Mapping Chromatin Structure: Isolation of a Specific Chromosome

By

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Abstract:

Chromatin, the DNA-protein complex is believed to play a crucial role in the regulation of genes. The main focus of this study is to investigate the post-translational modifications (PTMs) of histones, the major class of protein associated with DNA in chromatin. Study of chromatin structure has been based on the context of Prader-Willi Syndrome (PWS), an imprinting genetic disorder. All the genes associated with this disease are clustered on the 15q11-13 region of chromosome 15.

As a preliminary step, an immunocytogenetic approach was taken to study the PTMs, especially acetylation in a specific genomic locus. Immunofluorescence (IF) was used as a tool to analyze the histone modifications while fluorescence in situ hybridization (FISH) with a single copy FISH probe was used to identify the genomic locus of interest. The resolution obtained by these immunocytogenetic tools was insufficient to provide information about the chemistry of chromatin at a molecular level.

These preliminary observations and the more recent findings about the possibility of multiple types of modifications of histones contributing to the regulation of genes led us to think in the direction of mass spectrometric analysis of histones. However, in order to analyze histones in a specific genomic locus such as the PWS region, histones need to be extracted from that particular region of the genome. Thus, sample preparation is the most critical step in such an analysis. In order to extract histones from a particular region of a chromosome, that region will have to be clipped from the rest and separated. This step would be a much easier task

if the chromosome of interest is enriched. Therefore, the bulk of this dissertation is focused on the enrichment of chromosome 15 by two different approaches.

In one approach, the flow cytometric method, the main problem of lack of specificity, associated with the state-of the-art sorting of chromosomes by flow cytometry, which is based on the identification of chromosomes by fluorescent DNA binding dyes is addressed. Due to the poor specificity of this approach, there are many unresolvable groups of chromosomes that end up being sorted as a clump. In attempting to improve the purity of an individual chromosome by this method, the efficiency of sorting decreases dramatically. As a solution to this problem, an effort was made to identify the chromosome of interest with a specific DNA probe tagged with a fluorescent label. The population of fluorescent chromosomes were sorted and the purity verified by reacting an aliquot of the sorted pool of chromosomes with a chromosome 15 specific paint probe. The percentage of enrichment was 92%. A control experiment was performed by reacting chromosomes prior to fractionation, with the same chromosome 15 specific paint probe and the painted/total number of chromosomes was 6%. This indicates the feasibility to employ this method to obtain a highly pure population of chromosomes without losing the efficiency of sorting.

In the second approach where an affinity-based fractionation process is utilized, the two main short-comings of the state-of-the-art method of sorting chromosomes, specificity and efficiency are addressed by designing a massively parallel approach as opposed to a serial approach adopted in flow cytometric analysis. In attempt to isolate chromosome 15 from the rest of the human genome in a

lymphoblastoid cell line, a chromosome 15 specific FITC labeled DNA probe that targets the centromere was reacted with the chromosomes. Magnetic beads bound to anti-FITC antibody, were reacted with the labeled pool of chromosomes and separated by exposing to a magnetic field. Chromosome 15 could be enriched to about 75% within a maximum of 3-4 days regardless of the amount of material.

Dedicated to my parents

Mr.Piyasena Vitharana & Mrs.Nalini Vitharana

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Chapter 1: Introduction

The challenge taken up is to unravel the chemistry of chromatin, which is chromosomal DNA associated with mainly histones, a class of proteins which should shed light on understanding regulation of gene expression. Therefore, the objective of this work has been to set the foundation for analysis of post-translational modifications (PTMs) of histones associated with DNA in specific genomic intervals. As a preliminary approach to the problem, cytogenetic and molecular biological tools such as Fluorescence in situ Hybridization (FISH) and Immunofluorescence (IF) are utilized. These experiments have led to the conclusion that in order to gain adequate resolution to map the PTMs of histones on a residue to residue basis in specific locations in the genome, they need to be isolated from the corresponding genomic regions. In order to facilitate the isolation of a specific genomic locus, it is important to isolate the corresponding chromosome of interest from the rest of the genome, as the first step. Thus, methodology is developed to fractionate a specific chromosome by two novel approaches.

Concerning the structure of chromatin, chromosomal DNA is associated with basically two different types of proteins: histones and High Mobility Group (HMG) proteins. Out of these two categories of proteins, histones that are highly conserved across the species have drawn much of the attention of the scientific community because of the important structural and functional role they play in the organization

and regulation of chromosomal DNA. HMG proteins are associated with the transient dynamic behavior of chromatin by forming metastable complexes (Catez *et al*, 2003).

Approximately 146 base pairs of DNA are wrapped around an octamer of four different types of histones, H2A, H2B, H3 and H4. This entity is referred to as the nucleosome and is the primary level of organization of chromatin, composed of DNA associated with histones. The octamer of histones consists of 2 heterodimers each of H2A-H2B and H3-H4. Each histone comprises a globular domain residing in the core of the nucleosome, N-terminal and C-terminal tails protruding out of the core (Luger *et al*, 1997, Luger *et al,* 1998). For a very long time, it has been known that specific residues on the N-terminal tails of these histones undergo post-translational modfications such as acetylation, methylation, phosphorylation, ribosylation and ubiquitination. A fifth type, histone H1, is associated with the linker DNA between 2 nucleosomes (Wolffe, 1998).

The next level of organization is the solenoid, a 30 nm chromatin fiber resulting from the folding of nucleosomal chromatin into loops as shown in Fig 1.1. Electron microscopic images of the chromatin fiber are shown at the top part of Fig 1.1. It is believed that histones are involved in the compaction of the human DNA, whose total haploid length is 2 m long into a nucleus of 10 μm in diameter by forming primarily nucleosomes, secondarily solenoids and at a tertiary level by 3D arrays of solenoids. This is an example of the structural role played by histones.

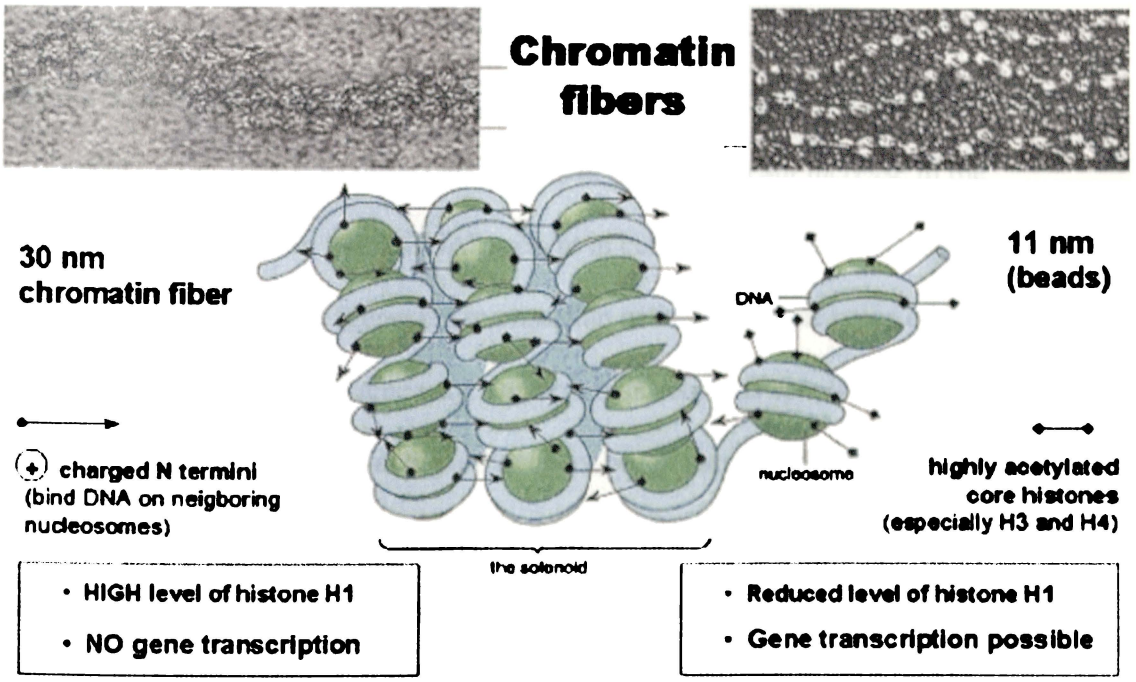

Fig1.1: Organization of chromosomal DNA into chromatin fiber

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Functionally, histones are believed to be involved in epigenetic regulation of genes. Epigenetics is a word coined to illustrate any regulation of DNA expression controlled by factors other than the DNA sequence that are heritable (Wolffe *et al*, 1999). Such epigenetic regulatory mechanisms could involve modifications such as DNA methylation and PTMs of histones. For example, in the context of this study, the genes responsible for Prader-Willi Syndrome (PWS), are all clustered in a genomically imprinted region (Ohta *et al*, 1999). This imprint, which is a mark to indicate expression of genes based on the parental origin, has been found to be associated with methylated DNA (Jones *et al*, 2001). This type of information

gathered over the years has led to various hypotheses involving regulation of genes and the modifications of histones, which will be described in the next few paragraphs.

Interestingly, with evolution, there seems to be a significant increase in the amount of non-coding or "junk DNA" present in cells, resulting in only 1-2% of total DNA being coding DNA, (genes) in mammalian cells. Both these types of DNA however, are packaged the same way, nucleosomes acting as spools at the primary level, extending to 3D arrays at higher levels of organization. Michel Grunstein and Fred Winston discovered in the late 1980s by genetic mutation studies performed with *Saccharomyces cerevisiae* (yeast) that histones do play an integral part in the regulation of genes (Han *et al*,1988). Yeast cells were genetically modified to produce a known amount of histone so that the degree of nucleosomal packaging could be controlled. This study proved that increasing numbers of nucleosomes have a negative impact on transcription. Transcriptionally active chromatin domains are structurally dynamic; the distribution or the spatial organization of nucleosomes changes when the state of transcription in a particular domain switches from a non-active to an active state. It has also been known that transcribed chromatin is more susceptible to nucleases such as DNase I and micrococcal nuclease, indicating the sensitivity to nucleases reflected by the exposed, nucleosome free chromatin in such domains that can recruit protein complexes required for regulation (Gross *et al*, 1988). DNase I hypersensitivity in particular is considered a property of actively transcribed domains of chromatin. The key point is that despite an enormous extent of

compaction of DNA with the assistance of histones, transcription still does take place in cells.

Acetylation of ε-amino and α-amino groups on lysine residues on the N-terminal tails of histones is a post-translational amidation reaction, taking place at the nucleosome. For about three decades, since Allfrey's pioneering work (Allfrey *et al*,1964) acetylation of histones has been known to favor transcription. In fact, by immunofluorescence and immunoprecipitation studies it has been concluded that acetylated lysine residues on histone H4 tails function as a marker for euchromatin, actively transcribed domains of chromatin (Jeppesen *et al*, 1992 and O'Neil *et al*, 1995). Hyperacetylation has been observed in euchromatin regions, whereas hypoacetylation has been observed in heterochromatin or non-transcribed chromatin domains. Immunofluorescence studies have shown that the inactive X chromosome in female cells is completely non-acetylated (Jeppesen *et al*,1992). Another important finding, in addition to the above, is the presence of histone acetyltransferase activity in the transcription machinery, providing evidence for direct association of acetylation to transcription (Brownell *et al*, 1996). This brings out two facets of acetylation: global or non-targeted acetylation indicates the heterochromatic and euchromatic blocks of the genome and the targeted acetylation at specific sites, especially the promoter regions when transcription is in progress. Interestingly, contrary to what has been observed most frequently, acetylation seems to be involved in the repression of the mouse mammary tumor virus promoter (MMTV), adding another layer of confusion and complication (Strahl *et al*, 2000). Not only is the

chromatin fiber in a cell dynamically equilibrating among the different levels of folding, but the acetylation - deacetylation status is continuously changing as well. The latter is a consequence of 2 classes of enzymes: Histone Acetyltransferases (HATs) responsible for acetylation, and Histone Deacetylases (HDACs) that remove the acetyl groups from the histones.

Two important types of methylation exist in epigenetic modifications; Cytosine methylation on DNA templates and Lysine and Arginine methylation on histones. Since the focus here is on post-translational modifications (PTMs) of histones, the discussion will be mostly confined to proteins. However, it is worth pointing out that it is the cytosines on clusters of cytosines and guanines, referred to as CpG islands (p indicating the phosphate groups that bridge the Cs to Gs) that get methylated, which again alters DNA-protein interactions in chromatin resulting in an alteration of the proteins that bind to the template favoring transcription or otherwise. Abnormal methylation of the promoter DNA regions that causes the corresponding genes to be silent, results in a wide array of diseases including cancer. The importance of differential DNA methylation patterns is emphasized by the initiation of a project called the human epigenome in 2000, in which an attempt is made to map DNA methylation patterns across the entire human genome (http://www.epigenome.org).

Methylation of histones involves an addition of a methyl group to lysine and/or arginine residues on N-terminal tails of histones H3 and H4. Lysine methylation occurs on residues 4, 9, 27, and 36 on H3 and lys 20 on H4. Unlike

acetylation and phosphorylation, methylation is a very stable modification and a demethylase has yet to be discovered. Until very recently, this was the least understood PTM of histones due to the lack of analytical tools to probe this modification. However, with antibodies generated against methylated and unmethylated lysine by Allis and coworkers (Allis *et al*, 2002) information about its function has started to trickle in.

Annunziato has first established that methylation is preferred on acetylated templates of chromatin (Annunziato et al, 1995). Another clue was provided by Strahl and co-workers when histone methyl transferase (HMT) activity was found in transcriptionally active macronuclei in *Tetrahymena* and not in inactive micronuclei. HMT activity was also found in a transcriptional coactivator-associated arginine methyl transferase (CARM1) (Chen *et al,* 2001). Methylation of H3 lys9 is considered a marker for transcriptional repression whereas methylation of H3 lys 4 marks active domains in *S. pombe* (Noma *et al*, 2001) and chicken β-globin locus control region (Litt *et al*, 2001). The inactive X-chromosome in female mammalian cells are also found to be H3-K9 methylated. (Boggs *et al*, 2001 and Peters *et al*, 2001) An important piece of evidence supporting the Histone Code hypothesis, which is described later, is the requirement of a histone H3 deacetylase for methylation of lysine 9 on histone H3, suggesting the deacetylation of lysine 9 and 14 is taking place prior to methylation of the lysine 9 residue (Nakayama *et al*, 2001). Interestingly, histone methylation seems to trigger DNA methylation as well.

Phosphorylation occurring at serine and threonine residues on the histone tails is a highly reversible process. This modification has long been believed to be associated with chromosome segregation during mitosis (Sauve *et al*,1999). However, there is also evidence for the involvement of H3 phosphorylation at Ser 10 in the induction of immediate early genes, by a decondensation mechanism of the chromatin fiber, contrary to the reputation of H3 as a chromatin condensing element (Clayton *et al*, 2000).

The contradictory observations made on the consequences of the same PTM led to thinking beyond the simplistic view of the influence of histone modifications on regulatory processes. As a result of this, the Histone Code hypothesis was proposed by Allis and coworkers (Jenuwein *et al*, 2000) and Turner (Turner, 2000), suggesting that instead of a single type of modification on the tail, the different modifications of the histone tails either act sequentially or combinatorially to create a code that is read by the regulatory proteins prior to their recruitment to the chromatin template. It has already been shown that acetylation and phosphorylation act synergistically to induce certain genes and methylation and acetylation are correlated as well, as supporting evidence for the histone code hypothesis (Nakayama *et al*, 2001).

Another important piece of evidence that shows the existence of a linkage between different processes is the demonstration of a histone H3 methyltransferase controlling DNA methylation in *Neurospora crassa* (Tamaru *et al*, 2001). An interesting suggestion to answer the question of how such a complex mark is

established and maintained is that one modification on the tail can enhance the efficiency of binding of an enzyme responsible for a second modification, using the previously modified tail as a substrate. This hypothesis highlights the continued need to study all the different PTMs possible on all histone tails in order to understand the actual underlying mechanism.

A plethora of techniques have been used so far to study chromatin structure, and new techniques are being tried as well. Some believe that the less the perturbation made to the system, the closer one will get to see the real picture of what is happening inside a cell. While that is exactly the case, non-invasive techniques provide very little information about molecular level chemistry of cellular processes. Green Fluorescent Fusion protein (GFP) has been used to tag the chromosomes to track their pathway during the cell cycle (Kanda *et al*, 1998). Fluorescence *in situ* Hybridization (FISH) is another popular technique that has been used to study chromosomal rearrangements and genomic deletions, especially as a clinical diagnostic tool. Both of these techniques are perfectly suited to obtaining a qualitative picture. However, to understand the mechanism at a molecular level and also to compare histone modifications of different individuals, such techniques do not provide sufficient spatial resolution. Numerous genetic and biochemical studies such as site directed mutagenesis have provided insight about the enzymatic activities of HATs and HDACs. A brief discussion about the most commonly used techniques to study acetylation of histones is described below.

Much of the information gathered about acetylation of histones on metaphase chromosomes in the early 1990s was by immunofluorescence. A primary antibody against the specific acetylated lysine residue is reacted with the sample to identify the PTM of interest and a secondary antibody tagged with a fluorophore recognizes the primary antibody, which is then detected by fluorescence microscopy. As mentioned earlier, this technique has allowed one to conclude that hyperacetylated regions correspond to actively transcribed domains while, hypoacetylation is a marker for non-transcribed regions in that tissue. Different patterns of acetylation have been observed with antibodies raised against different sites of acetylation on the same N-terminal tail as shown in Chapter 3.

A more recently discovered technique, which is widely used currently to study PTMs, especially acetylation, is the Chromatin Immunoprecipitation Assay (ChIP assay).

In this method, histones are cross-linked to DNA by treating the culture medium with 1% formaldehyde. Chromatin is sheared by sonication to obtain DNA lengths of 200-1000 base pairs, prior to immunoprecipitating the fragments with antibodies specific for a certain modification of interest, eg, anti-acetylated histone antibodies to probe acetylation of histones. After removing the cross-link, the precipitated DNA fragments are amplified by Polymerase Chain Reaction (PCR) with specific primers designed for the region of interest. PCR products are then analyzed by gel electrophoresis in which a product band will indicate the precipitation of that genomic fragment by the ChIP assay. This would mean that a particular DNA fragment was

bound to a histone with the modification of interest. Initially, Southern blotting was used instead of PCR to identify the genomic region associated with the precipitated fraction of histones (Simpson, 1999). One of the major drawbacks in this technique is the potential cross reactivity that results in co-precipitation if the antibodies are not characterized thoroughly against all the possible modifications. Thus, the antibodies need to be characterized exhaustively against all possible PTMs and combinations of them to make certain that there is no cross-reactivity, especially because most of these antibodies are generated against the corresponding peptide fraction, which increases the possibility for cross-reactivity. Any mutations in the N-terminal tail may affect the antibody binding capability as well (Smith *et al*, 2003). Another problem is the inablity to quantify the extent of a certain modification. This technique was first used to study protein-DNA interactions by Varshavsky (Solomon *et al*, 1988) and became more popular in chromatin research after Grunstein started using it for telomere studies (Hecht *et al*, 1995). It is noteworthy that the ChIP assay is an indirect way to study histones, because it is actually based on the analysis of DNA that has reacted with the histones.

Grunstein has moved one step further in this route by combining the same principle used in ChIP with DNA microarray technology to study genomewide histone acetylation. After PCR amplification and fluorescent labeling of the precipitated fraction of the ChIP assay, the products are hybridized to an array of DNA strands that cover intergenic regions, open reading frames (ORF) or both from the entire genome to identify the precipitated fragments. The fluorescent intensities

are normalized with respect to a control to get an idea about the extent of acetylation in a particular fragment (Robyr *et al*, 2003). This study has been performed on a wild type yeast cell line (control) and an HDAC mutant cell line and their ratios were being obtained to understand any differences in the level of acetylation. However, if the purpose is to find out the absolute level of acetylation in a normal individual, this method will not be capable of doing so. Thus, a major drawback is the inability to quantify the extent of a PTM in a cell. In addition, to explore the Histone Code Hypothesis, this technique needs to be extended using an analytical method capable of measuring multiple PTMs simultaneously. As pointed out earlier, the specificity of antibodies is a crucial factor for the ChIP component of this assay. Resolution of this technique is defined by that of the DNA microarrays, which is usually the length of an open reading frame or an inter-genic region of about 1 kb. This indicates the unlikelihood of detecting a change in acetylation in a nucleosome or even a few nucleosomes. Another exciting discovery made by this same technology of a combination of ChIP assay and DNA microarrays is the differences observed in the PTMs in the promoter and the coding regions of genes. This work done on histones of *Saccharomyces cerevisiae*, revealed that acetylation of histones H3 and H4 in the promoter regions correspond to transcriptional activation and it is dimethylation of histone H3 Lys 4 that activates transcription in coding regions (Bernstein *et al*, 2002).

Acetylation turnover rates have been extensively studied by Waterborg and coworkers in *Saccharomyces cerevisiea*, *Medicago* and *Chlamydomonas* by pulse or continous labeling of the acetate groups with ^{3}H and chasing its specific radioactivity

with time (Waterborg, 2002). The half-lives for post-translational acetylation of yeast histone H4 turns out to be about 15-20 min and for histone H3, 8-10 min. The turnover rates seem to change depending on the species, histone tail of interest, and also whether it is mono or multi acetylated . Overall the conclusion may be that the acetylation of histones has a half life from a few minutes to about half an hour. A summary of the results obtained by Waterborg and co workers is shown in Table 1.1. This is a steady-state labeling technique performed in vivo to study acetylation in bulk chromatin, the sole intention being to study the rates of turnover. However, with this method it is not possible to target a specific location in the genome to study the PTMs.

Histone: (half life in minutes)	**H4**	**H3**	**H2B**	**H2A**
Chalmydomonas (algae)	3.5 ± 1.1	1.7 ± 0.2	1.4 ± 0.3	2 ± 1
Saccharomyces (yeast)	~17	~10	~4	~6
Medicago (Alfalfa - plant)	30 ± 11	35 ± 13 26 ± 9	28 ± 5	-

Table 1.1: A summary of the acetylation turnover rates for histone tails in three different species (http://sgi.bls.umkc.edu/waterborg/DynAc02_files/frame.htm)

There is increasing evidence that the definitive solution to the important problem addressed in this work is going to be provided by mass spectrometry. Several groups in the chromatin research community have done mass spectrometric studies on bulk chromatin. Acetylation of lysine residues and methylation of lysine and arginine

of histone H4 tails have been the primary focus using a variety of mass spectrometric techniques. Acetylation and methylation were studied for the very first time in histones extracted from HeLa cells and chicken erythrocytes by MALDI-MS and nanoLC-ESI on tryptic digests to identify the positions of the modifications on the N-terminal tails (Zhang, 2000). Collision induced dissociation (CID) has been implemented to study the PTMs of interest. It was confirmed by this study that the order in which acetylation takes place in mammalian cells is Lys 16, 12, 8 and then 5. Positions for lysine methylation has been found to be 9 and 14. Another significant advance in this field is the discovery by Kim and coworkers (Kim *et al*, 2002) of a specific and more sensitive marker ion to detect acetylated lysines. This novel marker is at m/z 126.1, which is apparently 9 times more sensitive than the already established marker for acetylated lysine, the immonium ion at m/z 143.1. Tetra, tri and di-acetylated forms have been identified using this novel marker by HPLC-ESI-MS on tryptic digests. A giant step towards what we strive to achieve, quantification of acetylation at individual lysine residues in the H4 N-terminal region has been reported recently by ESI-MS on yeast cells (Smith *et al*, 2003). The presence of a large number of acetylatable lysines in a short stretch of amino acids, has been a challenge to developing this methodology. A combination of isotopic labeling and CID-MS/MS along with an algorithm to select fragments with multiply acetylated lysine residues in one ion, this first report on detection of endogenous levels of acetylation has proved quantification of histone PTMs to be feasible. With nano-flow HPLC-ESI-MS, 200 fmol of H4 has been sufficient for the analysis.

Another huge leap in the mass spectrometric determination of PTMs on histones has been reported very recently where peptide mass fingerprinting (PMF) is used to map the different modifications (Zhang *et al*, 2003). Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer with an electron ionization (EI) source has been utilized for this work. While most of the previously identified modifications on histone N-terminal tails have been confirmed by this method, some novel sites of modifications especially on the core histones and the COOH terminal domains have also been identified. The acid extracted calf-thymus histones have been first separated and purified by reverse phase High Performance Liquid Chromatography (HPLC) and then analyzed by Q-TOF (Time-Of- Flight) mass spectrometry to identify the individual histones. The purified histones have then been digested with proteolytic enzymes such as pepsin, trypsin, V8DE, V8E and Arg-C to generate peptide fractions of which the masses are measured and compared to theoretical values. This work is focused on determination of the smaller modifications such as methylation, acetylation and phosphorylation ignoring the larger ADP-ribosylation and ubiquitination modifications. One of the important new discoveries is the methylation of histone H4 lysine 59 which lies on the surface of the nucleosome crystal. Since it is located very close to H3 lysine 79, which has been found to repress gene activity, it has been predicted that H4 K59 modification has a similar function due to the easy accessibility of enzymes such as methyltransferases to those two sites. The fact that this method is successful in identifying a significant number of unidentified sites and types of modifications leads to the question as to

why they were not detected by the previously used techniques. In the case of immunofluorescence and most of the ChIP experiments, the focus has solely been on the N-terminal domain as antibodies raised against N-terminal peptides are used as tools for such studies. On the other hand, with Edman degradation sequencing, which has been used in a few cases, only the first 20-30 amino acids are studied. Pulse labeling techniques are more suitable for modifications with high turnover rates and some of the core modifications that take place are fairly stable. The other important possibility is that the detection limits of the method used in this work appear to be very much lower than the previously used techniques (Zhang *et al*, 2003). Although there is the possibility that some of those stable PTMs may not be directly linked to any regulation of genes, this encouraging piece of work certainly sheds light on a complete picture of the PTMs of histones and is definitely a stepping stone to an era of mass spectrometry on histones. Clearly, significant advances have been made in chromatin related mass spectrometric research in the past couple of years.

However, most of the mass spectrometric work is performed on bulk chromatin, extracted from the entire genome. This type of analysis would not allow us to compare the different types of modifications and the extent to which they are modified in a specific region of the genome. Such a comparison would be important to study the role of PTMs of histones in genetic disorders. Therefore, an attempt was made to set the foundation to carry out such investigations and allow comparison between control and diseased cell lines of various PTMs of histones, by the work in this dissertation.

Chapter 2: Production and Characterization of Anti-Histone Polyclonal Antibodies

2.1 Introduction:

Antibodies, a class of molecules generated as a result of an immune response to the presence of a foreign body in higher animals, have been widely used as an affinity tool that recognizes and binds to a variety of molecules, referred to as antigens. Such antigens can vary from low molecular weight compounds to macromolecules such as proteins and microorganisms. Antibodies were used for the first time, as molecular recognition elements by Yalow and Berson in a radioimmunoassay to detect insulin (Yalow *et al*, 1959). Using the same technique, thyroxine was measured by Ekins in the following year (Ekins *et al*, 1960). Antibodies, also known as immunoglobulins (Ig), generated in the body are divided into five classes depending on the types of heavy chain and their subunit structure; IgG, IgM, IgA, IgD and IgE (Voet, 1998). Except for IgM, which is a pentamer of about 800,000 Da in molecular weight, the other 4 types of immunoglobulins are similar in structure with a "Y" shape single molecule of about 150,000 Da in molecular mass. Out of the 5 categories, IgG is the type mainly used to carry out affinity based recognition as it is the most abundant type of Ig in serum. Fig 2.1 shows a schematic of an antibody. An antibody molecule consists of two identical Fab fragments that contain the antigen-binding site of about 15Å x 20 Å x 10 Å, and an Fc portion (constant region), that acts as the "stem" of the molecule (Ausbel *et*

al,1990). The variable region at the N-terminal end of each Fab fragment is what allows the antibody to recognize different epitopes, due to its variability in the amino acid sequences in the heavy (V_H) and light chains (V_L). The population of antibodies generated in the body against an antigen is specific to an antigen, but may bind to different epitopes. The immune response to a given protein targets a range of epitopes. The antibodies to these epitopes each derive from separate clones of B cells and therefore are referred to as polyclonal. Spleen cells from an immunized animal when fused with myeloma cells result in a hybrid cell line (hybridoma) that can be grown in a selective medium to obtain a homogeneous pool of antibodies referred to as monoclonal antibodies. Such a homogeneous and mono-specific pool of antibodies can be preferentially used to purify macromolecules, and as a diagnostic tool for infectious diseases, drugs and other substances present in tissues and body fluids (Voet, 1998).

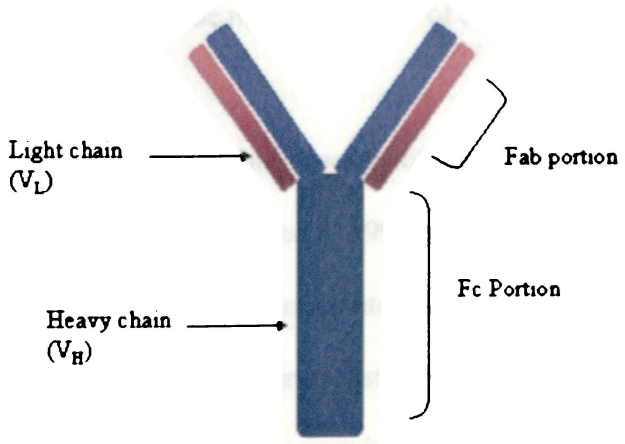


Fig 2.1: Structure of an Antibody. The constant and variable regions are indicated by Fc and Fab portions, respectively. Heavy chain (V_H) indicated in blue and light chains (V_L) indicated in pink.

In chromatin research, antibodies have been used as a probe to study histones. As mentioned in Chapter One histones are a highly conserved category of proteins associated with the DNA in a chromosome. The basic structural unit of chromatin or chromosomal DNA associated with histones, is the nucleosome. It is believed that the nucleosome performs two primary functions: firstly, to help package about 2 m of DNA into a nucleus with a diameter of only 10^{-5} m and secondly, to set a platform for the basic functions of chromosomal DNA to occur. The elegance of this whole compacting process is that while such a tight packaging of DNA takes place in the nucleus, it still allows recruitment of large protein complexes and enzymes to carry out the regulatory functions on the DNA template in a chromosome, such as transcription. Nucleosomes are not, as once believed, not the "most boring" proteins that help only in the packaging of DNA. In fact, with increasing evidence to support the idea that nucleosomal histones play a vital role in the function of chromosomal DNA, there is a burst of interest in the chromatin research community to study the underlying mechanisms at the molecular level of a nucleosome and its influence on biological function.

The X-ray crystallographic structure of the nucleosome (Fig 2.2) at a high resolution of 2.8 Å , was documented in 1997 by Luger and co-workers, and shed light on the nucleosomal composition in atomic detail (Luger, 1997). About 146 bp of DNA is wrapped around an octamer of histones comprising two of each, histone H2A, H2B, H3 and H4. The histone core is defined by two histone folds of each, H3-H4 and H2A-H2B dimerized with each other.

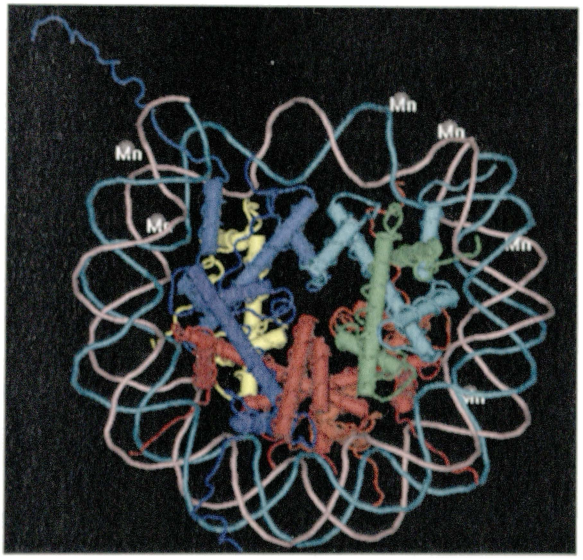


Fig 2.2: X-ray crystal Structure of the Nucleosome at 2.8 Å resolution (NCBI protein database, MMDB structure summary: http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6&db=t&Dopt=s&uid=8530)

Each histone consists of a 3-helix domain, referred to as the histone fold, and two unstructured tails, the N and C termini. These tails, which comprise 28% of the mass of the core histones, extend out of the nucleosomal particle. Some segments of the tails do not appear in the X-ray crystal structure for either of two possible reasons: their high degree of mobility or to the existence of multiple structures. The sequences of the N-terminal tails for four different types of histones are shown in Table 2.1, which also illustrates the heavy representation of positively charged lysines and arginines on these tails. Initially the belief was that these lysine and arginine rich tails that have a positive charge on the surface, non-specifically interact with the

negatively charged backbone of DNA. However, this line of thought started to change rapidly with emerging evidence of a significant amount of sequence conservation of histone N-termini, across organisms from yeast to humans. The question that arose then is why should there be so much conservation across species, if it is merely a non-specific electrostatic interaction? Thus, dawned an era of chromatin research on these enigmatic histone-tails.

