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ORIGINAL PAPER

Quantification of Low Concentrations of DNA Using Single Molecule Detection and Velocity Measurement in a Microchannel

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Abstract We present a novel method for quantifying low concentrations of DNA based on single molecule detection (SMD) for molecular counting and flow measurements inside a microchannel. A custom confocal fluorescence spectroscopic system is implemented to detect fluorescent bursts emitted from stained DNA molecules. Measurements are made one molecule at a time as they flow through a femtoliter-sized laser focal probe. Durations of single molecule fluorescent bursts, which are found to be strongly related to the molecular transit times through the detection region, are statistically analyzed to determine the in situ flow speed and subsequently the sample volume flowing through the focal probe. Therefore, the absolute concentration of a DNA sample can be quantified based on the single molecule fluorescent counts from the DNA molecules and the associated probe volume for a measured time course. To validate this method for quantifying low concentrations of biomolecules, we tested samples of pBR322 DNA ranging from 1 pM to 10 fM (~3 ng/ml to 30 pg/ml). Besides molecular quantification, we also demonstrate this method to be a precise and non-invasive way for flow profiling within a microchannel.

Keywords Single molecule detection · DNA quantification · Flow profiling · Microchannel

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Introduction

Advances in molecular technologies is changing the face of disease diagnosis from laborious phenotypic analysis of cells to more rapid and accurate analysis of molecular signatures [1, 2]. In particular, the analysis of nucleic acid markers such as copy numbers of genomic DNA [3, 4], single nucleotide polymorphisms [5–7], DNA methylation [8] and gene expression have assumed an essential role in modern in vitro diagnosis. Currently employed nucleic acid technologies such as Southern and Northern blotting may be useful for qualitative assessment of disease diagnosis. However, blotting technologies require large quantities of clinical samples. In addition, they lack high sensitivity and the quantitative capability necessary for many applications in disease diagnosis. Although highly sensitive measurements of DNA and RNA can be achieved through the use of polymerase chain reaction (PCR) for target amplification, PCR-based methods are often associated with several technical difficulties such as false positive amplification due to contamination, variations in amplification efficiencies in different experimental settings and PCR bias [3, 9, 10]. In order to precisely quantify the nucleic acid targets using PCR, it usually requires extra effort in both the design

of control experiments and the optimization of experimental conditions.

Recently, there has been increasing interest in using single molecule detection (SMD) technologies for directly analyzing nucleic acid targets in low abundance [11–17]. Such progress is motivated by both the high sensitivity of SMD to detect rare targets without the need for amplification and the potential for precise quantification through single molecule counting. SMD is mainly based on the use of a confocal fluorescence spectroscopy that detects the emission from a single fluorescent molecule when it traverses through a femtoliter laser focal probe. Fig. 1a depicts the small focal probe defined by the confocal fluorescence spectroscopic system (Fig. 1c). The convolution of this illumination region is given by a high numerical-aperture objective (typically N.A. $\geq$ 1.2). The use of a confocal pinhole (typically 50–100 μm) provides extremely high signal-to-noise ratio (SNR) by rejecting out-of-focus signals. This thereby allows for high sensitivity and the ability to detect biomolecular targets at concentrations of picomolar or lower [12, 15]. Quantitative measurements of biological samples containing targets of very low concentrations can be conducted by flowing the sample in a microchannel or capillary [12, 18]. This is followed by target quantification based on the number of detected single molecule bursts and the total volume of samples being probed.

Traditionally, SMD has not been routinely used for biomolecular quantification as it is hampered by the fact that the streamwise velocity varies across the flow channel

