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EWOD-based microfluidic chip for single-cell isolation, mRNA purification and subsequent multiplex qPCR

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Single cell analysis circumvents the need to average data from large populations by observing each cell individually, thus enabling the analysis of cell-to-cell variability. The ability to work at this scale presents many new opportunities for life science and biomedical applications. Microfluidics has become a tool of choice for such studies and electrowetting on dielectric (EWOD) technology is well adapted for samples of reduced size and biological studies at the single cell level. In the present manuscript, for the first time, we present an integrated and automated system based on EWOD that can process the complete workflow on a single device, from the isolation of a single cell to mRNA purification and gene expression analysis.

Introduction

Since the 2000's, expression profiling has become routine in many laboratories in basic biology, but it has also been used for biomedical applications, from oncology to infectious diseases, in order to stratify patients, to discover new therapeutic targets, to characterize new biomarkers that would enable early diagnosis, prognostic or facilitate therapeutic orientation. However, recent studies have demonstrated that gene expression is very heterogeneous, even in genetically similar cells (Huang[1], Li[2]). This heterogeneity originates from differences in epigenetic regulation, circadian clocks, cell cycle phases, microenvironment cues as well as intrinsic transcriptional stochastic behavior. To understand the molecular basis of gene expression heterogeneity, it would be extremely useful to analyze the transcriptome at the single cell level. This is particularly relevant for stem cells and embryonic cells which expression is highly variable depending on their microenvironments. It is also relevant for tumor cells, where the understanding of both interindividual variability and intratumoral variation has turned into a major challenge that many laboratories are attempting to address to identify more specific and more efficient treatments. This is a key issue in translational research, where there is a pregnant need for the analysis of expression profiles in a reduced number of cells, either healthy or disease-related, that are directly obtained from patients. In the last decade, many studies have also described cell-to-cell variability within a homogenous cell population in culture. Although genetically homogenous, the microenvironment and neighboring contexts can trigger epigenetic variations within the cell population that should be taken into consideration through transcriptome analysis at the single cell level. Therefore, there is an increasing need to analyze gene expression in a reduced number of cells and, ideally, within a single cell. Several groups have been attracted by this challenge (Bahcall[3], Longo & Hasty[4]) and numerous solutions have been proposed to achieve such transcriptome analysis on a reduced number of cells (Marcus[5], Hong[6]).

To address this issue, biologists first tried to amplify small quantities of cDNA exponentially, either using PCR (Brady[7]) or through the linear amplification of cDNA by T7 RNA-polymerase-based in vitro transcription (Eberwine[8], Van Gelder[9]). Several methods have been proposed, and efficient results have been obtained; nevertheless, the amplification step may introduce bias, as the efficacy of amplification depends on the length of the transcripts.

Within the last decade, microsystems in general and microfluidic systems in particular, have appeared as promising tools to achieve transcriptome analysis at the single cell level (Lindstrom[10], Lecault[11]). Several groups have developed microsystems to perform at least part of the process of genome-wide expression profiling from a small quantity of RNA. Toriello's group proposed a PDMS chip to analyze the gene expression of single cells (Toriello[12]). Soon after, Pottier's group also developed a PDMS chip to enable cell capture, cell lysis and RT on the same chip (Bontoux[13]). Some microfluidics systems were specifically designed for RNA extraction (Zhong[14]). Another chip, developed in 2008, enabled RNA capture but not cell lysis (Dimov[15]). In 2010, a microfluidic chip was developed to capture cells into 125 pL wells, to which the PCR mix was added (Gong[16]). Recently, two systems enabled complete protocols, from cells capture in microwells (White[17]) or in picoliter droplets (Mary[18]) to qRT-PCR analysis performed directly on the cells without any mRNA extraction and purification step. To date, most existing microsystems perform only a few steps of the entire process, limiting their potential applications in genomic analysis because the extraction of either coding or non-coding RNA might be necessary

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to study the cell-to-cell variability in gene expression and the stochastic nature of gene networks. Furthermore, these microsystems, which are only partially integrated, have a limited number of applications at the bed side of patients or in the field for micro environmental controls.

Therefore, there is a need for a highly integrated device that includes cell capture, cell lysis, mRNA extraction and reverse transcription from a single cell. Quake's group has proposed such devices (Marcus[5], Marcus[19]) using flow microfluidics but with a complex surrounding instrumentation. Among microfluidics technologies, digital microfluidics (DMF) is becoming a powerful approach, whereby individual droplets of a few tens of nanoliters are moved along an array of electrodes by electrostatic forces, the so-called electrowetting phenomenon (Pollack[20], Fouillet[21], Fair[22]). This technology, today industrialized by Advanced Liquid Logic, Inc., has proven its ability to handle complex biological protocols, from manipulation of cells suspensions (Shah[23], Park[24],) or even cell harvesting (Eydelnant[25]), to highly multiplexed enzymatic reactions (Sista[26]) such as PCR (Schell[27]) sample preparation for next generation sequencing (Amores[28]). However, again, the devices only address of few steps of the complete process. The EWOD-based microfluidic chip we have developed in the present study enables, for the first time, the entire process of transcriptome analysis, on the same chip. Starting with a reduced number of cells, this chip can isolate a single or a few cells in a droplet, extract mRNA from the cell, catalyze the RT reaction and perform RT-qPCR. Alternatively, this device could be utilized for the preparation of cDNA ready for deep sequencing.

