Integrated microdroplet workflow for high-throughput cell-free transcription in double emulsion picoreactors

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Abstract

Single-molecule nucleic acid assays offer a powerful route to understand gene regulation. To enable rapid and precise characterization of regulatory sequences such as promoters within a pool of synthetic $5'$ regulatory sequences, we present an integrated microdroplet workflow for high-throughput cell-free transcription in double emulsion (DE) picoreactors. For this, we utilize the T7 promoter as a positive control and the Mango III RNA aptamer for fluorescent readout of transcription in an *in vitro* setup. First, DNA templates are compartmentalized into picolitre droplets along with rolling circle amplification reagents and incubated off-chip. The resulting emulsion is then reinjected into a second device that integrates three operations on a single chip : (i) emulsion reinjection, (ii) electric-field-mediated StepInjection of IVT reagents and (iii) downstream water-in-oil-in-water (W/O/W) double emulsification. Post IVT incubation, the fluorescent DE droplets are selectively isolated by FACS, enabling sorting as well as the classification of droplets on the basis of their fluorescent intensities. We characterize the workflow with proof-of-principle experiments with three input DNA concentrations that yield distinct fractions of fluorescent DEs and also demonstrate intensity-based binning and indexed sorting of droplets into defined wells of a microtiter plate. This workflow establishes a practical route to high-throughput, single-template transcription assays in FACS compatible double emulsions and provides a foundation for future screening of synthetic regulatory libraries and promoter variants with fluorescence encoded readouts.

Keywords: *in vitro transcription, ultra high-throughput screening, stepinjection, double emulsions, flow cytometry, integrated systems, mango III aptamer*

1. Introduction

Single-molecule nucleic acid assays and single-cell gene expression studies have become indispensable for understanding the complexity and heterogeneity of biological systems [1–3]. By analyzing molecular events at the individual molecule or single-cell level, subtle variations that ensemble measurements often obscure can be detected [4]. These high-resolution methods provide critical insights into gene regulation, protein synthesis, and cellular responses to stimuli [5]. Furthermore, the rapid advancements in mRNA therapeutics fueled by urgent efforts during the COVID-19 pandemic highlight the need of precise control and characterization of gene expression [6]. The ability to fine-tune expression levels is, therefore, essential for designing optimized constructs, ensuring therapeutic efficacy, and minimizing unwanted side effects [7].

A key factor in regulating gene expression is the strength of the promoter, the *cis*-acting DNA sequences that initiate transcription, determining when, where, and at what level genes are transcribed [8, 9]. Characterizing promoter strength enables researchers to engineer genetic circuits with predictable expression levels, which is particularly relevant in synthetic biology and therapeutic applications [10]. One straightforward and effective method for gauging promoter strength is through fluorescence-based assays [11]. In such assays, the downstream reporter can emit a fluorescent signal proportional to the promoter's activity. By encapsulating these reactions in microdroplets, it is possible to generate a large population of individual "bioreactors," each containing a single promoter variant. These droplets can then be sorted according to fluorescence intensity, thereby allowing researchers to bin promoters into categories from weak to strong using a standard fluorescence-activated cell sorting (FACS) platform.

Droplet-based microfluidics has revolutionized high-throughput screening by enabling formation and manipulation of millions of picolitre- to nanoliter-scale compartments [12–14]. It offers several advantages such as ultra-high throughput, since billions of droplets can be generated rapidly; compartmentalization, which isolates individual biochemical or cellular reactions; and the ability to perform complex, multistep protocols in a controlled manner. Despite this, the practical adoption of droplet microfluidics in bioengineering labs is limited by complex instrument setups requiring sophisticated optical alignments. One promising alternative is the generation of double emulsions (W/O/W) that are compatible with commercial FACS instruments [15]. By converting W/O single emulsions into W/O/W double emulsions, droplets become more stable and can be screened on standard benchtop equipment without FADS sorters. Although single-emulsion droplet sorters exist and work at excellent throughput, they often need specialized knowledge of optics and programming (e.g., LabView) to implement multiplexed sorting [16]. In contrast, double emulsion droplets phenotyped on commercial FACS machines can democratize access to high-throughput droplet screening for a broader range of biologists, facilitating more streamlined workflows and reducing the expertise barrier [17].

In the current work, we demonstrate a modular droplet-based workflow that leverages two separate microfluidic devices to screen in-vitro transcription reactions using a fluorescence-based RNA aptamer readout. The schematic overview of the workflow is illustrated in Figure.1 (panels a–f). Specifically, it includes: (a) compartmentalization of DNA templates (plasmids) inside picolitre droplets, (b) off-chip incubation for nucleic acid amplification, (c) Emulsion reinjection, (d) reagent Step-Injection (SI) for introducing in-vitro transcription (IVT) reagents, (e) a double emulsification step to create stable W/O/W double emulsion droplets and finally (f) sorting double emulsions with transcripts via FACS based on fluorescence intensities. A key novelty here is the seamless integration of three critical droplet operations: emulsion re-injection, reagent StepInjection, and double emulsification into a single microfluidic device, which significantly reduces the emulsion handling and possible mismanagement. This integrated approach not only ensures droplet integrity throughout the process, but also enables high-throughput sorting on a

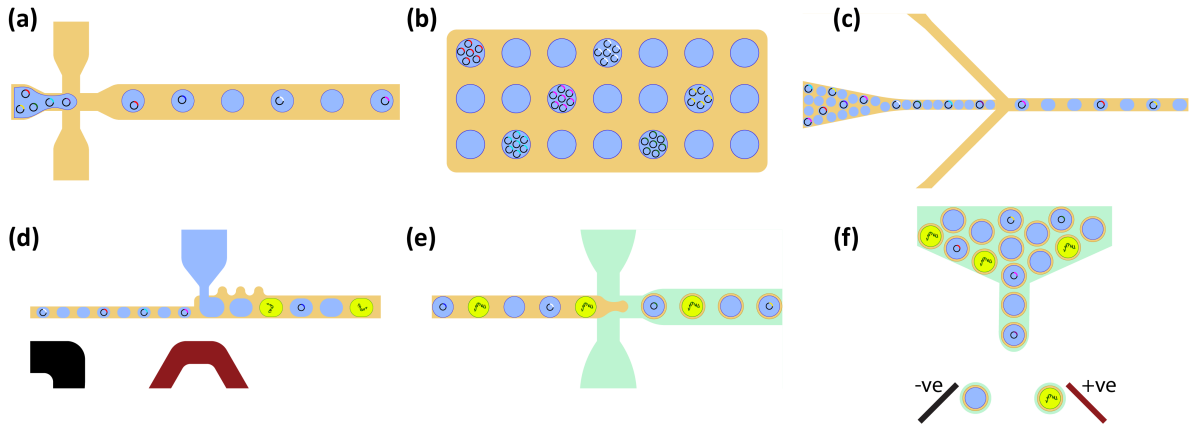

Figure. 1. Schematic illustration of the integrated, six-step droplet-based microfluidic workflow for high-throughput screening of nucleic acids using a fluorescence reporter. Firstly (a) plasmid DNA templates are compartmentalized into picolitre droplets. (b) The droplets are incubated off-chip for isothermal rolling circle amplification (RCA) of plasmid DNA. (c) After amplification, the monoclonal droplets are re-injected into a second device and spaced by oil after which (d) In vitro transcription (IVT) reagents are introduced by electric-field-mediated step-injection to ensure minimal contamination. (e) Each droplet is subsequently converted into a stable water-in-oil-in-water (W/O/W) double emulsion downstream. Finally, the double emulsions are sorted according to fluorescence signal (e.g., Mango-III aptamer readout) on a commercial FACS instrument, enabling intensity-based binning and pooling based on droplet intensity from mRNA formation.

standard benchtop FACS instrument with the possibility of binning droplets based on intensity inside double emulsions. Furthermore, we employ mango III RNA aptamer as fluorescence readout to directly monitor transcript formation, which can also be used to assess promoter strength. While previous work [18] has introduced the cross contamination free step injection system for ultra-sensitive assays, our workflow extends this capability by performing single-molecule in vitro transcription followed by a double emulsification step on the same device. By consolidating three critical droplet operations into a single device, this workflow offers a robust, and user-friendly platform that expands the accessibility and practicality of droplet-based functional screening of regulatory sequences.

2. Materials & Methods

2.1. Microfluidic device fabrication

Device design

The first microfluidic chip, designed for plasmid compartmentalization, featured a simple dual-inlet flow-focusing (FF) junction for droplet generation. The nozzle of the junction was 10 μm wide, and the main channel width was 20 μm. The second microfluidic device incorporated three functional components: (a) emulsion reinjection, (b) a step-injection junction, and (c) a downstream flow-focusing junction for W/O/W double emulsification. Three different microfluidic devices (Design – A, B & C) were designed to test and characterize step-injection and double emulsification (see Figure.3). The channel dimensions for design A and design B have been mentioned in Electronic Supplementary Information (ESI). Design C was chosen for cell free transcription experiments in droplets. Here, the main channel was 13 μm wide upstream of the SI junction and expanded to 25 μm downstream. The SI junction nozzle was 10 μm wide. The corrugated, teeth-shaped structures were integrated adjacent to the nozzle to prevent partial wetting during reagent injection. A serpentine resistor channel was introduced between the stepinjection and double emulsion junctions to electrically isolate the SI junction and prevent corona discharge induced hydrophilicity. The detailed designs and dimensions of both microfluidic devices are provided in Figure.S1 (a & b) of the Electronic Supplementary Information (ESI).

Photolithography

Both microfluidic devices were fabricated using standard photolithography techniques. Briefly, the designs were drafted in AutoCAD 2018 (Autodesk) and converted into direct-writeable DXF files using Layout Editor and Clewin 4.0. The plasmid encapsulation (FF - droplet formation) device was constructed as a single layer microfluidic structure, whereas the second integrated device was constructed in two layers. A negative tone photoresist (mr DWL 5, Micro-Resist technology GmbH) was spin coated on clean 4" silicon wafers as per the manufacturer's guidelines, followed by soft baking (at 50 and 90°C) to ensure complete solvent evaporation. The structures were patterned via direct laser writing with MLA 150 (Heidelberg instruments) at 405 nm with exposures of 300 - 375 mJ/cm^2. The post exposure bake was performed at 90°C. To remove any un-exposed photoresist, the resulting microfluidic structures were then developed in mr-DEV 600 (Micro-Resist technology GmbH) and subsequently washed in IPA and dried with nitrogen. Before using the wafers as molds for PDMS devices, their surfaces were silanized by exposing them to vapours of 1H, 1H, 2H, 2H - perfluorooctyltrichlorosilane in a desiccator. The resulting channel heights for the droplet formation devices were measured to be 10.5 μm, while for the step-injection devices, the heights were 10.75 μm for Layer-01 and 10 μm for Layer-02, as determined using a profilometer (Dektak).

