Tumor-Infiltrating Myeloid Cells – Using MultiOmyx™ to Distinguish between MDSCs, TAMs and TANs in the Pancreatic Tumor Microenvironment

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Background

Myeloid cells are the primary recruited effector cells during inflammation. A subset of these, consisting primarily of tumor-associated M1/M2 macrophages (TAMs), tumor-associated neutrophils (TANs), and myeloid-derived suppressor cells (MDSCs), accumulate in tumors where they establish an inflammatory tumor microenvironment (TME) that is favorable for tumor progression. Targeting tumor-infiltrating myeloid cells (TIMs) to either eliminate them or to convert them from their immune-suppressive to an immune-stimulatory state has emerged as a new strategy complementing current cancer immunotherapy strategies (Engblom et al. Nat Rev Cancer, 2016 Jul;16(7)). However, a major impediment to understanding the complexity of the distinct functions of subsets of TIMs and their spatial distribution within the TME is the phenotypic characterization of TIMs in FFPE tissues by standard immunohistochemistry, as most IHC studies do not utilize a panel of antibodies broad enough to characterize multiple myeloid cell subsets in the same sample.

MultiOmyx, a novel hyperplexed multi "omic" technology, enables visualization and characterization of multiple biomarkers across multiple assays on a single 4µm tissue section. MultiOmyx protein immunofluorescence (IF) assays utilize a pair of directly conjugated Cyanine dye-labeled (Cy3, Cy5) antibodies per round of staining. Each round of staining is imaged and followed by novel dye inactivation chemistry, enabling repeated rounds of staining and deactivation for up to 60 protein biomarkers. In this study, MultiOmyx hyperplexed IF assay was utilized to measure CD11b, CD14, CD15, CD16, CD33, CD68, CD163, HLA-DR, Arginase1, and PanCK protein expression from a single 4 µm FFPE section in order to identify different subsets of TIMs in tumor tissue from patients with pancreatic ductal adenocarcinoma (PDAC), one of the most aggressive forms of cancer with a 5-year survival rate below 5% and no current specific therapies. An increasing number of studies show accumulation of immune suppressor cells such as MDSCs and TAMs in PDAC patients. Hopefully, a greater understanding of the phenotypes and functions of subsets of these cell types, will result in new cancer immunotherapy strategies for PDAC.

Overview of Assay Workflow

Figure 1. Assay Workflow. For MultiOmyx IF study, slides were prepared and stained using MultiOmyx multiplexing IF staining protocol. For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by imaging acquisition of stained slides. The dye was erased, enabling a second round of staining with another pair of fluorescent antibodies.

TIM Panel
CD11b
CD14
CD15
CD16
CD33
CD68
CD163
HLA-DR
Arginase1
PanCK

Round #	Cy3	Cy5
1	CD15	CD163
2	PanCK	CD16
3	CD14	Arginase1
4	CD11b	CD33
5	CD68	
6	HLA-DR	

Table 1. Antibody Staining Sequence for MultiOmyx multiplexing staining.

Nomenclature	Tumor Tissue Phenotype
Monocytes	CD33+CD11b+CD14+CD16+HLA-DR+CD68+
M1 TAMs	CD68+CD33+CD11b+CD14+HLA-DR+CD163-
M2 TAMs	CD68+CD33+CD11b+CD14+HLA-DR+CD163+Arginase1
M-MDSC	CD33+CD11b+CD14+Arginase1+CD16+CD15-HLA-DR^low/-
G-MDSC (PMN)	CD33+CD11b+CD15+Arginase1+HLA-DR^low/-
TANs (PMN)	CD33+CD11b+CD15+CD16+Arginase1+HLA-DR^low/+/-CD14-

Table 2. Phenotyping of human tumor-associated myeloid cells. Cell surface markers associated with some of the main myeloid cell subsets found in the tumor tissue. While there is a large variability in the expression of myeloid markers in different tumor types, this table summarize some of the general markers identified in IHC and flow cytometry studies. TAM: tumor-associated macrophage; MDSC: myeloid-derived suppressor cell; M-: monocytic; G-: granulocytic; PMN: polymorphonuclear; TAN: tumor-associated neutrophil.

