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Fast and continuous-flow detection and separation of DNA-complexes and DNA in nanofluidic chip format

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Fast separation of DNA and detection of protein/DNA-complexes are important in many state-of-the-art molecular medicine technologies, like production of gene vaccines or medical diagnostics. Here, we describe a nanofluidic chip based technique for fast, efficient and virtually-label-free detection and separation of protein/DNA- and drug/DNA-complexes and topological DNA variants. The mechanism is based on continuous-flow dielectrophoresis at a nanoslit and allows efficient separation of small DNA fragments (<7000 base pairs) and fast detection of DNA-complexes within 1 minute.

Introduction

The standard methods separation of DNA compounds is gel electrophoresis, in either a slab gel or capillary format, or the different types of affinity chromatography. Although

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widespread and used in many laboratories, some intrinsic systematic disadvantages are obvious and remain unsolved: 1) preparation and separation take at least 2 h **[1]**; 2) the parameters of the separation cannot be adapted or optimized during the separation; 3) considerable shear force effects during separation may affect analyte integrity and functionality (topology) and 4) sample elution and postprocessing is difficult and often relies on the use of toxic and mutagenic reagents. Nevertheless, capillary electrophoresis and electrophoretic mobility shift assays (EMSA) are standard analytical techniques for purification of small and large DNA fragments along plasmid vaccine production, for determination of binding affinities of new cytostatic drugs and rational drug design **[2]**, and for protein-DNA interaction screenings [**3-5]** like screening for specific antibody-DNA-complexes. Additionally, there is huge requirement of novel tools that allow fast processing with low analyte volumes in low cost devices suitable for real time process control.

Here, we present a new chip-based method for fast, continuous-flow detection and separation of DNA-complexes and topological DNA variants. The detection and separation mechanism is based on electrodeless dielectrophoresis at a nanoslit. Dielectrophoresis (DEP) refers to the migration of polarizable objects within an inhomogeneous electric field [6]. Advantages of DEP are that it is non-invasive, label-free and allows separation due to inherent molecules characteristics, namely the electric polarizability.

For the experimental performance, we applied ac and dc electric fields to a channel system consisting a crossinjector and a bowed ridge that generates a nanoslit (see fig.1). At the ridge, selective dielectrophoretic forces were adjustable via the applied electric fields [7]. These effect two possible pathways at the nanoslit: either the molecule passes the nanoslit unaffected or it is deflected until it reaches the second channel wall (*cf.* fig.1). The respective pathway a molecule follows depends on the ratio of the electrophoretic and dielectrophoretic forces [8].

DNA follows the second pathway, *i.e.* is deflected at the ridge, if the dielectrophoretic

potential $W_{DEP} = \frac{1}{2}\alpha\vec{E}^2$, with α polarizability, is higher than the potential energy of the electrophoretic motion. For separation of two DNA species, either varying in size or DNA and DNA-complex, it was exploited that α differs significantly between the species [7]. Hence, downstream of the nanoslit two molecule streams could be observed (see fig.1).

A monolithic fabrication of the microfluidic device *via* soft lithography [9] allows an easy fabrication in most biology-focused laboratories, as no clean room is required, once a masterwafer, the original negative relief of the actual fluidic network, was fabricated. A clean bench is sufficient for chip assembly. For detection, fluorescence microscopy was chosen, since this method is most often available. As a power supply, a cost-effective high voltage amplifier is sufficient.

With the chip-based method, we could detect complex-formation of DNA and Actinomycin D in varying concentrations and complex-formation of DNA and Escherichia coli RNA polymerase core enzymes within 1 min. Therefore, only 0.9 ng DNA (about 10 pM) were necessary. Additionally, fast separation of topological DNA variants was performed in continuous-flow process with application in in-production quality control of gene vaccines.

Materials

Prepare all solutions using deionized water and analytical grade reagents. Store all pure reagents as suppliers recommend. Working buffer and incubated DNA are stored at 7 °C. Diligently follow all waste disposal regulations when disposing waste materials.

Production of Masterwafer

1. 4” silicon wafer.

2. Wafer tweezer.
3. 1 l glasses.
4. Caroic acid: one part hydrogenperoxide and two parts acid sulfur.
5. SU8 photoresist and its thinner and developer.
6. 200 ml vials.
7. Waterproof marker.
8. Programmable hotplate (PR 5 SR, Detlef Gestigkeit, Germany).
9. Centrifuge (Ble-Laboratory Equipment GmbH, Germany).
10. Chromium photomasks (Delta Mask, The Netherlands).
11. Maskaligner with UV light source (MJB3, Süss Micro Tec, Germany).
12. DekTak-Profilometer 3030 (Stanford Nanofabrication Facility Equipment, USA).
13. Nitrogen gas and adapter with duster.
14. Acetone and isopropanol.
15. Adhesive tape.
16. Tridecafluor-1,1,2,2-tetra-hydrooctyl-trichlorsilane (TTTS).
17. Hourglass.
18. Exsiccator.

