ARL-TR-9991 ● SEP 2024

Microfluidics for Army Synthetic Biology—DNA ENTRAP: A Microfluidic Tool for Enhanced DNA Transfer

by Jose A Wippold, Monica Chu, Rebecca Renberg, Margaret Hurley, and Bryn Adams

NOTICES

Disclaimers

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Citation of manufacturer's or trade names does not constitute an official endorsement or approval of the use thereof.

Destroy this report when it is no longer needed. Do not return it to the originator.

ARL-TR-9991 ● SEP 2024

Microfluidics for Army Synthetic Biology—DNA ENTRAP: A Microfluidic Tool for Enhanced DNA Transfer

Jose A Wippold, Rebecca Renberg, Margaret Hurley, and Bryn Adams
DEVCOM Army Research Laboratory

Monica Chu
DOD Science, Mathematics, and Research for Transformation (SMART) Scholarship-for-Service Program

REPORT DOCUMENTATION PAGE

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED	
		START DATE	END DATE
September 2024	Technical Report	9/16/2021	8/27/2024

4. TITLE AND SUBTITLE
Microfluidics for Army Synthetic Biology—DNA ENTRAP: A Microfluidic Tool for Enhanced DNA Transfer

5a. CONTRACT NUMBER	5b. GRANT NUMBER	5c. PROGRAM ELEMENT NUMBER
5d. PROJECT NUMBER W911NF-17-2-0144	**5e. TASK NUMBER**	**5f. WORK UNIT NUMBER**

6. AUTHOR(S)
Jose A Wippold, Monica Chu, Rebecca Renberg, Margaret Hurley, and Bryn Adams

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
DEVCOM Army Research Laboratory ATTN: FCDD-RLA-HD Adelphi, MD 20783	ARL-TR-9991

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
DISTRIBUTION STATEMENT A. Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES
ORCIDs: Jose A Wippold, 0000-0003-3607-1962; Rebecca Renberg; 0000-0003-0640-5087

14. ABSTRACT
Droplet microfluidic platforms have been broadly used to facilitate DNA transfer in mammalian and bacterial hosts via methods such as transformation, transfection, and conjugation, as introduced in our previous work. Herein, we recapitulate our method for conjugal DNA transfer between *Bacillus subtilis* strains in a droplet for increased conjugation efficiency and throughput of an otherwise laborious protocol. By co-incubating the donor and recipient strains in a droplet, our method confines cells into close proximity and allows for increased cell-to-cell interactions. This methodology is advantageous because it has the potential to automate and accelerate the genetic modification of undomesticated organisms that may be difficult to cultivate. This device is also designed for modularity and can be integrated into a variety of experimental workflows in which fine tuning of donor-to-recipient cell ratios, growth rates, and media substrate concentrations may be necessary.

15. SUBJECT TERMS
droplet microfluidics, bacterial conjugation, DNA transfer, automation, synthetic biology, genetic engineering, Biological and Biotechnology Sciences

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES
a. REPORT	b. ABSTRACT	C. THIS PAGE		
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UU	58

19a. NAME OF RESPONSIBLE PERSON	19b. PHONE NUMBER *(Include area code)*
Jose A Wippold	(301) 394-0453

STANDARD FORM 298 (REV. 5/2020)
Prescribed by ANSI Std. Z39.18

Contents

List of Figures

List of Tables

Acknowledgments

This project was supported in part by the US Army Research Laboratory (ARL) cooperative agreement with Texas A&M Engineering Experiment Station (TEES) (W911NF-17-2-0144). Jose A Wippold was supported via the Department of Defense Graduate SMART Scholarship program. The authors would like to thank the TEES AggieFab Nanofabrication Facility and the US ARL Specialty Electronic Materials and Sensors Cleanroom micro- and nano-fabrication facilities for the microfabrication support. Authors of this work have applied for patent protection of the developed microfluidic DNA transfer technology.

1. Introduction

Significant advances have been made in engineering microorganisms using a variety of synthetic biology tools and methods (Adams 2016). However, the introduction of DNA into nonmodel novel chassis organisms, particularly those that are uncharacterized, remains a major obstacle in advancing the state of the art in the field. There are several commonly used and efficient methods to introduce DNA into microorganisms, including chemical competence, protoplasting, electroporation, and *Escherichia coli*-mediated conjugation.

However, these methods only work for a handful of domesticated chassis microorganisms, thus limiting the range of microorganisms that may serve as synthetic biology host cells. To address this barrier, Brophy et al. (2018) developed a broad host conjugation-based DNA transfer tool named XPORT (Fig. 1). In this approach, the conjugation machinery, including the Integrative and Conjugative Element (ICE) endogenous to *Bacillus subtilis*, was engineered to controllably deliver genetic circuits to nontraditional and undomesticated chassis bacteria.

Although XPORT introduces a promising method for engineering undomesticated bacteria, several challenges remain for their widespread and practical use. First, XPORT-mediated conjugation requires the DNA donor and recipient cells to be in direct physical contact to facilitate DNA transfer, and thus the ratio of donor/recipient cells frequently must be quite high (>104:1) to ensure success. Second, the conjugation efficiency is dependent on the donor/recipient cell ratios as well as co-incubation time, requiring multiple experiments to be conducted with accurate control over the assay conditions to identify an optimum DNA transfer condition, which is very time and labor intensive. Droplet microfluidic systems—where cells can be encapsulated within a pico-liter-volume bioreactor (diameters in several tens to hundreds of micrometers), which thus naturally forces cells to be in close proximity to each other—in addition to the high-throughput automated nature of the system have the potential to overcome these challenges. In droplet bioreactors, the conjugation efficiency is expected to increase because donor and recipient cells co-encapsulated in the droplets are in close proximity to each other, which increases the potential for successful conjugation. Furthermore, integrating the multistep process that makes up the XPORT protocol into a fully automated droplet microfluidic system enables high-throughput experiments.

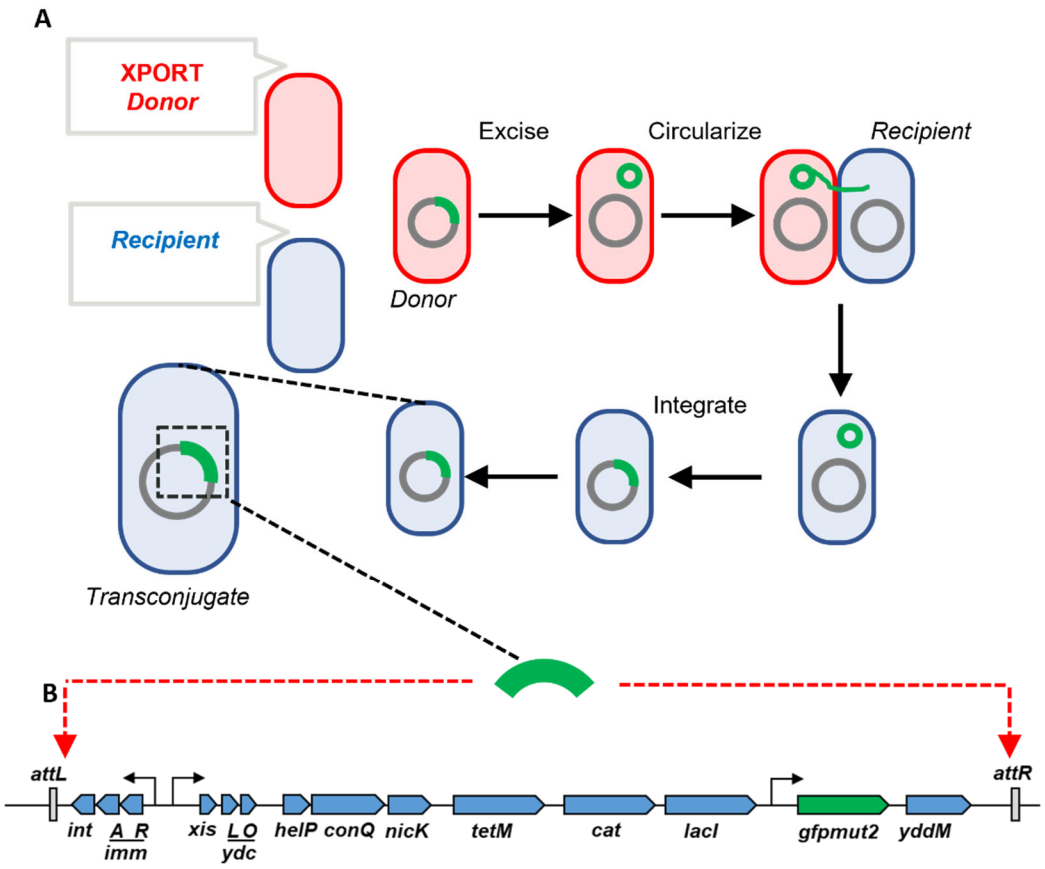

Fig. 1 An illustrative schematic depicting principles of XPORT-mediated conjugation. (A) Molecular mechanism of horizontal gene transfer via conjugation as described by Johnson and Grossman. The donor cell depicted is the XPORT donor described in Brophy et al. (2018). (B) The relevant genes excised from the donor genome being transferred from the XPORT donor cell to the recipient cell. The sites attL and attR are attachment sites located at the *trnS-leu2* genetic locus. These sequences are conserved across many *Bacillus* species and are critical to homologous recombination process. (Reprinted with permission from Wippold et al. [2024].)

Droplet microfluidics technology has demonstrated its utility as a screening method for numerous microbiological applications using broad ranges of bacterial cells encapsulated in water-in-oil emulsion droplets (Kaminski et al. 2016). For example, this technology has been used to characterize bacterial growth and the products they produce, degrees of bacterial pathogenicity, and mechanisms of bacterial antibiotic resistance, to name a few example applications (Akselband et al. 2005; Wang et al. 2014; Kim et al. 2016, 2017; Terekhov et al. 2017; Chen-Vestergaard et al. 2017, 2018; Ota et al. 2019). Droplet microfluidics has also been widely used in a range of cell and molecule screening applications (Huebner et al. 2008; Brouzes et al. 2009; Mazutis et al. 2013). The prolific use of droplet microfluidics technology can be attributed to several advantages, such as single-cell-resolution analyses capability, low reagent consumption, generation of highly monodisperse droplet

bioreactors at extremely high throughput, and the ability to link multiple liquid handling steps to complete complex liquid, cell, or particle manipulation steps in a single chip format with high degree of automation and speed.

In recent years, droplet microfluidics technology has emerged in a few synthetic biology applications, but its use in facilitating DNA transfer and genetic engineering remains quite limited (Martino et al. 2016; Gach et al. 2017; Luu et al. 2022). For example, to the best of our knowledge, the study conducted by Lam et al. (2019, 2020) is the only published demonstration of in-droplet DNA transfer between bacterial strains. In this publication, the authors described the encapsulation of *Streptococcus pneumoniae* strain capable of DNA transformation via pneumococcal cell-cell lytic attacks using three different antibiotic-resistance genes. In-droplet transformation was successfully demonstrated, and the recombinants were recovered by breaking up the droplets followed by selective plating. The successful use of droplet microfluidic technology in this application was due in part to the close proximity of cells provided by the cell-encapsulated droplet format. However, in these works, a high (5%) surfactant concentration was used to maintain high droplet stability, which led to lower than expected cell viability (40%–60%) in droplets. In addition, from a microfluidics platform standpoint, the system design did not enable on-chip in-droplet viability assessment that allows for a true, in-droplet microbial strain viability check. It also required multiple off-chip sample preparation steps before cells were introduced into the microfluidic platform that prevented accurate control over the co-incubation and reagent addition timing. These are critical parameters that influence the conjugation efficiency. In summary, to date, no droplet microfluidic platform has fully demonstrated, characterized, or optimized a bacterial conjugation system within microdroplets, nor has it been applied to enable the powerful XPORT DNA conjugation method in a droplet microfluidics format.

In this report, we present a droplet microfluidics platform named DNA ENTRAP (DNA ENhanced TRANsfer Platform), a system that can simplify, enhance, and increase the throughput and efficiency of XPORT-mediated DNA transfer. This system has the potential for broad utility, such as rapid screening of host cell compatibility to DNA transfer as well as rapid and automated assay parameter optimization (e.g., donor-recipient cell ratios, co-incubation length) to maximize the DNA transfer efficiency. Here, the feasibility and efficiency of this microfluidic DNA ENTRAP system compared to the traditional benchtop XPORT conjugation protocol was tested using *B. subtilis*. The platform represents the first iteration of a suite of microfluidic-adapted synthetic biology tools that has the potential to increase our ability to genetically modify diverse microorganisms and rapidly

accelerate the domestication of such untapped microorganisms for broad ranges of biotechnology and biomanufacturing applications.

