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Multicolor Fluorescence Detection for Droplet Microfluidics Using Optical Fibers

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Abstract

Fluorescence assays are the most common readouts used in droplet microfluidics due to their bright signals and fast time response. Applications such as multiplex assays, enzyme evolution, and molecular biology enhanced cell sorting require the detection of two or more colors of fluorescence. Standard multicolor detection systems that couple free space lasers to epifluorescence microscopes are bulky, expensive, and difficult to maintain. In this paper, we describe a scheme to perform multicolor detection by exciting discrete regions of a microfluidic channel with lasers coupled to optical fibers. Emitted light is collected by an optical fiber coupled to a single photodetector. Because the excitation occurs at different spatial locations, the identity of emitted light can be encoded as a temporal shift, eliminating the need for more complicated light filtering schemes. The system has been used to detect droplet populations containing four unique combinations of dyes and to detect sub-nanomolar concentrations of fluorescein.

Video Link

The video component of this article can be found at http://www.jove.com/video/54010/

Introduction

Droplet microfluidics provide a platform for high throughput biology by compartmentalizing experiments in a large number of aqueous droplets suspended in a carrier oil [1]. Droplets have been used for applications as varied as single cell analysis [2], digital polymerase chain reaction (PCR) [3], and enzyme evolution [4]. Fluorescent assays are the standard mode of detection for droplet microfluidics, as their bright signals and fast time response are compatible with detecting sub-nanoliter droplet volumes at kilohertz rates. Many applications require fluorescence detection for at least two colors simultaneously. For instance, our lab commonly performs PCR-activated droplet sorting experiments that use one detection channel for the result of an assay, and uses a secondary background dye to make assay-negative droplet countable [5].

Typical detection stations for droplet microfluidics are based on epifluorescence microscopes, and require complicated light manipulations schemes to introduce excitation light from free space lasers into microscope to be focused on the sample. After fluorescence is emitted from a droplet, the emitted fluoresced light is filtered so that each detection channel utilizes one photomultiplier tube (PMT) centered on a wavelength band. Epifluorescence microscope-based optical detection systems provide a barrier to entry due to their expense, complexity, and required maintenance. Optical fibers provide the means to construct a simplified and robust detection scheme, since fibers can be manually inserted into microfluidic devices, removing the need for mirror-based light routing, and allowing light paths to be interfaced using optical fiber connectors.

In this paper, we describe the assembly and validation of a compact and modular scheme to perform multicolor fluorescence detection by utilizing an array of optical fibers and a single photodetector [6]. Optical fibers are coupled to individual lasers and are inserted normal to an L shaped flow channel at regular spatial offsets. A fluorescence collection fiber is oriented parallel to the excitation regions and is connected to a single PMT. Because a droplet passes through the laser beams at different times, data recorded by the PMT shows a temporal offset that allows the user to distinguish between the fluorescence emitted after the droplet is excited by each distinct laser beam. This temporal shift eliminates the need to separate emitted light to separate PMTs using a series of dichroic mirrors and bandpass filters. To validate the efficacy of the detector, we quantitate fluorescence in droplet populations encapsulating dyes of different color and concentration. The sensitivity of the system is investigated for single color fluorescein detection, and shows the ability to detect droplets with concentrations down to 0.1 nM, a 200x sensitivity improvement as compared to recent fiber based approaches reported in the literature [7].

