STUDIES IN DETECTION LIMITS AND SELECTIVITY OF PROBES USED FOR THE ASSESSMENT OF HISTONE ACETYLATION AT SPECIFIC LOCI

by

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ABSTRACT

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Highly sensitive and selective probes for screening histone acetylation at particular loci are necessary. This work describes the design of selective DNA probes for the identification of specific chromosomal regions and highlights the importance of signal amplification for probes of less than 2 kilobases (kb), and the effect that particle size used for the amplification may have on its ability to bind to the target. Single copy FISH probes were designed for the identification of target regions, and generated by PCR amplification. Probe characterization was done by nick translation labeling with biotin or digoxigenin and detection with avidin or anti-digoxigenin. The probes proved to be selective, with detection efficiencies between 50 and 90%. Different labeling approaches were evaluated for the use of 3D DNA structures (dendrimers) to amplify the scFISH probe fluorescent signal. Low signal amplification of a 2.4 kb probe with anti-biotin dendrimers suggested that steric hindrance might keep dendrimers from binding to target probes. Qualitative studies of samples immobilized in polyacrylamide by near confocal microscopy demonstrated that after incubation with dendrimers there were biotins still available for binding. Furthermore, detection of higher number of quantum dots (15 nm in diameter) bound to probe than dendrimers (90 nm diameter) suggested that particle size will have to be considered when designing a method for signal amplification.

For the detection of histone acetylation, antibodies against H4-Ac16 and H4-nonAc were generated by conjugation of the target peptide to PPD. After affinity purification, the cross-reactivity of these antibodies to the non-antigenic peptide was ~8%. Immunofluorescence experiments with commercial and in-house antibodies on chromatin extended fibers agreed with the "zip" model of acetylation for H4, where the order of acetylation is from lysine 16 to lysine 5. It was infered from theoretical calculations and previous reports that the study of acetylation changes at regions of ~2.4 kb would require detection of modifications in 28 histone molecules, indicating the need for signal amplification for the determination.

To my parents

Gerardo Alberto Mora López y Flor de María Hererra Quesada. For their endless love and support. For molding me into the person I am today.

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"Puedo escribir los versos más tristes
esta noche...
...Porque en noches como esta la tuve
entre mis brazos mi alma no se contenta
con haberla perdido.
Aunque este sea el ultimo dolor que ella
me causa, y estos sean los ultimos
versos que yo le escribo."
Pablo Neruda (Poema No. 20)

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1. INTRODUCTION

1.1 DNA Organization in Eukaryotes

Almost all the DNA from eukaryotes is found in the nucleus as chromatin. In chromatin the DNA is associated mostly with histones and smaller amounts of other types of proteins. Histones have an unusually high content of the basic amino acids lysine and arginine that are positively charged near neutral pH. The positive charges from the histones interact with the negative charges from the DNA phosphate backbone stabilizing the DNA structure through the formation of nucleoproteins.[1]

There are five types of histones, distinguished by their size, net charge, relative content of lysine and arginine and solubility properties: H1, H2A, H2B, H3 and H4. Two molecules each of H2A, H2B, H3 and H4, form the structure around which the DNA superhelix (approximately 146 bp) wraps 1.8 times.[2] This DNA-protein complex is referred to as the nucleosome.[3] Nucleosomes are particles about 10 nm in diameter,[4] linked to each other by H1 bound to 40 to 70 bp of linker DNA, helping to further compact the "beads on a string" into solenoidal or cross-linker structures (fibers) of 30-33 nm in diameter. At this point the DNA has been compacted ~40 fold compared to its linear dimension. The fibers then form loops, and the loops fold into metaphase chromosomes,[5] for an overall packing ratio of about 10,000. Figure 1.1 shows the different levels of DNA compaction up to the metaphase stage. These different states of condensation will become important in the determination of histone modifications.

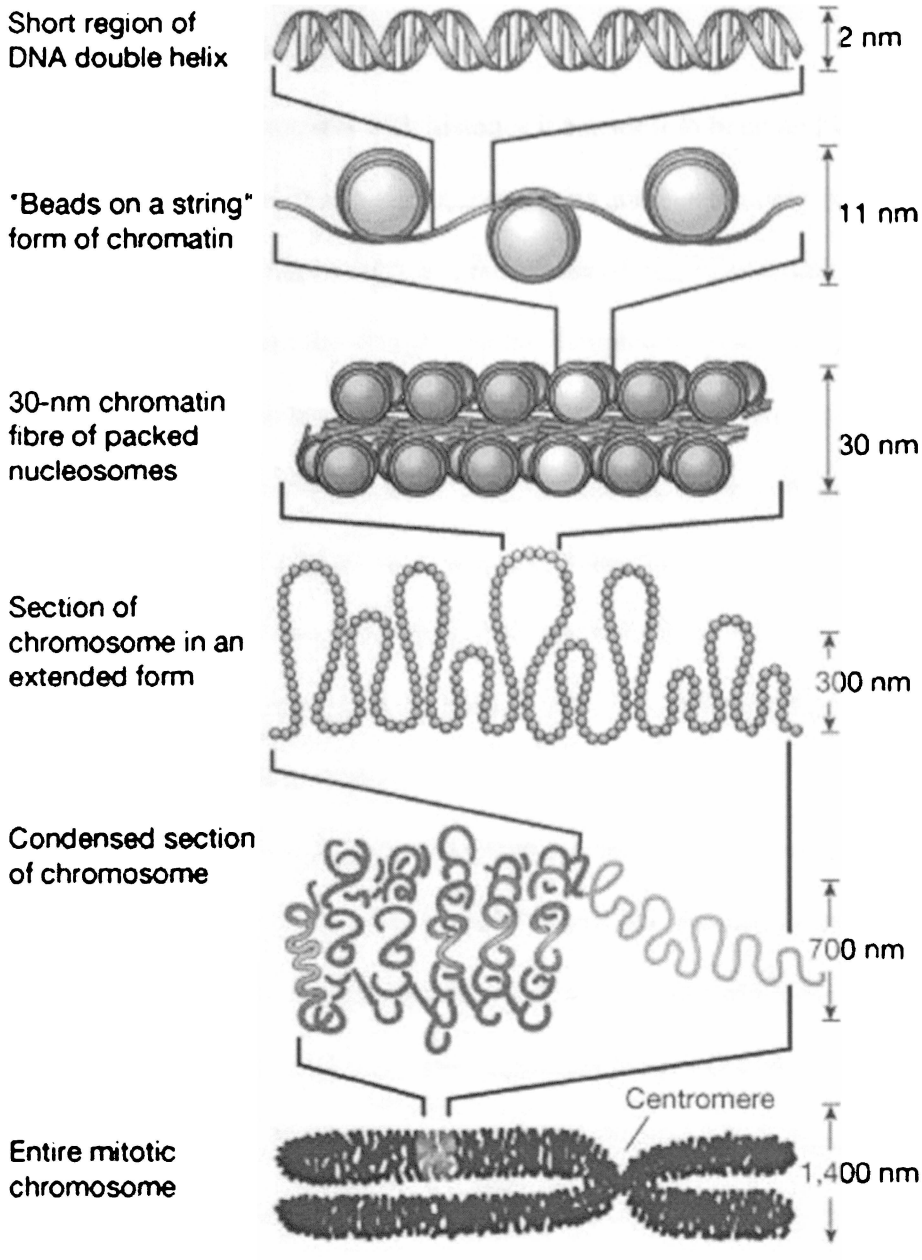

Figure 1.1. Different levels of DNA compaction, from double helix to metaphase chromosome. Printed with permission from Felsenfeld, G. and Groudine, M *Nature*, **2003**, *421*, 448-453.

1.2 Impact of Histone Modifications

When DNA interacts with histones it causes it to bend and bulge, changing the periodicity of the base pairs. These interactions and electrostatic shielding by the positively charged histones constrain the access of non-histone proteins to the DNA. But the interactions and the charges can be disrupted by post-translational modifications of some amino acid residues. The reactions involved are: acetylation, methylation, phosphorylation, and ubiquitination. Of these modifications acetylation has generated a lot of interest since it was correlated to gene activity.[6] When the lysines in histone tails are acetylated, their positive charge is neutralized. Without a charge the histone tails bind less closely to the DNA, allowing the transcription machinery to have better access to the DNA.

The amino acids in the histone tails can be modified *in vivo* by specific enzymes: histone acetyltransferases (HATs), and the histone deacetylases (HDACs). There are many types of each class, for example cytoplasmic histone acetylation is done by B-type HATs, and the A-type HATs are involved in nuclear and transcription related acetylation.[7] There are also many HDACs known to interact with repressors of gene transcription and factors required for chromosome and gene silencing.[8] Acetylation or deacetylation occurs as part of a balance between the activity of the two classes of enzymes. Katan-Khaykovich and Struhl[9] found that recruitment of HDACs or HATs to a well defined promoter caused acetylation to change in the promoter region, but once the recruiting protein was dissociated, the region returned to a steady-state of histone acetylation in a few minutes.

1.3 Detection of Histone Acetylation

1.3.1 Antibody Assays

Briggs and coworkers[10] analyzed nuclear extracts from mutant yeast carrying specific gene deletions and probed them with antibodies against methylated H3 lysine residues, and identified a gene product essential for methylation of lysine 79 of H3. Belyaev and colleagues[11] studied histone H4 acetylation in plant heterochromatin during the cell cycle by indirect immunofluorescence of chromosome preparations dropped on slides. With this approach they were able to study acetylation at the nucleolus organizing region (NOR), and heterochromatin regions. Boggs and coworkers[12] performed immunofluorescence assays on metaphase chromosomes with antibodies against unacetylated or diacetylated H3 and found reduced levels of H3 acetylation on the inactive X chromosome in human females. This method would not be suitable for the study of specific chromosomal regions on metaphase spreads, due to low resolution; it is only suitable to assess overall acetylation.

1.3.2 Radiometric Assay

Waterborg and Matthews[13] looked at acetylation patterns in *Physarum polycephalum* (slime mold) during replication and transcription. To grow the cells, they used medium with sodium [^{3}H]acetate, and another batch in [^{3}H]lysine (to identify newly synthesized histones), then isolated the histones either in S or G phase, and separated them by gel electrophoresis. They found the acetylation patterns during replication to be quite different from those during transcription. Two years

later Pesis and Mathews[14] studied the turnover rate at different acetylation sites of H4 during replication and transcription by the same method.

1.3.3 Chromatin Immunoprecipitation (ChIP) Assay

Hecht and Grunstein[15] studied the association of a regulatory protein with specific loci. They crosslinked DNA and protein, sheared the cellular preparation to obtain small fragments, immunoprecipitated the fragments associated with the protein of interest and then used PCR to determine the presence of specific loci (See Figure 1.2). In this example the antibodies were against the regulatory protein and not histones, but other groups[16-18] have taken similar approaches to evaluate levels of histone acetylation at particular loci. Collas and coworkers[17] actually correlated their ChIP assay results to those obtained by analysis of chromatin extended fibers using immunofluorencence (detection of H4 acetylation) and FISH (detection of transcriptionally active genes).

1.3.4 Mass spectrometry (MS)

It has been well documented that histone acetylation occurs at different lysine residues, but the role of each modification remains unclear. As pointed out by many, it is important to correlate these site-specific modifications with cellular events, and even modifications other than acetylation. Prior to the development of mass spectrometry methods for the determination of acetylation sites, determinations were

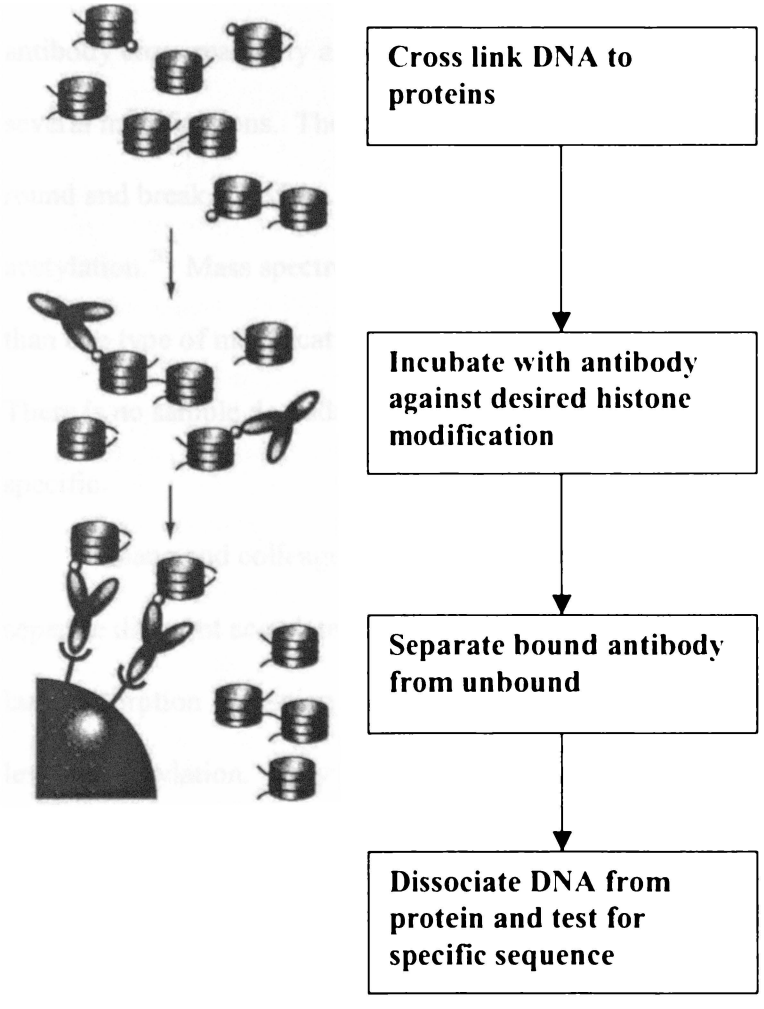

Figure 1.2. Flow chart and diagram indicating the steps involved in the chromatin immunoprecipitation assay (ChIP), used in the evaluation of histone acetylation levels at specific loci. Modified from Turner, B. M. *Chromatin and Gene Regulation: Molecular Mechanisms in Epigenetics*; Blackwell Science Ltd: Malden, 2001.

done with antibodies or by Edman microsequencing.[19] The former has the problem of antibody cross reactivity and does not allow for simultaneous determination of several modifications. The latter has problems due to decreased sensitivity with each round and breakage of the peptide backbone during removal of the terminal acetylation.[20] Mass spectrometry has the advantage of being able to determine more than one type of modification on the same sample (acetylation, methylation, etc.). There is no sample degradation during the determination and the method is very specific.

Zhang and colleagues[21] used reverse-phase HPLC and gel elctrophoresis to separate different acetylated isoforms of H4 from HeLa cells and used matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) MS to characterize the level of acetylation. They determined the sites of acetylation by nanoelectrospray tandem MS. Kim and coworkers[22] reported a similar study the same year, on HeLa cells treated with trichostatin A (an HDAC inhibitor). Cocklin and Wang[23] not only identified acetylation sites, but also methylation sites on mouse histone H3, also by MALDI TOF and nanoelectrospray ionization tandem MS. They used 10^8 cells for the assay; but the quantification of acetylation at specific lysines required sample analysis by collision-induced dissociation (CID) tandem MS, and 10-100 times more sample. Smith and colleagues[20] did such a study, they were the first to report direct measurement of endogenous levels of acetylation at individual lysine residues within the amino-terminal tail of yeast H4. Zhang and coworkers[24] used CID to differentiate

between peptides containing acetylated or tri-methylated lysines H3 in chicken erythrocytes and human HeLa cells.

1.4 Determination of Histone Acetylation at Particular Loci

Because of its linkage to gene activation, determination of histone acetylation at particular loci has become of great interest to the scientific community. The work presented in this dissertation will discuss and study the issues related to detection of the histone modifications and detection of a specific chromosomal region, separately, to understand their own limitations in terms of sensitivity, selectivity, and factors such as DNA condensation that may affect the results; and from there decide what is the best approach to combine both methods.

From the methods mentioned above for detection of the histone modifications, only two can be used to study acetylation at particular loci, the ChIP assay and immunofluorescence on stretched chromatin fibers. The other methods only give information about the histone acetylation of the entire population, and analysis by MS would require isolation of the locus of interest prior to the determination. Thus, chapter 5 focuses in the use of antibodies to assess histone acetylation, their characterization, the need to do the analysis on decondensed DNA (stretched chromatin fibers), and requirements for MS analysis.

A good model disease for the study of histone acetylation at particular loci is acute promyelocytic leukemia (AML-M3). There have been reports of patients going into remission with retinoic acid and HDAC inhibitor therapy.[25-27] Therefore, it is

important to understand how the genes involved in the translocation are regulated by acetyl-modifications in the histone environment. As mentioned above, the ChIP assay can be used to assess histone acetylation at particular loci. The disadvantage is that the method is based in the precipitation of all DNA associated with acetylated or non-acetylated histones (according to the antibody used), so finding the specific loci among the precipitated histone pool is like trying to find a needle in a haystack. Because many genes are associated with acetylated/non-acetylated histones, it might be easier if done with organisms with a genome simpler than the human. As mentioned by Turner,[2] for some genes the transient changes in acetylation might be in 1-2 nucleosomes and involve specific histones. Therefore the importance of generating short and specific DNA probes for colocalization of DNA sequence and changes of histone acetylation is obvious. The detection of specific genomic regions is discussed below, but prior to that it is important to introduce the model disease to understand which regions have to be analyzed and why.