Histone	Sequence
H3	ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPG
H4	SGRGKGGKGLLKGGAKRHRKVLRDNIQGIT
H2A	SGRGKQGGKTRAKAKTRSSRAGLQFP
H2B	PEPAKSAPAPKKGSKKAVTKAQKKDGKKRRKTRKES

Table 2.1: Sequences of the N-terminal tails of the core histones shown in single letter codes. Lysines and arginines are indicated in red (Turner, 2001)

Histone tails can undergo covalent and non-covalent modifications as mentioned in the previous chapter. The covalent modifications can be listed as acetylation, methylation, phosphorylation, ribosylation and ubiquitination. The chemistry behind some of the modifications N-termini undergo is depicted in Table 2.2. A ground-breaking discovery made in 1964 by Allfrey and co-workers was that acetylation of histones favors transcription (Allfrey *et al*, 1964). Later on, immunofluorescence experiments showed that hyperacetylation of histones is

associated with actively transcribed regions of the genome (euchromatin) whereas hypoacetylation is associated with non-transcribed genomic regions (heterochromatin) (O'Neill *et al*,1995). Due to these early observations made with regard to acetylation, the first hypothesis to explain its biological significance is as follows. Upon acetylation of lysine residues, as the N-terminal tails lose their positive charge, the electrostatic interaction between the tails and the negatively charged backbone of DNA is lost resulting in the loosening of chromatin enabling the transcription machinery to perform its functions on the DNA template. However, in 2000 Allis and co-workers presented a hypothesis suggesting that it may not be a single modification which influences the function of the genomics, but a combinatorial effect of one or more modifications taking place on the histone tails (Strahl *et al*, 2000 and Turner, 2000).

The non-covalent modifications are believed to be associated with the recruitment of chromatin remodeling complexes that change the conformation of chromatin by breaking and remaking histone-DNA contacts, thereby allowing the movement of nucleosomes on the DNA template (Lee *et al*, 2000). All such chromatin remodeling complexes, (ie: Swi/Snf and Rsc in yeast) require ATP for their performance.

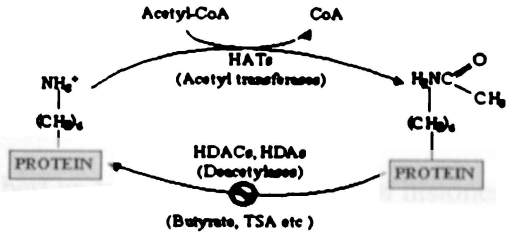
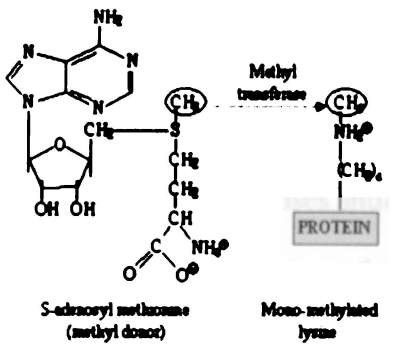
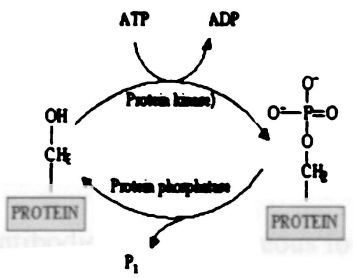
Acetylation and Deacetylation	
Methylation	
Phosphorylation	

Table 2.2: Chemistry of some covalent modifications of Histone tails (Turner, 2001).

The question is whether any of these histone modifications bear a correlation to the function of the genes in a particular region of interest in the genome. This is the problem that is being addressed and focus is exclusively on the covalent modifications of histones. The best understood modification of histones is acetylation. The effects of the other modifications, such as methylation and phosphorylation, on the regulation and organization of chromatin are poorly understood. To investigate any combinatorial effect the different histone modifications may have on the DNA template driven processes such as transcription, as opposed to the simplistic view that acetylation alone may enhance transcription and vice versa, it is essential to have tools to simultaneously probe the different modifications. One of the simplest and most commonly used approaches to probe modified histones uses antibodies. Therefore, antibodies against histones are key players in the arena of chromatin research, especially for qualitative analysis of histones.

Antibodies generated against such modified histones are employed for implementing techniques such as immunofluorescence, with a fluorescently labeled antibody, and chromatin immunoprecipitation (ChIP) assay, based on immunoprecipitation, as described in Chapter One. For these techniques, the high homogeneity of monoclonal antibodies is not required and, in fact, it could very well be that the heterogeneity of polyclonal antibodies is advantageous for immunoprecipitation, due to the presence of multiple antibodies binding to different epitopes on the antigen, resulting in a network that could easily be precipitated. Anti-

histone antibodies have been produced against acetylated histones, in which the different lysine residues are the targets of acetylation (Turner, 1989 & Pfeffer, 1986). For instance, Lysines 5, 8, 12 and 16 on histone H4 and lysines 9, 18, and 23 on histone H3 can be acetylated. In fact, in some cases, antibodies have been generated to tri or tetraacetyl derivatives of histones as well (Muller, 1987). Most recently, Allis and co-workers have generated antibodies for methylated lysine residues on histone H3 and H4 using a cassette of amino acid residues specific for their N-terminal tails.

For each modification of the residue a purified N-terminal fragment or the synthetic equivalent of that sequence, is used as the antigen and it is linked to a carrier molecule, cationized Bovine Serum Albumin (BSA) to make the immunogen. It has been found that the standard carrier molecule used to make anti-acetylated histone antibodies, Keyhole limpet hemocyanine is ineffective in generating anti-methylated histone antibodies (Allis *et al*, 2002). In other words, there is a broad spectrum of antibodies available to study modified histones at present. Using these methyl specific antibodies, which have been characterized for any cross reactivity with non-methylated lysine, it has been demonstrated that methylated lysine 9 of histone H3 is associated with heterochromatic regions in fission yeast (Nakayama *et al*, 2001). As pointed out previously, the topography and the architecture of chromatin is believed to influence the function of the associated genes. Thus, this recent burst in the production and availability of anti-histone antibodies goes hand in hand with the recently enhanced enthusiasm of the role of chromatin in regulation of cellular genes and also the development of techniques such as the ChIP assay.

Unlike the core histone domains, because the tails are freely moving, and lack a specific structure an antibody reacted with a nucleosomal particle will most likely bind to these tails. This very fact provides the basis for the generation of antibodies against covalently modified and unmodified histones. The assumption then is that the antibodies produced by an immune response elicited against a portion of the N-terminal peptide tail of a histone will react similarly against the native form of the entire protein as well. When this study was initiated the limited number of commercially available antibodies was extremely expensive and also not completely characterized. Therefore, attention was focused on generation of polyclonal antibodies against acetylated and non-acetylated histones, and purification and characterization of these antibodies to the point that the results obtained with them are meaningful.

Although it has been demonstrated that a portion of the peptide sequence of the N-terminal tail of interest can be used to generate antibodies against the corresponding whole protein of interest, the peptide by itself will not trigger an immune response. Therefore, the peptide is conjugated to a carrier molecule (eg: purified protein derivative, PPD) via a cross-linker, Sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane-1-carboxylate (sulfo-SMCC, Pierce) as shown in Fig 2.3. A key factor in this conjugation process is to retain the native structure of the antigen. A majority of the carrier molecules used for preparing immunogens tend to elicit an immune response by the host animal, resulting in the generation of antibodies against the carrier. PPD on the other hand, has been reported not to generate any such

immune response, thereby making all or most of the antibodies produced in the host specific for the antigen of interest (DeSilva et al, 2000). Sulfo-SMCC specifically was chosen as the linker for the conjugation of the antigen to the PPD carrier, due to its chemical characteristics; its water solubility makes it easier to handle in the experiments with the antigen and the carrier, which are highly water soluble too. It is also an uncleavable, heterobifunctional cross-linker that reacts with amines and sulfhydryl groups. Heterobifunctionality of the cross linker provides more control over the conjugation process. For example, conjugation in this case is designed such that NHS-ester group in the linker reacts with amines in the PPD and the maleimide groups react with the sulfhydryls in the peptide. The cyclohexane bridge on the linker adds extra stability to the maleimide groups. In order to generate anti-histone antibody for acetylated Lys 16 [H4 (K16)] and anti-histone non-acetylated antibody, the following procedure was adapted.

(a) PPD------Sulfo-SMCC------antigenic peptide

(b)

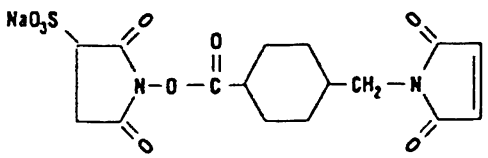


Fig 2.3: (a) Schematic of immunogen (b) Structure of Sulfo-SMCC

2.2 Experimental:

2.2.1 Buffers and Solutions:

Buffer	Composition
PBS pH7.4	0.008M Na_2HPO_4, 0.002M KH_2PO_4, 0.15M NaCl
Coating Buffer (0.05M carbonate buffer, pH 10.0)	0.02M $NaHCO_3$, 0.03M Na_2CO_3
Wash Buffer	PBS containing 0.5% Tween 20
Blocking Buffer	PBS containing 0.5% Tween 20, 0.3% BSA

Table 2.3: Composition of buffers used in the chapter

2.2.2 Procedures:

(i) Synthesis of the Immunogen:

The antigenic peptides of the sequences shown in Fig 2.4 were synthesized in the Biochemical Research Service Laboratories, KU.

Fig 2.4: Sequence of the non-acetylated histone H4 peptide: NRGKGGKGLGKCC referred to as H4(K)nonAc peptide

Sequence of acetylated at 16 histone H4 peptide: NGKGGAAcKRHRKCC referred to as H4(K16)Ac peptide

The carrier molecule, PPD and the cross-linker, sulfo-SMCC were incubated for 60 min at room temperature or for 30 min at 37° C in a molar ratio of 10:1 in PBS, pH 7.2 Product was dialyzed in a dialysis membrane with a molecular weight cut-off of 14000, to remove any unreacted sulfo-SMCC. Assuming all PPD used was activated, peptide was mixed in a 10:1 ratio (peptide:PPD) and incubated at 4°C for 2 hours, followed by desalting into the storage buffer of choice, PBS, by dialysis. The resulting solution was passed through a 2um filter membrane to get rid of any particulate matter.

(ii) Immunizations:

One week prior to the immunization with the antigen, the rabbits were pre-sensitized intra-muscularly, with 500 ug of BCG (Bacillus-Calmette-Guerin, RIBI Immunochemical Research, Hamilton, MT) dissolved in 500 uL of PBS, pH 7.4, at one site on each rabbit in the lumbar muscle. This was followed by the first immunization of the antigen intradermally, histone N-terminal peptide-PPD conjugate combined with Freund's complete adjuvant in a 1:1 (v/v) ratio, the following week, 200 uL per site in a total number of 6-7 sites. Thereafter, every two weeks the rabbits were given subcutaneous booster injections of antigen combined with Freund's incomplete 1:1 (v/v).

About 15-20mL of blood was collected in glass tubes every two weeks by bleeding from the ear artery. The tubes were kept at room temperature for about 2-3 hours and are then stored at 4°C overnight. The serum is separated out with a Pasteur pipet and centrifuged at 3500 rpm to further purify the serum. The samples can then be stored at 4°C for a short period of time, and if it is long term storage, 0.01% sodium azide is added and for long term storage at –40°C.

(iii) Immunoassay for Titer Determination:

In order to best retain the conformation of the peptide if there is any, the dry-coat method (Jitsukawa *et al*, 1989) is adopted for the characterization as well as titer determination assays. The first 32 wells in a microtiter plate are coated with 50 uL of a solution containing antigenic peptide (7ug/mL): BSA (7ug/mL) in a 1:1 ratio. Both peptide and BSA solutions are prepared in 0.05M coating buffer. The next 32 wells are coated with 50 uL of BSA in 0.05M coating buffer and the remaining 32 wells are coated similar to the first 32 wells, except that antigenic peptide is replaced with non-antigenic peptide. Thus, the three dilution series are: BSA with antigenic peptide, BSA alone as the positive control, and BSA with non-antigenic peptide to check any cross reactivity. The uncovered plate was incubated overnight or till all the liquid is evaporated at 37°C. Plate was washed with wash buffer four times and patted dry on a stack of paper towels. The surface of the well was blocked by incubating the titer plate with 200 uL of blocking buffer (freshly prepared everyday) for 1.5 hours at 37°C. Next, the plate was washed four times with wash buffer and patted dry.

Blocking buffer (100uL) was added to all the wells except for the first two in each of the three dilution series. A volume of 200uL of diluted serum (1:100 diluted in blocking buffer), containing the primary antibody was added to the first two wells in the first column, and then serially diluted down the columns through the fourth column by taking out 100uL from the previous two wells and adding to the following two wells, so that every serum concentration is in duplicate. 100 uL were taken out and discarded from the last two wells of each of the three dilution series. After an incubation of 1 hour at 37°C, the plate was washed with wash buffer four times and patted dry. Peroxidase labeled goat-anti-rabbit IgG was diluted 1:12000 in blocking buffer; 75 uL of this labeled secondary antibody was added to each well and incubated for 1 hour at 37° C. Plate was washed with nano pure water and patted dry. 100 uL of the substrate for the enzyme, TMB:peroxidase B (1:1 v/v) was added to each well and incubated at room temperature for 20 minutes. The reaction was then quenched by adding 50 uL of 1M HCl, and mixing thoroughly. Absorbance was measured by the kinetic plate reader at 450nm.

(iv) Immunoassay for Characterization of the Polyclonal Antibodies:

The same assay as above was employed with the following modifications:

(1) BSA in the blocking buffer was replaced with 1% Tween 20 as the blocking reagent.

(2) Diluted serum was replaced by affinity purified, primary antibody solution.

(v) Purification of Polyclonal Antibodies

(a) Gamma-Cut

A mass of 0.2g of finely ground ammonium sulfate powder is added slowly per mL of serum over a period of 2 hours with slow constant stirring. The mixture is then centrifuged at 2500 rpm for 15 minutes and the supernatant is discarded. The precipitate is resuspended in approximately the original volume of serum measured out of 40% saturated $(NH_4)_2SO_4$ (192g/mL). Mixture is then centrifuged and supernatant discarded. About 1/3 to 1/2 of the original serum volume of PBS is added to redissolve the supernatant. The solution is then dialysed in PBS at 4°C with several changes of PBS. The resulting gamma-cut can be stored at 4°C for short-term storage. The rabbits were bled prior to any immunizations to obtain serum that can be used as a negative control.

(b) Affinity Purification:

(i) Preparation of the affinity columns:

Two separate affinity columns were prepared for the two different antigens, K(Ac16)H4 peptide and K(nonAc)H4 peptide

1 mg of peptide is dissolved in 1 mL of 0.1M sodium phosphate, 5 mM EDTA-Na, pH 6.0. To reduce the peptide, 6 mg of 2-mercaptoethylamine is added to this solution and incubated at 37 °C for 1.5 hours. Mixture is then cooled to room

temperature and dialyzed against 50mM Tris, 5mM EDTA-Na, pH 8.5 in order to remove excess 2-mercaptoethylamine. A volume of 3 mL of the slurry of support material for the column, Ultralink™ iodoacetyl (Pierce, catalogue # 53155) was loaded into a column with a frit at the bottom. The column is then equilibrated with 6 column volumes of 50mM Tris, 5 mM EDTA-Na, pH8.5. The bottom cap is replaced prior to adding the peptide solution. Since the slurry contains 50% of the actual gel, only 1.5 mL is the active material. According to the manufacturer's instructions, 8 mg (5.33×10^{-8} mol) of reduced protein or peptide per 1 mL of gel can be reacted. Since in our case, it is a peptide that reacts with the column support material, moles of peptide per 1 mL of gel would be $5.33 \times x10^{-8}$ mol. For non-acetylated peptide this amount means 0.0834 mg should be loaded per 1 mL of gel. Each peptide is dissolved in 3mL of Tris-EDTA (50mM Tris, 5 mM EDTA-Na, pH 8.5) solution. Then the 3 mL of peptide solution is introduced to the column. After replacing the top cap the column is mixed by end over end rotation, at room temperature for 15 minutes. The column was then incubated at room temperature for 30 minutes without mixing. Buffer is drained and the column is washed with 3 column volumes of Tris-EDTA. Coupling efficiency is determined by standard Bicinchoninic Acid (BCA) protein assay (Pierce) by comparing an aliquot of the initial peptide solution to a fraction of the final wash. Coupling efficiency of the columns were found to be almost 100% and 95% for the K(Ac16)H4 column and K(nonAc)H4 columns, respectively. The non-specific binding sites on the gel, are blocked by adding 3 mL of cystein solution (50 mM cystein in 50 mM Tris, 5 mM EDTA-Na, pH 8.5) and mixing the column, by

end-over-end rotation for 15 minutes at room temperature. Nest, the column is incubated at room temperature for another 30 min without mixing. Liquid is then drained and the column washed with 16 column volumes of 1M NaCl, and 16 column volumes of degassed 0.05% sodium azide. An additional 0.05% sodium azide is added prior to storage at 4°C with top and bottom caps replaced.

(ii) **Purification Process:**

The key point in the purification process is that the column should contain at least 1000 times more sites than the amount of IgG introduced for purification. Therefore, amount of IgG introduced to the column per 1 mL of iodoacetyl support is 5.33×10^{-11} mol.

The storage solution is drained carefully and 5 mL of 0.01M Tris (pH8.0) is passed through the column in order to condition it. 2 mL of 5×10^{-8}M gamma-cut purified antibody is passed through the column and the eluate collected (E1). Next, 4 mL of Tris is passed through and the eluate is collected (E2). E1 and E2 were combined and recycled through the column 3 more times, to allow for any specific antibodies to react with the peptide in the column. Then to wash the non-specifically interacting antibodies off the column 10 mL of Tris is passed through it.The eluting 16 mL, including 6 mL from the previous step and 10 mL of Tris, was collected as the unbound fraction of antibodies.

To elute the bound fraction of antibodies, 8 mL of Immunopure IgG elution buffer, pH 2.8 (Pierce, cat # 1851520 in a kit or 21004 separately) was passed and the

eluate was collected to a testube containing 2 mL of Tris buffer, pH 8.0 The reason for doing this is to minimize any possible denaturation of the antibodies due to the low pH of the eluting immunopure buffer. By collecting the antibodies into a buffer of higher pH will somewhat raise the pH of the solution, helping the antibodies to preserve their native form. Another 10 mL of Tris was passed through the column, and the first 2 mL collected was added to the bound fraction of antibodies while the remainder was discarded. The column is stored in 0.01% sodium azide at 4^0C. Affinity purified antibodies should be dialyzed against PBS prior to use in an assay simply because assays are usually based on PBS or an equivalent around pH 7.4.

(iii) Dialysis:

The bound and the unbound antibody fractions were dialyzed separately against PBS pH 7.4 in a dialysis membrane with a molecular weight cut off of 10000 Da. The buffer was replaced with fresh buffer every 4 hours.

(iv) Reconcentration:

As dialysis tend to dilute samples, a reconcentration step is necessary. This was done by exposing the samples contained in a membrane with a MW cutoff of 14000 Da, to constant suction in a suction flask. The antibodies are ready to be used after this step.

2.3 Results and Discussion:

The dry coat method was employed as the procedure for all immunoassays concerning the antibodies generated, so that the peptides are laid down on the microtiter plate without changing their conformation. The uniqueness of this method lies in the fact that BSA added to the peptide solution coats the titer plate well surface to form a "soft feather bed" on which the peptides sit, so that denaturation of their native form is avoided.

The titer, which is a measure of the presence of the antibody of interest in the blood, was different for anti-H4(K16)Ac and anti-H4(K) nonac; they were 2^7 and 2^9 respectively, calculated by the results shown in Fig 2.5 and 2.6. There is a clear difference between the titer prior to and after immunizations in both types of antibodies. Prior to immunization, titer for anti-H4(K16)Ac is around 2^4 while, that for anti-H4(K) nonac is 2^2. With H4(K16)Ac antigen, the optimal titer was reached only after the second boost.

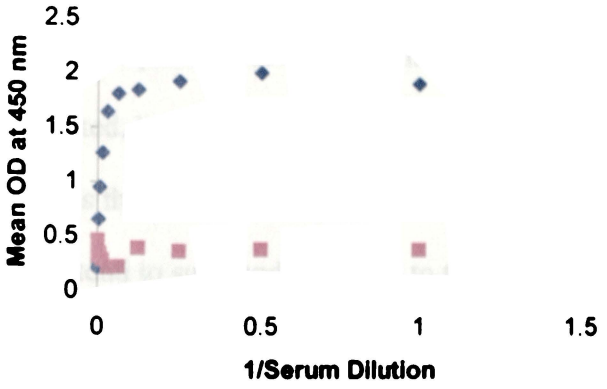


Fig 2.5: Titer curves for anti-H4(K16)Ac after (♦) and prior (■) to immunization are shown in the figure. Calculated Titer for after immunization is 2^7

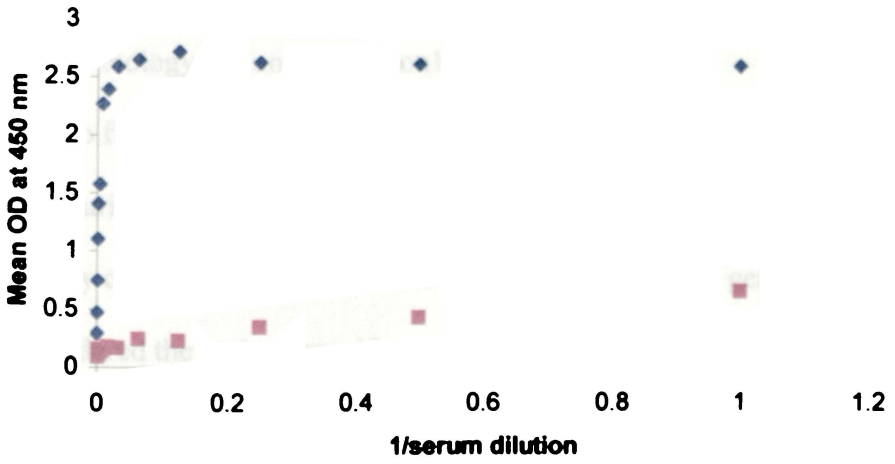


Fig 2.6: Titer curves for anti-H4(K)non-Ac after (♦) and prior (■) to immunization are shown in the figure. Calculated Titer for after immunization is 2^9

In order to improve the analytical utility of polyclonal antibodies, they can be purified by various methods. The first step of purification of antibodies from serum is the gamma-cut procedure. When ammonium sulfate is added slowly, most of the IgG is precipitated, leaving the other contaminating proteins in solution. The underlying chemistry is that when the concentration of SO_4^{2-} ions increases in solution, water molecules tend to surround them due to the affinity of SO_4^{2-} to water. Thereby, leaving an insufficient amount of water around protein molecules to keep them in solution. This results in a precipitation of the proteins. It is important to add ammonium sulfate slowly because otherwise, other contaminating proteins could coprecipitate as well (if the local concentration of ammonium sulfate is too high). This precipitation needs to be done between 4° and 25° C; outside this range the degree of saturation of ammonium sulfate deviates > 3%. (Current protocols in molecular biology, volume 2, section11.13.3)

To further purify the IgG fraction collected from gamma-cut they can be affinity purified by passing through a column, in which the antigenic peptide is covalently coupled to a support material. The antibodies that get bound to the column are considered the antigen specific antibodies. They can be eluted by varying the conditions such as decreasing the pH, high ionic strength and using chaotropic agents, to disrupt the antigen-antibody complex formed in the column. It is important to note that a method that does not denature the eluting antibody is by far the most preferable. The eluting fractions of antibodies were collected to a tube containing a high pH buffer (pH 8.0) in order to raise the unfavorable low pH of the eluting buffer (pH

2.0). There are commercially available elution buffers that perform at or close to neutral pH values (Gentle ImmunoPure Elution buffers, Pierce). However, the above strategy was adopted to increase the amount of recovery of antibodies binding to the affinity column.

Affinity purified antibodies showed an enormously high background (50% of the signal) when procedure 2.2.2 (ii) was used for characterization as shown in Fig.2.7. The optimal conditions turned out to be 1% Tween 20 as the blocking reagent instead of BSA in the original protocol and for the initial dry coat step, the amount of BSA used had to be increased to an equi-molar mixture of peptide: BSA instead of a mixture of equal mass solutions, used in the titer assay. (10^{-10} moles of BSA was mixed with 10^{-10} moles of peptide in the initial mixture). Just as there is no universal blocking reagent to all antigens, blocking reagents may also have to be changed depending on the conditions. Although the exact reason is not known, it could be speculated that the enrichment of one type of antibody by affinity purification causes the population of antibodies to react differently against the antigen. The evidence for this is that the background could be decreased to 5-8% of the signal with Tween 20.

The higher concentration of BSA required to reduce the background dramatically, in the initial coating step with the purified antibodies, indicates that low amounts of BSA resulted in insufficient coverage of the surface. As the concentration of specific antibodies was increased upon purification, non-specific adsorption of antibodies may have taken place when the surface coverage was low. Since the increase in concentration of BSA accounts for better coverage of the surface, that

minimizes non-specific interaction of the antibodies with the well surface. Therefore, this modified procedure was used for the characterization of antibodies.

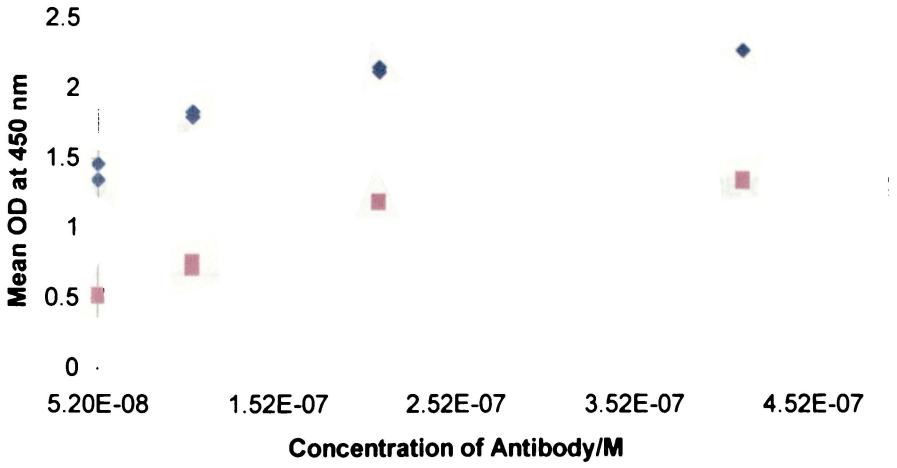


Fig 2.7: High background with BSA as the blocking reagent and with 7 μg/mL BSA: 7ug/mL peptide in 1:1 mixture. Binding curves for bound Ab (Anti-H4(K)Ac) ♦ and BSA ■ shown in figure.

Contrary to the titer results, anti-H4(K16)Ac showed a high binding constant, 0.14E9 M^{-1} against the antigen while anti-H4(K) nonac showed a binding constant of only 0.2E7M^{-1} (Fig 2.8 and 2.9 respectively). The fact that needs to be emphasized with such polyclonal antibodies is that the above numbers represent avidity of the antibody rather than the affinity, because it is a heterogeneous population of antibodies with different affinities to the antigen.

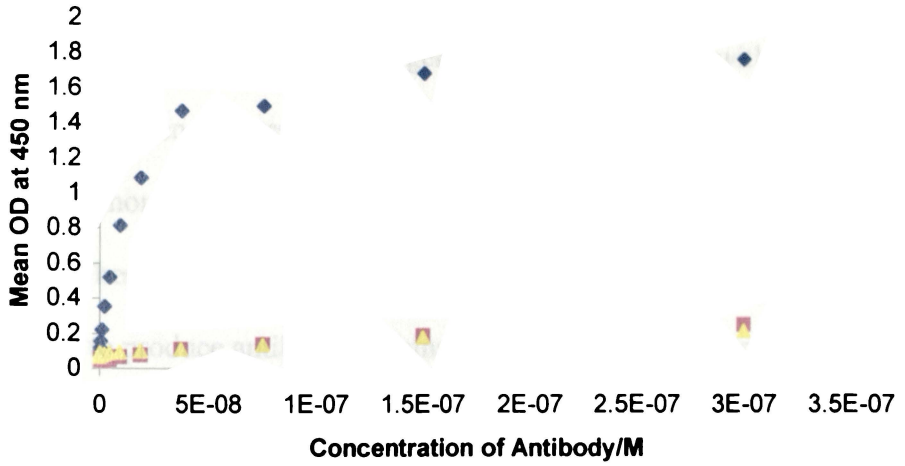


Fig 2.8: Binding curves for Anti-H4(K16)Ac ♦, BSA (control) ■, non-acetylated peptide (control) ▲ shown in figure. Binding Constant for Anti-H4(K16)Ac = 0.14E9 M^{-1}

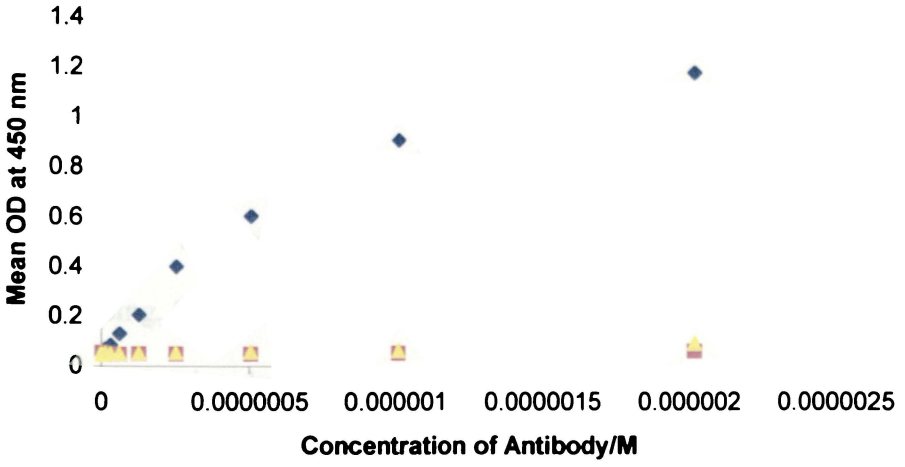


Fig 2.9: Binding curves for Anti-H4(K)nonAc ♦, BSA (control) ■, acetylated peptide (control) ▲ shown in figure. Binding Constant of Anti-H4(K) nonac Antibody is 0.2 E7 M^{-1}. The cross-reactivity of each antibody to the non-antigenic peptide is less than 8% and comparable to the control, BSA response.

2.4 Conclusion:

Based on these results, the generation of antibodies with the procedure adopted has been successful. The affinity purified, polyclonal anti-H4(K16)Ac and anti-H4(K)nonAc antibodies show great specificity towards the corresponding antigen. Therefore, this procedure for generating polyclonal antibodies can be extended to produce antibodies against the other acetylated isoforms of histones. Extending the method to generate antibodies for other modifications is another possibility. These polyclonal antibodies can then be utilized in techniques such as the ChIP assay or immunofluorescence for chromatin analyses. It is important to note that the antibodies will be capable of functioning as a qualitative marker for specific chromatin domains for example, to differentiate active domains from inactive domains etc, rather than a quantitative tool. This will be further illustrated in Chapter 3.

Using antibodies as tools to investigate the core problem, identifying which modifications of histones have taken place in a specific genome locus, does not look very promising, for multiple reasons. It is becoming increasingly evident that, it is not just one modification, but a combination of modifications that govern the epigenetic regulation of the genome. The resolution provided by antibodies to be employed in an imaging technique will not be sufficient to look at multiple modifications on a sample, simultaneously. The capability of antibodies to be used as tools in a ChIP assay will be determined by how well they are characterized with respect to the cross reactivity to similar modifications. For example, even the antibodies that were

generated in our lab would have to tested against the other acetylated histone H4 sites, such as H4(K5), H4(K8), H4(K12) and other types of modifications as well, in order to be able to use them in a ChIP assay, because otherwise the precipitated fraction of chromatin may not contain only the expected modification of histone. Most of the commercially available antibodies are not exhaustively characterized in this manner, which makes the interpretation of results obtained by assays such as ChIP very difficult.

Chapter 3: Detection of Acetylated Histones in a specific genomic locus: A Cytogenetic Approach

3.1 Introduction:

Analysis of the post-translational modifications of histones in a specific genomic locus being the focus of this work, a cytogenetic approach was resorted to as the preliminary step to achieving the goal. Using fluorescence as the method of detection, two well-established techniques, fluorescence in situ hybridization (FISH) and immunofluorescence (IF), are used separately for genomic identification and for protein structural analysis, respectively. The idea is to combine the two results to determine any co-localization of the signals, which would indicate the presence of a particular histone modification in a specific genomic locus. The two events can be differentiated from each other by labeling the two probes with two different fluorophores and co-localization can be determined by the resultant merging of the colors of the two fluorophores, in an overlay of the two individual events.

To locate a particular genomic region, a fluorescently tagged DNA probe complimentary to the target region of interest is utilized to perform fluorescence *in situ* hybridization (FISH). In conventional FISH technology, mega base sized probes are used to hybridize target DNA (Lichter *et al*, 1988). These probes consist of repetitive sequence elements as well as single copy intervals of the specific region of the genome. When such FISH probes are used to locate a gene or genes, they should be pre-annealed with human low-Cot DNA (Sealey *et al*, 1985) to eliminate binding

to repetitive elements elsewhere, which would otherwise result in the probe binding to those elements scattered in the entire genome. The alternative method is to enrich the single copy intervals when performing the experiment (Craig *et al*, 1997). Such large DNA probes are generated by cloning in cosmids, Bacterial Artificial Clones (BACs), PACs and Yeast Artificial Chromsomes (YACs), that can accommodate large DNA inserts.

In this work an attempt was made to generate single copy FISH (scFISH) probes, as opposed to conventional FISH probes to investigate specific genomic loci (Rogan *et al*, 2001). As the name implies, single copy FISH probes target only the single copy regions, which are unique sequences specific to one particular genomic locus in the entire genome. These scFISH probes result in an enhancement of the specificity due to the lack of frequently occurring repeat sequences. Mapping the genome at a higher resolution can be achieved with the scFISH probes as the length of the probes are significantly shorter compared to the conventional probes.

