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Optical Microscope Based on Multispectral Imaging Applied to Plasmodium Diagnosis

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Abstract: We have adapted an optical transmission microscope to multispectral imaging in order to diagnose *Plasmodium falciparum* without using fluorescent labels for parasites as is the case for the thick and thin blood smears. We show how a set of monochromatic Light Emitting Diodes (LEDs) has provided an efficient contrast and spatial resolution to identify the parasites inside the erythrocytes. The obtained images have been processed using MATLAB software to improve the contrast. This method is a promising alternative for malaria diagnosis.

Key words: Optical instrument, spectroscopy, malaria diagnosis, imaging process, falciparum, erythrocyte

INTRODUCTION

Malaria which is one of the planet's deadliest diseases is also known as a major cause of sickness and death in the developing world. Africa in particular pays a high tribute with not only 90% of the million of people killed each year but also with 70% being children under the age of five (Frosch *et al*., 2007). The accuracy of the biological diagnosis of malaria is very important especially as it plays an important role in the treatment of the patients. Much has been done and even if many of the techniques developed, are currently being used, some of them still remain only in research laboratories. The thick and thin blood film techniques already available in dispensaries and hospitals of countries concerned by the disease are still misused because of difficulties faced during their realization. The optical methods (Bremard *et al*., 2007; Borisova *et al*., 2007) which today appear as of powerful means for diagnosis are still insufficiently applied to diagnose malaria, but for drug design in the treatment; Resonance Raman Spectroscopy has been applied to study the malaria pigment (*Hemozoin*), a by-product of hemoglobin catabolization by the malaria parasite (Ong *et al*., 2002; Wood *et al*., 2003; Webster *et al*., 2008). Recently Belisle *et al*. (2008) have developed a sensitive detection of malaria by Third Harmonic Generation (THG) imaging. They established that malaria parasite infections can be specifically detected in infected red blood cells by imaging THG emission from the *Hemozoin* using infrared femtosecond pulsed laser excitation. This technique is expensive for the developing country and can not be used in the dispensaries. Recently Brydegaard *et al*. (2007) demonstrated a new technique of optical transmission microscopy based on broad-band multispectral imaging transmission spectroscopy employing an array of Light Emitting Diodes (LEDs). The illumination white source of a classical transmission microscope is replaced by a set of LEDs, with emission ranging from UV to Near-IR wavelengths. Using spectral isolated emission band to illuminate the sample allows higher contrast based on the specific absorption properties of distinct objects in the sample. A higher contrast can be achieved by processing the images acquired by using a set of wavelengths. This technique has been used successfully to identify the red cells and the *Plasmodium falciparum* parasites inside the trophozoides. We report here this new approach based on multispectral optical spectroscopy and color image processing, which aims at overcoming the difficulties faced during the realization of the thick and thin blood film techniques using fluorescent labels for parasites.

MATERIALS AND METHODS

This study had been conducted at National Polytechnic Institute of Yamoussoukro (Ivory Coast) from September 2007 to January 2008. The samples have been prepared in Parasitological and Mycology Laboratory of

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Abidjan (Ivory Coast). The imaging techniques were suggested by the Atomic Physics Division Lund University (Sweden).

The microscope: The type of microscope used in this experiment has been largely described by Brydegaard *et al.* (2008). In this experiment, it is a commercial microscope (ZEISS 47607-9901) which has been modified. Our images are acquired by a commercial USB digital ocular (Microcular) mounted on the microscope as shown in Fig. 1. We have used five LEDs covering the visible range, which spectra are given in Fig. 8. The spectra have been recorded with Ocean Optics USB4000 spectrometer. The change of the LEDs is done manually. The blood sample is prepared by spreading a drop of fresh blood, from finger of a sick patient, on glass slides, with typical size 75×25 and 1 mm in thickness. The image is recorded by the USB digital ocular and processed by MATLAB software. The images are separated in their RGB components in order to obtain a three dimensional image in a grayscale I = f(x,y). These RGB components correspond to the three spectral channels (R = 700 nm, G = 546 nm and B = 435 nm) in transmission measurements through the sample objects using three LED sequentially. The contribution to the intensity I_C in a pixel for the channel C is given by the relation,

$$I_C = \int_0^{+\infty} E(\lambda)T(\lambda)S(\lambda)d\lambda$$

where, E is the emission spectrum of the illumination, T the transmission spectrum, S the sensitivity of the detector and λ the wavelength (Brydegaard *et al.*, 2007).

