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Label-Free DNA Sequencing Using Millikan Detection

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Abstract

A label-free method for DNA sequencing based on the principle of the Millikan oil drop experiment was developed. This sequencing-by-synthesis approach sensed increases in bead charge as nucleotides were added by a polymerase to DNA templates attached to beads. The balance between an electrical force, which was dependent on the number of nucleotide charges on a bead, and opposing hydrodynamic drag and restoring tether forces resulted in a bead velocity that was a function of the number of nucleotides attached to the bead. The velocity of beads tethered via a polymer to a microfluidic channel and subjected to an oscillating electric field was measured using dark-field microscopy and used to determine how many nucleotides were incorporated during each sequencing-by-synthesis cycle. Increases in bead velocity of ~ 1% were reliably detected during DNA polymerization allowing for sequencing of short DNA templates. The method could lead to a low-cost, high-throughput sequencing platform that could enable routine sequencing in medical applications.

Keywords

DNA sequencing; tethered beads

Introduction

While rapid technological progress has been made since the original sequencing of the human genome using Sanger sequencing (1, 2), further reductions in cost and improvements in read length and data quality will be needed (3) before whole genome sequencing can be routinely used to assess gene function by comparative genome analysis across species (4, 5), to understand human genomic variation and its relationship to disease (6) and to characterize the somatic mutations that underlie cancer (7, 8). Although cyclic array technologies based on pyrosequencing (9), incorporation of fluorescent nucleotides (10), pH-sensing via field-effect transistor-based sensors (11) and ligation of fluorescent oligonucleotides (12) are currently widely used, and single molecule systems based on nanopores (13, 14) may hold

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promise for the future, new detection technologies need to be explored in order to ensure continued increases in DNA sequencing performance.

We tested the feasibility of a DNA sequencing method with the potential to greatly lower sequencing cost by enabling highly parallel, accurate, label-free sequencing-by-synthesis. The method is analogous to the Millikan oil drop experiment, which used opposing electrical, gravitational and hydrodynamic forces on a charged particle to measure the quantized electron charge with a precision of $<0.5\%$ (15). Using simple instruments, Millikan was able to distinguish drops that differed by one electron charge up to hundreds of electron charges per drop. More recently, McLaughlin and coworkers demonstrated the ability to use the opposing force concept for measuring enzyme-dependent changes in the number of charges from bead-attached molecules (16). Phospholipase kinetics were quantified by measuring the motion of charged beads held in an optical trap and subjected to an oscillating electric field. The use of optical traps as the restoring force limits the number of beads that can be analyzed in parallel since systems have been developed for forming arrays of tens of optical traps (17) but there is no clear path to obtaining a low cost array of millions of traps. We propose to replace the restoring force from an optical trap with the “entropic force” exerted by stretching a polymer chain from its termini (18). It is possible to form large arrays of tethered beads for highly parallel measurements (19).

In Millikan Sequencing, the incorporation of unlabeled, charged nucleotides onto bead-attached DNA templates is monitored by virtue of changes in the balance of forces on a bead. Beads with surface-attached clonal DNA templates are tethered to the surface of a microfluidic channel. In an oscillating electric field, the forces acting on the bead are: (1) an electrical force which is directly dependent on the total electrical charge of DNA on the bead, and (2) opposing forces consisting of a hydrodynamic force and a restoring spring-like force provided by a tethered polymer (see Figure 1A). In the presence of a DNA polymerase, the four dNTPs are added sequentially to the beads while dark-field microscopy is used to monitor the motion of the beads. In this sequencing-by-synthesis approach, incorporation of nucleotides into the bead-bound DNA results in a change in the balance of the forces, leading to a measurable change in bead position or velocity. The method can be used for the label-free sequencing of DNA.

Materials and Methods

Tether preparation

A bifunctional dsDNA tether was made with biotin on one end, to attach to avidin beads, and bovine serum albumin (BSA) on the other end, to attach non-specifically to a glass surface. A polymer tether was constructed by PCR using labeled primers to generate a 10,051 bp amplicon from nucleotides 7,179 to 17,179 of a λ DNA template (N3011, NEB, Ipswich, MA) with a free amine at one end and a biotin at the other end (20). The amplicon was purified with a QiaQuick PCR Purification kit (Qiagen, Valencia, CA) and the amplicon size was verified by micro-gel electrophoresis using the Bioanalyzer 2100 (Agilent, Santa Clara, CA). BSA (Pierce, Rockford, IL) was attached to the amine on the tether by activating 30 nM dsDNA with 250 μ M succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL) in 20 mM sodium phosphate (pH 7.25) at 4 °C for 4

hours. Bovine serum albumin was reduced with TCEP (Pierce, Rockford, IL) and added to the activated DNA at a final concentration of 67 μ M and allowed to react at 4 °C overnight.

