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Dual approach using unbiased proteomics and multiplexed immunofluorescence for the detection of markers predictive for immunotherapy in melanoma patients

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Background: Despite the clinical advances in recent years of immune checkpoint inhibitor (ICI) therapy no durable responses are observed in 40-60% of melanoma patients and therefore, a growing focus of immuno-oncology (IO) research is focused on identifying novel biomarkers that are predictive for ICI treatment response. Due to the complexity of the interactions between cancer cells and the immune system, the identification of predictive biomarkers for patient response requires a combination of tools and efforts. Therefore, we have designed a multi-modality approach for the protein analysis of tumor tissue samples from late-stage melanoma patients treated with ICIs, consisting of an unbiased deep proteomic analysis followed by a multiplexed immunofluorescence (mIF) spatial tissue analysis.

Methods: FFPE tumour samples provided by the University of Zürich were firstly used for unbiased quantification of proteins using data-independent acquisition (DIA) LC-MS technology and Biognosys' Spectronaut software. Baseline patient samples were classified as responders (n=9) or non-responders (n=15) based on the response at 3 months post ICI-treatment. Subsequently, the same patient samples were analyzed by MultiOmyx™, a proprietary and high-plex immunofluorescence (IF) multiplexing assay. A custom panel of 18 markers was generated in order to verify key markers identified by proteomics analysis in a spatial context. After multiplexing images were analysed by applying the proprietary deep-learning based cell classification platform NeoLYTX.

Results: Unbiased proteomics analysis of 24 FFPE samples from metastatic melanoma patients treated with ICI therapy stratified into non-responders and responders led to the identification of 76 differentially regulated proteins. MultiOmyx mIF analysis using a custom 18-marker panel produced a strong separation of non-responders and responders with significantly more T cells, M1 TAMs, and APCs in the responder group.

Conclusions: In this study we demonstrate the power of a dual proteomic and mIF profiling for a comprehensive characterization of melanoma patients and the discovery and detection of markers predictive for response to ICI-therapy

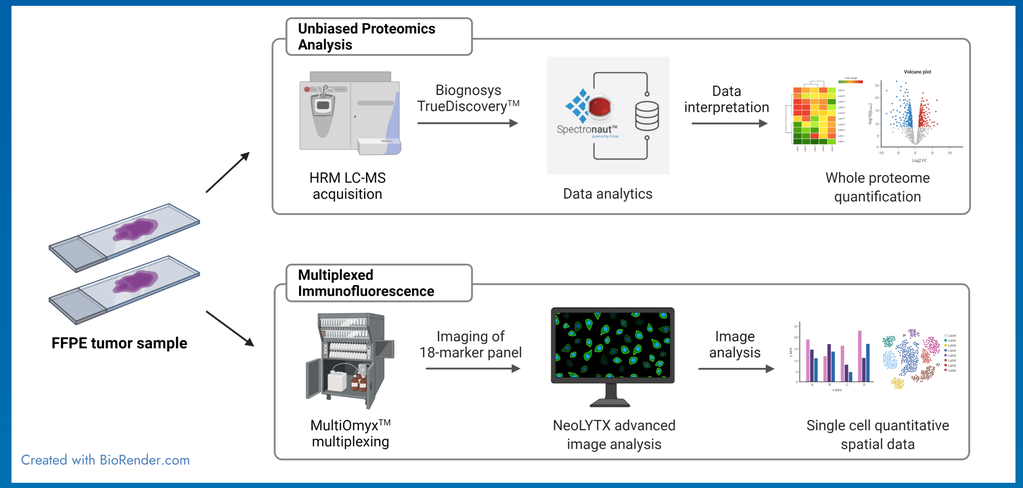
MultiOmyx™ panel and co-expressions

Round	Cy3	Cy5
1	CD31	PAK4
2	SOX10	LAG3
3	PD-1	Tbet
4	CD3	PD-L1
5	CD4	CTLA-4
6	CD8	FoxP3
7	CD68	CD11b
8	GrzB	TIM3
9	HLA-DR	CD163

