Automated Macrophage Quantification Program for Tissue-Engineering

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Abstract

Volumetric muscle loss injuries occur when the degree of tissue loss exceeds the endogenous regenerative capacity of skeletal muscle. These injuries result in permanent cosmetic and functional deficits, and current treatment options are ineffective for restoring muscle function. To solve this issue, new tissue-engineered solutions are being developed to improve skeletal muscle regeneration. To design an effective treatment, skeletal muscle repair must be better understood. Specifically, macrophages play an important role in the timing and mechanisms of functional recovery. The goal of the project was to develop an automated quantification program for macrophages that would permit more rigorous establishment of metrics for evaluating the participation and role of macrophages in wound healing following implantation of tissue engineered constructs. An automated quantification method was designed on MathWork's MATLAB using image thresholding techniques and a density based spatial clustering algorithm that could quantify macrophages on a slide image, and distinguish between the M1 and M2 phenotypes. To test the accuracy of the code, 20 digital slide images of macrophages stained with CD68 (pan macrophage marker), and CD163 (M2 macrophage marker) were analyzed using a manual counting method on NIH's ImageJ software, and using the developed automated method. The outcomes, including number of M1 macrophages, number of M2 macrophages, and the ratio of M2/M1 macrophages, were compared between the two methods. There was not sufficient evidence to suggest differences between automatic and manual measurements for M1 macrophages (P = 0.07; paired sample t-test) or M2 macrophages (P = 0.92; paired sample t-test). However, further analysis indicated that the automated program produced approximately equal numbers of false positive and false negative values. There was not sufficient evidence to suggest differences between automated and manual measurements for the M2/M1 ratio (P = 0.76; paired sample t-test), indicating that the M2/M1 ratio was conserved between methods.

Keywords: macrophage, volumetric muscle loss, skeletal muscle regeneration

Introduction

War fighters suffer extensive loss of skeletal muscle tissue during traumatic injuries suffered on the battlefield. When the degree of tissue loss exceeds the endogenous regenerative capacity, permanent cosmetic and functional deficits result which is referred to as volumetric muscle loss (VML). There are few current treatment options for restoration of VML injuries, and the efficacy of present therapies for skeletal muscle healing is extremely limited. The standard of care for VML injury involve physical rehabilitation, orthotics, or the placement of free or rotational flaps of muscle onto the site of large skeletal muscle defects. These procedures have proved ineffective in replacing lost skeletal muscle tissue and in restoration of skeletal muscle function[1]. A report detailing the wounding patterns in recent US conflicts found that 54% of combat wounds were located in the extremities, which often involve severe musculoskeletal injury[2]. These injuries contribute the most to the long-term disability of service members[3], and the lack of effective therapies presents a significant need for a regenerative solution that will restore the function of skeletal muscle for those suffering from these injuries. To

address this problem, we seek to develop new tissue engineered technology to improve muscle regeneration.

In order to develop effective tissue-engineered solutions for VML injuries, the physiology of skeletal muscle regeneration and wound healing environment must be better understood. Specifically, macrophages are a major player in functional regeneration of muscle due to their remarkable plasticity with diverse functions in tissue repair. There are two main macrophage phenotypes which can be observed at the wound site after skeletal muscle injury. These are the 'classically activated' macrophage phenotype, also known as 'M1' and the 'alternatively activated' phenotype, or 'M2'. Their presence depends on different external stimuli. M1 macrophages are produced in response to inflammatory stimuli and promote myoblast proliferation while also reducing fibroblast collagen production[4]. About 1-2 days after an injury, skeletal muscles recruit monocytes from the blood, which become inflammatory M1 macrophages[5]. 1-3 days after their recruitment, a phenotype switch takes place, and the macrophages transition to an anti-inflammatory M2 type[6]. Studies that demonstrated this sequence of macrophage phenotypes during skeletal muscle regeneration provided insight as to the benefits of anti-inflammatory drugs during the first days after muscle injury[7]. Similarly, the ability to accurately quantify and compare macrophage phenotypes at early timepoints after administration of a tissue-engineered treatment will provide useful data for recording the progression of the VML injury and the efficacy of the different applied therapies.

