

Sizing of single fluorescently stained DNA fragments by scanning microscopy

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ABSTRACT

We describe an approach to determine DNA fragment sizes based on the fluorescence detection of single adsorbed fragments on specifically coated glass cover slips. The brightness of single fragments stained with the DNA bisintercalation dye TOTO-1 is determined by scanning the surface with a confocal microscope. The brightness of adsorbed fragments is found to be proportional to the fragment length. The method needs only minute amount of DNA, beyond inexpensive and easily available surface coatings, like poly-L-lysine, 3-aminopropyltriethoxysilane and polyornithine, are utilizable. We performed DNA-sizing of fragment lengths between 2 and 14 kb. Further, we resolved the size distribution before and after an enzymatic restriction digest. At this a separation of buffers or enzymes was unnecessary. DNA sizes were determined within an uncertainty of 7–14%. The proposed method is straightforward and can be applied to standardized microtiter plates.

INTRODUCTION

Polymerase chain reaction (PCR) as well as DNA digestion by restriction enzymes result in DNA-fragments of specific lengths. The determination of the size of the fragments accurately is important for many applications in DNA fingerprinting, restriction mapping, etc. Here gel electrophoresis is by far the most commonly used technique, because it is simple to use and its results are widely accepted. However, gel electrophoresis is time-consuming and difficult to automate. Although appropriate for laboratory research, it is rather disadvantageous for highly desirable high-throughput measurements. Therefore, numerous promising sizing techniques have been developed, including mass spectroscopy (1–3), flow cytometry (4–8), optical microscopy (9,10), capillary electrophoresis (11,12) and atomic force microscopy (13,14).

In this paper we report on a new approach to size DNA-fragments based on the detection of individual DNA molecules adsorbed on a coated glass slide. We detect the fluorescence signal of surface-adsorbed TOTO-1 labeled fragments with a conventional confocal scanning microscope with single molecule sensitivity. DNA-sizing experiments,

based on flow cytometry, proved directly, that the amount of intercalation dye TOTO-1 in single fragments is proportional to their lengths (5). The fluorescence intensities obtained from single DNA molecules result in intensity histograms. By means of calibration experiments these can be transformed directly into histograms of size.

Standard glass cover slips show a very weak tendency to adsorb TOTO-1 labeled DNA non-specifically. A suitable coating should attract stained fragments and repel free dye molecules, thus it should be positively charged at a neutral pH. We selected poly-L-lysine (PLL) for the DNA-sizing experiments, because of its high adsorption yield of stained DNA and low adsorption yield of free TOTO-1.

To examine the dependence of molecular brightness on fragment length and for calibration, we measured DNA samples of defined sizes. On the basis of the obtained calibration curve, we performed DNA-sizing of five different fragment lengths in a mixture. Further, by performing a restriction digest of m13mp18 RF1 DNA with the restriction enzymes EcoRI and PstI we demonstrate the higher speed of our technique in comparison with gel electrophoresis of the same sample. Additionally DNA-sizing at the single molecule level naturally requires lower amounts of DNA compared with gel electrophoresis. Typically electrophoresis requires 5 ng DNA per band, the DNA-sizing method described here has been carried out with just 1 pg per fragment length. The higher sensitivity is of advantage, in order to avoid time-consuming amplification steps with the PCR technique (5,6,9).

MATERIALS AND METHODS

Preparation of DNA solutions

DNA-fragments of different lengths were produced either by PCR technique, by isolating plasmid DNA from bacterial cells or by DNA restriction. 1985 and 4120 bp fragments were obtained by performing PCR with λ -DNA (48 502 bp, Roche, Switzerland) as template, selected DNA primers (Tib Molbiol, Germany) and Taq PCR Core Kit (Qiagen, Germany). 14 200 bp plasmid DNA was isolated from EcoRI cells with the Plasmid Mini Kit (Qiagen, Germany). m13mp18 RF1 DNA (7250 bp), the plasmids pBR322 DNA (4361 bp) and pUC19 DNA (2686 bp) were purchased from MBI Fermentas, Germany.

m13mp18 RF1 DNA was digested at 36°C for 4 h by the restriction enzymes EcoRI and PstI (MBI Fermentas,

