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AUTHOR(S) Mitchell E. Johnson, CLS-2
Peter M. Goodwin, CLS-2
W. Patrick Ambrose, CLS-2
John C. Martin, LS-1
Babetta L. Marrone, LS-1
James H. Jett, LS-1
Richard A. Keller, CLS-2

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Bellingham, WA 98227-0010

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Los Alamos Los Alamos National Laboratory
Los Alamos, New Mexico 87545

Sizing of DNA fragments by flow cytometry

Mitchell E. Johnson, Peter M. Goodwin, W. Patrick Ambrose, John C. Martin, Babetta L. Marrone, James H. Jett, and Richard A. Keller

Chemistry and Laser Sciences and Life Sciences Divisions, Los Alamos National Laboratory, Los Alamos, NM 87545

ABSTRACT

Individual, stained DNA fragments were sized using a modified flow cytometer with high sensitivity fluorescence detection. The fluorescent intercalating dye ethidium homodimer was used to stain stoichiometrically lambda phage DNA and a *Kpn* I digest of lambda DNA. Stained, individual fragments of DNA were passed through a low average power, focused, mode-locked laser beam, and the fluorescence from each fragment was collected and quantified. Time-gated detection was used to discriminate against Raman scattering from the water solvent. The fluorescence burst from each fragment was related directly to its length, thus providing a means to size small quantities of kilobase lengths of DNA quickly. Improvements of several orders of magnitude in analysis time and sample size over current gel electrophoresis techniques were realized. Fragments of 17.1, 29.9, and 48.5 thousand base pairs were well resolved, and were sized in 164 seconds. Less than one pg of DNA was required for analysis.

1. INTRODUCTION

The use of a modified flow cytometer to size individual fragments of DNA is described in this paper. This approach yields substantial savings in time and sample size over current gel electrophoresis technology, and has the potential for sizing very large fragments with much higher accuracy and precision than electrophoresis.¹

Mixtures of lambda phage DNA (λ DNA) and a *Kpn* I digest of λ DNA were stained stoichiometrically with the cyanine intercalating dye ethidium homodimer (EthD).^{2, 3} Individual stained fragments were passed through a focused laser beam, and the fluorescence of each fragment was measured. Hydrodynamically focused flow was used to confine the sample flow to a small, central region of the laser beam. The distribution of individual fluorescence intensities yielded information about the sizes of fragments present in the mixture and their relative abundance.

Figure 1. Schematic diagram of experimental apparatus. See experimental section 2.2 for identification of components. Components of the flow system, except the flowcell cross-section (FC) are omitted for clarity.

2. MATERIALS AND METHODS

2.1 DNA staining

Solutions of λ DNA and a *Kpn* I digest of λ DNA were mixed separately with EthD dye solution at a DNA concentration of 400 pg/ μ l. The dye concentration was approximately 10^{-7} M, and the average molar base pair to dye ratio (bp:dye) was 5. The analyzed mixture was prepared by adding equivalent aliquots of each staining mixture to buffer diluent for a final total fragment concentration of approximately 10^{-13} M. All solutions were prepared in pH 8 TE buffer (10 mM Tris-HCl, 1 mM EDTA). The *Kpn* I digest contains fragments of 1.503, 17.053, and 29.946 thousand base pairs (kbp) in length. λ DNA (48.052 kbp) was obtained from GIBCO BRL (Life Technologies, Inc., Gaithersburg, MD), the digest was obtained from New England Biolabs (Beverly, MA), and the EthD was obtained from Molecular Probes (Eugene, OR). All materials were used without further purification.

