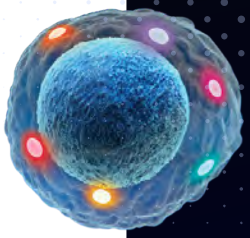
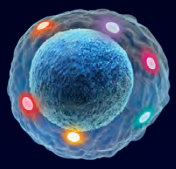
LASE Innovation

TALON Biomarkers

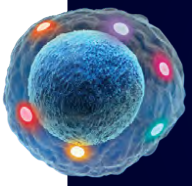


A New Frontier in T Cell Analysis: Dynamic Measurements that Precisely Characterize Phenotype and Function



Accelerate your T cell research

Introduction



T cell therapies offer new hope for tackling cancer and autoimmune disease. However, despite notable success in certain blood cancers, achieving consistent efficacy and durability in other disease areas has proven challenging.[1,2]

A major barrier is the significant heterogeneity within T cell populations, which complicates efforts to accurately characterize and assess the function of these therapeutic products. It is well-established that T cells can respond differently—even to the same activation signals—resulting in diverse behaviors and functional outcomes.[3,4] Factors such as activation protocols, culture conditions, and handling all contribute to this variability. However, most current single-cell analysis tools rely on endpoint measurements, capturing only a static snapshot after perturbations like activation, transduction, or checkpoint inhibition. These methods miss key transitional states—how cells change from '*before*' to '*after*'—as well as the dynamic regulation of protein markers over time. As a result, researchers lack critical phenotype-to-function (P2F) data needed to identify truly effective T cells and build predictive models of therapeutic efficacy.

Here we introduce a novel flow cytometry-based application, Time-lapse flow™ and three (3) examples of how looking at the temporal changes in biomarkers at a single-cell level can provide powerful insights into T cell biology.[5]

Time-Lapse Flow™: Measure and Track single cells over time at scale

We have recently developed a novel T cell assay called the Time-Lapse™ TITAN assay (**Figure 1**) that reveals dynamic, time-resolved changes in human T cell protein expression during activation, enabling deeper insights into functional diversity and therapeutic potential. This workflow involves barcoding T cells with laser particle (LP) optical barcodes™, stimulation(s) with drug(s) of choice, and repeated measurements at several timepoints using a high-throughput flow cytometer.

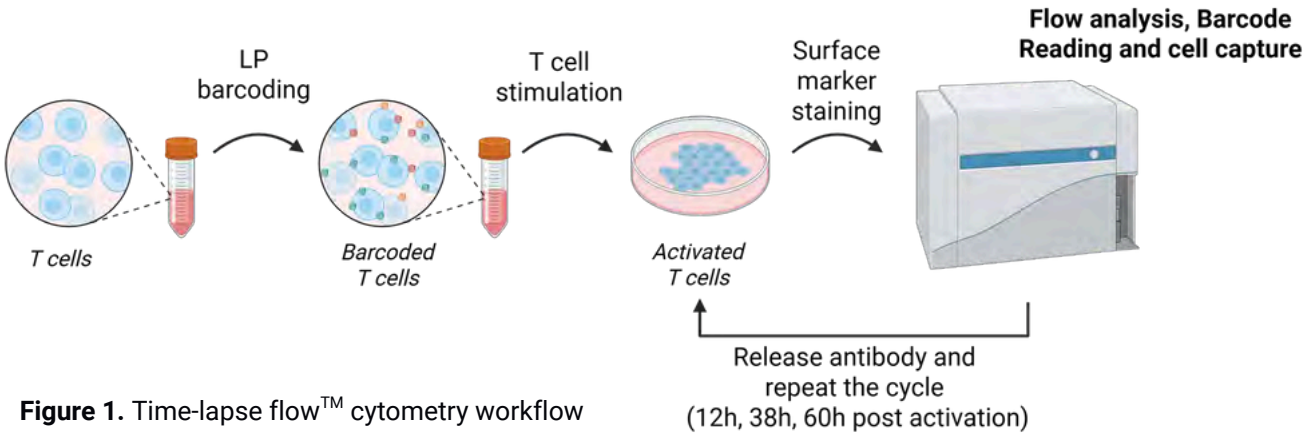


Figure 1. Time-lapse flow™ cytometry workflow

(Table 1) Optical barcodes are read on each cell at every measurement to track individual cells over time and construct unprecedented single-cell kinetic profiles of marker expression.

