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An *in vitro* microfluidic approach to generating protein-interaction networks

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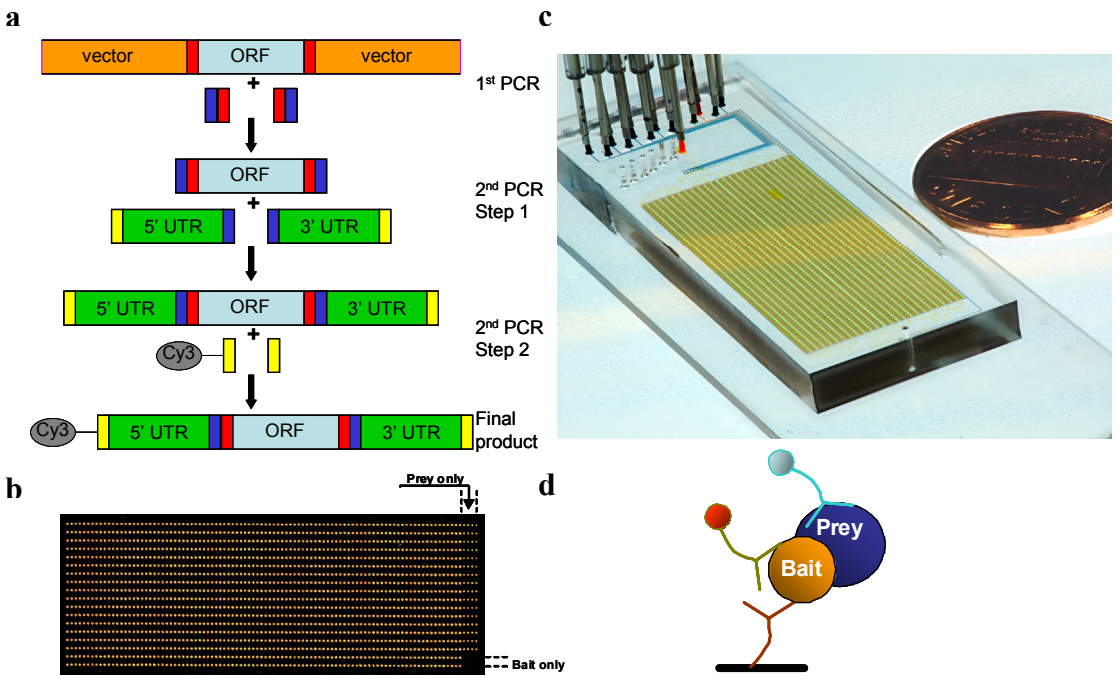
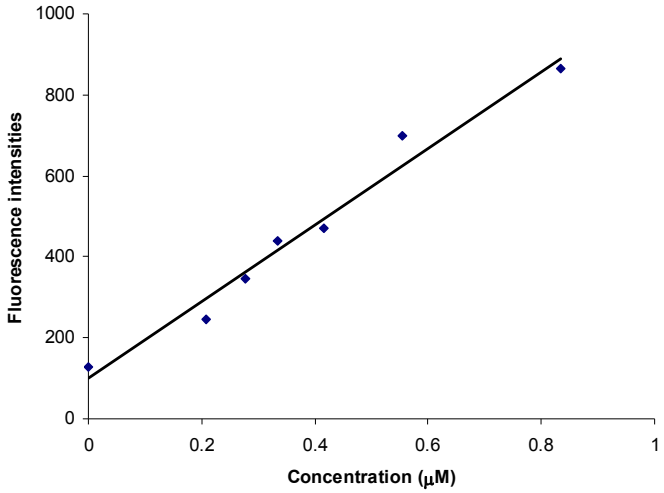
Note: Supplementary Data is available on the Nature Methods website.

Supplementary Figure 1. Experimental design.



