A computer vision system for biological specimen image analysis.

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A COMPUTER VISION SYSTEM FOR BIOLOGICAL SPECIMEN

IMAGE ANALYSIS

by

SRINIVAS S. MANTRIPRAGADA

A thesis
Submitted to the
Faculty of Graduate Studies and Research
through the
Department of Mechanical Engineering
in partial fulfillment of the requirements
for the degree of
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at the
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1988

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ABSTRACT

A video based micro-computer system (VISION System) was developed for the analysis of biological specimen images. The system comprises of a video camera connected to an IBM PC-XT compatible micro-computer through a digitizer board installed in the computer. For microscopic specimens, the image was magnified using microscope optics. The image was digitized and subsequently analyzed by the micro-computer. In the analysis stage, the digitized image was first segmented into its constituent parts (background and specimens) by subjecting the image to a number of image processing operations. Selected features of the specimens such as area, mean optical density and total optical density were extracted from the image and classification of the specimens was made on the basis of these features.

Several biological specimen images (pollen cells, hybridoma cells, red blood cells (DNA analysis), stigmas and protein gels) have been analyzed on the Vision system. Good results have been obtained for DNA and stigma image analysis. The results for other specimens have been found to be promising.

Software for this application was developed in Turbo Pascal R using the basic PC-EYE R image processing software. The software has been developed in a modular
form to facilitate additions, deletions and modifications. Software flexibility is one of the features of this system in that, it can be adapted for use in many other biological applications.
ACKNOWLEDGEMENTS

I am grateful to Him, without whose blessings this project would never have been completed. I would also like to express my appreciation to all those who have helped make this project a success.
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LIST OF ABBREVIATIONS

r .......... Rectangularity Factor
af .......... Aspect Ratio
w .......... Width
l .......... Length
c .......... Circularity Factor
p .......... Perimeter
a .......... Area
d .......... Distance
p .......... Pixel
m .......... Slope
b .......... Intercept
x .......... Mean
σ .......... Standard Deviation
HXV .......... Horizontal and Vertical Resolution
P .......... Pollen Cell Image
G .......... Hybridoma Cell Image
W .......... Red Blood Cell Image
S .......... Stigma Image
C .......... Protein Gel Image
Chapter I

INTRODUCTION
The study of growth and change in cells is an important area of research in the biological sciences. Research of this type involves recognition and counting of various specimens and extraction of different parameters such as density and area. This is a very tedious process for the biologist as it involves long hours of examining the specimens, often under a microscope. Thus, the examination and analysis of biological specimens is a good candidate for automation.

Digital image processing techniques have been used during the last three decades for automatic analysis of biological images. These systems were based on main frame computer systems and mini-computer systems. With the fast advancement of micro-computers, it is now possible to use micro-computer based vision systems for biological specimen image analysis. Micro-computer systems are relatively fast and more flexible when compared to main frame or mini computer systems and are limited only by the amount of memory required for such systems.

Most of the work that is done in a biological laboratory is done manually. Computer vision systems offer some advantages over manual techniques in that, quantitative measurements and analyses that cannot be
performed using manual techniques because of the complexities involved, can be performed using digital image analysis techniques. In comparison to manual systems, computer systems are fast, less expensive, and being non-operator dependent, yield more consistent results than manual systems. Since the type of analysis may vary from specimen to specimen, a vision system for biological specimen analysis should be flexible enough to be adaptable for use in different applications.

1.1 Specimens: Several biological specimen images of pollen cells, hybridoma cells, red blood cells, stigmas and protein extract stains have been analyzed on the micro-computer based vision system that has been developed. The following sections briefly describe these specimens and constraints, if any, involved in the analysis of these specimen images.

1.1.1 Pollen Cells: To determine the reproductive capability of a plant, the ratio between the live (viable) and dead (non-viable) pollen produced by the plant has to be calculated. Visually it is difficult to distinguish between live and dead cells. For this reason, the cells are stained with vital dyes resulting in the live cells being stained dark purple and the dead cells being stained lightly. This results in good
contrast between the live and dead cells and also between the cells and the background and because of this contrast difference, simple gray level partitioning techniques can be used to segment the image into its constituent parts. Parameters such as area or mean optical density of each cell can be extracted and classification can be made on the basis of these parameters. Since the number of cells in each slide is not high, and there is no need for any complicated procedures to recognize the cells, the computer time needed for analysis of each slide is minimal.

1.1.2 Hybridoma Cells: Hybridoma cells are hybrid cells formed by the fusion of two other biological cells, lymphocytes and myelomas. Lymphocytes make antibodies but cannot survive in a tissue culture medium very long. On the other hand, myeloma cells grow and multiply readily in tissue culture. By fusing these cells to form hybridoma cells, we get cells having the characteristics of both the lymphocyte and myeloma, i.e., they multiply and secrete antibodies into the tissue culture. To make hybridoma cells, approximately 1 million lymphocytes and 5 million myelomas are mixed together and fused with polyethylene glycol in a tissue culture medium. The fluid-cell mixture is then transferred to wells, each well containing approximately
100 µl of the fluid-cell mixture. Wells are tiny cups approximately 5mm in diameter. Wells are moulded on plates, each plate containing 96 wells. Theoretically, each of these wells should contain at least one hybridoma cell, which multiplies to form a colony or cluster. In order for the hybridoma cells to multiply, a special environment has to be created by adding a catalyst. Spleen cells are added to the wells to act as the catalyst and allow the hybridoma cells to multiply and form colonies. Now the job of the biologist is to retain only those wells which have a single hybridoma cell colony and discard all other wells. Typically, a biologist will screen up to 10,000 wells per experiment.

The problem of developing a vision system for the identification of hybridoma colonies is compounded by the following facts:

Each well might contain four types of cells, myeloma, lymphocyte, hybridoma and spleen cells. Except for some of the spleen cells which are elongated in shape, all other cells are approximately circular and highly irregular in shape and non-uniform in size. The only differentiating factor between a hybridoma and other cells is that hybridoma cells multiply to form colonies whereas other cells do not form colonies. The contrast difference between the background and the cell
boundaries is very low making it difficult to use any gray level partitioning techniques for segmentation. The difference in contrast between the cell boundaries and the cytoplasm (interior of the cells) is also very low making it virtually impossible to detect the edges of cells within a colony. Since the cell shapes are highly irregular and sizes are non-uniform, using template matching to recognize the colonies is ruled out. The computer time needed for analysis of hybridoma images is high because of the need to use complicated procedures to segment the images.

1.1.3 Red Blood Cells: The DNA content present in individual cells is of importance to the biologist. Red blood cells obtained from certain species of reptiles or fish are considered to be standard specimens as they contain known amounts of DNA and hence have been chosen for this experiment. To calculate the density of the DNA present in a cell, the cells are stained with a staining agent, the Feulgen stain. The region of the cell containing DNA is stained dark. The higher the DNA content, the lighter is the stain and vice versa. By calculating the optical density of a stained cell region and comparing it with that of a standard DNA stain with known concentration, the DNA content in the cell can be calculated. A wavelength filter is used to increase the
contrast between the the stained region and the background. The wavelength filter causes only the light of a certain wavelength (reflected by the stain) to pass through it. As with the pollen cells, gray level partitioning techniques can be used for segmentation of the cell image.

1.1.4 Stigmas: Stigmas are cone like structures produced by a plant for reproduction. The surface area of stigmas has to be calculated in order to evaluate the fertility of a plant. A larger surface area means a greater probability of pollen attaching to the stigma and therefore, greater fertility.

As the stigma are highly irregular in shape, and tiny in size, it is very difficult to calculate the surface area by conventional means. An easier way would be to capture the image of the stigma and then calculate the area of the stigma from the digital image. Stigmas being dark in color, can be easily segmented from the background. The next step after segmentation simply involves counting the number of pixels within the boundary of the stigma. By measuring the width of the stigma at the base and the tip, and using standard geometrical formulae, the surface area of the stigma can be calculated. The area in pixels can be converted to standard units by correcting for the magnification of
the microscope and taking into account the actual area of the pixel.

1.1.5 Protein stains: Analysis of protein stains often involves identification of the proteins on the basis of their molecular weights. The protein present in a biological sample is extracted, transferred onto gels and electrolyzed. Electrolysis causes the protein to migrate. The distance of migration is inversely proportional to the molecular weight. Figure 1.1 shows a typical protein stain gel. The protein being colorless, is stained to make it visible against the gel. In order to determine the molecular weights of the unknown proteins, proteins with known molecular weights are also transferred to the gel and stained as standards. The migrations of the stains from the top edge of the gel are measured and regression analysis is done on the data obtained. A linear regression for the data points obtained for a standard is determined and the slope and intercept are calculated and these values are used to calculate the molecular weights of the unknown proteins.

Image analysis of gels involves a similar procedure as described above. Distances are calculated by drawing the intensity profile. Protein stains being darker than the background, appear as depressions in the profile.
Figure 1.1 A typical protein gel with standard and unknown protein stains
distribution and their migration from the top of the gel can be measured in terms of pixels. The molecular weights for the standard can be inputted to the computer, which then calculates the slope and intercept of the best fitting straight line. The molecular weights of the unknown protein can then be calculated by substituting for known values in the standard equation of a straight line,

\[ y = mx + b \]

where \( y \) is the molecular weight, \( x \) is the distance, \( m \) is the slope and \( b \) is the intercept of the straight line.

1.2 Objectives: The following are objectives of this research work:

(a) To develop a micro-computer based vision system for analysis of biological specimens,

(b) To build a system that can be used for both qualitative and quantitative analysis of biological specimens, and

(c) To build a flexible system that can be adapted for use with different specimens.
Chapter II

THEORETICAL BACKGROUND
The first step required for analysis of any digitized image using digital image processing techniques is to isolate the objects in the image from the background. This process, called image segmentation, may be defined as a process that divides a digital image into different non-overlapping regions. In the case of a digital image, a region is a connected set of pixels.

The quality of the image dictates the segmentation method. The most common methods are thresholding or clustering, edge or boundary detection and region analysis. An image with good contrast between the background and the objects in the image, may be segmented using simple thresholding techniques, whereas an image with low contrast may need a more complicated approach such as boundary detection. After the image has been segmented, classification of the objects of interest may be made on the basis of the area, optical density, color or shape.

2.1 Image Segmentation: Image segmentation techniques can be categorized into three classes, thresholding or clustering, edge detection and region extraction [1]. Figure 2.1 gives an overview of the various segmentation techniques.
Image Segmentation Techniques

- Edge or Boundary Detection
- Thresholding or Clustering
- Region Analysis

- Spatial Filters
- Gradient Operators
- Laplacian Filters
- Region Growing
- Relaxation

Figure 2.1 Image Segmentation Techniques
2.1.1 Thresholding or Clustering: Thresholding is a technique widely used for segmenting digital images. Most thresholding algorithms involve gray level partitioning in order to select appropriate thresholds. Thresholding involves segmentation of images based on the value of the pixel at each location. Based on certain decision rules, each pixel is labeled individually and assigned to a region such as an object or the background. Thresholding is a useful technique for images containing solid objects resting on a contrasting background. It is computationally fast and simple to implement and is very effective in defining disjoint regions with close connected boundaries. Thresholding works well if objects have a uniform interior gray level with a background of unequal but uniform gray level [2].

In thresholding, characteristic features are used to define or segment an image. The characteristic feature that is commonly used is gray level values. If the characteristic feature is gray level, then one assigns all pixels at or above a certain threshold level to the object. All pixels with gray level below the threshold fall outside the object. The boundary can then be defined as that set of points belonging to the object which have at least one neighboring point belonging to the background [2].
Two types of thresholding, global and local, may be used for segmentation. In global thresholding, the value of the threshold is constant throughout the image. If the objects have an appreciable contrast difference which is reasonably uniform throughout the image, global thresholding will work well.

In variable or local thresholding, the threshold level is a varying function of position in the image. The threshold level is selected by considering a local neighborhood rather than the whole image. This method is useful when the background gray level is not constant and object contrast varies with the image.

Threshold levels may be set for local or global thresholding by examining the histogram of the image or a part of the image and determining the gray level range of the background and the objects [2].

Clustering is an extension of thresholding. Instead of only one characteristic feature, two or more characteristic features are used to differentiate between regions. Depending on these features, pixels are grouped together to form regions [3].

A detailed review of threshold techniques can be found in Ref. [4].
2.1.2 Edge or Boundary Detection: In edge detection, one attempts to locate the boundaries that exist in an image between the objects and the background.

A boundary or an edge is defined as the point where an abrupt change in gray level occurs or the point where a high gradient occurs. The gray level is relatively constant in each of two adjacent regions and changes abruptly as the border between the two regions is crossed [5].

An image can be segmented into regions by detecting the edges of the various regions that compose the image. The connected components of the non-edge points are then the regions. This approach is useful if there are regions having many different ranges of gray level so that the regions are not easily extractable by pixel clustering and classification [5].