Marker	Function
CD4	Cell Lineage marker
CD8a	Cell Lineage marker
CD25	Activation Marker
CD38	Activation/Differentiation Marker
CD62L	Central Memory Differentiation Marker
CD69	Activation Marker
PD-1	Activation/Exhaustion marker
Viability Dye	Viability

3 Timepoints
e.g. Pretreatment (0h), Post-treatment (e.g. 12, 36, 60h)

Table 1: LASE Time-lapse TITAN Assay

Time-lapse flow cytometry provides information on the cell states *before*, *during*, and *after* a trigger or perturbation. Because of the incredible diversity and plasticity of immune cells, dynamic measurements are crucial for a true understanding of function and mechanism.

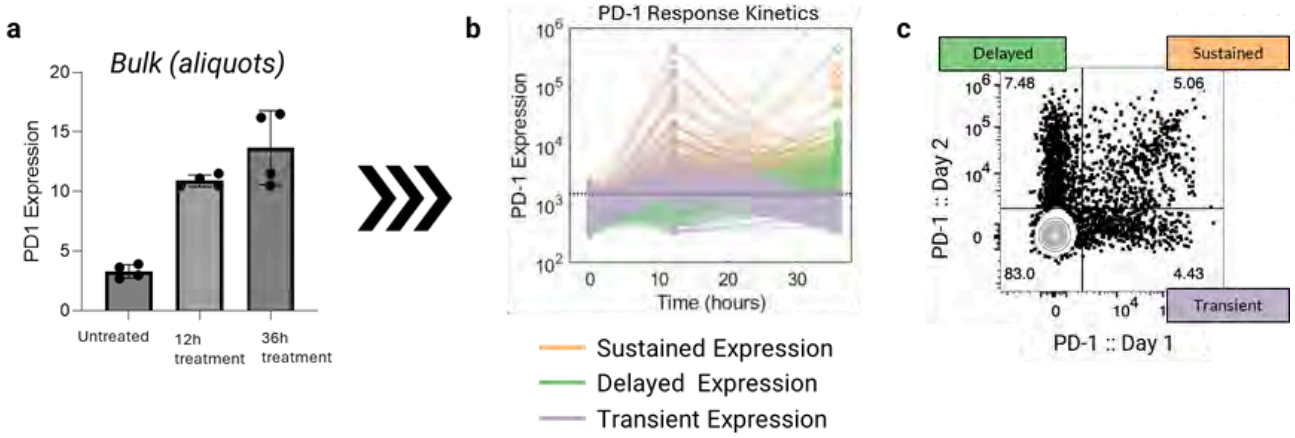
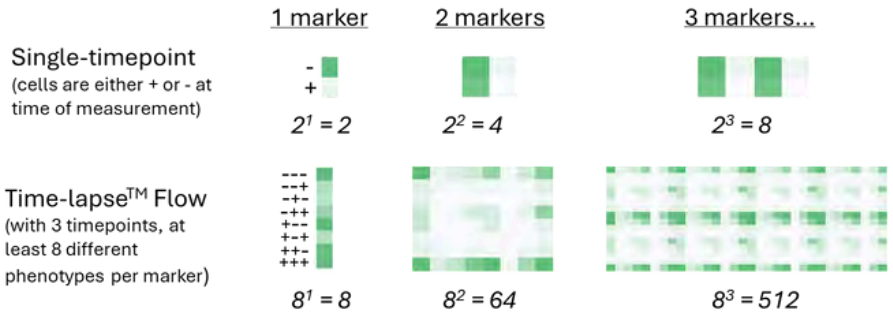


Figure 2. Conventional flow vs. Time-lapse flow. Example shown here is of PD1 expression of human T cells activated with CD3/CD28 over time. While conventional flow can detect an increase in PD1 expression in the bulk sample over time (a), time-lapse flow can differentiate between cells that have sustained, delayed or transient expression of PD1 (b and c).

Measuring individual cells over time means that **each cell can be used as its own control**. This differs from from conventional approaches in which treated and untreated samples are compared in bulk (**Figure 2**). This unprecedented resolution enables observation of subtle changes in activation response and identification of unique kinetic phenotypes.

The ability to measure marker expression trajectories enables **exponentially more phenotypes** can be identified. For example, for a 1-timepoint, 3-marker measurement, assuming each marker is + or -, this provides 2^3 potential phenotypes for classification. For 3-timepoints, each marker can have 8 possible trajectories (**Figure 3**) for each of the 3 markers, or 8^3 potential phenotypes. The TITAN assay (Table 1) can identify over 2 million potential phenotypes, providing a highly comprehensive dissection of cellular heterogeneity.



