Chapter 2
Chromosome Heteromorphism

The term heteromorphism is especially applicable to normal variants observed by chromosome banding techniques. However, normal variations in morphology in certain regions of the human genome were noted even before the advent of chromosome banding. In the first Conference on Standardization in Human Cytogenetics in Denver in 1960 [1], chromosomes were divided into Groups A-G based on their relative sizes and positions of the centromeres. The X chromosome fell somewhere in the C-group. The Y was distinguishable from the G-group by its lack of satellites and somewhat distinctive morphology. At the London Conference in 1963 [2], prominent secondary constrictions were identified near the centromeres in the no. 1 chromosome pair in the A-group, in a chromosome pair (no. 9) in the C-group and in a pair (no. 16) in the E-group. By the Chicago Conference in 1966 [3], it was generally recognized that these regions and the Y varied in length, and that there were morphological variations in the short arms of the D- and G-group chromosomes.

In the early 1970s, Q-, G- and C-banding techniques became widely used. Q- and G-banding introduced a new era in which individual chromosomes could be definitively identified. With this capability, it also became possible to localize regions variable in size and staining to specific chromosomes. In particular, Q- and C-banding revealed distinct classes of heteromorphisms that were not necessarily detectable in non-banded chromosomes, but could be shown to be heritable in banded chromosomes. The most distinctive heteromorphism by Q-banding was the brightly fluorescent distal long arm of the Y chromosome. The size of this brightly fluorescent segment varied from being almost negligible in size to being the longest segment on the Y long arm. Q-banding (Fig. 2.1) also revealed variations in staining of chromosomes 3, 4, 13–15, and 21–22 [4–8].

Although G-banding techniques became widely used for chromosome identification (Fig. 2.2), C-banding revealed size variations of heterochromatin (h) around the centromeres of every chromosome that could be more easily quantitated than in non-banded chromosomes. The h regions of chromosomes 1, 9, 16 and in the distal long arm of the Y, evident in non-banded chromosomes, were especially visible by C-banding (Fig. 2.3) [8–12]. A system to describe variations observed by Q- and C-banding by intensity and size was incorporated into the cytogenetic nomenclature (Table 2.1).
Additional specialized techniques quickly followed, including R-banding [13], silver staining for nucleolar organizing regions (NORs) [14], G-11 staining [15], and staining with various fluorescent DNA-binding fluorochromes, either singly or in combination. Some of these techniques revealed additional subclasses of variants [16] so that a complex system of characterizing variants by band intensity and stain-
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Fig. 2.3  

(a) Normal female karyotype by CBG banding by barium hydroxide treatment followed by Giemsa staining (c41).  

(b) CBG-banded metaphase from normal male. Arrows point to 1, 9, 16 and Y chromosomes, which typically show the greatest amount of heteromorphism in different individuals.
ing technique was proposed in a Paris Conference Supplement [17]. However, the system was not widely used, and is not included in subsequent versions of ISCN [18].

Early molecular studies showed C-band heteromorphisms to be composed of different fractions of DNA, referred to as satellite DNAs based on their differing AT/GC content and buoyant densities in CsCl or Cs₂SO₄ gradients [19–21]. Alkaline Giemsa and DA/DAPI [22, 23] techniques stain components of 1qh, 9qh, D-G-group short arms, 16qh and distal Yqh. In situ hybridization studies revealed different but overlapping distributions of satellite DNA fractions to the various heterochromatic regions in the human karyotype, with a loose correlation between alkaline Giemsa staining and sites of the “classical” satellite III [24–28]. In the 1980s and early 1990s, molecular techniques more accurately characterized various satellite DNA sequences [29], while fluorescent in situ hybridization (FISH) [30, 31] allowed virtually any DNA sequence to be visually localized to specific chromosomal sites. Current FISH and molecular technologies define satellite DNAs somewhat differently, but sequences in the satellite III family localize to similar chromosome regions.

FISH and DNA sequencing have shown considerable shuffling of satellite sequences. These technologies provided the means not only to characterize heteromorphisms detected by classical techniques with greater accuracy and precision, but to also identify new chromosomal variants. A handful of what might be termed “FISH variants” has been reported.