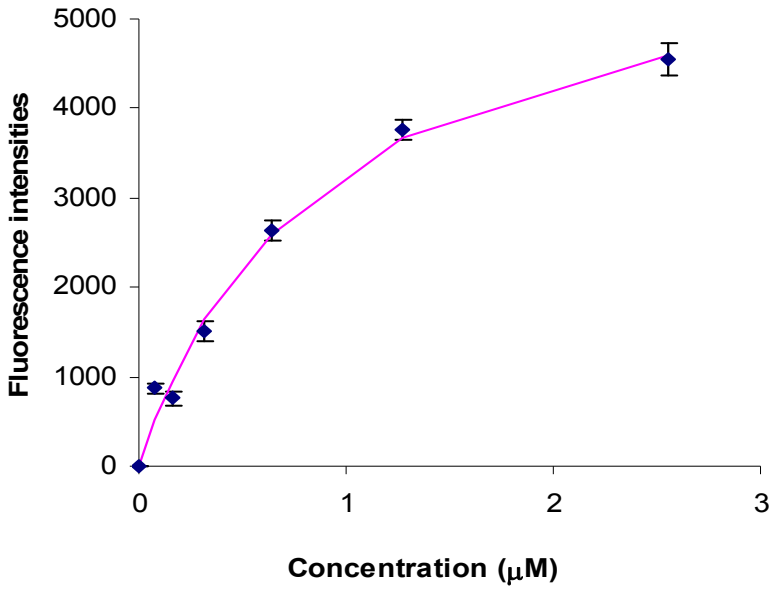
(**a**) ORF was PCR amplified for 30 cycles and extended to overlap with the 5' and 3' UTRs (Blue and Red primers) directly from bacteria, avoiding the need for plasmid purification. A second PCR step (10 cycles) assembled the ORF with the UTRs (step2) to produce an expression ready template. The template was then amplified for 30 cycles using final primers (yellow). A cy3 label was added to allow for quality control of the programmed devices. Hi Fi PCR (Roche) was used to maintain the fidelity of the amplified DNA. (**b**) An example of a DNA array printed on an epoxy glass slide ready for chip mounting. DNA is labeled with Cy3. (**c**) A photograph of a typical device aligned on a glass substrate. Control and flow lines are illustrated with food dyes. (**d**) Pull down / detection scheme for on-chip protein interactions assay. Bait c-terminal tag is used for pull down. Cy3 labeled anti c-*myc* is used to detect protein expression and $Alexa_{647}$ labeled antibody is used to detect prey interaction.

Supplementary Figure 2. GFP calibration curve.



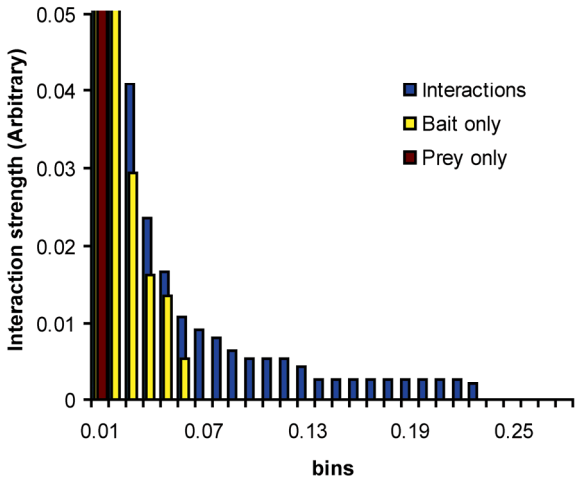
We calibrated the concentration of 4-HIS GFP in the extract using a calibration curve. For this purpose, we used a pure stock of eGFP 1mg/ml (Biovision). The eGFP fluorescence were measured on an RF3501 (Shimadzu) set with excitation at 490nm emission at 512nm and slits set to 10nm. The 4-HIS GFP proteins were measured in the E. coli extract in which they were expressed and diluted 10 fold in Hepes 50mM.

Supplementrary Figure 3. PING has sensitivity of about 1 μM.



We measured binding of 4-HIS tagged eGFP to the Penta-HIS antibody (Qiagen). A non-linear least square fitting was used to calculate the binding constant $K_d = 884 \pm 158$ nM ($R^2 = 0.99$; P value < 0.003). Each data point represents between 10-20 replicates.

Supplementary Figure 4. Protein interactions Histogram.



A histogram of the interaction strengths (ratios of interaction/expression signals) demonstrates a cutoff ratio of about 0.07 for non-specific interactions according to bait only expression. Interestingly, this cutoff results in a network of similar size as when no normalization is used.

