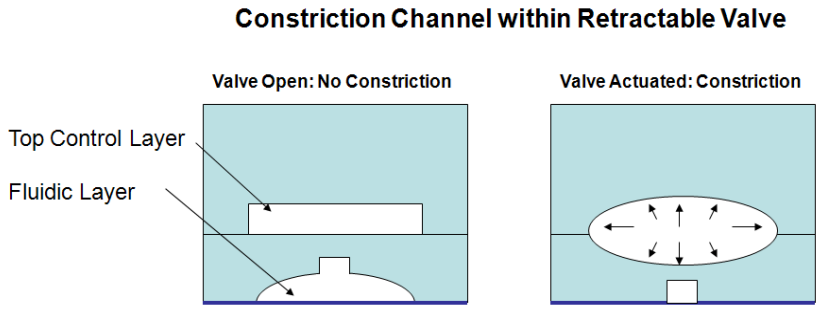
SUPPLEMENTAL DATA

S.1 Device Fabrication and Operation

As discussed in the text, all devices were fabricated using multilayer soft lithography. Two layers of polydimethylsiloxane (PDMS) were constructed, one for droplet generation and transport and another for the pneumatic valve. The mold for the device was made using three separate photoresist layers. First, a 10 μm tall pad of SPR220-7.0 (Shipley) was used to create the portion of the valve that would close tight against the underlying glass coverslip. After development, a reflow process at 200 °C for 120 minutes resulted in the semi-circular cross-section necessary for effective valve closure. SU-8 was then used to pattern a small constriction channel (20 μm wide with varying heights of 2.5 – 20 μm) on top of SPR220-7.0 pad that would remain open while the valve is in the actuated state. Finally, the 35 μm tall layer containing the droplet generator module was patterned to connect the constriction to the rest of the device. A separate mold for the pneumatic valve (control) layer was made at a 40 μm height, again using SU-8 3025 photoresist (See Sup. Fig. 1 for a cross-section of the constriction within the retractable valve).



Sup Fig. 1: A cross-section of the constriction within the retractable valve. Upon pressurization of the top control layer, the actuated valve caused the main portion of the channel to close while the smaller constriction channel was left as the only open path for droplet travel.

PDMS prepolymer (SYLGARD® 184, Dow Corning) was prepared at 1:7 mixing ratio and cast onto the control layer mold to result in a final thickness of ~ 3 mm upon curing at 80 °C for 10 minutes. The fluidic layer molds were silanized using vapor deposition of chlorotrimethylsilane (Sigma-Aldrich) before spin coating with PDMS prepolymer at a mixing ratio of 1:15 (1300 rpm for 60 seconds). The excess curing agent in the thick control layer (1:10 is the standard mixing ratio) was added to ensure fusion with the second PDMS layer. The thin PDMS layer was then partially cured at 80 °C for 5 minutes before the thick PDMS sheet containing the mechanical valve was peeled off its mold and aligned to the fluidic layer. The two layers were fused together upon curing at 80 °C for 30 minutes. During these steps holes for fluidic and valve access were punched using gauge 20 syringe needles (McMaster-Carr). Finally, the entire chip was bonded to a coverglass (Fisherfinest, 12-548-5R, 35x50-1) after activation of the PDMS surface via plasma oxidation. Samples were delivered to the

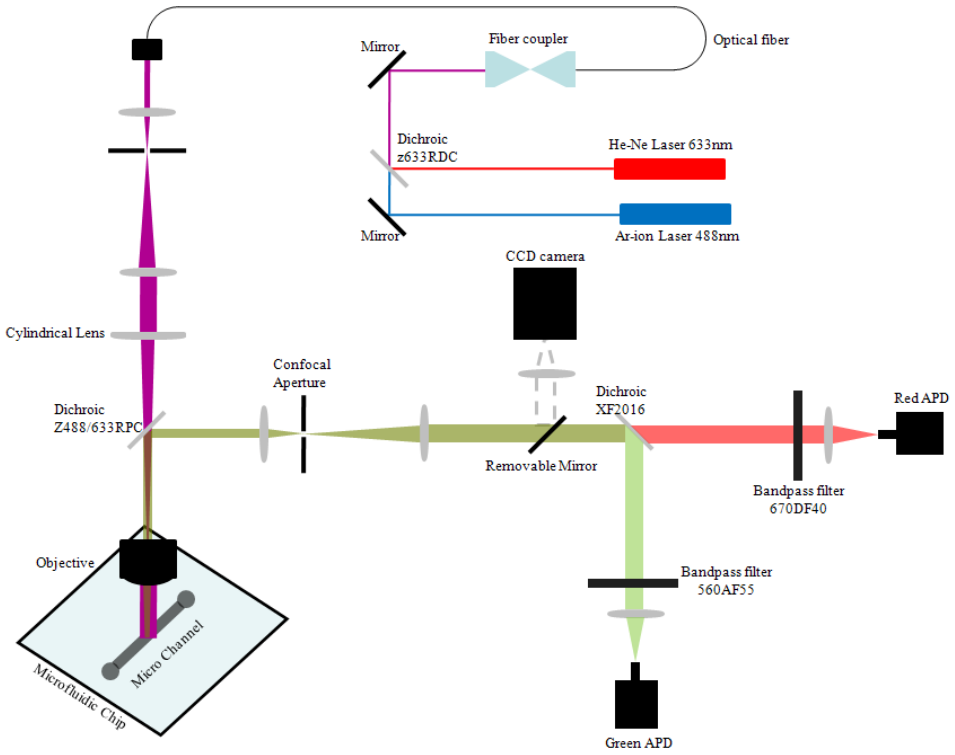
microfluidic chip using 0.02 inch I.D. Tygon tubing (Cole-Parmer) fitted with 23-gauge needle tips (McMaster-Carr).

