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Spatial multiplexing of protein biomarkers for immune cell profiling of the tumor microenvironment with ChipCytometry

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Abstract

Background: Understanding the spatial distribution of key immune cell populations is critical in advancing our understanding of cancer and the development of novel therapeutics. Historically, the spatial analysis of the tumor microenvironment (TME) has been limited to low-plex immunohistochemical (IHC) or immunofluorescent (IF) assays, which were inadequate for deep immune cell profiling. Here, we present the development and validation of a highly multiplexed IF assay for quantitative immune cell phenotyping in FFPE human tissue.

Methods: Experiments were performed using CellScape™ for precise spatial multiplexing, featuring ChipCytometry™ technology. The platform utilizes automated cyclic IF staining with open-source antibodies for detection of highly multiplexed protein biomarkers as well as high dynamic range imaging to facilitate quantitative phenotyping with single-cell resolution. To develop and validate a ready-to-use assay for tissue immune phenotyping, candidate antibodies were first screened for specificity and optimized for sensitivity in singleplex. Then, validation of the combined multiplex panel included determination of suitability for appropriate tissue types, specificity for targets without background noise, and reproducibility.

Results: The results show precise detection of each of the protein biomarkers in the developed 15-plex assay panel while maintaining spatial information about each cell. Dozens of immune cell subtypes were identified and quantified based on protein expression profiles. The 15-plex panel was validated on lung, breast, and colon tissues, and has also been tested on ovary, spleen, and tonsil tissues. Multiplex assay development and validation was a multi-stage, iterative process to evaluate antibodies for suitability, specificity, and reproducibility.

Conclusions: The ChipCytometry platform enables simultaneous detection of multiple protein biomarkers on a single tissue section for deep immune cell profiling in the TME. Combined with the single-cell spatial information, such datasets provide an opportunity for the discovery of new complex multiplexed biomarker signatures to inform therapeutic development and personalized medicine.

Assay development process

Goal: Create and validate a highly multiplexed immunofluorescence assay for precise immune phenotyping in human tumor tissues

Each antibody in the immune phenotyping panel completed a multi-step screening process:

1. Identification based on manufacturer-reported specificity and fitness for application
2. Assessment of specificity and sensitivity of candidate antibodies in single plex with appropriate counterstains to ensure that they localize to the expected structures and subcellular regions
3. Development of antibody panels in multiplex, including optimization of antibody titers and staining protocols

The multiplex assay panel was then validated for the following performance features:

1. Suitability for target tissues
2. Specificity of signal
3. Reproducibility of immune phenotyping

All assays were performed using the CellScape platform, featuring ChipCytometry technology.

Table 1. Optimized multiplex assay staining protocol

Cycle	Target	Filter Set	Incubation Time
1	Ki-67	FS560	60 minutes
	CD3	FS488	
2	CD45	FSPerCP	60 minutes
	CD8	FS560	
	AF488 quencher	None	
	CD45RA	FS395	
3	CD68	FS488	60 minutes
4	CD27	FS560	60 minutes
	AF488 quencher	None	
5	EpCAM	FSPerCP	60 minutes
	FOXP3	FS560	
	CD4	FS488	
6	CD20	FSPerCP	60 minutes
	CD279	FS560	
	AF488 quencher	None	
7	CD274	FS560	60 minutes
	Granzyme B	FS488	
	DNA	FS395 & FS421	

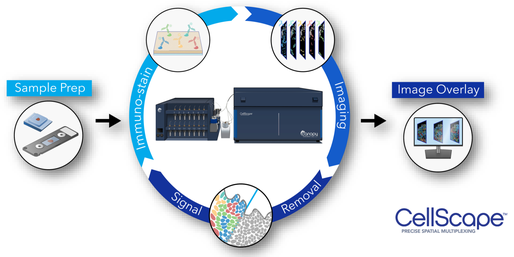

Figure 1. After sample preparation, the ChipCytometry workflow consists of successive rounds of immuno-staining, imaging, and signal removal to profile many biomarkers on a single sample. High dynamic range (HDR) imaging uses multiple exposure times to capture a wide range of biomarker expression. An image overlay is created, and cells are then phenotyped based on marker expression patterns. The cyclic immunofluorescence staining and imaging process is fully automated for up to four samples at a time with CellScape.

Multiplex assay panel is suitable for human FFPE tissues

The 15-plex assay panel was validated for suitability in duplicate using human FFPE breast cancer, colorectal cancer, and lung cancer samples. Each marker was assessed for presence of measurable signal and specificity in each tissue type. The assay panel was also tested with favorable results on healthy tonsil, healthy spleen, and head and neck cancer samples (not shown).

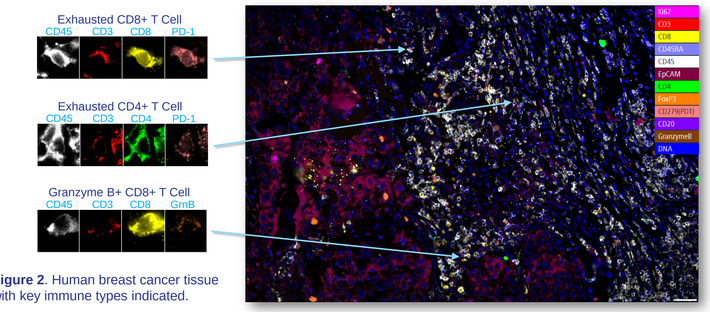

Figure 2. Human breast cancer tissue with key immune types indicated.

