Compensation of illumination inhomogeneities in multi-resolution image acquisition in confocal microscopy

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Abstract

A multi-resolution application in modern confocal microscopy is based on a set of objectives with different resolution and motorized XY stage. An image captured through a low-resolution objective serves as navigation map where the user points to positions or areas of interest in which a high-resolution image will be captured. This allows e.g. automated acquisition in time lapse experiments, generating random, or systematic random positions for stereological counting frames, image tiling etc. However, captured image series representing a volume of interest suffers from illumination inhomogeneities.

Algorithms described in this paper compensate both uneven illumination of captured images and light attenuation with depth. The first problem was solved by using modified morphological operation called the upper Lipschitz cover. To compensate the light attenuation with depth, a novel, very fast algorithm based on matching of distribution functions has been proposed.

Keywords: *Confocal microscopy, Light attenuation, Distribution function matching*

1. INTRODUCTION

A great number of image processing algorithms are based on a hierarchical top-down principle where the knowledge obtained in the higher (coarse) level is exploited in the lower (fine) level. A cooperation between the levels results in a significant increase of efficiency. Specific data structures (pyramids, quadtrees) simplify implementation of such multi-resolution algorithms in image processing.

A microscope itself represents a multi-resolution device because it is supplied with a set of objectives with different resolution. A typical task exploiting a coarse/fine resolution is navigation when the image captured using a low-resolution objective serves as a map where the user points at positions of interest from which a high-resolution image is to be captured. The system must be calibrated to determine the correspondence between the screen-coordinates and the real units required by the motorized stage. The navigation algorithms allow various applications like time lapse experiment, generation of stereological probes or image tiling and stitching.

2. PROBLEMS

Each objective has unique mechanical and optical properties leading to inhomogeneous illumination. A confocal laser scanning microscope (CLSM) is able to scan images of optical sections from different depths of a sample, which represents additional source of inhomogeneities.

Alignment of objectives. The exchange of objectives (manual or motorized) is usually accompanied by a misalignment of views. The image captured by a high-resolution objective represents an affine transform of the image captured from the same position by a low-resolution objective. This transform includes the coefficient of magnification and also a shift caused by imprecisely aligned objectives, which can be easily compensated by software.

Light attenuation with depth. It is well known that in image acquisition by CLSM, images obtained from deep layers of a specimen are often darker than images from the top layers due to absorption and scattering of both excitation and emitted fluorescent light. This effect causes problems in subsequent analysis of biological objects. Thus, an algorithm for the compensation of the light attenuation with depth has to be applied.

Uneven illumination. We assume that image regions corresponding to the same concentration of fluorescent material in the specimen should be mapped to the same grayscale levels. In practice, unfortunately, even ideally homogeneous specimens like artificial calibration grids exhibit darkening of image edges, lightening of the centre etc.; the effects are yet more pronounced in images of real biological specimens. A host of reasons account for the effect: optical imperfections of the objective (e.g. vignetting), surface of the specimen being non-perpendicular to the laser beam which makes the rays bend depending on the angle of incidence, locally curved surface of the specimen which concentrates or fans out the light, non-planar, curved, surface of focus, rather than a plane, which may miss a thin fluorescent layer, and the like. All these contribute to spatially uneven illumination of the specimen producing eventually the aforementioned artefacts. Besides making CLSM images unappealing, a spatially varying greyscale map complicates image post-processing. If, e.g., in image stitching of neighboring fields of view, overlapping regions possess spatially varying grayscale maps, similarity measures that assume spatial independence of the grayscale relationship may fail, with the result that a stitching algorithm sticks to a wrong spot.

Both, compensation of the light attenuation with depth and the light correction in the focal plane are solved in the next chapters.

2.1 Compensation of the light attenuation with depth

Image intensities from a CLSM suffer from light attenuation with depth caused by light loss due to two main effects—light aberration and photobleaching. As a result, images captured from deep layers of the specimen are darker than images from superficial layers, which make subsequent image analysis, segmentation, and 3D visualization of biological objects difficult.

Some methods for compensation of this effect has been addressed lately [1], [2]. They are based on rather complicated heuristics. However, a simple method with straightforward implementation

giving comparable results is often preferred in real laboratory practice.

In this article we present an efficient and fast method for compensation of the light attenuation based on matching the brightness of an image to be enhanced $\bar{I}$ with the apriori chosen reference image I_{ref}, using grey-level distribution functions ("cumulative histograms") of both images. While histogram matching [3] leads to a difficult optimization problem solved, e.g., by dynamic programming, matching distribution functions exploits their monotonicity resulting in simple optimization and fast execution. The brightest image of the stack should be chosen as a reference. If no satisfactory image is found (e.g. the whole stack is too dark) then a user-chosen image enhanced by a suitable software serves as a reference one. A short description of the algorithm follows.

