Chapter 2

Compact, low cost and field portable bright field microscope

Since its invention, the bright field microscopy proves to be an inevitable tool used in many fields of sciences such as biology, hematology, microbiology, biotechnology, bacteriology etc [3-4, 128-129]. In its most common form, bright field microscopes use a broadband source to trans-illuminate the specimen and an objective and an eye-piece lenses to magnify the object. The intensity pattern (absorption of light) resulting from the interaction of light with sample under investigation is either viewed by user through an eye-piece or is recorded using a digital array. Bright field microscopes have helped scientists and researchers to examine microscopic specimens such as parasites, bacteria etc to study cell structure in microorganisms, animal cells and plant cells. In hematology analysis, the bright field microscope is used for complete blood cell counting (CBC) such as Red blood cells (RBC), White blood cells (WBC) and platelets [128]. Also, in urine analysis, it helps to sense presence of WBCs and crystals which could indicate bladder infection. The bright field microscope (compound microscope) still stands as gold standard method in hospitals and pathology labs for diagnosis of many diseases such as tuberculosis, malaria, thalassemia, sickle cell anemia etc as well as primary inspection of illness [129]. Many advances medical science would not have been accomplished without the use of bright field microscopes.

2.1 Basics of bright field microscopes

A conventional compound microscope incorporates two to three lenses, namely, condenser lens, objective lens and eyepiece (Fig. 2.1). The condenser lens takes the light from source and focus a narrow light beam onto sample (it also increases the numerical aperture of the system). The light trans-illuminating the sample is then transmitted through objective lens which forms a magnified real image just inside the back focal plane of the eyepiece (inside the microscope tube). This leads to formation of a magnified virtual image of the real image formed by the objective lens (for finite conjugate objective lenses). To view this virtual image a converging lens (eye lens or camera lens) is required. A conventional compound microscope usually has three objective lenses (for creating the primary real image) of different magnification and numerical aperture (10×, 40× and 100× oil immersion) attached to front end of the tube. Usually the eyepiece at the back end of the microscope tube has 10× magnification. A 10× eyepiece used with 40× objective, will lead to a

total magnification of 400× total magnification (of the virtual image). This image is again imaged by the eye lens (converging lens) which de-magnifies it (by ~1/10). So the final image of approximately 40× magnification will fall on the retina/digital array.

