FIDELITY: A quality control system for droplet microfluidics

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Droplet microfluidic systems have been widely deployed to interrogate biological and chemical systems. The major limitations of these systems are the relatively high error rates from critical droplet manipulation functions. To address these limitations, we describe the development of FIDELITY (Flotation and Interdigitated electrode forces on Droplets to Enable Lasting system InteGrIT), a highly sensitive and accurate size-based droplet band-pass filter that leverages the natural buoyancy of aqueous droplets and highly localized dielectrophoretic force generated by interdigitated electrode arrays. Droplet manipulation accuracies greater than 99% were achieved at a throughput of up to 100 droplets/s and separation of droplets that differed in diameter by only 6 \(\mu\)m was demonstrated. Last, the utility of FIDELITY was demonstrated in a droplet size quality control application and also in a droplet-based in vitro transcription/translation workflow. We anticipate FIDELITY to be integrated into a broad range of droplet microfluidic configurations to achieve exceptional operational accuracy.

INTRODUCTION

Droplet microfluidics has shown promise in many different biological and chemical applications, including cell biology (1–3), drug screening (4–6), and nucleic acid analysis (7, 8) applications. Manipulation of droplets serves as a crucial component of droplet microfluidic systems. Thanks to the extensive technological advancements made over the past decade, individual droplets can now be transported, merged, dispersed, trapped, and sorted at very high throughput (9). In most of these applications, highly monodispersed droplets (in terms of their sizes) are required as each of these functions is designed and optimized for operation with droplets of a specific size range. If input droplet size varies or falls outside of the optimized size range, then high error rates in the droplet manipulation steps occur. This outcome, in turn, leads to lower performance of the entire droplet microfluidics platform (10, 11). However, droplets having unwanted sizes are difficult to avoid even in some of the most well-optimized systems due to undesirable droplet splitting or merging during droplet transport, two or more droplets merging during droplet merging operations, small satellite droplet generation during various droplet manipulation functions, and size variation over time during droplet generation. These issues are even more severe when operating such systems at high throughput or over long periods of time, such as during large library screening campaigns. Many operating conditions must be optimized every time a device is used, further hampering the widespread use of droplet microfluidic systems for routine biological and chemical assays. Yet, there has been little development in applying quality control (QC) measures to droplet microfluidic systems. A method of actively removing droplets of unwanted sizes can be the first step toward having a QC measure in droplet microfluidic systems and will also be critical for increasing their accuracy and efficiency.

Several size-dependent droplet separation techniques have been reported on the basis of deterministic lateral displacement (DLD) (10, 11), physical filtration (12), guiding tracks (13, 14), and hydrodynamic methods (15, 16). Although these methods have many advantages, each has substantial drawbacks. DLD-based methods typically have a large footprint due to their micropillar array geometries (10, 11). Furthermore, this method can only separate two distinct droplet populations that have at least twofold volumetric differences. These systems also have difficulties sorting larger droplets (>100 \(\mu\)m diameter) that are commonly used for cell-based assays. Physical filtration–based methods can, for example, use crossflow filtration structures to impede the flow of unwanted larger droplets and can distinguish droplets that have only 20% volume differences (12). However, this method has only been applied for separating two different droplet populations of different sizes. The cutoff size of filtered droplets is dependent on the flow conditions, an aspect that is not easily controllable and thus not suitable for QC purposes. The guiding track separation method uses guiding microstructures located at the top or bottom of a microchannel to guide droplets (13, 17). This method can achieve separation of droplets by size with a sensitivity of 15 \(\mu\)m (13). However, this is a relatively low throughput method (typically <10 droplets/s since higher throughput often results in droplet splitting due to the guiding pillar structures), rendering it unsuitable for high-throughput applications. This approach also requires the fabrication of complicated multilayered devices. Hydrodynamic methods, such as pinched flow fractionation (16, 17) and inertial force microfluidics, have also been reported for size-based droplet sorting, where separating small “satellite” droplets from target droplets that are more than five times larger has been demonstrated. However, this method has relatively low sensitivity to droplet size and therefore is not suitable for accurate size-based droplet sorting. In addition, microfluidic devices based on the balance of inertia and fluidic force for separation of hydrogel droplets were developed (15),
where droplets varying in size migrate to different size-dependent dynamic equilibrium positions across fluid streamlines under the combined effects of inertial forces and a wall effect lift force. However, the drawbacks are its relatively low sorting efficiency (~90%) and the fact that sorting a polydispersed population would require an extremely complicated device design. Acoustic-based systems, both bulk acoustofluidics and surface acoustic wave acoustofluidics, have been used for droplet manipulation (18–20). Both methods have been demonstrated to manipulate broad ranges of droplet sizes. However, in terms of size-based separation, the best-reported separation resolution so far is about 50-μm difference in droplet size. Thus, these previously developed systems do not have sufficient resolution to separate droplets with only small size differences or handle droplet populations with high polydispersivity, as presented here, and cannot be readily used in the droplet bandpass filter concept as proposed here. Although many different droplet microfluidic operations have been achieved using acoustofluidics, most droplet microfluidic systems are made of a polydimethylsiloxane (PDMS)/glass device structure due to the versatility of such a system, and thus, readily integrating the presented droplet bandpass filter concept into these diverse microfluidic systems is possible.

