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Automated Measurement of Haemozoin (Malarial Pigment) Area in Liver Histology Using Image J 1.6

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***Abstract*— Common histopathological changes in the liver due to *Plasmodium* infection is the presence of haemozoin (malarial pigment) in liver histology section. Identification of haemozoin generally done manually under microscope. Measurement of haemozoin area rarely done because it is quite difficult to separate the haemozoin area from other element in liver histology. Identification and measurement haemozoin area can be done by image analysis using ImageJ.** ImageJ is a public domain Java image processing program that **enables a plugin development. Plugins are small Java modules for extending the functionality of ImageJ by using a simple standardized interface. Aim of this research is developed ImageJ plugin to measure the haemozoin area in liver histology. Totally 60 random liver histology images were analyzed using our plugin. Algorithm of plugin contain several sequential stages, such as splitting channels, thresholding the image for detection haemozoin area in blue channel and measure haemozoin area. Average haemozoin area from 60 images defined with our plugin was 3884.5 µm^2. Our plugin succeeded in detecting and measuring the haemozoin area in liver images at approximately 3.91 seconds.**

***Keywords.*: Haemozoin, ImageJ, Liver Histology Malaria, Plasmodium berghei, Plugin**

I. INTRODUCTION

Malaria is considered as one of the most important infectious diseases in the worldwide. It affects 350 to 500 million people and causes more than one million deaths every year. Malaria is caused by protozoan parasites belongs to the genus *Plasmodium*, which are transmitted by blood-feeding Anopheline mosquitoes. The disease is characterized by a range of clinical features from asymptomatic infection to a fatal disease [1,2].

Malarial involvement of liver is now a known entity with specific histopathological changes. The commonly histopathological changes in the liver due to *Plasmodium* infection is the present of haemozoin [3]. Haemozoin or malaria pigment has a history in the scientific literature older than the malaria parasite itself, having first been described in the early 18th century by the noted Italian physician Lancisi [4]. Eventually, this pigment played a role in the discovery of the parasite and the elucidation of its life cycle [1,5]. Hemozoin is a polymer of heme produced by the parasite during hemoglobin breakdown inside the host red blood cells (RBC). Red blood cells lysis during infection results in release of merozoites with this pigment, which are phagocytized by circulating monocytes, neutrophils and resident macrophages [6,7]. The amount of haemozoin in tissues increases throughout infection, so the greater amount of pigment, greater degree of chronicity of lesion [1].

Liver histology is congested with a brown or black pigmentation as a result of accumulation of haemozoin [3]. Haemozoin identification in liver histology commonly does manually under the microscope. Measurement of haemozoin in liver histology can be done by measuring the brown area. Measurement of haemozoin can be done by image analysis using ImageJ. ImageJ is a public domain Java image processing program inspired by NIH Image for the Macintosh. It runs on any computer with a Java 1.1 or later virtual machine, either as an online applet or as a downloadable application. ImageJ has a large number of native functions supplemented by an ever increasing number of "plugins" (optional extras needing installation). A plugin is a file (named *.class) which needs to be in the "plugins" sub-folder of the ImageJ folder, otherwise ImageJ will not load it [8].

Aim of this research is to build plugin for ImageJ that can be use for measure the haemozoin area in liver histology of laboratory mice that already infected with *Plasmodium berghei*. The advantages of using laboratory mice as a model for malaria include a well studied immune system of the host, the opportunity to assess pathologic changes at all stages of the disease, and the availability of genetic variants [1].

II. MATERIALS AND METHODS

2.1. Mice

Male Swiss mice age 8 to 12 weeks was purchased from Pusat Penelitian dan Pengembangan Gizi dan Makanan, Kementerian Kesehatan Indonesia.

2.2. Parasites and infections

Mice were inoculated intraperitoneally with 10^6

erythrocytes infected by *P. berghei*. Mice were subjected to euthanasia at one week after inoculation. Fragments of the liver were fixed by immersion in 10% buffered formalin during 24 hours. These samples were then dehydrated, and processed for paraffin embedding. Five µm sections were cut and stained with hematoxylin-eosin (H&E).

2.3. Image acquisition

A Nikon Biophot microscope attached with Nikon D3000 digital single lens reflects (DSLR) camera system was used to capture images of the smears. The slides were examined under 40× objective lens. Images were captured at a resolution of 1936×1296 and saved as JPEG files.

2.4. Image analysis

A plugin for measuring the haemozoin area in liver histology was developed. Totally 60 random liver histology images were analyzed using our plugin in ImageJ 1.60. The algorithm of plugin can be divided into the following four sequential stages (Fig 1): (1) Splitting channels, (2) Detecting haemozoin area in blue channel, (3) Measuring haemozoin area, (4) Showing outlining haemozoin area in images, (5) Detecting total tissue area in green channel, and (6) Measuring tissue area.

