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Imaging processing settings of bright field staining images

Yishan Fu

College of Engineering and Computing, University of South Carolina, Columbia, USA

YISHAN@email.sc.edu

Abstract. Background: Clear images are vital to studying the cells and exploring the relationships between different parts of cells. Thus, histology is important to view the microscopic structures of cells by using different colors to show. However, the pipeline of getting a better and more useful image of bright field staining images is still a challenge. This article introduces different processed images to get a good condition to improve image quality. **Methods**: Four staining ways, including Periodic acid–Schiff (PAS) staining, Hematoxylin and Eosin (H&E) staining, Trichrome Staining, and immunohistochemistry (IHC), have been used on the liver, heart, and brain sections. Photoshop and Image J were used to process images of different conditions. The software settings have been compared. **Results**: 60X Oil immersion can provide nice details for the liver, heart, and brain with PAS, Trichrome, and H&E staining. By comparing different settings on software, the following settings can be optimal options: 300 PPI resolution for optimal size and clarity, gamma to adjust the light and dark, color balance to make the colors more comfortable to the eye, TIFF format to meet most requirements. Image J, compared to Photoshop, can solve many image processing and analysis problems with a smaller installation package and is free to download. **Conclusions**: By using optimized settings of image software, researchers can get better pictures and assist in solving more scientific problems.

Keywords: Histology, Staining, Image J, Bright Field Image.

1. Introduction

Histology is the microscopic research to study animal and plant cell and tissues through staining and sectioning. Histology is usually performed by examining a thin slice (section) of tissues or cells under a microscope (electron or light microscope) [1]. Its ability to visualize and identify microscopic structures [2]. Histology approaches are essential tools of biology and medicine [2]. However, there have been many pipelines to process images, such as using Photoshop (Adobe, San Jose, US) and Image J (National Institute of Mental Health, Bethesda, US), while some of the approaches have objective bias. Thus, it is vital to compare the images in different process conditions, including resolution, adjustment on a B/W plate, adjustment on a color plate, and staining methods.

Histological staining is a multistep procedure and involves a variety of stains and other chemicals. they interact with other compounds to change the results. Histological staining will easier, faster, cheaper, and more accurate by these changes [3].

This study used different staining approaches to stain the heart, liver, and brain, followed by Photoshop and Image J to process the images to compare the differences between imaging handling approaches.

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2. Materials and methods

2.1. Materials

The liver and heart tissue were frozen and paraffined. People use specialized equipment to cut thin slices of tissue [4]. Furthermore, the Reichert-Jung Leica Model 2030 Biocut Microtome (Oberkochen, Germany) was used to section the tissue with a thickness of 5-6μm. Four staining ways, Periodic acid–Schiff (PAS) staining, Hematoxylin and Eosin (H&E) staining, Trichrome Staining, and immunohistochemistry (IHC), were used in this study. Finally, a Nikon Eclipse E600 light microscope (Nikon Corp., Tokyo, Japan) was used to observe, and Q-Capture image software was used to take images.

2.2. Section preparation

2.2.1. Tissue fixation. Fixation's goal is to stop postmortem changes. Placing the tissue into a fixative allows optimal preservation of morphologic and cytological detail [5]. Fixation in 10% buffered formalin initially causes slight swelling of tissue specimens and stabilizes pH. Chemical or physical approaches can be used to fix tissues.

2.2.2. Paraffin. The heart and liver sections were picked and put in the mold. Fixed dissected tissues were placed in 10% formalin for at least 48 hours at room temperature and melted the paraffin prior to adding the tissue. The melted paraffin was poured into a paraffin block mold, which usually takes 15-20 min to be cooled down.

2.2.3. Microtome Sectioning. Microtome sectioning was used to decrease the thickness of the section and get thin sections for observation [6]. Before the tissue was embedded, the paraffin between the sample and the surface of the block was removed by trimming, first with the microtome by making sections until the first section with tissue appeared. Moreover, before cutting, the angle of the blade must be adjusted and placed in the ice box for smooth cutting. The section of the paraffin-embedded tissue was blocked in 4-10 μm thickness slides on a microtome and floated in a 37°C water bath containing deionized water. The sections were floated onto clean glass slides and dried in the air; then the tissue was bound to the glass. Slides can be stored overnight at room temperature and -80℃ for long-term storage.

