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Electrophoresis using ultra-high voltages

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Abstract

Optimization of electrophoretic techniques is becoming an increasingly important area of research as microdevices are now routinely adapted for numerous biology and engineering applications. The present work seeks to optimize electrophoresis within microdevices by utilizing ultra-high voltages to increase sample concentration prior to separation. By imaging fluorescently-tagged DNA samples, the effects of both conventional and atypical voltage protocols on DNA migration and separation are readily observed. Experiments illustrate that short periods of high voltage during electrophoretic injection do not destroy the quality of DNA separations, and in fact can enhance sample concentration five-fold. This study presents data that illustrate increases in average resolution, and resolution of longer fragments, obtained from electrophoretic injections utilizing voltages between 85 and 850 V/cm.

Keywords: Optimization; Ultra-high voltages electrophoresis; DNA

1. Introduction

The many adaptations of microdevices for numerous biology and engineering applications have made optimization of electrophoretic techniques a high priority. Electrophoretic separations of biomolecules are now routinely performed within microdevices due largely to the parallel analysis [1] and process integration [2] facilitated by microfabrication [1–3]. Microfabricated channels are particularly successful in electrophoretic applications because they dissipate heat effectively, enabling separation voltages of up to 30 kV to produce rapid, efficient separations. In addition, microdevices provide a unique opportunity for direct visualization, which facilitates our understanding of the dynamics underlying the electrophoretic process [4,5]. The present work seeks to optimize electrophoresis within microdevices by re-examining the protocols used during conventional separations. Specifically, this work focuses on sample stacking (or sample concentration effects) present during electrophoretic injection. Here, we utilize ultra-high voltages to increase sample stacking (sample concentration) prior to separation. The physical

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effects of conventional and atypical voltage protocols during electrophoretic injection are readily observed using fluorescently-tagged molecules. These results are then correlated with electropherograms to produce practical, experimental data that illustrate the benefits of increased stacking to DNA separations.

1.1. Microdevice operation

Microdevices used for DNA separations are comprised of four different reservoirs (cathode, anode, sample and waste), and three distinct channel sections (separation channel, cross-injector and channel tail) [1] as seen in Fig. 1. Conventional separations are performed using four consecutive steps: pre-electrophoresis, sample loading, electrophoretic injection and separation. During pre-electrophoresis, a large potential gradient of several hundred volts per centimeter, V_P, is first imposed between the cathode and anode ports of the device, and later the sample and waste reservoirs. The protocol is used to evenly distribute the ions of the buffer solution within all of the channels of the microdevice in order to facilitate subsequent loading of DNA [6]. Afterwards, a different loading potential gradient, $V_{L,}$ is applied along the sample and waste reservoirs of the cross-injector in order to draw DNA molecules into the microdevice. This process is called sample loading and is used to create a uniformly distributed DNA sample within the cross-injector offset. Lastly, a separate potential gradient, typically called the run voltage, $V_{R,}$ is imposed between the cathode and anode ports of the device in order to attract DNA towards the positively charged cathode. As a result, the run voltage initiates electrophoretic injection of DNA molecules into the channel, as well as their subsequent separation.

During the early stages of injection, DNA molecules migrate rapidly within the sample of the cross-injector, but experience an abrupt drop in velocity upon reaching the lower field within the high-conductivity electrolyte buffer [7,8]. The subsequent decrease in velocity creates a thin and concentrated zone of DNA molecules at the interface between the sample and separation buffer, called the stacked plug [9] or stacked sample [10]. This ‘‘stacking’’ mechanism [8,11,12] is a unique, physical process caused by a difference in potential gradient between the sample and the buffer solution. Sample stacking increases the sample concentration throughout electrophoretic injection and has generated high-resolution data in numerous subsequent electropherograms [5,13].