A mixture of Mineral oil (20% v/v), Tegosoft DEC (73% v/v), and ABIL WE09 (7% w/v)[S1] was used as the continuous phase for droplet generation. Upon generation drops were carried to a large volume (~2 uL) storage channel, where droplets could be collected for downstream analysis. During on-chip single molecule detection (SMD), the continuous phase was used to force drops through the constriction at a controlled speed. The discrete phase always consisted of high concentration of Alexa 488 dye, which served as an indicator dye to monitor droplet edges on the fluorescence spectroscopy platform. The signal from the indicator dye also provided feedback to control the speeds at which droplets traversed the constriction.

S.2 Experimental Setup for Single Molecule Detection

Spectroscopy data was acquired using a custom-built, dual laser excitation, dual emission channel, single molecule spectroscopy platform, as described previously (Sup. Fig. 2).[S2] The system was capable of Cylindrical Illumination Confocal Spectroscopy (CICS) with 488 nm (Melles Griot) and 633 nm (Thorlabs) laser illumination and detection in narrow wavelength bands centered on 560 nm and 670 nm (Sup Fig. 2). The two laser beams used in the setup were spatially aligned using beam steering mounts (Newport) and combined using a dichroic mirror (z633RDC, Chroma Technology). The joint laser beam was coupled with an optical fiber to isolate the laser optics from rest of the setup. The beam emerging from the optical fiber is collimated and expanded before hitting a cylindrical lens (f = 200 mm), which was used to shape it in the form of a sheet and focus it into the back focal plane of a microscope objective (100X, oil-immersion, 1.3 NA UPlanFl, Olympus). The objective tightly focused the beam into the microfluidic device, and collected emitted fluorescence in an epi-fluorescence configuration. The collected fluorescence signal was spectrally separated from the excitation light using a second dichroic mirror (z488/633RPC, Chroma Technology). Upon passing through a confocal aperture, the collected fluorescence was separated into two emission bands by a third dichroic mirror (XF2016, Omega), passed through bandpass filters (560AF55 and 670DF40, Omega Optical), and finally imaged on Silicon Avalanche Photodiodes (SPCM-AQR-13 and SPCM-CD2801, PerkinElmer Optoelectronics) using f = 30 mm doublet lenses (Thorlabs). The slit-like confocal aperture used in this configuration measured 750 μm by 50 μm. The actual size of the CICS illumination volume for the 633 nm laser in this configuration was estimated using the experimental data shown in Sup Fig. 3. The 633 nm laser beam was focused on a reflective silicon wafer surface and the reflected laser beam was imaged on a CCD camera (Allied Vision Technologies GmbH), after passing through the confocal aperture. The intensity profile of the image thus obtained was analyzed to estimate the width of the illumination volume (20.13 μm). This was followed by a z-scan to estimate the size of the illumination volume along the axis of the laser beam. During the z-scan, the reflective silicon wafer piece was moved along the axis of the laser beam, with respect to the laser beam focus and the reflected laser beam intensity was collected using the APD corresponding to the 670DF40 bandpass filter (Red APD).

The variation of the reflected laser intensity with changing position of the reflective surface respective to the laser beam focus is shown in the right panel in Sup Fig. 3. A Lorentzian function was fit to the data and $1/e^2$ radius of the illumination volume was estimated to be 3.2 µm. Hence, the overall cross section of the illumination volume corresponding to the 633 nm laser was found to be ~64.6 μm^2. The CICS setup in this configuration was used for the Lambda DNA experiments.



