# Bright-field multiplex immunohistochemistry assay for tumor microenvironment evaluation in melanoma tissues

P301

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## Background

Melanoma is one of the major causes of cancer-related death, and its incidence is increasing worldwide [1]. The immune system plays a crucial role in melanoma development and progression [2,3]. Standard single chromogenic immunohistochemistry (IHC) has been applied to immune contexture evaluation in melanoma tissues as part of retrospective studies or exploratory analyses [4], mostly focusing on the prognostic and predictive impact of density and distribution of immune cells, including tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs) [5,6].
Although most pathologists are familiar with the semiquantitative assessment of immune cells by singlepex IHC, bright-field (BF) multiplex IHC has been introduced to simultaneously visualize up to 8 markers labeling different cell populations on the same section [7]. However, there is currently limited knowledge on the best optimal combination and visual interpretation in variably pigmented melanoma tissues.

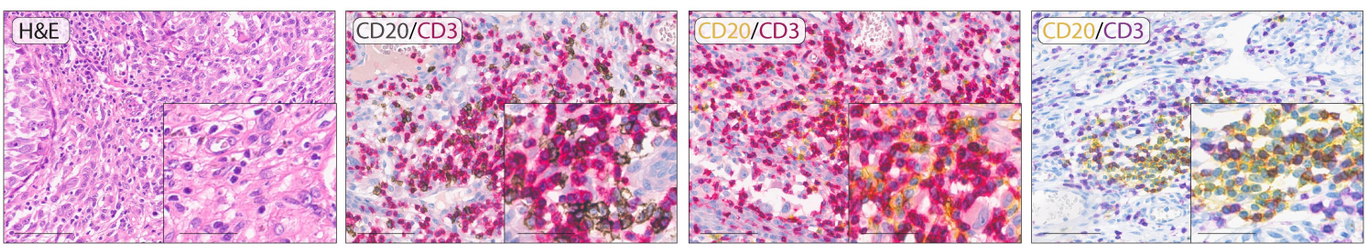
## Methods

Formalin-fixed paraffin-embedded (FFPE) in primary cutaneous melanoma tissues (n=20) tissue sections 3 µm in thickness, were obtained from paraffin blocks retrospectively selected from the Archive of the Section of Pathology, Department of Health Sciences, University of Florence, Florence, Italy. TME characterization was performed by evaluating different BF multiplex IHC staining using different combination of 6 immune cell markers (CD3, CD4, CD8, CD20, CD68 and CD163) and the melanoma specific cell marker (SOX10).

