COMPUTING AND STATISTICAL DATA ANALYSIS IN 3D IMAGING AND ANALYSIS OF A HUMAN CHROMOSOME

AUTHOR: SHERMAN LEIGH IP (1018526)
UNIVERSITY COLLEGE LONDON (UCL)
MSCI PHYSICS
PHASM201: PHYSICS PROJECT (MASTERS LEVEL)
SUPERVISOR: PROFESSOR IAN ROBINSON
SUPERVISOR: DR. GRAEME MORRISON
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Chromosomes store and compress DNA using a series of loops and coils called nucleosomes and it was suggested that they form a 30 nm chromatin fibre. A series of serial section images of human chromosomes were taken using serial block face SEM. The objective of this project was to analyse a stack of serial block face SEM images, of a human chromosome, using computational tools to identify the external shape and find statistically significant internal structure in chromosomes, as well to model how the 30 nm chromatin fibres were arranged.

The images were statistically analysed and were segmented using a threshold in ImageJ. The marching cubes algorithm was programmed, in Processing, to produce a volume visual using the threshold images to see the external shape of the chromosome.

ImageJ maxima search was used to find significant internal structure. Evidence of 30 nm chromatin fibres may have been found because the same value of 30 nm corresponded to the mean distance to the nearest neighbour for each maxima. Each maxima was modelled as a sphere to see if they form coils and to estimate the number of base pairs in the chromosome.
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II. LITERATURE REVIEW

All living organisms are made up of basic biological units called cells. [1, p. 49] [2, p. 3] Organisms, like bacteria, can be unicellular, meaning made up of only one cell, while complex organisms, like humans, are made of many cells and are therefore multicellular. [1, p. 49]

Cells produce a good environment for chemical reactions to take place to sustain life of the organism. [2, p. 3] They replicate themselves by dividing to produce two genetically identical daughter cells. [1, p. 81] [2, p. 83] [3] This process is called mitosis which is part of the cell cycle. [1, p. 81] [2, p. 83] [3]

Figure 3 shows the structure of the cell. The cell surface, called the plasma membrane, is a partially permeable wall which separates internal chemical reactions with the environment outside the cell. [2, p. 4] The most important features inside the cell, for this project, are cytoplasm, centrioles and the nucleus. Cytoplasm is a solution of many substances in water, mainly proteins, in which many chemical reactions take place. [1, p. 49]

The nucleus is the largest structure inside the cell. [2, p. 11] The surface of the nucleus is the nuclear envelope, consisting of two membranes. [1, p. 66] [2, p. 11] There are some gaps in the nuclear envelope, called nuclear pores, and these controls the exchange of biological substances between the nucleus and cytoplasm outside the nucleus. [1, p. 66] [2, p. 11] Genetic materials, deoxyribonucleic acid (DNA) and chromosomes, are stored inside the nucleus, to prevent damage to it. [1, p. 64] [2, p. 12]

A molecule of DNA, in humans, is stored within 46 different chromosomes inside the nucleus. [1, p. 88] [2, p. 80] DNA can replicate itself perfectly, before cell mitosis, and pass on its genetic material to the two daughter cells created during cell mitosis. [1, p. 82] [2, p. 67] DNA is extremely important as it provides instructions to cells, for example what proteins to produce, and therefore determines the characteristics of the cell and organism. [1, p. 82] [2, p. 70]

DNA consists of polymers, meaning that they are made up of molecular structures. [2, p. 65] These molecular structures are called nucleotides. [1, p. 97] [2, p. 66] Nucleotides are made up of 3 substances, a pentose sugar called deoxyribose, a phosphate group and one of the 4 nitrogen containing bases: adenine (A), thymine (T), guanine (G) and cytosine (C), as shown in Figure 1. [1, p. 97] [2, p. 67] [4]

A polynucleotide strand can be formed by linking many nucleotides to make a long chain as shown in Figure 2. [4] [5, p. 198] DNA is made of two polynucleotide strands, running anti-parallel to each other, wound in a double helix with 10 nucleotides per complete turn, held together by hydrogen bonds between the complementary base pairs A-T and C-G, as shown in Figure 4. [4] [5, p. 198]
Figure 3 The internal features and structure of a typical animal cell. [2, p. 11]
It is the sequences of these base pairs which provide instructions for the production of proteins. [1, p. 98] [2, p. 70] A group of three nucleotides, called a triplet, codes for an amino acid. [1, p. 98] [2, p. 71] By linking amino acids together, proteins are produced. The length of DNA which produces a single protein is called a gene. [1, p. 98] The 64 possible combinations of triplets can code for 20 different amino acids. [1, p. 98]

By using the results from Rosalind Franklin, James Watson and Francis Crick in 1953 deduced the double helix structure of DNA, and this discovery was significant in how DNA can replicate itself perfectly every time. [2, p. 67] [4] The hydrogen bonds between the two polynucleotide strands can be easily broken so that the polynucleotide stands can unwind. [1, p. 97] Free nucleotides then can pair up with their respective complementary bases in each polynucleotide strand to form a new polynucleotide strand, as shown in Figure 5. [1, p. 97] [2, p. 67] [5, p. 3]

The result is two identical DNA, one new DNA was formed from the old DNA. [1, p. 97] [2, p. 67] [5, p. 3] This method of replication is known as semi-conservative replication because half of the new DNA molecules comes from the old DNA. [1, p. 97] [2, p. 67]

The complete set of genetic material, or DNA, of an organism is called a genome. [5, p. 200] A human genome contains about 3.2 billion base pairs, stored across 46 chromosomes. [5, p. 200]

There are two types of chromosomes, autosomes and sex chromosomes. [1, p. 134] [2, p. 80] There are two sex chromosomes, X and Y, and they determine the sex or gender of the organism. [1, p. 134] [2, p. 80] A male human will have an X and Y chromosome and a female will have two X chromosomes. [1, p. 134] [2, p. 80] The remaining 44 chromosomes are called autosomes and they always comes in homologous pairs, where one comes from the mother and another from the father of the organism. [2, p. 80]
Chromosomes are most visible in mitosis form in which the chromosomes are at their densest and in the process of replicating itself. [5, p. 243] A technique called DNA hybridization distinguish the chromosomes, in mitosis form, by ‘painting’ them different colours and arranging them in order. [5, p. 202] A karyotype, a photograph of all the human chromosomes is shown in Figure 6. [5, p. 203] [6]

![Figure 6](image)

*Figure 6* A karyotype of male human chromosomes. DNA hybridization is a technique used to ‘paint’ each homologous pairs of chromosomes different colours, making them distinguishable. [5, p. 203]

Another technique, to distinguish the different chromosomes, involves staining the chromosomes with a dye which produces very clear and regular bands on chromosomes. [5, p. 203] [7] It is not well understood why these bands are as regular but it as shown in Figure 8. [5, p. 203] [7]

The structure of a mitosis chromosome is as shown in Figure 7. [5, p. 243] The most important features are the two sister chromatids, which contain an identical DNA each, held together by the centromere, which contains a protein complex called kinetochore that is used to separate the two chromatids during mitotic. [5, p. 210]

Otherwise chromosomes can be invisible with the light microscopic when in interphase form. [5, p. 209] Cells goes through a cycle in which sometimes chromosomes are condensed and sometimes they are not. [5, p. 208]

![Figure 7](image)

*Figure 7* (Left) A scanning electron micrograph of a mitosis chromosome. (Right) A drawing of a mitosis chromosome which the chromatid and centromere were labelled. [5, pp. 209, 243]

The cell cycle is the period of time from the birth of a daughter cell to the replication of itself. [2, p. 83] At the start of the cycle called interphase, the cell has just been formed and separated from its sister. [1, p. 81] [2, p. 83] The cell grows to its normal size and carries out normal functions just like its parent. [2, p. 83] At this stage, the interphase chromosomes exist as long tangled threads of a single DNA. [5, p. 209] The cell spends most of its time in interphase and this is when genetic information is being read out. [5, p. 209] However when a signal is received for the cell to divide, the DNA replicates so that the nucleus contains two strands of identical DNA. [2, p. 83]
Figure 8 By staining the chromosomes, very clear bands can be seen. The 24 chromosomes shown are lined up by their centromere. [5, p. 203]
Figure 9 The stages of division of a mitosis cell. a) Prophase: the replicated centrioles (surrounded by preicentriolar material to form centrosomes) move to opposite ends of the nucleus. b and c) Metaphase begins and the centrioles create spindles, made of protein microtubules, to organise the mitosis chromosomes. d and e) Anaphase: the centrioles pull the chromatids apart and drag them to the poles. f) Telophase: The nuclear envelope reform to segregate the nucleus. The chromatids in each nuclei begins to uncoil to form interphase chromosomes. [3]
Mitosis follows interphase and this is when the nucleus starts to divide to give birth to two identical daughter nuclei. [1, p. 81] [2, p. 84] Mitosis can be split into 4 continuous stages, prophase, metaphase, anaphase and telophase, as shown in Figure 9. [1, p. 81]

At prophase, the centriole replicates and starts moving to opposite ends, or the poles, of the nucleus. [2, p. 85] [3] The chromosomes replicate to form sister chromatids joined in the middle by a centromere, where each chromatids contains a molecule of DNA. [3] [5, p. 209] The nuclear envelope breaks down to allow the chromosomes for movement. [2, p. 85] [3]

Once the pair of centrioles have reached to the poles, metaphase begins and they create the spindle apparatus, made of protein microtubules which attach to each centromere, organising the mitosis chromosomes such that they line up at the equator of the nucleus. [2, p. 85] [3] [5, p. 208] At this stage, this is when chromosomes are most visible. [1, p. 82] [5, p. 208]

Then quite suddenly, the centrioles pull the chromatids apart by their centromeres and drag them to the poles by the spindles. [1, p. 82] [3] This process is called anaphase. [1, p. 82] [2, p. 85] [3]

At telophase, there are 46 chromatids and a centriole at each pole. [2, p. 85] The nuclear envelope reform at each pole, creating two nuclei. [2, p. 85] [3] The remains of spindles break down and cytokinesis, the separation of cells, begins. [2, p. 85] [3] The chromatids in each nuclei beings to uncoil, to form interphase chromosomes, to start interphase. [1, p. 82] [2, p. 85] [3] [5, p. 208]

Chromosomes in the cell cycle do a good job of not only organising replicated DNA so that each daughter cell is genetically identical, but also storing and compressing the human genome into a tiny volume. [5, p. 202] The nucleus is about 6 μm long in diameter but laying out the human genome in a straight line would reach 2 meters long. [5, p. 202]

Chromosomes are mostly made up of proteins, called histones, and DNA. [5, p. 211] The complex of histones and DNA is called chromatin. [5, p. 211] It was discovered in 1974 that DNA is packaged into chromosomes by nucleosomes. [5, p. 211] [6] Nucleosomes are a series of loops and coils in which DNA is tightly wrapped around histones. [5, p. 211] [6] Because there are about 30 million nucleosomes in a human cell, on average there about 650 thousand nucleosomes per chromosome. [5, p. 211]

![Figure 10 DNA is wrapped around histones to form nucleosomes. The nucleosomes form a 30 nm chromatin fibre to produce a very dense mitotic chromosome. [5, p. 244]](image-url)
It can be shown by treating nucleosomes such that they fold out, they appear to be like ‘beads on a string,’ as shown in Figure 10. [5, p. 211] [6] [8] These ‘beads’ are the nucleosome cores which consist of an octameric of two of each of these histones: H2A, H2B, H3, H4. [5, p. 211] [6] [8] [9] These nucleosome cores have diameter of about 11 nm and DNA wraps itself around each nucleosome core 1.7 times in a left handed coil, which is 147 base pairs, as shown in Figure 11. [5, p. 212] [8] [9] The strands of DNA between nucleosome cores is called linker DNA, which consists of between 0 to 80 base pairs. [9]

![Figure 11 DNA is wrapped around each nucleosome core, consisting of histones, 1.7 times in a left handed coil. [6]](image1)

It was suggested that nucleosomes form a 30 nm diameter chromatin fibre as shown in Figure 10. [5, p. 216] [9] It is not well known how the nucleosomes are arranged or packed in the 30 nm chromatin fibre but many experimental data have produced many suggestions and models about how nucleosomes are arranged in the 30 nm chromatin fibre. [5, p. 217] An experiment using x-ray crystallography suggests that nucleosomes are arranged in a zigzag model while another experiment using cryoelectron microscopy suggests a solenoid arrangements in the 30 nm chromatin fibre. [5, p. 217] [9] [10]

In the zigzag model, the linker DNA remains extended and straight throughout the whole arrangement in the chromatin fibres. [9] The nucleosomes are arranged such that they produce a zigzag formation in the chromatin fibres as shown in Figure 12 and Figure 13. [9] [10] In contrast the linker DNA in solenoid arrangements bends to produce a solenoid structure as shown in Figure 12 and Figure 14. [9] [10]

![Figure 12 (Left) Nucleosomes in an extended state. The linker DNA can remain extended and straight to form a zigzag formation (bottom right) or bends to form a solenoid (top right). [10]](image2)

![Figure 13 The zigzag formation of the chromatin fibres on one of the two chromatids using data from an x-ray crystallography. [5, p. 217]](image3)
However there is still some uncertainty in the arrangement of nucleosomes in chromatin fibres. [5, p. 217] One controversy is that the length of linker DNA varies throughout the arrangement and each model should be able to accommodate it. [9] The solenoid arrangements can easily accommodate for variable DNA length, but on the other hand there are some unanswered questions on how the zigzag model can deal with variable linker DNA length, whenever the size of the nucleosome fibres varies with linker DNA length. [9]

Many of the small scale biological substances discussed can be viewed using a range of equipment. [2, p. 3] One of the earliest equipment used is the light microscope. [2, p. 3] It works by focusing light onto the specimen, the light penetrates the specimen and then is refocused onto the eyepiece. [1, p. 51] The specimen must be thin enough to allow light to penetrate it and should be stained to allow transparent biological features to become visible under the light microscope. [1, p. 51] Often the specimens are submerged in liquid between a glass slide and a cover slip. [1, p. 51] The maximum resolution of the light microscope is about 200 nm, which is enough to see mitotic chromosomes. [1, p. 52] [2, p. 8]

To view objects smaller than 200 nm, the transmission electron microscope (TEM) or the scanning electron microscope (SEM) can be used. [1, p. 51] [2, p. 9] TEM works in the same principle as the light microscope but electrons are used instead of light, producing a monochromatic image where the brightness of a pixel corresponds to the energy of the electron detected in the fluorescent screen. [1, p. 51] Electrons are fired at the specimen and are focused using electromagnets. [1, p. 51] The specimen must be ultra-thin, thin enough for a fraction of electrons to penetrate it, and should be stained using metal ions. [1, p. 51] [11] Particular parts of the cell and other biological substances take in the metal ions, which are large and positively charged, so that they scatter electrons and will appear dark in the image. [1, p. 51] [2, p. 10] The specimen must also be in a vacuum to prevent air particles scattering electrons. [1, p. 51] [2, p. 11] As a result the specimen must be dehydrated therefore only dead material can usually be viewed in an electron microscope. [1, p. 51] [2, p. 11] In SEM, backscattered or reflected electrons are detected instead of transmitted electrons. [1] [2, p. 52] [12]

