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HEP-2 CELL IMAGES FLUORESCENCE INTENSITY CLASSIFICATION TO DETERMINE POSITIVITY BASED ON NEURAL NETWORK

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CERTIFICATION OF APPROVAL

HEP-2 CELL IMAGES FLUORESCENCE INTENSITY CLASSIFICATION TO DETERMINE POSITIVITY BASED ON NEURAL NETWORK

By
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Approved:

_____

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Project Supervisor

UNIVERSITI TEKNOLOGI PETRONAS

TRONOH, PERAK

CERTIFICATION OF ORIGINALITY

This to certify that I am responsible for the work submitted in this project, that the original work is my own except as specified in the references and acknowledgements, and that the original work contained herein have not been undertaken or done by unspecified sources or persons.

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Amin Fahim B. Abu Mansor

Electrical&Electronic Engineering

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ABSTRACT

Nowadays, the recommended method for detection of anti-nuclear auto-antibodies is by using Indirect Immunofluorescence (IIF). The increasing of test demands on classification of Hep-2 cell images force the physicians to carry out the test faster, resulting bad quality results. IIF diagnosis requires estimating the fluorescence intensity of the serum and this will be observed. As there are subjective and inter/intra laboratory perception of the results, the development of computer-aided diagnosis (CAD) tools is used to support the decision. In this report, we propose the classification technique based on Artificial Neural Network (ANN) that can classify the Hep-2 cell images into 3 classes namely positive, negative and intermediate,specifically to determine the presence of antinuclear autoantibodies (ANA).

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LIST OF ABBREVIATIONS

AAB	Autoantibody
ANA	Antinuclear Autoantibody
CAD	Computer Aided System
ELISA	Enzyme-linked Immunosorbent Assay
Hep-2	Human Epithelial Type 2
IIF	Indirect Immunofuorescence
MES	Multiple Expert Systems
MLPs	Multi Layer Perceptrons
RBF	Radial Basis Network
RGB	Red-Green-Blue

CHAPTER 1
INTRODUCTION

1.1 Background of study

In recent years, the improvement of imaging technologies strive the increasing interest of image processing and analysis technique for several of clinical diseases. The extraction of the image will be tested across laboratories by scientists with subjective analysis [1-3]. Connective tissue diseases (CTDs) such as Rheumatoid Arthritis, scleroderma, and Systemic Lupus Erythematosus (SLEs) show the presence of autoantibodies in human blood [4]. Antibodies protect the body cell from any foreign substances or also known as antigens. Nevertheless, autoantibody is a type of antibody (protein) that attacks body cells. There are many techniques to determine the autoantibody such as enzyme-linked immunosorbent immunoassay (ELLISA) and multiplexing technologies. The recommended laboratory technique to detect this autoantibody is by using indirect immunofluorescence (IIF) imaging because of its high sensitivity and the large range expression of antigens [5],[6].

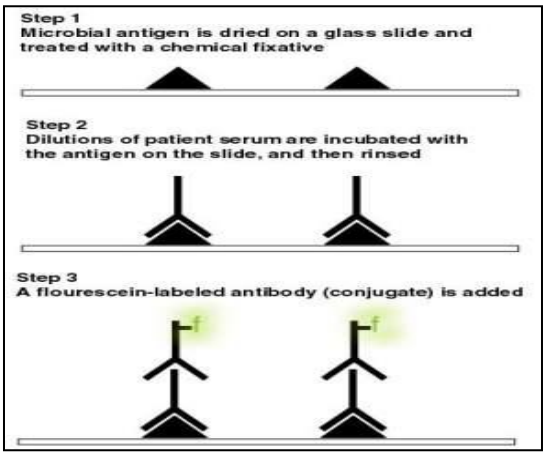


Figure 1: Indirect Immunofluorescence's method [7]

According to [5] , antigen or autoantibodies can give the feature of fluorescence images called fluorescence pattern and usually, direct immunofluorescence is used to detect antigen presence whereas for indirect immunofluorescence it is suitable for detecting

autoantibodies. As can be seen in Figure 1, the serum sample is prepared by adding mitotic cells into serum sample on the slides and will be detected by using fluorescent-labelled anti-immunoglobulin antiserum [7].

In order to detect the presence of autoantibodies by using indirect immunofluorescence (IFF), the first step is to dry the microbial antigen on a glass slide and treat with a chemical fixative. Next, the dried microbial antigen with the serum samples will be diluted. Basically, the standard dilution titration ratio is 1:80 titres and this dilution will prevent from background staining due to non-specific combination of clinically non-significant levels of circulating autoantibodies [5],[8]. In medical context, titre corresponds to concentration and titre employs serial dilution to obtain approximate quantitative information. Then, a fluorescent-labeled antibody is added and lastly, the slides are rinsed and dried. After that it will be observed by physicians under fluorescence microscope. The traditional method in observing immunofluorescence (IIF) has produced subjective result. This is due to physician's biased measurement, conservative and liberal during image classification on the basis of skill and background. To overcome this problem, Computer Aided Diagnostic (CAD) can automatically detect the Hep-2 cell pattern in the interest images [9],[10]. In this report, the focus points are for classification of positivity/negativity or intermediate of Hep-2 cells by using a ANN approach. Consequently, the process to classify the Hep-2 cell under CAD will undergo several processes which are image acquisition, image segmentation, feature extraction and fluorescence intensity classification.

1.2 Problem Statement

Classification of fluorescence intensity is essential in order to determine the positivity of the serum sample. To reduce the variability of substantial reading, the serum of sample is divided into three classes, namely negative, weak positive (intermediate) and positive. Recently, this IIF test is done manually leading to subjective result. The demand of autoimmune test has increased year by year, as the demand increases; consequently the test needs to carry out faster.Thus, this leads to non quality results and not standardized. Therefore, there is a need for automated classifications of fluorescence to improve standards and reduce the variability of results. The most common software used for classification and determine the positivity of Hep-2 serum are AKLIDES and SLIM [9]. So, there is a need to find the new technique in order to produce better results. In this report, the classification to determine positivity of serum will be based on ANN.

1.3 Scope of study and Objective

The main objective of this project is to classify the HEp-2 cell images fluorescence intensity to determine the positivity based on the ANN approach. Nonetheless, to complete the main objective, detail studies on some issues interrelated to the main objective are crucial for better analysis and understanding. To narrow down the project so that it is feasible and could be completed within the allocated time frame, the project will be focused on fluorescence intensity classification to determine positivity, negativity and intermediate of Hep-2 cell serum.

Peculiarly, this project will focus on:

i) Understanding and identifying different features in images that contributes to the positivity or negativity of the samples.
ii) Developing a classification algorithm using ANN to accurately divide the image into three classes: negative, positive and intermediate.
iii) Assessing the performance of classification algorithm and validating the accuracy of the classification results.

1.4 Significance of the project

This project will demonstrate the development of CAD. This is because, before this the anti-nuclear antibodies (ANAs) is tested manually by the experienced and specialized physician in the field. Moreover, when the incidences of autoimmune diseases are increasing, more tests are needed to carry out. Therefore, this often leads up the speed of observation of microscope resulting negative quality outputs. According to Rigon. A (2011), human will become effortless as the automation technology is used for this analysis and the great demand of diagnostic test for systemic autoimmune disease can be fulfilled [9]. This project will reduce the time consumption and also ease the human operator to classify the serum. Nowadays, the improvements in data and computer technologies can be seen clearly as many of improvement in a communication device, the relevancy of this project at this era clearly justifies [9].

1.5 The Relavancy of the Project.

The usage of automated classification in the modern medical industry has not being fully implemented. This is due to lack of awareness to the benefits of implementing automated classification of cells into three type positive,negative and intermediate that can assist doctor's decisions. In support of this, the automated system was developed which can interpret such images with accuracy and reproducibility greater than visual examination. Thus, the study on fluorescence intensity classification to determine positivity of cell is relavant in modern clinical system.

1.6 Feasibility of the project

This project is carried out using simulations, without employing prototype fabrication. Thus, the time frame of two semesters for FYP 1 and FYP 2 is feasible to complete the project. The final output will be the simulation of ANN classification technique together with detailed analysis. During FYP 1, the focus is to work on the literature review and understand the detailed characteristics of the ANN algorithm whereas for FYP 2 mostly to conclude data analysis.

CHAPTER 2
LITERATURE REVIEW

In order to complete this project, a set procedure is developed to obtain the desired output. The proposed approach to classify the HEp-2 images are illustrated in the Figure 2:

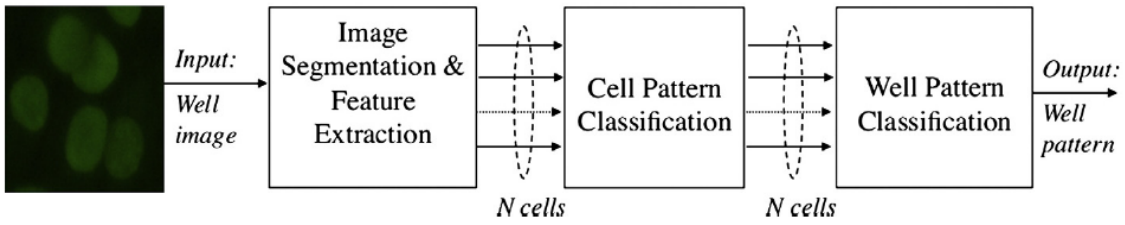


Figure 2: proposed approach to classify the HEp-2 images [11]

From Figure 2, the input of the system is well imaged and this image usually was taken from the image acquisition unit that already underwent the image acquisition process. Firstly, the cell image will be separated from the background and this process is known as image segmentation. The feature extraction of an image is the important method in order to simplify the numerous datasets to the amount of resources accurately. Next, *N cells* are the number of cells extracted and will become an input to cell pattern classification. At this stage, three classes of positivity, negativity and intermediate of the cells will be classiffied. For positive cells, it will go further process in well pattern classification. From this system, all the staining patterns have already obtained [9],[12] .

2.1 Image Acquisition

Basically, the HEp-2 cell is known as human epithelial cancer cell and more familiar with the name of Hela contaminant; caused by the least number of Y-chromosomes. The HEp-2 slides will be observed to determine fluorescence intensity on the cell body especially at the nucleus and cytoplasm [3]. The process of classification starts with image acquisition technique first, in a knowledge acquisition process with human operators, the distinctness conceptualization of fluorescence signal will occur because human vision contains different spectral sensitivity [13]. The often immanent selected image acquisition parameters are accordingly not optimal causing the computer-assisted analysis errors. The separate human perception and the automatic image acquisition are essential to gain the best quality of images, as all the parameters already directly come up from the image data [8].

According to [13], there are some requirements to improve image acquisition process:

i. The cells with fluorescence object must be very low or non fluorescence image matrix.
ii. For ideal analytic view, the gray-level of the image must be low as possible.
iii. Take into account the camera parameter such as exposure time and gamma functions.
iv. Use of image sensor for quality evaluation image data.
v. Assessment of image quality at standard RGB (red-green-blue channel)

The fluorescence object needs to be low or non-fluorescence image matrix in order to make sure the environment of background is dark and the region of interest show as bright fluorescence. In an ideal case, lower image gray level must be the lowest possible gray level as the image quality is inversely proportional to noise. So, as the image quality decreases, the noise in the image will increase. The camera parameter needs to be considered because during the image acquisition process, it needs to

determine whether the real value reaches ideal value. Besides, for visual improvement of images, the parameter of gamma correction is used. The correct sensor is needed to avoid from any overexposed image of the fluorescence signal as shown in Figure 3.

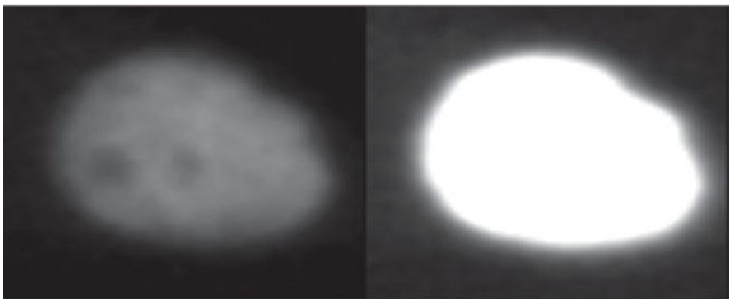


Figure 3:correctly exposed image and overexposed image [14]

Figure 3 shows the comparison between correctly exposed image and overexposed image to fluorescent signal. The left image shows correctly exposed whereas the right image is overexposed fluorescence signal. The other requirement to obtain better quality of fluorescence image data by using red-green-blue channel (RGB) in three primary colors with red, green and blue fluorescence.

In this project, all the images of the Hep-2 cell dataset were taken from the Mivia Hep-2 datasheet as there is not much reference database of the IIF image present. The images taken from acquisition unit include the microscope with a certain level of magnification, 50Watt mercury vapor lamp, 1388x1308 pixels of 24 bits in arrays and charge-couple design with the balance side squared pixel of 6.45μm [15].

