Chapter 6

Color Segmentation Based
Morphometric Evaluation of Nerves
Using TissueQuant Software

6.1 Introduction

The proposed algorithm for staining intensity quantification can be used as an efficient technique for color segmentation. TissueQuant software, which is an embodiment of this algorithm, was designed as a generic solution for color intensity quantification which is easy to use and is based on human perception. It was intended to be simple so that a medical researcher can use the software without requiring high level of computer skills. The use of TissueQuant software for segmentation of color images based on color scores is described in this chapter.

Segmentation of color images is a complex task since color is represented by three primary color components. Various approaches have been used for achieving efficient segmentation. Some of these approaches are histogram based thresholding, color clustering, color difference measurement, local/global region based techniques, edge based techniques, watershed segmentation etc. RGB color model is not very suitable since it does not represent the colors according to the perception of human eye. Hence, various
studies have tried different representations of color such as CMYK, CIELuv, CIELAB and HSI color models.

Color scores are calculated based on similarity of colors using the HSI color model. Since this color model represents the color in an intuitive manner, the user can select the color parameters more easily. Color segmentation using color scores is found to be more accurate in comparison with the segmentation obtained with Adobe Photoshop software, as described in Chapter 3.

Use of TissueQuant software for the evaluation of morphometric parameters of nerves of forearm is illustrated in the following sections. This analysis is based on color segmentation achieved using color scores. The morphometric parameters namely nerve cross sectional area, fascicular area, number of fascicles, amount of adipose tissue, number of sympathetic fibers and their areas are considered in this part of the study. Also, the comparison of changes in the amount of adipose tissue with aging in different nerves of forearm is made based on these measurements.

6.2 Use of TissueQuant Software for Nerve Morphometric Study

A peripheral nerve is a bundle of axons travelling together in the peripheral part of the body. Individual axons are enveloped in a connective tissue wrapping called endoneurium. Bundles of axons are wrapped in a connective tissue covering called perineurium. The nerve as a whole is enveloped in a connective tissue sheath called the epineurium. The peripheral nerve is composed of nerve fibers, fascicles and non-fascicular components. Group of nerve fibers collected into bundles and enclosed in connective tissue sheaths, forms a nerve fascicle. The number, size and pattern of fasciculi vary among different nerves and also along the same nerve at different locations. The non-fascicular area of a nerve is occupied by connective tissue, adipose tissue, blood vessels, lymphatics and nervi nervorum. A peripheral nerve fascicle has sensory,
motor and autonomic fibers and its components (Kuczynski 1980). If the nerve contains sensory axons only, it is called a sensory nerve. Thorough knowledge of fascicular and non-fascicular morphology of a nerve is essential for the diagnostic application of ultrasound and computed tomography scan, as well as for its successful surgical repair (Ikeda et al. 1996; Maravilla and Bowen 1998; Bendszuc et al. 2004).

The radial nerve (RN) is the largest peripheral nerve in the upper limb, arising from the branches of the posterior division of ventral rami of brachial plexus. The superficial branch of the radial nerve (SBRN) is a sensory branch of radial nerve that runs under the cover of brachioradialis along the antero-lateral aspect of forearm. The anterior branch of the medial antebrachial cutaneous nerve of forearm (AMACN) supplies skin over the medial aspect of the forearm and the lateral antebrachial cutaneous nerve of forearm (LCNF) is the terminal sensory branch of the musculocutaneous nerve which supplies skin over the lateral aspect of the forearm. The RN, SBRN, AMACN and LCNF injuries can result from direct nerve trauma, compressive neuropathies, neuritis or due to neuromas and intraneural mucous cysts (Naam and Massoud 2004; Tzeng et al. 2005; Nawrot et al. 2007; Paraskevas et al. 2008; Thomsen and Dahlin 2007; Tsai and Steinberg 2008). The efficient management of these nerve injuries needs a thorough understanding of cross sectional peripheral nerve anatomy. The area occupied by the fascicular part, non-fascicular part and its components play an important role in predicting recovery after nerve injuries. Obtaining precise quantitative measurements of the peripheral nerve is a very important and challenging task in the field of medicine.