A higher sample concentration is desirable because of the initial condition it creates. In a theoret-

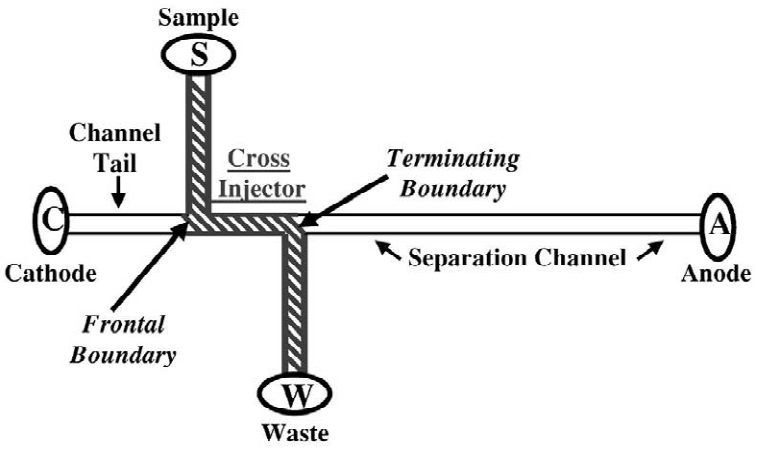

Fig. 1. A schematic of a conventional microdevice illustrates an 11-cm-long separation channel, a ‘‘double-T’’ cross-injector configuration, and channel tail section. The sample channel is depicted on the upper left-hand side of the image while the waste channel is shown on the lower right-hand side. Cathode and anode reservoirs are denoted by ‘‘C’’ and ‘‘A’’, respectively, as are the sample, ‘‘S’’, and waste, ‘‘W’’, reservoirs. The frontal and terminating boundaries of the DNA sample are also identified.

ical case of maximum stacking, the DNA sample could be one molecule wide due to extreme sample concentration. In this case, molecules of all sizes enter the separation channel with the same initial position. As a result, the number of pores initially available for migration is identical for each molecule, regardless of its size. This is an important point as electrophoretic velocity varies with molecular mass [14,15] and, hence, spans three orders of magnitude within a given sequencing reaction. In the opposite case of zero stacking, DNA molecules begin separation behind thousands of other molecules evenly distributed within the 250–500-μm dimension of the cross-injector. This creates a condition where the migration of smaller, fast-moving molecules is likely impeded by the slower-moving, larger molecules which occupy a large number of pores ahead of their paths. Optimal stacking enables faster molecules to migrate ahead of the sample, thereby reducing barriers to migration. This would likely increase the signal (and number) of DNA molecules detected, thereby increasing resolution.

In the present work, a new high voltage injection protocol is developed in order to optimize stacking within the channel prior to separation. Separations are still performed using four consecutive steps, but now four distinct voltages are used in lieu of three. The advantage of this protocol is that it enables a distinct injection voltage, V_I, to be used, specifically, to introduce a highly concentrated DNA sample into the separation channel. Here, pre-electrophoresis is performed using the voltage V_p followed by sample loading at the applied load voltage, V_L. Afterwards, a distinct injection voltage, V_I, is applied during electrophoretic injection, followed by a separate run voltage, V_R, imposed during separation. Values of V_I can be significantly greater than V_R, but are applied for a maximum of 5 s to avoid sample degradation. After this time, an electronic switch instantaneously reduces the voltage to a more conventional value, V_R, to be applied during separation.

In order to visualize the motion of DNA samples during electrophoretic injection, DNA molecules were fluorescently tagged with propidium iodide and observed using an epi-fluorescence microscope. Once the sample was introduced into the microdevice, detailed images of its migration within the cross-injector were captured using a CCD camera. We have quantitatively measured the width of the stacked sample using the values of intensity recorded for each pixel within the digital images. The results from video microscopy are then correlated with electropherograms to quantify the benefits of increased stacking to DNA read-lengths and electrophoretic resolution. This study performed DNA separations within microdevices imposing injection voltages, V_I, between 85 and 850 V/cm.

1.2. The mechanism of stacking

The phenomenon of stacking within a cross-injector operates on the same principle as stacking in a capillary [5,7,10,16]. When an electric field is applied along a channel, the flux of ions within the channel generates a current that is described by its current density, I [26]. This vector points in the direction of current flow and is strongly influenced by the ionic conductivity of the medium. Due to the chemical composition required for separations, the ionic conductivity of the buffer electrolyte solution, $\kappa_{B,}$ is typically much higher than that of the DNA solution, $\kappa_{D.}$ Hence, when the run voltage is applied along the channel, a disproportionate amount of the potential drop is found along the low-conductivity sample. However, as the DNA molecules migrate towards the buffer solution, they exhibit an abrupt drop in velocity upon experiencing the lower potential gradients present within the electrolyte. The sudden decrease in velocity creates a very thin and concentrated zone of DNA molecules at the injector exit via the mechanism called ''stacking'' [9,10,16,18].

