TECHNICAL NOTE

Getting Started with Your Single-Cell Projects by Following Key Sample Preparation Guidelines

The detailed guidelines ensure that your enrichment, stimulation and labeling will allow you to get physiologically relevant in vivo data right away

In this technical note we outline:

- Guidelines on enrichment, stimulation, and staining, the key sample preparation protocols, that will help achieve your project goals
- Specific stimulation plans that have been published in the past within various Bruker research areas
- A sample preparation methodology that will allow multiple types of users to begin to achieve data right away, and ensure success in their run

The IsoLight System: An Overview

Bruker's IsoLight system employs its IsoCode® chip, a single-cell, highly multiplexed chip consisting of highly multiplexed antibody capture chip from each single cell. The IsoCode technology uniquely addresses the cytokine detection limitations with its ability to measure a large panel of 40+ 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 instrument is a sample in, result out automation system which automates the imaging, incubation and ELISA fluidics steps of the IsoCode assay (Figure 1), and provides streamlined bioinformatics tools for subsequent data analysis.

www.brukercellularanalysis.com

Prep, Run, Analyze

3 Key Sample Preparation Steps Prior to Loading onto the IsoLight Enable Success

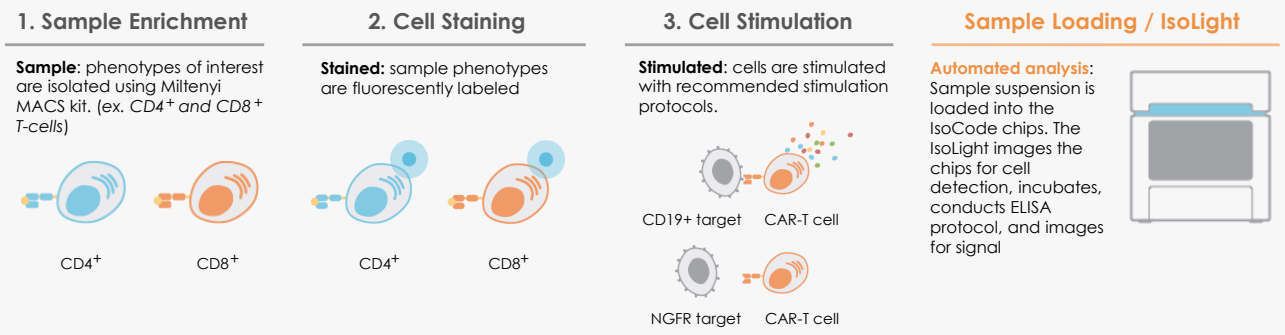

Figure 1 | Workflow of IsoLight for detecting secreted proteins from single cells. Step 1: Cells of interest are prepared by magnetic beads enrichment to enrich the phenotype of interest. Step 2: Enriched cells are either nonspecifically stimulated with anti-CD3 and anti-CD28 or specifically stimulated by co-culturing with antigen-expressing target cells overnight. Step 3: Stimulated cells are surface stained with General Stain provided by Bruker in each consumable kit.

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].

Recently, PSI™ 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].

Prep, Run, Analyze

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 (Figure 2a, 2b, 2c, 2d).

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 (See Technology Note: Sample Preparation Validation).

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

Similarly, 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 have been able to reveal differences that other methods were not able to uncover, using Bruker recommended protocols.

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 Staining
3. Cell Stimulation

Then, stimulated cells are loaded onto IsoCode chips, and automated proteomic analysis is performed on the IsoLight system. Choosing experimental conditions per Figures 2A to 2D will significantly improve the overall quality of single cell secretion analysis. There are four critically important elements for generating high quality of single cell proteomic data. (Enrichment, Staining, Stimulation, and Controls).

Utilizing Recommended Enrichment Methods.

ENRICHMENT GOAL

Capture phenotype that will provide enough polyfunctional cells to compare samples

REQUIREMENTS

- >80% viability before loading, minimum 25,000 cells per chip
- Retain recommended immune cell sub-types versus narrow multi-phenotype subsets
- Greater than 500 cells on chip of a given phenotype, enables recognition of polyfunctional subsets

Why Recommended Protocols Exist

- **Single sub-type phenotypic enrichment of T-cells (i.e. $CD4^+$) is recommended to reveal differences.** The cytokine producing subsets, around 5-25% of these cells, drive the correlative data. Multi-step phenotypic enrichment of a given subtype may miss polyfunctional sub-groups of an immune cell sample, i.e. the activity of key subtypes of $CD4^+$ or $CD8^+$ T-cells that drive correlates, simply based on cell frequency

- **Multi-step enrichment processes are to be avoided based on viability as well**: These multi-step enrichment processes additionally put greater stress on the sample and reduce overall cell counts for analysis.

