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A Review of the Different Staining Techniques for Human Metaphase Chromosomes

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Abstract

This review investigates the different staining techniques for human metaphase chromosomes. The higher order organization of chromosomes still remains unclear today. There are various imaging techniques that can be used to study chromosomes. Staining increases the contrast of chromosomes under these different imaging techniques while banding allows the identification of chromosomes and the abnormalities present in it, and provides information about the chromosomal substructures. The different stains that can be used for studying human metaphase chromosomes with light, fluorescence and electron microscopy, and coherent x-ray diffraction imaging are discussed. By understanding the underlying mechanism of staining and banding, an insight on the relation of the chromosomal bands with the chromosomal substructures is provided. Moreover, through this review, an understanding of how the structure of the stain affects its mode of binding to the chromosomes and how this mode of binding, in turn, affects the mechanism of band formation are achieved. The information obtained from this review can then be used to develop a suitable stain for studying the structure of human metaphase chromosomes with different imaging techniques.

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Introduction

Human cells each have 46 chromosomes, consisting of 23 nearly identical pairs. These chromosomes contain the genetic material of the cell, which determine how a person's body develops and functions. Hence, abnormalities in chromosomes such as deletions, insertions, and duplications can be fatal.

DNA and proteins in chromosomes are arranged into higher order structures. Chromosomes continue to condense until it reaches the metaphase state. This higher order organization of chromosomes is still not properly understood today and is currently of research interest. There are several imaging techniques that can be used to study chromosomes such as light, fluorescence and electron microscopy, and coherent x-ray diffraction imaging.

Staining allows the proper visualization of chromosomes under these imaging techniques. It increases the contrast of the chromosomes. Moreover, staining can lead to banding, which is a consequence of differential staining along the length of the chromosome. Banding provides more information about the chromosomes.

There are various stains that can bind to chromosomes and thus, can be used for chromosome staining. Visible light dyes such as giemsa are used in light microscopy while fluorochromes such as quinacrine are used in fluorescence microscopy. These stains are usually organic compounds with large aromatic groups. Meanwhile, heavy metal complexes are used as stains in electron microscopy and coherent x-ray diffraction imaging. The development of metal complexes that fluoresce is of great interest because it will allow the use of both fluorescence microscopy, and electron microscopy or coherent x-ray diffraction imaging on one sample preparation.

This review investigates the different staining techniques for human metaphase chromosomes with the intention of understanding the underlying mechanism of staining and banding so as to provide insight on the chromosomal substructures and organization, and develop a suitable stain for studying human metaphase chromosomes with different imaging techniques.

2.1 Chromosome Structure

Chromosomes are composed of chromatin, a complex of DNA and protein. The DNA in the chromosome is a long strand (~5 cm) that contains about 140 million nucleotides. These strands are condensed through coiling in order to fit inside the cell nucleus. Every 200 nucleotides, the 2-nm DNA helix coils around a core of eight histone proteins forming an organized structure called nucleosomes, which are arranged in 11-nm elementary fibers. The histone proteins, which consist of the basic amino acids arginine and lysine, are positively charged and therefore, interact strongly with the negatively charged phosphate groups of the DNA.

Higher order coiling is achieved when the string of nucleosomes start to coil into 30-nm fibers called solenoids. This is the structure of chromosomes during the interphase.

During mitosis, the chromosomes continue to condense until metaphase. The solenoids are arranged around scaffold proteins forming looped domains. The final organization of the chromosome is still unknown [1, 2]. A long-standing model for this final compaction involves further radial looping of the chromatin loops into rosettes around a protein scaffold, aided by a group of proteins called condensin [3]. Figure 2.1 presents the different levels of chromosomal organization.

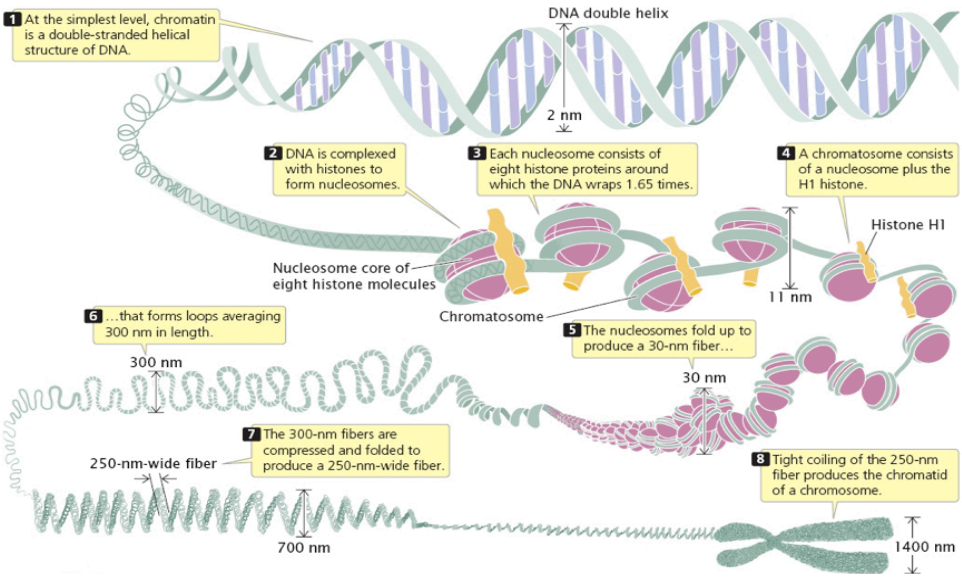

Figure 2.1. Levels of chromosomal organization [4].

There are various imaging techniques that can be used in studying the structure of chromosomes. These include light, fluorescence and electron microscopy, and coherent x-ray diffraction imaging.

Light and fluorescence microscopy both have limited resolution and thus, provide little information. The advantage of light microscopy is that it does little damage on the chromosomes [3] while the advantage of fluorescence microscopy is its high sensitivity and specificity [5].

Meanwhile, electron microscopy techniques include transmission electron (TEM) and scanning electron (SEM) microscopy. These techniques provide higher resolution than that of light and fluorescence microscopy. However, the 3-D structure of the chromosomes is hard to observe under these techniques. The sample thickness is limited to approximately 100 nm for TEM while SEM is only surface-sensitive [2].

Coherent x-ray diffraction imaging (CDI) provides high resolution and 3D-images of the chromosome structure. The main disadvantages of this technique are the hard sample preparation and the radiation damage induced on the chromosome samples [1].

2.2 Cell Cycle and Mitosis

The cell cycle consists of events involving cell growth, DNA replication, and cell division. It is divided into five phases (figure 2.2).

