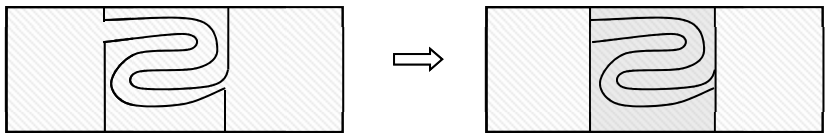
1) Assemble a chamber

a) To clean coverslips and slides, place them in racks in beakers or jars and agitate them gently in dilute laboratory detergent for 1 hour.
b) Place the beakers under the tap and rinse for 5 min.
c) Remove the racks and rinse the beaker with milliQ water. Place the racks in the beakers again and submerge in pure ethanol for storage.
d) Place a 22 X 22 mm square of parafilm on a 25 X 75 slide.
e) With sharp blade cut a parafilm spacer to create a serpentine channel as shown. The entrance should be as wide as the channel, but the exit should be narrower to reduce fluid flow rates in the final chamber.
f) Remove a slide and a coverslip from ethanol using forceps and dry them gently with an aerosol duster.
g) Place the parafilm spacer on a slide with the S-shaped channel openings toward the ends of the slide, and place a coverslip on top of the spacer as shown below.



Slide plus spacer **Slide plus spacer and coverslip**

h) Heat the crossed coverslips on a hot plate on the lowest setting until the parafilm becomes tacky and adheres to both glass surfaces. Gentle pressure applied using a broad, smoothly curved end of a pair of forceps helps to remove air bubbles, but too much force can break the coverslip!
i) Allow the sealed chamber to cool.

2) Prepare the surface for DNA

a) Introduce one volume (about 50 µl) of 0.2 µm filtered PBS near the larger opening allowing capillary action to draw it in. Then flush with two or more volumes of PBS by depositing a droplet of buffer at the opening and using a laboratory tissue twisted into a pointed cone-shape, touch the point to the narrow opening as a wick to draw the first volume of buffer out of the chamber. This will simultaneously draw in the second volume of buffer. If tissue is tightly twisted, the solution will quickly wet the narrow point and slowly wick into the tissue. All subsequent buffer exchanges are similarly performed.
b) Draw 2 volumes of 20 µg/ml anti-digoxigenin (Roche; in PBS) into the chamber and incubate room temperature in a closed container at high humidity for at least 2 hours.
c) Gently rinse the microchamber with 3 volumes of 0.2 µm filtered buffer with 200 mM salt (usually NaCl or KCl) and 0.5 mg/ml alpha-casein as a blocking agent and incubate for at least 5 minutes.

3) Link DNA to beads

a) Dilute 20 µl of Streptavidin-coated beads (Spherotech SVP-03-10) in 200 µl of buffer without casein and mix well in a 1.5 ml eppendorf tube. Although higher strength salt is desirable to remove

contaminants adsorbed to the beads, the polystyrene beads will float on solutions with higher salt concentrations.

b) Centrifuge at 16,000 G in a micro-centrifuge for 2 minutes.
c) Remove the supernatant (be careful not to disturb the beads).
d) Resuspend in 200 µl of buffer.
e) Repeat the preceding three steps (washes) two more times.
f) Add 15 ng of the bio-, dig-labeled DNA to 200 µl of buffer.
g) Add the DNA solution to the Streptavidin-coated bead solution in a 1.5 ml eppendorf tube and vortex on low speed (setting 1) for at least 10 minutes.
h) Spike the bead-DNA solution with 2 µl of 0.1 µM d-biotin (Sigma) in TE, mix, and incubate for 5 min.

4) Tether beads to chamber surface

a) Slowly inject the bead-DNA solution into the microchamber and incubate 5 min.
b) Gently rinse the microchamber with three volumes of buffer with 0.5mg/ml alpha-casein and 4 volumes of the experiment buffer.
c) Apply small drops of mineral oil to the openings to seal the chamber.
d) Place on the microscope for observation.

Alternative steps for reversed linkage with streptavidin-coated surface and anti-digoxigenin-coated beads

5) Assemble and flush chamber with PBS as above
6) Prepare surface for DNA
   a) Introduce one volume of 40 µg/ml solution of Bio-BSA diluted in PBS into the chamber and incubate in a closed container at high humidity at least 2 hours at room temperature or overnight at 4 degrees.
   b) Add one volume of 50 µg/ml streptavidin to the chamber and incubate for 1 hour
   c) Flush chamber twice with buffer.
7) Link DNA to beads
   a) Incubate 100 pM DNA in a 1:10 molar ratio with polystyrene microspheres (anti-dig-coated, 240 nm radius, Indicia, France) for no longer than 10 minutes to avoid producing beads with more than one DNA molecule attached.
8) Tether beads to surface
   a) Slowly inject the bead-DNA solution into the microchamber and incubate 5 min.
   b) Gently rinse as above.