1.5 Acute Promyelocytic leukemia AML-M3

1.5.1 Description of the Disease

Nearly all forms of cancer, leukemia among them, are caused by a series of malignant transformations (DNA mutations) in a single cell that alter its ability to grow and differentiate as a normal cell. In the case of acute promyelocytic leukemia, there is a blockage in the differentiation process of stem cells, at the promyelocyte stage, causing an increase in the amount of promyelocytes.[28] Figure 1.3 shows a diagram of

the differentiation process, where under normal circumstances, stem cells differentiate into cells such as platelets and erythrocytes. Promyelocyte proliferation in the bone marrow eventually diminishes erythrocyte and platelet production, causing anemia and thrombocytopenia, which may result in easy bruising and excessive bleeding.[29-32] Eventually if the bone marrow cannot hold all promyelocytes, they migrate into the peripheral blood from where the cancerous cells are able to spread to other organs.

Acute Promyelocytic Leukemia is a subtype of Acute Myeloid Leukemia (AML). It was designated M3 by the French-American-British classification.[33] Figure 1.4 shows the most prominent (50-80%) mutation among patients with AML-M3, a translocation between the long arms of chromosome 15 (15q22) and chromosome 17 (17q21),[34-37] identified in 1977 by Rowley and referred to as t(15;17). In most cases, it is a balanced reciprocal translocation. This means that there is a single break in the DNA in two different chromosomes resulting in an exchange of DNA between the two chromosomes.

1.5.2 Genes involved in the translocation

The genes involved in t(15;17) were identified in 1990 as the promyelocytic leukemia gene (PML) on chromosome 15 and retinoic acid receptor alpha gene (RARA) on chromosome 17.[38] The breakpoint on chromosome 15 occurs in the 3' region of the PML gene. Three breakpoints have been clearly defined: bcr1, bcr 2,

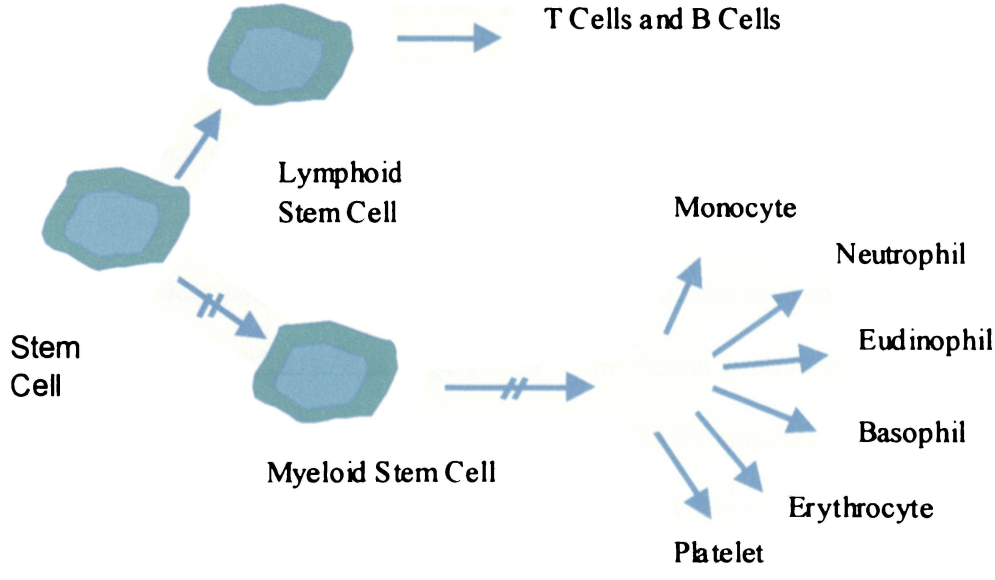

Figure 1.3. Cell differentiation in normal humans. The diagram shows only some of the cells product of the differentiation process. In AML-M3 there is a block in the differentiation process, represented in the diagram by the crossed arrows.

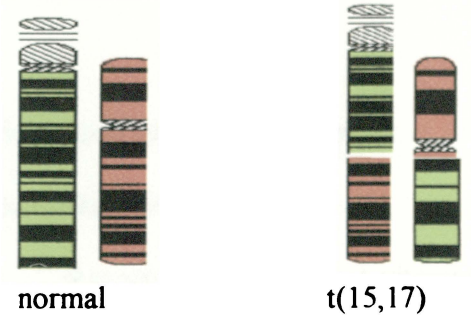

Figure 1.4. Representation of chromosomes 15 (green) and 17 (orange) from a normal cell and from a cell with AML-M3.

and bcr3. The most common is bcr1, between exons six and seven. The other breakpoints, bcr2 and bcr3, are located in exon 6 and intron 3, respectively.[39-43] The breakpoint on chromosome 17 occurs in the 5' region of the RARA gene, mainly in the first and second introns.[39, 41-44] The RARA gene is six exons long, so the break results in the retention of the last four or five exons, depending on the breakpoint location. In most cases the fusion gene consists of the first six exons of PML and the last four exons of RARA (See Figure 1.5). The fusion protein PML/RARA is the product of the translocation and is mainly localized in the nucleus. To understand the effects of this protein it is crucial to first understand the role of PML and RARA proteins alone.

There are reported cases of AML-M3 patients with other translocations. These translocations always involve RARA (chromosome 17), instead of PML, the other gene has been NPM (chromosome 5), or NuMA (chromosome 11), both in less than 1% of the cases, or gene PLZF (chromosome 11), present in nearly 5% of patients.[45]

1.5.3 Role of the normal genes

1.5.3.1 Role of RARA

Retinoic acid (RA) is a vitamin A derivative that plays an important role in cell differentiation, proliferation and development. Three RA receptors have been identified: RARα, ß, and γ. These receptors activate or repress gene expression, in a

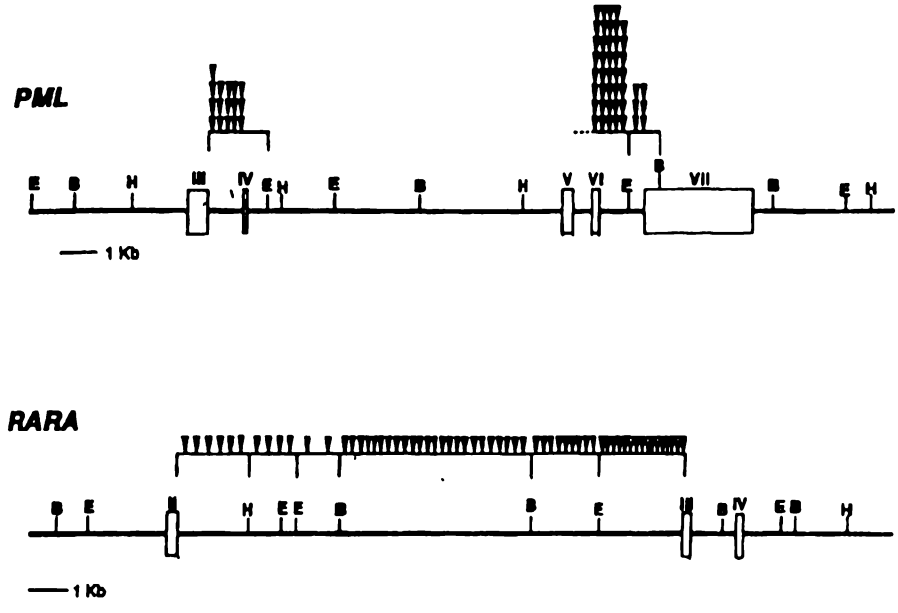

Figure 1.5. Location of the breakpoints in PML and RARA according to Yoshida *et al.*[39] The vertical arrows indicate the location of breakpoints determined by Southern blot and reverse transcription-PCR (RT-PCR). The white boxes indicate exons.

ligand-inducible way, through binding to hormone-responsive elements located in the promoter region of target genes.[44]

Retinoic acid receptor alpha (RARA) proteins usually homodimerize with other RARA proteins or form heterodimers with RXR (retinoid "X" receptor that binds 9-cis-RA) proteins and each dimer binds to RA response elements in the promoter regions of various genes. The RARA/RXR heterodimer binds to DNA, and then to N-CoR (nuclear Receptor Co-Repressor), which binds Sin3, an intermediary protein that serves as a bridge to bind HDAC (Histone Deacetylase). HDAC removes the acetyl groups and represses transcription.[46] RARA/RXR has been shown to be more responsive to transcription activation through binding of RA than the RARA homodimer.[31, 38]

1.5.3.2 Role of Normal PML

This gene is located in band 22 of the long arm of chromosome 15, and contains nine coding exons. There are 16 isoforms of the protein, product of alternative splicing of the mRNA, but their function is not known. PML is highly expressed in G1 and S phases, and it has been suggested that it inhibits cellular growth.[47] This implies that the lack of normal PML could result in cell proliferation.[38]

1.5.4 Fusion protein

In most cases the fusion protein has the amino acid sequence that corresponds to the first six exons of PML and the last four exons of RARA. There is evidence suggesting that the PML/RARA fusion protein binds the normal co-repressor complex (N-CoR / Sin3/ HDAC) with a higher affinity than RARA/RXR. This causes physiological levels of RA to be insufficient to trigger dissociation of the complex, resulting in continued repression of the RARA target genes and a blockage in myeloid differentiation.[38]

The transformed promyelocytes are somatic cells; this means they have two copies of each chromosome. Each leukemic cell will have a copy of the fusion gene, a copy of the reciprocal fusion gene (RARA/PML), one copy of the wild-type PML, and one copy of the wild-type RARA. These leukemic cells will also have two copies of the wild-type RXR gene, thus setting up a competition for protein binding. The dimer interactions divert the wild-type PML, RARA, and RXR from their desired DNA binding sites. It seems that treatment with therapeutic levels of RA induces a conformational change in the fusion protein that dissociates it from normal co-repressor complex, promoting gene transcription and eventually cell differentiation.[48]

1.6 Reverse Transcription Polymerase Chain Reaction (RT-PCR) for the Detection of t(15,17)

Because t(15;17) is present in almost 80% of the patients with AML-M3, its detection is important for diagnosis, specially because patients with t(15;17) respond

to all-trans retinoic acid therapy,[48, 49] while patients with t(11;17) do not.[50] The detection is also necessary for characterization of cell lines used as AML-M3 cell models.

Borrow and colleagues[51] developed a diagnostic test for AML-M3 by reverse transcription polymerase chain reaction (RT-PCR). They found that detection of PML/RARA mRNA by PCR was faster, more sensitive and required less material than detection by Southern blot.[41] PCR amplification of the PML/RARA junction is done first by in vitro transcription of the RNA to cDNA, and the cDNA is then amplified by PCR. The RT-PCR method identified different isoforms of transcript PML/RARA, depending on the location of the breakpoint in PML. The same method was used by others[32] to determine if molecular variants in AML-M3 could influence the clinical outcome of the disease.

1.7 Fluorescence in situ Hybridization (FISH)

For the purpose of this chapter and for comparison with other methods used in the detection of t(15;17), it is important to know that FISH (described in chapter 2)is a technique where DNA probes of sequences complementary to those of the chromosomal regions of interest are hybridized and then detected by fluorescence, thus enabling the localization of a region on interphase or metaphase chromosomes. Whole chromosome painting probes (see chapter 2 for description) or commercially available probes specific for the chromosome 15 and 17 breakpoints are frequently

used[36, 52] in the diagnosis of AML-M3 because of their ease in facilitating detection of the translocation.

1.8 Importance of FISH in diagnosis

As mentioned above, it is important to identify the type of translocation, since not all respond to RA therapy. Analysis by RT-PCR in human samples could be cumbersome due to the complexity of the genome, compared to yeast. For the detection of rearrangements by PCR, the breakpoints have to be clustered in a narrow genomic region, because the amplification of large fragments (greater than a few kilobases) is difficult, while FISH can be used to detect regions of several hundred kilobases.[53] FISH allows delineation of specific chromosomes in interphase cells[54] and facilitates the identification of metaphase chromosomes without the need of karyotyping. Even if the chromosomes involved in the translocation are not known, FISH can be used to identify them. The region of interest is microdissected, amplified with a universal primer, and the product is then hybridized to normal and abnormal cells to determine which chromosomes were present in the microdissected region.[55] FISH allows analysis of genomic regions from 1 cell, making it a very sensitive technique, but as discussed in chapter 2, the study of promoter regions, requires the use of short and specific DNA probes (~200 bp). It is currently difficult to detect regions of less than 2 kb. So, chapter 3 focuses on the amplification of the fluorescent signal of DNA probes shorter than 3 kilobases, required for the identification of short regions such as promoters. The results from the studies of

signal amplification raised many questions about the effect that particle size may have on binding to biotins incorporated into DNA probes, so this issue was studied on a third project by near confocal microscopy. The last project presented involves the generation, purification, characterization, and evaluation of polyclonal antibodies to determine the level of H4 acetylation on chromatin extended fibers from an AML-M3 derived cell-line.

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2. DETECTION OF t(15;17) WITH SINGLE COPY FISH (SCFISH) PROBES

2.1 INTRODUCTION

DNA probes can be utilized in assays to selectively recognize sequences of interest,[1-3] and in that way help understand cellular processes at the molecular level, in particular diseases. Fluorescence *in situ* Hybridization (FISH) is one of these assays. It is based on the hybridization of nucleic acid probes (labeled with fluorophores or haptens) to a DNA target. The target and double stranded DNA probes have to be denatured, in other words the hydrogen bonds present in the double helix have to be broken, so that probe and target can hybridize. After hybridization, unbound probes are removed. If the probes are labeled with fluorophores they can be directly visualized, but if the probes are labeled with a hapten, detection is done with a fluorophore labeled antibody, avidin, or streptavidin.[4]

FISH has played an important role in cancer diagnosis, particularly in the identification of subtle chromosomal rearrangements;[4] gene mapping;[1, 5, 6] study of specific chromosomal regions, or microdeletions;[1] and DNA sequence abundance (in the case of repetitive sequences). Conventional FISH commonly uses cloned genomic probes (14 kilobases (kb) to megabases in length),[7] tandem repeat probes and whole chromosome painting probes. They all include repetitive DNA sequences that require blocking during the hybridization and because of their length do not allow the study of sequences of less than a few thousand bases.

2.1.1 Probes

2.1.1.1 Large Recombinant Probes

These probes are prepared by inserting the sequence of interest into a cloning vector, in order to generate multiple copies. In this way, cloning has facilitated genomic mapping and the structural and functional analysis of large genes.[8] As mentioned above, the size of cloned probes ranges from 14 kb, if derived from a lambda phage, to more than 1 megabase (Mb), if prepared from yeast artificial chromosomes (YACs).[1, 4, 7, 9] These probes have the advantage of having an intense hybridization fluorescence and, due to their length, many fluorophores can be directly incorporated. There are few probes, available commercially, compared to the genome size, but they can save the investigator a great deal of time. Because of their large size recombinant clones are not sensitive to detection of small mutations/ rearrangements. deGuzzo *et al*[6] have pointed out another limitation of long probes: they result in very high background when used to study specific regions.

2.1.1.2 Tandem Repeats

Tandem repeat probes are designed from sequences known to be repeated head to tail, and that are adjacent to each other. These repeating units range from <5 to >200 bp, and are mainly found in centromeres and telomeres. Some of these repeat probes form clusters of up to 20 kb in length.[10] Therefore, their hybridization signal is very bright, but as with cloned probes, they are not sensitive to small mutations.

They are typically used as controls to identify chromosomes, or to study chromosomal organization, evolution, and role in cellular processes.[11, 12]

2.1.1.3 Whole Chromosome Painting Probes

Whole chromosome painting probes are derived from flow sorted or microdissected chromosomes, and as their name suggests they "paint" the majority of the chromosome, so they are very easy to visualize. These probes have been used to identify t(15;17),[13, 14] but just like cloned and tandem repeat probes they are not sensitive to small chromosomal changes.

2.1.1.4 ScFISH Probes

The concept of using the human genome sequence to computationally select single-copy segments within regions of ~100 kb was introduced and validated by Rogan and Knoll.[1, 15] Briefly, the DNA target sequence or gene of interest is compared to the rest of the human genome to eliminate sequences that show high homology (homologous genes show similarities between their sequences, and share a common evolutionary ancestor).[10] The sequence similarity could become a problem if the designed scFISH probe also binds to this homologous sequence. To avoid this problem, a homology search is performed utilizing software such as BLAST (Basic Local Alignment Search Tool). The sequence of interest is then compared against several databases. The output will show the regions of sequence that have homology to any other sequences. These regions are then avoided in the design of the scFISH

probe to make sure that the same sequence (or one of high homology) is not present in other regions, thereby increasing its selectivity.

Repetitive DNA constitutes more than 50% of the total human genome,[16, 17] and is simply DNA that is present more than once in the genome. In order to find the single copy regions, after selecting sequences that show low homology to others, the sequences are compared with a database of repetitive sequences. The regions with repetitive sequences are then discarded. The intervals left are sorted according to length, so that they can be selected according to the application. Intervals longer than 2 kb are easier to detect, but one could be interested in studying smaller segments of a particular gene. If no intervals longer than 2 kb are found, an alternative is to combine several probes that together will span chromosomal targets longer than 2 kb.

