A note on Gradient-Based Intensity Normalization

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Abstract. This paper presents an improvement on gradient-based intensity normalization introduced by Sintorn et al., which is used in microscopy especially in the evaluation of proteins and other cell elements in densitometry. In this method, images contain similar textures, but their density and intensity may vary dramatically. Two new profiles are introduced, and the Bhattacharyya distance is employed to find the best matching result. Results show the validity of the improved method.

Keywords: Intensity normalization, bivariate histogram, microscopy, densitometry, histogram profiles

1 Introduction

Densitometry is one of the most common methods employed in the evaluation of proteins and other chemical elements of cells. In this method, the optical density difference between several tissues stained with a specific marker is measured and compared. The original procedure involved exposing light-sensitive photographic films to light. Currently, films have been mostly replaced by electronic devices. Thus, to compare the intensity of different images, an initial approach consists of trying to acquire the images under similar light intensity without changing any other parameter. However, this procedure is not the best as it does not consider intensity differences due to changes in the tissue itself, differences in the quality of the staining, variations in light intensity, modification of the intensity due to the movement of the lens during focusing, and change of the distance from the lamp to the sample and the lens. Several image processing techniques have been proposed to accomplish this function. Alvarado et al. [1] proposed a simple method, which has several deficiencies that we present here. Using an image with the best quality as a reference is common in densitometry. This image normally shows the best contrast, is not saturated, and occupies all the available dynamic range $[0, L-1]$. The other images are compared against the reference image, and transformed to resemble the original as much as possible, assuming that they have very similar tissue. Considering that all images show the same kind of tissue and are acquired under similar conditions under ideal conditions, the gray level distribution in all images will be the same, and therefore the histograms should be the same. Based on this idea, several methods have been employed to match the histogram. The method proposed by Capek et al. [2] divides the histogram of the reference image in several parts and tries to transform the histogram of another image so as to resemble these parts. Another popular technique was proposed by Gonzalez et al. [3], and it uses the accumulative histogram to match the histograms of the images. More recently, Sintorn et al. [4] proposed the use of bivariate histograms, from which three profiles can be employed to match the tissues' brightness values. This method produces good results, which can further be improved. More complex techniques, not based on the histogram, have also been proposed, but they are beyond the scope of this paper and are not covered here. The described methods are based on the similarity of the histogram. In this paper, an improvement of the original Sintorn technique is proposed, including two additional profiles based on a different bivariate histogram, which in general produces better results than the original ones. Additionally, the distance between histograms is employed to compare these profiles, providing the better profile automatically.

2 Review

Alvarado et al. [1] did not give a mathematical formulation of their method; however, a formulation can be extracted from it. In this method, images are modified independently without using a reference image. This method consists of two steps. From Eq. (1), the first step is applied to the size of the histogram bins according to the value of alpha a:

$$R(q) = P(q) \cdot a \tag{1}$$

where $R(q)$ and $P(q)$ are the histograms of the reference image and the image to be fixed, and q is the number of gray levels in the image. The second step is simply an expansion of the histogram to the maximum range, i.e., between 0 and $L-1$, being L the maximum number of gray levels in the images of size 256. Clearly, the first step is useless and can in fact even degrade the image quality by introducing a quantification error. When $a < 0$, several gray levels of the original image are combined in just one gray level in the modified image. Equally, if $a > 0$ and

$$P(q) \cdot a > L - 1 \tag{2}$$

several gray levels of the original image are saturated, and the information provided by the higher gray levels are lost in the resultant image [Eq. (2)]. Subsequently, rejecting the first step, the Alvarado method consists only of a stretching step, which does not consider the reference image.

