Reports

Acoustic microstreaming increases the efficiency of reverse transcription reactions comprising single-cell quantities of RNA

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Correlating gene expression with behavior at the single-cell level is difficult, largely because the small amount of available mRNA (<1 pg) degrades before it can be reverse transcribed into a more stable cDNA copy. This study tested the capacity for a novel acoustic microstreaming method ("micromixing"), which stirs fluid at microliter scales, to improve cDNA yields from reverse transcription (RT) reactions comprising single-cell quantities of RNA. Micromixing significantly decreased the number of qPCR cycles to detect cDNA representing mRNA for hypoxanthine phosphoribosyl-transferase (Hprt) and nuclear receptor–related 1 (Nurr1) by ~9 and ~15 cycles, respectively. The improvement was equivalent to performing RT with 10- to 100-fold more cDNA in the absence of micromixing. Micromixing enabled reliable detection of the otherwise undetectable, low-abundance transcript, Nurr1. It was most effective when RNA concentrations were low (0.1–1 pg/µL, a "single-cell equivalent") but had lesser effects at higher RNA concentrations (~1 ng/µL). This was supported by imaging experiments showing that micromixing improved mixing of a low concentration (20 pg/µL) of fluorescence-labeled RNA but not a higher concentration (1 ng/µL). We conclude that micromixing significantly increases RT yields obtainable from single-cell quantities of RNA.

Understanding the molecular basis of cell biology usually begins by correlating gene expression with cell behavior or function. Unfortunately, current technologies for quantifying gene expression, particularly where large numbers of genes are assessed simultaneously, require large amounts of RNA to be extracted from many cells (e.g., 1–0.1 µg total RNA or RNA from ~50,000 cells is recommended for Affymetrix whole-transcript expression analysis; http://media.affymetrix.com/support/technical/appnotes/wt_appnote.pdf) (Santa Clara, CA, USA). The resulting gene expression profile represents an average across a very large number of different cell behaviors, and correlating gene expression back to the behavior of any particular cell is thereby problematic. A way around this is to "purify" the cell sample using, for example, laser-capture microdissection (LCM) or fluorescence-activated cell sorting (FACS). However, these techniques are expensive and time-consuming, exhibit a degree of contamination with other cells, and—in the case of FACS—may affect gene expression.

Ideally, gene expression would be measured in a single cell. Although this can be achieved (1–4), the data are largely qualitative and only a small number of genes can be assessed per cell. This is due to the limiting amount of mRNA that can be harvested from a single cell (<1 pg). Essentially, the tiny amount of input mRNA from a single cell degrades before it can be converted into a stable cDNA copy by the RT reaction, the efficacy of which rapidly diminishes as RNA concentrations decrease (5,6). One way to increase the cDNA yield from single-cell RT reactions would be to increase the rates of interaction between the input mRNA and RT reagents through better mixing.

Microliter volumes of solution are not easily mixed using standard methods such as shaking, triturating, or vortexing because they fail to produce turbulence at very small length scales (7,8), so that small volumes must be stirred artificially (8–10). Recently, a method of acoustic microstreaming has been developed, which uses sound waves in the audible spectrum to rapidly and effectively mix two or more solutions together

