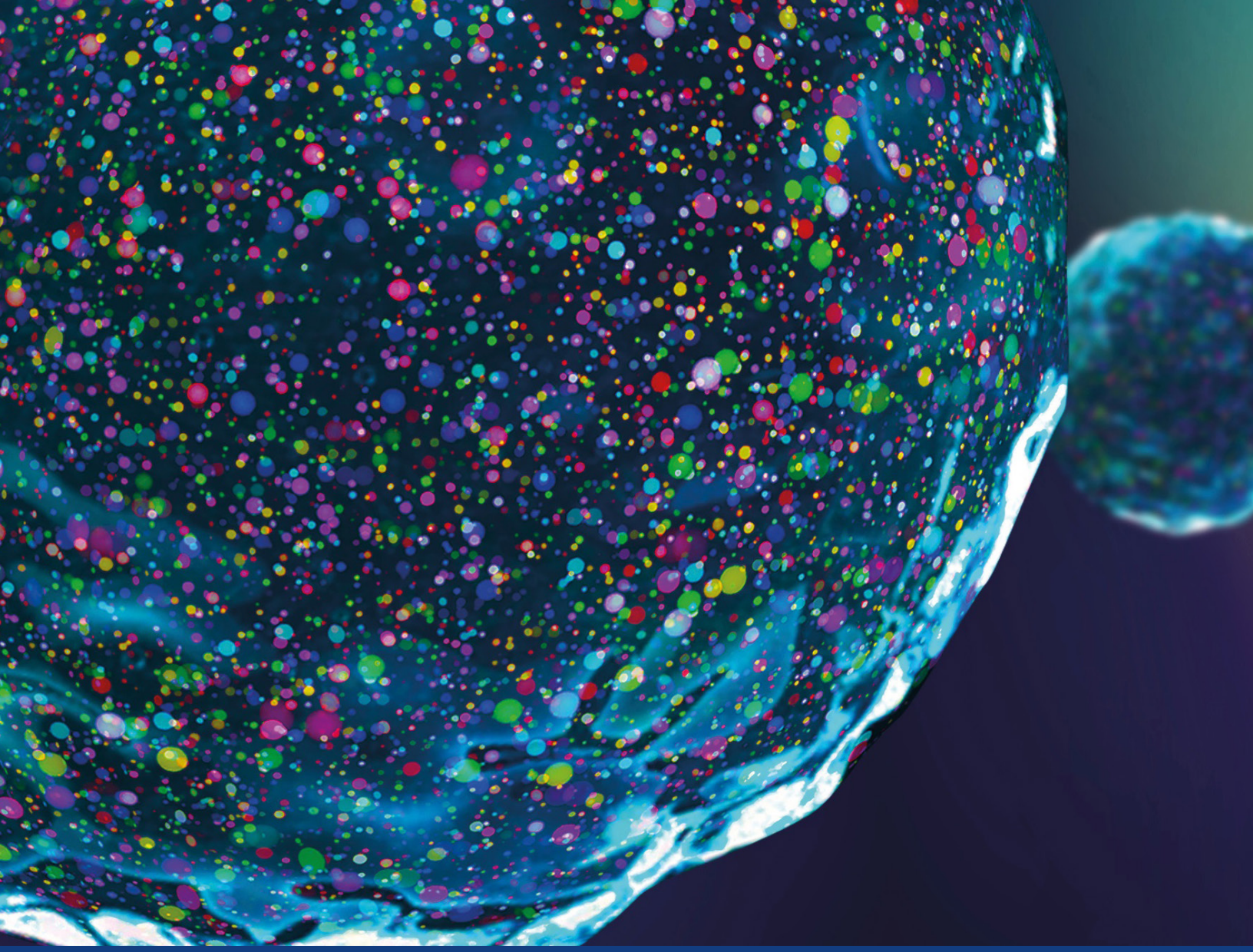

Exploring Cellular Complexity: The Power of Spectral Flow Cytometry

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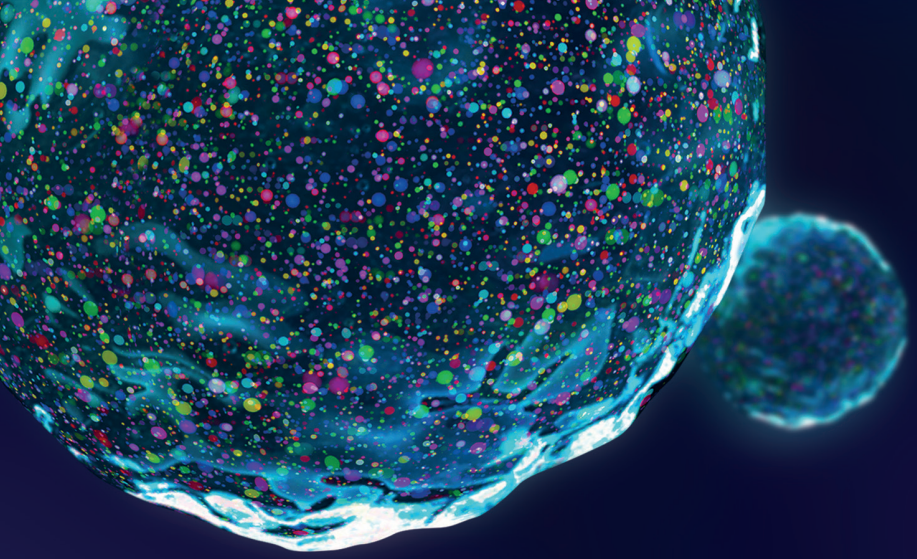

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Contents

Introduction

Spectral flow cytometry is a cutting-edge technology transforming research in immunology, cancer biology, oncology, and drug screening. By enabling high-dimensional data analysis it provides insights into cellular interactions and metabolic pathways. This technology allows for the simultaneous identification and analysis of multiple cell types, offering a comprehensive view of complex biological systems.

In cancer biology and oncology, it facilitates the study of tumor microenvironments and the immune landscape, contributing to the development of targeted therapies and personalized medicine approaches. This technology is particularly impactful in exploring the roles of cellular metabolism and regulatory mechanisms in cancer progression and treatment resistance. Previous studies have highlighted the significance of metabolic and regulatory pathways in cancer. For instance, research has shown how glucose metabolism in tumor-associated macrophages (TAMs) can enhance pro-tumor functions, promoting cancer metastasis and chemoresistance [1]. These insights into the tumor microenvironment reveal potential therapeutic targets for disrupting cancer progression. Similarly, the role of circular RNAs (circRNAs) in modulating cell death pathways, such as ferroptosis, has been explored in endometrial cancer [2]. The interaction between circRNAs and RNA binding proteins affects alternative splicing and contributes to cancer cell survival, presenting new opportunities for therapeutic intervention.

In immunology, spectral flow cytometry enhances our understanding of immune responses, enabling the identification of rare cell populations and the characterization of immune cell heterogeneity. Spectral flow cytometry is invaluable in drug screening and research, allowing for the evaluation of drug effects on cellular functions and interactions. Its ability to integrate phenotypic, functional, and metabolic data makes it a powerful tool for uncovering novel therapeutic targets and biomarkers.

This Expert Insights eBook begins with a review by Brestoff (2023) [3], which highlights the transformative potential of spectral flow cytometry in immunological research. The review showcases a 31-color spectral flow cytometry panel designed to investigate intercellular mitochondrial transfer using adipocyte-specific mitochondria reporter mice. By identifying 21 distinct cell types in a single-tube assay, this article demonstrates the power of spectral flow cytometry in enabling high-dimensional data analyses and a systems biology perspective on immune cell interactions. This article emphasizes the advantages of spectral flow cytometry over conventional methods, including higher dimensionality, panel flexibility, and increased sensitivity, paving the way for its clinical applications.

The second article by Verheijen *et al.* (2024) [4], discusses the intricate relationship between metabolism and immune cell function in the context of inflammatory diseases. The study emphasizes the importance of considering the local microenvironment's impact on immune cell metabolism and highlights the challenges in studying metabolic crosstalk. By evaluating current technologies such as single-cell omics, flow cytometry-based methods, and mass spectrometry imaging, the authors provide insights into the applications of metabolomics in clinical research. The article concludes with a practical flowchart guiding researchers in developing effective strategies to investigate immunometabolism in disease-relevant contexts.

The third article by Njock *et al.* (2022) [5], explores the technical advances in immunometabolism research, focusing on the spatial heterogeneity of the metabolome and the development of advanced model systems. The study highlights the use of mass spectrometry imaging (MSI) techniques for spatial metabolomics, enabling direct tissue measurements while preserving spatial information. Additionally, the article discusses the development of sophisticated *in vitro* model systems, such as organ-on-a-chip platforms and three-dimensional co-culture models, to study metabolic crosstalk between different cell types. These

advancements, coupled with stable isotope tracing and flow cytometry-based methods, provide unique insights into the complex interactions within immune microenvironments.

Overall, spectral flow cytometry is a transformative technology that enhances our understanding of cellular interactions and metabolic pathways. By providing high-dimensional data and the ability to analyze multiple cell types simultaneously, it offers valuable insights into complex biological systems. This technology is crucial for advancing research, identifying therapeutic targets, and improving personalized medicine.

Through the methods and applications presented in this Expert Insights eBook, we hope to educate researchers on new technologies and techniques for spectral flow cytometry. To gain a deeper understanding of available options for improving your research, we encourage you to visit Sony Biotechnology.

Andrew Dickinson, Ph.D.
Content Strategist at Wiley

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Full Spectrum Flow Cytometry in the Clinical Laboratory

Adapted from Brestoff 2023.

Spectral flow cytometry enables high-dimensional analysis of cells in suspension, capable of detecting 35+ antigens simultaneously in a single-tube format. This review contrasts spectral and conventional flow cytometry principles, highlighting how spectral cytometers capture a "spectral fingerprint" of each fluorophore's emitted light using numerous detectors per laser. The technology distinguishes fluorophores with up to 98% similarity, allowing for significantly more parameters than conventional systems. The analytical power of spectral flow cytometry is demonstrated through examples of data analysis incorporating machine learning algorithms to extract meaningful information from complex datasets. Having recently received regulatory approval in China and Europe for clinical use, preliminary studies suggest spectral flow cytometry performs comparably to conventional systems while offering greater multiplexing capabilities, potentially transforming clinical flow cytometry applications.

Introduction

Flow cytometry is a powerful technology used to analyze and measure physical, chemical, and/or gene expression characteristics of cells or particles in suspension. This technique allows clinical laboratories and researchers to measure numerous properties of individual cells rapidly, quantitatively, and in a multiplexed manner. Given its high flexibility, flow cytometry has gained widespread adoption across scientific fields including immunology, cell biology, cancer research, and developmental biology.

In clinical settings, flow cytometry is most commonly used to support the diagnosis of primary and secondary immunodeficiencies and hematologic malignancies, monitor responses to therapy, detect disease progression or recurrence, and support cellular therapies, including stem cell enumeration for transplants and manufacturing engineered cells like chimeric antigen receptor (CAR) T cells.

While conventional flow cytometers have been used in clinical laboratories for decades, full-spectrum flow cytometry (spectral flow cytometry) has recently gained significant traction in research settings and is beginning to be used in clinical laboratories in some countries. The primary advantage of spectral flow cytometry is its ability to measure over 35 antigens per cell in a single-tube assay format.

Basic Principles of Conventional and Spectral Flow Cytometry

The fundamental principle of all flow cytometry technologies is passing a single-file stream of cells or particles in suspension through a laser beam to measure each cell's physical and chemical features. As cells pass through the laser light, they scatter light in the forward direction (related to cell size and refractive index), scatter light to the side (side scatter, indicating cell complexity), and emit light from attached fluorophores.

While some cell types can be identified based purely on their forward scatter (FSC) and side scatter (SSC) properties (as is done in complete blood counts), many lymphocyte subsets have similar FSC and SSC profiles. Therefore, flow cytometry's primary value is its ability to measure cell type-specific protein antigens using fluorophore-labeled monoclonal antibodies.

The key challenge in flow cytometry is "spectral overlap," which occurs when emission spectra of different fluorophores overlap considerably, and when different fluorophores can be excited by more than one laser. The fundamental difference between conventional and spectral flow cytometry is how spectral overlap is handled.

In conventional flow cytometry:

- Each laser has a set of light filters with photon detectors (typically photomultiplier tubes).
- Each filter allows only certain wavelengths to reach the detector.
- Only one detector is associated with each filter, which isolates "on-target" light and reduces detection of "off-target" light.
- A compensation matrix is calculated to adjust for the remaining spectral overlap.

In spectral flow cytometry:

- Essentially all emitted light from each fluorophore is captured by many detectors for each laser.
- The emission of each fluorophore is measured by dozens of detectors simultaneously, which establishes a "spectral fingerprint" for each fluorophore.
- Single-color reference controls allow the computer to perform spectral unmixing (analogous to compensation).

The major advantage of spectral flow cytometry is that fluorophores with up to 98% similarity can be distinguished from each other and used in the same tube. For example, APC and Alexa Fluor® 647, which have very similar emission peaks (660 nm and 665 nm) and cannot be distinguished on conventional cytometers, can be easily differentiated on spectral cytometers by detecting small differences in their emission spectra.

Theoretically, 5-laser spectral flow cytometers could detect over 60 fluorophores simultaneously, though practical limitations on available reagents mean that a conservatively designed panel could include over 35 distinct markers in a single-tube assay.

Spectral Flow Cytometry Allows for High-Dimensional Data Analyses

To illustrate the analytic power of spectral flow cytometry, this review describes laboratory work studying intercellular mitochondria transfer using adipocyte-specific mitochondria reporter mice (MitoFat mice). The author developed a 31-color spectral flow cytometry panel (one viability dye, one fluorescent reporter, and 29 antibody-fluorophore conjugates) that identified 21 distinct cell types in a single-tube assay.

To identify non-immune cell populations, batch-correction methods from single-cell RNA sequencing were adapted for spectral flow cytometry. Two panels were created with 60% marker overlap, with the remaining 40% of the second panel dedicated to novel non-immune markers and mitochondria capture receptors. The overlapping antibodies allowed computational mapping between panels, enabling confident identification of 5 non-immune populations. These populations were characterized as two distinct fibroblast lineage subsets (adipocyte progenitor cells and fibroinflammatory progenitors) and three heterogeneous endothelial cell populations. This bioinformatic approach demonstrates how spectral flow cytometry enables systems biology perspectives on immune cell interactions rather than examining cell types individually.

Advantages of Spectral Flow Cytometry

Several advantages of spectral flow cytometry compared to conventional flow cytometry include the following:

1. Higher dimensionality - spectral flow can detect and distinguish many more fluorophores simultaneously, allowing for more complex and informative multicolor analyses.

2. Panel flexibility - multiple panels can be stitched together for deeper phenotypic datasets, enabling core panels with add-on tubes for customized diagnostic analyses.

3. Systems biology approach - immune cell populations can be studied as an interacting network, potentially revealing hidden patterns of immune system dysregulation.

