AUTOMATED COUNTING OF CELL BODIES
USING NISSL STAINED CROSS-SECTIONAL IMAGES

A Thesis

by

ASWIN CLETUS D’SOUZA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2007

Major Subject: Computer Science
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Approved by:

Chair of Committee, John Keyser
Committee Members, Yoonsuck Choe
Vinod Srinivasan
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ABSTRACT


(December 2007)

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Chair of Advisory Committee: Dr. John Keyser

Cell count is an important metric in neurological research. The loss in numbers of certain cells like neurons has been found to accompany not only the deterioration of important brain functions but disorders like clinical depression as well. Since the manual counting of cell numbers is a near impossible task considering the sizes and numbers involved, an automated approach is the obvious alternative to arrive at the cell count. In this thesis, a software application is described that automatically segments, counts, and helps visualize the various cell bodies present in a sample mouse brain, by analyzing the images produced by the Knife-Edge Scanning Microscope (KESM) at the Brain Networks Laboratory.

The process is described essentially in five stages: Image acquisition, Pre-Processing, Processing, Analysis and Refinement, and finally Visualization. Nissl staining is a staining mechanism that is used on the mouse brain sample to highlight the cell bodies of our interest present in the brain, namely neurons, granule cells and interneurons. This stained brain sample is embedded in solid plastic and imaged by the KESM, one section at a time. The volume that is digitized by this process is the data that is used for the purpose of segmentation.

While most sections of the mouse brain tend to be comprised of sparsely populated neurons and red blood cells, certain sections near the cerebellum exhibit a very high density and population of smaller granule cells, which are hard to segment using simpler image segmentation techniques. The problem of the sparsely populated regions is tackled using a combination of connected component labeling and template
matching, while the watershed algorithm is applied to the regions of very high density. Finally, the marching cubes algorithm is used to convert the volumetric data to a 3D polygonal representation.

Barring a few initializations, the process goes ahead with minimal manual intervention. A graphical user interface is provided to the user to view the processed data in 2D or 3D. The interface offers the freedom of rotating and zooming in/out of the 3D model, as well as viewing only cells the user is interested in analyzing. The segmentation results achieved by our automated process are compared with those obtained by manual segmentation by an independent expert.
To my family
ACKNOWLEDGEMENTS

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CHAPTER I
INTRODUCTION

The human brain has long been an area of immense fascination for researchers who believe that fully understanding its structure and functions would lead to great strides in the field of science, from biology to artificial intelligence. In this pursuit to completely understand the brain, scientists look to solve smaller and simpler problems before attempting to solve more complex ones and this is the reason the mouse brain has gained much attention from research groups from all across the scientific world.

From a medical point of view, the study of the mouse brain could help in identifying and arriving at possible treatments for neurological disorders like Parkinson’s disease and clinical depression. An important metric in this study has been the count of certain cell bodies in the brain, like neurons, since the loss of neurons has been found to accompany the deterioration of many brain functions. Attaining accurate counts of these cell bodies is however not an easy task due to the microscopic size of these cells, and the vast numbers in which they are present.

Certain methods of estimation have been used over the years in laboratories, like the hemacytometer and the counting chamber, but these methods are heavily dependent on sample concentrations and the estimation formula used [1][2]. Fortunately, due to much technological advancement in the fields of microscopy and digital imaging, more accurate counting mechanisms are now possible. Detailed cross-sectional images of mouse brain tissue can be extracted using devices like the Knife-Edge Scanning Microscope (KESM), which can then be processed automatically by means of various algorithms, and visualized in 3-D.

This thesis will describe a software application that counts cells in Nissl-stained image data obtained from the KESM, and enables the user to visualize these cells in 3-D using a graphical user interface.

This thesis follows the style and format of IEEE Transactions on Visualization and Computer Graphics.
1.1 Objectives

The primary goal of this thesis is to describe a software alternative to neurobiologists for purposes of obtaining cell counts and visualizing the cells of interest from image data. The application described has the following features:

i. An ability to load a number of consecutive images in the stack
ii. An automated process that segments the cells of interest
iii. A provision for the user to set certain cell characteristics that might help in segmentation
iv. Ability to traverse through the image set one image at a time
v. Options to show the boundaries of the cells found, in 2-D
vi. A 3-D visualization showing the cell bodies identified
vii. Graphical user interface

1.2 Significance

Neuron loss has been shown to have a significant effect on functions like memory acquisition and illnesses like clinical depression [3] [4]. This correlation is also evident in patients suffering from various neurological disorders like Alzheimer’s and Parkinson’s disease. Scientists studying these disorders in mice can gain significant savings in time with the help of an automated process that supplies them readily with the cell count of the neurons in the tissues they are studying. Such a process can be used specifically by members of the Brain Networks Laboratory to study brain tissue.

1.3 Outline of the thesis

The problem of automated cell counting has been addressed before and many techniques have been put forth to solve it. In Chapter II, we provide the reader with background information needed to understand the context and the conclusions of this thesis.

The working of the Knife-Edge Scanning Microscope is briefly described in Chapter III, Image acquisition. The two prominent types of images that we shall term type A and type B are introduced here, and their differences explained. Since the cells found in these images vary so vastly in size, shape and density, the algorithms used to
tackle them vary as well. We use a combination of connected component labeling and template matching for type A segmentation, and a variation of the watershed algorithm for segmentation of type B.

Pre-processing of the images must take place before the application of either of the algorithms mentioned above, in order to aid in better segmentation. We introduce these techniques in Chapter IV, Pre-processing, while Chapter V, Processing, provides an in-depth description of the two algorithms for the respective types of images.

Post-processing is an important stage, as both these algorithms do not result in perfect segmentation immediately once they are run. Splitting of groups of cells into their individual constituents, merging of components that are part of the same cell, elimination of noise, and so on are important procedures that must be done before the final stage. These are discussed in detail in Chapter VI, Analysis and Refinement.

Once the cells have been segmented, a visualization of them is helpful to the neuroscientist, who can then view the dataset from any angle/zoom and study the structure and arrangement of these cell bodies. Marching cubes is used to generate the 3-D volumetric representation of this data set. We describe this algorithm as well as the other interface features in Chapter VII, Visualization.

To find out how well the results of the software application compare with those of manual segmentation, we describe the method used to validate the results in Chapter VIII. The help of an independent expert is taken for comparison.

Finally, the conclusions of the research work, and a list of possible improvements are provided in Chapter IX.
CHAPTER II
BACKGROUND AND OVERVIEW OF STAGES

In this chapter, we provide the reader with a brief description of the neuron, the staining mechanism that was used to obtain the samples, and a review of image segmentation literature.

2.1 Basic concepts in neuroscience

2.1.1 Neuron theory

The ‘neuron theory’ states that nerve tissue is composed of individual cells which are fully functional units. Although the morphology of various types of neurons differs in some respects, they all contain four distinct regions with differing functions: the cell body, the dendrites, the axon, and the axon terminals [5] [6] [7]. The structure of a standard neuron can be seen below in fig. 1.

![Fig. 1. Neuron anatomy.](http://en.wikipedia.org/wiki/Image:Neuron-no_labels.png)

2.1.2 Nissl cell bodies

Named after Franz Nissl who developed a staining mechanism to identify neuronal cell bodies throughout the brain, Nissl cell bodies were the terms used to describe dense granular masses often found in nerve cells [8].
2.2 Staining methods

Staining is basically the process of making a compound of interest (DNA, proteins) more easily identifiable by enhancing its visibility through certain chemical means. The online medical dictionary defines staining as ‘the use of a dye, reagent, or other material for producing coloration in tissues or microorganisms for microscopic examination’ [9]. For the study of neurons, the following staining methods have been widely used:

2.1.1 Golgi staining

The Golgi technique stains neurons by using silver chromate. The detailed structure of the neuron (axons, dendrites) is made visible via this stain and has been very useful in the study of these cells. The Golgi method is essentially a stochastic technique and its exact chemical mechanism remains unclear [10].

2.1.2 Nissl staining

Nissl staining stains the Nissl bodies in cells, technically called the endoplasmic reticulum. The staining procedure consists of sequentially dipping the mounted brain slices in about a dozen different solutions for specified amounts of time [11]. We see a section of a Nissl-stained image below in fig. 2.

![Fig. 2. Section of a Nissl-stained image.](image-url)
2.3 Literature review

The application of computer image processing to cell counting and recognition has drawn much attention from image processing and cell biology communities [13]. Early work was based on simple thresholding techniques which assumed that the cells were significantly darker/lighter than background pixels [14] [15]. In the case of colored images, different colors were used to segment different objects [16]. This segmentation method has been applied to the detection of cancer cell nuclei as well [17]. Active contour detection has at times also been used in cell identification [13] [18]. In general however, most work in this area of cell segmentation could be broadly classified into two techniques: interactive and automated.

2.3.1 Interactive cell segmentation

Here, the human visual system and expert judgment is employed to manually segment and classify cell regions from the images, using a computer graphics device such as a tablet or a mouse. In some techniques, the expert goes through several 2-D images, marking the boundaries of the cell/nucleus of interest as he/she goes along [19]. The algorithm finally generates a surface that uses these boundaries to visualize the cell.

Reasoning that this process above was too tedious for the expert, work has been done in the development of several tools like hole-filling, point-bridging and surface-dragging that work in 3-D instead of 2-D, thus claiming to save valuable time during the segmentation process [20]. Many of these techniques employ some basic automated segmentation techniques to get started, before the manual process begins.

2.3.2 Automated segmentation

There has been a lot of work done in the field of automated segmentation, as most of the time, medical image stacks run to hundreds of images, with cell sizes being extremely small. Expecting a user to manually demarcate the cell/nucleus boundaries is not only time consuming, but near to impossible considering the tens of thousands of cells that might be present in the volume. Automated techniques will consequently be much faster, and will not burden the examiner with any of the segmentation work, other
than the initialization of parameters. Prior work in the automated effort can be broadly classified as:

i. Segmentation by threshold values
ii. Pattern recognition
iii. Gradient flow techniques

i. Segmentation by threshold values

In image analysis, there needs to be a key criterion to differentiate between objects of interest and other objects, or in other words, ‘foreground pixels’ and ‘background pixels’.

![Pepper seeds on a white surface.](image)

For instance, in fig. 3, it is easy to identify the foreground pixels as shades of brown and background pixels as white. *Thresholding* is an image processing technique for converting a grayscale or color image into a binary image based upon a defined value. If a pixel in the image has an intensity value less than the threshold value, the corresponding pixel in the resultant image is set to black. Otherwise, the resulting pixel is set to white. Image thresholding is very useful for keeping the significant part of an image and getting rid of the unimportant part or noise. This holds true under the assumption that a reasonable threshold value is chosen [21].

Formally, if intensity thresholding is applied to the pixels of the pre-processed image $I$, with the threshold value $t$, the resulting image $I_r$ will be:
\[ L = \begin{cases} 
1 & I \geq t \\
0 & \text{else} 
\end{cases} \]

One of the key problems of thresholding is that it is very sensitive to imaging variations in light and noise, and thus must be used only in situations where the foreground/background pixels are clearly identifiable [13]. Also, as can be seen in fig. 4, objects of interest that touch each other are hard to separate. Thresholding when all cells are clearly separable is a simple and straightforward way to proceed, but when closely packed cells appear in images, like in some of the cases in our Nissl stained samples, this technique is of little help as a solution.

We use this technique in the pre-processing stage for images of cells that are sparsely populated.

Fig. 4. Image after thresholding.

