Processing, visualization and analysis of 3D confocal microscopic images

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Processing, Visualization and Analysis of 3D Confocal Microscopic Images

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Abstract

Confocal laser scanning microscopes have been widely employed in biology research for many years. The advancement of computer technology enables interactive visualization and quantitative analysis of volumetric confocal image datasets thus giving deep insights into cellular structures.

This thesis details the computer techniques developed by the author to facilitate 3D confocal image studies. The key contributions of this work are in (1) confocal image acquisition and management, (2) 3D image processing, and (3) Virtual Reality (VR) enhanced visualization and analysis of cellular structures.

With a large amount of confocal images produced with microscopes for different biological studies, the administration of these image datasets become crucial. We develop a solution enabling convenient storage and management of confocal image data with a central image administration database. Furthermore, the integration of Internet techniques with the database makes it possible for online data access in a collaborative research environment.

Raw confocal image data has various problems thus usually needs to be processed before visualization and quantitative analysis. A fast interpolation algorithm to produce

high resolution confocal images has been developed using a look-up table (LUT) technique. Image intensity degradation with depth is a major problem affecting the qualities of confocal images. In this project, an adaptive algorithm making use of the spatial coherence between consecutive confocal images has been developed to correct the image depth attenuation. A novel restoration model is proposed to recover the intensity value for each optical section.

Using the *Gaussian Divergence Theorem*, we have developed a method to measure the suitability of gradient magnitudes with a defined *Measurement Function (MF)* for identifying 3D boundary points of confocal objects. With the detected boundary information of gradient magnitudes for volumetric points, we propose a 2D transfer function design for volume rendering and a histogram-based method to derive the proper iso-value for surface rendering in order to improve the visualization of 3D confocal images.

As an enabling technology, VR extends the limits of the interaction between human and computer. Efforts exploring the application of VR techniques to better facilitate the analysis and manipulations of visualized 3D confocal images are covered in this project.

A software system “*CellStudio*” is designed and developed to provide consistent image processing, volumetric visualization and analysis of 3D confocal image data. We have applied *CellStudio* in studying the expression pattern of specific proteins that are closely related to cardiovascular heart disease.

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Abbreviations

Apo(a)	Apolipoprotein(a)
ApoE	Apolipoprotein E
BCM	Bio-Chemical Module
BIM	Basic Information Module
CHD	Cardiovascular Heart Disease
CIM	Confocal Imaging Module
CLUT	Contribution Look Up Table
CPM	Clinical Preparation Module
DMEM	Dulbecco's Modified Eagle Medium
FA	Functional Area
FBS	Fetal Bovine Serum
FIR	Finite Impulse Response
IIR	Infinite Impulse Response
LDL	Low Density Lipoprotein
LUT	Look Up Table
Lp(a)	Lipoprotein(a)
MF	Measurement Function
OME	Open Microscopy Environment
QAM	Quantitative Analysis Module

SNR	Signal-to-Noise Ratio
TF	Transfer Function
Voxel	Volume Element
VR	Virtual Reality
WWW	World Wide Web

1. Introduction

The study and analysis of biological structures requires 3D imaging capabilities. The advent of confocal laser scanning microscope since the 1980's has made it possible to record 3D microscopic images of biological specimens at a high resolution. The dramatic technical advancements in confocal technology open up a new chapter in visualizing cellular structures. Today, confocal microscope has become a versatile and effective tool for biology research. Furthermore, the coupling of confocal microscopy with modern computer techniques provides valuable quantitative information and thus allows deep insight into cell and organelle biology. This thesis is the culmination of the research work done by the author in managing, processing, visualizing and analyzing 3D confocal microscopic image datasets using advanced computer techniques.

1.1 Background and Motivation

There are currently a variety of confocal systems with differing configurations and capabilities, and all operate on the principle of illuminating a small portion of the tissue and restricting the collection of light from a rather narrow focal plane in the sample. With the capability to eliminate out-of-focus information, confocal microscopes can perform non-invasive 3D inspection and imaging of sub-cellular structures. The capacity afforded by these modern confocal microscopes to peer into a relatively thick biological specimen at high spatial resolution has impacted greatly neurobiology and many other areas of biological investigation. As a result, confocal microscopy is enjoying wide applications in biological sciences, enabling studies from the molecular level to that of the whole organism.

The outputs of confocal microscopes are a series of 2D digitalized images. The optical image stacks generated are normally contaminated with artifacts and noise due to improper specimen preparation, imperfect imaging instruments and non-linear optics. Redesigning the optics and instrument electronics to obtain better-quality images is an expensive and challenging task with limitations to achieving optimality. Hence, image-processing techniques have been applied to reduce noise and artifacts and correct some of the non-linearity present in the images.

Besides noise and artifacts, the uneven illumination along the depth of a specimen is another major problem for confocal imaging, resulting in a spatial variation of light intensity in the recorded optical sections. In confocal images, the variation in image intensity is not only due to photo-bleaching of the specimen, but also caused by some optical problems like light scattering and absorption during light transmission through opaque specimens. Degradation of image intensity along the depth of the

specimen can be approximately modeled as a decay function and hence computationally corrected. Many research works have been done to predict the decay function.

To serve the purpose of visualization and analysis of confocal image stacks, some data exploration work needs to be done in advance. Boundary detection is one of such works. Most edge detection algorithms developed for 2D images can be extended to 3D boundary detection. Many of these algorithms estimate the discrete approximations of some differential operators to measure the rate of changes of the intensity field (gradients), and then detect the surface points based on a magnitude threshold of the gradient magnitudes. The extracted boundary surface information can be utilized in volumetric visualization like transfer function design in volume rendering and iso-value derivation in surface rendering, and cellular object segmentation.

After image processing, volumetric visualization techniques can be applied to display 3D confocal images to enhance visual perception of cell structures. Based on volumetric visualization techniques, a variety of software systems have been developed for confocal data exploration. Two visualization techniques are commonly used for displaying volumetric confocal image stacks: surface rendering and volume rendering. In surface rendering, the surface of the desired structure is represented by a group of polygons extracted with algorithms like *Marching Cube* (Lorensen and Cline, 1987). With the application of modern accelerating computer graphics cards, the polygons can be rendered and displayed very quickly. The challenging work for surface rendering is to find the appropriate intensity value that can define properly biological structures for polygon extraction.

Volume rendering visualizes volumetric biological structures by shading each volumetric point and projecting the colored, semi-transparent point to a chosen projection plane. Unlike surface rendering, volume rendering does not need to generate

surface polygons. Compared with surface rendering, volume rendering achieves better performance in displaying complex structures. However, it has the disadvantage of slow processing speed due to intense computation. Fortunately, with the advancement in specific hardware design for volume rendering (Pfister *et al.*, 1999), real time rendering speed has been achieved without quality degradation. To achieve optimal visualization effect, an important issue in volume rendering is to assign appropriate color and transparency values to volumetric points based on their numerical values, which is called transfer function design.

Visualization techniques are able to produce 3D information from confocal datasets. To enhance the 3D information in the displayed images, advanced stereo display methods are applied in this work to create an immersive environment. In stereo display methods, based on the principle that human beings associate depth perception with stereopsis in the real world, a pair of images of the same structure are produced with a small shift in the viewing direction for the left eye and right eye, respectively. As a high-end user interface that involves real-time simulation and interactions through multiple sensorial channels, Virtual Reality (VR) has been introduced in 3D based image analysis. One of the VR applications is to assist the interactive quantitative analysis of cellular structures.

Confocal image stacks record the fluorescence information inside the specimen. However, information on specimen preparing, confocal imaging configurations, data interpretation and analytical result is separated from the confocal images. Current software for confocal microscopy provides processing, visualization and analysis solutions for 3D confocal images, but does not keep track of analytical data in a systematical way. This motivates us to design an integrated solution using information technology. The Internet, as a powerful communication medium, can also be exploited

in confocal image informatics. With the Internet, users are free to upload, download, view and edit the confocal images across different time and space zones. The application of the Internet is significant especially within collaborative confocal research environments. As a result, the downstream operations of processing, visualization and analysis of confocal images will be more efficient and effective.

1.2 Objectives

Although there have been fast advancements in the developments of confocal microscopes, most confocal image stacks generated with confocal microscopes are anisotropic in terms of scan intervals in the scan direction and its perpendicular plane. This may cause problems with some downstream operations like volumetric visualization. To obtain cubic volumetric elements (voxel), interpolation from the original datasets is required to insert new slices from existing images with high accuracy. Most existing interpolation algorithms are, however, computationally intensive and time-consuming. One of the objectives of this research is to speed up the interpolation operation without sacrificing the accuracy. The computational speed for interpolation with this algorithm should be enhanced for general confocal image stacks.

Depth intensity attenuation is a major problem that troubles the quality of confocal images and leads to fault or unprecise analysis results, especially for thick specimens. There is much research developed to solve this problem. The nature of light intensity changes between adjacent optical sections is important to measure the degree of attenuation, but it is often ignored in the prior art. Based on the physical model of light scattering and absorption (Wyszecki and Stile, 1982), the second objective of this research is to develop a novel method for adaptive compensation of attenuation of light

intensity for confocal microscopic images through detecting and employing the intensity changes between neighboring optical sections. This method should adaptively estimate the degree of intensity degradation and correspondingly deduce the individual correction factor for each optical section. In addition, the calculation for each point with the correction factor should also be designed to avoid the influence of noise.

Gradient is an important numerical property for volumetric points and is often used to provide support for boundary detection and visualization including volume and surface rendering. Selection of the proper gradient magnitude as the threshold for edge and boundary surface detection is usually implemented through trial-and-error. The third objective of this research is to develop a new solution to quantitatively and automatically measure the suitability of gradient magnitudes as the potential threshold for 3D boundary surface detection with an evaluation function. The suitability evaluation function should be defined based on the criteria of boundary detection. The values of the evaluation function should offer sufficient information to judge each gradient magnitude and thus the optimal gradient magnitude with the best suitability can be easily identified.

Besides identifying boundary surface points, the extracted gradient information can also be applied to improve the quality of volumetric visualization. This is another objective of this research. The results of boundary detection can help to derive the appropriate iso-value for surface rendering. Transfer function design in volume rendering helps to classify and display different structures with different color and transparency values, enabling users to perceive better visual effect. The combination of intensity and gradient magnitudes can be utilized to design the proper transfer function to visualize the location relationship between boundary surface points and internal points within the same cellular structure.

In conventional confocal imaging research, procedures of specimen preparing, image capturing, image processing, 3D reconstructing and visualizing are often conducted separately at different locations with different hardware and software, and by different people. As a result, data communication for collaborative confocal research between separate locations becomes very crucial to researchers. Therefore, another objective of this research is to develop an applicable solution for collecting, transmitting, processing, visualizing and analyzing 3D confocal images. This solution should also integrate Internet-enhanced database technology to enable remote access to the stored confocal image datasets for collaborative research.

The last but not least objective of this research is to explore the roles of VR for the analysis and manipulations of cellular structures.

1.3 Thesis Organization

This thesis is the culmination of the research work done by the author in the management, processing, visualization and analysis of 3D confocal images captured with confocal microscopes. Chapter 2 reviews literatures in related perspectives, namely, the development of confocal imaging, confocal image enhancement, depth attenuation correction, boundary detection, 3D reconstruction and visualization, VR-enhanced analysis and the applications of informatics in confocal imaging.

Chapter 3 introduces an Internet-based confocal image data management system which supports online collaborative confocal research. The three subsequent chapters present our methods for 3D based confocal image processing. Chapter 4 illustrates a fast intensity-based interpolation method designed to enhance the axial resolution of confocal image stacks. To solve the image intensity degradation problem for large

confocal image stacks, Chapter 5 introduces an algorithm which can adaptively generate the correction parameters using the intensity change rate between consecutive confocal images. Chapter 6 presents a novel method to quantitatively evaluate the suitability of gradient magnitudes for the detection of boundary surface points of 3D confocal image stacks. The optimal gradient magnitude obtained can be utilized for boundary point detection and volumetric visualization.

Chapter 7 illustrates the research work on the improvement of volumetric visualization of 3D confocal images. The emphasis here is placed on the applications of VR techniques in visualization and analysis of volumetric confocal datasets.

Chapter 8 presents an integrated system incorporating management, processing, visualization and analysis for 3D confocal image datasets. The architecture of the system and implementation of the system are described. This chapter also shows the application of this system for the study of cardiovascular heart disease.

Finally, summary and future improvements of the research work are discussed in Chapter 9.

2. Literature Review

Confocal imaging of single cells and their internal structures labeled with fluorescence probes is now routinely performed and provides valuable information in life science research. The coupling of modern computer technologies with confocal imaging has greatly enhanced the capabilities of confocal microscopes allowing further enhancement, manipulation and analysis of the confocal images obtained. As a result, analysis of 3D architecture of cells, which cannot be accomplished by conventional light microscopy, is now possible. This chapter reviews the developments of confocal microscopy techniques and computer techniques applied to improve confocal microscopy. In Section 1, a brief introduction of confocal imaging is given. In Section 2, we investigate the applications of information technology for confocal imaging. In Section 3, we discuss currently available confocal image processing techniques, including image enhancements, attenuation correction and feature extraction. The applications of volumetric visualization techniques in studying confocal images are given in Section 4. Section 5 summarizes this chapter.

2.1 Laser Confocal Microscopy

Laser confocal microscopy is considered to be the most significant advancement in optical microscopy in the 20th century (Shotton, 1989). In the past three decades, confocal microscopy has been developed and applied in life science research and has become a versatile tool enabling studies from the molecular level to that of the whole organism (DeBiasio *et al.*, 1988; Summers *et al.*, 1993; Dunn *et al.*, 1994; Lichtman, 1994; Rizzuto *et al.*, 1996; Turner *et al.*, 1996; Lichtenstein *et al.*, 2003; Akita *et al.*, 2004). The main advantage of confocal microscopy is the elimination of out-of-focus information through a pinhole device at the front of the signal detector (Pawley, 1995), thus allowing accurate and non-destructive optical sectioning in a plane perpendicular or parallel to the optical axis of the microscope. The basic principle of laser confocal microscope is illustrated in Figure 2.1. In a confocal microscope, visible or ultra violet laser emission is focused on the first confocal pinhole and then onto the specimen stained with fluorescent as a diffraction-limited light spot. The primary incident light is reflected from a particular point or emitted from fluorescent molecules excited by it. Emissions from the point return along the primary laser light pathway and depart from it by lateral reflection from a dichroic mirror onto the second confocal pinhole. The aperture is confocal with the in-focus point in the specimen. The virtual elimination by defocusing of all distal and proximal flanking emissions at this physical point assures that the light passing onto the detector, a sensitive photo-detector or camera, is specifically derived from in-focus point with a resolution. After scanning the specimen slice by slice, a series of images in the x - y plane are recorded along the z axis of a microscope, so that the entire labeling can be subsequently volume-rendered and analyzed quantitatively. These serial images are commonly referred to as the image stacks.

Figure 2.1 The illustration showing the data collection by a confocal microscope

There are some other advantages with confocal microscopy that make confocal imaging effective and efficient for investigating the 3D architecture of cells in tissues. Firstly, equipped with multiple light sources of different wavelengths, a variety of fluorescence probes can be excited. There are several commonly used sources of excitation including argon (Ar; 488nm and 514nm), krypton (Kr; 568 nm), argon-krypton (Ar-Kr; 388 nm, 514 nm, and 568 nm), and helium-neon (He-Ne; 633 nm) lasers. The variety of excitation wavelengths for a confocal microscope offers the power and convenience of using combinations of fluorescent probes in a single specimen. But one major problem with multi-probe confocal imaging is the potential artifacts introduced by chromatic aberration in the image system (Pawley, 1995). Secondly, the advantage with confocal microscopy is the high resolution in imaging sub-cellular structures. It is feasible to collect a single stack of optical sections at resolution of $0.1 \mu\text{m}$ per pixel. However, the useful images in confocal microscopy are limited to depths into the tissues of less than $100 \mu\text{m}$ due to the light absorption and scattering by the

tissues (Laurent *et al.*, 1994). Thirdly, the confocal microscopy also has the capabilities to reconstruct and render the 3D structures of cells in their native tissue settings once coupled with volumetric visualization techniques (Kaufman *et al.* 1990; Sakas *et al.*, 1996; Razdan *et al.*, 2001; Eils and Athale, 2003; Liu and Chiang, 2003). This helps researchers to better interpret the spatial relationships of cells and tissue structures. Fourthly, with time-lapse confocal microscopy, information about the dynamics of cellular structure over time can be collected and subsequently analyzed (O'Rourke and Fraser, 1990; Smith *et al.*, 1990; Chen and McConnell, 1995; Adams, 1996; Zimmermann and Siegert, 1998). 4D confocal imaging is achieved by collecting 3D confocal images at time intervals. The resulting time series of confocal image stacks can be used to reconstruct 3D views of dynamic cell and tissue development.

However, there are also several factors that affect the quality of 3D images generated using confocal microscopes. Firstly, the reflection, refraction and scattering of the incident beam by the parts of the specimen located between the focal plane and the objective lens can, in principle, reduce the intensity of incident radiation. Secondly, the fluorescence photons can be attenuated (again by reflection, refraction, scattering and absorption) by the same upper structures on their way back (Visser *et al.*, 1991). Thus both the incident laser beam and the fluorescence emission are unable to pass through tissues more than 100 μm thick. Recent advances in imaging systems using infrared lasers are designed to enhance the laser penetration and thus excite the fluorescent probes twice deeper than a conventional confocal microscope. Liu and Chiang (2003) proposed a new technology named "*FocusClear*", a water-soluble clearing agent, to increase the tissue transparency allowing more light transmission. Thirdly, due to the bleaching of fluorochromes, each slice in the specimens does not receive the same amount of irradiation as its scanning begins. The photo-bleaching problem is merely due

to a reaction of molecular oxygen with the triplet excited state of the dye to produce highly reactive singlet oxygen (Pawley, 1995).