2.2.4. PAS staining. PAS staining is widely used for staining structures. The slide was immersed in the PAS solution for 5-10 min and rinsed 4 times with water, followed by Schiff's solution for 15-30 min and rinsed. Then, the slide was stained with Hematoxylin for 2-3 min. Moreover, slides were incubated in Bluing reagent for 30 s and rinsed, then incubated in the Light Green Solution for 2 min and rinsed for dehydration.

2.2.5. H&E staining. H&E is the combination of two histological stains: hematoxylin and eosin. H&E staining can easily differentiate between the nuclear and cytoplasmic parts of a cell and provide a general overview of a tissue sample's structure [7]. In this study, the Paraffined sections were placed, and sections were immersed in the filtered Harris Hematoxylin for 10 seconds, followed by rinse with tap water. Sections were immersed in EOSIN stain for ~30 seconds, followed by rinse with tap water, and finally dehydrated in ascending alcohol solutions (50%, 70%, 80%, 95% * 2, 100% * 2) in Columbia staining dish. For each rinse step, it is important to make sure to rinse until the water is clear. To clear the slide, xylene was used in a Columbia staining dish and mounted coverslip onto the section on a glass slide.

2.2.6. Mason's Trichrome Staining. Masson's Trichrome Staining is a histological staining method. Deparaffinization and rehydration were performed using 100% alcohol, 95% alcohol, and 70% alcohol

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sequentially, and then the alcohol was washed in distilled water. For tissues fixed with Formalin, re-fix in Bouin Solution for 1 hour at 56°C. Slides were rinsed with running tap water for 5-10 minutes to remove yellow color. Weigert's iron hematoxylin solution was then applied for 10 minutes. Beibrich-Scarlet Acid Fuschin solution was then applied for 10-15 minutes. Differentiate in the phosphomolybdic-phosphotungstic acid solution for 10-15 minutes until the collagen loses its red color. The stained section was transferred to aniline blue solution and stained for 5-10 minutes. The stained section was rinsed briefly in distilled water and differentiated with 1% acetic acid solution for 2-5 minutes. Slides were dehydrated through 95% ethyl alcohol and cleared in xylene, and covered with the glass slides.

2.2.7. IHC. IHC is one of the most widely used protein detection techniques. The principle of this technique is based on the binding of a specific antibody to a matching specific antigen in tissue [8]. In this study, slides were deparaffinized slides in a chemical fume hood by merging in a) xylene 3 times, 5 minutes each, occasionally swirling; b) 100% EtOH twice, 5 min each; c) 90% EtOH, 70% EtOH, 50% EtOH, 10 min each; d) twice of diH2O, 2 min each. Antigen retrieval was applied through boiling for ~30 minutes using a microwave in Dako citrate buffer. Slides were added to hot buffer and boiled at power 2 for 30 minutes, and cooled for 20 min. Slides were washed 3 times with diH2O for 5 min on the shaker, and a hydrophobic barrier was around the tissue section with a PAP pen. Slides were laid flat and blocking buffer was added to the tissue section (5% BSA + 0.3% Triton in PBS) for 1 hour at room temperature. The drained blocking buffer redrew the hydrophobic barrier if needed and returned it to the humidified chamber.

The primary antibody (rabbit ki-67, MedChemExpress) was added. Antibody was diluted 1:200 in staining buffer (1% BSA + 0.3% Triton in PBS). Tissue section was incubated at 4 ℃ overnight and washed 3x with PBS + .05% Tween for 5 mins on shaker and redrew the hydrophobic barrier if needed. Secondary Antibody (Donkey anti-Rabbit, MedChemExpress) Antibody was diluted 1:400 in staining buffer and incubated at 1 hour at room temperature. Then the slides were dried off around tissue and were covered with the glass slide.

2.2.8. Coverslip. When finished these four staining, gently cover the slips with the glues to protect the damage and other bacteria.

2.2.9. Light Microscopy. The Nikon Eclipse E600 (Nikon Corp., Tokyo, Japan) was used to obtain images from 10X, 20X, 40X, and 60X lenses with oil immersion. The center of the field was set to adjust the brightness.