As mentioned in the introduction (chapter 1), the ultimate goal is to analyze histone modifications at the PML and RARA promoters. In order to do the analysis, and determine if any of the normal genes are disrupted, a key point is to differentiate the normal genes from the abnormal ones. Therefore, the objective of the work presented in this chapter is to design single-copy FISH (scFISH) probes of less than 4 kb, to identify the rearrangement between chromosomes 15 and 17 present in patients with Acute Promyelocytic Leukemia (AML-M3), and in that way help differentiate abnormal from normal genes. These scFISH probes range in length from 900 base pairs (bp) to 10 kb, which allows higher-resolution analysis. They also lack repetitive sequences, thus no Cot-1 DNA (repetitive fraction of genomic DNA) is required in the hybridization mixture. The signal from probes 2 kb or smaller in length is

difficult to detect; furthermore, amplification and purification of probes greater than 3 kb can be troublesome. Therefore the best strategy so far was to prepare different scFISH probes in the region of interest and combine them in order to obtain a signal of greater intensity than that of single 2 kb probes. Chapter 3 addresses the evaluation of strategies to amplify the signal of probes smaller than 2 kb, so that probes would not have to be combined in order to detect them, and smaller targets could be studied.

2.2 EXPERIMENTAL METHODS

A list of all buffers and their abbreviations is provided in Table 2.1. Unless otherwise stated, the water used to prepare the buffers was purified with a Barnstead Nanopure II system. If used for the generation of probes or any hybridization steps the water was also autoclaved.

2.2.1 Cell Treatment

HT93A cells,[18] kindly provided by Dr. K. Toba, Niigata University, Japan, were grown in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA), containing 10% fetal bovine serum (Irvine Scientific), 10 Kunits/mL Penicillin sodium, 10 mg/μL Streptomycin sulfate, and 200 mM L-Glutamine (Life Technologies,

Table 2.1. Composition of buffers, and buffer and dye abbreviations.

Abbreviation	Composition
20X SSC	3 M NaCl, 0.3 M Na_3Citrate
4X SSC	600 mM NaCl, 60 mM Na_3Citrate
2X SSC	300 mM NaCl, 30 mM Na_3Citrate
DAPI	4',6-diamidino-2-phenylindole
Tris-HCl	Tris-(hydroxymethyl)aminomethane-HCl
QG	Contains guanidine thiocyanate, proprietary of Qiagen
PE	Proprietary of Qiagen
EB	10 mM Tris-Cl, pH 8.5
TE	10 mM Tris, 1 mM EDTA, pH 8.0
PBST	0.1 M PBS and 0.1% TritonX-100
0.1 M PBS	80 mM Na_2HPO_4, 20 mM KH_2PO_4, 150 mM NaCl, pH 7.4
BSA	Bovine serum albumin
1X SYBR Gold	10^4 dilution of stock solution of a unsymmetrical cyanine dye proprietary of Molecular Probes, OR
FITC	Fluorescein isothiocyanate
SDS	Sodium dodecyl sulfate

Rockville, MD), at 5% pCO_2 at 37 °C in humidified air, at 5-10 $x10^5$ cells/mL.

2.2.2 Mitotic Chromosome Preparation

This procedure was modified from several protocols.[19-22] Briefly, the HT93A cell suspension (10 mL) was transferred to a 15 mL centrifuge tube and incubated for 50 minutes in 0.1 μg/mL colcemid (Life Technologies) under the same conditions used for cell growth. Cells were then centrifuged for 10 minutes at 1200 rpm, and most of the supernatant removed, a small amount was left to resuspend the cell pellet. The cell suspension was slowly taken to 8 mL with 75 mM KCl, and incubated at 37 °C for 20 minutes. The centrifugation was repeated, and the cell pellet was resuspended in 10 mL of Carnoy's fixative (methanol:glacial acetic acid, 3:1). These last two steps (centrifugation and resuspension in fixative) were repeated at least 3 times. The last time cells were reconstituted in 2 mL of fixative.

Prior to the preparation of chromosome spreads, microscope slides were cleaned with 70% ethanol and wiped dry. A few drops of fixative were spread on top of the slide, immediately followed by 2-3 drops of the cell-fixed suspension, dropped from a height of a few centimeters. The fixative was evaporated over steam for a few seconds, and the spread was examined with a phase contrast microscope to assess cell density and quality of the metaphase cells (i.e. metaphase spreads).

2.2.3 Characterization of HT93A

The presence of t(15;17) on HT93A cells was evaluated with LSI® PML Spectrum Orange™/ LSI RARA Spectrum Green™ DNA probe (Vysis, Downers Grove, IL). Briefly, the cytological preparation (sample) was denatured for 5 minutes at 73 °C in 70 % formamide (Fisher Scientific, Pittsburgh, PA), 2X SSC and then dehydrated 1 minute in 70% EtOH, followed by 1 minute in 85% EtOH, then in 100% EtOH until the probe hybridization mixture was ready to apply. The probe hybridization mixture consisted of 1μL of PML/RARA probe, 2 μL of H_2O, and 7 μL of LSI hybridization buffer (dextran sulfate, formamide and SSC, pH 7.0, proprietary of Vysis). This mixture was mixed, and heated at 73 °C for 5 minutes and kept at 45-50 °C until ready to apply to the target. The slides were removed from 100% EtOH and air-dried, the hybridization mixture was applied to the denatured cells and covered with a plastic coverslip. The slides were incubated at 37 °C overnight. The following day the coverslip was removed and the slides immersed in 50% formamide/ 2X SSC at 46 °C for 10 minutes. This was repeated 3 times. The slides were then immersed in 2X SSC for 10 min and 2X SSC/ 0.1 % Triton X-100 for 10 min. For all the washes the slides were shaken for the first 3 seconds. Slides were air dried in the dark; stained with 50 μL of 1 μg/mL of DAPI (Sigma, St Louis, MO) in 2X SSC for 5 minutes, rinsed in 2X SSC and mounted with anti-fade solution (Vectashield, Vector Laboratories, Burlingame, CA)

2.2.4 Single Copy FISH Probes

2.2.4.1 Probe Design from the Human Genome Sequence

The chromosomal regions were selected from the genes involved in the translocation (PML and RARA) and two other regions telomeric of RARA (a region from gene TOP2A and the region between LOC125110 and TOP2A). The location of the breakpoints in PML and RARA was obtained from the literature.[14, 23-29] The PML probes were designed centromeric of the breakpoint. The RARA probes were designed in the breakpoint region as well as telomeric of the breakpoint. The contiguous genome sequences of interest were obtained from the Human Genome Draft (April 2001 and April 2002) available at the National Center for Biotechnolgy website[30]. The computer program Repeat Masker[31] was used to determine the location of repetitive sequences within the selected chromosomal regions. Basic Local Alignment Search Tool (BLAST)[32] was used to compare the regions of interest to all databases (GeneBank, EMBL, DDBJ, PDB, but no EST, STS, GSS or phase 0, 1, or 2 HTGS sequences) and discriminate those sequences with high homology from other chromosomal regions. The sequences with very low homology to other regions were further analyzed by custom software[15] to obtain single copy intervals. Intervals $\geq$ 900 bases were selected as targets for probe generation. The same software is capable of designing the primers used for PCR amplification of the target sequences. The parameters used for the selection were: melting temperature of 60-70 °C, (40-55)% of guanosine and cytidine (GC), and (80-90)% of the single copy interval as

minimum interval length. The primers were ordered from Integrated DNA Technologies Inc. (Coralville, IA).

2.2.4.2 Probe Generation

Probes were prepared from high molecular weight Human Genomic DNA by PCR (polymerase chain reaction) amplification. The reaction mixture contained ~980 ng of Human Genomic DNA (Promega, Madison, WI), 0.4 mM dNTPs, 1X *Pfx* amplification buffer (Invitrogen, Rockville, MD), 1X PCR enhancer solution, 1 mM $MgSO_4$, 0.2 μM on each primer (see Table 2.2 for sequence), and 1.25 units of Platinum *Pfx* DNA Polymerase, in a final volume of 50 μL. A Mastercycler thermocycler (Eppendorf, Westbury, NY) was used, with the lid kept at 98 °C so that no oil was used to cover the reaction mixture. PCR conditions were as follows: 94 °C for 2 minutes and 3 s; followed by 28 cycles of denaturation at 98 °C for 20 s, annealing at the primer optimal temperature (Table 2.2) for 5 minutes; and extension at 68 °C for 10 minutes. The reaction products were separated on a 0.8 % agarose gel at 75 volts for 2 hours, and visualized with ethidium bromide. The product was identified based on its length (number of basepairs) by comparison with a *Hind* III and *Eco*R I digested lambda DNA marker or a 100bp marker (Promega, Madison, WI), excised and extracted from the agarose gel with a QIAquick gel extraction kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Briefly, the

Table 2.2. List of primer sequence and melting temperature.

Primer	Sequence[i]	Tm (°C)[ii]
626-F	GAGACAGGGAAAGGAGCTGAGGCAGGAGGG	69.9
626-R	CCGTAGTTGGGGGAGGGGGAGACGCTTATC	69.6
660-F	CTGATAAGCGTCTCCCCCTCCCCCAACTAC	68.0
660-R	CCTTTCCCCCTTCCTGCTTCTCTCCTCTCC	68.1
255-F	TAAAGCCGAGTGGTGTGTG	55.9
255-R	GCAGACAGACATTTAGGAGGG	56.1
354-F	ACAGGAGAGCTGAGAAAGAC	54.8
354-R	GAAAGGAAGGAGACCAGGAC	55.4
737-F	CTTTCTCCACCATTACTCTCAC	53.9
737-R	TTCTTTAGCTCTTTGGCTCG	53.7
827-F	GTCTACCCCACATCTAACCCCCACTGAACC	66.7
827-R	CAACCTTGTGTGTCCACAGCATCTACTGCC	66.3
978-F	AGGACAGTGGCTGTGGGATGCAGAGGAGGG	67.4
978-R	CGGGGAGCATGGGAGTGTAAGGAAGAAGGGG	66.0

[i] All sequences written from 5' to 3'

[ii] Calculated value by the manufacturer

gel was weighted, put in a microcentrifuge tube and 3 volumes of QG buffer were added per volume of gel (100 mg ≡ 100 μL). This mixture was incubated at 50 °C for 10 minutes until the gel dissolved, and was then pipetted onto the QIAquick column. The sample was centrifuged for 1 minute at maximum speed, the flow-through was discarded, and 750 μL of PE buffer were added to the column. The column was centrifuged again and the flow-through discarded, this process was repeated once, and the column was placed in a clean microcentrifuge tube. The DNA was eluted with 30 μL of EB buffer.

2.2.4.3 PCR Reamplification

The PCR amplification product was separated on a low melt agarose gel, as explained above. The product band was excised, melted at about 65 °C and used as a template (instead of Human Genomic DNA) on a second PCR amplification. The product of the reaction was isolated from the mixture by ethanol precipitation.[19] Briefly, 13 μL of 3M NaOAc and 125 μL of ice-cold 100% EtOH were added to a PCR reaction volume of 50 μL. The mixture was incubated over night at –20 °C, and spun in a microcentrifuge tube at 4 °C for 20 minutes at maximum speed. The supernatant was discarded, the pellet was washed with ice-cold 100% EtOH, and centrifuged again for 10 minutes. The supernatant was carefully removed with a pipette and the pellet was air dried and resuspended in water or TE buffer.

2.2.4.4 Nick Translation

The concentration of the gel-extracted probes was estimated by direct comparison of the band intensity with a *Hind* III and *Eco*R I digested lambda DNA (Promega, Madison, WI). The nick translation reaction consisted of approximately 1μg of probe (DNA). 1X solution I (0.02 mM dATP, 0.02 mM dCTP, 0.02 mM dGTP, 50 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 μg/mL BSA), 0.05 mM digoxigenin-11-dUTP or biotin-16–dUTP (Roche Diagnostics, Indianapolis, IN), 20-40 U of DNA Polymerase I (Invitrogen, Life Technologies, Rockville, MD), and 0.8 U of DNAse I (Ambion, Austin, TX) in a final volume of 100 μL. The reaction was carried at 15 °C for approximately 2 hrs or until the probe fragments ranged between 200-500 bases. The size of the fragments was checked on an agarose gel with a 100 base-pair marker (Promega). Once the fragments had the desired size, the reaction was stopped by inactivating the enzymes with 3 μL of 0.5 M EDTA and heating 5 minutes at 70 °C. The probe was isolated by ethanol precipitation as explained on section 2.2.4.3, except that 2.4 μL of tRNA (8 mg/mL), and 2 μL of salmon sperm DNA (10 mg/mL) were added to the mixture prior to the ethanol and the NaOAc. The labeled probe was resuspended in 10 μL of H_2O or TE buffer.

2.2.4.5 Evaluation of Digoxigenin Incorporation

Probe 978 (labeled by nick translation) was diluted in water and pipetted onto a 0.45 μm Hybond-C extra, nitrocellulose membrane (Amersham, Piscataway, NJ), in

the middle of 2 cm^2 sections (drawn by pencil). Two sections were used, one with 1.68 ng of probe, and the second with 0.294 ng. The membrane was dried at 70 °C for 10 minutes, and then incubated in 50 mL of blocking solution (1% bovine serum albumin in PBST buffer) at 42 °C for 14 minutes, followed by 26 minutes at room temperature. The blocking solution was substituted by detection solution, 1:2560 monoclonal anti-digoxin-alkaline phosphatase conjugate (Sigma, MO) in 0.1 M Tris, 0.15 M NaCl, 1% BSA, and 1% FBS; and the membrane was incubated for 30 minutes at 37 °C. This was followed by 3 washes in PBST with gentle shaking. After the last washing the membrane was removed from the PBST buffer and 30 µL of 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-2-indolylphosphate (BCIP/NBT-purple, an alkaline phosphatase substrate from Sigma) were added to each section.

2.2.4.6 Characterization of the Probes on Metaphase Spreads

The hybridization mixture consisted of labeled probe to give a final concentration of 7-10 ng/µL, 50% formamide, 20% dextran sulfate, and 2X SSC (0.3 M NaCl, 0.03 M Na_3Citrate). The metaphase spreads were treated with 2X SSC at 39 °C for 15 minutes, heated in denaturing solution (70% formamide, 2X SSC) at 73 °C for 2 minutes to denature the target DNA, and dehydrated in ethanol. The hybridization mixture was heated at 75 °C for 8 minutes and left on ice until the slides were ready. Then the mixture was applied to the target, covered with plastic coverslips and incubated in a box with towels soaked in 2X SSC at 37 °C overnight.

After incubation the coverslip was removed and the slides washed in the following solutions: 2X SSC for 2 minutes, 0.5X SSC for 3 minutes in 3 different jars, and 2X SSC for 3 minutes in 2 different jars. In order to detect the probes by fluorescence, the slides were incubated with blocking buffer and avidin conjugated with FITC (Vector Laboratories) or anti-digoxigenin conjugated with Rhodamine (Roche Diagnostics, IN), in 1:400 and 1:100 mixtures respectively. The blocking buffer consisted of 1-3 % BSA, and 4X SSC. The incubations were done with 50 µL of the detection solution for 45 minutes at 37 °C with parafilm on top of the slides. The slides were washed in 2X SSC for 10 min, 2X SSC and 0.01% Triton X-100 for 10 minutes and 2X SSC for 10 minutes, all at room temperature in a shaker. Slides were counterstained with 1µg/mL of DAPI in 2X SSC for 5 minutes, rinsed in 2X SSC and mounted with anti-fade solution (0.95 g/L 1,4-phenylenediamine in 1X PBS, pH 8.0).

2.2.4.6.1 Use of Monoclonal Anti-Avidin to Amplify Signal of 827

The procedure described above (section 2.2.4.6) for biotinylated probes was used. After the detection with avidin and washings, the slides were incubated with monoclonal anti-avidin (Sigma), diluted 1:2000 in blocking buffer (1-3% BSA, and 4X SSC), for 40 minutes at room temperature. After the incubation, the slides were washed, stained, and mounted as done in section 2.2.4.6.

Intensity profiles across the target region were obtained by image analysis with LisPix (see section below). The noise was calculated by averaging the intensity of 10-15 background spots around the specific-signal, and the signal to noise ratio by

dividing the maximum intensity of the specific-signal by the average noise. The signal to noise ratio (S/N) of probe 827 (n = 5) detected with avidin-FITC and monoclonal anti-avidin-FITC was compared to the signal to noise ratio of the same probe when avidin-FITC alone was used for detection. The comparison was done with a student t-test with individual samples as well as the mean S/N between samples treated with monoclonal anti-avidin versus avidin alone.

2.2.4.6.2 Image Acquisition and Analysis

Images were captured with a Zeiss Axioplan 2ie upright high-resolution epifluorescence microscope (Carl Zeiss, Thornwood, NY) with the appropriate filter set. Table 2.3 provides a list of the filters used and their wavelength cutoff. The microscope was equipped with an Orca ER cooled charged coupled device (CCD) camera from Hamamatsu. Camera and microscope were controlled with Openlab and Volocity software (Improvision's, Lexington, MA). The images were overlapped using Adobe Photoshop version 6.0. Lispix software,[33] version Lx29P was used to obtain the intensity profiles of sections where probes were visually detected.

2.3 RESULTS AND DISCUSSION

2.3.1 Mitotic Chromosome Preparation

The number of metaphase cells was defined by the exposure time to colcemid. Colcemid is a drug that binds to tubulin dimers causing polymerization of

Table 2.3. Set of filters available with Zeiss Axioplan 2ie upright microscope.

