ADVANCED HEALTHCARE MATERIALS
www.advhealthmat.de

"Cells-to-cDNA on Chip": Phenotypic Assessment and Gene Expression Analysis from Live Cells in Nanoliter Volumes Using Droplet Microarrays

Shraddha Chakraborty, Charlotte Luchena, Jonathan J. Elton, Marcel P. Schilling, Markus Reischl, Margaux Roux, Pavel A. Levkin, and Anna A. Popova**

In vitro cell-based experiments are particularly important in fundamental biological research. Microscopy-based readouts to identify cellular changes in response to various stimuli are a popular choice, but gene expression analysis is essential to delineate the underlying molecular dynamics in cells. However, cell-based experiments often suffer from interexperimental variation, especially while using different readout methods. Therefore, establishment of platforms that allow for cell screening, along with parallel investigations of morphological features, as well as gene expression levels, is crucial. The droplet microarray (DMA) platform enables cell screening in hundreds of nanoliter droplets. In this study, a "Cells-to-cDNA on Chip" method is developed enabling on-chip mRNA isolation from live cells and conversion to cDNA in individual droplets of 200 nL. This novel method works efficiently to obtain cDNA from different cell numbers, down to single cell per droplet. This is the first established miniaturized on-chip strategy that enables the entire course of cell screening, phenotypic microscopy-based assessments along with mRNA isolation and its conversion to cDNA for gene expression analysis by real-time PCR on an open DMA platform. The principle demonstrated in this study sets a beginning for myriad of possible applications to obtain detailed information about the molecular dynamics in cultured cells.

1. Introduction

The recent advances in the field of combinatorial chemistry and genomics have contributed to the popularity of cell screening experiments.[1–3] The most important advantage of in vitro cell culture is ensuring a controlled physicochemical culture environment for the cells that is free from systemic variations,[4,5] thereby serving as an ideal system to analyze individual biological response of cells to a broad range of external stimuli (i.e., temperature[6,7] or pH change,[8,9] drugs,[10–12] cytokines,[13,14] nanoparticles,[15,16] small interfering RNAs (siRNA)[17–19] etc.). Along with evaluation of phenotypic changes in cells during various biological experiments, it is important to obtain the information about alternations in the transcriptome of treated cells.[20–22] This is because not all stimuli can be reflected in morphological changes, but still can influence the expression pattern of a plethora of genes regulating a number of cellular pathways.[23,24]

S. Chakraborty, C. Luchena, J. J. Elton, P. A. Levkin, A. A. Popova
Institute of Biological and Chemical Systems-Functional Molecular Systems
Karlsruhe Institute of Technology
Hermann-von-Helmholtz-Platz 1, Eggenstein-Leopoldshafen 76344, Germany
E-mail: levkin@kit.edu; anna.popova@kit.edu

M. P. Schilling, M. Reischl
Institute for Automation and Applied Informatics
Karlsruhe Institute of Technology
Hermann-von-Helmholtz-Platz 1, Eggenstein-Leopoldshafen 76344, Germany

M. Roux
Cellenion SASU
Bioserra 2, 60 avenue Rockefeller, Lyon 69008, France

P. A. Levkin
Institute of Organic Chemistry
Karlsruhe Institute of Technology
Fritz-Haber Weg 6, Karlsruhe 76131, Germany

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adhm.202102493

DOI: 10.1002/adhm.202102493

Usually the workflows for phenotypic cell-based screening and gene expression analysis of cells are performed using different platforms and involve a number of steps. Traditional microtiter plates are suitable for cell culture, incubation with different compounds and subsequent phenotypic analysis in microliter volumes. Thereafter cells are usually taken out of microtiter plates or lysed for mRNA isolation in microtubes. Finally, the complementary DNA (cDNA) is synthesized and purified in PCR microtubes, and carried forward for subsequent gene expression analysis. However, in vitro studies are prone to inter and intraexperimental variation.[25,26] Therefore, a platform that allows to use the same set of samples for both microscopy-based assays and gene expression analysis could be very useful to increase the reliability and reproducibility of a cell-based experiment. Moreover, wells in microtiter plates and microtubes are surrounded by boundaries that are believed to hinder efficient isolation of genetic material from low number of cells by nonspecific adsorption of nucleic acids to polymer walls.[27,28] Also the multiple-step protocols and the need for transferring the input material to different wells and microtubes during the entire experimental procedure further increases the loss of nucleic acids, which is especially critical when working with low cell numbers or single cells. Miniaturization and parallelization of experimental steps for sample preparation starting from live cells are very important in order to increase the efficiency of nucleic acid isolation and reduce the experimental budget and time. Hence the combination of high throughput sample preparation for gene expression analysis with prior incubation of cells in the presence of various stimuli within miniaturized volumes would be a game-changer, opening numerous possibilities to analyze the changes in cellular transcriptome.

Impressive advancements have been made in developing the protocols for miniaturized and high throughput sample preparation, including solid-phase, droplet-based or nanowell-based microfluidic systems for direct isolation of nucleic acids from cell lysates. Nestorova et al. reported an automated technique for mRNA extraction, purification, and reverse transcription, based on solid-phase microfluidics.[29] Kim et al. developed a droplet microfluidics-based single-step chemical lysis approach for high-throughput single-cell reverse transcription polymerase chain reaction (RT-PCR), where the cells are lysed in buffer with high pH and merged with subsequent reagents for gene expression analysis.[30] As one step further towards assessment of global transcriptomic landscape, Gierahn et al. have established a nanowell-based platform "Seq-well", enabling massively parallel single-cell lysis, barcoding of mRNA, and transcriptomic analysis.[31] Macosko et al. reported the "Drop-seq" protocol that involves encapsulation of cells within water-in-oil droplets for parallelized single-cell transcriptomic studies.[32] However, to the best of our knowledge, till date no platform has been reported that can support the entire workflow of cell culture, screening, phenotypic assessment, mRNA isolation and cDNA preparation for gene expression analysis, in a miniaturized format. Microfluidic-based approach is indispensable for high throughput and single-cell resolution in transcriptome when a bulk cell population has to be analyzed. However, the use of this approach in combination with screening of large compound libraries can be cumbersome, due to the need to introduce multiple channels and/or complex droplet recognition systems, along with the risk of cross-contamination of the droplets.[33–35] In addition, droplet microfluidic approach is not always compatible with cultivation of adherent cells due to the absence of the surface to attach themselves.

In our laboratory, we have developed a "Droplet Microarray" (DMA) platform, formed by superhydrophobic–hydrophilic patterning.[36–43] Using this platform, various cell-based experiments are possible in hundreds to thousands of micro- or nanoliter cell culture reservoirs, separated by superhydrophobic borders on a single microscope glass slide. DMAs can be prepared on various types of substrates, e.g., nanostructured glass,[37] porous poly(2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) polymer (HEMA-EDMA),[38,39] poly(thioether) dendrimers[44] etc.—having a broad range of surface topography and roughness. We have demonstrated that HeLa-CCL2 cells cultured on different biomaterial-coatings used for fabrication of DMA, and in droplets of as low as 9 nL volumes exhibit no drastic transcriptomic alternations in comparison to cells grown in microtiter plates.[45] The DMA platform has enabled culturing and screening of variety of cells in thousands of separated nanoliter droplets, making it a versatile, miniaturized, wall-free, high throughput platform for stem cells,[37] bacterial cells,[41] 2D and 3D mammalian cell culture,[36,46] single cells,[42] embryoid bodies[43] and even zebrafish (*Danio rerio*) embryos.[47]

In this study, we for the first time established and evaluated "Cells-to-cDNA on Chip" methodology on DMA platform, which includes culturing cells, microscopy-based evaluation, mRNA isolation, and conversion to cDNA—all on an array of 200 nL droplets, followed by gene expression analysis from as low as a single cell per sample. Parallelization and miniaturization of the entire workflow of cell culture to cDNA synthesis down to nanoliter volumes greatly reduces the time of experiment and reagent consumption, thereby decreasing the experimental cost. Moreover, DMA platform used in this study does not have any solid boundary and allows for confinement of miniaturized reaction volumes within a small surface area. Both these characteristic features of DMA could contribute to reduce the chances of loss of nucleic acids from low number of cells due to nonspecific adsorption, as observed in PCR microtubes.[27,28] Thus, we demonstrate the possibility to combine phenotypic screening with gene expression analysis of treated cells on miniaturized arrays of nanoliter droplets.

