detected using a 575/25 nm bandpass filter. Collected cells were diluted in 10 volumes of Luria–Bertani (LB) medium containing Tet and Kn and grown to saturation. To begin the third positive selection, a 25 ml GMML culture containing Tet, Kn, and 1 mM *p*IF, *p*AF, *p*CF, or *O*AY was inoculated with cells from the negative screen (100 µl, pelleted and resuspended in GMML). After incubation for 3 h at 37°C, Cm was added to a final concentration of 75 µg/ml, and cells were grown to saturation (~24 h). Following the third positive selection, cells were plated on GMML–agar containing Tet, Kn, 0.002% Ara, 0, 75, or 100 µg/ml Cm, and 0 or 1 mM *p*IF, *p*AF, *p*CF, or *O*AY, and grown for 48 h at 37°C.

**Expression and analysis of an unnatural amino acid–containing protein.** A 100 ml GMML starter culture containing Kn and Tet was inoculated with DH10B cells containing plasmid pBAD/JYAMB-4TAG and an evolved synthetase variant carried on plasmid pBK-JYRS (ref. 7), and grown to saturation. A GMML culture (500 ml) containing Kn, Tet, 0.002% Ara, 5 µM $FeCl_3$, and 0 or 1 mM of a desired unnatural amino acid was inoculated with 50 ml of the starter and grown to saturation (~18 h). Cells were pelleted and sonicated, and the protein was isolated using the QiaExpressionist His-tag purification kit (Qiagen, Valencia, CA). Proteins were analyzed by electrophoresis on a 12–20% gradient sodium dodecyl sulfate–polyacrylamide gel with a premixed set of low-range molecular weight proteins markers (Boehringer-Mannheim, Indianapolis, IN) and electrospray-ionization ion trap mass spectrometry.

*Note: Supplementary information is available on the Nature Biotechnology website.*

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# A DNA prism for high-speed continuous fractionation of large DNA molecules

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The analysis and fractionation of large DNA molecules plays a key role in many genome projects. The standard method, pulsed-field gel electrophoresis (PFGE), is slow, with running times ranging from 10 hours to more than 200 hours. In this report, we describe a thumbnail-sized device that sorts large DNA fragments (61–209 kilobases (kb)) in 15 seconds, with a resolution of ~13%. An array of micron-scale posts serves as the sieving matrix, and integrated microfluidic channels spatially shape the electric fields over the matrix. Asymmetric pulsed fields are applied for continuous-flow operation, which sorts DNA molecules in different directions according to their molecular masses, much as a prism deflects light of different wavelengths at different angles. We demonstrate the robustness of the device by using it to separate large DNA inserts prepared from bacterial artificial chromosomes, a widely used DNA source for most genomics projects.

To improve upon the very time-consuming PFGE process[1–5], many alternative methods for sizing large DNA fragments have been developed. One approach, based on flow cytometry, measures fluorescence intensity as individual DNA molecules pass through a focused laser beam. DNA fragments up to ~200 kb have been sized in about three minutes by this method[6]. The DNA fragments can then be transported to different reservoirs according to size[7]. Another single-molecule sizing approach is to fully stretch DNA fragments and measure their contour lengths[8]. Alternatively, capillary gel electrophoresis using pulsed fields has been shown to separate megabase DNA molecules in ~10 min[9].

Recently, micron-scale structures fabricated using semiconductor technology have been used to separate DNA[10–14]. In one design, DNA

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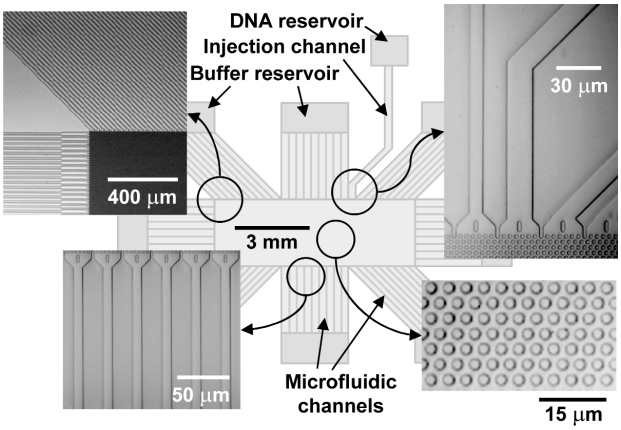


