Chapter 6

MITOMI: A Microfluidic Platform for In Vitro Characterization of Transcription Factor–DNA Interaction

Sylvie Rockel, Marcel Geertz, and Sebastian J. Maerkl

Abstract

Gene regulatory networks (GRNs) consist of transcription factors (TFs) that determine the level of gene expression by binding to specific DNA sequences. Mapping all TF–DNA interactions and elucidating their dynamics is a major goal to generate comprehensive models of GRNs. Measuring quantitative binding affinities of large sets of TF–DNA interactions requires the application of novel tools and methods. These tools need to cope with the difficulties related to the facts that TFs tend to be expressed at low levels in vivo, and often form only transient interactions with both DNA and their protein partners. Our approach describes a high-throughput microfluidic platform with a novel detection principle based on the mechanically induced trapping of molecular interactions (MITOMI). MITOMI allows the detection of transient and low-affinity TF–DNA interactions in high-throughput.

Key words: Microarrays, Transcription factor binding sites, High-throughput, Binding affinities, DNA array, Protein array, Surface chemistry, Two-step PCR, Microfluidics

1. Introduction

Transcription factors (TFs) are proteins that bind to specific DNA sequences and regulate the level of gene expression by either promoting or blocking the transcription of specific genes. These specific TF–DNA interactions are part of a dynamic gene regulatory network (GRN) which is beginning to be understood by generating integrated models from data of both experimental and computational methods (1, 2).

The comprehensive characterization of GRNs requires large-scale quantitative measurements of TF–DNA interactions in a high-throughput format. However, conventional experimental methods to study molecular interactions are limited by being either nonquantitative (3, 4) or relatively low-throughput (5). Generating microarrays of immobilized double-stranded DNA sequences and

Bart Deplancke and Nele Gheldof (eds.), *Gene Regulatory Networks: Methods and Protocols*,
Methods in Molecular Biology, vol. 786, DOI 10.1007/978-1-61779-292-2_6,

probing them with off-chip purified proteins has been widely used for the detection of TF–DNA interactions.

The more recent introduction of microfluidics in the field of protein and DNA microarrays is a promising approach to scale-down and parallelize biological assays and study individual molecular interactions in a miniaturized format. The use of microfluidics platforms has many advantages: samples can be detected with high-precision and sensitivity while at the same time decreasing the amount of consumables, and time needed, as compared to more conventional methods (6). With the development of multilayer soft lithography for the rapid prototyping of microfluidic systems (7, 8) and microfluidic large-scale integration (MLSI) (9) microfluidic technology has become appealing to the field of biology. These MLSI devices are generally fabricated from elastomeric materials, such as polydimethylsiloxane (PDMS) and harbor micron-sized channels with thousands of integrated micromechanical valves. Among the increasing number of applications in biology, microfluidic platforms emerged as powerful screening tools to study molecular interactions, which show the potential of realizing high-throughput and high-precision measurements (10, 11).

With the highly integrated microfluidic device described in this protocol, a novel detection method has been established based on the mechanically induced trapping of molecular interactions (MITOMI). MITOMI allows the capture of transient and low-affinity interactions between DNA sequences and TFs at equilibrium, and thus the measurement of absolute binding affinities. In short, picoliter-sized reaction chambers within the array of the microfluidic chip are aligned to spots of double-stranded DNA (dsDNA) sequences printed onto an epoxy-coated glass slide using standard DNA microarray instruments. After loading an in vitro transcription/translation (ITT) mixture together with genomic DNA coding for the TF, the TF of interest is synthesized on-chip and can bind to freely diffusing target DNA. These binding events are separated by micromechanical valves and thus will be detected independently by MITOMI.

2. Materials

2.1. Mask and Wafer Fabrication

2.1.1. Instruments for Mask and Wafer Fabrication

1. Mask writing on Heidelberg DWL200 laser lithography system (Heidelberg Instruments Mikrotechnik GmbH).
2. Mask and wafer development using DV10 (Süss MicroTec AG).
3. Wafer cleaning with oxygen plasma before processing using Tepla300 (PVA Tepla AG).
4. MA6 Mask Aligner (Süss MicroTec AG) for exposure of wafers.

5. Sawatec LMS200, programmable coater for negative resist and Sawatec HP401Z, programmable hot plate for soft bake (Sawatec AG).
6. Süss RC-8 THP, manual coater and hotplate for positive resist (Süss MicroTec AG).

2.1.2. Materials for Mask and Wafer Fabrication

Chemicals used in mask and wafer fabrication are from Rockwood Electronic Materials, Gréasque, France, and of Metal-Oxyde-Semiconductor (MOS) quality, unless otherwise stated.

1. Masks: square blank 5″ Nanofilm SLM 5 (Nanofilm).
2. Silicon wafers (diameter: 100 ± 0.5 mm, thickness: 525 ± 25 μm, conductivity type: P, dopant: Boron, resistivity range: 0.1–100 Ω cm; Okmetic).
3. Photoresists: AZ9260 positive photoresist (MicroChemicals GmbH); SU-8 negative photoresist GM1060 and GM1040 (Gersteltec).
4. Chrome etching of masks: CR7 consisting of $(NH_4)_2\,Ce(NO_3)_6$; $HClO_4$.
5. Developers: MP 351 for mask and AZ 400K for AZ9260coated wafers (AZ Electronic Materials); PGMEA (1-methoxy-2-propyl-acetate) for manual development of SU-8 wafers.
6. Solvents: Remover 1165 composed of 93% NMP (*N*-methyl-2-pyrrolidone; Sotrachem Technic) and 7% PGMEA for masks; isopropyl alcohol (IPA); acetone for wafers.