Soft Lithography

After fabricating the master molds, the PDMS devices were prepared by mixing the elastomer and curing agent (Dow Corning, Sylgard 184 elastomer kit) in the ratio of 10 : 1 (w/w). The mixture was thoroughly mixed, degassed, and poured over the master wafers placed in a 5-inch petri dish. The assembly was cured overnight in an oven at 65°C. Once cured, the PDMS layer was gently peeled off from the wafer and diced. All the inlets and outlets were punched with a 1 mm biopsy punch (Harris Uni-core) and the resulting PDMS molds were thoroughly washed with isopropyl alcohol (IPA) and ethanol to remove dust and PDMS debris. The resulting molds were then bonded to PDMS spin coated glass slides (25 mm × 75 mm), after exposing both the

surfaces to oxygen plasma (Diener Electronic) for 140 s at a pressure of 0.4 mbar. The bonded devices were subsequently baked at 150°C for at least 4 - 8 hours to restore the hydrophobicity of the channels.

Microelectrode fabrication

Microelectrodes for step-injection devices were fabricated by injecting a low-melting-point solder (Indalloy # 19 - 51In, 32Bi, 16.5Sn) into custom-made channels near the step-injection junction. Following device baking, the chips were placed on a preheated hot plate maintained at 95 - 100 °C. Approximately 100 - 200 μL of the molten solder was drawn into a syringe and slowly injected into the electrode channels to ensure bubble-free and uniform filling. Excess molten solder was carefully removed, and the metal connectors (1238060000, male header) were inserted to terminate the electrodes. The devices were thoroughly cleaned, and electrical connectivity was verified using a digital multimeter to ensure reliable and reproducible electrode functionality (see the schematic in Figure S.2 (a) in the ESI).

Device coatings

Surface wettability was carefully tuned across the two microfluidic devices to ensure optimal performance of each droplet operation. The microfluidic chip used for DNA compartmentalization and droplet formation was rendered hydrophobic to promote stable generation of monodisperse water-in-oil (W/O) droplets. This was achieved by filling the channels with 1 % (v/v) 1H, 1H, 2H, 2H - Perfluorooctyl - trichlorosilane dissolved in fluorinated oil (Picowave 7500, Sphere Fluidics). Excess solution was removed using compressed air and the devices were baked at 150°C prior to use.

In contrast, the second device which integrates emulsion reinjection, step-injection (SI), and downstream W/O/W double emulsification required spatially selective surface modifications. Firstly, the SI junction and the emulsion reinjection region were coated hydrophobic using the method described above. To enable reliable oil phase pinch-off and robust double emulsion formation, the right side of the W/O/W - FF junction was rendered hydrophilic. This asymmetry in surface wettability was essential for facilitating water-in-oil-in-water (W/O/W) droplet formation and preventing wetting failures. The hydrophilic surface functionalization was achieved in two steps. First, the junction was exposed to a locally generated corona discharge [19] by inserting the tip of the electrode through the channel outlet and activating the electrode for 10 seconds. Next, the junction was chemically modified using a sequential layer-by-layer coating with polyelectrolyte solutions to enhance hydrophilicity [20]. Briefly, after the corona discharged exposure, the channel was flushed with 2 mg/ml solution of PDADMAC (poly (diallyldimethylammonium chloride)) prepared in 0.5M NaCl for 10 minutes followed by rinsing with 0.1 M NaCl. Subsequently, the channel was flushed with 2 mg/ml solution of PSS (poly(styrene sulfonate)) in 0.1 M NaCl for 10 mins and finally, the channel was flushed with filtered MilliQ water. The entire procedure for preferential coating of step-injection devices has been illustrated in Figure S.2 (b) in the Electronic Supplemental Information (ESI).

2.2. Working fluids

Continuous phase

Fluorinated oil was used as the continuous organic phase for all the experiments. For droplet formation and DNA compartmentalization, 3.5 % Picosurf-1 (Spherefluidics) dissolved in fluorinated oil Picowave - 7500 (Spherefluidics) was used as the continuous phase. For spacing the emulsion during the re-injection and reagent step-injection, 1% (v/v) Picosurf-1 was used as

the continuous phase. For the formation of Water-in-Oil-in-Water (W/O/W) double emulsion droplets downstream, a 2 % Tween-80 solution in MilliQ water was used as the continuous aqueous phase. All the continuous phase solutions were filtered through 0.2 μm pore size filters before injection.

T-7 Mango plasmid construction

The Mango III aptamer, synthesized by Twist Bioscience, was first amplified using the Lib1a-F and Lib2a-R primers. The T7 promoter was then incorporated using the Lib1b-F and Lib2b-R primers. The resulting T7-Mango III product was further amplified with the Lib3-F and Lib4-R primers for Gibson assembly into the pSEVA2311 backbone, which had been previously amplified using the Lib5-F and Lib6-R primers. The assembly was performed using the NEBuilder HiFi DNA Assembly Master Mix. Successful integration of the T7-Mango III-pSEVA2311 construct was confirmed through colony PCR with the Lib7-F and Lib8-R primers before being used for the IVT reaction. The Primers has been listed in Table.1 of the ESI.

Isothermal RCA mix

For isothermal amplification in droplets, the illustra GenomiPhi V2 DNA Amplification Kit (Cytiva) was utilized. This kit provides the necessary buffers, random hexamers, and Phi29 DNA polymerase required for plasmid amplification. Briefly, the desired concentration of T7-Mango III-pSEVA2311 template was mixed with the sample buffer in a 1:9 (v/v) ratio. The mixture was heated at 95°C for 3 minutes and then immediately cooled on ice. Separately, the Phi29 DNA polymerase was combined with the reaction buffer in another tube also in the ratio of 1:9 (v/v). Both tubes were cooled on ice before being introduced into the microfluidic device for droplet emulsification.

In-vitro transcription in droplets

The MAXIscript™ T7 Transcription Kit (Invitrogen, Thermo Fisher) was used to prepare the in vitro transcription (IVT) reagent mix. The mix consists of nuclease-free water, 10X transcription buffer, 10 mM of each rNTPs, KCl, and T7 RNA polymerase. To visualize the mRNA transcripts generated within the droplets post stepinjection, TO1-3PEG-Biotin fluorophore (Applied Biological Materials) was included in the reagent mix. This dye specifically binds to the Mango aptamer incorporated into the mRNA, producing a strong fluorescence signal (Excitation wavelength of 505 nm and emission wavelength of 540 nm). A concentration of 150 mM KCl was required to facilitate the binding of the Mango aptamer to the Biotin-conjugated fluorophore, enabling sensitive detection of mRNA transcripts within the droplets. For droplet-based experiments, the IVT stoichiometry was adjusted to account for StepInjection dilution and to maintain a final 1X buffer strength inside the picodroplets. Accordingly, a 120 μl IVT master mix was prepared using the following component volumes: 44 µL nuclease-free water, 16 μl of 10X transcription buffer, 16 μl of 150 mM KCl, 4 μl TO-1 Biotin (1:16.5 stock), and 8 μl each of ATP, CTP, GTP, UTP (10 mM stocks), along with 8 μl T7 polymerase enzyme. This modified formulation ensured that, after injection, the reagent composition inside the droplet matched the required working concentrations without exceeding ionic strength limits or compromising droplet stability. If the injection volume or droplet size is changed, the IVT formulation must be recalculated proportionally to maintain the correct final 1X buffer condition and the required concentrations of rNTPs, KCl, and buffer components inside each droplet.

2.3. Microfluidic operations, image acquisition & analysis

The fluids were injected using the Nemesys S (Cetoni) and PHD Ultra (Harvard Apparatus) syringe pumps. Gastight luer lock glass syringes (1 ml : 1001 TLL and 5ml : 1005 TLL, Hamilton) were used to drive the fluids. Medical grade PTFE tubing (ID : 0.6 mm, OD : 1.07 mm, 24G, BB311-24, Scientific commodities inc.) was used to connect the syringes to the microfluidic chips via precision dispensing needle connectors (ID : 0.33 mm, 23G, L : 6.4 mm , 923025, TE Metcal). For injecting small volumes of the aqueous phases (e.g. 50 – 150 μl), the solutions were withdrawn into a pre-filled tubing and syringe with an air bubble gap to maintain separation. For the stepinjection, on-chip electrodes were connected to a high voltage amplifier (623B, TREK) via copper wires connected to a metal connector (RND 205-00181, female header, Elfa Distrelec). The high voltage amplifier amplified the signal from a waveform signal generator (33600A, Keysight) before transmitting them to the on-chip electrodes. The AC electric field was maintained at a frequency of 30 kHz and an amplitude of 120 V_{PP} to ensure a reliable injection of IVT reagents into the passing droplets. A high-speed camera (FASTCAM SA3, Photron, model: 120 K M1) mounted on an inverted microscope (Nikon Eclipse Ti2) was used to observe the droplet microfluidic experiments and record high-speed movies. Real time validation of droplet generation frequency and volume during the experiments was performed by recording at 30,000 FPS. The edge detection analysis for the droplet volume measurement was performed by Droplet Morphometry and Velocimetry (DMV) [21]. The droplet volumes before and after stepinjection were calculated using the $V = \left[HW - (4-\pi)\left(\frac{2}{H}+\frac{2}{W}\right)^{-2}\right]\left(L - \frac{W}{3}\right)$ [22], where the L represents the length of droplet along the major axis as detected by DMV and H, W are the height and width of the microfluidic channel respectively. The fluorescent images before and after sorting were acquired on Zeiss confocal microscope.