A three-dimensional (3D) image of the specimen can be reconstructed using a continuous parallel series of many TEM images of ultra-thin serial sections of the specimen. [13] Serial sections can be obtained by placing the specimen in an ultramicrotome and using a diamond knife to cut the specimen. [13] [14] Each image of serial sections can be stacked together as a series using the appropriate software to produce a 3D reconstruction of the specimen. [13] This method of serial
sectioning imaging is called serial-section TEM (ssTEM). [14]

ssTEM, however, can be very time consuming and labour intensive to obtain many fragile serial sections of a specimen in the ultramicrotome [11] [14]. By cutting the specimen using a diamond knife, the continuity between each section is destroyed and a lot of work is needed to precisely align each image of serial sections of the specimen. [14]

Another method of serial sectioning imaging is called serial block face SEM (SBFSEM). [11] [13] [14] Instead of viewing each serial section under a TEM, the surface of the specimen is viewed using a SEM. [15] An ultra-thin serial section is extracted, or sectioned, and discarded from the top of the specimen using a diamond knife which exposes a freshly cut block face. [15] The new face is then viewed and imaged using a SEM. [11] [15] This is done many times to obtain a stack of SEM images of the specimen surfaces after each serial section removal, as shown in Figure 15. [11] [15]

The main advantage of SBFSEM is hundreds of images can be generated in a few days and there is no need to handle fragile serial sections, they are simply discarded. [11] [14]

Experiments were done by J. Rouquette et al. to obtain a 3D reconstruction of chromatin arrangement of an interphase nuclei of rat hepatocytes using ssTEM and SBFSEM. [15] The specimen was treated, in addition to staining and dehydration as mentioned before, by embedding it in a plastic resin. [15]

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**Figure 15** SBFSEM consist of a series of sectioning of the surface of the specimen and imaging the new exposed surface using SEM. [11]

There has been some work on using SBFSEM to image human interphase chromosomes. The objective of this project was to analyse SBFSEM images using computational tools to identify the external shape and find statistically significant internal structure of chromosomes. Depending on the scale of the images, it may be possible to look at how nucleosomes are arranged in 30 nm chromatin fibres and also how the 30 nm chromatin fibre is coiled up and compressed in an interphase chromosome.
III. DEVELOPMENT OF A 3D RENDERING PROGRAM

The objective of this section was to render a 3D view of a chromosome, using a stack of SBFSEM images of a chromosome, using open source software.

Using open source programming languages was an advantage because they are free to be used and shared so no costs were involved, compared to commercial software and programs which can require yearly contracts. [16] [17]

ImageJ and Fiji are open source software, written in Java, designed for scientific image processing. [18, pp. 1, 8] They support stacks, meaning it can process images obtained from SBFSEM, making it very suitable for this project. ImageJ and Fiji also have many procedures, functions and plugins for image processing, for example cropping, noise removing and thresholds. [18] Fiji is just an extension of ImageJ with more plugins and features, making Fiji more useful to work with. [18, p. 101]

Processing is an open source programming language built on Java. [17] The main purpose of Processing is to make a suitable environment for programmers to produce computer graphics. [19, p. 1] Processing makes use of OpenGL compatible graphics card, which enables good rendering of 2D and 3D graphics. [19, p. 528]

ImageJ and Processing were used together to produce a 3D rendering of a chromosome using a stack of SBFSEM chromosome slices.

A threshold interval was set to select pixels, from the data, with certain intensity values to produce binary images. In a binary image, each pixels can only have two possible intensity values. An intensity value of one was given to pixels in the threshold interval, otherwise an intensity value of zero was given. This method of abstraction or segmentation was used to identify the shape of the chromosome.

All 22 slices from the SBFSEM images of a human chromosome are shown in Figure 17. The colour depth of these images was 16-bit grayscale, meaning each pixel can have 65546 different intensity values. Each slice had dimensions of 153 x 168 pixels where each pixel represent a scale of 27 nm x 27 nm and each slice was separated by 100 nm.

Figure 16 A screen shot of a simple Brownian motion simulation in Processing.
Figure 17: All 22 slices of a chromosome using SBFSEM where the 1st slice is on the far top left and the 22nd slice is the last image on the fourth row. The first sign of the chromosome is on slice 10. There were other signals in the images including a very bright object from slice 14 to slice 19. There was also another portion of a chromosome in slice 22.

Figure 18: The histogram of all the intensity values of all slices. (x-axis is the intensity values and the y-axis is the frequency.)

Outside the context of this project, it is meaningless to have 16-bit grayscale as 8-bit grayscale (256 possible intensity values) is sufficient enough to cover the full range of grayscale a human eye can distinguish.

[20, p. 118] However a 16-bit grayscale image in this project was extremely useful as each pixel contained more possible intensity values, and therefore more information, however the image will need more memory space to be stored. [18, p. 17]

The histogram in Figure 18 shows the distribution of each pixel intensity values in the stack. By inspection, the histogram looked very Normally distributed however a closer look showed there was a slight positive skewness in the distribution.

It was hypothesised that the skewness was the result of many lower intensity pixels in the background compared to the not many higher intensity pixels in the chromosome. Using the ImageJ histogram function, \( N = 10 \) samples of chromosome and background
intensity values were taken extracting the count $n_i$, sample mean $\bar{x}_i$ and the sample standard deviation $s_i$ of the intensity values, as shown in Figure 19.

![Figure 19 Using ImageJ histogram function to extract the count, sample mean and sample standard deviation of the intensity values in the selected area (yellow).](image)

The population mean and population standard deviation, $\mu$ and $\sigma$ respectively, were estimated using the estimators shown in Equation 1 and Equation 2. [21, p. 195] [22, p. 194]

Because the Normal distribution is a good approximation to the $t_v$ and $\chi^2_v$ distribution for large degrees of freedom $v$, the 95% confidence interval of $\mu$ and $\sigma$ were worked out using Equation 3 and Equation 4 respectively, where $\Phi^{-1}$ is the inverse cumulative distribution function of a standard Normal distribution. [21, pp. 195-198] [22, p. 194]

The estimated population mean and population standard deviation with their respective 95% confidence limits are shown in Figure 20.

<table>
<thead>
<tr>
<th></th>
<th>Chromosome</th>
<th>Background</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>17804 ± 24</td>
<td>16059 ± 5</td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td>647 ± 17</td>
<td>676 ± 4</td>
</tr>
</tbody>
</table>

![Figure 20 95% confidence limits of the population mean and standard deviation chromosome and background intensity values.](image)

Figure 20 shows more than sufficient evidence that the mean intensity for the background and chromosome were significantly different. This suggested that there are two distinct signals contributing to the histogram, such that the positive skewness in the histogram was due to the intensity values of the chromosome with mean 17804 and standard deviation 647.

A threshold interval was set centred at 17804, which was the estimated population mean of the chromosome intensity value, with a variable width. The events T, B and C were defined to be:

- T = The pixel intensity value is in the threshold interval
- C = The pixel represent the chromosome
- B = The pixel represent the background

Following from these definitions of these events, and assuming the intensity values of the background and chromosome pixels were Normally distributed with parameters from their estimations in Figure 20, the probability of a chromosome pixel or background pixel will be in a threshold interval, \( P(T|C) \) and \( P(T|B) \) respectively, were calculated as shown in Figure 21 and plotted in Figure 22.

| \( P(T|C) \) | Threshold lower limit | Threshold upper limit | \( P(T|B) \) |
|--------------|-----------------------|-----------------------|--------------|
| 70%          | 17133                 | 18475                 | 5.6%         |
| 75%          | 17059                 | 18548                 | 6.9%         |
| 80%          | 16974                 | 18633                 | 8.8%         |
| 85%          | 16872                 | 18736                 | 11.5%        |
| 90%          | 16739                 | 18869                 | 15.7%        |
| 95%          | 16535                 | 19073                 | 24.1%        |
| 99%          | 16136                 | 19471                 | 45.5%        |

Figure 21 \( P(T|C) \) and \( P(T|B) \) as the threshold centred at 17804 varies its width.

Figure 21 showed that increasing \( P(T|C) \) will also increase \( P(T|B) \). It was a trade-off between obtaining as many chromosome pixels in the threshold as possible and obtaining as least background pixels in the threshold as possible. The threshold was set at 16974 - 18633 because \( P(T|B) \) was under 10% while \( P(T|C) \) is at 80%.

The assumption that the distribution of the intensity values, for the chromosome and background, are Normally distribution was a good assumption, but unjustified because the histogram looked Normal but not tested to see if the data fitted with the Normal distribution. A \( \chi^2 \) goodness of fit hypothesis test could be conducted to test this assumption [23, p. 103] however it would be fruitless. The Normal distribution was only used to estimate the proportion of background or chromosome pixels which will be in the threshold interval.

A more useful statistics would be the portion of pixels in the threshold which are background pixels \( P(B|T) \) rather than \( P(T|B) \). Bayes’ theorem could be used to calculate \( P(B|T) \) as shown in Equation 5. [21, p. 20]

\[
P(B|T) = \frac{P(B)P(T|B)}{P(T)}
\]

The main problem with Equation 5 was that \( P(B) \), the proportion of pixels which are background in the stack, was needed. \( P(B) \) was estimated later on after analysing the threshold images. The threshold images are as shown in Figure 23. As expected, there were a few background pixels in the images. But by using the despeckle function and some manual editing in ImageJ, these were removed and the binary stack of the chromosome were restored, as shown in Figure 24.
Figure 23 The result of a threshold with the interval 16974-18633.

Figure 24 The result of the despeckle function. The slices were also cropped to only display the chromosome.
By treating each threshold pixel as a cube with dimensions 27 nm x 27 nm x 100 nm, the volume of the chromosome was estimated to be about 2.84 μm³.

| P(T|C) | P(T|B) | P(T) | P(B|T) |
|-------|-------|------|-------|
| 70%   | 5.6%  | 7.1% | 5.4%  |
| 75%   | 6.9%  | 8.4% | 5.7%  |
| 80%   | 8.8%  | 10.0%| 6.1%  |
| 85%   | 11.5% | 12.3%| 6.5%  |
| 90%   | 15.7% | 15.9%| 6.8%  |
| 95%   | 24.1% | 23.0%| 7.2%  |
| 99%   | 45.5% | 42.2%| 7.4%  |

Figure 25 The proportion of threshold pixels which are background pixels P(B|T) were worked out for each P(T|C)

From the binary stack, P(B) was estimated to be 6.9% and this was used together with Equation 5 to work out P(B|T) as shown in Figure 25. For the chosen threshold interval, P(B|T) was worked out to be 6.1%. This meant 6.1% of the threshold pixel will be background and this seemed to be a reasonable result.

These threshold images were then used to produce a 3D render of the chromosome using voxels. A voxel is a 3D equivalent of a pixel, such that a voxel is a cube instead of a square and a 3D object can be made up of many voxels. [24]

Firstly using Processing, each binary slices were analysed and the intensity values were stored in a 3D Boolean array, as shown in Figure 26. Then for each true value in the 3D Boolean array, a cube was drawn at the corresponding position as shown in Figure 27. The resulting render is as shown in Figure 30.

The first problem was that the program had a slow frame rate, of about 30 frames per second (fps). The main cause for such a slow program was because too often too many objects were being drawn.

In the 3D render of the chromosome, only voxels on the surface of the chromosome could be seen. Therefore voxels drawn inside the chromosome were meaningless because they cannot be seen. An algorithm was written to remove voxels with 6 connecting neighbouring voxels as shown in Figure 28. Because these hidden voxels were removed, the computer drawn less voxels and yet produced the same result.

At every frame, the program had to calculate the geometry of each voxel for it to be drawn. [25] This kind of algorithm is called immediate mode. [25] A faster way to render the chromosome was to work out the geometry of each voxel once and save it in memory, called a Vertex Buffer Object in lower-level OpenGL syntax, and draw it from memory at each frame without recalculating the geometry of each voxel. [25] This style of rendering is called retained mode and the code snippet is as shown in Figure 29. [25]

The final result resulted in an identical 3D render of the chromosome but at a frame rate of about 60 fps.
// for every pixel in the image  
for (int i=0; i<imageArray.get(z).pixels.length; i++)
    if (imageArray.get(z).pixels[i]==color(255)){ // if that pixel is white 
        pixelGrid[x][y][z] = true; // put true in the pixelGrid
    } // end if
    else{
        pixelGrid[x][y][z] = false; // else put false
    } // end else
    // increase x and y co-od accordingly 
    x++;
    if (x==imageWidth){
        x=0;
        y++;
    } // end if 
} // end for

Figure 26 A code snippet which analyses the pixels on a slice. It puts a true value for white pixels and a false value otherwise in a 3D Boolean array called pixelGrid.

// for every slice
for (int z=0; z<nSlices; z++)
    // for every pixel
    for (int y=0; y<imageHeight; y++)
        for (int x=0; x<imageWidth; x++)
            if (pixelGrid[x][y][z]==true){ // if the pixelGrid contain true
                fill(0); // set fill to black
                stroke(0,255,0); // set stroke to green
                // draw box at corresponding co-od 
                pushMatrix();
                translate(5*(x-imageWidth/2),5*(z-nSlices/2),5*(y-imageHeight/2));
                box(5);
                popMatrix();
            } // end if
    } // end for
} // end for

Figure 27 A code snippet which goes through all entries in pixelGrid and draws a voxel for every true entry.
for (int z=0; z<nSlices; z++){
    for (int y=0; y<imageHeight; y++){  
        for (int x=0; x<imageWidth; x++){

            //if the pixel is on the border, the pixel remains unchanged  
            if((x==0||x==imageWidth-1)||y==0||y==imageHeight-1||z==0||z==nSlices-1){  
                pixelGridHollow[x][y][z] = pixelGrid[x][y][z];
            }
        }
    }
}

Figure 28 A code snippet which goes through all the entries in pixelGrid and saves them in another 3D Boolean array pixelGridHollow but true values with 6 neighbouring true values were converted to false.
// chromosome is a group of voxel
chromosome = createShape(GROUP);

// for every slice
for (int z=0; z<nSlices; z++){
    // for every pixel
    for (int y=0; y<imageHeight; y++){
        for (int x=0; x<imageWidth; x++){
            // if the pixelGrid contain true save box at corresponding co-od
            if (pixelGridHollow[x][y][z]==true){
                PShape voxel;
                voxel = createShape(BOX,5);
                voxel.setFill(color(0)); // fill black
                voxel.setStroke(color(0,255,0)); // stroke green
                // translate voxel
                voxel.translate(5*(x-imageWidth/2),5*(z-nSlices/2),5*(y-imageHeight/2));
                chromosome.addChild(voxel); // add voxel to chromosome
            }
        }
    }
}

*Figure 29 (14) A code snippet which goes through all entries in pixelGridHollow but saves a voxel as a PShape, instead of drawing it, for every true entry.*
Figure 30 A 3D voxel render of a chromosome using voxels.
Voxels do produce a good 3D render of the chromosome however it was very crude to approximate a chromosome into many cubes. More work was done to obtain more details about the shape of the chromosome.