2.2. MITOMI Device Fabrication by Multilayer Soft Lithography

1. PDMS resin: Heat curable silicone elastomer (Dow Corning Sylgard 184).
2. Trimethylchlorosilane (TMCS) (Sigma-Aldrich).
3. Mixing and degassing of PDMS: Thinky Mixer ARE-250 equipped with adaptor for 100 ml disposable PP beakers (C3 Prozess- und Analysentechnik GmbH).
4. Degassing of PDMS control layer: Vacuum desiccator (Fisher Scientific AG).
5. Spin coating of PDMS flow layer: Programmable spin coater SCS P6700 (Specialty Coating Systems Inc.).
6. Stereomicroscope, SMZ1500 (Nikon AG).
7. Manual hole punching machine and pin vises, 21 gauge (0.04″ OD) (Technical Innovations, Inc.).

2.3. Epoxy Slide Preparation

Chemicals for epoxy-coating of glass slides are from Sigma-Aldrich.

1. Standard ($76 \times 26 \times 1$ mm) microscope glass slides (VWR).
2. Milli-Q water.
3. Ammonium hydroxide (NH_4OH; 30%).
4. Hydrogen peroxide (H_2O_2; 30%).

5. Solvents: Acetone, toluene, IPA.
6. 3-glycidoxypropyl-trimethoxymethysilane (3-GPS; 97%).
7. Nitrogen gas supply.

2.4. DNA Synthesis

All chemicals used for synthesis of DNA are from Sigma-Aldrich. For DNA primer sequences, see Table 1.

2.4.1. Synthesis of Linear Template DNA

1. DNA primers (Integrated DNA Technologies, IDT).
2. dNTPs (Roche Diagnostics AG).
3. Yeast genomic DNA (Merck Chemicals Ltd.).
4. Polymerase enzyme, Expand High Fidelity PCR system (Roche Diagnostics AG).
5. Elution buffer: 10 mM Tris–HCl, pH 8.5.

Table 1
Primer sequences used to generate ITT linear templates and target DNA library

Name (Comment)	Sequence
5′CompCy5 (Extension primer for target DNA synthesis)	5′-[Cy5]GTCATACCGCCGGA-3′
Target DNA (Design of target DNA library. Variable binding sites of interest are bracketed by constant linker sequences. 3′ ends consist of reverse complementary sequence of CompCy5 extension primer)	5′-[5′LINKER]-[BINDING SITE OF INTEREST]-[3′LINKER]-TCCGGCGGTATGAC-3′
Forward-ORF-HIS (Design gene-specific sequence to Tm of 60°C. Start codon is underlined. First five codons code for 5×His-tag. Alternatively, 5×His-tag can be added to Reverse_ORF primer)	5′-CTCGAGAATTCGCCACCATGCACCAC CACCACCAC-[GENE SPECIFIC]-3′
Reverse-ORF (Design gene-specific sequence to Tm of 60°C. Stop codon is underlined)	5′-GTAGCAGCCTGAGTCGTTATTA-[GENE SPECIFIC]-3′
Forward extension (T7 promoter sequence and transcription start site is indicated in italic and bold, respectively)	5′-GATCTTAAGGCTAGAGTA*CTAATACGA CTCACTATA***G** GGAATACAAGCTACTT GTTCTTTTTGCACTCGAGAATTC GCCACC-3′
Reverse extension (Poly(A) track is underlined)	5′-CAAAAAACCCCTCAAGACCCGTTTAGAGG CCCCAAG GGGTTATGCTAGTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTGTA GCAGCCTGAGTCG-3′
Forward final	5′-GATCTTAAGGCTAGAGTAC-3′
Reverse final	5′-CAAAAAACCCCTCAAGAC-3′

2.4.2. Synthesis of Target DNA

1. Primers, 5′CompCy5, (Integrated DNA Technologies, IDT).
2. dNTP (Roche Diagnostics AG).
3. Klenow fragment (3′→5′ exo-) (Bioconcepts).
4. Dithiothreitol (DTT).
5. Magnesium chloride ($MgCl_2$).
6. Buffer: Tris–HCl, pH 7.9.
7. 0.5% bovine serum albumin (BSA) in dH_2O (Table 1).

2.5. Microarraying/ Spotting

1. QArray2 microarrayer (Genetix GmbH) equipped with a NanoPrint™ microarray printer printhead and a 946MP3 microspotting pin (Arrayit Corporation).
2. BSA (Sigma-Aldrich) resuspended in DI water to 2 mg/ml.
3. Synthesized target DNA templates (see Subheading 2.4.2).
4. Epoxy-coated microscope glass slides (see Subheading 2.3).

2.6. Microfluidic Control Elements

2.6.1. Pressure Regulation and Control

1. Precision pressure regulator, BelloFram Type 10, 2–25 psi; 1/8″ port size (part. no. 960-001-000; Bachofen SA).
2. Bourdon tube pressure gauges, 0–30 psi (0–2.5 bar), G ¼ male connection (part. no. NG 63-RD23-B4; Kobold Instruments AG).
3. Custom-designed manual manifolds (rectangular metal casing: 14.5 × 1 × 1″) for control line regulation with 16 detented toggles and barbs for 1/16″ tubing ID; 1/4 NPT connection (Pneumadyne Inc.).
4. Fittings to connect regulators to gauges and to luer manifolds (Serto AG): tee union (SO 03021-8), male adaptor union (SO 01121-8-1/8).
5. Tubing: Tygon ¼″ OD × 1/8″ ID (Fisher Scientific AG).
6. Polycarbonate luer fittings (Fisher Scientific AG): multiport luer manifolds for flow inlet regulation (e.g., part. no. Cole Parmer 06464-87); male luer to luer connector (part. no. Cole Parmer 06464-90).

2.6.2. Fluidic Connections

Disposable stainless steel dispensing needles to connect to syringe 23 gauge, 1/2″ long, ID 0.33 mm (part. no 560014; I and Peter Gonano).

