A hardware approach for histological and histopathological digital image stain normalization

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Abstract
Advances in technology made the migration of pathological diagnosis to digital slides possible. As the need for objectivity and automation emerged, new computer software algorithms were proposed. Computer algorithms demand accurate color and intensity values in order to provide reliable results. The tissue samples undergo several processing steps from histological preparation to digitalization, which cannot be completely standardized. Thus, non-standardized input data generates unreliable output data. In this article, we discuss a new computational normalization algorithm for histopathological stained slides that uses a hardware color marker. The marker is added to the glass slide together with the tissue section, exposed to all the processing steps and altered in the same manner as the biological material of interest, thus becoming a solid color marker for image normalization. The results of the proposed method are numerically and perceptually tested in order to prove the advantages of the method. We conclude that our combined hardware-software technique for staining normalization of digital slides is superior to the existing methods based on only software normalization, and that its implementation will tackle not only the acquisition errors but also the technical errors that may occur during the staining process.

Keywords: hardware stain correction, hardware stain normalization, color correction, digital slide, histological slide.

1 Introduction
The pathologist is a specialized medical doctor, which establishes a histopathological diagnosis mainly based on visual examination, through optical transmission microscope assessment of a processed tissue section.

Advances in technology made possible the attachment of digital cameras to optical microscopes, thus providing the pathologist with a digital picture of the image observed under the microscope. Even more, the current trend is to replace the digital camera and the microscope with a specialized device, called slide scanner, which takes consecutive photographs of the glass slide and merges them into a so-called “digital slide”. The digital slide is a limited-by-resolution digital copy of the glass slide, which can be displayed on a screen and also can be analyzed with appropriate software.

Due to this technology, the need for computer-aided diagnosis (CAD) emerged, which has at least two distinct components – automation and objectivity – in contrast to the pathological diagnosis which can be a time-consuming task, sometimes with low reproducibility [1, 2].

The transition from unprocessed tissue to a glass slide and then to a digital slide requires many uncontrolled intermediary steps (explained below), thus making it impossible to have a “standardized stain” in practice:

- fixation – type of tissue fixative, concentration, penetration, proportional volume related to the biological material, temperature and the thickness and composition of the biological material;
- cutting – variability of the tissue section thickness;
- staining – staining protocol, stain manufacturer, concentrations, penetration, operating temperature;
- mounting – the manufacturer, type and quantity of mounting medium;
- the optical ax – starting from the light source, and passing through the slide, the mounting medium and the cover slip, objectives lenses and eyepieces/camera lenses, all the components having different light transmission properties;
- the digital cameras – have different properties and color estimation mechanisms.

Computational algorithms need accurate color and intensity values in order to provide reliable results. For this reason, most of the CAD software and research papers use a preliminary normalization step before the data is ready to be interpreted [3–11]. This is software stain normalization, an attempt for local optimization, designed only for enhancing the results of the algorithm that (empirically) alters the real proportion of the color composition in a way that it cannot be controlled. Simple algorithms, like contrast manipulation and histogram adaptation, seem to be overwhelmed by the complexity of the task, thus many papers present complicated algorithms for approximating the color composition, which are then followed by color corrections. This is an attempt to use a software solution to a hardware problem, which does not provide a reliable result that could be used in a generalized manner.

While acquiring digital images we set the “white balance”, which is the standard fully saturated color for all the channels in a digital image, by specifying an area within the image that should be represented as white (a fully transparent section). In a simple sense, this is the base of our method. In this particular case, we know that the selected area should be fully transparent, so normal light should pass unaltered. We propose adding a color marker to each slide having a standard concentration and
as we know that the area representing white is theoretically fully transparent, the area represented by the marker should be the same on all slides.

Our study aims, on one hand, to introduce a new computational normalization algorithm for histopathological digital slides using a hardware color marker added to the glass slide and, on the other hand, to assess the subjective perception of normalized images by the pathologist.

Materials and Methods

Materials

A color marker that stands as the ground truth for color normalization should be exposed and altered by all the processing steps in the same manner as the biological material. The color marker should have a protein (amino acid) structure that mimics stain fixation, as in real biological tissue. For this, egg albumin was mixed together with agarose. The obtained mixture was further thermally processed in order to start the agarose polymerization obtaining finally a uniform, semi-solid protein-agarose product, called the standard protein stain marker (SPSM).

