Nanopore Digital Counting of Amplicons for Ultrasensitive Electronic DNA Detection

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***Abstract*—**We demonstrate the feasibility of using the single molecule sensing nanopore as a digital counter to enumerate the amplicons for ultrasensitive electronic nucleic acid analysis. We show that the nanopore digital counting approach could capture the DNA replication dynamics in the LAMP reaction and has the potential to be used in a qualitative test as well as in a quantitative test. By keeping nanopores as simple as possible and by leveraging the amplification assay's high sensitivity, the amplification-coupled nanopore digital counting approach provides a promising optics-free method for highly sensitive and specific nucleic acid testing.

I. Introduction

Due to its conceptual simplicity, nanopore sensors have attracted intense research interest in electronic single molecule detection[1]. Although considerable success has been achieved, analysis of the analog quantity of the current dip events is still challenging due to poor signal-to-noise ratios[2, 3]. While the analog features of individual events are challenging to analyze, the event itself can be easily identified *i.e.*, nanopore digital counting of molecules is easier and more robust to perform. However, nanopore counting alone suffers from the lack of specificity[4] and extended analysis time at low analyte concentration[5, 6]. Fortunately, coding the specificity into the molecule quantities is readily achievable in nucleic acid amplification tests (NAATs), which has increasingly become the preferred method where sensitivity is needed due to its ability to identify extremely low target levels from the sample (down to a single copy)[7, 8]. We here demonstrate the feasibility of using the nanopore sensor to digitally count the amplicons from loop-mediated isothermal amplification (LAMP). We found that the amplification-coupled nanopore digital counting approach could capture the LAMP replication dynamics and has the potential to be used in a qualitative test as well as in a quantitative test. While we employed glass nanopores and the LAMP assay to demonstrate the proof-of-concept, this approach could be extended to other thermally robust nanopores and amplification strategies. Our findings open a new avenue for nanopore sensors towards a new form of compact, robust, low-cost electronic nucleic acid testing at the point of care.

II. Working Principle

While the amplicon abundance is conventionally determined by bulk fluorescence sensing, our approach relies on using the glass nanopore as the single molecule counting device to quantify the amplicons (Fig. 1). When a single DNA molecule is electrophoretically driven through the nanopore, a detectable ionic current blockade generates a digital '1' signal. Existing works[9, 10] have shown that the DNA molar concentration is linearly related to the translocation rate. Therefore, it is possible to infer the amplicon concentration by measuring the translocation rate. Fig. 1a shows the schematic diagram of the experimental setup. For a positive reaction (Fig. 1b), the increase of amplicons manifests itself as the increase of the translocation rate. For the negative reaction (Fig. 1c), the translocation rate remains unchanged or undetectable. The rate determined at certain time spots during the amplification is an electronic measurement of the corresponding amplicon concentrations (Fig. 1d).

III. Results

A. Single-molecule event rate as the readout for concentration

Before the amplification experiment, we first addressed whether the single molecule counting rate could be used as a reliable readout for DNA concentration in our glass nanopore. We performed studies on 5 kbp DNAs with a serial of concentrations. A quick eyeball on the current time traces in Fig. 2a shows that the translocation rate is faster at higher concentration. The extracted inter-arrival time distribution shows a remarkable exponential distribution for each concentration (Fig. 2b). Fig. 2c shows an expected linear relationship between translocation rate and the DNA concentrations.

B. Concept validation

We set out to test if the glass nanopore could detect the end-product of the LAMP reaction. First, we tested a no-template control (NTC) sample before (t=0 min) and after 35 min of LAMP reaction. As shown in Fig. 3a, no translocation events were observed. We then continued to test the positive control sample with *Plasmodium falciparum* genomic DNA. As shown in Fig. 3b, no detectable events were noticeable before the LAMP reaction (t=0 min) and clear translocation events were immediately observable after 35 min of LAMP reaction. Fig. 3c shows the gel image of the final LAMP products for both positive and negative controls which confirms the glass nanopore is able to qualitatively detect the LAMP end products.