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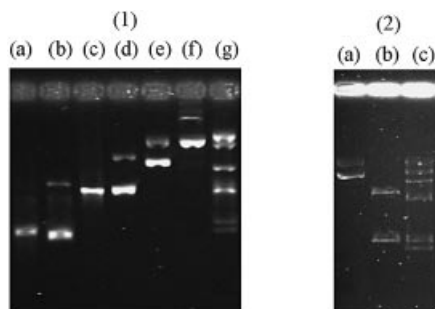


Figure 1. Gel electrophoresis of used DNA fragments (1) and an EcoRI and PstI digest of m13mp18 RF1 DNA (2), as described in Materials and Methods. (1) Lines: (a) 1985 bp linear DNA, (b) pUC19 plasmid DNA, (c) 4120 bp linear DNA, (d) pBR322 plasmid DNA, (e) m13mp18 RF1 DNA, (f) 14 000 bp plasmid DNA and (g) DNA molecular weight marker II [23 130, 9416, 6557, 4361, 2322, 2027 bp (Roche, Switzerland)]. (2) Lines: (a) m13mp18 RF1 DNA, (b) EcoRI/PstI digest of m13mp18 RF1 DNA, (c) DNA molecular weight marker II (Roche, Switzerland).

Germany) into 2318 and 4932 bp fragments. All fragment lengths were verified by gel electrophoresis (Fig. 1).

The restriction fragments, PCR products and plasmids were stained stoichiometrically with the DNA bisintercalation dye TOTO-1-iodide (15) (TOTO-1, thiazol orange homodimer, Molecular Probes, the Netherlands) according to the following procedure. At a DNA concentration of 10 ng/ μ l all fragment solutions were stained separately with 10^{-5} M TOTO-1 for 1 h in MES buffer (50 mM 2-Morpholino-ethansulfonic acid monohydrate, 50 mM NaCl, pH 6; Sigma, Switzerland) yielding a base pair to dye ratio of 1:1 in the solution. The analyzed sample was prepared by diluting DNA solutions with MES buffer to a final fragment concentration of $\sim 10^{-13}$ M. Staining solutions were prepared the same day, some hours before, dilutions were prepared immediately before the measurements. The success of all intercalation reactions was verified by taking absorption and emission spectra.

Preparation of surface coatings

Common glass cover slips (Menzel Gläser, Germany) with a thickness of 150 μ m were used for preparation of the coatings. Prior to use the cover slips were cleaned for 30 min in 70% ethanol in an ultrasonic bath and then dried in nitrogen flow.

The cellulose derivative aminopropyltrimethylsilyl ether-cellulose (ATMSC, MMI AG, Switzerland) (16,17) was dissolved in chloroform (Scharlau, Spain) at a concentration of 1 mg/ml and transferred onto the glass substrates by the Langmuir-Blodgett technique as described (17,18).

For coating with 3-aminopropyltriethoxysilane (APTES, Sigma) the substrates were cleaned in a 1:1 mixture of MeOH/HCl (Merck, Germany) for 30 min and in conc. H_2SO_4 (Merck) for 30 min. Afterwards they were rinsed with double distilled water and dried in nitrogen flow. The cover slips were treated with 2.5% solution of APTES in EtOH *pro analysi* (Merck) for 30 min, rinsed with EtOH *pro analysi* and dried at 45°C for 1 h.

For coating with polyaminoacids the substrates were cleaned for 2 h in a solution of 25 g NaOH (Erne-Chemie, Switzerland) in 250 ml 60% EtOH. Afterwards the substrates were rinsed with double distilled water excessively and incubated with a 0.01% w/v solution of PLL (Sigma) or

polyornithine (PORN, Sigma) in PBS-buffer (pH 7.4, Sigma) for 1 h. The coated glass slides were rinsed with double distilled water and dried in nitrogen flow. All films were stored in a desiccator over silica gel to remove remaining water and used within several days.

