

Spatial mapping of proteins and their activity states in cancer models by multiplex *is*PLA



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Introduction.

We demonstrate the utility and performance of multiplex *in situ* proximity ligation assay (*mis*PLA) across several biological models. *mis*PLA enabled simultaneous visualization of dynamic changes in protein phosphorylation states and protein interactions within the EGFR signalling network and immune cell interactions. Targeted multiplex panels were developed to efficiently characterize signalling cascades and immune-oncological interactions, providing a comprehensive view of protein and cellular activity states.

Technology and Method

Understanding protein function in cells and tissues requires improved analytical tools. *mis*PLA employs antibody–oligonucleotide pairs that form amplifiable DNA

upon proximal binding, revealing protein interactions and modifications. We applied *mis*PLA to cells and FFPE tissues to map dynamic changes in protein localization, interactions, and post-translational modifications (PTMs). Nine antibody-oligonucleotide pairs were applied and amplified when target epitopes were nearby. Detection was performed in three rounds using a standard microscope with three fluorescence channels and images were merged into a nine-plex map. Alternatively, a nine-channel microscope allowed single-round detection of all targets.

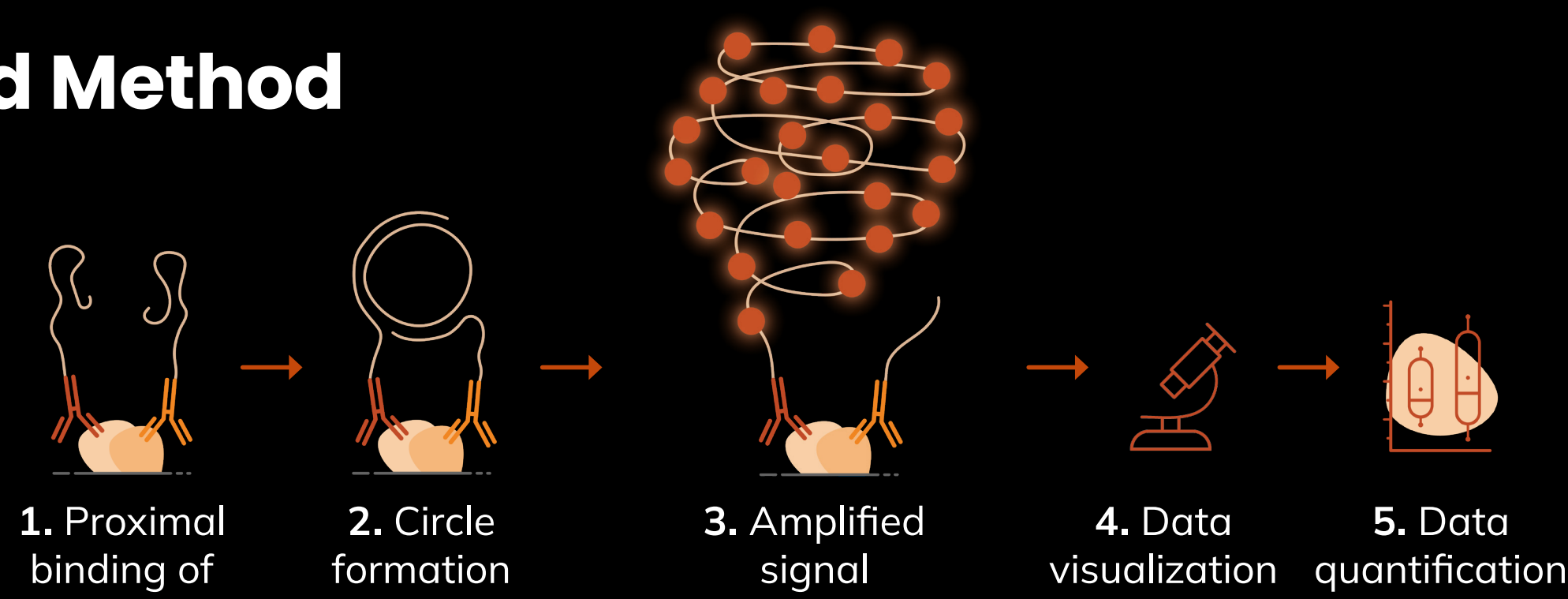


Fig. 1. Schematic of the *mis*PLA analysis workflow.

