Color performances of a hyperspectral imaging microscope for determining the color truth of histological tissue slides.

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**Abstract:** A hyperspectral imaging microscopy system was developed for obtaining the spectral transmittance of histological tissue slides on the pixel level.

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1. Introduction

Why do we do this work/ Motivation:

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

1. Material and method
   1. Experimental Setup

Zeiss AxioPhot 2 upright microscope, bright field illumination (Carl Zeiss Microscopy, NY, USA)

20x Zeiss plan apochromat microscope objective,

Zeiss achromatic-aplanatic condenser system ()

Illumination type? SHOW SPECTRUM, ARGUMENT FOR WHY THE T MISMATCH AT 380 AND 780 nm

OL490 Agile Light Source (Gooch and Housego, TX, USA).

Photo Research PR730 spectroradiometer equipped with FP-730 fiber probe (Photo Research, Syracuse, NY, U.S.A.)

Grasshopper3 9.1 MP Mono USB3 Vision (Point Grey Research Inc., BC, Canada), size and id of sensor, resolution

XYZ stage system MAC 6000, (Ludl Electronic Products Ltd., Hawthorne, NY, USA)

2.2 What did we measure

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. Samples

Kodak Warren gelatin neutral density (ND) filters with optical density (Edmunds Optics, Barrington, NJ, USA).

Kodak Wratten color gelatin filters #12 (yellow), #25 (red), #32 (magenta), #47 (deep blue), and #58 (green) (Edmunds Optics, Barrington, NJ, USA).

Roscolux transmission filters, no glass slide to prevent interference patterns due to air gap between film and glass. Glue on samples [1]

Tissue slide, comparison with WSI scanners measurements.

* 1. Measurements

Measurements of the transmittance were conducted using first the spectroradiometer with a broadband illumination and secondly the camera with narrow band illumination ( in steps of ). The transmittance of the sample is

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

were 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 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 [2-4], 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 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 and 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, [5, 6]. 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 [7]. 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 [8]

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

where

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

The resulting covariance matrix,,is of dimensions .

Finally, the uncertainty on the CIELAB coordinates can be propagated to the distance between 2 points of the CIELAB color space computed using Eq. (7). In that case, were is a block diagonal matrix

|  |  |
| --- | --- |
|  | (13) |

and is a vector whose elements are

|  |  |
| --- | --- |
|  | (14) |

with .

EXPLAIN

We limited our estimation to the type A uncertainty 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 Kodak Warren gelatin neutral density (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 1(a) shows that 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 2(a) illustrates this assumption by presenting the relative cumulative weight of the color matching functions, expressed as . Figure 2(b) presents data for sample for which the and overlap within the error bars at all wavelengths but for . 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 ND filters. For a broad range of wavelengths, , there is a linear relationship between . As an example, Fig. 1(b) presents the results of the linear interpolation at , with a slope and an intercept for a root mean square error . Table 1 presents the CILAB coordinates results for the set of neutral density filters. The values range from with uncertainties in the same range so CIELAB results computed from the and are identical.

Since neutral density filters have low chromaticity values, color filters were measured to assess the color performance of the setup. First, we measured a set of Kodak Wratten color gelatin filters (#12: yellow; #25: red; #32: magenta; #47: deep blue; #58: green). Figure 3 shows that for all samples and curves overlap within the error bars at all wavelengths but for . Specifically, Fig. 4(a) and 5(a) present the and spectra for color filter #32 and #47, respectively and show that the largest discrepancies are at . The CIELAB space representation of the results presented in Fig. 4(b) and 5(b) (#32 and #47, respectively) illustrates the proximity of the color coordinates issued from the and measurements. The pixel by pixel data points computed from the corresponding spectra show a good color uniformity of the sample. The CIELAB coordinates are presented in Table 2. The values range from with uncertainties of about to so there is a good agreement between the color results computed from the and measurements.

We extended the range of color, measuring a color phantom with dots on a glass slide

Table 1. CIELAB coordinates for the Kodak Wratten gelatin neutral density filters as measured by the spectroradiometer, , and the spatially averaged images acquired by the camera, . is the Euclidian distance in the CIELAB color space between both types of measurements. The uncertainties are presented with a coverage factor .

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ND | Transmittance |  |  |  |  |  |
|  |  |  |  |  |  |  |
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|  |  |
| --- | --- |
| (a) | (b) |

Fig. 1. (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 Kodak Wratten 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 .