From a practical standpoint, elimination of repetitive elements makes it unnecessary to use low-Cot DNA to pre-anneal the probe. scFISH probes are designed by scanning genomic intervals of about 100 kb in length for single copy regions running specialized software on the available genomic sequence data, to eliminate repetitive elements. Another appealing feature of scFISH technology is the fact that scFISH probes can be generated by Polymerase Chain Reaction (PCR) with appropriately designed primers to basically any gene, multiple genes or a fragment of a gene, corresponding to a disease. Conventional FISH probes are limited to certain

genes or genomic regions that are already available as clones. With the completion of deciphering the entire human genome in 2001, the designing of these scFISH probes to target any region in the genome is a much easier task.

The overall objective of this work is to investigate the post-translational modifications (PTMs) of histones in the Prader Willi Syndrome (PWS) region of chromosome 15. The focus of this chapter is to study the acetylation patterns of histones in this region. PWS, a neuro-behavioral pediatric genetic disease, is characterized by neonatal hypotonia, hyperphagia leading to obesity, short stature, small hands and feet and hypogonadism, and has some interesting features from a genetic standpoint. The disease is caused by a loss of expression of the PW genes clustered in the 15q11-13 region that extends for about 2 Mb of the paternal chromosome 15 (Schumacher, 2001). These genes are paternally imprinted, meaning that their expression is only by the complement of paternal genes in normal individuals and are repressed in the maternal chromosome. This imprint is believed to be passed from one generation to the other during gametogenesis, defies normal Mendelian Genetics in which case both maternal and paternal copies of genes are expressed. The reasons for the lack of expression of the paternal genes in PWS may be due to a deletion in the corresponding region on the paternal chromosome 15 (65-75%), the presence of two maternal copies of chromosomes referred to as maternal uniparental disomy (UPD) (20-25%), or a mutation in the imprinting center that regulates the expression of these imprinted genes.

A schematic of the distribution of the PWS genes on chromosome 15q11-13 is represented in Fig 3.1. It has been attempted to design scFISH probes to target ZNF127 and NDN genes from the centromeric end and IPW from the telomeric end of the imprinting center (IC) in this work. Most of the products of these genes are associated with the function of the brain. A list of some PW genes and their products are listed in Table 3.1. Some of these genes such as IPW (Imprinted Gene in the Prader-Willi syndrome region) are not translated at all while there are others such as PAR5 of which very little is known.

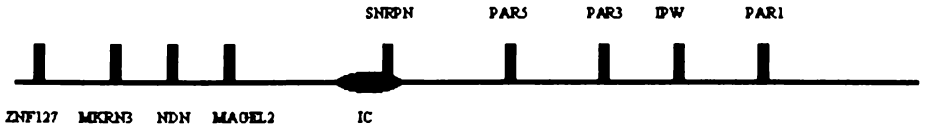


Fig 3.1: Schematic of the lay out of the genes associated with PWS in 15q11-13 region, left being the centromere end and right being the telomere end (Not drawn to scale)

IC –Imprinting center ( ), Each of the black bars indicate individual genes

Gene	Name	Appearance of Product
NDN	Necdin	Neuronal protein
ZNF127	Zinc Finger 127	Almost all adult tissues; highest expression levels found in testis
MAGEL2	Melanoma antigen gene expression like	Liver, kidney, placenta, brain & spinal cord

Table 3.1: Some PWS genes and their appearance in tissues (Schumacher, 2001)

One of the features that has baffled geneticists and aroused the interest of studying PWS is the imprinting mechanism associated with it. How are the genes marked as imprinted to be expressed monoallelically based on parental origin? This is a question the scientific community has been trying to address, as any abnormality in the expression of these genes would lead to various genetic disorders (Falls *et al*, 1999). It is believed that the imprint is set during gametogenesis by methylation of CpG islands based on a parent-specific nature. Experiments conducted with mice, knocking out the methyltransferase enzyme that methylates the CpG islands so that their imprint is lost suggests that methylation is directly linked with imprinting of genes (Li *et al*, 1993). However, the underlying mechanism is so complex that it is not entirely understood. With the evidence suggesting that chromatin structure is different in CpG islands than in other regions of the genome (Bird *et al*, 1999) and the possibility of a link between DNA methylation and histone PTMs (Ferguson-Smith *et*

al, 2001), it is worthwhile exploring any contribution of chromatin structure to establish this imprint mark in specific regions.

To investigate the different modifications of histones, immunofluorescence is adopted, where an antibody (primary) recognizes the modified or unmodified histone epitope, subsequently identified by a fluorescently labeled secondary antibody and finally detected by fluorescence microscopy (Jeppesen,1995). This is the most frequently used immunofluorescence method out of two options, referred to as indirect immunofluorescence. On the contrary, in direct immunofluorescence, the primary antibody recognizing the epitope of interest itself, is fluorescently tagged. Since conjugation of a fluorochrome may influence the capability of antibody recognition of the epitope and its binding affinity, the indirect method is preferred. Acetylation out of all the modifications is the PTM best studied by this method, one of the reasons being the availability of commercial antibodies that recognize acetylated histones. Immunofluorescence is a rather sensitive tool used by molecular and cell biologists to study sub-cellular components in cells and tissues. The most appealing feature of this technique is the ability to visualize the analytes directly in cells, tissues and sub-cellular compartments, *in situ*. Jeppesen and co-workers have applied the technique to study metaphase chromosomes extracted from cells growing in suspension as well as cells adhering to surfaces (Jeppesen, 1995). In order to study histone modifications, chromatin in metaphase chromosomes have been the sample of choice, possibly because transcription is at a standstill. In the cell cycle, metaphase chromosomes are the most condensed form of chromatin packaging. This feature

enables individual chromosome identification in most cases by their morphological features. Thus, while this high degree of condensation may be an advantage in identifying a chromosome by its shape, size and banding patterns, it has disadvantages as well, as discussed later.

Immunofluorescence studies on Drosophila polytene chromosomes have shown that acetylation of H4 shows unique patterns at the specific lysine residues (Turner, 1992). Blocks of heterochromatin (non-coding) lack acetylated histones compared to coding regions according to immunofluorescent data (Jeppesen *et al,* 1992) and immunoprecipitation data (O'Neil *et al*, 1995). In addition, immunofluorescence has also shown that inactive X chromosomes in female mouse and human cells lack acetylation (Jeppesen *et al,* 1993). Such inactivation is attributed to dosage compensation, which is the inactivation of the genes in one of the two X chromosomes present in female cells to equalize expression to that of the one X chromosome in the male cells. Jeppesen and co-workers have also demonstrated the variation of the acetylation patterns depending on the epitope in human metaphase chromosomes (Jeppesen *et al*, 1992).

The most critical aspect of immunofluorescence on metaphase chromosomes, as in many other techniques as well, is how the samples are prepared. For example, in the case of chromosomal analysis, metaphase chromosomes need to be extracted from cells arrested at metaphase. However, the standard conditions used for DNA analysis by FISH are not compatible with histone studies. The standard method to prepare metaphase chromosomal spreads for FISH studies is to expose cells to hypotonic

(0.075M KCl) treatment to swell the cells enabling cell membrane rupture, followed by (3:1) methanol : acetic acid fixation. As histones are basic proteins, this treatment could extract them or perturb the antigenicity of histone molecules, interfering with the antibody binding. This point is further elaborated in the discussion section. In order to get rid of this problem and preserve the histones intact, chemical fixation is completely eliminated. Instead, the chromosomes are spun on to a glass slide by a technique called cytocentrifugation after hypotonic treatment to extract the chromosomes on to the slide. Then immunofluorescence is performed on the cytospun metaphase spreads of chromosomes. If the two techniques, immunofluorescence and FISH, need to be combined, it is suggested that the former be performed first and then the cell spreads be fixed on to the slides prior to FISH analysis (Bickmore, 1998).

3.2 Experimental:

3.2.1 Buffers & Solutions:

Solution	Composition
Potassium chromosome medium (KCM)	120mM KCl, 20mM NaCl, 10mM Tris-HCl, pH8.0, 0.5mM EDTA, 0.1%(v/v) Triton X-100
0.1XSSC	0.015M NaCl, 0.001M sodium citrate, pH7.0

Table 3.2: Solutions and their compositions

3.2.2 Procedures:

(i) Cell Culture:

Lymphoblastoid cell lines provided by CMH were cultured in T25 flasks and fed with 10% FBS (Irvine Scientific) in RPMI 1640 (Irvine Scientific), 1% L-Glutamine (Gibco) and 1% Penicillin-Streptomycin (Gibco), regularly to maintain a healthy cell culture.

(ii) Preparation of Chromosome Spreads for Immunofluorescence:

Approximately about 24 hours prior to making the chromosome preparations, the cell culture is split and fed with medium in 1 :5 (culture:medium) ratio. The cultures are treated with colcemid (Gibco) at a final concentration of 0.1 ug/ mL of culture for 1-2 hours. Cultures are transferred to 15 mL centrifuge tubes and the cells are pelletted at 1000 rpm for 5 minutes. Supernatant is discarded leaving only about 0.2-0.3 mL, in which the cells are resuspended and kept on ice. A volume of 1 mL of hypotonic solution (0.075 M KCl) is then added dropwise to the culture, while gently

agitating the tube. Then another 2-3mL of hypotonic is added while mixing the suspension. Cells are incubated at 37 °C for 15 minutes to allow for swelling and then transferred back to ice until the cells are counted with a hemocytometer.

Next, the cells are exposed to cytocentrifugation in a Shandon Cytospin (Thermo Shandon, PA) using the "dry" technique by placing a filter card between the sample chamber and the microscopic slide as an absorbent. The swollen cells can be diluted with 75 mM KCl containing 0.1% Tween 20 immediately prior to cytocentrifugation in a 1:1 ratio, and the suspension is placed in the chamber and spun at a maximum speed for about 8 minutes. The swollen cells can be diluted with 75 mM KCl containing 0.1% Tween 20 immediately prior to cytocentrifugation. Slides are allowed to dry for a few minutes and are then transferred to a coplin jar with KCM and washed for about 10-15 min at room temperature without any agitation.

(iii) Immunofluorescence assay:

Antibody reactions are performed in KCM without fixation, as the cytocentrifuged chromosomes and nuclei are relatively stable. Both primary and secondary antibodies are diluted in 10% (v/v) serum in order to minimize non-specific binding. The primary antibody (Polyclonal rabbit IgG anti-histone antibody panel, (Serotec,Inc) is diluted 1:250 and the FITC labeled secondary antibody (Goat-anti-rabbit FITC conjugated polyclonal antibody, Sigma) is diluted 1:20 in serum.

After the KCM treatment, the specimen area is carefully blotted and 40 uL of diluted primary antibody of interest (anti histone H4 acetylated at different lysine residues) is applied. The parafilm-covered slide is then incubated at 37°C in a humidified chamber for 1-2 hours. The slide is then rinsed with KCM and incubated with 40 uL of secondary antibody for 30 min exactly the same way as before. It is important that the slide is not allowed to dry out at any point until the incubation is completed. Then the slides are rinsed in water and allowed to air dry. They are counterstained with 10 uL of 4',6-Diamidino-2-phenylindole, DAPI (0.125 ug/mL) for 10 min, washed briefly in water, air dried and mounted with antifade (Vectorsheild). The specimen area is covered with a coverslip and sealed with nail polish prior to analysis under a fluorescence microscope.

(iv) Designing scFISH Probes:

Designing the scFISH probes consists in choosing the gene or genomic interval of interest, eliminating any homologous sequences found elsewhere in the genome, and then designing primers for the selected unique genomic intervals for a given gene. This series of steps, are performed for the three PWS gene selected, ZNF127, IPW and NDN at Dr.Peter Rogan's laboratory in Children's Mercy Hospital Biomolecular Laboratories, Kansas City, MO. The corresponding genomic sequences are retrieved from http://genome.ucsc.edu, which is a database draft of the human genome, by providing the coordinates for the gene of interest.

In order to avoid any homologies, BLAST (Basic Local Alignment Search Tool) search was performed on the genomic sequences obtained from the draft of the human genome at http://www.ncbi.nlm.nih.gov/BLAST/, for the three sets of sequences. After eliminating the homologous sequences, the single copy intervals devoid of the repeating elements were obtained by running the Repeat Masker software on the selected genes and a representative scatter plot for the single copy intervals obtained for IPW region is shown in Fig 3.3. The data points indicate the single copy regions for IPW region, identified by the base coordinates. Primers were designed for selected intervals from the scatter plot for each of the three genes using integrated_scFISH, a unix script. A list of the probes designed for each gene with the corresponding forward and reverse primers are shown in Table3.2.

(v) Synthesis of scFISH probes:

There are three components to the synthesis of scFISH probes: PCR amplification of the target, extraction and purification of the amplified PCR product and finally, incorporation of labels to the product by nick translation or any alternative method. For the PCR amplification the program (Table 3.3) and the amounts of each reagent used are shown in Tables 3.4 and 3.5. The annealing temperature (step 3) of the PCR program is what changes depending on the primer. Two separate master mixtures are prepared as Solution A (Table 3.4) and B (Table 3.5), which are added to two separate PCR tubes for each reaction. They are

combined after step 1 of the PCR program to minimize primer-dimer formation and such non-specific amplification during PCR cycling.

(vi) Nick translation for labeling:

Reagents are combined according to Table 3.6 and incubated for about 90 minutes (depending on the conditions) at 15 °C. Test column in Table 3.6 is to work out the conditions for DNase dilution and timing with some known DNA and Probe column is for the PCR amplified product. One approach taken to determine the exact timing and the dilutions is to try different dilutions of DNase (1:100, 1:250, 1:500 etc) and incubate at 15°C while testing aliquots of the product after 60, 90, 150 and 180 min and run a 1% agarose gel to see what the product looks like.

Step number	Temperature/°C	Time
1	94	2 min
2	98	20 sec
3	65	5 min
4	Goto 2 repeat 14 times	
5	98	20 sec
6	65	5 min +15s/cycle
	Goto step 5 & repeat 14 times	
7	72	10 min

Table 3.3: PCR program

Reagent	Amount
DNA	3 uL
Betaine	12.5 uL
H_2O	10 uL

Table3.4: Amounts and reagents required for Solution A of PCR

Reagent	Amount
dNTP	8 uL
10XPCR buffer	5 uL
DMSO	1 uL
Forward primer*	5 uL
Reverse primer*	5 uL

* Primer sequences for each probe are shown in Table 3.7

Table 3.5: Amounts and reagents required for Solution B of PCR

Reagents	Test (total volume 50uL)	Probe (total volume 100uL)
DNA	500ng/uL	1000 ng/uL
Solution mix	5	10
DNA Polymerase	2	4
DTTP or dig-dUTP	1	3
DNase	1.5 (starting point)	3 (correct dilution)
H_2O	39	75

Table 3.6: The amounts and reagents for nick translation

(vii) Cell Harvesting for scFISH:

Colcemid (Gibco,10ug/mL) is added to the cell culture 100uL/mL of culture and incubated at 37 °C for 35 minutes. The culture is then transferred to a 15 mL polypropylene tube and centrifuged between 800-1000 rpm for 8 minutes. The medium is decanted and cell pellet is flicked to make a homogeneous suspension. Depending on the pellet size, 3.5-5.5 mL hypotonic solution (0.075M KCl) is added and incubated at 37°C for 10 min. The suspension is centrifuged at 800-1000 rpm for 8 minutes. The supernatant is decanted and the pellet flicked.

First fix: 3.5 mL of 3:1 Methanol (3): acetic acid (1) is added very slowly along the wall of the tube and incubated at room temp for 15 min. The suspension is centrifuged and the supernatant is decanted leaving less than 0.5 mL in the tube. The pellet is flicked to dissolve in the residual amount of fix.

Second fix: A volume of 3.5 mL of the same fixative solution is added as above and incubated at 37°C for 15 min. The suspension is centrifuged, supernatant decanted and pellet flicked as in the previous steps.

Third fix: The same volume of fixative is added and incubated for 10 min and centrifuged. After decanting the supernatant fresh fixative (about 2-3mL) is added and pellet is homogenized. (Depending on the size of the cell pellet, an appropriate volume of fixative is added to get the required cell density on the slide) The harvest is ready for dropping slides.

(viii) Slide Preparation for scFISH:

A glass slide (Richard Allen Scientific) is wetted with methanol (3):acetic acid (1) fixative solution and, before it is dried out, a few drops of cell suspension are dropped on to the slide held at an angle. The slides are dried on a tube rack placed on a hot water bath (to create a humid environment)

(ix) scFISH Assay:

Slides containing metaphase spreads prepared according to the procedure mentioned as above, are immersed in prewarmed denaturation solution (70% formamide, 2X SSC) at 72 °C for 2 min. The slide is then soaked in ice cold 70% EtOH for at least 1 min and then in 80%, 95% and 100% EtOH solutions at room temperature for 2 minutes each. The slide is then air dried. Hybridization buffer is prewarmed to 37 °C. To the probe 10 uL of formamide is added and heated at 70°C for 5 min. Tube is then placed on ice for 15 min. A volume of 10 uL of prewarmed hybridization buffer is added to the probe and mixed well. Then a total of 20 uL of probe mixture is applied to the slide containing metaphase spreads. A coverslip is placed on the slide and and sealed with nail polish. When the seal is dry, air bubbles are removed by gently pressing on the coverslip. The slide is then incubated at 37°C in a light proof humid chamber overnight to allow for hybridization.

During the last 30 min of hybridization, a solution of 50% formamide, 5X SSC was warmed to about 39°C. After removing the slide from the incubator, the coverslip is removed and washed in the above solution for 30 min at 37°C, shaking

every 10 min. The second post-hybridization wash is again at 37 °C in 2X SSC for 30 min. Finally, the slide is washed in 1 X SSC at room temperature for 30 min and then soaked in 4 X SSC for 5 min. Slide is then removed and the excess buffer is drained. Without letting the slide dry out, 50 uL of anti-Digoxigenin antibody (anti-Dig, Roche) solution (3.5 uL of anti-Dig in detection buffer, 4XSSC with 1% BSA) is added, placed a parafilm over the area, and incubated at 37°C for 45 min in a humidified chamber. Then the slide is washed in 4 X SSC for 20 min on a mechanical shaker, followed by washing in 0.1% Triton X in 4X SSC on the rocker for 20 min. Finally, it is washed again in 4X SSC for 20 min slightly shaking the solution and then equilibrated for 5-8 min in 1 X SSC on the rocker.

The slide is then counterstained with 10 uL of 0.1ug/mL DAPI for 10 min in the dark. The slide is then dried and mounted with antifade and stored at -20°C overnight prior to visualizing them under fluorescence.

(x) Preparation of chromatin fibers:

Slide preparation:

Glass slides are precleaned mechanically by repeated rubbing with kimwipes. They are rinsed several times with nanopure water. Slides are then immersed in boiling nanopure water for 10 min and air dried, prior to immersing in 18M H_2SO_4 for at least 30 min to remove any organic impurities. Finally, they are immersed in boiling water for 1-2 min, air dried and stored until further use.

Silane Modification:

Precleaned dry slides are immersed in a solution of 0.1% 3-aminopropyltriethoxy silane (APS) in 95% ethanol for 10 min. Slides are removed from the silane solution, and rinsed several times with water for 2 min. They are then dehydrated by immersing in absolute ethanol and are dried in air at 65°C on a hot plate, standing upright for 10 min. Slides are stored for 2-6 weeks at 4 °C in a sealed box prior to use under nitrogen. Coverslip preparation is similar to slide preparation as described above (Heinz-Ulli, 2001).

Fiber Preparation:

Cell suspension (50uL) is centrifuged at 3600 rpm for 5 min to obtain a cell pellet that is then resuspended gently in 50 uL of PBS. A volume of 1 uL of this suspension is then placed a the end of a cleaned, silanized microscopic slide and allowed to air dry. STE lysis buffer (10uL) is added on to the dried nuclei and incubated for 5 min at room temperature. The slide is then tilted at 45° and allowed for the chromatin to extend along the slide. The fibers are then fixed in methanol (3): acetic acid (1) for 2 min and allowed to air dry. The slides are incubated at 60°C for 30 min prior to staining with 10 uL of DAPI (100ug/mL) (Schwarzacher et al, 2000).

3.3 Results & Discussion:

scFISH:

In choosing primers for PCR amplification, there are two important things that should be borne in mind. The longer the primers, the more specific they will be for the selected target and the smallest difference in the annealing temperatures between the forward and the reverse primer is an indication of the compatibility of the two primers.

As shown in Fig 3.3 the PCR amplified scFISH product can be seen as bands in a 1% agarose gel. The PCR products are identified based on their expected size, by running a DNA marker in parallel with the products. The NDN product is seen as a single band indicating that it is a single copy gene and the stringency of PCR conditions is sufficient to amplify only that specific genomic locus. However, as the gel for IPW shows there are multiple products, with only the predominant band corresponding to the expected product. This indicates that the stringency of PCR has not been tight enough to amplify only the desired target in the latter case. Consequently, there are other products seen as faint bands in the agarose gel. In such a situation, the PCR conditions can be worked out so that only the desired product is being amplified. However, before proceeding to the next step of labeling the probes, as only the desired product is excised from the gel and purified, in the above case of IPW, it was decided to move on with these conditions. The gel band is excised, extracted using spin columns, and labeled by nick translation. The probes can be either directly or indirectly labeled. Direct labeling is when the fluorophore is directly

linked to a DNA base so that the fluorophore is incorporated to the probe in one step. In the case of indirect labeling, a different tag such as digoxigenin or biotin is attached to the DNA base that is identified by an antibody carrying a fluorescent tag, when the FISH experiment is being performed. Labeling by nick translation is based on the random introduction of nicks in one of the two strands of DNA by the enzyme, DNase I. Then, DNA polymerase synthesizes new DNA in the 5' to 3' direction using those nicks as primers and the intact strand as the template. The exonucleolytic capability of DNA polymerase to remove nucleotides in the 5' to 3' direction enables it to clear a path for the incorporation of labeled nucleotides into the new DNA strand that is being synthesized (Klett, 1968). Thus, the two purposes of this step, namely to label the PCR-amplified probe and then make shorter fragments of it, are being satisfied. It is important that the nick translated fragments be between 200-500 bases for hybridization to the target chromosomes. If the fragments are longer than the optimal range, it reduces the accessibility to the target, especially when the target is highly coiled DNA as in metaphase chromosomes and it would also lead to sticking of the probes to the glass slide non-specifically. On the other hand, if the fragments are too short, that would result not only in poor hybridization specificity to multiple target locations, but also in low hybridization efficiency, due to the tendency of the two strands of probe DNA to rehybridize to each other, making less probe available for hybridization to the target (Norgard,1985). Therefore, determining the right DNase dilution and the time is absolutely essential to obtain the fragments of the correct size. This needs to be done for each vial of DNase varying the timing at 15°C.

Fig 3.2: shows the products after nick translation in a 1% agarose gel. The smear between 200-500 bases shows the fragment sizes of the probe by this labeling procedure.

Fig 3.4 shows a scatter plot of the designed scFISH probe regions. The x-axis shows the position of the specific region in the genome by its coordinates and the y-axis shows the length of the probe. Theoretically any of these dots in the plot can be chosen to synthesize the probes. However, there are two aspects that need to be considered in choosing a specific region for amplification. The shorter the probe the fewer the number of fluorophores that can be incorporated, resulting in a less intense fluorescent signal. On the contrary, relatively larger probes will result in a brighter signal, but the extraction efficiency off the spin columns is very poor. Therefore, one needs to strike a balance between these two aspects when choosing the probes. The probes chosen for the genes that were selected for this work were in the range of 1000-2500 base pairs. The amplification and extraction procedure were very much easier for probes close to 1000 base pairs, as anticipated, and the larger probes gave poor recovery after extraction with the spin columns. Different methods of extraction to resolve this matter are being tried by Rogan and coworkers.

Fig 3.5 shows the characterization of one of the synthesized scFISH probes for the NDN region. This particular experiment was performed by combining three different probes (ac677f299r, ac095f039r and ac739f300r) to increase the size of the target length to 4300 bp so that the signal would be clearly seen. The NDN probe is bound to chromosome 15 in this image in the expected region of the long arm, which

indicated that the designing and the amplification of the scFISH probe has been successful. However, the probe is bound to only one of the two chromosome 15s in the metaphase spread. This is a frequent observation in this type of experiment that has a number of possible causes. Not seeing a signal on the other chromosome means that insufficient probe has bound to the target. This could be due to lack of accessibility of the target to the probe. The two DNA strands in a chromosome could be so compactly arranged that the conditions for denaturation are insufficient to unwind them, or it could simply be that not enough probe added to react with all the target molecules. Another reason why probes may not be able to access the target is the presence of residual cell debris making it difficult for the penetration process. This is a problem caused at the harvesting stage of the cell culture and might be able to be eliminated by washing the cell pellet more extensively with the appropriate fixative, which in this case is methanol (3): acetic acid (1). If such washing does not help, cells may have to be harvested from fresh cell cultures.

A challenge that needs to be addressed with scFISH technology is the sensitivity of the signal produced by the scFISH probes. As the probes are significantly shorter in length, only a few fluorophores can be incorporated at the labeling step resulting in a poor signal. This is especially the case when the probes are at the lower end size-wise, which are better in a resolution point of view to map the genome. So improving the sensitivity of the signal will widen the applicability of the scFISH technology significantly. Two promising approaches along these lines are to use 3D-Dendrimers (Genisphere,PA), which is a network of DNA to which about 50-

400 fluorophores can be attached and quantum dots (Molecular Probes), that are semiconductor (CdS) nano-particles into which hundreds of fluorophores are incorporated. As a single entity of the above contains a large number of fluorophores, sensitivity will be greatly enhanced. However, the size of the particles may be an issue when the target, such as a metaphase chromosome, is highly condensed. The smaller the particle, the better it would work in such cases due to easier accessibility.

IF:

The basic requirement for immunofluorescence to work is to retain the antigenicity of the analyte of interest during the preparation of chromosome spreads as this method is entirely based on how well the antibodies react with the corresponding antigens. This was proven to be the case when different fixatives were used to fix the metaphase spreads. For example, when methanol:acetic acid (3:1) was used as the fixative after dropping the slides in the conventional way for FISH experiments as described in section 3.2.2. (viii), the immunofluorescent signal (FITC labeling) was confined to the borders of each metaphase spread (Fig. 3.6). This is indicative of the lack of antigenicity or difficulty for the antibodies to access the antigens uniformly across the metaphase spread, with this particular fixative. Chemical fixatives were abandoned due to this observation which has also been noted in the literature (Jeppesen, 1995).

Cytocentrifugation is the method of choice for extracting metaphases for immunofluorescent work. The colcemid treated cells are arrested at metaphase and exposed to hypotonic (0.075M KCl) solution to solubilize the cell membranes. When the cell suspension is placed in the funnel chamber, and centrifuged in a cytospin, the metaphases and the nuclei are spun on to the glass slide. It is very important that an absorbent filter card is placed between the funnel and the glass slide in order to absorb the salt and buffer solutions, which would otherwise give rise to non-specific background signals due to the presence of salt crystals on the slide.

A significant level of method development was necessary to optimize conditions for good metaphase spreads. At the very preliminary stages, the slides not only contained a lot of salt crystals, but the chromosomes looked fuzzy as well, lacking distinct morphology (Fig 3.7). Sometimes, the spreads and chromosomes appeared to be connected to each other via thread-like structures, which could be cell debris. As mentioned earlier, the salt crystals on the slide can be eliminated to a significant degree with the use of the absorbent filter cards. A brief washing of slides in water immediately after cytocentrifugation, rinses off salts, further. This rinsing of cytospun spreads with water helps in retaining the distinct morphology of chromosomes as well; the fuzzy appearance of chromosomes in the initial experiments is partly a result of too much drying out before proceeding to the next step. The cell density of the culture is another very critical factor to get good spreads. The optimal cell density for this cell line is about 1×10^5- 10^6 cells/mL. In order to reach this cell density, the cultures need to be fed with medium regularly, and 24

hours prior to colcemid treatment, the culture is split and fed with medium in a 1:5 ratio of culture: medium. In order to obtain well spread metaphases without their being interconnected to each other via cell debris, about 100 uL of a culture, maintained at the optimal cell density, should be cytospun after the hypotonic treatment at 2000 rpm for 10 minutes. With these improvements, the quality of the spreads improved significantly (Fig 3.8). Such optimized cytocentrifuged spreads are then subjected to immunofluorescence. The morphology of the chromosomes prepared by cytocentrifugation appear somewhat different from those that are chemically fixed (compare cytocentrifuged spreads in Fig 3.8 to chemically fixed spreads in Fig 3.5).

Potassium Chromosome Medium (KCM) proves to be a good medium to carry out the antibody incubations for immunofluorescence and has been optimized by Jeppessen and co-workers. Three different secondary antibody dilutions (1:10, 1:20 and 1:40) were tested to determine the optimal concentration. At least the three dilutions tested did not seem to make a significant difference in the outcome. Therefore, 1:40 dilution was chosen for the analysis.

Fig 3.9 (B through I) demonstrates the differences seen in the acetylation patterns of histones based on which lysine residue was analyzed on PWS cells. Although not much useful information can be gathered about a specific genomic locus due to poor spatial resolution, it is interesting to see a distinct difference in the distribution of acetylation across the genome. Fig 3.9(A) shows the same experiment on a control cell line for acetylation at Lys 8. No difference can be detected between

the patient and the control cell lines for any of the modifications. These data are consistent with those reported in the literature (Jeppesen, 1993) and also with the hypothetical order in which acetylation takes place. Lysine 16 is believed to be acetylated first out of the four different positions of acetylation on histone H4 (Turner, 1993) and the immunofluorescent signal is basically a "paint" across the entire chromosome. Lys 12, postulated to be acetylated next, shows speckles throughout the chromosome, while Lysine 8, the next site, shows some sort of banding. Lysine 5, the last site in the order of acetylation of H4 gives a very faint signal. It is noteworthy at this point that deacetylation of histones is thought to take place in the reverse order (Thorne et al, 1990). Although transcription does not take place in the metaphase chromosomes during the cell cycle, histones turn out to be acetylated. This observation aroused the interest of many scientists and they revealed the fact that the acetylated domains in the chromosomes correspond to the gene rich euchromatin domains, which are also identified by the R-banding patterns (Jeppessen, 1993). The main problem associated with this approach to study acetylation or any other PTM of histones is the lack of resolution because the mitotic chromosomes are highly compact in nature. As the cell cycle progresses, chromatin undergoes different stages of condensation and decondensation. Decondensation of chromatin can be considered an important strategy for obtaining better spatial resolution.

Fig 3.10: shows extended chromatin fibers derived from mammalian lymphocytes. Such extended chromatin fibers, taking advantage of increasing spatial resolution, are mainly used for genomic mapping purposes. However, Collas and co-

workers (Collas *et al*, 2001) have demonstrated the feasibility of combining FISH with IF to determine acetylation in an inserted reporter gene, luciferase in Zebrafish. They have been able to qualitatively assess the presence of acetylated lysines on histone H4 at the luciferase gene. This provides evidence for the ability to detect histone modifications at a specific genomic locus with increased spatial resolution of the chromatin fibers, compared to metaphase chromosomes.

Gene	Probes designed for PWS region
NDN	ac582f181r
	ac095f039r
	ac677f299r
	ac679f300r
	ac675f300r
	ac739f300r
	ac677f300r
ZNF127	U788f035r
	U233f037r
	U233f030r
	U177f681r
IPW	IPW345f331r
	IPW262f070r
	IPW651f880r

Table 3.3: Single Copy FISH Probes designed for three genes in the PWS region, NDN, ZNF127, IPW.