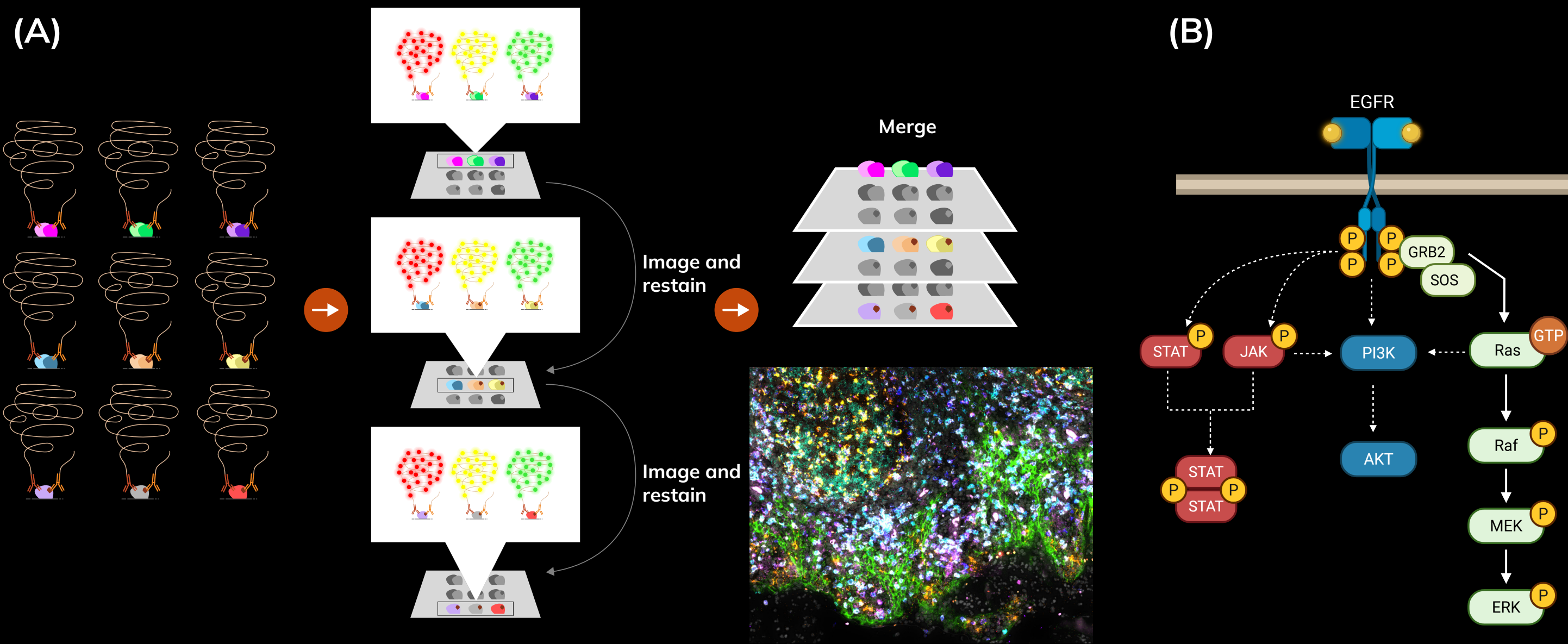


Fig. 2. (A) Detection strategy for *mis*PLA based on repeated cycling. (B) Simplified EGFR signalling network.

EGF-induced signaling dynamics captured by nine-plex *mis*PLA.

*mis*PLA offers an opportunity to map activation of elements in cellular signaling. SK-BR-3 breast cancer cells were stimulated with EGF resulting in markedly increased EGFR–GRB2 and GRB2–MEK1 interactions, indicating efficient formation of receptor–adaptor–kinase complexes. Moderate increases in JAK–STAT interactions suggest partial pathway engagement or regulation. These spatial measurements highlight *mis*PLA's ability to capture multi-protein complex formation, early adaptor recruitment, and selective pathway activation.