Chip fabrication

1. Vinylmethylsiloxane-dimethylsiloxane trimethylsiloxy terminated copolymer, platinum-divinyltetramethylsiloxane complex, 2,4,6,8-tetramethyl-2,4,6,8-tetravinylcycltetrasiloxane and (25-35% methylhydrosiloxane)-dimethylsiloxane copolymer.
2. Poly(dimethylsiloxane) Sylgard 184 and its linker.

3. Sharp tweezer and a bowed metal plate (15 cm x 30 cm) for peel-off the PDMS from masterwafer.
4. Scalpel.
5. Punching pattern according to channel dimensions.
6. Glass coverslips (24x60 mm thickness of 0.13-0.16 mm).
7. Ultrasonic bath (Transsonic460, Digitana AG, Germany).
8. Oxygen plasma chamber.
9. Working buffer consisting of 1 mM phosphate buffer (pH 7.4, 0.2 mM NaCl) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 0.25% N-dodecyl-β-D-maltoside (DDM) and 0.03% methyl cellulose (MC) (see **note1**).

Sample preparation

1. 11,1-(4,4,7,7-tetramethyl-4,7diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazol)-2-methylidene]-quinoliumtetraiodid (YOYO-1) was used for fluorescent labeling of DNA.
2. Pipettes of 2 µl, 20 µl, and 100 µl.
3. 2 ml Eppendorf vessels.
4. Vortexer (Vortexer-Genie 2, Scientific Industries, USA)
5. DNA of linear and circular conformation (lengths of 2.2-6.7 kbp).
6. Actinomycin D (ACTD) and Escherichia coli RNA polymerase core enzymes (RNAP) to form DNA-complexes.

Chip Experiments

1. Poly(methyl methacrylate) (PMMA) block with integrated reservoirs and electrodes corresponding to the chip layout.

Setup

1. Inverted fluorescence microscope with a motorized x/y-stage.
2. 100 x oil-immersion objective was used together with a mercury arc lamp and respective fluorescence filter sets (BP 450-490, BP 515-565, FT 510, Zeiss, Germany) and a grey filter (25% transmittance).
3. CCD interline-transfer camera with corresponding grabber card and software (DaVis 6.2) (see **note2**).
4. The voltage signal was created via a LabView 6i program and function generator DS345 (Stanford Systems, USA).
5. High voltage amplifier (AMT-1B60-L Matsusada, Japan) and three power supplies to deliver dc voltages (HCL 14-12500, FUG, Germany).

Methods

Diligently follow general safety instructions for handling with acids and solvents. Carry out all procedures at room temperature. Whenever the material is heated up or cooled down, use a temperature ramp with about 20 °C per 5 min (unless indicated otherwise).

Production of Masterwafer

1. The 4” silicon wafer is cleaned in caroic acid for 5 min twice and rinsed with water.

2. SU8 photoresist of 12%, 17% and 52% solid fractions are diluted from stock photoresist with thinner (see note3).
3. A first layer of SU8 photo resist (12% or 17% solid fraction, respectively) is spin-coated at 1000 rpm (12% solid fraction) or 900 rpm (17% solid fraction) for 30 s, (see fig.2). After spincoating the wafer is mounted onto a 65 °C warm hotplate for 1 min. Thereafter, the photoresist is exposed to UV light through a chromium mask in a mask aligner for 2 s (see **note4**). After postexposure bake at 95 °C for 3 min, the wafer is developed in developer for 1 minute. The developer is removed carefully (see **note5**) with acetone and isopropanol, successively, and dried with nitrogen. The height of the first layer is determined at several structures on the wafer, 180 nm (12% solid fraction) or 670 nm (17% solid fraction), with a Profilometer .
4. A second layer of SU8 (52% solid fraction) is spin-coated (3000 rpm for 30 s) and baked at 95 °C for 1 min (see **note6**). The photoresist is exposed to UV light for 7 s in the mask aligner. The exact alignment of the photomask to the first structures is of very high importance (see **note7**). After postexposure bake at 95 °C for 3 min, the wafer is developed in developer for 1 minute. Again, the developer is removed carefully with acetone and isopropanol, successively, and dried with nitrogen.
5. A terminal hardbake at 195 °C for 20 min is performed to close small cracks in the photoresist.
6. To prevent adhesion of the polymer to the photoresist structures, the wafer is silanized with TTTS in an exsiccator. Therefore, 10 droplets of TTTS are given on an hourglass and placed at the bottom of the exsiccator. The wafer is placed on a perforated plate over the hourglass. The vacuum is set to 0.1 mbar for 1 h. Afterwards, the exsiccator is slowly ventilated and the wafer can be removed.

