ORIGINAL RESEARCH PAPER

Anatomy

ESTIMATION OF AMOUNT OF GLYCOGEN IN HUMAN LIVER THROUGH HISTOMORPHOMETRY UTILIZING IMAGE J IN PAS STAINED SLIDES

KEY WORDS: Liver, glycogen, histomorphometry, PAS stain, Image J

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ABSTRACT

Hepatic glycogen is the main buffer for blood glucose levels. Following a meal, hepatocytes take up glucose from the portal vein and store it as glycogen, a branched polymer of glucose residues. Hepatic glycogen content varies 2 fold during the day and can constitute up to 10% of the weight of the liver in humans. During periods of fasting and increased energy demands, hepatic glycogen can be broken down into glucose, which is released into the circulation to maintain blood glucose levels.Histomorphometry is a digital image analysis approach that relies on the identification and analysis of morphological elements in a histological section. It is a technique used for diagnosis of neoplasms and to aid in tailoring cancer treatment.ImageJ is an essential tool for us that fulfills most of our routine image processing and analysis requirements. The near-comprehensive range of import filters that allow easy access to image and meta-data, a broad suite processing and analysis routine, and enthusiastic support from a friendly mailing list are invaluable for all microscopy labs and facilities-not just those on a budget.

INTRODUCTION

Hepatic glycogen is the main buffer for blood glucose levels. Following a meal, hepatocytes take up glucose from the portal vein and store it as glycogen, a branched polymer of glucose residues. Hepatic glycogen constitute up to 10% of the weight of the liver in humans.

Patients with poorly controlled type 1 diabetes can develop glycogenic hepatopathy characterized by the accumulation of large amounts of hepatic glycogen that ultimately can lead to fibrosis and cirrhosis of the liver. Genetic mutations in enzymes responsible for the synthesis or breakdown of glycogen cause glycogen storage diseases, a family of recessive disorders characterized by an abnormal quantity or quality of glycogen in the liver.

PAS (Periodic Acid Shiff) stain is the most versatile and widely used special stain technique for carbohydrate visualization.

Histomorphometry is a digital image analysis approach that relies on the identification and analysis of morphological elements in a histological section. It is a technique used for diagnosis of neoplasms and to aid in tailoring cancer treatment.

ImageJ is a readily available freely downloadable image analysis software package (http://imagej.nih.gov/ij/) developed by the National Institute of Health (NIH) and brings liver volumetry to the surgeon's desktop.

It enables accurate measurements of computerized images following predetermined calibration. This program has been used for histomorphometric measurements. Since it was developed in 2004, it has been widely used in many different fields of scientific research, including molecular and single cell evaluations.

AIMS AND OBJECTIVES

The aim of this study is to estimate the amount of glycogen in human liver through histomorphometry utilizing image J software in PAS stained slides.

MATERIALS AND METHODS

The present study was designed to be done in 20 liver specimens taken randomly from different age groups from mortuary. Then the tissue was fixed and processed by the automatic tissue processing method.

After preparing paraffin blocks, the block was cut with the help of rotary microtome into sections the size approximately 4 μ in thickness in the form of a ribbon.

The ribbon was gently placed on the surface of water in a preheated water bath whose temperature was set at 50-52 ° C for 2 hours or more.

After this, the slides were fixed with flaming over a spirit lamp, and the formalin fixed sections were stained with PAS stain.

All the slide images were photographed to document the amount of glycogen by using Image J software which was downloaded from https://imagej.nih.gov/ij/download.html.

Photographs were taken with the help of Lynx microscope at 10X and 40X magnification using C-mount adapter 0.7 X camera. All the data was collected and recorded.

Images were opened in ImageJ; and the glycogen highlighted by magenta stain was converted to binary image manually after thresholding hue, saturation & brightness and the area of no interest was removed by edit and clear option on each magnified field.

The area of the selected region of the liver occupied by glycogen was automatically calculated by image J software along with standard deviation and mean.

All these data were manually saved to excel. The estimated mean value of glycogen from 10X and 40X images of 4 thickness was compared with the estimated weight of glycogen 170gm(average) in normal liver of weight 1700gm(average).

Inclusion Criteria

- Liver tissue obtained from the mortuary, SCB Medical College, Cuttack.

Exclusion Criteria

- Patients treated for liver disease and already diagnosed neoplastic liver specimens.
- Liver showing gross or microscopic features of autolysis.