2.2. Experimental

The experimental apparatus used for detection of the stained fragments was a modification of the apparatus used for detection of single molecules and determination of their lifetimes^{4, 5} and is shown schematically in Figure 1. The fragments were excited at 514 nm with an Ar⁺ laser (LASER, Spectra Physics Model 171) at average powers between 10 and 150 mW. The laser was mode-locked at 82.5 MHz and had a 200 ps pulse width. Intensity was controlled with a half-wave plate ($\lambda/2$) and polarizer (P). The beam was horizontally polarized, spatially

filtered (SF), directed with mirrors (M), and focused into the flowcell (FC) with a 20 cm focal length lens (L) to a $1/e^2$ diameter of 40 μm . Fluorescence was collected at 90° to both optical and flow axes with a 40 $\times$, 0.85 NA microscope objective (MO, Nikon Fluor 40). The probe volume of ca. 10 pL was defined by the overlap of a spatial filter (vertical slit, S) at the image plane of the objective and the focused laser beam. The collected light was subsequently filtered with a Raman holographic edge filter (RHOE, Physical Optics Corp. RHE514.5) and a bandpass filter (DF) centered on 605 nm with a 30 nm bandpass. The transmitted fluorescence was focused on the photocathode of a cooled photomultiplier tube (PMT, Hamamatsu R636) operating in photon counting mode. The overall detection efficiency (number of photoelectrons counted relative to the number of photons emitted) was calculated to be about 2×10^{-3} .

The PMT anode pulses were amplified (A, Phillips Scientific 6954-S50), conditioned, and stretched with a constant fraction discriminator (CFD, Tennelec TC454). A portion of the laser beam was detected with a vacuum photodiode (VPD, Hamamatsu R1328U-02), the output of which was also amplified and conditioned with a CFD. The PMT and VPD CFD pulses, suitably delayed with respect to each other (Δt , Ortec 425A), were used as inputs to a very fast coincidence logic unit (FLU, Lecroy 365AL) for time gating. The output of the logic unit was then counted with a multichannel scaler (MCS, Lecroy 3521A), which was clocked at 10 kHz (CLK, Stanford Research Systems DG535). The MCS data was transferred to a memory unit (MEM, Lecroy MM8201 A) over a CAMAC bus, and was then transferred to a workstation (SUN, SUN Microsystems Sparcstation IPC) via a GPIB interface (National Instruments). All data processing was performed on the workstation.

The raw MCS data were processed by the following method. The average background was first determined from periods between fluorescent bursts. The background was subtracted, and MCS counts above a preselected threshold were accumulated to give the burst size. A typical background rate for the data discussed below was 20 photoelectrons per MCS bin (100 μs), and a typical threshold was 25 photoelectrons per bin.

Hydrodynamic focusing of the sample stream^{6, 7} was accomplished with a modified commercial square bore sheath flow cuvette (Ortho Diagnostics, Inc ; currently available from BDIS) with a 250 μm internal cross section. The collection side was cut down to a cover slip thickness to allow positioning of the microscope objective at its working distance from the sample stream. Ultrapure water (Burdick & Jackson) was used as the sheath fluid, and the flow was produced by gravity feed at approximately 30 $\mu\text{l}/\text{min}$. The sheath flowrate was adjusted for reasonable (1-5 ms) transit times (amount of time each fragment was in the probe volume). Samples containing 3 ng/ml of stained DNA were introduced by gravity flow, and the flowrate was adjusted for a sample detection rate of approximately 30 to 50 fragments per second. At this rate, the probability of two molecules being in the probe volume at the same time was between 5 and 10 %. Sample flow velocities ranged from 2-4 cm/s. One hundred and sixty-four seconds of fluorescence burst data was acquired, during which 4000 to 5,000 fragments, or less than 1 pg of DNA, were analyzed.

3. RESULTS AND DISCUSSION

3.1 Staining with EthD

In order to establish the best conditions for staining with EthD, fluorescence measurements were used to determine useful staining concentrations for both DNA and dye. The manufacturer provided guidelines for staining with the dye; however, these guidelines were developed under the assumption that the stained DNA would be separated by gel electrophoresis. It was expected that the staining requirements for our application might be somewhat different. For example, we found that either TE buffer or the recommended 0.1× TAE (4 mM Tris-acetate, 0.1 mM EDTA, pH 8.2) buffer were equally suitable.

Because of the flowrates used and the necessity for single fragment detection, the analysis concentration of stained DNA was a factor of 140 less than the optimum staining concentration; it was therefore necessary to determine whether the dye/DNA complex dissociated upon dilution. Fluorescence spectra obtained as a function of time after dilution (to 10^{-13} M DNA) for EthD stained λ DNA showed no appreciable decrease in intensity over a time periods of 4 hours, indicating no appreciable dilution.

