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Interrogation of STING induced cytokines in the tumor microenvironment using an Integrated MultiOmyx-RNAscope panel for spatial and quantitative profiling

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Abstract#94

The STING (Stimulator of Interferon Genes) pathway is a pivotal player in the innate immune response, responsible for detecting cytosolic accumulation of endogenous DNA and initiating a cascade of events leading to the production of pro-inflammatory type I interferons, which promote an antitumor environment through activation of T cells, dendritic cells, and natural killer cells. Therefore, STING agonists are currently being explored as a potential therapeutic for cancer treatment, often in combination with immune checkpoint inhibitors. While the STING pathway can be stimulated in multiple cell types, activation in antigen presenting cells, such as dendritic cells (DC), is crucial for antitumor activity in the tumor microenvironment (TME). Additionally, the type 1 interferon family consists of multiple subtypes, including IFNb and IFNa, which can produce differential biological responses. Although the STING pathway is known to induce expression of multiple cytokines, combined spatial characterization of STING and type I interferons in the TME has not been performed.

To characterize STING-interferon expression in the TME, we used the Integrated MultiOmyx-RNAscope platform. MultiOmyx™ (NeoGenomics Laboratories, Inc) is a proprietary multiplex immunofluorescence (mIF) platform for the visualization and characterization of up to 60 protein biomarkers in a single formalin-fixed paraffin-embedded (FFPE) section and offers high-resolution spatial and quantitative analysis of protein expression in tissue samples. RNAscope™ (Bio-Techne) Multiplex is a highly sensitive fluorescent in-situ hybridization (ISH) assay that can detect up to 3 RNA markers in a single FFPE section. The Integrated MultiOmyx-RNAscope assay allowed for simultaneous detection of both protein and RNA markers in a single sample. Herein we reported the design and use of a novel panel of commercially-available antibodies and ISH probes broad enough to characterize various immune subpopulations and cytokine expressing cells, including DCs and interferons, in the TME of various melanoma samples. Quantification and analysis were performed using NeoLYTX™, the proprietary MultiOmyx Analytics pipeline, to examine the spatial distribution and expression levels of key STING pathway induced cytokines to elucidate the dynamic communication of signaling molecules and their localization within specific cell populations. Understanding of the variety and phenotype of STING/cytokine expressing cells in the TME was crucial to define the populations being targeted by therapies for cancer treatment.

