TECHNICAL NOTE

Maximizing Bruker System Single-Cell Data by Referencing Precedent Sample Preparation Protocols

Precedent Protocols and Processes ensure that enrichment, stimulation and labeling will result in maximizing the correlative data achieved, while minimizing non-specific impact on the cells themselves.

In this Technical Note we outline:

- Published enrichment, stimulation, and staining protocols throughout immunotherapy types and indications help achieve your goals, in multiple publications and presentations
- Protocols that minimize impacts on the live cells that are analyzed on Bruker's IsoLight platform
- Guidelines for most commonly used cell types and applications

Bruker's IsoLight system employs its IsoCode® chip, a single-cell chip consisting of highly multiplexed proteomic barcode to analyze each single cell. The IsoCode technology uniquely addresses the cytokine detection limitations with its ability to measure a large panel of 32+ key secreted proteins from live single cells [1] and to deeply decipher the functional heterogeneity and key functional subsets of responding immune cells among individual patients. The benchtop IsoLight and IsoSpark instruments are sample in, result out automation systems which automate the imaging, incubation and ELISA fluidics steps of both single-cell and CodePlex assays as well as bioinformatics analysis (Figure 1), and provides streamlined bioinformatics tools.

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Prep, Run, Analyze

3 Key Sample Preparation Steps prior to loading onto the IsoLight enable success

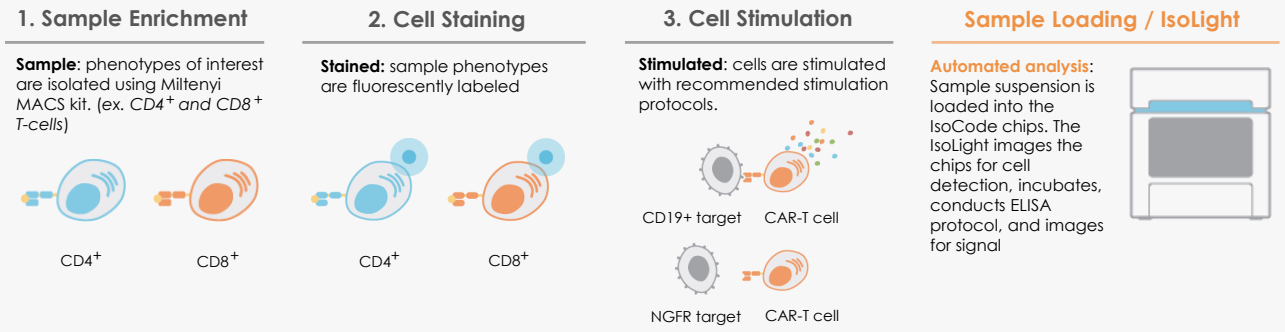

Figure 1 | Workflow of IsoLight for detecting secreted proteins from single cells. Step 1: Cells of interest are prepared by magnetic beads enrichment to enrich the phenotype of interest. Step 2: Stimulated cells are surface stained with General Stain provided by Bruker in each consumable kit. Step 3: Cells are stimulated with recommended stimulation protocols. Following, samples are loaded into the IsoLight for automated analysis.

In addition to quantitative assaying of a large array of secreted proteins per cell, the IsoCode chip is designed to fit various types of immune cells. The IsoCode chip measures Polyfunctional Strength Index (PSI™), a unique single-cell metric defined as the percentage of polyfunctional cells (2+ proteins per cell) in a sample, multiplied by the average signal intensity of the proteins secreted by these polyfunctional single cells (Figure 2).

Bruker's Single-Cell Polyfunctional Strength Application:

Bruker's polyfunctional strength index (PSI) consolidates high-dimensional, single-cell protein secretion data into a single metric that represents the overall activity of a sample. It captures two critically relevant factors uniquely: the percentage of polyfunctional cells (single cells secreting two or more cytokines) in a sample, and the intensity of all profiled secreted cytokines. Polyfunctional cells are recognized as key effector cells contributing to the development of potent and durable cellular immunity against viral infection, cancer, and other disease [2-4]. Bruker's ability to capture the range of relevant cytokines from each immune cell represents a unique secreted protein multiplexing capability. While the percentage of highly polyfunctional cells on its own is a meaningful indicator of potency, the Bruker system quantitates the intensity of the cytokines secreted by these highly polyfunctional cells. Having both of these key factors in tandem has helped capture the potency of important and highly functional T-cell and other immune cell subsets, which has correlated with in vivo response [5,6].