Protocol

1. SU8 Master Fabrication

1. Design the microfluidic structures for three layer fabrication using design software and have the designs printed by a vendor on circuit board film with 10 µm resolution. The details of device design are given in an attached reference [6] and the channel geometries are shown in **Figure 1**. The layers should include alignment marks to help collocate features from each fabrication layer [8].
2. Place a pre-cleaned 3 inch diameter silicon wafer on a spin coater and turn on the vacuum to affix it to the chuck. Apply 1 ml of SU8-3050 in the center of the wafer and spin for 20 sec at 500 rpm, then 30 sec at 1,750 rpm, providing a layer thickness of 80 µm.
3. Remove the wafer and bake on a 135 °C hotplate for 30 min. Allow the wafer to cool to RT before moving on to the next step.
4. Expose the coated wafer to the 1st layer mask under a collimated 190 mW, 365 nm LED for 3 min. After exposure, place the wafer on a 135 °C hotplate for 1 min, then cool to RT before proceeding to the next step.
5. Place the wafer on the spin coater and turn on the vacuum to affix it to the chuck. Apply 1 ml of SU8-3050 in the center of the wafer and spin for 20 sec at 500 rpm, then 30 sec at 5,000 rpm, resulting in a layer that provides an additional thickness of 40 µm.
6. Remove the wafer and bake on a 135 °C hotplate for 30 min, then cool to RT before moving to the next step.
7. Align the 2nd layer mask onto the geometry patterned in 1.3 and expose the coated wafer to a collimated 190 mW, 365 nm LED for 3 min. After exposure, place on a 135 °C hotplate for 4 min 30 sec, then cool to RT before proceeding to the next step.
8. Place the wafer on the spin coater and turn on the vacuum to affix it to the chuck. Apply 1 ml of SU8-3050 in the center of the wafer and spin for 20 sec at 500 rpm, then 30 sec at 1,000 rpm, resulting in a layer that provides an additional thickness of 100 µm.
9. Remove the wafer and bake on a 135 °C hotplate for 30 min, then cool to RT before moving to the next step.
10. Align the 3rd layer mask onto the geometry patterned in 1.3 and expose the coated wafer to a collimated 190 mW, 365 nm LED for 3 min. After exposure, place on a 135 °C hotplate for 9 min, then cool to RT before proceeding to the next step.
11. Develop the masks by immersing in a stirred bath of propylene glycol monomethyl ether acetate for 30 min. Wash the wafer in isopropanol and bake on a 135 °C hotplate for 1 min. Place the developed master in a 100 mm Petri dish for polydimethylsiloxane (PDMS) molding.

2. PDMS Device Fabrication

1. Prepare 10:1 PDMS by combining 50 g of silicone base with 5 g of curing agent in a plastic cup. Mix the contents with a rotary tool fitted with a stir stick. Degas the mixture inside a desiccator for 30 min, or until all air bubbles are removed.
2. Pour the PDMS to give a thickness of 3 mm over the master and place back into the desiccator for further degassing. Once all bubbles are removed, bake the device at 80 °C for 80 min.
3. Cut the device from the mold using a scalpel and place on a clean surface with the patterned side up and punch the fluidic inlets and outlets with a 0.75 mm biopsy punch. The device must be cut so that the 120 µm and 220 µm tall geometries are accessible from the side of the device.
4. Plasma treat the device, with feature side up, along with a pre-cleaned 2 inches by 3 inches glass slide at 1 mbar O_2 plasma for 20 sec in a 300 W plasma cleaner. Bond the PDMS device by placing the patterned side of the PDMS device onto the plasma-treated side of the glass slide. Place the device in a 80 °C oven and bake the assembled device for 40 min.
5. Render the channels hydrophobic by using a syringe to flush the device with a fluorinated surface treatment fluid. Immediately bake the device at 80 °C for 10 min to evaporate the solvent.

3. Preparation of Optical Components

1. Prepare the laser excitation fiber by removing the insulation from the last 5 mm of a 105 µm core, 125 µm cladding, NA = 0.22 optical fiber.
2. Prepare the 2nd color laser excitation fiber by repeating 3.1 for a 2nd identical fiber.
3. Prepare the fiber for collecting the fluorescence signal by repeating 3.1 for a 200 µm core, 225 µm cladding, NA = 0.39 optical fiber.
4. Inspect the tips of all the fibers — if the tips do not end in a flat surface, re-cleave the ends with a fiber scribe.
5. Attach a laser fiber coupler to a 50 mW, 405 nm laser and attach one of the 105 µm core fibers to the laser. Direct the stripped end at the sensor of the laser power meter, and use the fine adjustments of the laser coupler to maximize the laser power.
6. Repeat 3.5 for a 50 mW, 473 nm laser.
7. Mount a 446/510/581/703 nm quad bandpass filters on the PMT using lens tubes to block laser light and transmit emitted fluorescence. Attach fiber coupler so that light travels through the filters before hitting the PMT.
8. Attach the fiber from 3.3 to the unit assembled in 3.7.

