SUPLEMENTAL DATA 1 (S1) : Addendum to Experimental Methods

S.1.A Experimental Setup for Single Particle Detection (Figure 1A)

A 488 nm argon laser (Melles Griot) was used as the excitation source and the laser beam was focused using a 100X, 1.3 N.A., oil immersion apochromatic objective (Olympus). Throughout experiments excitation laser power was kept at 150 μW using neutral density filters. The emitted fluorescence was then collected by the same objective and an initial dichroic mirror (505DCXE, Chroma Technology) selected wavelengths > 505 nm. A 50 μm pinhole (Melles Griot) rejected out of focus fluorescence and a second dichroic mirror (D625LP, Chroma Technology) separated donor (QD605) and acceptor (Cy5) emission. Two avalanche photodiodes (APD, EG&G) detected signal from the two channels, which were filtered with separate bandpass filters (D605/20 and 660DF50, Chroma Technology). Acquisition software, written in Labview, and a digital counter (National Instruments) were used to collect data from the APDs.

S.1.B Device Fabrication and Operation (Figure 1A)

As discussed in the text, all devices were fabricated using multilayer soft lithography.[S1] Two layers of polydimethylsiloxane (PDMS) were constructed, one for sample delivery and another for pneumatic valves and pumps. For the sample delivery (fluidic) layer, SJR5740 (Shipley) was used as the mold material and patterned on a 4" silicon wafer using standard photolithography. After development, a reflow process at 200°C for 90 minutes resulted in a fluidic mold with a semi-circular cross-section (~ 14 μm max height). The valve layer mold was created using SU-8 2025 (MicroChem) in a similar fashion to make 20 μm features. PDMS prepolymer (Sylgard 184, Dow Corning) was prepared at 1:7 mixing ratio and cast onto the valve master mold to result in a final thickness of ~ 3 mm upon curing at 80°C for 9 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 (2000 rpm for 60 seconds). The thin PDMS layer was then partially cured at 80°C for 5 minutes before the thick PDMS sheet containing the mechanical valves was peeled off its mold and aligned to the fluidic layer. The two layers were fused together upon curing at 80°C for 25 minutes. During these steps holes for fluidic and valve access were punched using syringe needles (McMaster-Carr). Finally, the entire chip was bonded to a 170 μm thick glass slide 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 steel needle tips. The rotary chamber dimensions were 1 mm loop diameter, 14 μm depth, and 100 μm channel width, while the valve controls intersecting the fluidic channels had cross-sectional dimensions of 100 μm width by 50 μm depth. Loading needle tips with < 1 μL of sample enabled filling of multiple SMD-rotary pump elements, each allowing a separate measurement. The on-off valves in the device were controlled from separate compressed air sources (Air Gas) using an array of solenoid valves (Asco) actuated using a mechanical switchboard (Agilent). Valve control sequences and rotary pump frequencies were programmed using a Visual Basic (Microsoft) interface for the switchboard.

S.1.C QD-FRET – Hybridization Reaction (Supplemental Figure S1)

The hybridization experiments were performed using synthetic DNA targets that mathced a 32-nucleotide segment of the human β-globin gene in a buffered solution containing 5 mM Tris-HCl, pH 8.0. Breifly, two hybridization probes captured the DNA target, an initial biotin conjugated capture probe to couple the probe-target hybrid to streptavidin conjugated QDs (QD605, Invitrogen) and a second Cy5-labeled reporter probe (Supplemental Figure S1).[S2] This reporter probe acts as an energy acceptor, resulting in fluorescence emission by FRET, upon illumination of the fully conjugated QD-DNA nanoassemblies. Probe-target hybridization was carried out by mixing the biotinylated capture probe (Biotin-GTG CAC CTG ACT CCT G), Cy5-labeled reporter probe (AGG AGA AGT CTG CCG T-Cy5), and target DNA (ACG GCA GAC TTC TCC TCA GGA GTC AGG TGC AC; IDT DNA) at 90°C for one minute, followed by passive cooling to room temperature (RT). The molecular ratio of the probes was kept at 1:1, and after cooling to RT the probe-target hybrids (hybrids) were frozen and re-thawed. Streptavidin-functionalized QD (Invitrogen) probe-target capture was conducted in a 25 mM NaCl solution at 1 μM hybrid concentration, while the QD:DNA ratio was fixed at 1:30. Upon dilution for single particle experiments, QD-nanoassemblies were kept in 1 mM NaCl solutions. All tubes used for mixing throughout the reactions were coated with Bovine Serum Albumin (BSA) to minimize loss of the QD-nanoassemblies prior to confocal fluorescence detection.

