14 Speed, Resolution, Focus, and Depth of Field in Clinical Whole Slide Imaging Applications

Yukako Yagi and John Gilbertson

14.1 INTRODUCTION

The process of digital imaging in microscopy can be thought of as a series of operations each of which contributes to the quality of the final image displayed on the computer monitor. The operations include sample preparation and staining by histology, optical image formation by the microscope, digital image sampling by the CCD and camera, postprocessing and compression, transmission on the network and display on the monitor. Over the years, an extensive literature has developed on digital imaging, and each step of the process is fairly well understood. However, the development of automated, whole slide imaging systems for clinical applications has forced us to re-examine the relative significance of different parts of the digital imaging process. An obvious example is in the importance of compression. In a traditional single frame digital imaging environment, where a typical uncompressed image file may be one to several megabytes, efficient compression is a convenience. On the other hand, in a clinical whole slide imaging system which could generate several hundred, multi-gigabyte images a day, efficient compression is absolutely required for effective management.

Another area in which today's high-speed, automated whole slide imaging systems cause us to re-examine traditional thought is in the relationship between focus, optical (lateral) resolution and digital image sampling. In particular, today's high-speed whole slide imaging systems have traded precise field by field focus in exchange for overall capture speed. This trade off is highly appropriate given the requirements of high-speed image capture; however, it does force us to re-examine the nature and description of resolution and image quality in the context of whole slide imaging. This chapter does this by discussing the relationships between focus, image formation, image sampling and depth of field in the creation of high-quality images by high-speed image capture devices.

14.2 IMAGE FORMATION AND OPTICAL RESOLUTION

All pathologists realize that an image formed by the microscope is not a perfect

representation of the specimen under study. Instead, the microscope optics modulate the signal coming from the specimen in well-defined ways, and a skilled microscopist can guide or influence this modulation to fit particular needs. For example, one can "stop down" the microscope's condenser diaphragm to improve the contrast in the optical image (at the expense of resolution), but no microscopist and no microscope can produce a perfect representation of the specimen.

The most important image forming part of the microscope is its objective lens. All real objective lenses will convert a point source of light in the specimen to a blurry distribution in the image. At the back focal plane of the lens this distribution is known as an Airy pattern and has the appearance of a central disk surrounded by a series of concentric diffraction rings. As two point sources approach each other in the specimen, their corresponding Airy disks approach each other in the image. At some point the two disks touch and then merge such that they cannot be resolved into separate entities. That point, called the Rayleigh criterion, is often considered to be the sum of the radii of the two disks but actually is function of a number of factors including the contrast in the image. All factors being equal however, the larger the disk, the larger the Rayleigh criterion and the poorer the optical resolution of the microscope. To put this in another way, the larger the disk, the larger the distance between two points in the specimen that cannot be resolved in the image.

The size of the Airy disk and the optical resolution of the system is a function of the numerical aperture of the objective and condenser lenses as well as the degree of correction of aberrations built into the objective and the wavelength of light used to illuminate the specimen. When noncoherent light is used, the classical equation for optical resolution in a microscope is:

$$\text{Resolution} = 1.22 \ / \ (\text{NA[objective]} + \text{NA[condenser]}) \quad (14.1)$$

The factor 1.22 is based on objective lens design considerations (correction of aberrations) and will vary between lens. NA is the numerical aperture. NA is a function of the refractive index (n) of the media between the specimen and the lens (n = 1 in air) and the sin of the half angle subtended between lens and a point on the specimen. One can rationalize this by considering the angle between the specimen and the lens as a measure of the amount of light diffracted by the specimen and captured by the lens. The larger the angle, the more diffracted light captured and the more detail reconstructed in the image. When the NA of the condenser is equal to or greater than the NA of the objective, Equation 14.1 can be reduced to Equation 14.2.

$$\text{Resolution} = 0.61 \ / \ \text{NA[objective]} \quad (14.2)$$

Notice that optical lateral resolution is not a function of magnification. Consider a microscope with an objective lens (and matched condenser) with NA or 0.5 (this could be a 10× or 20× lens, depending on manufacture and design) illuminating a specimen with white light with an average wavelength of 0.5 microns. The system will have a lateral optical resolution of 0.61*0.5/0.5 – 0.6 microns. It will not resolve two high contrast points less than 0.6 microns apart.

