Parallel Evaluation of Melting Temperatures of DNAs in the Arrayed Droplets through the Fluorescence from DNA Intercalators

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Supporting Information

ABSTRACT: Parallel evaluation of melting temperatures ($T_m$) of DNA molecules in multiple floating droplets (20 $\mu$m in diameter) was demonstrated. The $T_m$ values were evaluated from the melting curves which were observed through the fluorescence from the DNA intercalators. The $T_m$ values measured in the droplets corresponded well to those measured in the bulk, indicating the validity of the measurement. The parallel evaluation of $T_m$'s was realized by observing melting curves of DNAs in the different droplets at the same time using the "droplet guide", which guided and fixed the floating droplets to the designated points in the observing plane. This demonstration would pave the way for the improvement of the precision of droplet digital PCR (ddPCR), whose state-of-the-art ascribes color and intensity of fluorescence to the base sequence of DNA in the droplet.

Since the discovery of DNA sequencing techniques by Sanger and Coulson1 and Maxam and Gilbert2 in the 1970s, they have constantly evolved, and their progress has changed where they stood. Reduction in time and cost of sequencing has made it easier for the market to accept DNA sequencing as a versatile tool for providing clues in applications such as criminal investigation, virus identification, and liquid biopsy. Liquid biopsy is one of the promising applications for the treatment of cancer; efficacy of the drugs can be monitored noninvasively by checking the concentration of mutant DNA molecules in the patient's blood.3 Digital PCR (dPCR)4−6 and droplet digital PCR (ddPCR)7−9 are the potential techniques to realize liquid biopsy. In ddPCR, each single DNA molecule in a sample is partitioned and amplified in a droplet. The existence of targeted DNA molecules in the droplet is indicated by fluorescence from dyes.7 The concentration of the targeted DNA molecules in the sample can be obtained by counting the number of the droplets in a fluidic tube that emit corresponding fluorescence. In principle, ddPCR has the potential for a single mutant DNA molecule in the sample to be detected. However, we have not reached that point, one of the reasons being the lack of precision in the ascription of the fluorescence to the corresponding DNA molecules.

In this paper, we report on the parallel evaluation of melting temperatures ($T_m$) of DNA molecules in the droplets floating on the oil surface. The $T_m$ value obtained would reduce errors in detecting targeted DNA molecules in ddPCR because the $T_m$ value is inherent to the base sequence of DNA molecules and helps one to precisely determine the DNA molecules in the droplet. The parallel evaluation of $T_m$'s was achieved by using the "droplet guide", which guided and fixed the floating droplets to the designated points in the observing plane. This technique would lead to the evaluation of the droplets at the same speed as that of the conventional ddPCR devices.

Table 1 shows base sequences of DNA molecules used in this study. Three types of double-stranded DNAs (dsDNAs) were used. These molecules were templated from the EGFR (epidermal growth factor receptor) gene, and their lengths were 16, 23, and 78 bp, respectively. These molecules were hybridized with the molecules with complementary base sequences to form the dsDNAs. A DNA intercalator (EvaGreen) was used for the melting curve measurements of the dsDNA in the droplets. The melting curves can be measured with the DNA intercalator because its fluorescence intensity increases with the increase in the number of dsDNA molecules. The droplets were prepared by using RainDrop (RainDance Technologies). The droplets were stabilized by the fluorocarbon surfactant and were floating on the surface of the fluorocarbon oil as prepared.10 In this study, dsDNAs were used instead of amplicons because PCR in the droplets might cause fluctuation in the concentration of the amplicons and make it difficult for the methodological evaluation of the $T_m$ measurement to be performed.

For the parallel measurement, the laminated fluidic cells with the droplet guide on the upper ceiling were prepared. The schematic drawing of the fluidic cells is shown in Figure 1a. These cells were prepared by simple lamination of commercially available tapes between the glass substrates. Figure 1b shows the optical microscope image of the droplet guide. The guide was an assembly of dimples of controlled size.
The droplets were introduced in the fluidic cell by injecting a mixture of the fluorocarbon oil from the inlet using a normal micropipette. Because the density of the droplets (around 1.0) was smaller than that of the fluorocarbon oil (around 1.6), the droplets were floating and mainly located at the interface between the upper ceiling and the oil. Therefore, when the droplets encountered the tubular wells, they were captured spontaneously according to the buoyancy exerted on them.

Figure 2a,b shows the optical image and the fluorescence image, respectively, of the droplets captured by the droplet guide and the distance in between, which was prepared by photoimprinting the mold with micrometer-sized pillars on the resin on the surface of the glass substrate.11 The droplet guide consisted of many tubular wells whose diameter, pitch, and depth were 18, 25, and 6 μm, respectively. The droplets were introduced in the fluidic cell by injecting a mixture of the droplets and the fluorocarbon oil from the inlet using a normal micropipette. Because the density of the droplets (around 1.0) was smaller than that of the fluorocarbon oil (around 1.6), the droplets were floating and mainly located at the interface between the upper ceiling and the oil. Therefore, when the droplets encountered the tubular wells, they were captured spontaneously according to the buoyancy exerted on them.

