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One-by-one single-molecule counting method for digital quantification of SARS-CoV-2 RNA

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A B S T R A C T

Digital counting individual nucleic acid molecule is of great significance for fundamental biological research and accurate diagnosis of genetic diseases, which is hard to achieve with existing single-molecule detection technologies. Herein, we report a novel one-by-one single-molecule counting method for digital quantification of SARS-CoV-2 RNA. This method uses one fluorescent micromotor functionalized with peptide nucleic acids (PNAs) to specially capture one target RNA molecule. The RNA-micromotors can be propelled by the electric field to target district and accurately counted. Moreover, the method can also clearly discriminate one-base mutation in the target RNAs, indicating the great potential for clinical diagnostics and virus traceability survey.

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Introduction

It has been a long-term desire to one-by-one count individual biological molecules, especially nucleic acids and proteins, which is of great significance for both fundamental life science and accurate clinical diagnostics. Therefore, various single-molecule counting methods, such as droplet digital PCR [1–3], microfluidic technology [4–6], flow cytometry (FCM) based methods [7,8], single-molecule fluorescence in situ hybridization (smFISH) [9,10], single-molecule enzyme-linked immunosorbent assay (digital ELISA) [11,12], fluorescence resonance energy transfer (FRET) based nanosensors [13,14] and special instrumentation for single-molecule detection, such as fluorescence correlation spectroscopy (FCS) [15] and total internal reflection fluorescence (TIRF) microscopy [16–18] have been developed. However, these methods are hard to achieve digital counting individual molecule, even difficult to detect the targets at single-molecule level because there are still some key problems that need to be addressed. Firstly, the existing methods are rare to establish the one-by-one relationship between the target molecule and the digitally counted signal readout. Secondly, the non-specific background signal is the most important aspect because it is impossible to one-by-one count the target molecules in the presence of the background signal, which is also the mainly principal cause that most of single-molecule detection methods can only detect the target molecules at sub femtomolar, but seldom reach single-molecule level.

Herein, we have developed a novel single-molecule counting method for one-by-one digital quantification of target RNAs. As well known, the global pandemic of novel coronavirus disease (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has already become a serious threat to people’s health [19,20]. Rapid and ultrasensitive detection of SARS-CoV-2 RNA is crucial to early diagnosing and preventing viral transmission. Therefore, in this work, the SARS-CoV-2 RNA was selected as the model target. Briefly, the PNA-functioned fluorescence microbeads (PFFM) with a diameter of 1.0 µm were employed as the micromotors. PNAs are oligonucleotide analogues in which the negatively charged sugar-phosphate backbone is replaced by a neutral backbone of 2-aminoethylglycine units, resulting in the high affinity and selectivity with the complementary RNA/DNA and the Tm values of PNA-RNA/DNA duplexes are independent of the salt concentration in the certain range [21–23]. Besides, PNAs show great stability against various chemicals and over a wide range of pH [22]. Under suitable conditions, one PFFM can specifically capture one target RNA, and thus well establish the one-by-one relationship between the target RNAs and the PFFMs. More importantly, the PFFM-RNA can be directionally propelled by the applied electric field to the target...
district due to the negatively charged sugar-phosphate backbone of RNA and thus the target RNAs can be digitally counted because 1.0 µm PFFMs are easily monitored one-by-one.

Results and discussion

The principle and workflow of single-molecule counting of SARS-CoV-2 RNA are illustrated in Fig. 1. Initially, the streptavidin-coated fluorescent polystyrene microbeads (FM) served as the single-molecule indicators and were modified with biotinylated PNA (see the sequence in Table S1) probes through biotin-streptavidin binding. It will be confirmed that the PNA probes can efficiently capture the target RNAs and the RNAs can drive the PFFMs to clearly separate from those without binding target RNAs.

PFFMs were firstly mixed with target RNAs to perform the hybridization between the PNA probes and target RNAs. When the number of the target RNAs is much less than the number of PFFMs, there will be either only one or no target RNA loaded on each PFFM according to the Poisson statistics [10, 17, 24]. In this method, the number of PFFMs input is 4000. From the Poisson statistics, we can calculate that when the number of the target RNAs is less than 700, the percentage of PFFMs that are loaded with one RNA is > 90% compared to those PFFMs that are loaded with two RNAs. And the percentage is as high as 97% when the number of the target RNAs is 200. The calculation process is shown in supporting information and the results are listed in Table S3. Soon afterwards, the mixture was put in the cathode terminal of the custom-made capillary chip (see Figs. 1B and S1-S3). Owing to the PFFMs themselves are uncharged or with a very low charge, the captured RNA makes PFFM-RNA negatively charged. Every PFFM captured one RNA molecule and PFFM-RNA could be propelled towards the anode through the capillary tube under applied electric field.