Nerve morphometry involves study of nerve bundle size, number or size of the axons, the number of fascicles and their sizes etc. It has been found to be of high importance in detection of developmental and pathological abnormalities (Jacobs and Love 1985; Dyck et al. 1984). In addition, nerve morphometric studies have been carried out in experimental nerve research to study the effect of diabetes on nerve morphology (Kamiya et al. 2005), to evaluate the effect of Memantine as treatment for glaucoma (Yu cel et al. 2006), to study the changes of laryngeal nerve associated
with aging (Tiago et al., 2008), to evaluate the recovery of hand after injury (Olave et al., 1996), to evaluate the contribution of acute inflammation to corneal regeneration (Li et al., 2011) and to study the effects of neonatal pinealectomy on microarchitecture of the sciatic nerve (Turgut et al., 2010). Most techniques used for estimating nerve parameters are based on manual measures which are highly time consuming and are error prone.

Computerized image analysis facilitates obtaining quantitative results which are accurate and highly reproducible. Manual judgment using the microscopic views of the stained sections of the nerves can only provide qualitative evaluation. Also, it suffers from inter-observer and intra-observer variations. The quantitative measurement is very much important to assess the nerve morphology. Hence, computerized image analysis plays a significant role in efficient and quantitative evaluation of morphometric parameters of nerves. Efforts on automation using image analysis for nerve morphometry have been reported (Jain et al., 1980; Garbay 1986; Ph et al., 1996). Thresholding is used to classify pixels between different tissue types which is followed by a structural analysis technique such as region growing, morphological operations or grouping of edge elements. Some other studies (Amini et al., 1990; Fok et al., 1996; Elmoataz et al., 1998) report use of Hough transforms and active contours. However these are computationally expensive and are not robust to variations in color and illuminations.

Use of TissueQuant image analysis tool to measure the morphometric parameters of nerves of forearm is described here. These parameters were used to establish a database for normal cases and also to evaluate the changes of adipose tissue with respect to age. Measurement of sympathetic fibre areas was also performed. This study was carried out on different nerves of forearm to observe the microanatomic variations among them.
6.2.1 Method

6.2.1.1 Sample Collection

From thirty three fresh human cadavers which were above 18 years, 30 samples each from RN, AMACN, LCNF and SBRN were collected for the study at cubital fossa. In this study, two centimeter long RN, SBRN, AMACN and LCNF tissue samples were obtained. From these samples, one centimeter proximal parts of each sample were subjected to paraffin sections (histological study) and one centimeter distal parts to frozen sections (immunohistochemical study) to identify sympathetic fibers in the fascicles of the nerves. The obtained nerve tissues were fixed immediately with 4% paraformaldehyde solution and embedded in paraffin (histological study).

The paraffin sections were used for Masson’s Trichrome staining to study collagen fibers in the non-fascicular area of these nerves. Serial six micron thick paraffin sections were taken from each nerve sample by using rotary microtome (Leica RM2125RT, Leica Biosystems Nussloch GmBH, Deutschland). From each paraffin block, consecutive three sections were selected and stained using Masson’s Trichrome. The morphometric analysis was performed under light microscope on these stained sections.

All the Masson’s Trichrome stained and immunohistochemically stained sections were focused under trinocular microscope (Olympus company), photographed (50x, 100x, 200x and 400x magnifications) with Motic live image program (Version 2.0, Motic China Group Co., Ltd.) for morphometric analysis. The images were of size 1024x768. All the images were analyzed using the TissueQuant software to measure the number of fascicles(Nf), total cross-sectional area(Asc), fascicular(Af), non-fascicular(Anonf), adipose(FAT), non-adipose(nFAT) and sympathetic fiber(Asym) areas.

6.2.1.2 Evaluation of Morphometric Parameters

For evaluating total cross-sectional area and fascicular area, contours were drawn manually around each of the fascicles in all the images. The contour was segmented out
of the image by appropriately adjusting the color settings. The same setting was used for all the images. Using the Batch Process option of TissueQuant software, the area covered by the contour was calculated in terms of number of pixels. The total nerve cross sectional area was also obtained in the same way by appropriately adjusting the color settings. For the purpose of calibration, a micrometer scale was photographed under the same magnification. Number of pixels representing a length of 1mm was calculated with the scale arranged in horizontal and vertical arrangements separately. This provided calibration for number of pixels representing one square millimeter of area. The non-fascicular area was derived from the difference between the total cross-sectional area and fascicular area. With these measurements, total cross-sectional area, individual and total fascicular area and non-fascicular area were calculated.

