Flexible and Comprehensive Phenotyping of Tumor and Peripheral Blood Mononuclear Cells in Endometrial Carcinoma

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Introduction

Cancer biomarkers have revolutionized management and treatment of the disease, leading to remarkable advancements in personalizing medicine and determining optimal therapeutic combinations. However, researchers face significant challenges in identifying biomarkers in the context of extreme biological heterogeneity, a common feature of many cancers. While immune profiling of peripheral blood is a common approach, many critical disease processes are only evident within the tumor environment. These processes can provide valuable prognostic and diagnostic insights or reveal therapeutic targets that are not detectable in the peripheral immune system.

Endometrial carcinoma (EC) is the most prevalent form of uterine cancer, with its incidence rising in developed countries due to factors such as population aging and increasing obesity rates. Despite a relatively favorable prognosis, with an 80% survival rate at 5 years post-diagnosis, the primary curative treatment remains the total removal of the uterus, ovaries and fallopian tubes. This underscores the need for identifying biomarkers that can lead to more personalized medicine approaches and the development of immunotherapies, particularly for aggressive EC subtypes.

Mass cytometry is a high-plex proteomic technology that simultaneously resolves phenotypic and functional markers, enabling researchers to implement large-scale immunophenotyping strategies that span biological heterogeneity and sample types. A combination of immune and functional profiling is key to elucidating disease mechanisms and revealing predictive biomarkers. Mass cytometry conducted on the CyTOF™ XT PRO system uniquely enables higher-parameter, precise immunophenotyping at greater throughput without the data artifacts introduced by compensation and spectral deconvolution. The ability to easily and rapidly design and modify 50-plus-marker panels, along with flexible sample staining and acquisition workflows and the use of sample multiplexing, makes mass cytometry the premier choice for large and complex clinical studies and drug discovery programs.

Objective

To provide a means to achieve deep phenotyping and functional characterization of multiple clinical samples in a single tube by leveraging modular, ready-to-use Flex-Fit™ panels and the enhanced throughput of the CyTOF XT PRO system. By showcasing characterization of both immune and non-immune cells in tumor tissue and PBMC, this method highlights a rapid, high-plex workflow that provides valuable insights for cancer research and potential therapeutic targets from minimal sample amounts.

Key advantages of mass cytometry on the CyTOF XT PRO system for clinical research

- Capturing phenotypic and functional variation in a single CyTOF panel generates unique biomarkers that can reveal mechanisms of disease activity, drug response and prognostic potential
- **Pre-optimized modular panels** combined with the enhanced throughput of the CyTOF XT PRO system enables fast implementation of large-scale immune profiling studies
- **Sample multiplexing** using a variety of barcoding reagents (Pd, CD45, TeMal) is a powerful method to harmonize sample sets, reduce batch effects, and improve standardization in multi-site and longitudinal studies
- The CyTOF XT PRO system addresses regulatory requirements with **21 CFR Part 11 compliance-enabling software** ensuring user management, user audit trails and integrity of output files