Finally, we observed that, in order to obtain the best results, all solutions should be prepared fresh daily. Stained DNA was found to be usable even after several days, but performance, in terms of peak spread (coefficient of variation or CV) and background, was always worse than when freshly prepared solutions were used.

3.2. Rejection of prompt scattering with coincidence gating

A major source of background in fluorescence detection of EthD stained DNA was Raman scattering from the water solvent. At 514 nm excitation, the 3400 cm^{-1} Raman stretch for water overlapped the EthD/DNA fluorescence spectrum, which had an emission maximum of 617 nm^2 . Time-gated detection was used to discriminate the prompt Raman scattering from the longer-lived fluorescence. A coincidence logic unit, encompassing a very fast AND gate, allowed discrimination against PMT pulses occurring at the same time as the laser pulse, as determined by the VPD pulse, and thereby rejected most of the Raman scatter. The delay and width of the PMT CFD pulse were adjusted with respect to the VPD CFD pulses to maximize the signal to noise ratio (SNR). In doing so, a portion of the initial fluorescence was also rejected. Figure 2 shows raw (unprocessed) MCS data both with and without coincidence gating. The data were obtained separately, so the fluorescence bursts do not match. Bursts were discernable without gating, but the SNR was far better when solvent scattering was rejected. Furthermore, the background was over an order of magnitude lower, reducing the load on the PMT.

Figure 2. Unprocessed fluorescence burst data from EthD stained λ DNA (a) without and (b) with coincidence gating for rejection of solvent Raman scattering. Data from (a) and (b) were obtained separately; burst occurrences are not matched in the two traces.

3.3. DNA fragment sizing with ethidium homodimer

Figure 3 shows a histogram of frequency of occurrence versus detected fluorescence burst size obtained with a mixture of λ DNA and a *Kpn*I digest of λ DNA stained with EthD. These data were obtained with an average laser power of 100 mW and a transit time of 1.7 ms. Three peaks can be clearly discerned; the fluorescence from the 1.5 kbp fragment was masked by background fluorescence. The identity of the peaks was confirmed by analyzing a sample containing only λ DNA, and also by gel electrophoresis. Also shown in Figure 3 is a non-linear least squares fit to the data using the sum of three Gaussians and an exponential background; the background derived from the fit is also shown.

The fluorescence burst size of each fragment (peak centroids) derived from the fit and their CVs, along with the theoretical shot noise limited CV is summarized in Table I. The shot noise limit is determined by counting statistics of detected photoelectrons, n , and the CV at that limit is $\sqrt{n}/n$. Plots of burst size versus known fragment length showed excellent linearity (correlation coefficient was 1.000), and the deviation of the burst

Figure 3. Histogram of the fluorescent burst sizes in photoelectrons from a mixture of λ DNA and a *Kpn* I digest of λ DNA. The histogram bin width was 5 photoelectrons. The DNA fragments were stained with ethidium homodimer (EthD). Bursts were observed from fragments of 17053, 29946, and 48502 bp in length. Laser power was 100 mW and the transit time was 1.7 ms. The open circles are the histogram data, the solid line is a nonlinear least squares fit using the sum of three Gaussians and an exponential, and the dashed line is the exponential background calculated from the fit.

sizes from those obtained from the fits were less than 0.1%. The quality of the fit and the small deviations document the usefulness of this technique for DNA sizing. The data in Table I show that shot noise heavily influenced the CV, causing the CV to decrease with increasing burst size. However, CVs were not determined entirely by photon counting statistics.