The product was cut in small fragments in order to be included in paraffin blocks together with the biological product.

As biological product, we used fragments of human placenta, due to the presence of different histological aspects within the same slide.

A paraffin block, containing one SPSM fragment and one placental tissue fragment was prepared, sectioned and stained with Hematoxylin and Eosin (HE) (Figure 1).

The obtained SPSM fragments have a monotonous, coarse granular aspect due to organic solvents washing away the agarose, the only remaining product being the protein matrix (Figures 2d and 3d).

Two sets of images, noted with A and B (Figures 2 and 3) were acquired with an Olympus ColorView II camera attached to an Olympus CX31 light microscope with 40× planC objective.

Each set consisted of six images: three for the biological product, noted with “a”, “b” and “c” and three for the SPSM, noted with “d”, “e” and “f”.

The images were acquired with different exposures in order to mimic some technical errors as follows: images “a” and “d” with the automatic camera adjustment exposure time (~400 ms), images “b” and “e” with under-exposed time (-350 ms) and images “c” and “f” with overexposed time (+350 ms).

Figure 1 – Slide aspect: top – the SPSM fragment; bottom – placenta fragment; green rectangles – Set A of images; yellow rectangles – Set B of images.

Figure 2 – Set A of acquired images (40× magnification): (a) Normal exposed snapshot of the placenta; (b) Underexposed snapshot of the placenta; (c) Overexposed snapshot of the placenta; (d) Normal exposed snapshot of the SPSM corresponding to the “a” image; (e) Underexposed snapshot of the SPSM corresponding to the “b” image; (f) Overexposed snapshot of the SPSM corresponding to the “c” image.
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Figure 3 – Set B of acquired images (40× magnification): (a) Normal exposed snapshot of the placenta; (b) Underexposed snapshot of the placenta; (c) Overexposed snapshot of the placenta; (d) Normal exposed snapshot of the SPSM corresponding to the “a” image; (e) Underexposed snapshot of the SPSM corresponding to the “b” image; (f) Overexposed snapshot of the SPSM corresponding to the “c” image.

Methods

Computational algorithm

Next step consists of the correction of a histopathological image together with its corresponding SPSM that will be named “source image” in relation to a histopathological image and its corresponding SPSM considered as normally stained that will be named “target image” using a computer algorithm.

The correction is based on a color transfer between images [12] modified implementation using Matlab workspace (Mathworks, USA). Using the LAB [13] color space, the algorithm first determines the standard deviation of the pixel values within the source image, then makes a normalization according to the standard deviation calculated within the target image. The mean values of the source and the target images are computed and, in the end, the source image mean value is replaced with the target image mean value. The computed image now has the same color and it is perceived as the target image.

However, the color composition of the images can vary depending on the biological product, not only on the different processing conditions. To overcome this limitation of the algorithm we first rectified each pixel value according to the SPSM differences thus modifying the standard variation estimation mechanism from the color transfer between images algorithm then we replaced the mean transfer with the median value calculated from the source and target SPSM images (subtracting the differences between the two SPSM median values).

The over and underexposed images together with their paired SPSM images from Set B were used as source images for the algorithms. The normal exposed images together with its SPSM image from Set A were used as target images. The computed images from both standard and proposed algorithms were then compared with the original normal exposed image from Set B, which stands out as control image.

The results of both the standard and the modified algorithms of in-between images color transfer were quantified by the average differences between computed images and the control image and paired pixel-level statistical testing between proposed and standard corrected images and the control image, and also between proposed and standard image correction results. For statistical validation of data, we used the Student’s $t$-test.

Perceptual assessment

The perceptual assessment of the corrected images was done with the help of six pathologists. For this, each image was sampled in four smaller regions noted with I, II, III, and IV as in Figure 4.

Figure 4 – Image sampling.

Corresponding sub-images were grouped in different sets, each set containing four sub-images:
- the original (normal exposed);
- the altered (sub/over exposed);
- the two corrected images:
  - one obtained with the original algorithm;
  - one obtained with the proposed one.
The pathologists were asked to rank each image by assigning a score from a scale ranging from “1” to “4” where “1” is for the image they consider having the worst appearance and details, while “4” is for the best one. There could not be two images with the same rank in the same set. For statistical validation of data, we used the Fisher’s exact test.

