Demo Data IA Report (8plex)

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Project: Demo Data (8plex)
Project Sponsor: NA
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Delivery Date: NA

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Project Overview

Executive Summary

Project Goals

The goal of this project was to analyze seven NSCLC samples stained with the FixVue Immuno8 Kit using the ‘Standard Image Analysis Service” to provide an example report and data-tables to be shared with prospective customers.

Project Results

The analysis was conducted successfully, results are summarized in this report.

Staining Report

This paragraph summarizes the high level outcome of the performed staining service. A detailed report can be ordered separately.
Samples were stained and scanned in 3 consecutive runs followed by co-registration of all images.

1. Run 1: DAPI, **FITC**: CD3, **TRITC** : CD4, **Cy5**: CD68, **Cy7**: CD8
2. Run 2: DAPI, **FITC**: CK, **TRITC** : FoxP3, **Cy5**: PD-1, **Cy7**: PD-L1
3. Run 3: H&E

Process Step	# Sections (pass/all)	Comment
Run 1	7/7	
Run 2	7/7	
Run 3	7/7	
Co-registration	7/7	

Image Analysis Report

This paragraph summarizes the performed image analysis.
Booked analysis:

- Standard Image Analysis with Tissue Segmentation

Phenotypes

Label Type	Class Type	Label	Phenotype	Description
Cell	Single-Marker Positive	CD3+ Cells	CD3+	Cells positive for CD3, all other markers are ignored (+ or -)
Cell	Single-Marker Positive	CD4+ Cells	CD4+	Cells positive for CD4, all other markers are ignored (+ or -)
Cell	Single-Marker Positive	CD68+ Cells	CD68+	Cells positive for CD68, all other markers are ignored (+ or -)
Cell	Single-Marker Positive	CD8+ Cells	CD8+	Cells positive for CD8, all other markers are ignored (+ or -)

Cell	Single-Marker Positive	CK-Sox10+ Cells	CK-Sox10+	Cells positive for CK-Sox10, all other markers are ignored (+ or -)
Cell	Single-Marker Positive	FoxP3+ Cells	FoxP3+	Cells positive for FoxP3, all other markers are ignored (+ or -)
Cell	Single-Marker Positive	PD-1+ Cells	PD-1+	Cells positive for PD-1, all other markers are ignored (+ or -)
Cell	Single-Marker Positive	PD-L1+ Cells	PD-L1+	Cells positive for PD-L1, all other markers are ignored (+ or -)
Cell	Phenotype	All Cells	CD3+-/CD4+-/CD68+-/CD8+-/CK-Sox10+-/FoxP3+-/PD-1+-/PD-L1+-	All detected cells. May be positive or negative for any marker.
Cell	Phenotype	CD3+ Cells	CD3+	Cells positive for the CD3+ phenotype. Unspecified markers are ignored (+ or -)
Cell	Phenotype	CD3+/CD4+ Cells	CD3+/CD4+	Cells positive for the CD3+/CD4+ phenotype. Unspecified markers are ignored (+ or -)
Cell	Phenotype	CD3+/CD4+/CD8+ Cells	CD3+/CD4+/CD8+	Cells positive for the CD3+/CD4+/CD8+ phenotype. Unspecified markers are ignored (+ or -)
Cell	Phenotype	CD3+/CD4+/FoxP3+ Cells	CD3+/CD4+/FoxP3+	Cells positive for the CD3+/CD4+/FoxP3+ phenotype. Unspecified markers are ignored (+ or -)
Cell	Phenotype	CD3+/CD8+ Cells	CD3+/CD8+	Cells positive for the CD3+/CD8+ phenotype. Unspecified markers are ignored (+ or -)
Cell	Phenotype	CD3+/CD8+/FoxP3+ Cells	CD3+/CD8+/FoxP3+	Cells positive for the CD3+/CD8+/FoxP3+ phenotype. Unspecified markers are ignored (+ or -)

