Qualitative and quantitative evaluation of the tissue micro-environment by high-resolution 17-plex immunofluorescence reveals distinct cell populations

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Abstract

Background. Inflammatory tumor micro-environments contain cells of various types and sub-types. The composition and spatial location of the cell populations reflects the host reaction to the inflammatory stimulus and increasingly is understood to influence responsiveness to tumor immunotherapies. Multiplexed imaging technologies allow identification of cell types and states within the spatial context of tissue architecture. We present here a prototype workflow that combines rapid high-resolution, whole-slide highly multiplexed immunofluorescence imaging with advanced image analysis tools for 1) segmenting tissues, cells, and quantifying cellular phenotypes based on multiple markers and 2) determining regional densities and proximity between cells. We apply the workflow to comparative assessment of three lymphoid tissues: tonsil (follicular hyperplasia); lymph node (quiescence); lymphoma (architectural effacement).

Methods. Formalin-fixed, paraffin-embedded 5 micron sections of tonsil, lymph node and chronic lymphocytic leukemia /small lymphocytic lymphoma were deparaffinized, subjected to alkaline pH epitope retrieval, and then manually stained with a 17-plex immunofluorescence panel including CD45 (leukocytes); CD20 (B cells); CD3d, CD4, CD8 (T cells); FOXP3 (T reg cells); CD68, CD163 (macrophages); CD45RO (activated cells); PD-L1, PD-1 (checkpoint markers); CD31 (vascular and lymphatic endothelial cells); cytokeratin, E-cadherin (epithelial cells); PCNA, Ki-67 (proliferating cells); and a nuclear dye. Stained slides were coverslipped and imaged on the Orion Instrument (RareCyte) to generate open microscope environment tagged image files (.ome.tiff). The image analysis workflow was performed by HALO® and HALO AI (Indica Labs). A HALO AI-based Tissue Classifier was developed for each sample, and a HALO AI nuclear segmentation algorithm was developed that performed across all three samples. Both algorithms were embedded into the HighPlex FL module of HALO for a cell-based phenotypic characterization reported by tissue class. User defined thresholds were applied to each of the biomarkers to define positivity for the appropriate subcellular localization (nuclear, cytoplasmic, and/or membrane) for phenotypic analysis.

Results. Regional masks that were defined by predominance of B-cells (CD20) or T-cells (CD3d) matched known lymphoid micro-anatomy of follicles and inter-follicular cortex respectively. Within the regions, populations and sub-populations of B-cells, T-cells, macrophages and vessels were measured, and their densities calculated and compared between tissues. Rare cell types of potential importance in immuno-oncology were investigated. The results demonstrate differences between the tissues at a phenotypic level that correspond to the morphologic differences seen by light microscopy.

Conclusions. Orion imaging combined with HALO image analysis provides a powerful and intuitive workflow for visualization and quantification of distinct microenvironment populations for use in translational and clinical research.

17-plex Panel	
1	PCNA
2	Nucleus
3	CD31
4	CD45
5	CD68
6	CD4
7	FOXP3
8	CD45RO
9	CD8a
10	CD20
11	PD-L1
12	CD3d
13	CD163
14	E-cadherin
15	PD-1
16	Ki67
17	cytokeratin

Multiplexed imaging workflow

Figure 1. Workflow steps. FFPE slide sections were stained with the complete 17-plex panel and imaged on the Orion instrument in a single scan. Images were qualitatively analyzed using Artemis visualization tools and quantitatively assessed using HALO AI.

