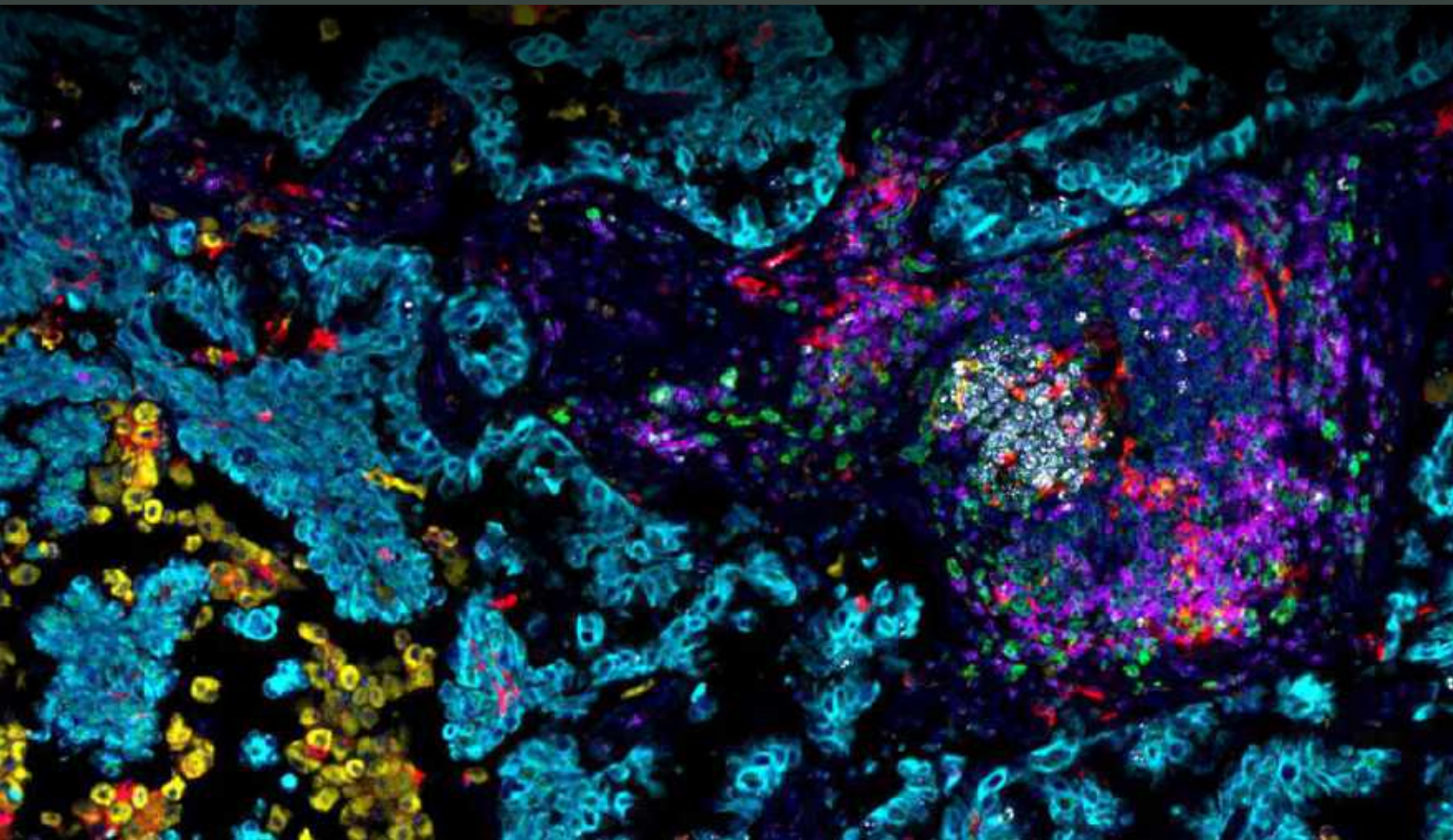
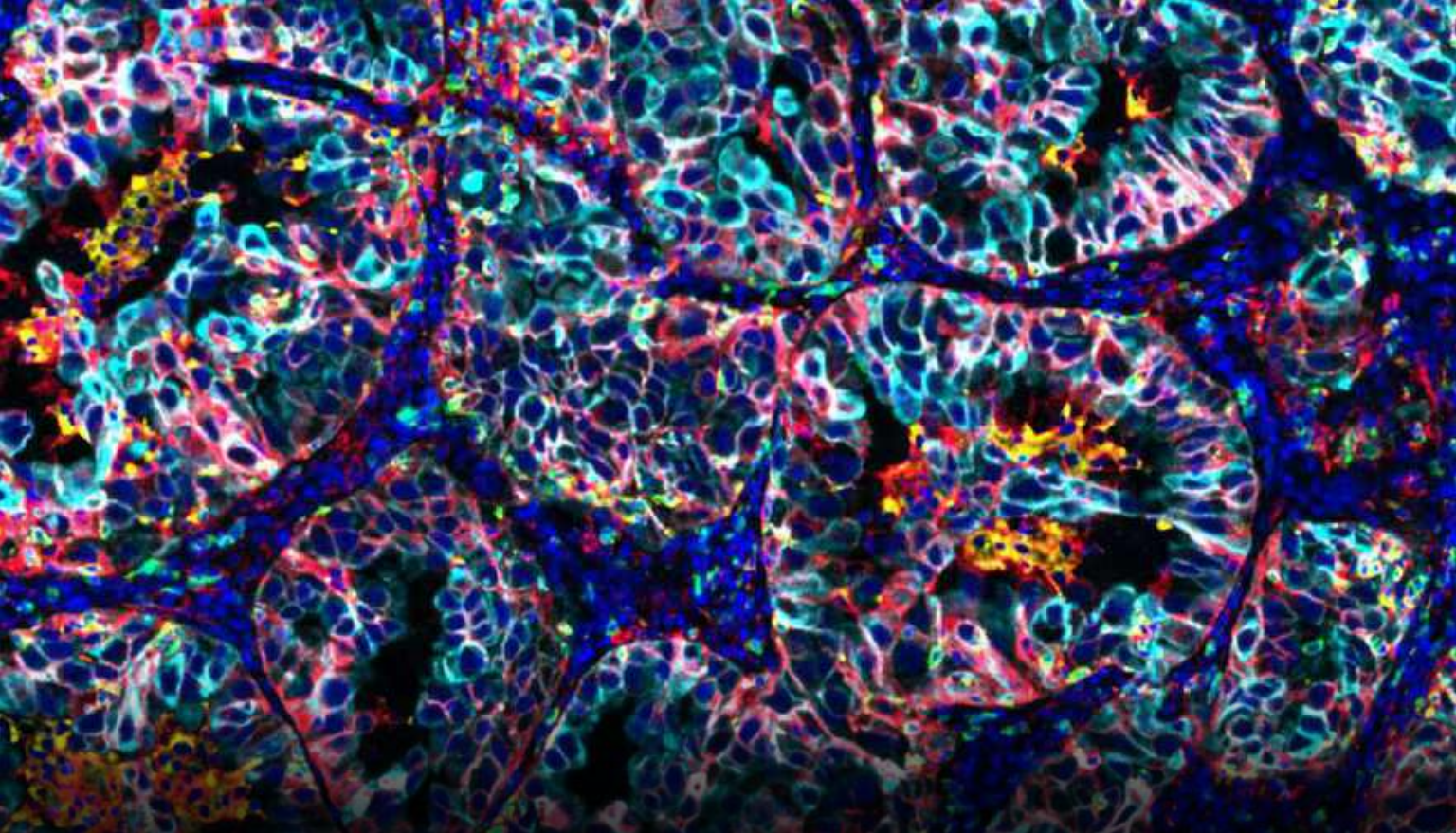