**Figure 1.** Structure of the microfabricated device illustrating the sieving matrix integrated with the microfluidic channels. The post array is 3 mm × 9 mm, and the posts are 2 μm in diameter, 2 μm apart, and 2 μm tall. A single channel connecting to the DNA reservoir injects DNA through a 28-μm opening. The many microfluidic channels connecting to buffer reservoirs produce uniform electric fields over the sieving matrix by acting as electric-current injectors[17].

molecules are sorted by diffusion as they flow through a microfabricated array of asymmetrically arranged obstacles[11,12]. In a second design, entropic trap arrays consisting of many submicron-scale constrictions separated by wider regions reduce the separation time to about 30 min[13]. In a third, arrays of micron-scale posts separate DNA molecules in the 100 kb range in a few seconds[14]. This last device consists of a microfabricated sieving matrix and a narrow constriction for sample concentration and launching. Pulsed fields are created with two pairs of electrodes connecting to the edge of the array. However, this device is limited in the amount of material that can be analyzed, and the electrodes cause severe electric field distortion, further limiting the usefulness of this design[14].

The DNA prism we report here presents three major advances over the microfabricated designs reviewed above. First, continuous-flow operation removes the limitation of the amount of sample the device can analyze. Second, a new microfluidic design has been used to generate and maintain uniform electric fields and precise sample injection. And third, the speed, resolution, and robustness of the DNA prism have been demonstrated using bacterial artificial chromosomes (BAC) and P1-derived artificial chromosome (PAC) inserts isolated by standard protocols[15]. The prism separated 61–209 kb DNA molecules in 15 s with ~13% resolution[16].

The DNA prism, fabricated by conventional photolithographic techniques on a fused silica wafer, consists of a hexagonally packed array of micron-scale posts, sample injection and extraction channels, and structures for shaping uniform electric fields (Fig. 1). Our goal was to inject DNA continuously into the post array using electric pulses, to separate DNA fragments as they flowed through the array, and finally to collect the sorted DNA in microfluidic channels for further downstream analysis. The continuous-flow separation is achieved using alternating electric fields of different strengths or durations (Fig. 2A). Microfluidic channels surround the array and connect it to fluid reservoirs, where voltages are applied. The channels provide sample loading and collection ports and create uniform electric fields across the entire array[17], which are necessary to form straight bands of injected molecules (Fig. 2B, C). Conventional PFGE apparatuses create uniform fields by surrounding the gel matrix with many electrodes[3]. This technique is, however, not suitable for the DNA prism because bubbles generated by electrodes interfere with resolution and reproducibility. The DNA prism thus relies on the realization that microfluidic channels can be used to shape electric fields[17].

We sorted BAC and PAC inserts of 61 kb, 114 kb, 158 kb, and 209 kb (Figs 2, 3). A wide range of pulsing conditions was tested, including field strengths from ~20 V/cm to ~250 V/cm, and square pulse durations from 10 ms to 500 ms. Because of the small scale of this microfluidic device (2 μm deep), high fields can be applied without generating much heat. Separation occurred under pulse conditions of low field strength combined with long duration, or higher field strengths with short durations (Fig. 2). By observing the molecular size in each separation stream at high magnification, we found, as expected, that small molecules move along the average field direction, whereas large molecules migrate toward the strong-pulse direction (Fig. 2A). Further, the migration direction and band sharpness depends on the exact pulse conditions. Whereas low-strength pulses (~50 V/cm) combined with long duration typically separate the four species into only two bands (Fig. 2B), higher field strengths (~200 V/cm) with shorter duration (~50 ms) resolve all four species (Fig. 2C). The separated molecules are collected in different channels at the edge of the device (Fig. 2D). Under high fields (~200 V/cm), the separation time over 3 mm is 15 s.