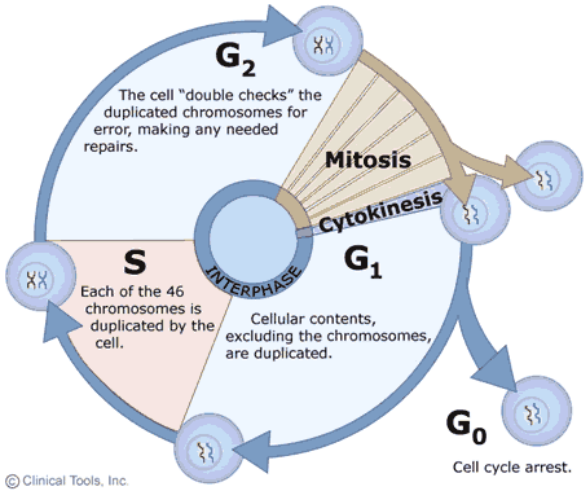

Figure 2.2. Stages of the cell cycle.

Gap phases 1 and 2 (G_1 and G_2), and synthesis (S) phase constitute the interphase. The G_1 and G_2 phases are the growth phases of the cell where proteins are synthesized and cell organelles are produced. During the S phase, replication of the DNA occurs. After replication, each chromosome is now composed of two sister chromatids that are held together at their centromeres (figure 2.3).

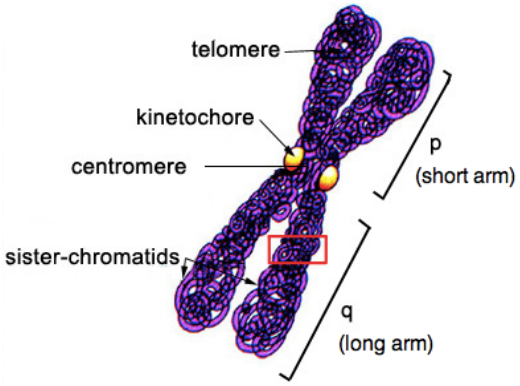

Figure 2.3. Features of the chromosome after the S phase.

The centromere is a point of constriction on the chromosome that binds specific proteins, which in turn make up a disk-like structure called the kinetochore. The kinetochore is an attachment site for spindle fibers, which play an important role during mitosis.

In the G_2 phase, the chromosome continues to condense from the 30-nm fiber. As the chromosomes become more condensed, they become visible with the light microscope. This marks the start of mitosis.

Mitosis (figure 2.4) is the phase of the cell cycle where the sister chromatids are separated by spindle fibers. It is subdivided into prophase, prometaphase, metaphase, anaphase, and telophase.

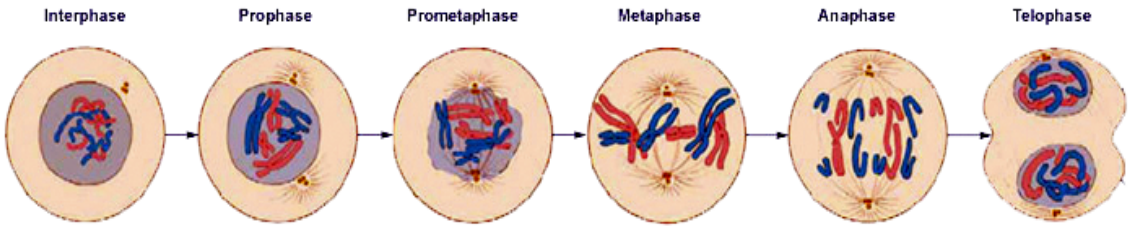

Figure 2.4. Stages of mitosis [6].

During mitosis, the chromosomes continue to condense until metaphase. In metaphase, the chromosomes align at the metaphase plate in preparation for the separation of the sister chromatids by the spindle fibers. The sister chromatids are finally separated in anaphase and the chromosomes begin to decondense in telophase preparing the DNA for replication as the cell cycle starts again.

Overview of Staining and Banding

Staining is used to enhance the contrast between different cellular components thus allowing the proper visualization of chromosomes with different imaging techniques. Differential staining along the length of a chromosome leads to the production of bands (figure 3.1).

Figure 3.1. Banding in chromosome 3 with its corresponding ideogram.

These bands provide further information about the chromosomes. It can be used to identify individual chromosomes since each chromosome number produces unique bands [7]. Furthermore, banding can be used to study abnormalities in the chromosome such as deletions, insertions, or translocations (figure 3.2) [8].

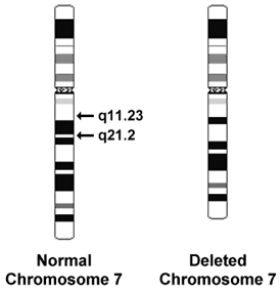

Figure 3.2. Deletion of a DNA sequence in chromosome 7.

In addition, studying the mechanism of banding can lead to an understanding of the chromosome structure since banding may be a reflection of the difference in the structure along the length of a chromosome [9-11].

3.1 Chromosomal Substructures

The bands produced by different banding techniques reflect a specific region or substructure of a chromosome. The different regions of a chromosome and the banding techniques that reveal them are presented.

Chromatin can either be heterochromatin or euchromatin [11-13]. Heterochromatin are chromatin regions that remain condensed throughout the cell cycle and are inactive in transcription. They are late replicating and have a low density of genes, which are mostly inactive. Only 20% of the mapped human genes are found in the heterochromatin. Meanwhile, euchromatin are chromatin regions that are decondensed in the interphase and are active in transcription. They are early replicating and contain most of the housekeeping genes.

Furthermore, heterochromatin can be classified as constitutive or facultative [13, 14]. Constitutive heterochromatin consists of satellite DNAs, which are repetitive sequences of DNA and can be found in the centromere and telomere of the chromosome. It remains condensed all throughout the cell cycle. Meanwhile, facultative heterochromatin is a condensed and transcriptionally silent chromatin region like constitutive heterochromatin but can decondense into euchromatin if triggered by several factors (figure 3.3).