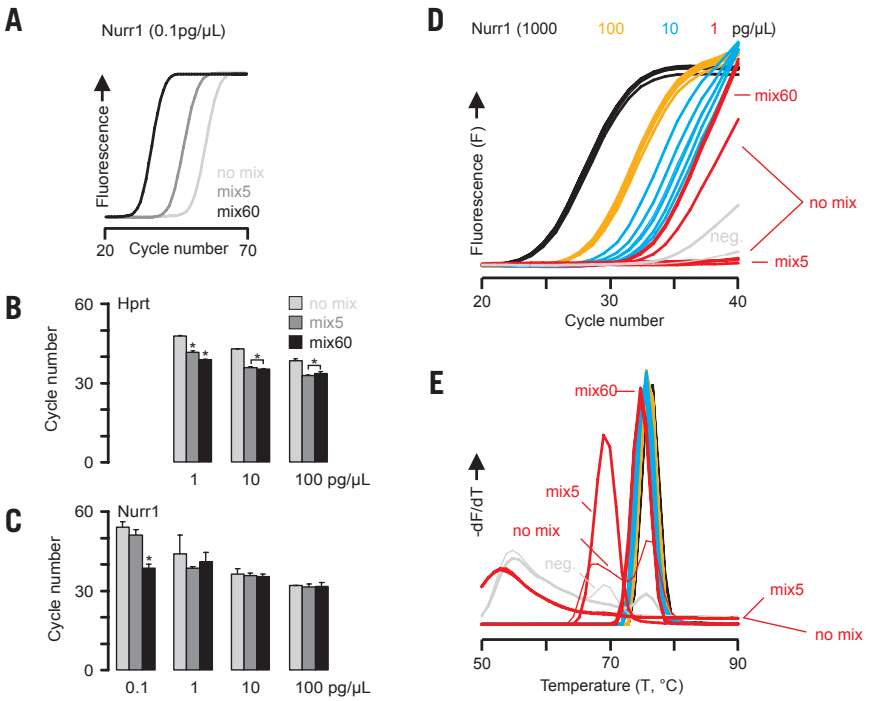

Figure 1. Effects of micromixing during 25 µL RT reactions on subsequent real-time quantitative PCR (qPCR). (A) Example qPCR traces detecting Nurr1 cDNA following three RT reactions comprising a "single cell–equivalent" concentration of RNA (0.1 pg/µL). "No mix," previous RT reactions mixed via trituration or repeated aspiration of the reaction into and out of a pipet, without micromixing; "mix5," micromixing for the initial 5 min of RT; "mix60," micromixing for the entire 60 min of RT. (B and C) Mean ± SEM number of qPCR cycles to reach 50% maximum fluorescence for detection of Hprt (panel B, $n = 2$ experiments) and Nurr1 (panel C, $n = 3$ experiments) as a function of RNA concentrations in prior RT reactions that were mixed as indicated. Asterisks denote significant ($P < 0.05$) differences compared with the no mix samples at the same concentration (Tukey multiple comparisons test). (D) qPCR traces detecting Nurr1 cDNA following RT reactions comprising different RNA concentrations [1000 (black), 100 (yellow), 10 (blue), and 1 pg/µL (red)]. (E) Melting curve analyses of qPCR products from the experiment shown in panel D.

in volumes of ~10–100 µL (8) (US Patent No. 20090034360). The aim of the present study was to determine whether acoustic microstreaming—hereafter also referred to as "micromixing"—can increase the yield of cDNA obtainable from RT reactions performed in conventional laboratory volumes (microliters), particularly those comprising amounts of RNA equivalent to that present in a single cell (hereafter referred to as a "single-cell equivalent").

Materials and methods

Acoustic microstreaming

As detailed elsewhere (8,11) acoustic microstreaming is a phenomenon where sound waves propagating around a small obstacle create a mean flow near the obstacle. Here we present an acoustic microstreaming–based device (Supplementary Figure S1) with a key simplification: acoustic microstreaming can be achieved at audio frequencies by ensuring the system has a liquid-air interface with a small radius of curvature, causing the entire drop to oscillate (8). The meniscus of a drop in a small well or vial provided an appropriately small radius. Most significantly, existing PCR vials or other standard laboratory consumables can be utilized. A frequency of 150 Hz was selected. In previous work, 150 Hz resulted in a simple vortex pattern in an open well (8). In the present work, it was found that a vortex was also created inside PCR vials.