Distinguishing between M1 or M2 phenotype can be done through immunohistochemical staining. CD68 and CD163 are used to identify macrophages in tissue sections. CD68 is a membrane protein used as a pan-macrophage marker[5]. In vitro studies suggest that CD163 is an M2 marker[6]. To stain, tissue sections are subjected to antigen retrieval, autofluorescence reduction protocol, and overnight blocking. Samples are incubated with antibodies against CD68 and CD163, followed by incubation with fluorescence dye conjugated secondary antibodies. The stained sections are then digitized using confocal microscopy, and are can be quantified. For this project, CD68+/CD163- macrophages will be considered M1 macrophages, and CD68+/CD163+ macrophages will be considered M2 macrophages. Macrophages contain a central round nucleus, which is identified using DAPI, a fluorescent stain that visualizes nuclear DNA in living and fixed cells[8].

There is currently no standardized approach for quantifying M1 and M2 macrophages. A common method is to manually count cells through the microscope or produce digital images of the stained sections and manually counting and marking the cells on ImageJ. This time-consuming method is subject to inter-user variability and error from repeated human judgement. The use of digital imaging in pathology for quantification of macrophages has been used in a recent study for rapid quantification of the number of macrophages in heart tissue sections immunostained with macrophage marker F4/80[9]. However, this method required several calibration steps for each individual image in order to provide a macrophage count within 5% of the true value, suggesting that the most reliable method for quantification is manual identification by lab personnel. In addition, this method is unable to distinguish between M1 and M2 macrophages. The automated quantification program developed in this project herein is novel in that it will be able to accurately quantify M1 and M2 macrophages in skeletal muscle sections. This will be a significant improvement over other methods because of its potential to reduce the time required for analysis, improve accuracy by eliminating human bias and inter-user variability, and provision of information about the macrophages' phenotype.

There are several digital pathology techniques that can be used for imaging of digital pathology slides. For this project, a combination of image thresholding and spatial analysis was used to create a MATLAB program that can identify locations of M1 and M2 macrophages. Image thresholding is a technique that segments a stained digital slide image by quantifying the staining intensity at each point on the image. If the intensity is above a certain threshold, a value is added to this area, and if the intensity is below the threshold, no value is given to this area on the image[10]. This approach was used to separate stained regions of the slide images from unstained or background regions. Spatial analysis is a broad range of imaging techniques that includes spatial clustering, which can be used to detect groups or clusters on an image. This approach was also used while developing this program to automatically identify clusters of macrophages, and then identify the phenotype based on the staining present in the clusters[11].

The goal of this project was to design an automated method for quantification of M1 and M2 macrophages by developing a MATLAB code capable of segmenting

images based on CD68+ immunofluorescent stain, CD163+ immunofluorescent stain, and DAPI stain, and identify individual macrophages on the segmented images through the use of a density-based spatial clustering algo

rithm. The goal of the program was to output data including the number of M1 macrophages, and the number of M2 macrophages. Then, using images from different timepoints, these data would be used to produce a graph of the change in M2/M1 macrophage ratio overtime. This analysis can be used in computational models that can predict the macrophage phenotype response for tissue-engineered treatments for VML injury in animal model studies.

Results

The MATLAB program developed requires the user to input a digital slide image stained with DAPI, CD68 and CD163. The program then will use the mentioned strategies to

Table 1. Comparison Between Manual and Automated Measurements of CD68+/CD163- macrophages and CD68+/CD163+ macrophages.

Measurement	CD68+/CD163-		CD68+/CD163+	
	Manual	Automated	Manual	Automated
Mean	30.75	38.81	12.44	12.38
SD	33.59	48.67	10.95	10.44
P-Value	0.07		0.92	

