Utilizing Multiplex Chromogenic IHC and Digital Image Analysis to Evaluate Immune Cell Content and Spatial Distribution within NSCLC Tumor Tissue

L. Sherry[1], M. Anderson[1], M. Cowan[1], L. Dawson[2], R. Bystry[2] and S. N'Dure[2]

[1]OracleBio, BioCity Scotland, ML1 5UH, UK, [2]Asterand Bioscience, Royston, SG8 5HD, UK

2937/8

Introduction & Aim

Chromogenic-based immunohistochemistry (IHC) is an established technique utilized within oncology R&D, allowing cells to be visualized within the context of their tumor microenvironment. However, when quantifying chromogenic stained sections via digital image analysis, the number of targets is usually limited due to constraints in deconvolving more than 3 chromogens present on the same tissue section image.

Evaluating immune contexture within the tumor microenvironment is of paramount prognostic and predictive value and it has the potential to help identify novel targets for immunotherapies in immuno-oncology R&D. The purpose of this study was to apply a customized algorithm, including a 5-color deconvolution process to triplex chromogen IHC plus hematoxylin stained Non-Small Cell Lung Carcinoma (NSCLC) tumor tissue in order to quantify CD8 and FoxP3 immune cell content and spatial distribution within both tumor and stroma regions of interest (ROI).

Methods & Materials

An automated multiplex IHC assay was developed using the Ventana Discovery ULTRA platform and applied to formalin-fixed paraffin-embedded NSCLC tumor tissue (gender: male, age:75yr, significant clinical diagnosis: adenocarcinoma of the lung) to stain epithelial tumor cells (Pan-CK, yellow), CD8+ cells (purple) and FoxP3+ nuclei (brown), counterstained with hematoxylin (blue). Antibody details are outlined in Table 1.

Immunostained whole slide images for image analysis were generated using the Aperio ScanScope AT system. A customized algorithm, which included a 5-chromogen color deconvolution process, was utilized within the Indica Labs HALO platform to separate the four IHC chromogens (including counterstain) and a fifth color (black) representing carbon deposit artefacts. Nuclear objects were formed by applying weighted optical density values for the purple, brown and blue colors, which were positive for either CD8 or FoxP3 and counterstain. A classifier was developed to automatically segment tumor from stroma (Figure 1). Each positive cell type was identified, using defined size and shape parameters, and quantified within each ROI.

Table 1. Antibody details

Antibody	Supplier and Description
Anti-CD8 (SP239) antibody	Spring Bioscience #M5394, monoclonal rabbit IgG
Anti-FoxP3 (SP97) antibody	Spring Bioscience #M3974, monoclonal rabbit IgG
Anti-Cytokeratin, pan (mixture) antibody	Sigma-Aldrich #C2562, monoclonal mouse IgG1

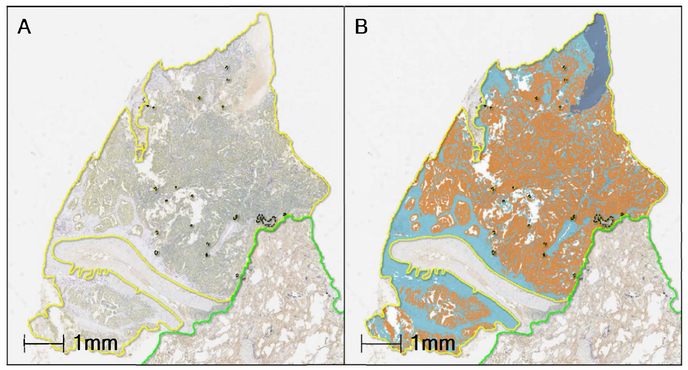

Figure 1. Whole tissue classification within multiplex IHC stained tumor section showing (A) annotated tumor (yellow outline), normal lung tissue (green outline) and (B) ROI classification overlays within the tumor annotated area (tumor – orange; stroma – light blue; necrosis/artefact – dark blue; white space – white). Black dotted lines represent artefacts that have been manually excluded.

Results & Discussion

IHC multiplex staining highlighted CD8+ cells and FoxP3+ nuclei to be present in both tumor and stroma compartments (Figure 2). Analysis data demonstrated the number of tumor and stroma CD8+ cells was 120 and 554 cells/mm^2, while the number of tumor and stroma FoxP3+ nuclei was 20 and 19 nuclei/mm^2 (Table 2). This gave a CD8+:FoxP3+ ratio of 6 within the tumor and 29 within the stroma.

Quantitative spatial distribution analysis for CD8+ cells and FoxP3+ nuclei is summarized in Table 3. The average distance of CD8+ cells in the tumor or stroma to their nearest FoxP3+ nuclei was 174µm and 149µm, respectively (Figure 3). The average distance of CD8+ cells, present in tumor and stroma, to their nearest tumor cell was 39µm while the average distance of FoxP3+ nuclei, present in tumor and stroma, to their nearest tumor cell was 29µm (Figure 4).