Bead Preparation

Low electrophoretic mobility avidin-labeled beads were made from 1.5 μ m diameter carboxypolystyrene beads (PC04N, Bangs Laboratories, Fishers, IN). Fifty μ L of beads were washed 2 $\times$ in 200 mM 2-(N-morpholino)ethanesulfonic acid (MES, pH 6) with 0.1% sodium dodecyl sulfate (SDS) and resuspended in 500 μ L of MES buffer and mixed with 500 μ L of 10 mg/ml EDC (Sigma-Aldrich, St. Louis, MO) to activate the carboxy groups. After 15 minutes, beads were washed with 200 mM sodium phosphate buffer (pH 7.5) with 0.1% SDS. The washed beads were resuspended in 150 μ L and reacted with 1 μ M avidin (Sigma-Aldrich, St. Louis, MO) and 25 mM 1 kDa amino-PEG (#26116, Pierce, Rockford, IL) for 3 hours at room temperature. Beads were washed and resuspended in 10 mM Tris (pH 8) with 50 mM NaCl and stored at 4 °C. Where indicated, 1 μ m diameter streptavidin coated beads (CP01N, Bangs Laboratories, Fishers, IN) having high electrophoretic mobility were used in place of the custom beads.

Bead Labeling

Biotinylated tether and biotinylated primed DNA oligo were added to avidin-labeled beads before tethering the beads within a glass microfluidic chip. Avidin beads were labeled with a tether by washing the beads 2 $\times$ in 10 mM Tris (pH 8) with 50 mM NaCl and 0.1% triton X-100, resuspending in 10 mM Tris (pH 8) with 1 M NaCl and incubating with the dsDNA tether at a ratio of ~1 tether per bead for 30 minutes. Beads were then incubated with 6 μ M of a biotinylated primed template (TEG or photocleavable PC-biotin-5'GAAGAGCCAAGGACAGGTAC3' (primer) and either 5'ATCGCTGCCGTACCTGTCCTT GGCTCTTC3', or 5'AGCTGTACCTGTCCTTGGCTCTTC3' or 5'AAAAAATGGAAAAA AAAAAAATCCCCCCCCACCCCGTACCTGTCCTTGGCTTTC3' (templates) from IDT) for 2 hours, washed in 10 mM Tris (pH 8) with 50 mM NaCl and 0.1% triton X-100 and resuspended in 10 mM Tris (pH 8) with 50 mM NaCl and 2% 5K DNA gel (Caliper Life Sciences, Mt. View, CA) containing polydimethylmethacrylate to suppress electroosmotic flow in the microfluidic channels. The number of templates bound per bead was estimated by binding oligos with photocleavable biotin groups (Integrated DNA Technologies, San Diego, CA) to a known number of avidin beads, washing away unbound oligos, photolyzing the biotin linkage and measuring the released oligo concentration using the DNA1000 assay on the Agilent Bioanalyzer system.

Microfluidic Chips

Custom designed glass microfluidic chips (Caliper Life Sciences, Mt. View, CA) with a 6 μ m $\times$ 312 μ m, 12.6 mm long channel were washed with 1 M NaOH for 30 minutes and rinsed with 10 mM Tris with 50 mM NaCl (pH 8) before labeled beads were flowed into the channel. Beads were incubated for 15–60 minutes, washed with 10 mM Tris 50 mM NaCl and 2% DNA 5K gel (pH 8) to remove untethered beads from the channel. Finally the channel was washed with water and the desired polymerization buffer was flowed into the channel for polymerization and sequencing-by-synthesis experiments.

