Supporting Information

Ultrasensitive On-Column Laser-Induced Fluorescence in Capillary Electrophoresis using Multiparameter Confocal Detection

Amir Mazouchi[1], Bryan J. Dodgson[2], David W. Wegman[2], Sergey N. Krylov[2*] and Claudiu C. Gradinaru[1*]

[1]Department of Physics, University of Toronto and Department of Chemical and Physical Sciences, University of Toronto Mississauga, Mississauga, Ontario L5L 1C6, Canada

[2]Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto, Ontario M3J 1P3, Canada.

Capillary alignment

The capillary was washed sequentially, for 10 minutes each time, using 95% methanol, 10 mM HCl, 10 mM NaOH and finally MQ water. It was then dried by passing air through it using a modified syringe pump compressed to 14 psi. The ECI was mounted on the 3-D piezo stage along the y-axis in the confocal microscope, with the capillary roughly aligned along the fast scanning (*x*-) axis, for imaging through the high NA objective. A flashlight was used to illuminate the ECI from the top and the transmission image of the capillary was projected on a white paper on the other side of the objective lens. This magnified image aided the placement of the capillary approximately in the centre of the scanning range of the *x*-axis. The axial (*z*-) position was adjusted to focus on the inner bore and sharpen the capillary image. Fine lateral and axial alignments of the ECI were done by maximizing the backscattering signal from the glass-

air interface. We used this method to find the bottom of the inner bore (**Figure S1**), with the edge and bandpass filters removed from the detection line to make the scattering more prominent.

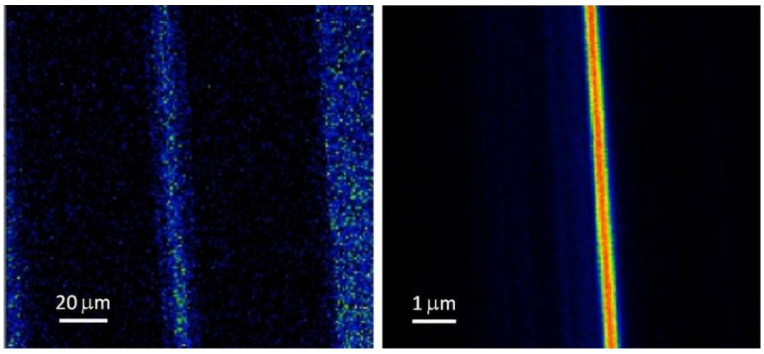

Figure S1 Backscattering from a dry capillary. Images were acquired by scanning the stage in the transverse plane (x and y). Left: focal plane in the centre of the capillary. The centre line is the capillary channel and the dark region is capillary wall. Right: Focal plane at air-glass interface from capillary inner bore. Because of the capillary curvature only along a narrow line, air-glass interface is in focus.

Focal volume – imaging beads

To investigate the cylindrical lensing effect of the curved capillary wall on the detection volume, polystyrene fluorescent beads were immobilized to the inner wall of the capillary as described in Material and Methods. The centre of the focal spot was moved to the glass-air interface by maximizing the backscattering. Immobilized beads were imaged by scanning the piezo in the *xy* plane (**Figure S2, upper right**) . One bead was selected and brought to focus laterally. A series of images were taken at different axial positions (**Figure S2, down**). Right on the interface the bead is in focus along the *x*-axis, while in the *y* direction it comes into focus almost 4 μm within the inner bore. At each focal point the bead was elongated nearly 4-6 times in the other direction. Upon filling the capillary with MQ water the astigmatism reduced considerably such that two separate focal points were no longer distinguishable. In this case, the bead shape was almost round (**Figure S2, upper left**) like one immobilized to a cover slip

under similar conditions, however the image of the ECI-immobilized bead is ~1.5 times larger, in both directions, than one from a bead immobilized to a thin (170 μm) coverslip.

