Application Of Morphology Filters To Compensation Of Lateral Illumination Inhomogeneities In Confocal Microscopy Images

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Abstract. Due to multiple distortion effects, confocal laser scanning microscopy (CLSM) images of even homogeneous specimen regions suffer from irregular brightness variations. Grayscale map variations hamper image postprocessing e.g. in alignment of overlapping regions of two images, in 3D reconstruction etc., since most measures of similarity assume a spatially independent grayscale map. In this paper, a fast correction method based on estimating a spatially variable illumination gain, and multiplying acquired CLSM images by the inverse of the estimated gain, is presented. The method does not require any special calibration (reference) images since the gain estimate is extracted from the CLSM image being corrected itself. The proposed approach exploits two types of morphological filters: the median filter and a morphological operator called the upper Lipschitz cover.

1 Introduction

In images acquired by confocal laser scanning microscopy (CLSM), regions corresponding to the *same concentration* of fluorophores in the specimen should be mapped to the same grayscale levels. In practice, due to multiple distortion effects, CLSM images of even homogeneous specimen regions exhibit irregular intensity variations, e.g. darkening of image edges and lightening of the centre (Fig.1).

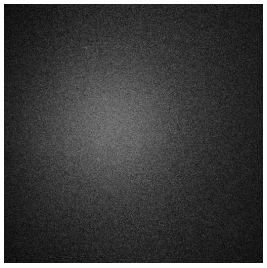


Fig 1.CLSM image of homogeneous solution of DIOC(3) fluorescent dye captured by a Leica SPE CLSM using an oil immersion 20x objective, NA=0.15.

A spatially varying grayscale map complicates image postprocessing e.g. in image stitching of neighbouring fields of view or in registration of images of successive sections for three-dimensional (3D) reconstruction. We present a fast correction method based on estimating a spatially variable gain which models the adverse effects of uneven illumination, and multiplying acquired CLSM images by the inverse of the estimated gain. The estimation is done from each CLSM image itself, not from some kind of a reference image. Two types of morphological filters are used in the approach: for the *estimation of the spatially varying gain,* a morphological operator called the *upper Lipschitz cover* is exploited. In images corrupted with noise, immediate use of the Lipschitz-cover estimate produces undesirable artifacts. These can be eliminated when *noise is removed* first using a fast implementation of the *median filter* before the gain is estimated.

2 State of the art

Various authors addressed the issue of spatially varying brightness mapping in different fields of biomedical imaging.

Mangin [1] presented an entropy minimization-based method for automatic correction of intensity nonuniformity in MR images. The approach models the intensity nonuniformity as resulting from a smooth multiplicative field. An image quality criterion is defined from an information theory perspective. The optimal correcting field is defined by the minimum of a functional combining the restored image entropy and a measure of the field smoothness. Hence, the optimal correcting field provides a trade-off between field smoothness and image quality. The functional is minimized by a fast annealing process. The smooth multiplicative field is modeled using spline functions; the process runs iteratively and processing time for a single image is in the range of minutes, i.e. the process is relatively slow. Mangin [1] assumes a thin unimodal intensity distribution for each tissue class (ideally a spike), with the result that the entropy of a perfect MR image should be very low. This assumption is not necessarily fulfilled - even for the problem statement in the original paper [1], the author himself admits that the entropy based approach may not be universal. Moreover, the algorithm is too slow (the order of minutes for a single image) to be of practical interest for processing of large stacks of CLSM images.

Lee and Bajcsy [2] share our problem statement. They address the problem of lateral intensity correction of images acquired by the fluorescent confocal laser scanning microscope (CLSM). They propose an intensity correction technique called mean-weight filtering, with data-driven parameter selection. The intensity heterogeneity correction problem is formulated as a search for an optimal, spatially adaptive, intensity transformation that maximizes intensity contrast with respect to background, minimizes overall spatial intensity variation for large area, and minimizes distortion of intensity gradient for local features. To correct spatial intensity variations, a pixel value recorded by the CLSM is multiplied by a local weighting coefficient computed over a pixel neighborhood. For the image foreground, the weighting coefficient is the ratio of the intensity mean of the whole image and the local mean in the respective neighbourhood; for the background (defined by a pixel brightness threshold), it is 1. Surprisingly, image quality criteria formulated in [2] do not enter the optimization process; their values are merely calculated and compared for various parameter choices after the correction has been made. The authors present several results obtained on synthetic images, and only one result for a single real image acquired with the Leica SP2 laser scanning confocal microscope using a 40X objective. No theory substantiating the choice of mean-weight filtering as a method for correction of illumination inhomogeneities is presented. Besides, the size and shape of the filtering kernel has to be found interactively by maximizing the global contrast while minimizing the gradient distortion, which prevents the method from being used for automatic processing of large CLSM image stacks.

