SUPPORTING INFORMATION FOR

Multi-parameter Screening on SlipChip used for nanoliter Protein Crystallization combining Free Interface Diffusion and Microbatch Methods

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Chemicals and Materials

All solvents and salts purchased from commercial sources were used as received unless otherwise stated. 1,2,3–Heptanetriol (high melting point isomer) was purchased from Fluka Biochemika (St. Louis, MO); FC–40 (a mixture of perfluoro–tri–n–butylamine and perfluoro–di–n–butylmethylamine) and FC–70 (perfluorotripentylamine) were obtained from 3M (St. Paul, MN). Food dyes were purchased from Ateco (Glen Cove, NY). Tridecafluoro–1,1,2,2–tetrahydrooctyl–1–trichlorosilane was purchased from United Chemical Technologies, Inc. (Bristol, PA). Alexa Fluor ® 488 dye (Alexa–488) was purchased from Invitrogen (Eugene, OR). 8-methoxypyrene-1,3,6 trisulfonic acid (MPTS) were purchased from Invitrogen (Eugene, OR). Soda–lime glass plates with chromium and photoresist coating were purchased from Telic Company (Valencia, CA). Amorphous diamond coated drill bits were obtained from Harvey Tool (0.030 inch cutter diameter, Rowley, MA). Fluorescence reference slides were purchased from Microscopy/Microscopy Education (McKinney, TX). Binderclips (5/32’ inch capacity, 1/2’ inch size) were purchased from Officemax (Itasca, IL). Pipettors were obtained from Eppendorf Inc. (Westbury, NY). Fisherbrand pipettor tips were from Fisher Scientific (Hanover Park, IL). N,N-Dimethyldodecylamine N-oxide (LDAO) was purchased from Fluka, Switzerland.

Experimental Procedures:

Fabrication of SlipChip with nanopatterning

We followed the procedure previously described[1] with the following modifications (Figure S1). A blank glass plate (Soda-lime glass, thickness: 0.7 mm; chromium coating: 1025 Å; AZ photoresist: 1 μm) was first cut to be 3 in × 1 in. A schematic diagram showing the step-by-step fabrication of the hybrid SlipChip with nanopatterning is presented in Figure S1.

Step 1: The glass plate with photoresist coating was aligned with a photomask containing the design for the wells and marks to align the mask with the plate (Karl Suss, MJB3). The photomask and plate were exposed to UV light for 1 min. Immediately after exposure, the photomask was removed from the glass plate and the glass plate was developed by immersing it in 0.1 mol/L NaOH solution for 2 min. Only the areas of the photoresist that were exposed to the UV light dissolved in the solution. The exposed underlying chromium layer was removed using a chromium etchant (a solution of 0.6:0.365 mol/L $HClO_4$ / $(NH_4)_2Ce(NO_3)_6$). The plate was thoroughly rinsed with Millipore water and dried with nitrogen gas, and the back of the glass plate was taped with PVC sealing tape (McMaster-Carr) to protect the back side of glass. On the edge of the glass plate, we designed cross marks for aligning the second photomask; these marks were also taped to prevent etching. The taped glass plate was then carefully immersed in a plastic container with a glass etching solution (1:0.5:0.75 mol/L $HF/NH_4F/HNO_3$) to etch the glass surface that was exposed after the chromium coating was removed. A 40 °C constant-temperature water bath shaker was used to control the etching speed. By control the etching time (~30 min), wells that were 40 μm deep were etched into the glass plate. The plate was thoroughly rinsed with Millipore water and dried with nitrogen gas.

Step 2: Using another photomask containing the design for the ducts and an etching time of ~15 min, 20 μm deep ducts were etched on to the glass plate using the same procedure as in Step 1. Care was taken to align the glass plate with the photomask. During this step, the 40 μm deep wells were further etched to be 60 μm deep. The plate was thoroughly rinsed with Millipore water and dried with nitrogen gas.

Step 3: After ducts and wells were etched into the plate, the plate was aligned with a nanopatterning photomask and the same procedure was followed as in Step 1. After removing the chromium coating, the glass plate was immersed in 50:25:37.5 mmol/L $HF/NH_4F/HNO_3$ etching solution, and etched for 20 min at room temperature (~23 °C) to produce ~250 nm deep patterns over the surface. Finally, the glass plate was rinsed with ethanol to strip the undeveloped photoresist, and immersed in the chromium etchant to remove the chromium coating. The glass was then rinsed with ethanol and Millipore water and dried with nitrogen gas.

