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**AUTOMATED COLOR SEGMENTATION IN QUANTITATIVE
MICROSCOPY**

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

the Degree of Doctor of Philosophy in the Graduate School of

The Ohio State University

By

Ajeetkumar Gaddipati, M.S.

* * * * *

The Ohio State University

1998

Dissertation Committee:

J. Fredrick Cornhill, D.Phil., Adviser

D. Geoffrey Vince, Ph.D.

Kimerly A. Powell, Ph.D.

Approved by

Adviser

Graduate Program in
Biomedical Engineering

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ABSTRACT

Over the past decade, there has been increased interest in quantifying cell populations in tissue sections. Image analysis is now being used in limited pathological applications, such as PAP smear evaluation, with the dual aim of increasing the accuracy of diagnosis and reducing the review time. These applications primarily use gray scale images and deal with smears in which cells are well separated. Quantification of routinely stained tissue represents a more difficult problem in that objects can not be separated in gray scale, as part of the background could also have the same intensity as the objects of interest. Many of the existing semiautomatic algorithms are specific to a particular application and are computationally expensive. Hence, we have investigated the general adaptive automated color segmentation approaches which alleviate these problems.

Three adaptive segmentation algorithms were compared using synthetic images and clinical microscopy slides. Both qualitative and quantitative performance comparison was performed with the clinical images. The competitive learning algorithm had the best performance, considering both time and accuracy of segmentation. Fuzzy C-means used the solution from competitive learning as the starting point and found the locally optimal solution. Hence, in some of the experiments, the fuzzy algorithm resulted in more accurate segmentation. Median split algorithm performed worse than both competitive learning and

fuzzy C-means algorithms. Overall the automated segmentation methods achieved an accuracy of 85% in tissue component separation. Four demonstrative applications of the segmentation as an integral part of quantitative tissue analysis were also presented. Finally the merits and feasibility of including contextual information in the segmentation were discussed along with future directions.

The research presented in this dissertation showed that automated color segmentation can achieve high accuracy (~85%) in tissue component separation on microscopy images at interactive speeds on workstations (~15 seconds for 1k x 1k image). Automated segmentation is the first step towards completely automated quantitative analysis. Quantitative histological studies in biomedical field tend to involve huge quantities of microscopy images. Automated quantitative analysis is the only feasible method of obtaining reliable and accurate results in these applications.

Dedicated to dear Ammamma & Tatayya

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VITA

July 31, 1970.....Born: Ponnuru, Andhra Pradesh, India.

July, 1988 - July, 1992.....BTech., Electrical and Electronics Engineering

Indian Institute of Technology, Madras, India

September, 1992 - March, 1995.....M.S., Biomedical Engineering

The Ohio State University, Columbus, Ohio, U.S.A

September, 1992- August 1993.....University Fellow

The Ohio State University, Columbus, Ohio, U.S.A

September 1993 - present.....Graduate Research Associate,

Biomedical Engineering

The Ohio State University, Columbus, OH

The Cleveland Clinic Foundation, Cleveland, OH

PUBLICATIONS

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FIELDS OF STUDY

Major Field: Biomedical Engineering

Computer Vision and Computer Graphics

Minors: Image and Signal Processing

Cardiovascular Physiology

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CHAPTER 1

INTRODUCTION

1.1 Problem statement

The visual inspection of cellular specimens and histological sections by light microscopy plays an important role in clinical medicine and biomedical research. The human visual system is very good at the recognition of patterns, such as cells partially covered by other structures on microscopy slides, but less efficient at quantitative assessment of these patterns. Diagnostic tests such as PAP-smears and large biomedical research studies, such as study of atherosclerosis genesis and progression, generate thousands if not millions of microscopic slides which need to be quantitatively analyzed each year. For this reason, both pathologists and researchers would benefit from a completely automated microscopy image analysis system. This system would act as a quantitative aid to the human visual system, relying on as little interaction from user as possible. This would alleviate the tediousness of such a repetitive task while maintaining high accuracy.

Microscopy analysis systems are becoming more of a reality with the recent advancements in technology. Typical microscopy analysis system comprises of three major components. i) image acquisition from well stained microscopy slides, ii) tissue component separation on the image (segmentation), iii) feature analysis for disease diagnosis or

research. The first component has improved with the advancement in staining technology and the high resolution, accurate, fast CCD cameras available today. Second and third components involve computationally intensive tasks and would not be possible without the rapid development of computers and the field of computer vision. Over the years histologists have developed special stains and procedures for bringing out the contrast between targeted tissues and surroundings. Dyes with contrasting colors have been used to enhance the visual difference between structures of interest. For example, hematoxylin and eosin (H&E) stain is used to differentiate between nucleus and cytoplasm. Nucleus is stained blue with hematoxylin and cytoplasm is stained different shades of pink by eosin. In addition to routine stains such as H&E, various special stains have been developed to identify specific histological structures. In this study, we investigated separating structures stained with Movat pentachrome. Movat pentachrome stain is used to label multiple tissue components such as smooth muscle cells, collagen, elastin and nucleus in the same section. In the early 60's immunohistochemical techniques were developed which, by the addition of antigen specific antibodies, allow histopathologists to identify molecular markers on cells and cell structures. We studied segmentation of the positive expression of numerous antibodies using 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen.

Early image analysis systems used monochrome images, as color image capturing hardware was expensive and not reliable. Since these systems did not use color, separate staining of histological sections into dark and white objects was required. As color technology developed, image analysis systems began including chromatic information also to aid in identification of objects present on the slide. The chromatic information was

necessary, especially when the intensity distributions of stains overlap. To illustrate this, color and gray scale images of a microscopy section are compared below. Figure 1 shows a Movat pentachrome stained image in gray scale and in color. The five different stains are easily distinguished in the color image. The same can not be said of the gray image.

In the past, researchers refrained from using segmentation approaches involving huge amount of computation as the computers were expensive and small in terms of computational power. The computing power of workstations has steadily increased making the use of sophisticated and computationally expensive quantitative analysis algorithms feasible.

Figure 1 Comparison of color and gray scale images of a Movat stained microscopy slide.

1.1.1 Hypothesis:

The goal of this research was to develop a completely automated color segmentation technique that could be used to separate a variety of stained tissue components on a microscopy slide based on color and contextual cues. In addition to being automated, this technique should be accurate, adaptive, and reproducible .

1.2 Significance

Currently quantitative analysis is used in basic research for applications such as cell counting, distribution and area determination. In the clinical field, quantitative analysis is used in such diverse applications as PAP smear analysis, quality assurance of microscopy slide processing, and as a general diagnostic aid to pathologists. The biomedical industry is also interested in using quantitative analysis to study the effects of biomaterials introduced into the body and the immunological responses after administration of new medicines. In due time, when the quantitative analysis is sufficiently advanced, it will become a part of the product development cycle, in particular a part of quality control process.

Commercially available color image analysis systems traditionally use a method of global thresholding to segment tissues of interest. This leads to inaccurate segmentation due to the variation in the natural stains and the complex composition of the tissue. The commercial systems tend to be very specific and limited in their application. In order to overcome the fundamental limitations of color segmentation, we have evaluated a number of unsupervised adaptive clustering algorithms for performing separation of color stained tissue components.

1.3 Brief overview of the reminder of the thesis

A review of histology particularly with regard to the staining procedure is presented in chapter two. This is then followed by a brief overview of color concepts. In chapter 3, a brief description of image acquisition and preprocessing is presented. Previous and contemporary research in the fields of color segmentation and quantitative analysis of microscopic images is described in chapter four. In chapter five, a complete description of the adaptive color segmentation algorithms used in this research is provided. In the sixth chapter, the performance of the algorithms compared to each other and to manual segmentation is illustrated. In chapter seven, several applications of the adaptive color segmentation approach are described. In chapter eight, the research is summarized and possible future directions listed.

CHAPTER 2

Histology and Color Theory

In this chapter, the basics of histology and color theory are reviewed before embarking on detailed study of color segmentation of histological sections in future chapters.

2.1 HISTOLOGY [1]

Histotechnology pertinent to the quantitative analysis of stained histological sections is first described. Then an outline of slide preparation is presented followed by a brief overview of routine staining such as H&E, and immunological staining with DAB.

2.1.1 Introduction

Preparation of a slide for microscopic examination can be listed in steps as tissue fixation, processing, sectioning, staining and finally mounting the slide. These steps are explained in the following paragraphs.

The initial stage in the preparation of histological sections is fixation. Fixatives serve to prevent decay and autolysis of tissues. They alter the tissue by stabilizing the protein so that it is resistant to further changes. Fixatives also bring out differences in refractive indexes and thus increase the contrast between different tissue elements. A properly chosen fixative can also enhance staining. Routine fixation is performed using formaldehyde in most laboratories. In the Whitaker Biomedical Image Processing laboratory, Histochoice is used for fixation of most tissues. A recent staining comparison

study [2] had proved that fixing in Histochoice yielded higher or equal quality staining on the tissue section slides compared to formalin. Histochoice is non-carcinogenous and thus easier to handle.

The next stage of the preparation is processing. Processing includes dehydration, clearing and infiltration. Dehydration is performed to prepare tissues for embedding in a non-aqueous medium such as paraffin. Alcohol solutions with sequentially lower percentages of water are usually used for dehydration. The hydrophilic action of alcohol removes water from fixed tissues. Clearing is then performed to remove the alcohol used for dehydration and to make the tissue receptive to the infiltration medium. Xylene is the most common clearing agent. After dehydration and clearing, tissue is infiltrated with the supporting medium. This step maintains the morphological structure of the tissue when it is cut in to thin sections. In the Whitaker Biomedical Image Processing laboratory, paraffin is used as the embedding medium. The tissue is then submerged in paraffin(embedding) to provide a supporting medium for sectioning.

Embedded tissue sections are cut into thin 5 μm sections using a microtome. Orientation for sectioning is carefully chosen based on the tissue block being prepared and the purpose of the histological study. For example, skin sections are always cut so that all the layers of dermis are visible in each section. Coronary artery sections are cut perpendicular to their axis to present a circular cross section on the mounted slide. Histotechnologists acquire the art of cutting tissue sections with uniform thickness through years of practice. Sections are extracted from the microtome in the form of serial ribbons and floated on a water bath. The ribbons are gently stretched to remove wrinkles in the

sections. Tissue sections are picked up on individual slides using electro-static attraction. The slides are then dried in a oven for a short period of time to remove the water and also to make the tissue sections adhere more securely to the slide. The paraffin remaining on the section is removed by immersing the slide in Xylene solution for a short duration.

The slides are now ready to be stained. Staining is discussed in detail in the following sections of this chapter. Once the slides have been stained, they are mounted using a mounting medium and cover slip. This is done to preserve the stained tissue section and facilitates microscopic examination at varying magnifications. The refractive index of the mounting media is kept slightly different from that of the unstained tissue for the purpose of locating unstained tissue on the slides. Unstained tissues have an average refractive index of 1.53 to 1.54. Most mounting media have a refractive index ranging from 1.51 to 1.55.

2.1.2 Routine Staining

Routine staining involves both physical and chemical reactions. Most stains depend on the tissue component of interest attracting minute dye particles from the surrounding solution. This first step is the physical reaction. The dye is then bound to the tissue by chemical reaction resulting in ionic or covalent bonds.

Routine staining usually deals with contrasting the nucleus with the cytoplasm. Nuclear staining is done with basic dyes or with dyes combined with or followed by metal mordants. Basic dyes stain by attaching to nucleic acids. In tissues where nucleic acids have been removed such as decalcified tissue, metal mordant staining must be used. Cytoplasmic staining occurs primarily by dyes reacting with charged groups on the side chains of amino acids constituting the proteins present in the cytoplasm. The two groups responsible for the charges are -NH_2 (amine) and -COOH (carboxylic).

Dye binding is affected by factors such as solution pH, temperature, salt content and fixative used. Solution pH determines the charges on both tissue element and the dye molecule and thus affects the formation of ionic bonds. A temperature increase will increase the rate of staining by increasing the diffusion rate of the dye molecules. Salt ions compete with dye ions for the same binding site and thus alter staining. Fixatives alter the staining character of the tissue by reacting with certain chemical groups in tissue and making those groups unavailable for dye binding.

The most commonly used nuclear stain is hematoxylin. Hematoxylin is a natural abstract from the heartwood of the logwood tree. Hematoxylin is not a dye, but its oxidation product hematein is a weak anionic dye. Hematein is combined with a metallic mordant to make it a strong dye with particular affinity for nuclei. Hematoxylin stains the nuclei between blue and dark purple.

Cytoplasmic staining is often anionic. (*i.e.* dyes that combine with positively charged tissue groups are used.) These dyes usually bind to sites containing aminoacids such as arginine, histidine, and lysine. Eosin is the most commonly used cytoplasmic dye. It is a sodium salt of a color acid. The anionic part of the eosin molecule imparts color to tissue proteins. Thus optimum staining with eosin is achieved at a pH of approximately 4.6 to 5.0. Eosin imparts *three* shades of pink to the non-nuclear proteins; one each for erythrocytes, collagen, and the cytoplasm of muscle or epithelial cells.

In most routine staining, hematoxylin is used together with eosin to stain tissue nuclei blue and the cytoplasm various shades of pink. The combination is called H&E staining. This staining assists in looking at changes in cell structure and morphology.

2.1.3 Immunohistochemical staining

Quantitative analysis of immunohistochemically stained slides is increasingly used for accurate diagnosis of disease and assistance in choice of therapy. For example, in breast cancer treatment, if the biopsy analysis determines that the breast tumor has large amount of estrogen receptors, drug therapy to reduce the estrogen levels may be prescribed instead of mastectomy. In this section, an overview of immuno staining is given.

Immuno staining involves antigen-antibody reaction. Antigen is any substance that can induce detectable immune response. Antibodies are produced by B-lymphocytes in response to antigenic stimulation. Antibodies attach to antigens on foreign cells and thus mark them for removal by macrophages. Thus, antibodies play a crucial role in protecting the body from potential foreign organism invasions. When an antigen triggers lymphocytes, they proliferate and form a clone of cells. Cells from a single clone produce identical antibodies. But, when we consider cells from different clones, antibodies produced can be of different classes with attraction towards different molecular sites on the antigen. Antigenic stimulation usually results in the production of a mixture of antibodies from many clones of lymphocytes. The collection is known as a *polyclonal* antibody. Current technology allows development of cells cloned from a single lymphocyte or *monoclonal* antibodies in cell line cultures using *invitro* fusion. Monoclonal antibodies provide high homogeneity, absence of nonspecific antibodies, and a low batch to batch variability. These antibodies can be used for the identification of specific antigens in tissue sections.

Often an indirect method is used for detecting antigen present in a patient's tissue using bright field microscopy. First an unlabeled antibody is used to bind to the antigen. Then a

second labeled antibody is used for localizing the first antibody. An Avidin-Biotin complex is bound to the second antibody. A chromagen such as Diaminobenzidide (DAB) is used to color the complex. Figure 2 explains this method.

Figure 2 Avidin-biotin complex immunohistochemical staining method

In the Whitaker Biomedical Imaging Laboratory, the following indirect staining procedure is used [3]. Slides prepared for immunohistochemical staining are first exposed to 3% hydrogen peroxide solution in phosphate buffered saline (PBS) for five minutes to remove any endogenous peroxidase present in the tissue. Normal rabbit serum at 1:10 dilution in PBS was added for 10 minutes as a blocking solution. Monoclonal antibody prepared at the adequate dilution using 1% bovine serum albumin in PBS was then applied for antigen marking. The slides were then incubated at thirty degrees for one hour. The

antibody was labeled using an Elite avidin/biotin peroxidase complex (Vector laboratories, Burlingame, CA) applied for 30 minutes. 3-3 diamine benzidine (DAB), activated by 3% hydrogen peroxide solution was added to impart a brown color to the peroxidase complex.

Figure 3 shows a sample DAB stained tonsil section.

Figure 3 Immuno stained tonsil section with nuclei counter stained using hematoxylin dye.

2.2 COLOR THEORY

As explained in introduction, the use of differently colored stains enables simultaneous separation of multiple tissue components on a slide. Understanding the basic color theory and choosing a suitable color space for automated segmentation of tissue components is the first step towards achieving the goal of this research.

The color we perceive depends not only on the color properties of the object, we are viewing, but also on the source of illumination, the color of the background, and on the human visual system. Before we delve into an in depth discussion of chromaticity, a brief overview of achromatic light sensation is given.

The amount of light is the only quality of achromatic light. It is measured as *intensity* or *luminance* when dealing with the light in the physical energy terms, where as *brightness* is used to describe the perceived light intensity or quantity. For our purposes, *brightness* is obtained by multiplying the spectral energy density function of the incident light with the luminous efficiency function of the eye. (Figure 4)

Physiological experiments have proven that the rod type sensors in our retina primarily perceive achromatic light. Chromatic perception involves three additional cone type sensors each of which has spectral response functions corresponding to the familiar red, green and blue colors. This is known as the *tristimulus* theory. Although, this may lead one to think that additive mixtures of red green and blue lights (the primary colors) could be used to obtain all colors, this is only approximately true.

(Figure 5) shows that negative values may be needed for achieving few of the colors in the visible spectrum. This means mixing red, green and blue can not produce these colors. To circumvent this problem, the Commission Internationale de l'Eclairage (CIE) defined three standard primaries X, Y, Z to be used for specifying color. The three corresponding matching functions for obtaining all the colors in the visible spectrum at a constant luminance are shown in (Figure 6).

Figure 4 Luminous-efficiency function for the human visual system

Figure 5 Color-matching functions, showing amounts of red, green, and blue needed to match all wavelengths of the visible spectrum.

Figure 6 Color-matching functions for the CIE X, Y, Z primaries

Figure 7 Typical spectral distributions of red, green and blue colors.

Each color specified by its dominant wavelength is described by color-matching functions, showing amounts of red, green, and blue needed to match all colors of the visible spectrum in Figure 5 and color-matching functions for the CIE X, Y, Z primaries in Figure 6. The primary Y is chosen deliberately to have a matching function identical to the Luminous-efficiency function for the average human eye. The CIE XYZ color space is used for specifying the color gamuts of all the color display, recording, and printing devices. This facilitates reproduction of identical colors on widely varying devices.