Sintorn et al. [4] proposed the use of a bivariate histogram. The 2D histograms of the reference and input images were obtained using the gray level of the images as abscissa and their gradient as ordinate. They showed that 2D histograms exhibit more similarity between images than 1D histograms do, by providing better matching results. Once the bivariate histograms were obtained, two projections or profiles of each 2D histogram in both axes were used as new 1D profiles and employed to match the input image to the reference image. Additionally, they proposed the relation between both projections to produce a third profile. Finally, the profiles were matched; thus, the resulting transformation of the input image was now similar to the reference image. The three profiles proposed by Sintorn et al. are the following:

Ordinary Histogram Matching (OHM). The simplest profile utilizes the information from the intensity in a standard gray scale from a histogram.

$$P_i^{OHM} = \sum_{g=0}^{L-1} b_{ig} \tag{3}$$

where P_i^x is the profile derived from the bivariate histogram, each profile is a function of intensity i, and x represents the abbreviation of the current method. b_{ig} is the bivariate histogram computed as the number of occurrences of image pixels with intensity i and gradient g.

Gradient weighted (GW). This profile combines intensity and gradient information.

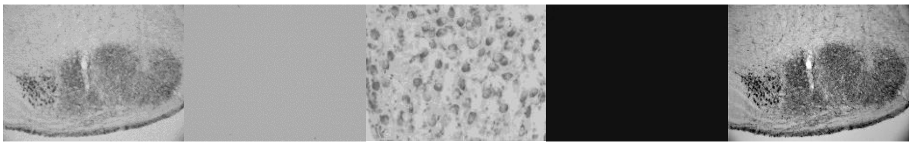
$$P_i^{GW} = \sum_{g=0}^{L-1} g b_{ig} \tag{4}$$

Average gradient (AG). The average (mean) gradient is the ratio of the first two profiles: the OHM and gradient weighted.

$$P_i^{AG} = P_i^{GW} / P_i^{OHM} \tag{5}$$

3 Material

In this study, 8 pairs of light microscopy images of rat brains with a size of 4080 × 3072 pixels and 25 pairs with a size of 1388 × 1040, 10 images of a transparent slide taken under different illumination conditions with size of 1382 × 1034 pixels were used, as shown in Fig. 1. Additionally, other 24 images were obtained by modifying the brightness and contrast of the light microscopy images. The image processing methods were implemented in Java as plugins for the open-access software ImageJ [5].



(a) (b) (c) (d) (e)

Fig. 1. Images of a rat brain and a transparent slide taken under different illumination conditions: (a, b) Original high contrast images taken as reference; (c, d) Low contrast images; (e) Modified image with doing a manual adjust of contrast.

4 Methodology

Sintorn et al. [4] proposed the use of bivariate histograms of the reference and input images, using the gradient as ordinate. Instead of the gradient, here we propose the use of a smoothed image, showing that in general, it produces better matching results, as shown in Fig. 2. In this way, two new profiles can be obtained:

Smooth weighted (SW). This profile combines intensity and smooth information, b_{is} is the bivariate histogram with intensity i and smooth s.

$$P_i^{SW} = \sum_{s=0}^{L-1} s b_{is}. \tag{6}$$

Average smooth (AS). Average smooth is the ratio of the two profiles: the OHM and smooth weighted.

$$P_i^{AS} = P_i^{SW} / P_i^{OHM}. \tag{7}$$

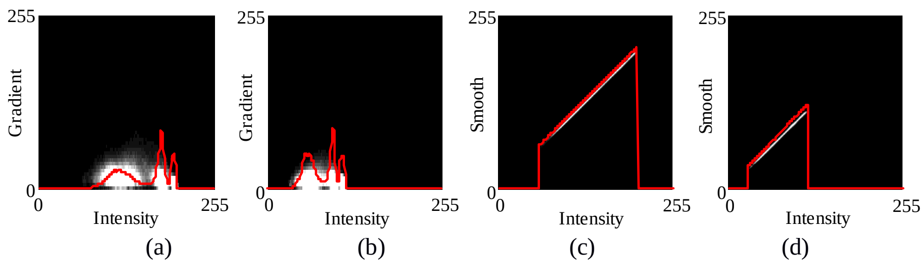


Fig. 2. Images of intensity-gradient bivariate histograms and intensity-smooth bivariate histograms: (a) intensity gradient of reference image; (b) intensity gradient of input image; (c) intensity smoothening of reference image; (d) intensity smoothening of input image.

