2094 / 30 - Development of a multiomic workflow using Oncore Pro X to stain tissues for high performance Dual Channel™ Validation of RNA expression with downstream quantitation using AI-based image analysis with Oncotopix Discovery™.

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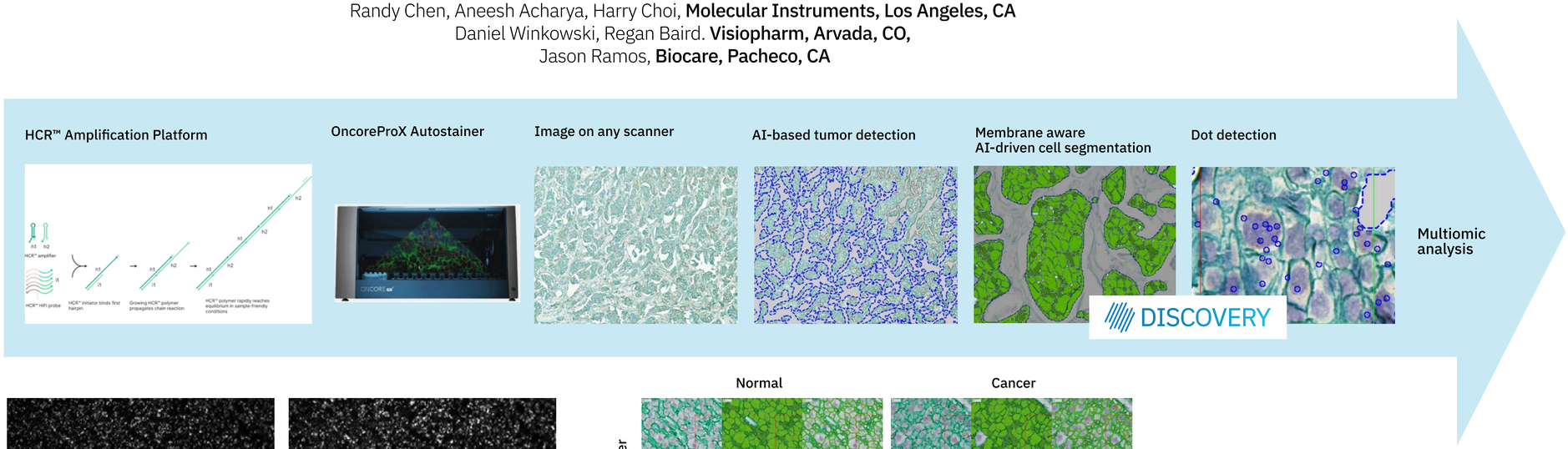
Abstract

Accurate quantification of RNA expression remains a critical challenge in advancing RNA-ISH multiomic technologies for translational and clinical applications. Current barriers include lack of guidelines for assay validation and difficulties in correlating RNA abundance with spatial localization. In this study, we introduce a robust pipeline leveraging Dual Channel™ Validation (DCV) of HCR™ HiFi Probes and AI-enabled analysis to enable relative quantification of RNA abundance.

The DCV approach uses two spectrally distinct fluorescent channels to confirm the specificity and sensitivity of RNA detection. This dual-channel strategy minimizes false positives while providing a reliable proxy for RNA transcript abundance through fluorescent intensity correlations. To enhance RNA quantitation, our pipeline integrates automated tissue staining for consistent and high-quality labeling plus advanced AI-based algorithms trained on combined cellular nuclear plus membrane segmentation markers to precisely delineate cell boundaries. This dual-marker framework addresses limitations of rule based nuclear expansion-based segmentation methods and improves the accuracy of RNA spot assignment.

Methods

- Staining Method: Membrane stains were utilized in both chromogenic and fluorescent formats of the HCR™ Pro RNA-FISH assay, enabling strong signal detection in brightfield and fluorescent imaging modes. This integrated assay design allows for concurrent visualization of gene transcripts – detected as individual puncta – alongside well-defined cell borders marked by membrane stains. The enhanced spatial resolution provided by the HCR™ assay leads to more accurate gene expression quantification through improved precision in cellular segmentation.
- All HCR™ Pro RNA-ISH + IHC/IF assays were performed on Biocare Medical's Oncore Pro X autostaining instrument. The HCR™ Pro RNA-CISH + IHC stains were imaged using the Olympus VS200 scanner, and the HCR™ Pro RNA-FISH + IF stains were imaged using the Thunder Live Cell Fluorescent Compound Microscope from Leica Microsystems.
- Analysis: Visiopharm's Discovery, a powerful AI-driven platform, was used to develop an automated mutliomic workflow which accurately detects nuclear stains and membrane markers, further enhancing the identification of cells by delineating their membrane-stained boundaries. This membrane-aware segmentation led to improved RNA dot assignment to specific cells. Expert user-created annotations were utilized to train an AI-driven algorithm to accurately recognize cell boundaries based on ground-truth chemical stains. This method greatly enhances cell boundary detection and RNA dot assignment, leading to more precise spot counts and a deeper insight into RNA expression within the complex tumor microenvironment.