Ethical Issues

This study will abide by the ethical principles of medicine by

the institutional ethics committee SCB Medical College, Cuttack.(IEC Appln.No:-756)

Procedure of Pas Staining

Reagent's :

Reagent Preperation :

Schiff's Reagent Preparation:

Procedure Of Periodic Acid Schiff Staining

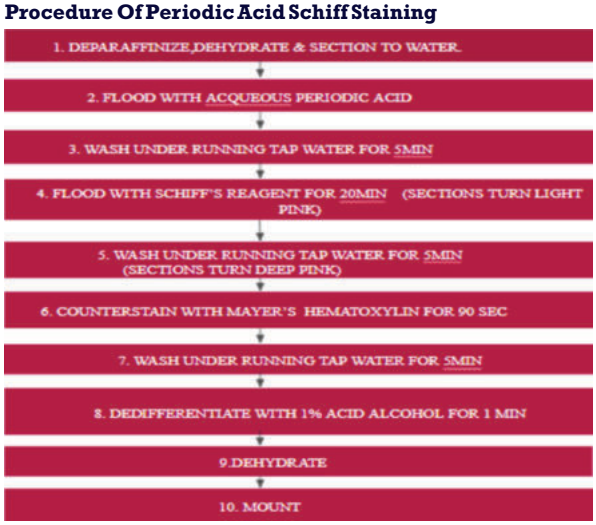


Figure 1: PAS staining station

Observations:

Image Analysis For Percentage Of Glycogen In Human Liver:

1) Initially, images of an objective micrometre with a 1mm scale having the exact resolution and size as the image of interest were taken in 10 X and 40 X magnification in the microscope and the distance of 50 μ and 10 μ were measured in pixels, respectively.
2) Next, the scale was set before measuring the area of interest in each magnified field with obtained respective values.
3) Then, the image analysis was done after making sure the image's size and resolution, which were to be measured, and the objective micrometre image was the same.

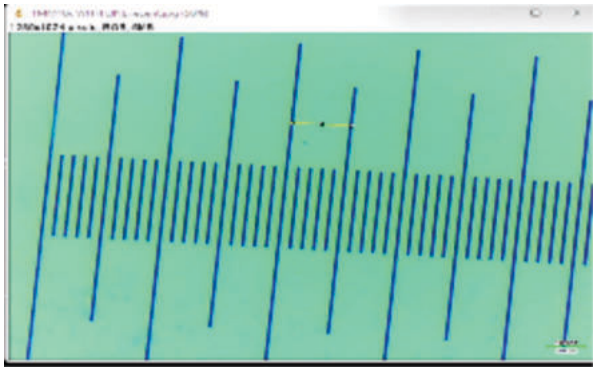


Figure 2: Objective micrometre (10X)

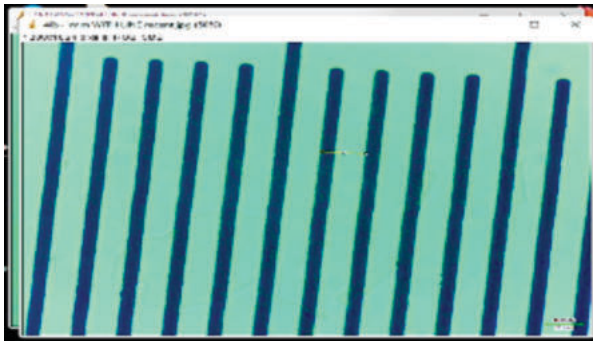


Figure 3: Objective micrometre (40X)

Image Analysis:

1) Images were opened in image J

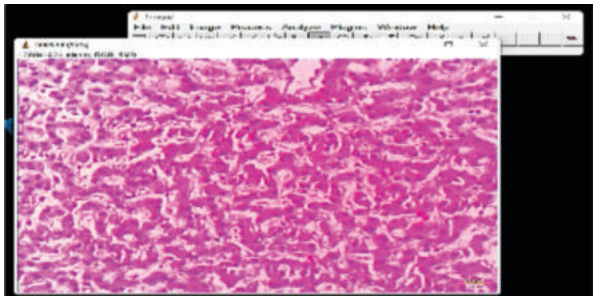


Figure 4: Image opened in image J(10X)

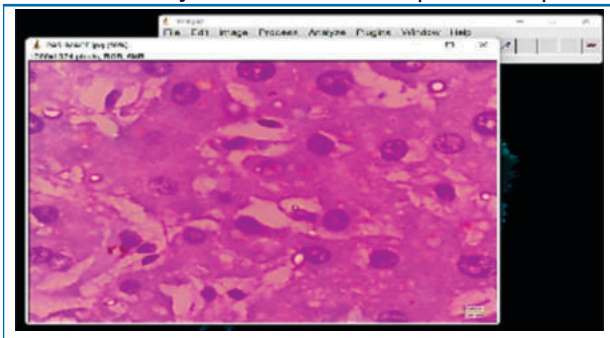
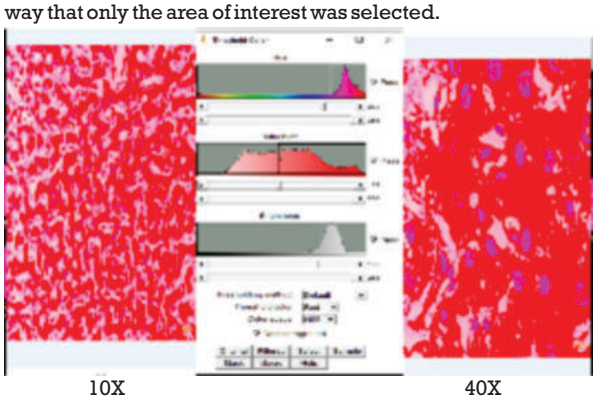