The method described here integrates nanometer–deep designs and various micrometer-deep designs on one glass substrate. It could also be used to create nanometer/micrometer hybrid channels for other nanofluidic/microfluidic applications.

The etched patterns were measured with a Veeco Dektak 150 profilometer (Figure S2). The glass plates were cleaned and subjected to an oxygen plasma treatment, and then the surfaces were rendered hydrophobic by silanization in a vacuum dessicator for 3 hours with Tridecafluoro–1,1,2,2–tetrahydrooctyl–1–trichlorosilane as previously described[2] . After silanization, the glass plates were baked in a 120 °C oven for 30 min, rinsed by immersing in a tank of FC-3283, and dried in a 60 °C oven overnight.

FEP spin coating

FEP coating was used for the Microbatch SlipChip before we developed the nanopatterning method. An aqueous emulsion of fluorinated ethylene propylene (FEP TE-9568, Dupont) was diluted 4 times with Millipore water before use. After plasma cleaning of the Slipchip, the FEP solution was evenly spread over the surface of the SlipChip device with a plastic pipet and spin-coated. The spin speed was set to be either 1500 rpm/30 second or 2000 rpm/30 second. Once the coating was finished, the SlipChip was transferred to 120 °C oven and incubated for 10 minutes. After incubation, the Chip was baked on a 250 °C hot plate for 10 minutes, followed by 265 °C for another 10 minutes. After baking, the Chip was sintered on a 340 °C hot plate for 1 minute. The sintered Chip was then cooled to room temperature.

Measuring Contact Angles of nanopatterning

Glass plates were etched with nanopatterns by using the nanopatterning photomask described in Step 3 of *Fabrication of SlipChip with nanopatterning*, and different nanowell depths were obtained by controlling the etching time. All glass was silanized and cleaned before measuring contact angles. The glass plate was immersed into fluorocarbon in a glass tank. The plate, with patterned surface facing down, was clamped by two micro binderclips on each end to create a gap between the plate and the bottom of the tank. 5 μL of the measured aqueous solution was pipetted into the gap, and the aqueous droplet with 0.1% LDAO contacted the plate due to its buoyancy in the surrounding fluorocarbon (FC-40). The contact angle of the droplet on the substrate was then measured by using an optical contact angle meter (Ramé-Hart Instrument Co., Model 500). The contact angle was measured immediately after the droplet contacted the glass plate and again 5 min after contact.

Food dye experiment in a FID device

A FID device was made with the method described above without nanopatterning or FEP coating. The two plates of the device were assembled under FC-40. In the resulting orientation, fluidic channels for all 16 reagents and one sample were formed. All the solutions used for food dye experiments were filtered with a 0.45 μm PVDF syringe filter before use. Four food dyes (yellow, pink, red, and blue) were diluted ~10 times from their stock solutions and were pipet-loaded into 16 reagent channels. To load each channel, 4 μL of dye was first pushed through the inlet using a pipette until the dye solution emerged from the outlet. A green dye was diluted 20 times and was mixed with 0.04% (w/v) LDAO to mimic a protein sample. The green dye was then loaded through the sample inlet. Using a pipette, 10 μL of the dye was loaded into the Chip until all the sample channels were fully filled. Once the sample was loaded, the SlipChip was slipped such that the connections between adjacent wells were disconnected and the vertical ducts formed a bridging diffusion channel for the sample wells and relative reagents wells under it. Sequential images (time interval of 3 min) were were taken with a Leica MZ 16 Stereoscope with a Plan APO 0.63× objective. Images taken at time points of 0 min, 24 min and 141 min were presented as Figure 2A, B and C.

