MultiOmyx™: A multiplexed immunofluorescent assay capable of profiling protein expression and phosphorylation, in combination with next-generation sequencing from a single FFPE tissue section

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Background

A comprehensive signaling pathway profile in combination with mutational analysis may be a critical guide for selecting effective clinical strategies for targeted drugs in combinations or in sequential regimens. In the United States, colorectal cancer (CRC) is the third most common cancer and the third leading cause of tumor associated death in men and women. CRC is a heterogeneous disease defined by different receptor tyrosine kinase (RTK) activation, signaling through phosphatidylinositol 3-kinase (PI3K)/AKT and RAS/MAP2K pathways. In addition, activating mutations in RTK and/or activating or loss-of-function mutations in downstream intracellular signaling proteins, can alter the efficacy of targeted drugs resulting in an ineffective treatment.

GE Healthcare, through its affiliate Clarient Diagnostic Services Inc., has developed a novel hyperplexed multi-omic technology, MultiOmyx, to enable 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. The same protein IF processed slide is then used to perform DNA FISH assay, followed by DNA extraction using laser capture microdissection (LCM) from region(s) of interest for next generation sequencing.

Herein, we report an analysis of the key receptor tyrosine kinases (EGFR, HER2, HER3, and cMET) and their downstream signaling proteins (PI3K, phospho AKT, and phospho ERK1/2) in 10 colon tumor samples using the MultiOmyx technology. Mutational analysis was then performed on tumor and tumor adjacent regions, and sequenced using the Ion AmpliSeq™ cancer panel, consisting of 50 targeted genes.