One way was to use the marching cubes algorithm. This algorithm approximates voxels into a triangular mesh and this produced a more detailed shape for the chromosome. [24] [26]

The marching cubes algorithm works by analysing 4 neighbouring voxels at a time, throughout the binary stack, and draws a polygon according to the values of the 4 voxels. [24] [26] Because each voxel can only have 2 possible values, there are a possible of 256 possible polygons to be drawn as shown in Figure 31. [24] [26]

It was more convenient and more organized to start using object oriented programming to program the marching cubes algorithm. Figure 33 shows the class definitions of the ‘marching cube,’ called Cube, and of a polygon to be drawn, called Triangle, with their corresponding member variables and methods.

Knowing which polygon to draw was tackled by using a lookup table, written by Paul Bourke (see [26]), containing the vertices for all 256 possible polygons, with a lookup index corresponding to an 8-bit number where each bit shows the Boolean value of the voxel, as shown in Figure 32. [26] The code snippet used for working out the lookup index and rendering the corresponding polygon is shown in Figure 34.

Figure 37 and Figure 38 shows a wire frame and coloured filled render using the marching cubes algorithm respectively. The results are very good because the shape of the chromosome was more defined and less ‘voxelated’.

The main problem was that the colour in the coloured filled render of the chromosome was very flat and many multi-coloured spotlights were used to make the render look as 3D as possible. This was because the normal for each polygon were not defined, therefore light interacted with each polygon in the same way, regardless of the orientation of each polygon.

The normals were calculated using the cross product between two vertices of the polygon as shown in the code snippet in Figure 35. This resulted in an image in Figure 39, which clearly shows the vertices and edges of the chromosome, making it look 3D. However because the normals were defined using the cross product, the normals were perpendicular to the surface of the polygon, instead to the original surface. [26] This created a very triangular and unsmooth render of the chromosome.

Bourke’s suggested approach was to use a weighted average of normals of the polygons sharing its vertex, where the weight is the reciprocal of the area of the polygon. This meant smaller polygons have greater weight because they may occur in regions of high surface curvature. [26] The code snippet using Bourke’s approach is shown in Figure 36.

By averaging the normals, Figure 40 was produced. The vertices and edges were certainly much smoother than before.

Figure 41 shows the centromere and one of the chromosome’s chromatid labelled in the 3D render of the chromosome.
Figure 31 The 256 possible polygons which could be drawn in the marching cubes algorithm. These can be generalised into 15 polygons. The spheres represent a true value voxel in the binary stack of the chromosome. [27]

Figure 32 The Boolean value at each voxel was saved as an 8-bit binary number. This was then used to look up what polygon to draw. [26]
Class Cube{
    MEMBER VARIABLES
    • PVector position
        o Position vector of the centre of the marching cube
    • ArrayList <Triangle> triangleArray
        o Contains an ArrayList of triangle objects in the marching cube
    CONSTRUCTOR ARGUMENTS
    • int x, int y, int z
        o position = new PVector(x,y,z);
    METHODS
    ➢ void addTriangles(verticesIndex)
        o Add a new triangle object, with vertices according to the verticesIndex, to triangleArray
    ➢ PShape getShape()
        o Returns the PShape of all the triangles in the marching cube
    ➢ ArrayList <Triangle> getTriangleArray (required for recalculateNormal())
        o Returns triangleArray
    ➢ void recalculateNormal()
        o Recalculate the normals of all the triangles in the marching cube by taking the weighted average of the normals
}

Class Triangle{
    MEMBER VARIABLES
    • PVector a,b,c
        o Position vectors of the vertices of the triangle
    • PVector cube_centre
        o Position vector of the centre of the marching cube the triangle is in
    • PVector n
        o Normal vector to the surface of the triangle
    • PVector nBar
        o Weighted average normal
    • float area
        o The area of the triangle
    CONSTRUCTOR ARGUMENTS
    • verticesIndex, int x, int y, int z
        o Calculates a,b,c according to verticesIndex
        o Cube_centre = new PVector(x,y,z)
    METHODS
    ➢ PShape getShape()
        o Returns the PShape of the triangle
    ➢ PVector getNormal()
        o Returns n (required for recalculateNormal())
    ➢ void newNormal(PVector parameter)
        o Set nBar = parameter
    ➢ float getArea()
        o Returns area (required for recalculateNormal())
    ➢ PVector[] getVerticesVector()
        o Returns an array of position vectors of the 3 vertices of the triangle (required for recalculateNormal())
    ➢ boolean isShareVertex(Triangle that)
        o Returns true if this triangle shares at least one vertex with that triangle (required for recalculateNormal())
}

Figure 33 Class definitions of the marching cube, called Cube, and Triangle.
```java
shape = createShape(GROUP);

// for every slice
for (int z=0; z<=nSlices; z++)
{
    // for every pixel in the slice
    for (int y=0; y<=imageHeight; y++)
    {
        for (int x=0; x<=imageWidth; x++)
        {
            // declare a variable called verticesIndex
            // this variable indicates which vertices has a true value...
            // ...in the pixelGrid as an 8 bit binary number
            int verticesIndex = 0;
            // each digit in the binary number represent a vertex

            // work out verticesIndex by analysing each corner
            if (pixelGrid[x][y+1][z]==true){ //0
                verticesIndex += 1;
            }
            if (pixelGrid[x+1][y+1][z]==true){ //1
                verticesIndex += 2;
            }
            if (pixelGrid[x+1][y+1][z+1]==true){ //2
                verticesIndex += 4;
            }
            if (pixelGrid[x][y+1][z+1]==true){ //3
                verticesIndex += 8;
            }
            if (pixelGrid[x][y][z]==true){ //4
                verticesIndex += 16;
            }
            if (pixelGrid[x+1][y][z]==true){ //5
                verticesIndex += 32;
            }
            if (pixelGrid[x+1][y][z+1]==true){ //6
                verticesIndex += 64;
            }
            if (pixelGrid[x][y][z+1]==true){ //7
                verticesIndex += 128;
            }

            // look up what polygon to draw using vertices...
            // ... and draw it at x,y,z
            drawPolygon(x,y,z,verticesIndex);
        }
    }
}
```

Figure 34 A code snippet used for working out a lookup index, called verticesIndex. This was then used to lookup what polygon to draw in the marching cube.
//CONSTRUCTOR
//Parameters: 3 edge indexes,
//and which edges the vertices of the triangle should be on
Triangle(int index1, int index2, int index3, float x, float y, float z) {

    //convert the edge indexes to position vectors
    a = getEdgeCo(index1);
    b = getEdgeCo(index2);
    c = getEdgeCo(index3);

    //get the position vector of the center
    this.cube_center = new PVector(x, y, z);

    //work out the normal vector (n) and the area
    PVector r1, r2;
    r1 = PVector.sub(b, a);
    r2 = PVector.sub(b, c);
    n = r1.cross(r2);
    area = n.mag() / 2;
    n.normalize();
    nBar = n; // nBar will be the averaged normal later on

    //default colouring
    wantStroke = false; // no stroke
    wantFill = true; // fill
    stroke = color(0, 255, 0); // green stroke
    fill = color(255); // white fill
} // end constructor

Figure 35 A code snippet, from the Triangle class constructor, used to work out and define the normal of the instantiated triangle.
For each triangle in the cube

```java
for (int i=0; i<triangleArray.size(); i++) {
    // Prepare this triangle and declare statistical variables
    Triangle triangle = triangleArray.get(i);
    PVector nBar; // Average normal vector
    nBar = new PVector(0, 0, 0);
    float weight = 0; // The sum of all weights used for averaging

    // Compare this triangle to the triangles in neighbouring cubes
    // For every neighbouring cube
    for (int u=xLower; u<=xUpper; u++) {
        for (int v=yLower; v<=yUpper; v++) {
            for (int w=zLower; w<=zUpper; w++) {

                // Get all the triangles in the neighbouring cube
                ArrayList<Triangle> thatTriangleArray =
                    cubeArray[x+u][y+v][z+w].getTriangleArray();

                // For every triangle in the cube
                for (int j=0; j<thatTriangleArray.size(); j++) {
                    Triangle that = thatTriangleArray.get(j);
                    // If this triangle share a vertex with that triangle
                    if (triangle.isShareVertex(that)) {
                        // Update the normal statistics with the reciprocal area as the weight
                        nBar.add(PVector.mult(that.getNormal(), 1/that.getArea()));
                        weight += 1/that.getArea();
                    }
                }
            }
        }
    }

    // Work out the mean normal and update that triangle’s normal
    nBar.mult(1/weight);
    triangle.newNormal(nBar);
}
```

*Figure 36 A code snippet from the calculateNormal() method in the Cube class. It calculated the weighted averaged normal for each triangle in the instantiated cube.*
Figure 37 A wire frame render of the chromosome using the marching cubes algorithm.
Figure 38 A render of the chromosome using the marching cubes algorithm with no defined normals.
Figure 39 A render of the chromosome, using the marching cubes algorithm, with normals defined perpendicular to each triangle.
Figure 40 A render of the chromosome, using the marching cubes algorithm, with each normal defined as the weighted average normal for each polygon it shares it vertices with.
Figure 41 A render of the chromosome, using the marching cubes algorithm, with the centromere and one of its chromatid identified.
IV. ANALYSIS OF INTERNAL STRUCTURES

Another stack of SBFSEM slices of a chromosome was worked on in the project, as shown in Figure 44. The stack had 122 slices which had dimensions of 266 x 314 pixels and, again, had a 16-bit grayscale colour depth. Each pixels represent a scale of 11 nm x 11 nm and the slice separation is 20 nm. Platinum blue was used to dye the chromosome, embedded in an epoxy resin, which meant the chromosome will show up with low intensity in the obtained images as shown in Figure 44.

It was noted that a few slices at the beginning of the stack were distorted, as shown in Figure 44 by a red arrow. It was also noted there were noticeable consistent lines in the orthogonal views of the stack, as shown in Figure 45, and it looked as though each imaging of each surface took up more than just one slice. This may be the result of alignment issues during sectioning of the specimen using a diamond knife. However these problems were ignored because it would be extremely difficult to remove such distortions and alignment issues.

Figure 42 shows the histogram of all the intensities in the stack. From inspection, the distribution was very negatively skewed with 3 maximas. This suggested there may be 3, not necessary Normally distributed, random variables contributing to the overall distribution. There were 3 distinct signals in the stack: the background, the chromosome and the internal & external features of the chromosome as shown in Figure 46.

A sample of the background, chromosome and the internal features intensities were taken. Their population mean and population standard deviation were estimated as shown in Figure 43.

Figure 43 showed more than sufficient evidence that the 3 objects’ mean intensity values were significantly different. Their estimated means were also at least 4 standard deviations away from each other, making them even more significantly different.

The threshold interval were chosen to be 2 standard deviations away from the mean of the chromosome and the internal features intensity values. As a result, the threshold was set at 11920 - 23662, to obtain the external shape of the chromosome, results in Figure 47.

After threshold, it was noticed that quite a few features in the slices were fused together, causing them to look like one single object. Using the watershed function in ImageJ, these were separated as shown in Figure 52. The threshold binary stack was edited using the despeckle function to remove noise, the medium function to smooth the edges, and some manual editing in ImageJ, as shown in Figure 48.
Figure 44 Slices 1, 6, 11, ..., 121 of a human chromosome taken using SBFSEM. A red arrow on slice 26 points to a distortion.
Figure 45 Orthogonal views (right and bottom) of the stack viewed from top. (top left).
Figure 46 Three thresholds were applied to the stack to segment the chromosome (blue) and its features (red) and the background (green) with the following intensity values:

- Blue: 11920 – 15824
- Red: 17142 – 23662
- Green: 27674 - 31146
Figure 47 A binary stack as a result of a threshold set at 11920 – 23662 to segment the external shape of the chromosome.
Figure 48 The binary stack in Figure 47 was manually edited using a medium filter, watershed algorithm and some manual editing.
Figure 49 The binary stack in Figure 48 was used to crop out the chromosome intensity values from the original stack in Figure 44. The stack was also inverted so that the chromosome will show up as high intensities.
Figure 50 A 3D render of a human chromosome using the binary stack in Figure 48.
By using the binary stack in Figure 48, a 3D render of the chromosome was made using the marching cubes algorithm, as shown in Figure 50. The centromere and chromatid were easily identified, as shown in Figure 51. By summing the volume of all the threshold voxels, the volume of the chromosome was estimated to be about 3.95 μm³. The binary stack was used to cut out the data with the shape of the chromosome as shown in Figure 49.

The scale of the slices was 11 nm per pixel and this unfortunately meant individual nucleosome, of diameter 11 nm, cannot be resolved because the scale is not small enough. It also meant the 30 nm chromatin fibre would be about 3 pixels thick in these images, which could be too small to resolve. However it may be possible to see structure, of size larger than the 30 nm chromatin fibre, in these images.

ImageJ has a function called ‘Find Maxima’ which finds the positions of maxima in an image. [28] By using the ‘Find Maxima’ function on an inverted cropped stack, Figure 49, maxima were found which should represent parts of the chromosome which are dense. The noise tolerance, a value equal are greater than zero, could be adjusted such that maxima were only accounted for if its intensity is more than this value from the ridge to a higher maximum. [29]
As a test, slice 59 (Figure 53) was used to see what results the ‘Find Maxima’ function could produce. An ImageJ macro was written to see how the number of maxima found, using the ‘Find Maxima’ function, varied with noise tolerance. The results are shown in Figure 55.

By treating the number of maxima as a frequency, the noise tolerance was treated as a random variable with probability proportional to the number of maxima. As a result, the sample mean and sample standard deviation noise tolerance were calculated. These were used to estimate the shape and scale parameters of a Gamma distribution, using the method of moments, as shown in Equation 7 and Equation 8. [21, p. 263] These parameters were used to see if a Gamma distribution, with probability density function shown in Equation 6, could fit with the data as shown in Figure 55. [21, p. 258]

\[ f(x) = \frac{\lambda^\alpha x^{\alpha-1} e^{-\lambda x}}{\Gamma(\alpha)} \]  
\text{(p.d.f. } f(x), \text{ noise tolerance } x, \text{ shape parameter } \alpha, \text{ scale parameter } \lambda)\]

\[ \alpha = \overline{x}^2/s^2 \]  
\text{(estimated mean } \bar{x}, \text{ estimated standard deviation } s)\]

\[ \lambda = \overline{x}/s^2 \]

Equation 6

Equation 7

Equation 8

It was clear that visually a Gamma distribution did not fit with the data because the distribution tended to zero as the noise tolerance goes to zero, which did not agree with the data. As a result of the sample mean and sample standard deviation being significantly different, an exponential distribution, which is a special case of the Gamma distribution, would not fit the data anyway. [21, p. 53] As a result, the Gamma distribution cannot be used to model the number of maxima as a function of noise tolerance. Another model was used to compare the data with.