Materials and methods

Electrowetting chip

The whole fabrication process has been optimized to be realized in standard microelectronics and MEMS cleanrooms, using industrial machines at a 200 mm wafer scale.
The closed EWOD microsystem (figure 1a) consists of 2 parts: a silicon active device and a glass cover. All photolithography steps were performed with a stepper machine with 0.5 µm precision. For the active device, starting with the 200 mm silicon wafer, a 1 µm silicon oxide (SiO_2) insulating layer was grown by thermal wet oxidation. A 200 nm Ti/AlCu layer was then deposited by sputtering. Electrode patterns were defined by photolithography, followed by the dry etching of the Ti/AlCu layer. A 300 nm dielectric layer of silicon nitride (Si_3N_4) was deposited by Plasma Enhanced Chemical Vapor Deposition (PECVD). This layer was etched through a mask defined by photolithography to create openings used as electrical vias. A second level of electrodes was created using the same materials (Ti/AlCu, SiN) and patterning processes, enabling more complex architectures. A 100 µm thick Ordyl dry film resist (Elga Europe, Italy) was laminated on the wafer and patterned by a contact UV-photolithography step, creating the spacer between the chip and the cover. A hard bake at 160 °C was performed to ensure an inert behavior towards the solvent. Ordyl walls are also used to isolate different zones on the chip, such as the reservoirs or reaction zones. The glass cover plate was made from a 200 mm glass wafer coated with ITO (Indium Tin Oxide) and used as a transparent conductive layer. Part of the silicon chip remains uncovered by the glass to provide access to 40 open pads for electrical contact with the instrument to address the 230 electrodes. This glass wafer was sand-blasted (Anteryon Eindhoven, The Netherlands) to form through-holes of 1.6 mm to create inlets for the different biological solutions. Individual chips and covers were obtained by dicing the wafers into the respective sizes of 22 x 22 mm^2 and 18 x 22 mm^2. Finally, the surfaces of the chips and covers were made hydrophobic by respectively depositing 1 µm and 250 nm of an original and proprietary SiOC material (Thery[29]). SiOC is obtained by PECVD process (P5000, Applied Materials, Santa Clara, CA, USA) from OMCTSO precursor (octamethylcyclotetracyloxane, Sigma Aldrich, St Louis, MO, USA). The elevated hydrophobicity (>110°) and very low hysteresis (<7°) values of the chip make it a convenient hydrophobic layer for EWOD. Moreover, because it is a deposition in vapor phase, it is fully compatible with microelectronics industrial-scale cleanrooms.
Electrodes designed on the chip (see figures 1b and c) have been developed using generic architectures already presented (30) and were optimized using know-how acquired from previous configurations (Delattre[30]). The DMF chip design and the dedicated benchtop instrument are presented in the next section.
Fluidic operations, such as droplet movement, droplet splitting, droplet dispensing from a larger reservoir and droplet merging, have been described many times in previous studies (Cho[31]) and are detailed in the supplementary information.

Laboratory instrumentation used for the electrowetting setup

A universal voltage generator was used to send an AC voltage to the electrodes of the silicon chip. The generator sends a voltage of up to 20 V, usually 12 V at 3000 Hz. A homemade amplifier was designed to multiply the signal by a factor of 5 to reach a typical voltage of 48 V after amplification. Then, the signal passed through relays (ER-16, National instrument, Austin, TX, USA) to address each open pad on the chip and to place the electrodes either at a ground state or an "ON" state when the voltage is applied.
The thermal components, the Peltier module and the temperature probes, were controlled by a sourcemeter (Keithley 2510, Keithley Instruments, Cleveland, OH, USA).
To facilitate the control of the voltage generator, of the sourcemeter and of the electrode actuation, high density data acquisition devices (USB-6509 96 and GPIB-USB-US, National Instrument, Austin, TX, United-States) were used, making the whole system connectable to any computer by USB. All the components are controlled using homemade dedicated software that enables the electrode actuation programming and live actuation.

Reagents

The filler fluid used to fill the electrowetting chip was 5cSt silicone oil (PDMS, 200®, Dow Corning, Midland, MI, USA).
The cells used in the present study were human HaCat adhering cells harvested in suitable medium (DMEM, Gibco with 1 % Penicillin-Streptomycin antibiotics, Gibco, Life Technologies Carlsbad, CA, USA and 10 % FBS, Perbio, Thermo Scientific, Rockford, IL, USA). The cells were passaged at 80 % confluence, washed with DPBS (Dulbecco's Phosphate Buffered Saline, Gibco, Life Technologies Carlsbad, CA, USA) and individualized by back and forth pipetting. Individualized cells were then fluorescence labeled (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen, Life Technologies Carlsbad, CA, USA) to enable easy observation using a 488 nm excitation wavelength.
The source of the analyzed mRNA was either commercial total RNA (Stratagene® qPCR Reference Total RNA human, Agilent, Santa Clara, CA, USA) or mRNA extracted and purified from the Hacat cells using an Oligo dT functionalized magnetic bead kit (mRNA micro, Invitrogen, Life Technologies Carlsbad, CA, USA). The same magnetic bead kit was used on chip. In addition to the beads, the kit

comprises a lysis buffer, washing buffers A and B, and an elution buffer.

The qRT-PCR kit (SuperScript™ III Platinum® One-Step Quantitative RT-PCR System, Invitrogen, Life Technologies Carlsbad, CA, USA) is a one-step protocol, as both enzymes are present in the same tube: the first enzyme for the RT reaction becomes deactivated at 95 °C, while the second enzyme for PCR is activated at that temperature. Both enzymes were also used separately for independent concentration optimization (SuperScript™ III Reverse Transcriptase and Platinum® Taq DNA Polymerase, Invitrogen, Life Technologies Carlsbad, CA, USA). The primers and probes sequences are provided in table 1. A unique set of temperatures and cycle durations were used for all qRT-PCR reactions performed (table 2).

The qRT-PCR results on chip were compared to the protocol realized in tube on a commercial real time thermocycler (MX3005P, Stratagene, Agilent, Santa Clara, CA, USA).