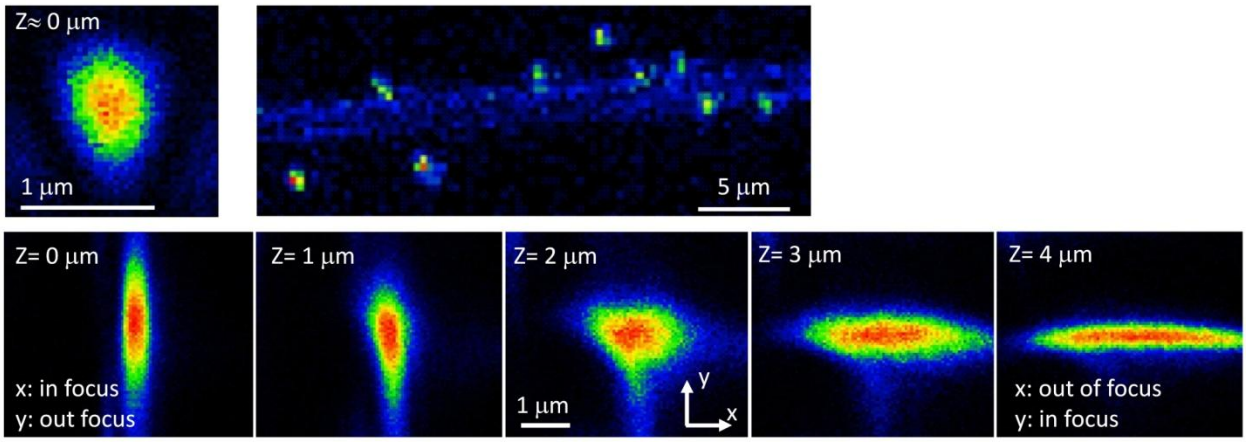

Figure S2. Upper right: An image of immobilized beads in a dry capillary (image is rotated 90 degree). The centre line is the backscattering from the capillary (long-pass and band-pass filters are removed). **Down**: Images of a fluorescent bead in a dry capillary at different axial positions of the focal point. Z is the distance between centre of the focal point and the glass-air interface. **Upper left**: An image of a fluorescent bead when the capillary is filled with MQ water.

Focal volume – FCS analysis

The intensity correlation function for free 3D Brownian diffusion is given by:

$$G_{3D}(\tau) = \frac{1}{N}\left(1+\frac{4D\tau}{r^2}\right)^{-1}\left(1+\frac{4D\tau}{r_z{}^2}\right)^{-\frac{1}{2}}\left(1+\frac{f}{1-f}exp\left(-\frac{\tau}{\tau_t}\right)\right)$$

where N is the number of molecules in the effective detection volume, D is the diffusion coefficient of the molecule, τ_t is the triplet lifetime, f is the average fraction of molecules in the triplet state and r and r_z are the respective lateral and axial radii ($1/e^2$) of the detection ellipsoid.

In case the diffusion is restricted in axial direction the correlation function takes the form of

$$G_{2D}(\tau) = G(0)\left(1+\frac{4D\tau}{r^2}\right)^{-1}\left(1+\frac{f}{1-f}exp\left(-\frac{\tau}{\tau_t}\right)\right).$$

Fluorescence correlation spectroscopy (FCS) analysis of 10 nM Rhodamine dyes within a capillary was performed to estimate the geometry parameters of the detection volume. The resulting FCS curve was best fitted with a 2D diffusion model (**Figure 3**), implying that the detection volume spans across most of the inner capillary bore. Thus the axial size of the detection volume is estimated to be around10 μm. In addition, the lateral radius of $r \sim 430$ nm was found from the fitting of the correlation curve using the 2D diffusion model. This represents a ~1.3 times expansion of the detection volume in the transverse directions compared to the same measurement on coverslips and it leads to a molecular counting efficiency of ~13% in our ECI. The calculated detection volume is ~5.8 fL , which is ca. 3.3 times larger compared to that found by the same experimental procedure on a coverslip.