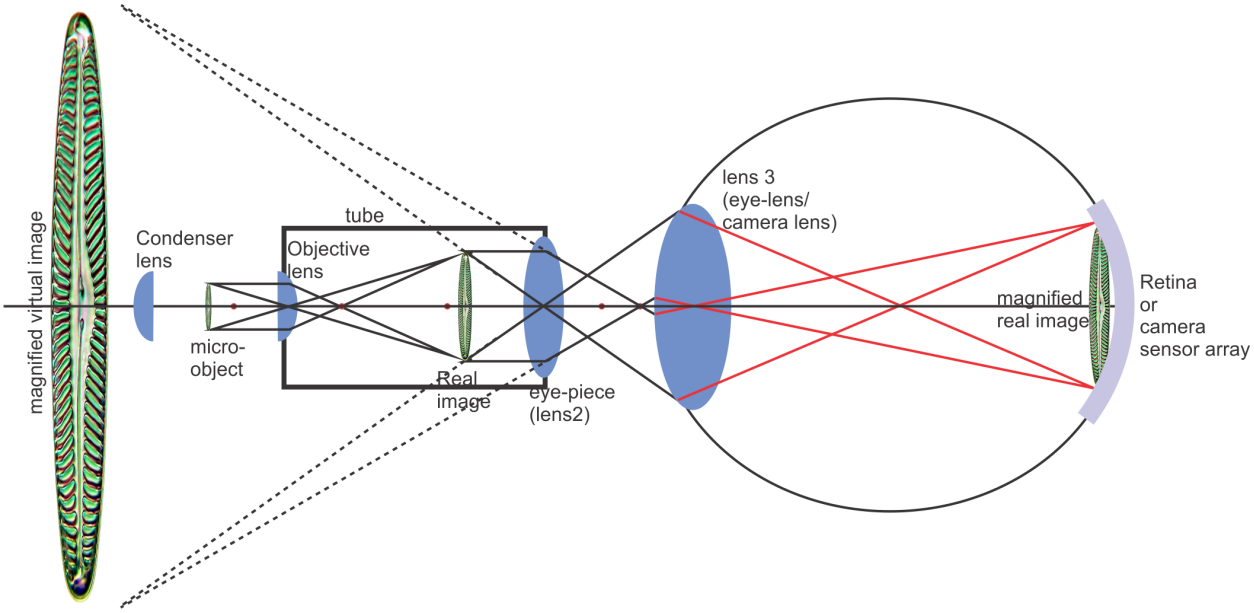

Fig. 2.1: Basic concept of a compound microscope

The conventional compound microscope works either in the transmission mode where light from the source will trans-illuminate the sample or in reflection mode where light reflected from the sample is collected. Contrast in the image is produced by higher absorption of the transmitted light in denser regions (or regions with higher thickness) of the sample compared to rarer regions (or regions with lower thickness). But most of the biological samples are transparent to visible light and hence produces low contrast images in the case of bright field microscopes [3]. So in the case of bright field microscopy biological samples must be stained with proper chemical reagents which changes the absorption cross section of the sample, making the cells or certain regions of the sample absorb more light compares to surrounding regions increasing the contrast of the images [4]. Also the tagging agents are used to pin-point certain cells or regions of the sample, to which these chemicals gets attached [3-6, 128-129].

2.2 Single lens brightfield microscope

But is it necessary to have a relay of lenses to capture the image of a sample? Apparently, a simpler system can be developed. It involves the use of a single positive lens to create a magnified real

image of the sample, that can be projected on to a screen (Fig. 2.2). If a digital array is used instead of the screen, it will lead to recording of bright field images of the sample.

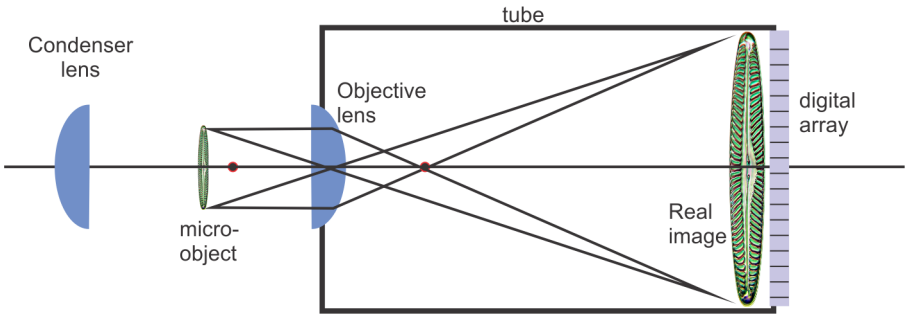

Fig. 2.2: Schematic of single lens bright field microscope

A single lens system is easy to realize. A single lens compact, low cost, pocket sized bright field microscope was fabricated by 3D printing its structure and then incorporating the light source, optical elements and the digital array. This microscope has been specifically designed for remote field applications, where basic disease diagnosis facility may not available. The developed bright field microscope is easy to use and doesn't requires trained personnel for its operation. To achieve better contrast while imaging stained samples, the microscope is equipped with RGB LED and white LED for sample illumination. The microscope can be connected to the android device or PC for capturing the sample images. The user can send the captured images to the offsite server where image is processed and analyzed automatically using in-house developed algorithms and the computed results can be send back to the user.

2.3 Development of compact field portable bright field microscope

A simple compact, lightweight digital bright field microscope based on single lens system was developed with low cost components which yields similar results compared to traditional compound microscopes. As depicted in Fig. 2.3, the developed bright field microscope consists of an array of red, blue, green and white LEDs for the illumination of sample. Three LED sources (LuxeonStar) having peak wavelengths of 627nm ($\Delta\lambda$=20nm), 520nm ($\Delta\lambda$=30nm) and 460nm ($\Delta\lambda$=20nm) were used as the source. All the sources had power less than 100mW. A diffuser was placed just below the sample holder so as to make the illumination uniform. The purpose of using different colored LEDs was to realize better contrast while imaging certain stained samples. Fig.

2.3 a shows the schematic of the microscope and 2.3b is the photograph of the assembled microscope. Fig. 2.3c shows the schematic of the control unit of the microscope.