3. Results

Using the PAS, H&E, Trichrome, and Immunohistochemistry stains. Adobe Photoshop (Adobe, San Jose, US) and Image J (National Institute of Mental Health, Bethesda, US) was used for editing microscope images.

3.1. Objective lens

Nikon Eclipse E600 microscope (Nikon Corp., Tokyo, Japan) with 10X, 20X, 40X, and 60X with oil immersion objective lenses was used on the liver (Figure 1) or heart (Figure 2, Figure 3, Figure 4) sections, which were 5-6 microns thick. From all of the sections, the 60X with oil immersion objective lenses appeared to be the optimal clarification for subcellular details. 10X to 40X helped to identify the tissue structure.

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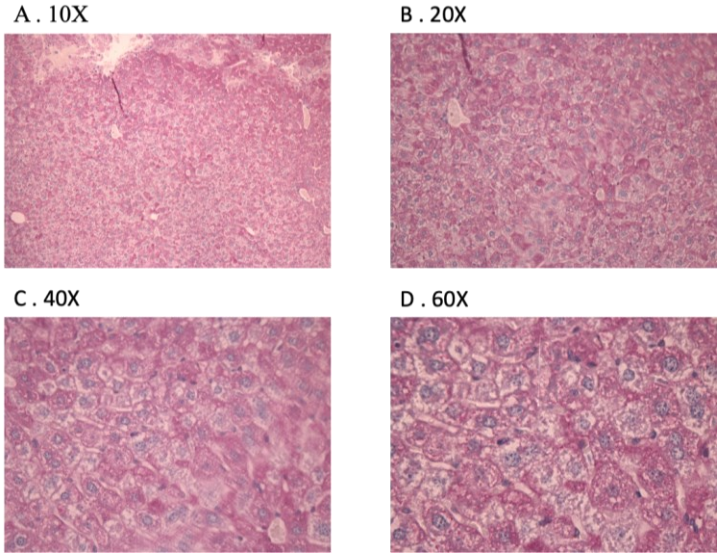

Figure 1. PAS staining images of liver at objectives (10X (A), 20X (B), 40X (C), and 60X Oil immersion (D)). Figure credit: original.

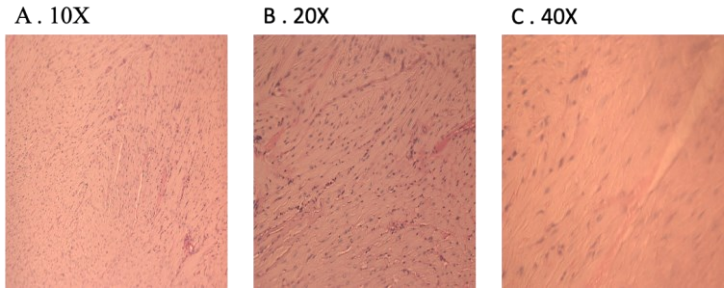

Figure 2. PAS staining of heart at various objectives (10X (A), 20X (B), 40X (C)). Figure credit: original.

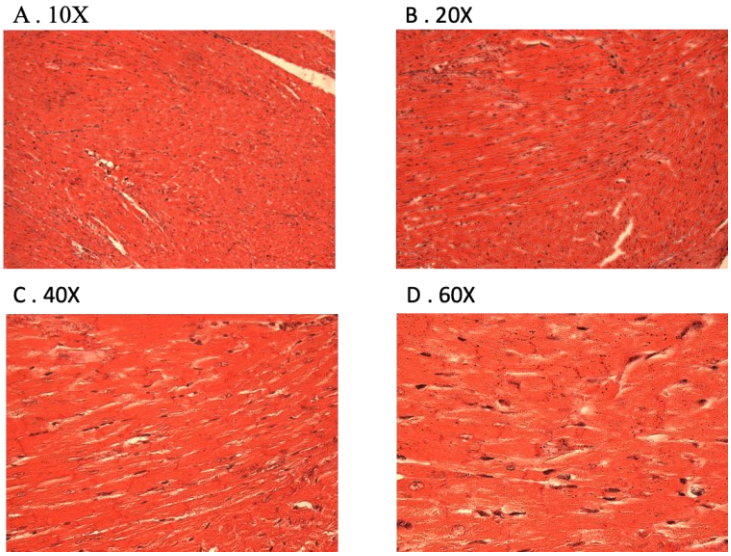

Figure 3. Trichrome staining of heart at various objectives (10X (A), 20X (B), 40X (C), and 60X Oil immersion (D)). Figure credit: original.

