Droplet Microfluidics Hot Paper

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Quantitative Single-Cell mRNA Analysis in Hydrogel Beads

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Abstract: *In recent years, technologies capable of analyzing single cells have emerged that are transforming many fields of biological research. Herein we report how DNA-functionalized hydrogel beads can serve as a matrix to capture mRNA from lysed single cells. mRNA quantification free of pre-amplification bias is ensured by using padlock probes and rolling circle amplification followed by hybridization with fluorescent probes. The number of transcripts in individual cells is assessed by simply counting fluorescent dots inside gel beads. The method extends the potential of existing techniques and provides a general platform for capturing molecules of interest from single cells.*

Single-cell analysis has the potential to bring new insight into long-standing biological questions. Protein and mRNA levels show heterogeneity even in cells bearing the same genotype.[1] The detection of these cell-to-cell variations is not possible in traditional bulk studies, where thousands to millions of cells are pooled together and their average gene expression is studied. The desire to understand cellular heterogeneity has been the driving force for the development of instrumentation, protocols, and methods for analyzing single cells on many different levels, including transcription.[2] Most single-cell gene expression analyses are based on RNA sequencing,[2c,3] reverse transcription quantitative real-time PCR (RT-qPCR) combined with microfluidics,[2b] or in situ hybridization methods.[4] None of those methods is free of limitations. RNA sequencing is still too expensive for high-throughput single-cell measurements.[5] Microfluidic RT-qPCR, which solves the problem of low-throughput, still suffers from additional noise in complementary DNA (cDNA) quantification due to a pre-amplification step.[6] The main drawback of mRNA fluorescence in situ hybridization (FISH) is the relatively small number of genes than can be simultaneously measured, and difficulties with resolving highly similar sequences. Detection of individual transcripts in situ with padlock probes and target-primed rolling circle amplification (RCA)[7] allows us to distinguish between expressed single-nucleotide sequence variants, but its dynamic range is limited.[8]

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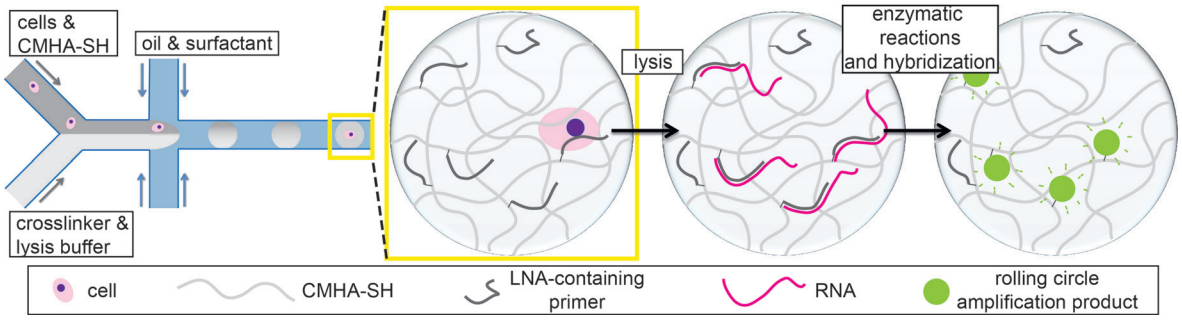
Herein we report the development of a new method, free of amplification bias, based on padlock probes and RCA. The method extends the dynamic range of previously published methods by lysing cells and immobilizing the mRNA within an increased volume. We employed droplet-based microfluidics as a relatively low-tech, low-cost, general, and potentially high-throughput procedure for capturing molecules from single cells.[9] First, individual cells are compartmentalized in picoliter droplets. These droplets contain lysis buffer and crosslinkable polymers with binding sites for the molecules of interest, in this case primers containing locked nucleic acid (LNA; Scheme 1). Upon crosslinking and cell lysis, mRNA will hybridize to the newly formed functionalized hydrogel, and the remaining cell debris can be washed away after breaking the emulsion. The individual mRNA molecules are then available for amplification and detection.