### 2.1 Chromosome Banding Techniques and Mechanisms

#### 2.1.1 Q-banding

Caspersson and colleagues at the Karolinska Institute with an American team of biochemists at Harvard Medical School headed by S. Farber and G. Foley set out to...
test or design fluorescent molecules that would preferentially bind to specific nucleotide pairs in DNA, which they hoped to be able to detect spectrophotometrically. One molecule tested was quinacrine mustard dihydrochloride (QM), a nitrogen mustard analog of the anti-malarial drug, quinacrine. The dye, first applied to *Vicia faba* and *Trillium erectum*, revealed brightly fluorescent bands that distinguished the individual plant chromosomes. The findings led Caspersson et al. [32] to apply QM staining to human chromosomes X, with the discovery that the end of the long arm of the Y chromosome was brightly fluorescent—bright enough that the human Y chromosome could be easily detected in interphase as well as in metaphase cells. With refinements, QM produced banding patterns that were specific for each human chromosome and revealed heritable variations in size and/or intensity of certain regions, especially of distal Yq, of the centromeric regions of chromosomes 3 and 4, and of the centromeric and short arm regions of the acrocentric chromosomes (Fig. 2.1). Several investigators showed that the AT-rich regions of DNA corresponded to the bright fluorescent bands obtained with quinacrine mustard [33–35]. Weisblum and DeHaseth [33] showed that rather than preferential binding, this difference in intensity of fluorescence reflected a difference in quenching of the QM molecule. AT-richness alone, however, is not the sole determinant of the intensity of Q-banding. The actual differences in relative percentages of AT vs. GC in different regions are not as great as might be implied. The periodicity of interspersed GC, within short, highly repetitive AT-rich sequences, as well as the presence of specific nucleoproteins appears to play a significant role [36].

### 2.1.2 G-banding

G-banding, introduced in 1971 by Sumner et al. [37] overcame two significant problems of Q-banding (stability and cost) and thus became the more widely used banding technique in clinical laboratories. G-banding acronyms GTG, GTW, GTL and GAG all represent variations used to obtain the same banding pattern that can be seen and analyzed by standard light microscopy. While the original G-banding method used acid fixation with saline treatment followed by Giemsa staining (GAG) [38], application of proteolytic enzymes such as trypsin [39, 40] or pancreatin [41, 42] were simpler and improved the banding pattern. The blood stains, Wright’s or Leishman’s, are often used instead of Giemsa, depending on the laboratory’s experience and preference. G-banding patterns are identical, however, irrespective of how they are obtained (by enzymatic or chemical pretreatment) or the blood stain used (Fig. 2.2). Similar to bright Q-bands, dark-staining G-bands are AT-rich regions of chromosomal DNA that are more condensed, and replicate their DNA later than GC-rich regions which are less condensed (Table 2.2) [43]. DNA-binding proteins thought to be involved in maintaining chromosomal structural integrity form the nuclear matrix and include topoisomerases that have a basic role in the control of gene activity [44–46]. It may be that nuclear matrix proteins that hold
AT rich regions together make them less easily available for DNA replication and at the same time allow dye to bind only in monomer form so that they stain more intensely. Conversely, GC-rich regions that are gene-rich and transcriptionally active may be more loosely bound and consequently bind dye in polymer form with less intense staining. Giemsa, Leischman, Wright or Romanowski blood stains all contain mixtures of thiazin dyes, each of which can produce banding under the right conditions. It is evident from the variety of treatments that produce G-banding that more than one mechanism is involved. The most reliable and widely used treatment is mild proteolytic digestion with trypsin [39, 40]. However, the precise role of nucleoproteins in G-banding has not been determined [47–50]. Extraction of histones also seems to have little effect [51–54]. In fact, very little protein is lost from chromosomes in various G-banding treatments [48]. Furthermore, it is evident that there is an underlying structural integrity of the chromosome that is revealed in the “chromomere pattern” of very long chromosomes in meiosis [55, 56]. This pattern in non-banded meiotic chromosomes is identical to the pattern of G-banded metaphase chromosomes (see ISCN [18]).

The relationship between DNA structure and the binding of components making up Giemsa dye mixtures also is not totally understood. Treatments that loosen the integrity of underlying DNA structure appear to be most effective, suggesting that certain Giemsa components bind to condensed DNA in monomeric form and to looser DNA structure in polymeric form. The more the individual dyes components become stacked, the greater the shift to lower absorption spectra (purple or pink). In monomer form the shift is to the blue end of the spectrum. Such a shift in color, based on a dye’s ability to become stacked in polymer form, is referred to as metachromacy. Some Giemsa components are more metachromatic than others. Methylene blue, Azure A, Azure B, and Thiazin show varying degrees of metachromacy determined by the number of methyl groups present on the dye molecule [57–59]. Eosin, which

<table>
<thead>
<tr>
<th>Table 2.2</th>
<th>Techniques for recognition of different classes of chromatin and properties of chromosome bands. (Modified from Sumner [43])</th>
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<tbody>
<tr>
<td><strong>Class</strong></td>
<td><strong>Heterochromatin</strong></td>
</tr>
<tr>
<td>C-banding</td>
<td>G-banding</td>
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is also a component of Giemsa dyes, shows no metachromacy but appears to have a differential staining effect when combined with the other components.

### 2.1.3 R-banding

Utrillaux and Lejeune [60] introduced a banding technique involving treatment of chromosomes in saline at high temperature (87°C) that resulted in a reverse pattern of G- or Q-bands. They called this “reverse (R) banding” and, since the method involved staining with Giemsa, it is described as a “RHG” banding. R-bands are most useful in identifying abnormalities involving the terminal regions of chromosomes, which are lighter staining by G- and or Q-banding. Alternate methods to produce R-banding use various fluorescent chemicals such as acridine orange and chromomycin A3/methyl green [61–63] (Table 2.2). However, because of technical difficulties or fluorescent requirements, R-banding is still not used in many laboratories.

