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Automated Microfluidic Droplet-Based Sample Chopper for Detection of Small Fluorescence Differences Using Lock-In Analysis

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Abstract

Fluorescence is widely used for small-volume analysis and is a primary tool for on-chip detection in microfluidic devices, yet additional expertise, more elaborate optics, and phase-locked detectors are needed for ultrasensitive measurements. Recently, we designed a microfluidic analog to an optical beam chopper (μ Chopper) that alternated formation of picoliter volume sample and reference droplets. Without complex optics, the device negated large signal drifts ($1/f$ noise), allowing absorbance detection in a mere 27 μm optical path. Here, we extend the μ Chopper concept to fluorescence detection with standard wide-field microscope optics. Precision of droplet control in the μ Chopper was improved by automation with pneumatic valves, allowing fluorescence measurements to be strictly phase locked at 0.04 Hz bandwidth to droplets generated at 3.50 Hz. A detection limit of 12 pM fluorescein was achieved when sampling 20 droplets, and as few as 310 zeptomoles (3.1×10^{-19} mol) were detectable in single droplets (8.8 nL). When applied to free fatty acid (FFA) uptake in 3T3-L1 adipocytes, this μ Chopper permitted single-cell FFA uptake rates to be quantified at $3.5 \pm 0.2 \times 10^{-15}$ mol cell $^{-1}$ for the first time. Additionally, homogeneous immunoassays in droplets exhibited insulin detection limits of 9.3 nM or 190 amol (1.9×10^{-16} mol). The combination of this novel, automated μ Chopper with lock-in detection provides a high-performance platform for detecting small differences with standard fluorescence optics, particularly in situations where sample volume is limited. The technique should be simple to implement into a variety of other droplet fluidics devices.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information

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Experimental details; fluidic channel cross-section in μ Chopper device; manually applied voltage drifts for proof-of-concept of

μ Chopper drift correction; titration of adipose tissue explants with FFA* (PDF)

Video depicting automated, continuous operation of the μ Chopper device (AVI)

To maximize sensitivity in optical measurements, noise reduction is an important issue to address. When signal are low, at similar magnitude to the noise floor, background drifts due to optical system fluctuations or detector noise begin to complicate signal collection. Low frequency noise ($1/f$ noise or drift) is particularly detrimental to measurements and is often indistinguishable from signals. A fitting and well-established solution to this problem is the lock-in amplifier, which effectively modulates the signal of interest to a higher frequency, removes low frequency noise, and then demodulates the signal for recovery.¹⁻⁴ Signals at levels several orders of magnitude lower than the noise can be recovered using this technique, which typically employs optical beam choppers and electronic phase-locking of photodetectors.⁵

In the field of microfluidics, particularly for droplet fluidics, fluorescence is ideally suited, as optical probe volumes can be reduced to match channel and droplet dimensions.⁶ However, practical limitations exist, since high numerical aperture lenses are limited to submillimeter working distances and are often unsuitable to interrogate a variety of fluidic and substrate architectures. By integrating optics onto the microdevice, the subfield of optofluidics has addressed many of these challenges. Integrated waveguides, lenses, filters, detectors, etc., in microfluidic systems, have been applied in fields ranging from cell biology to energy conservation.⁷⁻¹¹ On the other hand, typical optofluidic systems oblige fabrication complexities that can serve as barriers to widespread use. What remains is a need for alternative, generalizable approaches to reduce noise in more standard optical detection systems used with microfluidic devices.

A common detection system used for droplet microfluidics is a standard fluorescence microscope, based on the similarity of optical probe volume and droplet volume. It is certainly possible to implement optical beam choppers and phase-locked detectors into microscope optics, yet this approach has been rare and is unlikely to gain widespread use due to its impracticality. Recently, our group and others have shown that constant droplet referencing can be used in lieu of optical choppers, allowing lock-in detection to be applied to both absorbance¹² and Raman spectroscopy¹³ with relatively simple optical systems. In our passively controlled microfluidic device (μ Chopper), alternation of sample and reference droplets at ~ 10 Hz each permitted the use of phase sensitive detection, i.e., lock-in detection, and $1/f$ noise was greatly reduced. This μ Chopper device promoted quantitative absorbance measurements at nanomolar concentrations through a path length of only $27\ \mu\text{m}$.¹² Oil phase referencing was also proven useful by our group using fluorescence¹⁴ and by März et al.

using Raman spectroscopy.¹³ However, such passively controlled systems^{15,16} are inherently limited by the frequency-domain bandwidth of droplet formation. The main sensitivity limitation then shifts to the precision of droplet generation frequency, for which passively operated devices are prone to frequency-domain drifts.