# of markers	1	3	7
1 timepoint	2	8	256
3 timepoints	**8**	**512**	**2 million**

Over 2 million possible phenotypes with 7 markers and 3 time-points

Figure 3. Schematic showing potential phenotypes identified by single-timepoint or Time-lapse flow. Green colors represent relative frequency of phenotypes. Exponentially more phenotypes can be identified with a multi-timepoint approach.

A. Characterize novel kinetic heterogeneity of activation response in human T cells

Using the Time-lapse TITAN assay, we compared the activation response in Human T cell samples across three (3) different stimulation conditions and three (3) different time-points (**Figure 4**). We were able to identify unique activation-dependent response patterns for PHA, Cytostim, bsAB CD3/CD28 compared to media control.

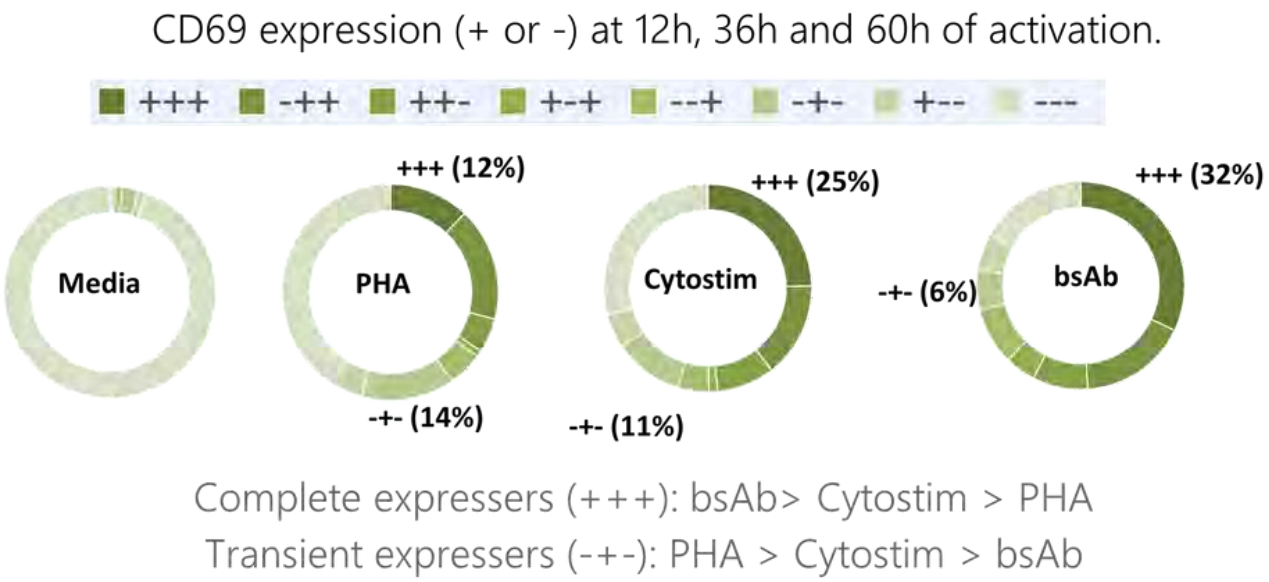


Figure 3. T cells exhibit distinct CD69 expression patterns across 3 time points depending on the stimulation conditions (PHA, Cytostim™, bsAB).

Measuring the expression changes of the early-activation marker CD69 at 12h, 36h and 60h within each cell, we are able to resolve at least 8 different kinetic patterns (+ or – for each of 3 timepoints with a single marker), providing greater sensitivity in measuring differences in activation response. These kinetic patterns are unique to a given stimulus and are not able to be resolved with conventional flow techniques. The biological mechanisms underlying this heterogeneity in activation response may hold clues to developing more potent and durable T cell therapies.

B. Identify *de novo* expression of markers with single-cell resolution

Understanding the mechanism and responses to activation signals is a key step in the development of T cell-based therapies. Expanded engineered T cells are often banked in a highly activated baseline state, which makes it difficult to quantify and distinguish *de novo* expression of biomarkers above background at the population-level. Conventional methods cannot identify stimulation-specific responses at the single-cell level and may miss biomarker signals from smaller subpopulations of T cells. We used the Time-lapse TITAN assay on thawed and rested TCR-engineered T cells to measure the CD69 early activation signature from specific- and non-specific peptide in a co-culture system. Cells were measured at baseline (0h) and 12h post-activation.