Table T1

	160 nm (radius) beads					
	λ buffer					
Tween (%)	-	-	-	-	-	0.5
glycerol (%)	0	20	30	50	70	0
DNA tether length (bp)	Numbers of beads					
336	33	15	19	16	11	56
590	37	22	14	19	6	51
817	16	8	8	9	9	58
1093	28	27	21	32	10	63
1394	26	22	17	9	7	28
1749	22	18	24	15	14	24
2103	9	13	12	17	10	24
2739	-	-	-	-	-	22
3015	-	-	-	-	-	31

Table T1. The number of 160 nm radii beads observed for 5 min each in Lambda buffer with or without glycerol and Tween

Table T2

	240 nm (radius) beads				
	λ buffer				
glycerol (%)	0	20	30	50	70
DNA tether length (bp)	Numbers of beads				
957	4	9	9	4	6
1460	16	13	12	10	9
2211	12	13	6	10	3
3477	10	7	4	8	7

Table T2. The number of 240 nm radii beads observed for 5 min each in Lambda buffer with or without glycerol.

Table T3

	240 nm (radius) beads			
	10 mM Tris			
KCl (mM)	10	50	100	200
DNA tether length (bp)	Numbers of beads			
711	17	3	3	4
1052	2	5	10	11
1267	5	8	8	10

Table T3. The number of 240 nm radii beads observed for 5 min each in 10 mM Tris buffer with various concentrations of added KCl.

Table T4

	240 nm (radius) beads			
	10 mM Tris with 10 mM KCl			
magnesium chloride (mM)	0	3	5	7
DNA tether length (bp)	Numbers of beads			
711	17	24	24	8

Table T4. The number of 240 nm radii beads observed for 5 min each in 10 mM Tris buffer + 10 mM KCl and various concentrations of added $MgCl_2$.

Table T5

	240 nm (radius) beads
	TR buffer
DNA tether length (bp)	Numbers of beads
225	15
590	8
1064	24
1555	20
1898	16
2974	19

Table T5. The number of 240 nm radii beads observed for 5 min each in TR buffer.

Table T6

Contour length (bp)		336	590	817	1093	1394	1749	2103	Stokes-Einstein
Glycerol percent (v/v)	Viscosity η (cP)	Diffusion coefficient D (x10^5 nm^2/s) R=160 nm							
0	1.006	4.57±0.26	5.89±0.29	6.01±0.60	7.21±0.65	7.32±0.47	8.91±0.66	9.55±0.60	13.33
20	2	2.43±0.41	3.24±0.14	3.44±0.42	3.90±0.27	4.20±0.22	4.97±0.33	4.72±0.33	6.71
30	3	1.66±0.15	2.27±0.12	2.50±0.08	2.71±0.12	3.12±0.18	3.35±0.16	3.33±0.10	4.47
50	8	0.71±0.04	0.84±0.07	0.87±0.06	0.97±0.08	1.01±0.06	1.24±0.06	1.13±0.08	1.68

Supplementary Table T6. Diffusion coefficients of tethered, 160 nm radii beads. Diffusion coefficients, D, for beads with 160 nm radii tethered by different DNA lengths and at different viscosities (glycerol percentages), were calculated using equation 3 and 4 from the main text. In each case, the right most column gives the free diffusion coefficient obtained using the Stokes-Einstein relation, $D = \frac{k_B T}{6\pi\eta R}$.

Table T7

Contour length (bp)		957	1460	2211	3477	Stokes-Einstein
Glycerol percent (v/v)	Viscosity η (cP)	Diffusion coefficient D (x10^5 nm^2/s) R=240 nm				
0	1.006	4.02±0.18	4.67±0.24	5.19±0.14	5.49±0.34	8.89
20	2	2.09±0.07	2.26±0.12	2.60±0.14	2.70±0.15	4.47
30	3	1.48±0.08	1.66±0.09	1.68±0.13	1.99±0.22	2.98
50	8	0.66±0.07	0.71±0.03	0.75±0.06	0.79±0.09	1.12

Supplementary Table T7. Diffusion coefficients of tethered, 240 nm radii beads. Diffusion coefficients, D, for beads with 240 nm radii tethered by different DNA lengths and at different viscosities (glycerol percentages), were calculated using equation 3 and 4 from the main text. In each case, the right most column gives the free diffusion coefficient obtained using the Stokes-Einstein relation, $D = \frac{k_B T}{6\pi\eta R}$.