Integrated MultiOmyx-RNAscope Assay Workflow

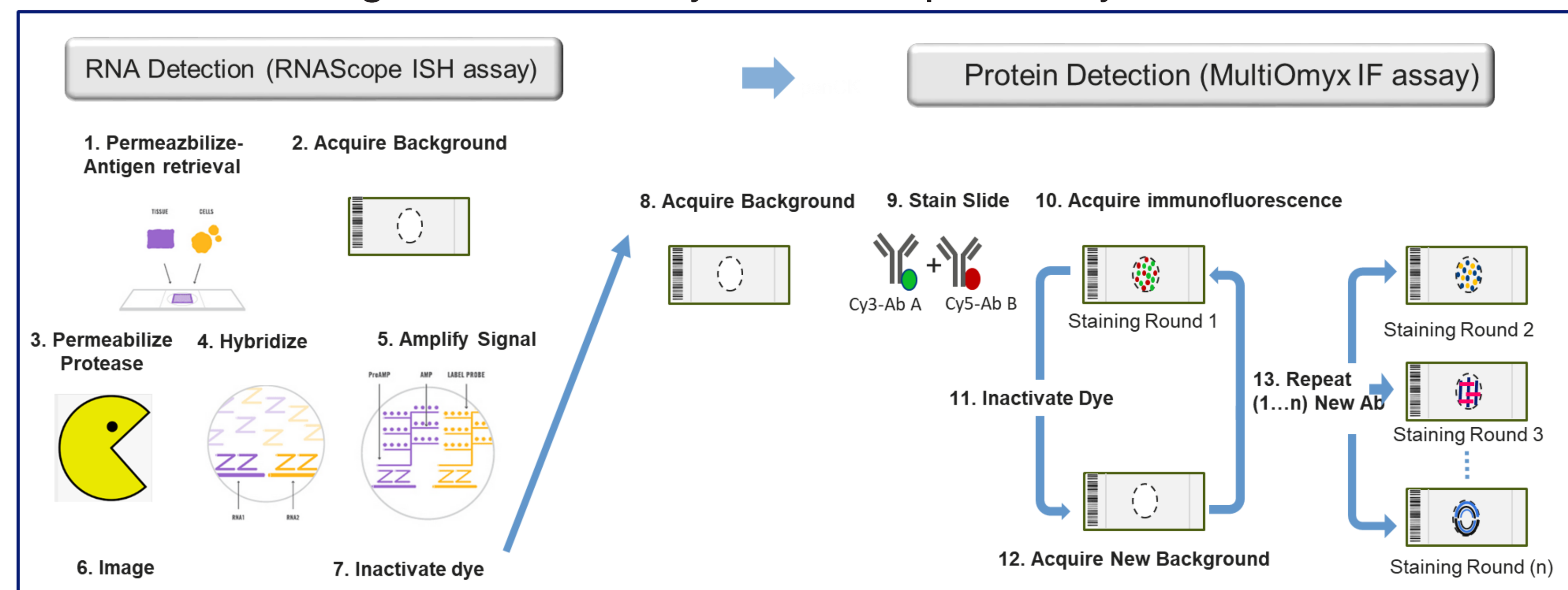

Figure 1. Integrated MultiOmyx-RNAscope Assay Workflow. The integrated workflow combines RNAscope ISH and MultiOmyx multiplexing IF staining protocols. Slides were first cleared and undergo pretreatment, which includes both heat activated epitope retrieval and protease steps. Then RNAscope ISH probe hybridization and staining was performed before proceeding to IF multiplexing. For each round of IF 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. Heatmap shows normalized marker abundance across the samples in this study.

cGAS-STING Panel Biomarkers	
IFNB1 ISH	IFNA2 ISH
CD3	CD4
CD8	CD11c
CD14	CD20
CD40	CD68
cGAS	Clec9A
DC-LAMP	DC-SIGN
HLA-DR	NKp46
PD-1	PD-L1
SOX-10	STING