2.2 Image Segmentation

Image segmentation is the first step of image analysis, image processing and computer vision study. According to J. Zhang (2008), image segmentation is crucial to understand the images and extract information as well as a key factor in image analysis, understanding and description [16]. In general, The basic idea of the image segmentation process is the partitioning a digital image into multiple segment, having sets of pixels and these pixels are based on homogeneity properties such as colour, intensity and body texture [17]. The segmentation method is chosen based on the type of image and characteristic problem. To understand the images, different methods are used

for different image segmentations techniques. For this project, the HEp-2 images having characteristics of light cell and dark background are chosen. Thus, the suitable method of image segmentation is thresholding method as it is simple but powerful approach. From [18], thresholding method can be divided into four type which are stable count thresholding, Otsu's thresholding, Isodata thresholding and mixture modeling thresholding. Stable count thresholding use 3D segmentation on nuclear compartment and have an error about 5.52 %. Otsu's thresholding technique different from others technique because it can minimize the overlapping between two cells and the error about 1.55 %. Next, Isodata thresholding use the technique of computer run algorithm through many iteration unti the threshold is reached, this technique gain an error about 2.43 %. Lastly, mixture modeling thresholding use Gaussian parameter and have error about 4.98 %. Thus, the applicable thresholding segmentation technique in this project is Otsu's thresholding because it having least error.

The images certainly have several levels of light intensity; the region of interest usually has higher intensity of lighter rather than the image background. The process of thresholding segmentation is by converting the multilevel image into binary image using a proper threshold value, *T*, to divide the image pixels into several regions and separate it from their background [19]. Otsu's thresholding segmentation is one of most popular segmentation as the segmentation performs accurately [16].

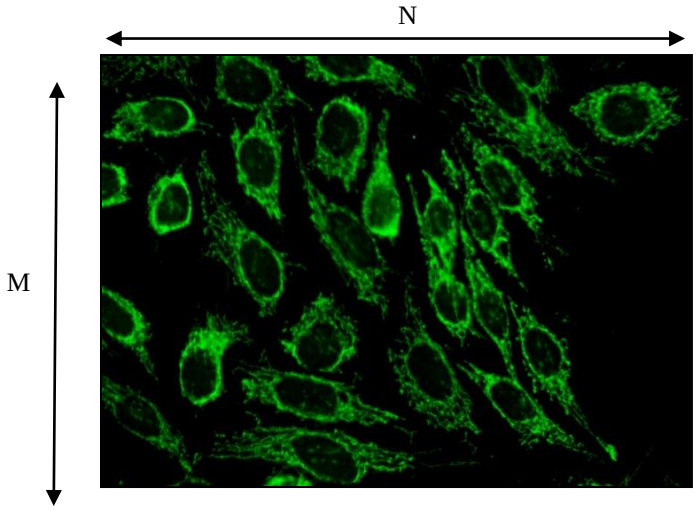


Figure 4: MxN image size

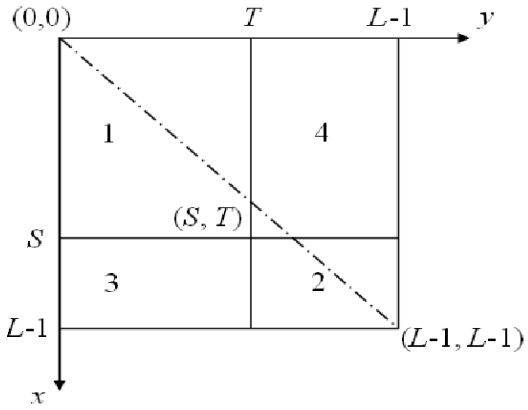


Figure 5: 2D Otsu histogram [16]

Figure 4 shows the x-coordinate (*i*) represents gray level and y-coordinate (*j*) represents the local average gray level of an image with of *M x N* . Figure 5 shows the two dimensional Otsu's histogram with (*S*,*T*) is the vector where *S* is the image pixel at that point and *T* is the threshold value. The 2D histogram is divided into four quadrants with the range of $0 \leq (S,T) \leq L\text{-}1$, with *L* as the gray levels. The dash-dot line represents diagonal of 2D histogram and usually, the pixels of the region of interest and background will distribute evenly to the near-diagonal element. This is because of homogeneity properties where the pixels of the region of interest (ROI) and background having similar gray level of pixel and local average gray level. Otherwise, the pixel's neighborhood of an edge between the region of interest and background, the local average gray level will be fairly different. Therefore, quadrant 1 and 2 contain distribution of object and background whilst quadrant 3 and 4 contain the distribution of noise and pixels near the edge [16].

In addition, erosion and dilation techniques are used to remove any disturbances such as noise [20]. Circulatory measure is computed to remove the overlapping cells. According to P. Elbischger (2009), the process of segmentation begin with subdivide the images in the scale of 16250x250 pixel blocks with the average of cell includes are four cells. Furthermore, the Otsu's algorithm is applied to each region of interest and new bonding box shape region of interest is formed around each tentative segmentation result [21]. This will be illustrated in Figure 6:-

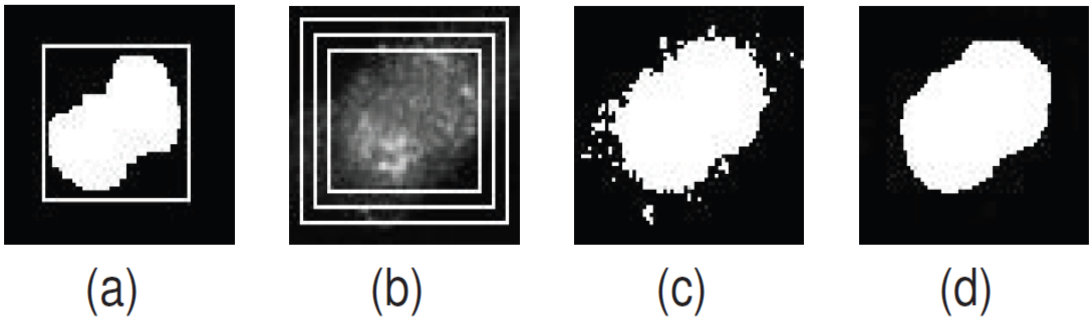


Figure 6: a) before iteration b) cell with increasing region of interest c) after iteration d) after morphology [21]

Figure 6(a) shows the region of interest before iteration using of Otsu thresholding methods. Figure 6(b) explains about the new bounding box shaped on region of interest and the Otsu algorithm is repeatedly applied to each object by increasing the size of the box with two pixels in each iteration. Figure 6(c) shows the image after iteration and lastly Figure 6(d) where the morphological operation applied to the segmentation result to obtain compact final representation of object [21].

2.3 Feature Extraction

After the images have been completely segmented, they will undergo a process known as feature extraction. The set of features is extracted based on the expertise and knowledgeable of a physician. The most important in classification system is choosing the suitable feature extraction that can strongly against change to size, intensity and shape of fluorescent cells. According to P. Soda (2006) a set of features is extracted based on first and second-order gray level histogram of whole images based on the result of [11], the detail information about the meaning of these histogram [22]. From [23], it compare the performance of two feature extraction techniques which is Principle component analysis and local binary pattern. Research found that the accurancy of principle component analysis is higher than local binary pattern with the percentage 97 % and local binary pattern acurracy percentage is 93 %. Principle component analysis is powerfull tool to compress data and also it can decrease the amount of redundant

information whereas local binary pattern will compute the histogram based on each pixel of image and compare the pixel with it neighbour.

Principal component analysis is used to extract the features as there are some dimensionalities of the feature space [24]. Feature of positive and negative control equation is given by Eq (1) and Eq (2):

$$RG_{sample/positive} = \frac{G_{medium\ sample}}{G_{medium\ positive\ control}} \quad (1)$$

$$RG_{sample/negative} = \frac{G_{medium\ sample}}{G_{medium\ negative\ control}} \quad (2)$$

From Eq (1) and Eq (2), $RG_{sample/positive}$ and $RG_{sample/negative}$ are variance of sample image between positive or negative control. The statistical mean of fluorescence intensity over all the serum samples are denoted by $G_{medium\ sample}$, $G_{medium\ positive\ control}$, and $G_{medium\ negative\ control}$.Every feature gets in the feature extraction need to obey these four image characteristic [11],[12],[25]:

i. The true image with the original image and original background
ii. Underline the difference between uniform and variable pattern in contrast-enhanced version of previous image
iii. Minimize the contribution and maximize cell borders by set original image background to zero.
iv. Emphasized pixel variation by contrast-enhanced version of previous image.

Basically, the method of image enhancement such as image segmentation and feature extraction can be used to determine the characteristic of the image that contributes to positivity or negativity of serum samples. Consequently, image segmentation and feature extraction (first objective) is used to determine the features of

the cell that have an antinuclear autoimmune disease (ANA). Usually for the test accuracy improvement, the mitotic cells will be added in the serum sample to assist doctors in decision making. The importance of mitotic cells is that it as the benchmark whether the slides of serum are correctly prepared or not by detecting one or two of mitotic cells appear in the serum samples. Mitotic cells also provide the information about the staining pattern [15]. P. Foggia (2010) compares the different features in images that contribute to positivity or negativity of samples. For positive mitosis, higher fluorescence intensity will appear mostly in the chromosome region whether the cell body contains weak or no fluorescence and vice versa for negative mitosis [26].

Table I: Fluorescent intensity classification guidelines [24]

Subgroups	Description
4+	Maximal fluorescence (Brilliant green)
3+	Less brilliant green fluorescence
2+	Defined pattern but diminished fluorescence
1+	Very subdued fluorescence
0	Negative

Table I shows the scoring range of fluorescent intensity from "0" scores up to "4+". The maximal fluorescence is "4+" subgroup which has brilliant green at the nucleus, this serum sample is called strong positive. The strong positive at a subgroup of "3+" and "2+", as long as the fluorescence colour can be clearly observable and distinguish between nucleus and background. Weak positive or intermediate serum sample is at subgroup "1+" and negative serum sample will have "0" subgroup.

In this project, the focus is only on these four positive staining patterns of Hep-2 cell and their characteristics are [1] :

i. Homogeneous - evenly diffuse fluorescence covering the entire nucleuplasm
ii. Speckled - a fine or coarse granular nuclear staining.
iii. Centromere - uniform discrete speckles located throughout the entire nucleus.
iv. Nucleolar - solid staining, especially in the external of nucleus with the weaker fluorescence at center of nucleus.

2.4 Classification algorithm using Neural Network

As stated in the introduction part of this report, there are many types of classification method approach to this similar problem such as fuzzy logic, statistic classification, support vector machine (SVM) and k-nearest neighbor (KNN). For this project, the investigated approach on the classifier family name ANN architectures. The bottom rank of other approaches in artificial neural network including the decision trees, discriminating analysis and data not shown in prior studies is one of the factors why back-propagation neural network approach were chosen [27]. The idea of artificial neural network arises when the people recognize the modern computer failed people with many problems such as system error [28]. The advantages of using neural network are:

i. Massive parallelism
ii. Adaptively
iii. Fault tolerance
iv. Low energy consumption

Generally, there are two types of ANN which is feed-forward network and recurrent (feedback) network. Mostly the characteristic of feed-forward network is static and one-directional connection between the neuron whereas the characteristics of recurrent network are consisting of a loop (feedback), and dynamic system. Similar to human, learning is the factor trait of intelligence and ANN learning process as the problem updating network architecture and connection weight so that the network can perform specific tasks. The performances of ANN increase time by time as they're updating the weight after iteratively learn the connection weight from training data sets. Three learning algorithms are used which is supervised, unsupervised and hybrid [28]. Figure 7 illustrates about single layer perceptron of neural network:

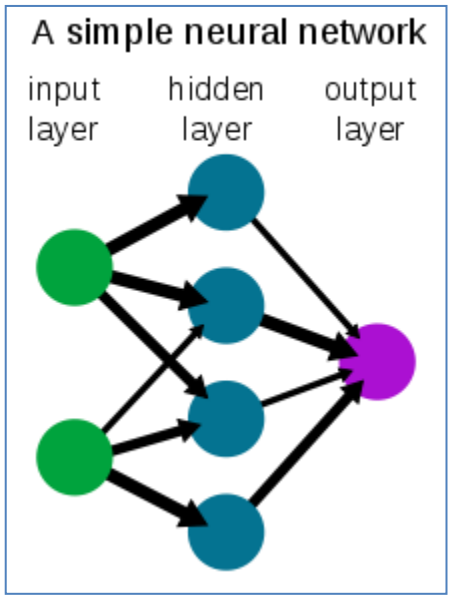


Figure 7: single layer perceptron [28]

Figure 7 is the single layer perceptron architecture network under the feed-forward network family; the colored circle represents neurons of the system which connected to each other via weighted link. The green circle represents the input layer of the system, blue circle represents hidden layer and purple circle represent the output layer. The bold arrows mean the connection has important values among the others, this connection known as weight.

According to P. Soda et.al (2006), the ANN classifiers have two outputs of neurons which is positive and negative classes. When the sample is positive class, the output neuron values will be coordinated (1,-1) and vice versa for the negative sample. The dubious or intermediate class will be underlying between these two classes [24].

