Introduction

In many cancers, tumor-infiltrating lymphocytes (TILs) indicate levels of tumor immunogenicity and are a strong predictor of survival. An understanding of the phenotype and spatial distribution of TILs within tumor regions would be advantageous for characterizing host response. However, visual TIL assessment is prone to error and multimarker quantitation is difficult with standard methods. Here we present a multi-marker, computer-aided event-counting method for determining the phenotypes of lymphocytes in melanoma sections using a novel multispectral imaging (MSI) approach, consisting of a Vectra 2 automated slide imaging system and the inForm® automated image analysis package. A section of a tissue microarray containing 120 melanoma cores was stained for CD3, S100, Foxp3, and hematoxylin. CD3 was used to label lymphocytes, Foxp3 was used to phenotype regulatory T cells and S100 was used to demarcate tumor areas. This TMA was imaged using Vectra and the individual staining of each marker separated from each other using spectral unmixing. The images were analyzed using inForm Tissue Finder™, which had been trained to recognize the tumor area based on the S100 and hematoxylin staining patterns. Then the Foxp3 status of each
CD3+ TIL was determined. Results indicate that machine-learning software can be trained to accurately recognize tumor regions within each core. MSI enabled the accurate quantitation of three immunostains in the sample without crosstalk. Within the tumor region of each core it was possible to count the CD3+ TILs and then determine the Foxp3 status of each, thus determining which cells were regulatory T cells (Tregs). This multimarker phenotyping and counting approach shows the potential for broad applicability in the assessment of many different kinds of TILs in many solid tumors.

Methods

A section of a tissue microarray containing 120 melanoma cores was stained for CD3, S100, Foxp3, and counterstained with hematoxylin. PerkinElmer’s Vectra 2 Intelligent Slide Analysis System was used to automatically acquire a multispectral image (MSI) from each TMA core. The use of MSI allowed for the individual staining of each marker to be separated from the others using spectral unmixing.1 The resulting multispectral images were analyzed using the inForm Tissue Finder Advanced Image Analysis software package from PerkinElmer. The software was user-trained to be able to automatically segment images into areas of tumor, stroma, and blank background (red, green, and blue overlays, respectively). The S100 staining was included in the set of markers used for training the area segmentation algorithm, to help the software recognize tumor, particularly at the margins. The Foxp3 status of each CD3+ lymphocyte located within the tumor area was determined through a thresholded co-localization analysis. Cells that were both CD3+ and Foxp3+ were considered Tregs. Scatter plots of tumor cellular co-expression of CD3 and Foxp3 were created in Microsoft® Excel®. Using the new interactive visual scoring features within inForm Tissue Finder, thresholds were set visually to determine the positivity cut off for both CD3 and Foxp3 individually (Figure 3). Based on these thresholds, cells were found to be either single positive for CD3, single positive for Foxp3, double negative, or double positive and were color-coded accordingly to be able to visually assess this scoring outcome.

Results

![Figure 1. (A) Color image and (B-D) unmixed images taken from multispectral data acquired from the multicolor IHC-stained sample to simulate a DAB and hematoxlyn staining for a single IHC marker in the (B) Foxp3, (C) S100, (D) and CD3 channels.](image-url)
Figure 2. (A) RGB representation of a multispectral image. (B) Image with automated tissue and cell segmentation masks separating tissue into tumor (in red), stroma (in green), and blank background (in blue); and tumor cells segmented and colored based on score. CD3+ only cells are shown in red, Foxp3+ only cells in green, and cells double positive for both CD3 and Foxp3 in yellow. Zoomed in views of (C) RGB image and (D) segmented image to show segmentation and scoring colors.

Figure 3. Screenshot of inForm interface for double positivity scoring.

Figure 4. (A) Scatterplot displaying per-cell quantitation of CD3 and Foxp3 intensity for tissue sample 13908. Red lines show where thresholds were set to determine positivity. (B) Histogram displaying the number of Foxp3 +ve T cells in each sample.
Of the 120 TMA cores scanned, 39 had sufficient tumor for data analysis. The TMA cores with insufficient tissue were rejected either during the scanning phase or during a Review and Merge step of the inForm analysis. The samples were automatically analyzed using inForm Tissue Finder to determine the number of Foxp3 +`ve T cells (Tregs) in each. This data is displayed in a histogram seen in Figure 4B. Across all samples, there were 27,998 tumor cells segmented with 1,808 of those found to be TILs, based on CD3 positivity. Of the 1,808 detected TILs, it was determined that 92 (5.09%) were Tregs (Foxp3 +`ve) In addition, there were 38 other cells which were Foxp3+ but which were not T cells. Had a single stain for Foxp3 only been used, these cells would have been counted as Tregs, which they are not. A scatter plot of the double positivity scores for a single sample (Sample 13908) and for the sum of all 39 samples can be seen in Tables X and Y.

As can be seen in Figure 4B, two samples had more than 25 Tregs in the tumor region and another 6 samples had 10 or more Tregs. As Tregs are thought to inhibit the body’s immune response, it is thought that the presence of a large number of Tregs will indicate a poor prognostic outcome and that tumor immunity can therefore be enhanced by depleting Tregs, attenuating Treg suppressive function, or rendering effector T cells refractory to Treg-mediated suppression.²

Conclusions

- Multispectral imaging enabled the quantitation of three immunostains (CD3, Foxp3 and S100) in the presence of hematoxylin and melanin, despite substantial spectral overlap among all five chromogens.
- Tissue segmentation and cell-counting accuracy was estimated at greater than 90%, based on visual review by pathologists.
- Automated multiplexed cytometric analyses while retaining tissue architectural context is feasible for routine clinical studies and works for both IHC and IF staining methodologies.
- This particular example, the enumeration of Foxp3 +`ve T cells (Tregs), demonstrates the power of these capabilities for elucidating tumor immunogenicity and supporting research into combinatorial therapeutic strategies that stimulate host immune response as well as target tumor cells.
- Using different combinations of markers, such as CD69 for activated T cells, or other T or B cell markers, one could easily extend this methodology to be able to phenotype and enumerate a wide range of lymphocytes.

References


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