Supplementary Table 1 - Description of Streptococcus pneumoniae clones

Clone ID	ORF length	Common Name	Gene	Oligomeric State
SP0438	300	glutamyl-tRNA(Gln) amidotransferase, C subunit	gatC	Heterotrimer
SP1278	519	pyrimidine operon regulatory protein	pyrR	Homodimer/Homohexamer
SP1577	510	adenine phosphoribosyltransferase	apt	Homodimer
SP1907	282	chaperonin, 10 kDa	groES	High Oligomer
SP1699	360	holo-(acyl-carrier protein) synthase	acpS	Homotrimer
SP0476	342	PTS system, lactose-specific IIA component	lacF	Homotrimer
SP1168	462	mutator MutT protein	mutX	Homotrimer
SP0516	522	heat shock protein GrpE	grpE	Homodimer
SP0959	555	translation initiation factor IF-3	infC	Monomer
SP0766	603	superoxide dismutase, manganese-dependent	sodA	Homodimer
SP0261	756	undecaprenyl diphosphate synthase	uppS	Homodimer
SP1877	738	integrase/recombinase, phage integrase family		Heterotetramer
SP1875	567	conserved hypothetical protein	scpB	Homodimer
SP0231	636	adenylate kinase	adk	Monomer
SP0702	630	orotate phosphoribosyltransferase PyrE	pyrE	Homodimer
SP1090	639	conserved hypothetical protein		Homodimer
SP1377	675	3-dehydroquinate dehydratase	aroD	Homodimer
SP0701	699	orotidine 5-phosphate decarboxylase	pyrF	Homodimer
SP1876	726	conserved hypothetical protein	scpA	Monomer?
SP0669	837	thymidylate synthase	thyA	Homodimer
SP1542	1341	asparaginyl-tRNA synthetase	asnS	Homodimer
SP0436	1440	glutamyl-tRNA(Gln) amidotransferase, B subunit	gatB	Heterotrimer
SP0437	1464	glutamyl-tRNA(Gln) amidotransferase, A subunit	gatA	Heterotrimer
SP0289	942	dihydropteroate synthase		Homodimer and Homotrimer
SP1014	933	dihydrodipicolinate synthase	dapA	Homotetramer
SP2112	984	maltose operon transcriptional repressor	malR	HTH (dimer)
SP1159	1068	integrase/recombinase, phage integrase family		heteroteramer
SP0519	1134	dnaJ protein	dnaJ	Homodimer
SP0019	1284	adenylosuccinate synthetase	purA	Homodimer
SP1128	1302	enolase	eno	Homodimer
SP1371	1281	3-phosphoshikimate 1-carboxyvinyltransferase	aroA	Monomer
SP0713	1473	lysyl-tRNA synthetase	lysS	Homodimer
SP0764	933	dihydroorotate dehydrogenase A	pyrDa	Homodimer
SP0894	2271	X-pro dipeptidyl-peptidase	pepX	Homodimer
SP1631	1941	threonyl-tRNA synthetase	thrS	Homodimer
SP2078	1689	arginyl-tRNA synthetase	argS	Monomer
SP0292	810	bifunctional folate synthesis protein		Homodimer and Homotrimer
SP1103	105	hypothetical protein		Homodimer
SP1362	735	putative transcriptional regulator MecA	mecA	Homodimer
SP1906	1620	chaperonin, 60 kDa	groEL	High Oligomer
SP2069	1458	glutamyl-tRNA synthetase	gltX	Monomer
SP2121	1287	histidyl-tRNA synthetase	hisS	Homodimer
SP2229	1023	tryptophanyl-tRNA synthetase	trps	Homodimer

Clones for the control set were obtained from TIGR (pfgrc.tigr.org).

Supplementary Table 2 - Statistical significance of the interactions

n1	n2	U	P (two-tailed)	P (one-tailed)
1273	25	31299	$<2 \times 10^{-06}$*	$<1 \times 10^{-06}$*
Normal approximation z=8.29			1.13×10^{-16}*	5.67×10^{-17}*

*** These values are approximate.**

Supplementary Table 3 - List of all the interactions found by PING.