The noise tolerance should be chosen such that it produce results which are interesting or significantly different from expected. One way was to compare the image with a
Monte Carlo simulated image which has the same shape as the chromosome but with completely no structure. This was done by treating the histogram of intensity values of the data as a probability distribution function to simulate the intensity values each pixel will have in the Monte Carlo image. A macro was written to generate these images, as shown in Figure 54 and Figure 58.

The number of maximas in the Monte Carlo image, \( f_{MC} \), was worked out and averaged over a sample of 5 Monte Carlo images. This was compared with the number of maximas in data, \( f_o \), as shown in Figure 56. There was a significant difference between \( f_{MC} \) and \( f_o \) at low noise tolerances, however a statistic, for each noise tolerance, should be calculated to put a measure on how different the number of maximas is compared with the data and the Monte Carlo images.

One possible statistic, \( z_1 \), was to compare the difference between the number of maximas with the sample standard deviation of the number of maximas in the Monte Carlo images, \( s_{MC} \), as shown in Equation 9. Another possible statistic, \( z_2 \), which could be calculated was to assume the occurrence of maximas, in the Monte Carlo image, is a Poisson process in space, thus the standard deviation of the number of maximas could be estimated as the square root of the number of maximas in the Monte Carlo images, as shown in Equation 10. [23, p. 61]

\[
\text{Equation 9} \\
z_1 = \left( \frac{f_o - f_{MC}}{s_{MC}} \right)^2
\]

\[
\text{Equation 10} \\
z_2 = \left( \frac{f_o - f_g}{f_{MC}} \right)^2
\]

Equation 9 and Equation 10 were chosen such that if they were summed over all possible values of noise tolerance and there was no significant difference between \( f_{MC} \) and \( f_o \), it would be follow a \( \chi^2 \) distribution. [23, pp. 61, 106] Therefore the noise tolerance with the highest one of these statistics would contribute the most to the \( \chi^2 \) statistic, therefore making it most significant, thus that noise tolerance should be selected. Figure 56 shows the two statistics, \( z_1 \) and \( z_2 \) plotted.

The two statistics were well behaved for high noise tolerances because the statistics tended to zero at high noise tolerance as expected. This was because there was little difference between the number of maximas compared with the data and Monte Carlo images at high noise tolerances. \( z_1 \) had much more random fluctuation than \( z_2 \) due to the statistical error in estimating the standard deviation of the number of maximas in a sample of Monte Carlo images. This can be seen in Figure 56 because the estimated standard deviation, shown as the thickness of the curve, varied randomly but decreased at higher noise tolerance. This statistical error could be reduced by taking a larger sample of Monte Carlo images, but at a cost of computing power and time.

\( z_2 \) was chosen to be worked with rather than \( z_1 \), even though the assumption of a Poisson process was not justified, because the curve of \( z_2 \) in Figure 56 was much smoother and made fitting an 8th order polynomial to the data much easier. By modelling \( z_2 \) as a function of noise tolerance as a polynomial, the noise tolerance with the highest \( z_2 \) was found by finding the maximum of the polynomial; this hopefully helped ‘average’ out random statistical fluctuations from estimating the mean of number of maximas in a sample of Monte Carlo images.
Figure 55 The number of maxima were worked out for each integer setting of noise tolerance on slice 59 (blue). By treating the number of maxima as a frequency, the mean and variance noise tolerance were estimated to attempt to fit a gamma distribution to the data (red).

Mean = 961, Variance = 776941, $\alpha = 1.19$, $\lambda = 0.00124$

Figure 56 The number of maxima at different noise tolerances, for slice 59 from the data (blue) and the Monte Carlo simulation (orange) were compared. The thickness of the orange line corresponds to the estimated standard deviation. The statistics $z_1$ (green) and $z_2$ (gray) were also plotted.
An ImageJ plugin was written to compare the number of maximas, for each integer noise tolerance setting from 0 to 5000, for each and all 122 slices in Figure 49 with a sample of 10 Monte Carlo slices per slice (Figure 58). It fitted an 8th order polynomial on the $z_2$ statistic as a function of noise tolerance for each slice, so that the noise tolerance which maximizes the polynomial was used to calculate maximas in that slice.

The result of the plugin, which took about 4 hours to run, found 32748 maximas as shown in Figure 59. A watershed algorithm was used on the maximas to connect them together and then cropped to fit in the shape of the chromosome as shown in Figure 60.

The problem with the watershed algorithm was that the algorithm only worked in 2D, thus only on each slice separately, therefore was unable to connect the whole stack of maximas together. A different approach was used to connect the maximas together.

A good statistics to look at is the estimated population mean and standard deviation of the distance to the nearest neighbour for each maxima. In Processing, an algorithm was written to calculate the distance to the nearest neighbour of every maxima in the stack. The algorithm was very naive because the algorithm calculated, for each maxima, the distances between every maxima, the smallest distance being the distance to the nearest neighbour. The histogram of the distances to the nearest neighbour is as shown in Figure 61.

The distribution of distances to the nearest neighbour in the histogram appeared to be very discrete and form bands on a certain range of distances. This was not a surprising result because this was the result of the discrete nature of voxels. Because voxels are arranged in a grid, this caused the distances to the nearest neighbour to have a discrete nature as well. All the possible distances to the nearest neighbour as are shown in Figure 57.

<table>
<thead>
<tr>
<th>z (nm)</th>
<th>x (nm)</th>
<th>y (nm)</th>
<th>d (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0</td>
<td>0</td>
<td>20.0</td>
</tr>
<tr>
<td>0</td>
<td>22</td>
<td>0</td>
<td>22.0</td>
</tr>
<tr>
<td>20</td>
<td>11</td>
<td>0</td>
<td>22.8</td>
</tr>
<tr>
<td>0</td>
<td>22</td>
<td>11</td>
<td>24.6</td>
</tr>
<tr>
<td>20</td>
<td>11</td>
<td>11</td>
<td>25.3</td>
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<td>20</td>
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<td>29.7</td>
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<td>11</td>
<td>31.7</td>
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<tr>
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<td>0</td>
<td>33.0</td>
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<tr>
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<td>11</td>
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<td>22</td>
<td>37.0</td>
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<tr>
<td>20</td>
<td>33</td>
<td>0</td>
<td>38.6</td>
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<tr>
<td>0</td>
<td>33</td>
<td>22</td>
<td>39.7</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

Figure 57 The smallest 14 possible distances to nearest neighbours (d) due to the discrete nature of the position of the voxels, the slice separation (z) of 20 nm and the pixel size (x and y) of 11 nm.

Equation 11

$$\bar{x} = \frac{\sum_{i=0}^{n} x_i}{n}$$

Equation 12

$$s = \sqrt{\frac{\sum_{i=0}^{n} x_i^2 - \left(\sum_{i=0}^{n} x_i\right)^2}{n - 1}}$$

Equation 3

$$\bar{x} - \Phi^{-1}(97.5\%)s < \mu < \bar{x} + \Phi^{-1}(97.5\%)s \sqrt{n}$$

Equation 13

$$\frac{\nu s^2}{\sqrt{2\nu} \Phi^{-1}(97.5\%)} < \sigma^2 < \frac{\nu s^2}{\sqrt{2\nu} \Phi^{-1}(2.5\%)} + \nu$$

where $\nu = n - 1$
Figure 58 Slices 1, 6, 11, ..., 121 of the Monte Carlo stack.
Figure 59 The result of a plugin of slice 5,10,15,...,120 which found maxima, shown as yellow dots, by using a noise tolerance setting which maximizes the $z_2$ statistic.
Figure 60 The watershed algorithm was applied and cropped on the points in Figure 59.
Figure 61 A histogram of the distances to nearest neighbour using the maxima obtained from the original data stack.

\( n_x = 32748 \)

95% confidence limits

Mean: \((29.008 \pm 0.084) \text{ nm}, \)

Standard Deviation: \((7.714 \pm 0.088) \text{ nm} \)
Figure 62 A histogram of the distances to nearest neighbour using the maxima obtained from the Monte Carlo stack.

$n_y = 109794$

95% confidence limits

Mean: $(23.823 \pm 0.019)$ nm,

Standard Deviation: $(3.130 \pm 0.013)$ nm
The sample mean, $\bar{x}$, and sample standard deviation, $s$, of the distances to the nearest neighbour, were calculated using Equation 11 and Equation 12 respectively. [21, p. 195]

Because a large sample of distances to the nearest neighbour was obtained, 32748, the sample mean and sample standard deviation approximately has a Normal distribution. [21, p. 181] As a result, the 95% confidence limit of the population mean and population standard deviation were calculated using Equation 3 and Equation 13 respectively.

The 95% confidence limit of the mean distance to the nearest neighbour is $(29.008 \pm 0.084)$ nm, which could be evidence of 30 nm chromatin fibre. However the result could be just a fluke and could be as a result of the resolution of the stack, for example maxima must be at least one voxel apart and this puts a lower limit on the distances to the nearest neighbour.

The same statistics, the mean and standard deviation of the distances to the nearest neighbour, were estimated using the maxima calculated from the Monte Carlo stack (Figure 58), and the 95% confidence limit of the mean distance to the nearest neighbour was calculated to be $(23.823 \pm 0.019)$ nm. The histogram of the data is shown in Figure 62. The mean distances to the nearest neighbour in the original data, $x$, and the Monte Carlo stack, $y$, were compared by calculating a $z$ statistics, which has a standard Normal distribution, as shown in Equation 14. [22, p. 149]

$$z = \frac{\bar{x} - \bar{y}}{\sqrt{\frac{s_x^2}{n_x} + \frac{s_y^2}{n_y}}}$$

The $z$ statistic was calculated to be $\sim 119$ and this was evidence that the mean distance to the nearest neighbour, obtained from the Monte Carlo stack, was extremely significantly different from the mean value obtained from the data.

Following from this, the justification that the obtained mean distance to the nearest neighbour from the original data being evidence of 30 nm chromatin fibre, was slightly strengthen because the statistic obtained from the data is significantly different from the simulation, ruling out the possibility that the statistics obtained was the result of the resolution of the stack. The results, however, are not yet reliable because the same method should be repeated and applied to other SBFSEM images, to see if the same results are obtained.

Another statistics calculated was the Pearson’s correlation coefficient, $r$, between the distances to the nearest neighbour with the distances to the centre of mass of the maxima. If evidence of such correlation existed, this could suggest that the maxima are denser closer to the centre of mass. Again, an algorithm was written in Processing to analyse the stack of maxima and to work out the distances to the centre of mass and the nearest neighbour. A scatter graph of the correlation using the data and the Monte Carlo stack is as shown in Figure 63 and Figure 64 respectively. Even though the values of $r$ were calculated to have magnitude of two decimal places, the degrees of freedom, which is essentially the number of data points $n$, must be considered to reach a statistical conclusion. [30, p. 56] Equation 15 was used to transform, $r$, to a random variable $z$ which is approximately Normally distributed with mean 0 and unit variance. [31] Following from this, the $z$ statistics were calculated as shown in Figure 65.
Figure 63 A scatter graph of the each maxima distance to nearest neighbour and distance to the center of mass using maxima from the data.

Figure 64 A scatter graph of each maxima distance to nearest neighbour and distance to the center of mass using maxima from the Monte Carlo simulation.
Equation 15

\[ z = \sqrt{n - 3} \arctanh(r) \]

<table>
<thead>
<tr>
<th>Data</th>
<th>Monte Carlo</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n)</td>
<td>32748</td>
</tr>
<tr>
<td>(r)</td>
<td>0.076011</td>
</tr>
<tr>
<td>(z)</td>
<td>13.781</td>
</tr>
</tbody>
</table>

Figure 65 Correlation statistics between the distance to nearest neighbour with the distance to the centre of mass using maximas from the data and the Monte Carlo simulation. The higher the value of \(z\) is, the stronger the evidence that there is a correlation.

The values of \(z\) shows that there was strong evidence of correlation between the distance to the nearest neighbour with the distance to the centre of mass. Although a correlation was found, nothing about the cause between the two random variables was found. Some suggestions to the cause of the correlation could be that the maximas are denser closer to the centre of mass or that maximas closer to the centre of mass have more neighbours than maximas on the surface on the chromosome. More sophisticated approaches should be used to further look into how the maximas are arranged inside the chromosome, rather than just statistically analysing random variables.

An approach used to connect maximas together was to fit 29.008 nm diameter spheres at every maxima, using Processing. Of course, it will be computationally intense if 32748 spheres were drawn, therefore only a sample of maximas were worked on in the project. The result is shown in Figure 66. At a glance, the 3D render did appeared to be spheres placed randomly. However after closer examination, some spheres overlap each other consistently to form a coil, as shown in Figure 67, Figure 68 and Figure 69. But at most times the spheres merged with each other to form a very tight cluster rather than a coil. The results could be evidence of 30 nm chromatin fibres, however more work or more accurate and precise data would be needed to find the structure or the arrangement of the maximas.

Finally, another approach was used to connect maximas together by simply drawing lines between maximas if the distances between maximas are in the order of \((29.0 \pm 7.7)\) nm. An algorithm was written in Processing to connect the maximas together as shown in Figure 70 and Figure 71.

The result was excellent because the X shape of the chromosome was much clearer than the result used from the marching cubes algorithm. The centromere and the pair of chromatids was easily identified.

Because the centromere was much more easily recognized, the position of the centromere could be estimated using Figure 70. The ratio of the length of the short chromatin over the length of the chromosome was estimated to be about \(\sim 0.4\). This information could be used to predict the specific chromosome which was worked on.

The volume of the chromatin fibres could be estimated by summing the volume of all 32748 29.008 nm diameter spheres. The diameter of the spheres, \(D\), was treated as a random variable with mean \(d\) nm and standard deviation \(\sigma_d\) nm, and as a result the volume of chromatin fibres, \(V\), was also a random variable as shown in Equation 16.

Equation 17 and Equation 18 is an approximate estimation for the mean and standard deviation of a function of a random variable, where dashes are derivatives with respect to \(D\). [21, p. 162]

These equations were used to approximately estimate the mean and standard deviation of \(V\) as shown in Equation 19 and Equation 20 respectively, where \(N\) is the number of spheres.
\textbf{Equation 16}
\[ V(D) = \frac{1}{6} N\pi D^3 \]

\textbf{Equation 17}
\[ E(V) = V(d) + \frac{1}{2} \sigma_d^2 V''(d) \]

\textbf{Equation 18}
\[ \sigma_V = V'(d)\sigma_d \]

\textbf{Equation 19}
\[ E(V) = \frac{N\pi d^3}{6} \left( 1 + \frac{3\sigma_d^2}{d^2} \right) \]

\textbf{Equation 20}
\[ \sigma_V = \frac{\sigma_d N\pi d^2}{2} \]

Taking the values of the mean and standard deviation of \( D \) as 29.008 nm and 7.714 nm respectively, as shown in Figure 61, the mean and standard deviation of \( V \) was estimated to be \( (0.51 \pm 0.33) \text{ \mu m}^3 \).

