**Indian Journal of Science and Technology,** *Vol 9(40), DOI: 10.17485/ijst/2016/v9i40/103279, October 2016*
*ISSN (Print) : 0974-6846*
*ISSN (Online) : 0974-5645*

# A Study on the Optimum Condition of Haematoxylin-Eosin using RGB Analysis

**JongHo Back[1], JeanSoo Kim[2] and DongYeop Lee[3*]**

[1]Department of Pathology, Eulji University Hospital, Korea; backjongho77@hanmail.net
[2]Department of Clinical Laboratory Science Daejeon Health Institute of Technology, Korea; jeansk@nate.com
[3]Department of Physical Therapy, SunMoon University, Korea; kan717@hanmail.net

## Abstract

**Objectives:** This research was conducted to look for the vest conditions of Hematoxylin-eosin staining. **Methods/ Statistical Analysis:** Method was investigated the Hematoxylin-eosin staining, Microscopy and RGB analysis. **Findings:** The result was as follows. The thickness of the optimal condition is 3 cm, the time of the optimal Hematoxylin 4 minutes 30 seconds and eosin 3 minutes 45 seconds. Also, RGB analysis was green. So green showed a significant difference in the change width to each stage, and the biggest change was 52.74 at 2㎛ and 3㎛. Improvements: Therefore, an appropriate fixed time is needed to be found to get staining intensity and sharpness, and the freshness of reagent for each stage should be maintained.

**Keywords:** Eosin, Hematoxylin, RGB, Microscopy, Stain

## 1. Introduction

For the accurate diagnosis and prognosis of the disease, surgically excised tissues from the surgery are essential for the examination. There are a number of methods diagnosing the disease using dissected tissues taken from the human body suspected to have a disease. The human body is composed of about 60 trillion cells. Not all the cells have the same conformation but each of the tissues and organs are uniquely composed, and cells are arranged to properly function they are responsible for. However, for the diseased organs or tissues, cells that make up the diseased organs or tissues are damaged which are not able to properly function, or they have abnormal shape and arrangement. With this theoretical background, biopsy is performed with mainly excised tissues by examining the shape and arrangement of cells of tissues under microscope to determine the disease status, cause, and prognosis[1]. The purpose of staining the tissue sections is to enable the microscopic examination by making differences in refractive index or color of particular cells, tissues, or tissue components. To examine the microscopic structure of collected tissues, the staining method using hematoxylin and eosin as main reagents is used which is most common and widely is used method[1]. This method is called "H&E staining" and the nucleus is stained in blue by hematoxylin and the cytoplasm is stained in red by eosin[2]. After treating cells with xylene and alcohol, the nucleus is stained in blue with hematoxylin and then the cytoplasm and connective tissues are stained in red by eosin according to the theory. There are significant discrepancies in staining intensity because some literatures show only the staining procedure without mentioning the time, and most books is described the time as "between a few minutes to a few minutes". Therefore, the purpose of this study is to find the optimal staining conditions (time and thickness of the slides) by staining the sectioned slides with various thicknesses in various times. The obtained optimal staining conditions including preferable thickness of the sectioned slides when observing under the microscope and optimal time for the samples being stained with objective and harmonious color borders will provide the basic reference to standardize H&E staining for the practitioners.

**Author for correspondence*

# 2. Study Methods

## 2.1 Subject of study

The normal tissue including glandular epithelium of mucosa membrane from the patients with gastric cancer referred to pathology after radical total gastrectomy was used and the samples were collected from January to June 2011 in Ulji University Hospital.