Fluorescent dye diffusion experiment in a FID device

A FID device was made with the method described above with nanopatterning. The SlipChip was assembled and solutions were loaded as described for the food dye experiment. 250 μM MPTS in PBS buffer (1×, pH 7.4) was loaded by pippetting into two reagent channels. 0.01% (w/v) LDAO solution was loaded into the sample channel to fill all sample wells. The SlipChip were slipped under a Leica MZ 16 Stereoscope to form 20 free interface diffusion experiments with 5 different duct geometries. The starting time point of FID was recorded with a timer. The device was quickly transferred to a Leica DMI6000 microscope (Leica Microsystems) with a 5 ×0.4 Leica objective and a Hamamatsu ORCAER camera. A DAPI filter with an exposure time of 20 ms was used to collect MPTS fluorescence. To calibrate the microscope, the fluorescent intensity of a fluorescence reference slide for the DAPI filter was recorded and used for background correction. Images were acquired and analyzed by using Metamorph imaging system version 6.3r1 (Universal Imaging) with multi-dimension acquisition function. Images were taken every 10 minutes. Figure S3 shows a bright field image and fluorescent images at different free interface diffusion time points. To obtain the average intensity in the sample well, we did linescan as shown in Figure S3B and C on each sample well. The intensity along the linescan was averaged, and the average intensity was plotted over time (Fig. 2D). The time was corrected by accounting for the delay between setting up the FID experimetns and start of imaging.

Food dye experiment in a hybrid device

A hybrid SlipChip was made by using the nanopatterning method described above. It was assembled under FC-40. In the resulting orientation, fluidic channels for both 16 reagents and one sample were formed. All the solutions used for food dye experiments were filtered with a 0.45 μm PVDF syringe filter before use. Four food dyes (yellow, pink, red, and blue, Ateco, Glen Cove, NY) were diluted ~10 times from their stock solutions and were pipet-loaded into 16 reagent channels. To load each channel, 4 μL of dye was first pushed through the inlet using a pipette until the dye solution emerged from the outlet. A green dye was diluted 20 times and was mixed with 0.04% (w/v) LDAO to mimic a protein sample. The green dye was then loaded through the sample inlet. Using a pipette, 10 μL of the dye was loaded into the Chip until all the sample channels were fully filled. Once the sample was loaded, the SlipChip was slipped such that the reagent wells overlapped with the sample wells in the microbatch sections, and the reagent wells were connected to the sample wells by the necks (ducts connecting the fluidic path of the sample before slipping) in the FID sections (Figure 4). Images of all sections were taken within five minutes, then after one hour, five hours, and 19 hours.

Crystallization of enoyl-CoA hydratase from Mycobacterium tuberculosis with microbatch SlipChip.

The protein sample was obtained from the Seattle Structural Genomics Center for Infectious Disease (SSGCID). As described previously,[3] the microbatch SlipChips were made by glass etching, surface-coated by fluorinated ethylene propylene (FEP), and assembled under lubricant fluorocarbon, a mixture of perfluoro–tri–n–butylamine and perfluoro–di–n–butylmethylamine (FC-40). 48 precipitants from a home-made screening kit (Table S1) were loaded into three assembled SlipChips, 16 precipitants in each Chip. Precipitants were combined with the protein sample by slipping. Each SlipChip was then

immersed into FC-70 in separate petri dishes. The petri dishes were were stored in a 23 ^{o}C incubator and the results were monitored for two weeks. Images of wells containing the crystallization trials were taken over the two weeks by using a SPOT Insight camera (Diagnostic Instruments, Inc., Sterling Heights, MI) coupled to a Leica MZ 16 Stereoscope.

Crystallization of enoyl-CoA hydratase with FID chip

The FID SlipChip for protein crystallization were made by using the nanopatterning method described above. 48 precipitants from a home-made screening kit (Table S1) were loaded into three SlipChips, 16 precipitants in each Chip; the loading procedure was the same as in the food dye experiments of FID Chip. After slipping, the precipitant wells and protein wells were connected in pairs by the protein neck to initiate FID experiments. Each SlipChip was then immersed in FC-70 in separate petri dishes. The petri dishes were stored in a 23 ^{o}C incubator and the results were monitored for two weeks. Images of wells containing crystals were taken over the two weeks.

Crystallization of enoyl-CoA hydratase with hybrid SlipChip.

