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VIGNETTING AND PHOTO-BLEACHING CORRECTION IN AUTOMATED FLUORESCENCE MICROSCOPY FROM AN ARRAY OF OVERLAPPING IMAGES

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ABSTRACT

We propose a novel acquisition scheme and non-parametric multi-image based method for correcting illumination in fluorescence images. Our approach measures changes in intensity of the subject by moving the microscope stage at regularly spaced intervals, and exploits this information to learn the correction function. The acquisition process and learning are performed prior to imaging, and take only a few minutes. Afterwards, images can be corrected for vignetting and photo-bleaching effects on the fly. Our approach can be implemented in any microscope with a motorized stage, and does not require a reference calibration slide. Experiments demonstrate that our method outperforms standard approaches to illumination correction.

Index Terms— automated microscopy, registration, mosaicing, illumination correction, image processing

1. INTRODUCTION

Every image acquired by an optical microscope is affected to some degree by *vignetting*, the uneven fall-off of image intensity. Vignetting is caused by a number of sources [1, 2], all of which can introduce errors in quantitative analyses or cause unwanted seams to appear in stitched image mosaics [3].

In optical microscopy, the most common approach to illumination correction is to estimate the so-called *vignetting function*, a normalization factor used to correct the image. The vignetting function is usually estimated using either the average of large collection of similar images [4] or a smaller set of images of a homogeneous fluorescent calibration slide [5, 6]. Several approaches combine the normalization function with an additive term modeling the background [7, 8]. Methods that correct the illumination of an image using only the normalization term [9] are only valid when the additive background term is zero. In most cases, modeling the background is necessary to properly correct the illumination level [6].

There are several limitations to methods that estimate the vignetting function from a fluorescent calibration slide. Images of the slide are typically acquired under different con-

ditions from the images to be corrected (e.g., different exposure time, lamp intensity, focus settings), which may alter the shape of the vignetting function. Furthermore, the optical properties of the calibration slide differ from that of the medium. Methods that rely on a collection of images require a statistically significant amount of reasonably confluent data to estimate the vignetting function and background model.

In this paper, we propose a non-parametric multi-image based correction method and acquisition scheme which can be implemented in any fluorescent microscope equipped with an *x-y* motorized stage. It relaxes constraints on the confluence of the data, does not rely on a fluorescent calibration slide, accounts for the effects of photo-bleaching, and is capable of modeling spatial and radiometric non-linear vignetting.

The central idea of our approach is to measure how the intensity of a particular object varies when it is re-positioned at regularly spaced locations within the boundaries of the image. This is accomplished by moving the microscope stage during an acquisition step (Fig. 1a). Our technique produces a set of precisely registered overlapping images surrounding a *central image*, as depicted in Fig. 1b. As a result of the acquisition procedure, any given pixel belonging to the central image also appears at regularly spaced locations in a series of other images. This collection of appearance variation provides us with a sparse representation of the vignetting. We exploit many such sparse representations to robustly estimate the vignetting function at every image location and intensity level by grouping data from similar intensity levels.

The entire learning process is performed in just a few minutes prior to the imaging campaign. Afterwards, acquired images can be corrected on-the-fly by applying a location- and intensity-dependent vignetting correction function.

To assess the effectiveness of our approach, we acquired pairs of partially overlapping images and corrected them using our method, background subtraction, and fluorescent calibration slides. Experiments measuring the Root Mean Squared Error (RMSE) between overlapping regions of the corrected images demonstrate that our method consistently outperforms these commonly used approaches.

2. ACQUISITION STRATEGY

The proposed acquisition strategy requires a microscope with a programmable x - y motorized stage. A collection of images is acquired at positions relative to the *central position* with location u, v . The image acquired at the central position is denoted the central image, with size $H \times W$. The central image is divided into $m \times n$ equal sized regions, defining the number of points used to sparsely represent the vignetting function. Images are acquired from $u - (W - \frac{W}{n}), v - (H - \frac{H}{m})$ to $u + (W - \frac{W}{n}), v + (H - \frac{H}{m})$ with horizontal intervals of $\frac{W}{n}$ and vertical intervals of $\frac{H}{m}$. This results in an array of $(2m - 1) \times (2n - 1)$ images, each of which partially overlap with the central image, as depicted in Fig. 1b. Additionally, c central images are acquired at regular intervals to correct for intensity decay due to photo-bleaching. In total, $(2m - 1)(2n - 1) + c$ images are acquired. To ensure that intensity decay due to photo-bleaching is as uniform as possible, the acquisition order of the images is randomized.

3. METHOD

3.1. Photo-bleaching correction

Because each point in the central image is imaged many times, photo-bleaching (the destruction of a fluorophore from exposure to light) becomes a serious issue. To account for this, prior to estimating the vignetting function, we model intensity decay due to photo-bleaching and correct for it.