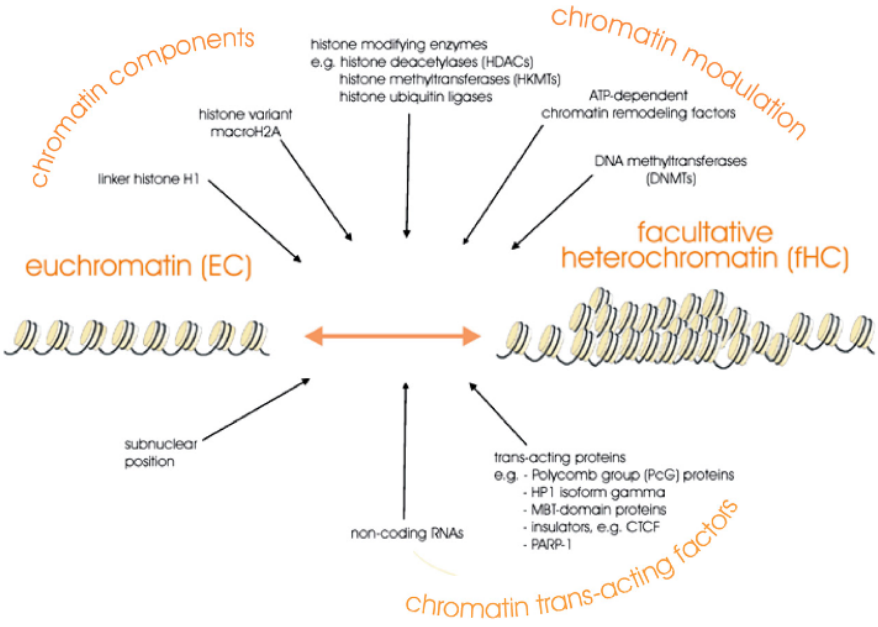

Figure 3.3. Interconversion between heterochromatin and euchromatin [13].

The common banding techniques that reveal these various chromosomal regions are summarized in table 3.1.

Table 3.1. Chromosomal regions and banding techniques that reveal them.

Chromosomal Region	Banding Technique
Constitutive heterochromatin	C-banding
Facultative heterochromatin	G- or Q-banding
Euchromatin	R-banding

3.2 Modes of Stain Binding

In order to understand how banding occurs and relate banding to chromosome structure, it is necessary to understand how stains bind to the chromosomes. There are several ways a stain can bind to a chromosome. Most of the stains discussed in this review are specific for DNA and hence, bind to the chromosomes through the DNA and not through the proteins. A stain can bind to the DNA through intercalation, minor groove or major groove binding, or external binding (figure 3.4). These modes of binding depend on the type of interaction the stain have with the DNA, which can either be covalent or noncovalent.

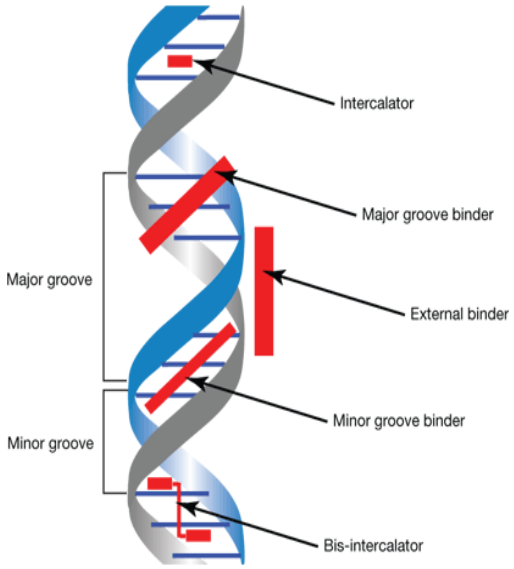

Figure 3.4. Modes of binding of a stain to DNA.

Covalent interactions usually involve metal stains wherein the metal coordinates with the nitrogen atoms of the base pairs of the DNA. Figure 3.5 shows the DNA base pairs with the atomic positions, and major and minor groove labels. The coordination of metal with nitrogen atoms often occurs at position 7 of adenine or guanine. Some platinum stains exhibit this type of interaction since platinum has a high affinity for nitrogen [15]. This type of interaction results to major groove binding.

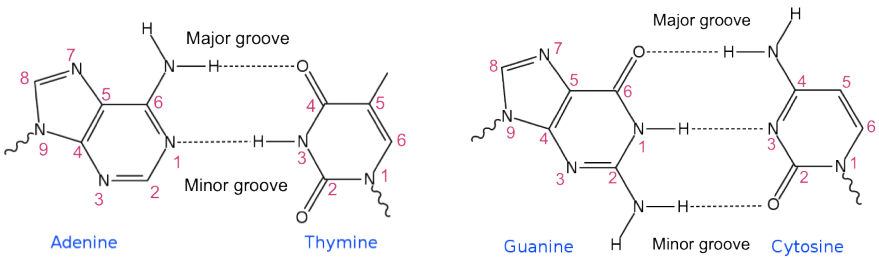

Figure 3.5. Base pairs of the DNA.

Noncovalent interactions include hydrogen bonding, electrostatic interactions, and π-π interactions. Hydrogen bonding (H-bonding) occurs with stains that have a functional group containing an electronegative atom such as nitrogen or oxygen. The electronegative atom can hydrogen bond with the H-donor sites of the base pairs. These sites are position 6 of adenine, position 4 of cytosine, and position 2 of guanine. Furthermore, the electronegative atom can be attached to a hydrogen and hydrogen bond with the H-acceptor sites of the base pairs. These sites are positions 3 and 7 of adenine and guanine, and position 2 of thymine. H-bonding leads to major groove or minor groove binding.

Electrostatic interactions involve stains that have cationic groups. The cationic groups form an ionic interaction with the negatively charged phosphate groups of the DNA. This type of interaction results to external binding.

π-π interactions occur with stains that have planar aromatic groups. The p orbitals of these aromatic groups overlap with the p orbitals of the aromatic groups of the DNA base pairs. As a result, stacking of the aromatic groups occurs and the electrons are delocalized throughout the aromatic groups of the stain and the base pairs. π-π interactions lead to an intercalation binding mode wherein the stain inserts between the base pairs of the DNA. Intercalation leads to the unwinding of the double helix and thus, leads to the lengthening of the DNA [15].

A stain can bind to the DNA through several binding modes if it contains functional groups that can produce more than one type of interaction [15]. For example, a stain can both have an aromatic group, which intercalates between the DNA base pairs, and a functional group capable of hydrogen bonding, which binds to the minor groove or major groove of the DNA. The H-bonding group should be able to rotate freely and orient itself out of the plane of the intercalating group in order for several binding modes to occur.

4.1 Giemsa Staining

Giemsa is a visible light dye that binds to DNA through intercalation and thus, is used for chromosome staining. It is a mixture of cationic thiazine dyes, most importantly azure B, and anionic eosin dyes such as eosin Y (figure 4.1) [16].