Going back to color perception from the colorimetry digression, color perception description usually involves three attributes known as hue, saturation and brightness. Hue describes the pigment of the color such as red, yellow, cyan etc. Saturation refers to how far color is from a gray of equal intensity. For example, navy blue is highly saturated, sky blue is relatively unsaturated. Lightness refers to the perceived intensity of a reflecting object. Some times Brightness is used instead of lightness to refer to the perceived intensity of self-luminous object, such as a star or a fire. In colorimetry, the term hue corresponds to dominant wavelength, saturation to excitation purity, and Lightness/brightness to luminance.

2.2.1 Color Spaces

So far, we have mentioned RGB and XYZ color spaces. We review these and other color spaces from the point of view of segmentation.

2.2.1.1 RGB color space:

Traditionally, color has been specified with RGB triplets in imaging community. As explained earlier, this model is based on the *tristimulus* theory. This model implicitly

represents colored light as the mixture of samples obtained using three spectral distributions^{3/4} one with a peak in the short wavelength region of visible spectrum (blue), one with a peak in the medium range (green), and one in long range (red) (Figure 7). If the spectral energy distribution of a light source is E_λ , then its color can be represented as a RGB vector multiplied with the color spectral distributions r_λ , g_λ and b_λ

(1)

The color matching functions are shown in Figure 5. R,G,B spectral distributions can vary from one color device to another. For example, printers can have substantially different RGB distributions when compared with computer monitors. Recently an International agreement has been reached on RGB primaries for high definition television (HDTV). These primaries are representative of contemporary monitors in video, computing and computer graphics. The white point used with this standard (D_{65}) closely resembles bright sunlight. The white point is the spectral energy distribution for which R,G and B values reach maximum. White point is crucial for calibration and determining the transformations between color spaces. The standard is called Rec. 709 and the primaries and white point are specified in CIE XYZ space as shown in Table 1. The numbers given in the table are normalized. (i.e. $x+y+z = 1.0$). Usually R, G, B values are normalized so that white point has the maximum value. For Table 1, R,G,B values range from 0.0 to 1.0.

	R	G	B	White
x	0.640	0.300	0.150	0.3127
y	0.330	0.600	0.060	0.3290
z	0.030	0.100	0.790	0.3582

Table 1 Rec. 709 primaries and white point values in CIE XYZ space.

As stated previously, one of the drawbacks of this space is that some of the visible colors can not be represented as additive mixtures of red, green and blue. Also the high correlation between the three features makes it less than optimal for the purpose of color segmentation.

2.2.1.2 XYZ COLOR SPACE:

As mentioned earlier, CIE (International Commission on Illumination) has defined X, Y, Z as a set of primaries with positive color matching functions across the length of the visible spectrum, as explained earlier in the chapter. Also, the fact that Y has a color matching function corresponding to the achromatic light sensitivity of our eyes is another advantage. We should note that XYZ primaries unlike RGB are not the colors we see in our daily life. This color space is the *defacto* standard for specifications of all color devices. Each manufacturer gives the transformation required for converting their device specific color coordinates into XYZ color space. This enables easy reproduction of a particular color on devices manufactured by various companies. The transformation from Rec. 709 RGB is given below.

$$\begin{bmatrix} X \\ Y \\ Z \end{bmatrix} = \begin{bmatrix} 0.41245 & 0.35758 & 0.18042 \\ 0.21267 & 0.71516 & 0.07217 \\ 0.01933 & 0.11919 & 0.95023 \end{bmatrix} \times \begin{bmatrix} R_{709} \\ G_{709} \\ B_{709} \end{bmatrix} \quad (2)$$

This color space by itself is not suitable for color segmentation as the distance from one color to another specified using XYZ does not correspond to the perceived difference between colors.

From the view of color image segmentation, the color spaces can be divided into three categories.

2.2.1.3 Perceptually uniform color spaces: Lab, Luv

These two color spaces were designed by CIE as approximations of perceptually uniform color space using nonlinear transformations from XYZ color space. A color space is perceptually uniform if a small perturbation to a component value is approximately equally perceptible across the range of that value. For example, the volume control on most audio systems is designed to be perceptually uniform. Rotating the knob by fifteen degrees produces approximately the same perceptual increment in volume anywhere across the range of the control. This is achieved by matching the logarithmic nature of our sound perception. If the scale were linear, our perceptual range would be limited to the bottom portion of the dial.

Both RGB and XYZ color spaces have perceptual nonuniformity of about 80:1. Lab and Luv spaces improve this ratio to about 6:1. The two spaces came into existence, as CIE could not agree on a single standard after decades of discussion. Luminance L is computed directly from the Y coordinate of XYZ color space.

Computation of Luv involves intermediate u' and v' terms.

$$u' = \frac{4X}{X + 15Y + 3Z}, \quad v' = \frac{9Y}{X + 15Y + 3Z} \quad (3)$$

u'_n and v'_n are computed next for the reference white coordinates X_n , Y_n and Z_n .

Then,

$$u = 13L(u' - u'_n), \quad v = 13L(v' - v'_n) \quad (4)$$

Lab coordinates are computed as follows,

for $(X/X_n, Y/Y_n, Z/Z_n) > 0.01$:

$$a = 500 \left[\left(\frac{X}{X_n} \right)^{\frac{1}{3}} - \left(\frac{Y}{Y_n} \right)^{\frac{1}{3}} \right], \quad b = 200 \left[\left(\frac{Y}{Y_n} \right)^{\frac{1}{3}} - \left(\frac{Z}{Z_n} \right)^{\frac{1}{3}} \right] \quad (5)$$

Both Lab and Luv color spaces are often used in segmentation.

2.2.1.4 Pigment based color spaces: HSV, HSI, HLS

HSV color space:

HSV is designed on the painter's notions of color using grays, tints and shades [Figure 8]. Tints are obtained by adding white pigment to 'pure' color pigment. Shades are obtained by adding black pigment to 'pure' color pigment. Tones result from adding both black and white pigments to 'pure' color pigment. Artists use these steps to create different colors of the same hue. Grays are obtained by adding just white and black pigments. This forms the basis of HSV color space.

Hue (H) explained in colorimetry terms corresponds to dominant wavelength in the spectrum. In terms of the painter, it is the color pigment. Hue (H) is measured as an angle (*i.e.* as a circular scale). Saturation (S) measures the purity of the pigment or how far the color is from the gray line. Value (V) is the intensity of the light. Figure 9 shows the HSV color space as a hexagonal cone.

The HSV coordinate system is cylindrical and all the colors are defined within a hex-pyramid subset. The vertical axis V is value. The top of the hex-pyramid or the V=1 plane contains relatively bright colors. Hue (H) is the angle measured around the V axis. Saturation (S) is a ratio going from 0 at V axis to 1 at the sides of the hex-pyramid. Foley *et al.* described HSV color space in detail in [4].

The conversion from RGB to HSV is nonlinear as it involves maxima and minima.

$$\text{Let } r = \frac{R}{256} \quad g = \frac{G}{256} \quad b = \frac{B}{256}$$

Where R, G, B are integer values with a range of 0 to 256. (6)

R, G, B corresponds to red, green and blue color values respectively.

V is calculated as $\text{maximum}(r, g, b)$. S corresponds to distance of the color from R=G=B line(gray line).

S is calculated as

$$S = \frac{\text{Maximum}(r, g, b) - \text{Minimum}(r, g, b)}{\text{Maximum}(r, g, b)} \quad (7)$$

H is the angle specifying the corresponding pure color and is calculated using a conditional statement.

$$\begin{aligned}
\delta &= \text{Maximum}(r, g, b) - \text{Minimum}(r, g, b) \\
\text{If } r &= \text{Maximum}(r, g, b) \quad \text{then } H = \frac{g - b}{\delta} \\
\text{ElseIf } g &= \text{Maximum}(r, g, b) \quad \text{then } H = 2 + \frac{b - r}{\delta} \\
\text{ElseIf } b &= \text{Maximum}(r, g, b) \quad \text{then } H = 4 + \frac{r - g}{\delta}
\end{aligned} \tag{8}$$

HLS Color Space

HLS is a deformation of HSV, in which white is pulled upward to form the upper hexcone from the $v=1$ plane. The terms hue, lightness and saturation are similar to the ones used in HSV. Lightness is 0 for black and 1 for white. $H=0$ corresponds to red as in HSV. Here the maximally saturated and separated hues are at $S=1$, $L=0.5$. HLS model, like HSV model is easy to use for animators and computer artists as it comes close to the way colors are perceived by human eye. The HLS color space is illustrated in (Figure 10).

2.2.1.5 Minimal correlation spaces: PCT, WT, I1I2I3

Principle Component Transform (PCT) is also some times called the discrete Karhunen-Loeve transform. Here first the covariance matrix of the three-color feature image is obtained. The three eigen vectors of the matrix are used as the new basis vectors to transform the image into the minimally correlated color space.

In the case of whitening transform (WT) each eigen vector is scaled down by its eigen value to transform the image into a color space with each of the new color features having unit variance.

I1I2I3 color space was designed by Ohta *et al.* [5], as a linear approximation to the Karhunen-Loeve transformation after studying a number of test images of colorful out door scenes. I1, I2, I3 are represented by the following equations,

$$I1 = (R+G+B)/3$$

$$I2 = (R-B)/2$$

$$I3 = (2G - R - B)/2 \tag{9}$$

where R = red, B = blue, G = green

Figure 11 shows a Movat stained image in six different color spaces with the three features given the colors red, green, blue. This allows qualitative assessment of the suitability of a color-space for segmentation.

Figure 8 Tints, tones and shades

Figure 9 HSV hexcone color model

Figure 10 HLS double-hexcone model

Figure 11 Movat stained coronary artery section shown in six different color spaces. In each space the first feature is shown in pseudo-red, second in pseudo-green and third in pseudo-blue.

In summary, chapter two defined histological terms used in this research and described typical histological staining procedures for obtaining stained microscopy slides. This chapter also described human visual system response to colored light and the suitability of numerous color spaces to the problem of color image segmentation.

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CHAPTER 3

Image Acquisition and Preprocessing

In this chapter, image formation and adequacy of optical imaging systems is reviewed first. Optimal adjustment of microscope for image capture using Koehler illumination principles is then described. The third section covers the correction of artifacts introduced during the process of image acquisition.

3.1 Light Theory

In this section, adequacy of resolution and sampling in digital optical imaging systems is considered with quantitative image analysis in mind.

Resolution determines whether the system can adequately reproduce the small detail in the objects of interest. Resolution is directly dependent on the point-spread function (PSF) of the optical imaging system. PSF is shown schematically in Figure 12. A point source at the origin of the object plane produces a spot image at the origin of the image plane of a simple single lens imaging system.

When the point moves slightly off the Z axis to a position (x_1, y_1) , the spot or point spread image moves to a lens magnified new position (x_{1i}, y_{1i}) given by

$$x_{1i} = -Mx_1 \quad \& \quad y_{1i} = -My_1 \quad \text{where } M = \frac{d_i}{d_o} \quad \text{is the magnification of the lens} \quad (10)$$

For high quality, aberrations free lenses the PSF can be assumed to be linear and shift invariant. In optical microscopy, the light source is often incoherent and the lens has a circular aperture. In this arrangement, PSF for a perfect lens would be

$$h(r) = \left[2 \frac{J_1 \left(\pi \left[\frac{r}{r_0} \right] \right)}{\pi \left[\frac{r}{r_0} \right]} \right]^2 \quad (11)$$

Where $J_1(x)$ is the first order Bessel function of the first kind. The constant r_0 , a dimensional scale factor is given by

$$r_0 = \frac{\lambda d_i}{a} \quad (12)$$

r is the radial distance measured from the optical (Z) axis of the image plane.

λ is the center wavelength of the narrow band incoherent light.

a is the lens aperture, d_i is the image distance from lens

Figure 12 A simple single lens optical system illustrating point spread function

The Optical Transfer Function (OTF) of a system is obtained by taking the Fourier transform of the PSF. For the PSF function shown above in equation 11, OTF is given as

$$H(q) = \frac{2}{\pi - 2} \left\{ \cos^{-1} \left[\frac{q}{f_c} \right] - \sin \left[\cos^{-1} \left(\frac{q}{f_c} \right) \right] \right\} \quad \text{where } q = \sqrt{u^2 + v^2} \quad (13)$$

u and v are spatial frequencies in x and y directions. f_c is the optical cutoff frequency.

$$f_c = \frac{1}{r_0} = \frac{a}{\lambda d_i} \quad (14)$$

Thus r_0 or the inverse of the spatial cutoff frequency can be said to be the resolution limit of the lens. More exactly the first zero of the PSF given in equation 11 occurs at a radius of $1.22 r_0$. This is called the radius of Airy disk and is often used in optics for describing resolution limits.

Two point sources are said to be resolved when their image peaks are at least $1.22 r_0$ apart according to often quoted Rayleigh criterion.

Since we are concerned with microscope systems that give their lens specifications in terms of numerical aperture (NA), the resolution limit is rewritten in terms of NA.

$$NA = n \cdot \sin(\alpha) = a/2f \quad (15)$$

Where n is the refractive index of the medium in between the object and the lens. α is the angle between the optical axis and the most obtuse/marginal ray originating at the focal plane intersection with the optical axis and passing through the lens aperture a . Focal length of lens is specified as f . Assuming the image to be formed at the focal plane, the resolution limit can be written as

$$1.22r_0 = 1.22\lambda \frac{f}{a} = 1.22 \frac{\lambda}{2NA} \quad (16)$$

3.1.1 Sampling

The optical image presented to the digitizer does not have frequencies above the cutoff frequency of the PSF of the imaging lens used. Thus the continuous image can be reconstructed from the discrete samples, if the samples are taken at twice the cut off frequency according to Nyquist sampling theorem. Thus in microscopes we require a samples to be taken at a spacing of $\lambda/4NA$. [1]

3.2 Microscope arrangement

Optimal adjustment of microscope is used for achieving the best possible resolution and contrast in the microscopy images. In this section, first a brief overview of the optical components in the microscope is given. This is followed by a description of adjustment of microscope for Koehler illumination, which has several desirable properties in image acquisition.

3.2.1 Components of microscope:

The optical train in a light microscope consists of six principle components. The illuminator comprising light source, collector and field diaphragm generates the bright light required for viewing the specimen. The condenser focuses the light from illuminator onto the specimen. The thin section specimen mounted on a slide and covered with a slip is the object of interest whose magnified image is viewed by eye or a camera. The objective lens, which is well corrected for chromatic and spherical aberrations, is used for the principal magnification step. The ocular or the eyepiece is used for forming a virtual image, which can be comfortably focused by the eye. Figure 13 shows the optical train consisting of these components.

Figure 13 Components of an optical train in a light microscope

3.2.2 Adjustments

In this section, adjustment of microscope to confirm with Koehler illumination principles is described. Koehler illumination leads to optimal contrast and resolution of the captured image. It also leads to almost uniform illumination of the microscopy slide. Uniform illumination is important, when we are comparing the color of one pixel in the image to another and deciding their classification.

In Koehler illumination, all the lenses are aligned and centered along the optical axis and arranged as illustrated in Figure 14 and Figure 15. In essence, the focal adjustment for Koehler illumination consists of three steps. First the collector lens (L_c) focuses an image of the light source onto the condenser iris diaphragm (D_c). Then the condenser lens (L_c) focuses an image of the field diaphragm (D_s) in the plane of specimen. In the third step, the objective lens, the eyepiece, and the refractive elements of the eye together focus an image of the specimen on to the retina.

The high-end microscope used in our laboratory has a built-in illuminator. Thus we started adjustments from the condenser onwards. A microscope slide containing a stained section is placed on the microscope stage. The specimen view is focused using a 10X objective lens. The condenser iris diaphragm is closed down and the height of the condenser is adjusted until a sharp image of the field diaphragm is superimposed on the specimen. The condenser center is aligned as follows. The field diaphragm is closed down and the condenser iris is moved to wide-open position. Now the color fringes around the image of the field diaphragm in the specimen plane are inspected for symmetry. The two centering screws are adjusted till the colored fringes become symmetrical.

After aligning and focusing the illuminator, condenser, and the objective lens, the field and condenser iris diaphragms are adjusted. This step needs to be performed every time, the objective lens is switched.

3.2.2.1 Field Diaphragm Adjustment

The field diaphragm's main function is to regulate the flare from the illuminator. Flare is the haziness superimposed on the image. Flare is caused by unwanted multiple reflections from surfaces of lenses and the sides of microscope tubes. For direct visual observation, the field diaphragm is set so that its image appears in the field of view, just surrounding the essential area of the specimen. In our imaging arrangement, the entire field of view is utilized for image capture. Hence, the field diaphragm is adjusted until it's image is just visible at the edge of the field of view.

3.2.2.2 Condenser iris adjustment

Image resolution increases proportionately with the sum of the objective and condenser NAs. However, flare also increases as the $NA_{\text{condenser}}$ is increased by opening the condenser iris diaphragm. Thus the $NA_{\text{condenser}}$ is chosen as a compromise between image resolution and contrast. For observation of stained specimens in bright field, the $NA_{\text{condenser}}$ is set to approximately 0.9 of the $NA_{\text{objective}}$. This is achieved by setting the diameter of the condenser iris so that the image of its opening is 0.9 of the diameter of the objective back aperture in the plane of back aperture. The ratio can be different when image capture instruments with contrast enhancement capabilities are attached to the microscope.

L_s : source lens
 L_c : condenser lens
 L_{ob} : objective lens
 L_{oc1} : ocular lens 1
 L_{oc2} : ocular lens 2

f_s : front focal length of source lens
 f_c : front focal length of condenser lens
 f_{ob} : objective back focal length
 f_{oc} : ocular back focal length

D_s : source back diaphragm
 D_c : condenser diaphragm
 D_{ob} : objective back aperture
 D_{oc} : ocular mid aperture

Figure 14 Koehler illumination source planes

L_s : source lens
 L_c : condenser lens
 L_{ob} : objective lens
 L_{oc1} : ocular lens 1
 L_{oc2} : ocular lens 2
 L_e : eye lens

Figure 15 Koehler illumination image planes

3.2.3 Koehler illumination benefits [2]

The advantages of Koehler illumination in a light microscope are described below.