\textit{ii. Pattern recognition}

Pattern recognition is defined as ‘the study of how machines can observe the environment, learn to distinguish patterns of interest from their background, and make sound and reasonable decisions about the categories of the patterns’ [22]. ‘Machine Vision’ is more closely related to our cell counting problem, as it is the area of study where images captured by an imaging system are analyzed using pattern recognition techniques to identify and classify objects of interest [23]. Some of the techniques are briefly described below:
• Template matching has long been used as a technique to help match objects of interest with the sample under supervision. Here, a template or a prototype, usually a shape that is a known representation of the object of interest, is searched for in the sample dataset and checked for how similar the two are, taking into consideration the various orientations the object could be in. A certain ‘score’ or ‘measure’ of similarity is calculated, and a threshold value of this score is used to decide the absence or presence of the object in the sample under inspection.

• Statistical classification basically represents each pattern in terms of a set number of features, and calculates how closely the sample matches these features. The pattern could be viewed then as a point in $d$-dimensional space, where $d$ is the number of features that have been enumerated [23]. Lin et al. use a feature set consisting of volume, texture, convexity, shape, circularity, area, mean radius and eccentricity [24]. The probability distributions of the patterns belonging to each feature class must either be specified explicitly during design or learned by means of a training set.

• Syntactic approach uses the combination of simpler features to describe complex features. The complexities of implementing grammar and noisy patterns result in large computational needs, due to the ‘curse of dimensionality’ [25].

• Neural networks use networks and combinations of simple processors to attack the problem of feature extraction. It is closely related to statistical pattern recognition, though its advantages as a result of parallel processing are noteworthy.

iii. Gradient flow techniques

The watershed algorithm is an algorithm that has been widely studied and used over the last couple of decades for the purposes of image segmentation [24] [26] [27] [28] [29] [30].

The biggest advantage of this algorithm is in attacking the problem of ‘connected cells’, which remained unsolved when thresholding techniques were used. It places great value on the gradient of the cells, which tend to go from dark to light at the borders (or
vice-versa if cells are stained white). If these areas where the gradient changes sharply are spotted and all the areas in between are connected to each other, a segmentation of the image is formed that is successful in distinguishing individual cells in a clustered section.

The key principle behind this algorithm is to consider that the gray levels of the pixels of the image are not just color values, but ‘height values’, where the greater the gray-scale value, the taller the peak and vice-versa. Thus, the image represents not mere pixels, but a topographical view of crests and troughs all across the image. At this point, we start filling the scene with water, up to a height $h$, incrementally from the lowest to the highest value.

The basins that are formed correspond to the connected components in the image, and the points where different basins meet are called the *watershed* points, much like dams. This algorithm tends to over-segment the image, and post-processing techniques are performed to ensure a good final segmentation.

*Gradient flow tracking* is a novel approach to segmentation. Instead of growing the catchment basins from the minima outwards, like the watershed algorithm, this algorithm points each voxel in the direction of its gradient flow. The gradient vector field is then diffused with an elastic deformable transformation, which smoothes the gradient field by propagating gradient vectors of large magnitude to vectors with weak gradients. Thus, at the end of the procedure, we have each voxel either pointing to another voxel by means of the gradient flow vector, or being a minimum in itself. All voxels that flow to the same center are then classified together as a cell, and this automatically separates individual cells from clusters [31].

### 2.4 Overview of stages

In fig. 5 we show a pictorial representation of the stages that will soon be described in the chapters to follow, with each chapter describing each of the stages in the process.

Though there are essentially 5 stages: *image acquisition, pre-processing, processing, analysis and refinement, and visualization*, the algorithms applied in each stage to the different types of images vary. The two types, *type A* and *type B* are
introduced in section 3.4. Though the *image acquisition* stage and the *visualization* stage are identical for both types of images, the three stages in between vary vastly, as depicted in fig. 5 by the branching off of the stages in two directions.

This is because, as we will explain in the chapters to follow, the same segmentation methods do not yield the best results for both types of images.

*Type A* image segmentation heavily depends on the post-processing stage, since much of the 3D grouping can happen only after the components at each image level are filtered appropriately for noise and mistakenly split components. *Type B* however attains a fairly good quality of segmentation after the processing stage itself.

We start with the first stage, *image acquisition* in the next chapter.
CHAPTER III

IMAGE ACQUISITION

The Knife Edge Scanning Microscope (KESM) is an instrument at the Brain Networks Laboratory of Texas A&M University that performs the simultaneous slicing and imaging of a mouse brain (fig. 6). It was designed by Dr. Bruce McCormick, and has since played a pivotal role as the bridge between the biological world and computer science, converting stained samples of mouse brain into its volume digitized representation that can then be used for various types of study using computer algorithms.

3.1 Features of the KESM

The KESM handles the following operations:

i. Slices layers of mouse brain embedded in solid plastic in a staircase like fashion.

ii. Lights up the imaging sample using light shone through the diamond knife that is used for slicing.

ii. Captures a high resolution, high magnification image of the layer being sliced currently

iii. Stores these images that were read into a digital storage device.

Fig. 6. Photograph of the KESM.
[http://research.cs.tamu.edu/bnl/static/galleryKesm.html]
3.2 Sub-systems of the KESM

This microscope is comprised of the following sub-systems, to enable it to provide for all the features mentioned above in 3.1. The sub-systems as mentioned in [12] are as follows:

i. The precision positioning system and ultra-microtome axis align the sample in X, Y and Z and keeps track of the position of the sample and the knife edge.

ii. The image capture system comprises of the microscope objective coupled with the high-resolution camera

iii. The Image analysis and Data storage system contains 5 clustered servers that store the information transferred over the gigabit network.

3.3 Working principle

The brain specimen which is in solidified form after being embedded in plastic is mounted using the precision positioning system. The diamond knife accurately cuts this solid block in a step-wise fashion to minimize resistance. At the same time, light is shone through the diamond knife and into the layer of tissue that is currently being sliced (fig. 7). This illuminates the region and provides better lighting conditions for the imaging system to capture the details as accurately as possible. The diamond knife thus serves dual use, one for the physical sectioning of the tissue and the other as an optical element through which light is shone during sectioning [12].
3.4 Types of images attained

We come across two types of cell images in our dataset. The first kind, which we call type A for the remainder of this thesis, is comprised of regular sized cell bodies – mainly neurons, glial cells, red blood cells and dying cells. They can be seen in fig. 8 (a). For the most part (at least 80-90% of the image space), the Nissl stained images attained comprise of regions of type A.

At certain portions of the brain, like the cerebellum, granule cells of very small size occur in very high densities in a band like fashion. We call these type B images and a sample can be viewed in fig.8 (b). Though the differences are obvious, they are enumerated here for clarity:

- Cells in type A are sparsely populated, while those in type B are very densely populated
- There are two main types of cells in type A: red blood cells / dying cells that are dark, small and compact in shape and neurons / glial cells that are larger, often hollow or exhibit a central dark spot inside a hollow ellipse, resembling an ‘eye’. There are three main types of cells in type B: granule cells that are tiny and compact, interneurons that are sparsely populated, and purkinje cells that are big and have a lighter texture than the rest of the cell bodies.

We analyze these two types in greater detail with the help of some magnified images, in the following sections.
3.4.1 Type A

The descriptions for each cell are provided below, with fig. 9 showing magnified sections of the image for better understanding:

- **Neurons / Glial cells**: are marked with red outlines in the figure above. They are identified by the central dark nucleus and an elliptical outer boundary, which makes them similar to the shape of the human eye.

- **Red blood cells / dying cells**: are darker and more compact than the rest of the cell bodies present. However, it is easy to mistake neurons for these cells because the central nucleus of the neuron is not visible in all cross-sections. Thus, the neighboring sections must be analyzed as well before classifying a cell as an RBC or a dying cell.

- **Endothelial cells**: occur rarely, but are still visible in Nissl images. They are found surrounding blood vessels.
3.4.2 Type B

Type B on the other hand consists of the following types, as seen in fig. 10:

- **Granular cells**: are highlighted by light green in the figure. They are densely populated and very tiny.
- **Purkinje cells**: are bigger, and exhibit neuron-like characteristics like those we find in type A. They are circled with yellow in fig. 10.
- **Interneurons**: are highlighted by red color. As is evident in the image, they are very sparsely populated in comparison to granular cells.
- **Endothelial cells**: are present in type B images again, and are found next to blood vessels.
CHAPTER IV
PRE-PROCESSING

In this chapter, we describe the various image processing techniques that were applied to the image stacks before the processing stage.

4.1. Image normalization

Normalization, also known as ‘contrast stretching’, is an image enhancement technique that ‘stretches’ the range of intensity values of the image. In our cross-sectional image stacks, due to variations in lighting and positioning on the KESM, the individual images often vary in their brightness and gray-scale range. This non-uniformity in range is problematic in the processing stage, where a high level of importance is placed on the gray-scale value of each pixel. Both, the watershed as well as the thresholding algorithms are based on the assumption that each image has the same brightness levels as the rest of the images in the stack.

To achieve this range stretching, we should first decide on the minimum and maximum gray scale value that the resulting image should have. Let us call these extremes $R_{\text{min}}$ and $R_{\text{max}}$. Usually, they correspond to the range of values that the data element can hold, for instance for an 8-bit integer, $R_{\text{min}} = 0$ and $R_{\text{max}} = 255$. We then find the minimum and maximum of the values of the pixels in image $I$ under processing. Let us call these $P_{\text{min}}$ and $P_{\text{max}}$. In order to prevent rare noisy pixels from distorting this range, the histogram of the image is analyzed to select better values for $P_{\text{min}}$ and $P_{\text{max}}$. [32]

Finally, for each pixel $P$ in the image $I$, the resultant pixel $R$ is calculated as:

$$R = (P - P_{\text{min}}) \left( \frac{R_{\text{max}} - R_{\text{min}}}{P_{\text{max}} - P_{\text{min}}} \right) + R_{\text{min}}$$

4.2 Median filtering

Median filtering is used to reduce noise in an image. While a similar technique called ‘Mean filtering’ is used to accomplish noise reduction as well, it suffers from the
Problem of blurring, as each pixel is replaced by the average intensity value of its neighbors. In median filtering though, no such average is taken, and the problem of blurring is thus avoided. This technique instead sorts the neighboring pixel values in a list, and the ‘median’ value, which is the value at the center of the list, is used [33].

To put it formally, if image I has dimensions $S_x \times S_y \times S_z$, $P_{i,j,k}$ is a pixel at location $(i,j,k)$ where $0 \leq i \leq S_x$, $0 \leq j \leq S_y$, and $0 \leq k \leq S_z$, and $N[0..n-1]$ is an array that holds the pixel values of $n$ neighbors of $P_{i,j,k}$ in sorted order, then the resulting median-filtered image $I_r$ contains at location $(i,j,k)$,

$$R_{i,j,k} = N \left[ \frac{n}{2} \right]$$

For instance, in the following $3 \times 3$ sample neighborhood, the central value $P_{1,1}$ is a noisy pixel whose value needs to be suppressed.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>100</td>
<td>97</td>
<td>104</td>
</tr>
<tr>
<td>102</td>
<td>6</td>
<td>104</td>
</tr>
<tr>
<td>101</td>
<td>94</td>
<td>99</td>
</tr>
</tbody>
</table>

Sorting the values, we have the neighborhood array N with n=9, as follows:

$N[0..8] = \{6, 94, 97, 99, 100, 101, 102, 104, 104\}$

The resultant pixel after median filtering will be

$$R_{1,1} = N \left[ \left\lfloor \frac{9}{2} \right\rfloor \right] = N[4] = 100$$
Sample median filtering results can be seen in fig. 11.