Protein A	Protein B
SP1907	SP0019
SP1907	SP0231
SP1907	SP0261
SP1907	SP0289
SP1907	SP0292
SP1907	SP0436
SP1907	SP0437
SP1907	SP0438
SP1907	SP0516
SP1907	SP0519
SP1907	SP0669
SP1907	SP0701
SP1907	SP0713
SP1907	SP0766
SP1907	SP0959
SP1907	SP1014
SP1907	SP1090
SP1907	SP1103
SP1907	SP1128
SP1907	SP1159
SP1907	SP1168
SP1907	SP1278
SP1907	SP1362
SP1907	SP1371
SP1907	SP1377
SP1907	SP1542
SP1907	SP1577
SP1907	SP1631
SP1907	SP1699
SP1907	SP1875
SP1907	SP1876
SP1907	SP1877
SP1907	SP1906
SP1907	SP1907
SP1907	SP2078
SP1907	SP2112
SP1907	SP0476
SP0516	SP0019
SP0516	SP0261
SP0516	SP0516
SP0516	SP0519
SP0516	SP0959
SP0516	SP1128
SP0516	SP1159
SP0516	SP1168
SP0516	SP1577
SP0516	SP1699

SP0519	SP0019
SP0519	SP0261
SP0519	SP0519
SP0669	SP1877
SP0669	SP0669
SP1014	SP1014
SP1875	SP1876
SP1875	SP1875
SP1875	SP1377
SP1875	SP1103
SP1875	SP0766
SP1875	SP0261
SP1875	SP0231
SP0766	SP1875
SP0766	SP0766
SP1876	SP1875
SP1128	SP0519
SP1128	SP2069
SP1128	SP1542
SP1128	SP1371
SP1128	SP1159
SP1128	SP1128
SP1128	SP0713
SP1128	SP0437
SP1128	SP0436
SP1128	SP0261
SP1128	SP0019
SP0701	SP1876
SP0701	SP1377
SP0701	SP0701
SP0701	SP0437
SP0701	SP0261
SP0701	SP0019
SP0702	SP0702
SP2112	SP0519
SP2112	SP2112
SP2112	SP1159
SP2112	SP0261
SP0289	SP2112
SP0289	SP0289
SP0437	SP1876
SP0437	SP0438
SP0436	SP1577
SP0436	SP0764
SP0436	SP0261
SP0436	SP0231
SP0438	SP1907
SP0438	SP0516
SP0438	SP0519
SP0438	SP0476

SP0438	SP2112
SP0438	SP2078
SP0438	SP1875
SP0438	SP1699
SP0438	SP1577
SP0438	SP1542
SP0438	SP1377
SP0438	SP1371
SP0438	SP1362
SP0438	SP1278
SP0438	SP1168
SP0438	SP1159
SP0438	SP1128
SP0438	SP1103
SP0438	SP1090
SP0438	SP1014
SP0438	SP0959
SP0438	SP0766
SP0438	SP0713
SP0438	SP0702
SP0438	SP0701
SP0438	SP0669
SP0438	SP0438
SP0438	SP0437
SP0438	SP0436
SP0438	SP0261
SP0438	SP0231
SP0476	SP1362
SP0476	SP0476
SP0476	SP0261
SP1090	SP1877
SP1090	SP1090
SP1278	SP0519
SP1278	SP1577
SP1278	SP1278
SP1278	SP1128
SP1278	SP1103
SP1278	SP0764
SP1278	SP0438
SP1278	SP0261
SP1577	SP1906
SP1577	SP1577
SP1577	SP1362
SP1577	SP1159
SP1577	SP0436
SP1577	SP0261
SP1377	SP0519
SP1377	SP1577
SP1377	SP0261
SP0261	SP1090

SP0261	SP0959
SP0261	SP0261
SP1168	SP0519
SP1168	SP0261
SP1699	SP0519
SP1699	SP1699
SP1699	SP0261
SP0019	SP0519
SP0019	SP0261
SP0019	SP0019
SP0894	-
SP2121	-
SP2229	-

The last 3 are proteins with no interacting partners.

Supplementary Table 4 - Expression levels do not bias the interactions.