C. Resolving the pore clogging by voltage cycling scheme

Intriguingly, in testing the end product of the positive control sample (Fig. 3b), two abrupt current drops sequential occurred and the current stopped returning to its baseline. This clogging issue is due to the high amplicon concentration. Another more representative current time trace was shown in

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Fig. 4a, which contains a full picture of different translocation scenarios. The normal single DNA translocation (Fig. 4a), the temporary clog (Fig. 4b) and the permanent clog case (Fig. 4c).

To resolve this clogging issue, we developed a voltage cycling scheme for long-term recording (Fig. 4d)The DNAs are driven into the glass nanopore when applied positive voltage and then drift in reverse direction by the following negative voltage. Fig. 4e shows the current time trace in two consecutive voltage cycles on the same LAMP product. Fig. 4f shows the overlay of the current traces over 5 s with a total of 487 translocation events. As shown, the reconstructed sensing current shows no baseline shift, which suggests the voltage cycling scheme resolves the clogging issue and is suitable for long-time measurement.

D. Probing LAMP reaction dynamics

After establishing a reliable approach for rate measurement, we tested if the nanopore digital counting could resolve the LAMP dynamics. Using the *P. falciparum* genomic DNA, LAMP assays were performed for a duration ranging from 10 min to 37.5 min at 65ºC, the product of which is digitally counted using the same glass nanopore. Fig. 5a shows segments of the current time trace for each reaction time. It is evident that the event rate increases with extended reaction time.

Fig. 5b shows the extracted rate as a function of LAMP reaction time. Interestingly, the translocation rate versus the reaction time can be fitted remarkedly well with a logistic growth model[11]

$$\mathrm{R}(t) = R_L + \frac{R_H - R_L}{1+e^{-\beta(t-t_0)}} \quad (1)$$

where R_L and R_H are the low and high bound of the translocation rate, respectively, t_0 is the time when the growth rate is at maximum, and β is a measure of the maximum steepness of amplification rate at the exponential growth stage.

Fig. 5c shows the current dip-dwell time scatter plot at each LAMP reaction time. As the amplification time increase, a substantial increase of population with higher current dip and longer dwell time was observed, indicating longer DNAs are produced when reaction continues. This is indeed expected for the LAMP final product[7].

E. Qualitative testing

To demonstrate the potential utility of the LAMP coupled nanopore digital counting approach for qualitative (yes/no) specific nucleic acid testing, we examined two of the most spread species of malaria: *P. falciparum* (*Pf*) and *P. vivax* (*Pv*). Each species-specific assay was tested with three different types of samples (*Pf, Pv, and NTC*). We used the nanopore to analyze the end product of the LAMP assay after 35 min of reaction at 65°C. Fig. 6a and Fig. 6b show the resulting current time traces for *Pf*-specific assay and *Pv*-specific assay, respectively. The digital events were observed when the assays match with the intended species. The reactions were then confirmed by gel electrophoresis

F. Quantitative testing

To evaluate the potential quantitative application of nanopore digital counting platform, we performed the nanopore-LAMP assay on the mitochondrial gene by using a 10-fold serial dilution of purified *P. falciparum* genomic DNA. The nanopore-LAMP performance (Fig. 7a) is benchmarked to the tube-based quantitative LAMP (Fig. 7b) on a benchtop real-time PCR instrument. Both the fluorescence-based method and the nanopore method show the expected right-shift of the amplification curve when reducing the gene copy numbers. Fig. 7c shows the extracted standard curves from both the nanopore and fluorescence methods. The threshold time is determined by the time corresponding to the reading of 500 RFU in the fluorescence method and 1 s^{-1} in the nanopore method, respectively. The amplification over a range of serially diluted DNA sample showed excellent linearity in both methods (R^2=0.98 for fluorescence method and R^2=0.99 for nanopore method). The linearity in the nanopore method suggests it could be used for quantitative analysis of DNA.

IV. CONCLUSIONS

In summary, our findings show the proof-of-concept of using single molecule sensing glass nanopore as an electronic 'eye' to 'digitally count' the number of specific amplicons from the loop-mediated isothermal amplification. We show that the nanopore digital counting approach can capture the DNA replication dynamics in the LAMP and has the potential to be used in a qualitative test as well as in a quantitative test. The amplification-coupled nanopore digital counting approach opens a new avenue for nanopore sensors, bypassing many challenges in the analog analysis of features within identified events (*e.g.*, dip magnitude, shape, and duration). By keeping the nanopore as simple as an orifice that lets a discrete number of molecules to pass through and coding the specificity information into the molecule numbers using the amplification chemistry, it provides a promising optics-free method for highly sensitive and specific nucleic acid testing at the point of care.