## InSituPlex® Technology Overview

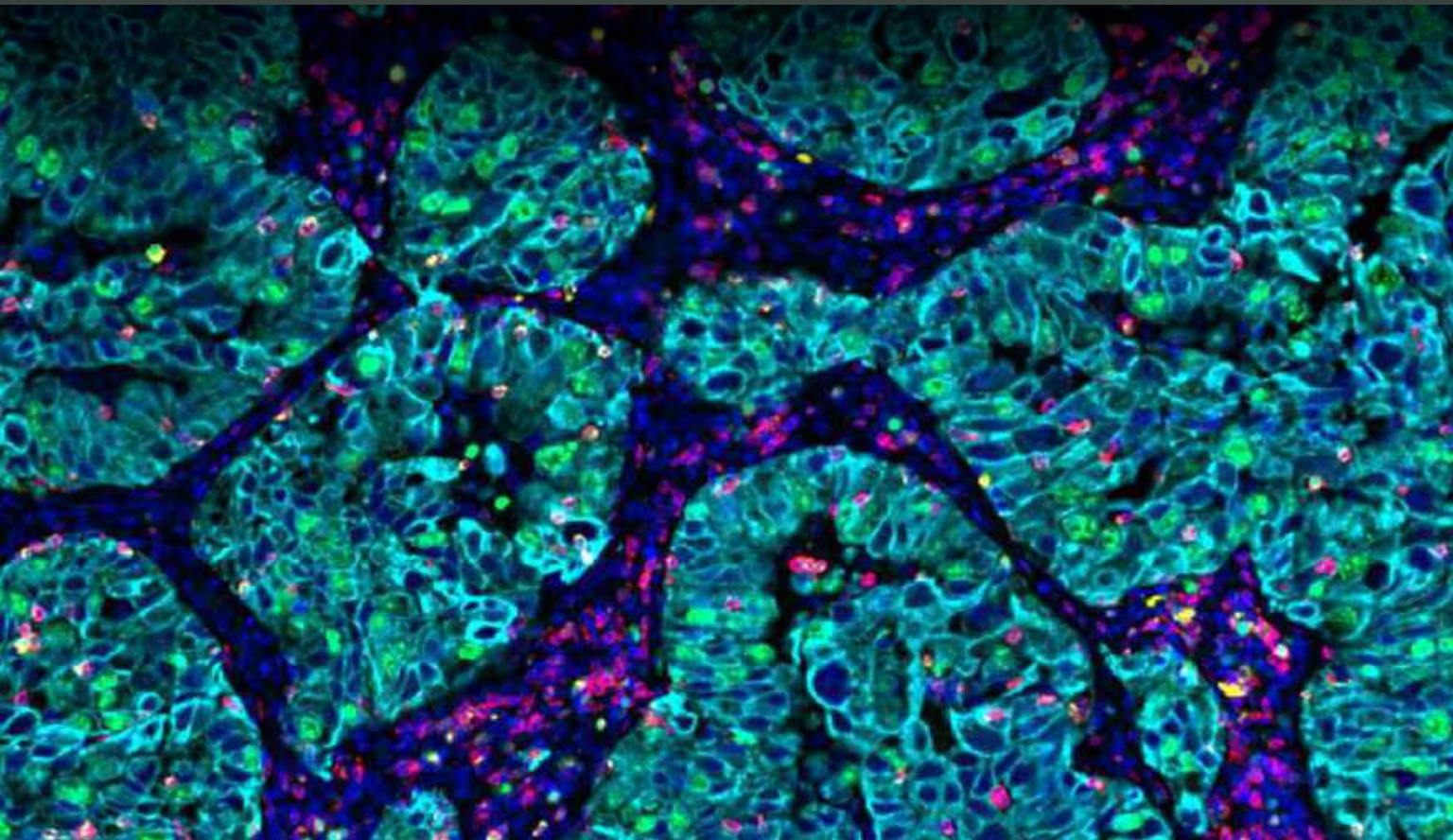






Above: UltiMapper I/O PD-L1 kit - CD8 (green), CD68 (yellow), PD-L1 (red), CK (cyan) in NSCLC

Below: UltiMapper I/O T-act kit - CD3 (red), Granzyme B (green), Ki67 (orange), CK (cyan) in NSCLC





# High Performance Tissue Multiplexing

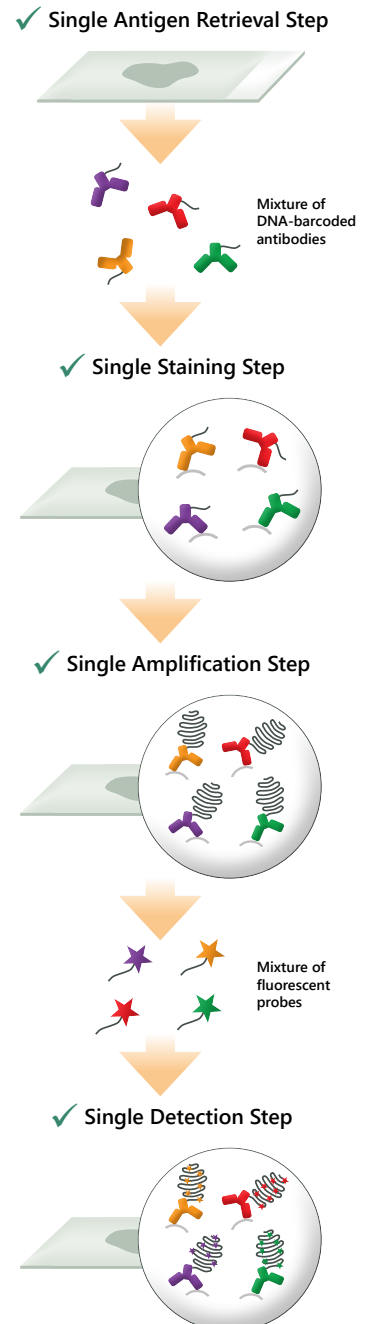
Immuno-oncology research has driven the development of more robust tools to interrogate the tumor microenvironment, specifically using multiplex immunofluorescence (IF). Multiplex IF allows investigators to visualize multiple biomarkers in tissue while preserving spatial context.

UltiMapper™ I/O assays utilize InSituPlex DNA-barcoding and antibody staining technology to enable whole-slide, high level multiplexing for cell phenotyping and spatial profiling of tissue biomarkers:

- 30 slides in 5.5 hours from staining to image acquisition and assessment
- Plug-in, reagent-based assays optimized for autostainers, slide scanners, and analysis software
- Whole-slide imaging offering true spatial context and eliminating bias of ROI selection
- More phenotypes per sample through single-cell co-expression and co-localization of markers
- Complete tissue and epitope integrity allowing for downstream -omics applications

## Assay Overview

With InSituPlex technology, UltiMapper assays support high-throughput multiplex tissue marker detection and analysis. The protocol enables single antigen retrieval, single staining, single signal amplification, and single detection steps for multiplexed, whole-slide images.



# High-throughput, optimized onto existing histology workflows

As a pre-optimized, reagent-based solution, UltiMapper kits seamlessly integrate on to existing equipment and software across the IHC workflow stream.

UltiMappers kits are ready-to-use on automated stainers including the Leica Biosystems BOND RX and BOND RX<sup>M</sup> systems. In addition, kits can be stained manually.

