AUTOMATED SOFTWARE TO COUNT STAINS IN IMMUNOSTAINING APPLICATIONS

A THESIS IN

Computer Science

Presented to the Faculty of the University
of Missouri-Kansas City in partial fulfillment of
the requirements for the degree

MASTER OF SCIENCE

By

KATIKA ANUSHA

B.TECH., Jawaharlal Nehru Technological University, 2012

Kansas City, Missouri

2019

AUTOMATED SOFTWARE TO COUNT STAINS ON IMMUNOSTAINING APPLICATIONS

Katika Anusha, candidate for the Master of Science degree

University of Missouri-Kansas City, 2019

ABSTRACT

Quantification of cells from immunostained images is a vital procedure in biomedical analysis, as it helps in the measurement of proliferation, immunodetection and differentiation of nuclear markers, which in turn play a significant role in the analysis of the cell functioning. Surgical pathology uses the quantified immunostained images as a diagnostic tool to differentiate between benign and tumor cells. However, manual quantification suffers numerous drawbacks, such as the lack of repeatability due to inter- and intra-observer variability, the lack of precision due to manual visual quantification and the larger time consumption for counting. This led to the introduction of the computerized image counting techniques as a measure to overcome these difficulties.

In this context, the present study proposes a software assisted GUI imaging technique and attempts to analyze its efficiency in the quantification of cells. The study adopted various analytical process, such as the comparison in the quantification between manual and automated in different stains, colocalization, to identify the number of active cells while images are spatially overlapped, fusion indexing and the comparison of the cell counts in myotubes with a control value. The comparative analysis between the proposed software assisted imaging technique and manual counting, using different stains, such as β-Gal, DAPI and sclerostin with the help of box plot, yielded a strong significant difference in DAPI and sclerostin stains. No

statistically significant differences were observed in β-Gal staining. The descriptive analysis in the quantification of overlapped cell using two overlapped images (β-Gal and DAPI) and three overlapped images (β-Gal, DAPI, sclerostin), demonstrated the improvement in the identification of active cells both in the case of two and three overlapped images. Furthermore, the comparative analysis of the fusion index value of Wnt3a images against control, using fusion bin range and fusion area methods, using box plot, revealed significant variation in fusion index value between Wnt3a and control in fusion area, whereas, the fusion bin did not yield any statistically significant outcome which help the study to reach into a conclusive inference. However, despite promising results, there is scope for improvement, which in turn opens the door for the future researchers to extend the study using more efficient automated imaging systems.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Computing and Engineering have examined a thesis titled “Automated Software to Count Stains in Immunostaining Applications” presented by Katika Anusha, candidate for the Master of Science degree, and certify that in their opinion it is worthy of acceptance.

Supervisory Committee

Thiagarajan Ganesh, Ph. D., P.E., Committee Chair

Department of Civil and Mechanical Engineering

Praveen Rao, Ph. D.,

School of Computing and Engineering

Mark L. Johnson, Ph.D.,

Department of Oral and Craniofacial Sciences

Cory Beard Ph.D.,

School of Computing and Engineering

CONTENTS

LIST OF ILLUSTRATIONS

Figure Page

ACKNOWLEDGMENTS

I would prefer to impart my thesis adviser Dr. Ganesh, Thiagarajan at University of Missouri - Kansas City. The door to Prof. Ganesh office was always open whenever I ran into a trouble spot or had a question about my research or writing. He systematically allowed this paper to be my very own work but steered me in the right direction whenever he thought I needed it.

I would also like to thank the experts who were involved in the validation survey for this research project: Dr. Mark Johnson, Nancy Hoover, Dr. Nuria Lara, Kun Wang, Dr. Sarah Dallas. Without their burning participation and input, the validation survey could not have been successfully conducted.

I would also like to acknowledge Dr. Mark Johnson of the UMKC School of Dentistry as the second reader of this thesis, and I am gratefully indebted to his very valuable comments on this thesis.

Finally, I must express my very profound gratitude to my parents, my spouse Karthik Akula and my brother Avinash Katika for providing me with unfailing support and continuous encouragement throughout my years of study and thru the method of researching and scripting this thesis. This accomplishment wouldn't be doable without their support.

CHAPTER 1

INTRODUCTION

Background

Immunostained image quantification is a vital procedure in the biomedical analysis as the quantification of proliferation, immunodetection, and differentiation of nuclear markers play a significant role in the study of cell functioning. The quantification of immunostaining helps to characterize the protein expression on normal or pathological slices of tissue.

Further, this approach aids morphological controls and carries out detailed tissue and cell localization, which in turn avoids the problems caused by cell and tissue heterogeneity. This helps in the identification of nuclear markers that are useful for the prognostic and therapeutic purposes (Decaestecker et. al., 2009). Hence, one of the prime uses of quantification of immunostaining is in oncology where the data obtained from tumor samples may have therapeutic or analytical impacts (Elie, 2003). The surgical pathology uses the quantified immunostained images as a diagnostic tool to differentiate between benign and tumor cells (Matos et al., 2006).

In the early ages, cell quantification was performed as a manual visual microscopic evaluation, which comprised of the assessment of ostensible density of immunostained cells for the assignment of the best representative category, through the investigator's observation. However, the lack of repeatability due to inter- and intra-observer variability (Walker, 2006), and the lack of precision due to manual visual quantification (Benali et al., 2003) remained the major drawbacks that restricted the application of the methodology. The counting of cells fused with muscle cells is extremely difficult and time consuming. The time taken to manually calculate the count of stains in the case of fused ones is usually 20-40 minutes.

Even after this, it is difficult to get an accurate count, as the total number of stains for an image can be in hundreds. Hence, in order to overcome these difficulties, the computerized image counting techniques are introduced wherein numerous software are used. These techniques effectively reduce the impacts of observer biases and increase the throughput and sensitivity of immunohistochemistry (Pham et al., 2007). In addition, the studies of Lin et al. (1996) and Seidal et al. (2001) have highlighted the fact that software-assisted immunostaining quantification has resulted in improved reproducibility and faster results. These results triggered other scholars, who conducted numerous researches and developed several software-assisted immunostaining quantification methods.

Related Studies

The shortcomings in the manual cell quantification motivated researchers to shift their focus to computer-assisted quantification tools, which were supposed to overcome the existing drawbacks. This resulted in the development of several automated methods with the help of software. Kohlberger et al. (1996) developed a true color RGB image analyzer on the basis of morphologically reduced instruction set computer processor. This analyzer was meant for estimating the area of stained endothelial cells and for comparing the percentage of factor VIII-related antigen-stained area with the counted micro-vessel density (CMVD) results. However, the tool could not overcome the drawbacks resulting in pixel dimension, which makes counting a difficult task and causes errors in the quantification of stained endothelial cells. Further, Lehr et al. (1997) designed a Photoshop-based image analysis tool by using commercially available and inexpensive software for the quantification of hormone receptor expression in breast cancer. The findings indicated the ability of the automated, software-supported imaging tool in the effective quantification of immunohistochemical

hormone receptor studies. However, the limited use of image databases was not enough to substantiate the tools' applicability in the quantification of staining in large image databases. The quantification of immunostaining by the measurement of cumulative signal strength by using Photoshop software and MATLAB was performed by Matkowskyj, Schonfeld and Benya (2000). The study highlighted the inefficiency of the available techniques for counting the image pixels and pointed out the necessity of a specific software for the quantification. Veltri et al. (2000) proposed a novel image analysis on the basis of a bio marker called quantitative nuclear grade (QNG), which uses quantitative nuclear morphometry data, derived from computer-assisted imaging for cancer detection. Mofidi et al. (2003) designed a digital imaging system using Adobe Photoshop software for the assessment of oestrogen receptor status. Even though the findings indicated a fair assessment of oestrogen receptor positivity, the inadequate number of the targeted population restricted the magnitude of the results. In a similar kind of study, Nabi et al. (2004) used a computer-assisted imaging system to classify the immunostaining heterogeneity of androgen receptors in metastatic carcinoma of the prostate. The findings demonstrated the image analysis of androgen receptor immunostaining. The digital imaging approach yielded vital prognostic information, which in turn helped to predict the response to hormone treatment in patients with a metastatic carcinoma of the prostate. Despite the fairly good outcome, the study was skeptical about the applicability of the imaging system in a wider context and suggested further research before its usage. The increased use of immunohistochemistry (IHC) in clinical research triggered more studies and one such attempt was done by Fray et al. (2003) to standardize the IHC analysis. The authors proposed a novel imaging technique, which involved the conversion of brightfield images of diaminobenzidene (DAB)-labeled antigens

to normalized blue images, thereby allowing the automated quantification of positively stained tissues. However, the possible misclassifications in the quantification, during the usage of two or more chromogens with overlapping absorption spectra, cast shadows over the applicability of the tool.

The importance of assessing androgen receptors in the detection of prostate cancer motivated Singh et al. (2005) to design an automated nuclear analysis tool using Java web start for the quantification of AR protein expression levels. However, the study failed to provide a satisfactory result regarding the performance of the proposed tool. Further, the quantification of protein expression levels was also performed by McCabe et al. (2005). The authors designed an automated imaging tool for the quantification. However, the study cast some doubts on the practical implication, due to the skepticism around the accuracy of the results. Decaestecker (2009) attempted to demonstrate the importance of proper imaging techniques in the quantification of immunostains on the tissue microarray materials. The study suggested certain pre-requisites for the imaging tool for the accurate quantification of immunostaining images. Diniz (2010) proposed a semi-automated computer-assisted image analysis for the quantification of immunostained receptors in vascular tissue sections. The findings of the study highlighted that the computer-assisted image analysis tools, meant for quantifying the immunostained receptors, must be specific to the applications. The importance of the quantification in the cancer detection process has widened its area of application and several researchers have attempted to propose different computer-assisted imaging tools for quantification. Following these, Sant'Anna, Sant'Anna and Parollini (2011) proposed an open-source image analysis software, called CellProfiler, to quantify the fibrosis induced in rats. CellProfiler permitted a simultaneous quantification of fibrosis in all of the

600 histological images, which were declared as malignant, at a rate of ~10 s/image. However, the study failed to highlight the quantification ability of the tool. Another attempt in this route was made by Duran and Arriazu (2013), who proposed a metamorphic image analysis system for the quantification of protein expression in the immunoflurescene-stained sections. Even though the results indicated the ability of the image analysis tool to quantify the protein expressions, the study highlighted the necessity of certain prerequisites, such as the interpretation of images by skilled technicians, for an accurate quantification. The findings suggested that the application of the image analysis tool enabled the easy and accurate prediction of cancer. However, the major drawback of the method was that the tool required simultaneous immunostaining of all the sections to avoid the resistivity index (Ri) variability.

Research Gap

Despite the advantages of the software-assisted immunostaining quantification, the applicability of the computer-based techniques is still limited due to the drawbacks highlighted in the comprehensive review conducted in the previous section. One of the prime shortcomings encountered by the review is the skepticism around the practical applicability of the imaging tool. Studies conducted by Nabi et al. (2003), McCabe et al. (2005), etc., have highlighted this shortcoming. The findings of the studies conducted by Lehr et al. (1997) and Mofidi et al. (2003) have highlighted the lack of sufficient data as a major drawback.

The inability to yield comprehensive conclusions about the efficiency of the proposed tools is another drawback revealed by the review. The studies conducted by Singh et al. (2005) and Sant'Anna, Sant'Anna and Parollini (2011) have highlighted this drawback.

The necessity of a specific software for quantification was highlighted by Matkowskyj, Schonfeld and Benya (2000) and Diniz (2010). The present study was significantly motivated by this necessity.

The next potential shortcoming found by the review was the requirement of adequate pre-requisites, such as skilled technicians, for the interpretation of images. Several researchers, such as Decaestecker (2009) and Duran and Arriazu (2013), have argued that lack of adequate image interpretation skills restricts the applicability of the software.

Furthermore, the difficulty in counting due to the pixel dimensions, (as pointed out by Kohlberger et al., 1996) and the possible misclassifications in quantification during the usage of two or more chromogens with overlapping absorption spectra (as indicated by Brey et al., 2003) form the other drawbacks that were discovered by the review, which in effect worked as the motivation for the present study.

Objectives

To develop an automated graphical user interface using MATLAB code for the quantification of cells from the immunostained images.

Chapter Description

The first chapter is the introduction chapter, which provides the overview of the research. The chapter contains the background of the study that briefs the requirement of quantification of immunostaining images and the reason behind the introduction of software-assisted quantification techniques. The chapter also contains a review of the studies that propose or use different automated tools for quantification and emphasize on shortcomings in the existing software. Finally, the objectives of the current study are presented.

The second chapter describes the software used for the development of automated Graphical User Interfaces (GUI). It explains about the toolbox used in MATLAB and also details the software design workflow and the steps taken in the development of the GUI.

The third chapter describes the program modules presented in the automated process. The chapter outlines the step-by-step process for the analysis of immunostained images to count the cells.

The fourth chapter illustrates the functioning of the entire analysis process through a step-by-step description and depicts the output presentation.

The fifth chapter briefly describes the results of statistical analysis used to measure the efficiency of the automated GUI software. The chapter depicts the output of the comparative analysis between the manual and the automated GUI cell counts for various stained images.

The sixth chapter concludes the study with a description of key inferences and the

final takeaway. The concluding chapter also shows the direction for future studies.

CHAPTER 2

SOFTWARE DESCRIPTION

Introduction to MATLAB®

MATLAB® allows matrix manipulations, plotting of functions and data, execution of calculations, creation of user interfaces, and interfacing with projects written in different dialects, including C, C++, Java, Fortran and Python. The MATLAB® application is built by using the MATLAB® scripting language. The common usage of the MATLAB® application involves using the Command Window as an interactive mathematical shell or executing text files containing the MATLAB® code.

Commonly, user interfaces (UIs) sit tight for a user to click a control button and respond to it. Every control and the UI itself, has one or more callbacks, named for the way they "get back to" to MATLAB® to request it to do things. A specific client activity, for example, squeezing a screen catch or ignoring the cursor a part, triggers the execution of every callback. The UI then reacts to these occasions. Several UIs have been developed in this thesis for various purposes.

Graphical user interfaces (GUIs), otherwise called graphical client interfaces, give point-and-click control of programming applications, killing the need to take in a dialect or sort orders with a specific end goal to run the application.

For more control over the configuration and advancement, one can write MATLAB® codes that characterize all part properties and practices. MATLAB® contains prebuilt codes to assist in the creation of the GUI for applications automatically. One can include dialog boxes, client interface controls, (for example, push catches and sliders), and compartments, (for example, boards and catch bunches).

Software Design Flow

With the end goal to utilize this automated counting process, the client to tally the aggregate number of cells must adopt a careful methodology. There are seven fundamental parts of this product. They are:

a. Queue

b. File Loading

c. Segmentation

d. Thresholding

e. Tools

f. Results

g. Help

The parts are briefly described below.

Queue

Queue tab is one of the new tabs added to Immunostaining GUI. It has several functionalities. Each functionality is for a different type of image. The main advantage of queue tab is to process the image faster than the normal flow process. The queue process stops whenever it needs input from the user.

File

After loading the required image, the user can browse and select the image. When a new image is loaded, its width, height, color type, and bits per sample are shown and the name of the image can be changed and saved.

Segmentation

Two actions are performed in this tab. The first action is choosing a channel from the four channels (Original, Red, Green, and Blue). The second action is to start the segmentation.

Thresholding

It is used as additional option to adjust the intensity of the stains. The low and high values can be manually entered in the box or can be done by dragging the bara. There are two types of thresholding.

Tools

Used to add some more criterion to the thresholded image and also to get count of stained cells. There are two types of functions under it. One for criterion another one is for counting. There are many types of criterions like, edge criterion, circularity criterion, Min and Maximum pixel area. In Cell counting there are box count, ROI count etc.,

Results

Along with count of stains and thresholding values the results are stored in an Excel sheet. These are cached for further reference.

Help

Provide software usage tips. If users have any doubts on the flow while working, then such doubts are resolved by this functionality.

CHAPTER 3

DESCRIPTION OF PROGRAM MODULES

Queue

β-Gal Queue

β-Gal queue is to process β-Gal images to identify the total number of blue stains on the image. Normal image processing needs user to follow many steps to get results, remember the steps to process and it is time consuming but, with the help of β-Gal Queue process you can overcome the above problems. It stops only when it really needs human entered values. This module reduces the manual work by loading each image from the images folder and process it until all images are processed.

This function load Images from selected folder, chooses the Original channel, asks user to manually segments the image, thresholds by using a double channel, calculates the minimum and maximum stain area, inter-cell distance, edge criterion, circularity criterion, calculates the total number of stains, and finally saves the results in a Microsoft Excel sheet.

DAPI Queue

DAPI queue is to process DAPI images to identify the total number of nuclei on the image. Normal image processing needs user to follow many steps to get results, remember the steps to process and it is time consuming but, with the help of DAPI Queue process you can overcome the above problems. It stops only when it really needs human entered values. This module reduces the manual work by loading each image from the images folder and process it until all images are processed.

This function loads the images folder, chooses the blue channel, uses the existing mask, thresholds by using a single channel, calculates the minimum and maximum stain area, inter-cell distance, edge criterion, circularity criterion, calculates the total number of stains, and finally saves the results in a Microsoft Excel sheet. Then the next image gets loaded and the above whole process is repeated. This process happens until, all the images in the folder are processed

Non-Immune Queue

It is to process non-immune images to identify the threshold value of non-immune images. Normal image processing needs user to follow many steps to get results, remember the steps to process and it is time consuming but, with the help of Non-Immune Queue process you can overcome the above problems. It stops only when it really needs human entered values. This module reduces the manual work by loading each image from the images folder and process it until all images are processed.

Non-immune queue is to process non-immune images. This function loads the images folder, chooses the red channel, uses existing mask, thresholds by using a single channel, and finally save thresholded values in an excel sheet. Then the next image gets loaded and the above whole process is repeated. This process happens until, all the images in the folder are processed

Sclerostin Queue

It is to process Sclerostin images to identify the total number of nuclei on the sclerostin image. Normal image processing needs user to follow many steps to get results, remember the steps to process and it is time consuming but, with the help of Sclerostin-

queue process you can overcome the above problems. It stops only when it really needs human entered values. This module reduces the manual work by loading each image from the images folder and process it until all images are processed.

This queue is to process Sclerostin images. This function loads the images folder, chooses the red channel, uses the existing mask, thresholds by using threshold value of non-immune images in single channel, calculates the minimum and maximum stain area, inter-cell distance, edge criterion, and circularity criterion, calculates the total number of stains, and finally saves the results in an Excel sheet. This process happens until, all the images in the folder are processed.

Fusion Index Area Queue

This queue is to process DAPI and Myotube images to get fusion Index value. i.e., ratio of total area of nuclei on the myotubes to the total area of nuclei on the whole image. Normal image processing needs user to follow many steps to get results, remember the steps to process and it is time consuming but, with the help of fusion index queue process you can overcome the above problems. It stops only when it really needs human entered values. This module reduces the manual work by loading each image from the images folder and process it until all images are processed.

Fusion index area queue is to process the DAPI + Myotube images. This function loads the images folder, chooses the blue channel, and chooses the threshold by using a single channel and get total are of nuclei on myotube by using fusion index area. The fusion index area repeats the similar procedure for nuclei area on myotube images by calling a green channel, choosing the entire image, removing myotubes having less than three nuclei,

thresholding by using a single channel and saving the thresholded image. Finally, it loads the blue image, chooses the blue channel, uses the myotube mask, chooses a single channel threshold and fusion index area, and saves the results in an Excel sheet. This process happens until, all the images in the folder are processed.

Fusion Index Bin Queue

This queue is to process DAPI and Myotube images to get fusion Index value by using binning procedure. i.e., ratio of total number of nuclei on the myotubes to the total number of nuclei on the whole image. Normal image processing needs user to follow many steps to get results, remember the steps to process and it is time consuming but, with the help of fusion index queue process you can overcome the above problems. It stops only when it really needs human entered values. This module reduces the manual work by loading each image from the images folder and process it until all images are processed.

This queue is to process DAPI/Myotube images. This function loads the images folder, chooses the blue channel, segments few smallest and biggest single and two fused nuclei which is helpful to get minimum, maximum and average size of the nuclei. To create myotube mask select the green channel and remove myotubes having less than three nuclei, thresholding by using a single channel, and saving the thresholded image. Finally, blue image is loaded, blue channel is chosen, existing mask is used, single channel threshold is chosen, fusion index with bin values are assigned and the results saved in an Excel sheet. The images are loaded only once, and the rest of the flow is same for all the images.

File

Fusion Index Indicator

The good feature of the immunostaining GUI software is that modules are independent of each other and can be reusable. There are few modules which should work slightly different for different images, so instead of rewriting whole module we have incorporated a few conditions on the exiting modules. Here Fusion index indicator notifies software that the current image is processed to get fusion index. It reduces the overwriting of the code.

[The function is used to indicate the GUI software that the user is working with the fusion index images and all functions should work in such a way that they should aid in fulfilling the fusion index requirements]

Upload an Image

The immunostaining GUI is developed to process images. In order to process the image, it must first be uploaded into GUI. When this function is executed, a box opens to select the image present in the system. When an image is loaded into GUI, its various details like width, height, color type and bits per sample are also displayed in GUI.

Reset All

On pressing the reset button, all variables, mat files, and images of the previous image are deleted. If the previous data is present, then there are some chances of affecting the current image like taking the previous image data for the current image. After removing, an

information dialog box appears with a message to the user that all previous data is removed.

Exit

Exit callback closes all the GUI windows and removes the variables and mat files. The GUI can also be deleted by clicking the close button, but the previous image mat files will not be removed. Therefore, the exit function has more advantages over the close button.

Segmentation

Selection of a Color Channel

Whenever an RBG image is loaded, it must be converted to binary image to perform a few MATLAB® operations. There are four channels in immunostaining GUI. They are original channel, blue channel, red channel, and green channel.

Original channel: Changes image from RGB to HSV image

Blue channel: It removes the blue color from the image and coverts into binary image

Red Channel: It removes the blue color from the image and coverts into binary image

Green Channel: It removes the blue color from the image and coverts into binary image

Start Segmentation

When the ‘start segmentation’ function is selected, it pops-up with a manual segmentation GUI. The user has many options to choose, out of which the fusion index requires only few options like push entire image, choose entire image, use the existing mask, select multiple regions, save the segmented image and exit.

Choose Entire Image

This copies the entire image into a region where an image is to be segmented. The users can select the entire image for segmentation by using the 'choose entire image' function. When a user clicks this button, the entire image is copied into a region where an image needs to be segmented.

Manual Segmentation

ROI of an image is selected by drawing with free hand.

Inner Segmentation

Manually removing a part of an image by drawing with free hand

Use Existing Mask

Used to mask an image with another image, so that, the masked area gets selected as the segmented image. For example, the original blue image is masked with myotube shape after the segmentation and thresholding.

Select Multiple Regions

With the help of the 'select multiple regions' function, an image can be segmented multiple number of times at different places of the image. When this function is selected, a dialog box pops-up to select multiple regions of an image. After the user is done with a left click and a drag, another dialog box with the options of accept, more and undo pops-up.

ACCEPT: If the user has finished by selecting multiple segments, then this button must be clicked to proceed further.

MORE: If the user wants to do more segmentation, then this option takes him to do

one more round of segmentation.

UNDO: If the user has selected different regions from the thought area, then this option helps him/her to correct his/her mistake.

The dialog box pops-up after every round of segmentation.

Create the Segmented Image Mask

'Create the segmented image mask' is used if a user wants to create a mask of the segmented image. It is used when a user is working with myotube, because this mask is used for choosing a region from the blue image.

Save the Segmented Image

The user can use the segmented image in a further process, only when 'save the segmented image' function button is clicked. Whatever the image being segmented, it must be saved.

Exit

Users can come out of manual segmentation of GUIs by using the 'exit' function. This is the final button used in the manual segmentation.

Thresholding

The users can remove the unwanted noise and background from the image and highlight only the required stains. The thresholding function is an additional option found in the menu bar, which is used to adjust the intensity of the stains. The low and high values can be manually entered in the box or can be done by dragging the bar.

Single Channel

The low and high intensity values are set to threshold the image.

Double Channel

The image is thresholded with hue saturation and threshold values.

Tools

Settings

Minimum and Maximum Pixel Areas

The minimum and maximum pixel areas are used to calculate the maximum, minimum and average of the thresholded image. When a function is selected by a user, a pop-up box appears with two buttons, single and double. The single button is used to calculate the average, minimum and maximum size of a single nuclei. The double button is to calculate the average, minimum and maximum size of a single nuclei. A single button needs to be selected when a single nucleus is segmented, and a double button needs to be selected when a two fused nucleus is segmented. There are two ways of using the maximum and minimum pixel areas. For images like β-Gal, DAPI, and Sclerostin, it gives information about the minimum and maximum areas of stains on the image and asks to manually enter in the dialog box for the removal of stains, less than the minimum value and more than the maximum value.

Whereas, it gives information about the minimum, maximum and average of stains for both single stains and two fused stains for the fusion index images. If the stain area for

this image is less than 50 pixels, then that area is not considered as the minimum.

Inter-Cell Distance

The function is called to get distance between two selected points. A line is drawn between these two points to represent the distance. Sometimes, the single stain may be divided into two or more. In such a case, the broken stain should be considered as one and the function of 'inter-cell distance' comes into use. The distance between the broken stains is considered and that value is entered in a dialog box to make the software to consider the broken stains as one.

Edge Criterion

Edge criterion is to remove the stains on the edges of the manually segmented region. There are two types of manual segmentation, namely, outer manual segmentation and inner manual segmentation. The stains found on the edges of both segmented regions are removed. This function is required when the segmentation line touches stains, which should not be considered.

Circularity Criterion

Circularity criterion is added to remove the nuclei whose eccentricity value is less than .975ab. When the circularity criterion button is pressed, the stains with an eccentricity value of less than 0.975 are removed from the image. These stains are not considered as noise because they are the nuclei or cells that do not have an eccentricity value of less than 0.975.

Cell Counts

The main aim of immunostaining GUI is to get the total number of stains on the image. Users can get a count of stains not only on the entire image, but also on free-hand regions of interest (ROI) and the selected box.

Entire Area

The function of 'entire area' is mostly used to count the total number of stains on the entire image.

Draw a Free-Hand ROI

Users can select their ROI by drawing with a free-hand.

Draw a Box

The ROI can be drawn in the form of a box.

Manual Counting

Counting an image with more than 500 stains is difficult without a marker because there might be a chance of counting the same stains a multiple number of times or a few of the nuclei may be skipped. The counting should be done with concentration because an interruption may result in a recount. However, it becomes easy with the help of a marker. Manual counting also works in the same way as a marker and an image. Once the user selects this function, a pop-up box appears with an instruction 'left click to select pixel, double click or right click when completed'. Thus, users can select the stain by clicking the left button after the marking is done and indicate that the marking is done by clicking on the right button. The backspace button is to undo the marks.

Add and Remove

Sometimes, the software does not recognize the stains that need to be considered and recognizes certain stains as noise. The 'add and remove' function is used for such stains.

Fusion Index Bin

Fusion index bin is the core function of the GUI. This function gives information like the minimum, maximum and average area of single and double nuclei and asks us to enter the bin range of the single and double nuclei with the information provided. Users must manually enter the bin range. The difference of the maximum single and double nuclei is added to the maximum double nuclei to get a triple fused nuclei maximum range and so on. The image is then displayed with the total number of nuclei count. Sometimes, a few nuclei may get skipped or may be counted more. Hence, a new method was incorporated to remove and add, respectively. Once the user is done with that, it gives a count of the nuclei and the fraction of the myotube over the blue nuclei.

Fusion Index Area

Since the area of individual nuclei is uniform, the total DAPI stained area generates a mask for what is a myotube and figures out what percentage of the DAPI-stained area is located within the myotube mask. This would still give an accurate measure of the fusion index (% nuclei located within myofibrils) without us having to know how many individual nuclei are present.

Quantitative Output

The total area of myotubes (i.e., the sum of areas of all individual myotubes within an image), the average myotube area (i.e., the average area of individual myotubes), and the average number of nuclei per myotube constitute the quantitative outputs. In order to get these outputs, it is required that each myotube is traced as an individual object and an individual mask is made for each myotube.

Results

Co-Localization

Co-localization is used to count the overlapped cells on two images (or three images). It joins images and count the overlapping cells.

Two Images

The two images are given here as the input to get the overlapped stains.

Three Images

The three images are given here as the input to get the overlapped stains.

Save to Excel

If the excel sheet is not created, then the program creates it and saves the results, such as date and time analysis (when it is copied into an excel sheet), single channel lower threshold level, single channel higher threshold level, single minimum pixel area, single maximum pixel area, double minimum pixel area, double maximum pixel area, number of nuclei in the manual count, total number of nuclei (blue), total number of nuclei (myotube

blue), fusion index fraction, comment, sum of areas, area 1, area 2,…area 20, etc.

Help

About

About is the information about the persons involved in the development of the immunostaining of GUI.

Content

It is not always easy to go to the document present in the folders to refer to the flow while working on GUI. Most of the time, users just copy the software into any device and start working in that situation if they want to refer the flow available in the content. It has all the flow charts. Whenever users need the flow, they can refer to this flow chart and understand how to perform the task. Flowchart is a pictographic representation of workflow. It is a step-by-step process and each step is represented with a box. The boxes relate to arrows that represent the flow process. These flow charts give information to users about how the flow goes. The step name itself denotes what each step does. The flowcharts used in the immunostaining of GUI are a bit different from the original flowcharts. There are many types of boxes, with each one of them having its own meaning.

Parallelogram box denotes processing

Rectangular box denotes name for a group of similar functions Rhombus box denotes that a decision is necessary

Flow lines denote the control that passes from and to Cloud box denotes the information to user

There is more than one flowchart for a process, such as fusion index bin and fusion index area. These flowcharts are just to make users understand the flow properly. There are many types of images with each having their own different flow. It is difficult for the user to remember all the flows accurately and if the user is new to immunostaining, then it would be difficult to know from where to start and end for obtaining the results. The flowcharts can be used in such situations.

We have created flowcharts for five types of images, i.e., β-Gal, DAPI, Sclerostin, non-immune by normal process and non-immune by queueing images and calling functions. Fusion index images have four ways to process, namely, fusion index bin (normal and queue) and fusion index area (normal and queue).

FAQ

1. Which image among DAPI, Sclerostin, and β-Gal is better to process first?

Answer: If you want to use the image mask of another image, it is better to work first on β-Gal image, because this image has clear borders enabling proper segmentation. Its mask can be used for DAPI and Sclerostin.

The borders of DAPI and Sclerostin images cannot be figured out properly when the channel is selected.

2. What is the suggested saturation value for β-Gal images?

Answer: The low saturation value for β-Gal images is 0.2 and the high saturation value is the default value.

3. What is the suggested threshold value for β-Gal images?

Answer: The default value is the low threshold value and 0.65 is the high threshold value.

4. What should be done, if the code is not working?

Answer: Click on the reset button and start again. It will reset all the values to empty.

5. When the code is run, should the folder be changed, or should the path be added?

Answer: It is better to change the folder because all results are saved in the folder that contains the code. Otherwise, it can be saved in the documents folder.

