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A high-throughput microfluidic method for generating and characterizing transcription factor mutant libraries

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Abstract

Characterizing libraries of mutant proteins is a challenging task, but can lead to detailed functional insights on a specific protein, and general insights for families of proteins such as transcription factors. Challenges in mutant protein screening consist in synthesizing the necessary expression ready DNA constructs and transforming them into a suitable host for protein expression. Protein purification and characterization are also non-trivial tasks that are not easily scalable to hundreds or thousands of protein variants. Here we describe a method based on a high-throughput microfluidic platform to screen and characterize the binding profile of hundreds of transcription factor variants. DNA constructs are synthesized by a rapid two-step PCR approach without the need of cloning or transformation steps. All transcription factor mutants are expressed on-chip followed by characterization of their binding specificities against 64 different DNA target sequences. The current microfluidic platform can synthesize and characterize up to 2400 protein – DNA pairs in parallel. The platform method is also generally applicable, allowing high-throughput functional studies of proteins.

Keywords: Microarrays; transcription factor (TF) binding sites; high-throughput; binding affinities; DNA array; protein array; surface chemistry; two-step PCR; microfluidics

1. Introduction

The sequence-specific binding of transcription factors (TF) determines in a large part the connectivity of gene regulatory networks as well as the quantitative level of gene expression. While technological advances increased our knowledge of DNA binding specificities across a number of TF families (1-7), relatively little is known about the functional consequences of how amino acid substitutions in the DNA binding domain impact these specificities. The lack of a simple correspondence between amino acid residues and specific DNA bases complicates the identification of such a sequence recognition code (8). The more recent development of microfluidic large-scale integrations (MLSI) offers new implementations of experimental approaches to elucidate the sequence – function relationship across a variety of TF families (3).

In this protocol we describe a novel detection method for the characterization of TF-DNA interactions based on the mechanically induced trapping of molecular interactions (MITOMI)(2). 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, MLSI devices are fabricated from polydimethylsiloxane (PDMS) an elastomeric material. These microfluidic chips harbor micron-sized channels with thousands of
integrated micromechanical valves to control picoliter-sized reaction chambers. Each reaction chamber is aligned to an array of dsDNA sequences printed onto an epoxy-coated glass slide using standard DNA microarray instruments. Here we modify our MITOMI protocol to map in parallel the contribution of amino acid substitution on TF DNA-binding specificity. By co-spotting PCR products coding for TF variants and cognate target DNA sequences and performing on-chip protein synthesis, MITOMI is capable of screening and characterizing the binding capabilities of several hundreds of TF variants to multiple target DNA sequences.

More generally, the protocol described here can be applied to the on-chip expression and functional characterization of hundreds to thousands of proteins in parallel. The expression templates can be readily derived either from cDNA libraries, generated by PCR, site-direct mutagenesis, or synthesized de novo using gene synthesis approaches.

2. Materials

2.1. Mask & Wafer Fabrication

2.1.1. Instruments for mask & 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 & 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 (NH₄)₂Ce(NO₃)₈; HClO₄
5. Developers: MP 351 for mask and AZ 400K for AZ9260 coated 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 (Polydimethylsiloxane) resin: heat curable silicone elastomer (Dow Corning Sylgard 184)
2. TMCS (trimethylchlorosilane) (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 × 26 × 1 mm) microscope glass slides (VWR)
2. Milli-Q water
3. Ammonium hydroxide (NH₄OH; 30 %)
4. Hydrogen peroxide (H₂O₂; 30 %)
5. Solvents: acetone, toluene, isopropyl alcohol (IPA)
6. 3-Glycidoxypropyl-trimethoxysilane (3-GPS; 97 %)
7. Nitrogen gas supply