Edge detection and image sharpening can be performed by using differentiation. For digital images, edges are detected by using difference operators or high emphasis spatial frequency filters that respond to changes in gray level or average gray level [6]. These operators take advantage of the fact that edges are local features and detect edges by examining local neighborhoods.
(a) **High-emphasis spatial filtering:** High spatial frequencies are associated with sharp changes in intensity such as at the edges of objects. The edges can be extracted or sharpened by performing high-pass filtering, i.e., by taking the Fourier transform of the image [3]. If \( f(x,y) \) is the original picture, \( F(u,v) \) its Fourier transform and \( F \) is the Fourier operator, then

\[
F(f(x,y)) = F(u,v)
\]

\( F(u,v) \) is then multiplied by a linear spatial filter \( H(u,v) \) and the inverse Fourier transform of the resulting image \( E(u,v) \) is the sharpened image \( e(x,y) \).

\[
E(u,v) = F(u,v) \cdot H(u,v)
\]

\[
e(u,v) = F^{-1}(E(u,v))
\]

(b) **Gradient operators:** If \( f(x,y) \) is a gray level function in a co-ordinate system with unit vectors \( i \) in the \( x \)-direction and \( j \) in the \( y \)-direction, the gradient is a vector function defined by

\[
\nabla f(x,y) = i \frac{\delta f(x,y)}{\delta x} + j \frac{\delta f(x,y)}{\delta y}
\]

15
\[ |\nabla f(x,y)| = \sqrt{\left(\frac{\delta f}{\delta x}\right)^2 + \left(\frac{\delta f}{\delta y}\right)^2} \]

and the direction of the gradient is given by

\[ \tan^{-1}\left(\frac{\delta f/\delta y}{\delta f/\delta x}\right) \]

Since the gradient takes on large values at the edges of objects, the gradient magnitude is taken as a measure of the edge strength [2]. Many digital approximations of the gradient are available in the literature. These produce a high magnitude where there is an abrupt change in gray level and a low magnitude where there is little change in gray level. For a digital image, differences are used instead of derivatives. For a 2X2 window,

\[ g(x,y) = \left[ (f(x,y) - f(x+1,y+1))^2 + (f(x+1,y) - f(x,y+1))^2 \right]^\frac{1}{2} \]

where \( f(x,y) \) and \( g(x,y) \) are the gray level function and magnitude of pixel \((x,y)\) respectively.

Examples of edge detectors based on differences of averages are the Sobel operator [6], Robert's operator
Examples of edge detectors based on differences of averages are the Sobel operator [6], Robert's operator [7], Prewitt operator [8] and the Kirsch operator [9]. These operators use 3x3 neighborhoods for edge detection. The principal difference between these operators is the weights assigned to each element of the 3x3 kernel used for the convolution operation.

(c) **Laplacian**: The Laplacian is the linear derivative operator

\[ \nabla^2 f = \frac{\delta^2 f}{\delta x^2} + \frac{\delta^2 f}{\delta y^2} \]

As with the gradient, the Laplacian takes on large values at the edges of objects. The discrete equivalent of the Laplacian for a digital picture is

\[ \nabla^2 f(x,y) = [f(x+1,y) + f(x-1,y) + f(x,y+1) + f(x,y-1)] - 4f(x,y) \]

which is the digital convolution of the function \( f \) with

\[
\begin{bmatrix}
1 \\
1 & -4 & 1 \\
1
\end{bmatrix}
\]
The magnitude $\nabla^2 f$ gives the edge strength at any point in the image.

When a Laplacian operator is applied to a digital image, low spatial frequencies are suppressed while the high frequencies remain relatively unchanged [2].

2.1.3 Region Analysis: In region analysis, one assigns pixels to particular objects—or regions. Most region finding algorithms involve gray level partitioning in order to select appropriate thresholds [1]. Each pixel is labeled individually based on certain decision rules. The grouping process involves connectivity analysis and fuses similar points into regions [5].

(a) Region Growing: Region growing processes may be used when some sub-part of the object of interest can be unambiguously detected. In this approach, the image is divided into many small regions. Then each region is assigned a set of parameters whose values reflect the object to which they belong. The parameters may include the average gray level, texture, or color of that region. Next, all boundaries between the adjacent regions are examined. The boundary strength is determined by computing the averaged properties of
adjacent regions. If the properties differ significantly on either side of the boundary, the boundary is considered to be strong and weak otherwise. Weak boundaries are dissolved and strong boundaries are retained. The process is iterated till there are no weak boundaries left in the image [2].

Region growing algorithms are computationally more intensive and need more a priori knowledge of the image than thresholding algorithms.

(b) Relaxation: Relaxation is an iterative approach to image segmentation. It groups pixels according to probabilistic decision rules at each iteration and then adjusts these rules at successive iterations, according to the decisions made at the preceding iteration at neighboring points [5].

In a digital image, different gray levels are attached to unambiguously labeled points depending on the class associated with them. The efficiency of this method depends greatly on the initial labeling process.

Like region growing processes, relaxation processes too are computationally very intensive.
2.2 Classification Criteria: In order to build a system that distinguishes objects of different types, in this case cells, one must first define the descriptive parameters of the objects to be measured, namely features. Examples of these parameters are size and shape. Figure 2.2 gives a brief overview of the classification criteria.

The following features or criteria are commonly used for classification:

2.2.1 Size Measurements:

(a) Area: The area of any object \( O \) in an image is the number of pixels in \( O \) including the boundaries. Area is a measure of the overall size of the object. Alternatively, if the length of the border around the object is known, then the area enclosed by the border can be calculated by using integration formulae [2].

(b) Length and Height: The horizontal and vertical extent of an object can be derived from its digital image. The length of an object is the horizontal distance between the leftmost and rightmost extreme points. The height or width, as opposed to the thickness, of an object is the vertical distance between the highest and lowest points. In general, the height or
Classification Criteria

Size Measurements  Optical Density  Color  Shape Measurements

Area  Length and Height  Perimeter  Rectangularity  Circularity

Figure 2.2 Classification Criteria
length of an object, in a given direction $\theta$ is the distance between the extreme points measured in a direction parallel to $\theta$ [5].

(c) **Perimeter**: The circumferential distance around the boundary is useful for classification purposes. The perimeter can be easily obtained by following the boundary of the object and counting the number of pixels [2].

2.2.2 **Optical Density**: Total and mean optical density of an object are the most commonly used classification criteria. The optical density of a pixel is the gray level value of that pixel. Classification is made based on the total value or the mean value of all the pixels within and including the boundaries of the object of interest. The optical density of an object is a measure of the brightness or darkness of that object [2].

2.2.3 **Color**: Partitioning of an image can be achieved on the basis of the color of the various regions that comprise the image [6]. Color can be represented by a vector that spans a three-dimensional space. The color co-ordinates of any point in an image can be represented as
\[ g(x,y) = [g_1(x,y), g_2(x,y), g_3(x,y)] \]

where \( g_1, g_2 \) and \( g_3 \) are the color co-ordinates at point \((x,y)\). The red, blue and green separations can be represented as \( g_R(x,y), g_G(x,y) \) and \( g_B(x,y) \) and the normalized color co-ordinates at any point are defined as

\[
g_R = \frac{g_R}{g_R + g_G + g_B}
\]

\[
g_G = \frac{g_G}{g_R + g_G + g_B}
\]

\[
g_B = \frac{g_B}{g_R + g_G + g_B}
\]

The image may be segmented into regions by assigning pixels having identical or close red, blue and green co-ordinates. The same criteria used for partitioning black and white images are used here. In the same way, objects can be assigned to different classes on the basis of their color co-ordinates. Image partitioning based on colors would be insensitive to changes of intensity but will be sensitive to changes in color [6].

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2.2.4 **Shape Measurements:** In many cases, the objects of one class can be distinguished from the objects of other classes by their shapes. The following are some of the commonly used shape factors:

(a) **Rectangularity:** Rectangularity factor \((r)\) may be defined as

\[
x = \frac{a_o}{a_r}
\]

This is the ratio of the area of the object \((a_o)\) and the area of the minimum enclosing rectangle \((a_r)\). This factor takes on a maximum value of 1 for rectangular objects and becomes small for curved objects. This factor varies between 0 and 1 [2].

A related factor is the aspect ratio \((a_f)\)

\[
a_f = \frac{W}{L}
\]

which is the ratio of the width \((W)\) to the length \((L)\) of the minimum enclosing rectangle [2].

(b) **Circularity:** Circularity factors \((c)\) are
minimized by circular shapes. The most common
circularity factor is

\[ c = \frac{p^2}{a} \]

which is the ratio of the perimeter (p) squared to the
area (a) of the object. This parameter takes on a
minimum value of 4π for a circular shape. Higher values
are yielded for complex shapes [2].

Another factor used to measure circularity is the
average distance from any point in the object to its
boundary [10]. This average distance is given by

\[ \bar{d} = \frac{1}{N} \sum_{i=1}^{N} x_i \]

\( x_i \) is the distance from the \( i^{th} \) pixel to the nearest
boundary point of the object and \( N \) is the number of the
points in the object. The circularity factor is given by

\[ c = \frac{a}{\bar{d}^2} \frac{N^3}{\sum_{i=1}^{N} x_i} \]

where \( a \) is the area and \( \bar{d} \) is the distance.
A detailed review of algorithms for shape factors may be found in Ref. [11] and Ref. [12].

2.3 Literature Review: The use of image processing techniques to identify biological components of an image may be traced back to the mid sixties [13]. Computers, mostly main frame and mini computers, have been used for segmentation and classification of a wide variety of biological specimens. Experiments have been done on segmentation and classification of chromosomes [14], bone marrow tissue cells [15], blood cells [16,17], cervical cells [18,19], liver tissue cells [20], pig muscle tissue cells [21], lung tissue cells [22], human muscle tissue cells [23], kidney cells [24] and many other cells. Many image processing techniques have been developed for segmentation and classification of biological specimens. There is no one common approach that can be applied to all kinds of biological specimen images. Researchers have used techniques like thresholding and edge detection or a combination of these for segmentation of the specimen images, with slight variations in each method depending on the images that were being dealt with. Most of the image analysis systems that have been developed basically involve digitization of the images, segmentation of the images
and extraction of classification parameters.

Prewitt and Mendelsohn [13] used gray level techniques to segment and classify white blood cells. The method involved a smoothing operation on the image histogram and placing the thresholds at the valleys between the peaks of the histogram. Castleman and Wall [14] used thresholding techniques to segment and classify chromosomes.

Brenner et al [15] have used thresholding techniques for analysis of bone marrow cell images. The analysis was done on an interactive image processing system, SCANT. A television camera was used to scan through a microscope and capture the images at different wavelengths and digitize them to a 64 gray level image. The analysis was performed on a large mini-computer. Parameters extracted include the cell area, nucleus to cell area ratio, and cytoplasm color.

Young and Paskowitz [16] developed a technique for analysis of red blood cell images. The technique involves scanning the cells with three different wavelengths of light and digitizing the image. The digitized images captured at different wavelengths are converted to binary images, combined and analyzed by a Tempo 16-bit mini-computer.
Young et al [17] have developed a system for classification of white blood cells. The methodology employed was that of digital image processing and involves digitization of the photomicrographs of the specimen images to a 108x72 (HXV) image with 64 levels of gray and extraction of such features as the size and color of the nucleus and cytoplasm. Lester et al [25] used both thresholding and gradient operations to segment and classify white blood cell images.

Cahn et al. [18] used thresholding and contour tracing techniques to segment cervical cell images. Initially, the cytoplasm was separated from the background using thresholding techniques. Subsequently, the cytoplasm and the nucleus were separated using clustering techniques.

Aggarwal and Bacus [19] developed a multi-spectral approach for analysis of cervical cytology smears. Multiple images of the specimen are digitized through color filters. Clustering techniques are used for extracting data from the images.

O'Gorman et al [20] developed an automatic system for liver tissue analysis. Images are captured in color and converted from the RGB co-ordinates to the intensity-hue-saturation (IHS) co-ordinates. Both
thresholding and edge detection techniques are used to segment the images. Parameters such as the size, shape and color of the image are used to classify the cells. An Interdata 3230 computer, connected to a diffr3-50 automatic light microscope was used for the analysis.

Jain et al [21] developed a multi-step algorithm to segment pig muscle cell images. This algorithm involves application of a number of low-level image processing algorithms including thresholding and edge detection to the muscle cell tissue images to segment the images into individual cells and cell clumps. The cell clumps are subsequently split into individual cells using hierarchical clustering techniques. The analysis was conducted on a PDP 11/34 mini computer.

Johnston [22] developed a simple automated procedure for counting the number of alveoli/unit area of human lung tissue. The images were digitized to a 96X128 (HXV) pixel image by means of a scanner and analyzed on a DEC PDP-8 general purpose computer. The procedure involves a smoothing operation using a low pass filter and thresholding of the image.

Sherman et al [24] developed an interactive video scanning system for identification of cancerous cells in the sediment of urine. The cell images were acquired by using an automatic scanning stage controlled by a micro-
computer. The system was interfaced to a PDP 11/23 computer. The method involves converting the cell images to binary images and extraction of parameters such as cell size, cell optical density, and nuclear area for classifying the cells.

Cellular logic machines which classify and segment several thousand cells/hour have been built [26]. These machines analyze the biological images by using cellular logic operations. The digitized image is analyzed by breaking it up into sub parts called cells hence the name cellular logic. Table look-up is used for these operations and the logic for all the operations is built into the hardware.

Preston [27] has developed techniques for analysis of human and animal tissues. The methodology employed is that of cellular logic. The operations were performed on the Carnegie-Mellon SUPRPIC image processing system using an Interdata 7/32 computer.

Yokoyama et al [28] used edge enhancement techniques to segment neuronal images. The images were digitized to a 480X640 (HXV) image with 256 levels of gray and analyzed on a DEC VAX 11/780 computer. Their method involves smoothing and extraction of the edges using a Laplacian filter. Classification was based on simple
parameters such as size and circumference of the neurons.