Overview of MultiOmyx Technology Workflow

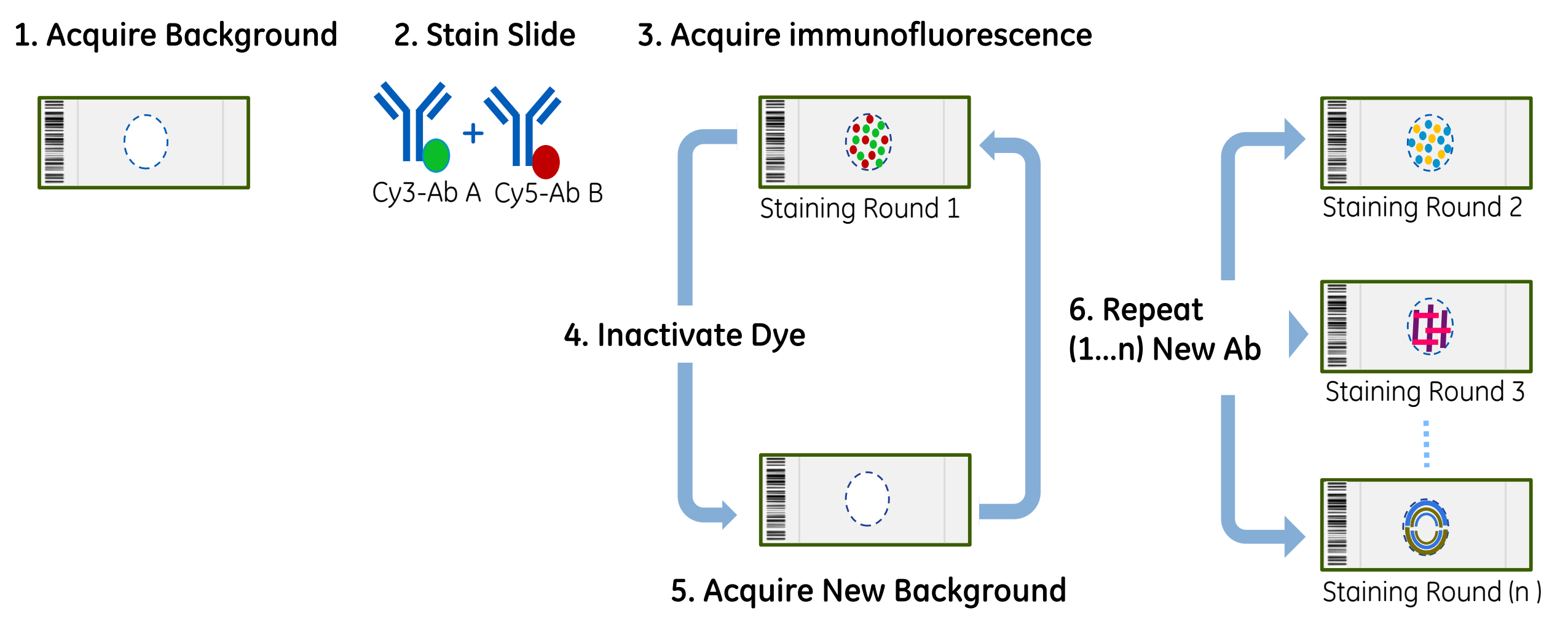


Figure 1. MultiOmyx IF multiplexing scheme from a single tissue section. Conjugated fluorescent antibodies were applied to a slide, followed by whole slide imaging. The dye was chemically inactivated, enabling a second round of staining with another fluorescent antibody. The process was performed multiple times from a **single slide.**

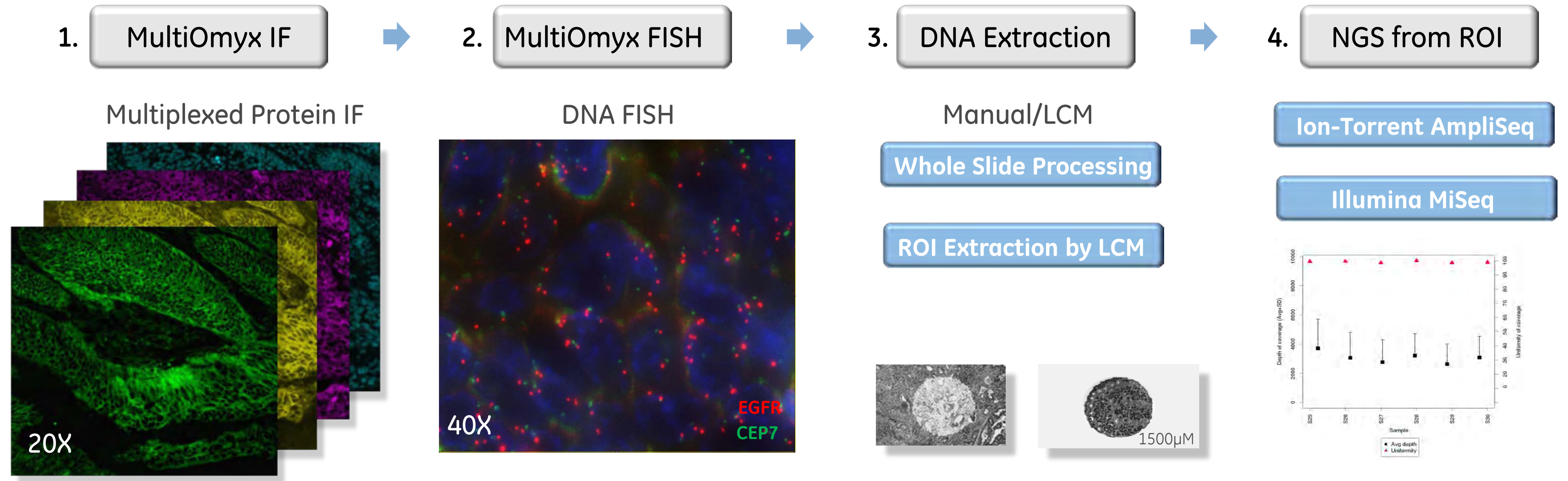


Figure 2. MultiOmyx multi "Omic" scheme for protein IF, DNA FISH, and NGS from ROI from a single tissue section.