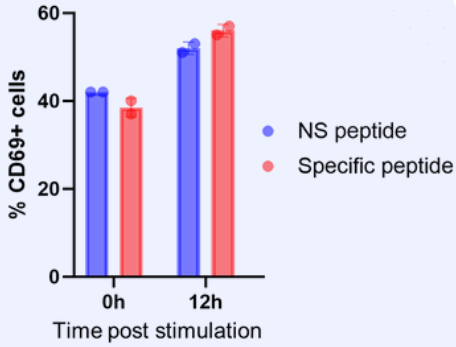


Figure 4. TCR-engineered T cells were stimulated in presence of specific or non-specific peptide and measured at baseline (0h) and 12h timepoints and measured with conventional (bulk) flow cytometry.

When measured at a population level, the pre-existing high background expression of CD69 makes resolving the antigen-specific induced expression challenging. (**Figure 4.**) Using Time-lapse Flow cytometry, we can resolve CD69 expression before and after peptide stimulation at the single-cell level. (**Figure 5**). Using this method, we were able to resolve de novo expression of CD69 as a result of specific peptide vs. non-specific peptide (**Figure 5, 'Q1'**). We were also able to observe cells that have downregulated CD69 (**Figure 5, 'Q3'**) from the initial timepoint. The improved resolution to subtle biomarker changes is due to the statistical power of having 10,000 to 1,000,000x more data points by virtue of the single-cell measurements.

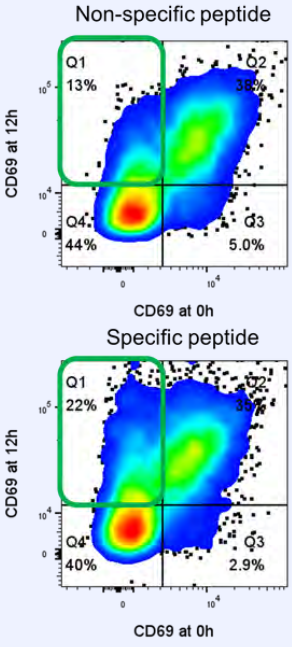


Figure 5. Barcoded TCR-engineered T cells were stimulated in presence of specific or non-specific peptide and measured at baseline (0h) and 12h timepoints.

C. Connecting phenotype to function (P2F) to train predictive ML models

A major hurdle to the development of improved adoptive T cell therapies is the variability of, and inability to predict, drug product function. Large scale datasets (e.g. sequencing profiles) are mostly single-timepoint snapshots of populations and cannot resolve the heterogeneity of response and connect it to a functional outcome at the single-cell level. Large scale data sets generated from Time-lapse flow cytometry are ideal for leveraging machine learning (ML) methods to train powerful predictive models. Unlike other modalities, our method provides single-cell data before, during and after perturbation, which are ideal training sets for developing predictive models.

We used the Time-lapse TITAN assay on bsAb CD3/CD28-activated human T cells and measured the dynamic biomarker panel changes at 2h, 22h and 45h post activation (**Figure 6**).

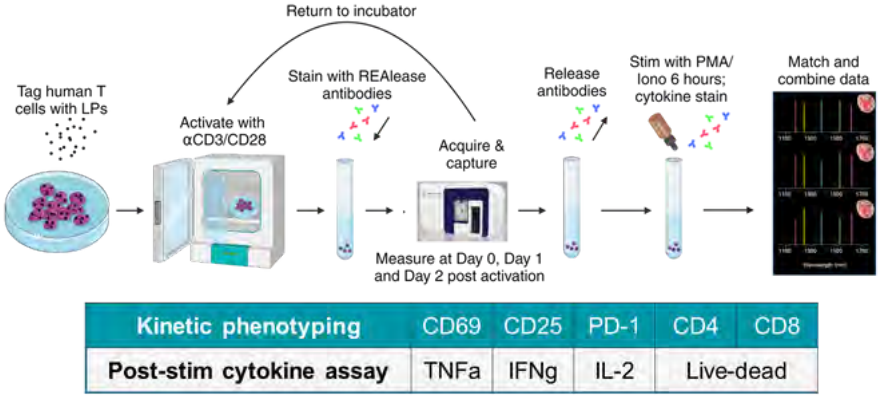
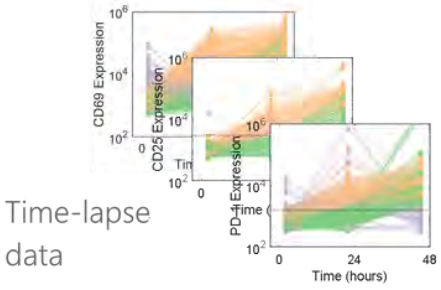


Figure 6. Single-cell P2F from bsAb CD3/CD28 activated human T cells using the TIme-lapse TITAN assay.

