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A nanoliter-scale nucleic acid processor with parallel architecture

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The purification of nucleic acids from microbial and mammalian cells is a crucial step in many biological and medical applications[1]. We have developed microfluidic chips for automated nucleic acid purification from small numbers of bacterial or mammalian cells. All processes, such as cell isolation, cell lysis, DNA or mRNA purification, and recovery, were carried out on a single microfluidic chip in nanoliter volumes without any pre- or postsample treatment. Measurable amounts of mRNA were extracted in an automated fashion from as little as a single mammalian cell and recovered from the chip. These microfluidic chips are capable of processing different samples in parallel, thereby illustrating how highly parallel microfluidic architectures can be constructed to perform integrated batch-processing functionalities for biological and medical applications.

Microfluidic approaches to DNA purification have been previously demonstrated, and the earliest results involved affinity capture and elution of purified DNA using a silicon microstructure fabricated by deep reactive ion etching (DRIE)[2]. Recovery of DNA molecules from dirty biological samples was achieved using a glass chip with a microchannel packed with silica particles and immobilized by a sol-gel method[3]. In both cases, cells were lysed outside the microchip with conventional methods before the on-chip experiment, and microliters of either lysate or a purified DNA sample were loaded for DNA isolation. Thus far there has been little integration or true small-volume scaling in these experiments.

Isolation of mRNA, either to measure gene expression or to construct a cDNA library, is another cornerstone of molecular biology. There are numerous techniques for mRNA isolation, but nearly all require hundreds to thousands of cells as starting material. Yet there are many situations in which it would be useful to know the gene expression profile, or to develop a cDNA library, from a single cell. Some primary cell types, like stem cells, are very rare, and expansion in culture to acquire more cells changes the expression profile of the initial cell population. Attempts to isolate primary cells from an animal or patient invariably results in a mixture of cell types because it is not possible to precisely identify, and therefore isolate in a pure form, any single cell type. Furthermore, there has recently been a renewed interest in understanding how epigenetic variations in gene expression between cells that have identical genotypes might play a role in development and other phenotypic differentiation processes[4–6].

There are well established methods for measuring expression of a few genes from a single cell[5,7], but these require a priori choice of a small number of targets. This limitation has been somewhat alleviated by recent progress in amplifying mRNA from a single cell for use in more highly parallel microarrays[8–10]; these methods allow parallel analysis of a larger number of targets, but the mRNA amplification process inevitably introduces some degree of distortion, and the microarrays themselves require choice of a finite set of possible transcripts. cDNA library construction from a single cell has not yet been demonstrated, but could provide a useful tool to study expression without a priori assumptions about which genes are being probed. Thus far, the minimum number of cells needed for cDNA library construction is 1,000, with 10,000 being more usual, and the virtues of using microfluidics to construct such a library have been recognized[11]. On-chip affinity purification of mRNA from total RNA has been done as a stand-alone step[12], without integrating other processes.

We fabricated microfluidic chips that can sequentially process nanoliter volumes to isolate variable numbers of microbial or mammalian cells, lyse them and purify their DNA or mRNA. All the steps in the process were carried out on a single microfluidic chip without any pre- or post-sample treatment. The chips are fabricated by multilayer soft lithography, which provides robust and accurate micromechanical valves that prevent any cross-contamination or leakage between steps of the processes. Such microfluidic plumbing is versatile and compatible with many biological assays, and has been used for protein crystallization[13], nanoliter-volume PCR[14], microfabricated fluorescence-activated cell sorting (μFACS)[15] and single-cell enzyme screening[16]. We further describe a general microfluidic architecture for parallelization of complex nanoliter processes, and show how this parallelization can be realized without substantially increasing the complexity of the control requirements. This architecture is illustrated in a microfluidic chip for parallel bioprocessing of DNA purification from small numbers of bacterial cells. The parallelization achievable with such technology is useful not just for increasing throughput in single-cell analysis but also in automation of reagent preparation on larger cell populations, as in industrial-scale microarray analysis. Similar