4. Offline Mixed Emulsion Generation

1. Obtain a flow focus microfluidics dropmaker device with a 60 µm x 60 µm orifice.
2. Fill a 1 ml syringe with HFE 7500 oils with 2% ionic fluorosurfactant [9].
3. Fill a series of 1 ml syringes with the following combinations of fluorescein (FITC) and dextran-conjugated blue dyes (CB) in PBS: 1 nM FITC/10 nM CB, 10 nM FITC/10 nM CB, 1 nM FITC/100 nM CB, and 10 nM FITC/100 nM CB.
4. Mount the dropmaker device on the stage of an inverted microscope and the aqueous and oil syringes on syringe pumps. Couple the syringes to the devices using PE-2 tubing.
5. Running syringe pumps at 500 µl/hr for the oil and 250 µl/hr for the aqueous solutions, create a mixture of 80 µm droplets containing the solutions from 4.3. For each type of aqueous sample, use a fresh dropmaker device to eliminate cross-contamination. Collect ~200 µl of emulsion from each dye combination into an empty syringe.
6. After the 4 droplets types have been collected in a single collection syringe, mix the emulsion by repeatedly rotating the syringe.

7. Repeat steps 4.2-4.6 with aqueous solutions containing 0.1, 1, 10, and 100 nM FITC in PBS.

5. Optical Fiber Insertion

1. Place the microfluidic chip fabricated in step 2 on the stage of an inverted microscope coupled with a digital camera capable of < 100 µsec shutter speeds.
2. Working carefully from the side, insert the fiber coupled to the 473 nm laser into the farthest upstream 120 µm channel, taking care not to puncture through to the main flow channel.
3. Insert the fiber coupled to the 405 nm laser into the farthest downstream 120 µm high side channel, providing fiber spacing of 300 µm.
4. Insert the PMT-coupled fiber into the 220 µm tall channel normal to the two laser excitation fibers.

6. Fluorescence Detection of Mixed Emulsions

1. Mount a 5 ml syringe filled with HFE 7500 with 2% ionic fluorosurfactant to the spacer oil inlet of the detection device with PE-2 tubing.
2. Mount the syringe containing the mixed FITC/CB emulsion on a vertically oriented syringe pump and couple to the device's droplet reinjection inlet using PE-2 tubing. Connect a length of PE-2 tubing from the device exit to a waste container.
3. Prime the device by running each of the pumps at 1,000 µl/hr until both oil and droplets are seen to be regularly combining in the device and flowing downstream.
4. Adjust the flow rates such that the spacer oil runs at 6,000 µl/hr and the droplets at 100 µl/hr, providing significant spacing between droplets traveling through the detection region.
5. Turn on the lasers, start the data acquisition program, and adjust the PMT gain to provide signals that are more than 100x the baseline noise floor. Adjust the laser power so that all of the doublet peaks are clearly visible on a single, linearly scaled timetrace.
6. Acquire 60 sec of the PMT voltage timetrace at at least 20 kHz. Import the data into computing program such as Matlab and measure the heights of peaks of the recorded doublets using the custom computing program script.
   NOTE: This is provided as supplementary information.
7. Repeat steps 6.2-6.7 using the FITC-only mixed emulsion created in 4.7, with the 405 nm laser turned off.

Representative Results

Fabrication of a PDMS device that allows for the insertion of optical fibers requires a multistep photolithography procedure to create channels of varying height (**Figure 1**). First, an 80 µm tall layer of SU-8 is spun onto a silicon wafer and patterned using a mask to create the fluid handling geometry. Next, an additional 40 µm layer of SU-8 is spun onto the wafer, and patterned using a second mask to create features that will form 120 µm tall laser fiber insertion channels. Last, 100 µm more SU-8 is spun onto the wafer, and patterned to give 220 µm tall detection fiber insertion channels. The master is developed and used to mold a PDMS device, which is in turn bonded to a glass slide. The final fabrication contains 80 µm tall flow geometry, accessed via holes punched in the top of the device and 120 µm and 220 µm tall optical fiber channels accessed through the side of the device.