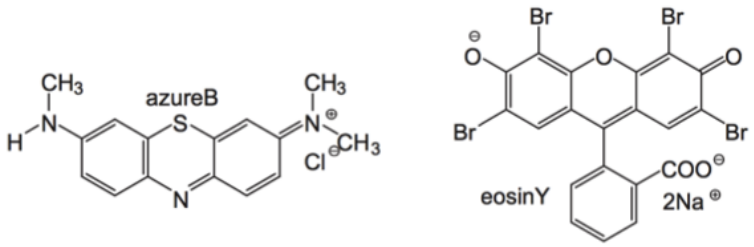

Figure 4.1. Structure of azure B and eosin Y.

Staining of the chromosomes involves the formation of a thiazine-eosin precipitate in a 2:1 molar ratio [10, 16]. Two molecules of the small, fast diffusing thiazine dye first intercalate between the base pairs of the DNA in a configuration that favors the binding of the large, slow diffusing eosin molecule. The chromosomes stain blue as a result of this. The eosin molecule then forms a precipitate with the thiazine molecules thus causing the chromosomes to stain purple. The formation of this precipitate is favored on a hydrophobic environment.

The type of interaction between the thiazine and eosin molecules is still a matter of debate [16]. A study by Zanker in 1981 [17] suggests the formation of a charge-transfer complex between the thiazine and eosin molecules with the thiazine as the acceptor and the eosin as the donor. Wittekind in 1985 [18] supports this study and suggests the formation of H-bonds, which facilitate the electron transfer, between the thiazine and eosin molecules. Furthermore, Wittekind considered that the thiazine and eosin molecules are associated in part due to hydrophobic interactions.

4.2 G-banding

G-banding is the most widely used banding method for cytogenetic analysis that was first developed by Seabright in 1971 [19]. This technique, which is nonfluorescent, is

advantageous in the aspect of stability and resolution of the bands produced [8, 20]. Visible light dyes are more stable and capable of producing clearer bands than fluorochromes. The bands obtained from G-banding are shown in figure 4.2.

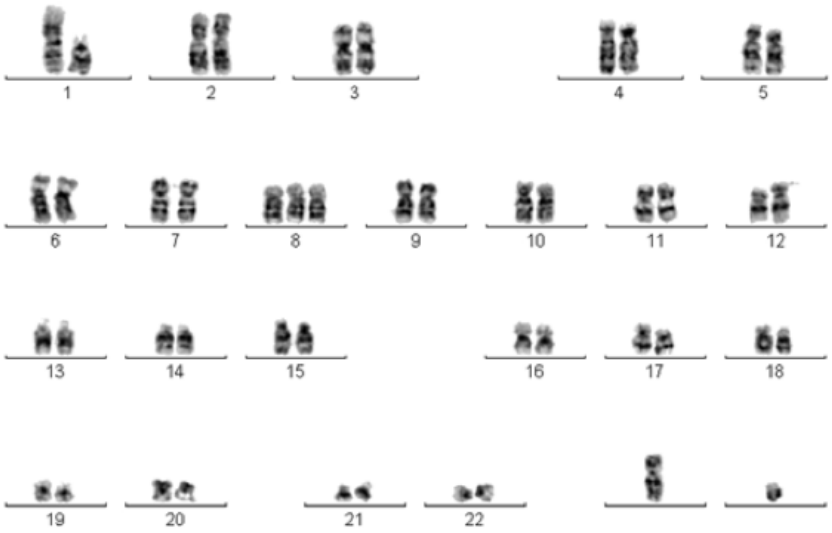

Figure 4.2. Karyotype of human chromosomes obtained from G-banding [21].

In order for banding to occur, the chromosomes must first undergo a pretreatment process before staining with giemsa [10]. The most common pretreatment method used is the digestion of the chromosomes with a protease such as trypsin. Other pretreatment methods include incubation of the chromosomes in hot-saline citrate or subjecting the chromosomes into a detergent or urea solution.

These pretreatment methods are known to extract a characteristic subset of proteins from the chromosomes [10, 22]. This differential extraction of the proteins throughout the length of the chromosome is responsible for the banding and is a reflection of the difference in the structure of the various chromosomal regions.

The positive G-bands, which are the dark bands, correspond to the hydrophobic regions of the chromosomes that favor the formation of the thiazine-eosin precipitate [10]. These regions are identified as the late replicating heterochromatin, which are characterized as condensed and rich in protein disulfide cross-links. Due to the condensed structure and richness in disulfide cross-links of these regions, the hydrophobic proteins needed for the formation of the giemsa complex are retained in position or appropriate conformation

during the pretreatment process. Since the heterochromatic regions are AT-rich, the positive G-bands can be revealed by fluorochromes that are specific for AT-rich regions of the chromosomes.

Meanwhile, the negative G-bands, which are the light bands, correspond to the less hydrophobic regions of the chromosomes that do not favor the formation of the thiazine-eosin precipitate [10]. These regions are identified as the early replicating euchromatin that have relatively loose structure and have their protein sulfur predominantly as sulfhydrils. Hence, the hydrophobic proteins in these regions are easily removed during pretreatment. Since the euchromatic regions are GC-rich, the negative G-bands can be revealed by fluorochromes that are specific for GC-rich regions of the chromosomes, a process called R-banding.

A study by Burkholder and Weaver in 1977 [22] shows the role of the nonhistone proteins in banding. Histone proteins are observed to have a uniform distribution and binding throughout the chromosomes and hence, will not be extracted differentially during the pretreatment process. On the other hand, the nonhistones, specifically, the most tightly bound nonhistones, are differentially bound throughout the chromosomes and may thus be responsible for the banding process. These nonhistones are more tightly bound in the condensed chromatin regions than in the less condensed regions and thus, will be extracted differentially during the pretreatment process.

4.3 Other Banding Techniques using Giemsa

Other types of bands, such as R- and C-bands, can be obtained using the giemsa stain. The type of bands produced depends on the extent of denaturation induced on the chromosome structure [10].

R-banding reveals the GC-rich euchromatin and produces positive bands that correspond to the negative G-bands. Banding (figure 4.3) is produced by incubating the chromosomes in an ionic solution at a high temperature (~ 87^{o}C) followed by staining with giemsa. The incubation process causes the denaturation of the AT regions of the chromosomes because of the low melting point of these regions (~ 65^{o}C) as compared to that of the GC regions (~ 105^{o}C).