The hybrid SlipChip for protein crystallization was made by using the nanopatterning method described above. 48 precipitants from a home-made screening kit (Table S1) were loaded into three hybrid SlipChips, 16 precipitants in each Chip; the loading procedure was the same as in the food dye experiments of the hybrid Chip. After one step of slipping, both microbatch and FID experiments were set up. Each SlipChip was then immersed in FC-70 in separate petri dishes. The petri dishes were stored in a 23 ^{o}C incubator and the results were monitored for two weeks. Images of wells containing crystals were taken over the two weeks.

Crystallization of dihydrofolate reductase/thymidylate synthase from Babesia bovis with microbatch SlipChip.

The protein sample was obtained from SSGCID. The screening experiments using microbatch SlipChips were performed in the same way as described for *enoyl-CoA hydratase*.

Crystallization of dihydrofolate reductase/thymidylate synthase with FID chip

The protein sample was obtained from SSGCID. The screening experiments using FID SlipChips were performed in the same way as described for *enoyl-CoA hydratase*.

Crystallization of dihydrofolate reductase/thymidylate synthase with hybrid SlipChip

The protein sample was obtained from SSGCID. The screening experiments using hybrid SlipChips were performed in the same way as described for *enoyl-CoA hydratase*.

Visualization of protein crystals using a UV-microscope

To confirm the crystals obtained in all of the crystallization experiments on SlipChps were indeed protein crystals, we used a UV-microscope (PRS-1000, Korima Inc., Carson, CA). Both brightfield images and images under UV-light were taken. The crystals were confirmed as protein crystals when UV signals from the crystals were detected (Figure 6B and C), and the corresponding crystallization conditions were identified as hits.

Crystallization of dihydrofolate reductase/thymidylate synthase in well plates. Once a crystallization condition for *dihydrofolate reductase/thymidylate synthase* was identified from the microbatch SlipChips, the experiment was scaled up in a sitting-drop well plate (Hampton research). Using the same mixing ratio identified by the screening experiments on the SlipChip, the protein sample was mixed with the precipitant to obtain a final volume of 3 μL in the well. In the reservoir, Millipore water was mixed with the precipitant to give the same precipitant concentration as in the well; the final volume was 600 μL. Each condition had one duplicate. The plate was then sealed with sealing tape (Hampton research) and incubated at room temperature. Images of crystals were taken by a SPOT Insight camera (Diagnostic Instruments, Inc., Sterling Heights, MI) coupled to a Leica MZ 16 Stereoscope.

X-ray diffraction and data processing

Crystals for x-ray diffraction were obtained from the well plate experiments. For precipitants that contained PEG-400, the mother liquor was used as a cryo-protectant, and the concentration of PEG-400 was changed to be 25% (w/v). For other precipitants, the mother liquor plus 20% (v/v) glycerol was used as a cryo-protectant. A crystal was first transferred from the original well to the well containing the cryo-protectant by using a nylon loop. Then the crystal was frozen in liquid nitrogen. The X-ray diffraction experiments were performed at GM/CA Cat stations 23 ID-B and 23ID-D of the Advanced Photon Source (Argonne National Laboratory). X-ray data were collected at 100 K using a wavelength of 1.0332 Å.

The data were processed and analyzed by using HKL-2000.[4]

X-ray structure determination of dihydrofolate reductase/thymidylate synthase. The structure of *dihydrofolate reductase/thymidylate synthase* was solved by molecular replacement using the PDBid 3I3R structure as a starting model and the MOLREP program[5] in CCP4 suite.[6] The data collected from crystals grown in the condition containing PEG-400 was used. Rigid-body, positional, and temperature factor refinements were performed using a maximum likelihood target with the program REFMAC5.[7] The SigmaA-weighted 2Fobs-Fcalc and Fobs-Fcalc Fourier maps were calculated using CCP4. The Fourier maps were displayed and examined in COOT.[8] The search for new solvent molecules was performed with help of COOT. The refinement statistics are summarized in Table S3. The the structure has been deposited in the Protein Data Bank (www.pdb.org), PBDid: 3KJR.

