Cascadable Hybridisation Transfer of Specific DNA between Microreactor Selection Modules

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Abstract. The paper demonstrates experimentally the basic principle of DNA transfer between magnetic bead based selection stages, which can be used in steady flow microreactors for DNA Computing [McCaskill, J.S.: Biosystems, 59 (2001) 125–138] and molecular diagnostics. Short DNA oligomers, which can be attached covalently to magnetic beads by a light programmable photochemical procedure [Penchovsky et.al.: Nucleic Acids Res., 22 (2000) e98], are used to bind matching ssDNA from a flowing solution. The beads are restrained in two reaction chambers (modules) by etched ledges in a bonded microreactor made of silicon and glass, with the solutions flowing in closed micro-channels. The action of a steady flow network of selection modules is studied in this two chamber microreactor using a succession of different buffer solutions at alternate pH to simulate the transfer between parallel flows in the former system. The pH changes cause successive hybridisation and dissociation of ssDNA to matching sequences on the beads. Detection of DNA is by fluorescence from rhodamine-labelled target DNA. The results demonstrate the successful selection of specific DNA in one module and its subsequent transfer to and pickup on the magnetic beads of a second module. This verifies the biochemical operation of the basic processing step for optically programmable DNA Computing in micro-flow reactors.

1 Introduction

One of the main attractions of DNA-based computation is that it allows a high level of customization as parallel processing hardware. In order for such fully customised approaches to parallel computation to be effective, however, attention must be devoted to ensuring that the DNA computer remains programmable for complex problems. A recent paper by one of the authors has demonstrated that optical programmability is an efficient choice for DNA Computing in conjunction with microreaction technology [1]. Microflow systems can implement a novel kind of dataflow architecture in which a stream of DNA molecules with different sequences are processed in parallel by a network of sequence-discriminating modules. A key example of such modules, as detailed in [1], is called the Strand Transfer Module (STM) and allows the selective transfer of DNA containing

N. Jonoska and N.C. Seeman (Eds.): DNA7, LNCS 2340, pp. 46–56, 2002.

a specific subsequence between two flows. In this paper, we demonstrate the ability to select DNA from a continuous stream in one module and pass it on isothermally to be picked up by a second module. On the basis of these experiments, we deduce appropriate chemical conditions for implementing strand transfer modules under constant flow conditions for DNA computations. The technology should also be of interest for molecular diagnostics such as expression and single nucleodite polymorphism analyses.

The transfer is implemented using DNA attached to paramagnetic beads and clocked by an external magnet, in parallel for all modules. A physical realization of such a module was presented previously [2]. Different buffer solutions in neighbouring channel flows are brought into contact by removing a small stretch of intervening wall (to allow the passage between them of beads under a magnetic field). Three different buffer solutions are used to achieve DNA hybridisation to, selective washing on and release from complementary matching oligomers immobilized on the super-paramagnetic beads. In this paper, we will demonstrate that these individual steps can indeed be performed in microflow reactors and will also show the range of buffer conditions necessary for this to be achieved.

A technique for optically programming such modules via the photochemically driven attachment of DNA oligomers to magnetic beads has been developed and tested by the authors [3]. In a parallel paper by our research group, we show how scalable, optically programmable microreaction technology, in steady flow, is being developed using STMs to allow a solution to combinatorial optimisation problems [4].

To quantify the efficacy of DNA selection according to this technique, a special two-chamber microreactor design was employed, allowing independently switchable flows for complete control. This design, shown in Fig. 1, shares the basic feature of STMs in that paramagnetic beads selectively capture DNA and are restrained by ledges positioned across the flow. However, it allows larger bead quantities and manual manipulation to be employed to assist in the quantitative characterization.

This work has direct bearing on our ability to integrate DNA Computing. In contrast with surface-based approaches to DNA Computing [5], the essential computational steps in the flow systems we are proposing are not dictated by off-chip pipetting robots. It can be regarded as a step towards a fully μTAS (micro Total Analysis System) conception of DNA Computing. Such a contribution is beneficial, not simply in terms of automation, but also for the scalable programmability of DNA Computing, since one hardware design is able to deduce the solution to any problem of a given class. We do not address the issue of constructing new solutions in the course of a computation in this paper, although this is the key to overcoming the fundamental limitation to DNA Computing as proposed by Adleman [6]. However, as will be addressed elsewhere, this is possible within the STM framework using evolution.

