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Effect of velocity on microdroplets fluorescence quantified by laser induced fluorescence

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Abstract

Microdroplets generated inside microfluidic devices have been widely used as miniaturized chemical and biological reactors allowing important reductions in experimental fluid volumes and making it possible to carry out high-throughput assays. Laser induced fluorescence (LIF) is commonly used to detect and quantify the product, marker or cell content inside each individual droplet. In this work, we employed this technique to characterize the response of in-flow microdroplets filled with fluorescein dye at different laser powers and flow velocities. Using two parallel laser beams closely focused inside a microchannel we determined microdroplet velocities and showed that droplet fluorescence intensity decreases exponentially with reducing velocities because of the effects of photobleaching. In contrast, fluorescence intensity increases linearly with laser power in the 4-10 mW range. When LIF is used for microdroplet measurements it is important to consider not just fluorophore concentration but also the droplet velocity and laser power in the development of quantitative assays.

Introduction

Microfluidic microdroplets are widely used for performing chemical and biological assays in reduced volumes in both low and high throughput regimes where individual droplets are filled with different reagents, cells or components.[1-5] Such experiments are relatively straightforward to implement and have been used in a great number of applications, ranging from bioanalytical assays;[6-8] enzyme kinetics;[9, 10] cell analysis, encapsulation and sorting;[1, 11, 12] chemical synthesis[13] and complex particle fabrication[14] to cite just a few. The large and growing number of applications with microdroplets can be found in recent reviews.[15-17]

Chemical analysis of the microdroplet contents can be evaluated by different methods such as Ramman spectroscopy,[18] mass spectroscopy,[19, 20] or electrochemical methods[21, 22] (other analytical detection techniques for microfluidic droplets have been recently reviewed[23]). However, fluorescence spectroscopy[24, 25] is probably still the most attractive method because of its extraordinary sensitivity. In particular, Laser induced fluorescence (LIF) provides one of the best non-intrusive methods for high spatial resolution measurements, which makes it extremely useful to precisely quantify the content of an analyte inside each droplet independently.[26, 27] In this technique a laser beam is focused by a microscope objective into a channel in such a way that only a small portion is illuminated, so only one droplet at a time is made fluorescent. The emitted light is collected through the microscope objective, filtered and recorded by a photodetector to assess the fluorescence of each droplet. Several variants of LIF systems for microfluidic droplets have been developed,[26-30] many of which were focused on detection limit improvements. For instance, employing an orthogonal laser arrangement technique, fluorescein dye concentrations up to 250 pM have been reported.[28] Using sensitive photomultiplier tubes, detection of a single molecule inside droplets has also been achieved.[29, 31] Besides fluorophore concentration, there are other potential factors that could affect the overall sensitivity of any LIF detection system that have not been evaluated for microfluidic droplets, such as laser power, sample size and volume, photobleaching effects and flow velocities.[32, 33]

In this work we analyze the LIF microdroplet signal under variations of flow velocity, and laser power. We implemented a straightforward method to measure the velocity of droplets traveling through a microchannel and confirmed that it has an enormous effect on the amount of fluorescent light measured at velocities below 2 cm/s. According to our experimental results we

propose that photobleaching effects are the cause of fluorescence decrease, even at low laser powers (4-10 mW).

Experimental Methodology

Microfluidic device fabrication and reagents. The microfluidic chips were fabricated in poly(dimethylsiloxane) (PDMS) (Kit Sylgard 184, Dow Corning) using a negative photoresist SU-8 (Micro Chem 2025) master mold created by soft lithography.[34] The PDMS replicas were sealed to glass slides using an oxygen plasma system (Femto, Diener Electronic GmbH). The microfluidic devices had an 80 μm square cross-section and a flow-focusing geometry to form droplets (Figure 1).[35] Microdroplets were filled with 10 μM fluorescein dye solution in 50 mM phosphates buffer pH 8.3. The continuous phase liquid was mineral oil with 0.1 % of Span 80.

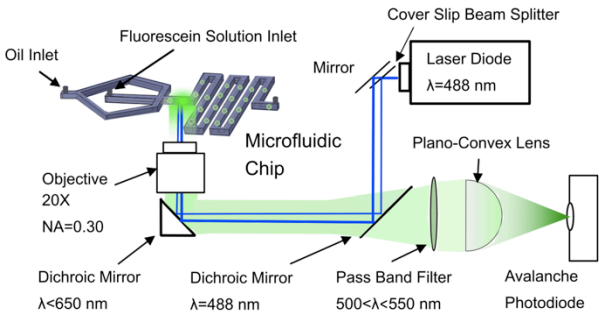

Figure 1. Schematic diagram of the experimental setup for microdroplet formation and LIF detection system.