To achieve digital counting of individual target RNA, there are several issues need to be addressed. Firstly, in the presence of electrolyte solution, the quartz capillary will generate electroosmotic flow due to the hydrolysis of -SiOH on the capillary surface to produce negative local charge (-SiO\(^-\)) [25]. The direction of the electroosmotic flow is opposite to the motion of the PFFM-RNA. By using the N gene (1260 nt, see Fig. S4 and Table S2) of the SARS-CoV-2 viral genome as the model target RNA, we have investigated the effect of the electroosmotic flow on the movement of the PFFM-RNA. As displayed in the Movie S1, the electroosmotic flow can bring the PFFM-RNA to the cathode direction with a velocity of about −16 µm/s (Fig. 2A). To eliminate the influence of the electroosmotic flow, we
treated the quartz capillary with polyacrylamide (PA) coating [26]. Firstly, the -SiOH on the surface of the quartz capillary reacts with γ-methacryloxy propyl trimethoxysilane (γ-MPS) to produce -SiO-γ-MPS (Fig. S5). -SiO-γ-MPS contained the active functional group olefinic bond and thus can react with acrylamide and N,N′-Methylene-bis-acrylamide (MBA) to produce polyacrylamide under the catalysis of Ammonium persulfate (APS) and N,N,N′,N′-Tetramethylethlenediamine (TEMED). Besides, MBA can act as the cross-linking agent and enable the cross-linking reaction between the polyacrylamide. Finally, a PA-coating is formed on the surface of the capillary. As shown in Movie S2, after the PA-treatment, the PFFM-RNA can move to the anode direction with a velocity of about 42 µm/s. Meanwhile, both the PFFM in the absence of the target RNAs and the non-functioned fluorescence microbead with PNA in the presence of the target RNAs (FM+RNA) are motionless under the applied electric field (Fig. 2A and Movie S3-S4), indicating that the electro-osmotic flow was effectively eliminated.

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Secondly, the pH value of the electrolyte solution is another key element to affect the movement of PFFM and PFFM-RNA. As demonstrated in Fig. 2B, by using phosphate buffers at pH 4.9 as the electrolyte solution, the PFFM-RNA can be propelled toward the anode with a velocity of about 42 µm/s. Meanwhile, both the PFFM in the absence of the target RNAs and the non-functioned fluorescence microbead with PNA in the presence of the target RNAs (FM+RNA) are motionless under the applied electric field (Fig. 2A and Movie S3-S4), indicating that the electro-osmotic flow was effectively eliminated.

Fig. 3. Digital counting results of PFFMs in the presence of different amounts of N gene RNAs. (A) Fluorescent images of PFFMs when the number of N gene RNAs ranges from 0 to 150 copies. The size of each image is 300 µm × 300 µm. (B) The linear relationship between the N gene RNAs and the monitored PFFMs. The linear regression equation is \( y_{\text{PFFM}} = 0.98x_{\text{N gene RNA}} - 3.79 \) and the corresponding correlation coefficient \( R^2 = 0.9988 \).

We further investigate the effect of the length of target RNA (namely, the charge number of the target RNA) on the movement of PFFM-RNA by theoretical analysis. When PFFM-RNA moves in the capillary, it is mainly subjected to the electric field force \( F_E \), viscosity resistance \( f_v \) and pressure drag \( f_p \) in the horizontal direction, and the gravity and buoyancy in the vertical direction. When it moves at a constant velocity in the horizontal direction, the forces in the horizontal direction reach a balance, that is, \( F_E = f_v + f_p \). In this
method, \( F_e = qU/d \) (i) \((U: \) the applied voltage; \( d: \) the distance between the cathode and anode, is 0.075 m; \( q: \) the amount of charge carried by PFFM-RNA). Since the charge of the PFFM-RNA comes from the sugar-phosphate backbone of the captured RNA, therefore, \( q = Ne \) (ii) \((N: \) the number of RNA bases, \( e: \) elementary charge, is \( 1.6 \times 10^{-19} \) C). According to Stokes’ law, \( f_v = 6\pi\eta rv \) (iii) \((\eta: \) viscosity coefficient, is \( 1.6 \times 10^{-3} \) Pa.s; \( r: \) the radius of PFFM, is \( 5 \times 10^{-7} \) m; \( v: \) moving velocity). The pressure drag \( f_p = 0.5\pi C_D \rho r^2 v^2 \) (iv) \((\rho: \) the electrolyte solution density; \( C_D: \) the drag coefficient). Because \( f_v \propto rv, f_p \propto r^2v^2 \), so the value of \( r \) and \( v \) has a greater effect on \( f_p \). In this work, \( r = 5 \times 10^{-7} \) m and \( v \) is at \( \mu \)m/s level, therefore \( f_p < f_v \) and \( f_p \approx f_v \) (v). Taking the formula (i), (ii) and (iii) into formula (v), we can get the PFFM-RNA moving velocity \( v = NUe/6\pi\eta dr \) (vi).

