Cell Therapy Manufacturing at Full Clinical Scale: Enhancing the Quality CAR-T Cell Therapy Starting Materials Through Massively Parallel Automated Microfluidic Cell Sorting

Alison M. Skelley*[1,2], Yasna Behmardi[1], Luke Peterson[1], David W. Inglis[3], Mabel Shehada[1], Laurissa Ouaguia[1], Khushroo Gandhi[1], Roberto Campos-González[1], Tony Ward[1]

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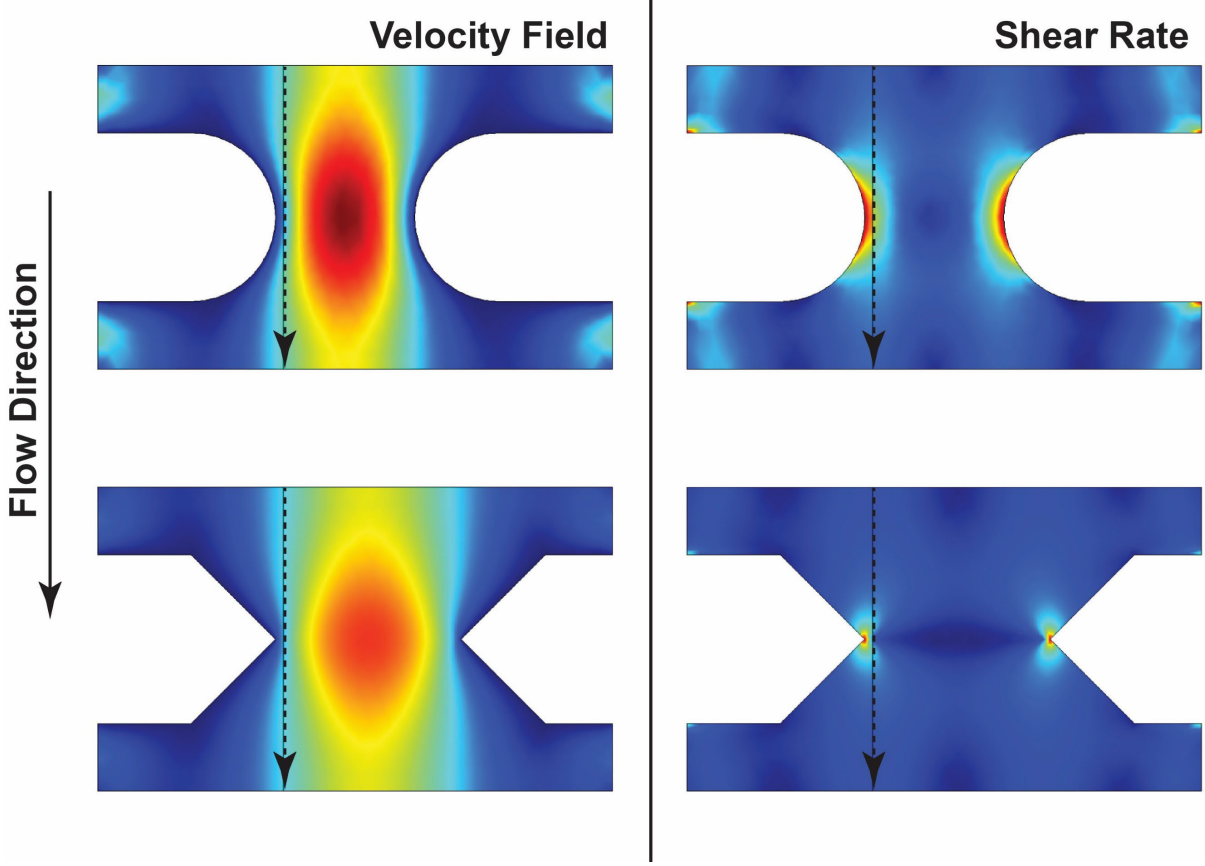
SUPPLEMENTAL INFORMATION