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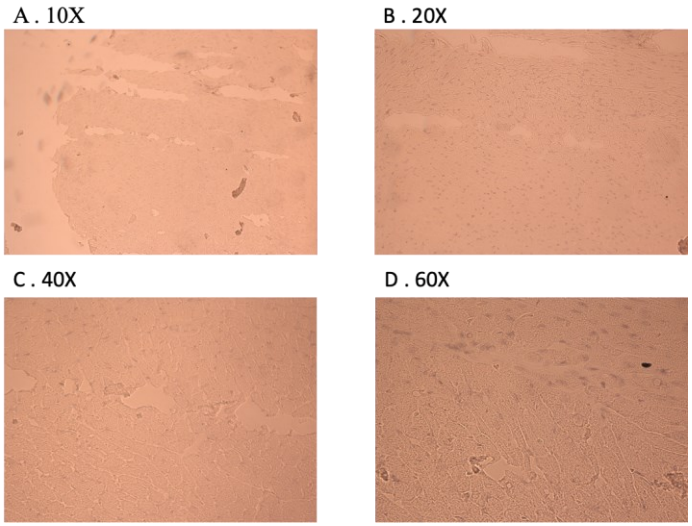

Figure 4. H&E staining of heart at different objectives (10X (A), 20X (B), 40X (C), and 60X Oil immersion (D)). Figure credit: original.

3.2. Resolution, file size, and format

Adobe Photoshop (Adobe, San Jose, US) and Image J (National Institute of Mental Health, Bethesda, US) are editing software, but with all of their great features, the manipulation can become an ethical issue if the images are edited beyond necessary contrast/brightness adjustments. Photoshop was used to edit images of the 10X liver PAS staining in different exposures, which were 72ppi (Figure 5A), 200ppi (Figure 5B), 400ppi (Figure 5C), and 1000ppi (Figure 5D). Both black and white and color images were saved with a resolution of 300ppi. In black and white, the original image was adjusted with contrast and brightness, gamma, sharpening filters, smoothing filters (blur), and median filters. The color plate was edited with level enhancements, color balance, and hue and saturation enhancements. Image J (National Institute of Mental Health, Bethesda, US) was then utilized to sharpen the image and make adjustments in brightness and contrast. A histogram of the 10X Trichrome staining of the colon was created, followed by a series of measurements of certain features within the image.

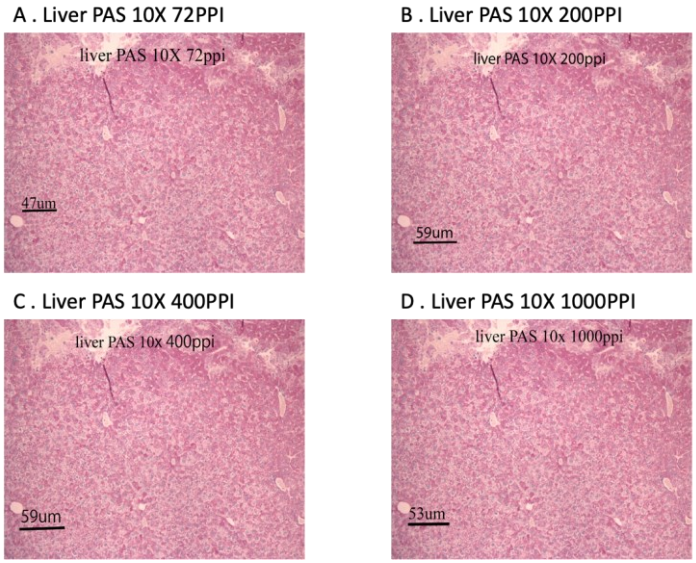

Figure 5. Liver PAS 10X staining in different resolutions. Images are setting at resolution of 72PPI (A), 200 PPI (B), 400PPI (C), 1000PPI (D). Figure credit: original.