Cell	Phenotype	CD3+/FoxP3+ Cells	CD3+/FoxP3+	Cells positive for the CD3+/FoxP3+ phenotype. Unspecified markers are ignored (+ or -)
Cell	Phenotype	CD3+/PD-1+ Cells	CD3+/PD-1+	Cells positive for the CD3+/PD-1+ phenotype. Unspecified markers are ignored (+ or -)
Cell	Phenotype	CD68+ Cells	CD68+	Cells positive for the CD68+ phenotype. Unspecified markers are ignored (+ or -)
Cell	Phenotype	CD68+/PD-L1+ Cells	CD68+/PD-L1+	Cells positive for the CD68+/PD-L1+ phenotype. Unspecified markers are ignored (+ or -)
Cell	Phenotype	CD8+ Cells	CD8+	Cells positive for the CD8+ phenotype. Unspecified markers are ignored (+ or -)
Cell	Phenotype	CK-Sox10+ Cells	CK-Sox10+	Cells positive for the CK-Sox10+ phenotype. Unspecified markers are ignored (+ or -)
Cell	Phenotype	CK-Sox10+/PD-L1+ Cells	CK-Sox10+/PD-L1+	Cells positive for the CK-Sox10+/PD-L1+ phenotype. Unspecified markers are ignored (+ or -)

Image Analysis Inventory:

Image ID	Customer ID	Customer Specimen ID	Image Analysis QC
ULT-2020-101-008_1	sample_1	case_1	passed
ULT-2020-111-012_1	sample_2	case_2	passed
ULT-2020-111-014_1	sample_3	case_2	passed
ULT-2020-185-008_1	sample_4	case_3	passed
ULT-2020-1973-009_1	sample_5	case_4	passed
ULT-2020-92-008_1	sample_6	case_5	passed
ULT-2020-94-009_1	sample_7	case_6	passed

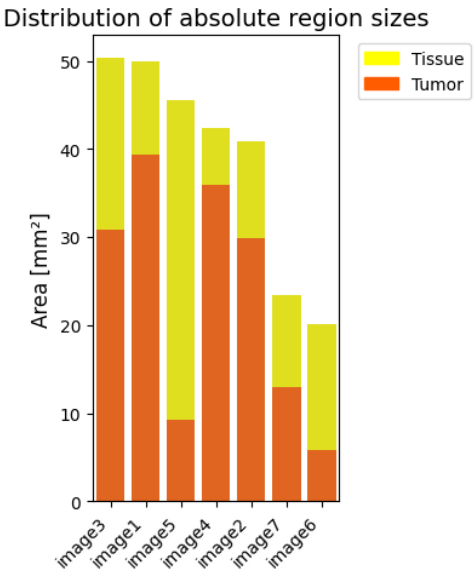
Data Overview

Analysis of high-plex stained tissue sections produces extensive amounts of quantitative data. In this section, a statistical overview of the project results is given to support initial analysis and hypothesis creation.

Distributions

Areas

The absolute size of the analyzed tissue and tissue regions gives an indication of how representative the generated cell quantifications are in relationship to the overall tumor sample. Small tissue samples (e.g. biopsies) or small subregions in the sample are less likely to produce data representative for the overall tumor mass.