Single molecule measurements

The coated coverslips were glued to aluminium slabs with six reaction vessels containing a volumetric capacity of 300 μ l and a coated surface area of 0.37 cm² each. The volume of applied DNA solution per well was 250 μ l. Scanning with a confocal microscope was started ~ 5 min later. This instrument consists of an argon ion laser (488 nm, Optlectra, Germany), an oil immersion microscope objective (N.A. 1.25, 100 \times ; Edmund, USA) a microscope stage (ScanIm120, Märzhäuser, Germany) and an avalanche photodiode (Perkin Elmer, USA). A surface area of 50 \times 50 μ m² was scanned with a resolution of 80 nm. The Gaussian shaped laser focus has a diameter of ~ 500 nm (full-width half-maximum), thus the signal of bound fragments spreads over a sufficiently large number of image pixels for the subsequent fits. The excitation intensity at the surface was reduced to 25 μ W by the means of a neutral density filter. One scan takes < 8 min and delivers image raw data of 640 \times 640 pixels. Although the start times of data acquisition and scan signal generation were perfectly synchronized, it was found that the resulting image showed a line-to-line mismatch, which is caused by a delay of the motion of the microscopic stage with respect to the driver signal. This delay depends on the mass of the moving part of the stage as well as on the mass of the sample. However, it occurs that this phase shift remains constant during one scan and the mismatches can be compensated by an alignment algorithm, described (19).

Subsequently we applied an algorithm, fitting 2D Gaussians onto the intensity maxima. The received amplitudes were used for the histogram plots. A potential source of error is the false interpretation of overlapping single molecules' images. In the majority of cases these incidences show distinct deviation from ideal 2D Gaussians and were neglected for the histograms. This procedure is able to sort out two fragments with a separation distance down to 100 nm. The occurrence of lower distances were found to be rare, as no ghost maxima of double intensity were observed when single fragment sizes were used for the calibration experiments.

Occasionally unbound DNA was detected, i.e. fragments diffuse through the detection volume without surface attachment. These maxima appeared in contrast to adsorbed fragments only on one image column and are sorted out by the algorithm described above. Because of their low frequency the probability of coincidence with the detection of a bound fragment was negligible.

Gel electrophoresis

For verification, all fragments were sized by conventional gel electrophoresis. The analysis was run on a 10 cm, 1% agarose gel at 70 V for 2 h in 0.5 \times TBE-electrophoresis buffer (0.045 M Tris-borate, 0.001 M EDTA; Fluka, Switzerland). Each lane was loaded with DNA in gel loading buffer IV (0.25% bromophenol blue, 40% w/v sucrose in water; Fluka) (20). After electrophoresis the DNA fragments were stained by immersing the gel in electrophoresis buffer containing

ethidium bromide (0.5 µg/ml; Fluka) for 30 min. See Figure 1 for details of lane composition.

RESULTS

The described method for sizing single adsorbed DNA molecules on a coated glass slide comprises staining of DNA-fragments with TOTO-1, immobilization of the sample DNA to the surface and imaging of adsorbed DNA-fragments.

Due to its spectroscopic properties, especially the increase of fluorescence quantum yield ($\sim 1000\times$) when intercalated in DNA, TOTO-1 is suitable for quantitative single molecule experiments (21). Current protocols use bisintercalation dyes to stain double-stranded DNA with a bp:dye ratio of typically 4:1 to 5:1 (22). According to the mass action equation, the portion of bound dye to available binding sites in DNA depends on the affinity constant and on the concentration of the educts. In respect thereof at low DNA concentrations, the concentration of the dye needs to be increased to obtain a fully occupied DNA. In accordance with literature (23) we reached an almost completely loaded DNA with a staining solution containing base pairs and dyes in a ratio of 1:1.

As is known TOTO-1 also binds to single-stranded DNA, but with distinctly lower fluorescence quantum yield (24). Hence only double-stranded DNA has been analyzed with our method. In acidic or basic solutions DNA denaturation occurs. It has been reported that at a pH of 5.5 and below, DNA molecules are partially denaturated (25). To investigate the best pH range we performed measurements between pH 6 and 8 using MES and Tris buffer.

To prevent time-consuming covalent coupling chemistry, we decided to use coated cover slips with strong non-specific binding properties for DNA instead. Due to the negative charge of the DNA backbone the coating needs to have a positive charge. The charge of amine-coated surfaces is affected by alteration of the pH and the density of the surface groups. By shifting the pH of the DNA solution below the pK_a , characterizing the basicity of surface, the non-specific adsorption of DNA molecules strongly increases (25,26).

