WHITEPAPER

4 Steps to Optimize the Optics in Automated Imaging Instruments

Optimize the performance and cost of your automated optical imaging system with 6 key equations for selecting an imaging sensor, objective lens, Z-focusing nano-positioning stage and XY sample positioning motion.

Topics covered include: optimal imaging sensor size and resolution, objective lens magnification and numerical aperture, and XYZ motion resolution and stability requirements to enable crisp images. Also discussed is how diffraction affects image resolution, and how to calculate the depth of field for a given objective lens.

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Introduction

Automated digital microscopes are central to a vast array of biomedical, life science, and diagnostic instruments from pathology scanners to DNA sequencers to cell imagers. These microscope instruments amalgamate rapid mechanical motion and precise optics in order to achieve breakthrough imaging resolution and throughput. Some of these systems employ simple brightfield imaging with epi-illumination (through the objective). Another approach to lighting or fluorescing the sample is transmissive illumination, where the light source is separate from the optical path, typically placed on the opposite side of the sample as the objective. In both cases, some simple optical rules can be used to configure a system and drive the requirements for the system's mechanics. This article will explore how the optics and mechanics interact and the 4 key steps to focus on when automating a microscope.

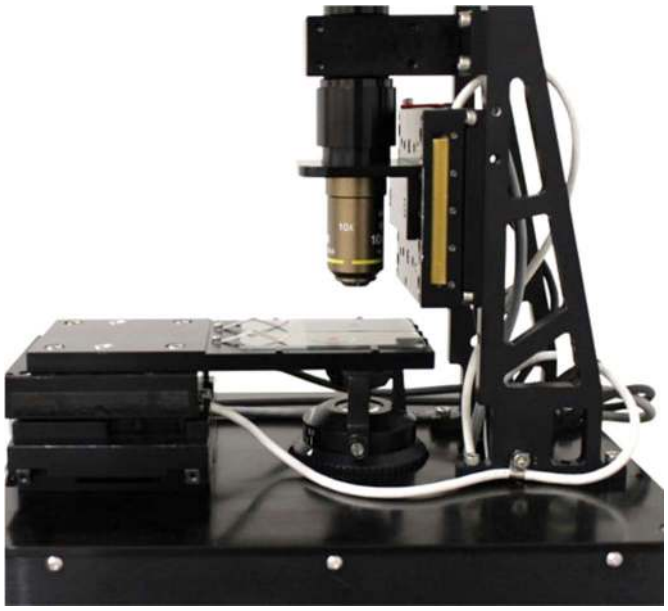

Figure 1 – Automated Digital Microscope with transmissive illumination

1 – Determine the Numerical Aperture (NA)

Objectives are specified with a value for the magnification and numerical aperture, and these values are usually printed on the side of the objective. The Numerical Aperture for a microscope objective is defined as how much light the objective is able to capture from the specimen at a fixed distance away from the sample. Since light exits the specimen at an angle, like water spraying out of a hose shown in Figure 2, it is prudent to capture as much of that light as possible through the objective. Catching as many orders of light as possible (shown in Figure 2) enables a high resolution image of the specimen. It is for this reason that you want to select the highest NA objective that is practical and affordable for your application.