4.3 Thresholding

As explained in section 2.3.2, *thresholding* is the process of classifying the image contents into foreground and background pixel classes, \( F_g \) and \( B_g \). On a gray scale image, this is done by comparing each pixel to a threshold value \( t \). Pixels that cross the threshold \( t \) are labeled \( F_g \) and the remaining \( B_g \). Formally,

\[
(x, y) \in F_g \forall I(x, y) \geq t \\
(x, y) \in B_g \forall I(x, y) < t
\]

The resulting image after thresholding can be expressed as a boolean matrix \( I_r \) as follows

\[
I_r = \begin{cases}
1 & \text{if } I \in F_g \\
0 & \text{else}
\end{cases}
\]

4.4 Gradient transform

The *gradient transform* serves to provide important cues about cell boundaries, which are helpful in the *watershed* algorithm when building ‘dams’ on the edges of the catchment basins. In the case of the Nissl images we obtained, the gradient from cell pixels (foreground) to background pixels is much higher than the corresponding intra-background or intra-foreground pixels. This important visual cue is exploited in the human cognition system as well, and is similarly applied to machine vision using this transform.

For an image function \( f(x, y) \), the gradient magnitude \( g(x, y) \) is computed as follows [34]:

\[
g(x, y) \equiv \sqrt{\Delta x^2 + \Delta y^2} \\
\Delta x = f(x + n, y) - f(x - n, y) \\
\Delta y = f(x, y + n) - f(x, y - n)
\]

where \( n = 1 \)
The Sobel operator is used as an approximation to calculate these equations. Since we have a 3D image, 3-dimensional operators are used for each of the x, y and z directions, as shown in fig. 12.

The Sobel operator is less sensitive to isolated noisy variations in pixel values, since the filter calculates a local average over sets of pixels in the immediate neighborhood. Fig. 13 shows the results of a 2-D Sobel operator on a sample portion of a Nissl image.

4.5 Distance transform

The distance transform runs through the image and returns the distance of each point from its nearest boundary. Many segmentation decisions can be made with the
knowledge of how ‘deep’ a pixel is in a cell, and this aids the watershed algorithm while creating catchment basins.

![Image](image_url)

**Fig. 14.** Chamfer 2-D Distance transform on sample image (a) Before (b) After.

Image thresholding is first applied to the image stacks to separate the Image $I$ into foreground and background pixel classes, $Fg$ and $Bg$. This binary image is then used to calculate the distance transformed image $I_d$ as follows [35],

$$I_d(x, y, z) = \begin{cases} 
0 & I(x, y, z) \in \{Bg\} \\
\min \left( |x - x_0, y - y_0, z - z_0|, \forall (x_0, y_0, z_0) \in Bg \right) & I(x, y, z) \in \{Fg\} 
\end{cases}$$

where the metric $|x - x_0, y - y_0, z - z_0|$ is a distance measurement used to quantify how near or far the point $(x, y, z)$ is from the closest background pixel. Euclidean distance, defined as

$$|x, y, z| = \sqrt{x^2 + y^2 + z^2}$$

is the best metric of distance, but due to its complexity, simpler approximations are generally used in its place. An algorithm known as the *chamfer distance transform* [36] accomplishes this in a 2-pass process to generate the distance transformed image $I_d$. The algorithm propagates information about boundaries from pixel to pixel, in a pre-defined direction in the first pass. Background pixels are assigned a distance of 0, and this value is incrementally passed on to the pixels that are adjacent to it. In the second pass, the same is done in the reverse direction, and we end up with $I_d$ consisting of an approximation of the distance between each pixel and its nearest boundary. A sample 2-D distance transform can be seen in fig. 14.
If the distance between the central pixel and its neighboring pixels in a $3 \times 3 \times 3$ filter are described by the constants $d_1$, $d_2$, $d_3$, $d_4$ and $d_5$, then the following is used to propagate boundary information in Pass 1, where the direction is chosen as left to right, top to bottom and back to front:

\[
I_\delta(x, y, z) = \min \begin{cases}
I_\delta(x-1, y-1, z-1) + d_5, & I_\delta(x, y-1, z-1) + d_4, & I_\delta(x+1, y-1, z-1) + d_5 \\
I_\delta(x-1, y, z-1) + d_4, & I_\delta(x, y, z-1) + d_3, & I_\delta(x+1, y, z-1) + d_4 \\
I_\delta(x-1, y+1, z-1) + d_5, & I_\delta(x, y+1, z-1) + d_4, & I_\delta(x+1, y+1, z-1) + d_5 \\
I_\delta(x-1, y-1, z) + d_2, & I_\delta(x, y-1, z) + d_1, & I_\delta(x+1, y-1, z) + d_2 \\
I_\delta(x-1, y, z) + d_1 \\
I_\delta(x-1, y+1, z) + d_5, & I_\delta(x, y+1, z) + d_4, & I_\delta(x+1, y+1, z) + d_5 \\
I_\delta(x-1, y-1, z+1) + d_5, & I_\delta(x, y-1, z+1) + d_4, & I_\delta(x+1, y-1, z+1) + d_5 \\
I_\delta(x-1, y, z+1) + d_5, & I_\delta(x, y, z+1) + d_4, & I_\delta(x+1, y, z+1) + d_5 \\
I_\delta(x-1, y+1, z+1) + d_5, & I_\delta(x, y+1, z+1) + d_4, & I_\delta(x+1, y+1, z+1) + d_5
\end{cases}
\]

In pass 2, the boundary information is propagated from right to left, bottom to top and front to back, as follows:

\[
I_\delta(x, y, z) = \min \begin{cases}
I_\delta(x+1, y, z) + d_1 \\
I_\delta(x-1, y+1, z) + d_2, & I_\delta(x, y+1, z) + d_1, & I_\delta(x+1, y+1, z) + d_2 \\
I_\delta(x-1, y-1, z+1) + d_5, & I_\delta(x, y-1, z+1) + d_4, & I_\delta(x+1, y-1, z+1) + d_5 \\
I_\delta(x-1, y, z+1) + d_4, & I_\delta(x, y, z+1) + d_3, & I_\delta(x+1, y, z+1) + d_4 \\
I_\delta(x-1, y+1, z+1) + d_5, & I_\delta(x, y+1, z+1) + d_4, & I_\delta(x+1, y+1, z+1) + d_5
\end{cases}
\]

### 4.6 Gradient weighted distance transform

Lin et al. describe a novel approach to combine the results of the gradient transform and the distance transform described above, into a ‘gradient weighted distance transform’ [24]. They define this new measure $D'$ as

\[
D' = D \times \exp \left( 1 - \frac{G - G_{\min}}{G_{\max} - G_{\min}} \right)
\]

where $G$ is the gradient transform described in 3.3, $D$ is the distance transform described in 3.4 and $D'$ is the gradient weighted distance transform.
CHAPTER V
PROCESSING

This chapter mainly deals with the techniques used to process the image stack and arrive at preliminary segmentations, which are then fed to the analysis and refinement stage of Chapter VI to further improve the results. We first explain the reasons for choosing certain algorithms for certain types of images, and then move towards a description of these algorithms.

5.1. Choosing the algorithm

As mentioned in section 3.4, type A and type B vary vastly in the shapes, sizes and densities of their cells. Having a unified approach to solve both types would compromise on the quality of segmentation results of both types, and hence they are tackled separately using algorithms whose strengths are best suited for one or the other.

For type A, we would need an algorithm that can find hollow, eye-shaped cells, as well as solid cells that are sparsely populated. Thresholding followed by connected component labeling is a good approach to this sparsely populated dataset, as they are easily distinguishable from each other.

As for type B, we need an algorithm whose strength is to be able to distinguish closely grouped components. This is where the watershed algorithm comes into play. We look at each of these types in the sections to follow.

5.2 Algorithms for segmenting images of type A

5.2.1 Connected component labeling

A novel approach to connected component labeling is introduced, which differs in many ways from the more classical implementations of the algorithm [37]. The algorithm works on images that have already undergone the threshold operation, i.e. binary images that store at every position the values 0 or 1, depending on the absence or presence of a Foreground pixel at that point.
A. Basic definitions

Definition 1: Image-volume $I$ is a 3-D array of voxels, that has dimensions $s_x \times s_y \times s_z$, along the x, y and z axis respectively, with $s_z$ equaling the number of Images in the image stack.

Definition 2: Index $i$ is a triplet $(x, y, z)$ of integer values that gives the position of a voxel in the image volume, where $0 \leq x < s_x, 0 \leq y < s_y, 0 \leq z < s_z$.

Definition 3: The operator $\text{ptr}()$ is defined to imply ‘pointer to’. $\text{ptr}(i)$ would thus imply ‘pointer to index $i$’.

Definition 4: Line $l$ is defined as a 4-tuple of values $(x_s, x_e, y, \text{ptrPatch})$, where $0 \leq x_s \leq x_e \leq s_x$, $0 \leq y \leq s_y$ and $\text{ptrPatch}$ is the pointer to a ‘patch’ $P$, whose definition will be provided shortly. $x_s$ represents the start position of the line along x-axis and $x_e$ represents the end position of the line on a particular row $y$ of an Image. In the algorithm, lines consist of a sequence of connected foreground pixels for a constant value of $y$ and $z$.

Definition 5: A patch $P$ is a group of lines that belong to the same connected component. Additional attributes describing the size and shape of the connected component are also contained in $P$. Formally, $P$ can be described as a 6-tuple $(L, i_{\min}, i_{\max}, z, A, \text{ptrBlob})$ where $L$ is a list of connected lines, $z$ is the image number on which the patch is contained, $\text{ptrBlob}$ is a pointer to a Blob data type(defined in 4.2.3), $A$ is the area of the component defined by the summation

$$A = \sum_{l \in L} (x_e(l) - x_s(l) + 1)$$

and the indices $i_{\min}$ and $i_{\max}$ are defined as:

$$i_{\min}(x, y, z) = \begin{cases} \min_{l \in L} x_s(l) \\ \min_{l \in L} y_s(l) \\ z = z(P) \end{cases}$$
Fig. 15 shows a connected component in a sample image with z value 0. The values of all the lines are shown in the $L$ list, and the other attributes of the patch are shown as well.

**B. The algorithm**

The main advantage of the algorithm that will be described is that it collects and builds information about the connected components in the image on the fly. Classical implementations tend to be multi-pass algorithms, where in the first pass, the pixels are labeled, and in the second pass, equivalence classes are used to ‘collect’ pixels that belong to the same component.

However, a variation of the algorithm is described that takes advantage of pointers and linked-lists to progressively augment the attributes of the patches as each row of the image is processed.

For a linked list $L$ of lines, we define the following operations:

- pushBack($L$, $l$) Inserts the pointer to the line $l$ at the end of the list $L$
- isEmpty($L$) Returns true if list $L$ is empty, false otherwise
• begin($L$)  Returns a pointer to the first line in the list $L$
• next($L$, $l$)  Returns a pointer to the line immediately succeeding $l$ in $L$

Each row of image $I$ is scanned from left to right. When a foreground pixel is found at position $(x, y)$, a line $l$ is created with starting position $x_s$ set as $x$. The scanning continues until we reach a background pixel at position $(x', y)$, or the end of the row. At this point, the end position $x_e$ of line $l$ is set to the value $(x' - 1)$ or $s_x$, whichever is smaller, and the $y$ value of $l$ is set to the current row number.