According to formula (vi), when \( U = 300 \) V, \( N = 1260 \) nt, the theoretical velocity of PFFM-RNA is \( 53.48 \) \( \mu \)m/s, which is consistent with the experimental results \( 42 \pm 6 \) \( \mu \)m/s. In general, when a micro-motor moves at a speed greater than three times its own size, its motion can be clearly observed. The diameter of PFFM is \( 1.0 \mu \)m, therefore when it moves at a velocity \( \geq 3 \mu \)m/s, we can clearly observe its motion. From formula (vi) we can see that \( v \propto N \), when the applied voltage \( U \) is constant. When \( U = 300 \) V, \( N = 1260 \) nt, the detected velocity of PFFM-RNA is \( 42 \pm 6 \) \( \mu \)m/s, thus we can calculate that when \( U = 300 \) V and the velocity \( v = 3 \mu \)m/s, the number of RNA bases \( N \) is 90 nt. Therefore, when the length of the target RNA is \( \geq 90 \) nt, PFFM-RNA can be propelled by the applied electric field and separated from the non-RNA banding PFFMs.

As described above, we have demonstrated that one target RNA can bind to one PFFM and can subsequently drive the PFFM toward the anode from the cathode under the applied electric field. The 1.0 \( \mu \)m fluorescent micromotor can be easily monitored by fluorescent confocal microscopy both in the manner of counting the moving PFFM-RNAs and imaging the PFFMs in the end point of electrophoresis (Fig. 1C). As demonstrated in Fig. 3 and Table S4, when the number of N gene RNAs increases from 10 to 150 copies, there is a good one-to-one relationship between the monitored PFFMs and the target RNAs. The linear regression equation is \( Y_{PFFMS} = 0.98X_{N_{gene \ RNA}} - 3.79 \) and the corresponding correlation coefficient \( R^2 \) is 0.9988, indicating that the proposed digital counting method can count the RNAs one-by-one. However, when the target RNAs are less than 10 copies, it is difficult to accurately pick up the RNA molecules by dividing a certain volume of the solution containing such low copies of RNA targets. Therefore, the monitored results show a higher deviation when the RNAs are less than 10 copies. However, a single cell can be accurately picked up to the sample solution. Theoretically, the proposed method can detect single RNA molecule in a single cell.

SARS-CoV-2 is a single stranded positive-strand RNA virus including four key genes to encode four structural proteins: Spike protein (S), Envelope protein (E), Membrane protein (M) and "Fig. 4. The ability to detect single-nucleotide mutation of this method. (A) Fluorescent images of PFFMs when 200 copies of S gene-mutant RNA and S gene-wild RNA serve as the target RNAs respectively, and the image of PFFMs when 200 copies of N gene RNA serve as the internal reference. The size of each image is 300 \( \mu \)m \times 300 \( \mu \)m. (B-C) The derivative of the fluorescence intensity signal as the function of temperature produced by the hybrids of S gene-mutant RNA/PNA (B) and S gene-wild RNA/PNA (C). (D) The number of monitored PFFMs when 200 copies of S gene-wild RNA and S gene-RNA serve as the target RNAs respectively."
Nucleocapsid (N) [27]. As described above, the specific sequences of N gene have been generally employed to detect the SARS-CoV-2 virus for diagnosis. On the other hand, SARS-CoV-2 viruses have been known with high mutation rates [28]. Several SARS-CoV-2 variants caused by the gene mutations such as Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) are considered variants of concern (VOCs) by the World Health Organization (WHO). The important mutations present in VOCs are located in the S gene [29,30]. For example, Delta harbors eight mutations in the S gene, including two key single-base mutations L452R and E484Q within the receptor binding domain [30]. The S protein generally determines the infectivity and the transmissibility in the host of SARS-CoV-2. Therefore, the S protein is the major antigen for all vaccines of COVID-19 and identification of SARS-CoV-2 variant is of great significance to prevent COVID-19 from spreading. The identification is usually performed by RNA sequencing to screen the mutations in S gene, which requires complex experimental steps, high consumption of reagents, long time and expensive instrumentations.

