Fluorescent detection upon droplet amplification of nucleic acids

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Abstract. Droplet microfluidics with amplification of nucleic acids allows to achieve sensitivity at the level of single molecules and is in demand in medical diagnostics, forensic science, and genetic research. The results of detection of nucleic acid amplification in liquid and water-in-oil emulsion are compared on the example of model solutions. Since the results of quantitative analysis are based on the calculation of the proportion of drops with a positive reaction result, one of the tasks was to study the features of automatic counting of drops in different zones of the reaction chamber.

1. Introduction
Droplet microfluidics allows manipulation of analyte droplets in an inert oil medium [1-2]. Special microfluidic devices with a droplet generator are used to “break” the sample into hundreds (thousands) of drops. At the same time, each drop can be effectively used for carrying out reactions, both at the molecular level, and in the study of cells or microorganisms. The advantages of the “droplet microfluidics” are: low consumption of reagents and samples, high droplet formation rates (with generation frequencies up to kHz), the ability to conduct high-performance studies in an array of identical droplets. In addition, it is possible to use both real-time polymerase chain reaction (PCR) techniques for high analyte concentrations and principles of digital PCR at low one. However, reducing the reaction volume to a droplet reduces the signal-to-noise ratio. The drop detection rate is limited when scanning with high resolution over a large area (array) of droplets.

The aim of the work was to compare the results of the analysis with amplification of nucleic acids in a liquid and in an emulsion using the example of model solutions. For this purpose, the oligonucleotide probe with fluorescent dye (without quencher) was used. Also of interest is the assessment of the legitimacy of selective analysis of digital PCR in emulsion from fragments of the image of the reaction chamber.

2. Experimental technique
Microfluidic chips for water-in-oil emulsion generation were made of polydimethylsiloxane by soft lithography [3]. Emulsions were generated by flow focusing method. Mineral oil with the addition of ABIL EM 180 (Evonik, Germany) surfactant was used as the continuous phase (of transport flow) for droplet generation. The flow rate of the continuous phase was 2.4 μl/min, and the discrete one was 0.6 μl/min. A solution of oligonucleotide probe (27 bp long) was used as amplified DNA fragments
obtained as a result of PCR. These probes were coupled to the FAM fluorescent dye and did not have a quencher.

The final concentration of amplicons after successful PCR is determined by the initial concentrations of the primers, which are the main components of the amplification reaction. The selected range of oligonucleotide probe concentrations (200 – 800 nM) corresponded to the real concentration of primers for amplification in droplets [4]. Emulsions were injected into a special reaction chamber, in which the droplets were distributed in one layer. For comparison, similar reaction chambers were filled with appropriate probe solutions and measured under the same conditions. To simulate the results of digital amplification, droplets with a fluorescent probe were mixed with non-fluorescent droplets from a buffer solution in a ratio (1 : 9).

A prototype fluorescent scanner with an excitation wavelength of 473 nm, was used for detection. The scanner was produced by Institute for Analytical instrumentation RAS. The result of the scan was a set of individual images of (460 × 350) μm in size that could be analyzed together or separately. For image processing was used special software.

3. Results and discussion

Figure 1 shows images of emulsions obtained from solutions with different concentrations of a fluorescent probe, as well as from a buffer solution. The average diameter of the resulting droplets for all images was 16 μm, which corresponds to a volume of ~ 2 picoliters. Observed large-diameter droplets make up less than 10% of the total number of droplets in the reaction chamber. Apparently, when the emulsion was injected into the reaction chamber by laboratory dispenser (pipette), some drops were combined under the action of external pressure, forming larger droplets.

Figure 1(a, b, c, d). Emulsions from solutions with different probe concentrations: (a) 800 nM; (b) 400 nM; (c) 200 nM; (d) buffer solution (without dye).
The value of the fluorescence signal was measured at the center of random 30 drops from three images. For the analysis of fluid data, a line was selected on the image frame (460 µm in length). Then, for each experiment, the average value of the fluorescence signal was calculated from the results of processing data from the lines from five images. The results obtained are presented in Table 1.

Table 1. Measured fluorescence signal at different probe concentrations (mean and standard deviation).

| Measurement conditions | Oligonucleotide probe concentration, nM | 0 (buffer solution) | 50 | 100 | 200 | 400 | 800 |
|------------------------|----------------------------------------|---------------------|----|-----|-----|-----|-----|
| Liquid                 |                                        | 214 ± 8             | 228 ± 9 | 247 ± 11 | 277 ± 11 | 392 ± 17 | 539 ± 30 |
| Emulsion               |                                        | 233 ± 7             | -    | -   | 265 ± 6 | 296 ± 9 | 476 ± 27 |

The threshold values of the fluorescence signal and the corresponding concentration value, interpreted as the detection limit, were determined. Based on the data in the table, the fluorescence signals from three low concentrations were modeled by a normal distribution with the integral characteristics presented in the table: mean and standard deviation. For intermediate concentrations, the characteristic values were obtained by linear interpolation.

For liquids, the average value of the fluorescence signal (228 relative units) at the lowest measured concentration of 50 nM corresponded to the upper limit of the 96.3% confidence interval for the buffer solution and at the same time the lower limit of the 96.3% confidence interval for concentration 100 nM. If a concentration of 100 nM was taken as the detection limit and 228 relative units was taken as the detection threshold, then the total probability of a false-negative and false-positive analysis was obtained around 7.5%. If we set the total probability of a false positive and false negative analysis result not exceeding 1%, then the detection limit is 160 nM (with a minimum signal value with a positive result of 235 relative units).