quantify the macrophages in the image, outputting CD68+, CD68+/CD163+, M1 and M2 values. To test the effectiveness of the MATLAB program, 20 digital images of skeletal muscle tissue sections stained with DAPI, CD68 and CD163 were tested. The images were obtained from in vivo rodent studies that were investigating the influence of a tunable Hyaluronic acid (HyA)-based biomaterial, alone or in combination with existing Tissue Engineered Muscle Repair (TEMR) technology on skeletal muscle regeneration after injury creation and biomaterial implantation. To simulate a VML injury, 20% of the rodent's tibialis anterior (TA) muscle was removed. The biomaterial was then implanted, and muscles were explanted and observed at various time points including 2 weeks, 6 weeks, and 12 weeks. Digital images were obtained from all three of these groups: 10 images from 12 weeks, 6 images from 6 weeks, and 4 images from 2 weeks. Manual macrophage quantification was performed on each image using ImageJ. Then, automated macrophage quantification was done using the MATLAB program for each image, and the results for
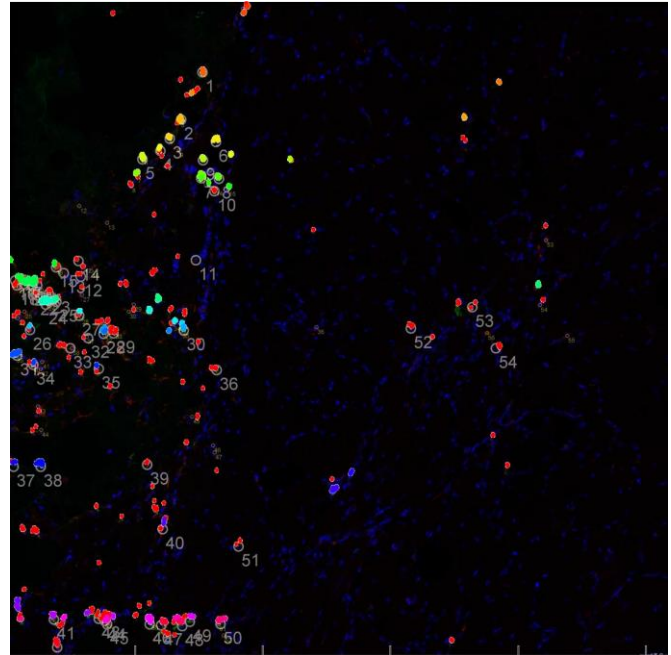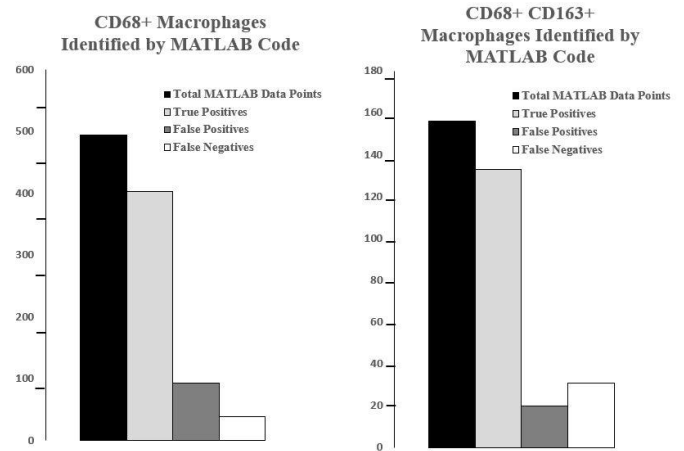

Fig. 1. Density-based clustering plot produced by MATLAB overlayed on Manual Quantified Image from ImageJ.

CD68+/CD163- (M1) macrophages and CD68+CD163+ (M2) macrophages were compared between methods.

Paired sample t-tests were performed to detect differences in quantified clusters between manual and automated measurements. P values below 0.05 were considered statistically significant. Table 1 summarizes the comparisons between manual and automated macrophage quantification of CD68+/CD163- macrophages, and CD68+/CD163+ macrophages. No significant differences were found between automatic and manual measurements for CD68+/CD163- clusters (P = 0.07; paired sample t-test). No significant differences were found between automatic

and manual measurements for CD68+/CD163+ clusters (P = 0.92; paired sample t-test). These results show that the automated program produces macrophage cluster counts comparable to the manual ones, because there is not sufficient evidence to coclude that there are significant differences in the counts between manual and automated methods.

