The Leading Investigator

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Leica MICROSYSTEMS

A Powerful Vision

To See or Not to See

By Rob Kimura, *Leica Product Manager*

As the evolution of digital photomicrography continues, new generations of high-resolution cameras have become available for forensic imaging. Digital camera technology is mainly driven by the consumer market where 'the more pixels the better' is the status quo. In recent years the consumer market has seen color cameras jump from 1.3 megapixels to 12 megapixels and higher. A common question people ask is, "How much higher will camera resolution get?" But the real question for forensic investigators should be, "What do I gain by using a high-resolution digital camera on my microscope?"

Understanding Image Formation

Due to the physics of the image formation process, even a perfect microscope objective will blur two adjacent objects into a single object when placed close enough together. One way to consider this 'limiting resolution' is to image a repeating pattern of adjacent black and white lines. When the number of 'line pairs' per millimeter (lp/mm) is increased beyond the optical resolution limit of the microscope, the image will no longer form lines, but instead will form a uniform gray background. In addition to blurring an image, an objective lens also magnifies an object. At the camera, this translates into an image spread across a larger area whenever magnification is greater than 1x. Also, a microscope may be configured with intermediate optical components to change the net magnification to the camera; for the purposes of this article, we will assume that the microscope has a 1x magnification c-mount attachment.

How Many Pixels?

One would expect that the ideal pixel correlation would place 2 pixels across each line pair so that one pixel can detect the white line and the other pixel the black line. However, this pixel ratio can produce a gray result because pixels can be placed between the white and black lines. To resolve all line pairs in all cases, there must be at least 3 pixels per line pair. As you can see in the ideal case shown in Diagram 1, with at least 3 pixels per line pair, the camera can now detect the line pairs, even if pixels shift to the left or right. It is important to note that further increasing the number of pixels can lead to 'over-sampling', where the additional pixels per line pair provide no gain in spatial information. However, the transition to over-sampling depends on the wavelength of light used, the objective's numerical aperture, magnification to the camera, and the camera's pixel size.

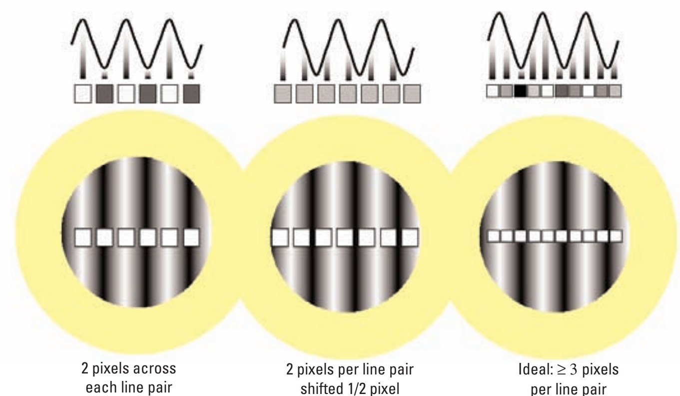

Diagram 1

Determining Resolution as Defined by Line Pairs

With simple assumptions, we can estimate the limiting resolution for a microscope objective, determine the number of line pairs across the field of view (FOV), and compare this to the number of pixels covering the same distance for a given camera. There are many mathematical definitions for optical resolution (R), but a simple

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approximation is:

$$R = \frac{\lambda}{2(NA)}$$

where λ is the wavelength of the light in nanometers (nm)

This relationship indicates that when using a high NA lens and white light illumination, the smallest resolvable distance is about 300nm or 0.3µm. In terms of line pairs per mm at a mid wavelength of the visible light spectrum, green 550nm:

Optical Resolution (lp/mm) = NA x 3000

To calculate the number of line pairs required to cover the objective's FOV, calculate the area visible through the lens by dividing the FOV of your microscope (let's assume 22mm) by the magnification factor.

A 5x objective allows you to observe an area of 4.4mm ϕ

$$\frac{22\text{mm FOV}}{5} = 4.4\text{mm}$$

A 50x objective allows you to observe an area of only less than 0.5mm ϕ

$$\frac{22\text{mm FOV}}{50} = 0.44\text{mm}$$

So how many line pairs can be observed with a 5x objective? Simply multiply the visible area by the optical resolution to calculate the FOV. But please note that there is one caveat in calculating FOV. While a microscope objective forms a circular image, a camera sensor is typically square or rectangular. If the FOV for the microscope is 22mm, then the FOV of the sensors reduced by the square root of 2.

