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A microfluidic approach to parallelized transcriptional profiling of single cells

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Abstract

The ability to correlate single-cell genetic information with cellular phenotypes is of great importance to biology and medicine, as it holds the potential to gain insight into disease pathways that is unavailable from ensemble measurements. We present a microfluidic approach to parallelized, rapid, quantitative analysis of messenger RNA from single cells via RT-qPCR. The approach leverages an array of single-cell RT-qPCR analysis units formed by a set of parallel microchannels concurrently controlled by elastomeric pneumatic valves, thereby enabling parallelized handling and processing of single cells in a drastically simplified operation procedure using a relatively small number of microvalves. All steps for single-cell RT-qPCR, including cell isolation and immobilization, cell lysis, mRNA purification, reverse transcription and qPCR, are integrated on a single chip, eliminating the need for off-chip manual cell and reagent transfer and qPCR amplification as commonly used in existing approaches. Additionally, the approach incorporates optically transparent microfluidic components to allow monitoring of single-cell trapping without the need for molecular labeling that can potentially alter the targeted gene expression and utilizes a polycarbonate film as a barrier against evaporation to minimize the loss of reagents at elevated temperatures during the analysis. We demonstrate the utility of the approach by the transcriptional profiling for the induction of the cyclin-dependent kinase inhibitor 1a and the glyceraldehyde 3-phosphate dehydrogenase in single cells from the MCF-7 breast cancer cell line. Furthermore, the methyl methanesulfonate is employed to allow measurement of the expression of the genes in individual cells responding to a genotoxic stress.

Keywords

Single-cell analysis; Microfluidics; Integrated transcriptional profiling; Parallelized RT-qPCR

1 Introduction

The ability to correlate genetic information with cellular phenotypes holds the potential to reveal disease pathways and has significant implications for biology and medical science. Traditionally, gene expression levels are measured by population-averaged assays in which millions of cells are lysed, and then, the released RNA is homogenized for downstream testing. However, most biological samples are heterogeneous as a result of the presence of multiple cell types, or differences in cell division cycle phases, extracellular environment, epigenetic regulation and the inherently stochastic nature of gene expression. Averaging over populations hence typically masks the variation in gene expression between cells, and population-averaged methods are insufficient for understanding the topology and dynamics of gene regulatory networks (Chattopadhyay et al. 2014). Single-cell transcriptional profiling involves measuring transcript levels in individual cells and is capable of resolving the interaction between intracellular processes and extrinsic stimuli (Levesque and Raj 2013; Klemm et al. 2014), providing insight into variations among cells in heterogeneous populations as ensemble measurements of cells can fail to detect differences in the molecular composition of individual cells. Existing methods for single-cell transcript measurements include RNA fluorescence in situ hybridization (FISH) (Lubeck et al. 2014), emulsion-based methods (Zhang et al. 2012; Ludlow et al. 2014) and in particular, quantitative reverse transcription polymerase chain reaction (RT-qPCR) (Bustin et al. 2009; Ståhlberg et al. 2013).

Microfluidic technology has been increasingly used for biochemical synthesis and analysis, allowing for automation, integration and parallelization (Sun and Morgan 2010; Kulkarni et al. 2014). In particular, RT-qPCR of single cells in microfluidic systems has involved on-chip integration of various analytical functions such as single-cell isolation and lysis (Nagrath et al. 2007; Stott et al. 2010), RNA purification (Dimov et al. 2008) and reverse transcription (Toriello et al. 2008; Han et al. 2014) and have been performed in a parallel fashion to achieve improved throughput, which is important for analysis of a large number of single cells (Eastburn et al. 2013; Rival et al. 2014; Kim et al. 2014; Yu et al. 2014; Thege et al. 2014). Polydimethylsiloxane (PDMS) microchips have been developed in which single cells were trapped by microwells (Kim et al. 2014), isolated in valve-controlled chambers (Yu et al. 2014) or encapsulated in droplets (Eastburn et al. 2013), followed by downstream-parallelized genetic analysis at the single-cell level. Alternatively, silicon-micromachined devices have been presented for single-cell gene expression analysis in which cells were fluorescently labeled (Rival et al. 2014; Thege et al. 2014) and immobilized in microwells or on antibody-coated microposts prior to genetic assays. These methods require a large number of functional units (i.e., multiple microwells, chambers or droplet processing units) to complete biochemical reactions on-chip, resulting in complex device designs and complicated off-chip control instrumentation and operation procedures. Additionally, the silicon devices are unable to lyse individual cells on-chip and, being composed of opaque materials (Rival et al. 2014; Thege et al. 2014), require potentially cell viability compromising labeling for accurate on-chip single-cell processing. Furthermore, these existing parallelized approaches only allow partial integration of the RT-qPCR process

(Wang et al. 2012; de Bourcy et al. 2014; Cheow et al. 2015) and still require considerable off-chip manipulation and manual handling of samples.

