Assessing colorimetrical uncertainty of a hyperspectral imaging microscopy system designed for measuring reference histological tissue slides

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**Abstract:**

The type A measurement uncertainty of a hyperspectral imaging microscopy system was assessed for a set of optical filters including neutral density and color filters based on the ground truth obtained from a reference spectroradiometer.

The hyperspectral imaging microscopy system was designed to measure the spectral transmittance of histological tissue slides at the pixel level. The per-pixel spectral data were converted into colorimetrical data for evaluating the color performance of whole-slide imaging (WSI) scanners.

In this study, the measurement uncertainty of was determined to be XXX, which was used to qualify the color performance evaluation of three WSI scanners.

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1. Introduction

Whole-slide-imaging (WSI) scanners are set to replace classical light microscopes for reviewing tissue slide by pathologists.

Tissue not observed directly, pre-scanned and displayed, high resolution images of the whole tissue area

Color reproduction by WSI is paramount for precision and reliability of clinical diagnosis.

Color measurement systems, color performances need to be assessed

Can be done with color checker slide but issues (WHAT ARE THEY?) Bautista [1] Clarke [2]

Recently, our group proposed to use tissue slide to assessment of color performances of WSI, color gamut, focusing.[3]

Hyperspectral microscope to measure the tissue slides

Color performance assessment of the microscope

Calibration of the hyperspectral microscope for colorimetric measurements

This setup is used to measure tissue slide further used as calibration samples for Whole-slide imaging microscopes

Transmittance spectra using the spectroradiometer and the camera.

Estimation of the uncertainties on the transmittance by propagation of the measurement uncertainty. Repeatability and reproducibility

Distance in the CIELAB color space between spectrometer and camera average measurements. Uncertainty on the results

1. Material and method
   1. Experimental Setup

Figure 1(a) presents the experimental setup used to estimate the color performances of the hyperspectral microscope recently implemented by our group. The hyperspectral microscope is based on a conventional Zeiss AxioPhot 2 upright microscope (Carl Zeiss Microscopy, White Plains, NY, USA) in bright field illumination mode. The conventional light bulb plus collector lens was replaced with an OL490 Agile tunable light engine (Gooch and Housego, TX, USA) equipped with a liquid light guide followed by a collector lens (Thorlabs, Newton, NJ, USA). The light engine uses a xenon lamp and provides a tunable illumination from to with a minimum band width . In this application is spanned in steps of and . Figure 1(b) presents the 41 spectral bands scaled to the same light intensity parameter used to obtain the hyperspectral images. The collector lens is followed by a Zeiss achromatic-aplanatic condenser system (). The sample is set on a motorized MAC 6000 XYZ stage system (Ludl Electronic Products Ltd., Hawthorne, NY, USA) and is imaged using a 20x Zeiss plan apochromat microscope objective (). The sample image is acquired by a Grasshopper3 9.1 MP Mono USB3 Vision monochrome camera (Point Grey Research Inc., BC, Canada) with a CCD sensor format of 1”, a pixel count of at 9fps and a pixel size of (ICX814, Sony Electronics, Newton, NJ, USA). The images collected here correspond to a pixels sub-region of the total available image size. The camera shutter time and light source intensity were optimized to prevent detector saturation while maximizing the detected signal for each measurement wavelength. Both the images manual focus and Kohler illumination were achieved in white light, . A Photo Research PR730 spectroradiometer (Photo Research, Syracuse, NY, U.S.A.) was used as a reference instrument for measuring the same region of interest (ROI) observed by the microscope. The tip of a FP-730 fiber probe attached to the spectroradiometer was located at the position of one of the eyepieces in one of the eyepiece tubes

RESOLUTION IN PIXELS???

The tunable light source, motorized stage, and camera were all controlled by programs written in Matlab 2015b (Mathworks, MA, USA) running in the Microsoft Windows 7 Professional 64-bit environment.

|  |  |
| --- | --- |
| (a)  (a) | (b) |

Fig. 1. (a) Hyperspectral microscope equipped with a PR730 spectroradiometer; (b) OL490 light engine output of the 41 spectral bands (scaled to the same light intensity parameter) used to obtain the hyperspectral images.