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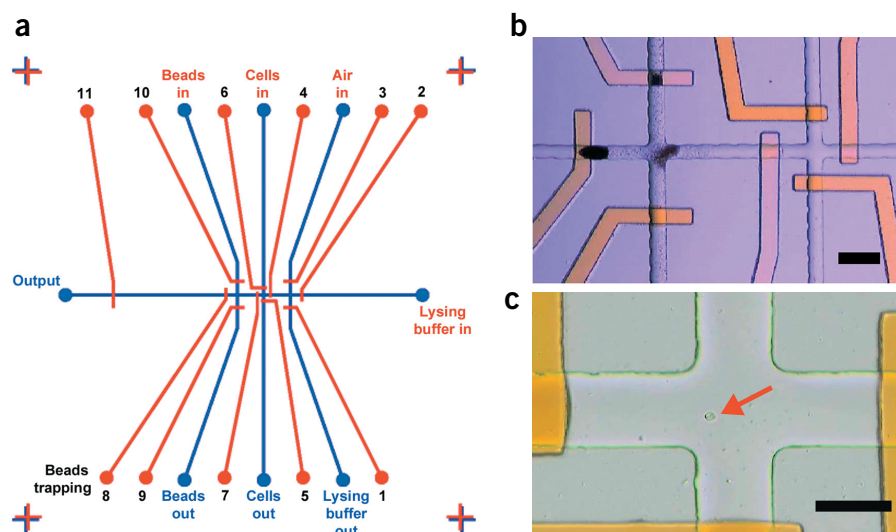
Figure 1 mRNA purification chip. (**a**) Layout of the microfluidic chip. The blue lines represent the 100-μm wide fluidic channels, the red lines are the 100-μm wide valve actuation channels. The fluidic ports are named; the actuation ports are numbered 1 to 11. The 'lysing buffer chamber' is composed of the channel space delineated by valves 1, 2, 3 and 4. The 'cell chamber' is composed of the channel space delineated by valves 4, 5, 6 and 7 (see also **b**,**c**). The 'bead chamber' is composed of the channel space delineated by valves 7, 8, 9 and 10 (see also **b**). (**b**) Photograph of the *in situ* affinity column construction. A column of 2.8-μm diameter paramagnetic beads covered with oligo dT is being built against a partially closed microfluidic valve (valve 8, see **Fig. 1a**). Scale bar, 200 μm. (**c**) One cell is loaded in the 'cell chamber' before the lysis step. (The channels are 100 μm wide). Scale bar, 100 μm.

processes have been previously automated in microfluidic format only in microliter volumes, with large numbers of cells and without parallelization[17].

The basic linear process that was implemented for mRNA purification consists of stacking the affinity column with oligo-dT polymer magnetic beads, isolating cells, metering reagents, mixing reagents and lysing cells, flushing lysate over the affinity column and recovering mRNA from the column (**Fig. 1**). The batch processing takes place in a linear fashion; valves and cross-junctions are used to load different segments of a channel with three reagents: cells, lysis buffer and dilution buffer. Opening the valve between the lysis buffer and the cell chamber allows diffusive mixing and cell lysis. The cell lysate is then flushed over the mRNA affinity column. mRNA is retained on the column, which is then recovered after a wash step.

Typically, chips are used for only a single experiment to obtain beads carrying mRNA from one or more cells. Reverse transcription and amplification were carried out directly on the beads, followed by gel electrophoresis of the products. Primers were used to identify two types of mRNA: the high-abundance β-actin transcript and the moderate-abundance zinc finger OZF transcript. Representative data are shown in **Figure 2a**. mRNA isolated from a single NIH 3T3 cell is shown in lane 3, and from nine cells in lane 4. A positive control, supernatant from lysed NIH 3T3 cells, is shown in lane 5, and several negative controls —PBS loaded on the chip (lane 2), lysis buffer and clean beads mixed in a test tube (lane 6), intact cells loaded on the chip but only supernatant solution trapped in the chamber (lane 7), water and beads in a test tube (lane 8) and PCR reagents only (lane 9)—are shown. None of the negative controls show a band in the β-actin DNA region, whereas the positive control shows a strong band.

