Flow Cytometry and Imaging Technologies

Predictive Modeling Leveraging Morphological Metrics with Invitrogen™ Attune™ CytPix™ Flow Cytometer for Enhanced Cell Therapy Manufacturing Research

Jasmine Padilla[1,#], Lina Zheng[1,#], Nicolas Rognin, Bethan M. Jones, Kim Ippolito, Weizhong Li, Kate Alford, Monique LaCourse, Yongchang Ji*, Steven Beeler*, Mark Powers*
Thermo Fisher Scientific, 5781 Van Allen Way, Carlsbad, CA 92008.
# Authors equal contribution. * Corresponding Authors

Abstract

Purpose: This project aims to improve the efficiency and predictability of cell therapy research workflows. By utilizing the Invitrogen™ Attune™ CytPix™ Flow Cytometer, we monitor T-cell morphology across various donors during ex vivo expansion and develop a predictive model. This model helps provide insights into the quality of donor-derived starting material on the day of T-cell isolation, enabling researchers to make informed decisions early in the process, efficiently increasing accessibility to research on cell therapies.

Methods: The Attune CytPix Flow Cytometer utilizes high-speed brightfield imaging and machine learning from the Attune Cytometric Software v7.1 to generate 36 morphological parameters and combines this data with conventional flow data. T-cell expansion was monitored from frozen peripheral blood mononuclear cell (PBMC) donor Leukopaks. Key data points, including cell proliferation, viability, surface marker expression, and morphological metrics, were collected on Day 0, Day 2, and Day 9 – time points aligned within CAR-T manufacturing framework. Expansion fold change was calculated by comparing the final cell count to the baseline on Day 0. Unsupervised community detection algorithms were applied to identify distinct subpopulations, followed by biomarker enrichment analysis to annotate clusters and associate them with downstream expansion potential.

Results: Integrating morphology with fluorescent marker clustering enables a comprehensive evaluation of starting material at the single cell level. Donors with favorable expansion outcomes can be grouped into a shared morphological cluster, clearly separated from poor expanders. Morphological traits such as circularity, cell size, and texture emerged as key indicators correlated with expansion fold change. Donors that expanded the best showed the most uniform morphology profiles on Day 0, while the donors that expanded worse showed highly diverse cell morphology profiles.

Introduction

The rapid evolution of gene-modified cell therapies has created a pressing need for streamlined, reliable analytics to support research and manufacturing. A major challenge in this space lies in the donor-to-donor variability that impacts T-cell expansion, editing efficiency, and performance. Gaining early insight into these biological differences can help optimize manufacturing workflows (Figure 1).

Traditionally, cellular characteristics are evaluated after culture initiation during expansion. However, this project highlights the potential of characterizing the donor material *before* the expansion begins. We present a research workflow that uses the Attune CytPix Flow Cytometer to capture morphological metrics from T cells during early ex vivo expansion. We present a research workflow capable of forecasting unmodified T-cell expansion capacity across multiple donors—paving the way for more informed, donor-specific manufacturing strategies.

Materials and methods

Sample Preparation

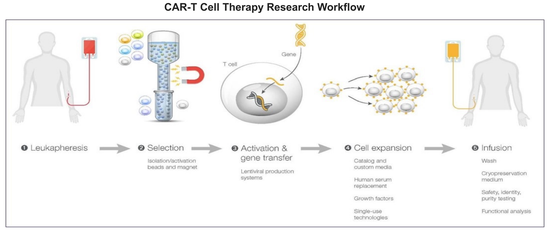
T cells were isolated from healthy donor PBMC Leukopaks using the Gibco Dynacellect Magnetic Cell Separation System along with Detachable CD3/28 Dynabeads. Isolated cells were cultured in a Grex vessel in OptMizer Pro media supplemented with Peprotech IL-2 (10ng/mL) and seeded at 1×10^6 cells/mL. After two days of activation, T-cells and Dynabeads were separated using the Dynacellect and expanded in a Grex vessel for a total of 9 to 12 days.

On Day 0, Day 2, and Day 9, approximately 100K-300K cells were collected per sample for image-enhanced flow cytometry analysis on the Attune CytPix Flow Cytometer. The collected cells were centrifuged at 400g for 5 minutes and washed once in **eBioscience™ Flow Cytometry Staining Buffer (00-4222-57)**. After the wash, each sample was resuspended and incubated in 20uL Fc Receptor Binding Inhibitor Polyclonal Antibody (14-9161-73) and 80uL of flow staining solution in a 96 well plate at 4C for 15 minutes. Each sample was then incubated in a selection of Thermo Fisher Scientific eBioscience™ monoclonal antibodies targeting various surface markers for immunophenotyping including Super Bright™ 600 CD45 (2D1, 63-9459-42), PE-Cyanine7 CD4 (OKT4, 25-0048-42), Alexa Fluor™ 700 CD8a (OKT8, 56-0086-42), Super BrightTM 780 CD56 (TULY56, 78-0566042), Super BrightTM CD2 (RPA-2.10, 63-0029-42), PerCP eFluor™ 700 CD19 (SJ25C1, 46-0198-42), APC eFluor 780 CD3 (OKT3, 47-0037-42), APC CD28 (CD28.2, 17-0289-42), PE-Cyanine7 CD14 (61D3, 25-0149-42), and SYTOX™ AADvanced™ Dead Cell Stain Kit (S10349).

