Improving Color Consistency, Color Integrity and Consequent Speed In Reading Slides

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Abstract: Color calibration of images from microscopes has emerged as both a desired method by the International Color Consortium and a means for increasing the speed of slide reading, and its implementation into Pathology and other medical specialties is anticipated. Until recently, no calibration standard was small enough to be imaged under a microscope. A new color calibration system named ChromaCal was released in February, 2014, and advantages to using this system lay in consistent colors among various camera/microscope systems. A method for measuring consistency and color intensity is shown with compelling results. Calibrated images were measured against the original images for color consistency and for color intensity among 3 scientific grade cameras. This study found the improvement in uniformity of images from camera to camera was nearly 50%; and greater color intensity increased, on average, by nearly 40%. Some before and after images increased in consistency upwards of 70%, and others increased in color intensity at 52%. This paper confirms that the use of the ChromaCal calibration system significantly improves both consistency and color intensity, with a resulting effectiveness in communicating results to colleagues and discrimination of specimen details.

Color images from microscopes, even with expensive cameras, are generally not a faithful representation of colors in the sample itself. [1] When these colors are consistently more precise among microscopy sessions, efficient high volume slide reading by pathologists follows. A result of seeing colors more precisely led to a 22% increase in speed in a study conducted by Elizabeth Krupinski, PhD [2]. When reading hundreds of slides per day in a typical clinical setting, that increase in speed translates to a cut in reading time by 1.75 hours in an 8 hour day.

Each camera shifts in color, whether it uses a single color matrix chip (CCD, CMOS), a rotating filter, or a piezo motor-driven chip. [3] This is true even after white balancing the camera for the other major contribution to incorrect colors: the nature of the light such as tungsten-halogen, LED, xenon, etc. (with the exception of user error, other contributions are minor, such as incorrect condenser settings, the variety of cover slips, and the various objectives).

Color calibration targets provide a standard set of colors to which the camera's colors can be calibrated.[4] In doing so, the color variation can be largely reduced across different brands of cameras, under varied lighting conditions, and with some extent of user error (with the exception of over-exposing the images). Professional photographers, printers, paint manufacturers, textile specialists and an assortment of other professionals use color standards daily, and rely upon these for consistent and faithful color representations in their digital images.

However, until recently, microscopists have not had a color standard small enough to be used even at the lowest magnification. Those in the scientific and medical professions where microscopes are used, including medical professionals such as pathologists and dermatologists, have operated without consistent or accurate colors for lack of a color calibration target since the inception of digital images. Before that, it was the color lab's responsibility when picture were printed from film, each lab responding to the request of the professional's perception of color.

The first International Color Consortium meeting at FDA headquarters in Bethesda, Maryland addressed color standardization in medical imaging, and spawned a concerted effort toward standardized color in medicine. Pathology is among the medical disciplines favored for standards, given that samples are stained largely with colors, such as Hemotoxylin-Eosin (H&E) and a limited number of other stains. When these sam-

ples are shared, such as in tele-medicine, color fitting to standards allows pathologists to view images with more consistent and reliable colors. A wider circle of observers demand greater uniformity, just as it has for medical practitioners in other disciplines, such as Radiology. In Radiology, not only were computer display designs put under a standard, but an image format was instituted and adapted to that discipline as well.

A COLOR STANDARD FOR MICROSCOPY

As a solution to the color conundrum in microscopy, a color calibration slide with a calibration target has been developed by Datacolor Inc. (Lawrenceville, NJ). The slide comes with calibration software and a monitor calibration sensor and software. The package is called "ChromaCal™."

A manuscript (in progress) investigates the accuracy of consequent color after the use of the ChromaCal calibration slide and software, and describes the color science behind the use of the color patches.[5] This paper investigates the robustness of the calibration slide's consistency of color among 3 cameras, and its improvement in color intensity. The latter improves the slide reader's ability to separate features by color, better done when colors are vibrant versus when colors are muted or "muddied."