We tested four different coatings with amino groups (PLL, PORN, APTES and, as control, ATMSC) for their adsorption capacity of DNA molecules. All these coatings are commonly used in biochemical and microbiological applications (27–29). Figure 2 illustrates the adsorption yield of the investigated coatings. ATMSC films usually prevent non-specific adsorption of biomolecules (17,18). As expected on ATMSC, no adsorbed DNA molecules were found. All others, PLL, PORN and APTES are in principle applicable for DNA sizing. Due to the largest density of adsorbed fragments observed and its easy handling we chose PLL for the sizing experiments. The measurements were carried out at a pH of 6 in MES buffer. Figure 3A shows the fluorescence image of intercalated pBR322 DNA fragments (4361 bp). The spots caused by single molecules emerge with very similar lateral extension and brightness. Adsorbed fragments appear to be tightly coiled. Their diameter equals the image diameter of a point source, i.e. the lateral spread of the DNA at the surface lies below the resolution of the microscope. The single molecule images were fitted with 2D Gaussians, the obtained maxima minus background are used for the histogram of Figure 3B. The intensity distribution of the fragments was fitted with a

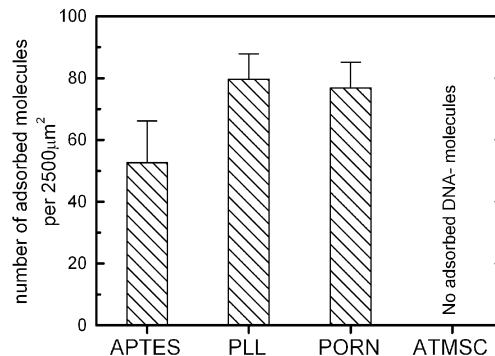


Figure 2. Amount of adsorbed DNA fragments onto different coatings at pH 6: DNA fragment concentration in the incubated sample was 10^{-13} M and the probed area $50 \times 50 \mu\text{m}^2$. While APTES, PLL and PORN films show a strong tendency to adsorb DNA molecules, ATMSC films resist as expected DNA adsorption.

Gaussian curve, resulting in an average fragment intensity of 49.9 photon electrons (PE) with a standard deviation of 3.7 PE or 7.4%.

DNA-fragments of 1.9, 2.6, 4.1, 7.2 and 14.2 kb were analyzed in the same way. The results of the Gaussian fits are summarized in Table 1. The coefficient of variation (CV) is defined to be the standard deviation of a distribution divided by its mean. For different sizes the CV varied from 7 to 14%, with tendency to obtain larger errors for shorter fragments. In Figure 4 the average fluorescence intensity is plotted versus the DNA fragment length. The line through origin was fitted to the data in a least square sense, with a slope of (10.81 ± 0.24) PE/kb and a correlation coefficient of $R = 0.998$. This straight line was used as the calibration curve for the following experiments.

An intensity histogram of five different DNA sizes is shown in Figure 5. The applied solution contained 2×10^{-14} M of the fragment lengths 1.9, 2.6, 4.3, 7.2 and 14.2 kb, respectively. To obtain adequate statistics, the histogram contains the data of approximately 160 molecules, found by scanning $5000 \mu\text{m}^2$. Table 2(a) gives the sizing results and error estimations comprehensively. Fragments of 2.6, 4.3, 7.2 and 14.2 kb are clearly resolved, but the signals caused by the 1.9 and 2.6 kb fragments are superposed. In calibration experiments the CVs obtained by these fragments are, with 14 and 12%, conspicuously higher than the CVs of the larger fragments (see Table 1). Consequently they cannot be resolved in two clearly separated frequency maxima. However, the high CV value (25%) of the leftmost Gaussian curve indicates the contribution of more than one fragment length.