To capture mRNA from lysed cells, thiolated carboxymethyl hyaluronic acid (CMHA-SH; Figure 1a) was functionalized with LNA-containing primers complementary to the mRNA of interest. For coupling we utilized amino-modified oligonucleotides and a sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) linker (Figure 1b). The coupling efficiency was tested by using fluorescently labeled oligonucleotides. Beads produced from CMHA-SH functionalized with fluorescent oligonucleotides showed bright, uniform fluorescence after washing (Figure 1c), as opposed to nonfunctionalized beads, which showed no fluorescence (data not shown). We estimated that a single bead bears between 4×10^8 and 5×10^8 copies of the LNA primer (for details see the Supporting Information). For detection and quantification of mRNA molecules captured on bead, we adapted a method previously developed by Weibrecht et al.[7b] Scheme 2 shows the mRNA quantification procedure in detail: after cell lysis the mRNA of interest hybridizes to the primer moieties in the gel bead. The oil is removed and remaining cell debris is washed away. The primer–mRNA pairs are subjected to reverse transcription yielding cDNA covalently attached to the thiolated carboxymethyl hyaluronic acid (Scheme 2, yellow strand). After mRNA digestion by ribonuclease H, the linear padlock probe can hybridize to the cDNA. Only upon perfect hybridization can the ends of the padlock probe be ligated by the DNA ligase Ampligase, yielding a circular product. This circular oligonucleotide acts as a template for target-primed RCA catalyzed by φ29 DNA polymerase, resulting in a long DNA concatemer. The number of captured transcripts is assessed at the end of the procedure by counting fluorescent dots that form following the hybridization with fluorescent detection oligonucleotides (Scheme 1). In order to decrease nonspecific background fluorescence (see Figure S2 in the Supporting Information), beads were treated with proteinase K prior to the hybridization step.



Scheme 1. Overview of mRNA quantification experiment workflow.

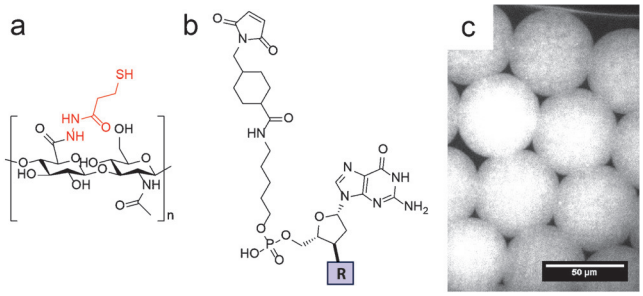
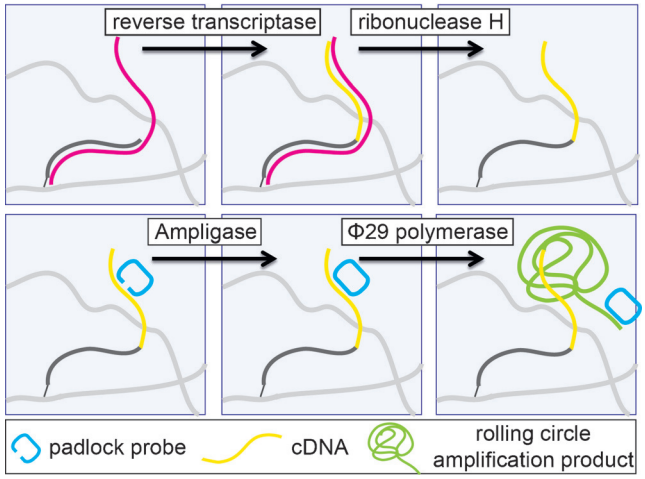


Figure 1. Hydrogel beads capable of capturing mRNA molecules of interest from single cells produced by means of droplet microfluidics. a) Structural unit of thiolated carboxymethyl hyaluronic acid (CMHA-SH). The modification of the hyaluronic acid is marked in red. b) Maleimide-activated LNA-containing primer. Heterobifunctional linker sulfo-SMCC reacted with a 5′ end amine-functionalized LNA-containing primer and the structure of the first base is shown. R = remaining bases of the LNA-containing primer. The maleimide group reacts with thiol groups on CMHA-SH. c) Confocal microscopy image of beads made on-chip of CMHA-SH functionalized with 20-base-pair-long fluorophore-labeled oligonucleotides. Scale bar in (c) = 50 µm.



Scheme 2. Detection of individual transcripts in beads with padlock probes (blue) and target-primed RCA.

To verify our detection procedure and to explore the dynamic range of the method, we performed RCA of padlock probes on beads modified with different amounts of single-stranded DNA complementary to the ends of the padlock probe (Figure 2a). We found that it is possible to quantify fluorescents dots from just a few copies up to 1000 DNA molecules per single bead (Figure 2b). In the previous work on in situ mRNA detection with padlock probes and RCA the signals started to coalesce at mRNA copy numbers over 100, thus limiting the dynamic range.[8] Our method allows for even spreading of mRNA from single cells during lysis over