1. Tubing (Fisher Scientific AG): For liquid/gas; flexible plastic tubing for fluidic connections, Tygon S54HL, ID 0.51 mm;
2. Steel pins for chip-to-tube interface: Tube AISI 304 OD/ID × L Ø0.65/0.30 × 8 mm, cut, deburred, passivated (USA).

2.7. Mechanically Induced Trapping of Molecular Interactions (MITOMI)

2.7.1. Surface Chemistry

1. Biotinylated BSA (Sigma-Aldrich), reconstituted to 2 mg/ml in DI water (referred to as: BSA-biotin).
2. Neutravidin (Thermo Scientific Pierce), reconstituted to 0.5 mg/ml in PBS (referred to as: NA/PBS).
3. 1:1 solution of biotinylated Penta-histidine antibody (Qiagen AG) in 2% BSA.

2.7.2. Transcription Factor Synthesis

1. TNT® T7 coupled wheat germ extract mixture (Promega AG).
2. FluoroTect™ GreenLys tRNA (Promega AG).
3. Linear template DNA coding for transcription factor (see Subheading 2.4.1).

2.8. Data Acquisition

1. Modified ArrayWorx scanner (Applied Precision) for detection and softWoRx® software.
2. Axon GenePix (Molecular Devices) for analysis.

3. Methods

The entire process involves seven distinct steps that are described in details (see Fig. 1). Molds for the two-layer microfluidic device are fabricated on silicon wafers patterned from laser-written chrome masks in a class 100 clean-room environment. A set of control and flow molds is used to produce a double-layer device by multilayer soft lithography (10).

Libraries of Cy5-labeled target DNA sequences are synthesized and spotted onto epoxy-coated microscope slides. The DNA arrays are aligned to the microfluidic device containing 768 unit cells and bonded overnight. Assembled chips are mounted on a microscope stage and connected to a pneumatic setup. Each unit cell of the device can be controlled by three individually addressable micromechanical valves, which allow compartmentalization of each unit cell, control of DNA chamber access and the detection area. A circular button membrane is used to mask a precisely defined area during surface derivatization and for the MITOMI.

Upon surface derivatization the device is loaded with a mixture of wheat germ ITT extract and the linear DNA template coding for the TF of interest. Spotted target DNA and the synthesized TF localize to an antibody deposited underneath the circular button area. MITOMI is performed by actuating the button membrane and trapping bound complexes in equilibrium. These complexes can subsequently be visualized by scanning the device with a DNA array scanner. Binding affinities are determined by quantifying the detected signals.

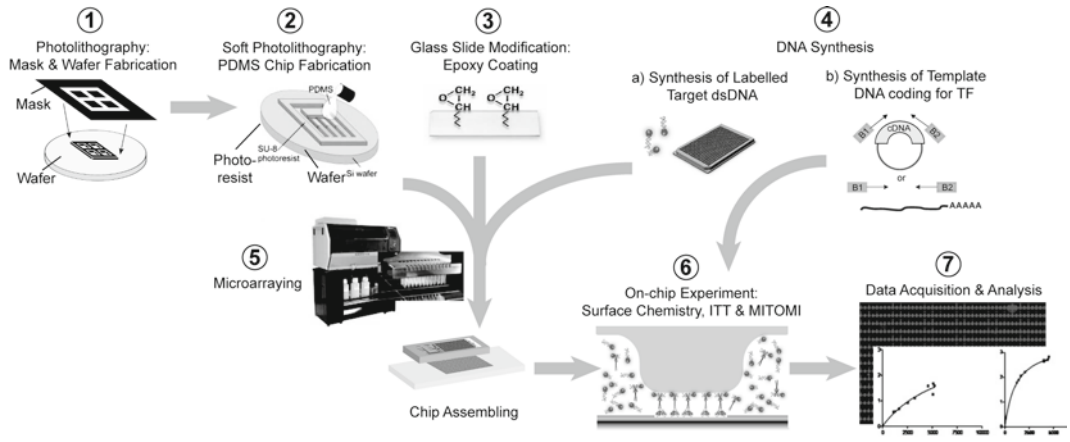

Fig. 1. Workflow of an MITOMI experiment. (1) Molds for the two-layer microfluidic device are produced on silicon wafers reproduced from chrome masks. (2) A double-layer device is fabricated of polydimethylsiloxane (PDMS) by multilayer soft lithography using a control and flow mold as template. (3) Microscope glass slides are surface modified with an epoxysilane coating. (4a) Short, fluorescently labeled target DNA sequences are synthesized and (5) spotted onto the epoxy-coated glass slides using a microarrayer before aligning and bonding them to microfluidic chip generated in step (2). (6) After surface derivatization of the glass slide, a mixture is loaded containing wheat germ in vitro transcription/translation (ITT) extract and the synthesized linear DNA template (4b) coding for the TF of interest. On-chip synthesized TFs are pulled down by immobilized antibody. TF–DNA interactions are captured by a mechanism based on mechanically induced trapping of molecular interactions (MITOMI) and (7) binding affinities quantified from detected interactions after scanning.

Note for researchers without clean-room and/or PDMS fabrication facilities: The MITOMI devices may also be obtained directly for a nominal fee from the California Institute of Technology (http://kni.caltech.edu/foundry/) and Stanford Microfluidic Foundries (http://www.stanford.edu/group/foundry/index.html).

Up-to-date protocols and microfluidic design files can be found on our laboratory homepage (http://microfluidics.epfl.ch).

3.1. Mask and Wafer Fabrication

All processes in this section are performed in a class 100 clean room.