Chip fabrication

1. A layer of h-PDMS (3.4 g Vinylmethylsiloxane-dimethylsiloxane trimethylsiloxy terminated copolymer, 18 µl platinum-divinyltetramethylsiloxane complex, 1 droplet of 2,4,6,8-tetramethyl-2,4,6,8-tetravinylcycltetrasiloxane and 1 ml (25-35% methylhydrosiloxane)-dimethylsiloxane copolymer (see **note8**)) is spincoated onto the wafer at 1500 rpm for 10 s (see fig.3) (see **note9**). The h-PDMS is precured 3 min on a hot plate at 65 °C (see **note10**).
2. A second layer of PDMS (6.5 g polydimethylsiloxane (PDMS) Sylgard 184 and 0.65 g silicone elastomer curing agent (PDMS-linker)) is poured on top of the h-PDMS and cured on a hot plate at 65 °C for 40 min.
3. The PDMS double-layer is peeled off (see **note11**) and the microstructured part of the slab is cut out with a scalpel with at least 3 mm distance to the channel ends.
4. Reservoirs of 2 mm diameter are punched into the PDMS slab at the channel ends (see **note12**). Afterwards, the PDMS slab is cleaned in acetone, ethanol and deionized water in each case for 10 s in an ultrasonic bath. PDMS (Sylgard 184) spincoated cover slips (see note "1 ml PDMS is poured onto the cover slip and spincoated by 1500 rpm for 10 s. If the PDMS layer was too thick the DNA could not be detected.") are cleaned the same way. The coated cover slips and the PDMS slabs are oxidized in oxygen plasma for 30 s and assembled (see **note13**).
5. 30 min after chip assembly (see **note14**) the channels are filled with working buffer and incubated for at least 30 min [10].

Sample preparation

1. The DNA is stained with YOYO-1, ratio YOYO-1 to DNA base pairs 1:10. Therefore, 1.6

µl of 10 µM YOYO-1 and 100 ng DNA are pipetted into an Eppendorf vessel and incubated on a vortexer for 2 h at lowest shaking level.

2. For the DNA cytostatics complex YOYO-1 and ACTD (MW 384 kDa, 2 µl, 1 µl or 0.5 µl of 0.3 mM solution) are added and incubated on a vortexer at lowest shaking level for 2 h.
3. For the DNA polymerase complex YOYO-1 and *RNAP* are added (100 ng DNA, 500 ng RNAP) and incubated on a vortexer at lowest level for 2 h).

Chip Experiments

1. The working buffer is removed out of the reservoirs before the PMMA-block with is placed on top of the chip to enlarge the reservoirs and simplify handling.
2. 9 µl of working buffer is pipetted into all reservoirs but reservoir 3 (see fig.1).
3. 9 µl of the DNA solution is pipetted into reservoir 3 of the prefilled chip (see **note15).**
4. The specific voltages for the separation experiments are chosen as follows. First, the dc voltages are set such that the DNA molecules continuously flow, parallel to the channel wall, towards the ridge (*cf.* fig.4) (see **note16**). It is assured that the inflowing molecules occupy less than a quarter of the total width of channel 2.
5. The ac voltage is switched on to reasonably small amplitudes (50 V) and its effect on the particle motion is observed while varying the frequency over an interval from 50 to 1000 Hz. If no deflection is observed the amplitude is increased, followed by varying the frequencies until a complete deflection of the DNA at the ridge is observed for the first time (see **note17**).

Image acquisition and processing

1. The channel is scanned over the whole width during running experiments with a constant

speed of 10 μm/s along the y-direction at different x-positions of interest. Hence, the width of the injected molecules stream and the efficiency of the separation/detection can be determined, respectively. The y-position of the recorded images within the channel is determined from scanning speed and time.