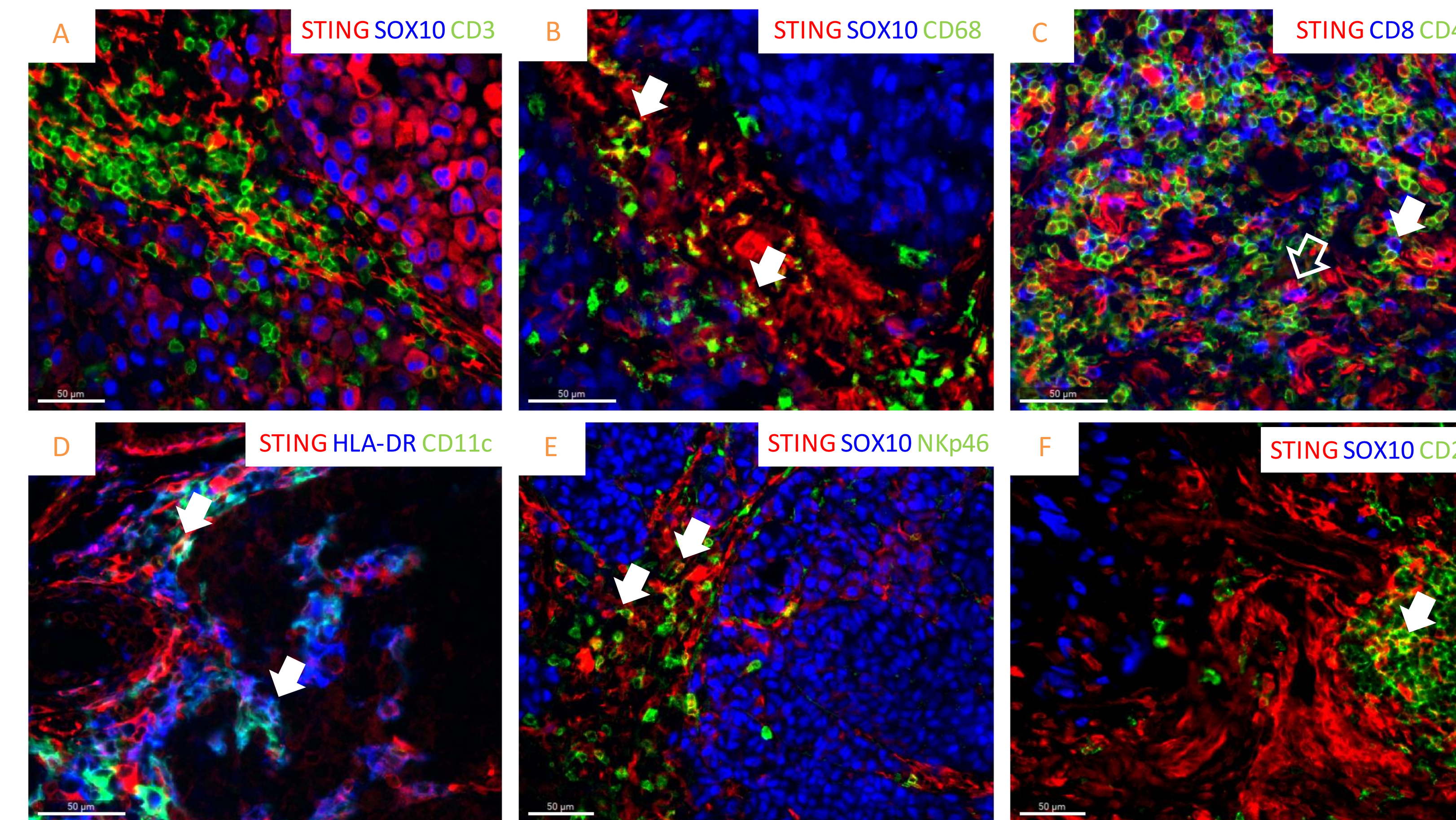

Figure 2. Characterization of STING expression in different cell type subsets in melanoma. Representative color overlay images showing expression of STING in melanoma samples. (A) Tumors expressing STING have a blue nucleus and red cytoplasm (STING+ SOX10+). (B) Arrows indicate examples STING+ macrophages (STING+ CD68+). (C) T cytotoxic cells expressing STING are shown in magenta (outlined arrow, STING+ CD8+) and T helper cells expressing STING are shown in yellow (white arrow, STING+ CD4+). (D) Examples of DC cells expressing STING are shown with white arrows (STING+ HLA-DR+ CD11c+). (E) NK cells expressing STING are shown in yellow with white arrows (STING+ NKp46+). (F) B cells expressing STING are shown in yellow with white arrow (STING+ CD20+). Scale bars = 50µm.