This was used as a model for the volume of the chromatin fibres in the chromosome. Following from this, the number of base pairs, or pairs of nucleotides, in the chromosome was estimated. By modelling nucleosomes as an 11nm diameter cylindrical disc with thickness 5.7 nm, [32] the number of nucleosomes was estimated by calculating the number of nucleosomes in the chromatin fibres, modelled by spheres. This was done, naively, by dividing the volume of the chromatin fibres with the volume of a nucleosome. It was estimated that there are \((940 \pm 620)\) thousands nucleosomes in the chromosome. This was an overestimation because the chromatin fibres consist of other material than just nucleosomes.

For each nucleosome, there are 147 base pairs wrapped around each histone with an additional 20 base pairs bounded by the linker histone H1/H5. [9] Furthermore, each nucleosome are separated by linker DNA which varies in length between 0 and 80 base pairs. [9] The distribution of the length of linker DNA, between each nucleosomes, was modelled with a uniform distribution from 0 to 80 base pairs, thus has mean 40 base pairs and standard deviation 23 base pairs. Following from this, it was estimated there are \((207 \pm 23)\) base pairs per nucleosome.

\textbf{Equation 21}
\[ \sigma_Y = y \sqrt{\left( \frac{\sigma_{x_1}}{x_1} \right)^2 + \left( \frac{\sigma_{x_2}}{x_2} \right)^2} \]

By assuming the number of base pairs per nucleosome and the number of nucleosomes in the chromosome are independent, the mean and standard deviation of the number of base pairs, in the chromosome, were estimated, with the aid of the error propagation formula Equation 21. [23, p. 22] It was estimated there are \((190 \pm 130)\) million base pairs in the chromosome.
Figure 66 A sample of maximas were used to model each maxima as a 29.0 nm diameter sphere.

Figure 67 Some spheres form a straight line.
Figure 68 Some spheres from a smooth curve.

Figure 69 Some spheres form a tight coil.
Red: (0.0 – 21.3) nm
Green: (21.3 – 36.7) nm
Blue: (36.7 – 44.4) nm

Figure 70 Different coloured lines were drawn connecting maxima together if the distances between them are in the order of (29.0 ± 7.7) nm.
Figure 71 The links between maxima viewed at different angles.
V. CONCLUSION

The SBFSEM images were segmented by a threshold. Firstly, samples of intensity values of different features or signals of the images were taken and were used to estimate the mean and standard deviation of the intensity values of each signal. Three significant signals were found: the chromosome, the background and other signals inside and around the chromosome (see Figure 46 p. 42). Fortunately the intensity values of the different signals were significantly different and a threshold was set to cover the range of intensity values within two standard deviations away from the mean intensity values of the chromosome and its features.

This was then used to estimate the volume of the chromosome to be about 3.95 $\mu$m$^3$. The volume calculation was dependent on the threshold used. The value calculated was suggested to be an overestimate because the distinguishable intensity values inside the chromosome may be vacancies, and should be accounted for in the volume calculation.

Following from the threshold, the volume of the chromosome was rendered in 3D using the marching cubes algorithm. [24] [26] The algorithm was programmed from scratch using an open source programming language called Processing. [19] This saved a lot in terms of cost, but more importantly a program specific for this project was built. The results were excellent and the centromere and chromatids were easily identified in the renders (see Figure 50 p. 46 and Figure 51 p. 47). The next step in visualization of the data should be looking at commercial software like Avizo [33] and Imaris [34]. Both software process 3D, and even 4D, images, using state of the art features. [33] [34]

Using commercial software would take the methodology of this project in a different direction. The marching cubes algorithm was programmed from first principles in this project, but using commercial software would require training to use the software instead.

Internal structures of the chromosome were attempted to be found using the ImageJ maxima finder. [29] The number of maximas found were compared using different noise tolerances and Monte Carlo images, which has the same shape as the data and uses the histogram of the intensity values of the data as a probability distribution function to generate an image (see Figure 58 p. 52). The noise tolerance was chosen which had the highest $\chi^2$ statistics (see Equation 10 p. 49) under the assumption that maximas occurring, in the Monte Carlo simulation, was a Poisson process in space.

The assumption was unjustified; for a Poisson process in space, each pixel in the image must have equal probability of a maxima occurring and independent of other pixels and maximas. [35, pp. 313, 327] This was, of course, not true because each maxima must be at least two pixels away. However, even under that assumption, the algorithm had produced smooth results which a polynomial can be fitted with (see Figure 56 p. 50), used to find the noise tolerance with the highest $\chi^2$ statistics. For the assumption to be dropped and Equation 9 (p. 49) to be used, it would need a lot of computing power to simulate and analyse a larger sample of Monte Carlo images. Larger samples of Monte Carlo images would produce more accurate and precise values of the mean and standard deviation of the number of maximas and thus should decrease the fluctuation seen in the statistics for different noise tolerances,
making finding the noise tolerance with the highest $\chi^2$ statistics easier.

The mean distance to the nearest neighbour was found for each maxima to be $(29.008 \pm 0.084)$ nm. This could be good evidence for 30 nm chromatin fibres. This was then used to model each maxima as a 29 nm sphere. The results do show coils forming (see Figure 68 p. 62) however not many. This was most likely due to the alignment problem with the diamond knife (see Figure 45 p. 41). Better data, without alignment issues, would be needed to produce more precise and accurate models on how the 30 nm chromatin fibres were structured.

By modelling the spheres as chromatin fibres, it was estimated that there are $(190 \pm 130)$ million base pairs in the chromosome. The standard deviation in the value was too big to make a judgement on what chromosome it is. Chromosome 4 also has about 190 million base pairs however chromosome 1, the biggest, has about 250 million pairs [36] which is within range of one standard deviation. The main source of error was from cubing the distance to the nearest neighbours to find the volume of the sphere (see Equation 16 p. 60).

A better statistics to use is the average of the distance to the first 2 nearest neighbours because for a chain, or fibre, to be formed, each point must be connected by two neighbouring points. This statistics should give a more accurate, and larger, estimation for the diameter of the 30 nm chromatin fibre.

Lines were drawn between maximas, where the distances between them are in the order of $(29.0 \pm 7.7)$ nm (see Figure 71 p. 64). The pair of chromatins were easily identified with most of the connections at the centromere. Using the figure, it was estimated that the ratio the length of the short arm over the length of the chromosome was ~0.4. This corresponds to chromosome 2 which has a ratio of 0.389, [37] suggesting the chromosome which was worked on may be chromosome 2.
VI. APPENDIX

The table below shows the information of the source code used in the project shown in the appendix.

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<tr>
<th>Name</th>
<th>Page Number</th>
<th>Language and Environment</th>
<th>Data Files</th>
<th>Classes</th>
</tr>
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<tbody>
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<td>68</td>
<td>Processing</td>
<td>Binary threshold .png images See Figure 24 p. 22</td>
<td></td>
</tr>
<tr>
<td>3D RENDER OF A CHROMOSOME USING THE MARCHING CUBES ALGORITHM</td>
<td>72</td>
<td>Processing</td>
<td>Binary threshold .png images See Figure 48 p. 44</td>
<td>Material Cube Triangle</td>
</tr>
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<td>MAXIMA FINDER BY USING NOISE TOLERANCES WHICH MAXIMIZES CHI-SQUARED</td>
<td>98</td>
<td>Java in an ImageJ Plugin environment</td>
<td>Cropped and inverted chromosomes .tiff stack See Figure 49 p. 45</td>
<td></td>
</tr>
<tr>
<td>NEAREST NEIGHBOUR SEARCH AND DISTANCE TO CENTER OF MASS</td>
<td>105</td>
<td>Processing</td>
<td>Binary .png images of the maximas</td>
<td>PointVector</td>
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<tr>
<td>MODELLING MAXIMAS AS SPHERES</td>
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<td>Processing</td>
<td>Binary .png images of the maximas</td>
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<tr>
<td>CONNECTING MAXIMAS TOGETHER USING LINES</td>
<td>113</td>
<td>Processing</td>
<td>Binary .png images of the maximas</td>
<td></td>
</tr>
</tbody>
</table>
3D RENDER OF A CHROMOSOME USING VOXELS

//import PeasyCam
import peasy.*;
PeasyCam cam;

//PShape for chromosome
PShape chromosome;

//declare a 3D array of booleans, true for white pixel
boolean[][][] pixelGrid;
boolean[][][] pixelGridHollow;
int imageWidth, imageHeight; //dimension of image
int nSlices = 26; //26 slices

//SETUP
void setup()
{
  //setup drawing
  size(1024,768,P3D);
  cam = new PeasyCam(this, 500); //makes a new camera and set distances

  //create the arrayList of slices
  ArrayList<PImage> imageArray = new ArrayList<PImage>();

  //for the fist 10 times, put the image into the arrayList
  for (int i=0; i<10; i++)
  {
    imageArray.add(loadImage("X0"+i+".png")); //load image
  }
  //for the last set of image, put the image into the arrayList
  for (int i=10; i<nSlices; i++)
  {
    imageArray.add(loadImage("X"+i+".png")); //load image
  }

  //get the dimensions of the image
  imageWidth = imageArray.get(0).width;
  imageHeight = imageArray.get(0).height;

  //create the pixelGrid of the correct size
  pixelGrid = new boolean[imageWidth][imageHeight][nSlices];
pixelGridHollow = new boolean[imageWidth][imageHeight][nSlices];

//fill in pixelGrid using the images
getPixelGrid(imageArray);
//hollow out the pixelGrid by removing voxels with 6 neighbours
hollowOutGrid();
//set up the PShape of chromosome
setupChromosome();

//DRAW
void draw(){
    background(0); //set background to black
    scale(1,50.0/27.0,1); //scale the matrix
    shape(chromosome); //show the chromosome
    println(frameRate); //print the frameRate
}

//Reads the binary images in imageArray and puts true in the pixelGrid for every white pixel
void getPixelGrid(ArrayList<PImage> imageArray){
    //for every image in imageArray
    for (int z=0; z<nSlices; z++){
        //load the pixels in the image
        imageArray.get(z).loadPixels();

        //declare x and y co-od
        int x = 0;
        int y = 0;

        //for every pixel in the image
        for (int i=0; i<imageArray.get(z).pixels.length; i++){
            if (imageArray.get(z).pixels[i]==color(255)){ //if that pixel is white
                pixelGrid[x][y][z] = true; //put true in the pixelGrid
            }
        }
    }
}
```java
else{
    pixelGrid[x][y][z] = false; // else put false
}// end else

// increase x and y co-od accordingly
x++;
if (x==imageWidth){
    x=0;
    y++;
}// end if
}// end for

// remove voxels with 6 neighbours from pixelGrid
void hollowOutGrid(){
    // for each slice
    for (int z=0; z<nSlices; z++){
        // for each pixel in the slice
        for (int y=0; y<imageHeight; y++){
            for (int x=0; x<imageWidth; x++){

                // if the pixel is on the border, the pixel remains unchanged
                if(x==0||x==imageWidth-1 || y==0||y==imageHeight-1 || z==0||z==nSlices-1){
                    pixelGridHollow[x][y][z] = pixelGrid[x][y][z];
                } // end if
                else if(pixelGrid[x][y][z]==true){ // if the pixel is true
                    if(pixelGrid[x-1][y][z]==true && pixelGrid[x+1][y][z]==true // if the pixel has 6 true neighbouring pixels
                       &&pixelGrid[x][y-1][z]==true && pixelGrid[x][y+1][z]==true
                       &&pixelGrid[x][y][z-1]==true && pixelGrid[x][y][z+1]==true){
                        pixelGridHollow[x][y][z]=false; // then that pixel shall be false
                    } // end if
                    else{
                        pixelGridHollow[x][y][z]=true; // else the pixel will remain true
                    } // end else
                } // end if
            } // end for
        } // end for
    } // end for
}
```
else{
    pixelGridHollow[x][y][z]=false; //if the pixel is not true, it will be false
} //end else
}
//end hollowOutGrid

//setup the PShape for the Chromosome
void setupChromosome(){

    //chromosome is a group of voxel
    chromosome = createShape(GROUP);

    //for every slice
    for (int z=0; z<nSlices; z++){
        //for every pixel
        for (int y=0; y<imageHeight; y++){
            for (int x=0; x<imageWidth; x++){

                if (pixelGridHollow[x][y][z]==true){ //if the pixelGrid contain true
                    PShape voxel;
                    voxel = createShape(BOX,5);
                    voxel.setFill(color(0)); //fill black
                    voxel.setStroke(color(0,255,0)); //stroke green
                    voxel.translate(5*(x-imageWidth/2),5*(z-nSlices/2),5*(y-imageHeight/2)); //translate voxel
                    chromosome.addChild(voxel); //add voxel to chromosome
                }
            }
        }
    }
} //end setup chromosome
3D RENDER OF A CHROMOSOME USING THE MARCHING CUBES ALGORITHM

Main.pde (functions and procedures)

```java
//import PeasyCam
import peasy.*;

//GLOBAL VARIABLES
PeasyCam cam;
Material chromosome; //PShape for chromosome

//SETUP
void setup(){
    size(1024,768,P3D);
    //make a new camera and set distance
    cam = new PeasyCam(this, 500);
    //image scale by 0.4
    //set up materials
    chromosome = new Material(58,"Z",true);
    chromosome.setStroke(false);
    chromosome.setFill(true);
    chromosome.saveShape();
}
//end setup

//DRAW
void draw(){
    background(0); //set background to black
    scale(1,20.0/11.0,1); //scale the chromosome
    lighting(); //set up the lights
    shape(chromosome.getShape()); //draw the chromosome
    println(frameRate); //print the frame rate
}
//end draw

//lighting
void lighting(){
```
ambientLight(50,50,50);
directionalLight(0,128,0,1,-1,0);  //green light
directionalLight(0,0,128,-1,-1,0);  //blue light
}//end lighting()
Lookup.pde (functions and procedures)

//GET EDGE COORDINATES
//Converts the edge index to a position vector (returned)
PVector getEdgeCo(int index){
    switch(index){
        case 0: return new PVector(0.0,5.0,-5.0); //0
        case 1: return new PVector(5.0,5.0,0.0); //1
        case 2: return new PVector(0.0,5.0,5.0); //2
        case 3: return new PVector(-5.0,5.0,0.0); //3
        case 4: return new PVector(0.0,-5.0,-5.0); //4
        case 5: return new PVector(5.0,-5.0,0.0); //5
        case 6: return new PVector(0.0,-5.0,5.0); //6
        case 7: return new PVector(-5.0,-5.0,0.0); //7
        case 8: return new PVector(-5.0,0.0,-5.0); //8
        case 9: return new PVector(5.0,0.0,-5.0); //9
        case 10: return new PVector(5.0,0.0,5.0); //10
        case 11: return new PVector(-5.0,0.0,5.0); //11
        default: return new PVector(0.0,0.0,0.0);
    }
}