Table T8

Glycerol percent (v/v)	0	20	30	50
Viscosity η (cP)	1.006	2	3	8
Contour length (bp)	Relaxation time, $\tau_0 = \frac{L_x^2}{\pi^2 D}$, in milliseconds (ms), R=160 nm			
336	20	37	54	127
590	27	49	70	190
817	32	55	76	219
1093	36	67	97	272
1394	42	73	98	302
1749	46	82	121	328
2103	50	101	143	422

Supplementary Table T8. Relaxation times for tethered, 160 nm radii beads in different viscosities. The decay time of the slowest mode, relaxation time, is given by $\tau_0 = \frac{L_x^2}{\pi^2 D}$ (Destainville and Salome 2006, Manghi, Tardin et al. 2010) where $D = D_x = {\sigma_x^2}/{2}$. L_x^2 and σ_x^2 were calculated using equations 3 and 4 from the main text. The relaxation times, τ_0, for beads of tethered by different DNA lengths and at different viscosities (glycerol percent), are given in milliseconds.

Table T9

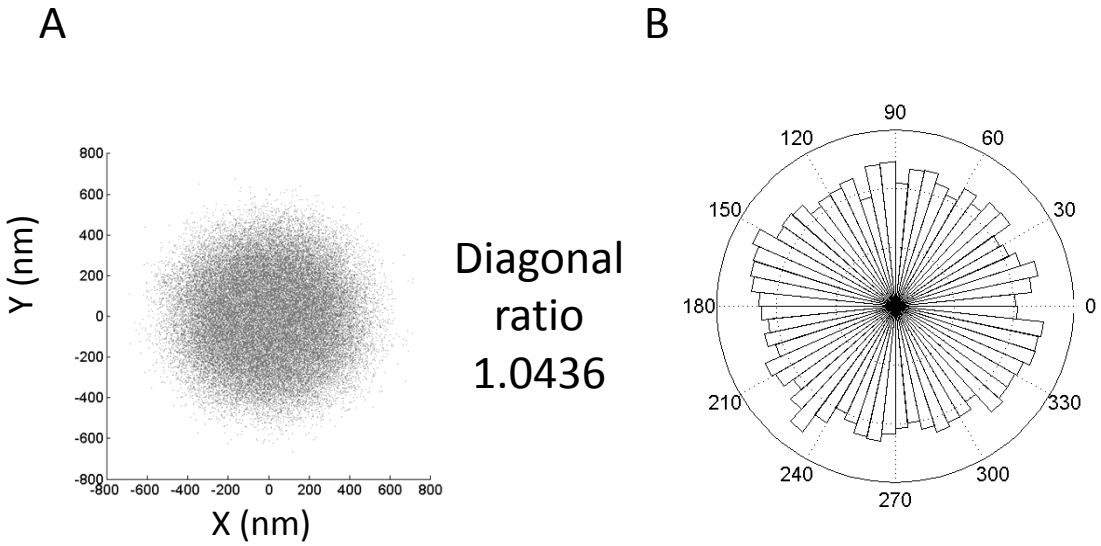
Glycerol percent (v/v)	0	20	30	50
Viscosity η (cP)	1.006	2	3	8
Contour length (bp)	Relaxation time, $\tau_0 = \frac{L_x^2}{\pi^2 D}$, in milliseconds (ms), R=240 nm			
957	70	85	112	153
1460	135	176	223	310
2211	190	239	344	422
3477	429	562	771	1058

Supplementary Table T9. Relaxation times for tethered, 240 nm radii beads in different viscosities. The decay time of the slowest mode, relaxation time, is given by $\tau_0 = \frac{L_x^2}{\pi^2 D}$ (Destainville and Salome 2006, Manghi, Tardin et al. 2010) where $D = D_x = {\sigma_x^2}/{2}$. L_x^2 and σ_x^2 were calculated using equations 3 and 4 from the main text. The relaxation times, τ_0, for beads tethered by different DNA lengths and at different viscosities (glycerol percent), are given in milliseconds.

