluorescence images from the top and middle part of the second chamber (**Fig. S4**) were obtained using a CH250 CCD camera. There was a clear border between the two pumped solutions in the top part of the second chamber (**Fig. S4b**). The border, between the two fluids disappears in the middle part of the chamber as a result of an almost complete mixing between the two solutions under the applied flow rate (**Fig. S4b**).

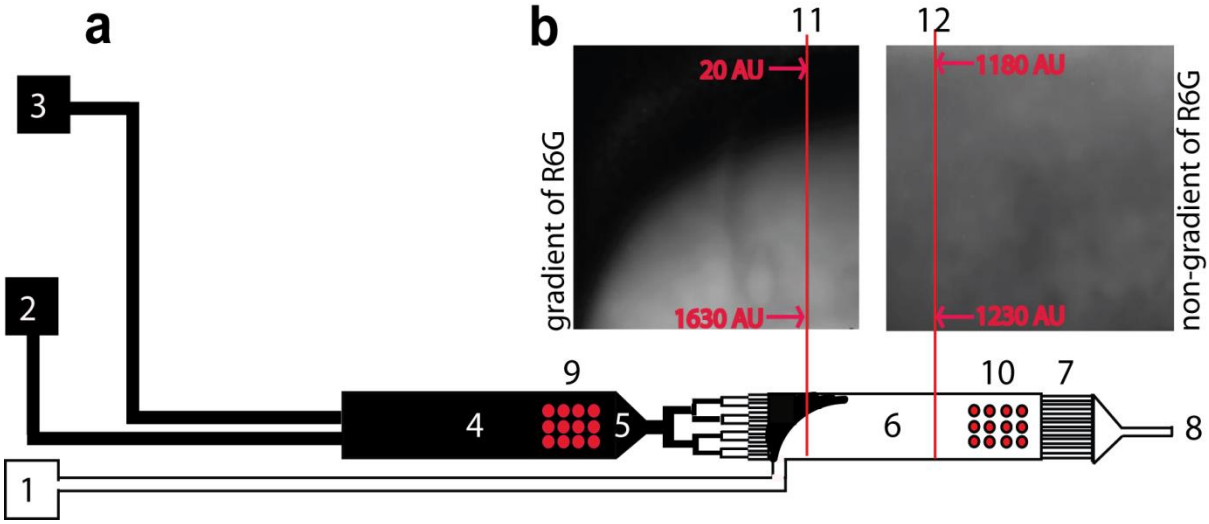

Fig. S4. Mixing efficiency in connected microchambers. *The common annotation for (a) and (b) is as follows: The microflow reactor used has three inlet channels (1-3), two cascade microchambers (4 and 6) filled with beads (9 and 10), and an outlet channel (8). The beads are kept into the chambers by bead barriers (5 and 7). For more details on the microflow reactor see Fig. 1C. (a) A Tris buffer solution, pH 8.3, containing R6G is pumped through inlet 1 at a flow rate of 0.01 µl/min and a R6G free solution is pumped through inlets 2 and 3 at a flow rate of 0.005 µl/min in the microreactor shown below: Fluorescence image from the top part of the second chamber (11). Fluorescence image from the middle part of the second chamber (12) with fluorescence intensity profiles are shown. (b) A white-light image of the microflow reactor used is shown.*

3. Programmable immobilization of amino-modified synthetic DNA to carboxyl-coated beads placed in cascade microfluidic chambers. The mixing efficiency in the microreactor (**Fig. S4a**), used for specially defined is very important not only for the programmable DNA immobilization but also for a cascade DNA hybridization transfer described in the next section. Therefore, the microflow reactor was, first, used to demonstrate that we can achieve a complete mixing between delivered solutions. To estimate the mixing efficiency in the second chamber, a 100 mM NaOH solution was delivered at a flow rate of 0.005 µl/min through inlets 2 and 3. A 500 mM Tris-acetate buffering solution, containing 50 mM NaCl and 1 µM R6G was delivered simultaneously through inlet number 1 at a flow rate of 0.01 µl/min (**Fig. S4**). Fluorescence images from the top and middle part of the second chamber (**Fig. S4**) were obtained using a CH250 CCD camera. There wqas a clear border between the two pumped solutions in the top part of the second chamber (**Fig. S4b**). The border, between the two fluids disappears in the middle part of the chamber as a result of an almost complete mixing between the two solutions under the applied flow rate (**Fig. S4b**).