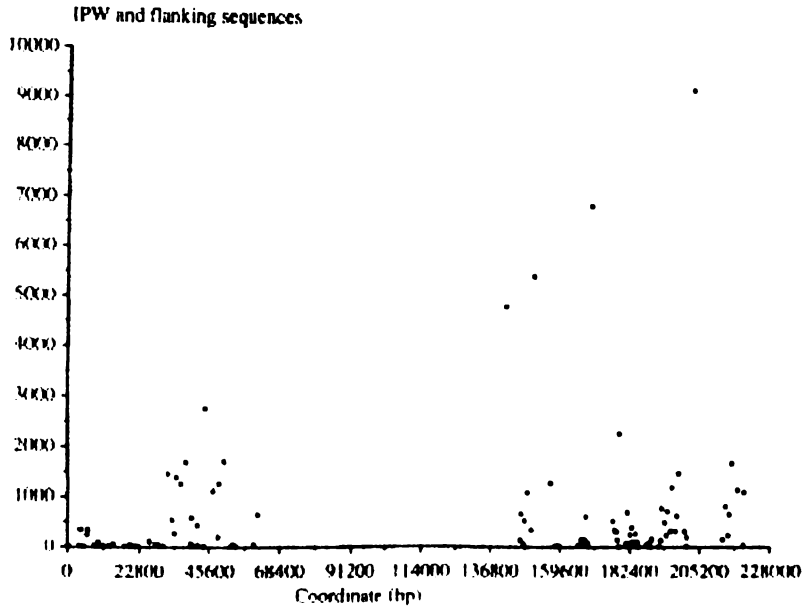


Fig: 3.4 Scatter Plot indicating the single copy regions for IPW region generated by the software

Probe	Primer Sequence
ac582f181r	F-TAAGGATCTGAGCGACCCTAACTTTGCAGC
	R- GGAGTAATCATGAGTAGCGATTTTTCCCACCC
ac095f039r	F-TACCCTGGTGAAGTGGGAAGCTTAAGAGTC
	R- GCTTTATGCGAACCAGTGAGAGGAACAGTC
ac677f299r	F-GTCCACCTGCCCCAAACTCTAATCTTACTC
	R-TAAAACTGCATTCTCACATGTCACCCTCCG
ac679f300r	F-CCACCTGCCCCAAACTCTAATCTTACTCTG
	R-TTAAAACTGCATTCTCACATGTCACCCTCC
ac675f300r	F-TTGTCCACCTGCCCCAAACTCTAATCTTAC
	R-TTAAAACTGCATTCTCACATGTCACCCTCC
ac739f300r	F-GACTCCTTAACTATCTGAGAGAGCTGCTCC
	R-TTAAAACTGCATTCTCACATGTCACCCTCC
ac677f300r	F-GTCCACCTGCCCCAAACTCTAATCTTACTC
	R-TTAAAACTGCATTCTCACATGTCACCCTCC
U788f035r	F-GCTCAGAGACTGAGAGGAAGCAGATGGCTGTG
	R-GCACTTGAAGGGGAAGGAGGGAAAGAACAAACT
U233f037r	F-GGAAGGGAAACAAAGGGCTGCCATGAATTAAAC
	R-GCACTTGAAGGGGAAGGAGGGAAAGAACAAAC
U233f030r	F-AGGGAAACAAAGGGCTGCCATGAATTAAAC
	R-GGGGAAGGAGGGAAAGAACAAACTGTAAAC
U177f681r	F-AGGATATGTTATGAAAGGCTGTCCTGCCACAACAC
	R-GGGCTGCCCTGGAACAAACTCAATGGCATC
IPW345f331	F-GGAGCACTGAGCAATTCCACCATTTTGAGC
	R-CAACACCCCTTAGACAAAAAGCCTCCTCACC
IPW262f070r	F-TCATTTTTTTCCCTCAATTTACACCTTTAACC
	R-TACTCAAGTAATTTTTCCTGTCCTTTTCCC
IPW651f880r	F-GTATCCTGTGCTGGGAAAACACGGAGGTTG
	R-GGCTGAAACACGCTGATTGCACTCTTTACC

Table 3.7: Forward (F) and reverse (R)primer sequences for each designed probe

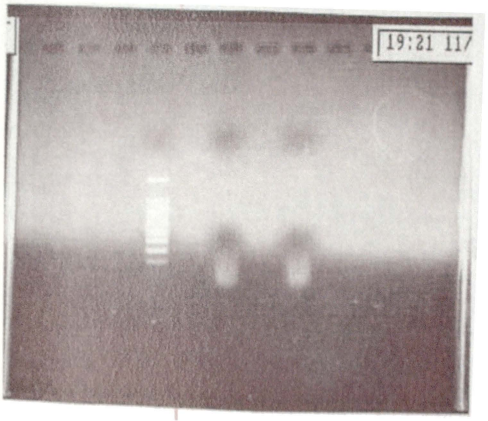


Fig 3.2: 1% agarose gel for the nick translated products. Fourth lane shows the 100 base pair marker; lane 6- Genomic DNA and lane 8 – ac677f299r product.

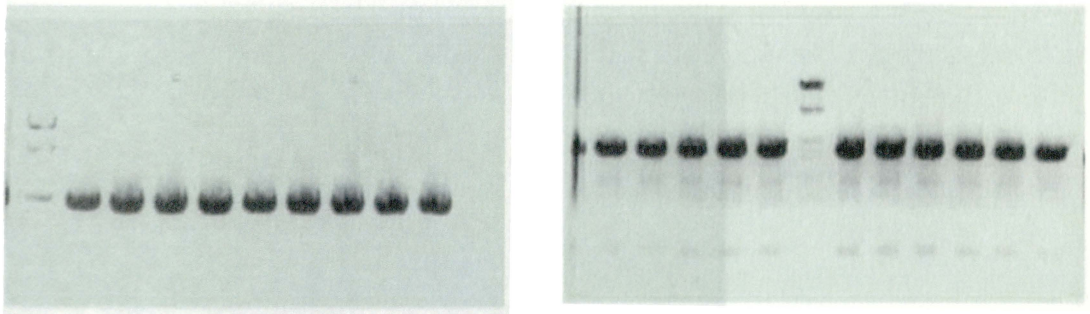


Fig 3.3: PCR products for NDN (ac677f299r) on left, and IPW (262f070r) on right, run in a 1% agarose gel. Lane 1 on the left gel and lane 6 on the right gel are markers (Lambda/EcoR1+HindIII marker) to identify the products based on the size.

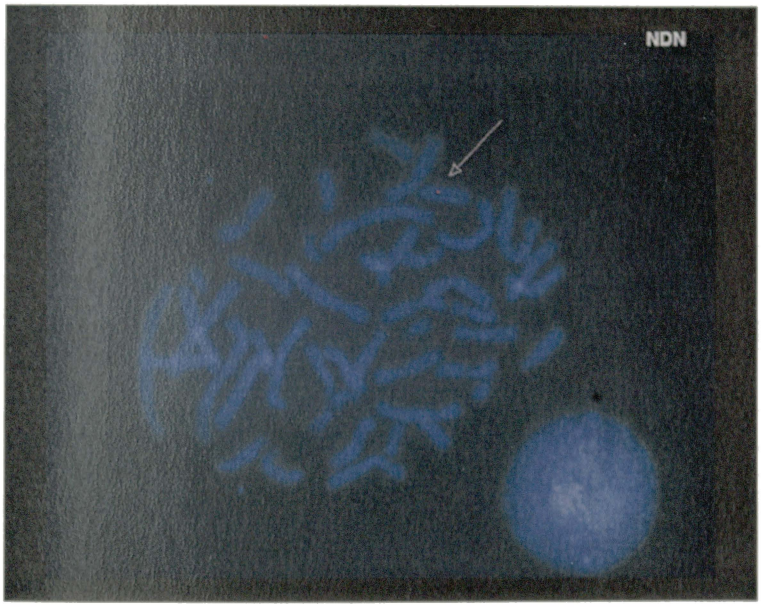


Fig3.5: The combined NDN scFISH probes reacted on a metaphase spread indicated by the red signal on chromosome 15

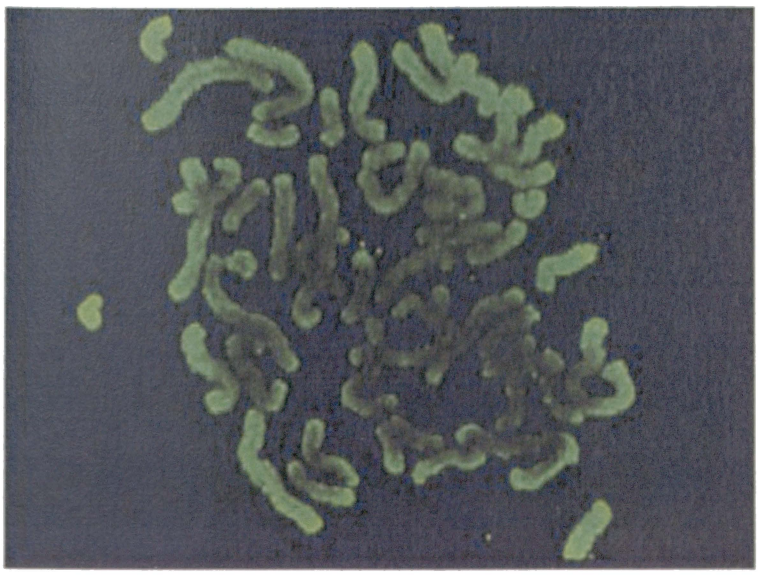


Fig 3.6: Metaphase spread of chromosomes fixed with methanol (3):acetic acid (1) subjected to immunofluorescence with polyclonal Rabbit-anti histone H4(K16) and FITC labeled Goat-anti-rabbit secondary antibody. Signal is confined to the borders of the chromosomes and the borders of the metaphase spread.

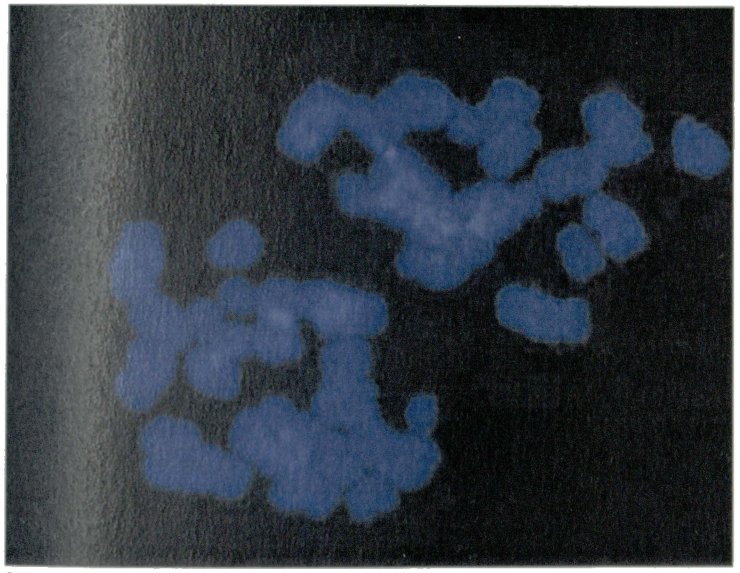


Fig 3.7: Prior to optimizing the conditions for cytocentrifugation of metaphases

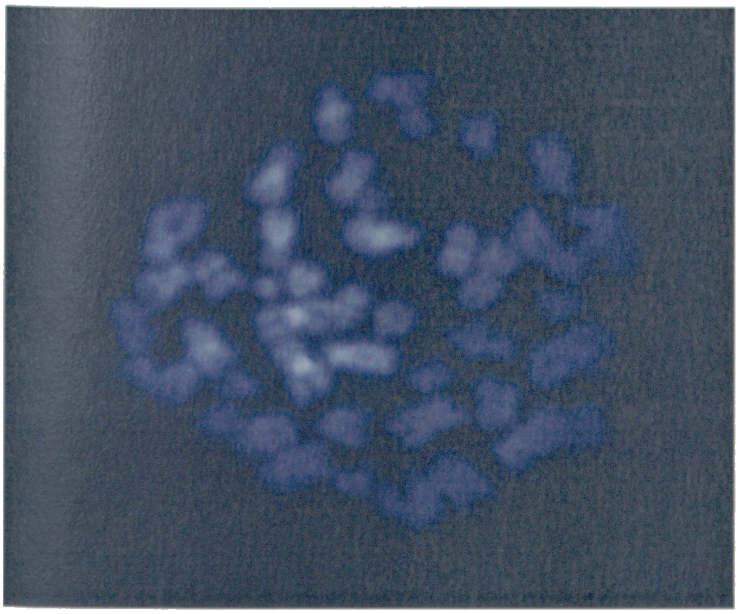
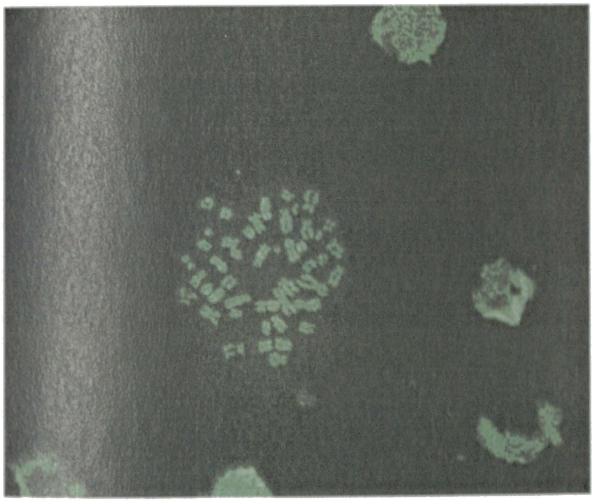
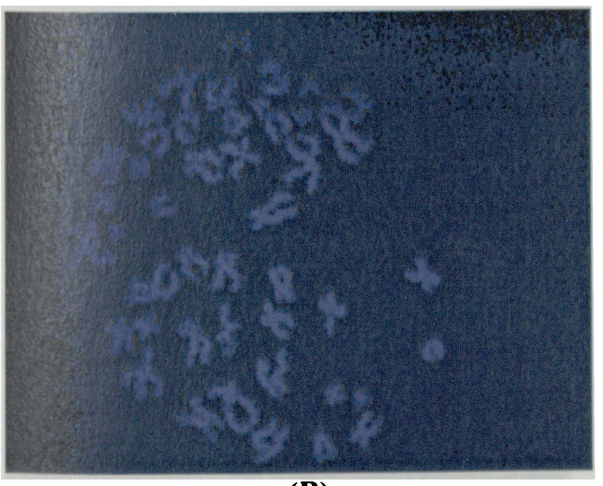


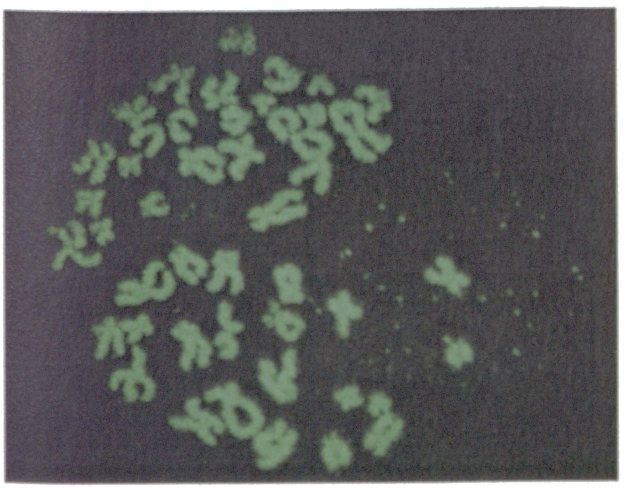
Fig 3.8: After optimizing the conditions for cytocentrifugation of metaphases



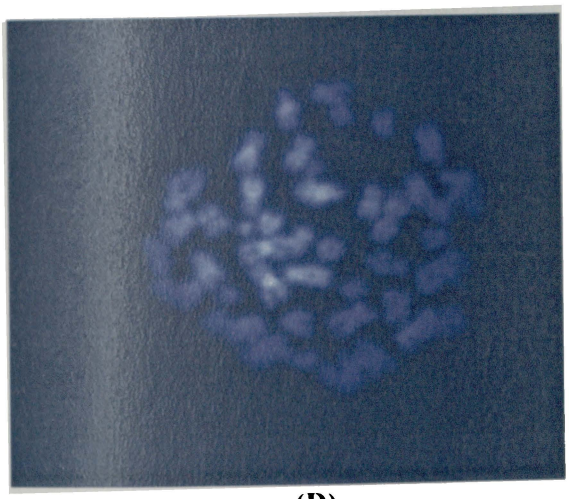
(A)



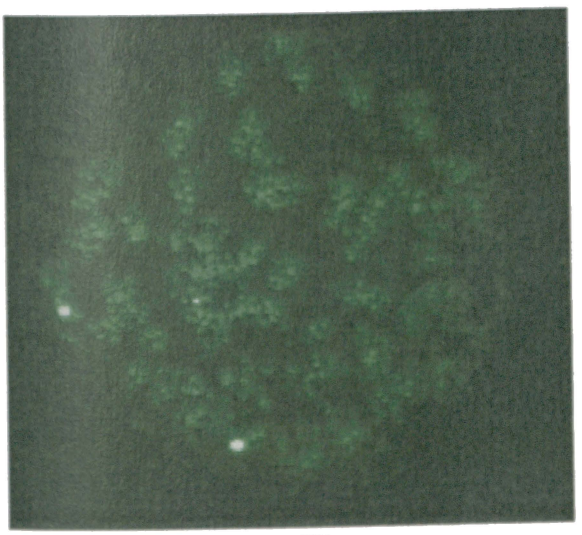
(B)



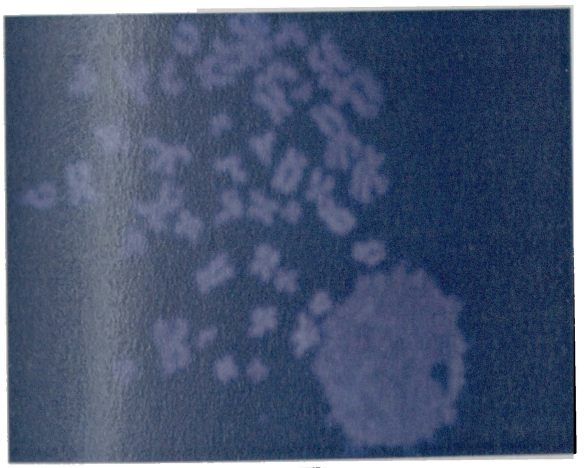
(C)



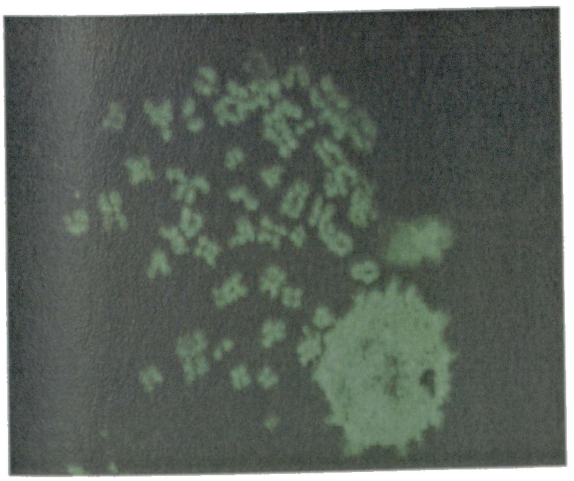
(D)



(E)

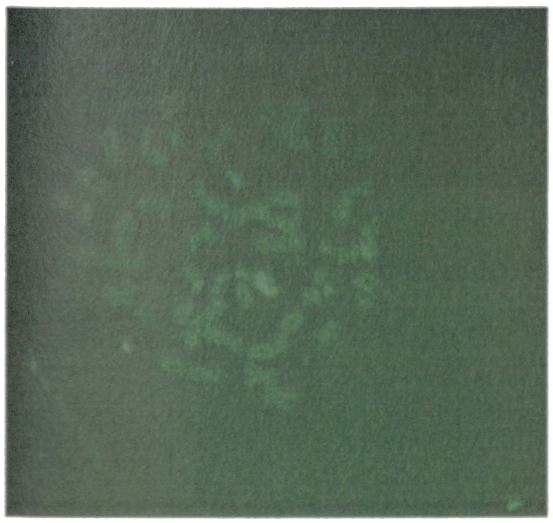


(F)



(G)

(H)



(I)

Fig 3.9: IF on different epitopes on control cell line and PWS cell line. The control cell line and the PWS cell line are treated exactly the same way with the corresponding primary antibody (anti his H4 lys) diluted 1:250 with 10%FBS and secondary antibody diluted with 1:40 with 10%FBS and examined under fluorescence.

(A) - lys 8 on control
(B) - DAPI stain of PWS spread shown on (C)
(C) - lys 16 on PWS

(D) - DAPI stain of PWS spread shown on (E)
(E) - lys 12 on PWS
(F) - DAPI stain of PWS spread shown on (G)
(G) - lys 8 on PWS
(H) - DAPI stain of PWS spread shown on (I)
(I) - lys 5 on PWS

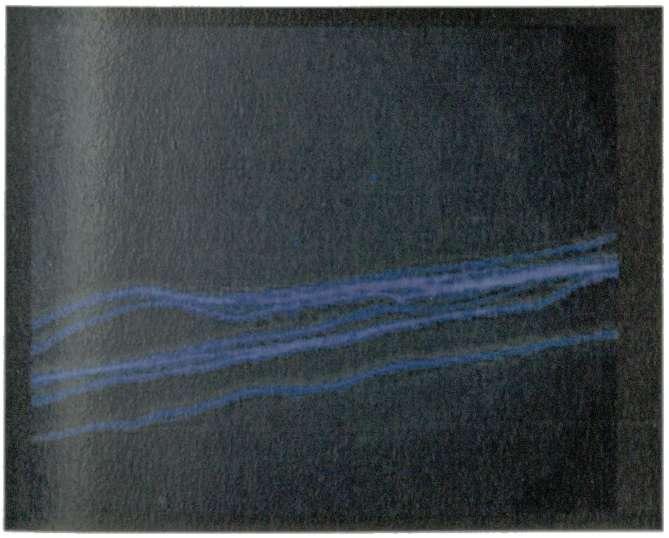


Fig 3.10: Extended chromatin fibers derived from mammalian lymphocytes stained with DAPI.

3.4 Conclusion:

The conclusions drawn from the immunofluorescence experiment results show that the acetylation patterns of histones obtained for the Prader-Willi cell line and the control cell lines are comparable to the results reported in the literature for the different histone H4 acetylation sites. However, poor spatial resolution makes it

impossible to gather any useful information about a specific genomic locus between the control and the patient cell lines. Consequently, a combination of the two techniques to find out the extent of acetylation at the molecular level associated with a gene or multiple genes does not accomplish the task, as the method suffers from lack of resolution. One of the ways to improve resolution is to unravel the compacted chromosome and form extended chromatin fibers. Although these extended fibers may be sufficient for a qualitative analysis of the PTMs, the resolution will still be insufficient for a molecular level investigation.

FISH is a technique mainly employed for clinical applications to gather genetic information such as a deletion or a translocation of a gene or genes. ScFISH probes have moved a step further to overcome some of the limitations associated with conventional FISH probes by the decrease in the size of the probe and eliminating repeating elements. The feasibility of design and synthesis of scFISH probes has been demonstrated. However, if one needs to adopt a cytogenetic approach, such as the one described in this chapter, for quantitative protein analysis, the probes may have to overcome the barrier of resolution even more - to the point of single molecule detection. In addition, strategies to improve the sensitivity of the scFISH probes need to be investigated as it is a main concern with regard to short scFISH probes. Use of quantum dots (Molecular Probes) and 3D-dendrimers (Genisphere) are two promising alternatives for classical fluorophores used to label the FISH probes. However, accessibility to compact metaphase chromosomal DNA of these particles with diameters in the nanometer range would be an issue that needs to be addressed. The

extended chromatin fiber may be a better target for probes labeled with such alternative fluorescent markers having their relatively large particle size. The overall conclusion of this approach of analysis is that the information gathered will qualitatively indicate the presence or absence of a certain modification of histones and may not be useful either as a quantitative tool or for an investigation of the modifications at a molecular level.

Chapter 4: Isolation of a Specific Chromosome

4.1 General Introduction:

Chromatin structure is believed to play a pivotal role in regulating genes, in addition to its function in packaging the DNA into the nucleus as described in Chapter 1 (Wolffe, 1999). The interest and effort previously devoted to exploring chromatin structure and its influence on genetic control have increased dramatically, with the rapid evolution of proteomics research after deciphering the Human Genome. Even though a single eukaryotic cell may contain thousands of genes, only those that are required for the function of a particular cell are expressed; the others are repressed by some regulatory mechanism. In other words, the complement of genes remains constant in all cells in a particular species, while certain genes are turned on and off depending on the cell type, tissue in which the cell is present and the developmental stage of the cell. That is one aspect of regulation. The second aspect is the underlying regulatory mechanism of molecular imprints, which are inheritable markers that indicate the transcriptional state of a gene based on parental origin.

It has been demonstrated that when a transcriptionally active gene is placed in a non-transcribing domain of the genome, the gene becomes repressed (Jenuwein *et al*, 2001). This phenomenon, which is called the Position Effect Variegation (PEV), illustrates the role of an epigenetic marker in these non-transcribing blocks of heterochromatin. There is ample evidence to support the fact that chromatin is an

integral part of such regulation of gene expression, although the details at the molecular level in entirety are not yet known. A map of chromatin structure therefore, in defined genomic intervals across the human genome, will be a useful tool to understand the impact of such epigenetic processes on the control of genes. What is not yet understood is what modifications of histones influence the regulation of transcription and how. It could either be as simple as loosening the grip of histones on DNA upon acetylation of histones that allows transcription machinery to access the DNA template in chromosomes, or a more complicated combinatorial process involving different types of modifications acting synergistically or sequentially, to mark a gene as transcribable or not. A quantitative analysis of the different modifications of histones in a region of interest in a particular chromosome is, therefore, the ultimate picture one would desire to see. A map that illustrates changes of histones, residue by residue, will enable a comparison of the modifications in a patient cell line with that of a control and determine if the gene regulation is influenced by such modifications in genetic diseases.

As discussed in chapter 1, a multitude of techniques have been used to study the different post-translational modifications (PTMs) of histones. Although tools such as immunofluorescence and fluorescence in situ hybridization will provide us with a qualitative picture about the protein modifications and the genetic information, respectively, such techniques lack the resolution required to understand the underlying chemistry of chromatin. This has led the chromatin research community in the direction of mass spectrometry to unravel the chromatin structure, on a residue by

residue basis. Although it is not debatable that mass spectrometry will be capable of providing more details than any other technique available and used so far to study chromatin, current mass spectrometric work performed in chromatin research involves the analysis of only bulk chromatin; that is to say, histones extracted from everywhere in the genome have been analyzed mass spectrometrically to study PTMs. Although this may give insight as to what positions and residues can be post-translationally modified by a specific functional group, this would not enlighten us about the specific modifications in a particular genomic region of interest. In my opinion, the latter is of far more importance, as it can shed light to what is happening genetically and epigenetically in a region of the genome that leads to a disease. However, to reach that point of analysis by mass spectrometry, there are some complicated barriers to overcome in the process of sample preparation, as the system in focus is a rather complex one. In the context of Prader-Willi Syndrome (PWS), the responsible genes for PWS are all clustered in a region 2 Mb in length on the long or q arm of chromosome 15. In order to compare the PTMs of histones associated with PW genes in patients with that of normal individuals, histones need to be extracted only from the region of interest. In order to do that the specific segment, which could be a single gene, a promoter region or multiple genes in the PW region needs to be clipped from the rest of the chromosome. The methodology for this has not yet been developed; some possible approaches to achieve this goal have been addressed in chapter 5. The amount of histone extracted from a specific region of the genome for MS analysis could seem to pose a problem due to sensitivity issues. However, with

the rapid increase in the availability of mass spectrometers with lower detection limits and higher resolution, this may soon be a resolvable problem. To clip the genomic region of interest utilizing Fenton chemistry, nucleases or using a fluorescent probe, as discussed in chapter 5, if the chromosome of interest is isolated from the rest of the genome or if at least one could start off with an enriched pool of the specific chromosome, the subsequent steps would be less complicated. Therefore, isolation of a specific chromosome or, in the context of PWS, isolation of chromosome 15 is the very first step in this crucial sample preparation to solve a complicated problem.

The current state-of-the-art technique for isolation of chromosomes is bivariate flow cytometry. Mitotic or metaphase chromosomes, the most condensed form of chromosomes in the entire cell cycle, are extracted by blocking the cells at metaphase. The extracted chromosomes are then labeled with two different fluorescent DNA binding dyes, i.e. Hoechst 33258 and chromomycin A_3. While the former binds to the DNA backbone non-specifically, the latter of the two dyes intercalates into the chromosomal DNA in different amounts depending on guanine:cytosine (G:C) and adenine:thymine (A:T) ratios. The flow cytometer is tuned to capture those 2 events, and to identify a specific chromosome when the two different fluorescent intensities coincide to uniquely define a particular chromosome. There are two major shortcomings of this method of isolation. Firstly, discrimination between certain chromosomes is not sufficient as it is mainly based on the A:T/G:C ratio of bases and the size or the total DNA content of chromosomes. Therefore distinguishing human chromosomes 14 from 15, 16 from 17 and 9,10,11 and 12 from

each other in order to get a pure population of a single chromosome species is almost impossible (Fawcett *et al*, 1994). Secondly, since this type of sorting of chromosomes is done in series it takes at least about 2500 operating hours (equivalent to several years of work time) to sort the number of chromosomes required to obtain sufficient amount of material for a mass spectrometric analysis (Calculation in Fig 4.1).

The important problem of isolating a specific chromosome as the first step of sample preparation for a thorough analysis of histones, is tackled by two methods. The two major setbacks in the flow cytometric approach to sort chromosomes are addressed by these 2 novel approaches. To improve the specificity of the current method we have proposed using a chromosome specific fluorescent DNA probe to identify the chromosome of interest as described in the first part of the chapter (Part I). To decrease the time scale required for the sorting process, a novel methodology has been developed to fractionate the chromosomes in a massively parallel way as opposed to the serial approach taken in flow sorting, which is described in the second part (Part II) of the chapter.

Fig 4.1: Calculation of the time required to sort a number of chromosomes that would give a sufficient amount of histones for mass spectrometric (MS) analysis

If the length of the gene of interest is 5000bp:

Since every ~150bp contain 2 histone H4 molecules, 5000bp will contain 66.67 molecules of H4, equivalent to 1.10×10^{-22} mol of H4

It was experimentally established that 1×10^{-12} mol is required for mass spectral analysis

Therefore, the number of chromosomes needed to extract this amount is about 9×10^{9}.

The speed of sorting of a state-of-the-art flow cytometer is 10000/s

Thus, the time taken to sort 9×10^{9} chromosomes is 900,000s equivalent to 250 hours.

This calculation assumes that the sorting process is 100% efficient. However, sorting efficiency is dependent on many factors such as the sort rate, which in turn is governed by the quality of the chromosome preparation. A more likely value for the sort rate for a chromosome preparation is about 1000 events /sec and the instrument will have an efficiency of about 90% at this rate. This would have the effect of increasing the sorting time to more than 2500 instrument hours.

Part I: A Novel Approach to Sort Chromosomes by Flow Cytometry

4.I.1. Introduction

Flow cytometry, a technique initially used to analyze cells and detect subpopulations of cells, has been fine tuned further in the past two decades to become a versatile technique, utilized for multiple applications. One such important application is sorting individual chromosomes to generate chromosome-specific DNA libraries (Fawcett, 1994). In the very initial stages of flow sorting chromosomes, they were stained with only one DNA intercalating dye and passed through the laser beam of the Fluorescence Activated Cell Sorter (FACS). The sorting was based only on the size of the chromosome, as the fluorescence was dependent on the extent of intercalation taking place in each individual molecule (Gray *et al*, 1975). Although chromosomes could be separated by this method, the resolution and, hence, the purity of the subpopulations obtained by sorting was rather poor and needed to be improved. With the use of dual lasers that based the separation on two measurements, this problem was addressed fairly successfully (Van Dilla *et al*, 1986). In this case, as mentioned earlier, two DNA binding dyes are used: one dye binds to the DNA backbone non specifically while for the second dye binding is based on the base pair (AT:GC) ratio. With the introduction of the second dye, the basis for separation is no longer only size dependent; it is also dependent on the composition of bases of the individual chromosomes. As the two fluorescent dyes can be chosen to have different fluorescent properties, sorting is processed by a coincident measurement of the two

events through bivariate analysis. This very feature, ability to make different measurements simultaneously through bi- and multivariate analysis, is the most appealing advantage in flow cytometric analysis as it can improve the specificity tremendously, and enables one to look at multiple constituents simultaneously, making it feasible to quantify different components within subpopulations.

The general principle behind flow sorting is shown in Fig 4.1. The analytes in a sample are pre-treated with fluorescent markers prior to running through the flow cytometer. The analytes in a sample traveling through a tube with a viscous drag on the boundaries created by the surrounding sheath fluid, are hydrodynamically focused into the coaxial plane (Fig 4.2). This results in the particles serially intercepting the laser beam individually, in a stream of fluid, generating different types of data such as fluorescence, diffraction, refraction, reflection and absorption based on their properties. Comparing the fluorescence and scatter data measured with the user-defined criteria for sorting, a decision is made as to whether a particular particle will be sorted or not. Based on this decision, the particles that are suitable for sorting, are given a charge (positive or negative) at the break off point. The charged droplets carrying an analyte go through a pair of oppositely charged high voltage plates (3000V). As the plates are also charged positively and negatively, the charged droplets are attracted to the plate of opposite polarity. An alternative to this electrostatic sorting is mechanical sorting in which case, interception of the laser to a hydrodynamically focused sample takes place inside a flow cell. When a particle of interest is encountered, a tube moves into the stream to collect the particles.