2. Results and Discussion

2.1. Concept of "Cells-to-cDNA on Chip" Method

The concept of "Cells-to-cDNA on Chip" method based on the droplet microarray platform is demonstrated in **Figure 1**. DMA platform is based on an array of hydrophilic spots on a superhydrophobic background that enables formation of arrays of separated and stable aqueous droplets of volumes ranging from microliters to nanoliters on plain wall-less and transparent surface. These droplets can be used as miniaturized wells for culturing and screening of live cells. As a first step of the "Cells-to-cDNA on Chip" methodology, cells of interest are dispensed onto hydrophilic spots using a non-contact liquid dispenser, where they can be introduced to various stimuli of interest, i.e., drugs, siRNA, nanoparticles, cytokines, biomimetic coatings etc. (Figure 1). As a second step, cell morphology and phenotypic changes in response to respective stimuli after incubation with the same

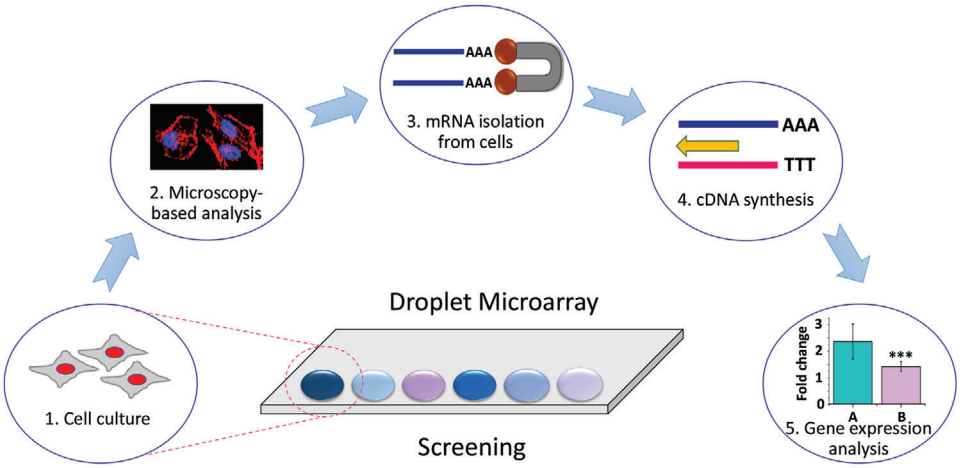

Figure 1. Schematic representation of "Cells-to-cDNA on Chip" method. 1 = cell culture and screening in micro/nanoliter droplets on DMA, 2 = phenotypic analysis of cells by microscopy-based methods, 3 = cell lysis and isolation of mRNA using poly-T magnetic beads, 4 = mRNA to cDNA conversion within the same droplet, 5 = collection of cDNA for qualitative and quantitative gene expression analysis.

can be assessed using optical microscopy. As a third step, cells are lysed in the same hydrophilic spots by addition of a suitable cell lysis buffer and isolation of mRNA from the cell lysate in the droplets is performed using poly-T magnetic beads. As a fourth step, mRNA in each spot is converted to cDNA directly onto the same droplet on DMA. Finally, the so-formed cDNA from individual spots is collected and used for gene expression analysis using PCR and real-time PCR (qPCR) techniques. "Cells-to-cDNA on Chip" method combines cell screening and gene expression analysis of cells in individual droplets. It enables parallel high-throughput sample preparation starting from live cells to cDNA synthesis in several hundreds of droplets with volumes as low as 200 nL. It can be applied either for screening applications, where cells in the droplets could undergo incubation with different stimuli followed by gene expression analysis; or as a sample preparation platform, where cDNA can be synthesized from low cell number, down to single cell. In this work for the first time, we have optimized and demonstrated the entire methodology for "Cells-to-cDNA on Chip", including the steps of cell culture, cell lysis, isolation of mRNA and cDNA synthesis—all in single separated droplets on a DMA chip in volumes ranging from 200 nL to 5 µL, using cell numbers ranging from 1 to 1000.

2.2. mRNA to cDNA Conversion on Droplet Microarray

As a first step, we have optimized the workflow for conversion of total RNA to cDNA on DMA chip in micro- and nanoliter volumes. Total RNA was isolated from cultured HeLa-CCL2 cells following standard protocol. DMAs of two formats were used for optimization: i) 80-spot (5×16) array with circular spots of 3 mm diameter accommodating 5 µL total reaction volume [3 mm DMA], and (ii) 672-spot (14×48) array of square spots with 1 mm side length accommodating 200 nL total reaction volume [1 mm DMA] (Figure S1, Supporting Information). The static water contact angles of the superhydrophobic borders and hydrophilic spots were previously calculated to be 157° and 16°, respectively.[48]

On the DMA platform, the droplet volume can be as small as few nL; therefore in order to avoid evaporation of the droplets, we first have optimized a set-up that allowed us to use a standard thermocycler machine for performing high temperature reaction steps required for nucleic acid manipulations, without a need to cover the sample with mineral oil. We have therefore fabricated a "humidity chamber" for DMA, optimizing few components that included i) a metal in situ adaptor for the heating block of thermocycler, ii) a thermostable 3D printed lid to cover the DMA placed on top of the metal adaptor, and iii) sterile tissues wetted with appropriate volume of nuclease-free water. Detailed description of the humidity chamber has been provided in the experimental section and in Figure S2, Supporting Information. This so-formed humidity chamber was evaluated for preventing evaporation of the droplets on DMA during high temperature steps (up to 80 °C) required for cDNA synthesis. To test the humidity chamber, we first performed the entire program for cDNA synthesis on 3 mm DMA in thermocycler, using 5 µL nuclease-free water on the hydrophilic spots. No merging and evaporation of the droplets on DMA chips were observed before or after the cDNA synthesis program was completed (Figure S3A,B, Supporting Information).

As the next step, conversion of DNase-treated total HeLa-CCL2 RNA to cDNA was attempted on DMA in 5 µL (3 mm DMA) and 200 nL (1 mm DMA) final reaction volumes. All reagents were manually added by pipetting (3 mm DMA) or dispensed by a nanoliter volume dispenser (1 mm DMA) onto the hydrophilic spots. **Figure 2**A shows a typical workflow of cDNA synthesis protocol on 1 mm DMA. Briefly, HeLa-CCL2 total RNA, dNTPs and oligo dT primers/random hexamers were pipetted/dispensed onto the hydrophilic spots of the respective DMA, and incubated at the thermocycler at 65 °C for 10 min. Next, the DMA was kept on ice for 2 min, reverse transcription cocktail was pipetted/dispensed onto the hydrophilic spots and it was incubated inside the thermocycler at 52 °C for 15 min (cDNA synthesis) and 80 °C for 12 min (termination of the reaction). As an optional step, RNase H was pipetted/dispensed onto the spots containing cDNA and incubated at 37 °C for 20 min. Finally, the cDNA

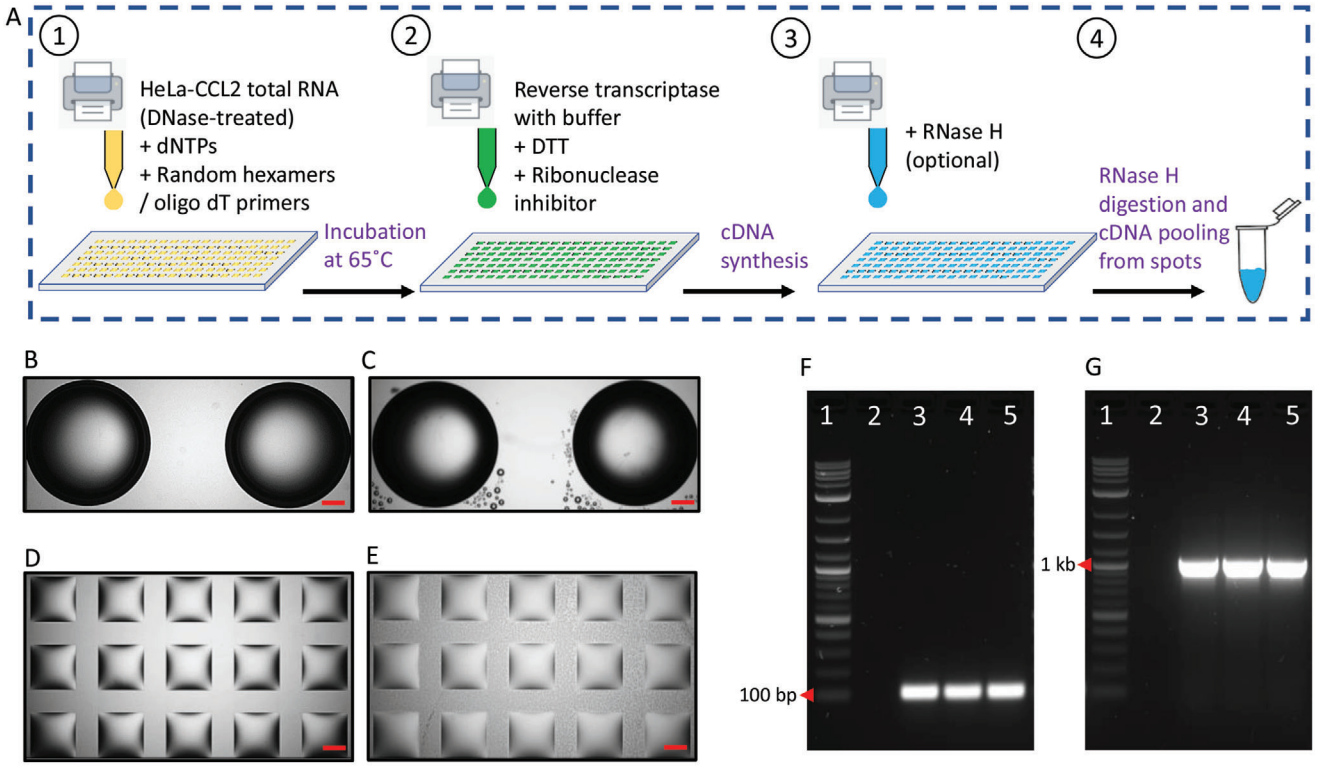

Figure 2. cDNA synthesis starting from total HeLa-CCL2 RNA on droplet microarray. A) Schematic protocol for cDNA synthesis on DMA. Bright-field microscope images of B,C) 5 µL and D,E) 200 nL droplets on hydrophilic spots of 3 mm and 1 mm DMA, respectively, B,D) before and C,E) after the cDNA synthesis reaction. Scale bar = 500 µm. F) Gel electrophoresis image of *GAPDH* PCR (product size = 112 bp) performed with cDNA synthesized from 50 ng HeLa-CCL2 total RNA. [Lane number 1 = 1 kb DNA ladder, 2 = no template (negative) control, 3 = PCR product of cDNA synthesized in microtube (positive control), 4 = PCR product of cDNA synthesized on 3 mm DMA in volume of 5 µL, 5 = PCR product of cDNA synthesized on 1 mm DMA in volume of 200 nL]. G) Gel electrophoresis image of *ACTB* PCR (product size = 1045 bp) performed with cDNA synthesized from 50 ng HeLa-CCL2 total RNA. Sample sequence in gel lanes is same as Figure 2F.

from each hydrophilic spot was collected into a PCR microtube, as described in detail at the experimental section. List of all the reagents and input volumes for 3 and 1 mm DMA have been listed in **Table 1**. Figure 2B–E presents bright-field microscope images of 3 and 1 mm DMA, before and after the cDNA synthesis reaction inside thermocycler. We observed no evaporation of the droplets after the temperature steps. Although there was a trace of condensation on superhydrophobic background of the DMA slides after the reaction (Figure 2C,E), no merging of consecutive droplets could be observed.