β-Gal Normal Flowchart

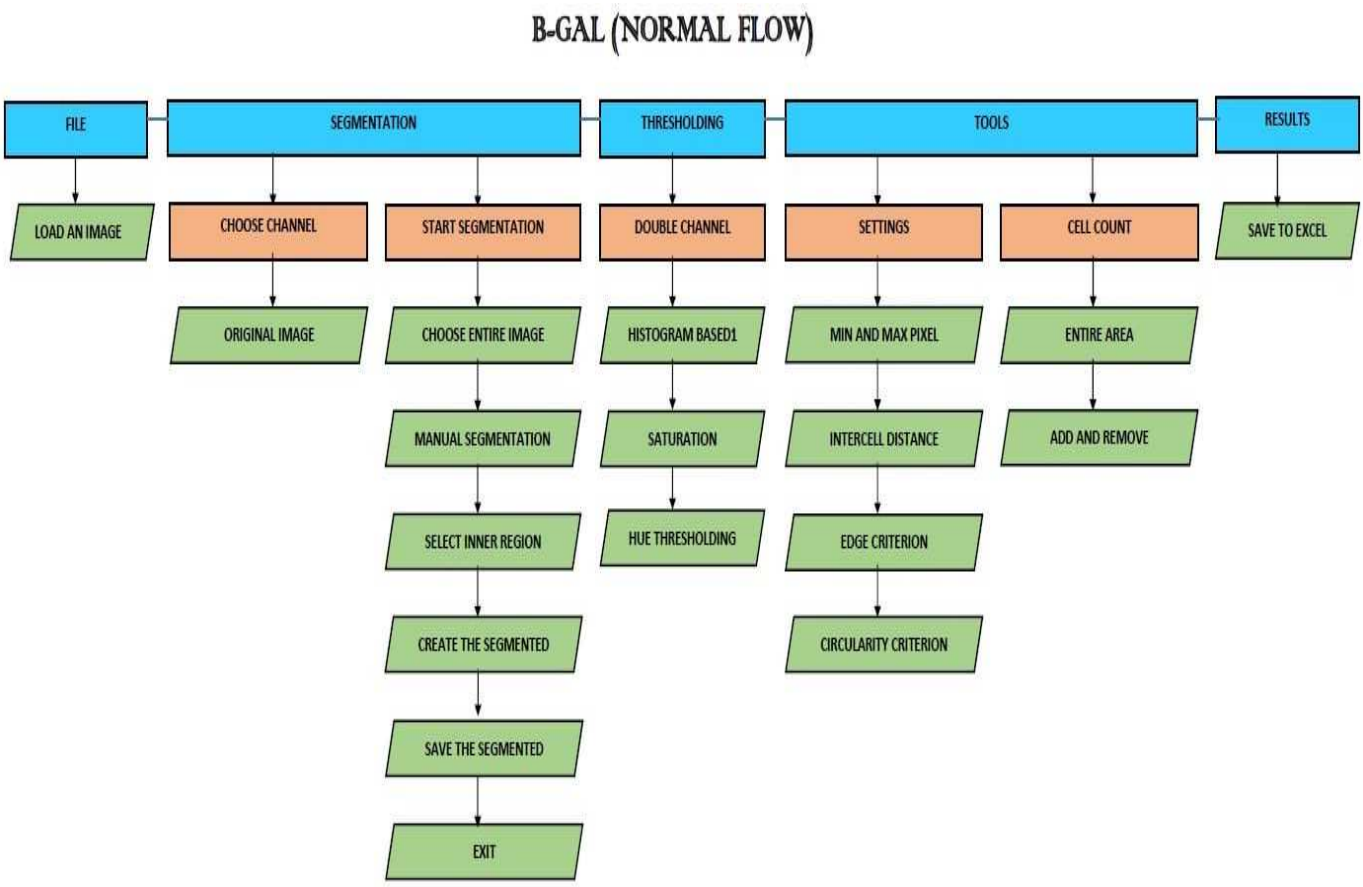


Figure 3.1. β-Gal Normal Flowchart

β-*Gal Queue* Flowchart

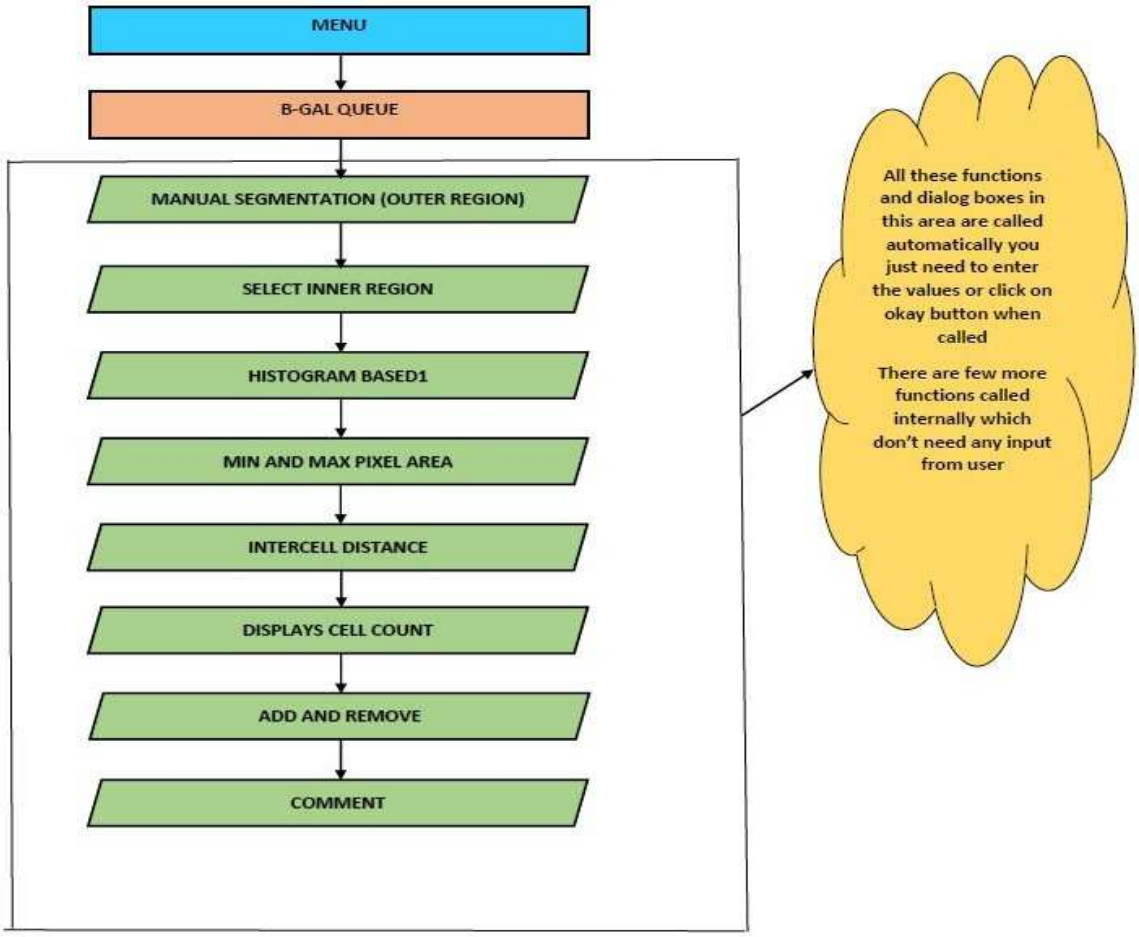


Figure 3.2. β-Gal Queue Flowchart

DAPI Normal Flowchart

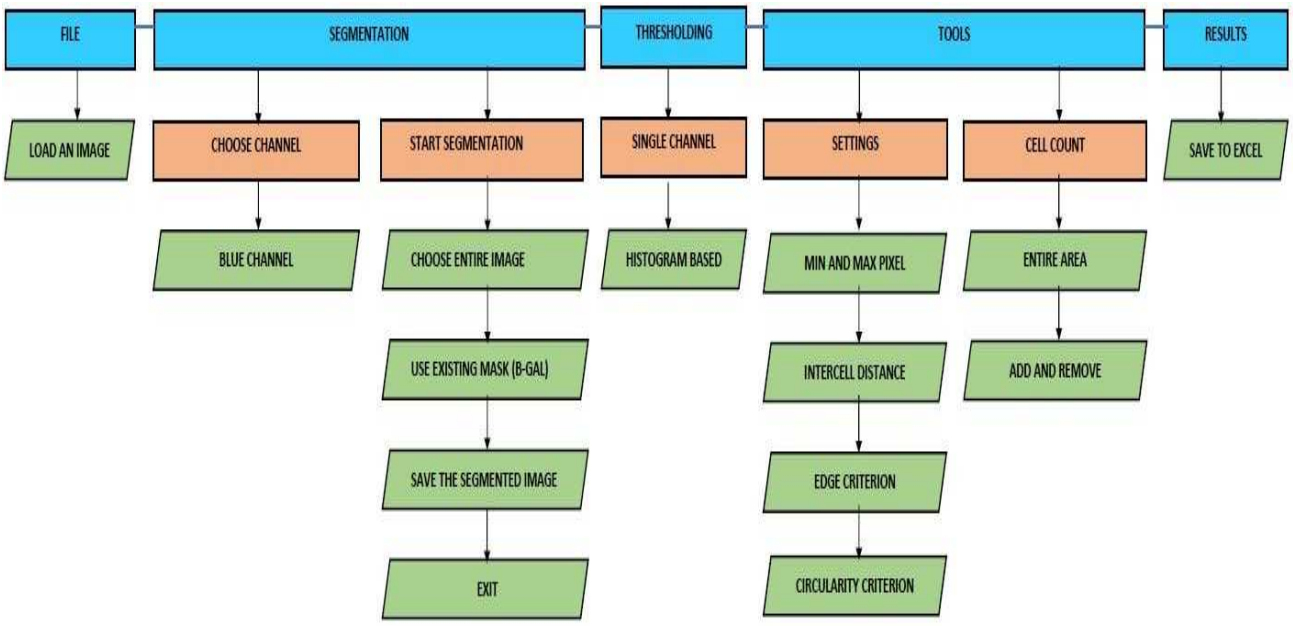


Figure 3.3. DAPI Normal Flowchart

DAPI Queue Flowchart

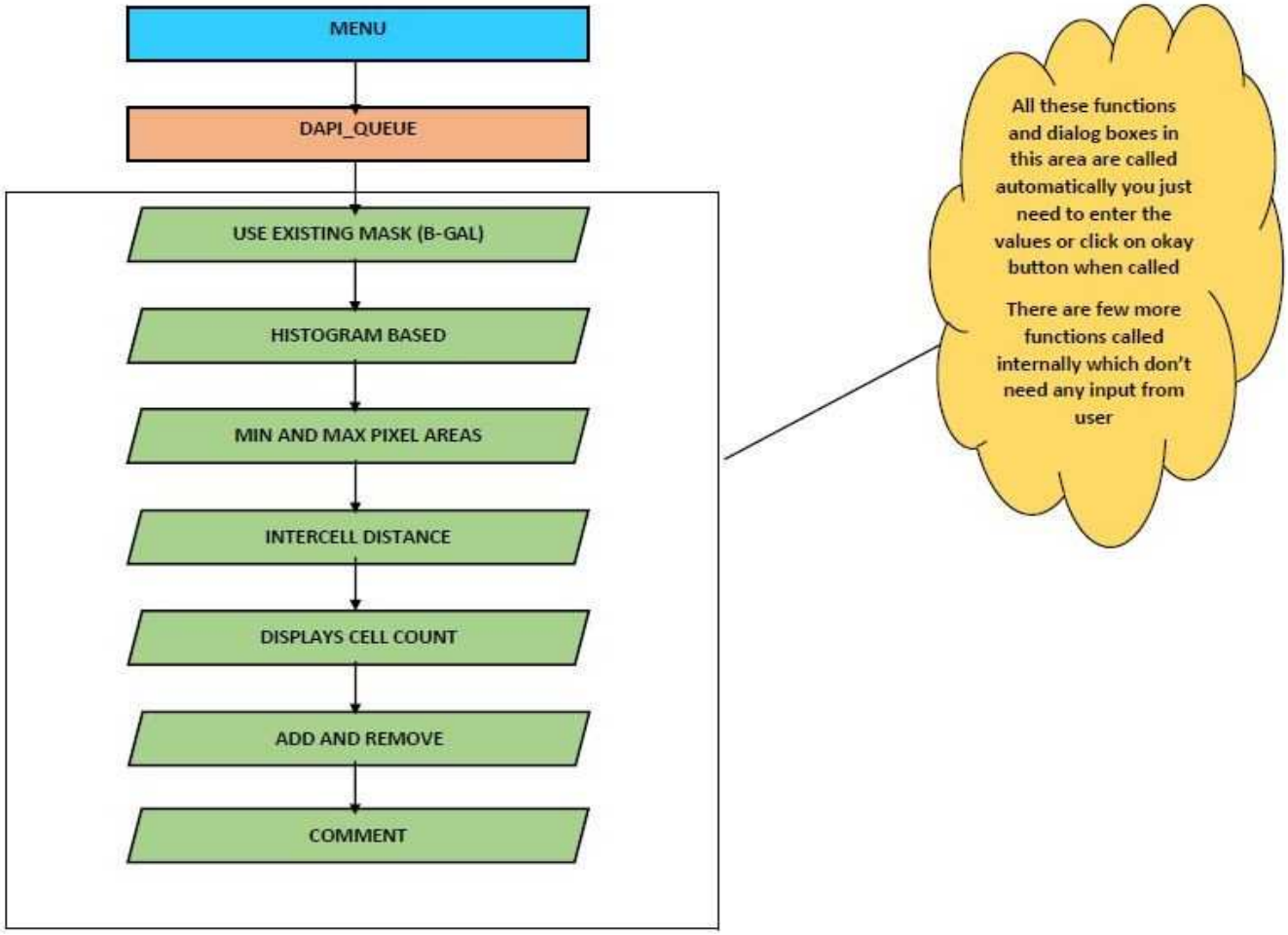


Figure 3.4. DAPI Queue Flowchart

***Non-Immune Normal* Flowchart**

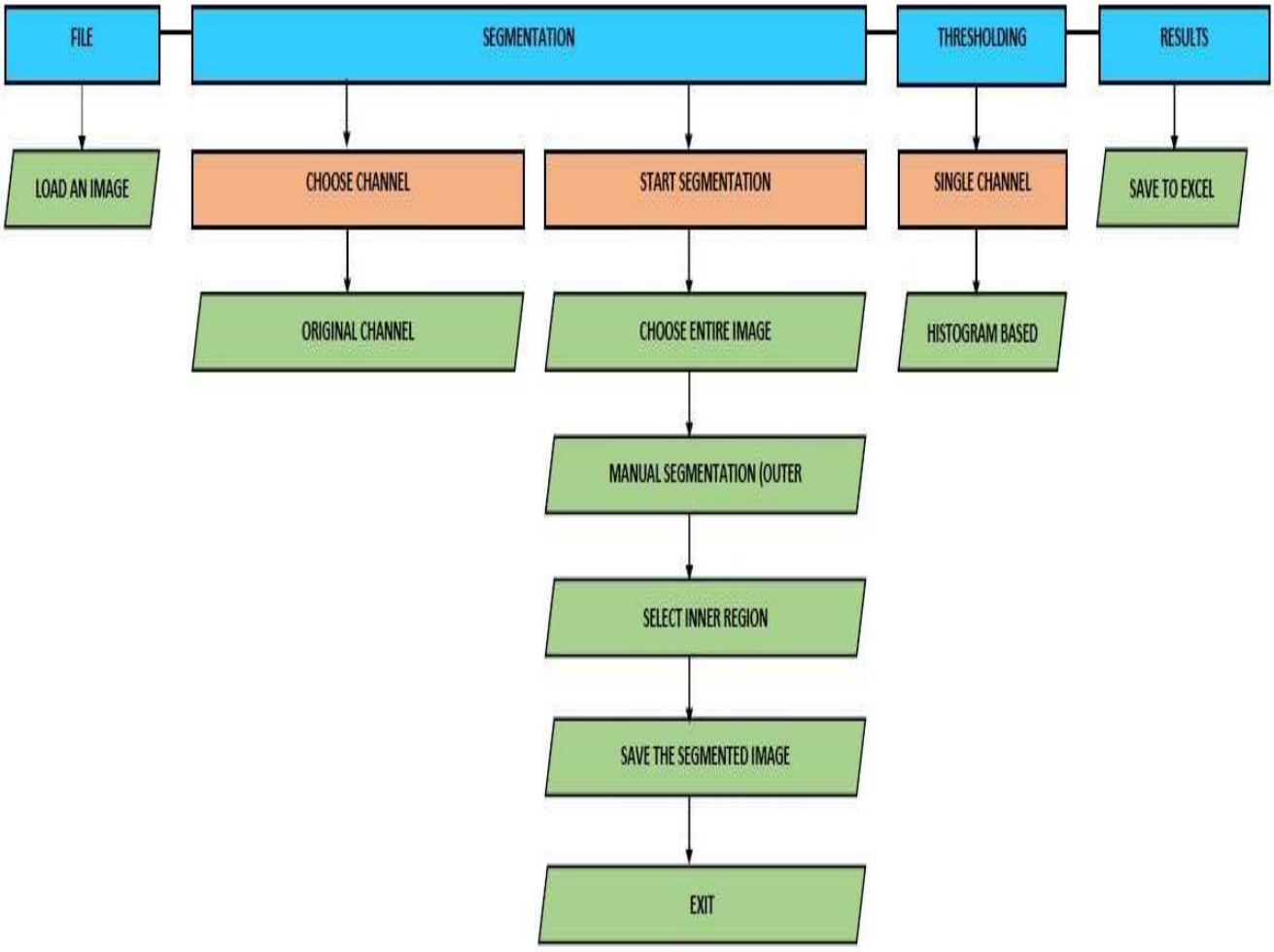


Figure 3.5. Non-Immune Normal Flowchart

***Non-Immune Queue* Flowchart**

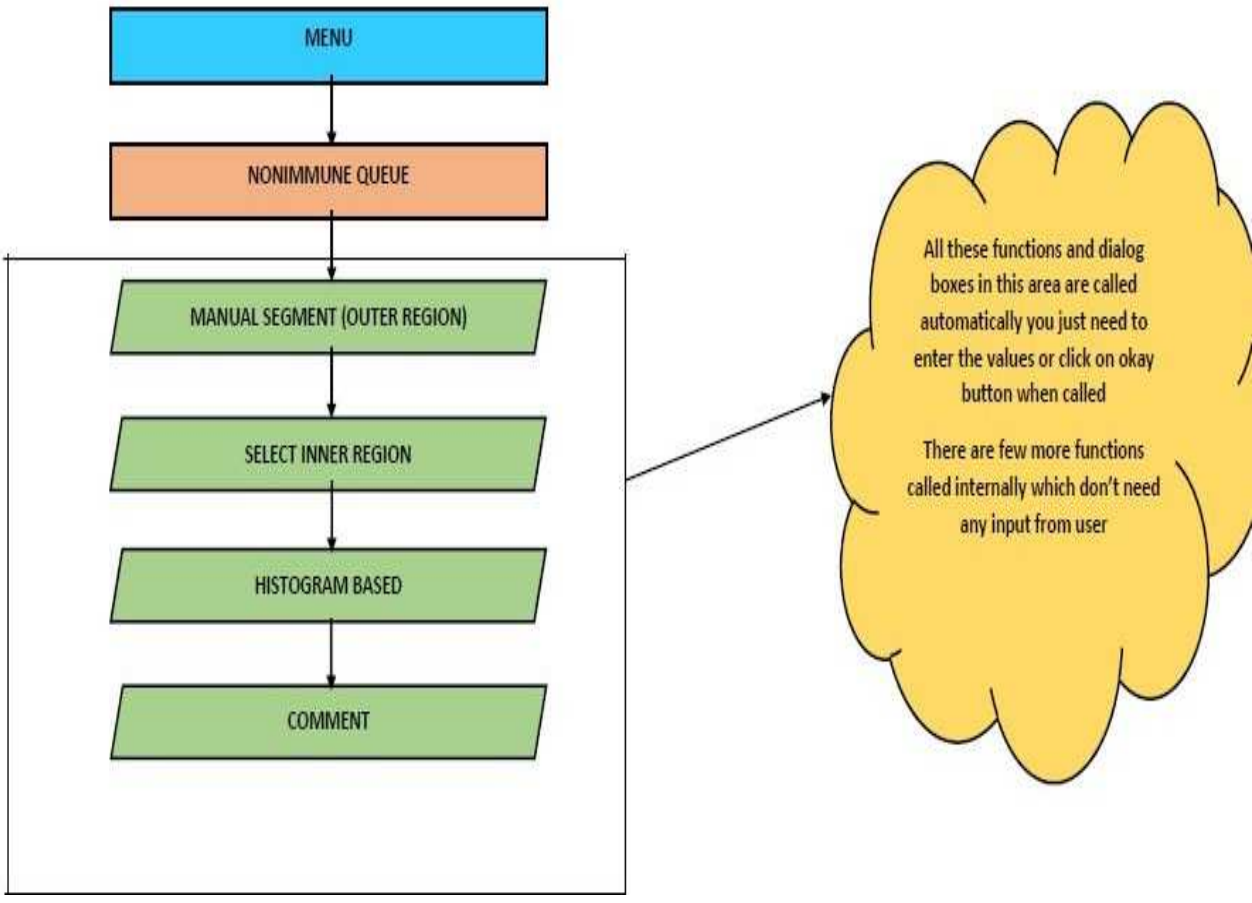


Figure 3.6. Non-Immune Queue Flowchart

Sclerostin Normal Flowchart

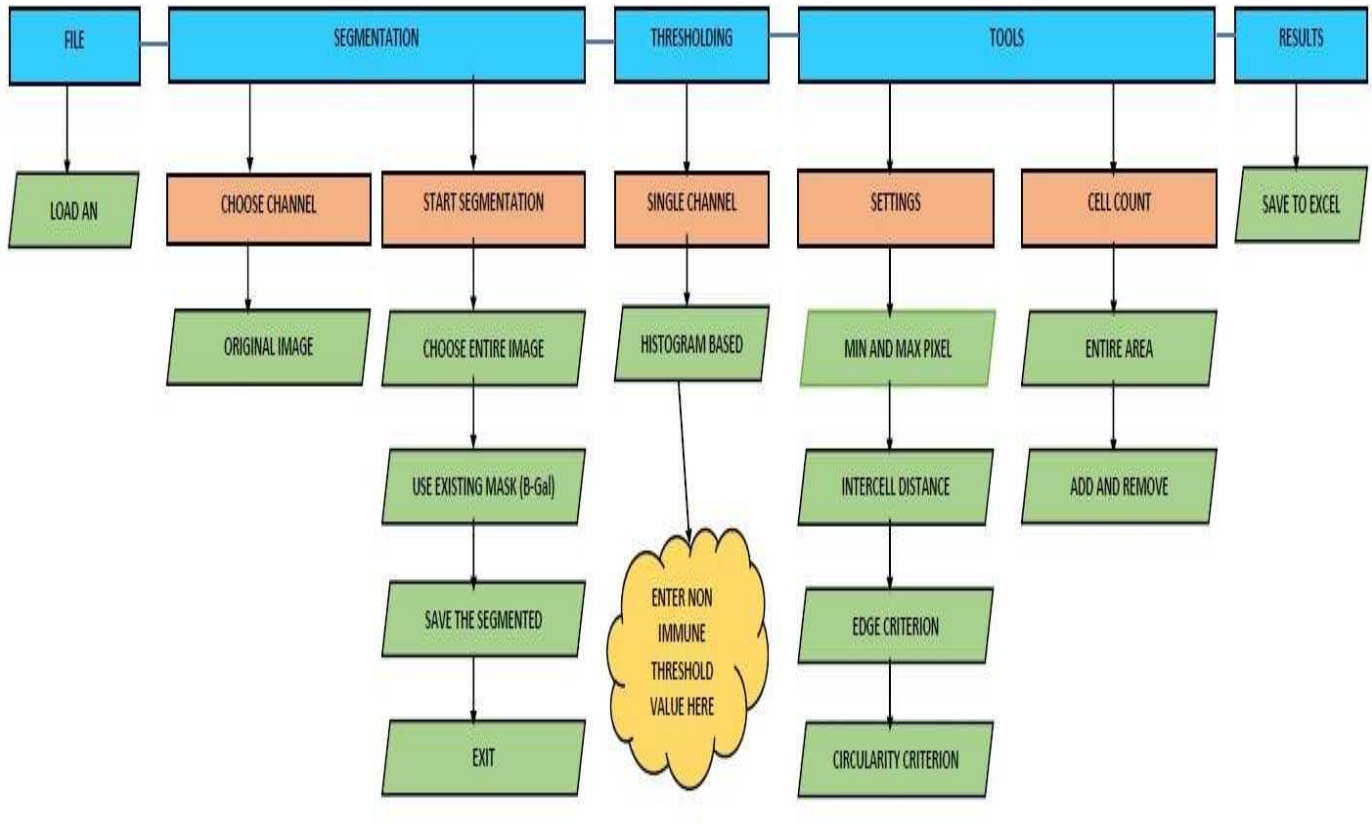


Figure 3.7. Sclerostin Normal Flowchart

Sclerostin Queue Flowchart

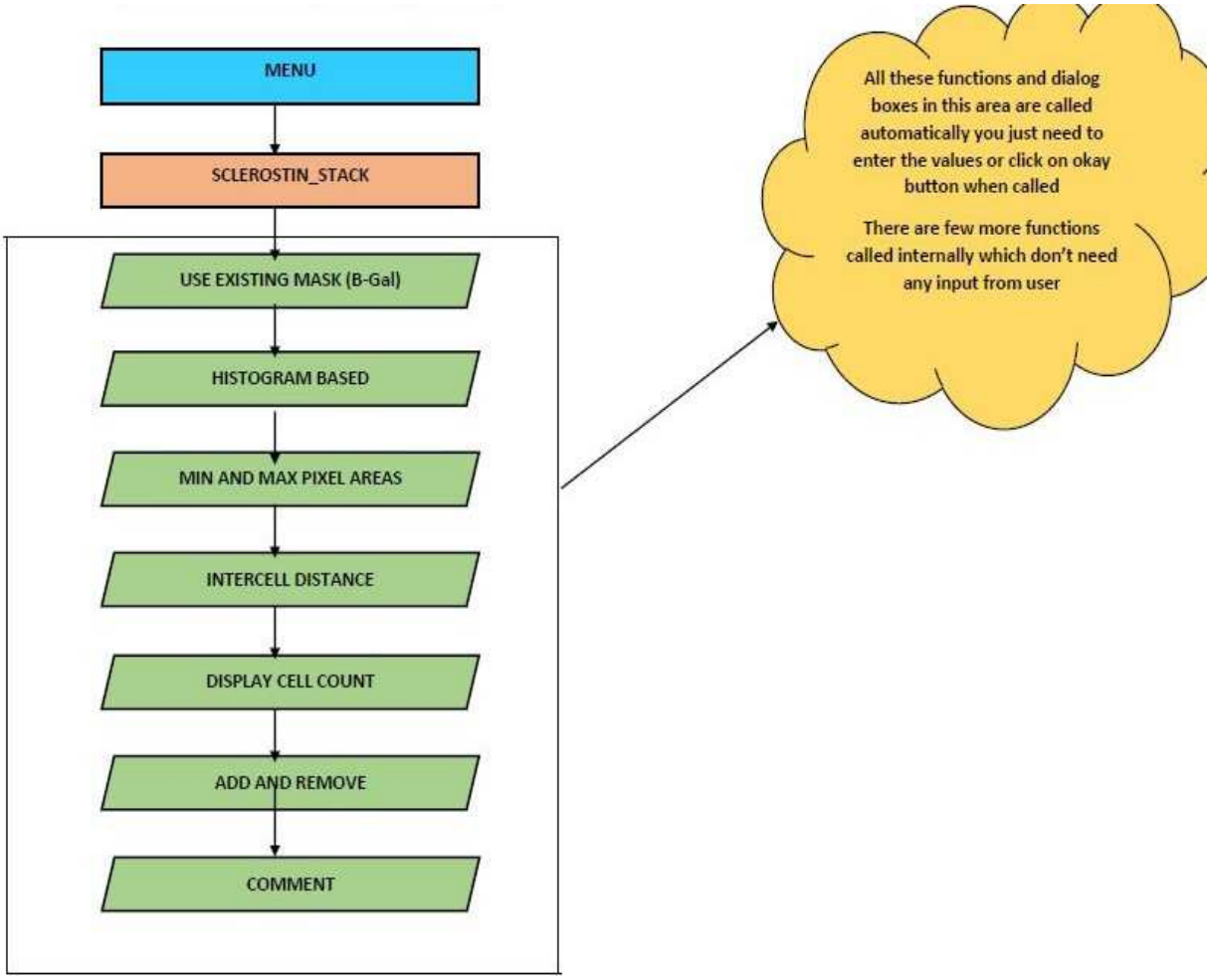


Figure 3.8. Sclerostin Queue Flowchart

Fusion Index Bin Normal Flowchart

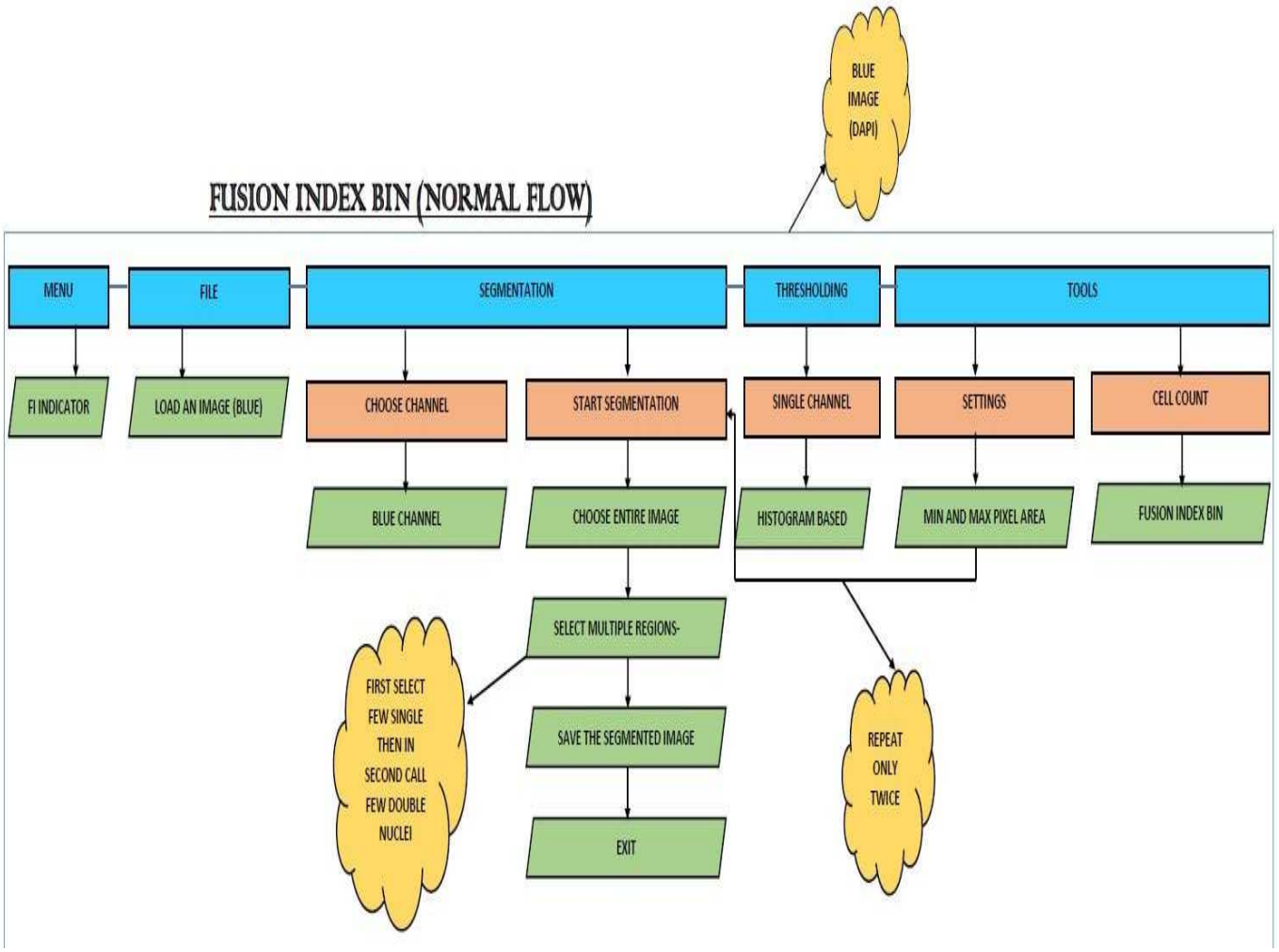


Figure 3.9. Fusion Index Bin Normal Flowchart – Blue Image

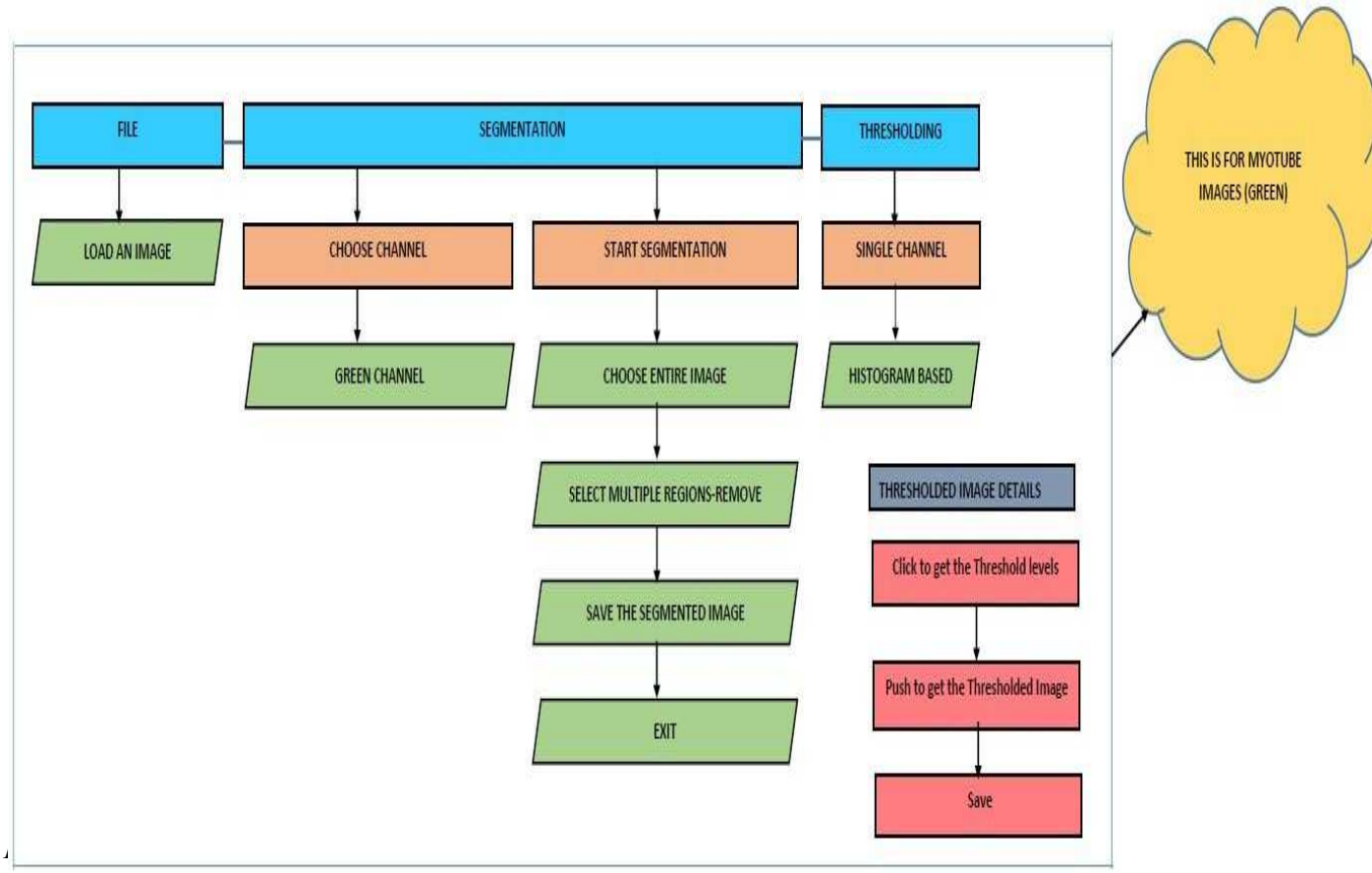


Figure 3.10. Fusion Index Bin Normal Flowchart – Green Image

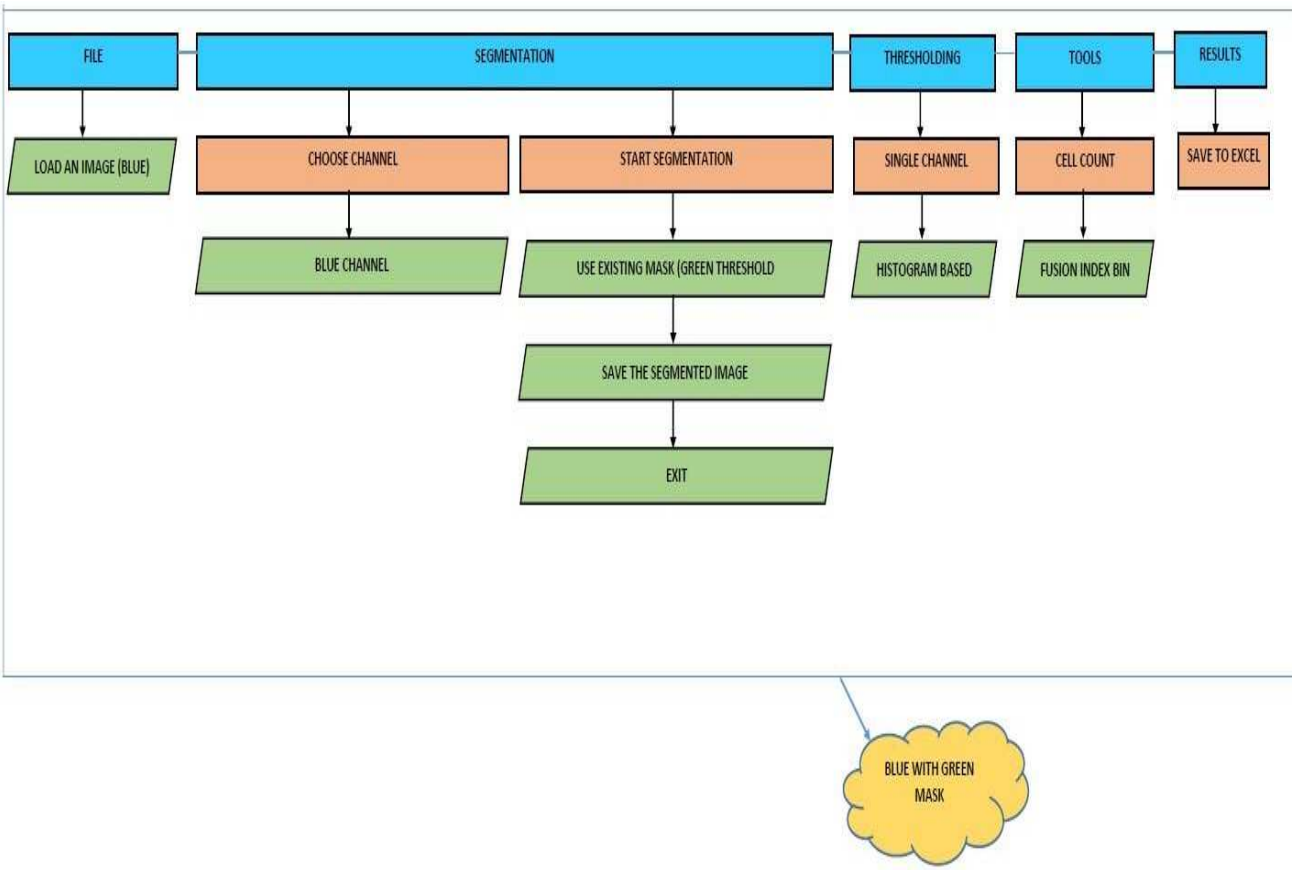


Figure 3.11. Fusion Index Bin Normal Flowchart – Blue with Green Image

Fusion Index Bin Queue Flowchart

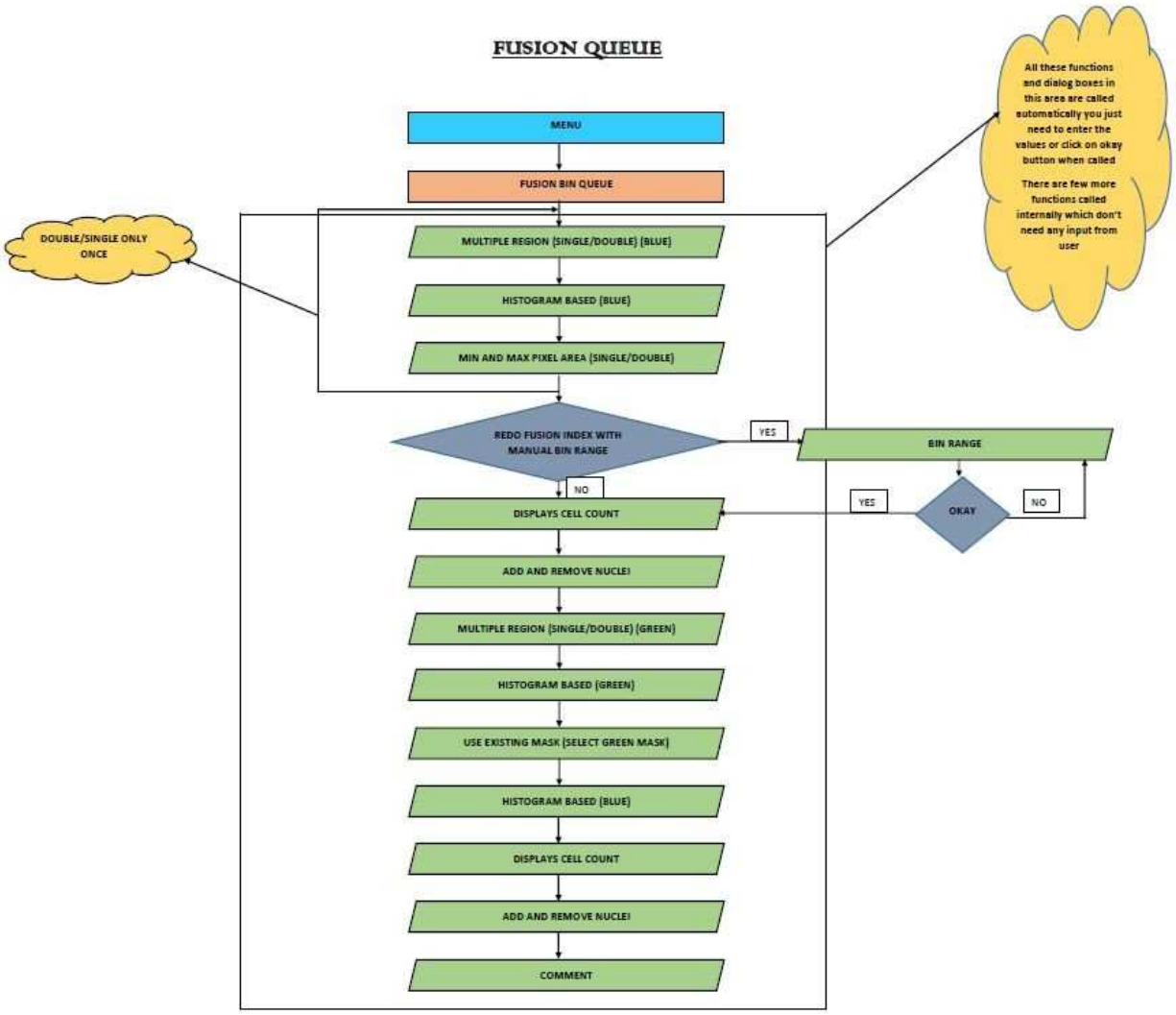


Figure 3.12. Fusion Index Bin Queue Flowchart

Fusion Index Area Normal Flowchart

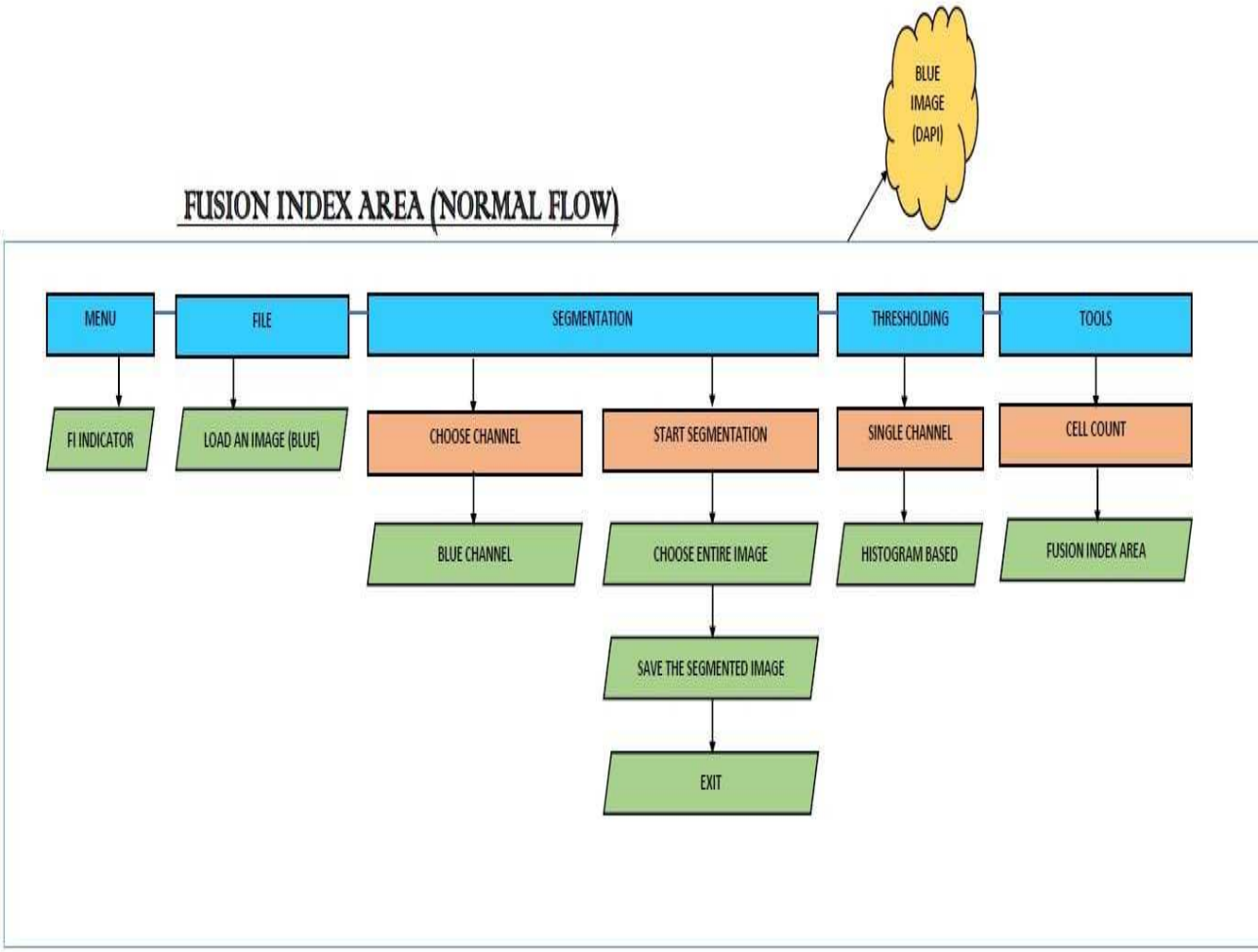


Figure 3.13. Fusion Index Area Normal Flowchart – Blue Image

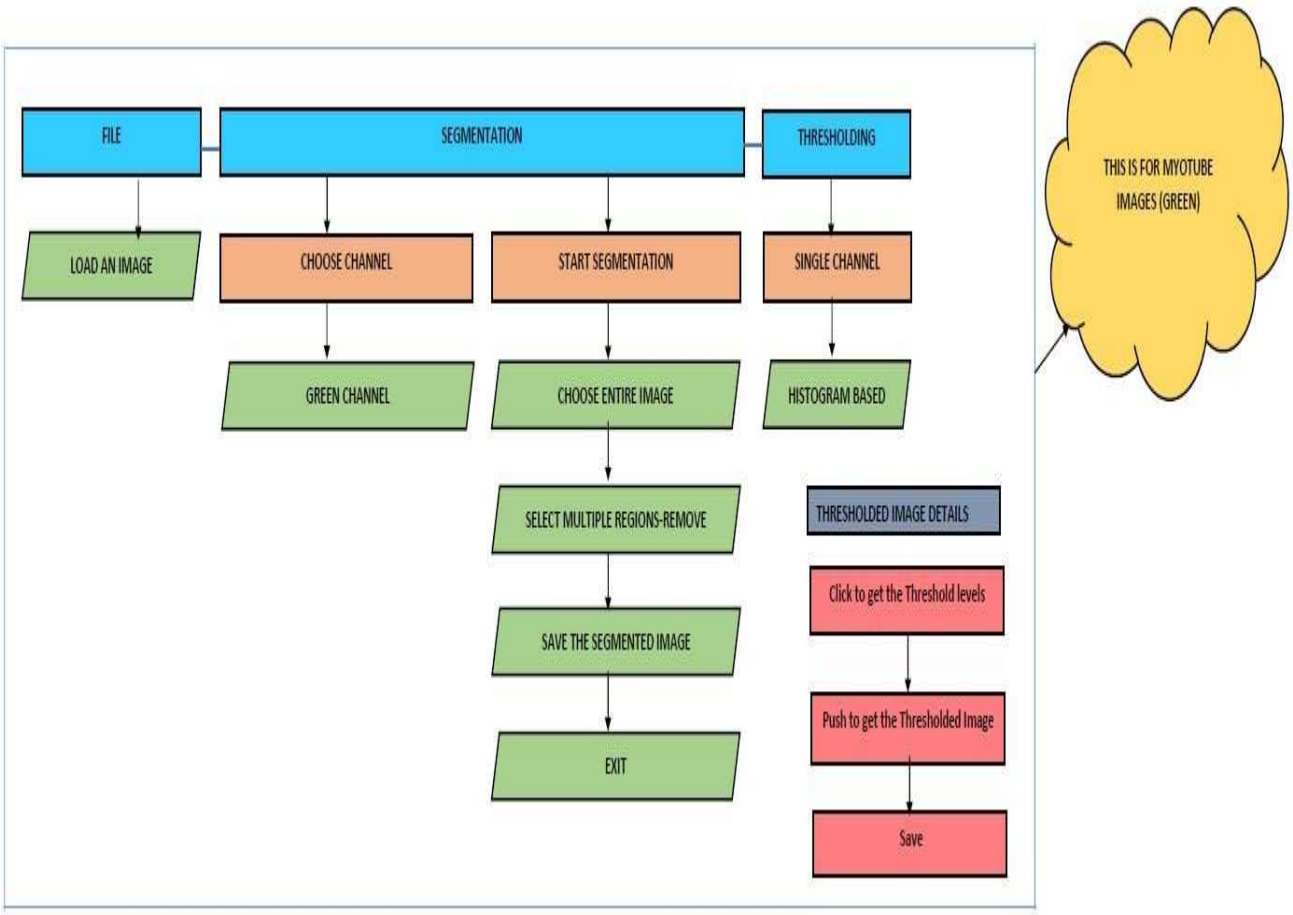


Figure 3.14. Fusion Index Area Normal Flowchart – Green Image

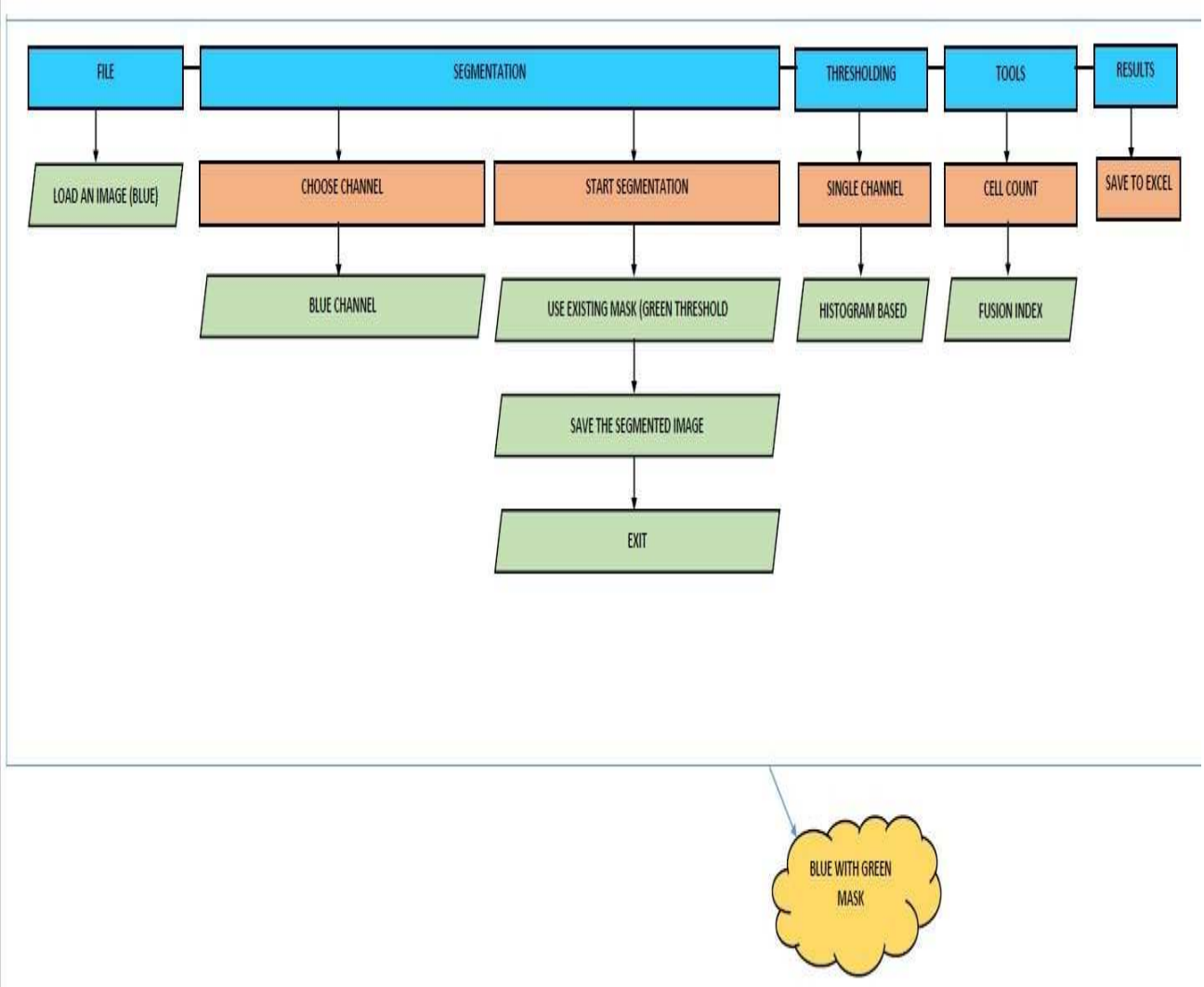


Figure 3.15. Fusion Index Area Normal Flowchart – Blue with Green

Fusion Index Area Queue Flowchart

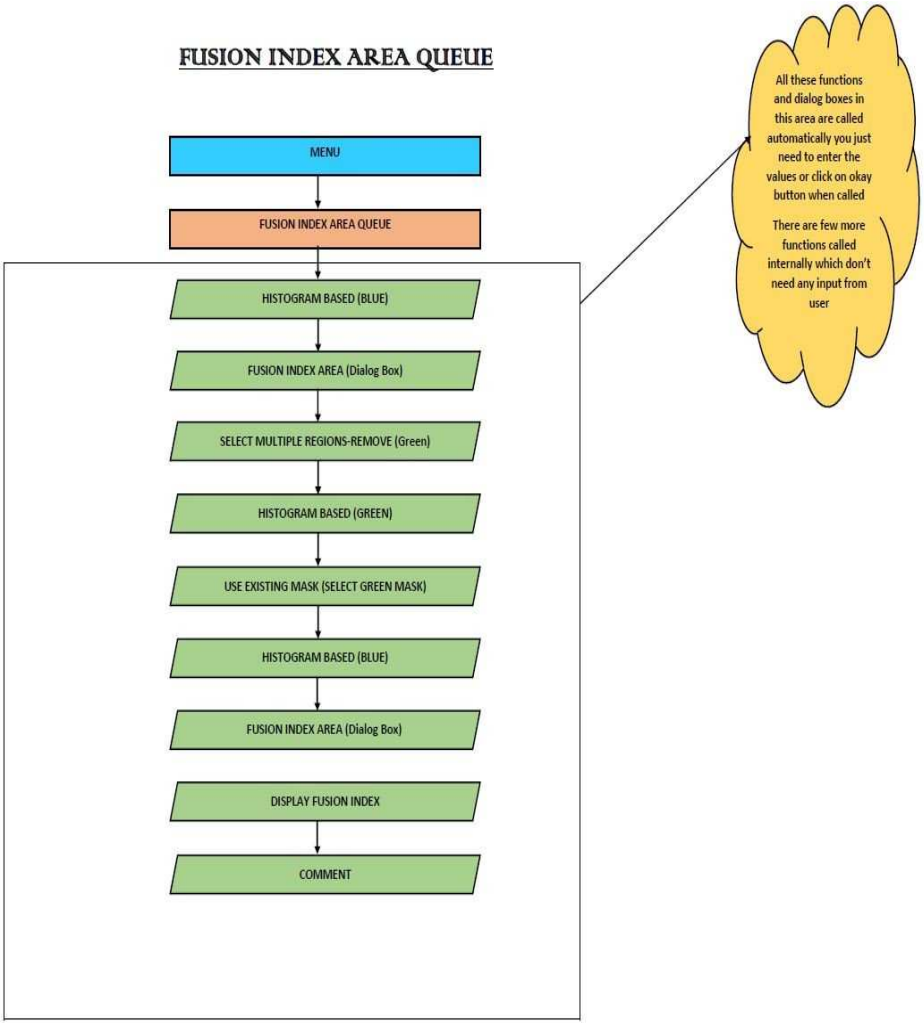


Figure 3.16. Fusion Index Area Queue Flowchart

Manual Counting β-Gal with Segmentation Flowchart

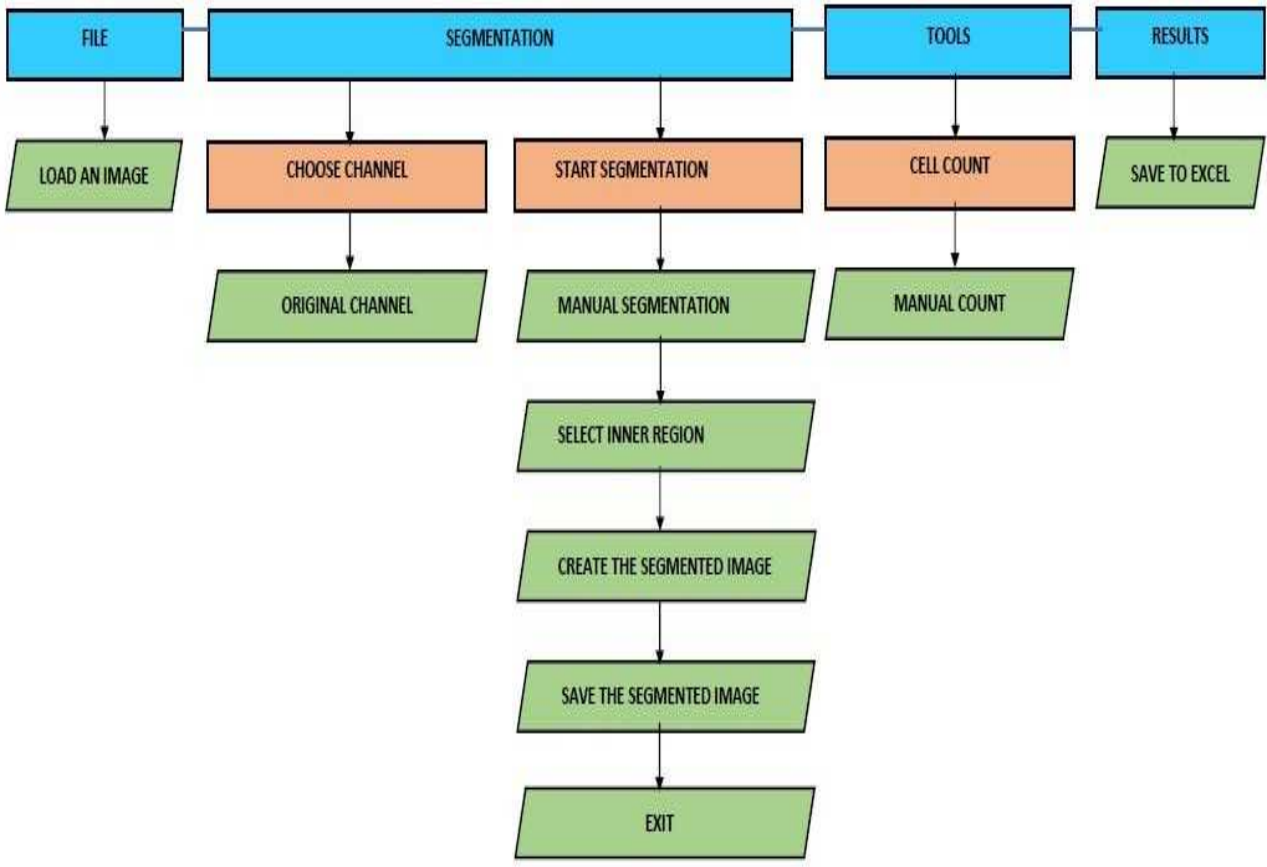


Figure 3.17. Manual Counting β-Gal with Segmentation Flowchart

Manual Counting DAPI/Sclerostin with Segmentation Flowchart

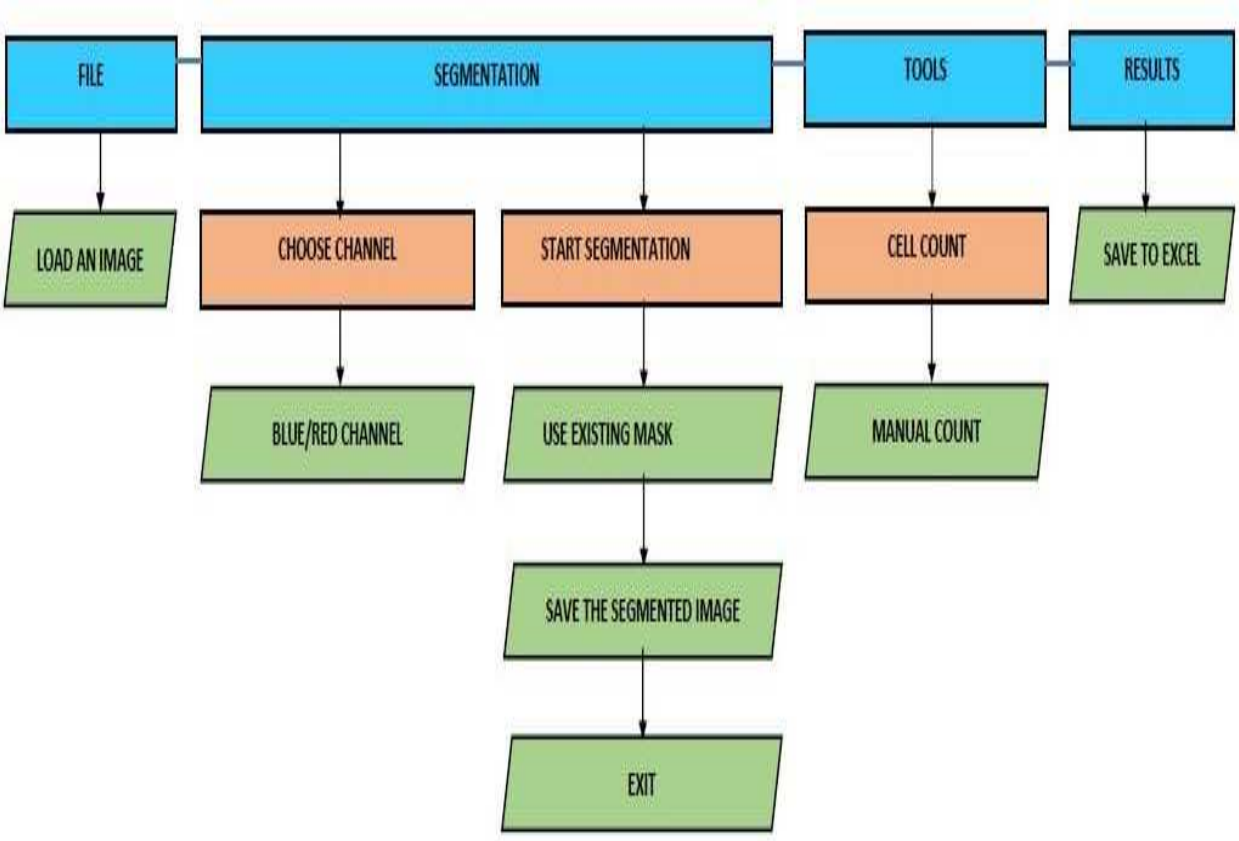


Figure 3.18. Manual Counting DAPI/Sclerostin with Segmentation Flowchart

Manual Counting β-Gal/DAPI/Sclerostin/Myotube without Segmentation Flowchar

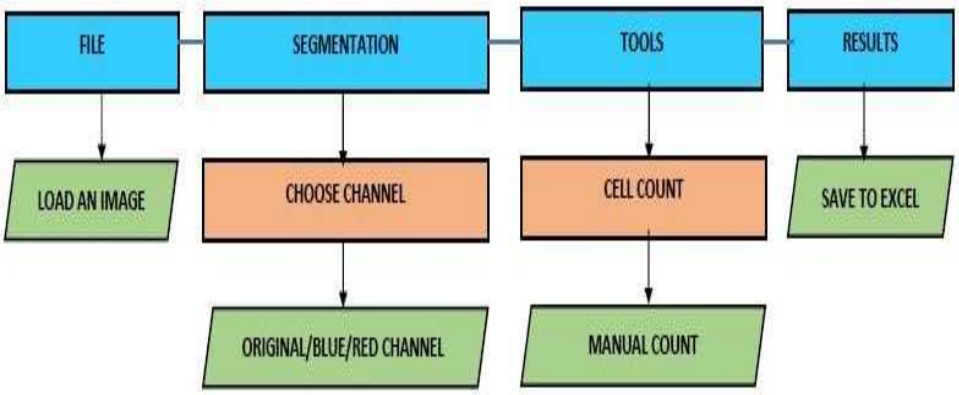


Figure 3.19. Manual Counting β-Gal/DAPI/Sclerostin/Myotube without Segmentation Flowchart