Equipment for LIF detection. A schematic diagram of the LIF detection system used in this work is shown in Figure 1. An optically pumped 488 nm semiconductor laser (Sapphire 488, Coherent) was used as light source to stimulate the fluorescence. The optical trajectory is described as follows: the laser beam was reflected on a 488 nm dichroic mirror (Di01-R488 Semrock) through a fluorescence port of an inverted fluorescence microscope (DM IL LED, Leica) and reflected on a second dichroic mirror (FF660-Di02, Semrock) placed on the microscope filter holder. The laser beam was focused by an objective (20x, NA=0.30, Leica) and pointed to the micro channel. The fluorescence light emitted by the microdroplets was collected by the objective, reflected by the dichroic mirror (FF660-Di02) and passed both through the first

dichroic mirror (Di01-R488) and through a band pass filter 500-550 nm (FF02-525/50-25, Semrock). Finally, a plano-convex lens (LA1951, Thorlabs) concentrated the light before being detected by an avalanche photodiode (APD-100, Hinds Instruments). The APD signal was recorded by a data acquisition card (USB-6351, National Instruments) and processed using both LabVIEW signal express and Matlab. To determine droplet velocities, a 0.3 mm thick glass cover slip was placed in front of the first mirror to split the laser beam into two parallel rays of different power (Figure 1). This is a standard technique in optics, described in the literature.[36] The relative intensities were approximately 8% and 92% for the glass reflected laser and the mirror reflected laser respectively. Both beams followed the same optical trajectory described above and were focused by the 20X objective with a distance of 210 μm measured by microscopy image.

Droplet fluorescence characterization. The fluorescein dye solution and mineral oil were pumped into the device using the aqueous and oil inlets (Figure 1) employing syringe pumps (NE-1000, New Era). The formed droplets were characterized by setting the laser power at 10 mW and setting the fluorescein and mineral oil flows at 30 μL/h each. When the microdroplet formation was stable, the signal was acquired for 10 s at a 1 kHz sample rate at the beginning of the microchannel. The flow of both fluorescein and mineral oil, were then increased by 15 μL/h keeping a 1:1 flow ratio. This procedure was repeated on each measurement up to a flow of 500 μL/h for each liquid. The fluorescence signal for each experimental flow was processed with an in-built Matlab program to determine the height of the peaks, which were then averaged. To evaluate our LIF system sensitivity to laser power changes, a second series of experiments were carried out in which a flow rate for both fluorescein and mineral oil was invariant at 60 μL/h each throughout the acquisitions. Droplet fluorescence was recorded and analyzed with different laser intensities from 4-10 mW with 0.5 mW intervals. To determine the effect of fluorescein dye concentration on the fluorescence intensity a flow-focusing geometry device with two aqueous inlets was used to introduce different amounts of buffer and fluorescein dye solution in the microdroplets.

RESULTS AND DISCUSSION

Variation of Droplet Fluorescence Intensity with Droplet Velocity. Figure 2 shows three distinct fluorescence traces at different flow rates of droplets filled with a 10 μM fluorescein dye solution. When the total flow rate was increased from 60 μL/h to 600 μL/h keeping the water:oil ratio 1:1, the fluorescence traces showed that droplet formation frequency increased from ~ 1 to 20 Hz, but most notably the fluorescence signal increased by about 60 %.

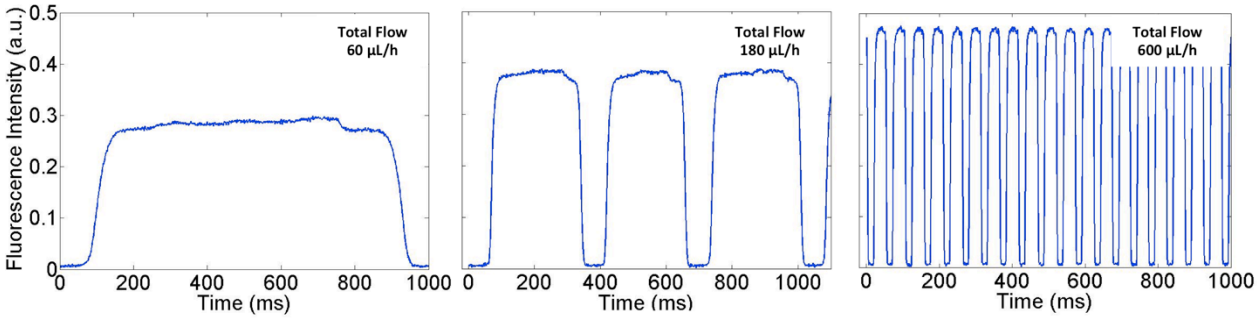

Figure 2. Experimental fluorescence signal of 10 μM fluorescein solution droplets in microchannels generated at different flow rates. Fluorescence amplitude and frequency both increase with total flow rate. The ratio of oil and fluorescein solution flow rates was kept constant at 1:1 for all cases.