### 2.1.4 C-banding

During experiments with in situ hybridization of tritium-labeled satellite DNA to mouse chromosomes, Pardue and Gall [64] noted that constitutive heterochromatin at the centromeres of mouse chromosomes stained darker than other chromosomal regions. In 1971, Arrighi and Hsu [65, 66] developed a modified technique in which they applied Giemsa staining to preparations that were first denatured with 0.07 M NaOH and then incubated in two times standard saline concentration (2 × SSC) for several hours. In a more recent modification, Sumner [67] substituted barium hydroxide for sodium hydroxide, producing the same C-banding pattern (CBG-banding) but with less distortion of the chromosome morphology. Both procedures result in intense staining of the heterochromatin around the centromeres, whereas the rest of the chromosome stains pale blue (Fig. 2.3). Arrighi and Hsu initially postulated this differential staining was due to faster re-annealing of repetitive DNA in heterochromatin than in the less repetitive DNA sequences elsewhere. McKenzie and Lubs [68] produced C-banding by simply treating chromosomes with HCl and prolonged incubation in 2 × SSC. Studies by Comings et al. [48] demonstrated considerable extraction of nucleoprotein and DNA from non-heterochromatic regions by various C-banding treatments, while heterochromatic regions were resistant to such extraction. Furthermore, they demonstrated that hybridization of repetitive sequences in solution was not required for enhancement of staining, but in fact those regions reassociated instantaneously when they were removed from the NaOH solution. Subsequent incubation in 2 × SSC extracted additional non-heterochromatic DNA. Since incubation that produces C-banding is done for times ranging from a couple of hours to overnight, it is unlikely that much single stranded DNA remains
to bind Giemsa components. Differential staining is more likely due to the greater amount of double stranded DNA remaining in the heterochromatic regions.

### 2.1.5 Cd Banding

The technique, first described by Eiberg [69], reveals pairs of dots at presumed centromere locations; hence, the term “centromere dots” (Cd). The technique involves the usual hypotonic treatment of chromosomes followed by a series of fixations starting with a 9:1 ratio of methanol: acetic acid followed by a 5:1 ratio and then the standard 3:1 ratio. One week old slides are then incubated in Earle’s balanced salt solution (pH 8.5–9.0) at 85°C for 45 min followed by staining in a dilute solution of phosphate-buffered Giemsa (0.0033 M, pH 6.5). The technique appears to specifically stain only active centromeric regions and not inactive centromeres, secondary constrictions or other variable heteromorphic regions [70, 71]. It has been used to identify the active centromere(s) in dicentric, pseudodicentric and Robertsonian translocations. The mechanism of this technique suggested by Eiberg was that it represented a specific DNA-protein complex. Evans and Ross [72] suggested the Cd-positive regions represent kinetochores. Nakagome et al. [70, 71] and Maraschio et al. [73] studied dicentric and pseudodicentric chromosomes and showed that the Cd-positive regions do appear to correspond only to active centromeres. The presence or absence of specific centromeric proteins associated with centromeric activity have been recently studied with specific fluorescent antibodies that distinguish particular proteins associated with active or inactive centromeres [74].

### 2.1.6 G-11 Banding

G-11 staining is used to selectively stain some heterochromatic regions on human chromosomes a deep magenta color in contrast to the pale blue color of the remainder of the chromosome. These include chromosomes 1, 3, 5, 7, 9, 10, 19 and Y. However, there is variability in the intensity of staining at the pericentromeric and satellite regions of acrocentric chromosomes. Such variability is dependent on the individual characteristics of these chromosomes. The G-11 technique utilizes modified Giemsa staining at an alkaline pH [75] and is useful in the study of human heteromorphic variants and pericentromeric inversions, especially on chromosome 9. Figure 2.4a shows a metaphase with typical G-11 banding. G-11 banding received its name from attempts to obtain differential banding of specific chromosome regions by staining in Giemsa at different pH values. The standard pH of the staining solution in G-banding procedures is 6.8–7.0. It was found by Patil et al. [77] that if the alkalinity of some Giemsa mixtures was raised to 9.0, G-banding could be achieved without any other special treatment. Bobrow et al. [76] showed that if alkalinity was raised to pH 11, subcomponents of C-bands, especially the secondary
constriction (qh region) of chromosome 9 stained a deep magenta color in contrast to the pale blue color of the euchromatic regions. Jones et al. [20] first showed that satellite III DNA, isolated on a silver cesium sulfate gradient, hybridized to the heterochromatic regions of chromosome 9 and to the acrocentric chromosomes. Buhler et al. [28] showed that this magenta-staining DNA which appears to be especially specific for 9qh, 15p and Yq corresponded to sites of hybridization of a specific class of highly repetitive satellite III DNA. Other classes of satellite DNAs, I-VII were found to be distributed in chromosome 9 and in other chromosomes [21], but satellite III was found mainly in these three chromosomes. The mechanism of G-ll banding is still uncertain. Wyandt et al. [77] tested various components of Giemsa and showed that G-ll banding could be achieved when the right proportions of Azure B and Eosin Y were mixed at pH 11. When mixed in equimolar amounts, most of the Azure B and Eosin Y precipitated as large highly reflective trapezoidal crystals of azure-eosinate (Fig. 2.4d). Finer crystals appear to be precipitated at magenta colored sites on chromosomes (Fig. 2.4b, c).

