Supporting Information

Streets et al. 10.1073/pnas.1402030111

SI Materials and Methods

Device Fabrication. Microfluidic devices were fabricated with polydimethylsiloxane (PDMS) using standard multilayer soft lithography (1). Two molds, a control mold and a flow mold, were patterned on silicon wafers with photolithography. The silicon wafers were first thoroughly cleaned using acetone and isopropyl alcohol. The wafers were then baked at 150 °C for 30 min to dehydrate the surface. For the control mold, negative photoresist (SU8-2025; MicroChem) was poured onto the wafer directly and then spun at 3,500 rpm for 30 s, yielding a 25-μm layer. Then, the wafer was baked on a hotplate for 3 min at 65 °C and 5 min at 95 °C. The resist-coated wafer was exposed to UV radiation through a negative mask (clear features and opaque background) imprinted with the control circuit using a photolithography aligner (JKG-2A; Shanghai Xueze Optics). After exposure, the wafer was again baked at 65 °C for 1 min and 95 °C for 2 min. The wafer was then submerged in SU-8 developer and gently agitated until the unexposed photoresist was removed, leaving the positive control features. Then, the wafer was carefully washed with isopropyl alcohol and blow-dried. The mold was baked at 150 °C for at least 3 h before further use.

The multiple-height flow mold was made from negative photoresist and positive photoresist. These wafers were treated with hexamethyldisilazane (Alfa Aesar) vapor for 5 min at room temperature after cleaning. The wafer was first spin-coated with the positive photoresist AZ4620 (AZ Electronic Materials) at 1,000 rpm for 30 s. After baking at 95 °C for 3 min and 105 °C for 6 min, the photoresist was then exposed to UV light through a high-resolution positive mask containing the flow circuit design and developed in AZ400K developer (diluted 1:2 developer: water). The positive photoresist was used for the channels with a height of 15 μm. We then baked the mold again by ramping the hotplate from 95 °C to 220 °C at 6 °C/h for a total of 20 h to reflow the positive resist and create rounded channels. Negative photoresist (SU8-2025) was then used for building the reaction chambers using the same protocol as above but spin-coating the resist at 2,500 rpm for 30 s to achieve a height of 30 μm.

PDMS (RTV-615; GE Advanced Materials) was cast on the master molds to make devices after all molds were exposed to trichloro-(1H,1H,2H,2H-perfluorooctyl) silane (Sigma-Aldrich) vapor for 10 min to facilitate PDMS releasing from the mold. These devices were designed in the push-down configuration. The PDMS mixture with a ratio of 20:1 (potting agent:cross-linking agent) was spin-coated onto the flow mold at 1,800 rpm for 60 s, and 30 g PDMS with a ratio of 5:1 (potting agent:cross-linking agent) was poured onto the control mold to make the thick upper layer of the device. The baking times of the flow and control layers were 15 and 30 min, respectively, at 80 °C. The thicker PDMS slab on the flow layer mold was then peeled off, and holes were punched for fluidic inlets using a 20-gauge rounded punch. The control layer slab was then aligned to the thin flow layer and baked at 80 °C for 60 min. The two bonded layers were peeled off from the flow mold, and then, holes for control line inlets were punched. A bottom dummy layer of PDMS was spun onto a clean silicon wafer at 2,000 rpm for 60 s and baked for 15 min at 80 °C. The dummy layer and two-layer chip were exposed to oxygen plasma for 30 s to remove all organic contaminants and prime the two layers for bonding. The chip was then quickly but carefully placed onto the dummy layer and baked for 2 h to complete the bonding. Finally, the assembled chip was cut from the dummy layer and bonded onto a glass coverslip with a thickness of 0.3 mm. The whole device was then baked at 80 °C for 15 min.

Cell Culture and Single-Cell Suspension Preparation. The feeder-independent mouse ES cells were from the 129 mouse strain. The cells were cultured in DMEM/F12 medium supplemented with 20% (vol/vol) FBS and containing 1% L-glucose, 1% non-essential amino acid, 1% Penicillin-Streptomycin (Invitrogen), 0.1% β-mercaptoethanol, 1% nucleic acids, 1% sodium pyruvate, 1.6% (vol/vol) sodium carbonate, and most importantly, 1,000 unit/mL ESGRO leukemia inhibitory factor (Chemicon). Mouse ES cells (mESCs) were cultured at 37 °C in a humidified incubator containing 5% (vol/vol) CO_2. The cell culture was maintained by regular passaging with a treatment of 0.05 trypsin with 0.1% EDTA (Invitrogen) and centrifugation at 250 × *g* for 3 min. The mouse embryonic fibroblast cells were cultured with DMEM supplemented with 10% (vol/vol) FBS and 1% Penicillin-Streptomycin. All cells were cultured at 37 °C in a humidified incubator containing 5% (vol/vol) CO_2. When cells became confluent, they were detached by 0.25% trypsin with 0.1% EDTA (Invitrogen; Life Technologies) and centrifuged at 250 × *g* for 3 min. Then, the supernatant was discarded, and cells were resuspended at a density of 2×10^7 cells/mL for passage. For the single-cell sequencing experiment, the concentration of the cell suspension was adjusted to 5×10^5/mL using a hemocytometer. After thoroughly vortexing, 10 μL this suspension was mixed with 40 μL cold acetylated (Ac)-BSA-PBS solution, which was prepared by dissolving Ac-BSA (20 mg/mL) in PBS at 1 mg/mL (Ac-BSA: B8894; Sigma—Aldrich; 1× PBS: 14249–95; Gibco). A typical cell suspension concentration for chip experiments was 10^5 cells/mL. In most experiments, around 5 μL single-cell suspension was injected into the chip, although the full 5 μL were rarely completely used, and it is possible to decrease this volume in situations where sample is limited.

Device Design and Operation. Before use, the microfluidic devices were baked at 150 °C for 30 min to inactivate any enzymes inside the channels. The single-cell suspension and all of the reagents used in the experiment were introduced into the device through the cell input and reagent input (Fig. 1*A*), respectively, by gel-loading aerosol-barrier tips (Thermo Fisher Scientific Inc.), which were pressurized with 10 psi. Before introducing the cells, flow channels were flushed with RNase/DNase free water (Ambion; Life Technologies) and incubated with 0.2% (wt/wt) Pluronic F-127 solution (P2443; Sigma-Aldrich) for 60 min at room temperature. After incubation, the flow channels were rinsed with RNase/DNase free water (Ambion; Life Technologies) and air-dried.