Laser excitation power

The effect of the laser excitation power on the S/N was studied by injection of long plugs of 100 pM fluorescein (peak width >10s) into the capillary by negative pressure. All photons were detected in a single SPAD and the intensity time trajectory was binned with 600 ms binning time. The optimum excitation power of ~200 μW (measured after the objective lens) was found by varying the laser power and measuring the resulting S/N from the sample **(Figure S3**). Assuming this laser power is uniformly distributed over the focal point with r = 0.43 μm found from FCS analysis, a focal plane intensity of 35 kW/cm^2 was estimated for optimal CE-CMPF experiments on our setup.

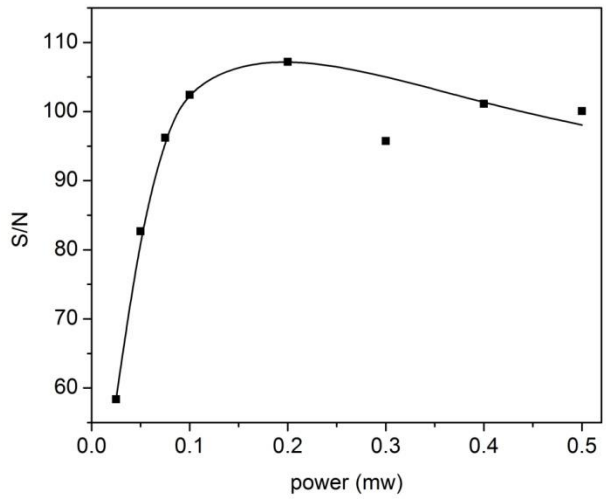

Figure S3 S/N versus the excitation power measured after the objective lens

Single-molecule bursts in the capillary

A capillary was filled by electrophoresis buffer (tetraborate, pH 9.3). Single-molecule bursts were recorded from a short plug of 100 pM fluorescein propagated through the capillary by pressure (**Figure S4a**). A 200-μs binning time was applied to the fluorescence intensity trajectory. This binning time resembles the diffusion time of a free dye through the detection volume found from FCS (107 μs). In order to determine the threshold level for the burst recognition, a 0.5 s data slice was analyzed in an interval without a clear peak (**Figure S4b)**. Three standard deviations above the average number of photons/bin resulted in the threshold level of 4 photons/bin, which was used in subsequent analysis. The 0.5 s time trajectory from tetraborate buffer resulted in 13 bursts (**Figure S4b)**.

In the centre of the plug (at 169 s in the full trajectory) the bursts are not distinguishable due to the high concentration (**Figure S4d)**. In fact, based on the capillary and detection region size we estimate to have

an average of 1 molecule in the detection volume at a concentration of ~300 pM. Far enough off the centre of the plug the concentration reduces due to the broadening of the plug during propagation through capillary and therefore burst analysis becomes feasible. **Figure S4c** shows a 0.5 s slice of the intensity-time trajectory at 167 s. The average concentration in this slice is ~5× lower than the concentration in the center of the plug, i.e., ~ 20 pM, and it contains a total of 117 bursts. An average S/N = 4.5 was calculated for these bursts. Here signal (*S*) is calculated by subtracting the average number of photons/bin found from the buffer slice (**Figure S4b)** from the average number of photons in these bursts. Noise (*N*) is taken as the standard deviation of the buffer slice. This low S/N ratio can be explained by the 6-fold reduction in the fluorescence intensity of the bursts in ECI compared to the sub-micrometer capillaries due to excitation beam expansion and reduction of the photon collection efficiency, as discussed in the main text. In addition, regular capillaries are prone to higher background fluorescence and backscattering because of curvature of capillaries, index mismatch, extended optical path in the glass and a larger detection volume.