Supplementary references

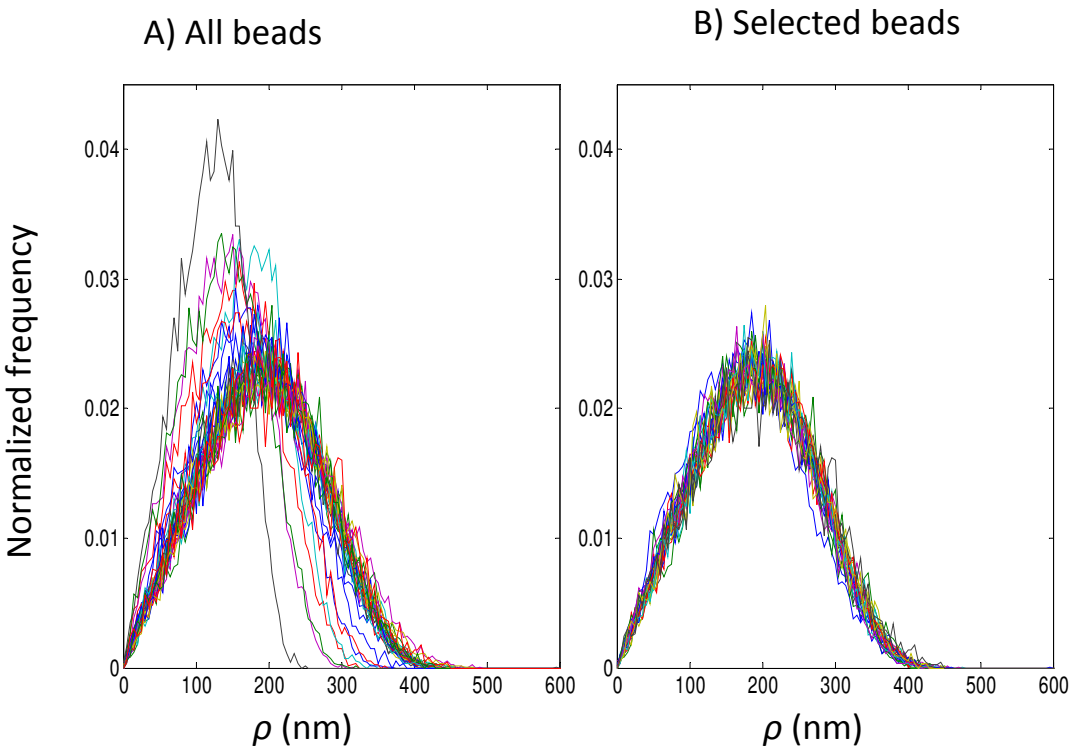
Destainville, N. and L. Salome (2006). "Quantification and correction of systematic errors due to detector time-averaging in single-molecule tracking experiments." Biophysical Journal **90**(2): L17-19.
Manghi, M., C. Tardin, J. Baglio, P. Rousseau, L. Salomé and N. Destainville (2010). "Probing DNA conformational changes with high temporal resolution by tethered particle motion." Phys Biol **7**: 046003.