4. Autofluorescence handling - cell autofluorescence can be eliminated as a variable, which is particularly important when analyzing myeloid and plasma cell populations.

5. Operational ease - setting up spectral flow cytometers and performing spectral unmixing is described as easier and faster than comparable processes on conventional cytometers.

Clinical Applications of Spectral Flow Cytometry

Some spectral flow cytometers have received regulatory approval for clinical use in China and Europe, but currently, there is no FDA *in vitro* diagnostic designation for any spectral flow cytometer in the United States.

Given the recent deployment of spectral flow cytometry in clinical settings, there are limited use cases to draw upon. However, several clinical studies and preliminary data are described:

1. A peer-reviewed study using a 27-color single-tube assay on a 3-laser cytometer for detecting measurable residual disease in acute myeloid leukemia patients, which achieved a detection limit of 0.0013% for abnormal myeloblasts and showed concordance with an 8-color multi-tube assay run on a conventional cytometer.

2. Non-peer-reviewed studies include:

- A 24-color single-tube assay that correlated highly with conventional 3- or 8-color flow cytometers
- A 23-color single-tube assay that yielded comparable results when compared against a 4-tube assay with the same markers for multiple myeloma diagnosis

These method-comparison studies provide preliminary evidence that spectral flow cytometers are functionally equivalent to conventional flow cytometers but superior in their ability to perform high-dimensional assays in a single-tube format.

Conclusions

Spectral flow cytometry is an exciting technology that allows for high-dimensional analyses of cells with dozens of fluorophores in single-tube assays. It has gained substantial traction in research settings and is now available to clinical laboratories in China and Europe.

Additional head-to-head method comparison studies are needed to ensure that spectral flow cytometers perform as well as or better than conventional flow cytometry platforms in various clinical assays. These studies will increase confidence in the clinical flow cytometry community regarding the adoption of this technology.

Spectral flow cytometry has the potential to transform capabilities in clinical flow cytometry laboratories by enabling the development and deployment of ultra-high-dimensional assays, potentially unlocking new diagnostic approaches in clinical cytometry.

References

[1] Brestoff, J.R. (2023). Full spectrum flow cytometry in the clinical laboratory. *International Journal of Laboratory Hematology.* DOI: 10.1111/ijlh.14098.

The ID7000™ Spectral Cell Analyzer from Sony Biotechnology represents an innovative solution for clinical and research laboratories seeking to enhance their flow cytometry capabilities with next-generation spectral technology. Listed below are ways this instrument can help address current research challenges in cancer biology, oncology, immunology, disease research, and drug screening.

Key Features and Advantages

High-Dimensional Analysis

- Supports up to 44 colors in a single panel, exceeding the conservatively estimated 35+ antigens discussed in the review.
- Enables simultaneous detection of multiple antigens in complex cell populations.
- Reduces sample volume requirements with single-tube assays.

Spectral Flow Cytometry Technology

- Captures full emission spectra using many detectors per laser.
- Creates unique "spectral fingerprints" of each fluorophore.
- Distinguishes fluorophores with up to 98% spectral similarity.
- Enables the use of fluorophores with overlapping emission spectra (e.g., APC and AF647).

Clinical and Research Applications

- Supports identification of rare cell populations with high sensitivity.
- Facilitates deep immunophenotyping of complex samples.
- Ideal for analyzing samples from hematologic malignancies, immunodeficiencies, and monitoring therapy responses.
- Enables development of high-dimensional laboratory-developed tests.

Addressing Clinical Needs

Operational Advantages

- Simplified panel design and setup compared to conventional flow cytometers.
- Reduced compensation complexity through spectral unmixing.
- Increased operational efficiency with single-tube assays versus multi-tube protocols.

Enhanced Analytical Capabilities

- Accounts for cellular autofluorescence, improving analysis of myeloid and plasma cell populations.
- Supports machine learning algorithms for advanced data analysis.
- Enables systems biology approach to immune cell population analysis.

Advancing Clinical Applications

- Comparable performance to conventional cytometers in method comparison studies.
- Potential for high sensitivity detection of residual disease (as low as 0.0013%).
- Enables the development of comprehensive immunophenotyping panels within a single tube.

Deciphering Metabolic Crosstalk in Context: Lessons from Inflammatory Diseases

Adapted from Verheijen, *et al.*, 2024.

Metabolism critically regulates immune cell function in health and disease, with alterations contributing to cancer and inflammatory disease pathogenesis. The local microenvironment significantly impacts immune cell metabolism, necessitating consideration of immunological and metabolic heterogeneity alongside spatial organization when investigating immunometabolism. This review addresses challenges in studying metabolic communication and evaluates current technologies for examining metabolism in inflammatory microenvironments, including single-cell omics, flow cytometry-based methods (Met-Flow, SCENITH, CyTOF, CITE-Seq), and mass spectrometry imaging. Given the importance of metabolism in regulating immune cells during disease, the authors discuss metabolomics applications in clinical research and obstacles to implementing these techniques in clinical practice. The review concludes with a practical flowchart guiding researchers in developing effective strategies to investigate immunometabolism in disease-relevant contexts, highlighting the need for integrated approaches to comprehensively understand metabolic crosstalk between immune cells.

Introduction

Impact of Immunometabolic Interactions

Metabolism plays a crucial role in regulating immune cell function, with metabolic changes accompanying immune cell activation. Naive T cells, for example, rely on oxidative phosphorylation before switching to aerobic glycolysis upon activation. Metabolic states also regulate immune cell differentiation and function. The tumor microenvironment exemplifies this relationship—tryptophan depletion in the tumor microenvironment can induce regulatory phenotypes in T cells.

Immune cells are profoundly influenced by their local microenvironment. They respond to tissue-specific signals, including locally produced metabolites, growth factors, adhesion molecules, nutrients, and oxygen availability. Immune cells demonstrate remarkable metabolic plasticity, expressing tissue-specific transcription factors to adapt their nutrient utilization strategies to local conditions. For instance, alveolar macrophages in the lipid-rich pulmonary environment express high levels of CD36 to support fatty acid uptake, while bone marrow-derived monocytes acquire Kupffer cell identity in the liver via factors like desmosterol.

Metabolic crosstalk between cells modulates inflammatory responses through metabolite signaling, cross-feeding, or nutrient competition. Nutrient competition between cancer and immune cells creates immunosuppressive environments, while autoimmune disease microenvironments often drive immune cell overactivation. Metabolites like itaconate, lactate, and succinate mediate inflammatory responses. For example, tumor-associated macrophage-derived itaconate reduces nucleotide synthesis and cytokine secretion in CD8+ T cells, while lactate can either suppress CD8+ T cell proliferation in tumors or exacerbate inflammatory responses in CD4+ T cells at inflamed sites.

This central role of metabolism in regulating immune cell functionality and inducing either inflammatory or immunosuppressive microenvironments makes cellular metabolism an interesting target to boost or dampen the immune response for the treatment of cancer or autoimmune diseases, respectively.

Challenges in Studying Immunometabolic Interactions

Several challenges persist in advancing our understanding of immunometabolism and immunometabolic crosstalk (Fig. 1). The current understanding is largely based on bulk metabolomics, which overlooks metabolic heterogeneity in microenvironments and misses metabolic features of minor but potentially important cell subsets. While single-cell RNA sequencing (scRNA-seq) has improved the characterization of immune landscapes, it does not provide information on actual metabolite levels, making metabolic crosstalk studies difficult.

Spatial resolution presents another challenge—the cellular organization of tissues determines if and how cells engage in metabolic crosstalk. Most metabolic techniques require cell isolation, losing information about cellular structure and metabolite distribution within tissues.

Additionally, *in vitro* models often inadequately represent *in vivo* conditions. Metabolic adaptation in immune cells differs between *in vitro* and *in vivo* systems, with *in vitro* conditions rarely matching the tissue-specific signals and nutrient levels of immune microenvironments. There is an urgent need for complex *in vitro* models that can be interrogated for metabolic status and crosstalk.

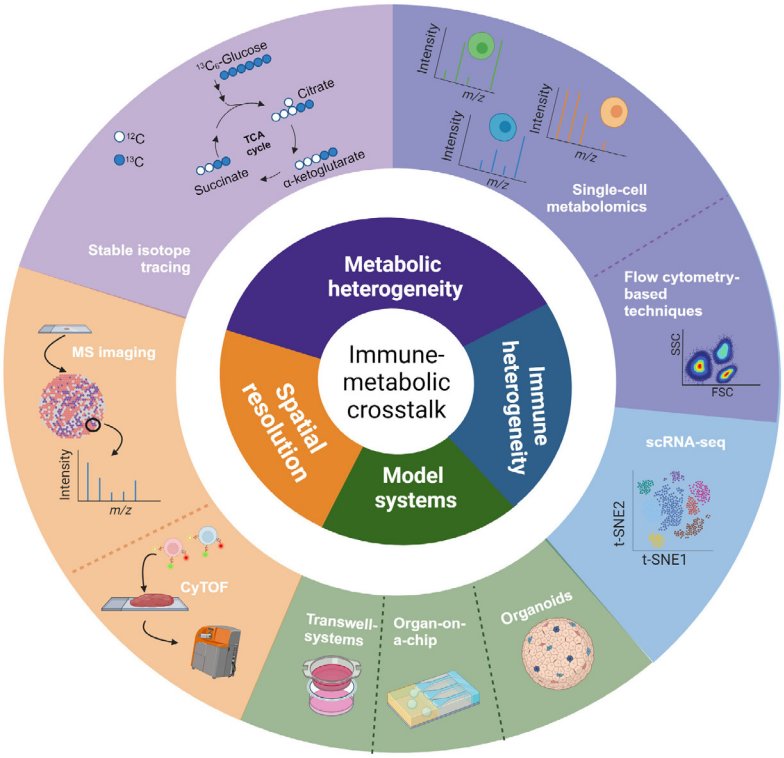


Figure 1. State-of-the-art techniques to unravel immune and metabolic heterogeneity as well as spatial resolution in the context of inflammatory microenvironments. Several challenges prevent us from obtaining a clear view of the complex interplay of (immune) cells within physiological conditions. Within one sample, heterogeneity can occur at the immune level, metabolic level, or a combination of the two. Several techniques can be utilized to unravel these levels of heterogeneity, such as single-cell metabolomics (SCM), single-cell RNA sequencing (scRNA-seq), stable isotope tracing, and flow cytometry-based techniques (Met-Flow and single-cell energetic metabolism by profiling translation inhibition (SCENITH)). Moreover, to acquire more information on a spatial level, mass spectrometry imaging (MSI), such as matrix-assisted laser desorption/ionization (MALDI-TOF), or cytometry by time of flight (CyTOF) can be utilized to measure metabolites or metabolic proteins, respectively. Finally, sophisticated model systems, such as Transwell systems, organ-on-a-chip, and organoids, can increase our knowledge of the impact of immunometabolism *in vivo*, as they more effectively mimic physiological conditions.

Technical Advances in Immunometabolism Research

Exploring Immune and Metabolic Heterogeneity on the Single-Cell Level: Single-Cell Omics Methods for Mapping Immune and Metabolic Heterogeneity: scRNA-seq and Single-Cell Metabolomics

scRNA-seq has provided insights into the transcriptional regulation of metabolic pathways. For example, scRNA-seq identified asparagine synthetase as an important modulator of CD8+ T cell differentiation. When combined with other single-cell technologies, scRNA-seq contributes to integrating metabolic and immune heterogeneity. However, scRNA-seq has limitations (Table 1): it does not provide information on protein expression or metabolic enzyme activity but serves only as an indirect measure of metabolic status.

Single-cell metabolomics (SCM) profiles metabolites in individual cells isolated by flow cytometry or other methods. This approach has revealed metabolic differences between circulating tumor cells from different cancer types and can measure drug levels in single cells. While SCM provides direct metabolite measurements with high resolution of heterogeneity, challenges include dependency on single-cell isolation (which affects sample quality and metabolic status), limited metabolite coverage, and low throughput.

Table 1. Advantages and disadvantages of advanced methods applicable to resolve challenges in studying immunometabolism.

Method (*Used in clinical samples)	Advantages	Disadvantages
Challenge 1: Studying immune and metabolic heterogeneity		
scRNA-seq*	• Unbiased • Combined metabolic and phenotypic information	• Limited sample size • Discordance between mRNA levels and protein levels/protein functionality • Indirect method to measure metabolism
SCM	• Low cell numbers needed • Direct metabolite measurements • High resolution of heterogeneity	• Limited number of metabolites covered • Costly and time-consuming • Single-cell isolation is needed, which might affect sample quality and metabolic status
SCENITH * Met-Flow	• Combined phenotypic and metabolic information • Fast • Most facilities have the appropriate machinery • High-throughput acquisition and resolution of low-expressed markers (spectral flow cytometry)	• Fluorescent spillover • Indirect metabolic measurements • Selection of targets needed
CITE-seq*	• Quantifies cell surface proteins alongside RNA molecules in a single-cell fashion • Applicable for tissue as well as body liquids and cultured cells	• Indirect metabolic measurements • Selection of the protein targets needed
Stable isotope tracing	• Information about metabolic pathway activity and metabolite sources	• Tracers are expensive • So far mostly been used in bulk analysis • Combinations of tracers require ultra-high-resolution equipment
Challenge 2: Resolving spatial resolution		
CyTOF*	• High dimensional • High throughput • Applicable for spatial as well as single-cell samples • Metabolic profile obtained on protein level	• Not applicable for weakly expressed markers • Limited number of protein markers • Advanced biostatistics and bioinformatics needed • Indirect metabolic measurement • Machinery not available at all research facilities • Lower sensitivity compared to flow cytometry
Imaging MS (MSI), such as MALDI-MS*	• Direct measurements of metabolism *in situ* • Retain intact tissue structure • Potential to measure a broad range of lipids/metabolites	• Must be combined with other histological or morphological information to couple metabolic to phenotypic characteristics • Sensitivity for low *m/z* (70–500 Da) remains limited (MALDI) • Challenging identification due to high matrix interruption (MALDI) • Advanced techniques/instrument are needed, which are not available at all research facilities
Advanced co-culture systems	• Method to study the metabolic interplay between specific cells • Especially suited for studying cell–cell interactions through secreted factors • Rapid separation minimizes disruption of the metabolome during isolation • Can be personalized by including patient materials • Valuable for pathogenetic/mechanistic studies	• Separation is needed before single-cell metabolism studies can be performed • Limited in the number of co-cultured cell types • Disregards circulation and lymphoid system • Artificial concentrations of nutrients

Antibody-Based Methods to Study Metabolism at Single-Cell Resolution: SCENITH, Met-Flow, CITE-seq, and CyTOF

There are several flow cytometry-based techniques for studying immune cell metabolism at the single-cell level. These methods allow researchers to combine metabolic data with phenotypic and functional information in a single measurement.