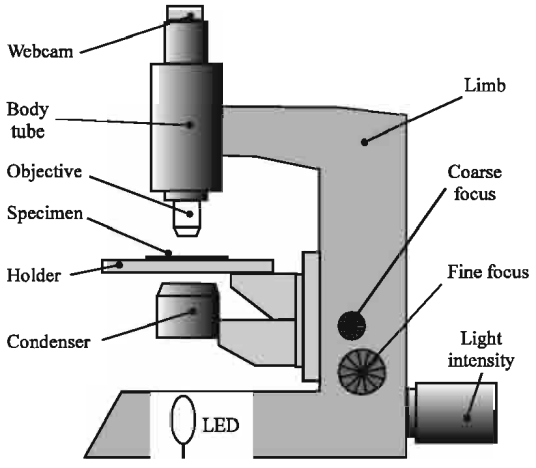

Fig. 1: Biological microscope modified by replacing the white light filament lamp by a set of differently coloured LED

The blood smear sampling: Venous blood can be collected on an EDTA coated tube by venous puncture. A capillary blood sample can also be used after a fingerstick. In this case, the finger must be carefully scrubbed with a 60° ethanol pad. By using a sterile disposable lancet, a skin puncture on the finger pad is made. The first blood drop is wiped away with a dry cotton pad. A small drop is then placed on the edge of a clean microscopy slide. Using another microscopy slide (spreader), place it on the first one (the angle between the two slides must be about 45°). Let it touch the drop, allow the blood to spread along the edge of the spreader by capillary action and then push it across the slide using an even motion. The blood must be spread on the first half of the slide. Allow smear to dry by carefully shaking it up. Label the end of the slide with a marker. The dried smear is then fixed with absolute methanol or MAY GRUNWALD solution.

Staining technique: The blood smear is stained by using a « rapid GIEMSA » solution 1/10th diluted by totally covering the L_1 slide with diluted GIEMSA solution for 10 to 15 min. Rinse the slide with tap water and let it dry. Place immersion oil on the smear for observation (x100 objective lens).

Microscopic examination: Smear screening for parasites will be made by focusing on the content of erythrocytes as *Plasmodium* is an intracellular parasite. The smear must be carefully observed for at least 30 min before calling it negative. Blood smear examination needs good training and coaching. Indeed, a good microscopist must be able not only to give true positive or negative results but also to correctly identify species involved in the disease. The Fig. 2 shows a positive blood smear picture.

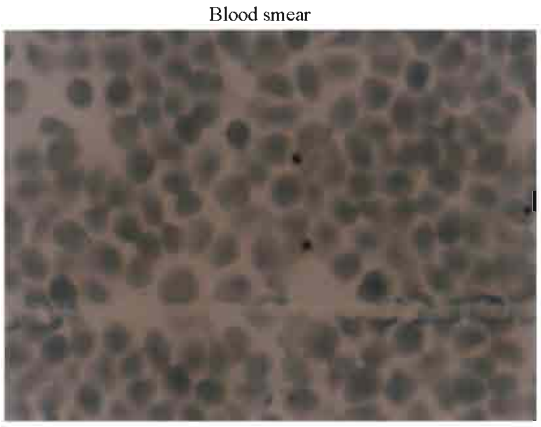

Fig. 2: Blood smear (white field)-Image dimension (160×120 μ)

The proposed method: In this case, the parasites are not labeled. The blood is spread on a clean glass slide; the immersion oil is placed on the smear for observation (x100 objective lens). The sample is then examined with microscope using a LED as an illumination light. The images are recorded on a computer via the USB digital ocular, as a function of the light wavelength (UV to IR). The Fig. 3 show six images, recorded with a white halogen light and a number of monochromatic LED lights (UV, Blue, Green, Yellow and Red). The sample image obtained with the white light illumination is compared to those obtained with the quasi-monochromatic LED lights.

RESULTS AND DISCUSSION

Figure 3 shows the multispectral images of positive blood sample.