14.3 DIGITAL SAMPLING AND THE SPACIAL SAMPLING INTERVAL

In a digital imaging system, the microscope forms an optical image, and the

camera's charge coupled device (CCD) samples the image. Both processes contribute to the resolution of the final captured image file.

Consider a simple imaging system made up of a square sensor (CCD) of area A, an optical system with a total magnification M and a sample. The Field of View, the area of the specimen that can be seen (and captured) by the sensor is A/M. This same physical relationship exists between the size of an individual CCD pixel, the optical magnification of the system and the area of the specimen subtended by the pixel. The area subtended by a pixel, often called the spacial sampling interval, is used by many digital slide systems as a measure of image quality and system resolution. For example, a system with square 5 micron pixels and a magnification of 20× would have a spacial sampling interval of 5/20 = 0.25 um. Note that the spacial sampling interval is based entirely on the magnification and the size of pixels, with higher magnification and smaller pixels resulting in better sampling.

The spacial sampling interval is a simple and useful metric for a whole slide imaging system; however, it must be used and understood in context. Consider the same system (5 micron pixels and a total magnification of 20). Assume for now that the microscope optical system is ideal and there is no dispersion or modulation of the optical signal from specimen to sensor (as discussed above, this is not the case for real systems). If two point sources of light where placed 0.4 microns apart on the specimen would the sensor be able to resolve them?

A system with ideal optics and a spacial sampling interval of 0.25 microns would not resolve two point sources 0.4 um apart. Each source would shine its light into adjacent pixels and the resultant captured image would contain one area of light two pixels wide. In fact, the CCD will not identify two spots of light until the spots are placed more than 0.5 microns apart on the specimen. This is a general result, if one defines resolution as the minimum distance between two objects in the specimen needed to identify those objects as being independent objects in the image, then a system's digital or pixel resolution is two times its spacial sampling interval. This is a statement of a basic theorem in information science known as Shannon's Sampling Theorem and the Nyquist criterion. The Nyquist criterion requires a sampling frequency be at least equal to twice the frequency of the signal being sampled to accurately preserve the nature of original (input) signal in the sampled output.

14.4 MATCHING OPTICAL RESOLUTION WITH DIGITAL SAMPLING

In pathology, we do not seek to image point sources of light through ideal optical systems. Rather, we image tissue specimens through real microscopes. The resolution of the final captured image is a function of both the optical resolution of the microscope (a function of the numerical aperture) and the sampling power of the CCD (a function of the spacial sampling interval). (This is actually a simplistic view, as factors such as contrast and dynamic range impact the final image quality.) In this situation, Shannon's Sampling Theorem requires that the camera have a spacial sampling interval equal to or less than half of the finest resolvable feature in the optical image.

Using the Shannon Sampling Theorem and our equations for optical lateral resolution and spacial sampling interval, we can match the relative resolving power of

the microscope and CCD (Equation 14.3a and Equation 14.3b):

$$\text{Optical Resolution} = 2 \times \text{Spacial Sampling Interval} \quad (14.3a)$$

$$0.61 \ / \ \text{NA[objective]} = 2 \times \text{Size of Pixel} \ / \ \text{Total Magnification} \quad (14.3b)$$

There are three possible outcomes in real systems:

- The spacial sampling interval under-samples the optical image — 2× Spacial Sampling Interval is greater than Optical Resolution.
- The sampling interval matches the optical resolution.
- The spacial sampling interval over-samples the optical image — 2× Spacial Sampling Interval is less than Optical Resolution.

Under-sampling the optical signal causes two disturbing effects. First, and most obviously, fine details in the optical image are not retained in the captured image file. Second, a phenomenon called *aliasing* can occur in which loss of high-frequency detail results in the creation of false low-frequency information in the captured image. This is a particular problem in samples with pronounced periodicity.

Over-sampling is far less of a problem. It will not cause image quality problems and may in fact improve image quality especially in areas of low contrast and in system with low dynamic range. Over-sampling will cause file sizes to be larger than necessary and will cause longer capture times in whole slide imaging systems.