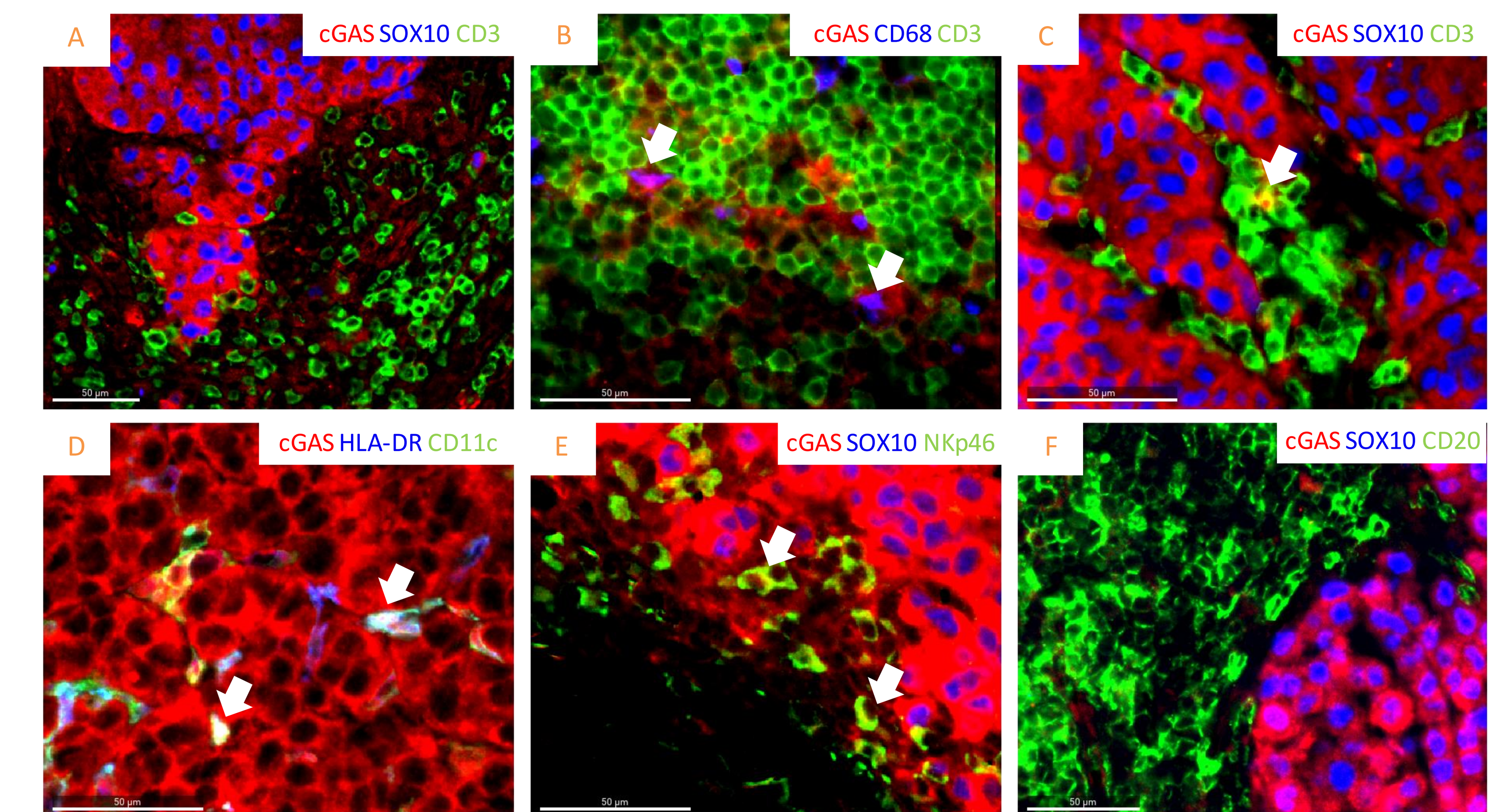

Figure 3. Characterization of cGAS expression in different cell type subsets in melanoma. Representative color overlay images showing expression of cGAS (red) in melanoma samples (A-F). (A, C, E, F) Expression of cGAS in tumor appears as red cytoplasmic stain with blue nuclear stain (cGAS+ SOX10+). (B) White arrows show examples of cGAS+ macrophages (cGAS+ CD68+). (C) Yellow stain shows example of a cGAS+ T cell (cGAS+ CD3+). (D) White arrows show examples of cGAS+ dendritic cells (cGAS+HLA-DR+CD11c+). (E) White arrows show examples of cGAS+ NK cells (cGAS+ NKp46+). (F) B cells (CD20+) are predominantly negative for cGAS. Scale bars = 50µm.

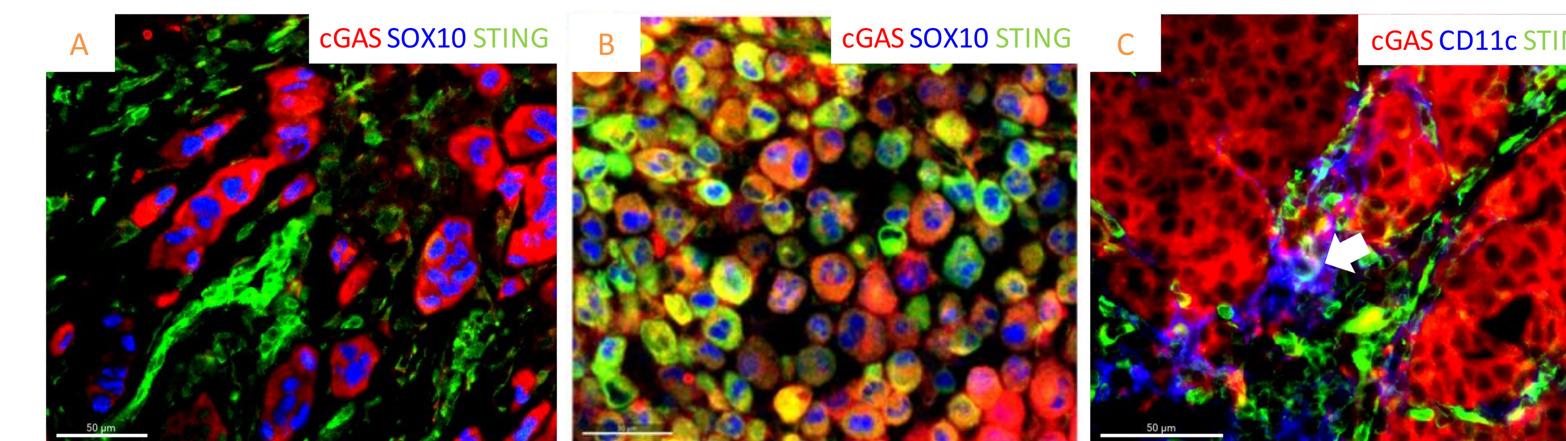

Figure 4. Expression patterns of cGAS and STING. (A-C) Representative color overlay images of cGAS (red) and STING (green) in melanoma samples. (A) STING and cGAS expression predominately observed in discrete populations. (B) Tumor cells expressing both cGAS and STING (blue nucleus and yellow cytoplasm). (C) DC expressing both cGAS and STING (white arrow). Scale bars = 50µm.