Sup Fig. 2: Schematic diagram of the optical components in our two color Cylindrical Illumination Confocal Spectroscopy (CICS) system.

In a second configuration for single fluorophore experiments, a separate cylindrical lens (f = 300 mm, Thorlabs) was used to shape the laser beam into a sheet and to focus it into the back focal plane of the objective (same as above). An aperture of size 600 µm by 150 µm was used in this configuration to obtain a smaller observation volume. The size of the actual illumination volume was estimated as described above and was found to be ~14.3 μm^2 in cross section. The single molecule burst data was collected from the APDs by a PC using a PCI 6602 counter DAQ card (National Instruments), controlled using a custom software written in LabView (National Instruments). The microfluidic devices were positioned using a combination of a piezo stage (BPC203, Thorlabs) and a manual XYZ stage (Newport), allowing focusing of the CICS illumination volume at desired positions on the devices.

S.3 Sample Preparation

For the Lambda DNA experiments, Lambda DNA (37 pM; Invitrogen) was stained with TOTO®-3 dye (Invitrogen) at a concentration of 1 µM for at least 30 minutes before droplet generation. The indicator

dye Alexa 488 was then added to the stained DNA and the sample was diluted to achieve the concentration of DNA required for a particular experiment. Final concentration of indicator dye in the discrete phase was kept to a constant of 100 nM in all the experiments with Lambda DNA.

The single fluorophore experiments were conducted using a DNA molecular beacon (MB; 5'-Cy5-CATCCGCTGCCTCCCGTAGGAGTG- BHQ2-3'; Integrated DNA Technologies) with the probe sequence complementary to a conserved region of the 16S rRNA in a wide-range of bacteria.[S3] Complementary DNA oligonucleotides were hybridized in 10 mM phosphate buffer (pH 7.8) and 900 mM NaCl at 100X of the MB concentration to saturate available probe. The MB-target mixture was heated to 95 °C and then gradually cooled to room temperature for 1 hour to ensure adequate hybridization, prior to loading into the microdevice for droplet generation. The final concentration of molecular beacons in the discrete phase was 40 pM, both in the 'MB only control' and the 'MB hybridized with target' experiment. The concentration of the indicator dye Alexa 488 in the discrete phase was kept to a constant of 10 nM in each case.

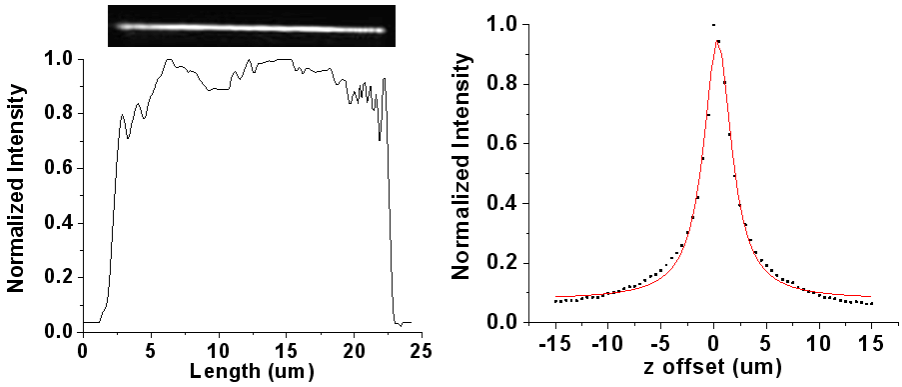
S.4 Single Molecule Data Analysis

An image of the droplets generated on each chip was taken prior to obtaining single molecule burst data using a custom stereoscope interface for the single molecule spectroscopy platform (Nikon SMZ1000). The average droplet size was estimated from a minimum of ten droplets for each particular experiment. This data allowed the droplet counts per droplet to be normalized to an average droplet size (44.5 pL for all experiments), so that slight experimental variation in droplet size did not effect overall results.

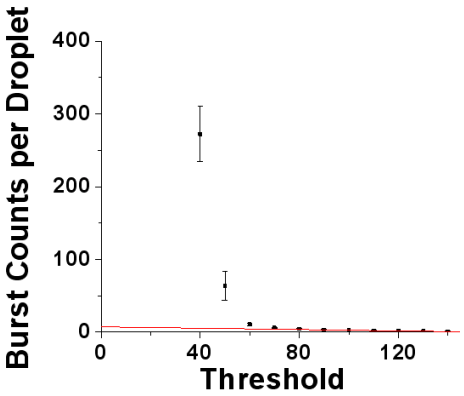
Data analysis was performed using custom software written in MatLab. Briefly, the leading and falling edge of each droplet were identified as the location in a data trace where the signal from the Alexa488 indicator dye reached three standard deviations above the background fluorescence of the continuous phase. A size filter was used to remove small satellite droplets from the analysis, while single molecule data from the Red APD (TOTO-3/Cy5 channel) was analyzed at each droplet location, as identified by the indicator dye signal (from the Green APD). Integration time for photon binning was set at 0.1 ms for all peak counting experiments.