The screenshots of the selection of fascicular area and total nerve cross sectional areas are shown in Figure 6.1 and Figure 6.2 respectively.

As described, the tool facilitates extraction of boundary of fascicles and nerves and calculates areas of the enclosed regions.
6.2.1.3 Sympathetic Fiber Area Measurement

Tyrosine Hydroxylase immunohistochemistry included counting of sympathetic fibers and measurement of area occupied by sympathetic fibers (Asym) in fascicular area. Upon staining, the sympathetic fibers express themselves in shades of brown color. Through the user interaction option of TissueQuant, the color settings to appropriately select sympathetic fibers were obtained. These color settings were used with ‘Batch Process’ option of TissueQuant to process all images. The area occupied by sympathetic fibers was automatically written to Microsoft Excel sheet. The screenshot of the color selection is shown in Figure 6.3.

6.2.1.4 Changes of Adipose Tissue with Aging

Masson’s Trichrome staining was used for measurement of non-adipose tissue area in non-fascicular area of the nerve cross section. The non-adipose tissue area comprises of collagen tissue and connective tissue areas. The images were analyzed for the presence of collagen tissue (non-adipose tissue) which expresses itself with bluish green shades and connective tissue (non-adipose tissue) which expresses itself in reddish shades.
These two color settings were used and areas occupied by these tissues were obtained. From the previous step, fascicular areas and total nerve cross sectional areas were obtained. Using this information, total area of non-adipose tissues in non-fascicular area was calculated. The adipose tissue area in the non-fascicular area was calculated by taking the difference between non-fascicular area and non-adipose area. The collagen and connective tissue identification in the non-fascicular area is shown in Figure 6.4.

Fig. 6.4: The different nerve components identified by the tool, A: Connective tissue, B:Collagen, C: Fascicles, D: Color coded representation of these tissues to indicate identification of the non-fascicular tissues and E:Original color image with reddish brown connective tissue and bluish green collagen.
6.2.1.5 Validation

Initially, ten images were randomly chosen from the image data set for manual evaluation. Total nerve cross sectional area and fascicle areas were measured using point counting technique. Similarly, adipose and non-adipose classification was also done on a set of randomly selected ten images from the Masson’s Trichrome stained images. The sympathetic index calculation was also similarly done on the immunohistochemically stained images. All these measurements were carried out by the same observer. The results of the automated measurements were compared with the manual evaluation. In all cases, the differences in measurement were well within 10%.

6.2.2 Results

The section of nerves (RN, SBRN, AMACN, LCNF) stained by Masson’s Trichrome stain was analyzed for number of fascicles, total cross-sectional area, total fascicular area, non-fascicular area and the number of fascicles per $mm^2$ ($Nf/mm^2$). From the non-fascicular area, adipose and non-adipose areas were also calculated. Immunohistochemistry method was used to study area occupied by sympathetic fibers ($Asym$) in fascicular area and the ratio between them.

6.2.2.1 Morphometric Parameters

The cadavers’ age ranged from 37 to 88 years, with mean $60.93 \pm 14.90$ standard deviation. The nerves studied did not show much difference in the values between right and left nerves. Hence, the mean values of right and left nerves have been combined together to give the final values. The nerve cross sections in which the fascicles are identified using TissueQuant image analysis tool are shown in Figure 6.5. Table 6.1 lists the morphometric parameters for RN, SBRN, LCNF and AMACN nerves.

Number of fascicles, total cross sectional area, fascicular area, non-fascicular area and amount of adipose tissue and non-adipose tissues were observed to be maximum
Fig. 6.5: Identification of nerve and fascicle areas for the nerves: total cross section area of the nerves shown as yellow and the fascicles shown in white, for the nerves namely RN(A), SBRN(B), LCNF(C) and AMACN(D)

Table 6.1: Mean values of morphometric parameters of nerves (in $mm^2$)