For this purpose, the subset of c central images collected during the acquisition step is used to model photo-bleaching decay. We assume that the effect of photo-bleaching is spatially independent, but does depend on the concentration of the fluorophore (and therefore, the intensity in the first acquired image). Intensity levels from the first image are grouped into K intensity bins, and functions mapping image acquisition order to intensity decay are learned for each bin by computing the median intensity of corresponding pixels in subsequent images (Fig. 2). Using these functions, it is possible to correct each acquired image for intensity decay due to photo-bleaching. In practice, we used lookup tables mapping the image acquisition number to intensity values to estimate the unbleached intensity.

3.2. Estimation of the vignetting function

Each image obtained during the acquisition step is first corrected for photo-bleaching, then spatially aligned using phase correlation [10]. As a result, the precise corresponding location of a given pixel x, y from the central image is known in an set of $m \times n$ offset images (Fig. 3a). The photo-bleaching corrected intensities measured from the corresponding locations in these images form a sparse, regularly spaced representation of the vignetting (Fig. 3b). Such a representation is produced for each of the WH pixels in the central image.

Fig. 1. Acquisition strategy. (a) Our approach learns the vignetting effect by observing the change in intensity of an object that results from moving the stage. In this illustration, a fluorescent cell with a homogeneous signal undergoes an appearance change due to vignetting when the position of the objective changes. (b) An array of $(2m - 1) \times (2n - 1)$ images are acquired at regularly spaced positions offset from the *central position* according to Section 2. The images are acquired in random order. This array of images is used to learn the vignetting correction. At regular intervals, the stage returns to the central position to acquire images to correct for photo-bleaching.

Unlike previous approaches which model vignetting as a function of only location $V(x, y)$, we model vignetting as a function of location and observed intensity $V(x, y, I(x, y))$, where I is the image to be corrected. To accomplish this, intensities from the central image are divided into K bins, and a separate vignetting function is learned for each bin. Each vignetting function, $V_k(x, y)$, is learned by fitting a thin plate spline to data from the sparse vignetting representations of pixels belonging to bin k (Fig. 3b). Finally, a linear interpolation is performed between each V_k , resulting in a function that maps an observed intensity at a given location to the appropriate correction factor $V(x, y, I(x, y))$.

3.3. Flat field correction

The standard model for illumination correction assumes a linear image formation model. Correction is accomplished by removing the additive background term $B(x, y)$, then normal-

Fig. 2. Intensity decay caused by photo-bleaching. These functions, learned from images acquired at regular intervals from the central position, model intensity decay due to photo-bleaching. Each line represents a lookup table mapping image intensity to acquisition number for a particular location. The unbleached intensity occurs at image number 0.

izing the image by the vignetting function $V(x, y)$ [7, 8, 6]

$$\hat{I}(x, y) = \frac{I(x, y) - B(x, y)}{V(x, y)} \cdot \bar{V}, \quad (1)$$

where $\hat{I}$ is the corrected image, I is the observed image, and $\bar{V}$ is the mean of the vignetting function [5]. However, many commonly used methods ignore the additive term and directly normalize the observed image [2, 5, 9].

Our method, on the other hand, directly learns a mapping function from the true image to the observed image. To obtain the corrected image from the observed image, the mapping is inverted

$$\hat{I}(x, y) = \frac{I(x, y)}{V(x, y, I(x, y))} \cdot \bar{V}(\cdot, \cdot, I(x, y)), \quad (2)$$

where $\bar{V}(\cdot, \cdot, I(x, y))$ is the mean of the vignetting correction surface for intensity $I(x, y)$.

4. MICROSCOPY AND IMPLEMENTATION DETAILS

Experiments were performed using fluorescence images of fixed HeLa cells, where the Actin filaments were stained with red fluorescent protein (RFP). Cells were seeded into a standard plastic 384-well plate. The biological assay was a human genome-wide RNA interference screen. The microscope used to acquire the images was an inverted ImageXpress Micro Widefield (Molecular Devices, United States), with a motorized x - y stage, a 12-bit CoolSNAP HQ digital CCD camera (Photometrics, United States), and a Plan Fluor ELWD objective with $20\times$ magnification. Images were acquired with a pixel resolution of $0.3225 \mu\text{m}/\text{pixel}$.

In our experiments, the image size was $H=1392$, $W=1040$, the number of intensity bins was $K=20$. The sparse vignetting

Fig. 3. Estimating the vignetting for intensity k . (a) As a result of the acquisition process and alignment step, the precise corresponding locations of a given point (red dot) in the *central image* (left) is known for the set of $m \times n$ offset images (right). (b) These points form a sparse regularly spaced representation of the vignetting function V_k . Many such sparse representations are combined from pixels with similar intensity (blue cross), and fitted with a thin plate spline to estimate the vignetting for intensity k , V_k .

representation contained $m=10$ and $n=10$ points, which required the acquisition of a 19×19 array of images, spaced 10% apart. Additionally $c=25$ center images were acquired to model photo-bleaching, making a total of 379 images.

To ensure robust vignetting estimation, V_k is only calculated when a sufficient amount of data (300 points) is available. Because minor registration errors can have an adverse impact on the estimation of the vignetting for pixels with a strong gradient, we only used sparse vignetting representations for pixels with the lowest 30% gradient values.