METHODS

Two Olympus BX51 microscopes were used with any one of the following: 1.25X (NA 0.04 plan apochromatic) 10X (NA 0.25, plan apochromatic), 20x (NA 0.85 oil immersion, plan apochromatic) and 40X (NA 1.35 oil immersion, plan apochromatic) lens. Each microscope illumination source was a tungsten-halogen lamp. Both scopes used a flip-top condenser (0.90/0.17). Aperture openings for the condensers allowed 90% of the light through to the camera, set by eye. The camera for Scope A is a QImaging EXi Aqua, Scope B used both a Dage XL MCT and a Lumenera Infinity 2.

Slides stained by Cornell University and stored for over 20 years were used. The set includes several conditions of disease (leishmaniasis, arteropathy, adenocarcinoma, and infarct) and organs (lung, lymph node, liver and intestine), all in mammals. Histochemical stains included H&E, PAS, Congo Red, and Ziehl-Neelsen. From 11 specimen slides, 7 slides were chosen for imaging based upon avoiding areas that overlapped in terms of colors used or disease conditions. From the 7 slides, one area per specimen was imaged, with the exception of two areas chosen on the lung specimen because it showed 2 conditions: an infarct and arteropathy. Four of the seven included H&E stains for this study.

Microscopes were set to a constant illumination. The daylight filter was inserted into the light path. Koehler Illumination was set with the 10x lens in place and left at that aperture opening, and the camera was white balanced only once at the beginning of the session. All images were taken during one microscopy session. Multiple exposures were taken of a single ChromaCal calibration slide, as well as specimen images from 2 of the 3 cameras. All images from the Dage, Lumenera and QImaging cameras were saved as TIFF files at either 8- or 16-bits. Images from the 2 cameras in which various exposures were taken of the same fields were sorted by how well these matched the 3rd camera's images in brightness levels to ensure that brightness levels do not affect appearance of colors. For the ChromaCal slide image, the brightest exposure without oversaturated values (at 255 for an 8-bit detector) was selected.

Because white balance shifted for 2 of the 3 cameras during the microscopy session, even though the white balancing was left at a manual setting (versus the automatic setting where the camera auto-white balances for each new location on specimen slides), specimen images as well as the calibration slide images were white balanced in post-processing to eliminate inconsistencies in white balance and any possible affect such inconsistencies could have on the color calibration.. A custom, auto-white balancing program provided white balancing.

It was critical that both the ChromaCal slide and specimen image exposures stay within the dynamic range of the camera. The consequence of a slightly underexposed ChromaCal slide image (when the white patch reads as low as 220 in any of the Red, Green or Blue channels) showed no perceptual difference in color rendition. An overexposed ChromaCal slide image, however, will darken the calibrated specimen image.

An array of nine images were acquired using the Lumenera camera and were subsequently stitched together using Photoshop (Version 10, Adobe, Mountain View, CA). See figure 1. The resulting composite image reveals the same region captured with the Dage and QImaging cameras.

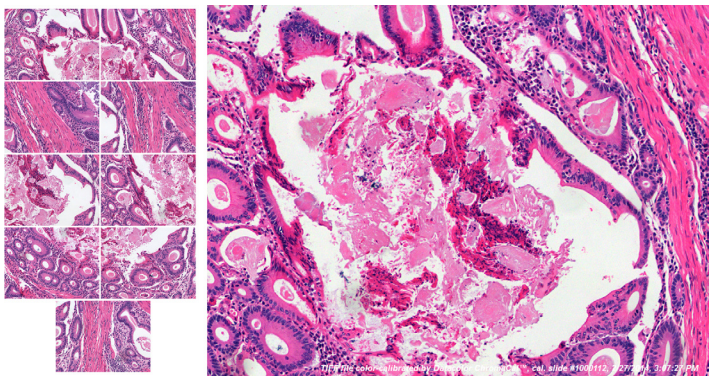

Figure 1. Nine discrete images of smaller fields were used to create a seamless photostitched image using the Panorama function in Photoshop.