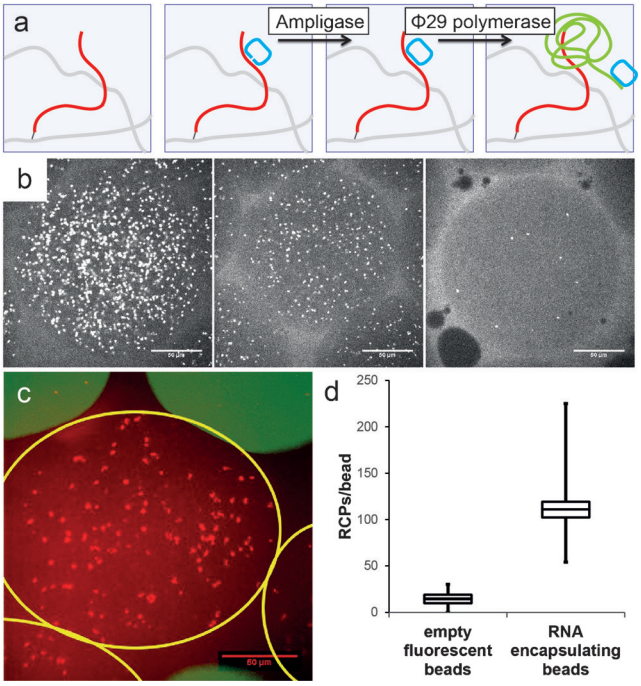


Figure 2. a) Schematic overview of DNA detection on a hydrogel support with padlock probes and RCA. Single-stranded DNA is shown in red. b) Confocal fluorescence microscopy images of beads containing fluorescent dots resulting from in-bead DNA detection. Concentration of immobilized DNA 10-fold diluted with each image from left to right. c) Estimation of cross-contamination between gel beads. A confocal microscope image representing a sum of 32 *z*-slices; rolling circle amplification products (RCPs; red) and beads functionalized with a maleimide reactive dye (green) are shown. Yellow lines were drawn around nonfluorescent beads as a visual aid. d) Quantification of cross-contamination between hydrogel beads. A box-and-whisker plot is shown, where the whiskers represent the minimum and maximum of all of the data. The bottom and top of the box are the first and third quartiles, the band inside the box is the median. 25 beads of each type were analyzed. Scale bars in all images in (b) and (c) = 50 µm.

the entire bead, increasing distances between molecules and enhancing the dynamic range of our method.

Next, we encapsulated single cells in primer-functionalized hydrogel microbeads for single-cell mRNA analysis. A suspension of cells, thiolated carboxymethyl hyaluronic acid in PBS, and crosslinker solution (poly(ethylene glycol) divinyl sulfone (PEG-DVS) in lysis buffer were injected into a microfluidic flow-focusing device with three inlets for microdroplet generation as shown in Scheme 1. Merging of the two aqueous streams just before droplet production ensures that cell lysates are confined to individual droplets and eliminates the risk of cross-contamination. Subsequently, these precursor droplets were gelled at 37 °C for 20 min and incubated at 60 °C for 40 min to facilitate lysis. The number of cells in each droplet follows a Poisson distribution when suspension cells were used, with slight deviations resulting from nonspecific clustering of the cells in experiments where an adherent cell line was used (Table S1). For a concentration of human myelogenous leukemia K562 cells corresponding to an average of 0.17 cells per bead, approximately 85 % of beads were empty (negative), and 90 % of positive beads contained a single cell, which is in perfect agreement with theoretical values calculated from a Poisson distribution. Using the human breast carcinoma SKBR3 cell line, 88 % of beads were empty and approximately 68 % of positive beads contained single cells (Table S2). It is important that the majority of positive beads contains only one cell, since it is not possible to determine the number of cells encapsulated in a bead once cells are lysed. Therefore suspension cells are a more convenient sample and were used in the majority of experiments. Adherent cells can also be used, but the increased encapsulation of multiple cells has to be taken into account during interpretation of results making analysis less straightforward.

We used a lysis buffer based on guanidine hydrochloride (for details on lysis buffer optimization see the Supporting Information), which is a potent protein denaturing agent and inhibits endogenous RNases. The buffer does not cause denaturation of mRNA–primer hybrids as the primers used in our experiments contain LNA nucleotides. LNA–mRNA hybrids are known to be stable even in high-concentration chaotropic salts solutions.[10] After lysis, no cellular remains were visible within the beads (Figure S3 d).

To verify possible leakage of RNA from gel beads, two populations of beads were mixed in a 1:1 ratio directly after on-chip bead production, preceding emulsion breakage. One set of beads contained RNA whereas the other beads were empty but made with fluorescently modified CMHA-SH. Both hydrogel bead populations were prepared out of CMHA-SH modified with primers complementary to the synthetic RNA used in the experiment. After pooling of the two populations, RNA molecules were detected as described above. The average number of spots counted in the negative control (reverse transcriptase omitted) was subtracted in all experiments from the results obtained for positive samples. In RNA-containing beads, an average of approximately 116 spots were counted whereas 14 spots were seen in control beads (Figure 2 c,d). These results indicate that leakage and transfer of mRNA after emulsion breakage is low. In cell-based experiments, the fraction of positive droplets is much lower than 50 %, making it highly unlikely that any fluorescent spots originate from mRNA of cells encapsulated in other droplets.