Instead of correlating the droplet fluorescence signals to the flow rate we associated them to the droplet velocities, which are independent of the channel size and geometries. An estimation of these velocities might be obtained from the dimensions and geometries of the channels and the total flow values. However, in many cases this figure can significantly vary with the real droplet velocity since other factors such as droplet size, surfactant content, relative viscosity of continuous and discontinuous phases, capillary number, temperature and channel geometry have a significant effect on droplet velocity.[37-40] A simple way to determine the droplet velocity without the need of a costly high-speed camera was to split the laser beam into two parallel beams of clearly different power using a glass cover slip. These beams were closely focused inside the microchannel device and were separated by a shorter distance than the length of a droplet. When a droplet passed through them it generated a characteristic fluorescent trace that was used to determine accurately the time it took one droplet to travel from one laser spot to the

second one. As the distance between the spots produced by both beams was measured from a microscope image (210 μm), droplet velocity was calculated by dividing this distance by the time obtained before (Figure 3).

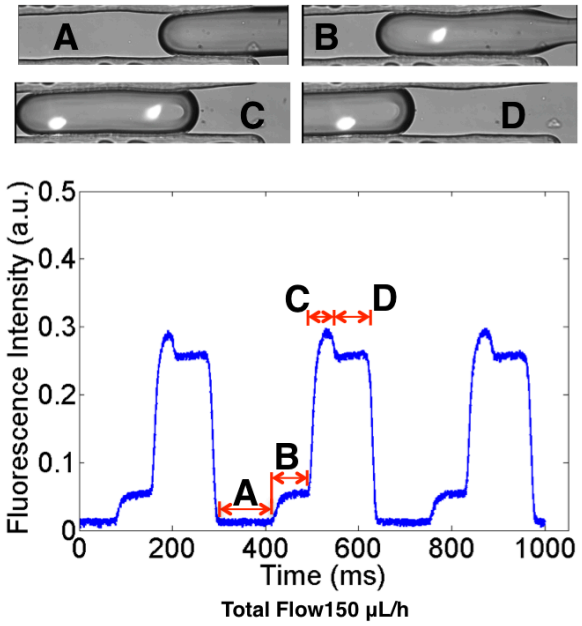

Figure 3. Droplet velocity determination in a microfluidic channel using two laser beams with a large intensity difference. (A) A droplet is formed and starts flowing through the microchannel in a right-to-left direction. (B) The droplet is first hit by the low-intensity laser beam generating a small fluorescent signal. (C) After moving downstream, both the high-intensity and the low-intensity beams hit the droplet and the fluorescent signal increases. The fluorescence is at its maximum as a result of adding both beams signals. (D) As the motion continues, only the high-intensity beam hits the droplet so the fluorescent signal slightly decreases. The droplet velocity can be obtained by dividing the distance between the two laser spots by the time it takes the droplet to cover this distance. The time can be calculated by subtracting, for example, the starting time of interval C minus the starting time of interval B.

Based on the latter procedure, a plot of the fluorescence intensity as a function of droplet velocity was obtained and is shown in Figure 4A. At very low velocities the fluorescence intensity is small and increases rapidly with droplet velocity, but then it starts to asymptote as the

velocity gets large. Figure 4A also shows the fluorescence signal of the same fluorescein dye solution in continuous flow inside a microchannel with identical characteristics as the one used to form droplets. The fluorescence intensity of the continuous flow follows the same pattern as for droplets, however, its intensity was ~8 % higher than with the droplets. The decrease of the signal from droplets with respect to a continuous flow has been also observed with other fluorescent molecules such as the green fluorescent protein and could be caused by the thin oil layer around the droplets, which might increase the stray light in the system.[30] These experiments show that the dependence of fluorescence intensity on the fluid velocity is not exclusive for continuous flow liquids as previously described,[32, 33, 41, 42] but for microdroplets as well. They also show that as droplet velocities rise, the dependence of fluorescence intensity in this parameter is less important, but at low velocities, significant variations on the fluorescent signal can occur with small changes on droplet velocities.