Materials and methods

Panel	Target	Clone	Metal	Staining Protocol	Part Number
Human TBNK+O CyTOF Panel, 9 Antibodies (201338)	CD45	HI30	89Y	Cytoplasmic	3089003
	CD56	NCAM16.2	146Nd	Surface	3146021
	CD3	UCHT1	170Er	Cytoplasmic	3170001
	CD16	3G8	209Bi	Surface	3209002
	CD19	HIB19	145Nd	Surface	3145020
	CD14	M5E2	148Nd	Surface	3148022
	CD66b	G10F5	152Sm	Surface	3152019
	CD8	SK1	142Nd	Cytoplasmic	3142021
	CD4	SK3	144Nd	Cytoplasmic	3144029
Human T Cell Profiling CyTOF Panel, 10 Antibodies (201340)	TCRαβ	T10B9.1A-31	141Pr	Surface	3141022
	CD45RA	HI100	150Nd	Surface	3150056
	CCR7	G043H7	167Er	Surface	3167009
	CD27	L128	155Gd	Surface	3155001
	CD28	CD28.2	160Gd	Surface	3160003
	CD38	HB-7	161Dy	Surface	3161034
	CD45RO	UCHL1	163Dy	Surface	3163032
	CD25	BC96	153Eu	Surface	3153032
	CD197/CCR7	G043H7	167Er	Surface	3167009
	CD127/IL-7Rα	A019D5	168Er	Surface	3168027
Human Immune Checkpoint Core CyTOF Panel, 9 Antibodies (201341)	CD366/TIM-3	F38-2E2	154Sm	Surface	3154010
	CD134/OX40	ACT35	158Gd	Surface	3158012
	CD137/4-1BB	4B4-1	173Yb	Surface	3173015
	TIGIT	MBSA43	166Er	Surface	3166033
	CD95/Fas	DX2	176Yb	Surface	3176029
	CD279/PD-1	EH12.2H7	156Gd	Surface	3156038
	CD274/PD-L1	MIH1	174Yb	Surface	3174027
	CD223/LAG-3	11C3C65	172Yb	Surface	3172035
	CD154/CD40L	24-31	142Nd	Cytoplasmic	3142033
Human Immune Checkpoint Expansion 1 CyTOF Panel, 3 Antibodies	CD272/BTLA	MIH26	175Lu	Surface	3175042
	CD244/2B4	PF05	166Ho	Surface	3166046
	CD223/LAG-3	11C3C65	172Yb	Surface	3172035
Human Cytokine Core CyTOF Panel, 5 Antibodies (201344)	IL-2	MQ1-17H12	112Cd	Cytoplasmic	3112002
	TNFα	MAb11	114Cd	Cytoplasmic	3114002
	IFNγ	B27	116Cd	Cytoplasmic	3116002
	IL-4	MP4-25D2	171Yb	Cytoplasmic	3171028
	IL-17A	BL168	169Tm	Cytoplasmic	3169002
Human Cytotoxic Mediators CyTOF Panel, 3 Antibodies (201345)	IL-6	MQ2-13A5	106Cd	Cytoplasmic	3106003
	Perforin	B-D48	196Pt	Cytoplasmic	3196002
	Granzyme B	GB11	198Pt	Cytoplasmic	3198002
Drop-in and custom	TGF-β	9206064	164Dy	Surface	3164033
	CD69	FN50	113Cd	Surface	3113002
	Ecad	24/E-Cad	115In	Surface	3115003
	EpCAM	9C4	139La	Surface	Custom
	127-IdU	NA	127I	Surface, 37 °C	201127
	Her2	24D8	148Nd	Cytoplasmic	3148011
	β-catenin	D10A8	147Sm	Nuclear	3147005
	Ki-67	B56	169Tm	Nuclear	Custom
Cell-ID Cisplatin- 194Pt	Viability		194Pt	Surface	201194
Cell-ID Iridium	DNA		191/193Ir	Intracellular	2011924
Barcoding	Barcode1		112Cd	Surface	
	Barcode2		114Cd	Surface	
	Barcode3		116Cd	Surface	
	Barcode4		106Cd	Surface	CHF-20100
	Barcode5		108Cd	Surface	
	Barcode6		110Cd	Surface	
	Barcode7		113In	Surface	

Table 1. CyTOF panel of 57 parameters composed of 47 antibodies, viability, DNA and barcoding reagents

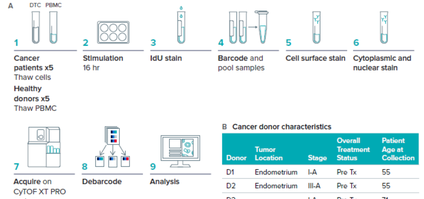


Figure 1. A) Sample stimulation, barcoding and staining workflow. B) Summary of the patient characteristics (age, tumor stage, tumor location if known) of the five sample donors

Sample stimulation and staining. Matched PBMC and DTCs from EC patients and healthy donor PBMC (n=5 each) were purchased from Discovery Life Sciences. Cells were stimulated with 25 ng/mL PMA and 500 ng/mL ionomycin in complete RPMI in the presence of brefeldin A and monensin (BioLegend) for 16 hours at 37 °C in a humidified incubator with 5% CO2. After labeling with 25 µM Cell-ID™ 127 IdU for 15 minutes at 37 °C, samples were harvested before live-cell universal barcoding using a tellurium-based 7-choose-3 strategy. The pooled and barcoded samples were then stained for viability, surface, cytoplasmic and nuclear targets before labeling with 15.6 nM Cell-ID Intercalator (iridium), followed by washing in Maxpar™ Cell Staining Buffer and Maxpar Cell Acquisition Solution (CAS) Plus according to the Maxpar Cell Staining with Fresh Fix User Guide (FLDM-01319).