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

Fig. 2. (a) Relative cumulative weight of the color matching functions; (b) Comparison of , the transmittance spectra measured with a spectroradiometer, and , the transmittance measured with the camera (spatial average over the image) for a Kodak Wratten gelatin neutral density filter () for which and overlap within the error bars for all wavelengths but for (coverage factor ).



Fig. 3: Transmittance spectra of five Kodak Wratten 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 .

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

Fig. 4. (a) Transmittance spectra of Kodak Wratten color gelatin filter #32 (magenta) measured by the spectroradiometer (, plain) and the camera (, dash); (b) CIELAB color space representation of results from pixel by pixel transmittance measurements, . The distance in the CIELAB space is small (). The CIELAB parameters are (): (i) (spectroradiometer), and (ii) (image spatial average).

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

Fig. 5. (a) Transmittance spectra of Kodak Wratten color gelatin filter #47 (deep blue) measured by the spectroradiometer (, plain) and the camera (, dash); (b) CIELAB color space representation of results from pixel by pixel transmittance measurements, . The distance in the CIELAB space is small (). The CIELAB parameters are (): (i) (spectroradiometer), and (ii) (image spatial average).

Table 2. CIELAB coordinates of five Kodak Wratten 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, . is the Euclidian distance in the CIELAB color space between both types of measurements. The uncertainties are presented with a coverage factor .

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



Fig. 6. 34 measured color filters in the CIELAB color space. ADD THE GAMUT OF THE TISSUE SLIDE TO PICK THE 2 EXAMPLES OF COLOR FILTER USED AFTER

|  |  |
| --- | --- |
| c |  |

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 Roscolux color filter #03. (b) CIELAB representation of the measurements for the spectroradiometer, the spatial average of the images captured by the camera and the pixel-by-pixel values. The distance in the CIELAB space is small (). The CIELAB parameters are (): (i) (spectroradiometer), and (ii) (image spatial average).

|  |  |
| --- | --- |
|  |  |

Fig. 8. (a) Comparison of , the transmittance spectra measured with a spectroradiometer, and , the transmittance measured with the camera (spatial average over the image) for Roscolux color filter #59. (b) CIELAB representation of the measurements for the spectroradiometer, the spatial average of the images captured by the camera and the pixel-by-pixel values. The distance in the CIELAB space is significant (). The CIELAB parameters are : (i) (spectroradiometer), and (ii) (image spatial average).

The multispectral imaging system was implemented by retrofitting a conventional light microscope. It comprised four components –– microscope, tunable light source, camera sensor, and control software. The hub of the multispectral image system was an upright light microscope (AxioPhot 2, Carl Zeiss Microscopy, NY, USA) in bright-field mode with a 10X objective (Carl Zeiss A-Plan 10X/0.25 Ph1). A motorized XY-stage (MAC 6000, Ludl Electronic Products Ltd., Hawthorne, NY, USA) translationally moved the glass slide under the objective to select the desired region of interest (ROI). The glass slide was illuminated by, in lieu of a conventional tungsten halogen lamp, a tunable light source (OL490 Agile Light Source, Gooch and Housego, TX, USA). On the detector side, a scientific monochrome CCD camera (Grasshopper3 9.1 MP Mono USB3 Vision, Point Grey Research Inc., BC, Canada) calibrated to a linear response was used to measure the luminance of each pixel in the field of view. After mounting the light source and camera, Kohler illumination was attained by refocusing the condenser (Zeiss achromatic-aplanatic condenser system, aperture 0.9) accordingly. The image was focused manually with 550-nm (green) light.

Inside the tunable light source, the broadband white light from a xenon lamp was dispersed into various wavelengths by prisms. In this study, the 150-micron aperture was selected to generate the narrowest bandwidth. A fast-switching MEMS-based Digital Light Processor (Texas Instruments Incorporated, TX, USA) with 1,024 columns was software-controlled to reflect a set of selected wavelengths. The mapping between the 1,024 columns and the wavelength was nonlinear and needed to be determined at factory as a calibration file. The factory software looked up the calibration file and actuated the corresponding columns based on the user's choice of wavelength. The wavelengths reflected by the actuated columns were then combined and delivered through a liquid light guide. The liquid light guide was coupled with the light microscope with a collimating adapter (LLG5A4-A, Thorlabs, Newton, NJ, USA).

The pixel count of the camera was 3,376×2,704 at 9 fps. The size of the CCD sensor (ICX814, Sony Electronics Inc., Park Ridge, NJ, USA) was the 1-inch format that covers a major portion of the field of view of the microscope. The resolution of the microscope system when using the 10X objective was 370 nm per pixel. 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.

