In situ multiplex analysis of resident microglia and infiltrating macrophages in glioblastoma

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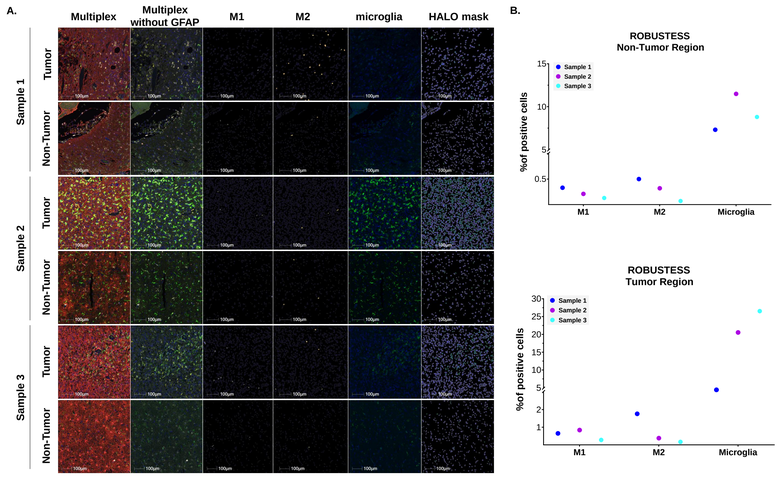
Background: Glioblastoma mutliforme (GBM) is the most malignant primary brain tumor. Resident microglia and peripheral infiltrating macrophages account for up to half of the non-neoplastic cells in a GBM. These tumor-associated macrophages have been implicated in proliferation, angiogenesis, and immunosuppression in GBM and can influence the efficacy of chemo-, radio-, and immunotherapies. However, certain cancer specific interactions have been associated with either microglia or macrophages, necessitating an approach that can delineate the two populations. Multiplex immunofluorescence offers a technical advantage that allows for the profound phenotyping of cells in the tumor microenvironment as well as their spatial organization.

Objective: Design a multiplex immunofluorescence protocol to differentiate microglia and infiltrating M1/M2 macrophages in GBM in situ.

Methods:

- Panel Design and Validation of HISTOPROFILE® Neuro M1/M2 panel
  - Targets: GFAP/TMEM119/CD68/c-Maf/CD163
    - ✓ Microglia: TMEM119$^+$
    - ✓ M2: CD68$^+$/CD163$^+$/c-Maf$^+$/TMEM119$^-$
    - ✓ M1: CD68$^+$/CD163$^-$/c-Maf$^-$/TMEM119$^-$
    - ✓ Tumor: GFAP$^+$
- Human Glioblastoma FFPE Blocks were sourced from the Cerba Research Montpellier Biobank
- Sequential multiplex protocol with Opal® (Akoya Biosciences) fluorophores on the BOND RX® (Leica) slide stainer
  - Whole slide images were acquired with the VECTRA® Polaris™ (Akoya Biosciences) slide scanner
  - Image Analysis with the HALO® (Indica Labs) Highplex module
    - ✓ Total Microglia, M2 and M1 marcophages were quantified
    - ✓ Infiltration at the tumor/non-tumor interface was analyzed

HISTOPROFILE® Neuro M1/M2: PHENOTYPING OF THREE INDEPENDANT SAMPLES



HISTOPROFILE®-Neuro M1/M2 on three independent GBM samples .
A) Example images of tumor and non-tumor regions of the three samples stained with the HISTOPROFILE® Neuro M1/M2 panel: GFAP (red), TMEM119 (green), CD68 (yellow), CD163 (white), c-Maf (orange). Representative images from left to right: merged multiplex, macrophage phenotyping targets (TMEM119, CD68, CD163, c-Maf). M1 HALO® masks, M2 HALO® masks, microglia (TMEM119), microglia HALO® masks. Scale bars 100 µm. B) Graphs showing percentage of positive cells for M1 (CD68$^+$/CD163$^-$/c-Maf$^-$/TMEM119$^-$), M2 (CD68$^+$/CD163$^+$/c-Maf$^+$/TMEM119$^-$) and microglia (TMEM119$^+$) in these three samples. Differences in these three samples in non-tumor (upper graph) and tumor (lower graph) regions can be appreciated.

