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Microfluidic Droplet Consistency Monitoring and Cell Detection via Laser Excitation

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Abstract

Microfluidic droplets formed in emulsions are used in a variety of analytical techniques and hold great potential for future scientific and commercial applications. Our experiments merge quantitative quality engineering methods into the microdroplet field. We present a unique microdroplet generation and consistency monitoring system with laser optics excitation and detection. Our setup analyzes each droplet with sub-millisecond signal resolution and single photon accuracy, and is compatible with process control methods. To demonstrate the consistency of microdroplet generation over time, we measure and examine the mean frequency of aqueous plug-shaped droplet (microplug) formation in oil phase, as well as the mean length of plugs, and the interval between consecutive droplets. We also demonstrate the detection of cancer cells encapsulated within aqueous microdroplets in continuous oil phase flow. Two-channel optical monitoring allows for the simultaneous and independent inspection of both microdroplet generation and identification of green fluorescent protein-labelled cancer cells within the droplets. Increased accuracy and consistency are central to many established and developing microfluidic technologies. A systematic, quantitative approach as demonstrated with our experiments may be essential in the development of advanced microfluidic concepts that require exacting reproducibility and would greatly benefit from incorporated automated measurement techniques for process control.

Keywords

microfluidic droplets; laser excitation; cell encapsulation; process control; quality assurance

INTRODUCTION

Microfluidic emulsion droplets hold great potential for new scientific insights and practical applications. They can be utilized in transport and manipulation of small liquid volumes for

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fundamental biological and chemical studies (Song et al. 2006; deMello 2006) and are also advantageous for a myriad of applications including microreactors (de Mas et al. 2003), integrated circuit cooling systems (Paik et al. 2004), digital microfluidic circuits (Cho et al. 2003; Ding et al. 2001), and bio-analytical devices (Srinivasan et al. 2004; Burns et al. 1998; You et al. 1997). Other microspheres such as multiple emulsions (Utada et al. 2005), vesicles (Thorsen et al. 2001), and liposomes (Locascio et al. 2002) have potential for use in an assortment of industries.

A paradigm shift may be helpful for microdroplets to quickly deliver their full potential. The realization of advanced concepts such as digital fluidic computing could benefit from the adoption of rigorous process control approaches, on-line monitoring techniques, and fundamental manufacturing engineering (time series analysis) methods used in the computer semiconductor industry. Although Honoré Blanc introduced precisely machined interchangeable parts in the 18th century, the concept was not fully applied for over 100 years when industrialists such as Ransom E. Olds and Henry Ford put the assembly line into practice, revolutionizing industry and transportation worldwide. Analogously, advanced process control methods may allow microfluidics to deliver the accuracy and reproducibility that are the cornerstones of modern technology.

This paper demonstrates a microdroplet generation and consistency monitoring system with laser optics excitation and detection. Studies have investigated droplet manipulation (Link et al. 2004; Tan and Yee 2005; Tice et al. 2004; Anna et al. 2003) and observed droplet size distributions by offline counting (Umbanhowar et al. 2000; Sugiura et al. 2001); this work engages *in situ* quality monitoring in the droplet-based microfluidics discipline. Using manufacturing engineering methodology and photon-accurate quantitative analysis, the novel setup uniquely characterizes each droplet and opens the window for forecasting, feedback, and control of droplet generation.

Previous studies on the formation of monodisperse microdroplets have observed the size distributions via image capture analysis (Umbanhowar et al. 2000; Sugiura et al. 2001) and many microdroplet papers in the open literature make limited mention of the methods, if any, used to observe the resultant sizes. On the other hand, methods in sundry fields have proven the accuracy, reliability, and reproducibility of laser optics based detection systems. For example, Gratton and colleagues were able to use dual-color single point measurements with a femtosecond multiphoton laser source to assess heterogeneity of lipid and crystalline phases, and to further measure changes in geometry of these domains (Gratton et al. 2001).

Compared to existing devices, our monitoring system offers increased accuracy and provides the possibility of high-speed quantitative output for manufacturing process control. In addition to the droplet generation and consistency monitoring experiments, this paper presents the detection of cancer cells encapsulated in aqueous microdroplets surrounded by continuous oil phase. Two-channel optical detection allows for the simultaneous monitoring of unlabelled microdroplet generation and identification of green fluorescent protein-labelled cancer cells within the droplets. These experiments demonstrate the applicability of our novel setup to medical diagnostics and other biological or chemical assays (Song et al. 2006; deMello 2006).

