Is an empty field the best reference to correct vignetting in microscopy?

Alessandro Bevilacqua\textsuperscript{1,2}, Filippo Piccinini\textsuperscript{2}

\textsuperscript{1}DEIS, Department of Electronics, Computer Science and Systems, University of Bologna, Italy; \textsuperscript{2}ARCES, Advanced Research Center on Electronic Systems, University of Bologna, Italy;

Abstract

The vignetting effect is the radial attenuation of light intensity from the center of optical axis to edges. The most common approach to compensate for vignetting is to build the vignetting curve from an empty scene (empty field). This approach works in case the linearity of the acquisition system is a reliable approximation of reality. This work aims at identifying possible situations where the empty field could not be the best choice. We take into consideration histological samples and cell cultures and measure vignetting correction built from empty field and from the background of each specimen, that in these cases is considered quite homogeneous. We compare the different vignetting curves with the empty field one and measure the differences. Besides, we apply the vignetting correction to build a mosaic and measure which curve yields the best outcome. The experimental results prove that for a wide class of samples small non-linearities hint at using a vignetting function built directly from the specimen itself.

Keywords Empty Field, Image Analysis, Microscopy, Mosaic, Vignetting

1 Introduction

Nowadays, the optical microscopes are part of the ordinary basis equipment of all biological laboratories. Analyses of Cell Culture (CC) and Histological Specimen (HS) underlie investigation and treatment of many diseases and they can derive great benefits from the improvements of the microscopic image analysis.

Typically, all the images acquired with optical microscopes are characterized from a radial fall-off intensity from the center of optical axis. This intensity curvature function is called vignetting and it mostly depends on the setup of the whole system, meant as light source, optics and digital camera’s sensor [1]. In the ideal condition of the microscope's sample being evenly illuminated (Köhler illumination [2]), the radiance exiting the objective’s lens decreases as cosine-fourth of the angle between principal ray and optical axis. On the contrary, the law of intensity fall off is hardly parameterizable [3]. Due to vignetting curvature, in each acquired images there are tonal artifacts that may create intensity drifts in the represented objects, thus misleading image analysis, such as in the algorithms designed to study cells proliferation and migration [4], to segment and characterize tissues [5] and cells [6], or to quantify the signal intensity of a protein expression [7]. A yet more evident effect is produced when more images are stitched together to form a mosaic that increases the microscope’s field of view: in the stitching regions, the seams can be perceptible [8].

To obtain an image’s curvature perfectly even, it is necessary to implement a correction of the vignetting function. Typically, to solve this problem a single Empty Field (EF) image, or a sequence, is acquired to characterize the vignetting function and to correct it [4, 5, 7]. However, EF images can be acquired only in case the microscope setup keeps unchanged [6] and the instrumentation is (still) at one’s disposal. In addition, acquiring images of EF every time the microscope setup changes could be time consuming [9]. Theoretically, the vignetting function should be characterized every time the focus changes and this is why there are many methods that computes vignetting function starting from real scenes. Nevertheless, characterizing the vignetting function starting from EF images is still the most straightforward approach.

In general, the vignetting function computed from EF does not take into account the interaction between light and matter being studied, here specimens. Accordingly, it could not be the best methods for any application. To our knowledge, this is the first work that aims to assess if and when the estimated vignetting function could take advantage of using the images containing the same background as the specimen. Or else, if EF could be really suitable to represent the vignetting in examinations of CC and HS, that together cover the main part of routine examinations. In this work, besides images of EF, we acquire images of the background of the specimen, where the background is the glass slide for HS and the Culture Medium (CM) for CC. One approach is then employed to characterize vignetting from the diverse sequences. The experiments provides a numerical confirmation that EF is not always the best approach to characterize the vignetting function used to correct the acquired samples.

2 Model and Methods

In optical microscopy, several factors (sketched in Fig. 1) contribute to form the vignetting function. First, we have the non-uniformity of the light field that illuminates the specimen: the Köhler illumination is just an approximation of reality. The interaction between light
and sample causes attenuation of the beam that crosses it and reaches the lens. Theoretically, the optics contributes according to the law of cosine-fourth but the non-idealities such as, for instance, lens’s impurities and thickness bring their contribution to distort the final image. Finally, the angular sensitivity of the digital sensor emphasizes the non-uniformity of the vignetting function [1].

