

QUANTIFYING MYOCARDIAL FIBROSIS ON MURINE MODEL USING IMAGEJ

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Abstract: *Quantification of histological images is a task often required in biomedical research. Many of the methods used are time-consuming, difficult to replicate and subjective. This work aimed to describe and validate a more objective, reproducible and easy to perform methodology to measure murine heart fibrosis, using ImageJ software. For this matter, a macro was created and validated for its intra and inter-observational reproducibility. No significant differences were found between the results obtained manually and with the macro ($p > 0.05$). Regarding the interobserver variability, the macro proved to be a less variable method by presenting higher correlation coefficients between observers when compared with the manual measurement. With these results, we were able to define and describe a methodology that outputs objective and replicable results in a less laborious way.*

Keywords: *Experimental pathology, Fibrosis Models, Heart, ImageJ, Macros, Morphometry.*

1. INTRODUCTION

One of the experimental pathologist challenges is to "translate" into numbers what was seen and described on a slide allowing posterior statistical analysis and comparison between groups /experimental models (Rahman and Itakura, 1996). This is of particular importance because of the growing discussion about the reproducibility of preclinical results obtained using animal models (Schofield, Ward and Sundberg, 2016). Given this and the fact that histopathological assessment continues to be one of the most used approaches for the phenotypical characterization of different model organisms, its replicability and correct description are extremely important

(Scudamore *et al.*, 2016). Obtaining this reproducibility is a constant challenge. All these factors can lead to morphometric tools gaining prominent importance because of their ability to extract exact measurements from slides instead of subjective descriptions. However, the usage of morphometric tools does have some counterparts because, in some cases, they are time-consuming, laborious and require the use of specialized instruments and infrastructures. (Soreide *et al.*, 2009). ImageJ is a freeware software that has the capacity to create and execute macros. Using its built-in command recorder (figure 1), it outputs lines of code that can be transformed into an executable file. Running this type of files on ImageJ allows repetitive tasks to be reduced to a small number of clicks

allowing to decrease the amount of work and time consumed in each task. (Hadi *et al.*, 2011). Fibrosis is the formation and deposition of excess fibrous connective tissue in an organ or tissue, in a reparative or reactive process (Wynn and Thirumalai, 2012). It can be linked to genetic diseases, inflammatory processes, exposure to toxins, ischemic changes or autoimmune diseases. Isoprenaline is a synthetic sympathomimetic non-selective β -agonist. The pathophysiological mechanism behind the myocardial damage caused by this compound is believed to be related to a dramatic drop in blood pressure after its administration leading to hypoperfusion damage. The histological characteristics of the lesions are the deposition of fibrotic tissue in the subendocardial/myocardial region, mainly in the left ventricle and in the papillary muscle zone as can be seen in figures 2 and 3. This pattern is similar to that found in other models of cardiac ischemia (ex: left anterior descending artery suture) (Brooks and Conrad, 2009).

2. MATERIALS AND METHODS

Experimental design

To validate the use of macros as a valid method for the quantification of fibrotic lesions two different approaches were taken. First, the intraobserver variation was assessed by comparing the results obtained for the affected percentage through two different methods, manually using only the Color Threshold tool and using an in-house developed macro, (Table 1 and 2) all being performed by the same operator. Second, in order to determine the interobserver variation, 6 sections clearly different from each other in terms of the affected area were selected and three different observers were asked to measure the percentage of affected tissue. The results were then compared between them.

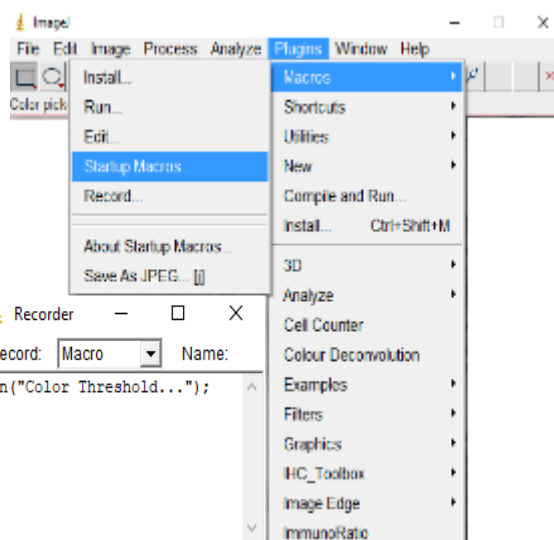


Figure 1 – Representative image on how to use ImageJ’s in-built recorder in order to develop your own macro.