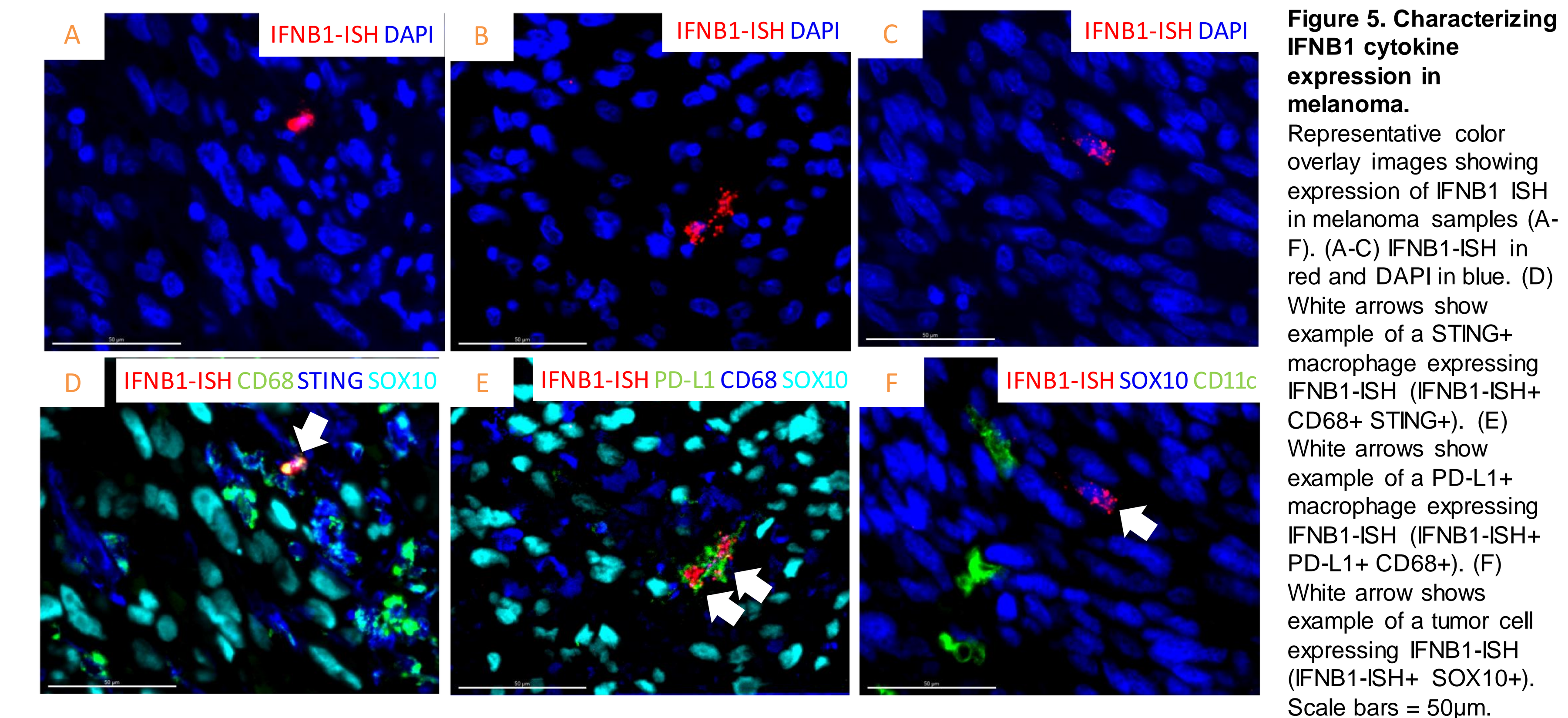

Figure 5. Characterizing IFNB1 cytokine expression in melanoma. Representative color overlay images showing expression of IFNB1 ISH in melanoma samples (A-F). (A-C) IFNB1-ISH in red and DAPI in blue. (D) White arrows show example of a STING+ macrophage expressing IFNB1-ISH (IFNB1-ISH+ CD68+ STING+). (E) White arrows show example of a PD-L1+ macrophage expressing IFNB1-ISH (IFNB1-ISH+ PD-L1+ CD68+). (F) White arrow shows example of a tumor cell expressing IFNB1-ISH (IFNB1-ISH+ SOX10+). Scale bars = 50µm.