2.4. Double emulsion sorting on FACS

The W/O/W double emulsions post IVT incubation were analysed and sorted on the SH800S Cell Sorter (Sony Biotechnology). Before the injection, a 50 μl DE emulsion was diluted in 500 μl of 1X sheath buffer (Sony) in a 12 x 75 round bottom FACS tube. The emulsion was interrogated using a standard blue laser (488 nm) configuration and a 130 μm sorting chip. This sorting nozzle size leads to approximately 8 nl drop post pinch off with the effective sorting frequency of around 12 kHz. FSC was used as the trigger with a detection threshold of 0.67 %. The FSC gain was 1, BSC gain 28 % and the gain for Mango signal was 40 %. The sample injection pressure was kept high (8 – 9 psi) in the initial phase. After a brief pickup delay time, double emulsions appeared as the events and were gated on SSC-A vs. FSC-A for gating and analysis. Once the events appeared the pressure was reduced to 4 - 5 psi. The event rate was kept between 300 - 800 events/s to minimize any shear induced breakup of the double emulsions. Since the DEs were denser than the sheath buffer, they were manually suspended intermittently during the analysis and sorting. Post the double emulsion gating on the SSC vs FSC plot, the DEs were subsequently gated on Mango-TO1-3PEG biotin signal on the fluoroscence histogram.

3. Results & discussions

3.1. Double emulsion pico-reactor workflow

Droplet formation and encapsulation of T7-Mango plasmids

The droplet-based screening workflow begins with co-encapsulation of T7-Mango plasmids and the isothermal RCA regents into picolitre-sized droplets using a dual-inlet flow focusing microfluidic device. Plasmids and sample buffer were injected via the first inlet, while enzyme and reaction buffer were introduced via the second (see Figure.S1 (a) of the ESI). Figure. 2 (a) shows the droplet formation process. The startup protocol involved prefilling the microfluidic device with the continuous oil phase at a flowrate of 100 - 200 μl/hr. Once the stable oil flow was established, both the aqueous phases were injected at 25 μl/hr ensuring that dispersed phases did not wet the PDMS channel walls. This approach consistently enabled stable emulsion production with uniform droplet volumes and no partial wetting. Following the initialization of the device operation, the steady state flowrates were optimized to 75 μl/hr for the continuous oil phase and 25 μl/hr for both the aqueous phases (50 μl/hr in total). Under these settings, the device reliably produced droplets of $\sim$ 2.5 pl at a generation frequency of $\sim$ 6.5 - 7 kHz in the *dripping* regime ensuring a high droplet monodispersity (<1 % COV). While the frequency and droplet volume during the experiment was confirmed by recording high speed movies, the movies were also analyzed via DMV for droplet volume calculations via edge detection [21] (see inset Figure. 2 (b)). The droplet size distribution shown in Figure.2(d) exhibits a narrow unimodal peak centered at 19.9 $\pm$ 0.05 μm, with a coefficient of variation below 0.5 %, confirming the high monodispersity of the emulsion. The corresponding effective confined volume within the channel geometry is estimated to be 2.3 pL, consistent with the designed droplet volume under stable dripping-regime operation.

Rolling circle amplification in picodroplets

Emulsion containing T7-Mango DNA plasmid templates and the RCA mix was collected in a 1 ml syringe (B-Braun) prefilled with 3.5 % Picosurf-1 in Picowave-7500. After being collected for approx. 2 hours, the droplets were incubated at 30°C overnight (16 hrs) to enable isothermal amplification. The Phi29 DNA polymerase in the mix utilizes random hexamers to initiate rolling circle amplification, generating long concatemeric DNA strands from the circular T7-Mango plasmid template [23, 24]. Post amplification, the enzyme was heat deactivated by incubating the emulsion at 65°C for 10 mins.

Compared to conventional thermocycling methods such as ddPCR, RCA offers a gentle and isothermal protocol that preserves droplet stability and integrity [25]. Initial attempts using ddPCR thermocycling with Q5 and TAC polymerases led to droplet coalescence and loss of monodispersity, likely due to surfactant instability at elevated temperatures ($\geq$ 90°C).This instability was consistently observed across multiple surfactant formulations and thermal profiles, as illustrated in Figure S.3 (ESI), highlighting the limitations of thermocycling for droplet-based PCR. Owing to a lower temperature of incubation the monodispersity of the emulsion remained intact post amplification. Figure.2 (c) shows a highly monodisperse emulsion post amplification which is critical for ensuring a stable emulsion reinjection and further downstream droplet operations.

Cell free transcription via stepinjection and double emulsification

Efficient and reliable injection of IVT reagents into DNA-containing droplets is central to the robustness of any droplet-based promoter discovery routine. Typically, in droplet microfluidics,

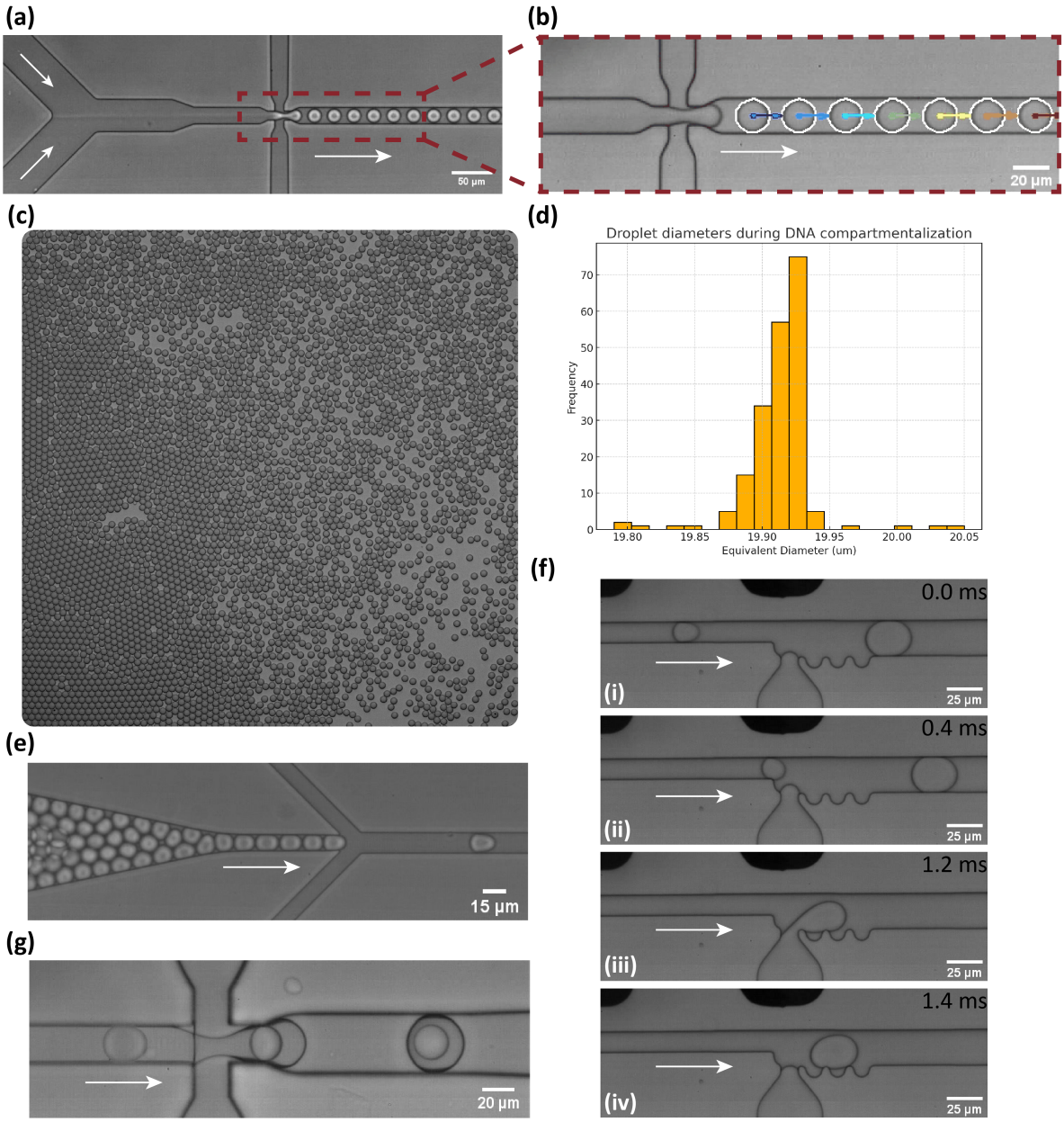

Figure. 2. Integrated microfluidic workflow for high-throughput cell-free transcription in double emulsion picoreactors. (a) Droplet generation using a dual-inlet flow-focusing junction for encapsulating T7-Mango plasmids and RCA reagents. (b) Frame from DMV analysis used for droplet size and monodispersity quantification via edge detection [21]. (c) Emulsion post-isothermal rolling circle amplification (RCA), confirming surfactant stability and droplet integrity. (d) Histogram showing droplet size distribution post-compartmentalization and RCA (n = 200 droplets), confirming <1 % COV. (e) Reinjection of droplets into the integrated chip, spaced by 1 % Picosurf-1 in HFE-7500. (f) Time-lapse series of electric field-mediated StepInjection of IVT reagents (T7 RNA polymerase, rNTPs, and TO-1 Biotin dye) into flowing DNA-containing droplets. (g) Downstream W/O/W double emulsification of injected droplets using a 2 % Tween-80 continuous aqueous phase, yielding stable double emulsion (DE) picoreactors suitable for off-chip incubation and FACS-based phenotyping.

pico-injection [26] is employed for this purpose; however, pico-injectors may be susceptible to inter-droplet cross contamination and can be suboptimal for molecular biology assays that demand high specificity and sensitivity [27]. To address this, we implement the "StepInjection" previously introduced by Hu & co-workers [18]. In this work, we extend this concept by inte-

grating it upstream of a W/O/W double emulsification module, enabling downstream compatibility with standard FACS-based double emulsion phenotyping. Figure S1 (b) in the Electronic Supplementary Information (ESI) shows the complete layout of this integrated device with all relevant channel dimensions. This integration facilitates three operations to be performed on a single microfluidic chip: (i) emulsion re-injection (see Figure.2 (e)), (ii) StepInjection of the IVT reagents (see Figure.2(f)) and (iii) double emulsification of droplets post StepInjection (see Figure.2(g)).

The integrated device coordinates four fluid streams to execute the overall multi-step workflow. Firstly, the single emulsion droplets post-isothermal amplification was reinjected into the chip and spaced using 1 % Picosurf-1 in Picowave 7500 forming an emulsion train with controlled droplet spacing (Figure.2(e)). High droplet monodispersity was critical for the stability of the subsequent reinjection. At the step-injection (SI) junction, IVT reagents—including T7 RNA polymerase, rNTPs, and the fluorogenic TO1-Biotin dye were delivered into each droplet via electric field mediated interface de-stabilization. Figure.2(f) shows a timelapse series of the SI process. The electric field (30kHz, 120 V_{PP}) was applied through on-chip microelectrodes to drive a contamination free merging of IVT reagents. Immediately downstream, the reagent-injected droplets entered a flow-focusing (FF) junction where a 2% Tween-80 aqueous stream sheared the oil phase to form stable water-in-oil-in-water (W/O/W) double emulsion picoreactors. This is shown in Figure.2(g). The resulting double emulsions in which the droplet consists of an oil shell and a post-injection core were collected and incubated at 37 °C prior to FACS based sorting.