SUPPLEMENTAL DATA 2 (S2): Velocity Calibration and Optical Probe Characterization

S.2.A Single Particle, In Situ Velocity Calibration

QD-FRET samples were injected into a glass microcapillary (ID 50 μm) using a gas tight 1 mL syringe and a high-precision syringe pump (Harvard Apparatus). A nanometer resolution translation stage (Physik Instrumente) was used to control the position of the optical probe volume for single particle fluorescence measurements. Briefly, an autocollimator was used to focus the laser beam at the water-glass interface giving a reference point for positioning the

optical probe volume. The lateral position of the microcapillary was held fixed by mounting the capillary on a glass slide. The slide could then be loaded into a port fabricated in the translation stage and held perpendicular to the objective mount. The optical probe volume was then set at the midpoint of the capillary by translation 25 μm away from the water-glass interface at the side surface. The 7 μm depth for all experimets could then be set utilizing the top surface as a second reference point. The duration of the fluorescence bursts recorded at this position represent the transit time of particles passing the optical probe region, which is altered under different flow conditions. Increased flow velocity resulted in an exponentially decreasing transit time, enabling establishment of the calibration curve in figure 2A. In situ velocity was determined using the analytical expression for Poiseuille flow in a circular channel,

Equation S.2.1: $$v_x = \frac{2Q}{\pi a^4}(a^2 - y^2 - z^2)$$

where Q is the volumetric flow rate, a is the radius of the circular channel, and y and z represent the radial position of the optical probe. All velocity measurements and calibrations were performed at QD-nanoassembly concentrations of 20 pM. Establishment of the calibration curve enabled measurement of the in situ flow velocity at defined rotary pump rates (Figure 2B) using the exponential curve fit for figure 2A:

Equation S.2.2: $$y = (0.351) + (0.472)e^{-x/2.28}$$

$$R^2 = 0.986$$

S.2.B Optical Probe Volume Dimensions (Fluorescence Correlation Spectroscopy, FCS)

FCS measurements were carried out with the same custom-built, confocal fluorescence spectroscope described above; however, a third channel was used to measure fluorescence from free Oregon green dye using the 488 nm, laser excitation probe (dichroic – 565DCXR (Chroma), bandpass – 520DF40 (Omega Optical)). The output electronic signal was fed into a correlator (ALV-5000/EPP, ALV-GmbH) for computing autocorrelation functions. Analysis of the autocorrelation curve was carried out with a least squares fit based on the Levenberg-Marquardt algorithm within Origin 7.0 (OriginLab) and the following analytical model,[S3]

Equation S.2.3: $$G(\tau) = \frac{1}{N(1+\frac{4D\tau}{\varpi_1^2})(1+\frac{4D\tau}{\varpi_2^2})^{1/2}}$$

where N is the average number of fluorophores diffusing in the optical probe volume, τ is lag time, D is the diffusion coefficient of the fluorescent molecule, and G is the autocorrelation function arising from fluorescence fluctuations due to translational diffusion. The half radii of the detection volume (ω_1 and ω_2) could then be approximated by fitting the autocorrelation function, using the 3D-guassian fit and the diffusion coefficient for Oregon green in water. Upon determination of the half radii in equation S.2.3 the cross-sectional area of the optical detection volume was estimated at 6.37μm^2 in the direction of flow.

SUPLEMENTAL DATA 3 (S3): Methods Applied to Fluorescence Burst Analysis

S.3.A Fluorescence Burst Counting

The background threshold for fluorescence burst analysis was determined using fluorescence data taken from no QD (QD channel) and no targets control samples (Cy5/FRET channel). The threshold values were determined using the equation:

Equation S.3.1: $$I_{Thr} = \mu + 3(\mu)^{0.5}$$

where μ is a single parameter related to the mean and variance of the Poisson distribution of fluorescence signal from control samples, yielding I_{Thr} values of 25 for the QD channel and 10 for Cy5/FRET channel. Burst count data within

the text is given as number of counts above background, which is defined as the number of burst counts from a negative control (no target) QD-FRET assay run in parallel with that experiment.