5. EXPERIMENTAL RESULTS

To assess the effectiveness of our approach, we collected a test set of 100 overlapping image pairs in which the second image is shifted by $\frac{1}{2}H$, $\frac{1}{2}W$ (Fig. 4). The images were precisely aligned using phase correlation. Each image pair was then corrected and the RMSE between the overlapping area of the two corrected images was computed to measure performance.

We compared the results of our method with results obtained from two other widely used approaches. For the first approach, *background subtraction*, we collected a set of 37 empty images of the culture medium, and computed the median image to model the background B . Images were corrected by subtracting the background, $\hat{I} = I - B$. For the second method, *calibration slide*, we estimated the vignetting function V as the median of a set of 252 images of an RFP fluorescence calibration slide (FluorRef, United States). Images

Table 1. Vignetting correction performance

	Uncorrected	Background Subtraction	Calibration Slide	Our Approach
RMSE	12.12 ± 2.48	11.75 ± 2.43	9.26 ± 1.82	8.96 ± 1.85
<i>p</i> -value	-	0.19	1.75×10^{-19}	1.12×10^{-20}
Average Improvement	—	3.02%	23.10%	25.58%

were corrected according to Eq. 1.

The RMSE was computed for each method on every image pair from the test set. The average RMSE for each method is provided in the top of Table 1. In the middle row, tests for statistical significance using a two-sample Kolmogorov-Smirnov test indicate that our approach improves over the uncorrected images with high confidence. At the bottom of Table 1, we can see that on average, our approach improved the RMSE over the uncorrected images by 25%, while *calibration slide* improved by 23%, and *background subtraction* by 3%. If we rank the methods by RMSE performance for every test pair, we find that in 86% of the cases, our method performed the best. The *calibration slide* performed best in 14% of the cases, while *background subtraction* performed best in 0% of the cases. A pair of uncorrected test images is shown in Figure 4a. A close-up in Fig. 4b reveals the effect of vignetting in the uncorrected images where the images are stitched. The effect is no longer visible in Fig. 4c, which shows the same region after correction using our method.

6. CONCLUSION

In this work, we present a non-parametric multi-image based method to correct for vignetting in fluorescent microscopic images. It is also capable of correcting for photo-bleaching when the amount of exposures is known. The approach can be implemented in any microscope with motorized stage. We demonstrate that our method outperforms background subtraction and correction using a calibration slide.

Source code and material related to this work are distributable upon request.

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7. REFERENCES

- [1] D. B. Goldman, "Vignette and exposure calibration and compensation," *IEEE Transactions on Pattern Analysis and Machine Intelligence*, vol. 32, no. 12, pp. 2276–2288, December 2010.
- [2] F. Piccinini *et al.*, "Multi-image based method to correct vignetting effect in light microscopy images," *Journal of Microscopy*, vol. 248, no. 1, pp. 6–22, October 2012.
- [3] L. Carozza *et al.*, "Mosaicing of optical microscope imagery based on visual information," in *Proc. 33th annual international conference of the IEEE Engineering in Medicine and Biology Society (EMBC 2011)*, August 30 - September 3 2011, vol. 1, pp. 6162–6165.
- [4] Thouis R. Jones, Anne E. Carpenter, David M. Sabatini, and Polina Goll, "Methods for high-content, high-throughput image-based cell screening," in *Proc. of the Workshop on Microscopic Image Analysis with Applications in Biology (MIAAB 2006)*, October 5 2006, pp. 65–72.
- [5] S. E. Weigum *et al.*, "Cell-based sensor for analysis of egfr biomarker expression in oral cancer," *Lab Chip*, vol. 7, no. 8, pp. 995–1003, 2007.
- [6] M. A. Model and J. K. Burkhardt, "A standard for calibration and shading correction of a fluorescence microscope," *Cytometry Part A*, vol. 44, no. 4, pp. 309–316, July 2001.
- [7] A. Burga *et al.*, "Predicting mutation outcome from early stochastic variation in genetic interaction partners," *Nature*, vol. 480, no. 7376, pp. 250–253, 2011.
- [8] M. Schwarzfischer *et al.*, "Efficient fluorescence image normalization for time lapse movies," in *Proc. 5th Microscopic Image Analysis with Applications in Biology (MIAAB 2011)*, September 2 2011, pp. 1–5.
- [9] T. Ragan *et al.*, "Serial two-photon tomography for automated ex vivo mouse brain imaging," *Nature Methods*, vol. 9, no. 3, pp. 255–258, March 2012.
- [10] C. D. Kuglin and D. C. Hines, "The phase correlation image alignment method," in *Proc. IEEE International Conference on Cybernetics and Society*, 1975, pp. 163–165.

Fig. 4. An example test pair. (a) A pair of overlapping uncorrected images used for testing. The red arrow points out a seam due to the vignetting effect. (b) A close-up of the uncorrected test pair reveals the effect of vignetting where the images are stitched. (c) Vignetting is noticeably reduced in the same region after flat-field correction using our approach. The contrast of the images has been stretched to improve visualization.