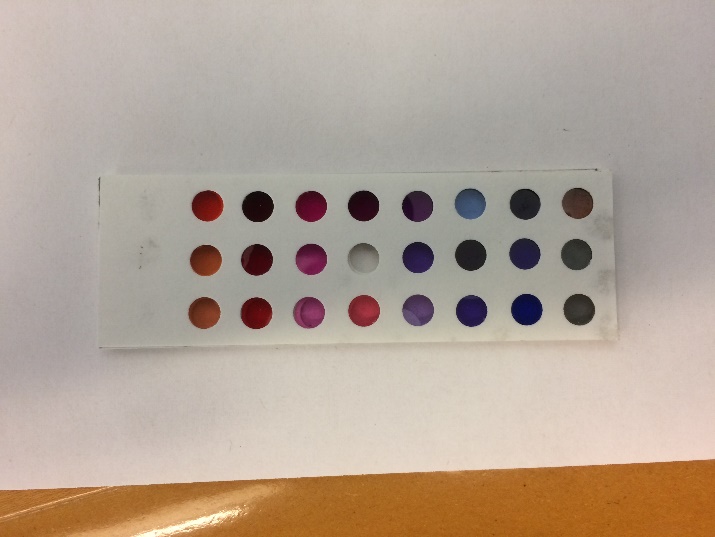
OL490 light engine

PR730 spectroradiometer

XYZ motorized stage

* 1. Samples

Both standard and non-standard transparent targets were used. The standard targets include Kodak Warren (KW) gelatin neutral density (ND) filters with optical density and color gelatin filters #12 (yellow), #25 (red), #32 (magenta), #47 (deep blue), and #58 (green) (Edmunds Optics, Barrington, NJ, USA). One should note that in order to prevent potential interference patterns due to air gap between film and glass[4], the KW filters were held laterally without setting them on a glass slide. As non-standard targets, we designed a color phantom populated with a choice of Roscolux color filters (Rosco Laboratories Inc., Stamford, CT, USA). The intent was to better represent the color gamut of the hematoxylin-and-eosin (H&E) stained tissue samples. 24 holes (diameter ) were punched on a 1 mm-thick cardboard slab with a dot puncher. A first thinner cardboard slab with holes was glued on the supporting slab and 23 locations were filled with a filter dot glued on the cardboard and the additional hole being left empty measuring the 100% transmittance. A second thin covering slab with 24 -punched holes was glued on top (Fig. 2).



#40

#316

#03

#46

#342

#24

#43

#336

#337

#39

#34

#48

#52

#51

#360

#347

#356

#59

#56

#68

#99

#398

#97

Fig. 2: Color phantom with 23 Roscoloux color filter dots glued on a cardboard slab set with aa adequate series of punched holes. The central position is 100% transmittance slot.

* 1. Measurements

Measurements of the transmittance were conducted first using the spectroradiometer with a broadband illumination and secondly using the camera with narrow band illumination. The numerical aperture of the spectroradiometer’s detector fiber probe averages out over the sample region of interest (ROI) and for comparison purposes, images of the same ROI captured by the camera are spatially averaged as well. Repeated measurements led to signal intensities from which a mean value and a standard deviation can be computed. The transmittance of the sample is then expressed as

|  |  |
| --- | --- |
|  | (1) |

where and are the intensities of the signal measured with the sample the light path and with no sample in the light path (), respectively. and are the background signals obtained setting the intensity of the light engine to zero and account for both persistent background illumination observed with the light source [Figure spectra??] and camera background noise. and were measured with an optimized value of the light source intensity value for obtaining the higher signal output without detector saturation. For the camera measurements, the light source intensity value and the camera shutter time were both optimized wavelength by wavelength. The International Commission on Illumination (Commission Internationale de l’Éclairage, CIE) tri-stimulus values were then computed as