Two flow cytometry-based methods are used to study metabolic regulation: SCENITH (Single-Cell Energetic Metabolism by Profiling Translation Inhibition) and Met-Flow. SCENITH uses metabolic enzyme inhibitors combined with puromycin to measure protein synthesis as a proxy for global metabolic activity. This technique has been applied to cultured cells, tumor biopsies, and blood samples, enabling researchers to determine cellular dependency on specific metabolic pathways. Met-Flow uses fluorophore-labeled antibodies against key metabolic enzymes and cell surface transporters, alongside lineage-specific markers to identify different cell populations.

Other, newer techniques with applications for immunometabolism include CITE-seq (Cellular Indexing of Transcriptomes and Epitopes by Sequencing), which combines single-cell RNA sequencing with antibody-based protein measurements, allowing simultaneous quantification of cell surface proteins and RNA molecules. This method has been used to study immune landscapes in various diseases including cancer and COVID-19. CyTOF-based methods utilize metal-tagged antibodies and mass spectrometry to analyze protein markers (Fig. 2). When performed on tissue slices, CyTOF provides spatial information about metabolic and immunological proteins. One such method, scMEP (single-cell metabolomic regulome profiling), has been used to study immune cell phenotypes in colorectal cancer.

While these techniques offer many advantages, including single-cell resolution, the ability to analyze rare cell populations, and integration with other data types, they also have limitations. These include the indirect nature of metabolic measurements, dependency on antibody availability, marker number limitations, and reduced sensitivity for weakly expressed proteins.

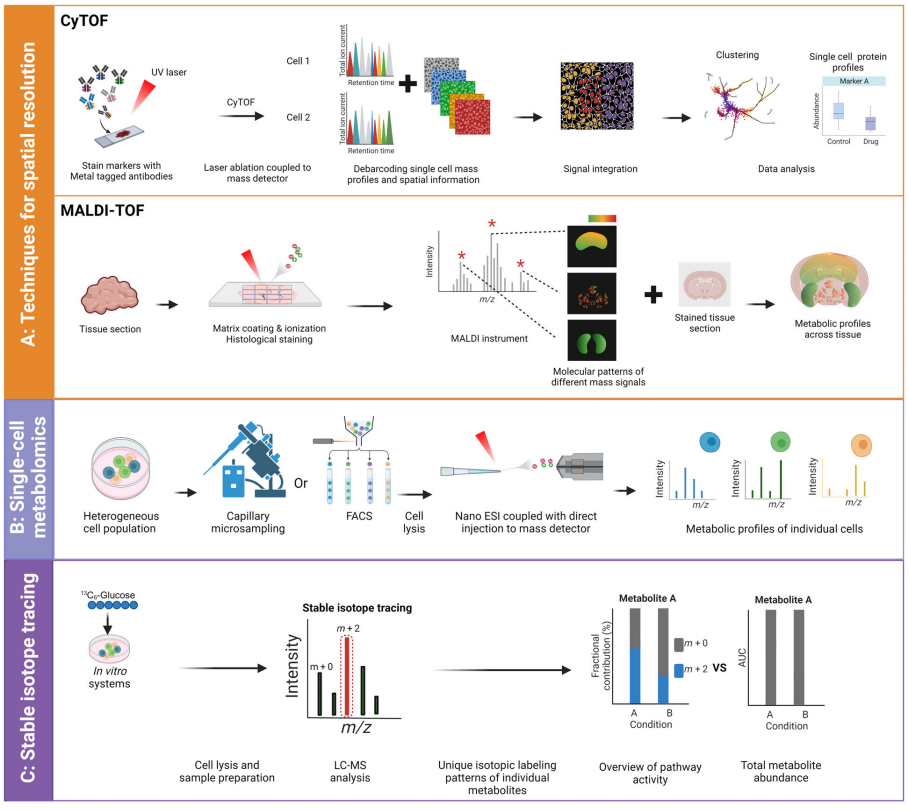


Figure 2. Workflows of selected state-of-the-art methods for studying metabolism. (A) Techniques for spatial resolution. Cytometry by time of flight (CyTOF) representative workflow: protein markers in samples are stained with stable heavy metal-tagged antibodies, ionized by laser ablation, and analyzed with a mass detector, often TOF. Single-cell mass profiles are de-barcoded and combined with spatial information, followed by downstream data analysis and visualization. In MS imaging, for example, in MALDI-TOF, a tissue section is coated with a matrix for extraction and ionization of metabolites which are subsequently measured with a TOF mass detector. Histological staining can be performed on consecutive tissue sections, to simultaneously allow for immune phenotypic characterization of (immune) cells within the tissue. Data analysis results in spatially resolved mass spectra, which can be combined with histological results. **(B)** For single-cell metabolomics (lilac panel), cells are isolated by either direct capillary microsampling or fluorescence activated cell sorting (FACS), followed by nano-electrospray ionization (nano-ESI) coupled to direct injection into a mass spectrometer, thereby, providing metabolic profiles of individual cells. **(C)** Unlike steady-state metabolomics, which only provides metabolite abundances, stable isotope tracing (dark purple panel) provides information on pathway activities. Incubation with tracers of choice, for example, $^{13}C_6$-glucose ***in vitro*** or ***in vivo*** is followed by sample collection, metabolite extraction, and mass spectrometry. Data are analyzed as fractional contribution (%) of tracer relative to the total intensity of a given metabolite. Differences in pathway activities in condition A versus B can be derived from differences in isotope fractions, which persist in the absence of differences in total metabolite abundances.

Understanding the Spatial Heterogeneity of the Metabolome

Mass spectrometry imaging (MSI) is a prominent technique for studying spatial metabolomics in situ. Methods like secondary ion mass spectrometry (SIMS), desorption electrospray ionization (DESI), and MALDI (Fig. 2) allow direct tissue measurements while preserving spatial information. When coupled with immunohistochemistry, MSI can spatially resolve metabolic and functional heterogeneity, revealing distinct lipid signatures in plaque-resident macrophages or metabolite distributions corresponding to immune cell subsets. Current MSI limitations include low sensitivity for certain mass ranges and matrix interference affecting compound identification (Table 1).

Development of Advanced Model Systems to Map Metabolic Crosstalk

Model Systems to Resolve Cell-Cell Metabolic Interactions

In vitro model systems have significantly advanced immunometabolism research by providing controllable experimental parameters. While simpler cell cultures lack the complexity of *in vivo* interactions, more sophisticated models offer opportunities to study metabolic crosstalk between different cell types.

Transwell systems enable the study of interactions mediated by secreted factors and allow quick cell separation for metabolite extraction with minimal disruption. Research using these systems has revealed how cancer cells can influence immune cell metabolism, such as thyroid cancer cells stimulating lipid synthesis in monocytes, which affects cytokine secretion and reactive oxygen species production.

More advanced platforms like tumor/organ-on-a-chip systems recreate key characteristics of *in vivo* microenvironments by preserving tissue mechanics, cellular compositions, and matrix signals. Examples include models studying inflammatory bowel disease and its connection to liver diseases, and devices investigating coronavirus effects on the human intestine.

Three-dimensional co-culture models, such as organoids, maintain microenvironment complexity and provide platforms to study multicellular metabolic interactions. These models can preserve the metabolic features of original tissues, as demonstrated in kidney spheroids, and can be personalized using patient materials.

Advances in Stable Isotope Tracing to Map Metabolic Reactions *in vitro*

Stable isotope tracing provides information on pathway dynamics rather than just metabolite levels. Cells are incubated with isotopically labeled nutrients, and the incorporation of labels into downstream metabolites is measured by GC/LC-MS. This approach has been pivotal in understanding metabolic switches in immune cells and linking metabolic regulation to functionality. Combining isotope tracing with advanced co-culture systems enables mapping of metabolic crosstalk in complex microenvironments. Limitations include high costs and the need for ultra-high-resolution equipment when combining multiple tracers.

Immunometabolism in Clinical Practice

Metabolomics analyses have gained prominence in clinical research for identifying drug targets and biomarkers, and for diagnostic and prognostic purposes (Fig. 3). Targeted metabolomics methods enable analysis of relevant metabolic pathways with reliable identification and quantification. For example, a targeted LC-MS method was developed to measure 220 metabolites associated with inborn errors of metabolism. Untargeted metabolomics extends coverage to discover novel biomarkers but faces challenges in metabolite identification.

Flow cytometry-based methods are particularly well-suited for clinical immunometabolism research due to their high throughput. SCENITH has been used to study metabolic regulation differences between CD4+ T cells in healthy volunteers versus COVID-19 patients. However, standard application of immunometabolism techniques in clinical research and practice remains challenging, requiring specialized equipment and trained personnel.

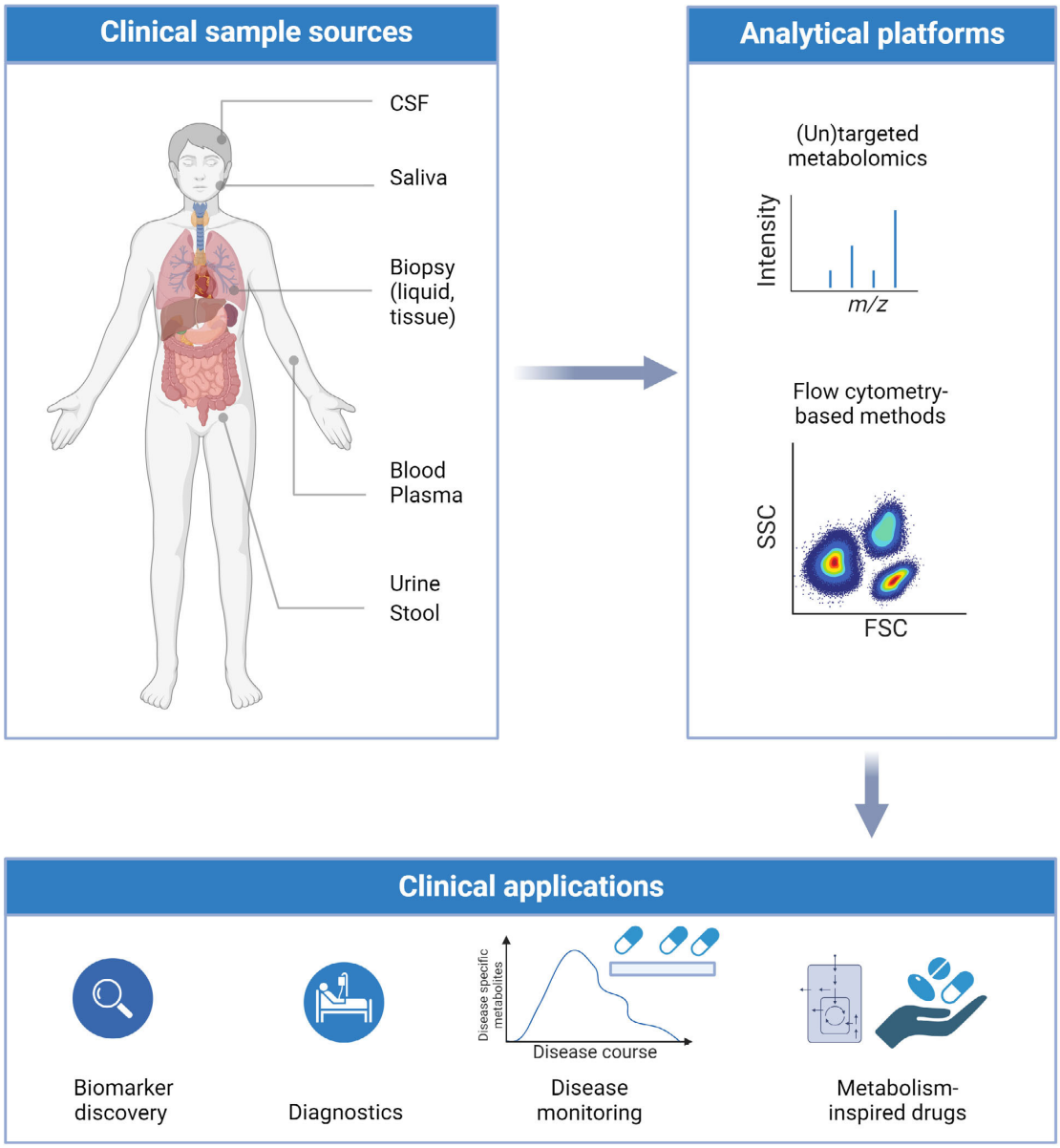


Figure 3. Immunometabolism techniques and their clinical applications. Increasing metabolic characterization of various sample types can yield important insights into the development of metabolism-inspired biomarkers and drug delivery platforms, and improving therapeutics. Moreover, this increased metabolic characterization can aid disease monitoring and the assessment of treatment efficacy. Sample material for metabolomics research in the clinic includes various body fluids, such as plasma, whole blood, urine, saliva, and cerebrospinal fluid (CSF), as well as tissue slices, stool, and biopsies from various tissues. These samples can be analyzed via different techniques including (un)targeted metabolomics or flow cytometry-based metabolic methods.

Conclusions

Understanding immune homeostasis requires deciphering fine-tuned metabolic crosstalk at cellular, tissue, and organ levels. Metabolites function as rapid messengers reflecting real-time cellular states and acting through various communication modules. Emerging technologies are helping untangle this complexity: advanced mass spectrometry methods like single-cell metabolomics, computational pipelines for spatial SCM, flow cytometry-based characterization, CyTOF, stable isotope tracing, and sophisticated co-culture systems all provide unique insights (Fig. 4).

For optimal results, researchers should combine multiple techniques to achieve a comprehensive view of heterogeneity. The integration of single-cell or spatial metabolomics with proteomics, transcriptomics, and genomics promises to advance understanding at a systems biology level. Current challenges include optimizing cell isolation procedures for single-cell metabolomics and expanding spectral flow cytometry applications to overcome marker limitations.

The parallels between cancer metabolism and immunometabolism suggest mutual benefits from shared discoveries and methodological advances. Integrating knowledge from both fields is essential for future research. Technological advancements will likely provide greater breadth (spatial and untargeted), depth (single-cell, isotopic characterization), and comprehensive (relevant models and multi-omics) resolution of metabolic crosstalk in health and disease, facilitating metabolism-targeted personalized medicine and improved therapeutics for inflammatory diseases and cancer.