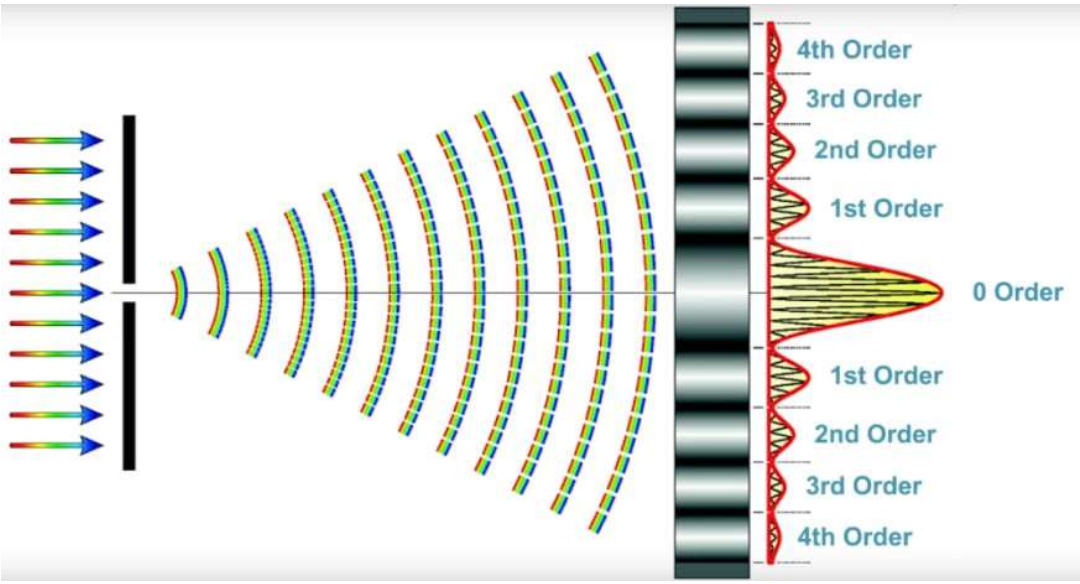

Figure 2– Diffraction of Light through a Pinhole

For this demonstration we define θ as the angle of the cone of light that exits the sample and is captured by the objective. Figure 3 illustrates how θ changes depending on how close the objective is to the sample. As you get closer to the sample, θ gets larger and you are able to capture more light from the sample. "n" in Equation 1 refers to the refractive index of the material between the objective and the sample. Typically, this medium is air and "n" = 1.

$$NA = \mathrm{n} \cdot \sin(\frac{\theta}{2})$$

Equation 1

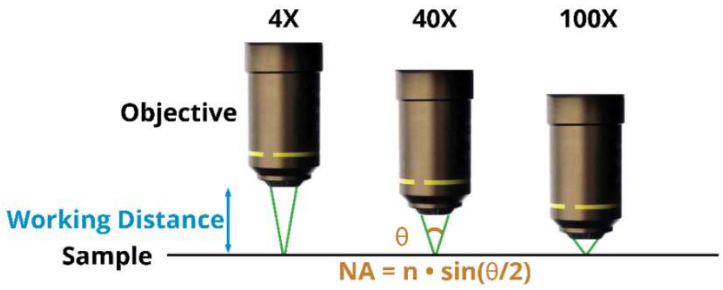

Figure 3 – Example of Objectives at Differing Working Distances

The first step, prior to calculating the numerical aperture (NA), is determining the optical resolution for the system. The optical resolution depends on the type of specimen being imaged, and the size of the features that need to be resolved. While a simple mammalian cell counting application may need a resolution of 2 µm, a pathology application targeting fine structural morphologies of cell nuclei may require 0.35 µm resolution. The NA is related to the optical diffraction limited resolution (R) as shown in Equation 2 and Equation 3:

$$R = \frac{(1.22 \times \lambda)}{(2\,x\,NA)}$$

Equation 2

Or

$$NA = \frac{(0.61 \times \lambda)}{R} \quad *$$

Equation 3

*Note: 0.5 is an acceptable substitute for the 0.61 coefficient (this is not absolute and varies by application)

By defining λ as is the wavelength of the illuminating light (typically around 0.5 µm) and R as the desired resolution, then the resulting unitless value of NA can range from 0.10 to 1.47. In the examples introduced above, the cell counting application requires an NA of only 0.12 while the pathology application requires an NA of 0.70.

2 – Select the Magnification

With the NA calculated the objective's magnification can now be chosen. The higher the NA, the higher the objective's magnification requirement. Typical NA ranges for microscope objectives are shown in Table 1 below, note the overlap in magnification options for a given numerical aperture value.

Objective Magnification	NA Range
4X	0.1 to 0.2
10X	0.25 to 0.45
20X	0.35 to 0.8
50X	0.5 to 0.9
100X	0.85 to 1.4

Table 1 – Example Objective Magnification and available NA Range (this varies by Objective Manufacturer)

In general, higher NA objectives within a given magnification are more expensive but the tradeoff is a higher image resolution.

Figure 4 – Example Objectives

Note that the material between the sample and the objective can affect NA. When imaging slides, in order to exceed an NA of 1 a liquid can be added between the coverslip and the objective. Typically, if an NA greater than 1 is required for the application, oil immersion or water immersion objectives are used. Water immersion objectives can have an NA ≤ 1.1 and oil immersion objectives can reach higher NA values of ≤1.47.