2.1.7 Silver Staining (AgNOR)

Silver staining is a method to stain the nucleolar organizer regions (NORs) on the human acrocentric chromosomes. NORs, which contain the genes for ribosomal RNA or proteins, were known early to stain with silver. Using this information, Howell et al. [78] showed that NORs on chromosomes could be stained with silver nitrate and called their technique “Ag-SAT”. Howell and Black [79] subsequently developed a simplified technique using a colloidal developer for better results. Many laboratories use this method with various modifications. Figure 2.5 shows a metaphase with typical AgNOR staining. There is still controversy as to the nature or exact location of this silver staining. Miller et al. [80] showed that the activity of NOR regions appeared to be responsible for the staining. Goodpasture et al. [81] showed the actual location of the staining was in the satellite stalks of acrocentric chromosomes.
chromosomes and not the satellites themselves, although the silver stained mass may appear to cover or extend into the satellite region if the stalk or satellite is small. Subsequent experiments by Verma et al. [82] showed that Ag-NOR positive chromosomes are those that are found frequently in satellite associations while the Ag-NOR negative chromosomes are not seen in such associations. Silver staining is an important banding method to study heteromorphic variations in the size and number of NORs, and to characterize marker chromosomes or other structural rearrangements involving the acrocentric chromosomes.

2.2 Other DNA-Binding Fluorochromes

A variety of different DNA binding fluorochromes will produce chromosome banding patterns or enhancement of AT or GC rich regions depending on absorption and emission spectra and how they are used in combination (Table 2.3). For instance, the combination of distamycin A (DA) and DAPI) produces bright qh regions on chromosome 1, 9 and 16 that correspond to G-11 bands and probably to satellite III DNA. The use of various fluorochromes and their mechanisms of action have been described by others [63] and will not be described in detail here.

2.3 Sister Chromatid Exchange Staining (SCE)

Sister chromatid exchanges (SCE) are the result of interchange of DNA between replication products at homologous loci [83]. SCEs at low levels are normally seen in humans and can be demonstrated in somatic cells by incorporating a thymidine
Table 2.3 Fluorescent DNA ligands used in human chromosome staining, base affinity and type of banding when used with counter stain. (Adapted from Verma and Babu [63])

<table>
<thead>
<tr>
<th>Primary dye</th>
<th>Affinity</th>
<th>Counter stain</th>
<th>Banding</th>
</tr>
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<tbody>
<tr>
<td>DAPI</td>
<td>AT</td>
<td>Distamycin A^a</td>
<td>DAPI/DA</td>
</tr>
<tr>
<td>DIPI</td>
<td>AT</td>
<td>Netropsin</td>
<td>DAPI/DA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pentamidine</td>
<td>DAPI/DA</td>
</tr>
<tr>
<td>Hoechst 33258</td>
<td>AT</td>
<td>Distamycin A^a</td>
<td>DAPI/DA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Netropsin</td>
<td>DAPI/DA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pentamidine</td>
<td>DAPI/DA</td>
</tr>
<tr>
<td>7-aminoactinomycin D</td>
<td>GC</td>
<td>Methyl green^a</td>
<td>R-bands (enhanced)</td>
</tr>
<tr>
<td>Chromomycin A&lt;sub&gt;3&lt;/sub&gt;</td>
<td>GC</td>
<td>Distamycin A^a</td>
<td>R-bands (enhanced)</td>
</tr>
<tr>
<td>Mithramycin</td>
<td>GC</td>
<td>Malachite green^a</td>
<td>R-bands (enhanced)</td>
</tr>
<tr>
<td>Olivomycin</td>
<td>GC</td>
<td>Distamycin A</td>
<td>R-bands (enhanced)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Netropsin</td>
<td>R-bands (enhanced)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methyl green^a</td>
<td>R-bands (enhanced)</td>
</tr>
<tr>
<td>Coriphosphin</td>
<td>GC (low)</td>
<td></td>
<td>Q-bands</td>
</tr>
<tr>
<td>Quinacrine/quinacrine mustard</td>
<td>GC (low)</td>
<td></td>
<td>Q-bands</td>
</tr>
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</table>