Comparison of MultiOmyx Multiplexed IF to IHC

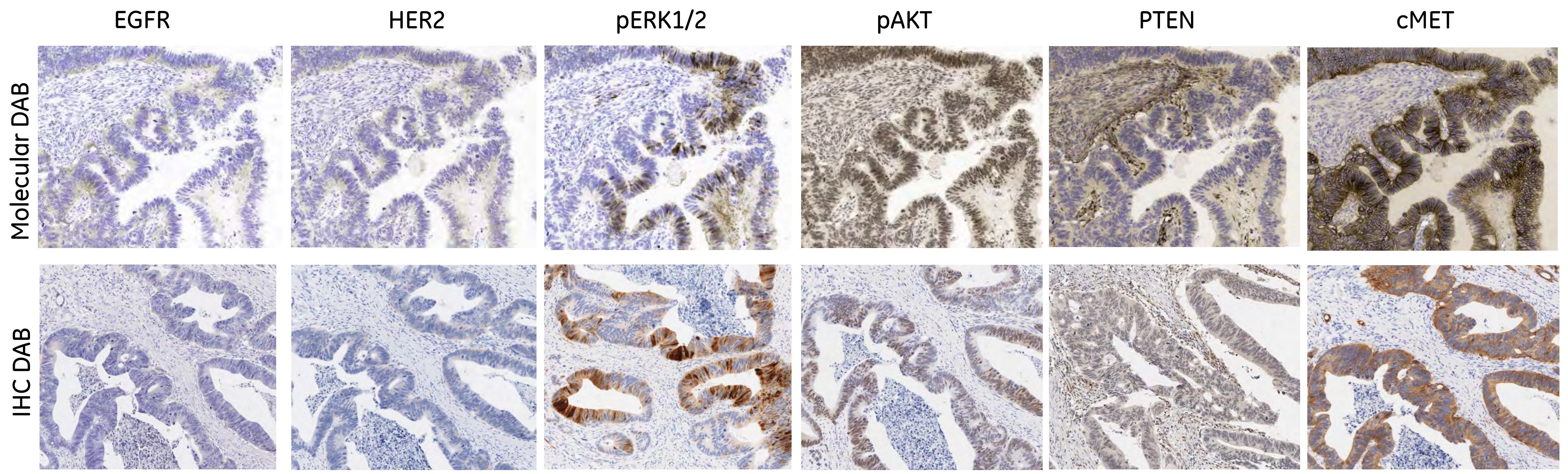


Figure 3. Comparison of MultiOmyx Multiplexed IF to IHC. The top row illustrates "Molecular" DAB or mDAB, which were generated from the IF images (data not shown), and demonstrates equivalent staining to the standard IHC DAB images in CRC tissues.