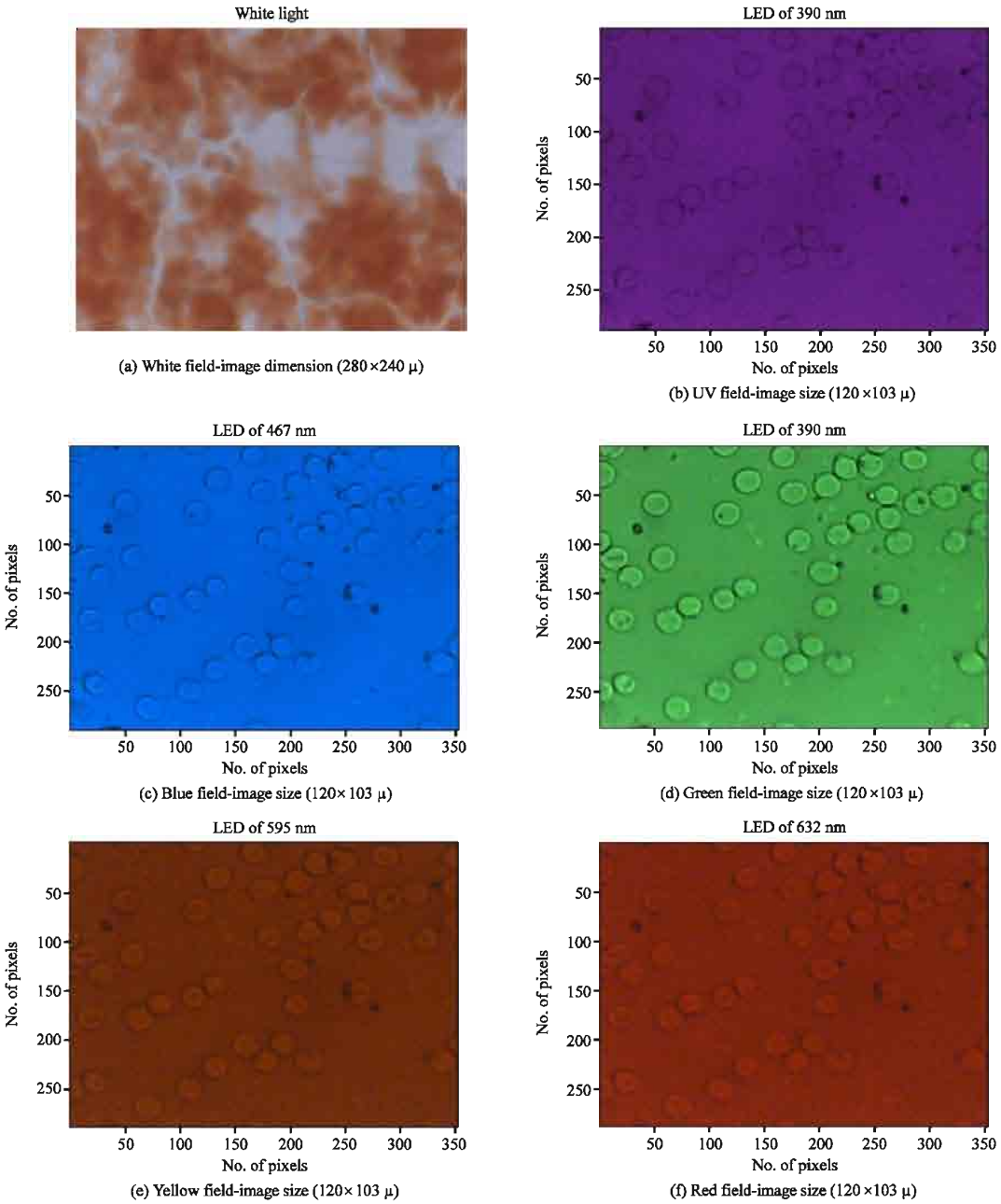

Fig. 3: Comparison between the white light blood smear image and multispectral images for quasi-monochromatic illumination

