#4336

Automation of Proximity Ligation Immunoassay for interaction between PD-L1 and PD-1 detection in the tumor microenvironment using microfluidic-based systems

Authors

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Introduction

We have developed an automated version of our NaveniFlex™ Tissue Proximity Ligation Technology, a well-established method that detects protein-protein interactions and post-translational modifications (PTMs) with high specificity. Automatization of the Proximity Ligation Technology opens up new possibilities for a standardized and reproducible multiplex analysis of protein interactions and PTMs with greatly reduced hands-on time compared to the manual method. In collaboration with the Spatial Proteomics National Facility at SciLifeLab and Lunaphore we have optimized our NaveniFlex™ Tissue Proximity Ligation Technology for compatibility with automated LabSat® and COMET™ staining platforms from Lunaphore. These platforms can perform sequential multiplex immunohistochemistry (IHC) and sequential immunofluorescence (seqIF™) staining in combination with RNA detection to study spatial biology in tissues. The Proximity Ligation technology's utility is demonstrated via the interactions between ß-catenin/E-cadherin and PD-1/PD-L1. ß-catenin/E-cadherin interactions are important for maintaining epithelial integrity. Aberrant expression of these proteins can cause metastases and is associated with a wide variety of malignancies[1, 2]. The interaction between PD-1 and PD-L1 is important for cancer cells to escape the immune system[3]. PD-1 and PD-L1 proteins exert most functions in cells and tissues by undergoing modifications and forming dynamic complexes – effects that cannot be explored by genomics, transcriptomics, or conventional immunostaining methods. Numerous cancer therapies are being developed that affect PD-1/PD-L1 signaling, and tools to study the PD-1/PD-L1 axis are, therefore, essential[4].

Method

The whole NaveniFlex™ Tissue protocol was run fully automated on FFPE tissue sections on the LabSat® instrument, followed by seqIF™ stainings performed on the COMET™ instrument. The NaveniFlex™ Tissue protocol is compatible with any primary antibody. For the purpose of the study, the following reagents were used in pairs: mouse anti-E-Cadherin and rabbit anti-ß-Catenin; mouse anti-PD-1 and rabbit anti-PD-L1. When the protocol was combined with seqIF™ on COMET™, the primary antibodies of the proximity assay were eluted, and the same tissue section was subsequently incubated with primary antibodies for the immunofluorescence staining of CD3, CD4 and CD8. The automated workflow is described in Figure 1.

Fig. 1a

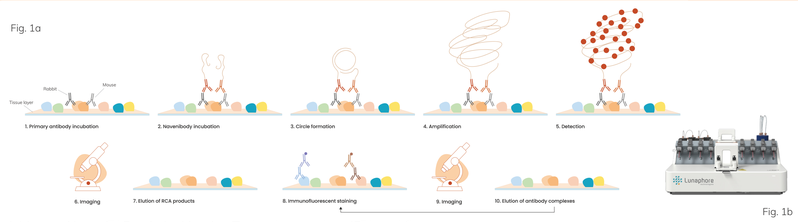

Fig. 1b

Fig. 1c

(a) Latest Naveni™ Proximity Ligation Technology and seqIF™ workflow.
1. Two primary antibodies bind their target epitopes
2. Navenibodies conjugated to oligonucleotides bind to the primary antibodies
3. The DNA oligonucleotides hybridize, and gaps are ligated to create a circular DNA template
4. The circular DNA template is amplified via rolling circle amplification (RCA)
5. Fluorophore-labelled DNA oligonucleotides hybridize to the amplified RCA products. Antigen retrieval and all *in situ* PLA steps from 1 to 5 were automated on LabSat® (b)
6. The fluorophore-labelled RCA products are visualized in a regular fluorescent microscope or with the built-in microscope of the COMET™ instrument (c)
7. The RCA products are removed by elution
8. New primary antibodies bind to their target epitopes, followed by fluorophore-labelled secondary antibodies
9. The immunofluorescent staining is imaged with the integrated microscope of the COMET™ instrument
10. Elution of primary and secondary antibody complexes
11. Step 8 to 10 can be iterated to detect 40 markers per automated run on COMET™

(b-c) Lunaphore automated platforms LabSat® (b) and COMET™ (c) are based on the Fast-Fluidic Exchange (FFeX™) technology, which allows to finely control all staining steps. Off-the-shelf primary antibodies can be used on both platforms.