Successful execution of this integrated workflow relied on a strict and deliberate startup protocol to prevent backflow and partial wetting. The downstream FF junction was first primed with the continuous aqueous phase (2% Tween-80 in MilliQ). Once filled, the spacer oil flow was initiated, producing stable oil-in-water droplets to establish the baseline flow. Only after both continuous phases were stabilized was the IVT reagent stream introduced at the SI junction. At this stage, transient droplet formation at the SI junction was observed until the emulsion re-injection was started. Once the emulsion enters, all four flowrates re-injection, spacer oil, IVT reagents, and continuous aqueous phase were fine-tuned to reach a stable operational regime. The steady state flowrates were $Q_{em} = 6$ μl/hr for the emulsion, $Q_{sp} = 65$ μl/hr for the spacer oil, $Q_{in} =$ 10-15 μl/hr for the IVT reagents and $Q_{aq} = 150$ μl/hr for the continuous aqueous phase (aqueous 2% Tween-80). In this final steady-state, a continuous train of monodisperse single emulsions entered the chip, received precise reagent addition, and were converted into W/O/W double emulsions at the FF junction.

3.2. Characterizing reagent stepinjection

To evaluate how StepInjection (SI) junction geometry influences reagent injection, we tested three microfluidic designs that varied in the width of the main channel downstream of the SI junction as well as nozzle width. All DNA-containing droplets originated from the same upstream generation protocol, ensuring consistent initial droplet volumes across designs. Timelapse microscopy of the SI region for each design is shown in Figure.3 (a), with corresponding channel dimensions annotated. Quantitative volume analysis was performed using edge detection via Droplet Morphometry and Velocimetry (DMV) on high-speed recordings (see Figure.3 (b)]. For each design, droplet volumes before and after reagent addition were extracted, and the injected volume was calculated as their difference. As shown in Figure.3 (c), the injected volume increased systematically from Design A to Design C to Design B, correlating with the progressive widening of the post-injection channel. This trend aligns with predictions from droplet formation physics in the squeezing regime at low capillary numbers, where a wider downstream channel prolongs the coalescence and pinch-off time, thereby permitting a larger

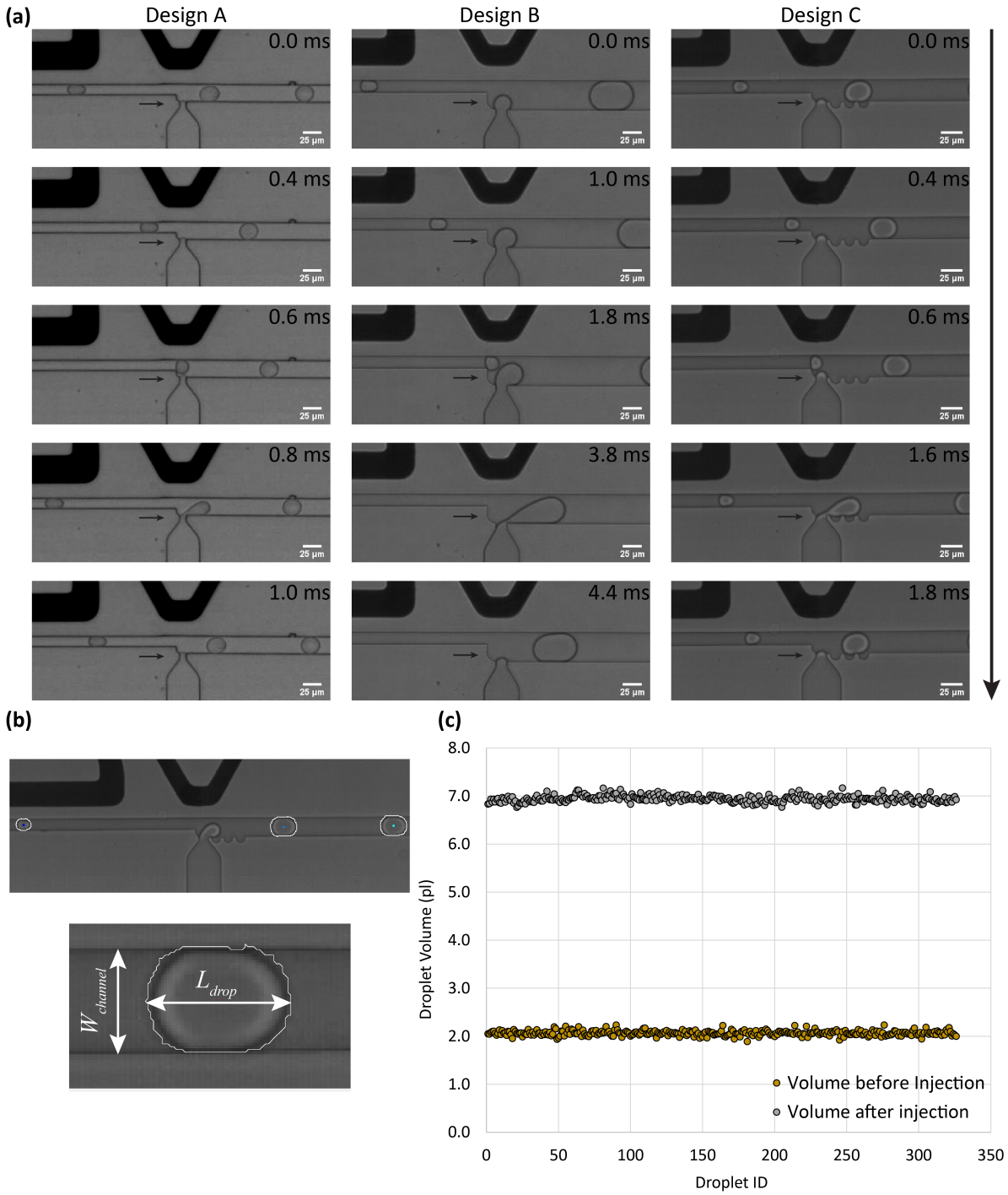

Figure. 3. Characterization of microfluidic reagent stepinjection for droplet based sensitive in vitro transcription assays. (a) Time-lapse microscopic images showing electric field-mediated stepinjection in three microfluidic devices (Design A, B, and C), where the post-junction channel width and nozzle dictates the injected reagent volume. (b) Representative frame from Droplet Morphometry and Velocimetry (DMV) analysis showing edge detection and droplet length measurement before and after injection. (c) Quantitative assessment of droplet volumes before and after StepInjection across 350 droplets per design. Volume consistency and monodispersity were maintained post-injection.

volume of reagent to be incorporated before detachment occurs [28, 29]. While Design A enabled high-frequency injections, the limited reagent volume ($\sim$ 6 pL) may be insufficient for robust IVT, increasing the risk of false-negative readouts. Conversely, Design B achieved the

largest injection volumes ($\sim$ 18 pL) but exhibited slower droplet processing and instability during W/O/W double emulsification, likely due to larger internal droplet volumes challenging the pinch-off at the final flow-focusing junction. Design C, with an intermediate channel geometry, achieved a favorable tradeoff: sufficient reagent addition for reliable IVT, stable double emulsion formation, and throughput compatible with large-scale screening. Importantly, all three designs maintained tight post-injection monodispersity, confirming the consistency of SI performance across geometries. These findings emphasize the critical role of post-junction channel width in tuning reagent delivery and throughput, allowing the SI architecture to be tailored for assay-specific demands.

3.3. Double emulsion phenotyping via FACS

To phenotype and sort droplets based on IVT-driven fluorescent signal, double emulsions were analyzed using a commercial FACS system (Sony SH800) equipped with a 488 nm laser and FITC filter set. Figure.4 outlines the flow cytometry gating strategy used throughout this study. DE droplets were first identified based on forward scatter (FSC) and side scatter (SSC) profiles, which distinguish them from smaller oil droplets and debris. Singlet droplets were then selected by gating on FSC height vs width, eliminating coincident doublets that may bias signal interpretation. Finally, fluorescence histograms were generated to quantify the proportion of droplets showing positive Mango signal in the FITC channel. This standardized gating pipeline enabled robust quantification of IVT activity at the single-droplet level and allowed downstream recovery of functional droplets by FACS-based sorting.

To validate the functional performance of the integrated DE droplet workflow, we performed a set of proof-of-concept experiments using a known positive control plasmid encoding the T7-Mango aptamer. In this approach, we systematically varied the initial plasmid concentration across three separate experiments, simulating different gene loading scenarios. These dilutions : 1:1000 (low), 1:100 (medium), and 1:10 (high) were applied during the initial DNA encapsulation step. Due to the stochastic nature of encapsulation, droplet occupancy follows a Poisson distribution, resulting in a large fraction of empty droplets, especially at lower input concentrations. Following isothermal RCA, step-injection of IVT reagents, and 3-hour incubation at 37°C, the resulting W/O/W DE droplets were analyzed via FACS (Sony SH800) using the FITC channel to detect Mango-TO1 Biotin fluorescence. As shown in Figure.5(a), the fraction of fluorescent-positive droplets increased with input DNA concentration: from 0.81% at 1:1000 to 18.59% at 1:10. These matched expectations based on Poisson statistics and confirmed that only DNA-loaded droplets exhibited Mango signal following successful transcription. To assess the ability of the platform to isolate functional droplets, we performed sorting of the positive population (based on Mango fluorescence) and re-imaged the sorted DEs under fluorescence and brightfield microscopy. Figure.5(b) demonstrates a high level of sorting fidelity: sorted positives consistently showed bright fluorescent cores, while the negative population exhibited no detectable fluorescence. Together, these experiments establish that our DE-based droplet platform enables robust, high-resolution phenotyping of IVT activity at the single-droplet level. Though these experiments employed a known positive control, they validate the key capabilities needed for future high-throughput screening of randomized promoter libraries—accurate reagent delivery, signal-specific readouts, and efficient recovery of functional variants based on phenotypic output.