S.3.B Expected Burst Frequency and Measurement Efficiency

The instataneous probability of n particles residing (r) in the optical probe volume (VE ~ 1 fL) follows Poisson statistics:[S4, S5]

Equation S.3.2: $$P_n^r(N) = e^{-N}\frac{N^n}{n!}$$

where the parameter is the relative concentration of particles (N = molecules / VE). In SMD, fluorescence is monitored from the VE over short time intervals, thus the probability for particle occupancy or fluorescence burst occurrences is altered due to the probability of n molecules entering (e) the VE within that interval (t):[S5]

Equation S.3.3: $$P_n^e(NT) = e^{-NT}\frac{(NT)^n}{n!}$$

where the parameter NT is the relative concentration (N) scaled by the relative measuring time (T = measurement time/characteristic particle transit time through VE). To obtain the n-particle probability within time interval t, the two probability distributions are convoluted:[S5]

Equation S.3.4: $$P_n = \sum_{i=0}^{n} P_i^r(N)P_{n-i}^e(NT).$$

Yielding a single-particle event probability of:

Equation S.3.5: $$P_{n=1} = N(1+T)e^{-N(1+T)},$$

and a multiple-particle event probability:

Equation S.3.6: $$P_{n>1} = 1 - e^{-N(1+T)}[1+N(1+T)]$$

Thus, the probability of single-particle events within the SMD-microdevice operated at a pump rate of 100 Hz (~0.67 ms transit time), quantum dot concentration of 5 pM, and 1 ms integration time is $P_{n=1} = 0.00742$, while the multiple-particle probability ($P_{n>1}$ ~0.00003) is relatively low and can be neglected in the following calculations.

After neglecting multiple occupancy events, the fluorescence burst rate (B_R) in traditional, continuous flow SMD platforms is proportional to both the volume flow rate (Q_F – m^3/s) through the flow cell and particle concentration (C – molecules / m^3).[S6]

Equation S.3.7: $$B_R \cong \varepsilon_M\, C\, Q_F$$

However, there exists an instrument dependent proportionality constant (ε_M), referred to as the measurement efficiency, to compensate for fluorescent particles that enter the SMD flow cell, yet do not yield a fluorescence burst. This measurement efficiency is defined as the probability that a given particle within the analyzed sample was detected as a fluorescence burst above background noise and is given by the equation:[S7]

Equation S.3.8: $$\varepsilon_M = \varepsilon_t\varepsilon_s\varepsilon_T\varepsilon_D$$

where ε_D is the detection efficiency or the probability that an analyte appearing in the probe volume produces a

detectable burst, ε_T is the transfer efficiency or the probability that an analyte makes it through the flow cell, while ε_s and ε_t are the spatial and temporal probing efficiencies, respectively. In traditional SMD platforms, burst heights vary dramatically due to the large range of particle trajectories available through the guassian optical probe volume, thus yielding trajectory dependent variations in signal-to-noise ratio. However, the extremely low background signal obtained by using femotliter probe volumes renders this effect on measurement efficiency negligible, yielding near unity detection efficiency values.[S8] The temporal probing efficiency is unity for continuous wave sources at high enough laser powers to ensure excitation of each particle entering the probe volume. Furthermore, transfer efficiency can be kept close to unity with proper care in choosing flow cell and buffer materials; indeed, figure 3 C shows that even in the 5 nL microdevice surface adsorption seems negligible up to 500 s measurement times. The spatial probing efficiency is by far the dominate factor in determining measurement efficiencies in traditional SMD platforms and accounts for loss of information due to the passage of analyte around or outside the optical probe volume.