The fluorescent dyes in UltiMapper kits are selected for optimal performance in whole-slide imaging and eliminate the need for spectral unmixing and completely remove the risk of marker bleed-through or cross-talk (when using Ultivue's recommended filter sets).

Currently, all UltiMapper kits can be used with a range of whole-slide scanners with Cy7 (near-IR) compatibility.

Existing image analysis software can execute unbiased analysis of cellular expression and spatial relationships on whole-slide data.

## Same-day Sample-to-Images

UltiMapper kits are developed to be run manually or automated on the BOND RX autostaining systems. The automated protocol for the BOND RX stains up to 30 slides within a 5.5 hour period.

### Automated Staining



Staining 5.5 hrs manually or automated (Leica Biosystems BOND RX systems)

### Whole Slide Imaging



RareCyte  
CyteFinder HT



Olympus VS-120



Leica Aperio Versa



ZEISS Axio Scan.Z1



Akoya Vectra  
Polaris v1



Hamamatsu  
NanoZoomer S60



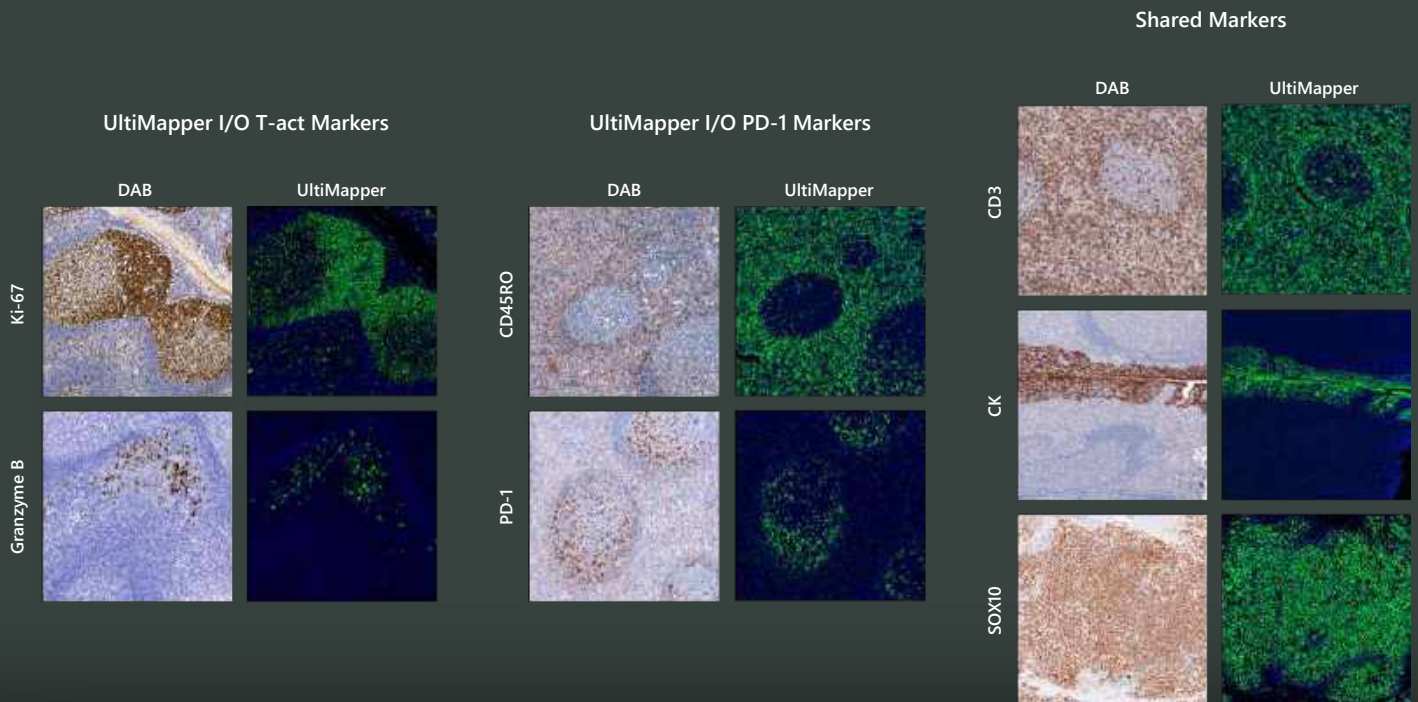
3DHistech  
Pannoramic 250

Imaging on Cy7-enabled slide scanners

### Image analysis

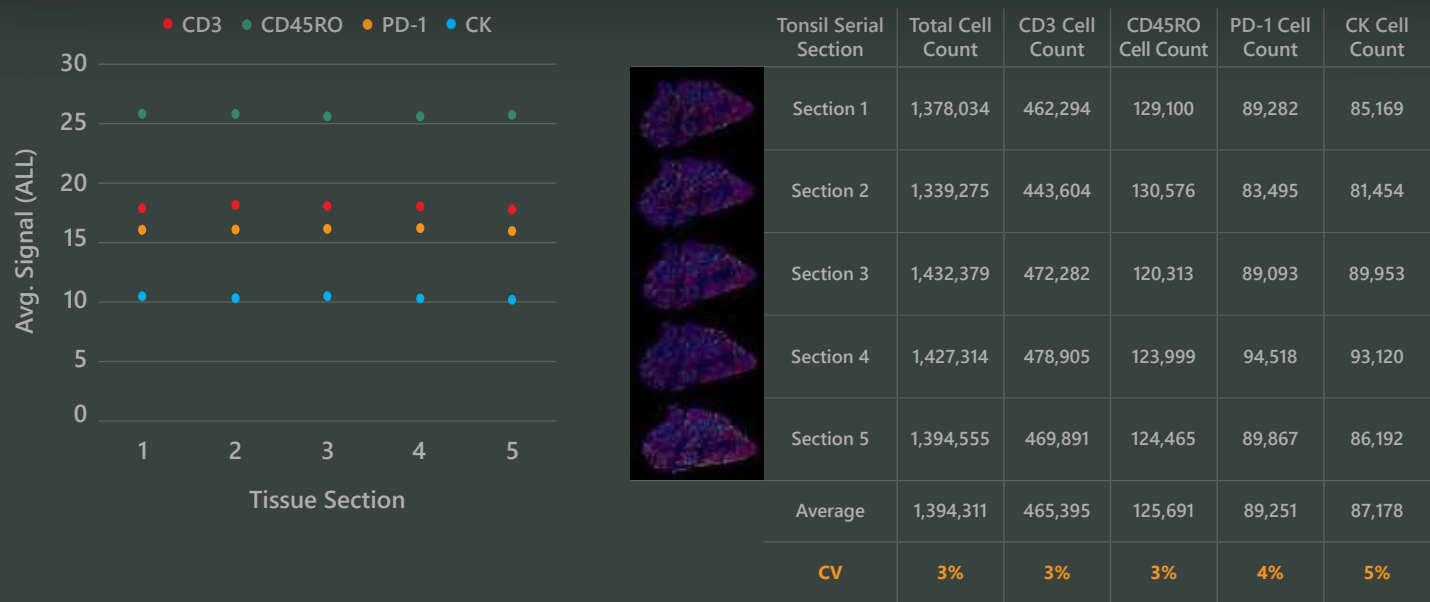


Analysis with user-dependent software  
(Indica Labs, VisioPharm, Definiens, ImageJ)



Above: DAB IHC screening of antibodies comprised in the UltiMapper I/O PD-1 Kit and UltiMapper I/O T-act Kit in tonsil and melanoma (Sox10). The same antibodies are then barcoded and stained in ISP to compare and confirm accurate staining patterns.