CHAPTER 4

PROCEDURE WITH IMAGES

β-Gal Procedure

Step 1: Load Image File → Load an image

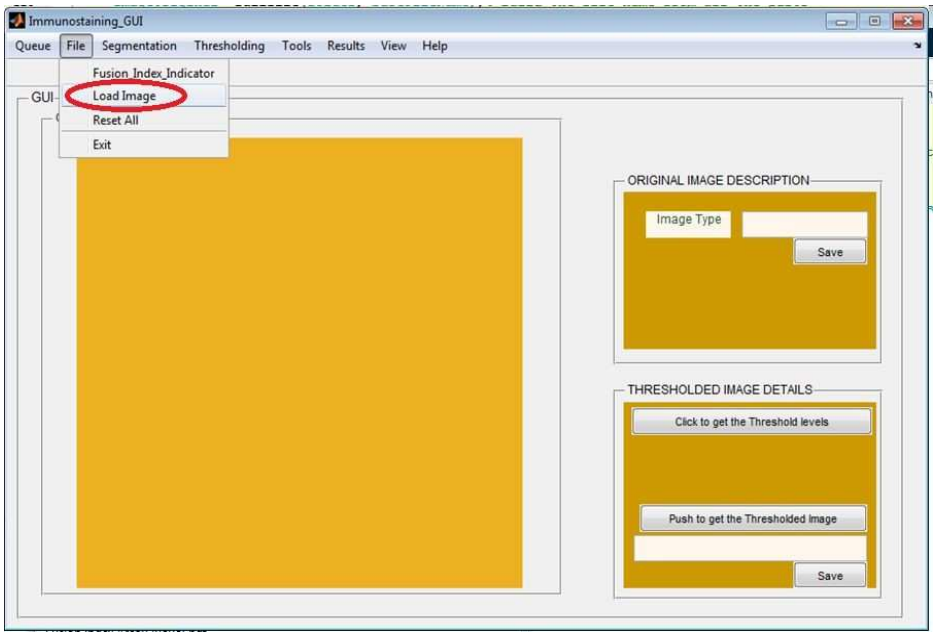


Figure 4.1. β-Gal Procedure - Manual Load an image

Step 2: Choose Channel

Segmentation → Choose Channel → Original Image

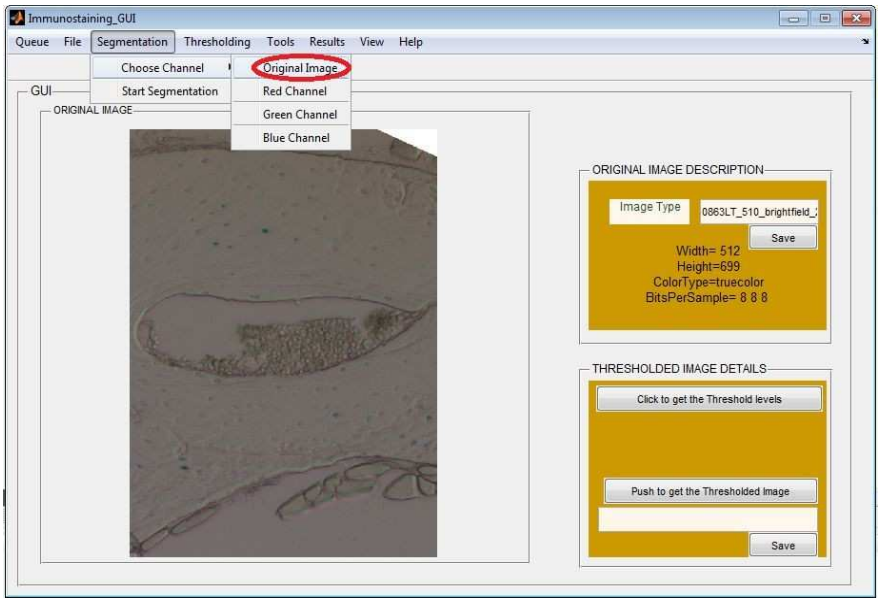


Figure 4.2. β-Gal Procedure - Choose Channel

Step 3: Segmentation

Segmentation → Start segmentation → Manual Segmentation → Select Inner Region →

Create the segmented Image mask → Save the segmented image → exit

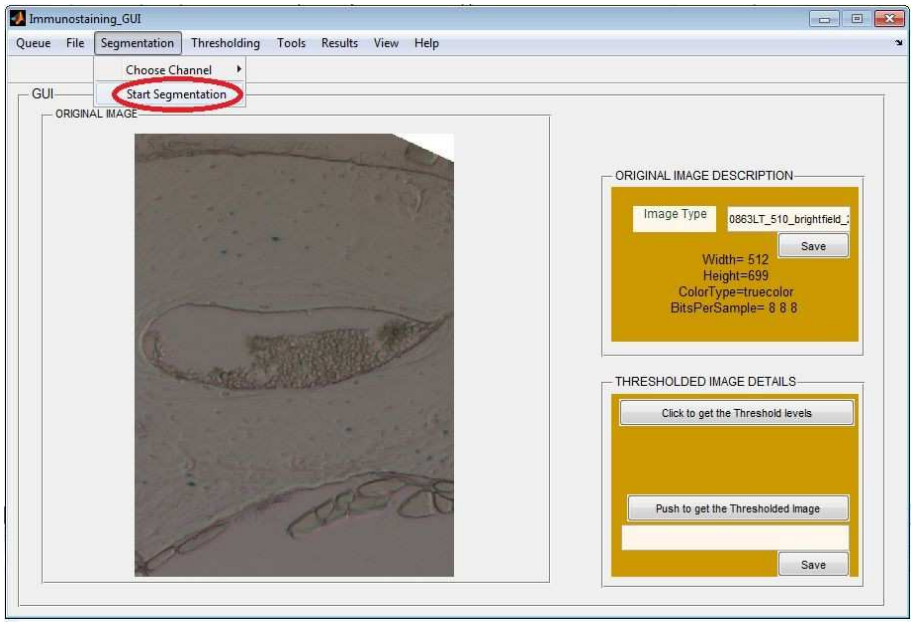


Figure 4.3. β-Gal Procedure – Start Segmentation

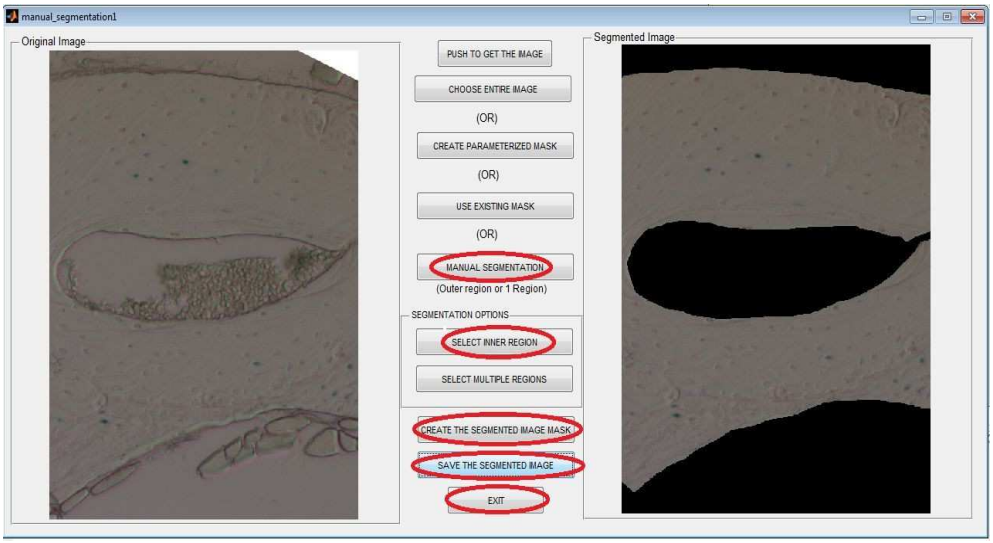


Figure 4.4. β-Gal Procedure – Segment Inner Outer region and create mask Step

Step 4: Threshold

Thresholding → Double Channel → Histogram Based1

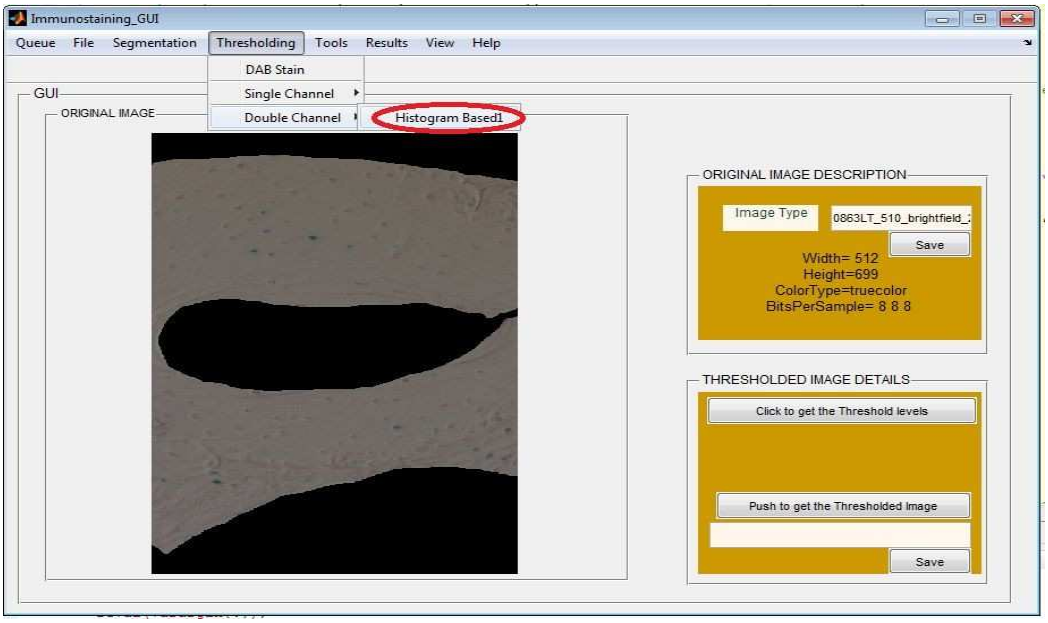


Figure 4.5. β-Gal Procedure – Select Double channel histogram

Step 4.1: Set Saturation Value

Change low saturation value around 0.15 to 2.5 based on image quality

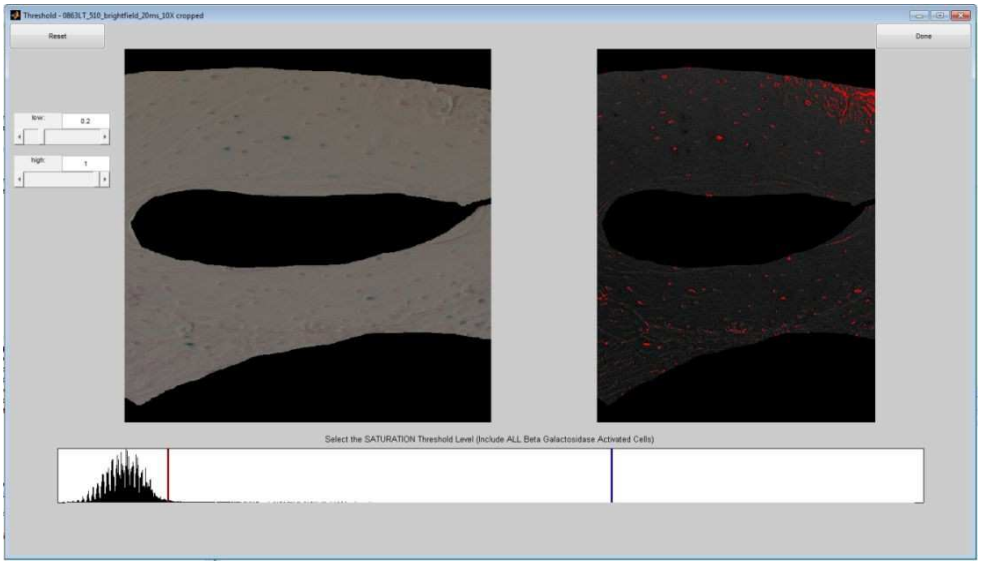


Figure 4.6. β-Gal Procedure – Set saturation value

Step 4.2: Threshold High value

Change threshold high value to .45 to .65 to highlight nuclei

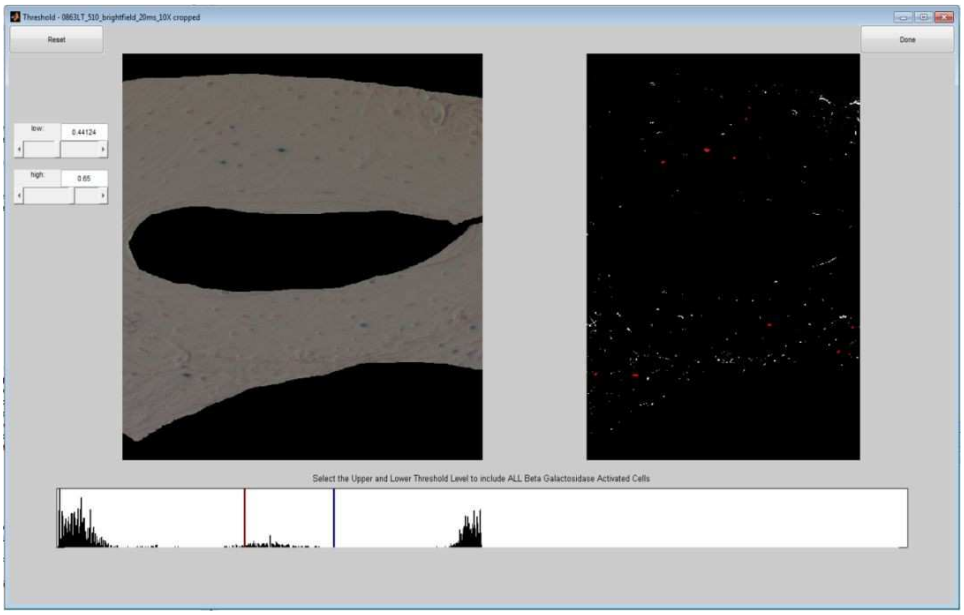


Figure 4.7. β-Gal Procedure – Set threshold value

Step 5: Save the Thresholded Image details

Click to get the Threshold levels → Push to get the thresholded Image → Save

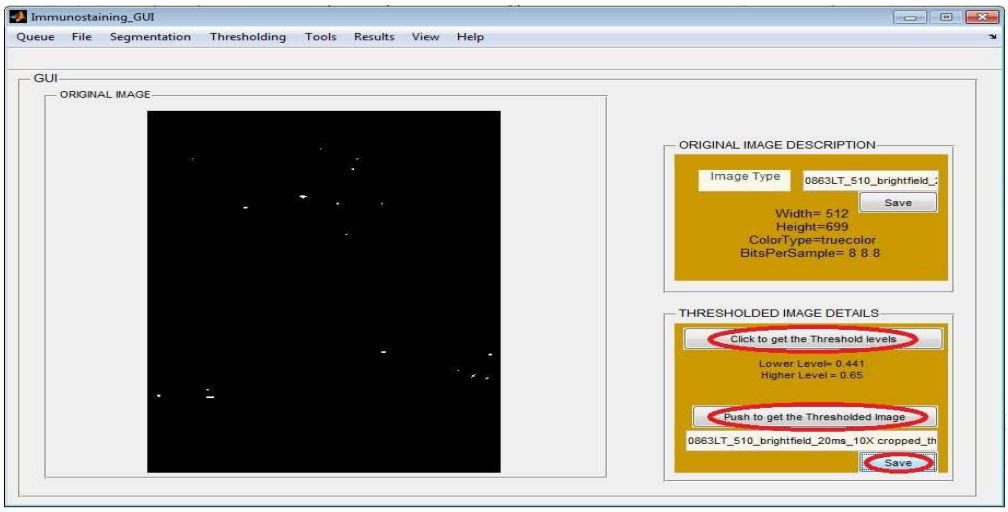


Figure 4.8. β-Gal Procedure – Save threshold image

Step 6: Minimum and Maximum pixel removes stains less than minimum value and greater than maximum value

Tools → Settings → Minimum and Maximum Pixel Areas

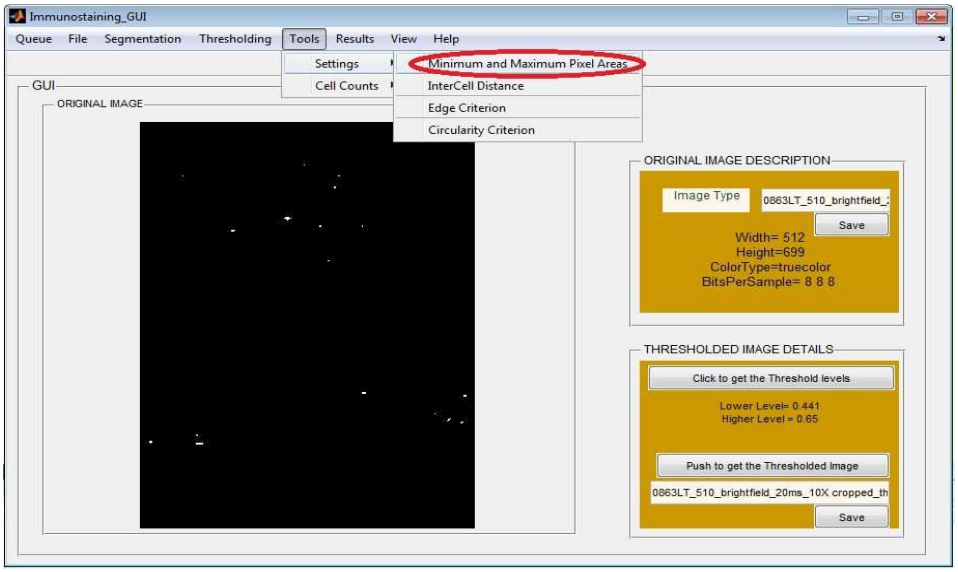


Figure 4.9. β-Gal Procedure – Select Minimum and Maximum Pixel Areas

The below images show smallest and biggest nuclei size in the image

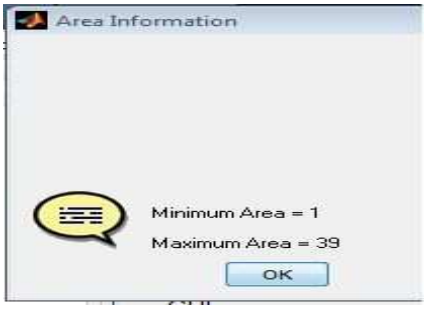


Figure 4.10. β-Gal Procedure – Smallest and biggest Nuclei Information

Below image shows stains less than 2 pixel

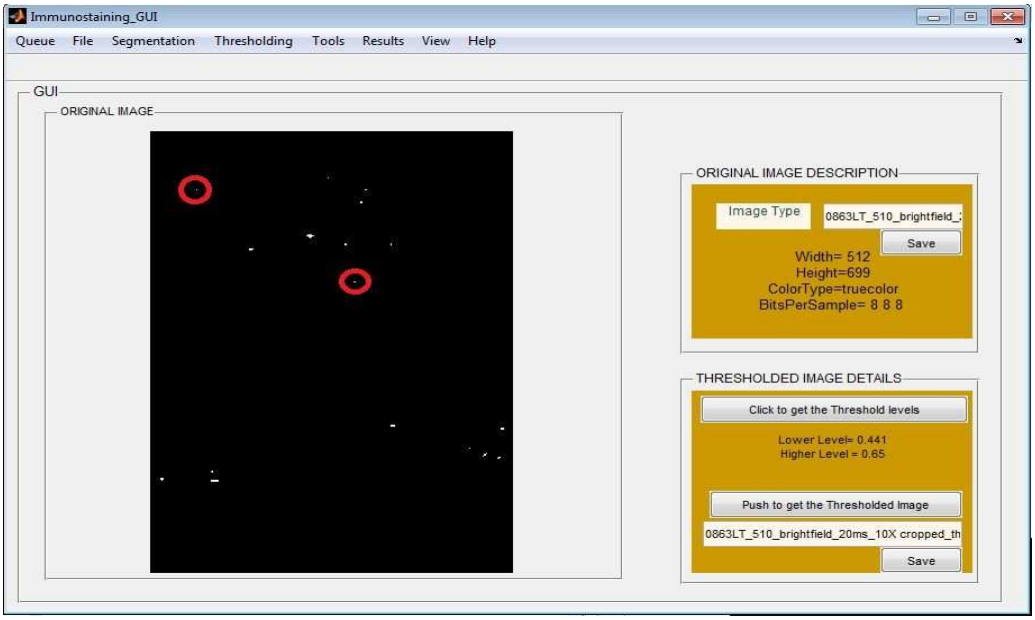


Figure 4.11. β-Gal Procedure – Highlighted smallest Nuclei < 2 pixels

Histogram Information

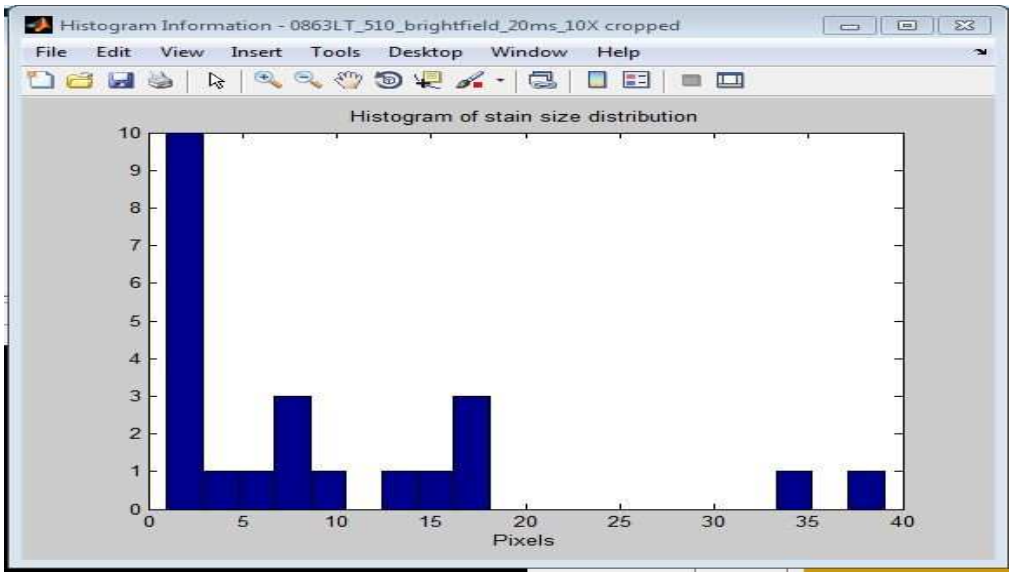


Figure 4.12. β-Gal Procedure – Histogram Information

set the minimum and maximum value in the input box

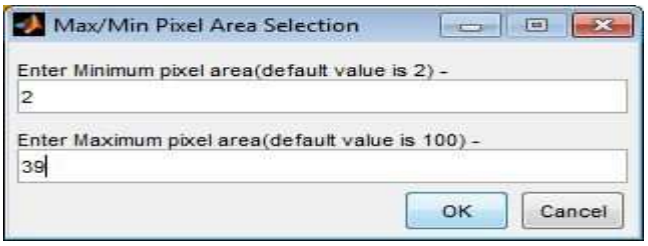


Figure 4.13. β-Gal Procedure – Minimum and maximum nuclei area selection

Step 7: Inter cell distance remove split single nuclei

Tools → Settings → Inter Cell Distance

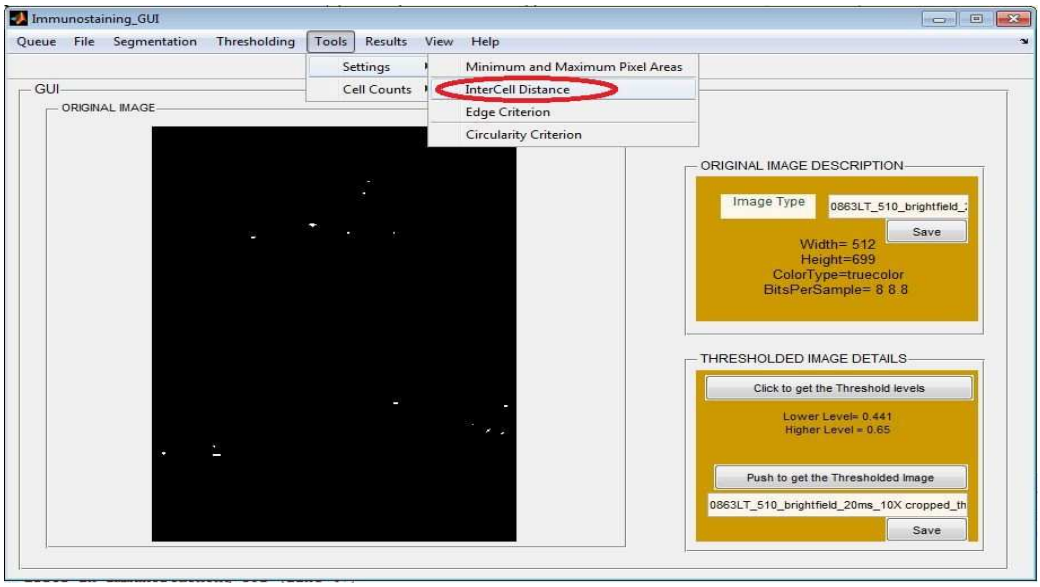


Figure 4.14. β-Gal Procedure – Select Inter Cell Distance

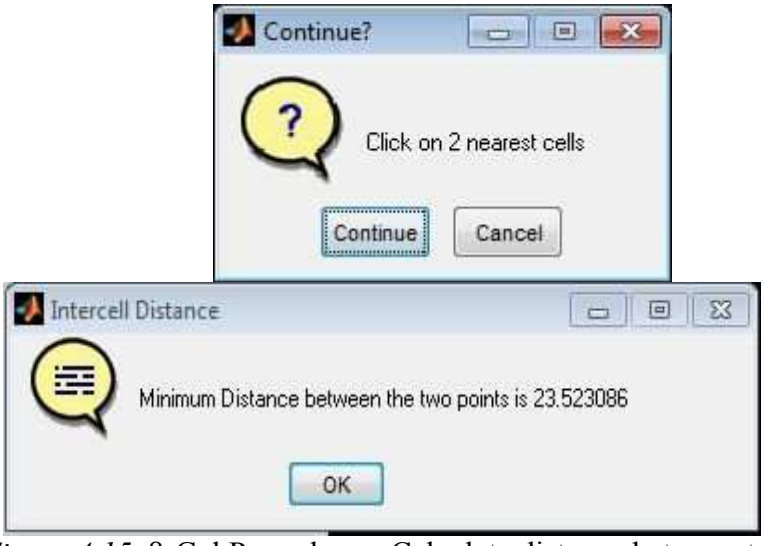


Figure 4.15. β-Gal Procedure – Calculate distance between two points

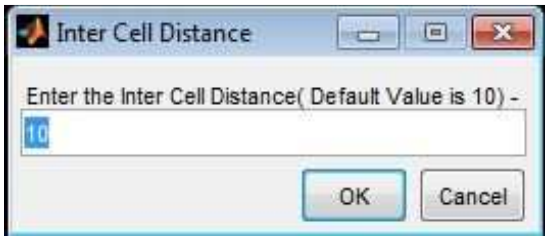


Figure 4.16. β-Gal Procedure – Enter Inter cell distance box

Step 8: Edge Criterion removes edge stains from image

Tools → Settings → Edge Criterion

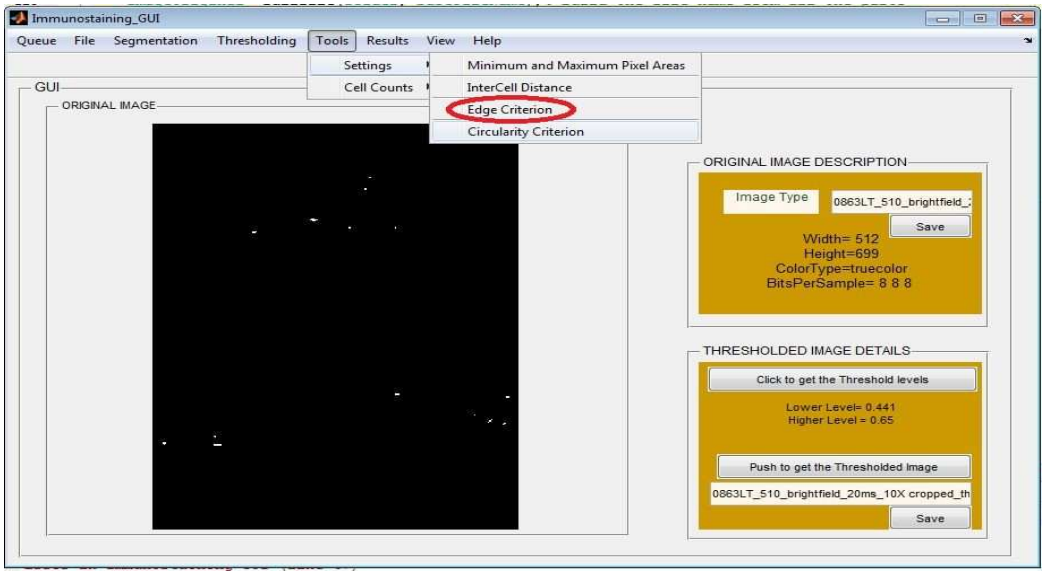


Figure 4.17. β-Gal Procedure – Select Edge Criterion

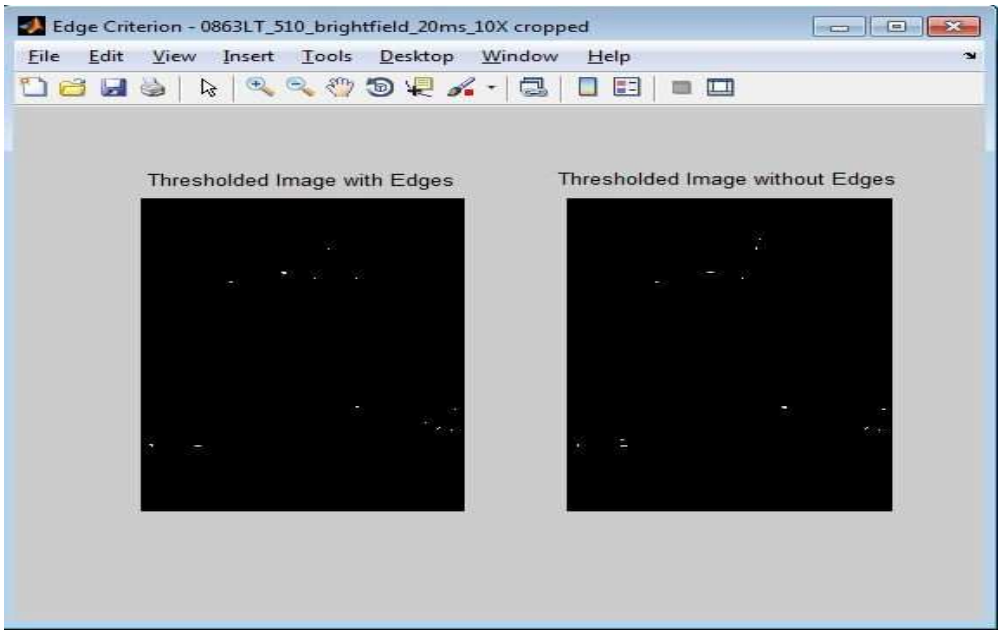


Figure 4.18. β-Gal Procedure – Comparison between before and after edge criterion

Step 9: Circularity Criterion removes stains which have eccentricity less than 0.975

Tools → Settings → Circularity Criterion

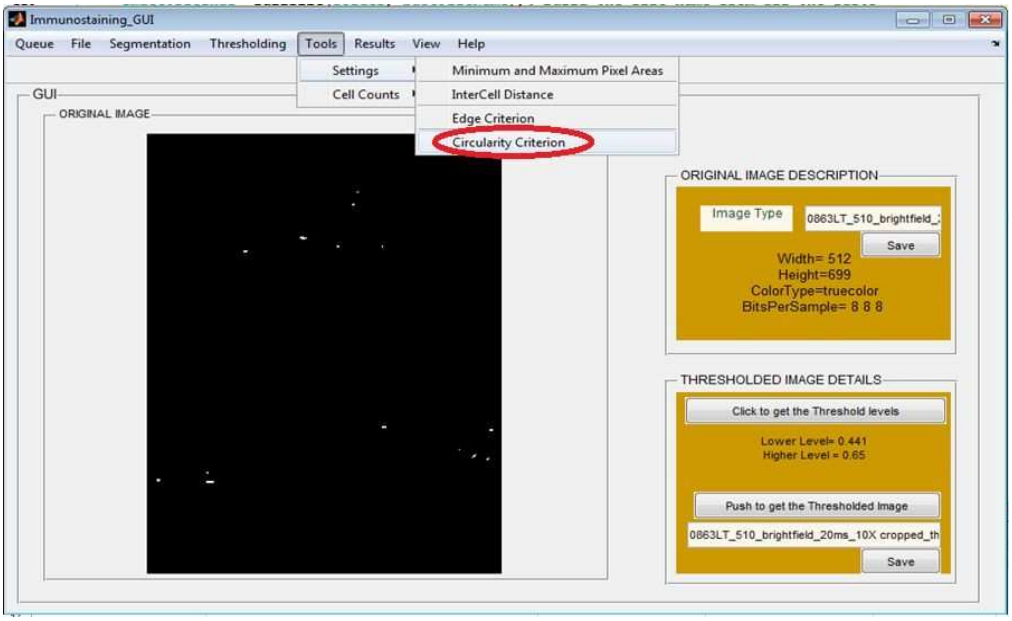


Figure 4.19. β-Gal Procedure – Select Circulatory Criterion

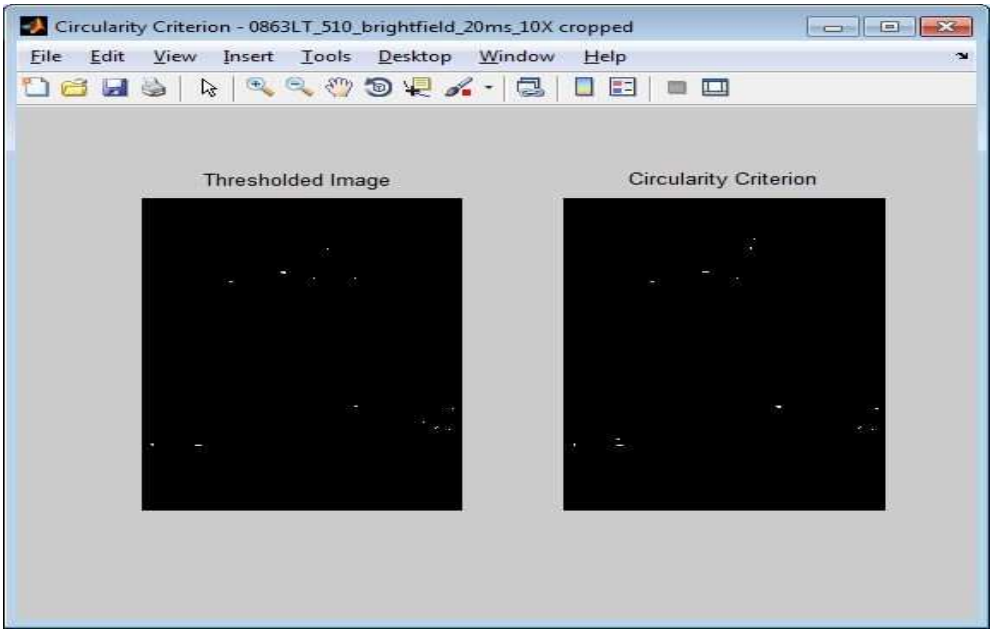


Figure 4.20. β-Gal Procedure – Comparison between before and after circulatory criterion

Step 10: Calculates total number of stains on the image

Tools → Cell Counts → Entire Area

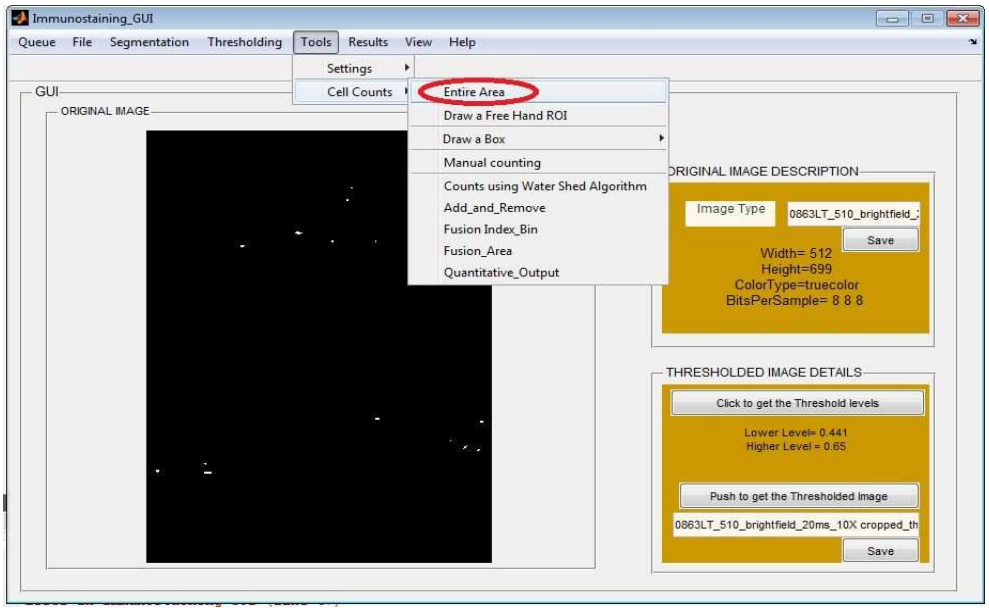


Figure 4.21. β-Gal Procedure – Select Entire Area cell count

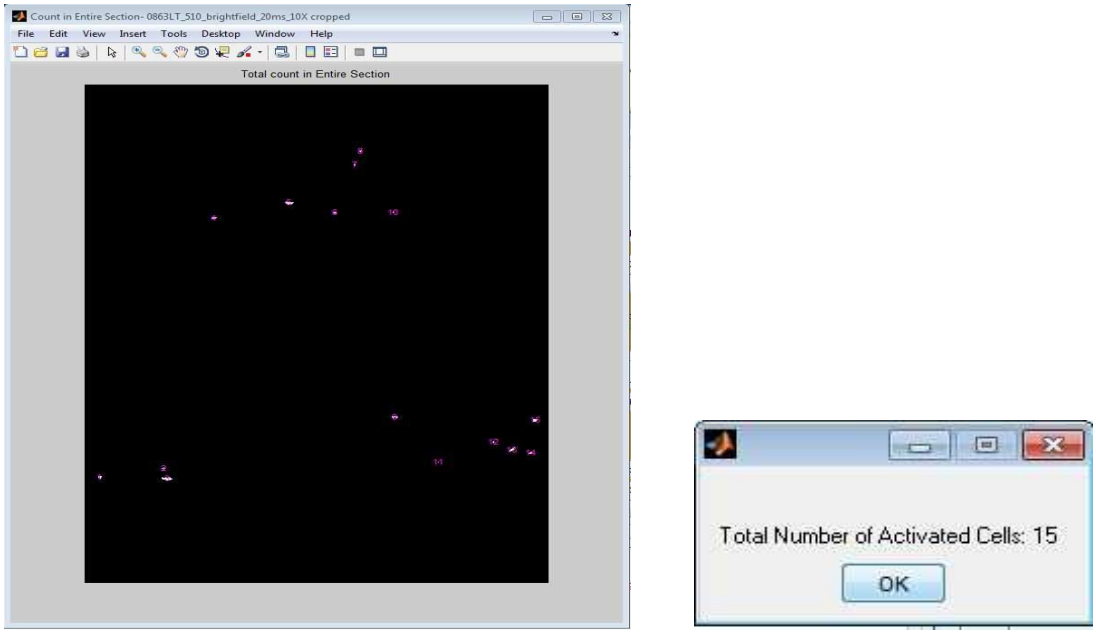


Figure 4.22. β-Gal Procedure – Total number of activated cells

Step 11: Save details to excel sheet

Results → Save to Excel

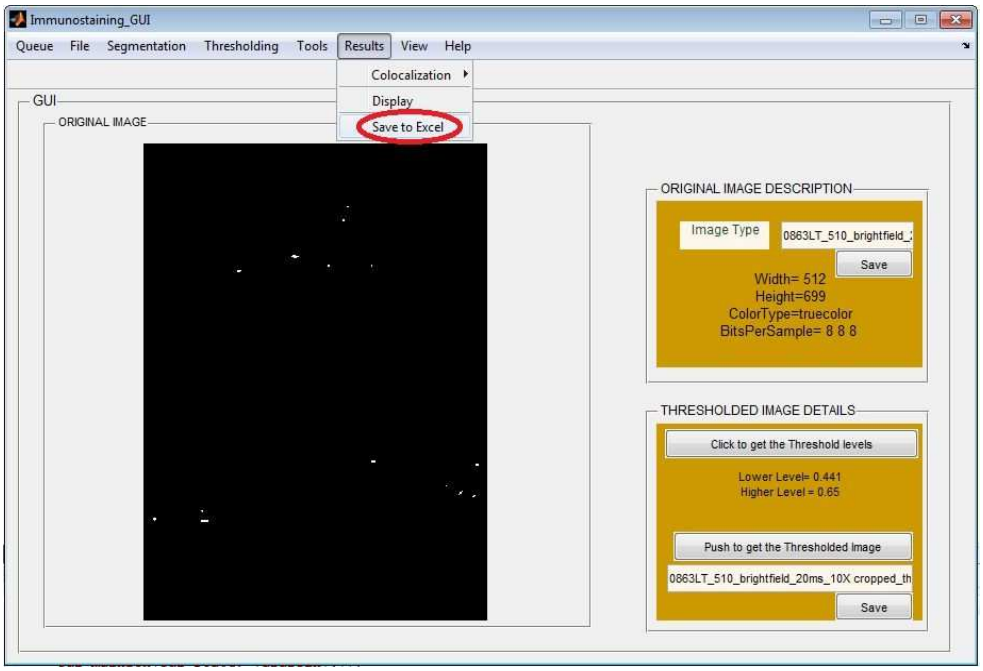


Figure 4.23. β-Gal Procedure – Select save to excel

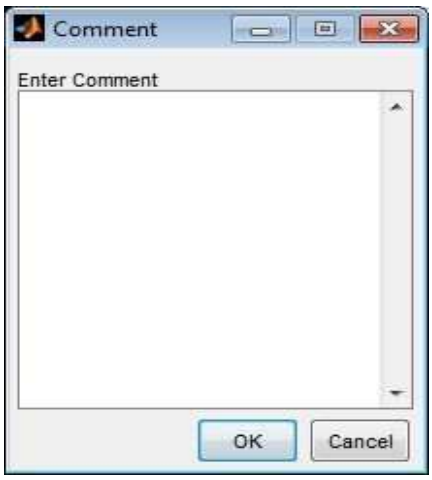


Figure 4.24. β-Gal Procedure – Comment box

	A	B	C	D	E	F	G	H	I	J
1	Date & Time of Analysis	File Name	Single Lower Threshlod	Single Higher Threshold	Double Low Hue	Double High Hue	Min. Pixel Area	Max. Pixel Area	Total No. of Activated Cells	Total No. of Activated Cells > 10
2	6/5/2016 15:10	0863LT_510_brightfield_20ms_10X cropped	0.2	1	0.441243506	0.65	2	39	15	15

Figure 4.25. β-Gal Procedure – Record in excel sheet

DAPI Procedure

Step 1: load an image File → Load Image

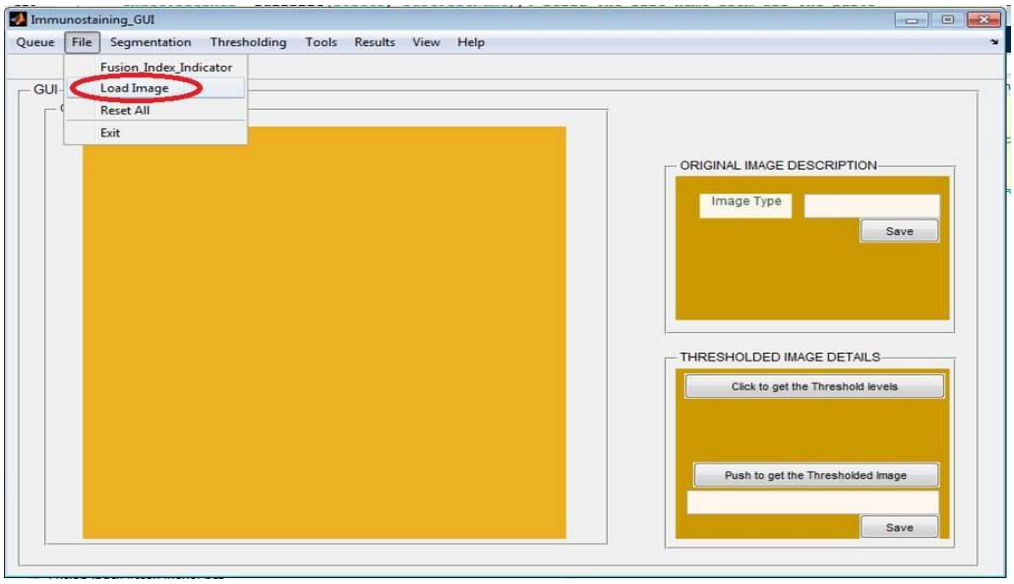


Figure 4.26. DAPI Procedure – Load an image

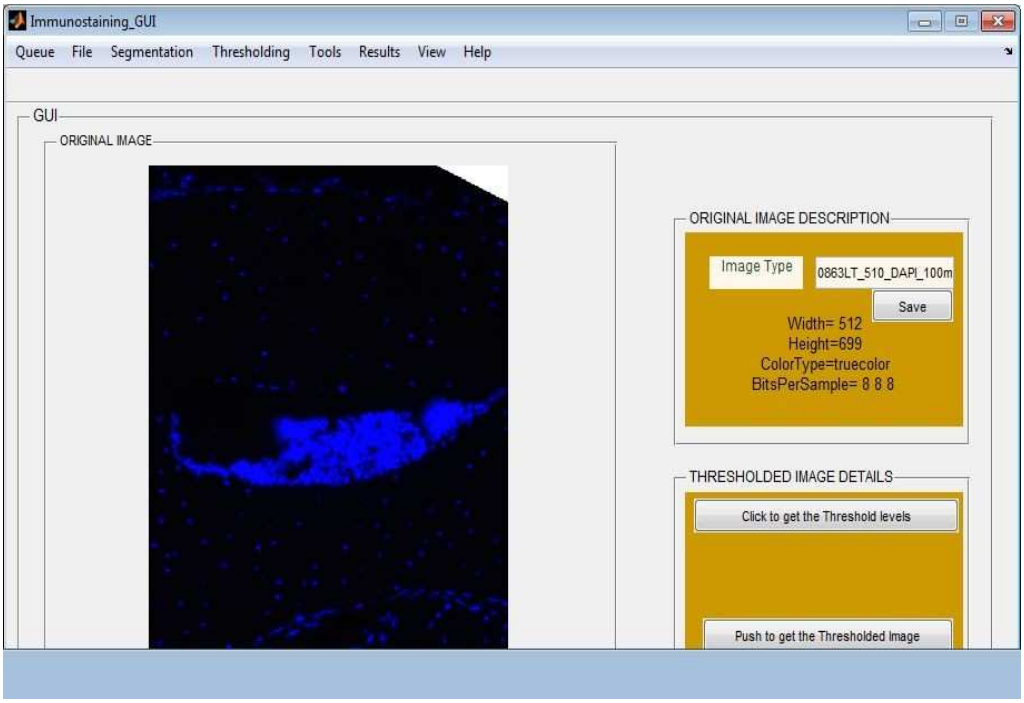


Figure 4.27. DAPI Procedure – Loaded a DAPI image Step 2: Choose Channel

Segmentation → Choose Channel → Blue Channel

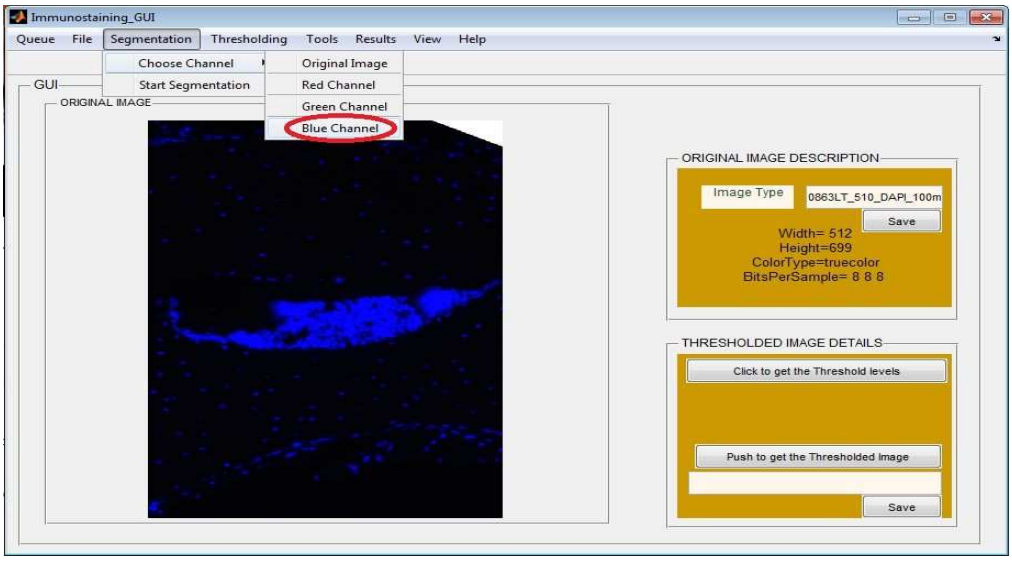


Figure 4.28. DAPI Procedure – Choose blue channel

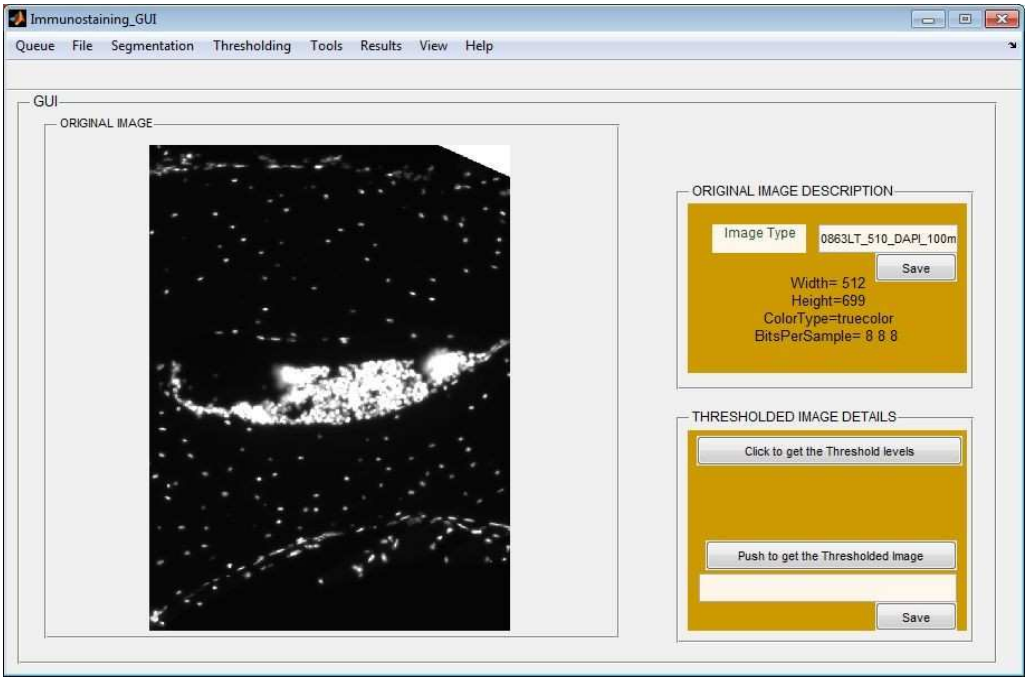


Figure 4.29. DAPI Procedure – Blue image changed to black and white

Step 3: Segment the Image

Segmentation → Start Segmentation → Use Existing Mask → Save the Segmented Image → exit

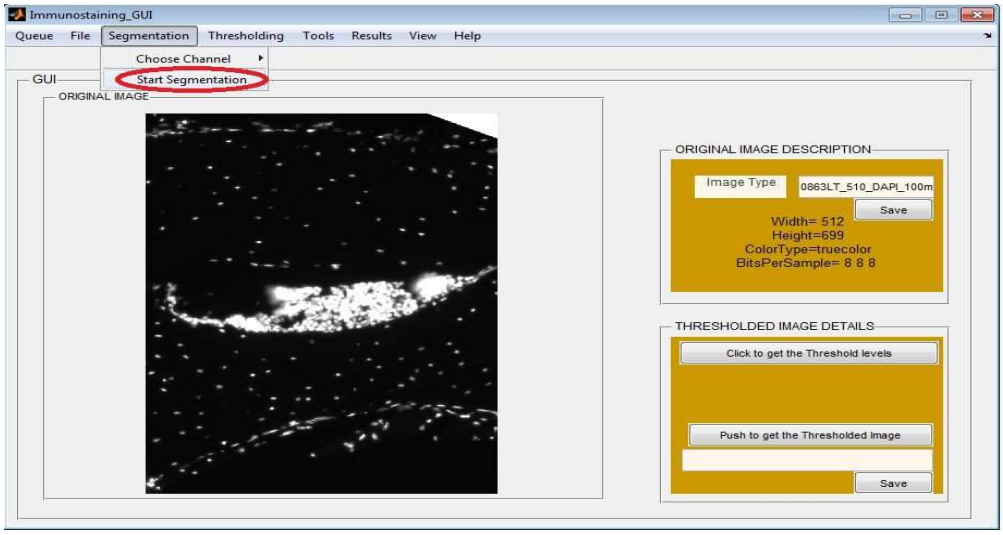


Figure 4.30. DAPI Procedure – Choose start segmentation option

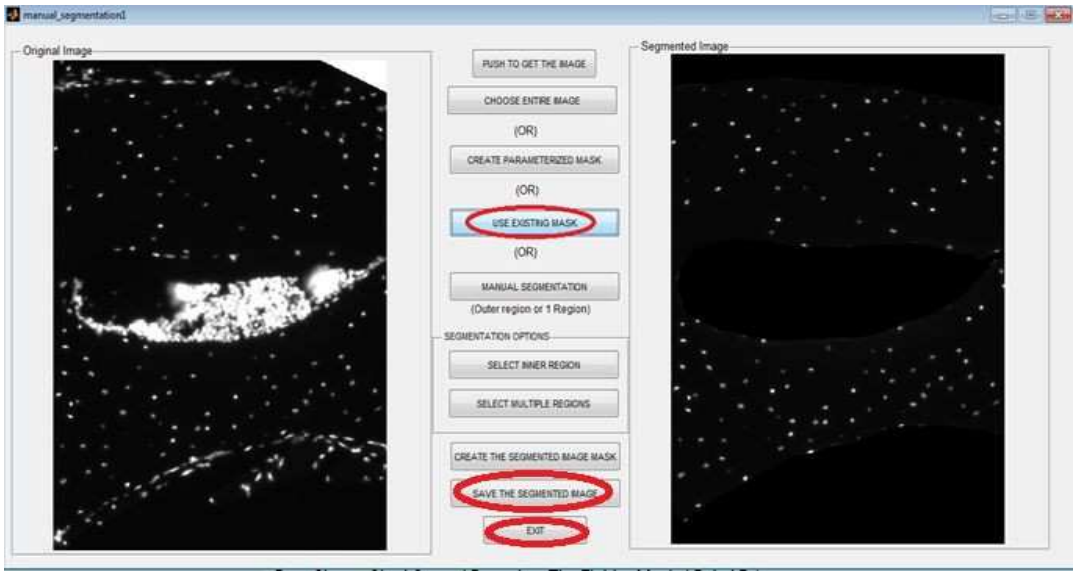


Figure 4.31. DAPI Procedure – Segment image by using existing mask and save

Step 4: Threshold Image by changing low and high threshold values but in most cases default value is sufficient