Resolving spectral overlap

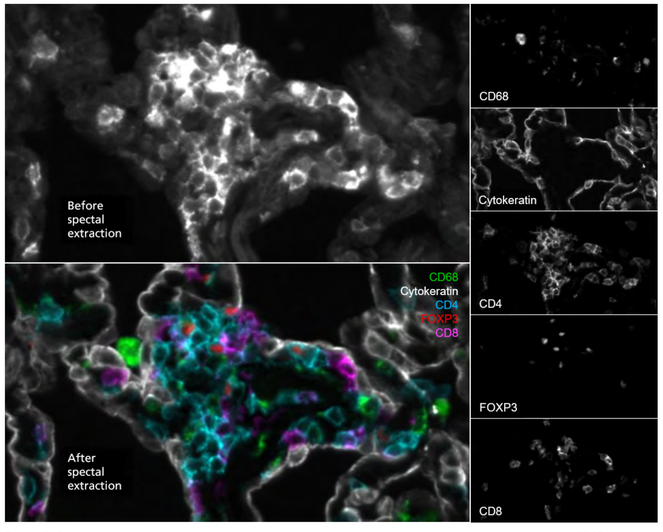
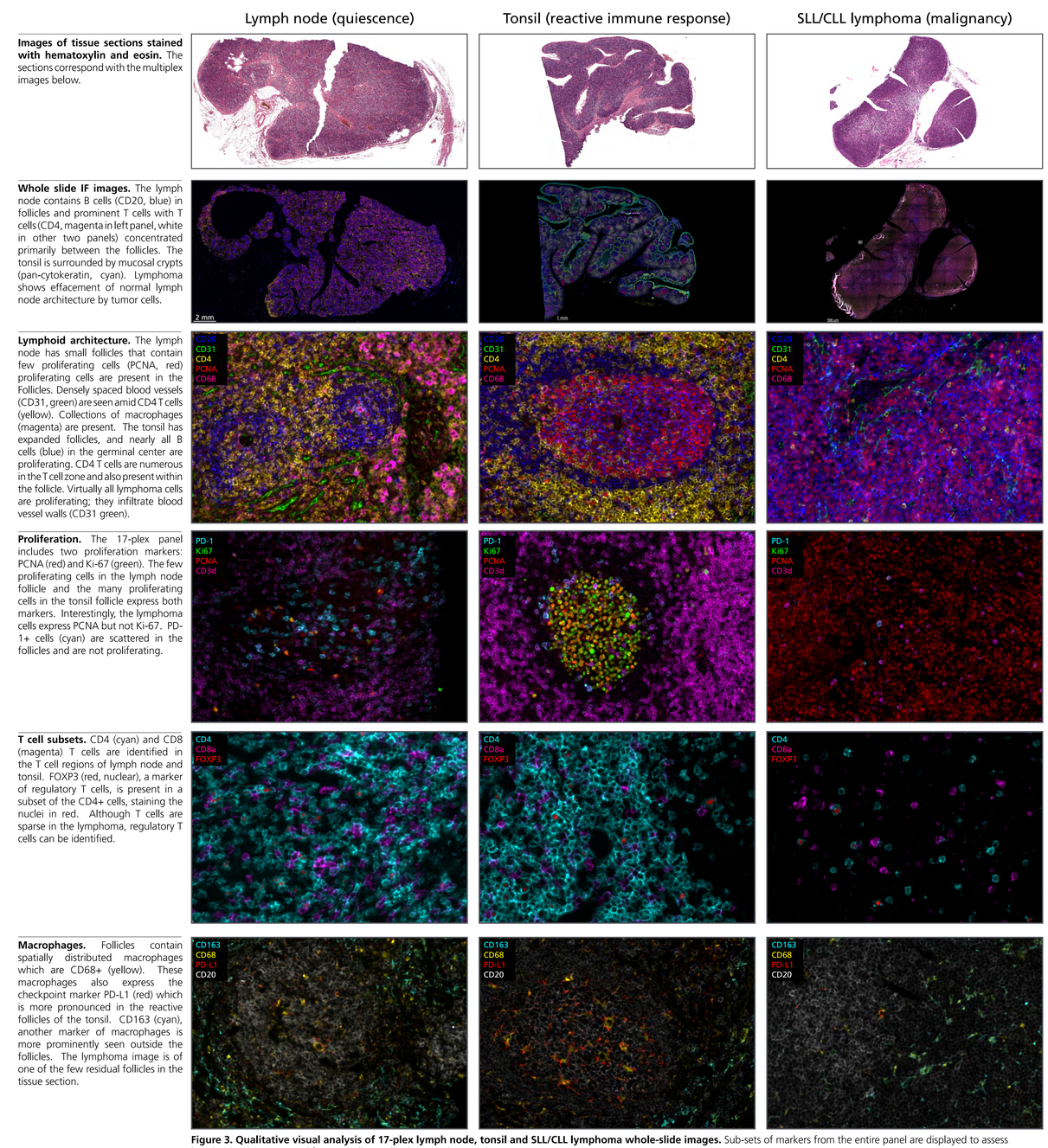


Figure 2. Spectral resolution of overlapping fluorescence signals. Lung section with emission signals from CD68, cytokeratin, CD4, FOXP3, and CD8 antibodies conjugated to fluorescent dyes that overlap within the orange region of the spectrum. Orion resolves the overlapping fluor signals into separate channels for visualization of the individual biomarkers.

Whole slides of three different lymphoid conditions stained with the 17-plex panel and imaged using the Orion system. Immunofluorescence images presented are displays of sub-sets of markers from the 17-plex set.



Images of tissue sections stained with hematoxylin and eosin. The sections correspond with the multiplex images below.

Whole slide IF images. The lymph node contains B cells (CD20, blue) in follicles and prominent T cells with T cells (CD4, magenta in left panel, white in other two panels) concentrated primarily between the follicles. The tonsil is surrounded by mucosal crypts (pan-cytokeratin, cyan). Lymphoma shows effacement of normal lymph node architecture by tumor cells.

Lymphoid architecture. The lymph node has small follicles that contain few proliferating cells (PCNA, red) proliferating cells are present in the Follicles. Densely spaced blood vessels (CD31, green) are seen amid CD4 T cells (yellow). Collections of macrophages (magenta) are present. The tonsil has expanded follicles, and nearly all B cells (blue) in the germinal center are proliferating. CD4 T cells are numerous in the T cell zone and also present within the follicle. Virtually all lymphoma cells are proliferating; they infiltrate blood vessel walls (CD31 green).

