Fully Automated proximity ligation assay enables high throughput, reproducible detection of protein-protein interaction in tissue for biopharma applications

[1] Kelly Hunter , [1] Mark Howard, [1] Chris Morse, [2] Hampus Elofsson

1)Propath. Hereford. UK

2)Navinci Diagnostics, Research and Development, Uppsala, Sweden

Background

Revealing protein-protein interactions (PPIs) enhances our understanding of the tissue milieu since PPIs are essential for the performance of biological processes such as signalling cascades, immune checkpoint activation, protein degradation, response to therapy etc. The Naveni® in situ proximity ligation (isPLA) technology can detect proteins located within an interaction range (<40nm) by means of antibodies conjugated to oligonucleotides that create amplified fluorescent or chromogenic signal only if the proximity requirement is fulfilled.

isPLA is a robust measurement of PPIs which are highly relevant to the biopharma industry when understanding mechanism of action and correlating with molecular and clinical response. Such applications require a high level of reproducibility and scalability. Here we demonstrate the throughput and reproducibility of a fully automated in situ isPLA method.

Methods laboratory

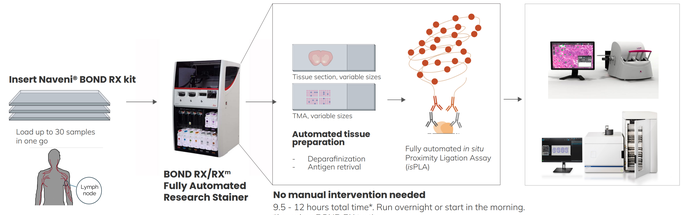


Figure 1. Laboratory workflow for automated isPLA staining using the NaveniFlex Tissue BOND RX kit on FFPE Human Hodgkins' Lymphoma tissue sections performed at 2 sites with independent autostainers and whole-slide scanners

35 4µM sections from a Human Hodgkin's Lymphoma FFPE tissue block were distributed to 2 laboratories and stained for MHC1-CD8 interaction using the Leica Bond RX and NaveniFlex Tissue BOND RX kit from Navinci.

We processed 3 independent staining runs of 5 samples and digitised them in a single scanning run using a Panoramic Scan II fluorescent scanner from 3DHistech, imaging in DAPI and Cy5 Channels. A subsequent batch of 10 slides were stained and scanned independently, 1 image was excluded following quality control.

10 further sections were processed at an independent location and digitised using an Olympus VS200, 1 image was excluded following quality control.

Methods data analysis

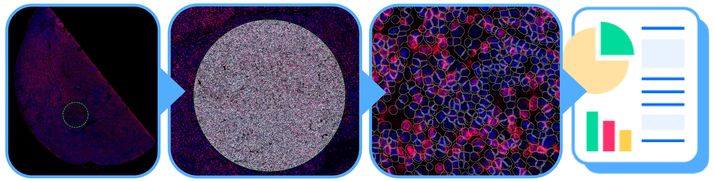


Figure 2. Pipeline for ROI manual annotation of each slide and automated cell segmentation using Oncotopix Discovery from Visiopharm, followed by data analysis in R using Posit Workbench

Methods data analysis (continued)

Image analysis was employed to extract data from comparable regions on each image using Visiopharm Oncotopix Discovery. Representative expression data from each image are presented in a Levey-Jennings plot with the overall coefficient of variation for the entire dataset.

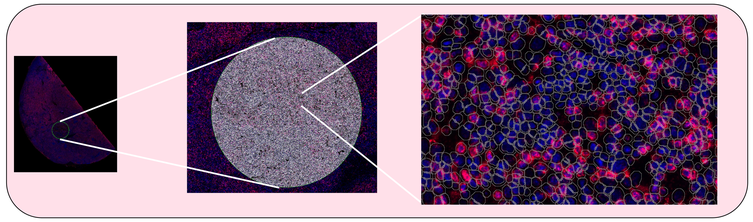


Figure 3. Representative example of ROI placement and cell segmentation. Pixel level expression data was extracted from within all cells in the ROI and an the mean value reported for each slide

A single Region of Interest (ROI) measuring 2000µM circumference was placed in the same location on each image. A central location was selected with easily identifiable morphology for accurate placement between samples, with relatively stable expression throughout the block to minimise biological variance, and away from the edges to avoid 'edge effect' present in many of the sections.

A deep-learning based cell segmentation algorithm was used to detect the nuclei, and estimate the cell boundary to exclude excluded extracellular tissue. Pixel-level fluorescence intensity was exported for each sample, taking only pixel inside cells, inside the ROI annotation and the analysed in R. The mean intensity value of each pixel was calculated per image and plotted on a Levey-Jennings plot and used assess trends and overall variance, along with calculated variance of the means from each image in a set.