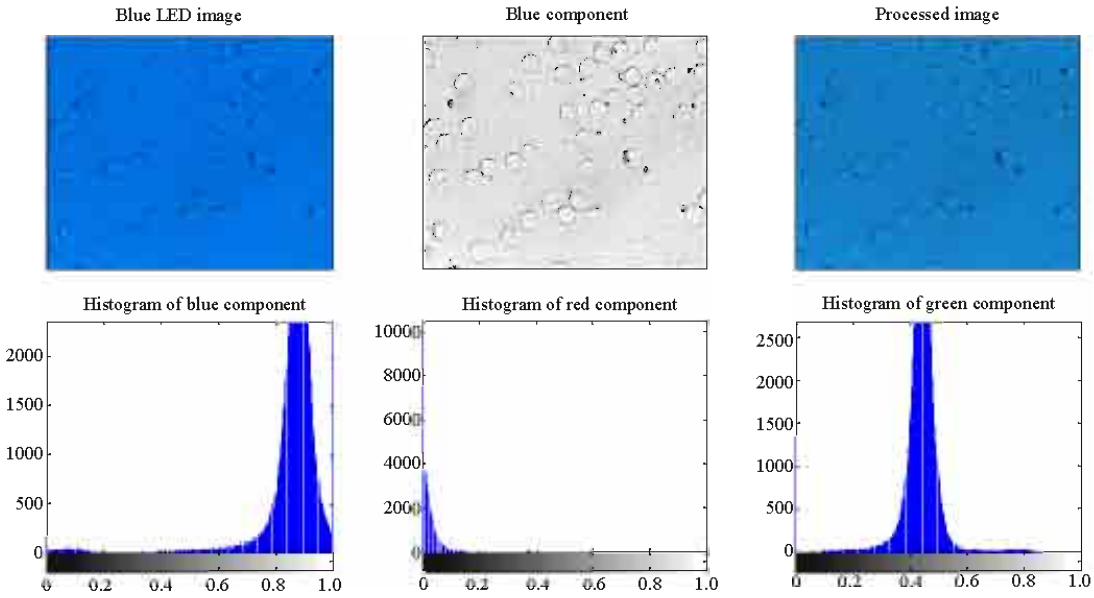

Fig. 4: Analysis of sample with the blue LED. Histogram (x for the grayscale level and y for the number of pixels)

The blood sample is coloured essentially by hemoglobin which yields the red color under white field. Comparing the Fig. 3a with Fig. 3b-f, one can observe that the images obtained with the quasi-monochromatic illumination provide a separation of distinct red blood cells and the parasites insides. This was not possible by using the classical white light. The image on Fig. 3a was acquired with the previous set-up using the non-digital camera. On the images of Fig. 3b-f, one can clearly distinguish the *Falciparum trophozoites* inside and outside the RBCs. The parasites outside the RBCs are due to explosion of some of the RBC during the preparation. In the Fig. 4, the image has been separated into its RGB components, the histogram of each component has been given and the processed image is the average of the five images through their RGB components. In Fig. 5, a fraction of the Blue image with two red blood cells (one parasitized and the second not parasitized) is analyzed using its histogram and 2D gray level repartition as a function of the pixel coordinates. This enables a clear differentiation of the RBCs and the parasites inside. After that, for more visibility, the image of the blue LED light illuminated sample has been processed using a contrast function (Fig. 6). The contrast function is an interactive algorithm, written in MATLAB, which allows a crop of selection object to highlight like the parasites. The Fig. 7 gives the simulated shape of the red blood cell to allow more understanding of the transmission function showed in the 2D gray level of the Fig. 5.

In transmission microscopy, the object image results from the transmitted photons intensity through the specimen on the detector. The incident photon intensity decreases with absorption, scattering, angular reflection, etc. The intensity of ballistic photons will decrease exponentially with distance as $I(z) = I(0)\exp(-z/l_s)$ (Liu *et al*., 1994), where z is the distance traveled and l_s is the scattering mean free path. In this case, the parasite is a hidden object inside the erythrocyte with smaller size. The parasite will appears on the image as a dark point due to the reduction of the intensity. The expected shape of the *Plasmodium falciparum* is an earlier stages in the asexual cycle, like a merozoite stage at the bottom of the erythrocyte or a ring stage of cup-like form showing a nucleus, surrounded by ribosome and some endoplasmic reticulum, with an average diameter of 1.3 μm (Bannister *et al*., 2000). The contrast appearance is a function of the complex refractive index (Sheppard and Gu, 1986) of each given object in the specimen and the wavelength-dependent diffuse transmission (Kaplan *et al*., 1994). A theoretical model of optical microscope based on the theory of partial coherence has been used to predict the image profiles (Nyyssonen, 1977). The illumination light characteristics are then crucial. The higher spatial resolution obtained with monochromatic lights is due to the reduction of the broadband effect in the diffraction patterns. The blue LED illumination image has been chosen to illustrate the physical processes involved in these images acquisition