To check if conversion of RNA to cDNA on the Droplet Microarray was successful, we have performed PCR for two housekeeping genes-*GAPDH* and *ACTB*. cDNA synthesized from total HeLa-CCL2 RNA (50 ng) in a PCR microtube according to the standard protocol was used as the positive control. Gel electrophoresis was performed for the PCR products from cDNA synthesized on 3 and 1 mm DMA, and prominent bands could be observed for both the genes at the right size [112 bp and 1045 bp for *GAPDH* and *ACTB*, respectively] (Figure 2F,G). Henceforth, we were able to optimize the setup for a critical step toward the establishment of "Cells-to-cDNA on Chip" method on DMA- the conversion of total RNA to cDNA on the DMA chip. This opens up an opportunity to do high-throughput manipulation of nucleic

Table 1. Reagent volumes for cDNA synthesis on 3 mm and 1 mm DMA.

Experimental steps/reagents	Reagent volume per hydrophilic spot	
	3 mm DMA (Total volume: 5 µL)	1 mm DMA (Total volume: 200 nL)
Annealing primer to template RNA		
HeLa-CCL2 total RNA	50 ng	1 ng
10 mM dNTPs	0.25 µL	10 nL
50 µM oligo dT primers or 50 ng µL^{-1} random hexamers	0.25 µL	10 nL
Nuclease-free water	to 3 µL	to 120 nL
Reverse transcription		
5× reverse transcription buffer	1 µL	40 nL
100 mM dithiothreitol (DTT)	0.25 µL	10 nL
Ribonuclease inhibitor (40 U µL^{-1})	0.25 µL	10 nL
Superscript IV reverse transcriptase (200 U µL^{-1})	0.25 µL	10 nL
RNase H (2 U µL^{-1}) [optional]	0.25 µL	10 nL

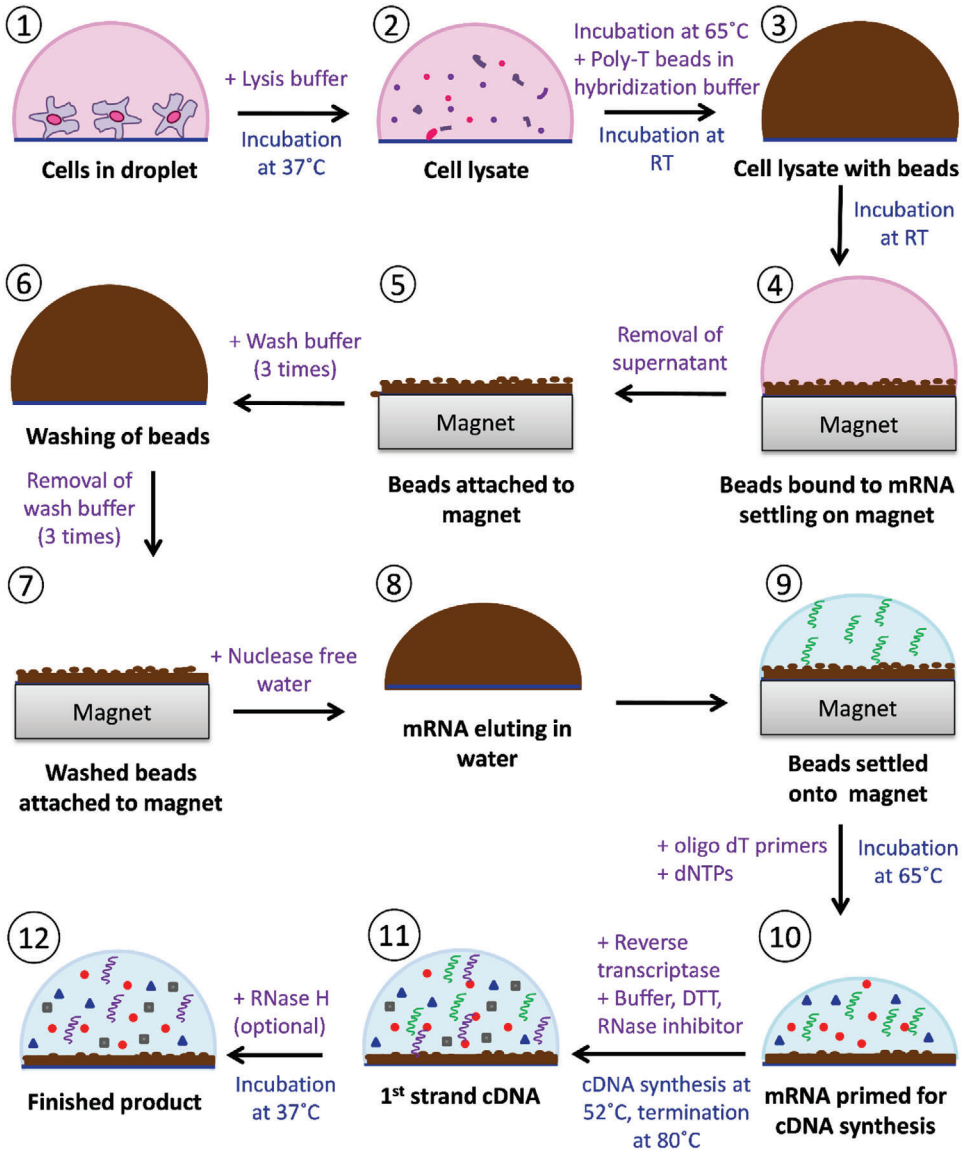

Figure 3. Detailed schematic workflow of "Cells-to-cDNA on Chip" method on Droplet microarray. RT = room temperature.

acids on open DMA chips for up to 672 droplets in volumes as low as 200 nL, without a need for any additional medium (e.g., mineral oil) to prevent evaporation.

As a confirmation to monitoring the extent of droplet evaporation during cDNA synthesis on DMA, static WCA of the droplets were measured both before and after the experiment (Figure S4A,B, Supporting Information). Moreover, total reaction volumes were measured from all 3 hydrophilic spots after cDNA synthesis (Figure S4C, Supporting Information). From the results, it could be observed that only a minute portion of droplet volume (less than 8%) is lost after the reaction (Figure S4C, Supporting Information), along with a subsequent reduction in static WCA. However, this evaporation/condensation does not seem to influence the cDNA synthesis reaction within droplets, as shown in Figure 2.

2.3. "Cells-to-cDNA on Chip" Method in 5 µL Droplets on 3 mm Droplet Microarray

Our envisioned "Cells-to-cDNA on Chip" methodology includes the following steps: 1) cell culture, 2) cell lysis, 3) mRNA extraction from cell lysate and 4) cDNA synthesis—all in single droplets on a DMA chip. The workflow of the entire "Cells-to-cDNA on Chip" method is demonstrated in **Figure 3**. In order to perform the steps of cell lysis and mRNA isolation on DMA according to "Cells-to-cDNA on Chip" method, a total of 1000 HeLa-CCL2 cells per hydrophilic spot on 3 mm DMA were manually seeded in 3 µL media, cultured for 24 h (Figure 3, step 1) and observed under a microscope (Figure S5A, Supporting Information). Next, the cells were lysed at 37 °C for 1 h by the addition of 2 µL RLT lysis buffer per hydrophilic spot (Figure 3, step 2), and were monitored every

Table 2. Reagent volumes for "Cells-to-cDNA on Chip" method on 3 mm and 1 mm DMA.

Experimental steps/reagents	Reagent volume per hydrophilic spot	
	3 mm DMA (Total volume: 5 µL)	1 mm DMA (Total volume: 200 nL)
Cell seeding		
Number of cells	1000	100
Culture medium	3 µL	100 nL
mRNA isolation		
Phosphate-buffered saline (×2)	–	200 nL
Lysis buffer	2 µL	150 nL
Poly-T magnetic beads in hybridization buffer	3 µL	100 nL
Wash buffer (×3)	5 µL	200 nL
Nuclease free water for mRNA elution	2.5 µL	100 nL

30 min under the microscope (Figure S5B,C in Supporting Information, respectively). Afterwards, the DMA was kept within the humidity chamber for 10 min at 65 °C inside a thermocycler to linearize the mRNA. Thereafter the DMA with the humidity chamber was put immediately onto ice to cool down. Next, the DMA was brought to room temperature within humified Petri dish and 3 µL poly-T magnetic beads in mRNA hybridization buffer were added to the cell lysate on DMA (Figure 3, step 3). Following a 5 min incubation at room temperature, the DMA was placed onto a magnet for 1 min to settle the mRNA-bound poly-T beads (Figure 3, step 4), and the supernatant was aspirated with a pipette (Figure 3, step 5). Next, beads were washed three times with 5 µL wash buffer, as described in the experimental section (Figure 3, step 6-7). Then the beads were resuspended into 2.5 µL nuclease-free water to elute the mRNA (Figure 3, step 8). Figure S5D, Supporting Information represents bright-field microscope images of the 3 mm DMA with cell lysate and magnetic beads on the hydrophilic spots at different experimental steps, indicating no merging of droplets during the entire workflow. Next, the beads were settled onto magnet again and reverse transcription was carried out with the isolated mRNA on the same hydrophilic spot (Figure 3, step 9–12), having the reagent volumes as described in Table 1. Volumes of reagents needed for cell seeding, lysis, and mRNA isolation steps according to "Cells-to-cDNA on Chip" method on 3 mm DMA are listed in **Table 2**. Upon completion of the reaction, the DMA was placed onto magnet again, and resultant cDNA (5 µL) from each spot was aspirated and taken into a standard PCR microtube for downstream analysis. To counteract the influence of any genomic DNA contamination in trace amounts during mRNA isolation, all the primers (especially the ones for qPCR) were designed on the exon–exon boundaries.