Shear stress analysis

The array geometry featured hexagonal microposts with pointed vertices to reduce the overall shear stress. One aspect of this strategy is that, while the shear rate at the corner is high, the cells experienced that shear rate for a short duration. We estimated the peak and average shear by simulating a 2D version of the DLD unit cell. We simulated the spatially averaged shear rate along a vertical line, 1 um from each obstacle. The gaps are set at 22 um for the hexagons (pointed vertex) and 16.5 um for the rounded obstacles (per Loutherback *et al.*[1], a 25% decrease in Dc is obtained by changing the obstacles from large rounded pillars to pillars with vertices facing the gap; therefore a 25% smaller gap between circular obstacles is required to achieve a comparable Dc to the geometry used here). The simulation was performed on COMSOL 4.4 using periodic boundary conditions and pressure controlled boundaries. Pressures were set to produce flow rates that matched system operation of 3.1 uL/min in each column of fluid. The spatially averaged shear rate was simulated to be 36% lower with the hexagon microposts than with the circular microposts, 4700 s^{-1} vs. 7400 s^{-1} respectively. The peak shear at the vertex in simulation was 20900 s^{-1}, but this is a significant overestimation because the fabricated device had rounding at the vertex of about 1-2 um. This high shear also only zooccupied an area of about 0.5 um^2.



Supplemental Figure 1: Comparison of DLD unit cells with the same flow rate and critical diameter. The upper version has a round obstacle and a gap of 16.5 µm, the lower has a pointed vertex and a gap of 22 µm. The left panel shows the velocity field, the right panel shows the shear rate. The scale is the same for each. A line is drawn vertically, 1 µm away from the gap edge, and we compute the average shear rate along this line (dashed arrow). The spatially averaged shear rate is 36% lower when using obstacles with sharp vertices.

To calculate average shear stress (τ) we used the formula:

$$\tau = \mu \cdot \gamma$$

Where µ is the dynamic viscosity of the sample. Apheresis collections have lower hematocrit than whole blood (µ~3.5 cP at high shear rates) but higher than saline (µ ~ 1.2 cP). This gives an average shear stress in the range of ~5-16 Pa. While this value is higher than physiological shear stress (average 3-5 Pa with peaks up to ~7 Pa in regions like the cerebral arteries)[2], the total exposure time is ~1s as the cells transit through the array.

Curate cassette and instrument

The cassette is a single-use, disposable, fully closed, sterile unit. Upon connection of the reagent, sample, and empty product and waste bags to the cassette, the user loads the cassette on the Curate system and hangs the bags on the corresponding load cells. Once the Curate door is closed, the system automatically processes the sample through a series of stages: DLD priming, run phase, and end of run product flush.

The system's in-line pressure detection upstream of the microfluidic array is used to confirm cassette sterility by testing the cassette seals. The cassette is pressurized using the peristaltic pumps to push fluid into the microfluidic component while keeping the outlet valves closed. Once the target pressure of 120 kPa is reached the pump stops pumping and the pressure sensors monitor the pressure drop over a period of 10s. If the pressure falls below 110 kPa then the system records an error and repeats the test. The system has to pass this pressure test 3 times; but if >2 errors are detected then the system aborts the run. All of this testing occurs before the sample is loaded into the cassette, so the sample is still preserved and can be loaded on to another cassette.

