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# Staining Strategies for Imaging of Metaphase Chromosomes

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# Abstract

The higher order structure of chromosomes remains unclear today, especially between the resolution of the nucleosome and that of visible light. There are various imaging techniques that can be used in studying chromosomes: x-ray, visible light, fluorescence, and electron microscopy. Staining increases the contrast of chromosomes under these different imaging techniques and allows the characteristic "banding" to become visualized, which in turn allows the identification of chromosomes by the pattern of segregation between euchromatin and heterochromatin. Fluorescent banding is advantageous over visible light banding because it allows the chromosomes to be simultaneously banded and hybridized in situ with probes. This study uses the principle of counterstaining to develop various combinations of fluorochromes that produce bands of higher resolution than that of a single fluorochrome. DAPI/Actinomycin D and SYTOX green/methyl green combinations were studied. The Q- and R-banding patterns of some of the 24 human metaphase chromosomes had been identified. Furthermore, a platinum blue complex was synthesized and applied as a heavy metal stain, together with a fluorescent platinum complex, for SEM characterization of chromosomes. These metal complexes increased the signal level and contrast obtained from SEM, with the best contrast achieved with platinum blue. The secondary electron images showed good topography wherein knot-like structures and parallel fibers were identified in the chromosome arm and centromere, respectively, while the BSE images showed the distribution and concentration of DNA along the length of the chromosomes.

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## 1.1 Chromosome Structure

Chromosomes contain the genetic material of the cell, which determines form and functions. Hence, abnormalities in the chromosomes such as deletions, insertions, and duplications may cause genetic disorders and may be fatal. Chromosomes consist of DNA and proteins, which are organized into nucleosomes. Nucleosomes are then arranged into 11-nm elementary fibers. Higher order coiling is achieved when these fibers start to coil into 30-nm fibers called solenoids. From this structure, the chromosomes continue to condense until it reaches the metaphase stage of mitosis (figure 1.1) and this higher order organization of the chromosomes remains unclear today [1, 2].

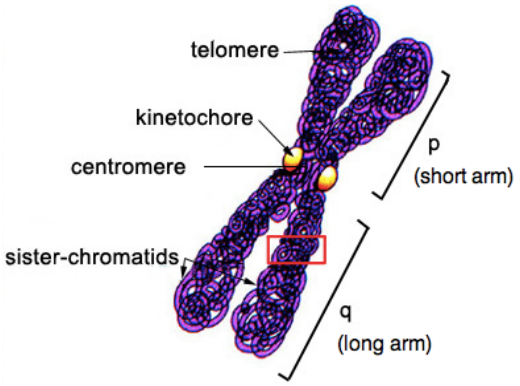


**Figure 1.1.** Features of a metaphase chromosome.

There are various imaging techniques that can be used in studying chromosome structure. These include x-ray, visible light, fluorescence, and electron microscopy. Light and fluorescence microscopy does little damage to the chromosomes but have limited resolution [3]. Electron microscopy provides higher resolution than the said techniques. However, 3-D information about the chromosome structure is hard to obtain as scanning electron microscopy (SEM) is only surface-sensitive and transmission electron microscopy (TEM) has a sample thickness limit of approximately 100 nm [2]. Coherent x-ray diffraction imaging (CDI), an x-ray-based imaging technique, provides high resolution and 3-D images of the chromosome structure but suffers from the radiation damage induced on the chromosome samples [1].

## 1.2 Overview of Staining and Banding

Staining increases the contrast of chromosomes under different imaging techniques. Differential staining along the length of a chromosome results in a characteristic striped appearance called “banding”. The characteristic banding pattern of each chromosome allows the identification of the chromosomes [4] and abnormalities [5] present in it (figure 1.2).

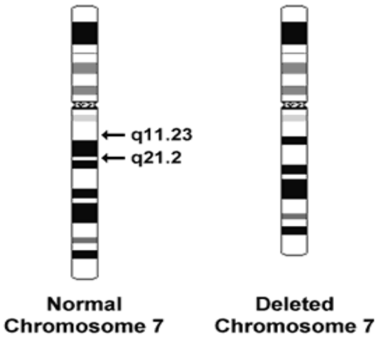


**Figure 1.2.** Deletion of a DNA sequence.

Furthermore, the bands provide an insight on the chromosomal substructures and organization wherein the heterochromatic and euchromatic regions of the chromosomes can be identified [6-8]. Heterochromatin are chromatin regions that remain condensed throughout the cell cycle and are inactive in transcription while euchromatin are chromatin regions that are decondensed in the interphase and are active in transcription [9-11]. Heterochromatin can be further classified as constitutive or facultative. Constitutive heterochromatin, consisting of repetitive sequences of DNA, can be found in the centromere and telomere of the chromosomes and remains condensed all throughout the cell cycle while facultative heterochromatin can decondense into euchromatin if triggered by several factors [11, 12].

## 1.3 Modes of Stain Binding

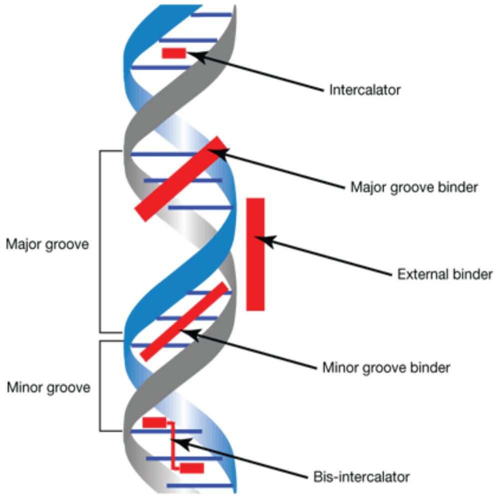


**Figure 1.3.** Binding modes of a stain with DNA.

There are several ways a stain can bind to the chromosomes. The stains that are discussed in this report are specific for DNA. A stain can bind to the DNA through intercalation, minor groove or major groove binding, or external binding (figure 1.3). These binding modes depend on the type of interaction between the stain and the DNA, which can either be covalent (metal coordination) or noncovalent (hydrogen bonding, electrostatic interactions, and π- π interactions). Table 1.1 summarizes the different binding modes with the corresponding type of interaction involved, the functional group of the stain responsible for the interaction, and the DNA site where the interaction occurs.

**Table 1.1.** Binding modes of a stain with DNA and the type of interaction involved.

| **Binding Mode** | **Type of Interaction** | **Functional Group of Stain** | **DNA site** |
|---|---|---|---|
| Major or minor groove binding | Hydrogen bonding | Groups w/ an electronegative atom (e.g. N or O) | H-donor and H-acceptor sites of the base pairs |
| | Metal coordination | Metal | N atom of the base pairs |
| External binding | Electrostatic interaction | Cationic groups | Phosphate groups |
| Intercalation | π- π interaction | Planar aromatic groups | Stain inserts between the base pairs |

Figure 1.4 shows the DNA base pairs with the atomic positions, and major and minor groove labels. The H-donor sites of the base pairs are positions 6 of adenine, 4 of cytosine, and 2 of guanine while the H-acceptor sites are positions 3 and 7 of adenine and guanine, and 2 of thymine.

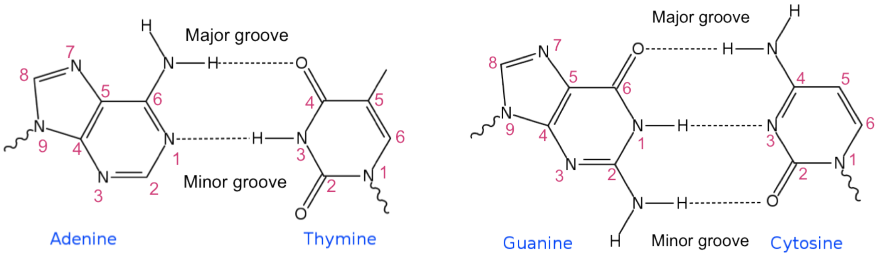


**Figure 1.4.** DNA base pairs.

Moreover, a stain can also bind to the DNA through several binding modes if it contains functional groups that can produce more than one type of interaction with the DNA [13].

## 1.4 Common Banding Patterns

The common banding patterns that can be produced are G-, R-, and C-banding pattern. G-banding is the most widely used banding method for cytogenetic analysis that was first developed by Seabright in 1971 [14]. This technique, which is nonfluorescent, involves the use of a visible light dye called giemsa and digestion of the chromosomes with trypsin before staining. The positive G-bands (dark bands) correspond to the heterochromatic regions of the chromosomes and are AT-rich [8]. Hence, a similar banding pattern can also be obtained through fluorescent staining with the use of fluorochromes that are specific for AT-rich regions of the chromosomes.

R-bands and C-bands can also be obtained using giemsa but with a different extent of denaturation induced on the chromosome structure before staining [8]. The positive R-bands correspond to the negative G-bands and reveal the GC-rich euchromatin. Hence, the R-banding pattern can also be obtained using fluorochromes that are specific for GC-rich regions of the chromosomes. C-bands, on the other hand, reveal the AT-rich centromere, which consists of constitutive heterochromatin. Table 1.2 summarizes the different chromosomal regions and banding techniques that reveals them.

**Table 1.2.** Chromosomal regions and banding techniques that reveal them.

| Chromosomal Region | Banding Technique |
|---|---|
| Constitutive heterochromatin | C- and G-banding |
| Facultative heterochromatin | G-banding |
| Euchromatin | R-banding |

## 1.5 Staining with Fluorochromes

Fluorochromes are organic molecules that are capable of undergoing fluorescence and are used for fluorescent staining. These molecules contain large conjugated systems such as aromatic or heterocyclic groups and are characterized by rigid and planar structures [15]. Although they are less stable than visible light dyes, they offer several advantages in banding [5, 16]. Unlike in giemsa banding, fluorochromes do not require a pretreatment process, which leads to the extraction of proteins from the chromosomes, in order for banding to occur. This is particularly important in studying the chromosome structure.