3.4. Intensity based droplet binning

Building on this paradigm, we demonstrate a fluorescence intensity based indexed sorting strategy for double emulsion (DE) picoreactors using a commercial fluorescence activated cell sorter (FACS). This workflow parallels the multiplexed droplet sorting concept reported by Caen

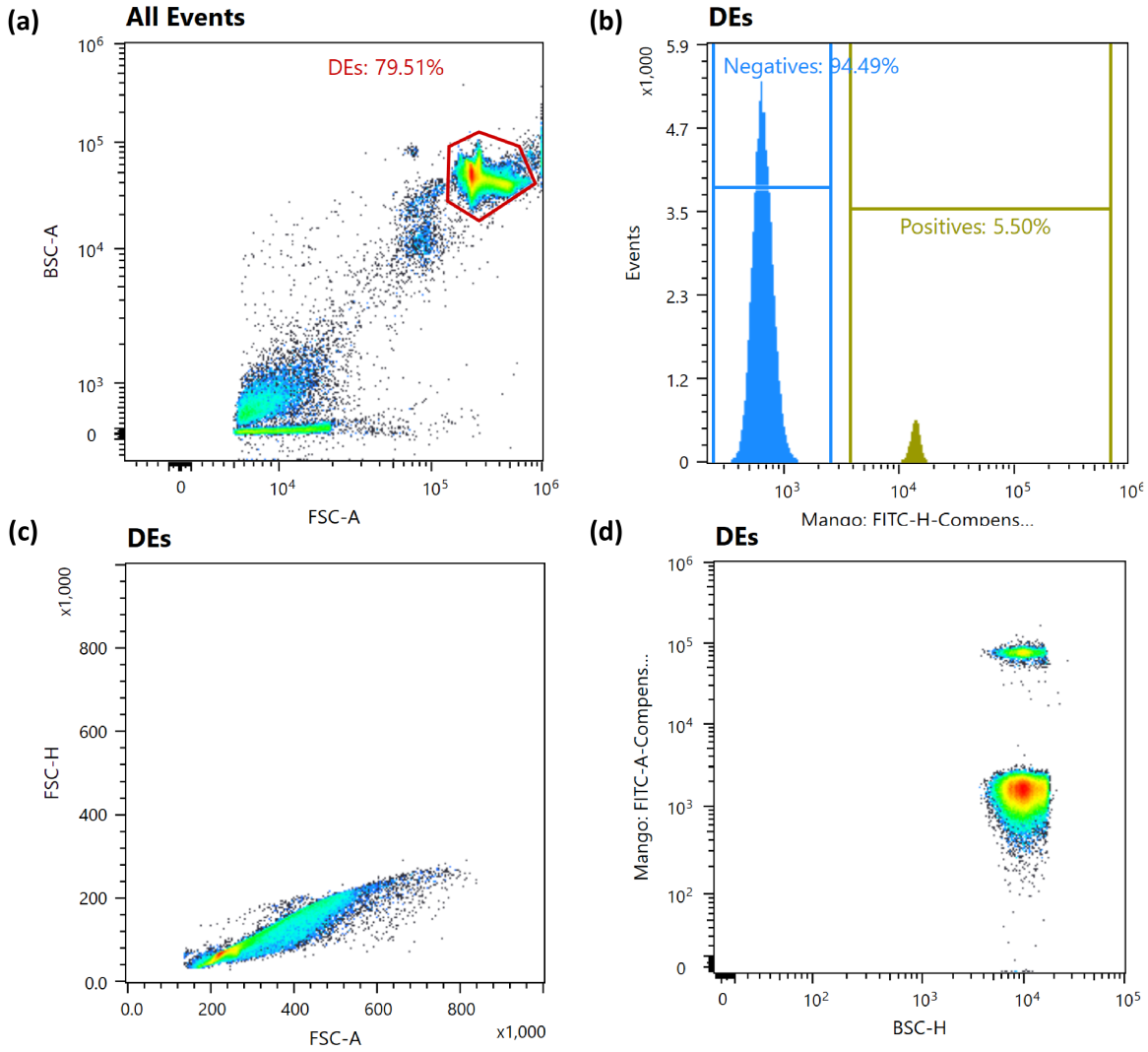

Figure. 4. (a) Side scatter (SSC-A) vs. forward scatter (FSC-A) plot of all detected events. The gated population (red polygon) corresponds to DE droplets based on their expected size and granularity, comprising 79.51 % of total events. Smaller oil droplets and debris appear as lower FSC-A/SSC-A populations and are excluded. (b) Fluorescence histogram (FITC-H channel) of gated DE droplets showing distinct negative (blue) and positive (olive) populations. Linear gating separates the fluorescently active DEs (5.5 %) from the non-fluorescent background (94.5 %) for downstream sorting and analysis. (c) Doublet discrimination plot of FSC-H vs. FSC-A for DE droplets. The diagonal trend indicates a predominantly singlet population with minimal doublet contamination. No additional gating was applied, as the population lies tightly along the diagonal. (d) Two-dimensional dot plot of FITC-A (fluorescence) vs. SSC-H, showing two distinct droplet populations. The lower cluster represents non-fluorescent DEs, while the upper cluster corresponds to highly fluorescent DEs, corroborating the histogram-based gating.

and co-workers [16] but extends it to water-in-oil-in-water (W/O/W) emulsion offering greater stability, and full compatibility with standard FACS instrumentation. Instead of performing on-chip single emulsion (O/W) microfluidic sorting, the DE droplets are electronically gated and deposited into predefined wells of a Microtiter plate according to their fluorescence intensity levels, enabling accurate binning of transcriptional activity. As a proof of principle, we used T7-Mango plasmids as a positive control template to generate fluorescence upon successful in vitro transcription (IVT) inside the droplets using the integrated workflow. The resulting Mango mRNA fluorophore complexes produced signals in the FITC channel. As shown in Figure.6 (a), DE droplets were categorized into three fluorescence intensity bins : high, medium, and low;

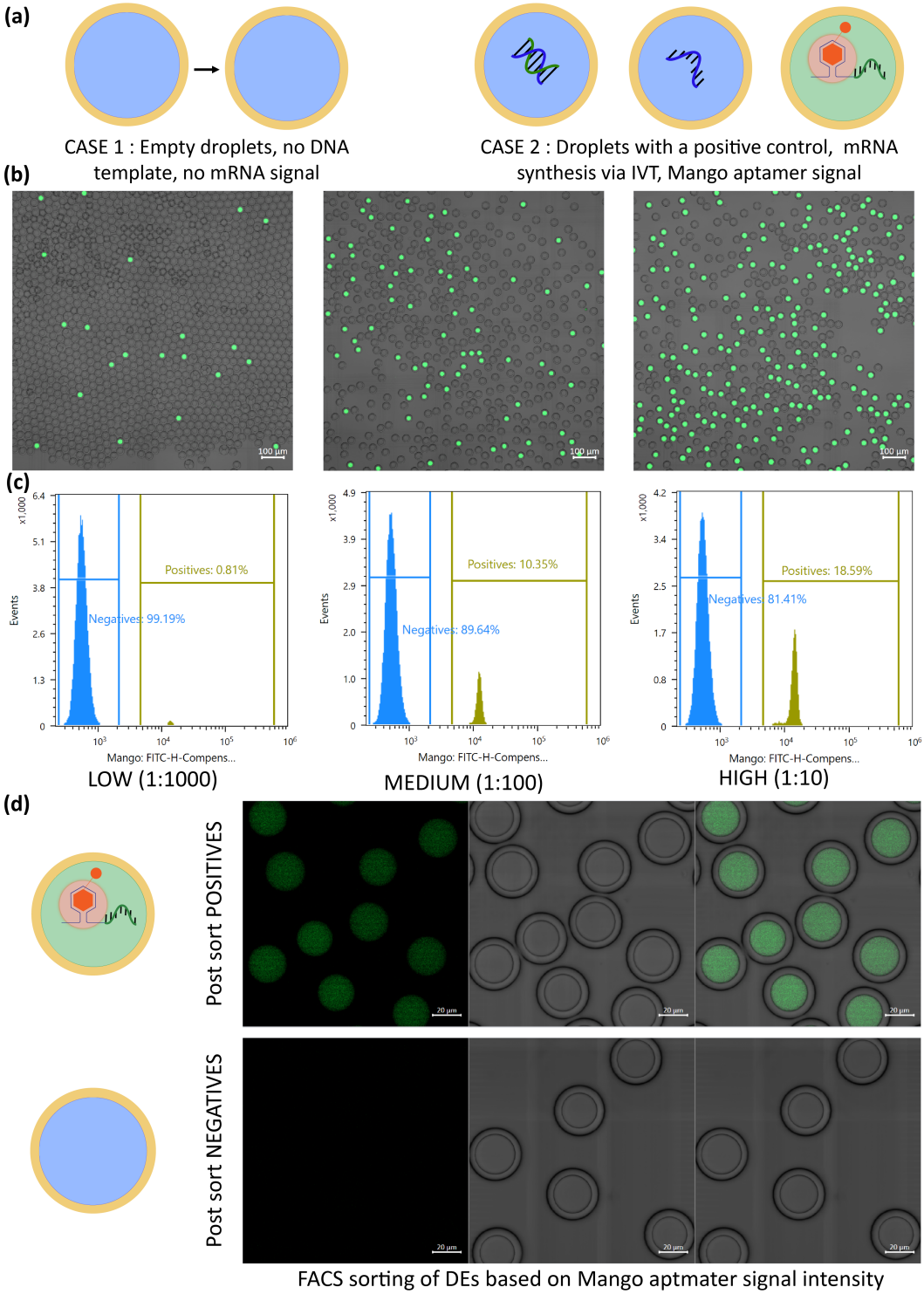

Figure. 5. Phenotyping and FACS sorting of double emulsion (DE) droplets based on IVT-driven Mango aptamer fluorescence. (a) Top row: Schematic illustrations of experimental cases—Case 1 (left) shows empty droplets lacking DNA template, while Cases 2 (middle, right) represent droplets encapsulating T7-Mango plasmids at increasing concentrations. Middle row: Overlaid fluorescence and brightfield microscopy images of post-IVT DE droplets reveal an increase in the number of fluorescent cores with higher input DNA. Bottom row: Corresponding FACS histograms (Sony SH800) showing clear separation between fluorescent-positive and negative droplets across three template loading levels—low (1:1000), medium (1:100), and high (1:10). As expected from Poisson statistics, the percentage of positive droplets increases with template concentration: 0.81%, 10.35%, and 18.59%, respectively. (b) Post-sort analysis of DE droplets based on Mango signal intensity. Top: Sorted positive droplets show clear fluorescent cores; Bottom: Sorted negative droplets show no fluorescence, confirming phenotypic separation.