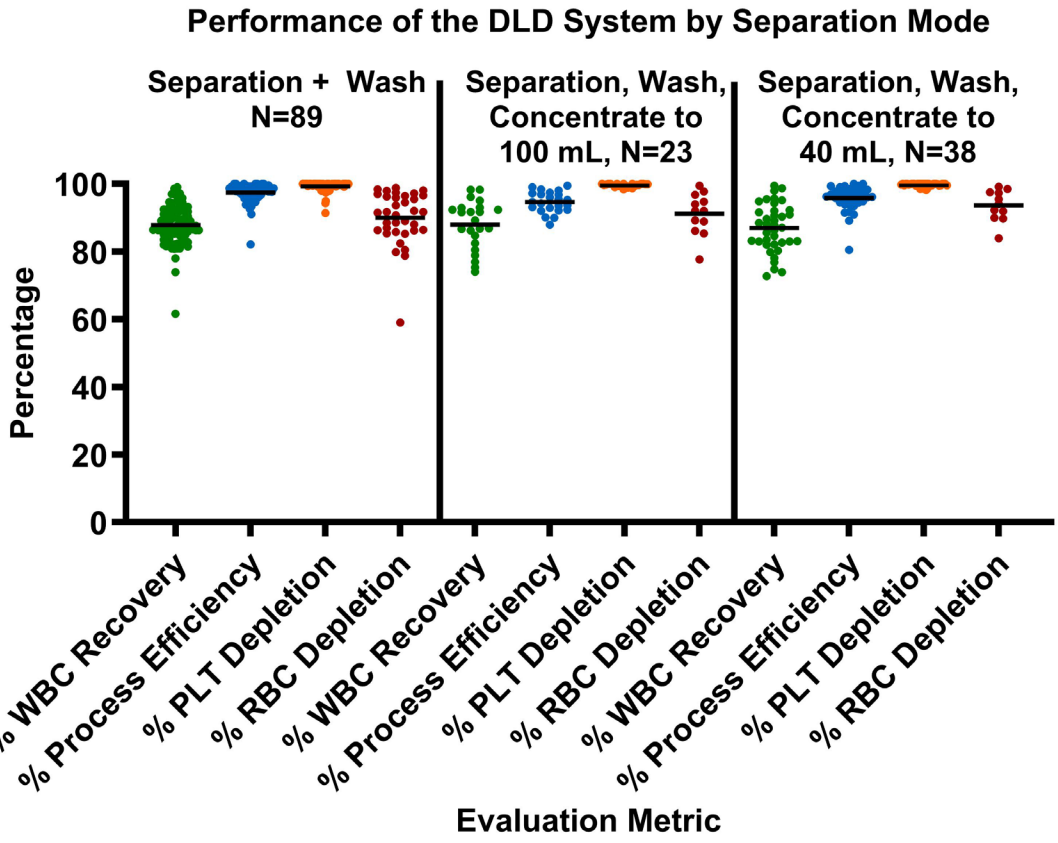
The system monitors the run performance through a series of onboard sensors, including bubble sensors and pressure sensors upstream of the DLD, and load cells (hangers) from which fluids (buffer, sample, product, waste) are hung. Sensor feedback during the run phase is used to both monitor the run progress and to automatically take action to preserve the viability of the sample and fidelity of the DLD microarray. Bubbles detected upstream of the DLD are shunted to a DLD bypass, while an increase in pressure results in an automatic scaling of all the flow rates to keep processing conditions below 25 psi (170 kPa), preserving sample viability while also continuing to process. Bag masses are also used to queue certain script actions depending on the run mode selected.

Mode of operation

Standard processing of a sample takes place in Separation and Wash mode. In this mode, sample and buffer are pumped into the DLD, and the target cells are deflected into the wash buffer and collected in the product channel. Cells smaller than the critical diameter are not deflected, and pass through the array into the waste channel. The final product characteristics (WBC concentration, volume) depend on the sample volume. Various running buffers can be used in this mode, without any modification of the run protocols, including albumin/ Plasma-Lyte®-A Injection, albumin/saline, common tissue culture medias such as Optmizer and TexMACs, and higher viscosity cryopreservation medias (up to 2 cP).