2.3.1. Qualitative and Quantitative Gene Expression Analysis with Different Number of Cells and Various Cell Types

In order to check if the cDNA could actually be synthesized from live cells on 3 mm DMA using our novel "Cells-to-cDNA on Chip" methodology, PCR and gel electrophoresis were performed for *GAPDH* and *ACTB* genes. **Figure 4**A,B shows the gel electrophoresis images for products from *GAPDH* and *ACTB* PCR, performed with the cDNA synthesized on 3 mm DMA along with the usual control PCR reactions. From our results, we can conclude that the entire workflow of "Cells-to-cDNA on Chip" method can be performed successfully on 3 mm DMA, in 5 µL reaction volume.

As a next step, we checked the possibility of quantitative gene expression studies followed by "Cells-to-cDNA on Chip" method on DMA. We selected qPCR technique to perform the quantitative assessment of cDNA synthesized on DMA in each hydrophilic spot. For this experiment, varying number of HeLa-CCL2 cells (10, 100, 500, and 1000) were seeded in a culture volume of 3 µL onto the 3 mm DMA. Samples with different cell numbers were placed in consecutive rows to ensure their close proximity to each other. Next, cDNA was prepared from the varying number of cells according to "Cells-to-cDNA on Chip" methodology. The resultant cDNA from each spot was collected into different wells of a PCR microplate and qPCR was performed with the same using *GAPDH* primers. Figure 4C shows comparison of Ct values from each group of cell numbers after performing qPCR. As expected, we observed an inverse relationship of Ct values with the number of cells per spot (Figure 4C). The differences between average Ct values (from 5 spots) for consecutive cell number groups were also statistically significant, [i.e., 10 vs 100 cells ($^{***}P < 0.001$), 100 versus 500 cells ($^{***}P < 0.001$) and 500 versus 1000 cells ($^{**}P < 0.01$)] with minimal spot-to-spot variability as depicted by the standard error of mean for each group (Figure 4C). This data indicates that—i) there is no cross-contamination between droplets during cDNA preparation, ii) there is uniformity between the amount of cDNA retrieved from a particular number of cells from different hydrophilic spots on DMA and iii) it is possible to synthesize qPCR-quality cDNA from a mere input of 10 cells on DMA.

We have compared the performance of "Cells-to-cDNA on Chip" method on DMA and PCR plate. Toward this goal, we performed "Cells-to-cDNA on Chip" method in a PCR microplate, using the same number of cells [10, 100, 500, and 1000 cells per well] in the same 5 µL volume. Here we observed higher Ct values for all cell number groups, since on average 2 more qPCR cycles were needed to detect a signal from each cell number group, as compared to that of the same on DMA (Figure 4D). Although we could observe a significant inverse relationship between cell numbers and Ct values, the difference in Ct values obtained from 500 and 1000 cells was not statistically significant (Figure 4D). This indicates that "Cells-to-cDNA on Chip" methodology on DMA has better efficiency compared to PCR microplate, which could be due to non-specific adsorption of nucleic acids on the walls of the plate, as shown in other studies.[27,28]

As additional experiment confirming no cross-contamination in between droplets, HeLa-CCL2 and HeLa-GFP cells were seeded onto consecutive spots on a 3 mm DMA. The cells were cultured for 24 hours followed by "Cells-to-cDNA on Chip" protocol. Figure S6A (Supporting Information) shows the fluorescence microscopy images of both the cell lines after 24 h of culture on DMA. In congruence with microscopy data (Figure S6A, Supporting Information), a 200 folds higher expression of GFP transcripts was observed in HeLa-GFP cells by qPCR, upon

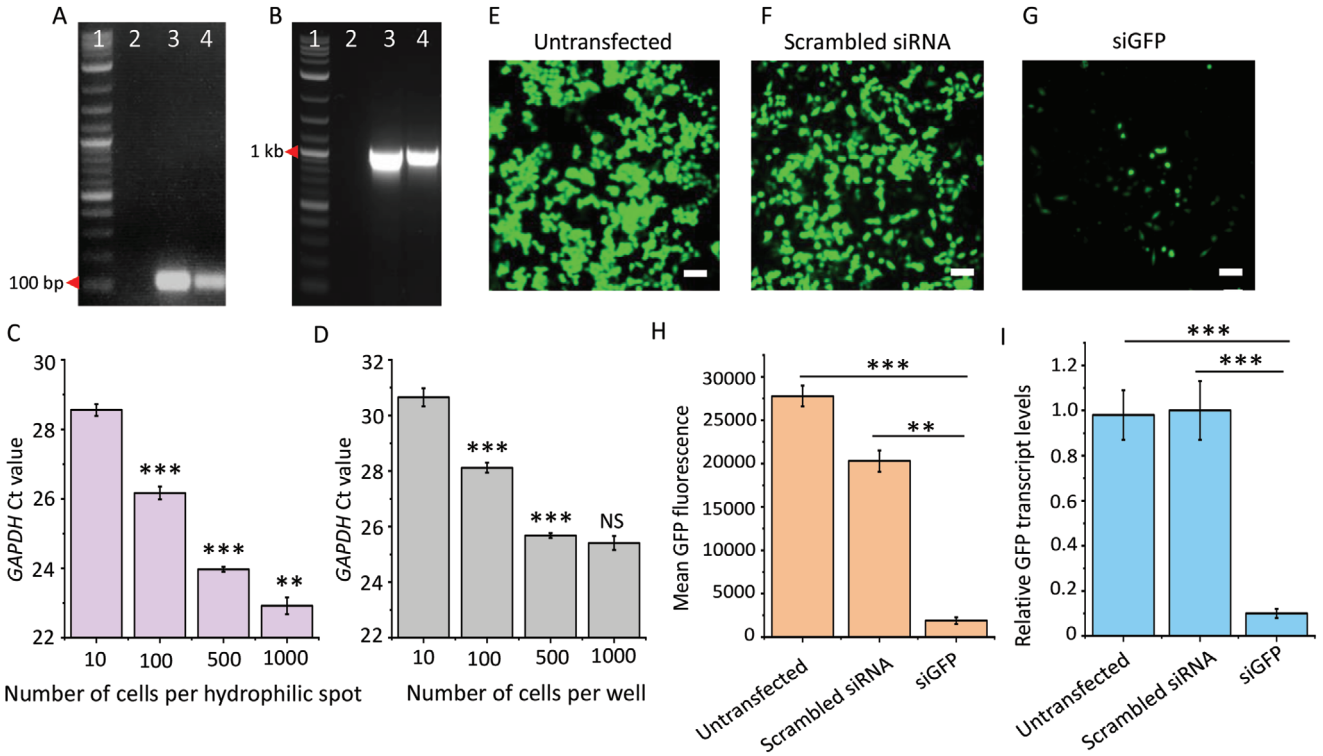

Figure 4. Qualitative analysis and gene expression quantification for different number of cells and siRNA-mediated targeted gene knockdown by "Cells-to-cDNA on Chip" method on 3 mm DMA. A,B) Gel electrophoresis image for *GAPDH* (product size = 112 bp) and *ACTB* (product size = 1045 bp) PCR respectively. [Lane number 1 = 1 kb DNA ladder, 2 = no template (negative) control, 3 = PCR product of cDNA synthesized in microtube from HeLa-CCL2 cells using standard protocol (positive control), 4 = PCR product of cDNA synthesized from HeLa-CCL2 cells on 3 mm DMA, via "Cells-to-cDNA on Chip" method]. (C-D) qPCR results of cDNA synthesized from HeLa-CCL2 cells via "Cells-to-cDNA on Chip" method on 3 mm DMA and PCR plate, respectively. Bar graphs from both C and D represent Ct values for 10, 100, 500 and 1000 cells, each with 5 replicates. Error bar represents mean ± SEM. $^{***}P < 0.001$, $^{**}P < 0.01$ according to two-tailed unpaired *t*-test, calculated as a comparison between consecutive groups. NS = not significant. Assessment of siGFP transfection to HeLa-GFP cells on 3 mm DMA: E–G) Fluorescence microscopy images showing GFP expression in untransfected, scrambled siRNA-transfected and siGFP-transfected cells, respectively (after 48 hours of transfection). Scale bar = 100 µm. H) Mean GFP fluorescence in HeLa-GFP cells post 48 h of transfection. Data presented as mean±SEM from 3 individual experiments. $^{***}P < 0.001$, $^{**}P < 0.01$ according to two-tailed unpaired *t*-test. I) qPCR for GFP transcript levels in cells post 48 h of transfection on 3 mm DMA; cDNA synthesized via "Cells-to-cDNA on Chip" method. Data normalized to *GAPDH* expression, and presented as mean±SEM, from 3 individual experiments. $^{***}P < 0.001$ as calculated by two-tailed unpaired *t*-test.

comparison with HeLa-CCL2 cell line which had no GFP expression (Figure S6B, Supporting Information).