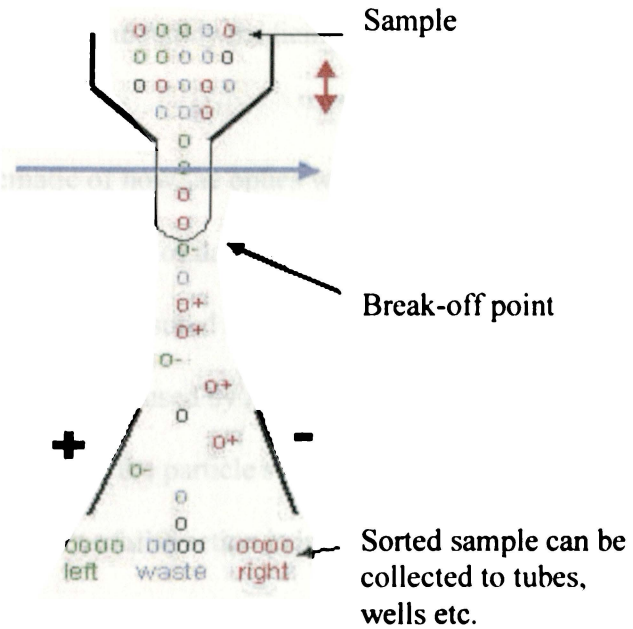


Fig 4.1: General Principle behind flow sorting
Reprinted with permission from Dr.Terry Hoy, Department of Haematology, University of Wales College of Medicine (http://www.uwcm.ac.uk/study/medicine/haematology/cytonetuk/introduction_to_fcm/cell_sorting.htm)

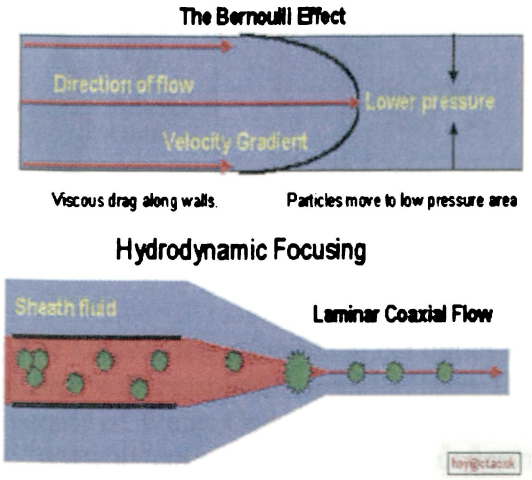


Fig 4.2: Hydrodynamic focusing of the fluid sample in a flow cytometer
Reprinted with permission from Dr.Terry Hoy, Department of Haematology, University of Wales College of Medicine

(http://www.uwcm.ac.uk/study/medicine/haematology/cytonetuk/introduction_to_fcm/fluidics.htm)

A schematic of how the optics work in a flow cytometer is shown in Fig 4.3. Out of the different forms of data generated by the interaction of the particle with the laser beam, scatter is measured at high angle (90^0) and low angle (10^0-15^0). High angle scatter, which is caused by reflection and refraction is referred to as side scatter (SSC), is a measure of the particle structure. Low angle scatter or Forward scatter (FSC) that is a result of diffraction is indicative of the cell size. Fluorescence is measured orthogonal to the incident laser beam and is filtered through the appropriate band pass filters and the signal multiplied by photomultiplier tubes.

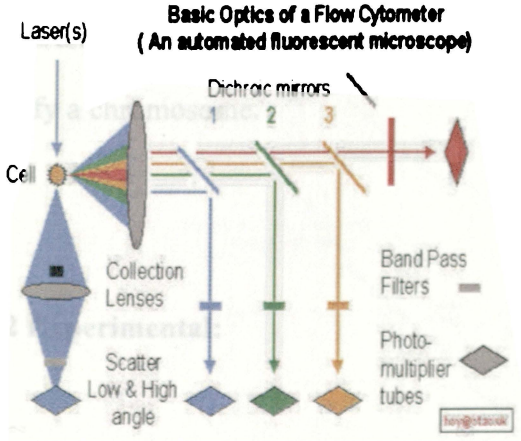


Fig 4.3: Underlying principle of the optics in a flow cytometer
Reprinted with permission from Dr.Terry Hoy, Department of Haematology, University of Wales College of Medicine
http://www.uwcm.ac.uk/study/medicine/haematology/cytonetuk/introduction_to_fcm/optics.htm

In this novel approach, a chromosome specific fluorescently labeled DNA probe will replace the fluorescent DNA binding dyes in the standard method. The main purpose is to demonstrate the ability to sort individual chromosomes by this method using flow cytometry so that a similar approach can be extended to the existing problem areas of overlap between individual chromosomes. The feasibility of this novel approach will enable the sorting process of any chromosome with the use of only one laser. This is advantageous due to the high cost of the dual laser beam systems employed currently including a UV laser, for bivariate flow sorting of chromosomes. With the availability of a new generation of benchtop flow cytometers that operate with a single laser beam in the visible range, sorting chromosomes by flow cytometry will become a fairly inexpensive process that can be carried out in any laboratory, by applying this developed methodology of using a specific probe to identify a chromosome.

4.I.2 Experimental:

Cell culturing, extraction of metaphase chromosomes and labeling of chromosome 15 with the fluorescently labeled DNA probe is performed as described in sections 4.II.2 (i) through 4.II.2 (iii). The specific probe used in this case is a Fluorescein isothiocyanate labeled (FITC) probe that targets the D15Z1 region of the

centromere of chromosome 15 (catalogue number: LPE015G, manufactured by Cytocell, UK; US distributors, Rainbow Scientific).

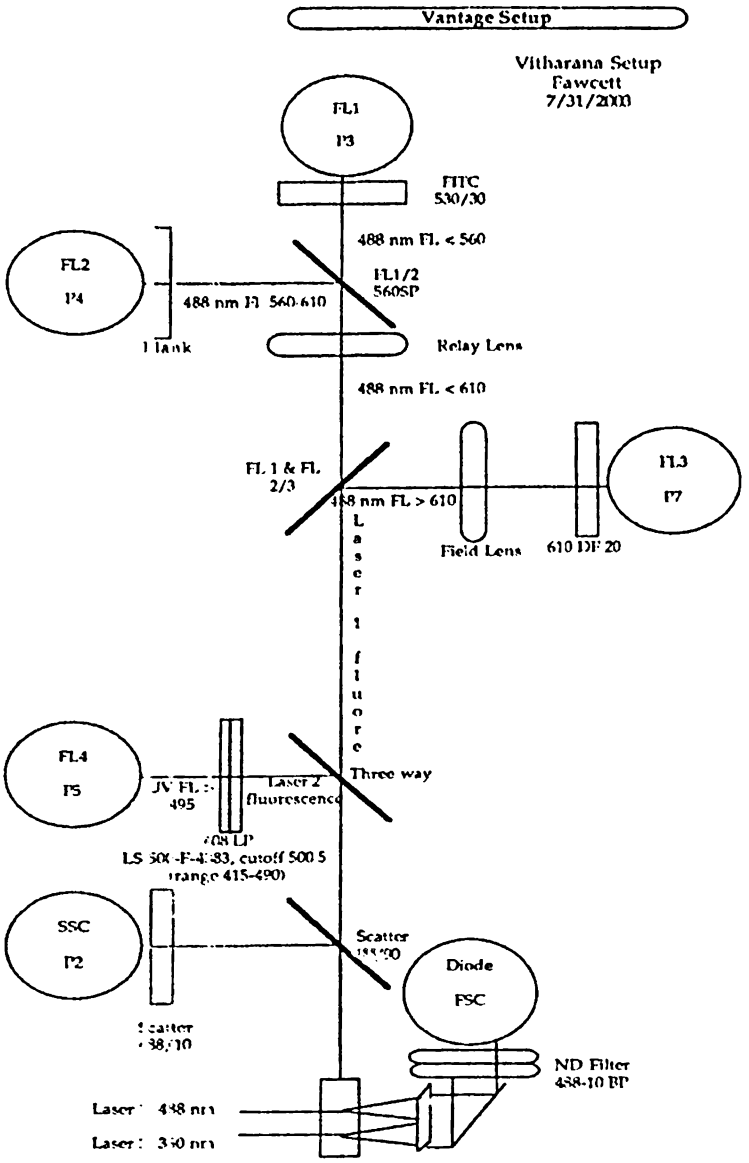


Fig 4.4: Vantage set up used for flow sorting of chromosomes by John Fawcett, at the Bio Sciences Division, Los Alamos National Flow Cytometry Resource, Los Alamos National Laboratories, NM.

The chromosome preparations for the analysis in part I was performed by Carolyn Bell with GM130 cell lines at the Bio Sciences Division, Los Alamos National Flow Cytometry Resource, Los Alamos National Laboratories, NM.

Fig 4.4 shows the Vantage set up used for the sorting of labeled chromosomes. Laser used for sorting is the 488 nm laser, forward and side scatter measured at 8^{o} and 90^{o} respectively. FITC fluorescence due to the label on the chromosome is measured with a 530 nm filter. Prior to running the samples, the cytometer is calibrated with non-fluorescent Duke scientific polyvinyltoluene latex microspheres (2.020 um) and fluorescent (FITC) polysciences Yellow-Green (YG) beads (1.75 um). After calibration, the labeled chromosome sample is run through the system and the following measurements are being made: fluorescence at 530nm corresponding to FITC, and non-fluorescent measurements, FSC and SSC. Filters FL-2, FL-3 and FL-4 shown in Fig 4.5 are not used in the sorting process because only one type of fluorescent labeling, FITC is used in this method. Therefore, only FL1-H fluorescence was measured.

4.I.3 Results & Discussion:

Figures 4.5 & 4.6 show the data obtained with fluorescent YG beads used for calibrating the flow cytometer. A majority of the beads give an FSC between channels 200 and 400 and an SSC between channels 300 and 500 (Fig 4.5). The FITC

fluorescence is between channels 575 and 625 (Fig 4.6).This calibration data generated by the YG beads helps in defining the gating windows for fluorescence, in the analysis of chromosomes. As a control experiment, unlabeled, fixed chromosomes were analyzed (Data shown in Fig 4.7 & 4.8). As indicated in Fig 4.7 the unlabeled chromosomes show an FSC and an SSC below channel 200. Low FSC of chromosomes indicate their smaller particle size compared to the calibrating beads. There was no FITC fluorescence observed in this sample, except for a very few events around channel 600 corresponding to the fluorescence given off by the residual YG beads left in the instrument (Fig 4.8). This is the anticipated outcome for this sample because the chromosomes are not labeled with the fluorescent DNA tag.

Fig 4.9 and 4.10 show the side scatter and the forward scatter data obtained for the entire population of chromosomes, which includes a small fraction of labeled chromosomes, respectively. Forward scatter (Fig 4.10) that is indicative of the particle size shows a large number of events between channels, 0 and 220, representing individual chromosomes as in the previous unlabeled sample (Fig 4.7). The small number of larger particles beyond channel 220 may correspond to clumps of chromosomes or nuclei present in the sample.

Fig 4.11 is a bivariate dot plot between the Forward Scatter Height (FSC-H) and the Side Scatter Height (SSC-H) of the entire chromosome population including the labeled chromosomes. The bivariate dot-plot for the PVT calibrating beads show them appearing beyond channel 800 for the SSC-H and between 250-400 on FSC-H (Data not shown). Based on the SSC-H value, it has been decided that chromosomes

would appear below channels 725 on SSC. Ideally, the boundary on FSC should have been chosen to be below 250. However, this has been chosen as 600, which results in the inclusion of larger events than chromosomes such as chromosome clumps. Should this boundary be at channel 250, only the individual chromosomes would be included. By having a larger window, it introduces contamination, which affects the analysis rate. Fluorescence of FITC has been measured in the R1 population as shown in Fig 4.13; corresponding FSC data is shown in Fig 4.12. The bivariate dot plot of the combination of Fig 4.12 and 4.13 is shown in Fig 4.14. The gated R2 window is decided to contain the fluorescently labeled chromosome population. The upper boundary of this window is decided based on the fluorescence of the YG beads used for calibration, which appear above channel 550 on FL-1 filter. The lower boundary of R2 is based on the total number of events in this window, which is 1.1%, a realistic number for a single chromosome in an entire population of chromosomes. Theoretically, although this number should be 4.3% as there are two chromosome 15s for every 46 chromosomes, only about 1-2% of individual chromosomes for the same type can be identified when processed by flow cytometry, in general. This loss can be attributed to clump formation and sample loss during processing. The value for FSC could be narrowed down to channel 400 as in the case of Fig 4.11, based on the same argument. Events occurring in the R2 window were sorted and subjected for verification.

Characterization of the sorted chromosomes was carried out by taking an aliquot, spreading them on a microscopic slide and performing a Fluorescent *in situ*

Hybridization experiment with a FITC tagged probe that hybridizes to most of the chromosome 15 (paint probe) as described in section 4.II.2 (vi).

Fig 4.15 (A&B) shows the control experiment performed with an aliquot of chromosome suspension, right after extracting from cells reacted with a paint probe specific for chromosome 15. The percentage of chromosomes painted is about 6%, calculated as follows: [painted (with FITC)/total number of chromosomes (DAPI stained)] x 100. Theoretically, this should be about 4.35% as there are 2 chromosome 15s for every 46 chromosomes. However, as this is a random sample, the figure obtained is not unreasonable for all the chromosomes are all mixed up at this point. Fig 4.16 shows some flow sorted chromosomes subjected to hybridization with a chromosome 15 specific paint probe on a slide stained with DAPI and the corresponding image taken with a FITC filter is shown in fig 4.17. Flow sorted individual chromosomes are shown in Fig 4.12 with the DAPI stain and the paint probe reacted chromosomes are shown in left and right, respectively. By scanning the slide and counting the number of chromosomes painted (under FITC filter) out of the total number of chromosomes (DAPI filter) the enrichment was determined to be about 92%, meaning 92% of the chromosomes sorted, have reacted with the paint probe. This number was arrived at, by counting a total of 114 chromosomes.

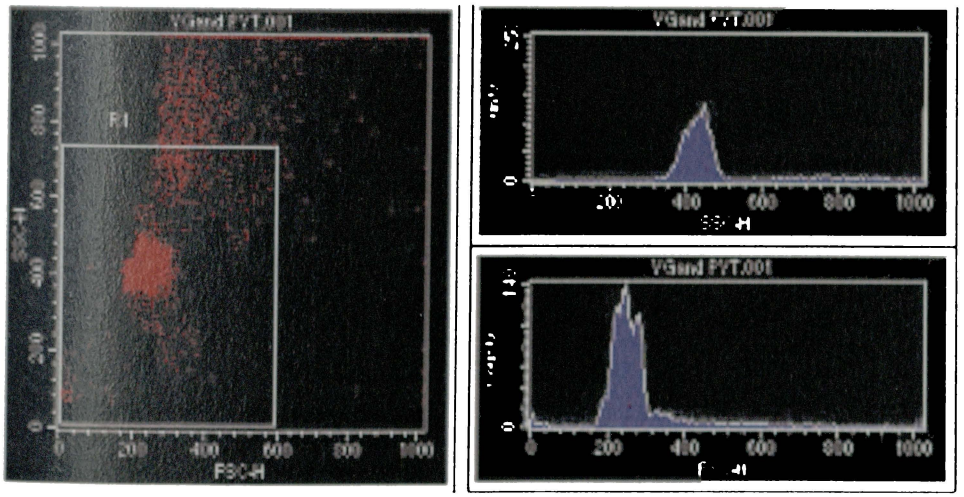


Fig 4.5: Calibration data produced by YG beads. Top right and lower right show the individual frequency distribution plots for FSC and SSC plotted against the number of events on y-axis. The bivariate dot-plot on the left (SSC vs FSC) shows the population of YG beads in window R1.

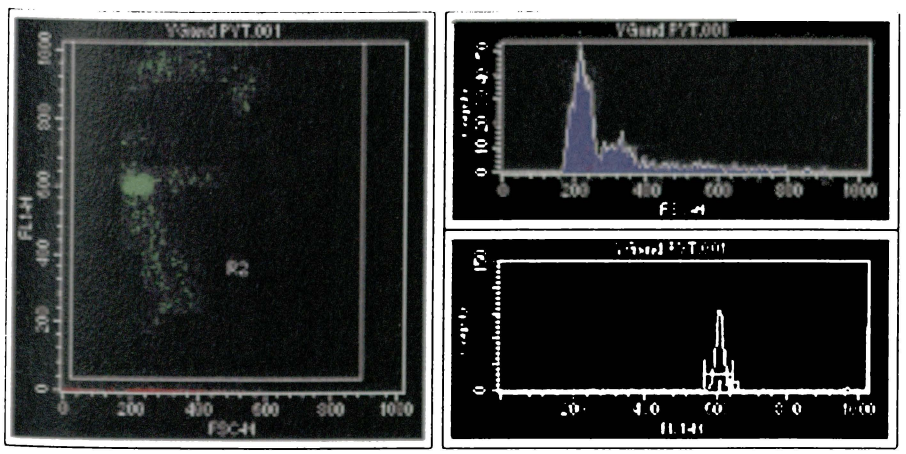


Fig 4.6: Frequency distribution plots for fluorescence (Lower right) and FSC (Upper right) vs the number of events produced by YG beads. The correlation between the two events, is shown in the bivariate dot plot (FL1-H vs FSC-H) on the left.

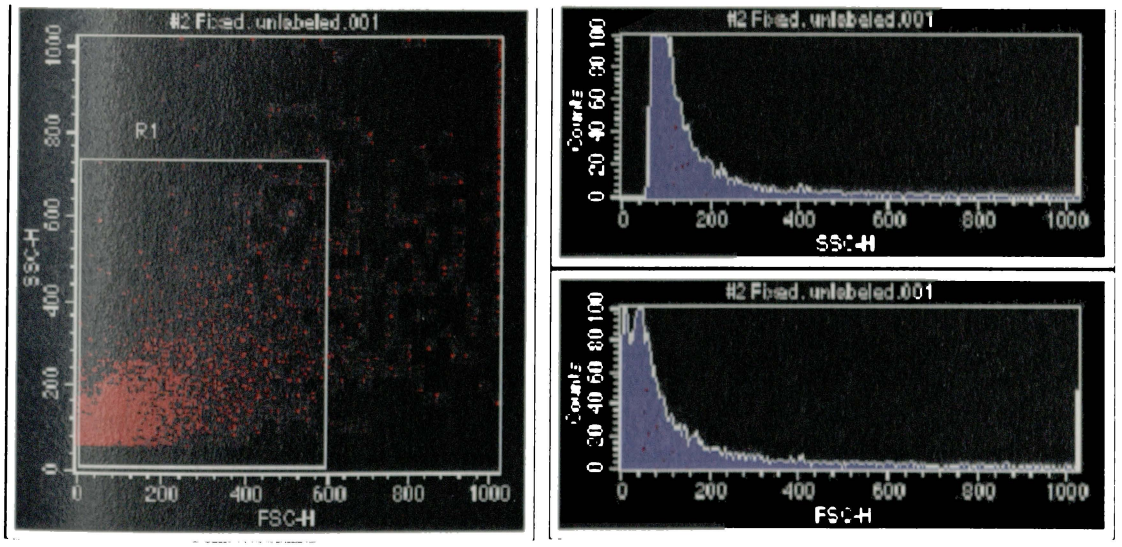


Fig 4.7: FSC (lower right) and SSC (upper right) plotted against the number of events, for an unlabeled sample of chromosomes. The bivariate dot plot for FSC vs. SSC is shown on the left.

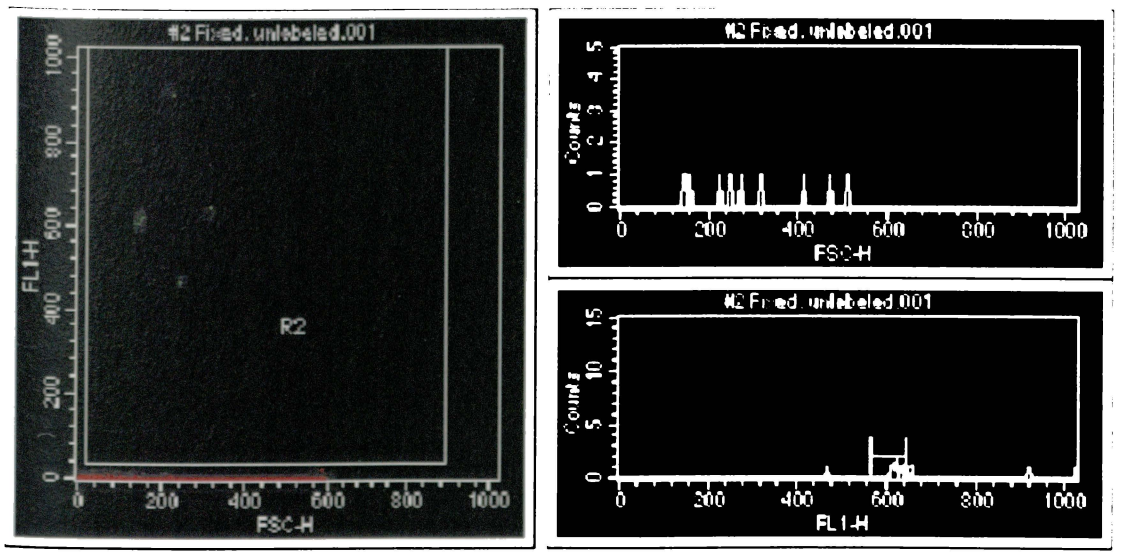


Fig 4.8: Fluorescence (lower right) and FSC (upper right) against the number of events for the unlabeled chromosomes. The fluorescence around channel 600 is due to some residual YG beads in the system. The bivariate dot-plot correlating fluorescence (FL1-H) and FSC is shown on left.

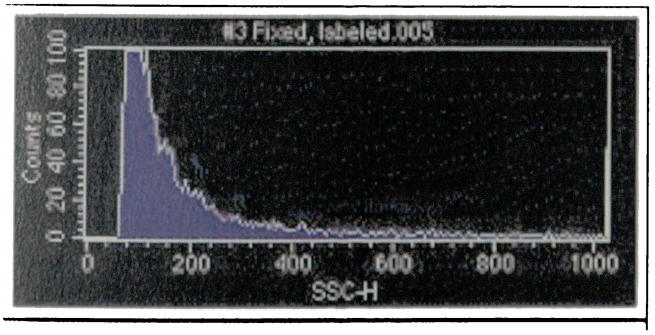


Fig 4.9: Acquisition histogram plot of SSC vs counts or number of events obtained for the entire population of chromosomes that include labeled chromosomes with a FITC tagged DNA probe

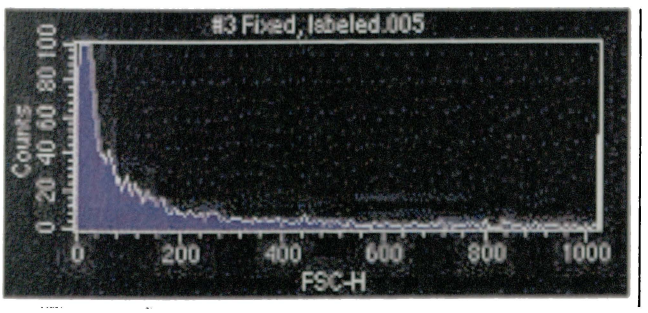


Fig 4.10: Acquisition histogram plot of FSC vs counts or number of events obtained for the sample containing the entire population of chromosomes that include labeled chromosomes with a FITC tagged DNA probe

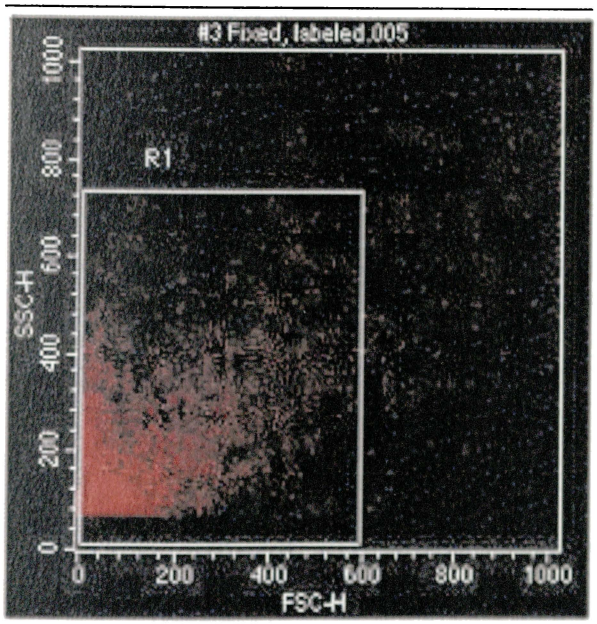


Fig 4.11: Acquisition dot plot of the two histograms shown in 4.9 and 4.10, correlating SSC and FSC for the labeled chromosome sample.

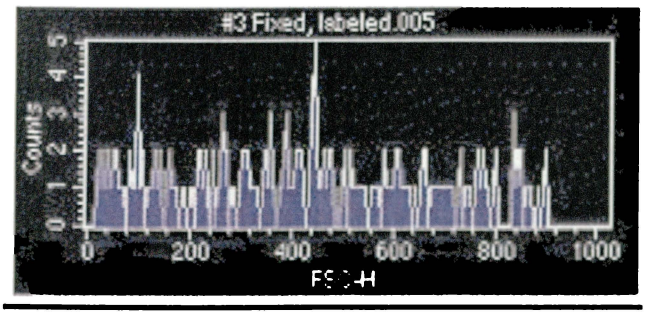


Fig 4.12: Acquisition histogram of FSC vs counts or number of events obtained for the gated population of sample shown as R1 window in Fig 4.11

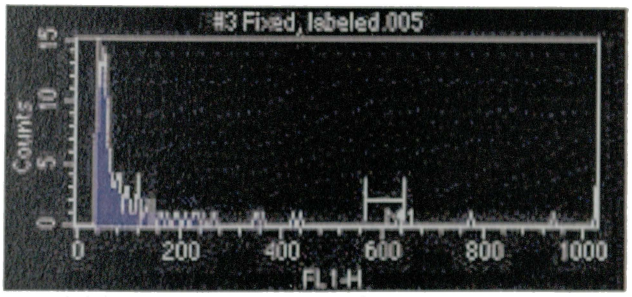


Fig 4.13: Acquisition histogram plot of FL1-H (FITC fluorescence) vs counts or number of events obtained for the gated population of sample shown as R1 window in Fig 4.11

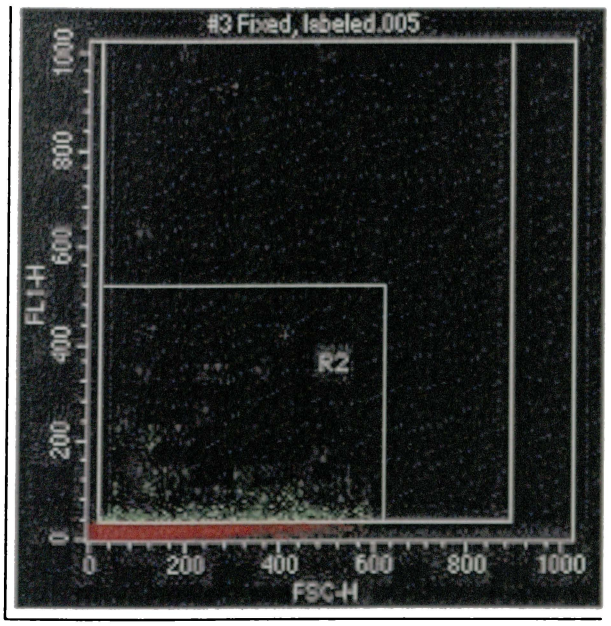
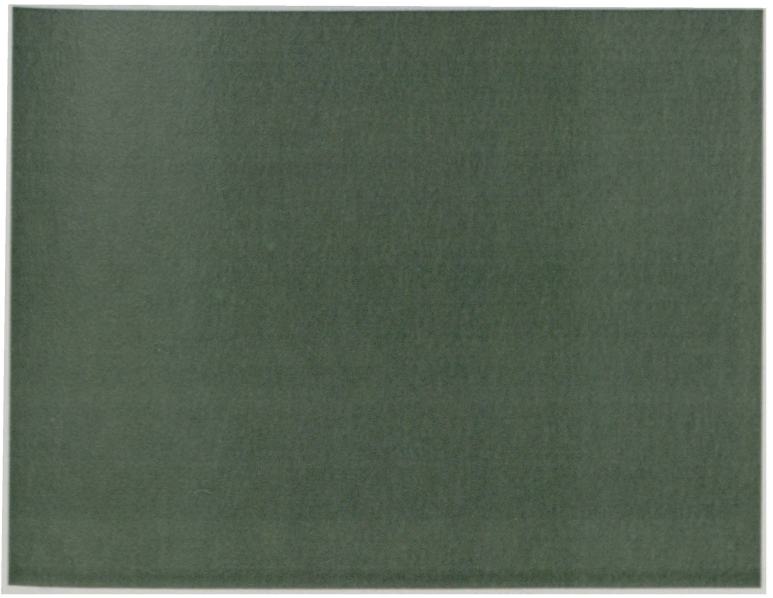


Fig 4.14: Acquisition dot plot of the two histograms shown in Fig 4.9 and 4.10 Window R2 shows the population gated for sorting.

4.15 (A): An aliquot of chromosome suspension right after extraction from cells, prior to fractionation subjected to FISH with paint probe for characterization. The chromosomes are stained with DAPI for identification 40X magnification



4.15 (B): An aliquot of chromosome suspension right after extraction from cells, prior to fractionation subjected to FISH with paint probe for characterization. The chromosomes are stained with DAPI for identification 40X magnification

Fig 4.16: DAPI stained flow sorted chromosomes (subjected to verification with chromosome 15 paint probe) under 360nm filter (63X magnification)

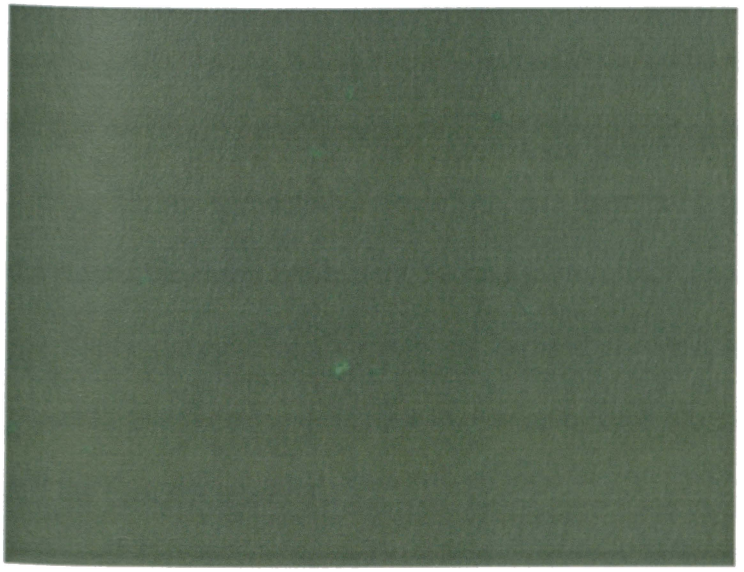


Fig 4.17: Flow sorted chromosomes subjected for verification by reacting with chromosome 15 specific FITC tagged paint probe under 530 nm filter (63X magnification)

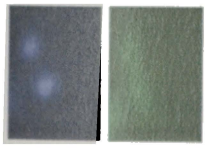


Fig 4.18: Flow sorted individual chromosomes. On left is the DAPI stained image of chromosome and on right is the painted chromosome with chromosome 15 specific paint probe, FITC labeled. (100 X magnification)

4.I.4 Conclusion:

First of all, this demonstrates the feasibility of using a fluorescently labeled DNA specific probe to sort chromosomes, instead of the current method employing two fluorescent dyes, which require two lasers including a UV laser making the sorting process extremely expensive. The developed methodology can be extended to sort any chromosome with a single laser and therefore can be done with the newly developed benchtop cytometers. Secondly, since this is a way of improving specificity, the developed strategy can be used in currently existing unresolvable populations of chromosomes due to overlap of each other due to the similarity of size and the base composition.

This work was supported by and the Los Alamos National Flow Cytometry Resource funded by the National Center for Research Resources of NIH (Grant P41-RR01315).

Part II: Fractionating chromosomes with Magnetic Beads

4.II.1 Introduction

The main objective of the strategy described below is to overcome the two major shortcomings associated with sorting chromosomes by flow cytometry. In this novel approach using magnetic beads, the chromosomes are sorted in a massively parallel way, as opposed to sorting in series by flow cytometry. Consequently, the time required to get a large quantity of material for any type of analysis is minimal. It is important to emphasize that the isolation time does not depend on the number of cells that need to be sorted as the experiment is running in parallel. Within a matter of about 3-4 days the complete procedure developed with the magnetic beads can be performed and a fractionated pool of the chromosome of interest can be obtained. Therefore, this is a practical solution to the severe problem of not being able to obtain sufficient material in a realistic time frame. Secondly, by the use of a specific probe to identify the chromosome of interest, the non-specificity of the standard method may be eliminated.

Chromosome fractionation may be performed in several ways other than flow cytometry. Laser Capture Microdissection (LCM) is one technique that could be used to separate a chromosome of interest. Labeling the chromosomes immobilized on a microscopic slide with a specific fluorescent DNA probe will enable selection of the fluorescently marked events by LCM into an appropriate container. However, as the selection process is done manually in a serial manner, this strategy is time consuming. The second approach involves an affinity-based separation in which the chromosome

of interest, recognized by a specific probe, is fractionated using magnetic particles. In both instances where this strategy has been adopted in the literature to isolate chromosomes, somatic hybrids such as human-hamster cell lines, in which case a single human chromosome is inserted to a hamster cell line, have been used (Dudin *et al*,1988 & Kausch *et al*, 1993). This insertion and subsequent growth in a different cell line may cause major changes in the regulatory functions of human chromatin. Thus, this would not be an appropriate method to pursue if the objective is to investigate the native state of human chromatin. Extraction and isolation of a specific human chromosome from an unperturbed system (cell line) is therefore pursued in this work.

The probe chosen to mark the chromosome of interest targets the D15Z1 genomic locus in the centromeric region of chromosome 15. The main reason why this probe was chosen over another specific probe is because blocking of the centromeric region of the chromosome does not interfere with further analysis of the chromosomal DNA and histones, eliminating a complication associated with the removal of the hybridized probe if it binds to some other region of the chromosome. It has been reported that probes generated against this region are mapping to the short arm of chromosome 14 (Stergianou et al 1991, Smeets *et al*, 1991). However, those results were based on studies done on a very small sample population and were seen on different cell lines than what was used in this work. Due to this controversy, this issue has been particularly addressed by characterizing the probe against the GM01056C cells worked with, and is discussed later.