Reverse transcription (RT)

Total RNA was isolated from snap frozen acutely prepared adult mouse midbrain slices using the PicoPure RNA Isolation Kit (Arcturus, CA, USA) and DNase treated according to the manufacturer's instructions. The concentration of this "stock" RNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). In a sterile, nuclease-free 200-µL thin-walled PCR tube, 0.5 µg random hexamer primers (Promega, Madison, WI, USA) was added to serial dilutions of the RNA stock in a total volume of 11 µL and heated to 70°C for 5 min. The tube was chilled on ice for 5 min and centrifuged briefly. After adding RNA, AMV reverse transcriptase (30 U; Promega), 5× reaction buffer, dNTP mix (1 mM final), RNasin RNase inhibitor (40 U; Promega), the RT mix was incubated for 60 min at 37°C, with or without micromixing.

qPCR

After reverse transcription, specific cDNA products were quantified using a Corbett Rotor-gene RG-3000 qPCR machine (Corbett Research, Sydney, Australia). The final qPCR reaction mix consisted of 1 µL RT mix, 0.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 1× Gold PCR buffer, 200 µM dNTPs, 1 mM $Mg(OAc)_2$, 5× SYBR Green (Invitrogen, Victoria, Australia), 5 µM specific primers (see Supplementary Materials), in a total volume of 20 µL. After the 5-min, 95°C hot start, cycling parameters were denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension and signal acquisition at 72°C for 20 s.

Optical detection and quantification of micromixing of single-cell quantities of RNA

After DNase I treatment, 1 µg isolated total RNA was fluorescent-labeled by coupling Alexa Fluor 488 to the purine bases using a ULYSIS Nucleic Acid Labeling Kit (Molecular Probes, Eugene, OR, USA). Briefly, the ethanol-precipitated (1/10 volume of 3 M sodium acetate and 2× volumes of ethanol) total RNA was resuspended in 24 µL ULS labeling buffer and denatured at 95° for 5 min. After chilling on ice, 1 µL labeling reagent was added, and then the reaction was incubated at 90°C for 10 min. The reaction was stopped by plunging the reaction tube into an ice bath. Uncoupled fluorophore was removed by passing the reaction mix through a Sephadex gel G-100 column (Sigma-Aldrich, St. Louis, MO, USA) by centrifugation at 1300× *g*. The recovered RNA was diluted to 1 ng/µL by RNase and DNase free water.

For the higher concentration of RNA (1 ng/µL) tested, an intensified CCD (ICCD) camera (PI-MAX, Princeton Instruments, Trenton, NJ, USA) was used to visualize the mixing. The ICCD camera software provided the grayscale mean and SD of each frame taken. However, the ICCD camera was not sensitive enough to enable visualization of the lower RNA concentration (20 pg/µL) tested. For the lower RNA concentration, a 488-nm Sapphire Laser (Coherent, Inc., Santa Clara, USA) was used for excitation of fluorescence-labeled

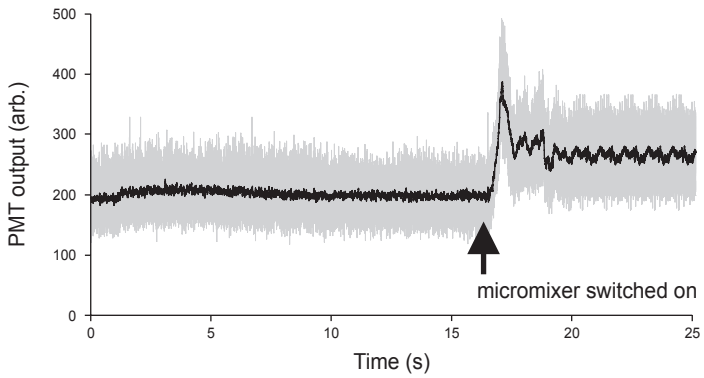

Figure 2. Effects of acoustic microstreaming on mixing of 20 pg/µL RNA in DI water. Without micromixing, RNA relies on diffusion only; the mixing process is slow and PMT output (gray line) is stable. As soon as the micromixing is switched on, RNA mixes quickly with DI water. The PMT average signal output (black line, 10-point average) increased, indicating rapid transport of RNA from the top to the bottom of the mixing chamber.