Example: Using a 5x objective with a .15 NA and a 22mm FOV

$$\frac{4.4}{\sqrt{2}} = 3.1\text{mm}$$

Achievable Optical Resolution = 0.15 x 3000 = 450 lp/mm

Line pairs covering camera sensor = 3.1mm x 450 lp/mm = 1400 lp

Monochrome Camera

The monochrome camera presents an ideal case as every pixel contributes equally to the resolution. The number of pixels required to capture every bit of spatial information coming from the objective is:

Pixels across line pair = 1400 lp x 3 pixels/lp = 4200 pixels

Camera resolution is specified in terms of the total number of pixels, so assuming a rectangular 4:3 format, the number of pixels needed for ideal digital image quality is:

4200 pixels x 3150 pixels = 13 megapixels

Color Camera

A pixel in a color (Bayer Matrix) camera performs two functions, spatial sampling of the image and measuring the intensity for a specific position of the spectrum, e.g., red, green, and blue. Consequently, it takes more pixels (approximately 25% more per line pair) to obtain the same resolution as a monochrome camera.

Required color camera resolution = (4 pixels/lp) => 23 megapixels

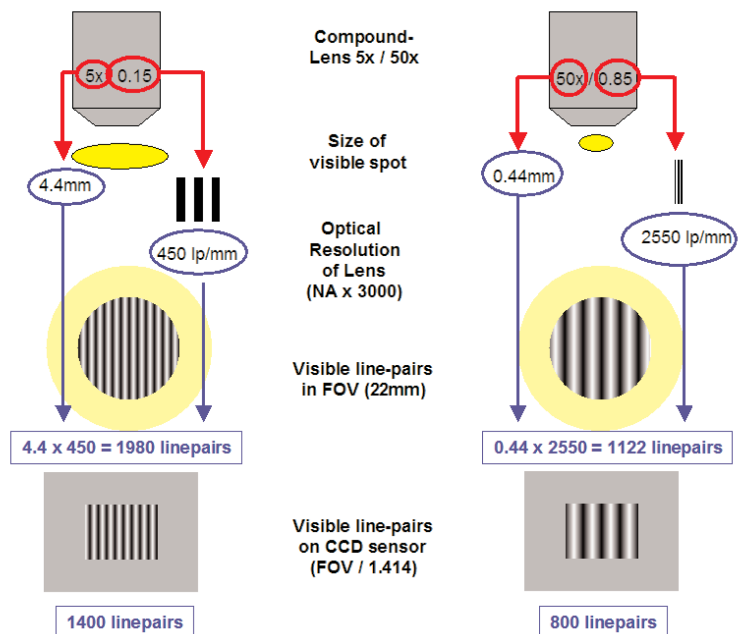

Diagram 2

In the above example, the lens is capable of better resolution than today's high-resolution cameras can capture.

At higher magnification, a lower megapixel camera is required. Lets take a 50x, 0.85 NA objective with a square FOV as an example:

$$\frac{\left(\frac{22\text{mm}}{50\text{x}}\right)}{\sqrt{2}} = 0.31\text{mm}$$

Achievable Optical Resolution = 0.85 NA x 3000 = 2550 lp/mm

Line pairs covering the camera's sensor = 0.31mm x 2550 lp/mm = 790 lp

Required monochrome camera resolution (3 pixels/lp) = > 4.2 megapixels

Required color camera resolution (4 pixels/lp) = >7.5 megapixels

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Do I Need a High-Resolution Digital Camera?

Now let's get back to our original question. High-resolution, 8-plus megapixel cameras provide a resolution benefit at low magnification. But as magnification increases, they provide less of an advantage. What is optimum depends on what kind of imaging you are doing. More pixels will increase the size of the image file. As the number of pixels increases, each pixel is typically smaller, which results in reduced dynamic range and light sensitivity.