Before operation, all valves were filled and closed with 30 psi operating pressure. To begin, the cell channel and reagent input channel were primed with Ac-BSA-PBS, filling the trapping channels but leaving the sorting chambers and reaction chambers empty. The cell suspension was then introduced from the cell input, and single cells were directed to the eight trapping chambers sequentially with a peristaltic pump downstream of the cell input channel (Fig. 1*B*). If multiple cells or unhealthy looking cells were trapped, the isolation valve was reopened, and the unwanted cell was discarded to the waste output. After trapping a single cell, the Ac-BSA-PBS was pressurized from the reagent inlet and pushed the cell into the neighboring sorting chamber. This trapping and sorting process was repeated for the eight reaction lanes (Fig. S1*F*). One or two of these lanes were often used for a no-cell negative control. In this case, suspension buffer without a cell was pushed into the sorting chamber. After sorting, the device was placed onto a temperature-controlled platform (Fig. S1*A*) made from thermoelectric Peltier coolers, which was set to 4 °C. Mineral oil was used to increase thermal conductivity between the platform and glass coverslip. For each subsequent reaction, the reagent mix was

injected into the reagent input line, which was flushed and primed with new reagent. Each lane was then simultaneously pressurized, and the newly added reagent pushed the contents of the previous reaction into the following stage, filling the chamber and replacing the air that was pushed through the PDMS. A stereomicroscope was used to ensure complete filling for every reaction step.

After filling, the added reagents were mixed using large valves positioned above the reaction chambers (Fig. 1*B*). For stages 2–4, a linear mixing strategy was used, in which mixing pumps were alternately actuated, driving the reactants back and forth between the chambers (Fig. S1*C*). For stage 5, a connective channel formed a closed loop in the reaction chamber, and the mixing pumps could be used in a peristaltic manner to circulate the contents through the ring and accelerate diffusive mixing (2) (Fig. S1*D*). Mixing times for each step were as follows: 3 min for stages 2 and 3, 5 min for stage 4, and 10 min for stage 5. The mixing schemes were characterized with optical absorption measurements. After filling the lysis chamber with distilled water, the stage 2 valve was opened, and a blue dye was injected into the lysis chamber, pushing the water into the reverse transcription chamber and simulating the experimental procedure (Fig. S1*E*). Linear mixing was then initiated, and images of the chambers were recorded every 20 s for 2 min. The mean pixel intensity (I') within small regions in both chambers indicated in Fig. S1*E* was calculated for every time point. Using the Beer–Lambert law (Eq. **S1**), the relative concentration of dye (c'/c_f) was calculated for each chamber and plotted in Fig. S1*E*:

$$\frac{I'}{I_0} = 10^{-\varepsilon l c'}, \quad \frac{c'}{c_f} = \frac{\log_{10} I'/I_0}{\log_{10} I_f/I_0}. \qquad \textbf{[S1]}$$

Here, ε is the dye's extinction coefficient, l is the depth of the chamber, I_0 is the incident intensity measured in a region outside of the chambers, and the subscript f denotes a chamber with pure dye. At least 100 s of linear mixing with a pumping frequency of 3 Hz were necessary to completely mix stages 1 and 2. To ensure thorough mixing, we extended the mixing times by two to five times the duration determined by this calculation. Placing a flexible valve above the reaction chambers increases the fluidic capacitance of the chamber. To prevent inflation of the chamber volume during filling, the pump valves were activated, and the operating pressure was reduced to match the flow pressure of ~10 psi. During mixing, the valve pressure was switched back to a nominal control pressure of 30 psi.

After mixing, each reaction step required a specific temporal temperature profile for denaturation, annealing, and enzyme inactivation, which is listed in the tables below. Otherwise, the default platform temperature was set to 4 °C. After the final stage, each isolated lane was flushed with nuclease-free water, and the double-stranded cDNA product was retrieved from the device into eight filtered gel loading tips along with 5 μL water and dispensed into 0.2-mL PCR tubes; 24 μL PCR mixture II of 1× ExTaq buffer, 0.25 mM dNTP, 1 μM V3-T24 primer, 1 μM V1-T24 primer, and 0.05 unit/μL ExTaq Hot Start Version was added for a first round of 20 cycles of PCR amplification with the following temperature profile: 95 °C for 30 s, 67 °C for 1 min, and 72 °C for 3 min with a 6-s extension per cycle. The second round of PCR was performed in a standard tube-based format to avoid evaporation of water from the microfluidic device, which is possible during long durations at high temperature. During this off-chip PCR amplification, negative control experiments were included using the same water that was used to flush the chip instead of cDNA, confirming that there was undetectable contamination during this procedure. Samples were stored at −80 °C until library preparation and sequencing.

cDNA Preparation Protocol. Below is a modified reagent list based on the single-cell transcriptome sequencing protocol from ref. 3 adjusted for the microfluidic platform. Here, we used two times the enzyme concentration as in the original protocol. For a typical experiment with a single device, we prepared 2–5 μL for each reaction mix.

(Stage 1) Cell lysis: 70 °C for 90 s; 4 °C.

Component	Mix concentration	Reaction concentration
10× PCR buffer II (without $MgCl_2$)	1.03×	0.76×
25 mM $MgCl_2$	1.55 mM	1.14 mM
0.1 M DTT	5.15 mM	3.8 mM
RNase inhibitor (40 units μL^{-1})	1.19 units μL^{-1}	0.88 units μL^{-1}
SUPERase-In (20 units μL^{-1})	0.6 units μL^{-1}	0.44 units μL^{-1}
0.5 μm UP1 primer	28.6 nM	21.2 nM
dNTP mix (2.5 mM each)	0.05 mM (each)	0.038 mM (each)
2% (vol/vol) Tween 20	0.2%	0.15%
10% Nonidet P-40	0.53%	0.39%
Nuclease-free water		

(Stage 2) Reverse transcription: 50 °C for 45 min; 72 °C for 20 min; 4 °C.

Component	Mix concentration	Reaction concentration
1× Lysis buffer (without 10% Nonidet P-40)	0.55×	0.24×
SuperScript III reverse transcriptase (200 units μL^{-1})	57.5 units μL^{-1}	24.4 units μL^{-1}
RNase inhibitor (40 units μL^{-1})	3.48 units μL^{-1}	1.48 units μL^{-1}
T4 gene 32 protein (5 mg mL^{-1})	0.15 mg mL^{-1}	0.065 mg mL^{-1}
2% (vol/vol) Tween 20	0.08%	0.034%

(Stage 3) Free primer removal: 37 °C for 33 min; 83 °C for 30 min; 4 °C.

Component	Mix concentration	Reaction concentration
10× Exonuclease I buffer	1×	0.23×
Exonuclease I (5 units μL^{-1})	1×	0.23×
2% (vol/vol) Tween 20	0.2%	0.046%
Nuclease-free water		

(Stage 4) 3′ Poly(A) tailing: 37 °C for 18 min; 72 °C for 15 min; 4 °C.

Component	Mix concentration	Reaction concentration
10× PCR buffer II (without $MgCl_2$)	1×	0.47×
25 mM $MgCl_2$	1.5 mM	0.7 mM
100 mM dATP	3 mM	1.4 mM
Terminal transferase (15 units μL^{-1})	1.5 units μL^{-1}	0.7 units μL^{-1}
RNase H (2 units μL^{-1})	0.2 units μL^{-1}	0.09 units μL^{-1}
2% (vol/vol) Tween 20	0.2%	0.09%
Nuclease-free water		

(Stage 5) Second-strand synthesis: 95 °C for 3 min; 50 °C for 2 min; 72 °C for 30 min; 4 °C.