MATERIALS AND METHODS

Oil-water microfluidics

In our experiments it is possible to observe the formation of aqueous droplets within oil at low flow rates, as Figure 1 (inset micrograph) shows. Wesson soybean oil (ConAgra Foods, Omaha, NE) and an aqueous liquid are directed into a horizontal microchannel. Deionized water with fluorescein (Sigma-Aldrich, St. Louis, MO) is the aqueous liquid used in preliminary experiments, whereas the secondary experiments employ phosphate buffered saline-cell suspension. Soft lithography rapid prototyping techniques (Duffy et al. 1998) are used to fabricate y-shaped 300 μm wide $\times$ 100 μm high polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) microchannels from photoresist (SU-8 50, Microlithography Chemical Corp., Newton, MA) master molds on 4-inch silicon wafers. Separate syringe pumps (KDS 100, KD Scientific, Holliston, MA) are used to inject the two liquids into the microchannel's flow focusing geometry. The aqueous solution is supplied via the middle inlet and oil via the two side inlets of the horizontal PDMS microchannel. At high flow rates, the channel geometry produces a stable water stream surrounded by oil sheath.

Optical Setup

The custom-built two-photon *in situ* microdroplet monitoring apparatus (Figure 1) uses a Titanium:Sapphire laser (Mira, Coherent Inc., Santa Clara, CA), which generates 50-femtosecond pulses at 800 nm with a repetition rate of 76 MHz. The femtosecond near infrared laser beam is focused into the microfluidic channel with the same objective (40X, Olympus America, Center Valley, PA) used to collect fluorescence from the microdroplets labeled with 10 mM fluorescein. Two-photon excitation enables efficient complete dichroic separation of the exciting and detected optical signals. A dichroic mirror reflects the laser into the microscope objective while transmitting the collected fluorescence. The fluorescein signal is recorded at the single photon level with a photomultiplier tube (PMT, Hamamatsu Photonics HC7421-40, Japan) via a multi-channel photon counting scaler (MCS SR430, Stanford Research Systems, Sunnyvale, CA). The signals are sent to a computer for data analysis.

Microdroplet Peak Analysis Algorithm

A MATLAB (The Mathworks, Inc., Natick, MA) program is used to extract fluorescent peaks above the background noise level from the multi-channel photon counting scaler trace signals. The data consists of a sequence of photon counts per 10.48 ms bin. An estimate of the background noise in a given experiment is performed by first analyzing the bins above the half-maximum photon count for the entire experiment. The final background bins are deemed to be all those bins with photon counts below the mean plus one standard deviation ($m_{initial} + 1\sigma_{initial}$) of the initial background estimate. The final background signal is taken as $m_{final} + 1\sigma_{final}$ of the final background bins. A peak is required to exceed this background signal by at least an additional standard deviation σ_{final} to be counted as a *bona fide* microdroplet. This threshold corresponds very well to the maximum signal level from the control trace (an experiment with 0 ml/hr water flow rate, 2 ml/hr oil flow rate). Once a peak is located, the peak characteristics, such as height (maximum fluorescence signal within the

peak), width (number of consecutive bins above the background threshold or full-width-at-half-maximum FWHM of signal above background) and location (the index of the maximum bin) are stored. A double-peak event is treated as a single event if the fluorescence signal between the two peaks does not fall below the background threshold value.

Cell Encapsulation

In the secondary experiments, we demonstrate the detection of cancer cells encapsulated in aqueous microdroplets surrounded by continuous oil phase. MCA 207 murine fibrosarcoma cells expressing green fluorescent protein (GFP) are suspended in a phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO) solution. The PBS-cell suspension is supplied via the middle inlet and oil via the side inlets of the microchannel. The flow rates of PBS-cell suspension and oil are adjusted with syringe pumps to produce microdroplet plugs as in Figure 1 (inset micrograph). In this manner, we generate microdroplets containing GFP-labelled cells surrounded by continuous oil sheath.