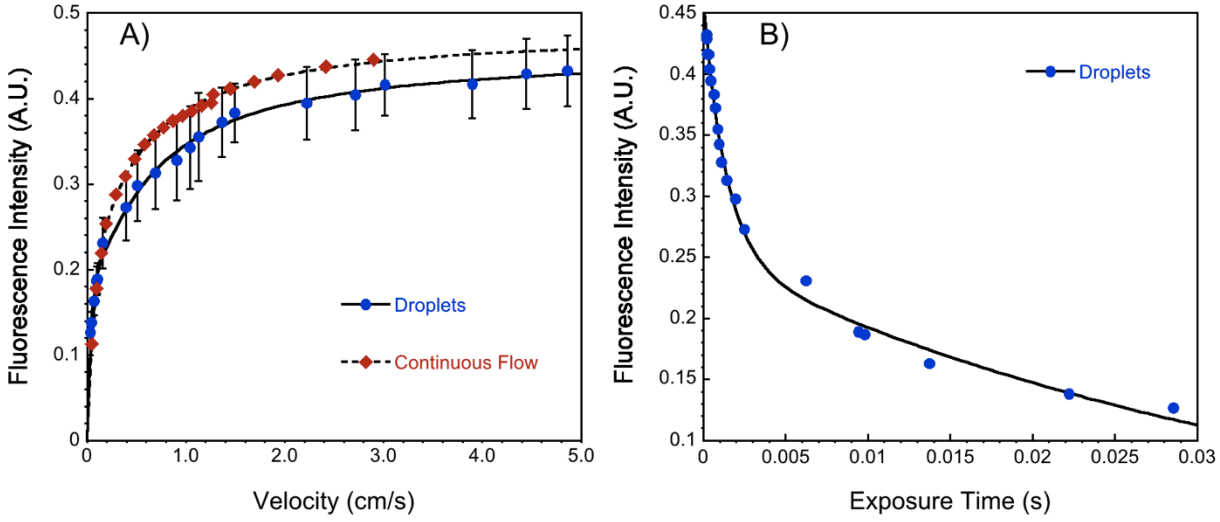

Figure 4. A) Experimental results of fluorescence intensity as a function of droplet or continuous flow velocity. The droplets were filled with 10 μM fluorescein solution and the points in the graph represent the average of three independent measurements in different microchips. Error bars show the standard deviation of these independent experiments. The solid and dotted lines correspond to the curve fitting of the experimental data to a double-exponential photobleaching process (Equation 2) (correlation with an R^2 value of 0.995). B) Droplet fluorescence intensity as a function of exposure time. The same data from Figure 4A was plotted substituting the velocity

by the exposure time using Equation 1. The curve fitting of the experimental data was done substituting the droplet velocity (*v)* by the exposure time on Equation 2. Error bars were omitted for clarity.

Figure 4B shows the same data from Figure 4A but plots the fluorescence intensity as a function of exposure time *t*, which is defined as:

$$t = \frac{d}{v} \qquad (1)$$

where *d* is the laser beam diameter (10 μm measured by microscopy image in this work) and *v* is the droplet velocity. The exposure time is an approximation of the maximum time that a fluorescein dye molecule is exposed to the laser (supposing no diffusion inside the droplet). In Figure 4B the short exposure times correspond to droplets at high-speed while higher exposure times are of those droplets traveling at low velocity fluids. It is clear that a larger exposure time of the fluorescein molecules leads to a reduction of the fluorescence intensity. The most likely explanation of this is that photobleaching of the fluorescein molecules leads to the decrease of the fluorescence intensities. Photobleaching is a phenomenon in which the constant exposure of a fluorescent molecule to light leads eventually to its inactivation and loss of its capacity to emit light.[43] Photobleaching of fluorescein has been studied in different systems.[44, 45] In some special cases the fluorescence intensity can decrease as a function of exposure time following an exponential decay,[46] but most commonly it decreases via a double exponential decay.[44, 47] Data from Figure 4A is indeed best described by the following double-exponential function:

$$I_f = I_{f1} \cdot e^{-k_1 \frac{d}{v}} + I_{f2} \cdot e^{-k_2 \frac{d}{v}} \qquad (2)$$

where I_f is the fluorescence intensity, I_{f1} and I_{f2} are amplitudes of the fluorescence intensity at very high velocities (t = 0) and k_1 and k_2 are first order rate constants of the photobleaching process. The I_{f1} and I_{f2} values for droplets are 0.25 A.U. and 0.21 A.U., while the respective values of the constants k_1 and k_2 are 27 s^{-1} and 710 s^{-1}. For continuous flow, I_{f1} and I_{f2} are 0.32 and 0.16 A.U. and k_1 and k_2 are 53 s^{-1} and 680 s^{-1} respectively. The observation that the decay

constants k_1 and k_2 are different for droplets compared to continuous flow suggests that differences in the microscopic fluid flow patterns and speeds in the two situations,[48] results in different exposure times at the molecular scale, and this has an effect on photobleaching. This clearly requires further investigation involving microscopic fluid flow,[49] beyond the scope of this experiment.