Stacking can be described analytically using the one-dimensional moving boundary equation first described by Longsworth [24] in the late 1950s. Since DNA molecules are constrained within the cross-injector prior to separation, the sample has two DNA boundaries, the frontal and terminating boundaries. The frontal boundary is formed between the sample and buffer region closest to the anode, while the terminating boundary is formed between the sample and buffer closest to the cathode. Molecules on the frontal boundary immediately migrate out of the sample and into the buffer electrolyte during injection because of their proximity to the anode [9,20]. In contrast, molecules on the termi-

nating boundary migrate towards the anode within the sample at all times during injection as a consequence of their position within the cross-injector. Using the moving boundary equation, stacking is described utilizing a Lagrangian reference frame that migrates concurrently with the sample's frontal interface. The stacking velocity can be determined from the expression [9,19]:

$$\frac{\mu C_{D,S}}{\kappa_S} - \frac{\mu C_{D,B}}{\kappa_B} = \frac{V_{ST}(C_{D,S} - C_{D,B})}{I} \quad (1)$$

where μ represents the electrophoretic mobility of DNA, $C_{D,S}$ is the concentration of DNA, D, present in the sample, S, and $C_{D,B}$ is the concentration of DNA in the electrolyte buffer, B. The ionic conductivity of the sample, S, is denoted by $\kappa_{S,}$ while κ_B represents the ionic conductivity of the buffer, B. The parameter V_{ST} represents the stacking velocity, i.e. the velocity of the terminating boundary with respect to the frontal boundary, and I is the total current density within the channel. The current density is defined by the total flux of ions within the channel, which in the general case [17] can be attributed to electromigration, diffusion, and convection as expressed in Eq. (2).

$$I = -\kappa\nabla\Phi - F\Sigma z_i D_i \nabla C_i + Fu\Sigma z_i C_i \quad (2)$$

where Φ is the electric potential in volts, F is Faraday's constant of value 9.65×10^4 C/mol, κ is ionic conductivity in S/cm, D is diffusivity measured in cm^2/s, z is the dimensionless valence number of the i-th ion, u is the bulk velocity (which is identically zero for a fixed sieving matrix), and C is concentration in $1/m^3$. For the general problem, the conductivity can change in time due to redistributions of ions within the channel. As shown previously [21,22], an expression for the stacking velocity, V_{ST}, can be obtained utilizing Eqs. 1 and 2 simultaneously.

One of the more complete stacking models proposed by Gebauer et al. [9] defined two types of stacking termed frontal- or terminator-type stacking. DNA exhibit frontal-type stacking when molecules accumulate near the frontal boundary of the sample, nearest the anode. Conversely, DNA exhibit terminator-style stacking when molecules accumulate near the terminating boundary of the sample, nearest the cathode. In the first type of stacking, molecules are concentrated on the frontal boundary. To first order, with constant potential gradients within the sample, DNA molecules experience an abrupt decrease in velocity only when they reach the frontal boundary. Here, molecules are influenced by the lower potential gradients of the electrolyte buffer almost immediately, and concentrate on the frontal boundary of the sample. In terminator-type stacking, molecules are concentrated on the terminating boundary of the sample. Here, the potential within the sample is continuously modified as the sample width diminishes, imposing the largest gradients near the terminating boundary [9] and the smallest potential gradients towards the frontal boundary. Accordingly, molecules accelerate during electrophoretic injection when the stacked sample reaches their position. A concentrated, stacked sample of DNA molecules is developed on the terminating boundary as a larger number of molecules are accelerated by the high potential gradient associated with this interface. Our previous work [4] has experimentally identified stacking of DNA molecules within microdevices as terminator-type stacking. The different potential gradients within the sample in the cases of frontal and terminator stacking are illustrated in Fig. 2.