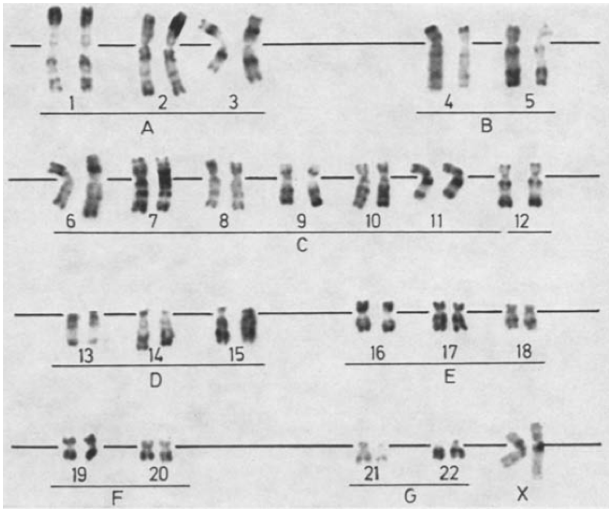

Figure 4.3. R-bands in human chromosomes [23].

C-banding, on the other hand, reveals the AT-rich centromere, which consists of constitutive heterochromatin. This technique involves acid treatment, hot saline incubation, and alkali treatment of the chromosomes. These treatments depurinate the DNA and break the DNA backbone, which then cause the extraction of the DNA from certain regions of the chromosomes.

C-bands (figure 4.4) are produced due to this differential extraction of the DNA. It was observed that the DNA in the C-bands is more resistant to extraction than the DNA in the other regions of the chromosomes. This is due to the stronger interaction of the proteins, which protects the DNA from extraction, with the DNA in the C-bands than in the other regions of the chromosomes [22].

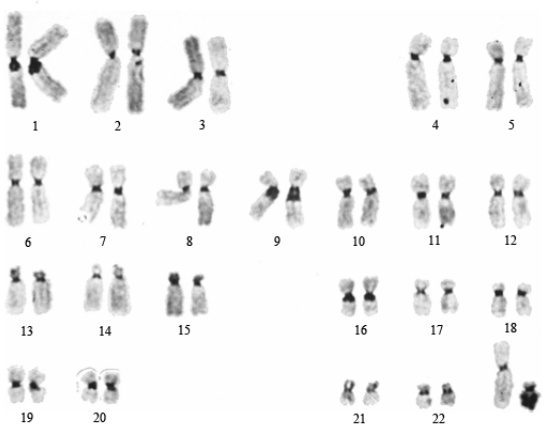

Figure 4.4. C-bands in human chromosomes [24].

5.1 Fluorochromes for Chromosome Staining

Fluorochromes are organic molecules that are capable of undergoing fluorescence. These molecules contain large conjugated systems such as aromatic or heterocyclic groups and are characterized by rigid and planar structures [5].

There are several parameters that are important in describing the fluorescence of a fluorochrome. These include the excitation and emission wavelengths, and quantum efficiency or yield. The excitation wavelength shows how much energy is required to excite the fluorochrome while the emission wavelength shows the energy of the photon emitted by the fluorochrome. The emission wavelength is usually longer than the excitation wavelength. Meanwhile, the quantum efficiency or yield, Φ, shows the probability of the excited fluorochrome to undergo fluorescence. It is defined as

$$\Phi = \frac{\text{\# of photons emitted}}{\text{\# of photons absorbed}} \qquad (5.1).$$

The fluorescence of a fluorochrome is affected by the fluorochrome structure and environment [5]. The quantum efficiency of a fluorochrome increases as the size of the conjugated system increases. Meanwhile, the environmental factors to consider include pH, ionic strength, temperature, viscosity of the medium, and presence of macromolecules. pH and ionic strength can change the structure of a fluorochrome thus affecting its fluorescence. Temperature, viscosity, and presence of macromolecules can affect the competition between radiantionless processes and fluorescence.

In chromosome staining, fluorochromes are used when the chromosomes are to be studied with a fluorescent microscope. They are also capable of producing bands in the chromosomes. Although fluorochromes are less stable than visible light dyes such as giemsa, as discussed earlier, they offer several advantages in banding. Giemsa requires the use of trypsin, which removes proteins from the chromosomes, in order for banding to occur. On the other hand, fluorochromes do not require this pretreatment process**.** Hence, the extraction of proteins from the chromosomes is avoided. This is particularly important

in studying the chromosome structure. Furthermore, fluorochromes allow chromosomes to be simultaneously banded and hybridized in situ with probes [8, 20]. Modern cytogenetic techniques that makes use of fluorescence, such as comparative genomic in situ hybridization (CGH), multicolor fluorescence in situ hybridization (M-FISH), and spectral karyotyping (SKY), require coordinated chromosomal banding analysis in order to be completely useful.

There are various fluorochromes that can be used in chromosome banding, some of which are presented in table 5.1.

Table 5.1. Commonly used fluorochromes in chromosome banding.

Fluorochrome	Binding Mode	Mechanism of Banding	Selectivity
Quinacrine	Intercalation	Differential fluorescence	AT
Daunomycin	Intercalation		AT
DAPI	Minor groove	Differential binding	AT
Hoechst 33258	Minor groove		AT
Chromomycin A3	Minor groove		GC

These fluorochromes produce bands either through differential quenching or fluorescence, or differential binding depending on the structure of the fluorochrome. Differential fluorescence or quenching is responsible for banding using fluorochromes that uniformly bind throughout the length of the chromosome. These fluorochromes usually bind to the chromosomes through intercalation and hence, do not produce a binding specificity [25]. Banding using these fluorochromes occur due to the quenching of their fluorescence at certain regions of the chromosomes. As discussed above, the presence of macromolecules can affect the fluorescence of a fluorochrome. 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 a major or minor groove binder and contain functional groups that are capable of forming H-bonds either with the AT or GC base pairs of the DNA [26].

The fluorochromes in table 5.1 are capable of producing bands that are similar to those produced using the giemsa stain. Fluorochromes that fluoresce brightly in the AT-rich

and GC-rich regions of the chromosomes have their positive light bands similar to the positive dark G-bands and R-bands, respectively.

5.2 Quinacrine

Chromosome banding using fluorochromes was first achieved by Caspersson in 1970 [7] with the use of quinacrine mustard (figure 5.1.a), an aminoacridine dye. Quinacrine mustard was then replaced by a less toxic quinacrine compound called quinacrine dihydrochloride (figure 5.1.b).

