In many cancers, tumor-infiltrating lymphocytes (TILs) indicate levels of tumor immunoactivity and predict survival. In particular, increased levels of regulatory T cells (Tregs) are associated with poorer prognosis, while CD8+ activated T-cells (Tacs) may also be prognostic. Understanding the phenotype and pattern of TILs in situ within tumors would be advantageous. However, usual TIL assessment cannot easily determine the type of lymphocyte in situ and multimarker quantitation is difficult with standard methods. We present a multi-marker, computer-aided event-counting method for determining the phenotypes of lymphocytes in a range of tumor types using a multiplexed imaging (MSI) automated tissue segmentation and counting approach. This paper will demonstrate the use of automated methods for counting Tregs, Tacs and other immune cells in follicular lymphoma, melanoma and lymph nodes. Automated, multiplexed tissue cytometric analyses are feasible for routine clinical studies, work with many multiplexed IHC staining methodologies for a range of immune cell types and are of importance for translational cancer studies in general and cancer immunotherapy in particular.

A tissue micrometry containing follicular lymphoma (FL) cores from 78 patients who were immunohistochemically immunostained for CD3, CD60 and FOXP3, counterstained with hematoxylin, of which 40 cores were informative for both triplex staining and clinical follow-up. Each core was imaged using MSI and the individual staining of each marker separated from each other using spectral unmixing. Images were analyzed using software trained to recognize different tissue compartments based on morphology, quantitatively based on CD3 rich (extra-follicular) and poor (intra-follicular) areas. The FOXP3+ or CD60+ ratio of each CD3+ TIL was then determined and number *Treg* (FOXP3+/CD3+) and CD60+ T-cells counted in the extra- and intra-follicular areas.

The intra-follicular (CD3 poor) and extra-follicular (CD3 rich) niches were accurately recognized within each core, based on abundance of CD60 cells. MSI enabled the accurate quantitation of CD3, CD60 and FOXP3 without manual counting of T-cells. The number of FOXP3+/CD3+ Tregs and CD60+ T-cells were counted in each core and used in Kaplan-Meier survival analysis, which demonstrated association of FOXP3+/CD3+ Tregs with favorable outcome in both the intra- (p=0.0173) and extra-follicular (p=0.0173) areas, as well as CD60+ T-cells in extra-follicular (p=0.017) areas. CD60+ T-cells were not prognostic in extra-follicular areas (p=0.4509).

This study demonstrates use of an automated method for counting Tregs in follicular lymphoma, showing association of FOXP3+ Tregs with good outcome. Given the generic nature of the method automated multiplex tissue cytometric analyses are feasible for routine clinical studies and work with many multiplexed IHC staining methodologies of importance for translational cancer studies in general and cancer immunotherapy in particular.

**Abstract**

In many cancers, tumor-infiltrating lymphocytes (TILs) indicate levels of tumor immunoactivity and predict survival. In particular, increased levels of regulatory T cells (Tregs) are associated with poorer prognosis, while CD8+ activated T-cells (Tacs) may also be prognostic. Understanding the phenotype and pattern of TILs in situ within tumors would be advantageous. However, usual TIL assessment cannot easily determine the type of lymphocyte in situ and multimarker quantitation is difficult with standard methods. We present a multi-marker, computer-aided event-counting method for determining the phenotypes of lymphocytes in a range of tumor types using a multiplexed imaging (MSI) automated tissue segmentation and counting approach. This paper will demonstrate the use of automated methods for counting Tregs, Tacs and other immune cells in follicular lymphoma, melanoma and lymph nodes. Automated, multiplexed tissue cytometric analyses are feasible for routine clinical studies, work with many multiplexed IHC staining methodologies for a range of immune cell types and are of importance for translational cancer studies in general and cancer immunotherapy in particular.

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**Conclusions**

- Multispectral imaging enabled the quantitation of three immunostains (CD3, CD60 & FOXP3) in intra- and extra-follicular compartments using automated event-counting.
- FOXP3+/CD3+ "Tregs" and CD60+/CD3+ "Tacs" were automated and used in Kaplan-Meier analysis, each associated with good outcome. The enumeration of FOXP3+/CD3+ and CD60+/CD3+ T cells in these clinical samples was effective and easy to perform.
- Need to test whether CD4+/CD25+/FOXP3+ T cells have same correlation
- This panel of immune markers can easily be changed to a panel of different or other markers according to interest (CD4, CD8, CD20, CD68, Ki67, etc.)

**Clinical correlation of results**

**Multispectral imaging of quadruplex-stained follicular lymphoma**

**Automated tissue and cellular segmentation**

**Multispectral imaging technology**

**Vector+™ Multiplex Imaging Systems**

- Images in different wavelengths
- Assemble the images into a single "cube" or RGB
- Spectrum of every (x,y) pixel

**RGB Representation of Spectral Cube**

- Mixtures of each channel (red, green, blue, etc.)
- Red = cancer mask
- Green = CD60
- Blue = FOXP3
- Yellow = CD69

**FOXP3 and CD60 analysis**

- Thresholding of CD69 (membrane) and FOXP3 (nuclear) was used to identify double FOXP3+/CD69+ T cells (shown as yellow cells).
- FOXP3+ (nuclear) and CD69+ (membrane) analysis was performed similarly.

**Isometric view**

- Tumors were classified as follicular, T-cell rich, or T-cell poor.
- Kaplan-Meier survival curves for T-cell rich tumors compared to T-cell poor tumors were calculated.
- Survival: 171+ months
- CD3+/FOXP3+: 12.3%
- CD3+/FOXP3-: 13.9%
- CD3-/FOXP3+: 0.66%
- CD3-/FOXP3-: 19.66%

**Sample 2**

- Extra-follicular cells: 1374
- Intra-follicular cells: 1316
- Survival: 133+ months

**Sample 1**

- Extra-follicular cells: 1473
- Intra-follicular cells: 1917
- Survival: 54 months

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