Establishment of an Immune Cell Phenotyping Multiplexed Immunofluorescence Assay and Digital Image Analysis Workflow to Investigate the Tumor Microenvironment of Solid Tumors

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INTRODUCTION

Multiplexed Staining Solutions in Clinical Trials

A promising approach for cancer immunophenotyping, in the context of clinical trials, is the usage of *in situ* multiplexed immuno-fluorescence (mIF) assays. We demonstrate Discovery's highly standardized approach to novel mIF assay implementation (**Fig. 1**), focusing on the key steps in the wet lab procedure. Detailed phenotyping of spatial distribution patterns within the tumor microenvironment e.g., localization of lymphocytes, myeloid cells, fibroblasts and blood/lymphatic vessels, is an increasingly important tool for the identification of novel prognostic and predictive biomarkers for personalized cancer therapy.

METHODS & MATERIALS

Samples, Staining Devices & Reagents

Formalin-fixed and paraffin embedded (FFPE) human normal tonsil and solid tumor tissues were stained on Ventana DISCOVERY ULTRA (Roche) and Leica BOND RX (Leica Biosystems) staining devices using primary antibodies specific for FoxP3 (SP97, Abcam), CD4 (SP35, Cellmarque), CD8 (C8/144B, Dako), CD68 (PG-M1, Dako), PD-L1 (SP263, Ventana) and panCK (AE1/AE3, Dako).

Scanning, Evaluation & Image Analysis

Qualitative evaluation of slides was performed by a pathologist using standard light microscopy. mIF slides were digitized at 20x magnification using Akoya's PhenoImager™ HT slide scanner. Spectral unmixing was done with the inForm® software version 2.7.0. The image analysis algorithm was set up as a sequence of custom apps in the Visiopharm® software, version 2021.09.2.11085.

RESULTS I

Primary Antibody Titration & Epitope Sensitivity

Immunohistochemistry (IHC) staining protocols for the selected markers (single-plex, 3,3'-Diaminobenzidin [DAB]) were set up to establish the ground truth staining with optimized primary antibody concentration (**Fig. 2**) and staining order according to each target epitope's sensitivity/robustness to repeated antibody complex removal ("stripping") conditions.

Stripping Efficiency & Target-Fluorophore Pairing

The target-fluorophore combination and fluorophore dilution was optimized in single-plex IF staining protocols. Additionally, stripping conditions were optimized and confirmed for all antibodies and successfully tested by sequential incubation with an alternative fluorophore (**Fig. 3**).

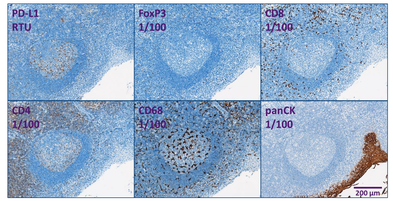

Figure 2: Single-plex chromogenic (DAB) IHCs for each selected marker.

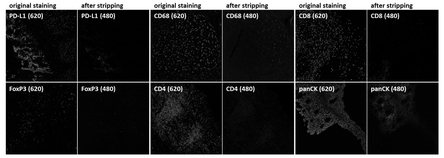

Figure 3: Confirmation of stripping efficacy for all markers of interest in the herein described panel. After stripping, no significant specific signal is observed when incubating with an alternative fluorescent dye.

Multiplex Immunofluorescence Assay

Optimized single-plex IHCs and IFs of each marker were combined into one mIF assay, which will be validated for different cancer indications afterwards for usage in clinical trials (control tissue: tonsil **Fig. 4**, tumor sample: gastric cancer **Fig. 5**).

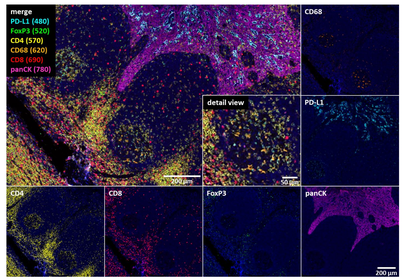

Figure 4: Final multiplex immunofluorescence assay on normal tonsil tissue.

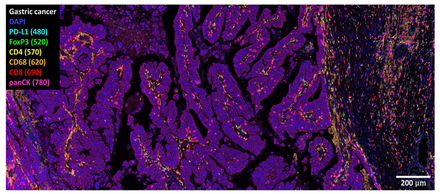

Figure 5: Final multiplex immunofluorescence assay on gastric cancer tissue.

RESULTS II – IMAGE ANALYSIS

App Sequence

A standardized image analysis app workflow was build using Visiopharm® software and Phenotyping module. The app sequence features tissue detection based on positive signals in the DAPI and autofluorescence channel, AI powered detection of nuclei in DAPI channel only, and phenotyping, i.e. labelling, of detected cells based on positive signals in the respective single channel combinations.
Gross annotation of regions of interest, i.e. whole tumor area, potential correction of sub-optimal detection of software and final release of data related to clinical trial samples remain in pathologists' hands.

Phenomapping

The Phenotyping module allows labelling of previous detected objects according to various cellular phenotypes based on positive signals in the single IF channels. These objects can then be subjected to future evaluation including counting and density measurements, distance measurements and advanced statistical evaluations such as t-SNE plots etc..
The described app workflow has been applied to several solid tumor samples, allowing for detection of cancer cells, macrophages and T_{reg} / T_{cyt} subsets (**Fig. 6**).

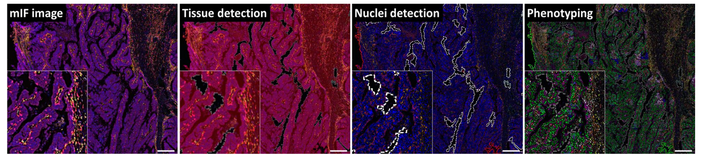

Figure 6: Image analysis workflow with detection of the tissue, nuclei and phenotyping according to biomarker positivity of single cells; scale bar 300 µm.

CONCLUSIONS

- Discovery Life Sciences has developed a state-of-the-art, GCLP compliant procedure for development and validation of custom multiplex immuno-fluorescence assays and associated digital image analysis algorithms
- The herein described 6-plex mIF assay (**Fig. 4, Fig. 5**) was successfully established by confirming epitope specificity/sensitivity and antibody complex stripping efficacy for each target throughout the whole staining process, while tissue morphology was not affected
- The selected marker panel provides a deeper understanding of the tumor immune microenvironment in clinical trials

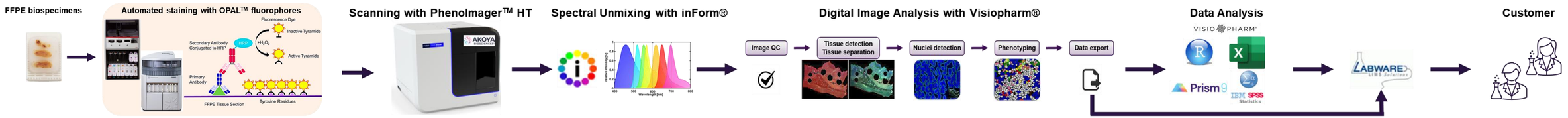

Figure 1: Immunohistochemistry assay development workflow. Formalin-fixed and paraffin-embedded (FFPE) tissue sections were stained on automated staining devices using Akoya's Opal™ fluorophores. Slides were scanned with the PhenoImager™ HT and spectral unmixing was done in inForm® prior to quantitative phenotypic digital image analysis in Visiopharm®. Data were either directly exported into LIMS and transferred to the customer or statistically analyzed, exported into LIMS and transferred to the customer.