

Media Cybernetics Applications Note

Using computerized image analysis to quantify CD68 positive macrophage cell populations in biopsies taken from transplanted kidneys

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Introduction

CD68+ is a 110 kDa transmembrane glycoprotein of 354 amino acids as predicted from the cDNA sequence and 9 potential N-linked glycosylation sites. The extra-cellular region consists of 298 residues with two domains connected by a proline-rich hinge. The N-terminal region represents a mucin-like domain comprised of short peptide repeats and highly enriched serine and threonine residues, which may bear O-linked glycan chains. The domain close to the membrane shows homology to a family of lysosomal/plasma membrane shuttling proteins (LGP) which include lamp-1 and lamp-2. CD68 stains the cytoplasm of non-hemopoietic tissues, especially in the liver, glomeruli, and renal tubules. Soluble forms have been found in serum and urine (Goyert, SM, 1999).

CD68 is expressed by monocyte / macrophage lineages, therefore, antibodies to CD68 are capable of staining monocytes, Kupffer cells, osteoclasts, dendritic cells, myeloid progenitor cells, a subset of CD34+ hemopoietic bone marrow progenitor cells, granulocytes and their precursors. Because of the specificity of CD68 immunohistochemical stains for macrophages, and the role of macrophages in inflammatory processes, CD68 immunostaining is widely used in immunological studies (Torrealba JR, et al; 2003 Liu ZH, et al 2003; Pizov G. and Friedlaender MM. 2002)

For example, Cockwell, et al (2002) was able to correlate CD68 expression patterns and show that fractalkine is a strong candidate for directing mononuclear cell infiltration in human renal inflammation. Hotta, O. et al, (2000) used the number of CD68+ cells in the urine of patients with glomerulonephritis (GN) as a diagnostic tool to differentiate proliferative GN from non-proliferative renal disease (e.g. hereditary nephropathy). Ozdemir BH, et al (2002) used the density of CD68, CD3, and HLA-DR-positive infiltrating cells to clarify the relationship between angiogenesis and mononuclear cell infiltration in renal allografts. Additionally antibodies to CD68 may be useful to differentiate myelomonocytic and histocytic tumors as well as fibrous histiocytoma from other pleomorphic sarcomas.

Efforts to quantify the amount of CD68+ and other immunohistochemically stained cells in tissue sections, by pathologists, has been shown to include large interobserver differences. Furthermore, semiquantitative grading scales such as 0-5+ are open to numerous errors and are quite subjective. Masserili M, et al (2000, 1998) clearly showed that image analysis was superior to semiquantitative data measurements and is not subject to the same errors. In addition, automatic segmentation (thresholding) further reduced intra- and inter-operator variability over manual methods. O'Brien, MJ, et al (2000) used image analysis to more accurately assess the amount of fibrosis in liver biopsies and correlate these measurements with qualitative assessments using various classification schemas. We chose automated computer analysis for our study over visual quantification methods to more accurately measure the amount of CD68+ stained macrophages and reduce operator subjectivity. Furthermore, computer image analysis allows us to distinguish subtle differences that may be lost using a more subjective analytical method.

FTY720 is a novel immunosuppressant that causes rapid and transient depletion of peripheral blood lymphocytes affecting T cells more than B cells. The exact action mechanism of this drug is unclear, but there is evidence that it causes lymphocyte sequestration in lymph nodes. It has been only recently recognized that humoral immune

mechanisms can be very important not only in hyperacute rejection, but also in later stages of acute rejection. Antibodies to CD68+, CD20+ and CD3+ react with monocytes/macrophages, B cells and T cells, respectively, in routinely processed archival tissue sections. Because FTY720 apparently significantly affects B cells, we were wondering if the composition of inflammatory cell infiltrate present in allograft biopsies showing humoral rejection from patients treated with FTY 720 is different from biopsies with C4d positive humoral rejection taken from patients treated with conventional immunosuppression (cyclosporine/CellCept/prednisone). Therefore, by using these antibodies we can determine the percentage of these inflammatory cell populations within an inflammatory cell infiltrate in biopsies of rejecting renal allografts. To avoid subjective errors, we introduced computerized morphometric methods to determine the amount of infiltrating positive CD68 macrophages.