NaveniFlex™ Tissue on LabSat® *vs* manual

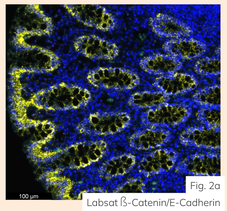

Fig. 2a
Labsat ß-Catenin/E-Cadherin

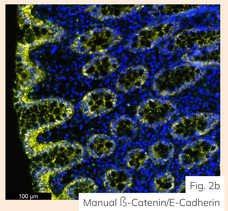

Fig. 2b
Manual ß-Catenin/E-Cadherin

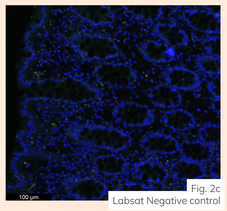

Fig. 2c
Labsat Negative control

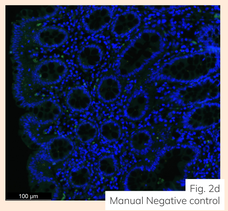

Fig. 2d
Manual Negative control

Fig. 2. Normal colorectal tissue section with *in situ* PLA signals from ß-catenin/E-cadherin interaction detected with FarRed fluorophore (yellow) in the mucosa. (a) *in situ* PLA (NaveniFlex™ Tissue) run on LabSat®. (b) Manually generated *in situ* PLA signals. Bottom panels are negative controls i.e., primary antibodies were excluded, performed on LabSat® (c) or manually (d). Top images (a-b) show that LabSat® and manually performed *in situ* PLA assays perform equivalently *in situ* PLA staining. Images were acquired with an epifluorescent microscope with a 20x (0.75 NA) objective.

Colon tissue microarray run on LabSat®

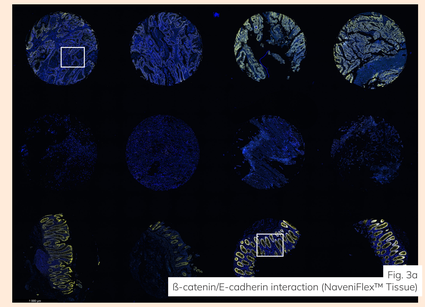

Fig. 3a
ß-catenin/E-cadherin interaction (NaveniFlex™ Tissue)

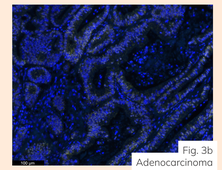

Fig. 3b
Adenocarcinoma

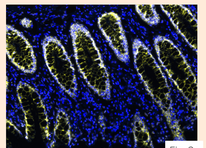

Fig. 3c
Colon cancer adjecent tissue

Fig. 3. Top image (a) shows a colon TMA with *in situ* PLA signals from ß-catenin/E-cadherin interaction detected with FarRed fluorophore (yellow). Tissue cores in the top row are adenocarcinoma, middle row mucinous adenocarcinoma, and bottom row cancer-adjacent normal colon tissue. Zoomed-in view of adenocarcinoma (b) and cancer-adjacent normal colon (c) tissue. Images were acquired with an epifluorescent microscope with a 20x (0.75 NA) objective.

PD-1/PD-L1 protein interactions in tonsil germinal center

Fig. 4. Tonsil tissue section stained on LabSat® automated staining platform with the NaveniFlex™ Tissue Technology and IF.
From left to right: (a) Negative control (primary antibodies were excluded) with unstained germinal centers of the tonsil tissue. (b) *in situ* PLA signals (red) visualizing proximity between PD-1 and PD-L1 proteins. (c) *in situ* PLA signals zoomed-in to one germinal center. (d-e) Images show the same germinal center with IF staining of PD-L1 (yellow) and PD-1 (green) performed on LabSat®. Images were acquired with an epifluorescent microscope with a 20x (0.75 NA) objective.