Holmquist et al [29] have developed an interactive system for measurements on microscopic cell images. The system allows the operator to isolate cells and extract parameters such as area and optical density. The system uses a PDP 8/F mini computer to analyze the 128X128 (HXV), 64 gray level image.

Olson et al [30] developed a method for detection of leukemia cells. Fuelgen stained cells are digitized and analyzed on a PDP 11/45 mini computer. Nuclear area, total optical density and average optical density are the parameters used for classification.

Ledley [31] outlines three methods for the analysis of cell images. The methods involve boundary and feature extraction using interactive techniques. The 700X500 (HXV) images were analyzed on an IBM 360/Mod 44 computer.

From the previous discussion, it is clear that the choice of a segmentation procedure depends on the quality of the image. More specifically, the contrast between the background and the objects present in the image. Except for the hybridoma cell images, all other specimen images have good contrast between the
background and the specimens, making it easy to segment those images. For pollen cell images, DNA analysis, stigma images and protein gel images simple thresholding techniques would be sufficient to segment the images. A more complicated approach involving boundary detection would be necessary for hybridoma cell images. Since there are no appreciable color or shape differences between different cells of a specimen image, the area, the average optical density and the total optical density were the only parameters that could be used for classification of the cells.
Chapter III

EXPERIMENTAL APPARATUS
& PROCEDURE
3.1 Experimental Apparatus: A block diagram of the system setup used in this work is shown in Figure 3.1. A picture of the experimental apparatus can be seen in Figure 3.2. An inverted microscope with an incandescent light source magnifies the specimens and an RGB camera attached to the microscope captures the image of the specimen. The analog signal from the camera is then digitized by the digitizer board to a 64,000 pixel image with 64 levels of gray. The digitized image is stored in the main memory or the display memory of the computer for subsequent analysis. The image may also be stored on the hard disk of the computer or on floppy disks. The digitizer board captures the image in 1/3 of a second. The captured image can be displayed on the screen of an RGB monitor. A printer is connected to the computer for hard copy output.

3.1.1 Equipment Description: The following sections give brief descriptions of the equipment.

(a) Microscope and Optical Systems: An inverted microscope based on the phase contrast principle magnifies the specimens (Figure 3.3). Five levels of magnification, 4X, 10X, 20X, 40X and 100X are possible with the objective lens. In combination with the
Figure 3.1 A schematic diagram of the system setup
Figure 3.2 A picture of the system setup
Figure 3.3 A picture of the microscope
eyepiece which has a magnification of 10X, magnification levels of 40X, 100X, 200X, 400X and 1000X are possible. The camera attachment has a lens with a magnification of approximately 4X. The microscope is equipped with a 30W tungsten lamp which serves as the light source. The lighting level can be adjusted by using a slide control lever, which controls the voltage supply to the light source.

The microscope specifications are given in Table 3.1.

(b) **Camera**: An RGB video camera with an RGB unit was used for the purpose of capturing the images of the specimens (Figure 3.4). Images can be captured in color or black & white. By connecting the RGB unit, true color images can be captured. Tables 3.2 and 3.3 give the specifications of the camera and the RGB unit.

(c) **Digitizer Board**: The analog signal from the camera must be converted to a digital form so that it can be analyzed by the computer. A digitizer board which uses the PC bus of the computer samples the incoming analog signal from the camera at regular intervals and converts it to a digital format. Images can be captured in color or in black and white. Table 3.4 gives the specifications of the digitizer board.
### TABLE 3.1

**Microscope Specifications**

<table>
<thead>
<tr>
<th>Make</th>
<th>Olympus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>IMT</td>
</tr>
<tr>
<td>Type</td>
<td>Inverted Phase Contrast</td>
</tr>
<tr>
<td>Light Source</td>
<td>30W Tungsten bulb</td>
</tr>
<tr>
<td>Magnification</td>
<td>4X, 10X, 20X, 40X</td>
</tr>
<tr>
<td>Objective</td>
<td>4X, 10X, 20X, 40X</td>
</tr>
<tr>
<td>Eye Piece</td>
<td>10X</td>
</tr>
<tr>
<td>Camera</td>
<td></td>
</tr>
<tr>
<td>Attachment</td>
<td>4X</td>
</tr>
</tbody>
</table>
Figure 3.4 A picture of the camera and the RGB unit
<table>
<thead>
<tr>
<th>Specification</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Make</td>
<td>Chorus</td>
</tr>
<tr>
<td>Model</td>
<td>CA-1600</td>
</tr>
<tr>
<td>Resolution</td>
<td>300 lines × 300 lines (HXV)</td>
</tr>
<tr>
<td>Signal System</td>
<td>NTSC Standard</td>
</tr>
<tr>
<td>Scanning</td>
<td>525 lines</td>
</tr>
<tr>
<td></td>
<td>2:1 Interlace</td>
</tr>
<tr>
<td>Minimum Illumination</td>
<td>10 lux (1 foot-candles)</td>
</tr>
<tr>
<td>Illumination Range</td>
<td>10 – 100,000 lux (1 fc – 10,000 fc)</td>
</tr>
<tr>
<td>Signal to Noise Ratio</td>
<td>45 dB or more</td>
</tr>
<tr>
<td>Power Source</td>
<td>DC 12V</td>
</tr>
<tr>
<td>Power Consumption</td>
<td>5.0 W</td>
</tr>
<tr>
<td>Weight</td>
<td>939 grams (2.07 lbs)</td>
</tr>
<tr>
<td>TABLE 3.3</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td></td>
</tr>
</tbody>
</table>

**RGB Unit Specifications**

<table>
<thead>
<tr>
<th>Make</th>
<th>Chorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>CA-1600</td>
</tr>
<tr>
<td>Power Source</td>
<td>DC 8.7V</td>
</tr>
<tr>
<td>Power Consumption</td>
<td>600mW</td>
</tr>
<tr>
<td>Weight</td>
<td>111 grams (3.9 ounces)</td>
</tr>
<tr>
<td><strong>Digitizer Board Specifications</strong></td>
<td></td>
</tr>
<tr>
<td>-----------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Make</strong> : PC-EYE</td>
<td></td>
</tr>
<tr>
<td><strong>Model</strong> : 3-3-2 Board</td>
<td></td>
</tr>
<tr>
<td><strong>Resolution</strong> : Dependent on the graphics card in use. Maximum resolution is 640X400 (HXV) pixels</td>
<td></td>
</tr>
<tr>
<td><strong>Bits/Pixel</strong> : Maximum of 6 bits/pixel</td>
<td></td>
</tr>
<tr>
<td><strong>Frame Buffer</strong> : No frame buffer is available on the board</td>
<td></td>
</tr>
<tr>
<td><strong>I/O Slot</strong> : Single IBM PC, XT, AT or compatible full length slot</td>
<td></td>
</tr>
<tr>
<td><strong>Video Input</strong> : 4 Channels, NTSC EIA RS-170, or EIA RS-130, Interlaced or Non-Interlaced</td>
<td></td>
</tr>
</tbody>
</table>
(d) **Computer:** An IBM XT compatible personal computer was used for the analysis of the specimen images. The computer is equipped with 640K of Random Access Memory (RAM), a 20 Mbyte hard drive, double floppy drives and an 8087 co-processor for floating point calculations.

(e) **Graphics Board:** A color graphics adapter (CGA) with two banks of display memory was used for displaying the digitized images on the video monitor. Each bank of memory is 64K in size. The display memory can store two images with one image being displayed at any given time. The board supports display of 256 colors from a palette of 262,000 colors. The graphics board specifications are shown in Table 3.5.

(f) **Monitor:** A three monitor configuration was used for displaying text and images at the same time, a digital/analog RGB TV monitor (SONY, KV-1311CR) for displaying the captured (digitized) images of the specimens, a monochrome monitor for displaying the text and an RGB color monitor for displaying the image directly from the video camera (live image). Figure 3.5 shows a digital image as seen on the monitor screen.

A more detailed description of the experimental apparatus can be found in Appendix A.
<table>
<thead>
<tr>
<th>Graphics Board Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Make</td>
</tr>
<tr>
<td>Resolution</td>
</tr>
<tr>
<td>Display Memory</td>
</tr>
<tr>
<td>Bits/Pixel</td>
</tr>
<tr>
<td>Color Display</td>
</tr>
</tbody>
</table>
Figure 3.5 A digital image as seen on the monitor screen
3.1.2 **Software**: Software algorithms for the analysis of the specimen images have been developed in Turbo Pascal\textsuperscript{R} which interfaces with the basic image acquisition software of the PC-EYE\textsuperscript{R} Video Capture System. The PC-EYE\textsuperscript{R} basic image processing software has over 35 routines for hardware configuration, software configuration and for image acquisition and other basic image manipulation operations.

Over 30 image processing routines incorporating filters, edge detectors, contrast enhancement techniques, histograms, thresholding, image size reduction and enlargement, image averaging, and classification and pattern recognition have been developed. The routines have been developed in a modular form to facilitate additions, deletions, and revisions whenever needed.

A detailed description of the software is found in Appendix B.

3.1.3 **Calibration**: Calibration of the system for area and distance calculations off the screen is done using a hemacytometer. A hemacytometer is a slide with horizontal and vertical lines etched on its surface. These lines have known distances (1/20mm) between them and form a square pattern. Figure 3.6 shows
a digitized hemacytometer pattern image (magnification - 20X) as seen on the monitor screen. The actual area within the squares is 1/400 mm². Figure 3.7 shows the pattern that is formed by the etched lines.

The image of the pattern is captured through the microscope at all possible magnifications and digitized. For each magnification, the number of pixels within a square is counted to express the area in pixels. This area in pixels can now be converted to standard units by taking into consideration the magnification at which the image has been captured and setting it equal to the actual area of the square. The same procedure is followed to calibrate the system for distances.

3.2 Experimental Procedure: The procedure for the acquisition and analysis of the biological specimen images of hybridoma cells, pollen cells and protein stains is described in the following sections. A flow diagram for the experimental procedure is illustrated in Figure 3.8. For microscopic specimens, the specimen image was magnified using a microscope. The image was then captured by the video camera, digitized by the digitizer board and then stored in the main memory of the computer for subsequent operations. Any magnification may be used for magnifying the specimen.
Figure 3.6 The image of the hemacytometer as seen on the monitor screen.
Figure 3.7 The pattern formed by the lines of a hemacytometer
Figure 3.8 A flow diagram for the experimental procedure
images as long as the clarity of the image is not sacrificed, but it should be noted that the greater the magnification, more number of images are needed for scanning the whole well or slide and hence more time is needed. The captured image has a resolution of 320x200 (HXV) and has a gray level range of 0-255.

3.2.1 Pollen Cells: Gray level partitioning or thresholding techniques are used for segmenting pollen cell images. The lighting intensity of the microscope is adjusted and the lens is focussed until a clear picture is obtained on the monitor screen. The image is then captured by the video camera, digitized and stored in the computer memory. About 30 images are needed to scan the whole slide at an objective magnification of 10X. Figure 3.9 shows the flow diagram for the acquisition and analysis of pollen cell images. Figures 3.10a and 3.10b show a typical pollen cell image and its histogram.

By adjusting the lighting level of the microscope and thresholding the cell image at a certain level, it is possible to segment the pollen cell images into cell and non-cell regions. The lighting and threshold levels depend on the image being processed and are set by trial and error, so that the background gray level is set to a value higher than that of the cells. It has been found
DIGITIZED IMAGE - P

SIMPLE THRESHOLDING OF IMAGE P (THRESHOLD)
P → T

FIND AREA & MEAN OPTICAL DENSITY OF COMPONENTS OF T

IF AREA > THRESHOLD OR MEAN O.D. > THRESHOLD THEN LIVE CELL

IF AREA < THRESHOLD OR MEAN O.D. < THRESHOLD THEN DEAD CELL

WRITE RESULTS TO SCREEN OR DATA FILE

Figure 3.9 A flow diagram for the analysis of pollen cells
Figure 3.10 (a) A picture of a pollen cell image and (b) its histogram.
that a threshold level of 40 on a 0-63 scale was sufficient to segment all of the pollen cell images. Once the image has been segmented, it is simply a matter of isolating each cell and counting the number of pixels in the stained portion within the cell boundary. Simultaneously, the mean optical density and the total optical density of the stained portion within the cell boundary can also be calculated. Since dead pollen cells are stained over a smaller area than the live cells and are stained more lightly than the live cells, this difference is reflected in the mean optical density and the stained area of the cells.

An interactive program has been developed for the analysis of pollen cell images. In the computer analysis, a window is created with an area slightly larger than the size of the cell. The size of this window limits the area in which the parameter extraction is performed. The size of the window may be changed to suit the user’s needs, however it should be noted that the larger the window, the greater is the time needed for the operation and vice versa. The cells are analyzed sequentially starting at the top left corner of the image. The number of pixels within the boundary of the cell is totaled to calculate the area and simultaneously, the intensity value of each pixel is
extracted to calculate the total and average optical density of each cell. The results of the analysis are output to the screen or saved in a text file. Figure 3.11 shows the results of the analysis superimposed on the original pollen cell image.

3.2.2 Hybridoma Cells: The procedure suggested by Jain et al [21] has been used for segmentation of the hybridoma cell images. Figure 3.12 shows the flow diagram for the analysis of hybridoma cell images. This procedure involves boundary detection of the cell regions. The lighting intensity of the microscope is adjusted until a clear image is seen on the monitor screen. The image is then digitized and stored in the computer. About 40 images are needed to scan the whole well at an objective magnification of 10X. Figures 3.13a and 3.13b show an image of a hybridoma cell colony and its histogram. Figures 3.14a and 3.14b show an image without any colonies and its histogram.

