A High-Resolution Multifocal-Plane Virtual Microscope for Teaching Histology

James A. Rhodes[1], Ronald P.J. Smircich[2], and John DeVore[1]

[1] Pacific Northwest University of Health Sciences, Yakima, WA, and [2] Bartels & Stout, Inc., Issaquah, WA

College of Osteopathic Medicine | Pacific Northwest University of Health Sciences

Abstract #R1941
Program Number 673.12

Virtual microscopy (using a computer to view digitally-scanned microscope slides) is widely employed in teaching histology labs. The increase in the memory capacity of computer servers now makes it possible for virtual microscopy to display virtual slides whose resolution equals that obtained using "real" microscope slides and microscopes with high numerical aperture (NA) oil-immersion objectives. Because high NA objectives have a very narrow depth-of-field, the ability to focus up-and-down through the specimen becomes very useful when examining high-resolution images. We describe here a process for creating multifocal-plane virtual-microscope slides whose resolution approaches the theoretical diffraction limit for a light microscope (~250nm) and the creation of a virtual microscope, intended for use in histology teaching labs, that allows the user to focus up-and-down through these high-resolution, multifocal-plane virtual slides.

Our first step in building a high-resolution, multifocal-plane virtual microscopy system was to scan histology slides using a Zeiss AxioImager.M2 microscope equipped with a 63x NA 1.4 PlanApo oil-immersion objective. The final digital magnification of these specimens is 14.4 pixels/µm, giving a theoretical "digital resolution" for these specimens of: 2 pixels ÷ 14.4 pixels/µm = ~140nm. The "digital resolution" is decreased somewhat by the RGB camera's Bayer filter, but the overall resolution obtained for virtual-slides scanned by this system is close to the theoretical "optical resolution" of ~250nm.

The second step in building our virtual microscopy system was to convert the scanned digital image into a format suitable for our microscope viewer; we chose to use jpeg images in an image-tile architecture similar to that used by Google Maps. Each focal plane of a virtual-slide is exported from the Zeiss (*.czi) format as a separate bigTIFF file, and the resulting TIFF file is then converted into a Google-Maps-compatible image pyramid using the libvips image processing library. Thus, a multifocal plane virtual slide is converted into a series of separate, but aligned, image pyramids.

The final step was to create a virtual microscope that is capable of displaying our multifocal plane digital specimens. HTML/javascript programming was used to create a client-side viewer that runs within the user's internet browser. This viewer allows the user to zoom-in/out to view the specimen at varying magnifications, and to focus up-and-down through the specimen. Most of the computing needed to display the specimen is done on the client-side by the viewer, with the viewer only calling on the server for information about the slide and for image tiles. To provide a responsive interface, the viewer maintains a buffer of images from the focal planes and zoom-levels adjacent to the image currently being viewed.

One of the keys to understanding the microscopic anatomy of an organ is being able to visualize the 3-dimensional relationships of the cells and tissues comprising the organ. The virtual microscopy system described here clearly demonstrates the utility of being able to focus through a histological specimen at high magnification when attempting to visualize these 3-dimensional relationships.

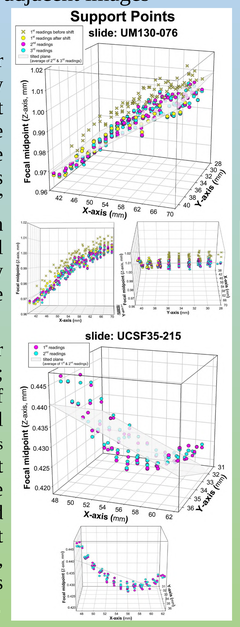
Scanning glass microscope slides

The specimens that have been scanned are standard histological specimens mounted on glass microscope slides (most of the slides are from student slide sets previously used for teaching medical-school histology). A Zeiss AxioImager.M2 microscope, equipped with a 63x N.A. 1.4 plan-apochromatic oil-immersion objective, an N.A. 0.9 aplanatic-achromatic condenser, a Zeiss 130x85 motorized stepping stage, and a Zeiss Axiocam 105 RGB CMOS camera, is used to scan the slides under bright-field illumination (microLED lamp at 3.6V).