Co-expression	Phenotypes	Co-expression	Phenotypes
CD3+CD4+	T helper	CD3+CD8+CTLA4+	Exhausted T cytotoxic
CD3+CD4+FoxP3+	T regulatory	CD3+CD8+LAG3+	Exhausted T cytotoxic
CD3+CD4+Tbet+	Effector Th1	CD3+CD8+GrzB+	Effector T cytotoxic
CD3+CD4+PD1+	Exhausted T helper	CD3+CD8+Tbet+	Effector T cytotoxic
CD3+CD4+CTLA4+	Exhausted T helper	CD11b+HLADR-	MDSC
CD3+CD4+LAG3+	Exhausted T helper	CD11b+HLADR+	APC myeloid
CD3+CD4+TIM3+	Exhausted T helper	CD68+HLADR+	M1 TAM
CD3+CD8+	T cytotoxic	CD68+CD163+	M2 TAM
CD3+CD8+PD1+	Exhausted T cytotoxic		

A. For MultiOmyx multiplexing two conjugated fluorescent antibodies are applied per round, followed by image acquisition of the stained slides. The dye is erased, enabling a subsequent round of staining with another pair of fluorescent antibodies. Table 1) 18-marker protein panel composition. Table 2) Phenotyping of human immune cells. GrzB: granzyme B, MDSC: myeloid derived depressor cell, APC: antigen-presenting cell, TAM: tumor-associated macrophage.

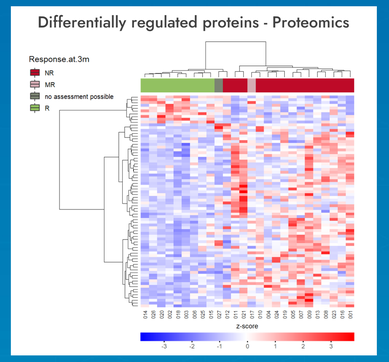
Demonstrating a multi-modality approach for a comprehensive protein analysis of tumors from ICI-treated melanoma patients



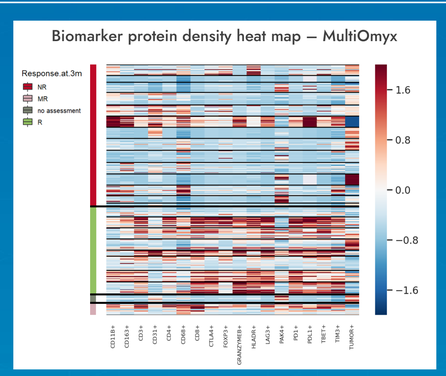
Key Take-aways

- NeoGenomics & Biognosys have designed a joint multi-modality approach for a complete protein analysis of 28 human melanoma FFPE tumor samples provided by the University of Zürich.
- This approach consists of an unbiased, deep proteomic analysis performed by Biognosys, followed by a multiplexed immunofluorescence spatial tissue analysis (MultiOmyx™) by NeoGenomics.
- Unbiased proteomics analysis resulted in the identification of 76 proteins that were changed significantly between responder and non-responder groups.
- MultiOmyx mIF single-cell quantitative analysis using a custom 18-marker immune panel resulted in the identification of 11 proteins and multiple immune phenotypes differentially regulated in responders. Further spatial analysis of these immune cells are now underway.

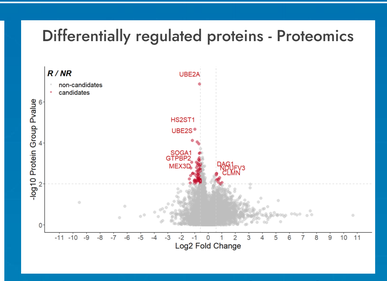
Analysis and multiplex images of samples from metastatic melanoma patients treated with ICI therapy



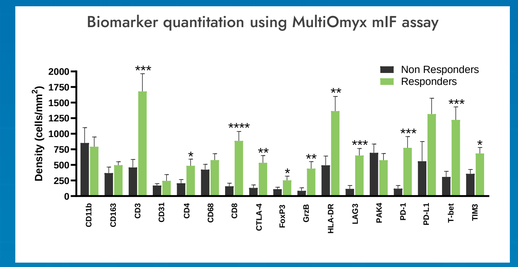
B. Hierarchical clustering analysis was performed by Biognosys using Manhattan distance measure of protein z-scores of the 76 protein candidates across all samples. Clustered data is displayed as heat map, and sample dendogram (top) displays strong separation according to Response at 3 months.



