Supporting Information

A Digital Microfluidic Platform for the Microscale Production of Functional Immune Cell Therapies

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Supplementary Methods

Reagents and Materials. Unless specified otherwise, general-use chemicals and kits were purchased from Sigma-Aldrich (St. Louis, MO). Device fabrication reagents and supplies included chromium-coated glass slides, and gold-coated glass slides with AZ1500 photoresist from Telic (Valencia, CA), MF-321 positive photoresist developer from Rohm and Haas (Marlborough, MA), chromium etchant 9051 and gold etchant TFA from Transene (Danvers, MA), AZ-300T photoresist stripper from AZ Electronic Materials (Somerville, NJ), Teflon-AF 1600 from DuPont Fluoroproducts (Wilmington, DE). Transparency masks for device fabrication were printed from ARTNET Pro (San Jose, CA) and polylactic acid (PLA) material for 3D printing were purchased from 3Dshop (Mississauga, ON, Canada). General chemicals for tissue culture were purchased from Wisent Bio Products (Saint-Bruno, QC, Canada). Electronic components were obtained from DigiKey (Thief River Falls, MI). eGFP mRNA was purchased from TriLink Biotechnologies (catalog #: L-7201). Human anti-CD19 CAR mRNA was purchased from Med Chem Express (catalog #: HY-153084A). Cas9 and sgRNAs were purchased from Synthego (Redwood City, USA).

Primary T-cell culturing. Primary human T cells were separated from fresh venous primary blood using LympPrep gradient centrifugation (Wisent Bio) and purified using either the EasySep Human CD4 T cell Isolation kit or EasySep Human T cell isolation kit to a purity of 95 % (STEMCELL Technologies, Canada, Catalog # 17952, and Catalog # 17951). All cells were kept in liquid nitrogen prior to use. Cells were thawed and cultured in complete culture medium consisting of RPMI-1640 with 10 % Fetal Bovine Serum (FBS), 100 IU / mL penicillin/streptomycin, and 200 IU / mL recombinant human IL-2 (STEMCELL Technologies, Canada, Catalog # 78220). Unless specified otherwise, cell culture was performed in U-bottom

96 well plates. After 24 hours post-thaw, the cells were activated with Human T-Activator CD3/CD28 Dynabeads (Fisher Scientific Ottawa, ON, #11131D) at a 1:1 cell to bead ratio and were incubated for 48 hours. After incubation, Human T-Activator beads were removed following manufacturers protocol by first gently pipetting up and down to release cells from the activator beads followed by transferring the cells to a magnetic tube rack for 1-2 minutes to allow for cells and beads separation and the supernatant containing cells was transferred to a fresh tube. Cells were electroporated within 24 hours of bead removal. Primary T cells were counted using the ViCell Blu automated cell counter (Beckman Coulter, Canada) and maintained at 0.25 - 1 x 10^6 cells / mL by daily addition of complete culture media. Post electroporation cells were recovered at 2.5 x 10^5 cells / mL in the same media cocktail as above but with IL-2 levels increased to 400 IU / mL.

Immortalized cell culture. Raji and MCF-7 cells were grown in cell culture media formed from RPMI (Raji) or DMEM (MCF-7), supplemented with 10% fetal bovine serum (FBS). Cells were grown to near confluency in complete media in T-25 flasks in an incubator at 37 °C with 5% CO_2. Prior to each experiment, cells were detached using a solution of trypsin (0.25% w/v) and EDTA (1 mM), centrifuged, then resuspended in complete media. Cell lines were passaged every 2-3 days by media removal, PBS wash, trypsinization (for adherent cells), resuspension in fresh culture media and split at a 1:10 ratio into fresh media. All immortalized cell lines were kept below passage 10.

Transfection protocols

triDrop Electroporation. Optimization and characterization of the triDrop platform and with different buffers for electroporation electrical parameters were tested in our previous manuscript.[1] Unless specified otherwise, 5 x 10^5 cells were centrifuged (300 g, 5 min), washed in PBS,

centrifuged, and resuspended in 10 µL of electroporation buffer with conductivity σ ~ 8 mS / cm (Neon Type T buffer containing 0.05% Pluronic F68 surfactant) and payload was added as described below. 7.5 µL of a buffer with conductivity σ ~ 16 mS / cm (PBS containing 0.05% Pluronic F68 surfactant) was pipetted into each of the device's flanking reservoirs and 7.5 µL of the cell solution was pipetted into the middle reservoir. DMF actuation was used to dispense three unit droplets, ~ 1 µL in volume, from each reservoir and actuate them to the three on-chip electroporation sites. Driving potentials (i.e. actuation) was used to merge the droplets into the triDrop configuration, and a pulse generation circuit was automatically triggered to deliver five, 550 V_{DC} pulses, 500 µs in duration to the droplet structure. After electroporation, cells were immediately removed from the chip via pipetting and transferred to recovery buffer. Cells from each of the three electroporation sites were loaded into their own well and served as technical triplicates.

Neon Electroporation. Neon electroporation was performed using manufacturer recommended protocols for the Neon Transfection System 10 µL kit (Thermo Fisher, Catalog # MPK 1025). For each unique condition, 4.4 x 10^5 cells were centrifuged (300 g, 5 min), washed in PBS, centrifuged again, and finally resuspended in 22 µL of type R buffer. 10 µL Neon tips were used a maximum of two times to serve as technical replicates. Three, 1600 V_{DC} pulses, 10 ms in duration were applied. Immediately, electroporation cells were transferred to recovery buffer.

To explore the effects of cell density on electroporation performance, 8 x 10^5 cells were washed in PBS and resuspended in 40 µL of Buffer R. The cell solution was diluted with fresh Buffer R in the following ratios, 5.5 µL: 16.5 µL and 11 µL: 11 µL, creating aliquots containing 1.1 x 10^5 cells and 2.2 x 10^5 cells respectively. These aliquots were used to perform two electroporation reactions each containing ~ 5 x 10^4 cells per reactions and ~ 1 x 10^5 cells per

reaction respectively. The remaining cell solution was used to perform two reactions with 2 x 10^5 cells per reaction (manufacturer recommended amount). All conditions were cultured at 5 x 10^4 cells per well for post-electroporation analysis.

Lonza Electroporation. Lonza electroporation was performed using manufacturer recommended protocols for the P3 Primary Cell 4D-Nucleofector X Kit S (Lonza, Canada, Catalog # V4XP-3032). For each condition, 1.1 x 10^6 cells were centrifuged (300 g, 5 min), washed in PBS, centrifuged, and resuspended in 22 µL of P3 buffer. 20 µL of cell solution was pipetted into the nucleofector cuvette and pulse code E0-115 was used for electroporation. Immediately following electroporation 100 µL of pre-warmed recovery buffer was added to each cuvette and the sample was incubated for 10 minutes prior to be transferred to a well plate.

To determine the effects of cell density on electroporation efficacy, 3 x 10^6 cells were washed in PBS and resuspended in 60 µL of P3 buffer. The cell solution was mixed with fresh P3 buffer in the following ratios, 2:38, 4:36, and 8:32, creating aliquots containing 1 x 10^5 cells, 2 x 10^5 cells, and 4 x 10^5 cells. These aliquots were each used to perform two electroporation reactions containing 5 x 10^4 cells per reaction, 1 x 10^5 cells per reaction or 2 x 10^5 cells per reactions respectively. The remaining cell solution was used to perform two reactions with 1 x 10^6 cells per reaction (manufacturer recommended amount). All conditions were cultured at 50,000 cells per well for post-electroporation analysis.

mRNA transfection. mRNA transfection was performed by preparing cells for electroporation using the techniques outlined above and adding mRNA immediately prior to electroporation. Unless specified otherwise, mRNA was added at 50 ng / µL for each system (50 ng per reaction for triDrop, 500 ng per reaction for Neon, and 1000 ng per reaction for Lonza). eGFP and anti-

CD19 CAR mRNA were stored at -80 ˚C in 5 µL aliquots with a stock concentration of 1 and 2 µg / µL respectively. The eGFP-mRNA sequence can be found in **Table S2**.