When the intensity of a band is normalized by the number of beads in the column, a monotonically increasing relationship is found between intensity and cell number for the highly abundant β-actin mRNA, down to the single-cell level (**Fig. 2b**, filled circles). We did 18 experiments with cell numbers between 1 and 20; in 14 of these β-actin was detected. Of these experiments, five were on single cells and four were successful. In some experiments, we also assayed for the moderately abundant zinc finger OZF mRNA. In those cases, we were able to detect signal from as little as two cells, but the typical sensitivity was between two and ten cells (**Fig. 2b**, open circles). These experiments established the sensitivity of this first-generation chip. Despite the fact that the RT-PCR is not a linear amplification process, these results are semi-quantitative in the sense that they are

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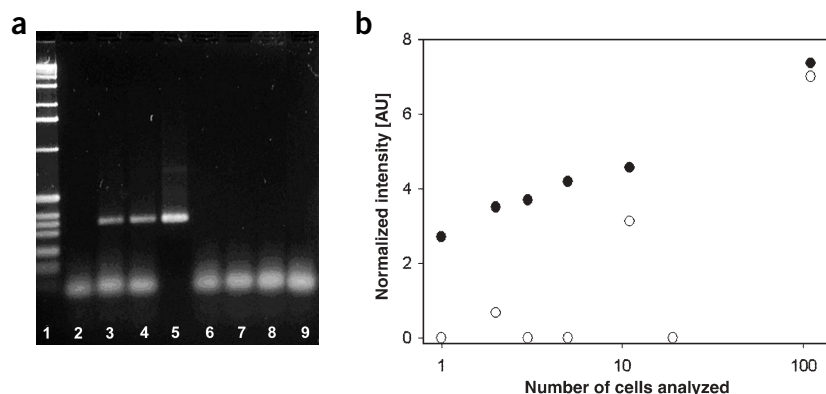
Figure 2 RT-PCR analysis of isolated mRNA. (**a**) A series of experiments, together with negative controls, were done to demonstrate single-cell sensitivity. The RT-PCR products were analyzed on a 2% agarose gel loaded with 5% of the reaction; the amplified gene is β-actin. Lane 1, 1 kb ladder; lane 2, PBS, no cells (on the chip); lane 3, one cell (on the chip); lane 4, nine cells (on the chip); lane 5, 200 μl supernatant from 1-d-old cells + 5 μl of beads (in a test tube); lane 6, 200 μl lysing buffer + 5 μl of beads (in a test tube); lane 7, cells loaded on the chip but none trapped in the chamber (on the chip); lane 8, 200 μl deionized water + 5 μl beads (in a test tube); lane 9, PCR reagents only. (**b**) A second series of experiments were done to test the chips with variable numbers of cells and both β-actin and OZF transcripts. The RT-PCR products for both transcripts were analyzed on a 2% agarose gel, whose bands were quantified, normalized and plotted on a graph. Zero values indicate the absence of a detectable band in the gel; neither transcript was detected in the experiment with 19 cells, possibly because of RNAase contamination or chip failure. For high-abundance actin mRNA (closed circles), detection is down to the single-cell level; for the moderately abundant OZF mRNA (open circles), detection is at the level of 2–10 cells.