2. Experimental section

Electrophoretic injections of DNA solutions were observed within the cross-injector portion of microdevices using the 10× objective of an inverted, epi-fluorescence microscope (Nikkon TE-3000). A CCD camera collected the intensity of the fluorescent signals emitted by the labeled molecules at a rate of eight frames per second using a time-lapse protocol (Openlab software). Intensity measurements obtained from pixels located in the channel centerline were then converted into 8-bit digital images using a 256 gray-level scale.

Microfabricated devices used in this study were manufactured from 150-mm-diameter glass wafers (Corning, NY) using techniques described in the literature [1]. The channels are hemispherical in cross-section, approximately 40 μm in depth and 90 μm in width, and have an effective separation length

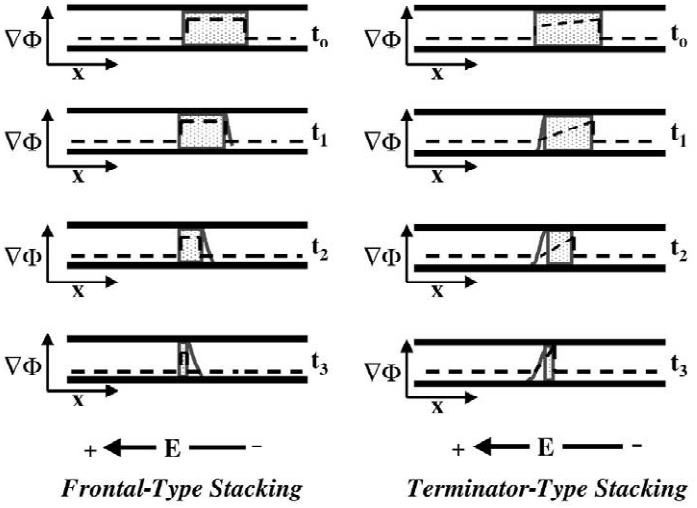

Fig. 2. The set of four images on the left-hand side illustrates a representative case of frontal-type stacking while images on the right-hand side illustrate terminator-type stacking. Potential gradients, $\nabla\Phi$, within the channel at different times, *t*, are represented by dashed lines. As seen, frontal-type stacking predicts constant potential gradients within the sample during stacking, while the model of terminator-type stacking predicts potential gradients that change with time and position of stacking.

of 11.5 cm. The sample and waste channels of each cross-injector are approximately 5.0 mm in length, and horizontally offset by a distance of 250 μm. Glass reservoirs (Ace Glass, Vineland, NJ) of 50-μl volume are affixed around the laser-drilled holes that access the electrophoretic channel in order to contain the appropriate volumes of sample and buffer solutions. The inner walls of the microfabricated channels were coated using a Hjerten procedure [23] while a polymeric sieving solution of 2% linear polyacrylamide (LPA, 9 MDa) was loaded into the channel center at rates that ensured minimum degradation [24]. Channels were re-loaded with a new volume of sieving solution prior to, and in between, successive sample loadings and injections.

Polydisperse reactions were prepared using 10^{-10} *M* concentrations of DNA sequencing reactions obtained from the M13mp18 vector [6]. Sequencing reactions were comprised of single-stranded DNA molecules ranging from 1 to 7300 bases in length, including the template molecule. Polydisperse samples were synthesized via standard cycle sequencing chemistry with AmpliTaq-FS, Big-Dye-Terminator labeling (Applied Bio-Systems/Perkin-Elmer, Foster City, CA) and desalted using Centri-Sep spin columns (Princeton Separations, Adelphia, NJ). Samples were also fluorescently labeled with 10^{-10} *M* propidium iodide to facilitate detection during experiments.