The double exponential decay has been interpreted as a complex mechanism in which at least two different photochemical decomposition reactions of excited fluorescein molecules occur simultaneously to yield non-fluorescent products. Examples of this phenomena are the photodestruction from the fluorescein excited singlet state, the reaction of a fluorescein molecule excited in its triplet state with an oxygen molecule, or the reaction between two triplet excited fluorescein molecules.[44, 47, 50]

Fluorescein dye is widely used for fluorescence measurements, either as a marker or as a product of a reaction.[5, 10, 28] However, other molecules show different photobleaching behaviors and could have a different effect on the relationship between velocity and laser power.[32, 51]

Implications of the fluorescence intensity dependency on droplet velocities could be significant in experiments where the velocities of a large group of droplets can differ between them. A possible example of this is when droplets incubated off-chip are reintroduced into microchannels for fluorescence measurements and sorting.[52] Also, in experiments where droplets move slowly, photobleaching can reduce significantly the detection limits, for example in point of care devices. Similarly, if the droplet velocity changes while traveling in a very long channel, comparison of the fluorescence intensities at different positions can lead to incorrect measurements. As mentioned before droplet velocity depends on several factors and an accurate method to determine droplets velocity should be considered when designing quantitative assays.

Variation of the Droplet Fluorescence Intensity with Concentration and Laser Power. Figure 5A shows the linear response of the fluorescence intensity as a function of fluorescein dye concentration on the μM range. This linear response has been reported for many other LIF systems.[5, 10, 28] Besides concentration and droplet velocities, we measured the effect of the laser power on the fluorescent signal generated by microfluidic droplets. Figure 5B shows how the fluorescence signal increases almost linearly with laser power from 4 to 10 mW, which is the highest power reached by the laser employed here, and only begins to show non-linearity.

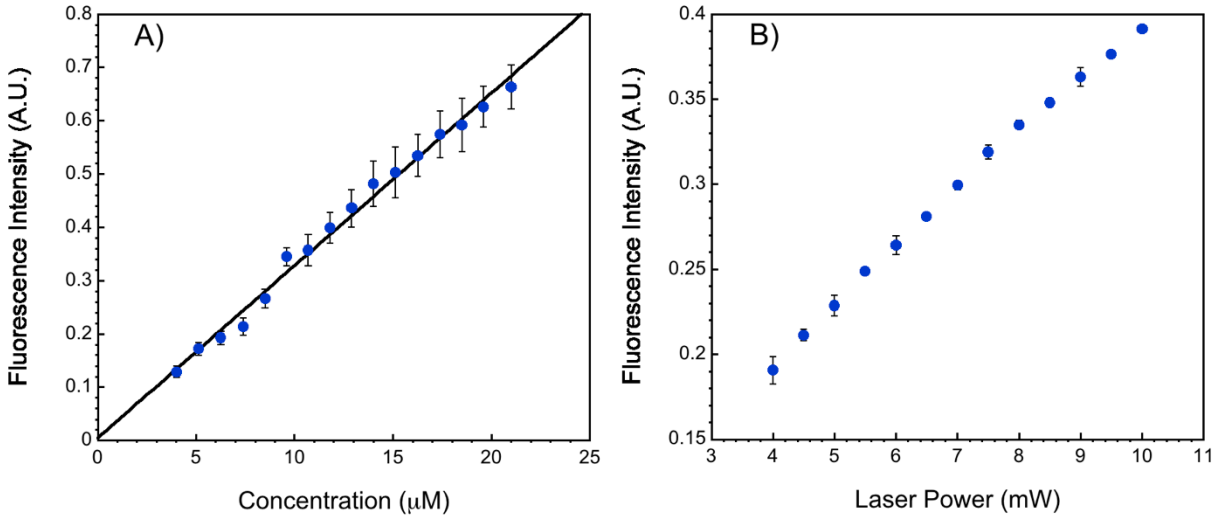

Figure 5. A) Droplet fluorescence intensity plotted against fluorescein dye concentration in 50 mM phosphates buffer pH 8.3, a laser power of 8.0 mW and droplet velocity of 2.5 cm/s. B) Droplet fluorescence intensity plotted against laser power. The droplets were filled with 10 μM fluorescein solution and their velocity was 2.5 cm/s. In both graphs the experimental points and their error bars represent the average of three independent measurements in the same microchip. Each of them was obtained by recording at least 10 seconds of flowing droplets.