Bruker's Single-Cell technology has been employed in the anti-CD19 CAR-T cell pre-infusion products manufactured from apheresis of patients with Non-Hodgkin lymphoma (NHL). It has demonstrated a statistically positive association predictive of objective response (OR) as well as CRS in these patients after anti-CD19 CAR-T therapy, outperforming other pre-infusion flow cytometry-based and bulk-level metrics in its ability to differentiate responders and non-responders [5].

Bruker PSI: polyfunctionality of a sample combined with the intensity of each cell's secreted cytokines

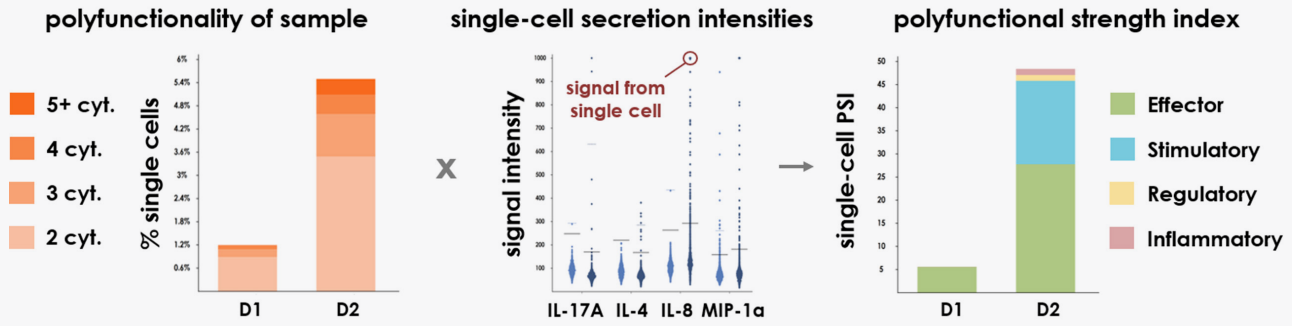

Figure 2 | PSI (Polyfunctional Strength Index) is defined as the percentage of polyfunctional single-cells (secreting 2 or more proteins, i.e. left panel) in a sample, multiplied by the average signal intensity of the secreted proteins from individual functional groups (middle panel) from each cell. Each cell's strength, across 1000+ cells, is then aggregated and simplified into the readout at right. This PSI measurement provides a comprehensible visualization of the potent cell subsets, and the cytokine types driving these potent cell subsets.

IsoLight Sample Preparation: Overview & an example

In contrast to technically challenging and time-consuming procedures typical of single cell systems, the IsoLight requires minimal amount of time and experience for successful measurement of true secretion from single cells. However, key guidelines should be followed to ensure success.

Before loading onto IsoCode chips and running on the IsoLight, in the example of analyzing engineering CAR-T cells, three simple steps are required:

1. Sample Enrichment
2. Cell Stimularion
3. Cell Staining

Then, stimulated cells are loaded onto Single-Cell Secretome chips and automated proteomic analysis is performed on the IsoLight or IsoSpark system. Choosing experimental conditions will significantly improve the overall quality of single cell secretion analysis.

Bruker's validated enrichment and stimulation methodologies demonstrate minimal impact on control, with highly specific stimulation of the antigen specific condition