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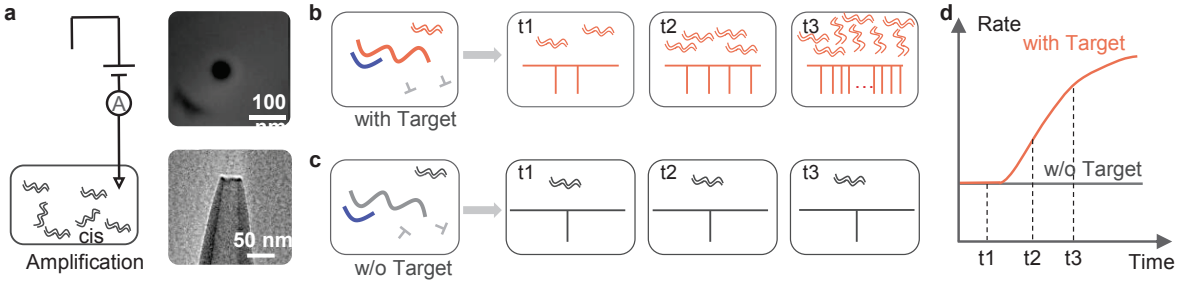

Fig. 1. Illustration of the working principle of nanopore digital counting of amplicons. (a) Schematic measurement setup as well as the SEM and TEM of the glass nanopore. Amplicons are electrophoretically driven through the nanopore one by one, resulting in discernible digital events of the ionic current blockade. The rate of digital events is proportional to the amplicon concentration (b) Digital events in a positive target case. (c) Digital events in a negative target case. (d) Schematic digital translocation event rate as a function of amplification time.

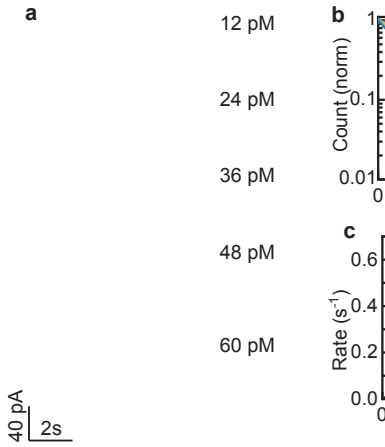

Fig. 2. Continuous recordings of current trace with 5 kbp-DNA through glass nanopore.(a) Segments of the current trace at different DNA concentrations. (b) The normalized probability distribution of the inter-arrival time at different concentrations, with corresponding exponential fits. (c) The average translocation rate as a function of DNA concentration, showing a linear dependence ($R^2 = 0.985$).

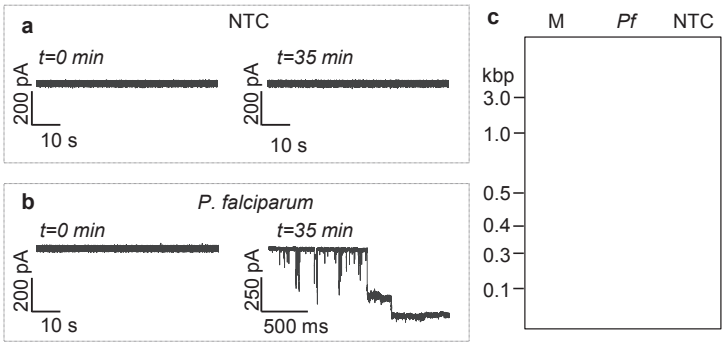

Fig. 3. Concept validation of nanopore digital counting of amplicons. Time traces for (a) negative no-template control (NTC), and (b) positive control before and after the 35 min LAMP reaction. The clogging issue was observed in the positive controls. (c) Gel electrophoresis image of the LAMP products (2% agarose gel).