The magnetic bead approach takes advantage of the specificity of the centromere probe. Mitotic chromosomes are extracted by blocking cells at metaphase by the same procedure as for flow sorting of chromosomes. Next, the chromosomes in suspension are reacted with chromosome 15-specific, centromere probes with a FITC label (Cytocell, UK), to mark the chromosome of interest that needs to be sorted. An anti-FITC antibody is covalently coupled to a magnetic bead (Bangs Labs) via a homo-bifunctional cross-linker, sulfo-EGS (Pierce), so that the antibody will recognize the chromosome marked with the centromere probe. Chromosome 15 can be isolated by exposure to a magnetic field and the crosslinker can be cleaved thereafter. A schematic of the entire fractionation process is outlined in Fig 4.19.

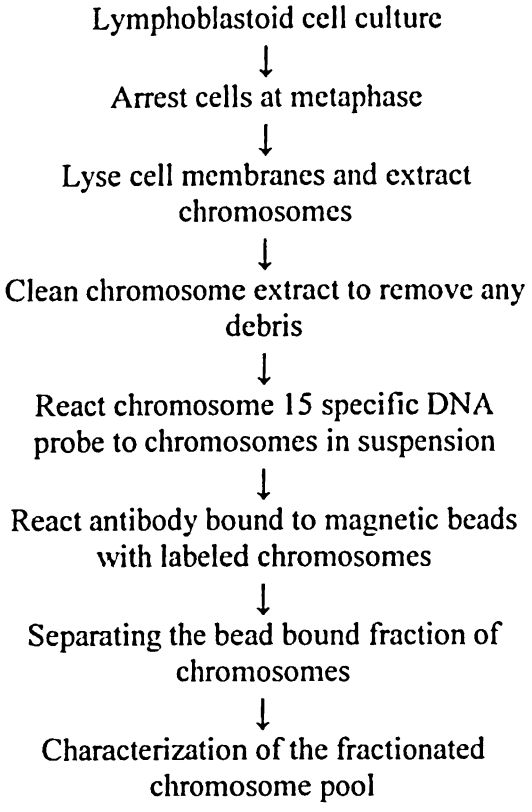


Fig 4.19: Flow chart indicating the steps involved in the procedure adopted for isolation of a specific chromosome

In order to determine if a homogeneous pool of chromosomes has been obtained, an aliquot of the isolated pool is spun onto a glass slide, or smeared on a microscopic slide and characterized with a FISH probe that paints chromosome 15 specifically. A control experiment was performed with an aliquot prior to isolation.

Other than mapping chromatin structure, the isolated pool of chromosomes can also be used for making extended chromatin fibers, which can then be used for high resolution physical mapping of the genome and to investigate chromosomal abnormalities such as translocations or gene organization in general. Interestingly, such extended chromatin fibers can also be used to gather a qualitative picture of PTMs of histones in a specific genomic locus, by performing immunofluorescence on them to identify a particular modification and FISH to identify the location. Using chromatin fibers will improve the spatial resolution significantly compared to this experiment being performed on metaphase chromosomes as shown in Chapter 3. Another potential application of FISH performed on fibers is to clip a certain region of the genome using a technique such as Laser Capture Microdissection (LCM), once it is identified by a fluorescent DNA probe as discussed in Chapter 5. Extended chromatin fibers that can be used for various applications such as the above are derived in various ways: lymphocytes separated from blood or cultured cells are two good sources for making chromatin fibers. However, this technology tends to be highly non-reproducible and not so robust. This could be attributed to the presence of a large amount of extraneous material in the cells from which, the fibers are derived. In addition, to make matters worse, a tremendous number of slides need to be

prepared to locate a certain genomic interval, as the probability of finding the particular region is very small due to the presence of the entire genome. Mann and co-workers have tried to address the first issue and to improve the robustness by making fibers from metaphase chromosomes extracted in polyamine buffer (Mann *et al*, 1997). They have demonstrated the feasibility of performing FISH on fibers derived from a pool of metaphase chromosomes. The second problem associated with the current fiber methodology can be addressed by starting off with an enriched pool of chromosomes. Therefore, the feasibility of making fibers from the fractionated pool of chromosomes is demonstrated.

Another important application of isolated pools of chromosomes is to generate chromosome specific libraries in cloning vectors. For this specific purpose, about 4-20 million chromosomes are needed (Fawcett *et al*, 1994), whereas certain other cytogenetic or molecular biological applications, such as preparation of paint probes specific to chromosomes, may require only a few hundred chromosomes. When it comes to large scale sorting, flow sorting of chromosomes becomes a highly time consuming process, as pointed out earlier. Thus, isolation of specific chromosomes in this massively parallel manner is advantageous for many applications other than for investigation of chromatin structure. In order to characterize the isolated pool of chromosomes to determine the enrichment of the fractionation process by magnetic beads, a commercially available fluorescent probe specific for chromosome 15 that hybridizes to the entire chromosome is reacted with an aliquot of the fractionated pool.

4.II.2 Experimental:

4.II.2.1. Buffers and Solutions:

Buffer or Solution	Composition
Ohnukis hypotonic solution	55 mM $NaNO_3$ 5 mL, 55 mM CH_3COONa 2 mL, 55 mM KCl 10 mL
Chromosome Isolation Buffer (polyamine)	5 mL of stock A and stock B + 40 mL nanopure water; pH 7.2 50 µL of β-mercaptoethanol. To 25 mL of the above 30 mg digitonin added & incubated for 40 min at 37^0C. Sterile filter and 12.5 µL each of 0.4 M spermine and 1.0 M spermidine added
Stock A	150 mM Tris-HCl, 20 mM EDTA, 800 mM KCl, 200 mM NaCl in 100 mL H_2O
Stock B	5 mM EGTA in 100 mL H_2O
1X SSC*	0.15 M NaCl, 0.015 M sodium citrate
IB+M solution	50 mM KCl, 5mM Hepes, 10 mM $MgSO_4$, pH 8.0
Phosphate Buffered Saline	137 mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.7H_2O$, 1.4mM KH_2PO_4, pH ~7.3
Denhardt's solution (100X)	for 500 ml: 10 g Ficoll 400; 10 g polyvinylpyrrolidone MW 360000; 10 g BSA fraction V; H_2O
Detergent Buffer	10mM Tris, 100mM EDTA, 0.5% SDS, pH 7.5
Denaturation Solution (for fiber FISH)	1.5 M NaCl, 0.5M NaOH
Neutralization Solution	0.5M Tris-HCl, 3M NaCl, pH 7.2

* 0.1X, 2X and 4X SSC are prepared the same way, but with the appropriate amounts of reagents. Eg: 0.1XSSC is a 10 times more dilute solution than 1X SSC.

4.II.2.2. Procedures:

(i) Cell Culture:

An apparently healthy GM01056C lymphoblastoid cell line (NIGMS cell repository) was cultured with 10% FBS (Irvine Scientific), 1% L-Glutamine

(GIBCO) and 1% Penicillin-Streptomycin (GIBCO) in RPMI 1640 medium (Irvine Scientific). The cells were fed once every 2 to 3 days depending on the cell cycle.

(ii) Extracting Mitotic Chromosomes (Polyamine Procedure)

Cells are blocked at metaphase by adding 0.1 ug of colcemid per mL of GM01056C lymphoblastoid cell culture for 12-13 hours. Cells are pooled on ice and counted. Cells aliquotted into 15 or 50 mL polypropylene centrifuge tubes aiming for a final concentration of 6-14 x 10^6 mitotic cells/tube. The number of mitotic cells is calculated assuming it is 30% of the total cell count. The cells are centrifuged between 800 and1000 rpm for 8 min at 2^o C. Supernatant is aspirated and the cell pellet flicked. Immediately prior to adding the hypotonic solution to swell the cells, 12.5 uL of spermine and spermidine each, is added to 25 uL of Ohnukis hypotonic solution. The volume of hypotonic added is dependent on the concentration of the cells; usually the volume ranges from 2.5 mL-5.5mL per 6-14 x 10^6 cells. Cells are allowed to swell for 70-90 min at room temperature and then centrifuged at 1000 rpm for 5 min at room temperature. Supernatant is aspirated and the cell pellet flicked. A volume of 1.0 mL of chromosome isolation buffer is added to each tube and then flicked. Each tube is vortexed vigorously for 15 sec and then placed on ice for at least 5 minutes. An aliquot of the suspension is examined under fluorescence by staining with 4',6-Diamidino-2-phenylindole (DAPI) or propidium iodide (PI), which are both DNA intercalating dyes. Until mitotic chromosomes are released by a majority of

cells, the suspension is vortexed for 45-90 seconds in total, in 15 sec increments. However, it is important to note that too much vortexing makes the longer chromosomes appear stringy and thus, it was avoided.

(iii) Labeling Chromosome 15 by FISH in suspension

Isolated chromosomes were centrifuged at 50 g for 3 min and the supernatant containing the chromosomes was transferred to a separate centrifuge tube. It was re-centrifuged at 50 g for another 3 min and the supernatant removed into a fresh centrifuge tube in order to get rid of most of the nuclei and cell debris present in the chromosome extract. To further purify the chromosomes, the suspension was drawn into a 1cc or 3 cc syringe through a 18G x 1 1/2" disposable needle, and then passed through a sterilized nylon mesh (Small Parts Inc,) wedged between the syringe and a fresh 18G x 1 1/2" needle.

The extracted chromosomes are then counted by staining with a DNA binding dye such as DAPI observing under fluorescence to determine the concentration. 1 uL of a chromosome suspension with a 10^6 chromosomes/mL sample is diluted in a total of 1 mL of polyamine buffer and fixed by adding 20 drops of 3:1 methanol: acetic acid fixative while gently swirling the suspension. Chromosomes were then centrifuged at 350 g for 10 min and the supernatant removed. A volume of 1 mL of the same fixative is added to the flicked pellet while vortexing the tube at the lowest setting and incubated at room temperature for 30 minutes. After centrifugation

on the same conditions, the supernatant was removed and 1 mL of fresh fixative was added. After centrifugation and removal of the supernatant, the chromosome pellet was resuspended in 160 uL of pre-warmed hybridization buffer (40% deionized formamide, 4 x SSC, 2x Denhardt's solution). A volume of 2.5 uL of pre-warmed alpha-satellite centromeric probe for chromosome 15 (Cytocell) was added to the chromosome suspension and the entire mixture was denatured for 3.5 min 73 ^{0}C. Then, it was kept on ice for 5 min and incubated for 2 1/2 hours at 37 ^{0}C in a shaking water bath. The labeled chromosome pellet was obtained by centrifuging and resuspended in 500 uL of prewarmed 0.1X SSC. Chromosomes were centrifuged and the pellet was resuspended in 2X SSC. An aliquot of 15 uL is stained with DAPI to make sure the labeling has taken place.

(iv) Linking Anti-FITC Antibody to magnetic beads

An amount of 8 x 10^{-13} mol each of rabbit anti-FITC antibody and magnetic beads in PBS (pH 7.5) was reacted with a 10 fold molar excess of cross linker, sulfo-EGS dissolved in nanopure water for 30 min at room temperature. The reaction is quenched with a volume of 1 M Tris (pH 7.5) for 15 min so that the final concentration of Tris is 20-50 mM. The reaction mixture is incubated for an additional 15 min. All the magnetic beads, reacted and not are collected by a magnet.

(v) Bead binding, Separation of bead bound fraction and cleavage of the bead

An amount of 1 x 10^6 anti-FITC antibody linked magnetic beads are suspended with 1 x 10^6 probe bound chromosomes in 100 uL of IB + M buffer containing 0.05% (v/v) Tween 20 and 5 % (v/v) non-fat dry milk. The suspension is incubated for 2 hours at 37 ^{0}C in a shaking water bath. The suspension is then transferred to a square cuvette and the bead bound fraction is collected by exposing one side of the cuvette to a bar magnet.

When needed the beads were cleaved by incubating equal volumes of sample and pre-warmed 2.0 M hydroxylamine.HCl solution (pH 8.5) at 37^{0}C with stirring for 4 hours. The hydroxylamine.HCl solution is prepared by adding hydroxylamine.HCl to a phosphate buffer of pH 8.5 and then raising the pH back up to 8.5. The cleaved beads can be collected by exposure to a magnetic field and the supernatant contains the cleaved chromosomes.

The entire bead containing entity is displaced from the chromosome by reacting the bead bound separated chromosome fraction with a 10000 molar excess of free fuorescein dye molecules for 40 min at room temperature, thus displacing the chromosomes and then collecting the supernatant containing free chromosomes upon exposure to a magnetic field, when required. Excess free-fluorescein can be removed by centrifuging the sample at 350 g and aspirating the supernatant to collect the centrifuged pellet, which contains chromosomes.

(vi) Paint FISH with commercial probe on isolated pool for verification

Fractionated pool of chromosomes is spread on to microscopic slides by two methods: cytocentrifugating 100 uL of the fractionated pool on a Shandon Cytospin for about 10 min at 7.5 speed or about 5 uL smeared on a glass slide. In the case of cytocentrifugation, an absorbent filter card is placed between the funnel containing the cell suspension and the glass slide, to absorb the excess liquid. The cytospun chromosomes are allowed to dry briefly in air and then transferred for 5 min to a coplin jar containing water. Next they are fixed in 3:1 methanol: acetic acid solution for 20 min at room temperature.

The paint FISH experiment is performed with a commercially available directly labeled paint probe specific for chromosome 15 (Vysis, Inc.) as follows. (This is a different probe than what is used for labeling the chromosomes by fractionation) Chromosome containing slides are denatured for 5 minutes in the denaturation solution at 73 ± 1 ^{0}C. Then slides are dehydrated by immersing in a series of 70%, 85% and 100% ETOH for 1 minute, each. Slides are then air dried and warmed between 45-50 ^{0} C to evaporate any remaining EtOH. The probe mixture is prepared by mixing 1 uL of probe, 7 uL of hybridization buffer, 2uL of H_2O to make the total volume 10 uL. The mixture is centrifuged for 1-3 seconds, vortexed and then centrifuged again. The tube containing the probe mixture is placed in a 73 ± 1 ^{o}C water bath for 5 min. The tube is then removed from the water bath and placed at 45-50 ^{0} C, until the probe is applied on to the slide. When both slides and probe are ready, 10 uL of probe mixture is applied to target DNA, placed a coverslip on the

specific area and sealed with nail polish. The slides are then incubated for 4-16 hours at 37 °C (preferably overnight) in a prewarmed humidified chamber for hybridization. Post-hybridization washes consist of 2 different washes: firstly, in 0.4 X SSC at 73± 1 °C, agitating the slide containing jar for 1-3 sec and then leaving the slides in the jar for another 2 min. Secondly, the slides are washed in 2X SSC at room temperature for a period of 5 sec-60 sec, agitating only for the first couple of seconds. Slides are allowed to air dry and counterstained with 10 mL of 0.125ug/mL of DAPI, prior to visualization under the appropriate filter set under a fluorescence microscope.

(vii) Preparation of fibers from an isolated pool of chromosomes:

A volume of 10 uL of the isolated pool of chromosomes is placed on one end of the slide and an equal volume of detergent buffer is added to the drop and mixed gently. The liquid drop is then spread on a line across the slide and tilted at an angle of 30^0 to unravel and extend the chromatin fibers. The slide is allowed to air dry and is then incubated in 3:1 methanol:acetic acid fixative for 20 min to fix them on to the slide. The slide is then stained with 10 uL of DAPI (100ug/mL) for about 30 min, mounted with anti-fade and detected under a fluorescence microscope.

4.II.3. Results and Discussion:

The objective of this novel approach is to address the two major drawbacks of the current state-of-the-art methodology of sorting chromosomes. Firstly, lack of specificity due to the non-specific dyes are replaced with a chromosome specific probe to identify the chromosome of interest, which would improve the specificity. Secondly, doing the fractionation process in a massively parallel way, rather than in series, would shorten the time of analysis tremendously.

GM01056C lymphoblastoid cell culture is chosen from the apparently healthy collection of cells at National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository, on the basis that if the fractionation could be demonstrated with this cell line, the methodology could be extended to any cell line of interest eventually.

The phase of the cell cycle at which individual chromosomes can be identified is metaphase. Chromosomes are in their most compact form in this stage of the cell cycle,

enabling them to be identified as individuals, which is key to isolation of a specific chromosome. In order to arrest a majority of the cells at metaphase so that the yield of mitotic chromosomes can be increased, they are exposed to chemicals such as colcemid that attack the microtubules and inhibit further cell division. Upon extended exposure to colcemid, which is about 12-13 hours for lymphoblastoids, chromosomes

get highly condensed and lose their native morphology, making it difficult to identify them. Unlike cytogenetic analysis that is entirely dependent on chromosomal morphology, this is not anticipated as a problem to achieve the expected goals.

Preparation of a clean chromosome extract of good quality from cultured cells is the most crucial factor in the entire process of isolation of a specific chromosome. The cell and nuclear membranes need to be lysed and the mitotic chromosomes then be extracted for the subsequent steps. Several problems have been encountered during extraction and maintaining mitotic chromosome preparations in the past (Dudin *et al*, 1988) The main problem has been the aggregation of chromosomes in the extract. This has been avoided by using two specific buffers in the literature: namely, $MgSO_4$ (Gray, 1989) and polyamine (Cram *et al*, 1990) as the buffer used to extract the chromosomes into, referred to as the chromosome isolation buffer. The polyamine method was adopted in this work mainly to preserve the total DNA content of the chromosomes as opposed to the $MgSO_4$ method, which increases the activity of endogenous nucleases due to the presence of divalent cations. In the polyamine procedure, spermine $(CH_2)_4[(NH_2(CH_2)_3NH_3)_2]^{4+}$ and spermidine $[H_3N(CH_2)_3NH_2(CH_2)_4NH_3]^{3+}$ which are linear, multivalent cationic polyamines are added to the buffer, to replace the divalent cations in the $MgSO_4$ protocol in order to stabilize the integrity of the chromosomes. Presence of polyamines in the extracting buffer tends to extract proteins such as High Mobility Group proteins (HMG), TATA binding proteins and transcription factors whereas the nucleosome array remains undisturbed (Wallrath *et al*, 1998). This assumption has been supported by

performing an immunofluorescence experiment with a primary anti-histone antibody for all acetylated isomers as shown in Fig 4.20, on intact chromosomes as well as extended chromatin fibers derived from chromosomes extracted in this manner. The secondary antibodies used to identify the primary antibodies carry an FITC fluorescent tag. Therefore, the signals seen with FITC labels indicate the presence of histones, confirming their integrity after exposure to the polyamine procedure.

In order to rupture the cell membranes for the extraction process, the cells need to be exposed to a hypotonic solution, preferably Ohnukis hypotonic for lymphoblastoid cell lines, which would swell the membranes. The amount of hypotonic solution used to enhance lysing of the membranes, turns out to be very critical. It is completely dependent on the number of mitotic cells present in the culture. For example, for a culture containing mitotic cells ranging from 6-14 x 10^6, the amount of hypotonic solution required varies from 2.5-5.5 mL. A total cell count is done and then the mitotic fraction is assumed to be 30% of the total cells. If too much hypotonic solution is used for the swelling of cells after the corresponding incubation period, the cells cannot be collected by centrifugation for the subsequent steps because the cell membranes rupture during the swelling step. On the other hand, using too little hypotonic solution will cause a drop in the efficiency of lysis and thereby extraction of chromosomes.

The swollen cells are then subjected to vortexing to break them open and release the chromosomes into the chromosome isolation buffer containing the polyamines. To reach this goal, applying a physical force by vortexing is carried out

for a brief period of time, 50-70 seconds in 15 second increments. One effect that was unanticipated at this point of the procedure is the extensive elongation of the chromosomes. They looked remarkably different, sizewise, that sometimes the question arose whether what is being observed is actually a chromosome. The lengths and the sizes seemed to vary depending on the vortexing strength used. Influence of the variation of conditions on the chromosome extracts is shown in Fig 4.21, where varying degrees of physical forces were applied to extract the chromosomes. It turned out that the greater the applied force, the longer the and more stretched the chromosomes appeared. This led to a brief glance at the biophysical properties of chromosomes consisting of chromatin fibers, which is a polymer of DNA and protein. Chromosomes or chromatin fibers are considered soft biophysical objects that can be reversibly and irreversibly changed depending on the extent of force applied. The elasticity of a material is expressed in terms of the elastic modulus, which indicates the amount of force per area that needs to be applied to elongate a material to double its length. For a mitotic chromosome the elastic modulus is about 500 Pa (Marko *et al*, 2003), which is considered a low number compared to substances such as agarose that has an elastic modulus of about 10000 Pa. This modulus gives an indication as to how strongly the polymer is linked internally within its monomer units. This means that chromosomes are somewhat loosely bound although the degree of compaction is extremely high. It has also been discovered that chromosomes can be extended 5 times their native length without causing any damage. Extensions beyond 5 times the native length are irreversible and any stretching beyond 30 times of native length

makes them look thicker as well due to "swelling" (Poirier, 2003). The force required for such extensions is only about 1 pN, (Bennink, 2001) and it has been demonstrated that histones are not affected by such extensions (Poirier, 2000).
This explained the somewhat unexpected observation made at the point of extraction.

The extracted chromosomes are coexisting with the lysed membranes and the remaining organelles of the cells, in suspension. For further analysis of this chromosome preparation it is important to get rid of all the other extraneous, unwanted cell debris from the extract. A specific protocol developed by the Bio Sciences Division, Los Alamos National Flow Cytometry Resource of Los Alamos National Laboratories (Fawcett, 1994) to remove most of the nuclei and other extraneous cellular debris was adopted. This procedure involves several centrifugation steps followed by filtering through a nylon mesh with 62 micron pores. However, it was not possible to completely eliminate all the nuclei in the extract. It is important to remember that the supernatant contains the chromosomes after the centrifugation steps at 50g, not the precipitated pellet, which contains the heavier nuclei and the other cell debris. Thus, supernatant should be preserved while the precipitate is discarded. An image of some extracted chromosomes that were subjected to this entire purification protocol under optimal conditions is shown in Fig 4.21 (C) as opposed to chromosomes present with nuclei and other debris in Fig 4.21(A)

Labeling of the specific chromosome to be isolated must be carried out in suspension to allow for the eventual magnetic bead separation. As the DNA probe

that binds specifically to the target chromosome is fluorescently labeled with FITC, probe binding to the chromosome of interest is a Fluorescence-*in situ*-Hybridization (FISH) experiment. Even though the FISH technique is widely used on solid phases such as glass slides, methodology for FISH in suspension was not fully developed until very recently. (He *et al*, 2001) The previous problems encountered due to clump formation and loss of chromosomes have been minimized in this procedure. Chromosome loss have been addressed by fixing the chromosomes in methanol : acetic acid (3:1) prior to exposing the chromosomes to the high temperatures of the FISH experiment and by omitting BSA and EDTA from the post-wash buffer. Clump formation has been minimized firstly by eliminating the use of 10% dextran sulfate, which increases the viscosity of the hybridization buffer and secondly, by decreasing the concentration of formamide from 70% to 40%. These revisions have resulted in preserving the morphology of the chromosomes and reducing sample loss through the procedure.

The probe used to label chromosome 15 targets the alpha-satellite III region in the 15 centromere, designated D15Z1. The company claims this probe to be specific to chromosome 15. However, there have been two reports in the past documenting mapping of a probe that targets this region to chromosome 14. In such a case three signals are seen, two of which are of intense brightness while the third signal binding to chromosome 14 produces a faint signal (Stergianou *et al*, 1991).To address this issue, the probe was characterized against metaphase spreads generated by the cell line used in this work, GM01056C on glass slides to perform solid phase FISH

experiments. They are performed both with the hybridization solution provided by Cytocell along with the centromere probe and also the hybridization solution that was prepared in the lab to carry out FISH in suspension. Results are shown in Fig 4.22 & 4.23. With both hybridization solutions only two signals of equal intensity were seen, indicating binding of the probe only to one type of chromosome. This may mean that either there is no cross-reactivity shown by this specific probe to this particular cell line used, or if there is any, it is undetectable under these conditions. The two instances previously reported have used different cell lines ; in addition it is also noteworthy that the conditions for washing after hybridization are less stringent (2X SSC) than the one used in this procedure (0.1XSSC). In one of the reports, the method of detection is not fluorescence making the comparison of results even more difficult (Smeets, 1991).

The chromosome 15 specific centromere probe is tagged with fluorescein isothiocyanate (FITC) fluorochromes after the probe is reacted with the chromosomes the 15. The labeled chromosomes are then identified by an antibody raised against FITC, which is covalently linked to a magnetic bead enabling the bead bound fraction to be separated from the remaining sample. The amine modified magnetic beads are encapsulated, meaning that iron oxide is added only during the initial stages of synthesis of the bead and decreases gradually towards the end. This results in a bead coated exclusively with the polymer with no iron oxide left on the surface, which is advantageous in many applications. In this case it is beneficial because the absence of positively charged iron reduces non specific binding of negatively charged DNA on

the bead surface. In other applications it would be advantageous due to the reduction of interference by iron in enzymatic reactions involving polymerases and certain types of cells. A homobifunctional crosslinker, Ethylene glycolbis(sulfosuccinimidylsuccinate) or sulfo-EGS (Fig 4.24) is used to link the antibody to the bead. Although homobifuntional linkers are not the most preferred for a situation like this as linking is less controllable than with a heterobifunctional linker, the above was the most suitable commercially available linker at the time for this specific situation. The linker was chosen because it is cleavable by hydroxylamine so that the bead can be cleaved from the chromosome/probe conjugate after the separation process. Another important feature of the linker is its water solubility, due to the sulfo group making the handling procedure less complicated.

The bead bound sample is then placed in a square plastic cuvete prior to exposing to a magnetic field in order to be able to do the separation on a flat surface. The sample is then exposed to a magnetic field for about 30-40 min as it has been observed that there is no settling of the particles during this time. A bar magnet is held against the wall of the cuvette instead of the bottom to get rid of any heavy extraneous particles such as unremoved cell debris settling at the bottom due to gravity. The supernatant is removed and the cuvete is washed extensively with IB+M buffer, especially the bottom to eliminate any chromosomes left out prior to removing the magnetic field. Then the bead bound fraction is collected for further characterization. Fig 4.25 (A) shows a pool of such isolated chromosomes and 4.25 (B) shows an individual isolated chromosome. It is important to note that clump

formation due to aggregation of chromosomes is reduced dramatically from what has been previously reported.

Usually flow sorted chromosomes are characterized by making a paint probe from the sorted fraction of the chromosomes and applying that to a metaphase spread to perform a FISH experiment, which would result in painting the corresponding pair of chromosomes in the spread. To make the paint probe, a method called DOP-PCR (Degenerate oligonucleotide primer polymerase chain reaction) is used where, as the name implies, a degenerate primer that can basically amplify any template is used so that the isolated fraction of DNA gets amplified in the PCR cycle. Then in a second step the amplified product is fluorescently labeled so that a fluorescent paint probe is obtained. If population of DNA were homogeneous, then a probe is generated to paint the specific chromosome that was sorted out in the first place. However, this does not give a statistical analysis of how much of an enrichment is obtained from this methodology. Even a slight contamination of any other DNA, will result in its amplification by PCR giving rise to non-specific products. Presence of such DNA is not a significant problem in our case because as long as we get sufficient enrichment of the chromosome of interest, the remaining purification will automatically be implemented at the next level, where the PW region is clipped as discussed in Chapter 5.

It turned out that the only way to get a statistical output for the enrichment by the two approaches of fractionation of chromosomes is by using the isolated pool as the target, and to perform a FISH experiment with a commercially available probe

specific for chromosome 15. The enrichment will be indicated by the specific signal counted and compared to a non-specific DNA binding signal. This specific probe is first characterized against a normal metaphase spread to confirm its specificity to chromosome 15 (Experimental procedure is similar to what is described in section 4.II.2.(vi)). The control experiment is performed with an aliquot of chromosomes immediately after extracting from cells, prior to isolation by the beads. A DNA binding dye, DAPI is used to identify the chromosomes on the slide. The results are shown in Fig 4.26 (A,B).The slide is scanned to count the number of total chromosomes and painted chromosomes in different locations to get the ratio of (painted/total) to calculate the percentage of enrichment. For the control experiment, as discussed in Part I of the chapter, the number obtained is about 6%. Ideally, if the sample is random, this number should be around 4.35% as there are 2 chromosome 15s for every 46 chromosomes in a single cell. However, the slightly higher number observed is not unreasonable considering the fact that the chromosomes are all mixed up in this preparation and do not remain as single cells like in a metaphase spread When the same experiment was performed for the isolated fraction, as shown in figure 4.27 (A & B), and a total of 239 chromosomes from 3 separate fractions are counted, 73% of the chromosomes identified by DAPI are painted meaning that the enrichment that has taken place by this fractionation procedure is approximately 73%. When this same approach was adopted with a different probe specific for the sub-telomeric region of chromosome 15 labeled with tetramethyl rhodamine isothiocyanate (TRITC) to verify the enrichment of fractionation, the percentage of

enrichment was calculated to be about 80% with a total number of 110 chromosomes being counted (Data shown in Fig 4.28 A & B). One of the reasons for this slight difference in percentage could be mainly due to the difference in the accessibility of the two probes to the target DNA. As the paint probe used in the first experiment covers the entire chromosome, it requires to hybridize to the entire chromosome as opposed to the second probe that targets only the sub-telomeric region in chromosome 15.

Fig 4.29 demonstrates the formation of extended chromatin fibers from the fractionated pool of chromosomes. The fractionation process increases significantly the probability of finding a certain genomic region. This can be further extended into a fiber FISH experiment where a genomic region of interest can be targeted with a fluorescently labeled specific DNA probe.

4.II.4. Conclusion:

Due to the shortcomings of the standard method for isolation of chromosomes and since it is essential to get rid of all the extraneous materials to reach for the histones from a selected region of a chromosome, a different method for chromosome isolation has been attempted. The efficiency of sorting by flow cytometry, reduces dramatically when dilute chromosome suspensions are being used, which is crucial to eliminate any aggregates of chromosomes. Therefore the time actually needed for sorting is very much more than the calculated number.

This novel methodology tries to overcome the problem of extracting sufficient material in a minimum amount of time, such as a few days as opposed to several years, and making the sorting process more specific to the chromosome of interest. In addition, it would also be worth pointing out that such an isolated or enriched pool of chromosomes will have a great degree of significance in general for many types of analysis such as making chromatin fibers and creating somatic hybrids.

Overall conclusion:

By flow cytometry, obtaining a 100% pure fraction of the chromosome of interest is feasible by narrowing the gate to be sorted at the expense of time. By the magnetic bead approach, may be due to non-specific binding of chromosomes to the sample container or non-specific binding of the probe, obtaining a fraction of such high purity may not be possible. However, the time required to get 75% enrichment is about 3-4 working days compared to several years needed for flow cytometry. Therefore, choosing the right method will be dependent on the application. The time factor will be more important for sample preparation and analysis of histones of a specific genomic region, as the required specificity of the particular region can be obtained at the next level, which is clipping of the region of interest.

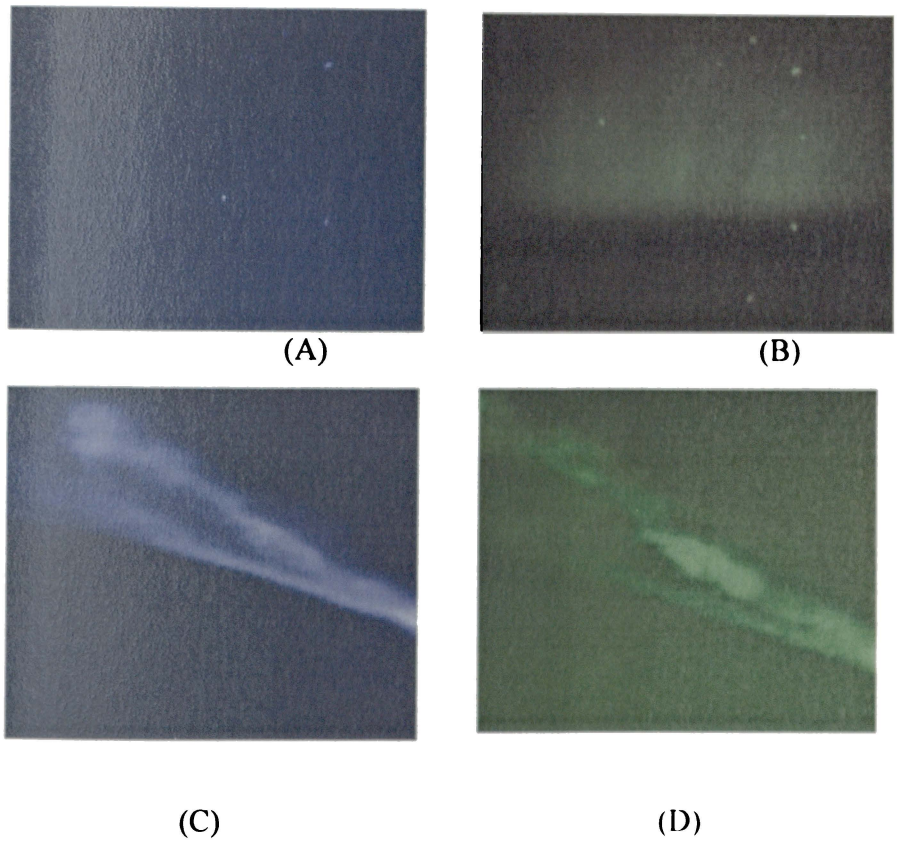


Fig 4.20: Immunofluorescence on extracted chromosomes to determine the integrity of histones upon exposure to polyamine procedure for chromosome extraction. Polyclonal rabbit anti-histone antibody that recognizes all isoforms of acetylated histones is used as the primary antibody. Secondary antibody is goat-anti-rabbit antibody that has a FITC tag on it. All images are taken under 40X magnification.