The migration angle of DNA molecules is also a function of the pulse duration (Fig. 3A). Using 55-ms square pulses of 240 V/cm and 150 V/cm, the four species of DNA were separated into three

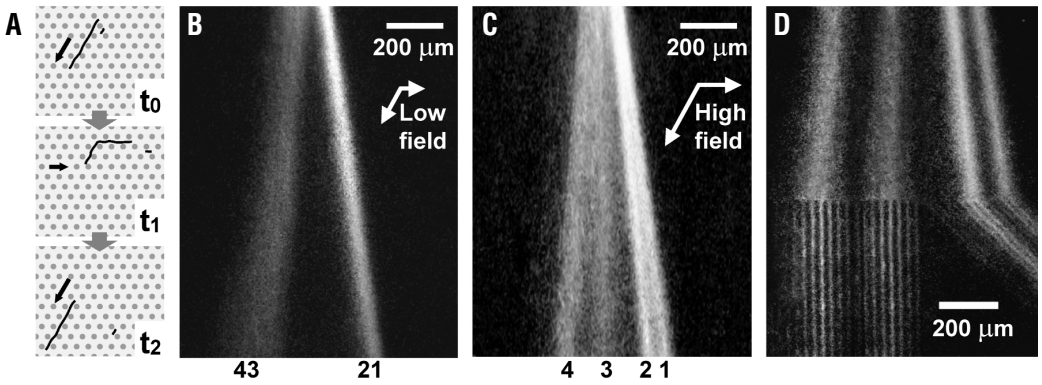


**Figure 2.** (A) Schematic showing the behavior of small and large DNA molecules in microfabricated arrays through a full cycle of asymmetric electric fields of alternating angles. Initially ($t_0$), the high field moves both small and large molecules at similar speeds (arrow shows direction of motion). Next ($t_1$), a low field rotated 120° causes reversal of the leading and trailing ends, and the low field (or short time) prevents the long molecule from sliding off the posts and reversing direction. With reapplication of the original field ($t_2$), the ends again reverse, and the large molecule resumes its original track while the small molecule is in a new track. The small molecules follow the average field while large molecules follow the stronger field. (B–D) Fluorescence micrographs of continuous DNA separation under different field strengths. Long and short arrows point to the DNA migration directions during strong and weak pulses, respectively. Band assignment for the BAC and PAC inserts: (1) 61 kb; (2) 114 kb; (3) 158 kb; (4) 209 kb. (B) Four species are separated into only two bands using 250-ms square pulses of 32 V/cm and 20 V/cm alternating at 2 Hz. (C) All four species are resolved using 40-ms square pulses of 240 V/cm and 150 V/cm alternating at 12.5 Hz. (D) Separated molecules are collected in different channels and routed to different reservoirs.