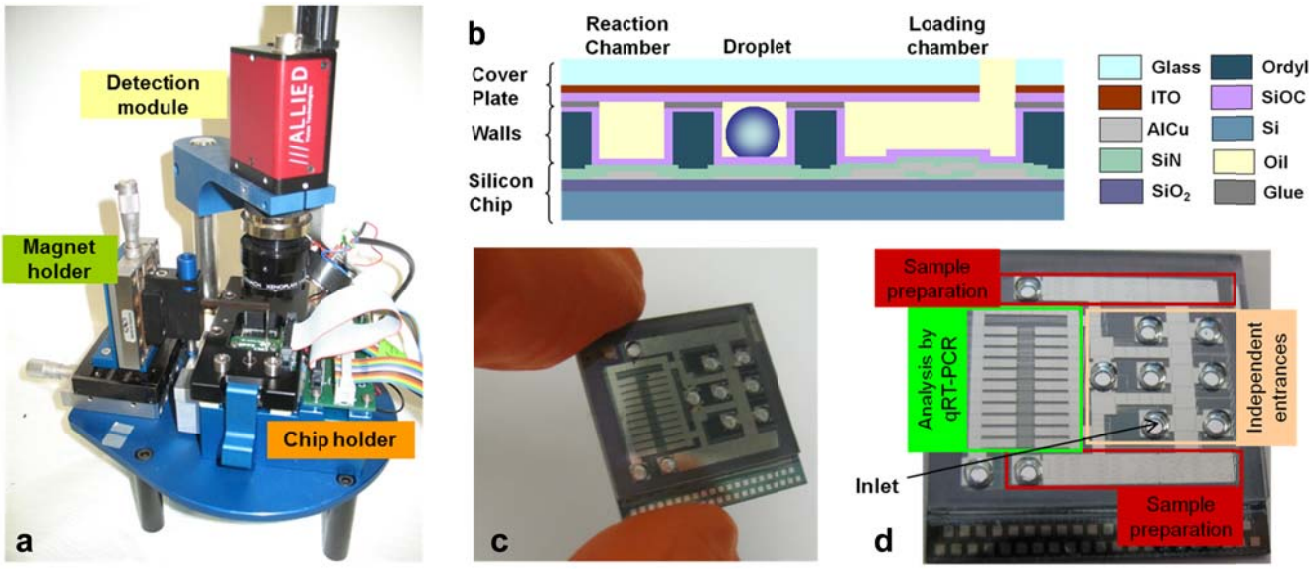

Figure 1. EWOD based microsystem developed for single cell analysis. a) A dedicated benchtop instrument that includes a chip holder for electrical connection and physical holding, a magnet holder to manipulate the magnets on top of the chip and a detection module for droplet observation and fluorescence detection. b) Chip structure and materials. c) The 22 mm x 22 mm chip developed for single cell analysis, handled between two fingers. d) Chip organization, including independent entrances, two sample preparation areas, and an isolated qRT-PCR area.

Table 1. Primers and probes sequences.

Myc	Reverse primer	TGCGACGAGGAGGAGAACTTC
	Forward primer	GTAGGAGGGCGAGCAGAGC
	Probe	ACCAGCAGCAGCAGCAGAGCGAGC
GAPDH	Reverse primer	TCCCTGCCACACTCAGTCC
	Forward primer	GAGCACAGGGTACTTTATTGATGG
	Probe	CCCTCCTCCTCACAGTTGCCATGTAG ACC

Table 2. RT and PCR parameters.

RT (1 cycle)		
50 °C	15 minutes	cDNA synthesis
95 °C	2 minutes	Inactivation of SuperscriptTM enzyme and activation of Platinum® Taq enzyme
PCR (40-50 cycles)		
95 °C	15 seconds	Denaturation of cDNA
60 °C	30 seconds	Elongation

Tween20 (Tween®20, for molecular biology, viscous liquid, Sigma-Aldrich, St Louis, MO, USA) surfactant at 0.01% was used in water-based reagents to reduce the surface tension of the droplet and make it compatible with electrowetting. In presence of cells, the surfactant added was Pluronic F68 at 0.1% (Sigma-Aldrich, St Louis, MO, USA).

Results

EWOD microsystem

To obtain a fully integrated sample preparation system that is able to generate a PCR signal from the input of a few cells, enabling gene expression analysis, both a microfluidics chip and a benchtop instrument were developed (figure 1).

The chip is an electrowetting, droplet based microfluidics chip specifically designed to enable both sample preparation steps and qPCR detection by utilizing dedicated and separated areas (figure 1d) contrary to previous systems we have developed that required sample preparation outside of the EWOD microsystem (Delattre[31]). The size of the chip (22 x 22 mm^2) is well adapted to small sample manipulation, but it can still be manipulated by hand (figure 1c). A specific area is dedicated to reagents and sample loading that utilizes seven independent inlets to avoid reagent contamination. The gap between top plate and electrode being fixed to 100µm by the Ordyl film, droplets volumes are only given by the area of the electrodes (more details on droplet dispenses are provided in the supporting information section). Each inlet leads to large electrodes that can dispense and manipulate droplets of approximately 256 nL. These large electrodes are linked to smaller electrodes for 64 nL and 36 nL droplet dispense and manipulation. To perform quality mRNA purifications, both small and large volumes are required for the sample preparation. Electrodes of the two sizes are connected to two

large sample preparation areas that can manipulate droplets from 256 nL to 1236 nL. The particular design of the electrodes (large interdigitations) enhances the mixing capability and reduces the risk of losing a droplet that would be smaller than expected (figure 2b). The end of the sample preparation area is an outlet used for removing used reagents. The sample preparation areas are then connected to the qRT-PCR area, which consists of an array of twenty-two 128 nL reservoirs (two columns of eleven reservoirs). The reservoirs are all connected through a four phase bus, ending with an outlet for sample recovery, if needed. Multiplexed addressing is an easy method used in the chip design to make each reservoir accessible independently by electrowetting (Fouillet[33]).

Prior to use, the chip is filled with an immiscible filler fluid, 5cSt silicone oil, used to prevent droplet evaporation at high temperatures and to ease droplet movements.

The compact benchtop instrument (figure 1a) consists of a central area and the chip holder. The chip is positioned on a copper plate and is mechanically clamped on the sides under a rigid cover that also makes electrical contacts in a robust way through spring loaded pins. The copper plate is glued on top of a Peltier module (Supercool PF-071-10-13) that is used for thermocycling the chip, and a temperature probe is inserted inside this copper plate (RTD Pt100, HEL 775-BT1 Honeywell, Morristown, NJ, USA). Temperature inside the chip was calibrated using miniature thermocouples (5TC-TT-K-40-36, Omega, Stamford, CT, USA) and set points were found to be 64°C to reach 60°C inside the chip and 102°C to reach 95°C. Located around and on top of the chip, the detection module for quantitative RT-PCR reactions analysis consists of a camera