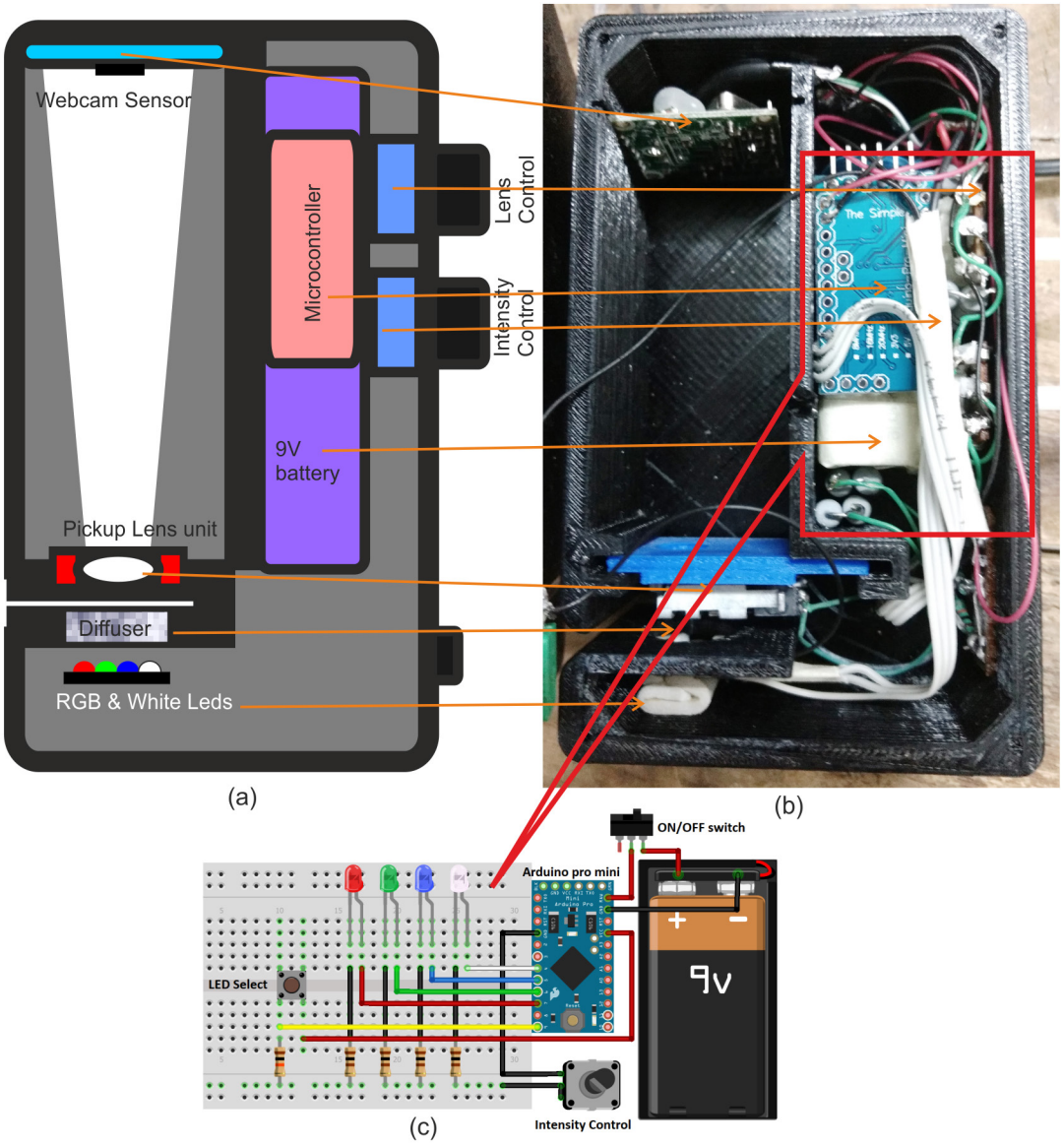

Fig. 2.3: Single lens, field portable bright field microscope. (a) Schematic of the microscope showing the position of various optical and imaging components. (b) Photograph of the microscope, showing various components mounted on a 3D printed structure. (c) Control unit of the microscope. It controls the focusing and lateral movement of the magnifying lens as well as the intensity of the LED sources and selection of LED source.

The magnification and focusing of the sample is carried out by electro-mechanically moving lens extracted from the DVD optical pickup unit having numerical aperture of 0.6 and focal length ~3.7mm. The lens rests between two permanent magnets on a platform having two voice coils which controls the position of lens in Z-axis and X-axis when varying current ranging from -20mA

to 20mA passed through them. A separate indigenous control unit was developed for controlling amount and direction of current with the aid of potentiometers which causes 1D translation motion and focusing movement of the magnifying lens. The translation motion of the lens renders ability to scan over the specimens. The single lens system formed the real image of the sample at the webcam. A VGA webcam (pixel pitch 3.2μm) as well as a 1MP webcam (pixel pitch 2.8μm) were used in the microscope. The magnification of the microscope was changed by changing the position of the sensor. The output from the webcam can be either coupled to a computer or to an android device supporting OTG connectivity. The use of quasi-monochromatic illumination not only improves the contrast but also the sharpness of the images. The whole system was run through the 9V battery and hence is stand-alone needing no additional power sources. This makes the system ideal for point-of-care applications.

2.4 Imaging with the compact bright field microscope

The developed microscope was used to image stained (chemically treated) blood specimen, intended towards numbering and identification of different blood cells. Two systems with magnification 20× and 50× were developed and tested. Fig. 2.4a shows the image of blood cells imaged with 50× microscope and Fig. 2.4b shows the images obtained with 20× microscope.