RESULTS AND DISCUSSION

Microdroplet Consistency Trends

Analyzing the flow data (Figure 2) with MATLAB, we can observe from the generated graphs if there are any fluctuations from the mean frequency of water droplet plug (microdroplet) formation, as well as the mean length of droplets, and the interval between consecutive plugs. In the graphs, the height of the peak corresponds to the fluorescence emitted by the excited region of the plug, which can be quantified. Our optical excitation and detection system exhibits submillisecond resolution and is capable of higher throughput rates than demonstrated; however, the microchannel design used in these experiments requires relatively low flow rates for the formation of droplets as opposed to a stable stream. These proof-of-principle experiments have been successful and alternative designs and material or fluid choices enabling higher droplet generation rates could be considered in the future. Our goal is to know the quantity and consistency of microplugs processed through the microdroplet generator.

Figures 3a and 3b help quantitatively demonstrate the consistency of the microdroplet generation over time. This on-line monitoring approach allows for statistical control via traditional Box-Jenkins modeling (Box and Jenkins 1970) or other ARIMA (autoregressive integrated moving average) and related methods.

Figure 3a confirms that our newly developed microdroplet generator maintains good consistency in both the plug length and plug separation, with a few occasional outliers. The plug separation outliers represent instances where two plugs are released in more rapid succession than average, or are slightly delayed. The plug length outliers are indicative of a shorter than average plug formation, while there is not a single case where the length of the plug is above the mode, represented by the nearly solid green line.

Physical Interpretation of Plug Length Trends

Figure 4 shows observed trends in individual plug length based on flow rate. The data points for the plot are the mean and standard deviation values corresponding to the histograms of individual plug length for varied oil and water flow rates.

It is interesting to correlate the average plug length (y) and the water flow rate (w). If the plug diameter did not change with water flow rate, one would anticipate direct proportionality to w (i.e., y varies as the 1st root of w). At the other extreme, if the plug expanded equally in all three dimensions with increasing water flow rate, the plug length would vary as the cube (3rd) root of w . Thus, a physically meaningful situation would bound the variance of plug length as the 1st – 3rd root of w .

The best power-law fit of plug length (y) versus relative oil/water flow rate (x) yields y proportional to $x^{-0.63}$. Thus, for a fixed oil flow rate, the plug length is directly proportional to the $1/0.63 = 1.6$ th root of water flow rate (w). This indicates that the plug expands quite preferentially in the direction of flow relative to any possible transverse expansion direction. As described in the next section, these elongated plugs are advantageous for optical analysis of intra-droplet objects such as cells; they exhibit less curvature at the oil-water interface and permit the laser beam to be at more normal incidence, resulting in less refraction.

Cell Detection via Laser Excitation

The cell-containing microdroplets from the secondary experiments are registered utilizing the laser optics excitation and detection setup (Figure 1), with the addition of a second photomultiplier tube (PMT) to detect long wavelengths that are emitted. In these experiments, the long wavelength channel detects in the 635 – 685 nm range and the short wavelength channel detects in the 505 – 555 nm range, which encompasses the GFP emission spectrum. The short channel signal represents fluorescence emitted by the GFP-labelled cells, whereas the long channel signal is used to indirectly detect the microdroplets by virtue of the soybean oil's fluorescence. Fluorescence emission from chlorophyll derivatives in vegetable oils has been recorded in the 640 – 800 nm range (Zandomeneghi et al. 2005; Kyriakidis and Skarkalis 2000; Casady 2000), and the long detection channel samples a subset of this region. Thus, areas where fluorescence is detected in the long channel can be attributed to regions of oil, and areas where no fluorescence is detected in the long channel correspond to the PBS-cell suspension droplets.

As a control experiment, we produced PBS droplet plugs without encapsulated cells. In Figure 5a one observes the uniform formation of PBS microdroplets; typical droplet formation data, consistency plots, and histograms were presented in Figure 3 (e.g., 15 droplets per 10 seconds). Figure 5b demonstrates the detection of PBS microdroplets without any fluorescent content via the long detection channel.