We have recently developed a technique to integrate trapping, lysis and RT-qPCR of a single cell on a micro-chip (Sun et al. 2015). Here, we build on this technique and present a microfluidic approach to rapid, parallelized, quantitative analysis of mRNA from single cells and apply this approach to measure the effects of genotoxicity on transcription profiles. The approach accomplishes arrayed RT-qPCR analysis of single cells by leveraging concurrent control of a set of parallel microchannels. Combined with bead-based reactions, this enables parallelized handling and processing of single cells in a drastically simplified operation procedure, using a significantly smaller number of microvalves and microchambers as compared with existing microfluidic methods for arrayed single-cell RT-qPCR. All steps for single-cell RT-qPCR, including cell isolation and immobilization, cell lysis, mRNA purification, reverse transcription and real-time PCR, are integrated on a single chip, eliminating the need for off-chip manual cell and reagent transfer and qPCR amplification. This high level of integration, which is highly desirable (Volpatti and Yetisen 2014) but has not yet been possible with existing microfluidic single-cell gene expression profiling methods (White et al. 2011; Taylor 2013), minimizes the risk of sample contamination and reduces time required to trap and lyse single cells (to maintain cell viability and avoid unintended changes in gene expression). Furthermore, rather than requiring the use of fluorescent labels (Rival et al. 2014; Thege et al. 2014), our approach allows label-free trapping of single cells prior to their lysis and analysis, which reduces the complexity of the protocol and the risk of gene expression changes induced by cell labeling.

We demonstrate our approach using a microfluidic device consisting of an array of six single-cell analysis units for use in the analysis of the genotoxic effects of the drug methyl methanesulfonate (MMS) on the induction of the gene cyclin-dependent kinase inhibitor 1a (CDKN1A) and housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in single cells from the MCF-7 breast cancer cell line. This device allowed single cells to be trapped and lysed in each unit within 10 min and analyzed via RT-qPCR within 2.5 h. By its use in the measurements of differences in the upregulation of CDKN1A and GAPDH in single cells exposed to MMS at dosing concentrations from 30 to 150 $\mu\text{g/mL}$ with treatment times from 0.5 to 4.5 h, the device demonstrates the potential of our microfluidic approach for genotoxicity studies in single cells.

2 Device design and operation

2.1 Design

To demonstrate our parallelized transcriptional profiling approach, we use a device consisting of six parallel channels, which, each serving as a single-cell RT-qPCR analysis unit, are connected to a single main inlet and main outlet. The RT-qPCR units of the device are identical in design, and each consists of a cell trap, a buffer outlet, a cell outlet and an elliptically shaped RT-qPCR microchamber. The cell trap is a neck-shaped flow constriction formed by a protruding microstructure in the channel between two valves (Fig. 1). The RT-qPCR chambers each have a poly-ethylene membrane embedded in the ceiling, which serves as a barrier to minimize evaporation and associated reagent loss during thermal cycling. A

thin-film resistive heater and a thin-film temperature sensor integrated on the device substrate allow all of the RT-qPCR chambers to be thermal cycled simultaneously. Eight individually pressurized elastomeric binary valves (Araci and Quake 2012) arranged in a combinatorial array achieve concurrent control of the parallel channels, allowing precise control of flow within a selected RT-qPCR analysis unit while preventing flow in the other five analysis units during single-cell trapping and lysis. As such, six single cells can be trapped and lysed successively in the individual units and then analyzed in parallel via RT-qPCR.

2.2 Operation

During the operation of the array chip, a single analysis unit is selected through the multiplexed valves, and then, 4×10^6 magnetic beads are introduced (100 nL/s for 20 s) until all analysis units have beads with a syringe pump while an external magnet is held under the chambers. The amount of beads is determined according to the manufacturer's reported bead density and verified by pipetting beads onto a glass slide and counting the total amount of beads with ImageJ software. Next, a single analysis unit is reselected with the multiplexed valves, and 1.25 μ L of cell-containing carrier buffer is introduced (concentration: 10^5 cells/mL, flow rate: 15 nL/s and infusion time: 83.3 s) into the microfluidic device and travel through the cell trap with the first cell to reach the trapping structure becoming trapped. The width of the constriction (5 μ m) is smaller than the diameter of the cells (18 ± 2 μ m), and therefore, the single cell can be immobilized in the trap.

