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Intensity normalization of two-photon microscopy images for liver fibrosis analysis

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ABSTRACT

This paper presents an intensity normalization method for analysis of liver tissue images, acquired using the two-photon microscopy system at different stages of fibrosis. Image informatics methods require precise intensity segmentation for analysis of collagen, vessel and cellular structures. Intensities of the images recorded at different time intervals corresponding to the progression of fibrosis could vary spatially and temporally depending on the experimental conditions. These variations significantly affect the image segmentation process and thus the final image analysis, especially when automatic computer-based methods are used for diagnostic parameters quantification. We propose an adaptive intensity normalization method that facilitates spatial and temporal intensity variations of the images before the segmentation process. The images are first portioned into a tessellation of regions with relatively uniform background pixels intensities and then the normalization is performed to make sure the intensity range is unified throughout the whole set of image data. This approach is further extended for montage of images acquired from multianode photomultiplier tube based multifocal multiphoton microscope (MMM) system. The proposed approach significantly improves the automated analysis of images with varying intensities without any user intervention.

Keywords: Intensity normalization, two-photon microscopy, liver fibrosis, bile duct ligation, image informatics.

1. INTRODUCTION

Fibrosis refers to the formation of scar during the tissue growth at the wound site. Liver fibrosis is scarring on or around the liver and results as the excessive accumulation of extracellular matrix (ECM) protein including collagen as a result of wound healing response [1]. Cirrhosis is usually defined in terms of excessive accumulation of ECM proteins which distort the hepatic construction. The hepatic stellate cell (HSC) is main cell type which basically contributes to liver fibrosis [2]. In the presence of liver damage, the HSCs start differentiates into myofibroblast and proliferate resulting in forming a collagenous scar through a fibrogenesis process. Image processing based methodologies have been presented for progression study of liver fibrosis [3-7]. The experimental procedures used optical fluorescence microscope to record the digital image of stained liver biopsy sections. Colour segmentation of the recorded image is performed and the segmented image is further processed to generate a score representing the stage of liver fibrosis. These image processing methods mainly include the fibrosis area segmentation and extraction of geometric information. The main limitation to use automated image analysis using these procedures is the variation in the staining because of various stains, protocols, and photobleaching etc. In contrast, nonlinear microscopy techniques have been used in biological research and provide certain unique advantages over conventional fluorescence imaging techniques [8-10]. Our group involved for the development of second harmonic generation (SHG) based microscope system for the study of collagen during the different stages of fibrosis [11, 12]. The beam of a pulsed Ti:sapphire laser is coupled into a beam-scanning mirrors and the imaging of the sample is performed through point by point scanning. Based on the images acquired from SHG microscope, a standardized, fully automated, quantification system is developed for collagen analysis during liver fibrosis progression.
The development of fully automatic quantification system (AQS) and its validation will lead the better ways to accurately understand the different stages of liver fibrosis. For long term analysis of the sample, intensities of the images recorded at different time intervals could vary spatially and temporally depending on certain experimental parameters e.g. change in laser power, PMT gain, and tilt in the sample stage etc. These varying intensities create complexities to use the standard segmentation methods for automated image analysis. To overcome such issues, normalization of the intensities of each pixel of the images is required so that intensity values are unified throughout the various stages of the fibrosis recorded by the same system. An example of local and global variation of intensities is shown in figures 1. Here, local intensity variation means the change in spatial pixels intensities and global intensity variation is the temporal pixels intensities representing background image.

Figure 1 Intensity variations in different images representing SHG and TPEF signals, recorded at different time. Intensities of the fluorescence signals are not uniformly distributed spatially and temporally.

Spatially varying intensities of pixels in an image are not suitable for direct implementation of the intensity normalization methods. It may provide varying pixel’s intensity corresponding to the same information and thus results as under/over segmentation during image informatics. Various techniques have been proposed to address such issues, e.g. space varying filtering to enhance image appearance [13], normalize image by disposing of low frequency luminosity drift using high-pass filter [14], and normalization based on the estimation of luminosity and contrast variability of background [15]. In this paper, we propose a simple and adaptive intensity normalization method that facilitates spatial and temporal intensity variations of the images acquired from SHG microscope system. The images are first portioned into a tessellation of regions with relatively uniform experimental conditions and then the normalization is performed to make sure the intensity range is unified. This approach is further extended for normalization and montage of images acquired from multianode photomultiplier tube based multifocal multiphoton microscope (MMM) system.