Analysis of RTK with Multiplexed IF and FISH

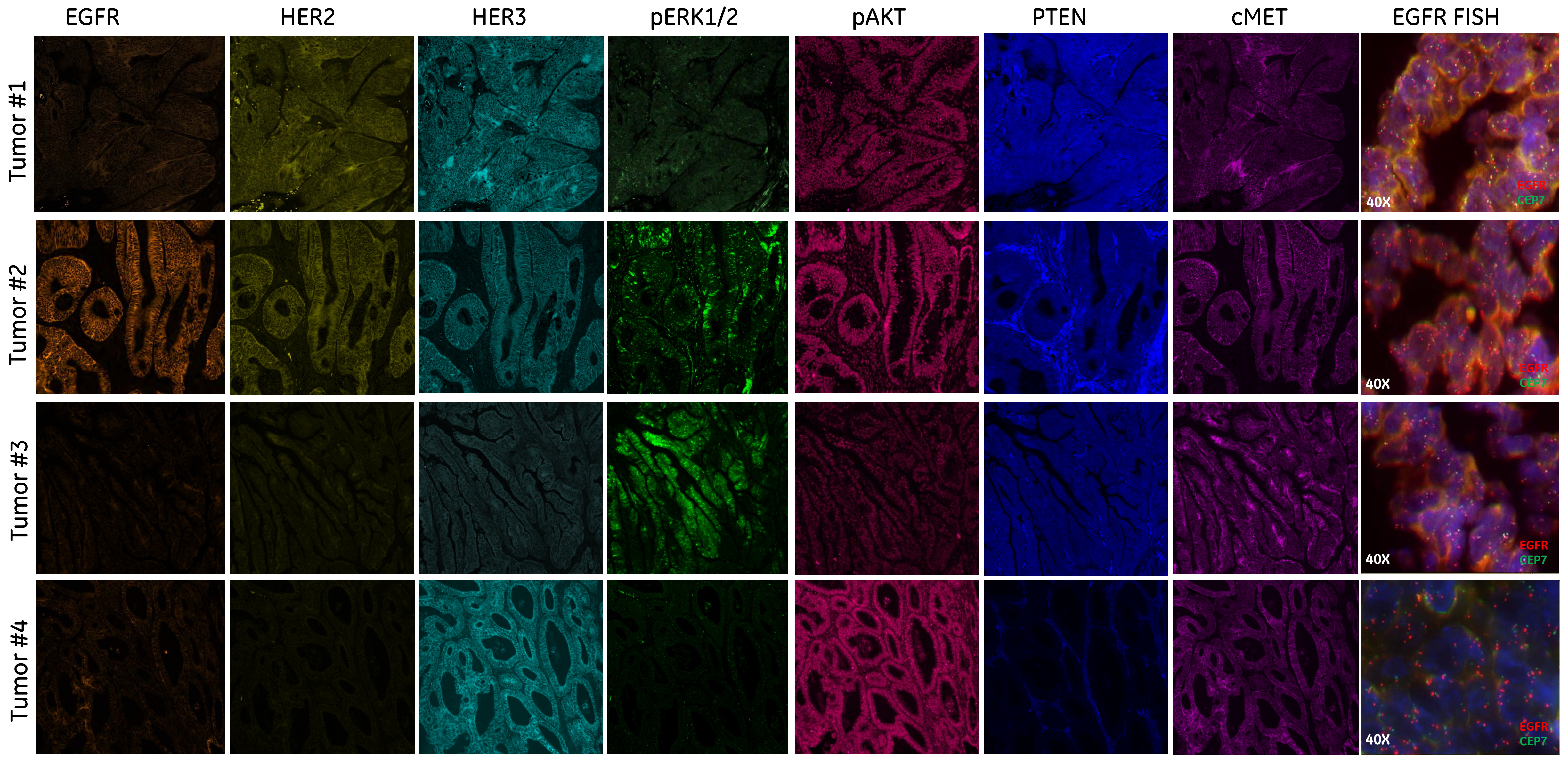


Figure 4. Characterizations of RTKs in CRC by MultiOmyx Multiplexed IF and FISH. The key RTKs (EGFR, HER2, HER3 and cMET) and downstream signaling proteins (PI3K, PTEN, pAKT and pERK1/2) were characterized in CRC tumors by MultiOmyx Multiplexed IF staining (20X). The EGFR FISH was performed after the completion of multiplexed IF staining on the same slide. Four distinctive patterns of biomarker expression are shown, indicating activation of different signaling pathway. MultiOmyx multiplexed profiling can categorize CRC into different subtypes.