For experiments exploring the effects of mRNA concentration on electroporation efficacy, 100 ng, 50 ng, and 10 ng per reaction conditions were explored using the triDrop. Three tubes were prepared, each containing 5 x 10^5 cells resuspended in 10 µL of electroporation buffer as described above. 1 µL of stock eGFP-mRNA (1 µg / µL) was added to the first tube, 0.5 µL of stock solution was added to the second tube, and for the final tube 1 µL of stock mRNA was diluted with 9 µL of type T electroporation buffer and 1 µL of the dilution was added to the cell mixture. For Neon, 500 ng, 100 ng, and 50 ng reaction conditions were explored. Three tubes each containing 4.4 x 10^5 cells resuspended in 22 µL of electroporation buffer were prepared as outlined above. 1 µL of stock mRNA (1 µg / µL) was added to the first tube. Next, 1 µL of stock mRNA was diluted in 9 µL of type R electroporation buffer. 2 µL of this dilution was added to the second tube, and 1 µL was added to the third tube. Each tube was used for two electroporation reactions. For the Lonza, 500 ng, 100 ng, and 50 ng per reaction conditions were explored. Three tubes each containing 2.2 x 10^6 cells resuspended in 44 µL of electroporation buffer was prepared as outlined above. 1 µL of stock mRNA (1 µg / µL) was added to the first tube. Next, 1 µL of stock mRNA was diluted in 9 µL of P3 electroporation buffer. 2 µL of this dilution was added to the second tube, and 1 µL was added to the third tube. Each aliquot was used for two electroporation reactions.

mRNA coding for a second-generation anti-CD19 CAR molecule with a 41BB-CD3z coactivation domain was used. For each electroporation system, > 4 x 10^5 activated Pan T cells were electroporated with anti-CD19 CAR mRNA using either the manufacturer recommended conditions or by performing eight reactions using 0.5 x 10^5 cells each (1 x 10^6 cells were used for

the Nucelofector). Immediately post electroporation cells were incubated in 200 µL recovery buffer at 0.5 x 105 cells per well.

Gene Editing. TRAC knockouts were performed by complexing TrueCut Cas9 protein V2 (Thermo Fisher, Canada, catalog #: A36496) with an sgRNA cocktail designed by Synthego to target the TRAC locus (Synthego, SKU: 052-1020-000-1.5n-0). Cas9 proteins and gRNAs were kept at -20 °C at a stock concentration of 30 pmol / µL and 100 pmol / µL respectively. The amount of gRNA and Cas9 protein added to each sample was controlled by either normalizing to the number of cells per reaction or by normalizing to the volume of the reaction. For normalization to the number of cells per reaction, 50 pmol of Cas9 was mixed with 100 pmol of gRNA for every 1 million cells used. For normalization to reaction volume, 50 pmol of Cas9 was mixed with 100 pmol of gRNA for every 20 µL of reaction volume (equivalent to volume required for one Lonza nucleofection cuvette). After mixing of the Cas9 protein with the gRNA, the mixture was left at room temperature for 10 minutes to allow for the formation of the ribonucleic protein (RNP) complex and either immediately added to the cell mixture or kept on ice for no more than 30 minutes before being added to the cell mixture. Knock-out efficiency was analyzed 4-days post transfection.

CRISPR knock-ins were performed by using the protocol originally described by Cloarec-Ung et al..[2] crRNA (200 pmol / µL, IDT) was mixed with tracrRNA (200 pmol / µL, IDT) at a 1:1 ratio and annealed by heating at 95℃ for 5 minutes followed by cooling to room temperature at a rate of 0.1℃ / s in a thermocycler to form sgRNA at a concentration of 100 pmol / µL. The resulting sgRNA was then mixed with Cas9 (30 pmol / µL) at a 2:1 ratio and left to complex for 10 minutes at room temperature to form RNPs. A single stranded oligonucleotide (ssODN) HDR repair template (400 pmol / µL, IDT) was diluted 1:8 in electroporation buffer prior to

electroporation. p53 siRNA (100 pmol / µL, Thermo Fisher, Catalog # 4390824) was diluted 1:1000 in nuclease free water. For every 20 µL of reaction volume, 50 pmol of RNP, 50 pmol of ssODN, and 20 fmol of p53 siRNA was added to cells already in electroporation buffer immediately prior to electroporation. Following electroporation cells were recovered in the recovery buffer described above with the addition of 0.5 µM AZD7648 (Cayman Chemical, Item # 28598). Knock-in efficiency was analyzed 2-days post transfection.

Genetic Analysis

RT-qPCR. RT-qPCR was performed in accordance with the Minimum Information for Publication of Quantitative Real Team PCR Experiments (MIQE) guidelines[3] and associated data (including primer sequences, qPCR validation, and data analysis) can be found in **Figure S11** and **Table S5**. RT-qPCR was performed on the Eco Real Time PCR System (catalog # EC-101-1001) using the Eco study V5.0 software. Cells from two donors were electroporated in technical replicates with each system using the methods outlined above using either the manufacturer recommended conditions or 5 x 10^4 cells per reaction. Post electroporation all cells were cultured at 5 x 10^4 cells cells per well in 200 µL of recovery buffer (recipe above). 6 hours after electroporation cells were washed in PBS, lysed and prepared for RT-qPCR using Power SYBR green cells-to-Ct kit (Thermo Fisher, Catalog #4402954). PCR cycling conditions were as follows: 95℃, 10 min; 40x (95℃ 15s, 60℃ 1 min). 18s rRNA was used as a reference gene.[4,5] For qPCR validation, primer specificity was confirmed, and experiments included pilot runs with endogenous controls, verifying linear decreases in CT values proportional to cell numbers, alongside Xeno RNA controls to confirm the absence of RT-PCR inhibitors. Each qPCR measurement was conducted in triplicate to assess technical reproducibility.

RNAseq Sample Preparation. Transcriptomic sequencing was performed using the Oxford Nanopore minION Mk1B with flow cell R9.4.1. Cells from three donors were electroporated using the optimized or manufacturer (Neon and Nucleofector) recommended condition. Per donor, 1.5 x 10^5 cells were treated with the triDrop (three technical replicates of 0.5 x 10^5). 6 x 10^5 were treated with the Neon (three technical replicates of 2 x 10^5), and 3 x 10^6 were treated with the nucleofector (three technical replicates of 1 x 10^6). All cells post-electroporation were cultured at 5 x 10^4 cells per well in 200 µL of recovery buffer. Six hours post electroporation cells were washed in PBS, lysed, and RNA was extracted using RNeasy Mini Kit (Qiagen, catalog #74104). As per manufacturer's recommendations, 200 ng of total RNA was used from each condition as an input to the kit with an RNA integrity number > 10. The RNA library was prepared using the PCR-cDNA Barcoding Kit (Oxford Nanopore, catalog #SQK-PCB111.24) following the manufacturer's protocol. Briefly, by taking full-length polyadenylated RNA, complementary strand synthesis and strand switching were performed using kit-supplied oligonucleotides. The kit contained 24 primer pairs, 12 of which were used to generate and then amplify double-stranded cDNA by PCR amplification using rapid attachment barcode primers with the following thermal cycle: 95℃ 30s; 18x (95℃ 15s, 62℃ 15s, 65℃ 120s), 65℃ 360s, 4℃ hold. These primers contained 5' tags and facilitated the ligase-free attachment of Rapid Sequencing Adapters and their sequences are listed in the **Table S2**. 1 µL of amplified cDNA from each condition was analyzed using a 4200 Tapestation System (Agilent) prior to sequencing to confirm sample quality and a concentration of at least 1000 pg / µL. Amplified and barcoded samples were then pooled together, and Rapid Sequencing Adapters were added to the pooled mix. The final pooled library loaded on to the flow cell contained 2 fmol of cDNA from each barcoded condition for a total of 24 fmol.

Thorough quality control was conducted during RNA extraction and cDNA library preparation. RNA quality was verified by Agilent Tapestation 4200, ensuring RNA integrity numbers (RIN) greater than 10 and A260/280 ratios between 1.8–2.2. For RNA sequencing, each donor sample was independently barcoded, prepared, and pooled. Sequencing was performed with adequate read depth (~18 million reads per sample) to ensure robust differential gene expression analysis. Gene expression data were analyzed using DESeq2 with rigorous criteria (log fold change > 1 or < -1, and p-value < 0.05) to identify significant gene expression changes. The Reactome database was used to identify genetic pathways. Genes were verified to ensure that unique genes were present in highlighted pathways and pathways with overlap were grouped together accordingly.