It is very interesting that the PNA probes not only have high affinity to capture the target RNAs but also possess high selectivity to identify single-nucleotide mutation in target RNAs [1,22]. To assess the ability to detect single-nucleotide mutation of this method, we selected the S gene of Delta SARS-CoV-2 variant as the model and prepared two RNA fragments: S gene-wild RNA and S gene-mutant RNA (1200 nt, Fig. S4 and Table S2). The S gene-mutant contains L452R and E484Q two single-base mutations, and we select L452R as the target site and prepared PNA probes complementary to the S gene-mutant RNA at L452R site. The PNA probe was designed specifically for the S gene-mutant RNA. First, we found that the hybridization of the PNA probes and S gene-mutant RNA, S gene-wild RNA is so specific that one base pair mismatch in the hybrid complex causes the Tm to reduce by 11 °C (Fig. 4B and C). Then we carried out the assay by using S gene-wild RNA and S gene-mutant RNA as the target RNA. The results showed that when S gene-mutant RNA input is 200 copies, 185 ± 19 PFFMs can be monitored in the end point of electrophoresis. However, when S gene-wild RNA input is 200 copies, only 68 ± 9 PFFMs can be monitored (Fig. 4A and D). Meanwhile, we select the N gene as an internal reference, when N gene RNA input is 200 copies, 189 ± 17 PFFMs can be monitored. By comparing the monitored PFFMs with the internal reference, we can easily judge whether the target site is a mutation site. Therefore, this method can clearly discriminate the single-nucleotide mutation in the RNA targets and thus can be applied to accurately trace the variants of the SARS-CoV-2 virus.

To evaluate the feasibility of this method, we used 83 copies of SARS-CoV-2 RNA extracted from the SARS-CoV-2 pseudo viruses as the model target. Then we carried out the assay to detect SARS-CoV-2 RNA by targeting the N gene sequence. As shown in Fig. 5, the
number of PPFMs monitored is 53 ± 7 which is lower than the theoretical value (83 copies). The reason is that the theoretical value is calculated by ignoring the loss in the extraction step. However, in the RNA extraction step, no matter what method or RNA extraction kit is used, RNAs loss cannot be avoided. From the monitoring results, we can further calculate that the extraction efficiency of SARS-CoV-2 RNA was about 64%. In addition, as shown in Fig. 5, there was no cross-reactivity to the related N gene sequences from other SARS-like coronaviruses, including SARS Tor2, HCoV-HKU1 and bat-SL-CoV-ZC45. Therefore, this method can be applied to detected SARS-CoV-2 viruses at very low concentrations with high specificity.

Conclusions

In summary, we present a new single-molecule counting technique for digital quantification of RNA targets. A key advance in this work is demonstrating that one RNA molecule can drive one fluorescent micromotor propelled by the applied electric field. The quantitative manner is unique that one fluorescent micromotor can indicate one target RNA molecule. And thus, we first realize to count the target RNAs one-by-one with a simple capillary electrophoresis, which has been successfully applied to counting SARS-CoV-2 RNAs. Moreover, the proposed technique can also accurately identify the single-nucleotide mutation in the target RNAs, which is of great importance for rapid traceability of SARS-CoV-2 virus and effective prevention of the COVID-19. Owing to the highest sensitivity with clear discriminating single-nucleotide mutation, the proposed method should be suitable to quantitatively detect DNA/RNA biomarkers in a single cell and the cell-free DNA/RNA biomarkers in the blood testing, including single-nucleotide mutation in mRNAs, fusion gene transcripts, mRNA splicing variants, long non-coding RNAs and so on. In fact, we have developed a universal platform for digital counting single-molecule. The applications of the digital counting single-molecule technique should be extended to all RNA/DNA targets when the nucleotides are greater than 90 nt.

On the other hand, the fluorescence micromotors should be easily encoded by their size and fluorescence color. Therefore, multiplexed DNA/RNA targets, especially relevant DNA/RNA molecules in a cell, all related cell-free DNA/RNA biomarkers in blood for specific cancer, and genes in a DNA/RNA virus, can be simultaneously counted with one step by immobilization of different PNA probes on the different encoded fluorescent micromotors. Next, we envision constructing a monitor system to accurately identify the microbead size with light scattering and decoding the different color fluorescence. Integrating the monitor system with the micromotor electrophoresis may open a new avenue for digital single-molecule counting and realize high-throughput digital counting single-molecule to simultaneously detect multiple targets.

CRediT authorship contribution statement

Weiliang Liu: Experiment design and implementation. Desheng Chen: Data analysis and image processing. Fengxia Su: Manuscript revision. Hongru Pian, Hui Wang, Pengbo Zhang: RNA target preparation. Zhengping Li: Experiment design, manuscript writing and project management. All authors reviewed and provided editorial comments on the manuscript.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Materials and methods

All experimental materials and methods are included in the Supporting Information.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nantod.2022.101664.

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