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Antibody-Drug Conjugate Immuno-Oncology Panel for Comprehensive Characterization of the Tumor and Associated Microenvironment

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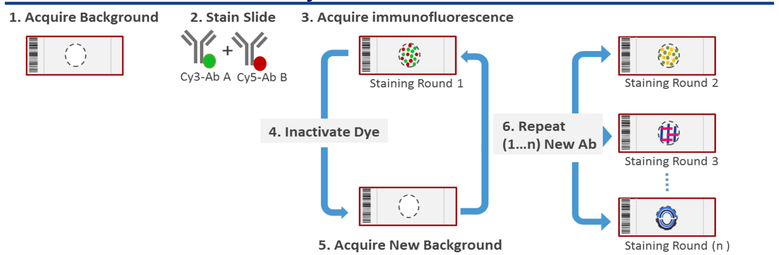
Antibody-drug conjugates (ADCs) represent a rapidly advancing class of targeted therapeutics that integrate the specificity of monoclonal antibodies with the potent cytotoxicity of small-molecule drugs. By coupling antibodies to highly active payloads through linkers, ADCs enable selective delivery of cytotoxic agents to tumor cells while minimizing systemic toxicity. Pivotal studies have shown compelling evidence that this strategy of anti-cancer therapeutics can dramatically improve outcomes for patients. Despite notable success, challenges such as tumor heterogeneity and predicting clinical efficacy have been difficult to overcome.

Because ADCs rely on precise spatial localization of target antigens to optimize treatment efficacy, subcellular resolution of protein expression is required. To provide insight into spatial patterns and heterogeneity within a tumor, we used the Paletrra™ (NeoGenomics Laboratories, Inc) platform. Paletrra is a proprietary multiplex immunofluorescence (mIF) platform for the visualization and characterization of up to 60 protein biomarkers in a single FFPE section and offers high-resolution spatial and quantitative analysis of protein expression in tissue samples. Herein, we report the design and use of a novel panel of commercially available antibodies to support ADC development in breast and lung cancer samples. The panel can measure common ADC targets such as B7H3, B7H4, c-MET, EGFR, HER2, HER3, and TROP2 combined with a membrane marker to enable subcellular differentiation between membrane and cytoplasmic expression. Quantitative image analysis enables normalization of protein expression and computation of membrane-to-cytoplasmic expression ratio, providing enhanced biological context. Moreover, because ADCs have been shown to modulate the tumor immune microenvironment, additional markers in the panel provide insight into immunogenicity, cellular relationships, and overall structural biology within the tumor samples. Application of this assay supports translational research and clinical development of ADCs, facilitating identification of patients with optimal target expression and localization.

Paletrra™ Assay Workflow and Biomarker Panel



ADC IO Panel Biomarkers	
B7H3	B7H4
CD3	CD4
CD8	CD11b
CD11c	CD31
CD45	CD56
CD68	CD163
c-MET	EGFR
FAP	FOXP3
HER2	HER3
HLA-DR	Ki67
PanCK	PD-L1
TROP2	Membrane marker

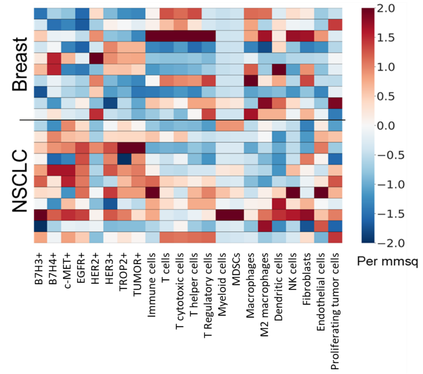


Figure 1. Paletrra assay workflow and panel information. Slides were first prepared and stained using Paletrra multiplexing IF staining protocol. For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by imaging acquisition of stained slides. The dye was erased, enabling a second round of staining with another pair of fluorescent antibodies. Table shows all markers that were stained, and heatmap shows normalized marker abundance across the breast and non-small cell lung cancer (NSCLC) samples in this study.