Below: Intra-assay testing with the PD-1 kit on tonsil sections resulted in whole-slide cell count CVs between 2.7% and 5.2% across markers.



# Staining Verification

The development of UltiMapper I/O assays involves rigorous screening of primary antibodies to ensure appropriate and accurate staining performance.

New antibodies are introduced into the InSituPlex platform through a thorough screening process involving an initial chromogenic IHC stain (DAB substrate) to confirm appropriate staining patterns and specificity.

Subsequently, antibodies are conjugated to DNA-barcodes and tested once again in chromogenic IHC to confirm that antibody performance has not changed post-conjugation.

Once this is confirmed, new antibody-DNA conjugates are run using the InSituPlex method to acquire immunofluorescence data which is directly compared to previous chromogenic screening.

Finally, antibody-DNA conjugates are tested in a multiplex format with other markers to confirm that monoplex and multiplex staining is comparable.

## Assay Reproducibility

UltiMapper assays are rigorously tested through multiple verification experiments to ensure assay reproducibility.

An example of this criteria is intra-assay reproducibility staining which whole-slide images were analyzed using HALO™ software to determine signal intensity and the number of positive cell counts for each marker in an specific UltiMapper kit.

In addition, each marker's average signal intensity was captured across serial sections. Signal intensities were consistent across the 5 serial sections for each marker during whole-slide analysis.

# Multiple Biomarker Co-localization and Co-Expression

InSituPlex technology offers the ability to tag and detect multiple markers on single cells even in the same cellular compartment.

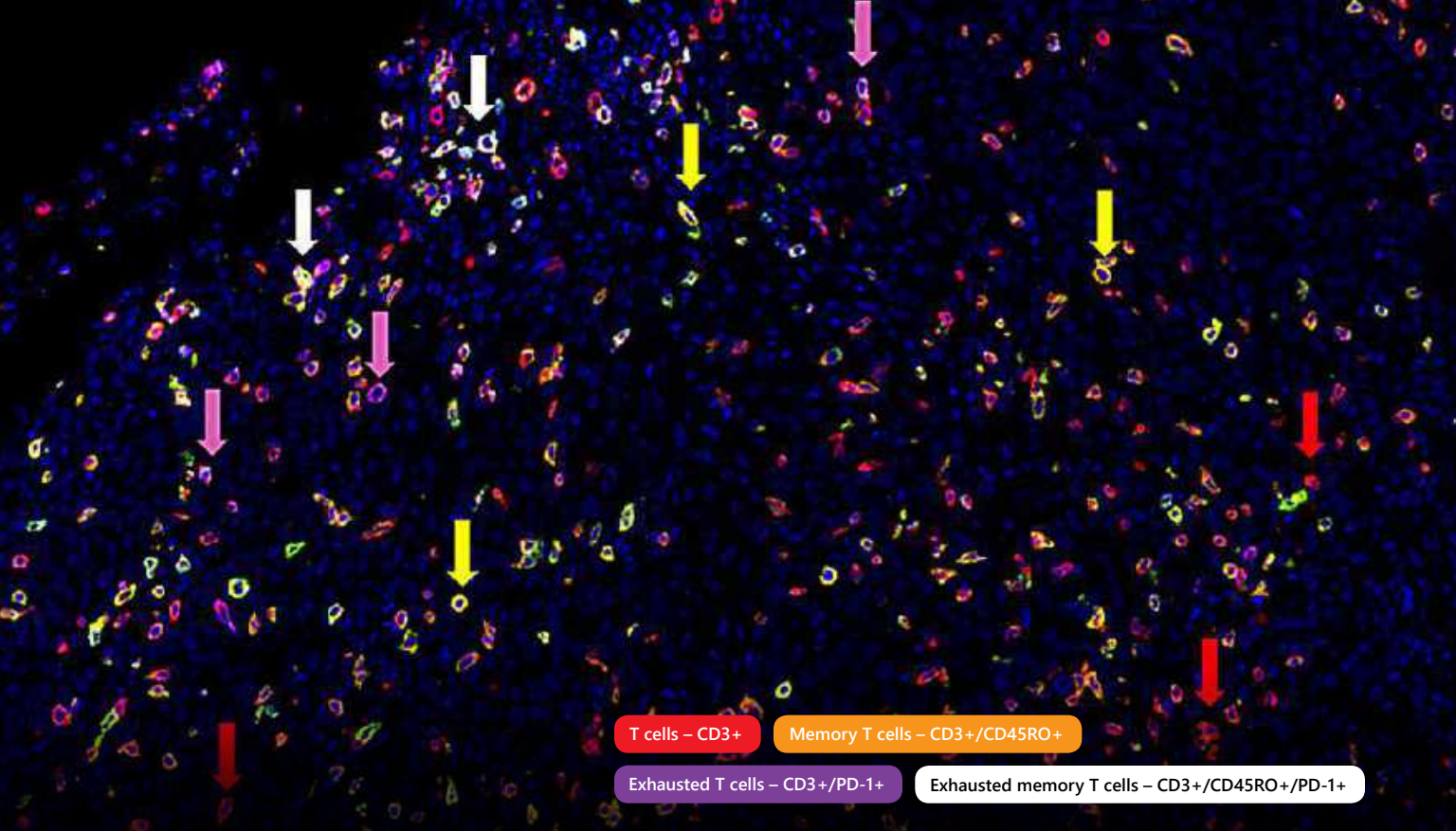
Markers can also be easily identified across multiple cellular compartments (e.g. plasma membrane, cytoplasm, and nucleus).

This allows for in-depth and accurate immunophenotyping in tissue through positive identification of markers.