Filter Set	Excitation (nm)	Emission (nm)
Zeiss CZ910 FITC	470 ± 40	535 ± 40
CZ902 UV	365	420
CZ915 Rhodamine	546	590
31019 GFP	425 ± 40	505 ± 40

the microtubules to stop, so that the cells stop at metaphase (mitotic stage where chromosomes are condensed).[34-36] The longer the exposure time to colcemid, the higher the number of cells arrested in metaphase. The problem encountered was that long exposure times caused the chromosomes to condense even more, yielding really small chromosomes that were harder to identify by their morphology. The optimal colcemid exposure time for HT93A dividing cells was found to be 1 hour.

There was great variability in the slide preparation process from day to day, and even among samples spread on the same day. Many authors[37-39] have reported on the effects that daily and seasonal weather, temperature, humidity, airflow, and cell type, just to mention a few, have on the preparation of slides. This step was found to be critical for the outcome of FISH experiments on metaphase chromosomes with single copy FISH probes, the effect was not as evident with tandem repeat probes due to the larger chromosomal target (even if some of the probes do not bind to the target, the signal is still strong enough for detection).

2.3.2 Cell line characterization

The presence of t(15;17) on HT93A cells was evaluated with LSI® PML/RARA, on 21 metaphase spreads and 200 nuclei. Table 2.4 shows the results in summary. More than 90% of the metaphase spreads showed one green signal (G) on chromosome 17, a red signal (R) on chromosome 15 and a yellow signal (F) on derivative chromosome 15. The yellow signal is observed due to the fusion between PML and RARA on derivative chromosome 15, which causes the red and green

Table 2.4. Analysis of HT93A cells with LSI® PML/RARA.

Signal	Total	F/G/R	2G/2R	F/G	F/R	2R/G	2G/R	2F/R	2F/G	2R/F
Metaphase	21	19	0	1	0	1	0	0	0	0
Interphase	200	177	1	8	1	3	1	2	4	1

Codes: F means fusion, and occurs when RARA is translocated to chromosome 15. The fusion is usually detected by a yellow signal, due to the close proximity of the green and red signals. G means green and indicates the location of RARA, and R means red and indicates the location of PML. F/G/R, 2G/2R, F/G, etc are combinations of those three signals.

signals to be so close that sometimes they appear yellow. Almost 90% of the nuclei had distinct green and red signals and a yellow signal or two very close green and red (represented in the table by F/G/R). Other combinations of signals were observed; F/R, F/G, 2R/G and 2G/R indicate inefficient hybridization. The ones with 2 fusions (2 yellow signals) are artifacts and constitute less than 1 % of the population.

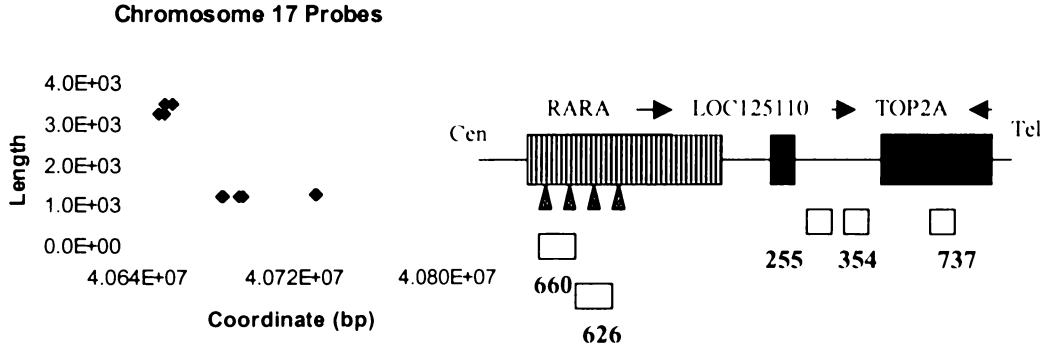
2.3.3 Probe Design

Figure 2.1 shows the localization of the probes in chromosomes 15 and 17; probes **827** and **978** are centromeric of the breakpoint region in PML, chromosome 15; **626**, and **660** are located in the breakpoint region, while **255**, **354**, and **737** are telomeric of the breakpoint region in RARA, chromosome 17. A list of the primers designed for the amplification of scFISH probes is provided in Table 2.3.

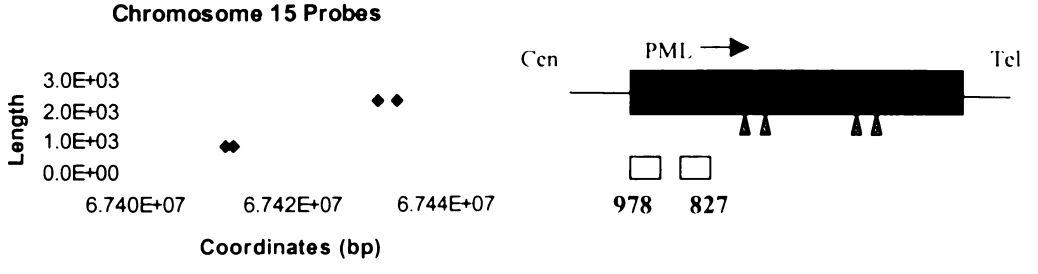
2.3.4 Probe Generation (PCR Amplification)

All probes were successfully amplified; Figure 2.2 shows a gel where 5 out of the 7 designed probes can be observed. Probe **978** was the easiest to amplify (always gave high yields) while the amplification of longer probes such as **626** and **660** was troublesome. This can be easily seen by the intensity of the bands in Figure 2.2, the intensity of **660** is low indicating low amplification yield, and **626** was not detectable. This result is not unexpected because longer probes would probably require a longer extension time to give similar yields as shorter probes, since in the former case the enzyme (DNA Polymerase) needs to add more nucleotides to extend the primer to the

Chromosome 17



Chromosome 15



Derivative Chromosome 15

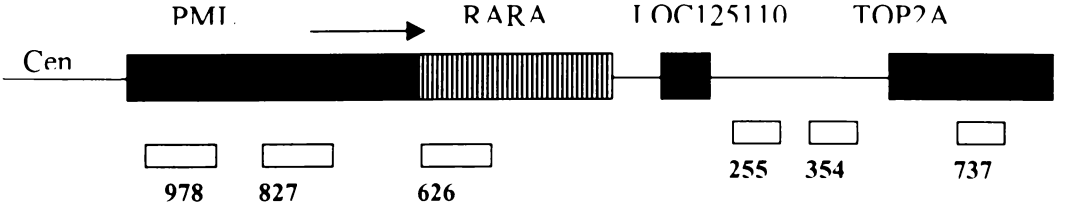


Figure 2.1. Partial map of 17q21 and 15q22, and derivative 15q22 showing the location of the breakpoint regions (triangles) and the scFISH probes (white boxes). The distances in the map are not to scale. The arrows indicate direction of transcription. Depending on the location of the breakpoint in chromosome 17 in HT93A cells, probes **660** and **626** might not hybridize to derivative chromosome 15.

Lane Number	Mixture Loaded
1	Lambda marker
2	**354** (chr 17, 1.2 kb)
3	**626** (chr 15, 3.5 kb)
4	**660** (chr 15, 3.5 kb)
5	**737** (chr 17, 1.3 kb)
6	**827** (chr 15, 2.4 kb)
7	**978** (chr 15, 0.9 kb)

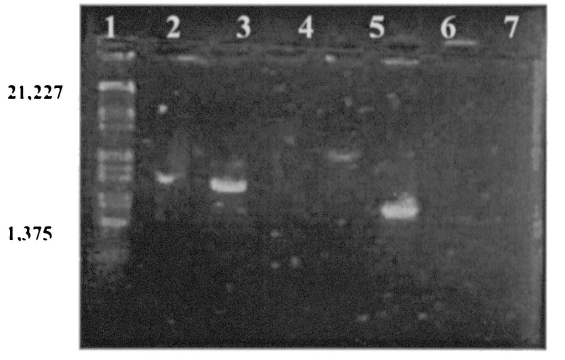


Figure 2.2. PCR amplification products on a 1% agarose gel run at 75 V for 1 hour. The gel was stained with SYBR Gold. 2-10 μL of each probe were loaded. The amount of probe 626 was not sufficient to be detected.

final fragment size. Another probe that was difficult to amplify was **255**; this probe has similar length as **737** and **354** as well as GC%. In this case, the lower yield could be due to the quality of the DNA. In both cases, the amplification gave higher yields when a new batch of Human Genomic DNA was used, presumably because of fragmentation of the DNA with use or changes in temperature when it was stored in the door of the refrigerator.

2.3.5 PCR Reamplification

As mentioned in section 2.3.4, probes $\geq$ 3 kb in length had lower amplification efficiency than probes $\leq$ 2.4 kb, and their recovery from gel extraction with the Millipore spin columns was low. Therefore, reamplification was applied to get around gel extraction. Unfortunately the reamplification products had faint smears above and below the main band. Because these smears could cause background (see experiments on arrays in chapter 3), since they would also be labeled, the bands were excised and extracted from the gel with QIAquick PCR purification kit (this kit gave higher recoveries than Millipore spin columns).

2.3.6 Characterization of Nick Translation Products

Incorporation of digoxigenin-11-dUTP was confirmed by visual detection of a purple substrate on the sections of a nitrocellulose membrane where probe **978** was applied. This purple compound is the product of the reaction of alkaline phosphatase with BCIP/NBT-purple. Fragment size after nick translation was always checked on

an agarose gel because the size of the fragments is critical to the hybridization. Incorporation of labeled nucleotides was only checked once, because the excess of labeled nucleotides in the reaction mixture assures their incorporation into the probe.

2.3.7 Probe Characterization on metaphase spreads

Three probes were chosen for characterization, **827** for PML in chromosome 15, and **354** combined with **737** for the region telomeric of RARA in chromosome 17. Even though probe **978** has higher yields than **827**; the latter is long enough (2.4 kb) so that combination with other probes in order to detect binding (hybridization) is not necessary. Thus it was selected for characterization. Probes **737** and **354** were selected because they had the highest yields among the chromosome 17 probes. The characterization was done by determining of the number of cells that had signal on the targeted chromosomal regions. This was described in terms of detection efficiency (D_{eff}).

$$D_{eff} = (H_{eff})\,(L_{eff})\,(P_{eff}) \qquad [2.1]$$

where H_{eff} is hybridization efficiency, L_{eff} is label efficiency, and P_{eff} is the photometric efficiency. The number of probe segments that are able to bind to the target region determines the hybridization efficiency (equation 2.2). This term is greatly affected by the preparation of the metaphase spread, and unfortunately there is enough variability between samples to cause changes in the hybridization efficiency.

Other factors that affect the hybridization efficiency are the sequence of the probe and size of the fragments; fragments of 200 - 500 bp in length yield higher hybridization efficiencies. The number of fluorescent labels attached to the probe segments (directly, or indirectly through avidin or antibodies) affects the detection efficiency (equation 2.2). This term is important because a high number of labels, or signal amplification of the labels, could compensate for a low number of probe segments binding. Finally, photometric efficiency refers to the collection of light from the probe, in this case done by the charged coupled device (CCD), and depends on the depth of focus, but for the purpose of this discussion it will be considered constant for all experiments, since the signal was manually focused prior to each determination and image capturing. The terms that affect the detection efficiency were not determined separately; instead, the detection efficiency is described in terms of the percentage of cells with detectable signal at the targeted region. Even though each term was not determined, it could be argued that H_{eff} is the one with more weight because if few segments bind to the target, the signal would decrease significantly.

$$H_{eff} = \frac{\text{bound probe segments}}{\text{total binding sites}}; \; L_{eff} = \frac{\text{labels attached}}{\text{total attachment sites}} \qquad [2.2]$$

The detection efficiencies are summarized in Table 2.5; the highest detection efficiency was observed for **827** when it was labeled with digoxigenin-11-dUTP;

Table 2.5. Detection efficiencies of scFISH probes characterized on HT93A metaphase spreads

Probe(s)	Target Region	Hapten	Metaphase Cells Analyzed	Detection Efficiency
827, 2.4 kb	PML, chromosome 15	Digoxigenin	102	>90%
827, 2.4 kb	PML, chromosome 15	Biotin	45	>50%
354, 1.2 kb, and **737**, 1.3 kb	centromeric of TOP2A, and TOP2A, chromosome 17	Digoxigenin	74	>70%
827, **354**, and **737**	t(15;17): chromosome 15, derivative 15 and 17	Digoxigenin and Biotin	29	>55%

Figure 2.3 shows the signal of **827** when it was detected with anti-digoxigenin Rhodamine conjugate (Roche), both chromosomes and both chromatids showed hybridization. As seen in Table 2.5, the detection efficiency when biotin-16-dUTP was used as a hapten was lower than when digoxigenin-11-dUTP was used. This could be explained by an increase in the background signal due to endogenous biotin, which could impair the ability to detect the probe. The binding of **827** to chromosome 15 was further corroborated by the hybridization of the probe in combination with control probe CEP-15. CEP-15 is a chromosome-specific tandem-repeat probe from Vysis, that hybridizes to the band region 15p11.2, locus D15Z1 of human chromosome 15, but due to a translocation, CEP 15 can hybridize to the centromere region of other acrocentric chromosomes, especially chromosome 14 (bands 14p11.1-q11.1) in HT93A cells, although the signal is not as bright as that on chromosome 15. Figure 2.4 shows probe **827** (in red) hybridized to chromosome 15 identified by CEP-15 (in green). The characterization results of **354** and **737** are shown in Figure 2.5, where chromosome derivative 15 shows signal in both chromatids, and chromosome 17 in one chromatid. The lower detection efficiency compared to **827** detected with the same system could be due to difference in label incorporation during nick translation, base composition, length and chromosomal location of the probes. These factors have been reported by Rogan and Knoll[1] to play an important role in the hybridization signal intensity. Another factor that affected

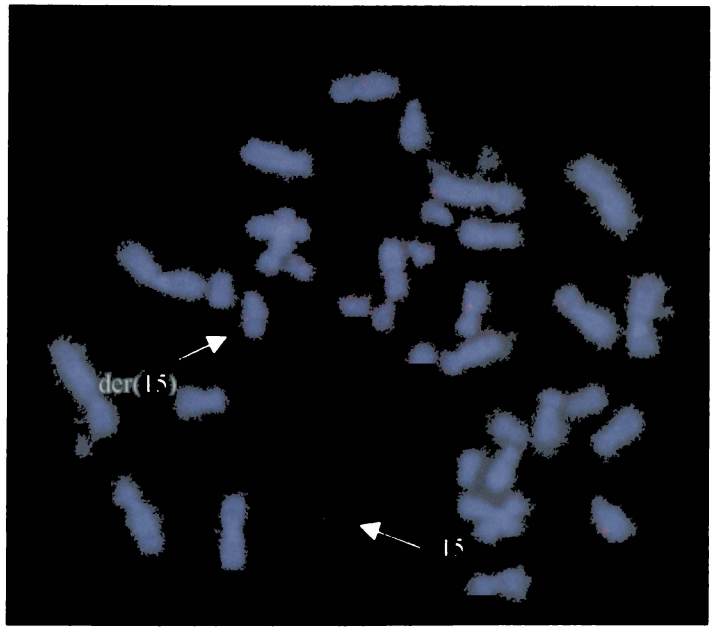


Figure 2.3. HT93A metaphase spread, hybridized to probe **827** (2.4 kb chromosome 15) labeled with digoxigenin, detected with Rhodamine anti-digoxigenin (red signal). The white arrows point at the red signal on both chromosomes 15. Both chromatids show hybridization. This was observed on ~ 90% of the cells, whereas the background was random.

