An integrated system for enzymatic cleavage and electrostretching of freely-suspended single DNA molecules†‡

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A novel polyacrylamide gel-based femtolitre microchamber system for performing single-molecule restriction enzyme assay on freely-suspended DNA molecules and subsequent DNA electrostretching by applying an alternating electric field has been developed. We attempted the integration by firstly initiating restriction enzyme reaction on a fluorescent-stained λDNA molecule, encapsulated in a microchamber, using magnesium as an external trigger. Upon complete digestion, the cleaved DNA fragments were electrostretched to analyze the DNA lengths optically. The critical parameters for electrostretching of encapsulated DNA were investigated and optimum stretching was achieved by using 1.5 kHz pulses with electric field strength in the order of 10^3 V cm^{-1} in 7% linear polyacrylamide (LPA) solution. LPA was adopted to minimize the adverse effects of ionic thermal agitation on molecular dielectrophoretic elongation in the microchamber. In our experiments, as the fragments were not immobilized throughout the entire protocol, it was found from repeated tests that digestion always occurred, producing the expected number of cleaved fragments. This versatile microchamber approach realized direct observation of these biological reactions on real-time basis at a single-molecule level. Furthermore, with the employment of porous polyacrylamide gel, the effective manipulation of DNA assays and the ability to combine conventionally independent bioanalytical processes have been demonstrated.

1. Introduction

Restriction enzymes, which serve as powerful tools for gene manipulation, bind to a specific recognition sequence in a DNA molecule and subsequently cleave the DNA molecule along the backbone of its double helix structure. Typically, a restriction enzyme reaction is carried out at a bulk level, where digestions are assayed by electrophoresis in agarose or polyacrylamide (PAA) gel.[1] Several problems of low throughput, as well as considerable time and sample consumptions associated with these methods, prompted the development of low cost miniaturized devices for biological assays, capable of allowing automated manipulation of samples as demonstrated by a pioneer integrated microdevice for DNA restriction fragment analysis, based on electrophoretic sizing.[2]

In a recent review,[3] the demand for further downscaling of these lab-on-chip devices has led to the exploration at a single molecule level. Single molecule detection and manipulation give fundamental insights of biological reactions, such as catalytic kinetics, conformational dynamics and intrinsic heterogeneity of biological molecules.[4] Therefore, single molecule analysis of a restriction enzyme reaction[5] has been attempted with the aim to achieve quick and highly sensitive analysis of a small amount of DNA, such as genomic DNA molecules derived from single cells.

Our previous investigations[6,7] have revealed that by using poly(dimethylsiloxane) (PDMS) femtolitre microchambers, it was possible to measure biomolecular reactions at a single molecule level without the need for immobilization. The fabrication technique was then adopted to produce polyacrylamide (PAA) gel-based microchambers.[8] This original approach of employing PAA gel offers several advantages over the PDMS microchambers. Due to its porosity, it has been demonstrated that this structure allows the migration of ions and enzymes, as well as the penetration of electric field through its gel matrix. In addition, typical use of PAA shows no unfavourable interactions with biomolecules. By exploiting these aspects, PAA gel-based microchambers were employed to encapsulate DNA molecules suspended in a solution containing restriction enzymes. Magnesium chloride was then applied to the exterior of the gel and with the subsequent diffusion of magnesium ions through the gel matrix, restriction enzyme reaction was initiated, and eventually complete digestion was observed in the microchamber. From the results, we learnt that by modifying parameters, such as the dimensions of the gel and introducing external dc current across the gel structure, the biological assays were performed with the capability of controlling the reaction.

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† The HTML version of this article has been enhanced with colour images.

‡ Electronic supplementary information (ESI) available: Supplementary data and three movie clips showing restriction enzyme reaction on single DNA molecules using *Nhe*I, *Pme*I and *Bam*HI, respectively, and subsequent electrostretching of cleaved fragments within the same reaction microchamber. See DOI: 10.1039/b711826k

Here in this study, we further extend our investigation by combining the restriction enzyme assay with subsequent measurements of DNA fragment length, which is generally indispensable in DNA analysis, within the PAA gel-based microchambers. Previous literatures have revealed that a number of analytical techniques involving the stretching of DNA have been developed and incorporated in single molecule restriction enzyme assays.[9] These stretching techniques, such as using optical tweezers[10] or dynamic molecular combing methods,[11] often required complete or partial[12] DNA immobilization on a solid surface or in a gel to retain stretched configuration of DNA molecules. When a DNA molecule is immobilized, only part of its surface is exposed to interact with reactants. This could sometimes cause severe streric hindrance for restriction enzyme molecules to access the recognition site and affect the reaction efficiency of restriction enzyme digestion of immobilized DNA.[13,14] On the other hand, several advantages of elongating completely mobile DNA molecules have also been reported.[15] Hence, for our system, we apply an alternating electric field across the PAA gel-based microchamber to observe the electrostretching[16] of freely-suspended DNA molecules under an optical microscope. Ultimately, with appropriate adaptation of reaction conditions, the integration of restriction enzyme assay and the direct measurement of the cleaved DNA length by means of electrostretching was attempted within the same single reaction microchamber to realize a simple, and therefore versatile, microdevice.