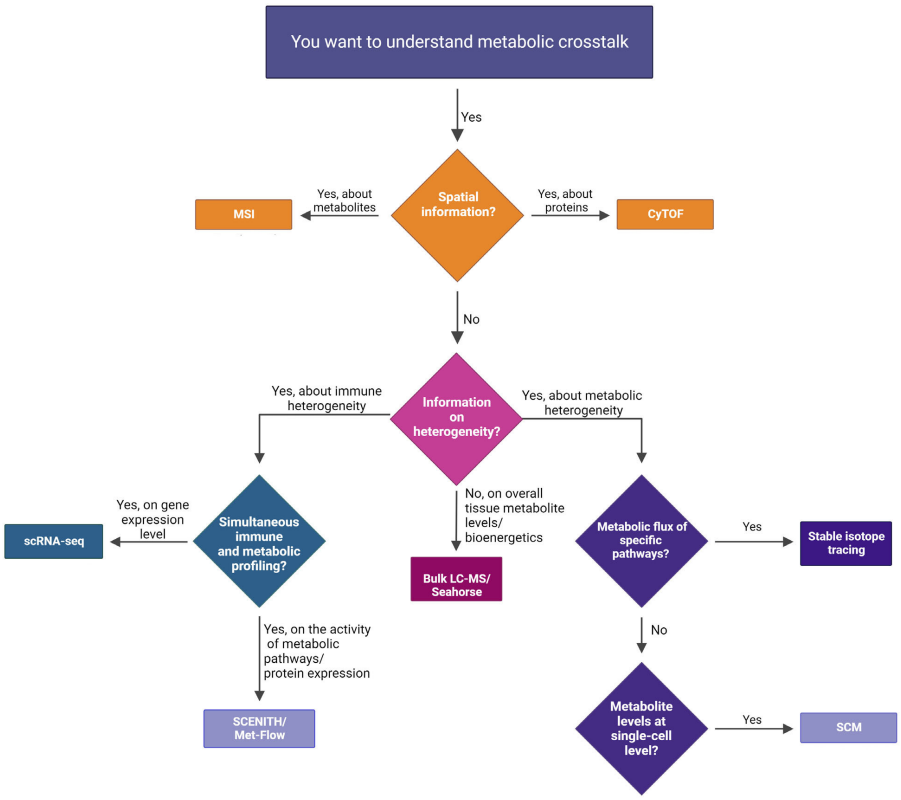


Figure 4. Flowchart displaying different techniques to study heterogeneity in an immunometabolic context. Starting with the need to tackle heterogeneity, the reader finds both CyTOF and mass spectrometry imaging (MSI) as options to study spatial heterogeneity within a sample. When interested in studying immune heterogeneity, flow cytometry-based methods (Met-Flow and single-cell energetic metabolism by profiling translation inhibition (SCENITH)) or single-cell RNA sequencing (scRNA-seq) are suggested, which can both give information on the expression of metabolic proteins, at both protein and RNA levels, respectively. Finally, stable isotope tracing and single-cell metabolomics (SCM) are proposed as methods to study metabolic heterogeneity at a metabolite level.

References

[1] Verheijen, F.W.M., *et al.* (2024). Deciphering metabolic crosstalk in context: lessons from inflammatory diseases. *Molecular Oncology.* DOI: 10.1002/1878-0261.13588.

The ID7000™ Spectral Cell Analyzer from Sony Biotechnology represents an innovative solution for clinical and research laboratories seeking to enhance their flow cytometry capabilities with next-generation spectral technology. Listed below are ways this instrument can help address current research challenges:

1. Resolving Immune and Metabolic Heterogeneity at the Single-Cell Level

- There is a critical need for techniques that can simultaneously assess both immune phenotypes and metabolic states of individual cells. The ID7000 is ideally suited for this purpose.
- Enhanced Flow Cytometry-Based Metabolic Profiling: The ID7000 could significantly improve "Met-Flow" approaches, allowing measurement of metabolic enzymes alongside immune cell markers with greater spectral resolution.
- Improved SCENITH Implementation: The ID7000's spectral technology could enable a more robust implementation of SCENITH, increasing the number of metabolic pathways that can be analyzed simultaneously in diverse immune cell populations.
- Reduced Fluorescence Spillover: As mentioned in Table 1, fluorescence spillover is a limitation of current cytometry-based metabolic methods. The ID7000's spectral unmixing capabilities would minimize this issue, allowing for clearer separation of closely emitting fluorophores.

2. Combining Metabolic and Immunological Profiling

- Integration of metabolic and immune heterogeneity is a primary research challenge.
- Expanded Panel Design: The ID7000's capacity for 44+ colors would allow researchers to develop comprehensive panels that simultaneously assess multiple metabolic pathways alongside detailed immune phenotyping, addressing the "limitation to 40 markers."
- Metabolic Enzyme Quantification: The analyzer's sensitivity would enhance the detection of weakly expressed metabolic regulators in rare immune cell subsets, which is crucial for understanding metabolic heterogeneity.
- High Resolution of Low-Expressed Markers: As noted in Table 1, the spectral flow cytometry approach offers "high-throughput acquisition and resolution of low-expressed markers"—a key advantage of the ID7000.

3. Studying Metabolic Crosstalk in Inflammatory Microenvironments

- Multi-Parameter Analysis of Co-Culture Systems: When analyzing advanced co-culture systems, the ID7000 could distinguish between cell types while simultaneously measuring their metabolic states, helping to unravel cell-cell metabolic interactions.
- Tracking Metabolic Changes in Response to Treatment: For studies examining metabolic adaptations of immune cells to therapeutic interventions, the ID7000 would allow for detailed before-and-after metabolic profiling.

4. Clinical Applications in Immunometabolism

- Immunometabolic analyses in clinical settings is an area of emerging importance.
- Clinical Sample Analysis: The ID7000's sensitivity would be valuable for analyzing precious patient samples from inflammatory diseases, enabling researchers to connect metabolic states with disease progression.
- Biomarker Discovery: As suggested in Figure 3, the analyzer could help identify novel metabolic biomarkers by detecting subtle changes in metabolic enzyme expression across patient cohorts.
- Treatment Monitoring: The instrument could aid in monitoring metabolic changes in immune cells during disease progression or in response to therapy, aligning with the "disease monitoring" application illustrated in Figure 3.

5. Integration with Other Research Methods

- Multi-modal approaches are advantageous for studying immunometabolism.
- Complementary to Single-Cell Techniques: The ID7000 could generate data that complement scRNA-seq and single-cell metabolomics approaches, providing protein-level validation of transcriptomic findings.
- Downstream Analysis: Data from the analyzer can be leveraged to inform and optimize cell sorting on platforms such as the FP7000 Spectral Cell Sorter. By identifying precise populations through high-resolution spectral analysis, accurate gating strategies can be defined to streamline sorting workflows, improving purity and yield.

Endothelial Extracellular Vesicles Promote Tumor Growth by Tumor-associated Macrophage Reprogramming

Adapted from Njock, *et al.*, 2022.

This study reveals that endothelial cells in mammary tumors release extracellular vesicles (EVs) containing specific microRNAs (miR-142-5p, miR-183-5p, and miR-222-3p) that influence tumor progression. RNA sequencing identified these miRNAs, which are conserved between humans and mice, as targeting genes involved in immune regulation. These endothelial-derived EVs transfer their microRNA cargo to macrophages within the tumor microenvironment. In mouse models, treatment with EVs enriched with these specific miRNAs promoted macrophage polarization toward an immunosuppressive M2-like phenotype, ultimately enhancing tumor growth. This result reveals a novel mechanism by which endothelial cells contribute to creating a tumor-permissive environment through EV-mediated macrophage reprogramming.

Introduction

Cancer is a dynamic and heterogeneous disease involving complex interactions within the tumor microenvironment (TME). Communication between different cell types in the TME occurs through direct cell-cell contact and via secreted factors, including EVs. EVs are membrane-enclosed structures secreted by most cell types and can be classified by their subcellular origin: microparticles/microvesicles (100 –1000 nm) are formed directly from the plasma membrane, while exosomes (50 –150 nm) are generated within multivesicular endosomal compartments. EVs contain various biomolecules including non-coding small RNAs, mRNAs, proteins, and lipids, and their content depends on the cell type and physiological state of the producing cell.

While endothelial cell (EC)-derived EVs are known to contribute to angiogenesis-related diseases, most studies have focused on EV communication involving only two cell types. This study investigated how EVs released by tumor-exposed ECs participate in the recruitment and reprogramming of macrophages within the tumor, creating an immunomodulatory phenotype that promotes tumor growth.

Results

TME Modulates Endothelial Phenotype

To investigate how the TME affects EC behavior, a coculture system of human umbilical vein endothelial cells (HUVECs) was established with human triple-negative breast cancer cells (MDA-MB-231). After 48 hours, HUVECs were purified using magnetic beads coated with anti-CD31 antibodies (Fig. 1). RNA sequencing analysis revealed significant differences between HUVECs from monoculture (mono-HUVECs) and coculture conditions (co-HUVECs).

Gene Set Enrichment Analysis (GSEA) showed that several inflammatory-related gene sets were enriched in co-HUVECs, including interferon-α and -γ responses, TNF-α signaling, and inflammatory response pathways. These findings were consistent with the upregulation of cell adhesion genes, including lCAM-1 and VCAM-1, in co-HUVECs, confirming that coculture with tumor cells alters the EC transcriptome and induces activation via inflammatory signaling pathways.

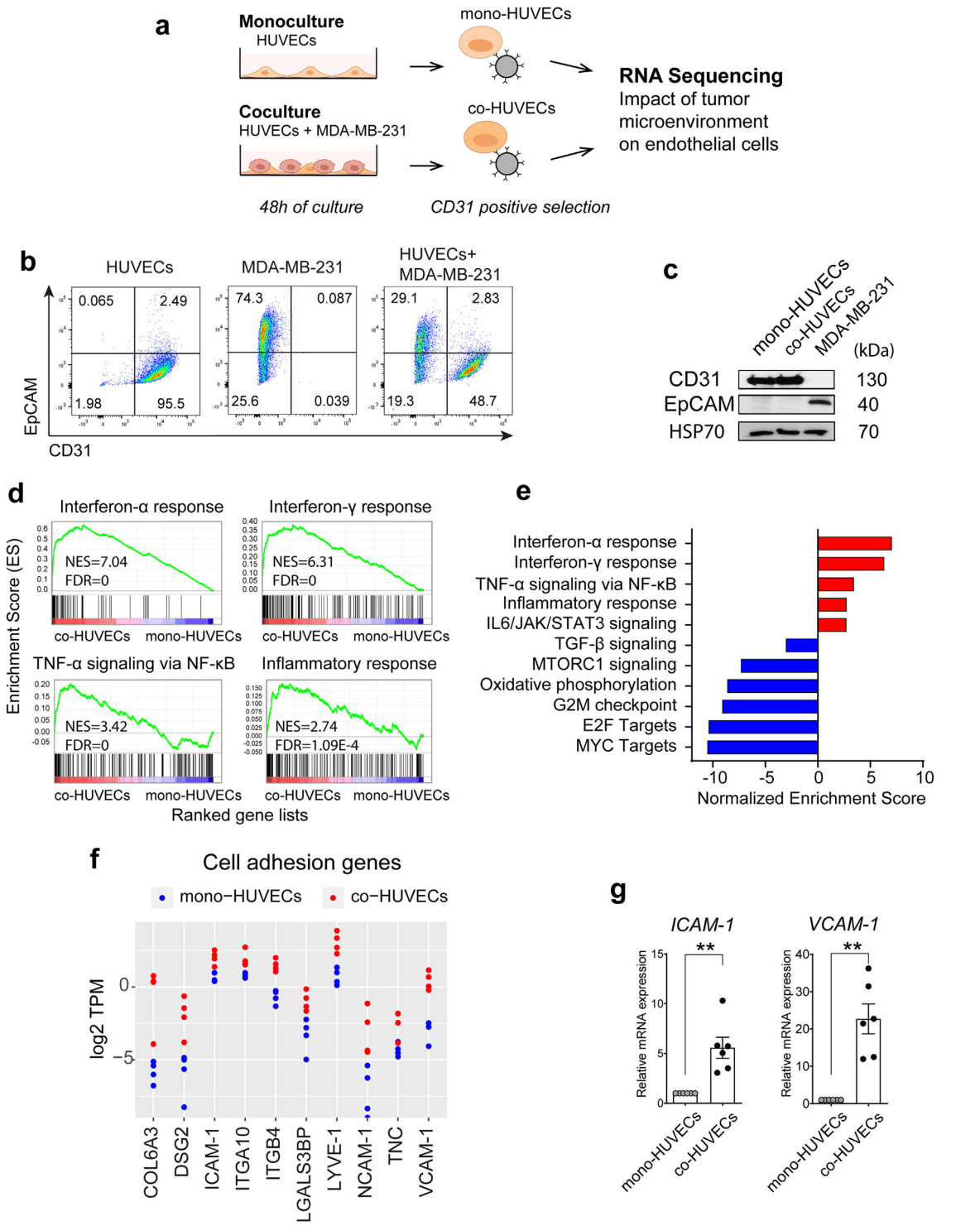


Figure 1. Co-culture with cancer cells induces an activation of ECs via inflammatory signaling pathways. (a) Schematic of the *in vitro* coculture system used to study the effect of TME generated by human breast cancer cells (MDA-MB-231) on human endothelial cells (HUVECs). HUVECs were cultured with/without MDA-MB-231 for 48 h (i.e., coculture vs. monoculture), followed by endothelial cell isolation using magnetic beads coated with anti-CD31 antibody. Then, RNA-Seq was performed to assess transcriptomic changes induced by MDA-MB-231. **(b)** Flow cytometry analysis of HUVEC, MDA-MB-231, and cocultured HUVEC/MDA-MB-231 cells stained for the CD31 endothelial marker and EpCAM. **(c)** Western blotting of endothelial marker CD31 and breast cancer marker EpCAM in lysates from HUVECs purified from mono or cancer cell coculture and from MDA-MB-231 cells. HSP70 is a loading control. **(d)**, Selected gene sets significantly enriched in co- versus mono-HUVECs from GSEA of RNA-Seq data obtained as in (a). **(e)**, GSEA enrichment of inflammation-associated pathways in co-HUVECs. **(f)**, Expression levels (log2 TPM) of several adhesion molecules in HUVECs monocultured or in HUVECs cocultured with MDA-MB-231 as shown by RNA-Seq data. TPM = Transcripts per Million. **(g)**, RT-qPCR analysis of expression of the adhesion molecules ICAM-1, VCAM-1 in HUVECs cocultured with MDA-MB-231 relative to HUVECs alone. Data are mean ± SEM. $^{**}P < 0.01$ calculated by One-Sample T-test ($n = 6$).

TME Modulates the Content of Endothelial EVs

To investigate how tumor cells affect the RNA content of EC-derived EVs, EVs isolated from human umbilical vein endothelial cells (HUVECs) were cultured alone (mono-EVs) or with cancer cells (co-EVs) using ultracentrifugation and CD31-based affinity capture (Fig. 2). Characterization confirmed these vesicles were primarily exosomes (~130 nm diameter) enriched with CD81, CD9, CD63, and syntenin markers. The presence of CD31 and absence of EpCAM verified their endothelial origin without tumor cell contamination.

RNA-seq analysis revealed 392 miRNAs in the EVs, with miR-126 (an endothelial-specific miRNA) being the most abundant. Differential expression analysis identified 22 significantly altered miRNAs between co-EVs and mono-EVs (16 upregulated, 6 downregulated), which was largely confirmed by RT-qPCR. Target gene analysis of upregulated miRNAs showed enrichment in cancer-associated pathways including Glioma Signaling, lL-3 Signaling, Molecular Mechanisms of Cancer, ErbB Signaling, and Breast Cancer Regulation by Stathmin1.

These findings were replicated using mouse endothelial cells (MS-1) cocultured with triple-negative breast cancer cells (4T1) and found similar miRNA regulation patterns. *In vivo* validation using a mouse allograft model with 4T1 cells demonstrated that miR-183-5p was significantly upregulated in CD31+ circulating EVs from tumor-bearing mice compared to controls.