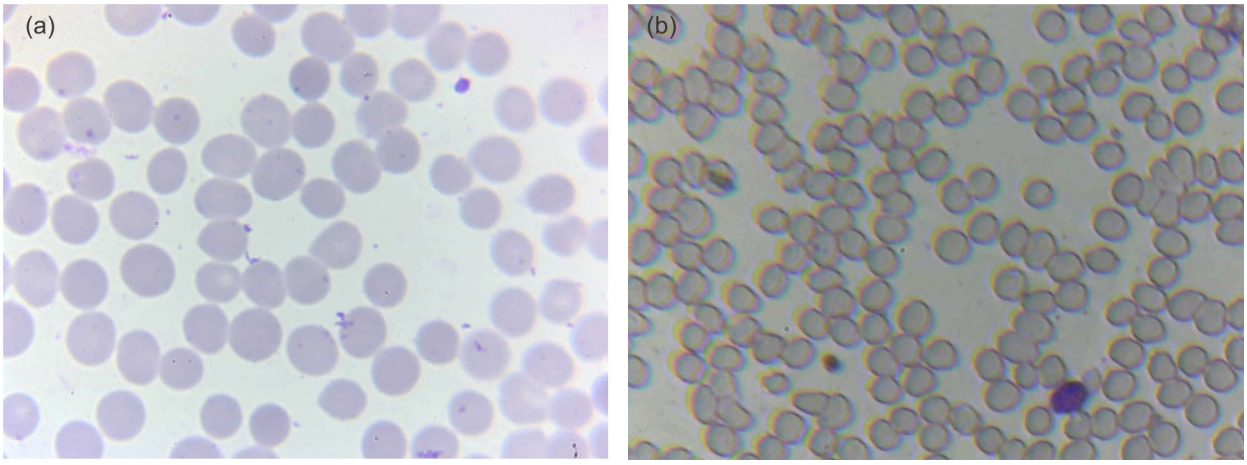

Fig. 2.4: Bright field images recorded with the field portable microscope with (a) 50× magnification and (b) 20× magnification. These images were then processed to yield different sample parameters such as the number of red blood cells, number of white blood cells as well as number of cells infected with malaria parasites. All these sample parameters were deduced using simple image processing steps, which makes the microscope suitable for use by an untrained person.

As mentioned in the previous section the microscope can be attached to an android based smart-phone. In the case of the device attached to a smart-phone (Fig. 2.5), the recorded images are send

to an off-site computer for post-processing and data mining. A web based application was developed to send the microscope images to the off-site computer. The computer contained the code developed in python to analyze the images.

Fig. 2.5: Portable bright field microscope attached to smart-phone.

The end user, uses the application and uploads the images to the images to the server. The processing of the data (images) takes place at the server and the extracted sample information (like number, species etc) is made available to the user, who may use it for informed decision making, which may include disease diagnosis and treatment.

2.5 Image analysis

As discussed in the previous section, the microscope was mainly used to image stained blood samples to identify different blood cells (red blood cells, white blood cells and platelets) in the field of view. An attempt was also made to use the microscope for identification of malaria by identifying red blood cells in the field of view with different absorption profile. Initially low magnification (20×) was used so as to create a high throughput device with 150 to 200 cells in a single field of view using a 1MP webcam array (2.8μm pixel pitch). The processing of the recorded images employed simple image processing steps but was found to yield accurate enough results. The processed data was used to obtain sample parameters.

In the first step, the microscope is calibrated to find the area (number of pixels) occupied by different types of blood cells (red blood cells, white blood cells and platelets) so that the number of cells of different category can be determined. Once the number of pixels occupied by a cell species is available, it can be used to identify the location of the cells and number of that species of cells. Table 2.1 shows the area (in number of pixels) of each cell species in the blood sample for the developed microscope.

Table 1.1: Area of blood cells

Cell type	Area (in pixels)	
	For 50× magnification	For 20× magnification
Red blood cells (RBC)	14270±2297	2736±242
White blood cells (WBC)	24320±5292	4237±845
Platelets	1342±498	198±68

Computed areas of the blood cells were used to discriminate between red blood cells and the white blood cells and to automatically count them. Two slightly different procedure were adopted for the 20× and 50× versions of the microscopes. Since the 50× version had slightly higher numerical aperture (since the sample is situated closer to the lens), it was used to count the number of different blood cells as well as to identify cells infected with malaria parasites, where as the 20× version was mostly used to discriminate between different blood cell types.

2.6 Elimination of background and identification of WBC

In the case of 20× microscope the recorded color images of chemically treated (giemsa stain) blood samples (Fig. 2.6a) were first converted into gray level images. From Fig. 2.6b it can be seen that the intensity (gray value) of the background (red box) is higher compared to regions having cells (black box). The background can be suppressed, and the location of the cells can be obtained by a simple thresholding of the gray level images by average gray level values and then replacing pixels with gray values greater than average value (which represents the background) by zeros. But it can be seen that the intensity distribution (even that of the background) is not constant across the field of the view. So a global thresholding of the image may lead to suppression of the cell information also (Fig. 2.7a). So instead of global thresholding a local thresholding by computing local mean gray values provides a better result (Fig 2.7b) [9, 10]. After eliminating the background from the

images, number of white blood cells were counted by further thresholding the image by the gray value of stained WBC (Fig. 2.8), which was pre-determined. The intensity values for a red blood cell having malaria parasite (which get stained by the same chemical) can also same as that of WBC, but the number of pixels it occupies will be less compared to WBC.