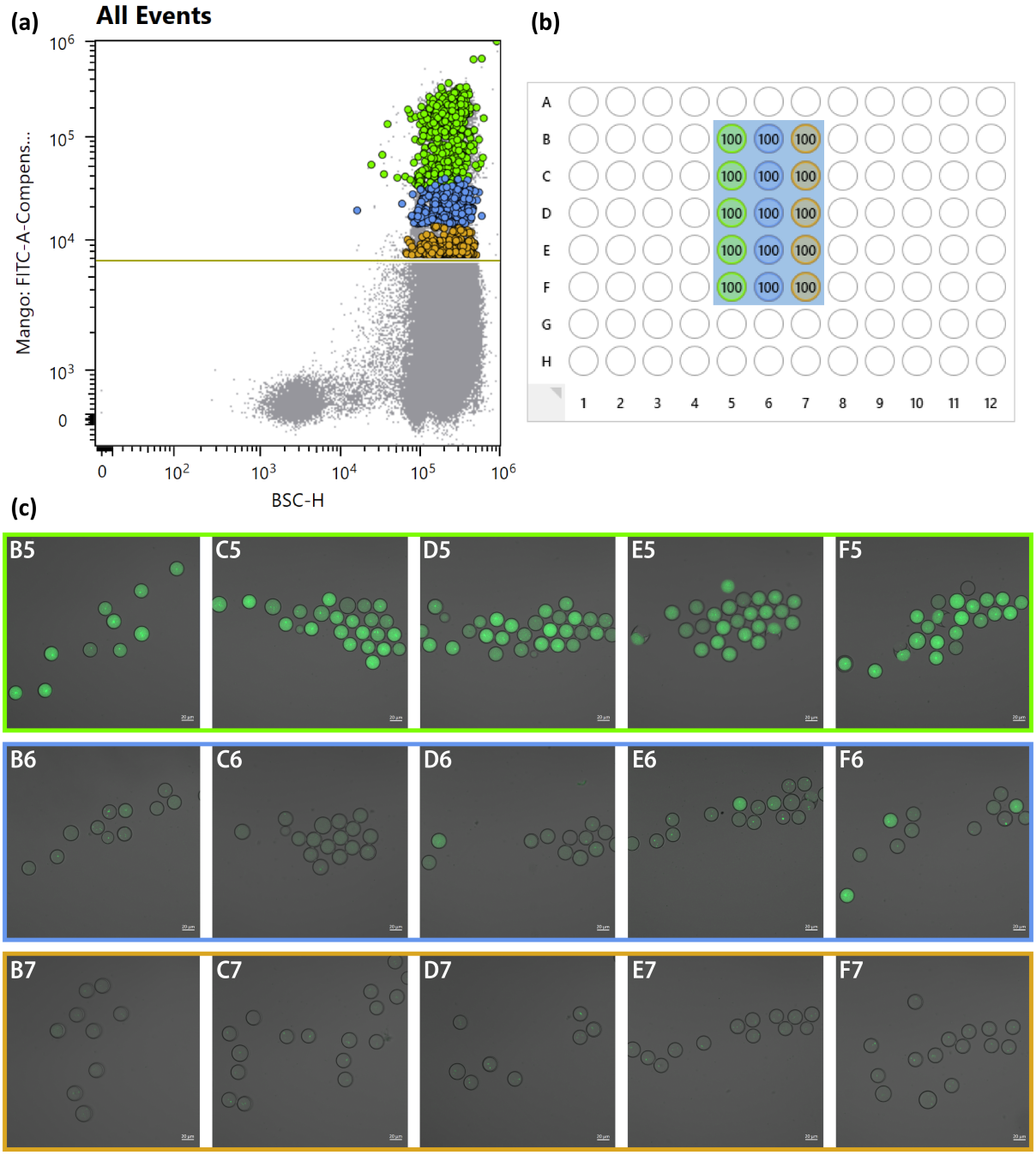

Figure. 6. Fluorescence intensity-based indexing of double emulsion (DE) droplets using FACS. (a) Fluorescence gating strategy used to partition DE droplets into discrete intensity bins (high, medium, low) based on Mango RNA fluorescence in the FITC channel. (b) Indexed deposition map showing the predefined well positions corresponding to each fluorescence gate during single-droplet sorting into a 96-well plate. (c) Representative post-sort microscopy images of W/O/W droplets retrieved from individual wells across the three fluorescence categories, illustrating clear separation of bright, intermediate, and dim droplet populations. The figure highlights the ability of the DE workflow to generate distinct transcriptional phenotypes and to recover rare high-intensity events through fluorescence-based binning.

each representing distinct mRNA yield distributions. These categories were indexed to specific regions of a 96-well plate (Figure.6 (b)), and post-sort microscopic imaging confirmed accurate fluorescence-based segregation (Figure.6(c)). Wells corresponding to high-intensity gates exhibited densely fluorescent droplets, while medium and low bins contained progressively dimmer

populations, validating the intensity-based categorization. This experiment establishes the foundation for functional screening of synthetic promoter libraries in which droplets would encapsulate a unique promoter variant driving transcription from a σ factor specific RNA polymerase complex. The resulting fluorescence intensities could thus report on promoter strength, allowing rapid categorization of artificial constructs into "strong," "medium," and "weak" regulatory elements. By operating under Poisson loading conditions (20 % occupancy with single variant), this approach would enable single template encapsulation and minimize crosstalk, facilitating genotype-phenotype mapping. This fluorescence-indexed DE sorting framework conceptually complements the bulk in vitro transcription based promoter characterization reported by Khan et al. [30], and illustrates how similar promoter activity measurements could, in the future, be adapted to a single-template droplet format to enable higher-throughput, finer-resolution functional screening.

4. Conclusions & perspective

In this work, we present an integrated microdroplet workflow for high-throughput cell-free transcription using double emulsion (DE) picoreactors, compatible with standard flow cytometry-based phenotyping and sorting. The platform brings together three microfluidic operations: (i) Picodroplet formation for co-encapsulating T7-Mango plasmids and RCA reagents followed by isothermal DNA amplification inside droplets, (ii) electric field-mediated StepInjection of IVT reagents and (iii) on-chip double emulsification. The emulsion re-injection, reagent injection and double emulsification operations were integrated on a single microfluidic device. This streamlined integration reduced device handling steps and minimized perturbations between assay stages, thereby offering enhanced robustness and reproducibility across experiments. While cross-contamination free stepinjection module has been introduced in earlier reports, here we characterize three different StepInjection device designs, where varying the width of the downstream channel and nozzle size influenced the volume of reagents being injected into the droplets after reinjection. To validate the performance of this workflow, we carried out proof-of-principle IVT experiments using a positive control plasmid (T7-Mango) at three different DNA input concentrations. This led to differences in the percentage of droplets showing fluorescent signal, as quantified via FACS. The experimental results aligned with Poisson statistics, reflecting the expected drop occupancy and enabling benchmarking of platform sensitivity across a range of template concentrations. Finally, by capturing cell-free transcription directly inside W/O/W double emulsions, our workflow circumvents the need for custom single emulsion-based FADS setups, enabling high-throughput phenotyping using standard commercial FACS instruments. DEs were sorted and binned based on aptamer-linked fluorescence readouts, providing a powerful and scalable route to indexing droplet phenotypes by fluorescence intensity.

Looking forward, this workflow lays a solid platform for functional screening of regulatory DNA libraries in vitro. For instance, a randomized promoter library can be encapsulated in droplets and a specific sigma factor- RNA polymerase can be injected into drops to identify active promoter elements responsive to that sigma factor. With fluorescence intensity serving as a proxy for transcriptional strength, sorted W/O/W double emulsions can be recovered, opened and ranked enabling quantitative promoter engineering. Operating at low template occupancy would enable single promoter variant encapsulation and minimize false positives from multiple encapsulations. Overall, this work establishes a robust, modular framework for genotype - phenotype coupling in droplets, unlocking new possibilities in synthetic biology, regulatory genomics, and enzyme evolution.