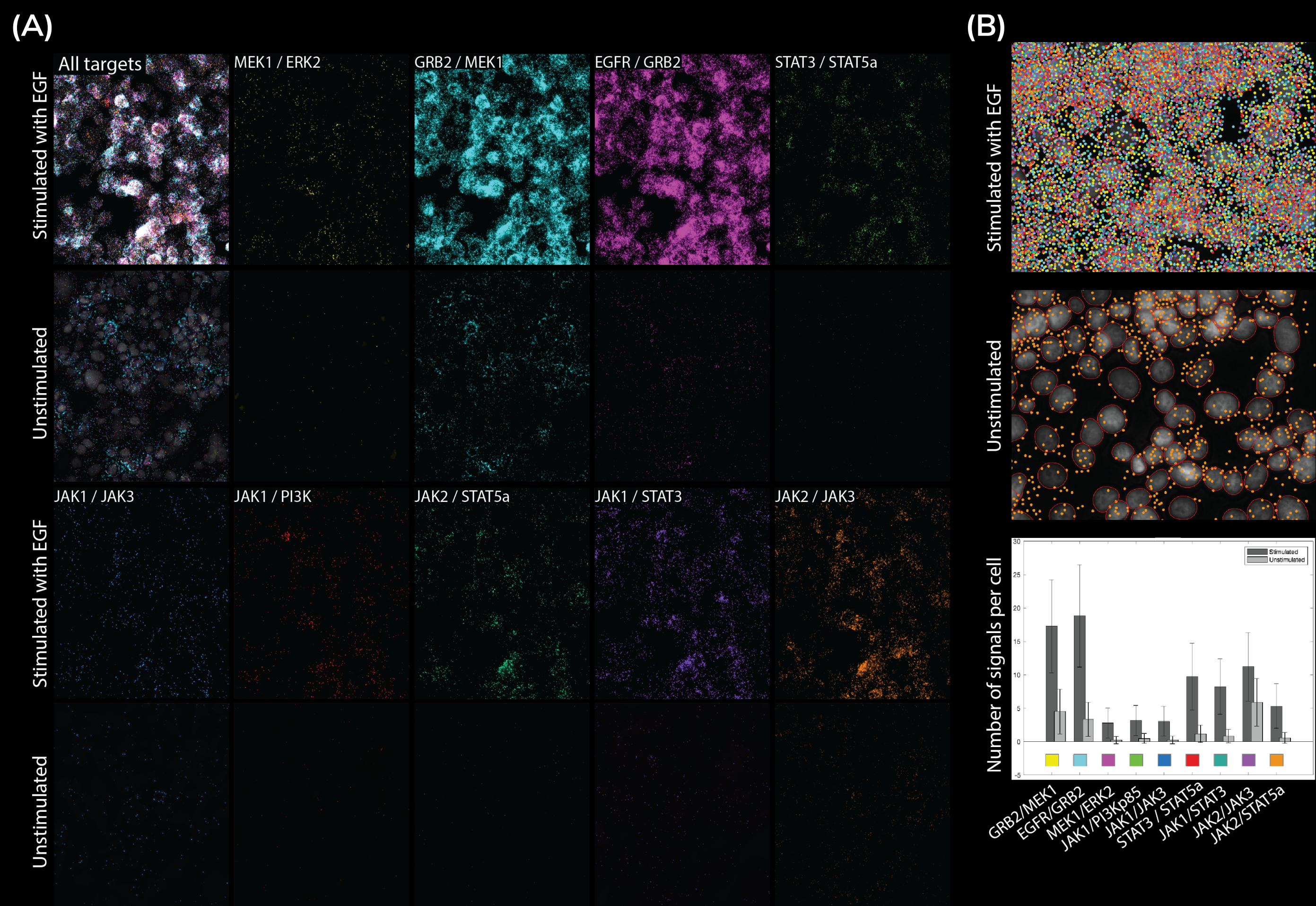


Fig. 3. (A) *mis*PLA analysis of protein interactions within the EGFR signaling network in the SK-BR-3 cell line with and without EGF stimulation. (B) Quantitative image analysis of the protein interactions based on signal detection.

Mapping signaling architectures across lymphoma subtypes.

*mis*PLA of the nine protein interactions was applied to FFPE lymph nodes from three classical Hodgkin lymphoma subtypes—mixed cellularity (MC), lymphocyte-depleted (LD), and lymphocyte-predominant (LP)—revealing distinct signaling profiles. MC and LD showed strong EGFR–GRB2 and GRB2–MEK1 signals, indicating active MAPK signaling. LP displayed weaker EGFR–GRB2 but retained GRB2–MEK1, suggesting truncated MAPK input. Prominent STAT3–STAT5a proximity signals were recorded in a MC section while LP signaling was sparse, consistent with its MAPK profile. These findings demonstrate *mis*PLA's ability to resolve active, partial, and silent signaling nodes in intact lymphoma tissue.