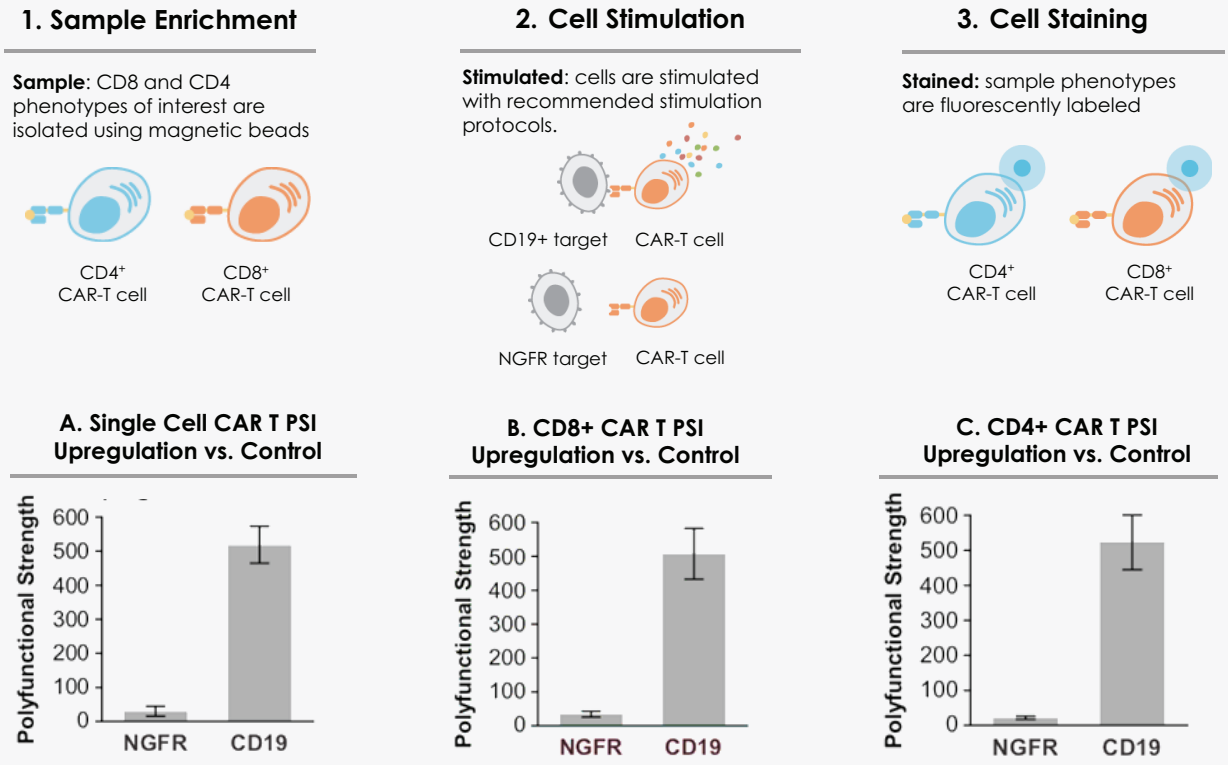

Figure 3 | (1-3) Schematic representations of CAR products enrichment, antigen-specific stimulation and surface labeling procedures. (A-C) Product T-cell polyfunctionality was assessed by using ELISA in combination with fluorescent detection of proteins from each single-cell chamber after T-cell stimulation. PSI of CAR T cells and $CD4^+$ or $CD8^+$ subset, ex vivo stimulated with $CD19^+$ as compared with $CD19^-$ cells (NGFR transfected).

Demonstrating minimal impact of enrichment on actual stimulation: Maximizing specificity to stimulus

Our data clearly demonstrated that post enrichment and stimulation with the NGFR (non-antigen specific) resulted in a baseline level PSI with almost no cytokine release, indicating that the enrichment and the sample preparation did not stimulate the CAR T cells. In contrast, CAR T cells stimulated with antigen-specific target cells secreted substantial amounts of cytokines and demonstrated high polyfunctionality (Figure 3A-3C,[2]). The multiple of antigen specific stimulation over NGFR stimulation further confirmed the conditions we used were optimized for evaluating CAR products. The majority of CARs we have profiled secrete low baseline levels of Granzyme B, but otherwise have minimal secretions without antigen-specific stimulation.

Bruker's recommended enrichment strategies, as seen in are based on similar published work that minimizes impact from the enrichment itself, and maximizes the connection between the stimulated cells and the *in vivo* physiology and cytokine mechanism.