|  |  |
| --- | --- |
| , | (2) |

where is the relative spectral power of one of the CIE standard illuminant, , and are the CIE 1931 color matching functions [5-7], and is the normalizing factor of corresponding to 100 for a perfectly transmitting sample. In this study, the CIE D65 standard illuminant was used. Converting the integral to summations, we have

|  |  |
| --- | --- |
| *,* | (3) |

where

|  |  |
| --- | --- |
| . | (4) |

The CIELAB (, , ) values were then computed as

|  |  |
| --- | --- |
|  | (5) |

where

|  |  |
| --- | --- |
|  | (6) |

and are the tri-stimulus values for a perfectly transmitting sample. The CIE 1976 color difference in the CIELAB color space can be computed as the Euclidian distance between two color points of coordinates and

|  |  |
| --- | --- |
| , | (7) |

where and .

* 1. Uncertainty propagation

The uncertainty on the transmittance, CIEXYZ coordinates, CIELAB coordinates were estimated using the law of uncertainty propagation which is based on the Taylor expansion of the functional relationship between the output quantities and the input quantities about mean values, [8, 9]. In matrix form, assuming that is the covariance matrix of the input quantities, the covariance matrix of the output quantities is

|  |  |
| --- | --- |
| , | (8) |

where is the Jacobian matrix with .

From Eq. (1) and Eq. (8), the uncertainty on the transmittance at each measurement wavelength is

|  |  |
| --- | --- |
| , | (9) |

since the measurements of the sample, the 100% transmittance and background are independent. Here is the standard deviation on the measured signals.

From Eq. (3), the tri-stimulus equations are linear functions of the transmittance measurements and Eq. (8) is not an approximation in that case, with

|  |  |
| --- | --- |
|  | (10) |

where is the number of measurement wavelengths. Since the transmittance measurements over the wavelengths are independent, is a diagonal matrix with elements , i.e. the variance of the transmittance measurements. The tri-stimulus values are correlated because the color matching functions overlap, and they depend on the transmittance spectrum [10]. The resulting correlation matrix is of dimensions .

From Eq. (5), the non-linearity of the relationship between the CIELAB coordinates and the CIEXYZ coordinates implies that Eq. (8) is an approximation, with [11]

|  |  |
| --- | --- |
|  | (11) |

where

|  |  |
| --- | --- |
| . | (12) |

The resulting covariance matrix,,is of dimensions .

From Eq. (7) the normally distributed CIELAB coordinates and can be used to form a Euclidian distance in the CIELAB space, that is not normally distributed. Hence, the statistical distributions of between both types of measurements were computed by Monte Carlo simulations of the color points positions using the covariance matrices of the CIELAB coordinates, and . The median of the statistical distribution, , is used as metric to estimate the proximity between and .

We limited our estimation to the type A uncertainty (uncertainty evaluated by the statistical analysis of series of observations[9]) by considering: i) the propagation of the uncertainty on a set of measured transmittances under the same measurement conditions, i.e. repeatability of the results, and ii) conducting the experiments several times under changed conditions to account of the reproducibility. The estimated variance, on the results of the reproducibility experiments is added to the square of the uncertainty on the repeatability experiments to compute the total type A variances, . The expanded uncertainty is where is the coverage factor. We used which correspond to a .confidence interval.

1. Results

To assess the linearity of the transmittance measurements with the camera, we compared the spatial average of the transmittance images, , to the spectroradiometer transmittance measurements, , for a set of KW gelatin ND filters with optical density . The uncertainties on both transmittance measurement channels are computed using Eq. (9). Ten reproducibility experiments were conducted for . The estimated variance from the reproducibility experiments, was used for all samples to compute the total type A variances, . Figure 3(a) shows that for most , and overlap within the error bars for over most wavelengths in the spectral range of measurements but that the differences are significant for . At these wavelengths, the values of the color matching functions , and are small enough that the impact of the transmittance values on the end results CIELAB coordinates is limited. Figure 4 illustrates this assumption by presenting , , and the Relative Cumulative Weight (RCW) of the color matching functions, expressed as . We computed a weighted linear interpolation of versus using the uncertainty on as weight parameters and considering as the ground-truth of the transmittance of the KW ND filters. For a broad range of wavelengths, , there is a linear relationship between . As an example, Fig. 3(b) presents the results of the linear interpolation at , with a slope and an intercept for a root mean square error (rmse) of .