Materials/Methods for Image Analysis

CD68+ immunohistochemistry stained slides from six cases were prepared from archival paraffin blocks of kidney needle biopsies or nephrectomies. Each slide contained 3 serial sections cut at 4-5 microns. Prior to scanning the slides for image analysis, section 2 (middle section on the slide) was arbitrarily chosen.

All images were quantified using Image-Pro[®] Plus version 4.5, a commercially available software package from Media Cybernetics (Media Cybernetics, Inc. 8484 Georgia Avenue, Suite 200 Silver Spring, MD 20910-5611 USA).

Hardware

Images were analyzed on a Dell[®] OptiPlex GX400 1.8GHz computer running the Windows[®] 2000 Pro operating system. Slides were imaged using a Hitachi HV-C20-SA 3CCD color camera mounted on an Olympus[®] BX-51 research microscope (Figure 1). The microscope is fitted with a Prior[®] H101 precision motorized stage driven by a Pro-Scan[™] advanced stage controller (Romer, Yearsley and Ayers 2003). The camera and stage are controlled via the Scope-Pro[®] plug-in for Image-Pro Plus software.

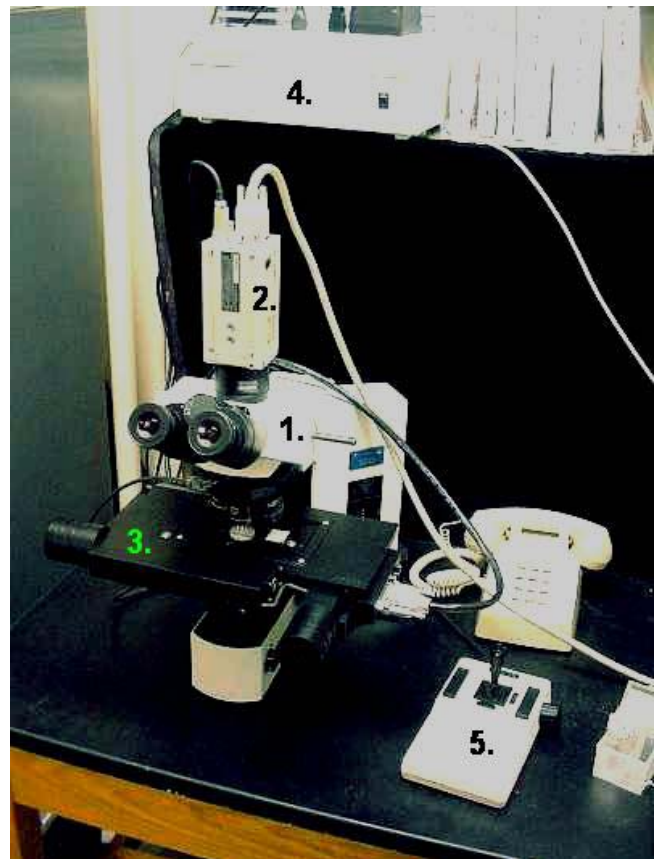


Figure 1. 1) Olympus[®] BX-51 research microscope fitted with a 2) Hitachi HV-C20-SA 3CCD color camera. Objectives available for imaging: 4x, 10x, 20x, 40x, and 60x. The microscope stage is controlled with a 3) Prior[®] H101 precision motorized stage driven by a 4) Pro-Scan[™] advanced stage controller. 5) Joystick for stage X/Y/Z axis manual control. The camera and stage are actuated using Image-Pro[®] Plus running the Scope-Pro[®] plug-in on a Dell Pentium 1.2 GHz computer.

Imaging tissue sections was automated using an Image-Pro Plus macro calibrated for each microscope objective. Initial objective measurement calibration was accomplished using a precision 0.01mm stage micrometer and saving the calibration settings for each objective within the macro to facilitate batch processing. The macro automatically captures 640x480 pixel blocks within the stage travel limits to build a complete full color image. Images scanned at x20 are stored as uncompressed TIF files on the local hard drive or burned to CD-R or DVD+RW media for further processing.