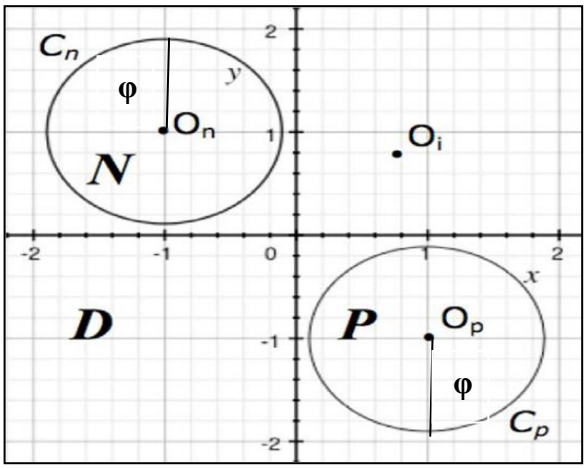


Figure 8: x-y plane of the classification rule [24]

Figure 8 shows the classification rule with the x-y-plane where x-coordinate indicate the output for the first neuron and y-coordinate indicate the output for second neuron. Based on Figure 8, The letter O_i represents the ith output vector, and letters O_p and O_n represent the positive and negative classification respectively. The accuracy of classification is measured by the distance between O_i output and positive or negative point, O_p or O_n. The shorter distance the more accurate the result is. To determine the intermediate classes, the radius of positive and negative point will be needed to become two circles which denoted as C_p and C_n . The radius is denoted as φ and the range in the interval [0, $\sqrt{2}$], to avoid these two circles from overlapping. The point of O_i outside these two circles are classified as intermediate or dubious class. The capital letter of *P*, *N* and *D* represent the located output of positive, negative and dubious of serum sample respectively.

The best and stable performance of ANN network is multi-layer perceptrons (MLPs) with the learning algorithm of back-propagation neural network (BPNN). A BPNN was chosen as a classifier primarily because of its ability to generate complex decision boundaries in a multidimensional space (Hornik et.al., 1989). The back-propagation algorithm followed by these steps [28]:

i. Initialize with applying the inputs to the network and work out the output, initial output could be anything as the initial weights were random number.

ii. Next, compute the error for neuron B. The error is the differences between the actual output of B, $\boldsymbol{O_B}$ and experimental output, $\boldsymbol{O_{EB}}$. The error for neuron B is given in Eq (3):

$$\boldsymbol{Error_B} = \boldsymbol{O_B}(\mathbf{1} - \boldsymbol{O_{EB}})(\boldsymbol{O_B} - \boldsymbol{O_{EB}}) \quad (3)$$

iii. Update the weight, Let $\boldsymbol{W_{AB}^{+}}$ be the new (trained) weight and $\boldsymbol{W_{AB}}$ be the initial weight. Let $\boldsymbol{O_A}$ be the output A. The update weight formula is given in Eq (4):

$$\boldsymbol{W_{AB}^{+}} = \boldsymbol{W_{AB}} + (\boldsymbol{Error_B} \text{ x } \boldsymbol{O_A}) \quad (4)$$

Notice that it is the output of the connecting neuron (neuron A). Update all the weight in the output layer in this way.

iv. Calculate the errors for the hidden layer neurons, unlike the output layer, cannot be calculated directly as target value not present. So, Back-propagate them from the output layer. This is done by taking the errors from output neuron and compute them back through the weight to get the hidden layer error. Errors for the hidden layer neurons is given in Eq (5):

$$\boldsymbol{Error_A} = \boldsymbol{O_A}(\mathbf{1} - \boldsymbol{O_A})(\boldsymbol{Error_B}.\boldsymbol{W_{AB}} + \boldsymbol{Error_C}.\boldsymbol{W_{Ac}}) \quad (5)$$

Let $\boldsymbol{Error_C}$, error of neuron C and weight connecting neuron A and neuron C is $\boldsymbol{WAc}$.

v. Having obtained the error for hidden layer neurons now proceed as in stage 3 to change the hidden layer weight. Repeating this method, so we can train a network of any number of layers.

Table II shows the summary of the literature review that was completed up to the time of writing.

Table II: Summary of literature review

No	Authors	Ref. No	Title	Methodology	Result
1	Hiemann, Rico Hilger, Nadja Sack, Ulrich Weigert, Martin	[12]	Objective quality evaluation of fluorescence images to optimize automatic image acquisition	The process of improving image acquisitions.	Image acquisition technique
2	Foggia, P Percannella, G Soda, P Vento, M	[14]	Early experiences in mitotic cell recognition on HEp-2 slides	Preparation appropriate technique for serum slide.	Presence of mitotic cell in serum indicates the serum is correctly prepared.
3	Elbischger, P Geerts, S Sander, K Ziervogel-Lukas, G Sinah, P	[19]	An algorithmic framework for HEp-2 fluorescence pattern classification to aid auto-immune disease diagnosis	Using Otsu's thresholding algorithms for image segmentation	Segmented image which the object had separated from the background
4	Jain, Anil K Mao, Jianchang Mohiuddin, K Moidin	[25]	Artificial neural networks: A tutorial	Artificial neural network algorithm.	Classification of sample to 3 classes which is positive, negative and dubious(inter mediate).

CHAPTER 3
METHODOLOGY

3.1 Research Methodology and Procedures

This project will cover the following activities towards the completion of FYP 1 and FYP 2:

1. Research and literature review

   The research basically was based on the theoretical and understanding the background of the project. During this phase, the scope of the project was determined and the related journals, books, research papers and coding manual was simplified. This is to ensure better understanding, and better view about the researched that will be carried out. The main resources of information are from the Coastal Engineering Manual, e-journal, e-thesis and several trusted links. Follow up with the literature review after that.

   Proposal writing

   The objectives and problem statement are stated clearly in the proposal. The scope of study must be relevant and feasible with the available duration.

2. Experimental design

   Gathering data and information for this project was done from studies together with a suitable method under ANN to be used.

3. Simulation testing

   Simulations are done by using MATLAB, Image Processing Toolbox. The results were then be analyzed.

4. Design improvement and modification

   Improvements on the design should be done if the preliminary result does not meet the requirements. This process was repeated until more satisfactory result was obtained.

5. Result analysis

   The final result were analyzed in order to understand the features of Hep-2 cells images which contribute to positivity, negativity of intermediate. In the end, the result image should be divided accurately into three classes: negative, positive and intermediate (weak positive) by using a ANN classification algorithm.

3.2 Proposed Topology

The proposed Hep-2 cell images classification system based on ANN design incorporated with three stages. A basic block diagram was designed for automated classification of Hep-2 cells positivity as shown in Figure 9. As this project is based on Matlab software simulation, each process algorithm need to be programmed in Matlab coding.

Figure 9: Basic design block diagram

The image segmentation is based on Otsu's Multi-thresholding, it will separates the object from the background. At this stage, Otsu's Multi-thresholding will create boundaries between overlapping cells as shown in Figure 10.

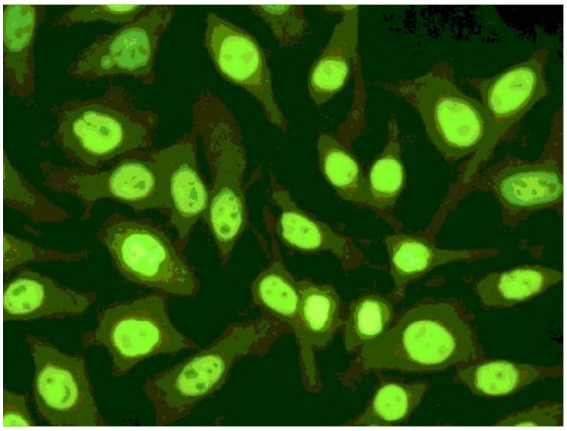


Figure 10: cell boundaries

Then, the cell image will be preprocessed in feature extraction, by this process all the important data about the image is taken including brightness, saturation and hue of images. In Matlab coding, the coding that will be used is based on mean value of green's channel and red's channel of its histogram. The data extracted from the cell image will become the input to the classifier system. In this project, the classifier is ANN which the algorithm for ANN train the data and learn are based on back-propagation algorithm.

The numbers of inputs in this ANN classifier are grouped into two inputs; including green's mean, and red's mean channels of the image. These two inputs will undergo to three hidden layers and the trained data for this back-propagation algorithm are more than hundred of images. The outputs of the classifier are positive, intermediate and negative anti-nuclear autoantibodies images.

3.3 Proposed Graphical User Interphase(GUI) in Matlab

After finished designing the block diagram, next task is to design Graphical User Interphase (GUI) for user to interact with computer with basic windows, menus and icons which can be manipulated by a person. The main purpose in designing this GUI is to make the Matlab software in computer more intuitive and user friendly. Figure 11 shows Matlab Graphical User Interphase (GUI) with menus and buttons corresponding to the previous block diagram.

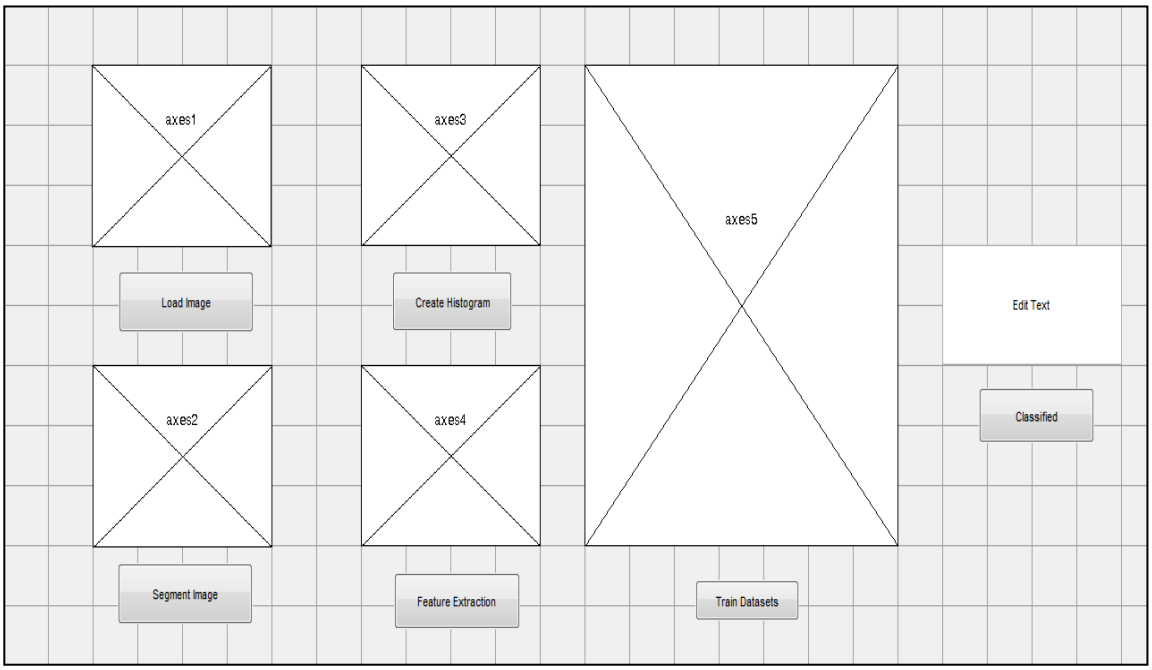


Figure 11: Matlab's Graphical User Interphase

There are six buttons to be selected by users in order to creating desired functions. First button is image selecting button, this button will direct the user to specific folder contains Hep-2 cells images. After selecting the image, the image will appear in the window known as axes 1. The images then will be used are in RGB colour space and sharpen for more clearer when the user press the 'image preprocess' button. The function code for Otsu's Multi-thresholding segmentation will be implemented in the button two which is image segmentation's button and the segmented image will be shown in axes two's window. Next, axes three will plot and show the histogram of the image which have red and green channel distributions.Different from others windows, axes four will not shows any images but it will form a table that contain numerical values which is green's and read's means. The train's button will call function articial neural network training code to training the datasets that have been collected during data mining, and axes five will plot the distribution of training data based on green and red mean values. Lastly, classification button will classify the image into three classes

which are positive, weak positive or negative. The axes five's window will shows the classes of images only.

3.4 Selection of Hep-2 cell images

The first phase of the project is to select the image which is want to be classified. All the Hep-2 cell images was taken from Mivia datasheet and will be stored in the specific folder . The matlab code below will give the basic function to select and show the image in the Matlab window.

```
figure(1);

[fn pn] = uigetfile('*.png','select png file');

complete = strcat(pn,fn);

im = imread(complete);

subplot(2,2,1);

imshow(im);
```

Functions of *uigetfile* is to direct the users to specific folder containing images which will be selected during image selection for testing. The function *imread* will read the input image and display the image by using function *imshow*. Subplot function used to plot the image into the Matlab window with placed at second row, second column and first quadrant. This code will be called once user press button 'select image' in Matlab GUI.

3.5 Image Pre-processing Matlab Code

The pre-processing techniques selected for this project is sharpening the image that can be programmed through Matlab software. Sharpening an image can be done in Matlab successfully by writing below codes in Matlab editor.

```
H = fspecial('unsharp', 0.15);

SImage = imfilter(ImageGray, H);
```

```
subplot(2,2,3);

imshow(SImage, []),
```

fspecial command is used to create blurred image for mask and the will undergo process filtering by function *imfilter*. This code will be called once user press button 'Image Preprocess' in Matlab GUI.

3.6 Image Segmentation programming

Based on design block diagram of classifier process, the function of image segmentation is to distinguish between object and background. Furthermore, colour segmentation will increase the segmentation quality, and reliable result. There are different way in which segmentation can be implemented in Matlab software. The easiest way is by call function *multithresh,* where the function is used Otsu's multi-thresholding technique by computes threshold of an image. The codes of Otsu's multi-thresholding are shown below. This code will be called once users press button 'Image Segmentation' in Matlab GUI.

```
figure(2);

threshRGB = multithresh(Image,7);


value = [0 threshRGB(2:end) 255];

quantRGB = imquantize(I, threshRGB, value);

figure, imshow(quantRGB,[]), title('Full RGB Image Quantization')
```

multithresh functions will return the threshold value *threshRGB* computed for input image which is Image using Otsu's method. Then, the input image will be converted into binary image by the function *imquantize*. The binary image will be shown in the new window by *imshow* code.