(A) DAPI stained metaphase chromosomes observed with filter for 470 nm emission

(B) Immunofluorescent signal on the metaphase chromosomes in (A) observed with filter for 525 nm emission wavelength as the secondary antibody contains FITC labels

(C) Extended chromatin fibers using the extracted chromosome preparation and observed with filter for 470nm emission wavelength

(D) Immunofluorescent signal on the extended fibers in (C) observed with filter for 525 nm emission wavelength.

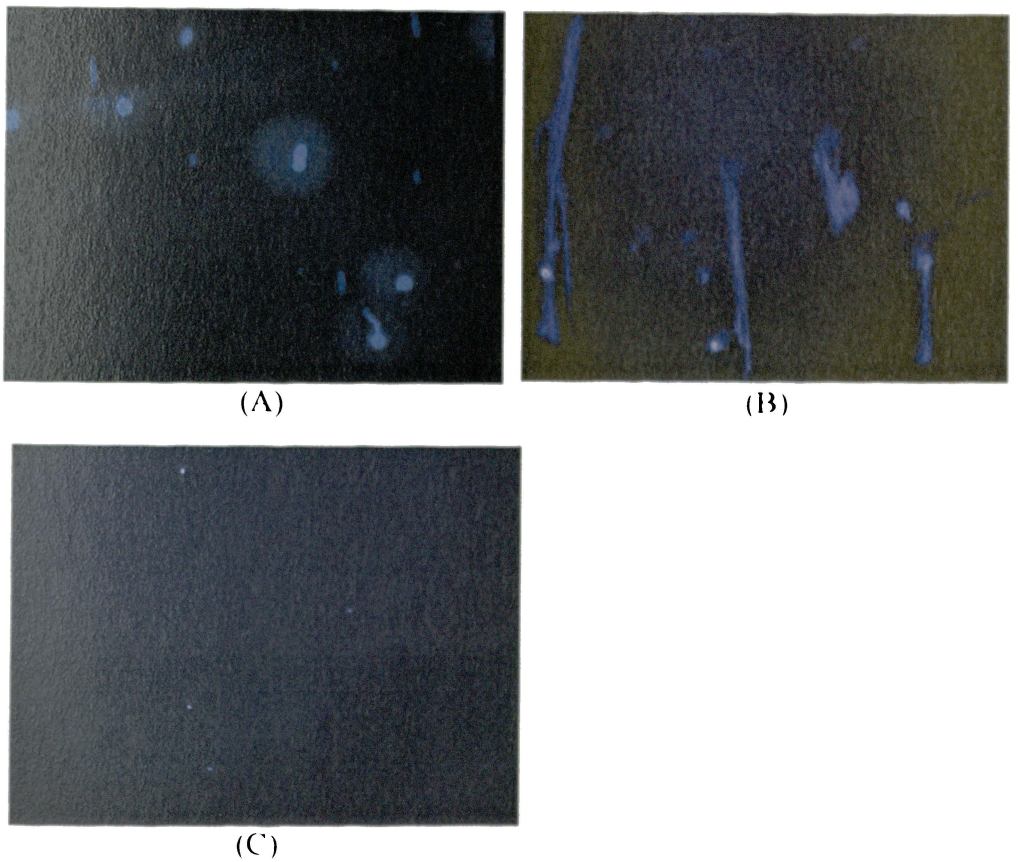
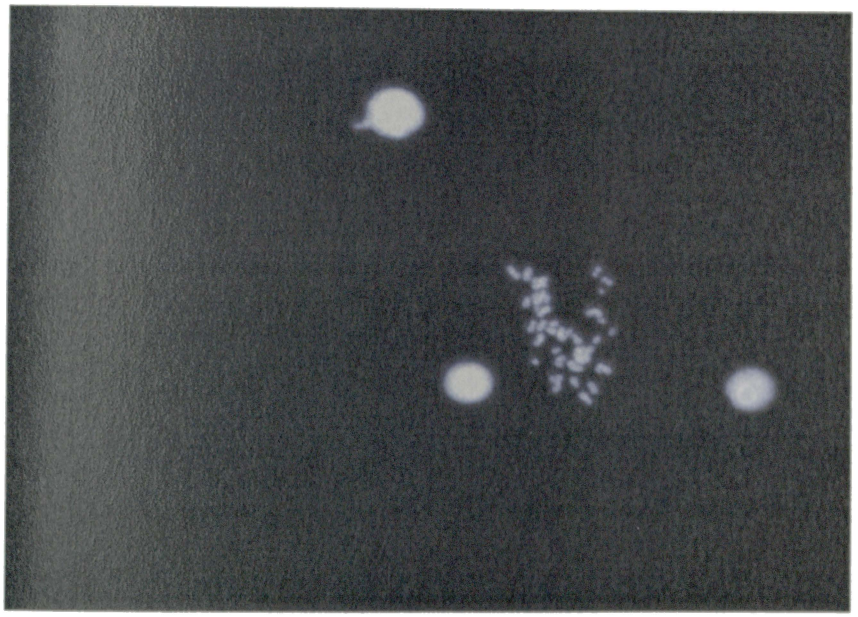


Fig 4.21: Metaphase chromosomes extracted from cells after 12-13 hour colcemid treatment to arrest cells as metaphase. Chromosomes are extracted by rupturing the swollen cell membranes applying a pressure by vortexing. DNA is stained with DAPI for identification. All images under 40X magnification.

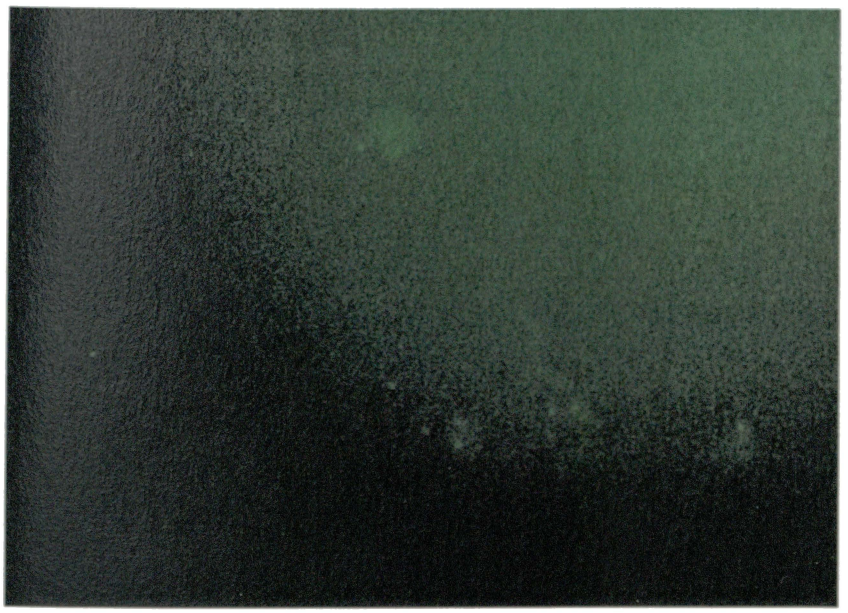
(A) Vortexing for 90 seconds elongates the chromosomes significantly. This chromosome preparation was done prior to the establishment of the entire cleaning protocol and that is evident in the high nuclei/chromosome ratio. DAPI staining is observed with filter for 470 nm emission wavelength.

(B) Vigorous vortexing for 2.5 min stretches the otherwise condensed chromatin in the native metaphase chromosome, dramatically. This extract also contains a high proportion of nuclei, as in (A).

(C) Chromosome extract prepared under optimal conditions: vortexing for 50-70 sec in 15 sec increements and entire protocol for cleaning described in section 4.II.2.(ii) is followed.



(A)

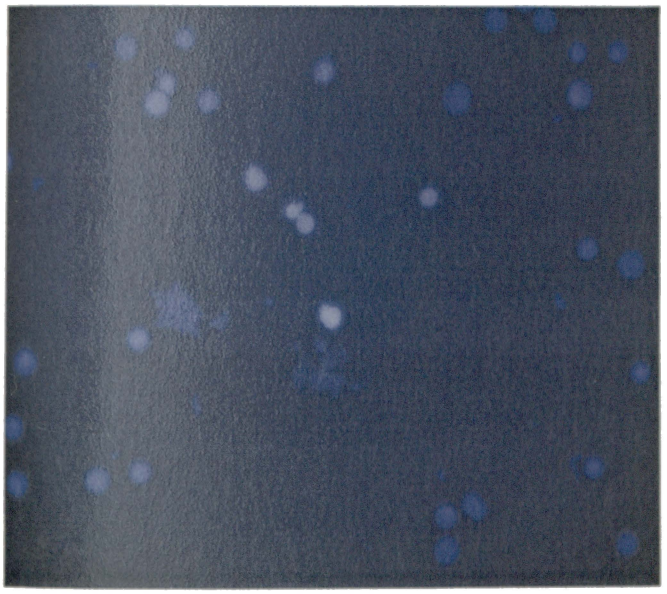


(B)

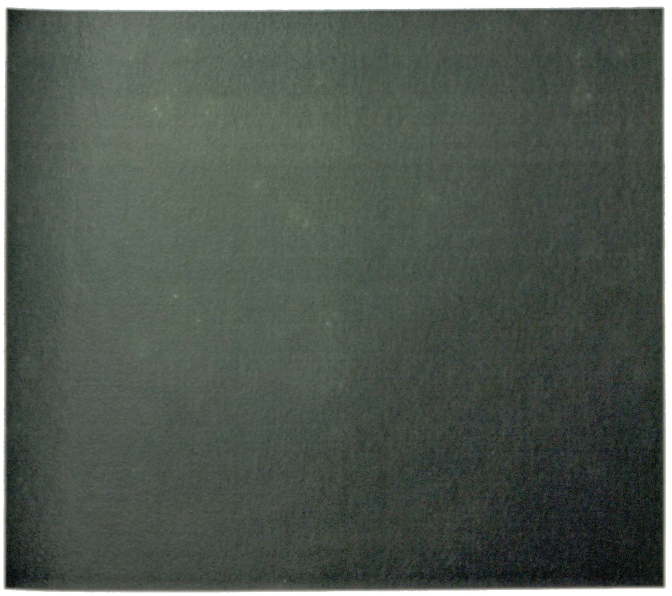
Fig 4.23: Characterization of the chromosome 15 specific FITC labeled centromere probe that targets the D15Z1 region with the hybridization solution used for labeling the chromosomes in suspension.

(A) DAPI stained metaphase spread with 470nm emission filter

(B) Centromere probe seen as the green signals with 525nm emission wavelength



(A)



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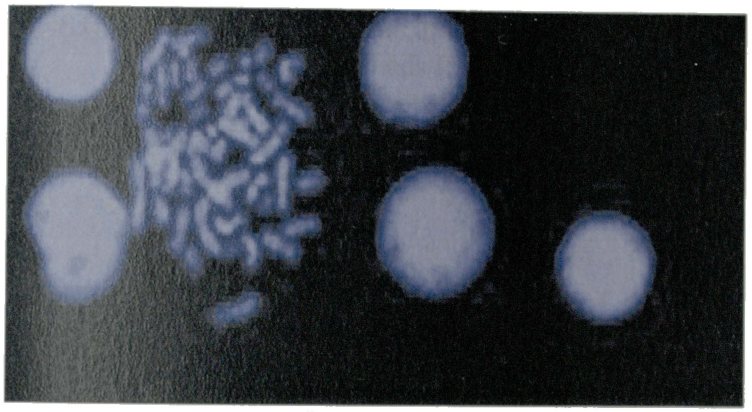
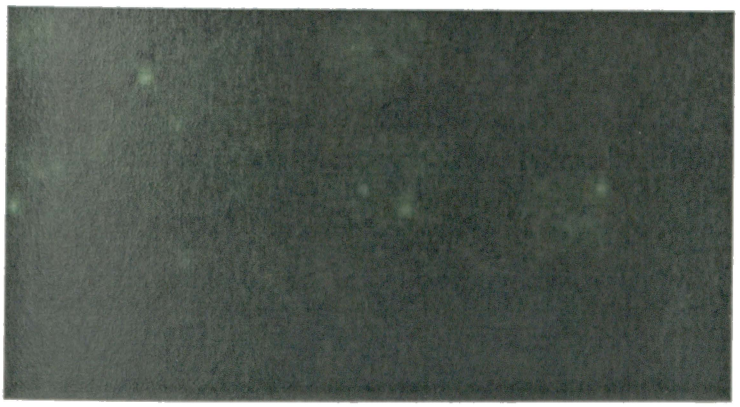
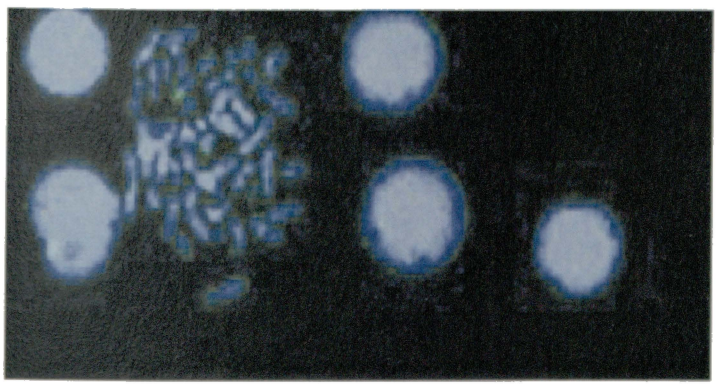


(C)



(D)



(E)

Fig 4.22: Characterization of the chromosome 15 specific centromere probe on metaphases and nuclei with the hybridization solution provided by the probe kit from Cytocell.

(A) & (C) are DAPI stained metaphase spreads under 40X and 100X magnification respectively with 470 nm emission wavelength filter.

(B) & (D) the green signals indicate hybridization of the probe to chromosomes and nuclei on the spreads (525 nm wavelength). It is clear that only two signals per spread can be observed.

(E) Overlay of (C) & (D) to show binding of the probe to the chromosomes

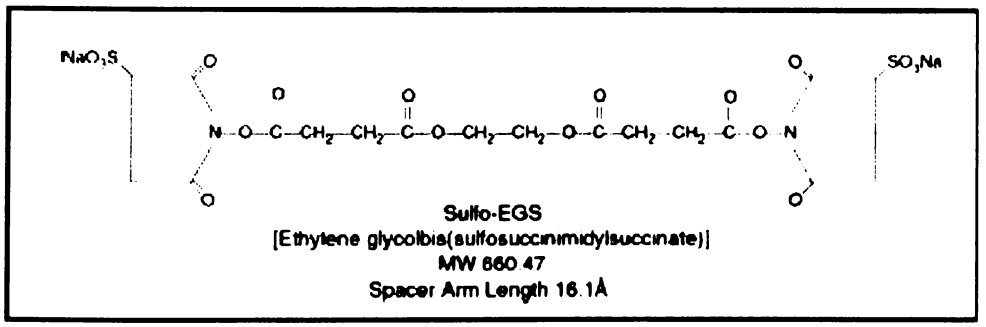
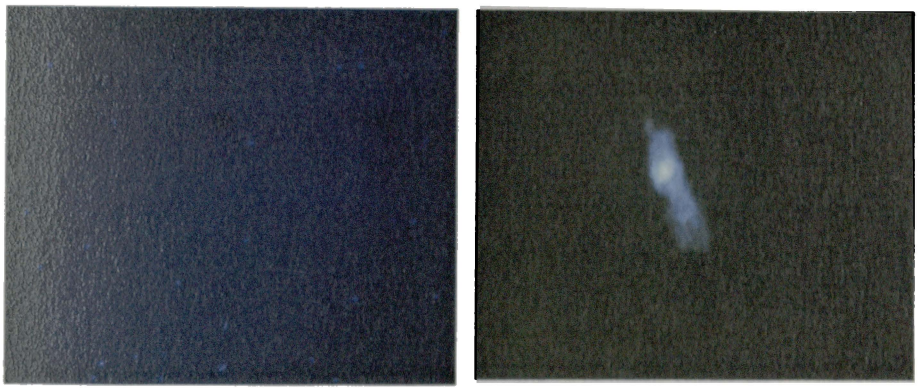


Fig 4.24: Structure of Sulfo-EGS, the homobifunctional cross linker that conjugates the magnetic bead to the anti-FITC antibody

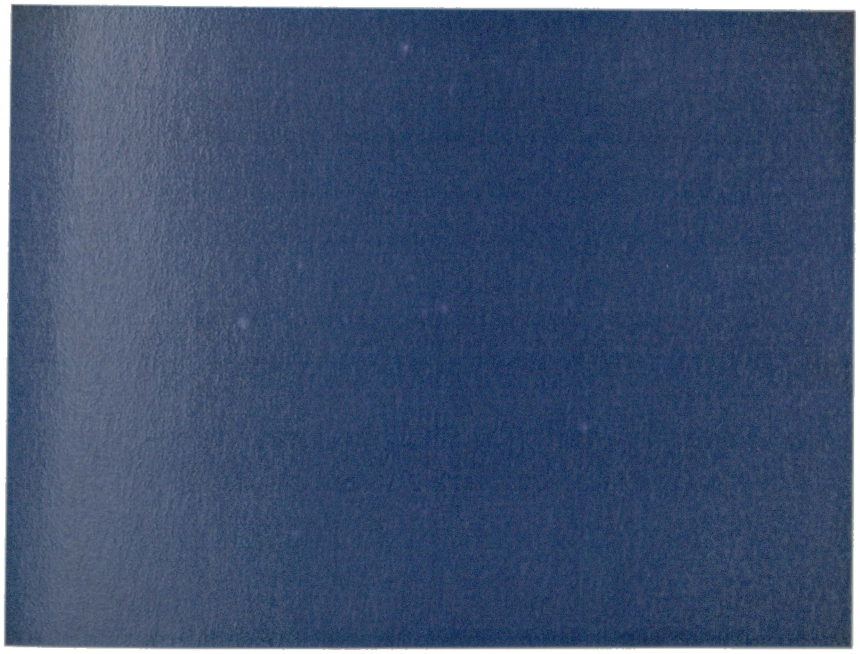


(A) (B)

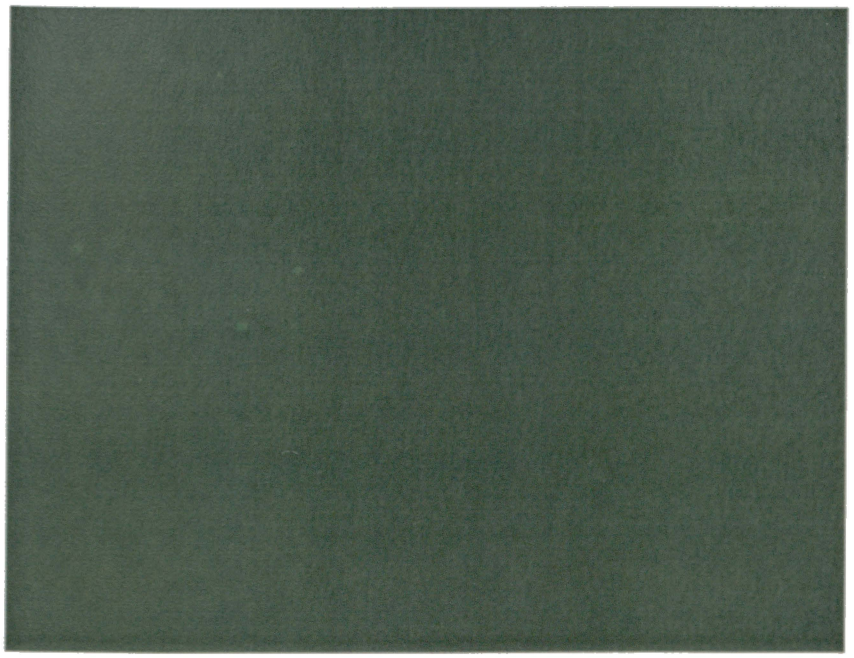
Fig 4.25: DAPI stained magnetically fractionated chromosomes with 470nm emission filter

(A) Bead bound isolated pool of chromosomes under 40X magnification

(B) Fractionated single chromosome with bead (Zoom in of a 100X magnification)



(A)



(B)

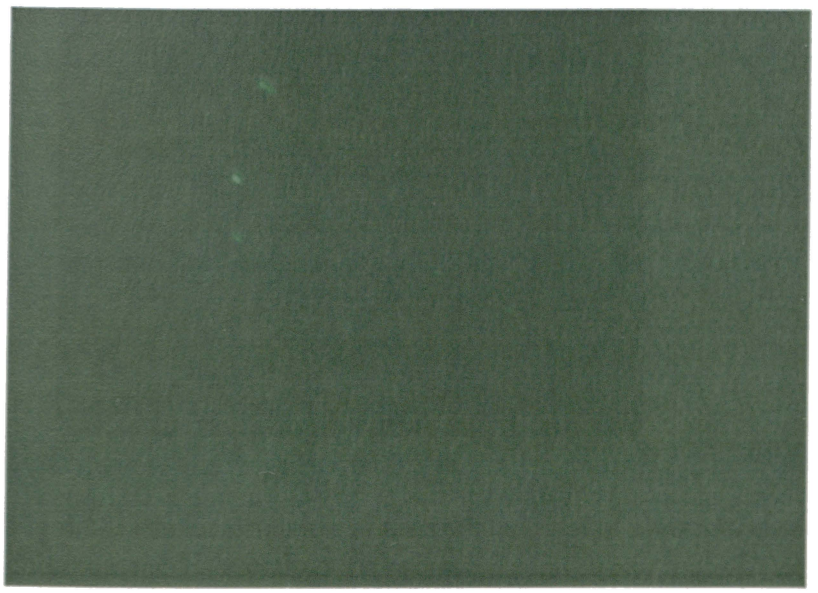
Fig 4.26: Control experiment for verification with the paint probe (40X magnification)

(A) An aliquot of chromosome suspension right after extraction from cells, prior to fractionation subjected to FISH with chromosome 15 paint probe. The

chromosomes are stained with DAPI for identification of the entire population of chromosomes

(B) The above chromosomes under FITC filter to detect the painted chromosomes; 40X magnification *Painted/total ratio = 6%

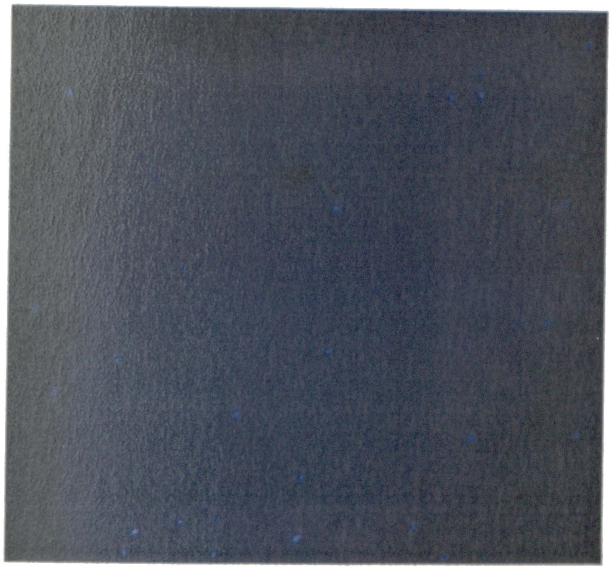
(A)



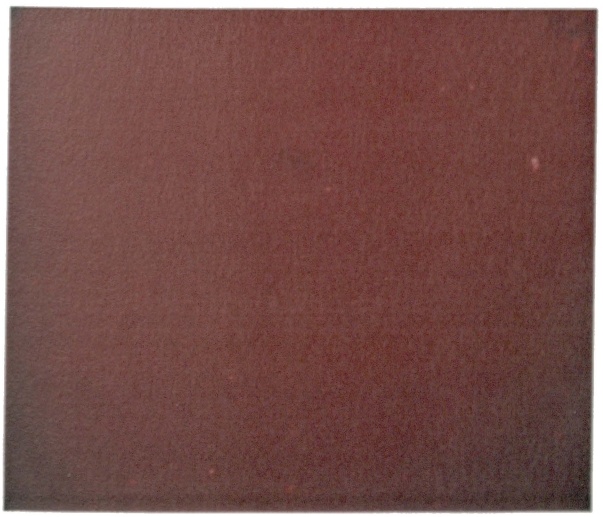
(B)

Fig 4.27: Characterization of the isolated pool of chromosomes with FITC labeled chromosome 15 paint probe.

(A) DAPI stained fractionated pool of chromosomes (40X magnification)
(B) FITC labeled chromosomes out of the total population shown in Fig 4.27 (A)



(A)



(B)

Fig 4.28: Characterization of the isolated pool of chromosomes with TRITC labeled chromosome 15 subtelomeric probe.

(A) 4.28 (A) Isolated pool of chromosomes stained with DAPI observed under filter for 470 nm emission wavelength; 40 x magnification

(B) Isolated pool in (A) characterized with a chromosome 15 telomere probe binding to the q arm telomere. The probe has a TRITC label that is observed under filter for 576 nm emission wavelength; 40 x magnification

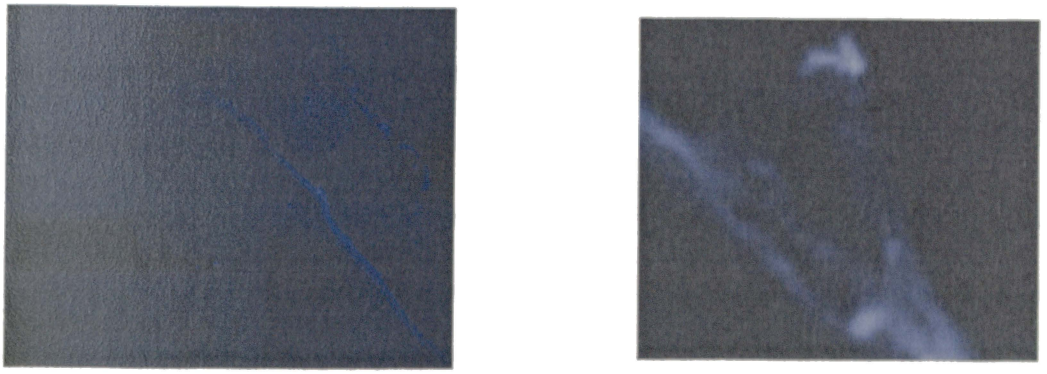


Fig 4.29: DAPI stained extended chromatin fibers made from
(A) fractionated pool of chromosomes (20X)
(B) extracted metaphase chromosomes from cells (63X)

Addendum to Chapter 4: Quantitative PCR

A4.1 Introduction:

Quantitative or real time Polymerase Chain Reaction (PCR) is a technique that has recently evolved as a derivative of PCR to make quantitative measurements. It is generally used to quantify the level of gene expression and copy number of genes. Gene expression is quantified by indirectly measuring the messenger RNA (mRNA) levels in cells in a two step procedure: reverse transcribing mRNA to cDNA by Reverse Transcription PCR (RT-PCR) and then PCR amplification of the resulting cDNA with either non-specific DNA intercalating fluorescent dyes such as SYBR Green (Morrison *et al*, 1998) or SYBR gold or specific fluorescent probes (Taveau *et al*, 2002) as tools for quantification.

The principle behind the technique with non-specific fluorescent dyes is that the dye intercalates to double stranded DNA, and as PCR amplification progresses the amount of product (ds-DNA) increases, resulting in an increase in fluorescence. The concentration of the initial template can be calculated based on a threshold value (C_t), where the fluorescence rises above the background. The fluorescence is measured at the annealing step of each PCR cycle as indicated in Table A4.1. One of the major issues associated with such dyes lies in its capability to bind to any ds-DNA, not necessarily the product, giving rise to non-specific fluorescence. As primer-dimer formation is a fairly common problem in PCR, this is an issue of great significance, which has been addressed by the use of more specific fluorescent probes that bind to

the DNA template. In this case, a probe has to be designed depending on the template sequence incorporating fluorophores, the downside of which is the cost when it comes to analyzing multiple templates.

In order to determine the enrichment of the fractionated chromosome pools, quantitative PCR was adopted as a tool to quantify the template DNA present in the sample. Primers were chosen to target a unique gene in each individual human chromosome to cover the full complement of chromosomes as shown in Table A4.3, with the hope that by comparing C_t values one would be able to determine the percentage of enrichment, normalizing the data with proper controls. Although SYBR green method suffers from giving rise to non-specific fluorescence, it was used over the specific probe method as design and synthesis of a specific probe for each individual chromosome is expensive.

A4.2 Experimental:

SYBR Green was purchased from Sigma (Cat no: S-4438, SYBR green JumpStart™ Taq ReadyMix™) with PCR program (Table 4.2) was slightly modified from the protocol provided by Sigma. The PCR machine used was the Opticon 2 set up from MJ research.

Step	Temperature/ ^{0}C	Time
Initial denaturation	94	10 min
40 cycles: denaturation	94	15 sec
☼ Annealing	55	1 min
Extension	72	1 min
Hold	4	

Table A4.1: PCR program for quantitative PCR
☼ Fluorescence is measured at this step.

Volume/uL	Reagent
25	SYBR green Jumpstart Taq readymix
-	Forward primer, 0.2 uM final concentration
-	Reverse primer, 0.2 uM final concentration
varies	Template DNA (genomic DNA or sample)
	water
50	Total volume

Table A4.2: Amounts and reagents used for quantitative PCR; template DNA has to be optimized for different controls and samples

Chr	Target gene	Forward primer (5'→3')	Reverse primer (5'→3')
1	p55CDC	AACTCAAAACAGCGCCAT AG	CACCAGTGATCGACACA TTC
2	(UBE1)	CAAACAAGGTAGAAGGC AGTGG	GCGTGTCCATGCTCTATT CCTT
3	Evi-1 mRNA	AAGAGCCATGCTACTGTT GG	GGAACTTCACAGATAGC CTC
4	*Humig*	GAGAAAGGGTCGCTGTTC CT	TTTGGCTGACCTGTTTCT CC
5	Histidyl tRNA synthetase	GGTTGAGGCATTTTATTG GA	GCATCTGCTGAACTGAA CAA
6	Major histocompatibilit y complex, class II, DM alpha	GGGGAAGGGATGTGGTTG TGAT	CAGCAATTCCCTATTGG GTACC
7	mRNA for prepro-alpha2(I) collagen	TTTAATTTTTCTGCTTGCC CA	CAAAACACTTTCCCATG AGTG
8	Translational inhibitor protein p14.5 (UK114) mRNA	TGTCGTCCTTGATCAGAA GGG	TGTCAGTGGTCCTTGGAT AGC
9	Spleen tyrosine kinase (SYK),	TGCACTGGTCAAACTTGA TTT	TGGCCTAGAGCACTCTC ACC
10	Dock180 mRNA	TCAATTTACTTGCAGCTCT GGG	CTTTTGGGAAAATGAGG ACTGA
11	Interferon-inducible 1-8D	TGACGATGAGCAGAATGG TC	ATGTCGTCTGGTCCCTGT TC
12	Sodium and Chloride dependent betaine transporter	GAAGTCAGGGGTTTGCGT TA	ATGCCGTCAGTCTGGAG TTT
13	TGF-beta stimulated protein TSC-22	TGAAAGTTGACAAGTGCA ACAG	CATTGGCTGTCTGAGGA GGT
14	mRNA for immunoglobulin lambda heavy chain	GCACTCATTTACCCGGAG ACA	ACAAGTGCAAGGTCTCC AACA
15	eIF-2 p67 homolog mRNA	AGCCTGGGATGACAATGA TA	CCTCACCAACATCACAC AGA

16	ATP-binding cassette mRNA	AGGGTGCAGTGCTGGAAACA	GGAAAACCAGCCCAGGTTGA
17	Phosphatidylinositol (4,5) bisphosphate 5-phosphatase	AAAGCCACGCTAATAATTACCC	CAGGCAGAAGGGAGAACCAGCT
18	Twisted gastrulation (TSG)	TTTGGAGTGAACATCATCTTGAA	ACCAGCTTGTACCCCTGGTAGG
19	CLPP like protease	TGATGACATCTACTCGCGG	GATCATGATACGGGAGTTGG
20	Retinoblastoma binding protein 3	GAGGGAACAGAGCTGTTAGGAA	TTGAAGCTTTAATGGAGCGTTA
21	ETS-2 Oncogene	CCCCATGAAACTCCTTCTTT	GAAAACTGAGGAAATCGGGT
22	PRAME	GTTTGTGGGGTTCCATTCAG	GGGCAAGGAGCACATCAAGT
X	NADH-Ubiquinone Oxidoreductase MFWE Subunit	CACTGTAACCTTCTAGCAGGGG	GTTCGAGATTCTCCCCGGACTC
Y	TSPY	AAAGGCAACGTCCAGGATAG	AATTCTTCGGCAGCATCTTC

Table A4.3: Primers for each individual chromosome for the entire set of human chromosomes. Primers for chromosomes 1 through X were kindly donated by Dr.Steven Johnson at University of Pennsylvania School of Medicine, PA. Primer sequences are given in http://www.realtimeprimers.org/SYBR%20Green/Human%20SYBR%20Green%20Primers/JS-50.html

A4.3 Results:

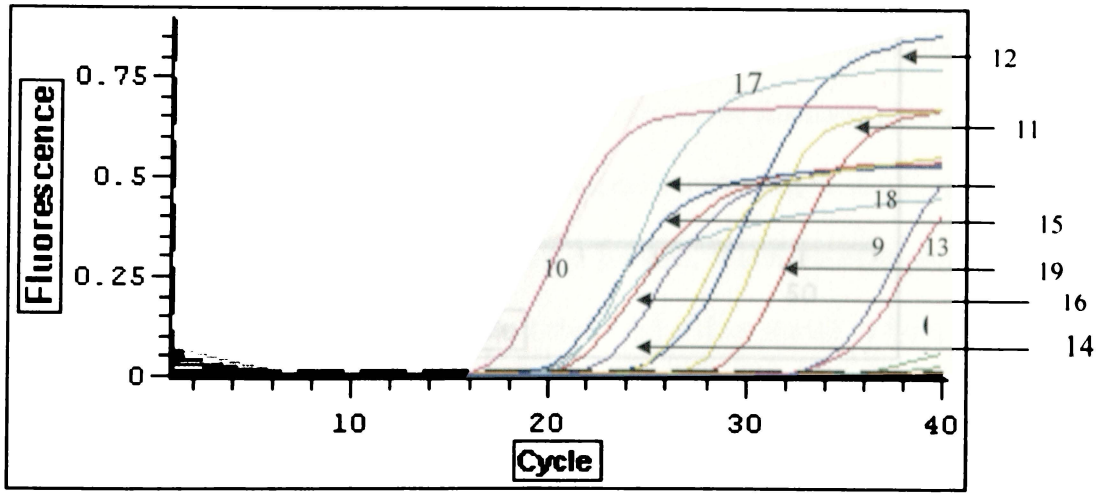


Fig A4.1: Quantitative PCR performed with primers for chromosomes 9 through 20 using genomic DNA as the template. (Numbers associated with the lines indicate the specific chromosome that was amplified in each case).