Furthermore, fluorochromes allow the chromosomes to be simultaneously banded and hybridized in situ with probes.

Fluorochromes produce bands either through differential fluorescence or differential binding, depending on the fluorochrome structure. Differential fluorescence is responsible for banding using fluorochromes that uniformly bind throughout the length of the chromosome, which are usually intercalators [17]. Banding occurs as a result of the quenching of the fluorochrome's fluorescence at certain chromosome regions. Meanwhile, differential binding is responsible for banding using fluorochromes that have a binding specificity and attach only to certain regions of the chromosomes. Such fluorochromes are usually major or minor groove binders and contain functional groups that are capable of forming H-bonds either with the AT or GC base pairs of the DNA [18].

## 1.6 Counterstaining

Counterstaining is a technique that is used to induce banding with fluorochromes that bind and fluoresce uniformly throughout the chromosome. It is also used to enhance banding patterns that do not have a very high resolution. This technique involves the use of a primary fluorescent stain and a fluorescent or a no fluorescent counterstain. If a fluorescent counterstain is used, the emission wavelength of the counterstain must differ from that of the primary stain.

Two mechanisms are responsible for the formation or enhancement of the bands: electronic energy transfer and direct binding competition. Electronic energy transfer is a process wherein the counterstain absorbs the fluorescence of the primary stain. In order for this process to occur, there must be a spectral overlap of the fluorescence emission of the primary stain and the absorption of the counterstain [19]. Meanwhile, direct binding competition involves the selective displacement of the primary stain by the counterstain. The primary stain and the counterstain should have the same binding mode [20].

There are various possible combinations of primary stains and counterstains that can be used in counterstaining. The important factors to consider based on the mechanisms involved in counterstaining are the binding specificity and spectral properties of the stains [20].

## 1.7 Staining with Heavy Metals

The use of a heavy metal complex or compound as a stain for biological specimens such as the chromosomes is particularly important when imaging the specimens with electron microscopy or x-ray-based techniques. The heavy metal coating increases the scattering of electrons and x-rays, thus enhancing the signal obtained from the said imaging techniques. Furthermore, through heavy metal staining, specimen conductivity is increased thus charging effects observed in SEM, especially for nonconductive samples, are avoided.

The most commonly used heavy metal stains for biological specimens are uranyl acetate ($UO_2(CH_3COO)_2$) and osmium tetroxide ($OsO_4$). They are known to scatter electrons well and adsorb to biological matter easily [21, 22]. Uranyl acetate can interact with chromosomes by binding ionically with the phosphate groups of the DNA [23]. Meanwhile, $OsO_4$ can stain chromosomes through its reaction with the basic amino acids of the histones. However, the chromosomes must be first treated with dilute acetic acid to expose these basic amino acids, which are linked to the DNA [24]. Both uranyl acetate and $OsO_4$ are very toxic compounds thus the need for safer and easily obtainable heavy metal stains.

Metal complexes such as those containing Pt, Ru, and Ir can also be applied as heavy metal stains for biological substances. In particular, platinum-organic complexes such as platinum blue, which are specific for DNA, have been used as stains for chromosomes. Platinum blue is a polymeric compound of deep blue, green, or purple in which platinum is coordinated with amide groups [25]. These platinum-organic complexes are relatively safer than uranyl acetate and $OsO_4$, and can be easily synthesized.

## 1.8 Objectives of the Study

The general objective of this study is to investigate different staining strategies to improve the imaging of chromosomes, which in turn will bring advancements in the study of the chromosome structure.

**The specific objectives are:**

1. To develop various fluorochrome combinations for counterstaining to produce bands of higher resolution than that of a single fluorochrome;
2. To obtain the banding patterns of human metaphase chromosomes;
3. To synthesize platinum blue and apply it as a heavy metal stain for SEM characterization of chromosomes;
4. To determine the efficiency of platinum blue and a fluorescent platinum complex as heavy metal stains; and
5. To obtain information on the structure of metaphase chromosomes from SEM studies.

## 2.1 Reagents

All reagents used in the experiments were supplied by Sigma-Aldrich Ltd., UK except for SYTOX green, which was supplied by Life Technologies Ltd., UK. The reagents were used as supplied and without further purification. Barley metaphase chromosomes fixed in a solution of 3:1 ethanol:acetic acid were provided by Dr. Elizabeth Schroeder-Reiter of the Ludwig-Maximillians University, Martinsried, Germany. PtLCl (L = 1,3-di(2-pyridyl)benzene) in phosphate buffer saline solution (PBS) was provided by Dr. Stan Botchway of the Science and Technology Facilities Council, UK and Dr. Julia Weinstein of the University of Sheffield, UK.

## 2.2 DAPI/AMD Banding

Prior to staining, human metaphase chromosomes fixed in a solution of 3:1 methanol:acetic acid were dropped onto glass slides from a height of at least 30 cm. The prepared glass slides were preincubated in a McIlvaine (citric acid/sodium phosphate dibasic) buffer at pH 6.9-7.0. The chromosomes were then stained with 300 μL of 0.25 mg/mL actinomycin D in methanol/McIlvaine buffer for 15-20 min and rinsed with the buffer. Afterwhich, the chromosomes were stained with 300 μL of 0.1 μg/mL DAPI in McIlvaine buffer for 5-10 min and rinsed with the buffer. Optimization of the actinomycin D and DAPI staining times were performed. The prepared DAPI solution is stable for at least 1 month at 4°C.

## 2.3 SYTOX Green/Methyl Green Banding

Human metaphase chromosomes prepared on glass slides were stained with 300 μL of 0.3 μM SYTOX green in phosphate buffer at pH 6.5 for 5 min and then rinsed with a PBS solution. Afterwhich, the chromosomes were stained with 300 μL of 1% methyl green in phosphate buffer at pH 6.5 for 1-3 hours and then rinsed twice with PBS solution. Optimization of the methyl green staining time was performed. The prepared SYTOX green solution is stable only for 1 hour at room temperature due to surface adsorption while the methyl green solution is stable for at least 1 month at -20°C.

## 2.4 Synthesis of Platinum Blue

Potassium tetrachloroplatinate (2 g) was mixed with acetonitrile (3 mL) in 40 mL of water at room temperature. The reaction was left for 10 days afterwhich yellow crystals were obtained. The liquid was decanted, and the crystals were air-dried and weighed. The crystals were then mixed vigorously with the same amount of silver sulphate in a fivefold volume of water until the blue color reached its maximum intensity afterwhich a 10-fold volume of methanol was added to the solution. The solution was filtered followed by the addition of diethyl ether to the filtrate to precipitate the platinum blue. The platinum blue was then filtered out from the solution and air-dried. As a powder, or in water, platinum blue is stable for at least 1 month at room temperature.

## 2.5 Chromosome Preparation for SEM

Marked glass slides were cleaned with ethanol and kept inside the freezer at -20°C prior to the sample preparation. Barley metaphase chromosomes fixed in a solution of 3:1 ethanol:acetic acid were dropped from a height of 40-60 cm onto a cold glass slide with a wetted surface. 45% acetic acid was dropped onto the glass slide just as the drop of chromosome solution had almost disappeared. The glass slide was then immediately covered with a coverslip and placed upside down on dry ice for 15 min afterwhich the cover slip was flickered. 2.5% glutaraldehyde in 75 mM cacodylate buffer was added onto the glass slide. The glass slide was left for 15 min and then rinsed with cacodylate buffer and dehydrated with three washes of acetone for 5, 10, and 15 min. Hexamethyldisilazane (HMDS) was added onto the glass slide and the glass slide was left until the HMDS had evaporated.

For chromosome samples that were to be stained, 60 μL of stain (2 mM platinum blue in water or 10 mM PtLCl in PBS solution) was added onto the glass slide after the washing of the glass slide with cacodylate buffer. The glass slide was left for 30 min and then rinsed three times with water for 5, 10, and 15 min, dehydrated with acetone, and dried with HMDS.

## 2.6 SEM Characterization of Chromosomes

The prepared chromosome samples for SEM were sputter coated with 3-4 nm layer of carbon and characterized in a bright-field light microscope to map the positions of the chromosomes in the marked slides. The samples were characterized with a Jeol JSM-7401F field-emission scanning electron microscope operated at high vacuum mode and a voltage of 4 kV. Secondary and back-scattered electron images of the samples were obtained from this.

## 3.1 DAPI/AMD Banding

The principle of counterstaining was used to enhance the banding patterns of human metaphase chromosomes obtained with the fluorochrome 4’-6-diamidino-2-phenylindole (DAPI). DAPI (figure 3.1) is a nucleic acid-specific fluorochrome that is widely used in chromosome staining because of its high quantum yield ($\phi = 0.92$) when bound to chromosomes [26]. Free molecules of DAPI in water have no fluorescence thus no background fluorescence is observed and proper visualization of the chromosomes is achieved. Furthermore, the fluorescence of DAPI does not fade easily as compared with that of other fluorochromes such as quinacrine and chromomycin A3 [5].

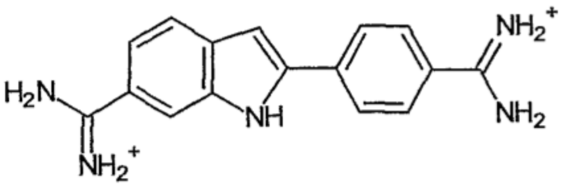


**Figure 3.1.** Structure of DAPI.

DAPI was used in combination with a nonfluorescent counterstain called Actinomycin D (AMD). Human metaphase chromosomes prepared in glass slides were stained with AMD followed by DAPI. Optimization of the AMD and DAPI staining times were performed (table 3.1). Furthermore, a slide with chromosomes stained with DAPI alone was also prepared as a control (figure 3.2).