After aligning the microscope for Köhler illumination, the camera is white-balanced on an "empty" field of the slide with the camera's exposure time set so that the pixels in this ("empty") field have an average value around 220. Scanning of the slide is done using Zeiss Zen software (to control the microscope & camera); in order to achieve correct alignment of the overlapping images, the slides are scanned at 20% of the maximum stage speed, stage & focusing backlash-correction is on, and adjacent images have a nominal overlap of 10%.

Support points. When inserted into the slide-holder on the microscope stage, the glass slide is not perfectly perpendicular to the optical path, and the slide is not perfectly flat. It is necessary to adjust the focal plane to account for these imperfections when scanning the slide. With Zeiss' Zen software, this adjustment is achieved by creating an array of "support points" whose z-axis component is the middle of the specimen (or the optimum focus) at each x,y location; a polynomial regression through these "support points" apparently defines a surface that is used as a reference for the focal planes when the slide is scanned.

A larger number of "support points" allows for better fitting of the scanned focal planes to the specimen; however, manually determining the z-component of each point is very time-consuming, and mechanical instability in the system (e.g., shifting or flexing of the glass slide) can invalidate previously-established support points (necessitating repeating of the entire process). The support points usually are spaced on a rectangular grid with ~1 to ~2mm between points; ~40 to ~80 support points usually are used for multifocal-plane specimens, while, in some cases, more than 150 support points have been used for large single-focal-plane specimens.



http://viewer.pnwu.edu – Zoom-in & Focus up-and-down

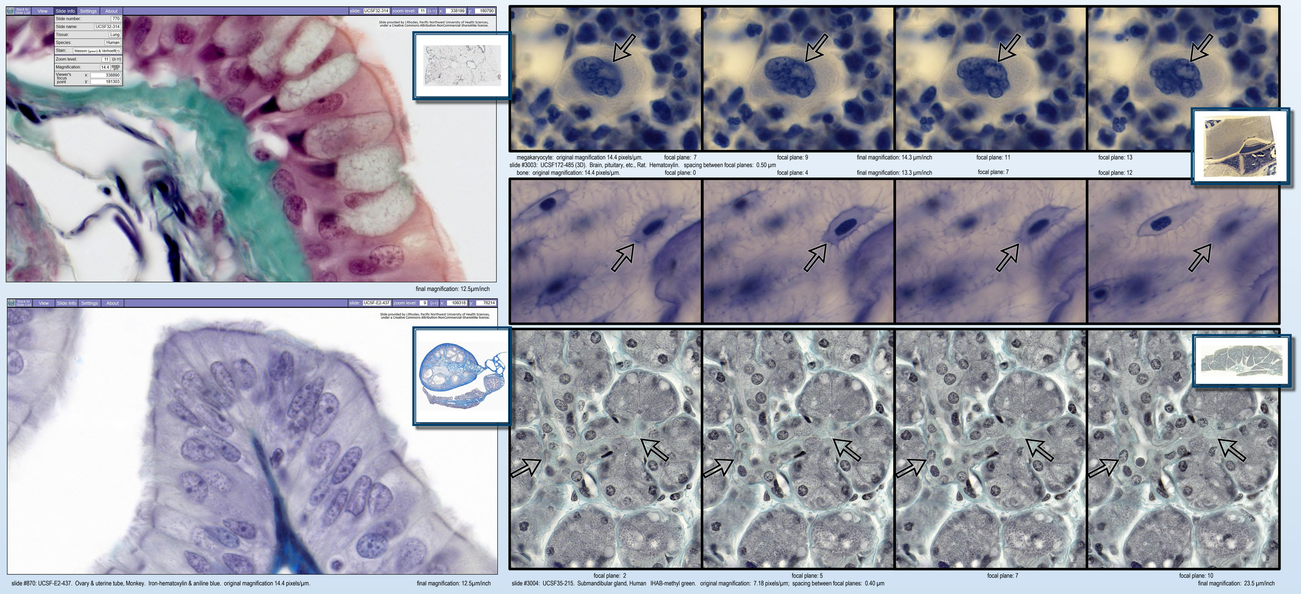
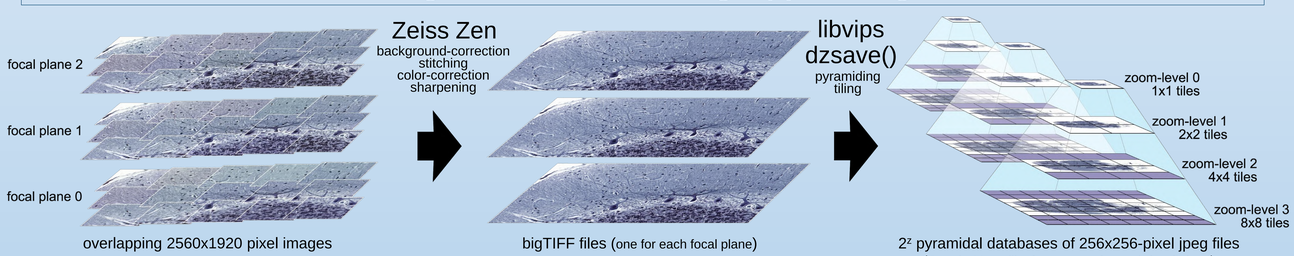