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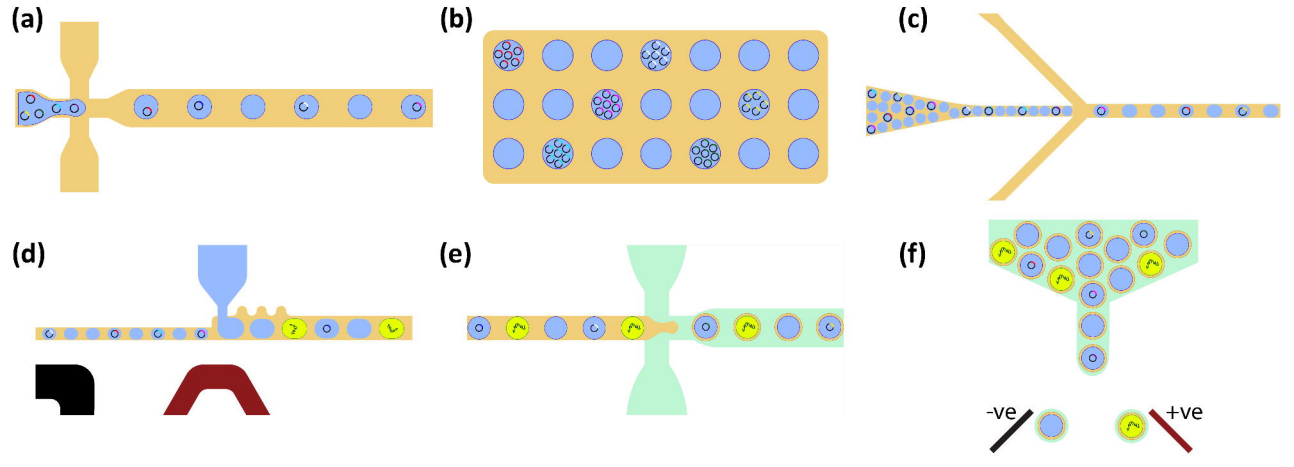
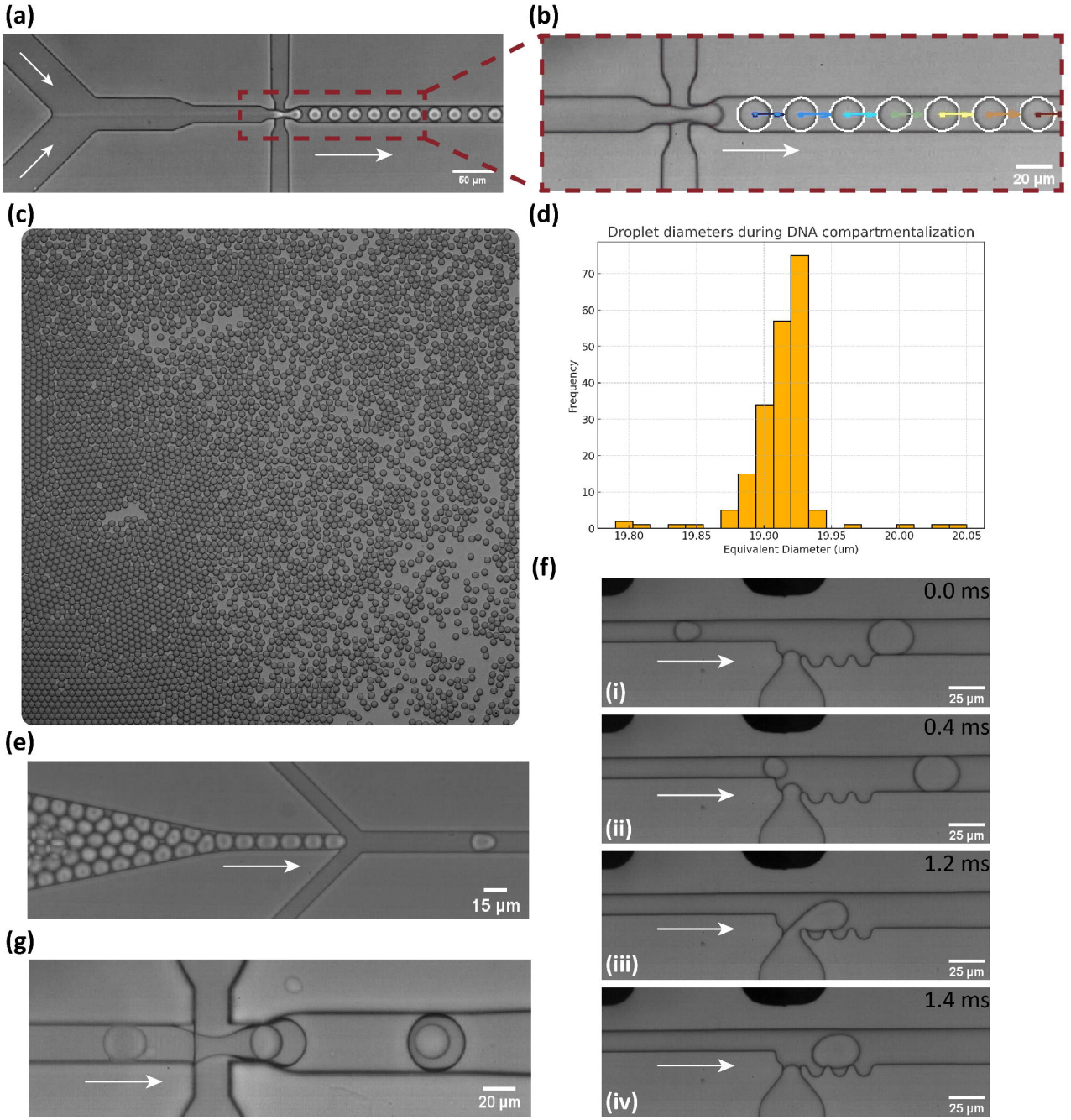
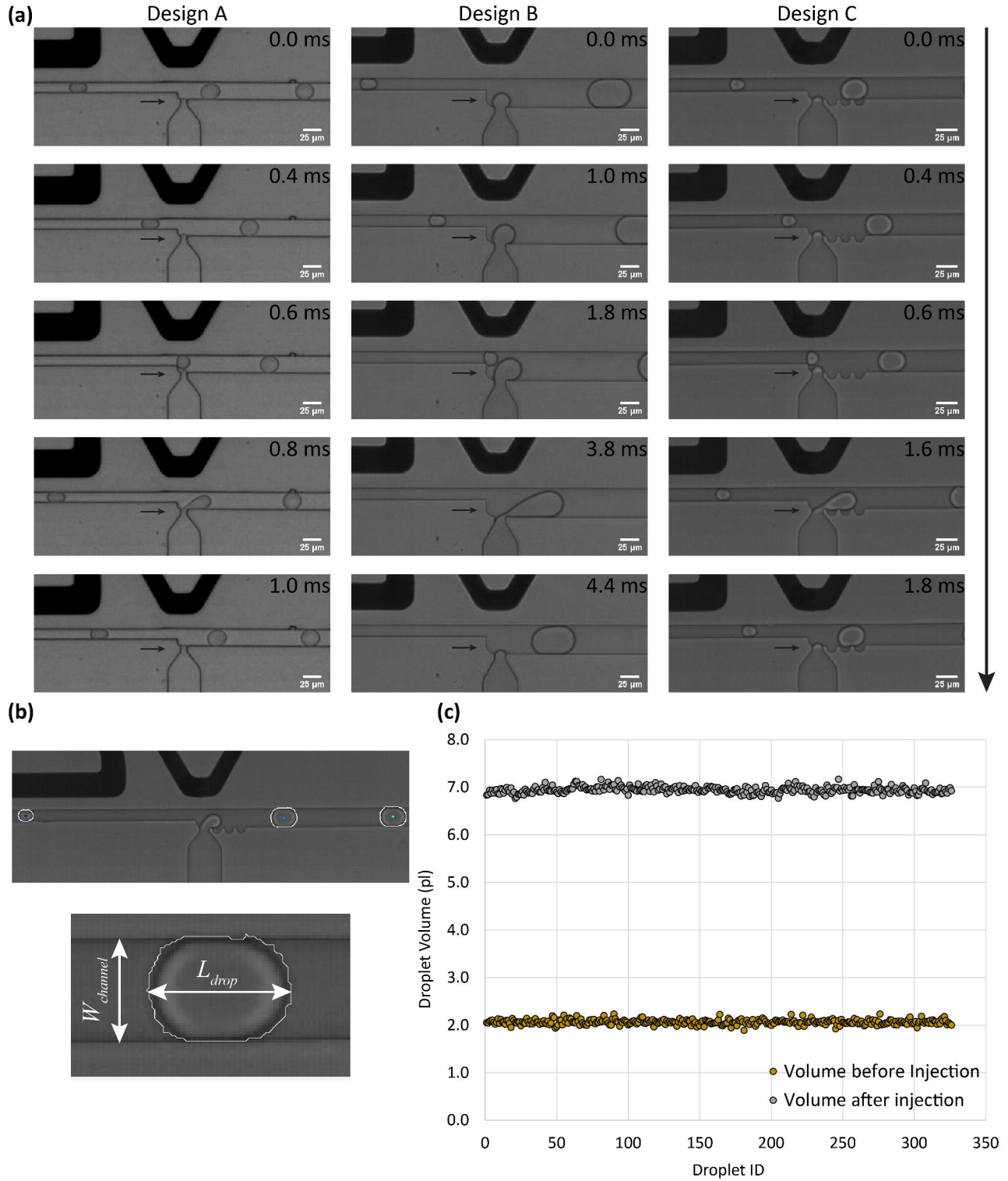
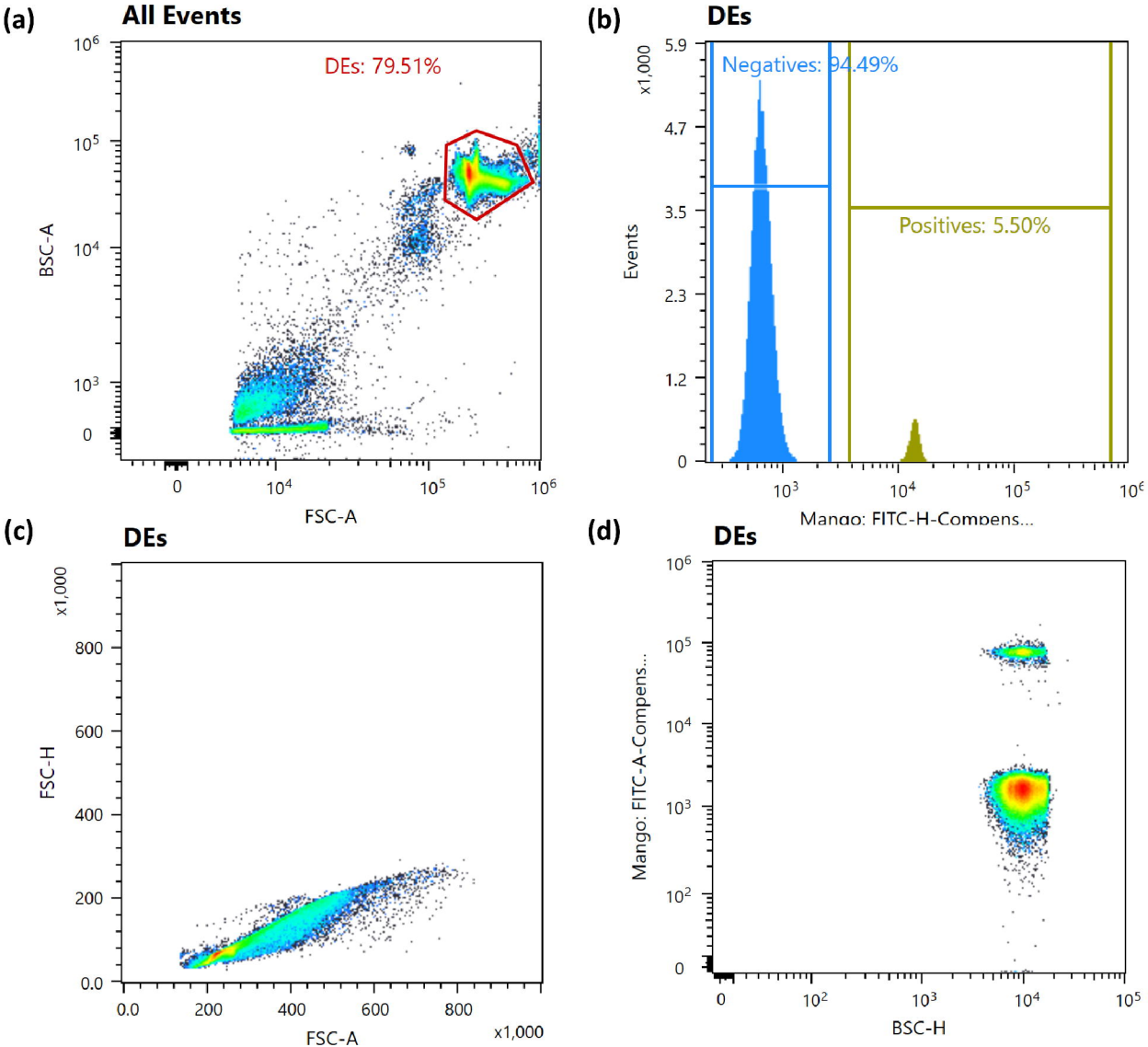
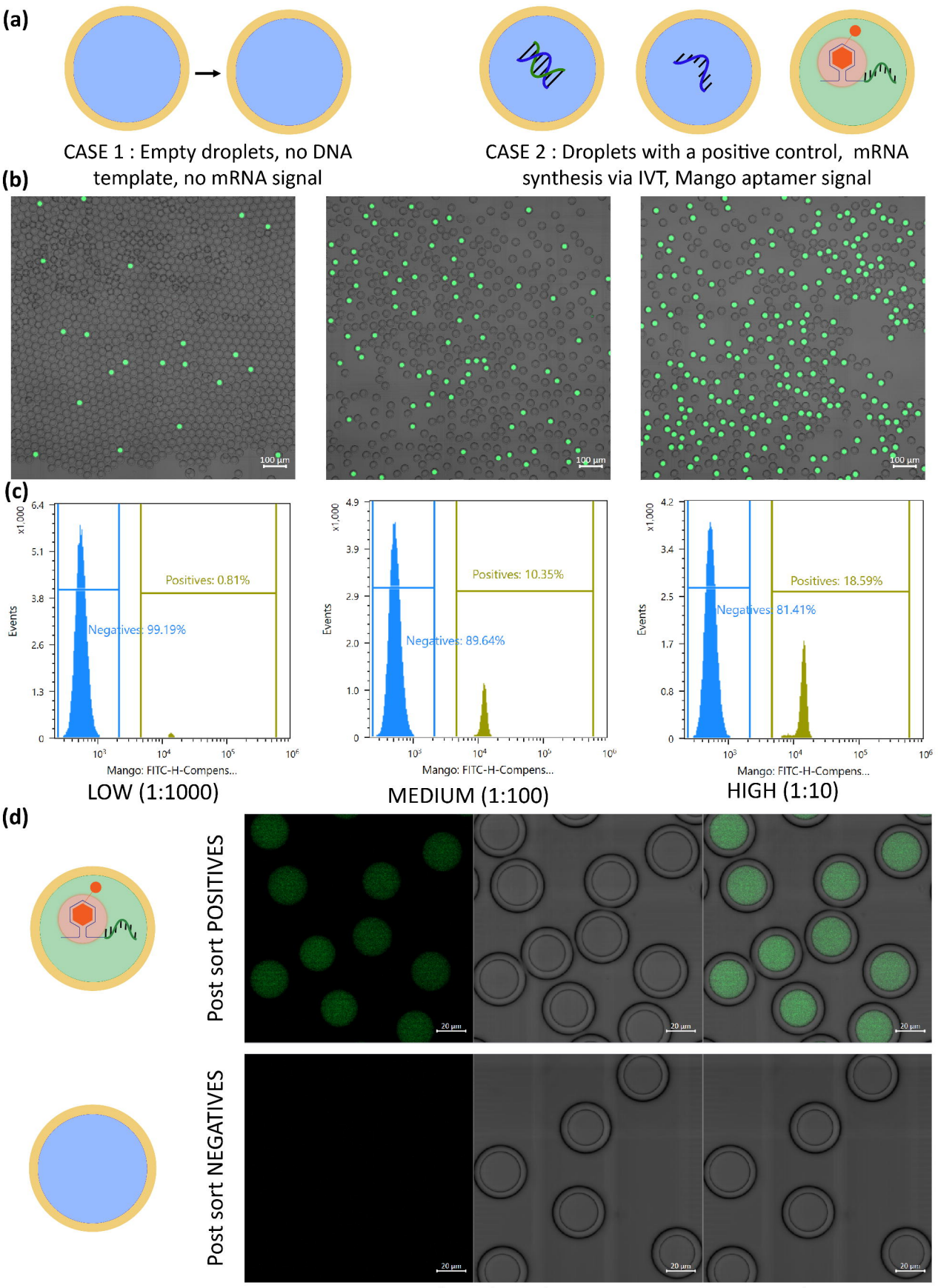
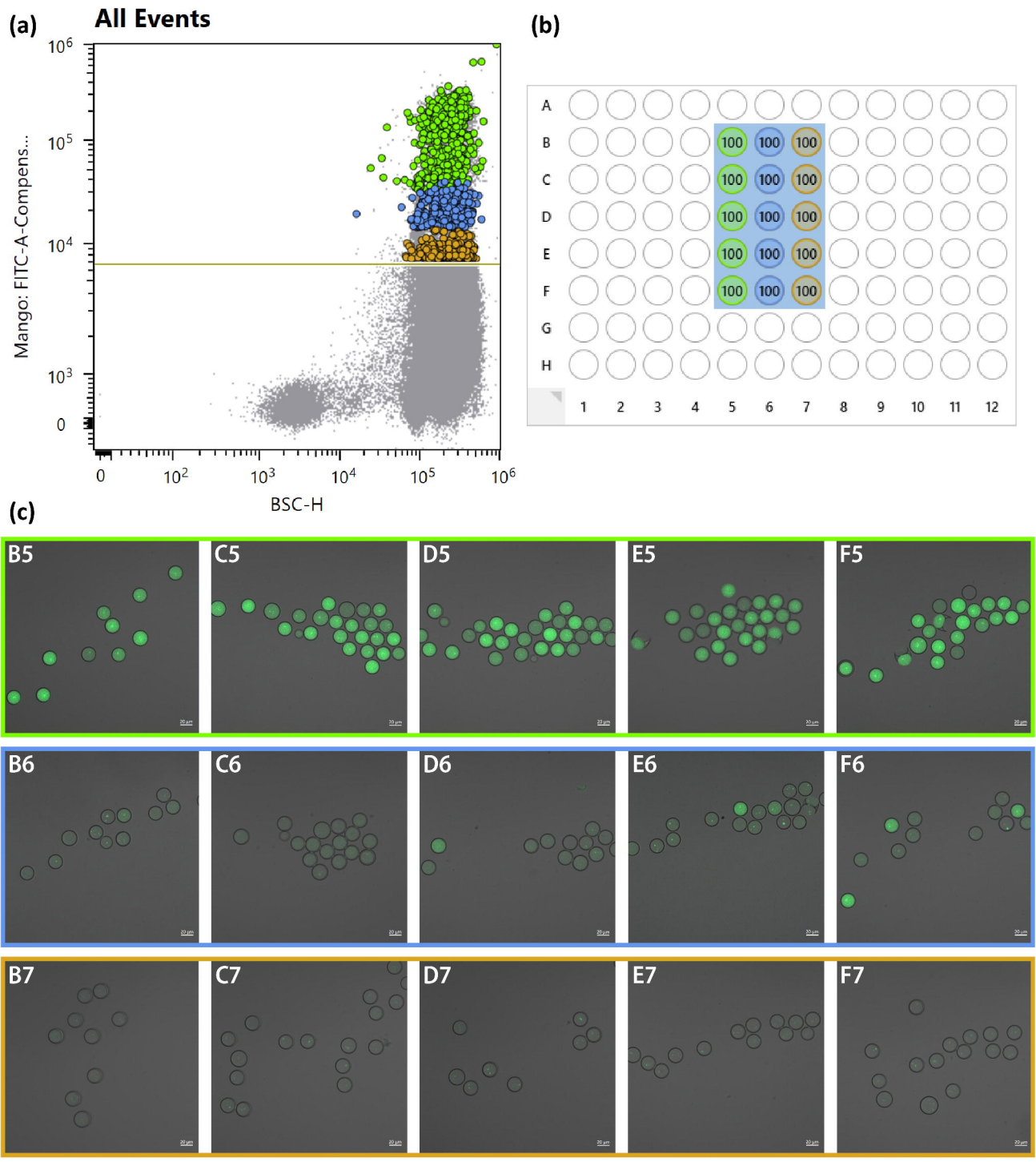