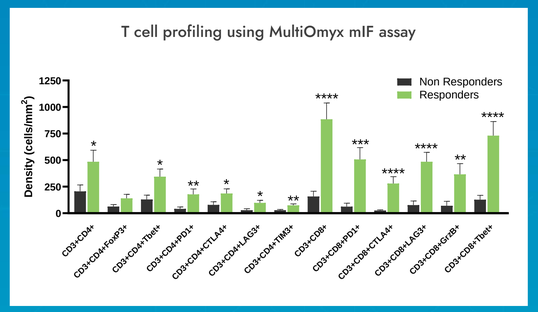
C. Heatmap showing density of the 18 biomarkers analyzed by MultiOmyx. For this heatmap density of positive cells were normalized to the scale indicated on the right of the graph. Each row is a region of interest (ROI) of a corresponding tumor sample. There is a strong separation according to Response at 3 months.



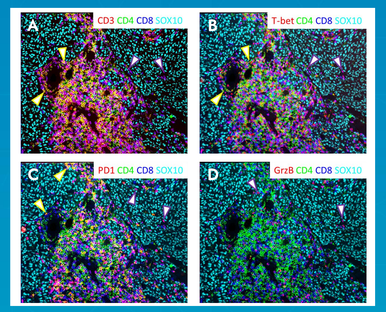
D. Volcano plot showing quantification of the 76 proteins identified as differentially regulated between responders and non-responder, with red dots showing protein candidates.
Differentially regulated proteins were identified using the following criteria: p-value < 0.01. Average fold change > 1.5.



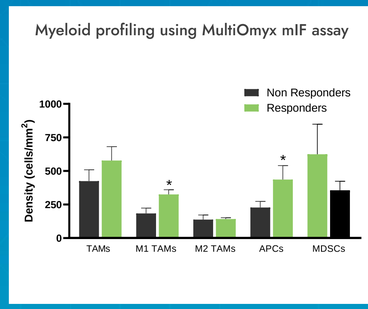
E. Quantification results of the density of the 18 biomarkers in the MultiOmyx panel were generated by applying the proprietary deep-learning based cell classification platform NeoLYTX to MultiOmyx multiplexed IF images.
The density of CD3, CD4, CD8, CTLA-4, FoxP3, GrzB, HLA-DR, LAG3, PD-1, T-bet, and TIM3 were all found to be significantly increased in responding patients. Error bars show SEM, and significance was calculated by a two-tailed, unpaired t-test.



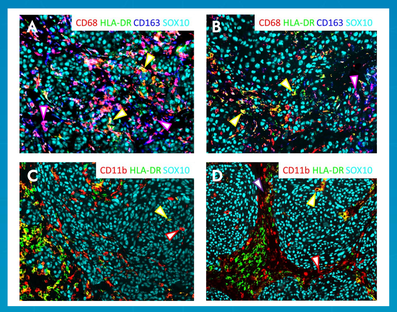
F. Quantification and classification results of T cells were generated by applying the proprietary deep-learning based cell classification platform NeoLYTX to MultiOmyx multiplexed IF images.



G. Representative color overlay images of a tumor from a responding patient. Yellow arrows indicate examples of T helper cells (**A**), Th1 cells (**B**), or PD-1 (**C**), while purple arrows indicate examples of cytotoxic T cells (**A**), cytotoxic effector cells (**B**), PD-1 (**C**), or granzyme B (**D**).



H. Quantification and classification results of myeloid cells were generated as in figures E+F. Density of M1 TAMs and APCs were found to be significantly increased in responding patients. Error bars show SEM, and significance was calculated by a two-tailed, unpaired t-test.



I. Representative color overlay images of a tumor from a responding patient. In **A+B** yellow cells are M1 TAMs and magenta cells are M2 TAMs. In **C+D** yellow cells are APCs and red cells are MDSCs.

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