2. Experimental

2.1 Strains and Growth Conditions

For the XPORT donor, we used a tetracycline-resistant, xylose-inducible conjugative *B. subtilis* strain derived from *B. subtilis* JH642, which is denoted as JAB 981 in Brophy et al. (2018). The recipient strain used in this work is a derivative of *B. subtilis* PY79, denoted as JAB 545. This strain has chloramphenicol resistance because the antibiotic marker was introduced when interrupting the comK gene. Thus, this strain is unable to uptake DNA through chemical competence or natural competence. The donor strain, JAB 981, was cultured in Luria broth (LB) media supplemented with 100 µg/mL D-alanine and 10 µg/mL tetracycline. The recipient strain was cultured in LB media with no additional supplementation. Both strains were grown at 37 °C on slanted racks, shaking at 225 rpm (New Brunswick Innova Shaker). Additional details for all methods are described in Appendices A–C.

2.2 Droplet Cell Viability

Microdroplet cell viability in our system was analyzed using a two-step approach. First, donor strain (JAB 981) cell viability was demonstrated and characterized in a droplet culture. For these experiments, a single colony from streak plate was used to inoculate 3 mL LB media supplemented with 100 µg/mL D-alanine and 10 µg/mL tetracycline for 3 h in an incubator, when the culture reached an OD600 (Biowave CO8000 Cell Density Meter, VWR) of 0.95–1.10. Next, 2.5 mL of the culture was centrifuged at 3000× *g* for 5 min and washed with phosphate-buffered saline (PBS) twice, repeating the pelleting process between each wash step. The cells were suspended in 500 µL sterile PBS, and a 1 mM blue HOESCHT 33342 fluorescent stain was used to label the cells for in-droplet identification. After this staining period, the cells were centrifuged, washed two times, and then resuspended in 500 µL fresh sterile PBS. The cells were then added to 3 mL fresh media containing equal parts 1× TSS media + LB media in addition to 100 µg/mL D-alanine and 1 µM YoYo-1 iodide cell impermeant, dead-cell green (pseudo-colored red for images) fluorescent stain, and adjusted to a total OD600 of 0.6. The suspension was then used to generate droplets of 60 µm in diameter. Fluorinated oil (Novec 7500, 3M) with surfactant (1.25 wt/wt%; Pico-Surf 1, Dolomite Microfluidics, Charlestown, MA) was used to generate and stabilize the

microdroplets. The droplets flowed for the droplet generator component into the culture and observation device. The droplets were monitored over 12 h using fluorescent microscopy to distinguish between the live and dead cells.

Second, the intended recipient strain (JAB 545) was assessed using a similar approach. To confirm the ability to detect dead cells, the JAB 545 recipient cells were co-encapsulated in droplet with tetracycline in parallel with viability staining. Briefly, JAB 545 recipient cells were cultured as previously described. Immediately after the final suspension into 3 mL LB media, 300 µg of tetracycline was added to the media to demonstrate not only the effectiveness of YoYo-1 at staining dead cells but also the ability to distinguish the fluorescent signal over any background LB media fluorescent noise (Fig. 2).

Approximately 25–30 cells were encapsulated per droplet for all experiments and viability was monitored for 12 h, the maximum expected duration of the entire in-droplet assay. All experiments were completed in triplicate. Through these assays, we demonstrated DNA ENTRAP's capability of maintaining cellular functionality, and we assume that this capability extends beyond the biological organisms presented here to various distinct bacterial species.

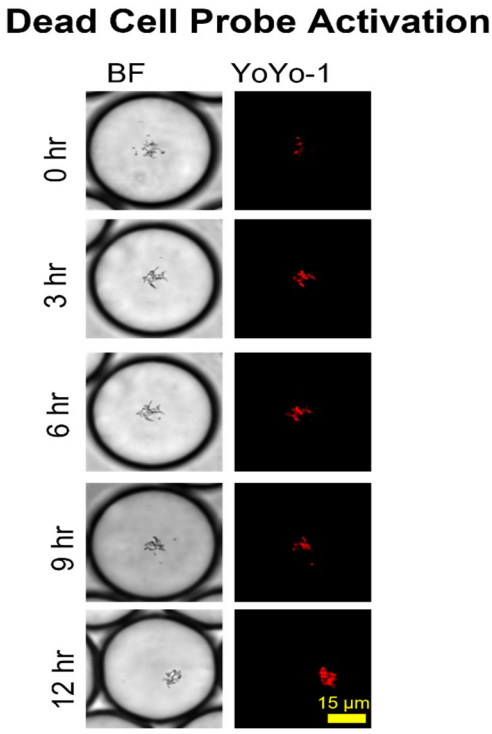

Fig. 2 Demonstration of dead cell probe functionality in *B. subtilis* cell model. Receiver cells in the proof-of-concept model, *B. subtilis* JAB 545 strain, were co-encapsulated into microdroplets with tetracycline (100 µg/mL) along with YoYo-1 fluorescent dead cell probe (pseudo-colored here red to elucidate cell death). (Reprinted with permission from Wippold et al. [2024].)

2.3 Benchtop and Droplet-Based Conjugation Approaches

For both the benchtop and droplet-based conjugation assay, single colonies of both the JAB 545 and JAB 981 strains were picked and cultured in 3 mL LB media and LB + D-alanine (100 μg/mL) + tetracycline (10 μg/mL), respectively. Both cultures were incubated until achieving an OD600 >1.2, then they were subcultured into 3 mL LB media and LB + D-alanine for recipient and donor, respectively. The cultures were diluted such that the starting OD600 = 0.025. The subcultures were set in a shaking incubator at 225 rpm and 37 °C for 1.5 h until the JAB 981 culture reached an OD600 = 0.15. At this time, 60 μL of 50% xylose was added to induce conjugative element production. The strains were then cultured for another 1.5 h until OD600 = 0.9 × 1.1. Next, 2.5 mL of the culture was centrifuged at 3000× *g* for 5 min and washed with PBS twice repeating the pelleting process between each wash step. The cells were suspended in 500 μL sterile PBS. Then, cells were added to fresh LB media supplemented with 100 μg/mL D-alanine to achieve an OD600 = 0.6 at 3:1 and 1:1 donor/recipient OD ratios.

For the benchtop protocol in this work, we did not use filter papers for conjugation, which is a deviation from the protocol originally established in Brophy et al. (2018). To provide a more accurate comparison of droplet conjugation versus benchtop conjugation, the cells were not concentrated onto a filter. Instead, equal volumes (50 μL aliquots) of each resuspended culture (at OD600 = 0.6) were combined into a separate sterile microcentrifuge tube and incubated at 37 °C with no shaking for 2 h. At each time point (0.5, 1, and 2 h), cells were dilution plated onto selection media, LB + tetracycline (10 μg/mL) as well as LB + tetracycline (10 μg/mL) + D-alanine (100 μg/mL) for counting donors and transconjugants (TCs). Selection media excluding D-alanine selects only for TCs while also preventing the donor strain from growing due to its D-alanine auxotrophy.

For the droplet-based approach, the cell suspension (OD600 = 0.6) was then used to generate droplets of 60 μm in diameter by injecting the solutions into the DNA ENTRAP platform via the two distinct cell port inlets at equal flow rates. The droplets flowed for the droplet generator device into the designed microvessel system and incubated for the designated set periods of time before spread plating. For the 1:1 comparison, both the donor JAB 981 strain and recipient JAB 545 strain were diluted into fresh media to an OD600 = 0.30. The suspension was then used to generate droplets of 60 μm in diameter using the same approach mentioned in Section 2.2. Fluorinated oil (Novec 7500) with surfactant (1.25 wt/wt%, Pico-Surf 1) was used to generate and stabilize the microdroplets. The droplets were flowed from the droplet generator device into the designed microvessel system and incubated for the designated periods of time before spread plating. After the set co-

incubation periods (necessary for conjugation to occur), the cultures were removed from the incubator and 50 µL of droplets were plated and spread onto selection agar. The experiments were conducted in triplicate over three consecutive days.

2.4 Conjugation Efficiency Analysis

To accurately account for conjugation efficiency in microdroplets, we used a selective media strategy to shed insight into conjugation event efficiency. At each timepoint, 50 µL of each test ratio was spread on an LB agar plate to count JAB 545 and successful TCs, an LB + D-alanine100 + tetracycline10 to count JAB 981 colonies and TCs, and an LB + tetracycline10 to select for TCs only to determine conjugation efficiency. To plate the droplets, the aliquot of collected droplets were pipetted onto the center of an agar place and then spread using an L-shaped disposable spreader and put into a 37 °C incubator for 16 h. The experiments were conducted in triplicate over three consecutive days.

The LB + D-alanine 100 µg/mL + tetracycline 10 µg/mL agar plates were used to quantify the number of colony-forming units (CFUs) that could be attributed to both JAB 981 and successful TCs. The LB agar plates were used to quantify the number of CFUs that could be attributed to both JAB 545 and successful TCs. The LB + tetracycline 10 µg/mL agar plates were used to quantify the number of CFUs that could be attributed to successful TCs. To get accurate JAB 981 CFU counts, the number of CFU counted on LB + tetracycline 10 µg/mL agar plates were subtracted from the number of CFUs counted on LB + D-alanine 100 µg/mL + tetracycline 10 µg/mL agar plates. To get accurate JAB 545 CFU counts, the number of CFUs counted on LB + tetracycline 10 µg/mL agar plates were subtracted from the number of CFUs counted on LB agar plates. This same approach was used for both the benchtop conjugation efficiency analysis and the droplet-based conjugation efficiency analysis.

For all statistical analyses, a two-way Analysis of Variance (ANOVA) assuming sphericity with an alpha of 0.05 and a Šídák's multiple comparison test were conducted to obtain an adjusted p-value. The Šídák's method was preferred to the Bonferroni method because it provides more power to the calculated p-value.

2.5 Device Fabrication

In this work, we established the first working prototypes for devices that are proficient in evaluating XPORT-mediated DNA transfer using microdroplet technology. DNA ENTRAP was fabricated using standard poly(dimethylsiloxane) (PDMS)-based soft lithography techniques (Xia and Whitesides, 1998). The droplet microfluidic devices we used to perform the experiments in Sections 2.2 and 2.3

consist of two functional components on separate but fluidically tethered chips: a droplet generation component and a droplet incubation chip. Droplet generation (60-µm diameter) was accomplished using a single cross-junction droplet generator comprising a horizontal carrier oil channel (55 µm wide) and a perpendicular water channel (50 µm wide), both with a height of 65 µm.

A separate droplet culture and observation device was used to assess the cell viability and co-encapsulation cell/droplet numbers using a Zeiss Colibri Axiovert 200 optical microscope with fluorescent imaging capabilities. The culture and observation device exploited 400 × 400-µm pillars spaced 20 µm apart, which served as an in-house designed, massive, basket-like trapping chamber for a monolayer of droplets to be collected, analyzed, and observed within a microfluidic chamber (10 × 3 mm). The height of the culture chamber was set to 100 µm to allow for single droplet layer observation of 60-µm droplets while ensuring minimal pressure buildup. The system used a syringe-based, flow-driven pump (Fusion 200 model; Chemyx, Sugarland, TX) and medical grade tubing (Tygon ND-100-80 01.X.03X500; catalog no. 89404-300, VWR) for injecting cell samples to the microfluidic devices and for transferring droplets between tethered microfluidic devices. For the droplet generation device, we used two inner inlets to introduce cells and another inlet to introduce fluorinated oil (Novec 7500) with surfactant (1.25 wt/wt%, Pico-Surf 1) to generate and stabilize the microdroplets.

To obtain 100-µm-thick microchannel patterns, a master silicon wafer was spin-coated with negative photoresist (SU-8 2075; Microchem Corp., Newtown, MA) at 2100 rpm, followed by soft baking in two steps at 65 ℃ and 95 ℃ for 24 h and 20 min, respectively. After the soft bake, the master was exposed to ultraviolet light using a standard photolithography mask aligner (EVG 610; EVG Group, Neuhaus, Germany) and post-exposure baked at 65 ℃ and 95 ℃ for 40 and 20 min, respectively. After developing the unexposed photoresist (EBR 10A, catalog no. 10018079, Microchem Corp.), the patterned wafer was (tridecafluoro-1,1,2,2 tetrahydrooctyl)trichlorosilane (United Chemical Technologies, Inc., Bristol, PA) coated for 60 min using a conventional desiccator, to prevent pattern removal during PDMS replication.