Once a single cell becomes immobilized, the back control valve is opened to allow carrier fluid, potentially containing additional cells, to bypass the trapping region and to flow directly to the outlet. To further ensure that no cells remain in the outlet well, buffer is injected into the inlet well with a syringe with a 25-gauge stainless steel needle. Lysis buffer is introduced into the cell trap region of each analysis unit at a flow rate of 2 μ L/min over a 5-min period to lyse the trapped cell (Sun et al. 2015). After cell lysis, the beads were mixed with the cell lysate by manually moving the magnet and captured the mRNA in the cell content, based on complimentary base pairing between the polyA tails of the mRNA and the oligo(dT)₂₅ residues covalently coupled to the bead surfaces. Potential cross talk between different analysis units (i.e., unwanted micro-bead movements in chambers not active in manipulation at a given time) was prevented by closing the associated valves and placement of a second, larger magnet above the inactive chambers as an added precaution. The beads were moved into the active chamber and mixed with the sample (mRNA in cell lysate or XenoRNA) by magnet-driven motion. This process is then repeated for the remaining units of the array chip until five of the RT-qPCR chambers each contain beads with bound mRNA. The sixth chamber provides a no-template control. Operation of a single unit is shown in Fig. 2a.

Reverse transcription is performed in all of the six chambers to convert the mRNA into cDNA, using the bead-bound oligo(dT)₂₅ as a primer and with the chamber temperatures controlled simultaneously in closed-loop by the integrated heater and temperature sensor (RT: 10 min at 25 °C and 50 min at 42 °C). The resulting cDNA template for PCR is amplified (qPCR: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at

60 °C) while the accumulation of products is imaged on a fluorescence microscope using a hydrolysis probe/primer set (TaqMan®). The reagent probe/primer consists of a fluorescein amidite (FAM) reporter dye, a minor groove binder and a nonfluorescent quencher (NFQ). Initially, the fluorescence of FAM is suppressed by NFQ. During the PCR annealing process, the probe binds to a complementary region of the target template. Then, during the elongation process that follows, NFQ is cleaved from the probe, causing the fluorescent intensity of FAM to increase. To correct for fluorescent fluctuations due to batch-to-batch changes in cavity volume and PCR component concentrations, a passive reference dye (ROX) is employed to normalize the FAM signal during real-time measurements. Fluorescent intensities of ROX and FAM are acquired and measured at the end of each PCR cycle (Fig. 2b).

3 Experimental

3.1 Materials

The MCF-7 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Minimum essential medium (MEM), fetal bovine serum (FBS), penicillin–streptomycin (P/S, penicillin 10⁴ unit/mL, streptomycin 10⁴ mg/mL), 0.25 % trypsin–ethylenediamine-tetraacetic acid (EDTA), Dulbecco's Phosphate-Buffered Saline (D-PBS), cell lysis buffer (100 mM Tris–HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, 1 % LiDS, 5 mM dithiothreitol), Vybrant Multicolor Cell Labeling Kit (DiI, DiO and DiD), Dynabeads® mRNA kit, TaqMan® reverse transcription reagents, oligo(dT)₁₆ (50 µM) primer, CDKN1A and GAPDH primer/probe set (product number: Hs99999142_m1 and Hs99999905_m1) XenoRNA control (10⁵ copies/µl), TaqMan® Gene Expression Master Mix, thin-walled RNase-free PCR tubes (0.2 mL), RNase-free water, RNaseZap wipes and MicroAmp® optical adhesive film for the evaporation barrier were purchased from Thermo Fisher Scientific Inc. (Grand Island, NY). MMS (~99 %) and bovine serum albumin (BSA, 98 %) were obtained from Sigma-Aldrich (St. Louis, MO).

3.2 Microchip fabrication

The microchip was fabricated using standard microfabrication techniques (Sun et al. 2015). Chrome (99.9 %) and gold (99.99 %) were deposited and patterned onto a glass substrate. AZ 4620 and SU-8 photoresist were patterned on a wafer to form the molds of the flow layer and the pneumatic control layer. A vapor barrier was embedded on top of the reaction chamber. A featureless thin film was bonded to the surface of the control layer and together bonded with the flow layer. Finally, the PDMS device was bonded to the heater, resulting in a packaged device. Prior to each test, the device was incubated in BSA for 5 min, and the beads were washed and were injected into the reaction microchamber. More details of the fabrication process are described in the Supplementary Material.

