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Simultaneous and spatially-resolved analysis of T-lymphocytes, macrophages and PD-L1 immune checkpoint in rare cancers

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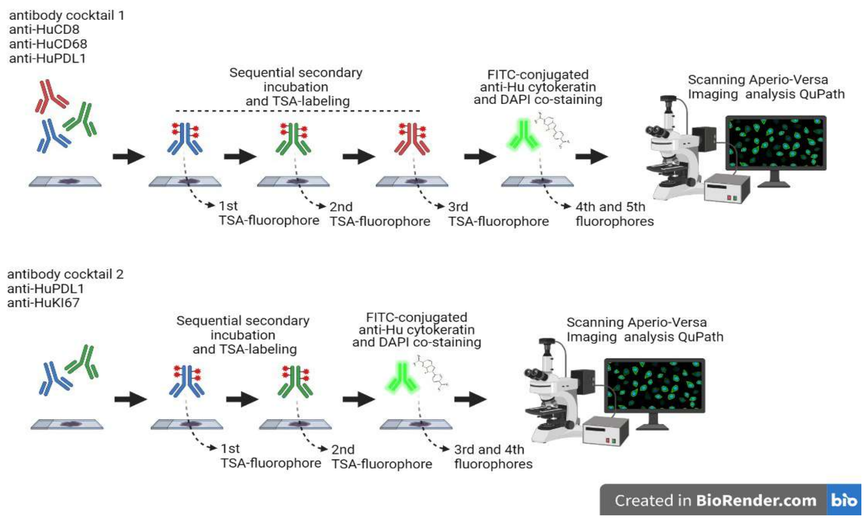
Introduction:

Rare cancers such as penile, vulvar and anal neoplasms show an incidence lower than 0.5% per year in Chile, however the impact of these neoplasms in quality of life and survival is high. These three types of cancer are related to human papilloma virus (HPV) infection and are characterized by a multistep progression from intraepithelial lesions to carcinoma, and immune infiltration. In this work, we established a multiplexed immunofluorescence for the simultaneous detection of CD8, CD68, PD-L1, Cytokeratin (CK) and Ki-67 in FFPE and applied tissue segmentation and cell phenotyping to interrogate the tumor microenvironment in retrospective cases

Methodology:

Multiplexed immunofluorescence: After antigen retrieval (97°C 20min), each slide was incubated with an antibodies cocktail (4°C ON). Isotype-specific HRP-conjugated antibodies (RT 60min) and tyramide-based amplification systems (Perkin Elmer) was used for signal detection. Quality control included human tissue as naturally positive and negative controls (skeletal muscle, tonsil and placenta).
All slides were scanned at 20X of magnification using an Aperio VERSA 200 microscope (Leica Biosystems, Vista, CA, USA), using sequentially the filters for DAPI, FITC, Cy3, Cy5 or Cy7. For cell segmentation and profiling all acquired images were analyzed using QuPath software.



Imaging analysis: 1) Cell segmentation used DAPI and considered a pixel size of 0.5 µm; background radius of 8.0 µm; median filter radius of 0.0 µm; minimum and maximum area of 10 and 200 µm2, respectively, and a threshold of 10.
2) Cell profiling used each fluorescent channel separately (excluded DAPI). We used the default configuration for the cell classifier of Multiplex QuPath to ensure an objective and unsupervised analysis. At least 400 cells were used for pixel training and object classification training. The automatic analysis was applied for each TMA separately. All images were previously visually to note staining artefacts.

Conclusions:

- We developed a suitable approach to measure up to 4 biomarkers simultaneously and optimized the use of limited samples for the comprehensive study of tumor microenvironment, and it may be apply for the study of many other solid tumors.
- Our tool showed to be robust, reproducible and accurate to detect cells and tissues with spatial resolution.
- QuPath is an open resource which allows cell profiling and segmentation in multiplexed fluorescent images.

Results:

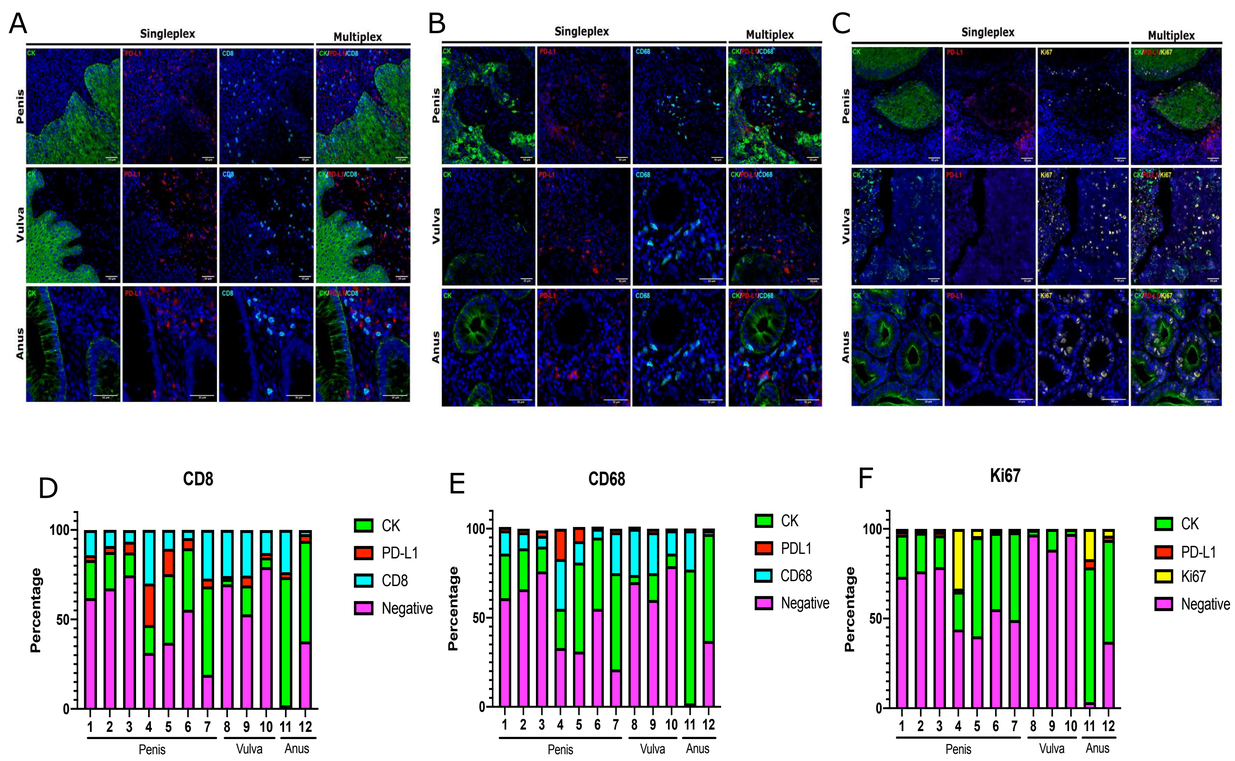


Figure 1. Multiplexed mmunofluorescence in Penile, Vulvar and Anal cancer. A-C) Images are representative for panels 1 (CK, PDL1, CD68, CD8) and 2 (CK, PDL1, KI67). For convinience some channels were separately processed (singleplex) or in combination (multiplex). Scale bar represents 50 µm. Nuclei were stained with DAPI.
Percentage of positive cells for CK, PD-L1, CD8, CD68, KI67 and unknown cells (negative for any marker) are shown in a combined-bar graph (D-F). Total number of individuals was estimated using DAPI-positive cells.

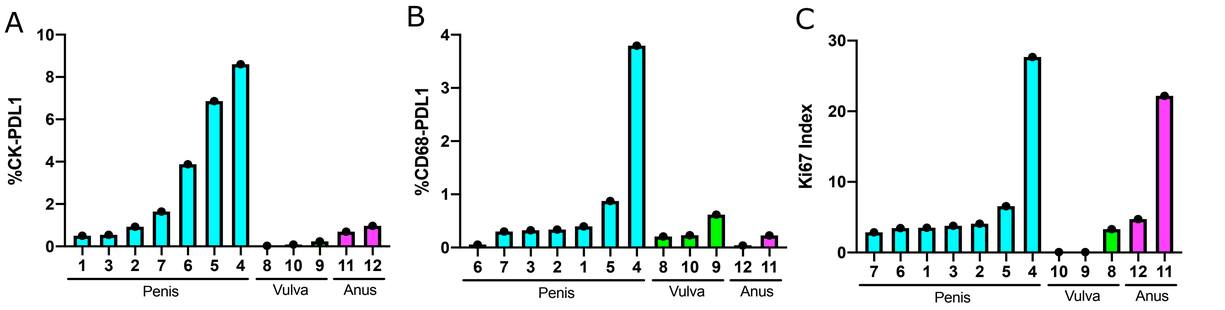


Figure 2. Biomarkers co-detection in Penis, Vulvar and Anal cancer. Percentage of individuals positive for two biomarkers, such as CK and PD-L1 (A),[B] CD68 and PD-L1 (B), and Ki67 Index (KI67-DAPI) were calculated.

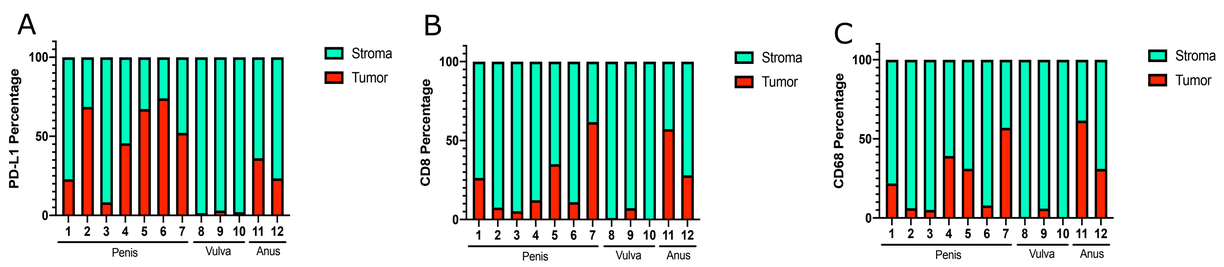


Figure 3. Biomarkers localization in Penis, Vulvar and Anal cancer. Percentage of positive cells for PD-L1 (A), CD8 (B) and CD68 (C) was calcutaed within the Tumor area (CK positive región) or Stroma (non-CK región). The individual value was determined using the location of a specifc biomarker withing a topographic map based on CK signal.

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