3.7 Cell's green colours mean and histogram computation

Positivity of Hep-2 cells depends on green colours intensity of IFF test, it is essential to calculate the mean of green colours of cells and determine its histogram to extract the colour features of the cells wether it is positive, weak positive or negative. After segment the image, each threshold value can be seen clearly, usually the higher intensity colour on the images. This colour intensity will indicate whether it is positive or negative, thus, the histogram of green colour channel will be plotted to analyze the distribution of green colour in the cell. The codes for computing green colour's histogram are shown below.

```
[x,y,z]=size(quantRGB);

rhist = ones(1,256);
if(z>1)
ghist = ones(1,256);
end

%Scanning Image Pixels
for i = 1:x
        for j = 1:y
            rhist(quantRGB(i,j,1)+1)=rhist(quantRGB(i,j,1)+1)+1;
            if(z>1)
            ghist(quantRGB(i,j,2)+1)=ghist(quantRGB(i,j,2)+1)+1;
            end
        end
end

ghist = double(log(ghist));
rhist = double(log(rhist));


%Ploting histogram
%Green pixels
show_hist = figure('Name',' Histogram','NumberTitle','off');
figure(show_hist);
 hold on;
    level=0:1:255;
    bar(level,ghist,'Barwidth',1,'Facecolor',[0 1 0],'Edgecolor',[0 1
0]);
    bar(level,rhist,'Barwidth',1,'Facecolor',[0 0 1],'Edgecolor',[0 0
1]);
```

As the image's colour space used is RGB colour, therefore the three channels need to be split. In this project, the green's and red's channel is take into account to calculate the mean values. After complete computed the histogram, each discrete value of histogram

is used to calculate mean of both channels. The codes to calculate mean are shown below.

```
M=mean(ghist,2);
m=mean(rhist,2);
```

3.8 Development of Multi-Layer Perceptron(MLP) artificial Neural Network Model

Based on the general structure of MLP, there are 3 main layers to be considered namely input layer, hidden layer and output layer. Apart from that, the spread coefficient of the MLP model needs to be determined as well. Prior to the development of MLP model, the data need to be properly selected, and partitioned to reduce the complexity of the learning process of MLP model, hence providing prediction with high accuracy.

3.8.1 Selection of Training Data

Data for image classification are from three different types of images in Mivia online image for researches. These images were chosen because of their different distribution of green and red colour means in its cell. Having a complete data is essential in establishing a pattern to be identified by MLP model, in order to produce a highly accurate estimation. Loopholes or missing data will resulted to high skewness and scattered data, which consequently increasing the complexity of the learning process of MLP model.

3.8.2 *Partition of Training Data and Data Analysis*

The partitioning of data for training and testing will be based on the data trend. Based on figure below, there are gaps between the mean values green and red between 0.03-0.08 for red and 0.15- 0.21 for green colour. The others gaps are from 0.15-0.21 for green mean and 0.03-0.05 for red mean. These data set show the two classes which is negative and positive based on mean colour of green and red. However, there is another gap between these two classes which is from 0.11-0.149 for green mean and from 0.03-0.08 for red mean . Hence it was decided that data prior fall under intermediate classes.

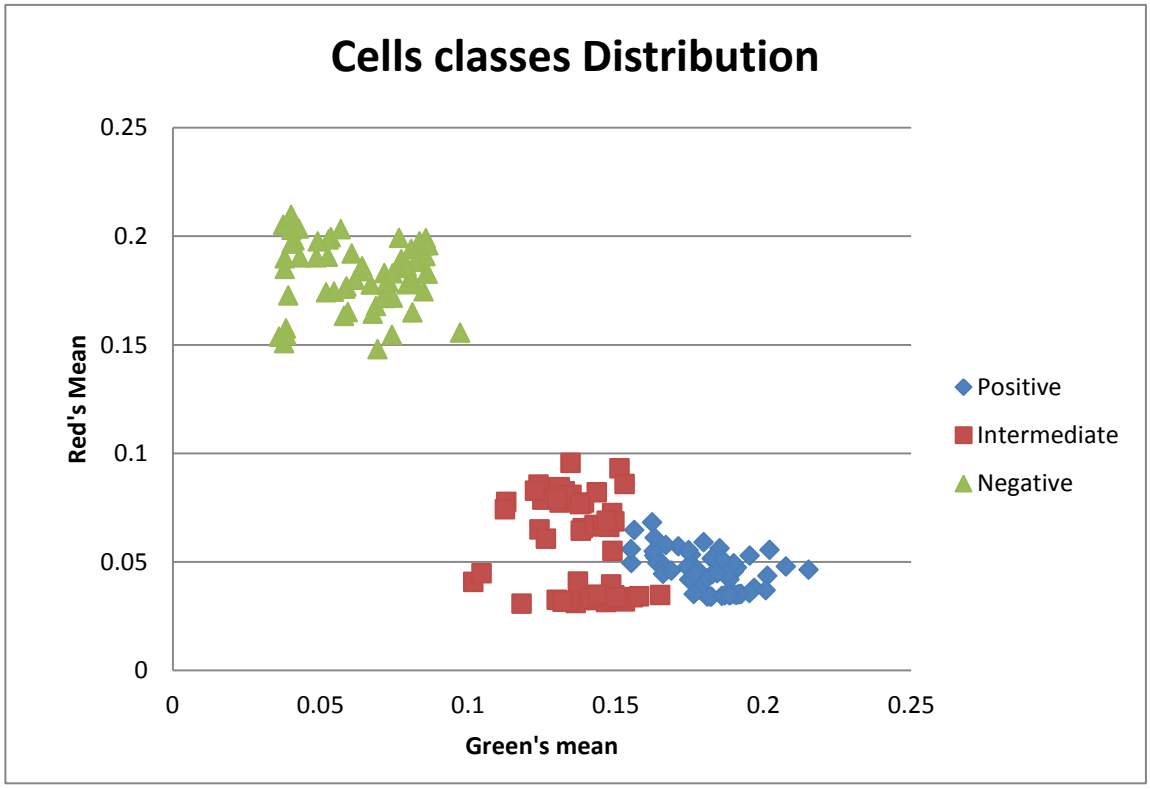


Figure 12: Training Data Distributions

Figure 12 shows, the distributions of three main classes of hep-2 cell images repective to its green and red means values. From the graph, it can be seen clearly the positive classes(blue) and negative(green) is separated far away. This is because for negative class, the green mean is lower while for positive the green mean is higher and vice versa for red mean. The intermediate class(red) is having at the middle of the graph, it has intermediate values of green mean and red mean. However, there are some overlapping between intermediate class and positive class. Total available data is 514 data and 372 data will be used for the purpose of training and the remaining 142 will be used for testing purpose, for classification on positive, intermediate and negative Hep-2 cell images.

3.8.3 *Artificial Neural Network Architecture*

The determination of input layer of MLP model depends on the number of input and the type of input variables. There are 2 inputs variables for this study and they are images's green and red means. The determination of these input variables was based on the colour features of cells images and recommendation of previous research papers. There are 3 hidden layers for this study, The method to determine the number of neuron in the hidden layer is trial and error method. The chosen of 3 hidden layers is based on distribution of training datas in this project. As there some overlapping between positive and intermediate classes, thus the best no of hidden layer to overcome meshed region problem is by increase hidden layer up to three. MSE denotes statistical parameter of mean square error. For the trial and error method, mean square error is chosen as the criteria to justify the best number of neuron in the hidden layer. Based on the trial and error method, the number of neurons that yield the best result, which is the lowest MSE, is 10 neurons for training stage. There are 1 output layer for this MLP model. The outputs are classification of positive, negative and intermediate classes. Summary of MLP model is as follows:

- Input Variables =2(Green and red means)
- Hidden Layer =3 hidden layer with 10 neurons
- Outputs Neurons =1 outputs(Either Positive, negative and Intermediate classes)

3.9 Flow Chart

Figure 13 shows the project flowchart for the whole duration of FYP 1 and FYP 2. The project procedures will be developed based on this flowchart.

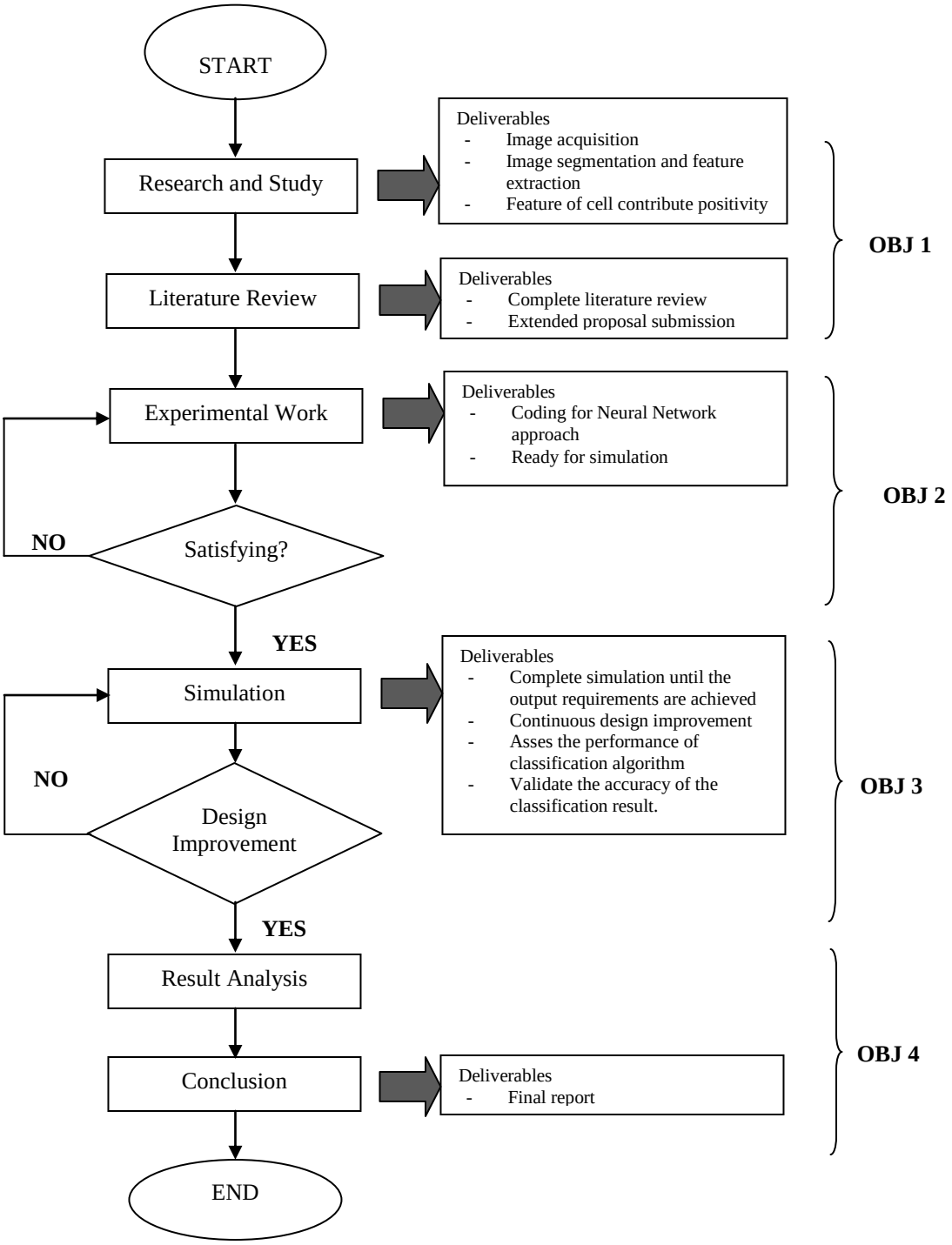


Figure 13: Project Flowchart for FYP 1 and FYP 2

After finished working on the literature review, the next task was experimental work. During design the coding for each process such as Otsu's Multi-thresholding, PCA and ANN coding ensured that no coding error. The coding afterwards were simulated by Matlab software where the output images is analyzed. Once the simulation results met the requirement, classifier will be asses by its performance by grouping the images into positive, weak positive and negative. If the classifier can classified the image into these three classes with high accurancy (e.g 80 %) the proposed design can be finalized, but if the classifier is not working as per required then the changes has to be made in design of coding.

3.10 Gantt Chart

The Gantt chart for this project (FYP1&FYP2) can be illustrated in Table III respectively. As per Gantt chart by end of this semester, all the preprocessed images coding and ANN coding has to be completed. In addition, the simulation has to be started within this semester and continued in the semester during FYP2. There are total of four submissions to be made for FYP1, begin with extended proposal submission, following with proposal defence presentation and lastly, end with draft interim and final interim report submission.

Table III represents the combination of Gantt Chart and Milestone for FYP 1. The schedule is important in order the project progress on track. The current position of this project is at week 6.