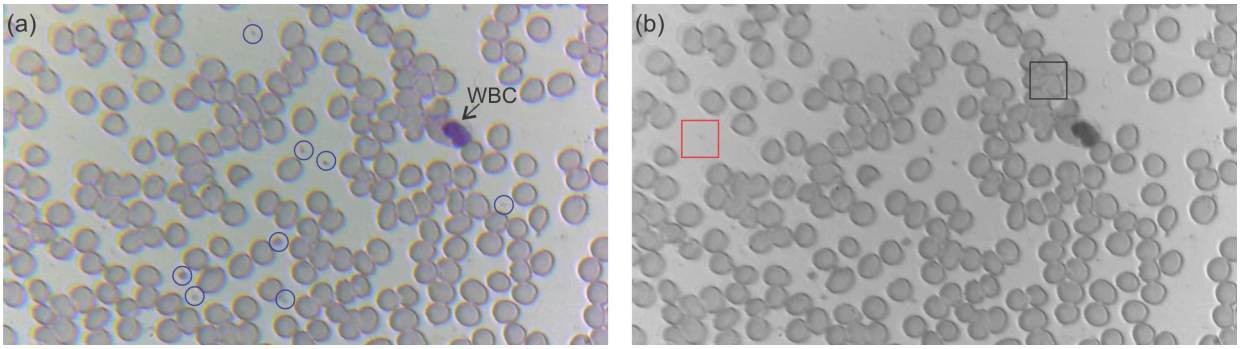

Fig. 2.6: (a) Color images recorded with portable bright field microscope with 20× magnification. (b) Image converted to gray scale. In Fig. 2.6a shows a stained WBC. Cells indie the blue circles are platelets and rest of the cells are RBCs

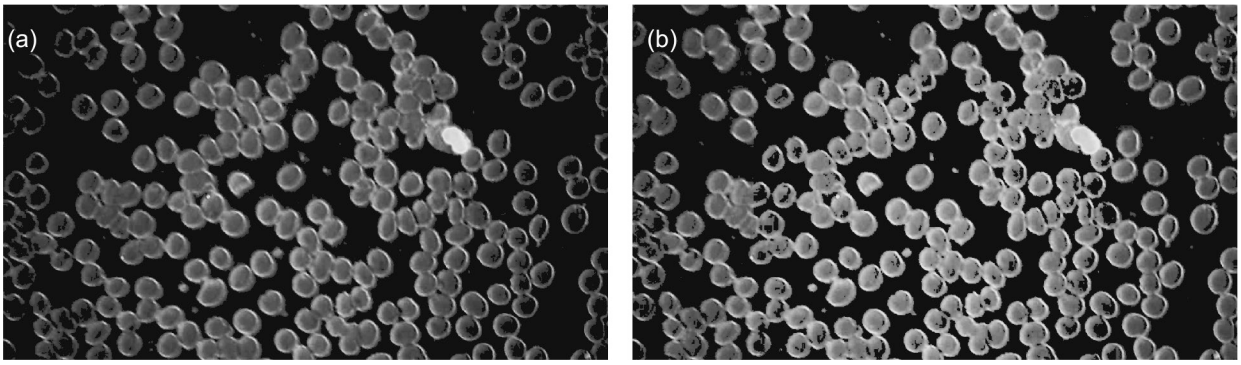

Fig. 2.7: Identified cell locations (a) using global thresholding. (b) using local thresholding.

Fig. 2.8: Counting of WBC after thresholding the image shown in Fig. 2.7b with the gray value of stained WBC. This leads to isolation of WBCs in the image.

2.7 Identification of RBC

Next step involves identifying the number of red blood cells. This is done by finding the edges in the image obtained after background suppression. The edges were determined by finding the partial derivatives in the *x* and *y* directions, which peaks at the edges (Fig. 2.9a). One the edges were determined, the regions inside the closed edges were filled and a binary mask was created and used for isolating the regions containing cells (Fig. 2.9b). To remove platelet information, regions which occupies less than 30% of pixels compared to RBCs were also eliminated. Plates can have a diameter of up to 3μm and change absorption when treated by Giemsa stain [129]. Since their diameter is less than half of the diameter of RBCs, it is safe to assume that regions having area less than 30% of RBC as platelets. Fig. 2.10 shows the image after detection of the red blood cells.