The geometry of the device used for detection is shown in **Figure 2**. Externally generated 80 µm emulsions are re-injected into a drop reinjection port and spaced by spacer oil before they flow into a L shape detection channel. Two laser-coupled fibers are inserted into 120 µm tall channels provide excitation at discrete spatial locations in the flow channel. Because the multimode fibers inserted into these channels have a 125 µm external diameter and a 105 µm core diameter, the entire cross section of the flow channel is illuminated by laser light. In our device the detection flow channel makes an abrupt 90 degree turn after the last laser excitation region to create close optical access for a 225 µm OD fiber used to detect emitted fluorescence. The larger fiber is used because of its high numerical aperture (NA = 0.39), which is similar to a standard microscope objective.

A droplet flowing through the detection channel encounters discrete excitation regions in front of each of the laser-coupled optical fibers (**Figure 3A**). The single PMT coupled to the detection fiber records emitted fluorescence with a temporal shift the maps to the excitation source. For a droplet that contains dyes that excite at 473 nm (region "1" in **Figure 3A**) and at 405 nm (region "2" in **Figure 3B**), the resulting PMT signal contains a signal doublet with a peak separation that correlates with the time it takes a droplet to move from one excitation region to the next. By temporally encoding spectral data in this manner, the need for further light filtering and separate PMTs for each fluorophore color is eliminated. **Figure 3B** shows signal doublets for a train of 4 droplets with different dye combinations. The first peak in the double corresponds to the fluorescence emitted after excitation by the 473 nm laser in region "1" and the second peak corresponds to fluorescence emitted after excitation by the 405 nm laser in region "2".

The efficacy of the detection scheme was tested by detecting a mixed emulsion of droplets containing dyes that excite at 405 nm (dextran-conjugated blue, CB) and at 473 nm (fluorescein, FITC). Droplets were flowed through the detection regions at roughly 50 Hz and data was recorded for 60 sec, then post-processed with a computing program script. The data is plotted in **Figure 4** and shows clear separation between the four re-injected droplet types. Because the detection system does not differentiate between different colors of emitted light, each droplet is described by its maximum total fluorescence after being excited in region "1" (peak 1) and in region 2 (peak 2).

The dynamic range and absolute sensitivity of the detector is investigated by turning off the 405 nm laser and measuring the fluorescence of an emulsion containing fluorescein-only droplets (**Figure 5**). The data shows the ability to detect dye concentration that range from 0.1 to 100 nM FITC on a single detector with the same detection settings.

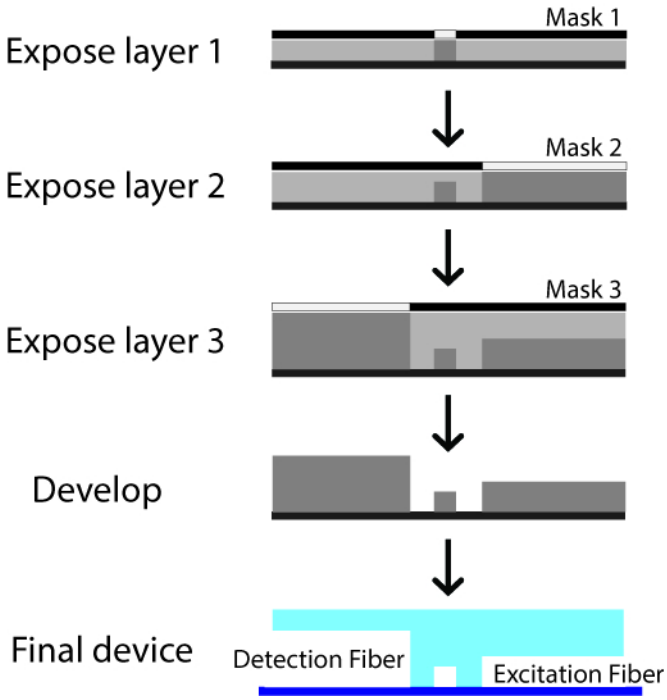

Figure 1. Multi-layer photolithography. The microfluidic device is fabricated by creating a master with three different feature heights. First, a 80 µm tall layer of SU-8 is spun on and patterned using a mask for the fluid channels. Next, 40 µm more SU-8 is spun on and 120 µm tall features are patterned with a 2nd mask through both of the SU-8 layers to yield geometry to accommodate 125 µm tall optical fibers. After this, 100 µm more SU-8 is spun on and patterned with a 3rd mask to yield 225 µm tall features. After developing, PDMS molding, and plasma bonding to glass, the resultant device contains large channels accessible from the side for fiber insertion, in addition to standard enclosed fluid channels. Please click here to view a larger version of this figure.