Flow Cytometry. Flow cytometry was performed using the BD FACS Melody (BD Bioscience, Canada). The FACS was equipped with three excitation lasers (405 nm, 488 nm, 561 nm) in a 2B-2V-4YG configuration. TRAC knockouts were detected via staining with anti-human alpha/beta TCR FITC (Thermo Fisher Scientific, catalog #11-9955-42). Anti-CD19 CAR expression was detected using FITC-labelled human CD19 protein (Acro Biosystems, catalog # CD9-HF251-25ug). CD3 expression was detected using PE-CD3 monoclonal antibody (Thermo Fisher Scientific, catalog #12-0038-42). CD19 expression was detected using a FITC Mouse anti-Human CD19 antibody (BD Bioscience, catalog #560994). All staining was performed in accordance with manufacturer guidelines, cells were incubated with the appropriate stains for 30 – 60 minutes at 4 $^{\circ}$C followed by three PBS wash steps to remove any unbound antibodies. For all samples, viability was assessed by staining dead cells using DAPI (50 ng / µL) added to the sample immediately prior to FACS and mixed thoroughly with the sample by pipetting. Lymphocytes were separated

from debris using forward scatter vs side scatter dot plots and singlets were separated from doublets by plotting side scatter – pulse width vs side scatter – pulse height.

DMF 'tri-drop' device fabrication

triDrop devices were composed of a top plate and a bottom plate. The bottom plate consisted of chromium electrodes divided into an array of 30 actuation electrodes (2 mm by 2 mm), 12 reservoir electrodes (2.9 mm by 5.5 mm) arranged into 3 reservoirs, 6 active dispensing electrodes (2 mm by 2 mm), and 3 splitting electrodes (3.8 mm x 3 mm). All electrodes were separated by a 150 µm gap. Bottom plates were coated in an SU8-5 dielectric layer (~ 5 µm thickness) and further coated in Teflon-AF 1600 in 2 % w/w in Fluorinert FC-40 to serve as a hydrophobic layer.

TriDrop top plates bearing gold electrodes (0.2 mm wide) were formed from a glass substrate coated with 100 nm gold adhered to seed chromium layer (~12 nm). To form the gold electrodes, top plates were spin-coated (10 s 500 rpm, 30 s 3000 rpm, 20 s 5000 rpm) in S1811, exposed through a transparent mask, developed using Microposit MF321 (2 min), washed with DI water, submerged in gold etchant (2 min), washed with DI water, and submerged in AZ stripper to remove the remaining photoresist before being washed with acetone, IPA, and DI water, and dried with nitrogen. To disconnect the chromium from the gold wiring, we followed the above protocol except using CR-4 etchant to remove the chromium. To insulate the gold electroporation electrodes from the Cr-grounding layer, the top plate was surface treated for 45 s in a plasma cleaner (Harrick Plasma PDC-001, Ithaca, NY) before coating a 5 µm dielectric of SU8-5. Briefly, the photoresist was spin-coated (10 s 500 rpm, 30 s 2500 rpm), followed by a soft bake (65 °C 2 min, 95 °C, 10 min), exposed to UV light through a custom mask (5 s), post-exposure baked (65 °C 2 min, 95 °C 10 min), developed in SU8 developer (15 s), rinsed with IPA and DI water, dried

with nitrogen, and then hard baked (180 °C, 10 min). Top plates were spin-coated with Teflon-AF 1600 in 2 % w/w in Fluorinert FC-40 (10 s 500rpm, 30s 1500rpm). To assemble the completed triDrop device, the top and bottom plates were assembled using two layers of double-sided tape (180 μm total thickness, 3M) and the gold electrode on the top plate were aligned directly above electroporation sites on the bottom plate.

DMF 'tri-drop' device assembly

The bottom plate of the triDrop device was placed on a pogo pin holder that has been propped to a height 20 cm above the benchtop using a chassis constructed from T-slotted aluminum extrusions purchased from McMaster-Carr (catalog #: 47065T101, Aurora, OH) and machined and assembled in-house. The system is connected to a 720 pixel, 30 frames-per-second camera (Skybasic, Houston TX.) to visualize droplet movements on the device. A 12-input card edge connector from Digikey (catalog #: 151-1410-ND, Thief River Falls, MI), was attached to the top plate of the triDrop device and connected via three leads (DMF ground, High Voltage DC, DC ground). The two DC leads were connected to an electroporation pulse circuit and one lead was used to provide the electrical connection for the DMF ground. The electroporation circuit consisted of an 8 pin optocoupler (Model #: AQW216EH) purchased from Digikey was connected to a Z650-0.32-U DC power source (TDK-Lambda) and controlled by an Arduino Uno running a custom pulse generating program, creating custom pulses of varying amplitudes and durations (100 - 600 V_{DC}, 0.2-10 ms in duration).

DMF 'tri-drop' device operation

For automating droplet movement on the device, see our previous published work for circuit and connectivity.[6] The electroporation and DMF actuation circuit were controlled by our in-house software which is available on our bitbucket registry: (https://bitbucket.org/shihmicrolab/littleleung_2023). Droplet movements were programmed by application of AC potentials (300 – 400 V_{RMS}) at 15 kHz between the top and bottom plates. The DMF actuation software was also used to initiate the electroporation pulse circuitry (**Figure S3**) to ensure immediate and uniform pulse application after triDrop merging.

Supplementary Tables

Table S1: Cost of Genetically Engineering Primary Human T cells

Process	Material	Material Cost (CAD)	Nucleofector		Neon		triDrop	
			Amount Needed per reaction	Cost (CAD)	Amount Needed per reaction	Cost (CAD)	Amount Needed per reaction	Cost (CAD)
Pan T Cell Isolation	Leukopack	4757 (~ 5 x 10^9 cells)	1 x 10^6 cells (20 µL)	0.95	2 x 10^5 cells (10 µL)	0.19	5 x 10^4 cells (1 µL)	0.05
	Isolation Kit	979 (1 x 10^9 cells)	1 x 10^6 cells	0.98	2 x 10^5 cells	0.2	5 x 10^4 cells	0.05
Cell Culture	Media + Serum w/ cytokines	85 (500 mL)	1 mL	0.17	0.2 mL	0.03	0.05 mL	<0.01
	Activation Beads	1195 (2 mL)	0.025 mL	15	0.005 mL	3	0.00125 mL	0.75
Gene Editing	Cas9 Nuclease	1294 (500 µg/3nmol)	50 pmol	21.50	10 pmol	4.32	2.5 pmol	1.08
	sgRNA	95 (1.5 nmol)	100 pmol	6.33	20 pmol	1.24	5 pmol	0.31
TOTAL COST FOR 1 REACTION			**$44.90**		**$8.98**		**$2.25**	

NOTE: Reagent costs are validated as of June 2024 and may change with bulk purchasing or institutional pricing. It is assumed that ~50% of cells in a Leukopak are pan T cells. Costs will change depending on what cell line is being engineered. Leukopak – StemCell Technologies (catalog # 70500); Isolation Kit – StemCell Technologies (catalog # 17951); serum – Thermo Fisher (catalog # A5670701); cytokines – Thermo Fisher (catalog #200-02-1MG); activation beads – Thermo Fisher (catalog #11161D); Cas9 Nuclease - Thermo Fisher (catalog #A36496); gRNA – Synthego (SKU: SKU: 052-1020-000-1.5n-0)