2. To obtain the experiments result, the intensity distributions downstream of the ridge is evaluated. Therefore, series of images are recorded during the scan in y-direction downstream of the nanoslit. Afterwards, each image is partitioned in six slices of height 11.4 μm (in y-direction see fig.5), in order to reduce convolution effects. Then, the total fluorescence intensity within these slices is determined. Successively recorded images are shifted by 1 μm in y-direction, so that in every eleventh image those slices overlap within a precision of less than 1 μm.
3. The measured fluorescence intensity is averaged over up to six such overlapping slices and plotted as a function of the y-position. These data are fitted with Gaussian curves and the resolution is calculated according to $\mathrm{Re}\,s = \frac{x_2 - x_1}{2(\sigma_1 + \sigma_2)}$ with x_2, x_1 the position of peak maximum and σ_1, σ_2 the corresponding peak width [42]. Therefore, values of $\mathrm{Re}\,s > 1$ indicate baseline separated resolution.

Notes

1. “Surface coatings DDM and MC were added to reduce adhesion of samples to the surfaces and for control and reproducibility of the electroosmotic flow.”
2. “By data acquisition with an 8 by 8 binning and 10 frames per second (fps) it was possible to detect single DNA molecules migration quite well.”
3. ”First the respective amounts of photoresist (12%: 23.21 ml, 18%: 35.3 ml, and 52%: 100

ml) are measured with water and filled into a vial. After the filing heights are marked the respective amount of thinner (12%: 100 ml, 18%: 100 ml, 52%: 32.7 ml) is measured with water, added into the vial and the filling height is marked. Then the vials are cleaned with caroic acid for 5 min, rinsed with water, twice and dried on a hotplate (180 °C for 30 min). After the vials are cooled to room temperature photoresist and thinner are filled into the vials according to the respective marked filling heights and carefully mixed."

4. "The wafer as well as the photomask was aligned such that the position could easily be reproduced along the second illumination. Thus, the time for alignment was minimized."
5. "To prevent disturbation of the photoresist structures the wafer was hold horizontal and slowly flooded with acetone first until it was totally covered. Then the acetone was slowly replaced by isopropanol, which was gently removed with nitrogen."
6. "It is important that the positioning crosses are covered with adhesive tape before the second layer of photoresist is poured. The tape is removed the moment the hotplate reaches 65 °C. We tried earlier and found the photoresist was to sticky to be removed without damages. If tried at higher temperature the adhesive became sticky due to the heat. "
7. "Even small malpositioning at the center of the wafer effects large displacement at the structures in the border area of the wafer. Thus, the positioning was checked at four positioning crosses to ensure perfect alignment of the second photomask to the first structures."
8. "The 2,4,6,8-tetramethyl-2,4,6,8-tetravinylcycltetrasiloxane and 1 ml (25-35% methylhydrosiloxane)-dimethylsiloxane copolymer was added and mixed up just before the h-PDMS was pured over the masterwafer due to the fast polymerization after adding."
9. "The first layer of h-PDMS had a thickness of about 40 µm and thus completely

contained the microstructured channel formed by the master wafer."

10. "The h-PDMS must be fully polymerized to ensure stable molding of the nanoslit. It was found that stable structures were formed if no imprint is visible when a tweezer is pressed onto the baked h-PDMS."
11. "The h-PDMS is very brittle, although the double-layer with PDMS allows better handability. To ensure that the h-PDMS does not crack during removement, the wafer is mounted on a hot plate at 65 °C for 5 min. The following steps are performed with the wafer still on the hot plate. Keep attention not to be burned!

    First, stickings were loosed by rounding the masterwafer with a tweezer in between the h-PDMS and the masterwafer. Then a bowed metal plate, that is warmed, too, is placed on top of the PDMS. When holding the PDMS double-layer and the metal plate with a tweezer the wafer is slowly peeled-off, following the curvature of the metal plate."
12. "To ensure a reproducible distance between the reservoirs a punching pattern, consisting blanks for all reservoirs, was used "
13. "Due to the very small height of the structures these are very sensible to pressures. To prevent a collapse of the nanostructures along assembly, the PDMS slab was gently laid onto the coverslip without bending the slab. If still air is in between the PDMS and the coverslip the PDMS must not be pressed but softly swiped with a tweezer."
14. "30 min was found to ensure that covalent bindings were formed between the PDMS slap and the PDMS coated cover slip. For shorter times the channel system was permeable."
15. "The DNA solution is filled last. Otherwise DNA would flood the other channels, which could prohibit controlled DNA injection as a narrow stream."
16. "The dc voltages were set such that the velocity of the DNA molecules was about 30 µm/s. Otherwise, higher ac voltages would have to be applied."