Definition 1. Distribution function. Let

$$h = \{h_0, h_1, ..., h_{b_{\max}}\} \tag{1}$$

be the numbers of occurrence of respective image greyscale values $\{0,1,...,b_{max}\}$. We define the distribution function of an image as a mapping

$$S : b \in \{0,1,...b_{\max}\} \to \{0,1,..., n_{pixels}\} \tag{2}$$

$$S(b) = \sum_{i \le b} h_i \,. \tag{3}$$

Obviously, the distribution function is monotonic non-decreasing. From here on, we will assume that the reference image and the image being matched are of the same size. This implies that the respective distribution functions have the same final values. These two properties simplify the brightness matching task: for any value of S, we simply need to shift the corresponding brightness value until the value of S coincides with the value of S_{ref}.

Definition 2. Brightness matching of images. We will say that the brightness of an image $\bar{I}$ matches the brightness of an image I_{ref} within the resolution of the distribution function S_{ref} if, for any two brightness levels $\bar{b}_1, \bar{b}_2$, the number of pixels of $\bar{I}$ whose brightness lies between $\bar{b}_1, \bar{b}_2$ differs from the number of pixels of the reference image I_{ref}, whose intensities lie between $\bar{b}_1, \bar{b}_2$, by no more than the sum of two histogram values of I_{ref} at, or neighboring to, $\bar{b}_1, \bar{b}_2$.

Definition 3. Brightness matching of distribution functions.

Let S_{ref} be an image distribution function,

$$S_{ref} : b \in \{0,1,...b_{\max}\} \to \{0,1,..., n_{pixels}\} \tag{4}$$

.

Define an auxiliary boundary value $S_{ref}(-1) = 0$. Let S be an image distribution function. We define a brightness mapping g of the image distribution function S onto S_{ref},

$$g : b \to \bar{b}\ ,\ b, \bar{b} \in \{0,1,...b_{\max}\} \tag{5}$$

by the relationships

$$\begin{aligned} S(b) = S_{ref}(b) \ &\Rightarrow\ g(b) = b \\ S(b) < S_{ref}(b) \ &\Rightarrow\ g(b) = b_{ref} : S_{ref}(b_{ref} - 1) < S(b) \le S_{ref}(b_{ref}) \\ S(b) > S_{ref}(b) \ &\Rightarrow\ g(b) = b_{ref} : S_{ref}(b_{ref}) \le S(b) < S_{ref}(b_{ref} + 1). \end{aligned} \tag{6}$$

2.2 Compensation of illumination inhomogeneities

Inhomogeneous light is a frequently solved problem in image stitching [4]. We developed a specific solution of this problem based on estimating a spatially variable gain which models the adverse effects of uneven illumination, and correcting images by inverting the estimated gain. The proposed approach exploits a morphological operation called the upper Lipschitz cover [5].

In confocal laser scanning imaging, the image brightness is proportional to the concentration of the fluorescent agent in the specimen; for specimen regions having the *same concentration* of the fluorescent agent, corresponding regions of the resulting image should be *equally bright*, since the brightness obeys the formula [6]

$$I_{em}(x, y) = I_{ex}(x, y).Obj(x, y), \tag{7}$$

whereby I_{em} denotes the intensity of the emitted light, I_{ex} stands for the excitation intensity, and *Obj* represents the concentration of the fluorescent agent at the location *(x,y)* of the specimen. If constant, excitation intensity can be assumed across the specimen, emitted light intensity for object regions having the same fluorescent concentration should *in an ideal case* be the same.

In order to be able to treat the problem of compensation of illumination inhomogeneities, we will make additional assumptions pertaining to CLSM imaging: First, the excitation intensity is constant across the whole specimen. Second, the emitted light at different pixel positions *(x,y)* is amplified or attenuated by diverse effects which we will summarize into a single function called *gain(x,y)* yields the relationship between the fluorescent concentration and the recorded light intensity I_{rec}:

$$I_{rec}(x, y) = gain(x, y).I_{em}(x, y) = gain(x, y).Obj(x, y).I_{ex}. \tag{8}$$

If we knew *gain(x,y)*, we could easily correct the *recorded image* I_{rec} to obtain the *emitted light* I_{em}, which is what we are really interested in:

$$I_{corr}(x, y) = (1/gain(x, y)).I_{rec}(x, y) = I_{em}(x, y). \tag{9}$$

Therefore, we will attempt to get an *estimate* of *gain(x,y)*, the function that summarizes all adverse effects on the recorded greyscale values, without even knowing the exact physical causes of these effects in detail.