Figure S1, Kumar *et al.* 2013



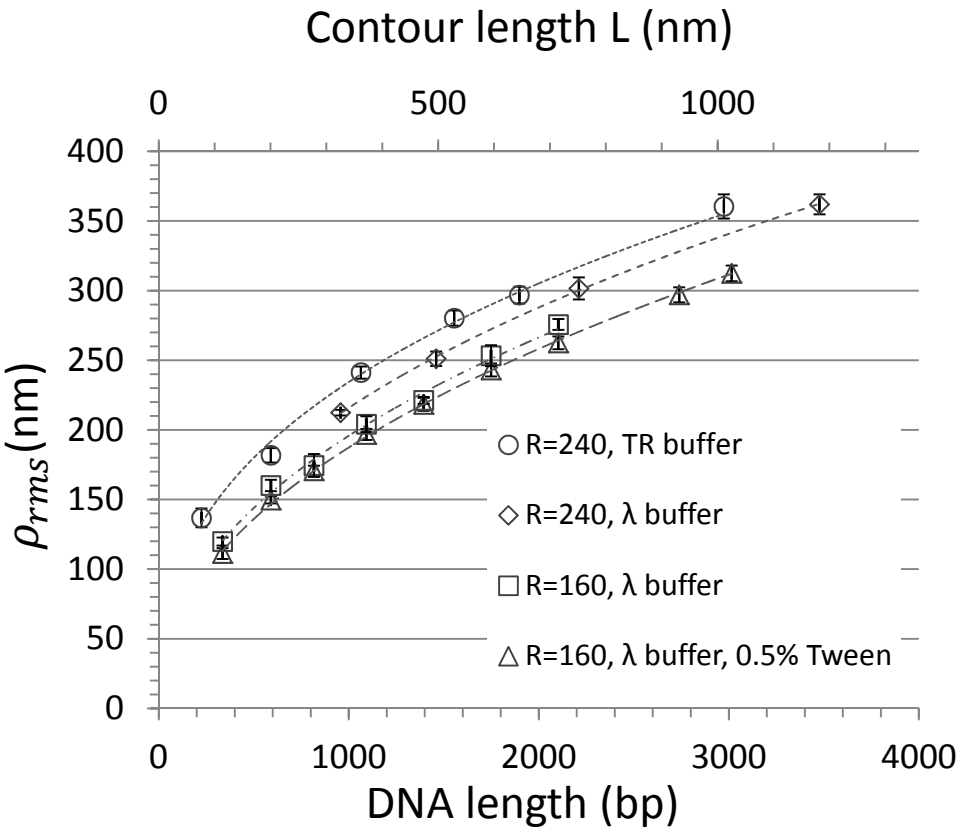
Supplementary Figure S1. Symmetry of bead motion. A) In 400 seconds a drift-corrected bead of 240 nm radius attached to a 2211 bp DNA tether was observed at these *xy* positions. B) A radial histogram of the angular coordinates for the same positions allowed judgment or whether or not the bead equally sampled all sectors (6 degrees) of the available hemisphere. The ratio of major to minor diagonals was 1.0436.

Figure S2, Kumar *et al.* 2013



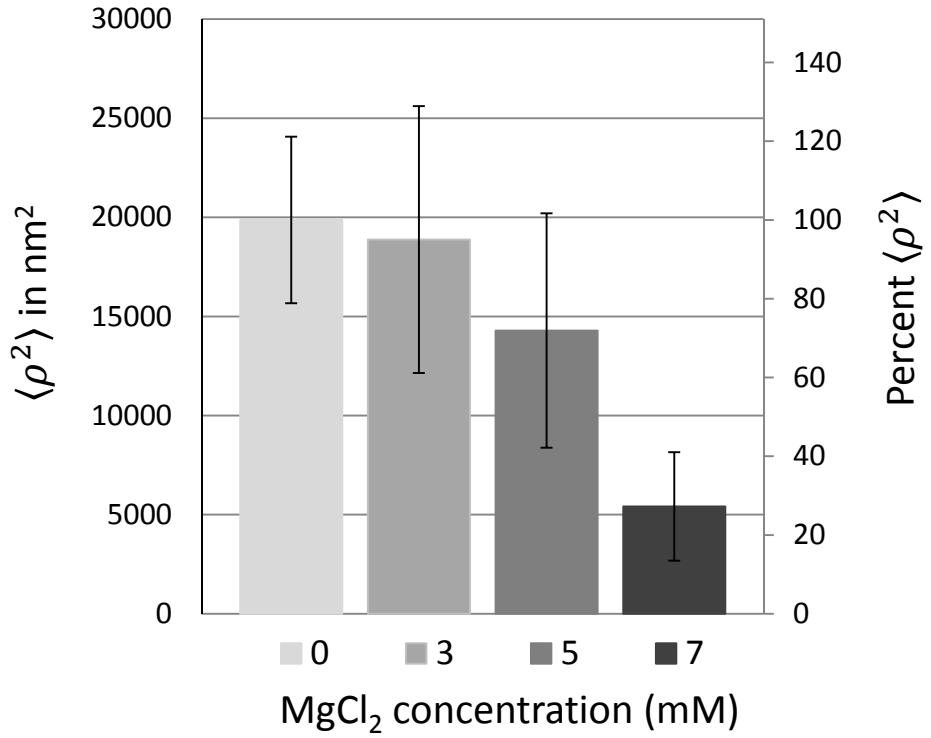
Supplementary Figure S2. Selection of beads based on ρ distributions. (A) Normalized frequency distributions of the observed excursions, $\rho = \sqrt{x^2 + y^2}$, in one field of view were plotted for beads with 160 nm radii attached to DNA tethers of 1093 bp. (B) Beads exhibiting similar excursions were selected by hierarchical clustering of frequency distributions using a centroid method.