To obtain 65-µm-thick microchannel patterns, a master silicon wafer was spin-coated with negative photoresist (SU-8 2050; Microchem Corp.) at 2600 rpm, followed by soft baking in two steps at 65 ℃ and 95 ℃ for 24 h and 20 min, respectively. After the soft bake, the same master mold preparation procedure was carried out as described for the completion of the 100-µm height master mold. A thin PDMS (Sylgard 184; Dow Corning Corp., Freeland, MI) layer (thickness: 30 µm) was spin-coated on the patterned glass slide at 3000 rpm for 30 s to obtain a hydrophobic bottom surface that is necessary for droplet generation. For both

functional devices, a PDMS microchannel was replicated from the master by pouring 20 g of PDMS mixture (1:10 curing agent to polymer) onto the master secured in a plastic petri-dish. After polymerization, the merging device was bonded to the PDMS-coated glass slide using conventional oxygen plasma treatment (Plasma Cleaner; Harrick Plasma, Ithaca, NY) for 90 s. After polymerization, the droplet generator and culture chamber microchannels were bonded to a PDMS-coated glass slide (Micro Slides 2947-75X50; Corning Inc., Oneonta, NY) using the same oxygen plasma treatment protocol. All PDMS microchannels were filled with a commercial hydrophobic surface coating agent (1H, 1H, 2H, 2H-Perfluorododecyltrichlorosilane; Sigma-Aldrich, St. Louis, MO) mixed with fluorinated oil (Novec 7500) at 1% wt/wt and flushed before use. The fabrication of the working DNA ENTRAP prototype marked an important milestone in the development of a fully automated, high-throughput, DNA-enhancing platform.

2.6 Genetic Confirmation and Population-Based Sequencing

Two primers flanking the insertion site on the recipient genome were used to perform a polymerase chain reaction (PCR) confirmation of a successful droplet conjugation event. The forward primer sequence is 5'-AAAGTCTTTTATTCTGCGCCG-3' and the reverse primer sequence is 5'-ATCATTTAGTAAGGCAGCTGCTA-3'. The PCR amplification was performed with Q5 polymerase (New England Biolabs), gel purified, and sequenced using the MinION (Oxford Nanopore Technologies). To confirm the fidelity of the conjugation, we performed droplet conjugation and scraped all the TC colonies off the selection media (LB + tetracycline10), washed and pelleted the biomass, and then isolated genomic DNA using the ReadyLyze Lysozyme (part no. R1804M, Lucigen) and epicenter MasterPure Total Nucleic Acid Isolation Kit (catalog no. MC85200, Lucigen) on the Covaris E220 evolution with microtube-130 AFA Fiber (catalog no. 520216, Covaris). The genomic DNA was prepared following the Genomic DNA by Ligation (catalog no. SQK-LSK109) protocol through Oxford Nanopore and then sequenced on the GridION (Oxford Nanopore Technologies).

2.7 Flow Cytometry for Transconjugant Induction

Both droplet TCs and benchtop TCs (generated via benchtop methods) were struck onto LB + tetracycline 10 μg/mL and incubated for 16 h at 37 °C. The following day, three colonies from the plate were picked at random and grown in 3-mL volumes of LB + tetracycline 10 μg/mL media at 37 °C, shaking at 225 rpm. After 2 h of growth, subcultures were started with an OD600 of 0.05. The cultures were

grown to an OD600 of approximately 0.5 and induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cultures were shaken at 37 °C for 1 h. After induction, 5-µL samples were diluted into 500-µL NERL Blood Bank Saline for flow cytometry analysis. Flow cytometry was performed on the Sony SA3800 Spectral Analyzer with the following parameters: threshold forward scatter (FSC) value, 0.5%; FSC gain, 17; side scatter (SSC) voltage, 20%; fluorescence photomultiplier tube (PMT) voltage, 69.8%; acquisition of 10,000 events.

2.8 Off-Chip Conjugation Methods

Three parameters of the XPORT-mediated conjugation experiments were evaluated to optimize for the conjugation efficiency: (1) donor/recipient ratio, (2) incubation time, and (3) cell density.

2.8.1 Optimizing Donor/Recipient Ratio

In these conjugation experiments, donor/recipient ratios of 10:1, 5:1, 3:1, and 1:1 were assessed for conjugation efficiency. In each case, the total combined volume of the cells remained the same while varying volumes of donor/recipient cells were suspended together in buffer. The conjugation efficiency was evaluated at a fixed time and cell density. Of the four evaluated conditions, a donor/recipient ratio of 3:1 was found to be optimal.

2.8.2 Optimizing for Incubation Time and Cell Density

In a previous reported, Brophy et al. (2018) optimized incubation time and cell density. Conjugation efficiency was arbitrarily evaluated at a co-incubation time of 3 h and cell density of 0.9 (concentrated onto a filter paper). In these optimization experiments, the conjugation efficiency was evaluated at cell densities of 0.6 and 10, and at incubation times of 1, 2, and 3 h, whereas donor/recipient ratio was kept constant. We hypothesized that increased cell density would improve conjugation efficiency due to closer proximity between neighboring cells. However, conjugation efficiency of cultures combined at an OD of 0.6 was found to be more optimal than cultures concentrated to an OD of 10. In terms of co-incubation time, the highest conjugation efficiency was observed at the 3 h co-incubation time in the off-chip condition, whereas the 2 h co-incubation time exhibited the highest conjugation efficiency in the droplet conjugation.

A single colony of the donor strain (JAB 981) was inoculated into 3-mL LB supplemented with D-alanine (100 µg/mL) and tetracycline (10 µg/mL). At the same time, a single colony of the recipient strain (JAB 545) was inoculated into 3-mL LB without antibiotics. Both strains were grown at 37 °C and 225 rpm to an

OD600 of approximately 1.0 (New Brunswick Scientific Innova 44). At this point, appropriate volumes (no more than 60 µL) were transferred from these cultures to start fresh subcultures at an OD600 of 0.025. JAB 981 was cultured in 3 mL fresh LB with D-alanine (100 µg/mL) and no antibiotic. JAB 545 was cultured in 3 mL fresh LB with no antibiotic. When the JAB 981 reached an OD600 of approximately 0.12, 50% xylose (w/v) was added to the culture at a 1:50 dilution. Then, donor strain and recipient strain were both incubated and shaken for another hour to allow for expression of the ICE conjugation machinery, after which the cultures would reach OD600 of approximately 0.9–1.1 for both strains. Aliquots (500 µL) of each culture were centrifuged at 3000× *g* for 5 min. The supernatant was discarded and cells were washed in 500 µL of PBS and resuspended in D-alanine to a final OD600 of 0.6. Equal volumes (50-µL aliquots) of each resuspended culture were combined into a separate sterile microcentrifuge tube and incubated at 37 °C with no shaking for 2 h. At each time point (0.5, 1, and 2 h), cells were dilution plated onto selection media, LB + tetracycline (10 µg/mL) as well as LB + tetracycline (10 µg/mL) + D-alanine (100 µg/mL) for counting donors and TCs. Selection media excluding D-alanine selects only for TCs while also preventing the donor strain from growing due to its D-alanine auxotrophy.

3. Results and Discussion

3.1 Device Design and Microfabrication

Efficient XPORT-mediated conjugation requires extensive tuning of microbial growth parameters, growth state, and other microbiological conditions for successful conjugative DNA transfer in a broad spectrum of microorganisms. To overcome these hurdles, we designed the DNA ENTRAP, a droplet microfluidic system that can fully automate the DNA conjugation process that will enable the screening of a variety of microbial and conjugation conditions to identify those that result in maximum DNA transfer efficiency. As the first step in demonstrating the device to the scientific community, we developed the ENTRAP prototype and conducted a proof-of-concept study by demonstrating the XPORT assay in droplet microfluidics format.

The DNA ENTRAP system was designed to harness the spatial and temporal qualities unique to droplet microfluidics to enhance cell-to-cell DNA transfer, a process that requires physical contact between the donor and recipient cells. The system was designed to perform the following functions: 1) generation of a large number of water-in-oil-emulsion droplets co-encapsulating donor and recipient cells, where each droplet serves as an independent nano-liter scale bioreactor; 2) capability to change the donor-recipient cell ratios during this cell-encapsulated

droplet generation steps; 3) incubation of the cell-encapsulated droplets for varying durations to identify the ideal co-incubation period that maximizes DNA transfer; and 4) dispensing of the cell-encapsulated droplets onto an agar plate to recover the cells. Figure 3 illustrates the droplet microfluidics-based XPORT assay steps (top row) in comparison to the conventional benchtop XPORT assay steps. The system is composed of three inlets (donor cells, recipient cells, carrier oil), a cell mixing region where the mixing ratio can be changed by simply adjusting the flow rates of the donor/recipient cell input flow rate, a droplet generator, and a droplet mixing region, all on the same chip. On another fluidically connected chip, a droplet microfluidic chamber used for conjugation assays was bonded to a glass slide and connected to the first half of the DNA ENTRAP platform via fluidic connections. For the droplet viability assays, the first half of the DNA ENTRAP platform (sample ports, droplet generator, droplet mixing) was fluidically connected to another droplet observation chamber to enable a time-lapsed fluorescent microscopy over a monolayer of microdroplets. All components of this prototype system were microfabricated in PDMS using conventional soft lithography techniques.

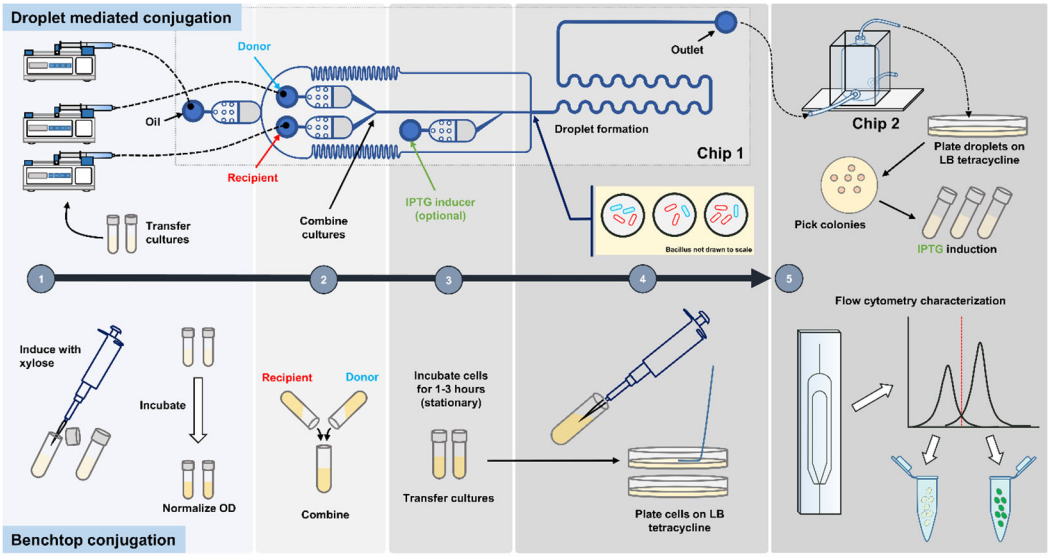

Fig. 3 Droplet microfluidics-based DNA transfer workflow (ENTRAP, top) compared to the conventional ENTRAP workflow (bottom). The ENTRAP approach uses a high-throughput droplet microfluidic workflow for all the liquid handling steps. Steps 1–4 from both approaches illustrate the different steps leading to a common read-out (step 5). (Reprinted with permission from Wippold et al. [2024].)

As discussed in Section 1, this microfluidic system integrates all of these functional steps, and it is capable of fully automated operation through a LabView-controlled custom interface. This enables precise control and modulation of the experimental parameters, such as the injection flow rates of each inlet and the ratio between those

flow rates, where such parameters could be entered into the control program before the start of the experiment. In addition, this setup allows us to address specific growth characteristics associated with different cell types and species. These features will enable the final DNA ENTRAP system to operate continuously with minimal human intervention. Furthermore, the overall platform stability is enhanced by minimizing manual sample and reagent handling steps, which improves aseptic conditions and reduces the possibility for human error. Each functional component of the DNA ENTRAP device, namely, the cell-encapsulated droplet generation component, droplet incubation component, and droplet content detection using an optical setup (currently based on microscopic imaging, to be changed to flow-through detection in the future), follows well-established techniques and instrumentation configurations widely utilized in the field of droplet microfluidics (Baret et al. 2009; Mazutis et al. 2013; Guzman et al. 2015; Kim et al. 2016, 2017; Wippold et al. 2020a, 2020b; Zhang H et al. 2020).