2. Materials and methods

2.1 Microchamber fabrication

The microchambers were circular, 17.5 μm in diameter, and approximately 1.5 μm in height. The molds were fabricated by photolithography using a chromium mask to pattern on a glass or silicon substrate.[6] A mixture of 30% w/v acrylamide (AA) solution containing *N*,*N*′-methylenebis(acrylamide) (Wako, Japan), ammonium persulfate (APS; Wako, Japan), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED; Wako, Japan), was poured onto the mold and cured at room temperature for 40 min. The PAA cured gel containing the microchambers was then cut into 3 mm circles for practical handling purposes. As relatively large DNA fragments (more than 5 kbp) were involved in the experiments, 14% PAA gel was found to be appropriate for the PAA gel-based microchamber.

2.2 Chemicals

λDNA molecules were purchased from Invitrogen (USA), SYBR Gold dye (10 000× concentration in anhydrous dimethyl sulfoxide) for staining DNA from Invitrogen (USA), *Nhe*I, *Pme*I and *Bam*HI from Takara (Japan).

Linear polyacrylamide (LPA). LPA (molecular weight of 0.7 ~ 1 MDa) was prepared with 5% AA solution, which contained acrylamide powder (Wako, Japan), 40 mM Tris-HCl (pH 8.0), 20 mM sodium acetate, and 1 mM EDTA. APS was then added to attain a final concentration of 0.1% (w/v). Then, 1/1000 volume of TEMED was added to the solution, and it was allowed to polymerize for 30 min at room temperature. When the solution increased in viscosity, the polymer was precipitated with 2.5 volumes of ethanol and subsequently recovered by centrifugation at 1000 rpm for 3 min. The recovered pellet was finally dissolved in 10 mM Tris-HCl (pH 8.0) to attain a PAA concentration of 10 wt%.

2.3 Operating procedures for the optimization of electrostretching

λDNA was digested with *Nhe*I and the resultant DNA fragments were separated with agarose electrophoresis, forming two distinct bands. The DNA fragments were then recovered from agarose and purified using Centri-Sep columns (Princeton Separation, Princeton, NJ, USA). Each of the DNA solutions was mixed with SYBR gold dye and LPA to adjust the DNA concentration to 3 pM.

DNA solution was pipetted onto a glass slide on a microscopic stage. A sheet of PAA gel with micron-sized holes on its surface was pressed against the glass slide by using a glass needle. This way, DNA molecules were entrapped in the microchambers. Individual DNA molecules were observed under a fluorescence microscope (IX71, Olympus, Japan) equipped with a mercury-arc lamp, an image intensifier unit (C8600-05, Hamamatsu Photonics, Japan), a 100× magnification objective lens, and a CCD camera (RC300, MTI, Michigan City, Indiana, USA) with a pixel size resolution of 8.4 μm (horizontal) by 9.8 μm (vertical).

To generate the alternating field for electrostretching, 300 nm thick titanium microelectrodes were patterned onto the glass slide and connected to an HP8114 pulse generator (Agilent, USA), as depicted in Fig. 1a.

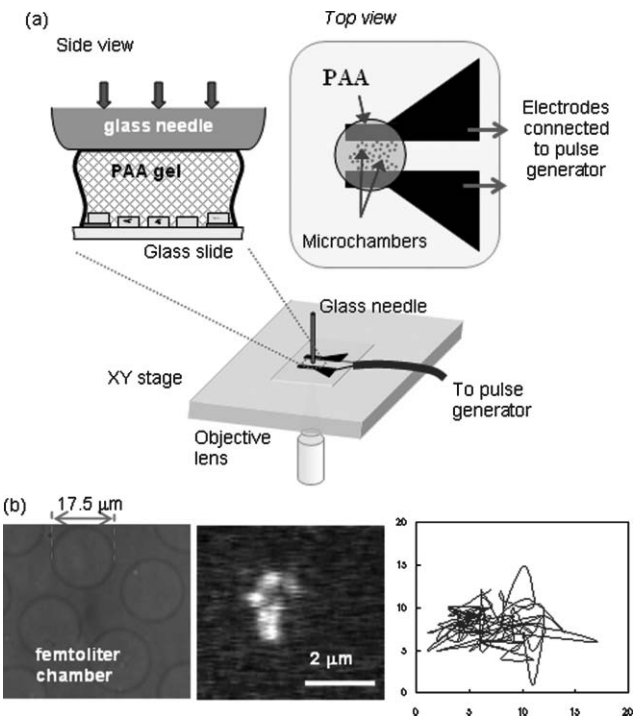

Fig. 1 (a) Schematic drawings of experimental setup. (b) Microscopic images of the microchamber and a single encapsulated λDNA molecule, and the corresponding restricted Brownian motion trace of center of mass.

Fig. 1b shows that with the captured fluorescent images of an encapsulated λDNA molecule, its motion in the enclosed microchamber environment could be traced, hence revealing the potential of this technique for further explorations in studying DNA kinetics.

2.4 Operating procedures for the integrated assay and analysis

A 50 μl solution containing 3 pM λDNA, 0.5 × concentration of SYBR gold dye, 5 mM Tris-HCl (pH8.0), 30 U of restriction enzyme and LPA was prepared. The initial solution was free of magnesium ions. DNA molecules suspended in the solution were encapsulated in the microchambers using the same procedures, and 7 mM magnesium chloride ($MgCl_2$) solution was then externally added to initiate the restriction enzyme reaction.