3 – Calculate the Field of View (FOV) for XY Motion

With the magnification selected the Field Of View (FOV) from the biological sample can be calculated. This calculation will help determine how much sampling, and therefore motion system movement, must be done to capture the entire area of interest of the specimen. An imaging sensor is typically referred to by its diagonal measure similar to how TV's are specified. Equation 4 is a simplistic calculation of FOV Diagonal: sensor diagonal measure divided by the magnification of the objective. The same equation can be used for the FOV Width and Length by substituting the Sensor Width and Length with the sensor diagonal measure in the equation. For example, FOV = 20mm/20x = 1mm, which is a typical FOV value for microscopy applications.

$$FOV\ Diagonal\ = \frac{(Sensor\ Diagonal\ Measure)}{(Magnification\ of\ Objective)}$$

Equation 4

This calculation can be used with pixels as well. For example, a sensor with 4 micron pixels and a 40X objective, yields 0.1 µm of geometric resolution or 100 nanometers. However, this simplistic approach of treating light as a particle going through a hole (Equation 4) may lead to significant oversampling of the specimen due to diffraction as shown in Table 2 below, and Figure 2– Diffraction of Light through a Pinhole, above.

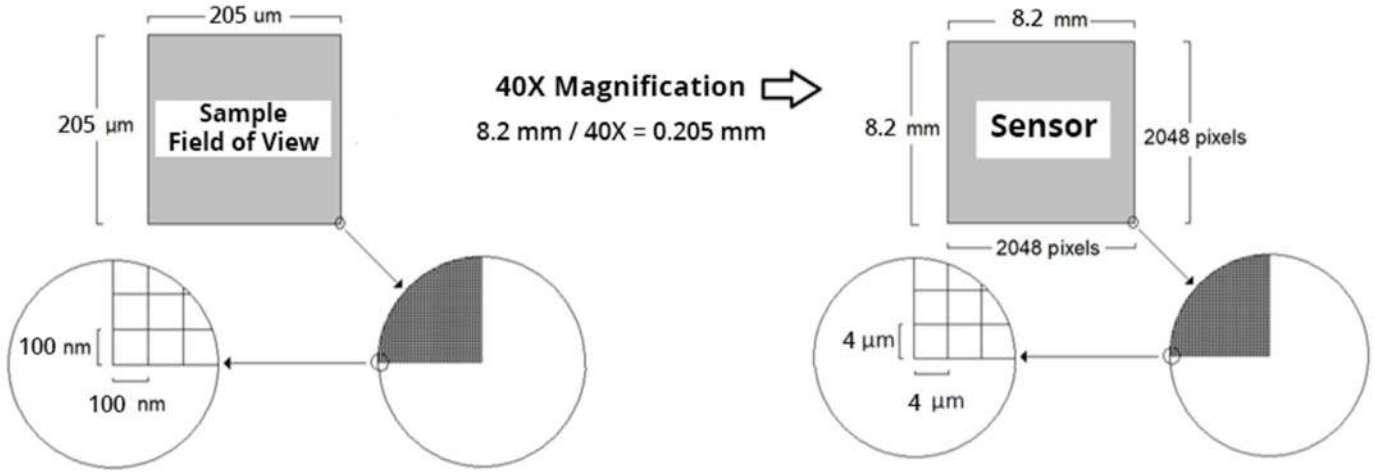

Figure 5 – Example Field of View (FOV) of a Sample

Since light is a photon, it turns out that the resolution limit of the microscope is based on the wavelength of light (λ) and the Numerical Aperture:

$$Diffraction\ Limited\ Resolution := \frac{(1.22\ \times\ \lambda)}{(2\ \times NA)}$$ Equation 5

Returning to the example with the 4 micron pixel sensor used with a 40X objective, it will be assumed that the numerical aperture is 0.8 and λ is 550 nm (yellow-green light). The calculated diffraction limited resolution is 419 nm, which is > 4 times the calculated geometric resolution of 100 nm. The implication is that if Equation 4 was used instead of Equation 5, the system would be designed to oversample the specimen by up to 4 times. Oversampling is more than digital waste – when system movement and settling is accounted for, sampling time increases and throughput decreases.

	Geometric FOV Calculation	Diffraction Limited Resolution
Equation	$FOV = \frac{(Sensor\ Diag)}{(Mag.of\ Obj.)}$	$Res: = \frac{(1.22\ \times\ \lambda)}{(2\ \times NA)}$
Inputs	Diagonal= 4 micron pixels Magnification = 40X	λ = 550 nm NA = 0.8
Calculated Resolution	100 nm	419 nm

Table 2 – Example of FOV Resolution vs. Diffraction Limited Resolution

A typical biological sample will be many times larger than this field of view. Now that the diffraction limited resolution (or corrected FOV) is known, the movements of a programmable XY stage can be determined. A precision XY stage allows many small fields of view across the total area of the sample to be imaged sequentially moving quickly from one FOV to the next.