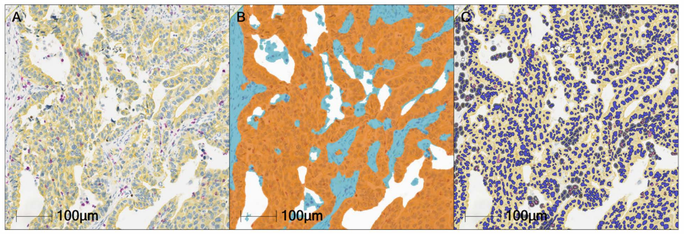

Figure 2. (A) CD8 (purple), FoxP3 (brown) and Pan-CK (yellow) multiplex IHC; (B) classified tumor (orange) and stroma (blue) ROI; (C) Cell detection overlay for CD8+ cells (purple), FoxP3+ nuclei (brown), Pan-CK cells (yellow) and hematoxylin positive nuclei (blue) in tumor and stroma ROI. Carbon deposit artefacts automatically excluded (white overlay circled in black).

Table 2. Quantitative analysis of ROI composition and IHC positive cell content within tumor and stroma ROI

Parameter	Tumor	Stroma
Content analysis		
ROI area (mm^2)	14.77	10.30
Total cell count	92,710	39,352
# CD8+ cells	1,773	5,710
# FoxP3+ cells	302	198
Calculated data		
# CD8+ cells/mm^2	120	554
# FoxP3+ nuclei/mm^2	20	19
CD8+:FoxP3+ ratio	6	29

Table 3. Quantitative spatial distribution analysis of CD8+ cells and FoxP3+ nuclei

Parameter	Analysis	
CD8+ cells to nearest FoxP3+ nuclei	**Tumor**	**Stroma**
Total CD8+ cells	1,773	5,710
Average CD8 to FoxP3 distance (µm)	174	149
Number of unique neighbors	282	356
CD8+ cells or FoxP3+ nuclei to nearest tumor cell	**CD8+ cells**	**FoxP3+ nuclei**
Total cells or nuclei across tumor plus stroma ROI	7,483	500
Average tumor cell distance (µm)	39	29
Number of unique neighbors	4,849	450

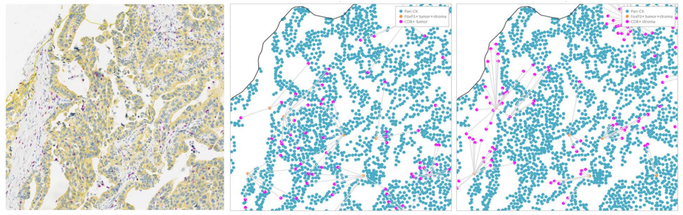

Figure 3. Spatial cell distribution analysis showing (A) multiplex stained IHC tumor section and corresponding spatial plots showing (B) the nearest FoxP3+ nuclei to every CD8+ cell within the tumor or (C) stroma. Grey lines connect CD8+ cells to their nearest FoxP3+ nuclei.

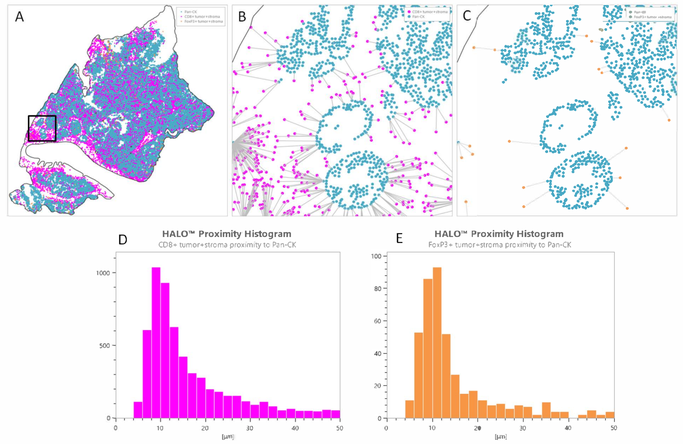

Figure 4. Spatial cell distribution analysis showing (A) CD8+ cells (purple), FoxP3+ nuclei (orange) and Pan-CK (blue) across the whole annotated tumor; high magnification example area showing (B) CD8+ cells and (C) FoxP3+ nuclei in the tumor plus stroma ROI to the nearest Pan-CK tumor cell (indicated by grey lines). Corresponding proximity histograms show the number of (D) CD8+ cells and (E) FoxP3+ nuclei that lie within 50µm of a Pan-CK tumor cell (shown in 2µm increments).

Conclusions

This study demonstrated the use of multiplex IHC combined with digital image analysis as a valuable approach for quantifying the content and spatial distribution of multiple cell types within the context of their tumor microenvironment and in relation to target positive tumor cells.

Furthermore, up to five chromogen colors can be separated utilizing a custom 5-plex analysis algorithm, leading to a more in-depth evaluation of immune cell types present and their spatial distribution. This propounds a greater potential for interpretation of therapeutic mechanistic responses in relation to the immune contexture within the tumor microenvironment.