3.3 Experimental setup

During experiments, the fabricated device was connected to a nitrogen tank with a pressure regulator (Concoa, Virginia Beach, VA), which controlled the pneumatic microvalves, and a digital multimeter (34420A, Agilent Technologies Inc., CA) and DC power supply (E3631, Agilent Technologies Inc., CA), which controlled the RT-qPCR reaction chamber

temperature. A syringe pump (New Era Pump Systems, Inc., Farmingdale, NY) was used to introduce cells, washing buffer, lysis buffer, RT reagents and qPCR reagents into the device. An epifluorescence microscope (IX71, Olympus, Center Valley, PA) was used to collect real-time data (Fig. 3) (Sun et al. 2015). The digital multimeter and DC power supply were connected to a personal computer to allow on-chip temperature monitoring and control through a proportional-integral-derivative (PID) algorithm implemented in LabVIEW (National Instruments Corp., TX). Each analysis unit was photographed at the completion of every PCR cycle with a CCD camera (c8484, Hamamatsu, Boston, MA) connected to the epifluorescence microscope.

4 Results and discussion

We first characterized on-chip thermal control (Supplementary Material). Then, we analyzed the capture capacity of the beads and validated the on-chip RT-PCR using synthetically homogenized XenoRNA. Next, we evaluated the on-chip RT-PCR, including the consistency of the approach, and compared it with the traditional in-tube-based RT-PCR using XenoRNA template. Finally, we used the device for single-cell RT-qPCR and studied the effects of the drug MMS on the induction of CDKN1A and the housekeeping gene GAPDH in single MCF-7 cells. Particularly, all steps for single-cell RT-qPCR, including cell isolation and immobilization, cell lysis, mRNA purification, reverse transcription and real-time PCR, are integrated on a single chip, completely eliminating the need for off-chip manual cell and reagent transfer and qPCR amplification as commonly used in existing approaches.

4.1 Characterization of on-chip RNA capture capacity

To assess the amount of mRNA that can be captured by the bead-bound oligo(dT), we tested capture of XenoRNA by the beads because of its similar properties to mRNA but with the advantage of being able to use a known amount of RNA. As a single cell contains approximately 3.6×10^5 RNA molecules (Alberts et al. 2002), we mixed varying amounts of XenoRNA (from 5×10^4 to 4×10^5 copies) with a constant quantity of oligo(dT) beads (4×10^6 beads), which have the capacity to capture 8.42×10^8 RNA molecules according to the manufacturer, and assessed the residual templates in the binding effluent. This was performed in four separate arrayed analysis units of the device, where XenoRNA of varying copy number was injected (1.5 μ l/min) and captured by the preloaded beads. The other two analysis units were used as a positive control (containing 4×10^5 XenoRNA previously mixed with beads off-chip) and a no-template negative control (containing only buffer). After mixing the beads with XenoRNA for 5 min, we collected the binding effluent in each analysis unit. For the positive control and no-template control, the entire contents of each chamber (including the beads) were transferred to tubes in an RNase-free environment for RT (10 min at 25 °C and 50 min at 42 °C). Finally, these RT products were analyzed by an ABI H7-7900 real-time PCR system (Fig. 4a) following established protocols (10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C). It was found that the signal in these effluents and no-template control was undetectable using 4×10^6 beads while an exponential amplification of the starting templates was observed in the positive control. Thus, we can conclude that after mixing, the XenoRNA was captured by 4×10^6 beads and an undetectable amount of free RNA templates were residual in the binding waste. In

addition, the lack of amplified products in the effluent verified that there was not a significant amount of bead loss because the presence of beads in the effluent, which would have bound XenoRNA, would generate detectable amplification during qPCR.

4.2 Validation of on-chip RT-PCR of model mRNA

For these studies, a similar procedure was followed as the single-cell studies except XenoRNA was injected directly into the reaction chamber through the inlet and captured onto beads, and a hydrolysis primer/probe set was used without the need for cell trapping and cell lysis.

4.2.1 Endpoint amplification—To validate the ability of the device to perform RT-PCR, we measured the background subtracted endpoint (after 40 PCR cycles) fluorescent intensity of the reaction chamber, which was compared to a no-template control. We first injected approximately 4×10^6 beads into all analysis units and 2×10^4 copies XenoRNA in three of the analysis units. RT and PCR reagents, without any XenoRNA templates, were introduced to the other analysis units to form no-template control analysis units. Following RT and PCR, the endpoint fluorescent intensity of the amplification was measured (background subtracted) and compared to the no-template control (Fig. 4b).

For the analysis units containing XenoRNA, the fluorescent images of reporter showed much greater fluorescent intensity than the no-template control. The mean fluorescent intensity value of three XenoRNA samples following PCR was 1.9 ± 0.26 . In contrast, there was negligible amplification in the no-template control chambers (mean fluorescent intensity 0 ± 0.1), verifying that cross contamination in the microfluidic channels was negligible when using the on-chip multiplexed microfluidic control.