Thresholding → Single Channel → Histogram Based

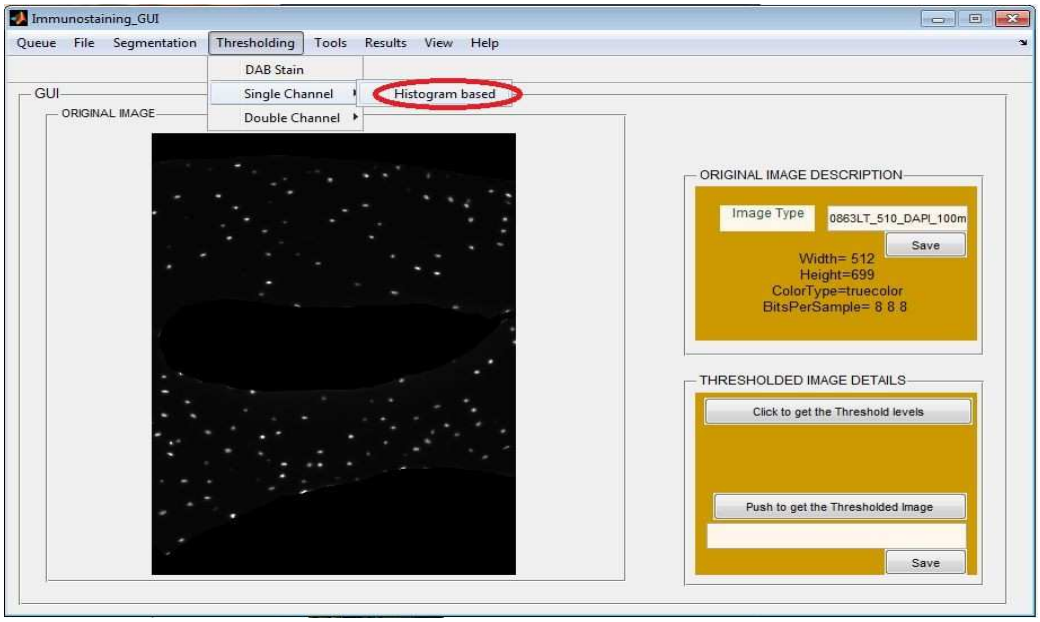


Figure 4.32. DAPI Procedure – Choose single channel histogram

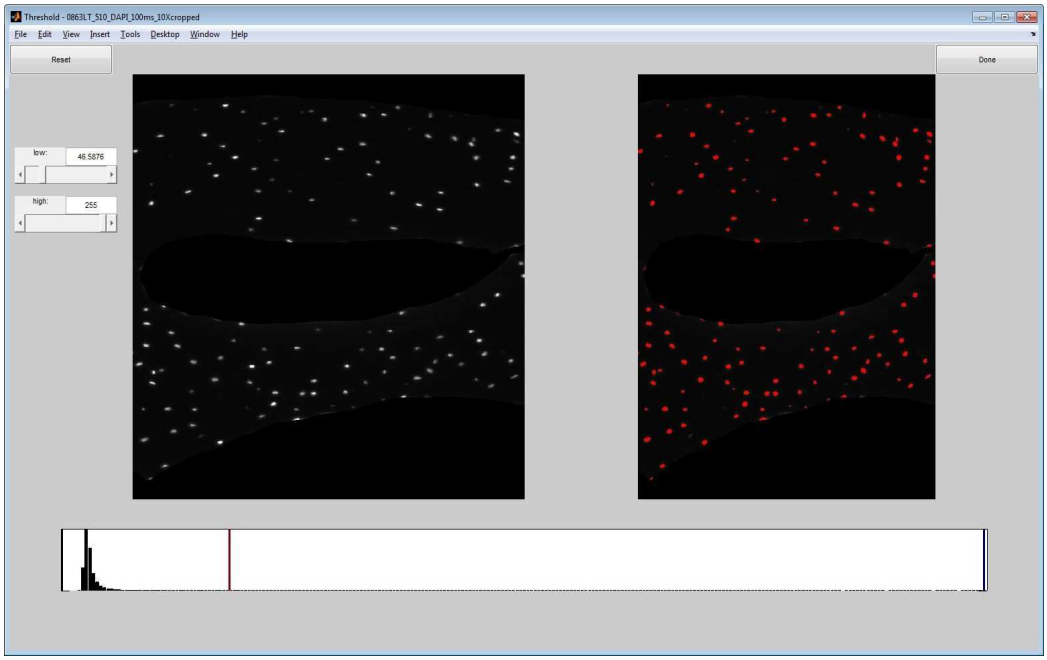


Figure 4.33. DAPI Procedure – Threshold the image with default values

Step 5: Save thresholded image details

Click to get the threshold levels → push to get the thresholded image → save

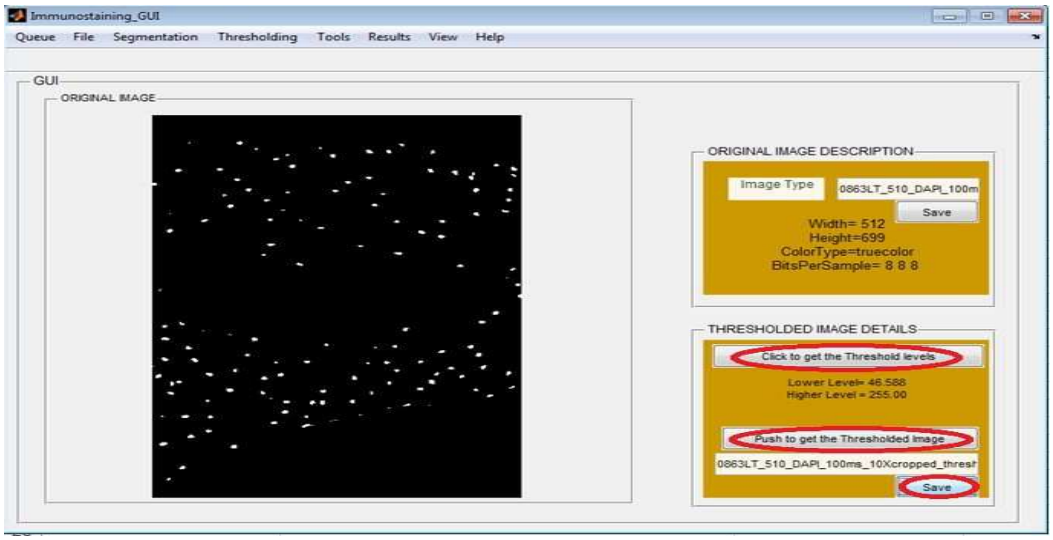


Figure 4.34. DAPI Procedure – Save thresholded image

Step 6: Calculate Minimum and Maximum pixel areas

Tools → Settings → minimum and maximum pixel areas

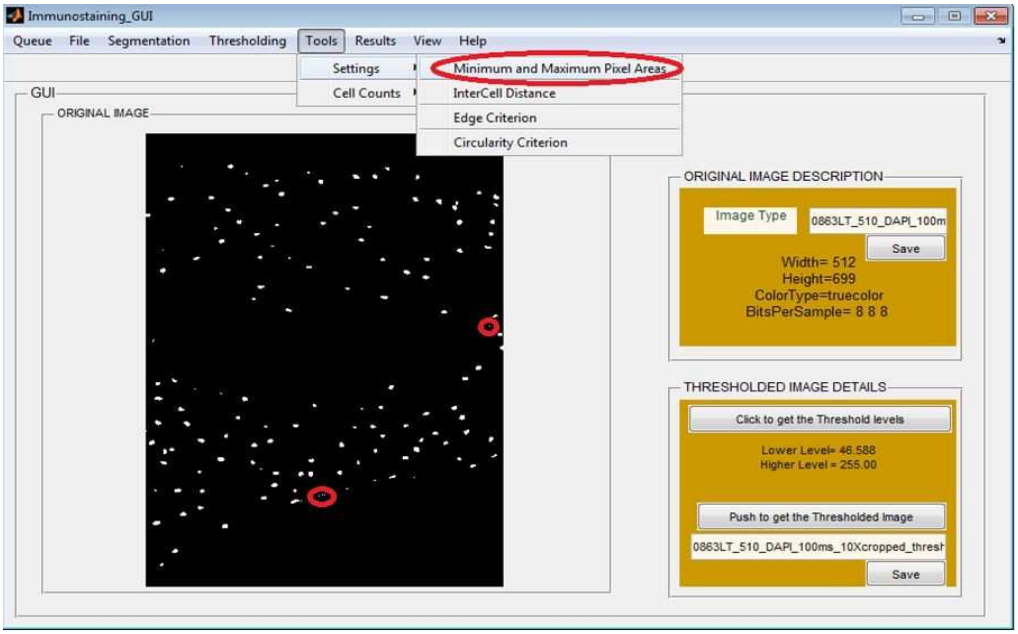


Figure 4.35. DAPI Procedure – Select minimum and maximum pixel areas option

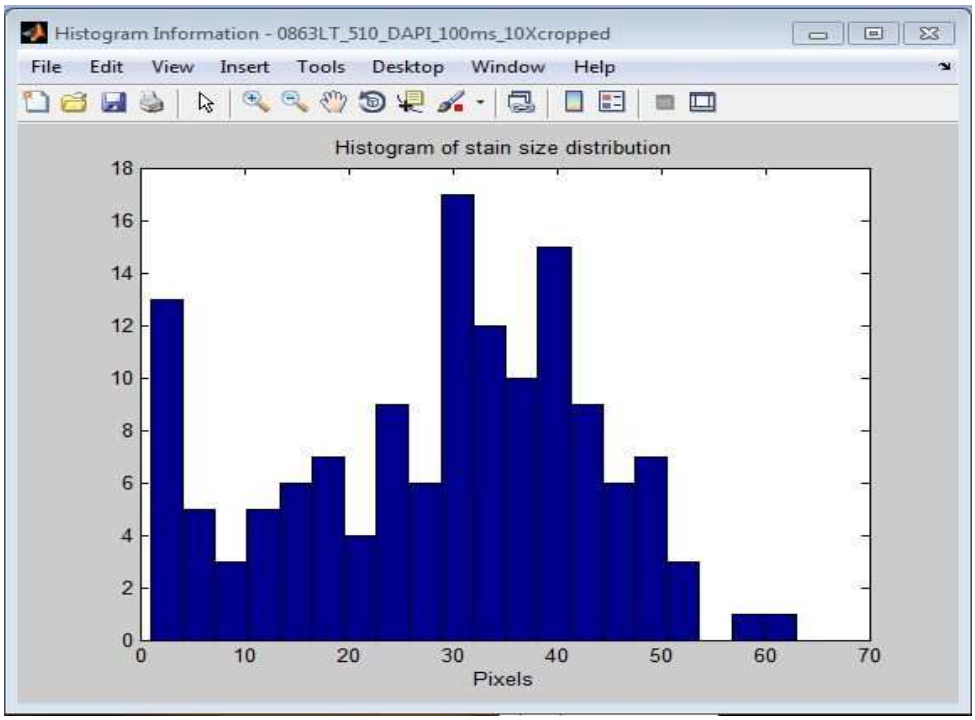


Figure 4.36. DAPI Procedure – Histogram Information

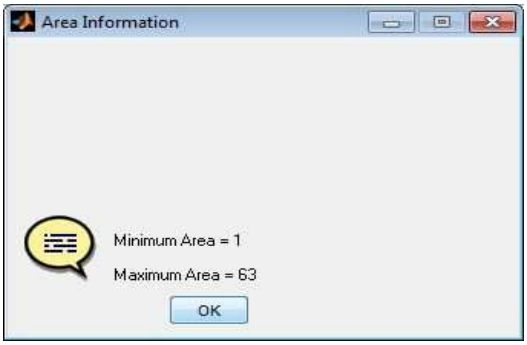


Figure 4.37. DAPI Procedure – Cells minimum and maximum area information

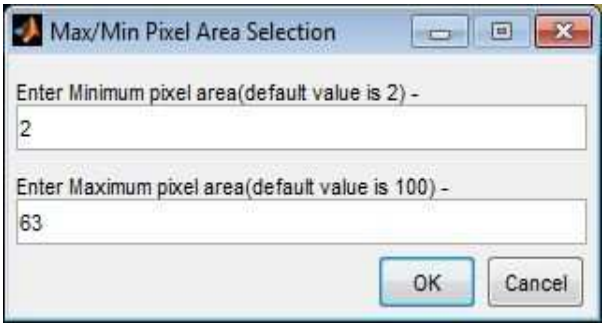


Figure 4.38. DAPI Procedure – Minimum and maximum area input box

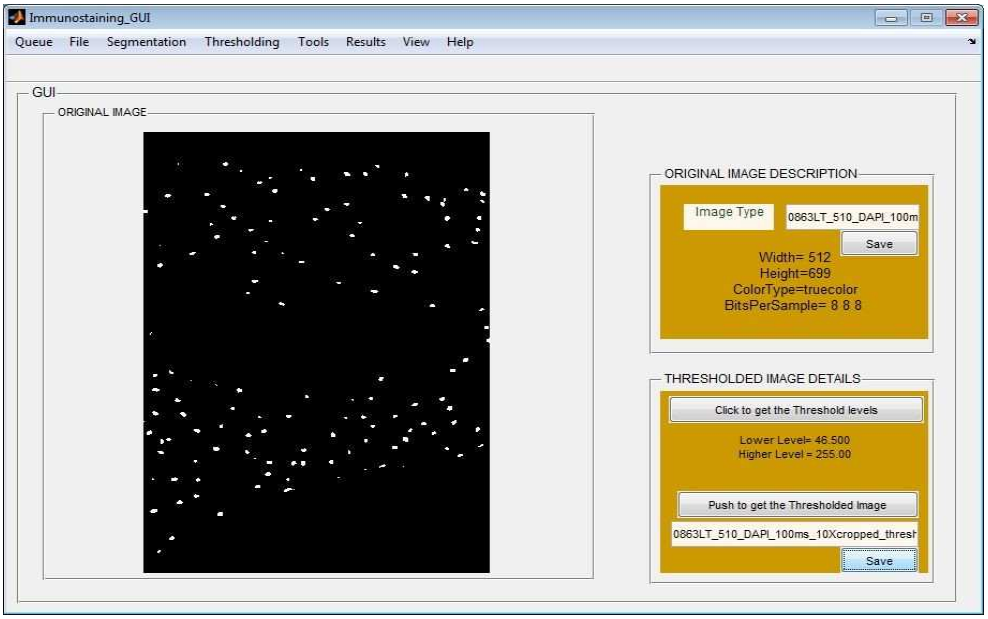


Figure 4.39. DAPI Procedure – Image after processing Minimum and maximum pixel areas

Step 7: Calculate Inter cell distance

Tools → Settings → Intercell distance

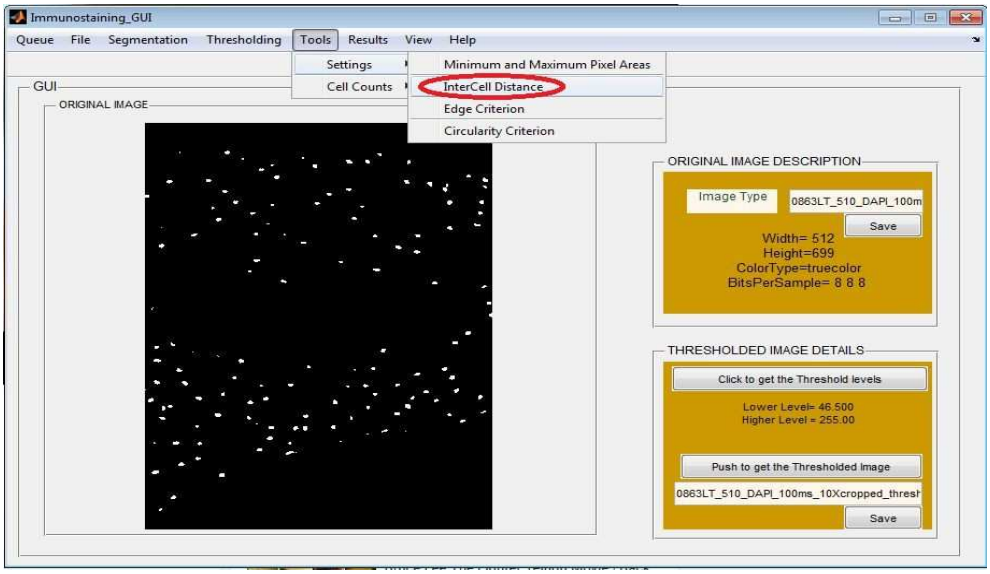


Figure 4.40. DAPI Procedure – Select Inter-cell distance option

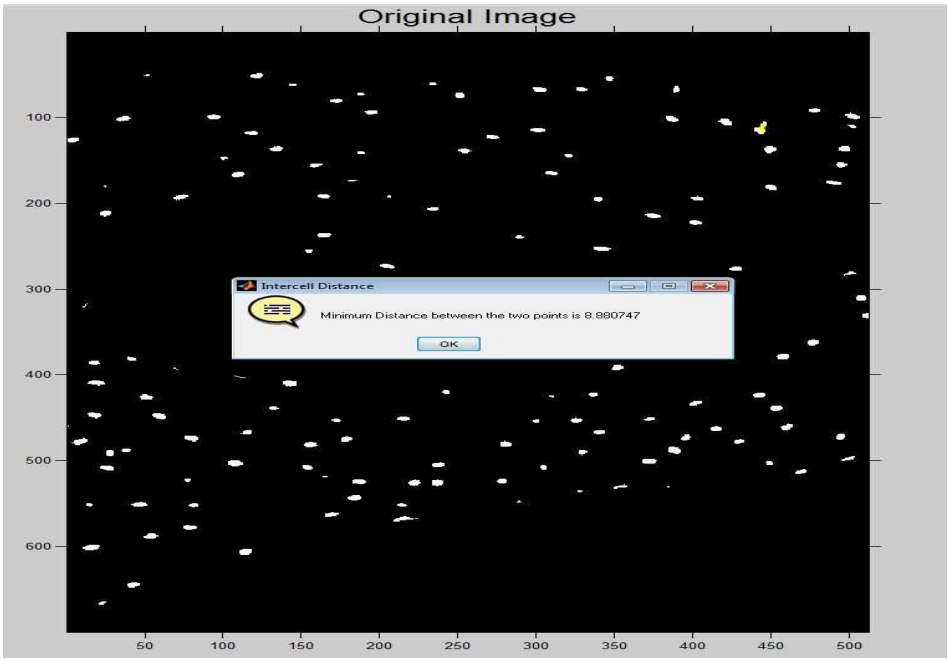


Figure 4.41. DAPI Procedure – Calculated Inter cell distance

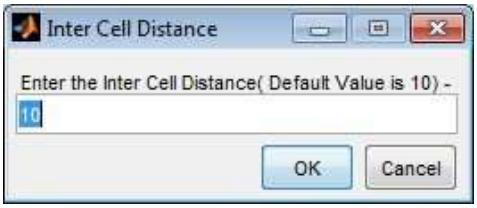


Figure 4.42. DAPI Procedure – Enter inter-cell distance manually

Step 8: Edge Criterion

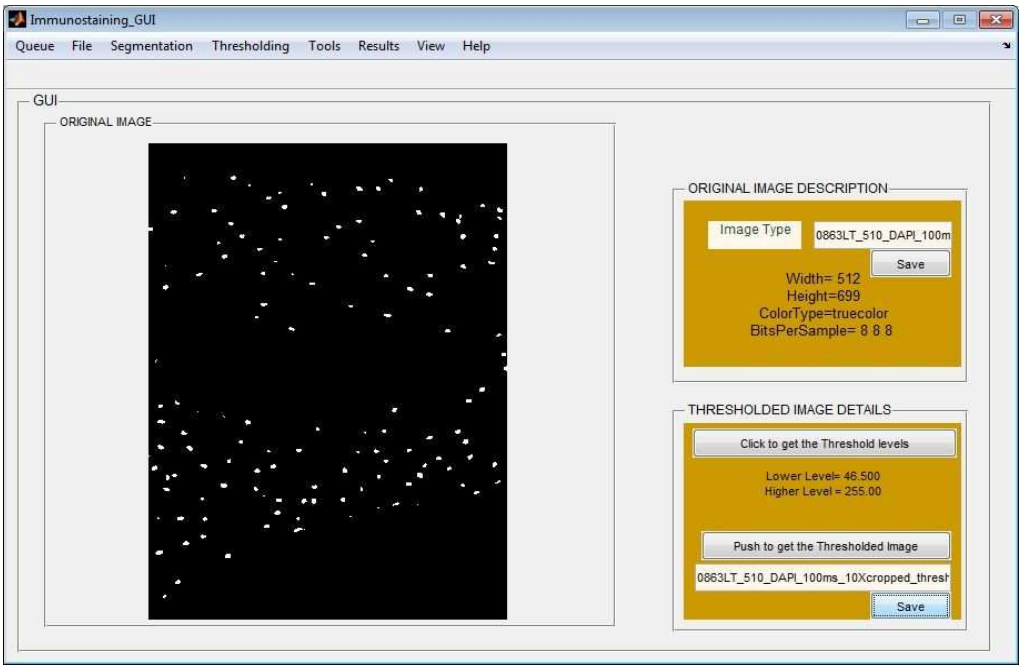


Figure 4.43. DAPI Procedure – Image before edge criterion

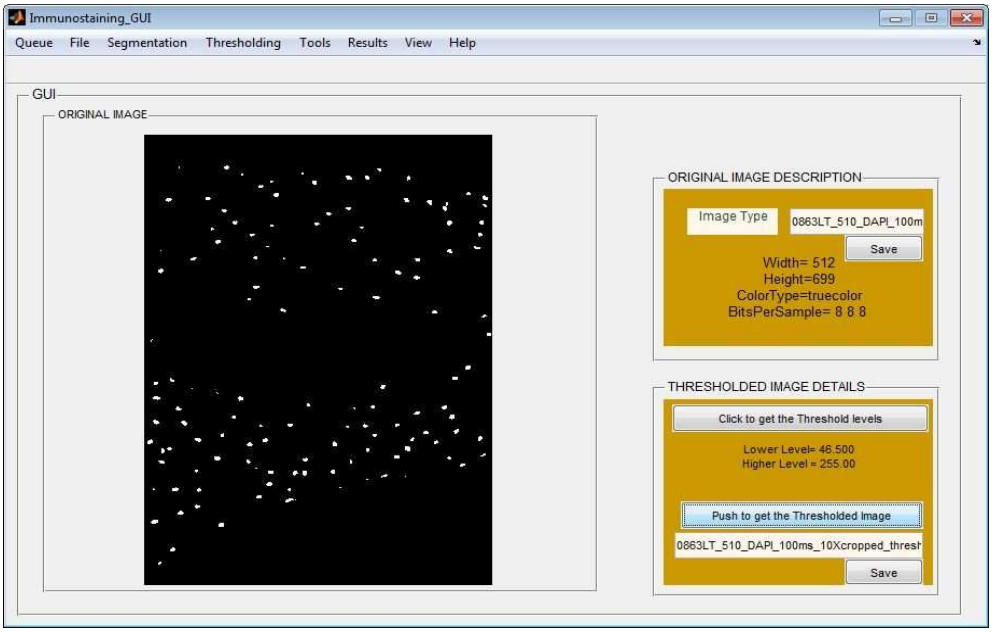


Figure 4.44. DAPI Procedure – Image after edge criterion

Step 9: Removes stains whose eccentricity is less than 0.975

Tools → Settings → Circularity Criterion

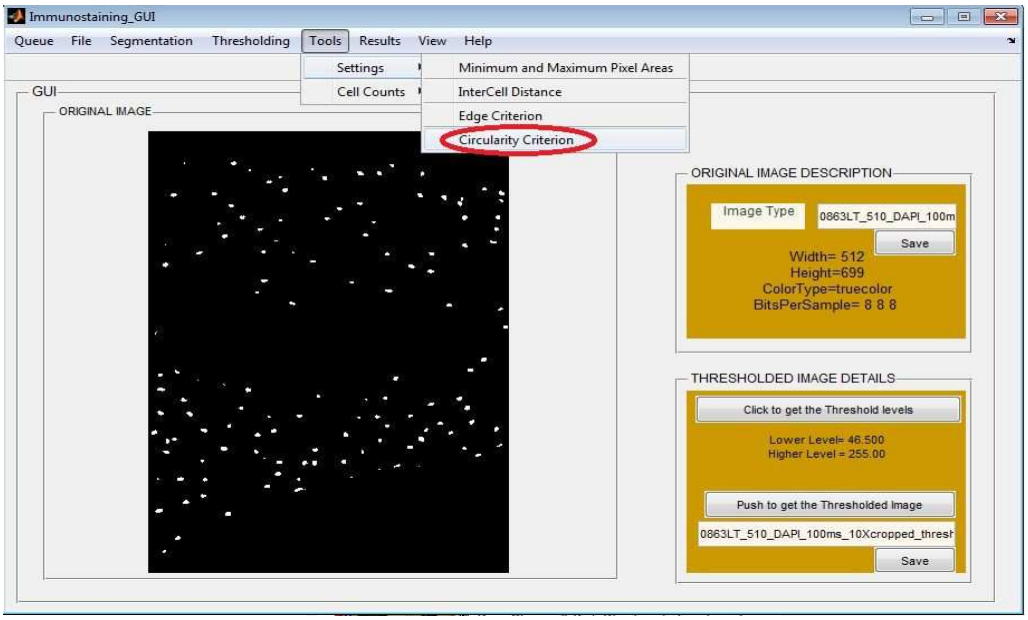


Figure 4.45. DAPI Procedure – Choose circularity criterion

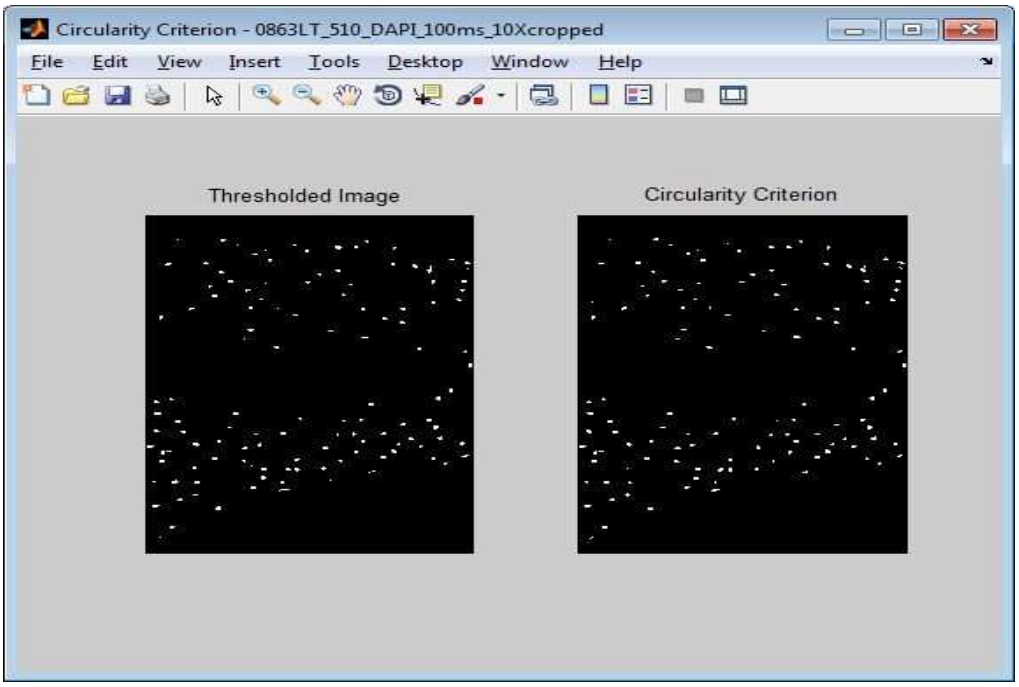


Figure 4.46. DAPI Procedure – Before and after circularity criterion

Step 10: Calculates total number of stains

Tools → Cell Counts → Entire Area

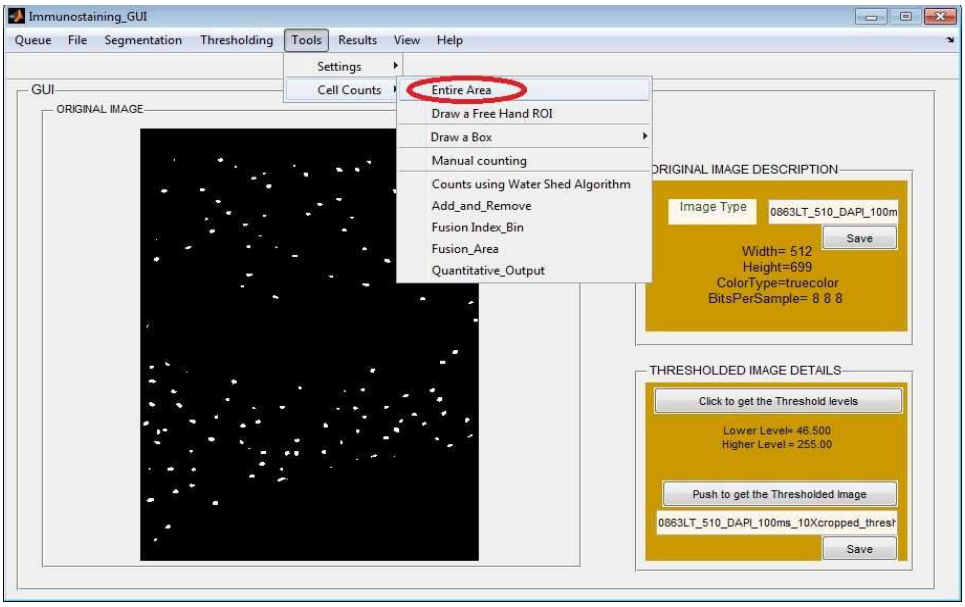


Figure 4.47. DAPI Procedure – Choose option to calculate entire area

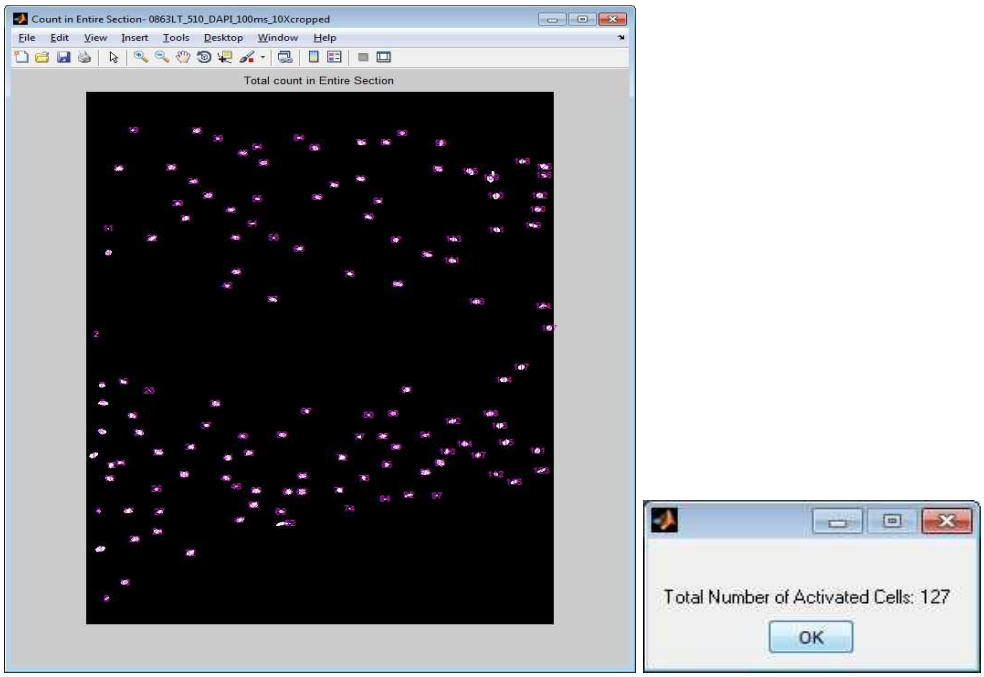


Figure 4.48. DAPI Procedure – Total number of activated calles image and count

Step 11: Save results to Excel Sheet

Results → Save to Excel

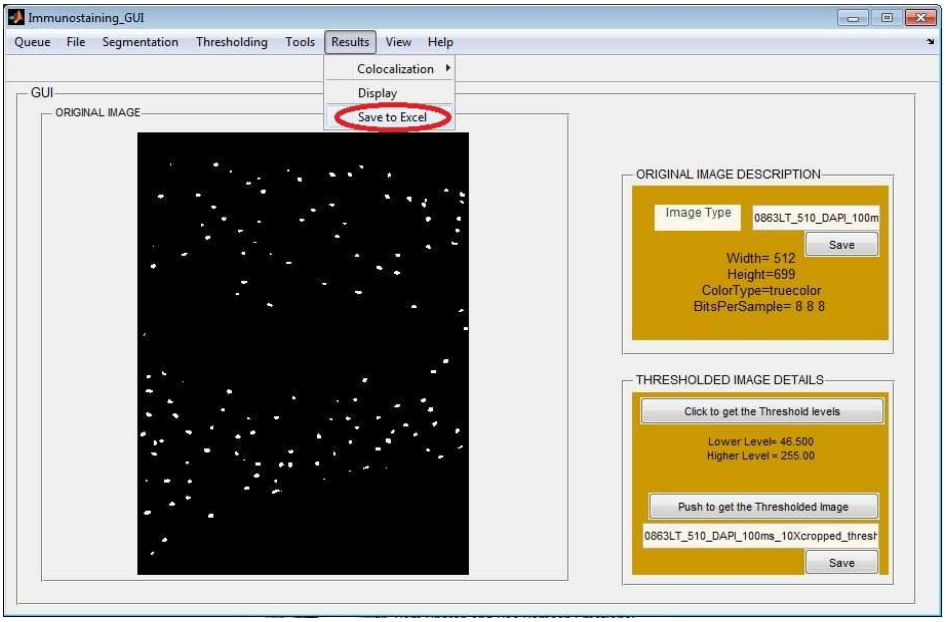


Figure 4.49. DAPI Procedure – Save results to excel

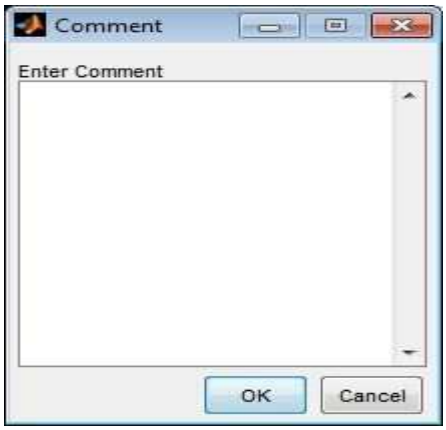


Figure 4.50. DAPI Procedure – Comment box

	A	B	C	D	E	F	G	H	I	J
1	Date & Time of Analysis	File Name	Single Lower Threshlod	Single Higher Threshold	Double Low Hue	Double High Hue	Min. Pixel Area	Max. Pixel Area	Total No. of Activated Cells	Total No. of Activated Cells > 10 p
2	6/5/2016 15:10	0863LT_510_brightfield_20ms_10X cropped	0.2	1	0.441243506	0.65	2	39	15	15
3	6/5/2016 16:57	0863LT_510_DAPI_100ms_10Xcropped	46.5	255			2	63	128	127

Figure 4.51. DAPI Procedure – Results in excel sheet

Non-Immune Procedure

Step 1: Load an Image File → Load Image

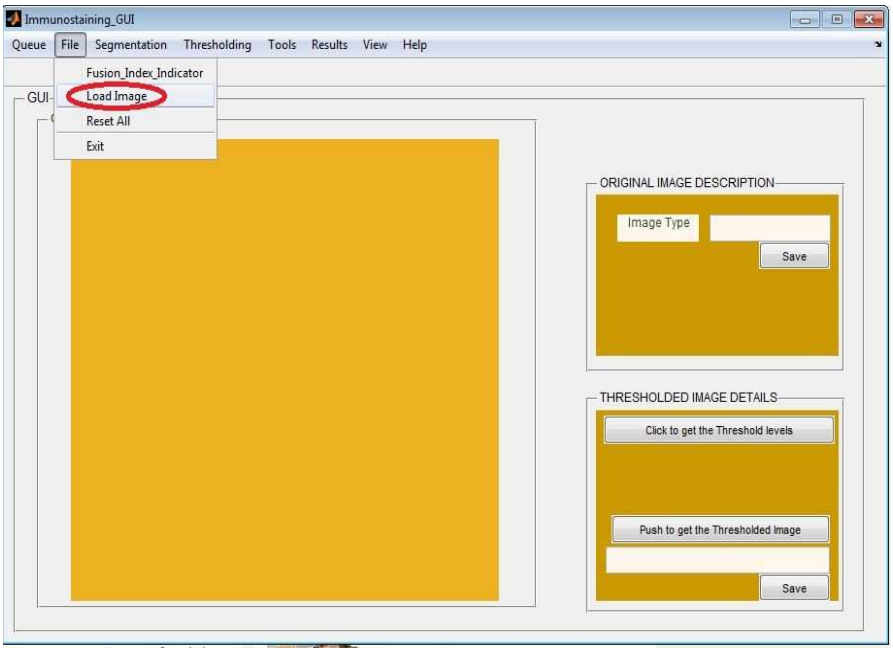


Figure 4.52. Non-Immune Procedure – Load an image

Step 2: Choose Channel

Segmentation → Choose Channel → Original Image

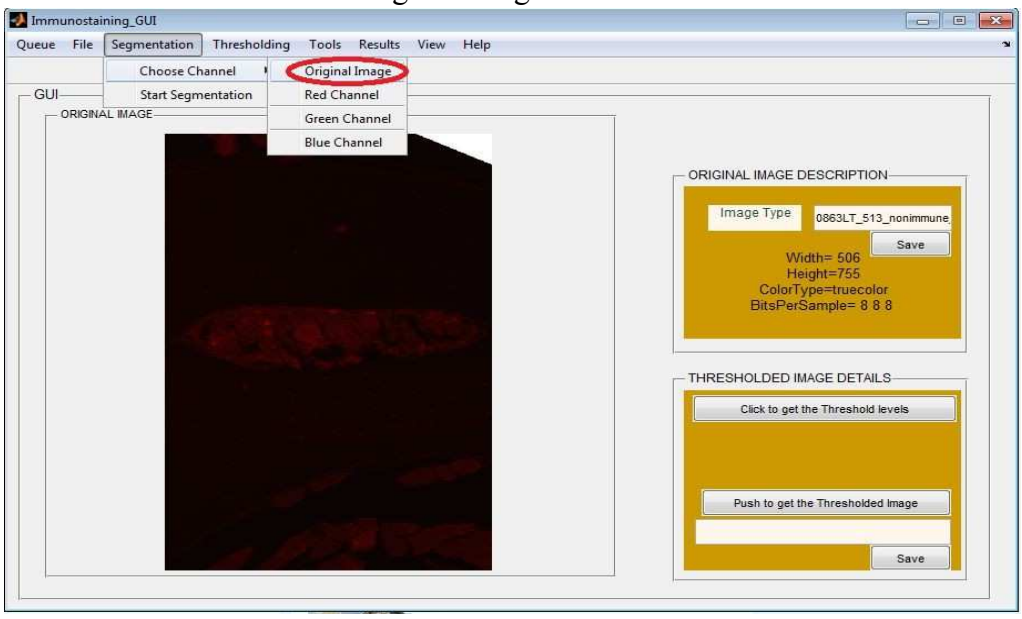


Figure 4.53. Non-Immune Procedure – Choose original image channel

Step 3: Segmented the image

Segmentation → Start Segmentation → Manual Segmentation → Select Inner Region → save the Segmented Image → Exit

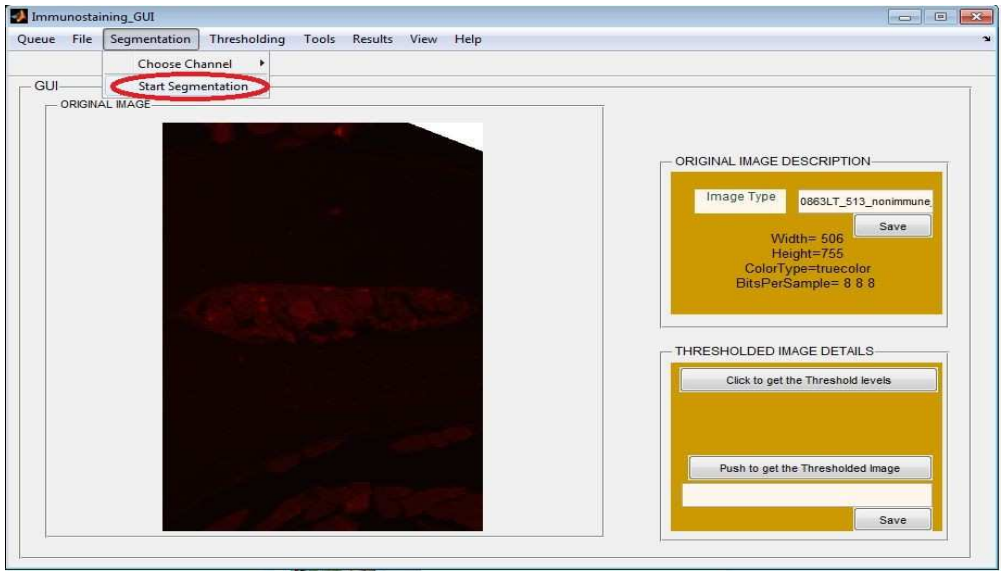


Figure 4.54. Non-Immune Procedure – Choose start segmentation option

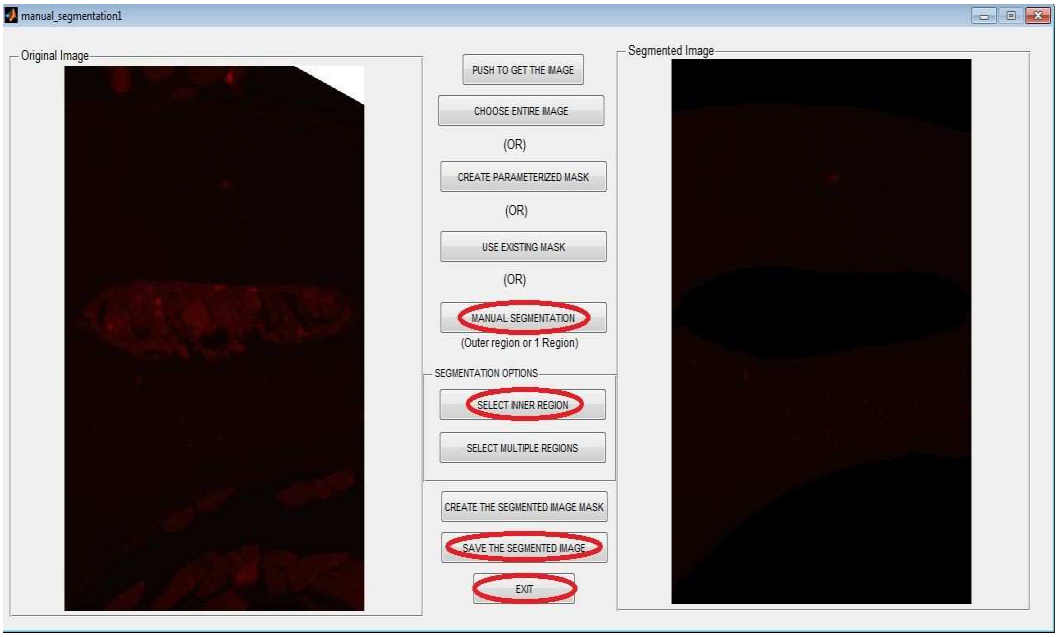


Figure 4.55. Non-Immune Procedure – Segment outer and inner region and save image

Step 4: Threshold image

Thresholding → Single Channel → Histogram Based

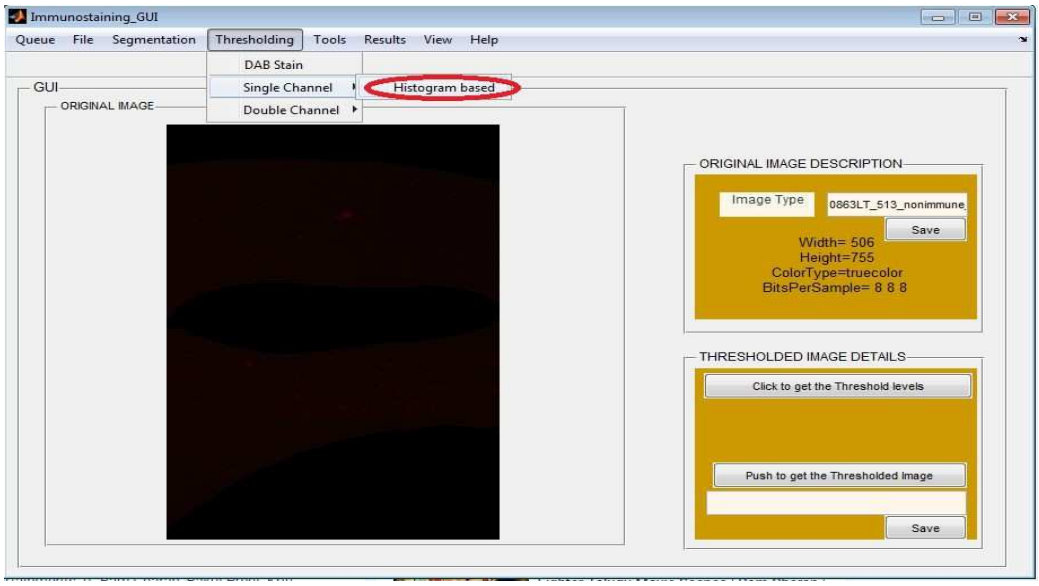


Figure 4.56. Non-Immune Procedure – Choose single channel histogram

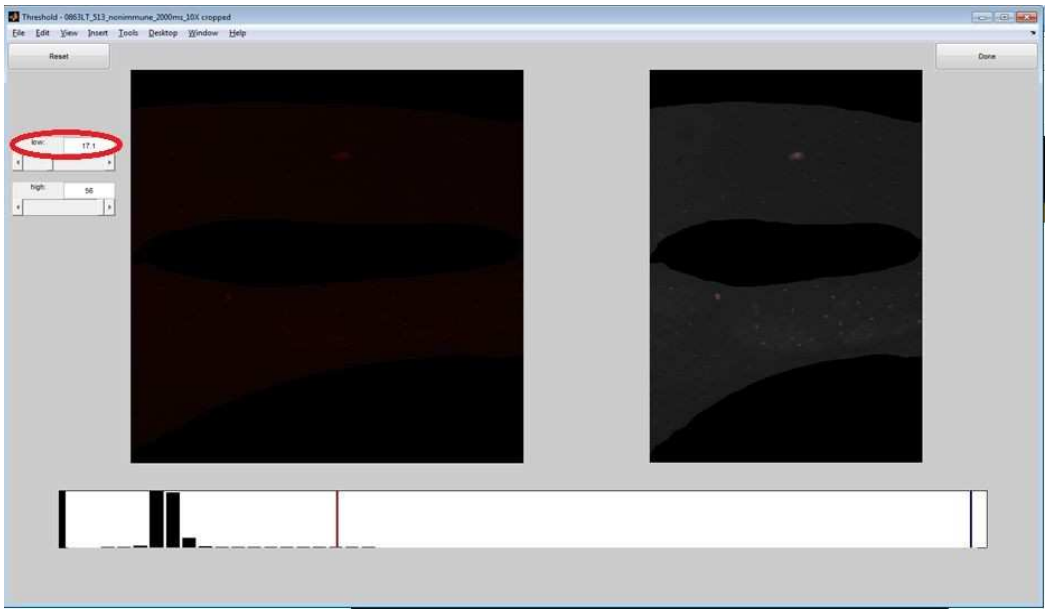


Figure 4.57. Non-Immune Procedure – Adjust low threshold value

Sclerostin Procedure

Step 1: Load an Image File → Load Image

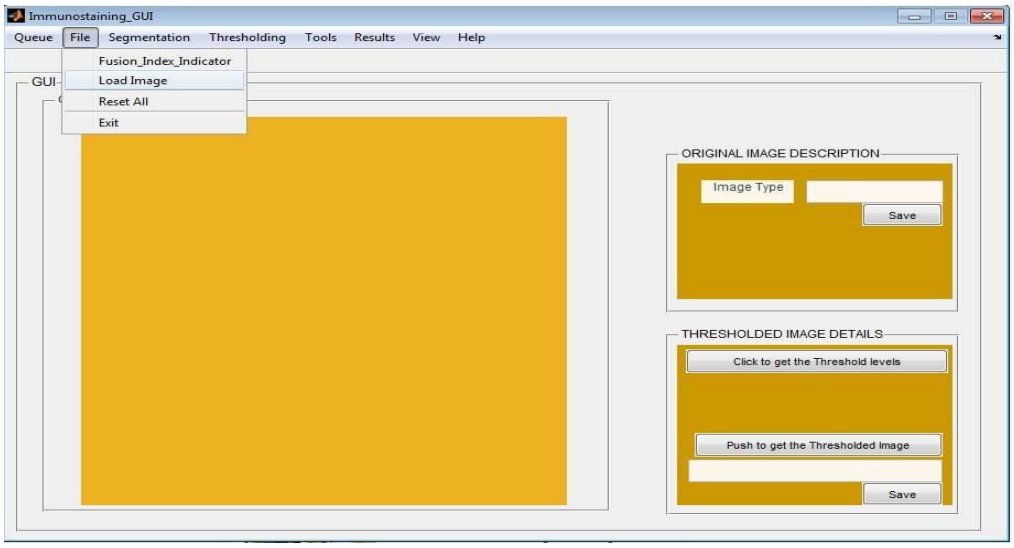


Figure 4.58. Sclerostin Procedure – Load an image

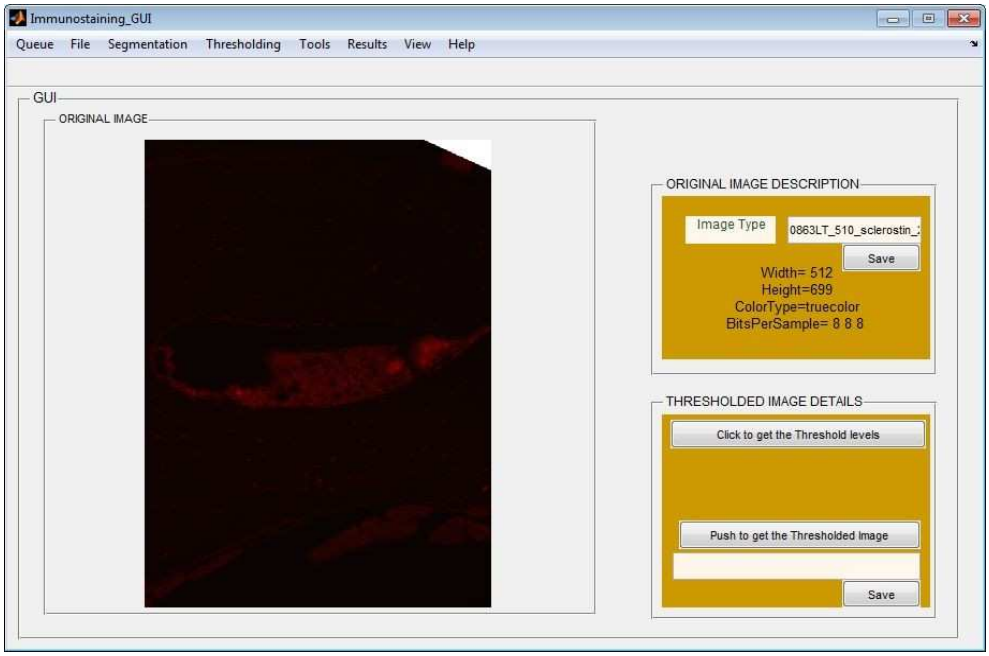


Figure 4.59. Sclerostin Procedure – Sclerostin Image

Step 2: Choose Channel

Segmentation → Choose channel → original Image

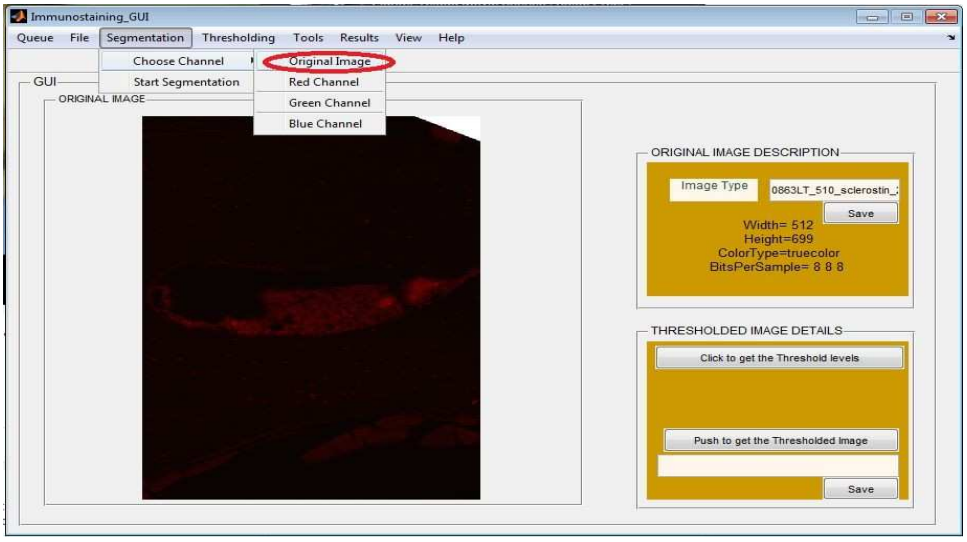


Figure 4.60. Sclerostin Procedure – Choose original image channel

Step 3: Segmentation

Segmentation → Start Segmentation → Use existing mask → Save the Segmented Image → Exit