Images were aligned and cropped in Photoshop, and saved as TIFF files without ICC profiles. Images were then calibrated in ChromaCal (Version 1.0, Datacolor Inc., Lawrenceville, NJ) and saved as TIFF files. For ease of visual comparison, the brightness in one set of images (arteropathy images) used Photoshop to add or subtract equal amounts from each pixel in the image. No contrast adjustments were made as these may lead to a change in color constituency. No other post-processing adjustments were made on any other images in the study.

ANALYSIS

Measurements for determining the variability of pre- and post-calibrated images relied upon a simple standard deviation of Red, Green and Blue (RGB) tonal values from specimen images at the same location. Red – (minus) Green and Blue – (minus) Green difference values were derived from identical sampling positions for each set of images, rather than using R, G and B values to eliminate any contribution from differences in brightness. The R-G and B-G values were averaged for each position, and then a standard deviation was found. Standard deviations for the 3 camera positions were then averaged and a final standard deviation was derived. In using this method, differences in variability among 3 camera images for each specimen before and after calibration could be determined as a percentage of improvement. This percentage describes an improvement in uniformity of appearance from the 3 cameras when images are calibrated.

Intensity of color is derived from color saturation. The saturation of a color from a Hue, Saturation and Brightness (HSB) color model refers to the greater absence of gray as the hue (specific color) becomes more pure. A pure color, in the HSB model, moves from 0 percent saturation (gray at a mid-brightness level) to 100 percent saturated, or pure color. See Figure 2.

Saturation can also be described as the absence of other colors that reduce a color's intensity.[6] The colors that reduce intensity are often referred to as "polluting" or "muddying" colors in the graphic arts industry. The result of polluting colors can be muted browns when using H&E as a stain in histopathology. See Figure 2.

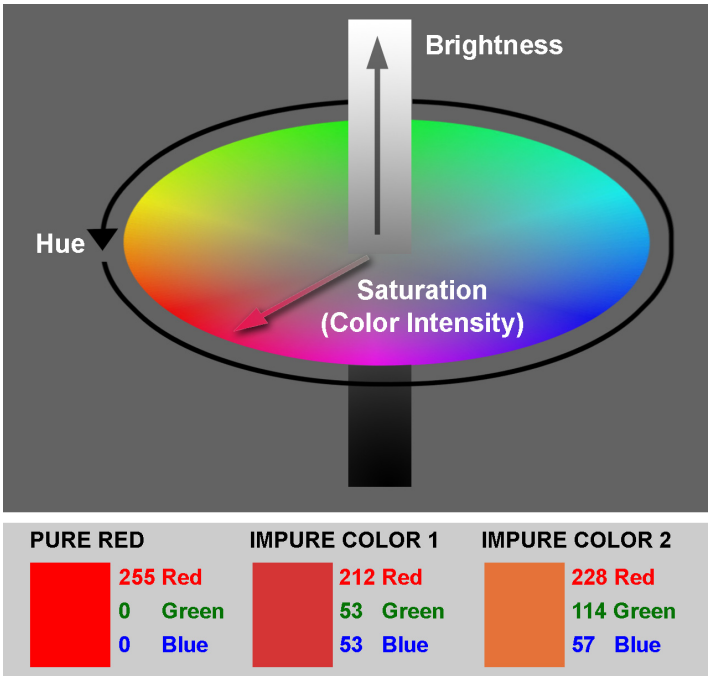

Figure 2. Top image shows the Hue, Saturation and Brightness (HSB) color model illustration. Hue refers to the color itself, shown as different colors depending on the color position along the 180 degree color wheel. Brightness refers to the color's lightness or darkness. Saturation refers to color intensity, with colors more intense at the periphery of the color wheel. Bottom image shows an intense (pure) color progressively muted by the addition of other colors.

Measurements were made in Image J (version 1.47t, Wayne Rasband, Bethesda, MD) from Regions of Interest (ROIs). ROI's were determined visually by 1) uniformity of color, 2) predominant hues, and 3) white areas (background) devoid of tissue. White areas were used for determining variability from one camera image to another only when background areas predominated and affected perceptual interpretation.