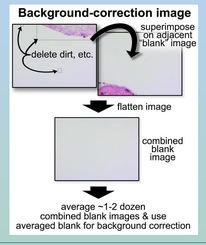
Image conversion: Stitching & pyramiding



Largest specimens (as of March 2020)

	specimen size	number of images	size of bigTIFF Files	
single-focal plane:	3.02 x 1.98 cm	30,832 images	348 GB	1 focal plane; 12 zoom-levels; 2,530,901 tiles (256x256-pixel jpeg files)
multifocal plane:	1.78 x 0.99cm (x 13 focal planes)	118,326 images	1,340 GB total (103 GB x 13 files)	13 focal planes; 11 zoom-levels; 9,745,801 tiles (256x256-pixel jpeg files)

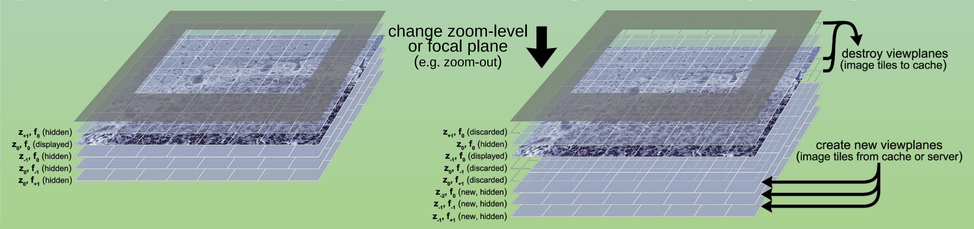
After scanning has been completed, Zeiss Zen software is used to apply a background correction (to each image) and to stitch the overlapping images into a single image for each focal plane, which is exported a bigTIFF file. The libvips dzsave() function (https://libvips.github.io/libvips/API/current/Making-image-pyramids.md.html) is used to convert each bigTIFF file into a Google-Maps-like pyramidal database of 256x256-pixel jpeg files.

