HIGH-THROUGHPUT MICROFLUIDIC RT-qPCR OF SINGLE CELLS
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ABSTRACT
We present microfluidic technology for high-throughput gene expression measurements in hundreds of single cells. Our device implements 300 parallel RT-qPCR assays, and performs all cell processing steps, including cell capture, cell lysis, reverse transcription, and quantitative PCR. We show that nanoliter volume processing achieves high precision, large dynamic range, single molecule sensitivity, and single nucleotide specificity in genotyping. We apply this technology to single cell studies of microRNA expression, the co-regulation of a microRNA and its target transcript during differentiation of embryonic stem cells, and the genotyping of primary tumour cells.

KEYWORDS: Single cell, microfluidics, RT-qPCR, high-throughput, lab-on-a-chip

INTRODUCTION
Transcription measurements with single cell resolution are critical to understanding variable responses in immunity, measuring stochastic noise in gene expression, and assessing the disease and developmental state of heterogeneous populations. The latter is particularly important in stem cell science, developmental biology, and cancer, where minority cells may be most significant. To see these populations requires the quick and cost-effective measurement of hundreds to thousands of individual cells. Quantitative real-time polymerase chain reaction (RT-qPCR) is a sensitive method for quantitative analysis of transcript levels that provides excellent sensitivity and dynamic range in the detection of transcripts. However, the use of RT-qPCR is generally limited to ensemble measurements of bulk cells or plasma, and is blind to minority cell populations. This aggregation obscures the underlying biological response and variability. To address this limitation, we exploit advances in scalable microfluidics to develop robust lab-on-chip technology capable of highly parallel and cost-effective measurements of transcript levels from single cells. The microfluidic device integrates single-cell capture, lysis, reverse transcription of contained RNA, and precise measurement of cDNA using RT-qPCR. We demonstrate this system in the study of microRNA expression in a cell line representing chronic myelogenous leukaemia, pluripotency markers in differentiated embryonic stem cells, and the detection of somatic mutations in a primary breast cancer sample.

EXPERIMENTAL
An integrated microfluidic device based on multilayer soft lithography that performs 300 parallel RT-qPCR assays and executes all steps of single-cell capture, lysis, reverse transcription, and qPCR is shown in Figure 1A. The prototype consists of 6 independent sample-loading lanes, each containing 50 cell-processing units. To reduce device complexity and obviate the need for RNA purification we optimized our device design to be compatible with standard and commercially available assays and “one-pot” RT-qPCR chemistries. The device architecture features 6 sample input channels, each divided into 50 reaction chambers having a volume of 60.6 nL each, resulting in 300 RT-qPCR reactions using a total of approximately 20 µL of reagents (Figure 1A). Each 0.6 nL cell capture chamber is connected sequentially to two larger chambers having volumes of 10 nL and 50 nL (Figure 1B) to implement RT-qPCR protocols including heat lysis followed by two-step RT-PCR, or chemical lysis followed by one-step RT-qPCR. All lanes are connected to a common feed channel which is used to inject reagents through the upstream chambers, thereby diluting the intermediate product (cell lysate or cDNA) and assembling the next reaction mixture. Temperature control and fluorescent detection during PCR amplification were performed using a CCD detector mounted above a flat-bed thermocycler plate. Fluorescent images of the entire chip during PCR cycles were segmented and analyzed using a custom MATLAB program, thereby generating real-time amplification curves.

A key element of our technology is the integration of hydrodynamic cell traps with upstream deflectors to achieve highly efficient single cell loading of each reaction chamber (Figure 1C). In a total of 8 experiments we successfully isolated single cells in 1518 of 1700 traps for an average single cell isolation efficiency of 89%. Immobilizing cells in traps facilitated on-chip washing of single cells, thereby removing additional non-trapped cells, and eliminating extracellular debris and RNA which may contribute noise in single cell analysis.

RESULTS AND DISCUSSION
We evaluated the sensitivity and precision of RT-qPCR in our device by performing measurements of GAPDH expression over an 8-fold dilution series of total RNA, ranging from 40 pg (~2 cell equivalents) to 10 fg. RNA was purified from K562
cells, a human cell line derived from a patient with chronic myeloid leukemia (Figure 2A-C). For the three highest concentrations (40 pg, 5 pg, 625 fg), cycle threshold (CT) values were found to differ by ~3, suggesting nearly 100% efficient amplification. The standard deviation of measured CT values for the three highest concentrations was less than 0.15, indicating that the technical variability in measuring mRNA abundance is less than 10% in absolute concentration. At concentrations below 625 pg we observed a digital pattern of amplification (49/50 for 78 fg, 19/50 for 10 fg) that titrated with concentration and was consistent with the detection of single cDNA molecules as determined by a binomial distribution [1].