DNA sequencing reactions were loaded into the cross-injector by applying a negative potential of 2300 V (corresponding to 150 V/cm) to the sample reservoir and keeping the waste reservoir at ground. During sample loading, the buffers in both the anode and cathode reservoirs were left floating. Leakage of excess sample from the cross-injector into the separation channel was prevented with a small electric pull back voltage (~40 V/cm) applied to both halves of the loading channel 10 s after injection, as described previously [4]. In all experiments, a run voltage between 85 and 850 V/cm was applied for 5 s during electrophoretic injection and then reduced to the standard 150 V/cm for full separation using a voltage relay switch. Additionally, pre-electrophoresis was performed at 300 V/cm for 3 min, before each sample was loaded into the channels. In order to obtain resolution data from electropherogram analysis, the G-traces of Big-Dye-Terminator labeled DNA sequencing reactions were used [1,4]. The G-traces were selected due to minimal cross-talk and ease of tracking isolated peaks over the entire range of fragment sizes. From the resulting electrophero-

grams, the migration time of the sequencing fragments were plotted against their base number and fitted with a Gaussian distribution using Microcal Origin 6.0 software (Microcal Software, Northampton, MA, USA).

3. Results and discussion

3.1. Digital images

Using the new high voltage injection protocol, the shape of the stacked sample at the exit of a 250-μm-long cross-injector offset is observed quantitatively. The effectiveness of this protocol to increase the sample concentration is evident in Fig. 3. The set of six digital images on the left-hand side of the figure displays the shape of different stacked samples obtained after electrophoretic injection. Images are arranged in order of increasing injection voltage, V_I, as indicated by the values shown near the top of each image. The set of plots on the right-hand side of the figure displays the corresponding fluorescence intensity within the stacked sample as measured along the channel centerline. These plots represent the molecular distribution of DNA molecules within the stacked sample, quantitatively, on a 256 gray scale.

As seen from Fig. 3, the distribution and overall width of the sample decrease quickly with increasing voltage during injection. Note, the sample width is defined by the distance between its frontal and terminating boundaries. In addition, molecules of the stacked samples in Fig. 3 do not appear to migrate in a Gaussian profile until very high injection voltages are applied. This is somewhat surprising, as the distribution of distinct DNA populations has been traditionally modeled using Gaussian profiles in electrophoretic analysis [13]. However, the distribution of molecules within the stacked samples is never Gaussian in Fig. 3 unless ultra-high voltages are used.

The first digital image in Fig. 3 illustrates the stacked sample generated by applying an injection voltage that is only slightly higher than the conventional run voltage, $V_I = 236$ V/cm. As seen, the width of the sample is fairly large and displays a sharply concentrated region of molecules in its center. Using an injection voltage of $V_I = 394$ V/cm, the width of the sample is slightly decreased and its overall DNA distribution is more compact. The electrophoretic injection performed using $V_I = 427$ V/cm is truly the first to illustrate the improved stacking dynamics desired. Here, ~92% of DNA molecules are concentrated into one profile, while only traces of DNA molecules are ''unstacked''. Further, the distribution of DNA molecules is near perfectly Gaussian when electrophoretic injection is performed using the ultra-high voltage of $V_I = 708$ V/cm.

The quantitative plots seen to the right of each digital image depict the width of the stacked samples proceeding injection. These plots display fluorescent intensity measurements from the corresponding digital images using a 256 gray scale. As seen in Table 1, the width of the stacked sample produced from an injection voltage of 236 V/cm was 73 μm, while the stacked sample developed via an injection voltage of 708 V/cm was only 27 μm wide. In addition, the digital images gathered during high voltage injection illustrate the inaccuracy of using the conventional full width at half maximum analysis, W_{FWHM}, to model distributions of DNA as Gaussian. As seen from Fig. 3, the parameter W_{FWHM} is not accurate unless ultra-high voltages are used.

Table 1 demonstrates that ultra-high voltages not only produce Gaussian distributions of DNA, but also decrease the full width at half maximum, W_{FWHM}, of the stacked sample. As seen, an injection voltage of 426 V/cm produces a stacked sample with $W_{FWHM} = 21$ μm, while a voltage of 708 V/cm

Fig. 3. The set of six digital images on the left-hand side represent the shape of a 10^{-10} *M* polydisperse sample following electrophoretic injection, as captured in real-time via video microscopy. In each image, the sample arm of the cross-injector is seen on the upper left-hand side (denoted by the letter ''S''), while the waste arm is shown on the lower right (denoted by the letter ''W''). The cathode and anode are located at the far left and right, respectively, of the main separation channel oriented horizontally in each image. Each image illustrates the stacked sample generated via high-voltage injection protocols. All experiments utilized a load voltage, V_L, of 300 V/cm and run voltage, V_R, equal to 150 V/cm. The values of injection voltage, V_I, used for each experiment are shown in the upper right-hand corner of the images. The set of six plots on the right-hand side of the figure illustrates the corresponding intensity profile of each stacked sample.