Results

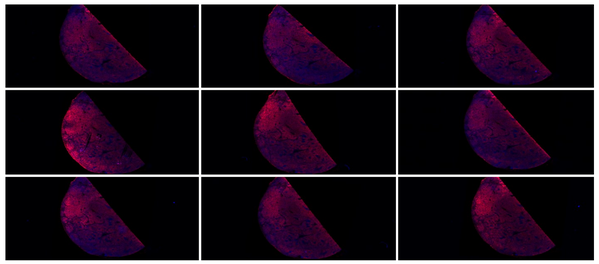


Figure 4. Whole slide scans of 1 batch of 9 FFPE Human Hodgkins' Lymphoma tissue sections (1 further slide from a batch of 10 was excluded following quality control).

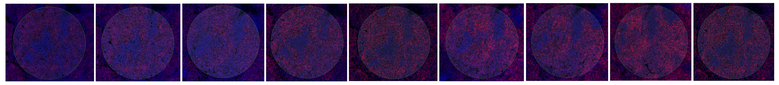


Figure 5. ROI annotations and cell segmentation from 1 batch of 9 FFPE Human Hodgkins' Lymphoma tissue sections (1 further slide from a batch of 10 was excluded following quality control).

Reproducibility Analysis Levey-Jennings plot

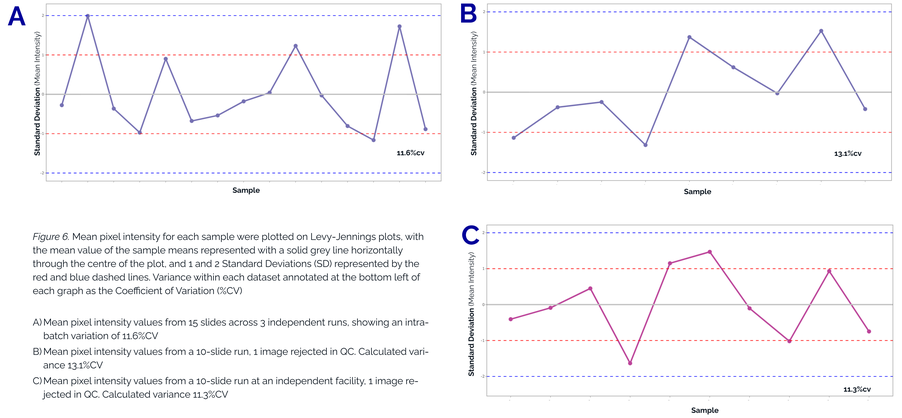


Figure 6. Mean pixel intensity for each sample were plotted on Levy-Jennings plots, with the mean value of the sample means represented with a solid grey line horizontally through the centre of the plot, and 1 and 2 Standard Deviations (SD) represented by the red and blue dashed lines. Variance within each dataset annotated at the bottom left of each graph as the Coefficient of Variation (%CV)

A) Mean pixel intensity values from 15 slides across 3 independent runs, showing an intra-batch variation of 11.6%CV

B) Mean pixel intensity values from a 10-slide run, 1 image rejected in QC. Calculated variance 13.1%CV

C) Mean pixel intensity values from a 10-slide run at an independent facility, 1 image rejected in QC. Calculated variance 11.3%CV

Similar MHC1-CD8 staining patterns were revealed across 35 Hodgkins Lymphoma sections with the Naveni® method run on Leica BOND RX over multiple runs and sites, where the staining pattern were assessed both qualitatively and quantitatively using Visiopharm.

Plot A represents 15 samples across 3 staining batches and a single scanning batch. This dataset demonstrates both inter and intra batch variability. Visual assessment of the scans together with review of this plot reveal no systematic trend either within or between batches.

Plot B represents a 10-slide batch stained and scanned independently, demonstrating intra-batch variability comparable variance to the first 3 batches.

Plot C represents a repeat of the 10-slide batch at a different laboratory with a different slide scanner. Variance is comparable to the 2 previous analyses, providing independent confirmation that this method can be reliably reproduced using the same sample and the same assay and method.

Published variance in gold-standard chromogenic Immunohistochemistry (IHC) assays typically range from 5-20%, reflecting our own experience. By controlling biological and technical variability, we have demonstrated a method for *in* situ proximity ligation assay with comparable reproducibility to chromogenic IHC.

Summary

- **We have shown that *In Situ* Proximity Ligation assays can be automated using the Leica Bond RX and Navinci NaveniFlex Bond RX kit.**
- **We have demonstrated a scalable method for detecting PPIs in tissue with a high level of reproducibility comparable to that observed in gold-standard chromogenic IHC, a method widely used in biopharma and the clinic**
  - Low assay variance enables quantification for differential expression analysis
- **Automation enables us to deliver an isPLA assay with high throughput and high reproducibility**
  - Protein-protein interaction assays in tissue is traditionally labour-intensive and can be unreliable
- **To fully assess the total expected variance including inter-site variability, imaging method standardisation would be required**
  - either centralised scanning, or data normalisation