References

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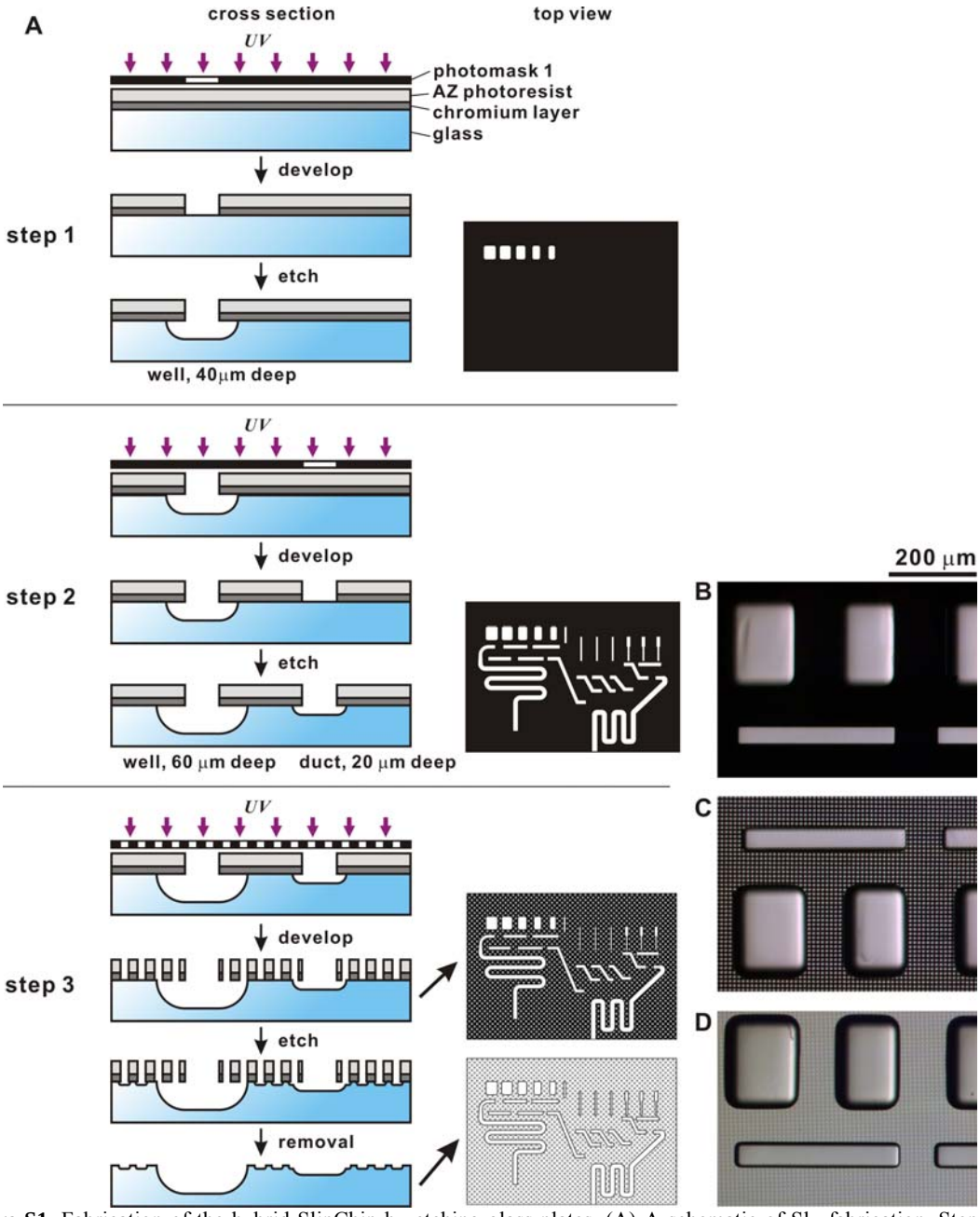

Figure S1. Fabrication of the hybrid SlipChip by etching glass plates. (A) A schematic of Slu fabrication. Step 1: A photomask is lain on top of the glass plate with chromium and photoresist coating. After UV exposure, the mask is removed and the exposed glass is etched to create 40 μm-deep microwells. The plate is washed and dried. Photograph shows the photomask used for this step. Step 2: A second photomask is aligned on top of the glass plate. After UV exposure, the mask is removed and the exposed glass is etched to create 20 μm-deep microchannels (ducts) and at the same time, the microwells are further etched to a final depth of 60 μm. The plate is washed and dried. Photograph shows the photomask for this step. Step 3: A nanopatterning mask is laid on top of the glass plate. After UV exposure, the mask is removed and the exposed glass is etched with a dilute etching solution to create 250 nm-deep microwells. The photoresist and chromium layers are removed, and the plate is washed and dried. Top photograph shows the plate before etching; bottom photograph shows the plate after the photoresist and chromium layers are removed. (B) Microphotograph of 40 μm-deep wells etched during Step 1. (B) Microphotograph of 60 μm-deep wells and 20 μm-deep ducts etched during Step 2. (C) Microphotograph of the device after developing with the nanopatterning mask. (D) Microphotograph of the device after 250 nm-deep microwells are etched and the photoresist and chromium coating are removed.

