QUANTIFYING HEPATIC FIBROSIS ON MURINE MODELS: HOW TO OBTAIN REPRESENTATIVE RESULTS IN A LESS LABORIOUS WAY

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Abstract: *In biomedical research, quantification of histological images is often required. Many of the methods used are time-consuming, laborious, originate variable results and are difficult to replicate. This paper is aimed towards finding a more objective, reproducible and easy to perform method to obtain representative results in murine models of hepatic fibrosis using ImageJ software. To do so on a liver fibrosis model, the percentages of fibrotic lesion obtained in an entire section and in several different magnifications were compared. No statistically significant differences were found (p> 0.05), but the correlation was stronger between the results obtained in the entire section photograph and the two photographs at 40x (r = 0.963).*

Using ImageJ facilitated the definition of a methodology that originated representative results on a liver section and at the same time allowed objective measurement in a reproducible and less laborious way.

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

1. INTRODUCTION

The importance of morphometric tools is on the rise, as they allow the experimental pathologist to obtain continuous variables from a histological slide instead of subjective descriptions. This increase is due to the continuous need to obtain results that, besides being accurate, are not variable among observers (Di Ieva *et al*., 2012). This leads to these tools being widely used in cases where the histological characterization is difficult or ambiguous in order to find and standardize objective and quantifiable parameters, thus avoiding confounding factors or observer introduced biases. This also facilitates a correct comparison between groups (Rathore *et al*., 2017). Nonetheless, these kinds of methods have some downsides because, in some cases, they are, laborious, time-consuming and require the use of specialized instruments and infrastructures that are not found in all laboratories (Soreide *et al.*, 2009). ImageJ is a freeware software that is widely used in biomedical research when image analysis is needed. On histological slides, it can perform many different morphometric studies namely measuring perimeters, areas, angles or distances. The measurement of areas can be a challenging task (Papadopulos *et al.*, 2007). The deposition of fibrosis is a complex phenomenon. It consists on the formation and deposition, by active fibroblasts and myofibroblasts, of fibrous connective tissue due to a reparative or reactive process (Borthwick, Wynn and Fisher, 2014). Histologically, the lesions observed are disorganized and challenging to quantify. On cases like these, the Color Threshold tool in ImageJ allows an easy and

trustworthy way to quickly quantify the percentage of affected tissue (Wang and Hou, 2015). Murine models of hepatic fibrosis may be induced in many different ways. Carbon Tetrachloride (CCl_4) is a compound that is metabolized by the p450 cytochrome present on hepatocytes and most active on those near the centrilobular space. This process originates a metabolite, Carbon Trichloride, that is toxic and induces necrosis. Microscopically, induced lesions are hepatocyte degeneration, necrosis and centrilobular fibrosis with or without bridging (Huang *et al.*, 2006; Kumar *et al*., 2014).

2. MATERIALS AND METHODS

Experimental design

To define a less laborious methodology that also outputs representative results of a liver section, percentages of affected tissue obtained at different magnifications were compared. After measuring the area of fibrosis and the total parenchyma area in two photographs with a magnification of 40x, three photographs at 100x and three 200x photographs, the results were compared to those obtained from a 40x full section photograph obtained through the stitching of five to eight overlapping photographs at 40x (figure 1) using the free software Image Composite Editor Version 2.0.3.0 (64 bit) 2015 from Microsoft. The two 40x photographs were taken on the section's middle area and only selected when they didn't overlap (figure 2). The photographs at 100x and 200x were selected randomly, without overlapping areas and only when all the photograph's area was occupied by parenchyma (figure 3 and 4). Only two or three photographs were chosen to ensure that it was the least laborious method possible compared to the photographs needed to make a complete collage of the section.

Sample collection

All samples were obtained in the Histopathology Unit of the Gulbenkian Institute of Science. Livers were sent by different research groups to the unit for histopathological analysis, being chosen for the present study ten (n=10) slides from CCl_4 induced hepatic fibrosis models. Samples were fixed in 10% buffered formalin and processed by routine methods for light microscopy. All selected samples were stained with Masson's Trichrome (according to the guidelines present in the NovaUltra Special Stain Kit) which stained the collagen blue and hepatocytes red.

Inclusion criteria

For the present study, a section per slide was selected based on the presence of fibrosis validated by the unit's pathologist and when Masson's trichrome had stained the collagen blue. Samples were excluded where the differential staining yielded unreliable results.