For each sample, three data traces were analyzed with each trace containing at least 10 droplets visible in the form of fluorescence signal from indicator dye. For each droplet analyzed, the number of fluorescent peaks from the Red channel was normalized with the average droplet volume estimated from the picture, as described earlier. The number of peaks per unit volume, thus obtained, was converted to number of peaks per drop of a standard size (44.5 pl). The peak counting algorithm introduced by Huang et. al[S4] was adopted to minimize thresholding artifacts. Traditionally, in single molecule counting experiments threshold values for fluorescent burst detection are set around three standard deviations from the mean background signal. Setting a lower threshold for detection in discrete volume droplets would result in lower false negatives, but cause a subsequent increase in false positive fluorescent bursts from background noise. However, as shown in Sup. Fig. 4 the same data

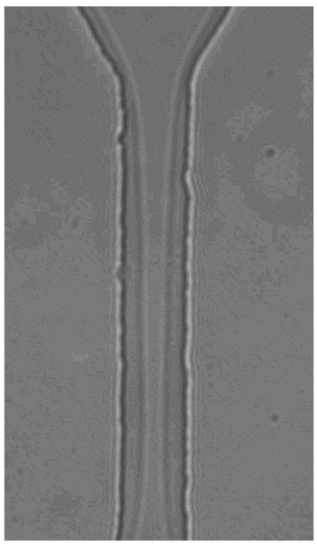
trace can be analyzed with different thresholds to obtain a linear trend (in which there is no interference from background noise) that can be interpolated to the zero threshold to estimate the true molecule count per droplet.[S4] In this manner, we were able to obtain molecular counts per droplet that closely matched the expected value, as determined by the maximum possible detection efficiencies (supplemental sections S1 and S2) and concentrations (supplemental section S3). Acquisition of single molecule data from the droplets with the laser probe focused through the thin layer of the continuous, oil phase (see Sup fig 5) was observed to cause a slight decrease in signal-to-noise ratio compared to that obtained for data acquisition from a fluid filled microfluidic channel (data not shown). However, this was not shown to have any effect on the estimation of intra-droplet molecular concentrations (figure 3 B and C).



Sup Fig 3: Estimation of the size of the CICS illumination volume. **Left panel:** The reflected image of the CICS illumination volume and the intensity profile of the image are shown. This image was acquired using the 633 nm laser on the CICS setup focused on a reflective silicon wafer surface and imaged with a CCD camera, after passing through the confocal aperture. The width of the illumination volume was measured to be 20.13 μm**. Right panel:** The reflected intensity data from a z-scan conducted to estimate the size of the CICS illumination volume along the axis of the laser beam is plotted. The X axis shows the location of the laser beam focus relative to the surface of the silicon wafer, while the Y axis shows the normalized reflected intensity of the laser beam collected using an APD.



Sup Fig 4: A simple linear interpolation using the fluorescence burst counts with the threshold between 90 and 120 gives a molecule count of 7.38 ± 0.56, which is close to the expected number of 9.92 molecules per droplet (0.37 pM Toto-labeled lambda DNA within 44.5 pL droplets; 50 μm^2 constriction within the 64.6 μm^2 illumination volume for a maximum possible detection efficiency of 100%). This number corresponds to the 50 μm^2 data point in figure 3C.



Sup Fig 5: A high magnification image of a droplet squeezing through a 20 μm by 10 μm constriction. This lateral view clearly shows the continued presence of the continuous phase surrounding the droplet as it is pushed through the constriction.

References for Supplemental Material

S1 Frank Diehl, Meng Li, Yiping He, Kenneth W Kinzler, Bert Vogelstein & Devin Dressman, Nature Methods 3, 551 - 559 (2006) doi:10.1038/nmeth898

S2 K. J. Liu and T. H. Wang, Biophys. J., 2008, 95, 2964-2975 (DOI:10.1529/biophysj.108.132472).

S3 C. M. Puleo and T. H. Wang, Lab. Chip, 2009, 9, 1065-1072 (DOI:10.1039/b819605b).

S4 Bo Huang, Hongkai Wu, Devaki Bhaya, Arthur Grossman, Sebastien Granier, Brian K. Kobilka, Richard N. Zare, Science 5 January 2007: Vol. 315. no. 5808, pp. 81 – 84