TROP2 IF Staining is Highly Concordant with IHC

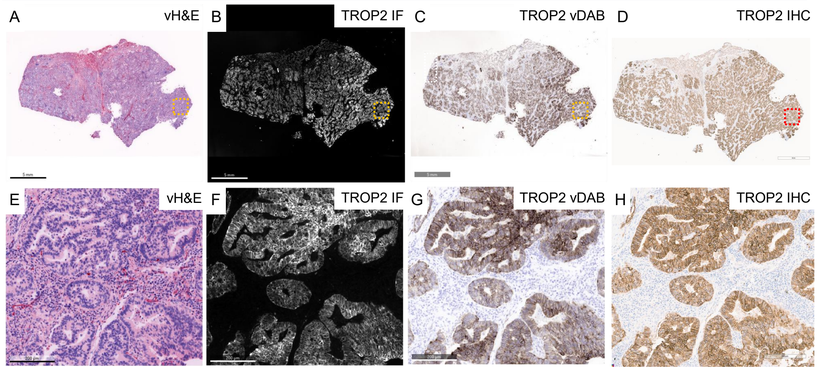


Figure 2. TROP2 IF-IHC Concordance. Sequential slides of NSCLC samples were stained with TROP2 using IF (A-C, E-G) or immunohistochemistry (IHC) (D and H). Representative virtual H&E (vH&E) (A, E), TROP2 IF (B, F), TROP2 virtual DAB (vDAB) (C, G), and TROP2 IHC (D, H) images are shown. Magnified views (E-H) correspond to dotted boxes. (I) TROP2 IF staining shows robust concordance with IHC, with a Pearson's correlation coefficient of 0.91.

TROP2 Co-expression with ADC Markers in Tumor Cells

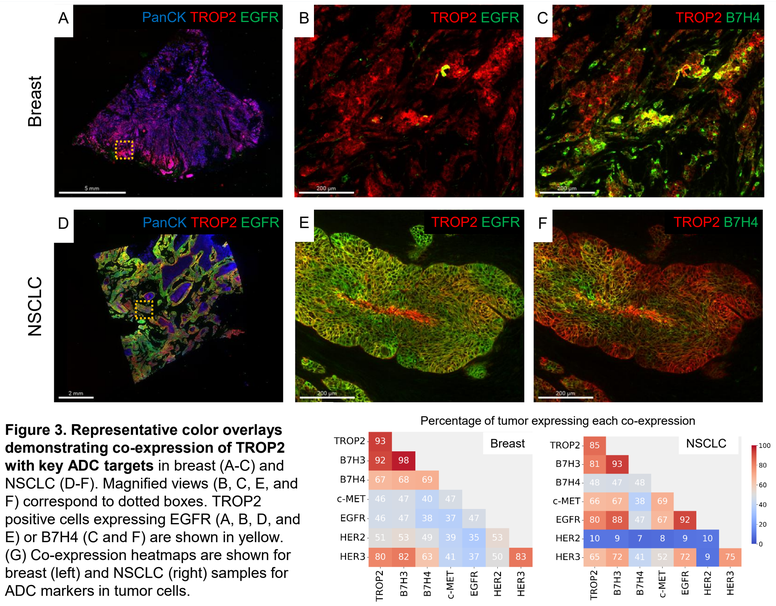
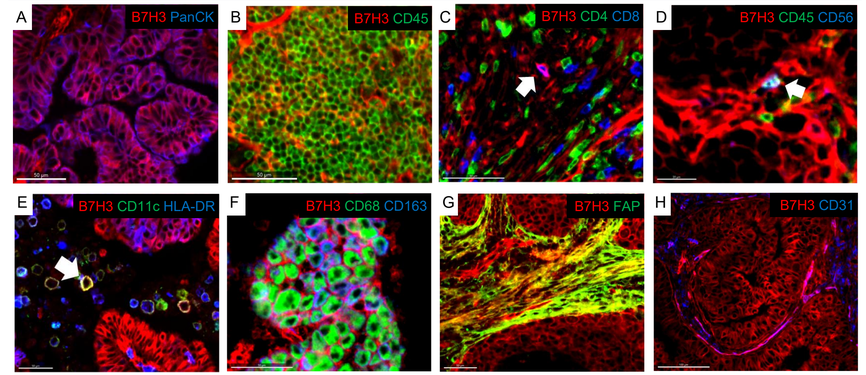


Figure 3. Representative color overlays demonstrating co-expression of TROP2 with key ADC targets in breast (A-C) and NSCLC (D-F). Magnified views (B, C, E, and F) correspond to dotted boxes. TROP2 positive cells expressing EGFR (A, B, D, and E) or B7H4 (C and F) are shown in yellow. (G) Co-expression heatmaps are shown for breast (left) and NSCLC (right) samples for ADC markers in tumor cells.