Having established the conditions for complete mixing of the delivered solutions in the second chamber, we demonstrate programmable immobilization of 5'-end amino-modified DNA (**Fig. S5a**) to carboxyl-coated polystyrene beads incorporated in the cascade microchambers (**Fig. S5**). To immobilize a DNA4 oligomer (**Fig. S5a**) to the beads placed in the second chamber, we used a separate delivery of the DNA molecules and the cross-linking reagents. A buffer solution of 50 mM MES, pH 6.1, in the presence of 10 µM 5'-end amino-modified synthetic DNA4 and 100 mM NaCl was pumped with a flow rate of 0.005 µl/min through inlets 2 and 3. A 50 mM MES solution in the presence of 50 mM EDAC was delivered simultaneously through inlet number 1 with a flow rate of 0.01µl/min (**Fig. S5b**). A MES buffer with pH between 4.7 and 6.1 is the best medium for this immobilization reaction.

Next to the immobilization procedure, the beads were treated with a hybridization buffer with 500 mM Tris pH 8.3 and 10 µM 5'-end R6G-labeled for 15 min. After washing for 5 minutes with the same buffer without DNA, fluorescence images were taken from the beads placed in the two chambers using the CH250 camera as described in M&M. The results obtained, are plotted in **Fig. S5e**. The data

demonstrate a fluorescence signal on the beads placed in the second chamber (SC) (image 8 in **Fig. S5b**) while there is no fluorescence signal on the beads in the first chamber (FC) (image 7 in **Fig. S5b**). As expected, DNA immobilization took place on the beads situated in the second chamber because EDAC was presented there, but not in the first chamber.

To demonstrate an inhibition of the immobilization reaction a buffer solution of 50 mM MES, pH 6.1, in the presence of 10 μM 5'-end amino-modified synthetic DNA4, 50 mM EDAC and 100 mM NaCl was delivered through inlets 2 and 3 with a flow rate of 0.005 μl/min. As a result, DNA4 was immobilized to the beads placed in the first chamber. A 250 mM NaOH solution was delivered simultaneously through inlet 1 with a flow rate of 0.01 μl/min that prevents DNA immobilization reaction on the beads at the second chamber due to the observed pH value of 12. The reaction was allowed to proceed for 1 hour at room temperature (**Fig. S5c**). The same hybridization and washing procedures are employed as described above. The results obtained are plotted in **Fig. S5e**. The data demonstrate, a fluorescence signal on the beads placed in the first chamber (image 7 in **Fig. S5c**) while there is no fluorescence signal on the beads in the second chamber (image 8 in **Fig. S5c**). As expected, DNA immobilization took place to the beads situated in the first chamber but not in the second chamber because pH-dependent inhibition of the immobilization reaction in the second chamber. Finally, to demonstrate DNA immobilization by reversible pH alteration, a solution of 50 mM NaOH in the presence of 10 μM 5'-end amino-modified DNA4 was delivered with a flow rate of 0.005 μl/min through inlets number 2 and 3. A 250 mM MES solution, pH 4.7, in the presence of 50 mM EDAC was pumped simultaneously through inlet 1 with a flow rate of 0.01 μl/min (**Fig. S5d**). After the same hybridization and washing procedures described above, fluorescence images were taken from both chambers. The results obtained are plotted in **Fig. S5e**. The data demonstrate a fluorescence signal on the beads placed in the second chamber (image 7 in **Fig. S5d**) while there is no fluorescence signal on the beads in the first chamber (image 8 in **Fig. S5d**). As expected, DNA immobilization took place to the beads situated in the second chamber but not in the first chamber because of reversible pH alteration of the immobilization reaction in the second chamber. The immobilization procedures demonstrated herein can be employed for programmable DNA immobilization in parallel as described in the supplementary section (**Fig. S7**).