17. “If the applied parameters were close to appropriate parameters the migration behavior of the DNA at the nanoslit altered. First, the DNA passed the nanoslit totally unaffected. When the parameters were close to the appropriate parameters it looked like the DNA briefly stopped before passing the nanoslit.”

Acknowledgement

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Figure & Legends:

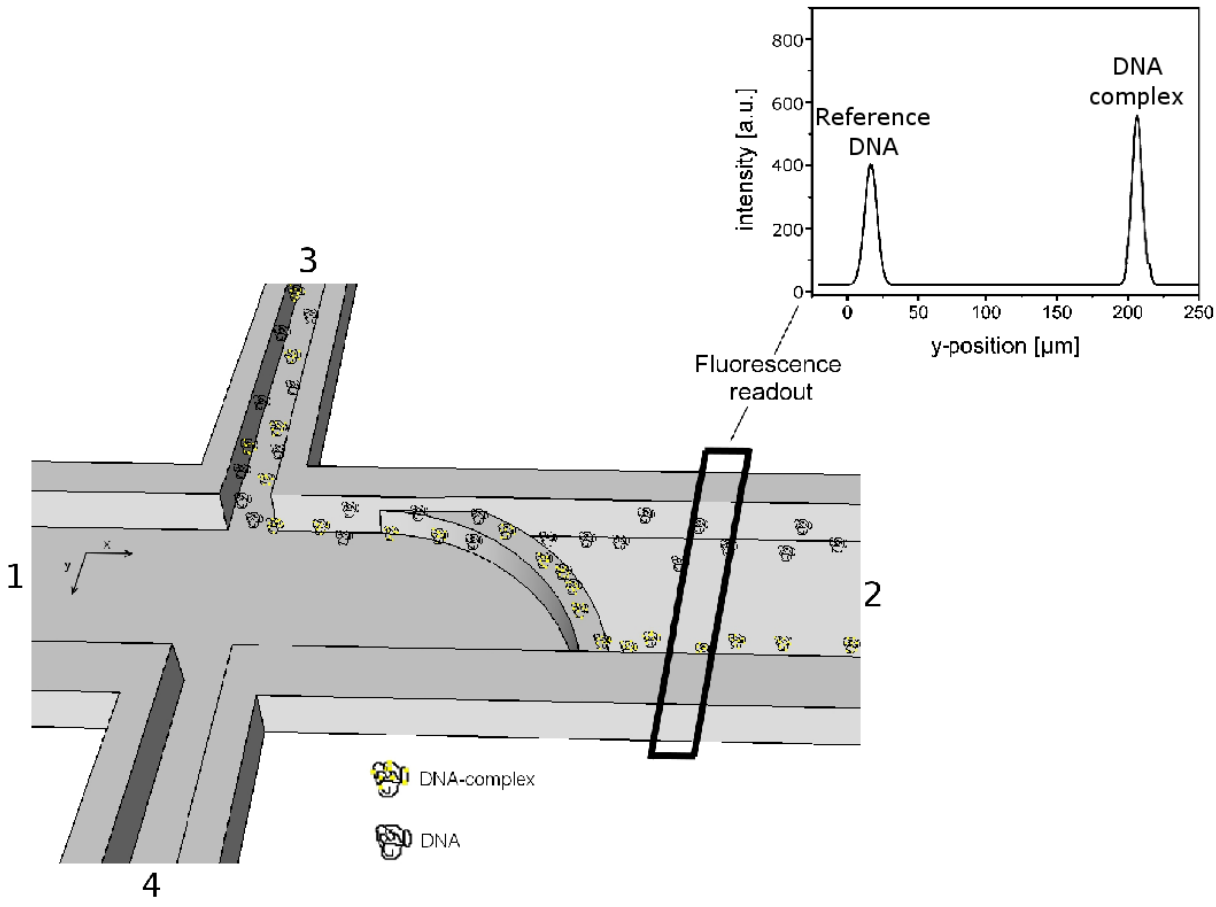

Fig.1: Scheme of the continuous-flow separation and analysis. The analyte is injected towards the ridge as a narrow stream. At the ridge, one species is deflected; the second species passes the ridge unaffected. Downstream of the ridge the response is monitored by fluorescence intensity distributions.