3.1.1. Mask Fabrication

1. The two-layer device is designed in CleWin4 (WieWeb software).
2. Each layer is reproduced as a chrome mask using a Heidelberg DWL200 laser lithography system with a 10 mm writing head and a solid state wavelength stabilized laser diode (max. 110 mW at 405 nm).
3. For the development of masks, first the dispenser arm within the DV10 development chamber is purged for 10 s, then a developer mixture (MP 351:DI water 1:5) is applied twice to the mask (15 s), agitated for 45 s and drained, before rinsing and drying (50 s).
4. Developed masks are chrome etched for 110 s, rinsed, cleaned twice 15 min in 1165 remover bath, quick rinsed and air dried.

3.1.2. Flow Mold Fabrication

1. A 3″ silicon wafer is cleaned in a plasma stripper (Tepla 300) with 400 ml/min oxygen gas at 500 W and a frequency of 2.45 GHz for a period of 7 min.
2. A 1–2 μm thin layer of GM1040 negative resist is deposited on the oxygen plasma cleaned silicon wafer by spin coating first for 10 s at 500 rpm (ramp 100 rpm/s), then for 46 s at 1,100 rpm (ramp 100 rpm/s), followed by a short quick spin for 1 s at 2,100 rpm, and finally for 6 s at 1,100 rpm.
3. The precoated wafer is baked for 15 min at 65°C and 15 min at 105°C with a ramp of 4°C/min, and allowed to cool down to room temperature.
4. The wafer is exposed for 2 s in flood exposure mode with an alignment gap of 15 μm, using a lamp with a light intensity of 10 mW/cm² (further settings: WEC type: cont, N2 purge: no, WEC-offset: off).
5. The exposed wafer is baked on a hotplate for 35 min at 100°C.
6. Positive resist AZ9260 is spin coated on the precoated wafer for 10 s at 800 rpm, followed by 40 s at 1,800 rpm (ramp 1,000 rpm/s) to yield a substrate height of around 14 μm.
7. The coated wafer is then baked on a hotplate for 6 min at 115°C.
8. The soft-baked positive resist is allowed to rehydrate for 1 h.
9. The wafer is exposed on an MA6 mask aligner for two intervals of 18 s with 15 s waiting time in photolithography (soft contact) mode at 360 mJ/cm² with a light intensity of 10 mW/cm² (broad band spectrum lamp). The alignment gap is set to 15 μm (further settings: WEC type: cont, N2 purge: no, WEC - offset: off).
10. Following 1 h relaxation time, the wafer is developed in a development chamber (DV10) for 8–12 min based on visual observation after each cycle of the following routine with a total time of 4 min: A development mixture (AZ 400 K:DI water 1: 4) is dispensed and agitated on the wafer in three cycles, drained, rinsed (total time: 3:15 min), and finally dried.
11. In a final step, channels of the flow mold are annealed and rounded at 160°C for 20 min to create a geometry that allows full valve closure.

3.1.3. Control Mold Fabrication

1. Negative photoresist SU-8 GM1060 is spin coated on an oxygen plasma cleaned silicon wafer (see step 1 in Subheading 3.1.2) first for 10 s at 500 rpm (ramp 100 rpm/s), then for 50 s at 1,500 rpm (ramp 100 rpm/s), followed by a short quick spin for 1 s at 2,500 rpm, and finally for 6 s at 1,500 rpm to yield a height of ~14 μm.
2. The coated wafer is baked for 30 min at 130°C and 25 min at 30°C on a hotplate.

3. The wafer is exposed on an MA6 mask aligner for three intervals of 20 s with 15 s waiting time (all other settings see step 9 in Subheading 3.1.2).
4. The exposed wafer is developed manually for 2×5 min in PGMEA, rinsed with IPA and dried with an air gun.

3.2. MITOMI Device Fabrication by Multilayer Soft Lithography

1. The control layer mold is placed in a glass Petri dish lined with aluminum foil to facilitate easy removal. Care must be taken that the aluminum foil lining does not contain any holes.
2. To generate a hydrophobic surface, both flow and control mold are exposed to vapor deposits of TMCS for 30 min by placing them into a sealable plastic container with 1 ml TMCS filled into a plastic cap. TMCS treatment is repeated for 10 min each time prior to PDMS chip fabrication.
3. For the control layer, 60 g of a 5:1 Sylgard mixture (50 g Part A:10 g Part B) is prepared, mixed for 1 min at 2,000 rpm (~400×*g*) and degassed for 2 min at 2,200 rpm (~440×*g*) in a centrifugal mixer.
4. The mixture is poured onto the control layer mold and degassed in a vacuum desiccator for 10 min.
5. For the flow layer, 10.5 g of a 20:1 Sylgard mixture (10 g Part A:0.5 g Part B) is prepared, mixed for 1 min at 2,000 rpm (~400×*g*) and degassed for 2 min at 2,200 rpm (~440×*g*) in a centrifugal mixer.
6. The mixture is spin coated onto the flow layer with a 15 s ramp and a 35 s spin at 2,200 rpm.
7. After removing the control layer mold from the vacuum chamber any residual surface bubbles are destroyed by blowing on top of the PDMS layer. Any visible particles on top of the control channel grid are carefully removed using a toothpick.
8. Both layers are cured in an oven for 30 min at 80°C.
9. Following polymerization, both molds are taken from the oven and allowed to cool for 5 min.
10. The control layer is then diced with a scalpel and holes (1–8 and B, S, C, O in Fig. 2a) are punched at the control input side using a hole puncher or a 21 gauge luer stub.
11. The channel side of the control layer is thoroughly cleaned with Scotch Magic Tape.
12. The cleaned control layer is then aligned to the flow layer on the stereomicroscope.
13. The device is bonded for 90 min at 80°C in an oven.
14. Bonded devices are removed from the oven and allowed to cool for 5 min.
15. Following the outline of the control layer each individual device is cut with a scalpel and peeled off the flow layer.