There are two approaches to imaging a sample, TDI Imaging (where velocity stability is key) and Sequential Field Imaging. Most imaging applications use sequential field imaging, and the XY stage has two roles: the first to accurately (and quickly) jump from one FOV to the next and the second to settle quickly at the new FOV. As soon as the stage has settled within the optical resolution, image exposure can begin. The travel of the XY stage should at least equal the length and width of the sample with additional travel for sample loading and unloading.

Figure 6 – Example of an XY Stage: Dover Motion SmartStage™ XY

There are several ways to implement an XY stage, with direct drive linear motion options like Dover Motion's SmartStage XY delivering the fastest and most accurate sample positioning (which is especially useful when a high NA objective is required). The drawbacks of using a traditional stepping motor and a leadscrew are slower speed and worse accuracy and repeatability. With a leadscrew additional time must be budgeted after the end of each movement for the high-Q ringing of the payload mass and nut/bearing compliance to damp out. For high throughput applications, direct drive stages with linear servo motors and linear encoders provide the highest levels of performance. For a typical move size (FOV) of 0.75 mm, a direct drive stage such as the Dover Motion SmartStage XY [1] series can move and settle to under 0.5 µm in about 50 milliseconds. Depending on the integration time of the digital camera and the illumination intensity, this can permit as many as 16 images per second to be acquired. The actual (not empty) resolution of the XY stage must be several times finer than the optical resolution; in the above 20X, 0.70 NA example, the stage resolution should be at least 0.1µm. To understand the actual stage performance, make sure to check on the Minimum Incremental Move that the stage can repeatably perform as the resolution of the feedback device can be misleading.

4 – Determine the Depth of Field for Z-Motion

Next, the Depth of Field (DOF) needs to be calculated in order to determine the Z-motion requirements. DOF is the distance along the optical axis that is required to move the sample into focus. In Figure 7 below, having the objective outside of the dashed blue lines will result in a blurry image. See Equation 6 for the DOF calculation:

$$DOF := \pm \frac{\lambda \sqrt{(1 - NA^2)}}{NA^2}$$

Equation 6

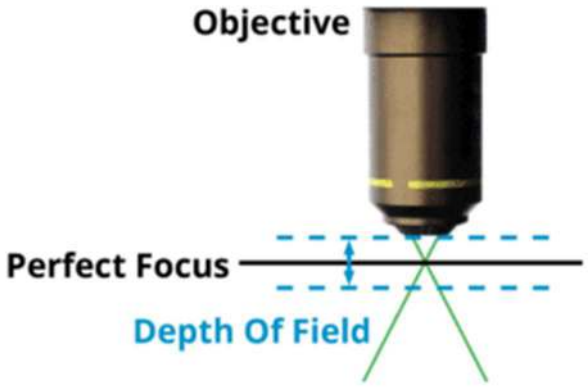

Figure 7 – Depth of Field (DOF) from an Objective

For high NA systems the demands on the DOF (or Z-axis) become much tighter. Using a 20X magnification (0.80 NA) apochromatic objective the depth of field is only +/- 0.48 µm.

Similar to the X and Y axes, the Z axis must move quickly and settle quickly. If an application is using software based contrast autofocus techniques there is typically many iterations of moving in Z then analyzing an image to determine if it is in focus at each image location on the sample. In response to high precision and small DOF requirements, Dover Motion released their Nanopositioning Stage for Objective Focusing, the DOF-5 [2]. The DOF-5 has a patented design with a built-in high performance motion controller, and is a new paradigm in focusing. Prior to the DOF-5, focusing motion was by default a piezo stage. Compared to the typical flexure based piezo stage, the DOF-5 offers greater travel, higher bandwidth, providing fast step and settle while maintaining image stability all at a very low cost.

Figure 8 – Dover Motion DOF-5 Nanopositioning Stage for Objective Focusing

Conclusion

Automating biological sample imaging requires an understanding of how the optical system requirements drive the mechanical system inputs. By establishing the 4 key optical requirements for the system, the required X-Y-Z movements can be determined. The first step was to determine the Numerical Aperture (NA) which was driven by the required optical resolution. This led to a determination of the system Magnification. Third, the Field of View (FOV) was calculated which determines the XY movement requirements for the system. Finally, the Depth of Field (DOF) requirement was calculated to determine the systems Z axis specifications.

References

1 – SmartStage™ XY – Direct Drive Linear XY Stages (https://dovermotion.com/products/linear-stages/direct-drive-linear-motor-stages/smartstage-xy/)
2 - Nanopositioning Stage for Objective Focusing – DOF-5 (https://dovermotion.com/products/linear-stages/microscope-stages/objective-focusing-stage-dof-5/)

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