To further explore the relationship between the manual
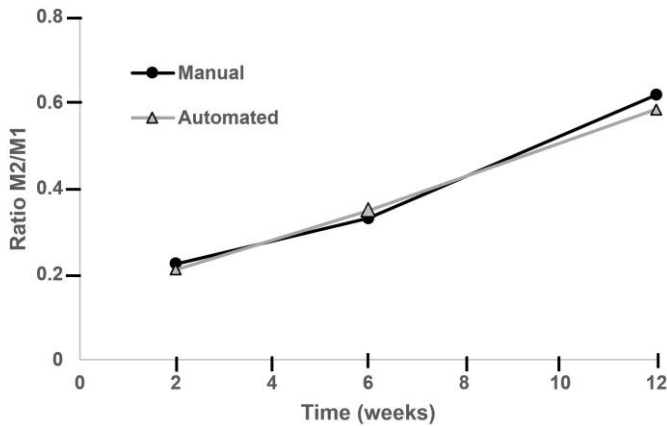
macrophage counts and the automated quantification program, data points for true positive counts, false positive counts, and false negative counts were taken. This was done by overlaying the density-based clustering plot produced by the MATLAB program with the manual quantified image from ImageJ, shown in Fig. 1. True positives were recorded at points where a manually counted cluster was correctly identified by the MATLAB program. Points where a manually counted cluster was identified, but the MATLAB code failed to quantify were classified as false negatives, and points where a manually counted cluster was not identified, but the MATLAB code quantified one was classified as a false positive. Fig. 2 shows these numbers for the CD68+ Clusters identified by the MATLAB code, and these numbers for the CD68+/CD163+ macrophages identified by the MATLAB code. These results show the code's tendency to overcount CD68+ clusters, and undercount CD68+CD163+ macrophages. Due to the automated nature of the MATLAB program, the numbers determined for M1 and M2 macrophages did not differ significantly from the manual counts. However, the false positive and false negative data show that the code does not correctly identify every single macrophage.

One important application of macrophage quantification, mentioned previously, is the change in M2/M1 ratio overtime. Using the quantification data obtained, the ratio

of M2/M1 macrophages was calculated for each image using both manual and automated counts. Fig. 3 shows how this ratio changes at 2-, 6-, and 12-week timepoints in the manual and automated counts. The MATLAB program's accuracy in determining the M2/M1 ratio was tested using a paired sample t-test to detect differences in the ratio obtained by the manual quantification method and automated method. No significant differences were found between automated and manual measurements for the M2/M1 ratio (P = 0.76; paired sample t-test). This result shows that the automated program produces M2/M1 ratios comparable to the manual ones.

Along with accuracy, the time required to quantify macrophages using each method was recorded. On average, it took around 13.5 minutes to complete the manual macrophage counts, and on average it took about 4.2 minutes for the MATLAB code to run. Significant differences were found between automated and manual measurements for the time required to obtain M1 and M2 counts. This shows that the user of this program could conduct the same analysis in about a quarter of the time. In

Fig. 3. Average M2/M1 Ratio over time for both manual and automated count. Data points from 3 different time points.

addition, during the time that the MATLAB code is being run, the user does not need to be monitoring the program; most of this time is saved by the user as well.

Discussion

Initial results comparing automatic and manual measurements for CD68+ clusters, CD163+ clusters, CD68+/CD163- macrophages and CD68+/CD163+ macrophages showed that there were no significant differences in the manual and automated quantification. However, when examining the results closer, it became evident that this may have been due to the consistency of the automated program, rather than its ability to actually identify each individual macrophage. When the density-based clustering plot produced by the MATLAB program

Fig. 2. CD68+ Macrophages Identified by MATLAB Code and CD68+/CD163+ Macrophages identified by MATLAB Code. True positive, false positive, and false negative shown for both groups.

was overlayed with the manual quantified image from ImageJ, it became clear that not all the points identified by the MATLAB code were accurate, or true positives. Instead, many of the points produced by the MATLAB code were false positives, meaning the code counted areas that were not actually negatives. In addition, many of the actual points that were manually counted on the images were not

identified by the MATLAB code, resulting in false negatives. Since the percentage of false negative points is close to the percentage of false positive points, these errors cancel each other out, resulting in the code outputting values that were not significantly different between manual and automated counts. Because of this, the way that the automated program is being used will affect the accuracy of the results. The automated program has proved to be accurate when the overall number of identified macrophages on an image is used. One important application of that quantification is the ratio of M2/M1 macrophages overtime. Testing showed that there were no significant differences in this ratio between the automated method and the manual method. The ability to obtain these values in a fraction of the time it would take for the manual counting method makes this program an extremely valuable tool for determining the modulation macrophage phenotype has on skeletal muscle regeneration for VML treatments. This data can be paired with data on muscle regeneration or functional recovery to show the effects that macrophage phenotypes have on the efficacy of the tissue-engineered solution.

Despite the abilities of the program to accurately quantify the ratio of M1/M2 macrophages, there are limitations to this program. If a user wanted a precise depiction of the location of each individual macrophage on the image, the program would not produce accurate results because of the numbers of false negatives and false positives that the program identifies. If the goal of the project being done was to explore the locations of macrophages in relation to the wound site, or to each other, this program would not provide accurate results. Instead, the program would need to be used as a semi-automated method for macrophage quantification. The program is able to successfully identify 82.3% of the manually counted macrophages, but human judgement would still be required to get rid of false negative and false positive counts in the final data set.