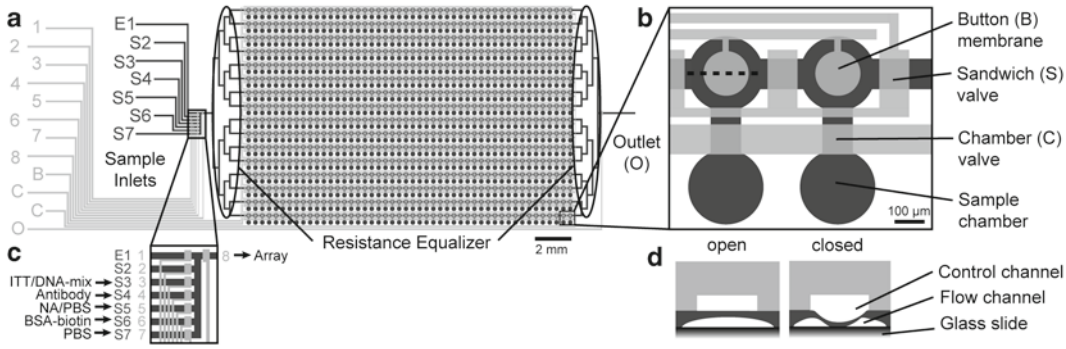

Fig. 2. (**a**) Drawing of an MITOMI device with 768 unit cells in the flow layer (*dark grey*) which are controlled (*light grey*) by 2,388 valves. Resistance equalizers toward solution inlets and outlet ensure equal flow velocities and even derivatization in each row of the channels. (**b**) The magnified view shows two individual unit cells each controlled by three separate micromechanical valves: the chamber in each dumbbell-shaped unit cell hosts a different target DNA sequence and is isolated with the chamber valve during surface modification steps for subsequent pull-down to an immobilized antibody in the detection area underneath the button membrane. For diffusion of samples to the immobilized antibody chamber valves are opened while sandwich valves between individual unit cells are closed in order to prevent cross-contamination between different samples. (**c**) Tygon tubing is loaded with different sample solutions and connected to a metal pin that is plugged into a hole at the end of each channel within the sample inlet tree (also see magnified insert of Fig. 3b). Loading of the device with samples via the inlets (S2–S7) is controlled by opening and closing the corresponding control valves. (**d**) A cross-section of one of the unit cells is shown to illustrate the detection mechanism based on mechanically induced trapping of molecular interactions (MITOMI). A deflectable membrane can be pushed down by actuating the water-filled control channel and consequently physically trap surface bound target DNA.

16. Holes are punched for the sample inlet and outlet (S1–S7 and O in Fig. 2a) using a hole puncher.
17. The flow channel side is cleaned thoroughly with tape before aligning the device to a spotted glass slide (see Subheading 3.5).
18. The flow mold is cleaned of any residual polymerized PDMS either by peeling off the thin layer of PDMS using a pair of tweezers or by an additional PDMS layer. For the latter, 11 g of a 10:1 Sylgard mixture (10 g Part A:1 g Part B) is mixed for 1 min at 2,000 rpm (~400 × *g*), degassed for 2 min at 2,200 rpm (~440 × *g*), poured on the flow mold cured in the oven for 30 min at 80°C, and peeled off after cooling down to room temperature. The control mold is cleaned with a nitrogen air gun of any PDMS debris.

3.3. Glass Slide Preparation

3.3.1. Cleaning Procedure

1. All glassware is prepared by rinsing with Milli-Q water.
2. 750 ml Milli-Q water and 150 ml ammonium hydroxide are heated to 80°C in a staining bath.
3. 150 ml hydrogen peroxide is carefully poured to the ammonium solution.
4. Glass slides are added into the staining bath and incubated for 30 min.

5. After removal from the staining bath, the glass slides are allowed to cool for 5 min.
6. Glass slides are then rinsed with Milli-Q water in the staining bath.
7. Clean glass slides are dried with nitrogen and stored in a dust free box.

3.3.2. Epoxysilane Deposition

1. Before epoxysilane deposition, all glassware is rinsed with acetone and dried at 80°C.
2. Cleaned glass slides are incubated for 20 min in 891 ml toluene with 9 ml 3-GPS.
3. After rinsing with fresh toluene to remove unbound 3-GPS, the glass slides are dried with nitrogen.
4. Glass slides are baked at 120°C for 30 min.
5. Following sonication in toluene for 15 min, glass slides are rinsed with fresh IPA.
6. Coated glass slides are dried with nitrogen and stored in a dust-free box under oxygen free conditions until usage.
7. In case of systematic PDMS chip delamination: Prior to DNA spotting, glass slides are rinsed with toluene and dried with nitrogen.

3.4. DNA Synthesis

3.4.1. Synthesis of Linear Template DNA

Generation of linear templates from genomic DNA or cDNA (see Note 1) of the TF of interest by a two-step polymerase chain reaction (PCR) method in which the first step amplifies the target sequence and the second step adds required 5′UTR and 3′UTR for efficient ITT.

1. For the first PCR step, a mixture of the following components is prepared in a final volume of 50 μl:

1 μM	Forward-ORF primer
1 μM	Reverse-ORF primer
100 ng	Yeast genomic DNA
200 μM	of each dNTP of a nucleotide mix
2.5 units	HiFi Polymerase enzyme mixture

2. After initial denaturation for 4 min at 94°C, the first PCR amplification is performed with 30 cycles as follows:

(a) Denaturation	94°C for 4 min
(b) Annealing	53°C for 60 s
(c) Elongation	72°C for 90 s and finished with a final extension at 72°C for 7 min

The correct product of this step should be ascertained on a 1% agarose gel.