2.4.1. Splitting channels

The purpose of this method is the separation of the red, green and blue channels of the RGB image. Haemozoin area is easy to identify in blue channel compared to red and green channels. In blue channel, the haemozoin area color is dark and the other component is light. Splitting channel also used for converting the RGB image to monochrome image for thresholding process. After splitting the channel we look at each channel individually to determine which one of the channel creates better contrast than another. The channel containing the highest contrast is the best one to choose for use for thresholding later on [9].

2.4.2. Detecting haemozoin area in blue channel

This method is performed by thresholding the image and making the binary image with ImageJ. Thresholding is quick method to identify areas of an image to include and areas of an image to ignore.With sufficient contrast, objects of interest may then be "detected," resulting in masking binary image components, where each pixel is either "on" or "off" [9].

Thresholding an image is a special type of quantization that separates the pixel values in two classes, depending upon a given threshold value a_{th}. The threshold function $f_{\text{threshold}}(a)$ maps all pixels to one of two fixed intensity values a_0 or a_1; i.e.,

$$f_{\text{threshold}}(a) = \begin{cases} a_0 & \text{for } a < a_{\text{th}} \\ a_1 & \text{for } a \geq a_{\text{th}} \end{cases}$$

with $0 < a_{\text{th}} < a_{\max}$. A common application is binarizing an intensity image with the values $a_0 = 0$ and $a_1 = 1$ [10]. In this case we used 160 as a threshold value (a_{th}), because with this value all the haemozoin area can be convert to black area in a binary image. Commonly 1 value will showed as a black color in a binary image. After thresholding process we selected the black area in binary image as a region of interest (ROI) using CreateSelection command so the black area can be measure using Measure command

2.4.3. Measuring haemozoin area

To measure the haemozoin area, we used the Measure command in ImageJ Analyze menu. Measure command will calculates and displays area statistics, line lengths and angles, or point coordinates the ROI. ROI defined as a black area in binary image (Fig 2).

2.4.4. Showing outlining haemozoin area in images

To show the outlining haemozoin area in original image, we used Add Image function in ImageJ Overlay menu.

2.4.5. Detecting total tissue area in green channel

This method is performed by detecting the total tissue area by threshold the image and making the binary image with ImageJ. Different with the haemozoin area, total tissue area is strongly easy to determine in green channel. We used 180 as a threshold value (a_{th}), because with this value total tissue area can be convert to black area in a binary image. After that we selected the total black area using CreateSelection command.

2.4.6. Measuring total tissue area

The purpose of this method is measuring the total tissue area using Measure function in Analyze menu in ImageJ. Detail script and flowchart of the plugin is show in Fig 1 and 2.

```
import ij.*;
import ij.text.*;
import ij.process.*;
import ij.gui.*;
import java.awt.*;
import ij.plugin.*;
import ij.plugin.filter.*;

public class Hemozoin_Analysis implements PlugIn {

	public void run(String arg) {
		ImagePlus image = IJ.getImage();
		IJ.run("Duplicate...", "title=Duplicated.jpg");
		IJ.run("Duplicate...", "title=Duplicated-1.jpg");
		IJ.run("Subtract Background...", "rolling=50 light");
		IJ.run("Split Channels", "");
		IJ.run("Threshold...");
		IJ.setThreshold(0, 160);
		IJ.run("Convert to Mask", "");
		IJ.run("Close");
		IJ.run("Create Selection", "");
		IJ.run("Measure", "");
		WindowManager.putBehind();
		IJ.run("Threshold...");
		IJ.setThreshold(0, 170);
		IJ.run("Convert to Mask", "");
		IJ.run("Close");
		IJ.run("Create Selection", "");
		IJ.run("Measure", "");
		IJ.run("Close");
		WindowManager.putBehind();
		WindowManager.putBehind();
		IJ.run("Close");
		WindowManager.putBehind();
		IJ.run("Close");
		WindowManager.putBehind();
		IJ.run("Add Image...", "image=Duplicated.jpg x=0 y=0 opacity=100");
		IJ.run("Put Behind [tab]", "");
		IJ.run("Close");
	}
}
```

Fig 1. Script Haemozoin Analysis Plugin

III. RESULTS

Average haemozoin area from 60 images defined with our plugin are 3884,5 µm^2, graphics of haemozoin area in 60 images are show in Fig 3. Our plugin success detected and measured the haemozoin area in a liver histology in approximately 3, 91 seconds.