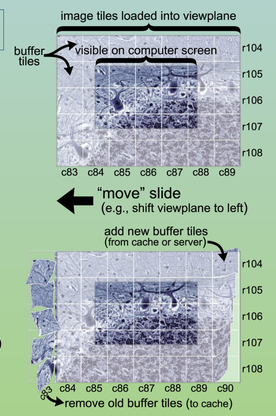


Background correction. To achieve seamless stitching, a "blank" image is necessary to correct for illumination intensity and other optical-system issues. A method was devised to generate the "blank" image by manually editing & combining images of adjacent empty fields (in Photoshop) and then averaging about 1-2 dozen of these combined images (using ImageJ) to create an ideal, averaged, dirt-free "blank" image for background correction prior to stitching.

Viewer design: Viewing multifocal-plane virtual slides

The image displayed on the computer screen is a grid of image tiles (a "viewplane") from the zoom-level and focal plane specified by the user. To improve responsiveness, the viewer software attempts to anticipate the user's needs by: (1) preloading a rim of "buffer tiles" (at the current zoom-level & focal plane) around the region currently being displayed, (2) preloading viewplanes for zoom-levels and focal planes adjacent to the current viewplane, and (3) maintaining a cache of recently used image tiles.





Introduction

This poster describes a virtual microscope that allows the user to focus up-and-down through high-resolution virtual slides.

Triola & Holloway built a single-focal-plane open-source virtual microscope that utilizes the Google-Maps API (Triola MM, Holloway WJ. (2011) BMC Med Educ. 11:4 (https://doi.org/10.1186/1472-6920-11-4)). Although the multifocal-plane virtual microscope described in this poster was created *de novo* and does not rely on Google-Maps software, this new virtual microscope uses the same tiling architecture as Google Maps and is backwardly-compatible (for single focal planes) with the Triola & Holloway virtual microscope.

There were three principal steps in creating this new virtual microscope:

1. Optimization of the technique for scanning slides.
2. Conversion of the scanned images to an appropriate format.
3. Construction of a viewer for the virtual microscope.

Conclusion

The virtual microscope presented in this poster allows the user to view histological specimens at a resolution approaching the limiting resolution of a light microscope, and allows the user to focus through the specimen to get a sense of its three-dimensional structure.

It is hoped that this virtual microscope will be generally useful for teaching histology. The virtual microscope is publicly available (at: http://viewer.pnwu.edu). The software for the microscope's viewer is open-source (at: https://github.com/Pacific-Northwest-University/virtualmicroscope/) and free (under a GNU GPL3 license). Most of the work of displaying the virtual slides is done on the client-side by the viewer, and specialized server software is not needed (in addition to the image files, the server uses several PHP files (available on github) and a SQL database). The high-resolution virtual slides created at Pacific Northwest University of Health Sciences (PNWU) are free and available under a Creative Commons license.

Acknowledgements

The microscope and computer used for scanning glass microscope slides were purchased with internal (start-up) funds provided by Pacific Northwest University of Health Sciences (PNWU). Microscope slides scanned at PNWU from the UCSF and UM student slide sets were generous gifts provided by Steven Rosen, PhD (University of California, San Francisco) and Michael Hortsch, PhD (University of Michigan) when their institutions discarded their glass teaching slides.

Disclosure

Mr. Smircich is a sales representative for Zeiss microscopes; both his current employer, Micro Optics of Florida, Inc., and his former employer, Bartels & Stout, Inc., are Carl Zeiss Authorized Dealers.

Contact Information

James A. Rhodes, PhD. Associate Professor of Histology
Pacific Northwest University of Health Sciences
200 University Parkway, Yakima, WA 98901 jrhodes@pnwu.edu

Ronald P.J Smircich, BS. Microscopy Support Specialist & Imaging Specialist
Micro Optics of Florida, Inc.
2840 Scherer Dr. N #400, St.Petersburg, FL 33716 ron@microopticsfl.com

John DeVore. Director of Network Services
Pacific Northwest University of Health Sciences
111 University Parkway, Yakima, WA 98901 jdevore@pnwu.edu