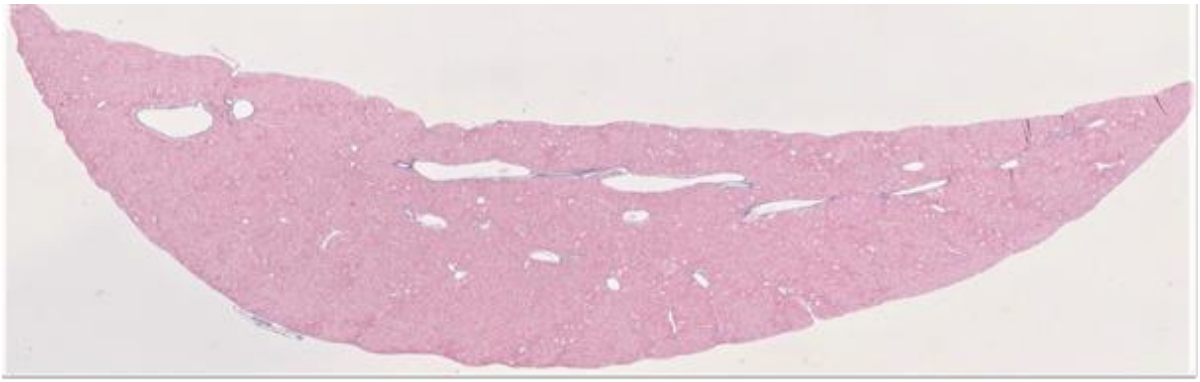


Figure 1 Liver 40x Masson's Trichrome - photograph of an entire section of mouse liver, after the collage of overlapping photos at 40x

Fibrosis Quantification

To quantify the extension of fibrosis, photographs were taken with a LEICA DMLB2 microscope connected to a Mac OS X Version 10.5.8 2.4ghz Intel Core 2 computer via the LEICA FIRECAM software, obtaining files in TIFF format. Photographs obtained had a resolution of 7844x 2576 pixels for the total section photos and 2088x1550 pixels for the remaining.
Images were analysed using ImageJ software. The scales used were: for photographs at 40x 0.734 pixels / mm; at 100x 1.842 pixels / mm and for 200x 3.673 pixels / mm. The quantification of fibrosis and the total area of the section / parenchyma present in the photograph was performed manually with the aid of the Colour threshold tool present in ImageJ. Subsequently, to obtain the affected percentage, the area of fibrosis obtained was divided by the total area, multiplying the result by one hundred. The number of liver samples analysed led to the obtaining of one hundred and fifty-one photographs. Forty different percentages were obtained.

Statistical analysis

Descriptive and inferential statistics were performed using the SPSS program version 22.0.
To determine if there were statistically significant differences between groups, an ANOVA test was performed after confirming the normal distribution of results through a Shapiro-Wilk test. To detect correlations between the results of the distinct groups a Pearson test was performed. P values <0,05 were considered significant

3. RESULTS

Liver samples used on this study presented CCl_4 induced lesions namely centrilobular foci of hepatocyte degeneration, necrosis and fibrosis deposition with occasional bridging. Table 1 presents the averages obtained for the measurements of liver fibrosis on the different magnification chosen to this study. The total section at 40x group averaged 1.87% (s= 0.9%), the 2 photos at 40x group 2.14% (s = 0.92%), the 3 photos at 100x 2.15% (s =0.68) and the 3 photos at 200x 2.17% (s =0.7%). On Table 2, it is shown every result obtained for the ten samples, allowing a case-by-case analysis. We highlighted the measurements that were considered disparate when compared with the total liver section. No statistically significant differences were found between groups (p> 0.05).

Table 1 Averages and Standard Deviation, in percentage, obtained after measuring the areas affected by fibrosis. Results obtained on the whole section (total 40x), on 2 central photos taken at 40x and on 3 random photos at 100x and 200x

	Average (%)	Standard Deviation (%)
Total 40x	1.87	0.9
2 photos 40x	2.14	0.92
3 photos at 100x	2.15	0.68
3 photos at 200x	2.17	0.7

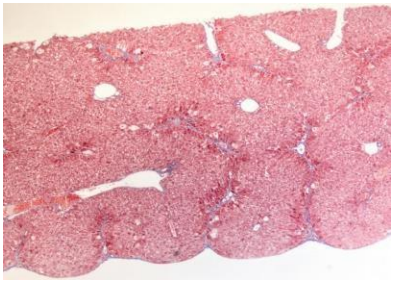


Figure 2 Liver 40x Masson's Trichrome- example of a photograph used for the measurement of fibrosis at 40x

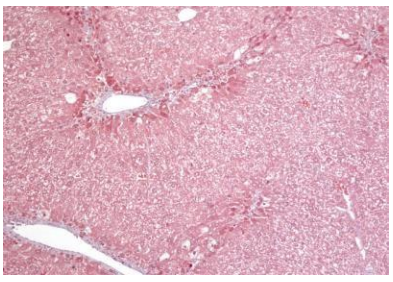


Figure 3 Liver 100x Masson's Trichrome- example of a photograph used for the measurement of fibrosis at 100x

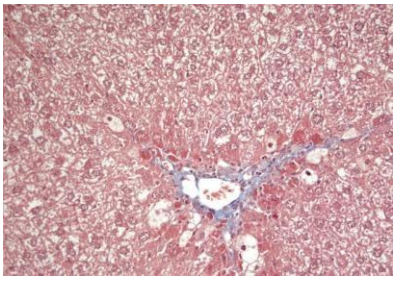


Figure 4 Liver 200x Masson's Trichrome- example of a photograph used for the measurement of fibrosis at 200x