<table>
<thead>
<tr>
<th>Nerve</th>
<th>Nf</th>
<th>Asc</th>
<th>Af</th>
<th>Anonf</th>
<th>FAT</th>
<th>nFAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN</td>
<td>11.35</td>
<td>7.19</td>
<td>2.66</td>
<td>4.53</td>
<td>2.84</td>
<td>1.69</td>
</tr>
<tr>
<td>SBRN</td>
<td>3.63</td>
<td>2.68</td>
<td>0.71</td>
<td>1.97</td>
<td>0.91</td>
<td>1.05</td>
</tr>
<tr>
<td>LCNF</td>
<td>8.85</td>
<td>2.99</td>
<td>0.55</td>
<td>2.44</td>
<td>1.44</td>
<td>0.99</td>
</tr>
<tr>
<td>AMACN</td>
<td>3.63</td>
<td>1.21</td>
<td>0.29</td>
<td>0.92</td>
<td>0.30</td>
<td>0.63</td>
</tr>
</tbody>
</table>

in RN and minimum in AMACN among all the four nerves. Non-fascicular area and FAT in LCNF was more when compared to SBRN and AMACN.

6.2.2.2 Sympathetic Fiber Area Measurement

Sympathetic fiber area (Asym) was maximum in fascicular area of RN and minimum in AMACN among all the four nerves. There was no significant correlation with age in all the four nerves. Sympathetic index (SI) was obtained by dividing the area of the sympathetic fibers (Asym) by the fascicular area (Af) as $SI = \frac{Asym}{Af}$. SI was more in SBRN and less in RN among all four nerves. Figure 6.6 shows the plot of sympathetic indices of RN, SBRN, LCNF and AMACN nerves.

6.2.2.3 Changes of Adipose Tissue with Aging

The non-fascicular area was well developed in all the nerves studied. The non-fascicular area was more when compared to fascicular area due to adipose or non-adipose tissue deposition in it. In RN, SBRN and AMACN, total cross sectional area was found to be more in elderly individuals compared to younger individuals due to increased amount
of adipose tissue in it. More adipose deposition was observed in LCNF, without definite relationship with age.

Figures 6.7, 6.8, 6.9 and 6.10 show the changes of adipose tissue with respect to age in the nerves namely RN, SBRN, LCNF and AMACN respectively.

To assess the relationship between age and amount of adipose tissue, Pearson correlation test was carried out. The correlation coefficients and the P-values for all the four nerves are listed in Table 6.2.
Fig. 6.8: Plot of age versus FAT in SBRN

Fig. 6.9: Plot of age versus FAT in LCNF

Fig. 6.10: Plot of age versus FAT in AMACN
Table 6.2: Correlation of changes of adipose tissue with respect to age

<table>
<thead>
<tr>
<th>Nerve</th>
<th>Pearson correlation coefficient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN</td>
<td>0.963</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td>SBRN</td>
<td>0.96</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td>LCNF</td>
<td>0.016</td>
<td>$p = 0.932$</td>
</tr>
<tr>
<td>AMACN</td>
<td>0.869</td>
<td>$p &lt; 0.001$</td>
</tr>
</tbody>
</table>

6.2.3 Discussion

(Sladjana et al., 2008) reported the study on relation of fascicular pattern to microanatomic morphometric characteristics of connective tissue sheaths of sciatic nerve. In this study, they used double square lattice-B100 which was placed on the screen of a projection microscope and manually evaluated the morphometric parameters. Studies based on gray scale image analysis based on threshold, region grow or active contours (Jain et al., 1980; Garbay, 1986; Ph et al., 1996; Amini et al., 1990; Fok et al., 1996; Elmoataz et al., 1998) are not robust and are prone to error with variations in illumination which is a commonly observed problem with images obtained from microscopy.

To investigate whether antioxidants vitamin Z and vitamin C could retard development of hepatic fibrosis, authors in (Soylu et al., 2006) carried out histological analysis of liver tissue using SAMBA software to determine the collagen content. A custom made program was developed to automatically outline and measure the collagen area and the total liver area. SAMBA 4000 can differentiate between 256 different hues. These hue values were used to identify the positive staining. Many research groups have reported using SAMBA for image analysis for the immunohistochemical staining quantification (Charpin et al., 1989; Auger et al., 1993; Esteban et al., 1994; Guidozzi et al., 1996; Lin et al., 2004).