Let G be the digitized image of the specimen. The first step after capturing the image involves segmenting the image into the background and regions containing the cells and colonies. Subsequently the areas of all the components of the image are calculated in terms of pixels. This is achieved in the following steps:
Figure 3.11 A picture of a pollen cell image with the parameters superimposed
Figure 3.12 A flow diagram for the analysis of hybridoma cells.
Figure 3.13 (a) A hybridoma cell image with a cluster (b) its histogram
Figure 3.14 (a) A hybridoma cell image without a cluster and (b) its histogram.
(a) G is the original image (Figure 3.14a).

(b) **Edge Detection:** The image G is convolved with the Sobel operator to extract the edges of the cells and colonies. The Sobel operator computes the gradient of the differences of averages of the pixels in a 3x3 neighborhood. The kernels used for the convolution operation with the image array are the digital approximation of the gradient and are:

\[
X = \frac{1}{4} \begin{bmatrix} -1 & 0 & 1 \\ -2 & 0 & 2 \\ -1 & 0 & 1 \end{bmatrix} \quad \text{and} \quad Y = \frac{1}{4} \begin{bmatrix} 1 & 2 & 1 \\ 0 & 0 & 0 \\ -1 & -2 & -1 \end{bmatrix}
\]

The gradient magnitude is given by

\[
M = \sqrt{X^2 + Y^2}
\]

or \[M = |X| + |Y|\]

The next step involves thresholding the image to determine the edges and convert the image to a binary image. The threshold value is selected by setting the value as close to 0 as possible. Too high a threshold value will result in too few edges being detected and
too low a threshold value will result in too many edges being detected. For any lighting level, the threshold value, once set, need not be changed. A threshold value of 10 on a 0-63 scale was found to be sufficient for most of the hybridoma cell images with very few spurious edges being detected. Figure 3.15 shows an image with the edges detected by the above method.

\[
\begin{align*}
G & \xrightarrow{\text{Sobel}} G_g \\
& \xrightarrow{\text{Binary}} G_c
\end{align*}
\]

(c) Cleaning: The image \( G_g \) contains not only true edge points, but also many spurious edge points. The spurious edge points are sparser and are connected to a smaller number of other edge points. To eliminate the spurious edge points, a cleaning operation is performed on the binary image \( G_g \). Any pixel with a gray level of 63 which has less than 2 and greater than 6 8-connected neighbors which are of value 63 are set to 0. Figure 3.16 shows the 8-connected neighbors and 4-connected neighbors of a pixel. Figure 3.17 shows a cleaned image.

\[
\begin{align*}
G_g & \xrightarrow{\text{Clean}} G_c
\end{align*}
\]
Figure 3.15 A picture of a hybridoma cell image with the edges detected
(a) A pixel having 8-connected neighbours constitutes the neighboring pixels along the horizontal and vertical axes and also the diagonals.

(b) A pixel having 4-connected neighbours constitutes the neighboring pixels along the horizontal and vertical axes.

Figure 3.16 The 8-connected and 4-connected neighbors of a pixel.
Figure 3.17 A picture of a hybridoma cell image after the cleaning operation
(d) **Averaging:** The cleaning operation on \( G_g \) to give \( G_C \) will result in many spurious edge points being eliminated and also some of the true edge points being eliminated. As a result a few edges will be broken. To overcome this, a smoothing or averaging operation is performed on the image \( G_C \) to connect the broken edges. This smoothing is achieved by using a sliding average on \( G_C \), resulting in the binary image \( G_a \). Each pixel in \( G_a \) is the average pixel value in a 3X3 neighborhood around that pixel in \( G_C \).

For any pixel \( p \) having eight neighbors, \( P_1, \ldots, P_8 \),

\[
\begin{bmatrix}
  P_4 & P_3 & P_2 \\
  P_5 & P & P_1 \\
  P_6 & P_7 & P_8 \\
\end{bmatrix}
\]

after averaging, the new value of \( p \) will be

\[
p = \frac{P_1 + P_2 + \cdots + P_7 + P_8}{9}
\]

Figure 3.18 shows an image on which the above smoothing operation has been performed.

\[\text{Average} \quad G_C \xrightarrow{\text{Binary}} G_a\]
Figure 3.18 A picture of a hybridoma cell image after the averaging operation
(e) **Thinning:** The averaging operation results in an increase in the sizes of the cells and colonies. To bring the cells and colonies back to their original sizes, one pass of Hilditch's thinning algorithm [32] is applied to the image $G_a$. This approach eliminates many of the extra pixels and brings the cells and colonies back to their original sizes.

Based on certain conditions, pixels at the boundaries of the components of the image, i.e., pixels which are white (63), are eroded away. After one pass of the operation, image $G_a$ will be one layer thinner to give the image $G_t$. The following are the conditions for the removal of a pixel:

(i) It belongs to a component of the image,

(ii) Its removal is allowed, i.e., it lies on the boundary of the component, and

(iii) Its removal does not alter the connectivity of the boundary.

Figure 3.19 shows the effect of the thinning operation.

\[
\text{Thinning} \quad G_a \xrightarrow{\text{Thinning}} G_t
\]
Figure 3.19 A picture of a hybridoma cell image after the thinning operation
$G_t$ will be an image with the background set to 0 and the edges of the cells and colonies set to 63 and the interior of the cells set to either 0 or 63.

(f) **Segmentation:** The next step involves converting $G_t$ to a three level image with the background set to an arbitrary value of 32, the edges to a value of 63, and the interior to either 0 or 63. This is achieved by the process of region growing. A few points in the background are set to 32 and are allowed to grow over the entire background.

A white pixel in the image indicates the border of a region. By starting at the top left corner, the image is scanned along a row until a white pixel (edge) is encountered. In the process of scanning, all consecutive black pixels in a row, are converted to 32. This process is repeated for all the rows in the image. In the next few passes, all pixels with a value of 32 are searched for any neighbors which are not white and which are outside the cell boundary and are set to 32. The number of passes for this conversion process has been set at four. Some images may need less and some images may need more passes, but on an average, four passes should be sufficient to segment most of the image.

Figure 3.20 shows a segmented image.
Figure 3.20 A picture of a segmented hybridoma cell image
(g) **Classification:** By the time the wells are examined, any hybridoma cells that were present would have multiplied to form colonies. These colonies have the largest areas of all the components of the image. Hence, for this magnification, if the area of any component in the image $G_s$ is greater than 5000 pixels, then it would indicate the presence of a hybridoma micro colony in the well. The average area of a colony has been set at 5000 pixels by taking the average size of a cluster as 300 cells, with each cell having an area of 16 pixels at a total magnification of 40X. It is important to recall that the image $G_s$ has three regions, the background set to 32, the edges of the cells set to 63, and the interior of the cells set to 0 or 63. Consequently, to find the area of each cell or micro colony, the number of black(0) or white (63) pixels in that cell or micro colony are counted.

To find the area of each cell or micro colony, analysis starts at the top left corner of the image. The first white pixel to be encountered is set to an arbitrary value of 10. In the next four passes, all white or black pixels that are connected to this pixel and which are within the cell boundary are set to 10. As
the pixels are being converted, a counter is incremented. The value of the counter after each such process would be the area of that cell or micro colony. Simultaneously, the total optical density and the mean optical density can also be calculated. The same procedure is followed for computing the areas of other specimens.

The results of the analysis are displayed on the screen or written to a data file. This information allows the selection of only those wells that contain the hybridoma cell micro colonies and disposal of all other wells.

3.2.3 DNA Analysis: The DNA content in a red blood cell is reflected in the brightness of the stained region of the red blood cell. The darker the stain, the lower is the DNA content in the cell and vice versa. Thus the DNA content in a cell may be calculated by computing the total optical density or mean optical density of the stained portion of the cell. Consequently the same procedure used for the acquisition and analysis of pollen cell images is used in this case also. The only difference involves using a special wavelength filter to increase the contrast between the stained region of a cell and the background. This filter allows only the light reflected by the stain to pass through
it. The mean optical density, the total optical density and the area of the stained region are calculated as for pollen cells. By comparing the total optical density to that of a standard, the DNA content in the cell can be calculated. A flow diagram of the procedure is shown in Figure 3.21. Figure 3.22 shows a red blood cell image and its histogram and Figure 3.23 shows the results of the analysis superimposed on the original image.

3.2.4 Stigma: The procedure used for the analysis of pollen cells is used for stigma analysis. By a combination of lighting level adjustment and threshold level adjustment, the image may be segmented into the background region and the stigma region. The background is set to 63, with the stigma region having a lower gray level than the background. The area enclosed by the boundaries of the stigma is calculated by counting the number of pixels within the boundary of the stigma. The computer program developed for pollen cell analysis is used for stigma image analysis. Since the stigma sizes are several times greater than those of pollen cells, the size of the operations window must be increased accordingly. The results of the analysis are output to the screen or a text file. A flow diagram of the experimental procedure is shown in Figure 3.24. Figure 3.25 shows a stigma image and its histogram and Figure
Figure 3.21 A flow diagram for DNA analysis
Figure 3.22 (a) A picture of a red blood cell image and (b) its histogram
Figure 3.23 A picture of a red blood cell image with the parameters superimposed
Figure 3.24 A flow diagram for the analysis of stigma
Figure 3.25 (a) A picture of a stigma image and (b) its histogram
3.26 shows the image with the parameters superimposed on it.

3.2.5 Protein extract stains: Figure 3.28 shows a typical protein stain gel image and its histogram. Image analysis of protein gels is similar to the procedure followed when analyzing the gels manually. The manual procedure has been described in Chapter 1. Image analysis involves the measurement of the distances of the stain from the top of the gel by means of the computer. An interactive computer program has been developed for the analysis of protein stains. The image of the gel is captured with the video camera. Since gels are not microscopic specimens (size - 6cmX6cm), their images need not be magnified by the microscope. By proper adjustment of the lighting levels and the threshold levels, an image with the background that is much brighter than the stains can be obtained. The user is then required to select a window covering the width of the stain and running in length from the top edge to the bottom edge of the gel (Figure 3.29). The pixels within the window are averaged horizontally (intensity profile) and these average values are then stored in a one-dimensional matrix whose size is the length of the window. The next step involves finding those values in the matrix which are low enough to be classified as
Figure 3.26 A picture of a stigma image with the parameters superimposed
DIGITIZED IMAGE - C

SIMPLE THRESHOLDING OF IMAGE C (THRESHOLD)
C ------ C T

SELECT WINDOW TO INCLUDE THE WIDTH OF THE STAIN PATTERN

DETERMINE LOCATION OF ALL STAINS IN THE PATTERN AND THEIR DISTANCES FROM TOP OF GEL

IF STANDARD STAIN, INPUT MOLECULAR WEIGHT FOR EACH STAIN AND CALCULATE SLOPE AND INTERCEPT OF BEST FITTING STRAIGHT LINE

IF NON-STANDARD STAIN, FIND DISTANCES AND SUBSTITUTE FOR KNOWN VALUES IN STANDARD EQUATION OF A STRAIGHT LINE

WRITE RESULTS TO SCREEN OR DATA FILE

Figure 3.27 A flow diagram for analysis of protein gels
GRAY LEVEL HISTOGRAM

Mean = 59.97  Std. Deviation = 3.73

Figure 3.28 (a) A picture of a protein gel image and (b) its histogram
Figure 3.29 A picture of a protein gel image showing the standard stain pattern included in the operations window
stains. Figure 3.30 shows the intensity profile superimposed on the original image of the protein stains. In order to locate the stains, the matrix is searched for the lowest value which falls between two high values and these low values are classified as stains. The matrix subscript gives the distance of the stain from the top edge of the gel in pixels. This distance being relative, need not be converted to standard units. After the location of the stains and their distances, if the stain pattern is specified to be a standard stain, the user is prompted for the molecular weights of the stains. With this information, the slope and the intercept of the straight line that fits these data are calculated using standard regression formulae. The user is then required to move the window to the unknown stain pattern where the process of finding the distances is repeated. The new distances and the slope and intercept that have been evaluated previously are substituted in the standard equation for a straight line,

\[ y = mx + b \]

where \( y \) is the molecular weight, \( x \) is the distance, \( m \) is the slope and \( b \) is the intercept of the straight line. The value of \( y \) gives the molecular weight of the unknown
Figure 3.30 A picture of a protein stain gel with the intensity profile of the standard stain pattern superimposed.
stains in the stain pattern. The molecular weights that have been calculated are output on to the screen or to a data file.
Chapter IV

RESULTS AND DISCUSSION
About 200 images of different specimens have been acquired for analysis by the computer system. Suitable image processing techniques have been used for the analysis of these specimens. The following sections deal with the results of the analysis of these specimens.

(a) Pollen Cells: Thirty images of pollen cells from five different slides have been acquired for analysis. A total of 168 cells, 109 live (viable) cells and 49 dead (non-viable) cells were analyzed by the computer system. Tables 4.1a, 4.1b and 4.2 give the results of the statistical analysis of the data obtained from the computer analysis. The analysis was conducted under the assumption that there was a significant relationship between the viability of a cell and its mean optical density or the stained area of the cell.

The basic statistical parameters that have been calculated are shown in Table 4.1a, 4.1b. It is evident that there is an appreciable difference between both the mean stained area and mean optical density of a viable cell and those of a non-viable cell.