RNA. The emission light was collected with a photon multiplying tube (PMT; Hamamatsu Photonics, Hamamatsu City, Japan) which was focused at the bottom of the mixing chamber. Data from the PMT was logged with a M8784 counting board (Hamamatsu Photonics) and a PCI bus add-in board.

Results and discussion

RT reactions were performed in standard 0.2-mL PCR tubes with RNA concentrations ranging from 1 ng/µL to 0.1 pg/µL in a total reaction volume of 25 µL. At the lower end of this range, the RT reaction mix contains a single cell–equivalent amount of RNA. Two different micromixing protocols were tested: micromixing for only the initial 5 min of the 60-min reaction ("mix5") and micromixing throughout the entire reaction ("mix60"). We also compared against the absence of micromixing ("no mix") in which standard laboratory mixing (trituration of the sample by repeated aspiration into and out of a pipet) was performed. The cDNA yield from these RT reactions was assessed by performing qPCR using primers designed to amplify two test genes expressed by dopaminergic (DA) neurons of the midbrain: hypoxanthine phosphoribosyl-transferase (Hprt), a commonly measured "housekeeping" gene present at relatively low levels (low abundance) and constant amounts in all cell types, and the orphan nuclear receptor–related 1 protein (Nurr1), a low-abundance transcription factor present in adult midbrain DA neurons.

The number of qPCR cycles required to reach detection threshold (arbitrarily defined as 50% maximum fluorescence in this study) for both Hprt (Figure 1B) and Nurr1 (Figure 1C) was significantly reduced when micromixing was applied during the RT reaction ($P < 0.001$ for Hprt and $P = 0.03$ for Nurr1, two-way ANOVAs). Thus micromixing offers improvement above and beyond current standard laboratory practice. This implies micromixing increased the amount of RT reaction product (cDNA) and therefore improved RT reaction efficiency. The degree of improvement was dependent on RNA concentration (see below), but was maximal (on average) at ~9 qPCR cycles for Hprt (at 10 pg/µL, Figure 1B) and ~15 cycles for Nurr1 (at 0.1 pg/µL, Figure 1, A and C). The extent of this improvement in terms of cDNA quantity cannot be determined because we did not establish standard curves for these qPCR reactions. However, when the yields from different concentrations of RNA are compared (Figure 1, B and C) it is apparent that, in the absence of micromixing, ~100-fold more RNA was required to produce qPCR signals equivalent to those obtained when reactions were micromixed. In other words, for Hprt, RT of 100 pg/µL RNA without micromixing resulted in a qPCR signal equivalent to RT of 1 pg/µL RNA with micromixing (Figure 1B), and for Nurr1, RT of 10 pg/µL RNA without micromixing resulted in a qPCR signal equivalent to RT of 0.1 pg/µL RNA with micromixing (Figure 1C). Therefore we conclude that micromixing results in a ~100-fold increase in cDNA product.

The effects of micromixing depended on RNA concentration, with low, single-cell equivalent concentrations benefiting significantly more than higher concentrations (Figure 1, B–D). In the case of Hprt (Figure 1B), micromixing for 60 min provided a better yield from the lowest RNA concentration (1 pg/µL) than micromixing for 5 min, whereas micromixing for longer periods had no benefit for the two higher RNA concentrations ($P = 0.008$, two-way ANOVA). Although the interaction between concentration and treatment did not quite reach statistical significance for Nurr1 (Figure 1C; $P = 0.052$, two-way ANOVA), there did appear to be a benefit in micromixing the lowest RNA concentration (0.1 pg/µL) for a longer period. The same conclusion can be drawn from the data in Figure 1D, which illustrates 6 qPCR runs for Nurr1 cDNA detection at each of four different RNA concentrations (1 ng/µL, black traces; 100 pg/µL, yellow traces; 10 pg/µL, blue traces; 1 pg/µL, red traces). Duplicate samples were run under each of the 3 different treatments (no mix, mix5, and mix60) at each concentration (i.e., 6 traces/concentration). At the two highest concentrations (black and yellow traces) micromixing made no or very little difference to the signal. At the next lowest concentration, micromixing began to make a difference (10 pg/µL, blue traces), and micromixing made its greatest contribution at the lowest concentration (1 pg/µL, red traces). When 1-pg/µL samples (red traces) were micromixed throughout the entire RT reaction (mix60), fewer cycles were required to reach the detection threshold (50% maximum fluorescence) than unmixed samples or samples micromixed for 5 min, some of which were undetectable after 40 cycles. Moreover, melting curve analysis of the