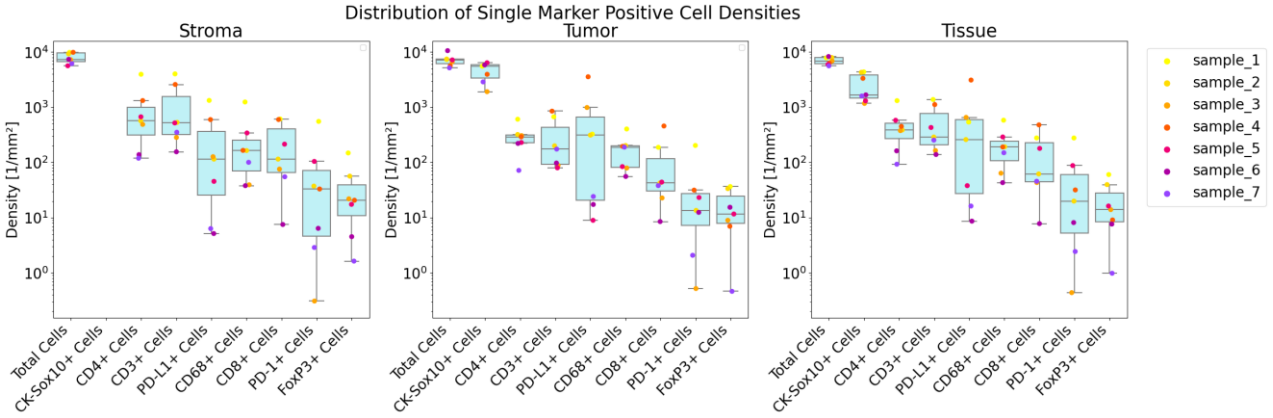


Cell Densities

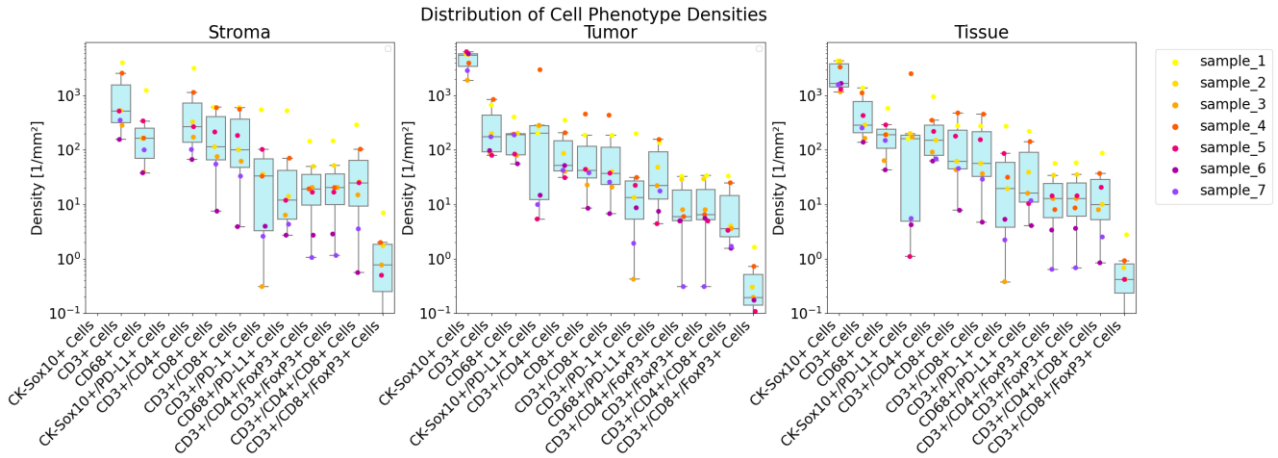
Similar to the analyzed areas, the frequency and distribution of measured cell classes can give important insights. Results for cell types which occur very often can be considered more representative than less frequent ones, however, rarely occurring cell types can be highly significant. The width of the distribution is another important factor. High variability indicates larger differences between samples, which could be related to differences in tumor biology. Importantly, the frequency of quantified cells strongly depends on the tissue

compartment in which they are quantified. Typically, immune cells are more frequent in stroma areas than in epithelium. Samples with high amounts of immune cells in the epithelium may indicate an active inflammation directed against the tumor and favorable prognosis in some tumor indications.