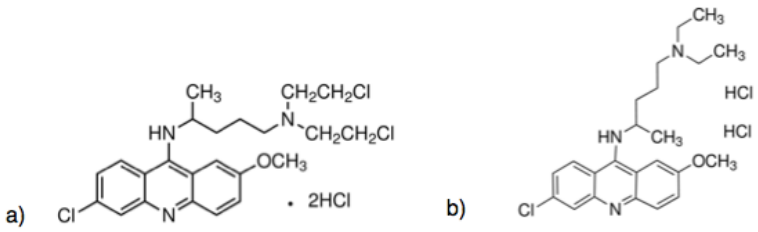

Figure 5.1. Structure of a) quinacrine mustard and b) quinacrine dihydrochloride.

At low dye/DNA ratio and high ionic strength, quinacrine binds to DNA through intercalation. Meanwhile, at high dye/DNA ratio and low ionic strength, quinacrine binds to DNA through an external ionic interaction. Quinacrine has a positive charge that is capable of interacting with the negatively charged phosphate groups of the DNA [25].

Quinacrine is observed to bind uniformly throughout the length of the chromosome. Hence, banding is produced due to differential fluorescence [25, 27]. The amino group at position 2 of the guanine bases of the DNA quenches the fluorescence of quinacrine thus causing the AT-rich regions of the chromosomes to fluoresce more brightly than the GC-rich regions. The bands produced are called Q-bands. The bright yellow-green positive Q-bands correspond to the positive G-bands minus the G-bands representing the centromeric constitutive heterochromatin. Thus, Q-bands represent the facultative heterochromatin.

The fact that quinacrine does not fluoresce brightly in the AT-rich centromere shows that other factors aside from base composition, such as base sequence arrangement, play an important role in banding. The fluorescence of quinacrine can be efficiently quenched even in AT-rich regions of the chromosomes if guanine residues are present and spaced with high periodicity [27, 28].

5.3 Daunomycin

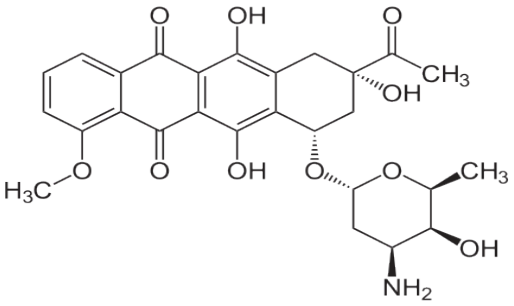

Figure 5.2. Structure of daunomycin.

Daunomycin (figure 5.2) is an anthracycline antibiotic dye. As a consequence of its structure, it binds to DNA through several ways (figure 5.3) [29]. About 3 to 3.5 base pairs are covered by one bound daunomycin molecule.

The aromatic ring system of daunomycin inserts between two adjacent DNA base pairs thus resulting to an intercalation binding mode. This results to the unwinding of the helix by 12^{o} and increase of the base-to-base distance by a factor of 2. Daunomycin can also form H-bonds with the DNA base pairs because of its hydrogen donor and acceptor functional groups, which bind to the minor groove of the DNA.

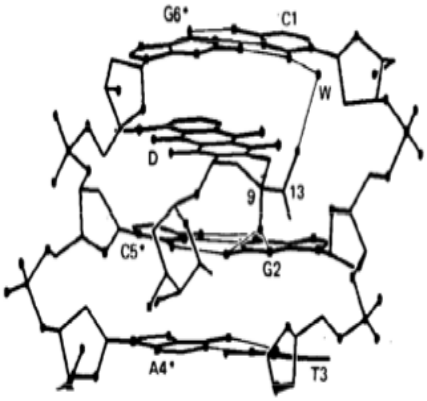

Figure 5.3. Binding of daunomycin to DNA.

There is no base preference in the DNA binding of daunomycin. Like in quinacrine, banding occurs as a result of differential quenching of the daunomycin fluorescence by the DNA base pairs [27]. Daunomycin fluorescence is almost completely quenched by DNA with AT-base composition of 0-60%. On the other hand, the fluorescence is only partially quenched when daunomycin is bound to DNA regions with a very high AT (>65%) content. This differential quenching causes the AT-rich regions of the chromosomes to fluoresce more brightly than the GC-rich regions thus producing a Q-like banding pattern for daunomycin.

5.4 DAPI

4',6-diamidino-2-phenylindole (figure 5.4), DAPI, is a fluorochrome that is widely used in chromosome staining. The reason for this is that DAPI has a very high quantum yield (~ 0.92) [30]. Furthermore, this high quantum yield is achieved only when DAPI is bound

to the chromosomes. DAPI is weakly fluorescent in water but its fluorescence is greatly enhanced upon binding to the chromosomes because it is shielded from the solvent. This protection from the solvent prevents intramolecular proton transfer in the excited state of DAPI, a process which results into less fluorescent species [26]. Since DAPI has a high quantum yield only when bound to the chromosomes, the background fluorescence from the DAPI molecules that are not bound is eliminated and proper visualization of the chromosomes is achieved. Furthermore, the fluorescence of DAPI does not fade easily compared with other fluorochromes, such as quinacrine, chromomycin A3, and distamycin A [8].

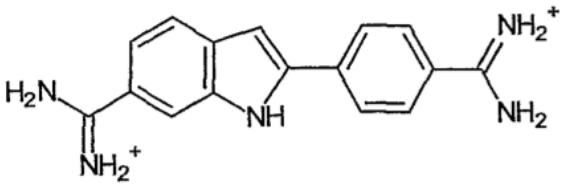

Figure 5.4. Structure of DAPI.

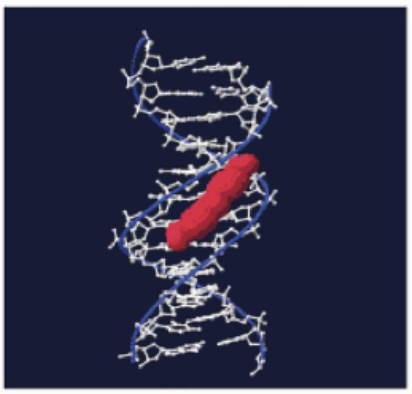

Figure 5.5. Binding of DAPI to the minor groove.

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 5.5) [31, 32]. It forms H-bonds with the nitrogen atoms at position 3 of the adenine bases and/or with the oxygen atoms at position 2 of the thymine bases. As a result of this sequence selectivity in the binding of DAPI, bands are produced. The banding pattern obtained from DAPI is similar to that obtained from G-banding.

However, at high DAPI/DNA ratio, heterogeneity in the banding mode of DAPI is observed. A study by Wilson in 1990 [33] shows that DAPI intercalates between GC base pairs in DNA regions that do not contain at least three consecutive AT base pairs. Another study by Kim in 1993 [26] shows that DAPI binds to the major groove of the GC-rich regions of the DNA. As a result of the possibility of DAPI binding to the GC-rich regions of the DNA, DAPI does not produce very clear bands as compared to those obtained from other fluorochromes such as quinacrine [34].