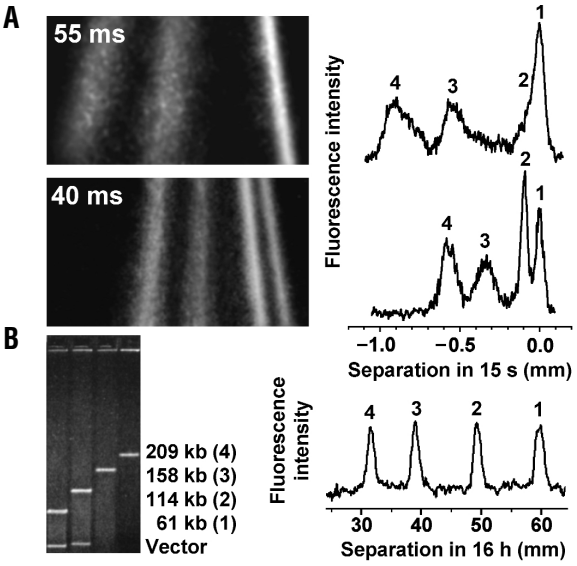


**Figure 3.** Separation of BAC and PAC inserts at different frequencies. (A) Separation in 15 s using the DNA prism. Fluorescence micrographs show the separated DNA bands from 2.5 mm to 3 mm below the injection point. The fluorescence intensity profiles are scanned at 3 mm from the injection point, with the origin of the horizontal axis defined as the average field direction. Peak assignment: (1) 61 kb; (2) 114 kb; (3) 158 kb; (4) 209 kb. The resolution in the 114–209 kb range is 11–15% at 55 ms and 16–19% at 40 ms. The separation time using a 3-mm-long sieving matrix is ~15 s. (B) Separation in 16 h using conventional pulsed-field gel electrophoresis. Pulsing conditions: 6 V/cm pulses separated by 120°, duration linearly ramped from 0.1–40 s over 16 h[15]. The resolution using this protocol is ~7%. The horizontal axis of the fluorescence intensity profile is defined as the DNA migration distance from the loading wells. Peaks for the P1 and BAC vectors are not shown.

bands (Fig. 3A, 55 ms). The brightest of the three contains 61-kb and 114-kb molecules. However, as we decreased the pulse duration to 40 ms (Fig. 3A, 40 ms), the large-molecular-mass bands (158 kb and 209 kb) shifted towards the average field direction, while the originally unresolved band of small molecules (61 kb and 114 kb) split into two. All four species were then resolved. Although the bands shifted with pulse duration in ways not yet fully understood, the changes were reproducible. The fact that separation depended on pulse duration is not surprising—it exists for standard PFGE as well. In fact, PFGE uses different pulse durations (typically 0.1–40 s) to resolve different molecular-mass ranges[15]. Although the continuous-flow prism requires a fixed pulsing duration to operate at steady state, different durations can be used in consecutive runs, each of which analyzes a different molecular-mass range chosen to optimize resolution.

The resolution is ~11–15% in the 100–200 kb range (Fig. 3A, 55 ms)[16]. It seems likely that the degree to which molecules fail to elongate fully during each pulse currently limits resolution. This is because fully elongated molecules backtrack further than molecules that are not fully elongated during each pulse, and the time spent backtracking is an important determinant of resolving power. To illustrate this, imagine choosing a pulse time (Fig. 2A) such that the fully stretched larger molecule spends precisely all of its time slithering back and forth in the channel defined by the posts over which it is draped, never moving in the direction of the average field. Any relaxation from the fully stretched state would then allow it to advance in the average field direction, thus reducing the distance between it and faster-moving molecules, and lowering the resolution. Because high fields stretch DNA molecules more than low fields, and short durations reduce the time for relaxation, molecules under these conditions elongate more fully (Fig. 2C). The post size of the array should also affect the stretching of DNA, particularly of small molecules. The 61-kb and 114-kb inserts are poorly resolved (Fig. 3A) because they are too small to interact with the posts and be stretched. In fact, 61-kb molecules never deviate from the average field direction, suggesting that these molecules do not elongate and back track. This result is supported by other experiments and theoretical calculations[18,19] showing that randomly coiled DNA molecules shorter than ~100 kb are smaller than the constrictions in the array (2 μm posts, 2 μm spacings), and thus little stretching should occur. Although the resolution is not yet as sharp as can be achieved by highly optimized conventional methods (Fig. 3B, resolution ~7%), it is clear that the resolution improves with separation distance and pulse tuning, neither one of which has yet been fully optimized (Figs 2 and 3A). In this regard, we point out that the resolution of the four inserts shown in Figure 3B required a linear duration ramp from 0.1 s to 40 s over 16 h. At constant pulse duration, conditions similar to those used with the DNA prism (Fig. 3A, 40 ms), these four species would separate poorly, if at all.