Conclusion

In this study, MultiOmyx technology, a platforms offered by NeoGenomics Laboratories, was utilized to analyze protein expression in 10 pancreatic ductal adenocarcinomas in an attempt to distinguish between different subtypes of myeloid cells present in the tumor micro-environment.

• Utilizing a panel of 10 antibody markers, we confirmed the presence of two major populations of myeloid cells; CD68+HLA-DR+ cells (TAMs expressing HLA-DR) and CD33+CD11b+HLA-DR- cells (MDSCs and TANs)

• We were able to distinguish between M1 subtypes of TAMs (CD68+CD163-) and M2 subtypes of TAMS (CD68+CD163+), the majority of which are M2

• We were able to differentiate between populations of HLA-DR-CD11b+CD14+ (e.g. M-MDSCs), and HLA-DR-CD11b+CD14-CD15+ (e.g. granulocytic MDSCs/TANs) cells, the majority of which are represented by cells with a granulocytic phenotype

General Markers for Myeloid Cells

Figure 2. Immuno-profiling of 2 FFPE PDAC tumors using MultiOmyx multiplexed IF assay. Representative color overlaid images of PanCK, CD11b, CD33, CD68, and HLA-DR in pancreatic tumors. A, CD11b+CD33+HLA-DR- cells (e.g. MDSCs or TANs) are magenta (indicated by magenta arrows) while CD11b-CD33-HLA-DR+ cells are green (indicated by green arrows). The white arrow points to an HLA-DR expressing cell co-expressing both CD11b and CD33. B, Green cells express the pan Mφ-marker CD68, while CD11b+CD33+CD68- cells (e.g. MDSCs or TANs) are magenta (indicated by magenta arrows). White arrows in 2B points to a Mφ co-expressing CD11b and CD33. Yellow arrow points to a Mφ co-expressing CD11b but negative for CD33. C, CD68+HLA-DR+CD11b- cells are magenta (indicated by magenta arrows), while the yellow arrow indicates a CD68+HLA-DR-CD11b+ cell.

M1-M2 Type Tumor-Associated Macrophages

Figure 3. Immuno-profiling of 2 FFPE PDAC tumors using MultiOmyx multiplexed IF assay. Representative color overlaid images of PanCK, CD68, HLA-DR, CD163, CD14, CD16, and arginase1. **A,** M1 type TAMs (CD68+HLA-DR+CD163-) are magenta (indicated by magenta arrow), while M2 type TAMs CD68+CD163+HLA-DR+ and CD68+CD163+HLA-DR- are white and yellow respectively (indicated by white and yellow arrows). **B,** CD68+CD16+CD14- (CD14-Mφ) cells are magenta (indicated by magenta arrow), and CD68+CD16+CD14+ cells are white (indicated by white arrow), while CD68+CD14+CD16- cells are detected as red cells with a green membrane (indicated by a red arrow with green outline). **C,** A small subset of CD68+ macrophages that co-express M2 marker arginase1 are seen in yellow (indicated by yellow arrow).

Myeloid-Derived Suppressor Cells and Tumor-Associated Neutrophils

Figure 4. Immuno-profiling of 4 FFPE PDAC tumors with MultiOmyx. Representative color overlaid images of PanCK, CD68, CD11b, CD33, arginase1, CD14, and CD15. **A,** CD68+ Mφ are purple (indicated by purple arrow), while CD68-CD11b+Arg1+ cells (e.g. MDSCs, and TANs) are yellow (indicated by yellow arrows). **B,** CD11b+CD33+Arg1- cells (e.g. monocytes, TAMs and M-MDSCs) are magenta (indicated by a magenta arrow), while CD11b+CD33+Arg1+ cells (e.g. MDSCs, and TANs) are white. **C,** CD11b+Arg1+CD14- cells (e.g. G-MDSCs and TANs) are yellow. **D,** CD11b+Arg1+CD15+ cells (e.g. G-MDSCs and TANs) are white (indicated by white arrows).