# Highly multiplexed single-cell spatial analysis of FFPE tumor tissues using CODEX®

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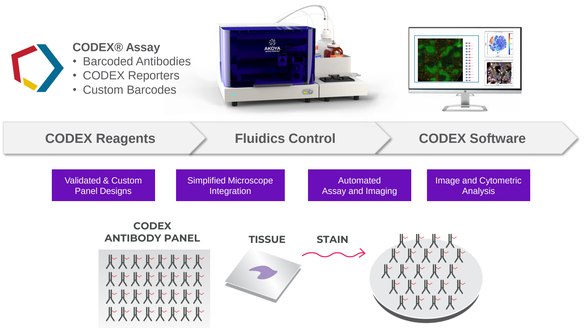
## Introduction

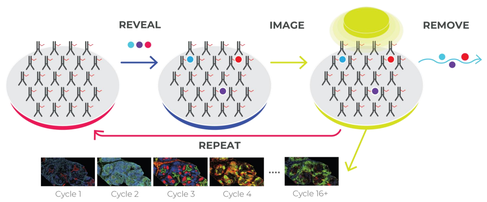
Characterizing the complexities of the tumor microenvironment is elemental to understanding disease mechanisms. The spatial relationships between infiltrating immune cells and the remodeling of the cellular matrix is widely recognized as a key component to defining tumor heterogeneity. Current methodologies for analyzing the spatial dimension in tissues, like traditional immunofluorescence (IF) and immunohistochemistry (IHC), are limited to a few parameters at a time, restricting the scope of identifiable cells. Conversely, single-cell technologies like mass cytometry and NGS-based tools provide multiplexing capabilities, but at the expense of the associated spatial information. Furthermore, some markers within the complex microenvironment are low expressing and difficult to visualize via IHC, thus necessitating signal amplification. Here, we present the 28-plex analysis of human formalin fixed paraffin embedded (FFPE) tissues with CODEX® to elucidate the multiparametric spatial interactions within the tumor microenvironment. We expanded our workflow to incorporated TSA-mediated dyes for amplification of key low-expressing markers, including FOXP3, PD-L1, and PD1.

## CODEX®: CO-Detection by indEXing




**CODEX antibody staining:** FFPE tissues are stained offline in a single step with the full panel of CODEX® antibodies or custom-conjugates using third party antibodies. This preserves sample integrity by avoiding excessing staining/stripping steps and reducing turnaround time.


**Basic Chemistry:** During each imaging cycle, three CODEX® Reporters with fluorophores are assayed to their corresponding barcodes conjugated to antibodies.

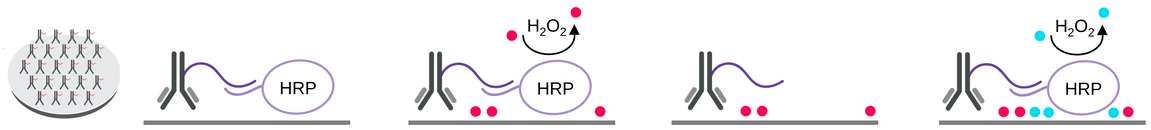



**Schematic of cyclical workflow:** Iterative cycles of labelling, imaging and removing reporters are performed via a fully automated fluidics system, until all biomarkers of interest are imaged. Images are collected and compiled across cycles to achieve single-cell resolution data.

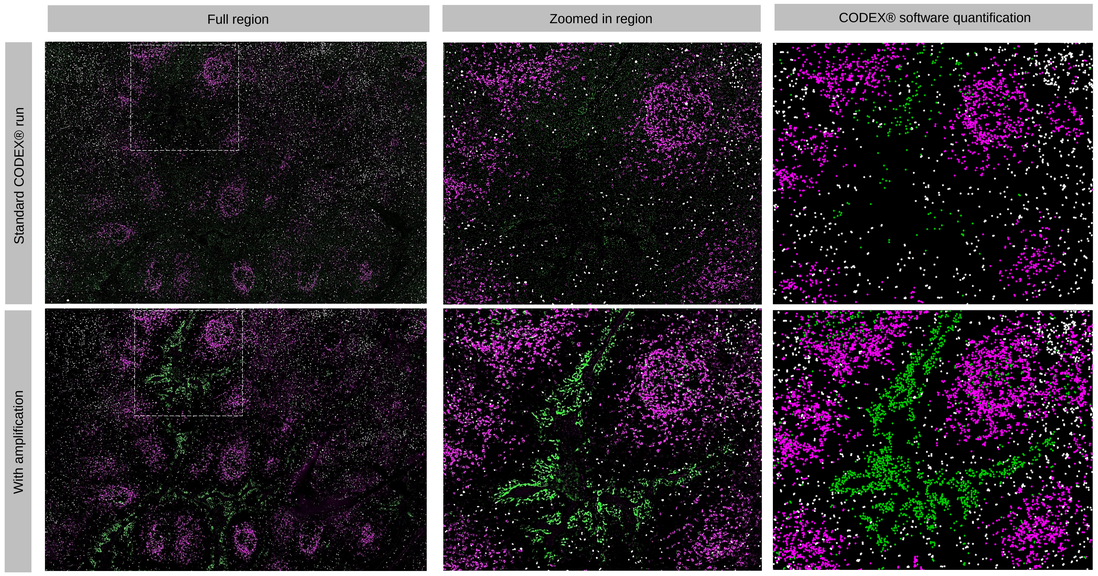
**Seamless microscope integration:** The CODEX® fluidics device integrates into microscope stages through a custom stage insert. The CODEX Driver Software is compatible with multiple microscope brands/types, including Keyence BZ-X710/800, Leica DMi8, Nikon TI2 & Zeiss Axio-Observer.