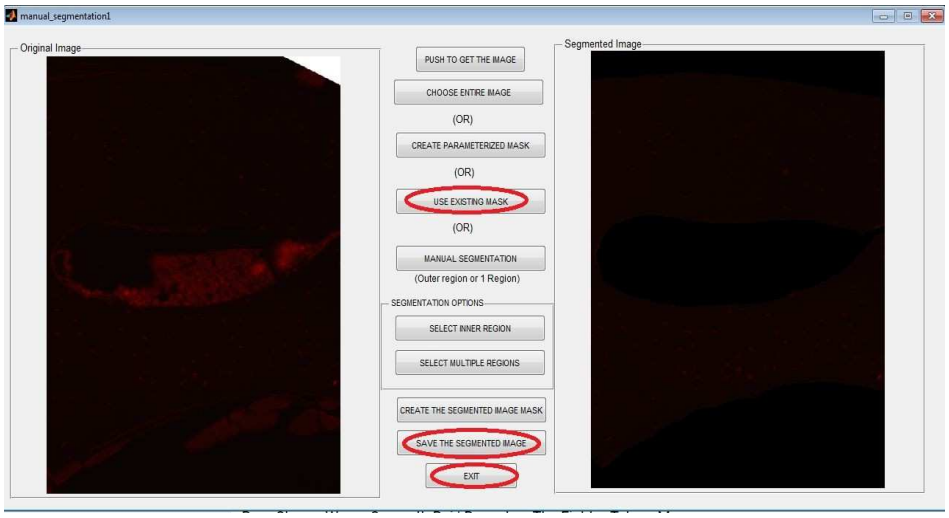


Figure 4.61. Sclerostin Procedure – start segmentation using mask and save image

Step 4: Threshold of non-immune is used for sclerostin image

Thresholding → Single Channel → Histogram based

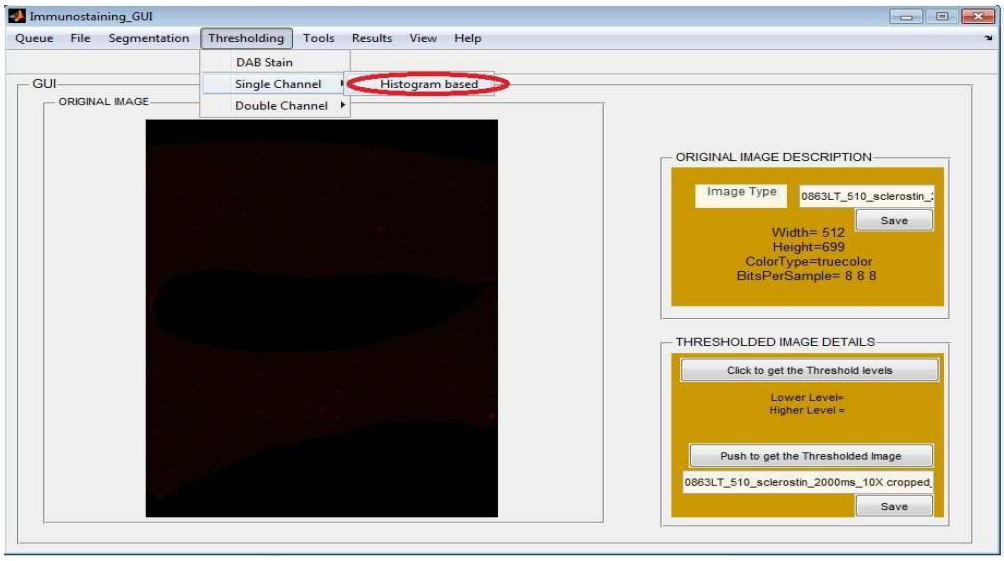


Figure 4.62. Sclerostin Procedure – Choose single channel histogram

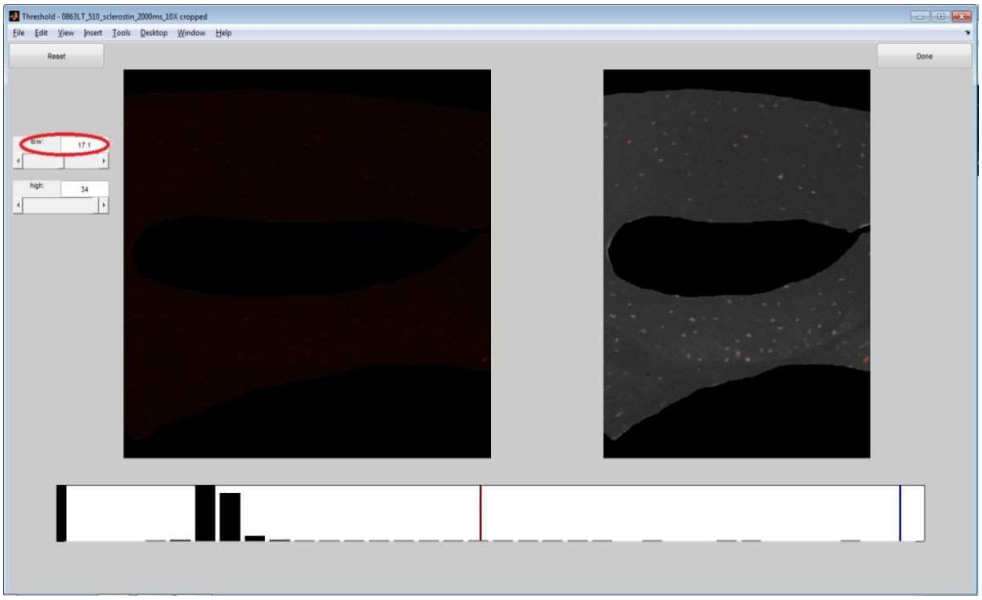


Figure 4.63. Sclerostin Procedure – Set threshold value same as non-immune threshold value

Step 5: Threshold the image details

Click to get threshold levels → Push to get the thresholded images → Save

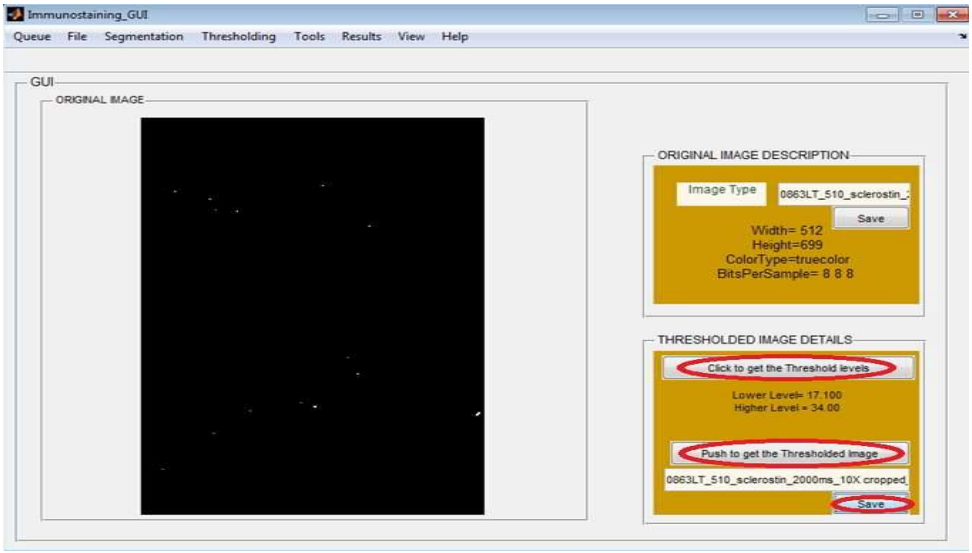


Figure 4.64. Sclerostin Procedure – Save threshold Image

Step 6: calculate total number of stains

Tools → Cell counts → Entire area

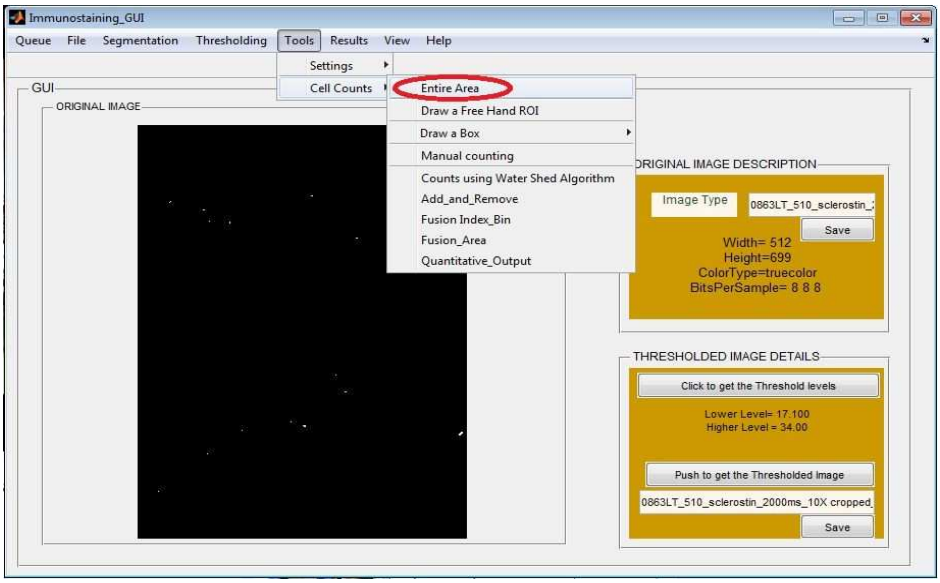


Figure 4.65. Sclerostin Procedure – Calculate total number of cells

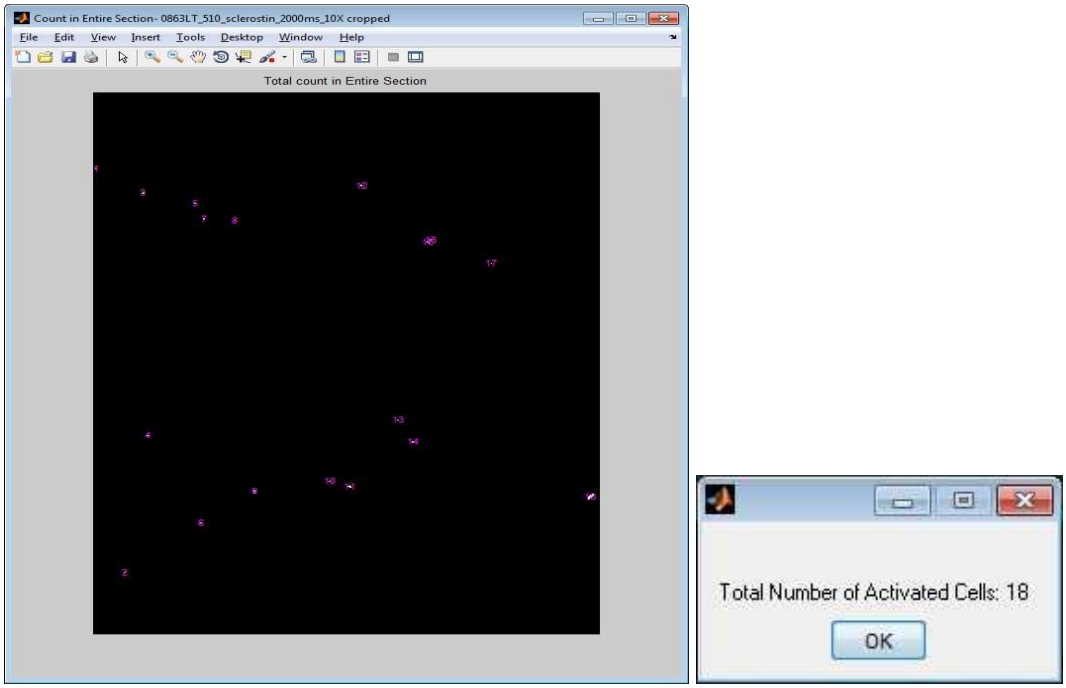


Figure 4.66. Sclerostin Procedure – Numbered cells on the image and its total

Step 7: Save the results in excel sheet Results → Save to Excel

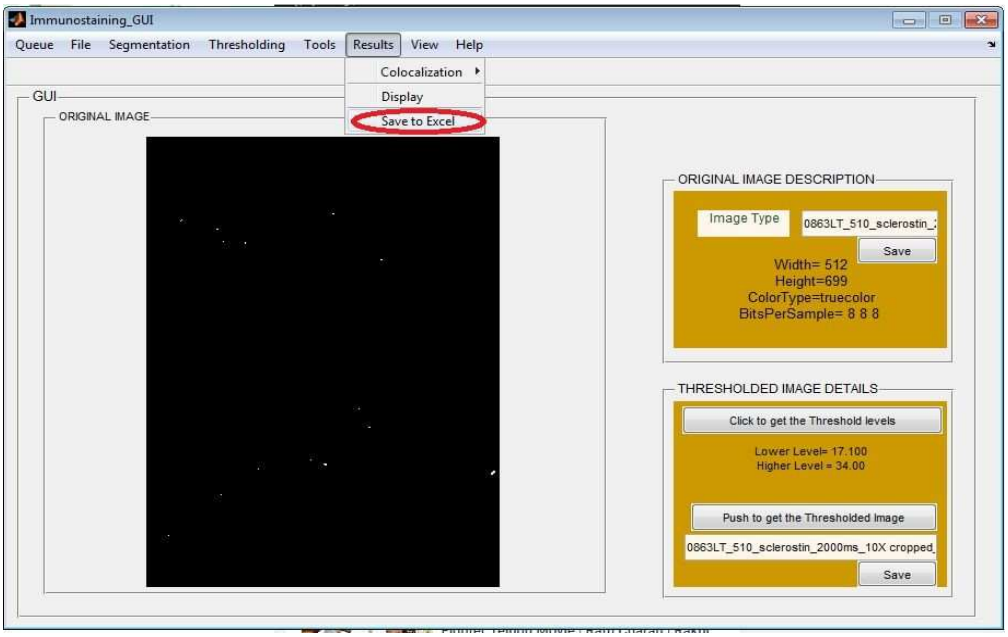


Figure 4.67. Sclerostin Procedure – Save image details to excel sheet

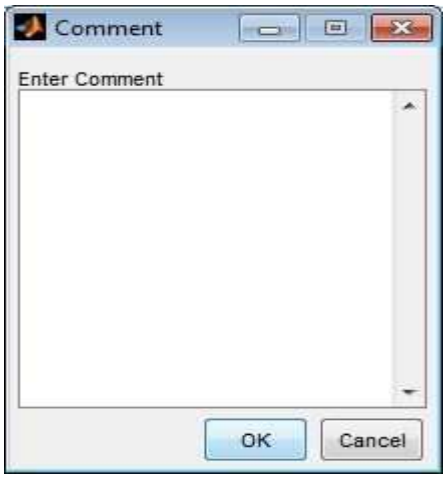


Figure 4.68. Sclerostin Procedure – Comment box

	A	B	C	D	E	F	G	H	I	J
1	Date & Time of Analysis	File Name	Single Lower Threshlod	Single Higher Threshold	Double Low Hue	Double High Hue	Min. Pixel Area	Max. Pixel Area	Total No. of Activated Cells	Total No. of Activated Cells > 10
2	6/5/2016 15:10	0863LT_510_brightfield_20ms_10X cropped	0.2	1	0.441243506	0.65	2	39	15	15
3	6/5/2016 16:57	0863LT_510_DAPI_100ms_10Xcropped	46.5	255			2	63	128	127
4	6/5/2016 17:42	0863LT_510_sclerostin_2000ms_10X cropped	17.1	34			2	10000000	18	18

Figure 4.69. Sclerostin Procedure – Details in excel sheet

Fusion Index Bin Procedure

Step 1: Set Indicator

From the File → Fusion Index Indicator. This is like an indicator just to know the program (Functions) that it is working on fusion Index. Because in Immunostaining each function performs many tasks based on the requirement.

example: Min Max Pixel area Function

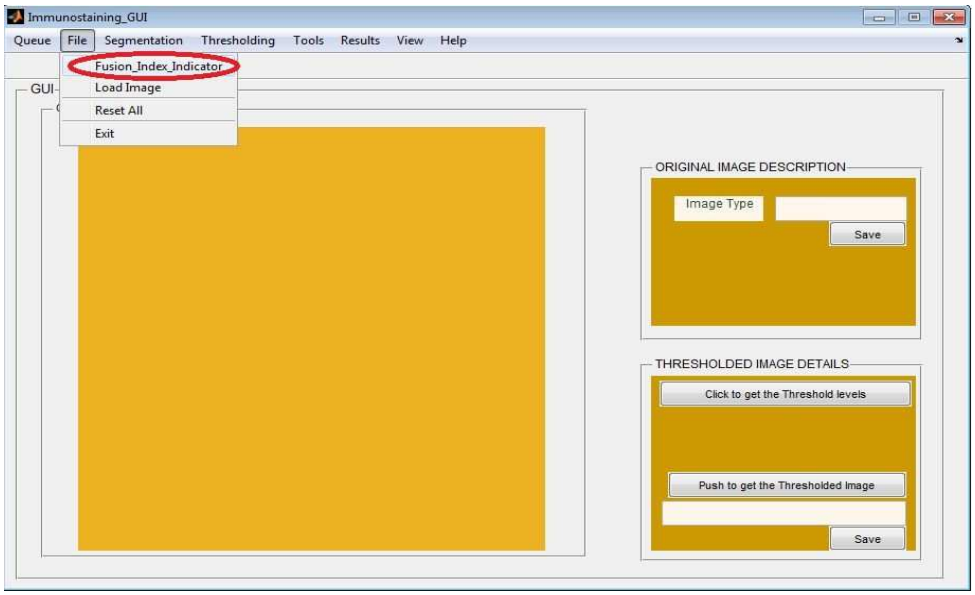


Figure 4.70. Fusion Index Bin Procedure – Set fusion index indicator

Step 2: Load Image

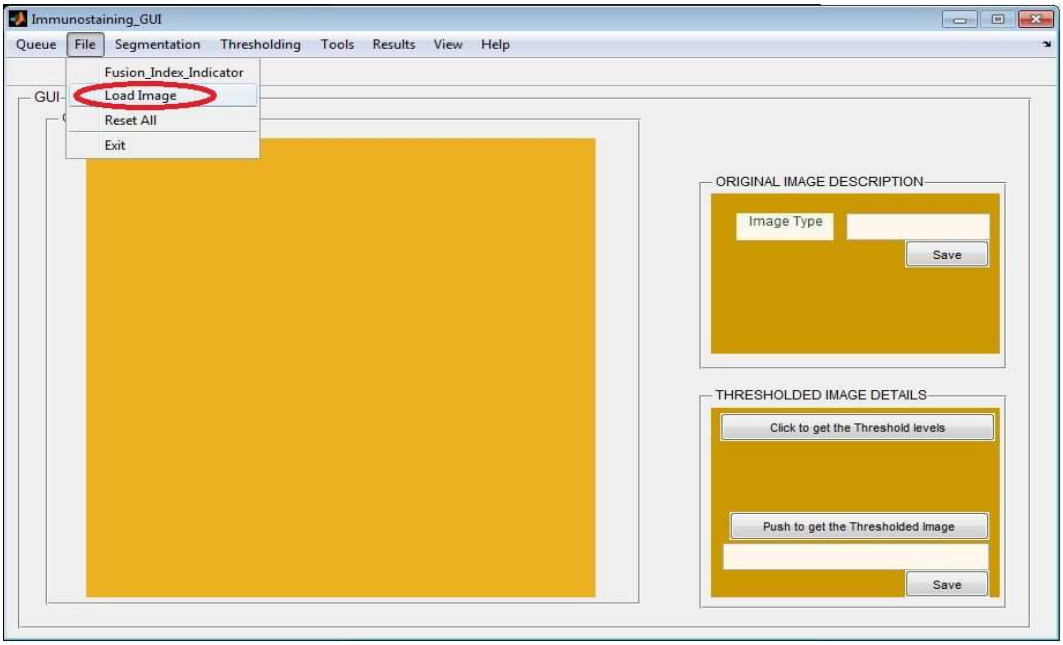


Figure 4.71. Fusion Index Bin Procedure – load fusion index image

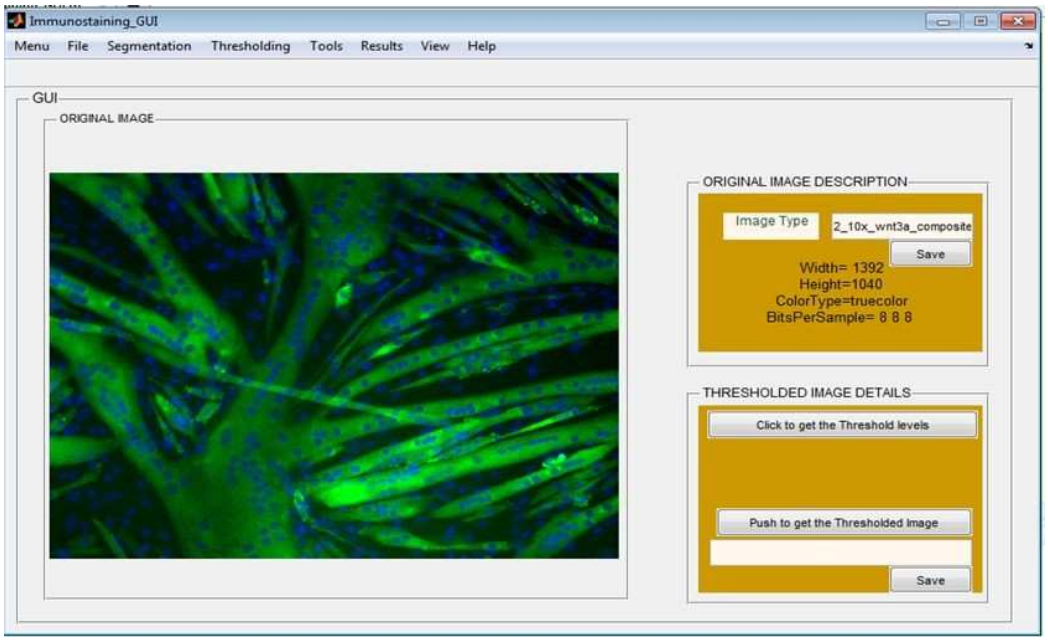


Figure 4.72. Fusion Index Bin Procedure – loaded fusion index image

Step 3: Choose Channel

Segmentation → Choose Channel→ Blue Channel.

It removes green (Myotube) from the image

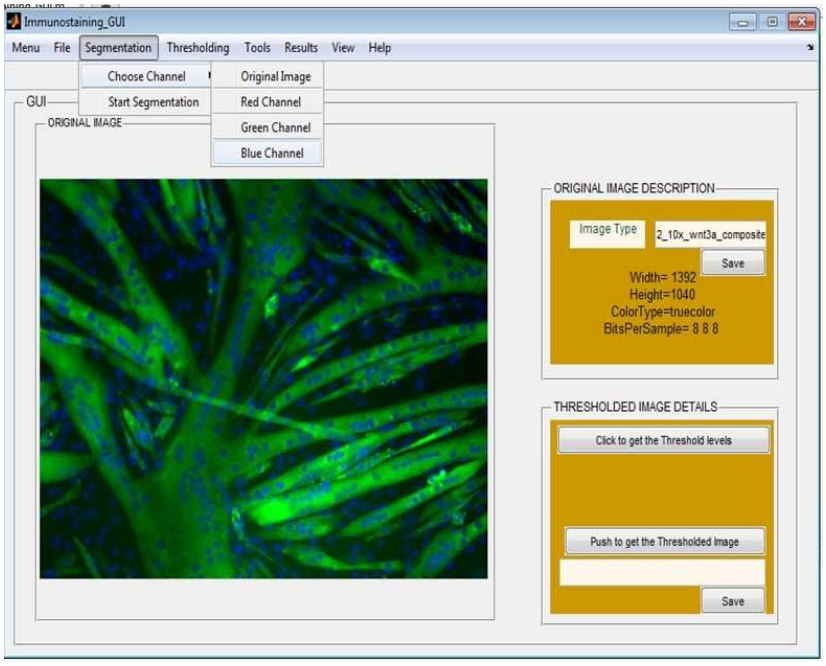


Figure 4.73. Fusion Index Bin Procedure – Choose blue channel to remove myotube

Figure 4.74. Fusion Index Bin Procedure – Changed blue image to black and white

Step 4: Segmentation

Segmentation → Start segmentation→ Choose Entire image → Select Multiple Regions

Select few smallest and biggest single nuclei which is helpful to get minimum, maximum and average size of the nuclei

Save→ Exit

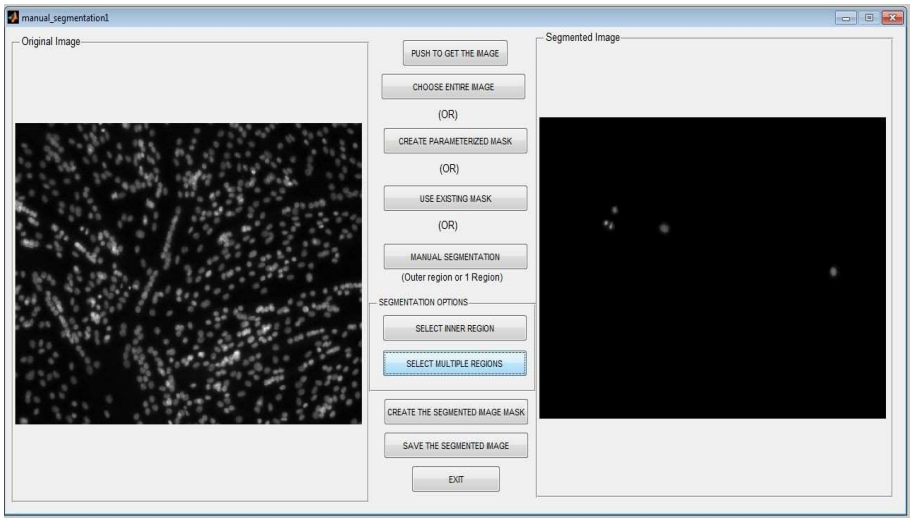


Figure 4.75. Fusion Index Bin Procedure – Select multiple single nuclei

Step 5: Threshold Image

Thresholding→ Single channel→ Histogram based

This is to adjust the threshold value of the nuclei selected, it is better taking the default values.

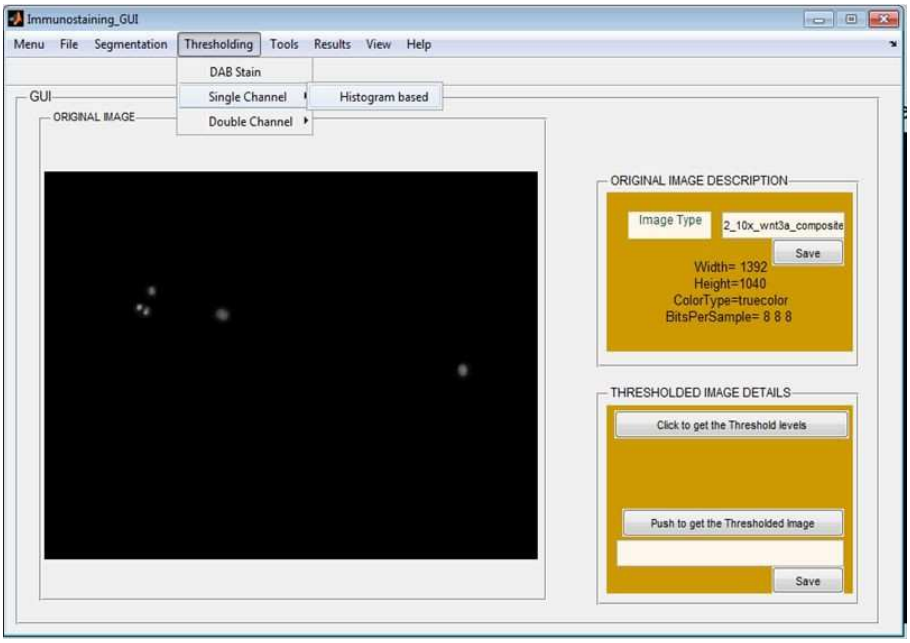


Figure 4.76. Fusion Index Bin Procedure – Select single channel histogram

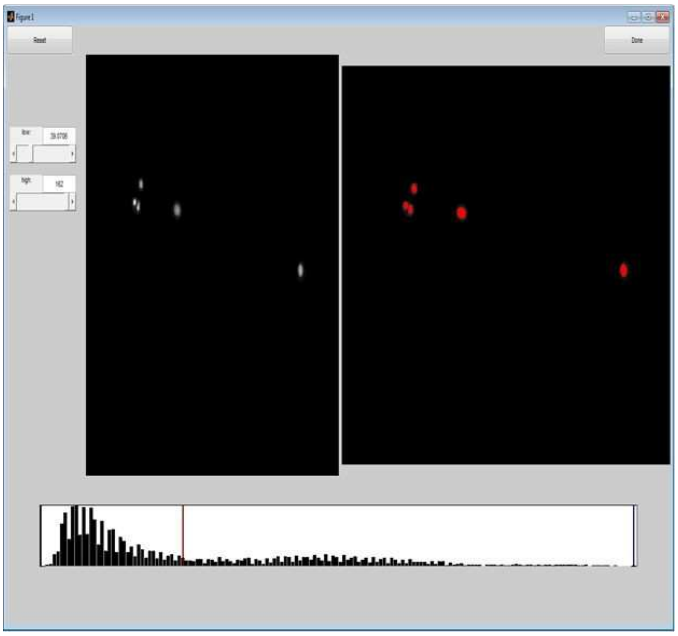


Figure 4.77. Fusion Index Bin Procedure – Set threshold value

Tools → Settings → Minimum Maximum Pixel Area → Single

It gives information like maximum, minimum and average of the single nuclei in a window and graph (Histogram) form

Step 6: Select single nuclei minimum, maximum and average values

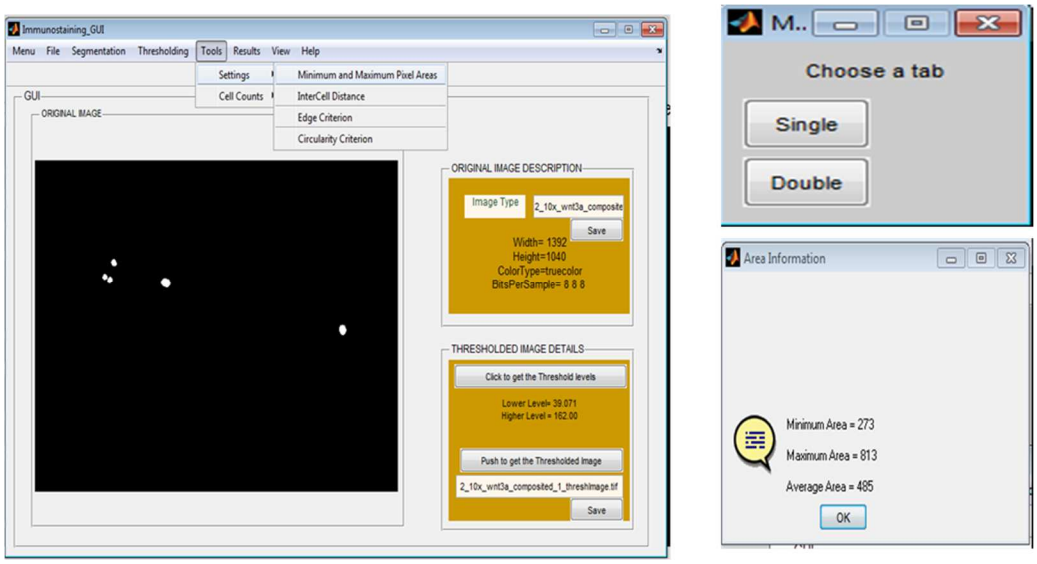


Figure 4.78. Fusion Index Bin Procedure – Calculate minimum maximum and average single nuclei area

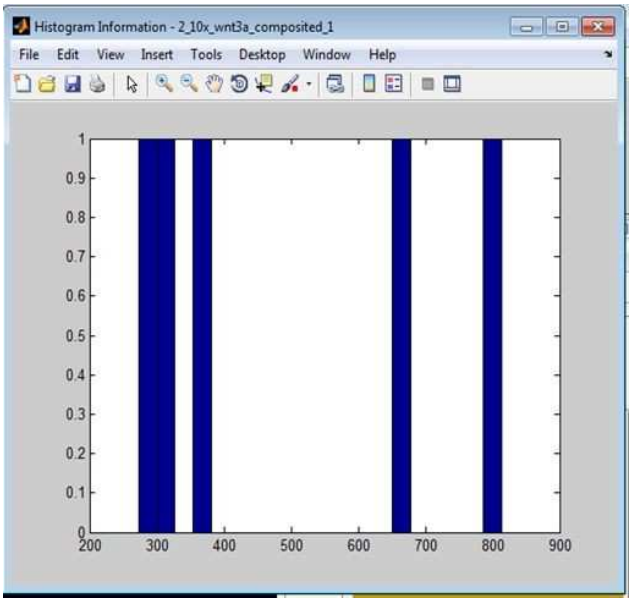


Figure 4.79. Fusion Index Bin Procedure – Histogram Information

Step 7:

Repeat the same procedure for double nucle from start segmentation till min max pixel area but, in last step instead of selecting single tab select ‘double’ tab

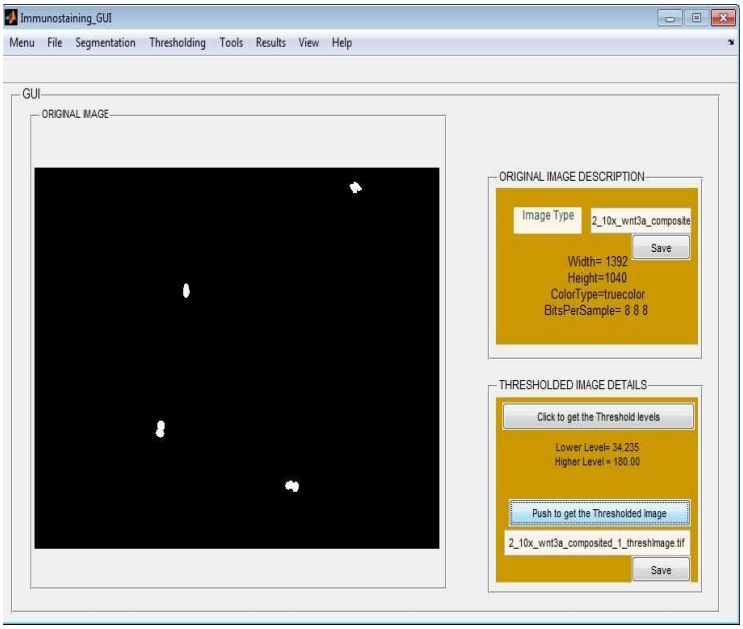


Figure 4.80. Fusion Index Bin Procedure – Select two fused nuclei

Step 8: Set Bin Range

Tools → Cell Counts → Fusion Index Bin

Select the bin range minimum, maximum and average of single and double nuclei based on the information displayed.

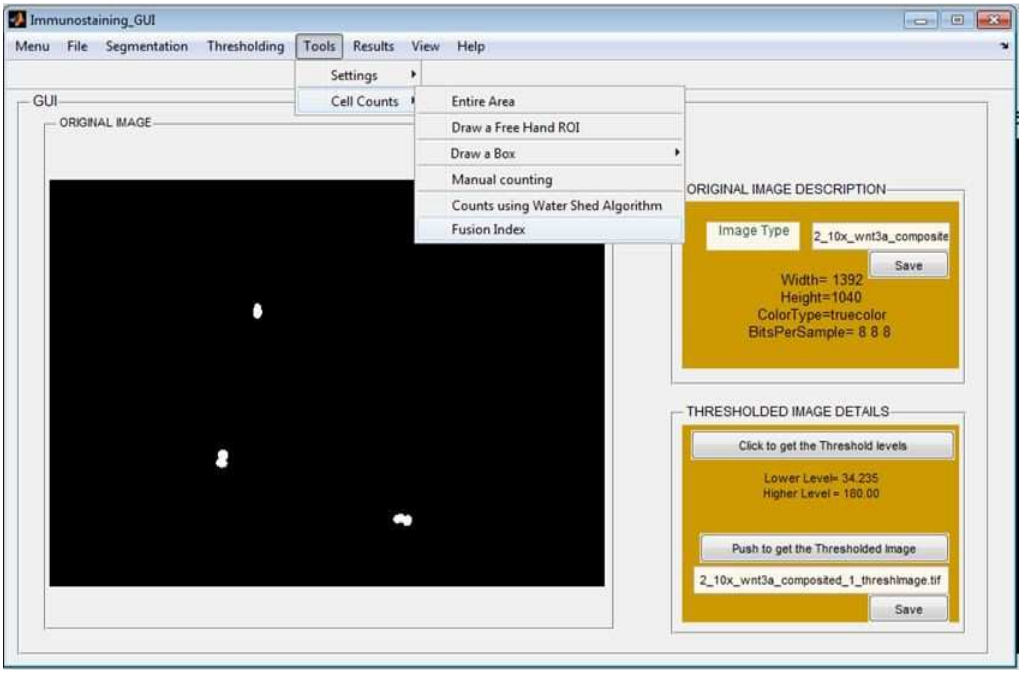


Figure 4.81. Fusion Index Bin Procedure – Select fusion index cell count.

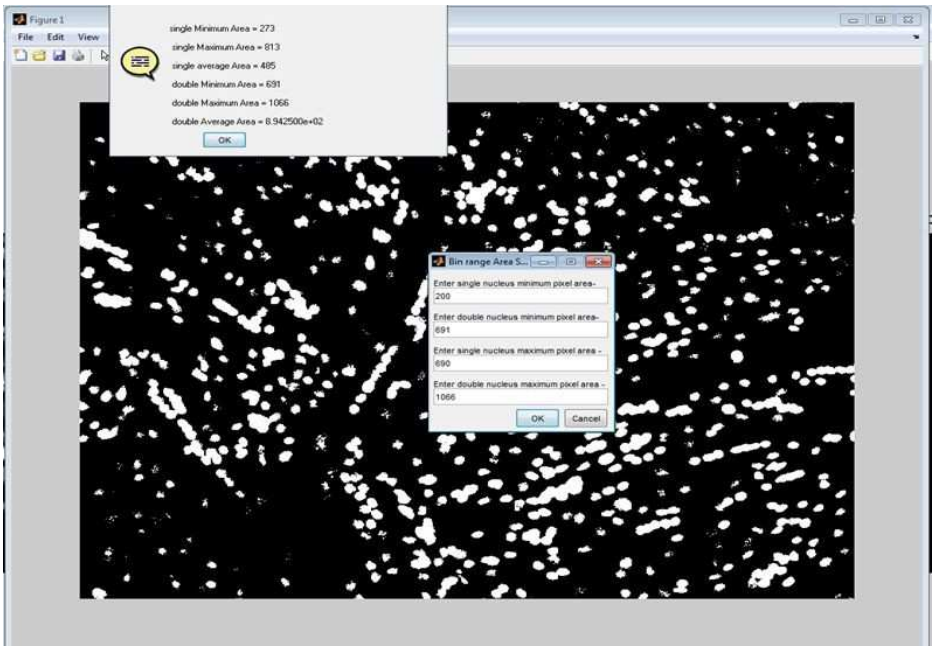


Figure 4.82. Fusion Index Bin Procedure – Set minimum and maximum area of single and double nuclei

Total number of nuclei in the pop-up box with numbering on the image

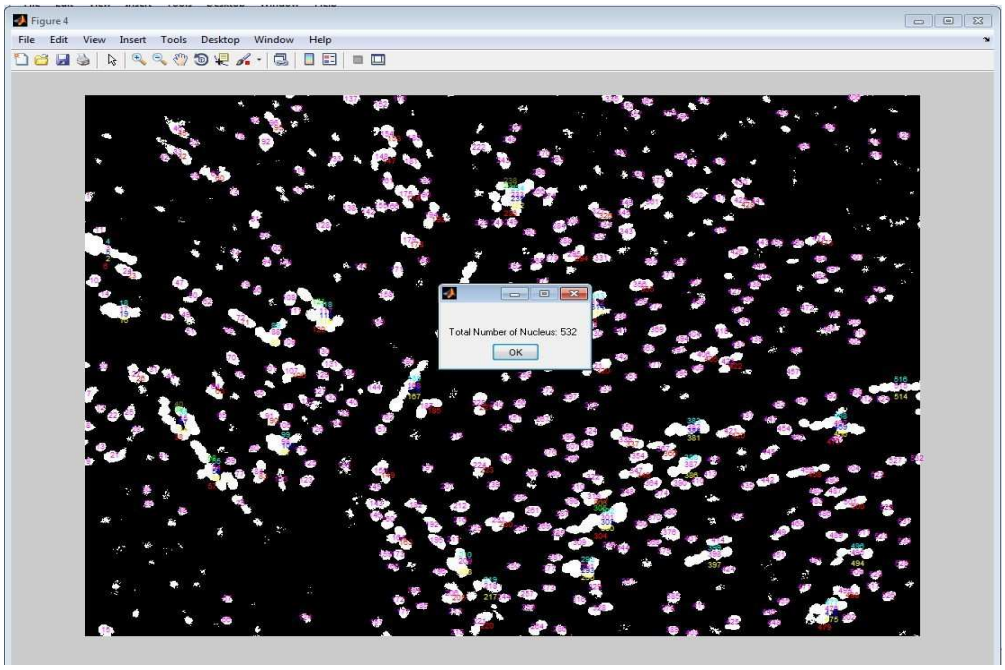


Figure 4.83. Fusion Index Bin Procedure – Numbered nuclei on the image and its count

You can reduce the nuclei count which are counted more than its number just by left click

once done right click.

Can undo by pressing backspace

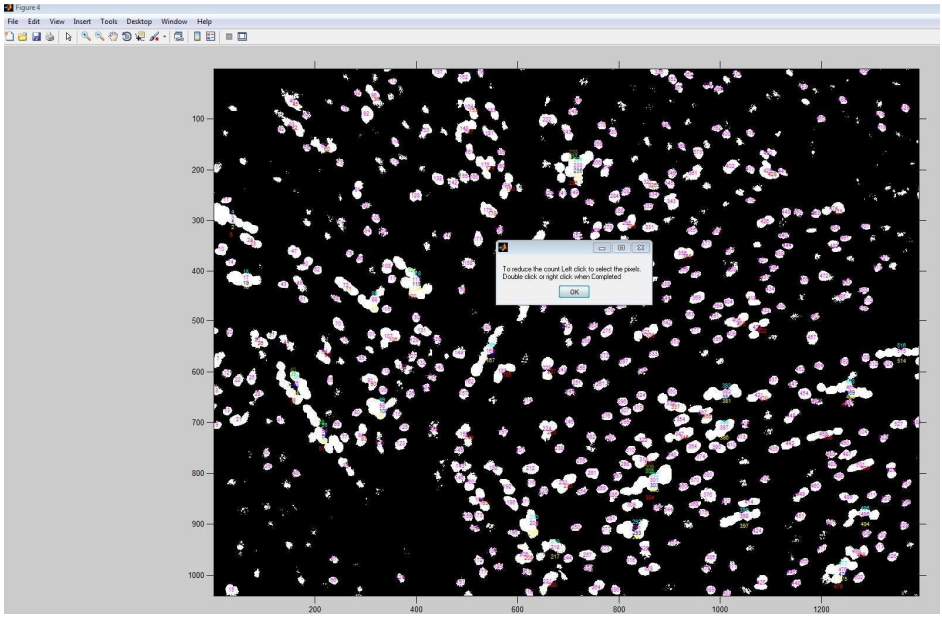


Figure 4.84. Fusion Index Bin Procedure – Removing excess nuclei count

You can increase the nuclei count which are counted less than its number just by left click once done right click.

Can undo by pressing escape

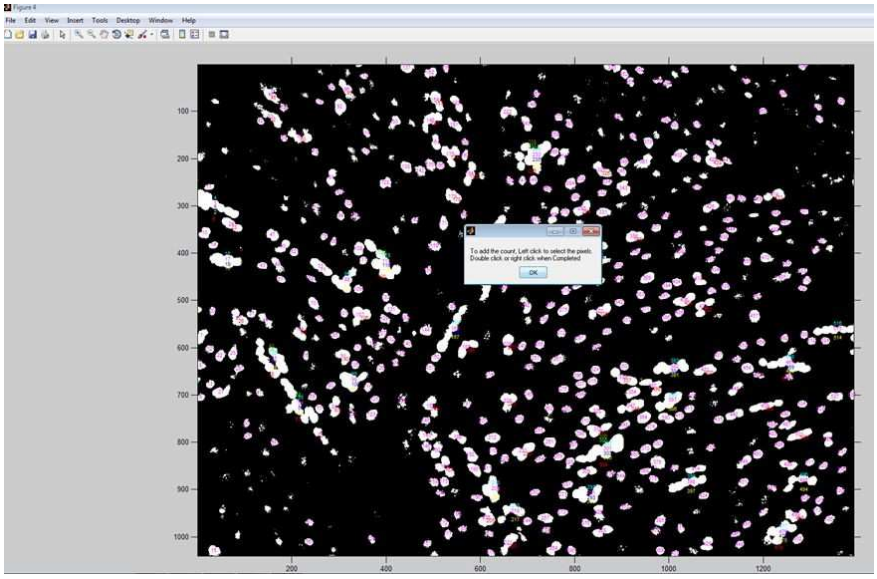


Figure 4.85. Fusion Index Bin Procedure – Add uncounted nuclei

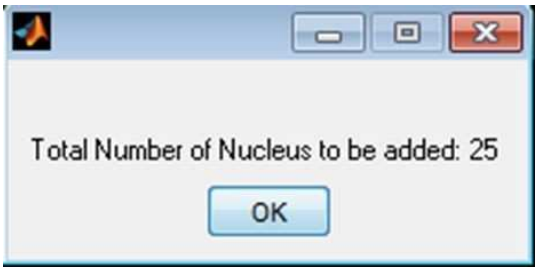


Figure 4.86. Fusion Index Bin Procedure – Total number of added nuclei count

Displays count of nuclei then choose Blue button from pop up window

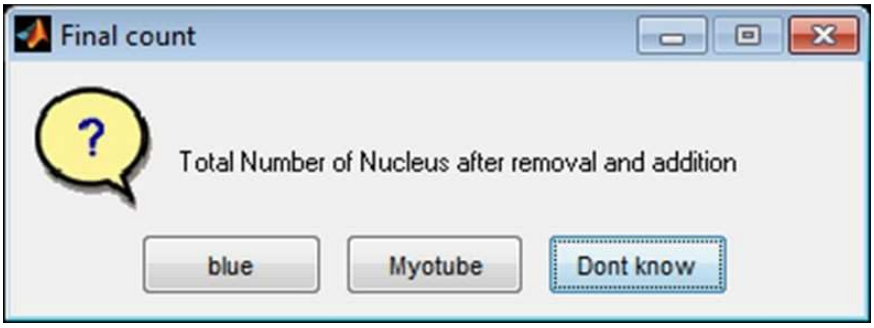


Figure 4.87. Fusion Index Bin Procedure – Choose the option blue

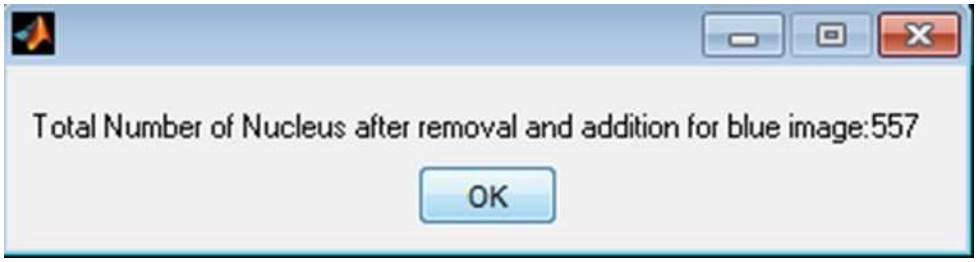


Figure 4.88. Fusion Index Bin Procedure – Total number of nuclei after removal and addition for blue image

Method to Process Myotube Images (Green)

Step 9: Load Image (same image)

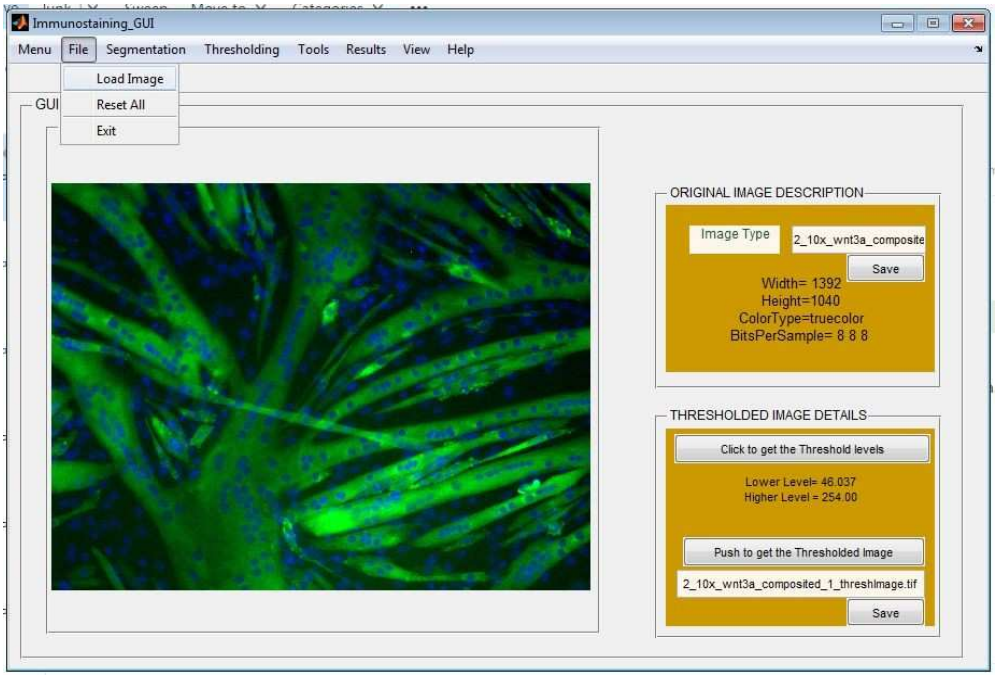


Figure 4.89. Fusion Index Bin Procedure – load fusion index image

Step 10: Segment myotubes

Segmentation → Choose Channel → Green channel (instead of blue)

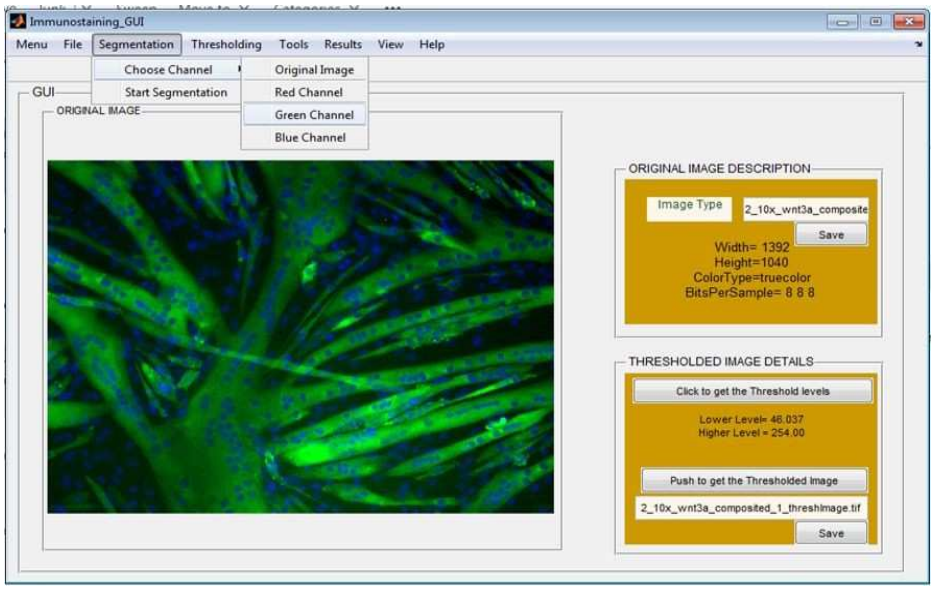


Figure 4.90. Fusion Index Bin Procedure – Choose the green channel to highlight myotubes