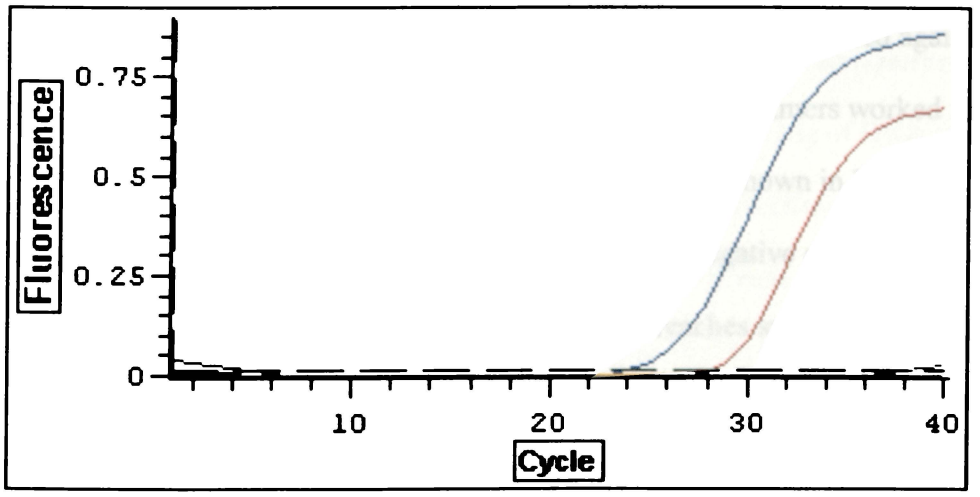


Fig A4.2: Quantitative PCR performed with primers for chromosomes 19 (red- positive control, green- negative control, C_t 28.36) and chromosome 12 (blue- positive control, orange – negative control, C_t 24.33)

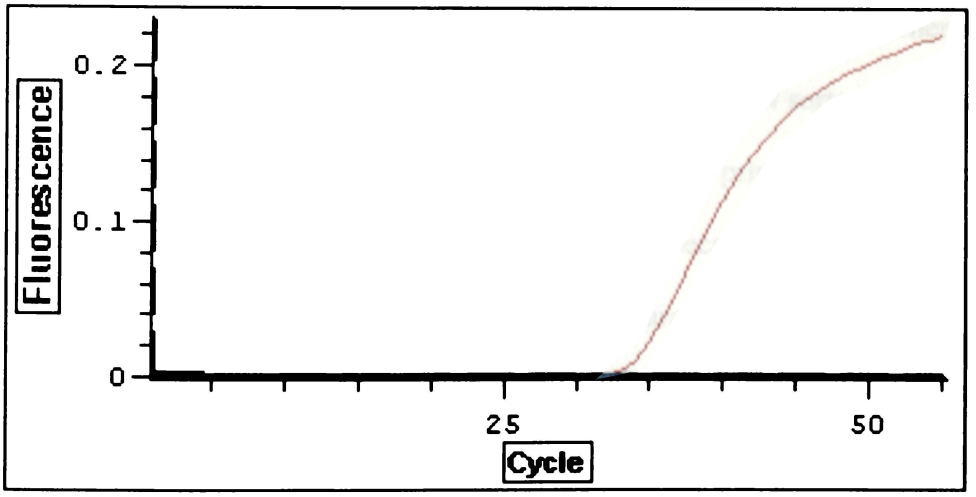


Fig A4.3: Quantitative PCR for chromosome preparation prior to isolation (red, C_t 32.27), flow sorted chromosomes (green), magnetic bead sorted (blue) with primers for chromosome 15.

A4.4 Discussion & Conclusion:

In order to verify the performance of primers, they were tested against genomic DNA with the Quantitative PCR reaction and all primers worked fine under the given conditions. Results for some of the primers are shown in Fig A4.1. Genomic DNA was used as the positive control and a negative control was run with no DNA template. What is expected is a curve that reaches saturation usually between 15 and 35 cycles for the positive control and no fluorescence for the negative control. Fig A4.2 shows two of the above primers in a separate plot to show this phenomenon clearly. At the beginning, when the primers were tested, a small hump was seen around the tenth PCR cycle in most of the primers. This turned out to be due to the secondary structures of the DNA template which was avoided by increasing the initial

denaturation from 2 min to 10 min. Fig A4.3 illustrates what happens when a chromosome preparation prior to fractionation, and both flow sorted and magnetically fractionated samples are used as the DNA template with primers that amplify a gene in chromosome 15. This specific primer was chosen because the fractionation was targeted at chromosome 15. The chromosome preparation works fine with a C_t of 32.27. However, neither of the samples gave a PCR product. When the PCR program was extended to 50 cycles instead of 40 as used above, a product started to form around cycle number 43 for the flow sorted chromosomes. An agarose gel confirmed that it is not a real product, indicating the possibility of primer-dimer amplification. Similar results were obtained for primers targeting at chromosome 21 as well.

The main difference between the chromosome preparation, for which a product was observed, and the sorted chromosomes is the high concentration of chromosomes in the former. The chromosome preparation contained about 10000 chromosomes whereas the sorted samples contained 300-500 chromosomes in the PCR reaction mixture. In the case of genomic DNA about 0.1ug of DNA is sufficient to see a PCR product. As the target is a unique gene in each chromosome, it could very well be that the copy number is so low that when there is not enough template, the product made does not give enough fluorescence to exceed background. This has been a commonly observed phenomena with low copy number targets (Teo *et al*, 2002). This was also confirmed by an agarose gel, which indicated little or no template for the samples. Increasing the concentration by lyophilizing the samples gave similar results with no fluorescence which could be still due to insufficient

template. Therefore, quantitative PCR experiment could not be used to determine the enrichment of the fractionated pools of chromosomes. If a large amount of material is fractionated and then concentrated, this experiment could work. As this is still a developing technique procedures may be modified and improved to increase the efficiency of amplification of low copy number targets.

Chapter 5: Future Work

The overall objective of this work is to analyze and compare PTMs of histones in specific genomic loci in order to assess the differences in the modifications related to genetic diseases. To accomplish this task, histones need to be extracted from the particular region of the genome and analyzed. Clipping and isolation of the specific genomic region from the remainder is a prerequisite to extraction of the histones of interest. An enrichment of the chromosome on which the particular gene or genes of interest are located, makes it easier to isolate the specific region in the genome. The work enclosed in the dissertation has demonstrated a 73% enrichment of the chromosome of interest utilizing the magnetic bead approach. Even if the fractionated pool of chromosomes is not a 100% pure population, at the subsequent clipping to isolate the specific region, the anticipated purity can be obtained. Some possible approaches to clip the fragment of interest, in order to extract and analyze histones, are outlined below.

Restriction enzymes, also referred to as microscopic scalpels, can cleave double stranded DNA (ds-DNA) within a specific DNA sequence. Out of the different types of restriction enzymes, type II enzymes or endonucleases, which are phosphoesterases, hydrolyze the internucleotide bond of DNA or RNA molecules (Mishra, 2002). The length of the DNA sequence identified by endonucleases, ranges from 4-8 base pairs. Two examples of such type II endonucleases are *Sau*3AI, is a 4-cutter enzyme identifying a 4 base long sequence, GATC, whereas *Eco*RI identifies a

6 base long sequence of GAATTC (Twyman, 1999). The frequency of occurrence of a sequence recognized by these enzymes is dependent on the length of the recognition site ($1/4^n$ where n is the length of the site of recognition). The shorter the recognition site, the more frequent is its occurrence in a genome. Therefore, if a 4-cutter is used to digest the DNA, it generates fragments of 250 bp and a 6 cutter would generate a fragment 4000bp in length. If one wishes to clip the entire PWS region, which is about 2 Mbp in length, from chromosome 15, these enzymes would not be the best choice. The closest one could get with endonucleases in performing such a cleavage in a complex, 3 billion base pair long genome such as the human genome, would be with an endonuclease that identifies a recognition site of 8 bases in length, which are referred to as a rare-cutter restriction enzyme. The probability of occurrence of an 8 base long sequence in the genome is about once every 65000 bases. *Not*I, although a rare-cutter enzyme, generates fragments of 95000 bp because its recognition site is fairly GC-rich and underrepresented in mammalian genomes (Twyman, 1999). There are two problems associated with using these enzymes to clip, for instance, the PWS region from chromosome 15. Firstly, there is little control over the points at which cleavage takes place and secondly, all the fragments generated are of equal size, making difficult the subsequent separation of the PW fragment from the remainder.

An alternative and more promising approach is to cleave the genomic region of interest, which improves the specificity of cleavage because the target length of DNA is increased, using the synthetic nucleases. These are semi-synthetic site specific DNA cleavage reagents, composed of two chemical moieties. First

component, a chelator and a suitable metal complex can cleave ds-DNA by Fenton chemistry as shown in Fig 5.1. A hydroxyl radical (•OH) is produced by the reaction of a chelator-metal complex such as Fe(II) EDTA complex or 1,10-phenanthroline-Cu(I) complex with H_2O_2, and cleaves the pentose sugar of the DNA backbone. Such metal chelator complexes are capable of cleaving double stranded DNA randomly, regardless of the sequence, is conjugated to the second component, a sequence specific DNA binding molecule that adds specificity to this reagent, thereby making the cleavage possible at a targeted specific DNA sequence. Such sequence specific DNA binding molecules include proteins (Chen *et al,* 1987, peptides (Sluka et al, 1987), drugs (Schultz *et al,*1982), polyamide ligands (Dervan *et al*, 1999) and homopyrimidine oligonucleotides (Moser *et al*, 1987) that can form triplexes. *Escherichia coli* Catabolite gene Activator Protein (CAP) is one such molecule that binds to DNA with extremely high affinity ($4x10^{10}$ M^{-1}) and the DNA cleavage moiety is introduced at the helix-turn-helix motif of CAP. The main advantage of using such a reagent over a type II restriction endonuclease is the improved specificity due to the increase in length of the target DNA sequence which is 22 base pairs in this particular example (Ebright *et al*, 1990) as opposed to 8 bases in restriction enzymes and data supports the specificity of the target sequence.

$$[Fe(EDTA)]^{2-} + H_2O_2 \longrightarrow [Fe(EDTA)]^{1-} + OH^- + \bullet OH$$

Fig 5.1: Fenton reaction between the Fe(II)EDTA complex and H_2O_2 (Wolffe,A., 1998)

Another possible approach along these same lines is to couple a random DNA cleavage reagent to a Zinc finger binding protein that binds a DNA sequence specifically. Such DNA binding zinc finger proteins can be conjugated through peptide linkers to increase the target DNA sequence (Liu et al, 1997). Therefore, depending on the sequence and the boundaries of the genomic region in focus, such exogenous DNA cleavage molecules can be designed. There are also some endogenous endonucleases such as I-TevI, a phage intron encoded endonuclease that is composed of a catalytic domain at the N-terminus and a C-terminus DNA binding domain with a long zinc finger linker that targets about 37 bases (Dean *et al*, 2002). Choosing the proper reagent or designing a new reagent will have to be determined by scanning the genomic sequence of interest and deciding the boundaries one would want to cleave the particular fragment, whether it be the entire PWS region or an individual gene in the PWS region.

A sucrose density gradient is one approach to separation of the clipped fragments from the remaining chromatin. The band expected for the clipped fragment in the sucrose gradient can be determined by its size and thus, isolated. Histones in the separated portion of chromatin can be extracted and further analyzed by mass spectrometry. Proteolytic digests of the extracted histones can be analyzed by either MALDI-MS/MS or ESI-MS/MS to study the PTMs (Zhang, 2000). These approaches that utilize DNA cleaving reagents perform the isolation of the specific fragments in a parallel way, that consumes less time.

Another possibility is to use a serial approach that may take a significantly longer time. A fractionated pool of chromosomes can be used to make extended chromatin fibers as shown in sections 4.II.2.(vii). Since this is an enriched pool of chromosomes, probability of finding the genomic region of interest is greatly enhanced compared to either fibers originating from cultured cells or the whole complement of chromosomes. As the latter two give rise to fibers from the whole genome, hundreds of slides will have to be made with extended fibers, to find a specific region in the genome. In the contrary, one may be able to see multiple targets on the same slide if an enriched pool is employed. DNA probes can be used to identify the region of interest, either by hybridizing a probe directly to the region or by marking the region by probes hybridized to the flanking regions. Using Laser Capture Microdissection (LCM), the demarcated region can then be cleaved and captured on to a surface and analyzed by two methods. The clipped regions, which are collected on to caps can be directly analyzed by mass spectrometry for PTMs of histones, by performing surface MALDI. Alternatively, histones can be extracted from the collected fragments using a lysis buffer contained in vials and then the histones be analyzed by MALDI or ESI-MS. This method although time consuming, leaves little or no room for contamination of histones by other regions. The downside of this approach is that to have sufficient amounts of material for MS analysis, a large number of cleavages will need to be done. All the above mentioned techniques are novel approaches to solve the problem at hand. Therefore, optimization of the experimental conditions may be necessary at each step. As this developed procedure

can be extended to fractionate any chromosome of interest, histones from any region of the genome can be extracted and analyzed to assess the corresponding PTMs.

References:

1) Allfrey, V. G., Faulkner,R.,Mirsky,A.E.; Acetylation and Methylation of Histones and Their Possible Role in Regulation of Rna Synthesis, *Proceedings of the National Academy of Sciences of the United States of America* **1964**, *51*, 786-794.
2) Allis, D. C., Strahl,B.D.; In *PCT*: US, 2002.
3) Annunziato, A. T., Eason,M.B., Perry,C.A; Relationship between Methylation and Acetylation of Arginine Rich Histones in Cycling and Arrested Hela Cells, *Biochemistry* **1995**, *34*, 2916-2924.
4) Ausubel, F. M., Brent,R., Kingston,R.E. et al; In *Current Protocols in Molecular Biology*; Wiley Interscience, 1990.
5) Belyaev, N. D. K., A.M.; Turner,B.M.; Differential Underacetylation of Histones H2a,H3 and H4 on the Inactive X Chromosome in Human Female Cells, *Human Genetics* **1996**, *97*, 573-578.
6) Bennink, M. L., Leuba,S.H., Leno,G.H. et al; Unfolding Individual Nucleosomes by Stretching Single Chromatin Fibers with Optical Tweezers, *Nature Structural Biology* **2001**, *8*, 606-610.
7) Bernstein, B. E., Humphrey,E.L., Erlich,R.L., et al; Methylation of Histone H3 Lys 4 in Coding Regions of Active Genes, *PNAs* **2002**, *99*, 8695-8700.
8) Bickmore, W.; Oxford University Press: Oxford ; New York, 1999.
9) Bird, A. P., Wolffe,A.P.; Methylation Induced Repression-Belts, Braces and Chromatin, *Cell* **1999**, *99*, 451-454.

10) Boggs, B. A., Cheung,P., Heard,E., et al; Differentially Methylated Forms of Histone H3 Show Unique Association Patterns with Inactive Human X Chromosome, *Nature Genetics* **2001**, *30*, 73-76.

11) Brownell, J. E., Zhou, J., Ranalli,T. et al; Tetrahymena Histone Acetyltransferase A: A Homolog to Yeast Gcn5p Linking Histone Acetylation to Gene Activation, *Cell* **1996**, *84*, 843-851.

12) Bustin, M. S., B.D.; Antigenic Determinants in Lysine-Rich Histones, *Biochemistry* **1973**, *12*, 1124-1129.

13) Catez, F., Lim,J.,Hock,R., et al; Hmgn Dynamics and Chromatin Function, *Biochemistry and Cell Biology* **2003**, *81*, 113-122.

14) Chen, C. H., Sigman,D. Chemical Conversion of a DNA-Binding Protein into a Site-Specific Nuclease, *Science* **1987**, *237*, 1197-1201.

15) Chen, X. W., Asahara,D.K., Emerson.T.M., et al; A Transcriptional Switch Mediated by Cofactor Methylation, *Science* **2001**, *294*, 2507-2511.

16) Clayton, A. L., Rose,S.,Barratt,M.J., et al; Phosphoacetylation of Histone H3 on C-Fos and C-Jun Associated Nucleosomes Upon Gene Activation, *EMBO journal* **2000**, *19*, 3714-3726.

17) Collas, P., Liang,MR.,Vincent,M.,Alestrom,P.; Active Transgenes in Zebrafish Are Enriched in Acetylated Histone H4 and Dynamically Associate with Rna Pol Ii and Splicing Complexes, *Journal of Cell Science* **1999**, *112*, 1045-1054.

18) Craig, J. M., Kraus,J., Cremer,T Removal of Repetitive Sequences from Fish Probes Using Pcr-Assisted Affinity Chromatography, *Human Genetics* **1997**, *100*, 472-476.

19) Cram, L. S., Campbell,M., Fawcett,J.J.,et al; Polyamine Buffer for Bivariate Human Flow Cytogenetic Analysis and Sorting, *Methods in Cell Biology* **1990**, *33*, 377-382.

20) Dean, A. B., Stanger,M.J., Dansereau,J.T. et al; Zinc Finger as Distance Determinant in the Flexible Linker of Intron Endonuclease L- Tev, *Proceedings of the National Academy of Sciences of the United States of America* **2002**, *99*, 8554-8561.

21) Dervan, P. B., Burli,R.W.; Sequence Specific DNA Recognition by Polyamides, *Current opinion in chemical biology* **1999**, *3*, 688-693.

22) DeSilva, B. S., Egodage,K.L.,Wilson,G.S.; Purified Protein Derivative (Ppd) as an Immunogen Carrier Elicits High Antigen Specificity to Haptens, *Bioconjugate chemistry* **1999**, *10*, 496-501.

23) Dudin, G., Steegmayer,E.W.,Vogt,P., et al.; Sorting of Chromosomes by Magnetic Separation, *Human Genetics* **1988**, *80*, 111-116.

24) Ebright, R. H., Ebright, Y.W., Pendergrast,P.S., Gunnasekera,A.; Conversion of a Helix-Turn-Helix Motif Sequence-Specific DNA Binding Protein into a Site-Specific DNA Cleavage Agent, *Proceedings of the National Academy of Sciences of the United States of America* **1990**, *87*, 2882-2886.

25) Ekins, R. P. The Estimation of Thyroxin in Human Plasma by an Electrophoretic Technique, *Clin.Chim.Acta* **1960**, *5*, 453-459.

26) Falls, J. G., Pulford,D.J., Wylie,A.A.; et al Genomic Imprinting: Implcations for Human Disease, *American Journal of Pathology* **1999**, *154*, 635-647.

27) Fawcett, J. J., Longmire,J.L., Martin,J.C., et al In *Methods in Cell Biology*; Darzynkiewicz, Z., Robinson, J.P., Crissman,H.A, Ed.; Academic Press: CA, 1994; Vol. 42, pp 319.

28) Ferguson-Smith, A. C., Surani,M.A.; Imprinting and Epigenetic Asymmetry between Parental Genomes, *Science* **2001**, *293*, 1086-1089.

29) Gray, J. W., Carrano,A.V.,Steinmetz,L.L.; et al Chromosome Measurement and Sorting by Flow Systems, *Proceedings of the National Academy of Sciences of the United States of America* **1975**, *72*, 1231-1234.

30) Gray, J. W., Carrano,A.V.,Steinmetz,L.L.; et al *Flow Cytogenetics*; Academic Press: San Diego,CA, 1986.

31) Gross, D. S., Garrard,W.T.; Nuclease Hypersensitive Sites in Chromatin, *Annual Reviews in Biochemistry* **1988**, *57*, 159-197.

32) Han, M., Grunstein,M.; Nucleosome Loss Activates Yeast Downstream Promoters in Vivo, *Cell* **1988**, *53*, 937-947.

33) He, H., Deng,W.,Cassel,M.J.,et al; Fluorescence in Situ Hybridization of Metaphase Chromosomes in Suspension, *International Journal of Radiation Biology* **2001**, *77*, 787-795.

34) Hecht, A., Strahl-Bolsinger,S., Grunstein,M.; Spreading of Transcriptional Repressor Sir3 from Telomeric Heterochromatin, *Nature* **1996**, *383*, 92-96.

35) Heinz-Ulli, G. W. In *Methods in Cell Biology*; Academic Press: CA, 2001; Vol. 64.

36) Jenuwein, T., Allis,D.C.; Translating the Histone Code, *Nature* **2001**, *293*, 1074-1079.

37) Jeppesen, P., Mitchell,A.,Turner,B., et al; Antibodies to Defined Histone Epitopes Reveal Variations in Chromatin Conformation and Underacetylation of Centric Heterochromatin in Human Metaphase Chromosomes, *Chromosoma* **1992**, *101*, 322-332.

38) Jeppesen, P., Mitchell,A., Turner,B., Perry,P.; Antibodies to Defined Histone Epitopes Reveal Variations in Chromatin Conformation and under Acetylation of Centric Heterochromatin in Human Metaphase Chromosomes, *Chromosoma* **1992**, *101*, 322-332.

39) Jeppesen, P., Turner,B., et al; The Inactive X Chromosome in Female Mammals Is Distinguished by a Lack of Histone H4 Acetylation, a Cytogenetic Marker for Gene Expression, *Cell* **1993**, *74*, 281-289.

40) Jeppesen, P., Turner,B.M.; The Inactive X Chromosome in Female Mammals Is Distinguished by a Lack of Histone H4 Acetylation, a Cytogenetic Marker for Gene Expression, *Cell* **1993**, *74*, 281-289.

41) Jeppesen, P. In *Chromosome Analysis Protocols*; Humana Press: NJ, 1994; Vol. 29.

42) Jitsukawa, T., Nakajima,S., Sangwara,I. et al; Increased Coating Efficiency of Antigens and Preservation of Original Antigenic Structure after Coating, *Journal of Immunology methods* **1989**, *116*, 251-257.

43) Jones, P. A., Takai,D.; The Role of DNA Methylation in Mammalian Epigenetics, *Science* **2001**, *293*, 1068-1070.

44) Kanda, T., Sullivan,K.F., Wahl,G.M.; Histone-Gfp Fusion Protein Enables Sensitive Analysis of Chromosome Dynamics in Living Mammalian Cell, **1998**, *8*, 377-385.

45) Kausch, A., Narayanaswami,S., Manning,J., et al; In *US patent*, 1993.

46) Kim, J. Y., Kim,K.W., Kwon,H.J., et al; Probing Lysine Acetylation with a Modification-Specific Marker Ion Using High-Performance Liquid Chromatography/ Electrospray-Mass Spectrometry with Collision Induced Dissociation, *Analtical Chemistry* **2002**, *74*, 74, 5443-5449.

47) Klett, R. P., Cerami,A., Reich,E.; Exonuclease Vi, a New Nuclease Activity Associated with E. Coli DNA Polymerase, Pnas, *Proceedings of the National Academy of Sciences of the United States of America* **1968**, *60*, 943-950.

48) Kurdistani, S. K., Grunstein,M.; Histone Acetylation and Deacetylation in Yeast, *Nature Reviews Molecular Cell Biology* **2003**, *4*, 276-284.

49) Lee, T. I., Young,R.A.; Transcription of Eukaryotic Protein-Coding Genes, *Annual Reviews in genetics* **2000**, *34*, 77-137.

50) Li, E., Beard,C., Jaenisch,R. et al; DNA Methylation, Genomic Imprinting and Mammalian Development, *Nature* **1993**, *366*, 362.

51) Lichter, P., Cremer,T., Borden,J.et al; Delineation of Individual Human Chromosomes in Metaphase and Interphase Cells by in Situ Suppression Hybridization Using Recombinant DNA Libraries, *Human Genetics* **1988**, *80*, 224-234.

52) Litt, M. D., Simpson,M., Gaszner,M., et al; Correlation between Histone Lysine Methylation and Developmental Changes at the Chicken Beta Globin Locus, *Science* **2001**, *293*, 2453-2455.

53) Liu, Q., Segal,D.Dj., Ghiara, J.B. et al; Design of Polydactyl Zinc-Finger Proteins for Unique Addressing within Complex Genomes, *Proceedings of the National Academy of Sciences of the United States of America* **1997**, *94*, 5525-5530.

54) Luger, K. M., A.W.; Richmond,R.K.; Sargent,D.R. et al; Crystal Structure of the Nucleosome Core Particle at 2.8a Resloution, *Nature* **1997**, *389*, 251-260.

55) Luger, K. R., T.; The Histone Tails of the Nucleosome, *Current opinion in genetics and development* **1998**, *8*, 140-146.

56) Mann, S. M., Burkin,D.J., Grin,D.K., et al; A Fast, Novel Approach for DNA Fibre-Fluorescence in Situ Hybridization Analysis, *Chromosome Research* **1997**, *5*, 145-147.

57) Marko, J. F., Poirier,M.G.; Micromechanics of Chromatin and Chromosomes, *Biochemistry and Cell Biology* **2003**, *81*, 209-220.

58) Mishra, N. C.; John Wiley and Sons, 2002, pp 5.

59) Morrison, T. B. Quantification of Low-Copy Transcripts by Continuous Sybr Green 1 Montioring During Amplification, *Biotechniques* **1998**, *24*, 954-962.

60) Moser, H., Dervan,P.; Sequence-Specific Cleavage of Double Helical DNA by Triple Helix Formation, *Science* **1987**, *238*, 645-648.

61) Muller, S. E., M.; Burggraf,E., et al; Immunochemical Detection of Changes in Chromatin Subunits Induced by Histone H4 Acetylation, *The EMBO journal* **1982**, *1*, 939-944.

62) Muller, S. I., A.; Couppez,M., et al; Specificity of Antibodies Raised against Triacetylated Histone H4, *Molecular Immunology* **1987**, *24*, 779-789.

63) Nakayama, J., Rice,J.C., Strahl,B.D.; Role of Histone H3 Lysine 9 Methylation in Epigenetic Control of Heterochromatin Assembly, *Science* **2001**, *292*, 110-113.

64) Noma, K., Allis, C.D., Grewal,S.I.; Transitions in Distinct Histone H3 Methylation Patterns at the Heterochromatin Domain Boundaries, *Science* **2001**, *293*, 1150-1155.

65) Norgard, M. V. Molecular Assessment of S1 Endonuclease-Resistant Snapback Hairpin Loops Generated by DNA Polymerase I During the in-Vitro Nick Translation Reaction, *Applied biochemistry and biotechnology* **1985**, *11*, 1-15.

66) Ohta, T., Gray,T.A., Rogan, P.K., et al; Imprinting Mutation Mechanisms in Prader-Willi Syndrome, *American Journal of Human Genetics* **1999**, *64*, 397-413.

67) O'Neill, L. P., Turner,B.M.; Histone H4 Acetylation Distinguishes Coding Regions of the Human Genomefrom Heterochromatin in a Differentiation-Dependent but Transcription Independent Manner, *The EMBO journal* **1995**, *14*, 3946-3957.

68) Peters, A. H., Mermoud,J.E., O'Carroll,D.; Histone H3 Lysine 9 Methylation Is an Epigenetic Imprint of Facultative Heterochromatin, **2001**, *30*, 77-80.

69) Pfeffer, U. F., N.; Vidali,G.; Availablility of Hyperacetylated H4 Histone Intact Nucleosomes to Specific Antibodies, *The Journal of Biological Chemistry* **1986**, *261*, 2496-2498.

70) Poirier, M., Eroglu,S., Chatenay,D., et al; Reverible and Irreversible Unfolding of Mitotic Chromosomes by Applied Force, *Molecular Biology and cell* **2000**, *11*, 269-276.

71) Robyr, D., Grunstein,M.; Genomewide Histone Acetylation Microarrays, **2003**, *31*, 83-89.

72) Rogan, P., Cazcarro,P.M., Knoll,J.M.; Sequence-Based Design of Single Copy Genomic DNA Probes for Fluorescence in Situ Hybridization, *Genome Research* **2001**, *11*, 1086-1094.

73) Rozalski, M. L., L,l Ruiz-Carrilo,A.; Monoclonal Antibodies against Histone H5, *The Journal of Biological Chemistry* **1985**, *260*, 14379-14385.

74) Sauve, D. M., Anderson,H.J., Ray,J.M.; Phosphorylation Induced Rearrangement of the Histone H3 Nh2 Terminal Domain During Mitotic Chromosome Condensat, *Journal of Cell biology* **1999**, *145*, 225-235.

75) Schultz, P., Taylor,J., Dervan, P.; Design Synthesis of a Sequence-Specific DNA Cleaving Molecule. (Distamycin-Edta)Iron(Ii), *Journal of American Chemical Society* **1982**, *104*, 6861-6863.

76) Schwarzacher, T., Heslop-Harrison,P., *Practical in Situ Hybridization*; Springer: Norwich, UK, 2000.

77) Sealey, P. G., Whittaker,P.A.,Southern,E.M.; Removal of Repeated Sequences from Hybridization Probes, *Nucleic Acids Research* **1985**, *13*, 1905-1922.

78) Simpson, R. T. In Vivo Methods to Analyze Chromatin Structure, *Current opinion in genetics and development* **1999**, *9*, 225-229.

79) Sluka, J., Horvath,S., Bruist,M., et al; Synthesis of a Sequence-Specific DNA-Cleaving Peptide, *Science* **1987**, *238*, 1129-1132.

80) Smith, C. M., Gafken,P.R., Zhang,Z.,et al; Mass Spectrometric Quantification of Acetylation at Specific Lysines within the Amino-Terminal Tail of Histone H4, *Analytical Biochemistry* **2003**, *316*, 23-33.

81) Solomon, M. J., Larsson,P.L., Varshavsky,P.; Mapping Protein-DNA Interactions in Vivo with Formaldehyde: Evidence That Histone H4 Is Retained on a Highly Transcribed Gene, *Cell* **1988**, *53*, 937-947.

82) Srebreva, L. Z., J.; Specificity Studies on Anti-Histone H1 Antibodies Obtained by Different Immunization Methods, *FEBS Letters* **1986**, *200*, 123-127.

83) Strahl, B. D., Allis, D.; The Language of Covalent Histone Modifications, *Nature* **2000**, *403*, 41-45.

84) Tamaru, H., Selker,E.U.; A Histone H3 Methyltransferase Controls DNA Methylation in Neurospora Crassa, *Nature* **2001**, *414*, 277-283.

85) Taveay, M. Quantification of Splice Variants Using Molecular Beacon or Scorpion Primers, *Analytical Biochemistry* **2002**, *305*, 227-235.

86) Teo, I. A. C., J.W.; Light Cycler Qpcr Optimization for Low Copy Number Target DNA, *Journal of Immunological Method* **2002**, *270*, 119-133.

87) Thorne, A. W., Kmiciek,D., Michelson,K.; Pattern of Histone Acetylation, *European Journal of Biochemistry* **1990**, *193*, 701-713.

88) Turner, B. M., Fellows,G.; Specific Cell-Cycle Related Use of Histone H4 Acetylation Sites in Mammalian Cells, *Eyr J Biochem* **1989**, *179*, 131-139.

89) Turner, B. M. Histone Acetylation and an Epigenetic Code, *Bioessays* **2000**, *22*, 836-845.

90) Turner, B. M. *Chromatin and Gene Regulation*, first ed.; Blackwell Science Ltd: Oxford, 2001.

91) Twyman, R. M. In *Adavanced Molecular Biology*; Bios Scientific: Oxford, UK, 1999.

92) Van Dilla, M. A., Deaven,L.L., Albright,K.L. et al; Human Chromosome Specific DNA Libraries: Construction and Availability, *Biotechnology* **1986**, *4*, 537-552.

93) Voet, D., Voet,J.G., Pratt,C.W.; ; John Wiley and Sons,Inc.: NY, 1998, pp pp188.

94) Wallrath, L. L., Swede,M.J., Elgin,S.C.R.; In *A Practical Approach*; Gould, H., Ed.; Oxford University Press: Oxford, 1998, pp 63.

95) Waterborg, J. H. Dynamics of Histone Acetylation in Vivo. A Function for Acetylation Turnover?, *Biochemistry and Cell Biology* **2002**, *80*, 363-378.

96) Wolffe, A.; Academic Press: Cambridge, 1998, pp 50-51.

97) Wolffe, A., Marjori,A.M.; Epigenetics:Regulation through Repression, *Science* **1999**, *286*, 481.

98) Yalow, R. S. B., S.A.; Assay of Plasma Insulin in Human Subjects by Immunological Methods, *Nature* **1959**, *184*, 1648-1649.

99) Zhang, K., University of the Pacific, 2000.

100) Zhang, L., Eugeni,E., Parthun,M., et al; Identification of Novel Histone Post-Translational Modifications by Peptide Mass Fingerprinting, *Chromosoma* **2003**, *112*, 77-86.