Bioinformatics Data Analysis

A time series single cell phenotyping database was built in CAR-T cell therapy research workflow. The single cell morphologic metrics and fluorescent markers intensities were collected from Attune CytPix Flow Cytometer. The cell types were identified by manual gating using FlowLogic software[1]. Bioinformatics graph algorithm and machine learning method have been implemented to identify cell subpopulations. Biomarkers to distinguish cell expansion performance have been identified through enrichment analysis.

Figure 1. Gene-modifed CAR-T research workflow. Donor samples are collected in Leukopaks and undergo the desired monocyte cell selection process, prior to CAR-T generation (activation and gene modification). After expansion of CAR-T cells and quality control measures, the therapies are ready for patient infusion.



Results

Characterization of Frozen Donor Samples from Day 0 to 9.

Figure 2. Brightfield Images from the Attune CytPix Flow Cytometer. Donor samples were analyzed on the Attune CytPix Flow Cytometer post sample prep, after debeading and then at harvest.

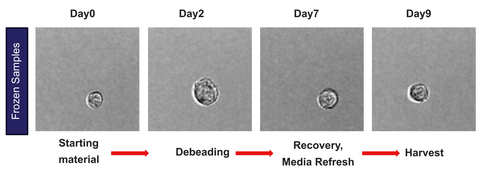
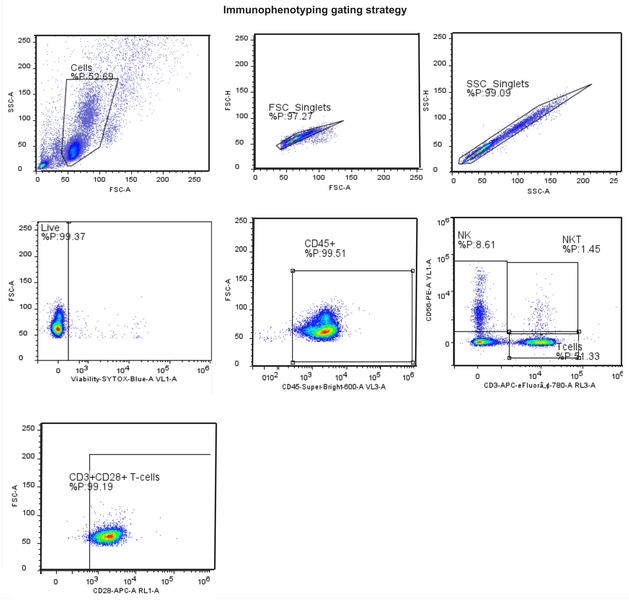


Table 1. Donor biometrics from for four healthy donor peripheral blood mononuclear cells. Ranking is based on Day 9 expansion. Fold expansion data was collected from Day 0 to 9. Donor samples were ranked by highest fold expansion, greater than 50-fold expansion represents a desirable metric for health of the sample.

	Age	BMI	Ethnicity	Gender	Day 9-Fold Expansion	Rank
Donor 6	30	26.1	African American	Female	40.6	1
Donor 9	37	26.4	Middle Eastern	Male	16.4	2
Donor 2	36	27.3	Caucasian	Female	14.6	3
Donor 7	37	27.2	African American	Male	13.7	4

Figure 3. Gating Strategy for Attune CytPix Flow Cytometer flow cytometry analysis in CAR-T cell research therapy. Immunophenotyping gating strategy used to identify CD3+CD28+ T-cells from activated monocyte population. Live singlets were gated according successive FSC and SSC bivariate plots and using viability stain. T cells comprised of at least 50% CD45+ cells from donor samples. Shown plots is a representation.



Bioinformatics analysis enabled forecasting T cell expansion capability in CAR-T cell therapy research

Figure 4. Bioinformatics workflow for Attune CytPix flow cytometry analysis in CAR-T cell therapy research. Bioinformatics data management, single cell database, and bioinformatics data analysis architecture.