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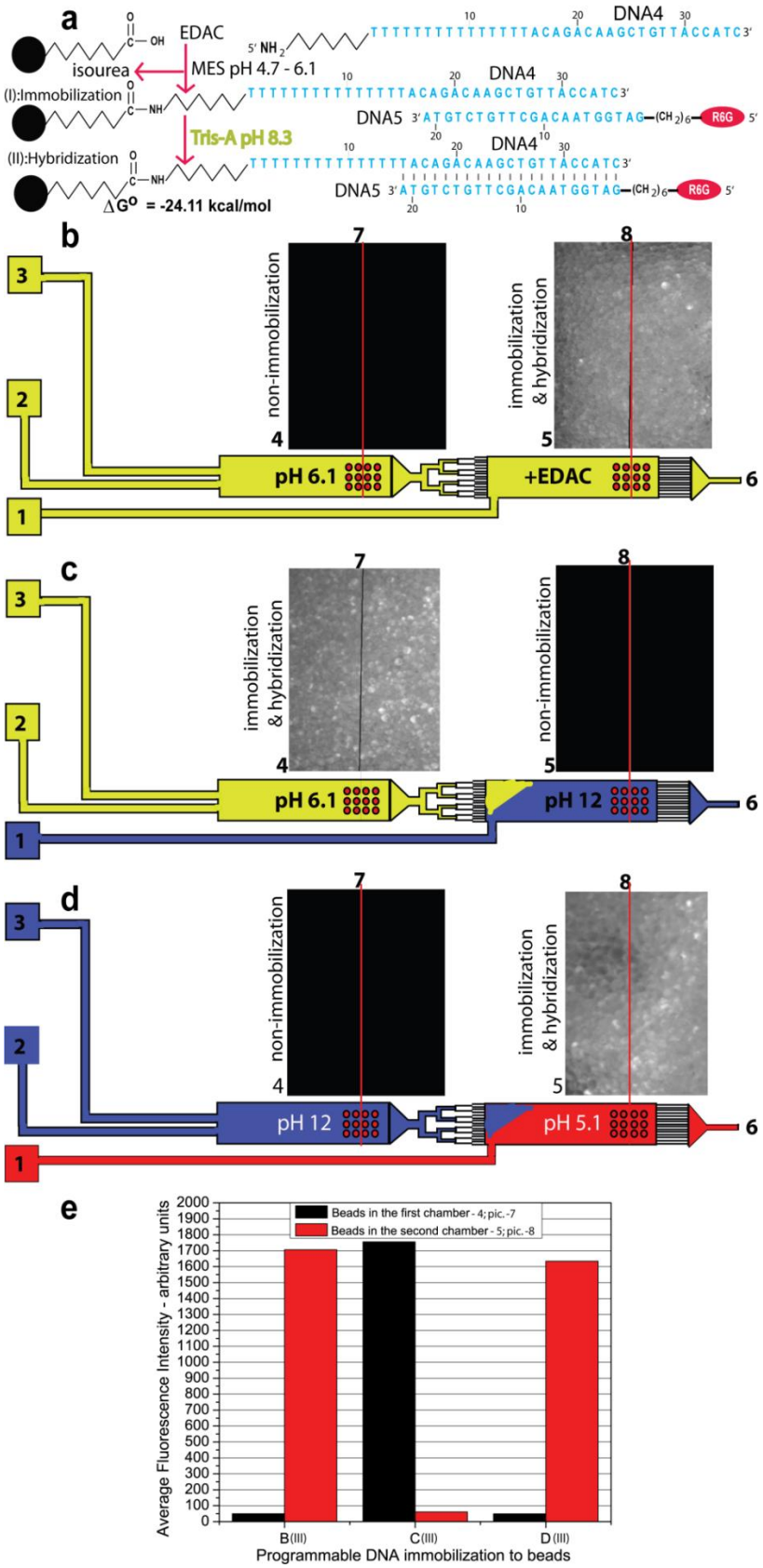

Fig. S5. Programmable DNA immobilization to beads placed in cascade microchambers with hybridization analysis. (a) Immobilization of 5'-end amino-labeled DNA4 to carboxyl-coated in MES solution in the presence of EDAC. The optimal pH range of

this chemistry is from 4.7 to 6.1. The DNA immobilization is visualized by hybridization with complementary 5'-end R6G-labeled DNA5. The common annotations for (b), (c), and (d) are identical with **Fig. 1b**. The depicted pH values in the microchambers are based on titration as well as mixing experiments (**Fig. S4**) (**b**) Programmable DNA immobilization by a separate delivery of the DNA oligomer and the cross-linking reagent. The cross-linking reagent EDAC is present only in the second chamber (6). As a result, DNA immobilization and hybridization take place only on the beads placed in the second chamber (8) but not on the beads in the first chamber (7) as shown on the fluorescence images. (**c**) Programmable DNA immobilization by a spatially inhibition of the immobilization reaction. A 250 mM NaOH solution is pumped through inlet number 1 that inhibits the immobilization reaction on the beads in the second chamber as shown in 8. The pH value is 6.1 in the first chamber that promotes DNA immobilization (7). (**d**) DNA immobilization by a reversible pH alteration. The pH value is 12 in the first chamber that inhibits DNA immobilization to the beads (7). A buffering solution is pumped through inlet 1 making the pH 5.1 in the second chamber. As a result, DNA immobilization takes place to the beads in this chamber (8). (**e**) Quantitative analysis of DNA hybridization to beads after the immobilization procedures as described above. Average fluorescence intensity (without the bias signal of the camera) is plotted in red for the beads in the first chamber and in black for those in the second chamber.

