A State-of-the-Art Procedure for Development and Validation of a Custom Multiplex Immunofluorescence Staining Panel and Image Analysis Algorithm

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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 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

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 sensitivity/robustness to repeated antibody complex removal ("stripping") conditions.

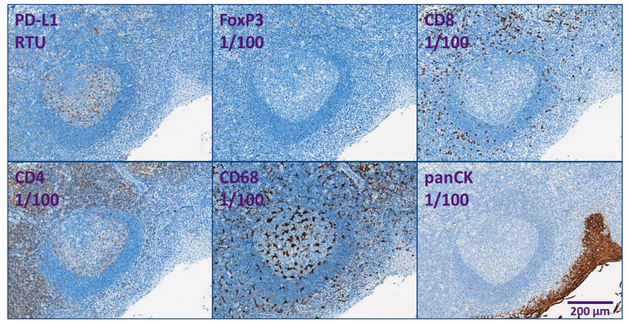

Figure 2: Single-plex chromogenic (DAB) IHCs for each selected marker.

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**).

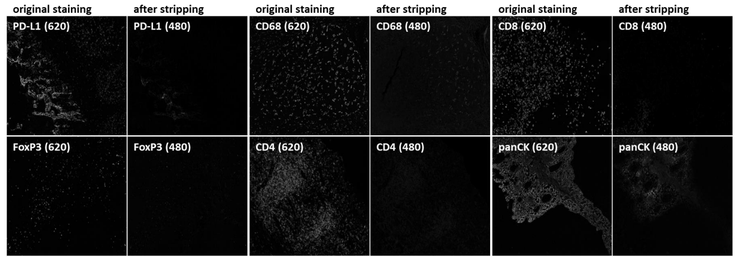

Figure 3: Confirmation of stripping efficacy for all markers of interest in the herein described panel. After stripping, no significant specific signal is observed when incubating with an alternative fluorescent dye.

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 (**Fig. 4**).

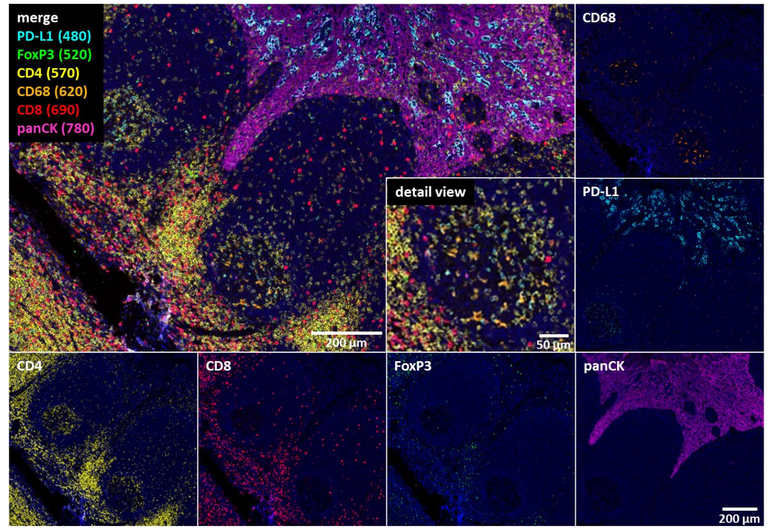

Figure 4: Final multiplex immunofluorescence assay on normal tonsil tissue.

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**) 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.