Integrated Analysis

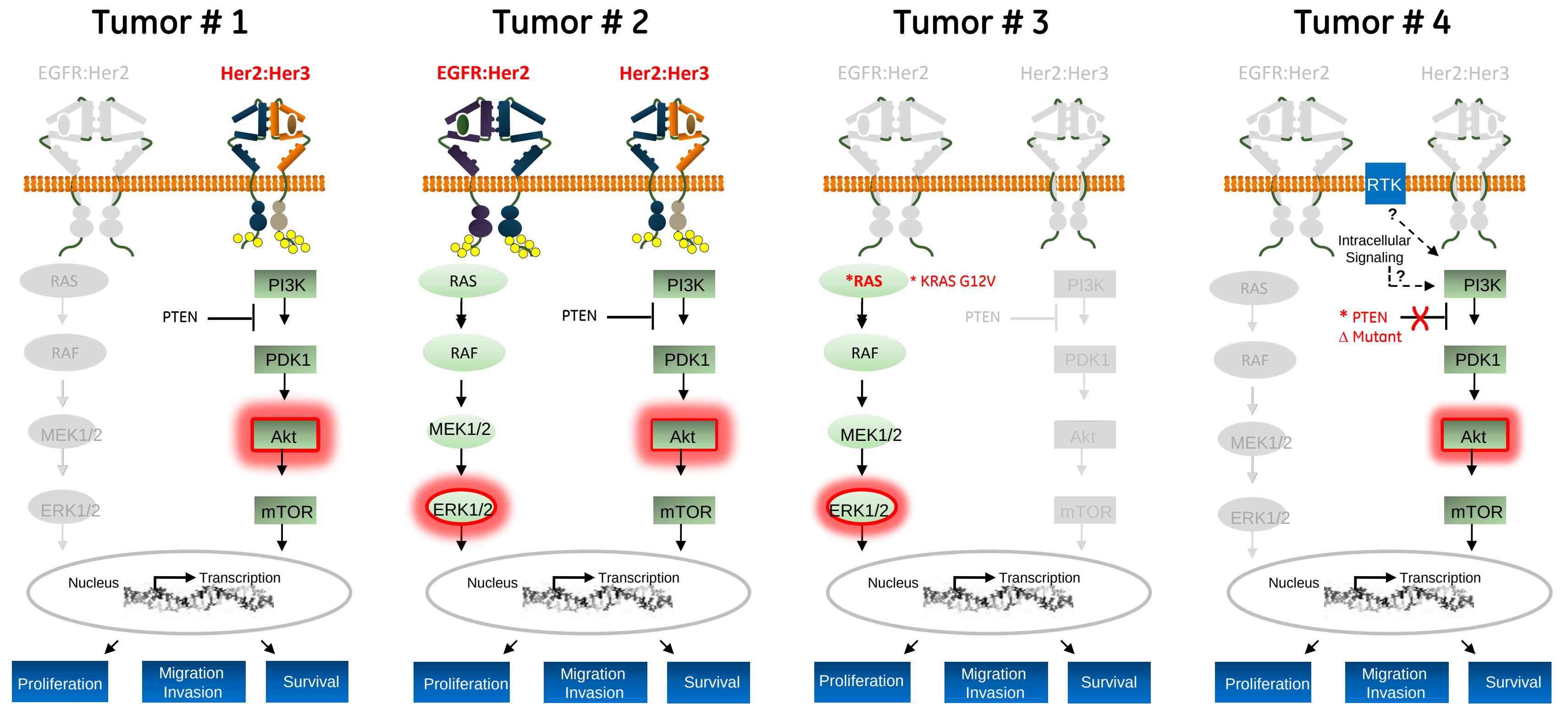
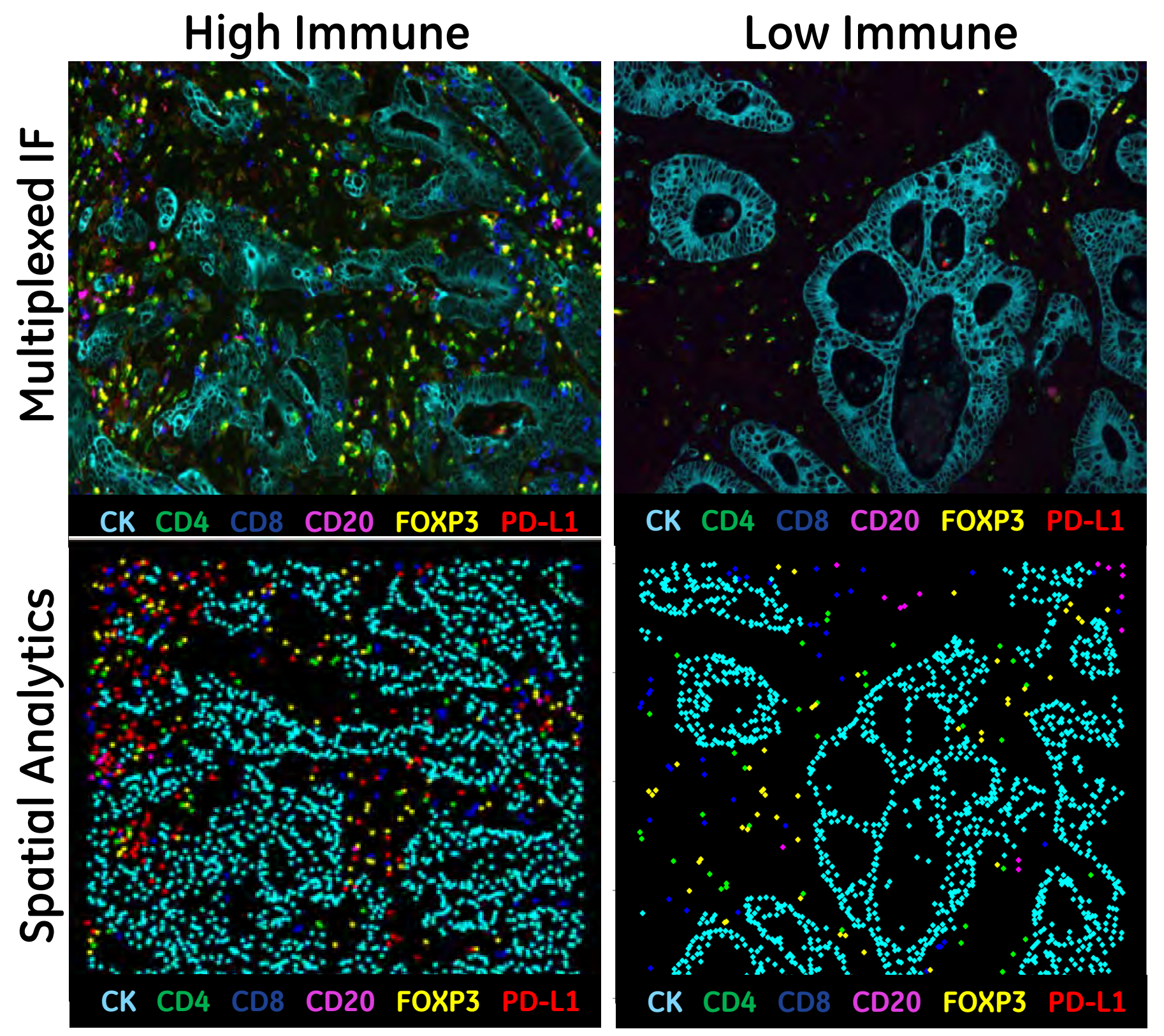