2.4. **DNA synthesis**

2.4.1. **Synthesis of linear template DNA**

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

1. MAX isoform B (NCBI accession #: BC003525) from Open Biosystems (Clone ID: 3607261)
2. DNA primers (Integrated DNA Technologies, IDT)
3. dNTPs (Roche Diagnostics AG)
4. Expand High Fidelity PLUS PCR system (Roche Diagnostics AG)
5. Elution buffer: 10 mM TrisCl, pH 8.5

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. Magnesiumchloride (MgCl₂)
6. Buffer: Tris-HCl, pH7.9
7. 0.5% BSA in dH₂O

Table 1. Primer sequences used to generate ITT linear templates and target DNA library.
5'CompCy5  
(Extension primer for target DNA synthesis)

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)

Forward-ORF-HIS  
(This primer primes 3' of the DNA-binding region of the transcription factor, adding a new binding region variant and a linker region)

Reverse-ORF  
(Adds a c-terminal 5xHis tag, MAX iso B specific region is in bold, linker is in talics)

Forward extension  
T7 promoter sequence is indicated in bold

Reverse extension  
(Poly(A) track is underlined)

Forward final

Reverse final

2.5. Microarraying / Spotting

1. QArray2 microarrayer (Genetix GmbH) equipped with a NanoPrint™ microarray printer printhead and a 946MP3 micro spotting pin (Arrayit Corporation)
2. Bovine serum albumin (BSA; Sigma-Aldrich) re-suspended 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: 14.5x1x1”) 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 x 1/8” ID (Fisher Scientific AG)
6. Polycarbonate luer fittings (Fisher Scientific AG): multi-port 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 & 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 x L Ø0.65/0.30 x 8mm, cut, deburred, passivated (Unimed S.A.)

2.7. MITOMI
2.7.1. Surface Chemistry
1. Biotinylated bovine serum albumin (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 will be described in detail (see Figure 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 (2, 9).
 Libraries of linear expression templates, coding for TF variants, and Cy5-labeled target DNA sequences are synthesized and spotted onto epoxy-coated microscope slides. The DNA arrays are aligned to the microfluidic device containing 2400 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 mechanical induced trapping of molecular interactions (MITOMI).
Upon surface derivatization the device is loaded with a mixture of wheat germ in vitro transcription/translation (ITT) extract to express TF variants from spotted linear templates. Bound complexes are trapped at equilibrium using MITOMI. These complexes can subsequently be visualized by scanning the device with a DNA array scanner and relative binding affinities are determined by quantifying the detected signals.

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 & 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. 110mW @ 405 nm).
3. For the development of masks, first the dispenser arm within the DV10 development chamber is purged for 10 sec, then a developer mixture (MP 351:DI water 1:5) is applied twice to the mask (15 sec), agitated for 45 sec and drained, before rinsing and drying (50 sec).
4. Developed masks are chrome etched for 110 sec, 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 sec at 500 rpm (ramp 100 rpm/sec), then for 46 sec at 1100 rpm (ramp 100 rpm/sec), followed by a short quick spin for 1 sec at 2100 rpm, and finally for 6 sec at 1100 rpm.
3. The pre-coated 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 sec 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 pre-coated wafer for 10 sec at 800 rpm, followed by 40 sec at 1800 rpm (ramp 1000 rpm/sec) 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 a MA6 mask aligner for 2 intervals of 18 sec with 15 sec. 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 400K: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 sec at 500 rpm (ramp 100 rpm/sec), then for 50 sec at 1500 rpm (ramp 100 rpm/sec), followed by a short quick spin for 1 sec at 2500 rpm, and finally for 6 sec at 1500 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 a MA6 mask aligner for 3 intervals of 20 sec with 15 sec. waiting time (all other settings see step 9 in Subheading 3.1.2.).

4. The exposed wafer is developed manually for 2 x 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 doesn't 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 2000 rpm (~400 x g) and degassed for 2 min at 2200 rpm (~440 x 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 2000 rpm (~400 x g) and degassed for 2 min at 2200 rpm (~440 x g) in a centrifugal mixer.