Image Analysis

Tissue features from selected areas of interest (AOIs) are analyzed for number of CD68+ stained cells. Ten semi-random AOIs were selected from each large image using areas specifically designated by the principal investigator or by using a macro to capture a pseudo-random series of 640x480 pixel images (Figure 2). A macro using the maximum pixel size of the image and a pseudo-random number generator places a 640x480 pixel box randomly on the tissue image. Once the box has been placed, the operator can move the box slightly to avoid large open areas and nonspecific artifacts (large arteries, tissue folds, etc).

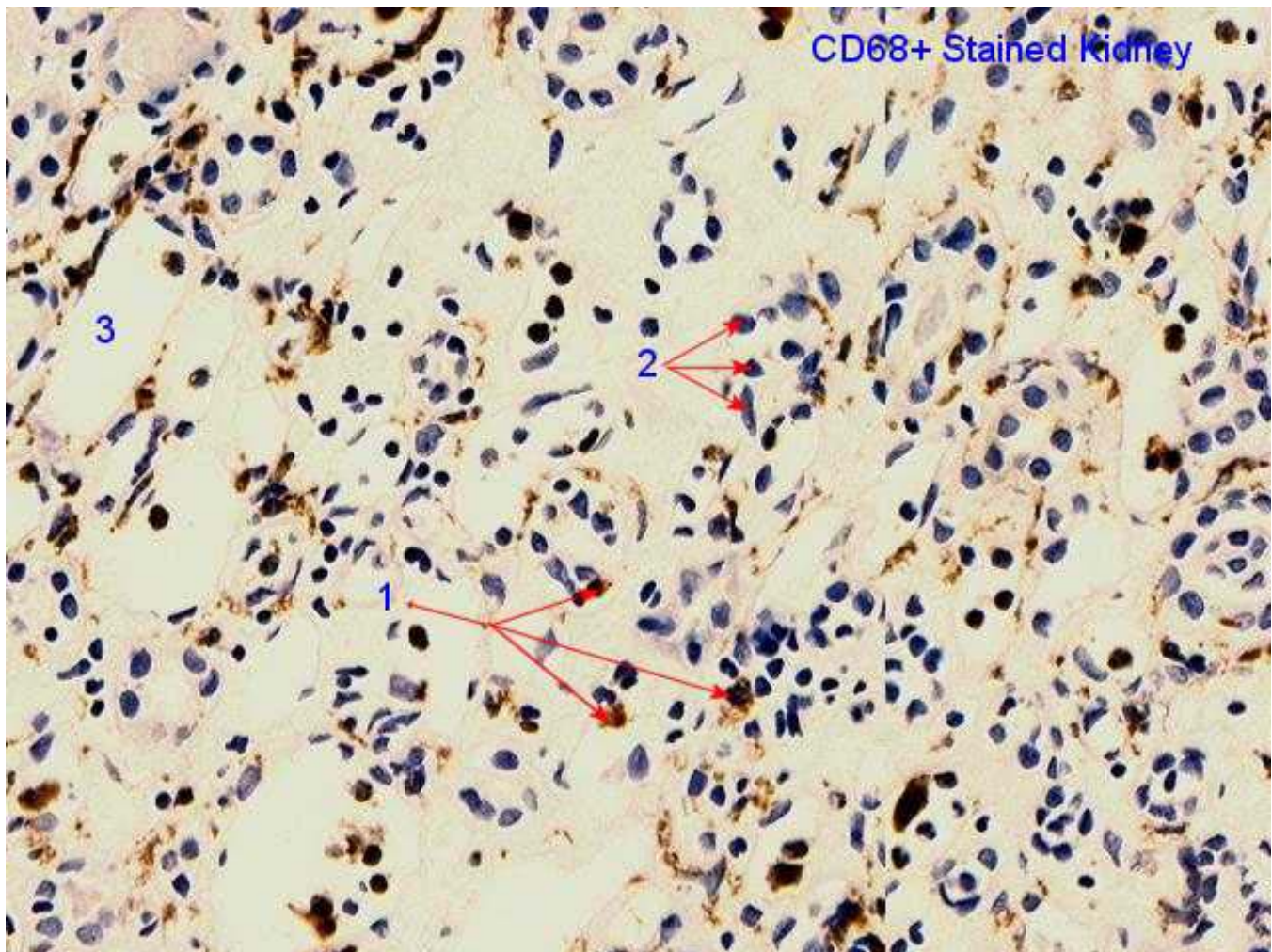


Figure 2. CD68+ immunohistochemically stained kidney tissue. The tissue has been counter-stained with hematoxylin to show cellular and morphological details. 1. Cells expressing the CD68 antigen on the cell membrane stain brown. 2. CD68 negative cells counter stained with hematoxylin exhibit dark nucleoli with little or no brown staining. 3. A renal tubule in cross section with little or no CD68+ staining.