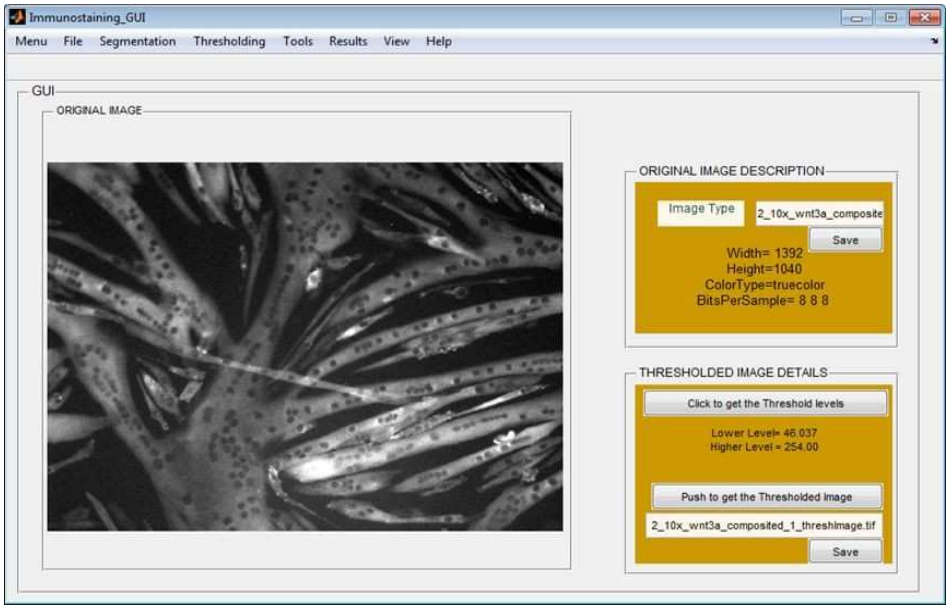


Figure 4.91. Fusion Index Bin Procedure – Image after channel selection

Segmentation→ Start Segmentation→ Choose Entire Image→ Select Multiple Regions

After segmentation create the segmented Image Mask→ save the Segmented Image→

Exit

Step 11: Segmenting Myotube

Segmentation → start segmentation → Select Multiple region → save the segmented Image

→ exit

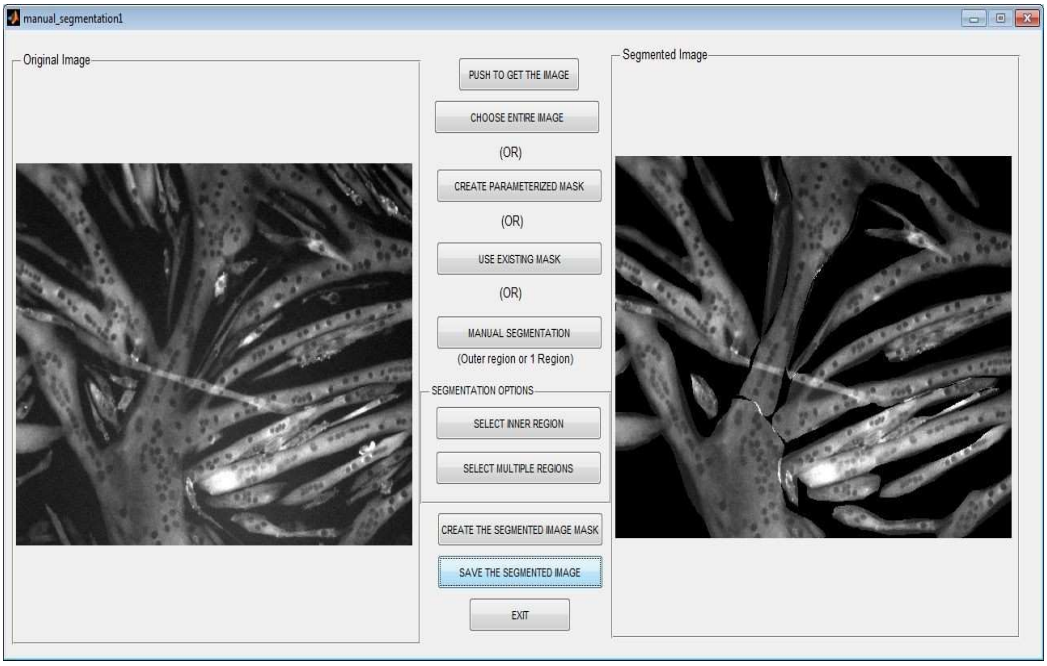


Figure 4.92. Fusion Index Bin Procedure – select multiple region having more than three nuclei of myotubes

Step 12: Threshold

Thresholding → Single Channel → Histogram Based

Change the threshold values until whole segmented image is red Save the thresholded image

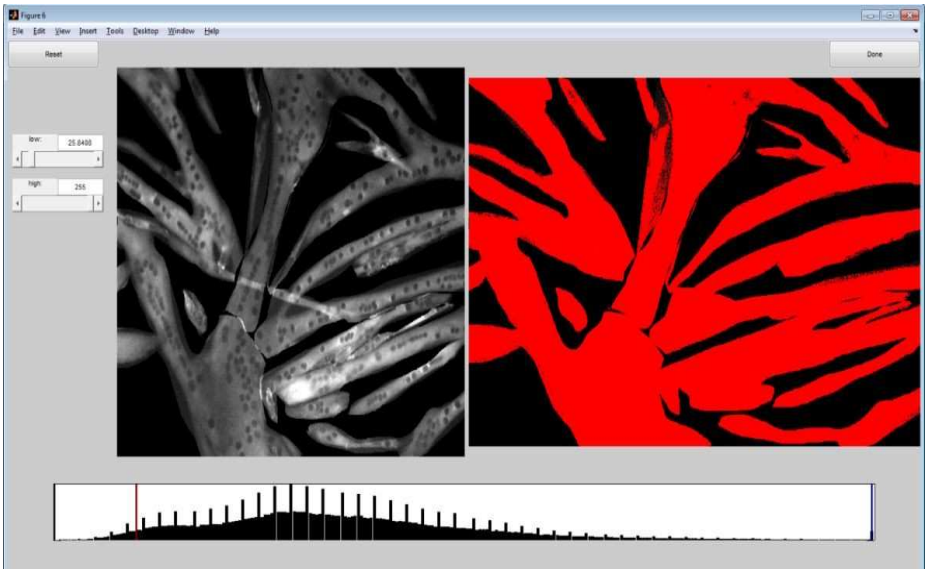


Figure 4.93. Fusion Index Bin Procedure – Threshold myotubes

BLUE + GREEN

Step 13: Load an Image

Load Image → Choose Channel → Blue Channel→ Segmentation → Start Segmentation→

Use Existing

Choose the mask (Green Image Mask) → Save→ Exit

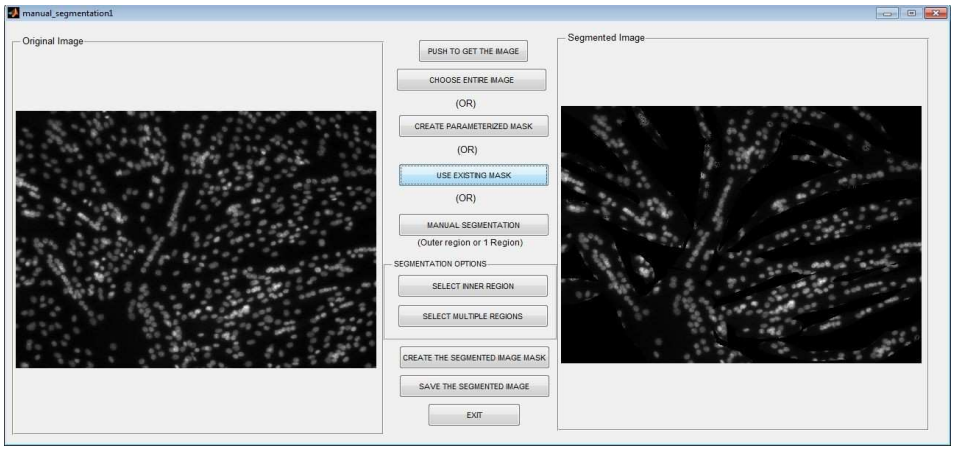


Figure 4.94. Fusion Index Bin Procedure – Use myotube mask on DAPI image

Step 14: Threshold Image

Threshold→ Single channel→ Histogram Based

Reduce the threshold so that it sizes matches with the original image nuclei size beside it

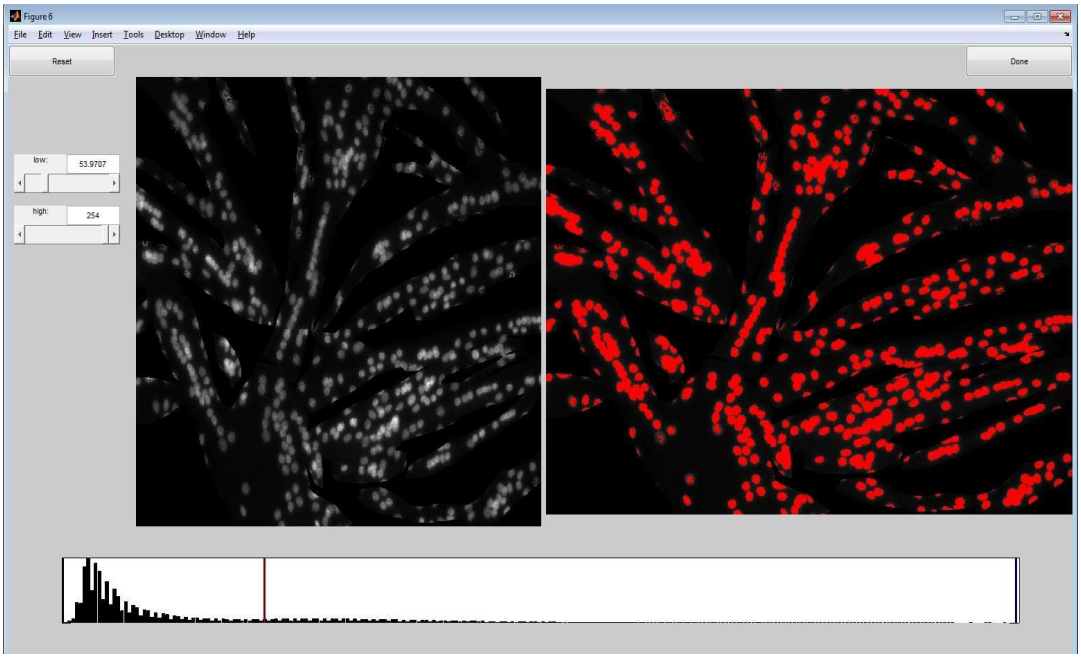


Figure 4.95. Fusion Index Bin Procedure – Threshold image after overlap

Step 15:

Tools → Cell Count→ Fusion Index Bin

Select the bin range minimum, maximum and average of single and double nuclei based on the information displayed and Total number of Nuclei

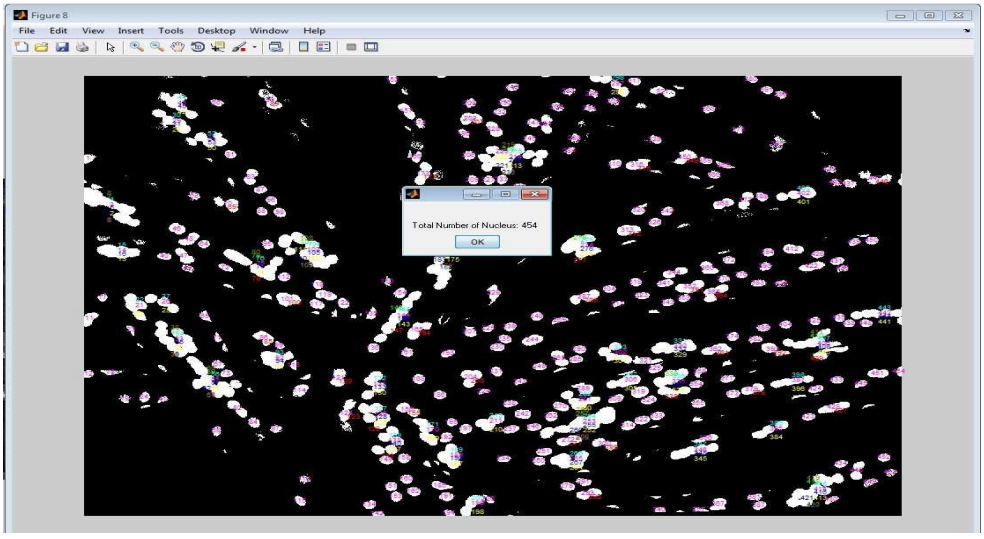


Figure 4.96. Fusion Index Bin Procedure – Total number of nuclei after using mask

Step 16:

Tools → Cell Count→ Add or Remove

Reduce: You can reduce the nuclei count which are counted more than its number just by left click on the image once done right click.

Can undo by pressing escape

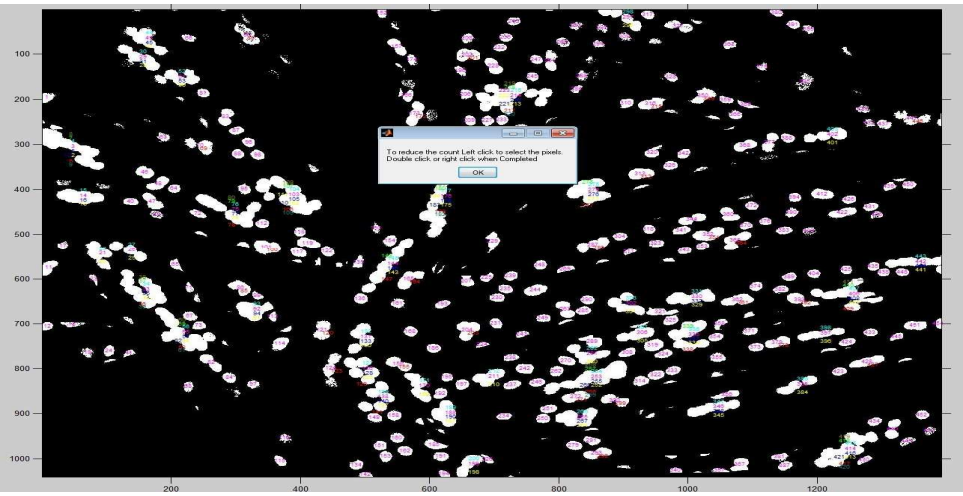


Figure 4.97. Fusion Index Bin Procedure – Reduce excess nuclei count

Increase: You can increase the nuclei count which are counted less than its number just by left click on the image once done right click.

Can undo by pressing escape

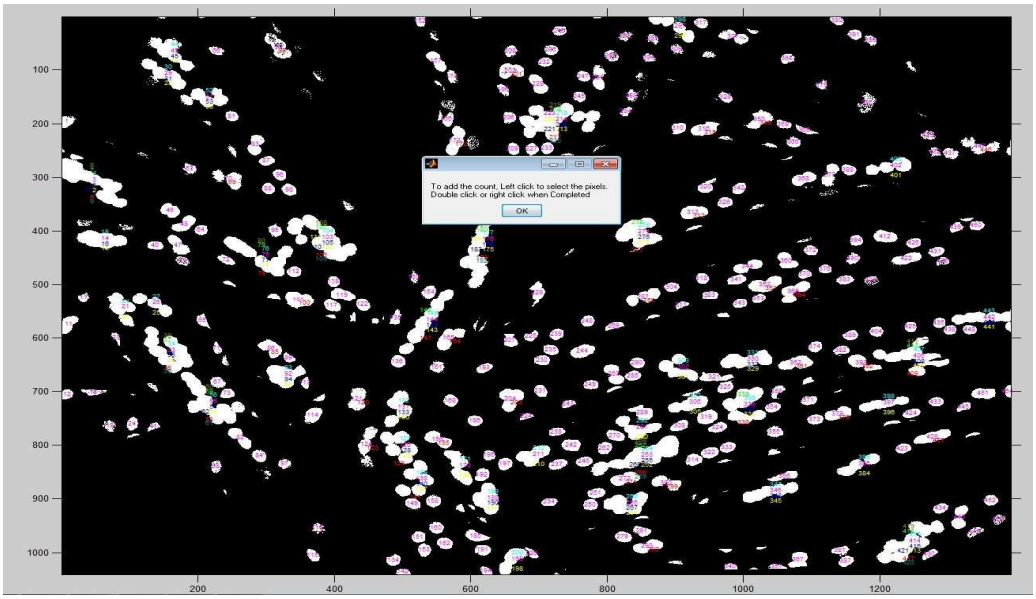


Figure 4.98. Fusion Index Bin Procedure – Add uncounted nuclei

Select Myotube button from the pop-up window, which gives total number of nuclei count after manual removal and addition of nuclei count for Myotube

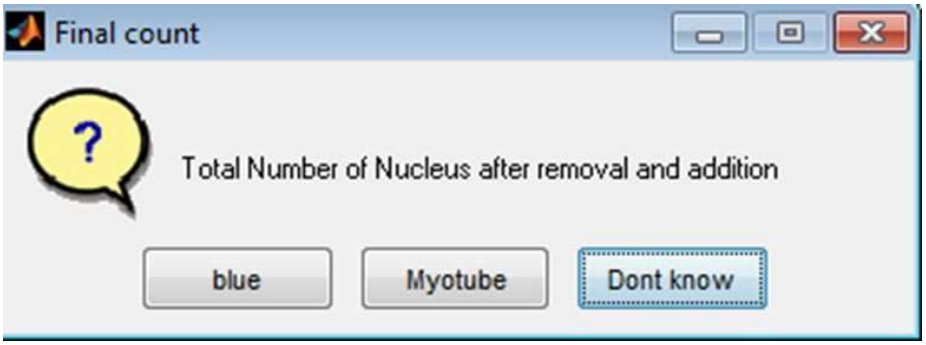


Figure 4.99. Fusion Index Bin Procedure –Type of image indicator

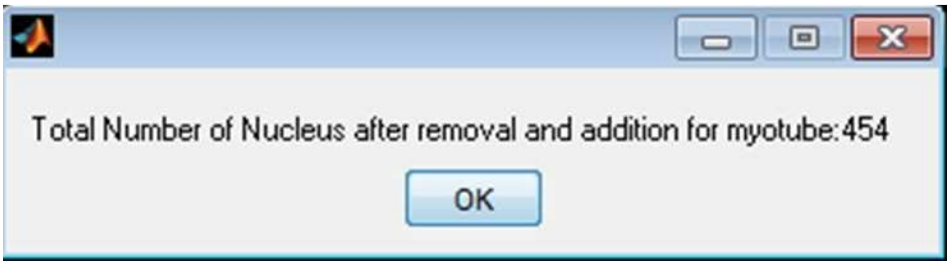


Figure 4.100. Fusion Index Bin Procedure – Total number of nuclei count

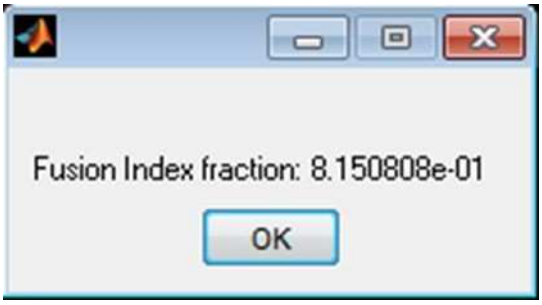


Figure 4.101. Fusion Index Bin Procedure – Fraction of fusion index

Results→ Save to excel→ comment

Step 17: Save results to excel sheet

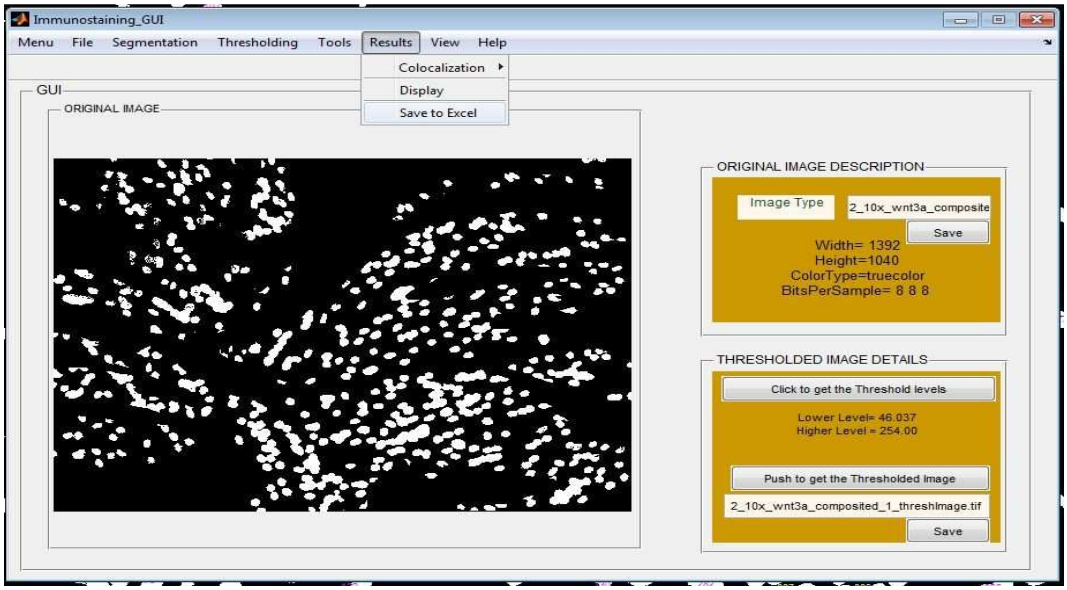


Figure 4.102. Fusion Index Bin Procedure – option to save details to excel

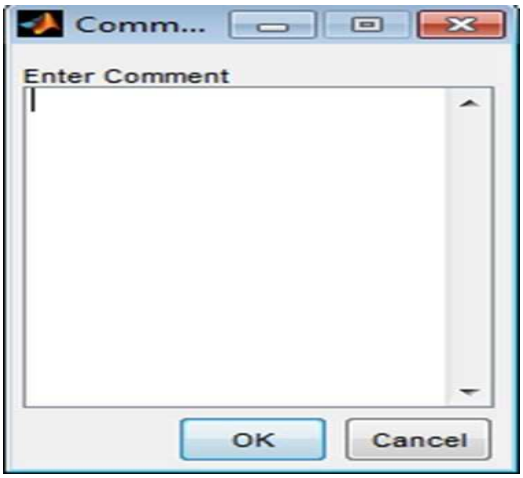
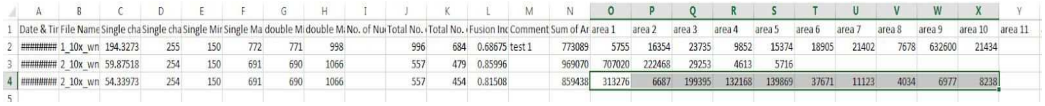


Figure 4.103. Fusion Index Bin Procedure – Comment box

Records in excel sheet



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
1	Date & Tir	File Name	Single cha	Single cha	Single Mir	Single Ma	double Mi	double Ma	No. of Nu	Total No.	Total No.	Fusion Inc	Comment	Sum of Ar	area 1	area 2	area 3	area 4	area 5	area 6	area 7	area 8	area 9	area 10	area 11
2	########	1_10x_wn	194.3273	255	150	772	771	958		996	684	0.68675	test 1	773089	5755	16354	23735	9852	15374	18905	21402	7678	632600	21434	
3	########	2_10x_wn	59.87518	254	150	691	690	1066		557	479	0.85996		969070	707020	222468	29253	4613	5716						
4	########	2_10x_wn	54.33973	254	150	691	690	1066		557	454	0.81508		859438	313276	6687	199395	132168	139869	37671	11123	4034	6977	8238	
5																									

Figure 4.104. Fusion Index Bin Procedure – fusion index details in excel sheet

Fusion Index Area Procedure

Step 1: Set Indicator

From the File → Fusion Index Indicator. This is like an indicator just to know the software modules that it is working for fusion Index.

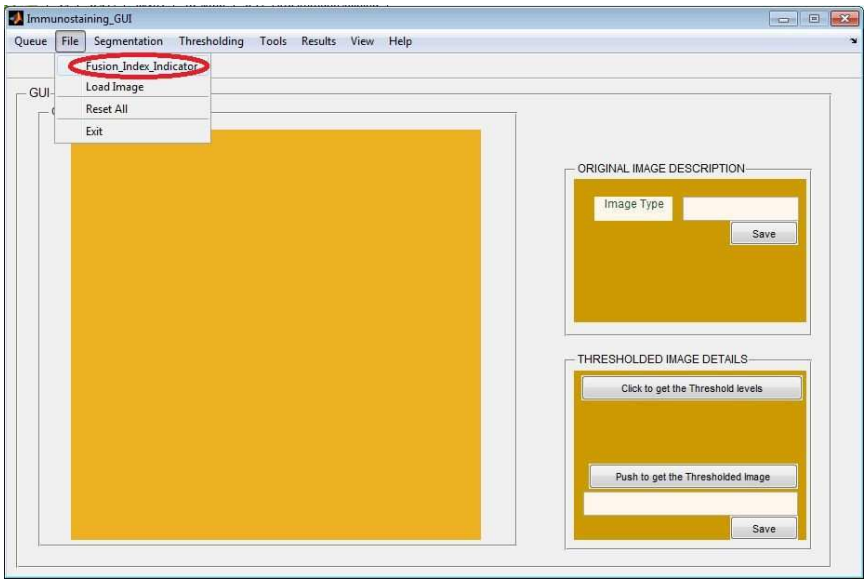


Figure 4.105. Fusion Index Area Procedure – Set fusion index indicator

Step 2: Load Image

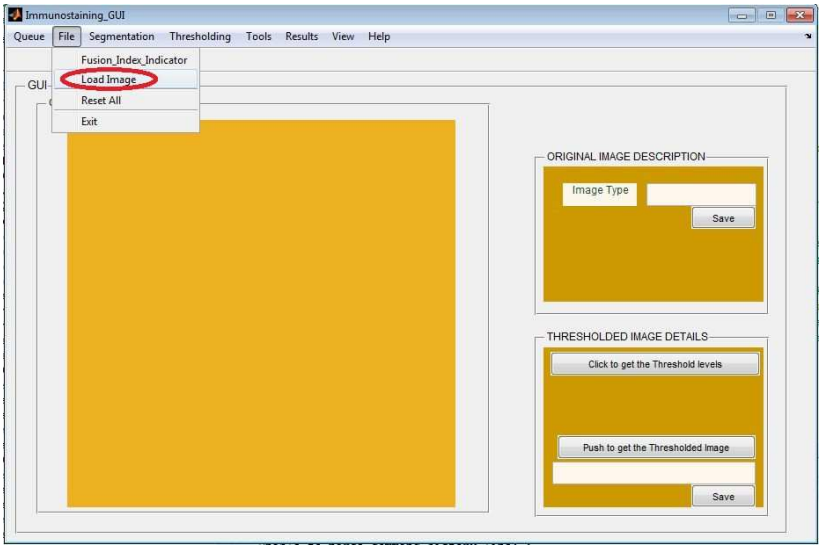


Figure 4.106. Fusion Index Area Procedure – load fusion index image

Figure 4.107. Fusion Index Area Procedure – loaded fusion index image

Step 3: Choose Channel

Segmentation → Choose Channel→ Blue Channel

It removes green(myotubes) from the Image

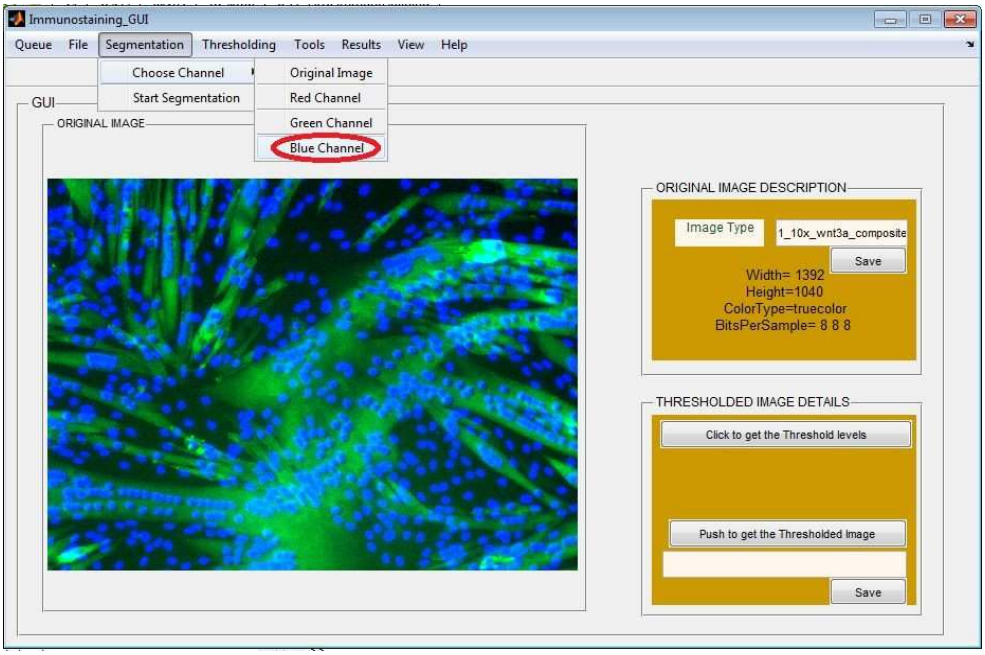


Figure 4.108. Fusion Index Area Procedure – Choose blue channel option for the image

Figure 4.109. 4.6 Fusion Index Area Procedure – Converted blue image to black and white image

Step 4: Segmentation

Segmentation → Start segmentation→ Choose Entire image → Select Multiple Regions

Select few smallest and biggest single nuclei which is helpful to get minimum, maximum and average size of the nuclei

Save→ Exit

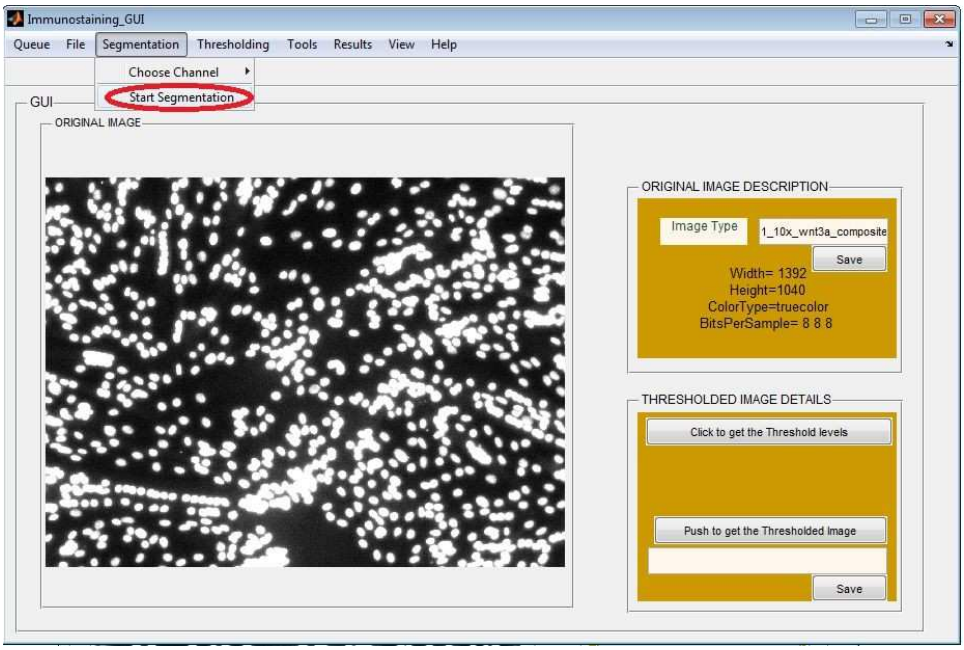


Figure 4.110. Fusion Index Area Procedure – Choose segmentation option

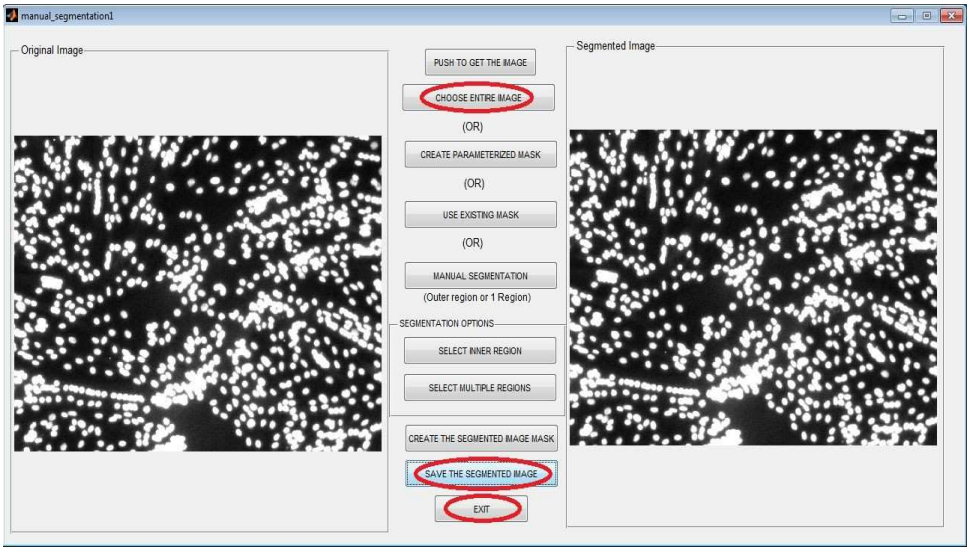


Figure 4.111. Fusion Index Area Procedure – save the whole image

Step 5: Threhsold Image

Thresholding→ Single channel→ Histogram based

This is to adjust the threshold value of the nuclei selected it is better taking the default values

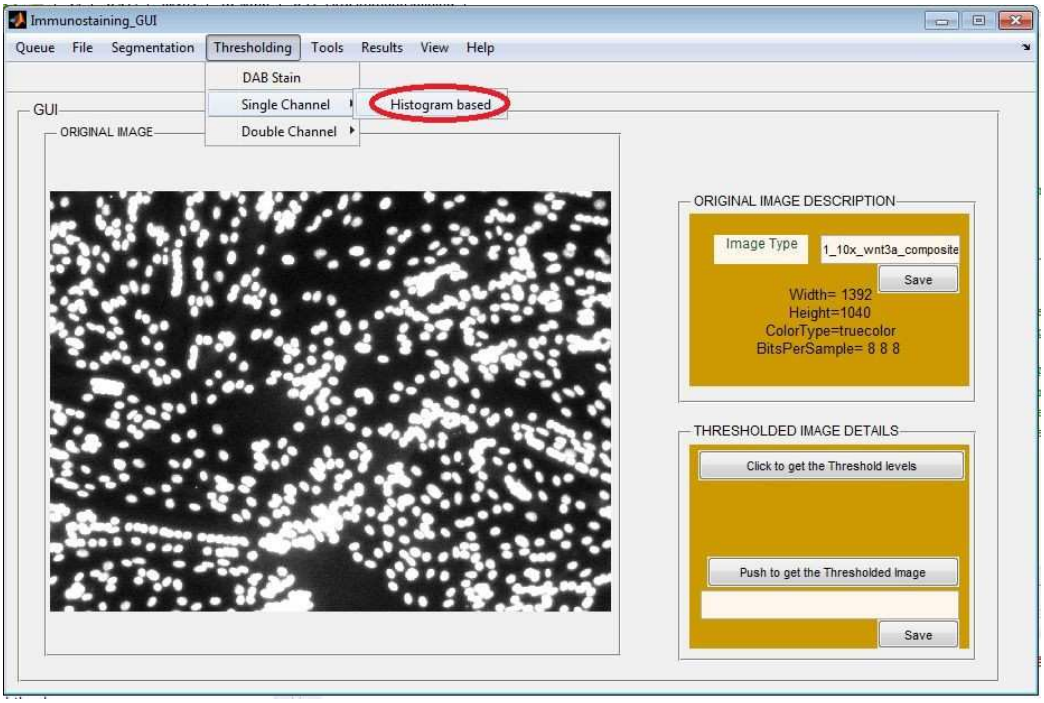


Figure 4.112. Fusion Index Area Procedure – Choose single channel histogram

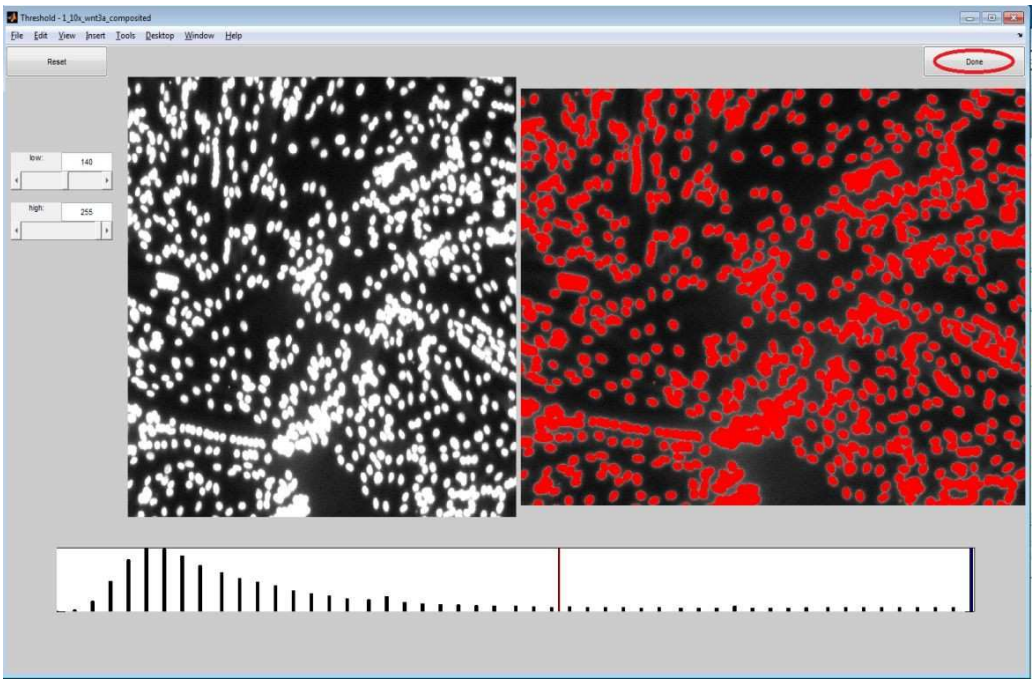


Figure 4.113. Fusion Index Area Procedure – threshold image

Figure 4.114. Fusion Index Area Procedure – Save thresholded image

Step 6: Tools → Cell Counts→ Fusion Index Area

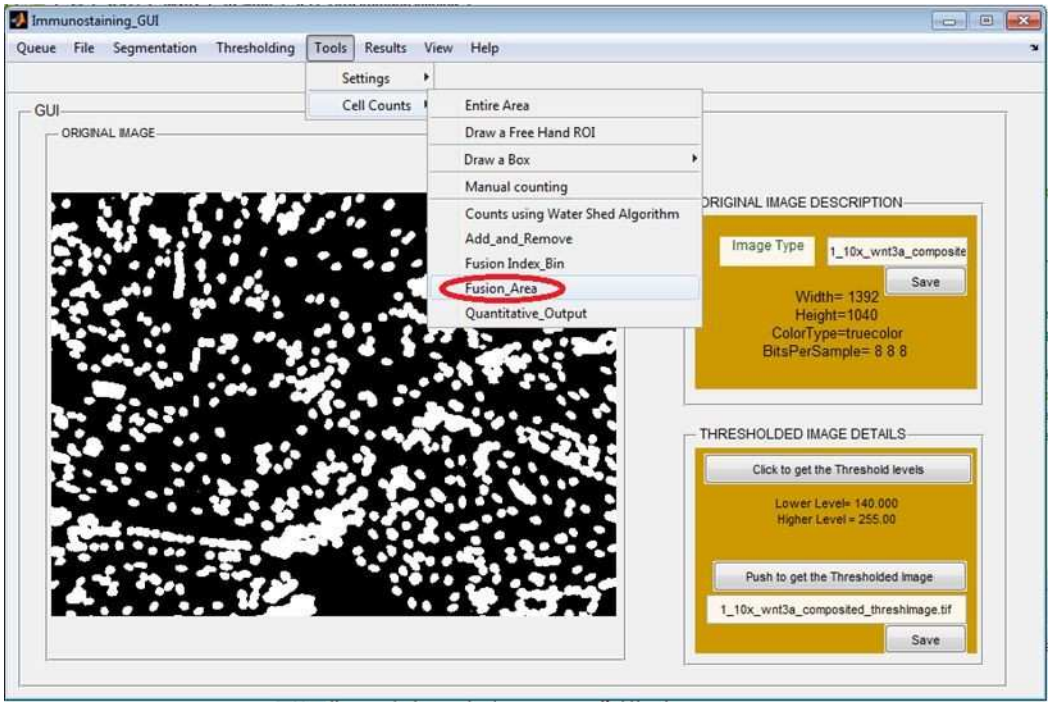


Figure 4.115. Fusion Index Area Procedure – Choose fusion area cell count option

Figure 4.116. Fusion Index Area Procedure – Choose type of image processing Load Image

Figure 4.117. Fusion Index Area Procedure – Load fusion index image

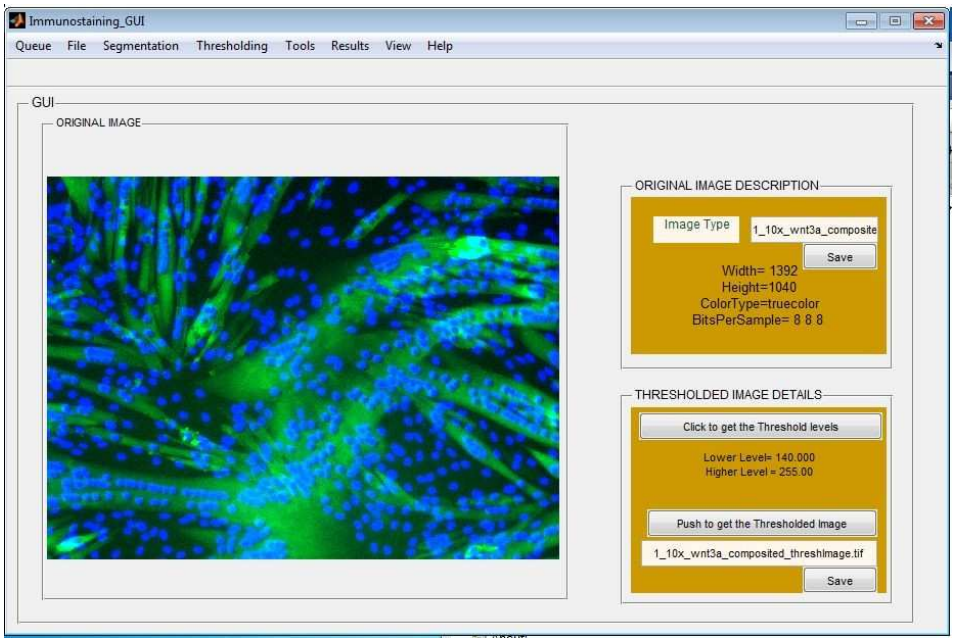


Figure 4.118. Fusion Index Area Procedure – Loaded fusion index image

Step 10: Segment myotubes

Segmentation → Choose Channel→ Green channel (instead of blue)

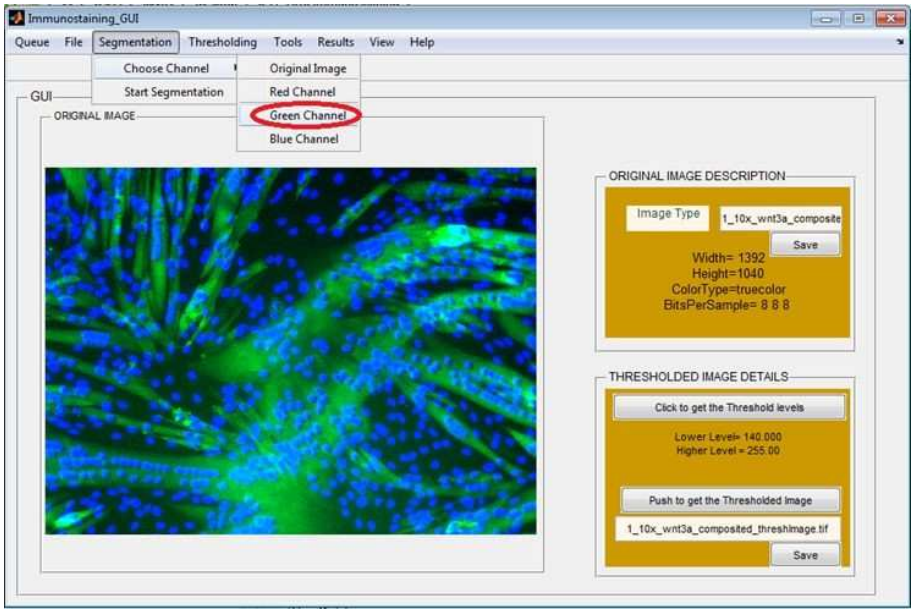


Figure 4.119. Fusion Index Area Procedure – Choose green channel option

Step 11: Segmenting Myotube

Segmentation → start segmentation → Select Multiple region → save the segmented Image → exit

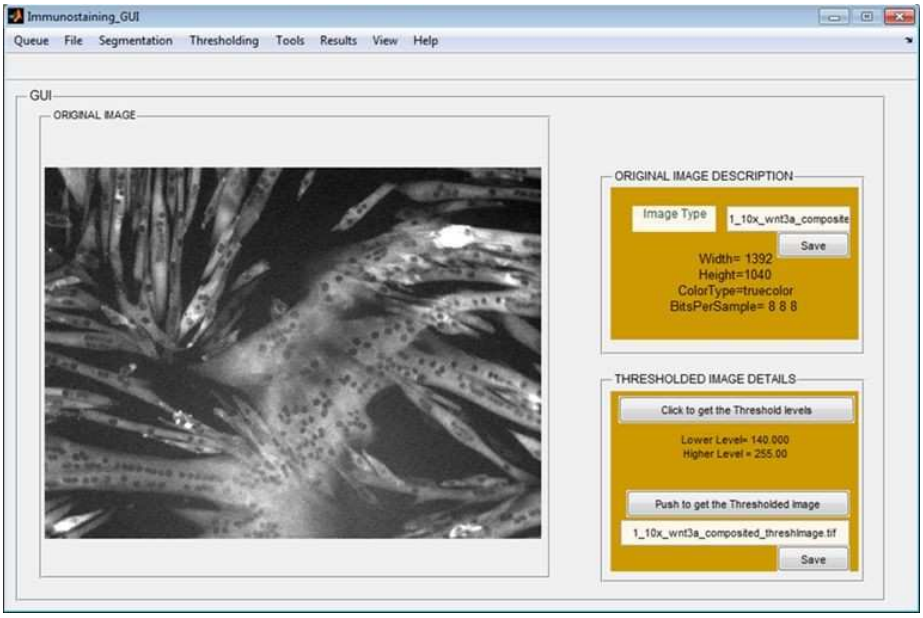


Figure 4.120. Fusion Index Area Procedure – Change green image to black and white images