In this work, we extend our μ Chopper concept to fluorescence detection with standard wide-field microscope optics. Notably, these devices show greatly improved droplet formation precision after integrating pneumatic valving,¹⁷ and we show herein that this enhanced precision leads to concomitant improvements in drift correction and limit of detection. Finally, we present biological validation of the methodology by quantifying free fatty acid uptake in single adipocytes and by homogeneous protein detection at the attomole level. Thus, a significant performance enhancement can be achieved by combining droplet fluidics with actively controlled valves that are phase-locked with the detection system, resulting in a novel microfluidic platform that should be well-suited to a variety of single-cell or small-volume analyses.

MATERIALS AND METHODS

Materials and Reagents

Polydimethylsiloxane (PDMS) precursors, Sylgard 184 elastomer base, and curing agent were purchased from Dow Corning (Midland, Maryland, USA). Silicon wafers were acquired from Polishing Corporation of America (Santa Clara, CA, USA). AZ-40-XT photoresist, AZ 300 MIF developer, and SU-8 photoresist and developer were purchased from Microchem (Westborough, MA, USA). Tubing (TGY-020-5C; 0.02 in. ID, 0.06 in. OD, 0.02 in. wall) and blunt needles (NE-223PL-C 22G) to interface syringes and devices were obtained from Small Parts (Logansport, IN, USA). Fluorescein was obtained from Alfa Aesar (Ward Hill, MA, USA). Fetal bovine serum (FBS) was purchased from VWR (West Chester, Pennsylvania, USA) and Aquapel from Pittsburgh Glass Works (Pennsylvania, USA). KH_2PO_4 , NaH_2PO_4 , 4–2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), phosphate buffered-saline solution (PBS), insulin, penicillin/streptomycin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and D-glucose were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). BODIPY FL C_{16} (termed as FFA*) and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Invitrogen (Thermo Fisher Scientific). Surfactant solutions were made by dissolving Pico Surf 2 (Dolomite Microfluidics, Royston, UK) surfactant in HFE-7500 oil (3M, St. Paul, MN, USA) to make 1.0% w/w solution. This not only helps to minimize exchange of compounds between aqueous and oil phases but also prevents adsorption to the PDMS.

Microfluidic Device Fabrication

Microfluidic devices were made of two layers of patterned PDMS following standard soft lithography guidelines.¹⁸ While standard methods employ photolithography using a mask aligner and UV light source, we employed an in-house built UV lithography source¹⁹ based on 365 nm LEDs, after a few modifications to the recent design detailed by Groisman and co-workers.²⁰ Two master wafers for fluidic and pneumatic channels were fabricated. All channel layouts were designed in Adobe Illustrator, and photolitho-graphic masks were

printed at 50 800 dpi resolution by Fineline Imaging (Colorado Spring, CO). For the pneumatic control channels (thin lower layer), 30 μm thick negative photoresist (SU-8 2050) was spin-coated onto a silicon wafer. The wafers were then baked on a hot plate at 95 °C for 5 min. UV exposure was carried out through the mask at $\sim 330 \text{ mJ/cm}^2$ on the in-house built UV LED exposure unit. The wafer was hard baked for 5 min at 105 °C and then developed for 5 min in the SU-8 developer. For the fluidic channel layer, 45 μm thick positive photoresist (AZ-40-XT) was spin-coated onto another silicon wafer. The wafer was then baked at 105 °C for 5 min followed by UV exposure at $\sim 330 \text{ mJ/cm}^2$. After another baking step at 105 °C for 5 min, followed by wafer development in AZ developer for about 5 min, the master wafer was baked at 115 °C for 6 min to anneal the AZ photoresist and create a more rounded cross-section of the fluidic channel template. All silicon wafers were exposed to trimethylsilyl chloride vapor for 30 min before use to enhance PDMS removal. Channels were later characterized by slicing an assembled PDMS device with a razor and imaging the channel cross section (Figure S-1).