We performed detection of the beta-actin gene (*ACTB*) transcripts in SKBR3 cells and compared the obtained values with literature data. The average number of spots corresponding to *ACTB* transcripts detected per cell of 108 is in good agreement with previous findings (Figure 3 a).[7a]

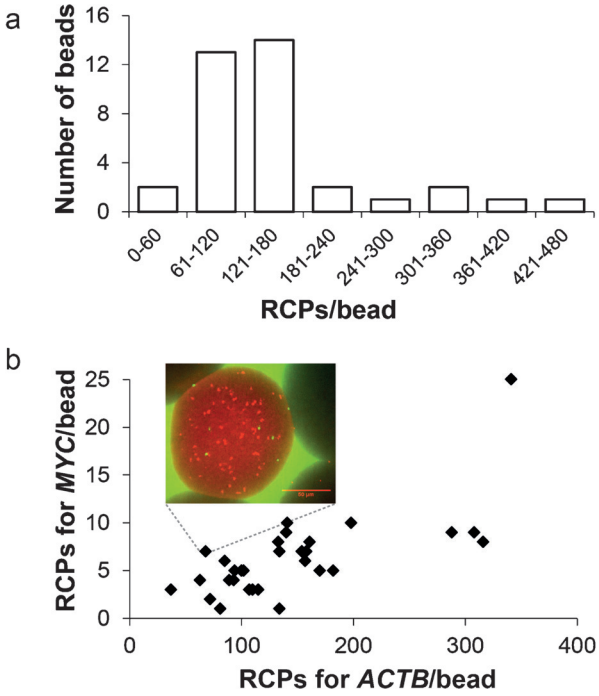


Figure 3. Quantification of mRNA from single cells. a) Histogram showing quantification of *ACTB* RCPs in 36 beads containing SKBR3 cells. b) Quantification of *ACTB* and *MYC* transcripts in 31 beads containing K562 cells. One gel bead corresponds to one point on the graph. The means of the independent experiments were: 145 and 108 transcripts for *ACTB* and 6 and 14 transcripts for *MYC*. Inset: a confocal microscope image representing a sum of 32 images from *z*-series showing RCA products corresponding to *ACTB* (red) and *MYC* (green) transcripts inside a hydrogel bead. Scale bar = 50 µm. a) and b) show the results of one representative experiment from two repetitions performed.

To test the ability of primer-functionalized hydrogel beads for capturing and detection of two transcripts from one cell we measured beta-actin (*ACTB*) and c-Myc (*MYC*) transcripts in the myelogenous leukemia K562 cell line. *ACTB* is widely regarded as a housekeeping gene, though there is a growing amount of evidence published lately for its up-regulation in tumor cells.[11] *MYC* is an oncogene that codes for the highly regulated and multifunctional MYC protein.[12] To enable analysis of two transcripts in one bead, CMHA-SH was modified with two different primers complementary to the transcripts of interest. The results of quantification of *ACTB* and *MYC* transcripts in K562 cells are presented in Movie S1 and Figure 3 b. The number of *MYC* transcripts

detected per cell in the analyzed sample ranged from 1 to 25 with an average and median of 6 transcripts per cell.

We observed considerable heterogeneity in the number of *ACTB* signals among cells. The number of transcripts detected per cell in the analyzed samples ranged from 51 up to 467 for SKBR3 cells and from 37 to 341 for the K562 cell line. The heterogeneity in the number of signals among cells is consistent with earlier literature reports (Figure 3).[7a] The *ACTB* transcript distribution is lognormal for both cell lines as confirmed by the Shapiro–Wilk normality test at a 95% significance level ($P = 0.05$) which is also in agreement with previous findings.[1c]

In summary, our method provides an alternative to other state-of-the-art gene expression assays that is suitable for analysis of mRNA from non-adherent cells. This method is a high-throughput, relatively straightforward, and low-cost procedure (Figure S4) with increased resolution in comparison to currently employed assays. We believe that after minor optimization, the method can be used for subsequent quantification of 4 different transcripts from single cells. We also introduce a new type of hydrogel bead, capable of capturing molecules from single cells, that can be utilized for isolation and quantification of other molecules from single cells after functionalization of thiolated carboxymethyl hyaluronic acid with different capturing molecules. Our work provides an interesting example how the marriage of material science and state-of-the-art technology can lead to the development of new and improved methods and protocols for biology.

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