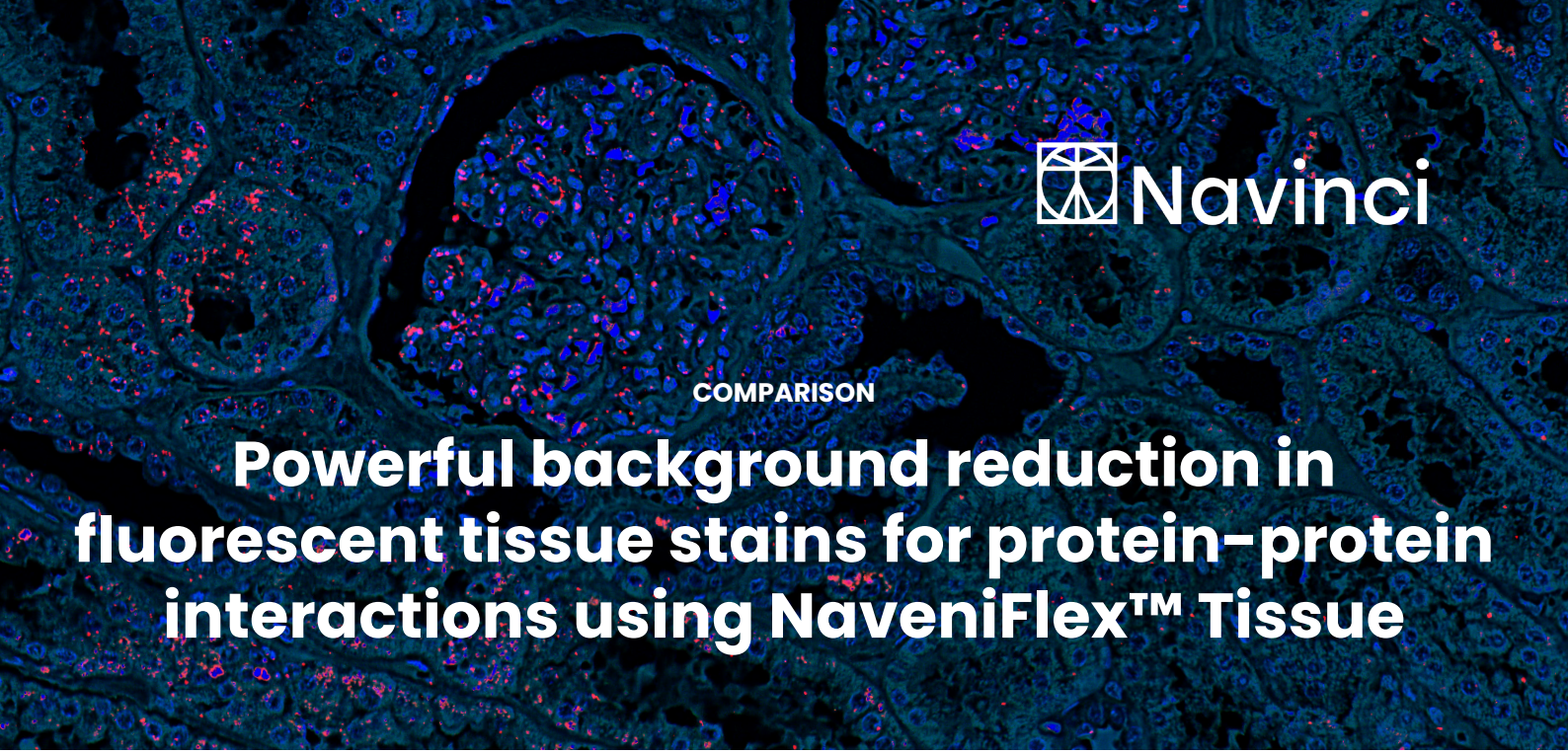


COMPARISON

Powerful background reduction in fluorescent tissue stains for protein–protein interactions using NaveniFlex™ Tissue

Introduction. Immunofluorescent staining of tissues via *in situ* proximity ligation assays is a well-established tool for highly sensitive detection of protein-protein interactions (PPI), post-translational modifications (PTM), and their localization in tissue. *In situ* proximity ligation assays find versatile applications in cell biology research, visualization of tissue architecture and identification of biomarkers, among others. However, standard immunofluorescence (IF) and *in situ* proximity ligation assays suffer from background signal originating from unspecific binding of fluorescently labeled detection reagents to certain types of cells. As a result, it can be difficult to distinguish true biologically relevant signal from background signal. To address this problem, we developed a new version of NaveniFlex, highly optimized for tissue use (learn more about the principles of our Naveni™ proximity ligation technology here: www.navinci.se/naveni-platform). NaveniFlex Tissue enables the specific and sensitive fluorescent detection of PPIs and PTMs in FFPE and fixed frozen tissues originating from either human or mouse. In contrast to previously available protocols, it can generate and visualize signal that would otherwise be obscured by background, thereby significantly increasing detection sensitivity.