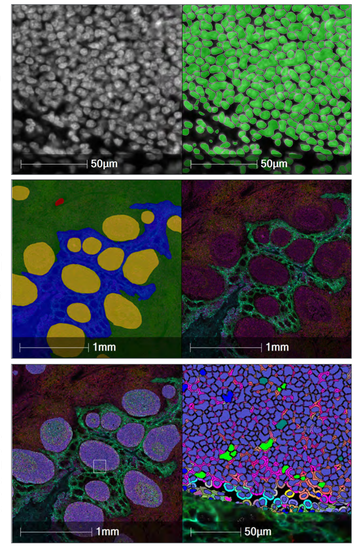
Proliferation. The 17-plex panel includes two proliferation markers: PCNA (red) and Ki-67 (green). The few proliferating cells in the lymph node follicle and the many proliferating cells in the tonsil follicle express both markers. Interestingly, the lymphoma cells express PCNA but not Ki-67. PD-1+ cells (cyan) are scattered in the follicles and are not proliferating.

T cell subsets. CD4 (cyan) and CD8 (magenta) T cells are identified in the T cell regions of lymph node and tonsil. FOXP3 (red, nuclear), a marker of regulatory T cells, is present in a subset of the CD4+ cells, staining the nuclei in red. Although T cells are sparse in the lymphoma, regulatory T cells can be identified.

Macrophages. Follicles contain spatially distributed macrophages which are CD68+ (yellow). These macrophages also express the checkpoint marker PD-L1 (red) which is more pronounced in the reactive follicles of the tonsil. CD163 (cyan), another marker of macrophages is more prominently seen outside the follicles. The lymphoma image is of one of the few residual follicles in the tissue section.

Figure 3. Qualitative visual analysis of 17-plex lymph node, tonsil and SLL/CLL lymphoma whole-slide images. Sub-sets of markers from the entire panel are displayed to assess lymphoid architecture, proliferating cells, T cell subsets and macrophages.

Workflow using HALO® image analysis



1. **Nuclear segmentation.**

   HALO AI was used to create a custom nuclear segmentation algorithm for analysis of the tonsil tissue to best segment the nuclei which are present at high density these samples.

2. **Tissue classification.**

   HALO AI tissue classifier algorithm was created and trained to recognize B cell zones (yellow), T cell zones (green), epithelium (blue) and stroma (red). Right panel corresponds to Orion image.

3. **Cell phenotyping.**

   The two HALO AI algorithms were embedded into the Highplex FL module which analyzed the tissue classes by cell phenotype. Here phenotypic analysis has been performed on the follicle region. The individual cell coloration is determined by phenotype.

Figure 4. Quantitative analysis using HALO platform

Tissue Regions

Tissue classification area (mm²)

Region	Lymph node	Tonsil
Whole section	139.3	126.8
B cell zone	**28.4**	**21.1**
T cell zone	**101.9**	**55.2**
Stroma	9.0	19.4
Epithelium	--	31.2
Ratio T : B zone	**3.6**	**2.6**

Region cell composition

Region	Cell type	Lymph node	Tonsil
B cell	CD20+	94.2%	93.5%
T cell	CD3+	81.9%	50.7%
	CD4/CD8 ratio	3.1	1.4

Vascularity and checkpoint

Analysis	Region	Lymph node	Tonsil
Vascularity			
CD31+ cells	B cell	3.1%	2.6%
	T cell	19.6%	12.6%
CD31 H-score	B cell	3.5	3.3
	T cell	25.4	19.4
Checkpoint			
PD-L1 H-score	B cell	34.4	24.0
	T cell	41.7	21.4
PD-1 H-score	B cell	85.5	48.5
	T cell	56.9	20.4

Complex phenotypes

Region	Phenotype	Lymph node	Tonsil
B cell	CD4+FOXP3+	1.1%	3.6%
T cell	CD4+FOXP3+	4.4%	3.0%
B cell	CD68+PD-L1+	4.4%	8.3%
	CD163+PD-L1+	0.46%	1.1%
T cell	CD68+PD-L1+	3.3%	17.7%
	CD163+PD-L1+	2.6%	12.7%

Quantitative conclusions

- Analysis integrates information from greater than 1 cm x 1 cm tissue
- B-cell zone in reactive tonsil is expanded relative to quiescent lymph node
- Ratio of areas of the T cell and B cell zones is decreased by 27% in the tonsil relative to the lymph node
- T cell region in tonsil has greater composition of non-T cells than lymph node
- Lymph node T cells are skewed toward helper T cells than cytotoxic T cells; tonsil is more balanced
- Greater number of CD31+ cells in T cell regions than B cell regions
- Higher proportion of CD31+ cells in T cell region of lymph node than in tonsil
- H-score combining intensity of staining and extent of staining yields similar results
- PD-L1 expression is greater in both B and T cell regions of lymph node than tonsil
- PD-1 expression is also greater in both B and T cell regions of lymph node than tonsil
- The lymph node contains a lower percentage of regulatory T cells (CD4+FOXP3+) in the B cell zone than in the T cell zone
- Regulatory T cell are evenly distributed between B and T cell zones in the tonsil
- PD-L1 expression is present on both CD68+ and CD163+ (M2) macrophages; reactive tonsil has much higher expression than lymph node