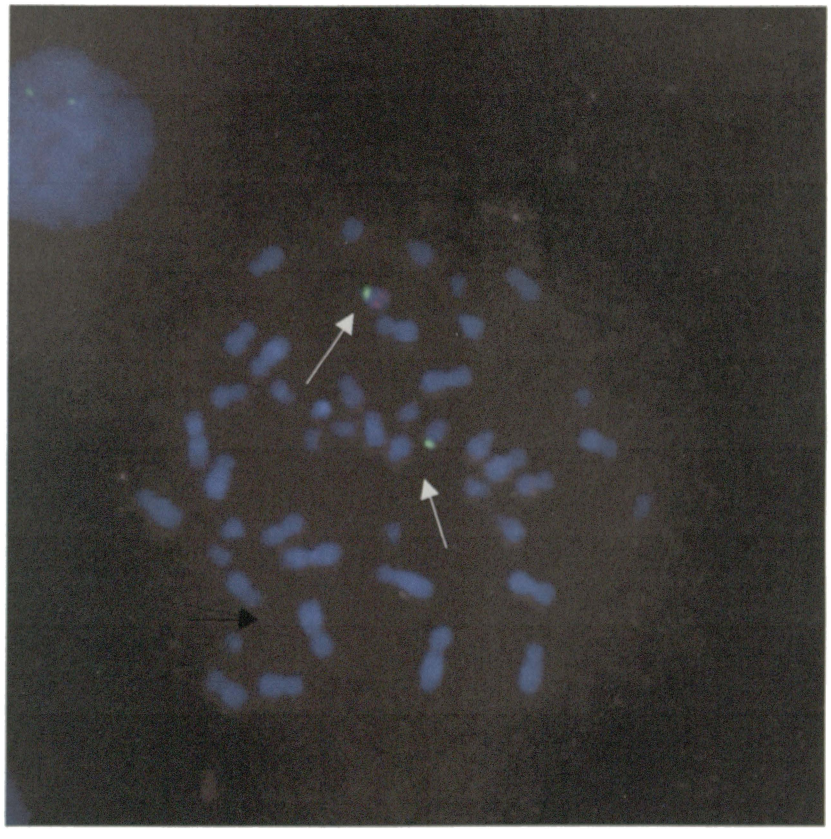


Figure 2.4. Probe **827** (2.4 kb chromosome 15) labeled with digoxigenin, detected with Rhodamine anti-digoxigenin (red signal). The white arrows point at both chromosomes 15. One chromosome shows signal in both chromatids. Probe CEP-15 (green signal) indicates the position of chromosome 15 and derivative 15

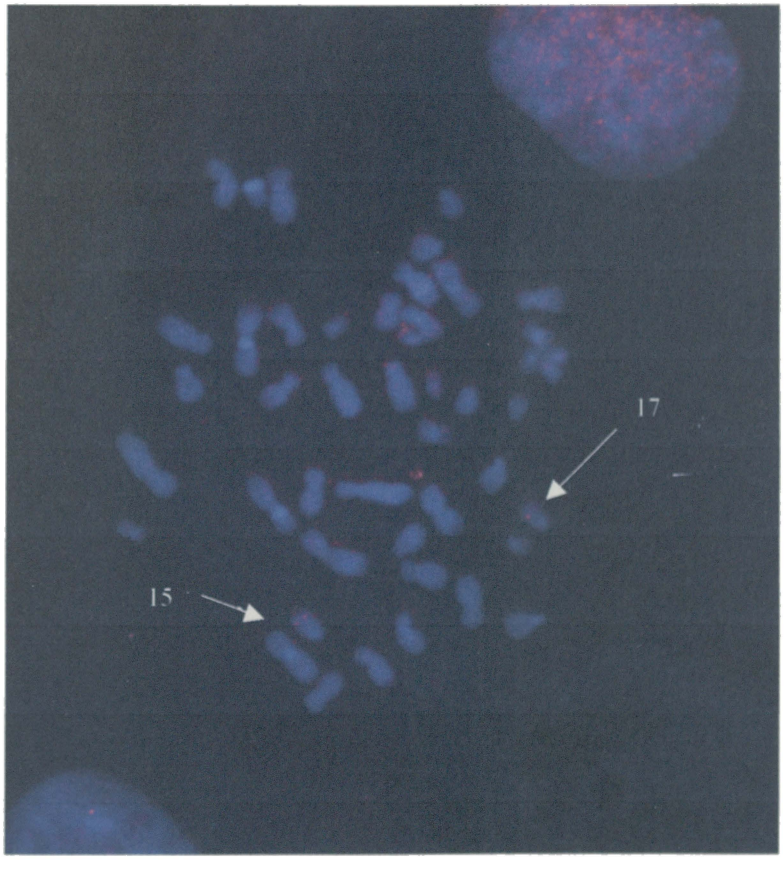


Figure 2.5. Probes **354** (1.2 kb chromosome 17) and **737** (1.3 kb chromosome 17) labeled with digoxigenin, detected with Rhodamine anti-digoxigenin (red signal). Chromosome derivative 15 shows signal in both chromatids, while chromosome 17 shows only one. Normal chromosome 15 and the derivative 17 could not be identified unequivocally due to banding and lack of probe hybridization.

detection of all probes was the presence of excess residual cytoplasm,[6] which increased background and decreased hybridization efficiency. As mentioned in section 2.3.1, the preparation of the chromosome spreads is crucial to the outcome of the hybridization efficiency.

Guzo and colleagues[6] reported on the difficulty involved in detection of small single copy targets (1-2 kb) and the necessity of cooled CCD cameras and digitally enhanced the faint signals, because direct microscopy images showed low S/N, and Viegas-Péquignot[40] reported 60% detection efficiencies for probes smaller than 1 kb. Thus when compared to detection efficiencies reported by others,[6, 40, 41] the results presented in this chapter are acceptable (>50% detection) or very good (>90% detection). Further optimization of the assay conditions is needed for those probes with low detection efficiencies because applications such as the detection of somatic mutations require detection efficiencies of 80% or better, and genotyping requires efficiencies of 100%.[41] Evans and coworkers[42] concluded that due to FISH failure to detect with 100% accuracy the presence of chromosomal regions, translocations, etc, further developments in the technique are required before it can be used as a replacement for karyotyping (description of total chromosome number and sex-chromosome configuration, and specific chromosome aberrations). However, conventional karyotyping is only sensitive to chromosomal changes that involve 10 Mb or more,[43] while FISH can detect chromosomal changes of a few kb, as established in this work.

2.3.7.1 Dual Color Detection of t(15;17) and Use of Monoclonal Anti-avidin for Signal Amplification

The identification of the translocation between chromosomes 15 and 17 t(15;17) is very important in order to differentiate abnormal PML and RARA from the normal ones. The translocation was detected in HT93A cells with probes **827**, **354**, and **737**. The dual color detection consisted of labeling the chromosome 15 probe (**827**) with biotin and detecting with avidin-FITC, and labeling the chromosome 17 probes (**354** and **737**) with digoxigenin and detecting with anti-digoxigenin Rhodamine conjugate. In this way a red-green signal (or yellow depending on the proximity of the probes) was expected on chromosome derivative 15 (the one with the translocation). Figure 2.6 shows the identification of derivative chromosome 15 and chromosome 15; the dual color detection facilitates the distinction between the two chromosomes, since only the former has red signal from the chromosome 17 probes.

The detection efficiency of der(15) by dual color FISH was only 7% when avidin-FITC was used for the detection of **827**-biotin, this increased to 56% when monoclonal anti-avidin-FITC was used following the detection with avidin. The detection of **354+737** was always done with anti-digoxigenin Rhodamine conjugate. Therefore, this result indicates an improvement in the ability to detect **827**-biotin. Probe **827** (2386 base pairs) is shorter than probes **354** and **737** combined (2538) by 152 bases; assuming an incorporation of one to two biotin-16–ddUTPs per 100 bases

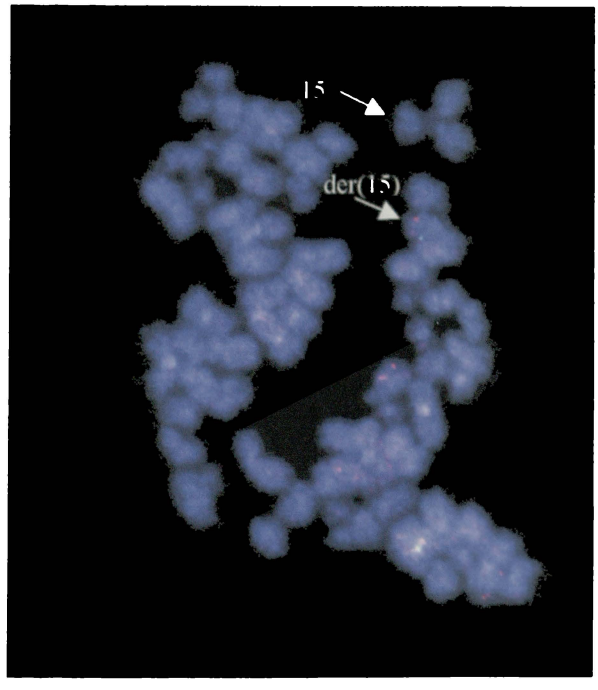


Figure 2.6. HT93A metaphase spread, hybridized to probes **827** (2.4 kb chromosome 15) labeled with biotin, and **354** (1.2 kb chromosome 17) and **737** (1.3 kb chromosome 17) labeled with digoxigenin. **827** was detected with avidin-FITC, followed by monoclonal anti-avidin FITC conjugate (green signal), while **354** and **737** were detected with Rhodamine anti-digoxigenin (red signal). Chromosome derivative 15 shows a green signal in one chromatid, and a red signal in the other one. Chromosome 15 shows signal in one chromatid (green), while chromosome 17 was not identified. The red signal in other chromosomes was determined to be random and part of background.

(according to Invitrogen), a maximum of 3 biotins would be incorporated in 152 bases; and if one avidin (fluorophore/protein ratio: 3.0-5.5)[44] binds to each one of those biotins, that would increase the signal by a maximum of 16 fluorophores. By themselves, 16 fluorophores are probably not enough to make a big difference in detection; another factors that might have affected the outcome is that avidin binds to endogenous biotin, increasing the background, therefore decreasing sensitivity.

The images of fourteen metaphase cells were analyzed with the program LisPix. Seven spreads were analyzed before the incubation with monoclonal anti-avidin, and another seven after incubation. The results are summarized in Table 2.6. Briefly, the S/N ratio before monoclonal anti-avidin incubation was 1.8 ± 0.2, and 2.4 ± 0.5 after incubation. When mean (average) S/Ns values were compared, "t" was 1.55. Since the tabulated "t" for 6 degrees of freedom, at 95% confidence is 2.45, it was concluded at the 95% confidence level, that there is no significant improvement between average S/Ns when the monoclonal anti-avidin FITC conjugate is used.

Analysis of individual S/Ns showed that in some cases S/N values doubled upon use of the monoclonal anti-avidin FITC conjugate, but overall S/N values increased 1.3 times. Figure 2.7 has a graphical representation of the change in **827**'s signal upon incubation with monoclonal anti-biotin. For these examples the peaks corresponding to **827**'s signal seemed sharper after incubation with monoclonal anti-avidin, and the background noise either remained constant or decreased. The blue trace shows the intensity profile of **827**-biotin detected with avidin-FITC. To the right of

Table 2.6. Comparison of signal to noise ratio of 827's detection signal, before and after detection with monoclonal anti-avidin FITC conjugate.

Parameter	Before Incubation	After Incubation
S/N for n = 7	1.8 ± 0.2	2.4 ± 0.5

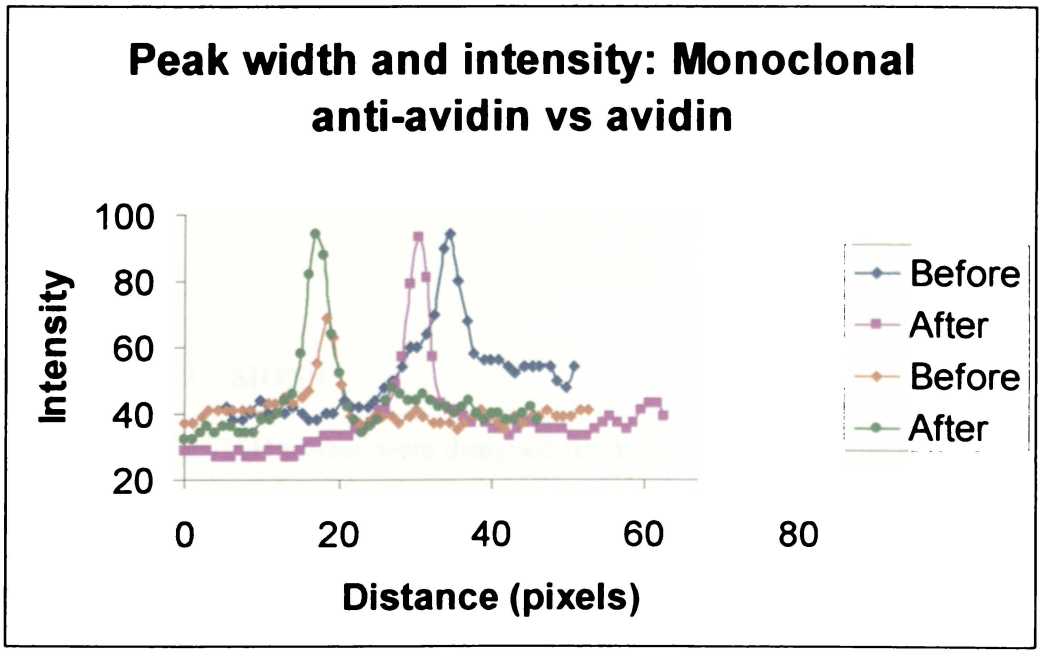


Figure 2.7. Intensity profile from D-group chromosomes cross section. The peak shows **827**-biotin detected with either avidin-FITC or avidin-FITC followed by monoclonal anti-avidin FITC conjugate. The blue and orange traces (before) represent the signal of 827 detected with avidin-FITC, while the pink and green traces (after) represent the signal of 827 detected with monoclonal anti-avidin FITC conjugate.

the signal the noise is higher than to the left, and even higher than other traces. Bleeding of the DAPI signal (from the chromosome) through the Rhodamine-filter, could be the cause of such high noise.

The fluorophore to protein ratio for the monoclonal anti-avidin FITC conjugate[44] is 4.9; so the expected increment in signal was close to 4.9. It could be possible that some of the signal from the monoclonal anti-avidin was quenched or maybe some avidins were not available for binding; if they are in close proximity, the antibodies might not be able to bind to all available avidins due to steric hindrance. This experiment cannot discriminate among these possibilities, but based on reports[45] where an increment in the signal is observed when the number of biotins incorporated into the sequence is increased, quenching seems the less likely of the two possibilities.

2.4 CONCLUSIONS

Seven scFISH probes were designed from the Human Genome Sequence, and successfully amplified by PCR. Two probes bind centromeric of the PML breakpoint region in HT93A cells, while another two were designed to bind around the breakpoint region of RARA, and the last three bind telomeric of RARA in HT93A cells.

Characterization of **827** on HT93A metaphase spreads, proved that the probe was specific for the region centromeric of the breakpoint in PML, chromosome 15. This was further confirmed by co-hybridization with control probe CEP-15 (15p11.2, locus D15Z1). When labeled with digoxigenin, **827** was detected with an efficiency

of >90%, but this number decreased to >50% when the probe was labeled with biotin. There are several factors that could have affected the outcome. First, there could have been a slightly better incorporation of digoxigenin. Second, avidin also binds endogenous biotin, and this could cause and increase in the background, making detection of **827** more difficult. Of the two possibilities, the first is the least likely because biotin-16-ddUTP is a smaller molecule (see Appendix) than digoxigenin-11-dUTP, so DNA Polymerase should have no problem incorporating the biotin-modified nucleotide.

Even though **354** and **737** were labeled with digoxigenin, their detection efficiency was >70%, 20% lower than **827**. This could be due to their difference in sequence, Rogan and coworkers[1] have reported that the length and chromosomal location of the probes play an important role in the detection of hybridization. Another factor could be the quality of the metaphase spreads. Throughout the experiments done for this work, it was found that the quality of the spreads could cause a decrease in the probe hybridization, and therefore, a decrease in the detection efficiency. Finally there is 4-5 °C difference in the melting temperature between **354** and **737**, which could decrease the hybridization efficiency.

Determination of the probe concentration after gel extraction was done by comparison with a lambda marker on a gel and staining with ethidium bromide (EtBr). However, Dutton and coworkers[46] found that the ratio of nucleic acid to EtBr must be within narrow limits in order to be used in the quantitative analysis of DNA probes. So if the probe concentration was incorrectly determined, it could have

affected the hybridization to the target (high concentrations of probe will cause fragments to reanneal instead of hybridizing to the target). There are studies about the optimum incorporation of biotin to get maximum signal.[45] Similar studies should be done for this type of probe, and they should be amplified in larger quantities that would allow for determination of the concentration by absorbance reading at 260 nm.

ScFISH probes are easy to design and generate by PCR, and they have proven useful for the analysis of chromosomal some regions ~100 fold smaller than those possible with common probes (tandem repeats, whole paint, and cloned). But signal amplification is required for the analysis of regions smaller than 2 kb. The increment in the S/N generated with use of monoclonal anti-avidin was not significant and other methods that provide a greater increment will have to be explored.

Even though the work presented in this chapter was successful in the design, and generation of single copy FISH probes, and characterization proved that the probes were specific, further optimization of the assay conditions for those probes with detection efficiencies lower than 80% is required for applications such as the detection of t(15;17), and RARA and PML promoter regions. It is very important to detect a high percentage of the sites for the histone acetylation determination in those regions, so that results reflect the overall cell population.

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3. SIGNAL AMPLIFICATION OF SCFISH PROBES BY 3DNA STRUCTURES (DENDRIMERS)

3.1 INTRODUCTION

Who does not want faster, more reliable assays that can detect smaller quantities of analyte? Everyone does, and, when dealing with DNA, in situ detection some groups have focused their attention on *in situ* target-amplification[1,2] rather than amplification of the signal from the DNA probes. The downfalls to this approach are, first, amplicon carryover that could cause false positives, second, poor reproducibility in cell preparations, and third, low amplification efficiency. Other researchers[3-7] have taken the approach of increasing by some means the number of fluorophores at the target site. Zhong and coworkers[8] utilized rolling circle DNA amplification (RCA)[9] to visualize target DNA sequences as small as 50 nucleotides. RCA utilizes a circularized oligonucleotide probe (ODN) that has a segment complementary to the target, and another segment that functions as a primer to initiate amplification of a circular DNA template. During amplification biotin-labeled nucleotides are incorporated, and the biotin is later detected by fluorescent-labeled streptavidin or avidin. This type of approach only works on decondensed DNA such as fibers and interphase chromosomes. This brings up a problem because the characterization of new DNA probes needs to be done on metaphase cells, where chromosomes can be identified by their morphology and banding patterns. Although it is not the subject of this chapter, it would be ideal to implement the signal-amplification method to detect

proteins, lipids and other small molecules, but RCA only works for DNA detection. Tyramide Signal Amplification (TSA) is a method used for the detection of probes labeled with biotin.[6, 10] First the probe is hybridized to the target, then a second reaction binds streptavidin conjugated to horseradish peroxidase (HRP), which on a third reaction binds tyramide-biotin to the biotinylated site. The biotin, coupled to tyramide, is detected on a fourth step with streptavidin-FITC. Schriml and coworkers[10] reported detection of probes as small as 608 bp, but the great number of steps involved in the detection makes the method less appealing. The presence of endogenous biotin could also lead to signal amplification of background. Other strategies are well reviewed by Qian and Lloyd,[3] and Andras *et al.*[11] A more recent approach has been the use of particles such as polystyrene microspheres[12] and quantum dots[13] as a means for signal amplification. Both approaches are addressed in chapter 4 in the study of possible steric hindrance problems related to the use of dendrimers.