## 2.2 Methods

### *2.2.1 Hematoxylin& eosin stain*

10% formalin fixed tissue was examined by naked eyes, cut into 2.0 X 1.0 X 3.0 and treated with alcohol and xylene. Then paraffin was used to interfuse between tissues, and paraffin block was made using paraffin again. To evaluate the changes by the thicknesses of slices, the slides were produced by 1 μm, 2 μm, 3 μm, 4 μm, 5 μm and 6 μm of the thicknesses. The micro-sectioned slides were produced by commonly used H&E staining from Ulji University. The micro-sectioned slides were stained in hematoxylin staining solution for 4 minutes and 30 seconds, and then in eosin staining solution for 3 minutes and 45 seconds. Then the samples were dehydrated in 70%, 80%, 95%, and 100% of alcohol respectively. Then each sample was placed in xylene for 2 minutes in 3 steps to increase the transparency of stained color. After staining, the sample was smeared with mounting medium and then covered with protective glass to prevent stained tissue damage and bleaching and to improve refraction index for the microscopic examination. In order to evaluate the changes depending on staining time, slide #1 was sliced in 3μm and stained in hematoxylin solution for 5 minutes and 24 seconds increasing 20% of the staining time and stained in eosin for 4 minutes and 30 seconds. Slide #2 was stained in hematoxylin for 4 minutes and 40 seconds without increasing the staining time and in eosin for 3 minutes and 45 seconds, and slide #3 was stained in hematoxylin for 3 minutes and 36 second and in eosin for 3 minutes reducing 20% of both staining time. Slide #4 was stained in hematoxylin for 2 minutes and 42 seconds and in eosin for 2 minutes and 15 seconds reducing 40% of the staining time. Slide #5 was stained in hematoxylin for 1 minute and 48 seconds and in eosin for 1 minute and 30 seconds reducing 60% of the staining time. Slide #6 was stained in hematoxylin for 54 seconds and in eosin for 45 seconds reducing 80 % of the staining time. Then after dehydration and transparent process, all the slides were smeared with mounting medium and covered with protective glasses.

### *2.2.2 Microscopy*

The stained tissue with damages or unevenly sliced glass slides under microscopy was excluded from the analysis. The stained slides of each condition depending on the intensity of hematoxylin and eosin staining read as "favorable" and are given point 0. Based on this point, the slides were classified by "weak" and "strong" according to the staining intensity of hematoxylin and eosin. Point -1 is given for a little weak staining intensity point -2 is given for a weak, point -3 for a very weak, point 1 for a little strong, point 2 for a strong, and point 3 for a very strong. All the slides were read and scored using an optical microscopy (Olympus BX51 microscope, Japan) by pathologists and technologists. The score was calculated from the average score marked by each individual read under 40 and 100 magnifications.

### *2.2.3 Image Analysis*

The slides stained by H&E staining according to each condition were examined and photographed using an optical microscope. Next, 30 mucous membrane cells per each slide were selected, the section was specified by the size of nucleus and cytoplasm, and then the RGB analysis was performed using Olympus DP71 digital camera

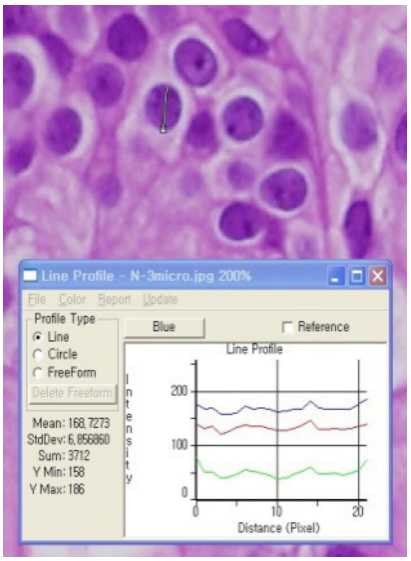


**Figure 1.** The image analysis program (Hematoxylin analysis)

(Olympus Corporation, Japan). The result of the staining intensity analysis was displayed on the graph with the numbers in order from the beginning to the end of the solid line minimum 0 to maximum 250 by red-green-red color Figure 1 and 2[3-6]. 30 cases of RGB results were calculated by the average of each individual result and then the standard deviation was calculated.