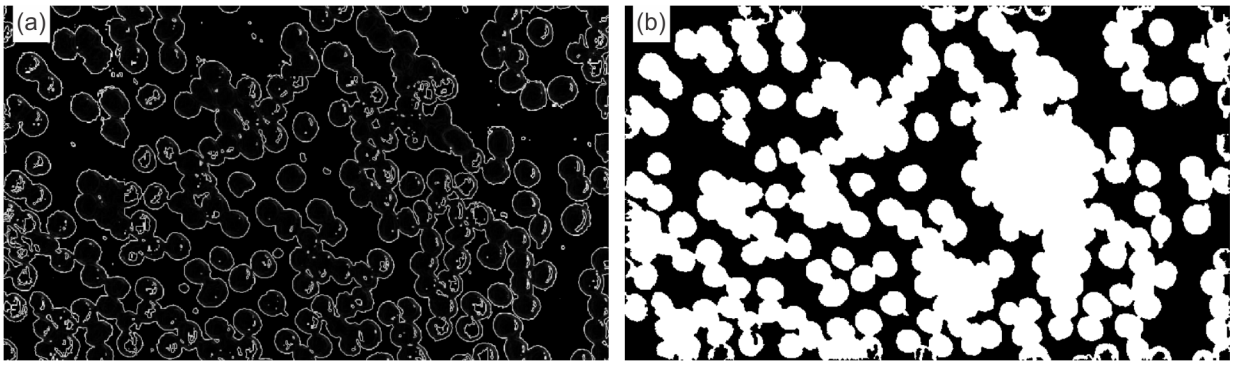

Fig. 2.9: (a) Edge detection by computing the partial derivatives of the image shown in Fig. 2.7b. (b) Binary image after edge detection the region inside the edges are filled. This may lead to regions not containing cells to be included in the counting of cells.

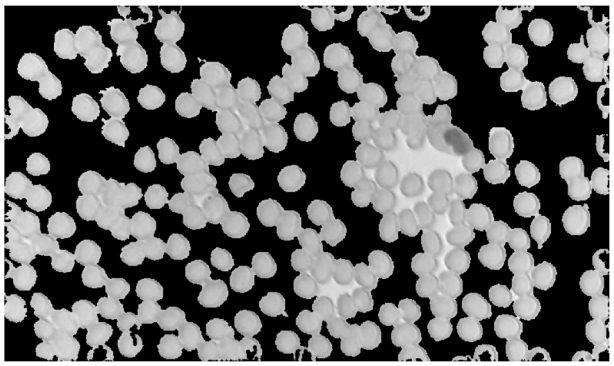

Fig. 2.10: Regions of the image identified as having red blood cells

Number of red blood cells (N_{RBC}) were determined by counting the total number of pixels having a value of 1 in the binary mask and using the following relationship

$$N_{RBC} = \frac{\left(S - N_{WBC} \times A_{WBC}\right)}{A_{RBC}} \tag{2.1}$$

where S is the total pixel having value 1 in the binary mask (Fig. 2.7b). NWBC is the number of WBCs detected in the field of view, A_{WBC} is the number of pixels occupied by a WBC and A_{RBC} is the number of pixels occupied by a RBC.

2.8 Identification of platelets

Plates are stained by the chemical and leads to higher absorption. So they can be determined from the absorption profile. Also they are smaller compared to RBCs. To identify the number of platelets in the field of view, while constructing the binary image from the edge detected images, after filling, regions with area larger than that occupied by RBC were set to zero (Fig. 2.11a) and the ensuing binary mask was used identify platelets (Fig. 2.11b).

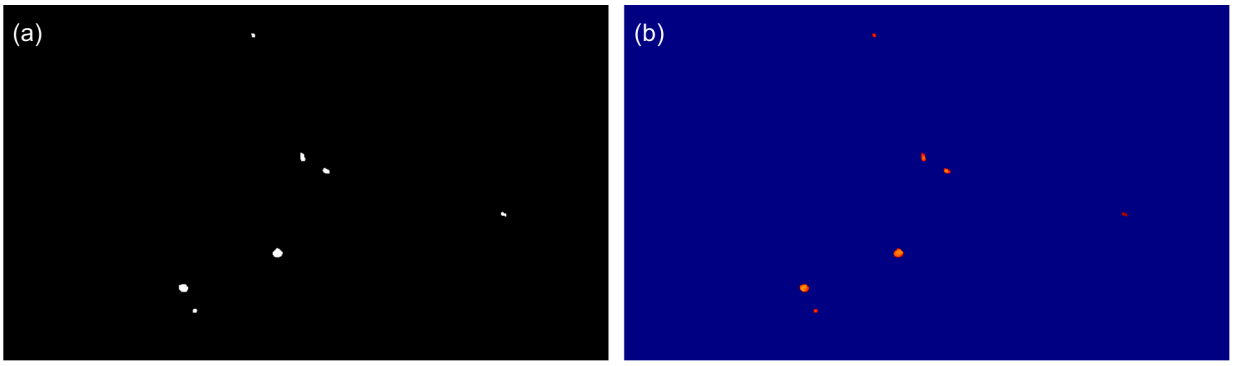

Fig. 2.11: (a) Binary mask for detection of platelets and (b) Identified platelets

Here also, dividing the total number of pixels with value 1 in the binary mask by the number of pixels occupied by a platelets leads to the count of the platelets. The image processing algorithm used in cell counting is shown in Fig. 2.12.