Dielectrophoresis (DEP)-based droplet manipulation is the most widely used method in droplet microfluidic systems (21–25), with three-dimensional (3D) electrode pair designs being the most common ones. Interdigitated electrode (IDE) arrays are widely used in microfluidic systems in general to perform functions such as particle manipulation (26) and cell separation (21, 27, 28) and have also been used in droplet microfluidic systems, mostly for droplet merging purposes (29–31). However, despite the fact that IDEs can provide a much more localized and finely controlled electric field (resulting in more precise control of droplets) compared to 3D electrode designs, they have not been used for droplet manipulations.

In this work, we present FIDELITY (Flotation and Interdigitated electrode forces on Droplets to Enable Lasting system IntegritY), an IDE-based droplet manipulation system that uses a highly localized electric field generated at the bottom of the microfluidic channel using IDEs and coupled with the fact that droplets float up due to their natural buoyancy in carrier oil, to selectively manipulate only droplets in the desired size range. Here, we demonstrate that FIDELITY can be used to separate droplets with as little as 6-μm difference in their diameters, either as a high-pass or low-pass droplet filter. By sequentially linking the high-pass filter and low-pass filter designs, selecting only a certain droplet size range becomes possible, with a filtration efficiency of more than 99% at throughputs of up to 100 droplets/s. The utility of this droplet bandpass filtration system was demonstrated in two applications. The first application was for droplet sorting efficiency (sensitivity = 6 μm in diameter) or for QC term droplet generation (sensitivity = 6 μm, the 95-μm droplet. This substantially different DEP force, which is about 14 times greater than the DEP force acting on the smaller droplets in the desired size range. Here, we demonstrate that FIDELITY can be used to separate droplets with as little as 6-μm difference in their diameters, either as a high-pass or low-pass droplet filter. By sequentially linking the high-pass filter and low-pass filter designs, selecting only a certain droplet size range becomes possible, with a filtration efficiency of more than 99% at throughputs of up to 100 droplets/s.

RESULTS

Working principle of the FIDELITY system

FIDELITY takes advantage of the natural buoyancy caused by the density difference between the aqueous solution and carrier oil of a water-in-oil emulsion droplet, combined with the highly localized DEP force generated by an IDE array at the bottom surface of a microfluidic channel. Here, because of the highly localized electric field at the bottom surface, droplets that are closer to the bottom DEP electrode experience a larger DEP force compared to droplets that are farther away from the DEP electrode (Fig. 1A). Since the densities of most commonly used carrier oils are greater than that of the aqueous droplets, the natural buoyancy results in all droplets floating upward and reaching the microchannel ceiling. Thus, if a droplet diameter is close to or greater than the microchannel height, the droplet is in close proximity to the IDE surface and hence receives a strong DEP force, making droplets easy to be manipulated by the DEP force and follow the trajectory of the IDE patterns (Fig. 1B). However, when the droplet size is less than the channel height, the buoyant droplet will float away from the IDE and will receive a smaller or negligible DEP force. Here, the trajectory of the smaller droplets will be dictated by the Stokes drag force and simply follow the flow of the carrier oil. By controlling the channel height in relationship to the droplet size, the IDEs can specifically influence and guide only the larger droplets to the collection outlet, while smaller droplets continue to flow to the “waste” outlet.

The electric field generated by the surface IDE was simulated using COMSOL Multiphysics simulation (fig. S1A). The results show that the intensity of the electric field 1 μm above the IDE’s surface was approximately 55 times higher than that at 100 μm above the surface. In the case of a microchannel with 100 μm height, droplet size versus DEP force received by the droplet was calculated and their relative DEP force was plotted (fig. S1B). This result shows that when two different droplets, 80- and 100-μm-diameter droplets, flow above the IDE-patterned microfluidic channel with a channel height of 100 μm, the 95-μm droplet receives approximately 13.4 nN of total DEP force, which is about 14 times greater than the DEP force acting on the smaller 80-μm droplet. This substantially different DEP force exerted on the droplets of different sizes enables highly selective size-based droplet manipulation.