The NaveniFlex Tissue workflow at a glance

- Deparaffinization and antigen retrieval of FFPE tissue samples
- Selection of primary antibodies for the proteins of interest (note that the two antibodies must be raised in different species and should be validated for use in IF or IHC)
- Blocking
- Primary antibody incubation
- Navenibody incubation (select the appropriate kit containing a combination of Navenibodies against the host species of the primary antibodies you used. Choose between mouse/rabbit, goat/rabbit and goat/mouse)
- Reaction 1
- Reaction 2
- Post-blocking
- Detection of fluorescent signal (choose between Red and Atto647N (far red, recommended for highly autofluorescent tissues) when ordering your NaveniFlex Tissue kit)
- Nuclear staining and mounting
- Imaging in a fluorescence miscroscope (note that you need a TexasRed filter cube set if using Detection Reagent NT Red, or a Cy5 filter cube set if using Detection Reagent NT Atto647N)

Assay 1. Detection of metastatic markers: Podocalyxin/Ezrin interaction. We compared the ability of NaveniFlex Tissue and another commercial *in situ* proximity ligation kit (which we refer to as kit X) to detect the interaction between Podocalyxin and Ezrin. The comparison was performed in human breast cancer in which co-expression is a prognostic marker for increased metastatic potential.

Result 1. FFPE breast cancer tissue was stained with either the complete set of proximity reagents (positive staining) or with all reagents except for primary antibodies, where no signal is expected (technical negative control). For kit X, we observed strong cell-specific background in both the positive (Fig. 1a) and technical negative control (Fig. 1b). The unspecific signal (large red areas in Fig. 1a, b) makes it impossible to determine the presence or absence of signal arising from proximity reactions (discrete red specks, up to 1 µm in size).

In contrast to the kit X results, the staining with NaveniFlex Tissue lead to dramatically reduced cell-specific background and protein interactions were clearly observed (Fig. 1c). Moreover, no signal was detected in the technical negative control (Fig. 1d).