2.3.2. siRNA-Mediated Knockdown of Target Gene and Quantitative Gene Expression Analysis

In order to further demonstrate applicability of "Cells-to-cDNA on Chip" method for different cellular assays, we performed targeted gene knockdown using siRNAs on 3 mm DMA, coupled with evaluation of the results by both microscopy-based readouts and qPCR for gene expression analysis. HeLa-GFP cells that are stably transfected with green fluorescent protein (GFP) were used for this experiment. A total of 1000 HeLa-GFP cells were seeded per hydrophilic spot onto a 3 mm DMA. Next, cells were transfected with an anti-GFP siRNA (siGFP) using ScreenFect A transfection reagent, via a one-step reverse transfection methodology as described in the experimental section. Untransfected cells and cells transfected with scrambled siRNA were taken as controls for the experiment. Figure 4E–G demonstrates the fluorescence microscope images of untransfected, scrambled siRNA-transfected and siGFP-transfected cells 48 h posttransfection, respectively. An efficient GFP knockdown in HeLa-GFP cells transfected with siGFP on DMA was indicated by the reduction in GFP fluorescence in the respective group of cells (Figure 4G). GFP fluorescence for each sample group was further quantified, and calculated as over 90% fluorescence reduction in the siGFP-transfected cells, in comparison to those transfected with scrambled-siRNA (Figure 4H). Next, cDNA was isolated using "Cells-to-cDNA on Chip" method from the same hydrophilic spots on DMA and qPCR was performed with the resultant cDNA for GFP expression. Figure 4I shows the relative GFP expression (with *GAPDH* as normalizing control) in all the three groups (untransfected, transfected with scrambled siRNA and siGFP), that confirms about 90% knockdown of GFP transcripts in the siGFP-transfected cells on DMA, with respect to both the other groups under consideration. These results on quantitative gene expression analysis post siRNA transfection in cells on DMA are important to re-emphasize the absence of cross-contamination between droplets on DMA during the entire course of experiments from

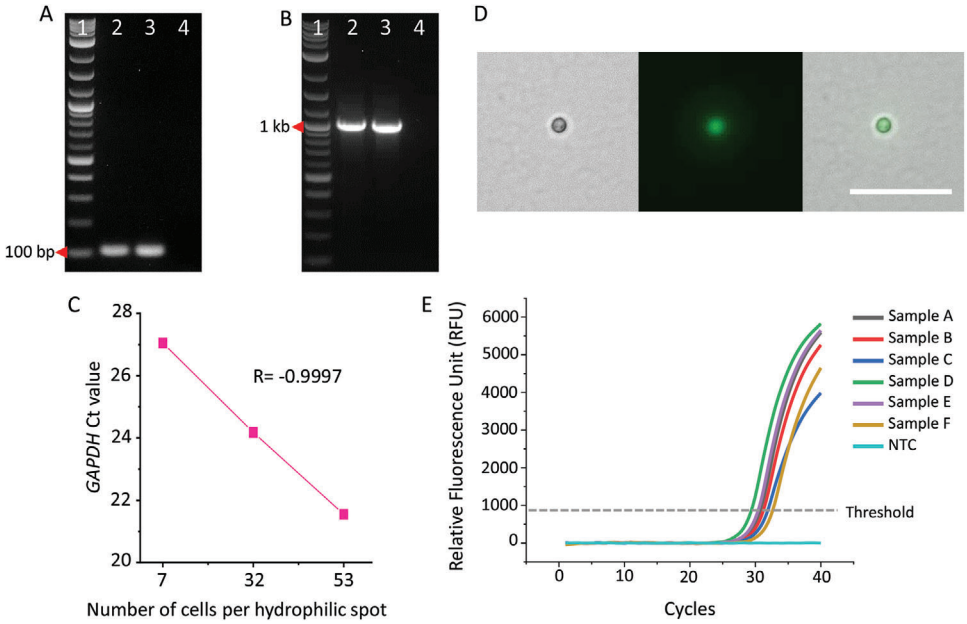

Figure 5. Quantitative and qualitative gene expression analysis with different number of HeLa-GFP cells by "Cells-to-cDNA on Chip" method on 1 mm DMA. A,B) Gel electrophoresis image for *GAPDH* (product size = 112 bp) and *ACTB* (product size = 1045 bp) PCR, respectively. [Lane number 1 = 1 kb DNA ladder, 2 = PCR product of cDNA synthesized from HeLa-GFP cells in microtube using standard protocol (positive control), 3 = PCR product of cDNA synthesized from HeLa-GFP cells on 1 mm DMA using "Cells-to-cDNA on Chip" method, 4 = no template (negative) control]. C) qPCR results of cDNA synthesized from HeLa-GFP cells using "Cells-to-cDNA on Chip" method on 1 mm DMA. Synthesized cDNA was first pooled from 168 spots and then diluted so the content corresponding to a single droplet was used for qPCR reaction. Pink squares represent the average Ct value per hydrophilic spot from each cell number group. R = correlation coefficient. D) Bright-field, fluorescent, and overlaid microscopy image of a single HeLa-GFP cell on 1 mm DMA. Scale bar = 100 µm. E) qPCR amplification curves for 6 technical replicates of cDNA synthesized by "Cells-to-cDNA on Chip" method from single HeLa-GFP cells. NTC = no template control.

live-cell culture to cDNA synthesis. Henceforth we confirm the applicability of "Cells-to-cDNA on Chip" methodology on DMA to perform various cell screening experiments followed by analyzing the phenotypic outcome by microscopy and gene expression analysis.

2.4. "Cells-to-cDNA on Chip" Method in 200 nL Droplets on 1 mm Droplet Microarray

As a next step, we further miniaturized the "Cells-to-cDNA on Chip" methodology down to 200 nL reaction volume on DMAs with 1 mm hydrophilic spots. For all our experiments on 1 mm DMA, we have imaged the DMA before the workflow to determine the exact cell number per spot. Figure S7A–D in Supporting Information show the bright field and fluorescence microscope images of HeLa-GFP cells on 1 mm DMA before and after cell lysis, respectively. In comparison with "Cells-to-cDNA on Chip" method performed on 3 mm DMA, we have optimized a step for parallel removal of liquids with sandwiching method, which is necessary in order to perform washing steps (Figure 3, steps 4–7), simultaneously from the whole array of 672 spots. In this method, the DMA slide containing droplets with magnetic poly-T beads was placed on a neodymium block magnet and sandwiched with a microscope slide coated with a layer of porous hydrophilic HEMA-EDMA polymer, which soaked all the liquid from each individual droplet without mixing of the droplets with each other and without removing the magnetic beads from the spots (Figure S7E–G, Supporting Information). Volumes of reagents needed for cell seeding, lysis and mRNA isolation steps according to "Cells-to-cDNA on Chip" method on 1 mm DMA are listed in Table 2.

2.4.1. Qualitative and Quantitative Gene Expression Analysis

We first performed "Cells-to-cDNA on Chip" method with about 100 HeLa-GFP cells cultured in 100 nL droplets for 24 h and checked the quality of synthesized cDNA by PCR. **Figure 5**A,B demonstrates the gel electrophoresis images of the PCR products for *GAPDH* and *ACTB*, respectively. The results indicate successful cDNA synthesis from cultured cells, in a total of 200 nL reaction volume on 1 mm DMA. Next, a possibility of a quantitative assessment of cDNA synthesized in nanoliter volume by "Cells-to-cDNA on Chip" methodology was tested. Pertaining to this goal, three groups of varying number of HeLa-GFP cells were seeded onto 1 mm DMA by the nanoliter volume dispenser and incubated for 24 h. The whole DMA slide was imaged by fluorescence microscope with 2× magnification before performing the cell lysis (Figure S8A, Supporting Information), and the actual number of cells per spot was estimated automatically using an in-house developed algorithm (Figure S8B, Supporting Information). Next, the cDNA was isolated on 1 mm DMA via "Cells-to-cDNA on Chip" method in 200 nL total reaction volume as described earlier. Figure 5C shows the average Ct values corresponding to different number of cells per hydrophilic spot onto

1 mm DMA. It confirms a distinct inverse relationship [correlation coefficient (R) = -0.9997] between qPCR Ct values and cell numbers on DMA. Our results confirm that "Cells-to-cDNA on Chip" method in 200 nL droplets on the DMA platform works efficiently to obtain cDNA from varying number of cells and distinguish between them (Figure 5C).

2.4.2. Quantification of Gene Expression from Single Cell per Droplet

As one step further, we have performed "Cells-to-cDNA on Chip" method with single cells on 1 mm DMA. Single cell per hydrophilic spot on DMA was dispensed by a single cell dispenser (Cellenion, France). Figure 5D shows bright-field, fluorescence, and overlaid image of single HeLa-GFP cell after dispensing onto DMA. For single-cell experiments, cell lysis with RLT buffer was carried out only for 5 min on ice. "Cells-to-cDNA on Chip" method was performed as described before for 1 mm DMA, with a few adjustments in the experimental methodology as explained in detail in the experimental section. qPCR was performed for GFP expression with cDNA obtained from single droplet (single-cell cDNA). As shown in Figure 5E, Ct values from single cells were ranging between 29 and 32, whereas there was no amplification for the negative (no template) control. Figure S9A,B in Supporting Information shows the melting curve and melting peak analysis of the same qPCR experiment, indicating no contamination in the samples. These results confirm the applicability of "Cells-to-cDNA on Chip" methodology on DMA for obtaining mRNA and cDNA from a single mammalian cell, in nanoliter droplets.