Design and working principle of the droplet bandpass filter

Figure 1C shows a schematic of the developed size-based droplet bandpass filter design. The device consists of three sections: (1) droplet reflow and lifting section, (2) large droplets removal section (low-pass filter), and (3) small droplets removal section (high-pass filter). In the first droplet reflow and lifting section, the height of the oil spacing channel (50 μm) was designed to be half of the droplet reflow channel (Fig. 1D). The purpose of this design, which is in contrast to conventional oil spacing channel designs having equal height as the main channel, was to create an oil sheath flow underneath the reflowed droplets while also spacing them out, thus lifting the droplets to the ceiling of the microfluidic channel before they approach the first IDE droplet manipulation region. This enabled faster lifting of the droplets, in addition to their natural buoyancy, enabling higher-speed operation of the system. In the second droplet low-pass filter section where the first IDE array was patterned (6-μm finger width and 6-μm spacing; Fig. 1E), large droplets (>100 μm) were siphoned away from the main flow by the DEP force and flowed out through outlet 1. To allow the detachment of large droplets from

Zhang et al., Sci. Adv. 8, eabc9108 (2022) 8 July 2022
the IDE array and prevent unwanted blockage (movie S1) of the outlet channel, the height of outlet 1 was gradually increased from 100 to 160 µm within a 500-µm distance (Fig. 1F) while the width also doubled. Then, the height of the main channel decreased from 120 to 80 µm before the droplets enter section 3 (Fig. 1G). For both sections 2 and 3, sideway bias flow was applied to push incoming droplets to the opposing side of the IDE sorting exit channels before the droplets reach the IDE patterns to minimize the droplet manipulation error. Figure 1H shows a micrograph of the fabricated microfluidic device and Fig. 1I shows the enlarged view of the IDE patterns.

Validation of size-specific droplet manipulation
To test the developed size-specific droplet manipulation method, a droplet population consisting of three different sizes of color dye droplets, 40 µm (black), 80 µm (blue), and 160 µm (red) in diameters,
was used. Here, the desired droplet size to recover was the 80-μm-diameter blue droplets. The droplet bandpass filter was designed (Fig. 2A) so that the 160- and 80-μm-diameter droplets are retrieved from outlets 1 and 2, respectively, as they can be strongly affected by the localized DEP force applied. The 40-μm-diameter droplets were expected to be unaffected by the IDE-generated electric field, thus flowing out straight through outlet 3. The experimental results shown in Fig. 2 (B and C) demonstrate selective size-based droplet manipulation. The performance of this droplet bandpass filter device was quantified under various throughputs (5, 20, and 100 droplets/s), where the FP and FN rates were analyzed (Fig. 2D). The FNs and FPs for all three outlets were less than 1% at all three flow speed conditions tested. Overall, the operation accuracy of the system was greater than 99% at throughputs of over 100 droplets/s. Video demonstration of the size-dependent droplet sorting process under the throughput of 20 droplets/s is shown in movie S2.

**Further characterization of the droplet bandpass filter performance using a highly polydispersed droplet mixture**

The top view of the droplet bandpass filter device having channel heights of 100 to 90 μm (i.e., 100 μm in the first filter region, reduced to 90 μm in the second filter region) is shown in Fig. 3A. The prepared highly polydispersed droplets generated through sonication of oil and water together (Fig. 3B) were flown through the device and droplets were collected from outlets 1, 2, and 3 (Fig. 3, D to F, respectively). Because of the high speed of sheath flow (200 μl/hour), droplets with sizes larger than ~180 μm were cleaved to become smaller droplets at the line of “a” and “a”’ (Fig. S2). This explains why extremely large droplets are not observed in Fig. 5C. The bandpass filter was able to target droplets with a very specific size range (70 to 80 μm in diameter), where the sizes of the collected droplets were measured at the circular observation chamber (“OB” in Fig. 1H) to quantify the performance of the developed droplet bandpass filter. Outlet 1, which was designed to remove any droplet larger than 80 μm in diameter, successfully removed those droplets (Fig. 3, D and G, red data points), with less than 1% (6 of 620 droplets) of the removed droplets being less than 80 μm in diameter. For outlet 3, which was designed to remove any droplet smaller than 70 μm in diameter, the filter successfully removed most of these small droplets (Fig. 3, G and H, green data points), with only 1% (6 of 620 droplets) of the droplets being larger than 70 μm in diameter. In summary, the droplet bandpass filter performance can be defined as having a 73- to 76-μm-diameter droplet passband (0 dB), while having a cutoff (~3 dB = ~70.7%) at 69 and 80 μm, making the passband 69 to 80 μm (Fig. 3H, blue line). The result also indicates that for a complete separation of two groups of droplets of different sizes, the size difference needs to be just 7 μm (x-axis distance from 66 to 73 μm in Fig. 3H) to achieve complete separation. Movie S3 shows the trajectories of droplets before and after the electrical field is turned on for outlet 1. Movie S4 demonstrates the continuous (30 min) filtration of the polydispersed droplets under a 24-V_{pp} applied voltage at a throughput of approximately 20 droplets/s.