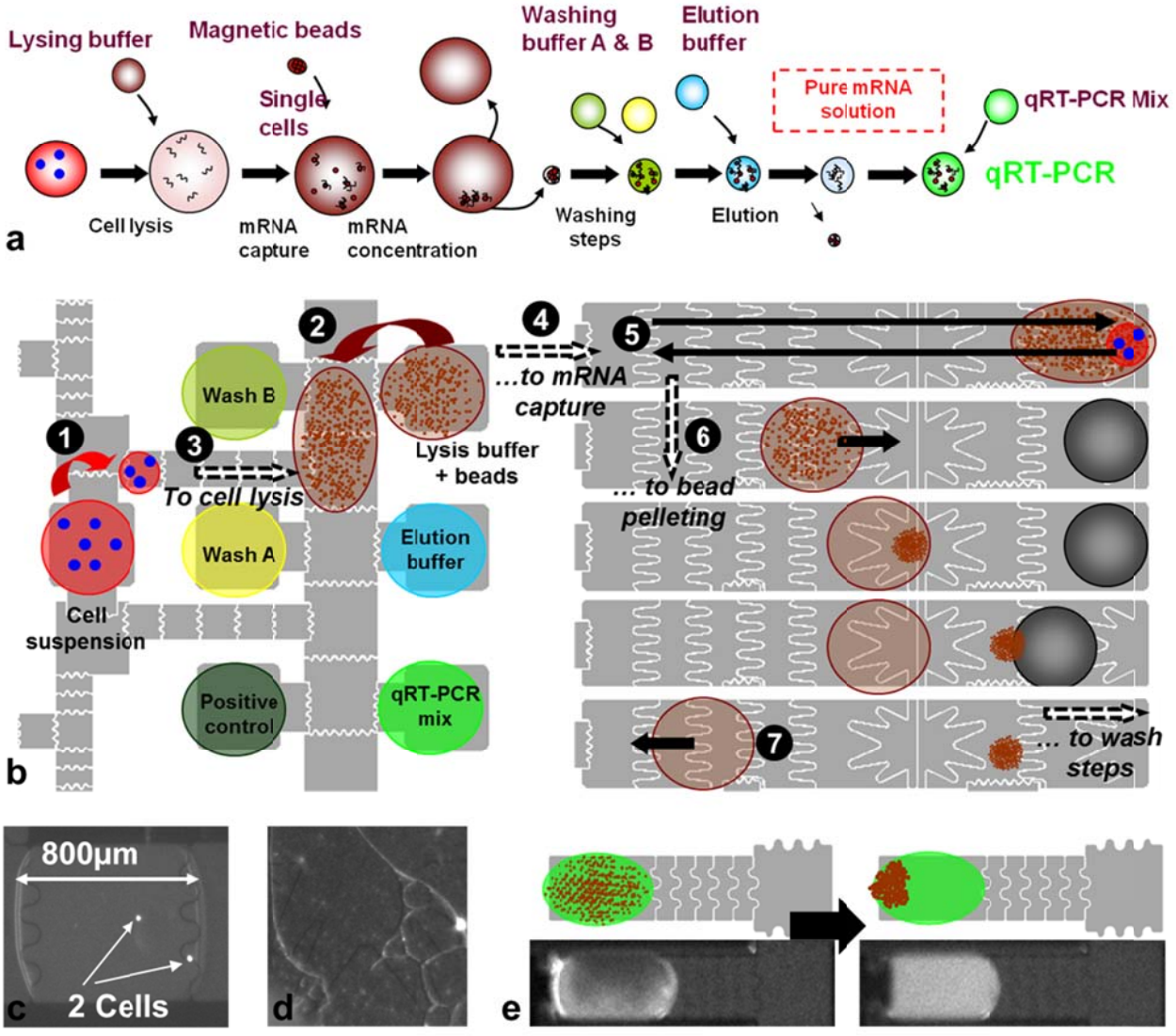

Figure 2. Protocol for mRNA extraction and purification from cells, followed by qRT-PCR for gene expression analysis.
a) Usual protocol, as described in the commercial manual, for in tube mRNA extraction, followed by qRT-PCR analysis.
b) Some steps of the protocol were adapted for on chip mRNA extraction: from cell lysis to mRNA capture. Left panel presents the lysis and mRNA capture steps: 1) droplet containing one or a few cells is dispensed 2) a droplet of lysis buffer and magnetic beads is then dispensed 3) droplet with the cells and droplet with the beads are merged resulting in cell lysis 4) this new droplet is moved to the "sample preparation" electrodes for operations on the magnetic beads. Right panel represents the operations on the beads that are similar for both mRNA capture and washing steps: 5) droplet is moved back and forth to enable bead mixing and mRNA capture 6) droplet is moved towards the magnet so that the beads are concentrated on one side of the droplet, then the magnet is moved towards the droplet resulting in the bead pellet extraction 7) empty droplet is moved towards the waist.
c) Two fluorescence-labeled cells in a 64 nL droplet.
d) DNA filaments adsorbed on the hydrophobic surface due to a cell concentration greater than 100 cells per droplet.
e) Final bead pelleting before the qPCR step to avoid fluorescence shading.

(Stingray, Allied Technology) with the proper filters to enable FAM emission wavelengths for detection. The 10 x objective was chosen to visualize all the droplets in the detection area at the same time. A LED is positioned on the side of the objective to illuminate the chip in the FAM excitation wavelength. Images taken by the camera were automatically analyzed, and the fluorescence levels were extracted as grayscales using ImageJ software. The Ct values from the PCR plots were calculated using the second derivative method (Guescini[34]). Next to the chip holder, a two axis (x, y) micro positioning plate was used to precisely manipulate a magnet maintained at the end of an arm. The magnetic force can then be applied on top of the chip surface to manipulate magnetic beads and can be removed out of the chip space when not needed. The benchtop instrument is about 20cm wide and 30cm high. It was connected to the standard laboratory instrumentations described in the materials and methods section.