We then correlated these changes to cytokine secretion post-stim with PMA/Ionomycin to generate P2F data sets. The P2F data where then used to train a Neural Network machine learning model to predict whether a given cell secretes different types of cytokines. (**Figure 7**).

Cytokine	AUC
TNFa	0.79
IFNg	0.88
IL2	0.74
Polyfunctional (IFNg+TNFa+IL2)	0.89



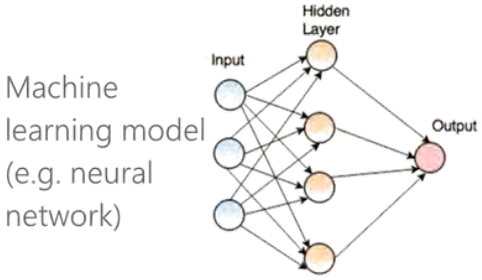
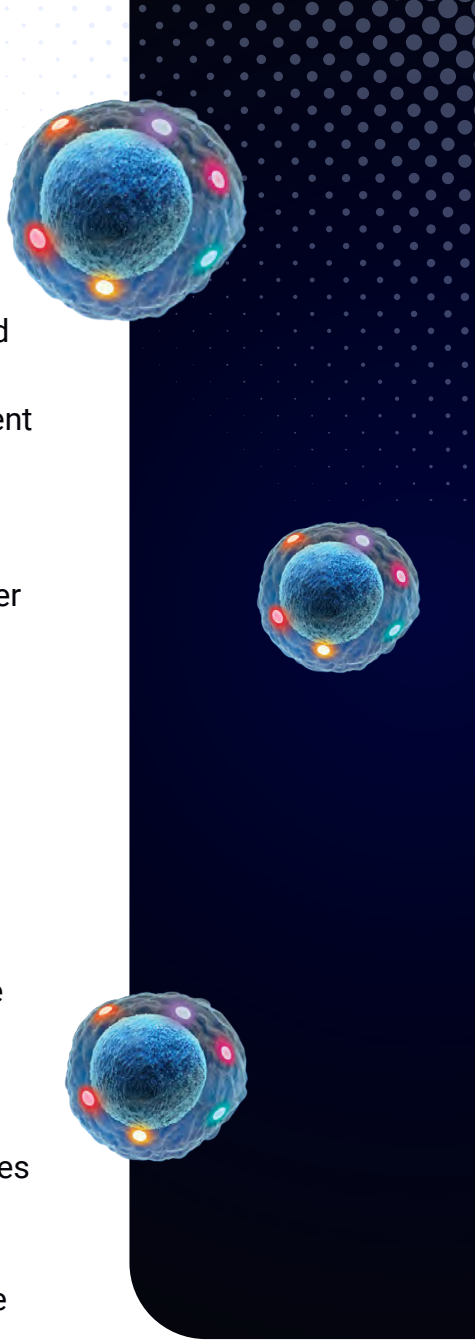


Figure 7. A classification model (via 5-fold cross validation) for predicting cytokine secretion using single-cell P2F data collected over 3 timepoints. Area under the curve (AUC) of the ROC curve is shown where AUC = 0.5 is a random classifer and AUC = 1 is a perfect classifier.

Conclusions



To improve the effectiveness and safety of T cell therapies, and expand their use beyond narrowly improved indications, we need to better understand the heterogeneity present throughout the entire development process. We have recently developed a novel assay technology called Time-lapse flow cytometry that can reveal dynamic, time-resolved changes in T cell biomarker expression during activation. Using our Time-lapse TITAN assay, we demonstrated the ability to track biomarker upregulation and downregulation at the single-cell level in response to perturbation such as activation and drug exposure.

Using this method, we were able to identify distinct biomarker profiles from three (3) different T cell stimulation methods, highlighting the heterogeneity of response to different activation conditions. We were also able to resolve up- and down-regulation of CD69 in a engineered TCR T cell model with very high baseline activation to distinguish antigen-specific responses from various peptides. Lastly, we were able to aquire large-scale P2F datasets to train an ML model to predict whether a given cell will secrete cytokine or not, prior to stimulation.

In summary, Time-lapse flow cytometry is a novel approach that enables tracking and measurements of individual cells over time, at scale. The Time-lapse TITAN assay is designed to resolve cellular heterogeneity that underlies the variability of T cell therapies, enabling more accurate predictions of desired cell functions and potentially cell drug product with improved performance.

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