3. Results and discussion

In our previous study, we primarily performed restriction enzyme reactions in a PAA gel-based microchamber;[8] however, electrostretching was not examined because the cleavage activity of restriction enzymes requires relatively high ionic strength and a divalent ion, Mg^{2+}, which prevented conventional electrostretching due to ionic thermal agitation under an alternating electric field. To accommodate restriction enzyme reaction and electrostretching under the same condition, linear polyacrylamide (LPA) was added to the reaction mixture. LPA was reported to support electrostretching of DNA molecules in a buffer with a high ionic concentration.[17–19] Although the actual mechanism of how linear polymer solution supports electrostretching was not clear, the feasible model was presented; condensed linear polymer solution with the average mesh size of approximately 20 Å, similar to the diameter of double-stranded DNA fragment, would entangle with DNA fragments to assist efficient stretching under the influence of an alternating field.[19,20] In this investigation, we first explored the optimum conditions for the DNA stretching in a PAA gel-based microchamber using our experimental setup (Fig. 1). A series of experiments was carried out to establish the relationships between the lengths of the stretched DNA molecules and each varying parameter, namely, the field strength and signal frequency of the electric field, and the concentration of LPA.

3.1 Optimization of experimental parameters for electrostretching

Three kinds of DNA fragments, namely λDNA (48.5 kbp) and the two *Nhe*I-cleaved fragments of 34.7 and 13.8 kbp, were separately stretched in the PAA gel-based microchambers. Fig. 2a–2c shows the relationships between the stretched lengths, as determined by direct measurement of the captured images, and various stretching conditions. In Fig. 2a and 2b, the influence of varying field strength at 1.5 kHz using 7% LPA, and signal frequency at 1200 V cm^{-1} using 7% LPA, were studied, respectively. The effect of LPA concentration on the stretched DNA length when subjected to an alternating signal of 1200 V cm^{-1} and 1.5 kHz was determined, as shown in Fig. 2c. To enable better comparison of the influence of stretching on the individual DNA fragment types, the data in Fig. 2a–2c was normalized as relative length against the theoretical lengths of fully stretched DNA (16.5 μm, 11.8 μm and 4.7 μm for λDNA, the 34.7 kbp and 13.8 kbp fragments, respectively) as presented in Fig. 2d–2f.

As shown in Fig. 2d–2f, the maximum stretched lengths were obtained within a field strength range of 1150 to 1300 V cm^{-1}, a frequency range of 1 to 2 kHz, and an LPA concentration range of 6% to 7%, for all three kinds of DNA fragments under test. Under the optimal conditions, the stretching efficiency raised over 80%. The imperfections of stretching were due to partial folding into a hairpin structure and kinking,[19,21] as seen in the heterogeneous fluorescent intensity along a DNA fragment (Fig. 3b). While the LPA concentration of 7% for the maximum stretching was the same as previously reported,[17] the strength and the frequency were much higher than the previously recorded values of 200 V cm^{-1} for the field strength, and 10 Hz for the frequency.[22] It could be assumed that the variations would be a direct result of the attenuation of the alternating electric field in the PAA gel. However, an exact mathematical representation of the electric field generated within each microchamber could not be established. This was due to some unknown factors, such as the parameters governing the dielectric properties,[23] the inconsistent number of induced DNA from neighbouring microchambers, as well as the deformation of the 3-dimensional PAA gel and chamber structures caused by the pressure exerted from the pushing glass needle.

Several observations made were to be noted. In our experiment, the position and contour length of DNA often fluctuated within the enclosed microchamber over the field strength of 1300 V cm^{-1}, where the physical collision of DNA against the chamber wall and the resultant bending of the DNA ends may attribute towards the suppression of effective stretching. Further, we noticed random segment breakage or tearing of the DNA fragments under the continuous application of high alternating field strength above 1700 V cm^{-1}, even if it was applied for a short duration of less than 2 min.

Although all three kinds of sample DNA experienced similar elongation, as seen in the graphical trends presented in Fig. 2d–2e, a distinct difference was observed in Fig. 2f. In the presence of 5% LPA, the 13.8 kbp fragment appeared to stretch to an averaged 45% of its theoretical length of 4.7 μm, while the λDNA managed to stretch to approximately 82% of its corresponding theoretical length. Our results were consistent with previous findings where DNA fragments with contour lengths of less than 5 μm exhibited "U-shaped" motion under "I-shaped" stretching conditions.[18,24] Due to the bending "U-shaped" effect, a shorter length would be measured from these short DNA fragments.

3.2 Standard line for the length of stretched DNA molecules

From Fig. 2d–2f, the most suitable alternating field setting can be estimated as a field strength of 1230 V cm^{-1}, a signal frequency of 1.5 kHz, and LPA concentration of 7%.

Here, in order to obtain the standard line of the stretched DNA molecules, several kinds of DNA fragments were stretched and measured for their apparent lengths. In addition to the three types of DNA used in the optimization of