3. Conclusion

With "Cells-to-cDNA on Chip" methodology, we have for the first time made it possible to perform screenings of live cells down to single cell per sample followed by isolation of mRNA and its conversion to cDNA in a parallel high-throughput manner on an open platform in volumes as low as 200 nL, without a need for using special reagents to prevent evaporation. No cross-contamination was observed between the droplets during the entire experimental workflow. We have demonstrated successful application of the resultant cDNA for quantitative gene expression analysis methods like qPCR, with down to single cells per droplet. "Cells-to-cDNA on Chip" methodology demonstrated superior efficiency on DMA, ascompared to PCR microplates, probably due to reduced nonspecific adsorption of nucleic acid. We could observe 90% reduction in GFP transcripts upon performing qPCR after siGFP transfection to HeLa-GFP cells on DMA, which was in perfect agreement with microscopy-based fluorescence readouts. The "Cells-to-cDNA on Chip" method established here on DMA can be used as a singular experimental strategy for cell culture, screening against various compounds or stimuli, imaging-based assays, isolation of mRNA and high-throughput gene expression analysis- all on one miniaturized platform. It is important because it allows us to obtain information about changes in cell morphology or functionality, and about gene expression level simultaneously from the same cell population. Also since both phenotypic and gene expression analysis can be done with cells in the same droplet, the data obtained is likely to be more reliable and less experimental repetitions are required. In addition, it is crucial to minimize the loss of nucleic acid, in this case mRNA, during isolation steps through nonspecific adsorption on tubes or pipette tips, especially when working with low cell numbers or single cells. Therefore, sample preparation within a single droplet in nanoliter volumes can reduce material loss during multiple experimental steps, resulting in increased sensitivity and at the same time requiring less cell numbers as input. "Cells-to-cDNA on Chip" methodology allows researchers to perform experiments in up to 100 folds miniaturized reaction volumes in comparison to usual lab-based protocols, thereby leading to a drastic reduction in experimental budget.

To the best of our knowledge, "Cells-to-cDNA on Chip" method is the first experimental workflow, which enables combination of high-throughput microscopy-based cell screening in nanoliter format with gene expression analysis of cells after the incubation with different stimuli. The DMA platform carries a number of advantages compared to conventional platforms for cell culture and sample preparation: i) parallelization and higher throughput, ii) possibility of combining microscopy-based phenotypic readouts with gene expression analysis with the same cells, iii) customizable reaction volumes according to the experimental needs, iv) miniaturization of culturing and reaction volumes down to nanoliters, which results in up to 100 fold reduction of reagent consumption, v) compatibility with multiple washing and reagent addition steps, as well as retrieval of sample from the droplets and vi) potential decrease of non-specific adsorption of nucleic acids, resulting in efficient retrieval of the reaction volume.

Our DMA with the established methodology can be used as sample preparation platform starting from live cells or cell lysates, down to single cell per droplet. mRNA isolated from the lysed cells on DMA chip as a part of "Cells-to-cDNA on Chip" methodology can be used for different applications, e.g., hybridization assays, northern blotting, structural analysis, nuclease protection assays and others. Since the DMA platform is compatible with various cellular models, it is possible to apply the "Cells-to-cDNA on Chip" methodology for isolation of nucleic acids from a variety of starting material on DMA including primary cells, patient derived cell lines, tumor spheroids, bacteria, and other microorganisms.

4. Experimental Section

Materials and Reagents: HeLa-CCL2 and HeLa-GFP cell lines were purchased from DSMZ GmbH, Germany and BioCat GmbH, Germany, respectively. Cell lines were tested for possible mycoplasma contamination using the kit from PromoCell GmbH, Germany. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, blasticidin, trypan blue stain, Superscript IV RT-PCR kit and nuclease-free water were purchased from Life Technologies GmbH, Germany. RNeasy kit with RLT cell lysis buffer and Taq master mix were bought from Qiagen GmbH, Germany. Poly-T magnetic beads for mRNA isolation were from GE Healthcare GmbH, Germany. Tris hydrochloride (Tris-HCl), lithium chloride (LiCl), ethylenediaminetetraacetic acid (EDTA), agarose and tris-acetate-EDTA buffer (TAE) were bought from Sigma-Aldrich Chemie GmbH, Germany. 1 kb DNA ladder and gel loading dyes were purchased from New England BioLabs GmbH, Germany. Gotaq qPCR mater mix was purchased from Promega GmbH, Germany. All primers were purchased from Integrated DNA Technologies Germany

GmbH. Anti-GFP siRNA (siGFP) and scrambled control siRNA were purchased from Dharmacon Research, Inc. (Lafayette, CO, USA) and Qiagen GmbH, Germany, respectively. Screenfect A transfection reagent was bought from Screenfect GmbH (Eggenstein-Leopoldshafen, Germany). Nanostructured nonpolymer-layered slides with various hydrophilic-superhydrophobic patterned surfaces were purchased from Aquarray GmbH (Eggenstein-Leopoldshafen, Germany). Reagents for preparation of HEMA-EDMA polymer layered slides were purchased from Sigma-Aldrich Chemie GmbH, Germany and Merck Chemicals GmbH, Germany. Standard microscope glass slides (75 mm × 25 mm) for preparation of HEMA-EDMA polymer layered slides were bought from SCHOTT Technical Glass Solutions GmbH, Germany. Parafilm roll was purchased from Fischer Scientific GmbH, Germany. The metal in situ adapter was bought from Antylia Scientific, Cole-Parmer GmbH, Germany. 3D printed PCR chamber lid was designed Rhinocerous 3D software[49] and produced by Creabis GmbH, Germany. Neodymium block magnet (60×30×15 mm) was purchased from Supermagnete, Webcraft GmbH, Germany. Standard polystyrene Petri dishes were purchased from Greiner Bio-One GmbH, Germany. qPCR plates with seals were bought from 4titude Ltd., United Kingdom. PCR microtubes and microplates were purchased from Axygen Scientific GmbH, Germany.

Cell Culture: HeLa-CCL2 cells were cultured in DMEM with 10% FBS supplemented with 1% penicillin–streptomycin solution, at 37 °C with 5% CO_2 in a standard cell culture incubator. HeLa-GFP cells were cultured under the same conditions, but in culture media containing DMEM with 10% FBS, 1% penicillin–streptomycin and 0.1% blasticidin solution. Both the cell lines were checked and found free of any mycoplasma contamination.

Humidity Chamber and Humidified Petri Dish: The "humidity chamber" for performing high-temperature reactions on DMA was built as described here. The DMA slide was placed onto the metal adapter for 96-well thermocycler machine (Bio-Rad Laboratories GmbH, Germany). Then, tissue strips wetted with 3–3.5 mL of nuclease-free water were placed around the DMA slide placed onto metal adaptor. Next, the whole setup was closed from top with the customized 3D-printed lid made of thermostable polyamide material. The so-formed "humidity chamber" was allowed to stay as it is for 10 min at room temperature before the experiment, in order to ensure proper humidity inside the chamber.

For culturing cells at 37 °C and incubation steps at room temperature, DMA was placed in a "humidified Petri dish" to maintain humidity and thereby prevent the evaporation of the droplets on DMA. For this, the lid of a sterile 100 mm Petri dish was layered with a tissue wetted with ~4 mL nuclease-free water. In addition, 2 mL of nuclease-free water was added to the Petri dish itself. The Petri dish was closed with lid containing wetted tissue and allowed to stay at room temperature for at least 15 minutes before starting the experiments.

cDNA Synthesis from Total RNA on Droplet Microarray: Total RNA from HeLa-CCL2 cells was isolated with RNeasy kit (Qiagen GmbH, Germany), according to manufacturer's protocol. For cDNA synthesis on DMA, the concentration of all reagents in the reaction cocktail was kept the same as per the standard protocol in microtubes, but the volumes were scaled down according to the hydrophilic spot size, as described in Table 1. RNA quantification was done with NanoDrop 1000 spectrophotometer (Fischer Scientific GmbH, Germany). To begin with, DMAs were sterilized with 80% ethanol, dried quickly using a nitrogen gun and placed inside a humidified Petri dish as described before.[45] A solution containing appropriate volumes of HeLa-CCL2 total RNA, dNTPs and random hexamers/oligo dT primers (Table 1) in nuclease-free water was pipetted (for 3 mm DMA) or dispensed by a nanoliter volume dispenser (Dispendix GmbH, Germany) onto the hydrophilic spots (for 1 mm DMA). The DMA slide with droplets containing the reaction mixture was then put into the humidity chamber and placed inside the thermocycler machine for incubation at 65°C for 10 min. On completion, the setup was taken out and placed onto ice for 2 min. Then a solution containing appropriate volumes of reverse transcription buffer, dithiothreitol (DTT), ribonuclease inhibitor and superscript IV reverse transcription enzyme (Table 1) was pipetted or dispensed (according to hydrophilic spot size) onto the DMA again. The setup was placed inside the humidity chamber and then into the thermocycler machine for reverse transcription reaction at 52 °C for 15 min, followed by inactivation of the enzyme at 80 °C for 12 min. As an optional step, an appropriate volume of RNase H enzyme (Table 1) was added (pipetted or dispensed, according to DMA type) to each hydrophilic spot of DMA. The DMA was then kept back into the humidity chamber for digesting the RNA inside thermocycler at 37°C for 20 min. Finally, the cDNA droplets were collected into a PCR microtube either using a pipette (3 mm DMA) or a piece of sterile parafilm to gently pool the droplets from the DMA spots (1 mm DMA).

Water Contact Angle (WCA) Measurement: The static water contact angles were measured for the total reaction volume on a 3 mm DMA, both before and after cDNA synthesis from total HeLa-CCL2 RNA, by a Drop Shape Analyzer machine (Krüss, Hamburg, Germany).