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In terms of the effects of adjusting resolution on image quality, as the number of pixels per inch increases, the resolution of the image increases. It is hard to distinguish the differences between these images by eye. However, they were in different inches when editing in Photoshop. Figure 1A was 35 inches*26 inches. Figure 1B was 12.8*9.6 inches. Figure 1C was 6.4*4.8 inches. Figure 1D was 2.56*1.92 inches. It is not worthwhile to prepare a print of 1000 PPI if the printer is 300 DPI; thus, the 300 PPI would be the better choice.

The same image saved in different formats has different sizes and colors. Figure 6A is 14.8 MB, Figure 6B is 5 MB, Figure 6C is 14.6 MB, and Figure 6D is 7.2 MB.

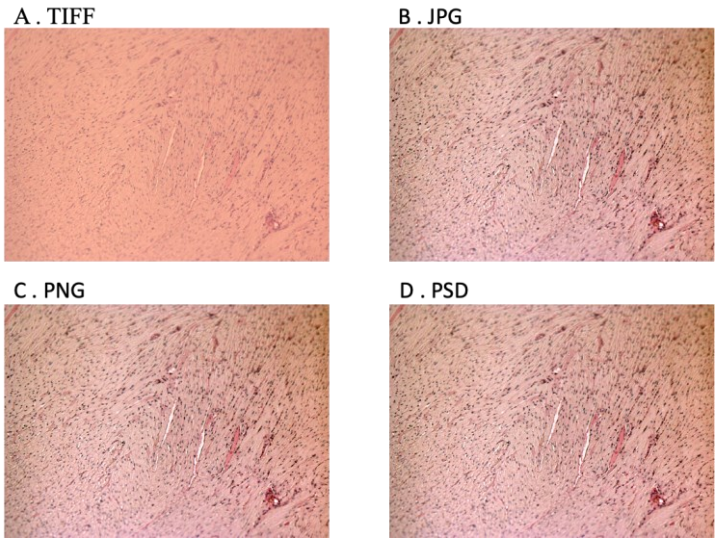

Figure 6. Heart 10X PAS staining images with different save formats. Images are setting at TIFF (A), JPG (B), PNG (C), PSD (D). Figure credit: original.

3.3. Factor of color adjustment

The Black and White plate of images at a 300 PPI each of a 10X PAS staining of liver with various adjustments have been compared, shown in Figure 7. Figure 7A is the 10X PAS staining with adjustments of brightness to 17 and contrast to 24. Figure 7B is the original image which with no adjustments. Figure 7C is that after the application of the median filter that was made with a radius of 16. Figure 7D is the 10X PAS staining with gamma adjustment correction of 0.74 that was made to the original photo. Figure 7E shows the application of sharpening filters to the 10X PAS stain with a radius of 2. Figure 7F depicts the application with a sharpening amount of 50%, 1.7 px radius, and noise reduction of 16%.

Brightness and contrast adjustment can increase the lightness and darkness of the image. Gamma increases the image tonal values. A sharpening filter allows for the emphasis of details in an object or the emphasis on edges. The median filter removes noise from an image or signal, and the blur/smoothing filter averages out rapid changes in intensity. The goal of a blur/smoothing filter is to reduce the amount of noise and detail in an image.