An additional limitation is the requirement of entering parameters for the density-based spatial clustering algorithm used in the program. This algorithm identifies clusters of points in an image based on the density within a certain region. Two parameters are set for the algorithm to start its analysis. First is epsilon, which is the distance around each point that the algorithm searches for another point. The other is the minimum number of points, which is used to determine core, border, and noise points. The algorithm begins by searching around each point, using the radius specified by the epsilon parameter. If there are the minimum number of points within that epsilon distance of

each other, the algorithm determines the point is a core point. Those that are within epsilon distance of a core point, but do not contain the minimum number of points with an epsilon radius, are labeled as border points. Those that are not within epsilon distance of a core point are considered noise, and are not counted as part of a cluster. The algorithm identifies clusters by looking at how many groups of core points, surrounded by border points, are contained in a single image. It then will output the number of clusters, which was used in the code to get the automated count for the clusters and macrophages. The two parameters in this algorithm, epsilon and minimum points, must be set by the user, and can be adjusted based on the size of the objects in the image. This provides more flexibility with the code, enabling it to be more precise for identification of smaller objects, however it also introduces human judgement and variability into the program. Adjusting the parameters even slightly has the potential to produce different quantities of clusters or macrophages on the images. Therefore, if a set of images from the same experiment was analyzed by several individuals, there is the possibility for variability in the results if the clustering algorithm parameters are not coordinated among the members working on the project. One of the original goals of the automated program was to remove the potential for human error when the manual counting was being done. The automated process mostly mitigates this error, but due to the parameter adjustments that must take place, it does not completely eliminate the need for human judgement.

This program was designed as a tool for quantifying macrophages in order to explore their effects on skeletal muscle regeneration after VML injuries. To do this, the program segments the images based on the color and intensity of the staining, and then clusters the objects to perform the automated count. Therefore, this program could be used for quantifying any type of cell or histological endpoint that can be stained using antigen retrieval, as long as fluorescence dye conjugated secondary antibodies are used at the end of the procedure. This has the potential to be used as a quantification tool for building predictive models. Macrophages are important for skeletal muscle regeneration, but they are not the only component that plays into muscle remodeling. Other inflammatory cells such as neutrophils, as well as the interactions of macrophages with native muscle cells such as satellite stem cells and fibroblasts all play roles in regeneration of skeletal muscle after injury[12,13]. With proper staining, detection and quantification of these cells would be possible with this program, and could be useful for future work with predictive modeling. The data obtained in this project about

the M2/M1 macrophage ratio overtime can be fed into a predictive model. Building a computational model of muscle regeneration using data collected about neutrophils and native muscle cells as well could provide an insightful basis for predicting muscle regeneration, which is useful information for designing or improving upon tissue-engineering solutions for skeletal muscle regeneration and functional recovery.

The automated program in its current state is useful for determining the overall number of macrophages on a slide image, or the overall ratio of macrophages phenotypes on a set of slide images, however it is not accurate enough to determine the precise location of each cell. This project is a great starting point for quantification of the number and location of macrophages, and future work could incorporate additional steps after the image thresholding and spatial analysis methods that were used. Future iterations to improve on this program could implement machine learning. This is the use of computer algorithms that automatically improve as the algorithm uses more data. As the program gains more experience, it would be able to accurately identify all macrophages on a slide image, and data regarding the macrophage's location in proximity to the injury site, and in relation to other macrophages could be extracted using the program.

Materials and Methods

20 digital images were obtained from in vivo rodent studies that were investigating the influence of a tunable Hyaluronic acid (HyA)-based biomaterial, alone or in combination with existing Tissue Engineered Muscle Repair (TEMR) technology on skeletal muscle regeneration after injury creation and biomaterial implantation. A VML injury model was created by surgically excising ~20% of the tibialis anterior muscle of Lewis rats. After