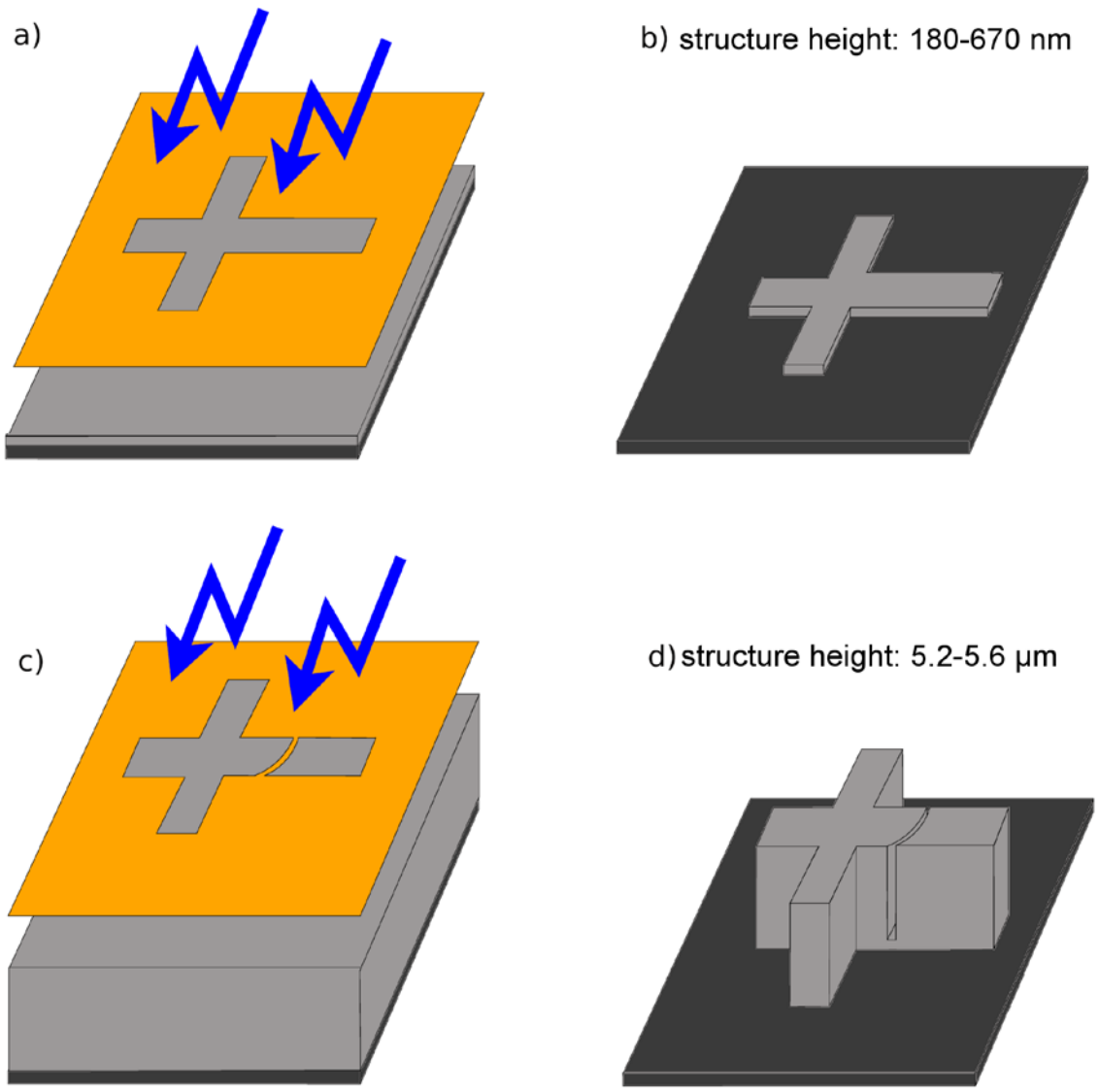

Fig.2: Scheme of masterwafer production. a) A thin layer of photoresist (dark grey) is spincoated on a silicon wafer (light grey) and illuminated through a chromium mask (orange), defining the nanoslit height and the general channel layout. b) Silicon wafer with first layer of photoresist after developing. The structure height is from 180-670 nm corresponding to the parameters of photoresist and spincoating. c) A second layer of photoresist is spincoated and illuminated through a chromium mask, defining the ridge shape and free channel height. d) Masterwafer, with negative relief structure, ready to be molded with PDMS.