An explanation of this behavior is as follows: As the first step of any fluorescence phenomena is the absorption of photons to excite the sample's electrons from their ground to higher excited states, the amount of emitted fluorescence is directly proportional to the amount of incident light.[24, 41] However, as the light intensity increases, the rates of the photobleaching reactions augment as well, establishing a ratio between absorption, fluorescence emission, intersystem crossing and photodestruction for each light irradiance.[41, 53] At low intensities, fluorescence increases linearly with light radiance until it reaches a maximum when the ground state of the molecules is depleted and triplet and other excited states are populated. But at high irradiances, fluorescence starts to decrease as higher rates of photodestruction occur.[53]

In this work 10 mW of laser power is equivalent approximately to an irradiance of 1.3×10^4 W/cm^2, which is still in the range of linear fluorescence increase. Similar excitation

intensities were tested with fluorescein isothiocyanate solutions giving constant photobleaching yields in this range.[50] For most LIF systems used for microdroplets the lasers employed are in the range of 10 to 100 mW,[3, 5, 10-12] so it would be interesting to know up to what extent the use of higher laser power employed gives a better response without causing thermal or chemical decomposition of the samples. Additionally, other factors as temperature, solvent polarity, pH and chemical components in the medium could be considered as the photobleaching reaction rates are dependent on them.[50]

CONCLUSIONS

The use of LIF for microdroplet fluorescence intensity quantification is an essential component of many lab on a chip systems that interrogate single droplets. In these measurements it is generally assumed that the concentration of the fluorophore is the main parameter that affects fluorescence intensity.[5, 10, 27, 30, 54-56] We show here that the flow velocity of droplets in microchannels has an important effect in the amount of fluorescent light emitted when interrogated by LIF. Especially at low velocities (for fluorescein dye, velocities lower than 2 cm/s), the effect of droplet speed cannot be overlooked in fluorescence measurements. As droplet velocities are difficult to predict, determining droplet velocities using the split laser system shown here is straightforward if the LIF system is already implemented. Additionally, laser power is an important factor to evaluate when higher sensitivity is needed. Quantitative fluorescence-based experiments on microdroplets can benefit when droplet velocity and laser power are taken into account in the design of assays.

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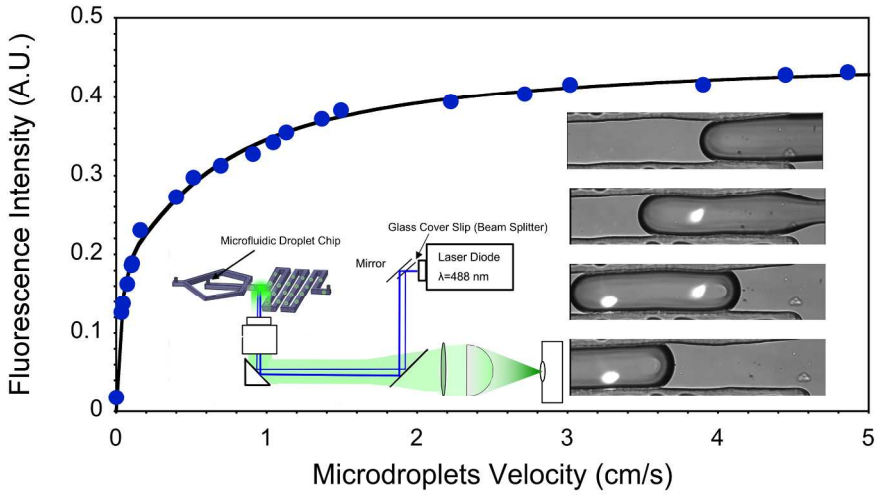
REFERENCES

1. E. Brouzes, M. Medkova, N. Savenelli, D. Marran, M. Twardowski, J. B. Hutchison, J. M. Rothberg, D. R. Link, N. Perrimon and M. L. Samuels, *Proc Natl Acad Sci U S A*, 2009, **106**, 14195-14200.