Instrumentation

As shown schematically in Figure 1B, platinum electrodes were placed in the wells of the microfluidic chip and voltages applied via a computer controlled programmable power supply (Agilent Bioanalyzer 2100). Reagents placed in the wells of the chip were introduced to the beads by applying an AC field to wiggle the beads for velocity measurement and a DC field to transport the charged nucleotides. The AC field had a frequency of 5 Hz, an amplitude of 60 V/cm AC and a square wave shape. A DC field with an amplitude of 10 V/cm was used. Dark field images of the oscillating, tethered beads were obtained on a Nikon Diaphot 300 microscope with a 10×, 0.3 NA objective using a 1 megapixel high-speed, digital, CMOS camera (SV643M, Epix, Buffalo Grove, IL) at 25 to 300 Hz. MATLAB (Mathworks, Natick, MA) code was written to convert the images into the bead position using a center-of-mass calculation. For user-identified beads in a field of view, the bead center, p_x , was calculated for each frame from:

$$p_x = \sum_{x=-m}^m s(x)x / \sum_{x=-m}^m s(x) \quad (1)$$

$$s(x) = \sum_{y=-n}^n I(x,y) \quad (2)$$

where $I(x,y)$ is the background subtracted pixel intensity at position x,y . The values of n and m were typically 3–7 pixels depending on the image magnification. The position of each bead was calculated for each frame to give a time course of bead position. For each electric field cycle, the absolute values of the maximum and minimum slope (over 4–16 frames, depending on the data acquisition rate) were averaged to obtain the bead velocity for each cycle. The velocities from a fixed amount of time were averaged (typically 20 seconds) and reported.

Polymerization Assay

Polymerization of the DNA templates on the beads tethered to the channel surface was performed with Klenow exo- DNA polymerase (Enzymatics, Beverly, MA) in 50 mM Tris, 300 mM NaCl, 10 mM MgCl₂ and 2% DNA 5k gel with 10 μM dNTPs (pH 8) at room temperature. Images were acquired for ~300 seconds as dNTPs were added to the beads using electrophoresis with a DC field. The velocity data was fit with a combination of linear (to account for drift) and exponential (to account for the polymerization) functions:

$$V = m \times t + b + a \left(1 - e^{-(t-t_i)/t_c} \right) \quad (3)$$

where m is the slope, t is time, b is the intercept of the linear function, a is the exponential amplitude, t_i the start time and t_c the time constant. The fit was performed twice, once with a set to zero and again with a unconstrained. The fit with the lower reduced sum of squares was used to calculate the magnitude of the change in velocity due to polymerization. For sequencing, the magnitude of the velocity change for each nucleotide addition was

normalized to account for bead-to-bead and oligo sequence differences. The number of incorporated nucleotides was called based on the normalized amplitudes.

Results

DNA tethers were made using PCR with biotin and amine containing primers yielding a dsDNA fragment 10,000 base pairs long (Figure 2A). Once coupled to BSA through the amine functionality, the tether was used to attach polystyrene beads to the surface of a glass microfluidic chip by non-specific adsorption of BSA to the glass. The right hand panels of Figure 2B show images of a typical tethered 1.5 μm bead imaged using dark field microscopy as the direction of an electric field (60 V/cm) along the channel is reversed. The bead moves until it reaches the end of its tether. The left panel of Figure 2B shows two superimposed images of 7 beads in a 100 $\mu\text{m} \times 100 \mu\text{m}$ area. The two images were captured 100 milliseconds apart as the field direction was reversed. Six of the beads were tethered while one (lower left) was non-specifically stuck to the surface. With a 640 $\mu\text{m} \times 480 \mu\text{m}$ field of view, up to ~100 beads attached randomly to the glass surface could be monitored simultaneously. A series of bead images acquired at 100 Hz was converted to bead position as a function of time. The bead position time series of a bead in the absence of an electric field shows diffusion of the bead (Figure 2C, top panel). When an oscillating electric field (60 V/cm, square wave at 1 Hz) was applied, the bead is observed to move quickly back and forth until the bead reaches the end of its tether (Figure 2C, bottom panel). The total distance traveled by the bead is consistent with the length of the dsDNA tether. Bead velocity was measured from the measured bead position as show in Figure 2D. For each electric field cycle, the velocity was calculated as the slope of the position trace over 4 points. The maximum absolute value of the velocity for each half cycle was averaged and those averages averaged again over 5 cycles to give the average velocity during this one second period. The averaging period used in this study ranged from 1 to 20 seconds.