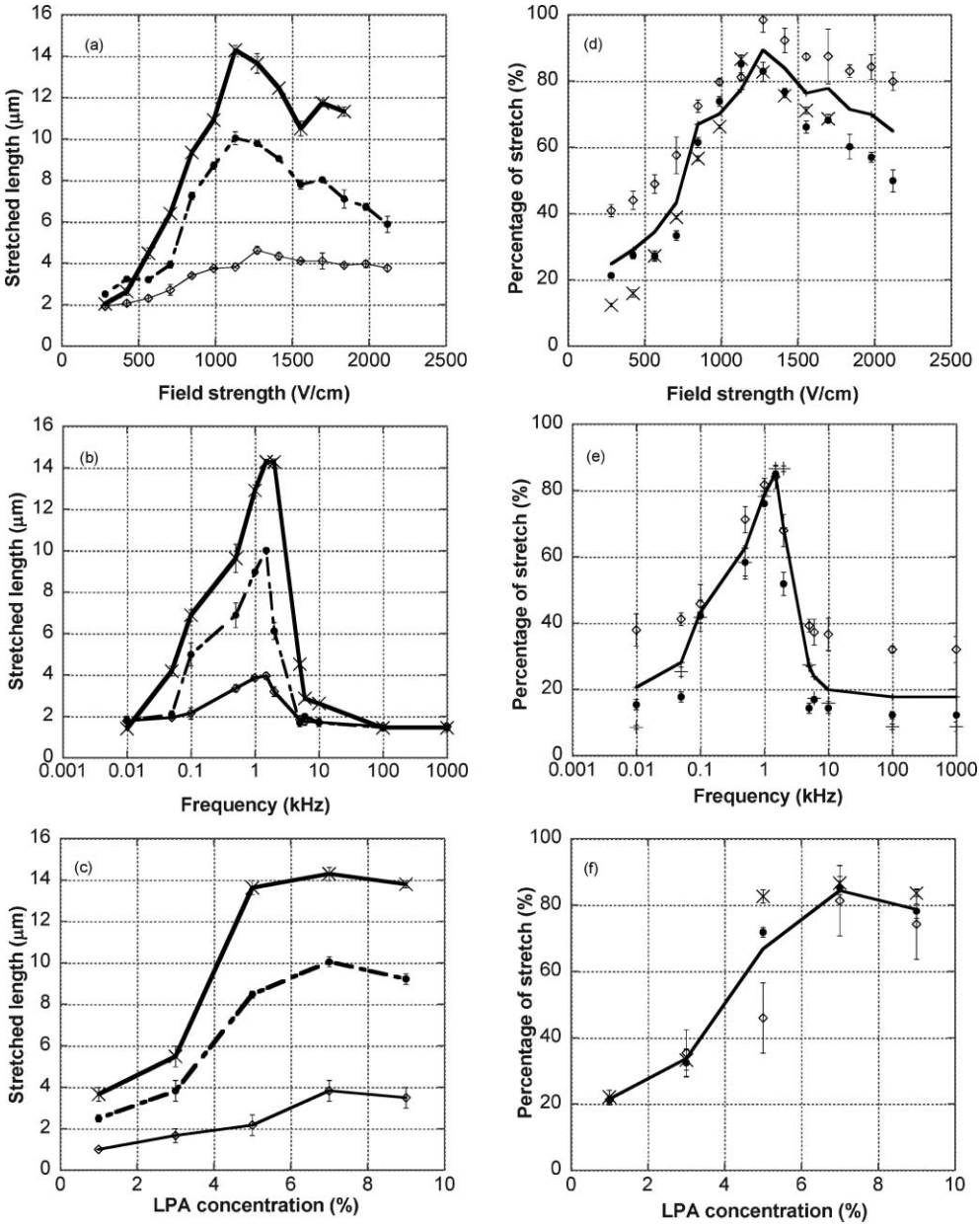

Fig. 2 Relationships between the stretched lengths and the different varying conditions. (a) & (d) Varying signal strength at fixed frequency of 1.5 kHz and 7% LPA; (b) & (e) Varying frequency at fixed signal strength of 1200 V cm^{-1} and 7% LPA; (c) & (f) Varying LPA concentration at fixed signal of 1200 V cm^{-1}, 1.5 kHz. Total number of molecules analyzed for each measurement is 5. (For the 'stretched length' plots: –×– λDNA (48.5 kbp), –●– 34.7 kbp fragment, –◇– 13.8 kbp fragment; for the 'percentage of stretch' plots: × λDNA (48.5 kbp), ● 34.7 kbp fragment, ◇ 13.8 kbp fragment, — average.)

electrostretching, three more fragments (7.8 kbp, 8.5 kbp and 32.2 kbp) were prepared by *Pme*I digestion of the λDNA and stretched. The lengths of the encapsulated DNA fragments were plotted against the DNA sizes (kbp), as shown in Fig. 3a. The length of the stretched DNA molecules displayed a good linearity with respect to the DNA size with a coefficient factor of 0.285 μm kbp^{-1}. In other words, conducting electrostretching in the PAA gel-based microchamber, the measured DNA carried 3.5 kbp per 1 μm. This factor was relatively consistent while the measured lengths were found to be repeatable, as indicated by the relatively small error bars observed in Fig. 2a–2c.

3.3 Integration of the restriction enzyme reaction and subsequent electrostretching analysis

Here, we attempted the integration of restriction enzyme reaction and the subsequent analysis of the digested DNA fragment length by electrostretching in a single microchamber. A λDNA molecule suspended in 7% LPA was encapsulated in a microchamber together with a restriction enzyme, *Nhe*I or *Pme*I. It was important to note that the addition of high SYBR gold dye concentration in the sample solution will inhibit the DNA-enzyme reaction, thus dilution to 0.5× concentration was found to be most appropriate and effective for visualising