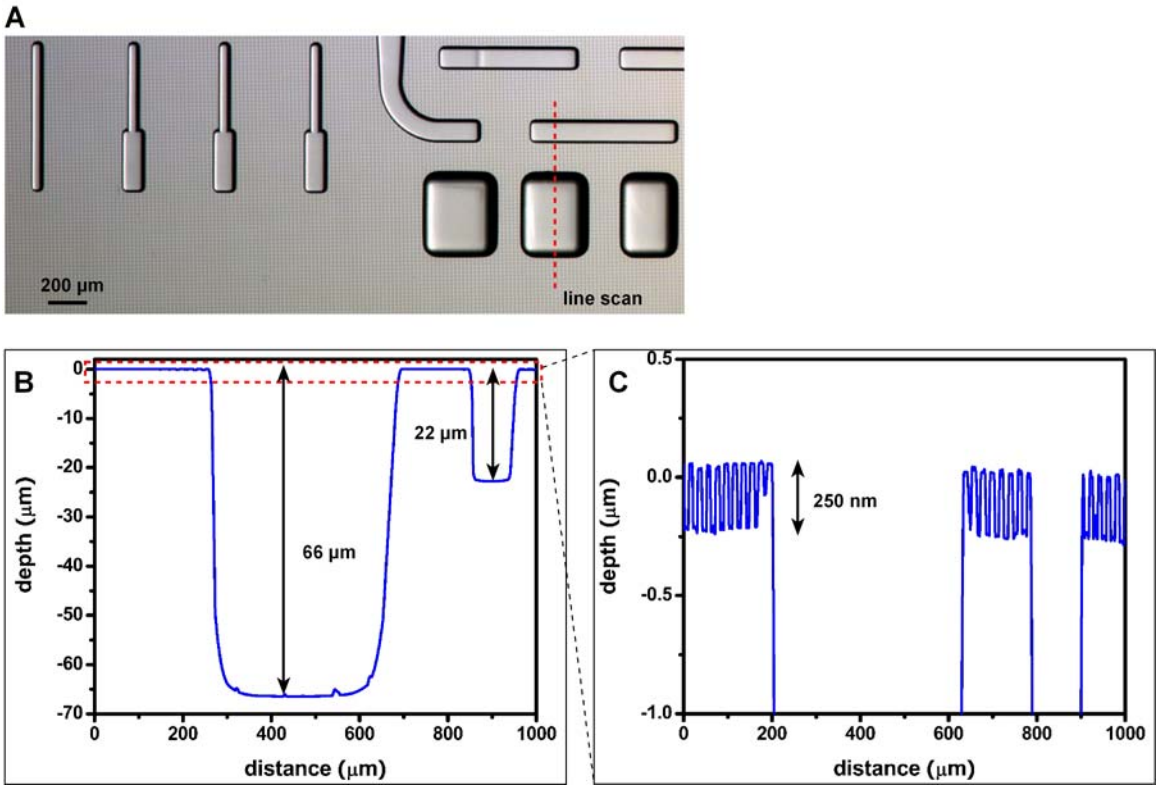

Figure S2. The etched SlipChip was characterized by surface scanning with a profilometer. (A) A microphotograph of a plate of the hybrid SlipChip with wells, ducts and nanopatterning. A line scan was taken with the profilometer along the dotted red line. (B) The line scan of the well and ducts with (C) a zoomed-in view for nanopatterning details.