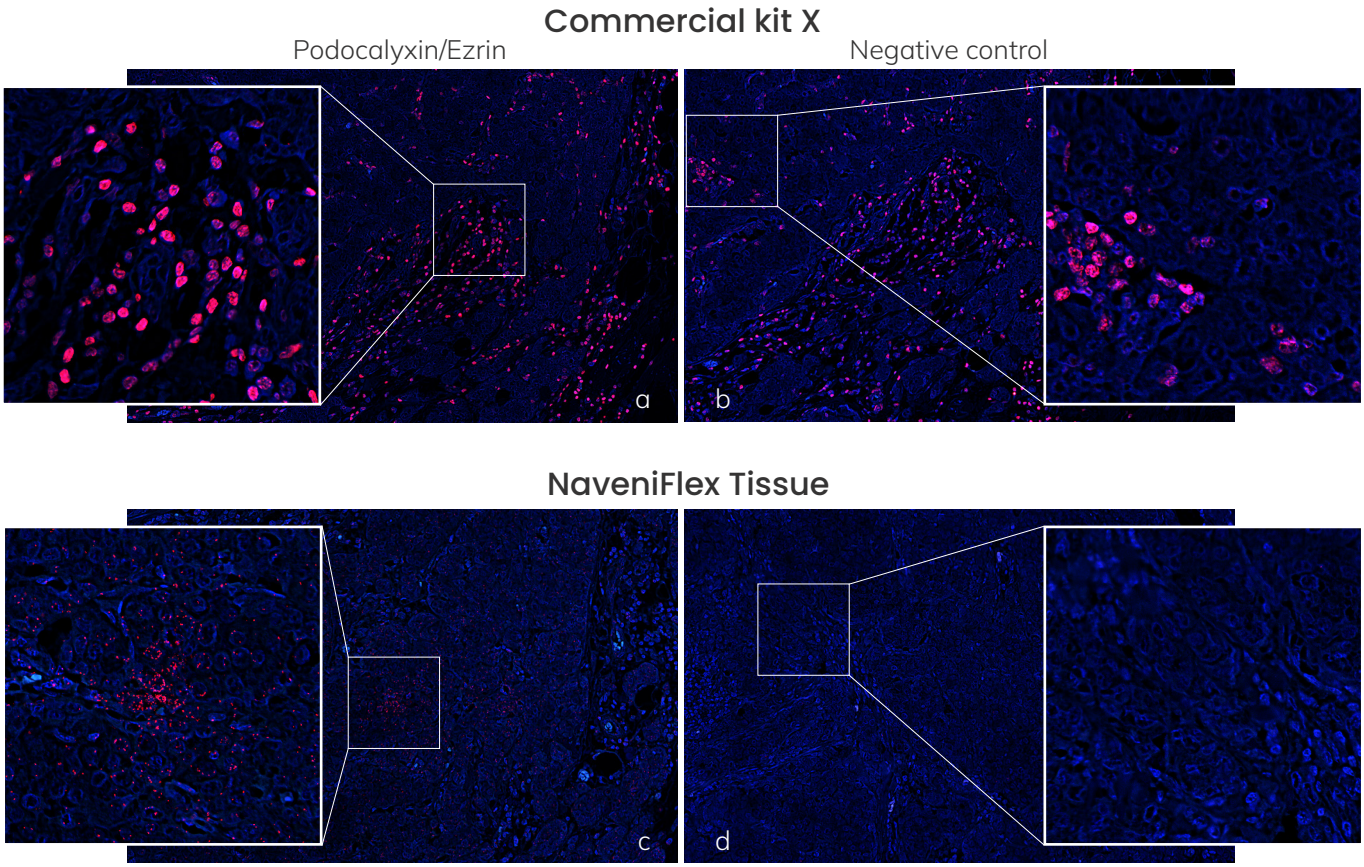
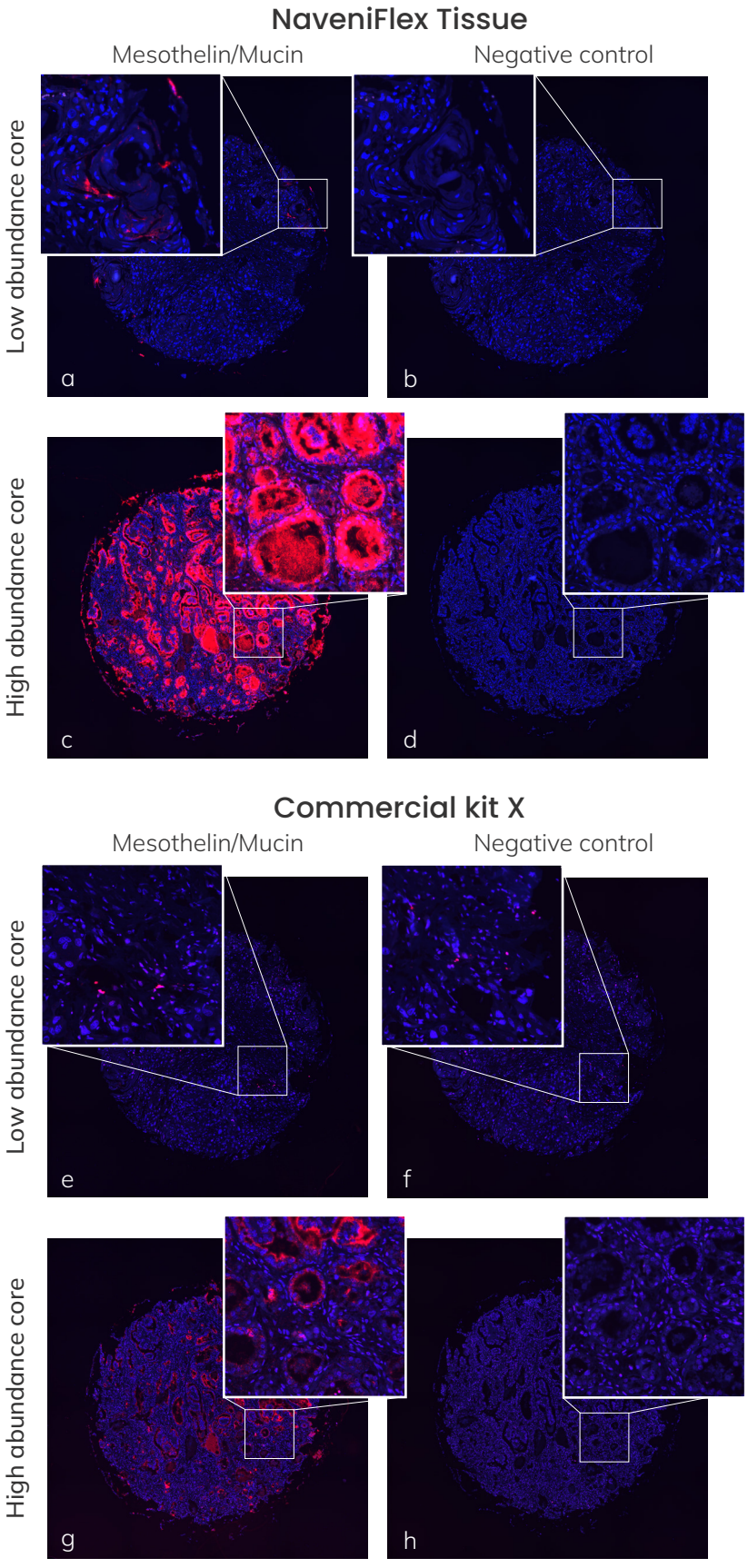