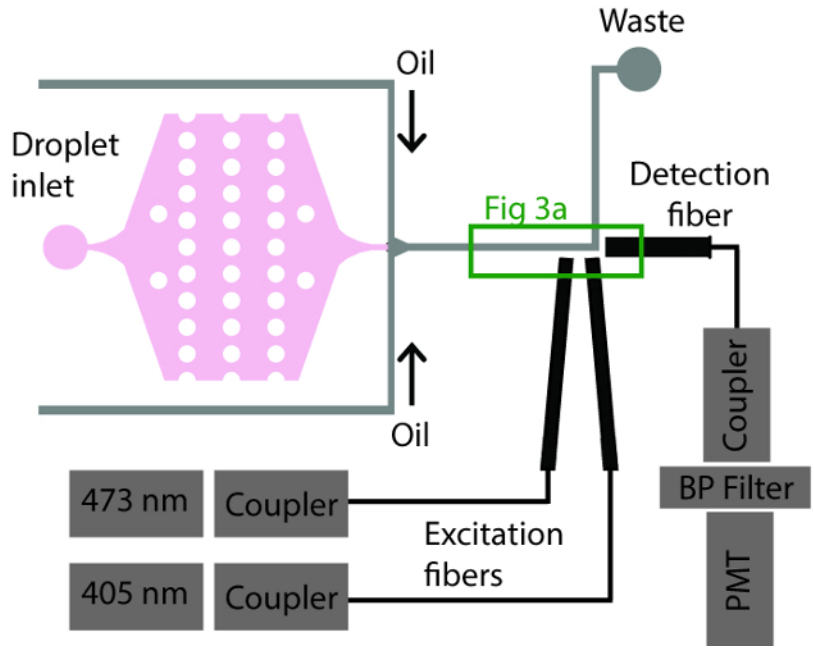

Figure 2. Device design and optical layout. The fluidic portions of the device are comprised of a droplet reinjector and an oil spacer, coupled to an L-shaped channel. Fibers coupled to individual lasers are inserted normal to the direction of flow. A detection fiber is inserted perpendicular to the laser fibers in order to collect fluorescent light. This light is filtered through a bandpass filter, before being detected by a single PMT. Please click here to view a larger version of this figure.