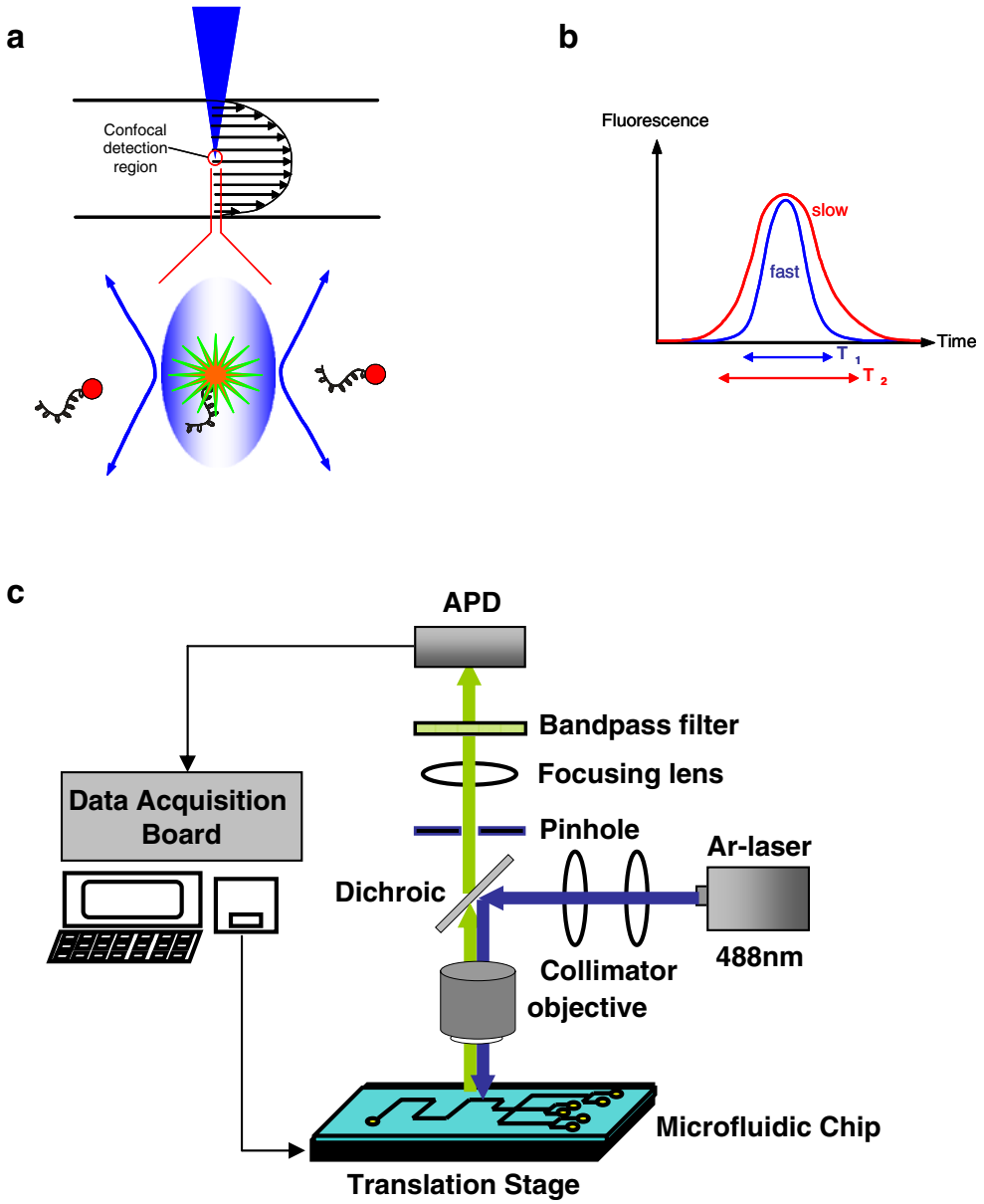


Fig. 1 **a** (*Top*) Due to the parabolic velocity profile in the microchannel, the in situ flow velocity at the laser focal probe region depends on its relative position within the microchannel. (*Bottom*) A stained DNA is excited and emits a fluorescent burst while it passes through the focal probe. **b** The molecular transit time decreases as flow velocity increases. **c** Setup of the confocal fluorescence spectroscopic system for SMD measurements