1. The field is homogeneously bright.
2. The working NA of the condenser and the size of the illuminated field can be regulated independently.
3. The specimen can be illuminated by a converging set of plane wave fronts as shown in Koehler illumination source planes. This gives rise to maximum lateral resolution and fine optical sectioning.
4. Image of objects at front focal plane of the condenser is formed at rear focal plane of the objective lens. This condition is needed for optimal contrast enhancement.
5. Flare, arising from the microscope optics and their barrels is minimized.

3.3 Artifact correction and Image restoration

In this section, a brief description of the image acquisition process is given followed by a description of steps taken to amiliate the effects of the distortions introduced in the image during the process of acquisition.

3.3.1 Image Acquisition

A digital image acquisition system typically comprises a light source, lenses, camera, and a computer interfaced with the camera to store and process the captured image. In this research project, a microscope is used as the light source and the lens system. A line scan charge-coupled device (CCD) camera is used for image capture. The camera was interfaced with a SUN workstation for image storage and control. Custom built software was used on the SUN workstation to control image acquisition, camera calibration and display. This image acquisition system is illustrated in Figure 16.

The imaging system was mounted on a floating table to isolate the system from mechanical vibrations. The microscope used was an Olympus BH-2 with objectives of S-plan apochromatic grade [3]. Images were normally acquired using a 10x objective lens (N.A. = 0.4) or a 20x objective (N.A. = 0.7) and a 2.5x projection lens to project the virtual image onto the camera image plane. The CCD camera attached to the microscope was an Ektron 1412 line –scan CCD camera. The CCD held 4096 elements arranged in a line 2.87 cm long. Each element was square and had a physical size of 7 μm .

The 7 μm element/pixel size mapped to 0.27 μm in the object plane when a 10x objective lens and a 2.5x projection lens were used. Since the diffraction limit (Rayleigh criterion) for visible light with a maximum $\lambda = 640 \text{ nm}$ passing through a lens system with NA = 0.4 is approximately 1 μm . (refer section 3.1), the spatial resolution of the system is not limited by the actual image element size. Similarly, the Nyquist sampling rate (see section 3.1) for minimum and maximum visible light wavelengths of 400 nm and 640 nm would be 4 pixels/ μm and 2.2 pixels/ μm respectively. The system-sampling rate was measured to be around 3.7 pixels/ μm [3]. Thus, aliasing artifact is present for wavelengths in the range of 400–430nm. Although, the more relaxed Rayleigh criterion specified a sampling rate of 1.85 pixels/ μm to be adequate for accurately conveying the information present in the analog image to the digital domain.

Figure 16 Image acquisition system

The stained tissue sections were digitized as color images using three color filters with separately controlled integration times for the CCD elements with each filter combination. The integration times were chosen so that the maximum 8 bit gray level value obtained was slightly less than 255 as a compromise between safeguarding against saturation and achieving maximum contrast. Blue filter integration time was almost double that of red or green filters. This is due to the relatively poor response of the CCD to violet light [4]. A beam splitter was used to display the image on a window mounted inside the CCD camera. This image was used to manually focus the microscope until a region of interest appeared in focus to the operator.

3.3.2 Non-uniform illumination and CCD gain artifacts

The charge-coupled device (CCD) elements used for digitizing the image give nonzero readings even when the light field was totally dark due to thermal electronic noise. This was known as dark current response. During calibration of the CCD camera, time averaged dark current reading was set as zero gray level. A blank clear slide was placed on the microscope stage and the integration times for the three color filters adjusted till the readings obtained at each CCD element are close to the maximum gray level, but never exceeding it. In the Ektron camera, the CCDs had an almost linear response to intensity levels in the bright light field range (thousands of photons). Hence, correction was not done for nonlinear response of CCDs to intensity variations. There was a small amount of spatial non-uniformity in the response of the CCD elements. The correction procedure for this is explained later in the section.

The illumination from the microscope light source is made almost uniform by following Koehler illumination principles. But unavoidable imperfections in the system such as lens aberrations, small misalignments in the optical train and lightsource nonuniformity result in residual spatial non-uniformity. This was seen on the captured image of a blank slide as roll off from a bright spot in the middle to slightly darker corners as shown in Figure 17 below. (gaussian shape).

Figure 17 Contrast enhanced blank slide image showing spatial non-uniformity as a gaussian roll off

A procedure for correcting for this spatial non-uniformity and the spatially non-uniform response of CCD camera is given below.

3.3.2.1 Illumination models

A simple overview of illumination models is given first as the correction approach can be different for different models. In the microscopy arrangement described above, the tissue is trans-illuminated (i.e. light passing through the tissue section is measured). For a trans-illuminated tissue, the light reaching the camera can be modeled as

$$f(x, y) = T(x, y) \times I(x, y) \quad (17)$$

Where $I(x, y)$ is the illumination pattern and $T(x, y)$ is the transmittance of the tissue at location (x, y) . $T(x, y)$ has the range $[0, 1]$ and is expressed as

$$T = \frac{I_{trans}}{I_{incident}} \quad (18)$$

Where I_{trans} is the light intensity transmitted and $I_{incident}$ is the light intensity incident on the tissue from below.

The tissue can also be illuminated from above to measure tissue reflectance R . In this case the $T(x,y)$ in equation becomes reflectance, $R(x,y)$.

$$f(x, y) = R(x, y) \times I(x, y) \quad (19)$$

Reflectance also has a range $[0,1]$. The reflectance of a fully transparent surface will be zero and the reflectance of a perfect polished reflective surface would be one.

Tissue light-absorbing property is represented by optical density D .

$$D = \log\left(\frac{I_{incident}}{I_{trans}}\right) = -\log(T) ; \quad 0 \leq D \leq \infty \quad (20)$$

If an optical density scanner is used for capturing images, the source illumination pattern would be superimposed on the tissue optical density as algebraic addition. In this case, subtractive correction is used to correct for the non-uniform illumination [1]. On the other hand, if one of the reflective or transitive models is used for image capture, the illumination pattern is superimposed by multiplication operation. The captured image $\hat{f}(x,y)$ is divided by the back ground image obtained by capturing a image of transparent object.

The background image is represented as $k \times I(x, y)$

Now the corrected image is obtained as

$$\hat{f}(x, y) = \frac{f(x, y)}{k \times I(x, y)} = \frac{T(x, y) \times I(x, y)}{k \times I(x, y)} = k' \times T(x, y) \quad (21)$$

3.3.2.2 Non-uniformity correction implementation

In the current research, the tissue section is mounted on the microscope stage and trans-illuminated. Thus the transmittive model is used for non-uniform illumination correction.

Let the dark current response of the CCD camera measured during calibration be $C(x,y)$.

Now, the corrected image, $f_c(x,y)$ can be expressed as

$$f_c(x,y) = \frac{f(x,y) - C(x,y)}{I(x,y) - C(x,y)} \quad (22)$$

The corrected image was normalized to 8-bit gray scale as our images were always captured at 8bits. The above discussion was for gray scale images. For color images captured with three filters (red, green, blue), the correction is repeated three times.

The same division process also achieves the correction for non-uniform gain of CCD elements. Let the non-uniform gain of CCD be $g_{nu}(x,y)$. The following equations explain the correction process.

$$f_m(x,y) = g_{nu}(x,y) \times [T(x,y) \times I(x,y) + C(x,y)] \quad (23)$$

Where f_m is the measured tissue image and $T(x,y)$ is the function being measured. $C(x,y)$ is the dark current response. The measured background image can be expressed as

$$I_m = g_{nu}(x,y) \times [k \times I(x,y) + C(x,y)] \quad (24)$$

Now, the corrected image $f_c(x,y)$ can be written as

$$f_c = \frac{f_m - C_m}{I_m - C_m} = \frac{g_{nu}(x,y) \times T(x,y) \times I(x,y)}{g_{nu}(x,y) \times k \times I(x,y)} = k' \times T(x,y) \quad (25)$$

Where $C_m = g_{nu}(x,y)C(x,y)$ is the measured dark current response.

In summary, the above analysis describes the need and procedure for removal of distortions caused by non-uniform illumination and CCD gain.

3.3.3 Imaging system PSF estimation and deconvolution [1, 5]

In this section experimental measurement of the PSF of the imaging system is described followed by a description of deconvolving the image with PSF to obtain the restored image. Assuming the system to be linear shift invariant, it can be represented as shown in Figure 18.

Figure 18 A linear imaging system with PSF denoted by $h(x,y)$

The simplest way to measure the PSF of a system would be to input an point source as $t(x,y)$ and measure the response $f(x,y)$. Then we could obtain the OTF $H(u,v)$ or the frequency domain version of PSF by taking Fourier transforms of $t(x,y)$ and $f(x,y)$

$$H(u,v) = \frac{F(u,v)}{T(u,v)} \quad (26)$$

Now the PSF can be obtained by taking inverse Fourier transform of $H(u,v)$. But, unfortunately this type of measurement is not possible for most of the optical systems and in particular bright-field microscopes.

3.3.3.1 Line targets

The next step would be to use line targets as input function $t(x,y)$. The perfect line target would be a line of infinitesimal width lying along the y-axis expressed as $t(x,y)=\delta(x)$.

The output is now given by the convolution

$$f(x,y) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} h(p,q) \delta(x-p) dp dq = \int_{-\infty}^{\infty} h(x,y) dy \quad (27)$$

The output spectrum $F(u,v)$ is equal to

$$F(u,v) = H(u,v) \delta(v) = H(u,0) \delta(v) \quad (28)$$

Thus $F(u,v)$ is the OTF $H(u,v)$ evaluated along the u-axis for line input target. If we assume circular symmetry, the complete OTF can be obtained by revolving the line-spread function around the origin.

3.3.3.2 Edge targets

Since, line targets are also difficult to obtain, edge targets which are commercially available are discussed next. Edge target can be described as a step function in the x direction times a constant in y-direction and is written as $t(x,y) = u(x)$, where $u(x)$ is the unit step function.

Since the edge function is the integral of line input, and convolution commutes with differentiation, the line spread function is obtained as the differentiation of edge spread function. Now the procedure for finding PSF described for line targets can be followed. Once the OTF is estimated as $H(u,v)$, the image $f(x,y)$ can be restored by convolving with the inverse PSF filter with transfer function $1/H(u,v)$. In practice, a pseudo inverse filter is used, where the zeros of $H(u,v)$ remain as zeros in the inverse filter. This avoids having to implement filters with infinite responses.

In this chapter, elements of optics theory required for understanding the modeling and distortion correction of the image acquisition system were first presented. This was followed by recommended microscope setup for optimal image acquisition. The third part of the chapter comprised a description of the image acquisition process, the elaboration of distortions present in the image acquisition system used and the corrective procedures adapted.

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CHAPTER 4

PREVIOUS AND CONTEMPORARY WORK IN COLOR IMAGE SEGMENTATION

Image segmentation splits an image into several perceptually distinct regions. Designing a general segmentation method to identify such regions in any given image is difficult.

Several criteria need to be satisfied in designing a good segmentation system. [1]

1. Regions of a segmented image should be uniform and homogeneous with respect to the significant features such as color and texture.
2. Regions should be simple and should not contain many holes.
3. Adjacent regions should have significantly different characteristics.
4. Boundaries of each region should be smooth and spatially accurate.

If a region is simple and does not contain many holes, it may violate the homogeneity criterion. Spatially accurate boundaries may be jagged. Stressing on large differences in feature values between adjacent regions will merge regions with smaller feature differences. Thus simultaneously satisfying all the criteria is difficult. Most segmentation implementations compromise by weighing these criteria according to their significance in specific applications. For example, in segmenting microscopy slides, regions tend to have many holes. Thus, criterion 2 is given less weight.

Color images describe each pixel location using three features compared to the single feature used by gray scale images. Making optimal use of the additional dimensionality and information is the goal of all color image segmentation algorithms.

4.1 Color segmentation in other fields

Color image segmentation implemented for natural outdoor scenes and industrial computer vision applications can be broadly divided into three groups. Feature space segmentation, context-based segmentation and physics based segmentation.

4.1.1 Feature space segmentation:

Feature based segmentation defines a partition in the feature space using clustering methods. Each pixel is assigned a label in the feature space partition. In this context, a region is defined as a connected set of pixels having the same label.

Feature space segmentation assumes that homogeneous objects in the image manifest themselves as clusters in the multi-dimensional feature space histogram. Image segmentation is accomplished by mapping the clusters back to the image domain where maximal connected components of the mapped-back clusters constitute the image regions. In one-dimensional histograms of grayscale images, clustering is accomplished by finding the valleys and defining the intervals between valleys as valid clusters. Some researchers have tried to directly extend this to color images by finding clusters separately in red, green and blue images. This approach does not utilize the information present in the simultaneous occurrence of the color features and results in sub-optimal segmentation. [2, 3]

Ohlander, Price and Reddy [3] used histogram analysis to recursively split the image into a set of regions. The recursive processing consisted of the following steps. First the

unsegmented portion of the image was selected using a bit mask. In the first iteration, this would be the whole image. Next, histograms of the selected pixels were calculated in nine redundant color features, red, green, blue (RGB), Luminance(Y), in-phase(I) and Quadrature(Q) chromatic coordinates, hue, saturation and value. The best peak formed in any of the nine histograms was selected for segmentation during current iteration. Upper and lower thresholds derived from the upper and lower bounds of the best peak were used to mark a subset of the selected pixels as belonging to one class/object. A new bit mask was formed from the remaining selected pixels. The whole process is continued till all the pixels in the image are classified. This method would fail when there are no clear peaks formed in any of the 1D histograms.

Witkin [4] proposes a method, called the Scale Space Filter (SSF), for histogram analysis, which enables the most prominent and stable peaks in the histogram to be found. Witkin denoted the gaussian convolution of a 1-D signal $f(x)$ as the scale space image of $f(x)$.

$$F(x, \tau) = f(x) * g(x, \tau) = \int f(u) \frac{1}{(2\pi)^{1/2} \tau} \exp\left[-\frac{(x-u)^2}{2\tau^2}\right] du \quad (29)$$

The scale space image of $f(x)$ is called F . F depends both on the signal independent variable x and gaussian deviation τ . Pairs of zero crossings of $F_{xx}(x, \tau)$ demarcate peaks and valleys in the function $f(x)$ at various scales represented by τ . Higher τ represents coarser scales. τ is called the scale constant. Here the subscript 'xx' denotes second partial derivative w.r.t. x . Plot of zero-crossing locations of $F_{xx}(x, \tau)$ on the scale-space was denoted as the finger print of the signal. Witkin defined an interval tree on the finger print in the

following manner. The scale space extrema at a certain scale τ partition the x-axis in to several intervals. Each of these intervals defined a rectangle in the scale-space, bounded above by the scale at which the pair of zero crossings emerged, bounded below by the scale at which the new different pair of zero-crossings appeared, and bounded on either side by the x-locations of the pair of zero-crossings. The rectangles in the interval tree with longest extent in τ direction at each x location were marked as active nodes by Witkin. An optimum scale constant τ_{opt} , where only significant peaks remained was computed from the set of active nodes. A more detailed explanation and justification for scale space was presented by Witkin *et al.* in [4] and Lindeberg *et al.* in [5].

Huang *et al.* [2] used SSF on one dimensional histograms in each of the red, green, and blue components of an color image to obtain the segmentation of image in to differently colored objects. This approach would work with gray scale images. Main drawback of this approach for color images is that the color clusters may not form clear peaks and valleys in any single one dimensional histogram. Lim and Lee [6] proposed a method of combining the peak thresholds from each of the 1D histograms in to a set of hexahedra in the three-dimensional (red, green, blue) color space. This method would not be accurate when the object clusters were aligned in a oblique direction to all the three feature axes (*i.e.* no distinct peaks can be observed).

Celenk [7] proposes a method that uses the uniform color space Lab. Image clusters are detected in cylindrical coordinates. Surfaces with constant lightness and constant chromaticity loci are used as decision elements. Each surface is obtained by using the histogram of only one of the L, H and C cylindrical coordinates of the image data. The

algorithm starts from the 1D projection histogram of the color space in a particular color component. It detects the two most prominent clusters, C1 and C2. These two clusters are projected onto the other two color coordinates and their respective 1D histograms are used to find the spatial distributions of the clusters. The estimated color distributions of C1 and C2 are projected onto the line of Fisher discriminant for 1D thresholding. The whole process is repeated until no decisive 1D peak exists.

Histogram based segmentation is well suited for unsupervised segmentation. But as this approach does not utilize the local contextual information, the regions produced are not smoothly bound and often contain numerous small holes.

4.1.2 Context-based segmentation

Statistical methods, such as the classical Bayes decision theory, have been quite popular in image segmentation. However, these methods depend on global *a priori* knowledge about the image contents and organization.

Stochastic relaxation and Markov Random Fields (MRFs) have been used in image segmentation and image restoration. MRFs provide an elegant means of specifying the interaction of neighboring pixels and embody the expected dependencies of neighboring pixels including both the prior and posterior probabilistic distributions. This local neighborhood-based specification of dependencies avoids *ad hoc* methods using global image knowledge. MRFs can be used to enforce properties such as smoothness and continuity of color regions over an entire image. The inclusion of both the prior and posterior distributions establishes a relationship between the noisy observed image and the segmentation result through Bayes rule.

Besag [8] uses the local MRFs for the reconstruction of the true scene from “dirty” pictures. The basic assumption is that pixels close together tend to have the same or similar colors. It is assumed that a non-degenerate MRF can represent the local characteristics of the true scene. A method called the Iterated Conditional Modes (ICM) is proposed to estimate the true color scene. ICM is equivalent to instantaneous freezing in simulated annealing. Although, ICM does not guarantee convergence to a global optimal solution, it is sufficient for many types of images. ICM’s rapid convergence has led to its use by many researchers.

Geman *et al.* [9] propose a statistical framework for finding boundaries and for partitioning scenes into homogeneous regions. Their model is applied mainly to the problem of texture segmentation. The model is a joint probability distribution for the array of pixel gray levels and an array of “labels”. In boundary finding the labels are binary; in partitioning, the label values are generic. The distribution incorporates a measure of disparity between certain spatial features of pairs of blocks of pixel gray levels, using the Kolmogorov-Smirnov non-parametric measure of difference between the distributions of these features. Large disparities encourage intervening boundaries and distinct partition labels. Forbidding label configurations that are inconsistent with prior beliefs, such as those defining very small regions or redundant or blindly ending boundary placements minimizes the number of model parameters. Probabilities of forbidden configurations are assigned with the value zero. The *maximum a posteriori* estimator of boundary placements and partitionings is examined. The forbidden states introduce constraints into the calculation of these configurations. Stochastic relaxation methods are extended to accommodate constrained optimization.