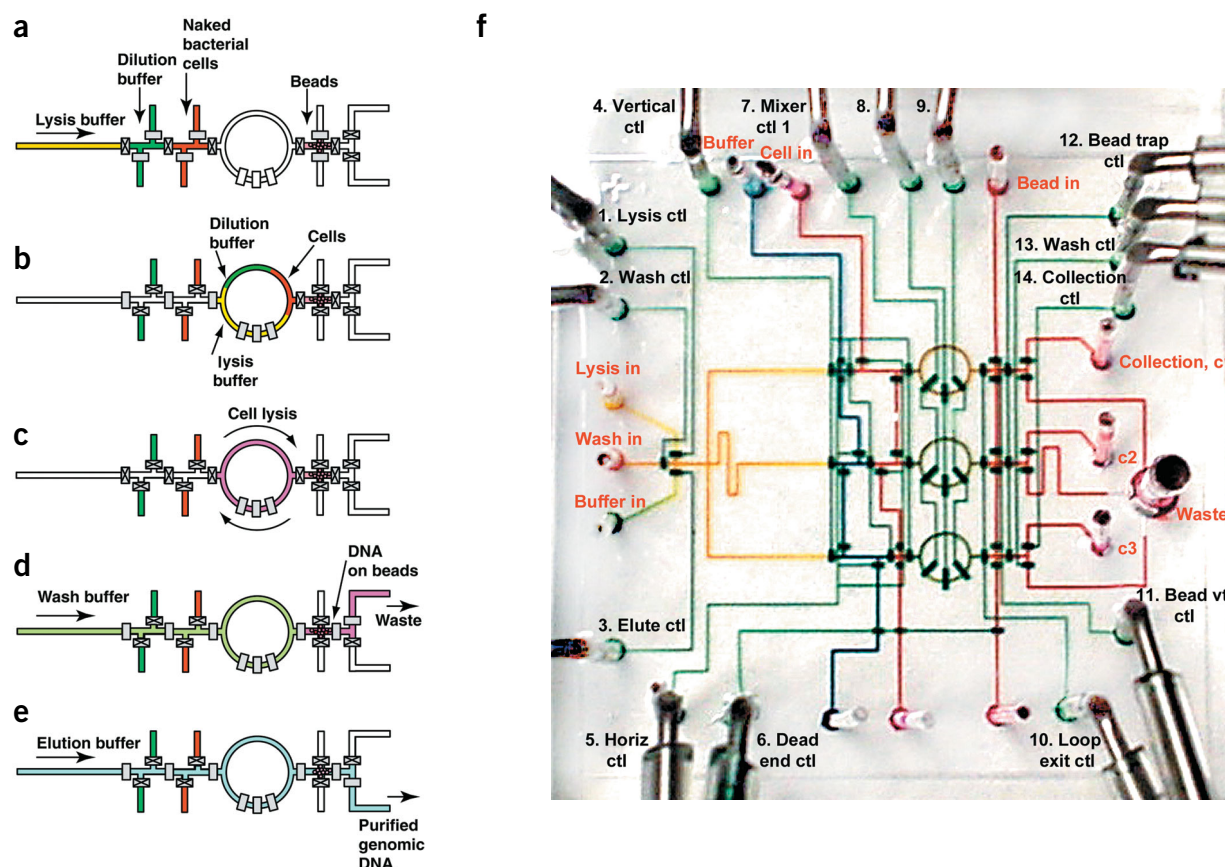
Figure 3 DNA purification chip and schematic diagram of one instance of the DNA isolation process. (Open valve, rectangle; closed valve, x in rectangle.) (**a**) Bacterial cell culture (red) is introduced into the microfluidic chip through the 'cell in' port located in the uppermost part of the chip. Buffer (green) for dilution of the cell sample is introduced through the 'buffer in' port located next to the 'cell in' port. The amount of dilution is determined by the ratio of channel lengths for cells and buffer; in this case, it is 1:1. Lysis buffer (yellow) is introduced from the left side of the chip. (**b**) The cell sample, dilution buffer and lysis buffer slugs are introduced into the rotary mixer. (**c**) The three different liquids are mixed thoroughly and consequently bacterial cells are lysed completely. Mixing is limited by diffusion, and without this rotary mixer complete mixing requires several hours to accomplish. For this reason, three different valves are operated sequentially to circulate the fluid inside the reactor, completely mixing the solutions after several minutes. (**d**) The lysate is flushed over a DNA affinity column and drained to the 'waste port.' (**e**) Purified DNA is recovered from the chip by introducing elution buffer from the left side of the chip and can be used for further analysis or manipulation. (**f**) Integrated bioprocessor chip with parallel architecture. This chip is made of two different layers, the actuation layer and the fluidic layer, which are bonded together. The actuation channels are filled with a green food coloring and the fluid channels are filled with yellow, blue and red food coloring according to their functionalities. The width of the fluid channels is 100 µm and that of valve actuation channels is 200 µm. Input and output ports and vias have been annotated. Multiple parallel processes of DNA recovery from living bacterial cells are possible in three processors with sample volumes of 1.6 nl, 1.0 nl and 0.4 nl. The chip has 26 access holes, 1 waste hole and 54 valves within 20 × 20 mm. The control channels are connected to polyethylene tubing through stainless steel pins.