## Dynamic Range of Expression

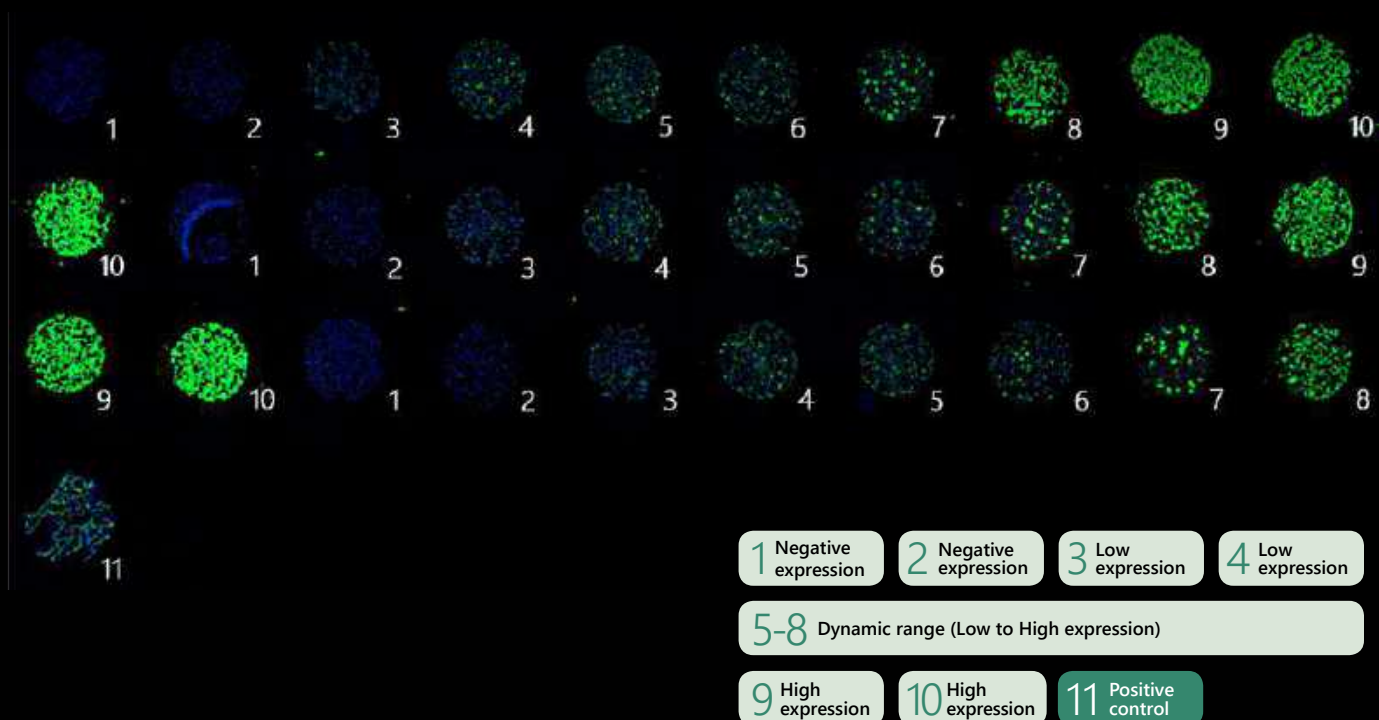
Conventional signal amplification techniques used in multiplex IHC employ a geometric amplification that can artificially increase expression of markers to equivalent levels of signal. InSituPlex technology employs a linear barcode amplification that increases the amount of hybridization sites for imaging while controlling for the differing levels of expression from marker-to-marker and cell-to-cell.



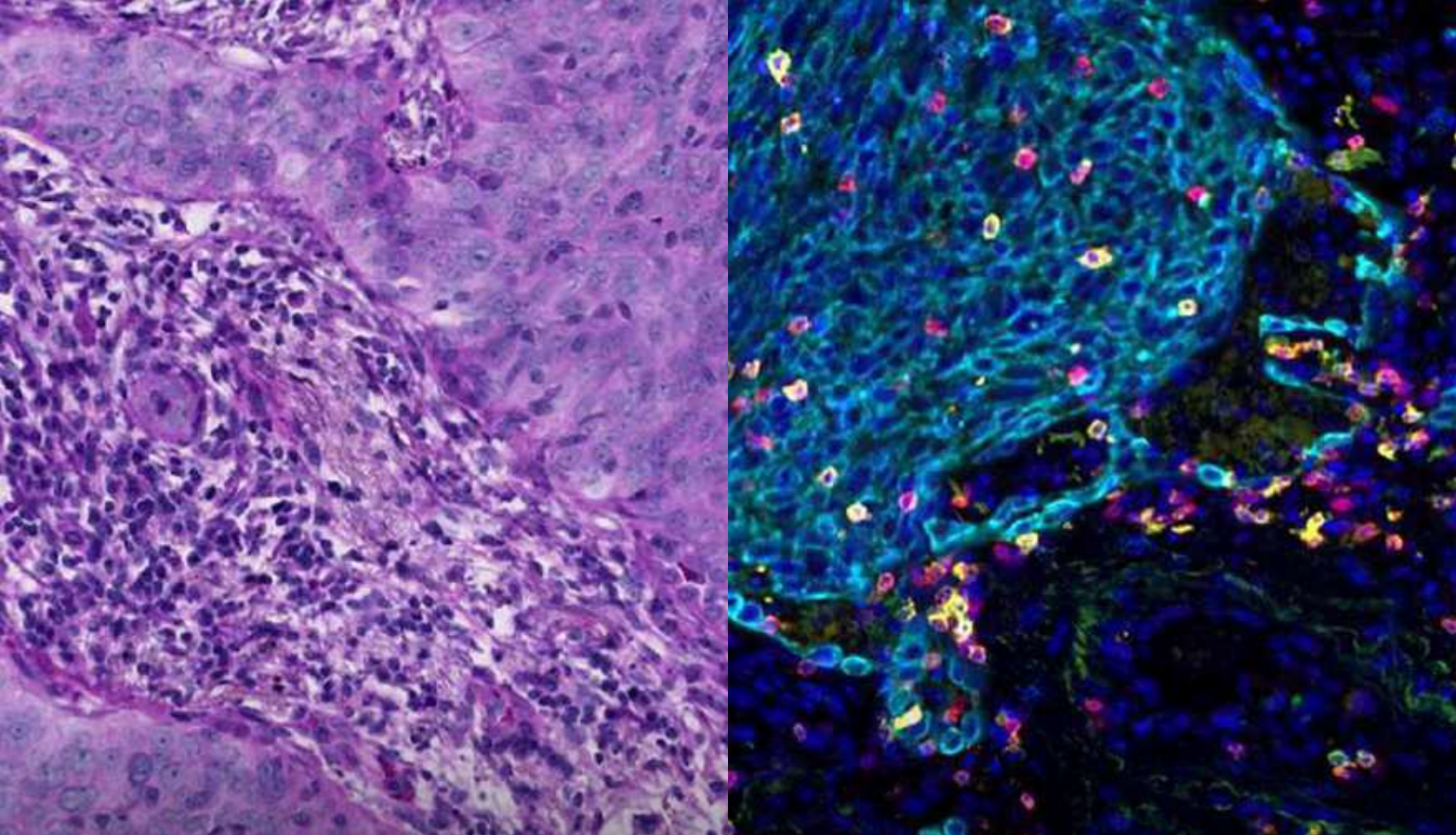


Above: Melanoma tissue stained with the UltiMapper I/O PD-1 kit.

Below: Cell line legend indicating varying levels of PD-L1 expression within the TMA.