To evaluate the effect of channel height on the passband of the droplet filter, Fig. 3I compares the size distribution of droplets collected from outlet 2 of a 100- to 90-μm-channel-height filter and a 100- to 80-μm-height filter. The results show that the smallest size of droplets that were collected from outlet 2 of the 100- to 80-μm bandpass filter was about 60 μm. Compared with the results from the 100- to 90-μm bandpass filter design where the minimum droplet size from outlet 2 was 70 μm, the lower bound of the bandpass filter could be extended by about 10 μm by simply reducing the main channel height of the second filter region by 10 μm.

Next, the effect of applied voltages on the bandpass filter performance was investigated by adjusting the voltage applied to the second IDE pattern (voltage applied to the first IDE remained constant at 24 V_{pp}). As can be seen in Fig. 3J, the filter tends to sort out larger droplets (75 to 82 μm) when the applied voltage decreases. The width of the passband under 12 V_{pp} was reduced by almost half compared to the case of 24 V_{pp}, meaning that a tighter size control was possible at a lower voltage. Movie S5 demonstrates the slight size difference of the collected droplets between the 12- and 24-V_{pp} conditions under the same device/flow conditions.

Both FC40 and Novec 7500 oils are the most commonly used carrier oil in droplet microfluidics but have different densities (1.85 versus 1.61 g/ml) (32). Here, heavier oil provides greater buoyancy force, which is expected to push the droplets more firmly and faster to the microchannel ceiling. The comparison is presented in Fig. 3K, where the mean droplet size of the FC40-surrounded droplets was slightly larger than that of the Novec 7500-surrounded droplets (P = 0.0136). Thus, both types of carrier oil can be used for the presented droplet bandpass filtration system.

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**Fig. 2. Proof-of-concept validation of the size-specific droplet bandpass filter design using droplets of three different sizes.** (A) Illustration of the expected result. (B and C) Experimental results at the sorting junction of (B) section 2 and (C) section 3. (D) Sorting efficiencies at all three outlets and at three different throughputs (n = 500 droplets analyzed for each outlet).
To assess the impact of throughput on the separation efficiency, the minimum voltage required for completely sorting three different sizes of droplets (42, 69, and 93 μm in diameter) under various throughputs was tested (Fig. 3L). As can be seen, there was a positive correlation between the voltages and throughputs, where the selection of smaller droplets required a higher voltage than that of larger droplets. However, in all cases, 100% success in separating the desired droplet sizes was achieved.
Application for QC in droplet microfluidic systems

The presented droplet bandpass filter can be used in many different droplet microfluidics applications. In this first application, we used the droplet bandpass filter for QC purposes. In droplet microfluidics, numerous factors can lead to unwanted changes in droplet sizes in the middle of a system operation, impeding the yield and error rates of the experiment. For example, precise control of monodispersed droplet generation is a critical first step in almost all droplet microfluidics systems; however, even with a fixed channel geometry and a fixed ratio of oil and aqueous flow rates, the size of generated droplets could still drift during long-term generation. Factors such as accumulated air bubbles in tubing and syringes, errors in mechanical pumps, and pressure controllers, as well as flow fluctuation due to imperfect fluidic connections, are some of the causes.

In the first scenario, we integrated our size-specific droplet filter with a typical flow-focusing droplet generator to create a droplet generation system with a droplet size QC feature (Fig. 4A, single-stage low-pass filter only). In this particular demonstration, the droplet generator was designed to generate droplets of approximately 90 μm in diameter. In the case that an undesired larger droplet is generated, it can be sorted out through outlet 1 of the device. Downstream of outlet 1 is an embedded impedance-based droplet sensing system to detect the presence of a droplet, which can trigger an alarm. To mimic the case of an unintended flow rate fluctuation, we expediently increased the flow rate of the aqueous phase from 15 to 25 μl/hour (while keeping the oil phase at 80 μm/hour). The size of the generated droplets was shifted from around 90 to 98 μm within 30 s (throughput 20 droplets/s). As can be seen in Fig. 4B, the initial droplets (90 μm) passed through the IDE without any interruption, while the droplets generated later with a size larger than 96 μm were completely filtered out through outlet 1 (Fig. 4C). Here, the droplet QC system was able to remove all droplets that were larger than 95 μm, which indicates that the sensitivity of the QC system is about 6 μm (in diameter) under such applied conditions (Fig. 4D). Meanwhile, a single large droplet flowing out from outlet 1 could trigger the impedance-based detection and alarming system (fig. S3), indicating an unwanted system fluctuation, which the user can then assess and intervene.