![Figure 1: Light path in optical microscopy. The rays emanating from the source light strike the specimen: the crossing rays are transmitted through the lens and reach the camera sensor.](image)

Let $I(x)$ be a function representing a generic image, where $x$ is a lexicographically ordered vector of coordinate points. Starting from the general camera image model proposed in [1, 10], $I(x)$ can be expressed according to Eq. 1:

$$I(x) = r(G \cdot V(x) \cdot L(x))$$  \hspace{1cm} (1)

where $r(I)$ is the camera response function, $G$ is the camera gain due to exposure, $V(x)$ is the (spatially variant) vignetting function and $L(x)$ represents the scene intrinsic propriety. In outdoor, $L(x)$ is the scene radiance while in microscopy it represents the transmitted light. Without loss of generality, $r(I)$ is considered as being linear and spatially invariant. Nevertheless, the model can be easily generalized to non linear functions, provided that they are strictly monotone and invertible. Supposing $G$ is a positive constant, to estimate the vignetting function $V(x)$ it is enough to acquire an image $B(x)$ of a scene with spatially uniform $L(x)$ and normalized that to the minimum value of $B(x)$. Then, dividing a generic image $I(x)$ by $V(x)$ yields the undistorted (vignetting free) image $I_{EF}(x)$. This pixel-wise division is known in literature as flat field correction.

In optical microscopy, most of routine examinations regard CCs and HSS. The vignetting function can be estimated starting from EF, CM, glass slide and even from the specimen itself. In particular, to estimate the vignetting function from images of specimen, several methods are proposed [1, 10]. We choose the algorithm proposed in [11], because it is a fast method to estimate vignetting functions from microscopic images, also fulfilling real time requirements.

To study the performance of different vignetting functions, we compare mosaics of sequences of images which differ just for the vignetting function used in the flat field correction. To build mosaics, we adopt the method proposed in [8] because it does not need any prior information, besides being very efficient. As the metric indexes, we employ the Mean Squared Error (MSE, Eq. 2):

$$MSE = \frac{\sum_{x=1}^{N}(R(x) - I(x))^2}{N}$$  \hspace{1cm} (2)

and the Signal to Noise Ratio (SNR, Eq. 3):

$$SNR = 10 \times \log_{10} \frac{\sum_{x=1}^{N} R(x)^2}{N \times MSE}$$  \hspace{1cm} (3)

calculated considering all the mosaic’s overlapped area, where $I(x)$ and $R(x)$ are the lower and the upper part, respectively.

### 3 Results

The experiments aim at assessing two different quantities. First, we want to assess how much the curves of the estimated vignetting functions differ from the EF’s one. To this purpose, the differences are “weighted” according to the dynamic range of the vignetting function estimated from EF via normalization. Second, we measure the effectiveness of the different corrections in a mosaicing application, where compensation for vignetting is commonly required. The experimental results have been performed with six test sequences of images of $640 \times 512$ pixel in size. All of them have been acquired in phase contrast with $100 \times$ magnification factor by using a non-automated microscope (Nikon Eclipse TE2000-U), equipped with a digital camera (Nikon D50). It is important to stress that all sequences have been acquired under the same environment and microscope’s set up conditions. $G$ has been kept constant for all the images of each sequence.

Four sequences of images randomly acquired are used to estimate the vignetting functions: $S_{EF}$, 68 EF images; $S_{CM}$, 29 images from a well with CM only, without any cell; $S_{HS1}$, 14 images selected from a HS, where the glass slide was with not any tissue; $S_{CC1}$, 25 images, from a culture of mesenchymal stem cells with an approximate confluence of 50%. In order to compare the correction performed using the flat field achieved through the different vignetting estimated functions, two sequences of partly overlapped images have been built: $S_{HS2}$, 7 images extracted from the same HS as above but not included in $S_{HS1}$; $S_{CC2}$, 12 images extracted from the same CC but not in $S_{CC1}$. Let $V_{EF}(x)$, $V_{CM}(x)$, $V_{HS1}(x)$, $V_{CC1}(x)$ be the vignetting functions estimated from $S_{EF}$, $S_{CM}$, $S_{HS1}$, $S_{CC1}$, respectively. Everywhere, but in $V_{CC1}(x)$, we have filtered the whole images using a pixel-wise mean filter. Since $S_{CC1}$ contains foreground objects (i.e., cells) too, the filtering is limited to the sequence of background regions segmented by utilizing the approach described in [11].