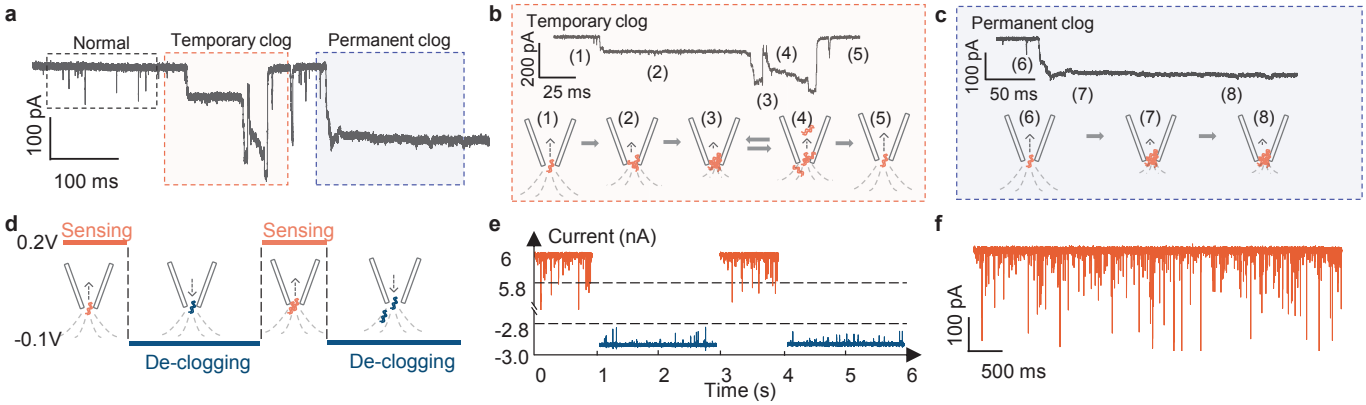

Fig. 4. Resolving the nanopore clogging by voltage cycling scheme. (a) A representative current trace showing normal, temporary clog (b) and permanent clog (c). (d) Illustration of the voltage cycling scheme. The voltage is cycled for sensing and de-clogging. (e) A typical current trace using the voltage cycling scheme. (f) Reconstructed 5 s current trace by sequentially combining the current obtained under the 200 mV sensing voltage. A total of 487 translocation events could be identified without clogging issue