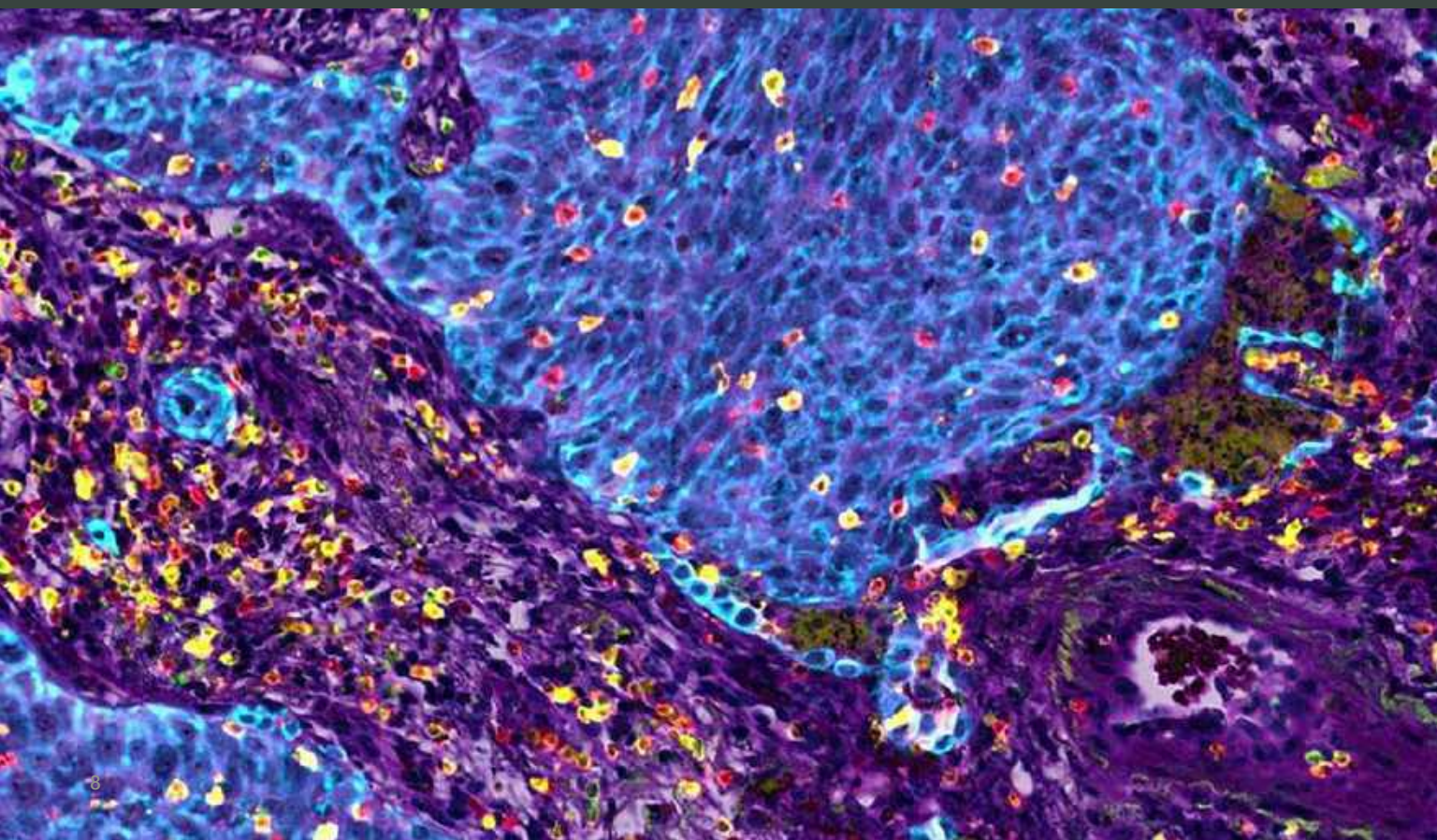






Above: UltiMapper I/O PD-1 Kit staining NSCLC followed by H&E staining. Transition from left to right showing brightfield staining to fluorescence staining on the same slide.

Below: Full merge of brightfield H&E image and multiplex IF image on the same slide.





# Tissue Multiplexing and Morphology on the Same Slide

The InSituPlex technology uses a gentle staining method that does not obstruct the integrity of the tissue sample.

Hematoxylin & Eosin (H&E) staining can be run post-UltiMapper staining to collect brightfield, tissue morphological data and fluorescent, multiplex data on the same tissue slide.

Using image analysis software, both images can then be co-registered and fused to produce a merged pseudo-brightfield/pseudo-fluorescence image for a comprehensive view into the tissue environment.



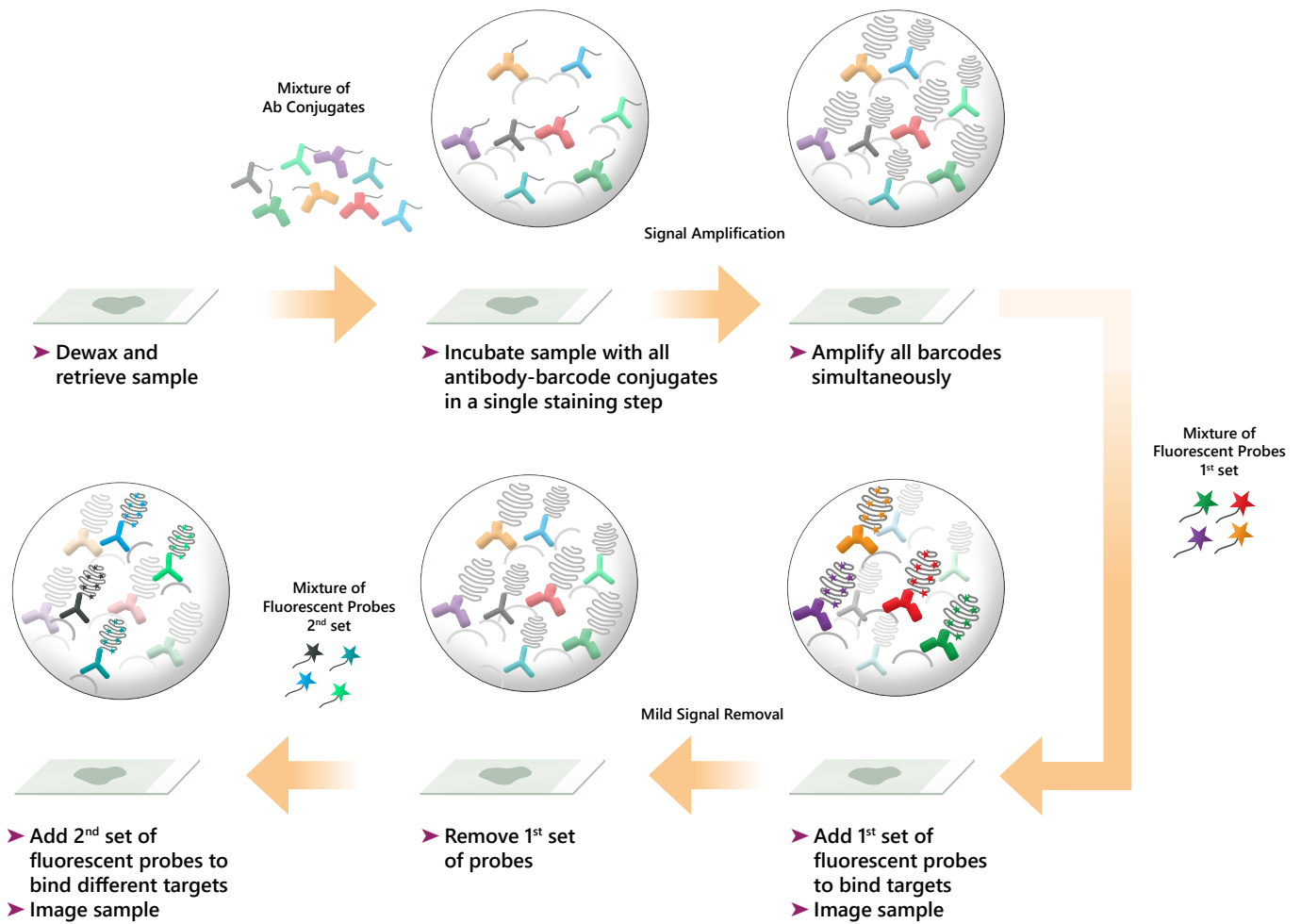
# Higher levels of multiplexing

InSituPlex technology can stain beyond 4 markers allowing for higher levels of multiplexing and in-depth characterization of individual cells.