Characterization of B7H3 in Different Cell Types in the Tumor Microenvironment



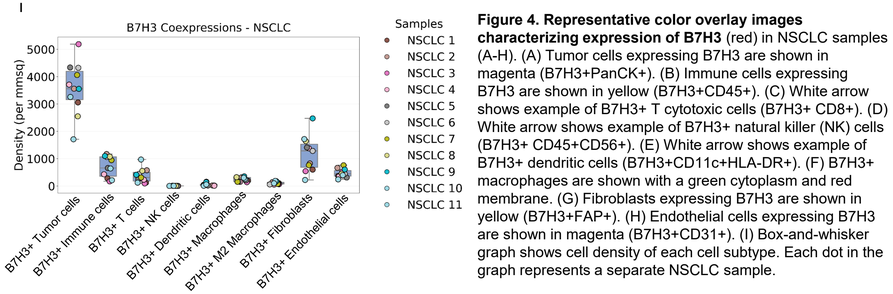


Figure 4. Representative color overlay images characterizing expression of B7H3 (red) in NSCLC samples (A-H). (A) Tumor cells expressing B7H3 are shown in magenta (B7H3+PanCK+). (B) Immune cells expressing B7H3 are shown in yellow (B7H3+CD45+). (C) White arrow shows example of B7H3+ T cytotoxic cells (B7H3+ CD8+). (D) White arrow shows example of B7H3+ natural killer (NK) cells (B7H3+ CD45+CD56+). (E) White arrow shows example of B7H3+ dendritic cells (B7H3+CD11c+HLA-DR+). (F) B7H3+ macrophages are shown with a green cytoplasm and red membrane. (G) Fibroblasts expressing B7H3 are shown in yellow (B7H3+FAP+). (H) Endothelial cells expressing B7H3 are shown in magenta (B7H3+CD31+). (I) Box-and-whisker graph shows cell density of each cell subtype. Each dot in the graph represents a separate NSCLC sample.

Spatial Analysis of TROP2 Expression

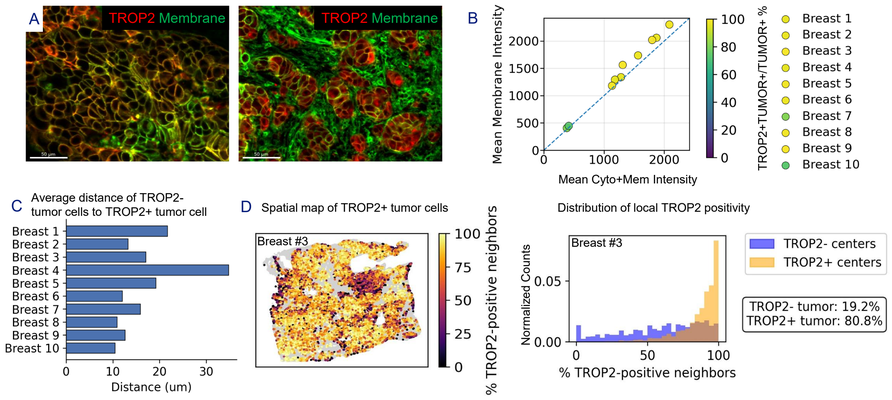


Figure 5. Spatial Analysis of TROP2. (A) Membrane expression of TROP2 is shown in yellow (TROP2 in red and membrane marker in green). Left image shows tumor cells primarily expressing TROP2 on the membrane and right image shows tumor cells expressing TROP2 in the cytoplasm. (B) Scatter plot shows TROP2 mean membrane intensity compared to cytoplasm intensity. (C) Horizontal bar graph shows average distance of TROP2- tumor cells to TROP2+ tumor cell. (D) Spatial map of TROP2 positivity demonstrates the location of the TROP2+ tumor cells (left), and overlapping bar graph shows the distribution of local TROP2 positivity in Breast #3 (right).

Summary

- The Paletrra assay was used to characterize ADC targets in tumor and associated microenvironment of breast and NSCLC samples.
- TROP2 IF staining was compared to IHC in NSCLC samples. Pearson's correlation coefficient of 0.91 indicates mIF is highly concordant with IHC validated staining.
- TROP2 was found to be highly co-expressed with key ADC markers. In breast cancer samples, TROP2 was primarily co-expressed with B7H3, HER3, or B7H4, while in NSCLC samples, it was mainly co-expressed with B7H3 or EGFR.
- While B7H3 was mainly expressed in tumor cells, it was also expressed in many cells in the associated microenvironment. The majority of B7H3 expression in the microenvironment was co-expressed with FAP.
- Spatial analytics were used to characterize ADC expression in cancer samples. TROP2, in most breast samples, had higher mean membrane intensity than mean cytoplasm intensity. Additionally, the average TROP2- tumor cell was observed to be within 35µm of a TROP2+ tumor cell, indicating these samples might be susceptible to the bystander effect.
- This assay provides a robust framework for advancing ADC translational research and clinical development through integrated characterization of target expression, spatial localization, and tumor microenvironment.

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