Huang *et. al* [2] use the MRFs in color image segmentation, and the relaxation process of using the simulated annealing and ICM are compared. Although the algorithm is used for color segmentation, each of the R, G, B components are processed individually and then combined as a segmented image.

Taxt and Bolviken [10] use single particle models from quantum physics to restore and segment images. The basic idea is to associate with each pixel I a physical system consisting of a single virtual particle with a certain amount of energy E_i and a potential function V_i between the pixel I and its neighbors. One of the main ingredients of quantum mechanics is that the behavior of such a particle is probabilistic. If its motion is confined to N_i (N_i includes pixel I and its neighbors), the position of this particle can be expressed as a probability distribution over N_i . The following restoration process is repeated iteratively. Suppose that the particle (pixel I) can move to a certain position j within N_i with probability p_{ij} , then a local restoration at pixel I has the form

$$\hat{g} = \sum_{j \in N_i} p_{ij} g_j \quad (30)$$

Where g_i is the estimated true value of pixel i .

The probabilities p_{ij} sum to 1 over all j ($j \in N_i$).

4.1.3 Physically based segmentation

Most color image segmentation methods were based on simple interpretation of color changes in an image. Hence these algorithms segmented images not only along material boundaries but also along other lines exhibiting color or intensity variations, such as

highlight and shadow boundaries, or object edges with significant shading changes. Klinker *et. al.* [11] identified this problem and presented an approach to segment surfaces with color variations due to highlights and shading. They established a model called dichromatic reflection model.

Materials were divided into two classes on the basis of their optical properties, optically homogeneous and optically inhomogeneous materials. Optically homogeneous materials

Figure 19 Schematic of light reflection at the surface of a plastic object

had a constant index of refraction throughout the material. Metals, glass, and crystals were the most common examples of homogeneous materials. Optically inhomogeneous materials were composed of a vehicle with many embedded colorant particles that differed optically from the vehicle. E.g. plastics, paper, textiles and paints.

A fraction of light incident upon the surface of an object was reflected. (Figure 19) The part of the reflection emanating from the interface in the direction such that the angle of incidence equals the angle of reflection was denoted as surface reflection. The fraction of the incident light that was reflected after traversing through the body of the object was labeled body reflection.

The Dichromatic Reflection Model (DRM) described the total reflection R , which was the amount of light reflected from the surface of a dielectric and non-uniform material, as a linear combination of the surface reflection and body reflection. The reflected light $L(\lambda, g)$ was expressed as

$$L(\lambda, g) = m_s(\lambda)C_s(g) + m_b(\lambda)C_b(g) \quad (31)$$

Where λ was the wavelength, g was the geometric factor, m_s and m_b were spectral power distributions of the surface reflection and body reflection, respectively. C_s and C_b were geometric scale factors. The points that were in the surface reflection areas were called high light points. The remaining object points were named matte points, the intensity of which was determined mainly by the body reflection process. Figure 20 shows the shape of the corresponding spectral histogram in the dichromatic plane. The combined spectral cluster of matte and highlight points resembled a skewed-T.

The segmentation method using DRM alternated between generating optical hypotheses from local image data and verifying whether the hypotheses fit the image. First, a linear hypothesis that corresponded mainly to matte areas was generated and exploited to segment the image coarsely. A planar hypothesis including the skewed-T feature was used to refine the segmentation into matte and highlight regions.

Figure 20 Spectral cluster on the dichromatic plane spanned by C_s and C_b .

Healey [12, 13] used the DRM and the normalized color space in performing color image segmentation. First he used conventional discriminant functions to segment the image, then the DRM was applied to examine whether or not a region corresponded to a highlight region. If it was a highlight region, it was merged into its neighboring regions. Healey admitted that the normalized color corresponding to a point in shadow would be different from the normalized color of a directly illuminated point of the same material. Thus normalized color still depended upon object geometry. This reduced its utility in creating geometry invariant segmentation.

Although DRM worked well with few inhomogeneous dielectric materials, it made several simplifying assumptions that restricted its application to general segmentation. For example, a single point source with a single spectral distribution was assumed to illuminate the imaging scene. Images of stained histological slides scanned with a microscope did not satisfy these assumptions. Hence this approach could not be pursued in current research.

4.2 Review of Color image segmentation in the study of histological section

Most of the recent applications of color image segmentation in histology appearing in current literature tend to be thresholding based, although few exceptions such as Sun *et al.* [14] and Umbaugh *et al.* [15] took the clustering approach.

4.2.1 Thresholding Methods:

Willmense *et al.* [16] have studied the use of color image processing in quantification of PS2 (an estrogen related protein with prognostic importance) and epithelial cell percentage in breast carcinomas. Breast tumor tissue was stained immunohistochemically for PS2 using a monoclonal antibody (specific to PS2). PS2 protein stained red. With each batch of slides, a control section of normal gastric mucosa was included. Willmense *et al.* used VIDAS 2.0, a commercial image analysis system for evaluating the percentage positive stained area on each slide. After digitizing images of tumor containing portions of the slide with a camera attached to the microscope, segmentation of PS2 positive areas was prepared using fixed preset thresholds in red, green and blue. The selection of threshold was performed by picking representative PS2 positive points from the control slide. The software calculated thresholds based on sample distribution in red green and blue.

For quantifying epithelial cell percentage in tissue sample, the author used an Azan stain. Azan stains epithelial cells red and stromal parts blue. Color segmentation was once again performed using fixed thresholds in red, green and blue. Epithelial cell percentage was used with PS2 fraction obtained from biochemical assay of tumor tissue as a whole to achieve a measure of epithelial PS2 expression.

This method shows the use of color image processing in enhancing the inference from biochemical assay. But the fact that authors did not use PS2 fraction obtained from image analysis shows their lack of confidence in accuracy of the method. Also using fixed thresholds can lead to error due to stain inconsistencies and variations in tissue composition which result in color dispersion on the slide.

Goto *et al.* [17] studied the relation between presence of abnormal H antigen and nuclear morphometry in colorectal adenocarcinomas. They used a monoclonal antibody C12 with DAB to stain for the antigen and hematoxylin(blue) for the nucleus. They converted RGB scanned images of the slides in to H (hue), S(saturation), I(intensity) color space. By plotting samples from DAB stained area, hematoxylin covered area and white background samples in HS space a chromaticity diagram was obtained. Since the three classes were well separated in chromaticity diagram, rules for separation of the classes were calculated in HS space and applied to segment the scanned images. After removing the C12 positive area, a density thresholding method was applied to obtain the nuclear cytoplasmic ratio. The color image analysis system used in this study was MAPPS-II. This system appears to assume total tissue color invariance and is very case specific.

Sandra *et al.* have performed a quantitative study of soft palate morphology using trichrome stained serial sections with the aim of aiding construction of soft palate biomechanical models. American Innovision's videometric 150 system was used for acquisition of color images from the slides and the subsequent interactive threshold based segmentation in to different tissues. Tendon, glandular tissue, connective tissue, muscular tissue and adipose tissue were segmented out from the image by interactive

thresholding and high lighting. The percentage area of each tissue type in the sections was used to arrive at the overall composition of the palate. Interactive thresholding is dependent on user.

Shiraishi *et al.* [18] studied the changes in volume of SA nodal cells during growth and aging using serial sections and a digital color image analyzer. Masson's trichrome stain was used to differentiate the SA nodal cells from collagen, elastin fibers, and other connective tissue components. Color images were taken with a video camera mounted on a microscope. The region of SA node was extracted on each image with a digitizer. A color range specific to SA nodal cell was picked with a digitizer and used for segmenting out all the SA nodal cells. Similarly, connective tissue was segmented after picking up samples with digitizer. The pixel numbers from all the serial sections were then added to get the volumes.

Masashi *et al.* performed an immunohistochemical study of cell composition of coronary and aortic atherosclerotic lesions in rabbits using a color image analyzer. Serial sections of coronary and aortic lesions were immunohistochemically stained with a muscle actin specific monoclonal antibody for smooth muscle and RAM-11 stain for macrophages. Both stains gave black reaction products and were thus performed on separate sections. Conventional staining such as Azan-Mallory was also performed to segment out blue staining collagen and white elastin. Histogram thresholding was used for classification by the color image analyzer (SP-500, Olympus Co).

Poston *et al.* [19] have performed a quantitative study of the expression of intra cellular adhesion molecule-1 (ICAM-I) on endothelial cells, macrophages, and smooth muscle cells

of the atherosclerotic plaque using immuno-histochemical staining. Sight Systems color image analyzer was used for quantitation of ICAM-I expression. This color analyzer interactively adjusted the thresholds in HIS color space to detect almost all of the staining.

Shapiro *et al.* [20] compared the staining properties of anti-desmin and anti-actin on prostate smooth muscle to determine the optimal antibody for quantifying the smooth muscle content of the human prostate. Prostate biopsy sections were stained with either anti-desmin or anti-actin and their activity was developed by DAB and peroxide as dark brown. Epithelium was stained red using alkaline phosphatase fast red chromogen. Finally tissue sections were counterstained with hematoxylin. Color assisted quantitative image analysis of the stained slides was done by Bioquant image analysis system. After setting the thresholds for identifying the tissue components in the prostate, area percentages of smooth muscle, fibrous tissue, glandular epithelium and glandular lumina were determined.

Defigueiredo *et al.* [21] applied color image analysis to quantification and sub type classification of the number of senile plaques in post-mortem Alzheimer's disease tissue. B-amyloid protein present in all senile plaques was stained brownish red and PHF-1 positive dystrophic neurite plaques were stained blue using specific immunohistochemical labels. Color images of the slides were captured using a video camera attached to a microscope with a color correction filter. Images were then converted from RGB to HSV space. Thresholds for dark brown amyloid, bluish PHF-1 dystrophic neurite, and light blue background pixels were semiautomatically calculated from hue and saturation histograms. Hue histogram thresholds were used to separate amyloid pixels from rest of the tissue. Saturation histogram was then used to separate blue PHF-1 positive tissue from blue tinged

background. Segmented plaques were then used to count number of plaques in each subtype. The authors acknowledge that their algorithm fails to separate two touching plaques, leading to error between manual counting and automated counting.

4.2.2 Histogram based clustering methods:

Sun *et al.* [14] segmented H&E stained liver tissue images into nuclei (blue), cytoplasm (purple), fat containing cells or background (white) and red cells (red) using a two tier procedure. They converted RGB image in to I1, I2, I3 color space to separate intensity from chromatic information. I1 corresponds to intensity. Intensity image was histogram normalized to make it invariant to illumination changes. Iterative probabilistic relaxation was used on intensity image to separate dark classes from white classes. In the second step, color information present in I2 and I3 was used to separate dark class into nuclei and red cells and the white class into cytoplasm and fat containing cells. A supervised Bayesian classifier was used for the second stage. In this method a set of samples are obtained manually for each class and used to model the class as a mixture of gaussian distributions. The automated segmentation results were in 90% agreement with the manual estimation of the four classes.

The two step process reduces the complexity of the segmentation problem from three dimensions to one and two dimensions. Separation and histogram normalization of intensity was a efficient way to remove effect of illumination changes. Probabilistic relaxation assumes uniform probabilities of white class pixels occurring next to dark class pixels, etc. and thus introduces errors in initial segmentation. Also the iterative relaxation computation is slow. Modeling the tissue color distribution as a mixture of gaussian

distributions means careful preview of slides for deciding how many gaussian distributions each class contains. Even then, gaussians may not correctly model the tissue color distribution. Using sampled points for constructing the gaussian makes the segmentation process subjective.

Umbaugh *et al.* [15] have made a interesting study of efficacy of unsupervised color image segmentation algorithms in detection skin tumor borders. The border finding algorithm starts with digitization of color photos of skin tumor. This is followed by number of empirical steps to remove noise and non-skin artifacts. Color segmentation was then used to segment out the tumor from normal skin. Six different algorithms namely, adaptive thresholding, Fuzzy C-Means, spherical coordinate transform/center split, principle component transform/median cut, split and merge segmentation, and multi resolution segmentation were used for color segmentation. This was followed by morphological operations for skin tumor boundary detection. Principle component transform followed by median cut algorithm gave the least deviation of border from the contour drawn by a dermatologist.

In this chapter, three different types of algorithms used for segmentation of color images of natural and industrial scenes were reviewed. These reviews were followed by an overview of the segmentation methods currently used in quantitative histology.

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CHAPTER 5

COLOR SEGMENTATION ALGORITHMS

The first section of this chapter describes the implementation of a semi-interactive algorithm. This algorithm labels clusters in the three feature histogram obtained from the image. The next section describes the implementation of three unsupervised clustering algorithms for automated separation of tissue clusters in the three feature histogram. The third section discusses the inclusion of contextual information in to the classification process. In particular, iterative conditional modes and modified split and merge algorithms are elaborated.

5.1 Semi interactive segmentation

The semi interactive cluster labeling algorithm was developed as a first step towards complete automation of tissue component separation problem [1]. This approach utilized the large color differences of tissue components achieved by histochemical staining and the tendency of pixels covered by same stained tissue component to form clusters (have similar colors) in color space.

First the image to be segmented was converted to HSV color space. Then the three dimensional histogram was calculated. Typically 8 bits were used for each color feature resulting in a 256^3 voxels(sub volumes/bins) in the 3D histogram. Three projected or accumulated views of the histogram were shown along the three principal axes. (Figure 21, Figure 22, and Figure 23).

Differently stained tissues appeared as separate clusters in the three views of the 3D histogram. The colors were well separated along hue axis (*i.e.* hue value is directly dependent on the color pigment). Tissue clusters were enclosed in hexahedra interactively by constraining in two dimensions in one accumulated view and using a second orthogonal direction to specify the constraints in the third dimension. Hexahedra were allocated pseudo colors based on the tissue component they were enclosing (Figure 23). The classified image resulting from the above cluster labeling was displayed on the screen (Figure 24). The user would then adjust the boxes to obtain the desired segmentation results. Comparison between original image and classified image could be made by clicking on a flicker button which switched from one image to the other. Figure 24 shows the original and segmented 'ORO' stained images.

As described in chapter two, the choice of color space is of critical importance to image segmentation. HSV color space was chosen for the classification for two reasons. Qualitative comparison of histograms of ten test images in RGB, HSV, HIS, I1I2I3, and Lab color spaces showed that HSV color space had the greatest tissue cluster separation. The tissue clusters were obliquely oriented in all of the color spaces. The clusters were aligned along the axes in HSV color space as hue and saturation are color pigment specifying parameters. This makes the task of interactively enclosing the clusters relatively easy. From the test images, it was also observed that the tissue clusters occurred in the same location of the color space. But the tissue clusters shifted slightly from image to image. Figure 25 illustrates the suitability of HSV color space by displaying three dimensional histograms of a CD68 immuno stained tonsil section in RGB and HSV color spaces. In

Figure 25. RGB histogram shows a single cluster and HSV histogram shows two distinct clusters. Thus HSV lead to better separation of brown(DAB) and blue(hematoxylin) clusters.

This interactive system was used to classify hundreds of 'ORO' stained coronary artery sections by slightly adjusting the tissue cluster enclosing boxes from image to image. Chapter 7 describes this application in detail.

Figure 21 Warping HSV hexagonal cone model to cube model for easier comprehension

Figure 22 Schematic of accumulated view of HSV color space histogram of an image along hue axis.

Figure 23 Labeled clusters in HSV color space histogram

Figure 24 Segmentation of 'ORO' stained coronary artery cross section image using labeled histogram.

Figure 25 RGB and HSV color space histograms of DAB stained cd68 tonsil section image.

5.2 Adaptive segmentation methods

Small variations in tissue component colors were noted from image to image in the implementation of the above semi interactive scheme. Tissue characterization studies described in section 6.1 also have shown that the cluster centers shifted from image to image. Thus we embarked on a quest to design and implement optimal automated adaptive segmentation schemes. Three adaptive clustering algorithms were implemented in slightly modified forms to optimally separate tissue components on microscopy slides. They were competitive learning, fuzzy C-means and median split algorithms. For all the three methods, the number of tissue components present was presumed to be known. In most of the microscopy slides, the number of stains (i.e. color pigments) used for preparing the slide is known. Thus the number of tissue components on a particular slide can be said to be the number of stains plus two. One class is added for each of the two unaccounted tissue components, namely unstained tissue and white background.

5.2.1 Fuzzy C-means algorithm

In this section, a formal definition of fuzzy C-partition is stated first [2]. This is followed by step wise description of fuzzy c-means algorithm [3] which minimizes the fuzzy c-means functional J_m .

Let R^p denote p dimensional real feature space. The elements of R^p are denoted as feature vectors x . Each vector x is a set of p real numbers $= (x_1, x_2, x_3, \dots, x_p)$.

Let X be a subset of R^p . A function $u: X \rightarrow [0, 1]$ is said to assign to each $x \in X$ its grade of membership in the fuzzy set u . The function u is called a fuzzy subset of X .

Fuzzy partitioning of X is achieved by defining several fuzzy sets on X such that for each $x \in X$, the sum of the fuzzy memberships of x in the fuzzy subsets is one. This is stated more formally below.

Given a finite set $X \subseteq R^p$, $X = \{x_1, x_2, \dots, x_n\}$ and an integer c , $2 \leq c \leq n$, a fuzzy C-partition of X can be represented by a matrix $U \in W_{cn}$ whose entries satisfy the following conditions.

- 1) Row i of U , say $U_i = (u_{i1}, u_{i2}, \dots, u_{in})$ exhibits the i th membership function (or i th fuzzy subset) of X .
- 2) Column j of U , say $U^j = (u_{1j}, u_{2j}, \dots, u_{cj})$ exhibits the values of the c membership functions of the j th datum in X .
- 3) u_{ik} shall be interpreted as $u_i(x_k)$, the value of the membership function of the i th fuzzy subset for the k th datum.
- 4) u_{ik} is characterized by the following conditions

$$\sum_i u_{ik} = 1 \quad \forall k; \quad \sum_k u_{ik} > 0 \quad \forall i; \quad \sum_k u_{ik} < n \quad \forall i; \quad (32)$$

As defined above, let U be a fuzzy C-partition of X , and let v be the set of c vectors $(v_1, v_2, \dots, v_c)$, with $v_i \in R^p$ being the initial center for class i , $1 \leq i \leq c$. Now, the fuzzy C-means functional J_m is defined as

$$J_m(U, v) = \sum_{k=1}^n \sum_{i=1}^c (u_{ik})^m (d_{ik})^2 \quad \text{where} \quad d_{ik}^2 = \|x_k - v_i\|^2 \quad (33)$$

$\|\cdot\|$ can be any inner product norm, and $m \in [1, \infty)$.