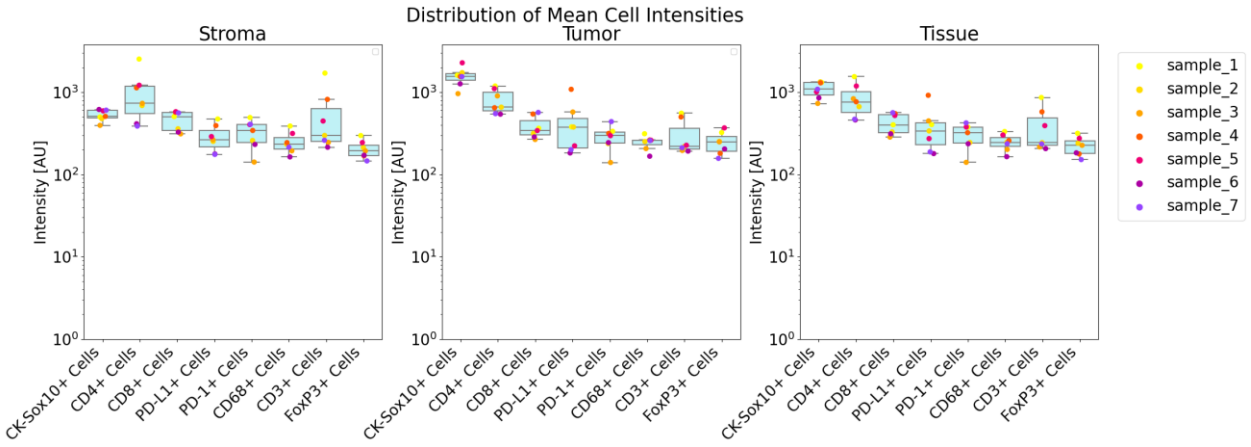
The following charts present an overview of the identified frequencies of marker positive cells in the analyzed tissue regions. Cell markers are sorted from high to low densities in the overall tissue region. Expression of markers can vary between different tissue regions. The y-axis of the chart is in logarithmic scale to allow evaluation of rarely expressed markers:



The following charts present an overview of the identified frequencies of cell phenotypes in the analyzed tissue regions. Cell phenotypes are sorted again from high to low densities in the overall tissue region. The y-axis of the chart is in logarithmic scale to allow evaluation of rare marker frequencies:



The next charts illustrates the distribution of staining intensities in cells in different regions:

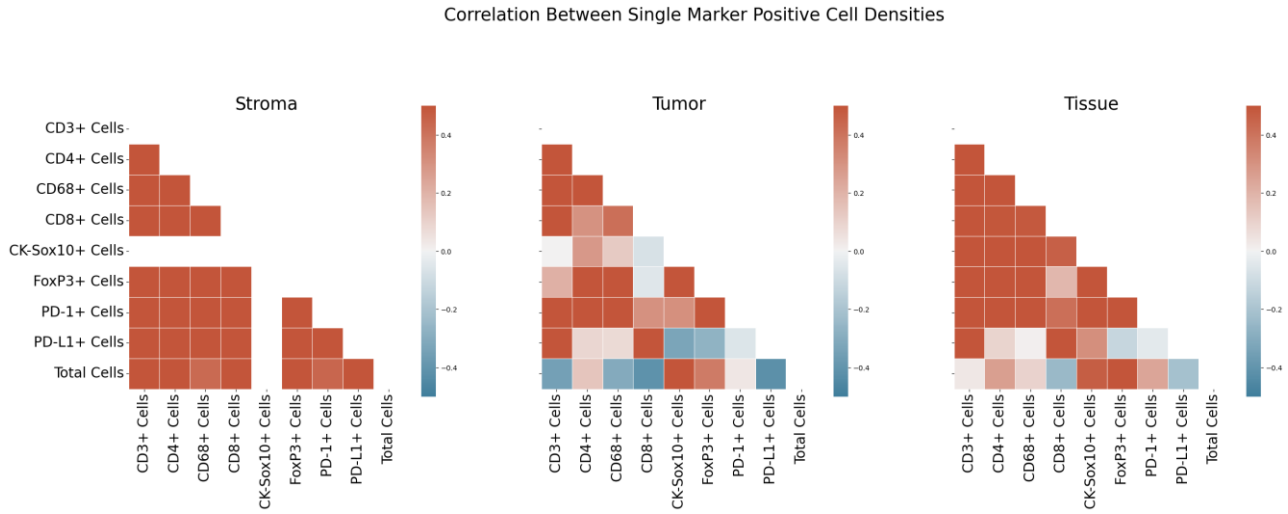


Correlation Analysis

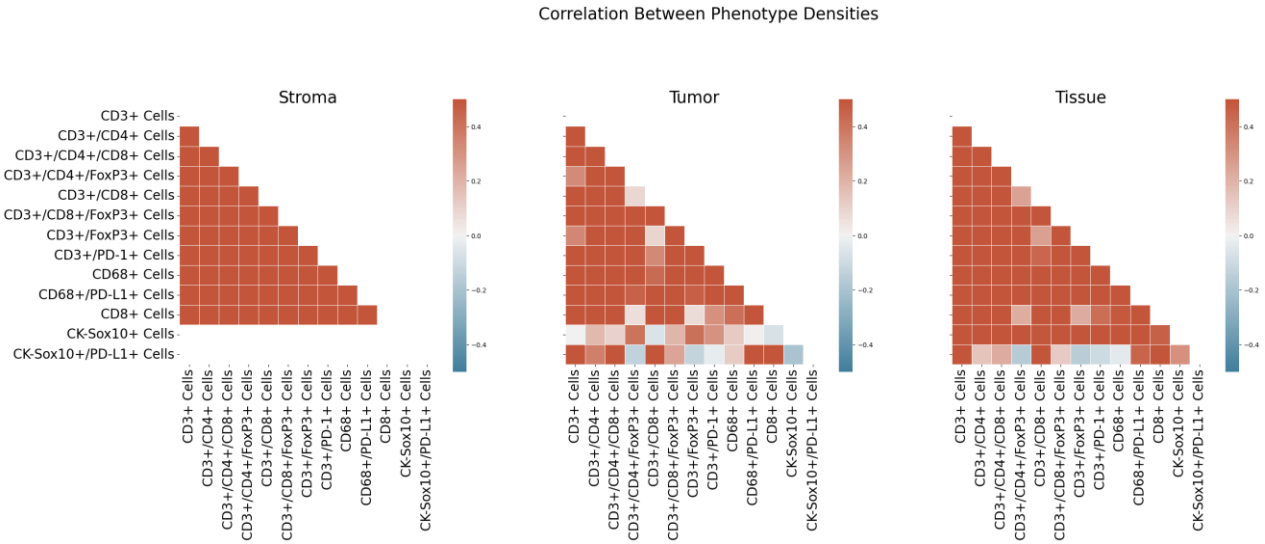
Inspecting correlations between marker or phenotype frequencies and samples can give interesting insights into the cohort in a hypothesis free and data driven way. It can reveal which cell types often co-occur or are mutually exclusive and help to group patient samples based on similar results.

Correlation between cell types

The following charts visualize the correlation between densities of marker positive cells. Positive correlations (e.g. an increasing amount of CD8 positive cells is often associated with an increasing amount of CD3 positive cells) are displayed in red, negative correlations in blue. Correlations can change depending on the analyzed region:



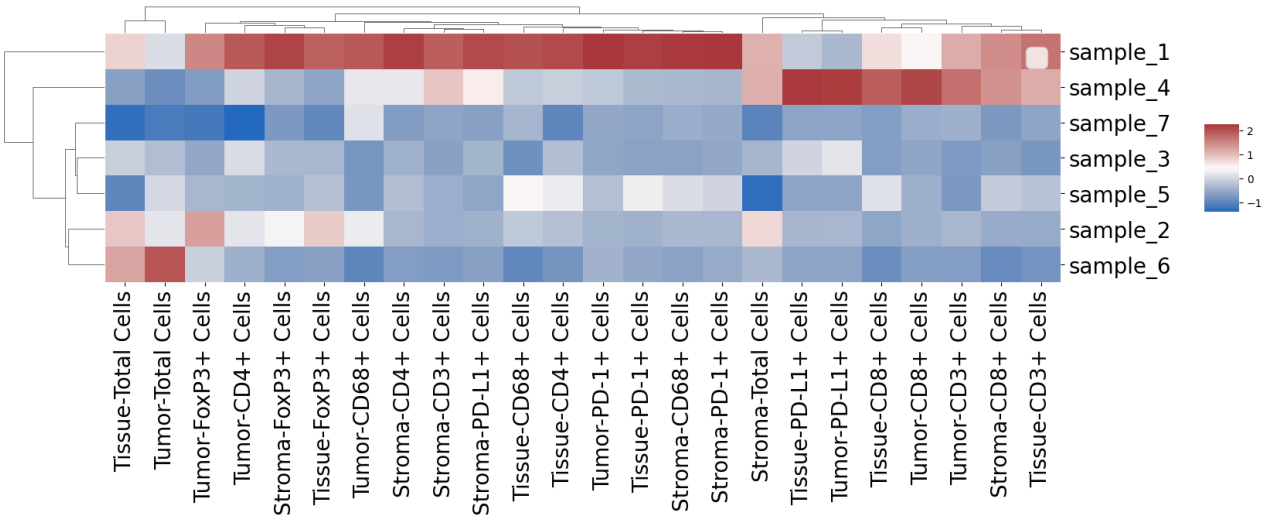
The following charts visualize the correlation between densities of cell phenotypes in the analyzed cases. Positive correlations are displayed in red, negative correlations in blue. Correlations can change depending on the analyzed regions:



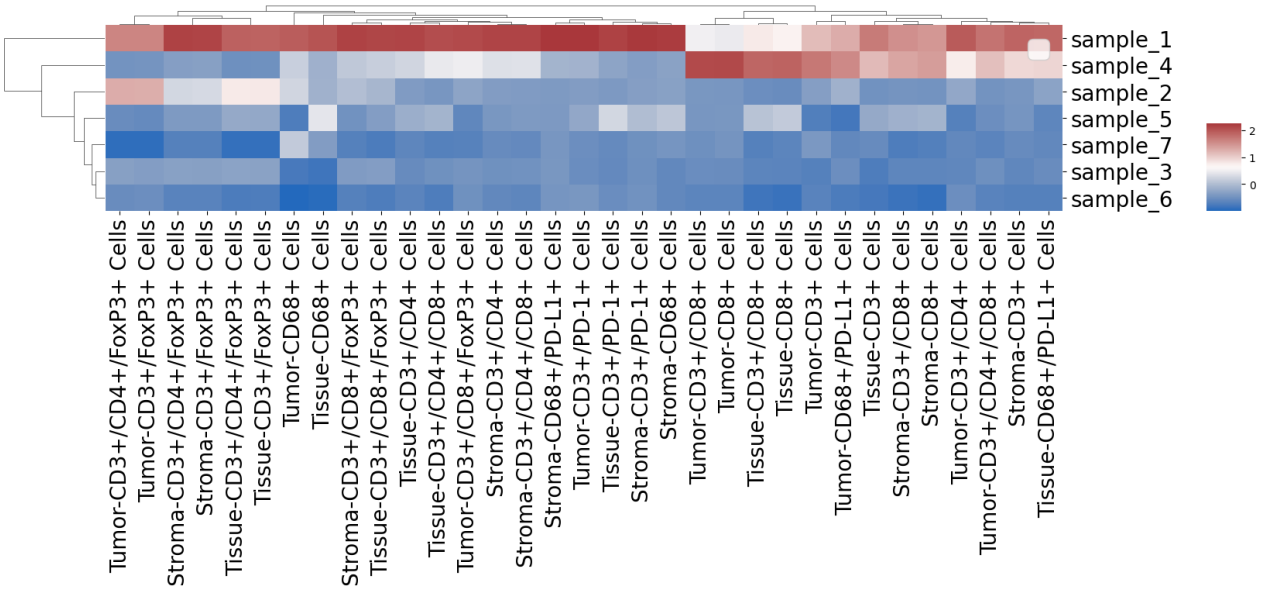
Correlation between cell types and samples

Cluster analysis is a useful tool to identify patterns in a dataset. Image analysis result can be clustered over the cases (i.e. cases with similar readouts are grouped together) and over the readouts (i.e. readouts with similar distributions across the cases are grouped together). In the following heatmaps, the densities are normalized by z-score, i.e. a case will appear red, if it has above average density, white for average density and blue for densities below average.