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4. Specific DNA hybridization transfer between cascade microchambers.

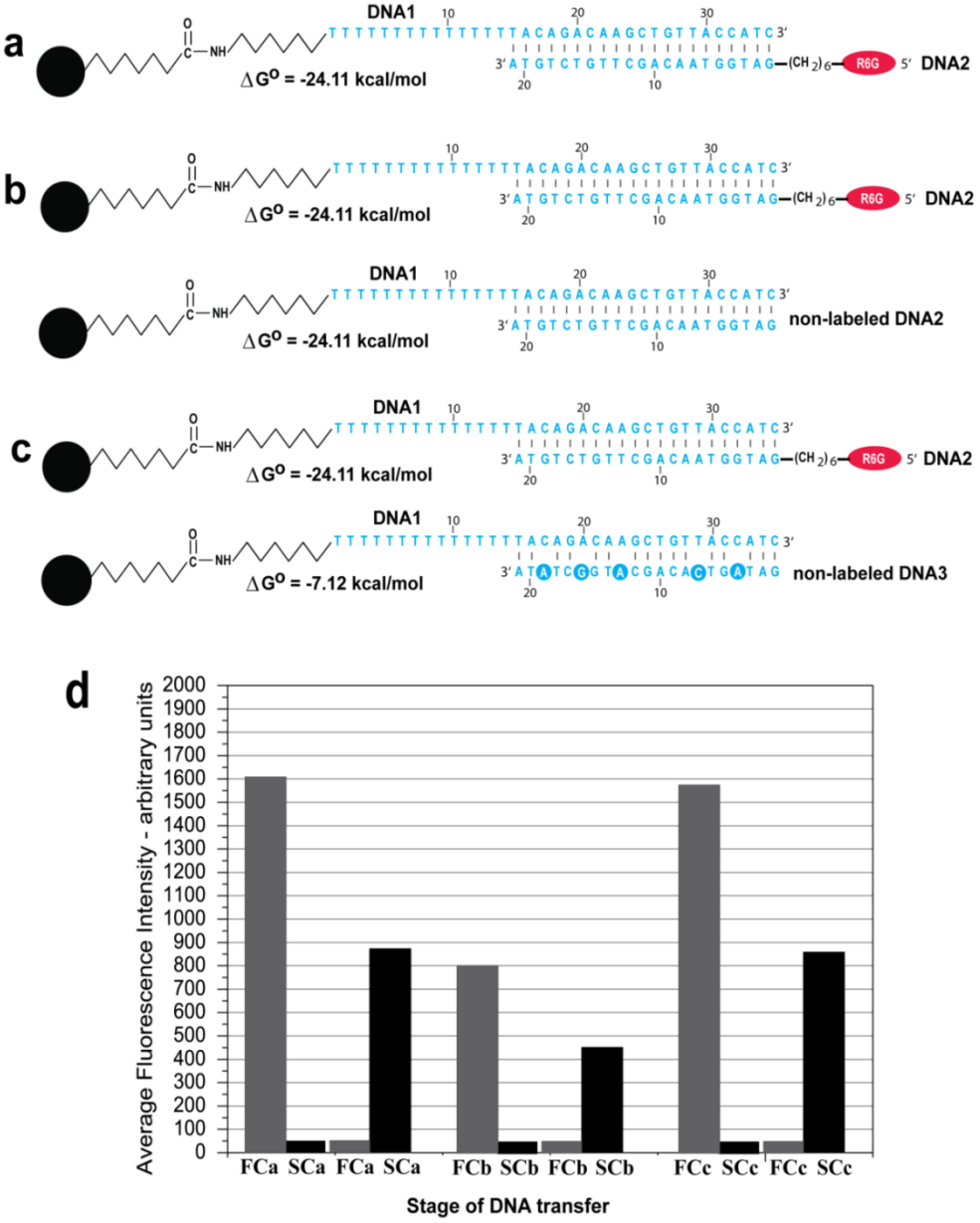

Fig. S6. Specific DNA hybridization transfer between cascade microchambers. *Beads with immobilized DNA1 are placed in the cascade microchambers (Fig. 1b). The DNA hybridized to the beads in the first chamber (FS) is transferred to the beads in the second chamber (SC) with about 60% efficiency as the amount of the beads in both chambers is equal (Fig. 3). (**a**) Perfectly matching 5'-end R6G DNA2 (500 nM) is used. (b) Half of the perfectly matching DNA was 5'-end R6G-labled (250 nM) while the other half was non-labeled (250 nM). This reduced twice the signal (d). (c) Perfectly matching 5'-end R6G DNA2 (500 nM) and non-labeled DNA3 (500 nM) with 5 mismatches are used at equal concentration. However, the signal on the beads is the same (d), which suggests that the DNA3 was not transferred from FC to SC under these conditions.*

5. Microfluidic platform for large-scale integration of programmable DNA immobilization and automated selection

A key advantage of DNA chip arrays is the opportunity to analyze many samples in parallel. A high level of automation, integration and programmability is required in the development of such devices. Here, novel microfluidic architecture is described for fully automated and integrated biopolymer arrays. The design is based on the pH-reversible chemistry for an isothermal DNA hybridization transfer between cascade microchambers and the programmable synthetic DNA immobilization demonstrated above. The established inhibition of the immobilization reaction guarantees high-specificity in DNA attachment. All procedures, including DNA