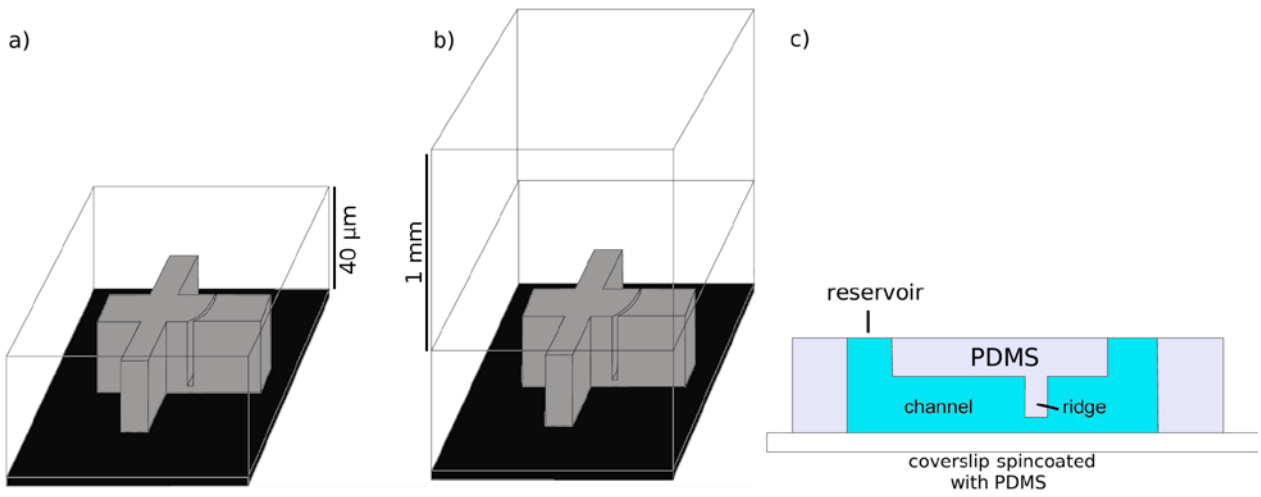

Fig.3: Scheme of double-layer soft lithography. a) First layer of h-PDMS 40 µm in height (not to scale), to stably mold the nanoslit. b) Second layer of PDMS 1 mm (not to scale), for better handability. c) Cross section of assembled chip with reservoirs and running buffer (blue).

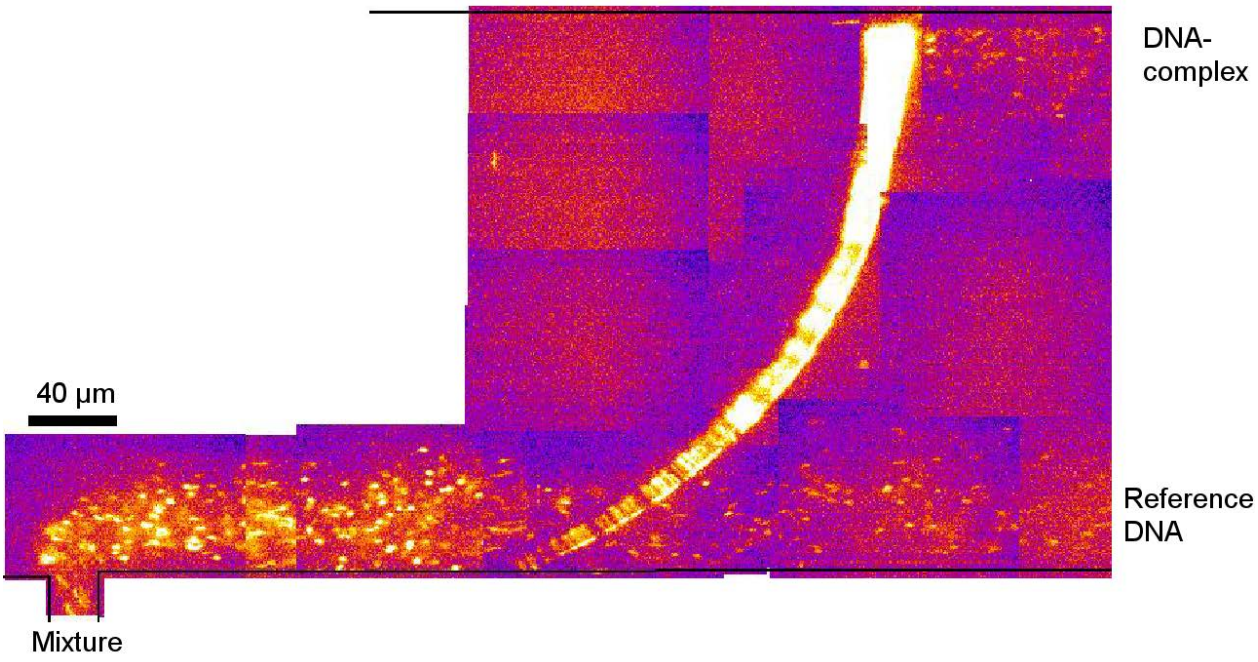

Fig.4: Detection of complex-formation and separation. Fluorescence images (collage) at the nanoslit. From the left, a mixture of DNA is continuously injected (yellow spots correspond to single DNA molecules. The reference DNA passes the nanoslit unhindered, whereas the DNA-complexes are trapped on the ridge and migrate towards the opposite channel side before they escape due to Brownian motion. Evaluation of the separation resolutions reveals a clearly baseline-separated resolution.