From the above equation, it is apparent that J_m is a squared error criterion, and its minimization produces fuzzy clusters (matrix U) that are optimal in a generalized least squared error sense.

The fuzzy C-means algorithm (FCM) arrives at a fuzzy C-partition of dataset X by iteratively finding the local minimum of J_m . The algorithm is described in steps below.

- 1) Fix the number of clusters c , $2 \leq c < n$. Fix m , $1 < m < \infty$.
- 2) initialize the c cluster centers $\{v_i^{(0)}\}$
- 3) at step b , $b=0,1,2,\dots$

calculate the partition $U^{(b)}$ as follows. For $k=1$ to n ,

- a) calculate I_k and $\tilde{I}_k$:

$$\begin{aligned} I_k &= \left\{ i \mid 1 \leq i \leq c, \quad d_{ik} = \|x_k - v_i\| = 0 \right\} \\ \tilde{I}_k &= \{1, 2, \dots, c\} - I_k \end{aligned} \quad (34)$$

- b) for data item k , compute membership values:

- i) if $I_k = \emptyset$,

$$u_{ik} = \frac{1}{\sum_{j=1}^c \left(\frac{d_{jk}}{d_{ik}} \right)^{\frac{2}{m-1}}} \quad (35)$$

- ii) else $u_{ik} = 0$ for all $i \in \tilde{I}_k$ and $\sum_{i \in I_k} u_{ik} = 1$

next k .

- 4) update the c cluster centers $\{v_i^{(b+1)}\}$ given $U^{(b)}$

$$v_i = \frac{\sum_{k=1}^n (u_{i,k})^m x_k}{\sum_{k=1}^n (u_{i,k})^m} \quad (36)$$

- 5) Compare $U^{(b)}$ and $U^{(b+1)}$ in a convenient matrix norm; if $\|U^{(b)} - U^{(b+1)}\| < \varepsilon$, stop; otherwise set $b=b+1$, and go to step 3).

The algorithm is shown as a flowchart in Figure 28. The current implementation differs from the one used in [3] in that color space histogram voxels were used as data element set X rather than the image pixels. This saves computation time as $1k \times 1k$ image has 1 million pixels and a 64^3 3D histogram has $\frac{1}{4}$ million voxels. Also the histogram is sparsely distributed (i.e. many of the voxels are empty and thus are not used in computation) further reducing the amount of computation. The results obtained by using fuzzy C-means algorithm are illustrated in chapter 6.

5.2.2 Competitive learning algorithm

Competitive learning has been used for vector quantization by Kohonen *et al.* [4]. Uchiyama *et al.* [5] used competitive learning as a tool for clustering based on the least sum of squares criterion. In this research, competitive learning was chosen as it yields an approximate global optimal solution to the clustering problem and can be implemented without the provision of initial cluster centers. Clustering based on least sum of squares criterion is explained in the next few paragraphs followed by a description of the competitive learning algorithm.

Let $X_j (j = 1, \dots, m), C_i (i = 1, \dots, n), W_i (i = 1, \dots, n) (m > n)$ be a finite number of input vectors ($= \chi$), clusters ($= C$), and weight vectors ($= W$), respectively. Figure 26 shows weight vectors and clusters.

Figure 26 Schematic of Voronoi tessellation and clustering

Partition function $C_i(W)$ is defined as the Voronoi tessellation determined by weight vector W sketched in Figure 26. Mathematically this can be stated as

$$X_j \in C_i \text{ iff } \forall k (k \neq i) \|X_j - W_i\|^2 < \|X_j - W_k\|^2 \quad (37)$$

The weight vector function $W_i(C)$, is defined as center of mass in cluster C_i .

$$\bar{\mu}_i = \frac{\sum_{X_j \in C_i} p_j X_j}{\sum_{X_j \in C_i} p_j}, \quad W_i(C) = \{\bar{\mu}_i | i = 1, \dots, n\} \quad (38)$$

Where p_j is the probability of X_j . In current implementation, $p_j = 1/m$.

Let the expected squared-error distortion be defined as

$$D(C, W) = \sum_{i=1}^n \sum_{X_i \in C_i} p_i \|X_i - W_i\|^2 \quad (39)$$

Now, given the number of clusters n and the data set X , the clustering satisfying least sum of squares criterion is the set of clusters C that minimizes the within cluster sum of squares S_w defined as

$$S_w(C) = D(C, W_i(C)) = \sum_{i=1}^n \sum_{X_i \in C_i} p_i \|X_i - \bar{\mu}_i\|^2 \quad (40)$$

Minimizing the within-cluster sum of squares is equivalent to maximizing between-cluster sum of squares S_B as shown below. Defining

$$S_T = \sum_{i=1}^n \sum_{j=1}^m p_j \|X_i - \bar{\mu}_0\|^2 \quad \text{and} \quad S_B = \sum_{i=1}^n \sum_{X_i \in C_i} p_i \|\bar{\mu}_i - \bar{\mu}_0\|^2 \quad (41)$$

where $\bar{\mu}_0 = \frac{\sum_{j=1}^m p_j X_j}{\sum_{j=1}^m p_j}$ is the global center of mass of X .

It is apparent that $S_T = S_w + S_B$

Minimization of S_w is equivalent to maximization of S_B as S_T is independent of C, W . Thus, least sum of squares criterion gives the most separable clustering.

The number $S(m, n)$ of possible partitioning C of length n of the m input vectors X is given by

$$S(m, n) = \frac{1}{n!} \sum_{i=1}^n (-1)^{n-i} \binom{n}{i} i^m \quad (42)$$

(see reference [5]). Since, this is a huge number of possible solutions to search through, it would not be practical to find an optimal solution by exhausting all the possible

partitions. The competitive learning algorithm is used as the means for finding approximately optimal solution by iteratively restricting the search. The algorithm is described in steps below using the following notations. N_R is the number of repetitions, N_{max} is the maximum number of repetitions, θ_i is the threshold of number of wins, and γ is the learning rate for the algorithm. The data set being clustered in current application being the pixels in the image.

- 1) Initialization: One unit is set up, with the weight vector W initialized to $\bar{\mu}_0$. The unit has an associated variable *wincount* that is initialized to 0. N_R is initialized to zero.
- 2) Competitive learning
 - a) one pixel color vector x is selected randomly from X using a uniform random distribution. The unit that is closest to x in the squared Euclidean distance $\|x - W_i\|^2$ is chosen as the winner W_{winner} . (If there is a tie, one unit is selected at random.)
 - b) Update the weight vector of winner W_{winner} by $\Delta W_{winner} = \gamma(x - W_{winner})$. Add 1 to *wincount* of winner.
 - c) If a winner's *wincount* = θ_i and number of units < n , then generate a new unit with the same W as the winner and clear the *wincount* of both to 0.
 - d) Add 1 to N_R . If $N_R = N_{max}$, then stop competitive learning phase, else continue competitive learning from a).
- 3) All the pixels in the image are assigned to the clusters with centers/weight vectors closest to their respective color vectors in the Euclidean sense.

The algorithm is shown in flow chart form in Figure 27. Using the mean of the image as the weight vector of the initial unit and splitting the units whenever they acquire too many winners as in 2 c) prevents any single unit from monopolizing all of the input pixels. In traditional competitive learning, desired number of units are created at the initialization stage itself and this could lead to single unit dominance and result in sub-optimal classification.

N_{max} should be adequate to guarantee generation of the desired n units by splitting. In the worst case, the n th unit will be generated when

$$N_R = (2(n-1)-1)\theta_i = (2n-3)\theta_i, \quad (43)$$

Thus, N_{max} should satisfy the relation

$$N_{max} \geq (2n-3)\theta_i, \quad (44)$$

to guarantee presence of n clusters in the final partition.

In the current implementation, empirical values recommended by Uchiyama *et al.* [5]

$$\theta_i = 400\sqrt{n}, \text{ and } N_{max} = (2n-3)\theta_i (n+7) \quad (45)$$

are used. Although Uchiyama used a constant learning rate, a hyperbolic learning rate was used in the current implementation. Yair *et al.*[6] have shown that hyperbolic learning rate yielded a constant weightage to the training data acquired throughout the learning process and thus was optimal.

5.2.3 Median split algorithm [7]

This method was popularized by Heckbert [7] for color quantization (i.e. for compressing 24 bit color images into 8 bit pseudo colored images). In this method there are two principal steps. In step one, the variance of the color space histogram/ sub volumes of

histogram are calculated. In step two, the histogram sub volume with maximum variance is split along the axis with maximum variance. The two steps are repeated till the required number of clusters are built. This is equivalent to trying to achieve the clustering with minimal within cluster variance using only rectangular box shaped clusters. Figure 29 shows the algorithm in flow chart form.

Figure 27 Competitive learning algorithm flowchart

Figure 28 Fuzzy C means algorithm flow chart.

Figure 29 Median split algorithm flow chart

The application of the above three algorithms to differently stained microscopy slide images is illustrated in chapter 6. The best segmentation achieved with any of the above algorithms was qualitatively determined to be around 85%. One reason for this could be tissue composition changes and changes in color pigmentation from one location on the image to another. Thus using contextual information or information about the pixel neighborhood should increase the accuracy of segmentation.

5.3 Context based adaptive segmentation using gaussian Markov random field models

In this section, two approaches for including the contextual information are described.

In the first approach, Gibb's random field (GRF) is used to impose a spatial connectivity constraint on the initial segmentation achieved using one of the above algorithms[8]. An in depth discussion on GRF can be found in Besag and Geman *et al.*[9, 10]. The image segmentation problem can be modeled as maximizing the probability of obtaining class label set $\mathbf{x}$ from observed image data $\mathbf{y}$. The posterior probability of obtaining $\mathbf{x}$ given $\mathbf{y}$ can be written as:

$$p(\mathbf{x} / \mathbf{y}) \propto p(\mathbf{y} / \mathbf{x})p(\mathbf{x}) \quad (46)$$

where $p(\mathbf{y}/\mathbf{x})$ is the conditional probability of the observed data given the segmentation. $P(\mathbf{x})$ is the *a priori* probability term used to model the spatial connectivity constraint using GRF.

$$p(\mathbf{x}) = \frac{1}{Z} \exp\left[\frac{-U(\mathbf{x})}{T}\right] \quad (47)$$

Where Z is the normalizing constant, T is the temperature constant, and $U(\mathbf{x})$ is Gibb's energy defined by

$$U(\mathbf{x}) = \sum_{c \in C} V_c(\mathbf{x}) \quad (48)$$

Where C is the set of all pair wise cliches in $\mathbf{x}$ and V_c is the individual cliché potential. *I.e.* We considered only eight nearest neighbors (second order neighborhood) for each pixel. Figure 30 illustrates neighborhood orders and cliches. Spatial connectivity was modeled by making

$$V_c(i, j) = \begin{cases} -\beta & \text{if } x_i = x_j, \\ \beta & \text{otherwise} \end{cases} \quad (49)$$

Where β is a positive number. As β is increased, probability of pixel pairs with same label increases and the probability of pixel pairs with differing labels decrease. This leads to formation of spatially connected regions in the image.

Now the conditional probability $p(y/x)$ can be modeled using the popular gaussian distribution as

$$p(y / x) \propto \exp\left[-\sum (y - \mu_x)^2 / 2\sigma^2\right] \quad (50)$$

Where μ_x is the mean of class x .

Iterative conditional modes (ICM) algorithm developed by Besag *et al.* [9] is used to obtain a computationally efficient sub-optimal solution to the probability maximization problem. In this algorithm, the class means μ_x and segmentation x are estimated in alternating steps to maximize the probability modeled by equation 15. Besag had looked at the empirical results of increasing β in linear steps with each iteration and also at using constant β . In the current implementation, numerical optimization procedures are used to find the β that maximizes the probability of the segmentation x at each iteration. This adds a modest amount of computation to each iteration. The result of using ICM to enhance the image segmentation is shown in Figure 31.

1st order

2nd order

5th order

Pixel neighborhoods

a)

b)

c)

d)

e)

Pixel cliches

Figure 30 Illustration of neighborhoods and cliches used in Markov random field image modeling.

a)Original b)Lab image c)Four class segmentation d)Enhancement using ICM

Figure 31 Pancreas islet segmentation enhancement using iterative conditional modes algorithm (ICM)

The second approach is a modification of the classical split and merge method. The method comprises two phases. In the first phase, the image is split into uniform regions. In the second phase, the gaussian Markov random field (MRF) model is used for merging similar adjacent regions minimizing the decrease in estimated pseudolikelihood function. This method was used by Liu *et al.*[11] to segment color images of outdoor scenes. Liu attempted to use simulated annealing, but found that a constant temperature of zero (i.e. no annealing) gave the best segmentation results. Healey *et al.*[12] enhanced the method to

include second order color texture parameters and used it for detecting color textures. Healey also made few simplifying assumptions to reduce the computation. The current implementation is a adaptation of Healey's method.

Without loss of generality, assume the segmentation is performed in RGB color space. In the first phase, image is first divided into blocks of 16X16. Each block is further divided in to smaller regions, if it does not pass the uniformity criterion. An image block S is assumed to be uniform, if each component of its estimated color mean $\mu_S = [\mu_R, \mu_G, \mu_B]$ differs from estimated color mean components of it's four sub blocks by less than a threshold ϵ_μ and if each component of its estimated covariance matrix $E = \left\{ [R - \mu_R, G - \mu_G, B - \mu_B]^T [R - \mu_R, G - \mu_G, B - \mu_B] \right\}$ also differs from the corresponding elements of estimated covariance of the sub blocks by less than a threshold ϵ_σ .

The second phase is described briefly. Detailed description can be found in Panjwani *et al.* [12]. Let a region S be described by it's estimated GMRF parameters (θ, Σ) . Let the image consist of regions $S_1, S_2, \dots, S_q$. If S_k and S_l are two spatially adjacent regions with GMRF parameters $(\theta_{,k}, \Sigma_{,k})$ and $(\theta_{,l}, \Sigma_{,l})$. Let S_M be the new image region obtained by merging S_k and S_l and let S_M be modelled by $(\theta_{,M}, \Sigma_{,M})$. Let $P_{ps}(S | \theta, \Sigma)$ be the pseudolikelihood of the region S given parameters (θ, Σ) . Pseudolikelihood can be viewed as the estimated probability of the region existence. The ratio R_{ps} of the pseudolikelihood of the image before merge and after merge simplifies to

$$R_{pv}(k,l) = \frac{P_{pv}(S_k | \theta_{,k}, \Sigma_{,k}) P_{pv}(S_l | \theta_{,l}, \Sigma_{,k})}{P_{pv}(S_M | \theta_{,M}, \Sigma_{,M})} \quad (51)$$

If $h(k,l)$ is defined as $\ln(R_{pv}(k,l))$, it can be simplified as

$$h(k,l) = \frac{(D_k + D_l)}{2} \ln(|\Sigma_{,M}|) - \frac{D_k}{2} \ln(|\Sigma_{,k}|) - \frac{D_l}{2} \ln(|\Sigma_{,l}|) \quad (52)$$

where D_k and D_l are number of pixels in regions S_k and S_l . Minimizing $R_{pv}(k,l)$ is equivalent to minimizing the log-likelihood ratio $h(k,l)$. Thus, at each iteration of the algorithm two adjacent regions S_k and S_l are merged if $h(k,l) = \min_{i,j} h(i,j)$ leading to minimal decrease in the pseudolikelihood of the whole image. Merging is stopped when the minimum likelihood ratio for merging exceeds a threshold T . Segmentation of a Movat stained coronary artery using this method has given promising results as shown in Figure 32. But the computation took 20 minutes on a SGI Indy workstation for a 300x300 image. The computation has to be reduced at least one order of magnitude for the segmentation to be at a viable rate. Hence this context based method was not considered in the validation and application stages of this research.

Figure 32 GMRF based region segmentation of 300X300 Movat stained coronary artery cross section image.

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CHAPTER 6

COMPARISON OF ALGORITHMS AND VALIDATION

In this chapter, the first section describes the tissue component color distribution characterization in four frequently used staining procedures. The second section uses the tissue characterization performed earlier in modeling a synthetic image. The synthetic image is used for testing the performance of three adaptive segmentation algorithms described in chapter 5. The third and final section of this chapter explores qualitative and quantitative comparison of the three algorithms. In this section, observations on computation time and reliability were also made.

6.1 Tissue characterization using test image sets

In this section, four test sets of optimally stained tissue sections were characterized. The four stains chosen were immuno staining using DAB as a chromogen, and routine stains such as Movat pentachrome, Picrosirius red and Hematoxylin and Eosin (H&E). Images of tissue sections were taken in areas of slides with homogeneous staining and maximum contrast selected by visual panning as the main purpose was characterizing the tissue color. The images were then corrected for image acquisition distortions and segmented into appropriate number of classes using the competitive learning algorithm. The mean color and 3x3 covariance matrix was calculated for each class in each image. Color distribution parameters such as mean and

standard deviation (S.D.) were obtained for each class by taking the average over the set of ten images. The segmentation and parameter calculation was performed in two color spaces namely, RGB and HSV.