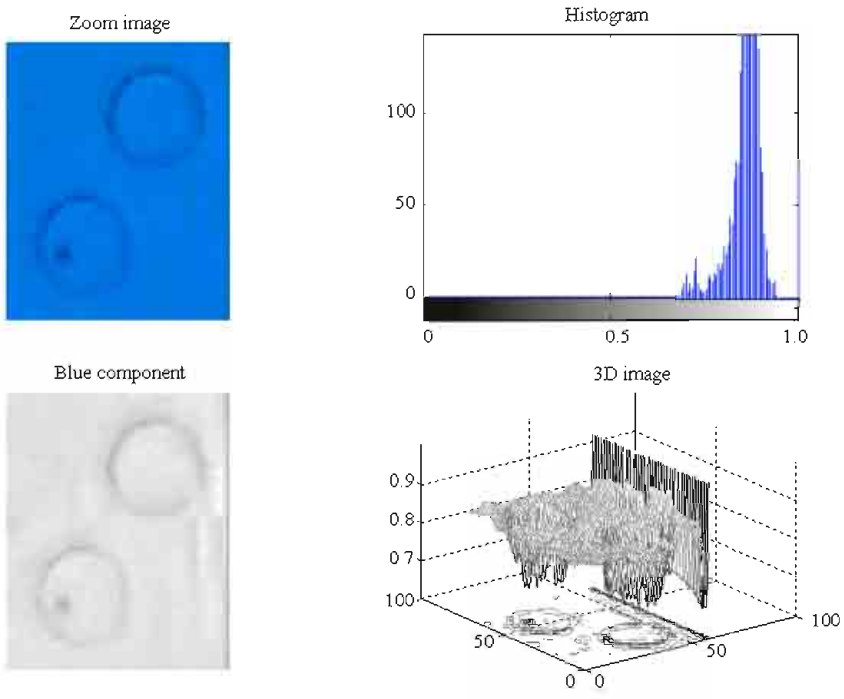

Fig. 5: Zoom image position (x:0-100; y:100-150). Histogram (x for the grayscale and y for the number of pixels) and 3D image axis are given in arbitrary units

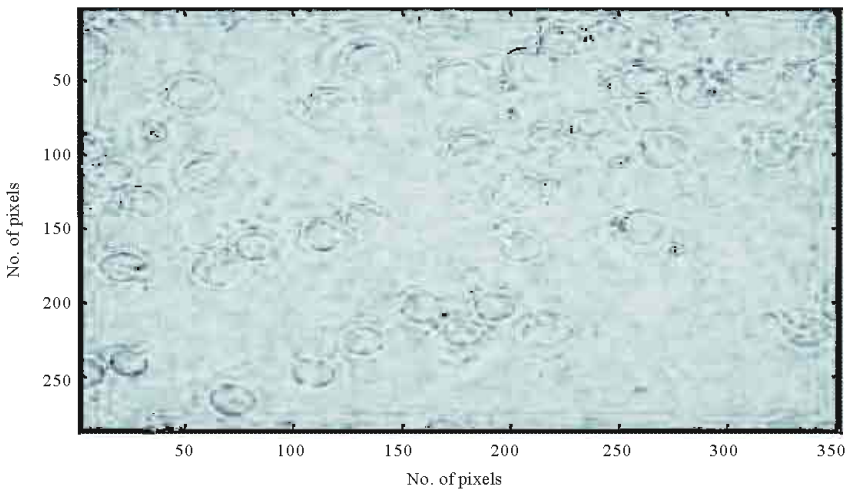

Fig. 6: Contrast function applied to the blue light sample

system. The reason is that the spatial resolution is limited to about the half wavelength used, according to the Abbe principle. The range sensitivity of the detector which had been used was 400 to 1000 nm. The UV LED wavelength is 390 nm. The Blue LED had then the shortest wavelength according to the detector range sensitivity. Hence, it gives the best resolved image. The Fig. 4 gives Blue LED illumination image, the blue component of this image, the processed image and the histogram of each component of the picture of the sample illuminated with a blue LED. The contrast obtained with the processed image is closer to the one obtained with the Blue LED. The histograms show that the blue and the green components are the most contrasted. The larger the base of the histogram, the higher contrast is the image. The red component has been used as a background image and was divided by the red and green one. In the reconstructed image, the red component was replaced by