I_{ex} is constant by assumption; what we see in a recorded image is the product of the fluorescent concentration in the object *Obj(x,y)* and the distortions *gain(x,y)*. If arbitrary types of objects are admitted, the functions *Obj(x,y)* and *gain(x,y)* cannot generally be distinguished. However, real CLSM objects show some features that are not to be expected in the empirical gain. So, for example, the gain changes slowly, is continuous and therefore has only a small number of minima or maxima, while the concentration of the fluorescent dye in the microscope specimen changes abruptly

on tissue boundaries, which in turn produces discontinuities and consequently high frequencies.

Our estimate of the *spatially variable gain* is based on the following reasoning: If the *concentration* of the fluorescent dye were *constant* (*e.g.* maximal) over the whole scanning layer of the object, the grayscale distribution of the captured image would possess form identical (up to a scaling constant) to that of the *gain* function. We will assume that local maxima (or at least some of them) in the acquired image correspond to specimen regions with the highest fluorescent dye concentration, and try to pad sub-maximal regions with this maximal concentration. Of course, this cannot be done physically on the specimen - instead, we *modify the brightness of the acquired image* (which frequently violates the condition of a slow change due to discontinuities at tissue boundaries and the like) so as to make it look like an image of an *object* with constant fluorescent dye concentration.

Because the form of the modified image should reflect the form of the *gain*, it must comply with the condition that *the speed of change* is slow. This requirement can be cast into the formula:

$$\left|gain\left(x_1,y_1\right)-gain\left(x_2,y_2\right)\right| \le K.\left|\left(x_1,y_1\right)-\left(x_2,y_2\right)\right| \qquad (10)$$

for any two pixels (x_1, y_1), (x_2, y_2) (Lipchitz condition). "Filling" the acquired image so as to satisfy the previous equation can be done very fast by subjecting the captured 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 $L(x,y)$ satisfying the conditions:

$$\begin{aligned} &\left|L\left(x_1,y_1\right)-L\left(x_2,y_2\right)\right| \le K.\left|\left(x_1,y_1\right)-\left(x_2,y_2\right)\right|, \\ &L\left(x,y\right) \ge I\left(x,y\right). \end{aligned} \qquad (11)$$

3. SOFTWARE

We developed a CLSM software package which can be used by the operator to capture images having different resolutions. The light compensation algorithm can be applied to captured stacks. The whole system is controlled by main dialog with a tree-like control. A manually entered (clicked) position on left image can be saved which adds a new node to a child level of a tree control. After entering the child nodes, the SCAN process moves the stage to saved positions and captures corresponding images. Any node can be defined as a new parent and the whole DEFINE/SCAN process can continue on the next level acquiring images at a higher magnification.

It is up to operator's decision what parents and how deep in the tree structure will he continue. Some nodes evidently represent regions that are not interesting and where the higher resolution image acquisition makes no sense. Automatically generated positions are very useful in applications like image tiling, stitching, generation of stereological probes, etc. Software was developed in C++ as a plug-in module of Ellipse image processing software package (ViDiTo Systems, Slovakia).

4. RESULTS

The images captured by CLSM from different depths of the same specimen suffer from the light attenuation with depth as well as the darkening of pixels in the edges of the fields of view. Compensation of both inhomogeneities is demonstrated on Fig. 1 using two different physical slices.

In our practical experiments we used a postcranial part of a 17-day-old Wistar rat embryo embedded in paraffine. The embryo was cut into thin, 30 μm thick, physical slices by a microtome. Slice1 was captured by CLSM in 3x5 neighboring positions with overlap 10%, and then a new image was created as a mosaic. Since CLSM is capable to focus through the physical slices, each mosaic consists of five images acquired from different depths (7.5 μm apart) of the same physical slice. Slice1 in 3D consists of 3x5x5=125 stitched images (first row on Fig.1). Smaller Slice2 consists of 3x4x5 images (third row). The second and fourth rows of images show the results of applying the respective two image enhancement algorithms. It is shown that both mentioned effects were removed.

The C implementation of the algorithm is very fast, it needs approximately 0.02 s to match a single pair of 512x512 8 bit images.

5. CONCLUSION

Despite the huge progress in technology, using microscopy for quantitative analyses is still very time-consuming. Most of the samples in biological research are too complicated to be acquired without the human interaction and therefore fully automated multi-resolution microscopy is successful only in very specific areas.