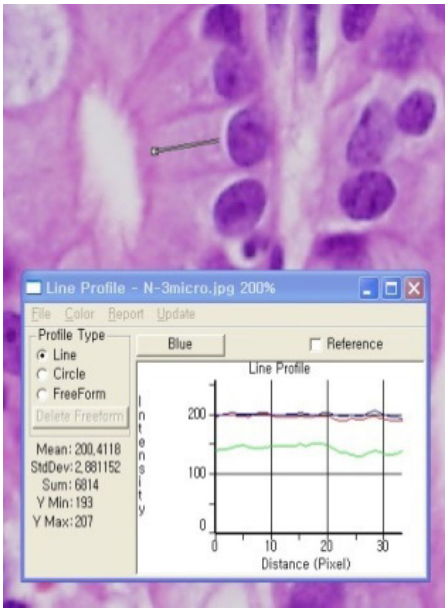


**Figure 2.** The image analysis program (Eosin analysis)

## 3. Results

### 3.1 Gross Readout Depending on Section Thickness and Staining Time

Each prepared slides were read under 40 and 100 magnifications of an optical microscope by 8 pathologists. The level of satisfaction was calculated by reading the result of the staining intensity of the hematoxylin and eosin. In the changes depending on the thickness of the slice, 3 μm thicknesses showed satisfied staining intensity under both 40 and 100 magnifications. The hematoxly in staining intensity showed the average value of 0.1 which is close to satisfied intensity and the eosin staining showed the average value of 0 which is satisfied intensity as well Table 1. Given the differences in staining time, hematoxylin staining time 4 minutes and 30 seconds and eosin staining time 3 minutes and 45 seconds showed the average value of 0.1 and 0 respectively which are favorable staining intensity Table 2.

**Table 1.** Microscopic analysis of overall staining intensity with different section thickness

| | X40 | | X100 | |
|---|---|---|---|---|
| | **Hematoxylin** | **Eosin** | **Hematoxylin** | **Eosin** |
| 1μm | -1.6 | -1.9 | -1.3 | -1.8 |
| 2μm | -1.0 | -1.1 | -0.9 | -1.1 |
| 3μm | 0.1 | 0 | 0.1 | 0 |
| 4μm | 0.3 | 0.3 | 0.4 | 0.1 |
| 5μm | 0.9 | 0.8 | 0.9 | 0.8 |
| 6μm | 1.5 | 1.3 | 1.6 | 1.2 |

〈0: weak, 0: good, 0 〈: strong

**Table 2.** Microscopic analysis of overall staining intensity with different staining duration

| | X40 | | X100 | |
|---|---|---|---|---|
| | **Hematoxylin** | **Eosin** | **Hematoxylin** | **Eosin** |
| 1 | 0.3 | 0 | 0.3 | 0 |
| 2 | 0.1 | 0 | 0.1 | 0 |
| 3 | -0.8 | -1.2 | -0.8 | -1.0 |
| 4 | -0.9 | -1.3 | -0.9 | -1.3 |
| 5 | -1.5 | -1.9 | -1.6 | -1.9 |
| 6 | -2.6 | -2.6 | -2.5 | -2.5 |

1. Hematoxylin: 5'24", Eosin: 4'30"
2. Hematoxylin: 4'30", Eosin: 3'45"
3. Hematoxylin: 3'36", Eosin: 3'
4. Hematoxylin: 2'42", Eosin: 2'15"
5. Hematoxylin: 1'48", Eosin: 1'30"
6. Hematoxylin: 54", Eosin: 45"

〈0: weak, 0: good, 0 〈: strong

**Table 3.** RGB image analysis of nuclear staining intensity with different section thickness

| | **Red** | **Green** | **Blue** |
|---|---|---|---|
| 1μm | 167.67±4.89 | 126.49±9.41 | 188.36±2.50 |
| 2μm | 162.18±6.61 | 109.19±13.02 | 185.91±4.18 |
| 3μm | 140.15±6.92 | 56.45±12.04 | 173.71±5.16 |
| 4μm | 142.99±6.53 | 50.05±8.90 | 176.19±4.90 |
| 5μm | 129.76±9.38 | 31.26±8.02 | 166.60±6.46 |
| 6μm | 131.45±7.98 | 28.92±6.37 | 166.04±6.32 |