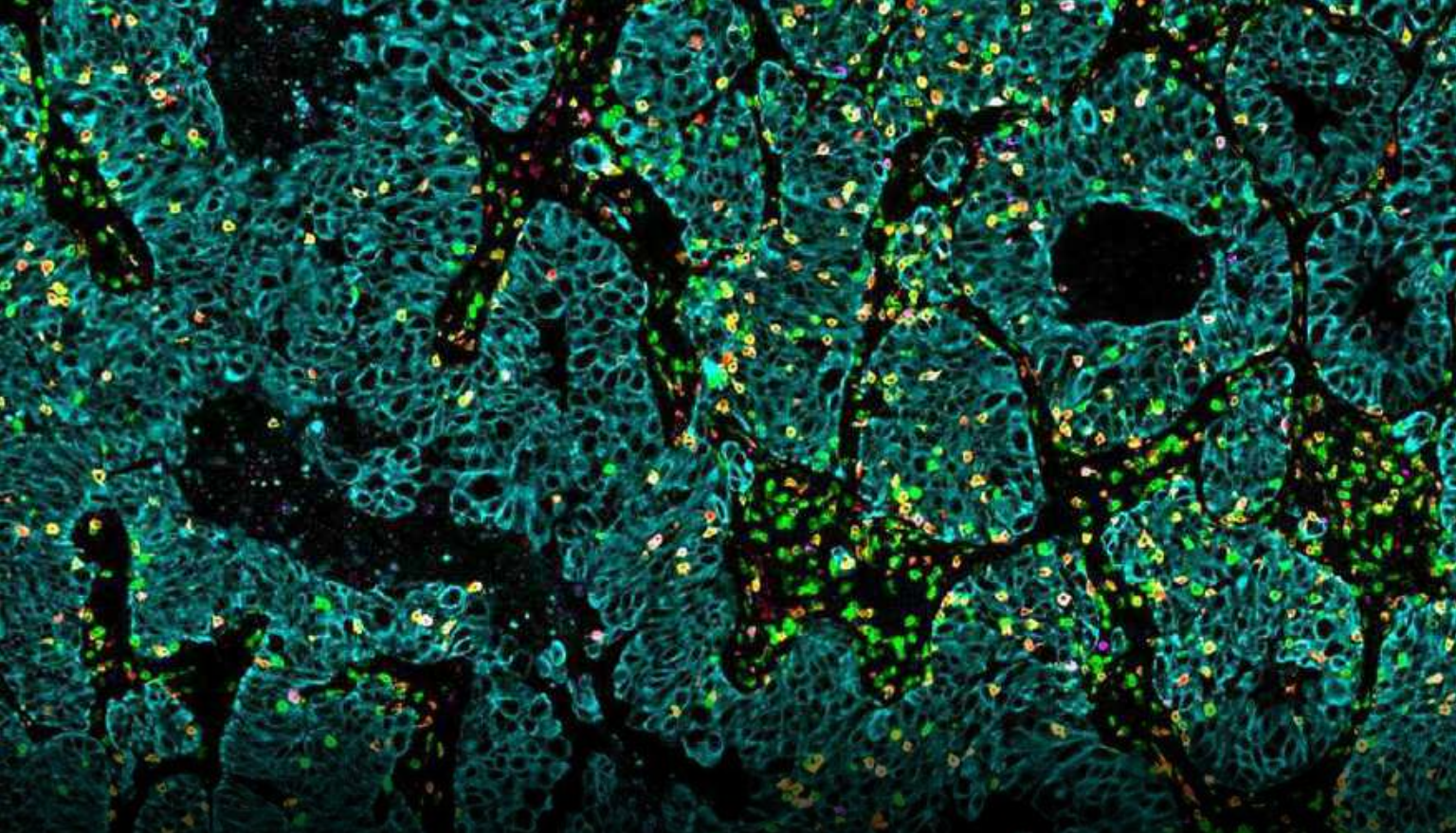
Fluorescent probes can be efficiently dehybridized from their complementary DNA-barcodes through a process called DNA-Exchange.

After a first round of probe removal, a different set of fluorescent probes can then be introduced onto the sample for another round of imaging leading to an increased detection of markers on the same slide.

Using image analysis software, images can be co-registered for full analysis of 8 markers in a multiplex setting.