4.2.2 Real-time amplification—We validated the on-chip RT-qPCR and evaluated the consistency of this approach (Fig. 4c) using model RNA. In five analysis units of the microchip, we introduced 2×10^4 copies XenoRNA and 4×10^6 beads and performed RT-qPCR. In the sixth analysis unit, the same procedures and reagents were used except XenoRNA was not introduced. Fluorescent intensities of reaction chambers were acquired at the end of each PCR cycle.

In Fig. 4c, the value of R_n , indicating the magnitude of the fluorescent signals and therefore amplification generated by PCR, demonstrates an exponential increase with the cycle number. Meanwhile, the quantification cycle, C_q , was set as the cycle number at which the measured fluorescence crosses a threshold of 20σ , where σ is the standard deviation (SD) of the fluorescence intensity for the first fifteen PCR cycles. Under these conditions, the threshold value was calculated to be 0.15, and accordingly C_q was found to be 31.3 using 2×10^4 copies XenoRNA. The results in Fig. 4c demonstrate that with the same amount of homogenized starting templates, the qPCR in all five analysis units generated significant amplification at almost the same cycle number (31.3 with a SD of 0.1). Based on this, we can conclude that for the same quantity of starting templates, the amplification in the separate array units was consistent. Furthermore, the consistency of normalized fluorescent intensity (R_n) values during the whole qPCR process implies that the reagent concentrations were stable throughout the process. Therefore, the reagent absorption and

evaporation during the thermal cycling process in the reaction chambers, which would affect the concentrations of the reagents and thus the fluorescent intensity of reporter dye (FAM), were effectively inhibited. These results served to verify the proper function of the device for integrated RT-qPCR and to the consistency among the individual units.

4.2.3 Comparison of on-chip and in-tube RT-qPCR—We compared the efficiency, sensitivity and amplification yield of our nanoliter-volume scale on-chip approach with the microliter-volume scale in-tube approach. XenoRNA with different copy numbers (from 10^4 to 10^5) was tested by in-tube and on-chip RT-qPCR, respectively, with results plotted and fitted linearly (Fig. 4d).

For on-chip RT-qPCR (black), the mean C_q values of XenoRNA with different copy numbers (from 10^4 to 10^5) were 32.6, 31.4, 30.2 and 29.1 with SD of 0.30, 0.26, 0.24 and 0.22, while the mean C_q values for in-tube tests (red) were 33.5, 32.4, 31.1 and 30.0 with SD of 0.39, 0.36, 0.35 and 0.34. Furthermore, we evaluated the PCR efficiency defined by $(10^{-1/k} - 1) \times 100\%$, where k is the slope of the C_q as a function of the logarithm of the template copy number (Fig. 4d) (Ramakers et al. 2003). The PCR efficiency for the on-chip qPCR (96.83 %) was similar to that for in-tube bead-based PCR (95.66 %). Additionally, the

R_n value after 40 cycles of qPCR increased from 1.7 to 2.5 on-chip (Fig. 4e) and from 1.6 to 2.4 (Fig. 4f) in-tube while the starting amount of XenoRNA copy numbers increased from 10^4 to 10^5 indicating increased efficiency of PCR on-chip. The lower mean C_q values (~ 1.0), the smaller C_q SDs (from 0.09 to 0.12), the comparable PCR efficiency and higher R_n (~ 0.1) value for on-chip PCR were likely attributable to the greater surface-area-to-volume ratios (which improve thermal homogeneity across the reaction volume and rapid heat transfer during RT and PCR), the smaller length scales of each analysis unit (which reduce the diffusion time and improve the mixing efficiency) and the greater degree of integration (which reduces the risk of sample loss during manual manipulation) (Wang et al. 2012; de Bourcy et al. 2014; Cheow et al. 2015).

4.3 Measurement of single-cell gene expression

Having validated the sub-functions of the device with XenoRNA, we then demonstrated the use of the device for single-cell studies.

4.3.1 Endpoint amplification—First, we validated the integrated on-chip single-cell RT-PCR by measuring the endpoint fluorescent intensity. Three analysis units (Fig. 5a) were used for cell studies, while the other three chambers were used as no-template controls. Single-cell RT-PCR for GAPDH was performed using the procedure in Sect. 3.3. The endpoint mean fluorescent intensity value of three single-cell samples was 2.5 ± 0.41 compared to 0 ± 0.1 with the no-template control. Thus, single MCF-7 cells were effectively lysed on-chip, and the released mRNA templates were successfully captured and synthesized to cDNA. In addition, the increased SD of the single-cell RT-PCR (0.41), when compared to the synthetically homogenized XenoRNA-based test (0.26), indicates that the variation in gene expression between cells is larger than that of homogenized RNA samples and suggests that the population-averaged reading of gene expression may be unable to reveal gene expression levels of individual cells.