The objective of the work presented in this chapter is to evaluate the use of DNA dendrimers as a method for signal amplification of scFISH probes.[14, 15] These probes (chapter 2) have the advantage of localizing a small section within a chromosomal region. The problem that arises is that not enough tags (fluorophores or hapten molecules) can be attached to some of the probes in order to detect binding. Dendrimers can have up to 38 times the number of fluorophores that can be attached to avidin or streptavidin giving rise to significant signal amplification. None of the

dendrimer-methods evaluated require target amplification, decondensed target, or more than two hybridization steps to detect the target DNA.

3.1.1 Dendrimers

The dendrimers used for this work are 3D structures made of DNA. The structure is assembled by hybridization of seven different types of oligonucleotides. The oligonucleotides hybridize only at a central region to form "X" like duplexes that have a double stranded central region and single stranded arms (Fig 3.1), and constitute the building blocks for the different dendrimer layers. The one-layer dendrimer is formed when the arms of other monomers bind to the four arms of the "initiating" monomer. As more monomers bind to other arms, the layers grow (Fig 3.2). After each layer is completed, the structure is covalently crosslinked. Dendrimers of the desired size are purified from denaturing sucrose gradients.[16]

The 4-layer dendrimers are made of 36000 bases and have a molecular weight of 1.2×10^7 g/mol.[17] The 2-layer dendrimers are made of 2700 bases and have a molecular weight of 9.0×10^5 g/mol. Hybridizing and crosslinking of Cy3, Cy5 or Alexa-488 labeled oligonucleotides to the outer arms of the dendrimers incorporates the fluorophores.[16] Each 4-layer dendrimer has 375 fluorophores for a diameter of 190 nm, while the 2-layer has 50 fluorophores and a diameter of 90 nm. The fluorophores are incorporated by attaching fluorophore-modified oligonucleotides (at the 5' end or both 3' and 5' end) to the single stranded arms of the outer layer.

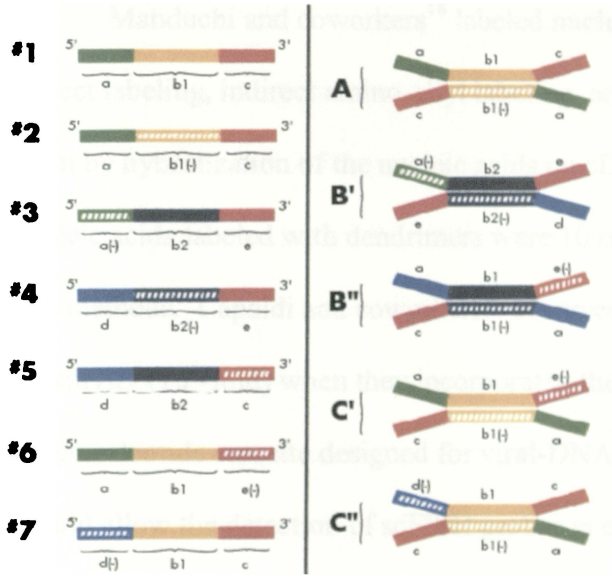


Figure 3.1. The seven unique oligonucleotides that constitute the building blocks of the monomers are illustrated on the left. Five different duplexes are depicted on the right. Solid lines represent positive strands, while broken lines represent complementary strands. Printed with permission from Genisphere (Hatfield, PA).

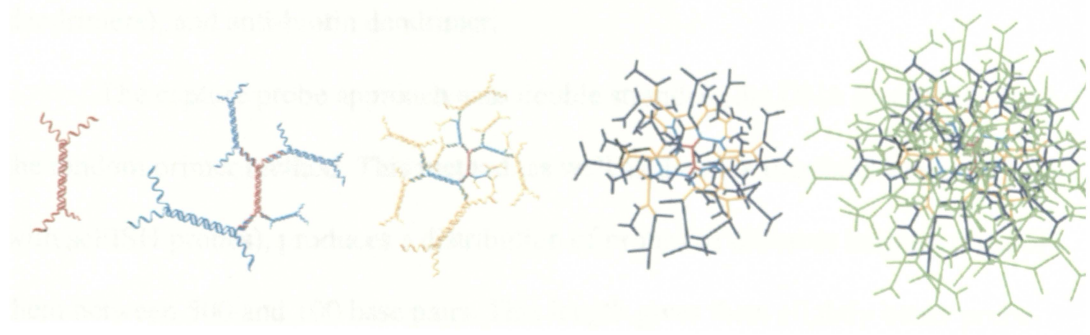


Figure 3.2. Schematic representation from left to right of 0-layer through 4-layer dendrimers. Each type of monomer is represented by a different color. The schematic is not to scale. Printed with permission from Genisphere (Hatfield, PA).

Manduchi and coworkers[18] labeled nucleic acids by three different methods (direct labeling, indirect amino-allyl labeling, and dendrimer labeling) and compared them by hybridization of the nucleic acids on cDNA arrays. They found that the nucleic acids labeled with dendrimers were 10 times more sensitive than the other two methods. Capaldi and coworkers[17] observed an even greater increase in sensitivity (10^6-fold) when they incorporated the use of dendrimers onto an oligonucleotide cassette designed for viral-DNA detection. So, in theory dendrimers should allow the detection of scFISH probes even smaller than 900 bp. It was the goal of this work to develop a methodology to increase the signal of scFISH probes by binding the dendrimer to the probe either before or after the probe is hybridized to the chromosomes. Three approaches were taken, as explained below: capture probe dendrimer, dendrimers covalently modified with specific sequences (specific dendrimers), and anti-biotin dendrimer.

The capture probe approach uses double stranded (ds) DNA probes labeled by the random primer method. This method, as well as the nick translation method (used with scFISH probes), produces a distribution of probes of different lengths, most of them between 500 and 100 base pairs. This length gives them slightly better probe-target complex stability (more base pairing interactions) than oligonucleotide probes. The great advantage of ds DNA probes is that they are less prone to degradation, but may be less sensitive because of reannealing of complementary-probe strands, instead of hybridizing to the target.

In the modified dendrimer approach, oligonucleotides specific to the region of interest are covalently attached to dendrimers. Oligonucleotide probes are often more specific than ds DNA probes but their short length limits the amount of label that can be incorporated resulting in a less intense signal, but the number of fluorophores per dendrimer considerably increases the sensitivity of these oligonucleotides. Another advantage of oligonucleotide probes is that because of their smaller size they can diffuse faster and are less prone to form secondary structures resulting in improved hybridization efficiency. However, in this approach the diffusion of the oligonucleotide probes is significantly reduced by the attachment to the dendrimer.

The third approach consists in attaching four anti-biotin antibodies to a dendrimer. The probes in this case are labeled with biotin by nick translation as standard scFISH probes (chapter 2) and detected by dendrimers on a second hybridization step. Detection of standard scFISH probes labeled with biotin is done with avidin or streptavidin that would have up to 7 fluorophores per molecule as compared to 50 fluorophores (at most) in a 2-layer dendrimer.

3.2 EXPERIMENTAL METHODS

A list of buffers, dyes, and abbreviations in addition to those from Table 2.1 is provided in Table 3.1.

Table 3.1. Composition of buffers, and buffer and dye abbreviations.

Abbreviation	Composition
PB	Contains guanidine hydrochloride and isopropanol, proprietary of Qiagen, CA
100X Denhardt's	2% BSA, 2% PVP, 2% Ficoll 400
4X Denhardt's	0.08% BSA, 0.08% PVP, 0.08% Ficoll 400
PVP	polyvinylpyrrolidone
Cy-3	Carboxymethylindocyanine-3 iodoacetamide
Cy-5	Carboxymethylindocyanine-5-*N*-hydroxy succinimidyl ester
10X TBE	108 g/L Tris base, 55 g/L boric acid, 20 mM EDTA
1X TBE	10.8 g/L Tris base, 5.5 g/L boric acid, 2 mM EDTA
6X ligation mixture	Contains capture probe and bridge oligonucleotide, proprietary of Genisphere, Hatfield, PA
DNA Ligase	T4 DNA Ligase solution

3.2.1 Capture probe Dendrimers

3.2.1.1 Preparation of the scFISH probe for ligation of capture probe

The process for the entire ligation procedure is illustrated in Figure 3.3. The desired probe is PCR amplified, purified, and labeled by random priming. Briefly, the probe (0.2 μg) is mixed with 4 μL of the random primer solution (1μg/μL) and enough water to make 16 μL. The mixture is boiled for 5 minutes and chilled on ice for 2 minutes. The following reagents are then added: 2μL of 10X buffer (500 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$, 500 mM NaCl), 1 μL dNTPs (10 mM in each dATP, dTTP, dCTP, dGTP, Promega, Madison, WI), and 1 μL of Large Fragment of DNA Polymerase I (3-9 units, Promega). The new mixture is incubated for 1 hr at 37 °C, 1 μL of 0.5 M EDTA is added to stop the reaction. The mixture is incubated at 65 °C to confirm deactivation of the enzymes and enough TE buffer is added to make 100 μL. The products of random priming are purified with a QIAquick® PCR purification kit (Qiagen, Valencia, CA). Briefly, the QIAquick column is placed in a collection tube and a mixture of 5 volumes of PB buffer to 1 volume of the random priming products is applied to the column and this is centrifuged for 30-60 s. The flow-through is discarded, PE buffer (0.75 mL) is added, and the column is centrifuged for 1 minute. The flow-through is then discarded and the centrifugation is repeated. The column is placed in a clean centrifuge tube and the DNA eluted with

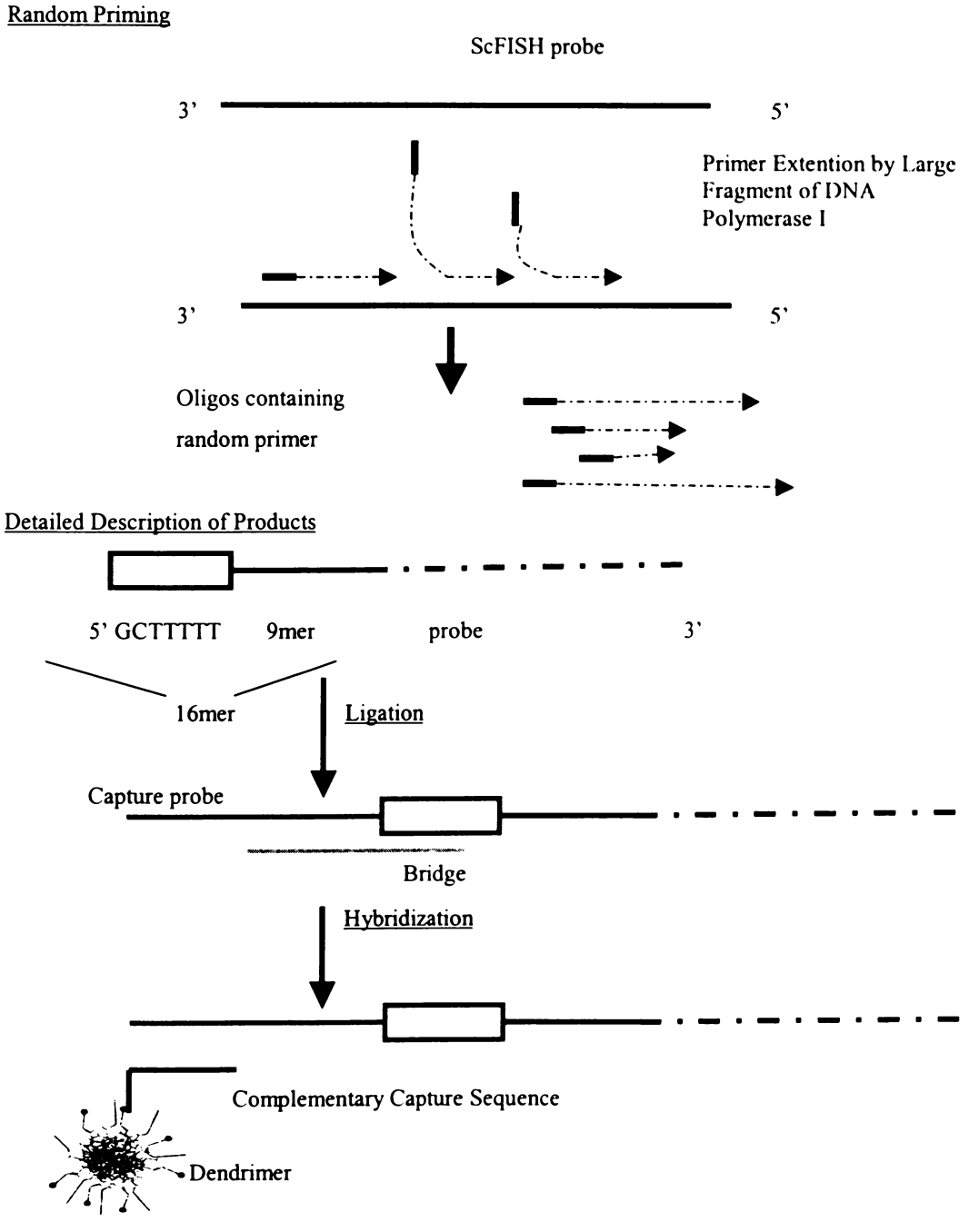


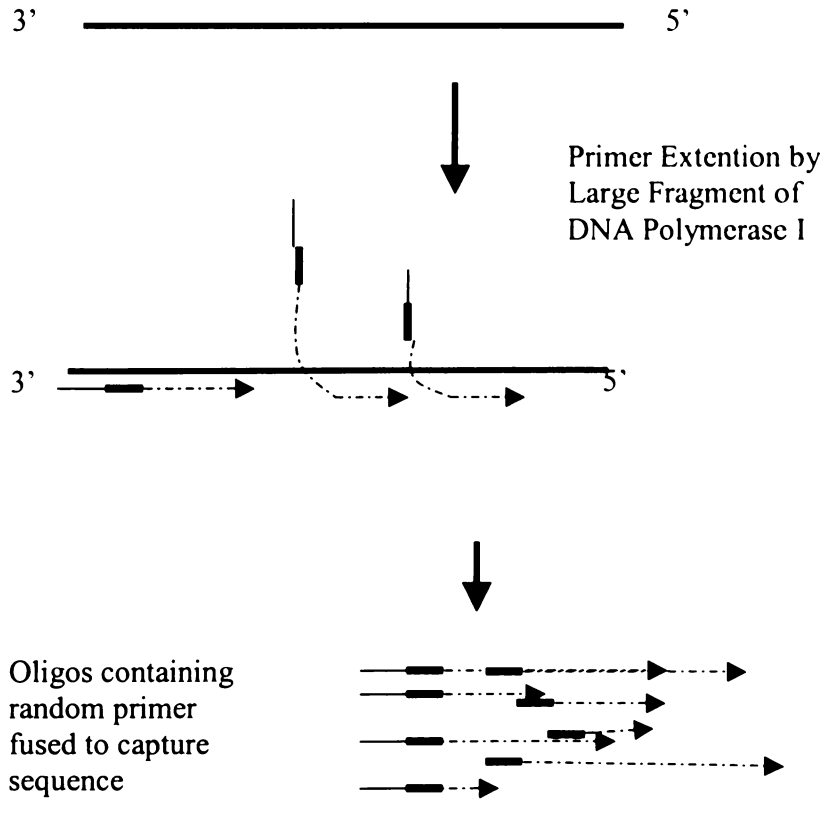
Figure 3.3. Diagram representing the various steps involved in labeling the single copy probes with capture probes and the dendrimer hybridization.

50 μL of EB buffer by centrifuging for 2 minutes. The eluent (random primed probe) is denatured by keeping it in boiling water for 10 minutes, and then chilling on ice for 2 minutes. For the ligation reaction, the 6X ligation mixture (10 μL) is added together with 2.5 μL of the DNA Ligase solution. The mixture is incubated at 15 °C for 1 hr and the reaction is stopped by addition of 7 μL of 0.5 M EDTA and heating at 65 °C for 10 minutes. The volume is taken up to 100 μL with TE buffer. The ligation product is purified with the QIAquick® PCR purification kit as described above and the DNA is concentrated with Millipore (Bedford, MA) Microcon® microconcentrators. Briefly, the reservoir is placed in a collecting tube and pre-washed with 100 μL of TE pH 8.0 by centrifuging at 13000 rpm for 3 minutes. The product of the ligation reaction is applied and centrifuged for 8 minutes. The sample reservoir is taken out, TE buffer (5 μL) is added. The reservoir is tapped to promote mixing, the reservoir is placed upside down on a clean collecting tube and centrifuged for 2 minutes. ScFISH probes ligated to capture probes isolated in this manner can be stored at –20 °C until use.