6.1.1 immuno stained tonsil sections

In this test set, tonsil sections immuno-stained for CD68, an antigen found on macrophages, were used. The images were captured using a 20x objective lens on the microscope giving a total magnification of 200. Each pixel of the captured image covered an area of $0.1225 \mu\text{m}^2$ on the microscopy slide. Ten images were obtained using custom engineered software, which allowed the calculation of optimal contrast and focusing. The images were classified in to four classes using the competitive learning algorithm. The classes were background white (weak saturation), DAB brown (strong saturation), blue hematoxylin (strong saturation), and unstained tissue (weak saturation). Figure 33 shows the RGB color space classification and Figure 34 shows HSV color space classification. Mean and standard deviation for each class in each of the ten images were measured. Figure 35 shows the standard deviations (S.D.) of DAB brown color pigment distributions in the image set. The S.D. of the three features red (R), green (G) and blue (B) have an approximate value of 20, when the features were measured using 8-bits (0-255 range). Figure 36 shows the S.D. of hematoxylin blue color pigment distributions in the image set. The blue feature has higher S.D. compared to green and red features and all the three S.D. oscillate around 15. From Figure 37 it can be observed that S.D. of DAB brown class were lower in hue(H) compared to saturation (S) and value (V). This implies that DAB brown pigment has a consistent hue with small variation and more varied intensity and saturation features. Figure

38 shows that S.D. of hematoxylin blue class is higher in hue compared to saturation and value. This could imply that the blue class picked up some of the surrounding “unstained tissue” causing large variation in hue.

The average parameters from the ten images listed in

Table 2 were used to represent (characterize) the class distribution.

Color	Component	Mean (Standard Deviation)		
		Red	Green	Blue
	DAB	126 (22)	92.4 (20.8)	80.8 (21.8)
RGB	Hematoxyli	159.6 (13.4)	144.6 (12.1)	158.4 (16.4)
	tissue	189 (11.2)	178 (10.7)	186 (11.3)
	Background	218 (9.62)	210.1 (10.6)	210.9 (10.4)
		Hue	Sat	Val
	DAB	11.3 (7.93)	97.9 (25.1)	134.2 (26.8)
HSV	Hematoxyli	199.9 (16.4)	34.2 (10.9)	165.6 (10.7)
	tissue	217 (22)	30 (10.9)	189.1 (8.8)
	Background	247.8 (28.6)	16.5 (12.4)	215.9 (10.6)

Table 2 DAB immuno stain tissue component parameters obtained from a set of ten images

CD68 immuno stained tissue characterization (RGB color space)

Figure 33 RGB color space characterization of immuno stained tonsil section images (1k x 1k pixels; RGB; pixel size = 0.135 μ m)

CD68 immuno stained tissue characterization (HSV color space)

Figure 34 HSV color space characterization of immuno stained tonsil section images (1k x 1k pixels; HSV; pixel size = 0.135 μ m).

Figure 35 Standard deviation of DAB brown class in different images in RGB color space

Figure 36 Standard deviation of hematoxylin blue class in the image set in RGB color space.

Figure 37 Standard deviation of DAB brown class in the image set in HSV color space.

Figure 38 Standard deviation of Hematoxylin blue class in the image set in HSV color space.

6.1.2 Picrosirius Red stained myocardial tissue sections

Myocardial tissue samples obtained from partial left ventriculectomy surgical procedure [1] were sectioned and stained with Picrosirius Red and mounted on slides. Collagen present in the samples stained red and the remaining muscle fibers stained yellow. These slides were utilized to obtain the test set of images. The images were captured using 20x objective lens on the microscope. Ten images were obtained using optimal contrast and focusing. The images were classified in to four classes using competitive learning algorithm. The classes were background white (weak saturation), Picrosirius Red (strong saturation), dark yellow tissue (weak saturation), and light yellow tissue (weaker saturation).

Figure 39 shows the RGB color space classification and

Figure 40 shows HSV color space classification. Mean and standard deviation for each class in each of the ten images were measured as described for the CD68 images. All the S.D. and mean plots are shown in appendix. The averaged class parameters are listed in

Table 3 below.

As evident from

Table 3, all the classes in the classification of Picrosirius stained slides had much lower S.D. in hue compared to saturation and value. This implies that the classes had consistent color pigments. Collagen acquired an average hue of 248. This corresponds to a reddish-pink pigment (i.e. Picrosirius red). Tissue1 and Tissue2 were light and dark tissue classes. These had average hues of 13.9 and 6.5 corresponding to yellow pigment. Background class had the highest value (203.6) as expected and relatively higher S.D. in hue.

Color	Component	Mean (Standard Deviation)		
		Red	Green	Blue
	Collagen	159.8 (16.7)	61.4 (15.8)	66.2 914.9)
RGB	tissue1	184 (11.4)	106.3 (13.6)	84.9 (13.5)
	tissue2	200 (10.7)	135.5 (13.2)	112 915.6)
	Background	218.1 (12.1)	177.5 (19.9)	172 (21.1)
		Hue	Sat	Val
	Collagen	248.1 (5.84)	15.6 (25.2)	163.2 918.8)
HSV	tissue1	13.9 (3.43)	144.4 914.4	191.1 (9.89)
	tissue2	6.5 (5.6)	133.3 (18.7)	184.9 (11.5)
	Background	255 (10.3)	74.6 (25.2)	203.6 (16.6)

Table 3 Picrosirius red stain tissue component parameters obtained from a set of ten images.

Picrosirius Red stained tissue characterization (RGB color space)

Figure 39 Picrosirius red stained tonsil section classification in RGB color space (512 x 512 pixel RGB images; pixel size = 0.27 μ m).

Picrosirius Red stained tissue characterization (HSV color space)

Figure 40 Picrosirius red stained tonsil section classification in HSV color space (512 x 512 pixel HSV images; pixel size = 0.27 μ m).

6.1.3 Hematoxylin & Eosin stained sections

Three different types of tissues were stained with H&E stain and mounted on slides. The tissues used were tonsil, breast biopsy samples, and coronary artery cross sections. Three sets of ten images were obtained from the slides using 20x objective lens on the microscope. The images were classified in to four groups, blue hematoxylin (nuclei), dark pink Eosin1 (one type of cytoplasm), light pink Eosin2 (another type of cytoplasm) in both RGB and HSV color spaces using competitive learning algorithm. The averaged means and S.D.s of the four classes were obtained for each of the three tissue types and are listed in Table 4, Table 5, and Table 6 .

Color space	Component	Mean (Standard Deviation)		
		Red	Green	Blue
	Hematoxylin	134.3 (16.8)	73.8 (10.0)	127.9 (11.5)
RGB	Eosin1	159.7 (8.55)	95.7 (7.27)	147.2 (7.61)
	Eosin2	174.1 (8.25)	118.3 (8.31)	163.7 (6.82)
	Background	193.1 (10.0)	150.8 (14.4)	183.9 (9.13)
		Hue	Sat	Val
	Hematoxylin	212.5 (6.96)	120.9 (13.6)	136.7 (13.9)
HSV	Eosin1	219.4 (4.53)	106.7 (12.1)	159.6 (7.39)
	Eosin2	219.1 (4.86)	94.7 (10.0)	172.5 (6.57)
	Background	219.4 (6.96)	68.5 (13.5)	190.4 (9.33)

Table 4 H&E stained coronary artery section characterization.

Color space	Component	Mean (Standard Deviation)		
		Red	Green	Blue
	Hematoxylin	62.9 (10.7)	26.7 (5.29)	88.2 (10.4)
RGB	Eosin1	100.3 (12.9)	44.5 (7.57)	111.6 (9.22)
	Eosin2	134.1 (12.8)	69.5 (12.4)	133 (10.2)
	Background	168 (14.3)	122.2 (21.8)	163.9 (11.3)
		Hue	Sat	Val
	Hematoxylin	194.7 (4.98)	178.2 (9.76)	86.4 (9.45)
HSV	Eosin1	202.2 (6.75)	153.6 (15.5)	113 (9.93)
	Eosin2	213.4 (6.54)	133.2 (19.9)	135.5 (10.7)
	Background	216.5 (8.81)	89.6 (24.8)	163.8 (13.1)

Table 5 H&E stained tonsil section characterization

Color	Component	Mean (Standard Deviation)		
		Red	Green	Blue
	Hematoxyli	136.6 (22.4)	75 (13.7)	179.3 (21.7)
RGB	Eosin1	200.4 (18.0)	111.6 (15.2)	216.7 911.6)
	Eosin2	230.4 (11.4)	156.2 (14.6)	230.8 (10.2)
	Background	241.3 (8.94)	209.1 (18.1)	243.2 (6.73)
		Hue	Sat	Val
	Hematoxyli	196.5 (6.41)	146.2 (17.5)	191.6 (18.9)
HSV	Eosin1	211.2 (5.67)	121.2 917.1)	225.9 (8.44)
	Eosin2	212 (6.29)	95.4 (18.2)	257 (5.88)
	Background	210.4 (14.9)	40 (19.4)	240.9 (10.5)

Table 6 H&E stained breast duct tissue component parametrization.

On comparing the class parameters from Table 4 and Table 5, it is evident that tonsil sections acquired stronger staining as all the classes had higher average saturations when compared with coronary artery sections. Hematoxylin had an average saturation of 178 and value of 86.4 for tonsil sections, where as hematoxylin in coronary artery sections had an average saturation of 121 and value of 137. Thus the hematoxylin stain was purer and darker in tonsil sections. The tissue component classes obtained from H&E stained breast duct sections had average saturation values in between those of tonsil and coronary artery sections. But the "value" measures were higher than those of both tonsil and coronary artery sections. The S.D.s for all the three sets of images were similar, except they differed slightly in value. The hues of the four classes were approximately same for all the three sets also. Thus, the above parameterization has shown that color pigment distributions vary from one type of tissue to another, although the colors of the tissue components remain approximately same.

H&E stained tissue characterization (RGB color space)

Figure 41 Hematoxylin (H) and Eosin (E) stained tonsil section classification in RGB color space. (1k x 1k pixel RGB images; pixel size = 0.135 μ m)

H&E stained tissue characterization (HSV color space)

Figure 42 Hematoxylin (H) and Eosin (E) stained tonsil section classification in HSV color space. (1k x 1k pixel HSV images; pixel size = 0.135 μ m)

6.1.4 Movat stained rat tail sections

Rat tail sections were stained with Movat pentachrome stain and mounted on slides.

Rat tail sections were chosen to obtain the purest strands of collagen feasible. A set of ten images were obtained from the slides using 10x objective lens on the microscope. The images were classified in to six classes, collagen, matrix proteins, immature collagen, blue stain, dark pink(muscle), and background in HSV color space using competitive learning algorithm. The averaged means and S.D.s of the six classes obtained are displayed in Table 7. Upon visual inspection, the collagen was accurately segmented in all the test images. The blue and dark pink classes were not well represented in some of the images and thus have inaccurate average values. Collagen and immature collagen have consistent hue of 20 corresponding to yellow color pigment. Immature collagen had fainter staining (lesser saturation) and was darker (less value). The background class had the highest value as expected.

Color	Component	Mean (Standard Deviation)		
		Hue	Sat	value
HSV	Collagen	21.4 (2.95)	164.2 (19.24)	145.9 (12.46)
	Matrix	133.1 (38.16)	111.5 (35.02)	58.8 (15.68)
	Immature	21.1 (10.58)	125.1 (30.86)	114.4 912.67)
	blue	206.8 (38.26)	68.8 (30.4)	119 (16.38)
	Dark pink	254 (27.54)	85.6 (34.07)	88.5 915.43)
	Background	11.3 (45.35)	26.2 (18.46)	186.8 (23.08)

Table 7 Movat stained rat tail section tissue component characterization.

Figure 43 Movat stained rat tail section classification in HSV color space. (1k x 1k pixel RGB images; pixel size = 0.27 μ m)

6.2 Synthetic image analysis

In this section, the color space distributions of stained tissue components were modeled using gaussian distributions in a synthetic image. The mean and S.D. parameters obtained in tissue characterization in earlier section were utilized in the gaussian modeling. The performance degradation of the three adaptive algorithms were studied under three different adverse scenarios. The synthetic image consisted of two circles superimposed on flat white background.

Figure 44 shows a synthetic image. The circle on the left was chosen to represent brown DAB stained tissue component and the circle on the right was chosen to represent the blue hematoxylin stained tissue component.

Figure 44 Synthetic image with DAB (brown) and hematoxylin (blue) color distributions modeled in RGB color space

6.2.1 stain color shift

First, the effect of shifting the colors towards each other along the straight line joining them was studied. Figure 45 shows the location of brown and blue color distributions in HSV color space. Figure 46 illustrates the shifting of color distributions progressively to increase the degree of overlap.

Figure 45 Location of experimentally measured blue and brown cluster means in HSV cone.

Figure 46 Schematic showing progressive overlap of the two color distributions by moving the distribution means along the straight line joining them.

The plots shown in Figure 48, and Figure 49 track the performance degradation of the three algorithms as the stain colors are brought closer. Maximum degradation is seen for the brown circle. This could be caused by higher standard deviation of the brown distribution. The median split algorithm performed worse than both competitive learning and fuzzy-C means algorithms as the percentage accuracy was shown to be less in both Figure 48, and Figure 49.

In HSV color-space plots, a reverse in the trend for median split can be seen. That is, it performs better for brown circle and worse for blue circle. This could be caused by the extensive overlap of the color distributions as the algorithm relies exclusively on feature space variance.

6.2.2 Standard deviation increase

In this experiment, the standard deviation of both brown and blue color distributions are progressively increased by the same factor. Significant performance degradation can be seen at a factor of two and beyond. Once again the median split algorithm performed worse than other two algorithms. In general the performance degraded more for the brown circle than the blue circle. Figure 50 and Figure 51 show the performance trends of the algorithms in both RGB and HSV color spaces.

6.2.3 Stain image fraction (percentage area) change

The effect of having only a small amount of one of the tissue components being separated present in the image is studied in the third experiment. The radius of the blue circle was progressively decreased. In RGB color space, competitive learning performed better consistently. Noticeable failure in labeling could be seen with the other two

algorithms when the area covered by blue circle became 2.15% of the total image area (Figure 47). In HSV color space, all the three algorithms performance degrade when the blue area becomes 2.15%. Figure 52 and Figure 53 illustrate the performance of the algorithms with area reduction. Median split algorithm performance is slightly better than competitive learning in the extreme case. In all other cases median split performed worse than competitive learning.

Figure 47 Synthetic image with blue circle area reduced to 2.15% of total area

RGB color space progressive color overlap performance plots

Figure 48 Progressive color overlap plots in RGB

HSV color space progressive color overlap performance plots

Figure 49 Progressive color overlap plots in HSV

RGB color space performance plots with progressively increasing standard deviations

Figure 50 Increasing standard deviation plots in RGB

HSV color space performance plots with progressively increasing standard deviations

Figure 51 Increasing standard deviation plots in HSV

RGB color space performance plots for regressive decrease in blue circle radius

Figure 52 Performance plots for progressive decrease in blue area in RGB

HSV color space performance plots for regressive decrease in blue circle radius

Figure 53 Performance plots for progressive decrease in blue area in HSV

6.3 Speed, accuracy and reliability

In this section, a brief discussion about the computational time for each of the algorithms is first presented. This is followed by a qualitative comparison, where an expert ranked the performance of the algorithms on a set of twenty test images. In-depth quantitative comparison of the performance of the three algorithms on three test sets of stained images using semi-automated segmentation as the “gold” standard is described next. Finally comparison of the three algorithms using manually traced collagen bundles in Movat stained rat tail sections is presented.

6.3.1 execution time

Computation time for segmenting a 1k x 1k CD68 immuno stained section image in to four classes on a SGI Indy workstation with R4400 CPU was 8 seconds for median split algorithm, 20 seconds for the competitive learning algorithm, and two minutes for the fuzzy C-means algorithm. The time for all the three algorithms will increase as the number of classes increase.

6.3.2 Accuracy:

In section 6.2, the performance of algorithms was analyzed using a synthetic image. In this section, routine images used in quantitative analysis are used for evaluation of the algorithms. Both qualitative and quantitative comparisons were performed.

6.3.2.1 Qualitative comparison

In this study, 18 test images stained using either DAB immuno staining for AE1/3 or Movat pentachrome stain were used. Tonsil sections were used for immuno staining and coronary artery sections were used for Movat staining. For each image, the original image and the pseudo colored classified images resulting from the three algorithms were displayed

on the computer monitor and an expert pathologist was asked to rank the classified images as first, second, and third in separating out the tissue components (Figure 54). First rank was given a value of four, second a value of two, and third assigned a value of one. The summed values for each algorithm were plotted as a chart (Figure 55). The median split algorithm had the smallest sum or the worst performance once again.

Figure 54 Comparison of segmentation algorithms in HSV color space using AE1/3 immuno stained tonsil section image. (1k x 1k pixel RGB images; pixel size = 0.27 μ m)

Figure 55 Qualitative comparison of the three algorithms

6.3.2.2 Quantitative comparison using semi automated segmentation as “gold” standard

Sets of ten images stained with Picrosirius red, DAB immuno stain, and H&E were first segmented in to tissue components described in section 6.1. The semi automated segmentation method described briefly in chapter five and in detail in Gaddipati *et al.* [2] was used to obtain visually agreeable results. An expert familiar with histological tissue component separation by observing through the microscope used the semi-automated segmentation algorithm to classify the three sets of ten images. The classification took approximately six hours. The segmented images were used as “gold” standard. Classification results from the three

algorithms were then compared with the “gold” standard. Figure 56 shows an image, its gold standard segmentation and segmentation from one of the automated algorithms.

The plots shown below give average relative performance of the algorithms with respect to the “gold” standard for each of the major tissue component in each of the three differing stains. The “gold” standard labeling was not perfect as the tissue clusters tend to be oblique and arbitrarily shaped. These clusters were not adequately modeled by hexahedral boxes used in the semi interactive labeling method. Thus, there were large discrepancies between the automated algorithms and the “gold” standard. Concentrating on the tissue component with the dominant stain in all the images, there was an overall agreement of 85% between the algorithms and the “gold” standard. The dominant stain was well separated from other tissue component clusters and was modeled with maximum care during the manual labeling process.

CD68 stained tonsil section tissue component separation comparison

Figure 56 Comparison of HSV colorspace classification of CD68 stained tonsil section image using semi interactive labeling and the automated competitive learning algorithm. (1k x 1k pixel RGB images; pixel size = 0.135 μ m)

Figure 57 Segmented area match between the “gold” standard classification and the automated algorithm segmentation for the brown and blue components in CD68 stained tonsil section images.

Figure 58 Segmented area match between the “gold” standard classification and the automated algorithm segmentation for the unstained and background components in CD68 stained tonsil section images.

Figure 59 Segmented area match between the “gold” standard classification and the automated algorithm segmentation for the Picrosirius red and drak yellow components in Picrosirius stained ventricle section images.