4.3.2 Real-time amplification—To validate real-time amplification in the device, we performed on-chip qPCR analysis of single cells for GAPDH and CDKN1A expression. We first prepared two microchips, one for studying GAPDH and the other for CDKN1A. Cells were introduced to the microchips following the same protocol as Sect. 3.3 except primer/probe sets for GAPDH and CDKN1A were used. One analysis unit of each microchip was reserved as a no-template control. On-chip analysis of six cells was completed in approximately 2.5 h [compared to 9 h with existing approaches (Fluidigm Corporation 2014)]. The mean R_n values of each test were measured and plotted (Fig. 5b). The FAM and ROX images acquired with the GAPDH primer/probe around the quantification cycle are also shown (inset of Fig. 5b). As the measured signal reflects the amount of dye per unit volume, an approximately constant fluorescence signal from the ROX reference dye, as observed in our experiment, would suggest negligible evaporation-induced volume decreases. The curves show exponential amplification for the targeted strands of CDKN1A and GAPDH, and the C_q difference from these curves can be used to infer differences in initial copy amounts between two genes. For GAPDH, the mean C_q value was 32.4, and for CDKN1A, it was 33.3, which indicates that GAPDH mRNA was more abundant than CDKN1A mRNA and is consistent with and supported by existing studies (Choudhury et al. 2006).

4.4 Measurement of drug-induced single-cell gene expression

The dosage and treatment time of a drug interacting with a cell are both essential for evaluating the efficacy of the drug in drug discovery and development. Microfluidic technology has generated great interest in this field by minimizing reagent consumption and costs while increasing the performance (Yin and Marshall 2012). We demonstrate the use of our device for this purpose by measuring the regulated transcription levels after treating individual human cancer cells with an alkylating agent and investigating the effects of drug concentration and treatment time on regulation levels in CDKN1A expression.

4.4.1 Gene expression profiling of drug-treated single cells—Stress-induced gene expression in single cells was investigated by treating cells with MMS, an alkylating agent, and then analyzing them by on-chip RT-qPCR. We first measured the transcript levels of CDKN1A and the housekeeping gene GAPDH in MMS-treated (120 $\mu\text{g/mL}$ for 2.5 h) and MMS-untreated single MCF-7 cells using the micro-fluidic array.

In each test, five MMS-treated or MMS-untreated single cells were isolated and immobilized in five separate analysis units of the array, and the remaining unit was used as a no-template control. Similar to the above-mentioned protocol, after cell trapping and lysis, the two-step RT-qPCR was initialized. The fluorescent intensity was detected and assayed using hydrolysis probe/primer sets for CDKN1A and GAPDH, respectively, during the qPCR process (Fig. 6a).

The mean C_q values of CDKN1A in untreated single cells were 33.5 with a SD of 0.54 (Fig. 6b). After treatment with 120 $\mu\text{g/mL}$ of MMS for 2.5 h, the value decreased to 30.5 with a SD of 0.23. In the no-template control samples, no fluorescent signal was detected. The difference between the C_q of treated and untreated cells, C_q , was determined to be 3.0 for

CDKN1A, and for the housekeeping gene GAPDH, C_q was found to be approximately 0.5. Encoded by the CDKN1A gene, which is located on chromosome 6 (6p21.2), p21/WAF1 can bind to and inhibit CDK activity, preventing phosphorylation of critical cyclin-dependent kinase substrates and blocking cell cycle progression (Abbas and Dutta 2009). By treating the single cells with MMS, the transcript levels of the CDKN1A were upregulated significantly. Conversely, the transcript level of GAPDH has been found to be very consistent regardless of the cellular condition (Schmittgen and Zakrajsek 2000), leading to its common use as an internal control for gene expression experiments.

Furthermore, we calculated the fold change (FC) of a target gene, defined by $FC = 2^{-(C_q)}$, where $(C_q) = C_{q,stimulated} - C_{q,GAPDH}$, $C_{q,stimulated}$ is the difference in the quantification cycle for a target gene between groups of modified and unmodified cells, and $C_{q,GAPDH}$ is the difference between the quantification cycle of a control (GAPDH) of modified and unmodified cells. That is, FC is the average multiplicative amount; the gene expression has changed from unmodified cells to modified cells and is calculated by using the amount of cycles needed to generate fluorescent signal. A higher FC indicates the gene expression having undergone a greater change. According to Fig. 6a, a fold change of 5.65 was obtained when our approach was used to analyze the CDKN1A gene in a single MCF-7 cells following 2.5 h of treatment with MMS. These results compare favorably with relevant data reported for existing microfluidic devices for single-cell analysis (Dun et al. 2014) and demonstrate the ability of our approach to analyze alterations in transcript levels in single mammalian cells in a parallelized fashion.