A modification of this mode is Separation, Wash with Concentration, a novel method of operating the DLD. This mode relies upon feedback from the product bag hanger to maintain a fixed product volume, as selected by the user at the start of the run. Once the target

volume/mass is reached, the product is recirculated back into the cassette and DLD elements during the run, taking the place of the buffer input during cell sorting. The sample continues to process with target cells deflecting into the recirculated product stream. Up to ~5-fold concentration of the sample is possible, with collected volumes as low as 40 mL, while still achieving the recovery and depletion metrics achieved during standard modes of operation.

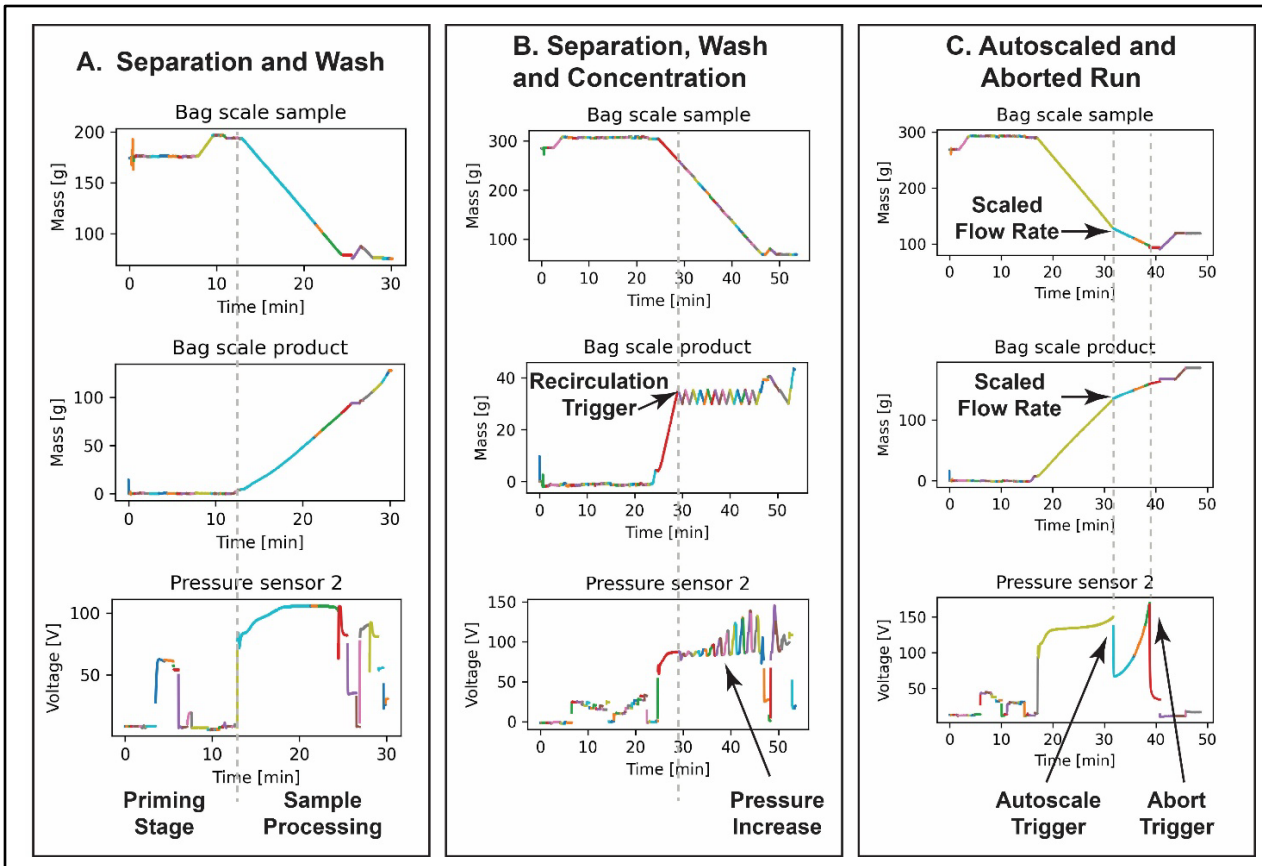


Supplemental Figure 2: Performance of the Curate system and DLD separation under different modes of operation. No difference in performance is observed between the 3 modes of operation.