The residual CV (i.e. in addition to shot noise) included contributions from non-uniform excitation intensity⁸, flow variations (sample stream wander and size changes), non-uniform collection efficiency, and, in digest mixture cases, from multiple fragment events. The flow variations were improved significantly by using gravity feed instead of stepper motor driven syringe pumps, but some flow instability still existed. Several other contributors to increased CV are possible, and are being investigated for relative importance. Nevertheless, the resolution approached that of gel electrophoresis. For example, analysis of published data show a CV of 4% for a 40 kbp band of T7 DNA.⁹

Table I. Summary of DNA sizing results shown in Figure 3.

fragment length, bp	burst size, photoelectrons (peak centroid)	CV, %	$\sqrt{n}/n \times 100\%$ ^a	δ , % ^b
48502	308.	9.8	5.7	0.021
29946	181.	15.	7.4	-0.084
17053	90.6	20.	10.	0.098

^a n is the burst size in photoelectrons (this column represents the shot noise limited CV)

^b deviation from linear fit of burst size to fragment length

While the method presented above worked very well for sizing DNA fragments, the method was not optimum. One disadvantage was the need for temporal discrimination against prompt scattering. Spectral discrimination is generally superior, from the standpoint of instrumental complexity. Furthermore, the absorbance maximum (528 nm) did not correspond to the laser wavelength used (514 nm). With longer wavelength excitation, for example 532 nm (Nd:YAG, 2 $\times$), the Raman band would be shifted out of the emission band, excitation would be more nearly optimum, time gating might be eliminated, and a continuous wave laser might be employed.

A flow cytometry method with sensitive detection offers several major advantages over gel electrophoresis. One distinct disadvantage to electrophoresis is the time required. Reasonable resolution of smaller DNA fragments (< 40 kbp) in conventional electrophoresis requires several hours, while larger fragments require pulsed field techniques with separation times ranging from tens of hours¹⁰ to days^{11, 12}. The histogram shown in Figure 3 was derived from burst data acquired over a 164 second interval. This translates to a 70 fold time savings over gel analysis of T7 DNA data at similar resolution.⁹ Another limitation of slab type gel electrophoresis measurements is the amount of sample required. Sizing DNA using conventional ethidium bromide staining and photographic detection typically requires tens of ng to μ g, though sophisticated detection techniques have reduced that requirement to tens of pg¹³ and capillary systems with fluorescence detection have reached zeptomole detection limits¹⁴. The histogram shown in Figure 3 was comprised of approximately 8800 bursts, 4500 of which were from DNA fragments. The latter corresponded to a total of 0.2 pg, or 7 zeptomoles (7×10^{-21} mol), of DNA.

The size limit for gel electrophoresis is on the order of 40 kbp without resorting to time-consuming pulsed field techniques.^{15, 16} Our technique has the potential to size large DNA fragments in a much shorter time and with better accuracy and resolution than gel electrophoresis. In contrast to gel based techniques, the precision of the burst size measurement increases (i.e. the CV decreases) with increasing fragment length, as long as staining continues to be stoichiometric and shot noise dominates the CV. The size limit for free DNA is expected to

depend on how large the DNA fragment can be before shearing from hydrodynamic forces during handling and analysis occurs.⁹

4. CONCLUSIONS

We have demonstrated sizing of individual, stained DNA fragments with resolution approaching that of gel electrophoresis for moderately large fragments, but with significant reductions in the analysis time and the amount of sample required. Furthermore, system response is linear with DNA fragment length, in contrast to the logarithmic response in gel electrophoresis. There exists the potential to perform this sizing using relatively simple instrumentation, i.e. a continuous wave laser of low power and current mode detection.

Future work will concentrate on improving CVs by improving collection efficiency, improving the flow system, and by shaping the laser beam profile to provide more uniform excitation. We will also attempt the use of different dyes to ameliorate the difficulties associated with EthD. We would ultimately like to be able to use the sorting capabilities of flow cytometry to sort individual DNA fragments according to size, in order to facilitate further analysis on isolated and purified DNA fragments.

Potential applications for flow cytometric sizing of DNA include DNA fingerprinting for forensic purposes¹⁷, DNA fragment sizing for basic biomedical applications such as genome mapping¹⁸, clinical DNA diagnostics, and sizing of intermediate size DNA samples as an alternative to current gel electrophoresis techniques. This work is an outgrowth of our work in single molecule detection.^{4,5}

5. ACKNOWLEDGMENTS

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