Figure 1: Design of presented microfluidic device for analyzing gene expression in single cells. (A) False-coloured fluorescent confocal scan of microfluidic device. Scale bar: 4 mm. Each one of 6 sample input channels is divided into 50 compound reaction chambers resulting in a total of 300 RT-qPCR reactions per device. White box denotes the enlarged unit region seen in (B). Control lines and fluid paths are depicted in red and blue, respectively. (B) Fluorescent confocal scan of array unit. Each consists of (i) a reagent inlet, (ii) a 0.6 nL cell capture region with integrated cell traps, (iii) a 10 nL RT chamber and (iv) a 50 nL PCR chamber. Scale bar: 400 μm. (C) Optical micrograph of a single cell trapped in the cell capture chamber (indicated by black arrow). Scale bar 400 μm.

Figure 2: Validation of microfluidic RT-qPCR. (A) Fluorescent image of chip showing positive amplification after 50 cycles of PCR. A limiting dilution results in a digital pattern of single molecule amplification. (B) 300 Real-time PCR amplification curves from processing sequences of images similar to (A). The threshold for determining CT values was determined by the maximum second derivative of the amplification curve. (C) RT-qPCR measuring GAPDH across a dilution series of purified total RNA. The dynamic range plateaus in the digital regime where single molecule amplifications are detected in 19 of 50 chambers for the 10-fg sample. Single cell measurements of GAPDH are shown in green, and correspond to approximately 20 pg of RNA per cell. Error bars represent standard deviation of measured CT values for all amplified reactions. (D) Histogram showing the distribution of GAPDH transcripts measured in single K562 cells (N = 233).

We next compared these results to measurements performed directly from single K562 cells. K562 cells were loaded directly from culture medium followed by washing and analysis using a chemical lysis and one-step RT-qPCR protocol (Cells Direct™, Invitrogen). Using a CT threshold of 31.5, corresponding to the mean CT of a single molecule of GAPDH (CT = 30.5) plus two standard deviations (s.d. = 0.5), we observed successful amplification in 100% of single cells (N = 233). Consistent with previous reports [2, 3], we observed a log-normal distribution of GAPDH in single cells with mean CT values of 20.3 (s.d. = 0.8) and an average of 1761 (s.d. = 648) copies per cell (Figure 2D). Taken together, these results establish the precise measurement of mRNA abundance with single molecule sensitivity and the dynamic range needed for single cell analysis.
We next applied our technology to the study of single cell miRNA expression in K562 cells, which are known to exhibit mixed characteristics of erythrocytes, granulocytes, and monocytes. miRNAs are short (~22 nucleotides), non-coding RNAs that have been shown to play important roles in regulating gene expression during development and oncogenesis. We performed a total of 1561 single cell measurements to examine single-cell variability in the expression of 9 miRNAs (Figure 3A) spanning a wide range of abundance (>16000 copies per cell to <0.2 average copies per cell).

To demonstrate the capability to measure multiple transcripts in single cells we designed an optically multiplexed assay to study the co-regulation of miR-145 and OCT4, a known target of miR-145, during the differentiation of a human embryonic stem cell (hESC) line (Figure 3B). Notably, single cell analysis at day 6 showed a bimodal distribution in both OCT4 and miR-145, revealing a transition of cellular state that is not apparent in population measurements.

Finally, to establish the specificity of our method we used multiplexed measurements of mRNA single nucleotide variants (SNV) to assess the genomic heterogeneity within a primary tumor sample. A total of 117 single cells isolated from a plural e-ffusion of a metastatic breast cancer were assayed for the expression of a SNV mutant of the transcription factor SP1, previously identified by deep sequencing [4]. Given that the frequency of tumor cells within the original sample was approximately 89% [4], but only 19% of cells contained the heterozygous SP1 mutation, single cell measurements suggest that the metastasis of this tumor is derived from multiple cancer cell lineages.

CONCLUSION
Here we have established the critical element of combining all single-cell and nucleic acid processing steps into an integrated platform. The core functionality established here provides the foundation from which a variety of on-chip single cell transcription analyses will be developed.

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