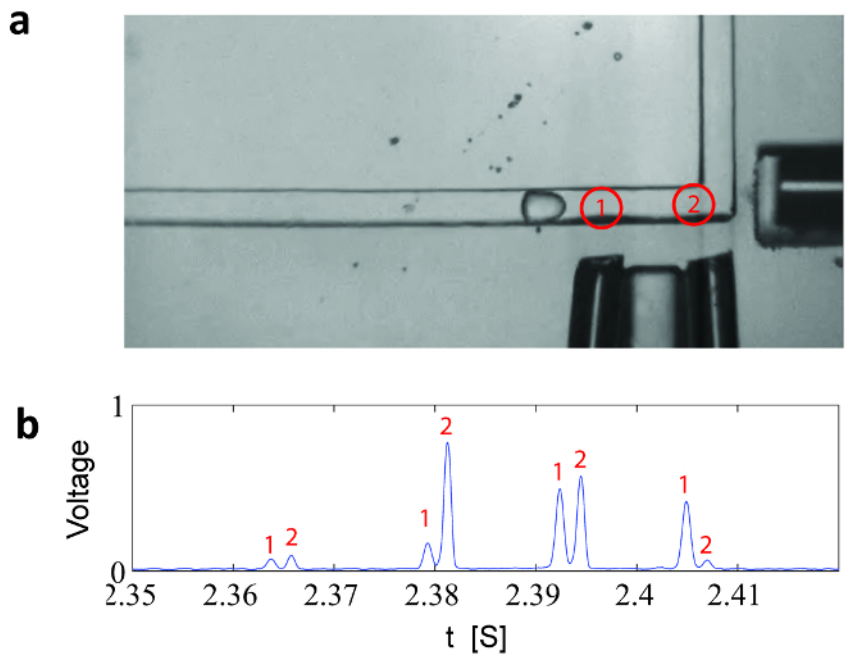

Figure 3. Temporal encoding of multicolor information. (**A**) Droplets flow through excitation regions "1" and "2" at different times, providing time shift that allows the identification of the emitted light. (**B**) Data from a train of 4 different droplets flowing through the detection channel. The first peak of each doublet corresponds to the fluorescence emitted after excitation in region "1" and the second peak in each double corresponds to the fluorescence emitted after excitation in region "2". The height of droplet peaks are proportional the amount of dye excited at a given wavelength, except when spectral bleed occurs due to the use of dyes with overlapping excitation spectra. The width of the peaks are determined by the amount of time that it takes a droplet to traverse an excitation region. Please click here to view a larger version of this figure.

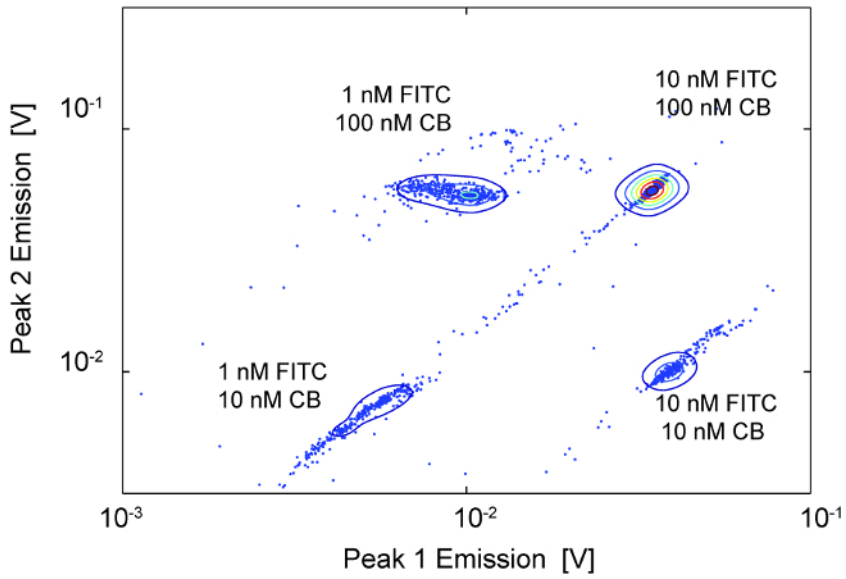

Figure 4. Multicolor detection of a mixed droplet population. Scatter plot showing maximum droplet intensities for a mixed droplet population after excitation and emission by a 473 nm laser (peak 1) and a 405 nm laser (peak 2). The droplets contain combinations of fluorescein (FITC) and cascade blue (CB) at nM concentrations. Please click here to view a larger version of this figure.

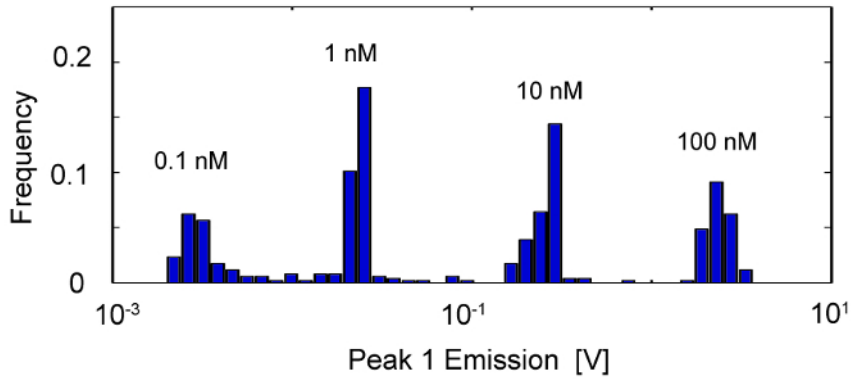

Figure 5. Single color detection on highly varied droplet population. Histogram of emissions by a mixed droplet population containing 0.1-100 nM of fluorescein after excitation by a 473 nm laser. Please click here to view a larger version of this figure.