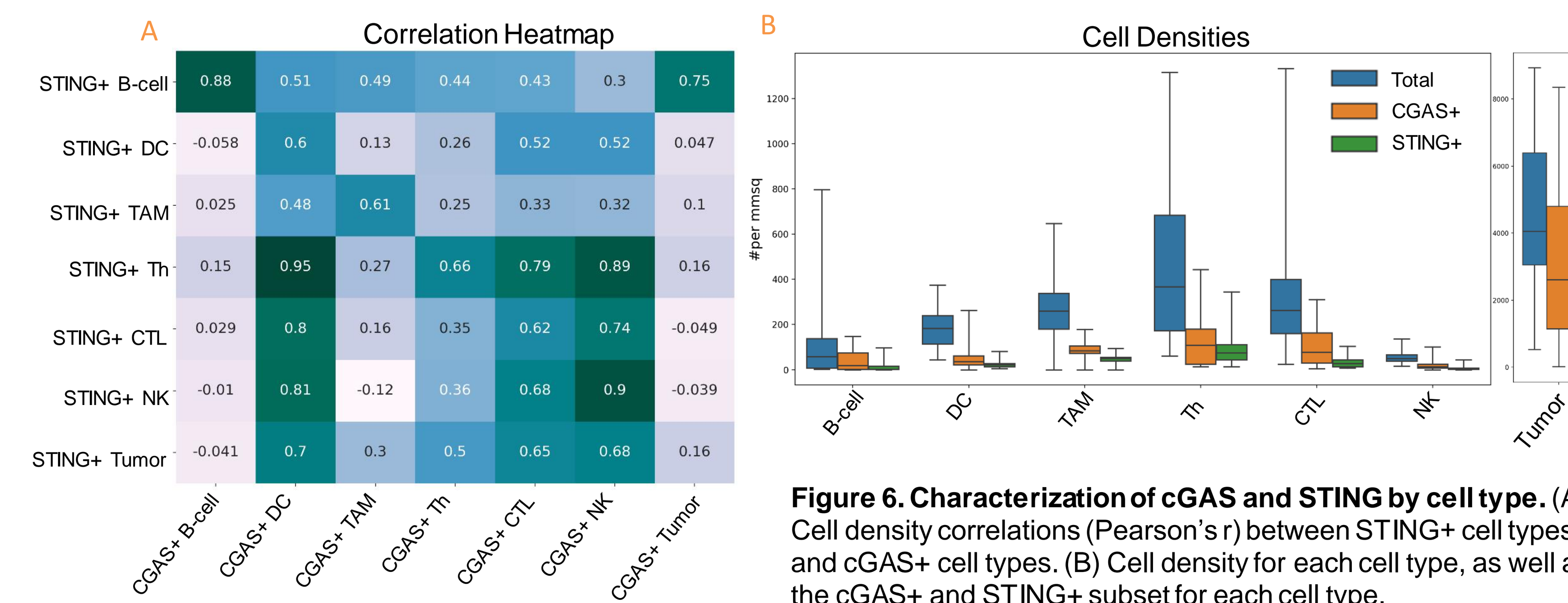

Figure 6. Characterization of cGAS and STING by cell type. (A) Cell density correlations (Pearson's r) between STING+ cell types and cGAS+ cell types. (B) Cell density for each cell type, as well as the cGAS+ and STING+ subset for each cell type.

Summary

- The Integrated MultiOmyx-RNAscope assay was used to characterize cGAS and STING expressing cells in melanoma samples.
- cGAS is primarily expressed in tumor while STING is primarily expressed in immune cells, including dendritic cells, macrophages, and T cells.
- IFNb1-ISH had low expression in all melanoma samples used in this study. IFNa-ISH positive cells were not detected. The Integrated MultiOmyx-RNAscope workflow is a sensitive tool to co-detect and characterize rare cytokine populations at single cell level.
- cGAS+ dendritic cell abundance is highly correlated with STING expression in Tumor cells as well as immune cells including T cells and NK cells. cGAS expression in Tumor has been observed to be correlated with STING expression in B-cells in this study.

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