due to the hydrodynamic shear (Fig. 1a). In addition, accurate estimation of the total probed volume requires additional effort to precisely align the laser focal probe, typically to the center of the flow stream, in order to determine the in situ flow velocity at the focal probe based on the volumetric flow rate used for sample injection. Slight deviation in alignment of the focal probe can cause substantial errors in determining the flow velocity and ultimately the quantity of biomolecular targets. Several techniques such as Micro-Particle Image Velocimetry (micro-PIV), [19, 20] Laser Doppler technique [21] and Fluorescence Correlation Spectroscopy (FCS) [22, 23] have been utilized for flow profiling. However, some of these techniques are either too invasive for bioanalytical studies or they interfere with the biomolecules. Further, they are mostly not efficient in measuring very low concentrations of the target biomolecule. More importantly, most of these flow profiling techniques are not compatible with the SMD setup and cannot be used to determine both the in situ flow rate and low concentrations of biomolecules.

In this report, we present a novel SMD-based method that quantifies DNA targets solely based on the kinetic information of the single molecule fluorescence bursts even without prior knowledge of the volumetric flow rate and the position of the focal probe. As the cross sectional area of the focal probe is known, the concentration of DNA can be determined through two other measured parameters: the total fluorescence bursts and the in situ flow velocity. Analyzing velocity-dependent durations of single molecule fluorescence bursts allows us to determine the in situ flow velocity at any random point where the focused laser probe is placed. This method therefore eliminates the need for precise flow rate control and tedious alignment of a focused laser probe, thereby simplifying the process of SMD-based molecular quantification. We demonstrate that the quantification of DNA targets based on this method is versatile and can be widely used for analysis of other biomolecular targets such as RNA or proteins as well. Additionally, beyond its application to biomolecular quantification, this SMD technique can be applied as a non-invasive method to measure flow profiles in biologically relevant microfluidic configurations.