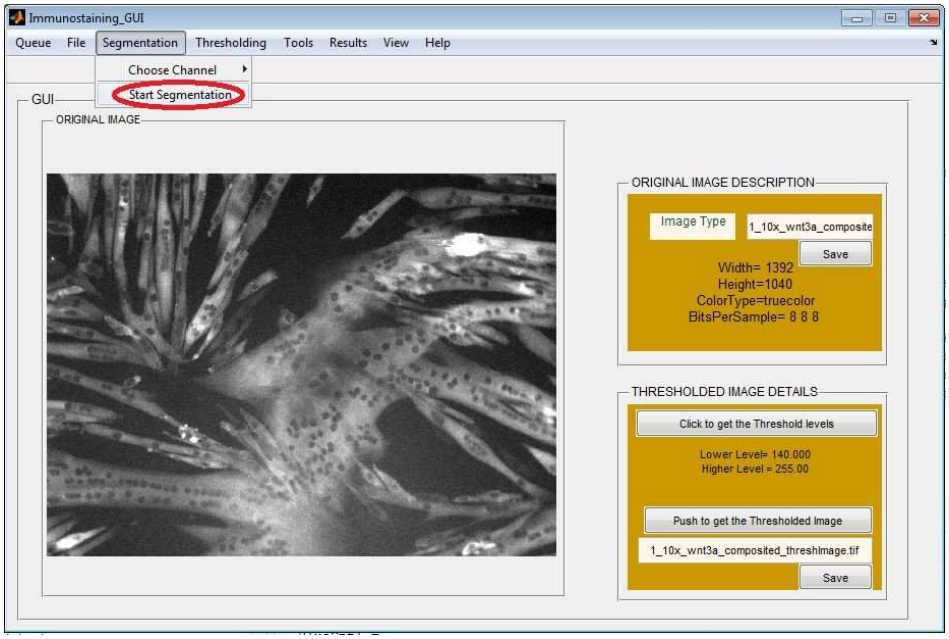


Figure 4.121. Fusion Index Area Procedure – Choose start segmentation option

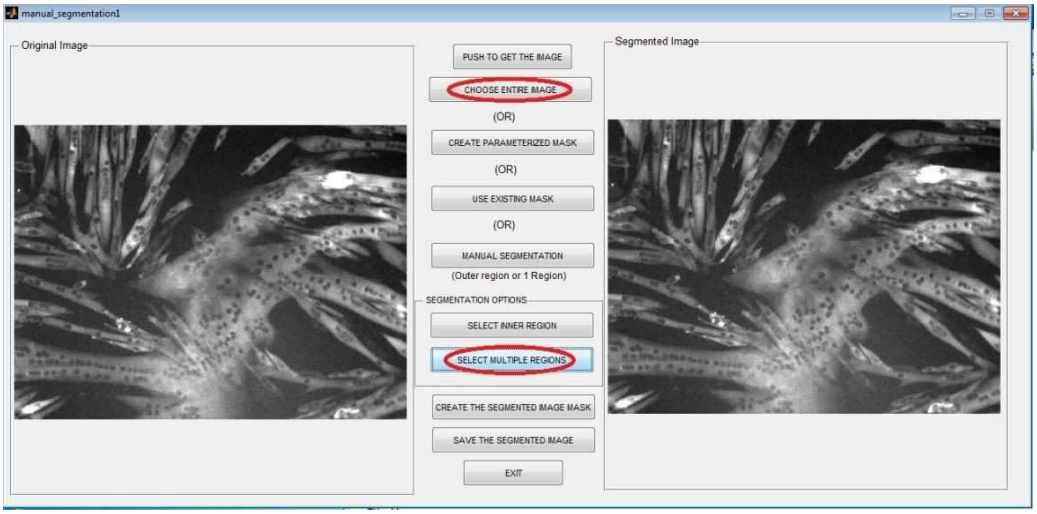


Figure 4.122. Fusion Index Area Procedure – Choose segmentation option

Figure 4.123. Fusion Index Area Procedure – Remove unnecessary myotubes

Step 12: Threshold

Thresholding→ Single Channel→ Histogram Based

Change the threshold values until whole segmented image is red

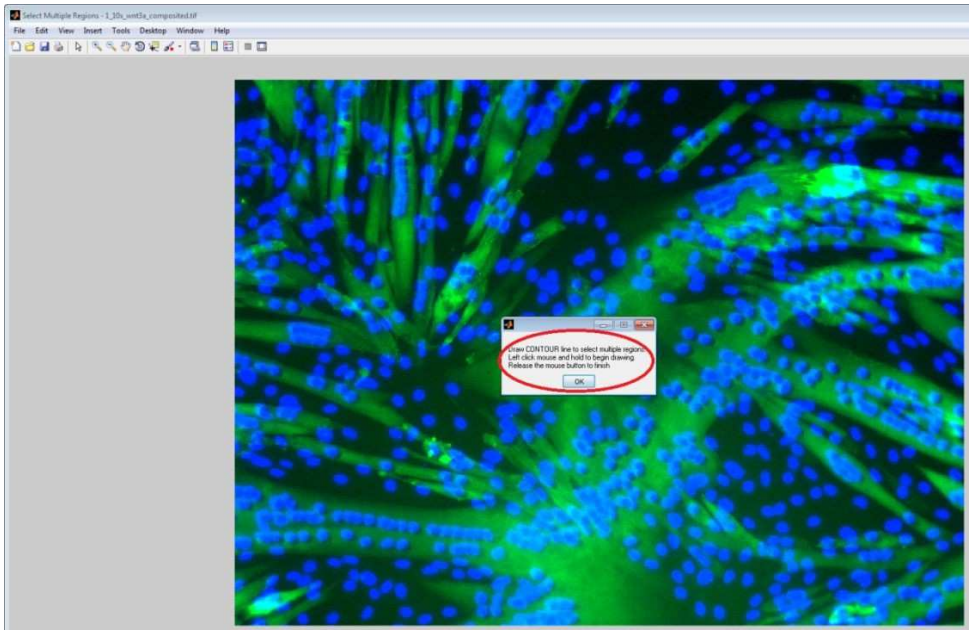


Figure 4.124. Fusion Index Area Procedure – Draw contour to remove myotubes

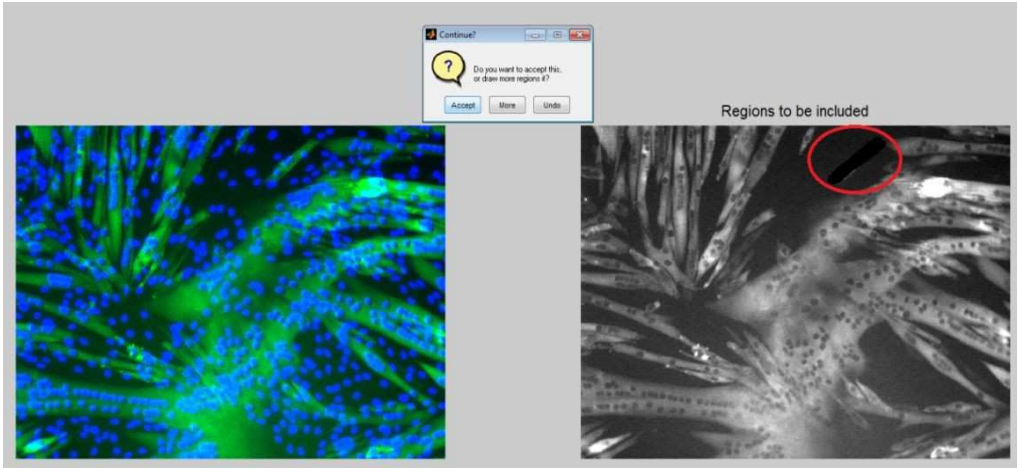


Figure 4.125. Fusion Index Area Procedure – Before and after removed myotubes

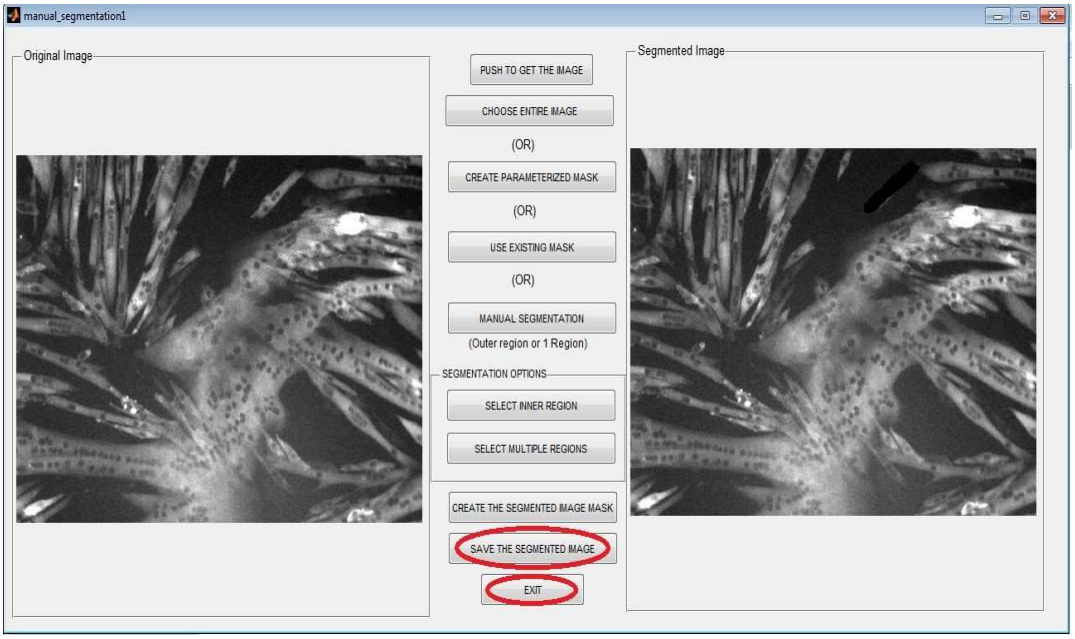


Figure 4.126. Fusion Index Area Procedure – Choose segmentation option

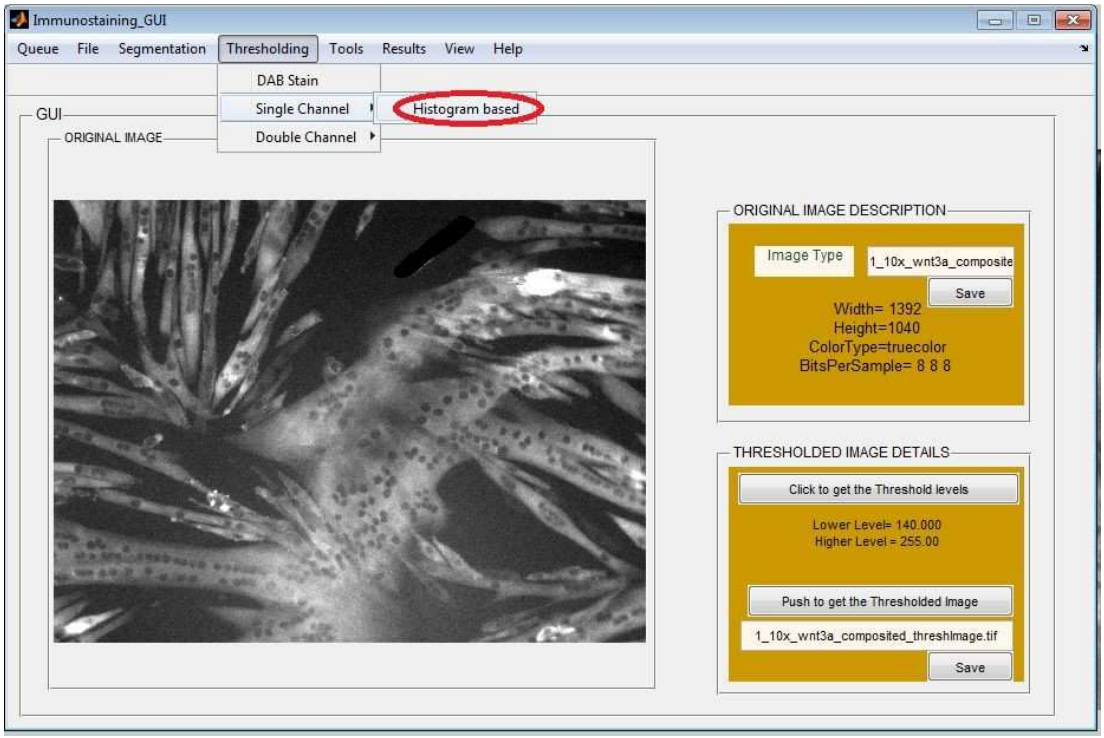


Figure 4.127. Fusion Index Area Procedure – Choose single channel histogram

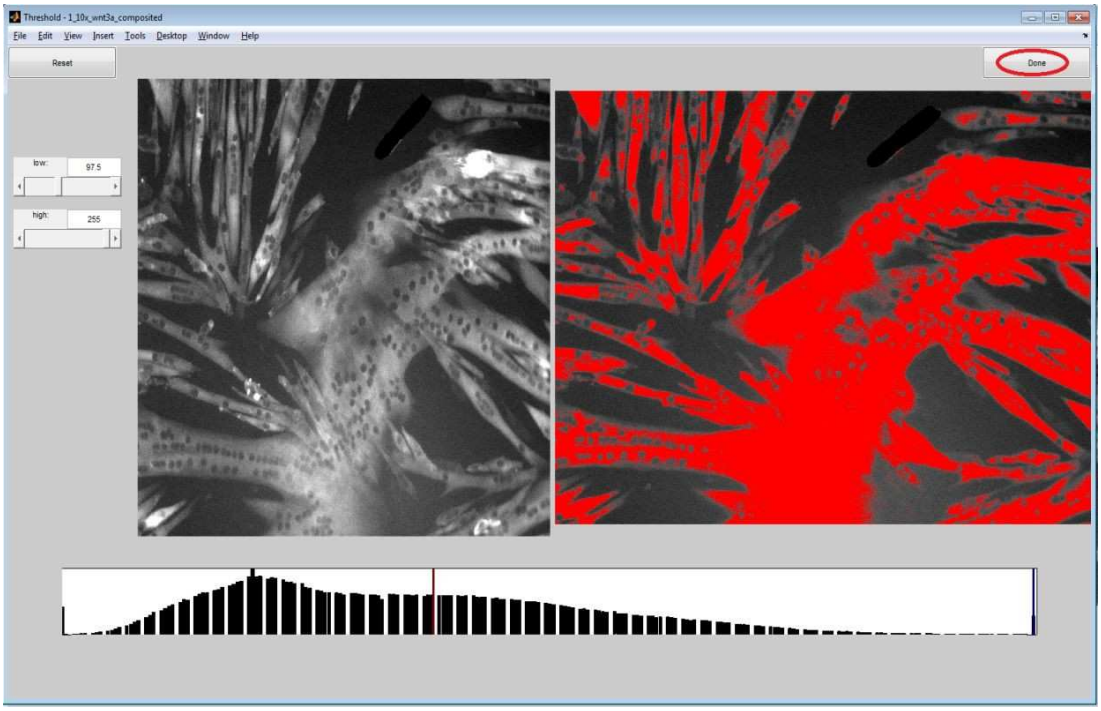


Figure 4.128. Fusion Index Area Procedure – Threshold segmented image

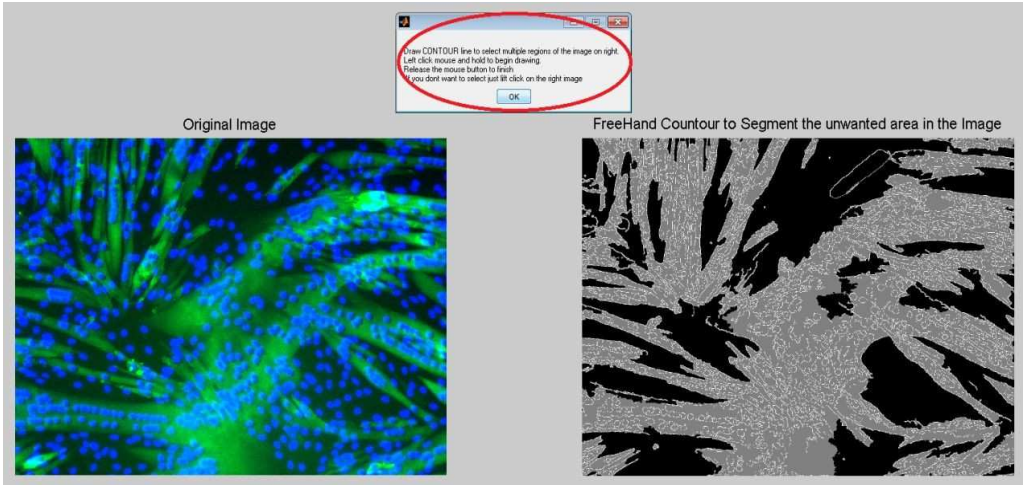


Figure 4.129. Fusion Index Area Procedure – Remove unwanted image

Figure 4.130. Fusion Index Area Procedure – Threshold unselected myotube

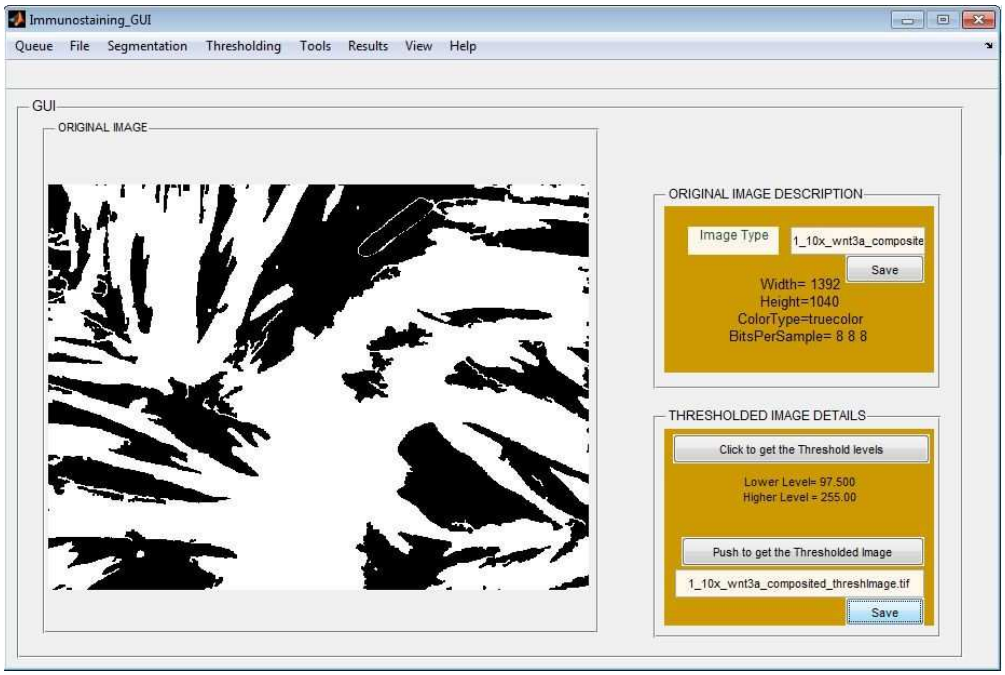


Figure 4.131. Fusion Index Area Procedure – Save thresholded image

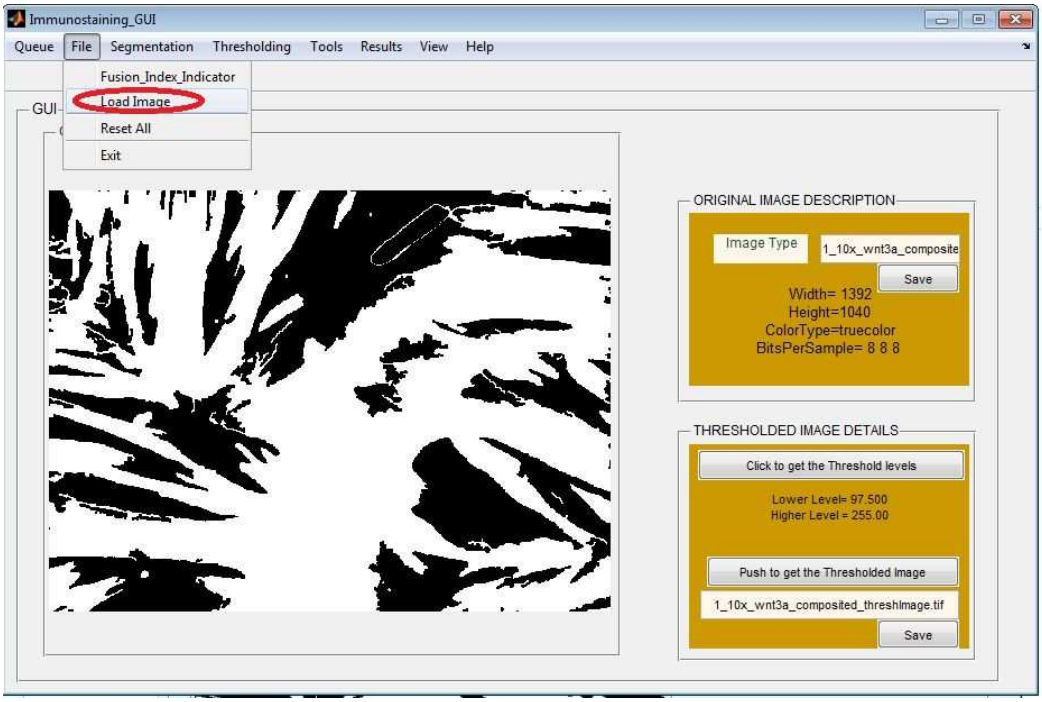


Figure 4.132. Fusion Index Area Procedure – Load fusion index image

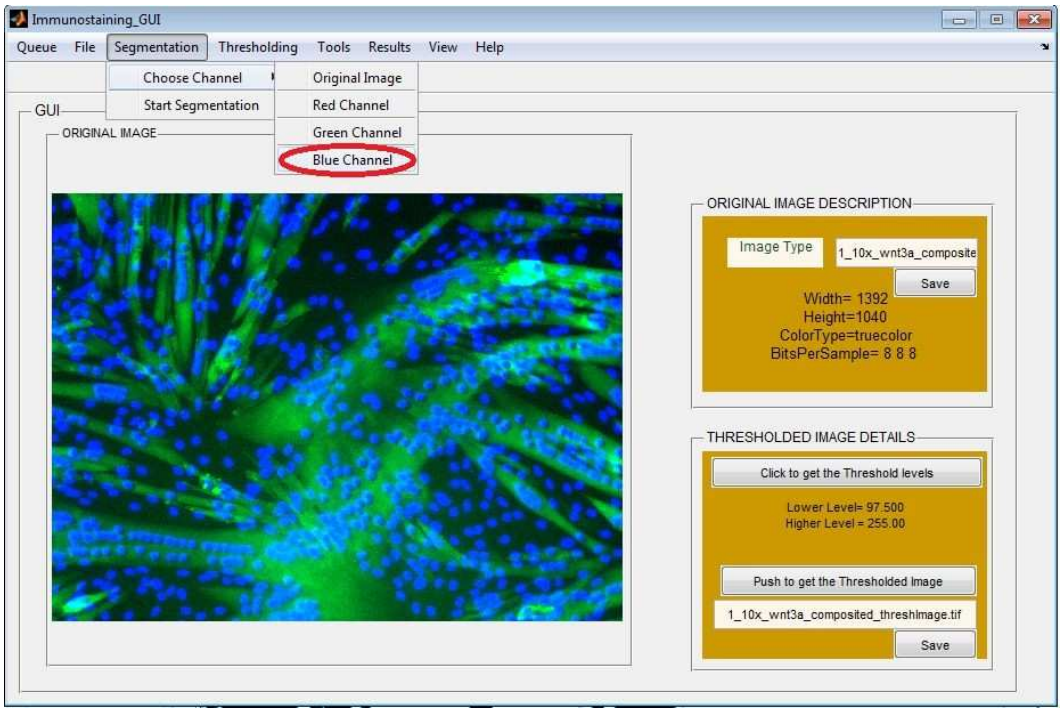


Figure 4.133. Fusion Index Area Procedure – Choose blue channel

Figure 4.134. Fusion Index Area Procedure – Image after channel selection

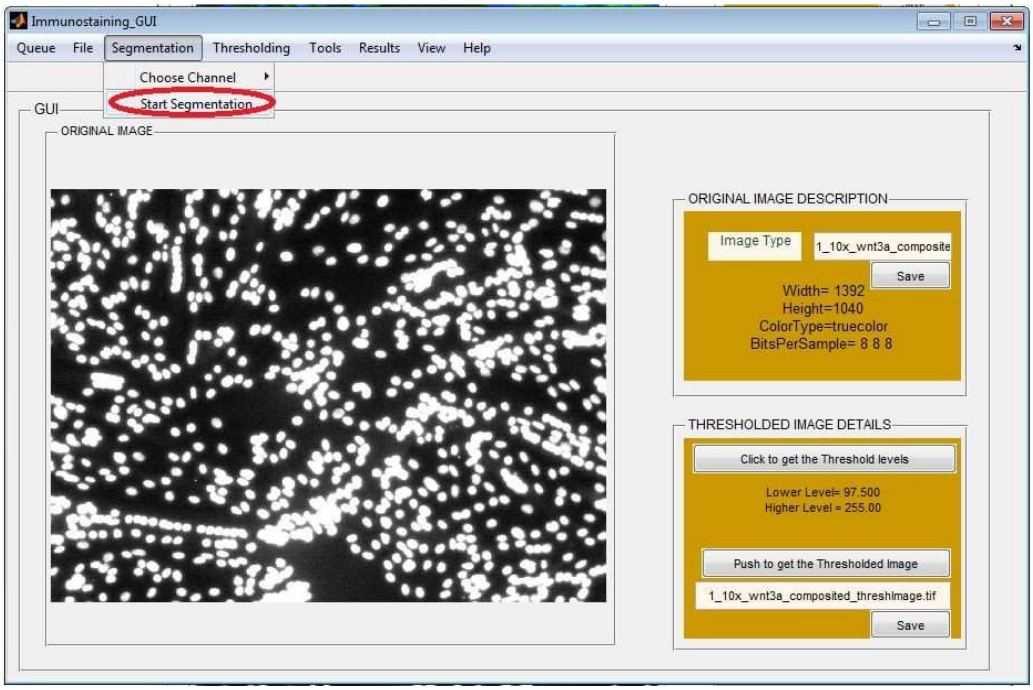


Figure 4.135. Fusion Index Area Procedure – Select start segmentation option

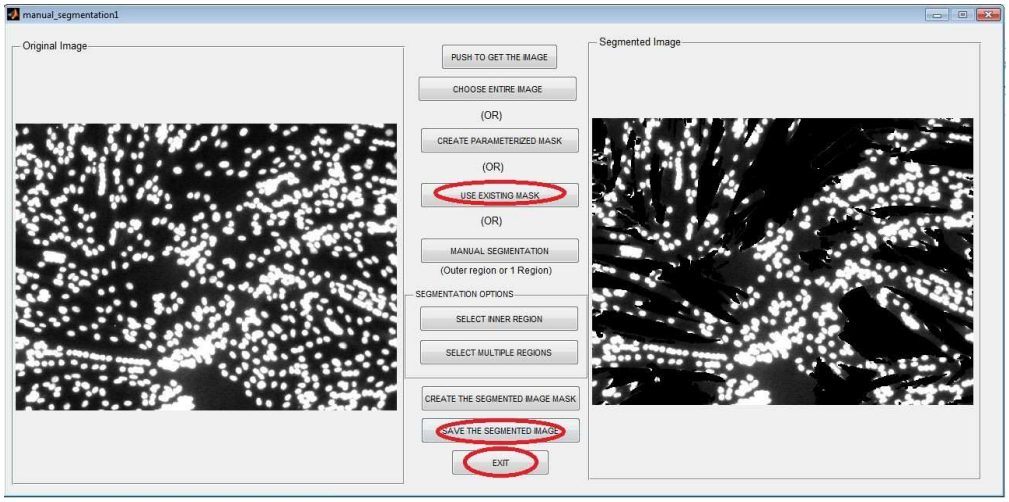


Figure 4.136. Fusion Index Area Procedure – Use myotube mask on the DAPI image

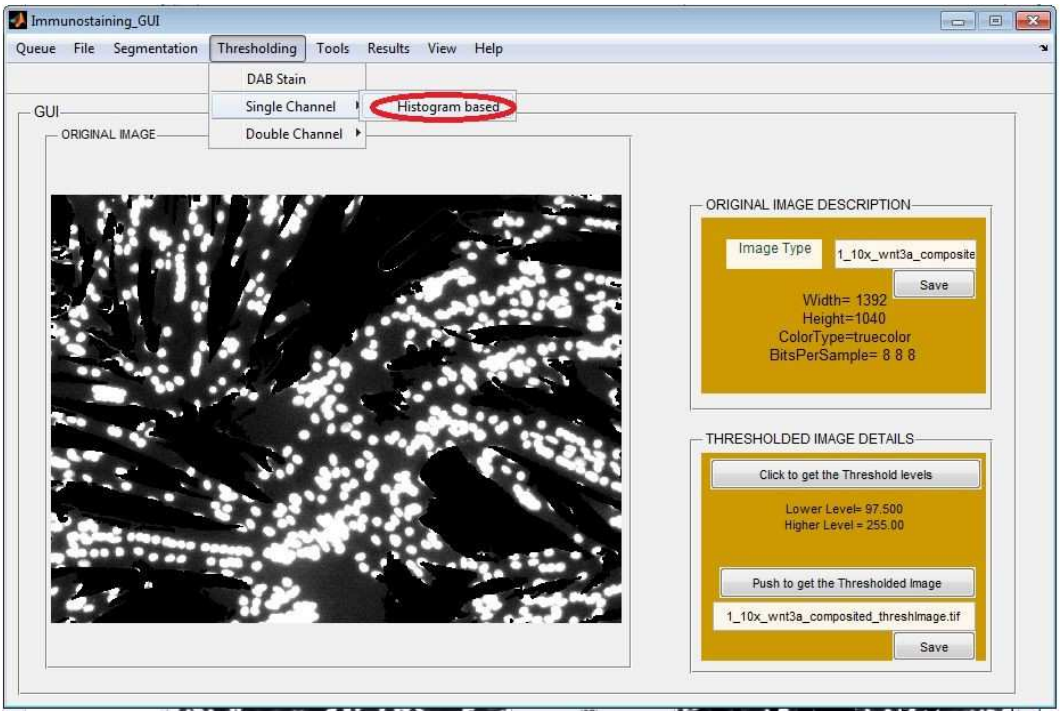


Figure 4.137. Fusion Index Area Procedure – Choose single channel histogram

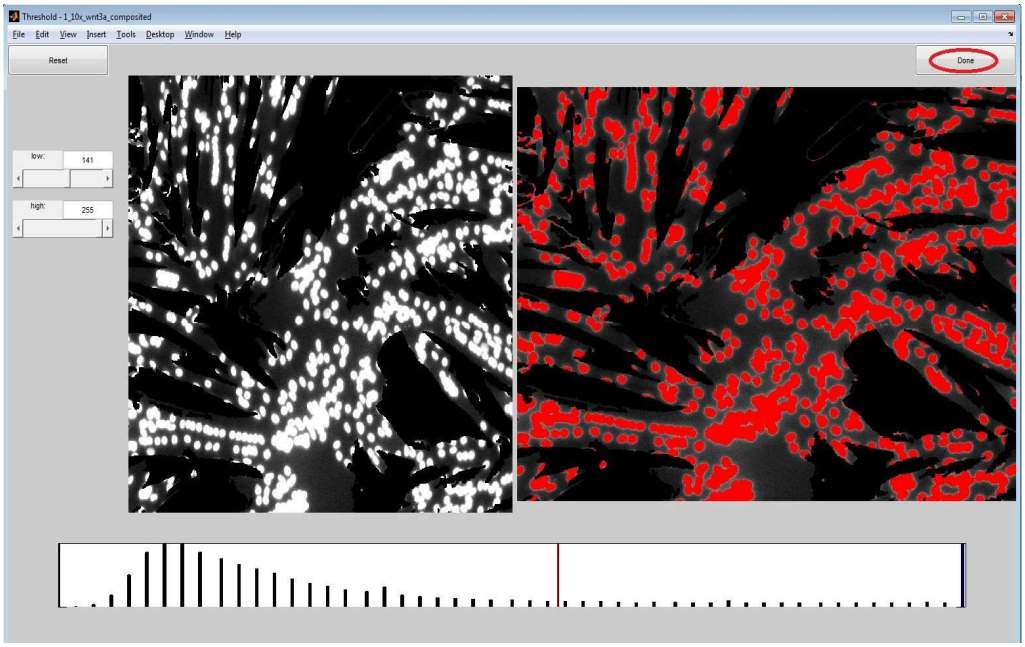


Figure 4.138. Fusion Index Area Procedure – Threshold segmented option

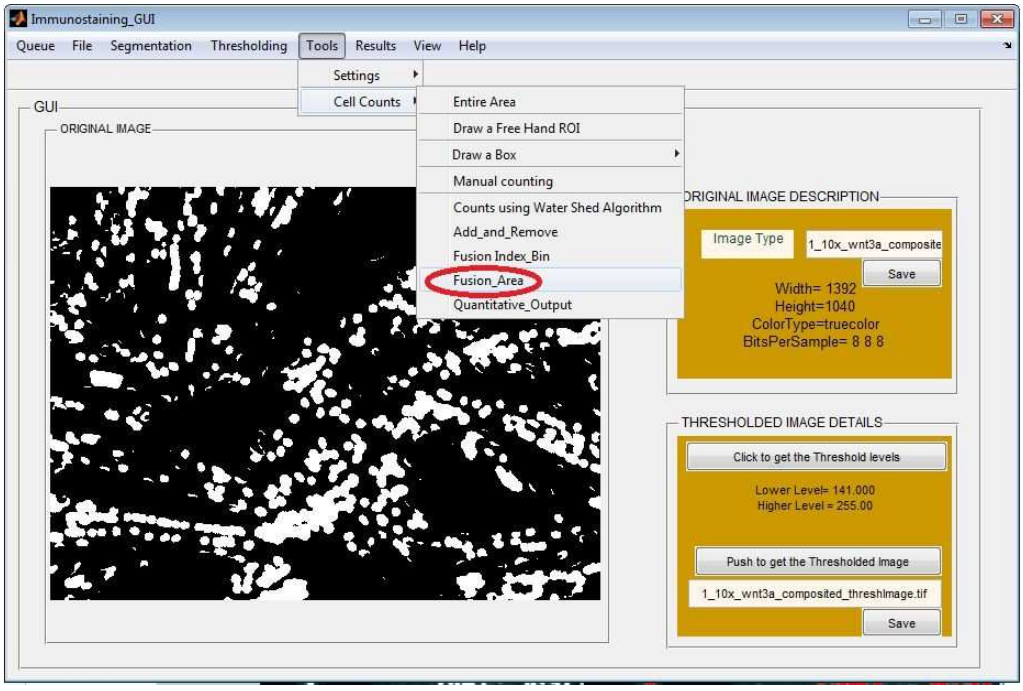


Figure 4.139. Fusion Index Area Procedure – Choose segmentation option

Figure 4.140. Fusion Index Area Procedure – Choose myotube option

Figure 4.141. Fusion Index Area Procedure – Fusion Index value

Co-localization

Co-localization is used to count the overlapped cells in two or more images. It combines the images and then count the cells that are overlapping (sitting one over the other). We have implemented two types of co-localization

i. Two images co-localization

ii. Three images co-localization

Following is the procedure to get Co-localized cell count for two images

i. Two images co-localization

Step 1: Load an image

File → Load Image

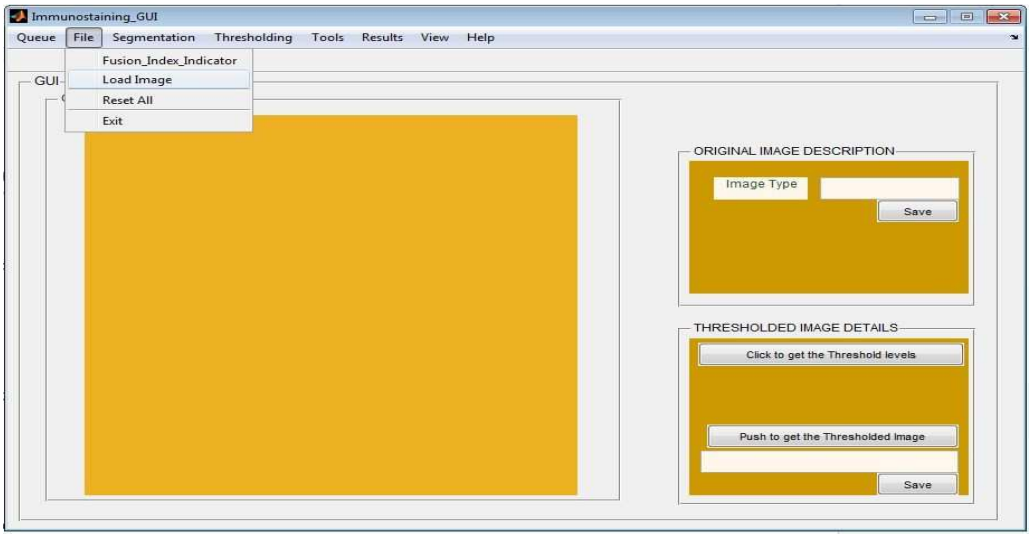


Figure 4.142. Co-localization two images: – Choose to load an image option

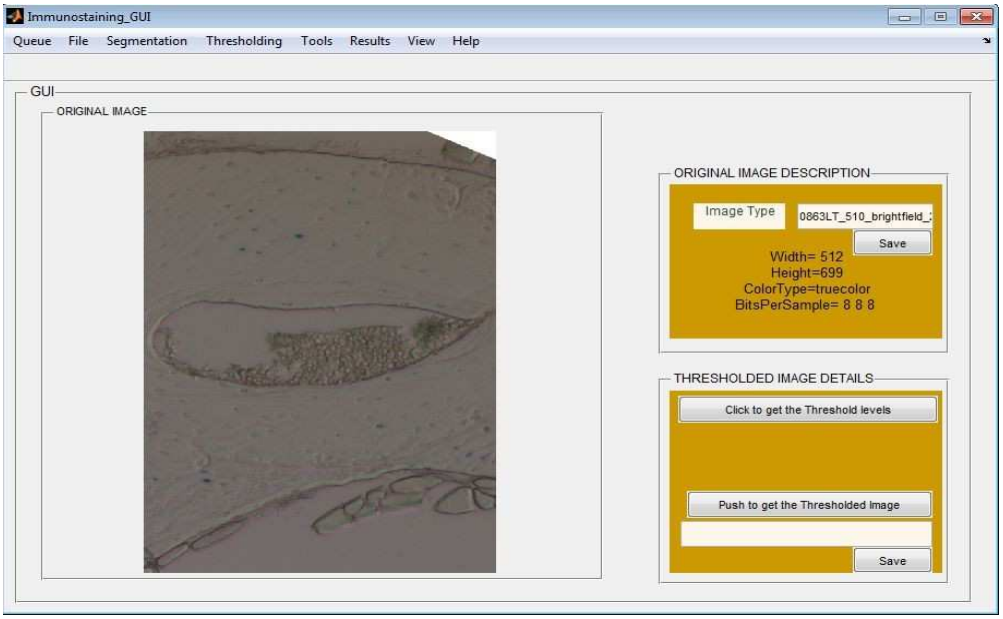


Figure 4.143. Co-localization two images – Loaded original image

Step 2: Choose Channel

Segmentation → Choose Channel → Original Image

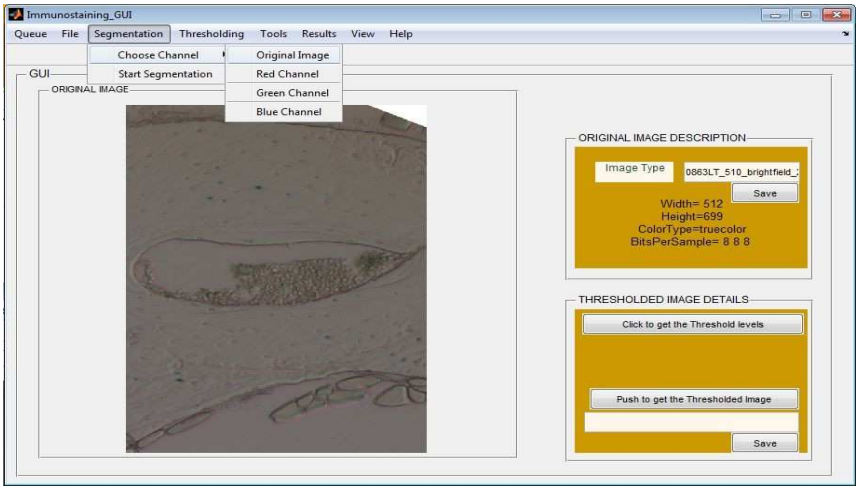


Figure 4.144. Co-localization two images – Choose segmentation option

Step 3: Segmentation

Segmentation → Start Segmentation → Manual Segmentation → Select Inner Region →

Create segmented Mask → Save results to Excel sheet → Exit

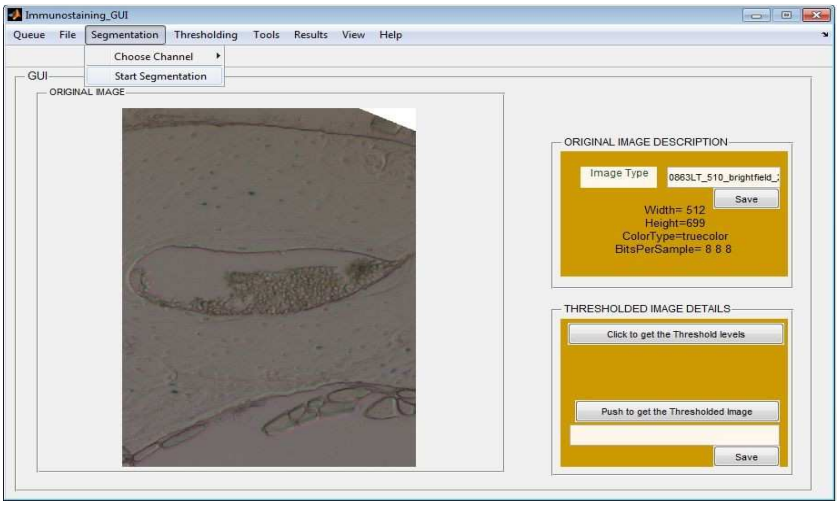


Figure 4.145. Co-localization two images – Select start segmentation

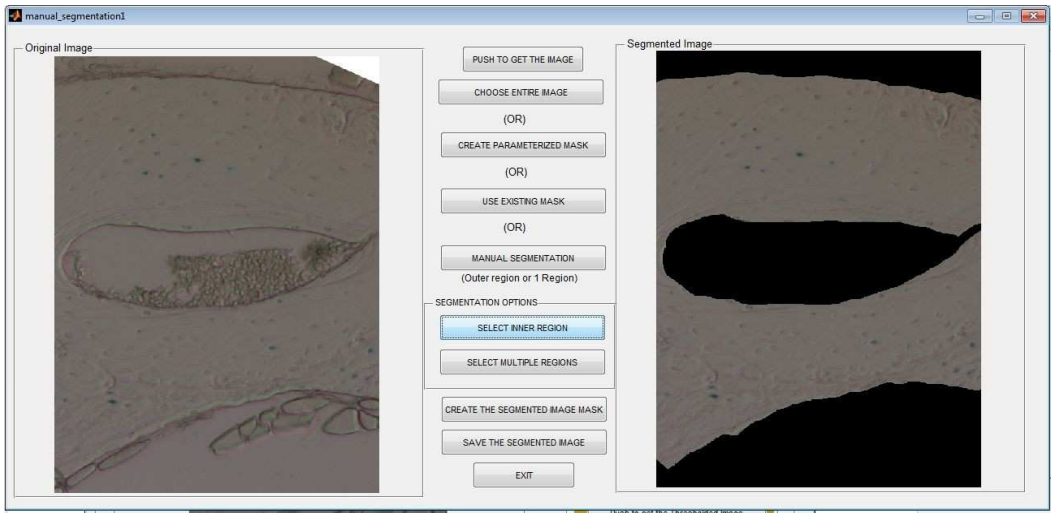


Figure 4.146. Co-localization two images – Segment Inner and outer region

Step 4: Threshold and save Image

Click to get the threshold levels → push to get the thresholded image → save

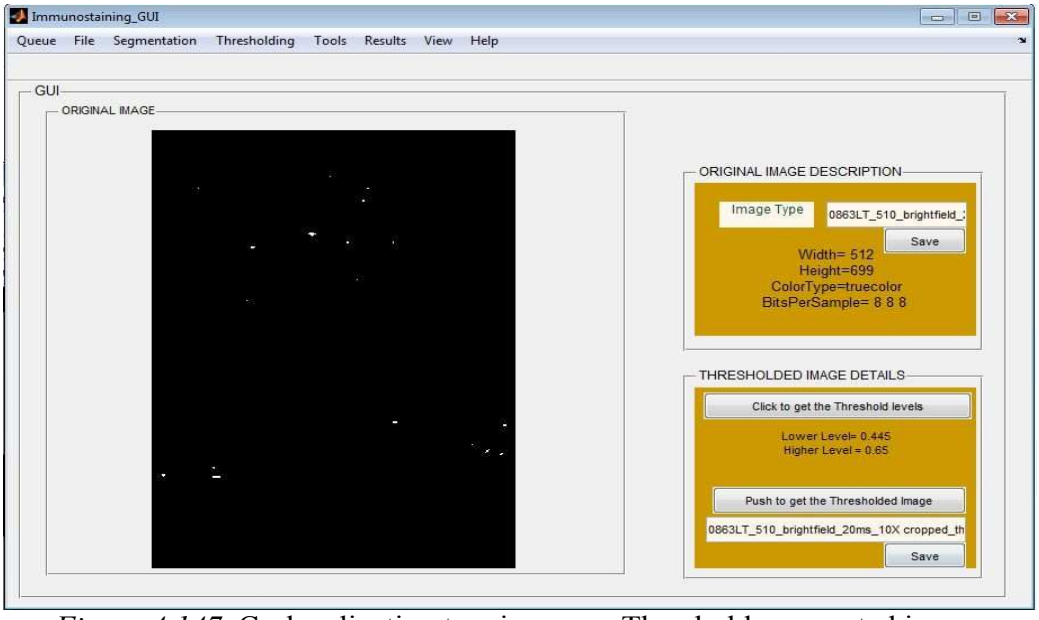


Figure 4.147. Co-localization two images – Threshold segmented image

Step 5: Repeat the above procedure for another image

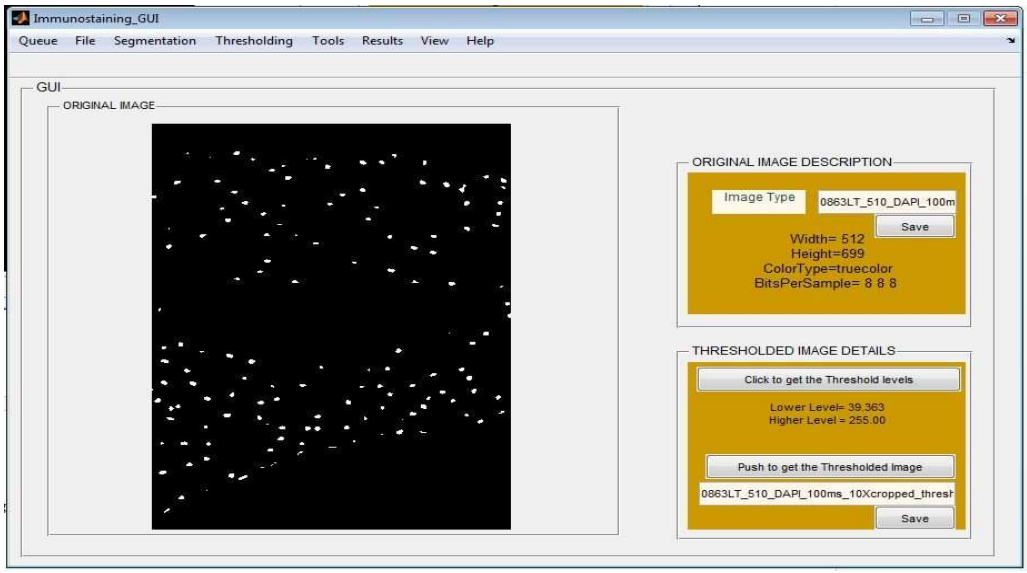


Figure 4.148. Co-localization two images – Thresholded DAPI image

Step 6: Co-localization of two images

Results → Colocalization → Two Images

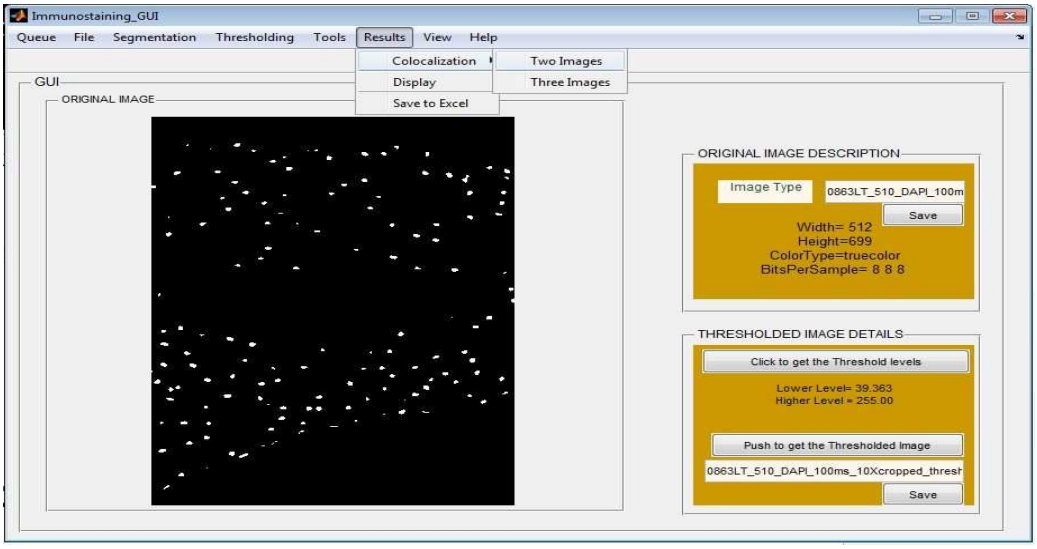


Figure 4.149. Co-localization two images – Choose segmentation option

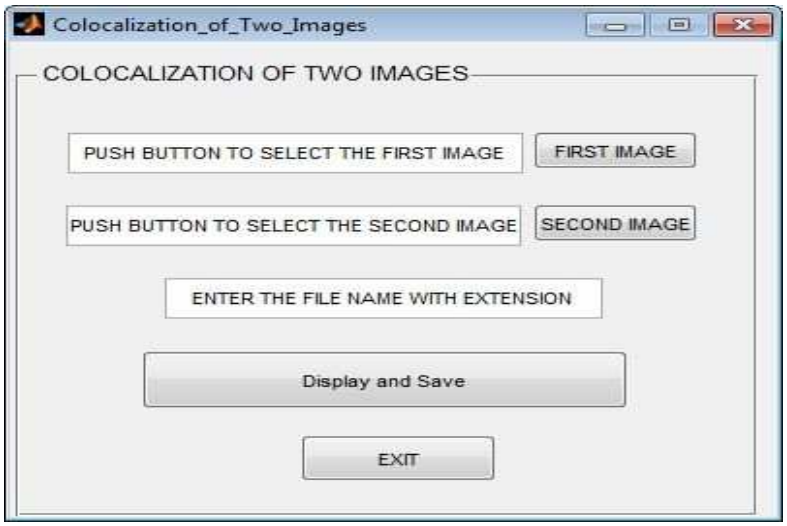


Figure 4.150. Co-localization two images – Colocalization screen

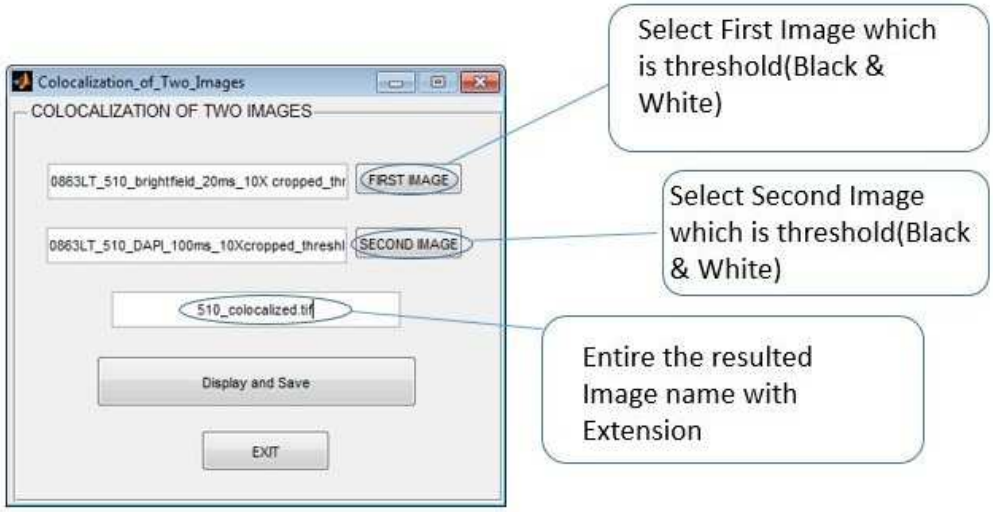


Figure 4.151. Co-localization two images – Option to choose first and second image