3.2.1.3 Preparation of the scFISH Probe for Direct Incorporation of Capture Probe

The scFISH probe was prepared in the same way as for ligation except that the primer used for random priming had the sequence of the capture probe directly coupled to the 3' end (see Figure 3.4). The products of random priming were purified

Random Priming



Detailed Description of Products

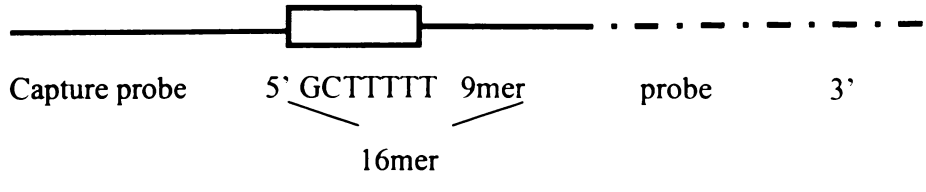


Figure 3.4. Diagram representing the various steps involved in the incorporation of capture sequence by random priming.

with a QIAquick® PCR purification kit and the DNA is concentrated with Microcon® microconcentrators as described above.

3.2.1.2 Characterization of the Capture Probe Ligation

3.2.1.2.1 Gel Shift Assay

The probe (120 ng) was dissolved in hybridization buffer (2X SSC and 0.06 % SDS) and denatured by incubating at 100 °C for 5 minutes, followed by chilling on ice for 2 minutes; the oligonucleotide complementary to the capture probe (~20 ng) was added to the denatured probe and the mixture was incubated at 37 °C for 20 minutes. The products were run on 6 % TBE buffer for 45 min at 150V. Gels were stained in 1X SYBR Gold for 20 min to view the DNA marker. The phosphor screen was exposed over night and scanned with a Lundlem ^{32}P survey meter model D.

3.2.1.2.2 Array Hybridizations

Amino silane and poly-L-lysine modified slides were spotted with 0.9 μg/μL human genomic DNA (Promega, Madison, WI), 1 μg/μL Cot-1 DNA (Invitrogen, Rockville, Maryland), 1 μg/μL porcine genomic DNA (ClonTech Laboratories, Palo Alto, CA), and 0.5 μg/μL HT93A DNA. All solutions were prepared by ethanol precipitation of the DNA and resuspension in 5M NaSCN. Solutions were spotted at a 300 μm center-to-center spacing with a Spotbot from TeleChem International, Inc (Sunnyvale, CA).

Pretreatment for arrays spotted on poly-L-lysine slides consisted in UV crosslinking at 55 mJ, followed by dipping slides in 0.2 % SDS for 15 minutes, in deionized water three times for a few seconds, and boiling water for 5 minutes. The arrays spotted on amino silane slides were kept for 15 minutes in isopropanol and 2 minutes in boiling water.

A mixture of 50 ng of random primed-ligated probe, 12 μL of 2X formamide buffer (50 % formamide, 4X SSC, 1 % SDS and 4X Denhardt's solution), and water to make 24 μL, was incubated at 100 °C for 10 minutes, while arrays were pre-warmed at 55 °C in an oven. The probe mixture was applied onto the slide, covered with a glass coverslip, and incubated in a humidity chamber at 50-55 °C over night. Coverslips were removed by dipping the arrays in 2X SSC, 0.2 % SDS until the cover floated. The arrays were then washed in same solution for 15 minutes at 55 °C, in 2X SSC for 10 minutes, and 0.2X SSC for 10 minutes, both at room temperature. The arrays were then dried by centrifuging at 1000 rpm for 2 minutes.

The dendrimer hybridization mixture consisted of 12 μL of 2X formamide buffer, 2.5 μL of dendrimer (20 ng/μL), and water to make 24 μL. The mixture was incubated at 75-85 °C for 10 minutes. The arrays were pre-warmed at 55 °C for the same amount of time, and the mixture was applied, covered with a glass coverslip, and incubated in a humidity chamber at 50 °C for 2-3 hrs. The arrays were washed in the same way as after probe incubation and scanned on a GenePix 4000B (Axon Instruments, Union City, CA).

3.2.1.2.3 Hybridization to Metaphase Chromosomes

Approximately 100 ng of each probe were diluted in 2 μL of H_2O, and 7 μL of LSI hybridization buffer (dextran sulfate, formamide and SSC, pH 7.0). This mixture was vortexed, centrifuged, and heated at 73 °C for 5 min and kept on ice until ready to apply to target. The slides were removed from 100 % EtOH and dried, the hybridization mixture was applied and the area covered with a plastic coverslip. The slides were incubated at 37 °C overnight. The coverslip was removed and the slide immersed in 50% formamide/ 2X SSC at 46 °C for 10 minutes. This was repeated 3 times. The slides were then immersed in 2X SSC for 10 min and 2X SSC/ 0.1 % Triton-X 100 for 10 minutes. For all the washings the slides were agitated for 3 s. The dendrimer hybridization mixture consisted of 12 μL of 2X SDS buffer (0.5 M Na_3PO4, 1% SDS, 2 mM EDTA, 2X SSC, 4X Denhardt's solution), 2 μL of dendrimer (20 ng/μL), and water to make 24 μL. The mixture was heated at 75 °C for 10 minutes prior to application to the slide, after which it was covered with a coverslip and incubated at 65 °C for 2 hours. The slides were washed in 2X SSC/ 0.2% SDS for 15 minutes at 65 °C, followed by 10 minutes in 2X SSC and 0.2X SSC both at room temperature. The slides were stained with 50 μL of 1 μg/mL of DAPI in 2X SSC for 5 minutes, rinsed in 2X SSC and mounted with anti-fade solution (Vectashield, Vector Laboratories, Burlingame, CA).

3.2.2 Dendimers Covalently Modified with Specific Sequences

3.2.2.1 Design of Oligonucleotides of 45 Bases

Segments of 45 bases were selected out of the already designed scFISH probes. OligoTech software program from Oligos, Etc. (Wilsonville, OR) was used to determine possible stem loops (secondary structures) and stability of the oligonucleotides, followed by analysis using Basic Local Alignment Search Tool (BLAST)[19] to confirm absence of homology to sequences other than the one designed for binding. The sequence TTT TTT TTT TCG was added to the 3' end of the oligomer to facilitate attachment to the dendrimer. The 57-oligomers were ordered from TriLink Biotechnologies, Inc. (San Diego, CA). The manufacturing department at Genisphere (Hatfield, PA) covalently attached the 57-oligomers to 2-layer and 4-layer dendrimers, to make "modified dendrimers". The 2-layer dendrimers have approximately 50 fluorophores, while the 4-layer ones have 375.

3.2.2.1.1 Tether Dendrimers

The Genisphere Manufacturing department made dendrimers with 100 bases between the outer arm and the specific sequence in the 4-layer format. The specific sequences used were: **978-765** (PML promoter region, chromosome 15) and **354-245** (centromeric of TOP2A, chromosome 17); both labeled with carboxymethyl-indocyanine-3 iodoacetamide (Cy-3).

3.2.2.2 Gel Shift Assay

The DNA probes were end-labeled with ^{32}P by Genisphere. Approximately 12.5 ng of probe and 60 ng of dendrimer (Table 3.2) were denatured and then incubated for 1 hour in 4X SSC and 0.125 % SDS. The experiment was repeated for the mixtures listed in Table 3.3; this time with ~6.5 ng of probe and 60 ng of each dendrimer; the hybridization conditions were kept as the previous experiment. The products were run on 6% TBE buffer and detected as gel shift assays for the capture probe approach.

3.2.2.3 Hybridization to Metaphase Chromosomes

Slides were pretreated with 2X SSC at 37 °C for 15 minutes and spun dried at 1000 rpm. Approximately 10 ng of dendrimer were mixed with 1 μL of Cot-1 DNA (1 μg/μL), 1 μL of salmon sperm DNA (10 μg/μL), 7 μL of LSI hybridization buffer (dextran sulfate, formamide and SSC, pH 7.0), and H_2O to make 10 μL of hybridization mixture. This mixture was vortexed, centrifuged, heated at 72 °C for 10 minutes, applied to target and covered with a plastic coverslip. Slides were then heated at 70 °C for 6 minutes to induce denaturing, and incubated at 37 °C overnight. The coverslip was removed and the slide immersed in 40% formamide/ 2X SSC at 37 °C for 5 minutes, followed by 10 minutes in 2X SSC at room temperature. Slides were stained with 50 μL of 1 μg/mL of DAPI in 2X SSC for 5 minutes, rinsed in 2X SSC and mounted with anti-fade solution (Vectashield, Vector Laboratories, Burlingame, CA)

Table 3.2. Mixture of probe and modified dendrimer(s) incubated to test hybridization.

Lane #	Mixture loaded	Gap*
1	**354** probe	na
2	**354** probe + 2-n **354-428**	na
3	**354** probe + 2-n **354-428** + 4-n **354-245**	183
4	**978** probe	na
5	**978** probe + 2-n **978-695** + 2-n **978-460**	235
6	2-n **978-695**	na
7	4-n dendrimer	na
8	**978** probe + 2-n dendrimer (negative control)	na
9	Lambda marker	na

*Gap: number of bases between the sites where the specific sequence dendrimers are designed to bind. It does not apply (na) to experiments where only one dendrimer was used

Table 3.3. Mixtures of probe and modified dendrimer(s) incubated to test hybridization of more than one dendrimer when the distance between the two hybridization sites is varied.

Lane #	Mixture loaded	Gap
1	**978** probe + 2-n **978-765** + 2-n **978-695**	70
2	**978** probe + 2-n **978-695** + 2-n **978-460**	235
3	**978** probe + 2-n **978-765** + 2-n **978-460**	305
4	**354** probe + 2-n **737-517** (neg control)	na
5	**354** probe	na
6	**978** probe	na
7	2-n **737-937**	na
8	Lambda marker	na

3.2.3 Anti-Biotin Dendrimers

The manufacturing department at Genisphere covalently attached approximately four anti-biotin antibodies to 2-layer and 4-layer dendrimers, and characterized them on DNA arrays.

3.2.3.1 Hybridization on Metaphase Chromosomes

Slides were pretreated with 2X SSC at 39 °C for 18 minutes, dehydrated by ethanol series (70 %, 85 %, 95 %, and 100 %, 1 minute each), denatured in 70% formamide 2X SSC at 73 °C for 2 minutes and 20 s and dehydrated again. Approximately 60 ng of **827** (2.3 kb, PML, chromosome 15) were diluted in hybridization buffer (20% dextran sulfate, 50% formamide and 2X SSC, pH 7.0). This mixture was vortexed, centrifuged, heated at 74 °C for 5 minutes, followed by cooling in ice for 2 min and then applied to target and covered with a glass coverslip. Slides were then incubated at 37 °C overnight. The coverslip was removed and the slide washed in 2X SSC for 2 minutes, 0.5X SSC for 3 minutes, 3 times and 2X SSC for 2 minutes twice, all at 39 °C. The detection solution consisted of 1μL of anti-biotin dendrimer (12 ng/μL, provided by Genisphere, PA) diluted in 400 μL of blocking solution (3% BSA, 4X SSC, 0.05% Tween 20, pH 7.0). The detection solution (100 μL) was applied to the target, which was covered with parafilm and incubated for 40 minutes at 37 °C. Washing consisted of 2X SSC for 10 minutes, 2X SSC/ 0.1% Triton X-100 for 10 minutes, and 2X SSC for 10 minutes, all at room

temperature. Slides were stained with 50 μL of 1 μg/mL of DAPI in 2X SSC for 5 minutes, rinsed in 2X SSC and mounted with anti-fade solution (Vectashield)

3.2.3.1.1 Evaluation of endogenous biotin blockage

Slides were pre-treated with 2X SSC at 39°C for 15 minutes, followed by ethanol dehydration and incubation in a 1:167 avidin:BSA solution at room temperature for 10 minutes. Slides were rinsed in 2X SSC and incubated for10 minutes in a 1:167 biotin:BSA solution to block the rest of the avidin binding sites, and finally rinsed twice with 2X SSC, removed by spinning the slides dried at 1000 rpm. Slides were immediately incubated with the DNA probe.

3.2.3.1.2 Pepsin Treatment

Slides were incubated in 10 mM HCl for 5 min prior to incubation in freshly made 50 μg/mL pepsin (3,200 – 4,500 units/mg, Sigma, St Louis, MO) in 10 mM HCl for 10 minutes at 37 °C. The reaction was stopped by placing the slides in H_2O for 1 minute, and then washing twice with 2X SSC for 5 minutes. Slides were treated in 4 % paraformaldehyde (m/v in 0.01 M PBS) for 10 min and washed twice in 2X SSC for 5 minutes, followed by dehydration in ethanol.

3.2.4 Image Acquisition and Analysis

Images were captured with a Zeiss Axioplan 2ie upright high-resolution epifluorescence microscope equipped with an Orca ER cooled charged coupled

device (CCD) camera from Hamamatsu, as described in section 2.2.4.6.2. The images were overlapped using Adobe Photoshop version 6.0 and analyzed with Lispix software.[20]

3.3 RESULTS AND DISCUSSION

3.3.1 Capture Probe Dendrimers

3.3.1.1 Characterization of Capture Probe Ligation

3.3.1.1.1 Gel Shift Assay

To confirm ligation of the capture probe to the scFISH probe a mobility shift assay was performed. This consisted of incubation of ligation product with an oligonucleotide complementary to the capture probe (same sequence as the arms in the dendrimer that bind to the capture probe). In some experiments the oligonucleotide was labeled with ^{32}P in order to increase the sensitivity of detection. Ligation of the capture probe to the scFISH probe could not be confidently confirmed when the complementary oligonucleotide was not ^{32}P labeled. The results of such an experiment can be seen in Figure 3.5. In this example a chromosome 17 probe (354) was ligated and characterized in parallel with a positive control. Some 354 was also random primed with a different type of primer that had the sequence of capture probe 03 (Genisphere designation based on the sequence) directly coupled to the 3' end of the random primer (see Figure 3.4). Therefore no ligation was necessary for this approach, the final product was designated as 354-cap 03. The shift in lane 5 is consistent with the size of the complementary capture probe (~47 bases), therefore

Lane #	Mixture loaded
1	**354**
2	Complementary cap35 + **354**
3	Complementary cap 35
4	Ligation control
5	Ligation control + complementary cap 35
6	**354**-cap 03
7	**354**-cap 03 + complementary cap 03
8	Complementary cap 03

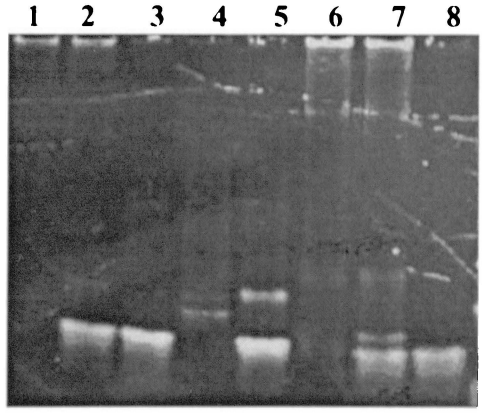


Figure 3.5. 10% TBE gel run for 50 minutes at 150V in 1 X TBE buffer and stained in 1X SYBR Gold. Lanes are numbered at top of the gel and the mixtures loaded described in the table. The positive control in lane 5 shows a band shift as compared to the position of the band in lane 4, indicative of a successful ligation. The slight shifts in bands in lane 7, as compared to lane 6 demonstrate that the capture sequence was incorporated by random priming.

the ligation reaction was successful. In lane 7 two similar shifts are observed, one at higher molecular weight than the other. The shifts indicate that probe **354**-cap 03 had bound to cap 03 complementary sequences. Lane 2 shows a band above that of the complementary capture probe. This shift indicates possible ligation of the complementary capture probe to the smaller fragments of the specific probe. Although encouraging, the mobility shift assay does not definitively confirm that the ligation is working, since we expected to see smears in lanes 2 and 7, caused by the different size fragments product of the random priming reaction, and not just a shift in the complementary capture probe.

One possibility is that only a fraction of the scFISH probes is being ligated to the capture probe and SYBR Gold or ethidium bromide staining cannot detect the shift.

The success of the ligation reaction was confirmed on a separate gel shift experiment by end labeling the complementary capture sequences with ^{32}P. This time, other labeled probes were checked along with **354**, see table on Figure 3.6. The first two lanes and lane 5 in the image show neither bands nor smear since neither marker nor probes were end-labeled with ^{32}P. The arrow in red indicates the position of the complementary capture probes in lanes 4, 7 and 10. Lanes 3, 6, 8 and 9 show a shift in the capture probe band (blue arrow) possibly due to the binding of the complementary capture probe to residues of ligation mix in the sample, there is also a smear above that shift (yellow bracket) indicating that the products from random priming were successfully ligated. The smear at higher molecular weight (orange

Lane #	Mixture loaded
1	100 bp marker
2	354
3	354 + Complementary cap35
4	Complementary cap35
5	978-cap 35
6	978-cap 35 + complementary cap 35
7	Complementary cap 03
8	737 + Complementary cap 03
9	978 + Complementary cap 02
10	Complementary cap 02

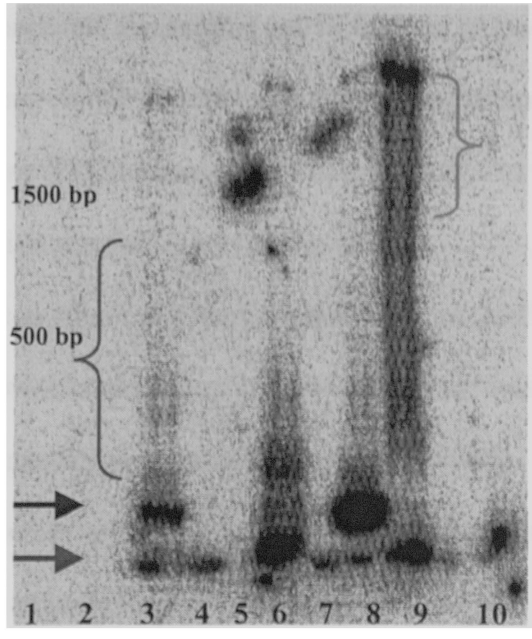


Figure 3.6. Scan of a phosphor screen exposed to a 6% TBE gel run for 45 minutes at 150V in 1 X TBE buffer. Lanes are numbered at bottom of the gel and the mixtures loaded are described in the table. Lanes 3, 6, 8 and 9 show the expected smear indicative of probe binding to the complementary capture sequence and therefore a successful ligation.

bracket) could be due to the formation of an aggregate between the probe and the ligase when the mixture was heated to inactivate the enzyme and in the case of lane 9, probe **978** was not purified before labeling, so that explains why there are products at lengths higher than the probe itself. These results demonstrate that probe labeling with the capture sequence either by ligation reaction or by random priming (differentiated in Figure 3.6 by **probe#-cap-#** instead of just **probe#**) was successful. Whenever ligated probes or probes known to have the capture sequence incorporated were incubated with ^{32}P labeled complementary sequences a smear was seen as a result of hybridization of probe to complementary capture sequence. It is important to notice that the signal of highest intensity in lanes 3 and 8 corresponds to possible residual ligation mixture. Lanes 6 and 9 have the desired smear of greater intensity.