4.4.2 Effects of drug treatment time on CDKN1A expression—We next assayed the effect of drug exposure time on transcript levels of CDKN1A in single MCF-7 cells. First, five cell suspensions were exposed to MMS for different time durations (from 0.5 to 4.5 h). Following exposure to MMS, the cell suspension from the five separate culture mediums was diluted and introduced to five analysis units on the microchip. C_q values of CDKN1A RT-qPCR in MMS-treated single cells and mean 3D qPCR amplification curves for this scenario (Fig. 6c) were plotted.

The mean C_q values decreased from 32.9 to 28.1 when the exposure time was increased from 0.5 to 4.5 h. Also, the SD of C_q decreased (from 0.5 to 0.2) as the exposure time increased. Furthermore, the exposure time did not have a linear relationship with CDKN1A expression, as seen by the increased inter-unit C_q differences with increasing exposure durations. As the exposure time increased beyond 2.5 h, the 3D amplification curves became saturated before 40 cycles of qPCR. We can conclude that the upregulation of CDKN1A expression caused by MMS was positively correlated with treatment time duration in the range of 0.5–4.5 h.

4.4.3 Effects of drug dosage on CDKN1A expression—To further investigate the effect of MMS treatment on gene expression levels of CDKN1A in single cells, we treated MCF-7 cells with different doses of MMS and then performed on-chip RT-qPCR. First, cells ($\sim 1 \times 10^6$ cells in a 10 cm dish) in five cultures were exposed to different doses of MMS (from 30 to 150 $\mu\text{g/mL}$) for 2.5 h at 37 °C in an incubator. After treating, cells were

introduced to the arrayed microfluidic device for single-cell processing and RT-qPCR following the above-described protocol.

In the five separate analysis units, with the dosage increasing from 30 to 150 $\mu\text{g/mL}$, the mean C_q value decreased from 33.5 to 29.3, while the SD of this C_q decreased from 0.5 to 0.2 (Fig. 6d). In addition, by plotting the mean qPCR data in a three-dimensional bar graph, it can be found that after 40 PCR cycles the amplification of samples which were treated by MMS with doses from 60 to 150 $\mu\text{g/mL}$ became saturated. While for the 30 $\mu\text{g/mL}$ MMS-treated sample, the amplification yield at the end of 40-cycle PCR was lower than the other four cases. Furthermore, the inter-unit C_q differences for CDKN1A were found to be 0.4, 0.7, 1.3 and 0.7, indicating that the intracellular DNA damage was greatest when MMS dosage increased from 90 to 120 $\mu\text{g/mL}$. As the cells were from the same generation of MCF-7 cell line, we can conclude that the upregulation of CDKN1A expression caused by MMS treatment was positively correlated with MMS dosage when it ranged from 30 to 150 $\mu\text{g/mL}$.

The results demonstrate that the presented microfluidic array is capable of detecting alterations in transcript levels of single cells and can potentially perform parallelized single-cell analysis.

5 Conclusions

A microfluidic approach has been presented for parallelized, quantitative analysis of mRNA from single cells via RT-qPCR. The approach leverages an array of single-cell RT-qPCR analysis units, which each include a cell trap integrated with an elliptically shaped RT-qPCR micro-chamber and are based on a single substrate equipped with a thin-film microheater and temperature sensor. These features, along with parallelized handling and processing of single cells through concurrently controlled pneumatic valves, allow integration of all steps of single-cell RT-qPCR, including cell isolation and immobilization, cell lysis, mRNA purification, reverse transcription and real-time PCR on a single chip, thereby eliminating the need for off-chip manual cell and reagent transfer and qPCR amplification commonly found in existing methods while allowing rapid analysis of single cells. Further, by employing a microfluidic bead-based RT-qPCR method, the approach is capable of a higher level of amplification of the targeted gene (i.e., the endpoint PCR yield) compared with conventional in-tube approaches.

To demonstrate the proof of principle for the approach, a microfluidic device has been tested for parallelized RT-qPCR analysis of six single cells. Thanks to the use of concurrently controlled parallel channels each housing an analysis unit, the device design allows for significant simplification in flow control requirements when compared with existing microfluidic methods (Pollen et al. 2014), which would require a much larger number of valves (8 vs. ~ 18) and chambers (6 vs. ~ 24) for analyzing six single cells. Also, this flow strategy can readily be scaled up to produce substantially larger arrays. For example, it is estimated that using a 200-mm wafer and standard soft lithography techniques, 20 RT-qPCR analysis units (footprint: 4 mm $\times$ 2 mm per unit) could be fitted in each of 20 parallel channels controlled by approximately 75 pressurized elastomeric binary valves. Thus, an

array of 400 single-cell RT-qPCR analysis units in a 20×20 layout could be created on a single substrate, again with much simplified flow control operations when compared with existing approaches (Fluidigm Corporation 2014). Such an array device would allow fully integrated and parallelized analysis of single cells for statistically relevant gene expression profiling.