Experimental methods

Fluorescence spectroscopy setup

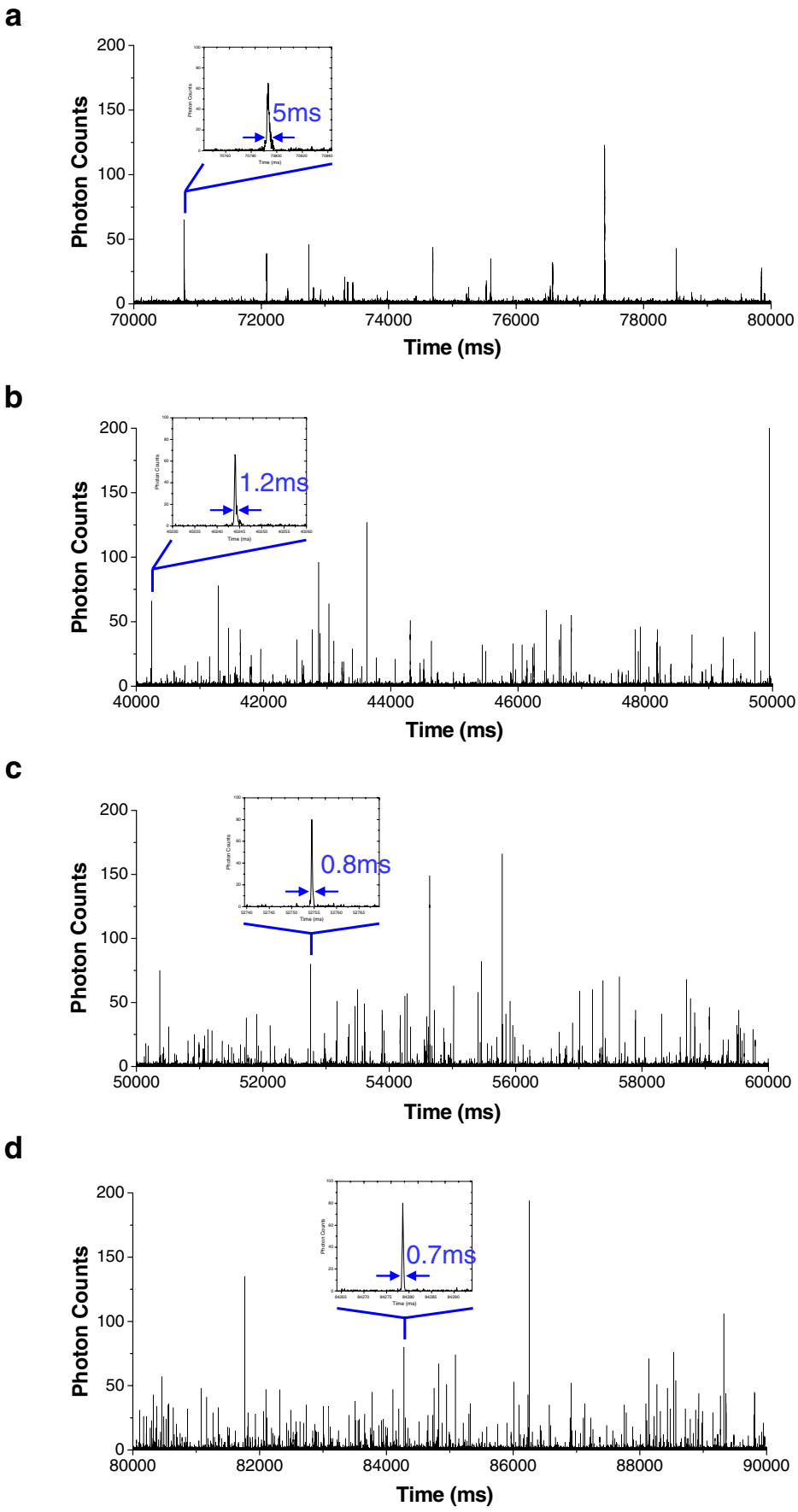
A custom single-line confocal fluorescence spectroscopic system for single molecule detection is shown in Fig. 1c. A 488 nm Argon laser (Melles Griot, Irvine, CA, USA) is used to excite the YOYO-1 dye. The laser beam is reflected by a dichroic mirror (505DCXR, Chroma Technology, Brattleboro, VT, USA) and focused into microfluidic chip or microcapillary by an oil immersion apochromatic objective (100×, N.A. 1.3, Olympus, Melville, NY, USA). The fluorescence emitted by the sample is collected by the same objective and transmitted through the dichroic mirror which allows light of wavelength greater than 505 nm to pass through. A 50 μm pinhole (Melles Griot, Irvine, USA) is used to suppress out-of-focus fluorescence and background noise, thereby increasing SNR. The fluorescence is then filtered by a band-pass filter (520DF40, Chroma Technology, Brattleboro, VT, USA) before detected by a single-photon avalanche photodiode (APD; SPCM-AQR-13, EG & G Canada, Vaudreuil, PQ, Canada). A program written in LabView (National Instruments, Austin, TX, USA) and a digital counter (National Instruments) are used to perform data acquisition and analysis. The average power density is kept at around 50 $\mu W/\mu m^2$ on the sample during the experiments.

Fluidic control and setup

DNA samples were injected into a microcapillary (ID 100 to 250 μm) through a 1,000 μl gastight syringe (Scientific Glass Engineering, Austin, TX, USA) by a high-precision syringe pump (Harvard Apparatus, Cambridge, MA, USA). The microfluidic channel used in the flow profiling measurement is fabricated in-house using a standard microfabrication process which includes standard photolithography, wet etching and wafer bonding technique [24]. The cross section of the microchannel is measured using a conventional upright microscope and found to be 60 μm wide and 50 μm deep. The silicon microchannel is anodic bonded to a borofloat cover glass which has a thickness less than 130 μm (working distance of the apochromatic objective is 150 μm). The silicon wafer is then mechanically punctured to give access to the microchannels with tubing. A nanometer resolution translation stage (Polytec PI, Tustin, CA, USA) is used to precisely control the position of single molecule fluorescence measurements. Flow profiles within the microchannel are then obtained by conducting in situ flow velocity measurements across the channel.