**Table 3.1.** Optimization of AMD and DAPI staining times.

| Slide | Time in AMD, min | Time in DAPI, min |
|---|---|---|
| 1 | 0 | 10 |
| 2 | 20 | 10 |
| 3 | 15 | 5 |
| 4 | 20 | 5 |
| 5 | 15 | 10 |

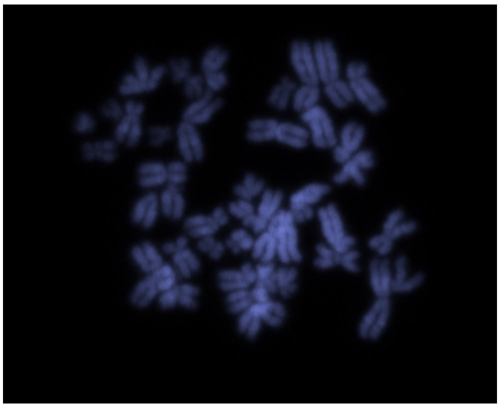


**Figure 3.2.** Human metaphase chromosomes stained with DAPI for 10 min.

It can be observed from figure 3.2 that bands are not evident in the chromosomes stained with DAPI only and the fluorescence intensity along the length of the chromosomes is almost uniform. At low DAPI/DNA ratio, DAPI binds to the minor groove of consecutive (3 to 4 base pairs) AT-rich sequences of the DNA (figure 3.3) through H-bonding with the nitrogen atoms at position 3 of the adenine bases and/or with the oxygen atoms at position 2 of the thymine bases [27, 28]. Hence, in theory, a banding pattern similar to that obtained from G-banding can be obtained with DAPI as a result of its binding specificity.

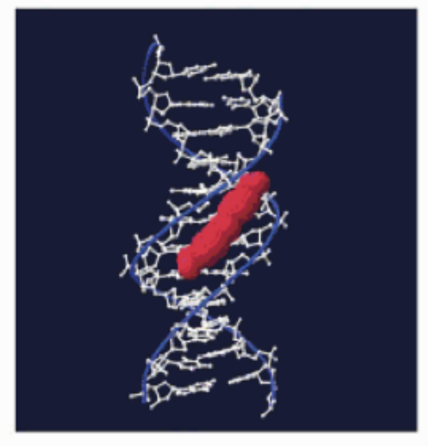


**Figure 3.3.** Binding of DAPI to the minor groove.

However, the bands produced with DAPI are not very clear, which is exactly what can be observed in figure 3.2, as compared with those obtained from other fluorochromes [29].

This is a result of the possibility of DAPI binding to the GC-rich regions of the DNA [18, 30]. At high DAPI/DNA ratio, heterogeneity in the binding mode of DAPI occurs. DAPI can either intercalate between GC base pairs in DNA regions that do not contain at least three consecutive AT base pairs or bind to the major groove of the GC rich regions of the DNA through H-bonding.

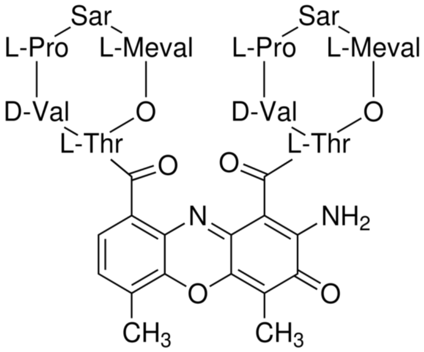


**Figure 3.4.** Structure of AMD.

By staining the chromosomes with AMD (figure 3.4) before DAPI, clear bands were produced (figure 3.5). AMD is an antibiotic that binds specifically to the GC-rich regions of the DNA. The spectral properties and DNA sequence specificity of DAPI and AMD are presented in table 3.2 to demonstrate how the banding works.

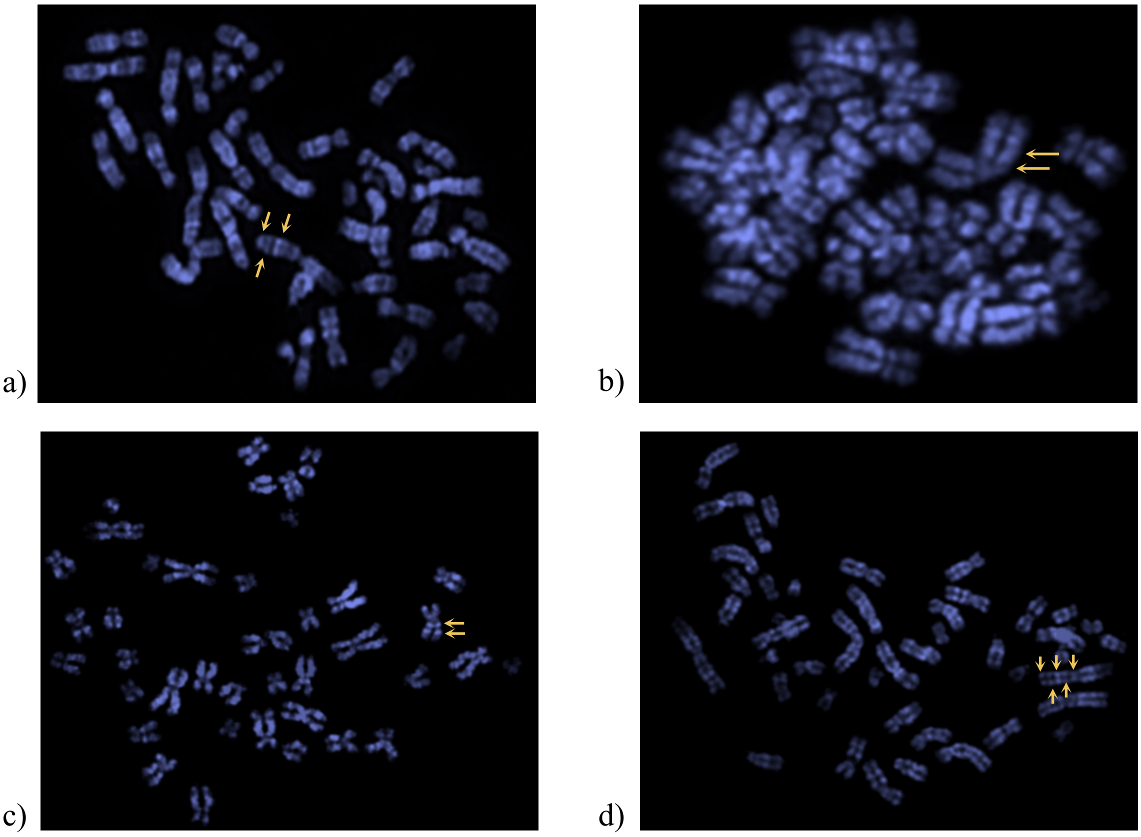


**Figure 3.5.** Human metaphase chromosomes stained with: a) AMD for 20 min and DAPI for 10 min, b) AMD for 15 min and DAPI for 5 min, c) AMD for 20 min and DAPI for 5 min, and d) AMD for 15 min and DAPI for 10 min.

**Table 3.2.** Spectral properties and DNA sequence specificity of DAPI and AMD.

| Dye | Specificity | Absorption max. (nm) | Fluorescence max. (nm) |
|---|---|---|---|
| DAPI | AT | 355 | 450 |
| Actinomycin D | GC | 455 | - |

Table 3.2 shows that there is an overlap between the emission wavelength of DAPI and the absorption wavelength of AMD, which then allows for electronic energy transfer or Förster resonance energy transfer (FRET) to occur. During FRET, the energy of the DAPI molecules, which are bound to the GC-rich regions of the DNA, in their excited state is transferred nonradiatively to the AMD molecules as a result of dipole-dipole interactions between DAPI and AMD. As a result, the fluorescence of the DAPI molecules bound in the GC-rich regions of the DNA is quenched and bands are enhanced. Moreover, the AMD molecules are bound to the GC-rich regions of the DNA. Thus, there is a possibility that the AMD molecules block the binding of the DAPI molecules to these regions, which then also leads to the enhancement of bands.

A Q-banding pattern is obtained from DAPI/AMD banding wherein the bright positive bands correspond to the AT-rich, late-replicating heterochromatic regions of the chromosomes and are similar to the dark positive G-bands minus the G-bands representing the centromere, which consists of the constitutive heterochromatin. Hence, the Q-bands represent the facultative heterochromatin.

From the optimization, the optimal AMD and DAPI staining times are 15 min and 10 min, respectively (figure 3.5.d). It can be observed from figure 3.5 that staining the chromosomes with DAPI for only 5 min did not produce very clear bands as compared with staining the chromosomes with DAPI for 10 minutes. If the DAPI staining time is too short, not enough DAPI is bound to the chromosomes thus causing the differences in the fluorescence intensity along the length of the chromosomes to be small and hard to discriminate. However, it is important not to stain the chromosomes with DAPI for too long as this may cause the DAPI molecules to displace the bound AMD molecules. Furthermore, it can be observed also that varying the AMD staining time from 15 to 20 min have little effect on the resolution of the bands produced. It is important, however, that enough time is given for the AMD molecules to bind in the GC-rich regions of the chromosomes.

After the optimization process, a software called ImageJ was used to obtain the banding patterns of the chromosomes. The chromosomes were separated and straightened. A plot of fluorescence intensity versus pixel for each chromosome was then generated. In order to identify what chromosome number the obtained banding patterns belong to, the experimental banding patterns were compared with literature Q-banding patterns. However, before comparing the obtained patterns with those from literature, the banding patterns from each metaphase spread were grouped based on the relative sizes (figure 3.6, table 3.3) and centromere positions (figure 3.7, table 3.4) of the chromosomes in order to make chromosome identification easier.