- **Bead enrichment is the recommended and validated enrichment protocol for IsoPlexis' systems:** While other enrichment methods, i.e. flow sorting, are compatible with IsoCode Technology, bead enrichment ensures higher viability when getting started with IsoPlexis' systems. Additionally, as seen in "Sample Preparation: Technology Validation" Technology Note, many of the key in vivo correlates have been obtained with bead enrichment on IsoPlexis' systems

Figure 2a | Enrichment Methods

Utilizing Recommended Enrichment Methods

The goals of utilizing the recommenced enrichment methods are to capture the phenotypes that will provide enough polyfunctional cells to compare samples.

Single sub-type phenotypic enrichment of T-cells (e.g., $CD4^+$) is recommended to reveal differences. The cytokine producing subsets, around 5-25% of these cells, drive the correlative data. Multi-step phenotypic enrichment of a given subtype may miss polyfunctional sub-groups of an immune cell sample simply based on cell frequency as well as put greater stress on the sample and reduce overall cell counts for analysis.

Bead enrichment is the recommended and validated enrichment protocol for Bruker's systems. Bead enrichment ensures higher viability when getting started with Bruker's systems.

The recommended Bruker protocols utilize bead based enrichement based on precedent success. See Figure 3 for enrichment framework.

Utilizing the Provided Cell Labels

CELL STAINING GOAL

Stain the enriched phenotype to comply with IsoPlexis' IsoLight imaging requirements

REQUIREMENTS

- Utilize IsoPlexis' cellular stains provided in Consumable Kits

Why Recommended Protocols Exist

- **General stain provided in kits:** IsoCode technology requires that each cell be fluorescently labeled for cell detection. IsoPlexis offers a general live cell stain to meet this requirement, which stains the cells post enrichment to achieve staining of the enriched phenotype. The stains offered in the kits comply with IsoPlexis' imaging requirements, where as external stains may not be compliant.
- **Recommended to run single phenotypes per chip:** It is recommended to run a single phenotype per chip to get started, and ensure the maximum single cell counts (at least 500 cells of a given phenotype to ensure detection of the polyfunctional cell subsets).

Figure 2b | Cell Staining

Utilizing the Provided Cell Labels

The goals of utilizing the recommenced cell labels are to stain the enriched phenotype to comply with Bruker's IsoLight imaging requirements.

IsoCode technology requires that each cell be fluorescently labeled for cell detection. Bruker offers a general live cell stain to meet this requirement, which stains the cells post enrichment to achieve staining of the enriched phenotype.

It is recommended to run a single phenotype per chip to get started and maximize single cell counts (at least 500 cells of a given phenotype to ensure detection of the polyfunctional cell subsets).

Utilizing Recommended Stimulation Protocols.

STIMULATION GOAL

Reveal the critical differences when comparing samples, which capture correlates to in vivo activity

REQUIREMENTS:

- Following recommended protocols avoids both under and overstimulation of the cells
- Greater than 5% of sample population should be expected to secrete at least one cytokine

Why Recommended Protocols Exist

- **Using IsoPlexis' recommended protocols to get started:** The goal on IsoPlexis' system is to detect the differences between samples that other platforms may miss. It is recommended that to achieve this type of difference data that is unique & specific to IsoPlexis, IsoPlexis' specific protocols should be used in the given cell type and indication type as recommended. Using other platforms protocols, are not recommended; for example, non-recommended stimulation concentrations may lead to an under- or over- stimulation of your samples.

- **CD3/28 and other receptor stimulation vs. antigen specific stimulation**: To differentiate sample response, a core goal of runs on the IsoPlexis platform is that greater than 5% of the population must be activated to see polyfunctional subsets. In multiple cases, immune cell receptor stimulations reliant on CD3, CD28 and other examples have reactivated key cytokine programs in-vitro and revealed correlates. See published protocols to use as a guide.

- **Stimulation time to uncover differences**: Selecting the ideal timing is a balance between ensuring optimal stimulation to active cell programs, and not overstimulating cells and masking true biological differences. See recommended protocols to use as a guide.

Figure 2c | Stimulation Protocols

Using Recommended Stimulation Protocols

The goals of utilizing the recommenced stimulation protocols are to reveal the critical differences when comparing samples, to capture correlates to in vivo activity.

The goal on Bruker's system is to detect the differences between samples that other platforms may miss. It is recommended that to achieve this type of difference data that is unique & specific to Bruker, Bruker's specific protocols should be used in the given cell type and indication type as recommended.

To differentiate sample response, a core goal of runs on the Bruker platform is that greater than 5% of the population must be activated to see polyfunctional subsets.

Selecting the ideal timing is a balance between ensuring optimal stimulation to active cell programs, and not overstimulating cells and masking true biological differences. See recommended protocols to use as a guide.