Going beyond 8 megapixels makes sense for very low magnifications typically used in comparison forensic macroscopy, if the entire available FOV is used. But even with fewer pixels, adjusting the magnification to the camera with an intermediate optic accessory such as a 1x magnification c-mount, you can match the camera to the microscope's resolution across a limited field of view.

Comparison Illumination

Achieving the Best Illumination for Your Comparison Microscope

by Wayne Buttermore, *Leica Marketing Manager, Forensic Microscopy*

One of the biggest challenges in comparison microscopy is balancing color and intensity of the light sources between the two microscopes. Unlike reflected light applications the transmitted light comparison microscope has many more variables, that affect both illumination intensity and color balance.

The setup of a compound microscope for diffraction-limited examination of trace evidence is best achieved by setting up Koehler illumination. Experienced microscopists can reproducibly establish the settings for the field and aperture diaphragms on an individual microscope to achieve the best resolution and contrast for a sample. However, when a second microscope is added to the system, matching the setup becomes far more complex.

First, let's look at the different illumination types that are used for comparison microscopy:

1. Two separate lamphouses controlled by separate power supplies: This was a common illumination setup found on older comparison microscopes from American Optical, Leitz, and Leeds. *Pre 1980*
2. Two separate lamphouses controlled by separate power supplies with continuous variable filter systems in the illumination path (Leica Variolux®): Leitz and Leica used this configuration for many years on comparison systems using the Laborlux, Dialux, Dialux 20, DM R, and DM4000 microscope platforms. *1980's to present*
3. Randomized bifurcated fiber optic light guides from a single cold light source (150W or 250W): Adapted to the illumination path in place of standard halogen light bulb sources. *Mid 1980's to present*

A microscope is designed with many optical elements apart from the illumination source that affect color. As light travels through the microscope, it passes through a collecting lens, diffuser, field diaphragm, glass cover for the light exit port, filter holder for colored glass or interference filters, aperture diaphragm, condenser element, slide, mounting media with sample, coverglass … and finally to the objective, eyetube lens, and eyepieces. Each of these components can be responsible for changing the light path, intensity, and color of the light passing through the optical system.

In the design and manufacture of modern comparison microscopes, care is taken to select matching optical components to reduce these

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Comparison Illumination

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influences by decreasing manufacturing tolerances and ensuring that components originate from the same production lot. Ideally, this leaves only lamp intensity, condenser height position, and field and aperture diaphragm settings as variables to contend with. In systems having a Leica Variolux® adjustable filter device, the process of balancing illumination and color is simplified.

How do I ensure balanced intensity and color?

Initially it is critical to set up the two light sources as uniformly as possible:

1. Prepare two identical slides with slides and coverglass from the same box. Place a slide on each microscope stage.
2. Place the comparison bridge in the side-by-side image mode.
3. Select the 10x objective for each microscope.
   a. When using a two-lamphouse system, adjust lamp intensity so the voltage is the same for both sides. Adjust the collector lens for each lamphouse so that intensity and homogeneity of the field of view is as uniform as possible.
   b. In microscopes with bifurcated fiber optic systems, adjust lamp intensity to a comfortable level for the objective in use. If intensity or homogeneity is not consistent, rotate the fiber bundle at its interface with the microscope. Then secure it in place. Intensity can be influenced by moving the fiber optic guide forward or backward in the mount.
4. Be sure to remove any colored glass filters, polarizers or polarization compensators from the light path.
5. Remove the first two slides from step 1, and place two new slides, each having a sample and coverglass, on the stage of each of the microscopes.
6. Establish Koehler illumination by setting the condenser height, centering the field diaphragm, and adjusting the aperture diaphragm to match the objective in use. (Leica users can refer to the Leica DM R user manual, Figure 49.) Discussion of Koehler illumination is outside the scope of this article.

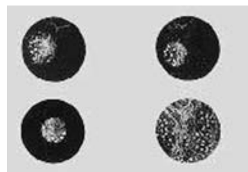


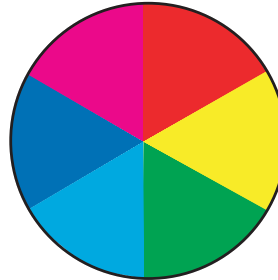
Figure 49: establish Koehler illumination

In microscopes with a bifurcated illumination system, these steps should be all that is required to obtain visually balanced light intensity and color.