Microfluidic platforms are being developed in a number of laboratories for various applications including enzyme assays [7], chemical synthesis [8], on chip PCR [9], and DNA analysis [10,11,12]. The research towards labs-on-a-chip is

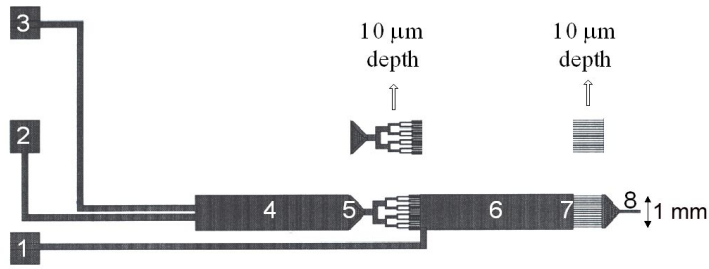

Fig. 1. Overall scheme of the micro-flow reactor employed to quantify DNA selection and transfer. The three inlet channels (1, 2, 3), the outlet channel (8) and the two chambers (4, 6) each with its own bead barrier (5, 7) are shown. Beads are introduced into the first and second chamber and restrained there by the bead barrier. The depth of the channels and the two chambers is 140 μm. The depth of the intervening bead barriers is 10 μm. Different structures for the various bead barriers were used for a functionality not relevant to the current experiments. The microreactor is sealed using anodic bonding of a pyrex wafer, with holes to allow access from polyethylene connecting tubing drilled in the pyrex above the inlets (1, 2, 3).

based on the idea of the integration on a single wafer of different chemical processes, all requiring different reaction conditions. DNA Computing places extreme demands on microfluidic systems, some of which are shared by molecular diagnostics. Recently microfluidic devices have been applied for implementing a parallel algorithm using fluid flow [13]. In contrast to a DNA computing based approach (as presented here) only limited parallelism achievable using only beads to store computational states. In our case we are developing microflow architectures for selection and sorting of DNA from complex populations which could open up new possibilities for integrated diagnostic applications with pipeline and conditional testing. The current work also lays the foundation for such applications.

2 Materials and Methods

2.1 Paramagnetic Beads and Oligodeoxynucleotides

Polystyrene super-paramagnetic beads were purchased from Micromod GmbH (Rostock, Germany). The beads are 15 μm in diameter, carboxyl-coated and essentially monodisperse. Oligomers were obtained from IBA-NAPS (Goettingen, Germany). The DNA was 5' amino-labelled using a C6 linker. All DNA was purified to HPLC grade. The oligomer sequences used are shown in Table 1.

2.2 DNA Immobilisation to Paramagnetic Beads

2.5 nmol of the 35 nt 5' amino-modified oligomer (see Table 1, line 1) was used for the covalent attachment to carboxyl groups on 5 mg beads dissolved in 500 μl 100 mM MES buffer pH 6.1 in the presence of 50 mM EDAC. The reaction was incubated for 3 h at 28°C under continuous shacking. Amino-coated beads could be employed for a light-directed DNA immobilization[3].

Table 1. Oligonucleotides used

N	Type of 5' modification	Sequence 5' - 3'	Length [nt]
1	Amino C6	TTTTTTTTTTTTTTTACAGACAAGCTGTGACCGTC	35
2	Rhodamine 6G	GACGGTCACAGCTTGTCTGTA	21
3	————	TACTGTCGCAGCTTGTCTGTATTTTT	26
4	Rhodamine 6G	TACTGTCGCAGCTTGTCTGTA	21

2.3 Microflow Reactor for Quantifying DNA Selection and Transfer

The microflow reactor used in these experiments was a two serially coupled chamber design with larger chambers than in the standard STMs to assist in quantitation, see Fig. 1. The ledges to restrain beads under fluid flow have a more complicated form than necessary, to allow the microreactor to be used in other contexts not discussed here. The construction of the microreactors is in the same materials (100 oriented silicon wafers bonded to pyrex glass) and micro-machining procedure employed in the full microflow DNA Computer [2,4].