consistent with the cell number. If the band intensities are not normalized by the size of the column, the results are not monotonic and do not obey any obvious functional relationship. We interpret this as an indication that the kinetics of mRNA binding to the beads were not fully equilibrated, and that the sensitivity of future devices can be improved by increasing the interaction time of the lysate with the column.

The loading geometry of the mRNA chip suggests an immediate route to parallelization that does not make the control of the chip more complex. It is possible to operate parallel bioprocessing with the same number of valve controls as a single process by aligning several linear processors and using the same cross-junction structures to load them simultaneously (**Fig. 3**). The conceptual advance is to use the chip in two different modes: 'loading' and 'processing.' In the loading mode, the control valves are actuated such that fluid flows only in the north/south direction, thus allowing simultaneous loading of the different batch processors. After the channels are loaded with their

respective reagents, the chip is switched to processing mode: valves are actuated such that flow is allowed only in an east/west direction, that is, along each batch processor, all of which are then operated in parallel (**Fig. 3**). We implemented this scheme in a chip that performs three parallel bacterial cell isolation and DNA purification reactions.

This architecture allows each processor to be customized. For example, to better characterize the ultimate sensitivity of the chip, we designed the three processors on the chip to meter different volumes of bacterial cells. The volumes of bacterial cell solution used in the top, middle and bottom processors are 1.6, 1.0 and 0.4 nl, respectively. The total volume of each rotary reactor is the same (5 nl), so 0.4, 1.0 and 1.6 nl of dilution buffer were metered on a chip and added to the bacterial cell volumes, respectively, along with 3.0 nl of lysis buffer. This metering process is illustrated with the use of food dyes (**Fig. 3f**). Different food colorings were applied through the 'buffer in' port and the 'cell in' port, metered and mixed together with the rotary pumps.
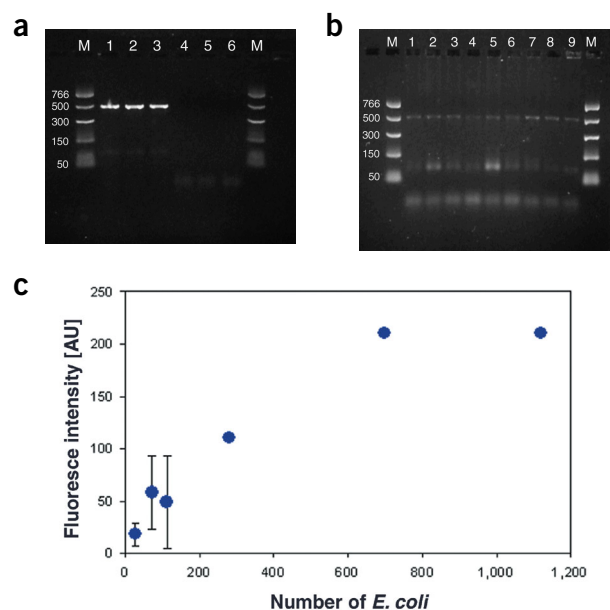
Figure 4 Verification of successful recovery of *E. coli* genomic DNA. The eluted samples were removed from the chip and amplified with PCR. The amplicons were run on a 2.0% agarose gel in 0.5× Tris-Borate-EDTA buffer. The target DNA is a 461-bp fragment of the *E. coli* gene *ppdD*. (**a**) Isolation of genomic DNA with an undiluted *E. coli* culture. Gel lanes as follows: M, PCR marker; lane 1, isolation of genomic DNA from 1.6 nl of culture with a corresponding bacterial cell number of ~1,120; lane 2, a 1.0 nl sample, cell number ~700; lane 3, a 0.4 nl sample, cell number ~280; lane 4, 5, and 6, negative controls for genomic DNA isolation. Instead of bacterial cell culture sample, purified water was used and all the other conditions were the same as lanes 1, 2, and 3. (**b**) Isolation of genomic DNA from a 1:10 dilution of bacterial cell culture. Lanes 1, 2, 3, lanes 4, 5, 6, and lanes 7, 8, 9 are the results from three different chips, respectively. Gel lanes as follows: M, PCR marker; lanes 1, 4, and 7, isolation of genomic DNA from an average of 112 bacterial cells; lanes 2, 5, and 8, isolation of genomic DNA from an average of 70 bacterial cells; lanes 3, 6 and 9, isolation of genomic DNA from an average of 28 bacterial cells. (**c**) Graph shows quantification of gel bands from **a** and **b** as a function of the number of bacteria trapped.

The mixing ratios of blue dye and red dye were 1:4, 1:1 and 4:1 for the top, the middle and the bottom, respectively. We also tested the loading efficiency of this scheme with bacteria. A culture of *Escherichia coli* expressing enhanced green fluorescent protein (EGFP) was verified to reach a final cell concentration of $5.3 \pm 3.5 \times 10^8$ cells/ml. It was then diluted 1:10 in nuclease-free water and loaded on the chip. The average number of bacteria in the 0.4 nl, 1.0 nl and 1.6 nl compartments were 27, 61 and 139, respectively, in fair agreement with the expected results of 21, 53 and 85. The transfer efficiency of the bacteria from the loading section to the rotary mixer ranged from 70% to 92%.