Traditional camera-on-microscope imaging systems must support multiple objective lenses (with different numerical apertures and magnifications) tied to a single CCD camera (with a single pixel size). It is hard therefore to balance optical resolution and spacial sampling interval in the design of these systems. Whole slide imaging systems in contrast use a single objective lens and a single camera for image capture. One can therefore use Equation 14.3 to balance optics and sampling in the design of whole slide imaging systems. For example, consider a system using a 20×, 0.5 NA lens and 5 micron pixels:

$$0.61 * 0.5/0.5 \text{ vs. } 2 * (5/20)$$

$0.61 > 0.5$ so this system slightly over-samples the optical image and should perform well. However, replacing the camera with one using 7 micron pixels will result in under-sampling.

14.5 HIGH-SPEED ROBOTIC IMAGE CAPTURE AND FOCUS

The Shannon Sampling Theorem, the Spacial Sampling Interval and the calculation of Optical Lateral Resolution through the Numerical Aperture are important and useful tools in the design and measurement of microscope digital imaging systems, including whole slide imaging robots, but blind use of these tools is can be misleading. Clearly, resolution is only one component of image quality. Contrast and dynamic range, color space, postprocessing, compression, and display fidelity are some of the many other parameters that affect a system's image quality and performance as measured by its Modulation Transfer Function. However, there is an even more important factor to consider in the evaluation of resolution in high-speed, automated whole slide imaging systems — estimation of optical resolution as defined in Equation 14.2 is relevant only if the specimen is in focus.

To create a digital slide in perfect focus is not difficult in principle. As one captures

the slide, field by field or line by line, one would run an auto-focus routine before capturing each frame. However, to capture a one-square centimeter tissue section at 20× with standard two-third inch CCD would require 700 fields. If one could focus and capture a field every second, it would take 2.43 hours to capture a single 5 × 2.5 cm coverslip. As discussed above, automated whole slide imaging systems such as those proposed for clinical use are designed for high-speed capture (several minutes/slide). Therefore, virtually all (if not all) of these systems have sacrificed precise focus in favor of maximum speed.

To provide high-speed capture, all systems today capture in two phases — a focusing phase (that calculates an in focus plane above the slide) and a capture phase (in which the objective moves rapidly and continuously, never stopping, following the in focus plane until all of the tissue is captured). An example prototype system was designed by Art Wetzel and one of the authors (JG) in 1999 at Interscope Technologies. Interscope no longer produces such a system, but the basic principles apply to all high-speed vendors.

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When a user presents a slide to the system, the system reads a bar code to identify the slide. Then a low resolution, single frame image is take of the entire slide. From this image, the system identifies the distribution of tissue on the slide and any areas of high contrast. This information is used to identify between 8 and 12 potential focus points. The system goes to each point and runs a standard auto-routine that identifies the ideal working distance for focus at that point by identifying the working distance that maximizes the contrast between pixels. With these points in hand, software attempts to calculate a plane over the slide that represents an estimate of best working distance over each point of the specimen (that would make the image in focus). If such a plane cannot be calculated within certain parameters, other focus points are added (and others dropped) until a satisfactory plane is generated. Then, in the capture phase, the objective lens moves rapidly and continuously over the slide, following the in focus plane and capturing the entire slide.

Prefocusing using an in focus plane works fairly well but does suffer from two serious problems. The first is the lever problem. When one generates a plane on the basis of a few measured points, areas outside of the points tend to be artificially raised up or pushed down as the measured points act as fulcrums and the area of the slide acts as a lever. Careful algorithm design can mitigate but not eliminate this problem. The second problem is worse and arises from the fact that tissue sections on slides are not flat planes but rather three-dimensional surfaces. The attempt to place an in focus plane over a three dimensional surface results in areas in which the working distance between the lens and the tissue is not optimal and the image loses focus.

Areas of poor focus are a significant limitation to the implementation of whole slide imaging in the clinical lab. In fact, in every study we have done looking at pathologist's acceptance of digital slides, areas of poor focus has been the number one concern. Numerous groups are attempting to develop a wide range of computational, mechanical and optical solutions to the problem of focus in high-speed whole slide image capture. Though these solutions are proprietary and therefore well beyond this discussion, it is worth noting that there are at least two basic types of solutions. One solution either better controls the working distance through either active feedback or better planning, or one solution gives the system greater leeway by increasing the depth of field.