These results suggest that cancer cells modulate the release of specific miRNAs into endothelial EVs, with many of these alterations conserved between mouse and human, potentially indicating functional relevance in cancer progression.

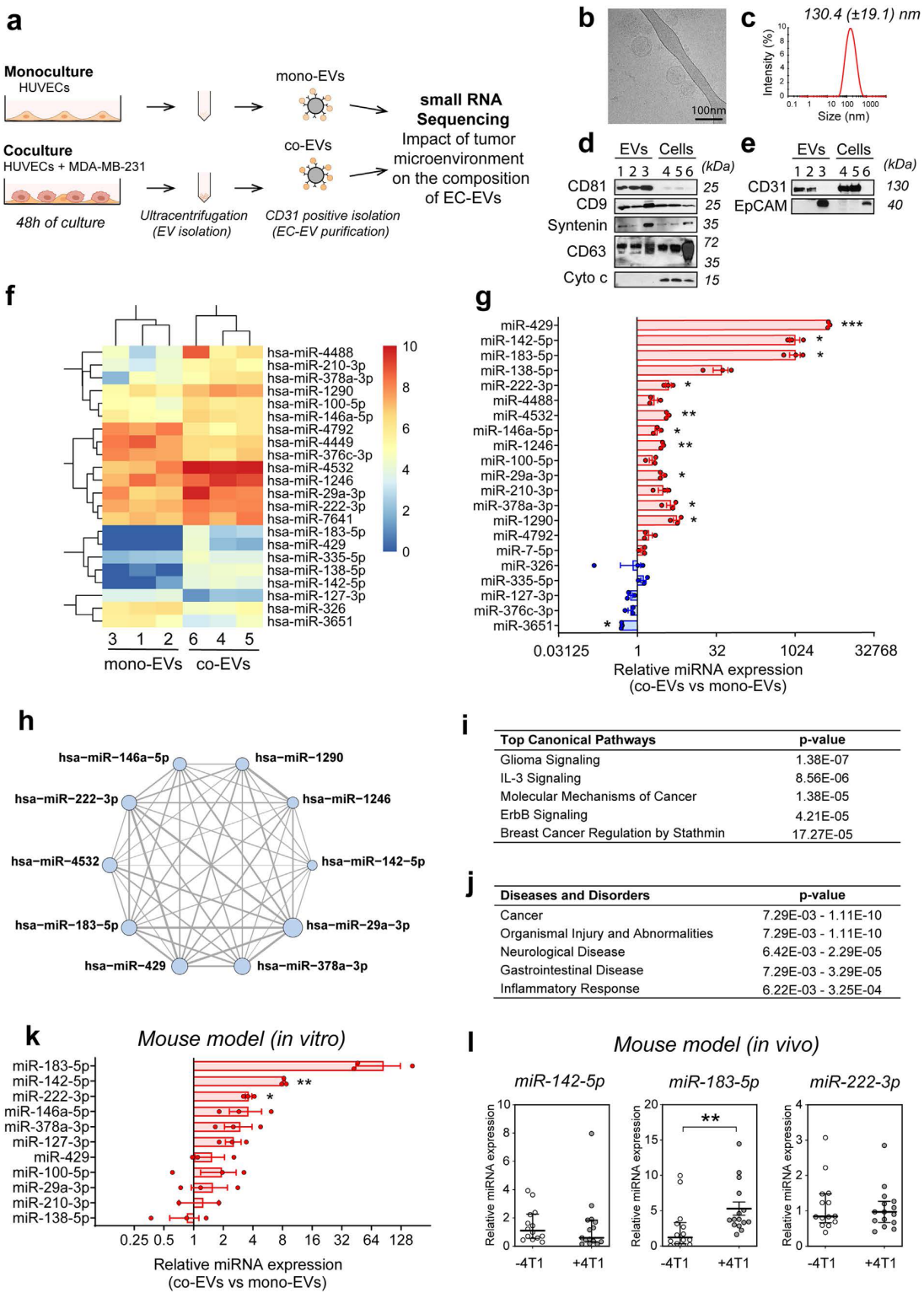


Figure 2. ECs release EVs with dysregulated miRNA levels when cultured in the presence of cancer cells, in particular miR-142-5p, miR-183-5p and miR-222-3p. (a), Overview of the procedure of endothelial EV isolation and small RNA profile analysis. **(b–e)** Characterization of HUVEC EVs by cryo-transmission electron microscopy analysis (bar represents 100 nm) **(b)**, dynamic light scattering analysis **(c)**, and western blotting of the exosomal markers CD9, CD81, CD63, and syntenin in lysates from purified HUVEC EVs and cells from mono- and coculture conditions **(d)**. EV lysates: 1: mono-HUVEC EVs, 2: co-HUVEC EVs, 3: MDA-MB-231-EVs; cell lysates: 4: mono-HUVECs, 5: co-HUVECs, 6: MDA-MB-231. **(e)**, Western blotting of endothelial marker CD31 and tumor marker EpCAM in lysates from purified HUVEC EVs and cells from mono- and coculture conditions. EV lysates: 1: mono-HUVEC EVs, 2: co-HUVEC EVs, 3: MDA-MB-231-EVs; cell lysates: 4: mono-HUVECs, 5: co-HUVECs, 6 :MDA-MB-231. **(f)**, Heatmap of miRNAs with differential abundance between mono-HUVEC EVs and co-HUVEC EVs. **(g)**, RT-qPCR analysis of the miRNAs dysregulated in co-HUVEC EVs. Data are mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ calculated by One-sample t-test ($n = 3$). **(h)**, Gene targets shared by miRNAs with increased abundance in co-EVs (nodes). Edge thickness is scaled by the number of genes targeted in common by the connected nodes. Node size is scaled by the number of predicted targets. **(i, j),** Top canonical pathways **(i)** and top diseases and disorders **(j)** by IPA analysis of genes targeted by at least 5 miRNAs upregulated in co-EVs. **(k),** RT-qPCR analysis of miRNAs dysregulated in EVs from MS1 cells in coculture (with 4T1 mouse breast cancer cell line) versus monoculture conditions. Data are presented as mean ± SEM. $*P < 0.05$ and $**P < 0.01$ calculated by One-sample t-test ($n = 3$). **(l),** Relative levels of miR-142-5p, miR-183-5p, and miR-222-3p in endothelial EVs isolated from plasma of mouse model of 4T1 breast cancer compared to those from control, uninjected BALB/c mice. Data are presented as median ± IQR. $**P < 0.01$ calculated by unpaired two-tailed Mann-Whitney test. -4T1 (empty circle) $n = 14$; +4T1 (black circle) $n = 15$.

Endothelial EVs Generated in Tumoral Context Deliver miRNAs to Macrophages

To determine the cellular targets of these miRNAs, the investigators focused on 3 miRNAs that were strongly upregulated in both human and mouse models: miR-142-5p, miR-183-5p, and miR-222-3p. Mouse endothelial cells, cancer cells, or macrophages were incubated with either mono-EVs or co-EVs, and the levels of these miRNAs in the recipient cells were measured (Fig. 3). The results showed that incubation with co-EVs significantly increased the levels of miR-142-5p, miR-183-5p, and miR-222-3p in both endothelial cells and macrophages, but not in cancer cells. This increase was confirmed to be due to direct transfer of the miRNAs rather than stimulation of endogenous miRNA production by demonstrating that the precursor miRNA levels remained unchanged. Additionally, using fluorescently labeled miRNAs it was shown that macrophages efficiently take up the miRNAs from EVs.

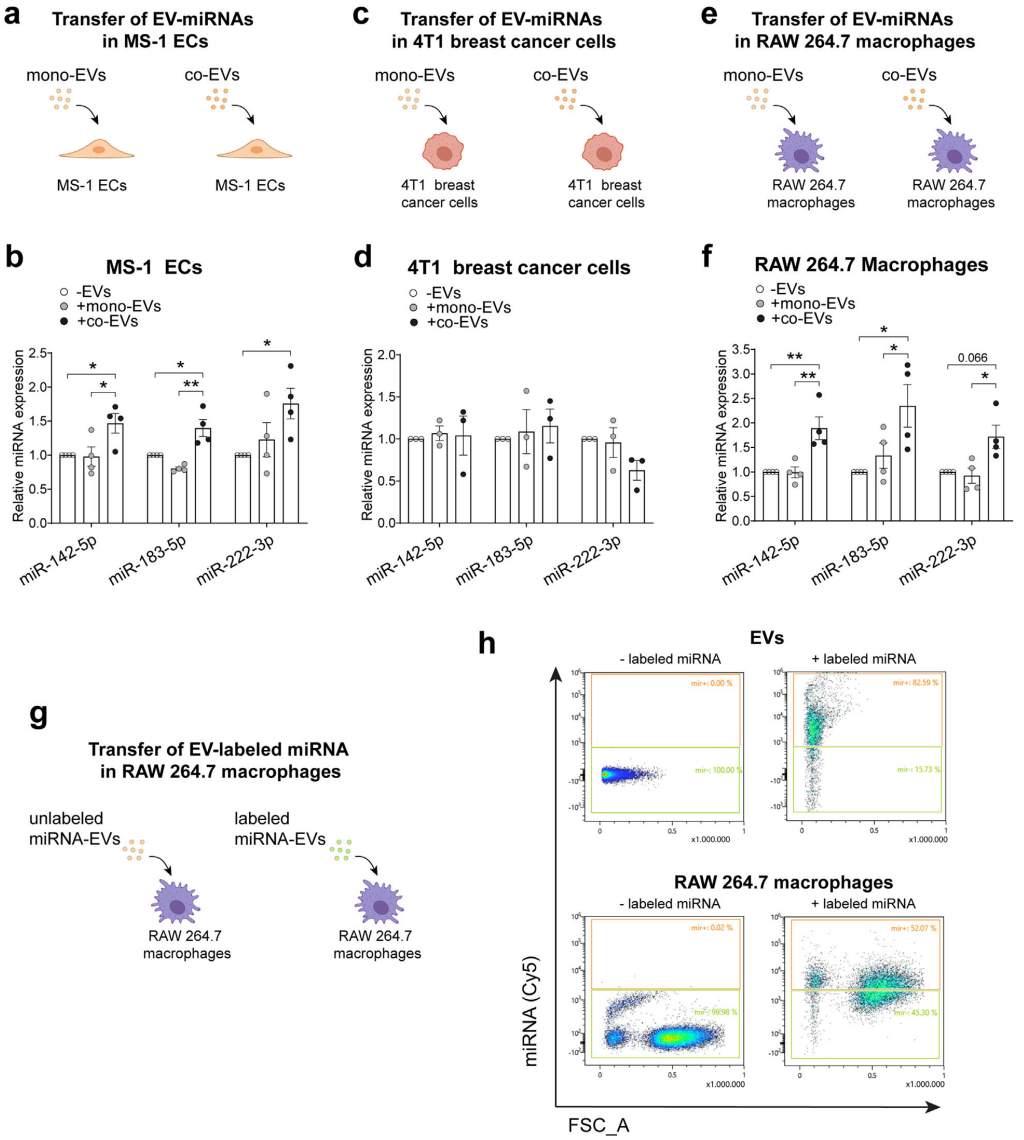


Figure 3. Endothelial EVs generated in the presence of cancer cells deliver miR-142-5p, miR-183-5p and miR-222-3p to RAW264.7 macrophages. (a, c, e), Overview of the protocol to assess the transfer of miRNAs from co-EVs to target cells. MS-1 ECs **(a)**, 4T1 breast cancer cells **(c)** or RAW264.7 macrophages **(e)** were incubated for 24 h with endothelial MS-1 EVs (3 µg/ml) purified from supernatants of MS-1 alone (mono-EVs) or MS-1 cocultured with 4T1 mouse tumor cells (co-EVs). **(b, d, f),** Relative levels of miR-142-5p, miR-183-5p, and miR-222-3p in MS-1 **(b),** 4T1 **(d)** or RAW264.7 **(f)** cells treated with mono-EVs and co-EVs compared to those without EV treatment, assessed by RT-qPCR. All data are mean ± SEM. RNA levels are expressed relative to the respective controls (-EVs condition). *$P < 0.05$ and **$P < 0.01$ calculated by one-way ANOVA with Tukey's test ($n = 3$-4). **(g),** Overview of the protocol to assess the transfer of Cy™5-miRNAs from EVs to macrophages. **(h),** Flow cytometry analysis of Cy5-miR-183-5p electroporated in MS-1 EVs and incubated with RAW264.7 macrophages for 24 h.

Altered Endothelial EV-derived miRNAs Promote Tumor Growth by Inducing M2-like Polarization of Tumor-Associated Macrophages

To investigate the impact of these miRNAs on tumor growth, tumor-bearing mice were injected with EVs electroporated with miR-142-5p, miR-183-5p, miR-222-3p, or an exogenous control miRNA (cel-miR-67) (Fig. 4). Treatment with EVs loaded with any of the 3 miRNAs led to significant increases in tumor volume compared to control EVs, with miR-183-5p and miR-222-3p also significantly increasing tumor weight. Importantly, direct injection of miRNA mimics without encapsulation in EVs had no effect on tumor growth, indicating that EV encapsulation was necessary for the pro-tumoral effect.

Flow cytometry analysis of the immune cell composition in the tumors revealed that treatment with miRNA-loaded EVs increased the percentage of M2-like macrophages ($Ly6C^{low}$-$MHCII^{low}$) without affecting other immune cell populations. Consistent with these findings, macrophages were shown to take up labeled miRNA-loaded EVs both *in vitro* and *in vivo*.

Gene expression analysis of tumors treated with miRNA-loaded EVs showed enrichment of tumor-associated macrophage (TAM) markers and M2-like macrophage gene signatures, along with depletion of M1-like macrophage markers. This indicated that the miRNAs in endothelial EVs promote tumor growth by inducing polarization of macrophages toward an immunosuppressive M2-like phenotype.