2.4 DNA Selection and Transfer Protocol on Beads in Microflow Reactor and Its Quantification

Beads with immobilised DNA (as described above) were incorporated in a microreactor using a precision syringe pump (Model 260, World Precision Instruments Inc., Sarasota, FL). The hybridisation was carried out in 500 mM tris-acetate buffer pH 8.3 and 50 mM NaOH in the presence of 1 μM 5' rhodamine 6G labelled DNA oligomers (see Table 1, line 2) at 45°C under flow conditions. After each hybridisation step, the beads were washed with 150 mM tris-acetate buffer pH 8.3 at the same temperature. Hybridised DNA on the beads was denatured using 100 mM NaOH. The denaturation solution is reversed to a hybridising solution by adding an equal volume of 1 M tris-acetate buffer pH 8.3. The capacity of the buffer is not exhausted by the NaOH solution, so that no change in the pH of the tris-acetate buffer upon mixing was observed.

2.5 Fluorescence Quantification of DNA In Situ

An inverted microscope (model Axiovert 100 TV, Carl Zeiss, Jena, Germany) was applied for detection of the hybridisation of 5' rhodamine 6G labelled DNA oligomers to immobilised DNA to polystyrene carboxyl-coated beads incorporated into a microflow reactor. The microscope was connected by an optical fiber to an argon-ion laser (model 2080-15S, Spectra-Physics Lasers, Inc., CA). The fluorescent images were detected by a CCD camera (CH250-KAF1400, Photometrics, Tucson, AZ) connected to the microscope by a C-Mount adapter. An emission fluorescent filter opening at 540 nm and closing at 580 nm (Oriel Instruments, Stratford, CT) was used. The images were obtained and analysed by PMIS image processing software (GKR Computer Consulting, Boulder, CO). The average fluorescence signal of regions of images containing beads was measured, subtracting background (dark plus bias) images.

3 Results and Discussion

3.1 Efficient Buffers for DNA Selection, Washing, and Release

Rather than moving the beads, as will be done in integrated applications using the STMs, we keep the beads stationary to facilitate better comparative quantification and switch the composition of the flow instead. A theoretical background for such selective hybridisation kinetics has been presented previously [1]. We postpone the important analysis of the efficiency of DNA selection to the following section. There are several other issues which must first be resolved experimentally.

- the ability to capture DNA on beads from a flowing solution
- the ability to discriminate mismatched DNA by washing
- the ability to dissociate hybridised DNA efficiently with a reversible buffer
- the ability to bind matching DNA selectively in the presence of mismatched DNA

The experiment presented in this section was designed to address these issues in practice. One chamber of the microreactor shown in Fig. 1 was filled with beads immobilized with DNA (see Table 1, line 1). The reactor was held at a temperature of 45°C while a series of different buffer solutions were pumped through the chambers as described below (see Fig. 2). The selective hybridisation in flow involved firstly exactly matching DNA (1 μM ssDNA, see Table 1, line 2) fluorescently labelled with rhodamine 6G in the hybridising buffer solution (500 mM tris-acetate pH 8.3 and 50 mM NaOH). This buffer was chosen since it is the solution in which DNA released from a previous module will be immersed. It arises in a previous module through neutralization of the dehybrising solution (100 mM NaOH) by mixing with an equal volume flow of buffer (1 M tris-acetate pH 8.3). In a separate measurement, it was confirmed that the pH change of the buffer on mixing is negligible. The series of fluorescence measurements, monitoring the concentration of hybridised DNA on the beads, is shown in Fig.2.

