Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information

On-Chip Dilution in Nanoliter Droplets

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Section 1: Pseudo programs for droplet sequences in the main text

The pseudo programs that generated the droplet dilution sequence in the main texts can be found below:

A) Pseudo code for main text figure 2(left):

```
Initialize:

Pump 1 for sample; Pump 2 for diluent buffer, Pump 3 for FC3283 oil

1) Set the number of Pump 1 units = 4
                              //  (Injected sample volume)
2) Set the number of droplets before next injection = 20
                              // determine the range of dilution
3) Set the number of repetitions= 1

Execute:
Loop 1 times                  //(replicates of a sequence)
{
      Pump Pump 1       4 units
                              // inject sample to be diluted

         Loop 20 times    // No. of droplets before next injection.
         {
         Pump Pump 2       1 unit   // Droplet production
         Pump Pump 3       1 unit   // using alternate pumping
         }
}
```

B) Pseudo code for main text figure 3 (top): Please note 5 repeated cycles per sample injection.

```
1) Set the Initial sample volume = 4 pump strokes
2) Set the number of droplets before next injection = 10
3) Set the number of repetitions= 4

Loop 4 times  // number of replicates
{
     //inject high concentration sample
     Pump Pump 1  ⟹  4 units

          // droplet production
          Loop 10 times // number of droplets
          {
          Pump Pump 2  ⟹  1 unit
          Pump Pump 3.  ⟹  1 unit
          }
}
```

```
1) Set the Initial sample volume = 3 pump strokes
2) Set the number of droplets before next injection = 10
3) Set the number of repetitions= 4

Loop 4 times  // number of replicates
{
     //inject high concentration sample
     Pump Pump 1  ⟹  3 units

          // droplet production
          Loop 10 times // number of droplets
          {
          Pump Pump 2  ⟹  1 unit
          Pump Pump 3.  ⟹  1 unit
          }
}
```

```
1) Set the Initial sample volume = 2 pump strokes
2) Set the number of droplets before next injection = 10
3) Set the number of repetitions= 4

Loop 4 times  // number of replicates
{
     //inject high concentration sample
     Pump Pump 1  ⟹  2 units

          // droplet production
          Loop 10 times // number of droplets
          {
          Pump Pump 2  ⟹  1 unit
          Pump Pump 3.  ⟹  1 unit
          }
}
```

```
1) Set the Initial sample volume = 1 pump stroke
2) Set the number of droplets before next injection = 10
3) Set the number of repetitions= 4

Loop 4 times  // number of replicates
{
     //inject high concentration sample
     Pump Pump 1  ⟹  1 units

          // droplet production
          Loop 10 times // number of droplets
          {
          Pump Pump 2  ⟹  1 unit
          Pump Pump 3.  ⟹  1 unit
          }
}
```

(c) Pseudo code for main text figure (bottom left):

Pseudo Program for Sequence 1

Initialization:

1) Set the number of Pump 1 units =3 (Injected sample volume)
2) **Set the number of droplets before next injection = 5**
3) Set the number of repetitions= 4

```
Loop 4 times
{
      Pump Pump 1  ⟹  3 units  // inject sample

            Loop 5 times
            {
            Pump 2  ⟹  1 unit   // Droplet production
            Pump 3.  ⟹  1 unit   // using alternate pumping
            }
}
```

OUTPUT: Max dilution ratio ~ 3 fold

Pseudo Program for Sequence 2

Initialization:

1) Set the number of Pump 1 units =3 (Injected sample volume)
2) **Set the number of droplets before next injection = 15**
3) Set the number of repetitions= 2

```
Loop 2 times
{
      Pump Pump 1  ⟹  3 units  // inject sample

            Loop 15 times
            {
            Pump 2  ⟹  1 unit   // Droplet production
            Pump 3.  ⟹  1 unit   // using alternate pumping
            }
}
```

OUTPUT: Max. dilution ratio ~80 fold

Section 2: Theoretical modelling, calibration and design considerations:

After the injection of a sample pulse, a concentration gradient is set up due to the combined effect of advection and diffusion. This phenomenon is known as Taylor-Aris dispersion and has been studies predominantly in channels with circular cross section. The concentration at a point sufficiently away from the injection is described by following convective-diffusion equation.

$$\frac{\partial C}{\partial t} + U\frac{\partial C}{\partial z} = D_{eff}\frac{\partial^2 C}{\partial z^2}$$

For above equation it is mandatory that the diffusion time scales in the transverse direction (normal to the flow direction) are small compared to advection time scales. The T junction should be placed sufficiently downstream such that transverse diffusion has sufficient time to smear out the concentration profile.