3. For the second PCR step, a mixture of the following components is prepared to yield a final volume of 100 µl:

   5 nM Forward extension

   5 nM Reverse extension

   1 µl PCR product (from previous step)

   200 µM of each dNTP of a nucleotide mix

   2.5 units HiFi Polymerase enzyme mixture

4. After initial denaturation for 4 min at 94°C, the second PCR amplification is performed with ten cycles as follows:

(a) Denaturation	94°C for 4 min
(b) Annealing	53°C for 60 s
(c) Elongation	72°C for 90 s and finished with a final extension at 72°C for 7 min

5. To each reaction, 1 µl of final primer mix is added (Forward final + Reverse final; each at 1 µM final concentration) and cycling continued for 30 cycles after 4 min at 94°C as follows:

(a) Denaturation	94°C for 4 min
(b) Annealing	50°C for 60 s
(c) Elongation	72°C for 90 sand finished with a final extension at 72°C for 7 min

6. The final product can be used directly in ITT reactions or purified on spin columns and eluted in 100 µl 10 mM Tris–Cl (pH 8.5).

3.4.2. Synthesis of Target DNA

1. Small Cy5 labeled, dsDNA oligos are synthesized by isothermal primer extension in a reaction of a total volume of 30 µl containing:

6.7 µM	5′CompCy5
10 µM	Library primer
5 units	Klenow fragment (3′→5′exo-)
1 mM	of each dNTP from a nucleotide mix
1 mM	Dithiothreitol (DTT)
50 mM	NaCl
10 mM	$MgCl_2$
10 mM	Tris–HCl, pH 7.9

2. All reactions are incubated at 37°C for 1 h followed by 20 min at 72°C and a final annealing gradient down to 30°C at a rate of 0.1°C/s.
3. After the synthesis, 70 μl of a 0.5% BSA dH_2O solution are added to each reaction.
4. The entire volume is then transferred to a 384 well plate and a sixfold dilution series prepared with final concentrations of 5′CompCy5 of 2 μM, 600 nM, 180 nM, 54 nM, 16 nM, and 5 nM (see Note 2).

3.5. Microarraying/ Spotting

Spotting target DNA onto epoxy-coated microscope slides is performed by a QArray2 DNA microarrayer.

1. Before each spotting routine, an Eppendorf or Falcon tube filled with DI water and the spotting pins is submerged in a sonicator water bath to clean the pins. During the spotting routine, a sterilizing loop (1 s DI water, followed by 1 s air drying) between different DNA samples keeps pins clean throughout the spotting procedure.
2. Sample plate(s) of target DNA are placed in an external source plate stacker before starting the spotting routine (see Note 3).
3. The dilution series for each target DNA sequence is spotted as a microarray with a column and row pitch of 373 μm and 746 μm, respectively.
4. Spotted arrays are aligned manually to a tape-cleaned PDMS device (see Subheading 3.2) on a Nikon SMZ1500 stereoscope and bonded overnight in an incubator at 40°C.
5. DNA arrays can be stored in a sealed box protected from light and dust at room temperature for several weeks.

3.6. On-Chip Experiment (Surface Chemistry and MITOMI)

3.6.1. Mounting MITOMI Device to Microfluidic Control Elements

1. The assembled device is mounted on a light microscope and connected to tubing (for details see Fig. 3).
2. Control channels are filled with DI water by actuation with ~5 psi of pneumatic pressure which forces the air from the dead-end channels into the bulk porous silicone. This procedure eliminates subsequent gas transfer into the flow layer upon valve actuation, as well as prevents evaporation of the liquid contained in the flow layer.
3. Devices are actuated with 15–20 psi in the control lines and between 5 and 8 psi for the flow line.
4. Upon actuation button membrane and sandwich valves are opened again; chamber valves remain closed during the following initial surface derivatization steps to prevent liquid from entering the sample containing chambers.

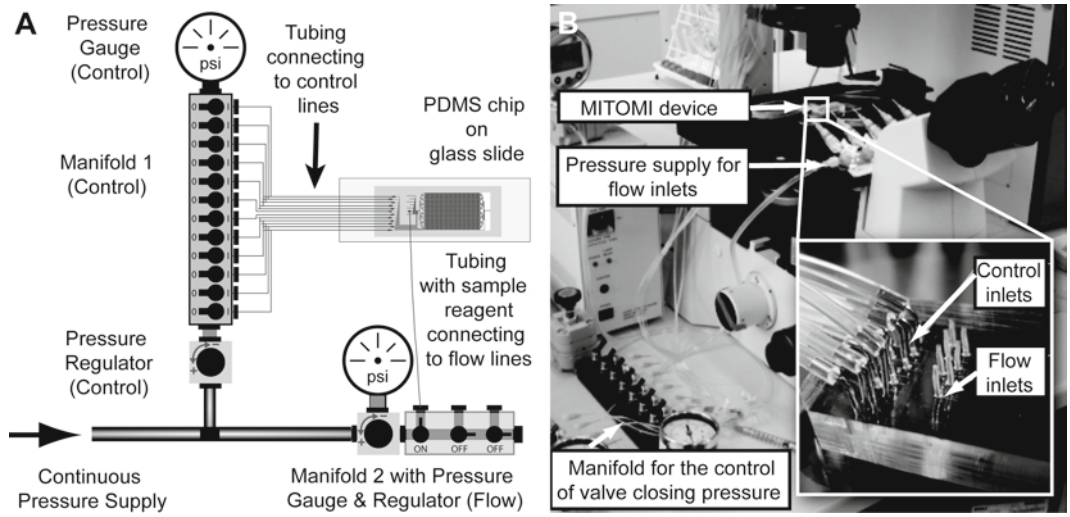

Fig. 3. Setup of the experiment. (**a**) Schematic of fluidic control set-up using regulated pressure and manually controlled manifolds. (**b**) Photograph of an assembled MITOMI device placed on the stage of a microscope, where flexible tubing (Tygon) is connected via metal pins to the inlets that actuate the control lines on the device (*magnified inset*). The tubing is filled with DI water displacing the air in the channels when actuated with pneumatic pressure that is controlled with manifolds. Each reagent for the on-chip experiment is filled into a pin-end flexible tube (Tygon) and connected to the flow inlets. The pressure of the flow can be controlled with a gauge.