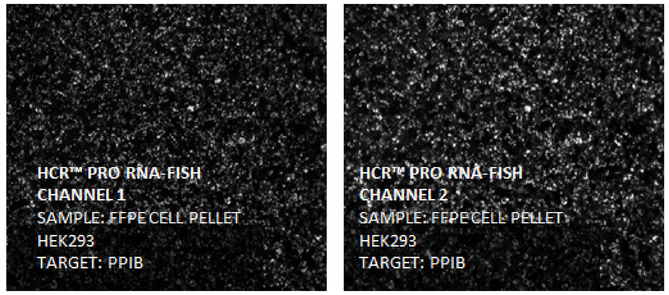


Figure 1. Dual Channel™ Validation of the PPIB HCR™ HiFi Probe in FFPE Human Cell Pellets. (A)HCR™ Pro RNA-FISH targeting PPIB in the 546 channel. (B) HCR™ Pro RNA-FISH targeting PPIB in the 647 channel. The analysis reveals a colocalization factor of 96.3% between the two channels, with an intensity correlation coefficient of 0.81. These findings validate the PPIB HCR™ HiFi Probe's ability to specifically bind its target RNA with high sensitivity and specificity, effectively detecting genuine RNA transcripts for precise and reliable dot counting in quantitative analyses. While this does not validate that individual dots are individual transcripts, it does validate that dots can be used as proxies for target abundance.

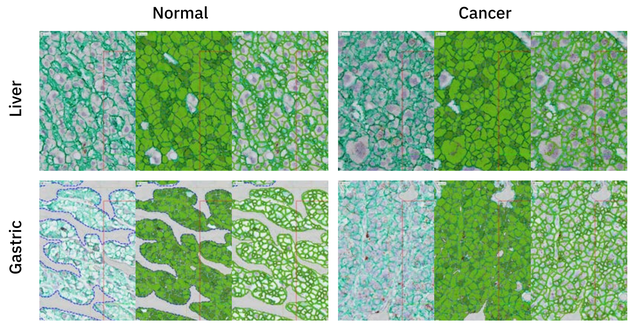


Figure 2. Dual Channel Multiomic staining and analysis by Visiopharm's Discovery of normal (A,C) and diseased (B, D) liver (A, B) and stomach (C, D). Images shown with Molecular Instruments HCR™ Membrane Green, HCR™ DAB, and Hematoxylin stained using the OncoreProX. Adjacent is the cell segmentation mask (nucleated cells: green, non-nucleated cells :Dark Green, Ki67 ISH, blue) followed by cellular outlines.

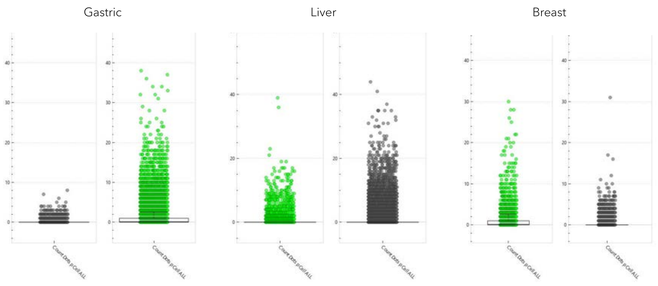


Figure 3. Dot counting distribution per cell of Ki67 ISH determined by AI-driven image analysis in Stomach (normal (black) and cancer (Green) samples), Liver (Normal (Green) and Cancer (Black) samples), and Breast (HER2+ (Green) and triple negative (Black) samples).

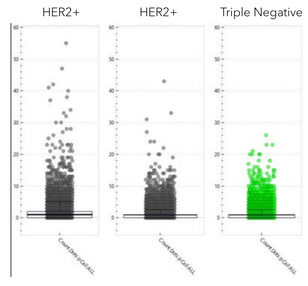


Figure 4. Dot counting distribution per cell of ppib ISH determined by AI-driven image analysis in HER2+ (black) and triple negative (green) samples.

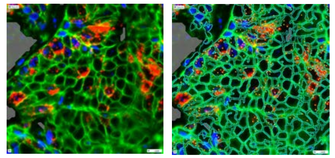


Figure 5. Membrane aware-AI multiomic workflow is also amenable to dual channel IF studies. A HER2+ Breast sample Ki67 ISH (red), Membrane (green), Dapi (blue). B. Nucleated Cells outlined with blue, Dapi negative cells outlined in dark blue, and Ki67+ ISH spots demarcated with Yellow.Not shown are HER2+ Breast samples stained with Ki67 and Triple Negative Breast Samples stained with either Ki67 ISH and PPIB ISH.

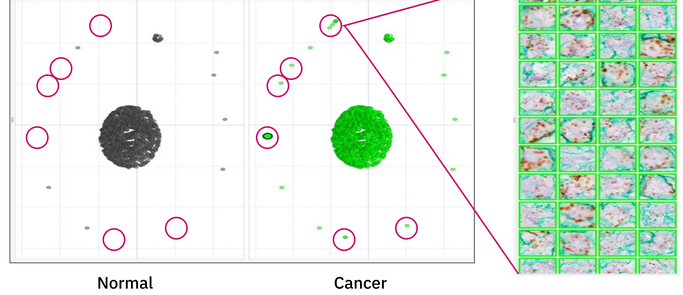


Figure 6. Interactive Informatics for Quality Control and Data Exploration. tSNE cluster analysis of dot per cell distribution in normal stomach (Black) and gastric cancer (green) samples. Red circles mark Ki67 ISH cluster present in gastric cancer but absent in normal stomach samples. Discovery's interactive plots will create a gallery of cellular thumbnails from selected points in the plot for verification.

Conclusions

Our results demonstrate that DCV, combined with autostaining and membrane aware AI-enabled cellular segmentation, achieves superior quantification fidelity. This approach not only validates RNA-ISH assay performance but also provides a scalable, reproducible method for relative RNA quantitation across diverse tissue types. By coupling high-performance probes with optimized automated tissue staining (on Oncore Pro X) and advanced computational analysis, our pipeline bridges critical gaps in RNA quantification, facilitating reliable, clinically meaningful insights into RNA expression within complex tissue environments.

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