**Title:Virtual Flow Cytometry of Tissue with CD30 + tumor cells and reactive CD8 T suppressors**

*Method for Virtual Flow Cytometry and Histopathology instruments:*

To gain the most objective quantification of different functional lymphocyte populations, we will employ a method known as "Virtual Flow Cytometry"which produces a dot plot histogram of stained cells in paraffin sections, analogous to flow cytometry of cell suspensions (see Figure 1 below). Formalin-fixed paraffin tissue sections will be cut at 2um thickness to minimize overlap of nuclei. IHC will be performed on a Benchmark automated IHC system (Ventana Systems, Tucson AZ). Slides will be examined with a 20x objective and images captured with an Insight color CCD (Diagnostics Instruments, Sterling Heights MI) running SPOT software version 4.7. The camera CCD photoreceptive field (1,060x 1020 pixels) is trimmed by software-mode treatment to 512x474 pixels for dimensionality reduction, optimal object size and computational efficiency. Images will be stored in JPEG files and analyzed using a previously developed advanced cell imaging software. The light intensity rheostat will be set to 7.0-12.0. The light source is a 30W 12 V incandescent bulb with a blue filter (80s Tiffen) collimated through a condenser aperture set at 0.5ph, under Kohler illumination, and using a 20x plan 0.4na objective without magnification in the trinocular adapter. The pixel size is 1.5 pixels per micron for the 512 x 474 pixel image frame. Image acquisition is manual on selected lymphoid rich areas. In these areas, 3-dimensional nuclei are tangentially cut in thin histologic sectional planes resulting in random nuclear tangents of varying diameter. The images are manually focused and saved. Results for 15 image frames will be recorded per antibody with an average of 7,500 total cells analyzed. A JPEG formatted image takes 2-3 seconds from start to display of results. The software used to analyze the images was developed by IHCFLOWGreenGreat. For image frames using the 20x objective as described, the pixel size converts to 2/3 um/pixel. The following formula was used to convert pixel area to cell diameter in microns (Y data). Cell diameter = 2x (sq rt of areas in pixels/m/1.5 - sq rt (area of pixels x P). The intensity component of the colored blue and brown objects are summed up per cell object, averaged, stored and converted as average stain density (X data). When tissue cytometry was compared with manual counting, the correlation coefficient and 95% confidence interval by linear regression analysis produces a concordance of 0.936518. An example of the analysis of quantification of immunostained tumor reactive CD8+ lymphocytes and CD30 + Hodgkin cells in a HL lymph node is shown in Figs. 1 and 2 below.

Fig 1. immunoperoxidase stained CD8+ lymphocytes (brown) in HL lymph node. At right is virtual flow cytometry dot-plot showing 576 (18%) CD8+ lymphocytes (red dot/circles) and 2747 (82%) CD8 negative (blue dot/circles) lymphocytes.

Fig 2. Hodgkin cells stained in heterogeneous manner by antiCD30 antibody and the insert panel showing the results of quantitation and intensity distribution of CD30 tumor cells.