3.2 Software

The goal of the multispectral imaging system was to measure the spectral transmittance, T, of the glass slide for each pixel. The glass slide was illuminated by the light source with spectral power distribution L. The spectral sensitivity of the camera, including both the sensor and optics, is C, which also depended on the camera parameters such as the exposure, gain, and brightness settings. The camera detects the luminance of the target Y and reports in digital count D. The detected luminance Y is proportional to C, L, and T over the visible light wavelength λ:

|  |  |
| --- | --- |
|  | (15) |

By fixing the settings of the camera and the spectrum of the light source, C and L can be considered constant so the detected luminance at a specific wavelength is proportional to the transmittance of the tissue sample on the glass slide only:

|  |  |
| --- | --- |
|  | (16) |

If the camera has a linear response between the detected luminance Y and the output digital count D, then the relationship between the transmittance and the digital count can be expressed as a linear function:

|  |  |
| --- | --- |
|  | (17) |

The two coefficients and can be found by measuring two different glass slides with known transmittance. In this study, a transparent target ( =1) and an opaque target ( =0) were used. The corresponding camera digital counts are, respectively:

|  |  |
| --- | --- |
|  | (18) |
|  | (19) |

The transmittance can then be calculated via interpolation:

|  |  |
| --- | --- |
|  | (20) |

Recall that the spectral power distribution of the tissue sample, , is the product of the spectral power distributions of the light source and the transmittance. Although the resulting spectral transmittance is the ground truth of the glass slide, it cannot serve as the color truth for comparison until a light source is applied. In this study, the CIE D65 illuminant was used.

(21)

The CIEXYZ tristimulus XYZ was calculated by

(22)

(23)

, (24)

where , , and are the CIE 1931 color matching functions.

Then the tristimulus of the CIE D65 illuminant was calculated similarly and used as the reference white, XnYnZn, to calculate the CIELAB L\*a\*b\* values.

(25)

(26)

(27)

(28)

(29)

The CIELAB L\*a\*b\* values would be used as the truth to evaluate the WSI scanners. For visualization and presentation purposes, the CIELAB data were also converted into the sRGB color space to reconstruct the truth image12. Notice that the sRGB color space was not used to process the truth data because some eosin-stained shades with high lightness values are out of the sRGB color gamut. Nevertheless, many commercial WSI scanners use sRGB to store and display images. In this study the CIE D65 illuminant was used to establish the truth. Alternative illuminants can be used for specific viewing environments or applications.

The conceptual procedure of multispectral imaging is described in the following pseudo-code.

Adjust the camera settings

For each wavelength λ=380:10:780

Adjust the tunable light source for λ

Image the opaque target to obtain Dmin for T=0

Image the transparent target to obtain Dmax for T=1

Measure the target slide to obtain D for each pixel

Linearly interpolate (D,T) from (Dmin,0) and (Dmax,1)

End

Reconstruct spectral transmittance for each pixel

3.1 Workflow

A series of 41 images were captured at wavelengths from 380 to 780 nm at the interval of 10 nm. The spectra of the light source are shown in Fig 2. The set of 41 images were compared with both flat-field bright and dark images to calculate the spectral transmittance. The flat-field bright image was acquired by imaging a blank area on a glass slide with coverslip mounted. The camera exposure settings were adjusted such that the bright pixel values did not saturate. These pixel values, Dmax, which may vary among pixels due to spatial non-uniformity, indicate the 100% transmittance, which already included the effects of the glass slide and the coverslip. The flat-field dark image was acquired by imaging a black plate that does not transmit light. These pixel values, Dmin, indicate the 0% transmittance. For each wavelength, each pixel reading was linearly interpolated between the bright and dark pixel values to obtain the per-wavelength transmittance (Equ 6). After collecting the per-wavelength transmittance data from 41 images, the complete spectral transmittance of each pixel was reconstructed (Fig 1, Spectral Transmittance). The spectral transmittance was multiplied by the spectrum of the CIE D65 illuminant (Equ 7) to obtain the spectral power distribution (Fig 1, SPD). The spectral power distributions of the tissue and the reference white were converted to CIEXYZ (Equ 8-10), which generated CIELAB (Equ 11-15). The procedure was implemented in Matlab conceptually but not verbatim because data vectorization was required to minimize the computation time for large images.

1. Accuracy Evaluation
   1. Paul’s uncertainty data
   2. Firdous’ data
2. Applications and Examples

4.1 Evaluate WSI scanners

4.2 Create ICC profiles

4.3 Evaluate staining variation

1. Funding, acknowledgments, and disclosures

5.1 Funding

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5.3 Disclosures

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