The device testing involved a study on the genotoxic effects of a mutagen on single-cell CDKN1A and GAPDH expression levels in MCF-7 cells. Single cells exposed to varying mutagen doses for varying durations could be differentiated through on-chip gene expression analyses via RT-qPCR. The on-chip mean value of C_q decreased from 32.9 to 28.1 when the exposure time increased from 0.5 to 4.5 h. Likewise, as the MMS dosage increased from 30 to 150 $\mu\text{g/mL}$, the mean C_q value decreased from 33.5 to 29. These results offer insight into genotoxic effects on single cells and demonstrate the potential of our parallelized single-cell RT-qPCR analysis approach in allowing fully integrated and efficient transcription profiling in basic biological research and clinical diagnostics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Microfluidic RT-qPCR array, experimental procedure and setup. **a** Schematic of the microfluidic array with a photograph of a packaged device in the *inset*. **b** Schematic of a single unit

Fig. 2.

a Experimental procedure. (1) Beads (brown) were introduced to an analysis unit and held in the chamber by a magnet located beneath the chip. (2) Cell suspension (blue) was introduced into the unit until a single cell was trapped by a neck-shaped flow constriction formed by a protrusion in the channel between two valves. (3) Excess cells were removed from the unit by washing with PBS buffer (green). (4) Lysis buffer (yellow) was introduced (through the buffer port) to lyse the trapped single cell, and then, the beads were moved to the trapping region and mixed with the cell lysate (by magnet-driven motion). (5) After repeating steps 1–4 for all analysis units, reagents (purple) for RT and qPCR were introduced to each unit for downstream assays. **b** Bead-based RT-qPCR principle and a typical temperature history for integrated single-cell RT-qPCR. Oligo(dT)₂₅ magnetic microbeads (mean diameter: $2.8 \pm 0.2 \mu\text{m}$) were used to specifically target and capture mRNA molecules from the cell lysate (color figure online)

Fig. 3. Schematic of the experimental setup with a photograph of a single pneumatic microvalve in the *inset*

Fig. 4.

a On-chip RNA capture capacity testing with varying XenoRNA copy numbers, no-template control (NTC) and positive control (PC). There was no detectable residual XenoRNA template in the binding effluent after 40-cycle qPCRs. **b** Validation of on-chip RT-PCR using synthetically homogenized XenoRNA templates and no-template control (NTC). **c** Validation and consistency of on-chip RT-qPCR testing using parallelized single-cell RT-qPCR of XenoRNA templates and no-template control (NTC). **d** Fitting curves of on-chip and in-tube RT-qPCR for XenoRNA templates of different copy numbers. Mean and SD are shown based on five repeated tests. Using identical starting copy numbers, the mean and SD of C_q for on-chip qPCR are lower than the corresponding in-tube tests, indicating that the efficiency and sensitivity of RT-qPCR have been improved by the presented nanoliter-volume reactions. **e** The endpoint fluorescent intensity of on-chip tests after 40-cycle qPCR. Data points represent ten repeated tests. **f** Endpoint fluorescent intensity of in-tube tests after 40-cycle qPCR. Data points represent ten repeated tests. The means of the fluorescent intensity show an increased RT-qPCR yield by the on-chip approach

Fig. 5.

a Mean fluorescent intensity of endpoint RT-qPCR of MCF-7 cells and no-template controls. Three samples were used for each, and *error bars* represent SDs. **b** Amplification during on-chip single-cell RT-qPCR for GAPDH, CDKN1A and no-template controls. The *inset* demonstrates ROX and FAM signals around the quantification cycle of GAPDH testing. The results were based on one test by an arrayed chip for each gene, respectively

Fig. 6.

a Amplification curves of CDKN1A and GAPDH in MMS-treated, MMS-untreated single MCF-7 cells and no-template control (NTC) by the microfluidic array. **b** qPCR C_q values of CDKN1A and GAPDH in MMS-treated and MMS-untreated single MCF-7 cells. **c** qPCR C_q values with amplification curves shown in the *inset* of CDKN1A in single cells exposed to MMS for different time durations. **d** qPCR C_q values with amplification curves shown in the *inset* of CDKN1A in single cells treated with different doses of MMS. Each experiment was repeated three times, and *error bars* represent SDs