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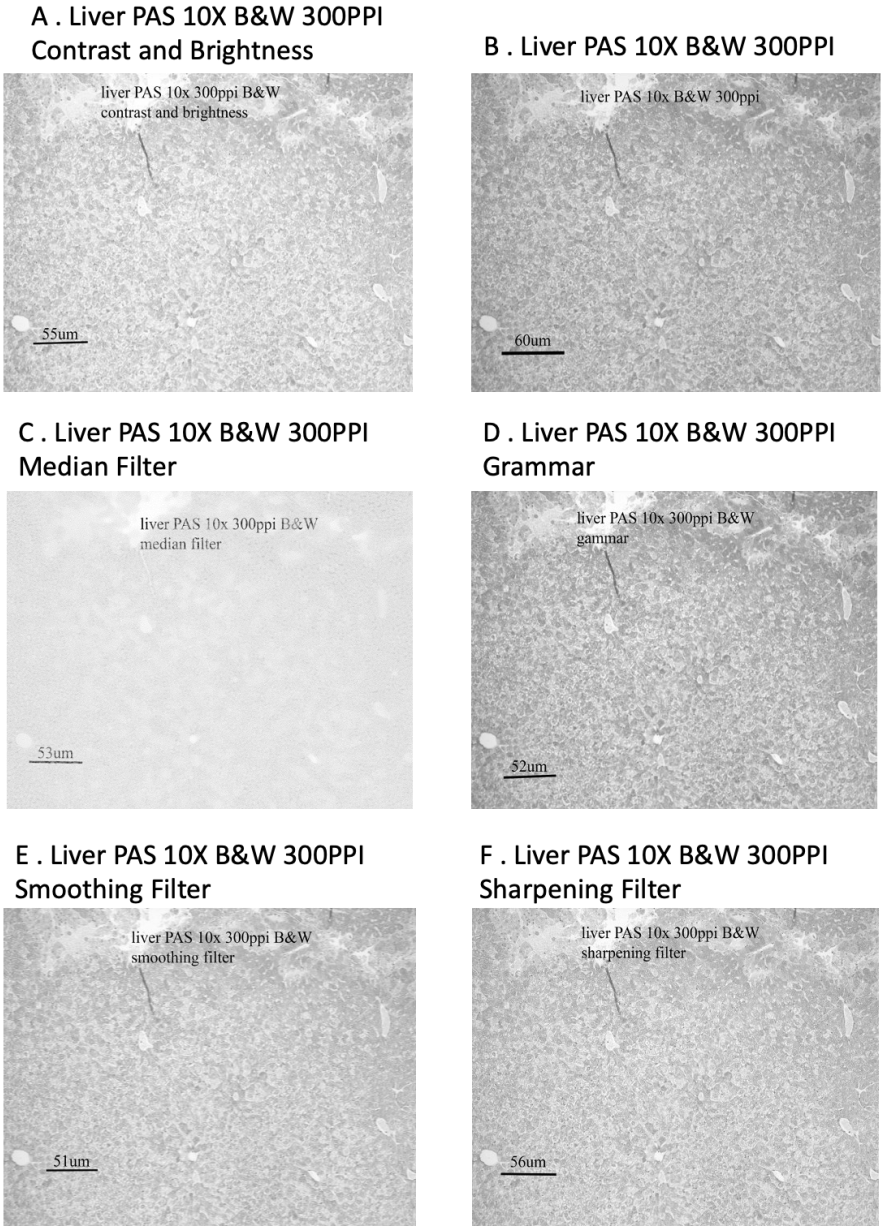

Figure 7. Black and White plate images of liver 10X PAS Staining with 300PPI with adjustments. Images are setting at contrast and brightness (A), original (B), median filter (C), grammar (D), smoothing filter (E), sharpening filter (F). Figure credit: original.

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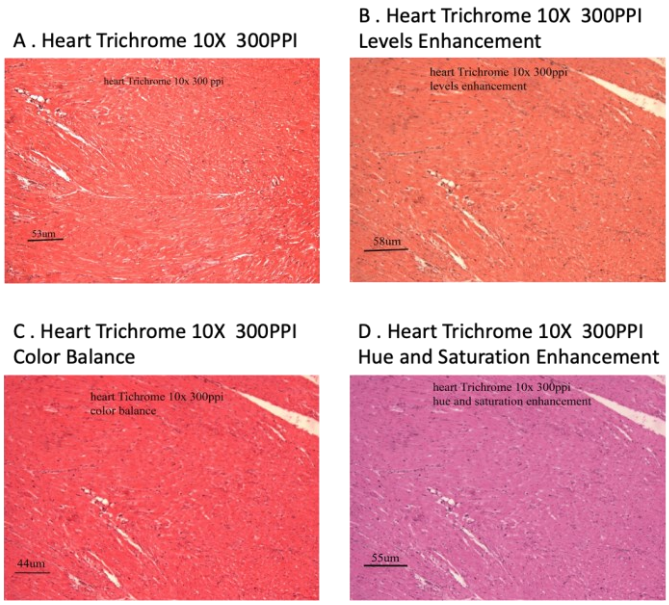

Figure 8. The color plate of 10X PAS of heart at 300 PPI that includes the original image (A), the image after levels enhancement (B), the image after color balance (C), and the image after hue and saturation (D). Figure credit: original.