^a Non-fluorescent with AT affinity
^b Non-fluorescent with GC affinity

analog, 5-bromodeoxyuridine (BrdU) into replicating DNA for two successive cell cycles and subsequent photodegradation of the resulting chromosomes. Staining of metaphases with Hoechst 33258 [84] or with Giemsa following this procedure results in faint staining of one chromatid and strong staining of the other chromatid. A reversal of staining intensity of the two chromatids occurs where there has been an exchange (Fig. 2.6). Because of the semi-conservative nature of DNA replication, after two complete pulses of BrdU substitution, one chromatid has both halves of

Fig. 2.6 Metaphase showing sister chromatid exchanges (arrows)
the DNA helix BrdU-substituted (bifilarly labeled) while the other chromatid has only one half of the DNA helix BrdU-substituted (monofilarly labeled). The latter is the basis of differences in staining of sister chromatids that allow detection of SCEs, mainly in non-heterochromatic regions. The technique has been extensively used for testing the mutagenic potential of various chemicals [85], to study cell cycle kinetics [86, 87] and to diagnose Bloom syndrome, in which there is a tenfold increase in SCE per cell [88].

### 2.4 Replication Banding

Replication banding is most useful in identifying the early and late replicating X-chromosomes in females or in patients with sex chromosome abnormalities. It is well known that one of the X-chromosomes in females is inactive, resulting in dosage compensation [89]. It is also known that X chromosome inactivation is random and that the inactive X chromosome initiates and completes DNA synthesis later than the active X and other chromosomes [90–94]. Replication banding, obtained by incorporation of 5-bromodeoxyuridine (BrdU) and subsequent staining with Giemsa or other stains [84], allows distinction of the active and inactive X-chromosomes. Variations in replication banding can also be achieved. In the “T pulse” procedure, BrdU is made available at the beginning of the cell cycle and then replaced with thymidine the for last 5–6 h before the harvest. With the RBG technique (R-bands by BrdU and Giemsa), the active or early replicating chromosome regions that inactive X chromosome, stain dark. The “B pulse” is the opposite. Thymidine, made available at the beginning of the cell cycle, is replaced with BrdU the last 5–6 h before harvest. Subsequent Giemsa staining will result in early-replicating chromosome regions appearing dark because they have incorporated thymidine, while the inactive or late-replicating chromosome regions appear pale due to the BrdU-incorporation.

**Banding patterns:** The equivalent of Q- and G- or R-banding patterns is achieved depending on whether a B or T pulse is used. If a B-pulse is used, a Q or G-banding pattern is achieved and if a T-pulse is used, an R-banding pattern is achieved. Subtle changes in pattern toward the earliest R-bands or latest G-bands can be achieved by shortening the length of the BrdU pulse. A short T-pulse at the very end of the S-period can produce what are referred to as T-bands (bright or dark bands at the terminal ends of some chromosome arms). These bright bands with a T-pulse also correspond to early replicating, GC-rich regions, whereas dull bands correspond to late-replicating AT-rich regions. The exception to this is the late-replicating X chromosome whose bright bands do not differ in AT: GC content from the less intensely stained bands at the same locations on the early-replicating X (Fig. 2.7).

**Lateral asymmetry:** An interesting variation of the BrdU labeling technique is the method of detecting lateral asymmetry. The latter is due to an interstrand
compositional bias in which one half of the DNA helix is predominantly T-rich and the complementary half is correspondingly A-rich [95]. Since BrdU substitutes for thymidine and not adenine, after one complete pulse of BrdU, the BrdU-rich strand stains less intensely than the T-rich complement, resulting in a block of heterochromatin that is more intensely stained on one chromatid than on the other. A more equal distribution of thymidine in both strands in either euchromatin or heterochromatin without interstrand compositional bias results in both chromatids staining similarly. Variation in the size and location of such blocks forms the basis of a subclass of variants in chromosomes 1, 9, 15, 16 and Y [96–98].

2.5 High Resolution Banding and Special Treatments

Other treatments and methods that have particular bearing on characterizing heteromorphisms include treatments such as methotrexate added to cultures to synchronize cells in G2 [99] and used for high resolution chromosome banding. Ethidium bromide intercalates into GC rich regions during cell culture, a property that is also used to produce elongated chromosomes for high resolution banding analysis [100, 101]. 5-azocytidine and a number of DNA analogs such as FudR, produce very long secondary constrictions such as shown by Balicek [102] or

Fig. 2.7 Metaphase with 47,XX,i(Xq) showing replication banding with a late T-pulse showing active X (A), lighter staining inactive X (small arrow) and extra i(Xq) (large arrow)
can enhance so-called “fragile sites” on chromosomes. Most of these are common fragile sites that can be induced in vitro in cells from anyone (See Sect. 5 on Fragile Sites). Other “rare” fragile sites are induced only in cells from certain individuals and are heritable.