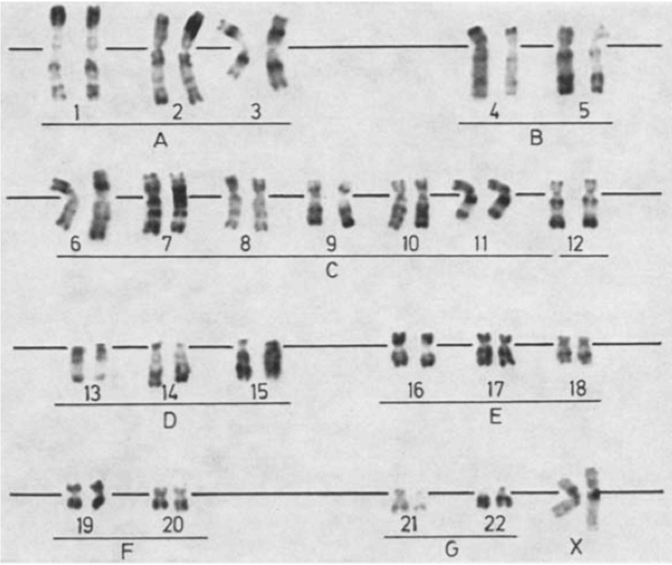


**Figure 3.6.** Relative sizes of human metaphase chromosomes [31].

**Table 3.3.** Human metaphase chromosomes grouped based on relative size.

| Chromosome | Relative Size, pixels |
|---|---|
| 1 & 2 | 100 |
| 3-6 | 80 |
| 7, X | 70 |

| | |
|---|---|
| 8, 10, 12 | 60 |
| 9, 11, 14, 15 | 50 |
| 13, 16 | 40 |
| 17 | 35 |
| 18 & 19 | 30 |
| 20-22, Y | 25 |

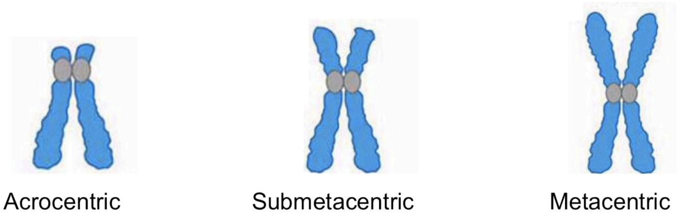


**Figure 3.7.** Types of chromosomes based on centromere position.

**Table 3.4.** Human metaphase chromosomes grouped based on centromere position.

| Chromosome | Centromere Position |
|---|---|
| 13-15, 21, 22, Y | Acrocentric |
| 2, 4, 5, 7-12, 16-18, X | Submetacentric |
| 1, 3, 6, 19, 20 | Metacentric |

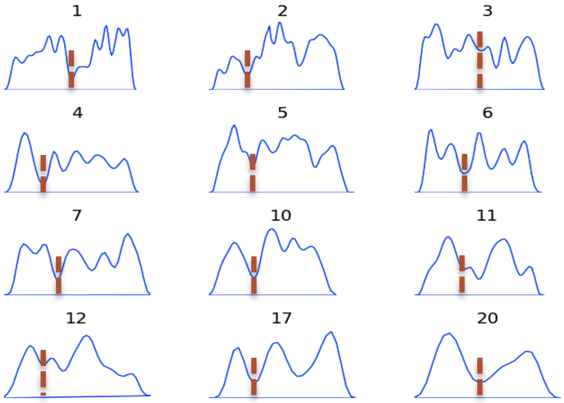


**Figure 3.8.** Q-banding patterns (intensity vs. pixel) of human metaphase chromosomes obtained from DAPI/AMD banding.

The banding patterns of some of the 24 human metaphase chromosomes obtained from the DAPI/AMD banding are shown in figure 3.8 while literature Q-banding patterns are shown in figure 3.9. Red dashed lines in figure 3.8 mark the centromere positions. The short arms of the chromosomes are in the left of the centromeres. It is also important to note that the x-axis of the patterns were not normalized into one scale thus signifying that the banding patterns do not represent the relative sizes of the chromosomes.

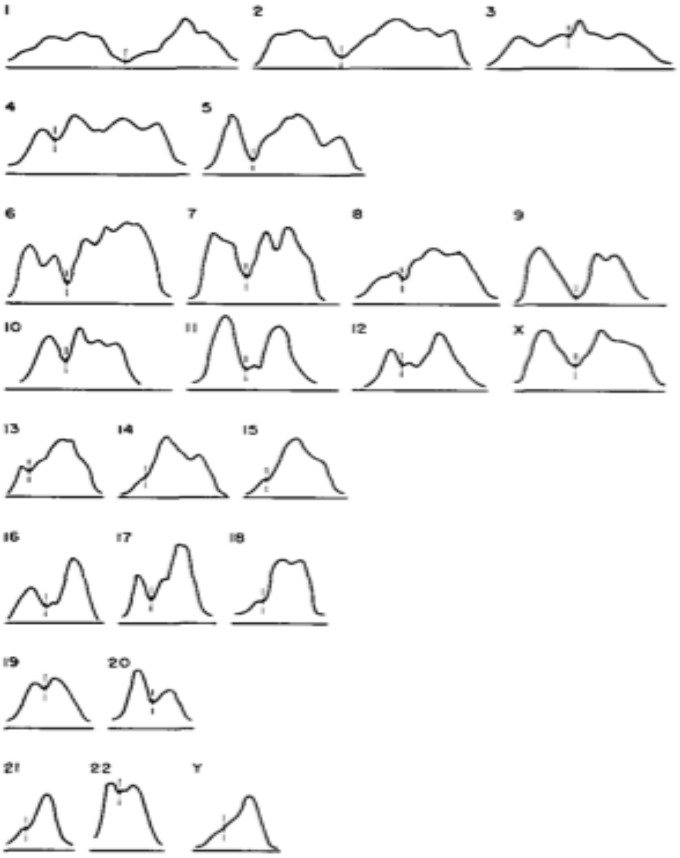


**Figure 3.9.** Q-banding patterns (intensity vs. pixel) of human metaphase chromosomes obtained from literature [4].

Figures 3.8 and 3.9 show that the banding patterns obtained from DAPI/AMD banding agree with the Q-banding patterns obtained from literature. The bands obtained from the DAPI/AMD banding have better resolution than those from the literature. Moreover, each chromosome number has a unique pattern, which can be used to identify the chromosomes. For example, the distinct features that can always be observed in the banding patterns of chromosomes 1 and 4 are shown in figure 3.10. It can be observed also that as the chromosomes get shorter, fewer bands are seen. For example, chromosome 1 has more bands than chromosome 20. This is because as the chromosomes get shorter, more bands are condensed into one band.

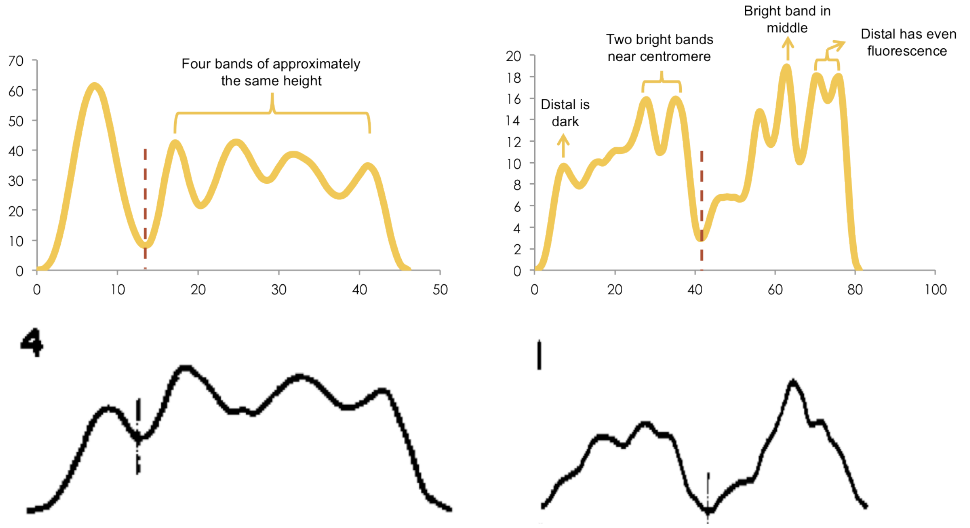


**Figure 3.10.** Distinct features present in the banding patterns of chromosomes 1 and 4. Top images are obtained from DAPI/AMD banding while bottom images are obtained from literature.

## 3.2 SYTOX Green/Methyl Green Banding

The principle of counterstaining was also used to induce banding with the fluorochrome SYTOX green. SYTOX green, like DAPI, is a nucleic-acid specific fluorochrome that has a relatively high quantum yield ($\phi = 0.53$) when bound to chromosomes as compared with other fluorochromes [10]. Free molecules of SYTOX green in water are weakly fluorescent therefore background fluorescence is minimized.

SYTOX green was used in combination with a nonfluorescent counterstain called methyl green. Human metaphase chromosomes prepared in glass slides were stained with SYTOX green for 5 min followed by methyl green. Optimization of the methyl green staining time was performed (table 3.5). Furthermore, a slide with chromosomes stained with SYTOX green alone was also prepared as a control (figure 3.11). Fluorescence images were inverted and converted into black and white images for better visualization of the bands.