Changing either the electric field strength or the number of charges on the bead surface, through the effect on the zeta potential, changes the electrical force acting on a tethered bead. The changes in the force acting on the bead caused by changes in the number of charges on the tethered bead can be monitored either from the bead amplitude or the peak bead velocity. We chose to monitor the bead velocity since it was approximately linearly dependent on the force acting on the bead over a large range of forces (see Figure 3A). Bead velocity measurements can be used to detect small changes in the forces acting on a bead. As seen in Figure 3B, changes in the electric field (and therefore the force acting on a bead) of <1% can be distinguished when bead velocity is measured over a 20 second time period with a field frequency of 5 Hz and field amplitude of 60 V/cm. As expected, the variability of the measurement decreases with the square root of the number of cycles used for averaging. Groups of avidin beads labeled with biotinylated oligos of increasing length show an approximately linear increase in bead velocity with the number of charges added to the beads by the oligos (Figure 3C). The velocity of an individual bead is normalized to the initial velocity of that bead to account for bead-to-bead differences in factors such as bead diameter. As seen in Figure 3C, such normalization results in low variability. Together, the linearity and sensitivity of the bead velocity measurement to the applied electrical force on

the bead indicated that bead velocity could be used to monitor enzymatic changes in the number of charges attached to a tethered bead.

The ability to detect enzyme-dependent changes in bead-associated charges by measuring bead velocity was tested. Oligo templates primed with biotinylated oligos were bound to Bangs streptavidin coated beads at a coverage of $\sim 10^4$ templates per bead. dNTPs were electrophoretically added to tethered beads by applying an oscillating electric field (amplitude of 60 V/cm at 5 Hz frequency) with a DC bias (10 V/cm). Images of the oscillating beads were acquired and converted to average velocity values over 100 cycles. As seen in Figure 4A, after addition of dNTPs bead velocity increased in the presence of Klenow exo- DNA polymerase but not in the absence of polymerase. The time constant decreased 4.8 fold as the enzyme concentration increased 5 fold. Bead velocity increased by $\sim 20\%$ as 38 nucleotides per template were added. Addition of only dGTP and dTTP before addition of all dNTPs yielded a stepwise increase in velocity (Figure 4B). The first step is 33% of the total increase in velocity which is consistent with the 34% increase expected by adding 13 of 38 total nucleotides added in the first step. For further experiments, beads were optimized to yield large changes in bead velocity per added nucleotide by reducing the bead charge.

Sequencing of short oligos was tried by sequential addition of single dNTPs followed by washout of the dNTP. Figure 5 shows bead velocity plots for individual beads with different template sequences. Superimposed on the data are the best fits using Eq. 3. As can be seen, each bead shows a sequence dependent change in bead velocity consistent with the known sequence. For example, Figure 5A shows a large increase in velocity as G is added to the initial two Cs in the template with subsequent smaller increases in velocity when a single complimentary base is added. Single nucleotide additions can clearly be distinguished (typical changes of 1–2% per nucleotide added) from lack of nucleotide incorporation. The observed change in velocity per nucleotide added varies between beads and is partly dependent on the local sequence. For example, the bead in Figure 5A showed a change in velocity of $\sim 2\%$ per added nucleotide while the bead in Figure 5B showed a 1% change per nucleotide. Additionally in Figure 5B, the change in velocity upon incorporation of a single G is ~ 1.5 times the change upon incorporation of a single C. While the underlying reason for the variability in the change in bead velocity upon dNTP incorporation is not known at this time, changes in bead velocity were normalized with respect to the change in bead velocity upon the first base addition in order to allow for base calling for sequencing.

Figure 6 shows sequencing results for 15 individual beads imaged in one field of view. The fit amplitude, a in Eq. 3 for each of the 13 nucleotide additions for each bead was normalized with respect to the fit amplitude for the first base incorporation (corresponding to addition of two Gs) and used to make base calls. The number of incorporated bases for each base addition was called with thresholds of ± 0.5 normalized units. For example, for the first C addition, the beads show a normalized amplitude ranging from 0.6 to 1.4 therefore all the beads have a C called for that position. The base calls for each of the 15 beads is shown at the right. As seen in Figure 6, base calls were made with 94% accuracy for the short 9 bp templates used in this study.