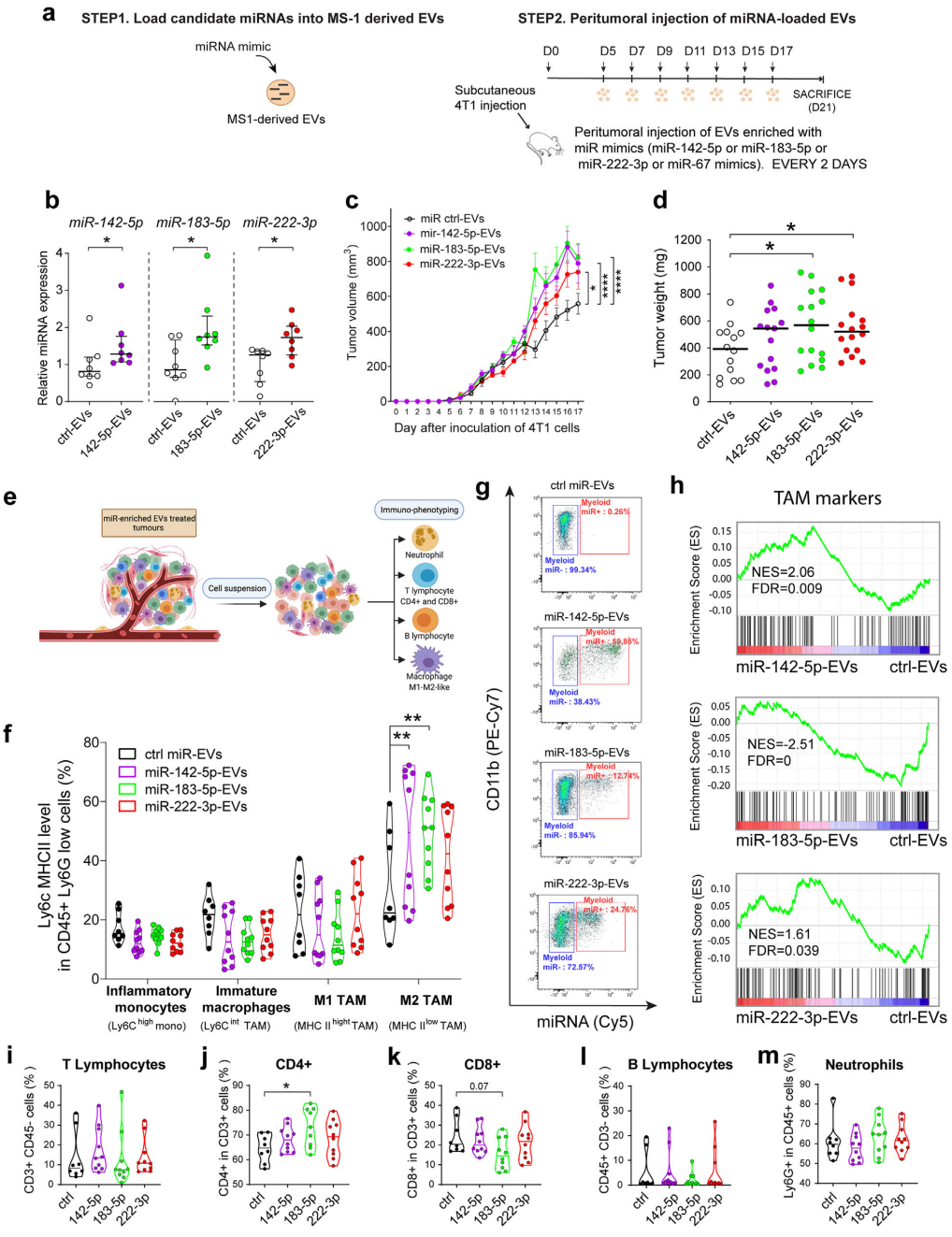


Figure 4. miR-142-5p, miR-183-5p and miR-222-3p increase tumor growth in a murine model of breast cancer by promoting M2-like macrophage polarization. (a), Overview of the 4T1-tumor bearing mouse model treated with EV-enriched with miRNAs: endothelial EVs were loaded with miRNA mimics and injected every 2 days in mice harboring 4T1 tumors. **(b)**, Relative levels of miR-142-5p, miR-183-5p and miR-222-3p assessed by RT-qPCR in tumors from mice injected with EVs loaded with corresponding miRNA mimic compared to those injected with EVs loaded with cel-miR-67 (ctrl) mimic. Data is presented as median ± IQR. *$P < 0.05$ calculated by unpaired two-tailed Mann-Whitney test. **(c)**, Kinetics of tumor growth in mice treated with miR-142-5p-EVs, miR-183-5p-EVs or miR-222-3p-EVs compared with those treated with cel-miR-67(ctrl)-EVs. *$P < 0.05$ and ****$P < 0.0001$ calculated by two-way ANOVA with Dunnett's test. **(d)**, Tumor weight at sacrifice of miRNA-EV-treated mice. Tumor weight at sacrifice (16 days after the first EV injection). Black bars indicate median values. *$P < 0.05$ calculated by unpaired two-tailed Mann-Whitney test. Data shown are from two independent experiments. **(e)**, Schematic diagram of isolation and characterization of immune cells from 4T1-tumor, **(f)**, Violin plots comparing the percentages of the four TAM subpopulations in 4T1-tumor bearing mice treated with cel-miR-67 (ctrl)-EVs, miR-142-5p-EVs, miR-183-5p-EVs, and miR-222-3p-EVs: inflammatory monocytes (Ly6C high MCHII low), immature macrophages (Ly6C high MCHII low), classically activated macrophages (M1) (Ly6C high MCHII low) and alternatively activated macrophages (M2) (Ly6C high MCHII low). Bars indicate median values. *$P < 0.05$ calculated by two-way ANOVA followed by Dunnett correction, $n = 8–10$ mice per group. **(g)**, Flow cytometry analysis of 4T1-tumor bearing mice treated every other day with cel-miR-67 (ctrl)-EVs, miR-142-5p-EVs, miR-183-5p-Evs or miR-222-3p-EVs and with the corresponding Cy5-miR-EVs the day before sacrifice (control was treated with unlabeled ctrl-EVs). **(h)**, GSEA showed enrichment of TAM markers in 4T1-tumors treated with miR-142-5p-EVs, miR-183-5p-EVs, or miR-222-3p-EVs compared to those treated with cel-miR-67 (ctrl)-EVs. Briefly, cells from mouse 4T1-tumors (treated with the different EV preparations) were collected, RNA-Seq and GSEA analysis were performed. $N = 3$ tumors per group. **(i–m)**, Quantification of flow cytometry analysis of T Lymphocytes **(i)**, CD4+ **(j)** and CD8+ **(k)**, B lymphocytes **(l)** and neutrophils **(m)** in 4T1-tumor bearing mice treated with EVs loaded with the indicated miRs. Bars indicate median values. *$P < 0.05$ calculated by one-way ANOVA, $n = 8–10$ mice per group.

miR-142-5p, miR-183-5p, and miR-222-3p Induce M2 Polarization by Targeting PTEN

In vitro experiments confirmed that treatment of macrophages with miRNA-loaded EVs increased the expression of M2 markers (Arginase-1, TGF-β) and decreased the expression of M1 markers *(iNOS, IL-1β)* (Fig. 5). Additionally, these miRNAs increased the migration capabilities of macrophages, suggesting they may promote macrophage recruitment to the tumor site.

Bioinformatic analysis identified 457 common targets of the 3 miRNAs, including PTEN (Phosphatase and Tensin homolog), a known regulator of macrophage polarization. All 3 miRNAs decreased PTEN expression in macrophages, suggesting a mechanism by which these miRNAs induce M2 polarization.

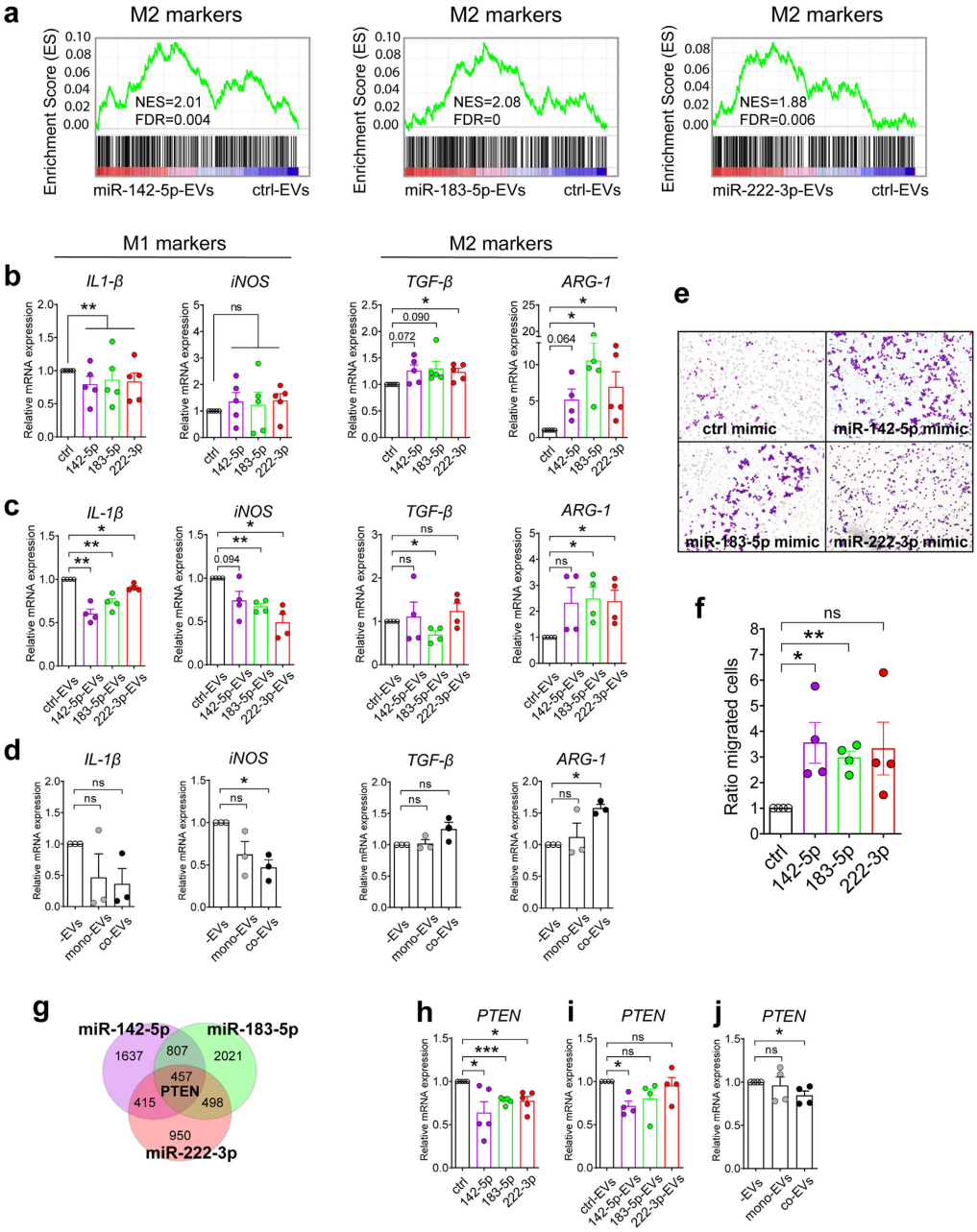


Figure 5. miR-142-5p, miR-183-5p and miR-222-3p alter the expression of M1/M2 markers of RAW264.7 macrophages and increase their migration capacity. **(a),** Depleted/enriched gene sets associated with M2 markers in RAW264.7 macrophages treated with miR-142-5p-EVs, miR-183-5p-EVs, or miR-222-3p-EVs compared to cel-miR-67(ctrl)-EVs from ranked GSEA analysis. **(b, c)**, Impact of miR-142-5p, miR-183-5p and miR-222-3p on the expression of M1 (IL-1β, iNOS) and M2 markers (TGF-β, ARG-1) in RAW264.7 macrophages **(b)** transfected with corresponding mimics (n = 5) or **(c)** treated with miRNA-enriched EVs (n = 4), assessed by RT-qPCR. **(d)**, Relative expression of M1/M2 markers in RAW264.7 macrophages treated with co- or mono-EVs compared to those without EV treatment, assessed by RT-qPCR (n = 3). **(e, f)**, Impact of miR-142-5p, miR-183-5p, and miR-222-3p on the migration of RAW264.7 macrophages. **(e)**, Representative images of transwell migration assay of RAW264.7 macrophages transfected with cel-miR-67 (ctrl), miR-142-5p, miR-183-5p and miR-222-3p mimics. **(f)**, Quantification of the migration of RAW264.7 macrophages shown in e. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 calculated by One-sample t-test. **(g)**, Venn diagram of mRNA targets of miR-142-5p (purple circle), miR-183-5p (green circle), and miR-222-3p (red circle). **(h, i)**, Impact of miR-142-5p, miR-183-5p, and miR-222-3p on the expression of their common target PTEN in RAW264.7 macrophages **(h)** transfected with corresponding mimics (n = 5) or **(i)** treated with miRNA-enriched EVs (n = 4), assessed by RT-qPCR. **(j)**, Impact of co- or mono-EVs on the expression of PTEN in RAW264.7 macrophages (n = 4). Data are presented as mean ± SEM. **(b, c, f, h, i,j)** relative to the respective controls (black bars). *P < 0.05, **P < 0.01, ***P < 0.001 calculated by One-sample t-test.

miR-142-5p, miR-183-5p, and miR-222-3p are Elevated in Breast Cancer Patients while PTEN is Decreased

Analysis of clinical data from breast cancer patients showed that miR-142-5p and miR-183-5p were significantly higher in tumor tissue compared to healthy control tissue (Fig. 6). For miR-222-3p, elevated levels were specifically observed in basal-like/ER(-) breast cancer subtype. As expected, PTEN levels were lower in tumors from breast cancer patients. Kaplan-Meier survival analysis indicated a decreased survival rate for ER+ breast cancer patients with high levels of miR-222-3p.