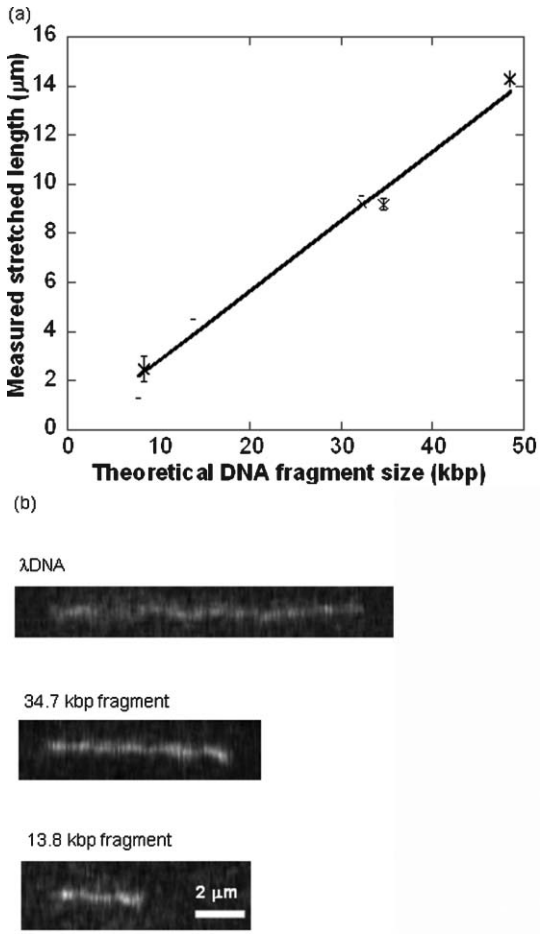

Fig. 3 Standard line of the stretched DNA. (a) The relationship between the measured stretched length and the DNA fragment size. Regressed linear equation of $y = 0.285x$ and regression coefficient of 0.993. (b) The fluorescent images of the λDNA (48.5 kbp), the 34.7 kbp and 13.8 kbp fragments, each corresponding to 16.5, 11.8, and 4.7 μm, respectively, stretched with an alternating field of 1230 V cm^{-1}, 1.5 KHz at 7% LPA.

the desired reaction. A 70 μl solution of 7 mM $MgCl_2$, an essential cofactor for the cleavage reaction, was then pipetted on the outer surface of the PAA gel to allow Mg^{2+} to diffuse into the chamber and consequently initiate the reaction.

Fig. 4a shows the setup used for initiating the restriction enzyme reaction and the resultant images of the cleaved stretched fragments obtained in two separate experiments, one using *Nhe*I (Fig. 4b) and the other using *Pme*I (Fig. 4c). The cleavage reaction was completed in several minutes. Repeated experiments showed that the observed DNA molecules were always cleaved into the expected number of fragments, two for *Nhe*I and three for *Pme*I digestion. Given the fact that DNA molecules were not immobilized on a surface or embedded in a gel matrix, but were suspended in a free solution, our experimental configuration allowed reactants to access the entire surface of the DNA structure. This method appeared to possess the potential of a possible alternative to address the previously reported problems on digestion efficiency.[14]

After the digestion, an alternating field with the optimum strength of 1230 V cm^{-1} at 1.5 kHz was applied to the titanium microelectrodes, and the electrostretching of the encapsulated cleaved DNA fragments was observed. As seen from the images in Fig. 4b–4c, the fragments were able to stretch effectively, independent of each other, within a microchamber (see supplementary movies in the ESI‡). This occurrence would probably be due to the entanglement network formed by LPA and the intrinsic negative charge of the DNA molecule. By using the optimum correction factor of 0.285 μm kbp^{-1}, we could now easily determine the lengths of the fragments. The determined lengths were compared to the expected values, as shown in Table 1. The averaged difference between the theoretical and experimental DNA sizes was 2.3 kbp or equivalent, to an accuracy of 77%, based on the calculations using theoretical lengths (as presented in Section 3.1). This variation would probably be the result of elevated thermal ionic agitation on the fragments due to the presence of increased Mg^{2+} ions, required by the restriction enzyme assay prior to DNA electrostretching. Nonetheless, the

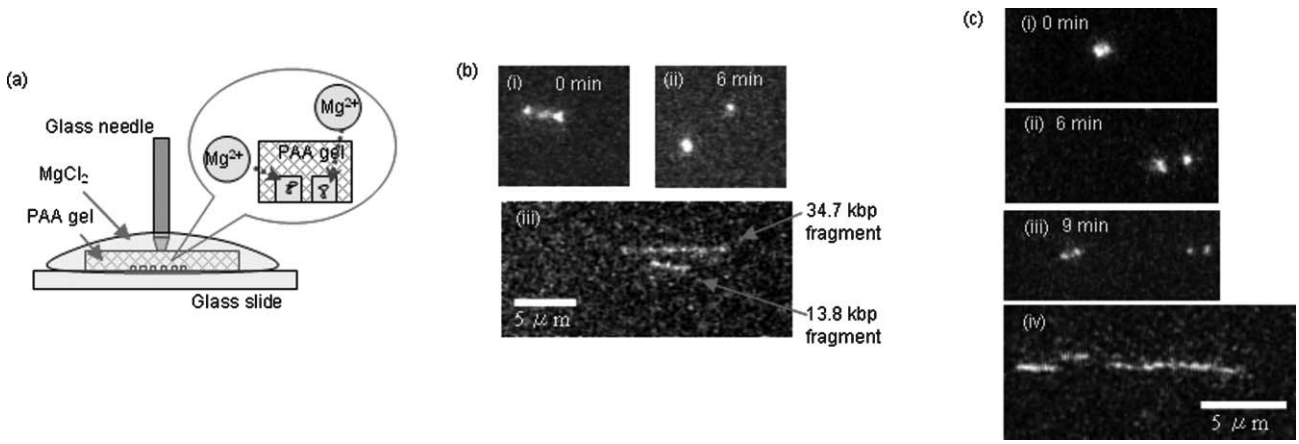

Fig. 4 (a) Experimental setup for introducing Mg^{2+} to initiate the restriction enzyme reaction in the PAA gel-based microchamber; (b(i)) Single encapsulated λDNA molecule, (b(ii)) Cleaving reaction using *Nhe*I—cut after 6 min, (b(iii)) Subsequent electrostretching of the 2 fragments within the same microchamber; (c(i)) Single encapsulated λDNA molecule, (c(ii)) Cleaving reaction using *Pme*I—1st cut after 6 min, (c(iii)) 2nd cut after 9 min, (c(iv)) Subsequent electrostretching of the 3 fragments within the same microchamber.