Table S2: Important Genetic Sequences

Name	Sequence
eGFP mRNA	AUGGUGAGCAAGGGCGAGGAGCUGUUCACCGGGGUGGUGCCCAUCCUGGUC GAGCUGGACGGCGACGUAAACGGCCACAAGUUCAGCGUGUCCGGCGAGGGC GAGGGCGAUGCCACCUACGGCAAGCUGACCCUGAAGUUCAUCUGCACCACCG GCAAGCUGCCCGUGCCCUGGCCCACCCUCGUGACCACCCUGACCUACGGCGU GCAGUGCUUCAGCCGCUACCCCGACCACAUGAAGCAGCACGACUUCUUCAAG UCCGCCAUGCCCGAAGGCUACGUCCAGGAGCGCACCAUCUUCUUCAAGGACG ACGGCAACUACAAGACCCGCGCCGAGGUGAAGUUCGAGGGCGACACCCUGG UGAACCGCAUCGAGCUGAAGGGCAUCGACUUCAAGGAGGACGGCAACAUCC UGGGGCACAAGCUGGAGUACAACUACAACAGCCACAACGUCUAUAUCAUGG CCGACAAGCAGAAGAACGGCAUCAAGGUGAACUUCAAGAUCCGCCACAACA UCGAGGACGGCAGCGUGCAGCUCGCCGACCACUACCAGCAGAACACCCCCAU CGGCGACGGCCCCGUGCUGCUGCCCGACAACCACUACCUGAGCACCCAGUCC GCCCUGAGCAAAGACCCCAACGAGAAGCGCGAUCACAUGGUCCUGCUGGAG UUCGUGACCGCCGCCGGGAUCACUCUCGGCAUGGACGAGCUGUACAAGUAA
SRSF2 gRNA	/AITR1/ CGGCGUGUGGUGAGUCCGGGGUUUUAGAGCUAUGC /AITR2/
ssODN HDR template	T*G*GACGGCCGCGAGCTGCGGGTGCAAATGGCGCGCTACGGCCGCCCTCCAG ATTCACACCACAGCCGCCGGGGACCGCCACCCCGCAG*G*T
Pri077 F	AGCGATATAAACGGGCGCAG
Pri077 R	TCGCGACCTGGATTTGGATT
Pri0003-A1	T*C*GGCGACGTGTACATCC
Barcode Flanking Sequence (Top Strand)	5' - ATCGCCTACCGTGA - barcode - TTGCCTGTCGCTCTATCTTC - 3'
Barcode Flanking Sequence (Bottom Strand)	5' - ATCGCCTACCGTGA - barcode - TCTGTTGGTGCTGATATTGC - 3'
Barcode 01	AAGAAAGTTGTCGGTGTCTTTGTG
Barcode 02	TCGATTCCGTTTGTAGTCGTCTGT
Barcode 03	GAGTCTTGTGTCCCAGTTACCAGG
Barcode 04	TTCGGATTCTATCGTGTTTCCCTA
Barcode 05	CTTGTCCAGGGTTTGTGTAACCTT
Barcode 06	TTCTCGCAAAGGCAGAAAGTAGTC
Barcode 07	GTGTTACCGTGGGAATGAATCCTT
Barcode 08	TTCAGGGAACAAACCAAGTTACGT
Barcode 09	AACTAGGCACAGCGAGTCTTGGTT
Barcode 10	AAGCGTTGAAACCTTTGTCCTCTC
Barcode 11	GTTTCATCTATCGGAGGGAATGGA
Barcode 12	CAGGTAGAAAGAAGCAGAATCGGA

Table S3: Mutually Dysregulated Genes (p < 0.05)

Gene Symbol	Gene Name	Ensembl Gene ID	Fold Change			Putative Function
			triDrop	Neon	Nucleofection	
ACBD3	acyl-CoA binding domain containing protein 3	ENSG00000182827	3.71228801	3.77293021	4.26957006	Involved in the maintenance of Golgi structure and function
MT1M	Metallothionein 1M	ENSG00000205364	2.87867827	2.77319454	3.70987145	Member of the metallothionein superfamily
SLC7A11	Solute carrier family 7 member 11	ENSG00000151012	4.36751455	4.49961256	3.7043796	Implicated in ferroptosis[7]
PMCH	Pro-Melanin Concentrating Hormone	ENSG00000183395	1.59762742	2.38135295	2.6530848	Expression in immune cells may inhibit proliferation[8]
CRIP1	Cysteine rich protein 1	ENSG00000213145	1.17007127	1.3629836	1.80771559	LIM/double zinc finger protein family
RPL38P4	Ribosomal Protein L38 Pseudogene 4	ENSG00000250562	1.19873041	1.38231401	1.49196731	Pseudogene

Table S4: Reactome Pathway Analysis

Reactome		Z-scores		
Pathway ID	**Brief description**	**Nucelofection**	**triDrop**	**Neon**
R-HSA-5661231	Metallothioneins bind metals	7.857664356	3.423042716	9.99969
R-HSA-5660526	Response to metal ions	6.395220981	2.653043923	7.833048
R-HSA-2028269	regulation of cell proliferation and apoptosis	4.41568806	1.176575666	4.359968
R-HSA-9614657	transcription of cell death genes	2.637279679	0.401525923	2.575192
R-HSA-9635465	suppression of apoptosis	2.544866109	1.800080837	2.624122
R-HSA-111458	formation of apoptosome	2.210876005	1.340514592	2.419572
R-HSA-9818027	cytoprotective genes	3.355447126	2.238439352	3.910091
R-HSA-9818035	endoplasmic reticulum stress associated genes	3.99123084	-0.123698357	2.488199
R-HSA-9648895	integrated stress response phosphorylation	3.915343024	1.7811285	4.282006
R-HSA-111995	lipid mediators involved in inflammatory responses	7.810607731	4.059175677	8.231199
R-HSA-9818026	inflammation associated genes	3.683541285	-0.287309368	2.024568
R-HSA-75876	resolution of inflammatory response	1.643061815	0.390802551	4.152934

Table S5: qPCR Primer Sequences

Gene Symbol	Gene Name	Ensemble ID	Primer sequence	PCR product (bp)	Product Melting Point (°C)
18srRNA	18s ribosomal RNA	NCBI ID: 106631781	**F:** 5'-CTC AAC ACG GGA AAC CTCAC-3' **R:** 5'-CGC TCC ACCAAC TAA GAACG-3'	110	82.3
IL-2	Interleukin-2	ENSG00000109 471	**F:** 5'-CACAGCTACAACTGGAGCATTTAC -3' **R:** 5'-TTCAGTTCTGTGGCCTTCTTGG-3'	133	76.6
IFNG	Interferon gamma	ENSG00000111 537	**F:** 5'-GAGTGTGGAGACCATCAAGGA -3' **R:** 5'-GGACATTCAAGTCAGTTACCGAA-3'	113	78.1
TNFα	Tumor necrosis factor	ENSG00000232 810	**F:** 5'- AACCTCCTCTCTGCCATCAA -3' **R:** 5'-GGAAGACCCCTCCCAGATAG -3'	100	84