We successfully isolated genomic DNA from *E. coli* culture samples on ten different chips (**Fig. 4**). For seven of these chips undiluted culture was used, whereas for three chips a 1:10 dilution was used. In the latter cases, the microchips provided purified genomic DNA from <28 bacterial cells (**Fig. 4b**). Because the amounts of purified DNA were too small to measure by conventional means, PCR amplification of the gene encoding prelipin peptidase-dependent protein D (*ppdD*) was used for verification of successful recovery. An additional seven chips were used as negative controls in which water was loaded instead of bacteria; no amplified signal was present in any of these cases. These results show that it is possible to reduce the number of bacterial cells needed for DNA isolation and thereby increase the sensitivity of this process three to four orders of magnitude over that of conventional methods.

There are several potential uses for a miniaturized DNA purification system. It can be used as a preparative step for environmental analysis or medical diagnostics, in which case the DNA purification may be preceded by a selection process or may be followed by integrated PCR, capillary electrophoretic analysis or a DNA cloning step. It could also be useful as a tool for microculture and analysis of slow-growing or unculturable bacteria, which make up 99% of the population in naturally occurring ecosystems. In this respect, the chip represents a general front-end processor for more complicated devices that could perform single- and few-cell analyses of the DNA and RNA in animal as well as bacterial cells. We envision further applications in genetic engineering as a rapid analysis tool that would eliminate overnight culture steps in analyzing clone inserts and bacterial cell lines, especially when combined with on-chip, single-cell biochemical assays[16].

An intriguing possible future application for microfluidic mRNA preparation is as a tool to generate subtractive libraries from single cells (or more accurately, pairs of single cells). This procedure is valuable in eliminating commonly expressed transcripts while enriching for differentially expressed transcripts, and is not feasible for small numbers of cells with conventional tools. In a standard preparation, mRNA isolated from roughly 10^6 cells is processed in a volume on the order of 10 μl. The same mRNA concentration would be attained (assuming no adherence of mRNA to vessel walls) if the mRNA from one cell were contained in a 10 pl volume. Although such volumes are smaller than those used in the present study, they are easily attainable with current microfluidic technology, and valves with displacement volumes an order of magnitude smaller have already been demonstrated[15]. The ability to carry out a range of lab-on-a-chip analyses, including mRNA expression patterns and cDNA libraries, from single mammalian cells will have a substantial impact on developmental biology as well as biomedical research.

In conclusion, we have shown that it is possible to parallelize integrated microfluidic processes on a single chip using mechanical microvalves to achieve parallel, nanoliter, fluid processing in a highly integrated system. More specifically, we have demonstrated parallelization of complex sequential bioprocesses comprising cell isolation, cell lysis, DNA affinity purification and the recovery of the purified DNA. The microfluidic architecture is not specific to this particular process and can be applied quite generally to the parallelization of many different biological procedures.

METHODS

Chip fabrication and operation. The chips were fabricated from polydimethylsiloxane using the methods of multilayer soft lithography. The microvalves are mechanical in nature, and are actuated hydraulically. Chip fabrication and operation details are described in **Supplementary Methods** online.

mRNA chip. The microfluidic chip is composed of different functional units that are integrated into a single structure. The processing architecture of the chip is linear (east-west) with reagents loaded orthogonally (north-south) (**Fig. 1a**); this scheme can be generalized to implement a large class of sequential, batch-processed reactions. The first functional unit consists of a chamber that can be loaded with a variable number of cells. The precise number is controlled by the concentration of cells in the loaded solution, and in this study we created conditions to trap between 1 and 100 cells. Adjoining the cell chamber is the 'lysis buffer chamber,' which can be loaded with a fixed amount of a solution of chaotropic salt. The valve separating the two chambers can be opened to allow diffusion of the lysis buffer into the cells. The total volume in which the cells are lysed (between valves 1, 2, 3, 5, 6 and 7) is about 20 nl (**Fig. 1b,c**). The next functional unit is designed to create a packed column of oligo-dT derivatized paramagnetic beads (Dynabeads Oligo(dT)25) through which the

cell lysate can be flushed. To trap a fixed number of beads, we used a microfluidic valve whose opening could be controlled precisely by a pressure regulator. Valve 8 (see **Fig. 1a**) is left slightly open so that a regulated fluid flow can pass through, but the large (2.8 μm) beads are trapped (**Fig. 1b**). All the other valves are on-off valves, controlled by individual pressure sources (Fluidigm). Those pressure sources are actuated by an NI-DAQ card (National Instrument) and a graphic interface (**Fig. 1a**) developed under Labview 6.0. At the end of each run, beads are collected from the chip and assayed for the presence of mRNA with benchtop RT-PCR.