	Clone	High end	Clone	Low end
	SP0019	5	SP1278	11
	SP1159	3	SP0959	4
	SP1168	3	SP1577	10
	SP0436	2	SP0894	0
	SP0437	5	SP2121	0
	SP1014	0	SP0764	2
	SP0231	5	SP1876	5
	SP0702	6	SP2229	0
	SP0289	7	SP1377	7
	SP0669	6	SP1362	3
Average		4.2		4.2
STD		2.2		4.0

We compared the average number of interactions per protein for 10 proteins from the low end and 10 proteins from the high end of the protein expression distribution. The average number of interactions per protein is the same.

SUPPLEMENTARY METHODS

Device fabrication

The device was designed in AutoCAD2004 (Autodesk, Inc.) and each layer reproduced as a chrome mask at 40,000 dpi (Fineline-Imaging). Flow molds were fabricated on 4" silicon wafers (Silicon Quest International) coated with hexamethyldisilazane (HMDS) in a vapour bath for 2min. The wafers were then spin-coated with SPR 220-7 (Shipley) initially at 500 rpm for 5s followed by 1800 rpm for 60s yielding a substrate height of around 9-11μm. The molds were baked at 115C for 90s followed by a 30s I-line exposure on a MA6 contact mask aligner (Karl Suss). Next the molds were developed with 1:5 2401 developer (Microposit) in H2O. Finally the molds were annealed at 120C for 20 min. Control molds were fabricated on 4" silicon wafers by spin coating SU-8 2025 (MicroChem) at 2700 rpm for 80s followed by a 65C bake for 2 min, 95C for 5min and a final step of 65C for 2 min. The wafers were then exposed for 10s on the I-line, followed by a post-exposure bake series of 65C for 2 min, 95C for 15min and 65C for 2 min. The wafers were then developed in SU-8 developer for 90s followed by an acetone and isopropanol wash. The microfluidic devices were fabricated essentially as described previously [1].

Linear expression template synthesis

Linear expression templates were generated by a three step PCR method (SI Figure 1) in which the first step amplifies the target sequence the second step adds required 5'UTR and 3'UTR for efficient in vitro transcription and translation and the third step amplifies the expression template. S. pneumoniae protein open reading frames were cloned by TIGR into pDONR221 Gateway entry vectors. Two libraries of prey and bait were generated: In the first the preys were tagged with a c-terminal 6-His tag and the baits were tagged with an N-terminal c-myc and C-terminal T7 tag. In the second set the 6-His and T7 tags were switched between prey and bait for detecting reciprocal interactions.

The first step PCR reaction was performed directly on bacteria clones and contained 1μM of each Gateway specific primer (table V), 200μM of each dNTP and 2.5 units of TAQ enzyme mixture (Expand High Fidelity PCR system, Roche) in a final volume of 25μL. The reaction was cycled for 4 min at 94C, followed by 30 cycles of 30s at 94C, 60s at 55C and 180s at 72C

and a final extension of 7 min at 72C. The products then served as templates in the second PCR reaction using 2μL first PCR product, 5 nM 5'ext1 primer (table V), 5 nM 3'ext2 primer (table V), 200μM of each dNTP and 2.5 units of Hi-Fi enzyme mixture (Expand High Fidelity PCR system, Roche) in a final volume of 100μL. The reaction was cycled for 4 min at 94C followed by 10 cycles of 30s at 94C, 60s at 55C and 90s at 72C. After this round of extension 2μL of 5μM Cy3-labeled 5'final and 5μM 3'final in dH_2O were added to each reaction and cycling was continued immediately for 30 cycles of 30s at 94C, 60s at 50C and 180s at 72C followed by a final extension of 72C for 7 min. The final product was then purified on a Millipore μ96 filter plate and eluted with H_2O 50μL. Bovine serum albumin (BSA) in H_2O was added to a final concentration of 1%. Fidelity of this method was previously demonstrated by Maerkl and Quake [2].