2.6 Satellite DNA in Heteromorphic Regions

Genes and gene-related sequences (promoters, introns, etc) constitute about 25% of the human haploid genome; only about 3% of the genome is transcribed. Repetitive sequences comprising most of the remainder are the basis both of heteromorphisms observed at the chromosomal level and polymorphisms detected at the molecular level. Tandemly repeated DNA sequences are classified by the length of the individual repeated unit and by total size [103].

Satellite DNA makes up approximately 10% of the genome [104, 105]. Consisting of large tandemly repeated DNA sequences, it is located mainly in heterochromatic blocks in the pericentromeric regions of human chromosomes, the short arms of acrocentric chromosomes and the distal long arm of the human Y chromosome [106–108]. Alpha satellite DNA is the principle component found at the centromere of every human chromosome. Other satellite DNAs distributed to various chromosomal locations include: (1) Beta satellite DNA, a 68 bp monomer that consists of different subsets that have been shown to be chromosome specific by FISH [106]; (2) Gamma satellite DNA, a 220 bp monomer, observed at the centromeres of chromosomes 8 and X [107–110]; (3) additional families that include a 48 bp satellite DNA on the acrocentric chromosomes, and the Sn5 family found in the pericentromeric regions of chromosome 2 and the acrocentric chromosomes [110].

Human satellite DNA fractions, consisting of heterogeneous mixtures of repetitive DNA sequences isolated from main band DNA by buoyant densities on CsCl (cesium chloride) [111] or CsSO₄ (cesium sulfate) gradients [21, 24, 111] are referred to as classical satellites I, II and III [25, 112]. In situ hybridization of these fractions to human chromosomes is to locations that correspond to heterochromatin viewed by C-banding or by the fluorescent dyes DAPI and distamycin. [25–27, 113].

Satellite DNA fractions have been further separated by restriction enzyme analysis into classical satellites 1, 2 and 3, found primarily in the large h regions of chromosomes 1, 9, 16 and Y [114–116]. Although, satellites 1, 2 and 3 are incorporated within density gradient fractions, they are distinct from satellites I, II and III [3]. By in situ hybridization, satellite 1 is localized to the pericentromeric regions of chromosomes 3 and 4, and the short arms of the acrocentric chromosomes, both proximal and distal to the rDNA of acrocentric stalk regions. Satellite 2 is localized to the large heterochromatic regions of chromosomes 1 and 16, with less prominent domains in the pericentromeric regions of chromosomes 2 and 10. Satellite 3 is localized to h regions of chromosome 1, 9, Y and the acrocentric chromosome short arms, proximal to the ribosomal DNA [114]. It is also found in the pericentromeric region of chromosome 10 [117].
2.6 Satellite DNA in Heteromorphic Regions

2.6.1 Alpha Satellite DNA

The fundamental unit of alpha satellite DNA is a monomer of ~171 bp. Monomers are tandemly organized into higher-order repeats (HORs) ranging from 2 to > 30 [118]. HORs at each centromere are in turn tandemly repeated up to several hundred times to form an array of several million base pairs. HORs that are specific for each chromosome and hence useful as FISH probes typically show less than 5% divergence. In addition to HORs, alpha satellite DNA contains a subset of monomers with a 9 bp degenerate motif that serves as the binding site for Centromeric Protein B (CENP-B) found in most mammalian centromeres and initially thought to be involved in recruitment of essential kinetochores proteins such as CENH3 (CENP-A), CENP-C and CENP-E. However, neither alpha satellite motif nor CENP-B are required for the formation of a functional centromere in all mammals and more recent investigation indicate epigenetic factors rather than sequence directed mechanisms in the formation of centromeres. The role of alpha satellite motifs and various centromeric proteins in centromere function is still an active area of investigation [119–121].

2.6.2 Minisatellites

Levy and Warburton [103] classify minisatellites into AT and GC rich. Tandemly repeated GC rich sequences [122] are at many different loci which vary in the size of the individual repeat (6 to ~100 bp) as well as in total length (100 bp to several kilobases). The widely variable number of tandem repeats (VNTRs) at these loci has made them a useful tool in forensic science for individual identification by DNA fingerprinting, and as highly polymorphic, multiallelic markers for linkage studies [123]. While most minisatellites are GC-rich, AT-rich minisatellites in humans are remarkably different from the GC-rich minisatellites [124–128]. The common features of these alleles include a predicted tendency to form hairpin structures and a domain organization with similar variant repeats commonly existing as blocks within arrays [123]. These loci may also share some mechanisms of mutation, with transient single-stranded DNA forming stable secondary structures which promote inter-strand misalignment and subsequent expansions or contractions in repeat number [128]. Telomeres are a special subset of minisatellites. The majority of hypervariable minisatellite DNA sequences are not transcribed, however some have been shown to cause disease by influencing gene expression, modifying coding sequences within genes and generating fragile sites [123].