AOIs were selected from a specific tissue section on each slide with the position of the tissue section recorded as counted from the slide label. Similar sections were then analyzed on each subsequent slide and the images saved for analysis. Each selected tissue feature of interest is assigned a unique color (segmentation) using the Hue, Saturation, and Intensity (HSI) color model (Figure 3). Segmentation allows individual features to be separated

from the background prior to measurement. In this case we were interested in the CD68+ stained cells. These cells stain a dark brown in the presence of the CD68 maker on the cell surface. By setting the threshold value for the amount of stain present (dark brown), nonspecific background staining (light brown) can be excluded.

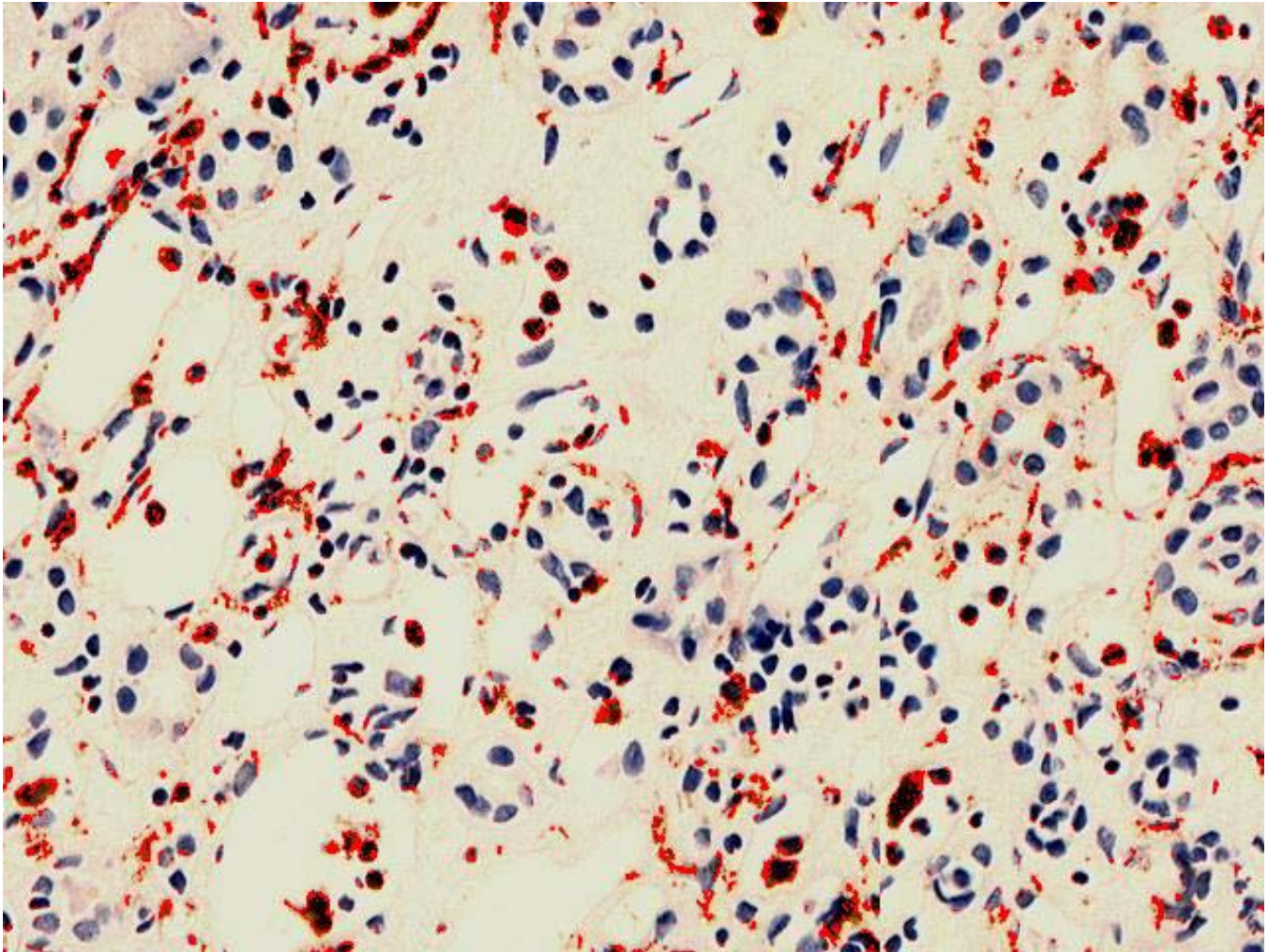


Figure 3. The same tissue section from Figure 2 with false color segmentation. Segmentation is set by a threshold color value on the CD68+ cells. The software assigns a false color (red in this case) so that each CD68+ cell is highlighted allowing an accurate measurement using Image-Pro Plus.

Automation of Image Analysis Using IPBasic Macros

The entire image analysis procedure from image acquisition to analysis has been automated using macros written in Image-Pro Basic programming language which is based on Microsoft® Visual Basic v5.0. Initial imaging of the tissue section is controlled by a macro that captures individual 640x480 AOIs. This macro first prompts the user to select the appropriate objective lens, then select the area of the slide to scan (either the entire slide or the user can select or limit the scan area). Once the area of travel has been selected, the stage is moved and the operator is asked to focus on the specimen. Three areas of focus are selected and the Z coordinates are stored. Focus of the specimen is maintained using a 3 point focal map and averaging the triangulation of focus over the selected area of travel. An option for capturing a background image is provided to correct for background illumination. The background image is stored as a temporary image and subtracted from each captured AOI. Each individual AOI (minus the background image) is stitched together into one