Sample preparation

pBR322 DNA (New England Biolabs, Beverly, MA, USA) is used as the target for the SMD-based flow measurement. pBR322 is a double stranded circular DNA of 4,361 base pairs (MW ~ 2.83 MDa) and is a common plasmid cloning vector in E. coli [25]. DNA is labeled with YOYO-1 iodide as suggested by the standard protocol [26] (Y-3601, Molecular Probes, Eugene, OR, USA) at a ratio of 1 dye molecule to 10 base pairs to maximize dye-DNA reaction


a
Photon Counts
Time (ms)
5ms
b
Photon Counts
Time (ms)
1.2ms
c
Photon Counts
Time (ms)
0.8ms
d
Photon Counts
Time (ms)
0.7ms

◀ **Fig. 2** Ten seconds trace of fluorescent bursts are obtained from YOYO-1 labeled pBR322 flowing in a microcapillary under four different volumetric flow rates. The corresponding volumetric flow rates are **a** 1 μl/min, **b** 5 μl/min, **c** 10 μl/min and **d** 15 μl/min. For each flow rate, a representative zoom-in (30 ms) of a single fluorescent burst is shown in the inset accordingly. The transit time of a molecule passing through the probe volume is inverse to the volumetric flow rate. The bin time of photon counting is 100 μs

efficiency and to minimize background noise level. YOYO-1 iodide has one carbon atom bridging the aromatic rings of the oxacyanine dye and exhibits absorption/emission maximum of 491/509 nm when bound to double-stranded DNA [26]. The reaction solution was incubated at room temperature for 30 min prior to measurements.

In situ flow velocity measurement

The technique adopted to measure the in situ flow velocity at the focal point is based on the statistical analysis of single molecule fluorescent transit time histograms obtained by the confocal spectroscopic system (Fig. 1c). The duration of a fluorescent burst represents the transit time of a molecule passing through the detection region (Fig. 1b). The molecular transit time is determined by both the in situ flow velocity at the focal point and the Brownian motion of the molecule. However, under a fast flow condition (>4 μl/min), the influence of Brownian motion on the molecular transit time can be neglected. In this regime, one would expect that increase in flow velocity would result in a corresponding decrease in transit time. The in situ flow velocity at the focal probe can then be estimated through its relationship with the molecular transit times.

To better quantitatively describe the relationship between the transit time and the in situ flow velocity at the focal probe, an assumption is first made that the average transit time, T_{avg},of a molecule passing through the probe volume is inversely proportional to the in situ flow velocity ν as,

$$\nu = k\left(1/T_{avg}\right) \tag{1}$$

where k is a proportionality constant. The value of k is dependent on the optics such as the numerical apertures of objectives and the size of the pinholes, and should be determined experimentally for each setup beforehand. Since the flow velocities across the flow channel for a pressure-driven flow with uniform pressure gradient has a characteristic parabolic profile [27], the proportionality constant k can be determined via a calibration process. The calibration is performed by quantifying the in situ flow velocities (ν_1 and ν_2) at two different positions (p1 and p2) with a known separation distance S, controlled by a nanometer resolution translation stage, inside the microcapillary. The relationship between in situ flow velocity and transit time is given by equation (2) [27],

$$\begin{aligned} \nu_1 &= 2V_{avg}\left[1-\left(\frac{r_1}{R}\right)^2\right] = \frac{k}{T_{avg1}}; \\ \nu_2 &= 2V_{avg}\left[1-\left(\frac{r_1+S}{R}\right)^2\right] = \frac{k}{T_{avg2}} \end{aligned} \tag{2}$$

where R is the inner radius of the microcapillary, r_1 is the distance, tuned by the translation stage, between the center and the first position p1, and V_{avg} is the average flow velocity depending on the volumetric flow rate applied [27]. After the single molecule fluorescent burst data at both positions is obtained, T_{avg1} and T_{avg2} are then calculated using the transit time histograms. The unknown variables r_1 and k can therefore be determined accordingly. Henceforth, one could estimate the in situ velocity by the average transit time solely by the single molecule fluorescent bursts, according to equation (1).