To fabricate the lower, pneumatic layer, a thin PDMS valving membrane ($\sim 15 \mu\text{m}$ membrane above control channels) was made by spin-coating a 20:1 ratio mixture of PDMS precursors and curing agent, respectively, onto the SU-8 defined control wafer. The upper, fluidic layer was made by pouring a 5:1 ratio mixture of PDMS precursors and curing agent to form a thick ($\sim 0.6 \text{ cm}$) substrate over the rounded, AZ-defined fluidic channels. Both layers were cured at 65 °C in an oven for $\sim 35 \text{ min}$. Thereafter, the thick fluidic layer was quickly peeled from the silicon wafer, cleaned with methanol, air-dried, and then aligned and bonded to the thin pneumatic layer. The assembled layers were again cured at 65 °C in an oven for $\sim 35 \text{ min}$ to facilitate interlayer bonding. Following this process, the resultant microfluidic devices were peeled from the wafer, cleaned with methanol, air-dried with a stream of N_2 , plasma oxidized, and bonded to a glass slide. The flow channels were treated with Aquapel then rinsed with methanol to repel aqueous solution, facilitating aqueous-in-oil droplet formation.

Flow Control

Microfluidic flow control was enabled by active pneumatic push-up valves, initially developed by the Quake group,¹⁷ which are formed at the intersections between the control and the flow channels. An in-house written application in LabVIEW (National Instruments) was interfaced to a control system of solenoid valves (Lee Co.; LHDA0533115H) to permit on-demand droplet formation. Valve actuation was powered by a regulated pressure source of $\sim 20 \text{ psi}$, and flow was promoted by vacuum applied to the outlet channel via a hand-held 100 mL glass syringe (SGE Analytical Science).

Droplet Fluorescence Measurements and Data Analysis

Fluorescence calibration standards and stocks were initially evaluated using a Nanodrop 1000 spectrophotometer or Nanodrop 3300 fluorospectrometer (Thermo Scientific, Grand Island, NY USA). These calibration standards were diluted in 20 nM HEPES buffer solution ($\text{pH} = 7.5$) and analyzed in triplicate. Wide-field or fluorescence imaging and measurements in the $\mu\text{Chopper}$ device were performed with either a cooled CCD camera (CoolSnap HQ2; Photometrics Scientific) or more typically using a PMT (Hamamatsu). These detectors were

interfaced to a Nikon Ti-E inverted fluorescence microscope via a green fluorescence filter cube (excitation: 470 ± 20 nm; emission: 525 ± 25 nm). The PMT was set to $10^{-3} \mu\text{A V}^{-1}$ sensitivity with a 0.5 ms time constant and a one-time, manually adjusted amplifier offset at the onset of measurements.

The device (μ Chopper) included a reference inlet (either buffer or high fluorescence), a sample inlet, and an oil channel inlet. Using pneumatic microvalves and the LabVIEW application, droplets were generated in an alternating fashion;¹² each droplet was moved to the region of interest (ROI) and stopped, and the fluorescence emission was collected with the PMT. This customized software allowed instantaneous separation of the sample and reference signals into two different data arrays, greatly simplifying data analysis compared to previous device iterations.¹² Droplets could be reliably formed at 3.50 Hz each (0.04 Hz bandwidth) while maintaining software-based phase-locking to the detector. Reference droplet signals were subtracted from sample droplet signals over time to correct for drift, and this analysis was carried out for up to 100 droplets for each sample. Alternatively, fast Fourier transform (FFT) analysis was carried out in Matlab, where sample and droplet data arrays were recombined in time, and the primary frequency-domain peak intensity at 3.50 Hz was used as the processed signal.

Adipocyte Culture, Treatment, and Lysis

Murine preadipocytes (3T3-L1; ATCC) were seeded on 35 mm culture dishes and kept in DMEM supplemented with 10% FBS and penicillin/streptomycin (5%) at 37 °C in an atmosphere of 5% CO₂. After confluency was reached, differentiation was induced by adding DMEM supplemented with 0.5 mM IBMX, 250 nM dexamethasone, 1.0 μM insulin, and 10% FBS. Three days later, DMEM supplemented with 1.0 μM insulin was added, and the cells were maintained for an additional 3 days in this media. Thereafter, cells were set in DMEM supplemented with 10% FBS before use, usually between 5 and 8 days.