If the same value of 160 nM was set as the limiting concentration for detecting emulsions, the total probability of a false-positive and false-negative result of the analysis increased to 4.5%. By reducing it to 1%, the concentration limit became the measured concentration of 200 nM (the minimum value of the signal with a positive result would be 251 relative units).

On the other hand, for detecting amplification in an emulsion, it is also important not only to identify the fluorescent droplets, but also to calculate their ratio to the total number of droplets. It was of interest to evaluate the legitimacy of the selective analysis of several individual images of reaction chamber.

A mixture of fluorescent and non-fluorescent drops was analyzed. The total number of drops was more than 20 000. The results of the automatic counting of drops were rectangular matrices of (14 × 18) cells. Each cell of this matrix contained information on one field of view measuring (460 × 350) µm. Two matrices were necessary for the analysis: one with the number of only fluorescent drops, the other with the total number of both fluorescent and non-fluorescent drops.

To assess the homogeneity of the samplings both matrices were divided into two parts (89 and 79 cells) by a vertical section. For each of the parts, the proportion of fluorescent droplets was calculated relative to their total number. In the first sample, the mathematical expectations was 0.165 and the standard deviation was 0.077; in the second sample - 0.161 and 0.062, respectively. According to the generalized Student’s criterion, these samples was homogeneous with high probability (P > 90%). This allowed us to conclude that the total number of droplets and the number of fluorescent droplets recorded in different areas of the reaction chamber are random variables with a sufficiently large coefficient of variation. Therefore, the mathematical expectations of division does not coincide with the division of mathematical expectations: if we consider the ratio of mathematical expectations (average number of fluorescent drops to the average number of drops) then it gives a value of 0.155. This value is not too different from previous estimates of mathematical expectations.
Evaluation of the law of the distribution of the number of drops in different regions of the reaction chamber showed that it approximately satisfies the normal (Gaussian) distribution with the mathematical expectation and variance estimated from the sampling. 92 cells were distributed over 7 intervals. Pearson's Chi-square criterion of agreement showed the confidence probability of accepting the hypothesis of a normal distribution of about 14-15%, which is sufficient reason to accept the hypothesis. Combining the two extreme intervals with a small number of drops will increase this probability.

Combinations of 9 cells with automatic counting of drops according to the \((3 \times 3)\) square scheme (essentially, smoothing of the selection elements) were considered. An estimate of the ratio of the number of fluorescent drops to the total number has a mathematical expectation of 0.146 and a standard deviation of 0.044. The generalized Student's criterion in assessing the homogeneity of the results gives a value less than 0.8, which indicates the homogeneity of the sampling. Consequently, combining areas with a corresponding change in the scanner settings will not lead to a significant change in the analysis result.

The ratio of fluorescent droplets to all droplets calculated over the entire reaction chamber is slightly higher than the expected ratio of 0.10. The reason may be the combination of droplets, which leads to an underestimation of the total number of droplets and an overestimation of the proportion of fluorescent droplets.

It is interesting how the calculated ratio of the proportion of fluorescent droplets depends on the location of the analyzed cell. 10 enlarged locations with different distances from the center of the reaction chamber were selected. This ratio ranges from 0.10 to 0.22. There is a positive correlation (the sample correlation coefficient is +0.51) between the distance from the center of the chamber and the proportion of fluorescent droplets — it increases in the regions corresponding to the edges of the reaction chamber. Fewer drops are recorded at the edges of the reaction chamber, and the counting error has a large effect. The decrease in the number of droplets recorded at the edges can also be explained by the characteristics of hydrodynamics. Additional experimental confirmation and theoretical substantiation of the observed effect is the subject of further research.

When analyzing another reaction chamber, it was proposed to combine the cells into groups and analyze the mean values, medians and standard deviation calculated by groups and by all cells. It turned out 9 groups: in the first group the cells contained 40 or less drops, in the latter - more than 180 drops. It is noted that if we exclude the measurements of the first group with the minimum number of drops (about 20% of measurements), then for the other groups the average estimates practically coincide with the median. It also significantly increased the average value for the sample (from 112 to 135) and reduced the standard deviation (the variance decreased more than twice). The measurement exclusion threshold can be selected as 100, 80 or 60. At the same time, the mean (and median) estimates changed by less than 10% (from 149 to 138), and the standard deviation — from 27.5 to 36.4 (less than 33%).

4. Conclusion

The possibility of fluorescence detection and automatic counting of a large number of drops using the developed devices and special software was shown. The same analysis algorithms for fluorescent droplets on the background of a large number of non-fluorescent ones can be used in analyzing the results of a digital polymerase chain reaction of a droplet at the end point.

According to the experimental data, the threshold value of the fluorescence signal and the corresponding value of the limiting concentration were determined. For the detection of emulsions under given conditions, the limiting concentration was 160 nM, and when detecting a liquid, it was about 100 nM.

The data obtained indicate that the main reliable information on the number of drops was concentrated in the central part of the reaction chamber. From this point of view, edge images having the interface between media (walls of the reaction chamber) can be neglected. This reduces the measurement area and reduces the scan time.
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