Miniaturization of the mRNA extraction and analysis protocol on the EWOD chip

The initial stage for the completion of the on chip protocol was the translation of a benchtop-based protocol to a format that fits the electrowetting chip design. The chip was designed according to the benchtop protocol, but because of the DMF low and unitary volumes, the protocol required adaptation. First, the protocol was decomposed into individual steps that could be reproduced in droplet operations by electrowetting (figure 2a). Cells from a suspension are lysed. The mRNA is captured using magnetic beads, which are then concentrated using a magnetic rack and the supernatant is removed. The beads are then washed successively with two washing buffers A and B, twice. Between each wash, the beads are concentrated and the supernatant is removed and replaced by another regent. Finally, the mRNa is released from the beads and collected in the elution buffer. The second stage was to make each reagent compatible with electrowetting, by adding surfactant (Tween 20). The right concentration was found experimentally when it was possible to dispense of droplets (0.01% Tween 20 in deionized water is a typical concentration). Then, the appropriate volumes were loaded to fit the reservoir electrodes sizes while keeping the right reagent ratios imposed by the benchtop protocol. Third, the schedule of reagents/sample dispense and droplet operations needed to be planned in detail, with the complete protocol being fully automated once the program launched. On chip, first, 2 µL of each reagent was manually loaded with a pipette into the inlets (figure 2b). The sample, a cell suspension of about 30 cells per microliter, was placed into an isolated well on the left side of the chip. The washing buffers A and B were then loaded. A positive control was added to ensure the RT-PCR worked well. The lysis buffer and the beads were loaded in the same well (the lysis buffer is also the bead binding buffer), as performing the lysis step and the mRNA capture at the same time was as efficient as performing these steps separately (DeKosky[35]). The elution mix and qRT-PCR master mix (containing all primers, probes, enzymes and salts) were loaded last. Because of the surface tension, the liquids did not enter the gap between the top plate and bottom plate but remained in the hole with the meniscus touching the inlet electrode so it could be pulled in once a voltage was applied. A 64 nL droplet of the sample was dispensed first. The camera was used to observe the fluorescencently labeled cells in real time and to numerate them. Individual single human cells could be detected (figure 2c, two individual cells are present in the droplet). If the cell concentration in the droplet was not suitable, the program was interrupted and serial dilutions were performed on chip by dispensing droplets of DPBS to reach the desired concentration. Alternatively, a smaller droplet of 36 nL of the cell suspension was dispensed. This step was clearly a limitation in the automation, which could be addressed with proper real time image processing. If a unique single cell is the lower limit, the greatest number of cells is physically limited to approximately one hundred cells. More than one hundred cells triggers problems during the lysis step, as genomic DNA aggregates in filaments and prevents further movement of the droplet (figure 2d). Once the desired cell concentration was obtained, a 512 nL droplet of lysis buffer and beads was dispensed, and the cell containing droplet was transported towards this large droplet by electrowetting on the 800 x 800 µm^2 electrodes until merging occurred. The large droplet was then transported to the sample preparation area, where it was moved back and forth along the line of electrodes in the zone. These movements enhanced the mixing process, which would be too slow by diffusion only (Fouillet[30]), and optimize the cell lysis and mRNA capture on the beads. Following this step, the magnet was placed on top of the top plate (in contact with the top plate) at the right side of the sample preparation area. The large droplet was brought to an optimal distance from the magnet: this distance, approximately 2 mm in our case, was the limit at which the magnetic force was sufficient for the beads to accumulate into a confined pellet but weak enough to avoid pellet extraction from the droplet. The meniscus assisted method which uses the droplet meniscus to sweep sedimented beads when moving and that is sometimes used to enhance the bead collection yield (Shah[36]) was found to be more problematic. Indeed, as moving droplets resuspends sedimented beads, it also tends to move beads away from the pellet because of the lateral flows present in a droplet when moving by electrowetting (Fouillet[30]). All the beads were collected after 30 s using the micro positioning plate; the magnet was then moved towards the large droplet, resulting in the bead

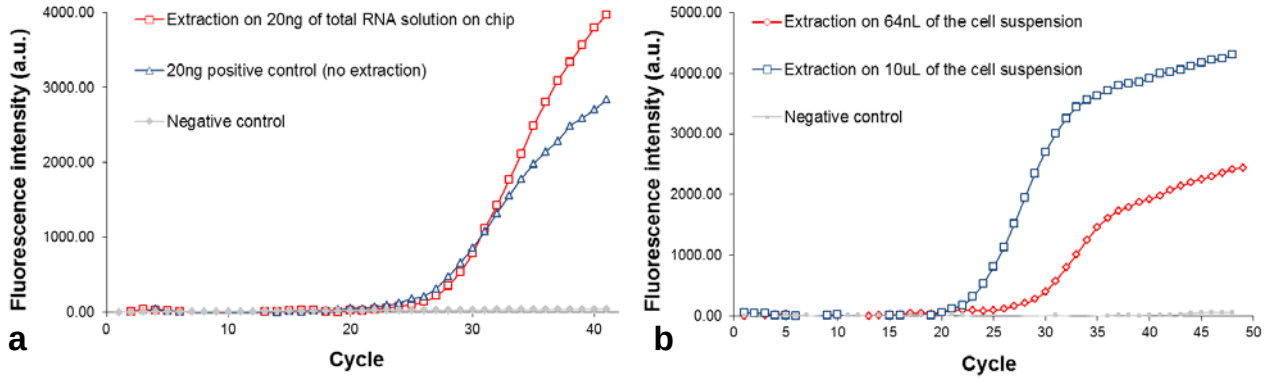

Figure 3. Validation of the on chip mRNA extraction step for the Myc gene by in tube qRT-PCR. a) Extraction performed on 20 ng of total RNA and controlled by 20 ng of total RNA. The superposition of the qPCR two plots, demonstrating that the same Ct values were obtained, shows that the extraction was performed correctly. b) Extraction performed on 64 nL of a cell suspension at 1,000 cells per µL and controlled by an in tube extraction on 10 µL of the same suspension. The differences in the Ct value on the qPCR plots, due to the different cell amounts in tube and on chip, are equivalent to the expected theoretical Ct differences.

pellet extraction. All the time, the droplet remained immobile. This method was found to be more efficient than placing the magnet on top of the droplet and either moving the droplet or moving the magnet to extract the beads. In particular, the residual supernatant around the 2 nL bead pellet was found to be only 3.9 nL with this method with 0.9 % CV, compared to up to 22 nL supernatant with up to 13 % CV for the two other methods (experiments repeated 5 times each). The supernatant was estimated from the picture of the droplet-pellet (beads and supernatant) knowing the initial amount of beads and therefore the volume they take. Reproducibility and low supernatant volume are critical to avoid inhibition during the qRT-PCR step. Bead recovery yield was assessed knowing the initial bead amount (from the concentration) and with bright filed microscopy by counting remaining beads in the droplet. The yield was greater than 99 % collected beads, which is more than any other bead collection reported. The remaining droplet was then moved to the outlets of the sample preparation area. In the meantime, a droplet of 512 nL washing buffer A was dispensed on the 256 nL electrodes and moved towards the bead pellet. The magnet was then removed and the droplet was moved along the sample preparation electrodes to resuspend the beads, as required by the protocol. In total, 2 washing steps with washing buffer A and 2 with washing buffer B were performed. Bead collection efficiency and low supernatant volumes are also important because of this large number of resuspension and bead concentration steps. The clean bead pellet was then moved by the magnet and the micro positioning plate and resuspended either into an elution droplet or directly into a qRT-PCR master mix droplet, which were both dispensed on a 64 nL electrode. If the next step was the collection of the purified mRNA for in tube analysis, the beads were placed into the elution buffer, elution was performed by heating the chip at 60 ºC and the beads were removed. If the next step was the qRT-PCR, then the beads were directly placed in a qRT-PCR master mix droplet. This droplet was then moved towards the qRT-PCR area and the chip was thermocycled for the RT step, and then, the qPCR was performed. The thermal ramps were approximately 2 ºC.s^{-1}, leading to a single PCR cycle of approximately 60 s, a duration similar to that achieved by most commercial thermocyclers. The droplet was kept immobile in the present study, whereas in other reported works, the droplet was shuttled by electrowetting from one temperature zone to another (Hua[37]). At this point in the present study, the beads were still present in the droplet, which was an efficient way to keep as much mRNA as possible for the RT step: elution was performed during the 95 ºC step for the inactivation of the RT enzyme. To enable a good detection of the fluorescent signal during the qPCR, the beads were concentrated by the magnet on one side of the droplet, rather than extracted.