Data acquisition and analysis. Samples were acquired on a CyTOF XT PRO instrument with a redesigned injector capable of increased throughput at >500 events/second, allowing for rapid acquisition of the barcoded sample after resuspension in Maxpar CAS Plus and addition of EQ™ Six Element Calibration Beads. Data normalization and debarcoding were performed to obtain sample-specific cell frequencies and marker expression profiles using Cytobank (Beckman Coulter) or CellEngine (CellCarta). Dimensionality reduction using an opt-SNE algorithm in Cytobank was performed for samples with at least 10,000 live singlets. Heat maps and correlation matrices were generated with Morpheus (Broad Institute).

Results

A 47-marker CyTOF panel for broad immune cell phenotyping, deep T cell profiling, cytokine production and cell proliferation

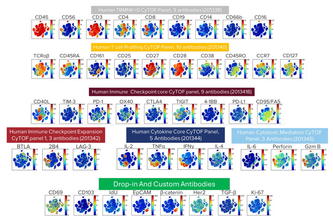
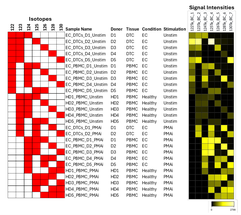


Figure 2. A comprehensive CyTOF panel measuring 47 markers was rapidly designed by combining pre-optimized modules for immune cell phenotyping, T cell profiling and cytokine/cytotoxicity detection. Eight additional antibodies relevant to endometrial cancer were integrated, creating a tailored panel that reveals immune and non-immune cell phenotypes, functions and interactions with minimal optimization effort. This panel expansion included assessment of cell proliferation using IdU incorporation and Ki-67 staining. Additionally, tumor cells and tumor-infiltrating leukocytes (TILs) were further characterized by incorporating the key markers EpCAM, HER2, β-catenin, CD69, CD103, PD-L1, FoxP3 and TGF-β1. In combination with the 39 markers distributed across six predefined panels, these add-on markers provide a highly customized panel for probing immune cell and non-immune cell phenotypes, functions and potential interactions.

Tellurium-based live-cell barcoding for sample multiplexing of immune and non-immune cells enables fast sample processing and reduces batch effects.



- 27 samples
- 10 individuals
- 0.3M–2M cells/sample
- Two stimulation conditions
- Two tissue types

Figure 3. Cell type-agnostic barcoding to reduce technical variation and batch effects across distinct sample types. Tellurium isotopologues (TeMal) were used to multiplex 27 samples using a 7-choose-3 barcode scheme, reducing hands-on time and ensuring consistent and comparable data across sample types. The samples were then pooled and stained in a single tube for surface, intracellular and nuclear targets before acquisition on a CyTOF XT PRO instrument. After acquisition, the multiplexed sample was debarcoded using CyTOF Software v9.2 with a barcode separation threshold of 0.3 and a Mahalanobis distance of 10 (representing no Mahalanobis filtering). About 15% of events were unassigned, including EQ6 Beads.

Cell composition and functional heterogeneity in PBMC and tumor-derived cells from endometrial cancer patients

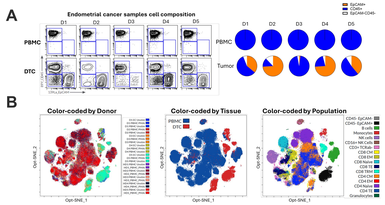


Figure 4. Cell composition and functional heterogeneity in PBMC and tumor-derived cells from EC patients. A) Cell composition in PBMC and dissociated tumor samples from five EC patients (D1–D5). B) Cells projected onto opt-SNE plots and colored based on donor identity, tissue type or gated population identity for samples with at least 10,000 live singlets. t-SNE plots were generated from 10,000 events per sample using all the Flex-Fit panels and drop-in antibodies (47 markers). C) Expression of EpCAM, β-catenin, HER2, PD-L1, CD95/Fas, IdU and Ki-67 in unstimulated dissociated tumor cells in the five EC patients.