(a)
(b)
(c)
(d)
(e)
(f)
-ve
+ve


(a)
50 µm
(b)
20 µm
(c)
(d)
Droplet diameters during DNA compartmentalization
Frequency
Equivalent Diameter (um)
0
10
20
30
40
50
60
70
19.80
19.85
19.90
19.95
20.00
20.05
(e)
15 µm
(f)
0.0 ms
(i)
25 µm
0.4 ms
(ii)
25 µm
1.2 ms
(iii)
25 µm
1.4 ms
(iv)
25 µm
(g)
20 µm


(a)
Design A
Design B
Design C
0.0 ms
0.4 ms
0.6 ms
0.8 ms
1.0 ms
1.0 ms
1.8 ms
3.8 ms
4.4 ms
0.0 ms
0.4 ms
0.6 ms
1.6 ms
1.8 ms
25 µm
(b)
$W_{channel}$
L_{drop}
(c)
Droplet Volume (pl)
Droplet ID
Volume before Injection
Volume after injection
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
0
50
100
150
200
250
300
350


(a)
All Events
DEs: 79.51%
BSC-A
FSC-A
(b)
DEs
Negatives: 94.49%
Positives: 5.50%
Events
x1,000
Mango: FITC-H-Compens...
(c)
DEs
FSC-H
x1,000
FSC-A
x1,000
(d)
DEs
Mango: FITC-A-Compens...
BSC-H


(a)
CASE 1 : Empty droplets, no DNA template, no mRNA signal
CASE 2 : Droplets with a positive control, mRNA synthesis via IVT, Mango aptamer signal
(b)
100 µm
(c)
Events
x1,000
Positives: 0.81%
Negatives: 99.19%
Positives: 10.35%
Negatives: 89.64%
Positives: 18.59%
Negatives: 81.41%
Mango: FITC-H-Compens...
LOW (1:1000)
MEDIUM (1:100)
HIGH (1:10)
(d)
Post sort POSITIVES
Post sort NEGATIVES
20 µm
FACS sorting of DEs based on Mango aptmater signal intensity


(a)
All Events
Mango: FITC-A-Compens...
BSC-H
(b)
A
B
C
D
E
F
G
H
1 2 3 4 5 6 7 8 9 10 11 12
100
(c)
B5
C5
D5
E5
F5
B6
C6
D6
E6
F6
B7
C7
D7
E7
F7