The critical step in this complete on chip protocol is the qRT-PCR and in particular the 95˚C step. Any air bubble present would quickly grow because of the elevated temperature and would eventually move the droplets away from the qRT-PCR reservoirs. A specific knowhow on the chip packaging acquired along the years in the laboratory was used to prevent this issue. It consisted in a precise bonding between top plate and bottom plate using screen printing to deposit the adhesive. This bonding prevented any air to enter the reaction chambers.

Validation of the on chip mRNA extraction protocol and of the on chip qRT-PCR

The sample preparation step, which consisted of mRNA extraction and purification, was first validated using a droplet containing 20 ng of commercial total RNA spiked in water. The mRNA extracted from the total RNA was obtained using the workflow described above, and the elution was performed on chip into the elution buffer. The droplet was then collected through the bottom left outlet (figure 1d), and transferred into a PCR tube with a qRT-PCR master mix containing primers and probes for the Myc gene (20 µL total

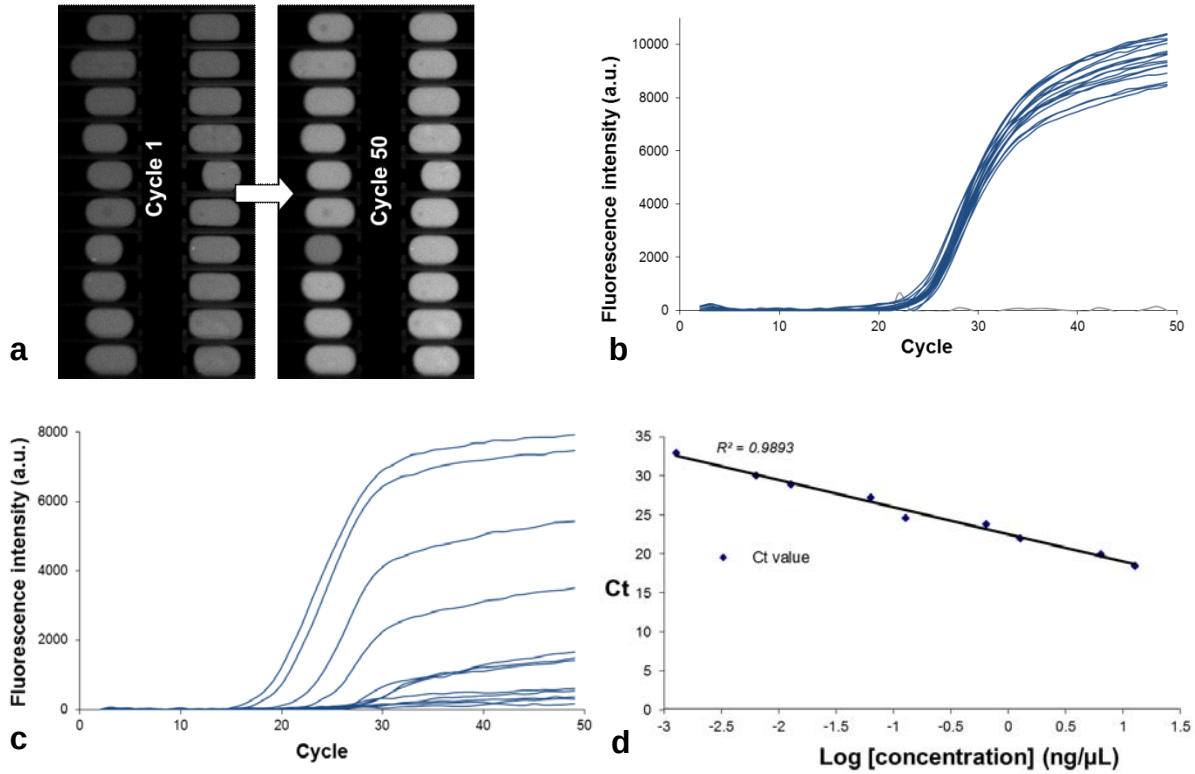

Figure 4. Different validations of the on chip qRT-PCR step for the GAPDH gene. a) and b) Assessment of qRT-PCR reproducibility from 20 qRT-PCR reactions on a single chip using 20 ng.µL^{-1} of total RNA. a) Pictures of the fluorescent droplets on the chip during the first and last PCR cycles. b) The corresponding qRT-PCR plots. c) qRT-PCR plots of a range of RNA concentrations (200 ng.µL^{-1}, 100 ng.µL^{-1}, 20 ng.µL^{-1}, 10 ng.µL^{-1}, 2 ng.µL^{-1}, 1 ng.µL^{-1}, 0.2 ng.µL^{-1}, 0.1 ng.µL^{-1}, and 0.02 ng.µL^{-1}). d) Standard curve obtained from these plots and used for PCR efficiency calculations.

reaction mix). As controls, a qRT-PCR was also performed on the Myc gene using the same amount of total RNA without extraction and purification, and a no template control (NTC) was used for non-specific amplification assessment. The normalized qPCR plots (figure 3a) demonstrate that both the mRNA from total RNA without extraction and the mRNA extracted on chip have similar Ct values (approximately 29), with a slight delay for the extracted mRNA. This result demonstrates that the mRNA extraction on chip was efficient, with a yield approaching 100 %. It is difficult to determine a precise yield knowing that the precision of the qPCR is approximately one Ct. This experiment was repeated 4 times with various concentrations giving similar yield results.

A second mRNA extraction and purification was performed on real human cells. The experiment was realized on chip on 64 nL of a cell suspension containing 1,000 cells per microliter (thus, on 64 cells) and compared to an extraction-purification performed in tube on 10,000 cells, which was used as a reference. The normalized qPCR plots (figure 3b) demonstrated that the experiments on chip and in tube had a difference in their Ct values that was close to the expected theoretical difference (7.2 Ct, details are provided in the supporting information section). The two experiments presented demonstrate that the mRNA extraction and purification step is efficient on chip and gave very good yields, similar to those obtained in tube but with fewer cells.