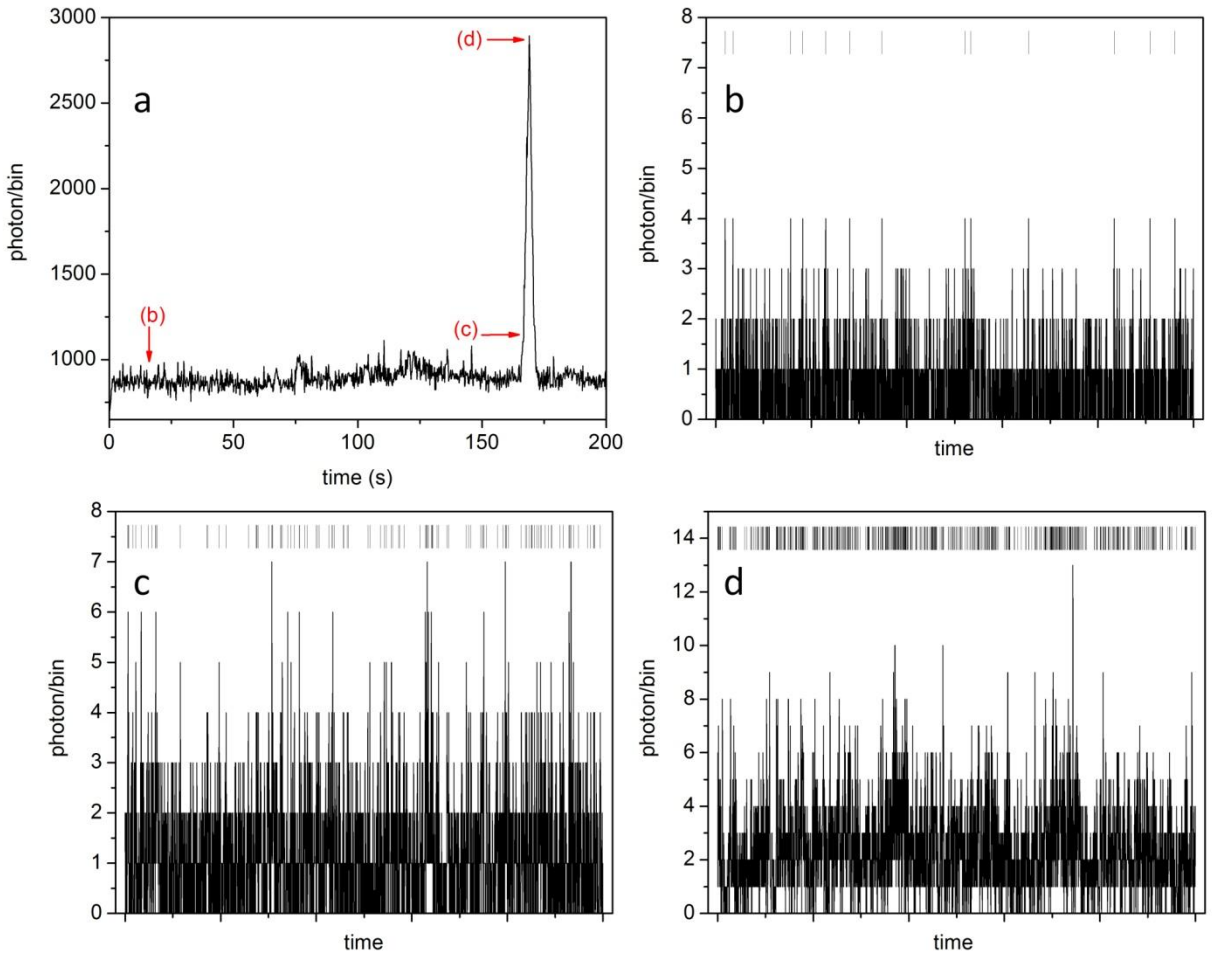

Figure S4 100 pM of fluorescein propagated through the capillary by pressure. In a) the full passage of the plug is shown, in b), c), d) 0.5 intervals at t = 16, 167, 169 s are shown, respectively.