Co-registration of plugs via the long detection channel (Figure 5b) and GFP fluorescence via the short detection channel makes it possible to detect cells encapsulated within PBS microdroplets, as shown in Figure 5c. This proof-of-encapsulation experiment incorporates simultaneous detection in two spectral channels to distinguish between three targets: cells, oil, and PBS. As expected, cells are detected only within PBS droplets.

Integrating the detection of cells within aqueous microplugs (Figure 5c) with the droplet consistency monitoring system would allow for quantitative analysis of the encapsulated cell suspension. Implementing soluble fluorophores could expand this method to biochemical applications such as screening of enzyme libraries engineered via directed evolution (Aharoni et al. 2005; Mastrobattista et al. 2005; O'Hare and Johnsson 2005). In addition to applications in droplet-based microfluidics, it is our hope that this laser-based high-speed monitoring method will be an enabling technology for automated microfluidic cell culture techniques.

CONCLUSIONS

We have developed a novel microdroplet generation and consistency monitoring system with laser optics excitation and detection. We have demonstrated *in situ* quality monitoring of microfluidic droplets and laser-based detection of cancer cells encapsulated in microdroplets. Our cell experiments draw upon two-channel optical detection to simultaneously and independently monitor both microdroplet generation and green fluorescent protein-labelled cancer cells within the microdroplets.

Compared to prevailing image-based offline counting techniques that are inherently qualitative or quasi-quantitative, this approach offers single photon accuracy, sub-millisecond signal resolution, and compatibility with advanced process control methods. The on-line monitoring algorithm is conducive to ARIMA and related statistical control methods for forecasting and quality assurance of microdroplet generation. This interdisciplinary solution is one example where adoption of manufacturing engineering process control methods may help propel the microdroplet field toward more advanced applications; fusing the disciplines of microfluidics and process control may help microdroplets quickly deliver their bountiful promise.

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Figure 1.
Laser-based optical setup used for microfluidic droplet plug consistency monitoring.

Figure 2. Raw data as registered by the PMT. Each peak corresponds to a microdroplet plug of fluorescein within the oil sheath.

Figure 3a

Figure 3b

Figure 3.

Figure 3a. Microdroplet plug production consistency for (0.15 ml/hr water, 2 ml/hr oil). The top graph shows the consistency of the plug production over the course of the experiment, averaging approximately 15 plugs per 10 seconds. The bottom graph shows the consistency of the plug length (green) and time between plugs (blue).

Figure 3b. This plot includes histograms corresponding to the data presented in the previous figure. The histograms show the distributions of the microfluidic droplet plug length variation (top graph) and the plug separation time interval (bottom graph).

Figure 4.

Observed individual microplug length as a function of relative oil/water flow rate in the microchannel. All data plotted use an oil flow rate of 2 ml/hr. The error bars show the standard deviation of ~200 plugs observed at each flow rate.

Figure 5a

Figure 5b

Figure 5c

Figure 5.

Figure 5a. PBS microdroplet plugs advance down the length of the microchannel. The droplets are surrounded by oil. The red peaks in **Figures 5b and 5c** correspond to regions of oil (red arrows above), and the red valleys in Figures 5b and 5c correspond to the PBS droplets (black arrows above). In Figure 5c, the purple data represent GFP-labelled cells suspended and detected in the PBS droplets.

Figure 5b. This plot indicates the ability to detect unlabelled microdroplet plugs of phosphate buffered saline (PBS) surrounded by oil sheath. The peaks correspond to areas of oil, and the valleys correspond to the PBS plugs. The signal is observed in the long detection channel, which detects in the 635 – 685 nm range, and is attributed to the fluorescence emission of soybean oil. The raw data is represented in blue and a Gaussian fit moving average is shown in red.

Figure 5c. This plot demonstrates the ability to detect GFP-labelled cells (purple data) within PBS microdroplets (red valleys), with oil as the continuous phase. By simultaneous detection in two spectral channels, it is possible to distinguish between three targets: cells, oil, and PBS. The raw signal from the cells (purple) is observed in the short detection channel, which detects in the 505 – 555 nm range and encompasses the GFP emission spectrum. The plug signal is observed in the long channel, which detects in the 635 – 685 nm range. As in Figure 5b, the red peaks correspond to areas of oil, and the red valleys correspond to the PBS plugs. This signal is based on fluorescence emission of soybean oil and is a Gaussian moving average fit.