immobilization, the selection, sequencing, and SNPs are fully integrated and automated, and take place in parallel. Microfluidic architecture of the microfluidic architecture described contains 2 supply horizontal channels, 12 horizontal channels, each with 8 cascade microchambers, crossed by 7 vertical supply channels at 84 crossing points (**Fig. S7a**). There is a mixing structure after each crossing point that combines vertical and chaotic mixers (**Fig. S7b, c**), followed by a chamber, being a part of the horizontal channels. The number of chambers is 96. Each chamber has a bead barrier at the bottom. Paramagnetic beads can be delivered to each chamber via the vertical channels except for the chambers of the first row, the beads being delivered by the horizontal channels. There is one valve at both end of each vertical supply channel as a total number of 14. Note that the number of valves, inlets and outlets increases only linearly while the number of chambers increases quandrantically. That makes the described design more scalable, since large numbers of chambers are easy to integrate.

The microfluidic architecture can be used various microfluidic arrays. The approach presented is universal as every type of biopolymer can be selectively immobilized to the beads placed in different chambers or to their surfaces by use of almost any cross-linking chemistry. That makes the biochip's production chemistry-independent. The general biopolymer immobilization procedure for the microfluidic device presented in **Fig. S7** can be described as follows. Different biopolymers (DNAs, polypeptides, and others) are delivered through the horizontal channels from 2 to 13 except for the first and the last supply channels (**Fig. S7a**).