This scanning and creation of lines is repeated till the end of the row is reached. We then compare all the new lines of this row with the lines immediately preceding it, and augment the new information to the patches that the lines point to, whenever we encounter an overlap. The algorithm is discussed in detail in the appendix (algorithm 1 and 2) but in brief is as follows:

1. Collect all lines of previous row in $UpperList$
2. Collect all lines of current row in $CurrentList$ (fig. 16)
3. Check for overlap between lines of $UpperList$ and $CurrentList$
4. If there is an overlap between line $l$ of $CurrentList$, and line $u$ of $UpperList$,
   i. if $u$ belongs to a patch, add $l$ to this patch
ii. else make a new patch \( p \), and add \( u \) and \( l \) to patch \( p \)

5. For every new addition of a line to a patch,
   i. update the attributes \( i_{\text{min}}, i_{\text{max}}, \text{area} \)

6. Update \( \text{CurrentList} \) and \( \text{UpperList} \) to their succeeding rows, and repeat all steps

At the end of the algorithm, we have a list of patches corresponding to all the connected components that were found in the image. A sample image and the resulting patch lists at various scan rows are shown in fig. 17.

5.2.2 Template matching

Thresholding and connected-component labeling are themselves not sufficient in attaining accurate segmentation results. This is due to the fact that the entire cell body of the neuron is not stained uniformly. Many Nissl cells are often stained more prominently near the cell boundaries, and at the center of the cell, which usually corresponds to the stained nucleus. Due to this non-uniformity in staining, thresholding often results in broken ‘eye’ shaped cells that only vaguely represent the overall elliptical shape of the cell. This problem is clearly illustrated in the images of fig.18.

Fig.18 (a) shows a section of a Nissl stained image, while fig.18 (b) shows the same image after a threshold has been applied to it. In fig.14 (c), we highlight in red two of the cells we have described above that face this problem. As is apparent in fig.14 (d),
connected component labeling results in convoluted shapes and smaller patches that do not reflect the actual shape of the cell in any way.

For the reasons showcased above, *template matching* is employed to find those positions in the image where these cells lie. Template matching is the process of comparing the image with a pre-defined sample, or *kernel* that contains the object of interest. The *kernel* moves all across the image like a moving window, comparing image pixels with *kernel* pixels at each position. The comparison is done by finding the difference between each pair of image / kernel pixels, and calculating the total sum of these differences. Points that correspond to low sums have a high probability of the occurrence of the object of interest.

Formally, a template $g(x, y, z)$ and image function $f(x, y, z)$ can be matched by running a distance metric $d(f, g)$ over the image with the template running across it like a box filter [38]. In its continuous form, the degree of matching could be calculated as

$$\int d(f, g)$$

where the distance metric is defined by

$$d(f, g) = |f - g|$$
In its discrete form, for an Image $I$ of dimensions $s_x \times s_y \times s_z$ and a template of dimensions $t_x \times t_y \times t_z$, the following formula sums up the ‘degree of matching’ $M$, at a particular point $(x, y, z)$:

$$M = \sum_{t_x} \sum_{t_y} \sum_{t_z} |f(x, y, z) - g(x-t_x, y-t_y, z-t_z)|$$

Fig. 19. (a) Kernel used for template matching (b) Original Image (c) Results of template matching.

Running this across the entire image volume, we get a new image volume $I'$ where each point contains the ‘degree of matching’ with template $T$. Running the $25 \times 25 \times 7$ template shown in fig. 19(a) of a sample neuron over the image volume, we get template matched results such as those seen in fig. 19(c).

Once the resultant image volume $I'$ is calculated, these results are used to make critical decisions regarding the patch segmentation. The details of how these cues from the template matching algorithm are used to provide better segmentation results are provided in Chapter VI.
5.3 Watershed algorithm for segmenting images of type B

5.3.1 Basic working principle

The watershed algorithm is a technique that makes use of image gradient cues to segment the image. It uses gray scale image data as a measure of ‘elevation’ rather than color value, and thus we attain a topographic representation of the data, also known as the Digital Elevation Model (DEM), as can be seen in fig. 20.

![Gray scale image and its Digital Elevation Model.](image1)

Now instead of using the gray scale image, if we used the gradient of the image to represent the topography, it would result in a DEM having ‘walls’ of separation between objects of interest, resulting from the edges that correspond to high values in the gradient.

![Original image, Gradient Image, Topographical representation of the gradient image.](image2)

This is depicted in fig. 21(b) and (c). These walls of separation are called *watersheds* and the regions they separate are called *catchment basins*. A minimum $M$ of a
catchment basin is the region within the basin that is at its lowest elevation, where any drop of water that were to fall inside in the basin would eventually flow to.

If now, the DEM is filled with water, with the water level slowly increasing per iteration, the catchment basins would steadily grow in size until they reach the watersheds which would be too high to scale. At the end of the iterations, we are left with a segmentation that corresponds to catchment basins and the watersheds that separate them from each other.

5.3.2 Definitions

**Definition 1**: A minimum $M$ of height $h$ is a connected region of pixels with the value $h$ from where we cannot reach a pixel of lower height without first traversing a point at a higher altitude [27]. Thus $M$ can be described as a region that is significantly darker (and hence at a lower elevation) that it’s neighboring pixels.

**Definition 2**: The neighborhood $N(p)$ of a pixel $p$ at index position $i(x,y,z)$ is defined by $N(p) = p(x+i, y+j, z+k) \forall i, j, k \in \{-1,0,1\}$. Since the algorithm is in 3-D, we have 26 neighbors for each point in the volume in the general case, with lesser number of neighbors along the edges and corners of the volume.

**Definition 3**: Each catchment basin $CB$ is associated with a minimum $M$ and denoted by $CB(M)$. It is defined as the set of all points in the image volume that are
topographically nearer to $M$ than any other minimum in the volume [29]. Thus, a water droplet falling at any point in $CB(M)$ will eventually flow into the minima at $M$.

**Definition 4:** If the image volume $I$ has value $I(x,y,z)$ at index $(x,y,z)$, then the range of gray scale values that $I$ contains is given by $0 \leq h_{\text{min}} \leq I(x,y,z) \leq h_{\text{max}} \leq 255$ where $h_{\text{min}}$ and $h_{\text{max}}$ are the minimum and maximum values, respectively. The catchment basin at height $h$, $CB_h(M)$, is defined in [27] as

$$CB_h(M) = \{ p \in CB(M), I(p) \leq h \}$$

and the threshold image $T_h(I)$ at height $h$ is similarly defined as

$$T_h(I) = \{ p \in I, I(p) \leq h \}$$

**Definition 5:** The geodesic distance between two points in a region $R$ is the length of the shortest path between the two points such that every point on this path lies within $R$. Formally, if $p_s$ and $p_d$ represent the start and destination points, the geodesic distance $gd_R(p_s,p_d)$ is given by

$$gd_h(p_s, p_d) = \min(|p_s, p_i, \ldots, p_d|) \quad p_s, p_i, \ldots, p_d \in R$$

A sample region $R$ and the geodesic path between two points within it can be seen in fig.22 (a).

**Definition 6:** Vincent and Soille’s definitions of geodesic influence zones and SKIZ are widely used across watershed transform literature as the standard terms to theoretically describe this segmentation technique [27]. Here, we provide the formal definitions that they introduced and proceed to explain the immersion procedure.

If $R_i$ is a subset of region $R$, the geodesic distance between the point $p_s$ and region $R_i$ is:

$$gd_R(p_s, R_i) = \min(gd_h(p_s, p_i)) \forall p_i \in R_i$$

If region $R$ consists of several connected components like $C_i$, the geodesic influence zone of $C_i$ within $R$, $iz_R(C_i)$ is defined as:

$$iz_R(C_i) = \{ p \in R, \forall j \in [1,k]/\{i\}, gd_h(p, C_i) < gd_h(p, C_i) \}$$
It is the locus of all points within $R$ that are closer to $C_i$ than any other component $C_j$. Fig. 19(b) shows three connected components, $C_0$, $C_1$ and $C_2$ and highlights the geodesic influence zone of $C_1$ in dark gray. The union of all the influence zones of $C$ is defined by:

$$IZ_R(C) = \bigcup_{i=1}^{k} iZ_R(C_i)$$

Now, the boundary points between the influence zones of two or more regions do not belong to the influence zones of any component in $C$ and these points are collectively called the **Skeleton by Influence Zones** or **SKIZ**$_R(C)$, given by

$$SKIZ_R(C) = A / IZ_R(C)$$

### 5.3.3 Recursive relation

Beucher and C. Lantuéjoul presented an algorithm that used *immersion* as an analogy to the watershed transformation [39]. This immersion technique has since been a popular approach, and is defined as a recursive relation which we shall describe shortly.

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**Fig. 23.** (a) Topography at Immersion level $h_{\text{min}}$ (b) $X$ at $h_{\text{max}}$ (c) Topography at Immersion level $h_{\text{max}} + 1$ (d) $X$ at $h_{\text{max}} + 1$ consists of two components, one that is growing from an earlier component, and the other as a new component.

If $X_h$ is the set of all points in the image volume at a height equal to or lower than $h$, then $X_h \subseteq T_{h+1}(I)$. If $C$ is one of connected components of $T_{h+1}(I)$, then $C$ is either an extension of a component in $X_h$ or is an altogether new component. This observation is depicted in fig. 23 at height levels $h_{\text{min}}$ and $h_{\text{min}} + 1$. The gray value in fig. 23(d) represents the new pixels arising from raising the immersion level by 1. The recursion is formally described in [29] as follows:
\[ X_{h_{\text{min}}} = T_{h_{\text{min}}} (I) \]
\[ X_{h+1} = \text{MIN}_{h+1} \cup IZ_{T_{h+1}(I)} \quad h \in [h_{\text{min}}, h_{\text{max}}) \]

5.3.4 Implementation

The implementation of the watershed algorithm takes advantage of many standard data structures like arrays and linked lists. The image volume is represented by a 3-D array of 8-bit integers, while storage of positions during the course of the algorithm is done using lists of indices. The terms ‘voxel’ and its representation ‘index’ are used interchangeably in this text.

Since the immersion procedure happens \( h_{\text{max}} - h_{\text{min}} \) times, with each iteration involving going through every pixel in the volume to see if its value is less than or equal to \( h \), a more efficient way was introduced [27]. All the voxels are instead sorted in increasing order of their heights, and stored in a data structure from where they can be retrieved readily by querying the data structure with the height \( h \). Once this is done, the ‘flooding’ step is where the recursion defined in 4.3.3 is implemented.

A. Sorting the image volume

Since we use 8-bit integers to store the values of the voxels, we know that the minimum and maximum values for a voxel are 0 and 255 respectively.

Fig. 24. Sorting data structure.
Thus, we initialize an array $VoxelsAtHeight[256]$ that stores a pointer to an index at each array location. The index pointer at $VoxelsAtHeight[h]$ points to the head of a linked list that contains all the indexes of voxels that have a height value $h$. This data structure is illustrated in fig.24. The pseudo-code for sorting by storage is available in algorithm 3 of the Appendix.

B. Immersion

By immersion, we mean the increasing of the ‘water level’ $h$ in the topography, thus ‘immersing’ the voxels that are below this level. Starting the process with $h_{\text{min}}$, the algorithm continues to iteratively increase the water level, while keeping track of catchment basins, geodesic influence zones, and watershed voxels along the way. The immersion stage is basically an implementation of the recursive relation described in sections 5.3.2 and 5.3.3. Access to all pixels at the $h$ and $h+1$ levels are easy with the data structure defined in 5.3.4 A.

Calculating geodesic influence zones is done using a distance matrix which updates distance information as and when the immersion takes place. A label matrix is also used to keep track of the various labels used. We maintain a running queue $Q$ that is used to traverse all the pixels that are to be analyzed in the current iteration.