**Table 3.5.** Optimization of methyl green staining times.

| Slide | Time in Methyl Green, hours |
|---|---|
| 1 | - |
| 2 | 1 |
| 3 | 2 |
| 4 | 2.30 |

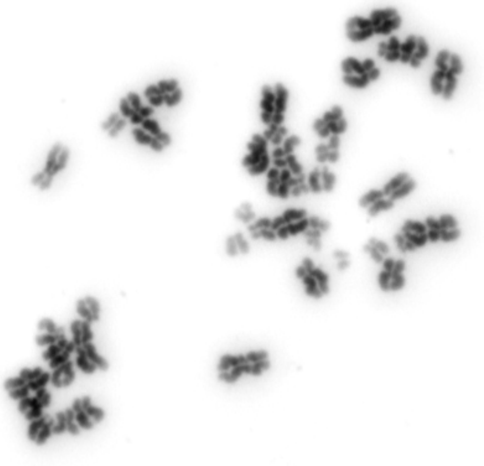


**Figure 3.11.** Human metaphase chromosomes stained with SYTOX green for 5 min.

It can be observed from figure 3.11 that bands are not present in the chromosomes stained with SYTOX green only. This is because SYTOX green is an intercalating dye with no functional groups that allow binding specificity and thus binds uniformly along the length of the chromosomes. Moreover, there is no differential quenching that occurs with SYTOX green thus its fluorescence intensity is also uniform along the length of the chromosomes.

By staining the chromosomes with methyl green (figure 3.12) after SYTOX green, clear bands were produced (figure 3.13). Methyl green is a minor groove binder that binds specifically to the AT-rich regions of the DNA. The spectral properties and DNA sequence specificity of SYTOX green and methyl green are presented in table 3.6 to demonstrate how the banding works.

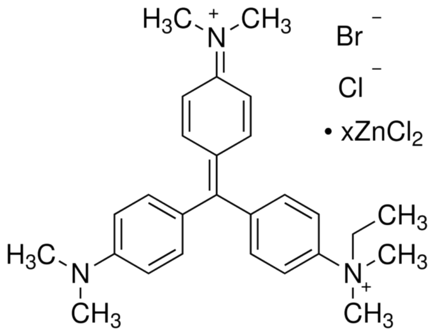
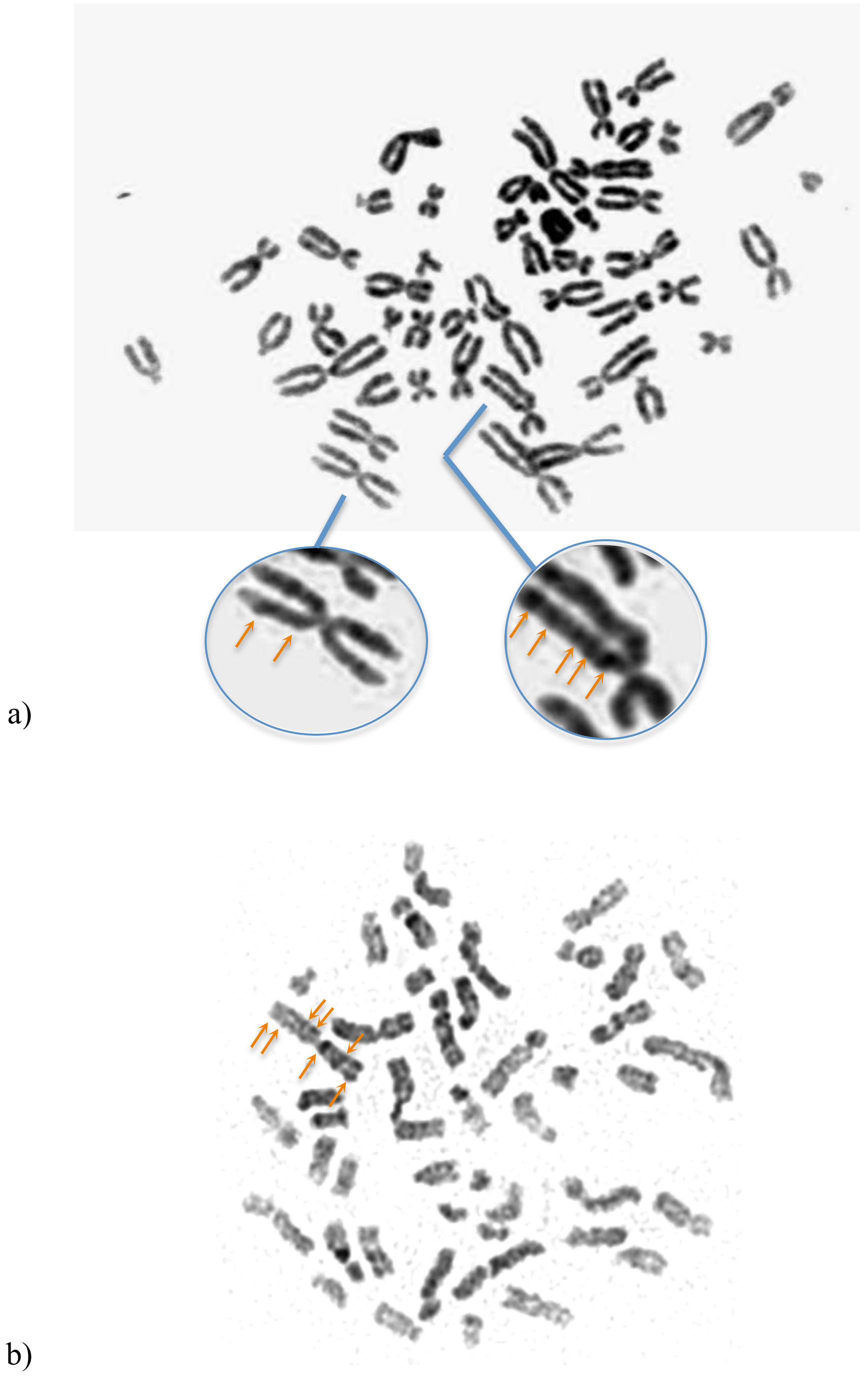


**Figure 3.12.** Structure of methyl green.



c)

**Figure 3.13.** Human metaphase chromosomes stained with SYTOX green for 5 min and methyl green for: a) 1 hour, b) 2 hours, and c) 2.30 hours.

**Table 3.6.** Spectral properties and DNA sequence specificity of SYTOX green and methyl green.

| **Dye** | **Specificity** | **Absorption max. (nm)** | **Fluorescence max. (nm)** |
|---|---|---|---|
| SYTOX green | - | 504 | 523 |
| Methyl green | AT | 638 | - |

Table 3.6 shows that the SYTOX green molecules in their excited state have enough energy to excite methyl green thus signifying that FRET can take place between these two molecules, like in DAPI/AMD banding, in the AT-rich regions of the chromosomes. Since, the energy of the excited state SYTOX green molecules bound in the AT-rich regions of the chromosomes is transferred nonradiatively to the methyl green molecules, the fluorescence intensity of SYTOX green is quenched at the AT-rich regions and thus, an R-banding pattern is produced. The bright positive R-bands correspond to the GC-rich, early-replicating euchromatic regions of the chromosomes and are similar to the light negative G-bands.

From the optimization, the optimal methyl green staining time is 2 hours (figure 3.13.b). It can be observed from figure 3.13.a that even though the chromosomes were only stained with methyl green for an hour, bands were still obtained. However, the contrast between the bands is poor. This is because the staining time is too short and thus, not enough methyl green is bound to the AT-rich regions of the chromosomes to quench the SYTOX green fluorescence in these regions. Meanwhile, figure 3.13.c shows that staining the chromosomes for 2.30 hours still produces bands. However, the long staining time resulted to a very weak fluorescence of SYTOX green along the length of the chromosomes.

The same procedure as in the DAPI/AMD banding was done to obtain the banding patterns of the chromosomes and identify what chromosome number the obtained banding patterns belong to. The obtained banding patterns of some of the 24 human metaphase chromosomes are shown in figure 3.14 while literature R-banding patterns are shown in figure 3.15. Red dashed lines in figure 3.14 mark the centromere positions. The short arms of the chromosomes are in the left of the centromeres. Again, the x-axis of the patterns was not normalized into one scale thus signifying that the banding patterns do not represent the relative sizes of the chromosomes.

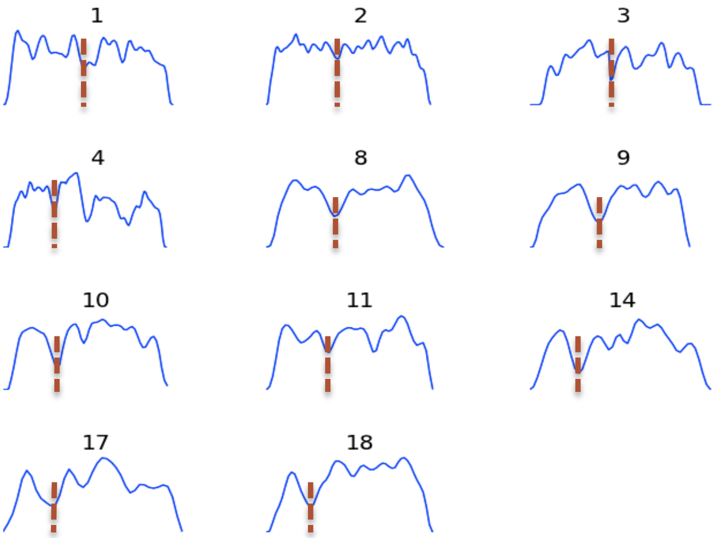


**Figure 3.14.** R-banding patterns (intensity vs. pixel) of human Metaphase chromosomes obtained from SYTOX green/methyl green banding.