### 3.2 RGB Analysis According to Section Thickness

The nucleus with the thickness from 1 μm to 6 μm was analyzed by RGB analysis. 1 μm showed the average value

of 167.6 in red, and then it gradually reduced to 131.45 in 6 µm of thickness. Green was analyzed by 126.49 in 1 µm and 28.92 in 6 µm. Blue was analyzed by 188.36 in 1 µm and 166.04 in 6 µm Table 3 and Figure 3. In cytoplasm analysis, red showed the average value of 190.36 in 1 µm and 189.80 in 6 µm. Green was analyzed by 196.27 in 1 µm and 187.03 in 6 µm Table 4 and Figure 4.

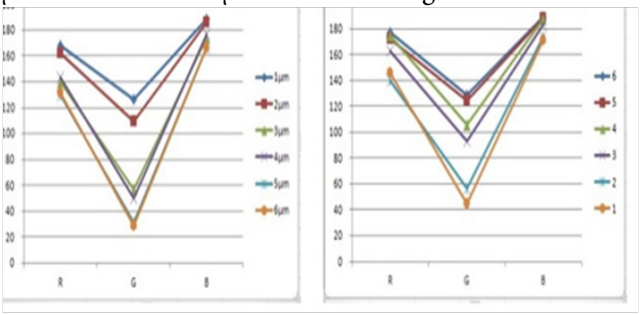

**Figure 3.** RGB image analysis of nuclear staining

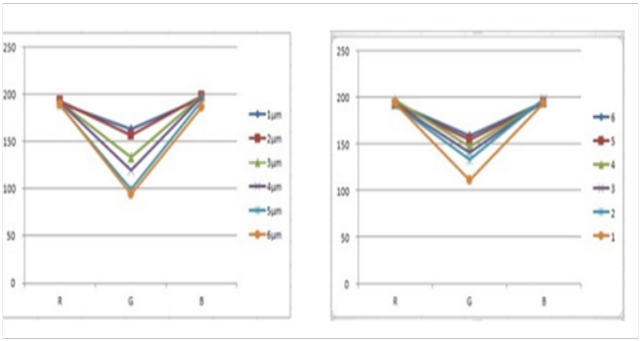

**Figure 4.** RGB image analysis of cytoplasmic staining

**Table 4.** RGB image analysis of cytoplasmic staining intensity with different section thickness

| | Red | Green | Blue |
|---|---|---|---|
| 1µm | 190.36±2.66 | 162.90±6.86 | 196.27±1.87 |
| 2µm | 192.85±2.92 | 157.23±7.42 | 198.03±2.52 |
| 3µm | 192.32±4.18 | 133.90±11.76 | 198.40±2.47 |
| 4µm | 191.96±2.99 | 119.98±8.20 | 197.11±1.87 |
| 5µm | 188.68±3.85 | 98.89±11.93 | 193.36±2.91 |
| 6µm | 189.80±4.13 | 94.29±22.83 | 187.03±20.04 |

## 3.3 RGB Analysis According to the Staining Time

The nucleus was analyzed using RGB analysis by increasing the staining time by 20% and decreasing by 80%. Red showed the average value of 145.54 when the staining time was increased by 20% and 177.19 when decreased by 80%. Green was analyzed by 44.57 when 20% of the staining time was increased and the average was increased to 128.99 when the staining time was decreased by 80%. Blue was analyzed by 171.05 when 20% of the staining time was increased and showed 189.33 when 80% of the staining time was decreased Table 5 and Figure 3. In cytoplasm analysis, the red showed the average value of 194.04 when 20% of the staining time was increased and 193.86 when 80% of the staining time was decreased. Green was analyzed by 111.16 when 20% of the staining time was increased and the value gradually increased to 158.91 when 80% of the staining time was decreased. Blue was analyzed by 193.64 in 20% increased staining time and 195.19 in 80% decreased staining time Table 6 and Figure 4.