2.2 Applications of IT in Cellular Imaging

To facilitate quantitative analysis and knowledge discovery from confocal imaging, Internet, database and information technologies have been applied to facilitate the confocal data administration and image processing (Carazo *et al.*, 1996, 1998; Carazo and Stelzer, 1999; Lindek *et al.*, 1999; Pittet *et al.*, 1999; Swedlow *et al.*, 2003; Bonetto *et al.*, 2004).

Carazo *et al.* (1996, 1998) introduced *BioImage* database which is an object-relational database for multi-dimensional microscopic images of biological specimens available through the World Wide Web (WWW). This object-relational system allows the handling of complex data using features such as collection types, inheritance, and user-defined data types. The Web Integration Option enables WWW access to the database. Pittet *et al.* (1999) added two nodes to the *BioImage* database: a real-time iso-surfacing node for VRML based on the *Marching Cube* algorithm and a texture-based volume rendering node. In this way, the *BioImage* database has been established as a database prototype for multi-dimensional microscopic data with the ability to view the data within a 3D interactive environment. In 2003, Swedlow *et al.* implemented a similar project by developing an *Open Microscopy Environment (OME)* as an informatics solution for the storage and analysis of optical microscope images. *OME* can automatically manage images captured from all optical microscopes including confocal, wide-field, and multi-photon systems.

Bonetto *et al.* (2004) presented a software package, named “*Power Up Your Microscope*”, designed and developed for the optimization of 3D optical microscopic image quality using the Internet and inverse problems computational approaches. The package is mainly devoted to 3D microscopy users, being operative for wide-field confocal and multi-photon microscopy. It provides the microscopy community with an extremely easy and comparatively powerful access to advanced image restoration methods. The core of the computational section is the optical system modeling and inverse deconvolution implementation, which is strongly linked to web-based software and technology. Their project constitutes a real and effective migration to the web, extending computational approaches to image restoration to the whole microscopy user community.

2.3 Confocal Image Processing

2.3.1 Image Enhancement

Usually, the images generated by confocal microscopes are in digital form. However, these raw confocal image data often contains noise, poor contrast, poor signal-to-noise ratio and unequal resolution in spatial and lateral directions, which adversely affect downstream visualization and analysis (Razdan *et al.*, 2001). Thus, a wide range of traditional and sophisticated digital image processing capabilities need to be applied to confocal image datasets to enhance the image quality (Gonzalez and Woods, 1992; Russ, 1995; Bovik, 2000; Gruber *et al.*, 2005).

Ancin *et al.* (1996) summarized the necessary processing work to enhance confocal image quality. To remove noise artifacts in general confocal images, Chen *et al.* (1995) introduced linear filters and nonlinear filters. They recommended applying linear

filters in frequency space and preferred the use of linear filters built from sums of *Gaussian* functions to avoid the introduction of ripples and contrast inversions that can otherwise be generated. Adiga and Chaudhuri (2001) presented some new techniques for confocal image processing. They developed an automatic and fast method to separate foreground from background of confocal images with non-distinct background by defining a good threshold in the intensity histogram curve with an empirical formula. They also presented an interpolation technique based on XOR contouring and morphing to virtually insert the image slices in the image stack to enhance the axial resolution. Their interpolation technique has the merits of both contour-based (Boissonat, 1988) and intensity-based interpolations (Lehmann *et al.*, 1999).

To achieve high quality visualization results, Fang *et al.* (2000) integrated image enhancement techniques with transfer function design in volume rendering in their image-based transfer function model. They summarized two types of image enhancement techniques commonly used: point enhancement and spatial enhancement. The point enhancement operations apply some functions to each intensity value individually to generate a new one. The most common point enhancement operation is intensity modifications in which the intensity curve of the input volume is altered in certain intensity intervals to increase or reduce the exposure of the corresponding regions. The spatial enhancement operations, including smoothing and sharpening operations, derive the new intensity values of a given point from its neighborhood points.

2.3.2 Depth Attenuation Correction

In a 3D confocal image stack, deeper sections in the specimen are produced with lower photon intensity due to scattering and absorption of both excitation and fluorescence light and photo-bleaching (Pawley, 1995). This effect severely reduces the quality of the

images and restricts their quantitative analysis. Therefore, much research has been carried out to correct the depth attenuation phenomenon in confocal images.

Early methods for attenuation correction are focused on predicting or measuring an attenuation function. The function will be applied directly to the images to minimize the attenuation influence. There are conventionally two basic approaches to predict the attenuation function: statistic modeling (Rigaut and Vassy, 1991; Liljeberg *et al.*, 1995; Adiga and Chaudhuri, 2001; Kervrann *et al.*, 2004;) and geometric modeling (Visser *et al.*, 1991; Roerdink and Bakker, 1993; Margadant *et al.*, 1996; Sun *et al.*, 2004). Both methods first compute a one-dimensional (along the z direction) spatial correction factor based on the statistical or geometric model, and then adjust the intensity for a slice at a given depth with a multiplicative correction factor.

The statistic methods model the distribution of the intensity along the axial direction as a function of depth by estimating histograms and fitting curves, assuming that the fluorochrome is distributed homogeneously in the stained tissues. As a simple example, Adiga (2003) introduced a simple iterative method of fitting a scale factor to each image slice to restore the image intensity. The difference between the average intensity value of each slice and that of the reference slice with the maximum average intensity value is calculated and used as the correction factor to enhance each point on each slice. The method introduced by Liljeberg *et al.* (1995) is based on studying the stack of 2-D histograms that can be formed from each consecutive pair of sections in a stack of optical sections. An attenuation factor is calculated interactively and a new compensated section series is computed. Capek *et al.* (2006) developed a two-stage histogram-based algorithm to correct the attenuation problem. In the first stage, from all images in the series they create a standard histogram maintaining relative frequencies of gray levels and improving brightness and contrast. In the second stage, individual image

histograms are warped according to this standard histogram. They tested their methods on real confocal image data and it was shown that their approach diminishes the light attenuation in images captured from deep layers of the specimen. The statistic modeling methods have an advantage of less computing time-consuming, however, because a correction factor is equally multiplied to all points including noise signals, the signal-to-noise ratio (SNR) is not improved after correction.

The geometric modeling methods are based on the assumption that the image intensity is a function of fluorochrome density. Visser *et al.* (1991) described the underlying optical geometry of confocal imaging and presented a reconstruction algorithm based on a layer stripping method. The light bundle introduced in their paper is assumed to travel as a spherical wave that converges to the focal point and forms a cone structure. The attenuation of excitation and fluorescence light is computed by integrating all light paths within this conical volume. However, this method is computationally expensive and time consuming especially for stacks with high resolution in the z direction. Following the model introduced by Visser *et al.*, Roerdink and Bakker (1993) reformulated the problem by 3D convolution integrals to reduce the computational time. To enhance the accuracy of Visser *et al.*'s model (1991), Roerdink (1994) reformulated the problem as a statistical estimation problem. By using a similar cone structure, Margadant *et al.* (1996) reduced the computational memory requirements. In the geometric based methods, the estimation of the light scattering coefficient is of high importance. Collier *et al.* (2005) presented evidence that the scattering coefficient of cervical epithelium varies by a factor of 3 within the epithelium owing to variations in nuclear density and to the presence of keratin. They estimated the scattering coefficient from regions of normal and precancerous cervical epithelium by fitting reflectance measurements from confocal images to an exponential function of depth based on Beer's

law of attenuation. However, the geometric modeling methods still suffer from the SNR problem noted above for statistic methods.

Besides the two approaches mentioned above, there are also some novel approaches for attenuation correction. Can *et al.* (2003) presented a two-view method for attenuation correction while improving the SNR. Their method is a combination of slightly modified specimen preparation, image registration, montage synthesis and signal reconstruction methods. The specimen is mounted in a symmetrical manner between a pair of cover slips, rather than between a slide and a cover slip. It is imaged sequentially from both sides to generate two 3D image stacks from two perspectives. An automated image registration algorithm performs a precise 3D alignment, and a model based minimum mean squared algorithm synthesizes a montage, combining the content the both the 3D views. Lee and Bajcsy (2006) developed an intensity heterogeneity correction technique that (a) adjusts the intensity heterogeneity of 2D images, (b) preserves fine structural details and (c) enhances image contrast, by performing spatially adaptive mean-weight filtering. Their solution is obtained by formulating an optimization problem, followed by filter design and automated selection of filtering parameters.

2.3.3 Object Feature Extraction

Feature extraction from volumetric confocal datasets is an important operation for the interpretation and analysis of the scanned specimens (Kyan *et al.*, 2001; Soltanian-Zedeh *et al.*, 2005, Huang *et al.*, 2006). The extraction operation can provide valuable information for downstream visualization and quantification. There are two categories of feature extraction operations: boundary detection and object segmentation.

Boundary detection operations find the boundary surface points from image data. For edge detection of 2D images, Canny (1986) developed an effective method by using both the first and the second derivatives of the intensity function. The automatic identification of 3D boundary points within 3D images such as confocal microscope images is analogous to the edge detection problem in 2D image processing. As a result, many existing surface detection techniques are 3D extensions of 2D edge detectors (Liu, 1977; Zucker and Hummel, 1981; Bomans *et al.* 1990; Monga *et al.*, 1990). Based on the method developed by Morrone and Owens (1987), Pudney *et al.* (1995) implemented a 3D local energy feature detector to extract surfaces from 3D confocal image datasets. The local energy of a 1D signal is obtained by convolving the signal with a quadrature pair of filters, and Pudney *et al.* extended it to 3D signal by convolving the 3D dataset with oriented pairs of 3D versions of *Morlet* wavelets. In order to increase the speed of the convolution, the filters are designed in frequency space and multiplied by the image's *Fourier* transform. Although Pudney *et al.*'s algorithm is able to accurately locate some feature types such as membrane-like structures in confocal microscope images, it is, however, time-consuming because of the calculation of *Fourier* transformations.

To realize boundary surface detection in confocal microscope datasets, Fang *et al.* (2000) studied two categories of approaches (iso-surface based and dynamic detection based). In the iso-surface based approach, a histogram of all the boundary points from the boundary detection operator is generated and smoothed with a *Gaussian* function in order to remove noise. The intensity values at which the histogram achieves a local maximum are used to locate the boundary surface points. To detect the surface which cannot be simply defined as a single iso-value surface, especially in confocal datasets that suffer from photo-bleaching problems, Fang *et al.* (2000) suggested that the gradient

is a more effective measure in which only points with gradient magnitudes higher than the gradient threshold can be considered as boundary points.

The segmentation operations of 3D confocal images aim to separate cellular structures in the same specimen from each other which can be achieved by interactive and automatic approaches. Interactive methods, based on drawing with a mouse around objects in sequence (Czader *et al.*, 1996; Tekola *et al.*, 1996), or orthogonal 2D slices (Lockett *et al.*, 1998; De Bruin *et al.*, 2004), are superior in performance of accuracy compared with automatic methods. Although interactive segmentation is simple and generally applicable, it is very time-consuming, monotonous, and demands constant precision from the operator. Therefore, these interactive measurements are not feasible for applications that have a large number of objects to be separated. As a result, automatic segmentation and identification methods have been developed. Rigaut *et al.* (1991) and Irinopoulou *et al.* (1997) developed an automated algorithm in which nuclei profiles are segmented using a global threshold and mathematical morphology operations. Clusters of objects are separated by combining a distance transformation with a *Watershed* algorithm (Vincent and Soille, 1991). Using this algorithm, they were able to measure the volume and DNA content of prostate nuclei, with corresponding coefficients of variation of 18.4-33.5% and 9.0-12.0%. Roysam *et al.* (1994), Ancin *et al.* (1996) and Mackin *et al.* (1998) applied an initial global segmentation algorithm on gradient processed images, followed by a locally adaptive threshold to separate connected objects using a 3D *Watershed* or cluster analysis algorithm. Adiga *et al.* (2001) developed a region-based segmentation method involving a *Watershed* algorithm to separate clustered objects, followed by a rule-based merging technique to reduce the over-segmentation of cells caused by the *Watershed* algorithm. According to them, applying this method to prostate cancer specimens resulted in 95% correctly segmented

nuclei. Luck *et al.* (2005) developed an algorithm for automatically segmenting nuclei in confocal images of cervical tissue. In their algorithm, a model that accounts for the properties of the imaging system and of the tissues is used in conjunction with a powerful image enhancement tool (anisotropic median-diffusion), appropriate statistical image modeling of spatial interactions (Gaussian Markov random fields), and a Bayesian framework for classification-segmentation. Their algorithm can detect 90% of hand-segmented nuclei with an average of 6 false positives per frame.

2.4 Volumetric Visualization

The applications of advanced volumetric visualization and display techniques in studying 3D confocal image data have facilitated knowledge exploration of cellular structures greatly (Choi *et al.*, 2005; Bassnett, 2005). With volumetric visualization, users are able to view, control, and manipulate 3D cellular structures through an interactive and intuitive way. Currently, there are two main commonly used visualization techniques: volume rendering and surface rendering.

2.4.1 Volume Rendering

Levoy (1988) first proposed the basic pipeline for volume rendering. In his paper, basic operators in volume rendering, such as shading, classification and re-sampling, were introduced. With its application to one molecular dataset and one medical imaging dataset, volume rendering was proved to be effective to display the surfaces from sampled volumetric data.

However, due to the large amount of computation, the volume rendering technique proposed by Levoy is time-consuming and resource demanding (Levoy, 1988).

As a result, in the past two decades, there have been three main approaches developed to achieve real-time visualization: highly optimized software methods, use of 3D texture mapping hardware, and special-purpose volume rendering hardware. The software solutions for volume rendering usually require pre-computation and additional data storage. An early development is the template-based ray traversal method proposed by Yagel *et al.* (1992). This work is based on the observation that, with parallel rays, the path through the volume element (voxel) structure is common to all rays. Levoy (1990) and Wan *et al.* (1998) presented another algorithm to enhance the computation speed by pre-processing the volume so that the regions of significance are identified, and volume rendering integration begins from the intersection with the first object rather than the intersection with the volume boundary. Originally developed by Cameron and Unrill in 1992 and later improved by Lacroute and Levoy in 1994, *Shear-warp* rendering algorithm achieves its fast rendering speed by exploiting coherence in the volume data and the intermediate image and so far is the fastest software solution for volume rendering. Cullip and Neumann (1993) used 3D texture mapping hardware to speed up volume rendering with a method called planar texture re-sampling. In this algorithm, a volume is stored as a 3D texture and re-sampled during rendering by extracting textured planes parallel to the image plane. With the texture mapping hardware, interactive rendering rates have been achieved on the SGI Reality Engine (Akeley, 1993; Cabral *et al.*, 1994). However, texture hardware does not support estimation of gradients which are required to identify surfaces for shading. Efforts to overcome these difficulties either require multi-pass rendering (Westerman and Ertl, 1998), or require significant pre-processing (Drebin, 1988). Over many years, Kaufman has led the research into the design of special purpose hardware architectures for volume rendering. In 1996, Pfister and Kaufman proposed the *Cube* architecture which was the earliest concept on the

hardware design for volume rendering. There has been a great advance in 1999 when Mitsubishi Electric enhanced the *Cube-4* architecture and created *VolumePro*, a commercial product as a PC-board which can be fit into a standard PC for real-time volume rendering (Pfister *et al.*, 1999). *VolumePro* is the first single-chip real-time volume rendering system for consumer PCs. It does not require any pre-processing and performs a brute-force re-sampling of all voxels for each frame. This makes changes to the volume data immediately visible and allows the integration of the *VolumePro* hardware into simulation systems (e.g., surgical simulation) or real-time acquisition devices (e.g., 3D ultrasound).

Research interests on volume rendering have moved to transfer function design in recent years. A transfer function assigns values of optical properties, such as color and opacity, to original values of the dataset being visualized. The most common approach in transfer function design is by trial-and-error. It allows users to arbitrarily and repeatedly manipulate the coefficients of the transfer function. To achieve automatic and optimal transfer function, He *et al.* (1996) employed genetic algorithms driven by user selection and objective image fitness function. Fang *et al.* (1998) presented an image-based transfer function model that integrates 3D image processing tools into the volumetric visualization pipeline to facilitate the transfer function design.

The rapid advancements of volume rendering have made it an effective tool in 3D confocal image visualization allowing deep insight into cellular structures. In a pioneering work, Kaufman *et al.* (1990) developed a software system called *BioCube* to render cellular structures. Sakas *et al.* (1996) applied volume rendering to visualize static and dynamic structures of 3D confocal microscopy datasets. They also illustrated the detailed pipeline for visualization using volume rendering. Razdan *et al.* (2001) investigated how to effectively visualize multi-color confocal microscopy data. They

developed volume rendering using volumetric ray casting based upon a perspective camera model and illustrated an innovative way to simultaneously visualize confocal datasets from three lasers using voxel level merging. A method to generate key frame animation sequence with an arbitrary flying through path was also included in their visualization tool. Clendenon *et al.* (2002) published a public domain software named “Voxx” supporting interactive inspection and manipulation of 3D confocal datasets. *Voxx* takes advantage of low-cost 3D graphics hardware and thus can be run on common PCs while achieving near real-time rendering speed.