A report on assessing biopsy specimen of terminal branch of posterior intraosseous nerve in the forearm can be found in (Thomsen et al., 2009). In this study, images of all fascicles were captured from the sections of biopsy specimen and Image Pro Plus
software was used to measure fascicular area and sub perineurial space. Image Pro Plus provides ‘Contrast Enhancement’ tool to enhance staining contrast, a ‘Flatten Background’ filter to provide correction to illumination variations. The ‘Irregular area of interest’ can be used to select the area to be measured.

Authors in (Yadav et al., 2009) report assessment of neural hypertrophy using Scion image analysis software. Images of relevant areas were analyzed, size and perimeter of the thickest nerve fiber in submucosa were measured and the count of nerve fibers was obtained.

Study on the endoneurial ECM content in dorsal and ventral spinal roots in relation to their axon type differences was reported in (Petr et al., 2001). An intensity of the immunofluorescence staining was assessed by computer-assisted image analysis tool called Lucia-G v4.21 (Laboratory Imaging, Prague, Czech Republic) using interactive segmentation in the HSI color space to select the measured areas of digitized pictures.

A color subtractive - computer assisted image analysis (CS-CAIA) system for quantification of cutaneous nerves in diabetic mouse model has been reported (Underwood et al., 2001). In this work, the authors used Adobe Photoshop and Image Processing Toolkit (Reindeer Graphics; Nashville, NC) to develop CS-CAIA to generate fast and consistent morphometric data.

However, these techniques would fail to manage the possible variations in staining variations and non-uniform illuminations. The proposed approach was designed to be able to manage wide variations in staining. As shown in Figure 6.11, the performance of this tool was tested on varying staining intensities and varying illumination effects. This approach works well, because it just segments out the circle which is drawn in a unique color and filters out the pixels with the specified color.

In the case of evaluation of the amount of collagen and connective tissues, this study considers the possibility of staining intensity variations due to non-uniform staining. The color segmentation algorithm developed for a particular set of images need not be suitable for another set in all cases. Color shades could vary though primarily the hue
might remain the same. For example, the collagen tissue takes up shades of blue every
time it is processed. But the shades of blue could be differing each time, depending on
the processing and staining variations and changes in acquisition parameters. The color
parameters for collagen tissue segmentation would then be chosen such that different
possible bluish green shades can be included. Similarly different possible shades of red
are included into segmentation as connective tissue. The color setting adjustment is
used to take care of this issue. TissueQuant image analysis tool can manage to include
all the shades of a color in an efficient manner and this helps in accurate segmentation
of the region of interest.

In the case of evaluation of sympathetic fiber areas, other components in the speci-
men may take up purplish shades. It is challenging to eliminate the purple pixels from
the brown pixels which represent sympathetic fibers because both are inseparable in
grayscale and in RGB domain. TissueQuant uses HSI color model and it facilitates
fine tuning with different weights for different shades of brown and purple and hence it
is possible to segregate the two and keep up only the ones with the pixels representing
the sympathetic nerve fibers.

In the case of evaluation of total nerve fiber area and the individual areas within
the nerve fiber, it is required to encircle these areas manually. This becomes necessary since the boundaries of fascicles and nerves are not well defined as can be seen in Figure 6.1 and Figure 6.2. Most of the previous studies too depend on the user interaction to define these regions.

Point counting is the most widely used manual technique for morphometric studies. A grid is superimposed on the projection screen and the intersection of the region of interest with the cells of the grid is counted to measure the area of the region of interest. This is tedious, time consuming and error prone. The time taken for a single measurement would be approximately 5 to 15 minutes for an experienced observer, depending on the size of region of interest. It is impossible to study large number of cases in this manner. Even though the approach taken in this study requires manual drawing of contours, it is much simpler and less error prone in comparison with the point counting technique. It provides easy, quick and efficient way for analysis of large number of images.

6.3 Conclusion

Color scores are useful for quantification of stained substances and for accurate segmentation. Medical research team depends on programmers or skilled technical people for use of this algorithm for their analysis. Hence, to facilitate the use of this algorithm for medical research applications, TissueQuant image analysis tool was developed which was designed to be simple and user friendly to be used by medical researchers themselves. This chapter has demonstrated the use of TissueQuant image analysis software for automation of measurements of morphometric parameters of nerve, in which the various components of the nerves are segmented and measured. It can be concluded that TissueQuant is useful as a simple and generic tool for color analysis in medical research applications.

The next chapter provides a conclusion to this thesis.