**Highly Multiplexed Analysis of the Tumor Microenvironment in Hodgkin Lymphomas by Imaging Mass Cytometry**

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**Introduction**

- In classic Hodgkin lymphoma (CHL) and nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), composition of the tumor microenvironment (TME) varies significantly among and within histologic subtypes and affects disease prognosis.
- Characterization of the TME is currently hindered by the phenotypic complexity of T cell subsets, macrophages and other myeloid subtypes, and stromal vascular components.
- Assessment of the spatial relationships of tumor cells and immune cell subsets is difficult using conventional techniques that either require tissue disruption (flow cytometry, gene expression profiling) or limit analysis to one to few markers per tissue section (immunohistochemistry, immunofluorescence).
- Using metal-labeled antibodies on FFPE tissue sections, the Fluidigm HypoCyte imaging mass cytometry (IMC) combines a mass cytometer with a laser ablation system, enabling simultaneous immunomapping of all markers on a single slide with subcellular resolution (1 μm).
- The complex multidimensional dataset generated by IMC can be analyzed by a variety of data analysis tools to segment and classify individual cells, cluster similar phenotypic subtypes and determine their spatial relationships.
- In this study, we demonstrate the feasibility of this approach to analyzing the TME of Hodgkin lymphomas.

**Antibody Panel**

- **BCL2**
- **BCL3**
- **CCL3 (CXCL1)**
- **CD206 (M2 macrophage)**
- **CD3 (Th cells)**
- **CD4 (helper T cells)**
- **CD68**
- **CD8 (cytotoxic T cells)**
- **CCL4 (M1 macrophage)**
- **CCL21 (CCL25)**
- **CCL5 (RANTES)**
- **CD31 (endothelial cells)**
- **CD45RA**
- **CD16 (NK cells)**
- **CD69**
- **PD1**
- **PD-L1**
- **Tbet**
- **ICOS**
- **CD505R**
- **pERK1/2**
- **pStat3 [Y705]**
- **EphrinB2**
- **Granzyme B**
- **Tim3**
- **Lag3**

**ICM Workflow**

1) Design panels using Antibodies conjugated to metal tags
2) Stain FFPE tissue sections using Immunohistochemistry
3) Laser Ablate/Image 1μm tissue sections using the HypoCyte Imaging System (Fluidigm). The data generated by the instrument is converted into 1 megapixel images.
4) Image Segmentation for Single Cell data
   - CellProfiler is utilized to preprocess the images for hands-free (live) cell identification. Using the Fluidigm framework, a supervised random forest based learning algorithm allows for dictating the pixels into different classes e.g. nucleus, cytoplasm/membranous and background.
   - The images along with the mask are analyzed with histocAT, developed for IMC data analysis. In addition, the features of each cell can be imported into the R environment for data visualization, statistical analysis, and clustering of cell phenotypes.
5) Data Analysis
   - Comparison of manually gated RS cells compared to backgating of Phenograph cluster 17 (PC17) on the source image. PC17 gating identifies higher numbers of RS cells in the image.

**Summary**

- IMC allows for successful multiplex imaging of the TME in FFPE tissues.
- Images can be resolved at the single cell level with simultaneous measurement of multiple membrane/cyttoplasmic and nuclear markers (including cell signaling and functional states), which can then be clustered into relevant phenotypic subtypes and re-analyzed in their spatial context on the original tissue section images.
- By identifying distinct immune subsets and mapping their interactions, this technology may potentially allow TME-based tumor subclassification and identification of novel biomarkers.
- Additional studies on larger sample sizes of CHL and NLPHL are ongoing, including "neighborhood" analyses to comprehensively define cell-cell interactions in the TME.

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**References**


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