2.4.2 Surface Rendering

When using surface rendering to visualize cellular structure and sub-structure, the surface of 3D biological structures are modeled and represented by a group of polygons (Chen *et al.*, 1995). Given an intensity threshold value, the points with intensity values around the threshold are considered to be located on the structure surface and subsequently utilized to generate the polygons. The classical approach for polygon extraction from volumetric datasets is the *Marching Cubes* algorithm, proposed by Lorensen and Cline (1987), with a similar suggestion from Wyvill *et al.* (1986). This algorithm assumes that data is on a structured grid and conceptually processes each cell independently, one after the other. Each cell in a structured grid is topologically equivalent to a cube, and the method focuses on the extraction of an iso-surface within a unit cube. Each vertex of a cube can be either greater than or less than the threshold value. With the tri-linear interpolation of the values at the cube vertices, the intersections of the iso-surface with the edges of the cube are easily and accurately calculated and located. Kaufman (1998) developed a software system named “*VolVis*” implementing *Marching Cube* algorithm to study nerve cells. Razdan *et al.* (2001) applied surface

rendering in confocal imaging. Based on the surface triangulation with *Marching Cube* algorithm, Kubinova *et al.* (2004) developed an algorithm to automatically capture geometric characteristics of cellular structures in confocal image data.

Compared with volume rendering, surface rendering is superior in running speed. The generation process of polygons only needs to be done once, and the resulting primitives can take advantage of modern acceleration graphics card enabling real time navigation through data containing well-defined surfaces. However, when dealing with fuzzy biological structures, surface rendering may have the problem of information loss because it is hard to accurately represent the complicated biological structure with only one iso-surface value (Razdan *et al.*, 2001). To find the right iso-surface value for surface rendering, Bajaj *et al.* (1997) introduced a method which provides signature curves that visually represent some important properties, such as surface area and mean gradient magnitudes, for a spectrum of iso-surfaces, so that users can choose the appropriate iso-values based on their rendering goals. Tenginakai *et al.* (2001) proposed a general model-independent statistical method to determine the material boundaries by calculating local higher order moments of the data distribution. A multi-scale iso-surface extraction algorithm extended from the histogram-based method (Fang *et al.*, 1998) was presented by Fang and Adada (2004). Using histogram analysis, the iso-values are extracted from histograms of boundary voxels defined by gradient magnitude thresholding. Multi-scale smoothing of the histogram using *Gaussian* filters of various sizes allows the iso-surfaces to be defined hierarchically over a scale space map.

2.4.3 Display and Interaction Methods

To achieve intuitive perception and interactive manipulation of visualized confocal image data, various displaying techniques and Virtual Reality (VR) techniques have

been adopted. Chen *et al.* (1995) introduced two useful techniques to display rendered images of 3D confocal datasets: stereo display and movie display. They also discussed issues related to overlaying multiple images, a powerful method for showing colocalization of different molecular components in the same specimen.

The understanding of structural characteristics of 3D confocal cell data requires spatial judgments that can be achieved by VR technology. VR is referred to as a technology that gives users the experience of being immersed in a computer-generated virtual world (Ellis, 1994). As an enabling technology, VR has long been applied in many fields (Burdea and Coiffet, 1994). Scharein *et al.* (1994) described their system applying VR for interaction and tracing 3D confocal data of neurons. Their interaction technique was based on head-coupling to free user's hand and the tracing task was done by marking a point of interest with the mouse in two different views controlled by the head. Brady *et al.* (1995) discussed the use of volume rendering techniques in a VR environment for the visualization of volumetric data and tracking of fiber structures. Their application, called "*Crumbs*", combines advantages from volumetric visualization and immersive environments in order to make the visualization and identification of complex biological structures imaged by confocal microscopy easier. De Leeuw (2000) and Liere *et al.* (2002) addressed the roles that VR can play in the analysis of time-dependent 4D cellular data with the "*Proteus*" system, a virtual environment they have developed for studying 4D confocal cell data. *Proteus* was equipped with a large fish tank display with a 6 DOF wand for interaction and also can be used in other environments, such as the CAVE, HMD, and desktop workstations.

2.5 Summary

This review covers a broad area of topics related to 3D confocal images. Literatures including developments of confocal microscopy, processing and visualization of captured 3D confocal images, and applications of information technology in confocal imaging, are discussed in this chapter. The review enables a better understanding of the state-of-the-art research in confocal imaging, thus provides the foundation for the subsequent investigations.

3. Confocal Image Acquisition and Management

Confocal imaging is a quantitative technique for probing cellular structures and dynamics. Nowadays it is widely and increasingly used for biology research. Although current software for confocal microscopy automates 3D image acquisition and provides solutions for 3D visualization and analysis, tracking the image operations and the analytical results is often ignored in a systematic fashion. This chapter describes an applicable and effective solution that we propose for the management of confocal images and their analytical information with the aid of database techniques. The employment of Information Technology in the solution enables the capability of online image administration.

3.1 Motivation

The development of confocal microscopy and computer techniques makes possible automatic quantifications of cells and sub-cellular structures (Zucker and Price, 2001; Phillips *et al.*, 2001; Kreitzer *et al.*, 2003; Akita *et al.*, 2004). Although current software for confocal microscopy automates image acquisition and provides solutions for image processing, they usually do not keep track of the images and their relevant analytical data. The huge amount of experimental and biochemical information poses a challenge for researchers. These background information are crucial to downstream operation with images, especially for analysis uses.

Database and Information Technologies can be employed to facilitate the data administration, image processing, or even quantitative analysis. An excellent example is the *Open Microscopy Environment (OME)* developed by Swedlow *et al.* (2003). *OME* is designed to store raw confocal image data together with the metadata that specify the context and meaning of an image stack. In addition, *OME* has the capability to provide a flexible mechanism incorporating new and existing image analysis routines.

Inspired by *OME*, we develop a management database to store both the confocal images and their relevant information. With this database, convenient administration of the confocal data can be achieved. Meanwhile, this database is also designed to store the analysis results after the confocal images are processed. Data communication between remote researchers is an important issue, especially for collaborative research. To solve this problem, we integrate the Internet techniques with the database, allowing online storage, manipulation and analysis of cellular images and relevant information across borders and time zones.

This chapter introduces the structure of our confocal image management database and the application of the Internet techniques for online confocal data

communications. It is organized as follows. In Section 2, we present the acquisition and formation of 3D confocal images. The basic structure of the management database is introduced in Section 3. In Section 4, we illustrate how the Internet techniques are employed for data communication. The summary of this chapter is given in Section 5.

3.2 Confocal Image Acquisition and Formation

As living cells and tissues are 3D structures, the spatial relationships between their components are often at issue. However, analysis of such issues is hampered by the inverse relationship between the resolution of a lens and its depth of focus. Fortunately, this problem has been significantly reduced by the development of confocal microscopy (White *et al.*, 1987; Shotton, 1989). After undergoing a period of rapid development, confocal microscope has now become a universal research tool in cell biology. It provides contrast capabilities and in-depth resolution that are simply not available in conventional field-illumination microscopes.

In confocal microscopy, the localization of multiple cellular components is determined with specific probes labeled with different fluorephores. The fluorescence-stained specimen is then illuminated by a laser point light source, and after passing a spatial filter (pinhole), the point will be imaged on a detector. The choice of lens, fluorophores, image recording device, and excitation intensity and exposure time are always governed by the specific application. Usually these configurations are set to minimize optical aberration and sample damage and to maximize resolution, total signal, and the signal-to-noise ratio. Several confocal systems are used in this project for confocal data generation. As an effort of international research collaboration, this project involves: (1) Nanyang Technological University, Singapore; (2) University of Toronto,

Canada; (3) Beijing University Medical Hospital, China; and (4) National Heart Center, Singapore. A Bio-Rad MRC-600 confocal fluorescence microscope equipped with a krypton/argon laser is used for fluorescence microscopy by the Toronto side. An Olympus FluoView^{TM} 500 confocal microscope is available with the Singapore side. A Leica TCS SP2 confocal laser scanning microscope is utilized by the Beijing side.

After scanning the specimen slice by slice, a series of images in the x - y plane are recorded along the z axis of a microscope. The format of the recorded images is usually decided by the confocal scanning system. All images in a stack in most cases are aligned along the x , y and z axis. Typical image sizes used in our applications are 256×256 , 512×512 and 768×512 . The selection of the image size is dependent on the desired size of the final 3D dataset. Figure 3.1 illustrates one confocal image slice of human cervical cells, HeLa, produced with a point-detection confocal laser scanning microscope (Olympus FluoView^{TM} 500, Figure 3.2). The cells are double labeled with primary antibodies against apolipoprotein(a) (mouse monoclonal anti-human apolipoprotein(a) followed by a TRITC-conjugated anti-mouse secondary antibody) and apolipoprotein E (rabbit polyclonal anti-human apolipoprotein E followed by a FITC-conjugated anti-rabbit secondary antibody).

Figure 3.1 HeLa cells imaged with confocal microscopy after double labelling with antibodies against apolipoprotein(a)(red) and apolipoproteinE (green).

Figure 3.2 The Olympus FluoView™ 500 Confocal Microscope.

3.3 Central Management Database

The first step to build up the database is the determination of the scope of the data to be stored. As mentioned earlier, for each raw confocal image stack, its relevant background information is crucial for users to better interpret the cellular structures. In addition, the database should also store the results from analysis and processing. Thus we design five modules as follows in the database to record the information.

(1) Basic Information Module (BIM).

BIM is used to record the general information for a dataset. It includes the general description, the name of the operator(s), the creation time and date, and others. Furthermore, for better management, we categorize the datasets into several groups according to the target proteins or cellular constituents. Thus each dataset is given a category name stored in BIM. In this way, users will be able to know the background information and the purpose of the experiments immediately.

(2) Clinical Preparation Module (CPM).

This module is employed to store the diagnostic description of the clinical procedure for datasets captured in disease-oriented experiments. For these datasets, the disease situation of the patients from whom the cell samples are obtained provides valuable resources to study the relationship between the disease and the specific protein(s). The aspects in this module include: the description of the patient's disease, the time and the venue of the clinical experiment, and the name of the clinical doctor and so on.

(3) Bio-Chemical Module (BCM).

This module is to record the information regarding the specimen preparation and the chemicals including antibodies used in the experimental process. The steps during the specimen preparation like fixation and permeabilization are recorded in details. The primary antibody for the target protein and the fluorescently labeled secondary antibody against the primary antibody are also used in this module.

(4) Confocal Imaging Module (CIM).

This is an important unit because it records the basic profiles about the imaging environment within which the confocal images are captured. The scan intervals in the x - y plane and z direction can be different from each other due to the variety of objective lens used. The data about the scan interval in each direction is required for downstream operations and it is recorded in this module. The whole physical size of the dataset provides a clue for quantitative analysis. Confocal microscopes can produce image stacks with up to 3 channels. In order to help the users to study the relationship among the data in different channels better, a brief introduction about the target protein in each channel is also recorded in this module.

(5) Quantitative Analysis Module (QAM).

Each time when users analyze a confocal dataset, some feature information may be extracted. These results can be shared among users. Therefore, QAM is designed to store and manage the information resulted from the processing and analysis conducted on the datasets.

After the data scope for the database is determined, we use *Microsoft Access* to create a relational database mainly based on these five modules. Five tables in charge of information storage for these five modules are created and each has an item “DatasetID” as the primary key to link them together. When a confocal image stack is captured with microscopes, a unique DatasetID is given to this image stack. The information for each module can be entered subsequently. Figure 3.3 illustrates the relationship among the five tables.

Figure 3.3 The relationship between the created tables in the management database.

3.4 User Interface and Connection to Internet

Our research project is conducted in a collaborative environment. The biological specimen are obtained in one site, the confocal scanning is conducted in another site, and visualization and analysis works on the captured datasets are done at a third site. Thus, online data communication among remote collaborators becomes important.

The Internet has become a powerful communication medium, increasingly exploited by businesses and research. To enable online storing, retrieving and editing the captured confocal images and relevant information, we design a graphic user interface to manage the database through a set of HTML pages (Figure 3.4). A server computer is equipped with high-performance configurations. The management database is maintained in the server and linked to the interface HTML pages. SQL code is incorporated into these HTML pages. When the browser at a client computer requests the HTML file, the SQL is extracted, passed to the management database and interpreted.

We will now look into the operations from the viewpoint of a remote user who has the privilege to access the database through the interface. When the user logs into the database, he/she is able to carry out the following operations.

(1) Upload the newly acquired dataset and create a new record for it. Since the confocal image stack is made up of a sequence of slices, for convenience, the slices need to be zipped to *.pic file format before uploading. The uploaded zipped files will be unzipped and stored as a series of *.tif images in the database. A rendered image using volumetric visualization techniques for this dataset can also be uploaded and used as the portrait.

(2) View and edit the record for a target dataset. A “Search” interface is implemented to help the user identify the target dataset. If found, all the information

belonging to the dataset will be displayed in the webpage and the user is free to view and update it.

(3) Check all the datasets under the same category.

(4) Download the dataset from the webpage.

(5) Record the analytical results. Each time after users finish the analysis on the dataset, the analytical results can be immediately stored in the management database through the webpage interfaces.

Figure 3.4 Two examples of the interface HTML pages linked to the management database.

3.5 Summary

In this chapter, we first briefly introduce the acquisition and formatting procedure for 3D confocal image stacks. To achieve efficient and convenient management of large amount of the acquired data, we develop a web-based image management system for collaborative confocal microscopy research. The captured raw confocal image data together with the metadata that specify the context and meaning of an image stack can be maintained and administered in a centralized management database. The analytical results after processing and quantification can be updated to the database as well. Furthermore, the web-based interfaces linked to the management database provide the solution allowing remote users to input, save, retrieve and edit confocal images and relevant information across the net.

4. 3D Confocal Image Restoration

Although there have been vast advancements in the development of confocal microscopes, most confocal image stacks generated with confocal microscopes are anisotropic in terms of scan intervals in the scan direction and its perpendicular plane. This may, however, result in unequal sizes in the three coordinate directions for the volume elements (voxels) within the reconstructed 3D dataset. Interpolation techniques can be applied to fix the problem. However, most existing interpolation algorithms are computationally intensive and thus time-consuming. We develop a method to speed up the interpolation operation without sacrificing the accuracy. With this algorithm, the computational speed for interpolation can be enhanced greatly for general confocal image stacks.

4.1 Background

High-resolution confocal imaging of cells and their internal structures labeled with fluorescence probes is now routinely performed in life science research. However, the captured 3D confocal image stacks usually have different resolutions in the x , y , and z directions and the actual distance between adjacent voxels may vary in each direction. Without correction, the anisotropy problem of the scan intervals in each direction will possibly lead to confusion and mistakes in downstream quantitative analysis and visualization.

To solve the anisotropy problem in the x - y plane for 2D images, many sophisticated methods have been developed and some of them are now available publicly (Schafer and Rabiner, 1973; Hou and Andrews, 1978; Parker *et al.*, 1983; Dodgson, 1997; Lehmann, 1999; Meijering and Unser, 2003; Blu *et al.*, 2004). For 3D confocal images, to minimize the influence between neighboring optical sections during confocal imaging, the scan interval in the z direction is usually larger than that in the other two directions. The intensity values of voxels are likely to vary discontinuously to a large degree along the z direction, which will affect the post-processing and analysis. Hence, it is necessary to enhance the axial resolution to generate cubic voxels by interpolation along the z direction (Adiga and Chaudhuri, 2001).

There are mainly three categories of traditional interpolation methods: intensity-based, contour-based, and shape-based interpolation. In the contour-based interpolation (Boissonat, 1988), a set of binary image slices that consist of cross-sectional object boundaries are used to generate a new set of interpolated binary sequence. Raya and Udupa (1990) developed a shape-based interpolation scheme for multi-dimensional images. This scheme comprises (1) segmenting the image data into a binary image, (2) converting the binary image back into a gray scale image wherein the gray value of a

voxel represents its shortest distance from the cross-sectional boundary, and (3) interpolating the gray image. The set of all voxels with nonnegative values associated with them in the interpolated image constitutes the interpolated object. In the intensity-based interpolation (Lehmann *et al.*, 1999), an interpolation kernel with finite width is convolved with the original sampled voxels to generate the voxels located between existing voxels under the assumption that the sampling frequency is higher than the *Nyquist* frequency (Oppenheim *et al.*, 1999). When using intensity-based interpolation, errors will occur when there is a shape variation between two adjacent optical sections. Because intensity-based interpolation is only able to accurately reconstruct band-limited signals in frequency domain and most natural objects are band-unlimited, there is always a loss of accuracy no matter what kind of interpolation kernel is chosen. A novel interpolation method having the merits of both contour- and intensity-based interpolations was presented by Adiga and Chaudhuri (2001) based on XOR contouring and morphing to insert new slices into the image stack.

Compared to the other two types of interpolation methods, intensity-based interpolation techniques are fast and easy to implement although they may cause loss of information when dealing with sharply varying datasets. In most cases, the signals along the z direction inside successive confocal optical sections change gradually. Therefore, in Section 2 of this chapter, based on the spatial coherence, we present a method which shortens the computation time dramatically for intensity-based interpolation without accuracy degradation. In this method, a look up table (LUT) is designed to accelerate the computation speed. The experimental results after applying the fast intensity-based interpolation are illustrated in Section 3. Section 4 gives the summary of this chapter.