Therefore, image processing is based on the following steps:

- Selection of the reference image and the input image, with or without ROI; both images must be in gray scale.
- Gradient image and smoothed image extraction.
- Creation of gradient-based bivariate histograms and the proposed smoothening-based histograms.
- Execute the five methods of intensity normalization, resulting in the three profiles proposed by Sintorn et al. and the two proposed herein.
- Generate the five resulting images: one from each method.
- Generate the histogram corresponding to each image and compare the distance between histograms with the reference.
- Evaluate the results to obtain the best and shows the result image.

Three of the corrections and improvements developed and a method to compare the results quantitatively are described below.

Tissues were analyzed, and image processing procedures were applied only from a region of interest, which was selected for this procedure from the whole image.

However, a common feature of tissue images is that part of them is occupied by the background. Given that the size of the area occupied by the background changes from image to image, the shape of the histogram changes accordingly, affecting the equalization method. To reduce the error introduced by the background, we selected the region of interest more accurately and excluded the intensity normalization section of the image that was not of interest (Fig 3). ROI selection was done manually.

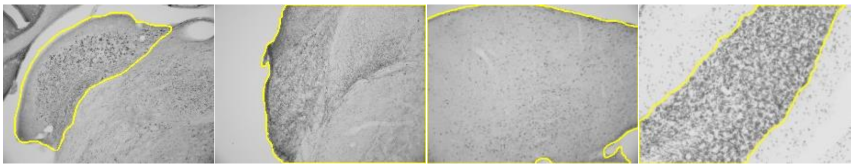


Fig. 3. Images of a rat brain with selection of ROI to exclude the background.

Saturation correction. This step comprises stretching the histograms of the images, maximizing the dynamic range of both images. In this way, the minimal and maximal gray levels on the images become the same. However, this method has several disadvantages: if the reference image is already saturated, i.e., many pixels have minimum or maximum intensity or both, the processed image will also have a high number of saturated pixels, which is not desirable. Therefore, instead of checking if there are peaks at the maximum and minimum intensity values of the histogram, as it was proposed by Sintorn et al. [4], in the solution proposed herein, we search if the reference histogram has saturated bins at the ends of the histograms and do not consider them when fixing the input image. Instead, the first and last values of the reference histogram are modified making them equal to the second and penultimate values of the reference histogram, and this modified histogram is then used to fix the histogram of the input image.

5 Results

We evaluate images of the same tissue under different conditions of light intensity, trying to reproduce the method usually employed in densitometry before the introduction of image processing techniques. Given that each pair of images has the same content, and only the light intensity is different, the normalized histogram of the input image should be exactly equal to the histogram of the reference image. Therefore, the quality of the normalization can be assessed by measuring the difference between the histograms of the reference and normalized images. Because the images resulting from intensity normalization are very similar, determining the measure that provides the best performance is difficult. Therefore, four distances employed to compare histograms were evaluated to determine the best measure [6]. The Bhattacharyya distance, used to assess equality between two distributions, was identified as the best one. This distance is closer to zero when histograms are more similar.

Figure 4 presents the results obtained with five pairs of images. Figure 5 shows the histograms of two of the images in Fig. 4. As it can be seen in Table 1, the profile of the ordinary histogram provides the best performance with the original images (twenty-three cases), followed by the average gradient profile in five cases. The ordinary histogram profile has the best performance with the contrast modified images (fifteen cases).

The use of a ROI histogram, as shown in Fig. 6, improves the result of the intensity correction, given that only the regions of interest are included in both images. Figure 7 shows the histograms of two of the images in Fig. 6. In this case, the SW profile gives the best performance in eighteen cases, followed by the ordinary histogram profile in fourteen.

Fig. 8 shows the results obtained with three pairs of images of the transparent microscope slides taken under different illumination conditions and Figure 9 shows the histograms of two of the images. As it can be seen in Table 1, the GW profile obtained the best performance in six cases, the SW profile in three cases and the ordinary profile in one. As it can be observed, the extension of the original method by Sintorn et al. provides better results in several cases, which is very important in biology to evaluate slight variations in tissues. Additionally, the use of the Bhattacharyya distance allows the automatic determination of the best profile.