$$L = longitudinal\ length\ scale$$

$$a = transverse\ length\ scale$$

$$Condition1: \tau^{L}_{advection} >> \tau^{a}_{diffusion} \Rightarrow \frac{L}{\overline{V}_{avg}} >> \frac{a^2}{D_{mol.}}$$

$$Condition1: \tau^{a}_{diffusion} >> \tau^{a}_{advection}$$

The above criterions give the design considerations for geometrical design and pump operating frequency (high Peclet number) to have droplet dilution workflow in a convection dominated regime. We found that Peclet number in our design is approximately of the order 40 which is large enough to ignore diffusion effects in the flow direction. A theoretical estimate for such convection dominated solute transport have been reported earlier in the literature. Even though a close form expression was obtained for circular cross sections, however obtaining a close form solution for solute transport for a pulsatile flow in a rectangular cross section is quite challenging. Nevertheless, all the concentration profiles at a point

sufficiently downstream of injection follow an error function form. Thus, to predict the dilution ratio, we formulated a calibration function with calibration constants, k_0, k_1, k_2, k_3 and k_4.

$$\frac{C(z,t)}{C_0} = k_0\left(erf\left(k_1 - \frac{k_2 t}{\sqrt{4D_{eff}t}}\right) - erf\left(k_3 - \frac{k_4 t}{\sqrt{4D_{eff}t}}\right)\right)$$

where error function is given by

$$erf\left(k_1 - \frac{k_2 t}{\sqrt{4D_{eff}t}}\right) = \frac{2}{\pi}\int_0^{k_1 - \frac{k_2 t}{\sqrt{4D_{eff}t}}} e^{-t^2}\,dt, \quad \frac{C(z,t)}{C_0} \Rightarrow Dilution\ \ factor,$$

$$D_{eff} \Rightarrow \text{Effective diffusion coefficient for rectangular cross-section}$$

By approximating D*eff* as 1.5 X10^-8 m^2/s, we get a theoretical curve fit as shown in the figure (2) below. The curve fitting coefficients are: k_0= 4.54; k_1=-2.66; k_2= 7.78e-5; k_3=2.54 and k_4= 6.94e-5. This curve fit was applied to the dilution factor profile as shown in figure 1. Further experiments are needed to establish a correlation between the calibration constants and operating conditions such as sample volume, number of droplets generated and frequency of droplet generation.

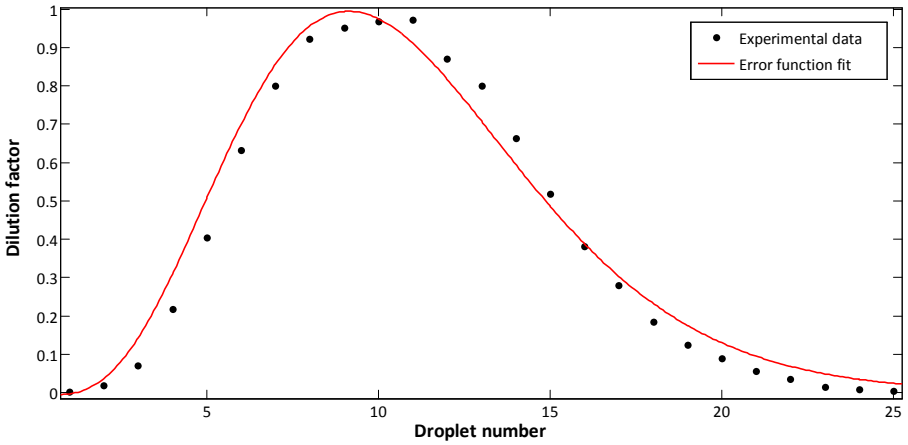

Figure S1 Theoretical fit to the dilution factor in a droplet sequence using a calibration function.

Section 3: Measurement of dilution factor

In order to measure dilution factor of a droplet sequence, we first calibrated our optical setup and CCD camera. First, a train of known concentration of CF488 dye was created with 0.03µL, 0.06 µL and 0.1 µL dye concentrations. These droplet were imaged using a CCD sensor and the recorded intensity is as shown in figure S2 (right). Next, a train of DI water droplets is generated and its greyscale intensity levels are captured as shown in figure S2 (left). Figure S3 shows linear relationship between measured greyscale intensity levels within droplets having known concentrations of CF488 dye. These intensity levels are used for background subtraction and subsequently for estimating dilution factor of a droplet sequence. For the purpose of this study, the spatial variations in the intensity resulting from the non-uniformity in the