Differentiated adipocytes were subjected to starvation for 1.0 h in DMEM without phenol red and FBS, supplemented with 3.0 mM glucose and 50 pM insulin at 37 °C in an atmosphere of 5% CO₂. Subsequently, this starvation media was replaced with DMEM without phenol red and FBS, containing 19.0 mM glucose, 2.0 nM insulin, and 1.0 μM BODIPY FL C₁₆ (FFA*). The cells were maintained for 1 h under the same conditions previously mentioned for starvation. Next, the differentiated cells were washed four times with 2.0 mL of phosphate buffered-saline solution (PBS). Carefully, ~100 adipocytes were detached with a 10 μL pipet tip and transferred into 200 μL of PBS. The solution was gently mixed with the pipet to disaggregate the cells. Using a tissue culture microscope and a 10 μL micropipette, 10, 3, 1, or 0 adipocytes were transferred into 30 μL of RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific). Each solution was mixed and kept in ice during the lysis process. Samples were then immediately centrifuged at 12 000 rpm for 10 min, and the supernatant was analyzed in our μ Chopper device.

For adipocyte imaging, differentiated 3T3-L1 cells were fixed and stained with lipid-staining LipidTOX and nucleus-staining DAPI. LipidTOX emission was collected at 525 ± 25 nm after excitation at 470 ± 20 nm, and DAPI emission was collected at 460 ± 25 nm after excitation at 350 ± 25 nm. Differential interference contrast (DIC) images were also

collected. Images were overlaid using ImageJ software,²¹ and false coloring was applied for visualization (green for LipidTOX, blue for DAPI, gray for DIC).

Homogeneous Immunoassays

Insulin was quantified in droplets using a homogeneous fluorescence assay (Human Insulin FRET-PINCER Assay Kit; Mediomix, St. Louis, MO), where higher insulin levels caused decreases in fluorescence due to target-specific probe quenching. Various concentrations of insulin (0, 11, 22, 44, 88, and 176 nM) were premixed with both antibody–oligonucleotide probes (16-fold larger than recommended in kit; ~160 nM each) and incubated for 30 min; then, fluorescence emission was measured using the Nikon Ti-E inverted fluorescence microscope via a green fluorescence filter cube (excitation: 470 ± 20 nm; emission: 525 ± 25 nm) interfaced to a CCD camera (Coolsnap HQ2; Photometrics). To provide a constant, unquenched emission, reference droplets consisted of ~80 nM of only the fluorophore-labeled probe. Unless otherwise noted, 30 droplet pairs (sample and reference) were analyzed for each concentration.

RESULTS AND DISCUSSION

Microfluidic Device Design and Operation

We previously reported a droplet-based μ Chopper for microfluidic absorbance detection consisting of three inputs (sample, reference, oil) and a single output. The device was passively self-regulated, alternating sample and reference droplets simply based on the opposing droplet T-junction geometries with manually applied vacuum at the outlet. Constant alternation of sample and reference droplets is a key feature of the lock-in analysis, because noise at frequencies and phases different from that of the droplets are largely rejected. This allowed nanomolar analyte detection limits (femtomole amounts) with absorbance,¹² even in short path-length microchannels. However, this passive approach lends itself to drifts in droplet frequency over time that must be tracked in data analysis. For this reason, we hypothesized that a more precisely controlled droplet formation system would permit further improvements in detection sensitivity and would simplify data analysis.

Following this logic, here, we have applied the μ Chopper device concept to fluorescence detection while also adding active control using pneumatic valving. Droplet formation was automated by software, and phase-locked detection was applied in real time. The device design and operation are depicted in Figure 1. With automated droplet formation and real-time, phase-locked fluorescence detection, a considerable reduction in data analysis was permitted compared to our prior work.^{12,14} Since the software was in control of the type of droplet being placed into the focal region, the fluorescence emission measurements by the PMT (green trace in Figure 1B) could be instantly stored into separate data arrays for sample and reference droplets, rather than relying on complex image analysis methods. Downstream data analysis required mere subtraction of reference traces from sample traces (or vice versa), which is easily built into the LabVIEW application.