qPCR products from the 1-pg/µL samples revealed that only those micromixed for 60 min (red mix60 traces in Figure 1E) yielded the appropriate product (i.e., an amplicon identical to that obtained in all the higher concentration samples). By contrast, the remaining 1-pg/µL samples (no mix and mix5), as well as the negative controls (gray traces), contained alternative amplicon products (i.e., false positives such as primer dimers). In other words, micromixing throughout the entire RT reaction permitted detection of an otherwise undetectable transcript from single cell–equivalent quantities of RNA.

Optical detection and quantification of micromixing of single-cell quantities of RNA

The above experiments led us to conclude that micromixing has beneficial effects at low RNA concentrations (a single-cell equivalent), but insignificant effects at higher RNA concentrations. The purpose of the next experiment was to visualize the RNA during the micromixing process to see whether it improved mixing of RNA and RT reagents, particularly at low RNA concentrations. If so, this was the likely source of the improvement seen at the qPCR level.

To do this RNA was fluorescence-labeled and imaged before, during, and after micromixing. Note that 25 µL deionized (DI) water was used to model the RT reaction mixture in these experiments. Although some components of the RT reaction mixture have higher viscosity, their concentration in the RT reaction mixture is small so this difference is unlikely to alter the conclusion drawn. At a higher RNA concentration (1 ng/µL), the RNA moved very slowly with the vortex once the micromixer was switched on. The SD of the image intensity did not change significantly before and after the micromixer was switched on. In contrast, at a much lower RNA concentration (20 pg/µL), micromixing produced a much faster and thorough mixing of the labeled RNA. The PMT was focused to collect the light from the bottom of the mixing chamber. It initially recorded some light emitted from the 1-µL drop of 20 pg/µL RNA placed on the top of the mixing chamber. With the micromixer switched off, the PMT output remained unchanged for 5 min (Figure 2). This indicates that diffusion alone is very slow, causing negligible transport of the introduced RNA from the top to the bottom of the mixing chamber over 5 min. However, as soon as the micromixer was switched on, the PMT output increased, indicating rapid transport of RNA from the top to the bottom of the mixing chamber (Figure 2). The labeled RNA then appeared to mix with the DI water, evidenced by an irregular and gradually reducing signal from the PMT. This is typical of any system in which a small quantity of introduced tracer abruptly begins mixing into a larger vessel (12). Decay of the irregularities and establishment of a new, stable output level, indicating mixed products, appeared to take ~3 s.

In conclusion, this study demonstrates that micromixing during RT reactions comprising low or single cell–equivalent amounts of RNA in small but conventional laboratory reaction volumes (25 µL) significantly enhances qPCR detection of two low-abundance mRNA transcripts (Hprt and Nurr1). This implies that micromixing increases the efficiency of such RT reactions over and above what can be achieved using standard laboratory mixing techniques (e.g., trituration), presumably by increasing the rates of interactions between reagents (mRNA, primers, reverse-transcriptase, and dNTPs). It is now well established that the quantity and quality of cDNA reverse transcribed from single-cell quantities of mRNA are greatly increased when RT is performed in nanoliter volumes using microfluidic technologies (13–16). Although there are many circumstances in which microfluidics or self-contained lab-on-a-chip devices performing RT or PCR would be a great advance, in other circumstances, including our own, micromixing alone provides significant and adequate benefit without the need for further specialized equipment and methods. Micromixing therefore provides a novel, simple, cheap, and easily implemented alternative to microfluidics-based approaches to enhance cDNA yield from single-cell amounts of RNA.

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Competing interests

The authors declare no competing interests.

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