Figure 5: Image opened in image J(40X)

2) Then, colour thresholding was done by adjusting the hue at magenta as glycogen highlighted by magenta in the P.A.S. stain; later, saturation and brightness were adjusted in such a way that only the area of interest was selected.



10X 40X

Figure 6: Colour thresholding in 10X and 40X

3)Then the image was made binary, and the white area, which was a region of interest, was measured after clearing any noise if present by edit and clear option.

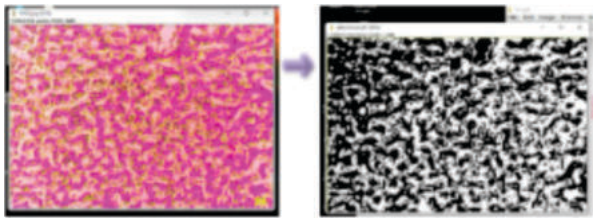


Figure 7: Binary image in 10X

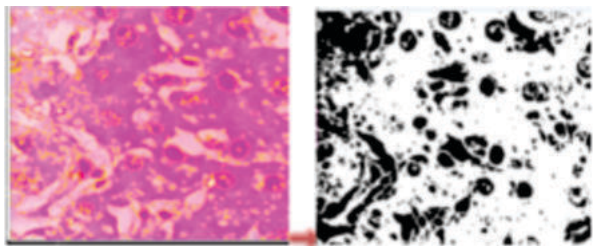


Figure 8: Binary image in 40X

4) Image J software automatically summarised the mean and standard deviation values of the area of glycogen. The values were saved in excel for further calculations.

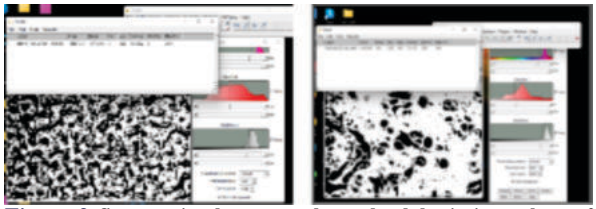


Figure 9: Summarized mean and standard deviation values of the region of interest in 10 X and 40 X

Calculations

For accurate histomorphometry the factors which has to be taken into account to reduce error is to consider : (1)The shrinkage of the the tissue processing (2) Proper staining. (3) Considering IHC & special stains preferentially over general stains & learning the different ways of measuring the ROI(region of interest) by adopting different workflows of specialised histomorphometry softwares including tips & tricks from other workers. (4)Quantification of histological structures by softwares requires not only art but also high level computer programming proficiency in python. (5)Sample size to be increased to 5times to see if it brings about any kind of difference. (6)The density of glycogen values varied from 0.5g/ml to 3g/ml in different papers and product monographs. So for convenience we have taken 1g/ml, a median value.

RESULTS

The total volume of estimated glycogen determined by histomorphometry came out to be

- In 10 X = 620.161g
- 40 X = 637.4g

Which shows to be 4 times more than the estimated gross weight of glycogen (170gm) indicates there were some limitations while estimating glycogen in liver using image J. The limitations can be taken into account and future study can be done by correcting whatever limitations we have encountered in current study so that correct amount of glycogen can be calculated.

Limitations

Accurate magenta staining of the glycogen in the tissue section was not proper leading to addition of noise to the microphotograph which lead to confusion on the part of the image analysis software to determine which was foreground and which was background.Therefore thresholding couldn't be done properly by the imageJ software, hence binary image included areas which should not be included. If there was proper contrast between the staining of the element of the cell there could have been proper difference in hue, saturation, and brightness then the software could have easily detected differences between different parts of the cell and carried out correct measurements. The nuclei of the hepatic cells were included during thresholding as they were stained deep magenta.

CONCLUSION

There are lot of other ways of selecting the region of interest in a microphotograph of H & E or other stained slides in the image J software like e.g. 1) Separating the colour photograph into different channels of RGB. 2)Manually selecting the areas of interest. 3)Application of different filters utlilising specific algorithms for different stains(plugins of image J). So in future we planned to utilise these features of the software to accurately measure the glycogen areas in the liver specimen. If specific immunohistochemistry staining is done which will lead to specific staining of the area of interest or cell constituents of interest there can be better threshold and better estimation.

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