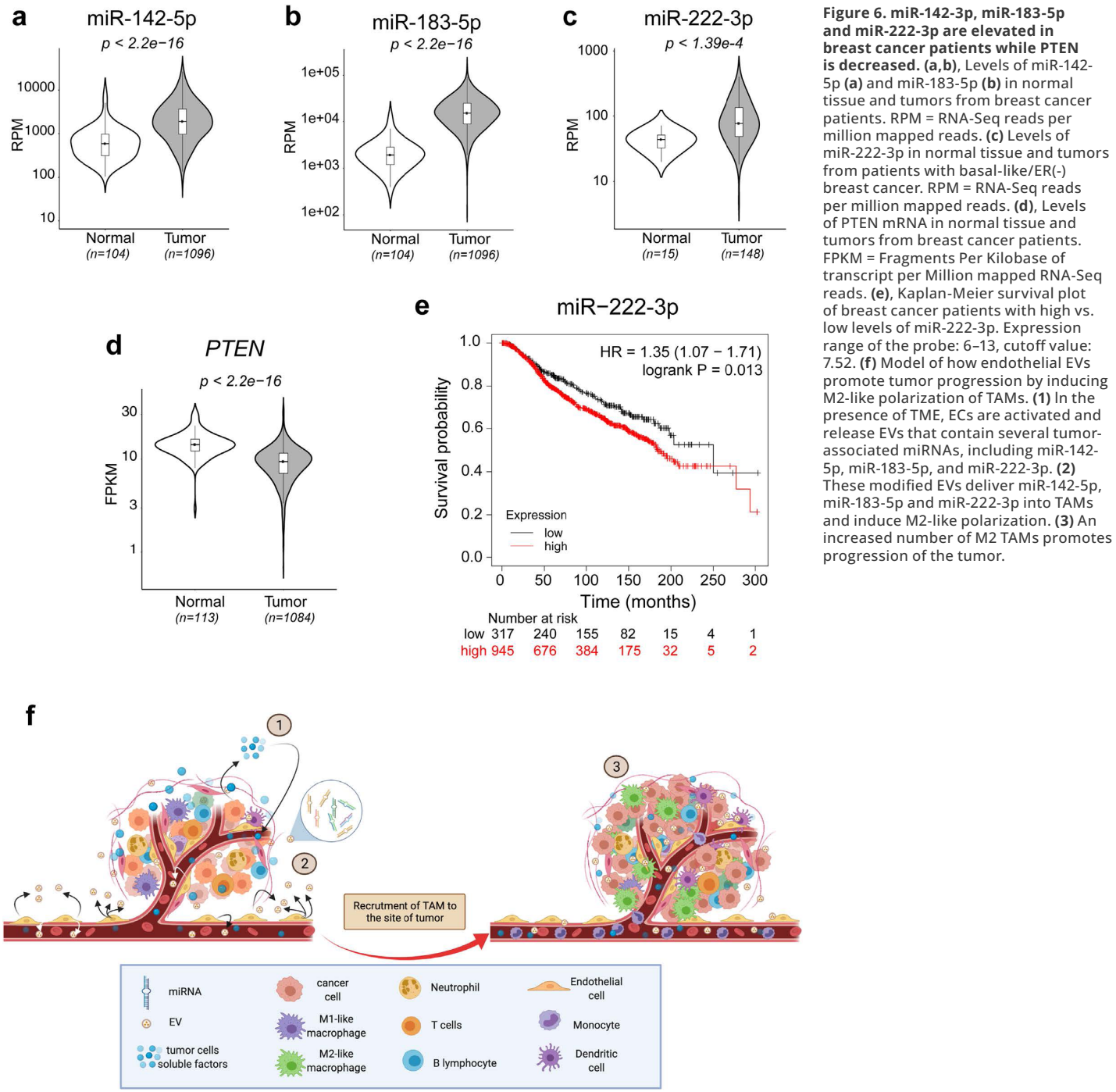


Figure 6. miR-142-3p, miR-183-5p and miR-222-3p are elevated in breast cancer patients while PTEN is decreased. (a,b), Levels of miR-142-5p **(a)** and miR-183-5p **(b)** in normal tissue and tumors from breast cancer patients. RPM = RNA-Seq reads per million mapped reads. **(c)** Levels of miR-222-3p in normal tissue and tumors from patients with basal-like/ER(-) breast cancer. RPM = RNA-Seq reads per million mapped reads. **(d)**, Levels of PTEN mRNA in normal tissue and tumors from breast cancer patients. FPKM = Fragments Per Kilobase of transcript per Million mapped RNA-Seq reads. **(e)**, Kaplan-Meier survival plot of breast cancer patients with high vs. low levels of miR-222-3p. Expression range of the probe: 6–13, cutoff value: 7.52. **(f)** Model of how endothelial EVs promote tumor progression by inducing M2-like polarization of TAMs. **(1)** In the presence of TME, ECs are activated and release EVs that contain several tumor-associated miRNAs, including miR-142-5p, miR-183-5p, and miR-222-3p. **(2)** These modified EVs deliver miR-142-5p, miR-183-5p and miR-222-3p into TAMs and induce M2-like polarization. **(3)** An increased number of M2 TAMs promotes progression of the tumor.

Discussion

This study reveals a novel communication pathway within the TME: endothelial cells, when activated by cancer cells, release EVs containing specific miRNAs (miR-142-5p, miR-183-5p, and miR-222-3p) that induce polarization of macrophages toward an M2-like phenotype, which in turn promotes tumor growth.

While previous studies have shown that tumor-derived EVs can direct macrophage polarization toward an M2 phenotype, this is the first evidence that endothelial-derived EVs can also affect macrophage polarization and tumor progression. The study demonstrates that miRNAs present in endothelial EVs collectively reprogram macrophages within the tumor to an M2-like phenotype by targeting PTEN, a key regulator of macrophage polarization.

The findings suggest that modulating endothelial EVs to shape macrophage polarization could represent a potential therapeutic approach in cancer treatment, complementing existing strategies that target tumor-associated macrophages directly.

References

[1] Njock, M., *et al.* (2022). Endothelial extracellular vesicles promote tumor growth by tumor-associated macrophage reprogramming. *Journal of Extracellular Vesicles*. DOI: 10.1002/jev2.12228

Increasing Panel Design Flexibility Using the 320-nm Laser on the ID7000™ Spectral Cell Analyzer

The Sony ID7000™ spectral cell analyzer may be equipped with a 320-nm laser to expand the way biological samples are analyzed, ensuring accurate visualization of fluorescent populations. In this article, we provide an overview of the method and benefits of spectral analysis with emission signals from 320-nm laser excitation, using currently available fluorochromes, compared to the same panel without the 320-nm laser.

Introduction to the ID7000 spectral cell analyzer optics

The ID7000 spectral cell analyzer is built using an innovative design that may include up to seven excitation lasers (320, 355, 405, 488, 561, 637, and 808 nm), optical fibers, diffraction grating, 32-channel array photomultiplier tubes (PMTs), single-channel PMTs, and a standard AutoSampler **(Figure 1A)**. A photograph of the excitation laser module is shown in **Figure 1B**. The optics schematic diagram is shown in **Figure 1C**. The excitation lasers are aligned as spots, which are spatially separated for all seven lasers. Fluorescence emission is captured by optical fibers and delivered to each detection deck. A diffraction grating was introduced to disperse the emission fluorescence signal. A custom micro lens array assembly then focuses each band of light onto a specific channel of the PMT array, which prevents loss of fluorescent photons to a boundary mask. **Figure 1D** shows a detection optics map, which highlights the relationship between wavelengths and PMT channels. The 32-channel PMTs and single-channel PMTs detect light from 360 to 920 nm. The detection range for 320-nm excitation is from 360 to 840 nm with a total of 35 PMT channels available.

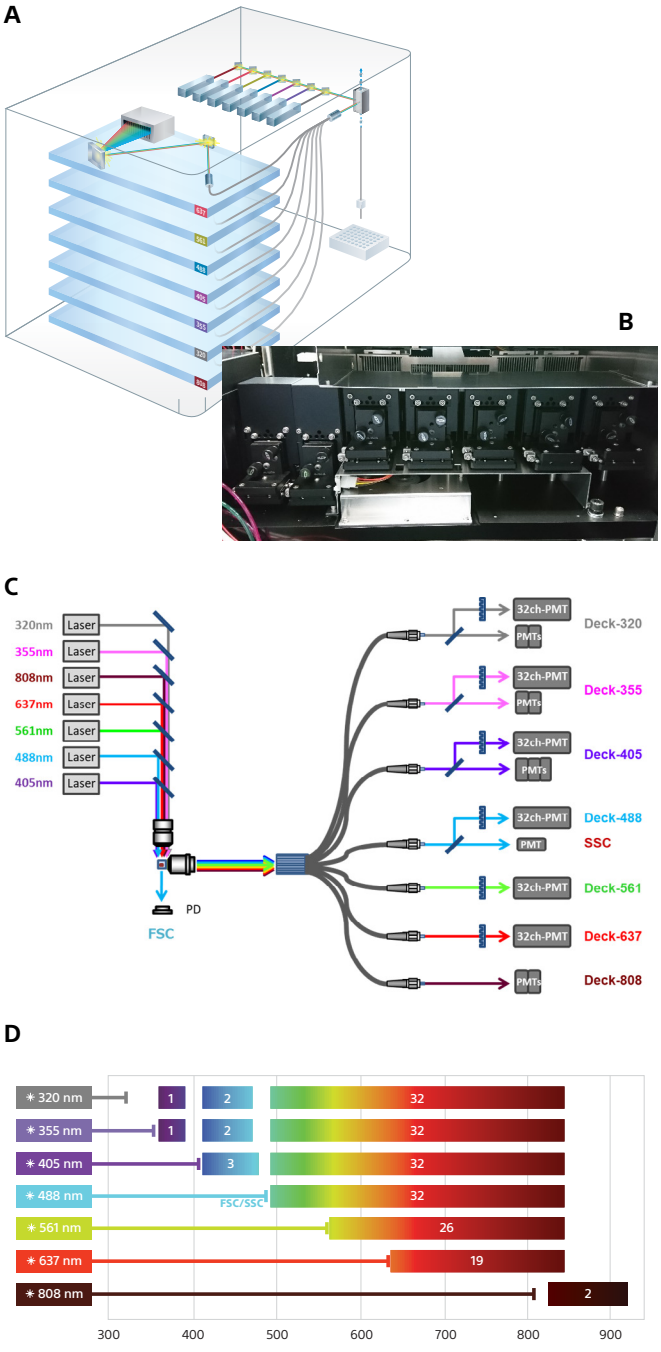


Figure 1. Technical overview of the ID7000 system optics

Stability of the 320-nm laser

The study was conducted using AlignCheck particles (Sony Biotechnology Inc. catalog no. AE700510). These particles were run on an ID7000 spectral cell analyzer with six lasers ON (320, 355, 405, 488, 561, and 637 nm) for 84 days. **Figure 2A** shows the emission spectra when excited by the 320-nm laser (20 mW at laser output) visualized in the ID7000 Software. AlignCheck particles have high fluorescence intensity over a wide range of wavelengths, from 360 to 840 nm. **Figure 2B** depicts Mean intensities of Area and Height of AlignCheck particles at a certain detection region, roughly 624 to 635 nm (red highlighted in (A)), calculated and tracked for 84 days. This region was selected to represent all 320-nm laser detection channels. These mean intensity values of both Area and Height show stable performance with the 320-nm laser. In addition, the rCV of these values was about 3.0%, which also shows good performance of the 320-nm laser for a long time.

Figure 2C shows the histogram plots generated using InSpeck™ Blue microspheres (Thermo Fisher Scientific catalog no. I7221). Intensities of Area of seven populations at roughly 419 to 472 nm with the 320-nm laser (right) and 355-nm laser (left) are shown.
Figure 2D depicts InSpeck Blue microspheres analyzed using the linear scale with the 320-nm laser excitation. The peaks were visualized using histograms and a virtual filter (roughly 419 to 472 nm). All peaks were set to 10^5 to normalize each population to the same region of the detector. The distribution of each peak was symmetrical, indicating low noise and a consistent beam shape for the 320-nm laser.

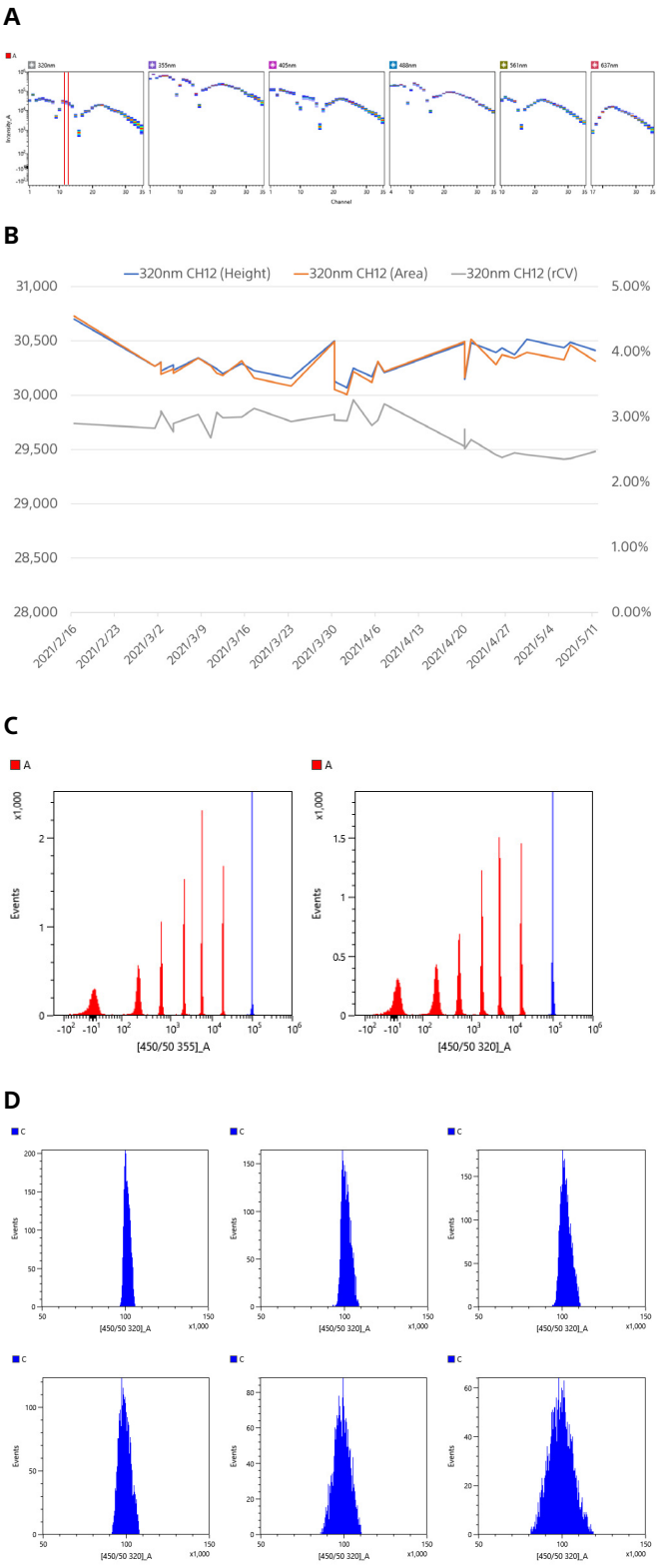


Figure 2. Demonstration of the stability of the 320-nm laser

Improvement of the spillover spreading values with 320-nm laser

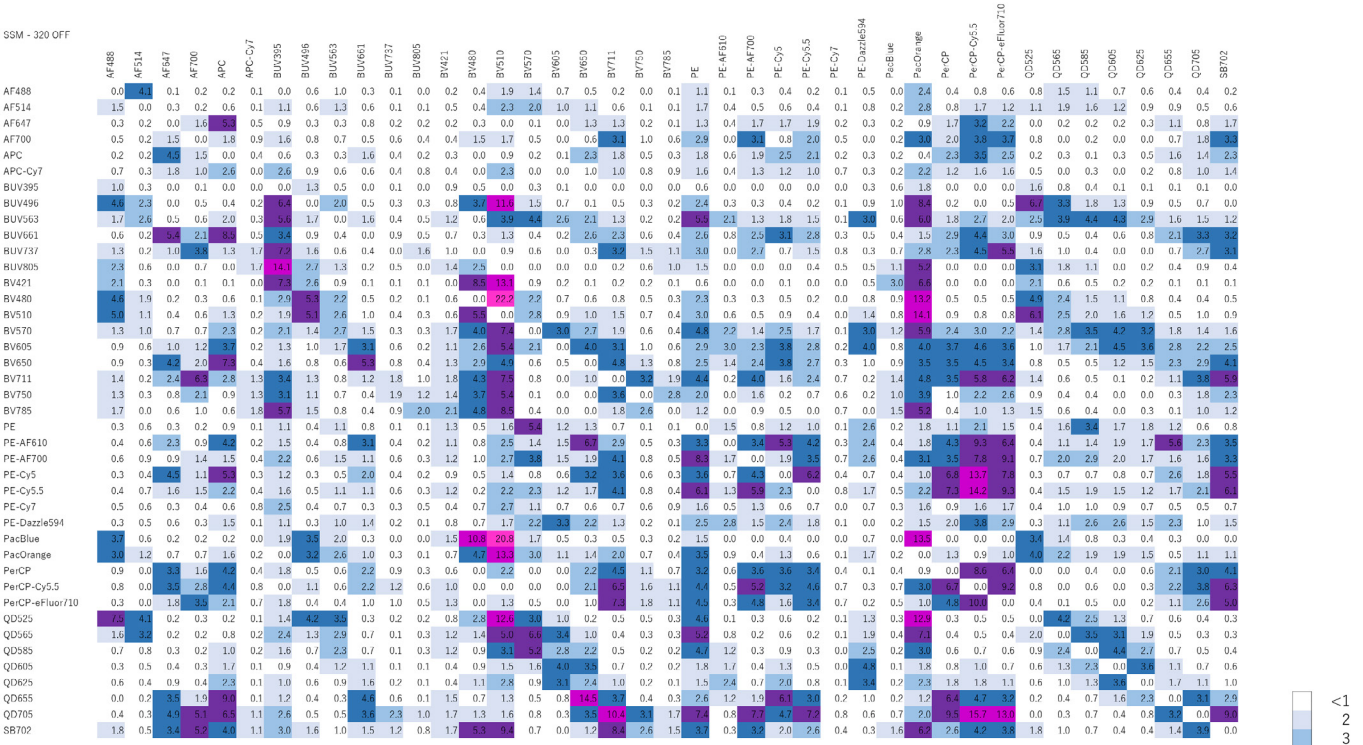
Spillover spread (SS) values were compared with and without the 320-nm laser to identify the benefit from emission spectra from the 320-nm laser excitation of currently available fluorochromes. The table shows the combination of fluorochromes for this testing. Forty-one colors and fifty colors were selected to compare the difference in the spillover spreading values between 320 ON and 320 OFF.