The results of an analysis of variance (ANOVA) for live and dead cells are shown in Table 4.2. According to this analysis, mean optical density has a more
<table>
<thead>
<tr>
<th>Results for viable cells</th>
<th>AREA</th>
<th>DENSITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells</td>
<td>109</td>
<td>109</td>
</tr>
<tr>
<td>Mean</td>
<td>256.679</td>
<td>20.844</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>47.494</td>
<td>2.446</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Results for non-viable cells</th>
<th>AREA</th>
<th>DENSITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>Mean</td>
<td>206.271</td>
<td>29.000</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>52.661</td>
<td>3.195</td>
</tr>
<tr>
<td>DEPENDENT VARIABLE</td>
<td>CORRELATION CO-EFFICIENT</td>
<td></td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------------------</td>
<td></td>
</tr>
<tr>
<td>1. STAINED AREA</td>
<td>0.194</td>
<td></td>
</tr>
<tr>
<td>2. OPTICAL DENSITY</td>
<td>0.677</td>
<td></td>
</tr>
<tr>
<td>3. REGRESSION</td>
<td>0.104</td>
<td></td>
</tr>
</tbody>
</table>
significant relationship with the viability of a cell than the stained area does. The mean optical density accounts for 67.7% of the variance, whereas the stained area accounts for only 19.4% of the variance. ANOVA for linear regression indicates that only 10.4% of the variance is accounted for by linear regression, the rest being due to higher degrees of regression. Please refer to Appendix C for the complete ANOVA.

It was hypothesized previously that there was a significant relationship between the viability of a cell and its mean optical density or stained area. From the results in Table 4.2, it can be concluded that there is a significant relationship between the mean optical density and the viability of a cell. However, area parameter does not show a strong relationship to the viability of the cells.

On the average, the computer time needed for the analysis of each image containing 6 cells is approximately 30 seconds.

(b) Hybridoma Cells: About 100 hybridoma cell images from three wells have been acquired and analyzed by the system. Table 4.3 provides a comparison of typical results (for 18 of the 100 images) obtained by computer analysis with those obtained by visual inspection.
<table>
<thead>
<tr>
<th>SLIDE ID-IMAGE NUMBER</th>
<th>CLASSIFICATION</th>
<th>DENSITY OF CELLS IN WELL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MANUAL</td>
<td>COMPUTER</td>
</tr>
<tr>
<td>E5-01</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>E5-07</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>E5-09</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>E5-15</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>E5-21</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>E5-30</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>D5-07</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>D5-11</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>D5-17</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>D5-21</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>D5-25</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>D5-32</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>D7-08</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>D7-14</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>D7-20</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>D7-28</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>D7-35</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>D7-42</td>
<td>-ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>
Visual inspection of the images (assuming that visual inspection is 100% accurate) showed that 84 of the images were negative i.e., did not contain any hybridoma micro-colonies and 14 images were classified as positive. By using the area as the classification parameter, computer analysis of the images showed that only 61 of the images were negative and that 39 of the images were positive. Thus, the computer made a false classification in 24 cases. The main reason for the false classification of these images was the density of the cells in those images. As shown in Table 4.3, when the images had a high density of cells, the computer made a false classification. Because of the high density of the cells, some of the individual cells are so close together that they tend to fuse and create pseudo clusters. Since area was the classification parameter, the computer would, obviously, classify these images as positive.

The time needed for segmentation of all images was approximately 6 minutes, but the time needed for extraction of the classifying parameter varied depending on the density of the image. The computer time needed for the analysis of images with high densities of cells was high because it is necessary to compute the area of numerous cells in the image. A reduction in
concentration levels of the cells might result in a more accurate classification.

(c) **DNA Analysis:** Thirty seven images of red blood cells have been acquired and analyzed by the computer system. These images have been taken from four different slides, each containing a different type of red blood cell, (Pisces, Xenopus, Mesotoma Lingua and M Ehrenbergin). The DNA content in each of these slides is a standard and is known. The optical density values obtained by the computer system have been standardized using the standard DNA values available for each slide. In addition, the computer system has been compared to a standard system which works on the same principle (the DADS system). The DADS system uses a photo diode to scan the slides and computes the mean optical density of each slide. The data obtained from both of these systems have been analyzed using linear regression techniques. Table 4.4a summarizes the optical densities obtained for the two systems. Table 4.4b shows the results of the regression analysis on these data. Inspection indicates that the computer system compares well with the DADS system. Figure 4.1 illustrates the relationship between the DNA content in each slide and the optical densities obtained for the two systems, using the values obtained from the regression analysis. The graph in Figure 4.2
### TABLE 4.4a
DNA Analysis

<table>
<thead>
<tr>
<th>SLIDE ID</th>
<th>MEAN OPTICAL DENSITY</th>
<th>DNA CONTENT (Picogram)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VISION SYSTEM</td>
<td>DADS SYSTEM</td>
</tr>
<tr>
<td>Pisces</td>
<td>40178</td>
<td>501</td>
</tr>
<tr>
<td>Xenopus</td>
<td>64772</td>
<td>969</td>
</tr>
<tr>
<td>M Lingua</td>
<td>150661</td>
<td>2285</td>
</tr>
<tr>
<td>M Ehrenbergin</td>
<td>414412</td>
<td>8003</td>
</tr>
</tbody>
</table>

### TABLE 4.4b
Results for DNA Analysis

<table>
<thead>
<tr>
<th>SYSTEM</th>
<th>CORRELATION CO-EFFICIENT</th>
<th>SLOPE</th>
<th>INTERCEPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>VISION</td>
<td>0.995</td>
<td>12386.91</td>
<td>9882.25</td>
</tr>
<tr>
<td>DADS</td>
<td>0.994</td>
<td>245.51</td>
<td>-308.21</td>
</tr>
</tbody>
</table>

94
VISION SYSTEM vs DADS SYSTEM

\[ y = 12386.91x + 9882.25 \] (VISION)

\[ y = 245.513x + (-308.216) \] (DADS)

O.D - Optical Density

Figure 4.1 Correlation Curves
shows that the mean optical densities of the two systems are related almost linearly. Please refer to Appendix D for the detailed regression analysis.

The computer time needed for the analysis of each image was found to be less than 30 seconds.

(d) **Stigmas:** Twelve stigma images have been acquired and analyzed by the computer system. The areas of the stigmas and the widths of the stigmas at the base and the tip were measured in terms of pixels and converted to standard units. The surface area of each stigma was calculated using geometrical formulae. The areas evaluated by the computer system have been found to be within reasonable limits [33]. Table 4.4 summarizes the surface areas that have been calculated for the twelve stigmas.

(e) **Protein Gels:** Two protein gel images have been acquired and analyzed by the computer system. The computer results were compared to the results obtained manually. The molecular weights of the standard stain are known. The molecular weights of the unknown stains have been calculated both manually and by the computer system. The results obtained from the computer were found to compare well with those obtained manually. Table 4.6 provides a summary of the results of the
<table>
<thead>
<tr>
<th>SLIDE ID</th>
<th>AREA IN PIXELS</th>
<th>LENGTH (mm)</th>
<th>SURFACE AREA mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLIDE</td>
<td>IMAGE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kuujjvaq</td>
<td>1</td>
<td>1710</td>
<td>0.6737</td>
</tr>
<tr>
<td>Set 1</td>
<td>2</td>
<td>1418</td>
<td>0.4149</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1451</td>
<td>0.3804</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1890</td>
<td>0.5866</td>
</tr>
<tr>
<td>Kuujjvaq</td>
<td>1</td>
<td>1998</td>
<td>0.4351</td>
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<tr>
<td>Set 2</td>
<td>2</td>
<td>2520</td>
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<tr>
<td></td>
<td>3</td>
<td>2492</td>
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<td></td>
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<td>7224</td>
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<td>2</td>
<td>6186</td>
<td>1.2873</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5642</td>
<td>1.1356</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5120</td>
<td>1.2744</td>
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### TABLE 4.6
**PROTEIN GEL STAIN ANALYSIS**

<table>
<thead>
<tr>
<th>STAIN PATTERN</th>
<th>MIGRATION (PIXELS)</th>
<th>MOLECULAR WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>55</td>
<td>63.0</td>
</tr>
<tr>
<td>2.</td>
<td>76</td>
<td>44.0</td>
</tr>
<tr>
<td>3.</td>
<td>89</td>
<td>31.0</td>
</tr>
<tr>
<td>4.</td>
<td>114</td>
<td>21.1</td>
</tr>
<tr>
<td>5.</td>
<td>132</td>
<td>14.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Unknown</th>
<th>COMPUTER</th>
<th>MANUAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>122</td>
<td>15.83</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>115</td>
<td>21.17</td>
</tr>
<tr>
<td>2.</td>
<td>126</td>
<td>14.35</td>
</tr>
<tr>
<td>3.</td>
<td>131</td>
<td>11.25</td>
</tr>
<tr>
<td>4.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>116</td>
<td>20.55</td>
</tr>
<tr>
<td>2.</td>
<td>127</td>
<td>13.73</td>
</tr>
<tr>
<td>3.</td>
<td>134</td>
<td>9.39</td>
</tr>
<tr>
<td>4.</td>
<td>139</td>
<td>5.67</td>
</tr>
<tr>
<td>5.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td></td>
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<tr>
<td>1.</td>
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<td>19.31</td>
</tr>
<tr>
<td>2.</td>
<td>127</td>
<td>13.73</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>128</td>
<td>13.11</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>123</td>
<td>16.21</td>
</tr>
</tbody>
</table>
analysis of one of the protein gel images (Figure 3.28). Some of the multiple bands have been identified by the computer as a single band because of their closeness, and because the gray level in the background area between these bands falls in the same range as that of the bands. Consequently, they appear as one band to the computer. This confusion may be avoided by magnifying the image to increase the distances between the bands.

Table 4.7 summarizes the image analysis techniques used for the specimens.
<table>
<thead>
<tr>
<th>Specimen</th>
<th>Objective</th>
<th>Segmentation Procedure</th>
<th>Parameters extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pollen Cells</td>
<td>Distinguish between live and dead cells</td>
<td>Thresholding</td>
<td>Mean Optical Density and area of stained region</td>
</tr>
<tr>
<td>2. Hybridoma Cells</td>
<td>Identification of micro‐colonies</td>
<td>Boundary detection</td>
<td>Area of all cells and micro‐colonies</td>
</tr>
<tr>
<td>3. Red Blood Cells</td>
<td>Quantification of DNA present in a cell</td>
<td>Thresholding</td>
<td>Mean and Total Optical Density</td>
</tr>
<tr>
<td>4. Stigmas</td>
<td>Surface areas of stigmas</td>
<td>Thresholding</td>
<td>Area and width at tip and base of stigma</td>
</tr>
<tr>
<td>5. Protein Stains</td>
<td>Molecular weights of the stains</td>
<td>Thresholding</td>
<td>Migration from top of gel</td>
</tr>
</tbody>
</table>
Chapter V

CONCLUSIONS
A semi-automatic system consisting of a video camera, digitizer board and a micro-computer has been developed for biological specimen image analysis. Software algorithms for the analysis of the specimen images were also developed. The specimen images were analyzed using digital image processing techniques. The system was tested for five different biological specimens.

The results obtained for most of the specimens compare favorably with results obtained either by visual inspection of the specimens or by other standard methods.

Based on the results of this work the following conclusions are made:

(a) For pollen cells, the computer results have been found to correlate well with those obtained by visual inspection. ANOVA of the data obtained for pollen cells has shown that there is a highly significant relationship between the mean optical density of a cell and its viability. It has been found that the mean optical density accounted for 67.7% of the variance. Consequently, the mean optical density can be used as a discriminating factor for distinguishing live cells from dead cells.
(b) Results from hybridoma cell analysis indicate that, in comparison to visual inspection, the computer system made correct classifications in 76% of the cases. The reason for the false classification was attributed to the density of the cells in the wells. The cells in the wells with higher densities were so close together that they appeared as clusters and thus were classified as clusters.

(c) DNA analysis of cells has given good results. The optical densities have been calibrated using standard. In addition, the system has been compared to a standard system (DADS) and has been found to compare well with the standard system.

(d) Surface areas of stigmas, which, because of the complex geometry are very difficult to measure manually, were calculated readily using image analysis techniques.

(e) Results of computer analysis for the calculation of molecular weights of protein stains were found to correlate well with results obtained manually.

The computer time required for analyzing most of the specimen images has been found to be less than 30 seconds. For laboratory applications, this speed can be considered to be very reasonable. The analysis of hybridoma cell images took more time to process (6 to 40
minutes), but, this time can be reduced as suggested in Chapter 6.

In terms of accuracy and repeatability, the DNA measurement was the most successful application of the system. The performance of the system in this case is comparable to a commercial system which costs 3 times as much. For other specimens (protein gels, stigmas, pollen cells and hybridoma cells) also, the system is of commercial value as the system proved to be a reliable and labor saving device.

In general, the computer vision system has been found to very flexible and adaptable for use with different biological specimens. The results obtained for the computer analysis of the specimens have been found to be very promising. Many interactive software algorithms have been developed for this application and this software can be modified to suit other applications.
Chapter VI

RECOMMENDATIONS
It is recommended that future experimentation should be concentrated in the following areas:

1. An 8-bit digitizer board with greater image resolution should be used for capturing the images. The advantage of using a digitizer board with higher bits/pixel will be that a greater range of gray levels would be available and subtle changes in light intensity could be detected. This improvement might lead to a reduction in the complexity of the task of segmenting the image. Also, the greater the resolution and gray level range, the closer is the digitized image to the original image.

2. Only two classification parameters, (area and optical density (mean and total)) have been implemented on the computer system. Extending the system to include other parameters such as color and shape will make the system more flexible.