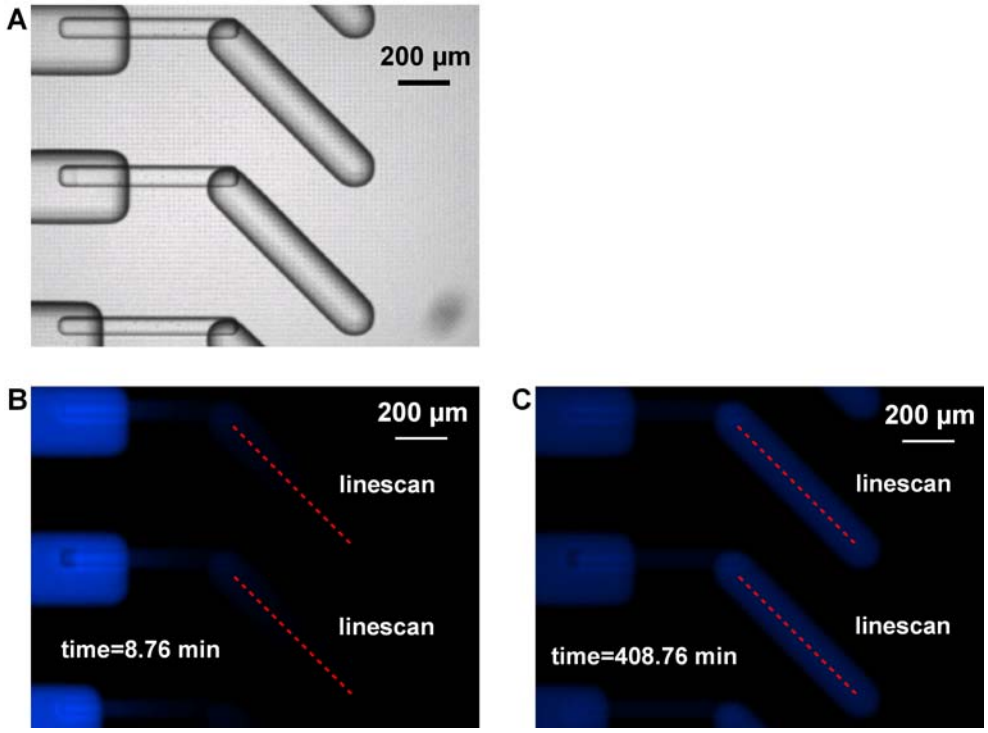

Figure S3. Free interface diffusion demonstrated with fluorescent dye. (A) A bright field image of FID SlipChip with sample and reagent wells connected by neck channels. The sample wells (right) are filled with water solution and reagent wells (left) are filled with 250 μM MPTS. The neck geometries are listed in Table S1, condition 3. (B) A fluorescent image taken with a Dapi filter indicates diffusion from the reagent well to the sample wells through the neck channels, at time point of 8.76 min. (C) A fluorescent image taken at 408.76 min indicates an equal distribution of the fluorescent dye in the sample and reagent wells.

Table S1 geometry of the neck channels in the FID SlipChip.

condition #	width (μm)	depth (μm)	Cross-section (μm^2)	Length (μm)	Length/Cross-section
1	103.92	21.25	2014.40	91.2	0.0453
2	92.31	21.25	1767.82	191.2	0.1082
3	80.71	21.25	1521.23	291.2	0.1914
4	69.10	21.25	1274.65	391.2	0.3069
5	57.50	21.25	1028.06	491.2	0.4778