An estimate of the spatial probing efficiency can be obtained by determining the ratio of the cross-sectional areas of the optical probe volume and flow cell. Using the probe volume measurement obtained in S2, leads to a ε_S value of 0.005 or a 99.5 % loss in measurement efficiency due to the mismatch between the probe volume and flow cell. In fact, this value is consistent with traditional SMD platforms and remains the major obstacle for combining SMD with low-volume and/or digital microfluidic systems. A simplifying assumption for analysis of the SMD-microdevice is that during the first revolution of the rotary pump particles enter the probe volume at most once and sampling is no different than traditional high-volume SMD platforms. An estimate for the expected number of bursts during this first revolution can now be obtained using equation S.3.7 and substituting the measured value of volumetric flow rate through the optical probe volume (Q_P = (In situ velocity at the probe volume (v – m/s) X (Cross-sectional area of the probe volume (V_P – m^2))) for Q_F, yielding the equation:[S6, S7]

Equation S.3.9: $$B_R = vV_PC$$

where ε_M can be neglected since the dominate term ε_S nears unity after substituting Q_P for Q_F. Substituting the calculated values from figure 2B (v ~ 8 x 10^{-4} m/s for 100 Hz pumping rate) and S2 (V_P = 6.37 x 10^{-12} m^2) yields an expected burst rate of 15.344 bursts per second for a 5 pM sample. By using the ε_S estimate obtained above and back-substituting the burst rate calcuataion into equation S.3.7 we obtain an estimate for the volumetric flow rate supplied by the rotary pump at 1.02 nL/s (1.02 x 10^{-12} m^3/s). Thus, fluid contained within the 0.00314 m long rotary pump completes one revolution every 4.9 seconds. However, continuous sampling and multiple revolutions lead to two main differences between the SMD-microdevice and capillary flow cells. First, the probability that any given particle within the sample has been detected goes up, due to resampling of the entire 5 nL's contained within the rotary pump. This leads to an increase in the relative measurement efficiency, enabling detection and quantification within the nL sample volumes. However, each successive revolution also increases the chance that one of those particles has been detected more than once.

SUPPLEMENTAL DATA 4 (S4): Poisson Statistics and Particle Resampling

S.4.A Poisson Sampling Statistics

Thus far two simplifying assuptions have been made regarding burst analysis within the rotary pump. First, at particle concentrations of 5 pM and below the probability of having double occupancy bursts was deemed neglible. Second, particles were assumed to enter the optical probe volume at most once during the first revolution of the rotary pump, thus allowing a sampling of particles contained within the 5 nL volume akin to sampling witin traditional capillary flow cells. In order to extend equation S.3.9 to fluorescence burst analysis throughout rotary pump operation (not just the first revolution) an additional assumption must be made, that is the mixing action of the rotary pump ensures random sampling of particles within the 5 nL volume in each successive revolution. Indeed, in its original use the microfluidic rotary pump was characterized as a dynamic mixer[S9] and for our intended use provides interfacial stretching and minimizes particle resampling due to measurements on recirculating laminar flow streams.

To examine the utility of this assumption the interarrival distribution of fluorescence bursts for the SMD-microdevice was compared to a standard microcapillary, in which fluorescence bursts are expected to follow the Poisson equation (S.4.1) since each measurement interval (integration time) becomes a Bernoulli trial when utilizing the aforementioned simplyfing assumptions.[S4, S10, S11]

Equation S.4.1: $$N(\Delta t) = \alpha e^{-\beta \Delta t}$$

where α is a proportionality constant and β is a characteristic frequency depending on concentration and sampling rates within the SMD platform. As discussed in the text, single particle detection within the microdevice (5 pM

QD-nanoasseblies, 100 Hz pump rate, 15 separate 100 s measurments) was described moderately well by Poisson statistics, as shown by the least squares fit to a single exponential for the interarrival frequency (Cy5/FRET channel) versus time graphs in figure 3D (R^2=0.992). The fit to this curve yields recurrence times (1/β) of 106 ms, compared to the expected recurrence time of ~126 ms This expected recurrence time can be obtained using the burst rate estimate (B_R) in equation S.3.9 to yeild:

Equation S.4.2: $$\tau_R = \frac{1}{\beta} \approx \frac{1000}{B_R F}$$

where F is a probe specific scaling factor that depends on both properties of the QD-FRET probe pair itself (e.g. QD to Cy5 probe ratio) and the user defined threshold for peak determination; Q was found empirically for the probe used in this report to be 0.51.