We first demonstrated that the developed platform produced uniform cell-encapsulated 60-µm diameter droplets containing *B. subtilis* cells (Fig. 4). We then characterized the initial in-syringe cell concentration to achieve the desired in-droplet cell count ranges (20–30 cells per droplet) (Fig. 5). Finally, we optimized the droplet generation rates to ensure tight control over the droplet incubation time. The final optimized overall assay speed (considering a single droplet being generated) ranged at approximately 180–200 droplets/s (Fig. 6).

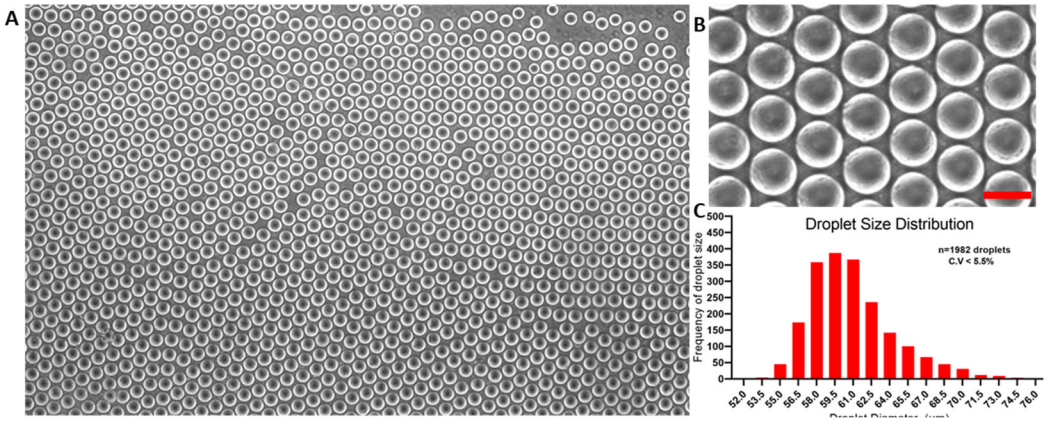

Fig. 4 On-chip droplet size confirmation using DNA ENTRAP. (A) The 5× phase contrast microscope image demonstrating large population (n > 100) with a homogeneous population diameter. (B) Enlarged microscope images extruded from large population of homogenous droplet diameters. (C) Histogram data of droplet population. Coefficient of variation, <5.56%. Red scale bar 60 µm. (Reprinted with permission from Wippold et al. [2024].)

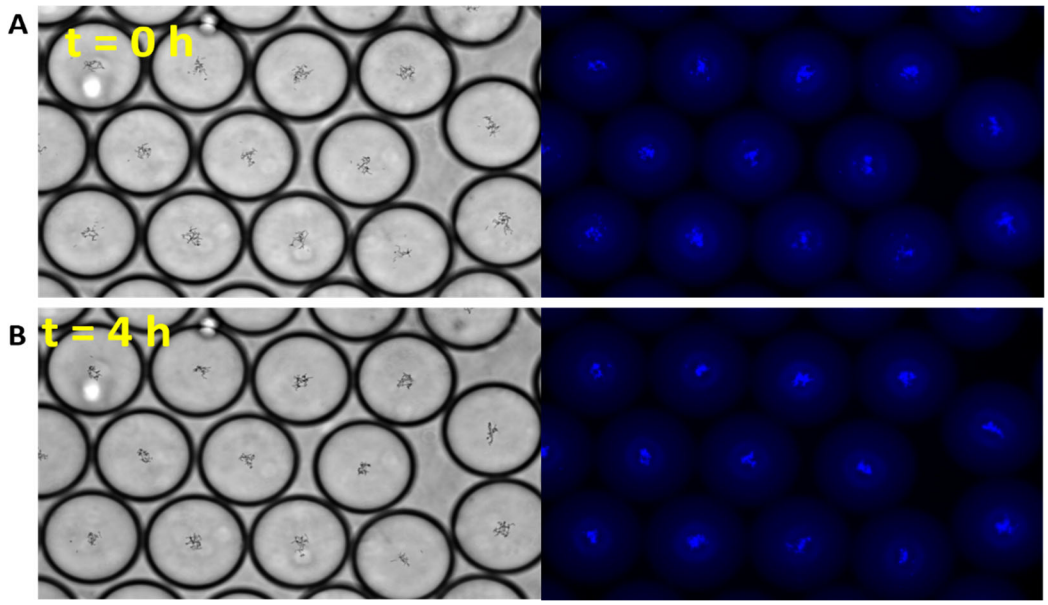

Fig. 5 **On-chip cell encapsulation verification of the DNA ENTRAP device. (A) Example of 10× phase contrast microscope image (left) and corresponding fluorescent image (right) used for characterizing in-droplet cell concentration achieved through device geometry (dimensions of droplet generation nozzle) and sample injection flow rates. (B) The 10× phase contrast microscope image (left) and corresponding fluorescent image (right) after 4 h of on-chip incubation. Scale bar = 50 µm. (Reprinted with permission from Wippold et al. [2024].)**

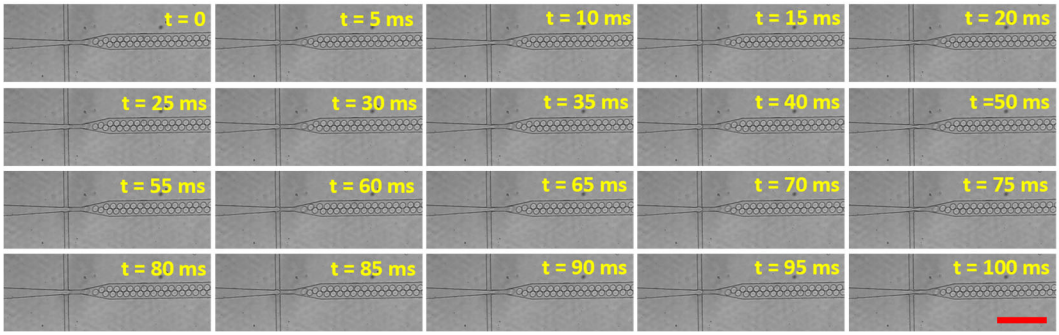

Fig. 6 **On-chip droplet generation characterization. Time lapse, high-speed images used for determining droplet generation rates. Scale bar = 400 µm. (Reprinted with permission from Wippold et al. [2024].)**

3.2 In-Droplet Donor: Receiver Cell Cocultivation and Viability Analyses

We used *B. subtilis* JAB 981 (DNA donor strain) and *B. subtilis* JAB 545 (recipient strain), two strains previously developed to perform the XPORT assay, in which the donor strain has the conjugation machinery including the ICE endogenous to *B. subtilis* and was engineered to controllably deliver genetic circuits to recipient chassis bacteria. These cells were encapsulated in droplets and their viabilities were

measured. Each strain was tagged with a HOESCHT blue fluorescent probe to stain the cells for later fluorescent identification and then encapsulated in a droplet with no additional washing steps. The fluorescent YOYO-1 dye was used to identify dead cells. Over the 12-h analysis period, neither donor JAB 981 nor recipient JAB 545 populations displayed any noticeable loss in viability (>98% viability) when encapsulated in a droplet. To first confirm the compatibility of YOYO-1 fluorescent stain in identifying dead *B. subtilis* cells in droplet, the JAB 545 recipient cells were co-encapsulated in droplet with the antibiotic tetracycline together with the YOYO-1 stain. The recipient strain showed decreased viabilities of 80%, 40%, and <5% at the 1, 2, and 3 h timepoints post-encapsulation with tetracycline, respectively. This result provided confirmation of the in-droplet bacterial cell viability assay strategy used here.

Based on this confirmed assay, we then used the DNA ENTRAP device to assess both donor and recipient cell viabilities in droplets. Both donor strain and recipient strain maintained high (>98%) viability over the course of 12 h when encapsulated in a droplet (Fig. 7), when *B. subtilis* DNA donor strain and *B. subtilis* recipient strain viability were monitored by co-encapsulating the YOYO-1 dead cell fluorescent stain (red color).

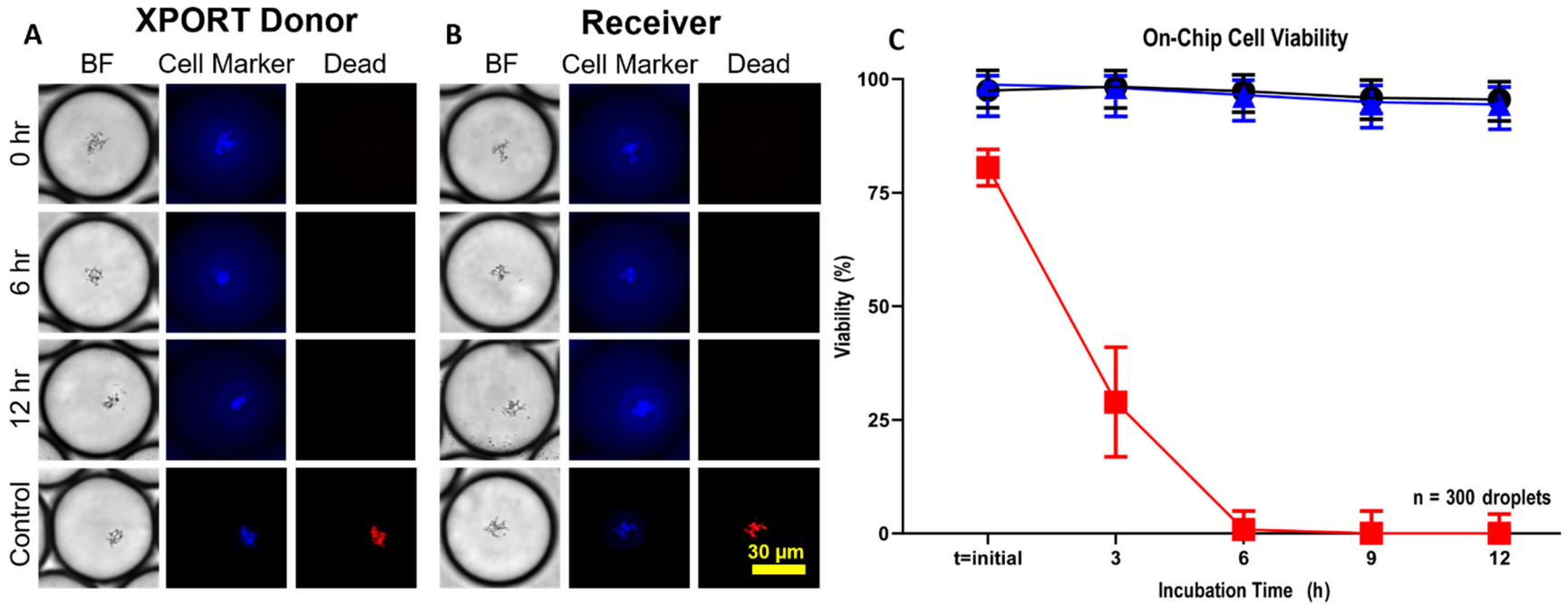

Fig. 7 **In-droplet viability of the donor and recipient cells. (A and B) Microscopic images of cell-encapsulated droplets over time (12 h) to analyze the cell viability. HOESCHT 33342 blue fluorescent stain was used to identify cells, and YOYO-1 dye (pseudo-colored as red) was used to identify dead cells. (A) The *B. subtilis* DNA donor cell viability in droplet over 12 h. (B) The *B. subtilis* receiver cell viability in droplet over 12 h. (C) Experimental results comparing the donor cell viability over the course of 12 h. Standard error of mean denoted by error bars obtained from triplicate experiments. (Reprinted with permission from Wippold et al. [2024].)**

3.3 In-Droplet DNA Conjugation

To demonstrate that the DNA ENTRAP platform can indeed automate the entire DNA transfer assay flow in a droplet microfluidics format and also enhance conjugative events with respect to traditional benchtop approaches, *B. subtilis* DNA donor strain and *B. subtilis* recipient strains were used in the droplet assay. To demonstrate the feasibility and versatility of DNA ENTRAP as a microfluidic conjugation platform, we evaluated various experimental parameters such as the donor/recipient ratio as well as the effect of co-incubation time on conjugation efficiency.