Choosing Appropriate Controls

Controls are critically important for interpreting the data acquired from the IsoLight platform. Depending on the scientific question and application for the designed experiments, blank, biological stimulus or a population of samples that are a control versus the diseased or treated population, are highly recommended. While not required, these controls are recommended for better understanding of the data. These controls will add to the number of chips, so it is advised to choose controls that are appropriate based on precedent data.

Controls when getting started

Proper controls ensure confidence in patient differences and stimulation success. A stimulation control is recommended to validate researchers' ability to replicate recommended protocols when getting started. A control population that helps researchers observe critical differences that reveal choice is recommended as well.

Based on the application, certain controls are recommended for more informative data analysis and to validate researchers' ability to detect upregulation versus that control. It is recommended to choose a protocol that is appropriate based on precedent to start with, which can reduce the need for large groups of negative and positive controls.

Framework for starting out with Bruker protocols: Starting point for running IsoLight system

	Cell Type / Sample	Enrichment Framework	Stimulation Framework
Cell Engineering & Therapy	Human CAR-T[5]	Bead Based: Broad Phenotype CD4 & CD8	4-20 hour, antigen specific stimulation with K562
	Human TCR-T[7]	Bead Based: Broad Phenotype CD4 & CD8	4-20 hour, antigen specific stimulation with K562
	Human NK cells[10]	Bead Based: Broad phenotype CD56	IL-15 O/N recovery + 1 hour IL-12/IL-18 stimulation
Checkpoint & Combination therapies	Human T-cells from PBMC with agonists[11]	Bead Based: Broad Phenotype CD4 & CD8	24 hour, CD3/CD28 stimulation
	Human TILs from Tissue, post therapy[12]	Bead Based: Broad Phenotype CD4 & CD8	24 hour, CD3/CD28 stimulation
	Human T-cells from PBMC: cancer vaccine[13]	Bead Based: Broad Phenotype CD4 & CD8	24 hour, CD3/CD28 stimulation
Inflammation & Autoimmunity	Human Monocytes[14]	Adherence purification	24 hour LPS stimulation
	Human NK cells[15]	Bead Based: Broad phenotype CD56	PMA/ Ionomycin stimulation
Mouse immunotherapy	Mouse TILs[16]	Bead Based: Broad Phenotype CD4 & CD8	24 hour, CD3/CD28 stimulation
	Mouse CAR-T[17]	Bead Based: Broad Phenotype CD4 & CD8	4-20 hour, antigen specific stimulation with K562
	Mouse TCR-T[16]	Bead Based: Broad Phenotype CD4 & CD8	4-20 hour, antigen specific stimulation with K562

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.

Requirements and recommendations to achieve goals

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

- To generate high quality of single cell proteomic profiles on the IsoLight system, it is necessary to follow the enrichment protocols provided by Bruker, e.g., utilizing bead enrichment
- Additionally, following the above stimulation plans will help ensure that the initial runs on the IsoLight systems achieve data in line with precedent data sets, i.e. connect to in vivo physiology
- Finally, utilizing the provided cellular stains, and determining how many cellular stains to use at a given time will ensure you get the data you are looking for out of your phenotype of interest