Table III: Gantt Chart and Milestone for FYP 1 and FYP 2

PROJECT SCHEDULE (MAY 2013-SEP 2013)

suggested milestone
process

	FYP 1														FYP 2														
ACTIVITIES/WEEKS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Research on topic: Fluorescence intensity classification to determine positivity																													
Analysis and comparison between the feature of cell in image that contribute positivity/negativity.																													
Planning and coding classification algorithm based on Neural Network																													
Testing the coding by using Matlab software (Laboratory work)																													
Analyzing the result obtain and classify the image into 3 classes (positive,negative, intermediate)																													
Validate the accuracy of the classification result and asses the performance of the classification Neural Network algorithm																													
MILESTONE																													
Selection project topic																													
Pre- research work																													
submission extended proposal						28-july																							
proposal defence								12-july																					
submission interim draft report																													
submission interim report														23-Aug															
project work continues																													
submission progress report																													
project work continues																													
pre-sedex																													
submission draft report																													
submission of dissertation(soft bound)																													
submission of technical paper																													
oral presentation																													
submission project dissertation (Hard bound)																													

3.11 Key Milestone

Figure 14 shows the key milestone that will be completed during FYP 1.

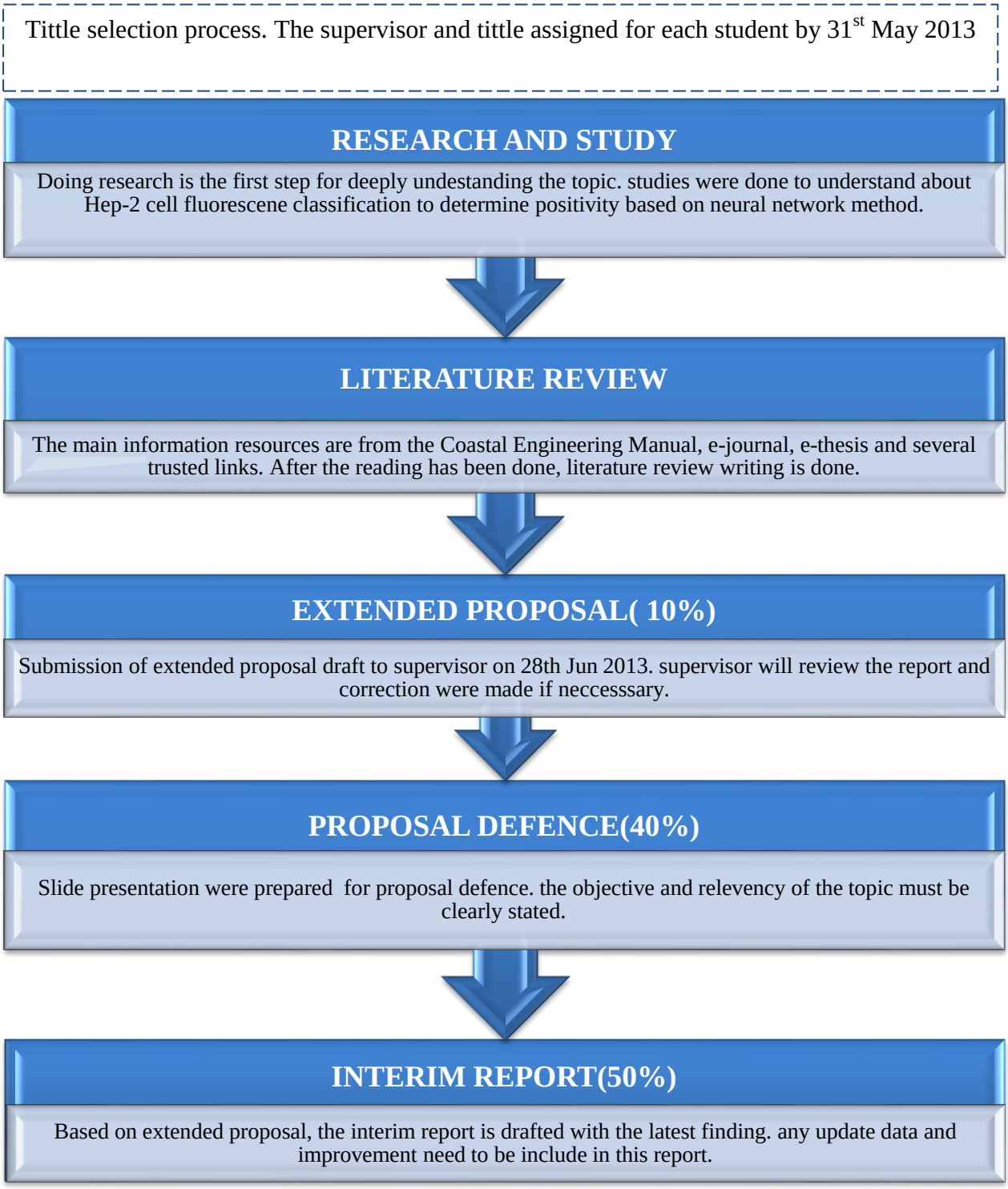


Figure 14: Key Milestone for FYP 1

Figure 15 shows the key milestone that will be completed during FYP 2.

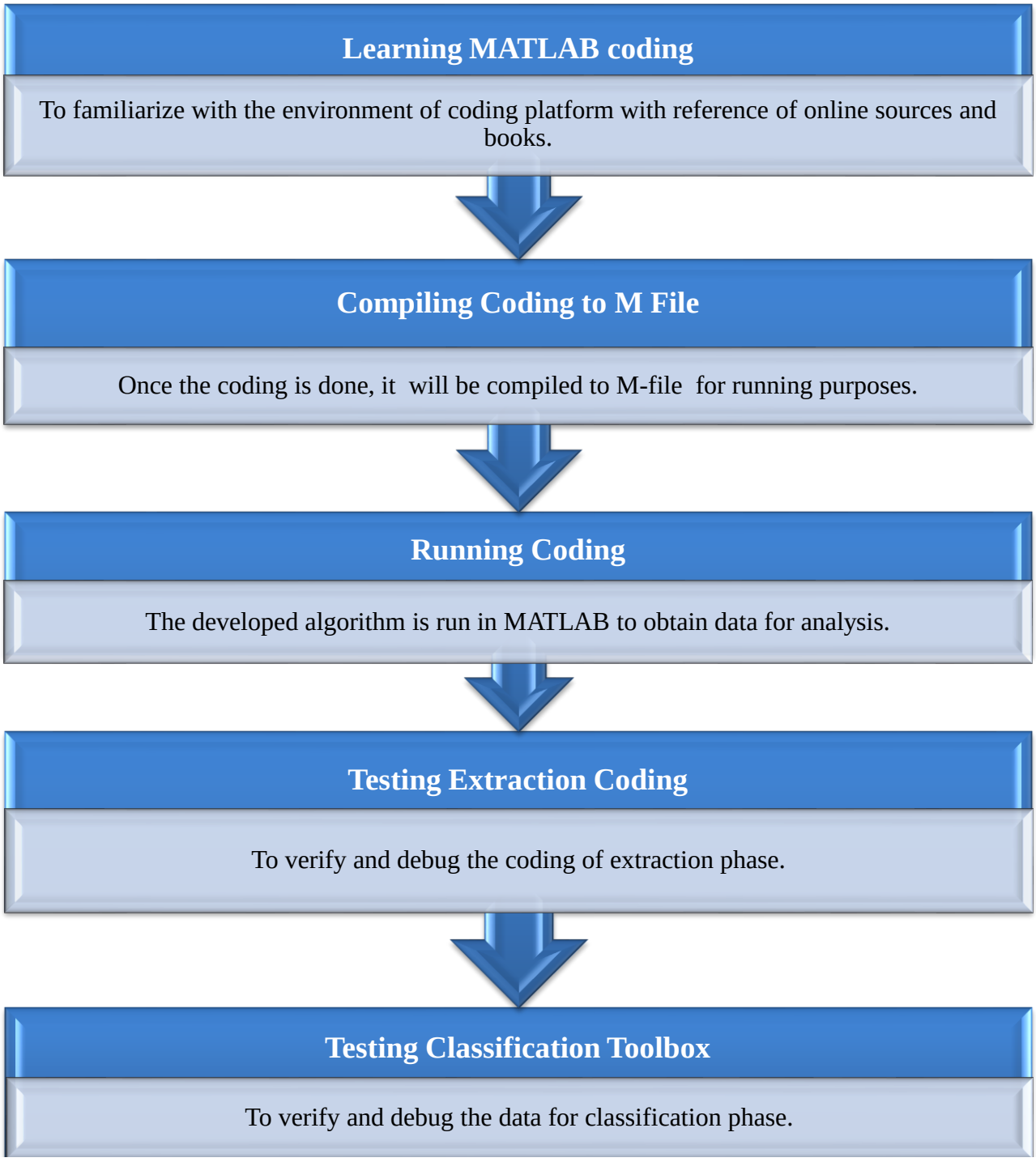


Figure 15: Key Milestone for FYP 2

3.12 Software required

For this project, the tools being used are as follows:

i. Matlab version R2013a

ii. Image Processing Toolbox version 8.2

iii. Microsoft Office Excel 2007

This project are based on simulation of software, working on algorithm of Matlab code for fluorescence intensity classification based on the ANN.

Microsoft excel is used to classify the three classes which are positive, negative or intermediate. It's also essential for plotting the graph for the result analysis later

CHAPTER 4

RESULT & DISCUSSION

During the period of FYP1, more and more progress were made towards completing this work. Begins with the literature review, a complete design of classification system were made to group HEp-2 cell images into three classes based on ANN. Follow up with completing the images which consist of three important phase; image segmentation, image feature extraction and ANN algorithm classifier. Each phase need to be simulated in Matlab software but in this FYP1, only images in segmentation simulation work are done. The coding of image segmentation is based on Otsu's Multi-hresholding but before the image undergo process image segmentation, the images is preprocessed by sharpening the image. There are more than 700 datasets were given by MIVIA research lab of the University of Salerno. However, the datasets are only consisting of positive and intermediate images. The project proceed with FYP2 which feature extraction work of images are done. Besides, to calculate mean values of each image channel, images histogram have been plotted. The given images have been labeled correctly by the experts, therefore the simulation of artificial neural network will be discussed in this section.

4.1 Image Pre-processing

Image pre-processing is essential to improve the image data which suppresses unwanted distortion and enhances the images features such as colour intensity. In this project, image pre-processing sharpening the images. This is important to achieve better images especially for the subject on edge. The RGB(red-green-blue) image is used because RGB colour space will determine the green colour intensity of cells. This will directly determine wether the cell infected by any auto-antibodies diseases. These RGB image and sharpening image are shown below(Figure 16 & Figure 17).

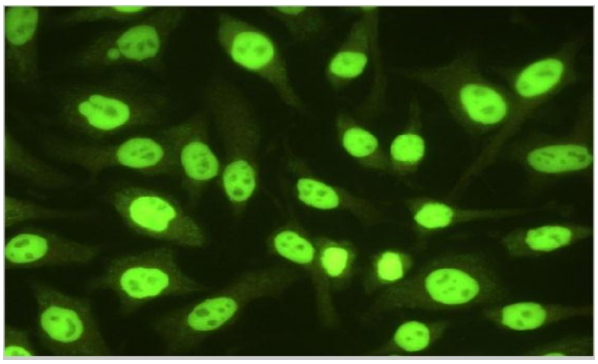


Figure 16: Original RGB image

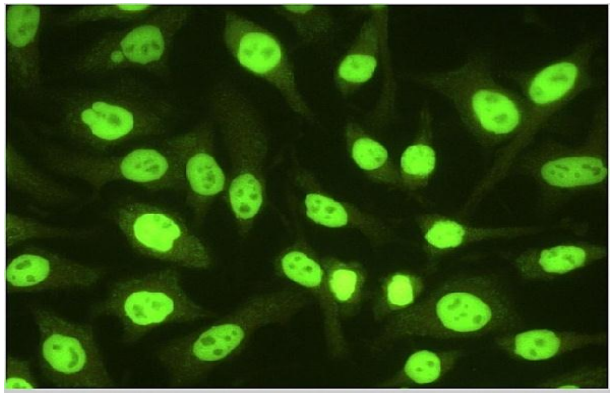


Figure 17: Sharpened image

In Figure 17, the enhanced RGB image after underwent process is known as sharpened image. From the image, there are not significant different between original image and the sharpened image because in the original image there are not presence of major noise. In Matlab code, the sharpened image use the function called unsharp masking. Unsharp masking is done by creating blurring to the original image and will be combined with the negative image. Negative image is totally inversion from original image, in which the lightest object will become dark and dark object will light. The combination creating that is less blurry and clarify image.

4.2 Otsu's Multi-Thresholding segmentation

Otsu's multi-thresholding method were applied to the image due to the characteristic of image having dark background and bright object suits well with this method. In such way, that object and background pixels have intensity levels grouped into two dominant modes. In Matlab, function of multithresh input an image and then computes the threshold value,T. The threshold is returned as a normalized value between 0 to 1. The calling syntax for multithresh in Matlab editor is shown below.

```
T = multithresh(I,7);
```

Where I is the input image and T is the resulting threshold. To segment the image, function i*mquantize* is used corresponding to T. This is because the Threshold normalized to the range [0,1]. Thus it must be scaled to the proper range before it is used.

```
quantRGB=imquantize(I, T, value);
```

Where *quantRGB* is segmented image, I is the input image and T is the threshold value from multithresh. Figure 18 shows the segmented image using Otsu's multi-thresholding segmentation respectively. The bright green shape is the threshold value of the cell image. Actually, the threshold value is chosen based on maximum between class variance. In the input image, the higher distributed of fluorescence intensity mainly at the nucleus of the cell. Therefore, the result of Otsu's multi-thresholding only focus on the nucleus of cell.