Figure 5. Heterogeneous protein expression/activation and mutational profiling. In tumor 1, strong HER3 expression correlated with activation of pAKT, possibly through HER2:HER3 dimer. In tumor 2, expression of both EGFR and HER3 in presence of HER2 correlated with positive staining for phospho ERK1/2 and phospho AKT, possibly through EGFR:HER2 and HER2:HER3 dimers, respectively. In tumor 3, mutational profiling revealed KRAS, G12V (c.35G>T), known to activate the RAS-MAP2K pathway and activation of pERK1/2. In tumor 4, loss of PTEN protein which acts as a tumor suppressor to dephosphorylate PIP_3 to PIP_2 correlated with pAKT activation, possibly through upstream RTK(s) or intracellular signaling protein(s) not profiled in this study. Normal EGFR copy number were detected across all four tumor samples shown.



Biomarker	High Immune			Low Immune		
	Cell Counts	Density in Tumor (#/mm²)	Density in Stroma (#/mm²)	Cell Counts	Density in Tumor (#/mm²)	Density in Stroma (#/mm²)
Tumor Cells	1692	NA	NA	1386	NA	NA
CD4+	231	238.1	675.4	64	23.3	177.4
CD8+	299	492.3	547.0	47	11.7	134.6
CD20+	14	2.7	61.8	13	7.8	33.6
FOXP3+	177	160.5	556.5	53	7.8	156.0
PDL1+	234	246.1	675.4	0	0	0

Figure 6. Characterization of Immune Cell Infiltration in CRC Tissue Sections. Two tumor samples exhibit different levels of infiltrating lymphocytes. Immunostaining of CD4, CD8, CD20, FOXP3 and PDL1 were quantified by MultiOmyx analytics metric. The densities of the infiltrating immune cells in the tumor region and surrounding stroma were also calculated and recorded as the number of positive cells per unit of tissue area.

Conclusion

MultiOmyx is capable of identifying various CRC tumor subtypes through combined analysis of both IF protein expression/activation and mutational profiling from NGS. The data demonstrates concordance between upstream RTK expression and pathway activation with mutations identified in the AmpliSeq panel. Additionally, Immunophenotyping revealed differences in lymphocytes infiltration and levels of immune infiltration has been shown in literature to correlate with clinical outcome. A comprehensive pathway signaling and immune profiling, combined with mutational analysis, may be necessary to select patients who may benefit from a single or combinational targeted therapy tailored to their individual tumor profile.

April 2015 JB29605US