PCR and Gel Electrophoresis: For PCR, cDNA synthesized on DMA was taken as the template and the reaction was done with primers for *GAPDH* or *ACTB* genes, according to manufacturer's protocol. Primer sequences for PCR have been mentioned in Table S1, Supporting Information. PCR with HeLa-CCL2 cDNA synthesized in microtubes according to standard protocol and nuclease-free water were taken as positive and negative controls, respectively. Resultant PCR products were run onto a 1.5% agarose gel with 1× TAE at 90 V for 75 min in a gel electrophoresis apparatus (Bio-Rad Laboratories GmbH, Germany). Gels stained with Sybr safe were visualized using an UV-transilluminator (Bio-Rad Laboratories GmbH, Germany).

cDNA Synthesis from Different Number of Cells on DMA ("Cells-to-cDNA on Chip" method): On 3 mm DMA:

i) For the experiment with different cell numbers, HeLa-CCL2 cells were trypsinized and the single cell suspension was prepared. Cellular viability was tested using trypan blue and per million concentration of cells was counted using an automated cell counter (Life Technologies GmbH, Germany). A 3 mm DMA was ethanol-sterilized as mentioned before and varying number of cells (10, 100, 500, and 1000 cells) were seeded onto consecutive rows of hydrophilic spots. Cells were manually seeded in a culture media volume of 3 µL volume, and 2 µL of RLT cell lysis buffer was added in each spot. Alternatively, cells may also be seeded onto the hydrophilic spots, kept inside the humidified Petri dish and incubated at the cell culture incubator at 37 °C for 24/48 h before performing cell lysis, according to the experimental need. For cell lysis, the DMA was placed inside a humidified Petri dish and incubated at 37 °C for 1 h inside a cell culture incubator. After that, the DMA was put inside the humidity chamber and incubated at 65 °C for 10 min. Thereafter the DMA with the humidity chamber was put on ice for 2 min. Next, 3 µL of poly-T mRNA isolation beads in mRNA hybridization buffer was added to each spot containing cell lysates, and incubated for 5 min at room temperature. Thereafter, the DMA was placed onto a magnet for 1 min to settle down the mRNA-bound beads, and the residual volume from each spot was aspirated by pipetting. Next, the magnetic beads bound to mRNA were resuspended into 5 µL of mRNA wash buffer, DMA was placed onto magnet for 1 min to settle the beads and the residual volume was removed as explained before. This process of washing the beads was repeated three times. Recipes for mRNA hybridization buffer and wash buffer have been provided in Table S2, Supporting Information. Next, the mRNA was eluted into 2.5 µL of nuclease-free water, and cDNA synthesis was carried out as described before, but with oligo dT primers. The metal adaptor for humidity chamber was thoroughly cleaned with a nitrogen gun to remove ice/water residues, before keeping it inside the thermocycler. Once the reaction was complete, the DMA was placed back onto magnet for 1 min and resultant cDNA (2.5 µL) from each spot was taken out into individual PCR microtubes or to different wells of a PCR microplate. 1 µL of cDNA per sample was used for qPCR.
ii) For experiments with different cell types, single-cell suspensions were prepared with both HeLa-CCL2 and HeLa-GFP cells, and cells from these two cell lines were seeded onto consecutive rows of a sterile 3 mm DMA with ~500 cells per hydrophilic spot. Cells were cultured for 24 h and "Cells-to-cDNA on Chip" was performed as described before.

On 1 mm DMA: HeLa-GFP cells were cultured as described before and single-cell suspension was prepared. Cells were tested for viability and counted as described above. Next, different numbers of HeLa-GFP cells were dispensed on DMA in 100 nL culture media per hydrophilic spot using the micro/nanoliter volume dispenser I-DOT One (Dispedix GmbH, Germany). For each cell number group, 168 hydrophilic spots were printed with cells onto DMA. Cells were then incubated within humidified Petri dish inside the cell culture incubator for 24 h before performing "Cells-to-cDNA on Chip" method. On the next day, the culture media from the hydrophilic spots onto DMA was removed by sandwiching the same with a hydrophilic HEMA-EDMA polymer layered slide. The hydrophilic HEMA-EDMA polymer coated slide was prepared in-house, as described previously.[38,39] Next, 200 nL PBS was dispensed onto hydrophilic spots containing cells, and removed by sandwiching method. The PBS washing steps were repeated twice. Thereafter, 150 nL RLT cell lysis buffer was added to the hydrophilic spots and the DMA was incubated for 1 h inside cell culture incubator to ensure cell lysis. Thereafter the DMA was briefly kept onto ice, as described earlier. All the following incubation steps were done inside the humidified Petri dish to prevent evaporation from the nanoliter droplets. Next, 100 nL poly-T magnetic beads in mRNA hybridization buffer was dispensed onto the spots and the DMA slide was incubated for 10 min at room temperature. Then the DMA was kept onto the magnet for 2 min to settle the beads and the residual volume was removed by sandwiching as before. Thereafter, three washing steps were performed by printing 200 nL of wash buffer to the hydrophilic spots followed by removal of the same each time by sandwiching. Next, 100 nL nuclease-free water was printed onto the bead-containing spots and DMA was incubated for 5 min at room temperature for eluting mRNA from the beads. The reagent volumes for this part of "Cells-to-cDNA on Chip" method have been mentioned in Table 2, for both DMA types. Next, cDNA synthesis was performed onto 1 mm DMA as described earlier. Upon completion of the experimental workflow, the resultant cDNA from each cell number group (168 spots) was collected by pooling the droplets from DMA with a piece of sterile parafilm. The cDNA was pooled into a PCR microtube and it was placed onto the magnet one last time for 2 min to settle any residual beads in the solution. Finally, the clear solution containing cDNA from 168 spots of respective cell number group was taken out into another PCR microtube for subsequent applications. Collected cDNA solution was diluted accordingly so that the volume corresponding to the cDNA from a single droplet was used for qPCR.

From a Single Cell: Single-cell suspension of HeLa-GFP cells in PBS was prepared as described before. First, 50 nL PBS was printed onto the hydrophilic spots of a sterilized 1 mm DMA using I-DOT One (Dispendix GmbH, Germany). Next, HeLa-GFP Cells were printed as single cells per hydrophilic spot within 50 nL droplets on DMA by a single cell printer cellenONE (Cellenion, France). For each technical repeat, 9 hydrophilic spots were printed with single cells on DMA. Next, 100 nL RLT cell lysis buffer was added by I-DOT One to each hydrophilic spot containing cells. The DMA was immediately kept on ice for 5 min within a humidified Petri dish to allow cell lysis. Next steps of "Cells-to-cDNA on Chip" method were performed exactly with the same reagent volumes and method as described before for 1 mm DMA. Upon completion of the reaction, 7 µL nuclease-free water was added on top of the 9 hydrophilic spots containing cDNA for each technical repeat. This allowed the cDNA from 9 cells to dilute into a total volume of ~9 µL [(200 nL×9) + 7 µL)]. The diluted cDNA was mixed with pipette to ensure complete retrieval of the reaction volume, and then collected into a PCR microtube. The tube was then placed onto magnet to get rid of any residual beads as described before. 1 µL of the cleaned cDNA (single cell cDNA) was used for qPCR.

Real-Time PCR (qPCR): qPCR was performed according to the manufacturer's protocol on a StepOne Real-time PCR system (Life Technologies GmbH, Germany). Gene expression data analysis was done as described before.[50]

siGFP Transfection to HeLa-GFP Cells on DMA: The 3 mm DMA was ethanol-sterilized as described earlier and 1000 HeLa-GFP cells in antibiotic-free complete medium were seeded onto each hydrophilic spot. Both siGFP and scrambled siRNA control were used at a final concentration of 100 nM. Sequences for primers and siGFP are provided in Table S1, Supporting Information. Cells were transfected by the one-step transfection method, according to Screenfect-A user protocol. After 48 h of transfection, cells were lysed, mRNA was isolated and converted to cDNA according to the "Cells-to-cDNA on Chip" methodology. The resultant cDNA samples were used for qPCR, to confirm GFP knockdown in cells. The transfection experiments were repeated thrice, with at least three technical repeats in each.

Microscopy: All bright-field and fluorescence microscopy images were taken with a Keyence BZ-9000 microscope (Keyence, Osaka, Japan), with 2× and 10× objective lenses. Nuclei were identified with Hoechst 33342 staining, according to manufacturer's protocol.

Statistical Analysis: Mean GFP fluorescence in different cell groups was estimated by ImageJ software.[51] Statistical differences within groups for real-time PCR data and fluorescence estimation were calculated by unpaired two-tailed t-test. Data for bar graphs were presented as mean±SEM. Quantitative gene expression data for different numbers of cells on 3 mm DMA and single cells on 1 mm DMA were calculated with ≥5 technical repeats. Quantitative expression of GFP transcripts after siGFP transfection to HeLa-GFP cells was performed with 3 biological experiments, each having 3 technical replicates.

To count the number of HeLa-GFP cells per hydrophilic spot on a 1 mm DMA, the whole DMA slide was imaged with 2× magnification and a stitching function of the Analyzer software was used to create a single image of the slide. Then the number of cells per spot was calculated using an individually developed automated workflow: First, a spot detection algorithm extracted each spot on the DMA slide. Thereafter, an automated approach based on Hough line estimations[52] was utilized to detect square-shaped spots. In order to provide robust processing in every experimental setup, a semi-automatic grid detection was available in parallel. Hereby, users could mark grid corners of the DMA slide as an alternative approach. The semi-automatic approach was used in cases where automated detection failed. Since there were separable cells within a spot, a blob detection algorithm was used as provided in the OpenCV library.[53] The algorithm is based on iterative filtering and merging of connected components in the image for detecting cells. To avoid the detection of false-positive cells, filtering was done with respect to properties like area, inertia, and convexity of each region. Subsequently, detections were counted, exported to result files, and visualized for inspection of results.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This research was primarily funded by the Deutsche Forschungsgemeinschaft grant (DFG), grant number PO1820/3-1 and partial funding was obtained from DFG (Heisenberg professor Projektnummer: 406232485, LE 2936/9-1). Furthermore, the authors thank the Helmholtz Program Materials Systems Engineering (MSE) for the support.

Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

S.C., C.L., J.E., M.S., M.R. and M.R. declare no conflict of interest regarding the publication of this article. P.L. and A.P., in addition to being employed by KIT, are also shareholders of Aquarray GmbH since March 2018.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords

cDNA synthesis on chip, cell screening, droplet microarrays, gene expression analysis, nanoliter volumes

Received: November 15, 2021
Revised: February 8, 2022
Published online: March 31, 2022

[1] W. F. An, N. Tolliday, *Mol. Biotechnol.* **2010**, *45*, 180.
[2] P. A. Clemons, *Curr. Opin. Chem. Biol.* **2004**, *8*, 334.
[3] P. Szymański, M. Markowicz, E. Mikiciuk-Olasik, *Int. J. Mol. Sci.* **2012**, *13*, 427.
[4] E. N. Willmer, *Cells and Tissues in Culture:Methods, Biology and Physiology*, Elsevier, Amsterdam **2013**.
[5] S. A. Hudu, A. S. Alshrari, A. Syahida, Z. Sekawi, *J. Clin. Diagn. Res.* **2016**, *10*, DE01.
[6] I. Watanabe, S. Okada, *J. Cell Biol.* **1967**, *32*, 309.
[7] M. Vergara, S. Becerra, J. Berrios, N. Osses, J. Reyes, M. Rodríguez-Moyá, R. Gonzalez, C. Altamirano, *PLoS One* **2014**, *9*, e93865.
[8] J. Michl, K. C. Park, P. Swietach, *Commun. Biol.* **2019**, *2*, 144.
[9] A. C. Taylor, *J. Cell Biol.* **1962**, *15*, 201.
[10] S. Shrivastav, K. R. Stone, R. A. Bonar, D. F. Paulson, *Cancer Res.* **1980**, *40*, 4438.
[11] S. Ozawa, M. Iino, K. Tsuzuki, *J. Neurophysiol.* **1991**, *66*, 2.
[12] P. Larsson, H. Engqvist, J. Biermann, E. Werner Rönnerman, E. Forssell-Aronsson, A. Kovács, P. Karlsson, K. Helou, T. Z. Parris, *Sci. Rep.* **2020**, *10*, 5798.
[13] E. Karsten, E. Breen, B. R. Herbert, *Sci. Rep.* **2018**, *8*, 3101.
[14] A. Rabinovitch, W. L. Suarez-Pinzon, K. Strynadka, J. R. Lakey, R. V. Rajotte, *J. Clin. Endocrinol. Metab.* **1996**, *81*, 3197.
[15] T. L. Moore, D. A. Urban, L. Rodriguez-Lorenzo, A. Milosevic, F. Crippa, M. Spuch-Calvar, S. Balog, B. Rothen-Rutishauser, M. Lattuada, A. Petri-Fink, *Sci. Rep.* **2019**, *9*, 900.
[16] X. Huang, D. P. Cavalcante, H. E. Townley, *J. Nanopart. Res.* **2020**, *22*, 23.
[17] A. Wittrup, J. Lieberman, *Nat. Rev. Genet.* **2015**, *16*, 543.
[18] M. R. Molla, S. Chakraborty, L. Munoz-Sagredo, M. Drechsler, V. Orian-Rousseau, P. A. Levkin, *Bioconjugate Chem.* **2020**, *31*, 852.
[19] S. Chakraborty, S. K. Vellarikkal, S. Sivasubbu, S. S. Roy, N. Tandon, D. Bharadwaj, *Biochem. Biophys. Res. Commun.* **2020**, *522*, 1022.
[20] J. A. Bittker, *Curr. Protoc. Chem. Biol.* **2012**, *4*, 49.
[21] C. Korherr, H. Gille, R. Schäfer, K. Koenig-Hoffmann, J. Dixelius, K. A. Egland, I. Pastan, U. Brinkmann, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 4240.
[22] S. C. Ramaiahgari, M. W. Den Braver, B. Herpers, V. Terpstra, J. N. M. Commandeur, B. Van De Water, L. S. Price, *Arch. Toxicol.* **2014**, *88*, 1083.
[23] R. J. DeBerardinis, C. B. Thompson, *Cell* **2012**, *148*, 1132.
[24] V. Lopes-Rodrigues, A. Di Luca, J. Mleczko, P. Meleady, M. Henry, M. Pesic, D. Cabrera, S. Van Liempd, R. T. Lima, R. O'Connor, J. M. Falcon-Perez, M. H. Vasconcelos, *Sci. Rep.* **2017**, *7*, 44541.
[25] C. Hirsch, S. Schildknecht, *Front. Pharmacol.* **2019**, *10*, 1484.
[26] M. Niepel, M. Hafner, C. E. Mills, K. Subramanian, E. H. Williams, M. Chung, B. Gaudio, A. M. Barrette, A. D. Stern, B. Hu, J. E. Korkola, L. Consortium, J. W. Gray, M. R. Birtwistle, L. M. Heiser, P. K. Sorger, *Cell Syst.* **2019**, *9*, 35.
[27] C. Gaillard, F. Strauss, *Tech. Tips Online* **1998**, *3*, 63.
[28] B. P. Belotserkovskii, B. H. Johnston, *Science* **1996**, *271*, 222.
[29] G. G. Nestorova, K. Hasenstein, N. Nguyen, M. A. DeCoster, N. D. Crews, *Lab Chip* **2017**, *17*, 1128.
[30] S. C. Kim, I. C. Clark, P. Shahi, A. R. Abate, *Anal. Chem.* **2018**, *90*, 1273.
[31] T. M. Gierahn, M. H. Wadsworth, T. K. Hughes, B. D. Bryson, A. Butler, R. Satija, S. Fortune, J. C. Love, A. K. Shalek, *Nat. Methods* **2017**, *14*, 395.
[32] E. Z. Macosko, A. Basu, R. Satija, J. Nemesh, K. Shekhar, M. Goldman, I. Tirosh, A. R. Bialas, N. Kamitaki, E. M. Martersteck, J. J. Trombetta, D. A. Weitz, J. R. Sanes, A. K. Shalek, A. Regev, S. A. McCarroll, *Cell* **2015**, *161*, 1202.
[33] D. T. Chiu, A. J. deMello, D. Di Carlo, P. S. Doyle, C. Hansen, R. M. Maceiczyk, R. C. R. Wootton, *Chem* **2017**, *2*, 201.
[34] M. Sonker, V. Sahore, A. T. Woolley, *Anal. Chim. Acta* **2017**, *986*, 1.
[35] Y. Liu, H. Lu, *Curr. Opin. Biotechnol.* **2016**, *39*, 215.
[36] A. A. Popova, K. Demir, T. G. Hartanto, E. Schmitt, P. A. Levkin, *RSC Adv.* **2016**, *6*, 38263.
[37] Y. Liu, S. Chakraborty, C. Direksilp, J. M. Scheiger, P. A. Levkin, A. A. Popova, *Mater. Today Bio* **2021**, *12*, 100153.
[38] W. Feng, L. Li, E. Ueda, J. Li, S. Heißler, A. Welle, O. Trapp, P. A. Levkin, *Adv. Mater. Interfaces* **2014**, *1*, 1400269.
[39] F. L. Geyer, E. Ueda, U. Liebel, N. Grau, P. A. Levkin, *Angew. Chem., Int. Ed.* **2011**, *50*, 8424.
[40] A. N. Efremov, E. Stanganello, A. Welle, S. Scholpp, P. A. Levkin, *Biomaterials* **2013**, *34*, 1757.
[41] W. Lei, J. Bruchmann, J. L. Rüping, P. A. Levkin, T. Schwartz, *Adv. Sci.* **2019**, *6*, 1900519.
[42] G. Jogia, T. Tronser, A. Popova, P. Levkin, *Microarrays* **2016**, *5*, 28.
[43] T. Tronser, K. Demir, M. Reischl, M. Bastmeyer, P. A. Levkin, *Lab Chip* **2018**, *18*, 2257.
[44] M. Benz, A. Asperger, M. Hamester, A. Welle, S. Heissler, P. A. Levkin, *Nat. Commun.* **2020**, *11*, 5391.
[45] S. Chakraborty, V. Gourain, M. Benz, J. M. Scheiger, P. A. Levkin, A. A. Popova, *Mater. Today Bio* **2021**, *11*, 100112.
[46] A. A. Popova, T. Tronser, K. Demir, P. Haitz, K. Kuodyte, V. Starkuviene, P. Wajda, P. A. Levkin, *Small* **2019**, *15*, 1901299.
[47] A. A. Popova, D. Marcato, R. Peravali, I. Wehl, U. Schepers, P. A. Levkin, *Adv. Funct. Mater.* **2018**, *28*, 1703486.
[48] Y. Liu, T. Tronser, R. Peravali, M. Reischl, P. A. Levkin, *Adv. Biosyst.* **2020**, *4*, 1900257.
[49] R. McNeel et al., Rhinoceros 3D, Version 6.0. Robert McNeel& Associates, Seattle, WA 2010. www.rhino3d.com (accessed: March 2020).
[50] M. W. Pfaffl, *Nucleic Acids Res.* **2001**, *29*, 45e.
[51] C. A. Schneider, W. S. Rasband, K. W. Eliceiri, *Nat. Methods* **2012**, *9*, 671.
[52] P. V. C. Hough, *US Patent 30696541962*, **1962**.
[53] G. Bradski, *Dr. Dobb's J. Software Tools* **2000**, *120*, 122.