Table 1 Comparison between experimentally measured DNA sizes (using the conversion coefficient of 0.285 μm kbp^{-1}) and their corresponding theoretical DNA sizes[a]

Theoretical DNA size/kbp	7.8	8.5	13.8	32.2	34.7
Experimentally measured DNA size ± standard deviation/kbp	5.8 ±1.6	9.4 ±2.0	11.1 ±1.3	29.3 ±0.8	31.5 ±0.9

[a] Total number of molecules analyzed for each measurement is 5.

measurements were highly repeatable with relatively small deviations, hence confirming the feasibility and effectiveness of this method.

It was important to note that with appropriate reaction conditions, the initiation of restriction enzyme digestion was found by previous bulk experiments to occur as quickly as 30 s or less. Thus, the cleavage times recorded, as shown in Fig. 4b(ii)–4c(ii), were mainly the times required for Mg^{2+} ion diffusion through the gel matrix and into the microchamber, although there was also the possibility of Mg^{2+} ion leakage through the interface between the underlying surface of the PAA gel and the glass slide. These values agreed well with a series of feasibility checks conducted with Mg indicator (Mag-fluo4; Molecular Probes, USA), evaluating the time needed for Mg^{2+} to diffuse through the gel matrix and detecting the first traces of Mg^{2+} ions in the microchamber was approximately 5.4 min. The dimensions of PAA gel and temperature condition for the feasbility checks were kept similar to that of the actual experiment for restriction enzyme assays. The detailed results of these feasibility checks are not presented in this paper.

This lag time could be shortened by reducing the dimensions of the PAA gel, or increasing the Mg^{2+} concentration. However, there existed physical limitations; the thickness of the PAA chamber sheet was required to be at least 1 mm to retain the stability for handling and encapsulation processes. Mg^{2+} concentrations could not be indefinitely increased because a high level of divalent ions would still lead to severe ionic thermal agitation, even in the presence of LPA solution, and exponentially decrease the fluorescent intensity of SYBR gold dye. An alternative way for reducing the reaction time was to apply an external DC current across the PAA gel, as performed in our previous study.[8] However, this technique was proven to be unsuitable at its current stage for integrated reaction-analysis protocol as it resulted in DNA "sticking" onto the chamber wall and glass surface. Hence, for now in this case, we compromised the rate of initiation of the restriction enzyme reaction in order to carry out consecutive processes within the same microchamber.

By employing the same experimental procedure and sample conditions as in the case for λDNA (a linear DNA), encapulated plasmids (circular DNAs) were also found to be equally responsive to the reactions, therefore reaffirming the capabilities of this device (see supplementary data in the ESI‡).

3.4 Analyzing large number of encapsulated fragments

Here, we explored the maximum number of fragments that could be analyzed in a single 17.5 μm reaction chamber by performing the digestion of λDNA with *Bam*HI, which would yield six fragments with theoretical sizes ranging from 5.5 to 16.8 kbp. First, we trapped a single λDNA molecule in a microchamber as shown in Fig. 5a. Using our encapsulation technique, the distribution of DNA molecules trapped within the microchambers was found to be relatively homogeneous. By examining neighbouring chambers, the percentage of having a ratio of 1 : 1 fragment per chamber was estimated at 72%.

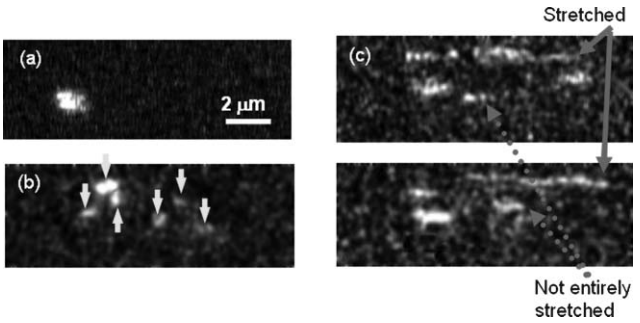

Fig. 5 (a) Mg^{2+} applied externally to trigger the cleaving reaction on the encapsulated single λDNA molecule. (b) Six fragments yielded by the cleaving reaction using *Bam*HI. (c) Electrostretching of the resultant cleaved fragments (2 separate frames of the captured image).