The algorithm takes care of both cases of voxels of height $h+1$:

Fig. 25. Watershed algorithm in progress (a) $h = 0$ (b) $h = 80$ (c) $h = 130$. 
(a) Voxels that are extensions of catchment basins identified at height $h$

(b) Voxels that form an altogether new minimum.

In fig. 25, we see the watershed algorithm in progress, with the light green color representing the ‘water’ that is slowly filling up the image volume. A scaled down and slightly modified version of the ‘fast watershed’ algorithm described in [27] is presented in algorithm 4 and 5 of the Appendix.

Below in fig. 26, we see the water filled image in 2-D at $h = 135$

Fig. 26. 2D image of image 3 in the stack, at $h = 130$. 
CHAPTER VI
ANALYSIS AND REFINEMENT

Post processing is an important stage in the filtering out of noise and segmenting the cells in the best way possible. As stated before, images of type A use the results from template matching to help further segment the results of connected component labeling, and the images of type B analyze the 3-D catchment basins that were formed to eliminate noise and split/combine cells.

6.1 Post-processing in images of type A

6.1.1 Hole-filling

Many of the components that we end up with after the processing of type A images have ‘holes’ in them. This is either due to the inherent nature of the staining of the cell as we discussed earlier in 5.2.2, or simply because a few of the pixels inside the cell fell short of the threshold value that was chosen to separate background and foreground pixels. For these reasons, an appropriate hole-filling algorithm is used, that is illustrated in fig. 27. For detailed pseudo-code, the reader is referred to algorithm 6 in the Appendix. In its simplest form, the algorithm works as follows:

i. Create a boolean matrix whose dimensions are based on the min and max values of the patch
ii. Fill this matrix with data from the patch, as in fig. 27(a)
iii. Complement this matrix (fig. 27(b))
iv. Identify those patches that are touching the boundaries of the matrix.
v. Delete this patches and their corresponding regions from the matrix (fig. 27(c))
vi. Complement the new image (fig. 27(d))
vii. Use this patch as a replacement for the original patch. (fig. 27(e))

6.1.2 Scoring

To decide if a component fits a certain amount of ‘similarity’ to a standard cell, few measures have been defined to help in scoring each component. The attributes we take into consideration during scoring are as follows:
A. Size

Though cross-sectional areas of a cell vary due to the shape in 3-D, it is fairly straight-forward identifying components that are too big, by comparing them to thresholds attained either by manual observation or a training data set. The ‘size’ of a component is defined by the number of pixels it contains. Since as defined in 6.2.1, each component is represented by a patch $P$ consisting of a list of lines $l$, the number of pixels $n$ is given by:

$$n = \sum_{l \in L(P)} (x_e(l) - x_s(l))$$

B. Compactness

Compactness is a region-based shape descriptor that is given as [40]

$$C_p = \rho / A$$

where $\rho$ is the perimeter of the region.

C. Circularity

The circularity measure is similar to the compactness measure, except that it is calculated by the following formula [41]:

$$Cr = \frac{4\pi \times A}{\rho^2}$$

where a value of 1 indicates a perfect circle, and deviation towards 0 implies an ellipse with progressively larger eccentricities.
D. **Height-to-width ratio**

The height-to-width ratio of a component represented by a patch $P$ is given by the following:

$$HTWR = \frac{y(i_{\text{max}}(P)) - y(i_{\text{min}}(P))}{x(i_{\text{max}}(P)) - x(i_{\text{min}}(P))}$$

The $HTWR$ measure is a good way to spot noise, which we shall discuss below.

6.1.3 **Segmentation refinement**

Now that the components have been ‘scored’ according to the metrics described above, we can proceed to derive inferences from them to help in the post-processing.

A. **Noise elimination**

There are two predominant types of noise found in Nissl images. Chatter noise, and Non-Chatter noise

- **Chatter:** is the term used to express the physical phenomenon of the vibration of the sectioning knife that occurs during the slicing of the mouse brain by the KESM. Due to this chatter, there are often imaging artifacts that are left behind in the image, which generally correspond to alternating lines of low and high intensities (fig 28.a).
Though a fair amount of image processing was done on the image set well before processing for an automated cell count, some artifacts are too prominent to ignore and often mistakenly get classified as foreground pixels. However, many of these components that arise out of mistaken segmentation of chatter artifacts can be successfully eliminated using the scores we mentioned earlier. Horizontal chatter that can be seen in fig 28.b is often eliminated after image processing, but some artifacts still remain (fig 28.c).

![Fig. 29. (a) Section with a group of close cells (b) Threshold results.](image)

The ‘height/width’ ratio helps identify components like these which are either too long compared to their height or too tall compared to their breadths. Since chatter usually occurs in the horizontal and vertical directions only, this measure is often very helpful and fairly accurate in determining noise components.

Thus if either of the following conditions for patch $P$ is satisfied,

$$HTWR(P) < HTWR_{\min}$$
$$HTWR(P) > HTWR_{\max}$$

$P$ is classified as noise and taken off from the $PatchList$.

- **Non-chatter noise**: Other noise that occurs in the image volume is eliminated fairly easily during the 3D Grouping process described in 6.1.4. Random noisy elements that occur in an image most often do not have a corresponding noisy element at the same location in the immediately preceding or succeeding images.
and hence are not grouped together. Thus, the inherent nature of the algorithm itself eliminates noise by preventing its propagation in 3D.

B. Incorrectly grouped cells

Though it occurs relatively infrequently when compared to images of type B, cells that are close to each other sometimes end up being grouped together as part of the same component due to the threshold level that was chosen for that image. At these points, some 3D cues are made use of as well as the results of the template matching algorithm to break the large component into its constituent cells. Fig. 29 illustrates this problem.

**Identifying the groups:** Since standard Nissl cells are not boundless in their sizes, it is fairly straightforward to identify which of the components arising after labeling comprise of multiple cells.

![Fig. 30. A mistakenly grouped cell is split using information from the preceding image.](image)

If the size of the patch $P$ is $n$ and $n_{\text{max}}$ is the size of the largest cross-section of a standard cell, then if $n > n_{\text{max}}$, patch $P$ is classified as a component that might possibly contain more than one cell. The methods used to help separate them are described below.

**Using patch list comparisons:** One option for tackling this problem is by comparing the identified patch $P$ with patches that it overlaps in the immediately preceding or succeeding image. In fig 30(a), we see that the upper cross-section consists of a component that is clearly a combination of two cells. We use this information for splitting $P$ into separate cells, and thus perform the split by performing binary AND operations between the two images.
**Template matching**: The above patch list comparisons described often do not work very well because of various reasons, namely

- Cells that are mistakenly grouped in the current image are likely to be mistakenly grouped in the preceding / succeeding image as well. This is because the distance between two cross-sections in the image stack is negligible.

- A component that in reality represents a single cell, but is incorrectly broken into two due to bad thresholding might further influence more components to incorrectly split up.

For these reasons, the results of the template matching algorithm are invoked to try and get better segmentation results and split the groups appropriately. In fig.31 (a) we see a standard section from a Nissl stained image, followed by its corresponding threshold image in (b).

![Fig. 31. (a) Original image (b) Thresholded image (c) 3D Template matching results (d) Local maxima of the matched results.](image)

The results from the 3D template matching algorithm are seen in fig. 31(c). Using a threshold that classifies based on the top 5% of the gray scale range of the matched image, we arrive at an image consisting of connected components that correspond to the peaks of the matching, in fig.31 (d). Connected component labeling is run over this image, and local peaks in the components are collected. These local peaks correspond to a high probability of occurrence of a cell, not just by local 2D pattern matching but by taking into account data from about 7 consecutive images at a time. Hence, a local peak very
strongly suggests the existence of a neuron / glial cell at this point. Local peaks for the template matched patches are shown as red dots in fig. 32(a), and blue dots in fig. 32(b).

To split a large cell, we see if it contains more than one local peak within it. If it does, we use simple boolean operations like AND and MINUS with a solid circle of standard size and arrive at the results, as shown below in fig. 32(b).

![Fig. 32. (a) Local peaks (b) Splitting of a group of cells using boolean operations.](image)

C. Incorrectly split cells

Just as we have incorrectly grouped cells, we have neurons and glial cells that are incorrectly split into many pieces due to bad thresholding, as discussed in section 5.2.2.
This is where the process of *patch replacement* is introduced. Just like in the case of splitting grouped cells, we analyze the data we have obtained using *template matching*. If there is a local peak at a point where there are many small patches (most likely pieces of the same cell), *all* these pieces are replaced by a cell of standard size. Fig. 33 shows some neurons that corresponded to bad thresholded components being successfully marked by cells of standard size (blue outlines in fig. 33 (b))

For a formal description of the process discussed in the last two sections, the reader is referred to algorithm 7 in the Appendix.

### 6.1.4 Three dimensional grouping

Now that all the low scoring patches in each image have been modified, replaced or refined by some means, we can begin to combine all these patches in 3D without having to worry about erroneous 2D patches propagating in 3D. A cell in 3-D usually spans 5-6 images, reaching its largest size at the central images and diminishing in size as we move further away due to its generally spherical shape (fig. 34).

The bounding box of a patch *P* is the rectangle whose top-left corner and bottom-right corner is defined by the co-ordinates of its minimum and maximum index positions, *i*\(_\text{min}\) and *i*\(_\text{max}\) respectively. Using the bounding box for patch comparisons, we can easily eliminate patches that do not overlap with each other.

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*Fig. 34. Cell and its respective cross-sections.*
Definition 1. A blob $B$ is defined as a representation of a 3-D cell, consisting of a collection of patches attached to each other. Formally, it is a 4-tuple $(L, i_{\text{min}}, i_{\text{max}}, S)$ where $L$ is a list of patches, and $S$, $i_{\text{min}}, i_{\text{max}}$ are defined as follows:

\[ S = \sum_{p \in L} A(p) \]

\[ i_{\text{min}}(x, y, z) = \begin{cases} \min_{p \in L} x(i_{\text{min}}(p)) \\ \min_{p \in L} y(i_{\text{min}}(p)) \\ \min_{p \in L} z(p) \end{cases} \]

\[ i_{\text{max}}(x, y, z) = \begin{cases} \max_{p \in L} x(i_{\text{max}}(p)) \\ \max_{p \in L} y(i_{\text{max}}(p)) \\ \max_{p \in L} z(p) \end{cases} \]

For a linked list $L$ of patches, the following operations are defined:

- **pushBack($L, p$)**: Inserts the pointer to the patch $p$ at the end of the list $L$
- **isEmpty($L$)**: Returns true if list $L$ is empty, false otherwise
- **begin($L$)**: Returns a pointer to the first patch in the list $L$
- **next($L, p$)**: Returns a pointer to the patch succeeding $p$ in $L$

A formal algorithm for the 3D Grouping process is available in Appendix, but in brief, it is as follows:

i. Compare a pair of patches in the upper patch list and current patch list, by checking if their bounding boxes overlap. If they do not, compare next pair.

ii. If the bounding boxes do overlap, check if they really overlap by using the line information of both patches

iii. If this is true as well, add the current overlapping patch to the blob being pointed to by the upper patch

This algorithm is very similar to the 2D case with lines and patches. Once 3D grouping is carried out using the algorithms described in algorithms 8, 9 and 10 of the
Appendix, we are left with a list $BlobList$ which contains all the blobs present in the volume.

### 6.2 Post-processing for images of type B

The *watershed algorithm* has a known problem of over-segmentation in the case of large foreground objects. This is because every local minimum ends up with a catchment basin of its own that is separated from other minima by watershed lines. In the case of large objects, more than one minimum often occurs within the same object, thus resulting in multiple segments per object though there ideally should be just one.