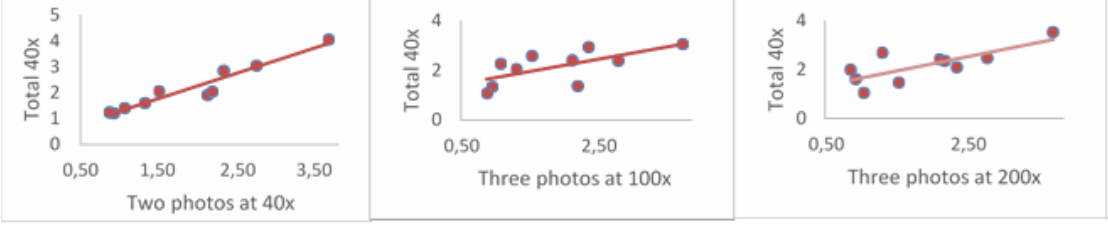
However, when analysing the results shown on the figures 5, 6 and 7, assessing the degree of correlation between the different methodologies, it was observed that there was a significant and greater correlation between the results obtained in the total photograph at 40x and the two photographs at 40x ($r = 0.963$, $p < 0.001$) when compared with the correlation coefficient of the remaining magnifications (for the three photographs at 100x $r = 0.664$ $p = 0.036$ and at 200x $r = 0.766$, $p = 0.01$)

Table 2 Percentages of hepatic parenchyma affected by fibrosis. Results obtained from measurements made at different magnifications and with different numbers of photographs. Highlighted the results that were disparate when compared with the total liver section.

Total Liver Section 40x (%)	2 photos at 40x (%)	3 photos at 100x (%)	3 photos at 200x (%)
2.10	1.9	2.41	2.41
2.75	3.05	2.38	2.47
3.66	4.06	3.06	3.54
2.33	2.85	2.94	2.1
1.31	1.6	2.05	**2.69**
1.05	1.4	**2.26**	1.05
0.93	1.2	1.33	**1.61**
0.86	1.24	1.07	**2**
1.52	2.06	**2.58**	1.47
2.17	2.04	**1.37**	2.36

4. DISCUSSION AND CONCLUSIONS

Quantifying disorganized lesions for comparative purposes is not always a simplistic task, can often become time-consuming and may give rise to disparate values among observers. On this work, we tried designing a method to overcome these difficulties. As far as quantification of fibrosis in livers is concerned, the current literature shows that the methodologies vary widely, being the most usual the quantification through a non-standardized number of images taken at random with different magnifications (ex: Amin *et al.*, 2016; Tong, *et al.*, 2009). This can originate problems because being random can in certain cases not be representative and lead to questionable results. A complete image of a section is the most correct point of comparison in terms of its representativeness. However, obtaining it and quantifying form it has the downsides of being quite laborious. Due to this fact, we tried to find if fewer photographs at different magnifications would be representative of the whole section. When comparing the percentages obtained on different magnifications, no statistically significant differences were observed between groups. However, when assessing the correlation coefficients, these were considerably higher between the results using the total section photograph and the two photographs at 40x. Given these results, we can affirm that in cases of hepatic fibrosis induced by CCl_4 two central photographs output results representative of the section. Using three random photos at 100x or 200x in cases with low percentages of affected parenchyma, can induce highly variable results when compared to those obtained on the total section. The fact that there are no such disparate cases between the first two groups of results could be explained by the fact that the photos were not totally random, and the magnification was not as high, making overvaluation / undervaluation more difficult. Increasing the number of photographs, as described on the studyof Amin *et al.*, 2016, to dilute the effect of a single photo is something to consider. However, in terms of workload may not be the best approach. The comparison of the results of this work with the existing literature



Figures 5, 6 and 7 - Scatter plots representative of the results obtained for the correlation between the different results at 40x, 100x and 200x respectively.

is complex. In most cases the quantification of fibrosis is done to address other topics and not to determine the most correct method to measure it. (Goodman *et al*., 2007; Carpino *et al.*, 2005). The only studies found to critically address the process of liver fibrosis quantification used human liver biopsy samples (Standish *et al*., 2006; Wang and Hou, 2015) which makes the comparison difficult, since, on these types of samples, the question of representativeness arises somewhat differently. It was also noticed that in several studies, instead of using Masson's Trichrome, *Sirius red* was used as the differential staining and more complex quantification methodologies were utilized, such as polarized microscopy, something that can certainly facilitate the process, but requires more specialized instruments. (Ex: Goodman *et al.*, 2007; Tong *et al.*, 2009). One type of study that may be of interest in the future will be to ascertain the correlation between the results obtained in a total section photograph at 40x with total section images obtained on slide scanners.

It is necessary to address some limitations of this study. First, although following a normal distribution, the sample size may be considered small (n=10). It would be interesting to deepen this study with a higher number of samples. Also, the affected areas observed were always very low (the maximum observed was 3.66%, where in cases of cirrhosis it can go up to 30%) (Amin *et al.*, 2016). Assessing how this variation behaves in strongly affected samples would certainly prove to be important.

Conclusion

We can affirm that two photographs taken as described at 40x are representative and output reliable results repeatedly. Ideally, in the future, this kind of work could be correlated with, for example, grading systems. As this work addresses to what is representative of the section, we hope it can be a starting point to discover how to obtain representative results of the whole organ.

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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