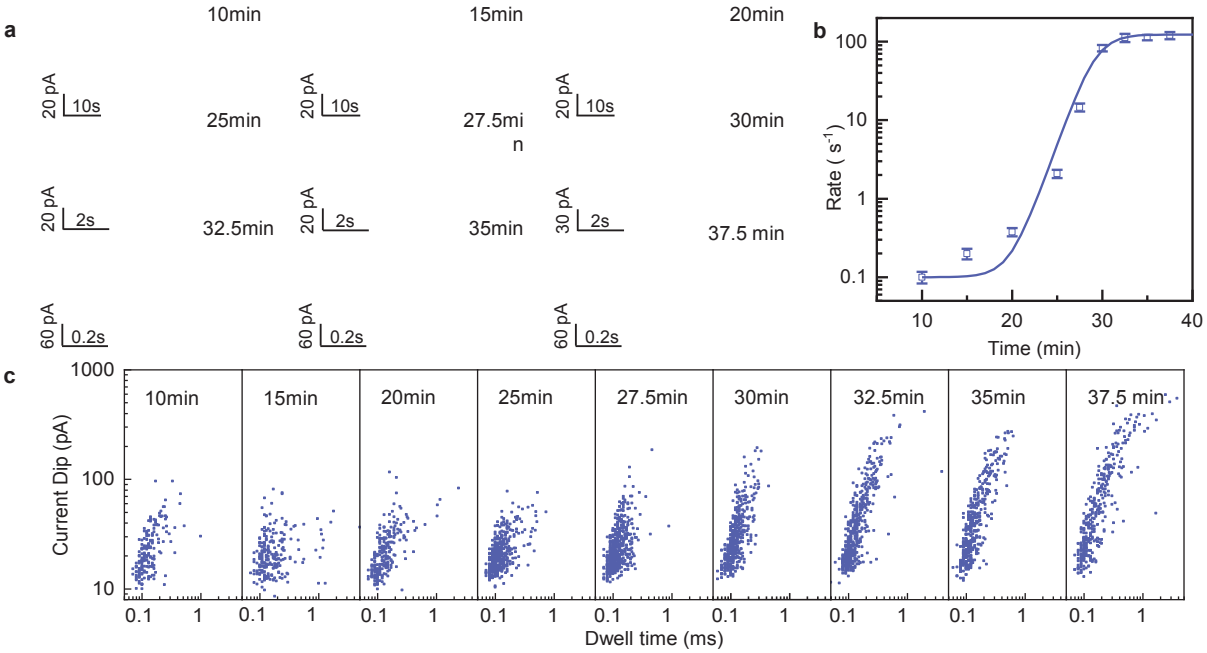

Fig. 5. Nanopore digital counting to probing the LAMP reaction dynamics. (a) Current traces at various amplification times. (b) The translocation rate as a function of the amplification time. The translocation rate increased exponentially before reaching a saturated level. (c) Scatter plots showing current dip magnitude vs. dwell time at various reaction times.

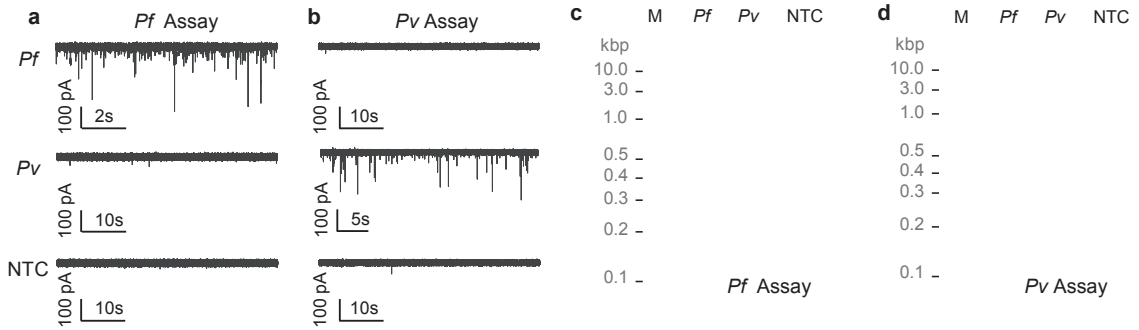

Fig. 6. Qualitative specific nucleic acid testing using the nanopore-LAMP. (a) Current traces obtained from nanopore reading for *Pf*-specific assay, and (b) for *Pf*-specific assay. The translocation rate difference between the positive and the negative is evident. (c) Gel electrophoresis image (2% agarose gel) for *Pf*-specific assay and, (d) for *Pv*-specific assay.

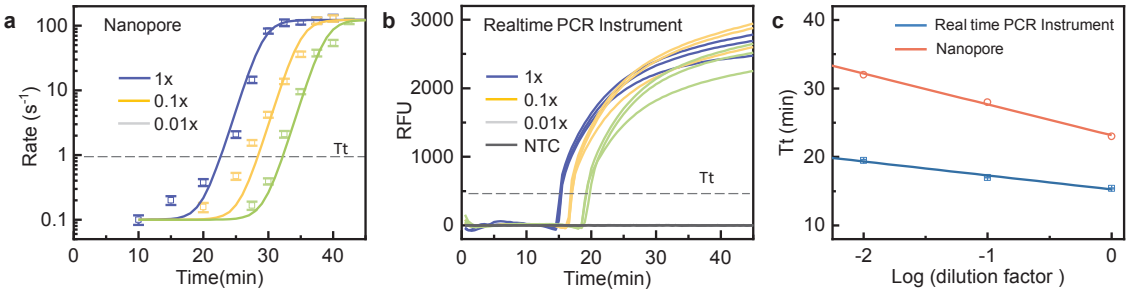

Fig. 7. Comparison between the nanopore method and fluorescence-based method. (a) The results acquired from the nanopore detection. 1X, 0.1X, and 0.01X denote the dilution factors of the templates. (b) Amplification curves obtained from the fluorescence method using benchtop real-time PCR machine. (NTC: no template controls). (c) Standard curves extracted from the nanopore platform and the fluorescence platform. The linearity in the nanopore method suggests it could be used for quantitative analysis of DNA.