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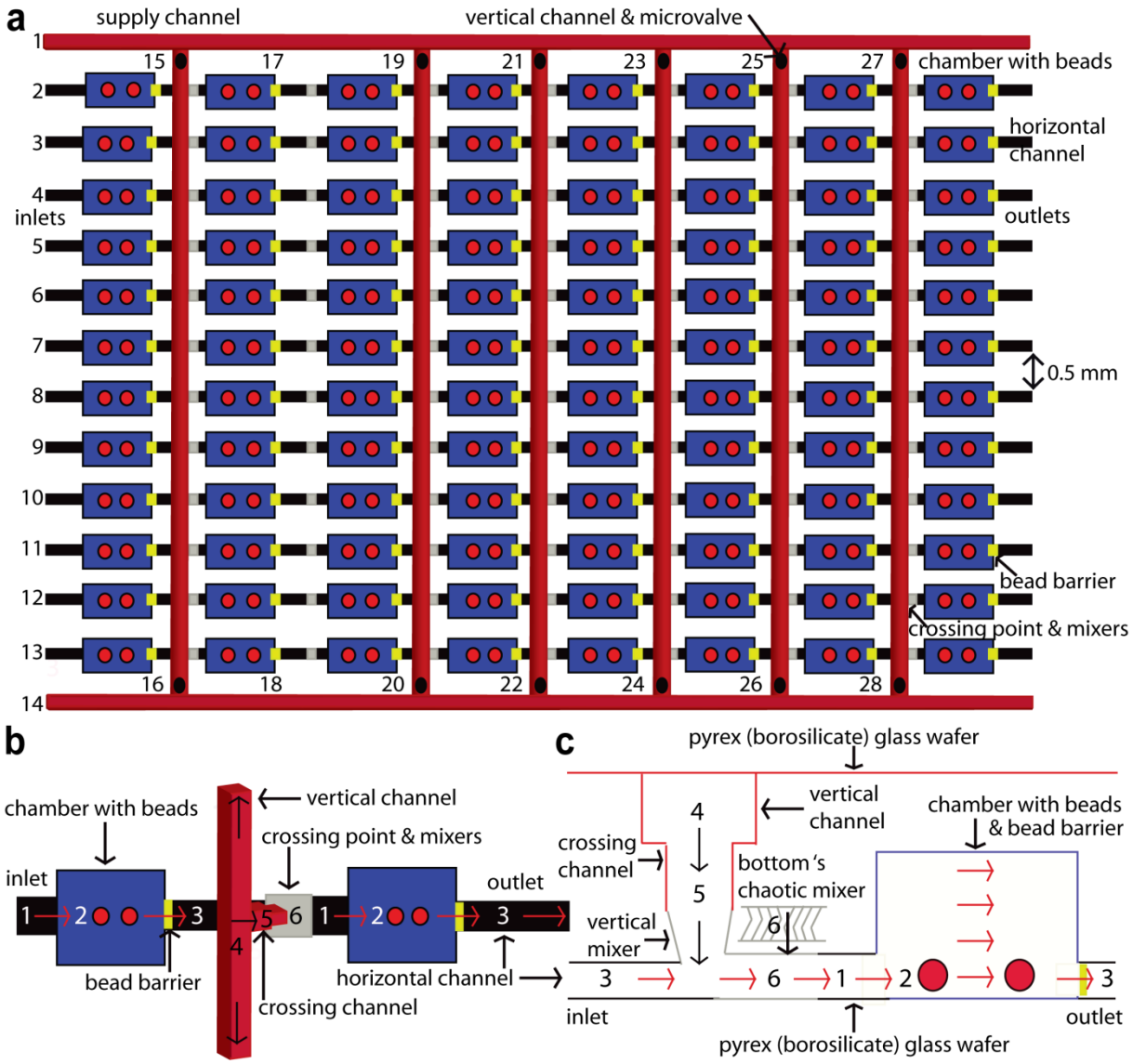

Fig. S7. A microfluidic architecture for programmable and parallel biopolymer immobilization and selection. ***(a)*** *Microfluidic architecture. There are 2 supply channels (1 & 14), 12 horizontal channels (2-13) crossed by seven vertical channels (84 crossing points secured by small connecting channels) with mixers and 14 microvalves (15-28). After each crossing point, there is a mixing structure. There are 96 chambers with an etched bead barrier at the bottom of each one. Carboxyl-coated magnetic beads are to be incorporated in all 96 chambers. (**b**) Crossing point between one horizontal channel (1 & 3) and vertical channel (4) secured by small crossing channel (5) with a mixer (6), and two microchambers (2) with beads and a bead barrier. (**c**) Vertical slice of the crossing point shown in (**b**) with the same notations. There are two mixer structures: vertical and chaotic mixers. The microflow reactor is sealed by pyrex glass wafer on both sides.*

The cross-linking reagent employed is pumped through the first horizontal channel while an inhibitor of the immobilization reaction is pumped through the last one. All

microvalves remain closed. As a result the cross-linking reagent is not present in any chamber and immobilization does not take place. If the valve number 27 is opened the cross-linking reagent enters the chamber of the last column (a vertical row) because of the flow direction. As a result all chambers in the last vertical row are immobilized with different biopolymer. After washing step new set of different molecular are delivered through the inlets. This time valves 25 and 28 are open. As results the beads from 7th vertical row are immobilized with the molecules of interest. The immobilization does not take place neither on the beads from the previous vertical rows due to the absence of the cross linking reagent nor on the beads from the last row because of the inhibition solution delivered through supply channel 14 (**Fig. S7a**). The reversible pH alteration in the immobilization solution described in above can be applied for programmable DNA immobilization as well. A similar flow procedure can be applied for multi-step DNA hybridization transfer in parallel applying the pH-reversible chemistry described above. Both processes of immobilization and selection are embedded into the same microfluidic architecture. Note that many different cross-linking procedures for biopolymer (DNAs, RNAs, and polypeptides) immobilization can be adopted for programmable immobilization using the described microfluidic design. The beads can be avoided for same applications as the chambers` surface can be functional and employed for immobilization. All these features make the discussed design an inexpensive and universal solution for various parallel biopolymer arrays.