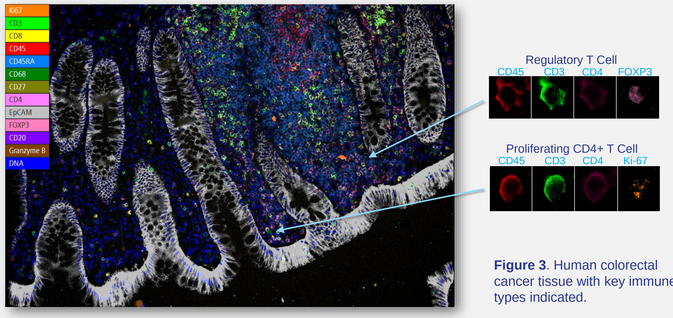

Figure 3. Human colorectal cancer tissue with key immune types indicated.

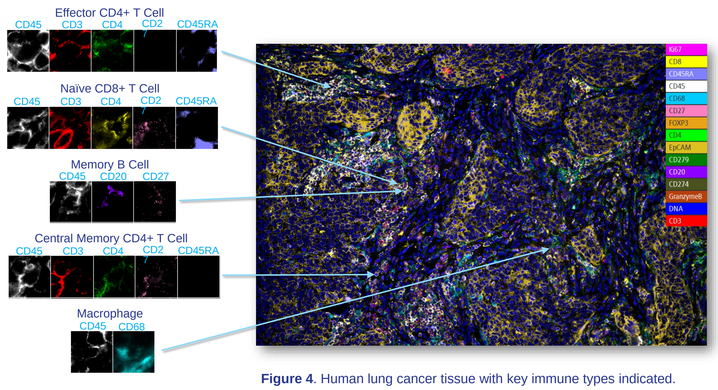

Figure 4. Human lung cancer tissue with key immune types indicated.

Assay panel demonstrates specificity

All antibodies in the 15-plex assay panel underwent testing for specificity with appropriate counterstains to confirm that each antibody stained the appropriate tissue structures, localized to the expected subcellular region, and did not exhibit significant background signal. Every antibody met the specificity criteria.

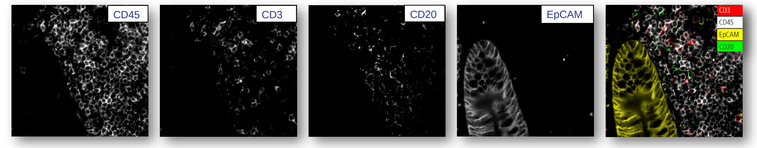

Figure 5. Representative specificity images from a colorectal cancer sample. CD45 staining overlaps with both CD3 and CD20 as expected for immune cells. CD3 and CD20 antibodies stain non-overlapping cell populations as expected for T cells and B cells, respectively. EpCAM staining is present on structurally distinct mucosal epithelia and does not overlap with immune cell markers.

Quantitative immune phenotyping is reproducible

The multiplex assay panel was tested for reproducibility on three tissue types (breast, colon, and lung) by quantifying immune cell populations. Replicates demonstrated consistency within samples and across days.

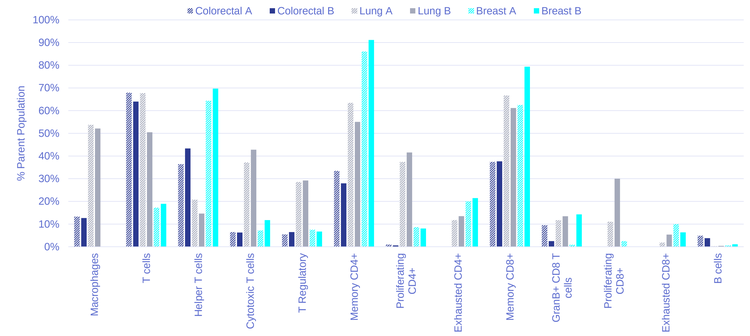

Figure 6. Technical duplicates from the same tissue cores were assayed on different days using the same CellScape instrument. Immune cell populations were identified by bivariate gating, quantified, and compared across replicates. Duplicates are indicated by shaded/solid of the same color.

Conclusions

- We developed and validated a robust assay for immune cell phenotyping in intact tissues utilizing CellScape, featuring ChipCytometry technology, for highly multiplexed cyclic immunofluorescence.
- Assay development included antibody screening, titer optimization, and staining protocol generation.
- Validation of assay panel included confirmation of suitability, specificity, and reproducibility.
- The multiplex panel developed here, commercialized as VistaPlex™ Spatial Immune Profiling Assay Kit, can be used to detect and quantify immune infiltration in fixed human tissues, both healthy and cancerous, with reliable results.
- Using a high quality pre-validated assay panel saves assay development time and increases reproducibility while still providing the flexibility to customize further.

Resources

Application note: Antibody validation

VistaPlex Assay Kits

ChipCytometry Technology

CellScape Platform

Contact

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