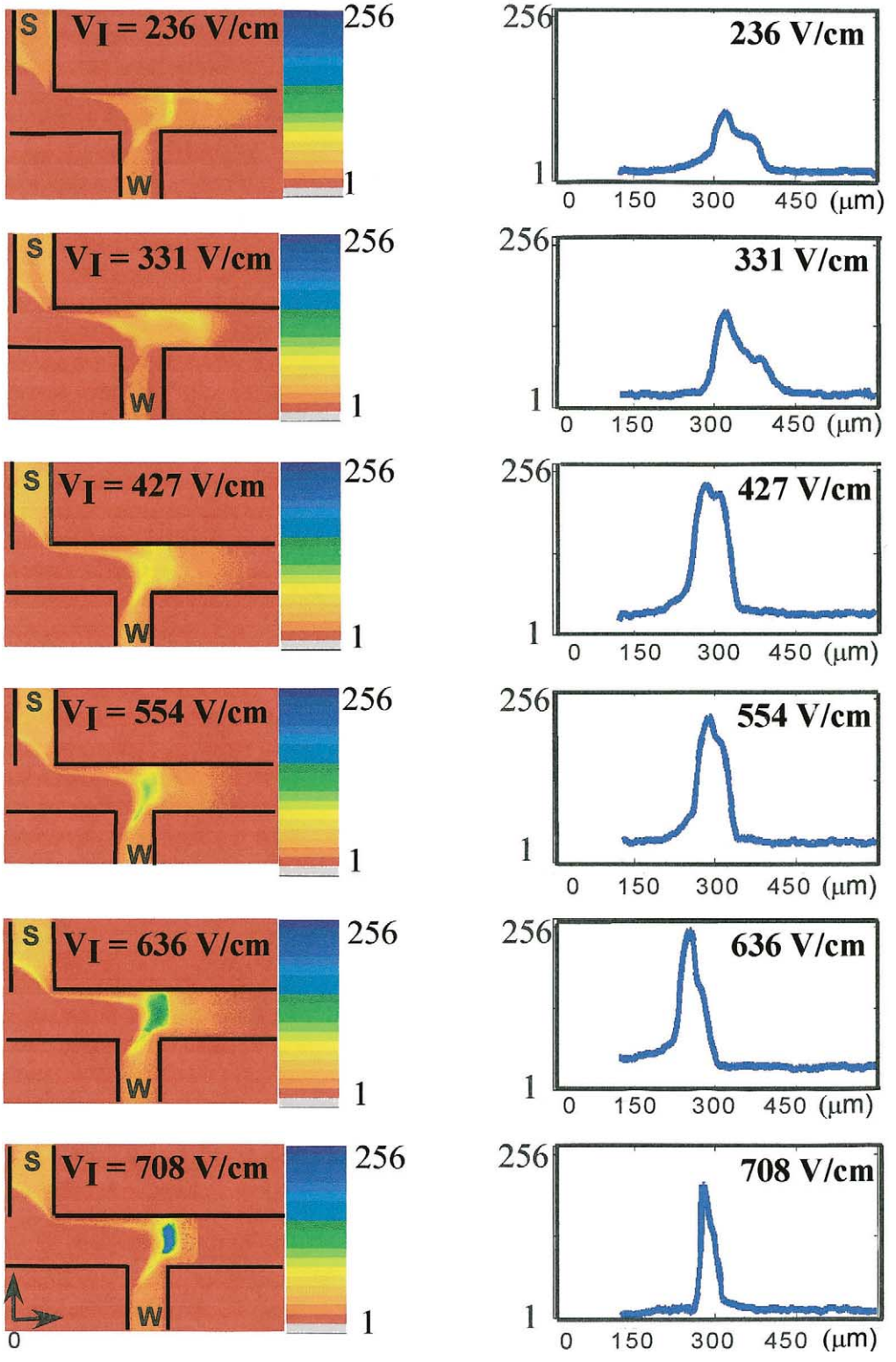
S
V_I = 236 V/cm
W
256
1
256
236 V/cm
1
0 150 300 450 (μm)
S
V_I = 331 V/cm
W
256
1
256
331 V/cm
1
0 150 300 450 (μm)
S
V_I = 427 V/cm
W
256
1
256
427 V/cm
1
0 150 300 450 (μm)
S
V_I = 554 V/cm
W
256
1
256
554 V/cm
1
0 150 300 450 (μm)
S
V_I = 636 V/cm
W
256
1
256
636 V/cm
1
0 150 300 450 (μm)
S
V_I = 708 V/cm
W
0
256
1
256
708 V/cm
1
0 150 300 450 (μm)