surgical excision, the animals were placed into one of the four treatment groups: (1) no repair, (2) repair with HyA biomaterial, (3) repair with TEMR implant, (4) repair with HyA + TEMR implant. Animals were allowed to recover before being euthanized at varying time points, including: 2 weeks, 6 weeks, and 12 weeks. TA muscles were explanted at sacrifice. Next, the muscles were sectioned and stained for CD68 and CD163. The sections were subjected to antigen retrieval, autofluorescence reduction protocol, and overnight blocking at 4ºC. Samples were incubated with antibodies against mouse CD68 (1:100, MCA341R; Bio-Rad) and rabbit CD163 (1:400, ab182422; Abcam) overnight at 4ºC. Secondary antibodies were then applied for 2-6 hours at 4ºC using Alexa Fluor 594 goat α-rabbit (1:500, A11017; Invitrogen) and goat α-mouse Alexa Fluor 488 F(ab')2 fragment (1:500, A11017; Invitrogen). The antibodies were diluted in Dako Antibody Diluent (Dako Antibody Diluent S0809; Agilent Technologies). The stained sections were then digitized using confocal microscopy, and the images were ready for analysis.

The first part of the analysis was the manual counts of the digitized images. This was done using NIH's ImageJ image processing program. Each digitized image was a Z-stack of confocal images, which displays 3D data. This was converted to a 2D image by using the maximum intensity projection tool. The 2D image for the DAPI stain and the CD68 stain were then merged to create a 2D image for quantifying CD68+ macrophages. Similarly, the 2D images for the DAPI stain and the CD163 stain were merged to create a 2D image for quantifying CD163+ macrophages. To manually count the images, ImageJ's point tool was used. This adds a circle with a number to the location on the image where the user clicks, so each macrophage was identifiable by a white (CD68+) or yellow (CD163+) circle on the image. After quantifying CD68+ and CD163+ macrophages, the images were overlaid to visualize which macrophages contained both CD68 and CD163 staining. From here, numbers of CD68+/CD163- macrophages and CD68+/CD163+ macrophages were produced. This was repeated for each of the digital images, and the amount of time to conduct this analysis was noted.

The second part for the analysis was the automated counts of the same digitized images. MathWorks's MATLAB program was used
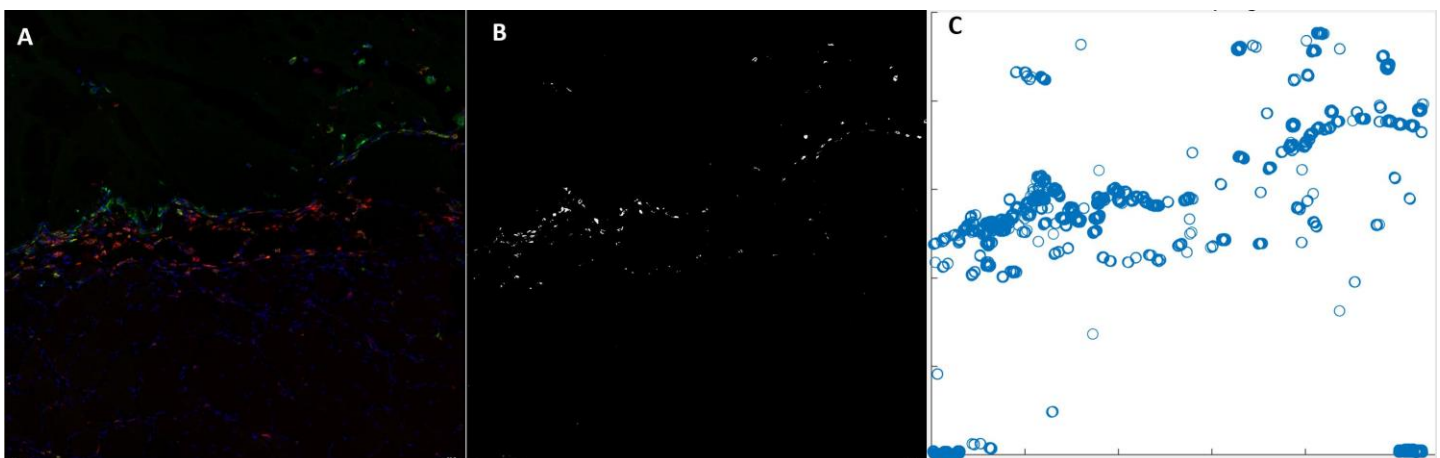

Fig. 4. Conversion of ORP15 Image 1 **(A)** into a binary image **(B)** and then into a plot of points that correspond to the binary image **(C).** All is done through the MATLAB code developed.

to write a code that would automatically quantify macrophage images. In designing the code, the first step was to create a section where the digitized image could be imported. This includes three steps to insert the file name for the images of the maximum intensity projected Z-stack of the DAPI stain, the CD68 stain, and the CD163 stain. Next, the code binarizes each of the images. This creates a binary image from the 2D image that was imported by replacing all the values above a globally determined threshold with 1s and setting all other values to 0s. This was done by the use of Otsu's method, which is an algorithm that determines a threshold that will minimize the variance between the image background and foreground. This threshold was computed using a 256-bin image histogram, and separates the image so that only the stained sections are visible. The logical array that the image is stored in consists of 1s where there was staining above the threshold, and 0s where there was staining below the threshold.