S.4.B Concentration Measurements

Upon validation of our final assumption, that is sampling rates will continue to follow Poisson sampling statistics after multiple revolutions, determination of both the flow velocity and the optical probe cross-section allows an estimation of particle concentration within the rotary pump.[S6] The calculated QD-nanoassembly (QD-NA) concentration (C – M) can be obtained using the expression:

Equation S.4.3: $$C = \frac{1}{1000} \frac{B_r}{V_p v N_A F}$$

where B_r , V_p , and v are as defined above and N_A is Avagadro's number. Table S.4.1 shows the measured concentration associated with the QD-NA input concentrations used in this report (50 fM data not included in text; B_R averaged from 5 separate 100 s rotary pump measurments (Figure 2 A and B)).

Table S.4.1

Input Concentration (M)	Measured Concentration (M)
5.00E-12	4.61E-12
5.00E-13	5.49E-13
5.00E-14	4.64E-14

The experimental burst rates measured with 5 pM (778.5 burst counts per 100 s, 782.68 expected), 500 fM (92.67 burst counts per 100 s, 78.27 expected) and 50 fM (7.2 burst counts per 100 s, 7.83 expected) show good agreement with the calculated or expected values. An interesting consideration for this SMD-microdevice is that longer acquisitions yield more information about the small sample through recirculation; at long acquisition times this results in a near 100% measurement efficiency. However, as the number of particles loaded into the well becomes small, Poisson sampling statistics begin to limit the precision of any single measurement. This is due to the large variation in trapping or valving off of small concentrations, such as the ~ 150 particles in the 50 fM case. Still, coupling this small volume SMD-microdevice to current single cell processing chips would eliminate this problem, by isolation of the entire discrete sample and removal of the particle sampling step. In addition, the number of particles counted within the 5 nL chambers in this report (50 fM – 150.575 expected particles per chamber, 138.5 measured) agrees well with the estimated number of mRNA transcripts isolated from single cells using enzymatic amplification techniques, such as PCR.[S12, S13]

S.4.C Resampling Statistics (Measuring Particles More Than Once)

As discussed throughout the supplemental section multiple revolutions of the rotary pump yeild an increasing relative measurement efficiency, which enables SMD assays within the nanoliter volumes. However, the price of this sample reduction is resampled particles or particles that pass through the optical detection volume 2 or more times. In homogeneous samples this may not significantly alter measurements, especially at concentrations yeilding 1000's of particles per SMD-chamber/rotary pump. However, this unique aspect of the SMD-microdevice may prove detrimental in samples with significant particle heterogeneity (i.e. individual particle QD:DNA in the QD-FRET assay). Still, the Poissonian nature of SMD measurements within the microdevice enables estimates of the number of resampled particles after a specified number of revolutions of the rotary pump, as shown in equation 3.4.4.

Equation S.4.4: $$P_{\#}(s) = e^{-R\varepsilon_S} \frac{(R\varepsilon_S)^s}{s!}$$

where the probability of a particle within the rotary pump being sampled s times is Poisson distributed with parameter $R\varepsilon_S$. The paramter is dependent upon the number revolutions of the rotary pump (R) and the spatial probing efficiency (ε_S) as calculated in S3. Supplemental figure S2A shows the probability that a particle is measured at least once (Relative Measurement Efficiency ($P_{s>0}$), triangles) and the probability that a particle is measured more than once (Resampling ($P_{s>1}$), circles) for specified numbers of rotary pump revolutions. In figure 2B it is apparent that the disparate rates of change of $P_{s>0}$ and $P_{s>1}$ leads to a relatively low percentage of measured partilces that have been resampled within the first 25 revolutions. The 100 s measurement periods utilized in this report took ~ 20 revolutions at 100 Hz.

SUPPLEMENTAL DATA 5 (S5): QD-FRET Efficiency at Varying Microfluidic Pump Rates

S.5.A FRET Efficiency versus Pump Rate

A QD-FRET specific benefit of conducting SMD in the recirculating microfluidic device is the ability to control FRET efficiency between the QD donor and Cy5 acceptor. FRET efficiency analysis was performed by evaluating peak by peak intensity of the Cy5 channel normalized to the sum of the QD605 and Cy5 intensities. Supplemental figure S3 shows histograms of this peak by peak FRET efficiency for 50 pM QD-nanoassemblies at three different rotary pump rates. This flow dependent improvement in FRET efficiency has been attributed to flow-induced reduction in donor-acceptor separation, due to deformation of DNA probes.[S14] This result suggests that detection sensitivity of the QD-FRET assay conducted within the low volume SMD-platform may be enhanced when operated at higher pump rates.

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