Whole Slide Imaging Modes and Curated Antibody Panels for Imaging Mass Cytometry Approach Reveal Extensive Spatial Heterogeneity of Human Glioblastomas

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Introduction

Glioblastoma (GBM) presents a complex form of brain cancer that is challenging to diagnose and treat. Gaining spatial insights into the cellular composition of GBM tissue has tremendous potential to inform clinicians and researchers about mechanisms behind spatial predictors of treatment success and disease etiology and progression.

Imaging Mass Cytometry™ (IMC™) technology is a high-plex spatial biology imaging technique that enables deep characterization of the diversity and complexity of GBM and other tumor microenvironments (TMEs). IMC systems support detailed assessment of cell phenotype and function using 40-plus metal-tagged antibodies simultaneously on a single slide without artifacts associated with fluorescence-based spectral overlap, tissue autofluorescence, multiple acquisition cycles and tissue degradation. Specifically designed for high-throughput applications and whole slide imaging (WSI) modes, the Hyperion™ XTi Imaging System with 40-slide loader permits automated and continuous imaging of more than 40 large tissue samples (400 mm^2 per tissue) per week. We showcase the application of WSI using curated antibody panels to study the complexity of the GBM TME.

Methods and materials

A 41-marker neuro-oncology IMC antibody panel (Figure II) was used to determine the cellular and structural landscape of GBM. We applied the panel on a tissue microarray (TMA) containing dozens of human GBM cores and whole GBM tumor tissue sections to spatially resolve over 40 distinct molecular markers.

We performed imaging using two features of the Hyperion XTi Imaging System (Figure IA) that provide whole slide scanning capabilities. **Preview Mode** (Figure IB, top panel) was applied to rapidly screen tumor cores for expression signatures associated with tumor immuno-oncology processes. This enabled biomarker-guided selection of areas in tumor tissue that were imaged at higher resolution and analyzed using single-cell analysis. In parallel, a high-throughput **Tissue Mode** (Figure IB, bottom panel) was applied to perform a detailed scan of the brain tumor TMA followed by pixel-clustering analysis to unravel the spatial composition of the TME.

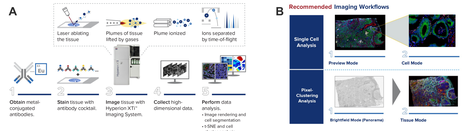

Figure I. Imaging Mass Cytometry workflows. (A) IMC technology offers a streamlined workflow that simplifies translational and clinical application of multiplexed tissue analysis. The five-step process, which consists of obtaining metal-conjugated antibodies, staining tissues with antibody cocktails, imaging tissues with the Hyperion XTi Imaging System, and the collection and analysis of high-dimensional data, can be accomplished in as little as 72 hours for the whole slide. Additionally, the slide loader can accommodate two cassettes of 20 slides each (40 slides total) to greatly increase throughput. (B) The WSI modes for IMC platforms offer a customized workflow for specific customer needs. Preview Mode offers a rapid scan of the sample and generates useful data for guiding region of interest (ROI) placement for Cell Mode acquisition for single-cell analysis application. Alternatively, Tissue Mode can be applied to generate a high-quality scan of entire tissue sections in a matter of hours with higher spot size ablations enabling entire tissue analysis using pixel-clustering methods. Both workflows offer unique advantages for specific research requirements.

Human Immuno-Oncology IMC Panel, 31 Antibodies (PN 201509)					Additional panels			
Human Stromal Cell IMC Panel, 4 Antibodies	Human Lymphoid IMC Panel, 4 Antibodies	Human Myeloid IMC Panel, 6 Antibodies	Human Cell Functional State IMC Panel, 5 Antibodies	Human Basic Immune IMC Panel, 4 Antibodies	Glioblastoma IMC Panel, 5 Antibodies	Human Neuro Expansion IMC Panel, 3 Antibodies	Maxpar™ Neuro Phenotyping IMC Panel Kit	Maxpar IMC Cell Segmentation Kit and Cell-ID™ Intercalator-Ir
PN 201511	PN 201512	PN 201513	PN 201514	PN 201518	PN 9100011	PN 9100012	PN 201337	PN 201100 PN 201192B
FAP Podoplanin αSMA CD44	CD4 CD8 CD45RO CD57	CD66b HLA-DR CD163 CD14 CD11b CD11c	Granzyme B PD-L1 PD-1 FoxP3 Ki-67	CD45 CD3 CD20 CD68	EGFR Vimentin SOX2 Nestin MMP9	Tubulin βIII Synaptophysin TMEM119	Iba1 GFAP NeuN S100β	MAP2 CD34 Olig-2	ICSK1 ICSK2 ICSK3 DNA1 DNA2