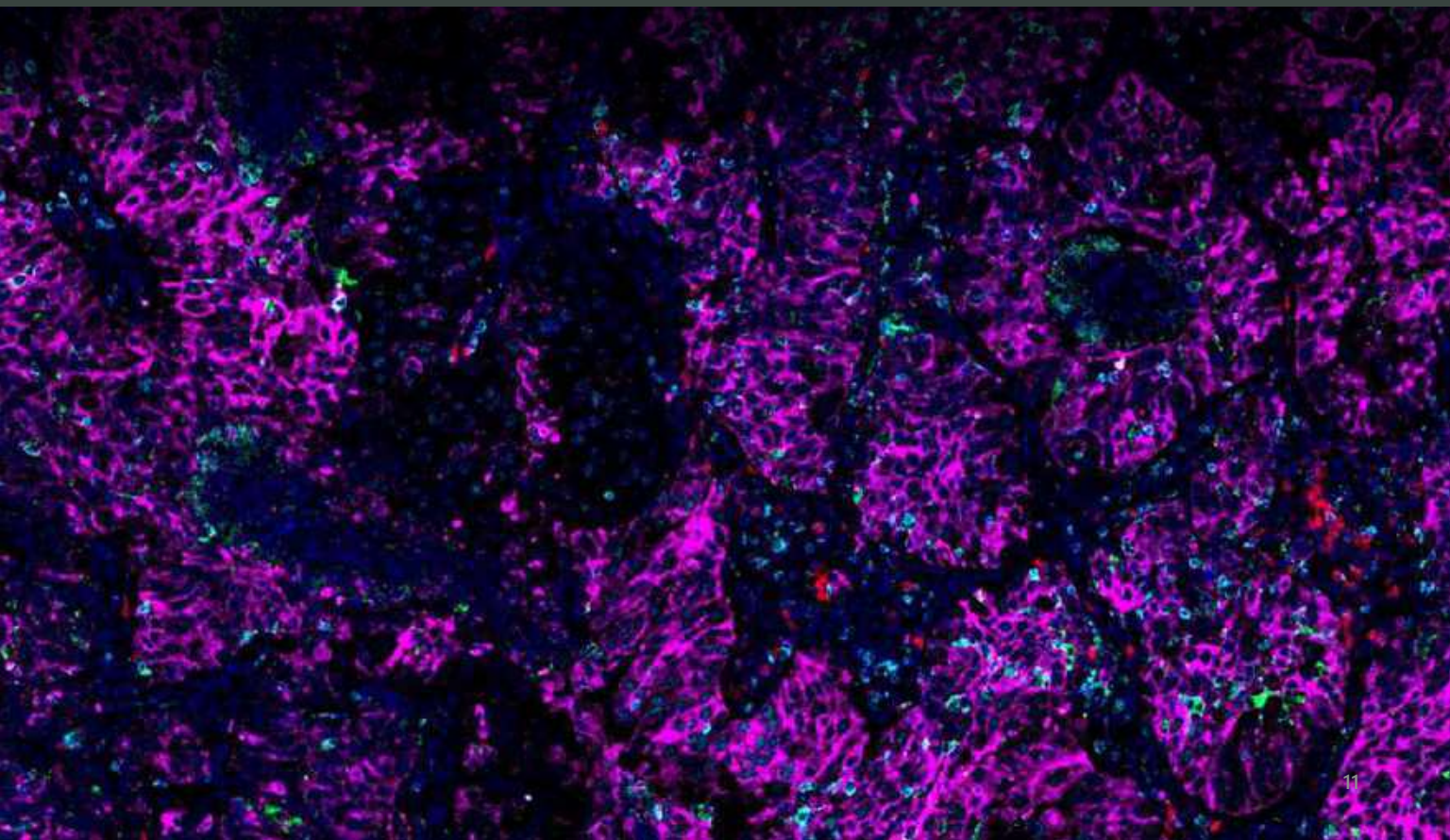




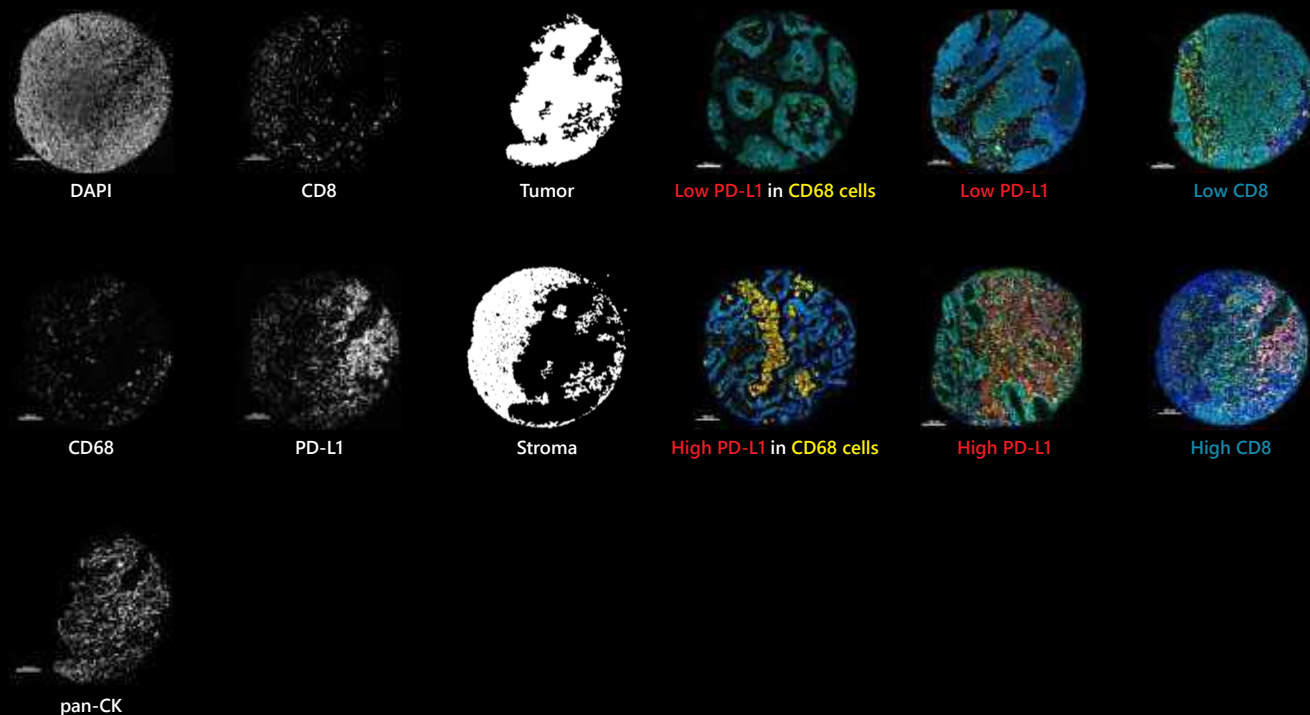


Above: 8-plex assay staining NSCLC tissue. Markers shown are CD3 (green), CD8 (red), Granzyme B (purple), and pan-CK (cyan).

Below: Same-slide visualization of above 8plex assay. Markers shown are CD4 (cyan), CD20 (red), CD163 (green), MHC II (purple), and nuclear counterstain (blue).



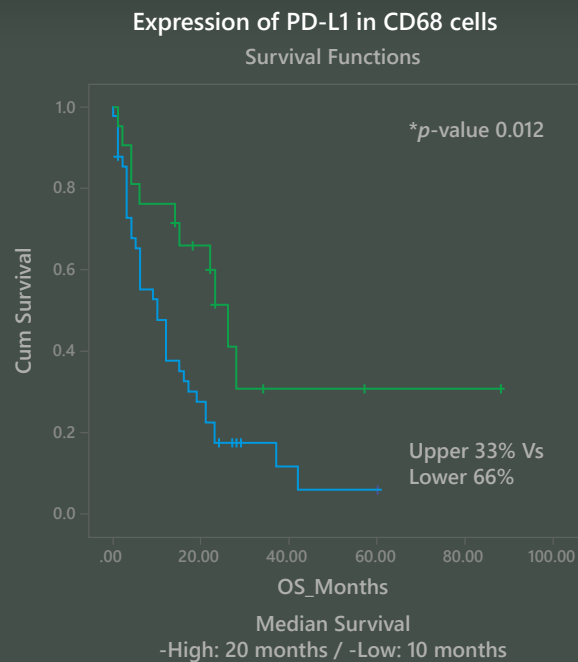
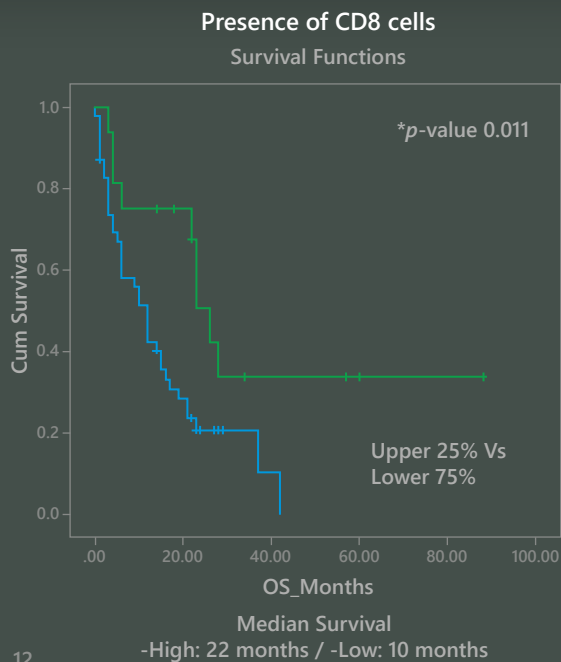




Above: UltiMapper I/O PD-L1 kit staining NSCLC FFPE TMA samples. CD8 (green), CD68 (yellow), PD-L1 (red), pan-Cytokeratin (cyan), and nuclear counterstain (blue).

## Clinical Cancer Research

Immune cell PD-L1 co-localizes with macrophages and is associated with outcome in PD-1 pathway blockade therapy  
Yuting Liu, Jon Zugazagoitia, Fahad Shabbir Ahmed, Brian S Henick, Scott Gettinger, Roy S. Herbst, Kurt A. Schalper and David L. Rimm





# Case Study:

## Measuring PD-L1 expression in immune cell and tumor cell compartments in a PD-1 pathway blockade treated non-small cell lung cancer sample cohort

The Rimm lab at Yale School of Medicine wanted to investigate PD-L1 expression in a multiplex setting to evaluate its localization across multiple cell types in the tumor microenvironment.

The team originally developed a panel using tyramide signal amplification (TSA) to assess CD8, CD68, PD-L1, and pan-Cytokeratin in a non-small cell lung cancer treated cohort.

In addition, the Rimm lab wanted to benchmark the performance and utility of the UltiMapper I/O PD-L1 kit to assess the same markers.

The group identified that PD-L1 on macrophages and CD8+ cells were better indicators of overall survival versus PD-L1 on tumors alone. Results were confirmed using both TSA and InSituPlex technology.

Findings were presented at the Society for Immunotherapy of Cancer Annual Meeting in 2018 and the study was published in Clinical Cancer Research.



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