illuminations source have been ignored. The droplet boundaries were determined using ImageJ software through the use of intensity threshold method. The dilution factor was then computed by averaging the greyscale intensity level over the pixels occupied within individual droplets. In order to measure the dilution factor over a large range, we used an enhanced fluorescent dye CF488 at a high concentration (0.1mM) and a high intensity illumination source. The concentration of CF488 and illumination intensity was adjusted such that it nearly saturated the CCC sensor array. This enabled measurement of dilution factors as large as 80 spread across 18 droplets as shown in main text figure (3). As the droplet number of was increased, concentration measurement for droplets beyond droplet#18 was obscured with noise levels of the camera. Thus, in this setting, measurements were limited by dynamic range of CCD. In order to boost the dynamic range of CCD, we used a neutral density filter with 1% transmittance. First, a sequence of 90 droplets was programmed with initial sample volume equivalent of 6 pump units. The resultant droplet array was imaged with and without the neutral density filter. For this measurement protocol, it is imperative to have high concentration of CF488 and sufficient illumination intensity that the CCD is nearly saturated even with 1% N.D. filter. We measured a dilution factor of ~6000 spread across 90 droplets. This confirms our hypothesis that the dilution range can be increased by increasing the number of droplets in a sequence (main text fig 3).

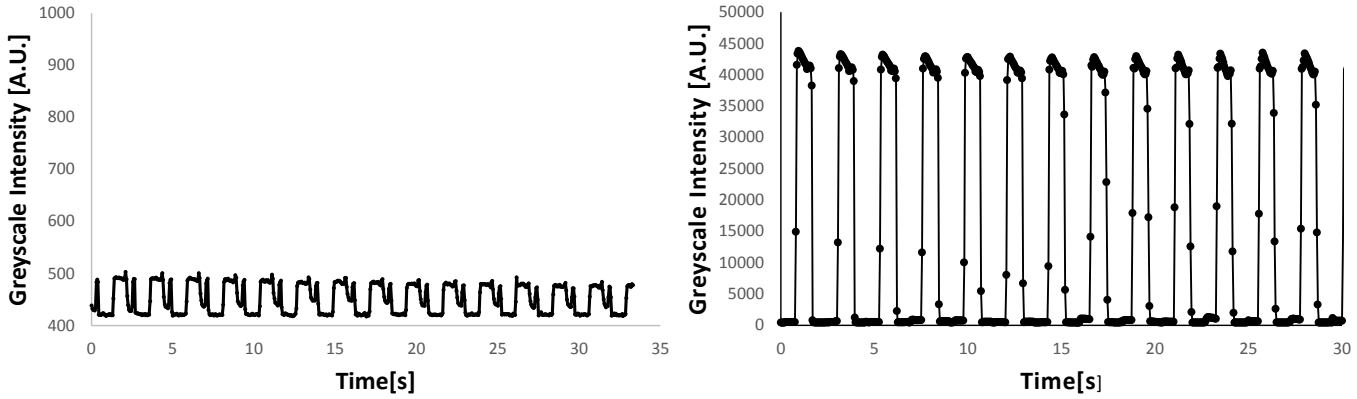

Figure S2 (left) greyscale intensity recordings for a train of pure DI water droplet. Even though the concentration of CF488 is zero, non-zero values emerge due to background noise of the camera. These values are averaged and used for background subtraction.(right) A train of CF488 droplets with known concentration (0.1mM) used for calibration of dilution factor.

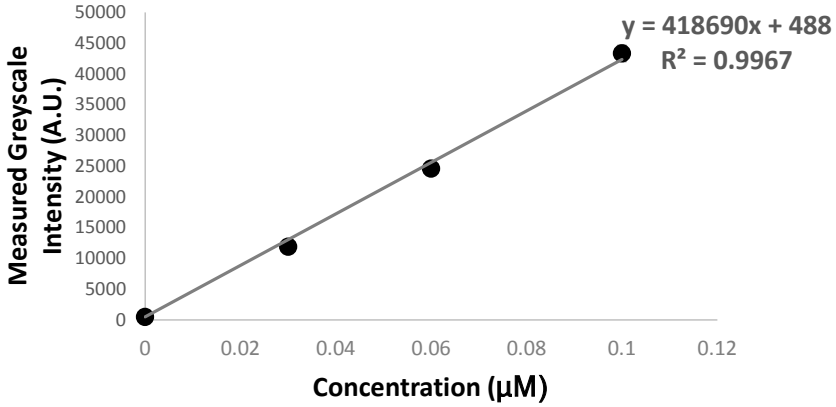

Figure S3: Calibration curve showing linear relationship between CF488 concentration and the measured greyscale intensity using a CCD camera. The non-zero y-intercept of the graph for DI water droplets corresponds to the dark noise in the camera. For subsequent calculation of dilution factor, this value is first subtracted from measured greyscale intensity within a droplet.