Component	Mix concentration	Reaction concentration
10× Ex Taq buffer (with $MgCl_2$)	1×	0.86×
dNTP mix (2.5 mM each)	0.25 mM	0.21 mM
UP2 primer (100 μM)	2 μM	1.7 μM
TaKaRa Ex Taq HS (5 units μL^{-1})	0.1 units μL^{-1}	0.086 units μL^{-1}
2% (vol/vol) Tween 20	0.2%	0.17%
Nuclease-free water		

Sequencing Library Preparation. Amplified cDNA was purified and size-selected (>0.5 kb) with magnetic beads (Agencourt AMPure XP); 1 ng product of each sample was amplified by a second round of 10 cycles of PCR [95 °C for 3 min and then 10 cycles of 95 °C for 30 s, 67 °C for 1 min, and 72 °C for 6 min (+ 6 s each cycle)] using amine-blocked primers. The samples were then column-purified (ZYMO) and size-selected (>0.5 kb) with magnetic beads (Agencourt AMPure XP). An aliquot of 50 ng DNA from each sample was used as the starting amount for library preparation to ensure sample consistency. The Illumina HiSeq 2500 sequencing system (Illumina) was used for sequencing, and the library preparation kits (E7370) were purchased from New England Biolabs. Sequencing library construction and template preparation were performed according to the New England Biolabs library preparation protocols. We constructed a paired-end library with insert size of ∼200 bp for each sample, and 12 cycles were performed during PCR amplification. Each sample was barcoded, and equal quantities of barcoded libraries were used for sequencing. All of the negative controls were treated similarly as above.

Sequencing Data Analysis. The original image data generated by the sequencer were converted into sequence data by base calling (Illumina pipeline CASAVA version 1.8.0). The raw reads were filtered to discard low-quality reads (reads containing more than 50 bases with quality value ≤ 5 and >10% bases as N). The results were clean reads. Adaptors with flanking polyA/T sequences were trimmed. Additionally, reads with AT content larger than 70% were also removed. Trimmed reads with length larger than 30 bp were kept. For paired-end reads, if only one end was kept, it was exported to another fastq file, which stored all single-end data. Both single- and paired-end reads were used in alignment. Filtered reads were mapped to the mouse reference sequence downloaded from the University of California, Santa Cruz table browser with Burrows–Wheeler Aligner (version 0.6.2-r126). The reads that mapped to Refseq genes were counted and converted to reads per kilobase transcript per million mapped reads using the length of the longest transcript of the gene. For long noncoding RNA detection, the filtered and trimmed reads were mapped to the transcript reference downloaded from GENCODE M2 annotation. The expression levels of long noncoding RNAs were counted and normalized in the same way as the coding genes. Hierarchical clustering was performed using the statistical programming language R. The $\log_{10}$-transformed reads per kilobase transcript per million mapped read values of the genes that showed significant variability between six mESCs and six mouse embryonic fibroblasts were used to generate a scaled matrix. Then, the function heatmap.2 from the R package gplots was used to perform hierarchical clustering and generate the heat maps in Fig. 5.

Bulk RNA Extraction and Quantification. Mouse ES cells from the 129 mouse strain were enumerated by a hemocytometer with three replications; $\sim 5 \times 10^5$ cells were used for RNA extraction following the standard instructions of the RNeasy Mini Kit (Qiagen), and the RNA samples were digested with DNase I on column to remove contaminating genomic DNA. The concentration of extracted RNA was quantified by Qubit (Life Technologies), and total yield was ∼10 μg, from which we estimated 20 pg total RNA per mESC on average; 100 ng total RNA was prepared for library construction following Illumina's TruSeq RNA sample preparation protocol and sequenced on the Illumina HiSeq 2500. This library was used for the bulk transcriptome analysis. Technical replicates were generated by diluting the extracted total RNA to desired final concentrations.

Exogenous RNA Spike-In. External RNA Controls Consortium RNA Spike-In Mix 1 was purchased from Life Technologies and stored at −80 °C. For single-cell spike-in experiments, the External RNA Controls Consortium Mix was diluted in buffer [RNase Inhibitor (1 unit μL^{-1}) and 0.05% Tween 20] and added to the lysis buffer for a total dilution of 1,000:1.

The spike-in containing genes encoding red fluorescent protein (RFP), green fluorescent protein (GFP), and cre recombinase (Cre) was prepared in the following manner. Full-length RFP, GFP, and Cre genes were inserted into a pCS2 plasmid vector with an 80-bp poly(A) sequence at multiple clone sites. To obtain transcripts of the RFP, GFP, and Cre genes with poly(A) sequence, the plasmids were linearized at the XbaI site downstream from the poly(A) sequence, and SP6 RNA polymerase (Promega) was used for in vitro transcription. All three targeted RNA products were purified according to the standard RNeasy Mini Kit's protocol (Qiagen) for eliminating DNA contamination. The concentration was measured by Nanodrop (Thermo Fisher Scientific), and the quantity of each gene was obtained with the known molecular weight. *RFP*, *GFP*, and *Cre* RNA were stored at the concentrations of 2×10^{10}, 2×10^9, and 2×10^8 molecule/μL, respectively, at −80 °C. For a single-cell experiment, the *RFP-GFP-Cre* spike-in was diluted and added to the lysis buffer with a total dilution of 300,000:1. Spike-in experiments were also performed on one device at five times the concentration stated (Table S1).

1. Unger MA, Chou HP, Thorsen T, Scherer A, Quake SR (2000) Monolithic microfabricated valves and pumps by multilayer soft lithography. *Science* 288(5463):113–116.
2. Hansen CL, Sommer MOA, Quake SR (2004) Systematic investigation of protein phase behavior with a microfluidic formulator. *Proc Natl Acad Sci USA* 101(40):14431–14436.
3. Tang F, et al. (2010) RNA-Seq analysis to capture the transcriptome landscape of a single cell. *Nat Protoc* 5(3):516–535.