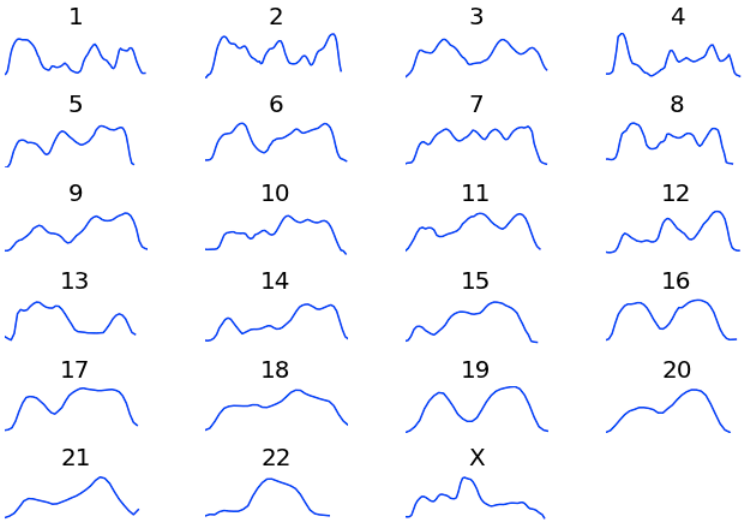


**Figure 3.15.** R-banding patterns (intensity vs. pixel) of human metaphase chromosomes obtained from literature [31].

Figures 3.14, 3.15, and 3.16 show that the banding patterns obtained from SYTOX green/methyl green banding agree with the R-banding patterns obtained from literature. Moreover, the bands obtained from the SYTOX green/methyl green banding have better resolution than those from the literature. Similar to DAPI/AMD banding, each chromosome has a unique pattern and as the chromosomes get shorter, fewer bands are observed.

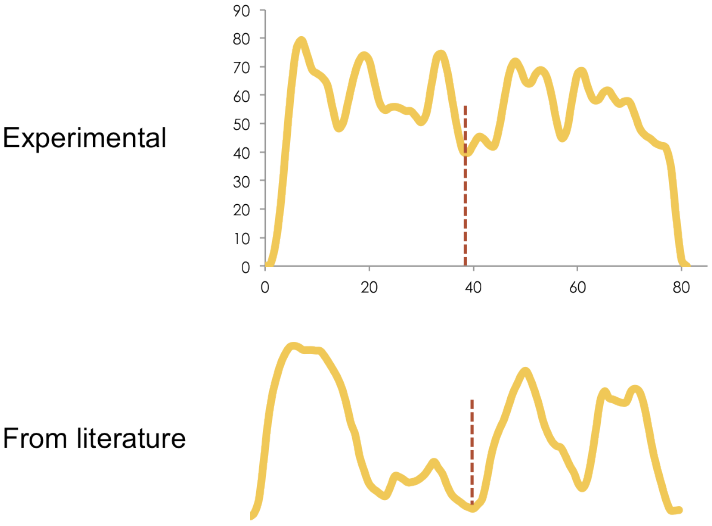


**Figure 3.16.** Comparison of the banding pattern of chromosome 1 obtained from the SYTOX green/methyl green banding (top image) and literature (bottom image).

Furthermore, the obtained R-banding patterns from the SYTOX green/methyl green banding are indeed the reverse of the Q-banding patterns obtained from the DAPI/AMD banding (figure 3.17).

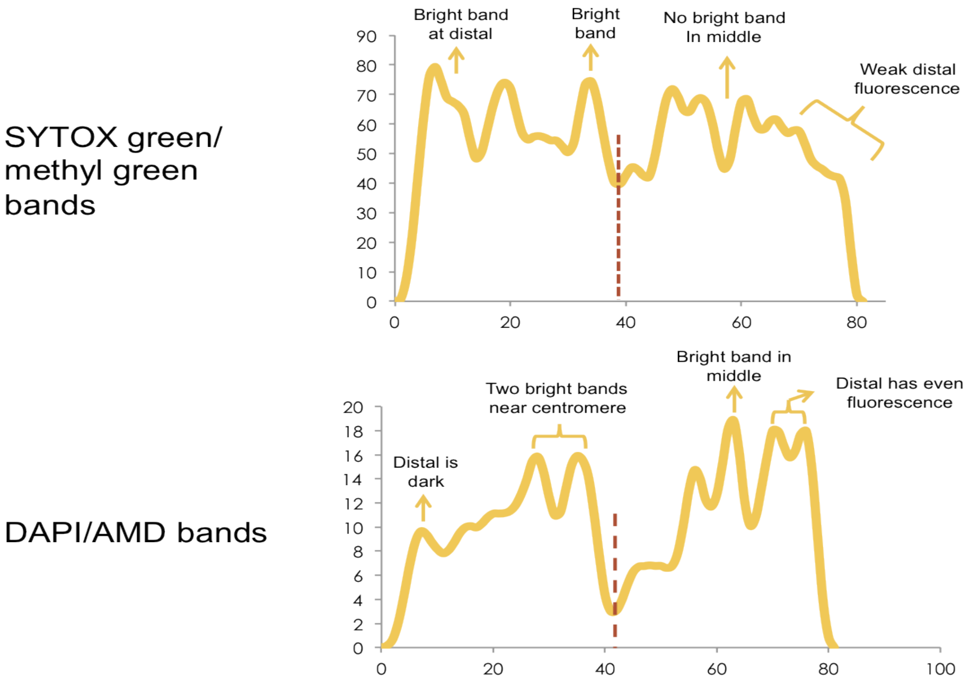


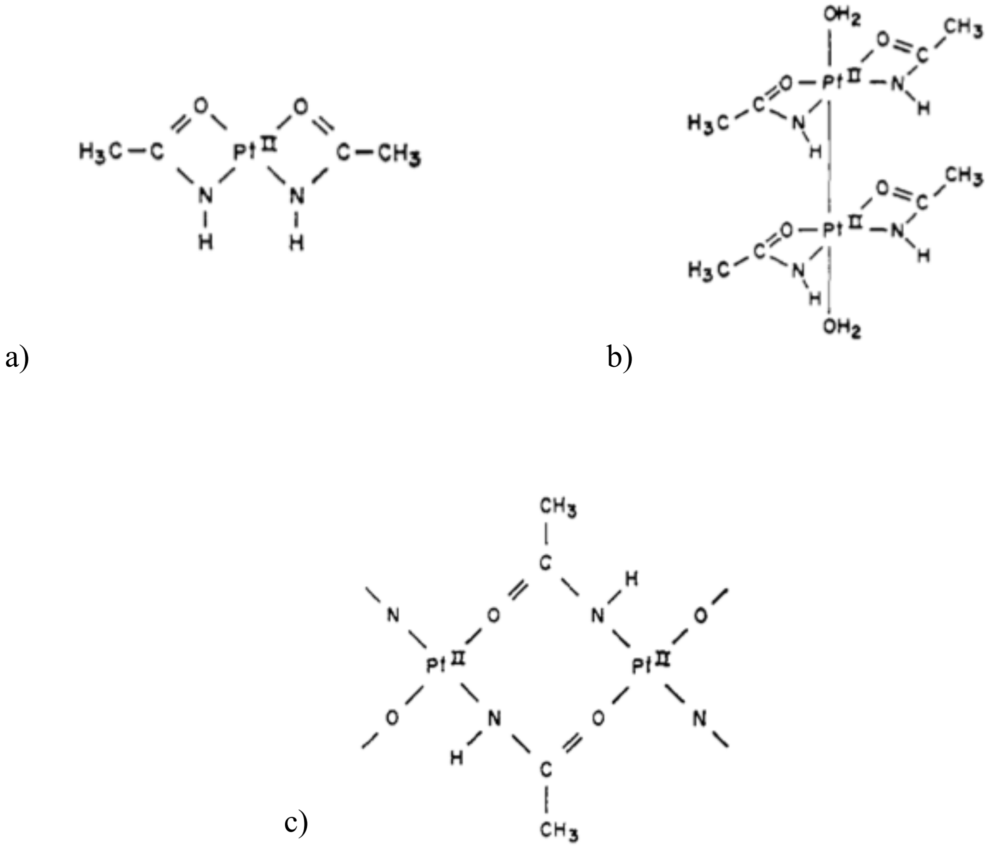
**Figure 3.17.** Comparison of the banding patterns obtained from SYTOX green/methyl green and DAPI/AMD banding.

It was observed that the SYTOX green/methyl green banding has several disadvantages when compared with the DAPI/AMD banding. First, SYTOX green is less stable to photobleaching than DAPI. Photobleaching is the process wherein a fluorochrome undergoes a photochemical reaction and is converted to another molecule that is not fluorescent or non-absorbent at the excitation wavelength. After prolonged exposures of light, the fluorescence intensity of SYTOX green was greatly reduced, unlike that of DAPI. Second, significant amount of background fluorescence was observed for the chromosomes stained with SYTOX green, unlike for those stained with DAPI. Third, the SYTOX green/methyl green banding has longer staining times as compared with DAPI/AMD banding.

## 3.3 Heavy Metal Staining

A platinum blue complex was synthesized, according to the method of Hofmman and Bugge [32], for heavy metal staining of chromosomes. Yellow crystals, dichlorobisacetonitrileplatinum(II) ($Pt(CH_3CN)_2Cl_2$), were obtained after reacting potassium tetrachloroplatinate with acetonitrile for 10 days. These crystals were then reacted with silver sulfate resulting to a blue solution. A blue amorphous powder, platinum blue, was precipitated from the solution through the addition of diethyl ether.

It is hard to investigate the structure of platinum blue due to the inability to crystallize them. The proposed structures for the synthesized platinum blue are shown in figure 3.18, all of which have the common feature that the platinum is divalent [25].



a) b)

c)

**Figure 3.18.** Proposed structures for the synthesized platinum blue: a) a monomeric bisacetamide complex, b) a dimeric six-coordinate complex, and c) an oligomeric chain involving acetamido bridges.