IV. DISCUSSION

In this plugin we choose to use splitting channels than ColorDeconvolution plugin. We used splitting channels because ColorDeconvolution plugin failing to produce an image that haemozoin area easy to identify. Color Deconvolution plugin commonly use for stain separation in histological images. This plugin assumes images generated by color subtraction (i.e. light-absorbing dyes such as those used in bright field histology or ink on printed paper). Our experiment showed that in the blue channel after we apply splitting channels process, a haemozoin is easy to identify because only the haemozoin area are coloring in dark and the other component showed in light color.

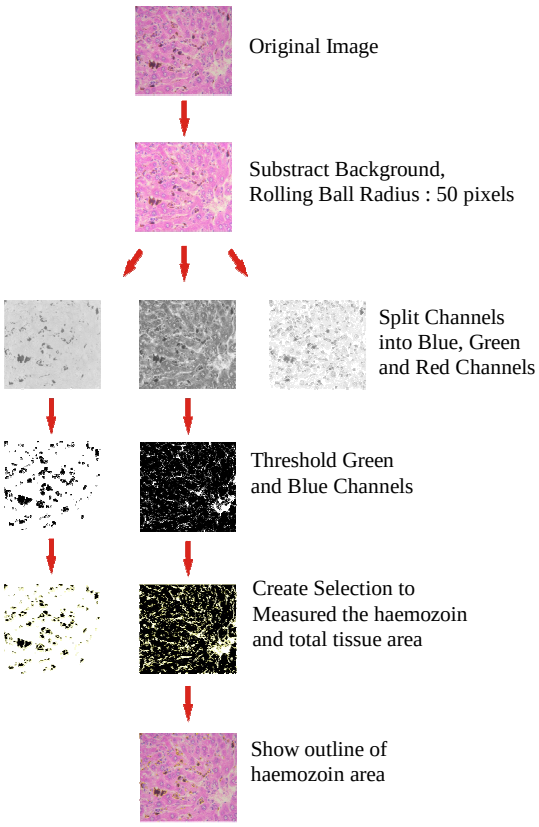

Fig 2. Flowchart of haemozoin area plugin

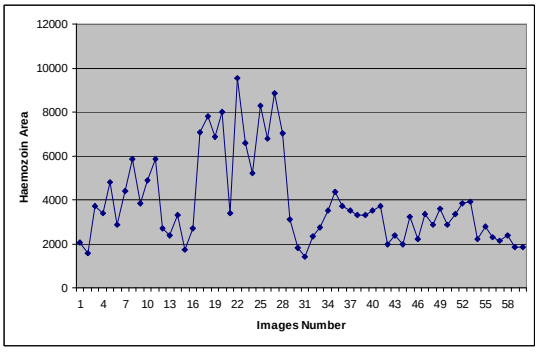

Fig 3. Chart of haemozoin area in 60 images

Images that comprise light objects on dark background or dark object on a light background can be segmented by threshold operation. Based on that we segmented and detected the haemozoin area in blue channel by thresholding the image using ImageJ. ImageJ automatically make binary image and then convert to mask after we apply thershold technique. The results is image that divides into objects in black color and background in white color.

To get measurement area result in µm not in pixel first the scale of the image must be set using Set Scale command in Analyze menu. A known distance should be measured by fitting a line to the known distance using the straight line selection tool in the ImageJ toolbar. Then open the Set Scale command, which will automatically register the distance from the straight line selection. Enter the Known Distance and the Unit of Length and after selecting Global and then OK, the scale will automatically be calculated from the registered distance [11]. A known distances we defined by capture a micrometer slide in under 40× objective lens. With the micrometer image then we define a scale using Set Scale command.

We also apply backround subtraction using rolling ball algorithm before we splitting channels to do background illumination correction in the images. Background correction can be applied while acquiring images (a priori) or after acquisition (a posteriori). The difference between these is that a priori correction uses additional images obtained at the time of image capture while in a posteriori correction, the additional images are not available and therefore an ideal illumination model has to assumed. Substract background using rolling ball algorithm is one of the a posteriori correction methods. Substract background function is removes smooth continuous backgrounds from images. Based on the a “rolling ball” algorithm described in Stanley Sternberg's article, “Biomedical Image Processing”, IEEE Computer, January 1983.

Overall our plugin success measured the haemozoin area in liver histology images, and the time need for analyze one images is approximately 3.91 seconds. Other research that conductes by Silva et al [1] also measured haemozoin area in liver histology images using ImageJ, unfortunatelly the details process is not explained so we can not compared with our methods.

V. CONCLUSION

We have developed ImageJ plugin that can be used measured the haemozoin area in liver histology images of mice infected with *Plasmodium berghei*. Time need for analyze one images is approximately 3. 91 seconds using our plugin. Overall, our plugin worked very well to measured the haemozoin area in liver histology images.

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