Fig.1. Comparison of the ability of NaveniFlex Tissue and a commercial *in situ* proximity ligation kit X to detect Podocalyxin/Ezrin in human breast cancer FFPE tissue. Kit X produced similar staining patterns in both the positive Podocalyxin/Ezrin staining (a) and the technical negative control (b). The high background (hazy red stain) in both images obscures any specific interaction signal. In contrast, NaveniFlex Tissue visualizes the intractions in the positive staining clearly (c, discrete red specks), background-free, and leaves the technical negative control (d) blank. Images were taken at 20x magnification; the white outlines depict zoomed-in areas. Proximity signals and cell-specific background fluorescence are visualized in red. DAPI was used as a nuclear stain (blue).



Assay 2. Mesothelin/Mucin-16 interaction in ovarian cancer. The binding of Mesothelin to Mucin-16 contributes to the metastasis of ovarian cancer to the peritoneum, which is a sign of malignant progression. Therefore the presence of the interaction may have prognostic significance in determining patient relapse-free survival. We tested the ability of NaveniFlex Tissue to detect this interaction in tissue microarrays (TMAs) containing tumors with high and low expression of the two proteins, respectively (data not shown), and compared these results to the performance of kit X.

Result 2. In a TMA staining for the Mesothelin/Mucin-16 interaction (Fig. 2), we successfully demonstrated a strong and clear signal with NaveniFlex Tissue in ovarian cancer (Fig. 2a, c), even in the tumor tissue where the expression of the individually measured proteins is low (Fig. 2a). In contrast, kit X detected significantly fewer interactions in the highly positive tumor (compare Fig. 2g with NaveniFlex Tissue data in Fig. 2c), whereas in the tumor with lower expression of these proteins it did not generate signals above background level (compare positive staining in Fig. 2e with negative control in Fig. 2f). Therefore, NaveniFlex Tissue improved the dynamic range of detection in tissues with varying protein expression levels, adding significant value to the staining.

Fig. 2. Comparison of the ability of NaveniFlex Tissue and a commercial *in situ* proximity ligation kit X to detect Mesothelin/Mucin-16 interaction in ovarian cancer. Consecutive sections of two TMA ovarian cancer cores – one with high expression, and one with low expression of Mesothelin/Mucin-16 – were stained with NaveniFlex Tissue (a, b, c, d) vs kit X (e, f, g, h). Negative controls were performed by omitting primary antibodies (b, d for NaveniFlex Tissue, and f, h for kit X). While both kits successfully stained the high abundance core (c, g), NaveniFlex Tissue produced stronger signal (c). It outperformed kit X in the low abundance core where it detected signal in the positive staining (a) but not in the negative control (b). Using kit X, results were similar between the positive staining (e) and the negative control (f). Images were taken at 20x magnification; the white outlines depict zoomed-in areas. Proximity signals and cell-specific background fluorescence are visualized in red. DAPI was used as nuclear stain in blue.

Assay 3. Detection of immune checkpoints in tonsil. Among the many molecularly defined immune checkpoint proteins, two of the most studied targets are the PD-1 receptor on T cells and its ligand PD-L1 expressed on antigen-presenting cells. The interaction between the two proteins plays an important role in initiating T cell inhibition and preventing autoimmune responses in healthy cells. In diseased tissue, the interaction may help tumors evade the immune system, which makes it interesting in the context of immunotherapy. We stained for PD-1/PD-L1 interaction in consecutive human FFPE tonsil sections with our NaveniFlex Tissue and NaveniBright kits.

Result 3. The two kits produced consistent results in detecting abundant and specific PD-1/PD-L1 interactions correctly localized in the germinal center of tonsil tissue (Fig. 3). Technical negative controls were also blank for both kits. As is generally the case with fluorescence *vs.* brightfield techniques, NaveniFlex Tissue performed slightly better when it comes to resolution, allowing for the better visualization of individual interaction signals and data quantification.