μ Chopper Device Characterization

Since droplet formation was now intimately phase-locked with detection using microvalves, we expected that the μ Chopper would permit quantitative measurements of small differences between the fluorescence emission from sample and reference droplets, even at levels far below the magnitude of the drift or $1/f$ noise at the detector. This concept could be implemented for a low concentration fluorescence measurement using buffer as the reference (“signal on” assay), or the reference solution could be defined as having a constant fluorescence emission (“signal off” assay), for example, with a quenching based assay. Our device was characterized in both situations.

First, we characterized the system with buffer (no fluorescence) as the reference and fluorescein as the sample (Figure 1A). Following lock-in analysis, we observed a highly linear response ($R^2 = 0.9997$) in the picomolar range. Using buffer as the reference, the PMT could be tuned to maximize sensitivity, at 9.65 V nM^{-1} . The LOD was determined to be 12 pM when sampling 20–70 droplets (180–620 nL). When only a single droplet was analyzed, the concentration LOD was higher at 35 pM, but this represents an amount of only 310 zeptomoles ($3.1 \times 10^{-19} \text{ mol}$) of fluorescein detected. Notably, when the same data was analyzed without the lock-in approach, the noise magnitude (and thus the LOD) was 54-fold higher, validating the use of the μ Chopper system for noise reduction (3σ magnitudes marked with horizontal dotted lines).

Interestingly, the inset graph in Figure 2A shows that, as the number of analyzed droplets was increased from 1 to 20, noise was reduced significantly, yet when analyzing >70 droplets, noise began to increase slightly. This trend was also observed in our prior work¹² and is likely due to buildup of the nonzero noise (white and $1/f$) in the narrow band at $3.5 \pm 0.04 \text{ Hz}$ with increasing signal collection time. The noise increase in this study is more attenuated compared to prior work, which is reasonable considering the much narrower bandwidth. Nonetheless, it is important to note the observation of an optimal, lower noise range of droplet number.

Next, we characterized the μ Chopper with a fixed fluorescence signal as the reference, which would be useful for quenching based, indirect assays. As shown in Figure 2B, this placed a limit on the PMT sensitivity settings; with higher reference concentrations, lower sensitivities must be used. Again, highly linear responses were observed ($R^2 > 0.99$). In these analyses, the independent variable was defined as a detectable change in fluorescein concentration (–[fluorescein]). With a reference of 50 nM, the μ Chopper could resolve as low as a 0.39 nM change in fluorescein; i.e., the system could discriminate 49.61 nM from 50 nM. With a reference of 10 nM, a 0.16 nM change was resolvable.

The frequency response of the system was also characterized, as shown in Figure 2C. FFT analysis was performed on the data from Figure 2A after recombining the sample and reference traces in the time domain, creating approximate square wave signals. The frequency domain in Figure 2C exhibited the standard square wave spectrum, with a fundamental peak at 3.50 Hz along with the odd harmonics at higher frequencies (10.5 and 17.5 Hz, etc.). The intensities of these peaks were clearly dependent upon the difference between the sample and reference concentrations. The inset log-scale spectra show a clear

peak at 3.50 Hz, even at the lowest tested concentration of 39 pM. Here, the bandwidth was calculated as 0.042 Hz, which confirms the exquisite control that was provided by automated droplet formation using on-chip valves. Using postprocessing via FFT, the LOD of the system for fluorescein detection was further improved to 5.4 pM (data from all 100 droplets analyzed), although the real-time processing described above is much more practical.

Real-Time Drift Correction

To fully appreciate the noise and drift correction achievable with the μ Chopper, it is instructive to view the time domain data, as shown in Figure 2D. Using standard microscope optics, the μ Chopper device allowed real-time drift correction by constantly referencing signal from the sample droplets (red trace) to that of the reference droplets (blue trace) at 3.5 Hz, in a phase-locked manner. It is clear from the difference signal (green trace) that larger system drifts (e.g., at the ~10 or ~70 s time range) were corrected using this approach. The difference signal trace was shown to be directly proportional to the sampled fluorescein concentrations. Further evidence of this drift correction is shown in Figure S-2, where the PMT offset was intentionally varied as proof-of-concept.