Table S2: Contents of the home-made screening kit

	precipitant	**buffer (0.1 M)**	**salt (0.2 M)**
1	30% (w/v) PEG-8000	CHES pH 9.5	
2	20% (v/v) 2-propanol	HEPES pH 7.5	NaCl
3	30% (v/v) ethanol	CHES pH 9.5	none
4	52.5% (v/v) 2-methyl-2,4-pentanediol	imidazole pH 8.0	$MgCl_2$
5	60% (v/v) PEG-400	CAPS pH 10.5	
6	30% (w/v) PEG-3000	citrate pH 5.5	
7	20% (w/v) PEG-8000	MES pH 6.0	$Zn(OAc)_2$
8	2.8 M $(NH_4)_2SO_4$	citrate pH 5.5	
9	1.5 M $(NH_4)_2HPO_4$	acetate pH 4.5	
10	30% (w/v) PEG-2000 MME	Tris pH 7.0	
11	40% (v/v) 1,4-butanediol	MES pH 6.0	Li_2SO_4
12	30% (w/v) PEG-1000	imidazole pH 8.0	$Ca(OAc)_2$
13	2.52 M $(NH_4)_2SO_4$	cacodylate pH 6.5	
14	1.4 M sodium citrate	cacodylate pH 6.5	
15	20% (w/v) PEG-3000	imidazole pH 8.0	Li_2SO_4
16	3.75 M NaCl	Na/K phosphate pH 6.2	
17	40% (w/v) PEG-8000	acetate pH 4.5	Li_2SO_4
18	1.5 M K/Na tartrate	imidazole pH 8.0	NaCl
19	30% (w/v) PEG-1000	Tris pH 7.0	
20	0.6 M NaH_2PO_4/1.6 M K_2HPO_4	imidazole pH 8.0	NaCl
21	30% (w/v) PEG-8000	HEPES pH 7.5	
22	20% (v/v) 2-propanol	Tris pH 8.5	
23	30% (v/v) ethanol	imidazole pH 8.0	$MgCl_2$
24	52.5% (v/v) 2-methyl-2,4-pentanediol	Tris pH 7.0	NaCl
25	45% (v/v) PEG-400	Tris pH 8.5	$MgCl_2$
26	20% (w/v) PEG-3000	CHES pH 9.5	
27	1.44 M NaH_2PO_4/0.64 M K_2HPO_4	CAPS pH 10.5	Li_2SO_4
28	30% (w/v) PEG-3000	HEPES pH 7.5	NaCl
29	20% (w/v) PEG-8000	CHES pH 9.5	NaCl
30	2.52 M $(NH_4)_2SO_4$	acetate pH 4.5	NaCl
31	30% (w/v) PEG-8000	phosphate-citrate pH 4.2	NaCl
32	20% (w/v) PEG-3000	Na/K phosphate pH 6.2	
33	2.0 M $(NH_4)_2SO_4$	CAPS pH 10.5	Li_2SO_4
34	1.5 M $(NH_4)_2HPO_4$	imidazole pH 8.0	
35	40% (v/v) 1,4-butanediol	acetate pH 4.5	
36	1.4 M sodium citrate	imidazole pH 8.0	
37	3.75 M NaCl	imidazole pH 8.0	
38	1.5 M K/Na tartrate	CHES pH 9.5	Li_2SO_4
39	30% (w/v) PEG-1000	phosphate-citrate pH 4.2	Li_2SO_4
40	20% (v/v) 2-propanol	MES pH 6.0	$Ca(OAc)_2$

41	45% (w/v) PEG-3000	CHES pH 9.5	
42	30% (v/v) ethanol	Tris pH 7.0	
43	52.5% (v/v) 2-methyl-2,4-pentanediol	Na/K phosphate pH 6.2	
44	30% (v/v) PEG-400	acetate pH 4.5	$Ca(OAc)_2$
45	30% (w/v) PEG-3000	acetate pH 4.5	
46	30% (w/v) PEG-8000	imidazole pH 8.0	$Ca(OAc)_2$
47	2.52 M $(NH_4)_2SO_4$	Tris pH 8.5	Li_2SO_4
48	30% (w/v) PEG-1000	acetate pH 4.5	$Zn(OAc)_2$

Table S 3: Statistics for data collection and structural determination for dihydrofolate reductase/thymidylate synthase

dihydrofolate reductase/thymidylate synthase	
Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a, *b*, *c* (Å)	83.1, 97.6,152.8
. α, β, γ (°)	90, 90, 90
Resolution (Å)	50-1.95 (1.98-1.95)
R_{merge} #	0.07 (0.72)
$I/\sigma I$	19.5 (2.5)
Unique observations	90329
Total observations	442010
Completeness (%)	99.8 (99.6)
Redundancy	4.9 (4.6)
Refinement	
Resolution (Å)	50-1.95
No. reflections	
$R_{work/}$ R_{free}	0.186/0.228
B-factors	
Protein	28.571
Ligand	41.063
Water	39.271
R.m.s deviations	
Bond lengths (Å)	0.013
Bond angles (°)	1.371

#: $R_{merge} = \sum_{hkl}\sum_{i} (I_i(hkl) - \langle I(hkl) \rangle)^2 / \sum_{hkl}\sum_{I} I_i(hkl)^2$