Approximately 25 min after the application of the Mg^{2+} trigger, the λDNA was completely cleaved into six fragments, as expected (Fig. 5b). On applying alternating signals of 1230 V cm^{-1} and 1.5 kHz to the microelectrodes, all the six fragments attempted to stretch within the microchamber, as shown in Fig. 5c. The increased number of fragments moved at a relatively fast pace and appeared to collide with one another very frequently within the microchamber. Since some of the fragments were similar in size, it was difficult to track the motion and location of individual fragments in each captured image. In addition to this setback, similar-sized fragments tended to overlap in the two-dimensional view for many cases (see supplementary movie in the ESI‡). As a result, after numerous trials, it was difficult to obtain clear images for the direct measurement of the fragment sizes. Thus, for simple analysis, the maximum number of DNA fragments for electrostretching analysis using the present microchamber design was limited to 3 or 4. Although the problem may be apparently solved by using larger chambers, the possibility of each chamber entrapping two or more DNA molecules will increase. If more molecules were initially encapsulated, the result would be more cleaved fragments after the restriction enzyme digestion. Hence, the original problem of "congestion" would once again be encountered.

4. Conclusions

In this investigation, the novel approach of a PAA gel-based microchamber used for restriction enzyme reactions and subsequent analysis by electrostretching DNA fragments at a single molecule level was developed, hence offering advantages in terms of reaction speed and efficiency.

Various types of restriction enzymes were employed to obtain cleaved fragments of different sizes. Furthermore, extensive experiments were carried out to determine the optimum conditions for combining the two processes—restriction enzyme reaction and electrostretching analysis—effectively within a single microchamber. Due to the porosity of the PAA gel, it was possible to externally introduce Mg^{2+} ions which diffused through the gel matrix into the microchamber, to initiate the restriction enzyme reaction. This feature facilitated the encapsulation of a single complete λDNA molecule prior to the assays. As a result, the entire reaction could be visualized in detail. We then analyzed the cleaved fragments by electrostretching them under the influence of alternating signals of 1.5 kHz and 1230 V cm^{-1}, and 7% LPA. The lengths of the freely-suspended stretched fragments were determined from the captured images; the results were plotted against the theoretical lengths, depicting a linear relationship with high regression coefficient.

For the current design, DNA fragments ranging from 3 to 60 kbp would be effectively accommodated. In the event of manipulating fragments out of this range, objectives lens with appropriate magnification should be employed for clear visualization. For fragments larger than 60 kbp, bigger microchambers should be used so that the full stretching of these fragments can be performed without spatial restriction between opposing chamber walls. As for fragments smaller than 3 kbp, down sizing the microchamber would allow the encapsulation ratio of 1 : 1 fragment per chamber to be maintained. However, it would be expected that there would still be some limitations in terms of fabricating extremely small chambers or maintaining the aspect ratio of the enlarged chambers to retain its structure during encapsulation. In the case of fragments, which are exceedingly different from the current operational range of 3 to 60 kbp, the concentration of PAA gel used for fabricating the microchambers should be revised to enable effective encapsulation. In addition, to achieve optimum stretching, the field strength and frequency of the alternating field should be re-evaluated accordingly.

This study demonstrated the PAA gel-based femtolitre microchamber system to be a precise and effective detection technique at a single molecule level. Although the experimental setup may appear to be slightly complex due to the increased number of instrumentation involved, the actual procedures proved to be simple and easily adopted. This system allowed the identification of fragments without immobilization and displayed potential to serve as an attractive and useful tool for other biological assays and further genomic explorations, such as DNA mapping.[25]

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References

1 F. Bastié-Sigeac and G. Lucotte, Optimal use of restriction enzymes in the analysis of human DNA polymorphism, *Hum. Genet.*, 1983, **63**, 162–165; B. Garcia-Barreno, T. Delgado and J. A. Melero, Identification of protein regions involved in the interaction of human respiratory syncytial virus phosphoprotein and nucleoprotein: significance for nucleocapsid assembly and formation of cytoplasmic inclusions, *J. Virol.*, 1996, **70**, 801–808; G. Leitz, C. Lundberg, E. Fällman, O. Axner and A. Sellstedt, Laser-based micromanipulation for separation and identification of individual Frankia vesicles, *FEMS Microbiol. Lett.*, 2003, **224**, 97–100.

2 S. C. Jacobson and J. M. Ramsey, Integrated microdevice for DNA restriction fragment analysis, *Anal. Chem.*, 1996, **68**, 720–723.

3 H. Craighead, Future lab-on-a-chip technologies for interrogating individual molecules, *Nature*, 2006, **442**(27), 387–393.

4 W. Min, B. P. English, G. Luo, B. J. Cherayil, S. C. Kou and X. S. Xie, Fluctuating enzymes: lessons from single-molecule studies, *Acc. Chem. Res.*, 2005, **38**, 923–931; S. Weiss, Fluorescence spectroscopy of single biomolecules, *Science*, 1999, **283**(5408), 1676–1683; L. C. Campbell, M. J. Wilkinson, A. Manz, P. Camilleri and C. J. Humphreys, Electrophoretic manipulation of single DNA molecules in nanofabricated capillaries, *Lab Chip*, 2004, **4**, 225–229.

5 R. Riehn, M. Lu, Y. M. Wang, S. F. Lim, E. C. Cox and R. H. Austin, Restriction mapping in nanofluidic devices, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**(29), 10012–10016.

6 Y. Rondelez, G. Tresset, K. V. Tabata, H. Arata, H. Fujita, S. Takeuchi and H. Noji, Microfabricated arrays of femtoliter chambers allow single molecule enzymology, *Nat. Biotechnol.*, 2005, **23**, 361–365.

7 Y. Rondelez, G. Tresset, T. Nakashima, Y. Kato-Yamada, H. Fujita, S. Takeuchi and H. Noji, Highly coupled ATP synthesis by F_1-ATPase single molecules, *Nature*, 2005, **433**, 773–777.