large composite TIF image. Stitching is done by actually capturing a slightly larger AOI which overlaps the previous AOI by 2 pixels. When individual AOIs are combined the overlap area is subtracted to insure a virtually seamless composite image. The large composite images are saved to the local hard drive or burned to DVD disks for image analysis (Romer, Yearsley and Ayers 2003).

Quantifying the data from large composite images is accomplished by sub-sampling and selecting an appropriate number of AOIs (usually 5-10 640x480 pixel AOIs). A macro to pseudo-randomly select those areas is used. The macro prompts the user for the file location, asks how many AOIs are needed, and the image file name. Using the total image area in pixels, a 640x480 AOI box is drawn on the image. The macro pauses to allow the user to check the AOI selected or move the AOI should it fall on an artifact or blank area of the slide. If the AOI is adequate, click the continue button to capture the AOI which will label and increment the AOI file information, then save that file to the appropriate subfolder.

Once the AOIs have been selected, another macro is used to quantify the data. The user is prompted to select the appropriate subfolder and enter the file information and select a prewritten Excel template. The macro loads the appropriate measurements and measurement parameters, filters, then loads any previous segmentation range files (AOI-1.rge). If no previous segmentation range file has been saved, the macro pauses to allow the user to adjust the segmentation thresholds as needed. Once the segmentation is set, the macro will measure and count the AOI saving the object outlines to an outline file (AOI-1.scl) in the AOI image subfolder.

Cell counts are automatically ported to the pre-selected Excel template using a sub routine written in Excel for tabulation and graphing (Figure 4) and the macro loops to analyze the next AOI in the series. The use of custom programmed macros enables us to assist the operator in properly selecting the correct parameters to identify and then consistently quantify the needed data. No “canned” software provided enough flexibility to adequately lead the user well enough to capture the data of interest.