//FUNCTION: GET EDGES
//EXTRACTED FROM: http://paulbourke.net/geometry/polygonise/
//Polygonising a scalar field
//return a row from the lookup table for which 3 edges the triangles' 3 vertices should be on based on the index
//the index shows which vertices has a voxel
//a -1 will indicate there are no more triangles to be rendered
int[] getEdges (int index){
    return edgeLookup[index];
}

int [][] edgeLookup =
    {{-1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
     {0, 8, 3, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
     {0, 1, 9, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
     {1, 8, 3, 9, 8, 1, -1, -1, -1, -1, -1, -1, -1}},
{2, 1, 5, 2, 5, 8, 2, 8, 11, 4, 8, 5, -1, -1, -1, -1},
{10, 3, 11, 10, 1, 3, 9, 5, 4, -1, -1, -1, -1, -1, -1},
{4, 9, 5, 0, 8, 1, 8, 10, 1, 8, 11, 10, -1, -1, -1, -1},
{5, 4, 0, 5, 0, 11, 5, 11, 10, 11, 0, 3, -1, -1, -1, -1},
{5, 4, 8, 5, 8, 10, 10, 8, 11, -1, -1, -1, -1, -1, -1},
{9, 7, 8, 5, 7, 9, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{9, 3, 0, 9, 5, 3, 5, 7, 3, -1, -1, -1, -1, -1, -1, -1},
{0, 7, 8, 0, 1, 7, 1, 5, 7, -1, -1, -1, -1, -1, -1, -1},
{1, 3, 3, 3, 7, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{9, 7, 8, 9, 5, 7, 10, 1, 2, -1, -1, -1, -1, -1, -1, -1},
{10, 1, 2, 9, 5, 0, 5, 3, 0, 5, 7, 3, -1, -1, -1, -1, -1},
{8, 0, 2, 8, 2, 5, 8, 5, 7, 10, 5, 2, -1, -1, -1, -1, -1},
{2, 10, 5, 2, 5, 3, 3, 5, 7, -1, -1, -1, -1, -1, -1, -1, -1},
{7, 9, 5, 7, 8, 9, 3, 11, 2, -1, -1, -1, -1, -1, -1, -1, -1},
{9, 5, 7, 9, 7, 2, 9, 2, 0, 2, 7, 11, -1, -1, -1, -1, -1},
{2, 3, 11, 0, 1, 8, 1, 7, 8, 1, 5, 7, -1, -1, -1, -1, -1},
{11, 2, 1, 11, 1, 7, 7, 1, 5, -1, -1, -1, -1, -1, -1, -1, -1},
{9, 5, 8, 8, 5, 7, 10, 1, 3, 10, 3, 11, -1, -1, -1, -1, -1},
{5, 7, 0, 5, 0, 9, 7, 11, 0, 1, 0, 10, 11, 10, 0, -1},
{11, 10, 0, 11, 0, 3, 10, 5, 0, 8, 0, 7, 5, 7, 0, -1},
{11, 10, 5, 7, 11, 5, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{10, 6, 5, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{0, 8, 3, 5, 10, 6, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{9, 0, 1, 5, 10, 6, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{1, 8, 3, 1, 9, 8, 5, 10, 6, -1, -1, -1, -1, -1, -1, -1, -1},
{1, 6, 5, 2, 6, 1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{1, 6, 5, 1, 2, 6, 3, 0, 8, -1, -1, -1, -1, -1, -1, -1, -1},
{9, 6, 5, 9, 0, 6, 0, 2, 6, -1, -1, -1, -1, -1, -1, -1, -1},
{5, 9, 8, 5, 8, 2, 5, 2, 6, 3, 2, 8, -1, -1, -1, -1, -1},
{2, 3, 11, 10, 6, 5, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{11, 0, 8, 11, 2, 0, 10, 6, 5, -1, -1, -1, -1, -1, -1, -1, -1},
{0, 1, 9, 2, 3, 11, 5, 10, 6, -1, -1, -1, -1, -1, -1, -1, -1},
{5, 10, 6, 1, 9, 2, 9, 11, 2, 9, 8, 11, -1, -1, -1, -1, -1},
{6, 3, 11, 6, 5, 3, 5, 1, 3, -1, -1, -1, -1, -1, -1, -1, -1},
{0, 8, 11, 0, 11, 5, 0, 5, 1, 5, 11, 6, -1, -1, -1, -1, -1},
{3, 11, 6, 0, 3, 6, 0, 6, 5, 0, 5, 9, -1, -1, -1, -1, -1},
{6, 5, 9, 6, 9, 11, 11, 9, 8, -1, -1, -1, -1, -1, -1, -1, -1},
{5, 10, 6, 4, 7, 8, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{4, 3, 0, 4, 7, 3, 6, 5, 10, -1, -1, -1, -1, -1, -1, -1, -1},
$$\{4, 9, 5, 7, 6, 11, -1, -1, -1, -1, -1, -1, -1, -1\},$$
$$\{0, 8, 3, 4, 9, 5, 11, 7, 6, -1, -1, -1, -1, -1, -1\},$$
$$\{5, 0, 1, 5, 4, 0, 7, 6, 11, -1, -1, -1, -1, -1, -1\},$$
$$\{11, 7, 6, 8, 3, 4, 3, 5, 4, 3, 1, 5, -1, -1, -1, -1\},$$
$$\{9, 5, 4, 10, 1, 2, 7, 6, 11, -1, -1, -1, -1, -1, -1\},$$
$$\{6, 11, 7, 1, 2, 10, 0, 8, 3, 4, 9, 5, -1, -1, -1, -1\},$$
$$\{7, 6, 11, 5, 4, 10, 4, 2, 10, 4, 0, -1, -1, -1, -1\},$$
$$\{3, 4, 8, 3, 5, 4, 3, 2, 5, 10, 5, 2, 11, 7, 6, -1\},$$
$$\{7, 2, 3, 7, 6, 2, 5, 4, 9, -1, -1, -1, -1, -1, -1\},$$
$$\{9, 5, 4, 0, 8, 6, 0, 6, 2, 6, 8, 7, -1, -1, -1, -1\},$$
$$\{3, 6, 2, 3, 7, 6, 1, 5, 0, 5, 4, 0, -1, -1, -1, -1\},$$
$$\{6, 2, 8, 6, 8, 7, 2, 1, 8, 4, 8, 5, 1, 5, 8, -1\},$$
$$\{9, 5, 4, 10, 1, 6, 1, 7, 6, 1, 3, 7, -1, -1, -1, -1\},$$
$$\{1, 6, 10, 1, 7, 6, 1, 0, 7, 8, 7, 0, 9, 5, 4, -1\},$$
$$\{4, 0, 10, 4, 10, 5, 0, 3, 10, 6, 10, 7, 3, 7, 10, -1\},$$
$$\{7, 6, 10, 7, 10, 8, 5, 4, 10, 4, 8, 10, -1, -1, -1, -1\},$$
$$\{6, 9, 5, 6, 11, 9, 11, 8, 9, -1, -1, -1, -1, -1, -1\},$$
$$\{3, 6, 11, 0, 6, 3, 0, 5, 6, 0, 9, 5, -1, -1, -1, -1\},$$
$$\{0, 11, 8, 0, 5, 11, 0, 1, 5, 5, 6, 11, -1, -1, -1, -1\},$$
$$\{6, 11, 3, 6, 3, 5, 3, 1, -1, -1, -1, -1, -1, -1, -1\},$$
$$\{1, 2, 10, 9, 5, 11, 9, 11, 8, 11, 5, 6, -1, -1, -1, -1\},$$
$$\{0, 11, 3, 0, 6, 11, 0, 9, 6, 5, 6, 9, 1, 2, 10, -1\},$$
$$\{11, 8, 5, 11, 5, 6, 8, 0, 5, 10, 5, 2, 0, 2, 5, -1\},$$
$$\{6, 11, 3, 6, 3, 5, 2, 10, 3, 10, 5, 3, -1, -1, -1, -1\},$$
$$\{5, 8, 9, 5, 2, 8, 5, 6, 2, 3, 8, 2, -1, -1, -1, -1\},$$
$$\{9, 5, 6, 9, 6, 0, 0, 6, 2, -1, -1, -1, -1, -1, -1, -1\},$$
$$\{1, 5, 8, 1, 8, 0, 5, 6, 8, 3, 8, 2, 6, 2, 8, -1\},$$
$$\{1, 5, 6, 2, 1, 6, -1, -1, -1, -1, -1, -1, -1, -1\},$$
$$\{1, 3, 6, 1, 6, 10, 3, 8, 6, 5, 6, 9, 8, 9, 6, -1\},$$
$$\{10, 1, 0, 10, 0, 6, 9, 5, 0, 5, 6, 0, -1, -1, -1, -1\},$$
$$\{0, 3, 8, 5, 6, 10, -1, -1, -1, -1, -1, -1, -1, -1\},$$
$$\{10, 5, 6, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1\},$$
$$\{11, 5, 10, 7, 5, 11, -1, -1, -1, -1, -1, -1, -1, -1\},$$
$$\{11, 5, 10, 11, 7, 5, 8, 3, 0, -1, -1, -1, -1, -1, -1\},$$
$$\{5, 11, 7, 5, 10, 11, 1, 9, 0, -1, -1, -1, -1, -1, -1\},$$
$$\{10, 7, 5, 10, 11, 7, 9, 8, 1, 8, 3, 1, -1, -1, -1, -1\},$$
$$\{11, 1, 2, 11, 7, 1, 7, 5, 1, -1, -1, -1, -1, -1, -1, -1\},$$
$$\{0, 8, 3, 1, 2, 7, 1, 7, 5, 7, 2, 11, -1, -1, -1, -1\},$$
$$\{9, 7, 5, 9, 2, 7, 9, 0, 2, 2, 11, 7, -1, -1, -1, -1\}.$$
{4, 0, 3, 7, 4, 3, -1, -1, -1, -1, -1, -1, -1, -1},
{4, 8, 7, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{9, 10, 8, 10, 11, 8, -1, -1, -1, -1, -1, -1, -1, -1},
{3, 0, 9, 3, 9, 11, 11, 9, 10, -1, -1, -1, -1, -1, -1},
{0, 1, 10, 0, 10, 8, 8, 10, 11, -1, -1, -1, -1, -1, -1},
{3, 1, 10, 11, 3, 10, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{1, 2, 11, 1, 11, 9, 9, 11, 8, -1, -1, -1, -1, -1, -1},
{3, 0, 9, 3, 9, 11, 1, 2, 9, 2, 11, 9, -1, -1, -1, -1},
{0, 2, 11, 8, 0, 11, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{3, 2, 11, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{2, 3, 8, 2, 8, 10, 10, 8, 9, -1, -1, -1, -1, -1, -1},
{9, 10, 2, 0, 9, 2, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{2, 3, 8, 2, 8, 10, 0, 1, 8, 1, 10, 8, -1, -1, -1, -1},
{1, 10, 2, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{1, 3, 8, 9, 1, 8, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{0, 9, 1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{0, 3, 8, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1},
{-1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1, -1};
Material.pde (class)

class Material{

    // contains an array of marching cubes, use getShape to get a PShape of all the polygons of the marching cubes

    // MEMBER VARIABLES

    PShape shape; // PShape
    Cube [[[[]]]] cubeArray; // array of marching cubes
    int imageWidth, imageHeight, nSlices; // dimensions of the stack

    // CONSTRUCTOR
    Material(int nSlices, String filename, boolean wantAverageNormal) {

        // LOAD IMAGES ------------------------
        // create an arraylist of PImages
        ArrayList < PImage > imageArray = new ArrayList < PImage > () ;

        // load all the images and add them in imageArray
        for ( int i = 0; i < 10; i ++ ) {
            imageArray.add(loadImage(filename + "0" + i + ".png")); // load image
        } // end for
        for ( int i = 10; i < nSlices; i ++ ) {
            imageArray.add(loadImage(filename + i + ".png")); // load image
        } // end for

        // get imageWidth, imageHeight, nSlices
        this.imageWidth = imageArray.get(0).width;
        this.imageHeight = imageArray.get(0).height;
        this.nSlices = nSlices;

        PVector center = new PVector(imageWidth / 2, imageHeight / 2, nSlices / 2); // work out the center of the image

        // CREATE ARRAY OF MARCHINGS CUBES---------

        // create the pixelGrid and cubeArray of size imageWidth+2 x imageHeight+2 x nSlices+2
        // fill pixelGrid with all entries as false
//fill cubeArray with cube with the position relative to the center
boolean[][][] pixelGrid = new boolean[imageWidth+2][imageHeight+2][nSlices+2];
cubeArray = new Cube[imageWidth+2][imageHeight+2][nSlices+2];
//fill the pixelGrid with falses
for (int i=0; i<imageWidth+2; i++){
    for (int j=0; j<imageHeight+2; j++){
        for (int k=0; k<nSlices+2; k++){
            pixelGrid[i][j][k] = false;
            cubeArray[i][j][k] = new Cube(10*(i-center.x),10*(k-center.z),10*(j-center.y));
        }//end for
    }//end for
};//end for

//for every image in imageArray
for (int z=0; z<nSlices; z++){
PImage slice = imageArray.get(z);
//load the pixels in the image
slice.loadPixels();

//declare x and y co-od
int x = 0;
int y = 0;
//for every pixel in the image
for (int i=0; i<slice.pixels.length; i++){
    if (slice.pixels[i]==color(255)){ //if that pixel is white
        pixelGrid[x+1][y+1][z+1] = true; //put true in the pixelGrid
    }//end if
    //increase x and y co-od according
    x++;
    if (x==imageWidth){
        x=0;
        y++;
    }//end if
};//end for
};//end for

//SETUP PSHAPE--------------------------------------------------------------------------------------
//set the shape is a group of PShapes
shape = createShape(GROUP);

// for every slice
for (int z=0; z<=nSlices; z++){

    // for every pixel in the slice
    for (int y=0; y<=imageHeight; y++){
        for (int x=0; x<=imageWidth; x++){

            // declare a variable called verticesIndex
            int verticesIndex = 0; // this variable indicates which vertices has a true value in the pixelGrid as an 8 bit binary number
            // each digit in the binary number represent a vertex

            // work out verticesIndex by analyzing each corner
            if(pixelGrid[x][y+1][z]==true){ // 0
                verticesIndex += 1;
            } // end if
            if(pixelGrid[x+1][y+1][z]==true){ // 1
                verticesIndex += 2;
            } // end if
            if(pixelGrid[x+1][y+1][z+1]==true){ // 2
                verticesIndex += 4;
            } // end if
            if(pixelGrid[x+1][y][z+1]==true){ // 3
                verticesIndex += 8;
            } // end if
            if(pixelGrid[x][y][z]==true){ // 4
                verticesIndex += 16;
            } // end if
            if(pixelGrid[x+1][y][z]==true){ // 5
                verticesIndex += 32;
            } // end if
            if(pixelGrid[x+1][y][z+1]==true){ // 6
                verticesIndex += 64;
            } // end if
            if(pixelGrid[x][y][z+1]==true){ // 7
                verticesIndex += 128;
            } // end if
        }
    }
}
// look up what polygon to draw using vertices and draw it at x,y,z
drawPolygon(x, y, z, verticesIndex);
}
// end for
}
// end for