**Table 5.** RGB image analysis of nuclear staining intensity with different staining duration

| | Red | Green | Blue |
|---|---|---|---|
| 1 | 145.54±9.47 | 44.57±11.32 | 171.05±7.12 |
| 2 | 140.15±6.92 | 56.45±12.04 | 173.71±5.16 |
| 3 | 162.95±7.48 | 92.98±15.07 | 183.62±5.09 |
| 4 | 174.50±4.31 | 105.63±8.97 | 187.58±2.88 |
| 5 | 172.34±4.42 | 124.83±7.78 | 187.99±2.57 |
| 6 | 177.19±3.87 | 128.89±9.34 | 189.33±2.48 |

1. Hematoxylin: 5'24", Eosin: 4'30"
2. Hematoxylin: 4'30", Eosin: 3'45"
3. Hematoxylin: 3'36", Eosin: 3'
4. Hematoxylin: 2'42", Eosin: 2'15"
5. Hematoxylin: 1'48", Eosin: 1'30"
6. Hematoxylin: 54', Eosin: 45'

**Table 6.** RGB image analysis of cytoplasmic staining intensity with different staining duration

| | Red | Green | Blue |
|---|---|---|---|
| 1 | 194.04±3.53 | 111.16±12.48 | 193.64±3.83 |
| 2 | 192.32±4.18 | 133.90±11.76 | 198.40±2.47 |
| 3 | 192.80±2.63 | 140.55±10.56 | 195.68±2.71 |
| 4 | 197.53±2.65 | 146.81±9.50 | 195.87±3.04 |
| 5 | 192.40±2.72 | 154.52±8.30 | 194.29±2.53 |
| 6 | 193.86±2.11 | 158.91±7.16 | 195.19±2.25 |

1. Hematoxylin: 5'24', Eosin: 4'30"
2. Hematoxylin: 4'30", Eosin: 3'45"
3. Hematoxylin: 3'36", Eosin: 3'
4. Hematoxylin: 2'42", Eosin: 2'15"
5. Hematoxylin: 1'48", Eosin: 1'30"
6. Hematoxylin: 54', Eosin: 45'

## 4. Discussion and Conclusions

Histopathology is the study of signs of disease using microscopic examination of surgical specimen[7]. The tissue sample manufacturing techniques have been developed as various forms of microscopy[8]. Particularly, with the development of an electron microscopy during the last 50 years, the knowledge of cell structure has remarkably expanded, but it has not yet exceeded the scope of an optical microscope. The ideal resolution of the optical microscope is about 0.2 μm, however, when observing the tissue, the actual resolution becomes lower than the resolution of the microscope. Therefore, it is important to produce thin and fine tissue samples. The purpose of staining tissues is to observe the composition of the cell, and staining with hematoxylin and eosin is a common staining method. Heavy metal's stain include careful. Heavy metals are potentially hurting humans[9] and the environment a huge health factor[10]. Particularly, the staining discrepancies depending on the sectioned slide thickness have become a very important factor in selecting section thickness. Therefore, preferred thickness of section, staining intensity, and color are difference by each observer[11]. Also, the damage and deformation during the sectioning process result in a number of problems in microscopic analysis. This study analyzed gastric mucosa membrane because many stomach biopsies are referred as gastro copy become active[12]. The previous studies described the appropriate thickness of the general sample as 4~6 μm[13], however, as a result of this study, the ideal thickness of the slide sample was 3 μm when staining for the microscopic analysis. In addition, the appropriate staining time for hematoxylin was 4 minutes and 30 seconds and for eosin was 3 minutes and 45 seconds. The result of RGB analysis of 3 μm thickness showed 140.15 in red, 56.45 in green, and 173.71 in blue. Also, in the analysis of nucleus thickness, the change range of red (167.67~131.45) and blue (188.36~166.04) were 36.22 and 22.32, but green (126.49~28.92) was 97.59 which showed significant reduction as the section become thicker. Also, green showed significant differences between each steps, and 2 μm and 3 μm of thicknesses showed the most significant changes which was 52.74. Therefore, when the sample thickness is diverse in H&E staining, the color determining the staining intensity of the nucleus is green while red and blue weakly contribute to the staining intensity. In H&E staining, it is important applying and practicing the sample staining with the above results to improve the quality of the staining.