The synthesized platinum blue complex was tested as a heavy metal stain for SEM, along with a fluorescent platinum complex (figure 3.19.a). Both metal complexes selectively interact with nucleic acids, especially with DNA. The interaction of platinum blue with DNA is not well studied. It is proposed that platinum blue interacts with DNA in the minor or major groove through the attachment of platinum with the free oxygen and nitrogen atoms of the DNA base pairs. Meanwhile, PtLCl interacts with DNA on a 1:2.3 PtLCl:DNA base pair ratio. It is proposed to intercalate between the DNA base pairs due to the similarity of its structure with that of platinum terpyridine, a well-established DNA intercalator (figure 3.19.b) [33].

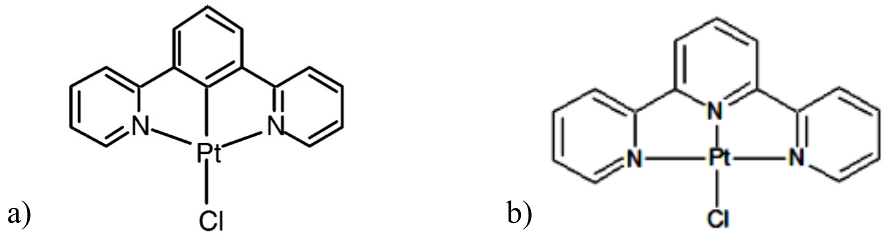
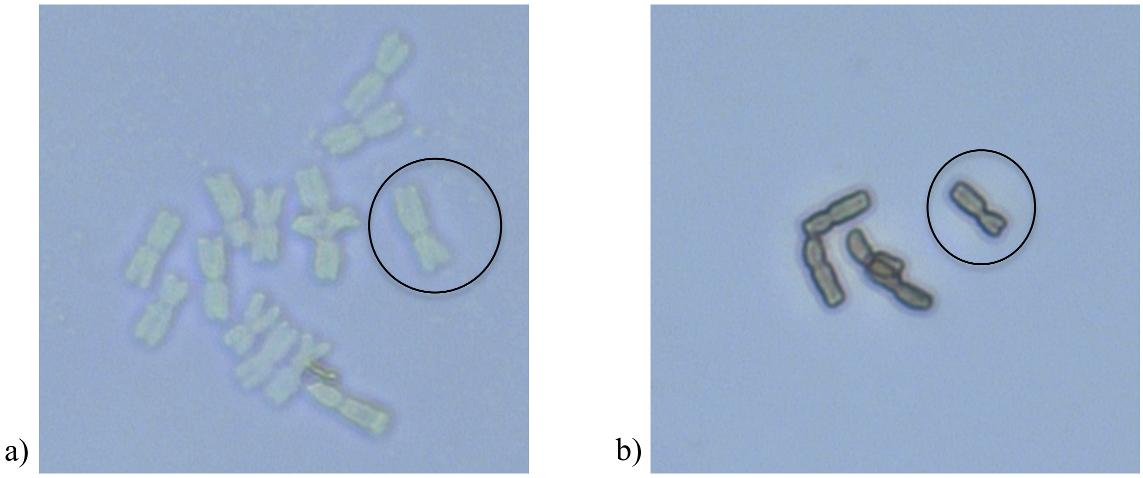


**Figure 3.19.** Structure of: a) PtLCl (L = 1,3-di(2-pyridyl)benzene), and b) platinum terpyridine.

Barley metaphase chromosomes were fixed in glutaraldehyde, stained with platinum blue or PtLCl, and dried with hexamethyldisilazane. Unstained chromosomes were also prepared as a control. The samples were first characterized with a light microscope (figure 3.20) before characterizing them with SEM.



c)

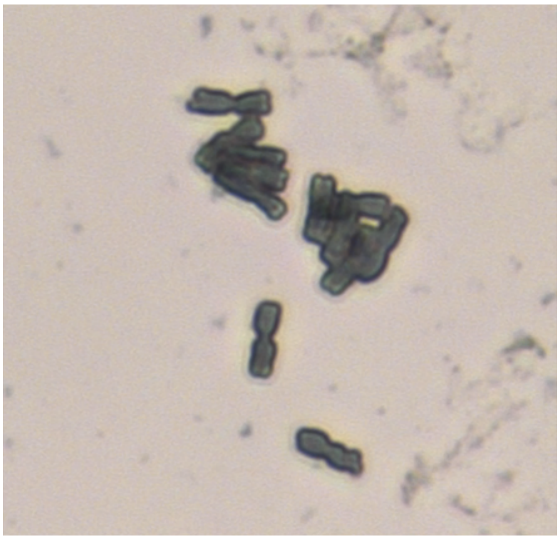


**Figure 3.20.** Light microscope images of barley metaphase chromosomes: a) unstained, b) stained with PtLCl, and c) stained with platinum blue. Encircled chromosomes were also characterized at high magnification with SEM.

The unstained chromosomes were very hard to find at low magnification in the light microscope and appeared transparent while the stained chromosomes were clearly visible and appeared dark. Figure 3.20 shows that staining the chromosomes gave them good contrast, with the highest contrast achieved with platinum blue. The same spreads of the unstained chromosomes and PtLCl stained chromosomes were characterized with SEM.

The secondary electron (SE) and back-scattered electron (BSE) images of the chromosomes were obtained (figure 3.21). In the BSE mode, the unstained chromosomes gave a weak signal and were hard to detect at low magnification while the stained chromosomes were clearly visible and appeared brightly. Figure 3.21 shows that staining the chromosomes with PtLCl and platinum blue increased the BSE signal, with the strongest signal achieved with platinum blue. This is because the BSE signal increases with increasing mean atomic number of the sample [34]. Platinum blue stained chromosomes gave the highest contrast because more platinum was incorporated onto the DNA for these chromosomes than for those stained with PtLCl, since platinum blue forms oligomers and has 2-4 platinum atoms while PtLCl has only one platinum. Meanwhile, in the SE mode, the stained chromosomes appeared brighter and showed more structural detail than the unstained ones. Figure 3.21 shows that there is also an enhancement in the contrast of the stained chromosomes in the SE mode although this enhancement is not as significant as that in the BSE mode and only a slight difference is observed between the contrast achieved with PtLCl and platinum blue. This is because the yield of SE also increases with the mean atomic number of the sample. However, the increase is less rapid than that in BSE [34]. Furthermore, it can also be observed from figure 3.21 that there is a good fit between the SE and BSE images.

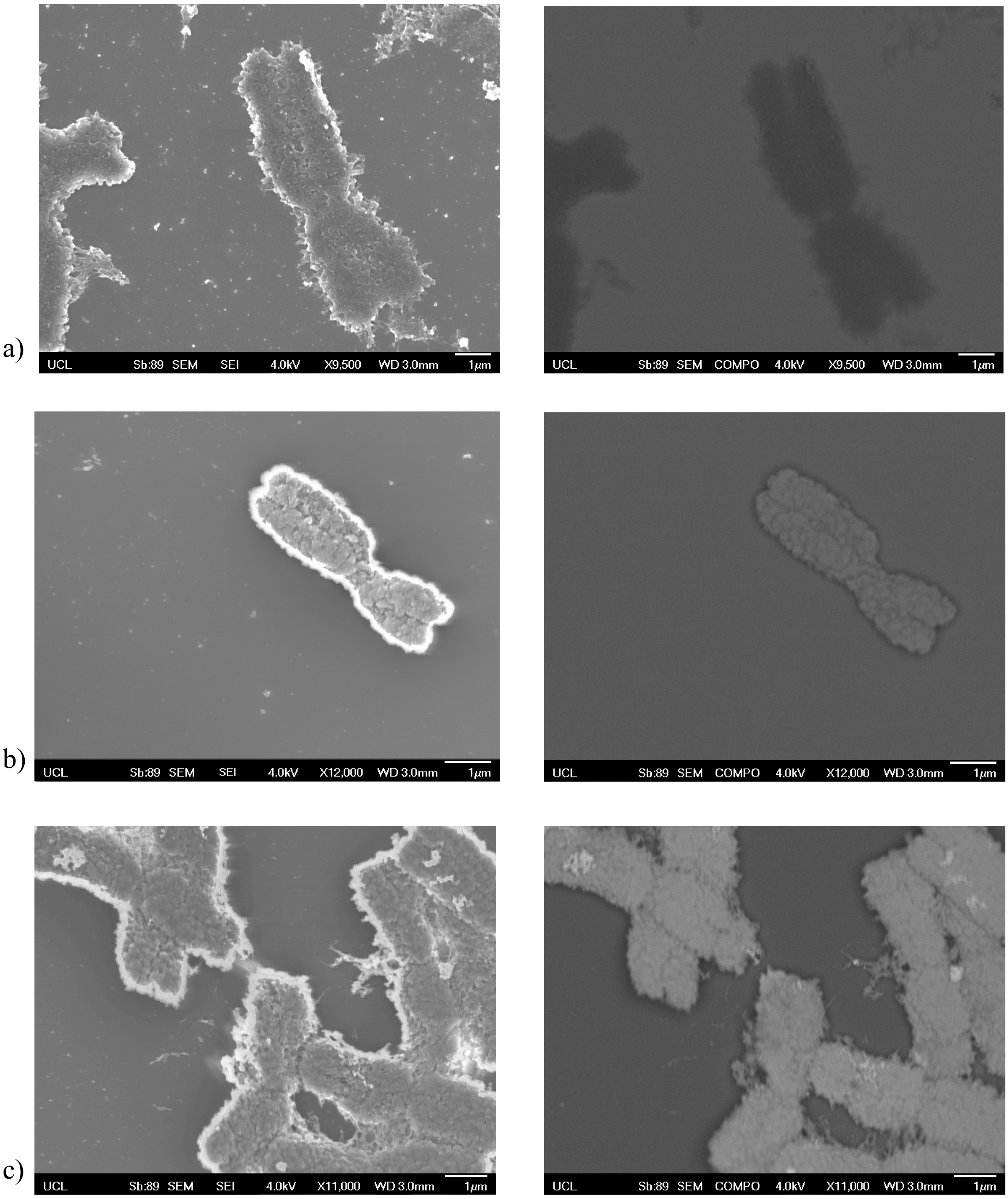


**Figure 3.21.** SEM images of barley metaphase chromosomes: a) unstained, b) stained with PtLCl, and c) stained with platinum blue. SE images are at the left while BSE images are at the right.