Bruker's recommended enrichment and stimulation methodologies are suited specifically to its platform and are meant to uncover differences between critical sample types that correlate to *in vivo* activity

While Bruker's customers tend to use a variety of platforms of stimulation and methodologies to achieve their goals, the goal of Bruker's platform is to detect sensitive difference unable to be captured by other technologies.

Researchers have developed and tested validated stimulation and enrichment protocols that work to the advantage of our platform.

Since the Bruker systems uncover these differences that are often missed by other platforms, the stimulation protocols recommended for other technologies are not sufficient to reveal sensitive differences and may not work on the Bruker Platform.

Likewise, Bruker's recommended protocols are specific to our system and are not recommended to be used on other platforms as well. Reference Figure 5 and Technical Note: Validation of Sample Preparation to see where Bruker's methods and recommended protocols have been able to reveal differences that other methods were not able to uncover.

PSI uniquely correlates to *in vivo* outcome in a variety of immunotherapy areas

PSI was found to uniquely correlate with CD19 CAR-T cell therapy clinical outcome, as published in the journal ***Blood***. To determine whether PSI is correlated with clinical outcome, researchers profiled pre-infusion $CD4^+$ and $CD8^+$ CAR-T cell samples from 20 of the 22 patients in a clinical trial [2]. Of these 20 patients, 14 had shown an objective response to CAR-T cell therapy. Using Bruker's systems, researchers found the PSI of pre-infusion CAR-T cells stimulated with CD19-K562 target cells, which combined $CD4^+$ and $CD8^+$ CAR-T response, showed significant correlation with the OR of patients (Figure 4A). Specifically, researchers found that the average PSI of the responder subgroup was more than twice as high as the PSI of the non-responders, a difference that was shown to be statistically significant ($p = 0.0119$).

In addition, researchers showed that PSI outperformed other pre-infusion metrics, including IFN-γ co-culture cytokine intensity, ratio of $CD4^+$ to $CD8^+$ T cells, and various T cell phenotype frequencies (Figure 4B). PSI was the only metric that statistically differentiated responding from non-responding patients. The correlation of PSI with clinical outcome indicates the metric's potential as a biomarker for guiding personalized CAR-T cell treatments and potentially predicting therapeutic efficacy.

PSI of pre-infusion CAR-T product is shown to uniquely associate with objective response, where existing potency metrics do not

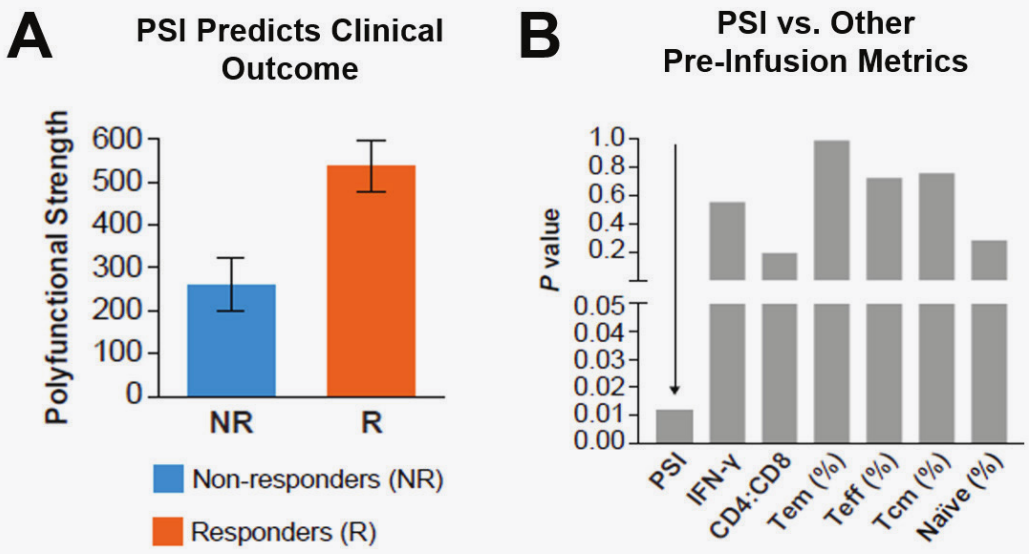

Figure 4 | Single-cell product PSI was determined for 20 patient donors by using Bruker's systems proteomic analysis of a panel of 32 secreted cytokines, chemokines, and cytotoxic molecules. Of the 20 patients, 14 had an objective response (OR) to the CAR T-cell therapy. (A) The results are shown as patient PSI mean ± standard error. All statistical values were computed using the Mann Whitney U test. (B) Pre-infusion CAR T cell product PSI outperformed other analyzed pre-infusion metrics, including IFN-γ co-culture cytokine intensities, $CD4^+$:$CD8^+$ T cell ratio, and various T cell phenotype frequencies, which did not show statistically significant associations with clinical response [2].