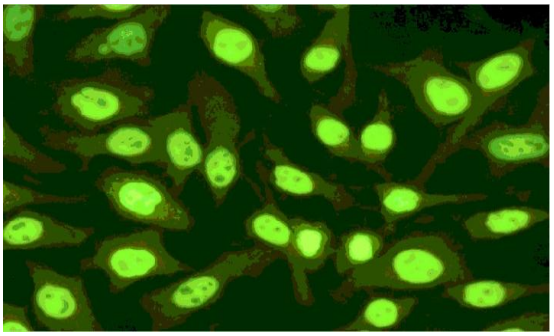


Figure 18: Otsu's Multi-Thresholding of cell image

4.3 Green and Red channel Histogram

Image histogram work as a graphical representation of tonal distribution including pixels, brightness, hue and saturation in a digital image. Every number tonal pixels in a image will be plotted accordingly. The image histogram give big pictures about entire tonal distribution in one image. By this, a viewer will be able to judge the image and analyze the distribution of its pixels. Basically, the x-axis represents the number of pixels in particular tones, while the y-axis represent the tonal variations of image. Figure 19 shows green's and red's channels histogram using Matlab simulation software respectively.

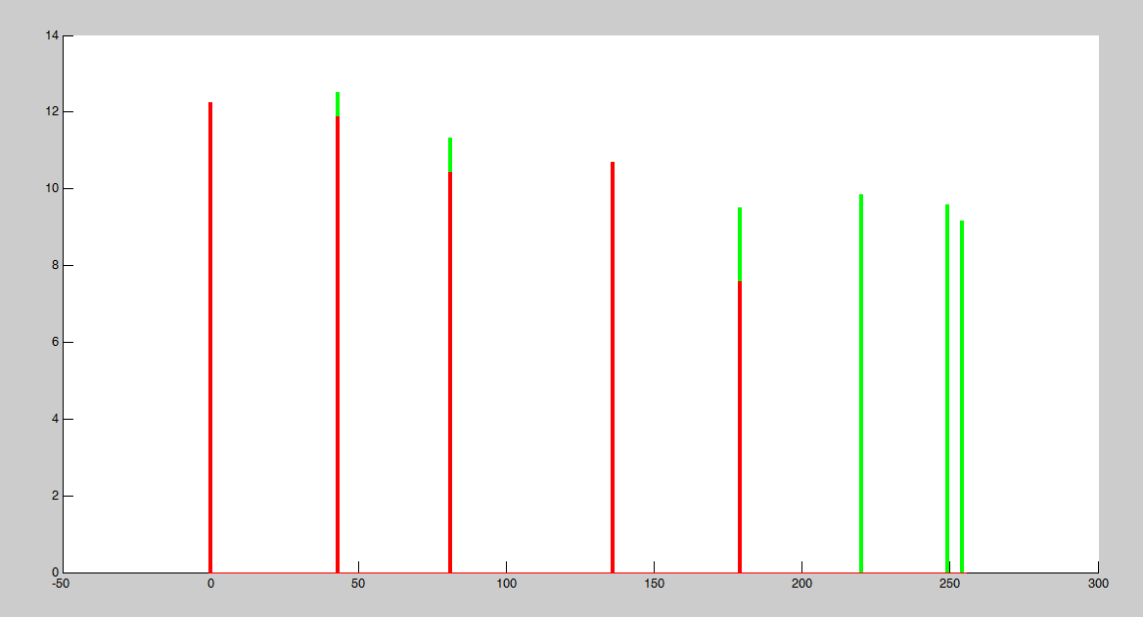


Figure 19: 2-channels histogram of a cell

Figure 19 shows two dimensional histogram with green and red channels. The red bar represents red colour intensity's distribution in an image while the green bar represents green colour intensity's distribution.

4.4 Feature Extraction

Table IV shows two means extracted from the RGB colour space of original image from types positive, intermediate and negative. The mean values of each layer which is mean values of green and red in histogram are calculated and extracted as shown. The calculated green's and red's mean value for positive and negative shows a distinct difference. However, the mean values for positive and intermediate have some overlapping on green intensity mean as both having green staining at the cells. The mean green of positive and intermediate intensities has higher values compared to red mean intensities of both classes . This is because the distribution of green fluorescence in the image is higher than red colour distributions. Nevertheless, the mean of red intensities is higher than mean green intensities in negative class as there approximately no presences of green fluorescence stain in the image. 186 train databeses have evaluated and all the means values is shown in APPENDIX B. The graph of positive, intermediate and negative intensity classes is plotted in APPENDIX C after train datas are tabulated in the table.

Table IV:RGB Colour Intensity Features Extractions

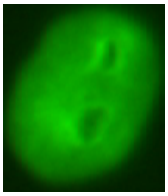
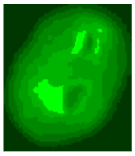
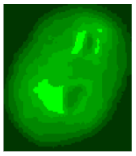
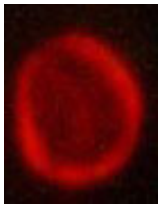
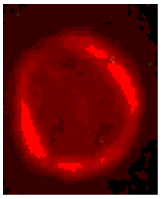
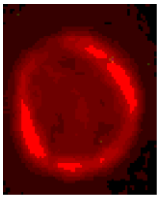
No.	Original Image	Red Intensity Mean	Green Intensity Mean	Fluorescence Intensity
1		 Mean = 0.0413	 Mean = 0.195	POSITIVE
2		 Mean = 0.03504	 Mean = 0.1617	INTERMEDIATE
3		 Mean = 0.1980	 Mean = 0.04138	NEGATIVE

Table V: Ranges of RGB Mean Values for Positive,Intermediate and negative Classes

	RANGES		
MEAN VALUE	**POSITIVE**	**INTERMEDIATE**	**NEGATIVE**
R(Red)	0.03-0.05	0.03-0.08	0.15- 0.21
G(Green)	0.15-0.21	0.11-0.149	0.03-0.08

Table V shows the mean range of positive,intermediate and negative classes. After obtaining the mean values for each class, the artificial neural network classification system is designed based on these means values range to update the connection between neurons and layers.

4.5 Artificial Neural Network Classification

Figure 20 below shows the artificial neural network fluorescence classification model with two inputs parameters; mean value R(red) and mean value G(green) with three hidden layers and one outputs with three membership functions which are positive, intermediate and negative.

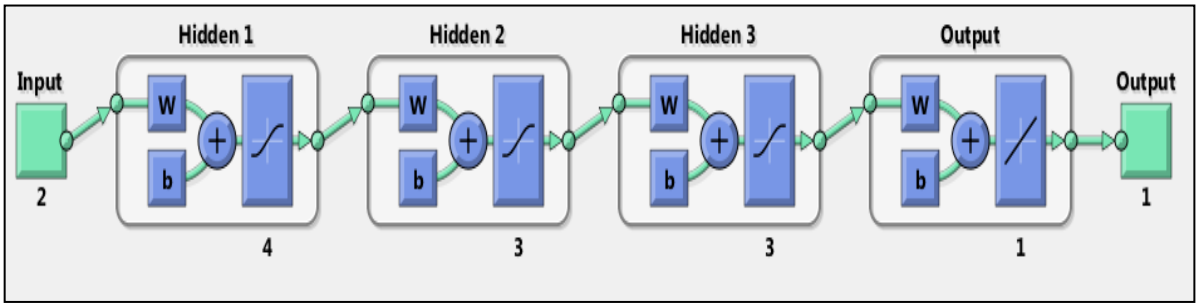


Figure 20: Artificial Neural Network Model

The algorithm behind this model is based on back-propagation algorithm or error correction principle. In order to determine best performance of this classification system, the neural network is trained by using train data as defined before. Term *W* in figure 20 denotes the term weight connections between the neurons while *b* is bias of these

connections. The weight connections will update its network architectures so that a network can efficiently perform the tasks. The performance of this classification system is improved over time as their updating the weight of network. In this artificial neural network classification system , sigmoids are used for hidden layers whereas piecewise linear is used for output layer.

As the classification system learns the training data, the mean square error(MSE) is decreased by iterations. Figure 21 shows the performance of neural network classification system after learn and updates its network architectures using training data based on back-propagation algorithm. Based on graph below, the maximum iterations is 1000 iterations; by this, the error for the system to misclassified is low and near to zero.

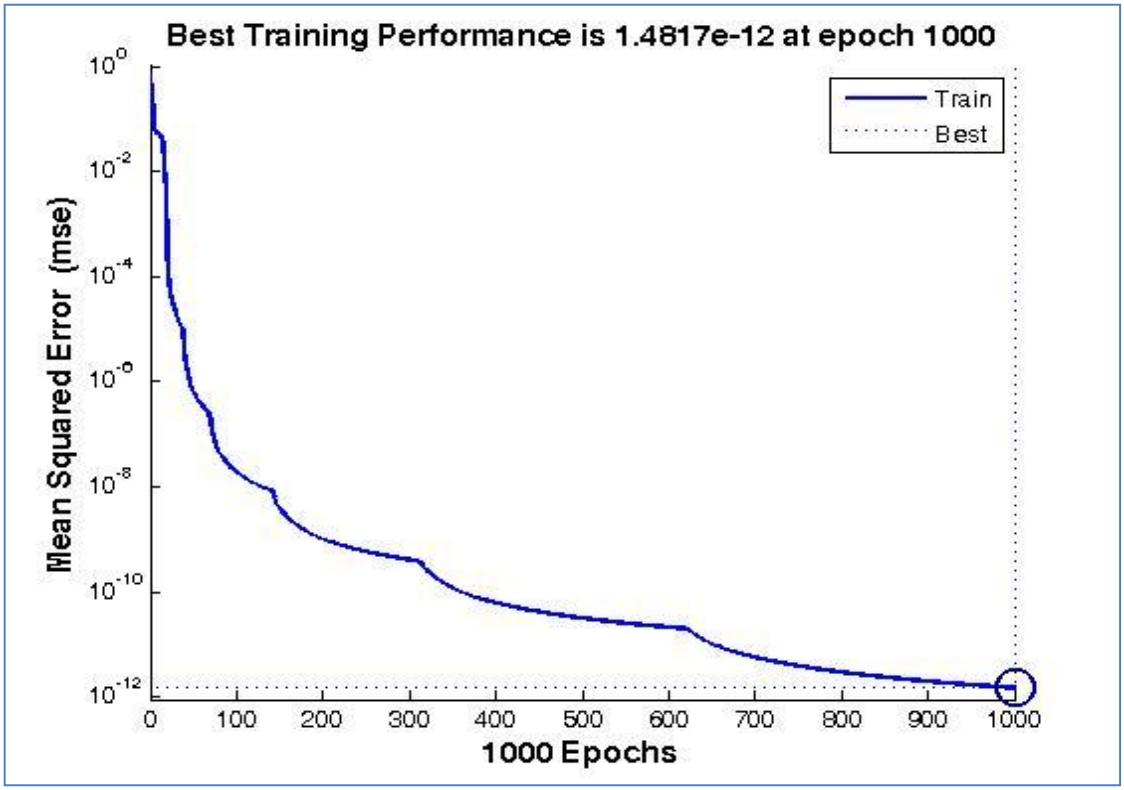


Figure 21: Training Performance of artificial neural network

After the networks have been trained using error-correction rules, 142 datasets are tested which are 69 positives data, 47 intermediates datas and 26 negatives data. The output variables are classified into 3 classes which are 1, 2 and 3. Positive class is

indicates by 1 respectively, whereas class 2 and 3 is correspond to intermediate and negative. During testing stages, the outcomes are from 69 positives data, there are 2 misclassified into intermediate's class, while from 47 intermediates data there are also 2 misclassified into positive's class. The testing on negative resulting all correctly classified. Figure 22 shows accuracy pie chart of neural network classification system after being tested. From the statistics, the proposed algorithm provides full information for the classification and show that the artificial neural network classification system is reliable with accuracy which is 97.2%. There are 3% misclassified between positive class and intermediate class because in training datas, there are overlapping between positive and intermediate datas.

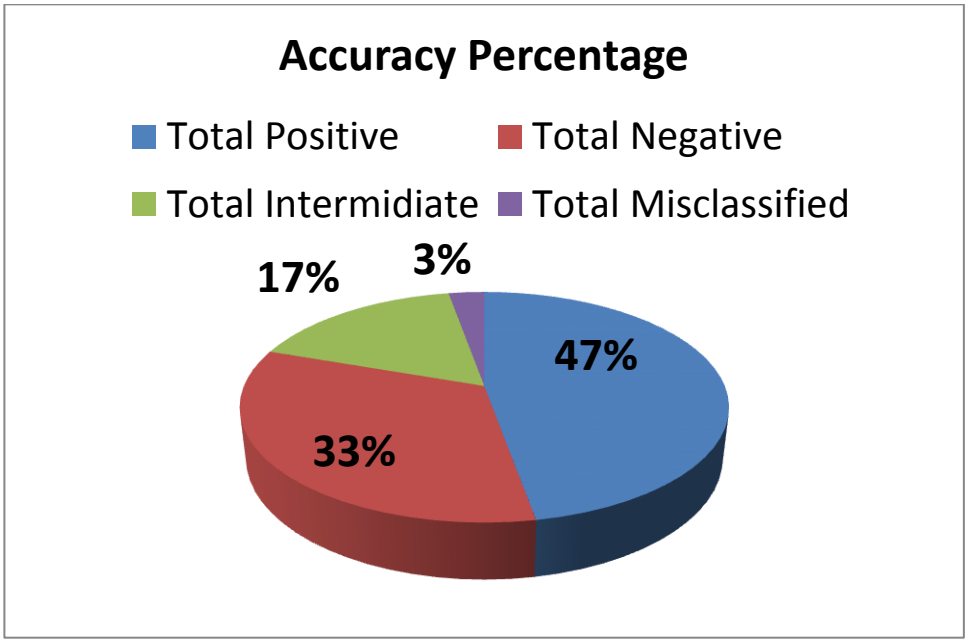


Figure 22: Accuracy's Pie Chart

CHAPTER 5
CONCLUSION

The pathology of antinuclear autoantibody (ANA) disease which applies automatic classification of fluorescent intensity of indirect immunofluorescence (IIF) samples is a very reliable technique in the modern clinical system. To achieve the objective, the suitable classification rule is proposed to allow varying the working point of the system. In this project, the process of classification of serum sample will undergo several methods such as image acquisition, image segmentation, features extraction and classification rule in order to achieve the objective 1 and 2 which are to understand the feature of serum that contribute to positivity of serum sample and develop classification algorithm based on Neural network .