2.6.3 Microsatellites

Microsatellites consist of units of two to four nucleotides repeated one to a few dozen times. Polymorphic alleles of such sites consist of a differing numbers of repeats, also referred to as short tandem repeat polymorphisms (STRPs). Several
hundred thousand STRP loci are distributed throughout the genome with many alleles for each locus in the population. Microsatellite polymorphisms are not usually implicated in disease, but are useful markers for determining the identity of a particular individual [129, 130].

2.7 Single Nucleotide Polymorphisms (SNPs)

The most common polymorphisms are single nucleotide polymorphisms (SNPs). In contrast to STRPs, SNPs usually only have two alleles for any specific location. They occur approximately every 1,000 bp, with approximately 3 million differences between any two genomes or an estimated 10 million alleles in all human populations [131]. A subset of approximately 1 million of the most frequent SNPs have been chosen for a high-density map called the “HapMap” or haplotype map of the human genome [132].

2.8 Fluorescence In Situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) has been a powerful adjunct in cytogenetics. In principal, any piece of DNA (or RNA) can be isolated, amplified and labeled. It can then be hybridized to intact chromosomes, nuclei, or other fixed target and detected. Labeled DNA segments, called probes, are prepared by a variety of techniques including (1) synthesis of cDNAs from mRNAs by reverse transcriptase [132]; (2) isolation of specific sequences by PCR amplification and/or gel electrophoresis [133, 134]; (3) propagation of larger DNA fragments in bacteria or yeast by insertion into cloning vectors such as plasmids, phage, cosmids, BACS, or YACS [135, 136]; (4) isolation and cloning of partial or complete DNA libraries from specific chromosome regions or entire chromosomes by microdissection [137, 138] or chromosome sorting [139, 140]. Whatever the source, labeling is usually completed by nick translation or random priming with nucleotides that either have fluorescent label attached directly or combined with a ligand that is recognized by a fluorescent-tagged protein.

Procedures for in situ hybridization, described in numerous reviews elsewhere [141, 142], involve a number of precisely controlled steps. The crucial steps are: (1) denaturation of probe and target DNA sequences to single strands; (2) incubation of probe and target under conditions that allow specific association (hybridization) of labeled probe DNA to complementary target sequences; (3) washing away non-hybridized probe; (4) detection of hybridized sequences in target cells. The rate of hybridization of probe in solution to complementary DNA targets bound on the glass slide follows first order kinetics [141]. This rate is dependent upon the labeled probe concentration in solution (number of copies of a specific sequence per unit volume) at given time. If the ratio of labeled probe to unlabeled target is too low, insufficient labeled sequence will anneal to the target to permit subsequent detec-
tion. If the ratio is too high, precipitation of probe or non-specific hybridization to imperfect complements may result in false-positive signals. Typical ratios of probe to target DNA are on the order of 100:1.

Other factors controlling rates and specificity of hybridization are salt and formamide concentrations and temperature, in both the hybridization and subsequent wash steps [141]. The melting or denaturation temperature (Tm) of DNA is 90–100°C. Such high temperatures applied to intact cells or chromosomes destroy their morphology and integrity. Formamide is used to lower the melting temperature of the DNA so that it does not unduly damage the target cells. Typically, denaturation is in 50% formamide in 2 × SSC at about 70°C. Hybridization is done in a mixture with precise concentrations of formamide, salt, buffer and probe in a humid environment at 37°C, for 4–24 h. Hybridization between closely related sequences with as little as 70% homology can occur. Therefore, precise conditions for washing away of excess probe, referred to as “stringency” of wash, are also crucial.

2.8.1 Types of Probes

FISH has become the technique of choice to detect chromosome abnormalities that are either too complex to be interpreted by banding or are below the resolution of standard chromosome banding techniques. Several types of probes, commonly in use, include:

1. *Satellite Probes*: These are probes that are homologous to repeated sequences around the centromeres of all chromosomes, the h regions of 1, 9, 16 and the Y and the satellites and short arms of acrocentric chromosomes. Alpha satellite probes for sequences that are specific to the centromeric regions of individual human chromosomes are commercially available. Exceptions are: chromosomes 1, 5 and 19; 13 and 21; 14 and 22. These three groups have probe sequences that cross-hybridize within each group and hence have been discontinued by at least one major probe distributor.

2. *Painting Probes*: These are libraries of probes that are specific for unique sequences isolated throughout the entire chromosome. Such libraries usually have non-specific repeated sequences repressed or removed. Paints specific for each human chromosome are commercially available.

3. *Locus-specific Probes*: Microdeletions that involve loss of segments of chromosomes less than a few megabases are usually not detectable by banding but are detectable by FISH when the appropriate probe is available. Several criteria should be met for such a probe to be useful: (1) it must be specific for a gene region associated with disease; (2) it must have been tested on enough cases to confirm specificity (frequency of association with the disease in question) and sensitivity (frequency of false positive and/or false negative results) [143]. Commercially available probes exist for about a dozen microdeletion syndromes and for an increasing number of chromosome regions involving oncogenes in cancer or leukemia.
4. **Subtelomeric Probes:** These are probes for sequences 70–300 kb in length that are immediately adjacent to the telomeres themselves and are specific for each chromosome arm (except the short arms of the acrocentric chromosomes) [144]. The forty-one different probes that are available commercially are typically used as a panel to rule out subtle structural deletions or rearrangements involving the ends of the chromosome arms.