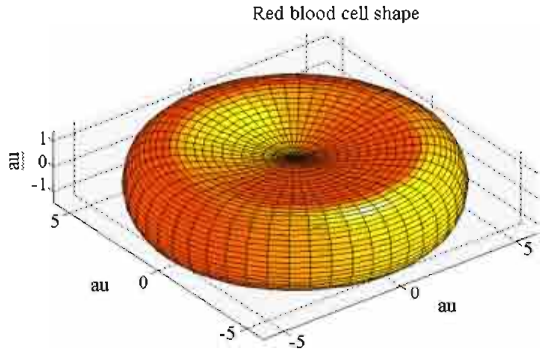

Fig. 7: Red blood cell shape in arbitrary unit (a.u)

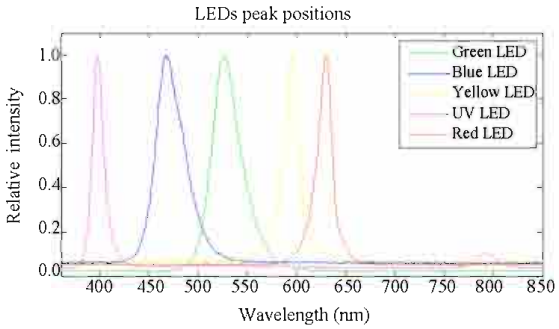

Fig. 8: Spectra of the illuminating LED

the blue one. The observed image results to the impact on the photo sensor, of the pixel to pixel intensity of the photons transmitted by the sample. One part of the photons is absorbed and scattered by the sample objects and the other one is lost by angular reflection due to the three-dimension morphological aspect of the RBCs. The red blood cells are like a biconcave disk (Fig. 7) with an average diameter of 6-8 μm inside a plasma area. It is composed of hemoglobin (32%), water (65%) and membrane tissue (3%). The shape of the RBC was described by Fung *et al.* (1981):

$$T(x) = 0.65d(1-x^2)^{1/2}(0.1583+1.5262x^2-0.8579x^4),$$

where, T is a thickness of the RBC (along the axis of symmetry), x is a relative radial cylindrical coordinate $x = 2\rho/d$ ($-1 < x <+1$), ρ is a radial cylindrical coordinate and d is the diameter of the RBC.

On the Fig. 5, the transmitted intensity of light in two dimensions shows a curve repartition of the intensity, which confirms the fact that, in addition to the absorption, the angular reflection contributes to reduce the transmitted intensity around the RBCs shape. The blue component of the blue LED gives a highest resolution; this is due to the fact that the blue component of the blue LED is the main contribution in intensity. The shadows around the erythrocytes are also due to the out-of-focus of three-dimension nature of the red blood cell (Streibl, 1984). The histogram gives the distribution of the photons intensity as a function the pixel density. The main density is related to the back image intensity and the lowest one to dark object. The light transmission as a function of the pixels area reveals the morphology of the red blood cell. The black regions inside the sample represent the parasites. The occurrence of hole inside the diagram (black regions) can be linked to the existence of the parasite. In the contrasted image of the Fig. 6, the main idea is to increase the dynamic range of the grey levels in the image being processed. The transformation used for contrast stretching is the number of gray levels; if the processed pixels before and after processing are the same the transformation becomes a "tresholding function" that create a binary image. Intermediate values of intensity produce various degrees of spread in the grey levels of the output image, thus affecting its contrast.

CONCLUSION

We have performed an original experiment which allows malaria diagnosis without labeling the parasites. Qualitative two-dimensional imaging by a transmission microscope has been demonstrated. The importance of the light parameters like the wavelength and coherence has been discussed. We have shown that some hidden biological object like a *Plasmodium falciparum* inside the red blood cell can be observed using monochromatic illuminating light in classical transmission microscope.

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