2. ADAPTIVE INTENSITY NORMALIZATION

Intensity normalization of image is the pre-processing operation on the image that reassigns the gray level of each pixel into another new gray level using the predefined transformation function. It is thus increase the dynamic range of gray levels and the resultant normalized image, also called histogram equalized image, is better suited for automated analysis. To perform such normalization process, consider an image \( I \) with total \( N \) pixels which contain of total number of gray levels equal to \( G \). For a gray level \( i \), let \( n_i \) be the number of its occurrence then \( \sum_{i=0}^{G-1} n_i = N \), and the probability of occurrence of a pixel with gray level \( i \) in the image is,

\[
P_i(i) = \frac{n_i}{N}
\]  

(1)
For the given range of gray levels, let the transformation of a gray level of any pixel \( i \) of the image \( I \) to the gray level \( j \) level of the normalized image \( I_N \) is defined as \( j = T(i) \). For the transformation of image \( I \) to \( I_N \), assume the gray values of the image \( I \) are within the definite range and transformation of gray level is a single values function which is monotonically increasing within the range of gray values. In such condition by choosing the transformation function as the cumulative distribution function (CDF) will produces the transformed image (normalized image) with gray levels of the uniform density [16]. For such case the transformation can be defined as,

\[
 j = T(i) = \int_{0}^{i} P_{I_N}(k) \, dk
\]  

(2)

Normalization is performed by using the transformation function as defined in eqn. (2). However, in case of spatially varying intensities, applying this method globally on the image is result as the unwanted effect of overemphasizing noise. To overcome this issue, here we propose a simple adaptive approach, based on local region preprocessing, for normalization of liver tissue images acquired from the SHG microscopy system.

Image from the SHG microscopy is acquired through two channels, one for SHG signals and the other for two photon emission fluorescence (TPEF) signals. Images from both channels are 12 bit grey-scale images. SHG signal image refer to collagen and TPEF images represent cellular morphologies. An example of local variation of intensities of a TPEF image (with an array of 8x8 scan sections) is shown in figures 2. We assume that the PMT gain and laser power is constant for the one scanned region (as highlighted by dotted lined in figure 2). The whole image is first portioned into a tessellation of regions. One region is the scanned section and represents a single image corresponding to the constant background pixels intensity. Thus the image shown in figure 2 is divided into 64 individual images. From each sub image, the transformation of gray values of the pixels is performed independently as described above and thus normalization of each individual sub image is performed independently. Final normalized image is obtained after placing the individual normalized images at their respective positions.

The normalization results, shown in fig. 3, present the outcome when conventional method and proposed method are used for image normalization. For conventional method, the normalization is performed based on the gray levels transformation by considering the entire image pixels. It can be clearly seen that the normalized image (fig. 3(a)) is only partially improved and the pixel intensity variations is not completely removed. However this issue is completely

![Figure 2: Example of intensity variations in background pixels for a recorded TPEF image](https://www.spiedigitallibrary.org/conference-proceedings-of-spie)
resolved when the normalization is performed with the proposed method as shown in fig 3(b). Thus the proposed approach provides the normalization of image along with simultaneous correction for the varying spatial intensities.

The automated segmentation method is applied on the normalized images for quantification of the bile duct region. Bile Duct Ligation (BDL) of rats was performed under general anesthesia with ketamine and xylazine for sample preparation as discussed in ref. 11. Bile duct region is represented by the dim region in the TPEF images. Intensity threshold values for automated BDL segmentation are calculated using the K-means clustering method [17]. For globally normalized image the threshold intensity values calculated for BDL region results as over segmentation as shown in fig. 4(a). The threshold values calculation for the normalized image with the proposed method is different because of the change in the gray level distribution and present the correction for over segmentation as shown in fig. 4(b)

Figure 3 Normalization of TPEF image by using (a) Conventional normalization method, and (b) Proposed adaptive normalization method

Figure 4 Automated segmentation of bile duct region from (a) Conventional normalized image and (b) Normalized image with proposed method
Proposed method of normalization is tested for automated bile duct proliferation analysis from large amount of image data set, recorded at different stages of fibrosis. A set of 32 images, corresponding to time week0, week2, week 4 and week6 (each week contains 8 images and images are recorded at different time for different tissue samples) have been used for the bile duct region quantification. The images show varying local and global intensities. The quantification results using the global segmentation from the conventional normalized image and using the proposed method is shown in figure 5(a) and (b) respectively. Because of the inaccurate segmentation of conventional normalized image, differentiation of the bile duct progression at first three weeks is not clear (fig. 5a). This limitation can be clearly addressed using the proposed normalization method (fig. 5b) where the bile duct region can be clearly distinguished.

The presented adaptive method is simple but computationally expensive compare to global normalization method. Depending on the local intensity variations, the size of the divided region can be adjusted accordingly and it will decide computational speed of the normalization. The increase in the size of the divided region mainly depends on the intensity variations of the background image pixels.