3. Calibration of the system for different magnifications which is being done manually at present, should be automated through appropriate software.

4. Images of hybridoma cells at lower concentration levels should be acquired and analyzed. Hybridoma
cells manufactured at a single concentration level were imaged and analyzed by the present system. By reducing the concentration levels of the cells, a reduction in computation time and more accurate classification would result as discussed in Chapter 4.

5. In the analysis of protein gels, only the molecular weights of the stains are calculated. The analysis should be extended to include other parameters such as Ferguson plots, free mobility and net charge.

6. Considering the fact that the computer system has been found to be extremely flexible, the system should be extended to other biological specimens where possible.


33. Dr. Michael Weis, Personal Communication, Department of Biological Sciences, University of Windsor, Windsor, Ontario, Canada.

34. Olympus Inverted Microscope Instruction Manual (Model IMT), Olympus Optical Co., Ltd., Japan.


37. Palette Capture Users Guide, December 1985, Quadram, Norcross, Georgia, USA.

38. Sony Trinitron Color TV (KV-1311CR) Operating Instructions, Sony Corporation, Japan.

Appendix A

EQUIPMENT DESCRIPTION
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CHORUS DATA SYSTEMS INC.,
P.O. BOX 370,
6 CONTINENTAL BOULEVARD,
MERRIMACK, NEW HAMPSHIRE,
03054.
(603) 424-2900

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Operations Manuel
For IBM PC, PC/XT, PC/AT, and compatible personal computers
Part Number of publication: 12-9401-01/REV. 3
Appendix B

SOFTWARE
Computer software for this application has been developed in Turbo Pascal\textsuperscript{R} 3.0 which interfaces with the basic PC-EYE\textsuperscript{R} Video Capture Software. The PC-EYE software has 35 routines for hardware and software configuration. Over a 100 procedures and functions dealing with many image processing operations have been developed. The following is a description of selected functions and procedures.

B.1 PC-EYE Routines: The PC-EYE routines accept a fixed number of parameters. The following are the routines available in the PC-EYE software package (Reproduced from the PC-EYE Technical Reference Manual and Programmer's guide, Release 2.24).

I. Hardware Configuration Routines:

(1) PCIADR: Sets the PC-EYE digitizer card I/O address.

(2) MMBADR: Sets the address of the main memory buffer. This buffer is used to store the image.

(3) GDMADR: Sets the base memory address of the graphics board display buffer.

(4) GIOADR: Sets the base I/O address of the
graphics board.

(5) SETCHN: Sets the DMA channel to use for DMA transfers.

(6) SETMUX: Sets the camera channel input mux. It activates any one of the four camera input ports on the PC-EYE board.

II. Software Configuration Routines:

(7) SETDTD: Specifies the data transfer method for the hardware environment.

(8) SETGID: Specifies the graphic interface driver to use with the graphics board in use.

(9) SETPRD: Specifies the printer driver to use. The printers supported are the HP Thinkjet and Epson FX and MX SERIES.

(10) SCAMRA: Specifies the current camera type.

(11) SETCRT: Specifies the video display mode, black and white or color.

(12) SETSRC: Selects the current source device for data transfer. PC-EYE supports the following devices:

   (i) The PC-EYE digitizer board.
(ii) The current graphics board.

(iii) The main memory buffer.

(iv) The current printer.

(v) The disk system or a file on disk.

(13) SETDST: Selects the current destination device for data transfer. See SETSRC for devices supported.

(14) CFGMEM: Selects the pixel packing format for the main memory buffer.

(15) AFRAME: AFRAME is used to select which portion of the camera's image appears within the display frame (DFRAM).

(16) DFRAME: DFRAME is used to specify the size and location of an image on the graphics monitor.

(17) THROTL: Specifies the rate at which lines are to be acquired during capture.

(18) SETCLR: Selects the active color for palette loading.

(19) SPALET: Selects the active palette for the graphics board.

(20) LODPAL: Loads the current palette of the current color to the graphics board driver.
(21) **GENPAL**: Loads a default black and white palette.

(22) **CALIBR**: CALIBR is used to adjust the black and white levels on the PC-EYE's A/D converter.

**III. Utility Routines:**

(23) **CAPTUR**: Captures an entire image frame to the current destination device set by SETDST.

(24) **DMXFER**: Transfers a display image from the current source device to the current destination device set by SETSRC and SETDST.

(25) **DPRINT**: Outputs an image to the current printer.

(26) **GETROW**: Moves a specified row of the image from the current source device to the program memory.

(27) **PUTROW**: Moves a specified row of the image to the current destination device.

(28) **GETPEL**: Extracts a specified pixel from the image for the current source device.

(29) **PUTPEL**: Replaces a specified pixel in the image for the current source device.

(30) **GETPIC**: Loads a PC-EYE image stored on disk to
the current destination device.

(31) PUTPIC: Saves a PC-EYE image to a file on disk from the current source device.

(32) CLRMOD: Specifies the mode for image capture, color or black and white.

B.2 VISION Routines: The following procedures have been developed as part of the applications program for biological specimen analysis.

(1) CURSOR: This procedure builds a graphics cursor on the video screen. The cursor may be used to specify the part of the image on which processing operations have to be performed.

(2) CONFIGURE: This procedure configures the hardware using the PC-EYE hardware configuration routines.

(3) ADD: This procedure adds or subtracts two images and displays the resulting image on the video monitor.

(4) IMAVERAGE: Different images of the same scene can be captured and averaged to reduce noise effects.

(5) KERNELS: This procedure builds kernels for edge detection operations. Kernels for horizontal, vertical
and Laplacian edge detectors have been included. The procedures EDGE and SHARP call the procedure KERNELS to initialize the kernels for the convolution operation. The following kernels are being used for the operations.

(i) Horizontal edge:

\[
\begin{bmatrix}
-2 & -2 & -2 \\
0 & 0 & 0 \\
-2 & -2 & -2
\end{bmatrix}
\]

(ii) Vertical edge:

\[
\begin{bmatrix}
-2 & 0 & 2 \\
-2 & 0 & 2 \\
-2 & 0 & 2
\end{bmatrix}
\]

(iii) Laplacian edge-I:

\[
\begin{bmatrix}
0 & -1 & 0 \\
-1 & 4 & -1 \\
0 & -1 & 0
\end{bmatrix}
\]
(iv) Laplacian edge-II:

\[
\begin{bmatrix}
-1 & -1 & -1 \\
-1 & 8 & -1 \\
-1 & -1 & -1 \\
\end{bmatrix}
\]

In addition the user may input any desired 3x3 kernel.

(6) EDGE: This procedure performs a convolution operations using the kernels in the procedure KERNELS. This operation is performed on a 3x3 block of the image matrix.

(7) SHARP: SHARP does a Laplacian image sharpening on any specified image. The following kernels are being used for the operation.

(i) Laplacian Sharpening-I:

\[
\begin{bmatrix}
0 & -1 & 0 \\
-1 & 5 & -1 \\
0 & -1 & 0 \\
\end{bmatrix}
\]

128
(ii) Laplacian Sharpening-II:

\[
\begin{bmatrix}
-1 & -1 & -1 \\
-1 & 9 & -1 \\
-1 & -1 & -1 \\
\end{bmatrix}
\]

(8) MEDIAN: Median implements the median filter. The value of the center pixel in a 3X3 block of the image matrix is the median of all the nine values in the 3X3 block.

\[
\begin{bmatrix}
a_4 & a_3 & a_2 \\
a_5 & a_0 & a_1 \\
a_6 & a_7 & a_8 \\
\end{bmatrix}
\]

The value of \(a_0\) is the median of all the values \(a_0\) to \(a_9\).

(9) SOBEL: The Sobel filter is implemented in this procedure. The kernels being used for the convolution operation are given below.

\[
\frac{1}{4}
\begin{bmatrix}
-1 & 0 & 1 \\
-2 & 0 & 2 \\
-1 & 0 & 1 \\
\end{bmatrix}
\quad \text{and} \quad
\frac{1}{4}
\begin{bmatrix}
1 & 2 & 1 \\
0 & 0 & 0 \\
-1 & -2 & -1 \\
\end{bmatrix}
\]
Please refer to Chapter 5 for a detailed description of the Sobel filter.

(10) HISTOGRAM: HISTOGRAM calculates the frequency of occurrence of each gray level, the mean gray level and the standard deviation of any image and plots the gray level vs. frequency graph on the video screen. In addition, the cumulative frequency and the percentage of the frequency of occurrence is also calculated.

(11) STRETCH: This procedure performs a contrast stretch on a given image and displays the resulting image on the screen. The value of any pixel within the specified limits is given by:

\[
\begin{align*}
  b_{\text{out}} &= \frac{\text{Pin} - \text{Plow}}{\text{Phi} - \text{Plow}} \times 63 \\
  &= 0 \quad \text{if Pin} \leq \text{Plow} \\
  &= \frac{\text{Pin} - \text{Plow}}{\text{Phi} - \text{Plow}} \times 63 \quad \text{if Plow < Pin < Phi} \\
  &= 63 \quad \text{if Pin} \geq \text{Phi}
\end{align*}
\]

where Pin is the input intensity value of the pixel, Pout is the output intensity value of the pixel, Plow is the lower threshold value and Phi is the higher threshold value.

Procedures 12 to 16 have been developed for hybridoma cell image analysis. Please refer to Chapter 5.
for a detailed review of the algorithms for these procedures.

(12) CLEAN: CLEAN procedure cleans up the image resulting in most of the spurious edges being wiped out.

(13) AVERAGE: AVERAGE procedure averages the pixels in a 3x3 neighborhood of the image, resulting in a thickening of the borders around the components of the image.

(14) SKELETON: SKELETON procedure thins the edges of the components of the image resulting from the averaging operation.

(15) GROW: GROW procedure segments a hybridoma cell image into the background and the cell regions.

(16) AREA: AREA procedure finds the areas of all the components of a hybridoma cell image and classifies the components of the image based on this area.

(17) THRESHOLD: THRESHOLD performs a thresholding operation on a given image. All pixels above a user specified higher threshold level and all pixels below the lower threshold level are set to white. This results in segmentation of the image into the background and its constituent components.

(18) PROFILE: PROFILE calculates the intensity
profile of any user specified portion of the image. The intensity profile is a one-dimensional representation of the image with the image row (horizontal intensity profile) or the image column (vertical intensity profile) on the x-axis and the average value of all the pixels along that row or column on the y-axis. The pixels are averaged along the row or column depending on the profile needed.

(19) PARAMEX: PARAMEX is the procedure used for pollen cell, stigma and red blood cell image analysis. This procedure extracts parameters of all the components of the image. The parameters that are extracted are the area, mean gray level and total gray level. Classification is then made on the basis of these parameters.

Procedures 20 to 23 have been developed for protein gel image analysis.

(20) FINDPEAK: FINDPEAK calculates the intensity profile of the protein stain pattern and locates the position of the stains with respect to the top edge of the gel. A search of the profile matrix for any drop in the gray level is made and all those points where there is an appreciable drop in the gray level value are classified as stains. These distances are subsequently
used to calculate the molecular weights.

(21) CURVE: CURVE calculates the slope and the intercept of the best fitting straight line for the data obtained for the standard stain pattern. The slope and the intercept are subsequently used for finding the molecular weights of the unknown stain patterns.

(22) CURVE_FIT: CURVE_FIT procedure uses the data obtained from the procedures FINDPEAK and CURVE. The known values are substituted in the standard equation of a straight line to calculate the molecular weights of the unknown stain pattern.