Red, Green and Blue values were measured from sampled ROIs, along with Saturation values after image was changed to a Hue, Saturation and Brightness

(HSB) model. The values were then imported into Excel (version 2010, Microsoft, Redmond, WA) to derive Standard Deviation and Saturation averages. The average standard deviation and saturation data was used to derive percentage difference between the original and calibrated images.

EXPERIMENTAL RESULTS

From a sampling of four different specimens all stained with H&E and at different magnifications, a significant improvement in both consistency (uniformity) of specimen images and increased color purity was found. All samples showed an increase in both uniformity of images taken with 3 different cameras, and in color intensity when compared with original images. On average, the improvement in uniformity of images from camera to camera was nearly a 50% improvement. Greater color intensity increased, on average, by nearly 40%. Some sets increased in uniformity upwards of 70%, and other sets increased in color intensity at 52% (see Figure 3).

DISCUSSION

While this study is conclusive about consistent and greater color intensity among 3 scientific grade cameras, more cameras will be included in future studies. More than one light source will be studied as well, to show consistency among varying types of illumination. Because images are subjectively evaluated person by person, it would be useful for medical practitioners to rate images according to perceived quality, based upon how effectively images represent specimen images as another study. That rating could include ratings tests with and without calibrated monitors, since the computer display plays an important function in assessment of digital images.

A study of a working environment where the ChromaCal system is used would be of particular interest to Clinical Pathologists and Health Administrators. This study would track slide reading speed before and after implementation of image calibration; measure speed of related activities such as image acquisition and post-processing; and provide hard numbers for session to session, histological specimen staining differences.

One of the advantages of consistent digital image colors lies in the ability to use automated, computer aided methods to sort slides. When colors are consistent, identification of disease conditions through the use of color can then be reliable. Specimen colors can be used to positively identify pertinent areas of the image, or the colors can be used as a reverse mask to identify other parts of the image. Graphic colors can be overlaid to alert Pathologists and medical professionals to pertinent image locations.

The need for more reliable colors can fulfill shortcomings in many areas. This standard can be used for Whole Slide Imaging systems and would be appropriate as part of Good Laboratory/Clinical Practices in settings complying with FDA regulations. Other disciplines where calibrated standards are necessary include forensics, R&D, academic research, and materials science.

CONCLUSION

Calibration of specimen images with the ChromaCal calibration system significantly improves both uniformity of images when different cameras are used, and color intensity. These improvements can provide a means through which colors are both more uniform from camera to camera, and a better representation of the specimen. When colors are a better representation, Pathologists and other medical professionals can reduce their viewing time and improve efficiency. Furthermore, improved consistency and color intensity will deliver a more ‘color-reliable” image, and therefore remove variability that can potentially distort the viewing and assessment of images when shared with others. Adopting ChromaCal within the medical and research industries could have consequences in improved efficiency as well as enhanced image reliability. With the fundamental need to collaborate and effectively communicate results, these consequences may deliver a profound advancement to the integrity of digital imaging. .