In the case of the Nissl data set however, over-segmentation is not much of a problem since the objects are extremely tiny (25-30 pixels in 2D). The minima and their catchment basins seem to represent the granular cells uniquely, i.e. one segment per cell. However, there are still noisy segments that need to be addressed. Just as in Section 6.1, we introduce certain metrics to score the segments to decide if the segments are on track. The scoring as described by Lin et. al is described as follows:

#### 6.2.1 Scoring

**A. Size**

The total number of voxels that form the component is considered the ‘size’ or ‘volume’ $V$.

**B. Uniformity of intensity**

Since the granular cells exhibit uniformity of intensity when compared to the cells of type A which due to their ‘eye’ shape exhibit vast variations in intensity, this measure

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![Image](https://via.placeholder.com/150)

*Fig. 35. Neighborhood connectivity for pixel that is (a) (a) 4-connected (b) 8-Connected (c) 26-Connected.*
is a good metric for scoring type B cells. We use the standard deviation equation,

\[ U = \sqrt[2]{\frac{1}{n-1} \sum_{i=0}^{n-1} (v_i - v_{avg})^2} \]

where \( v_{avg} \) is the average intensity of the cell, and \( U \) is the uniformity of intensity.

C. Shape

Similar to the circularity measure defined for 2D in section 5.1.2, the shape measure is defined for a 3D object as:

\[ S = \frac{|B|^3}{64\pi \times V^2} \]

where \( B \) is the number of boundary voxels in the image, and \( V \) is the volume defined in 6.2.1.A. A boundary voxel is defined as any voxel that is not completely surrounded by other foreground voxels. Since we use a 26-connected grid for our algorithm, we use the same to decide if the voxel is a boundary one or not (fig 35.c).

6.2.2 Merging

Often times a single cell gets broken up into different segments due to the formation of multiple minima and the growth of their respective catchments basins within this cell. To identify and rectify this incorrect splitting, we use the scoring mechanisms described in section 5.2.1 as well as some new merging techniques described below.

Fig. 36. (a) \( C_1 \) and \( C_2 \) before merging (b) After (c) \( C_3 \) and \( C_4 \) before merging (c) After.

The basic principle behind the decision of whether or not to merge is to compare the score of the merged segment with that of the pre-merged segments and see if the
score is significantly higher. If it is, then the watersheds separating the segments are broken, and the segments are combined. If not, they are left as they are. In fig.36, we see an example of cell segments where merging helps (fig.(a),(b)) and where it fails to make things better(fig (c),(d)).

The scoring metrics described above can be combined linearly, with a slightly higher priority given to the size of the segment. Overall score of a segment resulting out of the watershed algorithm is given by:

$$\text{Score}(C) = aV(C) + bU(C) + cS(C)$$

If cell segments $C_1$ and $C_2$ are candidates for the merging, and if $C_{1,2}$ is the combined cell segment, merging happens when

$$\frac{\text{Score}(C_{1,2})}{\text{Score}(C_1)} \times \frac{\text{Score}(C_{1,2})}{\text{Score}(C_2)} \geq \varphi$$

where $\varphi$ is a pre-defined threshold for merging decisions.

6.2.3 Classification

The watershed algorithm described in the last few sections is very helpful in identifying granule cells. However, interneurons and purkinje cells also get segmented using this algorithm. Classifying certain cells as granule cells and interneurons is done by analyzing the features that differentiate the two.

As mentioned in 3.4.2, interneurons are found in regions where the cells are very sparsely populated, while granule cells occur in regions of very dense concentration. We can use this fact to differentiate between the two.
A kernel of size $40 \times 40$ is used to run through each image, and generate a boolean matrix that is called $\text{DenseOrSparse}$. If the number of pixels that cross a certain darkness value in this kernel window is more than a pre-defined threshold value, the algorithm outputs ‘dense’ to the boolean matrix, else it outputs ‘sparse’.

When classifying the segments, the $\text{DenseOrSparse}$ boolean matrix is referred to decide how to label them. Purkinje cells are not identified using this matrix, but granule cells and interneurons have been consistently identified. The shades of red in fig. 37(c) correspond to granule cells, while the shades of cyan correspond to interneurons.
CHAPTER VII

VISUALIZATION

This chapter describes not only the algorithm that is used to convert the volumetric data set to a 3D polygonal model for viewing, but also the various features that the graphical user interface (GUI) provides the user.

7.1 Marching cubes

The Marching Cubes algorithm is used for converting the volumetric representation to a 3D Model [42]. This is briefly described in the following sections.

7.1.1 Basic principle

Rendering what is essentially a matrix of points where each point is either ‘in’ or ‘out’ of the foreground object of interest is not as straightforward as rendering a ‘pixel’ or a ‘dot’ at each foreground point. This might work when the object is at a distance from the observer and the rendering of the dots covers all the ‘holes’ between each pair of dots, but if we zoom into a rendering of such a kind, the flaws in the rendering become obvious.

If we were to analyze this in 2-D for simplicity, and the ‘foreground object’ was represented by the dots in fig. 38(a), a straightforward but unrealistic rendering would be attained by setting up ‘sprites’ at each position where a foreground pixel is seen, like fig. 38(b). We could improve on this by instead analyzing each ‘square’ of 4 positions each in the matrix, and looking at which of the corners of the square are classified as foreground, and which as background. Since the number of possible combinations of foreground-background pixels in each square is very limited, we can use this combination to look up a table of standard cases (fig. 38(c)), from where we can derive lines that cut across edges of the square.

Using these standard cases, we can depict the foreground object as a more solid combination of line segments that form a continuous ‘boundary’ for the object, enclosing the entire foreground object inside this boundary (fig. 38(d)). The essence of this algorithm is that a complicated boundary for this object is built \textit{incrementally}, one square
at a time, without having to worry about the overall object in its entirety at any point. The finer the grid, the smoother the boundary looks eventually, as in fig. 29 (e). To extend the case to 3D, we have the cases as enumerated in fig. 39, with the volume being traversed one cube at a time, rather than squares. The end product of this operation is a smooth boundary for the object, which is much less jagged than a sprite representation.

The modifications we made on the algorithm, as well as the images that were created are shown in the sections to follow. For a more formal description of the algorithm, the reader is referred to algorithm 11 in the Appendix.
7.1.2 Modifications

Since the volumetric dataset is anisotropic, i.e. distances along the z axis are much larger than distances along the x and the y axes, certain modifications were made to the resulting heights of the rendering. Apart from these, the implementation was a standard one as described in [42].

7.1.3 Visualization results

In fig. 40 and fig. 41, we see the results attained by using marching cubes on images of type A. Here, to help the user identify the distinction between cells, random colors were used. We see the rendering of images of type B in fig. 42, with a zoomed in view available in fig. 43.
Fig. 41. Alternate orientation of dataset using in fig. 40.

Fig. 42. Visualization of image stack of 10 type B images.
7.2 **Graphical user interface**

The interface for this application was developed using the OpenGL User Interface Library (GLUI), which is an open source library in C++ that can be downloaded and used by programmers. It provides an easy to use, intuitive and flexible platform from where the user can examine the image set and analyze the resulting segmentation, both in 2D and in 3D. Here are some of the main features of the interface.

7.2.1 Choosing segmentation type

Though we can use certain image cues like average intensity to decide if a certain image is of *type A* or *type B*, we leave this task to the user to tell the application what images in general it is going to be analyzing. This prevents algorithms of one type from unsuccessfilly trying to segment images from the other type.

7.2.2 Type A

1. **Choosing between 2D and 3D**

   While visualizing in 3D can be helpful in looking at the overall topology of the cells, 2D is helpful in analyzing the boundaries segmented in each image in detail. The 2D and 3D representations are shown in fig. 44.
Fig. 44. Interface shown for type A images in (a) 2D and (b) 3D.
ii. **Traversing the image stack**

Spinner controls are used to shift through the images in the stack, one by one. This helps the user track the segmentation of certain cells as he moves through the stack. We see this spinner control in fig. 44 and fig. 45 in the ‘current image’ panel.

iii. **Threshold control**

A slider is used to change the threshold value of the current image on the fly, in case the user feels that the automated threshold value chosen was too low or too high. *Type A* images with their threshold value at 140 and 133 are shown in fig. 45 and fig. 46 respectively.

iv. **Display type panel**

To switch between the regular image (fig. 44) and the thresholded image (fig. 45), the ‘display type’ panel is equipped with a radio button to enable the user to make this choice. Option to view *template matching* results are also provided after processing takes place.
v. **Functions panel**

At what point the algorithm segments the images, converts to a 3D representation and so on are in the hands of the user. These buttons are present in the ‘functions’ panel in last few images.

vi. **Analysis panel**

This panel is ‘activated’ only after the *process* operation has taken place. The algorithm analyzes the stack using the *type A* algorithms, and the user is then free to analyze the results. The preliminary classification before 3D grouping is: *compact*, *noisy*, *corrected* (replaced), and *prospective noise* cells. We see the boundaries of these respective types outlined in fig. 47(a), where *compact* cells are outlined in green, *noisy* in red and yellow, *corrected* in dark blue and *prospective noise* in light blue.

After 3D grouping, more useful information is now available. We are able to classify as *neurons / glial*, *red blood /dying cells*, *noisy cells* and *unidentified cells*,

---

**Fig. 46.** Type A thresholded image at threshold value $t = 133$. 
as seen in fig. 47(b), where neurons are outlined in red, red blood cells in green, unidentified in yellow and noise in white.

Fig. 47. Type A image shown with (a) preliminary classification: compact, noisy, corrected, prospective noise and (b) final classification: neuron/glial, rbc/dying, unidentified and noise.
vii. **3D panel**

The options of showing the image textures / marching cubes representation is available to the user in the 3D panel, as seen in fig. 48. We see the classification in 3D in fig. 49, where green represents *neurons*, red represent *red blood cells* and *blue* represents unidentified cells.

7.2.3 Type B

Most of the features are similar to *type A*, and so we only highlight those interface features here that are unique to *type B* segmentation.

i. **Display panel**

We are able to view not only the watershed growing through the image volume, but also see unique labels with random coloration, classified cells with a set color scheme, and various other representations in 3D. We see some of these features in fig. 50 and fig. 51.
Fig. 49. Type A images in 3D after classification.

Fig. 50. Type B image in 2D during the running of the watershed algorithm.
Fig. 51. Type B cells classified uniquely using (a) random coloration (b) coloration based on cell type: granule cells or interneurons.
We also see in fig. 52 the ‘representation’ option in 3D, which is basically a unique coloration applied to each cell and superimposed on the originally image textures.

\textit{ii. Functions}

The user is given the control to ‘start watershed’ algorithm and to ‘convert to 3D’ for purposes of 3D viewing.
CHAPTER VIII

RESULTS

The procedures described in this thesis lead to segmentation results that are very comparable with those arrived at by human cognition. The segmentation results of the independent experts are first described, and then a comparison and explanation of the results achieved by the techniques discussed in this thesis is provided.

8.1 Details of experiment

Since the best way of comparing the results from automated segmentation is by comparing them with those arrived at by human cognition, experts in the field of neuroscience were asked to mark the cell bodies they found in the image sets that were provided. They were given 3 each of type A and type B images. The type A images were of size 256\(\times\)256 px, while those of the latter were 128\(\times\)128 px.

The experts proceeded to mark with different colors, the various cell bodies they were able to identify in the individual sections. In type A, neurons and glial cells were marked by a red dot, red blood cells and cells that were dying were marked by yellow dots and endothelial cells by blue dots. In type B, granule cells were marked by red dots, purkinje cells by green dots, Interneurons by yellow dots and endothelial cells by blue dots.