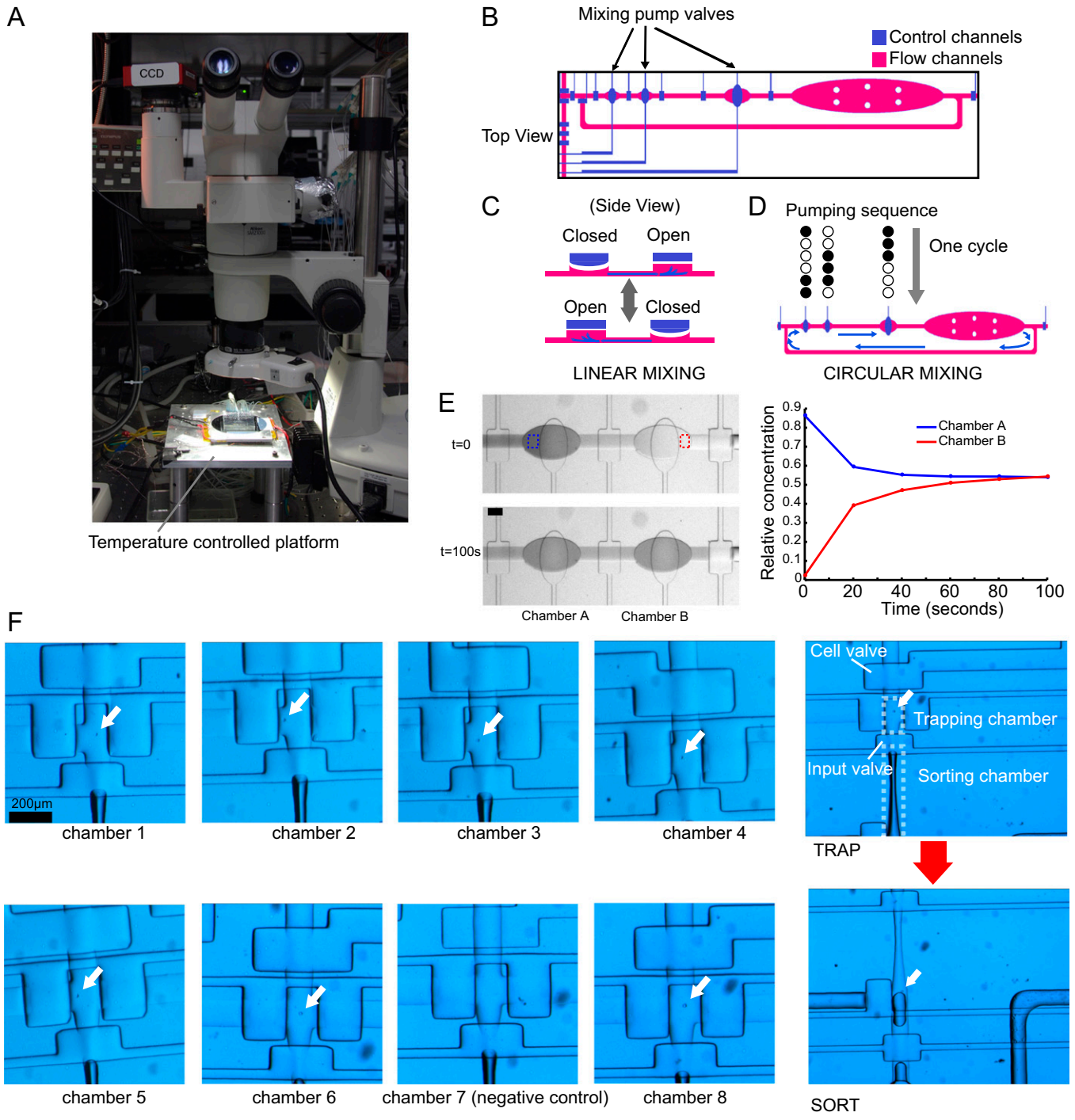

Fig. S1. Experimental setup. (*A*) A photograph of the experimental setup depicting the microfluidic device atop a temperature-controlled platform. (*B*) Three large valves are positioned over three of the reaction chambers. The control pressure of these valves can be adjusted independently from the rest of the control manifold and regulated to optimize mixing. (*C*) This linear mixing scheme is used in stages 2–4 of the reaction pipeline. Reactants were pushed back and forth between chambers to accelerate diffusive mixing. (*D*) In the final reaction stage, a ring path is open, and mixing can be achieved by using a peristaltic pumping sequence to circulate reactants around the ring and accelerate diffusive mixing. (*E*) Micrograph of the linear mixing scheme using dye to visualize reagents (*Left*). (Scale bar: 150 μm.) Mean transmitted intensity was measured in the regions indicated in chambers A and B every 20 s during mixing (*SI Materials and Methods*). The relative concentration of dye is plotted for both chambers during 100 s of mixing (*Right*). (*F*) Micrographs of the eight trapping events during a typical experiment. White arrows indicate the trapped cell. After trapping, a cell can be released if it looks unhealthy or if more than one cell was trapped. *Right* shows the sorting sequence in which, after a cell is trapped, the input valve and an independently addressable cell valve are opened, and the cell and surrounding media are pushed into an empty sorting chamber.

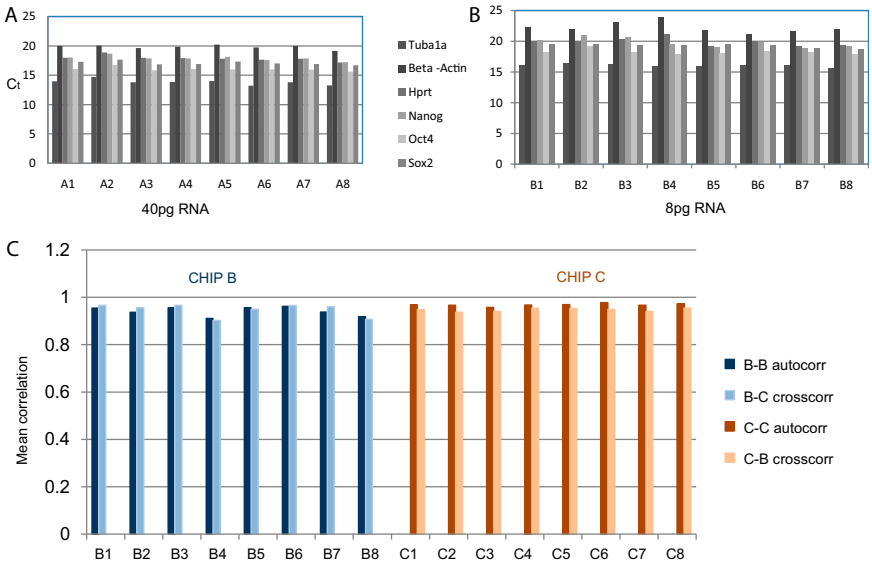

Fig. S2. Reproducibility of microfluidic reactions. Quantification of eight cDNA samples prepared in the microfluidic device from extracted RNA technical replicates of (*A*) 40 pg and (*B*) 8 pg. The threshold cycle (Ct) value of six genes [Tubulin alpha-1A chain (Tuba1a), Beta-actin, hypoxanthine phosphoribosyltransferase (Hprt), Nanog, octamer-binding transcription factor 4 (Oct4), and sex determining region Y-box 2 (Sox2)] was measured by quantitative real-time PCR. (*C*) Quantification of the correlation between reactions on a single device and between two devices. Technical replicates of 8 pg extracted RNA were prepared on two separate devices for a total of 16 cDNA libraries. The Pearson correlation coefficient for expression levels of all genes with reads per kilobase transcript per million mapped reads (RPKM) > 1 was calculated for every pair of libraries. For each library, the mean correlations between samples prepared on the same device (autocorrelation) and a different device (cross-correlation) are plotted on the bar graph.