In the second scenario, we focused on QC after a droplet merging step. When two droplets (carrying different cells/reagents) need to be merged into one single droplet, droplet pairing before merging is the most critical step to ensure all droplets are merged at a one-to-one ratio (22). This is one of the most error-prone droplet manipulation steps in droplet microfluidics where the typical droplet merging efficiency remains in the range of 80 to 95% in most cases, especially when the droplet sizes are relatively large (>100 μm). Considering a droplet library screening scenario in which the true-positive hit rates are low (e.g., <1% of the population), this 5 to 20% error will contribute dominantly to FPs and/or FNs in the droplet-based screening assay. Here, the 80- to 100-μm droplet bandpass filter design was used, and the experiments were conducted at a throughput of 20 droplets/s with 24 V_pp applied to both IDEs. For demonstration purposes, a droplet population with 80% of the droplets having successful one-to-one merging and ~20% of the population being either unmerged or overmerged was prepared by a conventional droplet merging microdevice. Figure 4 (E and F) shows the removal of the unmerged and overmerged droplets using a two-stage droplet bandpass filter (a low-pass filter followed by a high-pass filter; Fig. 4, E and F, respectively). After the removal of unwanted droplets using the integrated droplet bandpass filter, the collected droplets contained more than 99% of the desired 1:1 merged droplets (Fig. 4G). The droplet bandpass filter in action can be seen in movie S6.

Improving the efficiency of IVTT

IVTT assays, commonly used for in vitro expression of heterogeneous proteins, can be used to generate a combinatorial synthesis protein library for functional screening by droplet microfluidics. However, several issues will have to be resolved for reliable screening. First, although ddPCR has been realized in many previous studies, it is essential to add surfactants such as Tween 20 and Triton X-100 into the reagent buffer to ensure droplet stability during thermocycling (33, 34). However, this could substantially affect cell viability, which becomes unacceptable for many cellular functional assays using the IVTT library. Second, IVTT protein synthesis reagents need to be added to droplets after droplet PCR, so another droplet merging step is required. Here, unmerged or double-merged droplets will result in FPs or FNs during droplet content detection and thus is best to be removed before the droplet even goes through the droplet analysis function. Examples of unpaired or mispaired droplets from a conventional droplet merging step are shown in fig. S4.

Figure 5A shows the droplet-based IVTT workflow with images of the droplets in each stage, where the droplet bandpass filter was applied in two parts of the workflow. A template-encapsulated droplet library was generated (step ①) and droplet thermocycling was conducted for template amplification (step ②), followed by the application of the droplet bandpass filter to remove droplets with unwanted sizes (step ③, movie S7). This uniformly sized droplet library was merged with the IVTT substrate by droplet merging (step ④). Then, a second droplet bandpass filter unit was used to remove overmerged or unmerged droplets (step ⑤). Last, the IVTT protein expression was completed in droplet format (step ⑥), in this case, the production of green fluorescence protein (GFP), making the protein library ready for downstream screening. Here, the IVTT template library that contained different levels of GFP protein expression was used to generate a mock droplet library that can be easily visualized. As can be seen in the droplet images, a notable improvement in droplet size uniformity was achieved (steps ③ and ⑥). Without the two droplet bandpass filtration steps (Fig. 5B, without filtration), the resulting droplet library included many droplets without GFP expression (primarily due to failure in droplet merging), with only 67.1% of the droplets confirmed to have GFP and the correct droplet size. In contrast, after applying the two droplet bandpass filtration steps, 97.5% of the droplets were confirmed to be the correct size and 94.1% were GFP positive (Fig. 5B, with filtration). Thus, the developed IDE-based droplet bandpass filtration system could indeed substantially improve the efficiency of droplet digital IVTT assays. The droplet size change and distribution at each step during ddPCR and IVTT assay are illustrated in fig. S5. In addition, GFP protein expression was also verified using gel electrophoresis (fig. S6).

DISCUSSION

We have demonstrated that by combining a highly localized IDE-generated electric field and the buoyancy effect of droplets, selective manipulation of droplets based on their sizes can be achieved with high specificity (6-μm size difference) at a throughput of up to 100 droplets/s. Sequentially combining two of these IDE arrays enabled the creation of a droplet bandpass filter, which can be broadly
used for QC applications in droplet microfluidic systems. The bandpass filter is easily tunable in terms of what droplet size to filter out by simply controlling the microchannel height and the applied voltage. The applied voltage can also greatly affect the width of the passband, as they can provide extra or less DEP force acting on all droplets. For example, when using a 90- to 100-μm channel height configuration, as shown in Fig. 3J, the minimum sort size is inversely correlated to the applied voltage. Here, the lower boundary of the filtered droplet size shifts from 67 to 74 μm when the applied voltage decreases from 24 to 12 Vpp, which means reducing the voltages to both IDEs causes a shift of the passband from the 70- to 80-μm range to the 70 + λ₁ μm to 80 + λ₂ μm range. Here, λ₁ and λ₂ indicate the degree of passband shifts in micrometers. The minimum and maximum working voltages at a throughput of 20 droplets/s were 12 and 30 Vpp, respectively.