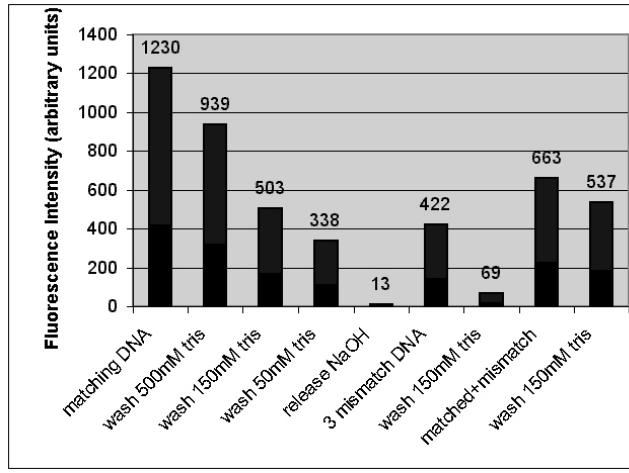

Fig. 2. DNA hybridisation fidelity analysis on beads placed in a microflow reactor. The average fluorescence intensity (minus background signal) of the region of the microreactor containing beads (cf. Fig. 4) is shown under various flow conditions. A single chamber of the microreactor shown in Fig. 1 was employed in this experiment. The measurements are averages over several images, after allowing time for reaction. For details, see text. The main features are successful hybridisation (column 1), release (column 5), the ability to separate matching and weakly mismatching DNA (cf columns 3 vs 7) with the appropriate buffer (tris 150 mM), and the ability to obtain high yield selective hybridisation of matched DNA in the presence of mismatched DNA (columns 8 and 9).

After switching to the hybridising solution, a rapid increase in signal (cf[1]), compatible with time scale induced by partial mixing lengthwise along the inlet tubes (upon buffer change from water), was followed by a slower increase towards saturation as more DNA is flowed past the beads. The DNA hybridisation on beads is taking place in less than a half of minute.

The beads were then washed with an identical buffer solution, not containing DNA, to remove unbound DNA and the fluorescence signal from the free solution. Washing continued for 40 min. The reduction in fluorescence signal (Fig. 2) was consistent with just removing the signal from DNA in the surrounding free solution. Next, the buffer solution was changed to 150 mM tris-acetate, which at lower ionic strength provides a more stringent test of DNA binding than the 500 mM solution. This buffer concentration was suggested as a wash solution by batch experiments in Eppendorf tubes (data not shown) and, as we shall see below, allows the discrimination of imperfectly matching DNA. The data reported in Fig. 2 confirms that significant signal is retained, with the exactly matching DNA, after a period of 30 min washing with this solution (Decreasing the buffer concentration further to 50 mM tris caused a steady depletion of

DNA, 50% over the next 30 min). Next the remaining DNA was dehybridised completely (see Fig. 2) using a solution of 100 mM NaOH. The fluorescent signal returned to its starting value. Separate measurements in a fluorimeter (data not shown) confirmed that there is no significant change of fluorescence signal of rhodamine-6G labelled DNA through the NaOH solution, so that the removal of fluorescence really implies that DNA is released from the beads.

After completion of this basic selection-wash-release cycle, a second round was initiated with an imperfectly matching DNA (1 μM, see Table 1, line 4) labelled with rhodamine 6G as above. This DNA contained three mismatches over a length of 21 nt, at separated locations in the sequence. A smaller amount of DNA bound to the beads was observed (even after 10 min hybridisation at 45°C) than in the case of perfectly matched DNA, as shown in Fig. 2. Washing with the discriminating buffer (150 mM tris-acetate, see above) was sufficient to remove the imperfectly matched DNA from the beads (see Fig. 2), so this buffer provides a good candidate for the intermediate wash channel in the STM modules in [1,2].

Finally, the ability of the wash buffer to discriminate in cases of simultaneous competitive binding between matched and imperfectly matched DNA was tested using a solution of hybridising buffer as at the outset but with 0.5 μM each of the two types of DNA (Table 1, lines 2 and 3), this time only the perfectly matching DNA being fluorescently labelled. The fluorescent signal on the beads rose to about 50% of its value in the first phase of the experiment (with 1 μM of labelled matching DNA), consistent with successful competitive binding of the matched form. After washing with the discriminating buffer (150 mM tris-acetate), the signal reduction was consistent with a removal of free labelled DNA as in the first phase of the experiment (see Fig. 2b). These results demonstrate that selective DNA hybridisation and release can be performed with appropriate buffer solutions and temperature, compatible with a steady flow implementation of DNA Computing as proposed in [1].