Sample collection

All samples were obtained in the Histopathology Unit of the Gulbenkian Institute of Science. Hearts were sent by different research groups to the unit for histopathological analysis, being chosen for the present study samples from models of myocardial fibrosis (isoprenaline model). Samples were fixed in 10% buffered formalin and processed by routine methods for light microscopy. All selected samples were stained with Masson's Trichrome which stained the collagen blue and cardiomyocytes red.

Inclusion criteria

For this study were included all sections that, after a careful histopathological analysis, were diagnosed with fibrotic lesions in the myocardium.

Quantification of fibrosis

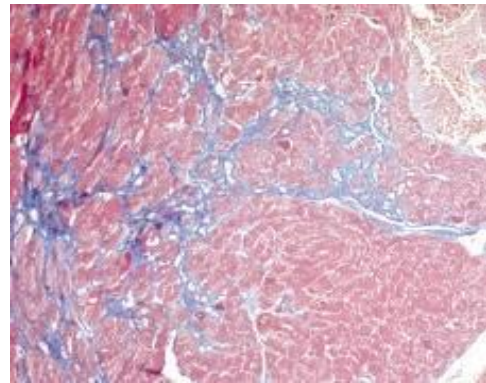
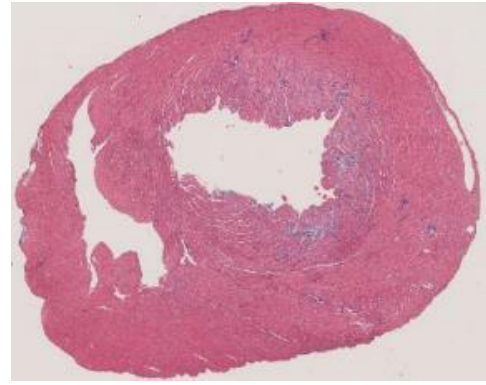
For the quantification of the fibrotic lesions, photographs were taken with the help of a LEICA MZ 12.5 stereomicroscope that was connected to a TOSHIBA Intel R Core i7-2677M 1.80ghz CPU portable computer with

8gbs of RAM and Windows 7 as the operating system through the UEyeCockpit software, obtaining files in JPEG format. In some cases, slides were scanned using the Hamamatsu Nanozoomer-SQ Digital Slide Scanner D12140 scanner and photographs were later acquired in JPEG format using the NDP.view 2 software. All the photographs were obtained with an ampliation of 2x, with a scale of 221 pixels/mm with a resolution of 2048x1536 pixels. For image analysis purposes, the ImageJ software was used. To obtain a percentage, the area of fibrosis obtained was divided by the total section area and then multiplied by one hundred. As described, the validation of the macro was divided into two phases. For the first phase, 45 sections were analyzed (n = 45) which led to the obtaining of 45 photographs in a magnification of 2x. For the second phase, six sections (n = 6) were chosen. In this phase, six different photographs were used.

Statistical analysis

Descriptive and inferential statistics were performed using the SPSS program (Statistical Package for the Social Sciences) version 22.0.

For the results obtained, the average, and standard deviation (descriptive statistics) were calculated. To determine if there were statistically significant differences between groups, a Mann-Whitney test was performed after confirming the non-normal distribution of results through a Shapiro-Wilk test. For the results of the different observers, the normality was verified through the same test and the differences between groups this time using an ANOVA test. To assess the correlations between the results using the two methods and between the different operators, Pearson (results with a normal distribution) and Spearman (results without a normal distribution) tests were performed. Values of $p < 0.05$ were considered as statistically significant.