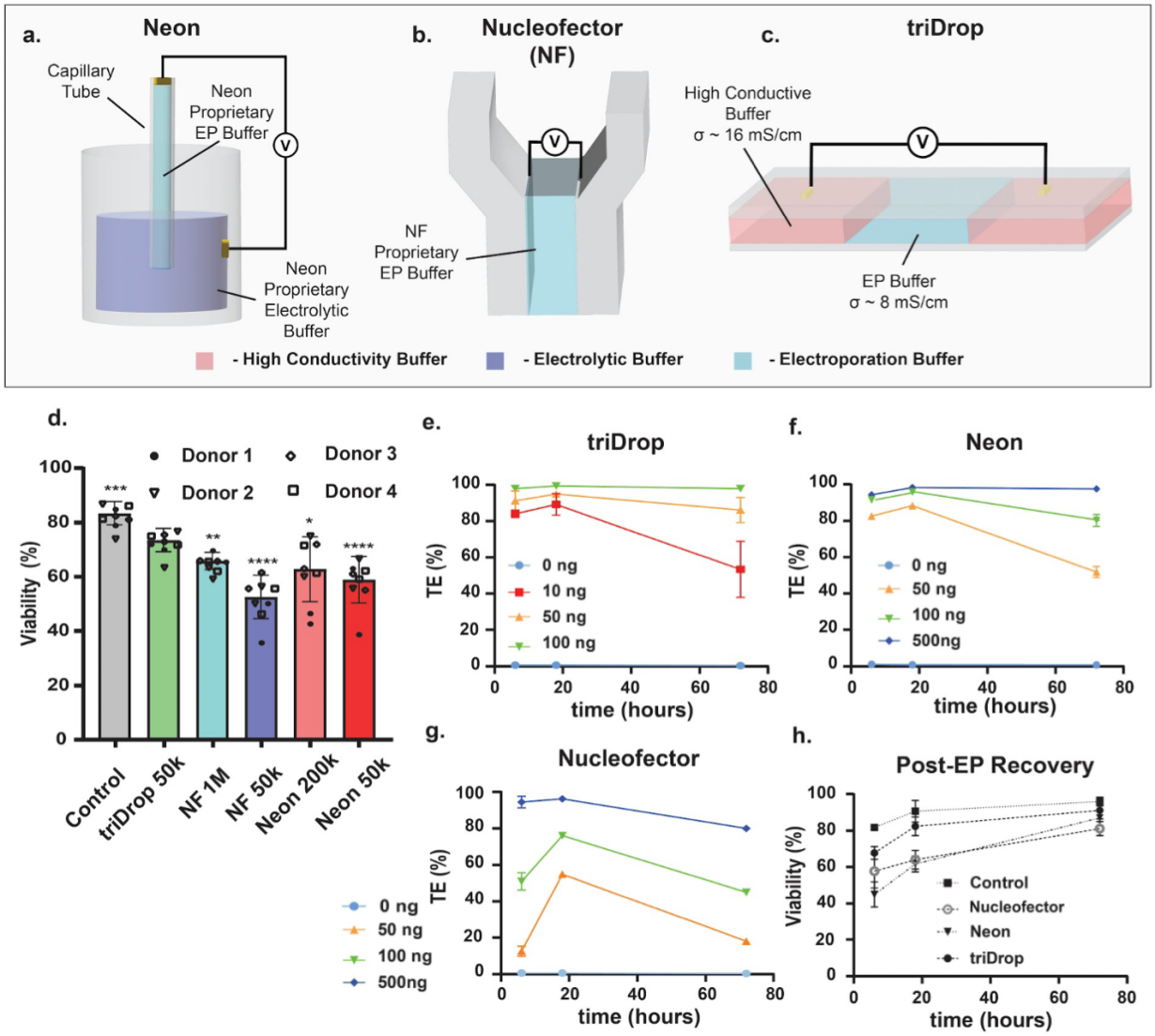

Figure S1: Schematic overview of three electroporation systems used in this work. a) Neon, b) Nucleofector, and c) triDrop. d) Viability measurements taken six hours post-EP for cells electroporated using either the manufacturer recommended number of cells or 0.5 x 10^5 cells per reaction. Four donors (shown as polygons) were used for this study with two technical replicates. Statistical significance markers represent comparison between triDrop and all other conditions (n = 8). Line graphs depicting transfection efficiency over 72 hours post-EP when using 10 ng (red line), 50 ng (orange line), 100 ng (green line) and 500 ng (blue line) of mRNA for e) triDrop, f) Neon, g) Nucleofector. h) Line graph depicting viability over 72 hours post-EP for cells electroporated with all three EP systems and a control. All error bars represent mean 1 SD. Statistical n.s indicates no significant difference, *, **,***, and **** represent p-values below 0.05, 0.01, 0.001 and 0.0001 respectively. Statistical analysis was performed using a Student's t-test.

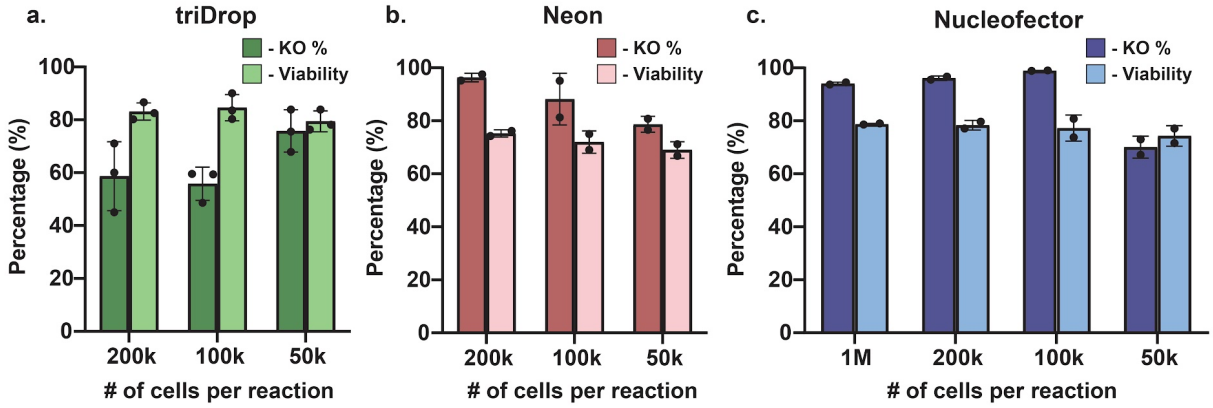

Figure S2: CRISPR knockouts in primary T cells. Bar graphs depicting knockout efficiency (dark colours) and viability (light colours) measured four days post EP when electroporating varying amounts of cells per reaction when using a) triDrop, b) Neon, and c) Nucleofector. Bar graphs showing d) knockout efficiency and e) viability for 0.5 x 10^5 cells electroporated with 2.5 pmol of RNP using the three EP systems (n = 4 - 6).

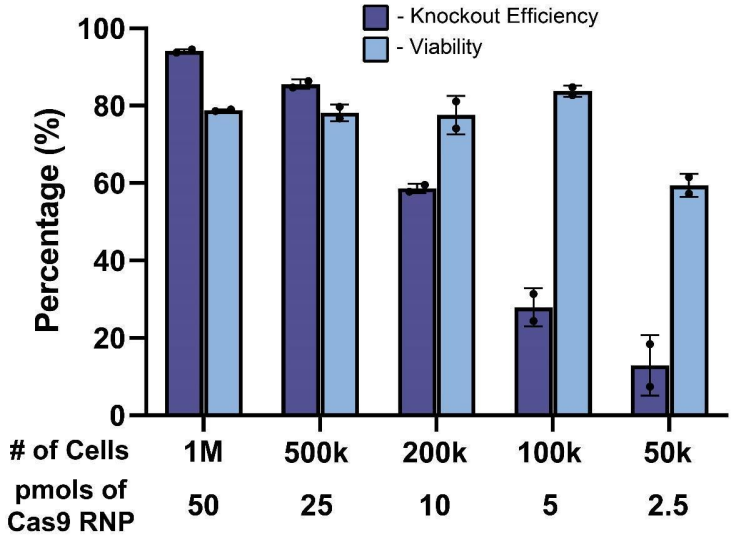

Figure S3. Effect of payload and cell count on viability and gene knockout using the Nucleofector. Bar graphs showing knockout efficiency (dark blue) and viability (light blue) measured four days post-EP for cells engineered with the Nucleofector. The number of cells and amount payload are indicated under each set of bars. All error bars represent mean +/- 1 SD.