Two Lamphouse Systems:

Systems having two light sources and Variolux continuous illumination control may require further adjustment. A Variolux contains three independent filter controls for continuous introduction of red, green or blue filters with increasing gradients. This introduces infinitely adjustable color to the illumination path on each microscope. With practice, it provides a perfect match of color and intensity during side-by-side examinations.

Procedure for Adjusting Variolux®



Color filter wheel

1. When starting, be sure to remove any colored filters from the optical path. Essentially this means neutralizing the Variolux by turning the filters to the open position. It is easiest to accomplish this by observing the field of view and turning each color filter control. If color is added while turning the knob, reverse direction until it reaches the stop position. This needs to be completed on both microscopes. If any colors were introduced by the Variolux during initial illumination setup as described above, repeat the illumination setup.

2. Carefully examine the color of the light on each microscope side. Keep in mind the complimentary color. If a yellow tint is viewed on one side, the addition of an equal amount of blue would be required to offset the effect. If the color match gets worse as the process continues, start over.

3. When any component in the optical path is changed, such as the sample, objective or aperture setting, you may need to readjust the Variolux.

Balancing illumination intensity and color takes practice. It is much easier to set up a system for visual examination. A camera system, whether 35mm or digital, is far more sensitive to color and density differences than the human eye, so fine tuning the camera may still be necessary.

Next issue: Achieving the best illumination for your digital camera

Industry News

The AAFS (American Academy of Forensic Sciences) will hold its 59th Annual Scientific Meeting on February 19-24, 2007 at the Henry B. Gonzalez Convention Center in San Antonio, Texas. The Academy's annual scientific meeting presents over 500 scientific papers, breakfast seminars, workshops, and other special events. The AAFS represents a wide range of forensic specialties.
More information: **www.aafs.org**

The 38th annual AFTE Training Seminar will take place at the Hyatt Regency, San Francisco, California on May 27-June 1, 2007. AFTE welcomes everyone to this beautiful city and to what promises to be a fantastic training conference. The host committee comprises not only the San Francisco Police Department – Firearms and Toolmark Unit, but also firearms examiners from various agencies all over Northern California such as Contra Costa County Sheriff's Office, Santa Clara County, Fresno DOJ, Sacramento DOJ, BATFE-Walnut Creek, Sacramento County, and Oakland PD.
More information: **www.afte.org**

The 2007 ASQDE Meeting will be held on August 11-16, 2007 at the Boulder, CO Millennium Harvest House. More information will be provided as it becomes available: **www.asqde.org**

The 2007 Annual Meeting of the Southern Association of Forensic Scientists will be on September 9-14, 2007 in Atlanta. A full program is planned including Advanced Structure Elucidation (DI), LC Tandem MS use in Post-mortem Cases, and Statistics in DNA Analysis. More information: **www.southernforensic.org**

Glossary

Koehler Illumination: A characteristic feature of the light path provided by all high-quality microscopes is the consistent imaging of the "luminous spot on the light source" and the "illuminated field" through all imaging stages of the microscope, from the light source to the final image. These conditions are met when the field and aperture diaphragm, positions in the microscope light path are conjugated to the object plane and to the rear focal plane of the objective, respectively.

White Point: A white point is one of a number of reference illuminants used in Colorimetry, which serve to define the color "white". Depending on the application, different definitions of white are needed to give acceptable results.

Color Temperature: Visible light is commonly described by its color temperature. A traditional incandescent light source's color temperature is determined by comparing its hue with a theoretical, heated, black-body radiator. The lamp's color temperature is the temperature, measured in kelvins, at which the heated black-body radiator matches the hue of the lamp. Color temperature is sometimes used loosely to mean "white balance" or "white point".

Line Pairs Resolution: Line "pairs" are often used to measure resolution instead of lines. A line pair is a pair of adjacent dark and light lines; while a line counts both dark lines and light lines. A resolution of 5 line pairs per mm means 5 dark lines alternating with 5 light lines, or 10 lines per mm.

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Note: We are interested in your comments and thoughts about the newsletter. Please feel free to email your comments to ***molly.lundberg@leica-microsystems.com.***