2. B. El Debs, R. Utharala, I. V. Balyasnikova, A. D. Griffiths and C. A. Merten, *P Natl Acad Sci USA*, 2012, **109**, 11570-11575.
3. B. Kintses, C. Hein, M. F. Mohamed, M. Fischlechner, F. Courtois, C. Leine and F. Hollfelder, *Chem Biol*, 2012, **19**, 1001-1009.
4. J. J. Agresti, E. Antipov, A. R. Abate, K. Ahn, A. C. Rowat, J. C. Baret, M. Marquez, A. M. Klibanov, A. D. Griffiths and D. A. Weitz, *P Natl Acad Sci USA*, 2010, **107**, 4004-4009.
5. A. Huebner, L. F. Olguin, D. Bratton, G. Whyte, W. T. S. Huck, A. J. de Mello, J. B. Edel, C. Abell and F. Hollfelder, *Analytical Chemistry*, 2008, **80**, 3890-3896.
6. Y. Schaerli and F. Hollfelder, *Mol Biosyst*, 2009, **5**, 1392-1404.
7. M. T. Guo, A. Rotem, J. A. Heyman and D. A. Weitz, *Lab on a Chip*, 2012, **12**, 2146-2155.
8. B. Kintses, L. D. van Vliet, S. R. A. Devenish and F. Hollfelder, *Curr Opin Chem Biol*, 2010, **14**, 548-555.
9. S. L. Sjostrom, H. N. Joensson and H. A. Svahn, *Lab Chip*, 2013, **13**, 1754-1761.
10. L. F. Cai, Y. Zhu, G. S. Du and Q. Fang, *Anal Chem*, 2012, **84**, 446-452.
11. L. Mazutis, J. Gilbert, W. L. Ung, D. A. Weitz, A. D. Griffiths and J. A. Heyman, *Nat Protoc*, 2013, **8**, 870-891.
12. J. C. Baret, O. J. Miller, V. Taly, M. Ryckelynck, A. El-Harrak, L. Frenz, C. Rick, M. L. Samuels, J. B. Hutchison, J. J. Agresti, D. R. Link, D. A. Weitz and A. D. Griffiths, *Lab on a Chip*, 2009, **9**, 1850-1858.
13. A. B. Theberge, E. Mayot, A. El Harrak, F. Kleinschmidt, W. T. S. Huck and A. D. Griffiths, *Lab on a Chip*, 2012, **12**, 1320-1326.
14. J. T. Wang, J. Wang and J. J. Han, *Small*, 2011, **7**, 1728-1754.
15. A. B. Theberge, F. Courtois, Y. Schaerli, M. Fischlechner, C. Abell, F. Hollfelder and W. T. Huck, *Angew Chem Int Ed Engl*, 2010, **49**, 5846-5868.
16. X. C. I. Solvas and A. deMello, *Chem Commun*, 2011, **47**, 1936-1942.
17. R. R. Pompano, W. S. Liu, W. B. Du and R. F. Ismagilov, *Annu Rev Anal Chem*, 2011, **4**, 59-81.
18. A. Walter, A. Marz, W. Schumacher, P. Rosch and J. Popp, *Lab Chip*, 2011, **11**, 1013-1021.
19. S. Sun, T. R. Slaney and R. T. Kennedy, *Anal Chem*, 2012, **84**, 5794-5800.
20. C. A. Smith, X. Li, T. H. Mize, T. D. Sharpe, E. I. Graziani, C. Abell and W. T. Huck, *Anal Chem*, 2013, **85**, 3812-3816.
21. S. Liu, Y. Gu, R. B. Le Roux, S. M. Matthews, D. Bratton, K. Yunus, A. C. Fisher and W. T. Huck, *Lab Chip*, 2008, **8**, 1937-1942.
22. S. Gu, Y. Lu, Y. Ding, L. Li, F. Zhang and Q. Wu, *Anal Chim Acta*, 2013, **796**, 68-74.
23. Y. Zhu and Q. Fang, *Anal Chim Acta*, 2013, **787**, 24-35.
24. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Springer, New York, 2006.
25. S. Das, A. M. Powe, G. A. Baker, B. Valle, B. El-Zahab, H. O. Sintim, M. Lowry, S. O. Fakayode, M. E. McCarroll, G. Patonay, M. Li, R. M. Strongin, M. L. Geng and I. M. Warner, *Anal Chem*, 2012, **84**, 597-625.
26. M. Srisa-Art, A. J. deMello and J. B. Edel, *Anal Chem*, 2007, **79**, 6682-6689.
27. F. Guo, M. I. Lapsley, A. A. Nawaz, Y. Zhao, S. C. Lin, Y. Chen, S. Yang, X. Z. Zhao and T. J. Huang, *Anal Chem*, 2012, **84**, 10745-10749.
28. G. D. M. Jeffries, R. M. Lorenz and D. T. Chiu, *Analytical Chemistry*, 2010, **82**, 9948-9954.
29. T. D. Rane, C. M. Puleo, K. J. Liu, Y. Zhang, A. P. Lee and T. H. Wang, *Lab Chip*, 2010, **10**, 161-164.
30. P. S. Dittrich, M. Jahnz and P. Schwille, *Chembiochem*, 2005, **6**, 811-814.
31. M. Srisa-Art, A. J. deMello and J. B. Edel, *J Phys Chem B*, 2010, **114**, 15766-15772.
32. J. P. Crimaldi, *Exp Fluids*, 1997, **23**, 325-330.
33. G. R. Wang and H. E. Fiedler, *Exp Fluids*, 2000, **29**, 257-264.
34. J. C. McDonald, D. C. Duffy, J. R. Anderson, D. T. Chiu, H. K. Wu, O. J. A. Schueller and G. M. Whitesides, *Electrophoresis*, 2000, **21**, 27-40.
35. R. Dreyfus, P. Tabeling and H. Willaime, *Phys Rev Lett*, 2003, **90**.
36. E. Hecht, in *Optics*, Pearson, New York, 4th edn., 2002, ch. 9.
37. V. Labrot, M. Schindler, P. Guillot, A. Colin and M. Joanicot, *Biomicrofluidics*, 2009, **3**.
38. M. J. Fuerstman, A. Lai, M. E. Thurlow, S. S. Shevkoplyas, H. A. Stone and G. M. Whitesides, *Lab on a Chip*, 2007, **7**, 1479-1489.
39. S. Jakiela, S. Makulska, P. M. Korczyk and P. Garstecki, *Lab on a Chip*, 2011, **11**, 3603-3608.
40. C. A. Stan, S. K. Tang and G. M. Whitesides, *Anal Chem*, 2009, **81**, 2399-2402.
41. R. A. Mathies, K. Peck and L. Stryer, *Anal Chem*, 1990, **62**, 1786-1791.
42. G. R. Wang, *Lab Chip*, 2005, **5**, 450-456.