Discussion

Fiber optic detection requires the alignment of optical fibers with respect to fluid channels. Because our device utilizes guide channels fabricated with multilayer photolithography, placement of masks with respect to each other is of great importance. If the fiber guide channels are too close to the fluid channel, there is a potential for fluid leakage; if the guide channels are located too far away or misaligned, the fluorescence signal gathered by the detection fiber may be significantly diminished. Proper alignment can be aided by designing alignment marks, such as concentric circles into masks to be co-located during photo patterning. Additionally, manual insertion of fiber optics into the device is a delicate process with the potential to break the tips of fibers within the device, rendering them unusable. Restraining fiber against the glass slide bonded to the PDMS device and inserting the fibers slowly while observing with the microscope are keys to successful fiber insertion. Last, reinjection of pre-formed emulsions should occur with a minimum of handling. Ideally, once the emulsion is made and deposited directly into an empty syringe, it should not be transferred until it is used for droplet reinjection.

Multicolor detection requires that the peaks in a signal doublet be distinct from one another and that signals from consecutive droplets do not overlap. The detection setup that we use here uses a pair of 125 µm optical fibers separated at a center-to-center distance of 300 µm, providing a non-illuminated region of ~175 µm between excitation regions. This allows an 80 µm droplet to be excited by only one light source at a time and ensures that each of the peaks in a doublet is solely a result of one type of laser excitation. Designs where the separation between the excitation regions is similar to the droplet diameter give problematic signals with overlapping emission peak doublets. The width of a signal doublet correlates to the time that it takes for the leading edge of a droplet to enter the first excitation regions to the time the trailing edge of the droplet to leave the final excitation region, a distance of ~500 µm in the device we describe. In order to maintain distinctness of adjacent detected droplets, signal doublets need to be spaced by a low signal region at least as wide as the signal doublet. For the configuration described here, 80 µm droplets are spaced at least 1 mm apart.

Although multi-color detection with a single photodetector allows for significant savings in terms of cost and complexity compared to standard techniques, there is some loss of robustness because emitted light is not filtered by wavelength. This can be problematic when detecting droplets that contain multiple fluorophores that excite at the same wavelengths, because the fluorescence source is indistinguishable. These limitations can be overcome by performing experiments with nonoverlapping excitation profiles, or by designing experiments where spectral bleed over does not occur into a "channel" where a sensitive assay is being performed. This is compatible with many droplet microfluidic detection applications, where a bright background dye is used to indicate the presence of a droplet, and a second fluorophore is used to report the result of a molecular biology assay [10,11].

Compared to standard techniques, our approach has two main advantages: 1) spatially registered excitation regions eliminate the need for complicated light filtering, and 2) use of fiber optics removes the needs for a microscope and optical hardware to direct light. Because we address common technical concerns with microfluidic detection systems, previous investigators have developed methods to address similar concerns. For instance, Martini *et al.* frequency-encode multicolor fluorescence recorded by a single detector by projecting an excitation source through uniquely patterned "bar code" filters [12]. Our use of an optical fiber array allows the utilization of a similar temporal encoding scheme using standard, off the shelf components. The literature shows optical numerous optical coupling techniques that are more controlled that the simple fiber insertion that we describe. Bliss *et al.* use liquid filled optical wave guides for the carefully controlled delivery and collection of light from specific microchannel locations [13], Martinez Vasquez *et al.* use waveguides laser etched into fused silica substrates to deliver excitation light [14], and Vishnubhatla *et al.* are able to precisely fabricate fiber insertion channels using a combination of laser irradiation and chemical etching [15]. Each of these techniques require additional steps beyond those used to fabricate the base fluidic architecture of a microfluidic chip. While these fabrication procedures may be necessary for highly sensitive detection applications, we have found that fiber insertion into a molded PDMS channel is adequate for most of the everyday applications in our lab. The detection system was able to detect FITC concentrations down to 100 pM in 80 µm droplets, roughly equivalent to the detection of ~17,000 fluorophore molecules. This sensitivity is adequate for most detection applications in our laboratory currently using an epifluorescence-based system, the most common of which being PCR-activated cell sorting of assay positive droplets containing the equivalent of 10^6 fluorophore molecules [11]. The detection sensitivity also compares favorably to recently reported sensitivities of other optical fiber based systems — for instance, it is 200x more sensitive than the detection limit of 20 nM of Alexa Fluor 488 reported in > 100 µm droplets[7].