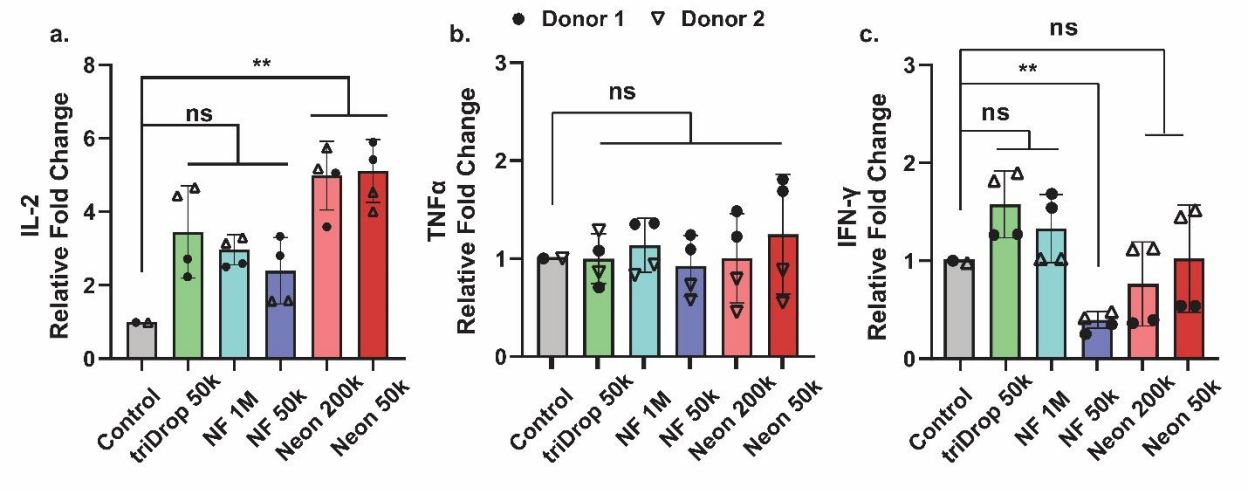

Figure S4. qPCR examination of key genes for cellular therapies. Bar graphs showing relative gene expression ($2^{-\Delta\Delta C_t}$) 6 hours post EP for two donors electroporated using manufacturer recommended number of cells or 0.5 x 10^5 cells per reaction for a) IL-2, b) TNF-α, and c) IFN-γ. All error bars represent mean +/- 1 SD. Significance n.s indicates no significant difference, and ** represents p-values below 0.01. Statistical analysis was performed using a Student's t-test. (n = 4).

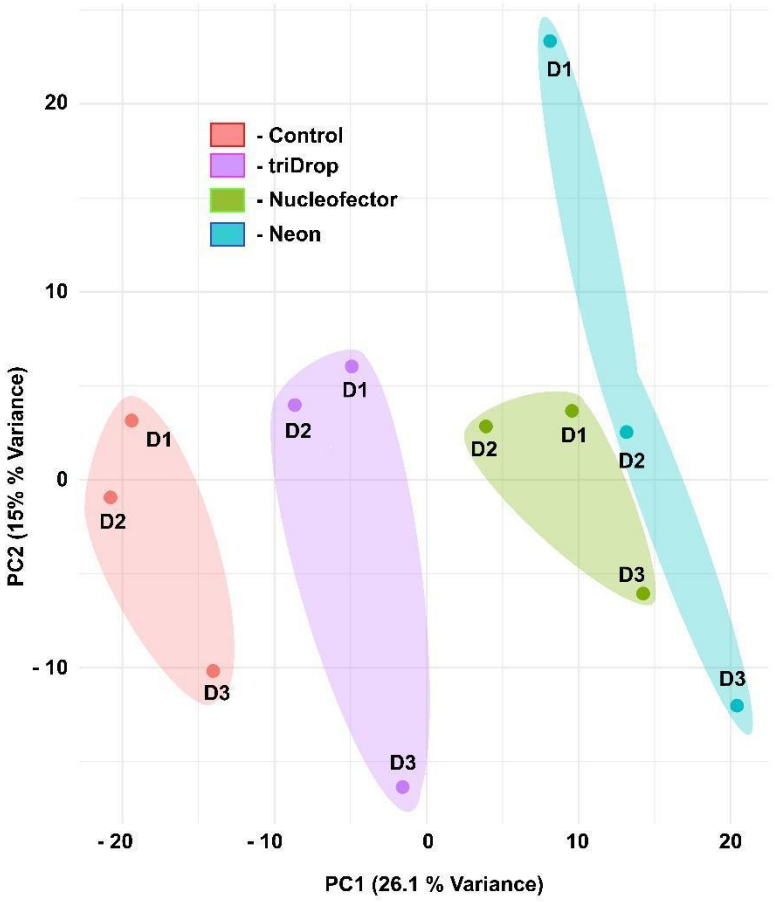

Figure S5. RNAseq data. Principal component analysis performed across all genes for three donors electroporated with the three EP systems. Control (red cluster), triDrop (purple cluster), Nucleofector (green cluster) and Neon (blue cluster) are all shown. Cluster proximity indicates increased similarity between samples meaning the triDrop is most similar to the control six hours post EP. Graph was generated using DESeq2.

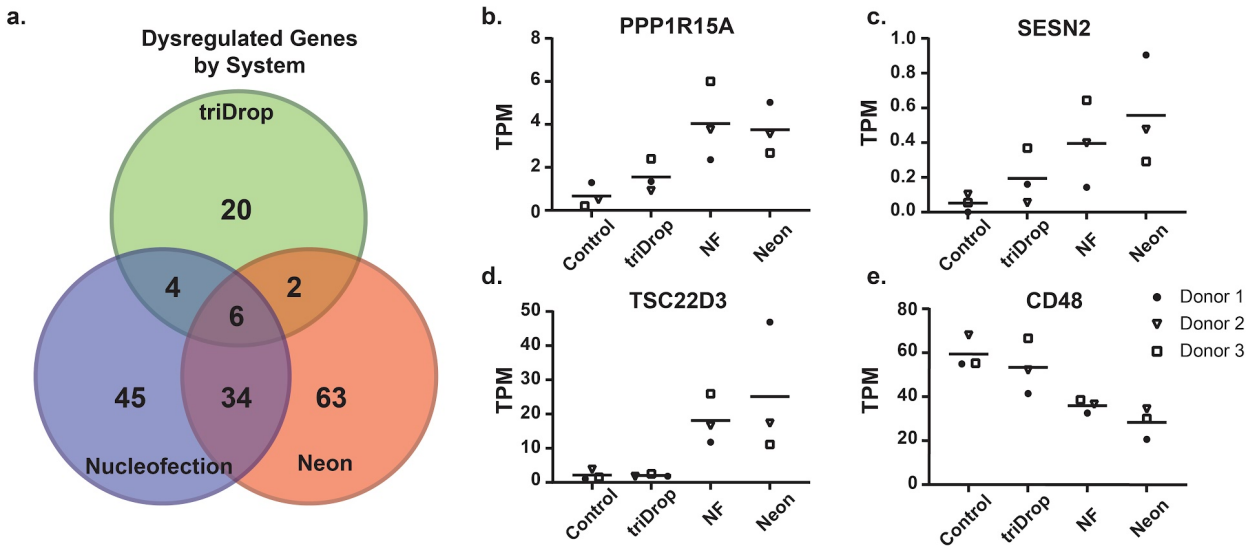

Figure S6: Transcriptomic Analysis. a) Venn diagram showing the number of genes that are uniquely dysregulated or mutually dysregulated between the three EP systems. Transcripts per million (TPM) values for three donors comparing the control (no electroporation), triDrop, Nucleofector, and Neon for b) PPP1R15A, c) SESN2, d) TSC22D3, and e) CD48.

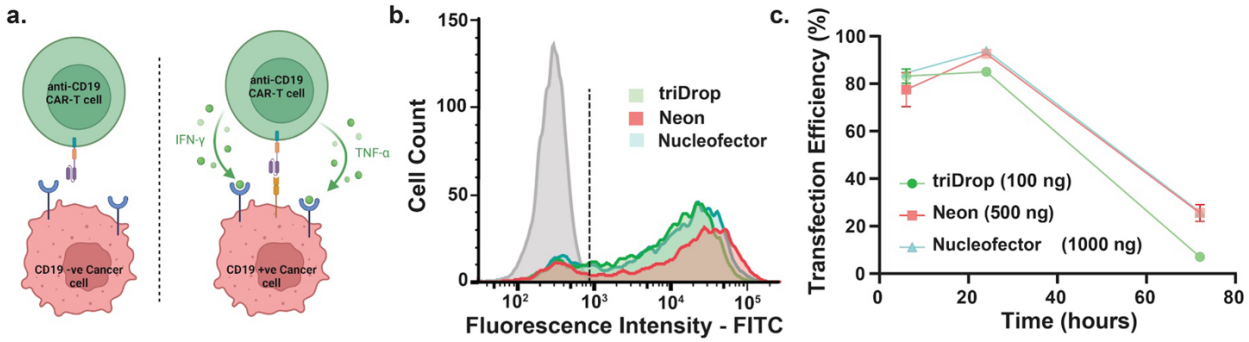

Figure S7: Functional CAR-T assay. a) Overview of immunotherapeutic testing assay detailing an anti-CD19 CAR T cell interacting with a CD19 negative or positive tumor cell line. Created with BioRender.com. b) Fluorescence intensity histograms showing FITC expression for cells stained with a FITC-tagged CD19 protein from a non-electroporation control (grey), triDrop (green), Nucleofector (blue), and Neon (red). c) Line graphs depicting expression of an anti-CD19 CAR molecule at 6, 24, and 72 hours post EP when using the triDrop (green, 100 ng of mRNA), Neon (red, 500 ng of mRNA), and Nucleofector (blue, 1000 ng of mRNA).