43. A. Diaspro, G. Chirico, C. Usai, P. Ramoino and J. Dobrucki, in *Handbook of Biological Confocal Microscopy*, ed. J. B. Pawley, Springer US, New York, 2006, ch. 39, pp. 690-702.
44. L. L. Song, E. J. Hennink, I. T. Young and H. J. Tanke, *Biophys J*, 1995, **68**, 2588-2600.
45. L. Song, C. A. Varma, J. W. Verhoeven and H. J. Tanke, *Biophys J*, 1996, **70**, 2959-2968.
46. T. Hirschfeld, *Appl Optics*, 1976, **15**, 3135-3139.
47. G. Szabo, P. S. Pine, J. L. Weaver, M. Kasari and A. Aszalos, *Biophys J*, 1992, **61**, 661-670.
48. C. N. Baroud, F. Gallaire and R. Dangla, *Lab Chip*, 2010, **10**, 2032-2045.
49. L. Jiang, Y. Zeng, H. Zhou, J. Y. Qu and S. Yao, *Biomicrofluidics*, 2012, **6**, 12810-1281012.
50. J. Widengren and R. Rigler, *Bioimaging*, 1996, **4**, 149-157.
51. C. F. Kuang, D. Luo, X. Liu and G. R. Wang, *Measurement*, 2013, **46**, 1393-1398.
52. J. Clausell-Tormos, D. Lieber, J. C. Baret, A. El-Harrak, O. J. Miller, L. Frenz, J. Blouwolff, K. J. Humphry, S. Koster, H. Duan, C. Holtze, D. A. Weitz, A. D. Griffiths and C. A. Merten, *Chem Biol*, 2008, **15**, 427-437.
53. C. Eggeling, A. Volkmer and C. A. Seidel, *Chemphyschem*, 2005, **6**, 791-804.
54. F. Courtois, L. F. Olguin, G. Whyte, D. Bratton, W. T. S. Huck, C. Abell and F. Hollfelder, *Chembiochem*, 2008, **9**, 439-446.
55. K. Churski, T. S. Kaminski, S. Jakiela, W. Kamysz, W. Baranska-Rybak, D. B. Weibel and P. Garstecki, *Lab on a Chip*, 2012, **12**, 1629-1637.
56. S. L. Sjostrom, H. N. Joensson and H. A. Svahn, *Lab on a Chip*, 2013, **13**, 1754-1761.



1057x530mm (72 x 72 DPI)

Using a split laser, we analysed how the velocity of in-flow microdroplets modify the droplet fluorescence signal when interrogated by LIF.