Based on the results that have been obtained, the proposed technique provides full information for classification as its shows the system is reliable with high agreement total accuracy measurement- 97% for all classes. However, 100% accuracy in this classification system could not be achieved as there are may be misinterpretation on datasets that determine by experts.

It is recommended for further research to include the outliers and use necessary method to eliminate outliers without affecting the good data in order to produce a reliable classification model and accurate classification. Apart from that, for a validation of classification models using datasets collected from Hospital Universiti Malaysia is highly recommended. The models will be used to classify HEp-2 cell fluorescence images into three classes based on artificial Neural Network(ANN).

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APPENDIX –A: PROGRAM CODE IN MATLAB

```
%%------------------Input Image--------------------------------------%%

%%original image
figure,
I= imread('php09arodAM.jpg');
imshow(I), title('Original image');

%%------------------Image segmentation-------------------------------%%

%%thresholding colour segmentation
threshRGB = multithresh(I,7);

value = [0 threshRGB(2:end) 255];
 %Quantize entire image using one threshold vector
quantRGB = imquantize(I, threshRGB, value);
figure, imshow(quantRGB,[]), title('Full RGB Image Quantization')



%%------------------Image Histogram----------------------------------%%

%initialize RGB binary
[x,y,z]=size(quantRGB);
rhist = ones(1,256);
if(z>1)
ghist = ones(1,256);
bhist = ones(1,256);
end

%Scanning Image Pixels
for i = 1:x
        for j = 1:y
            rhist(quantRGB(i,j,1)+1)=rhist(quantRGB(i,j,1)+1)+1;
            if(z>1)
            ghist(quantRGB(i,j,2)+1)=ghist(quantRGB(i,j,2)+1)+1;
            bhist(quantRGB(i,j,3)+1)=bhist(quantRGB(i,j,3)+1)+1;
            end
        end
end

rhist = double(log(rhist));
   if(z>1)
   ghist = double(log(ghist));
   bhist = double(log(bhist));
   end

%Ploting histogram
%Green pixels
show_hist = figure('Name',' Histogram','NumberTitle','off');
figure(show_hist);
 hold on;
    level=0:1:255;
```

```
    bar(level,ghist,'Barwidth',1,'Facecolor',[0 1 0],'Edgecolor',[0 1
0]);

%%-------------------Mean,variance,Std-------------------------------%%

M=mean(ghist,2);
M;

%%-----------------MLP NEURAL NETWORK TRAINING----------------------%%

% 62 samples of each class

% define 3 clusters of input data
Aa= xlsread('Mivia positive data.xlsx');
Bb= xlsread('Mivia negative data.xlsx');
Cc= xlsread('Mivia intermediate data.xlsx');

A=Aa';        %Transposed positive class data
B=Bb';        %Transposed negative class data
C=Cc';        %Transposed intermediate class data

% plot clusters
figure(1)
plot(A(1,:),A(2,:),'g+')
hold on
grid on
plot(B(1,:),B(2,:),'r*')
plot(C(1,:),C(2,:),'bd')

hleg = legend('Positive Class','Negative class','Intermediate
class','Location','Northwest');

%%Define output coding for all 3 clusters%%
% coding (+1/-1) of 3 separate classes

a = 1; %[-1 -1 +1]';     %output for positive class
b = 2; %[-1 +1 -1]';     %output for negative class
c = 3; %[+1 -1 -1]';     %output for intermediate class


%%Prepare inputs & outputs for network training%%
% define inputs (combine samples from all three classes)
P = [A B C];
% define targets
T = [repmat(a,1,length(A)) repmat(b,1,length(B)) repmat(c,1,length(C))
];

%%Create and train a multilayer perceptron%%
% create a neural network
net = feedforwardnet([4 3 3]);  % feedforward network with 3 hidden
layer, 1st hidden layer have 4 neurons, 2nd&3rd hidden layer have 3
neurons

% train net
```

```
net.divideParam.trainRatio = 1; % training set [%]
net.divideParam.valRatio = 0; % validation set [%]
net.divideParam.testRatio = 0; % test set [%]

% train a neural network
[net,tr,Y,E] = train(net,P,T);
% show network
view(net)
%%------------------MLP NEURAL NETWORK Testing-----------------------%%

p = [M;m];
v = sim(net,p);

O=round(v);

switch O
    case 1
        disp('positive');
    case 2
        disp('negative');
    case 3
        disp('intermediate');
end
```

APPENDIX-B: RESULTS OF RGB FEATURES FOR TRAINING IMAGES

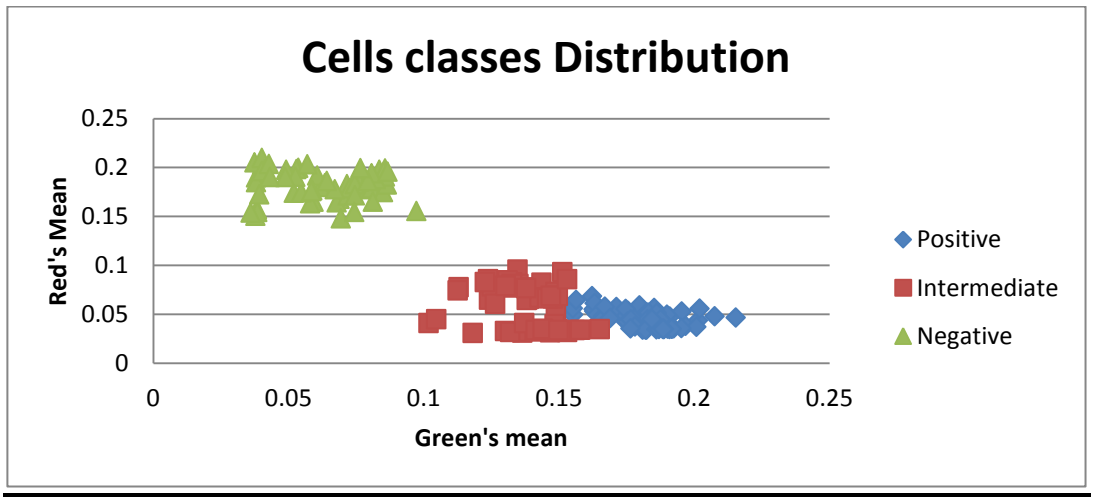
No	Red's Mean(R)	Green's Mean(G)	INTENSITY
1	0.051994793	0.163572502	**POSITIVE**
2	0.043571507	0.201428488	**POSITIVE**
3	0.053325661	0.175613013	**POSITIVE**
4	0.049494453	0.155373628	**POSITIVE**
5	0.055828515	0.155258987	**POSITIVE**
6	0.053027928	0.163157991	**POSITIVE**
7	0.054941589	0.162874028	**POSITIVE**
8	0.068264307	0.162344198	**POSITIVE**
9	0.041178322	0.179722643	**POSITIVE**
10	0.061173502	0.163263243	**POSITIVE**
11	0.064618605	0.156339033	**POSITIVE**
12	0.049351945	0.164188942	**POSITIVE**
13	0.048167051	0.166500837	**POSITIVE**
14	0.042412778	0.180965906	**POSITIVE**
15	0.045661738	0.177879709	**POSITIVE**
16	0.041889484	0.174966853	**POSITIVE**
17	0.047282444	0.176441496	**POSITIVE**
18	0.044519568	0.166009231	**POSITIVE**
19	0.046859542	0.177347375	**POSITIVE**
20	0.047712434	0.174169999	**POSITIVE**
21	0.04969813	0.184666145	**POSITIVE**
22	0.056327482	0.185243704	**POSITIVE**
23	0.055536682	0.202098965	**POSITIVE**
24	0.045704041	0.190079138	**POSITIVE**
25	0.057144724	0.171239069	**POSITIVE**
26	0.05903618	0.179845283	**POSITIVE**
27	0.051611193	0.182486491	**POSITIVE**
28	0.055362999	0.17468377	**POSITIVE**
29	0.042331682	0.17527548	**POSITIVE**
30	0.05136591	0.183264808	**POSITIVE**
31	0.057687122	0.167065001	**POSITIVE**
32	0.044392423	0.182789528	**POSITIVE**
33	0.045909315	0.168892342	**POSITIVE**
34	0.04455037	0.176647045	**POSITIVE**
35	0.046474319	0.215383471	**POSITIVE**
36	0.038043989	0.19688564	**POSITIVE**
37	0.047381848	0.191111525	**POSITIVE**

38	0.047826411	0.207638617	**POSITIVE**
39	0.052845813	0.195474586	**POSITIVE**
40	0.049374312	0.189996194	**POSITIVE**
41	0.033959626	0.181014448	**POSITIVE**
42	0.034753366	0.190896018	**POSITIVE**
43	0.033985733	0.18246282	**POSITIVE**
44	0.042046756	0.188640857	**POSITIVE**
45	0.035108678	0.191743713	**POSITIVE**
46	0.037303382	0.178155757	**POSITIVE**
47	0.036902314	0.200847426	**POSITIVE**
48	0.033959626	0.182039306	**POSITIVE**
49	0.035096949	0.190202059	**POSITIVE**
50	0.034316124	0.186048662	**POSITIVE**
51	0.035278646	0.192446305	**POSITIVE**
52	0.035084203	0.188541597	**POSITIVE**
53	0.035701127	0.195353541	**POSITIVE**
54	0.050917781	0.186198063	**POSITIVE**
55	0.034655962	0.186994304	**POSITIVE**
56	0.035137848	0.191762801	**POSITIVE**
57	0.044392649	0.187653676	**POSITIVE**
58	0.034753366	0.188970938	**POSITIVE**
59	0.034753366	0.188518358	**POSITIVE**
60	0.034651576	0.188617584	**POSITIVE**
61	0.04480435	0.184382798	**POSITIVE**
62	0.035270695	0.176463965	**POSITIVE**
63	0.03282997	0.143451869	**INTERMEDIATE**
64	0.064950518	0.124240219	**INTERMEDIATE**
65	0.033245992	0.152697933	**INTERMEDIATE**
66	0.034229086	0.146850462	**INTERMEDIATE**
67	0.033443722	0.148496406	**INTERMEDIATE**
68	0.033758745	0.145021492	**INTERMEDIATE**
69	0.032365286	0.14167547	**INTERMEDIATE**
70	0.032579906	0.130088965	**INTERMEDIATE**
71	0.031842272	0.153123405	**INTERMEDIATE**
72	0.033779373	0.155925095	**INTERMEDIATE**
73	0.03300608	0.139384691	**INTERMEDIATE**
74	0.0308182	0.118184474	**INTERMEDIATE**
75	0.095570577	0.134760983	**INTERMEDIATE**
76	0.07732048	0.131090222	**INTERMEDIATE**
77	0.076943671	0.138536982	**INTERMEDIATE**
78	0.065553345	0.13891872	**INTERMEDIATE**

79	0.080383295	0.130325102	**INTERMEDIATE**
80	0.080945339	0.135070465	**INTERMEDIATE**
81	0.084329477	0.129783395	**INTERMEDIATE**
82	0.082582531	0.132764523	**INTERMEDIATE**
83	0.077391833	0.139289966	**INTERMEDIATE**
84	0.083735015	0.130676015	**INTERMEDIATE**
85	0.082093992	0.143628129	**INTERMEDIATE**
86	0.084481505	0.131035998	**INTERMEDIATE**
87	0.085664628	0.123883214	**INTERMEDIATE**
88	0.080726706	0.132604891	**INTERMEDIATE**
89	0.093073563	0.151342802	**INTERMEDIATE**
90	0.078632085	0.125219573	**INTERMEDIATE**
91	0.077602659	0.112967579	**INTERMEDIATE**
92	0.034130047	0.144620791	**INTERMEDIATE**
93	0.034795361	0.165084173	**INTERMEDIATE**
94	0.06063424	0.126464914	**INTERMEDIATE**
95	0.03282997	0.146641953	**INTERMEDIATE**
96	0.033094515	0.150839072	**INTERMEDIATE**
97	0.031089557	0.136483322	**INTERMEDIATE**
98	0.031511075	0.146825894	**INTERMEDIATE**
99	0.032141316	0.134277778	**INTERMEDIATE**
100	0.031718726	0.132034106	**INTERMEDIATE**
101	0.04086088	0.101822307	**INTERMEDIATE**
102	0.032930114	0.144364328	**INTERMEDIATE**
103	0.064333978	0.138212478	**INTERMEDIATE**
104	0.055149455	0.148892675	**INTERMEDIATE**
105	0.072502789	0.148816264	**INTERMEDIATE**
106	0.044726007	0.10461254	**INTERMEDIATE**
107	0.06613813	0.14791481	**INTERMEDIATE**
108	0.066974172	0.142838985	**INTERMEDIATE**
109	0.066271722	0.145722366	**INTERMEDIATE**
110	0.06870134	0.149591267	**INTERMEDIATE**
111	0.068893048	0.1469953	**INTERMEDIATE**
112	0.076636871	0.13787377	**INTERMEDIATE**
113	0.040917211	0.137223738	**INTERMEDIATE**
114	0.032344548	0.148378262	**INTERMEDIATE**
115	0.034646087	0.149572208	**INTERMEDIATE**
116	0.033584635	0.141431177	**INTERMEDIATE**
117	0.03967292	0.148498325	**INTERMEDIATE**
118	0.034954906	0.14404313	**INTERMEDIATE**
119	0.0336997	0.149926116	**INTERMEDIATE**