Figure II. Glioma-specific human neuro-oncology IMC panel. This 41-marker panel is designed to uncover relevant immuno-oncological processes in human gliomas. The off-the-shelf modular structure of the panel offers excellent flexibility to customize IMC panels for application on translational and clinical samples. Metal assignments were carefully designated for each marker to extract the maximum performance from each individual antibody. The panel was optimized for FFPE tissues.

Conclusions

Multimodal visualization and analysis of high-plex **Imaging Mass Cytometry** has revealed numerous **biological outputs** and provided **new perspectives** on glioblastoma's **neuronal and mesenchymal origins, clonal differentiation of cancer stem cells and indicators of pro- and anti-tumorigenic immune responses**. This enhanced understanding opens avenues for **new diagnostic and therapeutic options**.

Results

Rapid visualization of all panel markers facilitates selection of relevant regions of interest (ROI) by revealing the spatial complexity of the entire GBM

The Human Immuno-Oncology IMC Panel, Neuro Phenotyping IMC Panel Kit, Glioblastoma IMC Panel and Human Neuro Expansion IMC Panel uncover the extensive heterogeneity of GBM for subsequent selection of ROIs that are relevant for the research questions.

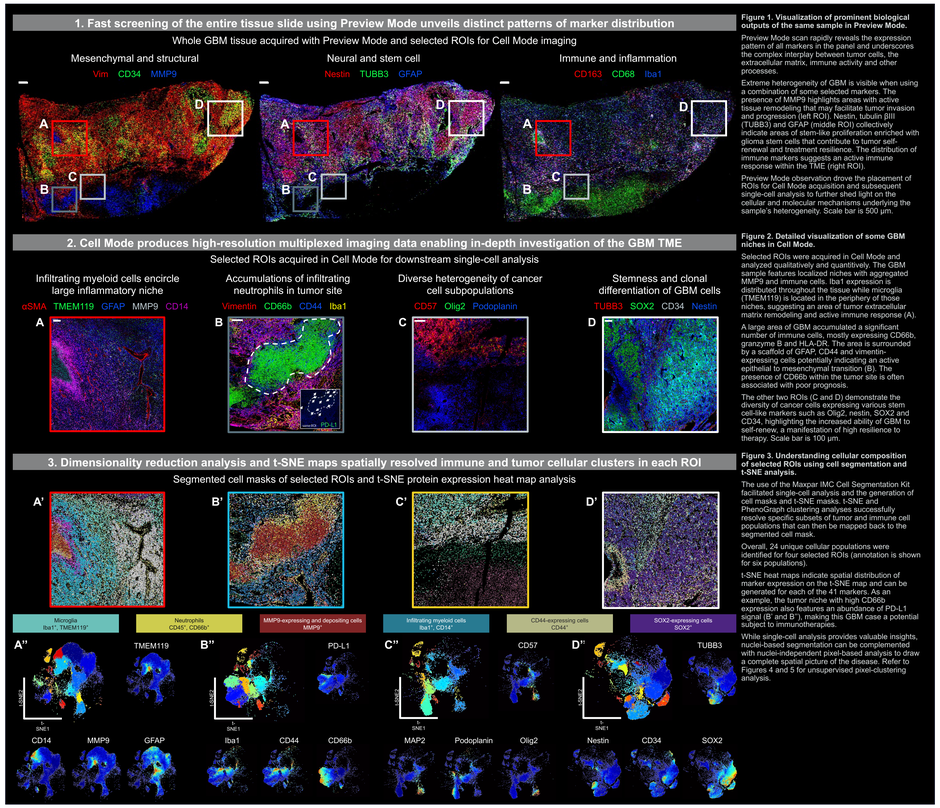

Figure 1. Visualization of prominent biological outputs of the same sample in Preview Mode.