3.2 Demonstration of Transfer of DNA between Two Selection Modules with Reversible Chemistry

In a second experiment, the ability to select, restrain, transfer and pick up DNA between two modules was examined. The experimental procedure is similar to that in the previous section (but at 40°C) except that both chambers are filled with beads and separate buffer solutions are used at the inlets to the two chambers at various stages. Whereas in the integrated STMs, operating under steady flow, switching buffers is induced by magnetic bead transfer, here the flows are switched in larger chambers to facilitate quantitation of the transfer yields with constant bead locations.

The series of events for a complete transfer round from one module to the next is:

1) Hybridisation of perfectly matching DNA (see Table 1, line 2) to beads in both chambers. The hybridising buffer (500 mM tris pH 8.3 and 50 mM

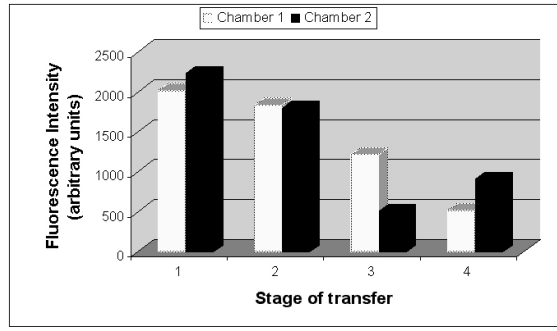

Fig. 3. DNA hybridisation transfer from beads in one module to those in a second module. Average fluorescence intensity (including the background signal of 490 units) images of two regions of the microreactor (see Fig. 1) containing beads (see Fig. 4) are shown under various flow conditions. White bars: fluorescence intensity from the first chamber of the micro-reactor. Black bars, fluorescence intensity from the second chamber. 1. DNA hybridisation on the beads in the first and second chamber. 2. Washing step of DNA hybridised on the beads in the both micro-reactor chambers. 3. Continuous washing step on the beads in the first micro-reactor chamber and denaturation step on the beads in the second chamber. 4. Denaturation of DNA from the beads in the first chamber and pick up of the same DNA by hybridisation on the beads placed in the second micro-flow reactor chamber. For details, see text.

NaOH) containing the DNA is pumped in parallel (4 μl/min) into all three inlets of the microreactor shown in Fig. 1.

2) Washing of the beads in both chambers (500 mM tris pH 8.3 and 50 mM NaOH without DNA), using inlets 1, 2 and 3.

3) Releasing DNA from the beads in the second downstream chamber and washing of the beads in the first chamber. NaOH solution at 100 mM is pumped through inlet 1 and 150 mM tris pH 8.3 through inlets 2 and 3.

4) Simultaneous release of DNA from the beads in the first chamber and pickup of the same DNA in the second chamber. 1 M tris pH 8.3 is pumped into the second chamber (inlet 1) and 100 mM NaOH into the first chamber (inlets 2 and 3). The outflow from the first chamber is neutralised by the tris buffer entering the second chamber from the side and then the DNA hybridises to the beads in the second chamber.

The results are summarized in Fig. 3, showing the quantitation of DNA on the beads in the two chambers at the four stages of the procedure. Figure 4 shows images of the fluorescence from DNA hybridisation to the beads in the two chambers at various stages (2, 3 and 4) of the above transfer procedure. Moderately high flow rates are employed, so that diffusive mixing is not perfect as indicated by the slightly inhomogeneous pickup (Fig. 4, image F). As seen in