5.5 Hoechst 33258

Hoechst 33258 (figure 5.6) is a bi-benzimidazole derivative. It binds to the minor groove of the DNA, specifically to the AT-rich regions [31, 35]. The mechanism of binding of Hoechst 33258 with DNA is very similar to that of DAPI at low DAPI/DNA ratio. Hence, the banding pattern produced with Hoechst 33258 is similar to that of DAPI. In addition to this differential binding of Hoechst 33258 with DNA, the fluorescence of Hoechst 33258 is enhanced to a greater degree in the AT-rich regions of the DNA as compared to the GC-rich regions thus further improving the resolution of the bands produced [28, 36].

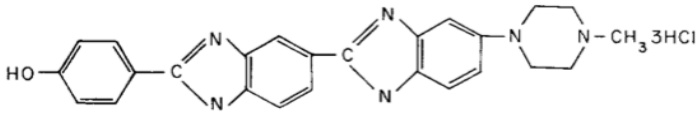

Figure 5.6. Structure of Hoechst 33258.

5.6 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 nonfluorescent counterstain. However, if a fluorescent counterstain is used, its emission wavelength 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. Electron energy transfer is a process where 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 [37]. 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 [35].

There are various possible combinations of primary stains and counterstains that can be used in counterstaining, some of which are presented in table 5.2 [35]. The important factors to consider based on the mechanism involved in counterstaining are the binding specificity and spectral properties of these stains.

Table 5.2. Possible combinations of primary stains and counterstains used in counterstaining.

Primary Stain	Counterstain	Type of Bands
DAPI	Actinomycin D	G
DAPI	Distamycin A	C
Chromomycin A3	Methyl green	R
DAPI	Chromomycin A3	G

Table 5.3. Spectral properties and DNA sequence specificity of some stains.

Stain	Specificity	Absorption max. (nm)	Fluorescence max. (nm)
DAPI	AT	355	450
Chromomycin A3	GC	430	570
Actinomycin D (AMD)	GC	455	-
Distamycin A (DA)	AT	340	-
Methyl green	AT	648	-

Based from these tables, it can be observed that the emission wavelength of the primary stains has enough energy to excite their corresponding counterstains. It can also be observed that the combination of the two fluorescent dyes DAPI and chromomycin A3 is possible because their emission wavelengths are different. Figure 5.7 shows the bands produced from counterstaining DAPI.

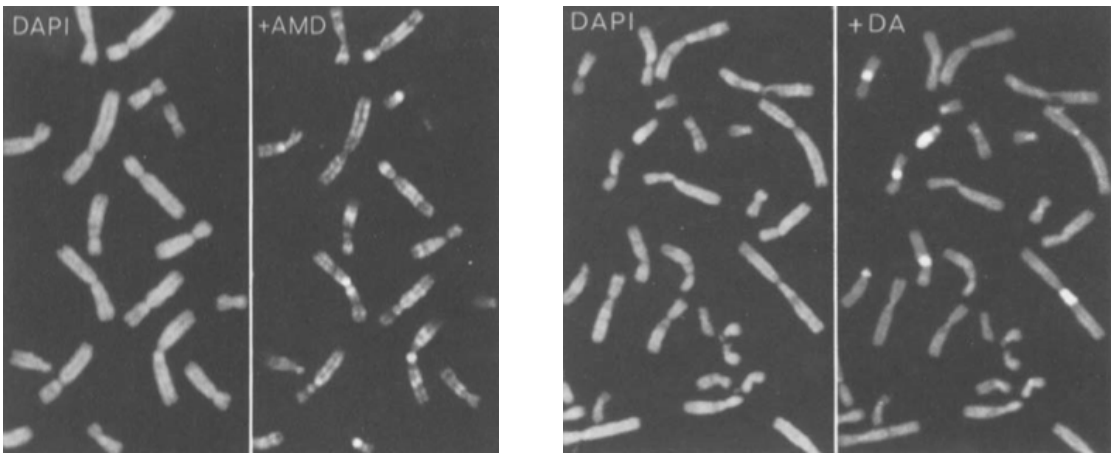

Figure 5.7. Enhancement of bands by counterstaining DAPI with AMD and DA [35].

The use of a heavy metal complex or compound as stains for chromosomes is particularly important when studying the chromosomes with electron microscopy or CDI. The presence of the heavy metal in the stains improves the visualization of the chromosomes under the said imaging techniques by enhancing the signal obtained from these techniques.

Heavy metal stains usually include DNA-metallointercalators and compounds containing Eu, Tb, Os, and Ur. DNA-metallointercalators are usually d8 platinum(II) complexes with a square planar geometry such as $[Pt(terp)Cl]^+$ and d6 octahedral metal complexes with aromatic diimine ligands such as $[Ru(diimine)]^{2+}$. Meanwhile, metallic compounds include osmium tetroxide and uranyl acetate.

6.1 Platinum Complexes

$[Pt(terp)Cl]^+$ (figure 6.1) is a well-known DNA-metallointercalator [38].

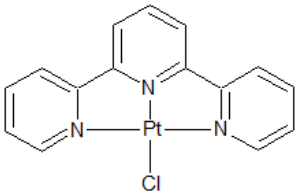

Figure 6.1. Structure of $[Pt(terp)Cl]^+$.

However, this complex is nonfluorescent. The fluorescence of a metal complex is dependent on the type of electronic transition involved upon irradiation of the complex with a photon [37]. $[Pt(terp)Cl]^+$ involves a metal-centered electronic transition. It is nonfluorescent because its d-d excited states are short-lived and easily subjected to radiantionless deactivation [39].

Enhancing the fluorescence of this metal complex is advantageous because the use of a fluorescent heavy metal stain allows the same chromosome specimen to be imaged using both fluorescence microscopy, and electron microscopy or CDI. The quantum efficiency of platinum complexes can be increased by raising the energy of the d-d excited states

and changing the type of the electronic transition involved into metal to ligand or intraligand charge transfer. This is achieved by using cyclometalating ligands such as 2-phenylpyridine [39]. Furthermore, adding aryl groups on the terpyridine ligand can also increase the quantum efficiency of platinum complexes [40].

A study by Williams in 2003 [39] shows that the use of a terdentate cyclometalating ligand, with an N^C^N coordination (figure 6.2), dipyridylbenzene specifically, greatly increases the quantum efficiency of platinum complexes (~ 0.7 [38]). It was also observed that terdentate ligands with the N^C^N coordination mode produces platinum complexes with higher quantum efficiencies than those with a C^N^N or C^N^C coordination mode.