4.2 Intensity-based Interpolation

Suppose that we have a dataset consisting of a stack of confocal optical sections with equal scan interval, r_{xy} , in the x - y plane and a larger scan interval, r_z , in the z direction. The ratio of r_z over r_{xy} is r . For convenience, we name the voxels already existing in the image stack as sample voxels. To obtain cubic voxels, $(r-1)$ voxels between two adjacent sample voxels along the z direction in the original dataset need to be generated by interpolation. In intensity-based interpolation, we use a symmetric interpolation kernel h with window size $2k$ to convolve the original intensity function I along the z direction. The intensity value of a voxel in the z direction can be obtained with:

$$f(z) = \sum_{|m \cdot r_z - z| \leq k \cdot r_z} I(m) \cdot h(z - m \cdot r_z) \quad (4.1)$$

where $I(m)$ represents the intensity value for a sample voxel on the m^{th} slice in the original dataset and $f(z)$ is the intensity value for a voxel at position z in the scan direction generated after interpolation. Suppose the original dataset has $M \times N \times L$ sample voxels, where M , N and L denote the number of voxels in the x , y and z directions respectively. After interpolation, the dataset will have $M \times N \times (r \times (L-1) + 1)$ voxels with $M \times N \times ((r-1) \times (L-1))$ voxels obtained by interpolation. As seen in Equation 4.1, for each voxel to be generated, we need to calculate the values of the kernel function for $2k$ sample voxels and multiply the outcomes with the scalar intensity values at those sample voxels separately. Finally, these $2k$ multiplied results will be summed up to generate the final result.

Figure 4.1 The Sinc kernel

As a result of *Information Theory* introduced by Shannon in the late 1940's, the interpolation kernel should approximate the *sinc* function and it should have infinite support size in order to restore the signals completely and precisely. However, this ideal interpolator has an infinite impulse response (IIR) and is not suitable for local interpolation with finite impulse response (FIR). Therefore, for practical reasons, usually we choose interpolation kernels with finite support size to reduce computation cost. The accuracy and the computational cost are determined by the size and the shape of the interpolation kernel. In intensity-based interpolation, there are several commonly used local interpolation kernels: *nearest neighbor kernel*, *linear kernel*, *cubic kernel*, and *truncated sinc kernel* (Lehmann *et al.*, 1999). To demonstrate our fast interpolation method, we have used the *truncated sinc kernel* (Figure 4.1) as our example which can be written in form of:

$$h(z) = \begin{cases} \text{sinc}(z) = \frac{\sin\left(\frac{\pi}{r_z} \cdot z\right)}{\left(\frac{\pi}{r_z} \cdot z\right)}, & |z| \leq k \cdot r_z \\ 0, & |z| > k \cdot r_z \end{cases} \quad (4.2)$$

Assume we are calculating the intensity value f for a random voxel located at $z = i \cdot r_z + j \cdot r_{xy}$, $0 < i < L$, $0 < j < r$ between two neighboring sample voxels that are on the i^{th} and $(i+1)^{\text{th}}$ slice in the original dataset. According to Equations 4.1 and 4.2, we obtain:

$$\begin{aligned}
 f(z) &= \sum_{\substack{m \leq (\frac{z}{r_z} + k) \\ m \geq (\frac{z}{r_z} - k)}}^{r_z} I(m) \cdot \text{sinc}(z - m \cdot r_z) \\
 &= I(i - (k - 1)) \cdot \frac{\sin\left((k - 1 + \frac{j}{r}) \cdot \pi\right)}{(k - 1 + \frac{j}{r}) \cdot \pi} + I(i - (k - 2)) \cdot \frac{\sin\left((k - 2 + \frac{j}{r}) \cdot \pi\right)}{(k - 2 + \frac{j}{r}) \cdot \pi} \\
 &\quad + \dots + I(i) \cdot \frac{\sin\left(\frac{j}{r} \cdot \pi\right)}{\frac{j}{r} \cdot \pi} + \dots \\
 &\quad + I(i + (k - 1)) \cdot \frac{\sin\left((-k + 1 + \frac{j}{r}) \cdot \pi\right)}{(-k + 1 + \frac{j}{r}) \cdot \pi} + I(i + k) \cdot \frac{\sin\left((-k + \frac{j}{r}) \cdot \pi\right)}{(-k + \frac{j}{r}) \cdot \pi}
 \end{aligned} \tag{4.3}$$

Equation 4.3 shows that the interpolation value for a voxel can be calculated by summing up the results of multiplying the intensity values with the returned values of *sinc* function for $2k$ sample voxels around the voxel whose intensity value is to be generated.

4.3 Fast Intensity-based Interpolation

As seen in Equation 4.3, the value of the *sinc* function at each sample voxel to be convolved in the original dataset is only dependent on two parameters: $2k$ and j (ranged from 1 to $r-1$). Given k and r , the *sinc* function can have $2k \times (r - 1)$ different values at these points at most. In a confocal dataset, the intensity values for voxels are digitalized

with 256 gray levels ranging from 0 to 255. Therefore, the contribution of each convolved sample voxel to the final value of the interpolation is limited to $256 \times 2 \times k \times (r-1)$ different choices at most regardless the position or the intensity value of the convolved sample point. Hence, we develop a method to improve the computation time for interpolation. Given the interpolation kernel size and the resolution ratio between the z direction and the x - y plane, we can first calculate these $256 \times 2 \times k \times (r-1)$ different values in advance before the interpolation, and then store them in a LUT with $256 \times 2 \times k \times (r-1)$ entries. This LUT is named Contribution Look Up Table (CLUT). During interpolation, to obtain the contribution of a sample voxel to the final result, we can just search the value already calculated and stored in the CLUT based on the position and the intensity value of this sample voxel. The calculation of the convolution contribution for each sample voxel can thus be avoided. Then we can sum up the searched results from the CLUT to produce the final value. With CLUT, the calculation for the *sinc* function and the multiplication operation in Equation 4.3 is avoided, so that the interpolation time is only spent on the entry searching and summing up the searched results. As a result, the computational time can be reduced substantially. Meanwhile, the accuracy of the operation is not sacrificed since all the possible cases are covered.

In the case when other interpolation kernel like cubic kernel is employed for the intensity-based interpolation, we can follow the same steps. That is given the size and shape of the kernel and the scan interval ratio, we first create a CLUT by taking into account all the possible contribution values for the sample voxels. During the interpolation, the remaining work is to search the contribution values in the CLUT for each convolved voxel and sum them up.

4.4 Experimental Results

The fast interpolation algorithm is implemented in Visual C++. To evaluate the performance of the fast interpolation method in terms of computation speed, we have implemented another intensity-based interpolation program without CLUT for comparison. The two programs run on a Pentium IV PC with 1.7 GHz CPU and 512 MB RAM. Several 3D confocal image stacks are used in the experiment to record the computation time. As an example, Figure 4.2a and Figure 4.2c show the rendered images of a confocal image stack of GFAP stained mouse brain vessels using volume rendering with different viewing angles. The scan step length along the z direction is $1.0 \mu m$ and the space between points in the x - y plane is $0.2 \mu m$. This image stack has $768 \times 512 \times 20$ points and the resolution for each slice is 72 dot/inch. Since the scan interval in the z direction, r_z , is 5 times of that in the x - y plane, r_{xy} , the resolution ratio r is set to be 5. The support size for the interpolation kernel is chosen to be 6. After interpolation, a new stack with $768 \times 512 \times 96$ voxels is generated and shown in Figure 4.2b and Figure 4.2d. The computation time for the interpolation is recorded. Table 4.1 shows the recorded interpolation time using *truncated sinc* kernel with and without CLUT for several 3D confocal datasets. As seen in Table 4.1, the computation time is greatly reduced without the reduction in quality with the application of CLUT.

Table 4.1 Comparison of computing time for interpolation with and without CLUT

Volume Size before Interpolation	Scan Interval Ratio (r)	Interpolation Kernel Size ($2k$)	Volume Size after Interpolation	Cost Time without CLUT (s)	Cost Time with CLUT (s)
768*512*91	3	6	768*512*271	398.2	19.7
768*512*50	3	6	768*512*148	209.03	10.01
768*512*19	3	6	768*512*55	72.46	3.68
515*512*50	5	6	512*512*246	310.19	14.97
512*512*30	5	6	512*512*146	188.39	8.92
512*512*20	5	6	512*512*96	128.37	6.02

Figure 4.2a The rendered image of a confocal stack of mouse brain vessel before interpolation

Figure 4.2b The rendered image of a confocal stack of mouse brain vessel after interpolation.

Figure 4.2c The rendered image of the confocal stack in Figure 4.2a from another view angle.

Figure 4.2d The rendered image of the confocal stack in Figure 4.2b from another view angle.

To test the accuracy of our interpolation algorithm, we choose a 3D confocal image stack of propidium iodide-stained nuclei from a frozen section of mouse brain

captured with a Bio-Rad MRC-600 confocal fluorescence microscope. This image stack has equal scan intervals in the x , y and z direction ($0.1 \mu m$) and its volume size is $768 \times 512 \times 141$. Figure 4.3a shows the rendered image of this image stack using volume rendering. We intentionally construct a testing confocal stack with $0.3 \mu m$ scan interval in the z direction and $0.1 \mu m$ scan interval in the x - y plane by extracting the 1st, 4th, 7th, ..., 139th slices from the original stack. We will apply our interpolation algorithm to this testing stack to generate a new stack with $0.1 \mu m$ scan interval in the x , y and z direction. This new generated stack will be compared with the original stack to verify the accuracy of our algorithm. The rendered image of the testing image stack is illustrated in Figure 4.3b. The volume size of the testing dataset with $0.3 \mu m$ scan interval in z direction is $768 \times 512 \times 47$. We first apply the intensity-based interpolation with the kernel size equal to 6 to the testing dataset and record the computation time which is 9.22 seconds. A new image stack is then generated with the scan interval in z direction equal to $0.1 \mu m$ and Figure 4.3c shows the rendered image of the new image stack. The rendering environments, including the lighting and the shading properties, for these three stacks remain the same. It is hard to compare the intensity value of each volumetric point inside the original stack and the new stack generated with interpolation. Instead, we estimate the average intensity values for the points on each optical section for the comparison. Figure 4.4a shows the two average intensity curves for the original and the new generated stack. As mentioned in Section 4.2, the support size of the interpolation kernel affects the accuracy of the final interpolation results. A larger support size improves the accuracy, but with a corresponding increase in the computation time. For example, for the testing stack shown in Figure 4.3b, we change the interpolation kernel size to 10 and obtained another new stack. The average intensity value for each section in the new stack is compared to the original stack and illustrated

in Figure 4.4b. As seen in Figure 4.4a and Figure 4.4b, the accuracy is improved with a larger interpolation kernel size. However, the computation time for the interpolation with the larger kernel size is increased to 33.1 seconds.

Figure 4.3 (a) The rendered image of a stack with equal scan intervals in x , y and z direction. The stack contains 141 image slices; (b) The rendered image of the stack artificially formed by evenly extracting 47 slices from the stack in (a); (c) The rendered image of the stack obtained through interpolating the slices inside the dataset shown in (b).

(a)

(b)

Figure 4.4 The average intensity curves for the stack in Figure 4.3a and the stack in Figure 4.3c which is obtained with interpolation. (a) The support size for the interpolation kernel is 6; (b) the support size for the interpolation kernel is 10.

4.5 Summary

In this chapter, we introduce a CLUT based method to speed up the computation for intensity-based interpolation without sacrificing accuracy. Thus, the computational time is greatly reduced with our method compared with traditional intensity-based interpolation methods. This method can also be extended to other interpolation applications in 3D confocal image restoration with different interpolation kernels. The results obtained with our method prove its superior performance and can be used to facilitate 3D reconstruction of confocal image stacks.

5. 3D Confocal Image Enhancement

Image intensity attenuation along the scan direction due to light scattering and absorption as well as photo-bleaching is a major problem for confocal microscopy. A variety of computer techniques have been developed to enhance the quality of 3D confocal images which suffer from depth attenuation. In this chapter, we introduce a novel method for adaptive compensation of attenuation of light intensity for 3D confocal microscopic images by detecting and employing the intensity changes between neighboring optical sections.

5.1 Introduction

As living cells and tissues are 3D structures, the spatial relationship among their components is often at issue. However, an analysis of this kind of data is hampered by an image of a specimen being degraded by out of focus information from planes below and above the focal plane. Fortunately, this problem has been solved by confocal microscopy (White *et al.*, 1987). Nowadays confocal microscopy has evolved into a versatile and quantitative tool allowing deep insight into cell and organelle biology with its fast and dramatic technical advancements.

In a confocal microscope, the specimen is imaged by the combination of point illumination and point detection which eliminates the bulk of the out-of-focus information thus allowing effective serial optical sectioning of a specimen (Laurent *et al.*, 1992). Optical sectioning, however, has been troubled by noise and light intensity degradation along the scanning direction, mostly due to scattering and absorption of both excitation and fluorescence light and photo-bleaching (Pawley, 1995). When a 3D confocal image stack is obtained, deeper layers in the specimen are imaged with lower photon intensity. Figure 5.1 shows two optical sections at different depth selected from a confocal image stack with the intensity attenuation problem. The light intensity attenuation may lead to loss of details. Consequently, post-processing and analysis of these images are limited. Earlier works on the restoration of the intensity of microscopic confocal images model the light intensity decay along the axial direction as an exponential function of depth (Rigaut and Vassy, 1991; Rigaut *et al.*, 1992). To generate the proper constant for the rate of intensity loss for the exponential function, Kervrann *et al.* (2004) employed an approach based on a robust estimation. To have precise corrections, an iterative correction method based on an approximated inversion of the confocal microscope transfer function was proposed by Visser *et al.* (1991) and later

improved by Roerdink and Bakker (1993). Adiga (2003) introduced a simple iterative method of fitting a scale factor to each image slice to restore the image intensity. The difference between the average intensity value of each slice and that of the reference slice with the maximum average intensity value is calculated and used as the scale factor to enhance each point on each slice.

Figure 5.1 Two optical sections selected from a 3D confocal image stack with intensity attenuation problem: (a) optical section #1 and (b) optical section #20 showing loss of details.

Most previous correction methods are more or less empirical and based on fitting a decreasing parametric function to the section mean intensity curve computed by summing all pixel values in each optical section. The fitted curve is then employed for the calculation of correction factors for each section. We believe it is important to investigate the nature of light intensity changes between adjacent optical sections which are valuable for measuring the degree of intensity degradation. In this chapter, we propose a novel method for adaptive compensation of attenuation of light intensity for confocal microscopic images by detecting and employing the intensity changes between neighboring optical sections. The method is deduced from the physical model of light scattering and absorption (Wyszecki and Stile, 1982), and is able to automatically and

adaptively generate the correction parameters for each optical section for intensity compensation. Once the correction parameters are available, we introduce a new model to carry out the intensity compensation featuring good noise reduction.

This chapter is organized as follows. In Section 2, we introduce the physical model for light scattering and absorption. Section 3 presents the automatic and adaptive method to derive the correction parameters for each optical section. Section 4 presents a novel correction model using the generated correction parameters. The experimental results after applying the attenuation correction method to two confocal datasets suffered from intensity degradation are illustrated in Section 5. Section 6 summarizes this chapter.

5.2 Physical Model of Light Scattering and Absorption

Light intensity degradation along the scanning direction due to scattering and absorption is a major problem for interpretation of confocal images. With the increase of scan depth, the intensity values of the optical sections decrease fast. Consider light with energy $L_0(x, y)$ propagating from a source point $(x, y, 0)$ to a voxel (x, y, z) in a transparent medium (Figure 5.2). The energy of light is attenuated (Wyszecki and Stile, 1982) by:

$$L_z(x, y) = L_0(x, y) \exp(-S_z(x, y)) \quad (5.1)$$

where $L_z(x, y)$ is the attenuated light energy at voxel (x, y, z) , and $S_z(x, y)$ describes the overall effect of absorption and scattering from the starting voxel $(x, y, 0)$ to the ending voxel (x, y, z) in the form of:

$$S_z(x, y) = \int_0^z A_z(x, y) d\bar{z} \quad (5.2)$$

Here, $A_z(x, y)$ represents the absorption and scattering power per unit length of the medium at depth $\bar{z}$.

From the above analysis, we can calculate the energy of the attenuated light $L_z(x, y)$ for light $L_0(x, y)$ from a starting voxel $(x, y, 0)$ to a location (x, y, z) in the medium as follows:

$$L_z(x, y) = L_0(x, y) \exp\left(-\int_0^z A_z(x, y) d\bar{z}\right) \quad (5.3)$$

We assume that $A_z(x, y)$ is associated with the voxel intensity, $I_z(x, y)$, at location $(x, y, \bar{z})$ measured in the confocal images. A larger value of $A_z(x, y)$ indicates stronger scattering power, which generates a brighter spot in the confocal image as well as attenuates light rays more significantly. Thus, this assumption can be expressed as:

$$A_z(x, y) \propto I_z(x, y) \quad (5.4)$$

Now $S_z(x, y)$ can be written as:

$$S_z(x, y) = \int_0^z \alpha_z(x, y) \cdot I_z(x, y) d\bar{z} \quad (5.5)$$

where $\alpha_z(x, y)$ is the attenuation coefficient for voxel $(x, y, \bar{z})$. We assume that $\alpha_z(x, y)$ is not a constant through the whole dataset but theoretically it has the same value for all the points on the same optical section.