Figure 8 shows the in-droplet DNA conjugation efficiency compared to the benchtop conjugation efficiency. The donor and recipient cells were co-encapsulated in droplets at ratios of 3:1 and 1:1 and the DNA conjugation efficiency evaluated for a period of up to 2 h. The conjugation efficiency was calculated by counting colony growth for the JAB 981 donor strain, the JAB 545 receiver strain, and successful conjugates from droplets plated on selection media agar, which provides the most accurate metric to compare the donor strain's capability to perform gene transfer events with respect to co-incubation time. The conjugation efficiency was found to be higher when XPORT-mediated DNA transfer was performed in-droplet compared to the benchtop approach (at the bench, by hand) at both donor/recipient cell ratios. Specifically, the conjugation efficiency at the 1:1 donor/recipient ratio improved over nearly 2 orders of magnitude for the 0.5-h incubation period and over 1 order of magnitude for the 1-h incubation period when compared to the benchtop method (Fig. 8A). For the 3:1 donor/recipient cell ratio, the conjugation efficiency improved nearly 1 order of magnitude for the 0.5-h co-incubation period and over 1 order of magnitude for the 1-h incubation period when compared to the benchtop method (Fig 8B). In summary, we demonstrated that this novel platform can conduct a highly customizable and tunable gene transfer assay that significantly enhances the DNA transfer efficiency from 2-fold to over 20-fold depending on the selected assay parameters.

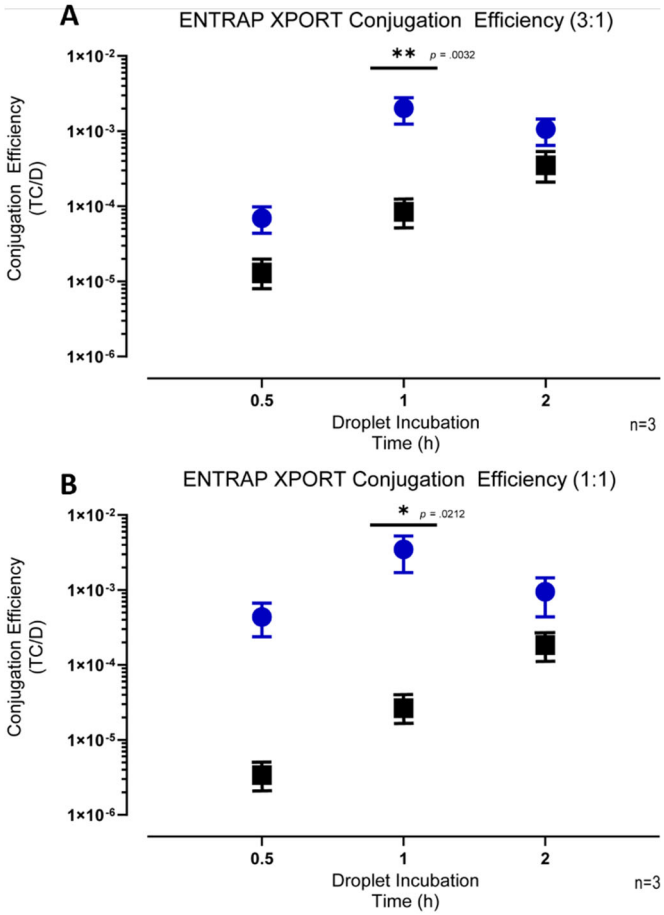

Fig. 8 In-droplet DNA conjugation compared to benchtop conjugation efficiency at two different donor/recipient cell ratios. (A) Results from the 3:1 ratiometric donor-to-recipient in-droplet cell pairing ratios. (B) Results from the 1:1 ratiometric donor to recipient in-droplet cell pairing ratios. (Reprinted with permission from Wippold et al. [2024].)

3.4 In-Droplet DNA Conjugation Confirmation Assays

Based on the successful in-droplet DNA conjugation assay monitored through fluorescent microscopy, we then verified successful conjugation through colony PCR amplification and subsequent sequencing of the miniICE insertion, followed by whole genome sequencing for a population of TCs. The miniICE insertion is a DNA fragment of the donor *Bacillus* that is excised from its own genome and subsequently passed to its recipient, then integrated into its genome. In the case of the XPORT donor, the miniICE region that gets transferred to the recipient cell has been engineered with an IPTG-inducible GFPmut2 fluorescent protein. The remainder of the miniICE region transferred contains helper proteins (e.g., nicK, xis, int) assisting in the integration of the DNA into the recipient genome. First, the cells collected from both in-droplet and benchtop XPORT-mediated conjugation were grown on selective media to allow TCs to be enriched, as previously

described. Three colonies from a droplet TC selection plate containing approximately 35 average total colonies were randomly picked to confirm the miniICE gene cassette insertion region by colony PCR amplification. The droplet conjugation system enables us to parallelize many different conjugation events through the encapsulation of the donor and recipient cells into individual droplets. Therefore, we hypothesized that with each individual reaction vessel (i.e., droplet), we could potentially recover TCs with genetic variability within the ICE region and the location at which it integrates. Although selective pressures on both the donor and recipient cells promote attachment site fidelity, earlier studies have provided evidence that the transference and integration of the ICE cassette into the recipient genome can occur at secondary (off-target) attachment sites (Lee et al. 2007). Although we were mindful of the possible occurrence of this phenomenon, we sought to confirm the location of the miniICE gene cassette in the recipient genome and the fidelity of the insertion by performing full genome sequencing on a population of TCs (colonies scraped directly from selection agar).

This was then followed by sequencing to verify the proper insertion of the miniICE gene cassette into the recipient genome. The long reads were also de novo assembled into a genome and aligned with the reference genome for the recipient, JAB 545. The gaps in the genome alignments suggested that the miniICE region was successfully integrated at the highly conserved leucine tRNA gene (*trnS-leu2*) in the recipient genome in the cells sampled. Although the sequences we analyzed confirm miniICE insertion in the expected region, we acknowledge that there is not enough read depth to exclude that the miniICE may be inserted into other regions at rare frequency levels. Observing such rare events is not the goal of this study, rather our primary goal for this study is to ensure that droplet-based conjugation can yield correct insertions in TCs. These same colonies were further cultured and used in the flow cytometry characterization described in Section 3.5.

Approximately 500 reads matching portions of the miniICE region were extracted from all the long reads that surpassed the quality threshold (approximately 2.4 million reads per replicate). These reads were mapped and aligned to the miniICE reference sequence using Geneious Prime 2021 (Biomatters Inc., Boston, MA). When analyzing the remaining base pairs flanking the miniICE region of the matched reads, we confirmed that the miniICE region had successfully integrated at the *trnS-leu2* locus in some portion of the total population sequenced.

3.5 Flow Cytometry Characterization of the In-Droplet DNA Conjugation Results

The miniICE cassette contains an IPTG-inducible green fluorescent protein (GFP) and tetracycline-resistance marker that should be integrated at the *trnS-leu2* region in the recipient genome (Menard and Grossman 2013; Brophy et al. 2018). To show that the droplet-based conjugation had not impacted the expression of the TCs and further confirm the presence of the miniICE gene cassette, we compared the GFP expression of the recipient cells with that of benchtop TCs. After a 1-h IPTG induction period, droplet TCs showed comparable fluorescence intensities with benchtop TCs (Fig. 9). These data suggest that a fully functional miniICE gene cassette was successfully integrated into almost all TCs tested and that there was no difference in expression compared to the conventional XPORT-mediated conjugation.

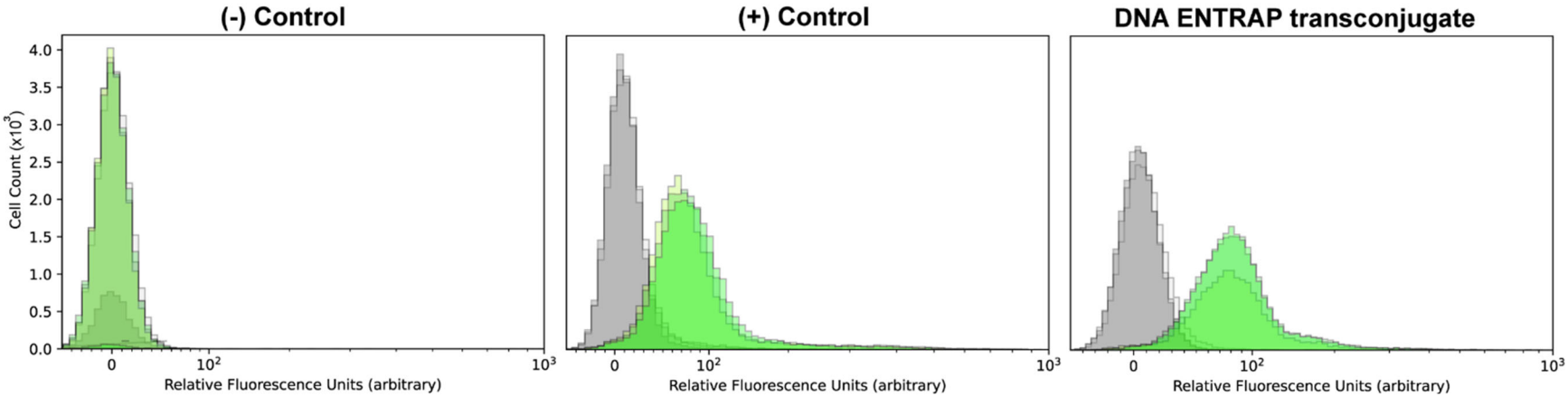

Fig. 9 In-droplet DNA conjugation confirmation through flow cytometry. Flow cytometry analysis of TCs created by the droplet conjugation method vs. traditional benchtop conjugation method. (Left) Negative control (unconjugated JAB 545 recipient). (Center) Benchtop TC. (Right) Droplet TC; green-shaded histograms represent cultures induced with 1 mM IPTG for 1 h. Gray-shaded histograms represent uninduced cultures. (Reprinted with permission from Wippold et al. [2024].)

3.6 Off-Chip Conjugation

To confirm whether the underlying technology of DNA ENTRAP is capable of performing the relevant biological assays, cells were co-encapsulated in droplets at two ratios 3:1 and 1:1 donor cells to recipient cell, respectively, for 0.5-, 1-, and 2-h co-incubation periods. Conjugation efficiency (TC/D) is determined by the ratio between the number of donor (D) colonies and the number of TC colonies on their respective selection media. This ratio is of particular interest because it provides the most accurate metric to compare the donor strain's capability to perform gene transfer events across various culture conditions. For all statistical analyses, a two-way ANOVA assuming sphericity with an alpha of 0.05 and a Šídák's multiple comparison test was conducted to obtain an adjusted p-value. The Šídák's method was preferred to the Bonferroni method because it provides more power to the calculated p-value. We determined the conjugation efficiency (TC/D) to be 1.31×10^5, 8.44×10^3, and 3.54×10^4 for a 3:1 ratio at 0.5, 1, and 2 h, respectively. We determined the conjugation efficiency to be 3.40×10^6, 2.65×10^5, and 1.84×10^4 for a 1:1 ratio at 0.5, 1, and 2 h, respectively.

4. Conclusions

The current status of the DNA ENTRAP platform has many advantages, but it still offers several interesting avenues for further advancement. First, the scope of the studies presented here was limited to two *B. subtili*s strains: a single donor *B. subtilis* strain and a single receiver *B. subtilis* strain. Given the extent of previous work demonstrating XPORT-mediated DNA transfer into environmental isolates and other non-*Bacillus* species, we believe the developed platform and technique here can be used to introduce exogenous DNA into a wide range of prospective recipients (Brophy et al. 2018). Second, culturing and scaling inconsistencies with growing the engineered XPORT strain resulted in a longer than expected assay development time to establish a results-driven link between off-chip and on-chip methods. We expect that a more robust, engineered donor strain could overcome some of these variability issues.

From a device perspective, we recognize two additional drawbacks. First, an in-line conjugation success read-out is desired for the final platform. This can be achieved through the integration of a droplet sorting module in such a future platform. Second, we performed the DNA ENTRAP method on two distinct yet fluidically tethered chips. As this body of experimentation demonstrates a biological proof-of-concept, we plan to consolidate the various microfluidic components in our future work. These engineering aspects will enhance DNA ENTRAP as a transformative genetic transfer tool. Here, we presented the DNA ENTRAP platform that

demonstrates enhanced and automated conjugative events with respect to traditional benchtop approaches, with efficiency increases ranging from 2-fold to over 20-fold depending on selected assay parameters. This work helps overcome the limitations of traditional genetic manipulation and DNA transfer approaches by confining the cells in nano-sized, water-in-oil emulsions to encourage cell-to-cell contact, which is needed for conjugation to occur.