For rapid DNA sizing, isolation and purification steps, e.g. by precipitation, should be avoided, therefore we investigated whether the presence of enzymes affects our sizing method. The restriction enzymes *PagI* and *EcoRI* digest m13mp18 RF1 DNA (7250 bp) in two fragments of 2318 and 4932 bp. To expedite the measurement, TOTO-1 was added before inactivating the enzymes. Figure 6 shows intensity histograms of m13mp18 RF1 before and after the addition of the enzymes. Before restriction only one maximum at 77.0 ± 1.1 PE appears, corresponding to a length of 7100 ± 260 bp. After restriction this peak is replaced by two maxima at 21.6 ± 0.6 and 56.3 ± 0.9 PE, corresponding to 2100 ± 100 and $5200 \pm$

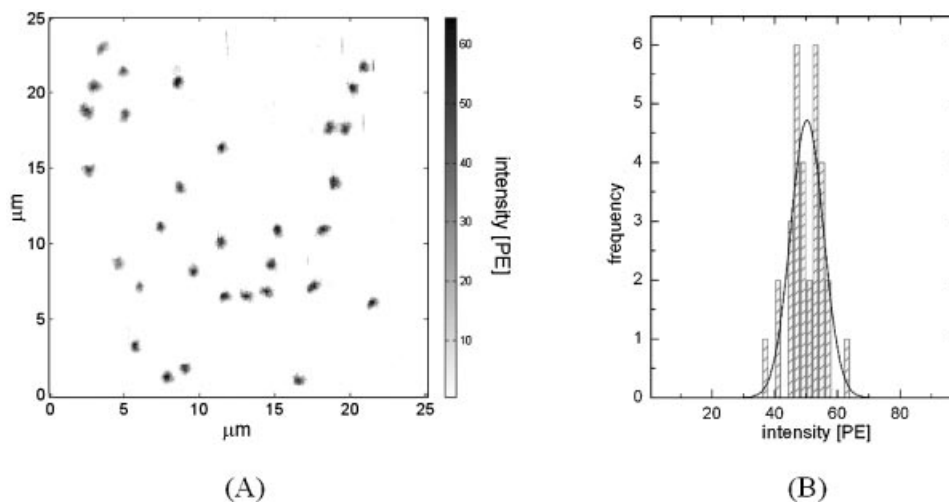


Figure 3. (A) Scanning image of intercalated pBR322 DNA fragments adsorbed on PLL-coated glass slides ($25 \times 25 \mu\text{m}^2$, pH 6, vertical scanning direction). Brightness of the molecules measured in PE. Adsorbed molecules cover a larger area than diffusing ones (small lines in the upper section of the image). (B) Histogram of the brightness of the adsorbed pBR322 DNA fragments, background of 12 PE subtracted. Bin width 2 PE. Histogram was fitted with Gaussian curve (black line). The reduced χ^2 of the fit is 0.75.

Table 1. Calibration data

Fragment size (bp)	Intensity centroid (PE)	σ (PE)	CV (%)
1985	18.8 ± 0.5	2.6	13.8
2686	26.7 ± 0.8	3.3	12.4
4120	45.3 ± 0.7	3.4	7.5
4361	49.9 ± 0.8	3.7	7.4
7250	80.5 ± 1.2	6.9	8.6
14 200	151.5 ± 1.7	10.3	6.8

200 bp as shown in Table 2(b). Hence it is not necessary to separate free dye or enzymes prior to the measurement. This experiment was verified by gel electrophoresis, where the fragment was determined to 2350 and 5200 bp as can be extracted from the photograph in Figure 1, (b). The fragment size analysis of the DNA restriction by single fragment analysis at the surface took <30 min, while a conventional gel electrophoresis required 2 h.

DISCUSSION

We have presented a new method to size DNA rapidly. A great benefit is the low amount of DNA needed, several orders of magnitude less than with gel electrophoresis. To ascertain the length of a particular fragment a quantity of $\sim 10^{-17}$ mol was sufficient. The required time for one experiment was ~ 30 min, 25% of the time needed to run a gel. Nevertheless, our method can be speeded up significantly. By conducting the focus with movable mirrors equipped with piezos instead of moving the sample, fluorescence images can be received within 1 min. Automated liquid handling will additionally save time and make it possible to size DNA within a few minutes.