The qRT-PCR step was also validated on chip. The master mix with primers and probes for the housekeeping gene GAPDH was mixed with commercial total RNA to obtain a final in tube concentration of 20 $ng.uL^{-1}$. An optimization of the enzyme concentrations was performed to obtain the smallest Ct value possible, when compared to normal conditions. The goal was to enable a workable Ct value for very low mRNA concentrations. With the optimized addition of 0.2 $U.\mu L^{-1}$ of Platinum® Taq and 8 $U.\mu L^{-1}$ of Superscript™ III, we managed to gain 6 Ct counts compared to standard conditions. BSA was also added, at a concentration of 0.8 $\mu g.\mu L^{-1}$, to prevent the loss of enzymes and RNA at the droplet interface. Primer, probe, and magnesium concentrations were maintained, as recommended in the kit. This solution was then introduced inside the chip, and 20 droplets were dispensed, with volumes voluntarily ranging from 64 nL to 128 nL, and moved to the qRT-PCR reservoirs. The chip was then thermocycled, and a picture of the droplets was taken at each PCR cycle under FAM excitation (figure 4a). All droplets were expected to provide the same Ct values, as the concentrations of mRNA in each droplet were the same. Figure 4a highlights the differences in the fluorescence signals between the beginning of the experiment and the last PCR cycle (50). The fluorescence signals in the last cycle for all the droplets were very comparable, except for the NTC, which did not show any fluorescence increase. The qPCR plots (figure 4b) demonstrate that the PCR behaved the same for all the droplets, despite the volume variation, providing similar Ct values, with a maximum difference between replicates of 1.6 Ct. This performance is as good as that observed with the usual benchtop PCR thermocyclers, and the Ct value obtained with this instrument was similar to the one obtained on chip for the same RNA concentration. A wide range of concentrations were also tested, from 200 $ng.uL^{-1}$ to 0.02 $ng.uL^{-1}$ of total RNA (figure 4c), and the extracted standard curve (figure 4d) gives a PCR efficiency of 94 %.

Complete on chip protocol with a reduced number of human cells

After the two steps of the complete protocol were validated independently, the entire protocol was performed on a single electrowetting chip, which was achievable because of the particular design of the chip and the automation that has been previously described. The total time for the complete protocol did not exceed 1.5 h due to the efficient automation. We compared qRT-PCR plots that were realized on chip for the Myc gene (figure 5a) from mRNA samples of different origins. The first qRT-PCR plot (red line, figure 5a) was previously obtained on the same chip from mRNA extracted from 20 human cells in suspension. The cells were individualized prior to the experiment and numerated on chip inside the dispensed droplet. The second plot (blue line, figure 5a) was the positive control, in which the extraction of mRNA was performed in tube and the equivalent RNA of 20 cells was introduced to the chip. The third plot (green line in figure 5a) was a second positive control using a commercial RNA equivalent to 20 cells (400 pg) in the droplet, which was also introduced on the same chip. All three plots provided similar Ct values of 22, which clearly demonstrates that the complete protocol realized on chip was successful for twenty cells, with a correct yield. The NTC, also on the chip, did not amplify.

The entire protocol was then performed on a single cell in a 64nL droplet obtained from a cell suspension at low cell concentration. To ensure that only a single cell was present in the droplet, the droplet was observed under a microscope. qRT-PCR was performed on the same chip for a range of total RNA concentrations to compare against the results of the single cell qRT-PCR experiment, following the mRNA extraction and purification. Contrary to the protocol performed on 20 cells, the protocol for a single cell did not provide qPCR plots that are comparable to the same amounts of total RNA (approximately 10 pg). We observed an approximately 5 Ct difference, which is later than expected. However, the NTC provided no PCR amplification, ensuring that the PCR signal from the single cell was derived from the amplification of the mRNA that was extracted from the single cell (Figure 5b).

Direct qRT-PCR on single cells

One reason to perform mRNA extraction and purification prior to the analysis of gene expression is to avoid the addition of potential

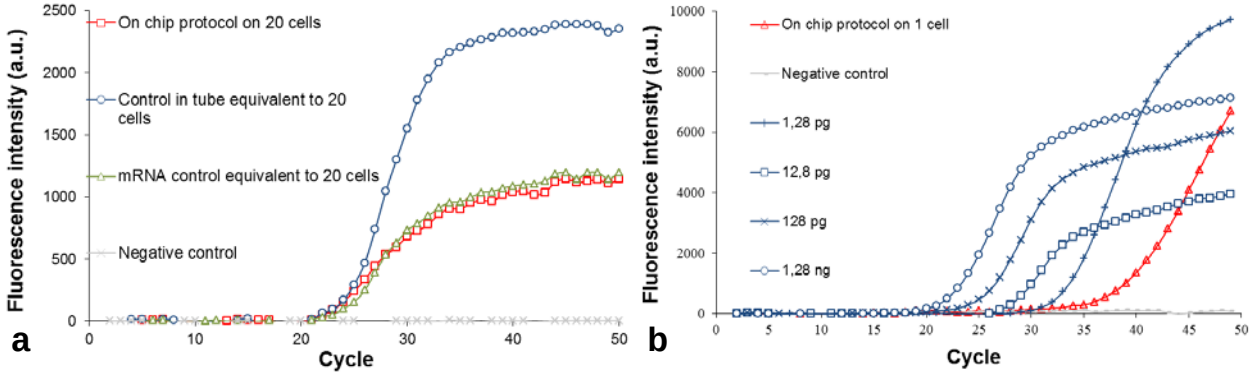

Figure 5. qRT-PCR plots for the Myc gene, showing the validation of the complete protocol for single cell gene expression analysis realized on chip using either a) 20 cells in the droplet or b) 1 single cell in the droplet.

inhibitors of the RT and PCR reactions that may originate from the cell itself. However, one can hypothesize that a single cell would not provide enough inhibitors to affect the enzymes, and many commercially available kits utilize protocols that perform the RT-PCR reaction directly on the single cell content. Thus, we decided to directly perform the on chip qRT-PCR reactions for single cells without any sample preparation. From a cell suspension at low concentration, a droplet was dispensed, containing a single cell and merged directly with another droplet containing the qRT-PCR master mix (2x concentration master mix with primers and probes for the Myc gene and enzymes). The cell is directly lysed when the entering into contact with the master mix at high temperature. The operation was repeated ten times to obtain ten reactions for ten single cells in individual droplets. The qPCR plots obtained (figure 6a) exhibited a nice exponential shape, confirming the absence of inhibition with the single cell. The Ct values were divided into two categories, as we were able to discriminate two Ct ranges (figure 6b). The first cell type had a Ct of approximately 27-28, whereas the second type had a Ct of approximately 31, which suggests that 2 subpopulations of cells were present in this experiment. The Cts obtained correspond to the range of expected value for the mRNA concentration in a single cell, i.e. 1 to 10 pg per cell, as shown in Figure 5b.