We have presented a compact and modular droplet microfluidic multi-color detection scheme as an alternative to more costly, complex, and bulky epifluorescence microscope based systems. The necessary detection components used here (lasers, fibers, fiber adapters, filter, and PMT) can be purchased for <$10,000, making the multi-color droplet detection accessible to a great number of investigators, and allowing

individual investigators to afford multiple detection stations in a laboratory. The system also remove some expertise-based barriers to entry, as a degree of familiarity is required to align free space optical systems in conjunction with an epifluorescence microscope. Additionally the small footprint of the detections setup makes it ideally suited for portable and diagnostic applications.

Disclosures

The authors have nothing to disclose.

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References

1. Teh, S.-Y., Lin, R., Hung, L.-H., & Lee, A. P. Droplet microfluidics. *Lab Chip.* **8**, 198-220, (2008).
2. Mazutis, L., Gilbert, J., Ung, W. L., Weitz, D. A., Griffiths, A. D., & Heyman, J. A. Single-cell analysis and sorting using droplet-based microfluidics. *Nat Protocol.* **8** (5), 870-891, (2013).
3. Hindson, B. J., Ness, K. D., *et al.* High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal Chem.* **83**, 8604-8610, (2011).
4. Agresti, J. J., Antipov, E., *et al.* Ultrahigh-throughput screening in drop-based microfluidics for directed evolution (vol 170, pg 4004, 2010). *Proc Nat Acad Sci USA.* **107** (14), 6550, (2010).
5. Eastburn, D. J., Sciambi, A., & Abate, A. R. Picoinjection Enables Digital Detection of RNA with Droplet RT-PCR. *PLOS ONE.* **8** (4), (2013).
6. Cole, R. H., de Lange, N., Gartner, Z. J., & Abate, A. R. Compact and modular multicolour fluorescence detector for droplet microfluidics. *Lab Chip.* **15** (13), 2754-2758, (2015).
7. Guo, F., Lapsley, M. I., *et al.* A droplet-based, optofluidic device for high-throughput, quantitative bioanalysis. *Anal Chem.* **84**, 10745-10749, (2012).
8. *Lithography.* at <https://www.memsnet.org/mems/processes/lithography.html>.(2015).
9. DeJournette, C. J., Kim, J., Medlen, H., Li, X., Vincent, L. J., & Easley, C. J. Creating Biocompatible Oil-Water Interfaces without Synthesis: Direct Interactions between Primary Amines and Carboxylated Perfluorocarbon Surfactants. *Anal Chem.* **85** (21), 10.1021/ac4026048, (2013).
10. Fallah-Araghi, A., Baret, J.-C., Ryckelynck, M., & Griffiths, A. D. A completely *in vitro* ultrahigh-throughput droplet-based microfluidic screening system for protein engineering and directed evolution. *Lab Chip.* **12**, 882, (2012).
11. Eastburn, D. J., Sciambi, A., & Abate, A. R. Ultrahigh-Throughput Mammalian Single-Cell Reverse-Transcriptase Polymerase Chain Reaction in Microfluidic Drops. *Anal Chem.* **85** (16), 8016-8021, (2013).
12. Martini, J., Recht, M. I., Huck, M., Bern, M. W., Johnson, N. M., & Kiesel, P. Time encoded multicolor fluorescence detection in a microfluidic flow cytometer. *Lab Chip.* **12** (23), 5057-62, (2012).
13. Bliss, C. L., McMullin, J. N., & Backhouse, C. J. Rapid fabrication of a microfluidic device with integrated optical waveguides for DNA fragment analysis. *Lab Chip.* **7** (10), 1280-7, (2007).
14. Martinez Vazquez, R., Osellame, R., *et al.* Optical sensing in microfluidic lab-on-a-chip by femtosecond-laser-written waveguides. *Anal Bioanal Chem.* **393**, 1209-1216, (2009).
15. Vishnubhatla, K. C., Bellini, N., Ramponi, R., Cerullo, G., & Osellame, R. Shape control of microchannels fabricated in fused silica by femtosecond laser irradiation and chemical etching. *Opt Express.* **17** (10), 8685-8695, (2009).