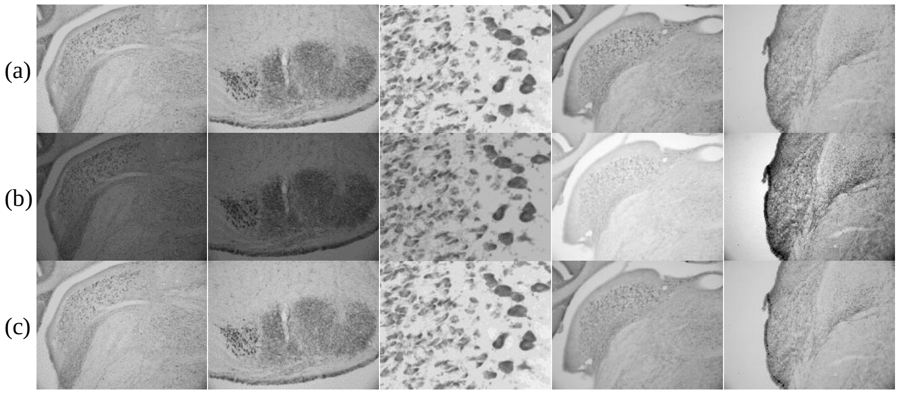


Fig. 4. Five pairs of light microscopy images of rat brains taken under different illumination conditions: (a) reference images; (b) input images; and (c) best result obtained of each one. The first three columns correspond to original images, and the last two to manually modified ones.

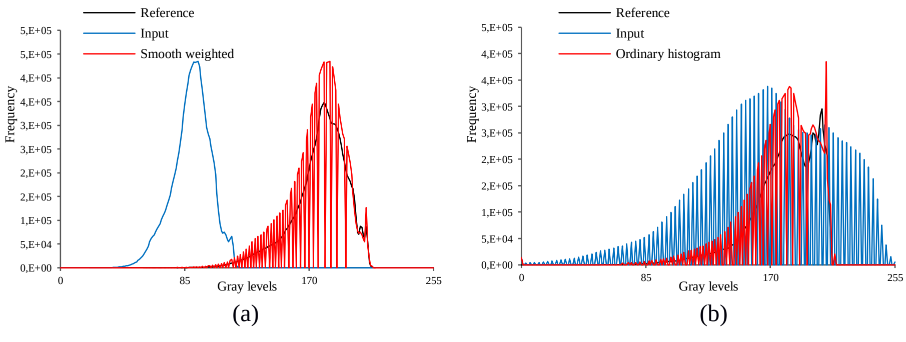


Fig. 5. Histograms of the images tested in Fig 4, reference image in black, input image in blue, and profile of the best result in red: (a) column 1 in Fig 4, and (b) column 5 in Fig 4.

(a)
(b)
(c)

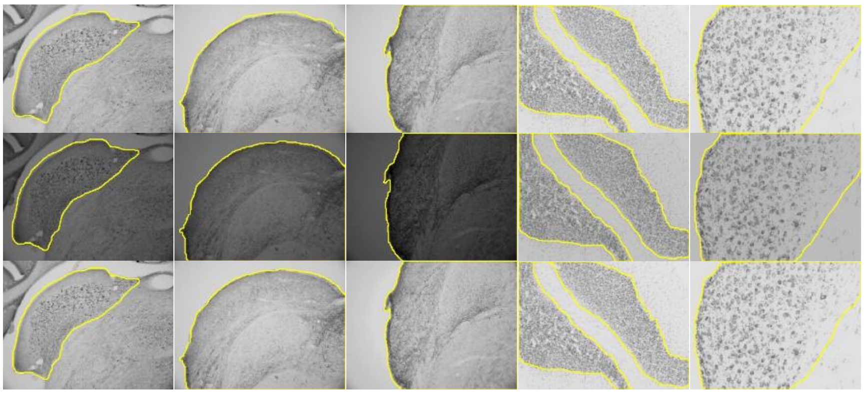


Fig. 6. Images of a rat brain with ROI: (a) reference images with ROI, (b) input images with ROI, (c) best result obtained of each one.