Next, the logical array was used to find the overlap of staining between DAPI and each of the macrophage marker stains. To do this, the logical arrays of the two groups were multiplied together. This works because any areas that contained staining on both images would be the product 1x1 = 1. Any other areas that did not contain staining on one image or the other would be multiplied by 0 and therefore result in 0. Before multiplication took place, the code first adds 1s values to pixels that are adjacent to pixels with a current value of 1. This was done so that areas where DAPI and CD68 stain were next to each other, for example, but did not completely overlap, were still present in the image being analyzed. The goal of this was to eliminate areas of staining that contained only CD68 or only CD163, which would be background staining because macrophages are identified by the nuclear DAPI stain surrounded by the CD68 and/or CD163 stain. Multiplying DAPI and CD68 stain together found areas that contained a nuclear stain and the CD68 pan macrophage marker and multiplying DAPI, CD68 and CD163 stain together found areas that contained a nuclear stain and the pan macrophage marker and the CD163 M2 macrophage marker, representing CD68+/CD163+ macrophages. Next, these logical arrays were converted into a set of points based on where values of 1 were located. This allows for a plot of points to be created that represents where staining exists. Fig. 4 shows the conversion of a CD68 stained image into the binary image, and then into a set of points.

With the points of interest that contain relevant staining plotted in a graph, the spatial analysis method of density-based clustering is then implemented. The code uses a density-based spatial clustering algorithm to automatically quantify the number of clusters on the DAPI and CD68 plot, and on the DAPI, Cd68 and CD163 plot. This algorithm performs clustering on an input data matrix and returns the cluster indices that indicates which pixels are core points, which are border points, and which are noise points. Core points are located in the middle of the cluster, border points are those that are on the outside of the cluster, neighboring the core points, and noise points do not belong to a cluster; they are outliers. These are important to consider in this automated method because there is often off target staining that occurs which should not be counted as a macrophage. Two parameters are set which will determine which points are classified as core, border, or noise. The first is the epsilon neighborhood, which is specified as a numeric scalar that defines a neighborhood search radius around the point. The second is the minimum number of neighbors required for a core point. This epsilon neighborhood of a core point

in a cluster must contain at least the minimum number of neighbors, but the epsilon neighborhood of a border point can contain fewer neighbors than the minimum number of neighbors. Each cluster detected after the entire image is analyzed is counted as a macrophage. This algorithm is run on the plots of CD68 and DAPI intersection, and also the plots for CD68, DAPI, and CD163 overlap. The result is numbers of clusters for CD68+ macrophages from the first plot, and CD68+/CD163+ macrophages from the second plot. From here, M1 macrophages are calculated by subtracting the CD68+/CD163+ clusters from the CD68+ clusters, and M2 macrophages are the number of clusters from the second plot. M1 and M2 macrophages counts appear as an output in the MATLAB command window once the analysis is complete. The time for each

As described in the results section, the accuracy of the automated code was tested by comparing the outcomes for M1 and M2 macrophages. Paired sample t-tests were performed to detect differences in quantified clusters between manual and automated measurements. The accuracy was further explored by identifying data points for true positive counts, false positive counts, and false negative counts. This was done by overlaying the density-based clustering plot produced by the MATLAB program with the manual quantified image from ImageJ. Lastly, the ratio of M2/M1 macrophages overtime were compared between the two methods. The MATLAB program's accuracy in determining the M2/M1 ratio was tested using a paired sample t-test to detect differences in the ratio obtained by the manual quantification method and automated method.

End Matter

Author Contributions and Notes

G.C. and J.P. designed research, G.C, J.P. and A.F. performed research, A.F. wrote software, A.F. and J.P. analyzed data; and A.F. wrote the paper. The authors declare no conflict of interest.

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