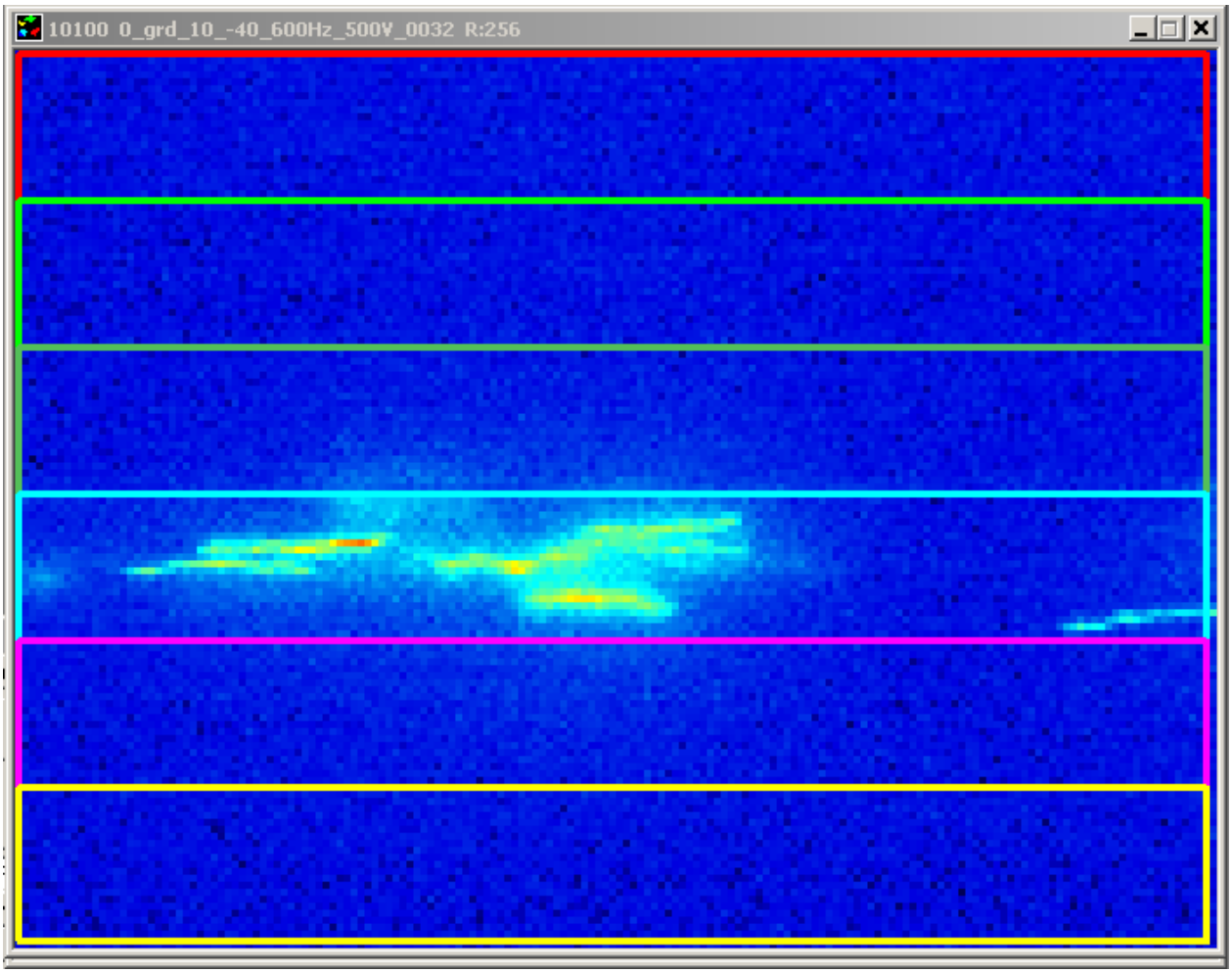

Fig.5: The region of interest is partitioned in six slices, each with height of 11 µm. To determine the analytes distribution the fluorescence intensity is measured in each slice separately, along the scanning procedure, and averaged over up to six overlapping slices.