Results and discussions

Fluorescent bursts obtained from YOYO-1 stained pBR322 DNA (2.3 pM) are measured for 100 s durations as they flow through the microcapillary under four different flow conditions (1, 5, 10, and 15 μl/min). The typical results are as shown in Fig. 2 over a span of 10 s The above results are obtained using our customized confocal fluorescence spectroscopic system. The focal probe of the confocal spectroscope was kept at the center of the microcapillary using a high-precision translation stage for the four different experiments. The average background signal in the absence of DNA was measured to be ~3 photon counts/100 μs. Each fluorescent burst shown in Fig. 2 represents a single DNA molecule passing through the laser focal probe. The single molecule events presented high levels of photon count rates that were unambiguously identified against the background. The inset in each of the four panels represents a single fluorescent burst with a constant peak photon count rate (80 photon counts/100 μs). The duration of the fluorescent burst decreased when the volumetric flow rate was increased, suggesting that the transit time of the single molecules through the focal probe was dependent on the flow velocity. Correspondingly, the total number of fluorescent bursts that were detected within the measurement time period increased with the volumetric flow rate.

The variation in intensity of fluorescent bursts (Fig. 2), which is typical to confocal single molecule measurements, is attributed to the different trajectories of molecules passing through the Gaussian intensity profile within the focused laser excitation probe. When a fluorescent molecule passes

near the maximal excitation intensity in the center of focused probe, a burst with higher intensity is detected and vice versa. The duration of a burst varies with the position at which the molecule enters the focal region of a Gaussian intensity profile. To characterize the overall transit time at the focal point, a statistical analysis of burst duration times is plotted for different flow conditions as shown in Fig. 3. Only bursts with a photon count rate above 8 photon counts/100 μs were considered true events to avoid complications from the background and spurious fluorescence noise. A program written in Matlab was used to determine peak duration time and perform the burst count analysis. As shown in Fig. 3, Gaussian fitting curves clearly show that the mean of the burst duration distribution decreased as the volumetric flow rate increased. In addition, the deviation and the width of the right tail in the distributions also decreased with the increase in flow rate, indicating that the influence of Brownian motion on the transit time was less significant under faster flow conditions.

The average duration obtained from the Gaussian fit was used as a characteristic transit time to determine the in situ flow velocity. Fig. 4 depicts the average transit time as a function of volumetric flow rate. The reciprocal of the average transit time (square) is the flow velocity (circle) which shows a linear relationship with volumetric flow rate demonstrating that the flow measurement based on average fluorescent durations is accurate. This plot can be used as a standard curve to determine the flow velocity at any detection position inside the micro-flow passage. The measured flow velocity starts to deviate from this linear behavior when the flow speed is lower than 1 mm/s (4 μl/min) because the Brownian motion becomes more significant under slow flow conditions. Therefore, more precise quantification of flow velocity is suggested under fast flow conditions.

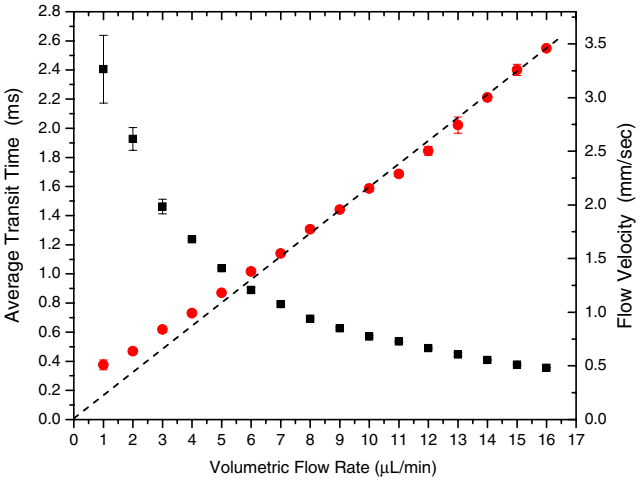


Fig. 4 Average transit time (*square*) and in situ flow velocity (*circle*) as a function of volumetric flow rate

Figure 5 shows a flow profile measured transversely across a 50 μm-deep microchannel. The experiment was performed by positioning the focused laser probe at various measurement points using a translation stage controlled by a computer. Each pitch between movements was 4 μm. The solid line represents the theoretical profile calculated according to the volumetric flow rate applied and the channel geometry. The distribution of measured flow velocities agrees well with the theoretical profile illustrating clear parabolic shape caused by the pressure-driven force. As mentioned previously, this SMD-based flow measurement technique is not ideal for measuring very low flow velocities comparable to the molecular diffusion velocities.