120	0.034117489	0.157922717	**INTERMEDIATE**
121	0.074235749	0.112555232	**INTERMEDIATE**
122	0.085901661	0.15307861	**INTERMEDIATE**
123	0.082876842	0.122675105	**INTERMEDIATE**
124	0.079009634	0.130349065	**INTERMEDIATE**
125	0.183944345	0.06352072	**NEGATIVE**
126	0.182977641	0.071695248	**NEGATIVE**
127	0.179857351	0.061881166	**NEGATIVE**
128	0.167905326	0.068894391	**NEGATIVE**
129	0.165013974	0.059400021	**NEGATIVE**
130	0.179071568	0.07316153	**NEGATIVE**
131	0.177835194	0.079397566	**NEGATIVE**
132	0.174922661	0.072512421	**NEGATIVE**
133	0.191951168	0.060636237	**NEGATIVE**
134	0.163302398	0.058082212	**NEGATIVE**
135	0.164324237	0.067823163	**NEGATIVE**
136	0.171536429	0.071978863	**NEGATIVE**
137	0.178830776	0.072571046	**NEGATIVE**
138	0.189236411	0.083014717	**NEGATIVE**
139	0.177718102	0.067111941	**NEGATIVE**
140	0.155572705	0.0973614	**NEGATIVE**
141	0.186132768	0.064196584	**NEGATIVE**
142	0.183113561	0.086018273	**NEGATIVE**
143	0.179032607	0.081599468	**NEGATIVE**
144	0.189293809	0.077404524	**NEGATIVE**
145	0.178583792	0.080803648	**NEGATIVE**
146	0.185437506	0.076050233	**NEGATIVE**
147	0.174667863	0.085048195	**NEGATIVE**
148	0.199063967	0.085748704	**NEGATIVE**
149	0.182777926	0.08647694	**NEGATIVE**
150	0.190870573	0.085716364	**NEGATIVE**
151	0.182886558	0.074564808	**NEGATIVE**
152	0.188134978	0.082254252	**NEGATIVE**
153	0.197662905	0.083650976	**NEGATIVE**
154	0.195842035	0.085262293	**NEGATIVE**
155	0.194077632	0.080802823	**NEGATIVE**
156	0.186271394	0.079316644	**NEGATIVE**
157	0.150711843	0.037815552	**NEGATIVE**
158	0.189832939	0.038025763	**NEGATIVE**
159	0.202951637	0.040238758	**NEGATIVE**
160	0.157564101	0.038396555	**NEGATIVE**

161	0.184944023	0.038051807	**NEGATIVE**
162	0.154433117	0.038533152	**NEGATIVE**
163	0.153695234	0.036009405	**NEGATIVE**
164	0.172640092	0.039213058	**NEGATIVE**
165	0.205264436	0.037464979	**NEGATIVE**
166	0.209865136	0.04020859	**NEGATIVE**
167	0.198006157	0.041388121	**NEGATIVE**
168	0.199504408	0.053621036	**NEGATIVE**
169	0.198485921	0.052906792	**NEGATIVE**
170	0.196502874	0.040234819	**NEGATIVE**
171	0.203502202	0.042820203	**NEGATIVE**
172	0.203132012	0.057005634	**NEGATIVE**
173	0.197579734	0.049152884	**NEGATIVE**
174	0.199234717	0.076673215	**NEGATIVE**
175	0.195774972	0.086706864	**NEGATIVE**
176	0.176822759	0.058930674	**NEGATIVE**
177	0.174239264	0.051969322	**NEGATIVE**
178	0.174429316	0.054688386	**NEGATIVE**
179	0.19048428	0.052616377	**NEGATIVE**
180	0.190198311	0.048772275	**NEGATIVE**
181	0.190198311	0.043081847	**NEGATIVE**
182	0.171616206	0.074645201	**NEGATIVE**
183	0.16484265	0.081204936	**NEGATIVE**
184	0.147958568	0.069383238	**NEGATIVE**
185	0.175822781	0.058507455	**NEGATIVE**
186	0.154626627	0.074336342	**NEGATIVE**



APPENDIX-C: RESULTS OF ARTIFICIAL NEURAL NETWORK FLUORESCENCE INTENSITY CLASSIFICATION

No	R	G	INTENSITY	RESULTS - AGREEMENT
1	0.0563	0.1852	POSITIVE	YES
2	0.0486	0.1952	POSITIVE	YES
3	0.0457	0.1901	POSITIVE	YES
4	0.0501	0.1886	POSITIVE	YES
5	0.0516	0.1825	POSITIVE	YES
6	0.05	0.1634	POSITIVE	YES
7	0.0452	0.1779	POSITIVE	YES
8	0.0481	0.1791	POSITIVE	YES
9	0.0512	0.1918	POSITIVE	YES
10	0.0599	0.1781	POSITIVE	YES
11	0.0534	0.2053	POSITIVE	YES
12	0.0516	0.1849	POSITIVE	YES
13	0.05	0.1764	POSITIVE	YES
14	0.0486	0.1971	POSITIVE	YES
15	0.0618	0.2116	POSITIVE	YES
16	0.056	0.1837	POSITIVE	YES
17	0.0348	0.1886	POSITIVE	YES
18	0.0465	0.1705	POSITIVE	YES
19	0.0496	0.1739	POSITIVE	YES
20	0.0625	0.1858	POSITIVE	YES
21	0.0429	0.1639	POSITIVE	YES
22	0.0389	0.1759	POSITIVE	YES
23	**0.0358**	**0.1547**	**POSITIVE**	**NO(INTERMEDIATE)**
24	0.0474	0.1911	POSITIVE	YES
25	0.0473	0.1808	POSITIVE	YES
26	0.0458	0.1814	POSITIVE	YES
27	0.0602	0.1625	POSITIVE	YES
28	0.0439	0.1751	POSITIVE	YES
29	0.042	0.187	POSITIVE	YES
30	0.0421	0.175	POSITIVE	YES
31	0.053	0.1767	POSITIVE	YES
32	0.0363	0.1804	POSITIVE	YES
33	0.0498	0.1782	POSITIVE	YES
34	0.0476	0.1916	POSITIVE	YES
35	0.0567	0.1884	POSITIVE	YES
36	0.0492	0.1803	POSITIVE	YES
37	0.0418	0.1894	POSITIVE	YES
38	0.0441	0.1576	POSITIVE	YES
39	0.0395	0.1791	POSITIVE	YES
40	0.0482	0.1665	POSITIVE	YES
41	0.0333	0.1791	POSITIVE	YES

42	0.0428	0.1749	POSITIVE	YES
43	0.0398	0.1762	POSITIVE	YES
44	0.061	0.1739	POSITIVE	YES
45	0.0473	0.1764	POSITIVE	YES
46	0.0406	0.1802	POSITIVE	YES
47	0.034	0.182	POSITIVE	YES
48	0.0477	0.168	POSITIVE	YES
49	0.0343	0.186	POSITIVE	YES
50	0.0474	0.2025	POSITIVE	YES
51	0.0426	0.1957	POSITIVE	YES
52	0.0382	0.1932	POSITIVE	YES
53	0.0469	0.1858	POSITIVE	YES
54	0.0458	0.1885	POSITIVE	YES
55	0.0378	0.1904	POSITIVE	YES
56	0.0501	0.1881	POSITIVE	YES
57	0.0374	0.1873	POSITIVE	YES
58	0.0355	0.198	POSITIVE	YES
59	0.0495	0.1714	POSITIVE	YES
60	0.0435	0.1727	POSITIVE	YES
61	0.0544	0.1549	POSITIVE	YES
62	0.0463	0.1661	POSITIVE	YES
63	**0.0479**	**0.1518**	**POSITIVE**	**NO(INTERMEDIATE)**
64	0.0497	0.1562	POSITIVE	YES
65	0.0357	0.2115	POSITIVE	YES
66	0.0449	0.1689	POSITIVE	YES
67	0.0409	0.1866	POSITIVE	YES
68	0.0428	0.182	POSITIVE	YES
69	0.0479	0.1648	POSITIVE	YES
70	0.1839	0.0635	NEGATIVE	YES
71	0.183	0.0717	NEGATIVE	YES
72	0.1799	0.0619	NEGATIVE	YES
73	0.1679	0.0689	NEGATIVE	YES
74	0.165	0.0594	NEGATIVE	YES
75	0.1791	0.0732	NEGATIVE	YES
76	0.1778	0.0794	NEGATIVE	YES
77	0.1749	0.0725	NEGATIVE	YES
78	0.1919	0.0606	NEGATIVE	YES
79	0.1633	0.05808	NEGATIVE	YES
80	0.1643	0.0678	NEGATIVE	YES
81	0.1715	0.07197	NEGATIVE	YES
82	0.1788	0.07257	NEGATIVE	YES
83	0.1892	0.08301	NEGATIVE	YES
84	0.1777	0.0671	NEGATIVE	YES
85	0.1555	0.09736	NEGATIVE	YES
86	0.1861	0.06419	NEGATIVE	YES
87	0.1831	0.08601	NEGATIVE	YES

88	0.179	0.08159	NEGATIVE	YES
89	0.1892	0.0774	NEGATIVE	YES
90	0.1785	0.0808	NEGATIVE	YES
91	0.1854	0.07605	NEGATIVE	YES
92	0.1746	0.085	NEGATIVE	YES
93	0.199	0.0857	NEGATIVE	YES
94	0.1827	0.08647	NEGATIVE	YES
95	0.1908	0.0857	NEGATIVE	YES
96	0.1828	0.0745	NEGATIVE	YES
97	0.1881	0.0822	NEGATIVE	YES
98	0.1976	0.0836	NEGATIVE	YES
99	0.1958	0.08526	NEGATIVE	YES
100	0.194	0.0808	NEGATIVE	YES
101	0.1862	0.0793	NEGATIVE	YES
102	0.1507	0.0378	NEGATIVE	YES
103	0.1898	0.03802	NEGATIVE	YES
104	0.2029	0.0402	NEGATIVE	YES
105	0.1575	0.0383	NEGATIVE	YES
106	0.1849	0.03805	NEGATIVE	YES
107	0.15443	0.0385	NEGATIVE	YES
108	0.1536	0.036	NEGATIVE	YES
109	0.1726	0.0392	NEGATIVE	YES
110	0.2052	0.0374	NEGATIVE	YES
111	0.2098	0.0402	NEGATIVE	YES
112	0.198	0.0413	NEGATIVE	YES
113	0.1995	0.0536	NEGATIVE	YES
114	0.1984	0.0529	NEGATIVE	YES
115	0.1965	0.0402	NEGATIVE	YES
116	0.2035	0.0428	NEGATIVE	YES
117	0.0772	0.1537	INTERMEDIATE	YES
118	**0.0362**	**0.1734**	**INTERMEDIATE**	**NO(POSITIVE)**
119	0.034	0.1576	INTERMEDIATE	YES
120	0.0349	0.1558	INTERMEDIATE	YES
121	0.0356	0.1555	INTERMEDIATE	YES
122	0.0763	0.153	INTERMEDIATE	YES
123	0.0669	0.1282	INTERMEDIATE	YES
124	0.0764	0.1509	INTERMEDIATE	YES
125	0.0356	0.1555	INTERMEDIATE	YES
126	0.0397	0.1485	INTERMEDIATE	YES
127	0.035	0.144	INTERMEDIATE	YES
128	0.0368	0.1669	INTERMEDIATE	YES
129	0.0341	0.1446	INTERMEDIATE	YES
130	0.0335	0.1704	INTERMEDIATE	YES
131	0.0357	0.1664	INTERMEDIATE	YES
132	0.0358	0.1651	INTERMEDIATE	YES
133	0.0331	0.1508	INTERMEDIATE	YES

134	0.0349	0.1673	INTERMEDIATE	YES
135	0.0345	0.1689	INTERMEDIATE	YES
136	0.0338	0.145	INTERMEDIATE	YES
137	0.0318	0.1531	INTERMEDIATE	YES
138	0.0338	0.1559	INTERMEDIATE	YES
139	0.0778	0.145	INTERMEDIATE	YES
140	0.0683	0.1479	INTERMEDIATE	YES
141	**0.0742**	**0.1601**	**INTERMEDIATE**	**NO(POSITIVE)**
142	0.0418	0.0665	INTERMEDIATE	YES