Preview Mode scan rapidly reveals the expression pattern of all markers in the panel and underscores the complex interplay between tumor cells, the extracellular matrix, immune activity and other processes.

Extreme heterogeneity of GBM is visible when using a combination of some selected markers. The presence of MMP9 highlights areas with active tissue remodeling that may facilitate tumor invasion and progression (left ROI). Nestin, tubulin βIII (TUBB3) and GFAP (middle ROI) collectively indicate areas of stem-like proliferation enriched with glioma stem cells that contribute to tumor self-renewal and treatment resilience. The distribution of immune markers suggests an active immune response within the TME (right ROI).

Preview Mode observation drove the placement of ROIs for Cell Mode acquisition and subsequent single-cell analysis to further shed light on the cellular and molecular mechanisms underlying the sample's heterogeneity. Scale bar is 500 µm.

Figure 2. Detailed visualization of some GBM niches in Cell Mode.

Selected ROIs were acquired in Cell Mode and analyzed qualitatively and quantitively. The GBM sample features localized niches with aggregated MMP9 and immune cells. Iba1 expression is distributed throughout the tissue while microglia (TMEM119) is located in the periphery of those niches, suggesting an area of tumor extracellular matrix remodeling and active immune response (A).

A large area of GBM accumulated a significant number of immune cells, mostly expressing CD66b, granuzyme B and HLA-DR. The area is surrounded by a scaffold of GFAP, CD44 and vimentin-expressing cells potentially indicating an active epithelial to mesenchymal transition (B). The presence of CD66b within the tumor site is often associated with poor prognosis.

The other two ROIs (C and D) demonstrate the diversity of cancer cells expressing various stem cell-like markers such as Olig2, nestin, SOX2 and CD34, highlighting the increased ability of GBM to self-renew; a manifestation of high resilience to therapy. Scale bar is 100 µm.

Figure 3. Understanding cellular composition of selected ROIs using cell segmentation and t-SNE analysis.

The use of the Maxpar IMC Cell Segmentation Kit facilitated single-cell analysis and the generation of cell masks and t-SNE masks. t-SNE and PhenoGraph clustering analyses successfully resolve specific subsets of tumor and immune cell populations that can then be mapped back to the segmented cell mask.

Overall, 24 unique cellular populations were identified for four selected ROIs (annotation is shown for six populations).

t-SNE heat maps indicate spatial distribution of marker expression on the t-SNE map and can be generated for each of the 41 markers. As an example, the tumor niche with high CD66b expression also features an abundance of PD-L1 signal (B' and B''), making this GBM case a potential subject to immunotherapies.

While single-cell analysis provides valuable insights, nuclei-based segmentation can be complemented with nuclei-independent pixel-based analysis to draw a complete spatial picture of the disease. Refer to Figures 4 and 5 for unsupervised pixel-clustering analysis.

Tissue Mode highlights tissue compartments and supports subsequent pixel-clustering analysis

Tissue Mode visualizes tissue compartments and indicates high heterogeneity of human GBM. The entire sample was used for subsequent pixel-clustering analysis.