|  |  |
| --- | --- |
| (a) | (b) |

Fig. 3. (a) Comparison of , the transmittance spectra measured with a spectroradiometer, and , the transmittance measured with the camera (spatial average over the image) for a set of KW gelatin neutral density filters with . and overlap within the error bars (coverage factor ) for for most ND filters; (b) versus fitted with a linear model for .



Fig. 4. The CIE 1931 color matching functions , and the relative cumulative weight.

Table 1 presents the CIELAB coordinates results for the set of neutral density filters, their uncertainties and the median value of the statistical distributions of , , obtained for each sample. Figure 5(a) presents the transmittance data for and shows that and overlap within the error bars at all wavelengths but for . Figure 5(b) (c) and (d) present the CIELAB coordinates and 95% confidence regions issued from and measurements and their uncertainties in the (), () and () projection planes, respectively. There is no overlap between the 95% confidence regions apart from in the () projection plane which points to a systematic error on the color coordinates. However, the median value of () is relatively small and the agreement between the color coordinates issued from and is considered reasonable. One should note that, for , despite the relative proximity of the CIELAB coordinates mean values their large uncertainties explain the larger obtained for this sample for which the and values are close to zero over the detection wavelengths.

Table 1. CIELAB coordinates for the KW gelatin neutral density filters as measured by the spectroradiometer, , and the spatially averaged images acquired by the camera, . The statistical distribution of the Euclidian distance in the CIELAB color space between both types of measurements was computed by Monte Carlo simulations of the color point positions using the covariance matrices of the CIELAB coordinates. The median value of these distributions are reported. The uncertainties are presented with a coverage factor .

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ND | Transmittance |  |  |  |  |  |
|  |  |  |  |  |  |  |
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|  |  |  |  |  |



(a)

(b)

(c)

(d)

Fig. 5. (a) Comparison of , the transmittance spectra measured with a spectroradiometer, and , the transmittance measured with the camera (spatial average over the image) for KW gelatin neutral density filter (error bars with a coverage factor ); (b) (c) and (d) CIELAB coordinates and 95% confidence regions from (blue) and (red) in the (), () and () projection planes, respectively.

Since neutral density filters have low chromaticity values, color filters were measured to assess the color performance of the setup. We first measured the set of KW color gelatin filters then we measured the color phantom. Ten reproducibility experiments were conducted for samples #32 and #47. The estimated variance from the reproducibility experiments, of sample #32 was used for all KW color filters but for sample #47 to compute the total type A variances, on the measured transmittances. The reason for that choice was that the resulting uncertainties on the CIELAB coordinates were higher in that case. Figure 6 shows that for all samples and curves overlap within the error bars at all wavelengths but for .



Fig. 6: Transmittance spectra of five KW color gelatin filters (#12: yellow; #25: red: #32: magenta; #47: deep blue: #58: green) measured by the spectroradiometer (, plain) and the camera (, dash). and overlap within the error bars (coverage factor ) for .

The CIELAB coordinates, their uncertainty and the median value of the statistical distributions of obtained for each KW color filters are presented in Table 2.

Table 2. CIELAB coordinates of five KW color gelatin filters (#12: yellow; #25: red: #32: magenta; #47: deep blue: #58: green) derived from the spectra measured by the spectroradiometer, , and the spatially averaged images acquired by the camera, . The statistical distribution of the Euclidian distance in the CIELAB color space between both types of measurements was computed by Monte Carlo simulations of the color point positions using the covariance matrices of the CIELAB coordinates. The median value of these distributions are reported. The uncertainties are presented with a coverage factor .