Figures 2 and 3 – Heart, Masson's Trichrome 2x and 200x respectively. Representative images of the isoprenaline-induced lesions in a mouse's heart. Photographs taken by the authors

3. RESULTS

Macro Validation for intra-observer variability

The results obtained when using the macro compared to those by the manual process were very similar. (table 3) This was corroborated by the fact that no statistically significant differences were found between groups. ($P > 0.05$) For this case, the results demonstrated in figure 4 representing the degree of correlation between the different results show that a strong significant correlation between both methods exists. ($r = 0.954$, $p > 0.001$)

Macro validation for inter-observer variability

In the second phase, the results obtained by the four different operators were compared. No statistically significant differences were found between groups. ($p > 0.05$). The results presented in tables 4 and 5 show a strong correlation between the results obtained by the different observers but, highlighted, there are cases where this correlation is higher when using the macro. All the correlations were considered statistically significant ($p < 0.05$)

Table 3 Comparison between the results of the descriptive statistic of the two different methodologies

	N	Average (%)	Standard Deviation
Macro	45	0.74	0.92
Color Threshold	45	0,76	0,87

Table 4 and 5 - Comparison between the Pearson's correlation coefficient obtained by the different operators using the two methods. It is possible to find some cases where the manual method presents lower correlation values when compared with the macro

Operator	% affected section - Macro			
	1	2	3	4
1	1	0,993	0,989	0,991
2	0,993	1	0,993	0,991
3	0,989	0,993	1	0,999
4	0,991	0,991	0,999	1

Operator	% affected section - Color Threshold			
	1	2	3	4
1	1	0,967	0,971	0,848
2	0,967	1	0,992	0,857
3	0,971	0,992	1	0,895
4	0,848	0,857	0,895	1

4. DISCUSSION AND CONCLUSIONS

Quantifying an image for comparative purposes is not always an easy task and can often turn into a time-consuming, laborious task that gives rise to disparate values among observers. Daunoravicius *et al* 2014 demonstrated that colorimetric methods were more precise and less variable in comparison to visual gradation, underlining the importance of this type of methods as an aid to the obtaining of precise and invariable data. Having this in thought, on this work, we tried to develop tools in order to overcome these difficulties.

The use of Color Thresholding is a well-described method to quantify myocardial fibrosis (Teekakirikul *et al.*, 2010). The use of macros for the same purpose is also described, as well as their validation in relation to the time saved and the results obtained with other methodologies, but in samples stained with *Sirius red*. (Hadi *et al.*, 2011; Ellims *et al.*, 2015). In this work, the samples used were stained with Masson's Trichrome and therefore we decided to create a macro that facilitated this type of work under these conditions. The results of the statistical analysis corroborate that, on this kind of samples, it is possible to quantify fibrosis using a macro, under the controlled conditions encountered, and that it outputs less variable results between observers in a less laborious way when compared with the manual method, being in concordance with what is described in the bibliography (Hadi *et al.*, 2011; Ellims *et al.*, 2015).

Our study does present some limitations, the main being the small number of samples that the different operators measured. Ideally, at least the 45 samples should be repeated.

Conclusions

After this work, we can affirm that the methodology presented is a valid tool for the conditions described from the point of view of the repeatability, precision, and reduction of workload. Since it is an editable file, it can be easily adapted to meet one's needs in different working conditions (ex: different

microscopes). As a future work idea, the repetition of measurements by more different operators with a larger number of samples undoubtedly has some interest.