Cell sample preparation. Freshly harvested NIH 3T3 cells were used for each experiment. NIH 3T3 cells were grown to near confluency on 100 mm tissue culture plates. The cells were rinsed with 1× PBS and then trypsinized. The trypsinized cells were resuspended in 1× PBS to adjust the final cell concentration to between 10^6 cells/ml and 10^7 cells/ml.

mRNA transcription and cDNA amplification and detection. The collected beads were washed twice in 100 μl 1× OneStep RT-PCR buffer (Qiagen) plus 1 U/μl Superasin (RNAase inhibitor, Ambion) using a 96-well magnetic stand (Dynal). The beads were then resuspended in 50 μl One Step RT-PCR reaction mix (1× OneStep RT-PCR buffer, 400 μM each dNTP, 0.6 μM each gene-specific primer and 2 μl Qiagen OneStep RT-PCR mix). The positive control was 50 ng of mouse spleen total RNA. The negative control was no RNA (water only). PCR conditions for the actin PCR were: 50 °C (30 min) (RT reaction), 95 °C (15 min) (Hotstart), then 40 cycles of 94 °C (30 s), 60 °C (30 s) and 72 °C (60 s). This was followed by a final cycle of 72 °C (10 min). The mRNA for two types of transcripts—high-abundance β-actin and moderate-abundance zinc finger OZF—was amplified. The primers used were:

    actin, forward: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′
    actin, reverse: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′
    OZF, forward: 5′-CTACGGGTGCAATGAATGTG-3′
    OZF, reverse: 5′-AATTCTCCGGTGTTCACGAC-3′

We loaded 5% of the reaction volume on a 2% agarose gel and poststained with SYBR Green. The reaction volume was removed from the beads and 100 μl Tris-EDTA 1× buffer (TE) was added to the beads, which were then frozen at −20 °C. After thawing, the beads were pelleted, TE was removed and 50 μl of OneStep RT-PCR reaction mix with zinc finger OZF primers was added (using the same concentrations of reagents as for the actin RT-PCR). The same RT-PCR program was run as with actin primers. We loaded 10% of the reaction mixture on a 2% agarose gel and post-stained with SYBR Green.

Genomic DNA chip. The affinity columns were constructed as before. A valve at the location on the chip where the column is to be constructed was cracked open, and 2.8 μm beads (Dynabeads DNA Direct Universal) were allowed to flow in. The beads are larger than the valve opening, so they cannot pass through and are quickly concentrated into a small column. Using the manufacturer's specified minimum capacity for each bead, we calculated that each column has a total capacity of 100 pg of DNA, enough to purify genomic DNA from some 20,000 bacteria. Because the three columns on each chip are eluted simultaneously from a single input, it is important that each processor have the same fluidic resistance. If the channel lengths vary, then the corresponding resistance will cause unequal fluid flow in the separate processors, thus affecting the elution rates. We therefore ensured that the overall microfluidic channel lengths for each processor were identical (**Fig. 3f**).

Preparation of bacteria for DNA isolation and gene amplification. *E. coli* (BL21–2⁺) expressing EGFP was grown at 37 °C for 12 h in Luria-Bertani liquid

medium containing 40 μg/μl ampicillin. After the DNA purification and recovery from the chip, a gene, *ppdD* (prepilin peptidase-dependent protein D precursor, 461 bp), was amplified with conventional PCR. Thermal cycling conditions were as follows. Initial denaturing, 95 °C (2 min); 30 cycles of DNA denaturing, 95 °C (30 s); primer annealing, 60 °C (30 s); dNTP polymerizing, 72 °C (2.5 min); final extension, 72 °C (10 min). The primer set for *ppdD* was 5′-GGTGGTTATTGGCATCATTGC-3′ for forward and 5′-GTTATCC-CAACCCGGTGTCA-3′ for reverse. The PCR cocktail was 5 μl of 10× reaction buffer, 1 μl of 10 mM dNTP mix, 1 μl each of forward and reverse primers, 0.5 μl of 5 U/μl Taq polymerase, and template and water to make 50 μl of total reaction volume.

Note: Supplementary information is available on the Nature Biotechnology website.

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