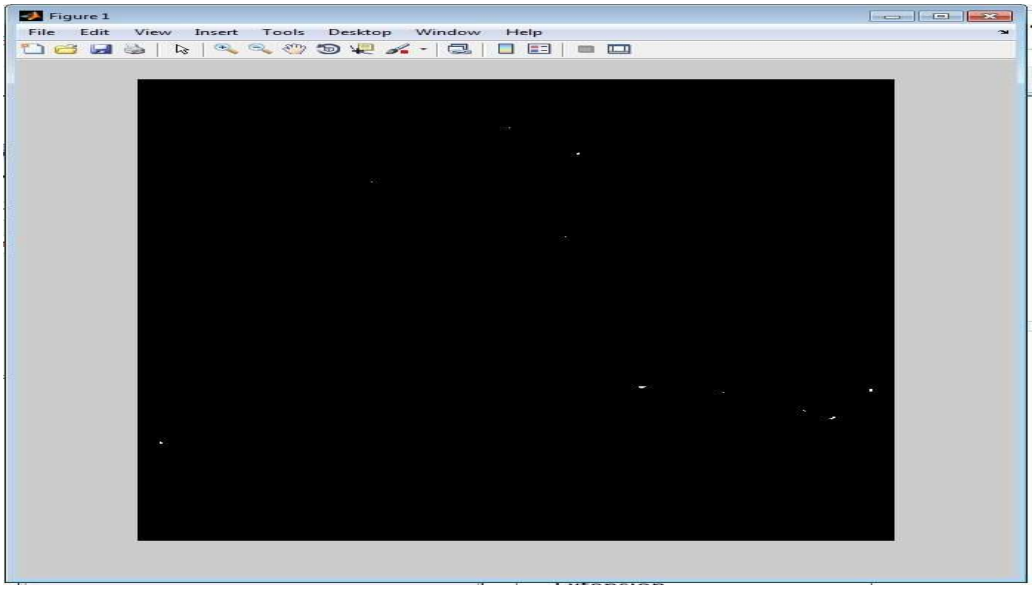


Figure 4.152. Co-localization two images – Image after overlapping

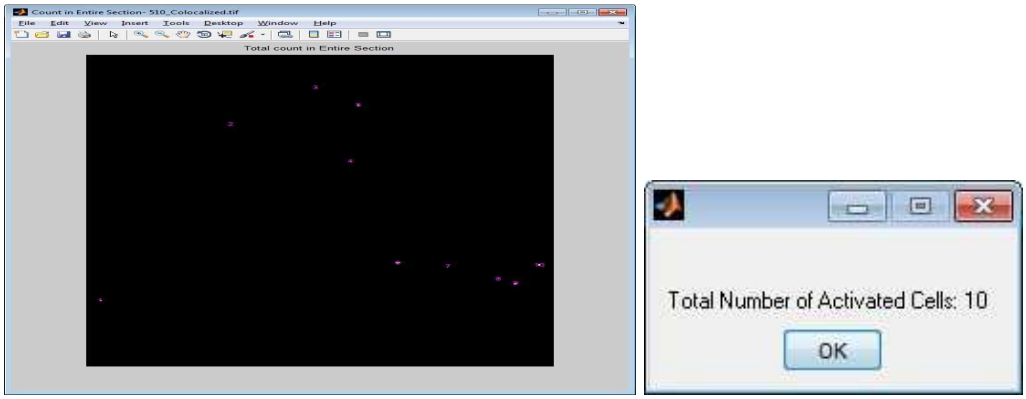


Figure 4.153. Co-localization two images – Total number of overlapped cells

Step 7: Save to Excel

Results → save to Excel

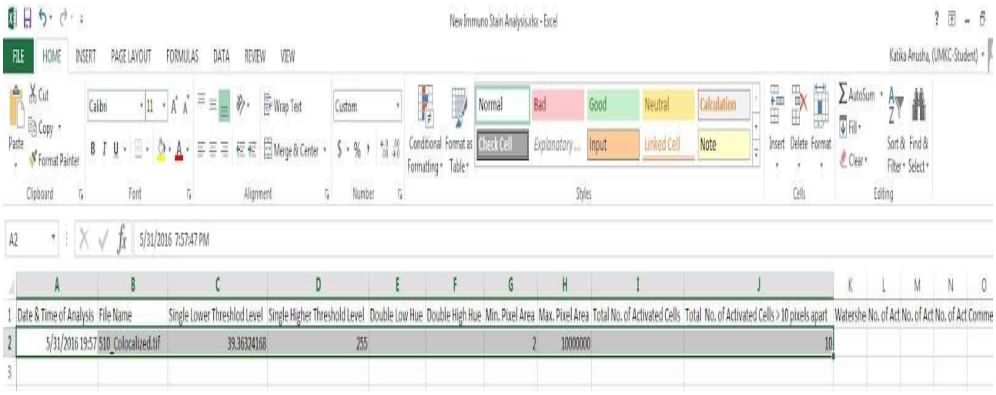


Figure 4.154. Co-localization two images – Colocalization results in excel

ii. Three images co-localization

Step 1: Load an Image File –> Load Image

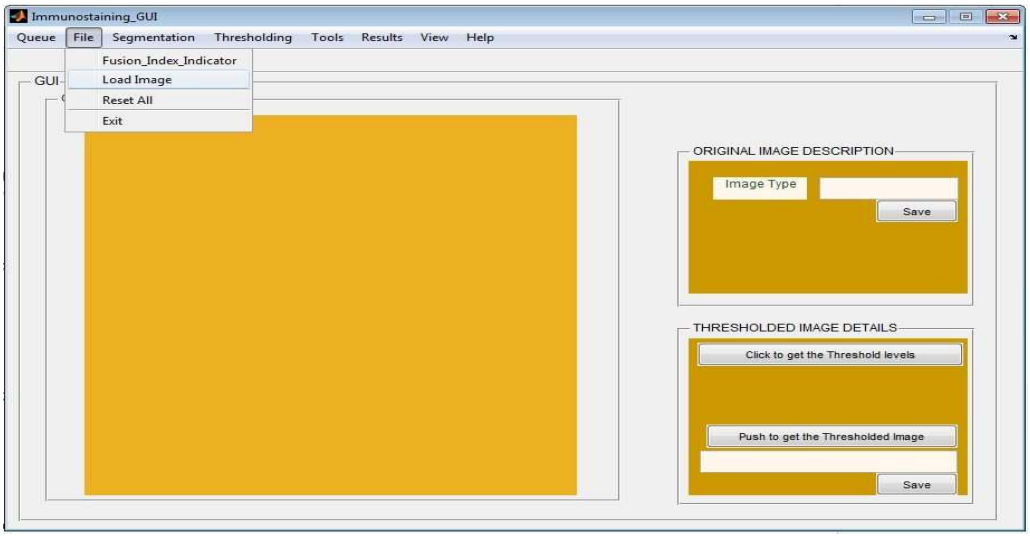


Figure 4.155. Co-localization three images: – Choose to load an image option

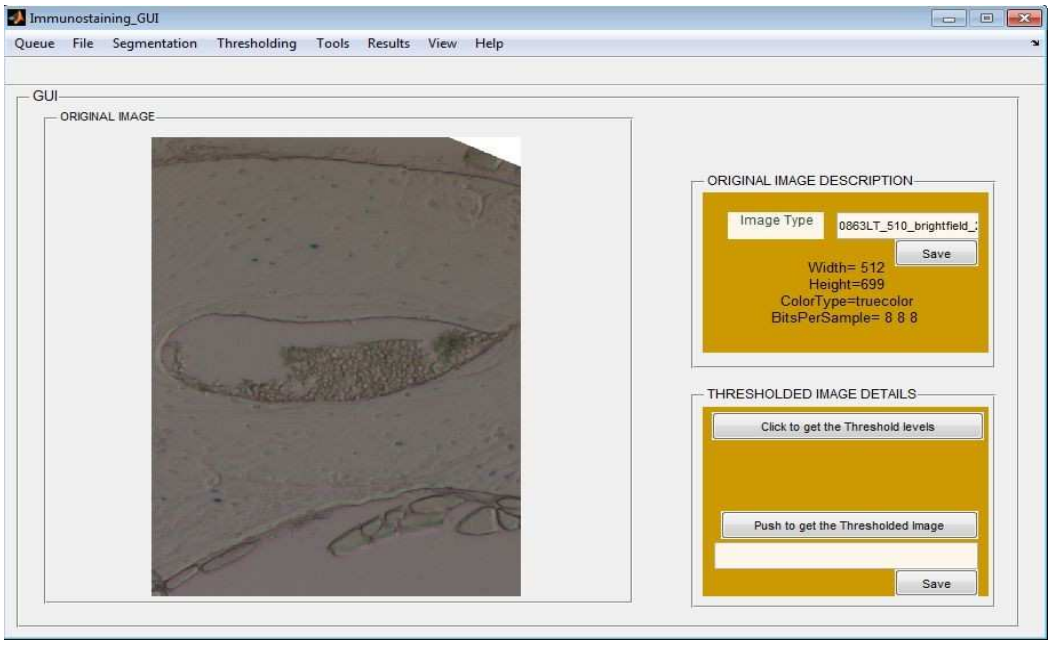


Figure 4.156. Co-localization three images: – loaded original image

Step 2: Choose Channel

Segmentation → Choose Channel → Original Image

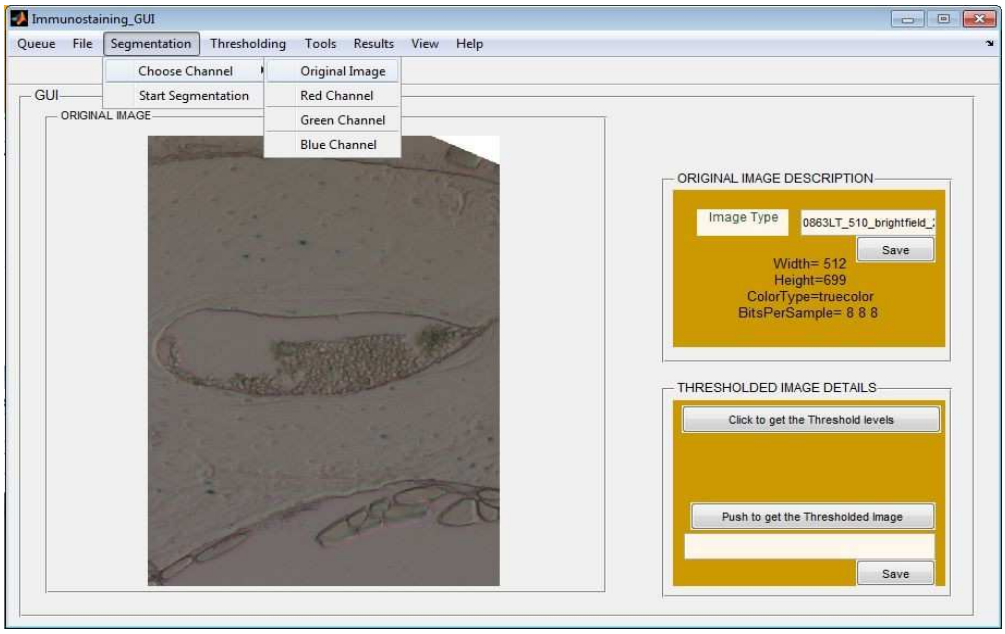


Figure 4.157. Co-localization three images: – Choose original image channel

Step 3: Segmentation

Segmentation → Start Segmentation → Manual segmentation → select the inner region → save the segmented image → Exit

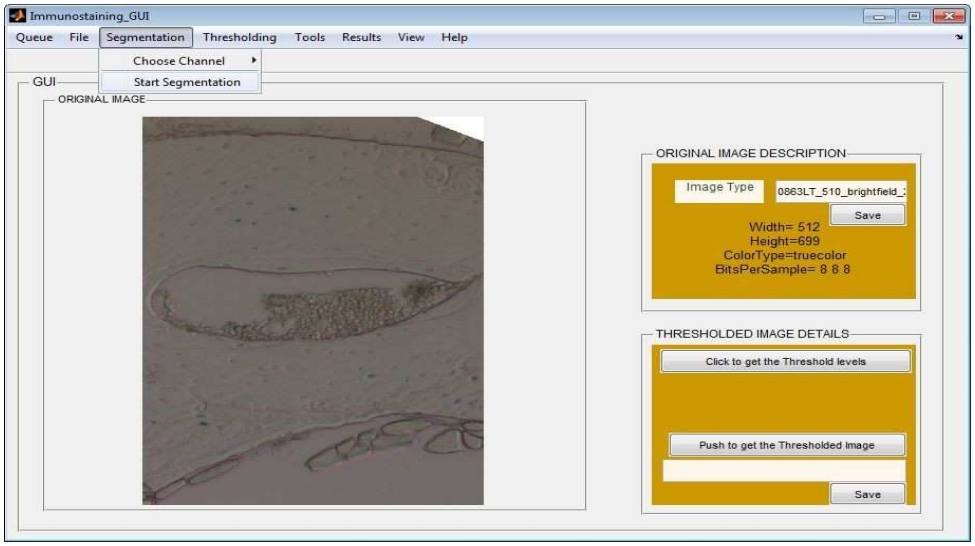


Figure 4.158. Co-localization three images: – Choose start segmentation option

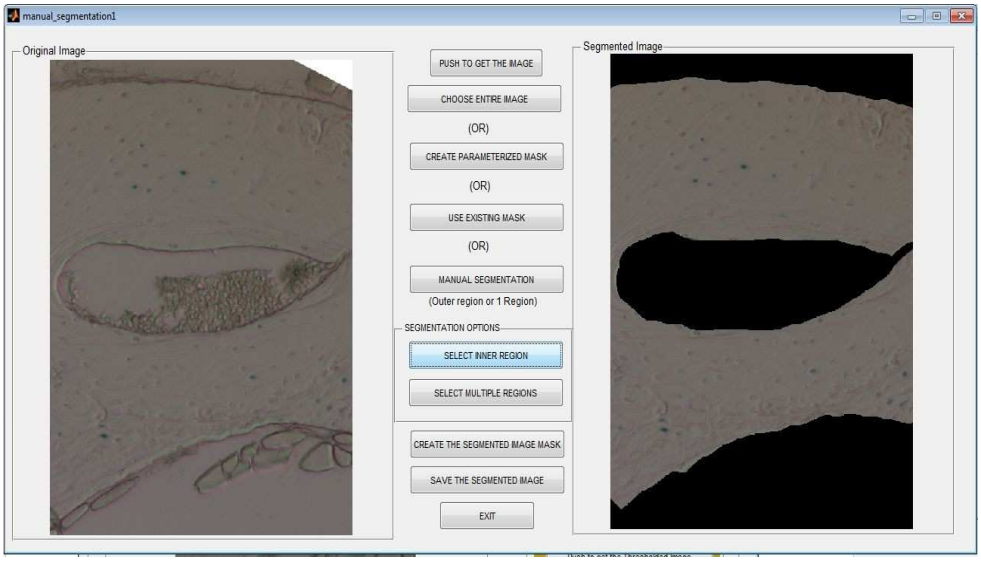


Figure 4.159. Co-localization three images – Segment inner and outer region of the image

Step 4: Threshold and save Image

Thresholding → Double Channel → Histogram Based1

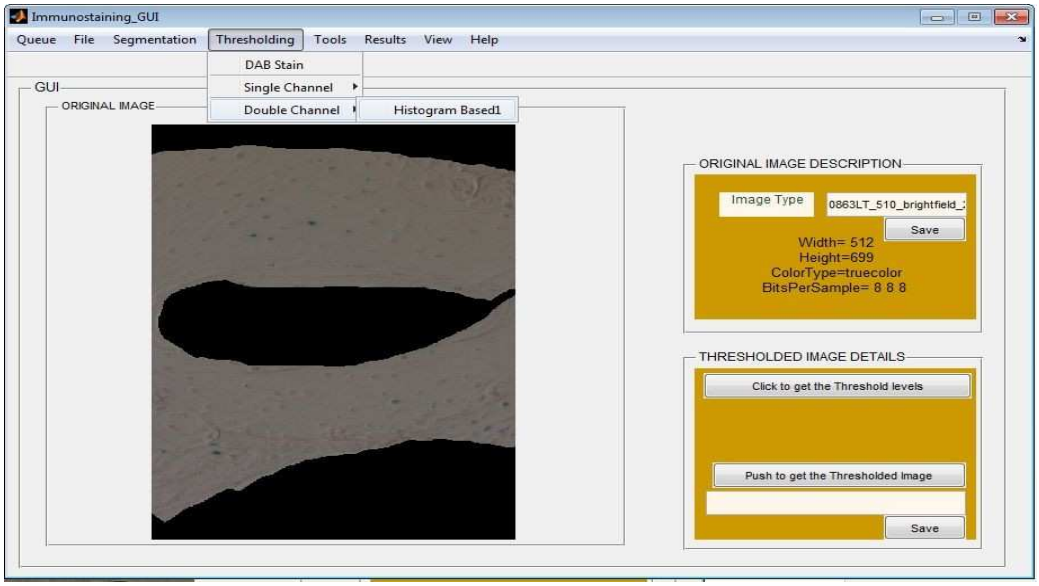


Figure 4.160. Co-localization three images: – Choose double channel histogram

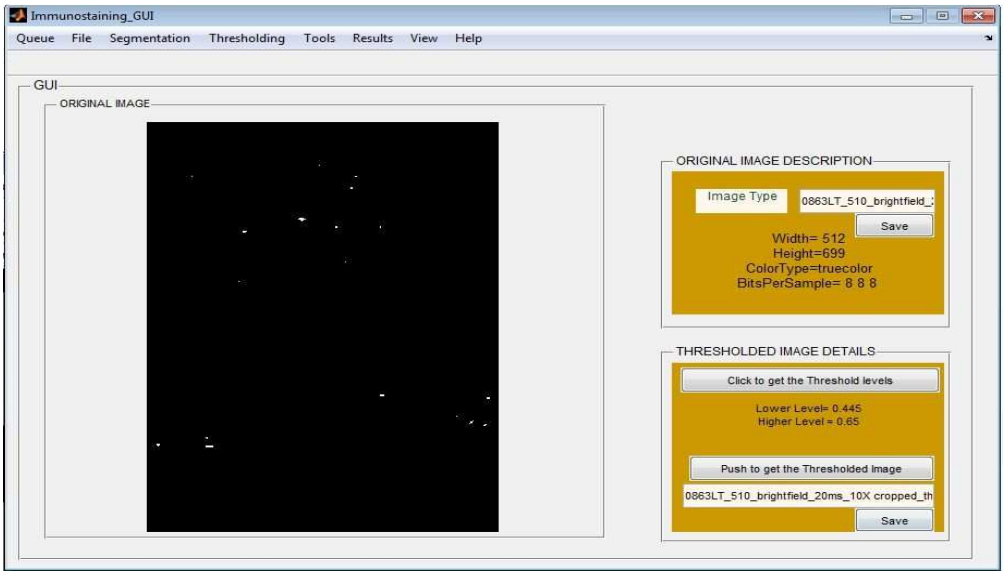


Figure 4.161. Co-localization three images: – Save thresholed image

Step 5: Repeat the above procedure for another two images

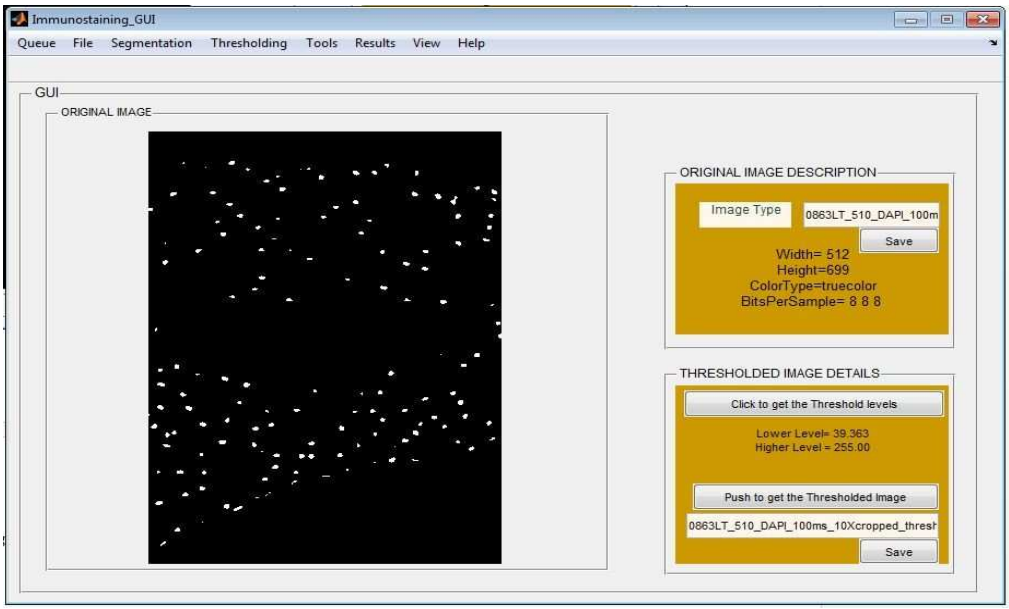


Figure 4.162. Co-localization three images: – Threshold DAPI image

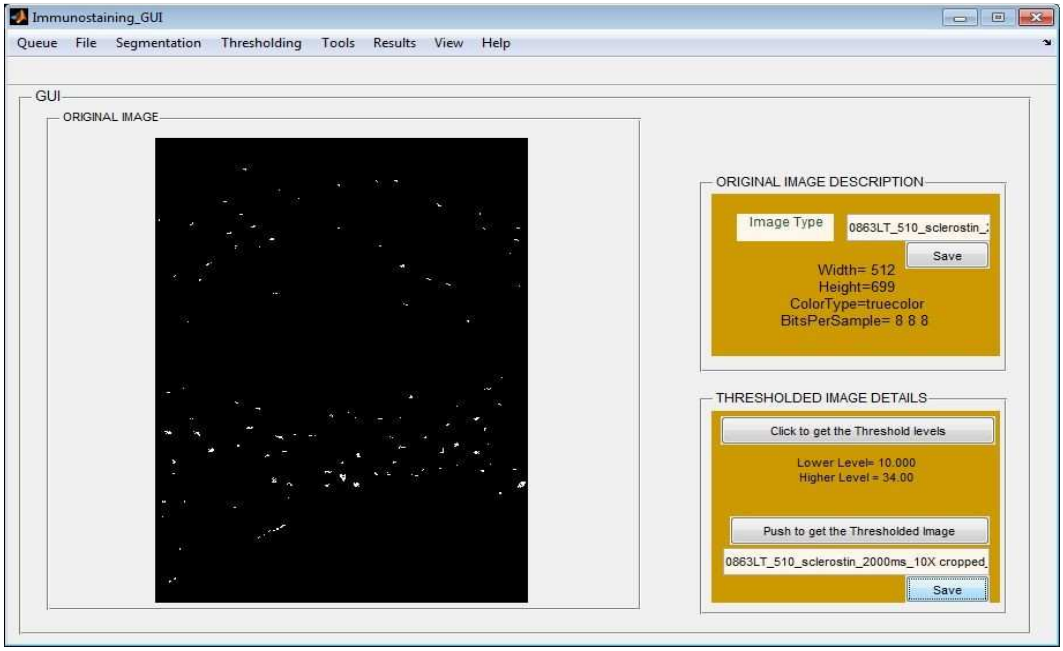


Figure 4.163. Co-localization three images – Thresholded sclerostin image

Step 6: Co-localization of three images

Results → Co-localization → Three Images

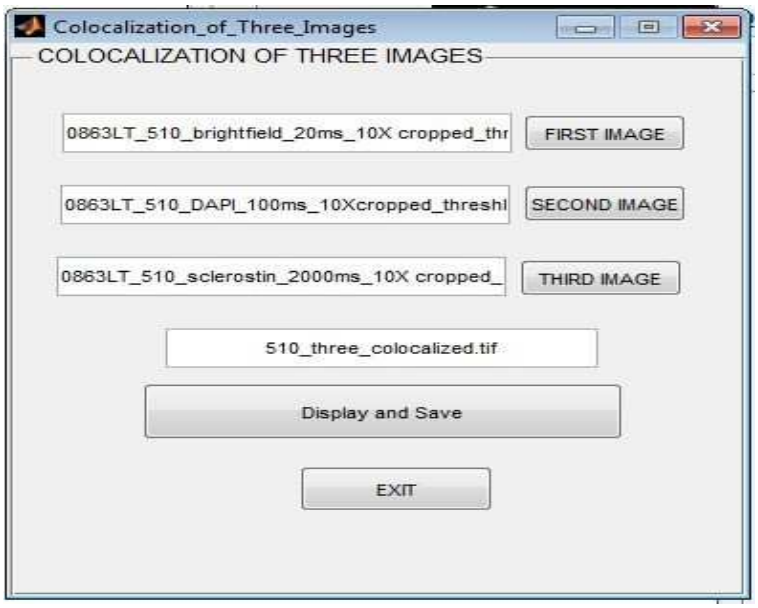


Figure 4.164. Co-localization three images: – Colocalization of three images screen

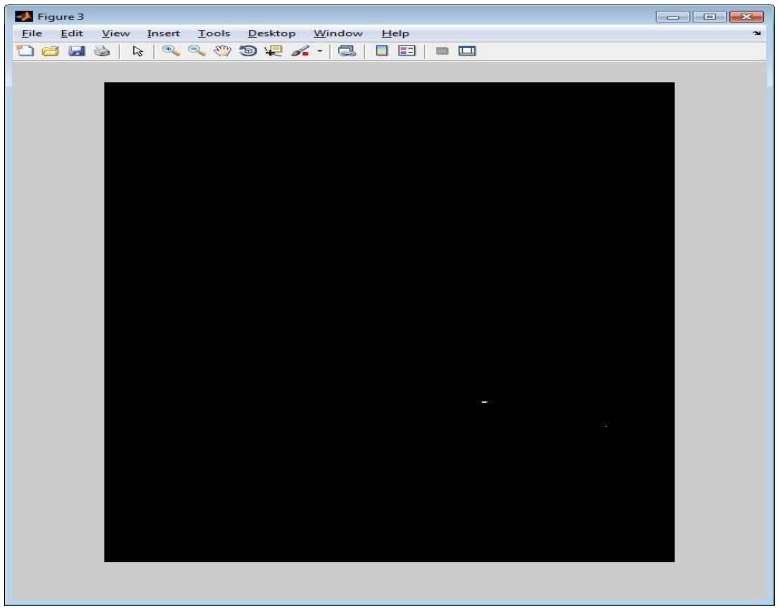


Figure 4.165. Co-localization three images: – Image after co-localization

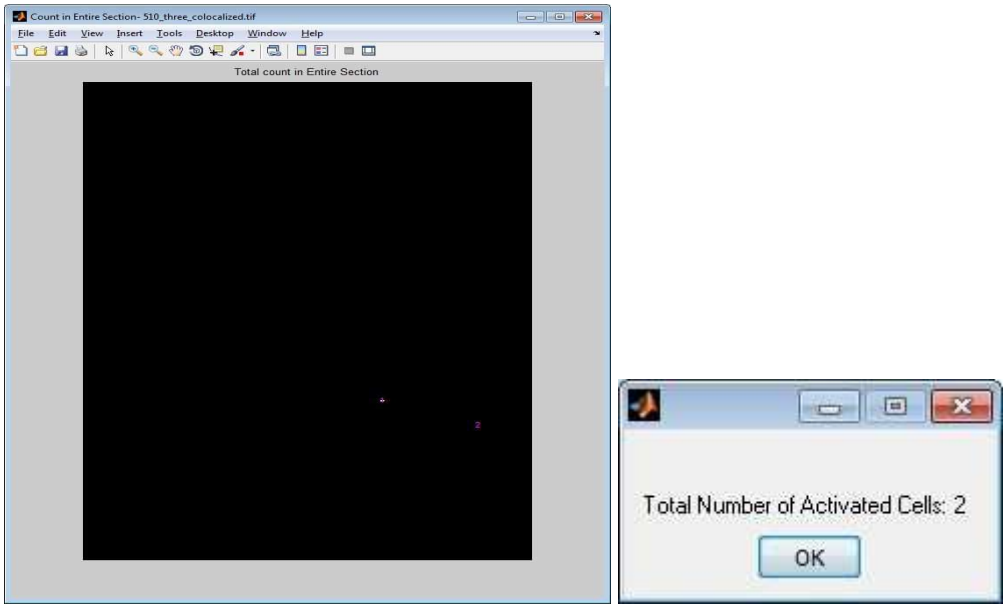


Figure 4.166. Co-localization three images: – Total number of activated cells

CHAPTER 5

RESULTS

Overview

This chapter explains the findings and observations of the analysis of the images by using the automated GUI software. The study compared the results of the automated software with manual counting and attempts to demonstrate the efficiency of the proposed GUI software assisted image technique. Different types of stained images, such as β-Galactosidase, DAPI and Sclerostin were used by the study for the analysis. The findings of the colocalization, which was performed to identify the number of active cells which had multiple staining modalities in the same cell, are also presented. The presentation of the findings of the fusion index using the GUI software and the comparison of the cell counts in the Myotube with a control value concludes the chapter.

Excel sheet was used for the statistical analysis of the data. The study used Box plots to find out the significant difference existing between the manual and automated counting.

Comparison between Manual and Software counting in different stains

This section provides the comparative results of the cell counts (manual and software) in different stains. The study used box plot to illustrate the statistical differences between manual and software counting of cells in different image stains. The study used stains of β-Galactosidase, DAPI and Sclerostin for the analysis and the results are presented.

Difference between Software and Manual counting of cells in β-Gal

Figure 5.1 provides the findings of the box plot, conducted to compare between the

outcomes of manual and software cell counting in the β-Gal stains. However, the box plot revealed no significant difference between the manual and software counting ($p=0.906>0.05$). Even though, the mean is marginally higher in the case of manual counting, it is not enough to yield any significant outcomes. Hence, the results indicated the fact that there is no significant impact is evident as a result of the use of software assisted cell quantification. The plot in Figure 5.1 depicts the graphical representation of the comparative results.

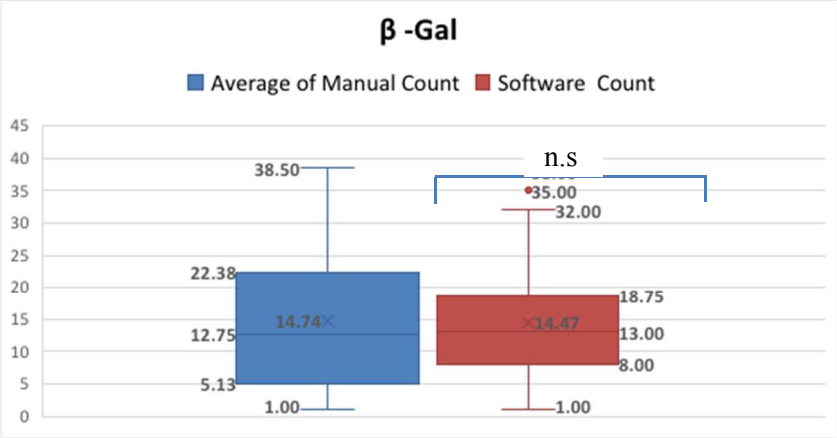


Figure 5.1. Difference between Software and Manual counting of cells in β-Gal

n.s. - No significant difference

Difference between Software and Manual counting of cells in DAPI

4′,6-diamidino-2-phenylindole (DAPI) is a blue-fluorescent DNA stain that is used to count the number of nuclei and to evaluate gross cell morphology (Tarnowski et.al.,1991). Its high affinity for DNA made it favorable for the counting of cells. Figure 5.2 yields the

findings of the box plot, conducted to compare between the outcomes of manual and software cell counting in the DAPI stains. The findings indicated the fact that the cell count varied significantly between manual and software counting ($p=0.00<0.05$) and the quantification using software showed higher mean value 117.32 compared to the manual counting 101.97. Hence, this result clearly indicated the difference of the proposed software assisted quantification technique in comparison with manual counting. Figure 5.2 demonstrates the differences in manual and software assisted cell counting.

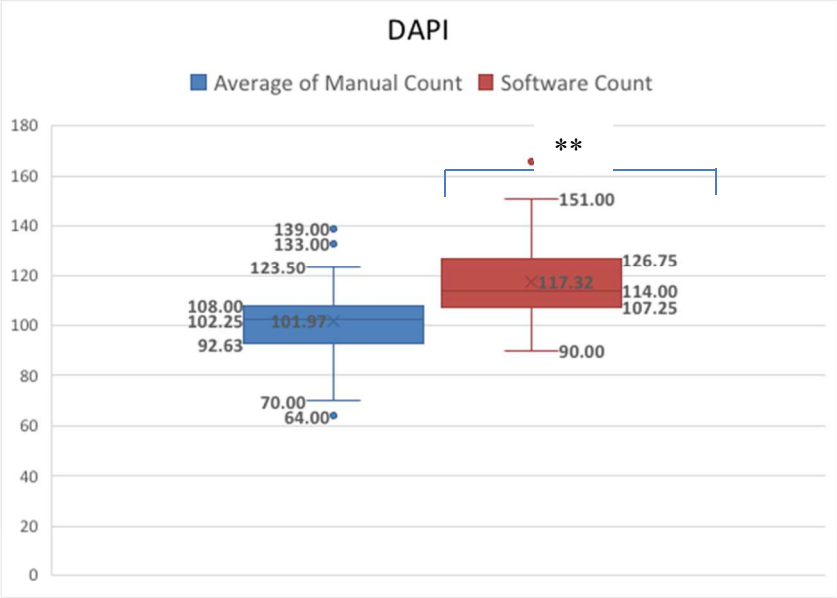


Figure 5.2. Difference between Software and Manual counting of cells in DAPI

** $p < 0.01$

Difference between Software and Manual counting of cells in Sclerostin

Figure 5.3 shows the findings of the box plot, conducted to compare the outcomes of

manual and software cell counting in the sclerostin stains. Sclerostin staining is widely used in fluorescence microscopy for the counting cells, especially bone cells, because the dysregulation of sclerostin expression highlights the pathophysiology of skeletal maladies indicated by loss of bone mass and the harmful effects of some cancers in the bone (Delegado,Satto and Bellido, 2017). An up-regulation of sclerostin stained cells can be an indicator of tumor (Zhu et al., 2017). The findings of the box plot demonstrated a clear and significant difference between manual and automated counting of stained cells($p=0.00<0.05$). Figure 5.3 clearly highlighted the differences between the means of manual an automated counting, with automated counting yielded a significantly higher mean (72.21) compared to the manual counting (44.41). Hence, the findings exhibit the greater efficiency of the proposed software assisted quantification technique in comparison with manual counting. Figure 5.3 demonstrates the differences in manual and software assisted cell counting

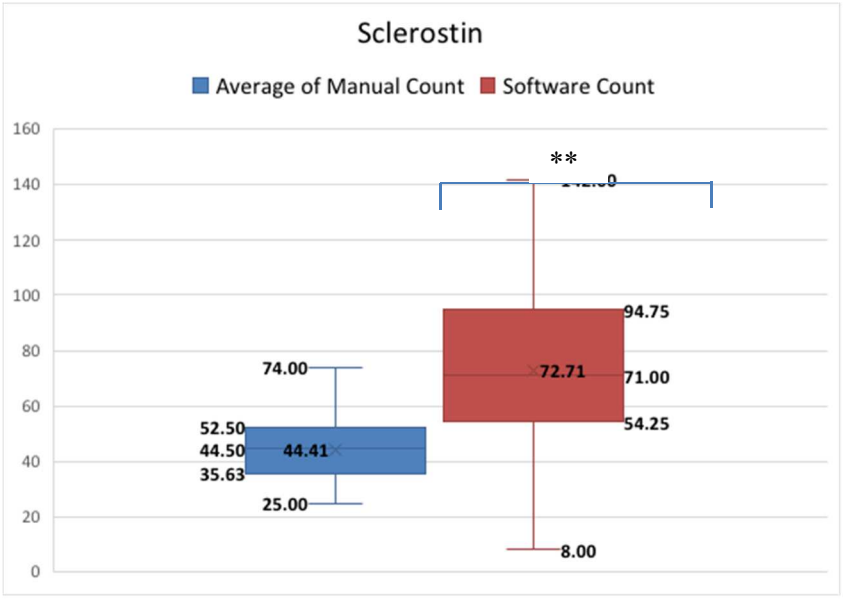


Figure 5.3. Difference between Software and Manual counting of cells in Sclerostin

** $p < 0.01$

Colocalization

The counting of overlapped cells on two or three overlapped images is performed by Colocalization. The present study attempted to count the overlapped cells on both two overlapped images (β-Gal and DAPI) and three overlapped images (β-Gal, DAPI, Sclerostin) using the proposed software assisted technique and results are presented in this section. The study has performed a descriptive analysis to yield the range and mean of active cells presented in the overlapped images. Figure 5.4 provides the findings of the descriptive analysis to identify the ability of the proposed software assisted imaging technique in the quantification of active cells during the spatial overlapping of images. The findings regarding to the colocalization of the two images demonstrated the fact that the software assisted imaging system identified the active cells, which ranges from 0.00 to 15.00 with a mean of 5±3 with respect to different images. Further, the analysis of three images indicated that the software identified the active cells with a mean of 0.6±0.7 and having the minimum count of 0.00 and maximum count of 3.00.

Summary about the samples and its measures

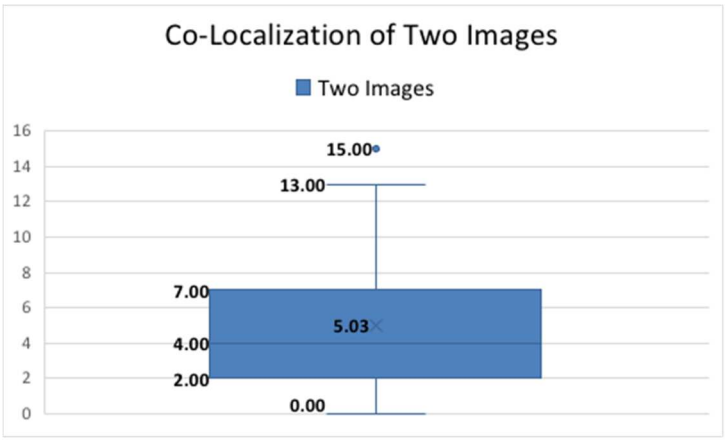


Figure 5.4 Box plot of Co-localization of two images

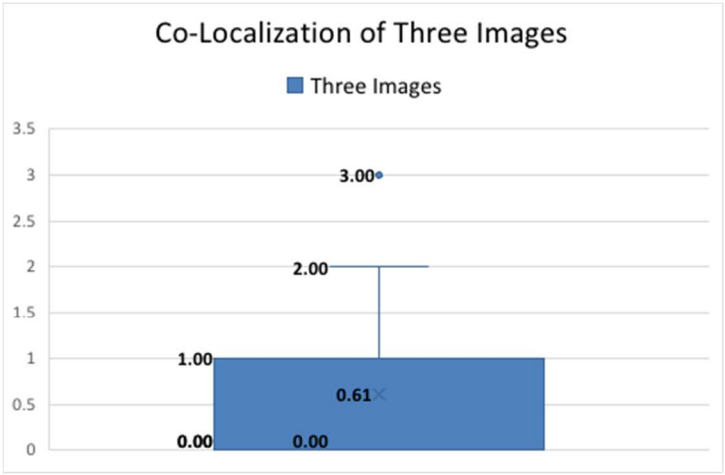


Figure 5.5 Box plot of Co-localization of three images

Fusion Index

Fusion index is the percentage value of the ratio between the number of nuclei incorporated in the myotube and total number of nuclei in the field of view. In other words,

Fusion index = (*number of nuclei incorporated in the myotube /total number of nuclei in the field of view*) x 100.

The present study, using GUI software, attempted to find out the fusion index value of Wnt3a images and compared it with the value of control cell. Wnt3a is a protein from the Wnt gene family, which enhances the signaling process and regulates the proliferation process (Shang et al.,2007). The study analyzed the fusion index across the fusion bin range and fusion area. The statistical tool box plot was used to find out the significant difference between the index values. The findings of these analyses are described in the following sections.

Fusion Index Bin

The fusion index bin will yield the information like minimum, maximum and average area of single and double nuclei. The findings of the box plot as indicated in the figure 5.5 exhibited the fact that, albeit Wnt3a showed higher mean index value (0.67) compared to control index (0.59), the differences were not significant enough to reach into any conclusive inferences ($p=0.18>0.05$). Figure 5.6 shows differences between Wnt3a and control index values.

Fusion index (bin range)

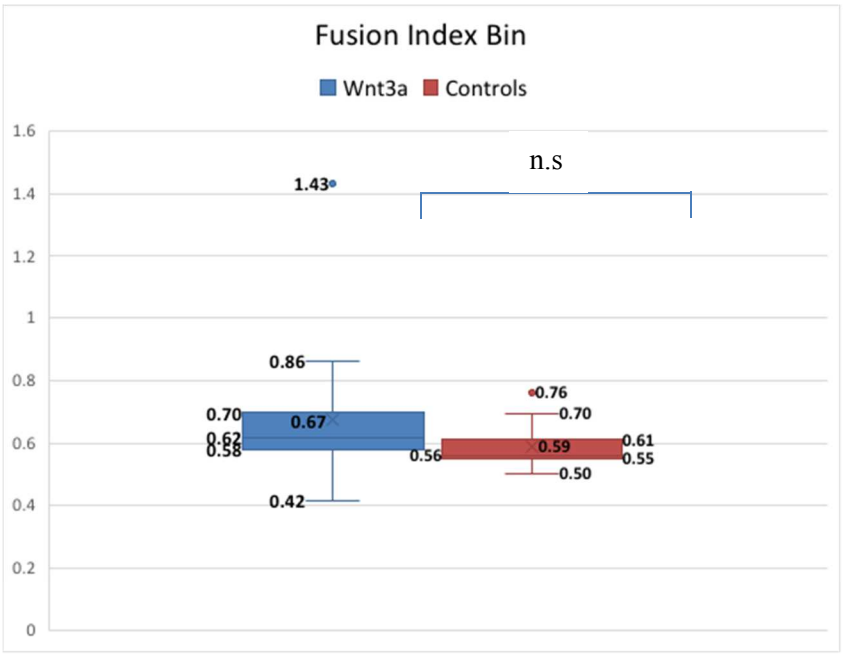


Figure 5.6. Differences between Wnt3a and control index values (bin range)

n.s. – No Significant difference

Fusion Index Area

The fusion index area will yield the percentage of the DAPI stained area located within the myotube. This in effect will help to provide an accurate measure of the fusion without the information regarding the how many individual nuclei are present.

The findings of the box plot analysis (as indicated in figure 5.6) indicated the fact that the index values are significantly varied between Wnt3a and control ($p=0.002<0.005$) with higher mean value exhibited by Wnt3a (0.66) compared to control (0.59). Figure 5.5 shows differences between Wnt3a and control index values. This result, in effect highlights the

efficiency of the proposed software assisted imaging technique to yield significant results in the quantification of cells.

Fusion index (area wise)

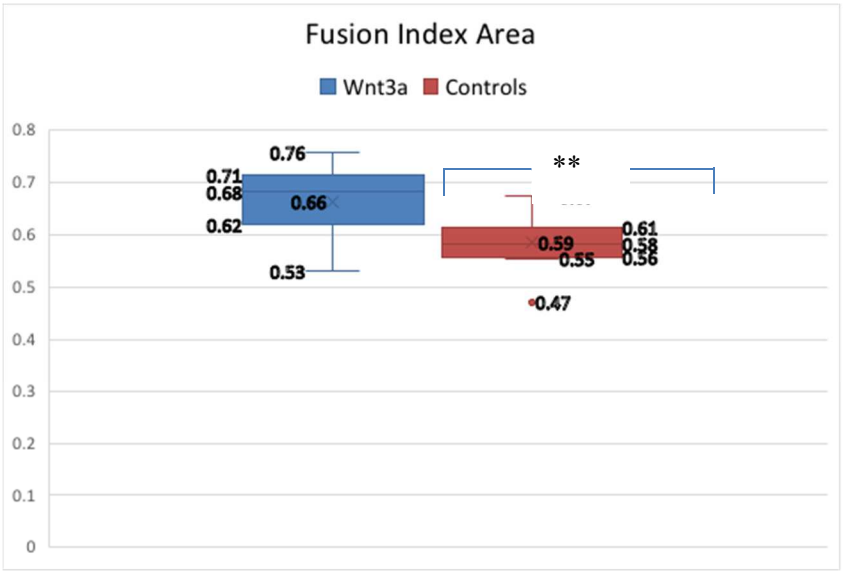


Figure 5.7. Differences between Wnt3a and control index values (area wise)

** $p < 0.01$

CHAPTER 6

CONCLUSION

Overview

Cell counting from immunostained images is a vital procedure in biomedical analysis as it helps in the quantification of proliferation, immunodetection, and differentiation of nuclear markers, which in turn have a significant role in analyzing the cell functioning. Hence, quantification of immunostained cells is largely used in oncology where the data obtained from tumor samples may have therapeutic or analytical impacts (Elie, 2003). Surgical pathology uses the quantified immunostained images as a diagnostic tool to differentiate between benign and tumor cells (Matos et al., 2006). Manual quantification has the drawbacks, such as the lack of repeatability due to inter- and intra-observer variability (Walker, 2006), the lack of precision due to manual visual quantification (Benali et al., 2003), the larger time consumption taken for counting and the lack of accuracy.

As a measure to overcome these difficulties, computerized image counting techniques was introduced. The studies of Lin et al. (1996) and Seidal et al. (2001) have highlighted the fact that software-assisted immunostaining quantification has resulted in improved reproducibility and faster results. In this context, the present study has proposed a software assisted GUI imaging technique and attempted to analyze its efficiency in the quantification of cells. The findings have been drawn on the basis of various analytical process, such as the comparison in the quantification between manual and automated in different stains, the colocalization, to identify the number of active cells while the image is spatially overlapped,

and the fusion indexing and the comparison of the cell counts in the Myotube with a control value. The significant outcomes drawn from these analyses are described in 'findings'.

Findings

The comparative analysis between the proposed software assisted imaging technique and manual counting in the quantification of stained cells, using different stains, such as β-Gal, DAPI and Sclerostin with the help of box plot, yielded a strong significant difference in DAPI and Sclerostin stains by demonstrating the higher cell count as a result of the application of proposed software assisted imaging technique. However, with β-Gal the analysis could not reach into a significant conclusion regarding the efficiency of the proposed automated imaging system.

The descriptive analysis performed to analyze the efficiency of the proposed software imaging technique in the quantification of overlapped cell on both two overlapped images (β-Gal and DAPI) and three overlapped images (β-Gal, DAPI, Sclerostin) demonstrated the fact in the case of two colocalized images, the software assisted imaging system identified the active cells, which ranges from 0.00 to 15.00 with a mean of 5±3 with respect to different images. However, the analysis of three images indicated that the software could only identify the active cells with a mean of 0.6±0.7 and having the minimum count of 0.00 and maximum count of 3.00.

The comparative analysis of the fusion index value of Wnt3a images against control, across the fusion bin range and fusion area using box plot, revealed significant variation in fusion index value between Wnt3a and control. Fusion area with higher mean value exhibited by Wnt3a (0.6±0.06) compared to control (0.5±0.04), whereas, despite showing higher mean

value for Wnt3a the fusion bin did not yield any proper significant outcome which help the study to reach into a conclusive inference.

Future Directions

Despite yielding the fairly good outcomes, the analysis depicted certain limitations that confined the scope of the study and in turn paved the way for the future studies. Albeit, the analysis highlighted the performance supremacy of the proposed automated imaging system in the quantification of the stained cells using DAPI and sclerostin stains, it could not repeat that vivid and favorable result in the case of β-Gal. Hence, the future researchers can extend this study using more efficient automated imaging systems which can yield a better and clear quantification results in β-Gal stain. The quantification of stained cells in the case of three colocalized images using the proposed imaging technique yielded less impressive results, which in turn opened the door for the future researchers to research and develop more advanced and refined technique which can yield higher counts from the three overlapped images. Finally, the researchers can also focus on developing a more powerful imaging technique, which can demonstrate a higher number of counts in the fusion bin.

REFERENCES

Benali, A., Leefken, I., Eysel, U. T., & Weiler, E. (2003). A computerized image analysis system for quantitative analysis of cells in histological brain sections. *Journal of Neuroscience Methods, 125*(1-2), 33-43.

Brey, E. M., Lalani, Z., Johnston, C., Wong, M., McIntire, L. V., Duke, P. J., & Patrick Jr, C.W. (2003). Automated selection of DAB-labeled tissue for immunohistochemical quantification. *Journal of Histochemistry & Cytochemistry, 51*(5), 575-584.

Decaestecker, C., Moles Lopez, X., D'Haene, N., Roland, I., Guendouz, S., Duponchelle, C., & Salmon, I. (2009). Requirements for the valid quantification of immunostains on tissue microarray materials using image analysis. Proteomics, 9(19), 4478-4494.

Delgado-Calle, J., Sato, A. Y., & Bellido, T. (2017). Role and mechanism of action of sclerostin in bone. *Bone, 96*, 29-37.

Diniz, C. (2010). Microscopic image analysis using computer-assisted methodology to quantify immunostained receptors. Microscopy: Science, Technology, Applications and Education. Eds. Méndez-Vilas A & Díaz J. Formatex Research Center, 1516-25.

Durán, E., & Arriazu, R. (2013). Quantification of protein expression on an immunofluorescence section by using the MetaMorph image analysis system. *Universal Journal of Applied Science, 1*(3), 86-94.

Elie, N., Plancoulaine, B., Signolle, J. P., & Herlin, P. (2003). A simple way of quantifying immunostained cell nuclei on the whole histologic section. Cytometry Part A: *The Journal of the International Society for Analytical Cytology, 56*(1), 37-45.

Fray, A.E., Ince, P.G., Banner, S.j., Milton, I.D., Usher, P.A. Cookson, M.R., & Shaw, P.J. (2003). The expression of the glial glutamate transporter protein EAAT2 in motor neuron disease: An immunohistochemical study.*European Journal of Neuroscience, 10*(8), 2481-9

Kohlberger, P. D., Obermair, A., Sliutz, G., Heinzl, H., Koelbl, H., Breitenecker, G., ... & Kainz, C. (1996). Quantitative immunohistochemistry of factor VIII-related antigen in breast carcinoma: a comparison of computer-assisted image analysis with established counting methods. *American Journal of Clinical Pathology*, *105*(6), 705-710.

Lehr, H. A., Mankoff, D. A., Corwin, D., Santeusanio, G., & Gown, A. M. (1997). Application of photoshop-based image analysis to quantification of hormone receptorexpression in breast cancer. *Journal of Histochemistry & Cytochemistry*, *45*(11), 1559-1565.

Lin, H. C., Sotnikov, A. V., Fosdick, L., Bostick, R. M., & Willett, W. C. (1996). Quantification of proliferating cell nuclear antigen in large intestinal crypt by computer-assisted image analysis. *Cancer Epidemiology and Prevention Biomarkers*, *5*(2), 109-114.

Matkowskyj, K. A., Schonfeld, D., & Benya, R. V. (2000). Quantitative immunohistochemistry by measuring cumulative signal strength using commercially available software photoshop and matlab. *Journal of Histochemistry & Cytochemistry*, *48*(2), 303-311.

Matos, L. L. D., Stabenow, E., Tavares, M. R., Ferraz, A. R., Capelozzi, V. L., & Pinhal, M.D. S. (2006). Immunohistochemistry quantification by a digital computer-assisted method compared to semiquantitative analysis. *Clinics*, *61*(5), 417-424.

McCabe, A., Dolled-Filhart, M., Camp, R. L., & Rimm, D. L. (2005). Automated quantitative analysis (AQUA) of in situ protein expression, antibody concentration, and prognosis. *Journal of the National Cancer Institute*, *97*(24), 1808-1815.

Mofidi, R., Walsh, R., Ridgway, P. F., Crotty, T., McDermott, E. W., Keaveny, T. V., ... & O'Higgins, N. (2003). Objective measurement of breast cancer oestrogen receptor status through digital image analysis. *European Journal of Surgical Oncology (EJSO), 29*(1), 20-24

Nabi, G., Seth, A., Dinda, A. K., & Gupta, N. P. (2004). Computer based receptogram approach: an objective way of assessing immunohistochemistry of androgen receptor staining and its correlation with hormonal response in metastatic carcinoma of prostate. *Journal of Clinical Pathology,57*(2),146-150.

Pham, N. A., Morrison, A., Schwock, J., Aviel-Ronen, S., Iakovlev, V., Tsao, M. S., ... & Hedley, D. W. (2007). Quantitative image analysis of immunohistochemical stains using a CMYK color model. *Diagnostic Pathology, 2*(1), 8.

Sant'Anna, L. B., Sant'Anna, N., & Parolini, O. (2011). Application of computer-assisted image analysis for identifying and quantifying liver fibrosis in an experimental model. *Journal of Computational Interdisciplinary Sciences*, *2*(2), 139-48

Seidal, T., Balaton, A. J., & Battifora, H. (2001). Interpretation and quantification of immunostains. *The American Journal of Surgical Pathology*, *25*(9), 1204-1207.

Shang, Y. C., Wang, S. H., Xiong, F., Zhao, C. P., Peng, F. N., Feng, S. W., ... & Zhang, C. (2007). Wnt3a signaling promotes proliferation, myogenic differentiation, and migration of rat bone marrow mesenchymal stem cells 1. *Acta Pharmacologica Sinica*, *28*(11), 1761-1774.

Singh, S. S., Kim, D., & Mohler, J. L. (2005). Java Web Start based software for automated quantitative nuclear analysis of prostate cancer and benign prostate hyperplasia.

Biomedical Engineering Online, *4*(1), 31.

Tarnowski, B. I., Spinale, F. G., & Nicholson, J. H. (1991). DAPI as a useful stain for nuclear quantitation. *Biotechnic & Histochemistry*, *66*(6), 296-302.

Veltri, R. W., Partin, A. W., & Miller, M. C. (2000). Quantitative nuclear grade (QNG): A new image analysis-based biomarker of clinically relevant nuclear structure alterations. *Journal of Cellular Biochemistry*, *79*(S35), 151-157.

Walker, R. A. (2006). Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment I. *Histopathology*, *49*(4), 406-410.

Zhu, M., Liu, C., Li, S., Zhang, S., Yao, Q., & Song, Q. (2017). Sclerostin induced tumor growth, bone metastasis and osteolysis in breast cancer. *Scientific Reports*, *7*(1), 11399.

VITA

Katika Anusha was born April 27, 1991 in Hyderabad, India. She did her undergraduate work at JNTUH, India. She received her Bachelor of Science and technology with Computer Science in 2012. After college she moved to United States of America to complete her master's in computer science at UMKC. She began on her work as a Software Engineer for the Food and Drug Administration with the purpose of combining her interests in technology and the problem-solving skills.