Discussion

Overall the performance of the system we describe in the present study is comparable to benchtop qRT-PCR instruments but with at least a 20-fold reduction in volume and a much smaller initial samples. The specific design of the microfluidics chip we have developed, as well as its automation, enables the manipulation and isolation of single cells from a larger population and the analysis of gene expression at the single cell level. One can even imagine coupling a FACS chip (Sun[38]) to the device we describe in the present study to provide the sorted single cells and obtain a complete system, enabling cell sorting and gene expression at the single cell level. Such a system would be particularly useful for oncology applications such as the monitoring of circulating tumor cells (Moste[39]).

The very low quantity of mRNA that can be extracted from a single cell could be lost at the droplet interfaces (know issue in microfluidics), preventing the capture by the beads, even with very small volumes. This could explain the 5 Ct difference between the experimental single experiment and the theory (the RNA dilution); however, by using small volumes, we strongly limited the potential inhibition of reverse transcriptase or polymerase by the cell extract. As a consequence, we favor the direct qRT-PCR protocol, even though using this protocol would prevent multiple analyses on the same cell. To perform multiple analyses on a single cell, mRNA extraction and purification would remain necessary. The chip was designed with 22 reservoirs for qRT-PCR, and the study of multiple genes in parallel is therefore physically possible. There is much room for further improvements in the field of single cell sample preparation, and one potential direction to investigate could be the pre-amplification of all the mRNA, prior to the analysis of specific genes.

For precious samples, the use of an automated, dedicated and integrated chip appears very attractive. Indeed, the protocol we describe limits the number of liquid pipetting events to a total of 7, reducing the risk of sample loss, whereas bench protocols with multiple pipette-mediated transfers increase that risk. The integration of the complete protocol on a single device that is completely automated is also advantageous for the prevention of sample contamination and sample degradation, while dramatically increasing robustness. We observed a very good reliability for qRT-PCR reactions performed from only 20 cells. Furthermore, the low Ct value of 22 that was observed with our device provides the opportunity for at least a 10-fold dilution factor of the sample, enabling more genes to be analyzed in parallel. The system we have developed in the present study will certainly favor the development of automated, dedicated, and disposable devices designed to perform multiplexed qRT-PCR analyses on very small samples, to monitor the environment either at the patient bed side or in the field. Its already very compact size could be further reduced with the use of miniaturized and integrated instrumentation to reach an overall system of the size of a desktop computer, thus becoming smaller than most commercial microfluidics systems.

Previous works reported on electrowetting based devices were used to control cell suspensions and to manipulate cells individually. For example, Shah et al. showed manipulation of cell suspensions by electrowetting and cell concentration with optical tweezers. But herein, for the first time on a single electrowetting chip, we demonstrated the integration and automation of a complete protocol of gene expression analysis, starting from a few human cells and even at the single cell level. To date, there are several types of microfluidic chips described in the literature that enable either cell isolation, some part of the mRNA extraction and purification step, or qRT-PCR; however, rarely are all three steps or even any combination of two steps performed on the same chip. However, some of these devices exhibit good specifications. For instance, a system based on droplet microfluidics in microchannels is capable of capturing 100 single cells in single droplets containing a qRT-PCR master mix and of completing the assay in less than 2 h (Mary[18]). Using a multilayer technology enabling microfluidics valves and, therefore, small chambers, White's group developed a system with an even higher throughput: 300 cells in 300 pL chambers, capable of

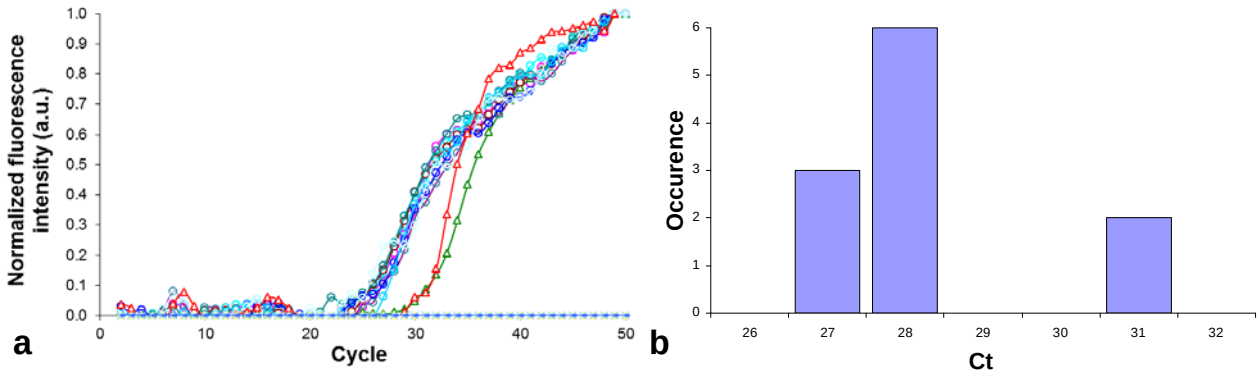

Figure 6. qRT-PCR of the Myc gene, realized on the same chip, from 10 individual single cells in 10 droplets (1 cell per reaction droplet). a) qPCR plots normalized between 0 and 1. b) Ct occurrence for the ten qRT-PCR droplets, showing two different populations among the cells.

cell capture followed by qRT-PCR, with each fluid being added step by step (White[17]). The electrowetting system we are presenting here provides a smaller throughput than continuous flow or flow focusing devices and provides analysis on a few tens of cells. However, this system is more adapted to precious samples or low throughput analysis. Indeed, a complete control of the process is possible because the digital microfluidics technology enables individual droplets to move independently, which also helps in the recovery of the droplets from unwanted operations. Moreover, more steps are possible, such as the extraction and purification of coding or non-coding RNA, which has not been proposed in the two previous systems. This additional step, the core of most sample preparation protocols, is usually avoided for very small samples because of the risk of losing material. Beyond gene expression, the device we describe here could open new avenues in genomic analysis, including sample preparation for next generation sequencing, RNA-seq, ChIP-seq, and genotyping from a limited number of cells.

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