In fig. 53, we see the type A images that were supplied in their raw form before segmentation, as well as the results after the expert identified the cells in each image. Similarly we see the raw and labeled type B images in fig. 54. Each image was labeled by what they look like to the observer in that one image alone. For instance, a solid dark region is labeled as a ‘red blood cell’ even though it might actually be a neuron on further investigation of the preceding and succeeding image. For this reason, certain labeling corrections were made to the set, which we describe in the section 8.2.

Cells of type B that are closely packed are labeled granule cells, while those that occur sparsely in the section are labeled Interneurons, as discussed previously in section 6.2.3.
Fig. 53. (a), (b), (c) Type A images provided for segmentation by independent experts
(d), (e), (f) Marked images where the cell types are labeled by the colors red, blue and yellow.

Fig. 54. (a), (b), (c) Type B images provided for segmentation
(d), (e), (f) Marked images where the cell types are labeled by the colors red, blue, green and yellow.
8.2 Labeling corrections

For comparison between our results and those of the expert, a 3D consolidation process has to take place, because the automated process is the result of a 3D combination and not merely 2D segmentation. Thus, the results of the experts are combined in 3D using certain rules that were agreed upon, as described below.

In type A, any cell that is classified as a ‘neuron’ in even one of the images must be labeled as a neuron in all the remaining images as well. This is because the key characteristics of the neuron (central dark nucleus and elliptical outer body) might be seen only in a few images that contain it. Thus, only a yellow dot (non-neuron) can be corrected to a red dot (neuron), and not vice versa. No corrections are made to endothelial cells. We see these corrections in fig. 55. Only a handful of cells change their label during this correction process.

No corrections were made to type B cells as all cells had the same label across the different images.
8.3 Type A results

In fig. 56, we see the results attained by human cognition compared with those of the algorithm. The color scheme for the expert’s results is the same as in fig. 53 while the color scheme for automated results is as follows: Red outlines indicate neurons and glial
cells, white outlines indicated tiny noisy elements, and green is used for red blood cells / dying cells.

### TABLE 1
Comparison of manual and automated results for segmentation of cell bodies

<table>
<thead>
<tr>
<th>Img</th>
<th>Manual $\text{num}(\text{cb}_m)$</th>
<th>Automated $\text{num}(\text{cb}_a)$</th>
<th>1-1 Correspondence $\text{num}(\text{cb}_{\text{corr}})$</th>
<th>Percentage identified $\frac{\text{num}(\text{cb}_{\text{corr}})}{\text{num}(\text{cb}_a)} \times 100$</th>
<th>Percentage extra / missed by expert $\frac{\text{num}(\text{cb}<em>a) - \text{num}(\text{cb}</em>{\text{corr}})}{\text{num}(\text{cb}_a)} \times 100$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>62</td>
<td>55</td>
<td>96.5 %</td>
<td>11.3 %</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>65</td>
<td>60</td>
<td>90.9 %</td>
<td>8.3 %</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
<td>59</td>
<td>57</td>
<td>90.5 %</td>
<td>3.4 %</td>
</tr>
<tr>
<td>3D</td>
<td>72</td>
<td>68</td>
<td>62</td>
<td>86.1 %</td>
<td>8.8 %</td>
</tr>
</tbody>
</table>

### TABLE 2
Comparison of manual and automated results for segmentation of neurons

<table>
<thead>
<tr>
<th>Img</th>
<th>Manual $\text{num}(\text{n}_m)$</th>
<th>Automated $\text{num}(\text{n}_a)$</th>
<th>1-1 Correspondence $\text{num}(\text{n}_{\text{corr}})$</th>
<th>Percentage identified $\frac{\text{num}(\text{n}_{\text{corr}})}{\text{num}(\text{n}_a)} \times 100$</th>
<th>Percentage extra / missed by expert $\frac{\text{num}(\text{n}<em>a) - \text{num}(\text{n}</em>{\text{corr}})}{\text{num}(\text{n}_a)} \times 100$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>40</td>
<td>25</td>
<td>83.3 %</td>
<td>37.5 %</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>45</td>
<td>27</td>
<td>84.4 %</td>
<td>40 %</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>40</td>
<td>26</td>
<td>83.9%</td>
<td>35 %</td>
</tr>
<tr>
<td>3D</td>
<td>33</td>
<td>45</td>
<td>28</td>
<td>84.8 %</td>
<td>37.7 %</td>
</tr>
</tbody>
</table>

Since the focus is on identifying neurons, we have a separate column of images in fig. 56 showing only the outlines of neurons that were identified by our algorithm.

We tabulate these results in Table 1 and Table 2. We find that the ability of our algorithm to find general cell bodies in the image set is very strong. Examining the numbers in the ‘1-1 Correspondence’ column, we see that a high percentage (86 %) of those cell bodies identified by the expert have also been successfully identified by our automated process. This result is encouraging, considering that many cell bodies,
especially neurons, tend to get split up into multiple pieces due to their non-uniform staining during the thresholding process. This shows that the patch replacement using template matching has been overall a successful approach to this problem of non-uniform staining of cells. In the case of further classifying these cell bodies into neurons and non-neurons, the results are promising as well. The percentage of neurons found by the expert that the algorithm was also able to identify is reasonably high as well (85%).

Fig. 57. (a) Original image (b) Segmentation: first pass (c) Corrections by expert during second pass.

However, there are many neurons that are ‘extra’ in the automated segmentation. Now, these extra neurons could be either of the two following cases:

- Cells that were incorrectly marked as neurons by our algorithm.
- Cells that were actually neurons but were missed by the human observer when segmenting the first time.

To find out which of these categories the extra neurons fell into, the automated results were shown to the expert for a second look, to confirm whether each cell was indeed incorrectly marked, or a real neuron. The results of the expert did not change much from that of the first time, though there were a few cells that the observer conceded to have missed during the first time. In fig. 57 we see one such sample, where the 3 white dots in fig. 57 (c) correspond to cells that the algorithm helped the observer find. In this sample, we find that out of the 18 extra cells, 3 were conceded to be actually neurons,
while the rest of the 15 were thought to be red blood cells that were incorrectly marked as neurons.

This excess of cells classified as neurons can be explained due to the following:

- The automated experiments were conducted using an image stack of 20 images with the kernel comprising of 7 cross-sections, out of which the subset corresponding to the images handed to the expert were extracted from this stack and compared. It is thus possible that the algorithm saw neurons in other sections (beyond those given to the expert) that then propagated its labeling into this smaller subset.

- Low value of threshold might have been chosen during the template matching phase, resulting in many more local maxima forming and influencing cells to be classified as neurons / glial cells.
Due to the high priority given to neurons in the 3D classification, this error is propagated across images and we get many more cells identified as neurons than those identified by a human observer.

8.4 Type B results

In fig. 58, we see the results attained for type B. The first column shows original images, the second the results of the human observer, the third shows the automated results highlighting the classification, i.e. granule cells (shades of maroon) and interneurons (shades of green and blue), while the final column randomly colors each cell to aid in the process of counting.

Purkinje cells and endothelial cells, which occur at the boundary of the densely populated granule cells and on blood vessels respectively, are not segmented by the automated algorithm and are consequently ignored in the counting process. The results are tabulated in Table 3 and Table 4.

<table>
<thead>
<tr>
<th>Img</th>
<th>Manual $num(gr_m)$</th>
<th>Automated $num(gr_a)$</th>
<th>1-1 Correspondence $num(gr_{corr})$</th>
<th>Percentage identified $\frac{num(gr_{corr})}{num(gr_a)} \times 100$</th>
<th>Percentage extra / missed by expert $\frac{num(gr_a) - num(gr_{corr})}{num(gr_a)} \times 100$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98</td>
<td>109</td>
<td>86</td>
<td>87.7 %</td>
<td>21.1 %</td>
</tr>
<tr>
<td>2</td>
<td>83</td>
<td>101</td>
<td>75</td>
<td>90.3 %</td>
<td>25.7 %</td>
</tr>
<tr>
<td>3</td>
<td>73</td>
<td>85</td>
<td>62</td>
<td>84.9 %</td>
<td>27.05 %</td>
</tr>
<tr>
<td>3D</td>
<td>112</td>
<td>134</td>
<td>103</td>
<td>91.9 %</td>
<td>23.1 %</td>
</tr>
</tbody>
</table>

Similar to type A, we have a very high percentage of cells segmented by the human observer that have also been spotted by the automated process, but many extra as well. The watershed algorithm works well and can be seen by the numbers of 1-1
correspondence in granule cells. Interneurons are spotted by the dense/sparse matrix discussed earlier.

<table>
<thead>
<tr>
<th>Img</th>
<th>Manual $\text{num}(i_m)$</th>
<th>Automated $\text{num}(i_a)$</th>
<th>1-1 Correspondence $\text{num}(i_{corr})$</th>
<th>Percentage identified $\frac{\text{num}(i_{corr})}{\text{num}(i_a)} \times 100$</th>
<th>Percentage extra / missed by expert $\frac{\text{num}(i_a) - \text{num}(i_{corr})}{\text{num}(i_a)} \times 100$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>15</td>
<td>9</td>
<td>100 %</td>
<td>40 %</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>31</td>
<td>11</td>
<td>100 %</td>
<td>64.5 %</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>17</td>
<td>10</td>
<td>100 %</td>
<td>41.1 %</td>
</tr>
<tr>
<td>3D</td>
<td>13</td>
<td>33</td>
<td>13</td>
<td>100%</td>
<td>60.6 %</td>
</tr>
</tbody>
</table>

The problem of extra cells occurs again, which can either be incorrectly marked cells or cells that the observer failed to see. However, ending up with extra segments is a known drawback of the watershed algorithm, and so the results were not re-verified for confirmation. These extra segments can be eliminated by some changes in the post-processing method and some tweaking of the threshold data used to classify noise.
CHAPTER IX
CONCLUSION AND FUTURE WORK

9.1 Conclusion

A software application was described that could serve as an alternative to the manual approach by neuroscientists to segment, count and visualize various types of cells present in the Nissl stained images obtained from the KESM. While most of the noise was eliminated from the images and most of the cell bodies were successfully identified, the classification process still had some false positives when identifying neurons and granule cells. The watershed algorithm is one of the only ways to approach the problem of granule cells due to their size and density, and this was successfully implemented to separate out these cells.

There is still room for better classification results, and we discuss this in the ‘future work’ section.

9.2 Future work

9.2.1 Automatic identification of type A and type B

While using an average intensity threshold was indeed considered to decide if the image being analyzed was of type A or type B, this was not implemented since it was likely to be prone to error. More complex analysis to decide on the type is needed for this classification.

9.2.2 Using faster techniques for matching

The 3D template matching that is currently implemented uses a 3D kernel that steps through the volume one at a time. This is computationally very costly, and can be improved by using faster techniques like Fast Fourier Transforms (FFT).

9.2.3 Smoothing on marching cubes visualization

The smoothing operation could serve to make the 3D polygonal representation using marching cubes more appealing visually.

9.2.4 More accurate classification techniques
The problem of false positives could be further alleviated using better post-processing techniques for this classification.

9.2.5 Better comparison method for validation

As described in section 8.3, there were certain issues with the set analyzed by the experts and those analyzed by the automated algorithms. A more consistent comparison method could be used in future.
REFERENCES


[34] “Edges: Gradient edge detection,”


[38] Rudolf K. Bock, “Template Matching,”


[41] D.P. Huijsmans, “Microscopy, Modeling Visualization Mathematical Morphology,”

APPENDIX

Pseudo-code for various algorithms referenced in the thesis are provided here.