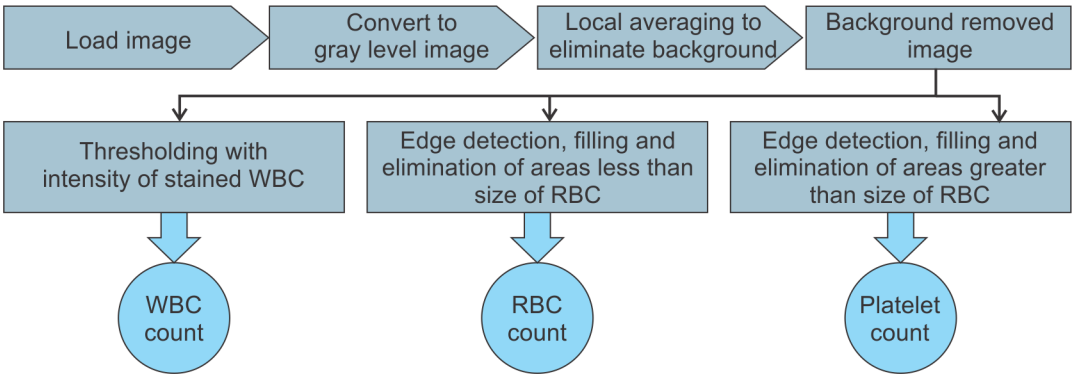

Fig. 2.12: Cell counting algorithm

Algorithm was tested on 122 field of views and the number of WBCs, RBCs and platelets were obtained. For each field of view the number of cells of each type were also manually counted. Fig. 2.13 shows the counts of the cells obtained using the described algorithm (circles) and obtained by manual counting (solid line) which is the ground truth for 30 of the recorded field of views.

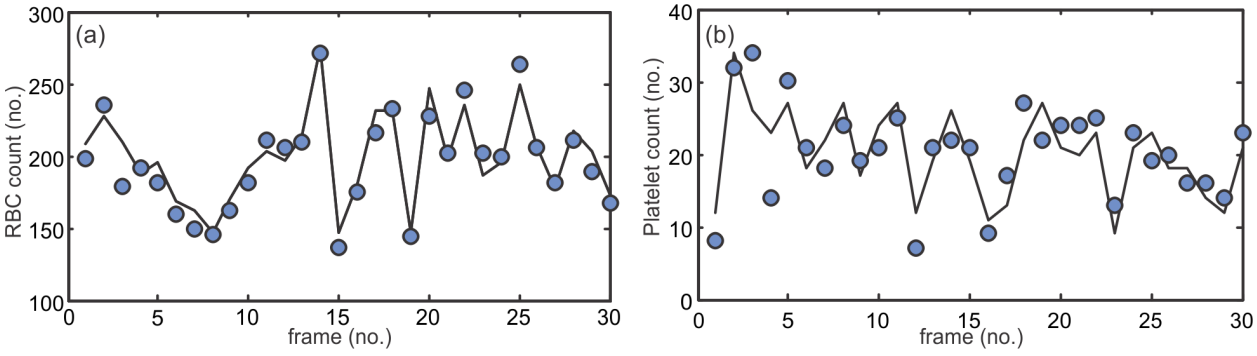

Fig. 2.13: Comparison of manual and automatic cell counting. (a) RBC count. (b) Platelet count. Solid line represent the manual count and solid circles represents automatic counting.

The average error in the counting of RBC was less than 5% over all the recorded frames. For platelets the average error was near 12%. Here the error is higher because even regions which may not have been washed properly after staining may be counted as platelets. For WBC which was very few in the field of view the error in counting was about 16%. Here also higher error resulted because some regions of the sample had large areas (compared to RBC) of the staining dye, which was not washed properly.

2.9 Image analysis of microscope with 50× magnification

Microscope with 50× magnification provided a better resolution compared to the one with 20× magnification as can be seen from Fig. 2.4. So it was used to identify, regions inside the red blood cells, which had different absorption profile after staining. Giemsa stain gets attached to malaria parasites (which are 1-2μm in size) and but not to RBCs. Fig. 2.14 shows a frame recorded using the higher magnification microscope in which RBCs containing malaria parasites are marked. Procedure shown in Fig. 2.12 was used to identify red blood cells and eliminate all other regions including platelets which is shown in Fig. 2.15a. Thresholding the resulting image (which contains only RBCs) with the lower intensity due to higher absorption of light by malarial parasites result in the identification of parasites in the field of view.