Figure 5.2 A diagrammatic illustration of light scattering and absorption. L_0 – light energy at the source, L_z – incoming light energy at the depth z , L_z^* – an emitted light energy at the depth z , L_z^c – emitted light energy at the collection point.

After incident light reaches voxel (x, y, z) with energy $L_z(x, y)$, another light $L_z^*(x, y)$, fluorescence emission, is excited. After passing back through the medium, emitted light $L_z^*(x, y)$ will be attenuated and eventually captured by a microscope photo-device, the camera, with energy $L_z^c(x, y)$. Suppose $Q_z(x, y)$ is the *Quantum Efficiency* of the fluorochrome at voxel (x, y, z) , thus $L_z^*(x, y)$ and $L_z(x, y)$ can be related by:

$$L_z^*(x, y) = Q_z(x, y) \cdot L_z(x, y) \quad (5.6)$$

Before $L_z^c(x, y)$ is captured by the camera, $L_z^*(x, y)$ undergoes attenuation. Hence, the relationship between $L_z^c(x, y)$ and $L_z^*(x, y)$ can be expressed in the form of (Rodenacker *et al.*, 2001):

$$\begin{aligned} L_z^c(x, y) &= L_z^*(x, y) \cdot \exp\left(-\int_z^0 \alpha_z(x, y) \cdot I_z(x, y) d\bar{z}\right) \\ &= L_z^*(x, y) \cdot \exp\left(-\int_0^z \alpha_z(x, y) \cdot I_z(x, y) d\bar{z}\right) \end{aligned} \quad (5.7)$$

From Equations 5.3, 5.6 and 5.7, we are now able to obtain the relationship between the source light and the captured light as:

$$I_z^c = Q_z(x, y) \cdot L_0(x, y) \cdot \exp\left(-2 \int_0^z \alpha_{\bar{z}}(x, y) \cdot I_{\bar{z}}(x, y) d\bar{z}\right) \quad (5.8)$$

In an ideal case without scattering or absorption, we cast light with energy $L_0(x, y)$ from the source point $(x, y, 0)$ to a voxel (x, y, z) . Because we have assumed no attenuation, light will keep its energy $L_0(x, y)$ when it excites the fluorochromes at voxel (x, y, z) and the emitted light caused by the excitation is $Q_z(x, y) \cdot L_0(x, y)$. Since we also have assumed no attenuation during the transmission back to the microscope camera either, the excited light will maintain its energy at $Q_z(x, y) \cdot L_0(x, y)$ as it is captured. The light energy captured in the ideal situation without attenuation is denoted as $L_z^i(x, y)$, and is related to $L_0(x, y)$ as:

$$L_z^i(x, y) = Q_z(x, y) \cdot L_0(x, y) \quad (5.9)$$

Therefore, from Equation 5.8 and Equation 5.9 we can obtain:

$$L_z^c(x, y) = L_z^i \cdot \exp\left(-2 \int_0^z \alpha_{\bar{z}}(x, y) \cdot I_{\bar{z}}(x, y) d\bar{z}\right) = L_z^i \cdot \exp(-2 \cdot S_z(x, y)) \quad (5.10)$$

Equation 5.10 illustrates the relationship between the “ideal” light intensity and the “practical” light intensity that can be captured by a photo-device of a confocal microscope after being attenuated through the medium. As seen in Equation 5.10, the degree of light attenuation for a voxel (x, y, z) is mainly decided by the attenuation coefficient for each optical section and the intensity values of the voxels above the optical section at depth z . Given the attenuation coefficient for each section, α_z , the ideal light intensity without attenuation can be easily restored. However, the trial-and-error manual selection of the attenuation coefficients is tedious and imprecise, leading to many problems like over-correction. As a result, we develop a method to solve this problem by calculating the voxels’ intensity change rate between two consecutive optical sections.

5.3 Generation of Correction Parameter

We first calculate the partial derivative of Equation 5.10 along the z direction and obtain the following equation:

$$\frac{dL_z^c(x, y)}{dz} = -2 \cdot \frac{dS_z(x, y)}{dz} \cdot L_z^c \cdot \exp(-2 \cdot S_z(x, y)) = -2 \cdot \frac{dS_z(x, y)}{dz} \cdot L_z^c(x, y) \quad (5.11)$$

For computational convenience, the derivatives of $L_z^c(x, y)$ and $S_z(x, y)$ can be approximated numerically as:

$$\frac{dL_z^c(x, y)}{dz} = L_{z+1}^c(x, y) - L_z^c(x, y) \quad (5.12)$$

and

$$\frac{dS_z(x, y)}{dz} = S_{z+1}(x, y) - S_z(x, y) = \alpha_{z+1}(x, y) \cdot I_{z+1}(x, y) \quad (5.13)$$

In a confocal microscope, the intensity of the captured light is proportional to the voxel's intensity value in a confocal image at a given confocal depth. Thus, we can obtain:

$$\begin{aligned} L_{z+1}^c(x, y) - L_z^c(x, y) &= -2 \cdot \alpha_{z+1}(x, y) \cdot (p \cdot L_{z+1}^c(x, y)) \cdot L_z^c(x, y) \\ &= -2 \cdot (p \cdot \alpha_{z+1}(x, y)) \cdot L_{z+1}^c(x, y) \cdot L_z^c(x, y) \end{aligned} \quad (5.14)$$

where p is the constant parameter illustrating the linear relationship between the recorded light intensity L and the voxel intensity I .

We set $\alpha_{z+1}^*(x, y) = p \cdot \alpha_{z+1}(x, y)$ and name it the correction parameter for voxel $(x, y, z + 1)$, and from Equation 5.14 we obtain:

$$\alpha_{z+1}^*(x, y) = \frac{L_z^c(x, y) - L_{z+1}^c(x, y)}{2 \cdot L_{z+1}^c(x, y) \cdot L_z^c(x, y)} \quad (5.15)$$

Equation 5.15 illustrates how to calculate the correction parameter for a voxel on an optical section at depth $(z+1)$ inside a 3D confocal image dataset. In our light absorption and scattering model, we have assumed that the correction parameters for all the voxels located on the same optical section should own the same value. Therefore, it

is necessary to find an optimal correction parameter for each optical section. To achieve fast optimization of the correction parameter for each slice, we employ the traditional least-square method. The least-square solution finds the optimal correction parameter by minimizing the sum over all voxels of the squared residual errors, defined as $r_z^2(x, y) = (\alpha_z^* - \alpha_z^*(x, y))^2$, where α_z^* is a random candidate of the correction parameter for the optical section at depth z . Among all the voxels on a section, there are some voxels whose intensity values change either irregularly or at a high rate, thus requiring very large values of the correction parameter. If these voxels are not filtered, they would distort the final correction result. To eliminate their influence on the optimization of α_z^* , these isolated voxels need to be filtered out by adopting a threshold T which can be obtained from a statistical analysis of the correction parameters distribution. In addition, because the light intensity value is supposed to decrease with depth, the available $\alpha_z^*(x, y)$ should have a positive value according to Equation 5.15. In this way, for all of the available voxels on an optical section located at depth z , the sum of all the residual errors squared for a given α_z^* can be expressed as:

$$R_z(\alpha_z^*) = \sum r_z^2(x, y) = \sum_{0 < \alpha_z^*(x, y) \leq T} (\alpha_z^* - \alpha_z^*(x, y))^2 \quad (5.16)$$

Suppose on the optical section at depth z , there are N_z voxels whose correction parameters are positive and below threshold T , then the optimal value of α_z^* at which $R_z(\alpha_z^*)$ achieves its minimum value is:

$$\alpha_z^* = \frac{\sum_{0 < \alpha_z^*(x, y) \leq T} \alpha_z^*(x, y)}{N_z} \quad (5.17)$$

5.4 New Restoration Model

From Equation 5.17, the optimal correction parameter for each optical section can be estimated by calculating the mean value of the available correction parameters. Once the correction parameters are obtained, it seems that the original light intensity value that is not attenuated can be restored directly from Equation 5.10 as in Sun *et al.*'s method (2004). However, although this method is straightforward and easy to implement, it is sensitive to noise and often leads to over-correction. Therefore, we propose a new method to do the attenuation compensation avoiding noise influence.

Figure 5.3 The light intensity attenuation correction model

First, among the optical sections in an image stack we select an optical section z_0 as the reference slice. The reference slice can be the section with the maximum mean intensity value in the image stack and we assume that it does not suffer from attenuation thus no correction is needed. Suppose the captured light intensity for a point located at $(x, y, z_0 + n)$ on the $(z_0 + n)^{\text{th}}$ optical section is $L^c_{z_0+n}(x, y)$ and its restored intensity value at depth z_0 is $L^r_{z_0+n}(x, y)$. As seen in Figure 5.3, $L^r_{z_0+n}(x, y)$ undergoes attenuation slice by slice from the reference slice to the $(z_0 + n)^{\text{th}}$ slice, generating a series of attenuated intensity values denoted as $L^{c'}_{z_0+1}(x, y), L^{c'}_{z_0+2}(x, y), \dots, L^{c'}_{z_0+n-1}(x, y)$ before it

finally becomes $L_{z_0+n}^c(x, y)$. Our solution is to use the relationship between adjacent slices (Equation 5.15) to recover the attenuated light intensity values for the intermediate slices and restore $L_{z_0+n}^r(x, y)$ at the end. From Equation 5.15, we can obtain

$$L_{z_0+n-1}^{c'}(x, y) = \frac{L_{z_0+n}^c(x, y)}{1 - 2 \cdot \alpha_{z_0+n}^* \cdot L_{z_0+n}^c(x, y)} \quad (5.18)$$

where $L_{z_0+n-1}^{c'}(x, y)$ is the attenuated intensity value for $L_{z_0+n}^r(x, y)$ at the $(z_0 + n - 1)^{\text{th}}$ optical section. Similarly, we can get:

$$L_{z_0+n-2}^{c'}(x, y) = \frac{L_{z_0+n-1}^{c'}(x, y)}{1 - 2 \cdot \alpha_{z_0+n-1}^* \cdot L_{z_0+n-1}^{c'}(x, y)} \quad (5.19)$$

and therefore

$$L_{z_0+n-2}^{c'}(x, y) = \frac{\frac{L_{z_0+n}^c(x, y)}{1 - 2 \cdot \alpha_{z_0+n}^* \cdot L_{z_0+n}^c(x, y)}}{1 - 2 \cdot \alpha_{z_0+n-1}^* \cdot \left(\frac{L_{z_0+n}^c(x, y)}{1 - 2 \cdot \alpha_{z_0+n}^* \cdot L_{z_0+n}^c(x, y)} \right)} \quad (5.20)$$

If we recursively conduct all the calculations from $z_0 + n - 1$ to z_0 , the restored intensity value, $L_{z_0+n}^r(x, y)$, computed from the original $L_{z_0+n}^c(x, y)$ on the $(z_0 + n)^{\text{th}}$ section with reference to the slice at depth z_0 can be given by

$$L_{z_0+n}^r(x, y) = g_1 \circ g_2 \circ \dots \circ g_n(L_{z_0+n}^c(x, y)) \quad (5.21)$$

where

$$g_i(L(x, y)) = \frac{L(x, y)}{1 - 2 \cdot \alpha_{z_0+i}^* \cdot L(x, y)} \quad (5.22)$$

Equation 5.21 shows that the restoration of the attenuated intensity value at a voxel $(x, y, z_0 + n)$ with respect to the reference slice at depth z_0 can be achieved by calculating a series of intermediate values through Equation 5.22. Throughout the calculation procedure, only the light intensity at voxel $(x, y, z_0 + n)$ and correction

parameters for the optical sections are involved. Therefore, the influence from noise signals above voxel $(x, y, z_0 + n)$ can be avoided.

5.5 Experimental Results

The adaptive attenuation correction algorithm is implemented in Visual C++. Several confocal datasets suffering from intensity attenuation are employed for testing the algorithm and excellent results are obtained. The two images shown in Figure 5.1 are chosen from a confocal image stack consisting of 34 slices. This image stack shows Purkinje cells and brain vessels labeled with two different antibodies in frozen sections of an embryonic mouse brain. The slices inside are captured with 72 dot/inch resolution and 512×512 pixels using a Bio-Rad MRC-600 confocal fluorescence microscope equipped with a krypton/argon laser. Figure 5.4 illustrates the curve of the average intensity values of all voxels on each optical section. As seen in Figure 5.4, the voxels' average intensity value begins to decrease from section #1 due to intensity attenuation. We select section #1 as the reference slice and conduct the restoration operation for the voxels located on the slices behind the reference slice. Figure 5.5a shows some sections from the original image stack without correction, and Figure 5.5b shows the corresponding processed sections. We employ volume rendering to visualize the 3D projection of the dataset before and after correction to show the restoration effect (Figure 5.6).

Figure 5.4 The curve of the average intensity value for a 3D confocal image stack suffering from intensity attenuation.

Figure 5.5a Selected optical sections in a confocal image stack suffering from intensity attenuation.

Figure 5.5b The corresponding optical sections after attenuation correction. Compared to the image sections in Figure 5.5a, the intensity of these sections are enhanced.

Figure 5.6 (a) The rendered image of the confocal image stack before attenuation correction using volume rendering; (b) The rendered image of the confocal image stack after attenuation correction using volume rendering. The correction results in the visibility of more details in (b).

Figure 5.7 shows two optical sections selected from another confocal dataset showing Purkinje cells and brain vessels. This image stack consists of 25 optical sections with 512×512 pixels and 72 dot/inch resolution, and is troubled with intensity attenuation as well. To do the correction, we select section #1 as the reference slice since it has the maximum average intensity value. All the points on the optical sections behind the reference slice undergo the attenuation correction. The comparison of the images in the dataset before and after correction is illustrated in Figure 5.8. Figure 5.9 shows the rendered images for the dataset with and without correction respectively using volume rendering.

Figure 5.7 The optical section #1 (a) and #20 (b) in a confocal image stack. Attenuation has reduced the intensity for the image stack to a low level in section #20.

Figure 5.8a Selected optical sections in a confocal image stack suffering from intensity attenuation.

Figure 5.8b The corresponding optical sections after attenuation correction. Compared to the sections in Figure 5.8a, the intensity of these sections are enhanced greatly.

(a)

(b)

Figure 5.9 (a) The rendered image of the confocal image stack before attenuation correction using volume rendering; **(b)** the rendered image of the confocal image stack after attenuation correction using volume rendering. It can be observed in (b) that, with the correction, the quality of the rendered image is improved with more details visible.

5.6 Summary

The purpose of this study is to find a method to correct attenuation of light intensity caused by light scattering and absorption within a biological specimen that is imaged in a confocal microscope. In this chapter, we present an adaptive method to compensate the loss of intensity of light in confocal fluorescence microscopy image stacks. The method relies on depth information and the rate of intensity change between neighboring optical sections.

Over correction and insufficient correction are two common problems with some correction algorithms due to manually choosing the correction coefficients, like the algorithm introduced by Sun *et al.* (2004) and the algorithms using exponential function to fit the intensity decay curve. Compared with those algorithms, the method proposed in this chapter is able to adaptively and automatically generate the correction parameters for different image sections from calculating the intensity change rate between consecutive sections based on a physical model of light scattering and absorption. Therefore, the over correction and insufficient correction can be greatly diminished. Furthermore, the restoration model introduced can eliminate the noise influence during the procedure of attenuation correction.

6. Suitability Evaluation of Gradients for 3D Boundary Detection of Confocal Objects

Gradient is an important property of volumetric points and it has been used for boundary surface detection and object segmentation of 3D confocal microscopic images. Furthermore, gradient provides valuable information for volumetric visualization of confocal image datasets. However, the quantitative measurement of gradient remains a challenge. In this chapter, we present a method to automatically evaluate the suitability of gradients for identifying 3D boundary surface points from confocal image stacks.

6.1 Introduction

Confocal image stacks are usually huge, noisy and often contain superfluous information. Data segmentation is then necessary to identify and extract the objects of interest (Rzano *et al.*, 1999; Ponomarev and Davis, 2003). Once identified, these objects can be further processed by volumetric visualization techniques such as volume rendering and surface rendering (Eils and Athale, 2003).

Boundary detection is of great importance in the object identification and knowledge discovery from 2D and 3D images. For edge detection of 2D images, there are two main groups of approaches: gradient-based and Laplacian-based (Mlsna and Rodriguez, 2000). The gradient-based approach detects the edge by locating the points where the first derivative of a gray-level function reaches a local maximum. The Laplacian-based approach achieves the edge detection by locating the points where the zero crossing of the second derivative of a gray-level function occurs. However, both these methods suffer from noise, interference from nearby edges and inaccuracies resulting from the use of a discrete grid. Canny (1986) developed an effective method for edge detection in 2D images by using both the first and the second derivatives. The Canny operator is a classic application of the gradient-based approach in the presence of additional white *Gaussian* noise while incorporating elements of the Laplacian-based approach and works in a multi-stage process. Compared to a pure gradient-based or Laplacian-based approach, Canny's operator achieves three simultaneous goals: low rate of detection errors, good edge localization and only a single detection response per edge. The automatic identification of 3D boundary points within volumetric images such as confocal microscope image stacks is analogous to the edge detection problem in 2D image processing. There are works done for 3D boundary detection using existing 2D edge detection algorithms (Liu, 1977; Zucker and Hummel, 1981; Bomans *et al.*, 1990;

Monga *et al.*, 1990). There are also some other approaches for automatic boundary surface detection of confocal objects (Fang *et al.*, 2000). For example, based on the method developed by Morrone and Owens (1987), Pudney *et al.* (1995) implemented a 3D local energy feature detector to extract surfaces from 3D confocal image datasets. The local energy of a 1D signal is obtained by convolving the signal with a quadrature pair of filters, and Pudney *et al.* extended it to 3D signal by convolving the 3D dataset with oriented pairs of 3D versions of *Morlet* wavelets. In order to increase the speed of the convolution, the filters are designed in frequency space and multiplied by the image's *Fourier* transform.