1. Scan

Initialization:
/* upperList and currentList hold Lines, while patchList holds Patches */

\[\text{upperList} \leftarrow \text{NULL} \]
\[\text{currentList} \leftarrow \text{NULL} \]
\[\text{patchList} \leftarrow \text{NULL} \]

Scanning:
For \( y \leftarrow 0 \) to \( s_y \)

\[\text{upperList} \leftarrow \text{currentList} \]
\[\text{currentList} \leftarrow \text{NULL} \]

For \( x \leftarrow 0 \) to \( s_x \)

if \( I(x, y) \in F_g \)

Initialize \( l \)

\[x_e(l) \leftarrow x \]

while \( I(x, y) \in F_g \) and \( x \leq s_x \)

\[x \leftarrow x + 1 \]
\[x_e(l) \leftarrow x \]
\[y(l) \leftarrow y \]

pushBack (currentList, \( l \))

if \( y > 0 \) and isEmpty(currentList) = false and isEmpty(upperList) = false

augmentResults (upperList, currentList)

2. Augment results( upperList, currentList )

For each line \( ul \) in upperList and \( cl \) in currentList{

if \( x_e(ul) > x_e(cl) \)

Advance \( cl \) to the next line in currentList

else if \( x_e(ul) < x_e(cl) \)
Advance ul to the next line in upperList

else

/* Case 1 - cl overlaps with multiple lines from upperList */
while (isOverlap(cl, ul) = true and isOverlap(cl, next(ul)) = true)
    Combine ptrPatch(ul) with ptrPatch(next(ul))
    Advance ul to the next line in upperList

/* Case 2 – cl overlaps with a single line from upperList */
if ptrPatch(ul) = NULL
    initialize new Patch P
    pushBack(L(P), ul)
    pushBack(L(P), cl)
    ptrPatch(ul) ← ptr(P)
    ptrPatch(cl) ← ptr(P)
    fill all attributes of P based on values of ul and cl
    pushBack(patchList, P)
else
    Initialize patch P ← ptrPatch(ul)
    pushBack(L(P), cl)
    ptrPatch(cl) ← P
    Adjust the attributes of P based on the newly added cl
    Advance cl to the next line in currentList

3. Sort by storage

    for i ← 0 to sz
        for j ← 0 to sy
            for k ← 0 to sx
                Initialize index ind(x,y,z) to (k,j,i)
                if VoxelsAtHeight[I(k,j,i)] = NULL
                    VoxelsAtHeight[I(k,j,i)] ← ind
                else
Insert \textit{ind} at the end of the List pointed to by $VoxelsAtHeight[I(k,j,i)]$.

4. \textbf{Is marked (voxel $v(x,y,z)$)}

\begin{align*}
\text{if } & \text{Labels}[z][y][x] > 0 \text{ or Labels}[z][y][x] = \text{WSHED} \\
& \text{return true} \\
& \text{return false}
\end{align*}

5. \textbf{Fast watersheds}

Sort all the voxels in the Image Volume $I$ using the $\text{sortByStorage()}$ algorithm for $h \leftarrow h_{\text{min}}$ to $h_{\text{max}}$

for each voxel $v$ in the list $VoxelsAtHeight[h]$

set $\text{Labels}(v) \leftarrow \text{MASK}$

if $\exists v' \in N(v)$ such that $\text{isMarked}(v') = \text{true}$

set $\text{Distance}(v) \leftarrow 1$

$\text{pushBack}( Q, \text{ind} )$

set $\text{curDist} \leftarrow 1$

$\text{pushBack}(\text{dummyVoxel})$

while(true)

Initialize $v \leftarrow \text{begin}(Q)$

if $v = \text{dummyVoxel}$

if $\text{isEmpty}(Q) = \text{true}$

Break

else

$\text{pushBack}(\text{dummyVoxel})$

$\text{curDist} \leftarrow \text{curDist} + 1$

$\text{ind} \leftarrow \text{begin}(Q)$

For every voxel $v'$ in $N(v)$
if $\text{Distance}(v') < \text{curDist}$ and $\text{isMarked}(v')$

if $v'$ is named

if $\text{Labels}(v) = \text{MASK}$ or $\text{WSHED}$

$\text{Labels}(v) = \text{Labels}(v')$

else if $\text{Labels}(v) \neq \text{Labels}(v')$

$\text{Labels}(v) = \text{WSHED}$

else if $\text{Labels}(v) = \text{MASK}$

$\text{Labels}(v) = \text{WSHED}$

else if $\text{Labels}(v') = \text{MASK}$ and $\text{Distance}(v') = 0$

set $\text{Distance}(v')$ to $\text{curDist} + 1$

$\text{pushBack}(Q, v')$

//New minima

for every voxel $v$ in $\text{VoxelsAtHeight}[h]$

set $\text{Distance}(v) \leftarrow 0$

if $\text{Labels}(v) = \text{MASK}$

Assign new label $l$ at voxel $v$

$\text{pushBack}(v)$

while $\text{isEmpty}(Q) = \text{false}$

Initialize $v' \leftarrow \text{begin}(Q)$

for every neighbouring voxel $v''$ in $\text{N}(v')$

if $\text{Labels}(v'') = \text{MASK}$

$\text{pushBack}(v'')$

set $\text{Labels}(v'') \leftarrow l$

6. Hole fill(patch P)

Initialize a Boolean matrix $\text{Img}$ of dimensions $\text{rows} \times \text{cols}$, where

$$\begin{align*}
\text{cols} & = (x(i_{\max}(P)) - x(i_{\min}(P)) + 1) \\
\text{rows} & = (y(i_{\max}(P)) - y(i_{\min}(P)) + 1)
\end{align*}$$
Initialize every index in Img to false
for each $l \in L(P)$
    for each $x \in [x_l(l), x_r(l)]$
        set $Img[y(l) - y(i_{\min}(P))] [x(l) - x(i_{\min}(P))] = true$

//Complement the Img matrix
for each $(x, y)$ where $0 \leq x < cols, 0 \leq y < rows$
    $Img[y][x] = \overline{Img[y][x]}$

//Run Connected-Component labeling on the resulting Img, clearing the pixels of all
//patches that do not touch any boundary
newPatchList $\leftarrow$ returnPatchList($Img[][[]]$)
for each $nP \in newPatchList$
    if $x(i_{\min}(nP)) \neq x(i_{\min}(P))$ and $x(i_{\max}(nP)) \neq x(i_{\max}(P))$
        and $y(i_{\min}(nP)) \neq y(i_{\min}(P))$ and $y(i_{\max}(nP)) \neq y(i_{\max}(P))$
            for each $l \in L(nP)$
                for each $x \in [x_l(l), x_r(l)]$
                    set $Img[y(l) + y(i_{\min}(nP))][x(l) + x(i_{\min}(nP))] = false$

//Complement Image Img again
for each $(x, y)$ where $0 \leq x < cols, 0 \leq y < rows$
    $Img[y][x] = \overline{Img[y][x]}$

//Resulting patch $P$ is one without any holes (fig. 22 e)

7. Patch refinement (PatchList)

/* This algorithm inputs a list of Patches in the current image and corrects by means of
modification or replacement, all Patches that do not satisfy certain score requirements */
for each patch $P \in PatchList$
    if $HWR(P) < HWR_{\min}$ or $HWR(P) > HWR_{\max}$ or $n(P) < n_{\min}$
        Classify $P$ as noise. Remove $P$ from PatchList
    if $C_p(P) < C_p_{\min}$ or $Cr(P) < Cr_{\min}$
        Analyze Convolved Image $I_c$ and get local maxima near $P$
        if $\exists (x, y, z) \in (i_{\min}(P), i_{\max}(P))$ such that $I_c(x, y, z) \geq Threshold$
            Modify or Replace patch $P$ so its attributes are within acceptable limits
8. **Bounding-box overlap** \((patch \ P_1, \ patch \ P_2)\)

If \(y_s(i_{\min}(P_1)) > y_e(i_{\max}(P_2))\) or \(y_s(i_{\min}(P_2)) > y_e(i_{\max}(P_1))\)

Return \(false\)

Else if \(x_s(i_{\min}(P_1)) > x_e(i_{\max}(P_2))\) or \(x_s(i_{\min}(P_2)) > x_e(i_{\max}(P_1))\)

Return \(false\)

Else

Return \(true\)

9. **Patch overlap** \((patch \ P_1, \ patch \ P_2)\)

Initialize Boolean matrix \(M\) of size \((\max i \max y)(\max x \max i)\)

for each line \(l \in L(P_1)\)

for \(i \leftarrow x_s(l) \to x_e(l)\)

set \(M[y][i] \leftarrow true\)

for each line \(l \in L(P_2)\)

for \(i \leftarrow x_s(l) \to x_e(l)\)

if \(M[y][i] = true\)

Return \(true\)

Return \(false\)

10. **Group in 3D** \((list[] \ Patches)\)

for \(i \leftarrow 0 \text{ to } s_z - 1\)

Initialize patch \(curPatch \leftarrow begin(Patches[i])\)

Initialize patch \(upPatch \leftarrow begin(Patches[i+1])\)

while \(y(i_{\min}(upPatch)) < y(i_{\max}(curPatch))\) and \(upPatch<> end(Patches[i+1])\)

if isBoundingBoxOverlap(\(curPatch, \ upPatch\)) = false

Advance \(upPatch\) to next patch in \(Patches[i+1]\)

else

if isPatchOverlap(curPatch, upPatch) = false

Advance \(upPatch\) to next patch in \(Patches[i+1]\)

else

/* Patch is overlapping. Add to the Blob list */

if \(ptrBlob(curPatch)<>NULL\)

\(ptrBlob(upPatch) \leftarrow ptrBlob(curPatch)\)
\textit{pushBack}(L(ptrBlob(upPatch)), upPatch)

Adjust attributes of \textit{ptrBlob(upPatch)} with new data

\textbf{else}

Create a new Blob \textit{B}

\textit{pushBack}(L(B), \textit{curPatch})

\textit{pushBack}(L(B), \textit{upPatch})

\textit{ptrBlob}(\textit{curPatch}) \leftarrow \textit{B}

\textit{ptrBlob}(\textit{upPatch}) \leftarrow \textit{B}

Fill attributes of \textit{B}

Add \textit{B} to \textit{BlobList}

Advance \textit{upPatch} to next patch in \textit{Patches}[\textit{i+1}]

Advance \textit{upPatch} to next patch in \textit{Patches}[\textit{i+1}]

11. Marching cubes()

for each image \textit{I}_k in the data set, where \textit{k} \in (0, \textit{s})

Read image \textit{I}_k and next image \textit{I}_{\textit{k+1}}

for each position \((x, y, z)\) in \textit{I}_k

Read in the values of the four neighboring voxels of \((x, y, z)\) in \textit{I}_k:

\begin{align*}
  v_3 &= (x + 1, y + 1, z) \\
  v_2 &= (x, y + 1, z) \\
  v_1 &= (x + 1, y, z) \\
  v_0 &= (x, y, z)
\end{align*}

Read the values of the corresponding voxels in \textit{I}_{\textit{k+1}}:

\begin{align*}
  v_7 &= (x + 1, y + 1, z + 1) \\
  v_6 &= (x, y + 1, z + 1) \\
  v_5 &= (x + 1, y, z + 1) \\
  v_4 &= (x, y, z + 1)
\end{align*}

Calculate the table index \(TI\) as described using the values of the corner voxels of the cube as values for \((i_7, i_6, i_5, i_4, i_3, i_2, i_1, i_0)\)

Use the calculated \(TI\) to extract information about edge intersections and corresponding triangles from the table

Calculate the normal for the triangles so that lighting calculations are accurate
VITA

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