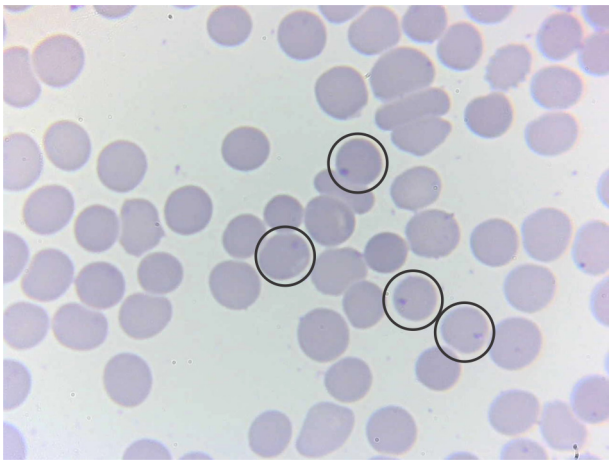

Fig. 2.14: Recorded image using microscope with 50x magnification. RBCs infected with malaria parasites are marked.

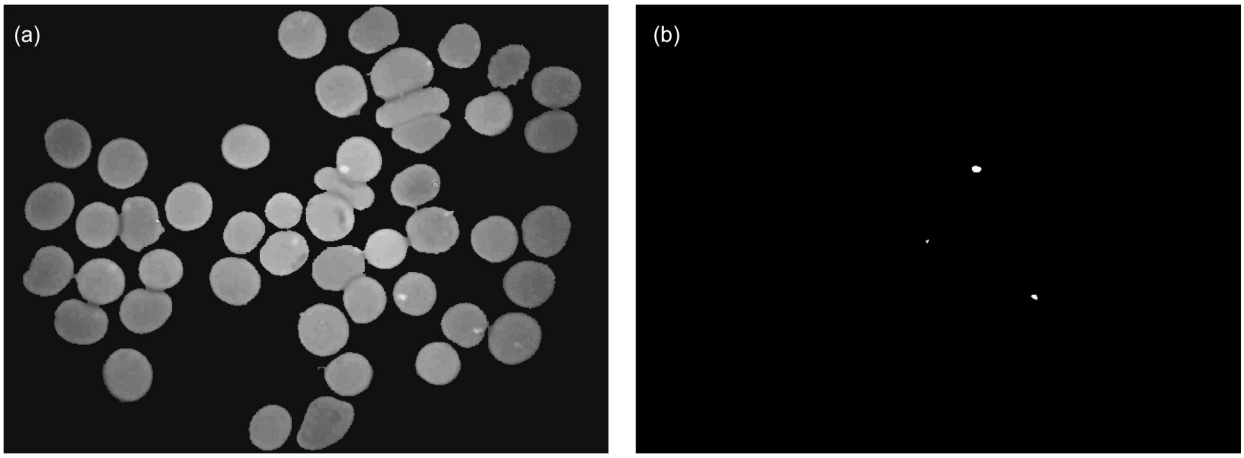

Fig. 2.15: (a) Identified red blood cells after background removal. (b) After edge detection, this image is gain thresholded with gray level value of the malaria parasite, leading to their detection.

Multiple field of views (52) were recoded with the large magnification microscope were recorded and were used to identify red blood cells infected by malaria parasites and it was found that the developed algorithm was able to identify 126 of the 148 malaria (counted manually) infected red blood cells which were present in the field of view.

2.10 Conclusions

A portable compact microscope, which can be attached to a smart-phone was developed using off-the shelf low cost optical and imaging components. The developed microscope was found to yield image quality comparable to that of clinical versions. A simple image processing algorithm was used to count, red blood cells, white blood cells and platelets. The image processing algorithm was

found to be powerful enough to accurately identify, classify and count the cells. It was verified by manually counting the cells. The main drawback of the system is that, it requires a chemical treatment of the sample to change the absorption profile of cells (especially white blood cells, platelets and malaria parasites), which was then be used to identify and count them. In the absence of staining, it may become difficult to identify and classify these cells from the recorded intensity images. But the compactness, ease and stand-alone nature of the microscope makes it a potential candidate for point-of-care applications. Also the microscope can be converted into a fluorescent microscope by introduction of filters in the system (these could be easily integrated into the microscope). The use of monochromatic LEDs (with narrow line width) also allows the use of gel filters, which will make the microscope cheap. But a microscope which is compact, field-portable and can produce high contrast images without staining might be better to identify and classify cells. The following chapters in the thesis deals with development of quantitative phase microscopes, which provides high contrast quantitative images of otherwise transparent specimen.