Appendix

The linear property of the phasors allows for spectral unmixing of up to three components [41]. The vertices of a reference triangle are manually selected or obtained from reference compounds. Next, the relative contribution of the three components is calculated for each pixel in the image and multiplied by the intensity in that pixel. This results in three separate images of the individual components. The accuracy of the analysis is determined by the signal-to-noise in the spectra. Pure spectra with high signal-to-noise ratio will appear as a single point in the phasor plot. However, usually spectra contain significant levels of noise (or additional uncharacterized spectral components) that introduces variations in the spectrum. As a result, the spot in the phasor plot approaches a diffuse circular cloud. As a special case, the presence of just two spectral components in different admixtures will result in an ellipsoidal cloud. In order to analyze the images recorded in bright field transmission configuration, we first convert transmission data into optical density units as follows: (1) Standard 8-bit RGB color images are separated into red, green and blue channels and optical densities estimated by the following equation:

$$OD = \log\left(\frac{255}{I}\right)$$

(2) Regions corresponding to the most representative appearance for the counterstain signal for blue (hematoxylin), Fast Red (B cells) and DAB (brown, T cells) were manually selected and phasor transformed (Figure A.1). The phasor coordinates from this conversion are used as references on the phasor plot to unmix all the images. As described above, fractions of each component are calculated which is a direct measure of their intensity/abundance.

A region of interest is created on the images to include the T cells and B cells and to exclude the regions with hematoxylin only. After un-mixing bleeding through from other components observed as a background signal at both T cells and B cells images. In order to remove this artifact we subtracted the minimum of T cells and B cells at each pixel from the individual images. This is to make sure that the signal was coming only from the cells and not from the background. Next we thresholded the images and counted the pixels above the threshold as an estimate for number of the cells at each images. The fraction of each component that is the measure of number of the cells is calculated by dividing the number of pixels from each image to sum of the pixel numbers of both images.

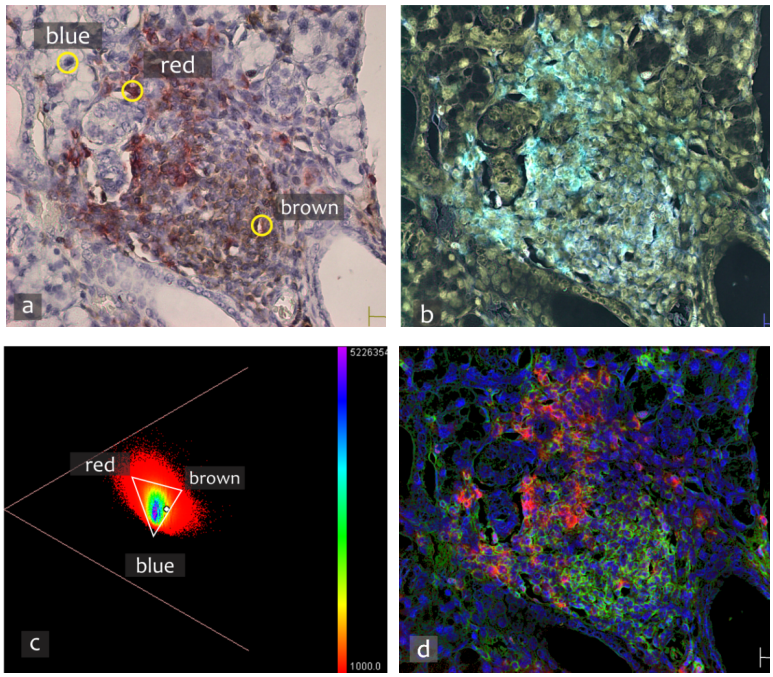

Figure A.1 (A) The RGB bright field image is shown. Region of interests that are phasor transformed and used in the phasor plot are indicated (yellow circles). (B) The optical density map is constructed by converting the brightfield image shown in (A) using the above equation. (C) Unmixing of the 3 components. (D) Overlay of unmixed image. Blue represents the hematoxylin signal, red corresponds to B cells, and green indicates the distribution of T cells.