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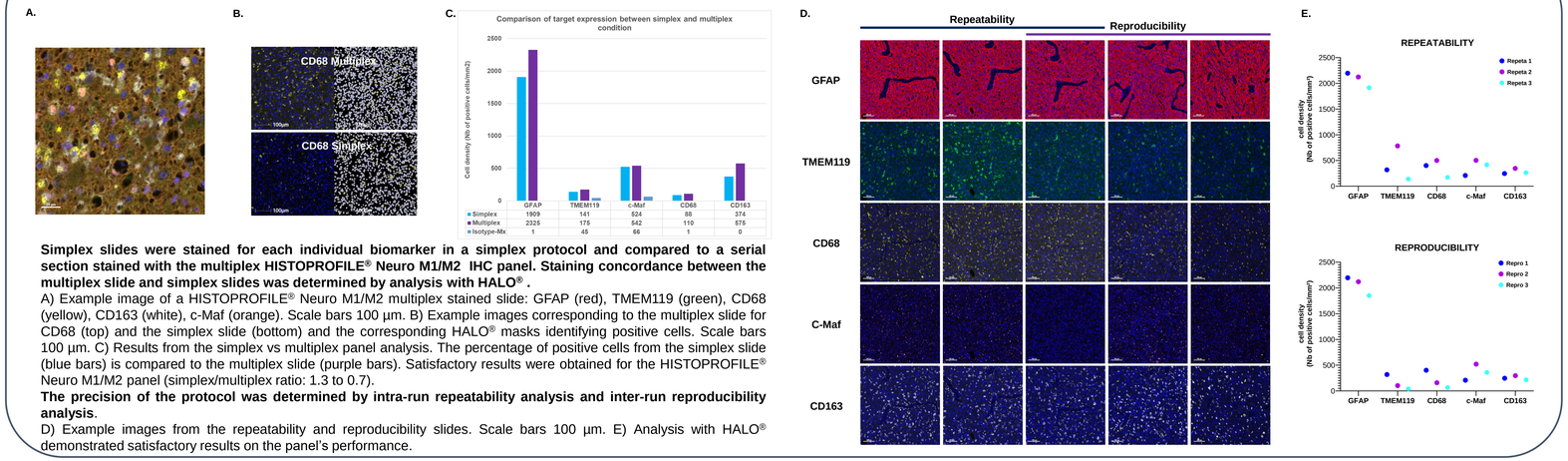
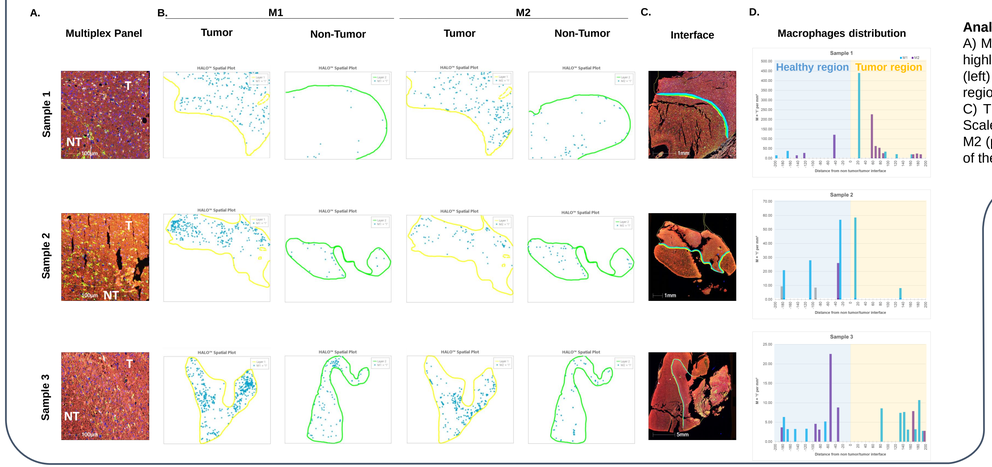
HISTOPROFILE® Neuro M1/M2: PANEL VALIDATION



Simplex slides were stained for each individual biomarker in a simplex protocol and compared to a serial section stained with the multiplex HISTOPROFILE® Neuro M1/M2 IHC panel. Staining concordance between the multiplex slide and simplex slides was determined by analysis with HALO® .
A) Example image of a HISTOPROFILE® Neuro M1/M2 multiplex stained slide: GFAP (red), TMEM119 (green), CD68 (yellow), CD163 (white), c-Maf (orange). Scale bars 100 µm. B) Example images corresponding to the multiplex slide for CD68 (top) and the simplex slide (bottom) and the corresponding HALO® masks identifying positive cells. Scale bars 100 µm. C) Results from the simplex vs multiplex panel analysis. The percentage of positive cells from the simplex slide (blue bars) is compared to the multiplex slide (purple bars). Satisfactory results were obtained for the HISTOPROFILE® Neuro M1/M2 panel (simplex/multiplex ratio: 1.3 to 0.7).
The precision of the protocol was determined by intra-run repeatability analysis and inter-run reproducibility analysis.
D) Example images from the repeatability and reproducibility slides. Scale bars 100 µm. E) Analysis with HALO® demonstrated satisfactory results on the panel's performance.

HISTOPROFILE® Neuro M1/M2: INFILTRATION ANALYSIS



Analysis of the infiltrating macrophages at the tumor/non-tumor interface.
A) Merged image at the interface. The interface of the tumor (T) and non-tumor (NT) regions is highlighted with a yellow trace. Scale bars 100 µm. B) Halo spatial plots demonstrating the M1 (left) and M2 (right) cells at the interface of the tumor (yellow region) and the non-tumor (green region). The blue circles allow for visualization of the phenotyped populations in the landscape. C) The region included in the analysis is appreciated in the highlighted line at the interface. Scale bars 100 µm. D) Results from the infiltration analysis. The density of M1 (blue bars) and M2 (purple bars) are represented at the interface of the healthy to tumor regions. The variability of the macrophage distribution at the tumor/non-tumor interface can be appreciated.

Conclusion: Human GBM tissues were investigated with a multiplex panel consisting of GFAP, TMEM119, CD68, c-Maf, and CD163, the HISTOPROFILE® Neuro M1/M2. After multi-spectral acquisition, the microglia and macrophage populations could be easily phenotyped and localized in the tissues examined. Microglia and peripherally recruited M1 and M2 macrophages were quantified. The spatial distribution of the populations within the tumor core, edge, or margin was evaluated. The approach presented here demonstrates the power of multiplex immunohistochemistry in the phenotyping and spatial analysis of resident and recruited immune cell populations on a single tissue section and the potential application of this method in clinical studies.

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