Designation and sequence of DNA oligo's used for creating the linear expression templates:

(1) 5'-ORF, TTGTTTAACTTTAAGAAGGAGATATACCATGCCAACTTTGTACAAAAAAGCAG;

(2) 5'-cMyc,
GTAGCAGCCTGAGTCGTTATTAGTGATGGTGATGGTGATGAACTTTGTACAAGAAAGCTGGGT

(3) 3'-T7,
TTGTTTAACTTTAAGAAGGAGATATACCATGGAACAAAAATTGATAAGTGAGGAAGATTTACCAACTT
TGTACAAAAAAGCAG

(4) 3'-HIS-ORF,
GTAGCAGCCTGAGTCGTTATTATCCCATTTGTTGACCCCCTGTCATACTTGCCATAACTTTGTACAAGA
AAGCTGGGT

(5) 5' 4-HIS-GFP,
TTGTTTAACTTTAAGAAGGAGATATACCATGCATCACCATCACGTGAGCAAGGGCGA

(6) 5' 5-HIS-GFP,
TTGTTTAACTTTAAGAAGGAGATATACCATGCACCATCACCATCACGTGAGCAAGGGCGA

(7) 3' GFP, GTAGCAGCCTGAGTCGTTATTACTTGTACAGCTCGTCCAT

(8) 5'UTR,
GATCTTAAGGCTAGAGTACTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAA
TTTTGTTTAACTTTAAGAAGGAGATATACC

(9) 3'UTR,
CAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTTTTTTTTTTTTTTTTTTT
TTTTTTTTGTAGCAGCCTGAGTCG

(10) 5'Final-Cy3, GATCTTAAGGCTAGAGTAC

(11) 3'Final, CAAAAAACCCCTCAAGAC

First 4 oligo's were used for extracting the open reading frames out of the Gateway vector (Invitrogen). Oligos 5-7 were used to create a linear template for the expression of 4- and 5-HIS tagged eGFP. Oligo's 8-9 were used for adding the 5' and 3' UTRs onto the linear templates. The last 2 oligo's were used to amplify the linear expression template.

DNA arraying and device alignment

Expression templates were spotted with an OmniGrid Micro (GeneMachines) microarrayer using a CMP3B pin (TeleChem International, Inc.) for delivery onto epoxy coated glass substrates (CEL Associates). Each sample solution contained 1% BSA in H_2O to prevent covalent linkage of the target DNA to the epoxy functional groups as well as for visualization during alignment. After spotting the arrays were quality controlled on a GenePix4000B (Molecular Devices). The arrays could then be stored in the dark at room temperature until aligned to a microfluidic device. Device alignment was done manually on a SMZ1500 (Nikon) stereoscope and bonded overnight on a heated plate at 40C.

Surface chemistry, protein synthesis and MITOMI

All devices were driven between 10 and 15 psi in the control line and 6 psi for the flow line. For the initial surface derivatization steps the chamber valves remained closed to prevent liquid from entering the chambers containing the spotted DNA targets. First, all accessible surface area was derivatized by flowing a solution of biotinylated BSA (Pierce) resuspended to 2 mg/mL in H_2O for 30 min followed by a 5 min PBS wash. Next a 0.5 mg/mL Neutravidin (Pierce) solution in PBS was flown for 30 min, followed by a 5 min PBS wash. The "button" membrane was closed and the PBS wash continued for an additional 5 min. Then all remaining accessible surface area was passivated with biotinylated BSA for 30 min, followed by a 10 min PBS wash. Finally a 1:4 solution of biotinylated-penta-histidine (Qiagen) or biotinylated anti T7 antibody (EMD Biosciences) in 2% BSA in PBS was loaded for 2-5 min, after which the "button" membrane was opened and flow continued for 30 min, again followed by a 10 min PBS completing the surface derivatization procedure.

Next a 25µL T7 coupled RTS HY Escherichia coli extract mixture (Roche) spiked with an extra 0.5 µl of T7 RNA polymerase (Promega) was prepared and loaded onto the device. The device was flushed for 5 min, after which the chamber valves were opened allowing for dead end filling of the chambers with extract. The chamber valves were again closed and flushing continued for an additional 5 min. Next the segregation valves separating each unit cell were closed followed by opening of the chamber valves allowing for equilibration of the unit cell by diffusion. The entire device was heated to 30C on a temperature controlled microscope stage and incubated for up to 2.5hrs.