Table 1
Width of the stacked DNA sample during electrophoretic injection using high voltage protocols

V_I (V/cm)	W_F (μm)	W_{FWHM} (μm)
236	73	–
394	62	–
426	55	21
554	51	19
630	40	15
708	27	9

Measured width, W_F, is defined as the distance between the frontal and terminating boundaries of the sample. These values were experimentally determined using intensity values from the channel centerline. Further analysis of intensity measurements provided a full-width at half-maximum representation, W_{FWHM}, where applicable.

develops a sample with $W_{FWHM} = 9$ μm. If we were to represent the distribution of the stacked sample obtained from using $V_I = 236$ V/cm as a Gaussian with a W_{FWHM} of 45 μm (as would normally be done lacking digital images), these data indicate high voltage injection increased stacking by a factor of five.

3.2. Correlation with sequencing results

Data obtained from fluorescent imaging have clearly demonstrated the increased sample concentration resulting from high voltage injections. These data are now correlated to separation data obtained from electropherograms performed using identical injection protocols. Although numerous researchers [13,14,25] have documented the negative effects of high run voltages during separation, these newest experiments indicate that short periods of high voltage during injection do not destroy the quality of DNA separations.

Results of separations of DNA sequencing reactions performed under identical experimental conditions described in the Experimental section are summarized in Table 2. All separations were performed using a conventional run voltage, $V_R = 150$ V/cm, applied after electrophoretic injection. The data illustrate that implementation of the high voltage injection protocol increased DNA resolution by 25%, as anticipated in Section 1.1. However, although each separation produced approximately the same overall read-lengths (50–510 bases), Table 2 also indicates that higher values of injection voltage increased the resolution of larger DNA molecules. As seen, when an injection voltage of 236 V/cm was used, an average resolution of 0.42 was obtained, with molecules 150–250 bases in length exhibiting the highest resolution. When the injection voltage was increased to 708 V/cm, a higher average resolution of 0.57 was measured, but the highest resolution was exhibited by DNA 198–325 bases in length. Although the 25% increase in overall resolution is significant, the fact that the highest resolution region was shifted to larger molecules is perhaps an even larger discovery. These preliminary experiments indicate that higher levels of stacking may be the key to sequencing larger DNA fragments, or possibly increasing overall read-lengths during separations. Although the optimal benefits of stacking will surely depend upon a detailed match of the channel geometry and experimental conditions with the desired assay, the modified high voltage protocol will certainly assist in this effort.

Table 2
Read-lengths and resolution measurements, R_L, obtained from separations performing high-voltage injections

V_I (V/cm)	Read-lengths (bases)	Highest R_L (bases)	Average R_L
236	50–510	150–250	0.42
554	52–512	178–310	0.55
708	55–510	198–325	0.57

All separations were performed using spin-column-purified 10^{-10} *M* sequencing reactions, 2% solutions of 9 MDa LPA, a load voltage of $V_L = 300$ V/cm and a run voltage of $V_R = 150$ V/cm within identical 250-μm-length injectors. Actual electrophoretic injections were performed at the elevated injection voltages, V_I, denoted in the table.

4. Conclusions

In this study, high voltage injection protocols were used to increase sample concentration during electrophoretic injection, prior to separation. The effectiveness of this protocol was well described by digital images and intensity measurements that documented increased levels of stacking induced by high voltage injection protocols. These data were also supported by electropherograms which confirmed that stacking

effects resulted in a higher average resolution per separation, as well as increased resolution of larger DNA fragments.

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