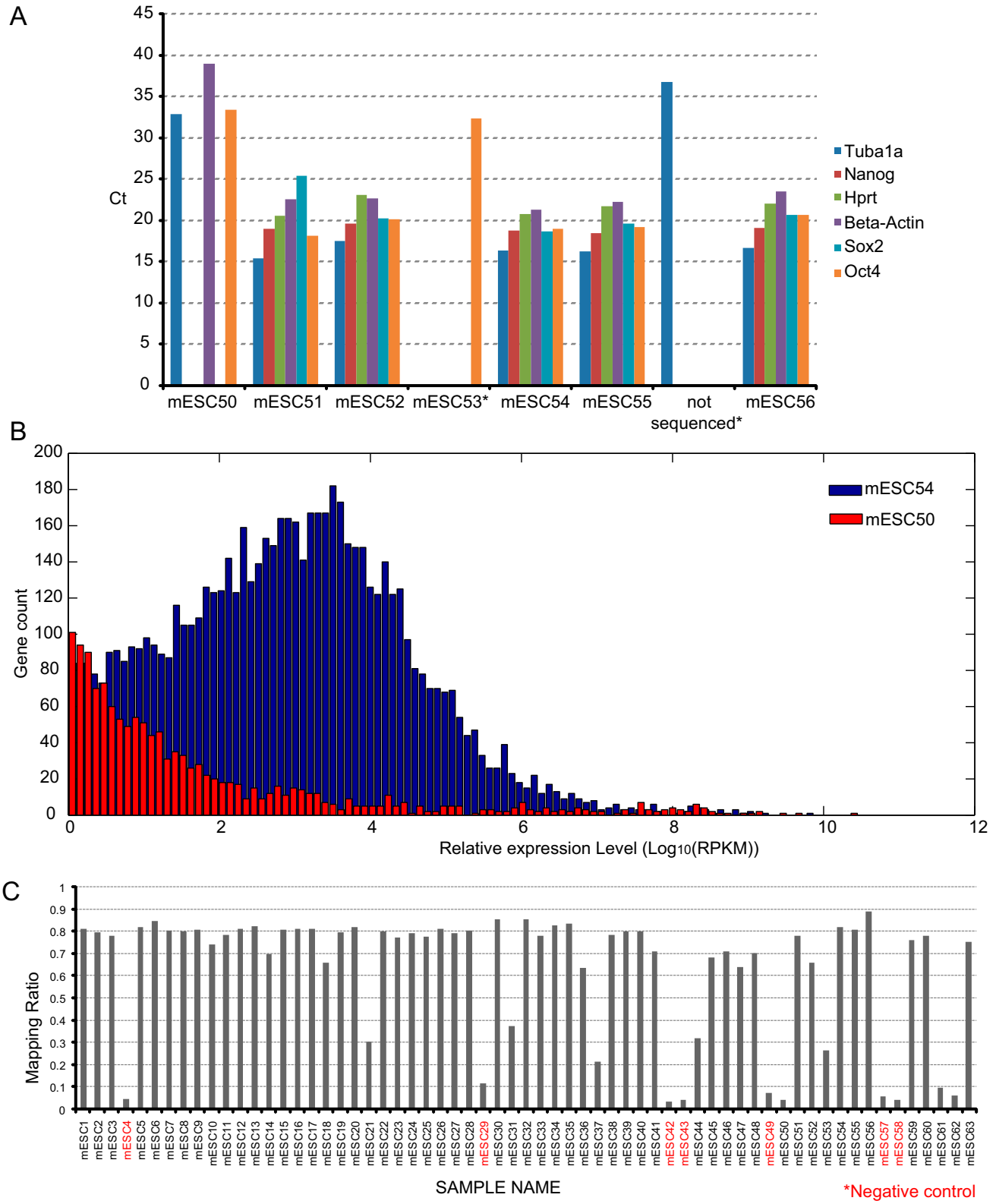

Fig. S3. Determination of dead or unhealthy cells to be discarded. (*A*) Quantitative real-time PCR was used to assess cDNA obtained from microfluidic preparation. The Ct value for six genes in eight samples is plotted, including two negative control samples. mESC50 was identified as possibly dead or unhealthy. (*B*) Histograms of gene expression distributions for two cells from the experiment in *A*. The distribution of gene expression in cell mESC50 is nonrepresentative of a healthy cell and was discarded from additional analysis. (*C*) Fraction of reads mapped to the mouse reference sequence for all 63 mESC libraries, including seven negative control samples. Cells with less than 40% of reads mapped were considered dead or unhealthy and discarded.

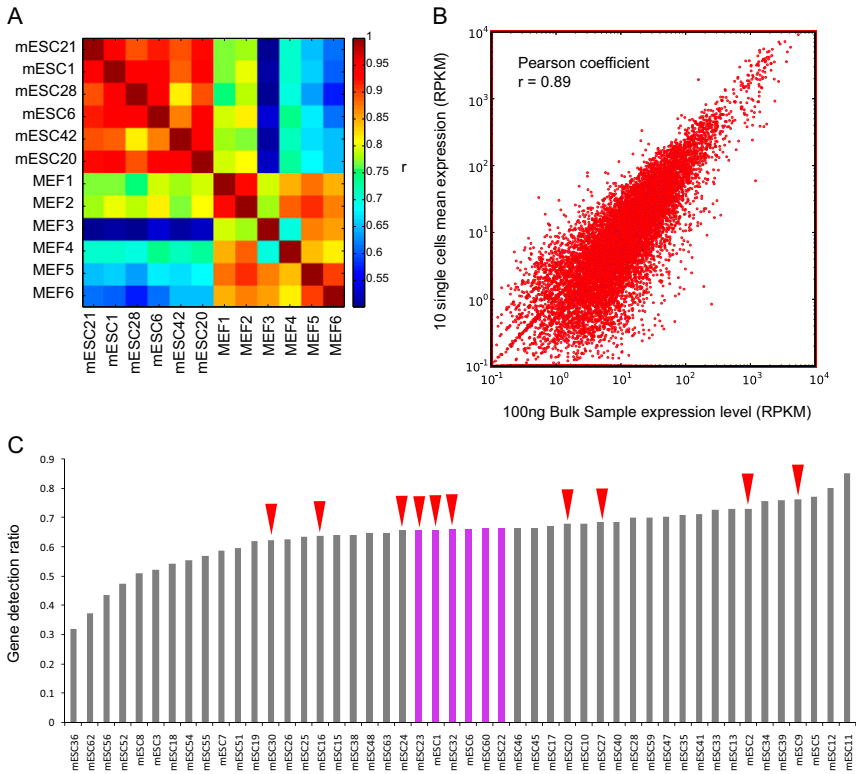

Fig. S4. Global analysis of mESC and mouse embryonic fibroblast (MEF). (*A*) Correlation of gene expression between 12 single cells, including 6 mESCs and 6 MEFs. Correlation is measured by the Pearson correlation coefficient. The six mESCs represent typical cells, because the number of genes detected was around the median for all mESCs. These six cells are labeled with purple in *C*. (*B*) Correlation of expression levels between genes pooled together from 10 cells identified in *C* and the bulk 100-ng sample; 200,000 reads were randomly sampled from each single-cell library and mapped to the reference sequence. The mean RPKM value for each gene averaged across all 10 libraries is plotted against the RPKM of genes mapped from 2 million randomly sampled reads in the bulk sample. (*C*) mESC cells ranked by gene detection as in Fig. 2*B*. Red arrows indicate 10 cells used in the ensemble comparison with Fig. 2*D* and above in *B*. Purple bars indicate six typical mESCs used in comparisons between mESCs and MEFs (Fig. 5*E*).