(23) GELANALYSIS: GELANALYSIS is the main procedure for protein gel analysis. This procedure calls the procedures FINDPEAK and CURVE for the standard stain pattern and the procedures FINDPEAK and CURVE_FIT for calculating the molecular weights of the unknown stain pattern.
Overlay Procedure Sobel;  {PROCEDURE FOR SOBEL OPERATOR}

Var
    SumX, SumY: Real;

Begin
    FileRead;
    Write('Enter threshold value:');
    Readln(Thresh);
    Readln;
    Locate;
    Wait;
    CRT_ON;
    Transfer;
    If Errs=True then Exit;

    For Row:=0 to 197 do
        Begin
            Reply:=GetRow(Buffer1, Row, 0);
            Reply:=GetRow(Buffer2, Row+1, 0);
            Reply:=GetRow(Buffer3, Row+2, 0);
            For I:=1 to 320 do
                Buffer4[I]:=0;
            For Column:=1 to 320 do
                Begin
                    [VALUE OF GRADIENT IN X-DIRECTION]
                    SumX:=Abs(1/4*(Buffer1[Column+2] +
                        (2*Buffer2[Column+2])+Buffer3[Column+2])- (Buffer1[Column]+(2*Buffer2[Column])+ Buffer3[Column+2]));
                    [VALUE OF GRADIENT IN Y-DIRECTION]
                    SumY:=Abs(1/4*(Buffer1[Column]+
                        (2*Buffer1[Column+1])+Buffer1[Column+2])- (Buffer3[Column]+(2*Buffer2[Column+2])+ Buffer3[Column+2]));
                    Buffer4[Column+1]:=Round(Abs(SumX+SumY));
                    If Buffer4[Column+1]>Thresh then
                        Buffer4[Column+1]:=Round(Buffer4[Column+1]/4);
                    If Buffer4[Column+1]<Thresh then Buffer4[Column+1]:=0;
                    end;
                    Reply:=PutRow(Buffer4, Row+1, 0);
                end;

            For I:=1 to 320 do
                Buffer4[I]:=0;
            Reply:=PutRow(Buffer4, 0, 0);
            Reply:=PutRow(Buffer4, 199, 0);
{ROW #1, ROW #199 AND COLUMN #1 AND 319}
{OF THE IMAGE ARE SET TO 0}
For Row:=1 to 198 do
  Begin
      Reply:=PutPel(Row,0,0,0);
      Reply:=PutPel(Row,319,0,0);
  end;

Source_Display1;
NoKey;
Windows;
Save;
end;

{*******************************************************************************}

Overlay Procedure Profile;
{DRAWS THE HORIZONTAL AND VERTICAL PROFILE OF}
{A SPECIFIED PART OF THE IMAGE}

Var
  Sbuff:Array[0..319] of Integer;
  Averag:Real;
  GrandTot:Integer;
  Rep,Pro:Char;

Begin
  FileRead;
  Locate;
  Wait;
  CRT_ON;
  Transfer;
  If Errs=True then Exit;
  NoKey;
  Rep:='N';

  Repeat
    If Rep='Y' then
      Reply:=Dmxfer;
    Windows;
    Write(' "H" for horizontal profile, "Y" for vertical profile ');
  Repeat
    Read(Kbd,Pro);
    Pro:=Uppercase(Pro);
    Until Pro in ['H','V'];
    WriteLn(Pro);
    Cursor;
    WriteLn;

135
Locate;
Wait;

For I:=0 to 319 do
Sbuff[I]:=0;
GrandTot:=0;

If Pro='V' then
 {VERTICAL PROFILE ADD ALL PIXELS IN A COLUMN}
Begin
Column:=StartCol;
Repeat
For Row:=StartRow to EndRow do
Begin
PelValue:=GetPel(Row,Column,0);
Sbuff[Column]:=Sbuff[Column]+PelValue;
end;
Column:=Column+1;
Until Column=EndCol+1;
For Column:=StartCol to EndCol do
Sbuff[Column]:=Round(Sbuff[Column]/(EndRow-StartRow+1));

If,StartCol=0 then
I:=StartCol
Else I:=StartCol-1;
If EndCol=319 then
L:=EndCol
Else L:=EndCol+1;
For Row:=130 to 195 do
For Column:=I to L do
Reply:=PutPel(Row,Column,0,63);

If (I>2) and (K<317).then
Begin
For Row:=130 to 195 do
Begin
For Column:=(I-2) to I do
Reply:=PutPel(Row,Column,0,31);
For Column:=L to (L+2) do
Reply:=PutPel(Row,Column,0,31);
end;

For Row:=127 to 129 do
For Column:=(I-2) to (L+2) do
Reply:=PutPel(Row,Column,0,31);
For Row:=196 to 198 do
For Column:=(I-2) to (L+2) do
Reply:=PutPel(Row,Column,0,31);
end;
Column:=StartCol;
Repeat
  PelValue:=Sbuff[Column];
  If PelValue>0 then
    Begin
      For Row:=194 downto (194-PelValue) do
        Reply:=PutPel(Row,Column,0,0);
      end;
    Column:=Column+1;
  Until Column=EndCol+1

For Column:=StartCol to EndCol do
  GrandTot:=GrandTot+Sbuff[Column];

Averag:=GrandTot/(EndCol-StartCol+1);
NoKey;
Windows;
Writeln(' Average= ',Averag:3:2);
end;

If Pro='H' then
{HORIZONTAL PROFILE, ADD PIXELS IN A ROW}
Begin
  Row:=StartRow;
  Repeat
    For Column:=StartCol to EndCol do
      Begin
        PelValue:=GetPel(Row,Column,0);
        Sbuff[Row]:=Sbuff[Row]+PelValue;
      end;
    Row:=Row+1;
  Until Row=EndRow+1;

  For Row:=StartRow to EndRow do
    Sbuff[Row]:=Round(Sbuff[Row]/(EndCol-StartCol+1));

  If StartRow=0 then
    I:=StartRow
  Else I:=StartRow-1;
  If EndRow=199 then
    L:=EndRow
  Else L:=EndRow+1;
  For Row:=130 to 195 do
    For Column:=I to L do
      Reply:=PutPel(Row,Column,0,63);
  If (I>2) and (L<317) then
    Begin
      For Row:=130 to 195 do
        Begin
          
137
For Column:=(I-2) to I do
  Reply:=PutPel(Row,Column,0,31);
For Column:=L to (L+2) do
  Reply:=PutPel(Row,Column,0,31);
end;

For Row:=127 to 129 do
  For Column:=(I-2) to (L+2) do
    Reply:=PutPel(Row,Column,0,31);
  For Row:=196 to 198 do
    For Column:=(I-2) to (L+2) do
      Reply:=PutPel(Row,Column,0,32);
  end;

Column:=StartRow;
Repeat
  PelValue:=Sbuff[Column];
  If PelValue>0 then
    Begin
      For Row:=194 downto (194-PelValue) do
        Reply:=PutPel(Row,Column,0,0);
    end;
  Column:=Column+1;
Until Column=EndRow+1;

For Row:=StartRow to EndRow do
  GrandTot:=GrandTot+Sbuff[Row];

Averag:=GrandTot/(EndRow-StartRow+1);
NoKey;
Windows;
Writeln(' Average= ',Averag:3:1);
end;

Writeln('Save intensity distribution data? (Y/N)');
Repeat
  Read(Kbd,Dum);
  Dum:=Upcase(Dum);
  Writeln(Dum);
Until Dum in ['Y','N'];
Writeln;

If Dum='Y' then
  Begin
    Write(' Enter Filename: ');
    Readln(Filename);
    Assign(Outfile,Filename);
    Rewrite(Outfile);
    If Pro='V' then
      Begin
        For Column:=StartCol to EndCol do
          ....
....
138
Writeln(Outfile, Column:6, ', Sbuff[Column]:6);
end;
If Pro='H' then
Begin
  For Row:=StartRow to EndRow do
    Writeln(Outfile, Row:6, ', Sbuff[Row]:6);
end;
Close(Outfile);
Writeln;
end;

Write('Press "y" to clear screen and continue or "q" to quit ');
Repeat
  Read(Kbd, Rep);
  Rep:=Upcase(Rep);
Until Rep in ['Y', 'Q'];
Writeln(Rep);
Writeln;
Locate;
Windows;
Locate;
CirScr;

Until Rep='Q';
end;

{*******************************************************************************}

Overlay Procedure ObjectArea;
{PROCEDURE FOR FINDING THE AREAS, MEAN AND}
{TOTAL OPTICAL DENSITY OF POLLEN, STIGMA & DNA}

Var
  Found:Boolean;
  Counter, Count, Counts, Wind_Width1, Wind_Width2, Wind_Height: Integer;
  Height, Width, Passes, Pass, GLThresh: Integer;
  ASum, ASum1, ASum2, GLAverage, GLSum: Real;
  Option: Char;

Begin
  FileRead;
  Locate;
  Wait;
  CRT_ON;
  Transfer;
  If Errs=True then Exit;
  NoKey;

  Height:=30;
  Width:=40;

139
Passes:=1;
{DEFAULT SIZE OF OPERATIONS WINDOW AND AREA}
{THRESHOLD AND NUMBER OF PASSES AND OPTICAL}
{DENSITY THRESHOLD}
ASum1:=100;
If ASum1>200 then
ASum2:=50
Else ASum2:=ASum1/2;
GThresh:=50;

Windows;
Write(' Change options (Y/N)?');
Repeat
   Read(Kbd,Option);
   Option:=Upcase(Option);
Until Option in ['Y','N'];
WriteLn;

If Option='Y' then
Begin
   Write(' Current window height is ');
   WriteLn(Height);
   Write(' Enter new window height: ');
   ReadLn(Height);
   Write(' Current window width is ');
   WriteLn(Width);
   Write(' Enter new window width: ');
   ReadLn(Width);
   Write(' Current number of passes is ');
   WriteLn(Passes);
   Write(' Enter number of passes: ');
   ReadLn(Passes);
   Write(' Current threshold for area is ');
   WriteLn(ASum1:5:0);
   Write(' Enter new threshold: ');
   ReadLn(ASum1);
   Write(' Current threshold for gray level is');
   WriteLn(GThresh);
   Write(' Enter new threshold: ');
   ReadLn(GThresh);
end;

Locate;
Wait;
Window(1,1,80,25);
ClrScr;
Reply:=Setsrc('t');
Reply:=Setdst(1);

Counter:=0;
Count:=0;
Counts:=0;
For Row:=0 to 199 do
  {SET ALL VALUES WHICH ARE 0 TO 1 TO AVOID}
  {COUNTING PIXELS WHICH HAVE BEEN COUNTED BEFORE}
  Begin
    Reply:=GetRow(Buffer1,Row,0);
    For Column:=1 to 320 do
      Begin
        If Buffer1[Column]=0 then
          Buffer1[Column]:=1;
        end;
        Buffer1[1]:=63;
        Buffer1[320]:=63;
        Reply:=PutRow(Buffer1,Row,0);
      end;
    For Column:=1 to 320 do
      Buffer1[Column]:=63;
    Reply:=PutRow(Buffer1,0,0);
    Reply:=PutRow(Buffer1,199,0);
  Repeat
    ASum:=0.0;
    GLSum:=0.0;
    Found:=False;
    Row:=Counter;
  Repeat
  {SELECT ALL PIXELS WHICH ARE CONNECTED AND WHICH ARE BELOW THE}
  {THRESHOLD SET BY THE USER OR DEFAULT THRESHOLD CALCULATE THE}
  {AREAS OF CELLS AND OPTICAL DENSITY}
  Reply:=GetRow(Buffer1,Row,0);
  Column:=1;
  Repeat
    If ((Buffer1[Column]<50) and
      (Buffer1[Column]<>0)) then
      Begin
        Found:=True;
        GLSum:=GLSum+Buffer1[Column];
        ASum:=ASum+1;
        Buffer1[Column]:=0;
      end;
      Column:=Column+1;
      Until (Found=True) or (Column=321)));
    Row:=Row+1;
    Until (Found=True) or (Row=200));
  Column:=Column-1;
  StartRow:=Trunc((Row-1)/8)+2;
  StartCol:=Trunc((Column-1)/4)+2;
  If StartCol>76 then StartCol:=75;
Counts:=Row-1;
{FIND LOCATION OF EACH CELL IN PIXELS AND CONVERT}
{THE LOCATION TO TEXT SCREEN LOCATIONS. EACH}
{CHARACTER IS 4 PIXELS IN WIDTH AND 8 PIXELS IN HEIGHT}
If Counts=0 then
    Counter:=0
Else Counter:=Counts-1;
If Counter<=2 then
    Count:=2
Else Count:=Counter;

If Column>=Round(Height/2) then
    Wind_Width1:=Column-Round(Height/2)
Else Wind_Width1:=1;

If Column<=(320-Round(Height/2)) then
    Wind_Width2:=Column+Round(Height/2)
Else Wind_Width2:=320;

If Counter>(199-Width) then
    Wind_Height:=199
Else Wind_Height:=Counter+Width;

If Found=True then
    Begin
        Reply:=PutRow(Buffer1,Row-1,0);
        For Row:=Counter to Wind_Height do
            Begin
                Reply:=GetRow(Buffer1,Row,0);
                Reply:=GetRow(Buffer2,Row+1,0);
                Reply:=GetRow(Buffer3,Row+2,0);
                For Column:=Wind_Width1 to Wind_Width2 do
                    Begin
                        If Buffer2[Column+1]=0 then
                            Begin
                                If ((Buffer1[Column]<50) and
                                    (Buffer1[Column]<>0)) then
                                    Begin
                                        GLSum:=GLSum+Buffer1[Column];
                                        Buffer1[Column]:=0;
                                        ASum:=ASum+1.0;
                                    end;
                                If ((Buffer1[Column+1]<50) and
                                    (Buffer1[Column+1]<>0)) then
                                    Begin
                                        GLSum:=GLSum+Buffer1[Column+1];
                                        Buffer1[Column+1]:=0;
                                        ASum:=ASum+1.0;
                                    end;
                                If ((Buffer1[Column+2]<50) and
                                    (Buffer1[Column+2]<>0)) then
                                    Begin
                                        GLSum:=GLSum+Buffer1[Column+2];
                                        Buffer1[Column+2]:=0;
                                        ASum:=ASum+1.0;
                                    end;
                            End;
                    End;
    End;

142
GLSum := GLSum + Buffer1[Column + 2];
Buffer1[Column + 2] := 0;
ASum := ASum + 1.0;
end;

If ((Buffer2[Column] < 50) and (Buffer2[Column] <> 0)) then
Begin
  GLSum := GLSum + Buffer2[Column];
  Buffer2[Column] := 0;
  ASum := ASum + 1.0;
end;

If ((Buffer2[Column + 2] < 50) and (Buffer1[Column + 2] <> 0)) then
Begin
  GLSum := GLSum + Buffer2[Column + 2];
  Buffer2[Column + 2] := 0;
  ASum := ASum + 1.0;
end;

If ((Buffer3[Column] < 50) and (Buffer3[Column] <> 0)) then
Begin
  GLSum := GLSum + Buffer3[Column];
  Buffer3[Column] := 0;
  ASum := ASum + 1.0;
end;

If ((Buffer3[Column + 1] < 50) and (Buffer3[Column + 1] <> 0)) then
Begin
  GLSum := GLSum + Buffer3[Column + 1];
  Buffer3[Column + 1] := 0;
  ASum := ASum + 1.0;
end;