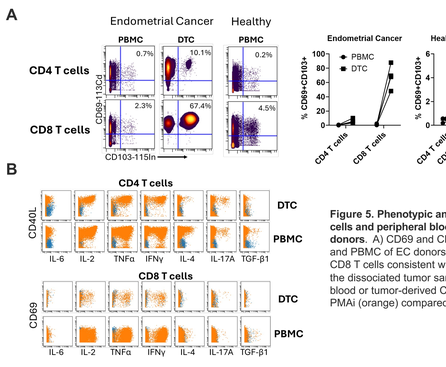


Figure 5. Phenotypic and functional profiling of T cells in tumor-derived cells and peripheral blood from EC donors and in peripheral blood healthy donors. A) CD69 and CD103 expression on CD4 and CD8 T cells from DTC and PBMC of EC donors and PBMC of HDs. An increase in CD69+CD103+ CD8 T cells consistent with tissue residency phenotype is observed only in the dissociated tumor samples. B) Cytokine response in EC donor peripheral blood or tumor-derived CD4 and CD8 T cells after *in vitro* stimulation with PMAi (orange) compared with unstimulated samples (blue)

Activation and checkpoint markers in tumor and peripheral blood T cell subsets captured the unique enrichment of CD69+ CD103+ tissue-resident memory T cells in tumor samples.

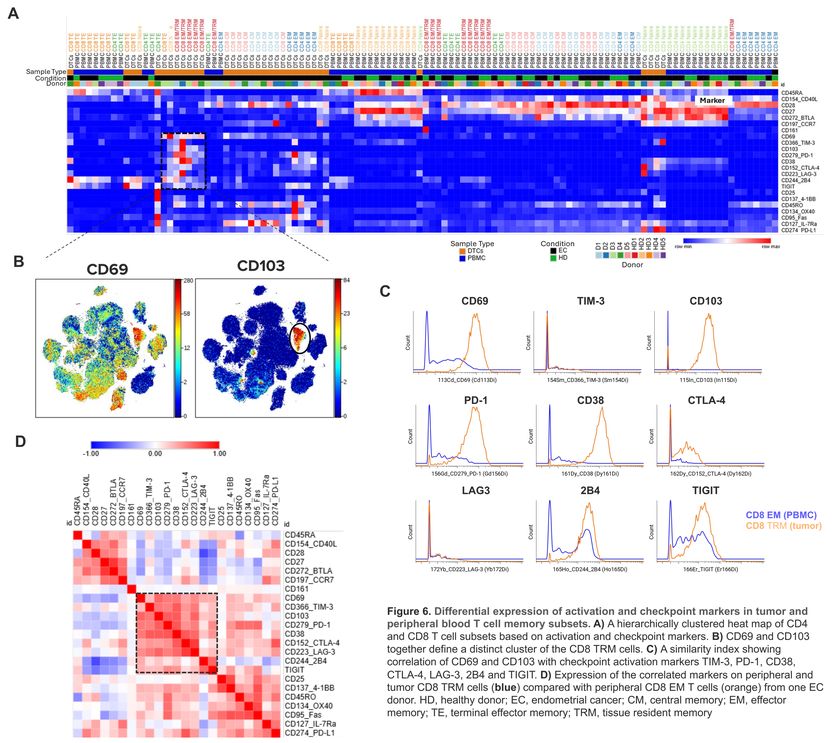


Figure 6. Differential expression of activation and checkpoint markers in tumor and peripheral blood T cell memory subsets. A) A hierarchically clustered heat map of CD4 and CD8 T cell subsets based on activation and checkpoint markers. **B)** CD69 and CD103 together define a distinct cluster of the CD8 TRM cells. **C)** A similarity index showing correlation of CD69 and CD103 with checkpoint activation markers TIM-3, PD-1, CD38, CTLA-4, LAG-3, 2B4 and TIGIT. **D)** Expression of the correlated markers on peripheral and tumor CD8 TRM cells (**blue**) compared with peripheral CD8 EM T cells (orange) from one EC donor. HD, healthy donor; EC, endometrial cancer; CM, central memory; EM, effector memory; TE, terminal effector memory; TRM, tissue resident memory

Conclusions

- This panel enables cell composition analysis of immune and non-immune cells including frequencies of T cells, B cells, NK cells, granulocytes, monocytes and epithelial cells
- By using modular and flexible panel design, deeper characterization of T cells and NK cells (memory, exhaustion, response to stimuli, proliferation) revealed disease- or tissue-specific processes
- This approach can be used for tracking the homing, activation and functional responsiveness of engineered cell therapy products such as TILs and CAR T cells at the tumor site
- The response to other immune-directed therapies such as checkpoint inhibitors, antibody-drug conjugates, cancer vaccines or bispecific T cell engagers can be monitored
- Tellurium-based barcoded enabled sample-sparing multiplexed staining and acquisition to reduce batch effects and streamline the sample preparation, staining and acquisition workflow

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CyTOF XT PRO

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