Figure 2a,b shows the optical image and the fluorescence image, respectively, of the droplets captured by the droplet guide at 30 °C. The droplets contained the dsDNA (78 bp, 0.4 μM) and the DNA intercalator (1.2 μM), and these images were observed using the inverted fluorescence microscope (IX70, Olympus Corporation) with the excitation and observed wavelength being 490 and >500 nm, respectively. Bright spots were observed from the positions where the droplets were captured, indicating that the DNA intercalators were inserted in the dsDNA and emitted fluorescence. The bright spots were also observed from the droplets containing the DNA intercalator and the dsDNAs (16 and 23 bp), but their intensities were smaller than that of the 78 bp dsDNA when compared under the same concentration (0.4 μM), since shorter double-stranded molecules have fewer intercalated sites.

The parallel evaluation of melting curves of dsDNAs in different floating droplets was demonstrated by measuring the temperature dependence of fluorescence intensity of the multiple droplets in an observing plane. In the measurement, the temperature was raised at 0.1 °C/s and controlled with a transparent glass stage (Thermo Plate, TOKAI HIT).

Figure 3 shows an example of the melting curve of the 78 bp dsDNA obtained from a single droplet: measured data (dots), fitting curve (solid line), and derivative of the fitting curve (dashed line). The arrows show the corresponding axes for the plots and the line.

![Figure 3](null)

Figure 3. Melting curve of the 78 bp dsDNA obtained from a single droplet: measured data (dots), fitting curve (solid line), and derivative of the fitting curve (dashed line). The arrows show the corresponding axes for the plots and the line.

**Table 1. Base Sequences of DNA Molecules Used in This Study**

<table>
<thead>
<tr>
<th>length (bp)</th>
<th>base sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>5′-CTGGCCGTCGTTTAC-3′</td>
</tr>
<tr>
<td>23</td>
<td>5′-AATCTGTGTCCTTGACATGCTG-3′</td>
</tr>
<tr>
<td>78</td>
<td>5′-GCAGCATGTCAGATCAAGATTTTGGGGCGGGCCAAACTGCTGGGTGCGGAAGAGAAAGAATACCATGCAGAAGGAGG-3′</td>
</tr>
</tbody>
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standard deviation of the $T_m$ value was 0.6 °C. The deviation in $T_m$ would come from various factors such as the environmental difference around the DNA molecules.

Figure 5 shows the fluorescence images of the samples where the droplets with different dsDNAs (16 and 23 bp) coexisted (1:1). At 45 °C, two kinds of droplets with different fluorescent intensities were observed (Figure 5a). Figure 5b shows the image at 57 °C. The droplets with smaller intensity in Figure 5a almost disappeared, while the droplets with larger intensity in Figure 5a remained fluorescent. At 70 °C, all the fluorescence disappeared. (The movie of the fluorescence images for this measurement is available in the Supporting Information.)

Figure 6 shows a histogram of $T_m$’s obtained through the same analysis in Figure 3 for the sample with the 16 and 23 bp dsDNAs. The total number of the droplets analyzed was 2046. In Figure 6, two distinctive peaks were observed; one was centered at 53.4 °C and the other at 59.4 °C, which corresponded to the $T_m$’s of the 16 bp (57.1 °C) and 23 bp (64.0 °C) dsDNAs in bulk, respectively. This result suggests that the DNA in each droplet can be distinguished through the $T_m$ as well as the fluorescence intensity. The breadth of the peak in Figure 6 might be due to the weak emission and/or the inadequate acquisition of fluorescence from the droplets, which was also one of the possible reasons for the difference of peak surfaces between two samples; the brightness of some droplets with the 16 bp dsDNA did not reach the threshold for the analysis because the shorter dsDNA has fewer intercalating sites for the dyes. We are currently working on this issue to avoid ambiguity in the interpretation of the results. The achievement will be reported elsewhere in the future.

In summary, the parallel evaluation of $T_m$’s of DNAs in multiple floating droplets was demonstrated by observing the temperature dependence of fluorescence from the DNA intercalator. The droplet guide was successfully applied for the fixation of the droplets at designated points in the observing plane. The $T_m$ values evaluated in this method corresponded to those measured in bulk, suggesting the validity of the measurement. Furthermore, the dsDNA with the different base sequence in the droplet could be distinguished in terms of the $T_m$’s, which would lead to the contribution to the reduction of errors in detecting targeted DNA molecules in the ddPCR format.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b01343.

Movie of the fluorescence image of the $T_m$ measurement for the sample with the 16 and 23 bp dsDNA (AVI)

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Notes

The authors declare no competing financial interest.

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REFERENCES