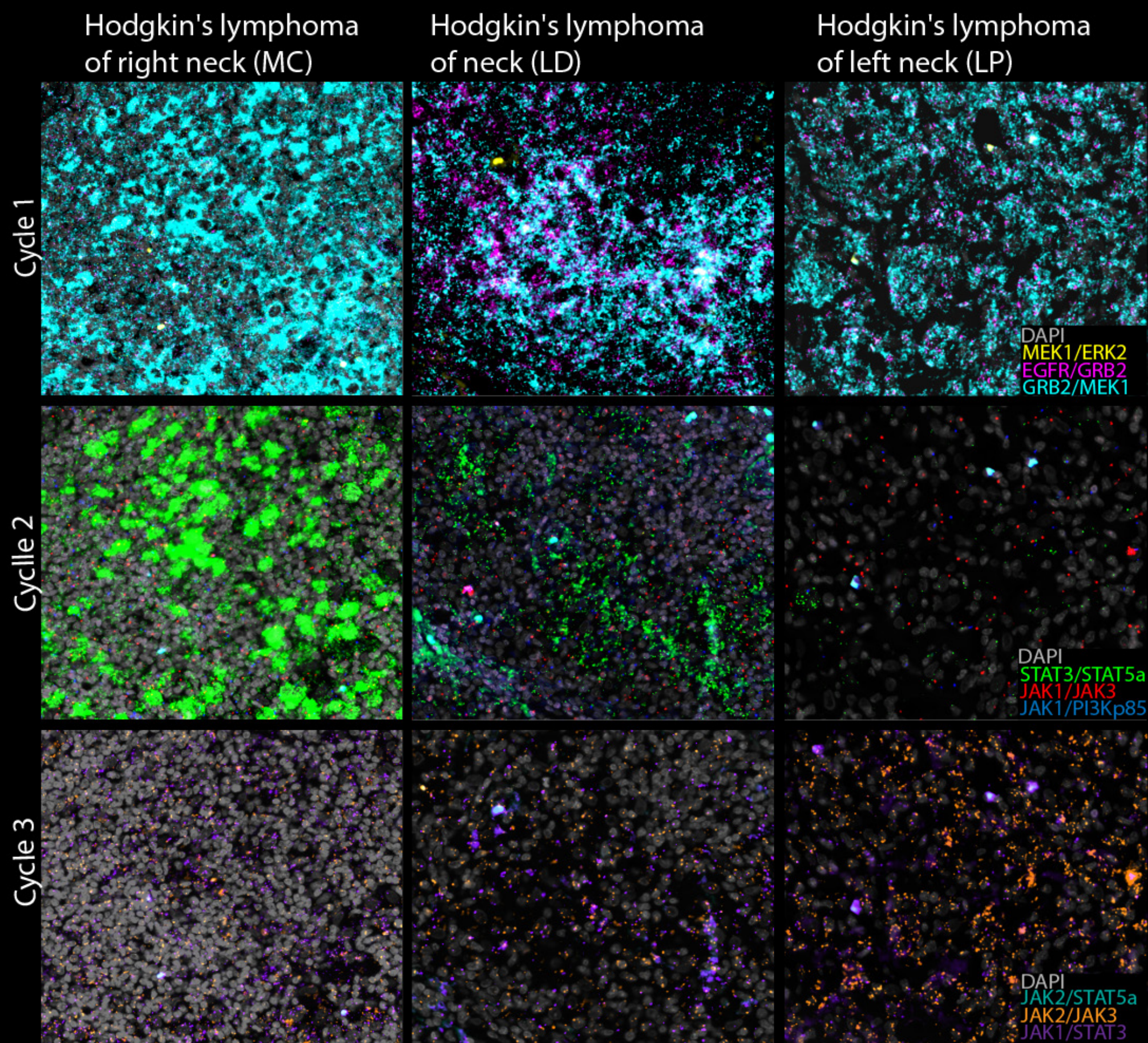


Fig. 4. Protein interaction along the EGFR signaling pathway across the Hodgkin lymphoma subtypes mixed cellularity (MC), lymphocyte-depleted (LD), and lymphocyte-predominant (LP).

Spatial Mapping of Immune Architecture in Tonsils.