5. **Telomeric Probe Sequence:** One specific repeated sequence, (TTAGGG)n is present at the end of every chromosome arm. The number of repeats (n) for each arm varies greatly from a minimal modal number of 300 bp to 17 kb, depending on tissue, differentiation, age and genetic factors. A critical number of repeats on each arm are necessary for the chromosome to be stable and for DNA replication of both strands to be completed without gradual loss of DNA over time [145].

### 2.8.2 Applications

A wide variety of applications of *in situ* hybridization techniques have been developed. The principles as outlined above are the same for all of them. Only the combinations of probes and their targets are changed. With increasing complexity of the technology, detection and data analysis is typically augmented by special computer software.

1. **Dual-colored Probes:** In the case of microdeletions or other locus-specific probes, a control probe of a different color is typically included in the probe mixture and hybridized at the same time. Detection can either be with a triple band-pass filter that allows detection of three different wavelengths (three different colors) simultaneously, or with three different single band-pass filters that each allows detection of only a single color at a time. In the latter case, individually collected digitized images are typically superimposed by computer software to generate a single three-colored image [146, 147].

2. **Multiple-colored Probes:** Several available systems allow visualization of the entire genome in multiple colors, accomplished by labeling DNA representing a particular chromosome in three or more colors and combining these colors in different ratios to give a different color for each chromosome [148]. Such combinatorial labeling can be achieved by superimposing narrow band-pass filter images that allow distinction of the various color ratios (so-called M FISH) [149] or by quantitatively measuring the pixel-value of each color and assigning a new color for each of the ratios (so-called SKY-FISH) [150, 151]. A third more esoteric method combined color ratio labeling of individual orangutan chromosomes and inter-species hybridization of the multicolored orangutan DNA to human metaphases with a resultant multi-colored banding pattern on the human chromosomes representing rearrangement of the orangutan genome in its evolution to the human karyotype (so-called Rx-FISH) [152].

3. **Comparative Genomic Hybridization:** This method of hybridization has been used mainly to characterize complex multiple chromosome abnormalities in
tumor cell lines [153–155]. DNA from the tumor line is extracted and labeled with a green fluorescent tag such as FITC and is mixed in equal molar amounts with DNA extracted from a normal cell line that has been labeled with a red fluorescent tag such as Texas red or rhodamine. The probe mixture is then hybridized to metaphases prepared from normal cells. Segments of chromosomes or entire chromosomes that are either in excess or deleted will have more or fewer green- vs. red-labeled sequences competing for complementary sites on the normal chromosomes. A segment that is in excess will have more green than red sequence (3:2 ratio) and produce a signal on the target chromosome that is correspondingly more green; conversely, a segment that is deleted will have less green than red sequence (1:2 ratio) and produce a signal that is correspondingly more red. Normal diploid segments have equal numbers (1:1 ratio) of red- and green-labeled sequences in the probe mixture and hence produce a yellow signal over chromosome regions that are not lost or gained. Such differences in ratios may not be seen easily in a single metaphase by eye and so are typically measured spectrophotometrically and the results combined from multiple metaphases.

2.8.3 Studies of Heteromorphisms by FISH

This section deals directly with the molecular characterization, particularly of the satellite DNA's that make up the most visible, structurally variable regions of the human genome. Such studies, for the most part, are based on results from a few cases and do not attempt to correlate molecular and cytological findings in any significant population of normal individuals. With one or two exceptions [156–158], the characterization of heteromorphisms at the molecular level is more anecdotal than systematic. However, more accurate characterization of heteromorphisms detected by banding is greatly augmented by the application of molecular cytogenetic techniques. FISH allows identification of specific segments of DNA in ways that are not possible with any of the standard ways of studying chromosome by conventional banding techniques. At the same time, they can detect new forms of heteromorphism in the human genome that were not detectable by previous methods. In fact, one of the drawbacks of FISH technologies is that variation in signal size or the apparent lack of a signal with a probe that is associated with a certain disease could be reflecting normal variability instead. Therefore, care must be taken in the interpretation of results when a new probe is used or the disease has not been well characterized [143]. It is also important to realize that differences in signal size by FISH, is more qualitative than quantitative. The smaller the signal, the more it may vary in size and frequency of detection.

Regions in the human genome that are variable in size and staining properties are also heterogeneous in their repetitive DNA make-up. Secondary constrictions of chromosomes 1, 9 and 16 are good examples of such heterogeneity, where reshuffling of repetitive sequences frequently occurs and can be detected by FISH. Because such sequences are closely related, there also can be frequent cross-hybridization.
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