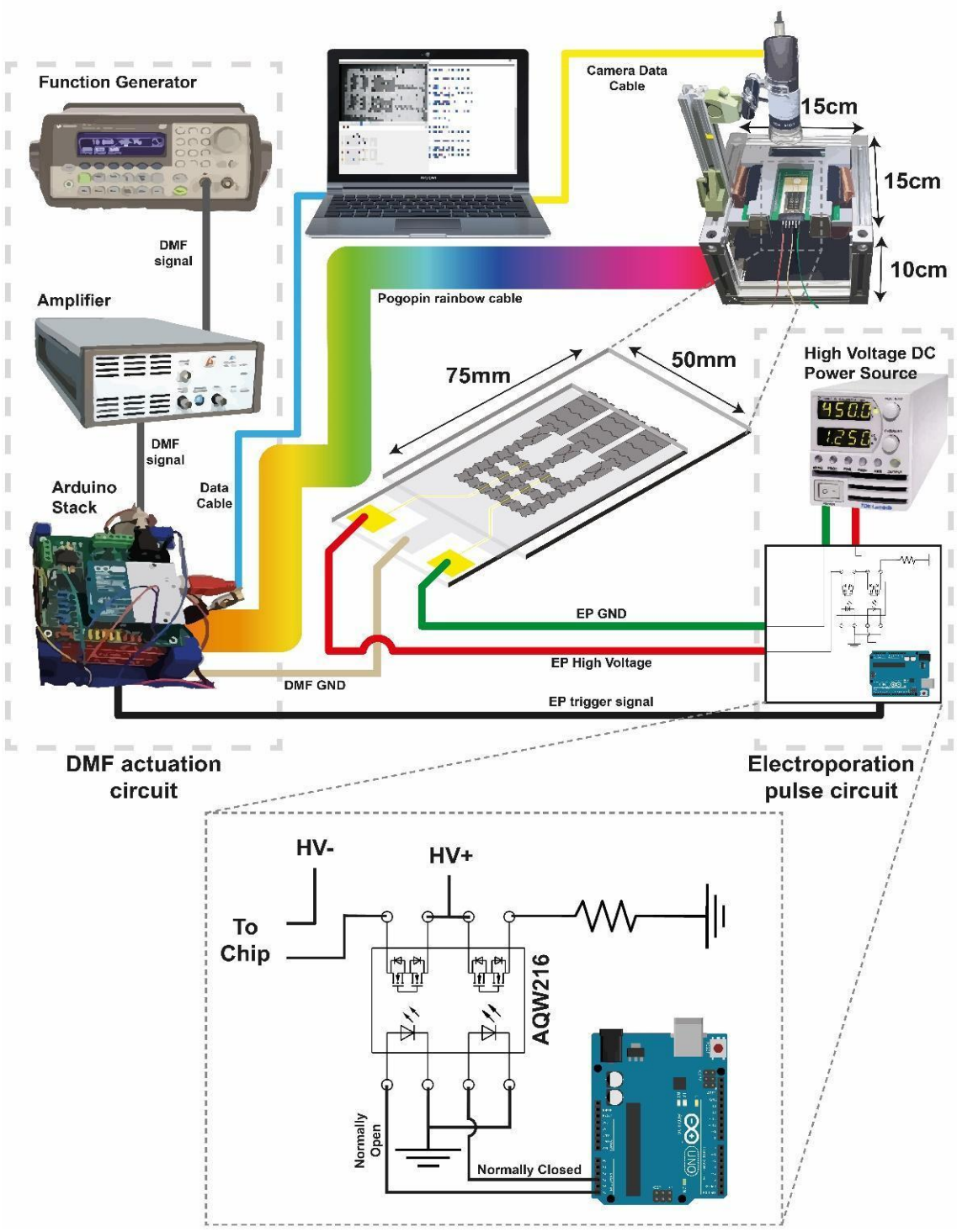

Figure S8. DMF and electroporation circuit diagram. Schematic overview of the complete triDrop automation setup detailing the DMF actuation hardware, automated electroporation pulse generation circuit, and chip holder.

Wild Type Sequence

tggacggccgcgagctgcgggtgcaaatggcgcgctacggccgccccccggactcacaccacagccgccggggaccgccaccccgcaggt

ssODN HDR Template Sequence

tggacggccgcgagctgcgggtgcaaatggcgcgctacggccgcccTccAgaTtcacaccacagccgccggggaccgccaccccgcaggt

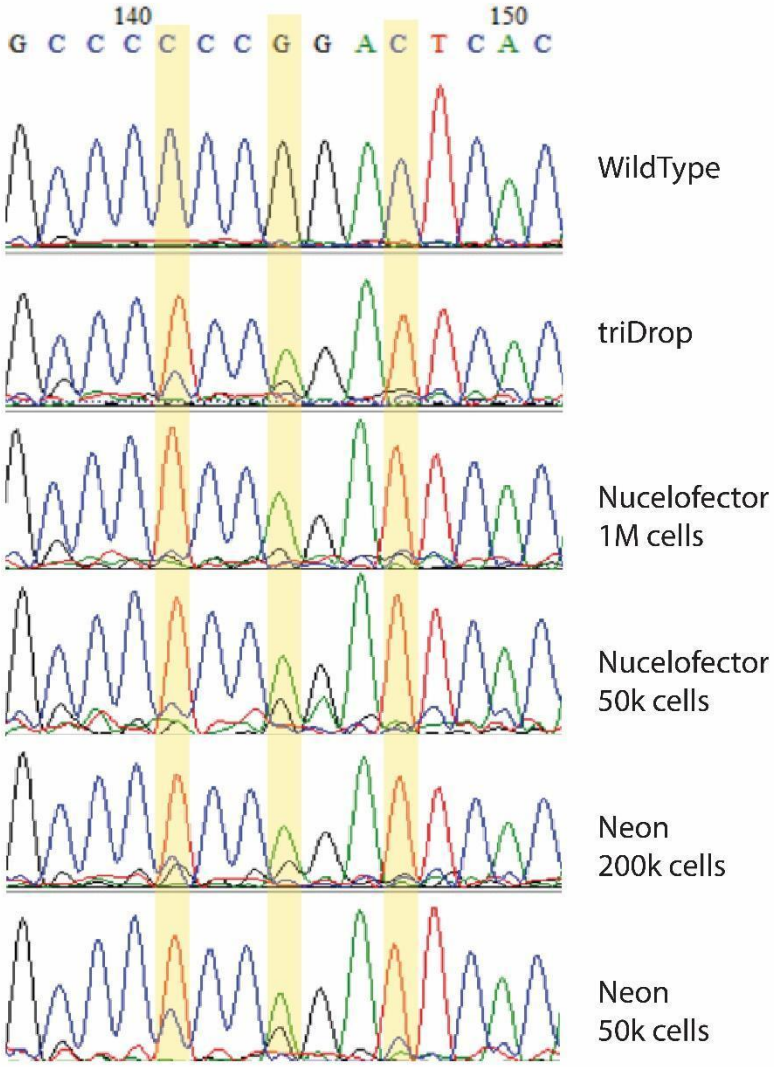

Figure S9. Sanger Sequencing. Wild type sequence and ssODN HDR template sequence with difference between the sequences highlighted. Chromatograms produced via Sanger for cells electroporated with all three EP systems using varying amounts of cells per reaction.