// tell the user the progress of rendering each slice
println("Analyzing " + filename + " images: " + ((float) z) * 100 / ((float) nSlices) + ";
}
// end if for

if (wantAverageNormal) {
    // for every slice
    for (int z = 0; z <= nSlices; z++) {
        // for every pixel in the slice
        for (int y = 0; y <= imageHeight; y++) {
            for (int x = 0; x <= imageWidth; x++) {
                // recalculate the normal in the cube
                cubeArray[x][y][z].recalculateNormal(x, y, z, imageWidth, imageHeight, nSlices, cubeArray);
                // add the cube shape to the shape
            }
        }
    }
    // tell the user the progress of rendering each slice
    println("Calculating " + filename + " normals: " + ((float) z) * 100 / ((float) nSlices) + ");
}
// end if
// end constructor

// METHODS

// saveShape
public void saveShape() {
    shape = null;
    shape = createShape(GROUP);
    for (int z = 0; z <= nSlices; z++) {
// for every pixel in the slice
for (int y=0; y<=imageHeight; y++){
    for (int x=0; x<=imageWidth; x++){
        shape.addChild(cubeArray[x][y][z].getShape());
    }//end for
}//end for
}//end for
}//end saveShape

// getShape
public PShape getShape(){
    return shape;
}//end getShape

// drawPolygon at (x,y,z) by looking up what triangles to draw using verticesIndex
private void drawPolygon(int x, int y, int z, int verticesIndex){
    // make an array of edgeIndexes from the lookup table
    // this contains which 3 edges the 3 vertices of the triangles are on, each group of 3 entries are for each triangle
    // the edgeIndexArray will contain a -1 to indicate when there are no more triangles to be drawn
    int [] edgeIndexArray = getEdges(verticesIndex);
    // pointer points to entries in edgesArray
    int pointer = 0;

    boolean trianglesToRender = true; // boolean to indicate when there is a triangle to render

    // while there are triangles to render
    while (trianglesToRender){
        if (edgeIndexArray[pointer]!=-1){ // if the edgeIndex is not -1
            // add a new triangle with 3 vertices on the 3 edges in the edgeIndexArray to the cube
            cubeArray[x][y][z].addTriangle(edgeIndexArray[pointer], edgeIndexArray[pointer+1], edgeIndexArray[pointer+2]);
        }
        pointer += 3;
    } // end while
} // end drawPolygon
trianglesToRender = false;
} //end else
} //end while
} //end drawPolygon

// COLOUR METHODS
public void setStroke(boolean wantStroke)
{
for (int z=0; z<=nSlices; z++)
{
  //for every pixel in the slice
  for (int y=0; y<=imageHeight; y++)
  {
    for (int x=0; x<=imageWidth; x++)
    {
      cubeArray[x][y][z].setStroke(wantStroke);
    } //end for
  } //end for
} //end for
} //end stroke

public void setFill(boolean wantFill)
{
for (int z=0; z<=nSlices; z++)
{
  //for every pixel in the slice
  for (int y=0; y<=imageHeight; y++)
  {
    for (int x=0; x<=imageWidth; x++)
    {
      cubeArray[x][y][z].setFill(wantFill);
    } //end for
  } //end for
} //end for
} //end fill

public void setStroke(color colour)
{
for (int z=0; z<=nSlices; z++)
{
  //for every pixel in the slice
  for (int y=0; y<=imageHeight; y++)
  {
    for (int x=0; x<=imageWidth; x++)
    {
      cubeArray[x][y][z].setStroke(colour);
    } //end for
  } //end for
} //end for
} //end fill
public void setFill(color colour){
    for (int z=0; z<=nSlices; z++){
        //for every pixel in the slice
        for (int y=0; y<=imageHeight; y++){
            for (int x=0; x<=imageWidth; x++){
                cubeArray[x][y][z].setFill(colour);
            }
        }
    }
} // end fill
} // end class
Cube.pde (class)

class Cube{

    //CUBE CLASS
    //A marching cube analyse 4 voxels and store/draw triangles according to the state of the voxels
    //The marching cube contains triangles to represent the surface of the chromosome according if the voxel is white
    //or not.

    //MEMBER VARIABLES
    private PVector position; //the position of the cube
    private ArrayList<Triangle> triangleArray; //arraylist of Triangles

    //CONSTRUCTOR
    //Position of the cube (x,y,z)
    Cube(float x, float y, float z){
        position = new PVector(x, y, z);
        triangleArray = new ArrayList<Triangle>();
    }

    //METHODS
    //addTriangle to the marching cube
    public void addTriangle(int index1, int index2, int index3){
        //add triangle object to the triangle arraylist
        triangleArray.add(new Triangle(index1, index2, index3, this.position.x, this.position.y, this.position.z));
    }

    //get the PShape for all the triangles in the cube
    public PShape getShape(){
        //create a PShape for the marching cube
        PShape cube;
        cube = createShape(GROUP);
        //for every triangle in the arraylist
        for (int i=0; i<triangleArray.size(); i++){
            //add triangle
        }
    }
}
//add the triangle to the cube PShape
    cube.addChild(triangleArray.get(i).getShape());
} //end for
//translate the cube according to its position and return it
    cube.translate(position.x, position.y, position.z);
    return cube;
} //end getShape

//get the array of triangles
public ArrayList<Triangle> getTriangleArray()
    { return triangleArray; }
} //end getTriangleArray

//recalculate the normals of each triangle in the marching cube
    public void recalculateNormal(int x, int y, int z, int imageWidth, int imageHeight, int nSlices, Cube[][][] cubeArray)
    { //recalculate normals for the triangles by taking the average of the normals of triangles sharing its vertices
        //set the boundary on which neighbouring cubes to analyze
        int xLower, xUpper, yLower, yUpper, zLower, zUpper;
        xLower = -1;
        yLower = -1;
        zLower = -1;
        
        xUpper = 1;
        yUpper = 1;
        zUpper = 1;
        
        //if the marching cube is on the boundary, than it has less neighbours
        if (x==0){
            xLower = 0;
        } //end if
        else if (x==imageWidth){
            xUpper = 0;
        } //end elseif
        if (y==0){
            //...
yLower = 0;
} // end if
else if (y == imageHeight)
    yUpper = 0;
} // end else

if (z == 0)
    zLower = 0;
} // end else
else if (z == nSlices)
    zUpper = 0;
} // end else

// for each triangle in the cube
for (int i = 0; i < triangleArray.size(); i++)
    // prepare this triangle and declare statistical variables
    Triangle triangle = triangleArray.get(i);
    PVector nBar; // average normal vector
    nBar = new PVector(0, 0, 0);
    float weight = 0; // the sum of all weights used for averaging

    // compare this triangle to the triangles in neighboring cubes
    // for every neighboring cube
    for (int u = xLower; u <= xUpper; u++)
        for (int v = yLower; v <= yUpper; v++)
            for (int w = zLower; w <= zUpper; w++)
                // get all the triangles in the neighboring cube
                ArrayList<Triangle> thatTriangleArray = cubeArray[x+u][y+v][z+w].getTriangleArray();

                // for every triangle in the cube
                for (int j = 0; j < thatTriangleArray.size(); j++)
                    Triangle that = thatTriangleArray.get(j);
                    // if this triangle share a vertex with that triangle
                    if (triangle.isShareVertex(that))
                        // update the normal statistics with the reciprocal area as the weight
                        nBar.add(PVector.mult(that.getNormal(), 1/that.getArea()));
                        weight += 1/that.getArea();
                    } // end if
work out the mean normal and update that triangle’s normal
nBar.mult(1/weight);
triangle.newNormal(nBar);
}
}
}
}
}
//end for
//end for
//end for
//end for
//end recalculateNormal

//COLOUR METHODS
public void setStroke(boolean wantStroke){
   for (int i=0; i<triangleArray.size(); i++){
      triangleArray.get(i).setStroke(wantStroke);
   }
}
//end stroke

public void setFill(boolean wantFill){
   for (int i=0; i<triangleArray.size(); i++){
      triangleArray.get(i).setFill(wantFill);
   }
}
//end fill

public void setStroke(color colour){
   for (int i=0; i<triangleArray.size(); i++){
      triangleArray.get(i).setStroke(colour);
   }
}
//end stroke

public void setFill(color colour){
   for (int i=0; i<triangleArray.size(); i++){
      triangleArray.get(i).setFill(colour);
   }
}
//end fill

}//end class
Triangle.pde (class)

class Triangle{
    // TRIANGLE CLASS
    // An instance from this class can return a triangle PShape according to the 3 edgeIndexes inputted in the constructor

    // MEMBER VARIABLES
    private PVector a, b, c; // position vectors of the vertices of the triangle relative to cube_center
    private PVector cube_center; // the co-od of the center of the cube
    private PVector n; // normal of the triangle
    private PVector nBar; // the mean normal of the triangle
    private float area; // the area of the triangle
    private boolean wantStroke, wantFill;
    private color stroke, fill;

    // CONSTRUCTOR
    // Parameters: 3 edge indexes, which edges the vertices of the triangle should be on
    Triangle(int index1, int index2, int index3, float x, float y, float z) {

        // convert the edge indexes to position vectors
        a = getEdgeCo(index1);
        b = getEdgeCo(index2);
        c = getEdgeCo(index3);

        // get the position vector of the center
        this.cube_center = new PVector(x, y, z);

        // work out the normal vector(n) and the area
        PVector r1, r2;
        r1 = PVector.sub(b, a);
        r2 = PVector.sub(b, c);
        n = r1.cross(r2);
        area = n.mag() / 2;
        n.normalize();
        nBar = n; // nBar will be the averaged normal later on

        // default colouring
        wantStroke = false; // no stroke
wantFill = true; //fill
stroke = color(0,255,0); //green stroke
fill = color(255); //white fill
} //end constructor

//METHODS

//getShape of the triangle
public PShape getShape(){

    //create a PShape
    PShape triangle;
    triangle = createShape();

    //set the colour of the stroke
    if(wantStroke){
        triangle.setStroke(stroke);
    } //end if
    else{
        triangle.setStroke(false);
    } //end else
    
    //set the colour of the fill
    if(wantFill){
        triangle.setFill(fill);
    } //end if
    else{
        triangle.setFill(false);
    } //end else
    
    //draw the triangle and return it
    triangle.beginShape();
    triangle.normal(nBar.x,nBar.z,nBar.y);
    triangle.vertex(a.x,a.z,a.y);
    triangle.vertex(b.x,b.z,b.y);
    triangle.vertex(c.x,c.z,c.y);
    triangle.vertex(a.x,a.z,a.y);
}
triangle.endShape(CLOSE);
return triangle;
 }//end display

//get the normal vector
public PVector getNormal(){
    return n;
}//end getNormal

//update nBar, the averaged normal
public void newNormal(PVector normal){
    nBar = normal;
    nBar.normalize();
}//end newNormal

//GET AREA of triangle
public float getArea(){
    return area;
}//end getArea

//get array of vertices vectors of this triangle
public PVector[] getVerticesVectors(){
    PVector [] verticesArray;
    verticesArray = new PVector[3];
    verticesArray[0] = PVector.add(cube_center,a);
    verticesArray[1] = PVector.add(cube_center,b);
    verticesArray[2] = PVector.add(cube_center,c);
    return verticesArray;
}//end getVertices

//IsShareVertex
//does this triangle share a vertex with that triangle?
public boolean isShareVertex(Triangle that){
    boolean share = false; //assume false
//get the 2 triangles vertices vectors
PVector [] vertexArray1, vertexArray2;
vertexArray1 = this.getVerticesVectors();
vertexArray2 = that.getVerticesVectors();

PVector r1, r2;

//for every vertex
for (int i=0; i<3; i++){
    //compare this vertex
    r1 = vertexArray1[i];
    //for every that vertex
    for (int j=0; j<3; j++){
        r2 = vertexArray2[j];
        //if the coord of both vertex is the same, then they share the same vertex
        if(r1.x==r2.x && r1.y==r2.y && r1.z==r2.z){
            share = true;
            //break the loop
            j=3;
            i=3;
        } //end if
    } //end for
} //end for
return share;
}//end

//COLOUR METHODS
public void setStroke(boolean wantStroke){
    this.wantStroke = wantStroke;
}//end

public void setFill(boolean wantFill){
    this.wantFill = wantFill;
}//end

public void setStroke(color colour){
    this.stroke = colour;
    this.wantStroke = true;
}
public void setFill(color colour){
    this.fill = colour;
    this.wantFill = true;
} // end

} // end class
import ij.*;
import ij.process.*;
import ij.measure.*;
import ij.gui.*;
import ij.plugin.filter.*;
import java.lang.*;
import java.util.*;
import java.awt.*;

public class MaximaAnalysisStack_Plugin implements PlugInFilter {
    ImagePlus imp;

    //GLOBAL VARIABLES
    private int background; //binary value of a background pixel
    private int width, height, nSlices; //dimensions of the image
    private int numberOfSimulations; //number of simulated images to be produced per slice
    private int maximumNoiseTolerance; //the maximum noise tolerance setting for the maxima function
    private double t; //t statistics

    //GLOBAL OBJECTS
    Random random = new Random(); //random number generator
    MaximumFinder maximum_finder = new MaximumFinder(); //maxima_finder object: finds the maximas of an image

    //SETUP
    public int setup(String arg, ImagePlus imp) {
        this.imp = imp;

        //set global variables
        width = imp.getDimensions()[0];
        height = imp.getDimensions()[1];
        nSlices = imp.getDimensions()[3];
        background = 0;
        maximumNoiseTolerance = 5000;