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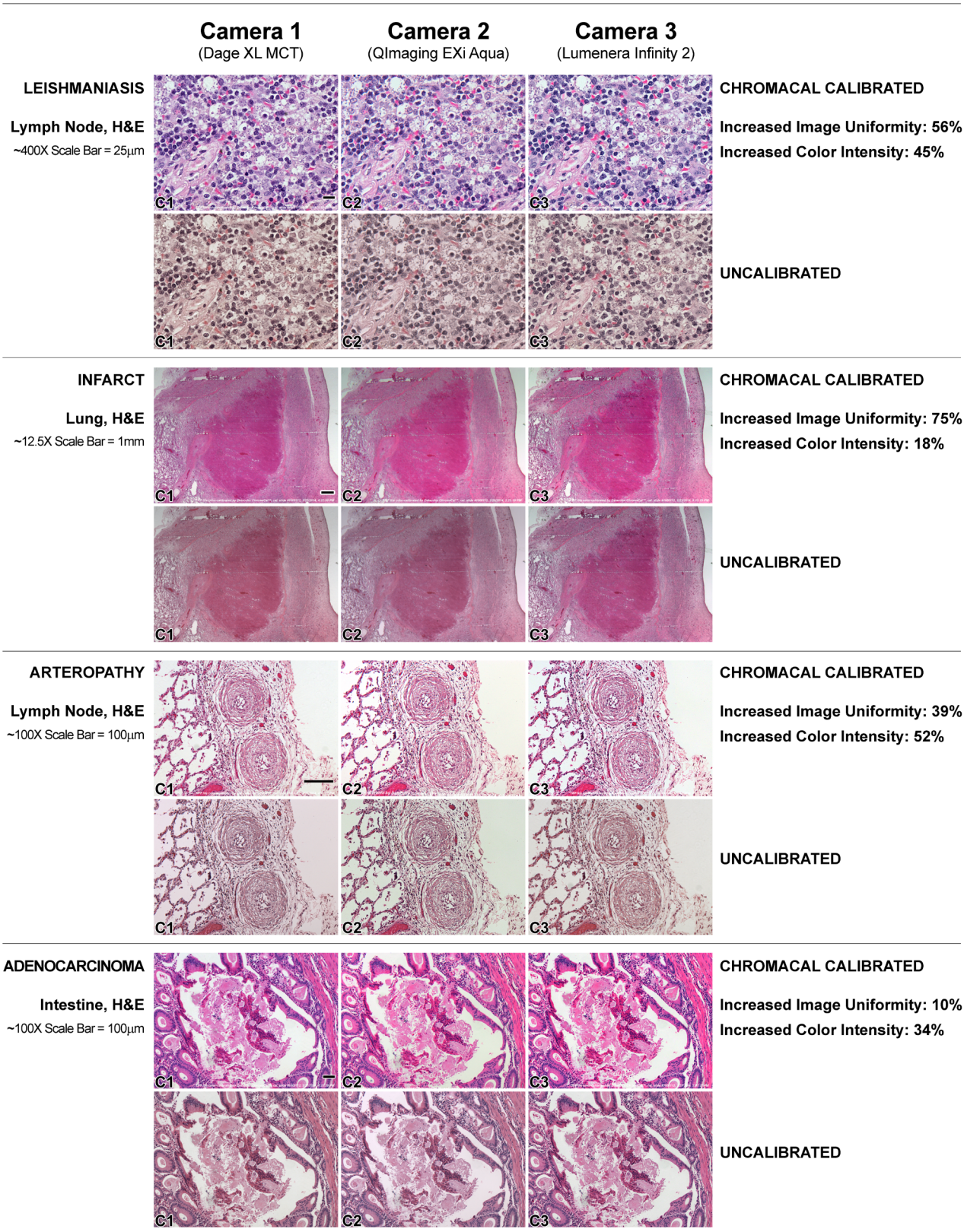

Figure 3. Figure 3. Four separate panels containing 6 images in each panel (denoted by the disease condition in the text on the left) are shown above. Within each panel 3 calibrated images (top) and 3 uncalibrated images (bottom) comprise the rows. Images taken with 3 different cameras are designated by the 3 columns. In each panel, images calibrated with ChromaCal calibration system demonstrate and increase in both uniformity of image appearance and color intensity. The increase is indicated by percentage in the text to the right of each panel. Image Uniformity was determined by averaging the standard deviation from Regions of Interest (ROIs) for both the calibrated and uncalibrated images. A decrease in standard deviation indicates greater uniformity. Color intensity was determined, by measuring and averaging hue saturation for calibrated and uncalibrated images. An increase in saturation indicated increased color intensity. The percentage increase in color intensity was calculated for both image uniformity and color intensity of the calibrated image averages versus the uncalibrated image averages. Printed image was matched as carefully as possible with a calibrated monitor, but subtle nuances in color may not have been reproduced, nor can intensity of color on paper match that of the same image on a monitor.