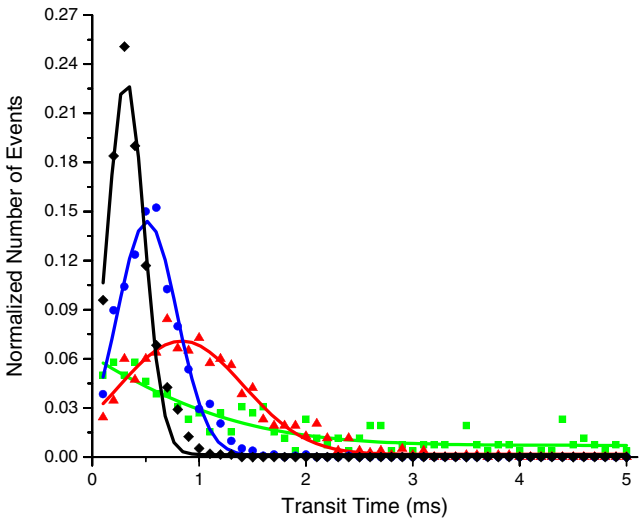


Fig. 3 Burst duration distributions for different volumetric flow rates. The corresponding flow condition is 1 μl/min (*square*), 5 μl/min (*triangle*), 10 μl/min (*circle*), and 15 μl/min (*diamond*). The *solid lines* represent Gaussian curve-fitting for these distributions

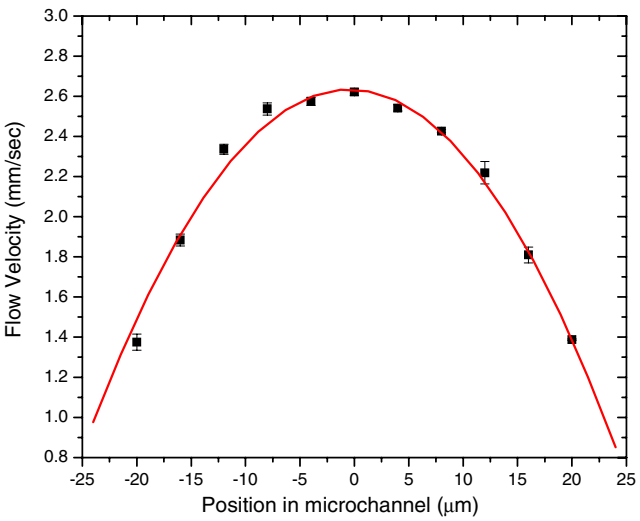


Fig. 5 Transverse direction flow profiling measured inside microchannel. The theoretical profile is represented by the *solid line*

Hence, measurements of flow velocities near the channel boundary were not conducted.

To validate the performance of molecular quantification with this method, we conducted measurements of seven different DNA samples of known concentrations from 1 pM to 10 fM. All the samples were injected into a micro-capillary and measured at a fixed point under 3 different volumetric flow rate settings (1 μl/min, 5 μl/min, and 10 μl/min). As shown in Fig. 6a, the number of counted single molecule fluorescent bursts events, during a fixed time period (100 s), varied with the volumetric flow rates for each DNA sample. Similar variations in burst counting were obtained when measuring a DNA sample at a constant volumetric flow rate at varied measurement positions (data not shown). It is usually difficult to determine the absolute concentration of DNA based on the counted single molecule events without knowing the relative position of the focal point in the microcapillary or specifically the in situ flow speed at the focal point. However, with the proposed statistical analysis of SMD transit time histogram, the in situ velocity could be determined directly. Along with the knowledge that the numbers of single molecule fluorescence bursts is proportional to the product of the in situ velocity, the sample concentration, the cross sectional area of the focused laser beam and the Avogadro's number, quantification of the biomolecules (i.e. the sample concentration) can then be realized. In the current experiment, the cross sectional area of the laser probe was determined to be ~6.37 μm^2 and confirmed by FCS analysis [22]. Fig. 6b demonstrates the resulting estimated concentrations. Although different burst counts were measured under varying volumetric flow rates for each DNA sample (Fig. 6a), the estimated concentrations remained consistent regardless of the different flow conditions. More importantly, the estimation agrees satisfactorily well with the experimentally prepared concentrations.