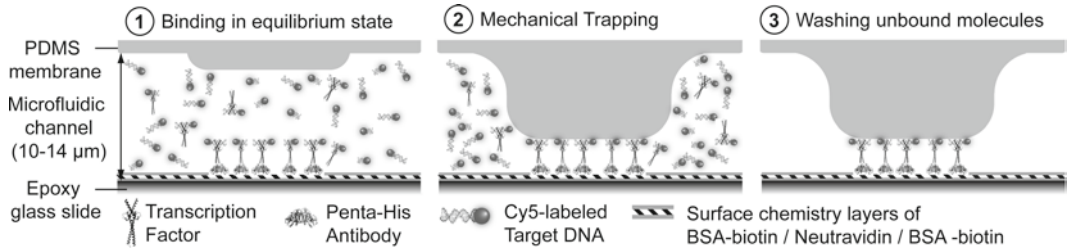

Fig. 4. Schematic of the MITOMI process. The *gray structure* at the top of each panel represents the deflectable button membrane that can be brought into contact with the glass surface (also see Fig. 2d). (1) His_5-tagged TFs are localized to immobilized penta-His antibody at the epoxy-coated glass slide. Specific binding between Cy5-labeled target DNA and TFs are at steady state when (2) the button membrane is actuated and brought into contact with the surface. Any molecules in solution are expelled while surface-bound material is mechanically trapped. (3) Unbound material that was not physically protected is washed away, and the remaining molecules are quantified.

3.6.2. Surface Chemistry

The surface area within the flow channels of the device is modified by depositing layers of BSA-biotin, NA/PBS, and biotinylated antibody onto the epoxy coated glass slide (see Fig. 4). Using a syringe, the different sample solutions are loaded into short pieces (30–40 cm) of clean Tygon tubing which are hooked to a metal pin that is then pushed into the corresponding flow sample inlet holes (see Fig. 2c).

1. Tubing with 30 µl of BSA-biotin is inserted into the sample inlet hole S6. The port on manifold 2 for flow inlet regulation (see Fig. 3) is actuated and valves are opened by switching the

corresponding toggles on manifold 1 in the following order: valve 6 controlling sample flow inlet S6, equalizer (1), array inlet (8).

2. Once the air in the channels of the array is displaced with liquid, the outlet valve (O) is opened and the equalizer valve (1) opened.
3. After flowing BSA-biotin for ~20 min the array inlet valve (8) and the sample valve (6) are closed again and the port on manifold 2 turned off.
4. The tubing is disconnected from the port of manifold 2.
5. The process of valve and port opening/closing is performed for the following samples.
6. After BSA-biotin derivatization, the surface area is washed for 2–3 min with 5 μl PBS (S7).
7. A 30 μl solution of NA/PBS (S5) is flowed for ~20 min and washed again for 2–3 min with 5 μl PBS.
8. The button membrane (B) is closed and PBS washing continued for 1 min (~2 μl) making sure button is closed.
9. The remaining surface area is passivated with 30 μl BSA-biotin (~20 min) and washed with 5 μl PBS (2–3 min) while button is actuated.
10. 30 μl of a 1:1 solution of biotinylated penta-His antibody in 2% BSA is loaded (S4). To ensure that all channels are saturated with antibody solution, the button membrane is opened only after flowing 5 μl.
11. After finishing the antibody deposition (total ~20 min), the surface is washed again with 5 μl PBS for 2–3 min.
12. The surface derivatization procedure is finished with a final 5 μl PBS washing step (see Note 4).

3.6.3. DNA Pull-Down and Detection of Interactions On-Chip

1. 25 μl TNT T7 coupled wheat germ extract is prepared and spiked with 1 μl $tRNA_{Lys}$-Bodipy-Fl and 2 μl of linear expression ready template coding for the appropriate transcription factor.
2. The mixture is immediately loaded onto the device (S3) and flushed for 10 μl (around 5–7 min) while the button membrane is closed.
3. Chamber valves (C) are opened and the outlet valve (O) is closed to allow for dead end filling of the chambers with wheat germ extract.
4. Chamber valves are closed and the outlet valve opened again and flushing is continued for an additional 10 μl (5–7 min).
5. Sandwich valves (S) that separate each unit cell are closed.
6. After ensuring that all sandwich valves are closed the chamber valves and button membranes are opened.

7. The device is incubated for 90 min at room temperature to allow for protein synthesis and diffusion of the samples to the immobilized antibody under the button membrane.
8. After the incubation period, the device is scanned on a modified ArrayWoRx microarray scanner.
9. Button membranes are closed to trap bound samples.
10. Chamber valves are closed, sandwich valves opened, and the channels washed with 10 µl PBS (5–7 min).
11. The washed device is scanned once more with closed button membranes to detect the trapped molecules.

3.7. Data Acquisition and Analysis

1. For each experiment, two images (see Fig. 5a) are analyzed with GenePix3.0 (Molecular Devices): The first image, taken directly after the 60–90 min incubation period before washing, is used to determine the concentration of solution phase or total target DNA concentration (Cy5 channel). The second image, taken after MITOMI and the final PBS wash, is used to determine the concentration of surface bound protein (FITC channel) as well as surface bound target DNA (Cy5 channel).
2. Dissociation equilibrium constants can be calculated for each experiment using a curve fitting software (e.g., Graphpad Prism4 or Mathematica) by performing global nonlinear regression fits using a one site binding model to the data plotted as surface bound target DNA (RFU) divided by surface protein concentration (RFU) (or effectively fractional occupancy) as a function of total target DNA concentration in RFU (see Fig. 5b).