Discussion

Previous work by McLaughlin (16) had demonstrated that the principle behind the Millikan oil drop experiment, observing the motion of a particle subjected to opposing forces to monitor the number of electrical charges on the particle with high precision, could be applied to measure enzymatic reactions in aqueous buffers. Our results indicate that the Millikan sensing approach can be used to sequence DNA using sequencing-by-synthesis on clonal populations of bead-bound templates. The method was sensitive enough to detect single nucleotide incorporations using beads with ~10,000 templates per bead. This is two to three orders of magnitude lower than that required for other sequencing methods such as pyrosequencing (9) and pH sensing (11). This opens up the possibility of using bead clonal template amplification methods other than emulsion PCR such as surface PCR (10) which could greatly simplify sample preparation. Since the electrical force acting on the bead depends on the surface density of charges (i.e. the zeta potential), fewer templates could be used if smaller beads were used. To visualize smaller beads with sufficient signal-to-noise ratio, gold nanoparticles can be used since they have a very high scattering cross section (22).

High throughput sequencing requires the ability to measure millions of beads simultaneously. While optical traps have been used to provide a force opposing the electrical force on the bead to measure enzyme dependent changes in bead charge, it is difficult to multiplex optical traps beyond hundreds or thousands (17). We demonstrated that a polymer tether could replace an optical trap by providing a restoring force that both opposes the electrical force on the bead and keeps beads localized to a defined area so that multiple rounds of dNTP addition can be conducted while individual beads are monitored. The tether can be stretched to provide a high restoring force to look for small changes in bead displacement as was done with optical traps. Alternatively, a long tether can be used without much stretching to monitor bead velocity as was reported in this study. Recent work has demonstrated the ability to generate large arrays of tethered beads that could be used for highly parallelized sequencing (19).

The read lengths (~10 nt) and call accuracy (~94%) obtained using Millikan sequencing were modest and would require at least a 3-fold improvement in read length and a 6-fold improvement in the error rate in order to make the approach viable for genome resequencing efforts. Assuming that all of the charge on the bead comes from the oligos, reading the last nucleotide of a template of length N requires a bead velocity measurement with a sensitivity of $1/2N$. Our current 1% detection sensitivity should allow read lengths of ~35 nucleotides assuming a 15 bp primer region. With our current beads, about two thirds of the bead charge comes from unreacted carboxy groups so our 1% sensitivity leads to the modest 10 bp read length. We are developing methods to reduce the bead charge and improve the bead coatings to allow us to work with more neutral beads while minimizing bead aggregation and bead sticking. The dsDNA tether also accounts for part of the bead charge (typically ~25%) so moving to uncharged polymers such as heterobifunctional PEGs could further reduce non-productive bead charges (22). Improvements in the velocity measurement should also yield an improvement in the read length by improving the detection sensitivity. The fundamental limit on the sensitivity of the bead velocity measurement is the diffusion of the bead since

even with perfect bead positional measurements bead diffusion causes a difference between the measured bead velocity and the electrophoretic bead velocity. Increasing the field strength minimizes the diffusion distance compared to the electrophoretic distance. Increasing the number of cycles of observed bead motion by either increasing the data acquisition time or increasing the field frequency could also reduce the noise by averaging. The signal-to-noise ratio of the velocity measurement is expected to be proportional to the product of the amplitude of electric field and the square root of the averaging time and the frequency of the electric field. Such improvements in detection sensitivity together with neutral beads would improve both the read length and call accuracy.

Currently available commercial next generation sequencing technologies typically use either fluorescently-labeled nucleotides, enzyme cascades or a microelectronic sensor to detect incorporation of nucleotides or the pyrophosphate product of nucleotide incorporation (23). The Millikan sequencing approach uses the charge on the phosphate of an added nucleotide to detect incorporation of nucleotides. Because no extrinsic labels or expensive enzymes or sensors are needed for detection, the cost of reagents for sequencing is expected to be low. In contrast to fluorescence-based detection that requires a narrow excitation source and high numerical aperture collection optics, Millikan detection uses simple, dark-field optics that should lead to a low cost instrument in addition to low per-sample costs.

Conclusions

We have demonstrated the feasibility of using the principle of the Millikan oil drop experiment to detect the charges added to clonal bead-bound templates in a DNA sequencing-by-synthesis approach. This label-free approach had sufficient sensitivity to detect single nucleotide incorporations and to sequence short templates. Future work to optimize bead and tether charges as well as tethered bead velocity sensitivity should improve the accuracy and read length. The method could lead to a low-cost, high-throughput sequencing platform that could enable routine sequencing in medical applications.