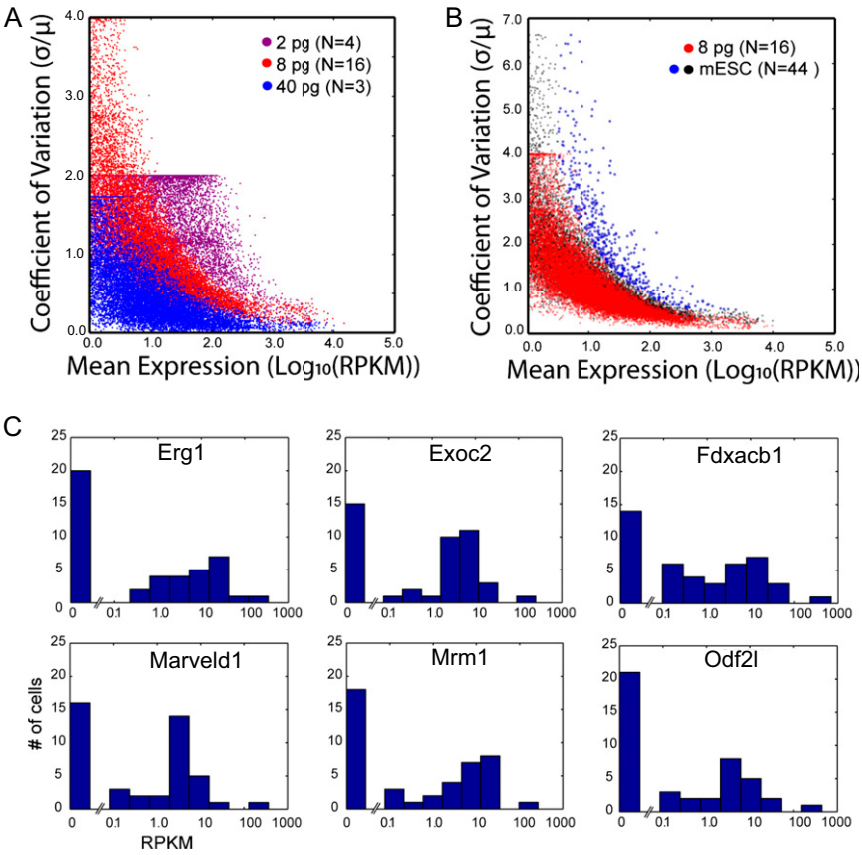

Fig. S5. Technical variation and biological variation. (*A*) Coefficient of variation (CV) plotted against $\log_{10}$-transformed mean expression of genes detected in technical replicates of 40 pg (blue), 8 pg (red), and 2 pg (purple) extracted RNA. The maximum CV is defined by $\sqrt{N}$, and data points that cluster at this cutoff represent genes that were only detected in a one of the samples. (*B*) CV plotted against $\log_{10}$-transformed mean expression of genes detected in 16 8-pg technical replicates and 44 single mESC libraries. Endogenously expressed genes in the mESC set that showed CV larger than 3 SDs from the mean CV in the 8-pg set were marked in blue and considered to show biological variability that was detectable above the technical noise. These 475 genes are listed in Dataset S1. (*C*) Histograms of the distribution of expression in 44 single mESCs for six highly variable genes listed in Dataset S1.

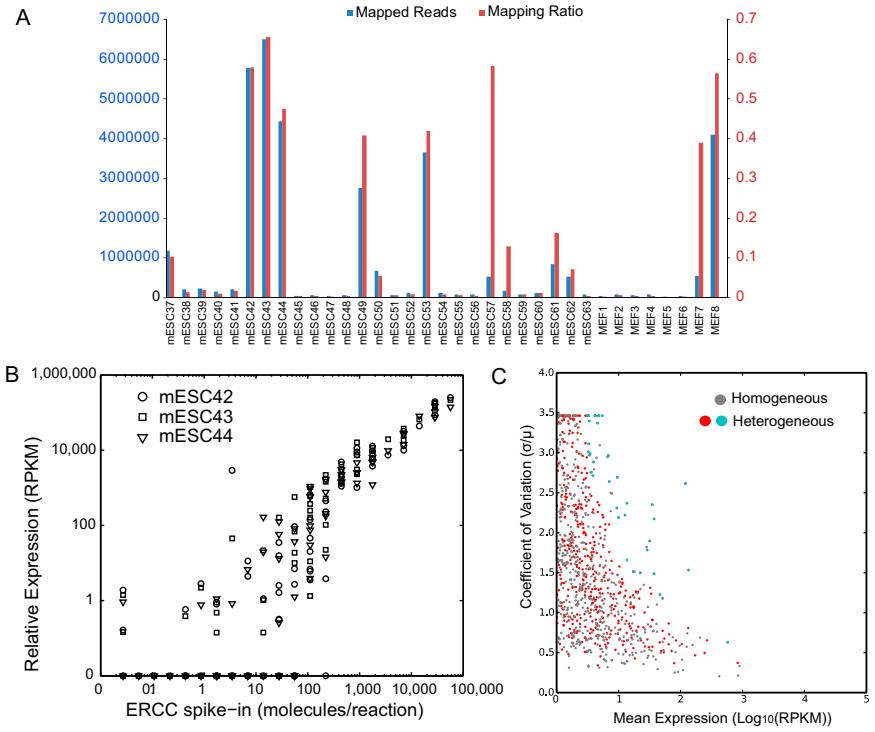

Fig. S6. (*A*) External RNA Controls Consortium (ERCC) transcript detection in 35 experiments that contained spike-in. Mapped reads (blue) are the total number of reads mapped to the ERCC reference. Mapping ratio (red) is the fraction of mapped reads to total reads obtained in each sample after trimming. Samples mESC42, mESC43, and mESC44 were used for the ERCC accuracy analysis in Fig. 4*A*, because they contained both a high number of mapped reads and a relatively high mapping ratio. (*B*) Relative expression of ERCC transcripts plotted against starting number of transcript molecules for the three samples plotted in Fig. 4*A*. (*C*) CV plotted against $\log_{10}$-transformed mean expression of noncoding genes mapped to the GENCODE M2 mouse annotation database from reads sequenced in two sets of 12 libraries. The first set is labeled homogeneous and contains 12 mESC libraries (gray dots; mESC9–mESC14 and mESC23–mESC28). The second set is labeled heterogeneous and contains six mESC libraries and six MEF libraries (red dots; mESC23–mESC28 and MEF1–MEF6). At each expression level, genes in the heterogeneous set that displayed a CV greater than 3 SDs above the mean CV in the homogenous set were considered to show large differential expression between mESCs and MEFs, and they are labeled in turquoise. These 38 genes are listed in Dataset S1.