This is important since the droplet z-axis position affects the DEP force acting on droplets, which is the main principle used here for size-based droplet manipulation. When droplet size exceeds the threshold where DEP force becomes dominant in compass to the buoyancy force, the entire droplet can be pulled down to the IDE surface, subsequently further increasing the DEP force on the droplet. If this occurs, the separation capability of the system is lost. Therefore, it is critical for the buoyancy force to be dominant to ensure the proper functioning of our system. Having said that, it is to be noted that the DEP force decreases exponentially in the z direction away from the electrode since the electric field drops exponentially; thus, a fairly large DEP force has to be generated for this undesired outcome to occur. In our presented design, droplets are initially lifted upward and come in contact with the ceiling by the sheath flow to ensure the DEP force applied to the droplets stays minimal at the beginning of the manipulation (fig. S2). Without such a lifting structure, any droplet position fluctuation in the z direction as they come in may not be properly manipulated. The relationship between the buoyancy force versus DEP force at different droplet sizes is shown in fig. S1B.

The droplet trajectory during IDE manipulation is mainly determined by the ratio between the Stokes drag force and the DEP force.
Stokes drag force is determined by the droplet size and flow rate, and DEP force is relative to the applied voltage (electric field) and droplet size. It is to be noted that for the DEP force, since the diameter of the droplet determines the \( z \) coordinate of the buoyant droplet, the DEP force received by the droplet will depend on the droplet size. To better understand how the flow pattern and DEP force can affect the separation efficiency of different sizes of droplets, we first calculated the droplet size versus DEP force/Stokes drag force ratio under one geometry setting. The flow rate and applied voltage were set to be constant throughout the simulation. As can be seen in fig. S7, when droplet size increases, the ratio also increases, which indicates that the DEP force gradually starts to have a dominant role in determining the trajectory of the target droplets.

Second, to better understand the impact of channel height (the \( x-y \) plane design was identical) on the minimum sorted droplet size (defined as minimal droplet size with >95% sorting efficiency), we summarized all successful droplet sorting conditions in table S1, along with the DEP force/Stokes drag force ratio. As can be seen in this table, the minimum ratio was 0.29, with the average ratio being 0.33. This ratio (0.33) can be used to predict the sorting or nonsorting of droplets with specific sizes under specific conditions. To compare, we have also summarized the results from the droplet merging applications, where experiment conditions were set as 1200 \( \mu \)l/hour flow rate, 24 V. During the experiment, we observed that in the first 100-\( \mu \)m channel, only 90-\( \mu \)m droplets were sorted, and in the second 80-\( \mu \)m channel, only 78-\( \mu \)m droplets were sorted, while 60- and

Fig. 5. Droplet-based IVTT workflow. (A) Droplet-based IVTT workflow with the integration of the droplet bandpass filter for droplet size QC. Droplet images in each step are shown. (B) Results of the IVTT droplets without and with the application of the droplet bandpass filter (\( n = 3465 \) for the "without-filtration" group and \( n = 2318 \) for the "with-filtration" group). "Size+" and "Size−" stand for droplets with correct and incorrect sizes. "GFP+" and "GFP−" stand for GFP-positive and GFP-negative droplets.
68-μm droplets remained unsorted (table S2). Their calculated ratios were in line with the sorting threshold we identified previously, where any ratio greater than 0.33 is expected to be a successful sorting condition, while any ratio that is less than 0.33 will result in a nonsorting condition. On the basis of this observation, we simulated the minimum droplet size depending on the channel height, as shown in fig. S8, where this graph can be used to design the appropriate channel height depending on the desired droplet size to be sorted. This simulated graph also shows the impact of applied voltages and flow rates, which can further help the design process depending on the need.

To check whether IDE-generated electric field could cause unwanted droplet merging at the IDE regions, we observed outlets 1 and 2 where two highly packed streams of droplets were collected and lined up (Fig. 2, B and C, from movie S2). However, even though the droplets were in direct contact with each other, no unwanted droplet merging was observed. For droplets to merge by the IDE, the system needs to meet one of the following requirements: (i) large enough spacing between the IDE fingers so that the generated electric field can reach the droplet-to-droplet contact area (condition 2, fig. S9); (ii) high applied voltage to achieve higher electric field so that sufficiently high electric field reaches the droplet-to-droplet contact area (more than 60 V_{pp} needed in our case; condition 3, fig. S9); and (iii) additional force (i.e., collision force) to compress the gap between the droplets so that even the surface-localized electric field can reach the droplet-to-droplet contact area (condition 4, fig. S9). The electric field generated by our IDEs having an electrode-to-electrode distance of 6 μm does not extend sufficiently from the electrode surface; therefore, merging (which is typically due to the depolarization of surfactant at the contact point) is less likely to occur in our design (condition 1, fig. S9). However, higher voltages combined with a relatively low flow speed could slow down or trap the sorted droplets and cause unwanted droplet merging when a droplet hit another droplet from the back. It is to be noted that in our design, as mentioned in the previous section, to prevent the trapping of droplets on the IDE surface or at the edge of the IDEs, we used an upward sloped microchannel so that the sorted droplets rapidly move away from the IDE surfaces right after they are sorted, as well as to also minimize compression between two consecutively sorted droplets to minimize accidental droplet merging coming from mechanical force. Figure 2 (B and C) from movie S2 can be referred to as an example, where two highly packed streams of droplets were collected and lined up at outlets 1 and 2, where no unwanted droplet merging was observed (throughput = 20 droplets/s).