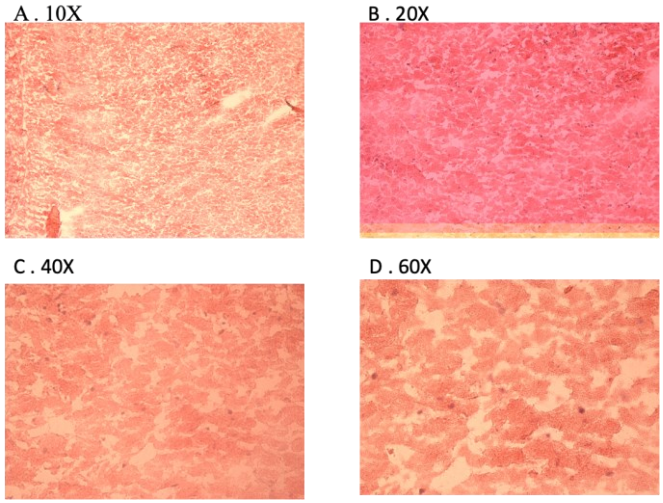

Figure 9. The color plate of IHC staining brain at various objectives (10X (A), 20X (B), 40X (C), and 60X Oil immersion (D)). Figure credit: original.

Figure 8 shows the color plate images at 10X PAS of the heart at 300 PPI. The original image is Figure 8A, and the image that contains level adjustments is Figure 8B with a levels enhancement of (0, 1.31, and 225) with output levels remaining 0 and 225. Figure 8C and Figure 8D after the color balance contained a -37 scale movement towards the cyan end of the spectrum, a +3 scale movement towards the green of the spectrum, and a -13 scale movement towards the yellow end of the spectrum. Similar settings on IHC staining (Figure 9) showed a clear view.

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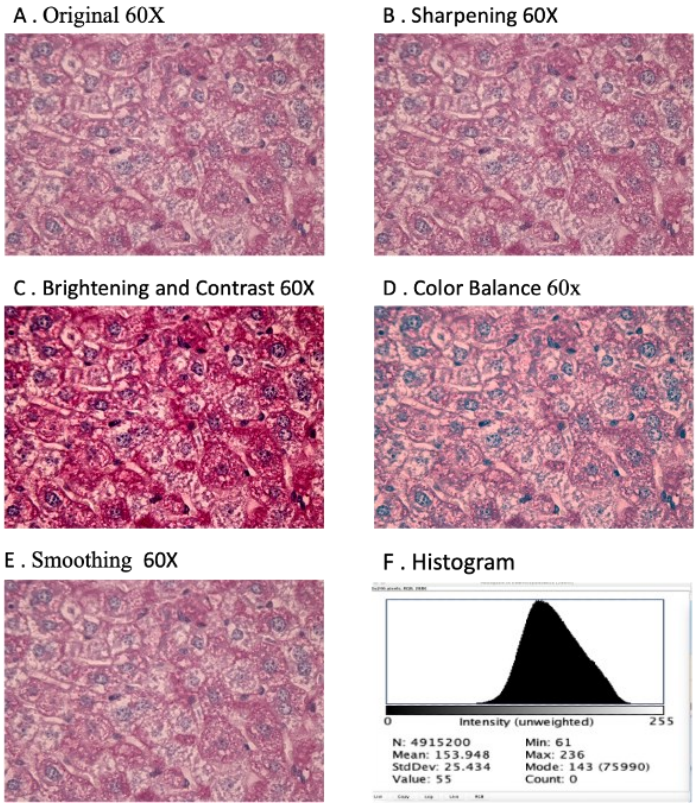

Figure 10. Liver PAS staining in 60X adjusted by Image J. The original image (A) was adjusted with sharpening (B), brightening and contrast (C), color balance (D) and smoothing filers (E) and histogram obtained from the original image (F). Figure credit: original.

By using all of the adjustment discussed in this section, the liver PAS staining in 60X was adjusted using Image J (Figure 10). Image J is dynamic range acceptable. Figure 10.F shows the min is 61 and max is 236, the StdDev is 25.434 and value is 55.