Results

Computational algorithm

For the overexposed data set, the computed differences between obtained images and the target image show an average difference of $9.0466/(256\times3)$ pixel value for the proposed algorithm and $12.5718/(256\times3)$ pixel value for the standard algorithm. Thus, the difference between the two methods is of $3.5252/(256\times3)$ pixel value. For the underexposed data set, the computed differences between obtained images and the target image show an average difference of $7.6470/(256\times3)$ pixel value for the proposed algorithm and $10.7756/(256\times3)$ pixel value for the standard algorithm. Thus, the difference between the two methods is of $3.1286/(256\times3)$ pixel value (Table 1).

Table 1 – Computational algorithm results performance

<table>
<thead>
<tr>
<th>Image correction results</th>
<th>Student’s t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overexposed set</td>
<td></td>
</tr>
<tr>
<td>Standard – control</td>
<td>12.5718</td>
</tr>
<tr>
<td>Proposed – control</td>
<td>9.0466</td>
</tr>
<tr>
<td>Difference</td>
<td>3.5252</td>
</tr>
<tr>
<td>Underexposed set</td>
<td></td>
</tr>
<tr>
<td>Standard – control</td>
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<tr>
<td>Proposed – control</td>
<td>7.6470</td>
</tr>
<tr>
<td>Difference</td>
<td>3.1286</td>
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</tbody>
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Perceptual assessment

The results from the perceptual assessment are presented in Table 2. Scores are shown for both under and over exposed images, together with the overall score. Each image is labeled, as mentioned above, from I to IV. Each pathologist is labeled from P1 to P6. Perceptual assessment shows overexposed image having the lowest score (as expected), followed by the standard corrected image. The best perception is on the image resulted from the proposed method, being superior to the normal exposed image. Perceptual assessment of the underexposed image shows similar trend with a better score on both reconstructed images when compared with the overexposed ones.

Discussion

Computational algorithm

Overexposed image normalization results show the differences between the proposed algorithm and the standard one: the RGB histograms show a higher similarity between the proposed correction result ("d" vs. "b") and the standard one ("c" vs. "d") (Figure 5). Student’s $t$-test statistically confirms the significant differences both between corrected images when compared with the control image and between the two corrected images.

The results of underexposed image normalization also prove the differences between the proposed and the standard correction method (Figure 6).

This time the wave shift is more clear (from left to right), the proposed method having wave shapes closer to the normal exposed image. Student’s $t$-test statistically confirms the significant differences both between our corrected images when compared with the control image and between our two corrected images (Table 1).
Figure 5 – Overexposed image normalization: (a) Source Image and corresponding histogram detail; (b) Control Image and corresponding histogram detail; (c) Result Image from the standard algorithm and corresponding histogram detail; (d) Result Image from the proposed algorithm and corresponding histogram detail.

Figure 6 – Underexposed image normalization: (a) Source Image and corresponding histogram detail; (b) Control Image and corresponding histogram detail; (c) Result Image from the standard algorithm and corresponding histogram detail; (d) Result Image from the proposed algorithm and corresponding histogram detail.
Perceptual assessment

In the case of overexposed image correction, the proposed method has better performance than the standard one, as confirmed by Fisher’s exact test statistical significance. The use of our technique could make possible quantitative appreciations within different set of slides provided by the same or by different laboratories. Computer aid diagnosis on digital slides should only be taken in consideration if the stain is normalized; otherwise, even if the implemented algorithm is reliable the data that feeds the algorithm is not and the result are prone to errors.

Further work

We are further developing the method, estimating to finally obtain an ideal color stain normalization implying that images could be corrected using only the SPSM. More dilutions (concentrations) of the SPSM will be used, ranging from zero to the maximum possible concentration, with as many intermediate concentrations (classes) as possible, at least 256 for an 8 bit/channel of color digital cameras. Different protein types will also be used for different color components of the staining (e.g., for the HE staining – acid proteins for the Eosin and basic ones for the Hematoxylin).

Conflict of interests

The authors declare that they have no conflict of interests.

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