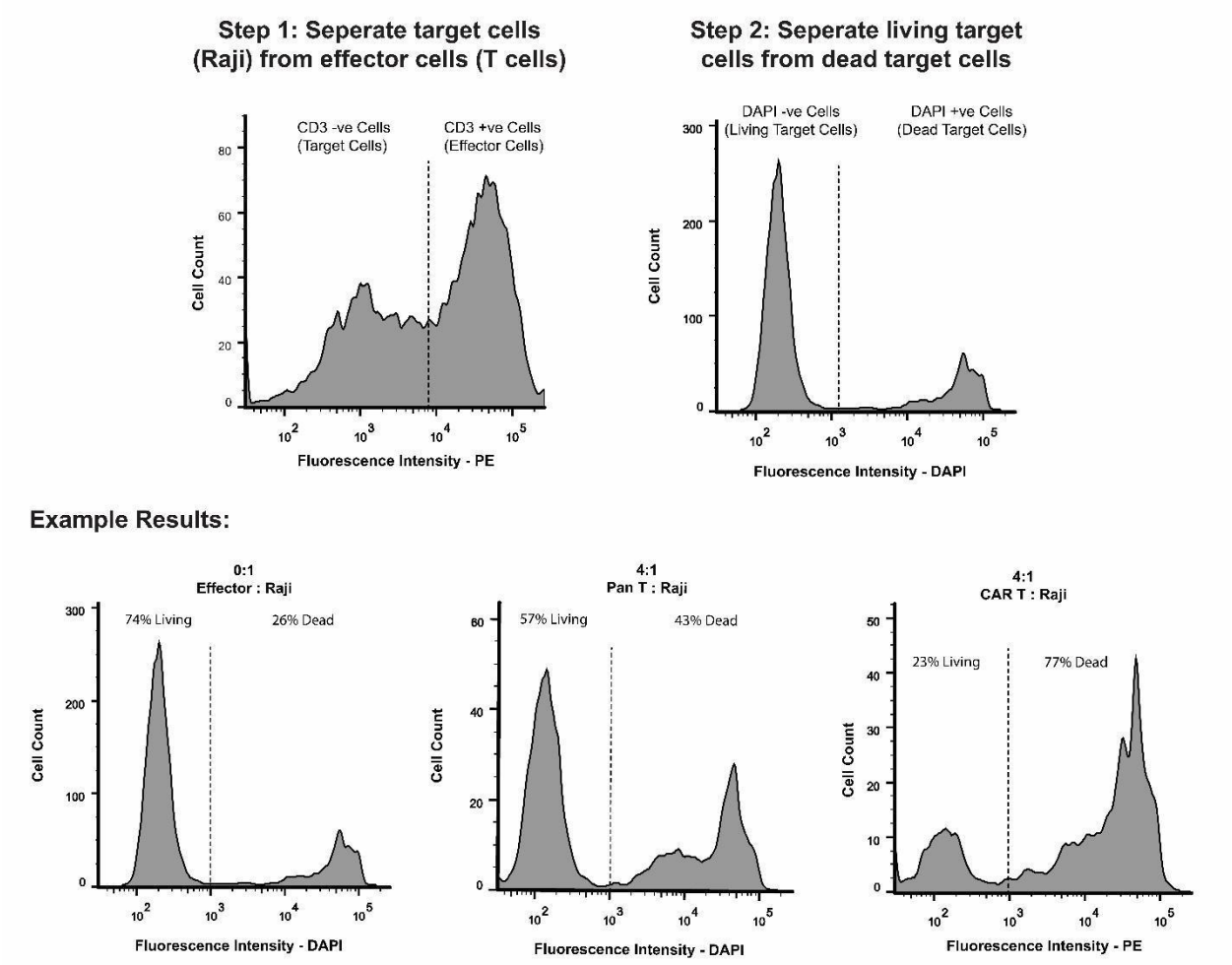

Figure S10. CAR killing-assay flow cytometry pipeline. Flow cytometry gating pipeline for target cell killing analysis. Cells are stained with CD3-PE (Phycoerythrin) antibody and DAPI prior to flow cytometry. Histogram showing PE fluorescence intensity is generated for all lymphocytes showing two peaks allowing for effector and target cells to be differentiated. CD3 negative target cells (i.e. PE negative) are isolated and a new histogram is generated showing DAPI fluorescence intensity allowing for the differentiation of living and dead cells. Sample live-dead plots are shown for control Raji cells, and Raji cells cultured for 24 hours at a 4:1 ratio with activated pan T cells and triDrop engineered CAR T cells.

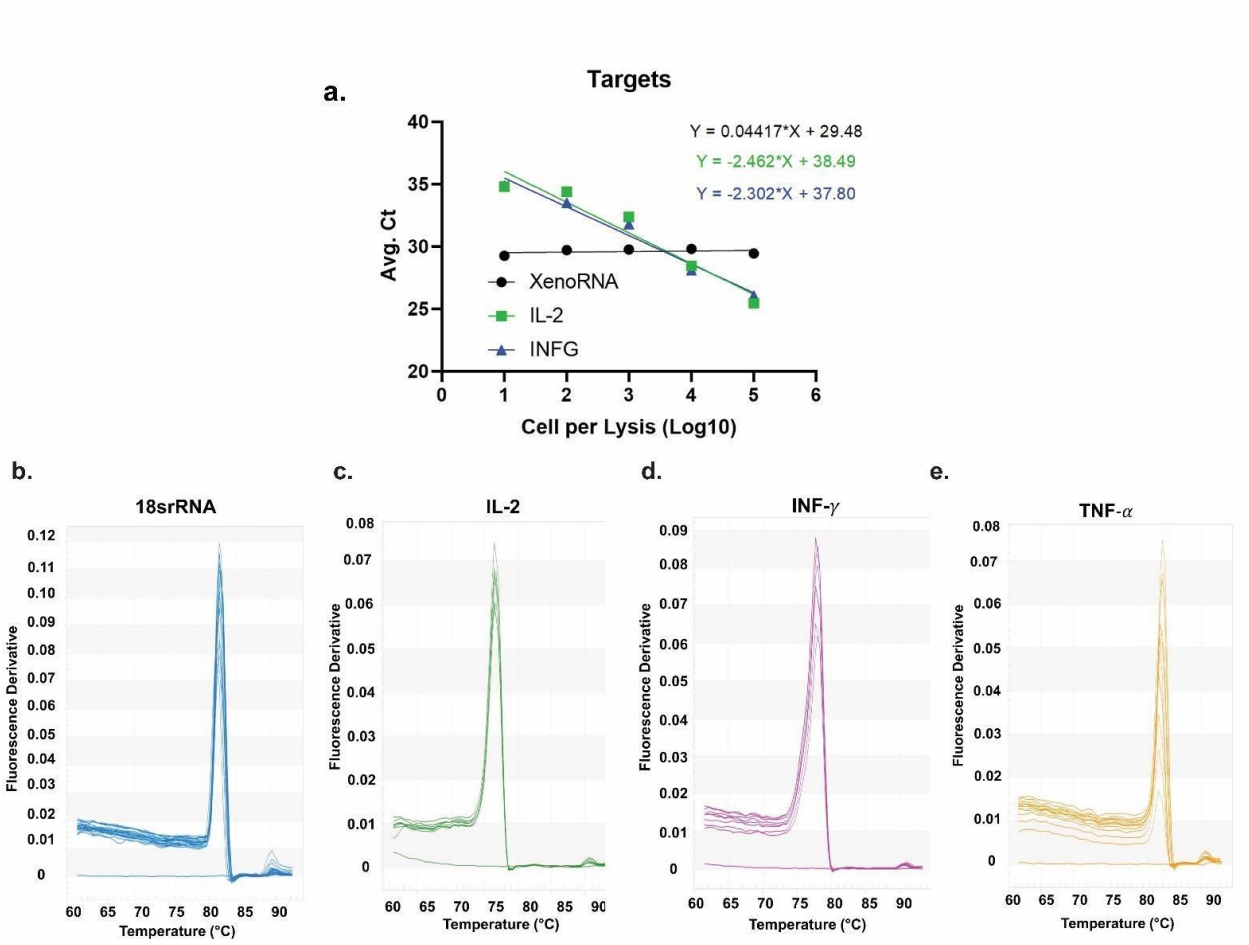

Figure S11. qPCR raw data. a) Standard curve depicting relationship between threshold cycle and number of lysed cells. Melting curves for b) 18srRNA, c) IL-2, d) IFN-γ, e) TNF-α showing product melting point is consistent with that predicted in **Table S5.**

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