In an article on image heterogeneity correction in three-dimensional multiphoton microscopy [3], Hovhannisyan et al. also present a method based on mutiplication of the acquired image by a lateral correction factor, but, in contrast to [1] or [2], they do not estimate the correction factor from the image itself. Instead, the correction factor is calculated from an image of a SRB (sulforhodamine B) uniform fluorescent sample. The necessity to capture calibration images for different image acquisition conditions to carry out lateral brightness correction limits the flexibility of the method of [3]. In addition, our own (unpublished) experiments with lateral brightness correction of CLSM images using calibration images taken from uniform fluorescent samples yielded results inferior to those achieved by the correction method presented in this paper.

3 A new method for lateral brightness correction

In contrast to all methods found in the literature, we propose a method for lateral brightness correction of *large stacks* of CLSM images which is *fast*, *fully automatic*, and *does not rely on uniform fluorescent samples*.

3.1 Estimation of spatially variable grayscale map using the morphology operator *the upper Lipschitz cover.*

Heintzmann [4], presents a formula for the intensity of the emitted light $I_{em}(x,y)$ in CLSM:

$$I_{em}(x,y) = I_{ex}(x,y) \cdot Obj(x,y) \qquad (1)$$

with $I_{ex}(x,y)$ the excitation intensity, and $Obj(x,y)$ the concentration of the fluorescent dye.

If excitation intensity is constant across the specimen, recorded light intensity for object regions having the same dye concentration should be the same. In real CLSM images as in Fig.1, however, image brightness varies depending on the pixel location within the frame. To model this dependence we suppose that the emitted light at different pixel positions is amplified or attenuated by a single function called $gain(x,y)$, that together with Eq.(1) yields the relationship between the dye concentration and the light intensity (cf. Fig.2):

$$I_{rec}(x,y) = gain(x,y) \cdot I_{em}(x,y) = gain(x,y) \cdot Obj(x,y) \cdot I_{ex} \qquad (2)$$

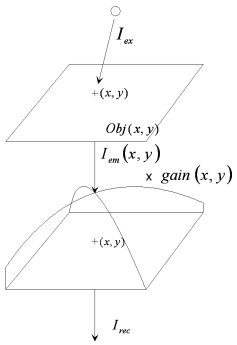


Fig 2.Formation of the recorded image according to Eq.(2): Excitation, emission, gain and recorded light.

If an estimate of the gain function, $\tilde{g}ain(x,y)$, were known, one could easily correct the recorded image to obtain the emitted light:

$$I_{corr}(x,y) = \frac{1}{\tilde{g}ain(x,y)} \cdot I_{rec}(x,y) \approx I_{em}(x,y) \qquad (3)$$

To separate in the recorded image the gain from the object, we need some qualitative features that distinguish the function $Obj(x,y)$ from $gain(x,y)$. Such distinguishing features are listed in Tab.1.

	$gain(x,y)$	$Obj(x,y)$
continuity	continuous	discontinuous
#of minima/maxima	small	large
rate of change	slow	fast

Tab 1. Comparison of the features of the object function and the gain function.

It is obvious from Eq.(3) that in a CLSM image of a specimen with *uniform* dye concentration such as in Fig.1 the grayscale distribution assumes shape identical to that of the gain function. To get the gain estimate for a *real, non-uniform*, specimen, we assume that local maxima in the acquired image correspond to specimen regions with the highest fluorescent dye concentration, and try to fill (pad) submaximal regions numerically with this

maximal concentration.