Several other factors can also affect the characteristics of this bandpass filter system. Different microchannel heights will result in two very distinct filtered droplet size profiles (Fig. 3I). When using different types of commonly used fluorinated carrier oil, specifically FC40 (density, 1.85 g/ml) and Novec 7500 (1.61 g/ml), the density difference results in a slight variation in the collected droplet size profile, but not notably (Fig. 3K). Overall, depending on the application need, the first and second IDEs can be turned on/off accordingly to simply use the device as a high-pass filter, low-pass filter, or bandpass filter. In addition, the cutoff droplet size of these filters can be readily tuned by increasing or decreasing the applied voltage, changing the channel height, the choice of carrier oil, throughput, or a combination thereof.

The main anticipated utility of this droplet bandpass filter concept is for droplet size QC, as having unwanted droplet sizes coming from errors in droplet manipulations steps or preventing droplet size variations over time is difficult to completely prevent even in the most well-designed and well-optimized droplet microfluidic components and systems. Through three application examples, we have demonstrated such utilities. In the first application, we have designed an alarm system that can be triggered if the droplet size varies during the droplet generation step (Fig. 4, A to D). In the second application example, we have demonstrated that the droplet bandpass filter can select only the perfectly 1:1 merged droplets after a droplet merging unit (Fig. 4, E to G). In the third application example, we have demonstrated the generation of a high-quality IVTT droplet library.

The droplet bandpass filter method here was operated when the carrier oil density was higher than that of the dispensed aqueous solution. However, the method can also be used in the case where the density of carrier oil is lower than that of the aqueous solution, which is the case when using mineral oil. In this case, the IDEs need to be placed on the ceiling of the microfluidic channel instead of the bottom so that naturally sinking droplets can be sorted on the basis of their sizes. It is important to note that the applied voltage did not cause any negative effect on the biological assays (shown in fig. S10), in terms of both cell viability and IVTT reaction. Overall, the developed droplet filter system can be used in combination with other parts of the microfluidic manipulation systems to substantially improve the overall performance of these systems. We expect that the developed technology will be impactful to the field of droplet microfluidics.

METHODS
Device fabrication
The master mold for the PDMS microfluidic device replication was fabricated using a two-photon polymerization instrument (2PP; Nanoscribe Photonic Professional GT, IP-Q photoresist). 2PP printing technology facilitates the fabrication of sloped structures that allow smooth height transition between different regions (22, 35) and is thus used here. Details of the 2PP master mold printing conditions are included in the ESI. The device was replicated from the master mold using conventional soft lithography techniques (23, 36, 37). IDE patterns (Ti/Au, 20 nm/200 nm) were prepared by conventional microfabrication techniques. To isolate and protect the IDE metal layer from direct contact with reagents flowing in the microfluidic channel, a 100-nm Si₃N₄ insulation layer was deposited using plasma-enhanced chemical vapor deposition (Oxford PlasmaLab 80 Plus). Then, the PDMS microchannel layer was bonded onto the Si₃N₄-coated IDE-patterned glass substrate following O₂ plasma treatment. The PDMS microfluidic channel was aligned with the IDE pattern and bonded together at 85°C for 8 hours. Right before the experiment, the fabricated device was rinsed with filtered fresh Aquapel (Pittsburgh Glass Works LLC., USA) to make the entire microchannel surface hydrophobic. The schematic of the entire fabrication procedure is shown in fig. S11. Details regarding the fabrication error and handling are further discussed in fig. S12.

Water-in-oil emulsion droplet preparation
For the initial proof-of-concept validation, droplets of three different sizes were generated separately using different food color dyes [40 μm (black), 80 μm (blue), and 160 μm (red) in diameter]. Novec 7500 (density = 1.61 g/ml) with 2% Pico-surf was used as the carrier oil. These droplets were then mixed as a three-color droplet pool. Next, a highly polydispersed droplet population was generated by
sonicating (Ultrasonic Cleaner, Branson 2800) a mixture of Novec 7500 (2% Pico-surf) with color dye (blue) at a 1:1 volume ratio in a 1-ml syringe for 10 min, and the prepared polydispersed droplet library was then directly reflowed into the droplet bandpass filter device for testing. For demonstration of the droplet merging QC application, the merged and unmerged droplets were prepared using a conventional droplet merging device having a merging efficiency of approximately 80%. Two reflow droplet populations having sizes of 60 and 70 μm in diameter were generated, with the successfully merged target droplet size being 82 μm in diameter and the overmerged droplets being much larger than 90 μm in diameter. The prepared droplets were then reflowed through the droplet bandpass filter device.