Table 1. point to point measurement results of Figure 11

	Area	Mean	Min	Max	Angle	Length
1	1170	173.008	107	255	-92.066	582.379
2	1930	166.261	110	255	-130.968	965.469
3	2181	170.906	90	255	-3.478	1088.00

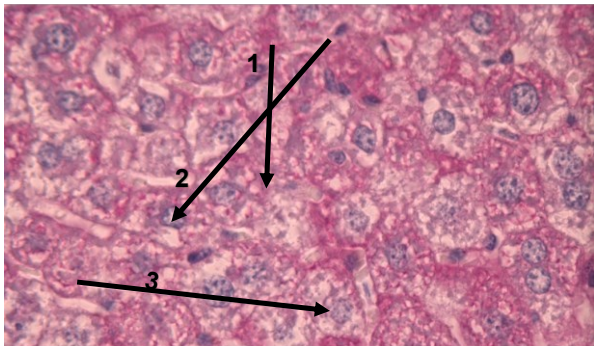

Figure 11. Point to point measurement of three lines (1, 2, 3) like picture shows. Figure credit: original.

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Besides adjusting images, Image J also provides measurement functions, such as point-to-point measurements (Figure 11 and Table 1), which can be useful for many applications.

4. Discussion

This study compared staining methods and imaging analysis with different software, such as Photoshop and Image J, in the liver, heart, and brain sections. The settings of different software, including resolution, contract and brightness, color balance, etc., have been explored.

For the liver, heart, and brain with PAS, Trichrome, and H&E staining, 60X Oil immersion gave a nice look. Using oil can reduce the refractive loss of light when passing through the interface between air and glass. Oil droplets were more resistant to evaporation than water droplets, and they resolved cellular structures more visible clearly [9].

Resolution is an important factor that affects image quality. However, it is not worthwhile to prepare a print of 1000 PPI if the printer is 300 DPI. Thus, 300 PPI is an optimal choice in many scenarios.

There are numerous image file formats nowadays, for example, JPG (Joint Photographic Experts Group), PSD (Photoshop Document), PNG (Portable Network Graphic), TIF (Tag Image File Format), etc. JPG is the most widely used compressed image format for digital cameras, different operating systems, and the Internet. PSD is an image file format native to Adobe's popular Photoshop Application. PSD files are commonly used for containing high quality graphics data. PNG is a particularly popular file type with web designers. Saving files in a lossy format will lose the details of images. Thus, using an uncompressed TIF format will present the original picture and details is the better way to use.

Brightness refers to the absolute value of colors (tones) lightness/darkness. Contrast is the distinction between lighter and darker areas of an image, and it refers to making more obvious the objects or details within an image. Gamma adjusts the midtones from the tonal scale but keeps the white and black. Gamma is about translating between digital sensitivity and human eye sensitivity. Thus, gamma can be used to adjust the light and dark [10]. Color balance and saturation adjustments are important to make the colors more comfortable to the eye and to complement the research goal from images. Over-adjustment should be avoided in case the images become too blue or too red.

People would not recommend using software filters or adjustments to improve image quality for biological images. Digital image filters destroy original functions. Thus, the original photos should be saved.

Layers of images can let users add components to an image and work on them one at a time, without permanently changing the original file. For each layer, a lot of parameters can be adjusted, including color and brightness, special effects, repositioning layer content, specifying opacity and blending values, etc.

Different from commercial Adobe products, ImageJ is a Java-based image processing program. Custom acquisition, analysis, and processing plugins can be developed using ImageJ to solve many image processing and analysis problems.

5. Conclusion

In this study, staining methods and imaging analysis in the bright field images from the liver, heart, and brain sections have been examined. For the liver, heart, and brain with PAS, Trichrome, and H&E staining, a microscope using 60X Oil immersion can have a good picture. A resolution of 300 PPI is an optimal choice in many scenarios. TIF format is one of the best formats for saving images. Gamma can be used to adjust the light and dark; however, avoid using contrast and brightness. Software filters or adjustments to improve image quality are not recommended for biological images. Color balance can be used to make images readable. Image J can solve many image processing and analysis problems, which is useful for solving biomedical problems. By understanding these settings in software, biological and medical researchers can save time and focus on scientific questions rigorously.

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