6. The mixture is spin coated onto the flow layer with a 15 sec ramp and a 35 sec spin at 2200 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 Figure 3 A) are punched at the control input side using a hole puncher or an 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.
16. Holes are punched for the sample inlet and outlet (S1 – S7 and O in Figure 3 A) 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 2000 rpm (~400 x g), degassed for 2 min at 2200 rpm (~440 x 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 coding for TF variants

Generation of linear templates from 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 substitutes the DNA binding domain of the transcription with designed variants. 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:
   100 nM Forward-ORF primer
   100 nM Reverse-ORF primer
   1 µL Plasmid of MAX iso B cDNA clone
   200 µM of each dNTP of a nucleotide mix
   0.5 units HiFi Plus Polymerase enzyme mixture

2. After initial denaturation for 7 min at 94 °C, the first PCR amplification is performed with 30 cycles as follows:
   a. Denaturation 94 °C for 30 sec
   b. Annealing 55 °C for 60 sec
   c. Elongation 72 °C for 90 sec
   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
   1 unit HiFi Plus Polymerase enzyme mixture

4. After initial denaturation for 7 min at 94 °C, the second PCR amplification is performed with 10 cycles as follows:
   a. Denaturation 94 °C for 30 sec
   b. Annealing 55 °C for 60 sec
   c. Elongation 72 °C for 90 sec
   and finished with a final extension at 72 °C for 7 min.

5. To each reaction 2 µl of final primer mix is added (Forward final + Reverse final; each at 5µM final concentration) and cycling continued for 30 cycles after 4 min at 94 °C as follows:
   a. Denaturation 94 °C for 30 sec
   b. Annealing 50 °C for 60 sec
   c. Elongation 72 °C for 90 sec
   and finished with a final extension at 72 °C for 7 min.
6. The final products were then purified on PCR spin columns (Qiagen) with the supplier protocol and eluted in 80 µL of dH2O, pH 8.0-8.5.

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 MgCl2  
   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/sec.

3. After the synthesis, 70 µl of a 0.5% BSA dH2O solution are added to each reaction.

4. The entire volume is then transferred to a 384 well plate (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. Prior to any template spotting 0.5% BSA dH2O is spotted to prevent binding of DNA templates to the epoxy surface.

3. Sample plate(s) of ITT template and target DNA are placed in an external source plate stacker before starting the spotting routine (see Note 3).

4. Two rounds of spotting are sequentially performed. In the first round ITT templates, coding for transcription factor variants, are spotted, followed by a second round of spotting of target DNA sequences. Generally 3 technical repeats of each TF mutant – target DNA combination are spotted.

5. 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.

6. 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 & 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 Figure 2).
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 to 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.

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 Figure 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 Figure 3 C).

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 Figure 2) 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. **TF synthesis, DNA pull-down & detection of interactions on-chip**
1. 25 µl TNT T7 coupled wheat germ extract is prepared and spiked with 1 µl tRNA<sub>lys</sub>-Bodipy–Fl.
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 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 or 30°C 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 & analysis
1. For each experiment two images (see Figure 5 A) 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).

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 TrisCl, 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

Figures & Captions

Fig. 1. Workflow of a 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 PDMS (polydimethylsiloxane) 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 labelled 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 pulls down and can bind spotted target DNA sequences. 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.

Fig. 2. 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 insert). 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. 3. (A) Drawing of a MITOMI device with 768 unit cells in the flow layer (blue) which are controlled (red) by 2388 valves. Resistance equalizers towards solution inlets and outlet ensure equal flow velocities and even derivatization in each row of the channels. (B) The magnified view shows two single unit cells each controlled by 3 different micro-mechanical 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 under 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. 2 B). 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 thin deflectable membrane can be pushed down by actuating the water-filled control channel and consequently physically trap any material under this area in the flow channel.

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. 3 D). (1) His5-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.
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).