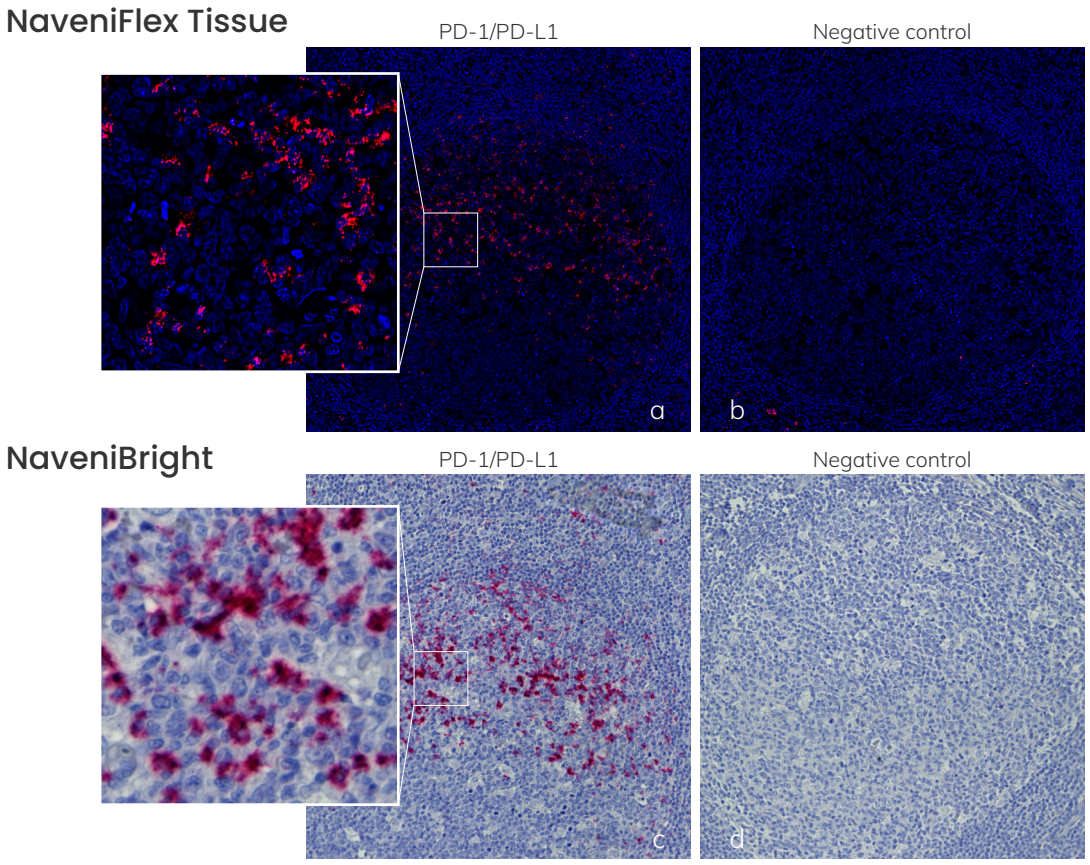


Fig. 3. Fluorescent vs. brightfield staining of PD-1/PD-L1 interactions in human FFPE tonsil. The flexible kits NaveniFlex Tissue (a) and NaveniBright AP (c) both detected activation of the PD-1/PD-L1 immune checkpoint pathway in the germinal center of human tonsil. The fluorescent readout of NaveniFlex Tissue allowed individual signals to be distinguished with improved clarity. Technical negative controls for both kits (b, d) were blank. Images were taken at 20x magnification; the white outlines depict zoomed-in areas. Proximity signals and cell-specific background fluorescence are visualized in red. DAPI was used as nuclear stain in blue.

Summary. Our data illustrate that NaveniFlex Tissue outperforms the *in situ* proximity ligation kit X by efficiently reducing cell-specific background, which improves visualization of the signal and signal-to-noise ratios in various healthy and diseased tissues. The kit is also the best fluorescence-based alternative to the NaveniBright kits, and due to the fluorescent readout it offers very high resolution. NaveniFlex Tissue allows flexibility and versatility, as it can be applied on both human and mouse tissue and is compatible with three combinations of primary antibody host species (mouse and rabbit (MR), goat and mouse (GM) or goat and rabbit (GR)). Additionally, one of two fluorophores for detection (red or far red) can be selected with each kit.

Navinci

FOR MORE INFORMATION OR TO PLACE AN ORDER, VISIT WWW.NAVINCI.SE/PRODUCTS
Email: contact@navinci.se