In 3D space, gradient is an important feature of volumetric points. It can be used for the 3D boundary detection of confocal image stacks. However, the precise and automatic identification of the appropriate gradient threshold still remains a challenge for 3D boundary detection due to the complexity of confocal images. It is crucial for the gradient threshold used in the boundary detection to locate as many correct boundary points as possible. Meanwhile it should be able to remove non-boundary points. Based on these criteria, in this chapter we present a method to quantitatively and automatically evaluate the suitability of the gradient threshold for 3D boundary points detection with a *Measurement Function (MF)*. In this method, after image processing, gradient calculation is conducted on each volumetric point inside the dataset. A *MF* derived from a boundary model is defined to quantitatively measure the suitability of all gradient magnitudes. The application of *Gaussian Divergence Theorem* provides a numerical solution for calculating the *MF*. The optimal gradient magnitude is considered to be the one at which *MF* attains its maximum. Once the optimal gradient magnitude is detected, it can be employed as the threshold to automatically detect boundary surface points. The

extracted boundary information can be further utilized in volumetric visualization techniques such as surface rendering and volume rendering.

This chapter is organized as follows. In Section 2, the biological background for our testing datasets is introduced. The details of our algorithm including the definition of *MF*, the boundary model of 3D microscopic objects, and the application of *Gaussian Divergence Theorem* are introduced in Section 3. In Section 4, we illustrate the experimental results after applying our algorithm to two confocal datasets. The summary of this chapter is given in the last section.

6.2 Confocal Imaging and Image Processing

To get the confocal images, 10 μm frozen sections of an embryonic day 13.5 mouse brain on slides are collected, and permeabilized in 0.3% TritonX-100 for 5 minutes and then washed in PBS three times. The sections are subsequently treated with RNase for 1 hour at room temperature before incubation with propidium iodide for 15 minutes. After a 2-minute washing in PBS, the sections are mounted in a Dako mounting medium. A Bio-Rad MRC-600 confocal fluorescence microscope equipped with a krypton/argon laser is used for fluorescence microscopy. The images are coded in 256 levels of gray and captured in a frame with a 72 dot/inch resolution. Figure 6.1 shows the rendered image of a confocal image stack consisting of 50 confocal images of propidium iodide-stained nuclei within a frozen section of the mouse brain and the size for each slice is 768×512 .

Figure 6.1 The rendered image of a 3D confocal image stack using volume rendering.

Although in confocal microscopy, only the in-focus light is imaged and the out-of-focus light is rejected by the confocal aperture (pinhole), noise still remains a major problem that can affect the detection result. In order to reduce noise, a median kernel filter is used to convolve with each point inside the image stack (Chen *et al.*, 1995). The median filter has proved a good tool because it eliminates the noise and surface artifacts while maintaining the sharpness of the contour (Sakas *et al.*, 1996). Here the intensity value of the original point (x, y, z) is replaced by the median value of the points contained in a window around that point. That is:

$$F(x, y, z) = \text{median}\{I(x-l, y-m, z-n), (l, m, n) \in W\} \quad (6.1)$$

where $F(x, y, z)$ is the output intensity value, I is the input intensity value and W is the chosen window. Figure 6.2a shows the optical section #30 from the stack shown in Figure 6.1, and Figure 6.2b shows the result of section #30 after applying a $3 \times 3 \times 3$ median filter to the whole dataset.

Figure 6.2 (a) The optical section #30 chosen from the image stack shown in Figure 6.1 before being processed with median filter; (b) the same optical section after being processed with median filter. Compared to figure (a), figure (b) is smoother and contains less noisy points.

6.3 Suitability Measurement of Gradients

Gradient is a vector giving the direction of the intensity changes. Frequently gradient is used as an estimation of the surface normal for shading in volume rendering and surface rendering techniques. The gradient of a 3D intensity function is the partial derivative of that intensity function with respect to the three principle directions. In a discrete volumetric dataset, the intensity value of each point is considered to be the value of the intensity function at that point. There are many commonly employed operators to estimate the local gradient for discrete datasets (Bentum *et al.*, 1996). We employ one commonly used operator, *Central Difference Method*, to estimate the local gradient. Figure 6.3a shows the rendered image for the calculated gradient magnitudes of the dataset in Figure 6.1 using volume rendering technique (Levoy, 1988), and Figure 6.3b shows the calculated gradient magnitudes for each point on the section #30 of the dataset.

The intensity values for a random point in a 3D confocal image stack cover a range due to a variety of technical problems related to data collection by fluorescence microscopy itself (Dobrucki, 2004). Therefore, the gradient magnitudes in confocal volumetric image datasets usually vary greatly as well. So it is difficult to decide the appropriate gradient magnitude as the threshold for boundary point detection. If the gradient threshold with a high value is chosen, the boundary will become more fragmented although fewer false boundary points will be obtained. On the other hand, if we choose a gradient threshold with a low value, some points not belonging to boundary points will be selected and a false boundary will be generated. We consider that, for the appropriate gradient threshold, it should satisfy two criteria: i) complete surface area; ii) large changing rate for each point on the surface. In order to find the most appropriate gradient threshold, we introduce a *Measurement Function* with which the suitability of gradient magnitudes as the potential threshold in 3D boundary detection can be

measured. The gradient magnitude where the MF achieves its maximum can be used as the optimal threshold and subsequently employed for boundary detection.

(a)

(b)

Figure 6.3 (a) The rendered image of a dataset formed by the gradient magnitudes of the points shown in Figure 6.1; (b) the image of gradient magnitudes of the points on the optical section #30 of the image stack shown in Figure 6.1.

Consider a random gradient magnitude G . Within a volumetric confocal image stack, there are some points whose gradient magnitudes are equal to G . To measure the suitability of G as the potential threshold for 3D boundary detection, we assume that those points with gradient magnitudes equal to G are located on the boundary surface. A good gradient threshold should be able to detect as many correct boundary points as possible. As a result, the detected boundary surface should have a large area. But this is not sufficient. Meanwhile, the points located on the boundary surface should have large data change rate. Thus, we define the MF for G to be the surface integral of the dot product of the gradient with the surface area for all the points whose gradient magnitude is equal to G . So the MF for G can be written in form of:

$$M(G) = - \int_{|g(x,y,z)|=G} g(x,y,z) \cdot ds \quad (6.2)$$

where $M(G)$ is the value of the MF for the gradient magnitude G , $g(x,y,z)$ is the gradient vector for a point and its magnitude is equal to G , and ds is the surface area vector at a point whose gradient magnitude is equal to G . The direction of ds is parallel to its normal direction. Figure 6.4 shows the simple model of a confocal fluorescence object in the x - y plane. Red circles indicate background points, while green circles represent points belonging to the object of interest. A yellow line connects the yellow boundary points and demarcates the boundary contour. The blue arrow N represents the direction of the contour normal at a boundary point, while the black arrow g represents the direction of the gradient vector at that point. In confocal fluorescence images, the intensity values of objects are always larger than those of background points. Therefore, the direction of the gradient vector at the boundary point is opposite to the direction of the contour normal. In 3D space, the direction of the gradient vector is also opposite to the direction of the boundary surface normal for a point located on the boundary surface. Thus there is a minus sign in the MF .

Figure 6.4 A model of a relationship between a gradient and the boundary surface in a fluorescence microscopical object. Red circles indicate background points, while green circles represent points belonging to the object of interest. A yellow line connects the yellow boundary points and demarcates the boundary contour. The blue arrow N represents the direction of the surface normal at a boundary point, while the black arrow g represents the direction of the gradient vector at that point. In confocal fluorescence images, the intensity values of objects are larger than those of background points. Therefore, the direction of the gradient vector at the boundary point is opposite to the direction of the surface normal.

According to *Gauss's Divergence Theorem* (Morse and Feshbach, 1953), the surface integral of a vector $g(x, y, z)$ over the boundary surface and the volume integral of the divergence of the vector $g(x, y, z)$, $\nabla \cdot g(x, y, z)$, over the region surrounded by the boundary surface are related by:

$$\int_{|g(x,y,z)|=G} g(x, y, z) \cdot ds = \int_V (\nabla \cdot g(x, y, z)) dv \quad (6.3)$$

In Equation 6.3, V is the group of points that is surrounded by the boundary surface (including the boundary surface points), dv is the volume size for each point within V ,

and ∇ is the gradient operator $\nabla = \left(\frac{\partial}{\partial x}, \frac{\partial}{\partial y}, \frac{\partial}{\partial z} \right)$.

The “dot product” of the vector operator ∇ with vector $g(x, y, z)$, namely the divergence of $g(x, y, z)$, can be calculated as:

$$\nabla \cdot g(x, y, z) = \frac{\partial g_x(x, y, z)}{\partial x} + \frac{\partial g_y(x, y, z)}{\partial y} + \frac{\partial g_z(x, y, z)}{\partial z} \quad (6.4)$$

Now the MF becomes:

$$\begin{aligned} M(G) &= - \int_{|g(x, y, z)|=G} g(x, y, z) \cdot ds \\ &= - \int_V (\nabla \cdot g(x, y, z)) dv \\ &= - \int_V \left(\frac{\partial g_x(x, y, z)}{\partial x} + \frac{\partial g_y(x, y, z)}{\partial y} + \frac{\partial g_z(x, y, z)}{\partial z} \right) dv \end{aligned} \quad (6.5)$$

In our confocal object boundary model (Figure 6.4), we assume that the points surrounded by the boundary surface have the same intensity value and thus the divergences of gradients for those points are zero (0). Only the points located on the boundary surface have valuable gradient divergences. In addition, because a volumetric confocal dataset comprises discrete intensity values, the divergences of gradients, $\nabla \cdot g(x, y, z)$, are discrete values as well. Usually we use a numerical method to evaluate integrals for discrete datasets. In volumetric dataset, each point is considered to have a unit cubic volume. Therefore, dv is equal to one (1). So now the MF can be rewritten as:

$$M(G) = - \sum_{|g(x, y, z)|=G} \left(\frac{\partial g_x(x, y, z)}{\partial x} + \frac{\partial g_y(x, y, z)}{\partial y} + \frac{\partial g_z(x, y, z)}{\partial z} \right) \quad (6.6)$$

The above equation shows that the MF for a gradient magnitude G could be calculated by summing up the divergences of the gradients for all the points that have the same gradient magnitude as G . For the other possible gradient magnitudes, the

values of their MF can be calculated in the same way and recorded as a quantitative clue to reflect their suitability of being the threshold for 3D boundary point detection. The gradient magnitude at which the MF achieves its maximum value can be used as the optimal threshold for boundary point detection.

6.4 Experimental Results

We implement our algorithm in Visual C++ on a common PC window platform. We test our algorithm on several stacks of images of fluorescently labelled nuclei (round objects), intermediate filaments (long thin objects), microvascular endothelium (long tubular objects) and endoplasmic reticulum (irregular cytoplasmic objects) and obtained excellent results in each case. For each existing gradient magnitude for the points in the image stack shown in Figure 6.1, we calculate and record the value of its MF by summing up the divergences of the gradients for the points with gradient magnitude equal to it. Figure 6.5 is a graph showing the MF values obtained for each gradient magnitude for the image stack shown in Figure 6.1. As seen in Figure 6.5, the maximum value of the MF is achieved when the gradient magnitude is equal to 47.

We conduct a basic boundary detection method on the dataset shown in Figure 6.1 by considering that the points whose gradient magnitude is equal to or bigger than 47 are located on the boundary surface. Figure 6.2 shows section #30 of the image stack shown in Figure 6.1 before boundary detection. Figure 6.6a shows the boundary points on section #30 detected using the optimal gradient threshold equal to 47. For comparison, Figure 6.6b and c show the boundary points on section #30 detected with another two gradient thresholds that have lower values of the MF . The boundary contour in Figure 6.6a is less fragmented than the one in Figure 6.6b and there are less false boundary

points in Figure 6.6a than in Figure 6.6c. Figure 6.6d illustrates the rendered image of the detected boundary points for the whole image stack using volume rendering technique.

Figure 6.5 The *MF* values obtained for all gradient magnitudes for the dataset shown in Figure 6.1.

Figure 6.6a The detected boundary surface points on the optical section #30 after we apply the basic boundary detection algorithm to the dataset shown in Figure 6.1 with the optimal gradient threshold value equal to 47 obtained with *MF*.

Figure 6.6b The detected boundary surface points on the optical section #30 after we apply the basic boundary detection algorithm to the dataset shown in Figure 6.1 with the gradient threshold value equal to 70.

Figure 6.6c The detected boundary surface points on the optical section #30 after we apply the gradient-based boundary detection algorithm to the dataset shown in Figure 6.1 with the gradient threshold value equal to 20.

Figure 6.6d The rendered image of detected boundary surface points using volume rendering after we apply the simple boundary detection algorithm to the dataset shown in Figure 6.1 with the optimal gradient threshold value equal to 47 obtained with *MF*.

Another testing dataset is a confocal stack consisting of 90 images of propidium iodide-stained nuclei (Figure 6.7). Figure 6.7a shows rendered image of the stack using volume rendering technique, and Figure 6.7b shows the optical section #50 after noise removal. Each image within the dataset has 512×512 pixels and the size of the whole image stack is about 18.7 MB. The *MF* values obtained for the gradient magnitudes of this dataset are illustrated in Figure 6.8. The *MF* achieves its maximum value when the gradient magnitude is equal to 28. We use this gradient magnitude as the threshold value to locate the boundary surface points with the basic boundary detected algorithm. Figure 6.9a shows the detected boundary points for the image section #50 shown in Figure 6.7b, and Figure 6.9b illustrates the rendered image of the detected points for the whole image stack using volume rendering technique.

Figure 6.7a The rendered image of a confocal stack consisting of 90 images of propidium iodide-stained nuclei.

Figure 6.7b The optical section #50 of the confocal dataset shown in Figure 6.7a after noise removal.

Figure 6.8 The recorded values of MF for all gradient magnitudes calculated from the dataset shown in Figure 6.7.

Figure 6.9a The detected boundary surface points on the optical section #50 after we have applied the basic boundary detection algorithm to the dataset shown in Figure 6.7 with the optimal gradient threshold value of 28 obtained with *MF*.

Figure 6.9b The rendered image of detected boundary surface points using volume rendering after we have applied the basic boundary detection algorithm to the dataset shown in Figure 6.7 with the optimal gradient threshold value of 28 obtained with *MF*.

6.5 Summary

In this chapter, we introduce an automatic method to quantitatively evaluate the suitability of gradient magnitudes for 3D boundary surface point detection of confocal microscopic datasets. A *MF* is defined to reflect the suitability of all gradient magnitudes based on an object boundary model. The application of *Gaussian Divergence Theorem* provides a practical solution to calculate the *MF* numerically.

Traditional boundary detection algorithms usually choose the gradient threshold in the trial-and-error manner or through experimental analysis, which is time-consuming and inaccurate. With our method, the suitability of gradient magnitudes for identifying the 3D boundary surface points can be quantitatively and automatically evaluated. Therefore, the computation time is reduced and the accuracy is enhanced. Furthermore, the information obtained about gradient magnitudes through our method can also be utilized in volumetric visualization which is introduced in the following chapter.

7. VR Enhanced Volume Visualization and Analysis of 3D Confocal Images

7.1 Introduction

One of the most challenging tasks in 3D microscopy is the analysis of the complicated datasets. Owing to the large amount of information in the datasets, it is necessary to extract useful information from them with the aid of data visualization. With the advancement and development of computer graphics technologies, real time volumetric visualization of large volumetric confocal image datasets becomes possible, allowing intuitive knowledge discovery from them.

Currently, there are two commonly used visualization approaches available: volume rendering and iso-surface rendering. Both approaches have been developed and applied in many scientific and medical applications for a long time. Particularly, the application of volumetric visualization techniques in confocal microscopy allows users to peer into the internal cellular structures for true 3D data viewing and analysis. Many software systems for 3D confocal image study have been developed incorporating these two visualization approaches (Kaufman *et al.*, 1990; Sakas *et al.*, 1996; Razdan *et al.*,

2001; Clendenon *et al.*, 2002). Moreover, with the capabilities of modern computer graphics hardware, the coupling of advanced display techniques with volumetric visualization techniques enhances the 3D feature of visualization results and facilitates the knowledge discovery from volumetric confocal datasets (Chen *et al.*, 1995). More advanced intuitive and interactive techniques, like Virtual Reality (VR), also provide a great potential for studying 3D confocal images in the future.

This chapter introduces a histogram-based approach to derive the proper iso-values for iso-surface rendering and a 2D transfer function design scheme for volume rendering. Both of these methods are based on the boundary information of gradient magnitudes obtained using the method introduced in the previous chapter. Some pioneering work exploring the roles that VR can play in the analysis and manipulation of volumetric confocal images are also covered in this chapter.