Declaration of interest: The authors state no conflicts of interest. The authors alone are responsible for the content and writing of this paper

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TABLES**Table 1** - The macro used to measure the myocardial fibrosis

<i>Line 1</i>	run("Smooth");
<i>Line 2</i>	run("Smooth");
<i>Line 3</i>	run("Color Threshold...");
<i>Line 4</i>	// Color Thresholder 1.50i
<i>Line 5</i>	// Autogenerated macro, single images only!
<i>Line 6</i>	min=newArray(3);
<i>Line 7</i>	max=newArray(3);
<i>Line 8</i>	filter=newArray(3);
<i>Line 9</i>	a=getTitle();
<i>Line 10</i>	run("HSB Stack");
<i>Line 11</i>	run("Convert Stack to Images");
<i>Line 12</i>	selectWindow("Hue");
<i>Line 13</i>	rename("0");
<i>Line 14</i>	selectWindow("Saturation");
<i>Line 15</i>	rename("1");
<i>Line 16</i>	selectWindow("Brightness");
<i>Line 17</i>	rename("2");
<i>Line 18</i>	min[0]=135;
<i>Line 19</i>	max[0]=226;
<i>Line 20</i>	filter[0]="pass";
<i>Line 21</i>	min[1]=28;
<i>Line 22</i>	max[1]=255;
<i>Line 23</i>	filter[1]="pass";
<i>Line 24</i>	min[2]=0;
<i>Line 25</i>	max[2]=203;
<i>Line 26</i>	filter[2]="pass";
<i>Line 27</i>	for (i=0;i<3;i++){
<i>Line 28</i>	selectWindow(""+i);
<i>Line 29</i>	setThreshold(min[i], max[i]);
<i>Line 30</i>	run("Convert to Mask");
<i>Line 31</i>	if (filter[i]=="stop") run("Invert");
<i>Line 32</i>	}
<i>Line 33</i>	imageCalculator("AND create", "0","1");
<i>Line 34</i>	imageCalculator("AND create", "Result of 0","2");
<i>Line 35</i>	for (i=0;i<3;i++){
<i>Line 36</i>	selectWindow(""+i);
<i>Line 37</i>	close();
<i>Line 38</i>	}
<i>Line 39</i>	selectWindow("Result of 0");
<i>Line 40</i>	close();
<i>Line 41</i>	selectWindow("Result of Result of 0");
<i>Line 42</i>	rename(a);
<i>Line 43</i>	// Colour Thresholding-----
<i>Line 44</i>	run("Convert to Mask");
<i>Line 45</i>	run("Analyze Particles...", " show=Masks display summarize");

Table 2 - The macro used to measure the total section area

Line 1	run("Smooth");
Line 2	run("Smooth");
Line 3	run("Color Threshold...");
Line 4	// Color Thresholder 1.50i
Line 5	// Autogenerated macro, single images only!
Line 6	min=newArray(3);
Line 7	max=newArray(3);
Line 8	filter=newArray(3);
Line 9	a=getTitle();
Line 10	run("HSB Stack");
Line 11	run("Convert Stack to Images");
Line 12	selectWindow("Hue");
Line 13	rename("0");
Line 14	selectWindow("Saturation");
Line 15	rename("1");
Line 16	selectWindow("Brightness");
Line 17	rename("2");
Line 18	min[0]=146;
Line 19	max[0]=255;
Line 20	filter[0]="pass";
Line 21	min[1]=44;
Line 22	max[1]=255;
Line 23	filter[1]="pass";
Line 24	min[2]=0;
Line 25	max[2]=255;
Line 26	filter[2]="pass";
Line 27	for (i=0;i<3;i++){
Line 28	selectWindow(""+i);
Line 29	setThreshold(min[i], max[i]);
Line 30	run("Convert to Mask");
Line 31	if (filter[i]=="stop") run("Invert");
Line 32	}
Line 33	imageCalculator("AND create", "0","1");
Line 34	imageCalculator("AND create", "Result of 0","2");
Line 35	for (i=0;i<3;i++){
Line 36	selectWindow(""+i);
Line 37	close();
Line 38	}
Line 39	selectWindow("Result of 0");
Line 40	close();
Line 41	selectWindow("Result of Result of 0");
Line 42	rename(a);
Line 43	// Colour Thresholding-----
Line 44	run("8-bit");
Line 45	setAutoThreshold("Default");
Line 46	//run("Threshold...");
Line 47	setOption("BlackBackground", false);
Line 48	run("Convert to Mask");
Line 49	run("Analyze Particles...", " show=Masks display summarize");