Because the form of the padded image should reflect the form of the gain, it must satisfy the condition that the rate of change is slow. This is guaranteed, if the padded function satisfies the Lipschitz condition:

$$\left|gain(x_1, y_1) - gain(x_2, y_2)\right| \le K \cdot \left|(x_1, y_1) - (x_2, y_2)\right| \qquad (4)$$

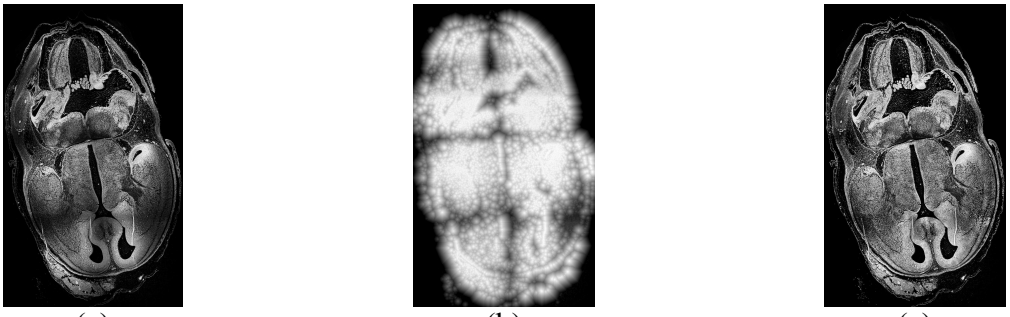
with *K*, the Lipschitz constant, limiting the maximum rate of change of $gain(x, y)$. Padding the acquired image numerically can be done very fast by subjecting the image to a morphological operator called "*the upper Lipschitz cover*".

The upper Lipschitz cover of an image $I(x, y)$ is the infimum of functions satisfying the conditions:

$$\left|L(x_1, y_1) - L(x_2, y_2)\right| \le K \cdot \left|(x_1, y_1) - (x_2, y_2)\right| \qquad (5)$$
$$L(x, y) \ge I(x, y)$$

The upper Lipschitz cover constructed in the process is used as the gain estimate $\tilde{g}ain(x, y)$. The Lipschitz constant *K* which bounds the rate of change of the gain estimate is a selectable parameter of the algorithm. A fast algorithm for numerical computation of the Lipschitz cover is presented in [5].

To validate the method for biological tissues, the proposed method was applied to correction of mosaics composed of multiple fields of view of a cross-section of a rat embryo head (captured by a Leica SPE CLSM using a HC PL FLUOTAR dry 5x, NA=0.15, objective, details are given in [6]). Fig.3 shows the results.

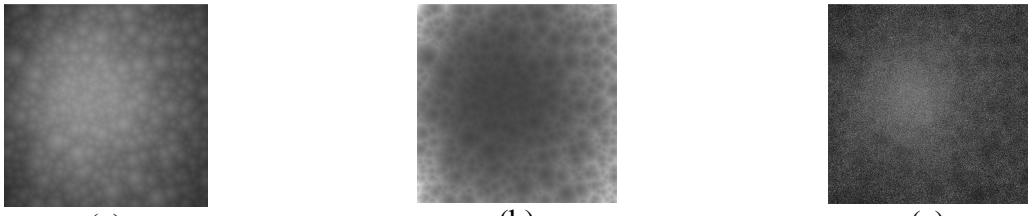


(a) (b) (c)

Fig 3.Correction of an eight-field mosaic of a rat embryo head: (a) recorded image, (b) Lipschitz-cover estimated gain, (c) corrected image.

3.2 Noise removal using the morphology operator *fast median filter.*

If the Lipschitz cover-based gain estimate is done from *noisy* images, the upper Lipschitz cover creates *cones* with vertices at noise peaks, which produce undesirable artifacts such as in Fig.4. Fig.4(a) shows the upper Lipschitz cover of the uniform sample image in Fig.1, Fig.4(b) the inverse gain, and Fig 4(c) the corrected image. The gain estimate and its inverse show circular artifacts which give rise to darker spots centered at the noise peaks in the corrected image.



(a) (b) (c)

Fig 4.Correction of the image of a uniform sample: (a) Lipschitz-cover estimated gain, (b) the inverse of the gain, (c) corrected image.