A seven-plex *mis*PLA was performed on FFPE human tonsil tissue, with all targets visualized in a single imaging cycle. The assay resolved T-cell zones and B-cell follicles, while capturing checkpoint engagement within active immune regions. The resulting spatial map reflected the native microenvironment and functional organization of lymphoid tissue.

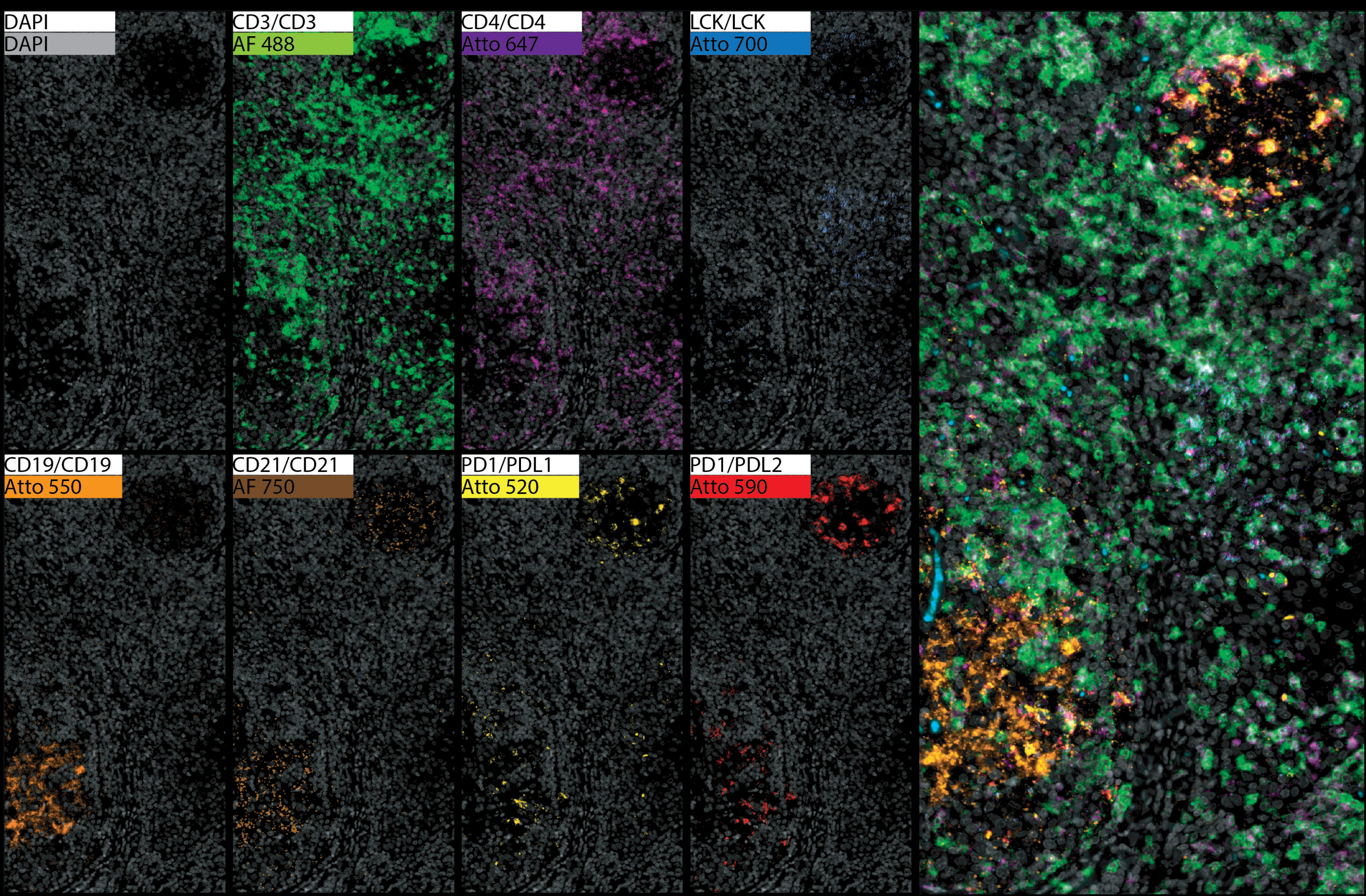


Fig. 5. Visualisation of tonsillar multiplex *mis*PLA staining of immune cell markers and checkpoint inhibitors.

Applications.