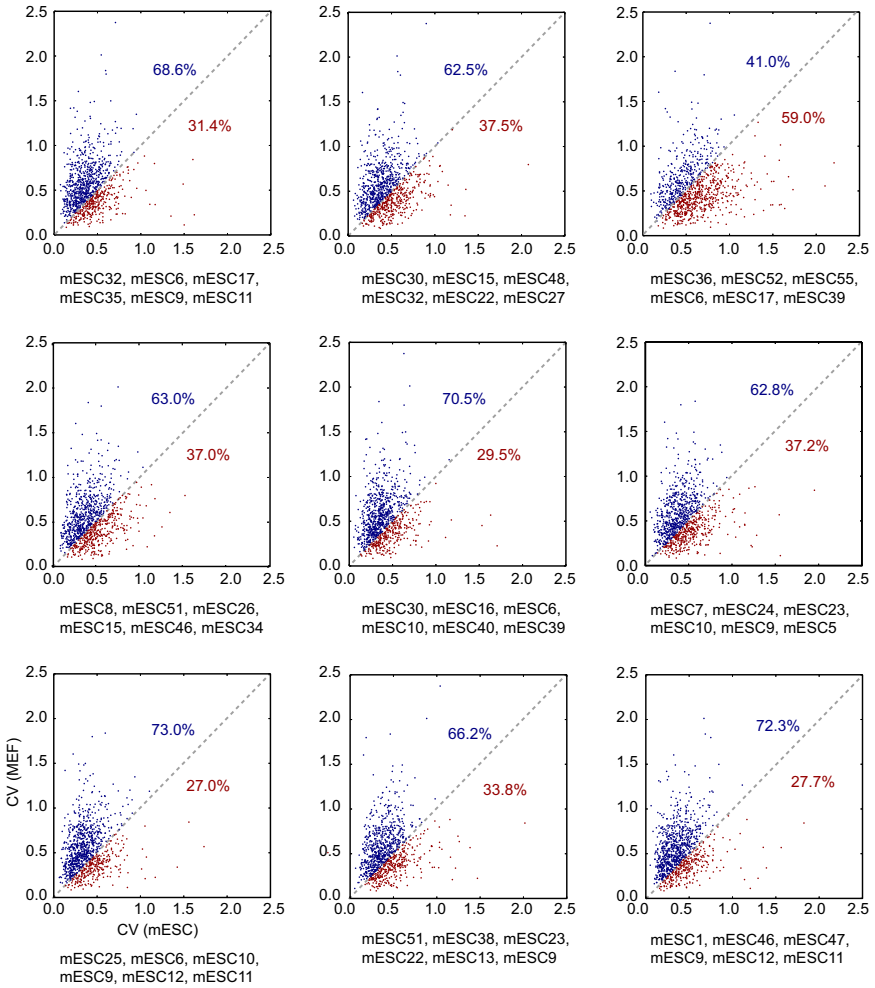

Fig. S7. Comparison of the CV for genes expressed with RPKM > 50 in six MEFs and nine sets of six mESCs. The percentages represent the numbers of genes above (blue) and below (red) the $x = y$ line.

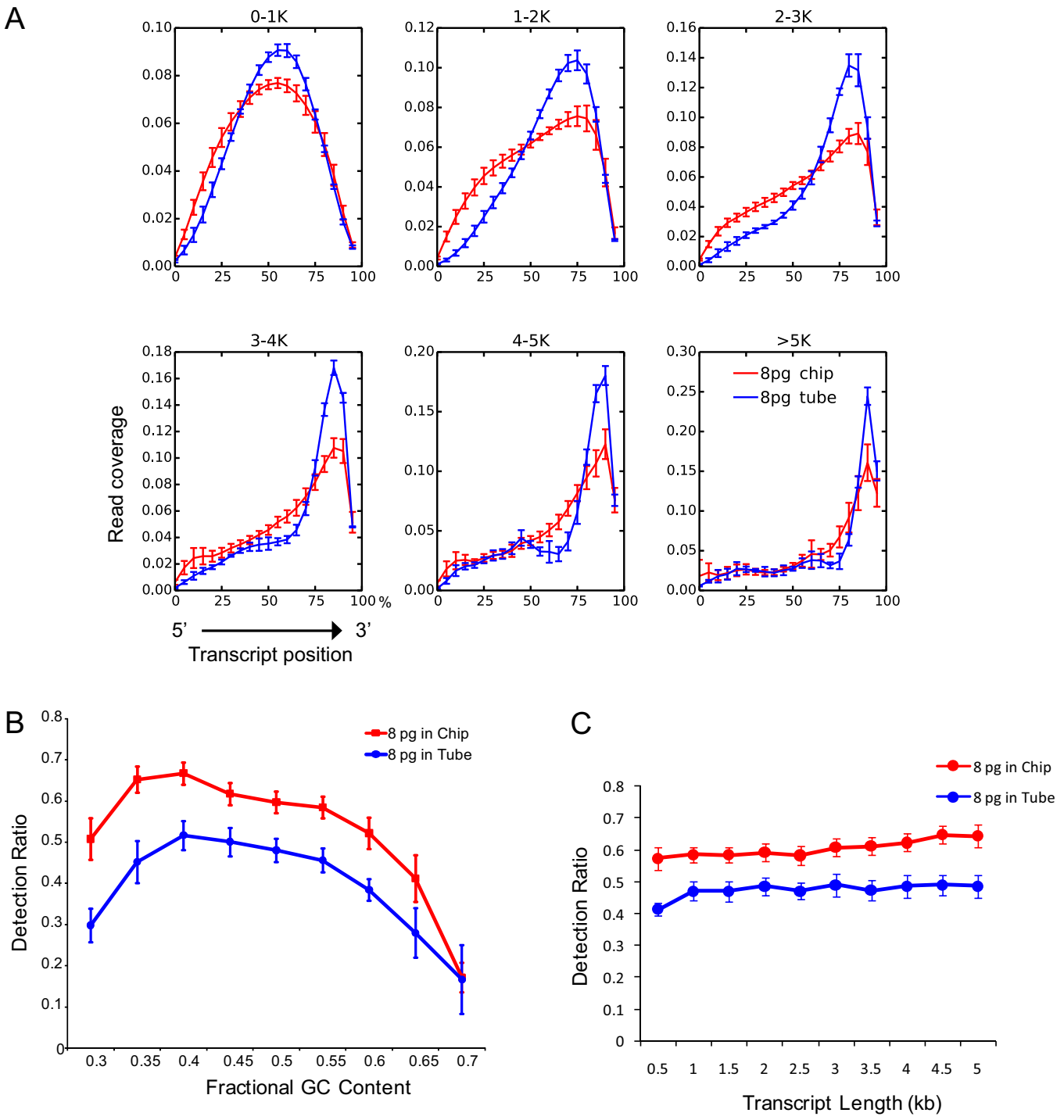

Fig. S8. Bias in microfluidic single-cell transcriptome sequencing: (*A*) 5′ to 3′ coverage distribution; (*B*) GC bias. The detection ratio represents genes detected in the 8-pg replicates divided by genes detected in the bulk sample. (*C*) Fraction of genes detected in the 8-pg sample prepared in the chip (red) and the tube (blue) to genes detected in the bulk sample as a function of transcript length.