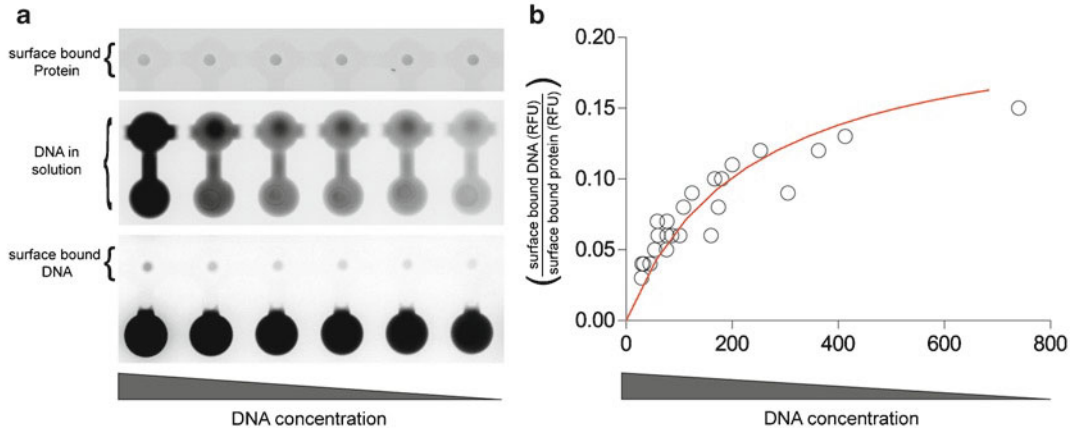

Fig. 5. Steps to analysis of MITOMI experiments. (**a**) Fluorescence scans of subsequent MITOMI steps. Scan of immobilized, fluorescent labeled protein (*Top*), solubilized target DNA (*Middle*), and surface bound target DNA after mechanical trapping and washing step (*Bottom*). (**b**) The fraction of surface bound target DNA is plotted against concentration of target DNA in solution. Dissociation constants are determined by performing a nonlinear regression fit using a one site binding model.

3. Relative K_ds (RFU^{-1}) must be transformed into absolute K_ds (M^{-1}) using a calibration curve previously established by measuring known quantities of 5′CompCy5 primer.
4. The change of free Gibbs energy (ΔΔGs) with $\Delta\Delta G = RT \times \ln(K_d / K_{d,ref})$ is calculated at a temperature of 298 K, where the highest affinity sequence serves as the reference.

4. Notes

1. Linear expression templates can also be synthesized from bacterial cDNA clones after lysing them in 2.5 μl Lyse n'Go buffer (Pierce) at 95°C for 7 min. The lysate serves as template in an Expand High Fidelity PCR reaction (Roche). The first PCR product is then purified using the Qiaquick 96 PCR purification kit (Qiagen) and eluted in 80 μl of 10 mM Tris–HCl, pH 8.5.
2. The on-chip DNA concentration can be increased by raising the numbers of repetitive stamps of sample DNA per spot during the spotting routine (multiple returns of spotting pin to the same spot).
3. The humidity inside the spotter is set to 65–80% to prevent the samples in the source plate from evaporating during long spotting routines (>2 h).
4. An additional passivation step can be included by flowing 5 μl BSA-biotin for 2–3 min after the antibody immobilization, then closing the button, followed by 2–3 min of 5 μl BSA-biotin. This additional BSA-biotin passivation step was found to reduce background signal.

References

1. Kim, H. D., Shay, T., O'Shea, E. K., and Regev, A. (2009) Transcriptional Regulatory Circuits: Predicting Numbers from Alphabets, *Science* **325**, 429–432.
2. Beer, M. A., and Tavazoie, S. (2004) Predicting gene expression from sequence, *Cell* **117**, 185–198.
3. Berger, M. F., Philippakis, A. A., Qureshi, A. M., He, F. S., Estep, P. W., 3 rd, and Bulyk, M. L. (2006) Compact, universal DNA microarrays to comprehensively determine transcription-factor binding site specificities, *Nat Biotechnol* **24**, 1429–1435.
4. Bulyk, M. L., Huang, X., Choo, Y., and Church, G. M. (2001) Exploring the DNA-binding specificities of zinc fingers with DNA microarrays, *Proc Natl Acad Sci USA* **98**, 7158–7163.
5. Majka, J., and Speck, C. (2007) Analysis of protein-DNA interactions using surface plasmon resonance, *Adv Biochem Eng Biotechnol* **104**, 13–36.
6. Whitesides, G. M. (2006) The origins and the future of microfluidics, *Nature* **442**, 368–373.
7. Duffy, D. C., McDonald, J. C., Schueller, O. J. A., and Whitesides, G. M. (1998) Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane), *Analytical Chemistry* **70**, 4974–4984.
8. Xia, Y., and Whitesides, G. M. (1998) Soft Lithography, *Angewandte Chemie International Edition* **37**, 550–575.
9. Thorsen, T., Maerkl, S. J., and Quake, S. R. (2002) Microfluidic large-scale integration, *Science* **298**, 580–584.

10. Maerkl, S. J., and Quake, S. R. (2007) A systems approach to measuring the binding energy landscapes of transcription factors, *Science* **315**, 233–237.

11. Maerkl, S. J., and Quake, S. R. (2009) Experimental determination of the evolvability of a transcription factor, *Proc Natl Acad Sci USA* **106**, 18650–18655.