The Spatial Biology Company™

## HRP-based amplification of low expressing markers

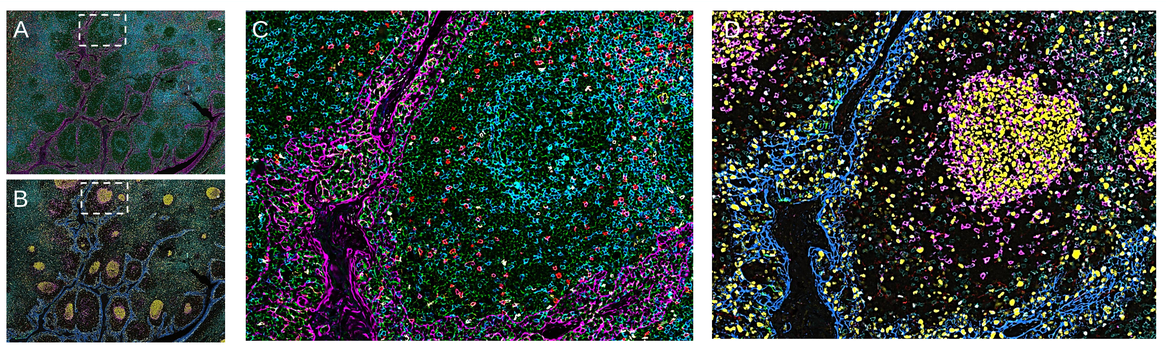



**CODEX® HRP workflow:** Tissues are stained offline in a single step according to standard CODEX® protocol. After iterative cycles of labelling, imaging, and removing via our standard CODEX® workflow, up to three cycles of HRP oligonucleotides, catalysis, deposition of a single TSA-mediated dye per cycle, and removal of HRP oligonucleotides can be performed and imaged with our standard microscope filters (Cy3, Cy5, AF750). TSA-mediated dye deposition is not removable and remains on the tissue after stripping the HRP oligonucleotide.



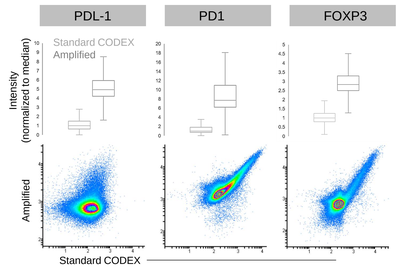

**Comparison of standard CODEX® run with amplification:** FOXP3, PDL-1, and PD-1 antibodies were first run and imaged with the standard CODEX® workflow and were then amplified using the HRP scheme described above with Opals 570, 780, and 690 respectively. Zoomed in regions indicate higher signal intensity of amplified signal compared to standard CODEX® run. Quantification of pre-and post-amplification markers using CODEX® Multiple Analysis Viewer (MAV) software reveal larger marker-positive populations with amplification.

## Multiparametric spatial profiling of FFPE tissue specimens

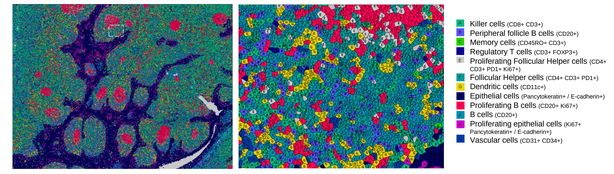



**Human FFPE Tonsil:** Tissue was stained with over fifteen CODEX® markers in a single step, revealed via a fully automated fluidics workflow through iterative cycles, and processed using our standard CODEX® processing software. The CODEX® processor aligns images across cycles, stitches tiles across large regions, subtracts autofluorescence, and segments and integrates marker intensities for each cell. Panel for A) and C) CD8 CD31 CD20 CD45RO CD4 Pancytokeratin CD34. Panel for B) and D) CD11c Ki67 PD-L1 E-cadherin CD3 PD1 FOXP3.

## Analysis using CODEX® software




**Comparison of standard CODEX run and amplified:** Signal intensity is higher with amplification compared to the standard CODEX run for PDL-1, PD1, and FOXP3 (top panel). Single cell dot blots of standard CODEX vs. amplified signal reveal larger populations with amplification than compared to standard CODEX.

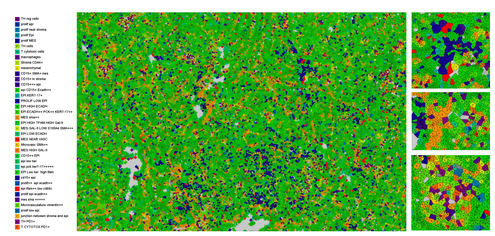



**Voronoi diagram reveals spatial interactions:** The CODEX® Multiple Analysis Viewer computes Delaunay graph of cell centers to estimate which cells are interacting with each other and uses Voronoi maps to display the abstract-level single-cell architecture of the tissue.

## Applications to cancerous tissues




We applied these techniques to FFPE non-small cell lung cancer samples. The tissue was stained with our 28-plex immuno-oncology panel with low-expressing markers (PD-L1 and FOXP3) amplified.



With the Voronoi map of this lung cancer sample, we can elucidate separate cell populations, such as CD15+ epithelial cells interacting with mesenchymal cells and microvascular cells

## Conclusions

- CODEX® enables multiplexed, spatial analysis of tissue specimens in a fully automated workflow.
- CODEX® is compatible with a variety of tissue specimens, including FF and FFPE formats.
- CODEX® data can be processed and analyzed using the CODEX analysis tools to characterize cell type, map the tissue architecture, and identify cellular niches.
- Amplification with TSA-mediated dyes will be integrated into the automated CODEX® workflow.

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