**Experimental setup for initial droplet bandpass filter proof of concept**

The droplet bandpass filter system was first evaluated using the droplet library containing droplets of three different sizes, 40 (black), 80 (blue), and 160 μm (red). The main channel heights for the bandpass filter were designed to be 120 μm (Fig. 1C, section 2) and 80 μm (Fig. 1C, section 3), aiming to sort out the 160-μm (red) and 80-μm (blue) droplets from outlets 1 and 2, respectively. The width of the main channel was designed to be 100 μm. The flow rates for the droplet reflow, spacer oil, bias oil infused from outlet 1, and bias oil infused from outlet 2 were set to 30, 200, 400, and 200 μl/hour, respectively. These flow rates should be scaled up on the basis of the channel height. The ratios of the outlet flow rates were set to 30% for outlet 1, 20% for outlet 2, and 50% for outlet 3. The throughput under this flow condition is ~15 to 20 droplets/s. Syringe pumps (Fusion 400, Chenyx Inc.) were used to control the volumetric flow rates of all input flows. The color images and videos were captured through a microscope using a Nikon F13 color camera (5K). A function generator (DG4102, Rigol) and a high-voltage power amplifier (Model 2210-CE, TREK) were used to apply the electrical field to the IDEs. In this experiment, a 24-Vpp 100-kHz square wave was used for generating the electric field for droplet manipulation.

**Experimental setup for droplet bandpass filter performance characterization**

To investigate the passband and bandwidth as well as the sensitivity and selectivity of the developed droplet bandpass filter method, a polydispersed droplet library containing a wide range of droplet sizes (see the “Water-in-oil emulsion droplet preparation” section) was used. Two different bandpass filter designs were fabricated to examine the effects of channel heights on the resulting passband. Here, the channel height combinations for device 1 and device 2 were set to be 100 μm (Fig. 1C, section 2) to 90 μm (Fig. 1C, section 3) and 100 μm (Fig. 1C, section 2) to 80 μm (Fig. 1C, section 3), respectively. In theory, both bandpass filters should sort droplet sizes smaller than 100 μm with bandwidths of around 10 and 20 μm, respectively. The initial voltages were set to 24 Vpp (100-kHz square wave). When investigating the effect of voltages on the resulting passband, the voltage applied to the second IDE was gradually decreased to 12 Vpp with a decrement of 6 Vpp. In addition, the minimum voltages needed to achieve ~100% sorting efficiency for different droplet sizes at different throughput rates were also evaluated using a similar setup.

**In-droplet IVTT workflow**

IVTT of GFP was carried out to demonstrate the utility of the developed droplet bandpass system. The plasmid pJL1-sfGFP was a gift from M. Jewett (Addgene plasmid no. 102634). The plasmid was extracted using the ZymoPURE plasmid miniprep kit (Zymo Research, CA, USA). Forward primer 5′ GCC AAT TAA TAG GAC TCA CTG TAG GG 3′ and reverse primer 5′ TTC TAA TCA GAA TTG GCT TTC AGC 3′ were used to amplify the GFP sequence to generate the DNA template for New England Biolabs (NEB) PURExpress in vitro protein synthesis reagent (NEB, #E6800L). Droplet generation (80 μm) and PCR amplification were carried out using the protocol described by Sukovich et al. (34) but without the addition of Tween 20. To carry out IVTT of GFP, PCR-amplified droplets were reflowed and merged with droplets (100 μm) containing the NEB PURExpress protein synthesis kit, which included the NEB PURExpress solution A (55% v/v), NEB PURExpress solution B (42%), and RNasin ribonuclease inhibitor (3%). The merged IVTT droplets were collected in a 3D-printed droplet cultivation chamber (fig. S13) and incubated at 37°C for 3 hours. The fluorescence intensity of the expressed GFP was measured at an excitation wavelength of 479 nm and an emission wavelength of 520 nm. GFP expression was also confirmed by comparison of the SDS–polyacrylamide gel electrophoresis bands with the well plate control group. The PCR products generated using high fidelity Q5 polymerase were cleaned and concentrated using the Zymo Research DNA clean and concentration kit and used as a positive control.

**Data collection and analysis**

The sizes of collected droplets were measured at the circular droplet observation chambers (“OB” in Fig. 1H) of the microfluidic system. An image analysis workflow was developed in Fiji/ImageJ to calculate the size of the droplets, and the same workflow was then applied to all the acquired images. In brief, the bright-field images were first converted to binary masks based on an arbitrary intensity threshold. Holes in the resulting droplet masks were filled and the bounding boxes of all the droplets were extracted. The height and width of each droplet’s bounding box serve as two measurements of its diameter. An average of the two was taken as the final measurement. A scaling of 0.241 μm/pixel was determined for the objective and camera being used, and based on this, the unit of the diameter measurement was converted from pixels to micrometers. At least 200 droplets were counted and measured for each analysis to provide statistical significance.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abc9108

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