The DNA prism showed good run-to-run reproducibility: no change in separation angles occurred when the device was operated overnight, and the separation angles were always the same for a given set of pulsing conditions, regardless of the conditions applied during sample loading. This is because the microfabricated post array replacing the conventional gel matrix has accurate pore sizes, and the thin array generates very little heat. Further, although we used small buffer reservoirs (typically ~20 μl per reservoir), the amount of buffer inside the device is only ~100 nl, and therefore the buffer strength remains constant over a long time. The device-to-device reproducibility is also good—using standard semiconductor-processing techniques, the variations in the post and channel dimensions are typically <5%. We tested four devices and observed similar separation patterns using running buffer and DNA samples freshly made and stained.

The current version of the DNA prism has a throughput of ~$10^4$ molecules per second (~10 ng/h or ~1 μl/h). This rate is high compared to other unconventional techniques, and certainly high enough for efficient sequencing-library preparation. Sample recovery from the device is currently limited by the ability to pipette small volumes of liquid; however, we believe that this problem is offset by our ability to direct fractionated DNA into collection channels (Fig. 2D) by which samples can be routed on-chip to other compartments for further analysis. By comparison, the entropic trap array[13] and our previous work on hexagonal post arrays[14] are limited in throughput (~10 pg/h) because of the small sample plug. Other continuous-flow techniques also have lower throughput—the asymmetric obstacle arrays[11,12] typically have a throughput of ~100 pg/h because of the low flow speed required for diffusion-based separation, and fluorescence-activated cell sorting methods typically process ~100 molecules per second (~100 pg/h).

In summary, the DNA prism technique has substantial advantages over other techniques. It sorts molecules at high speeds, with running times of ~15 s. This is more than 1,000 times faster than conventional PFGE[1–5] (10–240 h). The prism is about 9 times faster than flow cytometry[6,7] (15 s vs. 130 s[6]), 40 times faster than pulsed-field capillary electrophoresis[9] (~10 min), and over 100 times faster than entropic trap arrays[13] (~30 min) and asymmetric obstacle arrays[11,12] (~2 h). Further, the prism device has better resolution (~13%) than entropic trap arrays[13], asymmetric obstacle arrays[11,12], and an earlier version of the hexagonal post arrays[14], all of which exhibited resolutions 5–10 times lower in the 100–200 kb range.

## Experimental protocol

**Device fabrication.** Channels and posts were patterned on fused silica wafers by photolithography, and created using reactive ion etching. Holes for buffer and DNA sample were drilled and the device was sealed with a glass coverslip coated with silicone rubber (RTV-615 from General Electric, Schenectady, NY).

**DNA preparation.** BACs and PACs were isolated from *Escherichia coli* strains RPCI 21 168-F5, RPCI 21 539-K14, RPCI 22 49-E10, and RPCI 23 200-J16 by standard methods[15]. Plasmid preparations were digested with *Not*I and the digestion buffer was exchanged with 0.5× TBE using centrifugal filters (Microcon YM-100, Millipore, Bedford, MA).

**Electrophoresis conditions.** The electrophoresis buffer was 0.5× TBE[5] containing 0.1% (wt/vol) POP-6, a performance-optimized linear polyacrylamide (Applied Biosystems, Foster City, CA) and 10 mM dithiothreitol (DTT) added to suppress electro-osmotic flow and photobleaching, respectively. DNA was stained with TOTO-1 (Molecular Probes, Eugene, OR) at a ratio of 1 dye molecule per 10 bp. The starting DNA concentration was ~10 ng/μl. Images were recorded by epifluorescence microscopy.

**Resolution calculation.** The resolution between two peaks of molecular mass $L_1$ and $L_2$ ($L_2 > L_1$) is defined as[16]

$$1.5\ [(\sigma_1 + \sigma_2)\ \ln(L_2/\ L_1)]/\Delta x$$

where $\sigma_1$ and $\sigma_2$ are the standard deviations of the peaks, and $\Delta x$ is the distance between the two peaks. A device having 13% resolution can thus resolve 113-kb molecules from 100-kb molecules, 226-kb molecules from 200-kb molecules, and so on.

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