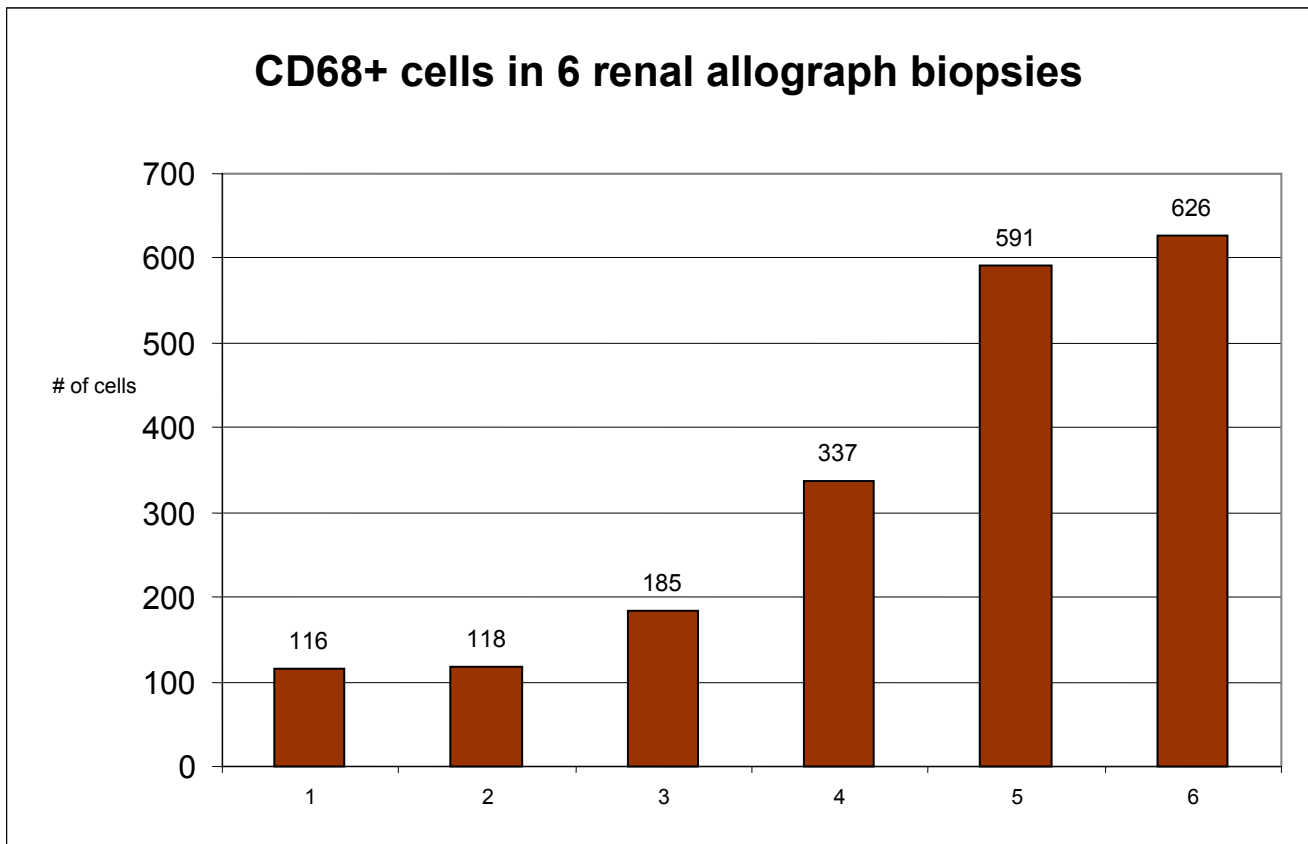


Figure 4. Bar diagram showing the number of CD68+ cells in 10 randomly selected areas of interest from 6 kidney transplant biopsies with humoral rejection. Results from Image-Pro Plus are automatically ported into a pre-programmed Excel template that automatically tabulates and graphs the raw data.

Preliminary Conclusions and Ongoing Investigation

Using Image Pro Plus has greatly increased slide throughput and data accuracy. Whereas it took a pathologist several hours to quantify the six cases listed in Figure 4, the same cases were automated and data collected in less

than half that time. Further errors were eliminated by automatically porting the data to Excel. When the same slides were submitted to several pathologists for evaluation of total overall CD68 staining using a 0-5+ scale, the results varied widely on the same slide. For example, one pathologist may rate a case as a 1+ and another would rate the same slide as a 3+ especially if there was a small area of cells on the slide that stained darker than the rest of the slide. By using Image Pro Plus, this variance was reduced to near zero because we could not only count stained cells but use the overall abundance of CD68 stain (as a percentage) to quickly spot any trends between several cases and/or treatments that were not easily observable. The software also gave us the control to set thresholds so that background staining (very light) could be eliminated. Furthermore, data could be filtered by size to also reduce background or artifact staining.