References

1. Lu Y, Xue Q, Eisele MR, Sulistijo ES, Brower K, Han L, Amir el AD, Pe'er D, Miller-Jensen K, Fan R (2015) Highly multiplexed profiling of single-cell effector functions reveals deep functional heterogeneity in response to pathogenic ligands. Proc Natl Acad Sci U S A 112 (7):E607-615. doi:10.1073/pnas.1416756112
2. Precopio ML, Betts MR, Parrino J, Price DA, Gostick E, Ambrozak DR, Asher TE, Douek DC, Harari A, Pantaleo G, Bailer R, Graham BS, Roederer M, Koup RA (2007) Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. J Exp Med 204 (6):1405-1416. doi:10.1084/jem.20062363
3. Ding ZC, Huang L, Blazar BR, Yagita H, Mellor AL, Munn DH, Zhou G (2012) Polyfunctional CD4(+) T cells are essential for eradicating advanced B-cell lymphoma after chemotherapy. Blood 120 (11):2229-2239. doi:10.1182/blood-2011-12-398321
4. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, Hoff ST, Andersen P, Reed SG, Morris SL, Roederer M, Seder RA (2007) Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med 13 (7):843-850. doi:10.1038/nm1592
5. Rossi J, Paczkowski P, Shen YW, Morse K, Flynn B, Kaiser A, Ng C, Gallatin K, Cain T, Fan R, Mackay S, Heath JR, Rosenberg SA, Kochenderfer JN, Zhou J, Bot A (2018) Preinfusion polyfunctional anti-CD19 chimeric antigen receptor T cells are associated with clinical outcomes in NHL. Blood 132 (8):804-814. doi:10.1182/blood-2018-01-828343
6. Xue Q, Bettini E, Paczkowski P, Ng C, Kaiser A, McConnell T, Kodrasi O, Quigley MF, Heath J, Fan R, Mackay S, Dudley ME, Kassim SH, Zhou J (2017) Single-cell multiplexed cytokine profiling of CD19 CAR-T cells reveals a diverse landscape of polyfunctional antigen-specific response. J Immunother Cancer 5 (1):85. doi:10.1186/s40425-017-0293-7
7. Ma C, Cheung AF, Chodon T, Koya RC, Wu Z, Ng C, Avramis E, Cochran AJ, Witte ON, Baltimore D, Chmielowski B, Economou JS, Comin-Anduix B, Ribas A, Heath JR (2013) Multifunctional T-cell analyses to study response and progression in adoptive cell transfer immunotherapy. Cancer Discov 3 (4):418-429. doi:10.1158/2159-8290.CD-12-0383
8. Lin L, Ma C, Wei B, Aziz N, Rajalingam R, Yusung S, Erlich HA, Trachtenberg EA, Targan SR, McGovern DP, Heath JR, Braun J (2014) Human NK cells licensed by killer Ig receptor genes have an altered cytokine program that modifies CD4+ T cell function. J Immunol 193 (2):940-949. doi:10.4049/jimmunol.1400093
9. Fujiwara M, Anstadt EJ, Flynn B, Morse K, Ng C, Paczkowski P, Zhou J, Mackay S, Wasko N, Nichols F, Clark RB (2018) Enhanced TLR2 responses in multiple sclerosis. Clin Exp Immunol 193 (3):313-326. doi:10.1111/cei.13150
10. Zhu H, Blum RH, Wu Z, Bahena A, Hoel HJ, Ask EH, Guan KL, Malmberg KJ, Kaufman DS. Notch activation rescues exhaustion in CISH-deleted natural killer cells to promote in vivo persistence and enhance anti-tumor activity. ASH Annual Meeting 2018.
11. Seo YD, Zhou J, Morse K, Patino J, Mackay S, Kim EY, Conrad III EU, O'Malley RB, Cranmer L, Lu H, Hsu FJ, Xu Y, Loggers E, Hain T, Pillarisetty VG, Kane G, Riddell S, Meulen J, Jones RL, Pollack SM. Intratumoral (IT) injection of the toll-like receptor 4 (TLR4) agonist G100 induces a clinical response and a T cell response locally and systemically. Journal of Clinical Oncology, 36, Suppl 5S (2018).
12. Mackay S, Flynn B, Morse K, Paczkowski P, Chen J, Liu D, Bacchiocchi A, Heath JR, Fan R, Sznol M, Halaban R, Zhou J. Single-cell PSI of $CD8^+$ TILs in melanoma shows uniquely sensitive correlates with response to anti-PD-1/CTLA4 therapy, where histology and serum cytokines were unable to detect significant associations. SITC Annual Meeting 2018.
13. Mackay S, Flynn B, Chen J, Paczkowski P, Jaffee E, Zheng L, and Zhou J. Single-Cell Polyfunctionality of CD4+ T Cells Shows Promise as a Predictor of Overall Survival of Pancreatic Cancer Patients Treated with GVAX Vaccine. FOCiS 2018.
14. Fujiwara M, Anstadt EJ, Flynn B, Morse K, Ng C, Paczkowski P, Zhou J, Mackay S, Wasko N, Nichols F, Clark RB. Enhanced TLR2 Responses in Multiple Sclerosis. Clinical and Experimental Immunology (2018).
15. Lin L, Ma C, Wei B, Aziz N, Rajalingam R, Yusung S, Erlich HA, Trachtenberg EA, Targan SR, McGovern DPB, Heath JR, and Braun J. Human NK Cells Licensed by Killer Ig Receptor Genes have an Altered Cytokine Program that Modifies $CD4^+$ T Cell Function. Journal of Immunology, 193, 940-9 (2014).
16. Parisi G, Saco J, Salazar F, Krystofinski P, Tsoi J, Zhang R, Puig Saus C, Zhou J, Hu-Lieskovan S, Comin-Anduix B, Wu A, Charych DH, Ribas A. Enhanced Expansion and Tumor Targeting of Adoptively Transferred T Cells with NKTR-214. Session PO.CL06.03 - Adoptive Cell Therapy 3. American Association for Cancer Research Annual Meeting 2018.
17. Kotani H, Li Gongbo, Yao Jiqiang, Mesa TE, Chen J, Boucher JC, Yoder SJ, Zhou J, Davila ML. Aged CAR T cells exhibit enhanced cytotoxicity and effector function but shorter persistence and less memory-like phenotypes. ASH Annual Meeting 2018.