5. Future Work

In addition to establishing the first droplet microfluidic-based bacterial XPORT conjugation platform, the work we presented addresses a major bottleneck limiting the development of novel synthetic biological chassis organisms. Limitations on assay throughput and testing capability created by the need for highly manual, benchtop protocols were addressed here by using microfluidics. Further downstream automation can be achieved through further adaptation of microfluidic technology to synthetic biology tools. This includes the use of LabView-controlled fluid flows along with incorporating various liquid handling steps, such as droplet merging and droplet sorting, while also achieving automated analysis via droplet content detection. Although this level of automation through complex droplet handling modalities falls beyond the scope of this work, when combined with our current demonstration, such automation is a possible future direction for our platform.

We anticipate the presented platform's true utility will be in conducting massively parallel genetic engineering of environmental isolates via XPORT. To this end, we hope to transition this device and experimental protocol toward screening environmental isolates. Furthermore, as we recognize the platform's capability in screening and analyzing highly complex DNA libraries with an abundance beyond the normal screening capability of benchtop researchers, we plan to incorporate droplet detection and droplet sorting functionalities while also advancing the complete automation of the system. At a modest droplet throughput of 10 s of droplets screened per second, we calculate that DNA ENTRAP can screen a population size of 106 environmental isolates in under 4 days.

In contrast, using the conventional agar-plate based selection process, with a day and night shift team of 25 scientists working 12-h shifts each (50 plates/day total), we expect this same 106 environmental isolates will take 54.8 years to screen. To fully realize this goal of full-platform automation, we hope to develop additional system hardware to incorporate droplet detection and sorting systems. To this end, we developed a novel platform capable of conducting horizontal gene transfer with highly customizable and tunable experimental parameters enabling efficiency

increases ranging from 2-fold to over 20-fold depending on selected assay parameters. Through the biological proof-of-concept demonstration presented herein, we hope to offer a complete platform that aligns with the need of providing an efficient, automated, reliable, and high-throughput system for synthetic biology applications (Gach et al. 2017; Li et al. 2018; Wang 2018).

6. References

Adams BL. The next generation of synthetic biology chassis: moving synthetic biology from the laboratory to the field. ACS Synth Biol. 2016;5(12):1328–1330.

Akselband Y, Cabral C, Shapiro DS, McGrath P. Rapid mycobacteria drug susceptibility testing using Gel Microdrop (GMD) growth assay and flow cytometry. J Microbiol Methods. 2005;62:181–197.

Baret JC, Miller OJ, Taly V, Ryckelynck M, El-Harrak A, Frenz L, Rick C, Samuels ML, Hutchison JB, Agresti JJ. Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. Lab Chip. 2009;9(13):1850–1858.

Brophy JAN, Triassi AJ, Adams BL, Renberg RL, Stratis-Cullum DN, Grossman AD, Voigt CA. Engineered integrative and conjugative elements for efficient and inducible DNA transfer to undomesticated bacteria. Nat Microbiol. 2018;3(9):1043–1053.

Brouzes E, Medkova M, Savenelli N, Marran D, Twardowski M, Hutchison JB, Rothberg JM, Link DR, Perrimon N, Samuels ML. Droplet microfluidic technology for single-cell high-throughput screening. Proc Natl Acad Sci USA. 2009;106(34):14195–14200.

Chen J, Vestergaard M, Jensen TG, Shen J, Dufva M, Solem C, Jensen PR. Finding the needle in the haystack-the use of microfluidic droplet technology to identify vitamin-secreting lactic acid bacteria. mBio. 2017;8(3):e00526-17.

Chen J, Vestergaard M, Shen J, Solem C, Dufva M, Jensen PR. Droplet-based microfluidics as a future tool for strain improvement in lactic acid bacteria. FEMS Microbiol Lett. 2018;365(23).

Gach PC, Iwai K, Kim PW, Hillson NJ, Singh AK. Droplet microfluidics for synthetic biology. Lab Chip. 2017;17(20):3388–3400.

Guzman AR, Kim HS, de Figueiredo P, Han A. A three-dimensional electrode for highly efficient electrocoalescence-based droplet merging. Biomed Microdevices. 2015;17(2):35.

Huebner A, Sharma S, Srisa-Art M, Hollfelder F, Edel JB, DeMello AJ. Microdroplets: a sea of applications? Lab Chip. 2008;8(8):1244–1254.

Kaminski TS, Scheler O, Garstecki P. Droplet microfluidics for microbiology: techniques, applications and challenges. Lab Chip. 2016;16(12):2168–2187.

Kim HS, Guzman AR, Thapa HR, Devarenne TP, Han A. A droplet microfluidics platform for rapid microalgal growth and oil production analysis. Biotechnol Bioeng. 2016;113(8):1691–1701.

Kim HS, Hsu S-C, Han S-I, Thapa HR, Guzman AR, Browne DR, Tatli M, Devarenne TP, Stern DB, Han A. High-throughput droplet microfluidics screening platform for selecting fast-growing and high lipid-producing microalgae from a mutant library. Plant Direct. 2017;1(3):e00011.

Lam T, Brennan MD, Morrison DA, Eddington DT. Femtoliter droplet confinement of streptococcus pneumoniae: bacterial genetic transformation by cell-cell interaction in droplets. Lab Chip. 2019;19.

Lam T, Maienschein-Cline M, Eddington DT, Morrison DA. Multiplex gene transfer by genetic transformation between isolated S. pneumoniae cells confined in microfluidic droplets. Integrat Biol. 2020;11.

Lee CA, Auchtung JM, Monson RE, Grossman AD. Identification and characterization of int (integrase), xis (excisionase) and chromosomal attachment sites of the integrative and conjugative element ICEBs1 of Bacillus subtilis. Mol Microbiol. 2007;66(6):1356–1369.

Li X, Hu J, Easley CJ. Automated microfluidic droplet sampling with integrated, mix-and-read immunoassays to resolve endocrine tissue secretion dynamics. Lab Chip. 2018;18(19):2926–2935.

Luu XC, Shida Y, Suzuki Y, Sato N, Nakumura A, Ogasawara W. A novel high-throughput approach for transforming filamentous fungi employing a droplet-based microfluidic platform. Nat Biotechnol. 2022;72:149–158.

Martino C, DeMello AJ. Droplet-based microfluidics for artificial cell generation: a brief review. Interface Focus. 2016;6(4):20160011–20160011.

Mazutis L, Gilbert J, Ung WL, Weitz DA, Griffiths AD, Heyman JA. Single-cell analysis and sorting using droplet-based microfluidics. Nat Protoc. 2013;8:870.

Menard KL, Grossman AD. Selective pressures to maintain attachment site specificity of integrative and conjugative elements. PLoS Genet. 2013;9(7):e1003623.

Ota Y, Saito K, Takagi T, Matsukura S, Morita M, Tsuneda S, Noda N. Fluorescent nucleic acid probe in droplets for bacterial sorting (FNAP-sort) as a high-throughput screening method for environmental bacteria with various growth rates. PLoS One. 2019;14(4):e0214533.

Terekhov SS, Smirnov IV, Stepanova AV, Bobik TV, Mokrushina YA, Ponomarenko NA, Belogurov AA Jr, Rubtsova MP, Kartseva OV, Gomzikova MO, Moskovtsev AA, et al. Microfluidic droplet platform for ultrahigh-throughput single-cell screening of biodiversity. Proc Natl Acad Sci USA. 2017;114(10):2550–2555.

Wang BL, Ghaderi A, Zhou H, Agresti J, Weitz DA, Fink GR, Stephanopoulos G. Microfluidic high-throughput culturing of single cells for selection based on extracellular metabolite production or consumption. Nat Biotechnol. 2014;32(5):473–478.

Wang Z. Detection and automation technologies for the mass production of droplet biomicrofluidics. IEEE Rev Biomed Eng. 2018;11:260–274.

Wippold JA, Chu M, Renberg R, Li Y, Adams B, Han A. XPORT ENTRAP: a droplet microfluidic platform for enhanced DNA transfer between microbial species. N Biotechnol. 2024;81:10–19.

Wippold JA, Huang C, Stratis-Cullum D, Han A. Enhancing droplet transition capabilities using sloped microfluidic channel geometry for stable droplet operation. Biomed Microdevices. 2020a;22(1):15.Wippold JA, Wang H, Tingling J, Leibowitz JL, de Figueiredo P, Han A. PRESCIENT: platform for the rapid evaluation of antibody success using integrated microfluidics enabled technology. Lab Chip. 2020b;20(9):1628–1638.

Xia Y, Whitesides GM. Soft lithography. Ann Rev Mater Sci. 1998;28(1):153–184.

Zhang H, Guzman AR, Wippold JA, Li Y, Dai J, Huang C, Han A. An ultra high-efficiency droplet microfluidics platform using automatically synchronized droplet pairing and merging. Lab Chip. 2020;20(21):3948–3959.

Appendix A. Materials and Reagents

Biological Materials

1) Donor strain JAB 981, *Bacillus subtilis* JH642 Δ(ydcS-yddM)::[(Pspank-GFPmut2) tet(M) cat] thrC::[(int-yddJ) ΔnicK mls] alrA::[(Psweet-rapI) spc]
2) Recipient strain JAB 545, *B. subtilis* PY79 cured of ICEBs1, ΔcomC:: aphA-

Reagents for Bacterial Culture

1) LB Miller (Fisher Scientific, catalog number: BP1426-2)
2) Phosphate-buffered saline (VWR, catalog number: 97062-336)
3) D-alanine (Sigma-Aldrich, catalog number: A7377)
4) Tetracycline hydrochloride (Calbiochem, catalog number: 583411; may alternatively use Sigma-Aldrich catalog number: T7660)
5) Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, catalog number: I5502)
6) NH_4Cl (Sigma-Aldrich, catalog number: A9434)
7) $MgCl_2$ (Sigma-Aldrich, catalog number: M8266)
8) $K_2HPO_4 \cdot 3H_2O$ (Sigma-Aldrich, catalog number: P5504)

Materials for Chip Fabrication

1) SU-8 2075 photoresist (Kayaku [formerly Microchem Corp.])
2) SU-8 2050 photoresist (Kayaku [formerly Microchem Corp.])
3) Microposit EBR 10A (Kayaku [formerly Microchem Corp.])
4) (Tridecafluoro-1,1,2,2 tetrahydrooctyl)trichlorosilane (United Chemical Technologies LLC, catalog number: T2492)
5) Poly(dimethylsiloxane) (Dow Corning Corp., PDMS Sylgard 184, catalog number: 761028-5EA)
6) 3-(trimethoxysilyl)propyl methacrylate (Sigma-Aldrich, catalog number: 440159)
7) Propylene glycol monomethyl ether acetate (PGMEA) (Sigma-Aldrich, catalog number: 484431)

8) 99% isopropyl alcohol (ULINE, catalog number: S20735)

9) EBR-10 A (Microposit Kayaku Advanced Materials)

Reagents for Confirmation Assays

1) Q5 polymerase (New England Biolabs [NEB], catalog number: M0491L)

2) Deoxynucleotide (dNTP) solution sets (NEB, catalog number: N04475)

3) Q5 Reaction Buffer (NEB, catalog number: B9027S)

4) 10 μM primers (IDT, exact sequences are noted in the protocol)

5) Sheath fluid for flow cytometry (Thermo Scientific, NERL Blood Bank Saline, catalog number: 8504)

6) ReadyLyze Lysozyme (LGC Biosearch Technologies, Lucigen, R1804M)

7) MasterPure Total Nucleic Acid Isolation Kit (LGC Biosearch Technologies, Lucigen, MC85200)

Solutions

1) Tris-Spizizen Salts (TSS) buffer (see Recipes).

2) Carrier (oil) phase for ENTRAP device (see Recipes).