Fragments larger than ~ 4 kb have been determined with errors below 10%, which is comparable with the performance of gel electrophoresis (30). The higher CV values of the 1.9 and 2.6 kb have prevented their clear identification in the mixture of five different fragments. It is comprehensible that

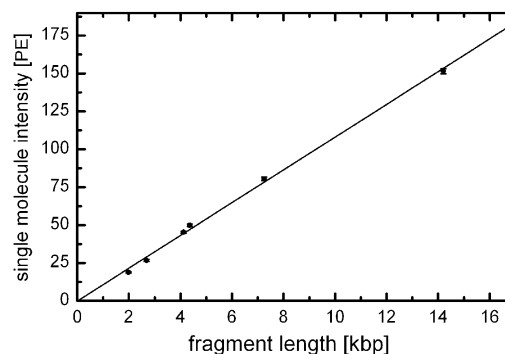


Figure 4. Plot of the single molecule brightness from the Gaussian fit versus fragment length in kilobases (cp. Table 1). Points were fitted by linear regression. Correlation coefficient of the fit is 0.998 and the slope is (10.81 ± 0.24) PE/kb. Background subtracted.

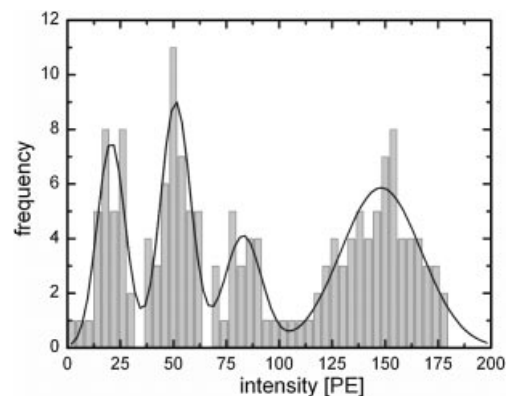


Figure 5. Histogram of the molecule brightness of ~ 160 DNA fragments from a mixture of different DNA molecules with fragment sizes of 1985, 2686 (pUC19 DNA), 4361 (pBR322 DNA), 7250 (m13mp18 RF1 DNA) and 14 200 bp. The fragments with 1985 and 2686 bp are superposed. Background of 12 PE is subtracted. The solid line is a fit with a sum of 4 Gaussians. The reduced χ^2 of the fit is 0.79; fit data are shown in Table 2.

Table 2. Data analysis

Fragment size (bp)	Intensity centroid (PE)	σ (PE)	Determined fragment size (bp)	CV (%)
(a) DNA mixture				
1985 + 2686	21.9 \pm 1.0	5.7	2000 \pm 140	25.9
4361	51.1 \pm 0.8	5.4	4700 \pm 180	10.5
7250	82.9 \pm 2.1	7.4	7600 \pm 360	9.4
14 200	149.1 \pm 2.1	17.2	13 800 \pm 500	11.5
(b) Restriction of m13mp18 RF1 DNA				
2318	21.6 \pm 0.6	4.5	2100 \pm 100	19.9
4932	56.3 \pm 0.9	10.6	5200 \pm 200	18.8
7250	77.0 \pm 1.1	11.9	7100 \pm 260	15.2

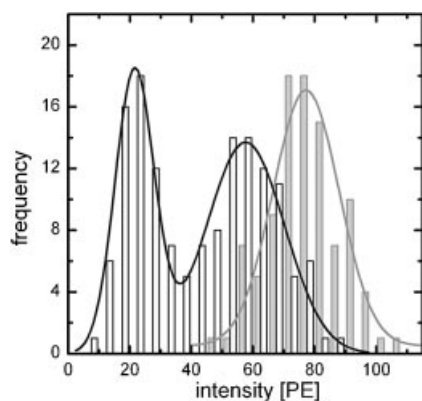


Figure 6. Histogram of the molecular brightness of m13mp18 RF1 DNA (grey) and m13mp18 RF1 DNA digested with EcoRI and PstI (black). The histograms are fitted with a Gaussian curve respectively with a sum of 2 Gaussians, respectively. Fit data are shown in Table 2.

the precision of our method increases with increasing fragment length, since the statistical deviation of the number of intercalated dyes decreases. However intensity fluctuations above 5% from one molecule to the next cannot be attributed to shot noise alone, but may be influenced by base specificity of the dye and by an inhomogeneous environment at the interface. Further optimization should be directed to the choice of the surface coating, especially in respect of homogeneity. Nevertheless, the achieved resolution is already sufficient for many applications, e.g. DNA fingerprinting for medical diagnostics and forensic studies. We believe that our sizing method has a high potential to reduce times and costs of many types of DNA analysis.

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