Bruker Protocol Frameworks to Provide Users with Precedent Workflows

Bruker systems have been used in a variety of research areas and contexts, as seen in Figure 5, to achieve understanding of correlates to *in vivo* activity in patients and mice. The below figure provides an overview of the variety of research areas, cell types, and indications where Bruker systems have been used to publish data to achieve correlates to response, i.e. patient differences, that explain mechanism *in vivo*. The Stimulation type and Enrichment type can act as a guide, with cited publications and presentations, that users can reference.

See Figure 5 for a variety of contexts in which our protocols for stimulation, enrichment, and labeling were used to achieve correlative outcome.

Protocol frameworks where Bruker systems achieved published correlates: precedent enrichment & stimulation workflows

	Immune Cell Type & Immunotherapy type	Indication	Enrichment	Stimulation type and Time	PSI Correlate
Cell Engineering & Therapy	Human CD19 CAR-T cells[5]	Cancer: Non-hodgkins Lymphoma	Beads	CD19 antigen	Responder correlate (p = 0.0117)
	Human TCR-Engineered Mart-1 T-cells[7]	Cancer: Solid Tumor	Tetramer	Mart-1 antigen	Tumor relapse correlate
	Human NK cells[10]	Cancer: Leukemia	Beads	IL-15 O/N + IL-12 / IL-18	Mouse tumor regression correlate
Checkpoint & Combination Therapies	Human PBMC, pre and post TLR Agonist Therapy[11]	Cancer: Sarcoma	Beads	CD3 / CD28	Responder correlate
	Human TILs, post checkpoint therapy[12]	Cancer: Solid Tumor	Beads	CD3	Responder correlate (p = 0.0294)
	Human PBMC, pre and post cancer vaccine[13]	Cancer: Solid Tumor	Beads	CD3 / CD28	Survival Correlate (p = 0.001)
Inflammation & Autoimmunity	Human Monocyte in healthy and diseased patients[14]	Autoimmune & CNS: Multiple Sclerosis	Beads	P3C or LPS	MS correlate (p = 0.0348)
	Human NK cells, no therapy[15]	Autoimmune: IBD and Crohn's disease	Flow Cytometry	PMA/Ionomycin	Inflammatory state correlate
Mouse Immunotherapy	Mouse TILS, post combination therapy[16]	Cancer: Solid Tumor	Flow Cytometry	CD3 / CD28	Mouse Response Correlate
	Mouse CD19 CAR-T cells[17]	Cancer, B-ALL	Beads	CD19 antigen	Product Analysis
	Mouse PBMC, post vaccine[16]	Infectious disease	Beads	CD3 / CD28	Survival correlate

Figure 5 | The goal for starting with a straightforward protocol that is recommended by Bruker is to ensure success and drive optimization. These protocols have been highly successful in detecting polyfunctional differences in the past (see Technology Note: Validation of Sample Preparation). We recommend not deviating from published protocols, but at the same time simplifying certain aspects that should not impact the success of the study, to ensure success for first time IsoLight users.

Conclusion

- Published enrichment, stimulation, and staining protocols throughout immunotherapy types and indications can help provide a framework to achieve goals of obtaining single-cell data, in multiple publications and presentations
- These enrichment and stimulation protocols minimize impacts on the live cells that are analyzed in Bruker's systems
- The type of sample preparation protocols and strategies have helped researchers achieve critical correlates to *in vivo* data
- While every user project is specific in terms of requirements, (e.g., stimulation time), precedent datasets can act as a rubric for choice of protocol.

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