Figure 60 Segmented area match between the “gold” standard classification and the automated algorithm segmentation for the light yellow and background components in Picrosirius stained ventricle section images.

Figure 61 Segmented area match between the “gold” standard classification and the automated algorithm segmentation for the blue and dark pink components in Hematoxylin and Eosin stained tonsil section images.

Figure 62 Segmented area match between the “gold” standard classification and the automated algorithm segmentation for the light pink and background components in Hematoxylin and Eosin stained tonsil section images.

6.3.2.3 Quantitative comparison using manually traced images as “gold” standard

In this section, collagen bundles traced in Movat stained rat tail section images are used as “gold” standard for comparing the three algorithms. As mentioned earlier, rat tail sections were chosen as they contained purest natural collagen bundles available in our laboratory. First the Movat stained rat tail section images were displayed on a computer screen and the yellow collagen bundles were traced using custom built tracing program. The traced images were then thresholded to remove the dark purple nuclei from the bundles. Figure 63 shows this process.

Figure 63 Generation of traced “gold” standard collagen bundle image from Movat stained rat tail section slides. (1k x 1k pixel images; pixel size = 0.27 μ m)

The three algorithms were then applied to the set of ten microscopy images to obtain images segmented into six classes. The class representing yellow collagen stain was identified in each of the segmented image and compared with the “gold” standard labeling.

Table 8 shown below lists the percentage matches of the algorithm segmentations. Median split algorithm performed erratically, resulting in good matches in some images and almost no match in other images. Fuzzy C-means performed better than competitive

learning algorithm with an average accuracy of 95%. In some of the images the yellow collagen color pigment was split into two or three classes resulting in the poor matching.

IMAGE	COMPETITIVE %	MEDIAN SPLIT %	FUZZY C%
1	83.68	79.97	89.3
2	80.51	05.43	92.9
3	73.63	77.26	96.34
4	76.56	03.75	95.63
5	70.87	06.32	96.16
6	65.68	04.17	97.23
7	54.94	96.80	96.89
8	60.24	02.73	95.24
9	36.94	07.83	95.87
10	65.51	95.93	97.83

Table 8 Table listing percentage match between manually traced collagen content of Movat stained rat tail section images and the collagen content obtained using automated segmentation algorithms.

6.4 Cluster number determination

All the algorithms validated above assume that the number of color-space clusters present in the image is known. In general, this is not true in practical applications and especially in unsupervised segmentation applications such as the current research. The number of clusters present can be estimated from the observed data (*i.e.* image) using stochastic image models and information theoretic criteria.

We modeled the cluster validation problem as a multiple decision problem, in that we try to find which model parameterized by K , the number of classes, best fits the observed data. We used gaussian mixtures to model the image data. We have used Akaike's information criterion (AIC) advocated by Modestino *et al.* [3] and an MRF based neighborhood agreement term for the purpose of measuring the goodness of the fit.

Let the image data be represented by N random vectors, denoted by $Y = \{y_1, y_2, \dots, y_N\}$. Assume that Y contains K data classes and can be modeled by a probability density function (pdf) $p_K(Y|a^{(K)})$ where $a^{(K)}$ is the model parameter vector. Now, the measure of fit, AIC can be defined as

$$AIC(K) = -\log \left\{ p_K \left(Y | \hat{a}_{ML}^{(K)} \right) \right\} + 2K' \quad (1)$$

where $\hat{a}_{ML}^{(K)}$ is the maximum likelihood (ML) estimate of the model parameter vector $a^{(K)}$ and K' is the number of independently adjustable parameters of the K class data model. The number of classes yielding the minimum AIC is said to best fit the model. The first term in equation 1 is the negative log likelihood and minimizing this term results in the maximum likely hood estimate of the number of classes. The second term is added as a

penalty for increasing number of parameters. This criterion was initially derived for autoregression(AR) models to one dimensional data. Thus, it did not account for neighborhood relations present in the image data. We modeled the pixel local neighborhood relations using a simple Gaussian Markov random field model and added the negative log neighbor-likelihood term to the goodness of fit measure.

We computed AIC in the following manner. Assume, the N pixels comprising the image are already segmented into K classes. Now, assuming the pixels have independent gaussian probability distributions with parameters specified by the class they are allocated, the segmentation likelihood can be written as

$$p_K \left(Y \middle| a^{(K)} \right) = \prod_{k=1}^K \pi_k^{N_k} \prod_{j=1}^{N_k} p_k(y_{kj}) \quad (2)$$

Where $N_k \leq N$ is the number of samples in the k^{th} class and y_{kj} , $j=1,2, \dots, N_k$ are pixel vectors allocated to this class. π_k is the prior probability of occurrence for the class k . In this implementation, this is taken to be the fraction of image pixels assigned to the class k . $p_k(y_{kj})$ is the probability of the pixel vector belonging to the class k . Using gaussian mixture model, this probability is written as

$$p_k(y_{kj}) = \frac{1}{(2\pi)^{m/2} |\Sigma_k|^{1/2}} \cdot \exp \left[-\frac{1}{2} (y_{kj} - \mu_k)^T \Sigma_k^{-1} (y_{kj} - \mu_k) \right] \quad (3)$$

where m is the dimensionality of pixel vector y . For color images, this is 3.

Now, the neighbor-likelihood term $NL(X_K)$ is calculated using a simple MRF model which penalizes for neighbors having differing classes. X_K is the maximum likelihood segmentation of the image into K classes. $NL(X_K)$ is calculated using equation 7 as explained in the section on ICM algorithm. Thus the complete measure of fit $GF(K)$ is

$$GF(K) = -\log \left\{ p_k \left(Y \middle| \hat{a}_{ML}^{(K)} \right) \right\} + 2K' - \log NL(X_K) \quad (4)$$

Now the estimate of number of classes in the image $K_{\min}$ is taken as the K satisfying the condition

$$K_{\min} = \arg \min_K \{GF(K)\}, \quad 1 \leq K \leq K_{\max} \quad (5)$$

We validated this measure using ten test images of Picrosirius stained microscopy slides. Each image was segmented using median split algorithm first into two classes, then three, four, and so on stopping at seven classes. Figure 65 shows classification of a test image into four classes. AIC criterion and measure of fit (GF) were calculated for each segmentation. Two experts (two of the committee members experienced in analyzing Picrosirius stain) were asked to find the number of classes resulting in the best segmentation of Picrosirius stain in each image. This task was performed by displaying the original test image and the psuedocolored segmented images side by side on the computer monitor.

Figure 64 compared the manual evaluation with the measure of fit and AIC evaluation. The AIC criterion agreed with one of the experts, where as the measure of fit (GF - AIC+MRF) performed poorly.

Figure 64 Comparison of best number of classes for segmentation of picro stained image.

Picrosirius Red stained tissue characterization (RGB color space)

Figure 65 Picrosirius red stained tonsil section classification in RGB color space (512x512 pixel images; pixel size = 0.27 μ m)

In summary, this chapter covered tissue component parameterization for four different stains. This was followed by validation and comparison of three automated segmentation algorithms using both synthetic and clinical images. Both qualitative and quantitative comparisons show median split algorithm to be worse than competitive learning and fuzzy C-means algorithms. Since fuzzy C-means algorithm requires almost three times the computation required for competitive learning, competitive learning would be the preferred method of automated segmentation. We have also illustrated and validated a method for finding optimal number of clusters present in the image using AIC criterion. could be used for determining The number of clusters the algorithms should divide the color space into could be decided by this criterion in future.

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CHAPTER 7

APPLICATIONS

In this chapter, the use of the system developed and validated in earlier chapters for tissue component separation in clinical and research applications is described. The semi-interactive segmentation system was used in laboratory of vascular diseases (LVD) at The Ohio State University for quantification of atherosclerosis in young people. The quality of staining when using Histochoice and formalin as fixing agents were compared in another application. In another study, histological sections of left ventricular wall removed during a partial left ventriculectomy were analyzed for collagen content. The technique was also applied to the study of the epithelial cell nuclei arrangement in breast ducts for histological subtyping of breast disease. [1]

7.1 Lipid content in arterial wall [2-4]

The semi-interactive segmentation system described in chapter five was used to quantify extent of atherosclerosis in the left anterior descending coronary artery (LAD) as a part of the study of risk factors causing atherosclerosis conducted at LVD in collaboration with 11 other institutes [2]. It was determined that measuring the lipid accumulation in the atherosclerotic plaques was a practical method of quantifying atherosclerosis. Thus, in this study, 630 LAD artery cross sectional slides stained with oil red "O" (ORO) was used for identifying the amount of intra cellular lipid (ICL) and extra cellular lipid (ECL) in the

sample. ICL is the lipid absorbed by macrophages and smooth muscle cells. ICL formed dark red portions of the ORO stained arterial images. ECL is the lipid dissolved in the intercellular liquid. ECL formed the light red portion of the images. Lipid quantification was achieved by classification of arterial images into ICL, ECL and normal tissue using the semi-interactive segmentation system as the manual segmentation was bound to be slow, tedious and subjective. The classified arterial image database was subsequently used in quantifying the statistical relations of lipid accumulation in arteries with risk factors such as age, thickness of lesion and smoking Gaddipati *et al.* [3].

The intima of the arterial section was divided into three layers for intimal lipid spatial distribution analysis. The layers were called inner, middle, and outer layers. The lipid accumulation in the three layers was plotted for ICL and ECL in separate graphs. These graphs clearly showed that inner layer had less lipid compared to middle and outer layers. Plots of lipid content with thickness of lesion showed a general increase of lipid area with thickness (Figure 67, Figure 68, Figure 69, and Figure 70). It was noticed that the inner layer had significantly less lipid than middle and outer layers and ICL was much lower than ECL. Plotting lipid content versus age showed a small increase of lipid area with age (Figure 71, Figure 72, Figure 73, Figure 74). But, this was much less significant than the thickness trend. On plotting a bar chart of smokers and nonsmokers it was found that smokers had higher lipid content than nonsmokers. Similarly the group with high “low density lipid” in their blood (LDL) had more lipid area than the low LDL group. For brevity, only the bar chart for smoking is shown in Figure 75. All the plots are illustrated in Gaddipati *et al.* [4].

Figure 66 Segmentation of 'ORO' stained coronary artery section. (1k x 1k pixel RGB images; pixel size = 0.27 μ m)

Figure 67 Bar charts showing intimal total lipid, extra cellular lipid (E), and intra cellular lipid (I) accumulation as a function of age.

Figure 68 Bar charts showing intimal inner layer total lipid, extra cellular lipid (E), and intra cellular lipid (I) accumulation as a function of age.

Figure 69 Bar charts showing intimal middle layer total lipid, extra cellular lipid (E), and intra cellular lipid (I) accumulation as a function of age.

Figure 70 Bar charts showing intimal outer layer total lipid, extra cellular lipid (E), and intra cellular lipid (I) accumulation as a function of age.

Figure 71 Bar charts showing intimal total lipid, extra cellular lipid (E), and intra cellular lipid (I) accumulation as a function of intimal thickness.

Figure 72 Bar charts showing intimal inner layer total lipid, extra cellular lipid (E), and intra cellular lipid (I) accumulation as a function of intimal thickness.

Figure 73 Bar charts showing intimal middle layer total lipid, extra cellular lipid (E), and intra cellular lipid (I) accumulation as a function of intimal thickness.

Figure 74 Bar charts showing intimal outer layer total lipid, extra cellular lipid (E), and intra cellular lipid (I) accumulation as a function of intimal thickness.

Figure 75 Bar chart showing lipid accumulation in coronary arteries of smokers and nonsmokers.

7.2 Comparison of Histochoice and formalin as fixing agents [5]

Formalin has traditionally been used as a tissue fixative because it imparts excellent morphological preservation, penetrates quickly, and facilitates sectioning by hardening the tissue. Formalin fixes tissue by cross-linking protein molecules, a process which can reduce immunohistochemical staining intensity. Thus formalin may be less than ideal fixative for immuno staining. Formalin is also becoming increasingly expensive to use because of the stringent laboratory safety procedures required due to its toxicity. Thus a number of proprietary fixatives have become commercially available for immunohistochemical studies. Histochoice is one such proprietary fixative made available by Amresco Inc., Solon, OH. Histochoice is much less toxic than formalin. Preliminary qualitative studies in the Whitaker Image Processing Laboratory had shown that Histochoice offered staining intensity equal to, and in many cases greater than, that of formalin. In this study, an

automated quantitative comparison of microscopy slide staining “quality” resulting from the two fixatives was performed. This study has been published in *Analytical Cellular Pathology*. [5]

Tissue samples from seven patients, comprising tonsil (n=3), colon (n=1), prostate (n=2), and pancreas (n=1), were obtained from the Department of Anatomic Pathology at The Cleveland Clinic Foundation. Each sample of tissue was bisected and fixed for eight hours, half in formalin and half in Histochoice. The tissue was then processed and paraffin embedded according to standard laboratory procedures. Sections were cut from each block at 4 μ m, and collected onto electrostatically charged slides. Immunohistochemical staining of the sections was performed utilizing fully automated temperature, humidity, time, and volume controlled immunostaining instruments. This ensured, that all the other factors affecting staining quality were identical for both procedures. Twenty-one different antibodies were used for immunostaining to emphasize extensive comparison.

Four high resolution color images were acquired from each section using the previously described image acquisition system. Figure 76 and Figure 77 show the pairs of images obtained using both the fixatives. Semi interactive segmentation algorithm was used to isolate pixels covered by positive DAB-stained tissue. An analysis of variance (ANOVA) was performed on the measurements of hue, saturation, and value at each positive pixel in each image.

The analysis revealed small differences in hue in eight out of twenty-one antibodies, although these differences were less than 7%, indicating a very small change in color had occurred. Histochoice-fixed tissue was found to give higher mean saturation than formalin

with 57.1% of the antibodies tested. No significant difference in the saturation was detected in 28.6% of the cases. Formalin gave higher mean saturation levels with 14.3% of the antibodies. The median mean saturation level was 62.2% for the histochoice-fixed tissue and 46.2 for the formalin-treated tissue. Histochoice-fixed tissue was found to give lower “values” in 66.7% of cases than those prepared with formalin. Formalin gave lower values than Histochoice in 14.3% cases. In 19% of antibodies there was no significant difference in the “value” of the stain.

In the context of immunostaining, the saturation depends upon the density of available antigens. Thus higher mean saturation with Histochoice implies greater availability of antigens for binding with antibodies. Similarly darker stain or lower “value” implies availability of more antigens for binding. The hue of the positively stained pixels did not vary much as the DAB chromogen will always result in the same color pigment. Thus the above results show that Histochoice fixative when compared with the more hazardous formalin, provides a more saturated, and intense immunostaining reaction product in many cases.

When evaluating new histology reagents or changes in traditional methodologies, it is vitally important to consider the methods of quantification and interpretation of the stained tissue. In relatively simple quantification tasks, such as cell counting, manual techniques have been shown to be time consuming and of questionable accuracy when compared to automated analysis methods. The parameters such as hue, saturation and value used in this study are more difficult to quantify manually. Automated image analysis was the only path for quantifying these parameters accurately.

CD64 antibody stained tonsil section images

Histochoice fixing

Formalin fixing

Figure 76 Images of CD64 staining in human tonsil. Tissue was fixed in Histochoice and formalin for comparison. (1k x 1k pixel RGB images; pixel size = 0.27 μ m)

Histochoice fixing

Formalin fixing

Figure 77 Section of pancreas stained for chromogranin. Tissue fixed in Histochoice and formalin for comparison. (1k x 1k pixel RGB images; pixel size = $0.27\mu\text{m}$)

7.3 Collagen content and fiber orientation in ventricular wall [6]

In this section, a description of the quantitative analysis of samples obtained from myocardial tissue sections removed from patients with dilated cardiomyopathy during the partial left ventriculectomy performed at The Cleveland Clinic Foundation is presented. [7]

Dilated cardiomyopathy may be caused by alcoholism, viral infection or be idiopathic. It is characterized by large dilated left ventricle with poor systolic contractile function.

Dilation results in increased end diastolic and end systolic left ventricular volumes. The left ventricular mass and volume/mass ratio are also increased. The wall stress goes up as the size of the ventricle is increased. Decreased apical-base shortening and decreased chamber stiffness are also observed in dilated cardiomyopathy. All these factors lead to the patient not being able to pump sufficient blood through the cardiovascular system. This leads to degradation of other body functions and body adjustments, which causes further dilation of heart. Eventually the patient is caught in an ever worsening loop and will face premature demise unless a corrective procedure is followed. Currently heart transplant/artificial heart replacement are prescribed in most tertiary hospitals. Recently surgeons are adapting partial ventriculectomy procedure for this purpose. The Cleveland Clinic Foundation is one of the leaders in performing this procedure.

The physicians believe the collagen matrix of the ventricle undergoes a change during dilation. The collagen content and type may change during dilation. The collagen fiber orientation also may deviate from normal leading to development of abnormal wall strains. This can have a significant effect on the recovery of the ventricle after the surgical procedure.

The fiber orientation has been studied previously in canine hearts. But this is the first time this type of analysis is being performed on human hearts due to the limited availability of human myocardial tissue.

The myocardial section samples were prepared for quantitative analysis in the following manner. The ventricular section was cut into three 2x2x1.5cm tissue samples corresponding to base, mid, and apex regions shown in Figure 78. Each of the samples was

sectioned in to ten 5 μm thick cross sections using a microtome (Figure 79). Each of the cross sections were stained using Picrosirius red stain. This stain imparts a pink color to collagen fibers. The stained cross sections were carefully mounted on slides. The microscopy slides were then digitized into 1024x1024 (4 μm /pixel) RGB images using the image acquisition system described in chapter 3.

The images were then segmented in HSV color space using competitive learning algorithm developed in chapter five to separate out the collagen fibers. The segmented image was skeletonized to form thin lines corresponding to the fibers. The skeleton image was subjected to Hough transform to obtain dominant fiber orientations. Figure 81 illustrates the above described processing. The collagen content in each of the images was also measured. The number of images being processed for this study is of the order of 8000. Thus a completely automated quantitative analysis is almost a necessity for this application. It is hoped that this analysis will help in determining whether partial left ventriculectomy procedure is suitable for a patient with dilated cardiomyopathy.