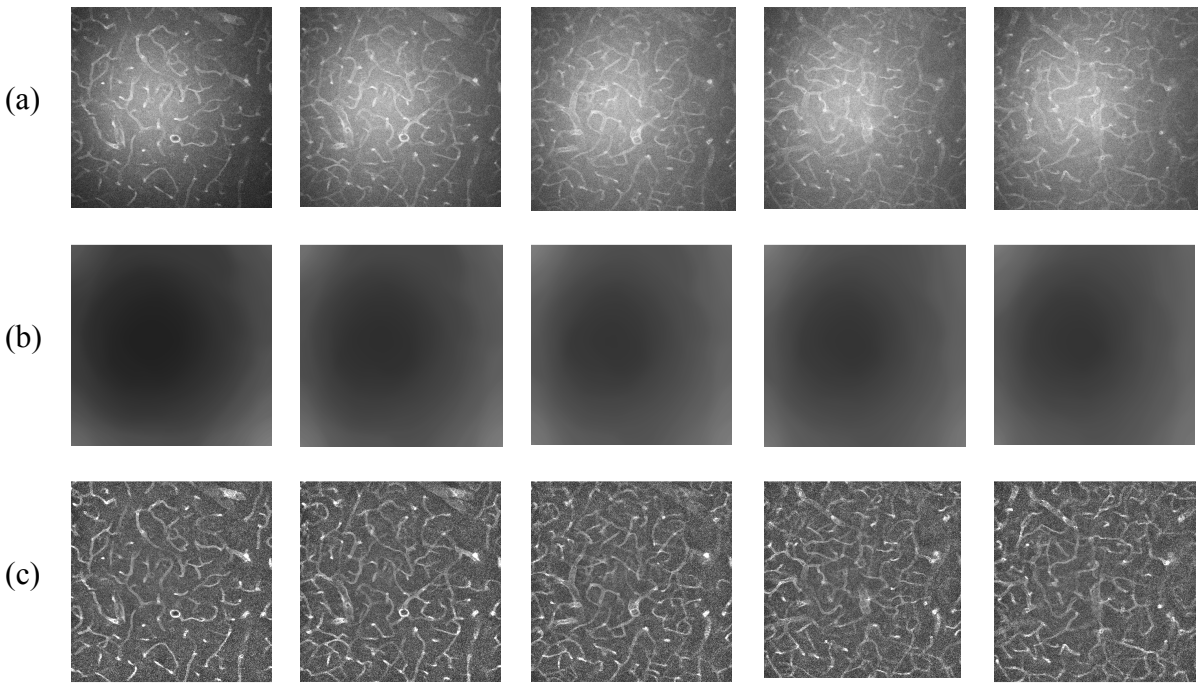
This issue can be remedied when noise is reduced using a median filter before applying the upper Lipschitz cover. Fig.5 shows the result when the original image Fig.1 is median-filtered prior to constructing the upper Lipschitz cover.

(a) (b) (c)

Fig 5.Correction of the image of a uniform sample: (a) Lipschitz-cover estimated gain from median-prefiltered image, (b) the inverse of the gain, (c) corrected image.

4 Results

As an example of a real CLSM stack lateral brightness correction, Fig.6 shows the steps in correcting images of a cross section of a rat brain cortex with stained capillaries captured by a Leica SPE CLSM using a HC PL FLUOTAR 20x objective, NA=0.15.



(a)

(b)

(c)

Fig 6.Correction of a CLSM stack of cross section of a rat brain: (a) the original images, (b) the reciprocal of the Lipschitz-cover estimated gain, (c) corrected images.

5 Discussion

The method for lateral brightness correction presented in this paper is based on the assumption that the distortions are due to a multiplicative gain modeled by Eq.(2). The results shown in Fig.3, Fig.5 and Fig.6 suggest that the multiplicative assumption captures well the image formation process. Median filtering removing noise peaks in the raw images enables the upper Lipschitz cover to reconstruct the slowly varying gain precisely enough to yield corrected images that - besides significant visual improvement - are much better suited for further processing.

6 Conclusions

Fully automatic processing of a whole stack of sixty CLSM images (512x512x8 bit) takes typically about 11 seconds on a 3GHz PC when the fast C-coded algortihm for the upper Lipschitz cover described in [5] and the fast algorithm for the median filter running in O(1) time published in [7] are used. Therefore, our method is well suited for routine lateral brightness correction of large CLSM stacks. A preliminary version of the algorithm has been incorporated in the *Ellipse* (ViDiTo, Slovakia) software image analysis environment.

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