Recipes

Table A-1 TSS buffer

Reagent	Final concentration (mM)	Amount (g/L)
NH_4Cl	150	2
$K_2HPO_4 \cdot 3H_2O$	20	0.35
Tris base (pH 7.5)	49.5	6
$MgCl_2$	125	11.9

Table A-2 Carrier phase for ENTRAP device

Reagent	Final concentration	Amount (mL)
PicoSurf (5% in Novec 7500)	1.25%	1
Novec 7500	N/A	4
Total	N/A	5

Laboratory Supplies

1) 15 mL culture tubes (Corning, Falcon, catalog number: 352059)
2) 5 mL plastic syringe (BD, catalog number: 309646)
3) 1 mL plastic syringe (BD, catalog number: 309628)
4) 0.2 µm Whatman Puradisc 13 syringe filter (Sigma-Aldrich, catalog number: WHA67801302)
5) Microtube-130 AFA Fiber for sonication (Covaris, catalog number: 520216)
6) Non-DEHP Tubing, 30 Gauge (Saint-Gobain Performance Plastics, Tygon ND-100-80, catalog number: VWR 89404-300)
7) Micropipettes 100–1000 µL (VWR, Eppendorf, catalog number: 89125-306)
8) Pipette tips 1000 µL (USA Scientific, TipOne, catalog number: 1122-1830)
9) 96-well microplate (Corning, catalog number: 353072)
10) Glass slides (Corning, catalog number: 2947, 75X50)

Equipment

1) Microcentrifuge (Eppendorf, Centrifuge 5420)
2) Shaking incubator (New Brunswick, Innova 42R, catalog number: M1335-0080)
3) Spectrophotometer (Biowave CO8000 Cell Density Meter, VWR)
4) Stationary incubator (Thermo Fisher Scientific, Heratherm, catalog number: 50125590)
5) Focused ultrasonicator for genome extractions (Covaris E220 Evolution, catalog number: 500429)
6) Desiccator (Zoro, catalog number: G4655463)
7) Spin coater (Laurell Technologies Corporation, Model H6-23)
8) Photolithography mask aligner (EVG Group, EVG 610)
9) Syringe pump (Chemyx, Fusion 200-X, catalog number: 0720X)

10) Plasma cleaner (Harrick Plasma, catalog number: PDC-32G)

11) Optical microscope (Zeiss, Colibri Axiovert 200)

Software and Datasets

1) Geneious 2023 (paid software for genetic mapping)
2) Alternative genetic mapping software: Benchling (free online interface for gene editing)
3) AutoCAD (paid software for designing master molds)
4) Alternative CAD software for silicon mold design: Blender, FreeCAD
5) Filtlong v0.2.1 (free)
6) Raven v1.7.0 (free)
7) medaka v1.4.2 (free)
8) prokka v1.14.5 (free)

Appendix B. Procedure

Device Fabrication

1) Option 1: traditional photolithography method to fabricate master mold on silicon wafer

    a. Spin coat SU-8 2050 onto a silicon wafer at 2100 rpm (see Appendix C, General Note 1).

    b. Apply the first soft baking step at 65 °C for 24 h.

    c. Apply the second soft baking step at 95 °C for 20 min.

2) Expose the coated Si wafer in the photolithography mask aligner using a mask design (either screen printed or using chromium mask processing) wafer using standard i-line (365 nm) and a total dose of 1000 mJ/cm^2

    a. Apply a post-exposure baking step at 65 °C for 40 min, then 95 °C for 20 min.

    b. Remove the unexposed photoresist using EBR 10A (commercial Edge Bead Remover solution engineered to eliminate the any unexposed negative photoresist).

    c. Using a conventional desiccator, apply a coating of (tridecafluoro-1,1,2,2 tetrahydrooctyl) trichlorosilane for 60 min (see Appendix C, General Note 2).

        i. For this, place all wafers to be silanized oriented feature-side up.

        ii. Apply 2–4 drops of trichlorosilane (using a plastic pipettor; approximately 100 μL total) to a small weigh boat.

3) Option 2: Two-photon lithography method to fabricate master mold on silicon wafer.

    a. For the devices used in this work, two approaches can be used for the fabrication of the microfluidic master molds that were used to conduct the soft lithography steps (i.e., the replica molding step to create the poly(dimethylsiloxane) [PDMS] chips). We used a typical SU-8 photolithography approach along with a two-photon polymerization approach, depending on the overall geometry and footprint of the microfluidic chips that were designed (see Appendix C, General Note 3).

b. Silanizing the silicon wafer

i. Make a bath of 1% solution of coupling agent (3-(trimethoxysilyl)propyl methacrylate; Sigma-Aldrich) in ethanol

ii. Submerge substrates overnight

Submerging can be expedited using only a 4-h period, if timing is an issue.

iii. Remove the substrate from the chemical bath, rinse with DI water, and dry using N2 gun

iv. Store wafers in airtight container at room temperature for up to 3 months (see Appendix C, General Note 4).

4) Adding IP-S photoresist

a. Apply a volume of IP-S photoresist sufficient to cover the intended footprint of the microfluidic geometry to be fabricated.

b. Gently spin the photoresist (500 pm) for 60 s to ensure an even spreading.

5) Running Nanoscribe

a. Run fabrication using the Nanoscribe two-photon polymerization (2PP) tool

i. Upload the .STL file to the 2PP software tool: DeScribe.

ii. Fabricate the master mold pattern on the Nanoscribe Photonics Professional GT (Nanoscribe GmbH, Karlsruhe, Germany) using the pretreated silicon wafer as the substrate and negative photoresist (IP-S, Nanoscribe GmbH) using a power scaling of 1.0, tetrahedron inner scaffold, base scan speeds of 50,000, base laser power of 60%, shell/scaffold scan speeds of 100,000, and shell/scaffold laser power intensities set at 70%.

6) Developing

a. After the fabrication run, remove the wafers from the tool and develop in propylene glycol monomethyl ether acetate (PGMEA, Millipore Sigma, MA) for 6 min.

b. For fine development, place the wafers containing the master mold in 99% Isopropyl alcohol (VWR, IPA) for 10 min.

c. After the IPA bath, dry the masters gently with nitrogen gas and inspect under a microscope for quality assurance.

7) Post-fabrication silanization of microfabricated structures

a. After the inspection, coat the patterned wafers with (tridecafluoro-1,1,2,2 tetrahydrooctyl)trichlorosilane (United Chemical Technologies, Inc., Bristol, PA) for 20 min.

b. This prevents pattern removal during PDMS replication.

Generating the ENTRAP Device Using PDMS

1) Mix 22 g of PDMS (2 g of curing agent and 20 g of polymer material; 1:10 ratio as specified by the manufacturer)

2) Place silicon wafer mold (fabricated in Step 1) into a petri dish or any clean container large enough to fit the silicon wafer. Pour 20 g of material over the mold (see Appendix C, General Note 5).

For ease of removal of the PDMS post-curing, secure the Si-SU-8 master mold to the petri dish using Kapton tape along the edges of the mold.

3) Allow PDMS to cure over the mold either at room temperature (~25 °C) overnight on a level surface or at 65 °C for approximately 1–4 h in an oven.

Fabricating the Bottom Surface of the Channels

1) Using the PDMS made in Step 2, coat a clean glass slide with a thin layer of PDMS (30 μm thickness) by spin coating at 3000 rpm for 30 s.

Allow this PDMS to cure under the same conditions in Step 3c (see Appendix C, General Note 6).

2) Bonding the ENTRAP device to the PDMS-coated glass slide and preparing for experimentation

a. Carefully, using a scalpel or razor, cut a large rectangle in the PDMS around the channel patterns.

b. Remove the PDMS rectangle (which contains the channels) from the silicon wafer and place it on the PDMS-coated glass slide (see Appendix C, General Note 7).

3) Introduce tubing for the inlet and outlets of the device

    a. Use a needle to poke holes in the device where the inlets and outlets are designed.

    b. Place Tygon tubing in the holes for each of the inlets (one for the oil inlet, one for the cells) and one for the outlet.

4) Oxygen Plasma Bonding

    a. Place the device in the plasma cleaner and apply a 90-s plasma treatment at power of 150 W and a pressure of 150 mTorr.

    b. Connect the outlet tube to the second chip containing the microvessel system for incubation of the cells.

Cell Culture Preparation

1) Streak strains from glycerol stock onto Luria broth (LB) agar plates with selection antibiotics. *Bacillus subtilis* donor strain JAB 981 should be struck onto LB agar supplemented with tetracycline (10 µg/mL) and D-alanine (100 µg/mL). *B. subtilis* recipient strain JAB 545 should be struck onto LB agar, supplementation with chloramphenicol (10 µg/mL) is optional.

2) Incubate these streaked agar plates overnight (>14 h) at 37 °C. Pause point.

3) Pick a single large colony from the plates and suspend this colony in 3 mL LB liquid media supplemented with the same selection antibiotics that were on the agar media (see Appendix C, General Note 8).

4) Culture the donor and recipient strain to an OD600 between 0.8 and 1.2, as read on the spectrophotometer.

5) At this point, subculture these strains into fresh media with the same selection antibiotics (culture volumes: 3–5 mL).

6) Allow the donor strain to grow to an OD600 of 0.2: at this point, xylose should be added to the culture to a final concentration of 1%. Critical. This step serves to induce the conjugation machinery in the donor strain. No conjugation will occur without this.

7) Culture both strains to an OD600 between 0.8 and 1.0. (see Appendix C, General Note 9).

8) Pellet cultures by centrifugation at 3000× *g* for 3 min.

9) Wash pellets with 1 mL PBS and recentrifuge at 3000× *g* for 3 min. Repeat twice.

10) Resuspend both pellets in either TSS media (Recipe 1) or LB media supplemented with D-alanine (100 µg/mL) to a final OD600 of 0.6. (see Appendix C, General Note 10).

Conjugation of Cells in the ENTRAP Device

1) Prior to experimentation, the fully fabricated PDMS DNA ENTRAP microdevices were baked at 80 ºC for 4 h to ensure a state of ensured hydrophobicity.

2) Combine the donor cells with recipient cells at a 1:1 ratio. This step is optional, should you wish to keep the donor and recipient cells separate and use separate inlets for each. In such a case, use the chip designed with two reagent inlets (see Appendix C, General Note 11).

3) Load at least 500 µL of the donor/recipient cell mix into a 1-mL BD plastic syringe.

4) Prepare the oil phase solution according to Recipe 2. Filter the solution through a 0.2-µm filter before use.

5) Prime all microfluidic channels and devices with the oil phase solution before operation.

6) Load 5 mL of the carrier oil/surfactant mixture (Novec 7500/PicoSurf) into a 5-mL BD plastic syringe.

7) Place the loaded syringes into separate Chemyx Fusion 200 pumps. For the syringe containing the cell suspension, set the syringe pump at a flow rate of 400 µL/h. For the syringe containing the carrier phase (oil + surfactant), set the syringe pump to a flow rate of 2000 µL/h (see Appendix C, General Note 12).

8) Run both syringe pumps to begin droplet formation. Ensure that the outlet tube of the ENTRAP chip is connected to the second chip containing the microvessel system, which enables accurately timed incubation periods. Allow the droplets to accumulate in the microvessel system.

9) While droplets are being formed, you may perform imaging under an inverted optical microscope. For the Zeiss AxioObserver Colibri, use the following parameters:

    a. For brightfield imaging, use the 10× objective with a phase contrast (Ph1) ring and 10-ms exposure time at 9V total light power.

b. For fluorescent imaging, the corresponding LED (to excite the fluorophore or fluorescent protein) should be used in combination with an appropriate dichroic mirror/beam splitter and emission filter (to remove signal not corresponding to the excited target). For example, with GFP imaging, the BP 469/38 LED should be used to excite the fluorophore, while paired with a triple band pass splitter 405+493+610. LED power should be set to 95%, and exposure times ranged from 300 to 3000 ms. Select the appropriate exposure time that provides satisfactory signal-to-noise feedback. Keep the exposure time consistent across all experimental runs.

10) You may stop the syringe pumps when you have achieved the desired volume (or expected droplet number). Clip the end of the tube (stopping flow) by placing approximately 0.5 cm of the outlet tube into a microcentrifuge tube and closing the lid.

11) Incubate droplets for at least 1 h at ambient temperatures or 37 °C (depending on your recipient strain's culture constraints).

12) After the desired co-incubation period, flow the droplets out from the microvessel chip.

13) Pipette 50 µL of the droplets that are in oil suspension within the microvessel chambers used for conjugation onto a selection agar plate, which may be any media appropriate for transconjugant growth supplemented with 10 µg/mL tetracycline. Spread the droplets (breaking them) using a sterile L-spreader. For the recipient strain, JAB 545, we use LB Miller as the base media (see Appendix C, General Note 13).

14) For controls and conjugation efficiency calculations, plate the same volume of droplets onto (1) selection agar containing 100 µg/mL D-alanine and 10 µg/mL tetracycline, which will capture both donor and transconjugant, and (2) agar media with no antibiotic or D-alanine supplementation, which will capture untransformed recipients and transconjugants, while preventing JAB 981 donors from growing due to their auxotrophy.

15) Incubate the plates (~16 h) in a 37 °C stationary incubator or at the preferred temperature of the recipient strain. Most of the *Bacillus* recipient strains we have used with the XPORT donor can tolerate 37 °C, but one should take heed to the culture conditions of the recipient when plating for transconjugants. Pause point.

Transconjugant Selection and Evaluation

1) After the incubation period, inspect the transconjugant plates (plates with 10 μg/mL tetracycline) for colonies. If colonies have not yet grown, you may need to incubate for longer.