We then performed a modified version of MITOMI by closing the "button" membrane as well as the chamber valves. We washed for 5 min with PBS, followed by 5 min with detection antibodies (cy3-labelded anti c-myc (1:1000) and either Alexa647-labeled anti T7 or anti penta-Histidine (1:1000)) mixed with PBS and 2% BSA. We then lifted the "button" and closed the segregation valves and the chamber valves. The proteins were allowed to equilibrate in the presence of the antibodies for 20 min, after which the "button" and chamber valves were closed again and the segregation valve lifted. The device was washed for 5 min with PBS and imaged to detect trapped molecules on an ArrayWorx^{e} (Applied Precision). Overall, we performed two scans of the 43 by 43 matrix with 4 repeats each using 8 devices (2400 chambers each), a total of 14792 unit cells excluding controls for bait only and prey only.

Image and Data Analysis

For each experiment two images where analyzed with GenePix3.0 (Molecular Devices). The first image (cy3 channel) was used to determine relative bait concentrations and the second image (Alexa647 channel) was used to determine bound prey concentrations. Columns of prey only and rows of bait only were used to assess non-specific binding of prey and bait proteins to the surface. Each row and column was then normalized by subtracting the non-specific baseline signal of its prey and bait respectively. A signal that was 2 SD above the average noise was considered an interaction. Only bidirectional interactions (interactions that were positive in reciprocal scans with both 6-his and T7 tagged baits) were considered positive. Reproducibility of positive interactions was approximately 95%. A protein network was constructed using the Cytoscape software [3].

Co-immunoprecipitation experiments

Prey and bait proteins were co-expressed in a 50μL of Roche RTS HY extract for 2.5hrs at 30C and 600 rpm (Roche RTS Proteomaster). As a control for non-specific binding of prey and antibody to the beads, preys were expressed in the absence of bait. Next, 1μL of Ni-NTA agarose magnetic beads (Qiagen) and Alexa647-labeled anti T7 antibody (final dilution 1:1000) were added. Mixture was incubated for an additional 15 min. Beads were then pulled down with a magnet and washed once with 250μL of PBS. Proteins were then eluted from the beads using 200 mM imidazole in PBS. The soluble Alexa647 probe from each reaction was read on a fluorimeter (Shimadzu RS 5301PC) in duplicates. Excitation was set to 647 nm and emission set to 670 nm, both with 5 nm slits. Alexa647 signal after subtraction of the respective control reaction is proportional to the strength of the interaction between the corresponding prey and bait. Overall, we tested 13 prey and bait pairs with 6 known interactions, 6 unknown interactions and 1 non-interacting pair as a negative control. The experiment was repeated twice.

Determining eGFP concentrations

A linear template for 4- and 5- HIS tagged eGFP were constructed using the PCR method described above (SI table V). The constructs were expressed in vitro with the Roche RTS 100 kit in a 50 ul volumes and their concentrations in the extract were determined using the eGFP fluorescence. For this purpose we made an eGFP calibration curve from a stock of pure eGFP 1mg/ml (Biovision) SI Figure 2.

Determining 4-HIS eGFP binding constant

In order to find the sensitivity of PING we did the following experiment: We flowed a series of increasing 4-HIS GFP (or 5-HIS eGFP) concentrations on a device with a Penta-HIS antibody (Qiagen) surface chemistry. Each concentration was flowed into two channels for 5 min each with a total of 8 different concentrations. Hepes 50mM was used as concentration 0. We then incubated the eGFPs in the presence of the antibody for 30 min. MITOMI was performed and the results were imaged. The binding constant of 4-HIS eGFP to the Penta-HIS antibody was determined by a non-linear least square fitting to $F=Fmax*[C]/(Kd+[C])$, where F is the

fluorescence intensities obtained from PING, C is the concentration of 4-HIS eGFP and K_d is the binding constant.

Supplementary References

1. Thorsen, T., Maerkl, S.J. & Quake, S.R. Microfluidic large-scale integration. *Science* **298**, 580-584 (2002).
2. Maerkl, S.J. & Quake, S.R. A systems approach to measuring the binding energy landscapes of transcription factors. *Science* **315**, 233-237 (2007).
3. Shannon, P.*, et al.* Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research* **13**, 2498-2504 (2003).