Autoscaled and Aborted Runs

During testing of the N=150 samples there were 7 runs that autoscaled and ran to completion, and 2 runs that autoscaled then aborted. There was some correlation between the runs that autoscaled and the instrument run mode. Concentration mode runs comprised 78% of the runs that autoscaled, but only comprised 38% of the runs that processed without issue. During the recirculation phase, product volume was recirculated back in the DLD device as running buffer, with increasingly higher concentrations of cells flowing through the device as the run

progressed. Higher cell concentrations in the array increased resistance and higher pressures were detected on the Curate system upstream of the DLD. Examples of sensor readings from a normal run and a recirculation run are shown in Supplemental Figure 3. The system performs recirculation mode by setting upper and lower mass limits on the product bag. When the upper mass is triggered the system enters recirculation mode, when the lower mass is triggered it returns to normal run mode. If these two trigger values are relatively close together (i.e. 38 and 42 g) then the system frequently flip flops between run modes. As the run progresses more cells are recirculating through the DLD due to the increased product concentration. The pressure spikes match when the system is in recirculation mode; once the system returns to run mode and is using running buffer the pressure drops. The pressure during the recirculation phase continues to get higher as the concentration increases and can eventually trigger the system to autoscale.



Supplemental Figure 3. A) Sensor data from a normal Separation and Wash protocol. The system performs a priming sequence (including priming into the sample bag to clear tubing of all air) and then moves into run mode. The pressure sensor shows a higher pressure when running but it stabilizes well below the Autoscale threshold. B) Sensor data from a Concentration run. Once the product bag reaches the threshold values the system automatically initiates recirculation. The system flip flops between upper and lower mass thresholds. As the system enters recirculation mode, more and more cells are flowing back through the DLD, and the pressure increases. The small pressure spikes match when the system is recirculating, and when the system returns to run mode the pressure drops. C) Sensor data from an aborted run (sample part of N=150 data set). Once the pressure reached 150 kPa the system autoscaled the flow rates. At 170 kPa the system aborted the run and flushed the remaining product into the product bag, while backflushing any unprocessed sample back into the sample bag.

The metrics of the 2 aborted runs also indicate that cell concentration played a role. The Separation and Wash run that aborted had an input volume of 200 mL and a WBC concentration of 91 M/mL (net 18.3 B WBCs). The run data is shown in Supplemental Figure 3C, showing an initial trigger at 150 kPa followed by an autoscaling of flow rates (visible as a change in slope of hanger mass vs. time). The second trigger of 170 kPa was also passed, at which point the system executed a recovery protocol. Similarly the aborted Separation, Wash and Concentration to 45 mL run had an input volume of 189 mL at a WBC concentration of 42 M/mL

which would yield a theoretical final concentration of 176 M/mL in 45 mL, The actual recovered product was 54 mL at 107 M/mL due to additional flush protocols after the system aborted.

Panels for Phenotypic Analysis in FACS

Apoptosis - Annexin V-FITC, 7-AAD, CD45-BV421, CD3-APC with cells in 0.5% BSA/PBS + 2.0mM $CaCl_2$

%T cell determination - CD3-BV-421, CD4-PerCP, CD45-FITC and CD8-PE; CD45-FITC, CD14-PE, CD3-BV421, CD19-BV-605 with 0.25 ug/tube 7-AAD added for viability.

T cell subtype and activation: CD11b-BV670, CD95-FITC, CD69-BV510, CD3-BV421, CD4-BV650, CD45RA-BV605, CD25-APC, CDD62L-PE/Cy7, CCR7-PE, CD8-APC/Cy7 and 3.0µM DRAQ7.