If ((Buffer3[Column + 2] < 50) and (Buffer3[Column + 2] <> 0)) then
Begin
  GLSum := GLSum + Buffer3[Column + 2];
  Buffer3[Column + 2] := 0;
  ASum := ASum + 1.0;
end;

end;

Reply := PutRow(Buffer1, Row, 0);
Reply := PutRow(Buffer2, Row + 1, 0);
Reply := PutRow(Buffer3, Row + 2, 0);
end;

If ASum > ASum2 then
Begin
  Pass := 1;
  Repeat
    For Row := Counter to Wind_Height do
      Begin
        Reply := PutRow(Buffer1, Row, 0);
        Reply := PutRow(Buffer2, Row + 1, 0);
        Reply := PutRow(Buffer3, Row + 2, 0);
      End;
  End;
End;
Reply:=getRow(Buffer1,Row,0);
Reply:=getRow(Buffer2,Row+1,0);
Reply:=getRow(Buffer3,Row+2,0);
for Column:=Wind_Width1 to Wind_Width2 do
begin
  if Buffer2[Column+1]=0 then
    begin
      if ((Buffer1[Column]<50) and
          (Buffer1[Column]<>0)) then
        begin
          GLSum:=GLSum+Buffer1[Column];
          Buffer1[Column]:=0;
          ASum:=ASum+1.0;
        end;
      if ((Buffer1[Column+1]<50) and
          (Buffer1[Column+1]<>0)) then
        begin
          GLSum:=GLSum+Buffer1[Column+1];
          Buffer1[Column+1]:=0;
          ASum:=ASum+1.0;
        end;
      if ((Buffer1[Column+2]<50) and
          (Buffer1[Column+2]<>0)) then
        begin
          GLSum:=GLSum+Buffer1[Column+2];
          Buffer1[Column+2]:=0;
          ASum:=ASum+1.0;
        end;
      if ((Buffer2[Column]<50) and
          (Buffer2[Column]<>0)) then
        begin
          GLSum:=GLSum+Buffer2[Column];
          Buffer2[Column]:=0;
          ASum:=ASum+1.0;
        end;
      if ((Buffer2[Column+2]<50) and
          (Buffer2[Column+2]<>0)) then
        begin
          GLSum:=GLSum+Buffer2[Column+2];
          Buffer2[Column+2]:=0;
          ASum:=ASum+1.0;
        end;
      if ((Buffer3[Column]<50) and
          (Buffer3[Column]<>0)) then
        begin
          GLSum:=GLSum+Buffer3[Column];
          Buffer3[Column]:=0;
          ASum:=ASum+1.0;
        end;
      if ((Buffer3[Column+1]<50) and
          (Buffer3[Column+1]<>0)) then
        begin
          GLSum:=GLSum+Buffer3[Column+1];
          Buffer3[Column+1]:=0;
          ASum:=ASum+1.0;
        end;
    end;
GLSum := GLSum + Buffer3[Column + 1];
Buffer3[Column + 1] := 0;
ASum := ASum + 1.0;
end;

If ((Buffer3[Column + 2] < 50) and
    (Buffer3[Column + 2] > 0)) then
Begin
  GLSum := GLSum + Buffer3[Column + 2];
  Buffer3[Column + 2] := 0;
  ASum := ASum + 1.0;
end;
end;

Reply := PutRow(Buffer1, Row, 0);
Reply := PutRow(Buffer2, Row + 1, 0);
Reply := PutRow(Buffer3, Row + 2, 0);
end;

For Row := Wind_Height downto Counter do
Begin
  Reply := GetRow(Buffer1, Row, 0);
  Reply := GetRow(Buffer2, Row + 1, 0);
  Reply := GetRow(Buffer3, Row + 2, 0);
  For Column := Wind_Width1 to Wind_Width2 do
  Begin
    If Buffer2[Column - 1] = 0 then
    Begin
      If ((Buffer1[Column] < 50) and
           (Buffer1[Column] > 0)) then
      Begin
        GLSum := GLSum + Buffer1[Column];
        Buffer1[Column] := 0;
        ASum := ASum + 1.0;
      end;
      If ((Buffer1[Column - 1] < 50) and
           (Buffer1[Column - 1] > 0)) then
      Begin
        GLSum := GLSum + Buffer1[Column - 1];
        Buffer1[Column - 1] := 0;
        ASum := ASum + 1.0;
      end;
      If ((Buffer1[Column - 2] < 50) and
           (Buffer1[Column - 2] > 0)) then
      Begin
        GLSum := GLSum + Buffer1[Column - 2];
        Buffer1[Column - 2] := 0;
        ASum := ASum + 1.0;
      end;
      If ((Buffer2[Column] < 50) and
           (Buffer2[Column] > 0)) then
      Begin
        GLSum := GLSum + Buffer2[Column];
        Buffer2[Column] := 0;
        ASum := ASum + 1.0;
      end;
    end;
  end;
end;
Buffer2[Column] := 0;
ASum := ASum + 1.0;
end;
If ((Buffer2[Column-2]<50) and (Buffer1[Column-2]>0)) then
Begin
GLSum := GLSum + Buffer2[Column-2];
Buffer2[Column-2] := 0;
ASum := ASum + 1.0;
end;
If ((Buffer3[Column]<50) and (Buffer3[Column]>0)) then
Begin
GLSum := GLSum + Buffer3[Column];
Buffer3[Column] := 0;
ASum := ASum + 1.0;
end;
If ((Buffer3[Column-1]<50) and (Buffer3[Column-1]>0)) then
Begin
GLSum := GLSum + Buffer3[Column-1];
Buffer3[Column-1] := 0;
ASum := ASum + 1.0;
end;
If ((Buffer3[Column-2]<50) and (Buffer3[Column-2]>0)) then
Begin
GLSum := GLSum + Buffer3[Column-2];
Buffer3[Column-2] := 0;
ASum := ASum + 1.0;
end;
end;
Reply := PutRow(Buffer1, Row, 0);
Reply := PutRow(Buffer2, Row-1, 0);
Reply := PutRow(Buffer3, Row-2, 0);
end;

Pass := Pass + 1;
Until Pass = Passes + 1;
end;

For Row := Counter to Wind_Height+2 do
Begin
Reply := GetRow(Buffer1, Row, 0);
For Column := (Wind_Width-2) to (Wind_Width+2) do
Begin
If Buffer1[Column] = 0 then
Buffer1[Column] := 63;
end;
Reply := PutRow(Buffer1, Row, 0);
end;
If ASum>ASum1 then
{WRITE RESULTS TO SCREEN}
Begin
   GLAverage:=GLSum/ASum;
   GotoXY(StartCol,StartRow);
   Write(ASum:5:0);
   GotoXY(StartCol,StartRow+1);
   Write(GLAverage:5:0);
   GotoXY(StartCol,StartRow+2);
   Write(GLSum:5:0);
end;
Until Found=False;

Reply:=Setsrc(2);
Reply:=Setdst(1);
Reply:=Setcrt($E);
Reply:=Dmkfer;
Finish;
end;

{*************************}
Appendix C

ANALYSIS OF VARIANCE
(POLLEN CELLS)
The complete analysis of variance and the results obtained for pollen cells are shown in Tables C.1a, C.1b, C.1c. Please refer to Ref. [39] for a detailed explanation of analysis of variance.
### TABLE C.1a

**POLLEN CELLS (Analysis of Variance)**

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DEGREES OF FREEDOM</th>
<th>SUM OF SQUARES</th>
<th>MEAN SQUARE</th>
<th>F-RATIO</th>
<th>F - PROBABILITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable</td>
<td>1</td>
<td>972664.482</td>
<td>97266.482</td>
<td>39.921</td>
<td>0.000</td>
</tr>
<tr>
<td>Error</td>
<td>166</td>
<td>404453.422</td>
<td>2436.466</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>167</td>
<td>502719.904</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dep Var: AREA  
N=168  
Correlation Co-efficient = 0.194
<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DEGREES OF FREEDOM</th>
<th>SUM OF SQUARES</th>
<th>MEAN SQUARE</th>
<th>F-RATIO</th>
<th>F - PROBABILITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable</td>
<td>1</td>
<td>2546.35970</td>
<td>2546.3597</td>
<td>341.338</td>
<td>0.000000</td>
</tr>
<tr>
<td>Error</td>
<td>166</td>
<td>1238.34862</td>
<td>7.4588</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>167</td>
<td>3784.70832</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE C.1b**

**POLLEN CELLS (Analysis of Variance)**

Dep Var: DENSITY  N=168  Correlation Co-efficient = 0.677
**TABLE C.1c**

**POLLEN CELLS (Analysis of Variance)**

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DEGREES OF FREEDOM</th>
<th>SUM OF SQUARES</th>
<th>MEAN SQUARE</th>
<th>F-RATIO</th>
<th>F - PROBABILITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td></td>
<td>54889.137</td>
<td>54889.137</td>
<td>20.392</td>
<td>0.00</td>
</tr>
<tr>
<td>Residual</td>
<td>166</td>
<td>446830.768</td>
<td>2691.752</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>167</td>
<td>501719.905</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N=168  Correlation Co-efficient = 0.104
Appendix D

REGRESSION ANALYSIS (DNA)
The regression analysis and the results obtained for the comparison between the Vision system and the DADS system for the DNA analysis are shown in Tables D.1a and D.1b.

The co-efficient of correlation and the slope are given by the following formulae:

\[
\text{CO-EFFICIENT OF CORRELATION} = \frac{\sum XY}{\sqrt{\left(\sum X^2 \times \sum Y^2\right)}}
\]

\[
\text{INTERCEPT} = \left(\text{CO-EFFICIENT OF CORRELATION} \times \sigma_Y\right) / \sigma_X
\]
### TABLE D.1a

**REGRESSION ANALYSIS FOR DNA (Vision System)**

<table>
<thead>
<tr>
<th>SLIDE ID</th>
<th>DNA CONTENT (x)</th>
<th>x̄ = X</th>
<th>OPTICAL DENSITY (y)</th>
<th>ȳ = Y</th>
<th>X²</th>
<th>Y²</th>
<th>X*Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pisces</td>
<td>1.7</td>
<td>-11.02</td>
<td>40178</td>
<td>-127327.7</td>
<td>121.55</td>
<td>1.6x10⁻⁴⁰</td>
<td>1403788</td>
</tr>
<tr>
<td>Xenopus</td>
<td>6.3</td>
<td>-6.42</td>
<td>64775</td>
<td>-102733.7</td>
<td>41.28</td>
<td>1.1x10⁻⁴⁰</td>
<td>660064</td>
</tr>
<tr>
<td>M. Ling</td>
<td>10.2</td>
<td>-2.52</td>
<td>150661</td>
<td>-16844.7</td>
<td>6.38</td>
<td>2.8x10⁻⁴⁰</td>
<td>4931952</td>
</tr>
<tr>
<td>M. Ehre</td>
<td>32.7</td>
<td>19.97</td>
<td>414412</td>
<td>246906.2</td>
<td>399.00</td>
<td>6.1x10⁻⁴⁰</td>
<td>4931952</td>
</tr>
<tr>
<td></td>
<td>TOTALS</td>
<td>0</td>
<td>670023</td>
<td>0</td>
<td>568.21</td>
<td>8.8x10⁻⁴⁰</td>
<td>7038338</td>
</tr>
</tbody>
</table>

**MEAN**

- $\bar{x} = 12.72$
- $\bar{y} = 167505.7$
- $\sigma_x = 11.91$
- $\sigma_y = 148334.9$

**CO-EFFICIENT OF CORRELATION = 0.995**

**SLOPE = 12386.91**

**INTERCEPT = 9882.25**

The regression line for the Vision system is given by

$$y = 12386.91x + 9882.25$$
### TABLE D.1b
REGRESSION ANALYSIS FOR DNA (DADS System)

<table>
<thead>
<tr>
<th>SLIDE ID</th>
<th>DNA CONTENT (x)</th>
<th>x-x = X</th>
<th>OPTICAL DENSITY (y)</th>
<th>y-y = Y</th>
<th>X²</th>
<th>Y²</th>
<th>X*Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pisces</td>
<td>1.7</td>
<td>-11.02</td>
<td>501</td>
<td>-2438.5</td>
<td>121.55</td>
<td>5946282</td>
<td>26884.4</td>
</tr>
<tr>
<td>Xenopus</td>
<td>6.3</td>
<td>-6.42</td>
<td>969</td>
<td>-1970.5</td>
<td>41.28</td>
<td>3882870</td>
<td>12660.4</td>
</tr>
<tr>
<td>M Lingua</td>
<td>10.2</td>
<td>-2.52</td>
<td>2285</td>
<td>-654.5</td>
<td>6.38</td>
<td>428370.3</td>
<td>1652.6</td>
</tr>
<tr>
<td>M Ehreng</td>
<td>32.7</td>
<td>19.97</td>
<td>8003</td>
<td>5063.5</td>
<td>399.00</td>
<td>25639032</td>
<td>101143</td>
</tr>
</tbody>
</table>

**TOTALS**

<p>| | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50.9</td>
<td>0</td>
<td>11758</td>
<td>0</td>
<td>568.21</td>
<td>35896555</td>
<td>142340</td>
</tr>
</tbody>
</table>

**MEAN x = 12.72**

**MEAN y = 2935.5**

σₓ = 11.91

σᵧ = 2995.6

CO-EFFICIENT OF CORRELATION = 0.994

SLOPE = 245.51

INTERCEPT = -308.21

The regression line for the DADS system is given by

\[ y = 245.51x + (-308.21) \]
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