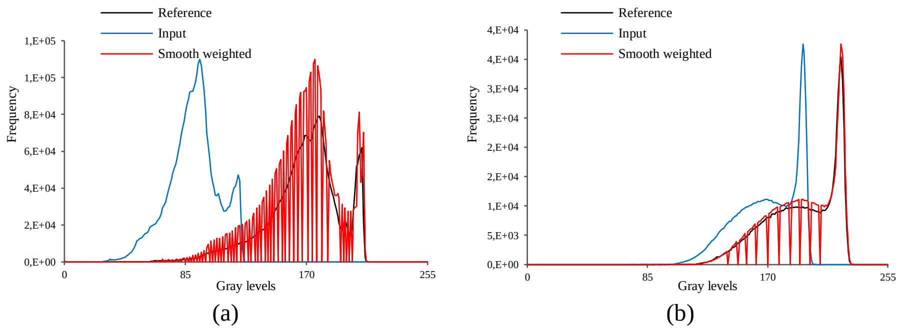


Fig. 7. Histograms of two pairs of images tested in Fig 6, with the reference image in black, input image in blue, and profile of the best result in red: (a) column 1 in Fig 6, (b) column 4 in Fig 6.

Table 1. Distances between histograms in all analysed images.

Images	Ordinary histogram	Gradient weighted	Average gradient	Smooth weighted	Average smooth	Total pairs of images
Original	23	0	5	4	1	33
Contrast modified	15	1	0	7	1	24
ROI selection	14	4	1	18	1	38
Transparent slide	1	6	0	3	0	10

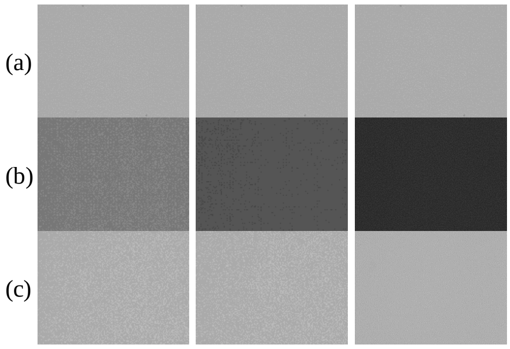


Fig. 8. Images of a transparent slide taken under different illumination conditions: (a) reference images with intensity 0.5; (b) input images with intensity 2.5, 4.5 and 6.5; (d) best result obtained of each one.

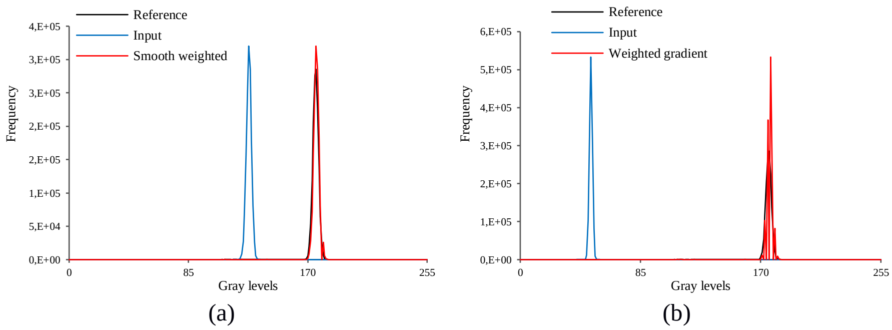


Fig. 9. Histograms of two pairs of images tested in Fig 8, with the reference image in black, input image in blue, and profile of the best result in red: (a) column 1 in Fig 8, (b) column 3 in Fig 8.

6 Conclusions

In this work, three improvements over Sintorn et al.'s gradient intensity normalization were introduced, including saturation correction, the selection of the region of interest, and two new profiles based on the use of a smoothening-based bivariate histogram, obtaining good results. Alvarado et al.'s method was also shown to be inappropriate for this task.

In some cases, wherein microscopic images have much non-useful information (background), the selection of the region of interest is a great alternative for optimizing the results of the intensity normalization.

One of the two proposed profiles of smoothening-based bivariate histogram, called smooth weighted, in most cases exhibited a better approximation in the intensity normalization with respect to the reference, than original proposed profiles.

Since the best profile for adjusting two images depends on the type of image, Bhattacharyya's distance is adequate for measuring the quality of the adjustment and automating this task.

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