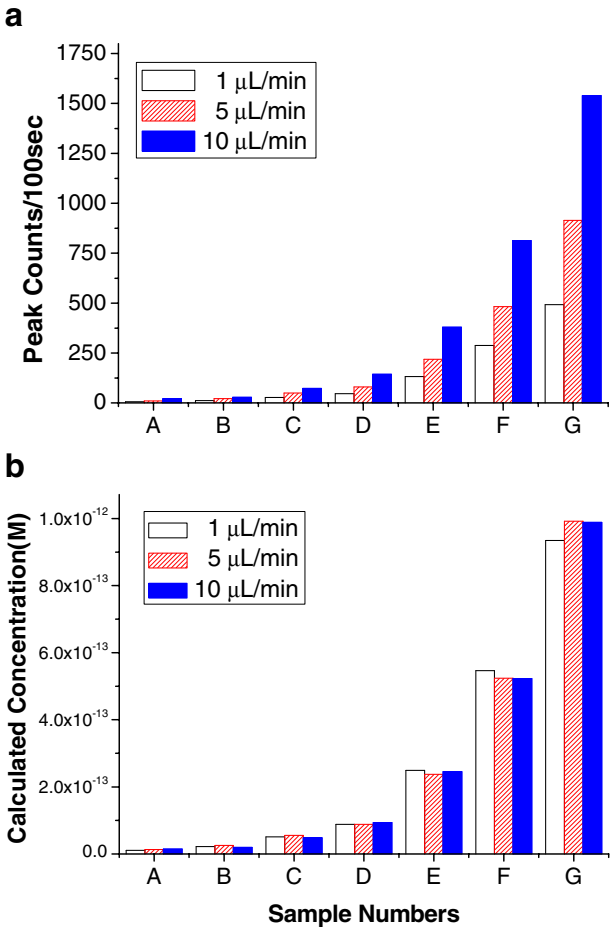


Fig. 6 **a** Fluorescent burst counts measured from DNA samples of seven known concentrations under three different volumetric flow rates (1 μl/min, 5 μl/min, 10 μl/min). **b** Concentration of DNA samples measured based on the burst count and the calculated in situ flow velocity. For each sample, the measured concentrations are fairly consistent, regardless of the volumetric flow rate setting and the position of the focal probe. The measured concentrations for the different samples agree well with the actual concentrations which are *A*, 10 fM; *B*, 25 fM; *C*, 50 fM; *D*, 100 fM; *E*, 250 fM; *F*, 500 fM; and *G*, 1 pM

Conclusion

Quantification of biomolecules based on single molecule detection in a micro-flow system provides several advantages over conventional methods. This method allows for extremely high sensitivity and resolution (both at the single molecule level), requires very small amounts of sample (<5 μl) and also allows for rapid analysis (<2 min). The development of in situ flow velocity measurement enables accurate quantification with high reliability. We have demonstrated that this method is capable of quantifying DNA (pBR 322) sample at an extremely low concentration of ~10 fM (or 30 pg/ml), based on a 100 s measurement. The actual limit of detection can be further decreased by using longer measurement times to obtain a sufficient number of burst counts with high statistical accuracy [15]. The SMD-based quantification platform is versatile to be applied for analysis of a variety of biomolecules such as RNA, peptides, and protein using different fluorescent molecular probes and fluorescent labeling techniques. In addition, the SMD-based flow technique measures the in situ flow velocity directly using the fluorescence data from the biomolecules of interest. Hence, it can be used as a non-invasive method to profile the flow in biologically relevant micro-flow configurations.

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