        //t-statistics
        numberOfSimulations = 10;
    }
\[ t = 2.262; \quad //2.5\% \; percentage \; point \]

```java
    return DOES_16; //does only 16 bit images
} //end setup

//RUN
public void run(ImageProcessor image) {
    ImageStack maximaStack = new ImageStack(width, height); //stack of maxima point images
    ByteProcessor sliceMaximas; //imageProcessor object for each slice

    //for every slice
    for (int i = 1; i <= nSlices; i++){
        //obtain the optimized maxima point image of a slice and add it to the stack
        sliceMaximas = getMaximas(image, i);
        maximaStack.addSlice(sliceMaximas);
        //save a copy of the maxima point image
        IJ.save(new ImagePlus(String.valueOf(i), sliceMaximas), "C://Users/Sherman/Documents/chromosomes/Z/term1/maxima_analysis/data/" + i + ".tif");
    } //end for

    //create an image of the stack, show it and save it
    ImagePlus result = new ImagePlus("Result", maximaStack);
    result.show();
    IJ.save(result, "C://Users/Sherman/Documents/chromosomes/Z/term1/maxima_analysis/maximas.tif");
} //end run

//GET MAXIMAS
//Returns an image of the maxima points using a noise tolerance setting which maximizes chi-squared
//Arguments: stack to work on, which slice to work on
private ByteProcessor getMaximas(ImageProcessor image, int slice){

    //set the slice on the stack
    IJ.setSlice(slice);

    //Create an array of simulated images
    ImageProcessor [] simulationArray = new ImageProcessor[numberOfSimulations];
    for (int i=0; i<numberOfSimulations; i++){

    ```
simulationArray[i] = simulate(image);
} //end for

/*/DEBUG: Show all simulation images
for (int i=0; i<numberOfSimulations; i++){
    ImagePlus imp = new ImagePlus(String.valueOf(i), simulationArray[i]);
    imp.show();
} //end for*

//create arrays to store data
double [] noiseTolerance = new double[maximumNoiseTolerance+1]; //noise tolerance settings
double [] dataCount = new double[maximumNoiseTolerance+1]; //number of maxima in the data
double [] simulationCount = new double[maximumNoiseTolerance+1]; //average number of maxima in the simulated images
double [] simulationCountError = new double[maximumNoiseTolerance+1]; //std of maxima in the simulated images
double [] sumSimulation = new double[maximumNoiseTolerance+1]; //sum(x)
double [] sumSquaresSimulation = new double[maximumNoiseTolerance+1]; //sum(x^2)
double [] chiSquared = new double[maximumNoiseTolerance+1]; //poisson chi-squared
double [] chiSquaredError = new double[maximumNoiseTolerance+1]; //estimated standard deviation of chi-squared

//for every noise tolerance setting, find the number of maxima on the data image and put it in the array dataCount
for (int i=0; i<=maximumNoiseTolerance; i++){
    dataCount[i] = maximum_finder.getMaxima(image,(double)i,false).npoints;
    noiseTolerance[i]=i;
} //end for

//for every noise tolerance setting, find the number of maxima on the simulated images and update statistical arrays
double value;
for (int j=0; j<numberOfSimulations; j++) { //repeat simulations for n times
    for (int i=0; i<=maximumNoiseTolerance; i++){
        value = maximum_finder.getMaxima(simulationArray[j],(double)i,false).npoints;
        sumSimulation[i] += value;
        sumSquaresSimulation[i] += value*value;
    } //end for
} //end for
//calculate statistics
for (int i=0; i<=maximumNoiseTolerance; i++){
    //mean
    simulationCount[i] = sumSimulation[i]/numberOfSimulations;
    //standard deviation
    simulationCountError[i] = Math.sqrt((sumSquaresSimulation[i] -
    Math.pow(sumSimulation[i],2)/numberOfSimulations)/(numberOfSimulations-1));
    //chi-squared
    chiSquared[i] = Math.pow(simulationCount[i]-dataCount[i],2)/simulationCount[i];
    //estimated standard deviation of chi-squared
    chiSquaredError[i] =
    chiSquared[i]*simulationCountError[i]*(simulationCount[i]+dataCount[i])/(simulationCount[i]*Math.abs(simulationCount[i]-dataCount[i]));
}

//end for

//develop a curve fitter which fits a curve for noiseTolerance VS chiSquared
CurveFitter curve = new CurveFitter(noiseTolerance,chiSquared);
curve.doFit(CurveFitter.POLY8);
double [] parameters = curve.getParams(); //array of parameters

//create a new results table which will display statistics
ResultsTable results = new ResultsTable();

//declare variables to find the optimal noise tolerance
double maxChiSquared = 0;
double optimalNoiseTolerance = 0;
double f; //value of curve at a certain point

//get the data from arrays and put it on the ResultsTable...
//...find the optimal noise tolerance setting...
//...while there is data in the arrays
int i;
do {
    //increment the counter and store it in i => noise tolerance = i-1
    results.incrementCounter();
i = results.getCounter();
    //put data into results
results.addValue("Noise tolerance",i-1);
results.addValue("Data",dataCount[i-1]);
results.addValue("Simulation",simulationCount[i-1]);
results.addValue("Standard Deviation",simulationCountError[i-1]);
results.addValue("95% confidence error",t*simulationCountError[i-1]/Math.sqrt(numberOfSimulations));
results.addValue("Chi-squared",chiSquared[i-1]);
results.addValue("+/-",chiSquaredError[i-1]);
f = curve.f(parameters,i-1);
results.addValue("Curve fit",f);

//update optimalNoiseTolerance if found a new maxChiSquared
if (f>maxChiSquared){
    maxChiSquared = f;
    optimalNoiseTolerance = i-1;
}

} while(results.getCounter()<=maximumNoiseTolerance);
//end do-while

//show results table and save it
results.addValue("Optimal", optimalNoiseTolerance);
results.show("Results");
try{
    results.saveAs("C:/Users/Sherman/Documents/chromosomes/Z/term1/maxima_analysis/data/results"+slice+".csv");
} catch (Exception exception){
    IJ.showStatus("Cannot save results in slice "+slice);

} return
maximum_finder.findMaxima(image, optimalNoiseTolerance, ImageProcessor.NO_THRESHOLD, MaximumFinder.SINGLE_POINTS, false, false);
//end get maximas

//SIMULATE
//Creates a Monte Carlo simulated image
// Each non-background pixel is arranged randomly WITH REPLACEMENT

private ImageProcessor simulate(ImageProcessor image) {

    // count number of signal pixels n
    int n = 0;
    for (int x = 0; x < width; x++) {
        for (int y = 0; y < height; y++) {
            if (image.getPixel(x, y) != background) {
                n++;
            } // end if
        } // end for
    } // end for

    // store pixel values in array of length n
    int[] pixelArray = new int[n]; // array of pixel values in the original image
    int i = 0;
    for (int x = 0; x < width; x++) {
        for (int y = 0; y < height; y++) {
            if (image.getPixel(x, y) != background) {
                pixelArray[i] = image.getPixel(x, y);
                i++;
            } // end if
        } // end for
    } // end for

    // replace signal pixels with a random signal pixel in the array
    ImageProcessor result = new ShortProcessor(width, height);
    for (int x = 0; x < width; x++) {
        for (int y = 0; y < height; y++) {
            // If not background, put random pixel from array
            if (image.getPixel(x, y) != background) {
                result.putPixel(x, y, pixelArray[random.nextInt(n)]);
            } // end if
            // else put a background pixel
            else {
                result.putPixel(x, y, background);
            } // end else
        } // end for
    } // end for
}
//return the image

return result;
}
}//end procedure

}//end class
NEAREST NEIGHBOUR SEARCH AND DISTANCE TO CENTER OF MASS
Main.pde (functions and procedures)

//SETUP
void setup()
{
    //make csv output
    PrintWriter output = createWriter("data.csv");

    float xScale = 11; //11 nm per pixel
    float yScale = 11; //11 nm per pixel
    float zScale = 20; //20 nm per pixel

    int nSlices = 117; //number of slices (117)

    //save slices in array
    PImage [] imageArray;
    imageArray = new PImage[nSlices];
    //for every slice, load and save image in the array
    for (int i=0; i<10; i++){
        imageArray[i] = loadImage("maximas00"+i+".png");
    } //end for
    for (int i=10; i<100; i++){
        imageArray[i] = loadImage("maximas0"+i+".png");
    } //end for
    for (int i=100; i<nSlices; i++){
        imageArray[i] = loadImage("maximas"+i+".png");
    } //end for

    //set size of image
    size(imageArray[0].width,imageArray[0].height);

    ArrayList<PointVector> PointVectorArray = new ArrayList<PointVector>(); //array list of PointVectors
    PVector COM = new PVector(0,0,0); //center of mass
    int n = 0; //number of points

    //for every slice
    for (int z=0; z<nSlices; z++){
        PImage slice = imageArray[z]; //get slice
//load the pixels in the slice
slice.loadPixels();
color colour;

//for every pixel in the slice
for (int x=0; x<slice.width; x++){
    for (int y=0; y<slice.height; y++){
        //get the colour of that pixel
        colour = slice.pixels[x+y*width];
        //if white
        if (colour == color(255)){
            //make new PointVector
            PointVectorArray.add(new PointVector(x*xScale,y*yScale,z*zScale));
            //update COM statistics
            COM.x += x*xScale;
            COM.y += y*yScale;
            COM.z += z*zScale;
            n++;
        } //end if
    } //end y for
} //end x for

//compute the center of mass
COM.div(n);

//find distance to nearest neighbours
//for every pointVector
for (int j=0; j<PointVectorArray.size(); j++){
    //find and print the distance to the nearest neighbour
    output.print(PointVectorArray.get(j).findDistanceToNearestNeighbour(PointVectorArray));
    output.print(", ");
    //find and print the distance to the center of mass
    output.println(PointVectorArray.get(j).distanceTo(COM));
} //end for

//flush and close output
output.flush();
output.close();
println("done");
} // end setup

void draw()
} // end draw
PointVector.pde (class)

class PointVector{

    // MEMBER VARIABLES
    private PVector r; // position vector

    // CONSTRUCTOR
    PointVector(float x, float y, float z){
        this.r = new PVector(x,y,z);
    } // end constructor

    // METHOD
    private PVector getPVector(){
        return r;
    } // end getPVector

    public float distanceTo(PVector u){
        return r.dist(u);
    } // end distanceTo

    private float distanceTo(PointVector u){
        return r.dist(u.getPVector());
    } // end distanceTo

    // find distance to nearest neighbour using an array of PointVector in the parameter
    public float findDistanceToNearestNeighbour(ArrayList<PointVector> array){
        float dMin = pow(width,2)+pow(height,2); // distance to nearest neighbour, set to to the length of the diagonal squared first
        // for every maxima
        for (int i=0; i<array.size(); i++){
            // get the position vector of that maxima
            float d = this.distanceTo(array.get(i));
            if (d < dMin && d!=0){ // if d is less than dMin and not equal to zero
                dMin = d;
            }
        } // end for
        return dMin; // return the distance to nearest neighbour
    }
}
};//end findDistanceToNearestNeighbour
};//end class
MODELLING MAXIMAS AS SPHERES

//import camera
import peasy.*;

//GLOBAL VARIABLES
int nSlices = 29; //number of slices
PeasyCam cam; //camera
PShape chromosome; //PShape for chromosome
float R = 29.0078145178338; //mean distance to nearest neighbour
float xScale = 11; //scale
float yScale = 11; //scale
float zScale = 20; //scale

//SETUP
void setup(){
  size(1024,768,P3D);
cam = new PeasyCam(this,1000); //camera
ArrayList<PVector> maximaArray = new ArrayList<PVector>(); //arrayList of position vectors of maxima
  //save the slices in an array
  PImage [] imageArray; //array of images
  imageArray = new PImage[nSlices]; //nSlices in the array

  //save the slices in an array
  for (int i=0; i<10; i++){
    imageArray[i] = loadImage("maximas0"+i+".png");
  }

  //get dimensions of the image
  int imageWidth = imageArray[0].width;
  int imageHeight = imageArray[0].height;

  //for each slice
for (int z=0; z<nSlices; z++) {
    // get PImage of the slice and load the pixels
    PImage slice = imageArray[z];
    slice.loadPixels();

    // for every pixel
    for (int x=0; x<slice.width; x++) {
        for (int y=0; y<slice.height; y++) {
            // if that pixel is white
            if (slice.pixels[x+y*slice.width] == color(255)) {
                maximaArray.add(new PVector(x, y, z)); // add the position vector to maximaArray
            }
        }
    }
}

// create PShape for chromosome
chromosome = createShape(GROUP);
sphereDetail(20); // set level of detail to be used to draw spheres

// for every maxima
for (int i=0; i<maximaArray.size(); i++) {
    noStroke(); // noStroke
    PShape sphere = createShape(SPHERE, R); // create a sphere
    sphere.setFill(color(255, 255, 255)); // fill the sphere white
    PVector r = maximaArray.get(i); // get position vector of the maxima
    sphere.translate(r.x*xScale, r.y*yScale, r.z*zScale); // translate the sphere according to the position vector
    sphere.translate(-imageWidth*xScale/2, -imageHeight*yScale/2, -((float)nSlices)*zScale/2);
    chromosome.addChild(sphere); // add the sphere to the chromosome PShape
}

// DRAW
void draw() {
    background(0); // black background
//lights
ambientLight(50, 50, 50);
directionalLight(255, 255, 255, 0, 1, 1);
directionalLight(255, 255, 255, 0, -1, -1);

//draw chromosome
shape(chromosome);
//print frameRate
println(frameRate);
}//end draw
CONNECTING MAXIMAS TOGETHER USING LINES

//import camera
import peasy.*;

//GLOBAL VARIABLES
int nSlices = 117; //number of slices (117)
PeasyCam cam; //camera
PShape chromosome; //PShape for chromosome
float R = 29.0078145178338; //mean distance to nearest neighbour
float STD = 7.71405915186672; //standard deviation distance to nearest neighbour
float xScale = 11; //scale
float yScale = 11; //scale
float zScale = 20; //scale

//SETUP
void setup(){
  size(1024,768,P3D);
cam = new PeasyCam(this,1000); //camera
ArrayList<PVector> PVectorArray = new ArrayList<PVector>(); //array list of position vectors of maximas

  //save the slices in an array
  PImage [] imageArray; //array of images
  imageArray = new PImage[nSlices]; //nSlices in the array

  //load all the slices
  for (int i=0; i<10; i++){
    imageArray[i] = loadImage("maximas00"+"i"+.png);
  } //end for
  for (int i=10; i<100; i++){
    imageArray[i] = loadImage("maximas0"+"i"+.png);
  } //end for
  for (int i=100; i<nSlices; i++){
    imageArray[i] = loadImage("maximas"+"i"+.png);
  } //end for

  //get imageWidth and imageHeight
float imageWidth = imageArray[0].width;
float imageHeight = imageArray[0].height;

//for every slice
for (int z=0; z<nSlices; z++){
    PImage slice = imageArray[z];  //get slice
    slice.loadPixels();  //load the pixels in the slice
    color colour;

    //for every pixel in the slice
    for (int x=0; x<imageWidth; x++){
        for (int y=0; y<imageHeight; y++){
            colour = slice.pixels[x+y*slice.width];  //get the colour of that pixel
            //if white, add maxima position vector to PVectorArray
            if (colour == color(255)){
                PVectorArray.add(new PVector(x*xScale,y*yScale,z*zScale));  
            }  
        }
    }
}

//create PShape chromosome
chromosome = createShape(GROUP);

//for every pair of maxima
for (int i=0; i<PVectorArray.size()-1; i++){
    for (int j=i+1; j<PVectorArray.size(); j++){
        PVector a = PVectorArray.get(i);
        PVector b = PVectorArray.get(j);
        float r = a.dist(b);  //get distance between maxima

        //if less than 2 standard deviations away from the mean
        if (r<=R+2*STD){
            if (r<R-STD){  //if less than mean-1*standardDeviation
                stroke(255,0,0);  //set stroke to read
            }  
        } else if (R-STD<=r && r<=R+STD){  //if within one standard deviation from mean
stroke(0,255,0); //set stroke to green
} //end elif
else { //else (between one and two standard deviation as away from mean)
    stroke(0,0,255); //set stroke to blue
} //end else
PShape line = createShape(LINE,a.x,a.y,a.z,b.x,b.y,b.z);
line.translate(-imageWidth*xScale/2, -imageHeight*yScale/2, -((float)nSlices)*zScale/2);
chromosome.addChild(line);
} //end if
} //end j for
} //end i for
} //end setup

//DRAW
define draw(){
    background(0); //set background to black
    shape(chromosome); //draw chromosome
    println(frameRate); //print the frameRate
} //end draw
VII. REFERENCES