Quantification of CD68+ stained macrophage infiltrate is part of a larger work in progress that will include CD3 and CD20 immunohistochemistry stained kidney tissues from the same archival blocks. At this time, further refinements to the initial image analysis procedures, quantification of date, and macros noted above, are ongoing as well as gathering data from control specimens as a baseline.

References

- Cockwell P, Chakravorty SJ, Girdlestone J, Savage CO. Fractalkine expression in human renal inflammation. *J Pathol.* 2002 Jan;196(1):85-90.
- Boehler T, Schuetz M, Budde K, Neumayer H, Waiser J., FTY720 alters the composition of T-lymphocyte subpopulations in the peripheral blood compartment of renal transplant patients. *Transplant Proc.* 2002 Sep;34(6):2242-3
- Bohler T, Waiser J, Schutz M, Friedrich M, Schotschel R, Reinhold S, Schmouder R, Budde K, Neumayer HH. FTY 720A mediates reduction of lymphocyte counts in human renal allograft recipients by an apoptosis-independent mechanism. *Transpl Int.* 2000;13 Suppl 1:S311-3.
- Gourishankar S, Turner P, Halloran P. New developments in immunosuppressive therapy in renal transplantation. *Expert Opin Biol Ther.* 2002 Jun;2(5):483-501.
- Goyert , SM. NCBI Website http://www.ncbi.nlm.nih.gov/prow/guide/2088387162_g.htm
- Hotta O, Yusa N, Kitamura H, Taguma Y. Urinary macrophages as activity markers of renal injury. *Clin. Chim. Acta.* 2000 Jul., 297(1-2):123-133.
- Liu ZH, Chen SF, Zhou H, Chen HP, Li LS. Glomerular expression of C-C chemokines in different types of human crescentic glomerulonephritis. *Nephrol Dial Transplant.* 2003 Aug.;18(8):1526-34.
- Masseroli, M. Caballero, C., O'Valle, F., Del Moral, RMG., Pérez-Milena, A., Del Moral, RG. Automatic quantification of liver fibrosis: design and validation of a new image analysis method: comparison with semi-quantitative indexes of fibrosis. *Journal of Hepatology* 2000; 32:453-464.
- Masseroli, M., O'Valle, O., Andujar, M., Ramírez, C., Gómez-Morales, M. de Dios Luna, J., Aguilar, D., Rodríguez-Puyol, M., and Del Moral, RG. Design and validation of a new image analysis method for automatic quantification of interstitial fibrosis and glomerular morphometry. *Laboratory Investigation* 1998, 78(5):511-522.
- Goyert , SM.1999 NCBI Website http://www.ncbi.nlm.nih.gov/prow/guide/2088387162_g.htm
- O'Brien, MJ.; Keating, NM., Elderiny, S., Cerda, S., Keavens, AP., Afdhal, NH., and Nunes DP. An assessment of digital image analysis to measure fibrosis in liver biopsy specimens of patients with chronic hepatitis C. *Am J*

Clin Pathol 2000; 114:712-718.

Ozdemir BH, Ozdemir FN, Gungen Y, Haberal M. Role of macrophages and lymphocytes in the induction of neovascularization in renal allograft rejection. Am J Kidney Dis. 2002 Feb;39(2):347-353.

Pizov G, Friedlaender MM. Immunohistochemical staining for proliferation antigen as a predictor of chronic graft dysfunction and renal graft loss. Nephron. 2002;92(3):738-42.

Romer, DJ, Yearsley, KH, and Ayers, LW. Using a modified standard microscope to generate virtual slides. The Anatomical Record (Part B: New Anat.) 272B:91-97, 2003.

Torrealba JR, Fernandez LA, Kanmaz T, Oberley TD, Schultz JM, Brunner KG, Peters D, Fechner JH Jr, Dong Y, Hu H, Hamawy MM, Knechtle SJ. Immunotoxin-treated rhesus monkeys: a model for renal allograft chronic rejection. Transplantation. 2003 Aug 15;76(3):524-30.

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