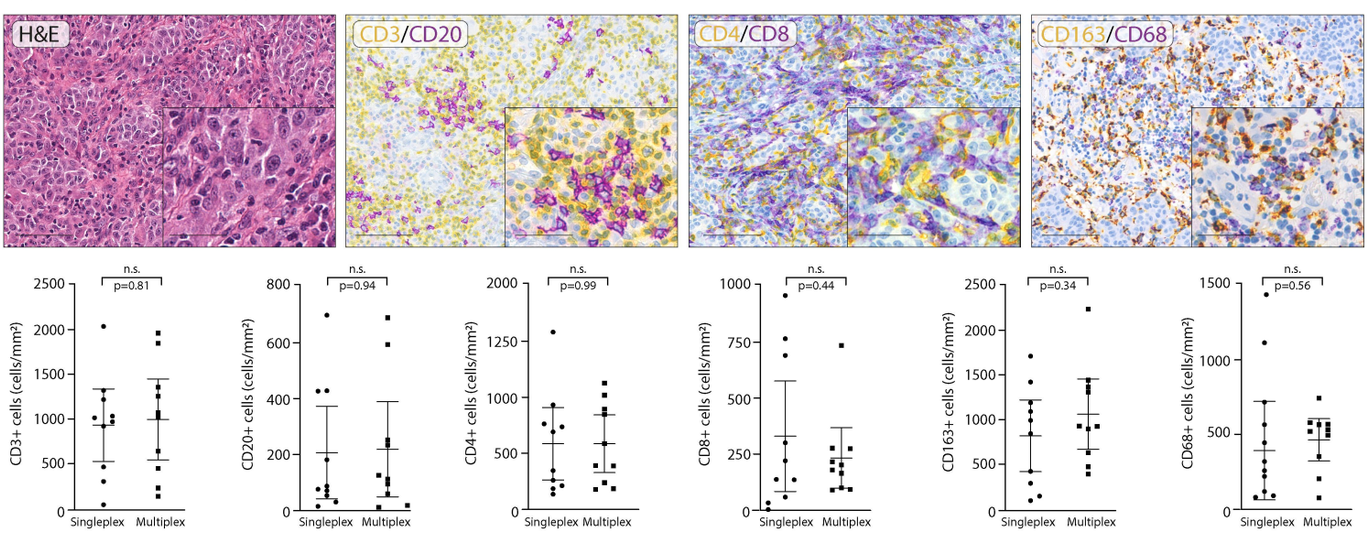
## Results

*Optimization of double labeling protocols*

Starting from single-plex validated staining protocols routinely used, we first assessed whether these traditional BF-IHC could combine with each other, in particular we combine 6 immune cell markers protocols for CD3, CD4, CD8, CD20, CD68 and CD163. These protocols allowed us to visualize two different immune cells population on the same section, CD3/CD20 (T and B lymphocytes), CD4/CD8 (T helper and T cytotoxic), CD68/CD163 (M1 and M2 macrophages). We started to optimize the protocol from the CD3/CD20 staining. To characterize the distribution of B-cells and T-cells in the TME of melanoma samples, we performed 3 different staining protocols using the following chromogens combinations Red/DAB, Yellow/Red, Yellow/Purple. As shown in the figure BF multiplex IHC Yellow/Purple protocol guarantees the maximum contrast in comparison with the previous combinations tested Red/DAB and Red/Yellow. Moreover, we applied this protocol for CD4/CD8, CD68/CD163 staining protocols.



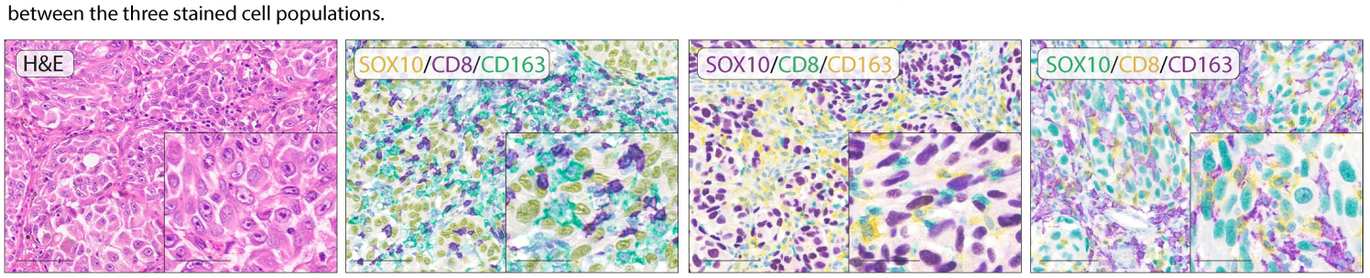

Representative double labelling CD20/CD3 BF-multiplex IHC of melanoma tissue. Magnification 200X and 400X (scale bar: 100µm, inset 50µm).




Representative double labelling CD3/CD20, CD4/CD8 and CD163/CD68 BF-multiplex IHC of melanoma tissue. Magnification 200X and 400X (scale bar: 100µm, inset 50µm) and pooled data of CD3, CD20, CD4, CD8, CD163 and CD68 positive cells densities comparison between single and multiplex IHC.

*Optimization of triple labeling protocols*

We introduced the nuclear marker specific for melanoma cells SOX10 to our optimized double labelling protocols. In figure we reported one of the possible combinations for triple labelling BF IHC SOX10/CD8/CD163. To achieve this goal, we introduced the green chromogen for the third stain. We tested three different chromogens' combinations: SOX10 (Yellow), CD8 (Purple), CD163 (Green); SOX10 (Purple), CD8 (Green), CD163 (Yellow) and SOX10 (Green), CD8 (Yellow), CD163 (Purple). Our results showed that the combination SOX10 (Green), CD8 (Yellow), CD163 (Purple), guarantees the best contrast and sharp discrimination between the three stained cell populations.




Representative triple labelling SOX10/CD8/CD163 BF-multiplex IHC of melanoma tissue. Magnification 200X and 400X (scale bar: 100µm, inset 50µm).

## Conclusions

In conclusion, we herein compared different BF IHC multiplex protocols for the study of TME in primary cutaneous melanoma tissues and offered the best optimized protocol for visualization and evaluation. These methodologies are studied to maximize the quality of staining (choice of chromogens) considering the tissue characteristics under examination (variably pigmented specimens), maintaining a high level of standardization and reproducibility thanks to the complete automation of the process developed in Ventana Discovery Ultra immunostainer.

## References

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