2) Pick several colonies from the transconjugant plate. Inoculate 3-mL cultures, supplemented with 10 μg/mL tetracycline. After reaching mid-log phase, subculture these strains for improved consistency (across replicates) in expression.

3) When grown to an OD600 of approximately 0.2 (or anytime during early log phase—this OD value is heavily dependent on the growth behavior of the recipient strain), induce cells with 1 mM IPTG, then shake for 1 h at 37 °C (or whatever temperature suits your recipient strain). Be sure to aliquot a portion of the cultures out as a negative (untreated) control.

4) After this incubation period, you may choose to perform various analyses to confirm successful conjugation of DNA into the recipient strain.

    a. Option 1: Flow cytometry. For this type of analysis, prepare a sample by diluting the induced sample and uninduced control in sheath fluid (100-fold dilution). Run the samples using the Sony SA3800 Spectral Analyzer with the following parameters: threshold FSC value, 0.5%; FSC gain, 17; SSC voltage, 20%; fluorescence PMT voltage, 69.8%; acquisition of 10,000 events.

    b. Option 2: Plate reader for fluorescence measurement. For this type of analysis, prepare the following dilutions of your samples: undiluted, 2-, 4- and 8-fold dilutions. Pipette 200 μL of each sample into a 96-well plate. Read absorbance at 600 nm and fluorescence in accordance with the excitation/emission parameters of the expressed fluorophore. In this work, we measure GFPmut2, which has an excitation/emission of 485 and 508 nm, respectively. Divide the relative fluorescence units by the absorbance at 600 nm of the same sample to normalize for the difference in cell densities across samples.

    c. Option 3: Fluorescence microscopy. One may use the sample parameters as stated above in Section titled, “Conjugation of Cells in the ENTRAP Device,” Step 9.

    d. Option 4: Colony polymerase chain reaction (PCR). Use the following primer sequences, flanking the insertion site, to confirm

conjugation success: 5′-AAAGTCTTTTATTCTGCGCCG-3′ and 5′-ATCATTTAGTAAGGCAGCTGCTA-3′. After obtaining the template DNA by boiling colonies for 3 min, set up the PCR reactions using Q5 polymerase and the aforementioned primers. Apply an annealing temperature of 64 °C and extension time of 8.5 min. Sequence the resulting amplicon.

e. Option 5: Whole genome sequencing. Obtain a cell pellet of the transconjugant. Isolate genomic DNA from the pellet using ReadyLyze Lysozyme (Lucigen R1804M) and MasterPure Total Nucleic Acid Isolation Kit (Lucigen MC85200) on the Covaris E220 Evolution with a microtube-130 AFA Fiber (Covaris 520216). After isolating gDNA, treat with the Short Read Eliminator XS kit (Circulomics [PacBio]) to remove fragments smaller than 10 kb. Because the integrative and Conjugative elements (ICE) insertion is larger than 10 kb, this procedure helps to ensure that DNA fragments corresponding to the ICE will contain at least one or both of the flanking sequences (upstream/downstream) of the integration site. Apply the Oxford Nanopore Ligation protocol (SQK-LSK109) to pretreat the gDNA, then sequence the fragments using the GridION (Oxford Nanopore Technologies) on a R9.4.1 MinION flow cell.

Data Analysis

1) To calculate conjugation efficiency, count the number of colonies on the tetracycline/D-alanine plate (i.e., number of donors + transconjugants) and then count the number of colonies on the tetracycline plate (i.e., number of transconjugants). Subtract the latter number from the former; this number estimates the total number of donor colony-forming units. Divide the number of transconjugants by the number of donors to obtain the conjugation efficiency. Be sure to use at least three biological replicates for this calculation.

2) Sequences may be analyzed with the following protocol:

    a. Filtering with filtlong (https://github.com/rrwick/Filtlong.git) to a minimum length of 1000.

    b. Assembly with raven (https://github.com/lbcb-sci/raven.git).

    c. Polishing with medaka (https://github.com/nanoporetech/medaka.git).

    d. Annotation with prokka (https://github.com/tseemann/prokka.git).

e. Resulting annotations can then be assessed with GENEIOUS. For further sequencing confirmation of transconjugants, whole genome sequencing on the transconjugants should be performed with the fragments de novo assembled into a genome. The de novo assembled transconjugant genome can then be aligned with the original genome (wild type). Gaps where the transconjugant sequence does not match the wild-type genome should reveal any potential site where the ICE could have inserted. Alternatively, the long-read fragments can be mapped to the theoretical sequence of the transconjugant genome to confirm the presence of the ICE.

3) For flow cytometry analysis, be sure to run at least three biological replicates and strive to induce all samples at the same point in their growth phase. This ensures better consistency in expression across samples. For the data acquisition, be sure to run at least 10,000 events. If there is enough sample volume and/or unusually heterogeneous population, an acquisition of 50,000 events is appropriate. Calculate the mean fluorescence of each sample and the standard deviation across three biological replicates. For multiple sample groups, one may apply Tukey's multiple comparisons (post hoc) test to determine whether there is any significant difference in expression between the controls and the experimental samples (see Appendix C, General Note 14).

Appendix C. General Notes and Troubleshooting

1) SU-8 2050 is used to obtain 65-µm channels (height). However, for 100-µm channels, use SU-8 2075 following manufacturer guidelines.

2) It is important to note that the layout of materials is consequential within the desiccator. The wafers to be silanized should be placed between the port connected to a vacuum source and the weigh boat with the trichlorosilane. This will enable a more thorough silanization of the wafers. If desired, rotate the wafer 180º after 30 min within the desiccators to ensure an even coating.

3) Both traditional photolithography and two-photon photolithography (2PP) was used during the timeline of experimentation for this work. This was the result of the sponsoring laboratory's cleanroom installing an upgraded system. Traditional photolithography is quicker, yet typically constrained to a "2.5D" fabrication. The height of a resultant structure is typically set at a single level and achieved through spin coat a wafer to a desired thickness (i.e., channel height). For 2PP, the channel height is fully dimensioned into the CAD drawing of the microchannel system. Thus, through 2PP, you can achieve multiple heights without the need of multiple spin-expose-develop-spin steps as is required through a traditional approach. The drawback of the 2PP approach when compared to a traditional approach is the overall fabrication time needed to create the master mold. In a traditional approach, exposure of the entire device usually takes <5 min. In 2PP, the overall footprint of a microfluidic device master mold can take anywhere between 6 and 36 h, depending on the total volume that needs to be polymerized. For this experiment, a 2PP approach was used for device's containing complex geometry (i.e., sub 10 µm feature sizes or multiple height critical geometries).

4) Wafers are tested stable for up to 3 months. After 3 months of shelf storage, the authors cannot ascertain whether the surface modification will be strong enough to ensure full attachment of the photoresist.

5) Be careful not to introduce any bubbles at this step. If there are any bubbles left over from the previous step, you may centrifuge the poly(dimethylsiloxane) (PDMS) mixture for 30 s at 2000× *g* to remove the bubbles.

6) It should be noted that this step is optional. PDMS can readily bond to an uncoated glass slide.

7) When placing the PDMS down on the glass slide, be sure that the PDMS is flush against the slide with no bubbles. Check for cracks and any channel defects in the PDMS under the microscope.

8) Alternatively, instead of pulling a colony from a plate, liquid cultures (>3 mL) may be started from the previous night (shaken at 22 °C) and sub-cultured the next morning by performing no less than a 250-fold dilution of the overnight culture into fresh media. If you choose this alternative step, you may skip Steps 1–3 of Appendix B, "Cell Culture Preparation," and use this culture as the final cultures to be used in the ENTRAP chip.

9) There is some flexibility in this portion of the protocol. Although the OD600 range is somewhat arbitrary, this fixed range is proposed to ensure that the cells are being collected before late exponential growth phase, where *Bacillus subtilis* cells may begin to enter a vegetative state or begin to sporulate. Every spectrometer is different, so a growth curve analysis on your preferred instrument is recommended.

10) This resuspension media is the media that will be used for co-incubation. Alternative media may be used in this step if the donor and recipients used in this protocol differ from JAB 981 and JAB5 45. For example, if the donor being used in this platform is an *Escherichia coli* S17 and the recipient is another *E. coli* or *Pseudomonas* spp., then any compatible media to sustain both strains (e.g., LB or M9) may be used.

11) Pre-mixing cells can add bias into the results because one cannot ascertain whether conjugation occurs in the culture tube prior to microfluidic injection or in the droplet system. Therefore, it is best practice to avoid any mixing of cell types until the droplet generation point. In addition, premixing cells will result in an in-droplet distribution that will require additional optimization steps to ensure target in-droplet cell concentrations and ratios are favorable for the conducted experiments.

12) For dual reagent (donor and recipient separate) experiments, each injection input syringe is controlled via a distinct syringe pump operated at 200 μL/h. Variable flow rates can be applied for different donor/recipient ratios. In general, as dictated in part by droplet geometry, a 1:5–1:10 ratio between the input phase(s) and the carrier phase is optimal for generating droplets at a stable diameter.

13) PicoBreak may be used to disperse the droplets, but it is usually not necessary as the carrier phase is volatile and will volatilize once plated onto agar.

14) Given that Wippold et al.[1] performed the first XPORT conjugation-based droplet microfluidic work, there is little known regarding the expected confidence and p-values.

Troubleshooting

1) What to do if there are bubbles in the channel or if the droplets are merging?

    a. Perform a surface treatment of the channels in the device. Prior to experimentation, flow Aquapel through the DNA ENTRAP droplet generator device in reverse flow (from the out to the reagent inlets). Apply a short (~5 mm) tubing into each of the inlets. After initial filling, sequentially clamp reagent inlets while leaving one open. This will ensure Aquapel flow is directed fully through the inlet channel. Repeat this process for all the inlets by releasing the clamp and replacing a clamp on the previously open inlet. In this case, 500-µL Eppendorf tubes were used to as clamps.

    b. Allow Aquapel to react with the PDMS/glass for 60 s. Remove syringe from the outlet and air purge with an air-filled, hand-actuated 10-mL plastic BD syringe. Repeat the Aquapel filling process another time to ensure full coating of the channel surfaces to enable a maximum hydrophobic treatment. After this second Aquapel treatment, repeat the air purge described above. Next, replace the Aquapel syringe with a 5-mL syringe with the carrier oil (0.2 µm filtered Novec 7500). Using the carrier oil, hand actuate the syringe to purge the channel of any residual Aquapel from the channels with 500 mL of solution, again repeating the inlet clamp/release method to ensure all inlets are cleared. Finally, repeat the air purge to empty the channels.

    c. Inspect the microfluidic device under a microscope using a 10× objective to complete a quality control step to identify any particles that have infiltrated the channels. If any particles are seen, repeat the carrier oil purge to dislodge and remove the particle. At this point, one can engage flow from any of the available inlet ports or again through the outlet port. If no particles are seen, apply Kapton tape to the inlet and outlet ports to protect the microfluidic device from airborne particles. Set aside for later use.

[1]Wippold JA, Chu M, Renberg R, Li Y, Adams B, Han A. XPORT ENTRAP: a droplet microfluidic platform for enhanced DNA transfer between microbial species. N Biotechnol. 2024;81:10–19.

d. The Aquapel-treated device will be stable at room temperature for 1 year. Immediately before experimental use, prefill all channels with the carrier oil. Once the channels are filled, begin engaging the reagent syringe pumps to inject the cell, reagent, and carrier oil + surfactant phases into the microfluidic device.

List of Symbols, Abbreviations, and Acronyms

ANOVA	Analysis of Variance
ARL	Army Research Laboratory
CFU	colony-forming unit
D	donor
DEVCOM	US Army Combat Capabilities Development Command
DNA	deoxyribonucleic acid
DNA ENTRAP	DNA Enhanced Transfer Platform
FSC	forward scatter
GFP	green fluorescent protein
ICE	integrative and conjugative elements
IPTG	isopropyl β-d-1-thiogalactopyranoside
LB	Luria broth
NEB	New England Biolabs
OD	optical density
PBS	phosphate-buffered solution
PCR	polymerase chain reaction
PDMS	poly(dimethylsiloxane)
PGMEA	propylene glycol monomethyl ether acetate
PMT	photomultiplier tube
2PP	two-photon photolithography
SSC	side scatter
TC	transconjugant
TSS	Tris-Spizizen Salts
wt%	weight percent

1 (PDF)	DEFENSE TECHNICAL INFORMATION CTR DTIC OCA
1 (PDF)	DEVCOM ARL FCDD RLB CI TECH LIB