## References


1. Filella X, Molina R, Grau JJ, Piqué JM, Garcia-Valdecasas JC, Astudillo E, Biete A, Bordas JM, Novell A, Campo E. Prognostic value of CA 19.9 levels in colorectal cancer. *Annals of Surgery*. 1992 Jul; 216(1):55-59.
2. Lee JH, Rhu WS, Park PJ, Kim OL, SON KS, Lee JB, Bea JW, KU BH. The Survival Analysis of Immunohistochmically Defined Basal and Luminal Subtype of Breast Cancer. *Journal of the Korean Surgical Society.* 2006; 70(1):7-13.
3. Pritt BS, Gibson PC, Cooper K. Digital imaging guidelines for pathology: A proposal for general and academic use. *Advances in Anatomic Pathology*. 2003 Mar; 10(2):96-100.
4. Leong FJWM, Leong ASY. Digital imaging applications in anatomic pathology, *Advances in Anatomic Pathology*. 2003; 10(2):88-95.
5. Vrekoussis T, Chaniotis V, Navrozoglou I, Dousias V, Pavlakis K, Stathopoulos EN, Zoras O. Image analysis of breast cancer immunohistochemistry stained sections using image: An RGB-based modal. *Anticancer Research*. 2009 Dec; 29(12):4995-98.
6. Pinco J, Goulart RA, Otis CN, Garb JG, Pantanowitz L. Impact of digital image manipulation in cytology, *Archives of Pathology and Laboratory Medicine*. 2009; 133:57-61.
7. Kim SM, Jo SL, Lee JS, Park SD, Hea KB, Ha HC, Kim SH, Hea MH, Kang KI. Investigation of Alpha 1-Proteinase Inhibitor in Serum and Specimen of Lung Cancer Patients. *The Korean Journal of Thoracic and Cardiovascular Surgery*. 1994; 27(5):364-73.
8. Mun CH, Kim YT. Average Numbers of Regional Lymph Nodes in Radically Resected Surgical Specimens. *Annals of Surgical Treatment and Research*. 1979 Jun; 21(6):63-68.
9. Rotterdam H, Sheahan DG, Sommers SC. Biopsy diagnosis of the digestive tract. *American Journal of Surgical Pathology*. 1982 Jul; 6(5):483.
10. Bancroft JD, Stevens A, Turner DR. Theory and practice of histological techniques. 6th (edn), Churchill Livingstone, 2007 Dec.
11. Afrifa CG, Ofosu1 FG, Bamford SA, Atiemo SM, Aboh IJK, Gyampo O, Ahiamadjie H, Adeti JP, Arthur JK. Health Risk Assessment of Heavy Metal Exposure from Soil Dustat Selected Fuel Filling Stations in Accra. *International Journal of Science and Technology*. 2015 Jul; 4(7):289-96.
12. Akoto O, Abankwa E. Heavy Metals Contamination and Speciation in Sediments of the Owabi Reservoir. *Environmental Research Journal*. 2014; 8(1):10-16.
13. Beevi KS, Bindu GR. Analysis of Nuclei Detection with Stain Normalization in Histopathology Images. *Indian Journal of Science and Technology*. 2015 Sep; 8(23):1-5.