Cases versus marker positive cells:



Cases versus cell phenotypes:

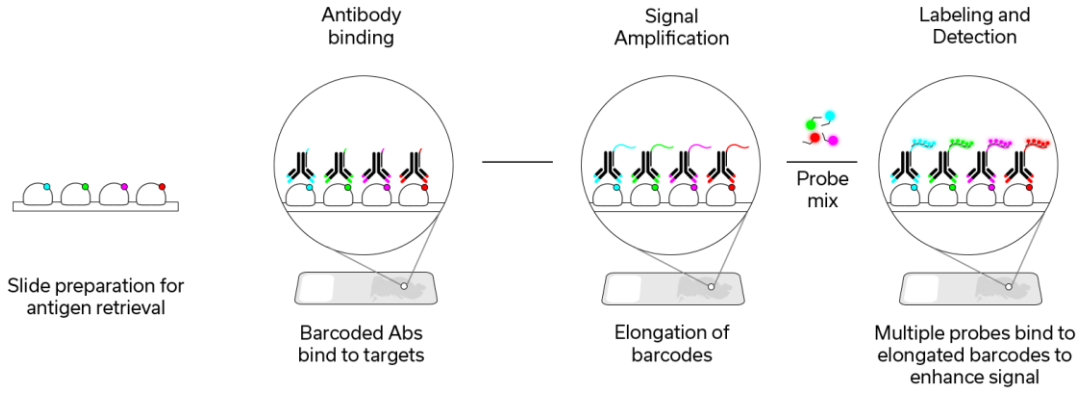


Materials and Methods

ISP assay workflow

Ultivue provides researchers and scientists with multiplex biomarker assays and advanced image informatics for tissue phenotyping and digital pathology. Our proprietary InSituPlex® technology enables advanced exploration and interrogation of tissue samples for precision medicine research. These highly customizable solutions and scientific consultative approach strengthen and accelerate biomarker discovery and drug development programs.

InSituPlex® technology requires only a single antigen retrieval step before applying a mixture of DNA barcoded antibodies, which then bind to the desired array of antigens. After amplification, fluorescent probes are attached to those barcodes, allowing visualization using fluorescent microscopy. The InSituPlex technology is available in 4, 8, or 12-plex panel options.



The workflow can be summarized in four to five steps:

Slide Preparation:

The first step involves slide preparation (dewaxing) and single antigen retrieval from FFPE samples. This simplified process requires only one retrieval step, preserving the tissue.

Antibody binding:

The antibody barcode conjugates are incubated on the slide to stain the biomarkers. Each antibody is labelled with a DNA barcode that uniquely identifies it for detection, eliminating the need for secondary antibodies. Up to 12 different antibodies can be added simultaneously. Only one single staining step is required.

Barcode Amplification:
During the next step the barcodes linked to the antibodies are amplified simultaneously and at the same rate. The extended barcodes do not interfere with the immediate surroundings so there is no steric hindrance or epitope masking.
Detection:
The complimentary fluorescent DNA probes are added and hybridize to the barcode targets. The samples are then ready for imaging.
Signal Removal (Optional):
For additional imaging rounds, fluorescent signal must be removed by using exchange technology. This involves the gentle removal of fluorescent DNA probes, while keeping barcodes and tissue integrity intact. The slide is now ready for additional fluorescent DNA probes.

Image QC workflow

To provide results optimally suited for scientific interrogation, tissue sections and scanned images undergo a multi-step QC process, which is outlined below:
Tissue Qualification:
Only tissue sections of good pre-analytical quality can be used to achieve good staining. Serial sections stained with H&E are visually checked by trained lab experts before performing ISP staining.
Staining intensity:
Image exposure times are optimized on a control sample to determine the dynamic range of fluorescence from the stained sections in the scanned images. Exposure times between 1-40 ms are considered optimal, higher exposure times may indicate insufficient staining quality.
Signal intensity
In addition to overall signal intensity, the ratio between specific staining (e.g. cells) to background regions is determined in control samples.
Staining specificity:
Tissue level: Images are visually inspected to confirm staining localization in the expected tissue regions (or absence thereof).
Cell level: Images are visually inspected to confirm the anticipated staining distribution in positive cells.
Background staining: Images are visually inspected for the absence of unexpected background staining artifacts.
Co-registration quality:

If more than one staining run was performed, images are aligned with UltiStacker software. Co-registration quality is determined by an automated score and confirmed visually.