8 L. Lam, K. Ishizuka, S. Sakakihara and H. Noji, Restriction enzyme assay in femtoliter microchamber, *Proc. μTAS*, 2006, **2**, 1429–1431.

9 S. Katsura, N. Harada, Y. Maeda, J. Komatsu, S. Matsuura, K. Takashima and A. Mizuno, Activation of restriction enzyme by electrochemically released magnesium ion, *J. Biosci. Bioeng.*, 2004, **98**, 293–297.

10 C. Hoyer, S. Monajembashi and K. O. Greulich, Laser manipulation and UV induced single molecule reactions of individual DNA molecules, *J. Biotechnol.*, 1996, **52**, 65–73.

11 W. Cai, H. Aburatani, V. P. Stanton, Jr., D. E. Housman, Y. Wang and D. C. Schwartz, Ordered restriction endonuclease maps of yeast artificial chromosomes created by optical mapping on surfaces, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 5164–5168; X. Michalet, R. Ekong, F. Fougerousse, S. Rousseaux, C. Schurra, N. Hornigold, M. Van Slegtenhorst, J. Wolfe, S. Povey, J. S. Beckmann and A. Bensimon, Dynamic molecular combing: stretching the whole human genome for high-resolution studies, *Science*, 1997, **277**, 1518–1523.

12 H. W. Li and E. S. Yeung, Direct observation of anomalous single-molecule enzyme kinetics, *Anal. Chem.*, 2005, **77**, 4374–4377.

13 M. Linial and J. Shlomai, Sequence-directed bent DNA helix is the specific binding site for crithidia fasciculata nicking enzyme, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 8205–8209.

14 E. Eriksson, J. Enger, B. Nordlander, N. Erjavec, K. Ramser, M. Goksor, S. Hohmann, T. Nystromb and D. Hanstorp, A microfluidic system in combination with optical tweezers for analyzing rapid and reversible cytological alterations in single cells upon environmental changes, *Lab Chip*, 2007, **7**, 71–76.

15 J. O. Tegenfeldt, C. Prinz, H. Cao, S. Chou, W. W. Reisner, R. Riehn, Y. M. Wang, E. C. Cox, J. C. Sturm, P. Silberzan and R. H. Austin, The dynamics of genomic-length DNA molecules in 100-nm channels, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**(30), 10979–10983.

16 M. Washizu, O. Kurosawa, I. Arai, S. Suzuki and N. Shimamoto, Applications of electrostatic stretch-and-positioning of DNA, *IEEE Trans. Ind. Appl.*, 1995, **31**, 447–456; R. Holzel, N. Gajovic-Eichelmann and F. F. Bier, Oriented and vectorial immobilization of linear M13 dsDNA between interdigitated electrodes–towards single molecule DNA nanostructures, *Biosens. Bioelectron.*, 2003,

18, 555–564; H. Oana, M. Ueda and M. Washizu, Visualization of a specific sequence on a single large DNA molecule using fluorescence microscopy based on a new DNA-stretching method, *Biochem. Biophys. Res. Commun.*, 1999, **265**, 140–143.
17 M. Ueda, Dynamics of long DNA confined by linear polymers, *J. Biochem. Biophys. Methods*, 1999, **41**, 153–165.
18 M. Ueda, H. Oana, Y. Baba, M. Doi and K. Yoshikawa, Electrophoresis of long DNA molecules in linear polyacrylamide solutions, *Biophys. Chem.*, 1998, **71**, 111–122.
19 N. Kaji, M. Ueda and Y. Baba, Molecular stretching of long DNA in agarose gel using alternating current electric fields, *Biophys. J.*, 2002, **82**, 335–344.
20 M. Doi and S. F. Edwards, *The Theory of Polymer Dynamics*, Oxford University Press, New York, 1986, ch. 5, pp. 156–160 and ch. 6, pp. 188–191.
21 T. T. Perkins, D. E. Smith and S. Chu, Single polymer dynamics in an elongational flow, *Science*, 1997, **276**, 2016–2021.
22 M. Ueda, K. Yoshikawa and M. Doi, Molecular motion of long deoxyribonucleic acid chains in a concentrated polymer solution depending on the frequency of alternating electric field, *Polym. J.*, 1999, **31**, 637–644.
23 D. N. Gavrilov, O. Kosobokova, V. Khozikov, A. Stepukhovitch and V. Gorfinkel, Electrophoresis in capillary cells with detection gap, *Electrophoresis*, 2005, **26**, 3430–3437; I. M. El-Anwar, O. M. El-Nabawy, S. A. El-Hennwii and A. H. Salama, Dielectric properties of polyacrylamide and its utilization as a hydrogel, *Chaos, Solitons Fractals*, 2000, **11**(8), 1303–1311.
24 S. J. Hubert and G. W. Slater, Theory of capillary electrophoretic separation of DNA using ultradilute polymer solutions, *Macromolecules*, 1996, **29**, 1006–1009; C. Wu, M. A. Quesada, D. K. Schneider, R. Farinato, F. W. Studier and B. Chu, Polyacrylamide solutions for DNA sequencing by capillary electrophoresis: Mesh sizes, separation and dispersion, *Electrophoresis*, 1996, **17**(6), 1103–1109.
25 D. Botstein, R. L. White, M. Skolnick and R. W. Davis, Construction of a genetic linkage map in man using restriction fragment length polymorphisms, *Am. J. Hum. Genet.*, 1980, **32**, 314–31.