Let $D_{V_\text{mean}}(x)$ be defined as the pixel-wise differences between $V_{EF}(x)$ and the remaining estimated vignetting functions $V_\text{mean}(x)$ (where $V_\text{mean}$ means one of $V_{CM}$, $V_{HS1}$ or $V_{CC1}$), normalized by the amplitude of the dynamic range of $V_{EF}(x)$. Fig. 2 shows the estimated vignetting functions ($z$ axis is reported in Normalized Units, N.U.), while Tab. 1 reports mean and standard deviation (std) of $D_{V_\text{mean}}(x)$. The best values, for both mean and std, reported in Tab. 1 refer to $V_{HS1}(x)$. As a matter of fact, the light rays passing through the thin glass substrate for tissue histology almost are neither absorbed nor reflected and the values show a slight difference with EF. On the
contrary, CM and CC represent a volume and the interaction with light may undergo non-linear behavior caused by heavier absorption and diffraction due to the plastic holder of the six wells. In addition, the medium contains impurities yielding outliers in the averaged curves (Fig. 2(c) and (d)), whose subsequent normalization to their minimum value makes the final curve higher or lower, even generating locally high absolute differences. These can be positive or negative ones, whether the vignetting curve is on average lower or higher, respectively. This appears as a bias in Tab. 1, but std gives the real impact of our operation. We can see that the best std (and mean) is for $V_{HS1}(x)$, while $V_{CM}(x)$ performs better than $V_{CC1}(x)$, due to the algorithm employed also performing a smart outliers removal.

Figures 3 and 4 show two couples of mosaics, built on $S_{CC2}(x)$ and $S_{HS2}(x)$, respectively. The first mosaic of each couple refers to simple stitching, while the second couple is built according to the flat field correction performed using $V_{EF}(x)$. As a matter of fact, using the other estimated vignetting functions bring not any visible differences, hence the related images are not reported. However, things change at numerical level. Tab. 2 reports the values of MSE and SNR in the overlapped area in the mosaics. As the first consideration, MSE for $S_{CC2}$ keeps always smaller than $S_{HS2}$, because the dynamic range of culture images is limited with respect to HS. Let us start with HS. As expected, the correction performed using EF is better, since the glass just introduces a Gaussian noise, as it can be seen by comparing Fig. 2(a) and (b). As for the sequence $S_{CC2}$, there here are non-linearities introduced by non-uniformities of the CM. Using for estimating the vignetting the same matter employed in the experiments permits to detect and face the non-linearities of the image formation system, where the model for vignetting always relies on a linear interaction between energy and matter. In fact, MSE and SNR computed using $V_{EF}(x)$ are not the best ones, rather, MSE is even the worst, while using $V_{CC1}(x)$ we always perform bet-
ter than $V_{EF}(x)$. Besides, using $V_{CC1}(x)$ we achieve the best SNR, while with $V_{CM}(x)$ we obtain the best MSE. This is probably due to the mean value of $V_{CM}(x)$ being the highest one, as it can been argued from Tab. 1. In general, we can state that the lower MSE arises from a pixel wise division with a vignetting function retaining a higher mean. However, SNR here is the worst, despite of the lowest MSE. As a matter of fact, the darkening of the flat field correction performed with $V_{CM}(x)$ prevails in SNR computation. Yet more, this gives emphasis to the improvement introduced by using $V_{CC1}(x)$ where, despite being on average a lower vignetting function, we obtain an even better result for MSE.

4 Conclusions

Using the empty field to characterize the illumination field of microscopes is the most common approach. This work compares different methods to correct vignetting, with the main purpose to assess whether using empty field really is the “gold standard”. The experiments have been carried out using cell cultures and histological samples, which both cover most of the routine examinations in optical microscopy. The vignetting functions have been estimated from a culture medium free of cells and from the glass slide and used to correct cell cultures and histological samples, respectively. The different vignetting curves have been numerically compared with that achieved using empty field. Besides, we have considered mosaicing as the target application for vignetting correction: the images have been mosaiced with the different vignetting corrections in order to emphasize possible seems. Differences are not at visual but at a numerical level. Experiments with histological samples show that using empty field is the best method. Instead, with cell cultures the lowest error is yielded using culture medium.

We have then performed experiments estimating the vignetting function from the cell culture itself. This proves that the best vignetting correction is achieved when the function is estimated from the sample, rather than from empty field.

We are now working at preprocessing of the vignetting curves, in order to remove outliers coming from object impurities and unevenness, without loosing signal.

Acknowledgements

We would like to thank Dr. Enrico Lucarelli and his staff of the Osteoarticular Research Laboratory of the Rizzoli Orthopedic Institute (Bologna, Italy) for providing the data used in this work.

References


senschaftliche Mikroskopie, 10:433–440, October 1893.


[7] S. Bolte and F. Cordelieres. A guided tour into sub-

[8] L. Carozza, A. Bevilacqua, and F. Piccinini. Mo-
saicing of optical microscope imagery based on vi-


[10] S. J. Kim and M. Pollefeys. Robust radiometric cal-
ibration and vignetting correction. IEEE Transac-

[11] A. Bevilacqua, F. Piccinini, and A. Gherardi. Vignetting correction by exploiting an optical micro-
scopy image sequence. In Proc. 33rd annual in-

Address for correspondence:
Alessandro Bevilacqua
DEIS, University of Bologna, Italy
alessandro.bevilacqua@unibo.it