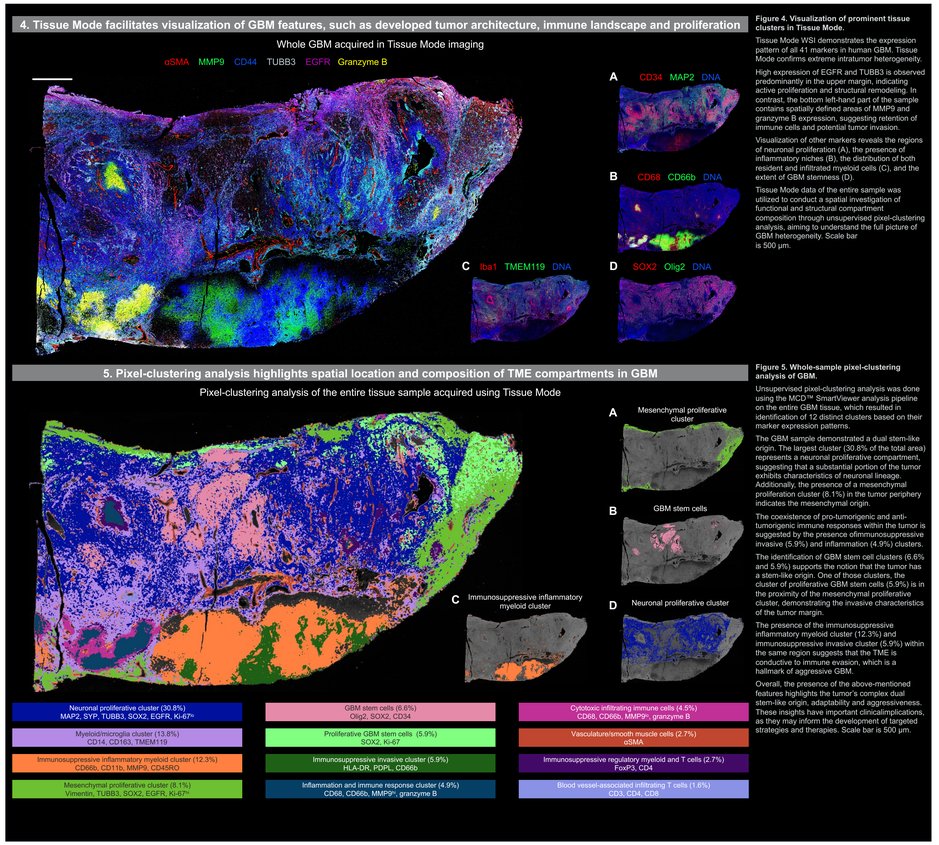

Figure 4. Visualization of prominent tissue clusters in Tissue Mode.

Tissue Mode WSI demonstrates the expression pattern of all 41 markers in human GBM. Tissue Mode confirms extreme intratumor heterogeneity.

High expression of EGFR and TUBB3 is observed predominantly in the upper margin, indicating active proliferation and structural remodeling. In contrast, the bottom left-hand part of the sample contains spatially defined areas of MMP9 and granzyme B expression, suggesting retention of immune cells and potential tumor invasion.

Visualization of other markers reveals the regions of neuronal proliferation (A), the presence of inflammatory niches (B), the distribution of both resident and infiltrated myeloid cells (C), and the extent of GBM stemness (D).

Tissue Mode data of the entire sample was utilized to conduct a spatial investigation of functional and structural compartment composition through unsupervised pixel-clustering analysis, aiming to understand the full picture of GBM heterogeneity. Scale bar is 500 µm.

Figure 5. Whole-sample pixel-clustering analysis of GBM.

Unsupervised pixel-clustering analysis was done using the MCD™ SmartViewer analysis pipeline on the entire GBM tissue, which resulted in identification of 12 distinct clusters based on their marker expression patterns.

The GBM sample demonstrated a dual stem-like origin. The largest cluster (30.8% of the total area) represents a neuronal proliferative compartment, suggesting that a substantial portion of the tumor exhibits characteristics of neuronal lineage. Additionally, the presence of a mesenchymal proliferation cluster (8.1%) in the tumor periphery indicates the mesenchymal origin.

The coexistence of pro-tumorigenic and anti-tumorigenic immune responses within the tumor is suggested by the presence ofimmunosuppressive invasive (5.9%) and inflammation (4.9%) clusters.

The identification of GBM stem cell clusters (6.6% and 5.9%) supports the notion that the tumor has a stem-like origin. One of those clusters, the cluster of proliferative GBM stem cells (5.9%) is in the proximity of the mesenchymal proliferative cluster, demonstrating the invasive characteristics of the tumor margin.

The presence of the immunosuppressive inflammatory myeloid cluster (12.3%) and immunosuppressive invasive cluster (5.9%) within the same region suggests that the TME is conductive to immune evasion, which is a hallmark of aggressive GBM.

Overall, the presence of the above-mentioned features highlights the tumor's complex dual stem-like origin, adaptability and aggressiveness. These insights have important clinicalimplications, as they may inform the development of targeted strategies and therapies. Scale bar is 500 µm.

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