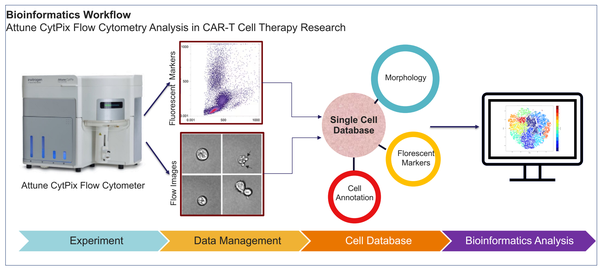
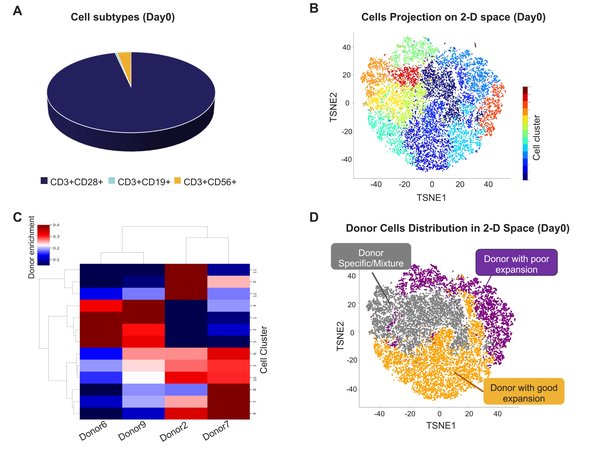


Figure 5. Bioinformatics analysis for Attune CytPix flow cytometry analysis in CAR-T cell therapy research for donor frozen samples on Day 0. (A) Donor frozen samples cells collected on Day 0 are mostly CD3+CD28+ T-cells. **(B)** Donor frozen samples cells can be clustered into 13 clusters with fluorescent markers and morphologic metrics by graph algorithm and unsupervised machine learning methods. The cell clusters allocated in distinct area in 2-D space. **(C)** Donor distribution across cell clusters. **(D)** Donor cells (Day 0) distribution in 2-D space. Donor with poor expansion located in purple zone, whereas donor with good expansion located in orange zone.



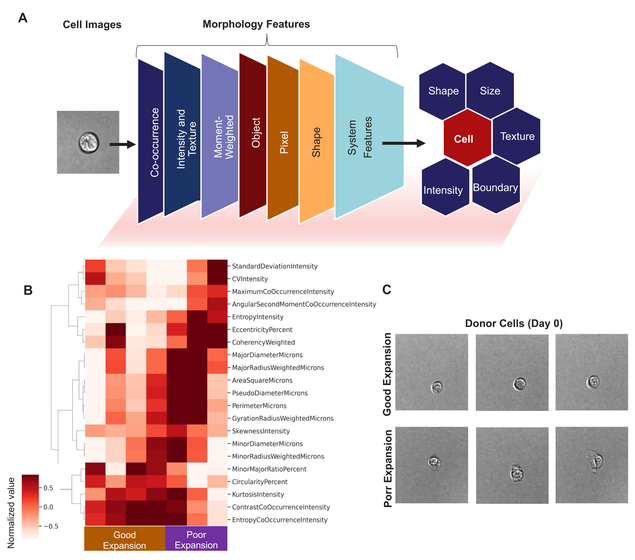
Conclusions

This project underscores the importance of early characterization of T-cell donor material to improve the efficiency and predictability of cell therapy research workflows. By utilizing the Attune CytPix Flow Cytometer, we were able to integrate detailed morphological measurements with conventional flow cytometry data, enabling a comprehensive evaluation of starting material. Our key findings demonstrate:

- Differences in expansion potential amongst donor CD3+CD28+ isolated T-cells show distinct morphological differences on Day 0 and through expansion.
- Morphological parameters related to cell shape, size, and entropy could serve as potential biomarkers for cell expansion on Day 0 before the drug manufacturing workflow begins
- Donors with favorable expansion outcomes were characterized by uniform morphological profiles, while those with poorer expansion outcomes exhibited diverse morphology.

This predictive model helps in making informed decisions early in the process. The bioinformatics analysis pipeline developed can be further applied to understand cell behavior through genetic engineering, extending its utility to other cell types such as NK cells. This approach paves the way for more refined, donor-specific manufacturing strategies, addressing the challenge of donor-to-donor variability and enhancing the overall success of gene-modified cell therapy research.

Figure 6. Morphologic indicators of frozen donor T-cell expansion potency. (A) Schema of morphologic features produced by Attune CytPix Flow Cytometer. **(B)** Morphologic features enrichment in donor with good/bad expansion cell clusters. **(C)**Examples of donor frozen sample cells with good/poor expansion.



References

1. Flowlogic software: https://flowlogic.software/

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