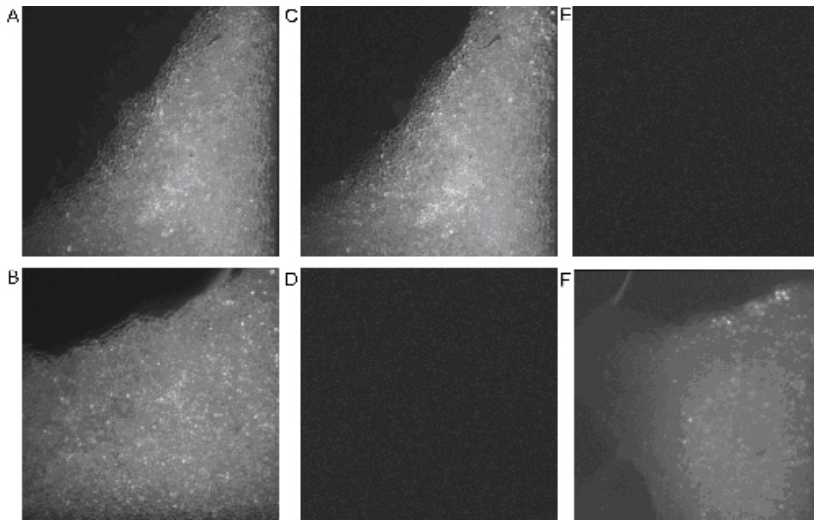

Fig. 4. Fluorescence images of DNA hybridisation on beads in micro-flow reactor showing the course of transfer from one module to the next. DNA hybridisation on the beads placed in the first chamber (A) and second chamber (B); Washing of the beads in the first chamber (C) and DNA denaturation on the beads in the second chamber (D); DNA denaturation from the beads in the first chamber (E) and pick up of the same DNA on the beads placed in the second chamber.

Fig. 3, about 50% of the DNA bound in chamber 1 is successfully picked up in chamber 2 in the transfer step (4, above).

4 Conclusions

This paper demonstrates that the basic processing and linkup steps of steady flow DNA Computing in microreactors can be experimentally verified. The key step of reversible chemical hybridisation and dehybridisation has been demonstrated under flow in microreactor modules similar to the integrated devices to be used in the complete DNA Computer [4]. Once a single complete round of selection, release and pickup on new beads has been achieved, as shown in the previous section, the procedure can be cascaded to many processors in series and in parallel. It should be noted that the problem of increasing salt concentration, on successive rounds of hybridisation and dehybridising by chemical means, does not occur for STMs [1] because the buffers are only used in one complete cycle before the beads are transferred to a fresh flow [1].

Experiments in Eppendorf tubes (data not shown) suggest that the discrimination between matched and non-matched DNA is significantly better with members of the DNA library [4]. This is because they have a modular structure, with unwanted hybridisation involving more than 3 mismatches over a length of

16 nt, so the experiments reported here provide an underestimate of fidelity. This issue will be addressed more completely with the full library in other work. The present paper establishes that a suitable wash buffer of 150 mM tris-acetate pH 8.3, compatible with the reversible hybridisation-dehybridisation solutions, can be employed in the steady flow integrated DNA Computer to enhance discrimination and hence fidelity of matching. The concentration of the denaturating solution (100 mM NaOH) could be reduced down to 50 mM for denaturation of small oligomers so that the concentration of the buffering solution of tris could be reduced to 500 mM instead of 1 M. It could be used a hepes buffer as a buffering solution as well (data not shown). Additional results (data not shown) imply that an alkali DNA denaturation to beads is more efficient than using temperature. This requires reducing to a minimum the non-specific attachment of DNA to the beads. Carboxyl-coated beads show less non-specific DNA attachment than amino-coated ones[3].

The efficiency of DNA transfer from one module to the next of 50% (see Fig. 3, stage of transfer 2 and 4) is not yet optimised, with values greater than 90% being sought. The flow rates employed in the current series of experiments were too high to allow sufficient time for either complete mixing or completed binding to beads. Optimisation of these parameters will be carried out using the network of STM microreactors actually to be employed in the computations[4]. We would like to stress that the current paper represents the first results in which selective bead to bead transfer of DNA with release and pickup has been quantitatively investigated, and that there is significant room for improvement.

Acknowledgements: The transfer and pickup experiments were quantified in a larger microreactor designed previously (to test switchable magnetic mixers) by Kristina Schmidt and reconstructed at the GMD by Marlies Gohlke. The authors wish to thank Jonathan Howard for helpful discussions. Assistance with the fluorescence imaging setup by Harald Mathis, Thomas Kirner and Danny van Noort was appreciated. The support of the GMD for DNA computing and German Ministry of Sience (BMBF) for BioMIP's start-up grant (#01SF9952) is gratefully acknowledged.

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