This chapter is organized as follows. Section 2 introduces the histogram-based iso-value detection for surface rendering. Section 3 is concerned with a 2D transfer function design scheme for volume rendering. Section 4 presents our explorations of VR techniques for interactive perception and manipulation of volumetric confocal image datasets. Section 5 summarizes the works introduced in this chapter.

7.2 Histogram-Based Iso-Value Detection for Surface Rendering

In surface rendering, a volume is represented by a set of geometric primitives, which are projected onto a plane perpendicular to the viewing direction. The most common method used to extract the geometric primitives from volumetric datasets for iso-surface rendering is the *Marching Cube* algorithm (Lorensen and Cline, 1987) in which each

point inside the 3D dataset is classified as either inside or outside the target 3D structure based on a given threshold intensity value (iso-value).

Compared to volume rendering, iso-surface rendering has an advantage in terms of computing speed. Once generated, the geometric primitives can be reused to reduce the computation time by taking advantage of modern 3D-graphics acceleration hardware to achieve real-time rendering. The iso-surface represents the contour of the dataset at a given iso-value. However, due to the complexity of most 3D confocal datasets, it is usually difficult and time-consuming to find the appropriate iso-value that can best approximate the boundary surface of confocal objects. For instance, Figure 7.1 shows some rendered images after applying iso-surface rendering to the confocal dataset shown in Figure 6.1 with different iso-values. Although there are some research investigating the effective solution to find the right iso-value (Bajaj *et al.*, 1997; Fang *et al.*, 1998; Tenginkai *et al.*, 2001; Fang and Adada, 2006), in practice the iso-value is usually decided through trial-and-error. We have developed a histogram-based approach to find the appropriate iso-value for iso-surface rendering of 3D confocal images. What follows are the steps in this method.

Figure 7.1a The rendered image of the dataset in Figure 6.1 using iso-surface rendering with the iso-value of 50.

Figure 7.1b The rendered image of the dataset in Figure 6.1 using iso-surface rendering with the iso-value of 67.

Figure 7.1c The rendered image of the dataset in Figure 6.1 using iso-surface rendering with the iso-value of 100.

Figure 7.1d The rendered image of the dataset in Figure 6.1 using iso-surface rendering with the iso-value of 150.

Figure 7.1e The rendered image of the dataset in Figure 6.1 using iso-surface rendering with the iso-value of 200. It is evident from these five pictures that different iso-values produce different contours in the image of the same dataset.

First, we detect the boundary surface points from the original dataset. We consider the boundary surface to be made up of points that have gradient magnitudes larger than the optimal gradient magnitude obtained with the method introduced in Chapter 6. This binary selection procedure can be written as:

$$f^*(x, y, z) = \begin{cases} f(x, y, z), & \text{if } |g(x, y, z)| \geq g_{opt} \\ 0, & \text{if } |g(x, y, z)| < g_{opt} \end{cases} \quad (7.1)$$

where $f^*(x, y, z)$ indicates the intensity value for a point located at (x, y, z) in the new volumetric dataset containing boundary surface points only, $f(x, y, z)$ is the original intensity value for the same point in the original stack, $|g(x, y, z)|$ is the gradient magnitude for the point in the original stack, and g_{opt} indicates the optimal gradient magnitude obtained with *Measurement Function (MF)*. For those points in the original dataset whose gradient magnitude is equal to or greater than the optimal gradient

magnitude, their intensity values are maintained in the new stack. However, for the other points with smaller gradient magnitudes, their intensity values are reduced to 0.

Figure 7.2 The graph showing the number of boundary points against intensity value for the dataset in Figure 6.1.

In the new dataset containing the boundary surface points only, the intensity values still cover a large range. We consider the intensity value at which the maximum number of boundary surface points is achieved as the appropriate iso-value for iso-surface rendering (Fang *et al.*, 2004). This can be done by generating a histogram for all intensity values of the boundary surface points. For volumes with well-defined surfaces, the histogram should have clear peaks and valleys. To remove noise from the histogram, a smoothing operation normally needs to be conducted. Figure 7.2 shows the smoothed histogram for the boundary surface points detected in the dataset shown in Figure 6.1. One local maximum is achieved at intensity value 67. Thus 67 is considered to be the right iso-value and employed for the iso-surface rendering of the original dataset (Figure 7.1b).

7.3 2D Transfer Function Design for Volume Rendering

Direct volume rendering is a technique of displaying a volumetric dataset as a 2D image. It does not involve the intermediate conversion of volume data to geometric primitives; rather the volume is directly visualized by projecting the data onto an image screen (Levoy, 1988; Lacroute, 1996; Pfister *et al.*, 1999). The most popularly employed volume visualization algorithm is ray-casting algorithm developed by Levoy in 1988. In the ray-casting algorithm, imaginary rays of light are cast through the volume from the center of pixels on the image plane. As a ray goes through the volume, it samples color (red, green, and blue) and opacity (a value indicating the opposite of transparency of the point) at points along the ray (called *sample points*). Then the color and opacity values for each ray passing through the volume are accumulated and stored in a pixel on the image plane (Figure 7.3). The compositing procedure can be written as:

$$\begin{aligned}
 F &= I_0 \cdot \alpha_0 \\
 \alpha_{accum} &= \alpha_0 \\
 \text{for } i &= 1 \text{ to } n \\
 F &= F + I_i \cdot (1 - \alpha_{accum}) \cdot \alpha_i \\
 \alpha_{accum} &= (1 - \alpha_{accum}) \cdot \alpha_i + \alpha_{accum} \\
 \text{end}
 \end{aligned}$$

where F represents the accumulated result for a pixel, α_i represents the opacity value assigned to a sample point, α_{accum} represents the accumulated opacity value along the ray, and I_i represents the intensity value assigned to a sample point.

Figure 7.3 The graphic illustration of the ray-casting equation.

Volume rendering is the process that directly displays a volumetric dataset as a 2D image on the screen without intermediate geometric calculations. Before being rendered, each voxel inside the dataset needs to be assigned opacity and color values based on its numerical values, like intensity value, and this mapping is done through a *Transfer Function* (TF). The procedure can be simply illustrated in the form as:

$$\begin{aligned} Color(x, y, z) &= TF_{color}(f(x, y, z)) \\ Opacity(x, y, z) &= TF_{opa}(f(x, y, z)) \end{aligned} \quad (7.2)$$

in which $Color(x, y, z)$ and $Opacity(x, y, z)$ indicate the color and opacity values returned by *Transfer Function* TF_{color} and TF_{opa} respectively for a voxel located at (x, y, z) whose intensity value is $f(x, y, z)$. TF plays an important role in volume rendering since it decides the final visualization result directly. Moreover, TF helps users to differentiate cellular structures by assigning variant color and opacity values to volumetric points based on their numerical properties.

Research on transfer function design for volume rendering started since the end of 1980's (Levoy, 1988; He *et al.*, 1996; Bajaj *et al.*, 1997; Fang *et al.*, 1998; Kindlmann and Durkin, 1998; Kniss *et al.*, 2001; Kniss *et al.*, 2002). However, most of the previous works are developed for medical datasets like CT/MRI images and few of them are concerned about 3D confocal microscopic datasets. Furthermore, some transfer function design methods must be implemented manually or semi-automatically, which limits their applications to some extent.

With the method introduced in Chapter 6, we are able to automatically obtain the information of gradient magnitudes for boundary point detection. Since gradient magnitude is also an important numerical property for each volumetric point, we employ it together with intensity value for the transfer function design. Now the transfer function becomes:

$$\begin{aligned} Color(x, y, z) &= TF_{color}(f(x, y, z), |g(x, y, z)|) \\ Opacity(x, y, z) &= TF_{opa}(f(x, y, z), |g(x, y, z)|) \end{aligned} \quad (7.3)$$

where $|g(x, y, z)|$ represents the gradient magnitude for a point located at (x, y, z) . As mentioned in Chapter 6, points with gradient magnitudes equal to or greater than the optimal gradient magnitude obtained with MF calculation can be considered to be boundary points while the other points are regarded as internal points. We can assign two different colors to these two groups of points based on their gradient magnitudes to visually differentiate them (Figure 7.4a). But either the boundary points or the internal points have their individual intensity values representing the fluorescence quantity at the point. Therefore, the values for the two colors are decided by the intensity values of points through a linear mapping (Figure 7.4b and Figure 7.4c). The detected boundary points form the surface surrounding the internal structures. As seen in the ray-casting algorithm, high opacity values assigned to boundary surface points will block users from viewing the internal structures. As a result, we can set the boundary surface points to be semi-transparent while giving high opacity values to internal points (Figure 7.4d).

We apply our 2D transfer function to the dataset shown in Figure 6.7. Two colors, yellow and pink, are assigned to boundary points and internal points respectively. Two linear look up tables storing color values are set up for these two point groups. The opacity value for the boundary points is set to be at a low level while the internal points are given a high opacity value. With this 2D transfer function, the final image is illustrated in Figure 7.5b. Figure 7.5c and Figure 7.5d are two close-up snapshots of two different sub-regions inside the whole dataset for better illustration.

Figure 7.4 The designed 2D transfer function for volume rendering.

Figure 7.5 (a) The rendered image of a confocal dataset using volume rendering without 2D transfer function; (b) the rendered image using volume rendering with the proposed 2D transfer function; (c) a close view of a sub-volume; (d) a close view of another sub-volume.

7.4 VR-enhanced Visualization and Analysis

VR is a form of simulation in which computer graphics is used to create a realistic world. Interactivity and the captivating power of VR contribute to the feeling of “immersion”, of being a part of the action happening on the screen. To some extent, VR can help enhance users’ perception of intracellular structures and the distribution of proteins in cells. In a virtual environment, users can feel the depth of the cell samples and thus will

view the cell samples as realistic 3D objects. Furthermore, with interactive tools, users are able to manipulate, quantify and interact with the cell samples.

7.4.1 Stereographic Display

The fundamental function of VR is the capability to create stereo images of rendered objects. People can perceive width, height and depth of 3D objects, but common 2D images can only convey the width and height information. Most people associate depth perception of 3D objects through stereopsis in which both eyes register an image and the brain uses the horizontal shift of the image positions registered by the two eyes to measure the depth. Therefore, based on the human eye stereo viewing principle, to produce a stereo image, two images of the volumetric dataset for the left and right eyes with a small shift of viewing angle are generated using volume rendering and then projected onto a stereo-ready monitor. Figure 7.6a shows the stereo image pair for a three-channel confocal dataset visualized using volume rendering. To display the stereo image pair on the monitor, there are several techniques like active stereo and anaglyph stereo. The active stereo display technique is most commonly used and it merges the two images together and alternates their display on the monitor. But this technique requires shuttle glasses and a synchronizing emitter (Figure 7.6b). Anaglyphs provide an alternative method to present two stereo views to the left and right eye. This is done by encoding the two views using two different colors (like red and blue), and then viewing through complementary colored glasses (Figure 7.6c).

Figure 7.6 (a) The stereo image pair for a 3D confocal dataset rendered using volume rendering; (b) display the stereo image pair using active stereo technique; (c) display the stereo image pair in anaglyph.

7.4.2 Interactive Manipulation With Data-Glove

Manipulations on the rendered datasets like transforming operations facilitate the study and research of the specimen and enhance users' perception of the cellular structure. Traditionally, mouse and keyboard are used in these operations for 2D images. Unfortunately, their range for interactivity in a 3D environment is limited. Hence, we utilize a 3D interactive tool, data-glove, to generate the necessary data to perform the manipulations (Figure 7.7). The volumetric datasets are projected onto a large display

screen in stereo mode in order to enhance the sensation of presence of cellular structures. The data-glove is a 3D interactive tool which can be used to generate spatial information. Then the spatial information produced can be subsequently employed for operations on the rendered datasets.

Figure 7.7 Manipulations on the rendered datasets with data-glove in a stereo environment.

7.4.3 Spatial Navigation and Localization

The quantitative analysis conducted on captured confocal microscopic image datasets provides the basis for mathematical modeling of protein kinetics and biochemical signaling networks (Eils and Athale, 2003). Usually identification and segmentation of confocal objects are needed before analysis since users want to isolate and concentrate on the object of interests only. Thus there exist many automatic segmentation algorithms (Ortiz de Solorzano *et al.*, 2000; Belien *et al.*, 2002; Adiga, 2003; Bajaj *et al.*, 2003; Lin *et al.*, 2003). Although these algorithms are able to separate different objects within the

same whole dataset from each other, the extraction of volumetric data belonging to the object of interest still remains a challenge. A straightforward solution to counter this challenge is to implement the segmentation and data extraction directly in 3D space, which can be achieved with VR techniques. What follows is the introduction of a method developed for interactive and intuitive localization and segmentation of 3D confocal datasets with VR techniques.

The volumetric confocal image stack is considered to be within a 3D space. The points belonging to the object of interests are distributed within a region around a central point. To extract these points from the whole volume, the contour surrounding the object of interests needs to be stretched. However, this is a time-consuming procedure. To simplify the stretching, we prepare several commonly used template shapes in advance. Users can select the template shape that matches the object best. Then the position and the orientation of the template shape are controlled through a tracking device, like Polhemus Patriot (a six-degree-of-freedom stylus tracking system). Users wearing the goggle observe the navigation of the template shape within the whole volume. Once the object of interest falls into the template shape completely, the volumetric points belonging to the object detected through comparing its position with the border will be extracted and used to reconstruct a new volume. The new volume can be subsequently visualized. In addition, statistic analysis and numerical calculation on the new volume can be done. Figure 7.8a shows a user wearing a goggle navigating through a confocal dataset with a tracking system, and Figure 7.8b shows an object of interest extracted from the whole volume using the VR technique.

Figure 7.8 (a) The user is navigating through a volume with a tracking device; (b) an object of interests extracted from the volume using VR techniques.

7.5 Summary

In this chapter, we introduce a histogram-based approach for the derivation of appropriate iso-value for surface rendering of 3D confocal image stacks. To enhance the visualization effect of volume rendering, we develop a 2D transfer function making use of the gradient magnitudes of volumetric points. We also develop a localization approach using VR technology for interactive manipulation and analysis on 3D confocal images. The work introduced in this chapter provides new solutions to enhance the visualization and manipulation of 3D confocal images. Especially, the navigation and localization approach highlights the parts that VR can play in the analysis and manipulation of volumetric confocal images.

8. System Development and Application

8.1 Introduction

Confocal imaging now has wide applications in analysis of complex medical and biological structures (Dailey *et al.*, 1999; Razdan *et al.*, 2001; Clendenon *et al.*, 2002; Karen *et al.*, 2003; Liu and Chiang, 2003;). As an input source, volumetric confocal data offers rich information for downstream analysis in 3D space. With the vast and fast available data, quantitative analysis on volumetric confocal data has become a meaningful but challenging endeavor (Zucker and Price, 2001; Lichtenstein *et al.*, 2003; Akita *et al.*, 2004). Fortunately, the marriage between advanced computer technology and confocal imaging has greatly enhanced the capabilities of image analysis for cellular research (Clendenon *et al.*, 2002; Eils and Athale, 2003).

Currently, capturing, processing, modeling, visualization and analysis on confocal microscopic datasets are, however, separate from each other, which may lead to lengthy processing time and a lot of inconvenience, especially for comprehensive research work. Furthermore, the operations conducted on the captured confocal images still more or less follow the traditional fashion that is 2D centric via a slice-by-slice strategy. Such image sequence based operations can potentially cause problems in data communication, interpretation, and knowledge discovery at a global 3D cellular level.

While treating confocal scanned volumes as a whole has its advantages, 3D processing and visualization of volumetric data is still challenging in terms of both computational load and time.

In this chapter, we introduce our integrated solution for comprehensive research on 3D confocal images with a system named “*CellStudio*” that we have developed. The result of a team effort, *CellStudio* is an easy-to-use software system written in Visual C++ that can be executed in a single PC/Window platform (Figure 8.1). It integrates the techniques and methods discussed in the previous chapters, and most of its capabilities are 3D-based by treating the confocal image stack as a unit. In this way, *CellStudio* enables consistent 3D confocal image processing, volumetric visualization and analysis.

Figure 8.1 The CellStudio system interface.

8.2 Structural Design and Implementation of CellStudio

There are four main functional areas (FA) in *CellStudio*: data reformatting, image processing, visualization and VR-enhanced manipulation and analysis (Figure 8.2). The “data reformatting” FA is used to extract valuable information and data from raw confocal images in various file formats. It is also responsible for building up linkage with the central management database based on the extracted information. The extracted image data are used to construct the volume which is the input for the downstream processing and visualization. The “image processing” FA implements the basic processing techniques to improve the quality of extracted image data. These processing techniques cover noise removal, contrast enhancement, resolution enhancement and attenuation correction. Processed image data are visualized in the “visualization” FA in which volume rendering and iso-surface rendering are implemented. To achieve real time performance of volume rendering, a *VolumePro* graphics card is utilized. For surface rendering, we employ *Marching Cube* algorithm to extract the polygons from confocal images and use *OpenGL* to render the extracted polygons. In “VR-enhanced manipulation” FA, four stereo display techniques are implemented, which makes it possible to create stereo views under different conditions. General interactive tools including the data-glove and the stylus are also supported in this FA. The navigation and localization algorithm introduced in the previous chapter is also employed to enhance the interactive manipulation and analysis on the rendered cellular structures. To better demonstrate the *CellStudio* system, we introduce the application of *CellStudio* for studying confocal images of cardiovascular heart disease related proteins in the following section.