Enumeration of RBC, PLT and WBC in the input, product and waste - CD3-BV-421, CD41-FITC, CD235a-PE and DRAQ5.

T cell senescence status: CD45-RO-BV570, TIM3-BV510, PD1-BV421, CD4-BV650, CD3-APC, KLRG1-PE/Cy7, CCR7-PE, CD8-APC/Cy7 and DRAQ7. Both the T cell subtype and senescence panels were washed by centrifugation prior to addition of DRAQ7.

Comparison of CD69+ Activation:

A set of 21 paired samples were used to compare CD69+ activation in DLD vs. Ficoll prepared cells. Of the N=21 samples, N=5 of the samples were processed using the same microfluidic array driven by constant pressure (Fluigent) instead of peristaltic pumps on the Curate system. Peristaltic-driven DLD showed on average 4.7 +/- 2% activation, while pressure driven flow showed on average 3.7 +/- 2% activation.

Cytokines

A 51-plex cytokine assay was performed post purification by either Ficoll or DLD. The table below shows values where the relative Ficoll/DLD values were >2x. Full results of the panel, along with information regarding the source and mechanism of action for each cytokine is in the supplemental data file.

Average of pg/mL* Row Labels	Column Labels Source	Ficoll	DLD	%REMOVED FI/Source	DLD/Source	RELATIVE Fi/DLD
PDGF-BB	13,936.6	17,238.5	1,958.4	-24%	86%	8.80
TGF B-1	46,020.6	15,465.6	969.3	66%	98%	15.96
NCAM	206,721.4	10,658.4	4,755.5	95%	98%	2.24
RANTES	22,657.2	5,829.3	1,065.2	74%	95%	5.47
PDGF-AB/BB	40,493.6	5,451.5	550.8	87%	99%	9.90
PAI-1 (total)	32,623.8	3,565.9	164.3	89%	99%	21.71
sCD40L	2,881.4	2,897.9	209.9	-1%	93%	13.80
GRO alpha	2,788.0	1,943.1	155.3	30%	94%	12.51
PDGF-AA	1,388.7	833.4	85.7	40%	94%	9.72
BDNF	8,248.9	470.6	141.2	94%	98%	3.33
EGF	216.8	215.0	8.8	1%	96%	24.45
FGF-2	376.1	114.3	27.8	70%	93%	4.11
MDC	1,527.5	58.7	16.0	96%	99%	3.67
VEGF-A	223.0	52.8	22.4	76%	90%	2.36
MIP-1B	263.2	37.8	ND	86%	100%	-
IL-8	12.7	35.1	0.3	-175%	98%	140.30
MCP-1	247.1	15.7	ND	94%	100%	-
IFNa2	5.3	12.7	5.3	-140%	0%	2.40
Fractalkine	5.6	9.9	4.0	-77%	29%	2.49
G-CSF	6.0	2.5	0.4	57%	93%	6.37
IL-1RA	702.3	1.8	0.5	100%	100%	3.88
IFNy	1.9	1.0	0.2	49%	88%	4.34
IL-5	0.1	0.0	0.0	80%	94%	3.33

Supplementary Table 1. Cytokines present both pre- and post-separation by Ficoll and DLD. N=2 samples were analyzed in duplicate. Green boxes highlight all ratios >1 (Ficoll value greater than DLD).

ND: not detected or out of range of linearity. Yellow boxes: Ficoll product value was equivalent to or exceeded value from input material. Full panel is presented in the data file.

References:

1. Loutherback, K. *et al.* Improved performance of deterministic lateral displacement arrays with triangular posts. *Microfluid. Nanofluidics* **9**, 1143–1149 (2010).
2. Cheng, C. K., Wang, N., Wang, L. & Huang, Y. Biophysical and Biochemical Roles of Shear Stress on Endothelium: A Revisit and New Insights. *Circ. Res.* **136**, 752–772 (2025).