The presented paper solves problems of the light correction in confocal microscopy as a part of software for interactive multi-resolution data acquisition. The proposed solution allows automated data acquisition from interactively selected positions having different resolutions. Acquired images have reduced light inhomogeneities in both axial and lateral axes.

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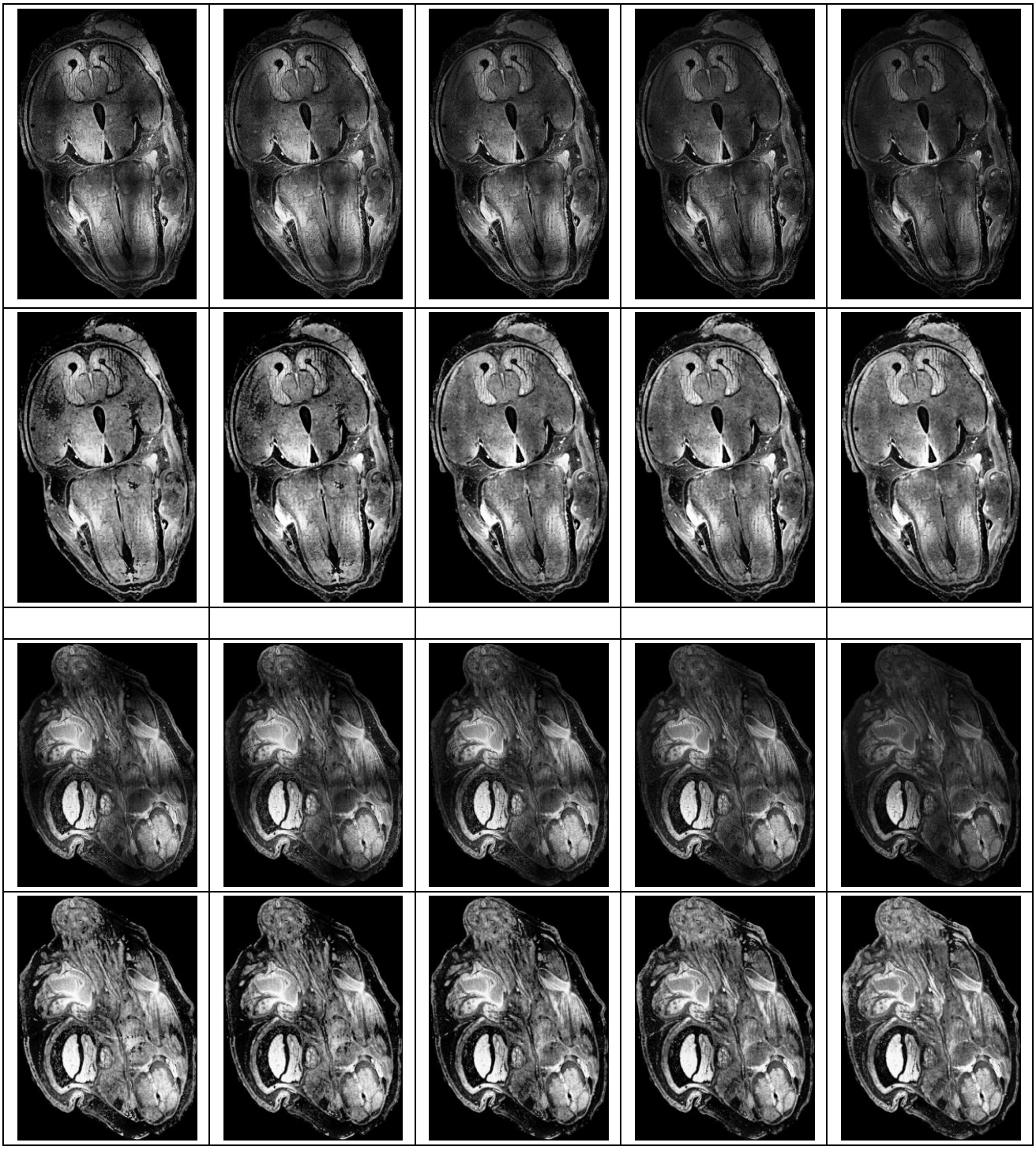

Fig. 1. Compensation of light in CLSM demonstrated on a rat embryo sample. Each image is created by stitching high-resolution images together (3x5 images from slice1 and 3x4 images from slice2). Rows 1 and 3 show the increasing light attenuation from left to right caused by increasing depth of acquisition and also the light inhomogeneities inside the individual images. Rows 2 and 4 represent the light compensation after the application of both algorithms.