High measurement precision was also achieved with the μ Chopper. Responses recorded in Figure 2A,B were highly linear ($R^2 = 0.9997, 0.9990$, and 0.9943) with minimal measurement error at each concentration. Interestingly, this high precision exposed what appears to be slight human errors in sample preparation in one of the samples from Figure 2B (3.125 nM data point with 50 nM reference). Considering the other data sets in Figure 2 match the linear models very well, this deviation is most likely human error related and not a result of a poor regression model. Moreover, it is to be expected that fluorescence emission intensity will be linear versus concentration, since consistent optical settings were applied.

Free Fatty Acid Uptake Quantification

Methodology for single-cell analysis is critical in studying the variability in cell populations,²² preventing inaccuracies in taking averages of entire cell populations. However, single cell studies are often limited by the difficulties associated with analyzing small amounts of starting material. Microfluidic approaches are ideal in handling small volumes, allowing manipulation of small numbers of cells and minimizing sample dilution, for instance, after cell lysis.²³ Single-cell analyses using droplet microfluidics have been previously reported.^{24–27} However, these studies were focused mainly on cell screening or gene expression analysis. Studies including breakdown, absorption, and uptake of metabolites are scarce and mostly not quantitative.

It has been reported that fatty acid accumulation is cell-to-cell heterogeneous;²⁸ therefore, it is critical to quantify the amount of uptake from single cells rather than averaging uptake of larger groups of cells in bulk conditions. Such information should contribute to gaining a better understanding of factors that lead to obesity^{29–33} and type 2 diabetes.^{34,35} Although our prior studies of adipose tissue on microdevices were quantitative in nature,^{19,36–38} single cell resolution was not achieved.

With all of this in mind, we validated the μ Chopper system by applying it to quantify the amount of labeled free fatty acid (FFA*) that was taken up by small numbers of 3T3-L1

adipocytes. As shown herein, due to its extraordinary noise reduction and ability to discriminate very small fluorescence differences, our μ Chopper system could successfully quantify FFA* uptake by even a single 3T3-L1 adipocyte by simply lysing the cell in bulk solution.

Fully differentiated adipocytes were incubated with a fluorescently labeled fatty acid solution (BODIPY FL C₁₆, or “FFA*”). Small numbers of adipocytes (0, 1, 3, or 10) were washed and then lysed in 30 μ L aliquots of lysis buffer. Following centrifugation, the supernatant was analyzed directly with our μ Chopper device and lock-in analysis method. As shown in Figure 3, our system permitted single-cell FFA uptake rates to be quantified at $3.5 \pm 0.2 \times 10^{-15}$ mol cell⁻¹. To our knowledge, this is the first report of single cell uptake rates, and the value correlates well with confirmatory experiments using primary adipose explants (see Figure S-3). Our approach was more sensitive compared to the most recently reported methods^{39,40} since we were able to quantitatively measure FFA* uptake by a single cell. Slight deviations from the linear regression model at low cell number are likely due to human error related to the multistep cell isolation and preparation, as discussed above.

Homogeneous Immunoassays in Droplets

Since the device was also shown to be useful with a fixed fluorescence signal as the reference (see Figure 2B), here, we show proof-of-concept that a fluorescence quenching based, homogeneous immunoassay can be used to quantify small amounts of proteins in droplets using the μ Chopper. The device allowed homogeneous quantification of insulin in droplets with a concentration LOD of 9.3 nM using 30 droplet pairs and a mass LOD of 1.9×10^{-16} mol (190 amol) using 2 droplet pairs (Figure 4). A four-parameter sigmoidal fit (see Supporting Information for details) was applied to the sandwich type assay; observed deviations from this model are likely a result of a human induced bias in sample preparation rather than device error. As shown in the inset plot, reference droplets (red trace) contained a constant fluorescence reference (unquenched antibody–oligonucleotide probes), while sample droplets contained continuously varying insulin concentrations mixed with both homogeneous immunoassay probes. Again, the reference trace (red) can be used to validate the usage of the μ Chopper device, since time-dependent optical system drift is readily observed at magnitudes that would be detrimental to the assay without correction. The lock-in detection approach used here allows for much of that drift to be corrected. Such a system would be ideal to apply to real-time cell secretion quantification for any protein with an available homogeneous immunoassay, and droplet sampling provides the potential for significant improvements in temporal resolution.¹⁴