14.6 DEPTH OF FIELD

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It has been mentioned in an early section that the objective lens modulates the light from a point source in the specimen into a blurry distribution (often referred to as a point spread function) in the lens's back focal plane. When the specimen is in focus, a section at the back focal plan perpendicular to the optical axis will reveal the familiar Airy Disk(s). However, the distribution is actually three-dimensional. It is radially symmetric around the optical axis and periodic along the axis, with increasing blur (and decreasing contrast transfer) as one moves away from the back focal plane.

Consider two point sources of light in a specimen. In this case the sources are placed not next to each other but one above the other. Using the same logic used to discuss lateral resolution and Airy disks, we can define the axial resolution of objective/condenser pair as the axial (z-axis) distance between two sources in the specimen at which their point spread functions cannot be distinguished.

Axial resolution can also be expressed as depth of field: the axial (z-axis) distance that an infinitely thin specimen can be moved and still remain in focus. Because the point spread function is complex (and the criteria for in focus ill defined), numerous authors have proposed equations to estimate the depth of field for microscope systems. The most accepted equation for depth of field in brightfield microscope using incoherent light (open condenser) is given by Equation 14.4:

$$\text{Depth of Field} = (* \text{ n / NA}^2) + (\text{n} * \text{e} / \text{M} * \text{NA}) \quad (14.4)$$

Where lambda is the wavelength, n is the refractive index, NA is the numerical aperture, M is the total magnification and e is the smallest feature that can be resolved by the CCD (2× pixel size). In our laboratory, we have measured the depth of field for a number of digital imaging system, including whole slide imagers and have verified that Equation 14.4 generally applies.

Optical designers have traditionally tried to minimize depth of field. Small depth of field results in thinner focal planes or optical sections, and thinner optical sections result in clearer sharper images. Essentially, the point spread functions from objects above or below the subject of interest interfere less with the image. However, given the fact that pathologists identify focal loss of focus as the main limitation to the use of digital slides in clinical practice, minimizing depth of field may not be the ideal optical design for today's high-speed whole slide imagers.

14.7 TRADE OFFS IN THE DESIGN AND SELECTION OF WHOLE SLIDE IMAGING SYSTEMS

Consider two optical setups, both using a CCD with square, 5 micron pixels. In the first system, there is a 0.6 NA objective lens. The objective has a magnification of 20×, and there is a 1× relay lens. If the specimen is in focus, the set up will have an optical lateral resolution of 0.5 microns (Equation 14.2) and a sampling interval of 5/20 = 0.25 microns. Using the Shannon Sampling Theorem, the lateral optical resolution and the digital sampling are matched (Equation 14.3). However, the depth of field with this set up will be approximately 2.2 microns (Equation 14.4).

The second set up uses the same CCD but a 0.3 NA, 10× objective lens with a 2× relay lens (for a total magnification of 20×). The optical lateral resolution

(Equation 14.2) will be 1.0 microns with the same sampling interval. The set up will over sample the image (Equation 14.3) but that should not cause a problem with image quality. The depth of field of this set up is 7.3 microns (Equation 14.4).

The first optical set up has better lateral resolution while the second has a more forgiving depth of field. Which one will perform better when applied to a whole slide imaging system will depend largely on the whole slide imaging system's ability to maintain the ideal working distance as it moves across the slide. Given the limitations of most current system in maintaining focus, depth of field must be considered an important parameter in the design and selection of whole slide imaging systems. Experiments ongoing at the University of Pittsburgh (to be published later this year) indicate that increasing depth of field at the expense of maximum lateral resolution can in fact result in better overall image quality in whole slide systems.

14.8 CONCLUSIONS

The development of automated, high-speed, high resolution whole slide imaging robots is forcing pathology to re-examine the significance of many aspects of digital microscopy. One of these aspects is the importance of depth of field as a measure of image capture consistency and quality. As high-speed capture systems struggle with focus versus speed, designers may consider trading maximum lateral resolution for the safety of increased depth of field.

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