In both cases, when the 320-nm laser was ON, the total value of spillover spreading was improved. For 41 colors, the total SS value without the 320-nm laser was 2,695.0 and with the 320-nm laser was 2,161.3. For 50 colors, the total SS value without the 320-nm laser was 9,661.6 and with the 320-nm laser was 7,806.5. This results in an improved signal-to-noise ratio after spectral unmixing, which allows users to have more flexibility in their panel designs.

Fluorochrome	Marker	41 color	50 color
Alexa Fluor® 532	CD19	N/A	✓
Alexa Fluor® 514	CD39	✓	✓
Alexa Fluor® 488	CD8a	✓	✓
Alexa Fluor® 647	CD4	✓	✓
Alexa Fluor® 700	CD123	✓	✓
APC	CD27	✓	✓
APC-Cy™7	CD16	✓	✓
APC-eFluor® 780	CD38	N/A	✓
BUV395	CD45RA	✓	✓
BUV496	CD4	✓	✓
BUV563	CD14	✓	✓
BUV661	CD4	✓	✓
BUV737	CD56	✓	✓
BUV805	CD4	✓	✓
BV421	CD19	✓	✓
BV480	IgD	✓	✓
BV510	CD3	✓	✓
BV570	HLA-DR	✓	✓
BV605	CD45RA	✓	✓
BV650	CD183 (CXCR3)	✓	✓
BV711	CD196 (CCR6)	✓	✓
BV750	CD4	✓	✓
BV785	CD197 (CCR7)	✓	✓
CF® 568	CD4 (EDU-2)	N/A	✓
eFluor® 450	CD161	N/A	✓
eVolve™ 605	CD4	N/A	✓
eVolve™ 655	CD27	N/A	✓

Fluorochrome	Marker	41 color	50 color
Pacific Blue™	CD3	✓	✓
Pacific Orange™	CD4	✓	✓
PE	CD45RO	✓	✓
PE/Dazzle™ 594	CD24	✓	✓
PE-Alexa Fluor® 610	CD24	✓	✓
PE-Alexa Fluor® 700	CD4	✓	✓
PE-Cy™5	CD95(Fas)	✓	✓
PE-Cy™5.5	CD8a	✓	✓
PE-Cy7	CD7	✓	✓
PerCP	CD45	✓	✓
PerCP-Cy5.5	CD127	✓	✓
PerCP-eFluor® 710	TCR g/d	✓	✓
Qdot™ 525	CD39	✓	✓
Qdot™ 565	CD39	✓	✓
Qdot™ 585	CD39	✓	✓
Qdot™ 605	CD4	✓	✓
Qdot™ 625	CD39	✓	✓
Qdot™ 655	CD4	✓	✓
Qdot™ 705	CD4	✓	✓
Qdot™ 800	CD4	N/A	✓
Super Bright 645	CD8	N/A	✓
Super Bright 702	CD4	✓	✓
Super Bright 780	CD4	N/A	✓

A



B

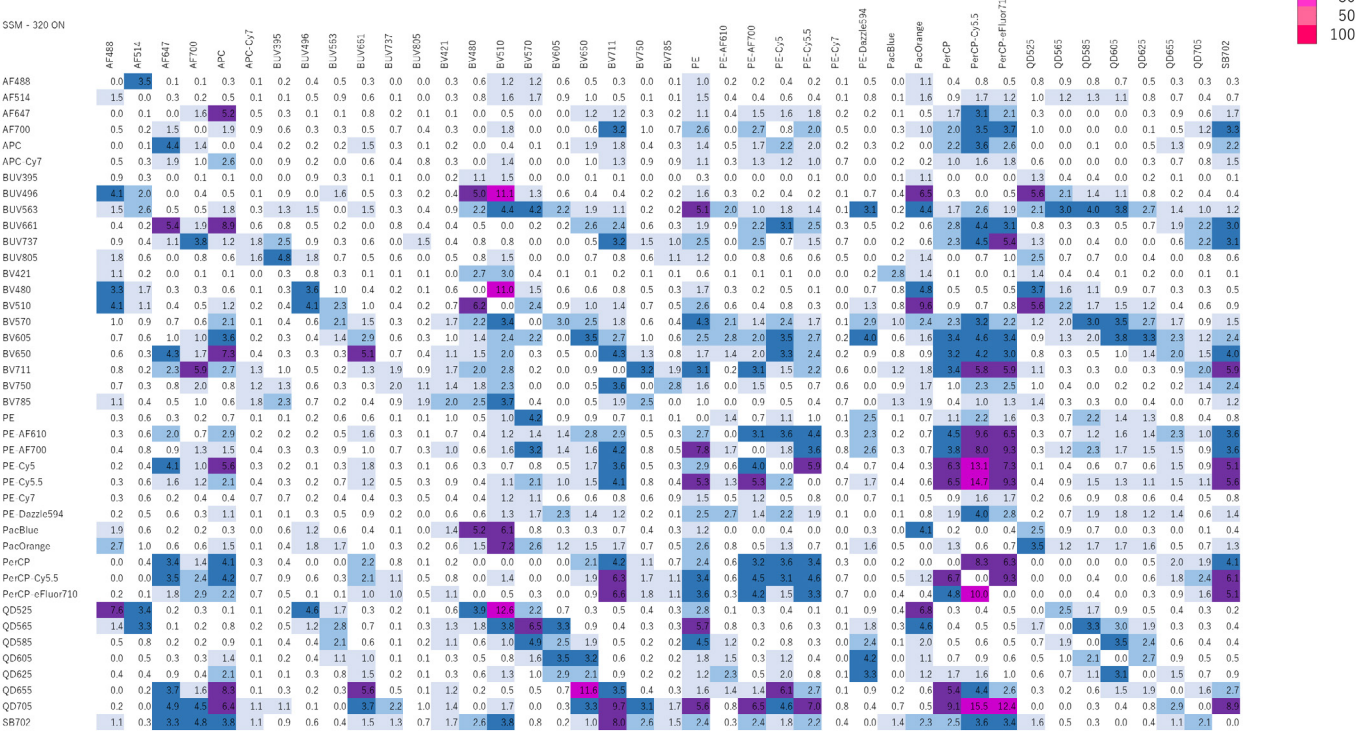


Figure 3. Spillover spread matrix for 41 colors without (A) and with (B) the 320-nm laser

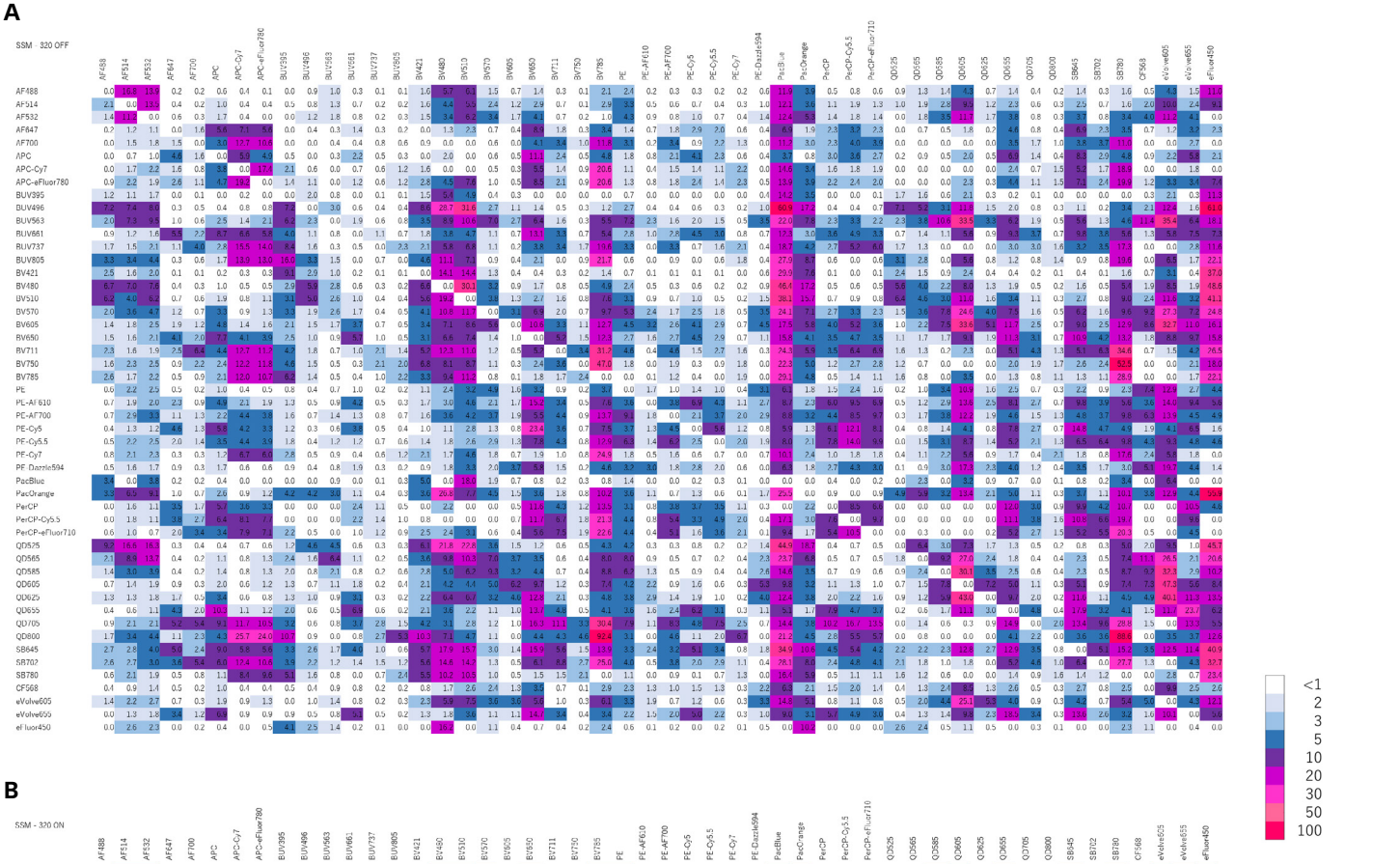


Figure 4. Spillover spread matrix for 50 colors without (A) and with (B) the 320-nm laser

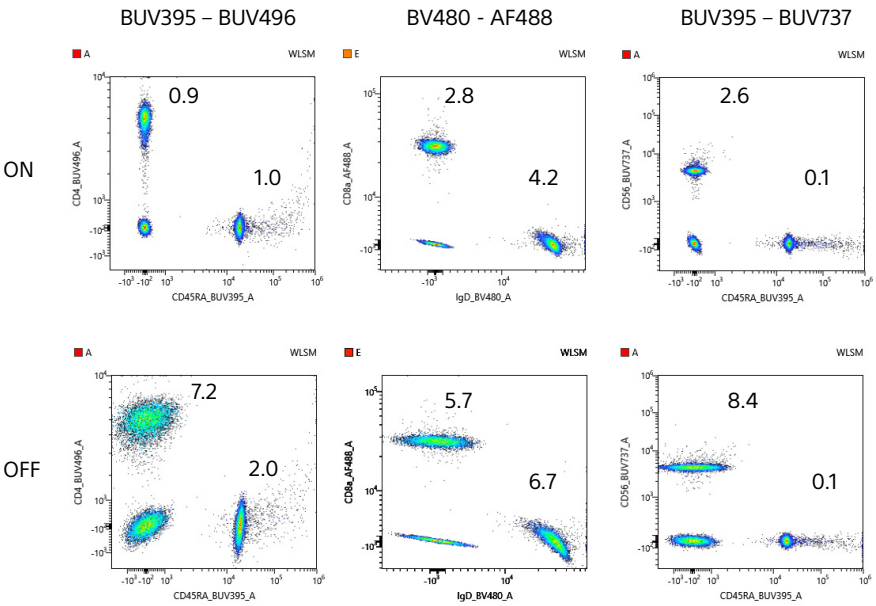


Figure 5. Examples of improvement with the 320-nm laser

Summary

The key advantage of spectral flow cytometry is the flexibility it provides for panel design for multicolor experiments. Spectral flow cytometry allows fluorochromes to be used that might be challenging to detect with non-spectral optics because of overlapping or highly adjacent emission spectra. Analysis of the separation of adjacent fluorochromes was carried out with the 320-nm laser to compare the signal-to-noise ratio after spectral unmixing and evaluate the ability of the 320-nm laser to separate individual fluorochrome signals in a multicolor experiment.

Experimental results show the ability to distinguish populations when using spectrally similar fluorochromes is improved by the 320-nm laser because the emission signals from the 320-nm laser excitation contribute additional information to improve spectral unmixing results.

References

Telford WG, Stickland L, Koschorreck M. Ultraviolet 320 nm laser excitation for flow cytometry. *Cytometry A.* 2017;91:314-325. Pub Med

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Further reading and resources

- **Take a Tour of the lD7000 System**
- **Spectral vs Conventional Flow Cytometry**
- **Learn More**

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