High magnification images of a single chromosome were obtained in the SE and BSE modes (figure 3.22). Figure 3.22.a shows that a good surface morphology of the chromosome was obtained with SE. In the SE mode, the contrast (figure 3.23.a) depends mainly on the topography of the surface features of the sample. The SE image shows that the chromosome arm is mainly composed of knot-like structures (enclosed with a square in the image), which are approximately 200-300 nm in size. It is proposed that these knot-like structures are chromomeres (coiled solenoids), which have been described before by several authors [2, 32]. Meanwhile, the centromere is characterized by the presence of parallel fibers (marked with an arrow in the image).

In the BSE mode, the contrast is a function of the elemental composition of the sample although topographic contrast is also present. However, the surface topography obtained from BSE is not as accurately resolved as for SE imaging (figure 3.22.b). The BSE image shows a fibrous network that is less compact than the structure observed in the SE image. Since the BSE mode provides contrast based on elemental composition, the distribution and concentration of DNA along the length of the chromosomes can be mapped if chromosomes are stained with DNA-specific stains. It can be observed from figure 3.23.b that there are only small variations in the BSE signal along the chromosome arm thus signifying that there are only small changes in the DNA distribution along this region and that the platinum blue is bound almost uniformly. Furthermore, a weaker signal was obtained at the centromere region thus signifying that the DNA concentration in this region is lower than that in the chromosome arms.

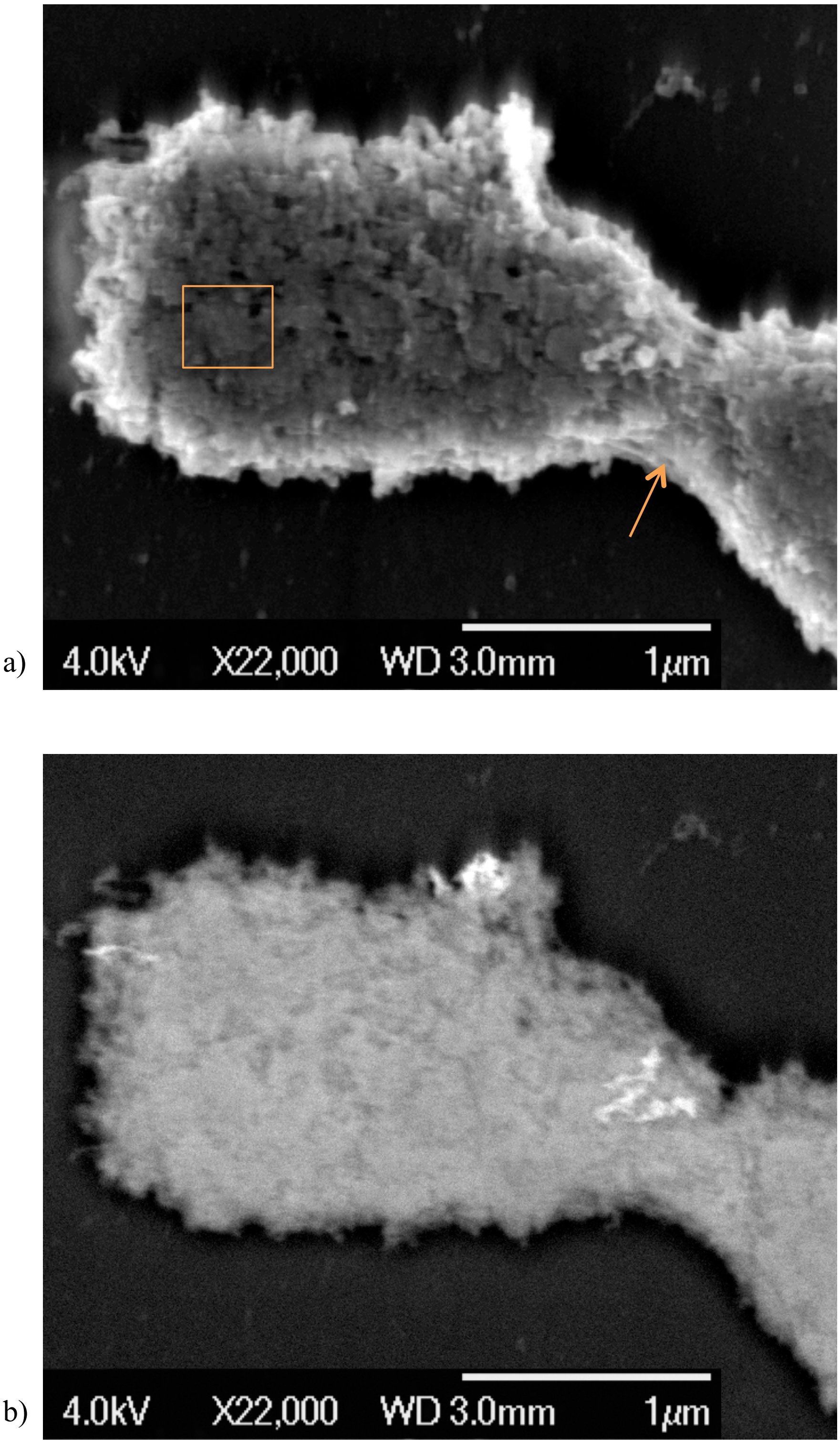


**Figure 3.22.** High magnification SEM images of a barley metaphase chromosome taken in the: a) SE mode, and b) BSE mode.

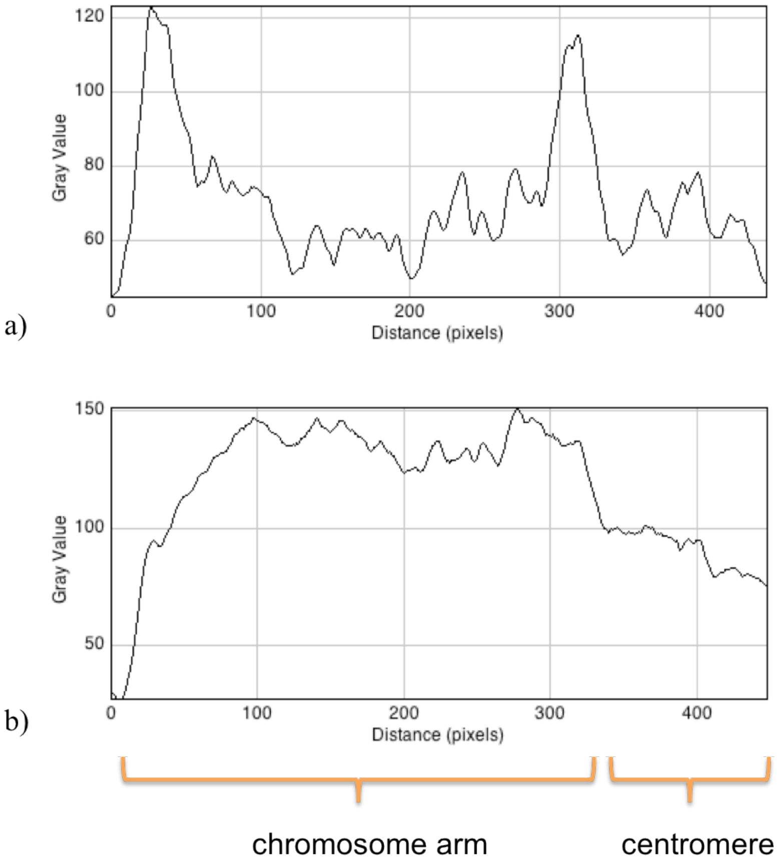


**Figure 3.23.** Contrast map (signal versus pixel) of a barley metaphase chromosome imaged in: a) SE mode, and b) BSE mode. Images in figure 3.22 were used to generate the maps.

# Conclusion

The principle of counterstaining was successfully used to enhance the banding patterns of human metaphase chromosomes obtained with DAPI and induce banding with SYTOX green. The Q-banding patterns of some of the 24 human metaphase chromosomes were obtained from DAPI/AMD banding while the reverse of this patterns, R-banding patterns, were obtained from SYTOX green/methyl green banding. The obtained banding patterns were of higher resolution than those from the literature. These patterns can be used in future experiments such as correlating bands with the 3-D chromosome structure and analyzing banding through the use of fluorescence lifetime imaging.

Meanwhile, platinum blue was successfully synthesized for heavy metal staining of barley metaphase chromosomes. The use of platinum blue and PtLCl increased both the SE and BSE signals thus resulting to a good contrast of the chromosomes. The best contrast was achieved with platinum blue. This allowed the production of SE images with good topography wherein knot-like structures and parallel fibers were identified in the chromosome arm and centromere, respectively. Moreover, the BSE images allowed the mapping of the DNA distribution and concentration along the length of the chromosomes wherein it was observed that the centromere has a low concentration of DNA and that the DNA distribution along the chromosome arms has very small variations.

Through the SEM studies, it was confirmed that platinum blue gives chromosomes good contrast and hence, in the future, will be applied as a heavy metal stain for coherent x-ray diffraction imaging of chromosomes. Furthermore, it was also determined that PtLCl gives chromosomes enough contrast. Thus, this stain, which is also fluorescent, can be used for correlative microscopy wherein fluorescence and electron microscopy can be simultaneously used on the same sample preparation. PtLCl will be combined with various counterstains such as AMD and will be tested for banding in SEM to correlate bands with the 3-D chromosome structure. Overall, the results of the studies performed will improve the imaging of chromosomes, which in turn will bring advancements in the study of the chromosome structure.

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