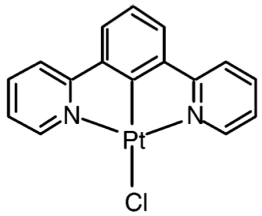

Figure 6.2. Platinum complex with an N^C^N cyclometalating ligand.

The platinum complexes discussed above do not have sequence specificity and bind uniformly along the length of the DNA, which can be attributed to their intercalation binding mode. Hence, these complexes are not capable of producing bands in chromosomes. Banding in chromosomes can be made possible for platinum complexes by adding a ligand with a functional group that is capable of forming H-bonds with the AT or GC base pairs of the DNA. For example, the platinum complex $[Pt(CNN)(4\text{-dpt})]^+$ (figure 6.3) contains two types of ligand [15]. The platinum metal together with the CNN ligand, which is responsible for the fluorescence of the complex, intercalates between the DNA base pairs. Meanwhile, the 4-dpt ligand can freely rotate and be out of the plane of the intercalating group, and contains hydrogen donor and acceptor groups that are specific for the AT base pairs of the DNA. This causes the 4-dpt ligand to bind in the minor groove of the DNA and the complex to have sequence specificity.

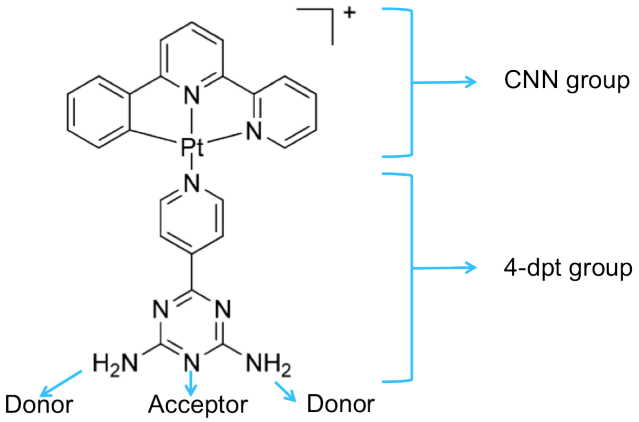

Figure 6.3. Structure of $[Pt(CNN)(4\text{-dpt})]^+$.

6.2 Uranyl Acetate and Osmium Tetroxide

Uranyl acetate ($UO_2(CH_3COO)_2$) and osmium tetroxide (OsO_4) are heavy metal stains that are widely used in studying biological samples with electron microscopy and can be used for both positive and negative staining. They are known to scatter electrons well and adsorb to biological matter easily [41]. Furthermore, these two compounds can also act as fixatives for biological samples [41, 42].

Uranyl acetate, a radioactive substance, is known to especially increase the contrast of nucleic acids. The staining solution is typically prepared as 2% or saturated aqueous solutions or alcoholic solutions with a concentration of 2-25% of pH 3.5-4 [43]. The acidic nature of the staining solution ensures the production of UO_2^{2+} ions, which are capable of interacting ionically with the phosphate groups of the DNA. It was observed that one uranyl ion is attached for every two phosphate groups of the DNA [44]. The treatment of DNA with uranyl acetate increases the dry weight of the DNA by a factor of two [45] and prevents the extraction of the DNA from its histone shell [46]. Furthermore, uranyl acetate is also known to interact with carboxyl groups [47]. Hence, it can stain not only the proteins in the chromosomes but also the proteins of other cellular components. This is problematic when studying chromosomes. However, uranyl ions have stronger affinity for phosphate groups than carboxyl groups and thus, preferentially staining DNA over proteins [44].

If the staining solution is used at high pH, precipitation of the DNA occurs. Furthermore, the high pH causes the production of anions from uranyl acetate that can bind to proteins [44, 47]. Hence, the preferential staining of DNA over protein is reduced.

Uranyl acetate is also commonly used in double staining with lead citrate [47]. It is added after the samples are stained with lead citrate. This procedure is known to increase the density of cell structures beyond the degree that can be obtained with either stain alone.

Meanwhile, osmium tetroxide is also widely used as a heavy metal stain in electron microscopy for biological samples primarily because of its property as a strong oxidant. It is also nonpolar thus allowing easy penetration inside the cell [42]. However, one disadvantage of this stain is that it is a highly poisonous compound.

Since OsO_4 is a strong oxidant, it is capable of oxidizing unsaturated double bonds in biological samples [48]. The reduced osmium imparts the high contrast. This heavy metal stain is usually used for staining lipids, which contain a lot of unsaturated double bonds.

Chromosomes are completely unstained by OsO_4 [49]. Even though OsO_4 stains nucleic acids by oxidizing the double bond at carbons 5 and 6 of thymine and cytosine, the double stranded DNA in the chromosomes remain inert. The reason for this is that this double bond is not accessible to OsO_4 in the double stranded DNA [42, 50].

Another possible site of interaction of OsO_4 with chromosomes are the proteins [49]. OsO_4 can react with the oxidizable nitrogen-containing groups of amino acids: e-amino group of lysine, guanidino group of arginine, imidazole group of histidine, pyrrolidine nitrogen of proline, and indole nitrogen of tryptophan. The histones in the chromosomes are rich with the basic amino acids arginine, histidine, and lysine. However, these basic groups of the histones are linked to the phosphate groups of the DNA. Hence, chromosomes fail to stain with OsO_4 due to the blocking of the reactive groups.

Upon treatment of the chromosomes with dilute acetic acid, however, chromosome staining becomes possible [49, 51]. The acetic acid weakens the nucleoprotein linkage thus exposing the basic amino acids of the histones and allowing these to react with OsO_4. Chromosome staining starts at pH 6.2 and reaches a maximum at pH 3.5.

Conclusion

By investigating the mechanism of staining and banding, an understanding of the relation of the chromosomal bands with the chromosomal substructures was achieved. Furthermore, through this review, the dependence of these mechanisms on the structure of the stain was presented. This review also examined the possibility of enhancing bands through counterstaining, and enhancing the fluorescence and sequence specificity of metal stains.

The information obtained from this review will be of use for future works in developing various combinations of stains for counterstaining to produce bands of higher resolution, and in developing a metal stain that scatters electron well and is capable of fluorescence and banding. These future works will improve the imaging of human chromosomes with fluorescence and electron microscopy, and coherent x-ray diffraction, which in turn will bring advancements in the study of the chromosome structure.

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