Image analysis workflow

Segmentation and initial classification

Image analysis is performed using state of the art strategies combining segmentation of DAPI stained nuclei and classification (Fig. 1):

Nuclei are detected and segmented, based on the DAPI signal (Fig 1A,B). Cells (represented by the nuclei) are classified based on signal in or around the detected nuclei (Fig1 B,C). Depending on the required accuracy, project size and other requirements, classification algorithms will be based on thresholding approaches or deep learning models. Generally, thresholding based approaches require more adjustments per image but can be created faster for smaller image sets and previously unseen markers. Deep learning based classification algorithm development requires more initial efforts to train, but can be applied and reused on large datasets with fewer or even without further adjustments.

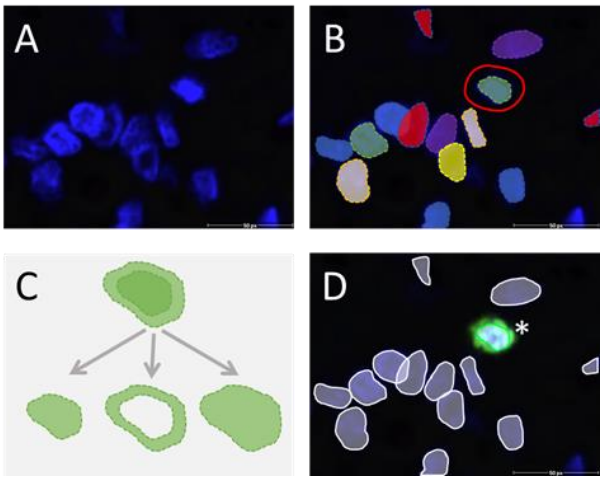


Figure 1: Image analysis strategy for cells.

Algorithm adjustments

Not all images are created the same. The interplay of tissue indication, pre-analytical sample quality and stained marker combinations introduce variability which needs to be accounted for during image analysis algorithm development.

Frequent adjustments on an image or image batch level are required to ensure optimal

quality for:

- mIF based detection of overall tissue regions
- Exclusion of regions unfit for image analysis (e.g. tissue folds, dust particles, …)
- Parameters and thresholds for threshold based cell or region classification

Pre-existing AI based algorithms are more robust and should be adjusted, if required, on a study level to provide consistent and comparable results of optimal quality. This typically entails adding supporting annotations to (re-)train deep learning algorithms for the classification of cells or detection of tissue sub-regions, like tumor-epithelium.

Result QC

Results of the region detection, cell segmentation and cell classification are visually inspected for accuracy. All steps are documented throughout the process and important findings are summarized in the image analysis report section.

Phenotyping and readout calculation

The described segmentation and classification strategy is used to generate binary classification of cells per marker. Biological interpretability is further improved by adding phenotyping analysis, such as:

- Biological knowledge about marker combinations or cell localization.
- Observation and reporting of implausible marker combinations produced by cells in close proximity. For example, a CD8 positive immune cell located in cytokeratin stained epithelium will often be labeled as CD8 and Cytokeratin positive.

By injecting expert knowledge, cells can be labeled into ‘phenotypes’ (e.g. ‘T-Helper Cell’) which give more accurate and intelligible insights into the biology of the analyzed cell populations.

Readout Generation

Quantitative readouts are generated for the analyzed tissue areas. The “Standard Image Analysis Service” includes absolute and relative cell counts (cell densities and percentages) as well as cell intensities in all analyzed tissue areas.
Spatial relationships between cell populations, heatmap analysis, tumor classification and many other readouts and analysis can be provided as part of a Spatial Phenomics Service.