*mis*PLA provides a powerful platform for spatial proteomics, enabling high-sensitivity detection of subcellular localization, protein interactions, and post-translational modifications. Its compatibility with several biological models and standard microscopy makes it broadly applicable in both basic and translational research. Potential applications include immune cell profiling, checkpoint interactions, and complex signaling networks in cancer and other diseases, using customizable multiplex panels tailored to specific biological questions.

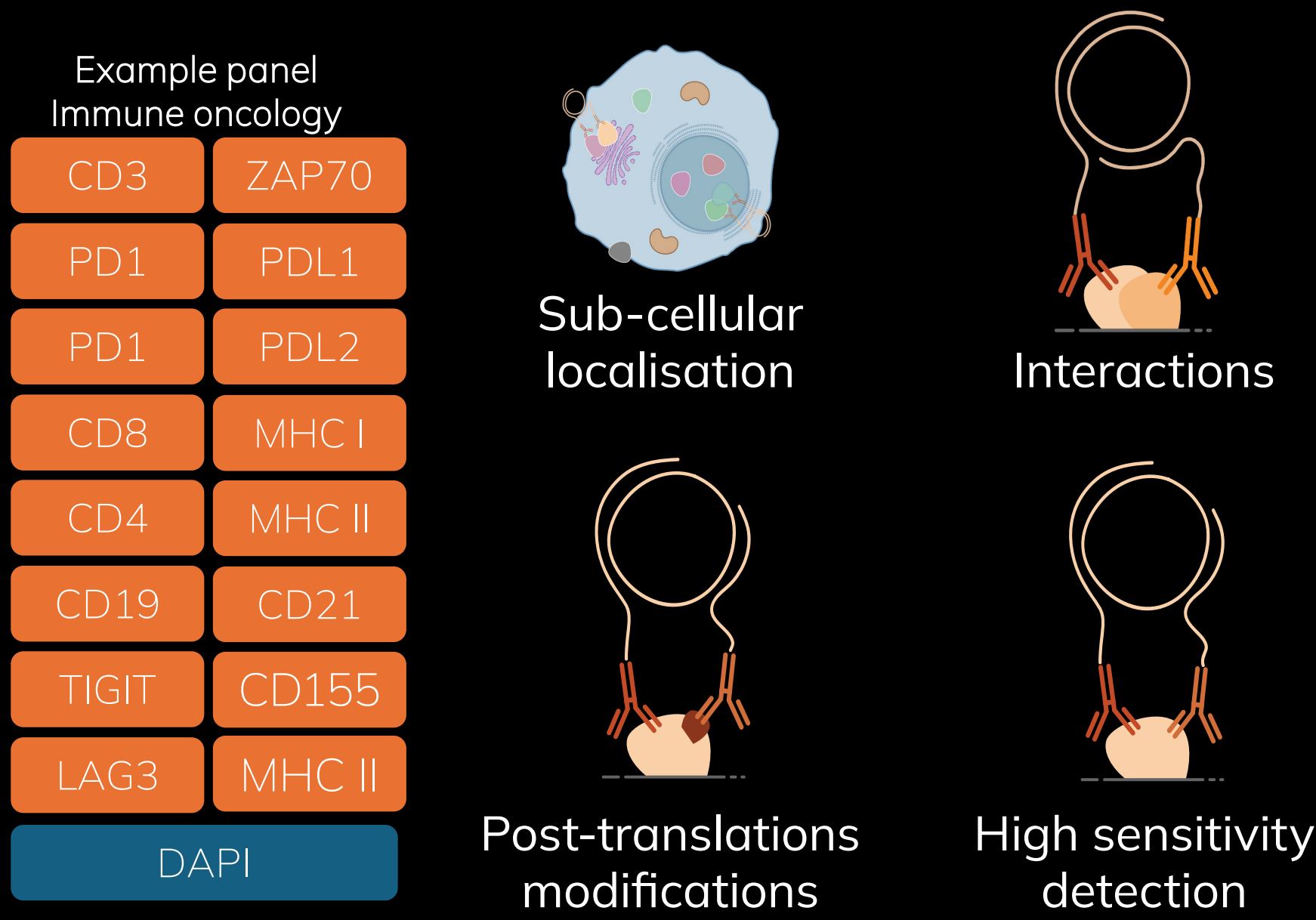


Fig. 6. Possible applications for the multiplex *mis*PLA technology.

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