Figure S3, Kumar *et al.* 2013



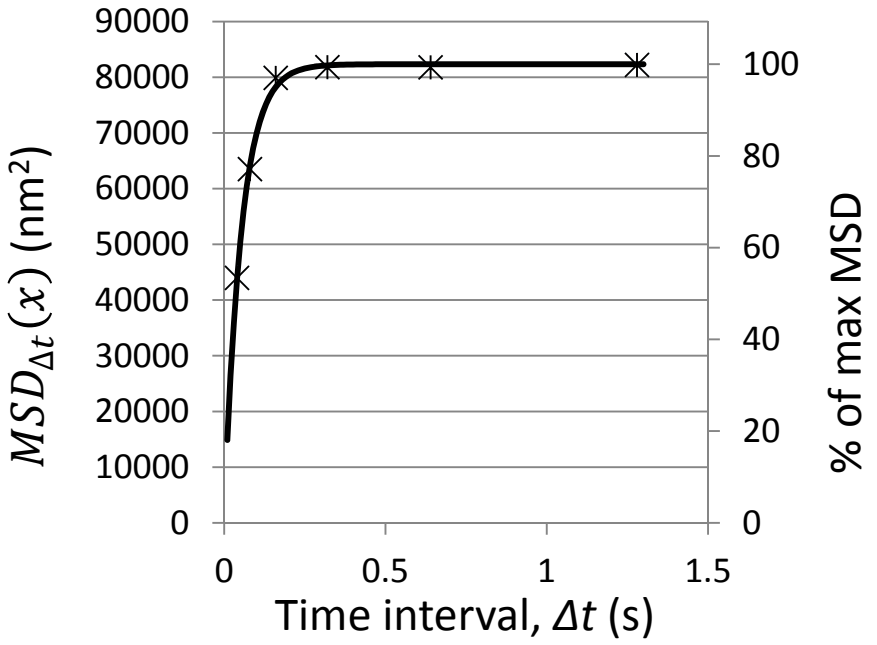
Supplementary Figure S3. Calibration of root-mean-square excursion versus DNA contour length. Excursions for two different sizes of tethered beads (radii of 160 and 240 nm) in different buffers were calculated using the formula $\sqrt{\langle \rho^2 \rangle_t} = \sqrt{\langle ((x - \langle x \rangle_t)^2 + (y - \langle y \rangle_t)^2) \rangle_t}$, with t = 20.48 seconds. The data are $\sqrt{\langle \rho^2 \rangle_{20.48s}}$ for 160 nm radii beads in λ buffer with added Tween (Δ), 160 nm radii beads in λ buffer (□), 240 nm radii beads in λ buffer (◊), or 240 nm radii beads in TR buffer (○). Connecting curves linking similarly sized beads in identical buffers are shown as guides. Error bars are standard deviations of the excursions for ensembles of identically assembled tethered beads.

Figure S4, Kumar *et al.* 2013



Supplementary Figure S4. Effect of magnesium chloride ($MgCl_2$) in a buffer with a low concentration of monovalent salt on mean-squared excursion. The average excursion was plotted for beads of 240 nm radii tethered by 711 bp DNA in low ionic strength λ buffer (only 10 mM KCl) supplemented with 0, 3, 5 or 7 mM $MgCl_2$. The mean-squared excursion of the beads decreased rapidly as the magnesium concentration was increased leading to a 70 % reduction at just 7 mM. Error bars are standard deviations of the excursions of ensembles of identically assembled tethered beads.

Figure S5, Kumar *et al.* 2013



Supplementary Figure S5. Mean-square displacement ($MSD_{\Delta t}(x)$) versus time interval (Δt). $MSD_{\Delta t}(x)$ was calculated for Δt = [0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12, 10.24, 20.48, 40.96, 81.92] using the formula

$$MSD = \langle r^2(n) \rangle_A = \frac{1}{N-n} \sum_{i=0}^{N-n-1} [\vec{r}(i+n) - \vec{r}(i)]^2$$

and fit with the equation

$$MSD_{\Delta t}(x) = \frac{L_x^2}{6} - \frac{16L_x^2}{\pi^4} \sum_{n=1(odd)}^{\infty} \frac{1}{n^4} exp\left\{-\frac{1}{2}\left(\frac{n\pi\sigma_x}{L_x}\right)^2 \Delta t\right\}$$

to determine the fitting parameters L_x and σ_x . L_x is a measure of the confinement and σ_x is proportional to the diffusion coefficient. A fit for a bead of 160 nm radius tethered to a DNA of length 2103 bp in a buffer with 0% glycerol is shown.