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FIGURE 1.

A. Schematic showing how sequencing-by-synthesis can be conducted by measuring the change in bead velocity as dNTPs are incorporated into bead-bound primed templates. Beads tethered to the surface of a microfluidic channel are subjected to periodic changes in the direction of the electric field along the channel causing the beads to wiggle back and forth a distance x_0 in the half cycle time of dt with a velocity (x_0/dt) . Addition of a non-complementary base leads to no change in the velocity. Addition of a complementary base, leads to an increase in bead charge and an increase in bead velocity $(x_0 + dx / dt)$. B.

Schematic of experimental setup. Tethered beads in a microfluidic channel are imaged with a dark-field microscope as the beads undergo motion caused by the time-varying electrical field applied via an Agilent Bioanalyzer power supply.

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FIGURE 2. Velocity of tethered beads

A. Amplification of 10 kilobase DNA tether by PCR is confirmed in Bioanalyzer gel microelectrophoresis result showing 10 kilobase dsDNA PCR amplicon which migrates slower than the 150 and 7,000 bp markers. B. Left panel shows two superimposed dark-field microscopy images of a group of beads moving in response to a field reversal. Images were taken 100 milliseconds apart. The right panels show sequential images of a tethered 1.5 μm bead moving in response to reversal of the electric field (60 V/cm). Images were taken 20 ms apart. The scale bars represent 5 microns. C. Measured position of a tethered bead in the

absence (top) and presence (bottom) of a 60 V/cm, square wave (1 Hz) field. D. Bead velocity is measured from the measured bead position as a function of time in a 60 V/cm, square wave (5 Hz) field. The velocity is calculated as the slope of the position trace over 4 points. The maximum absolute value of the velocity for each half cycle is averaged (shown at top for each cycle). The average velocities are averaged again over 5 cycles to give an average velocity of 32.5 $\mu\text{m}/\text{sec}$ for this one second period.

FIGURE 3. Tethered bead velocity depends on length of bead-bound oligonucleotides

A. The velocity of a tethered bead (average and standard error of the mean shown for 100 cycles) was measured as a function of the electric field amplitude and fit by a line. B. Variability in the average bead velocity in a 60 V/cm, 5 Hz field was measured as a function of the number of field cycles. The ratio of the standard error and mean of the velocity measured for each cycle is shown for 7 tethered beads. The ratio of the standard error and the mean is well fit by a function which depends on the square root of the number of cycles. C. The relative change in tethered bead velocity was monitored for beads after addition of

oligos with varying complimentary lengths. Oligos having 0, 15, 21 or 30 complementary nucleotides to a bead-bound template were transported by electrophoresis to the beads and changes of bead velocity monitored. Average and standard errors of the ratio of the final to initial bead velocities for 6 to 19 beads are shown.

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FIGURE 4. DNA polymerization

A. Bead velocity was measured in the presence of varying Klenow exo- DNA polymerase concentrations. To account for differences in the initial bead velocity, data is normalized to the initial bead velocity. The time constant of exponential fit decreased 4.8 fold as the enzyme concentration increased 5 fold. B. Bead velocity was measured after addition of all dNTPs (left) or stepwise addition of dGTP and dTTP followed by addition of all dNTPs (sequence is 5'AAAAAATGGAAAAAAAAAAAAATCCCCCCCCACCCC3').

FIGURE 5. DNA sequencing

Bead velocity was measured as indicated dNTPs were sequentially added. The velocity data was normalized to the initial bead velocity. Best fit curves to Eq. 3 are shown. A. The template sequence was 3'CCGTCG5'. B. The template sequence was 3'TCGA5'. Incorporation of a single base resulted in a change of bead velocity of 1 to 2 %.

FIGURE 6. DNA base calls

Beads containing bound oligos (template sequence: 5'ATCGTGCC3') were imaged for sequencing. Fitted amplitudes for 13 dNTP additions for 15 individual tethered beads were normalized to the amplitude for each bead's initial dGTP incorporation. Red lines indicate thresholds for base calls. Called sequences (along with the expected sequence in bold) are shown at right.