Schematic of left ventricle

Figure 78. Left ventricle Schematic showing anatomical areas [6]

Sample sectioning

Figure 79. Obtaining cross sections from a ventricular sample. [6]

Myocardial sections

DCM stained sections

Normal fiber orientation

endocardium

mid-wall

epicardium

Figure 80. Fiber orientation of left ventricular vertical sections in dilated cardiomyopathy (DCM) patients and normals. [6]

Picrosirius red stained collagen fiber segmentation

Figure 81. Collagen fiber segmentation from Picrosirius stained myocardial tissue section images. (512 x 512 pixels; RGB; 0.27 μ m)

7.4 Cell architecture analysis for aiding breast cancer diagnosis

In this section, quantitative analysis of stained breast tumor biopsy specimen slides is described. The purpose of this study was to identify morphological features used by pathologists in diagnosing malignancy and use these features in the design of automated analysis system which would aid the pathologist in diagnosis.

In this study, twenty biopsy specimens were obtained from pathology department at The Cleveland Clinic Foundation. These specimens were sectioned at 4 μ m and stained with hematoxylin and eosin. The previously described image acquisition system was used to capture 4kx4k 24 bit color images of the stained microscopy slides. Each pixel on the digitized image covered approximately 0.3 μ m x 0.3 μ m area on the slide.

A typical captured image of a malignant tumor biopsy specimen is shown in Figure 82. Following scanning, nuclei of the cells were segmented out using the competitive learning

color segmentation algorithm. The segmented image was subject to further processing to separate touching cells and to remove spurious objects. This was followed by morphological feature analysis of the labeled nuclei, namely area, eccentricity, orientation, and chromaticity. Figure 83 presents a overview of the entire image processing pipeline for this application.

Benign architectural patterns identified by pathologists are shown in Figure 84. Any pattern which deviates significantly from these patterns would be classified as malignant. Figure 85 shows a malignant tumor and the corresponding processed labeled cell image.

The purpose of this study was automating the diagnosis process to the maximum extent possible and thus reducing the strain on the pathologists.

Figure 82 Image of stained biopsy section showing a malignant tumor. (1k x 1k pixel RGB images; pixel size = 0.135 μ m)

Figure 83 Flowchart of the image processing pipeline for breast cancer histological analysis.

Breast Duct - Architectural Patterns

Figure 84 Schematic of benign breast duct architectural patterns.

H&E stained breast section

Processed binary cell image

Figure 85 Image of a malignant tumor in RGB colorspace and the corresponding processed labeled image. (1k x 1k pixel images; pixel size = $0.27\mu\text{m}$)

In summary, four applications of quantitative analysis of microscopy slides are presented in this chapter. The color segmentation algorithms developed during the current research were an essential part of the quantitative analysis. Automated quantitative analysis of microscopy slides can also be used in many other research and clinical applications.

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CHAPTER 8

DISCUSSION, FUTURE DIRECTIONS, AND CONCLUSION

In this chapter, the segmentation methods and their application to microscopy images are discussed. Possible future directions for this research are then listed. This is followed by a summary of the thesis.

8.1 Discussion:

The validation studies performed with the synthetic images show that median split had the worst performance. Competitive learning and the fuzzy methods were better than median split and were comparable to one another in performance. Competitive learning had better performance when the area percentage of a class was reduced. Fuzzy C-means method performed better with increase in standard deviations of the color pigment distributions. The qualitative validation study also showed that competitive learning and fuzzy algorithms had comparable performance. The quantitative study with rat tail collagen bundles implied that the fuzzy algorithm clearly had the best performance. Quantitative comparison with semi-interactive thresholding 'gold' standard were inconclusive. This could be due to lack of dexterity in enclosing oblique tissue component clusters with rectangular boxes in three dimensional color space. On the whole, comparison with the semi-interactive thresholding showed around 85% agreement with the automated methods for the dominant color pigment (i.e. DAB brown, picosirius red etc.) in each of the test

sets. Competitive learning took 25 seconds and the fuzzy algorithm took 90 seconds to segment 1kx1k image. Thus from computational point of view competitive learning would be chosen for color segmentation in an automated image analysis system. However, the competitive learning algorithm has been developed for obtaining an approximate global solution to the problem of achieving segmentation with maximal inter cluster separation and minimal intra cluster variance. Fuzzy C means algorithm was developed to find the local optimal solution given an initial solution. Thus, as demonstrated by our experiments, using the solution from the competitive learning algorithm as the starting point for the fuzzy algorithm yielded the best segmentation results. Also, cluster validation can be performed on the segmentation results from using a range of cluster numbers around the expected number of tissue component clusters present in the image using AIC criterion. The authors recommend this approach, when including color segmentation as one of the steps in automated image analysis as accuracy is the most important factor to be considered. The number of computations required is becoming less of an issue with the improvements in microprocessors and the specialized image processing hardware.

The author felt that the segmentation could be improved by utilizing additional contextual cues present in the image. Two GMRF based contextual segmentation algorithms were implemented, however running the GMRF based split and merge probability optimization algorithm on a 400x400 image took 20 minutes on a SGI Indy, even after simplifying the parameter estimation and thus reducing the amount of computation. The computation needs to be further reduced, for practical application of the method in segmenting large number of images. One way of doing this would be dividing the

image into smaller sub images and segmenting each of the sub images in turn. ICM algorithm is faster taking only 90 seconds for 1kx1k image on SGI Indy, with the β optimization step being the slowest part of the algorithm. ICM requires a good initial solution to obtain optimal results. The actual enhancement of segmentation achieved would depend on the validity of the simple GMRF model used and the pseudolikelihood approximation.

8.2 Future directions:

In this research, algorithms selected by the author were analyzed for their suitability for solving the problem of tissue component separation on color microscopy images. The incorporation of local neighborhood information using statistical models will become more feasible with the latest hardware enhancements made to desktop computers. Similarly hardware implemented neural network approaches such as LEGION [1] can be used for including the contextual information. Another late development has been the invention of multi-spectral analysis machines that can record the whole visible spectrum at each of the microscopy image pixels. Scientists at NIH are working with this costly instrument (\$80,000) to achieve better tissue component separation in clinical applications.

8.2.1 Neural networks:

Neural networks which attempt to model the human visual system in integrating local neighborhood information could be a viable alternative to statistical methods when implemented in hardware [2-4]. One such network is LEGION being developed by Wang *et al.* at Ohio State University [1, 4-6] . LEGION stands for locally excitatory, globally inhibitory oscillating neurons. In this network, each pixel is modeled by a neuron which

picks up excitatory input from local neighbors and when fired sends a small inhibitory signal to all neurons. Neurons oscillate from a inactive to active state continuously. Neurons/pixels belonging to a coherent region phase lock and oscillate as a group sustaining each others activity. This involves solving two differential equations at each oscillation (cycle). The amount of computation required for the current application makes the hardware implementation a necessity. This network is being implemented in hardware at Massachusetts Institute of Technology. Once the implementation is complete, it could be used for microscopy image analysis.

8.2.2 Hardware improvements

In the current research, modeling contextual information using statistical probability models was attempted. This approach was not persued to the full extent due to long computational times (~80 minutes for 1kx1k image) involved. Some of the digital image processing boards released in the last few months, such as Matrox millennium, have very high computational power. These boards are capable of performing one billion operations per second, compared to the few million performed by a mid range workstation such as SGI O₂. Thus, with the expected continued enhancement of the processing power, it should be possible in next few years to implement the contextual methods discussed in this research in real time.

8.2.3 Multispectral analysis

Multiple wavelength spectrophotometry was used by Zhou *et al.* [7] to estimate the amount of different dyes present at each pixel. Zhou *et al.* used Beer-Lambert absorption law. They acknowledge the invalidity of some of the assumptions needed for application of

Beer- Lambert law, hence their results were questionable. Recently researchers at the National Institutes of Health (NIH) [8], have started using “imaging spectrometers” to achieve as many as 300 channels in the visible spectrum. They refer to the huge amount of data available for each image as a hyper-cube. Principle component analysis is performed on the 300 element vector data to obtain the five most distinguishing features in the image. These features are then used to separate different tissue components. This approach could be more accurate as it uses a 300 element vector compared to three element vector given by routine color images. The problems with this approach currently are the huge amount of computation involved and the high cost of the instrument. Nevertheless, if the ongoing research yields promising results, this could be the direction future automated segmentation methods migrate.

8.3 Conclusion

In conclusion, the problem of separating tissue components in histologically stained tissue section microscopy images has been addressed. Three automated adaptive algorithms were implemented for performing the image segmentation with the goal of achieving acceptable segmentation with minimal computational burden (i.e. short processing time). The algorithmic performance was evaluated using synthetic and clinical test image sets. The competitive learning algorithm had the best performance, considering both time and accuracy of segmentation. However, competitive learning algorithm has been developed for obtaining an approximate global solution to the problem of achieving segmentation with maximal inter cluster separation and minimal intra cluster variance. Fuzzy algorithms were developed to find the local optimal solution given an initial solution. Thus, from theory and

as demonstrated by our experiments, using the solution from the competitive learning algorithm as the starting point for fuzzy algorithms should yield the best segmentation results. Also, cluster validation can be performed on the segmentation results from using a range of cluster numbers around the expected number of tissue component clusters present in the image using AIC criterion as explained in section 6.4. This approach is recommended, when including color segmentation as one of the steps in automated image analysis as accuracy is the most important factor to be considered. The number of computations required is becoming less of an issue with the improvements in microprocessors and the specialized image processing hardware. Four applications of the segmentation algorithms in research settings were described as integral parts of quantitative analysis.

This research has shown that automated segmentation can achieve an accuracy of 85% in tissue component separation. Automated segmentation is a necessary early vision process in automated quantitative analysis. In applications involving huge quantities of microscopy images such as the ventricular collagen content study described in chapter seven, automated quantitative analysis is the only feasible method of obtaining reliable and accurate results.

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APPENDIX

Graphs for tissue characterization

Unstained tissue -CD68 DAB

Figure 86 Standard deviation characterization of CD68 immunostained tonsil section unstained tissue component in HSV color space.

Figure 87 Standard deviation characterization of CD68 immunostained tonsil section background component in HSV color space.

Figure 88 Standard deviation characterization of CD68 immunostained tonsil section unstained tissue component in RGB color space.

Figure 89 Standard deviation characterization of CD68 immunostained tonsil section background component in RGB color space.

Figure 90 Mean color characterization of CD68 immunostained tonsil section DAB chromogen component in HSV color space.

Figure 91 Mean color characterization of CD68 immunostained tonsil section hematoxylin component in HSV color space.

Figure 92 Mean color characterization of CD68 immunostained tonsil section unstained component in HSV color space.

Figure 93 Mean color characterization of CD68 immunostained tonsil section background component in HSV color space.

Figure 94 Mean color characterization of CD68 immuno stained tonsil section DAB chromogen component in RGB color space.

Figure 95 Mean color characterization of CD68 immuno stained tonsil section hematoxylin component in RGB color space.

Figure 96 Mean color characterization of CD68 immuno stained tonsil section unstained component in RGB color space.

Figure 97 Mean color characterization of CD68 immuno stained tonsil section background component in RGB color space.

Figure 98 Standard deviation characterization of Picrosirius stained myocardial section collagen component in HSV color space.

Figure 99 Standard deviation characterization of Picrosirius stained myocardial section dark yellow component in HSV color space.

Figure 100 Standard deviation characterization of Picrosirius stained myocardial section light yellow component in HSV color space.

Figure 101 Standard deviation characterization of Picrosirius stained myocardial section background component in HSV color space.

Figure 102 Mean color characterization of Picrosirius stained myocardial section collagen component in HSV color space.

Figure 103 Mean color characterization of Picrosirius stained myocardial section dark yellow component in HSV color space.

Figure 104 Mean color characterization of Picrosirius stained myocardial section light yellow component in HSV color space.

Figure 105 Mean color characterization of Picrosirius stained myocardial section background component in HSV color space.

Figure 106 Standard deviation characterization of Picrosirius stained myocardial section collagen component in RGB color space.

Figure 107 Standard deviation characterization of Picrosirius stained myocardial section dark yellow component in RGB color space.

Figure 108 Standard deviation characterization of Picrosirius stained myocardial section light yellow component in RGB color space.

Figure 109 Standard deviation characterization of Picrosirius stained myocardial section background component in RGB color space.

Figure 110 Mean color characterization of Picrosirius stained myocardial section collagen component in RGB color space.

Figure 111 Mean color characterization of Picrosirius stained myocardial section dark yellow component in RGB color space.

Figure 112 Mean color characterization of Picrosirius stained myocardial section light yellow component in RGB color space.

Figure 113 Mean color characterization of Picrosirius stained myocardial section background component in RGB color space.

Figure 114 Standard deviation characterization of H&E stained tonsil section hematoxylin component in HSV color space.

Figure 115 Standard deviation characterization of H&E stained tonsil section Eosin1 component in HSV color space

Figure 116 Standard deviation characterization of H&E stained tonsil section Eosin2 component in HSV color space

Figure 117 Standard deviation characterization of H&E stained tonsil section background component in HSV color space

Figure 118 Mean color characterization of H&E stained tonsil section hematoxylin component in HSV color space.

Figure 119 Mean color characterization of H&E stained tonsil section Eosin1 component in HSV color space.

Figure 120 Mean color characterization of H&E stained tonsil section Eosin2 component in HSV color space.

Figure 121 Mean color characterization of H&E stained tonsil section background component in HSV color space.

Figure 122 Standard deviation characterization of H&E stained tonsil section hematoxylin component in RGB color space.

Figure 123 Standard deviation characterization of H&E stained tonsil section Eosin1 component in RGB color space.

Figure 124 Standard deviation characterization of H&E stained tonsil section Eosin2 component in RGB color space.

Figure 125 Standard deviation characterization of H&E stained tonsil section background component in RGB color space.

Figure 126 Mean color characterization of H&E stained tonsil section hematoxylin component in RGB color space.

Figure 127 Mean color characterization of H&E stained tonsil section Eosin1 component in RGB color space.

Figure 128 Mean color characterization of H&E stained tonsil section Eosin2 component in RGB color space.

Figure 129 Mean color characterization of H&E stained tonsil section background component in RGB color space.

Figure 130 Standard deviation characterization of H&E stained artery section hematoxylin component in HSV color space.

Figure 131 Standard deviation characterization of H&E stained artery section eosin1 component in HSV color space.

Figure 132 Standard deviation characterization of H&E stained artery section Eosin2 component in HSV color space.

Figure 133 Standard deviation characterization of H&E stained artery section background component in HSV color space.

Figure 134 Mean color characterization of H&E stained artery section hematoxylin component in HSV color space.

Figure 135 Mean color characterization of H&E stained artery section Eosin1 component in HSV color space.

Figure 136 Mean color characterization of H&E stained artery section Eosin2 component in HSV color space.

Figure 137 Mean color characterization of H&E stained artery section background component in HSV color space.

Figure 138 Standard deviation characterization of H&E stained artery section hematoxylin component in RGB color space.

Figure 139 Standard deviation characterization of H&E stained artery section Eosin1 component in RGB color space.

Figure 140 Standard deviation characterization of H&E stained artery section Eosin2 component in RGB color space.

Figure 141 Standard deviation characterization of H&E stained artery section background component in RGB color space.

Figure 142 Mean color characterization of H&E stained artery section hematoxylin component in RGB color space.

Figure 143 Mean color characterization of H&E stained artery section Eosin1 component in RGB color space.

Figure 144 Mean color characterization of H&E stained artery section Eosin2 component in RGB color space.

Figure 145 Mean color characterization of H&E stained artery section background component in RGB color space.

Figure 146 Standard deviation characterization of H&E stained breast biopsy section hematoxylin component in HSV color space.

Figure 147 Standard deviation characterization of H&E stained breast biopsy section Eosin1 component in HSV color space.

Figure 148 Standard deviation characterization of H&E stained breast biopsy section Eosin2 component in HSV color space.

Background std dev for breastHE20x in HSV

Figure 149 Standard deviation characterization of H&E stained breast biopsy section background component in HSV color space.

Hematoxylin mean color for breastHE20x in HSV

Figure 150 Mean color characterization of H&E stained breast biopsy section hematoxylin component in HSV color space.

Figure 151 Mean color characterization of H&E stained breast biopsy section Eosin1 component in HSV color space.

Figure 152 Mean color characterization of H&E stained breast biopsy section Eosin2 component in HSV color space.

Figure 153 Mean color characterization of H&E stained breast biopsy section background component in HSV color space.

Figure 154 Standard deviation characterization of H&E stained breast biopsy section hematoxylin component in RGB color space.

Figure 155 Standard deviation characterization of H&E stained breast biopsy section Eosin1 component in RGB color space.

Figure 156 Standard deviation characterization of H&E stained breast biopsy section Eosin2 component in RGB color space.

Figure 157 Standard deviation characterization of H&E stained breast biopsy section background component in RGB color space.

Figure 158 Mean color characterization of H&E stained breast biopsy section hematoxylin component in RGB color space.

Figure 159 Mean color characterization of H&E stained breast biopsy section Eosin1 component in RGB color space.

Figure 160 Mean color characterization of H&E stained breast biopsy section Eosin2 component in RGB color space.

Figure 161 Mean color characterization of H&E stained breast biopsy section background component in RGB color space.

Figure 162 Standard deviation characterization of Movat rat tail section background component in HSV color space.

Figure 163 Standard deviation characterization of Movat rat tail section blue component in HSV color space.

Figure 164 Standard deviation characterization of Movat rat tail section collagen component in HSV color space.

Figure 165 Standard deviation characterization of Movat rat tail section dark pink component in HSV color space.

Figure 166 Standard deviation characterization of Movat rat tail section immature collagen component in HSV color space.

Figure 167 Standard deviation characterization of Movat rat tail section matrix protein component in HSV color space.

Figure 168 Mean color characterization of Movat rat tail section background component in HSV color space.

Figure 169 Mean color characterization of Movat rat tail section blue component in HSV color space.

Figure 170 Mean color characterization of Movat rat tail section collagen component in HSV color space.

Figure 171 Mean color characterization of Movat rat tail section dark pink component in HSV color space.

Figure 172 Mean color characterization of Movat rat tail section immature collagen component in HSV color space.

Figure 173 Mean color characterization of Movat rat tail section matrix protein component in HSV color space.

IMAGE EVALUATION TEST TARGET (QA-3)

APPLIED IMAGE, Inc.
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

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