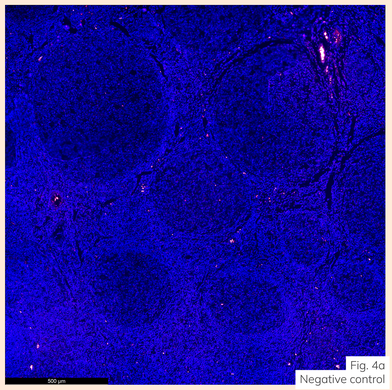

Fig. 4a
Negative control

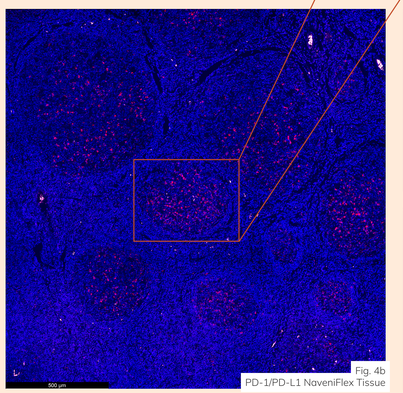

Fig. 4b
PD-1/PD-L1 NaveniFlex Tissue

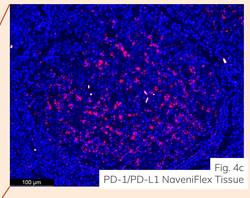

Fig. 4c
PD-1/PD-L1 NaveniFlex Tissue

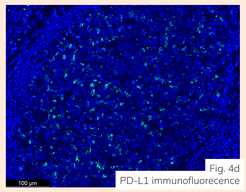

Fig. 4d
PD-L1 immunofluorecence

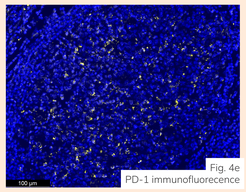

Fig. 4e
PD-1 immunofluorecence

Automated NaveniFlex™ Tissue combined with seqIF™ on COMET™

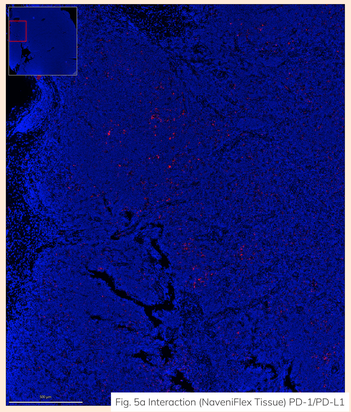

Fig. 5a Interaction (NaveniFlex Tissue) PD-1/PD-L1

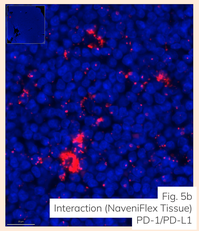

Fig. 5b
Interaction (NaveniFlex Tissue)
PD-1/PD-L1

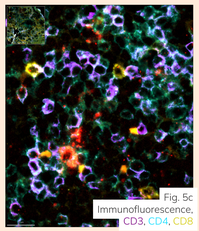

Fig. 5c
Immunofluorescence,
CD3, CD4, CD8

Fig. 5. (a) Lymphoid tissue section from a patient with Hodgkin's lymphoma stained on LabSat® automated staining platform with the NaveniFlex™ Tissue Technology to detect PD-1 and PD-L1 interactions in FarRed (red). (b) Zoomed-in tissue section. (c) Same zoom as in (b) with seqIF™ staining of CD3 (purple), CD4 (green), and CD8 (yellow) performed on the COMET™ instrument, run after *in situ* PLA on the same tissue section. All images were acquired with the COMET™ instrument with a 20x objective.

Conclusion

We have shown that our technology NaveniFlex™ Tissue can be fully automated on LabSat® with equivalent results to manual execution. Our data demonstrate the feasibility of eluting antibodies and detection reagents from the proximity assay, and using the same sample for subsequent seqIF™ stainings on COMET™. The method is truly flexible, giving the user the option to freely choose from any primary antibody, both for protein-protein interactions and single protein detection with sequential immunofluorescence. Automatization of the Proximity Ligation Technology opens up new possibilities for a standardized and reproducible multiplex analysis of protein interactions and PTMs with minimal hands-on time. A manual PLA protocol from tissue preparations to mounted slides usually takes two days day to perform. With automation, the hands-on time is greatly reduced with all steps being performed in less than a day.

We believe this approach will enable spatial and functional studies of the interface between tumor and immune system and provide necessary information about signaling pathway activation *in situ*, the latter representing a novel state-of-the-art in tissue diagnostics.

Acknowledgements
The work has been done in close collaboration with the Spatial Proteomics National Facility at Science for Life Laboratory. The Facility already has both LabSat and COMET in their instrument portfolio and offers seq IF on COMET as a service to their users.

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