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Filter | Transmittance |  |  |  |  |  |
| 12 |  |  |  |  |  |  |
|  |  |  |  |  |
| 25 |  |  |  |  |  |  |
|  |  |  |  |  |
| 32 |  |  |  |  |  |  |
|  |  |  |  |  |
| 47 |  |  |  |  |  |  |
|  |  |  |  |  |
| 58 |  |  |  |  |  |  |
|  |  |  |  |  |

As an illustration of the uncertainty analysis for the KW color filters, Fig. 7(a) presents the and spectra with their uncertainty for filter #32 (magenta) and shows that the largest discrepancies indeed occur at . The 95% confidence regions around the CIELAB coordinates derived from and and their uncertainties as presented in the CIELAB projection planes in Fig. 7(b) (c) and (d) do not overlap and again this points to a systematic error. The corresponding median value of () is relatively small and the agreement between the color coordinates issued from and is considered reasonable. Fort the whole set of KW color filter, ranges from to and the agreement between the color coordinates issued from and is considered reasonable.



(a)

(b)

(c)

(d)

Fig. 7. (a) Comparison of , the transmittance spectra measured with a spectroradiometer, and , the transmittance measured with the camera (spatial average over the image) for KW color gelatin filter #32 (magenta) (error bars with a coverage factor ); (b) (c) and (d) CIELAB coordinates and 95% confidence regions from (blue) and (red) in the (), () and () projection planes, respectively.

The gamut of the color filters composing the color phantom is represented in Fig. 8(a) with their CIELAB coordinates along with their color appearance. Figure 8(b) is a boxplot of the corresponding values computed from , and their uncertainties. Median value of are in the 0.5 to 1.0 range while most outliers are smaller than 3 with some exceptions for a small sub-selection of 5 filters (#24, #342, #46, #347, #59). However, these patches have upper whiskers smaller than 3. Again, there is a good agreement between the color results computed from the and measurements. The phantom will be used provide traceability of the measurements by the hyperspectral microscope.

|  |  |
| --- | --- |
| (a) | (b) |

Fig. 8. (a) CIE LAB representation of the 23 patches composing the color filter phantom; (b) Corresponding issued from (spectroradiometer) and (camera) measurements.

1. Conclusion

Tissue slides can be used for assessing the color performances of WSI scanners. A hyperspectral microscope was previously developed by our group to measure the color data of such reference tissue slides at the pixel level. The color performances of this microscope were assessed measuring the transmittance of a set of spatially uniform KW neutral density filters, KW color filters and a color phantom composed of 23 Roscolux color filter dots glued on a cardboard. The results were compared to a reference obtained measuring the same ROI with a spectroradiometer equipped with a fiber probe whose tip was set in the one of the microscope eyepiece tube. The hyperspectral microscope acquired 10 spatially-averaged images at each acquisition wavelength. Mean values and standard deviation of the corresponding signal intensities when measuring the sample and a 100% transmittance (no sample) with the light source on and off (background) were composed to estimate the transmittance spectra. Similar repeated measurements were conducted with the spectroradiometer. The CIELAB color coordinates and their uncertainties were then estimated and compared by computing a Monte Carlo simulation of the Euclidian distance in the CIELAB space. The 95% confidence region generated in the CIELAB space projection planes generally do not overlap for the sample measured pointing to some systematic error. However, the median values of were in the range of to for ND filters with an outlier of for at which the transmittance signal is close to zero. For the color filter, are in the 0.5 to 1.0 range while most statistical outliers are smaller than 3. These differences are deemed small enough for the color assessment of WSI scanners. The color phantom will be used to maintain a traceability on the measurements by the hyperspectral microscope. In a next step, we will extend the uncertainty study to the SRGB space and compare measurement of tissue slides to results obtained by a series of WSI scanner images.

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5.3 Disclosures

The mention of commercial products herein is not to be construed as either an actual or implied endorsement of such products by the Department of Health and Human Services.

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