Table S1. Annotated list of 102 libraries from cDNA prepared in the microfluidic device and a tube

Sample ID	Sample name	Description	Notes	Spike-in dilution (ERCC/Cre)
0	T0	Tube negative control	Negative control	
1	T8-1	8-pg tube		
2	T8-2	8-pg tube		
3	T8-3	8-pg tube		
4	T40-1	40-pg tube		
5	T40-2	40-pg tube		
6	T40-3	40-pg tube		
7	T100	100-ng tube		
8	B2-1	2-pg chip		
9	B2-2	2-pg chip		
10	B2-3	2-pg chip		
11	B2-4	2-pg chip		
12	B8-1	8-pg chip		
13	B8-2	8-pg chip		
14	B8-3	8-pg chip		
15	B8-4	8-pg chip		
16	B8-5	8-pg chip		
17	B8-6	8-pg chip		
18	B8-7	8-pg chip		
19	B8-8	8-pg chip		
20	B8-9	8-pg chip		
21	B8-10	8-pg chip		
22	B8-11	8-pg chip		
23	B8-12	8-pg chip		
24	B8-13	8-pg chip		
25	B8-14	8-pg chip		
26	B8-15	8-pg chip		
27	B8-16	8-pg chip		
28	B40-1	40-pg chip		
29	B40-2	40-pg chip		
30	B40-3	40-pg chip		
31	mESC1	mESC two-cell chip	Two-cell device	
32	mESC2	mESC two-cell chip	Two-cell device	
33	mESC3	mESC two-cell chip	Two-cell device	
34	mESC4	mESC two-cell chip	Negative control	
35	mESC5	mESC two-cell chip	Two-cell device	
36	mESC6	mESC two-cell chip	Two-cell device	
37	mESC7	mESC two-cell chip	Two-cell device	
38	mESC8	mESC two-cell chip	Two-cell device	
39	mESC9	mESC eight-cell chip		
40	mESC10	mESC eight-cell chip		
41	mESC11	mESC eight-cell chip		
42	mESC12	mESC eight-cell chip		
43	mESC13	mESC eight-cell chip		
44	mESC14	mESC eight-cell chip	Discarded	
45	mESC15	mESC eight-cell chip		
46	mESC16	mESC eight-cell chip		
47	mESC17	mESC eight-cell chip		
48	mESC18	mESC eight-cell chip		

Table S1. Cont.

Sample ID	Sample name	Description	Notes	Spike-in dilution (ERCC/Cre)
49	mESC19	mESC eight-cell chip		
50	mESC20	mESC eight-cell chip		
51	mESC21	mESC eight-cell chip	Discarded	
52	mESC22	mESC eight-cell chip		
53	mESC23	mESC eight-cell chip		
54	mESC24	mESC eight-cell chip		
55	mESC25	mESC eight-cell chip		
56	mESC26	mESC eight-cell chip		
57	mESC27	mESC eight-cell chip		
58	mESC28	mESC eight-cell chip		
59	mESC29	mESC eight-cell chip	Negative control	
60	mESC30	mESC eight-cell chip		
61	mESC31	mESC eight-cell chip	Discarded	
62	mESC32	mESC eight-cell chip		
63	mESC33	mESC eight-cell chip		
64	mESC34	mESC eight-cell chip		
65	mESC35	mESC eight-cell chip		
66	mESC36	mESC eight-cell chip		
67	mESC37	mESC eight-cell chip	Discarded	200:1/60,000:1
68	mESC38	mESC eight-cell chip		200:1/60,000:1
69	mESC39	mESC eight-cell chip		200:1/60,000:1
70	mESC40	mESC eight-cell chip		200:1/60,000:1
71	mESC41	mESC eight-cell chip		200:1/60,000:1
72	mESC42	mESC eight-cell chip	Negative control	200:1/60,000:1
73	mESC43	mESC eight-cell chip	Negative control	200:1/60,000:1
74	mESC44	mESC eight-cell chip	Discarded	200:1/60,000:1
75	mESC45	mESC eight-cell chip		1,000:1/300,000:1
76	mESC46	mESC eight-cell chip		1,000:1/300,000:1
77	mESC47	mESC eight-cell chip		1,000:1/300,000:1
78	mESC48	mESC eight-cell chip		1,000:1/300,000:1
79	mESC49	mESC eight-cell chip	Negative control	1,000:1/300,000:1
80	mESC50	mESC eight-cell chip	Discarded	1,000:1/300,000:1
81	mESC51	mESC eight-cell chip		1,000:1/300,000:1
82	mESC52	mESC eight-cell chip		1,000:1/300,000:1
83	mESC53	mESC eight-cell chip	Discarded	1,000:1/300,000:1
84	mESC54	mESC eight-cell chip		1,000:1/300,000:1
85	mESC55	mESC eight-cell chip		1,000:1/300,000:1
86	mESC56	mESC eight-cell chip		1,000:1/300,000:1
87	mESC57	mESC eight-cell chip	Negative control	1,000:1/300,000:1
88	mESC58	mESC eight-cell chip	Negative control	1,000:1/300,000:1
89	mESC59	mESC eight-cell chip	*	1,000:1/300,000:1
90	mESC60	mESC eight-cell chip	*	1,000:1/300,000:1
91	mESC61	mESC eight-cell chip	Discarded	1,000:1/300,000:1
92	mESC62	mESC eight-cell chip	Discarded	1,000:1/300,000:1
93	mESC63	mESC eight-cell chip	*	1,000:1/300,000:1
94	MEF1	MEF eight-cell chip		1,000:1/300,000:1
95	MEF2	MEF eight-cell chip		1,000:1/300,000:1
96	MEF3	MEF eight-cell chip		1,000:1/300,000:1
97	MEF4	MEF eight-cell chip		1,000:1/300,000:1
98	MEF5	MEF eight-cell chip		1,000:1/300,000:1

Table S1. Cont.

Sample ID	Sample name	Description	Notes	Spike-in dilution (ERCC/Cre)
99	MEF6	MEF eight-cell chip		1,000:1/300,000:1
100	MEF7	MEF eight-cell chip	Negative control	1,000:1/300,000:1
101	MEF8	MEF eight-cell chip	Negative control	1,000:1/300,000:1

Samples separated with and without gray shading represent experiments prepared on the same device. Samples marked with a (*) showed an abnormal gene expression profile and were excluded from the ensemble analysis. ERCC, External RNA Controls Consortium.

Table S2. Gene ontology analysis of mESCs and MEFs

Gene ontology ID	Gene ontology term	*P* value
Enriched in mESCs		
0019827	Stem cell maintenance	0.011
0048864	Stem cell development	0.012
0048863	Stem cell differentiation	0.022
0007281	Germ cell development	0.038
0001701	In utero embryonic development	0.047
Enriched in MEFs		
0001568	Blood vessel development	7.7×10^{-9}
0007155	Cell adhesion	1.7×10^{-8}
0008083	Growth factor activity	1.8×10^{-7}
0001558	Regulation of cell growth	1.1×10^{-6}
0051270	Regulation of cell motion	5.4×10^{-5}
0042692	Muscle cell differentiation	1.4×10^{-5}
0015629	Actin cytoskeleton	5.0×10^{-5}
0005581	Collagen	7.8×10^{-5}
0001501	Skeletal system development	2.8×10^{-4}

Other Supporting Information Files

Dataset S1 (XLSX)