CONCLUSIONS

We have now shown that our droplet phase-locking approach with microfluidics (μ Chopper) is applicable to both absorbance¹² and fluorescence detection systems, and others have recently used a similar strategy with Raman spectroscopy in droplets.¹³ Phase-locking with droplets can be done passively, but in our application to fluorescence herein, we demonstrated that significant bandwidth reduction was possible using on-chip pneumatic valving. Considering these results together, it should be relatively straightforward to apply

the technique to improve any optical readout method without modifying the optical assembly, particularly for groups already employing droplet microfluidics. Furthermore, it is theoretically possible to apply this droplet phase-locking technique to any droplet-integrated detection method to eliminate signal drifts. For example, ion collection in droplet-interfaced mass spectrometry^{41,42} could be phase-locked with droplet formation to help recover signal from low abundance analytes. Alternatively, electrochemical detection in droplets⁴³ could be phase-locked with droplet formation to recover lower analyte currents.

Although a >50-fold reduction in noise was exhibited here, in theory, this system could be further improved by phase-locking to higher frequency droplet formation. Droplets are now routinely generated at kHz frequencies,⁴⁴ a sampling range that is known as a “quiet” region of the noise spectrum.⁵ If kHz range pneumatic valve actuation frequencies could be achieved, this ultimate low-noise sampling bandwidth should be attainable. Overall, using the improved μ Chopper device presented here, we have further demonstrated the compatibility of droplet fluidics with phase-sensitive (lock-in) detection. In many existing droplet microfluidics systems, this approach should provide significant noise reduction with relatively few design modifications. Such microsystems are ideal for analyzing precious specimens such as cells or biological reagents and for quantifying samples of limited volume.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. μ Chopper device design and operation. (A) Channel layout depicts flow channels (black), control channels (blue, red, and purple), and the fluorescence detection point. (B) Schematic of valve-automated chip operation cycle: t_D is the delay time, t_S is the sample flow time, t_M is the measuring time, t_O is the oil flow time, and t_R is the reference flow time. Droplet position was phase-locked with the detector collection cycle. (C) An image montage showing segments of a video collected during automated droplet formation on the μ Chopper (full video file, Video S-1). Open valves are marked with asterisks. Scale bar represents 200 μ m.

Figure 2.

μ Chopper characterization with fluorescein. (A) With a buffer reference, high sensitivity and an LOD of 12 pM were possible. Inset graph shows dependence of LOD on droplet number (moving average shown as a guide). (B) With fluorescent reference droplets, lower PMT sensitivities were required, but the system was highly linear, resolving small signal differences (0.39 and 0.16 nM). (C) Fourier analysis showed the expected square wave spectrum, with the fundamental peak at 3.50 Hz showing linear concentration dependences. Inset plot shows log-scale intensities and the highly regulated bandwidth of 0.042 Hz . (D) Time domain data illustrates the benefits of real-time drift correction using the μ Chopper.

Figure 3.

Labeled free fatty acid (FFA*) uptake by 3T3-L1 adipocyte cells was quantifiable down to the single cell level using the μ Chopper system with lock-in analysis. Single cell FFA* uptake was quantified at $3.5 \pm 0.2 \times 10^{-15} \text{ mol cell}^{-1}$ for the first time. The inset image shows a confocal fluorescence scan of fixed and stained adipocytes, where lipid storage is stained in green and nuclei are stained in blue. Scale bar represents $20 \mu\text{m}$.

Figure 4.

Homogeneous quantification of insulin in droplets was possible at levels as low as 9.3 nM (30 droplet pairs) or 190 amol (2 droplet pairs) using the μ Chopper system. Reference droplets contained unquenched antibody–oligonucleotide probes. Calibration plot includes a 4-parameter sigmoidal fit curve obtained by nonlinear least-squares fitting. Inset graph shows a typical real-time data collection as insulin standards are sequentially added to the sample droplet reservoir.