Figure 8.2 The structure of CellStudio system.

8.3 Application of CellStudio for Studying Cardiovascular Heart Disease

8.3.1 Motivation

Cardiovascular heart disease (CHD) is a major cause of morbidity and mortality in the developed countries including Singapore, Canada and Australia. Research has discovered that CHD is primarily caused by atherosclerosis which is accumulated by fatty deposits in artery walls. Lipoprotein(a) (Lp(a)), a cholesterol-rich plasma lipoprotein, is shown to be an important risk factor implicated in the development of atherosclerosis. It is also a low density lipoprotein (LDL)-like lipoprotein which contains an additional protein called apolipoprotein(a) (apo(a)). So apo(a) can serve as a distinctive marker of Lp(a). Apolipoprotein E (apoE), a polymorphic protein, is another apolipoprotein closely related to the risk of CHD. Studying the cellular expression

pattern of apolipoproteins in CHD patients helps to better understand the relationship between apolipoproteins and CHD.

8.3.2 Materials and Method

Since apolipoproteins are mainly produced in the liver and white blood cells (Tenger and Zhou, 2003; Hilmer *et al.*, 2004), we use three kinds of cell samples. They are (i) a normal patient's white blood cells acting as control of the experiment; (ii) a CHD patient's white blood cells acting as study model; (iii) the liver cell-line, HepG2, which is the place of production of apolipoproteins. The HepG2 cells are cultured according to the instructions provided by the supplier. The culture medium Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) are used with anti-biotics penicillin/streptomycin added for the culture/subculture of the cells. Human white blood cells are collected from normal and heart disease patients from the National Heart Centre of Singapore under a consent agreement with the Singapore General Hospital. The cells are cultured with the same environment with HepG2 cells for the comparison of protein expression pattern between these two kinds of cells.

Primary antibodies for apo(a) [anti-mouse monoclonal], and apoE [anti-mouse monoclonal and anti-rabbit polyclonal] are used to target the intracellular proteins apo(a) and apoE respectively. Secondary antibodies TRITC (red color, anti-mouse monoclonal and anti-rabbit polyclonal) and FITC (green color, anti-mouse monoclonal and anti-rabbit polyclonal) fluorescence dyes (Dako, USA; Sigma, USA) are used for the binding of primary antibodies for color display during confocal imaging. The above primary antibodies are binded and stained in the cell model of liver cancer cells, HepG2 and human white blood cells in the study of its protein expression pattern as a model for the study of quantitative analysis. An Olympus FluoView^{TM} 500 confocal microscope

system (www.olympus.com) is used to capture confocal images. Figure 8.3 shows an optical section in a captured confocal image stack of HepG2 cells stained with antibodies against apo(a) and apoE. After confocal imaging, all the background information for the stacks can be restored in the management database through the Internet. Each image stack is given a unique name which is the enquiry ID to access the record of this dataset in the management database.

Figure 8.3 HepG2 cells stained with antibodies against apo(a) (red color) and apoE (green color).

8.3.3 3D Confocal Image Processing

Raw confocal image stacks are usually made up of a sequence of 2D images. Before a confocal image stack is processed, the valuable signals inside it need to be extracted, reformatted and stored in a single file for convenience. The file formats that *CellStudio* supports include: *.pic and *.vox. Inside *.pic and *.vox files, there are two main parts: header and data. The “header” is used to store the introductory information of the dataset, and the “data” part is a three-dimensional array employed to store the volumetric data. The difference between these two file formats is “*.vox” files can store multi-channel

confocal data while *.pic files cannot do it. Each point in the 3D array is assigned a 32-bit memory space within which 24 bits are used to store the intensity values for up to 3 channels and the remained 8 bits are utilized to store the transparency value.

To eliminate noise in raw confocal optical sections, median filters are recommended since they can reduce background signals while maintaining small features. The scheme of median filters is to choose a $n \times n \times n$ box (n is odd) surrounding a particular volume element (voxel) in 3D space and to find the median intensity value instead of calculating the mean intensity value. The position of the box is incremented by one voxel and the process is repeated until all the points have been processed. Figure 8.4 shows the result after applying a $3 \times 3 \times 3$ median filter to an apo(a) and apoE confocal image stack.

Figure 8.4 (a) An optical section in a noisy confocal image stack; (b) the optical section after noise removal.

Raw confocal images usually contain structures that are represented by only a portion of the display system. This causes some desired details to be suppressed. Image contrast enhancement is then necessary for visualization and analysis. We use Contrast Stretching for contrast enhancement (Razdan *et al.*, 2001). In contrast stretching, the

slope of the gray level histogram is changed to make it occupy the whole gray level range. This improves the overall visibility of the image (Figure 8.5).

Figure 8.5 (a) An optical section in a confocal image stack with low contrast; (b) the optical section after contrast enhancement.

For isotropic datasets, once formatted, they can be directly rendered. However, some 3D confocal images have different resolutions in the x , y , and z directions, hence the actual distance between adjacent points may vary in each direction. Without processing, the anisotropy may cause errors and faults to downstream visualization and analysis. As a result, a fast interpolation method introduced in Chapter 4 has been implemented in *CellStudio* to increase the axial resolution to get cubic voxels.

Another highly undesirable effect of confocal imaging is the degradation of light intensity along the depth of the image stack due to photo-bleaching and other optical problems. Often the last few slices of a raw confocal image dataset appear with too low image intensities. In order to correct the depth attenuation and restore good quality image slices, the adaptive attenuation correction program introduced in Chapter 5 is incorporated into *CellStudio* (Figure 8.6).

Figure 8.6(a) Some optical sections evenly extracted from a confocal image stack suffered from intensity attenuation.

Figure 8.6(b) The corresponding optical sections after depth attenuation correction.

8.3.4 3D Cellular Volumetric Visualization

Volumetric visualization enhances users' perception of cellular structures in 3D space.

CellStudio integrates the two most commonly used volumetric visualization techniques:

volume rendering and iso-surface rendering. Programming of these two techniques is done with *OpenGL*. For iso-surface rendering, we implement the *Marching Cube* algorithm to extract polygons from volumetric points based on a given iso-value. For volume rendering, we choose a *VolumePro* accelerating graphic board to achieve real-time performance (Figure 8.7).

Figure 8.7 (a) The rendered image of a confocal image stack showing the expression pattern of apolipoprotein E in HepG2 cells; (b) the rendered image from another view direction; (c) the rendered image after cross-sectioning the dataset; (d) the rendered image after zooming in the dataset.

In *CellStudio*, the classification for a one-channel confocal dataset is implemented in the form of a look up table. As the intensity values of voxels vary from 0 to 255, the size of the look up table is 256. The content for each entry of the look up table stores 4 bytes of data representing the color values of red, green and blue and opacity to be assigned to a specific voxel intensity value. With the mouse, users can randomly and interactively change the content of the look up table through a graphical interface (Figure 8.8).

Figure 8.8 The rendered images of a same image stack with different look up tables for the classification in volume rendering.

CellStudio also has the capability of rendering colocalized voxels for better study of the intracellular structures. Colocalization in confocal microscopy is defined as the presence of two or more different molecules residing at the same physical location in a specimen. To visualize the colocalization, a threshold for the intensity value in each channel is set to eliminate the influence of noise first. Then the voxels that own valuable intensity values in more than one channel are located. The detected colocalized voxels are set to be opaque and the remaining points are set to be fully transparent by changing their opacity value to 0 (Figure 8.9).

Figure 8.9 (a) The rendered image of apo(a); (b) the rendered image of apoE; (c) the rendered image of points stained with antibodies against both apo(a) and apoE simultaneously.

8.3.5 VR-enhanced Interactive Analysis and Information

Management

There are four stereo display techniques that *CellStudio* supports: active stereo, passive stereo, anaglyph and cross-eye stereo. The stereo images of confocal image stacks can be displayed on big screens and common PC CRT or LCD monitors. To enable interactive manipulations in the stereo environment, *CellStudio* provides supports for data-glove and Polhemus Fastrack tracking system. So far a group of general quantification operations have been completed in *CellStudio*, including identifying the maximum signal value, calculating the average signal value, producing the intensity-based histogram and calculating the average signal value for each optical section. A graphic interface showing the analysis results has been implemented (Figure 8.10).

Figure 8.10 A dialog box showing the result of quantitative analysis on an image stack showing the expression of apo(a) in HepG2 cells.

CellStudio implements the linkage between a dataset to the image data management database. Each time users want to have more background information about a dataset being processed, *CellStudio* will search the record of the opened dataset in the database and feedback the results to users. Meanwhile, the interface in *CellStudio* is also able to create a new record or update the record for the dataset being processed (Figure 8.11). In this way, full access to the database is allowed in *CellStudio*.

Figure 8.11 The interface showing the linkage between CellStudio and the management database.

8.4 Summary

The *CellStudio* software system and its application in studying cardiovascular heart disease are introduced in this chapter. *CellStudio* not only serves as the platform to implement the algorithms and techniques introduced in previous chapters, but also

provides an applicable solution for consistent 3D processing, visualization and analysis of volumetric confocal image data.

9. Conclusion

In this chapter, we summarize the results of the research work, highlight the contribution and discuss the possible future improvement of the research work.

9.1 Contributions

This thesis details the research work done by the author in the management, processing, visualization and analysis of captured 3D confocal images using advanced computer techniques. A common feature shared by these techniques is that the operations are conducted in 3D space by treating 3D confocal image stacks as a whole, instead of following the traditional slice-based solution. These techniques are also integrated and implemented in a software system named “*CellStudio*”, which can be employed to benefit microscopists for their knowledge discovery from large confocal image stacks.

9.1.1 Internet-based Confocal Images Management

To facilitate the data administration and image processing, we develop a management database to store both the confocal image data and their background information data. Five modules in charge of recording different aspects of each individual confocal image stack are included in the database. In addition, Internet techniques are integrated with the database, allowing online storage, manipulation and analysis of cellular images and

relative information across borders and time zones. In this way, besides providing an easy-to-use environment for remote collaborative confocal research, the success of the Internet-enhanced database provides a practical informatics solution for the administration of confocal data.

9.1.2 3D Processing Techniques for Raw Confocal Images

Raw confocal image stacks usually have poor quality due to the complexity of the specimen preparation and confocal imaging. There are problems with raw confocal image data troubling microscopists, like anisotropy of scan interval and depth intensity attenuation. When dealing with the anisotropy problem, we investigate a new method to speed up the interpolation operation based on the observation of the interpolation function. Given the size and shape of the interpolation kernel, a Contribution Look Up Table that stores the contribution value for each sample point to be convolved is built up before carrying out the interpolation. In this way, during the interpolation procedure, the huge computing load for each sample point is reduced, resulting in the enhancement of interpolation efficiency.

The light intensity attenuation problem in large confocal image stacks usually leads to a loss of information and adversely affects the downstream analysis. We develop an automatic and adaptive method to deal with the problem, which is based on detecting and employing the intensity changes between neighboring optical sections. The method is deduced from a physical model of light scattering and absorption. After obtaining the equation relating the ideal light intensity without light attenuation and the captured actual light intensity suffered from light attenuation for each optical section, we derive the solution to obtain the correction parameters for each individual optical section through partial derivative calculations. The correction parameters are closely related to

the intensity changes between neighboring optical sections. To remove the undesired effects due to noise signals during restoration, we develop a novel restoration model. In this model, to restore the intensity value for an individual volumetric point, only the attenuated intensity value of the point and the correction parameters for the optical sections above the point are involved in the calculation. Because the correction parameters are closely related to the intensity changes between neighboring optical sections, they are able to control the restoration for each optical section adaptively.

In 3D space, gradient is an important feature of volumetric points. It can be used for the boundary detection of confocal image stacks. However, the precise and automatic identifying of the appropriate gradient threshold still remains a challenge for 3D boundary detection of confocal image stacks due to the complexity of confocal images. It is crucial for the gradient threshold used in the boundary detection to locate as many correct boundary points as possible. Meanwhile it should be able to remove non-boundary points. Based on these criteria, we present a method to quantitatively and automatically evaluate the suitability of the gradient threshold for 3D boundary point detection with a *Measurement Function (MF)*. In this method, after image processing, gradient calculation is conducted on each volumetric point inside the dataset. A *MF* derived from a boundary model is defined to quantitatively measure the suitability of all gradient magnitudes. The application of *Gaussian Divergence Theorem* provides a numerical solution for calculating the *MF*. The optimal gradient magnitude is considered to be the one at which the *MF* attains its maximum. Once the optimal gradient magnitude is detected, it can be employed as the threshold to automatically detect boundary surface points. The extracted boundary information can be further utilized in volumetric visualization techniques such as surface rendering and volume rendering.

9.1.3 VR-enhanced Volumetric Visualization and Analysis

Volumetric visualization has been widely employed in exploration of scientific data. For studying confocal image data, volumetric visualization provides researchers an opportunity to intuitively view and manipulate the volumetric confocal datasets. In this thesis, we propose a histogram-based method to detect the appropriate iso-value for iso-surface rendering which is one of the two commonly used visualization techniques. In this method, we isolate boundary surface points from internal structure points using the binary boundary detection algorithm with the optimal gradient threshold detected through calculating the MF . Among the boundary surface points, we generate a histogram showing the number of detected boundary surface points for each intensity value. The histogram then is processed with smoothing techniques. The intensity value at which the histogram reaches its local maximum can be considered to be the proper iso-value for surface rendering.

As another important visualization technique, volume rendering has evolved for nearly two decades. With the fast development of algorithm and hardware, volume rendering has achieved real-time performance nowadays and become an efficient tool for knowledge discovery from volumetric confocal image datasets. In this thesis, we investigate one important issue in volume rendering, 2D transfer function design, which can be described as follows:

$$\begin{aligned} Color(x, y, z) &= TF_{color}(f(x, y, z), |g(x, y, z)|) \\ Opacity(x, y, z) &= TF_{opa}(f(x, y, z), |g(x, y, z)|) \end{aligned}$$

The information about gradient magnitudes obtained through calculating the MF is utilized to decide the mapped colors for the boundary surface points and internal structure points. A linear look up table is employed to generate the color values during rendering process. To better illustrate the spatial relationship between boundary surface

points and internal structure points, the opacity values for surface points can be set to a low level such that those boundary points are rendered in semi-transparent mode.

Based on volumetric visualization, we also conduct research into the roles that VR can play in the analysis and manipulation of volumetric confocal images. In the immersive VR environment, we develop effective interaction methods between users and cellular structures with various interactive tools. As a result, interactive and intuitive manipulations on the rendered confocal structures are achieved. Furthermore, to facilitate quantification on volumetric confocal data, a volume localization algorithm in VR environment is developed. In this algorithm, several templates are designed for extracting sub-volumes with different shapes from whole volumetric confocal dataset. Tracking device like Polhemus Patriot (a six-degree-of-freedom stylus tracking system) is used to produce the spatial data of the localization, guiding the navigation through the 3D space. Once the object of interests falls into the template shape completely, the volumetric points belonging to the object detected through comparing its position with the border will be extracted and analyzed subsequently.

9.2 Future Improvements

The work presented in this thesis has advanced the computer techniques for studying 3D confocal image data. But there is still work that can be done to further polish these techniques. A few directions are outlined here.

9.2.1 Enhancement of Central Administration Database

We have established a web-based database for online administration of confocal image datasets. The database facilitates the online storing, viewing, editing and retrieving

confocal images for collaborative research. In the future, data-mining or image-mining techniques can be integrated in the database in order to provide automatic analysis of large amount of image data. Besides image data and metadata, the database can be designed to support VRML data for surface modeling.

9.2.2 Precise Correction Parameters Calculation for Attenuation Correction

During the calculation of correction parameters for the depth attenuation of confocal image stacks, the least-square method is used to generate the optimal correction parameter for each section. However, some isolated points may cause reverse influence. Threshold techniques can be employed to filter out those isolated points before calculation. In the next stage, we plan to find a solution which is able to automatically and accurately generate the precise correction parameters by filtering out the isolated points.

9.2.3 Object Segmentation with Optimal Gradient Magnitude

In this thesis, we propose a method to evaluate the suitability of gradient magnitude as the potential threshold for boundary point detection of 3D confocal image stacks. With this method, the optimal gradient magnitude for 3D boundary detection can be identified. The boundary detection results can also be utilized for the segmentation of confocal objects like nuclei. Therefore, in the future, the optimal gradient magnitude can first help to detect boundary points belonging to different objects. Then we can separate connected objects using a 3D *Watershed* algorithm or a cluster analysis algorithm. The successful

segmentation can help to improve the quantitative analysis on individual confocal objects that are separated from each other.

9.2.4 Transfer Function Design for Multi-channel Confocal

Datasets

To improve the visualizing effect of volume rendering, we propose a 2D transfer function making use of the information of boundary surface points. But this algorithm is limited to single channel confocal dataset. It is much more complicated to handle the assignment of color and transparency values for multi-channel confocal datasets due to the variety of data values of volumetric points. In the future, we will make use of VR techniques to develop a semi-automatic transfer function for volume rendering of multi-channel confocal datasets. During navigating through the cellular structures in the stereo immersive environment, the data properties for points belonging to a specific structure can be obtained with the localization algorithm. The property information can be recorded along with the navigation. After collecting and analyzing the properties for different structures, a look up table can be built for storing the customized color and opacity values for these structures. Then during subsequent volume rendering, the look up table can be used.

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