![Figure 5 Bile duct proliferation analysis from 32 images (8 images corresponding to each week) with spatial and temporal intensity variations. Results are shown when automated segmentation method is applied for BDL region quantification from (a) normalized images using conventional method, and (b) normalized images using proposed adaptive method](image-url)

3. IMAGE MONTAGE FOR MMM SYSTEM

High throughput imaging can be accomplished with the MMM system. The MMM utilizes multiple foci with Diffractive Optical Elements for imaging multiple points simultaneously and maintains high resolution [18]. Image montage method is the process of combining multiple images, scanned through the each foci, of the sample. The scanned images contain limited overlapped regions and montage process also used to find the best overlap and produces single seamless overlapped image. This process of combining the images also called ‘tiling’ or ‘mosaicing’ which requires the algorithms for image stitching and blending to produce a single edgeless image. To do this, scanned image of the each foci is normalized as discussed in the previous section and the methodology is developed for stitching the acquired images from each foci. The aim is to explore the method for minimizing pixel-to-pixel dissimilarities while matching common overlapped regions of each scanned foci images to be stitched. It requires the appropriate mathematical model for image alignment relating to pixel coordinates in one scanned foci image to pixel coordinates in next scanned foci image. The transformation occur in the 2D plane is illustrated in fig. 6. Here the each scanned foci image section size is $N_y \times N_x$ pixels with overlapped region contain $n_y$ rows and $n_x$ columns pixels respectively.

The methodology proposed here is based on the calculation of cross correlation coefficients matrix of the overlapped region and find the shift in row and column vectors corresponding to the peak of cross correlation coefficient. The significance of the cross correlation coefficient will change depending on the size of the overlapped region from which it...
was computed. The cross correlation coefficient of an overlapped region with number of overlapped pixels $n_x$ and $n_y$ in columns and rows respectively can be defined as,

$$r_{mn} = \frac{\sum_{n_x} \sum_{n_y} (I_{1mn} - \bar{I}_1)(I_{2mn} - \bar{I}_2)}{\sqrt{\left(\sum_{n_x} \sum_{n_y} (I_{1mn} - \bar{I}_1)^2\right)\left(\sum_{n_x} \sum_{n_y} (I_{2mn} - \bar{I}_2)^2\right)}}$$

(3)

where, $\bar{I}_1 = \text{mean}(I_{1n,n})$, and $\bar{I}_2 = \text{mean}(I_{2n,n})$ are the mean’s of intensities values of the pixels of overlapped regions of the images to be stitched. The calculation of cross correlation coefficient is performed by sliding the common overlapped region in both directions, i.e., along rows and columns. The position of highest correlation coefficient represents the best match of edge features. The selection of too many pixels of the overlapped region makes the correlation coefficient calculation process time consuming, however lower the number of pixels by decreasing the overlapped region reduce the efficiency of finding the best match. The selection of the overlapped regions used is strongly related to the features to be visible in the overlapped region of the images i.e. the maximum spatial frequency present in the overlapped region.

In order to stitch an array of images, the vector of column and row pixels shifts are calculated corresponding to the overlapped regions, thus generating the matrix corresponding to each image by setup the first image $I(1,1)$ as reference image i.e. its shift in both rows and columns is set as zero. The calculation of the shift in columns and rows pixels corresponding adjacent image generate the rows and columns shift matrix. Let $R(i, j)$ and $C(i, j)$ be the shifted rows and columns corresponding to the image array element $I(i, j)$ respectively.

After generating the matrix of columns and rows shift, the next step is to first create an empty image array which contains the size of total image array plus the total maximum shift in columns and rows. If $m \times n$ be the image array size, then the size of the empty image become: $\{n \times N_x + n \times n_x\} \times \{m \times N_y + m \times n_y\}$. The pixel coordinates of the reference
image $I(1,1)$ is Row: $m \times n_x$ to $(m \times n_x) + N_x$, and Column: $n \times N_y$ to $(n \times N_y) + N_y$ and the adjacent images are placed based on their respective shift in rows and columns as shown in figure 7.

![Image montage procedure for MMM system](image)

**Figure 7 Image montage procedure for MMM system**

5. CONCLUSIONS

A region based adaptive image normalization method is presented in this paper to addresses the issues of spatial and temporal intensity variations of the two-photon microscopy images for liver fibrosis analysis. The method is presented for automated analysis of large amount of image data recorded with varying intensities according to experimental conditions. The advantage of the proposed method is presented to address the issue of variations in intensities that significantly affect the image segmentation process thus the present errors, especially when automatic computer-based methods are used for diagnostic parameters quantification. This presented normalization method is extended for for montage of images acquired from multianode photomultiplier tube based multifocal multiphoton microscope (MMM) system. The methodology of the image stitching of the normalized scanned foci images is presented.

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