3.3.1.1.2 Array results

The purpose in making these array hybridizations was to check for selective binding of the dendrimers to probes with complementary capture sequences. The arrays are easier to prepare and to use as compared to the metaphase spreads and the results could give a good starting point to set the conditions required for the hybridizations on metaphase spreads.

Poly-L-lysine arrays showed very high Cy-3 background and hardly any material (DNA) remained after pretreatment. The pretreatment for the poly-L-lysine arrays was modified without any success, so the use of this type of slide was discontinued. Probes were direct-labeled with Cy-5 or ligated to capture probes for Cy-5 dendrimer

detection, then hybridized to an amino silane array (Fig 3.7). Table 3.5 shows the positions where the different types of DNA were spotted on the amino silane arrays. An internal Genisphere human DNA array (unknown identity) was used as a control (Fig.3.8). Figure 3.7 shows hybridization (spots in red) of probe 978-cap02 to spots 3, 4, 21 and 22, which have Cot-1 DNA. In this case the probe was detected with a Cy-5 dendrimer. Probe 978-cap02 was impure. This means that the probe was PCR amplified using a previous batch of the same probe as a template and that after amplification no purification by gel electrophoresis was performed. This explains why the probe would bind to cot-1 DNA, which contains repetitive DNA. There is also very faint signal on HT93A and Human Genomic DNA spots. Porcine and empty spots have no trace or red signal, as expected since the sequence of 978-cap02 was designed from the Human Genome draft. The green spots on the array could be due to salt residues over the spotted areas. The sequence of the DNA on the control array is unknown. Results on this array only show that hybridization of the probe to some spots was successful and dendrimer was binding to probe.

To test if the hybridization of unpurified probe to cot-1 DNA was due to repetitive sequences, amino silane arrays were hybridized to impure probe 354 in the presence of Cot-1 DNA. Only a few spots had Cy-3 signal, but it was unclear if it came from the binding of probe or salt residues. Another batch of arrays was spotted with controls for the binding of dendrimers, and controls for the binding of the beta-2 microglobulin (β-2m) gene (Ambion, Austin, TX), labeled in parallel with

Figure 3.7. Enlarged area of an amino silane slide that shows positive hybridization (spots in red) of probe 978-cap02 to cot-1 DNA detected with a Cy-5 dendrimer. The green spots could be due to salt residues over the spotted areas

Table 3.5. DNA type and its position on amino silane spotted arrays, such as the one imaged in Figure 3.7.

DNA Type	**Spot Number**
Human Genomic	1, 2, 9, 10, 15-20
Cot-1	3, 4, 13, 14, 21, 22
Porcine	5, 7
HT93A	11, 12
Empty	6, 8

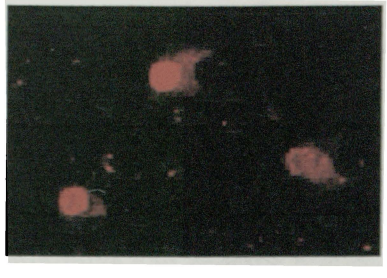


Figure 3.8. Enlarged area of the human DNA array (unknown identity) showing positive hybridization of probe **978**-cap02 detected with a Cy-5 dendrimer

chromosome 15 and 17 probes. The results from this experiment showed that the hybridization for β-2m was successful and that the dendrimer bound to the control spots, but no signal was detected from the chromosome 15 and 17 probes.

It was later determined that the amount of human genomic DNA on the spots (calculated based on the concetration) did not provide more than 13 copies of the specific sequence even before pretreatment of the slide, so that after washings this is not enough to detect with the scanner (about 250 fluorophores are needed). It was not possible to determine from this experiment whether the chromosome 15 and 17 probes hybridized to the spots with Human Genomic DNA.

3.3.1.1.3 FISH on metaphase spreads

The first experiments done on metaphase spreads showed that the probes prepared by reamplification (chapter 2) required further purification. Figure 3. 9 is a good example of the type of non-specific binding observed. For that experiment a chromosome 17 probe (354) was labeled with Cy-3 by random priming. This probe is only 1.2 kb in length, probably too small to be detected by itself, yet virtually every chromosome had red signal from Cy-3 aggregates (lower frame of the image). Probes were further purified by gel electrophoresis and hybridization conditions optimized to minimize non-specific binding. Results from 17 individual experiments, where a total of 126 metaphase spreads were analyzed, show that only 25% of the spreads had signals on at least one chromatid on each of the target chromosomes.

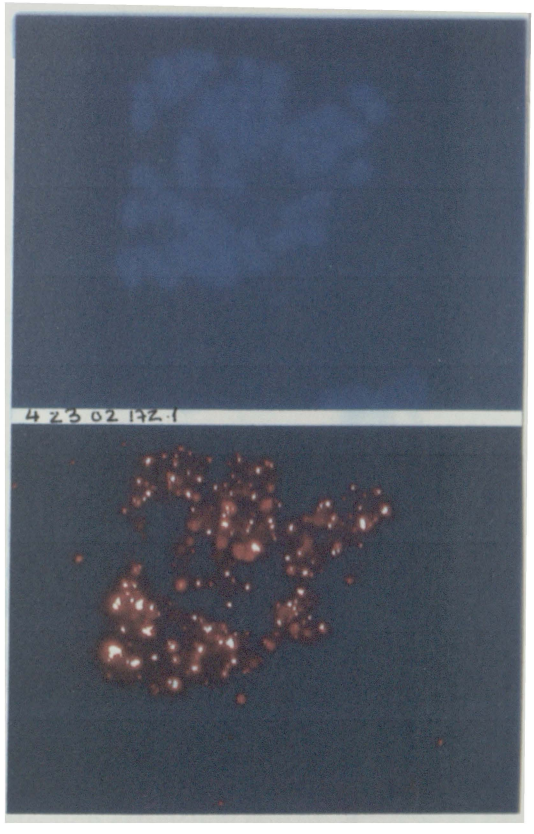


Figure 3.9. Metaphase spread (upper frame in blue) hybridized to ~200 ng of non-purified **354** direct labeled with Cy-3. The picture shows Cy-3 aggregate artifact (lower frame in red).

Less than 2% of the spreads had signals on both chromatids of the target chromosome. Because this percentage is too low, the event is considered random. All experiments done had moderate to high background. An example of the background observed in this experiments is shown in Figure 3.10. A slide of HT93A cells was hybridized to probes **354** (1.2 kb, 340 ng) and **737** (1.3 kb, 360 ng). These probes had a capture probe specific for a 2-n Alexa 488 dendrimer. The dendrimer showed signal at the expected location, but there were random signals on other chromosomes as well. The 4-layer dendrimer signal was usually in the border of the chromosomes (not necessarily chromosome 17). The same observation about 4-layer dendrimers was made in other experiments.

The effect of slide-pepsin treatment on dendrimer binding was not significant. Detection efficiency remained low with random binding. The section incubated with 4-layer dendrimer had signal mostly outside the chromosomes and random signal at what seem to be the right section of chromosome 17. This experiment should be repeated once hybridization conditions are worked out to produce specific binding. It is not possible to determine a difference between 2-layer and 4-layer dendrimers with low detection efficiencies and high background.

In an effort to determine if the probes were binding to the right chromosome a chromosome 15-control probe was used. The Vysis satellite III chromosome-15 enumeration probe (CEP) described in section 2.3.7 was used as a control to determine if the hybridizations were selective. Four slides (see Table 3.6) were

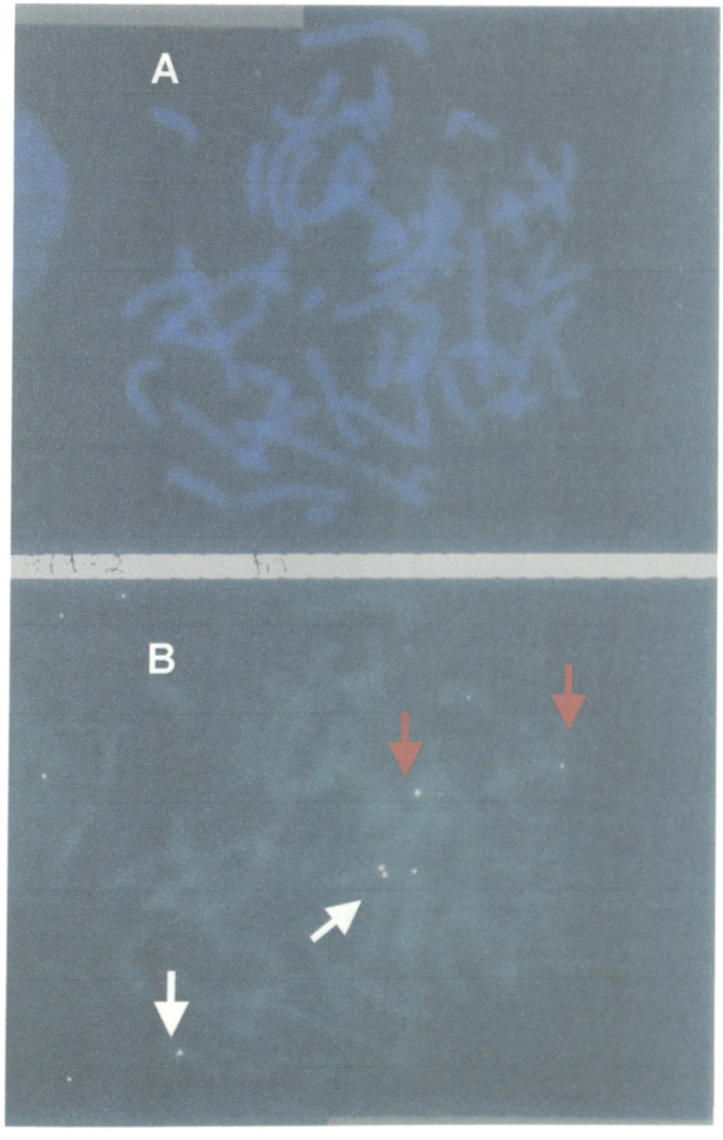


Figure 3.10. Chromosome spread from a female subject without t(15;17). The spread was hybridized to 330 ng of probe **354** (chromosome 17, 1.2Kb) and 360 ng of probe **737** (chromosome 17, 1.3 kb). Both probes had a tag for Alexa 488 dendrimer. A: Metaphase spread stained with DAPI. B: Signal from a 4-layer dendrimer. White arrows point to chromosomes of a size appropriate for chromosome 17. The red arrows show random hybridization to other chromosomes that is considered background.

Table 3.6. Slides used in different experiments with the CEP 15 probe from Vysis and other in-house probes. Probe **737** (1.3 kb) and **354** (1.2Kb) are for chromosome 17, and probe **827** (2.3 kb) is for chromosome 15.

Slide #	Probe mixture	Denaturing time/ min	Result (signal on target chromosome)
68230-1	CEP 15 + **737**	5	Non-specific
68220-2	CEP 15 + **737**	3	Non-specific
68220-1	CEP 15 + **827**	5	Specific on 6 out of 11 cells
220-1	CEP 15 + **737** + **354**	5	Non-specific

hybridized with 1μL of CEP 15 and 200 ng of scFISH probe labeled with capture probe. Probe **737** was random primed and ligated to a capture sequence for use with Alexa 488 dendrimers. The capture sequence ligated to **827** was for hybridization to Cy-3 dendrimers. No hybridization of **737** and **354** to chromosome derivative 15 was observed. The signal from the dendrimer was always weak and non-specific, on the border of chromosomes. The only difference between slides 68230-1 and 68220-2 was that the latter had weaker signal from CEP 15. This indicates that the denaturing time had an impact on the probe's hybridization to the target. Six metaphases out of 11 from slide 68220-1 had probe **827** hybridized to the right section of chromosome 15 (see Figure 3.12), five of the metaphases did not have signal from **827** on a chromosome 15. The metaphases in the middle of the slide showed more non-specific binding compared to the slide's periphery. None of the chromosomes had signal on both chromatids. The difference in results among the 4 slides relay in the denaturing time, length of target region, and probe sequence. The longer denaturing time and the probe with larger target region produced better detection efficiency. The lack of detection of probes **737** and **354**, when combined, could be related to their sequence, as determined in chapter 2.

Figure 3.11. Diagram of GTG banded human chromosome 15. The red dots represent the section of chromosome 15 where probe 827 is expected to bind (single copy region); the green spot is the section where CEP 15 binds (repetitive target).

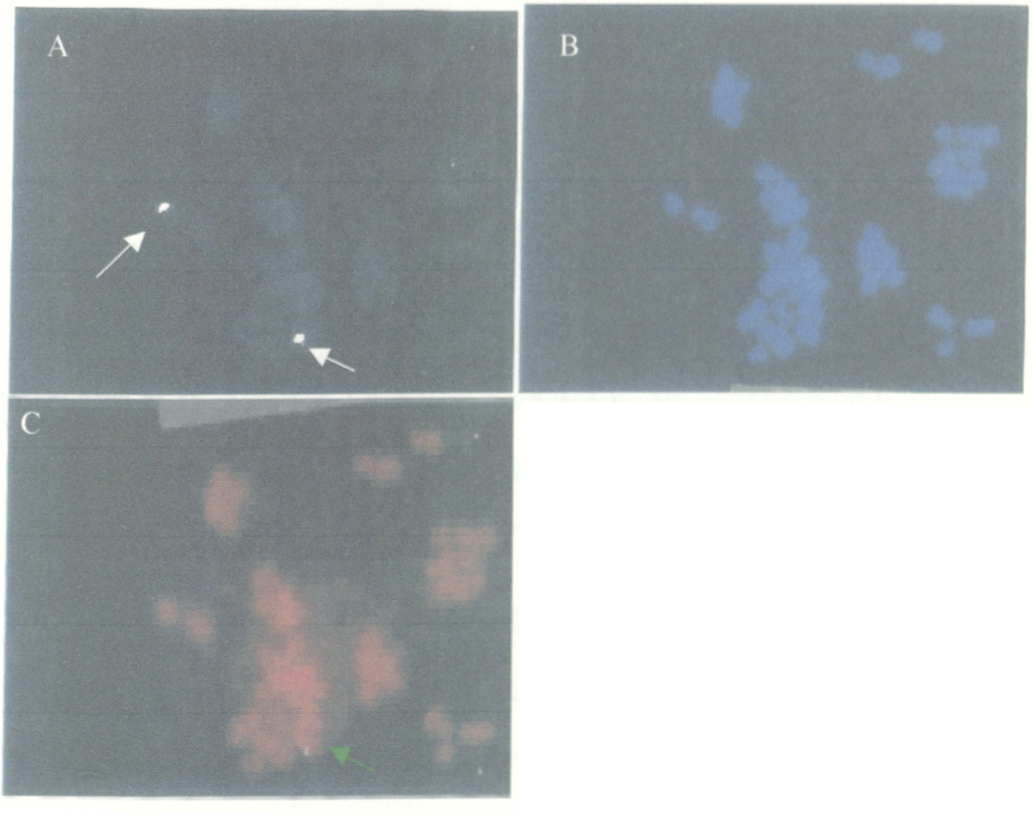


Figure 3.12. Chromosome spread of HT93A cells hybridized to 1 μL of CEP 15 and 200 ng of **827** (chr 15, 2.4 kb). A: White arrows point at CEP 15 hybridized to chromosome 15. B: Same spread stained with DAPI. C: The green arrow points at signal from a Cy-3 2-layer dendrimer hybridized to **827** on chromosome 15.

3.3.2 Specific Dendrimers

3.3.2.1 Design of Oligonucleotides of 45 Bases

Table 3.7 provides a list of the modified dendrimers and the number of bases between the oligonucleotide attached to the dendrimer and the closest oligonucleotide designed to bind to the same chromosomal region.

3.3.2.2 Specific Dendrimers Characterization

3.3.2.2.1 Gel Shift Assay

The objective of this experiment was to confirm hybridization of the modified dendrimers to the chromosomal region. Double stranded DNA probes were used as a simplified model of the chromosomes. This experiment was done to help determine if several of these modified dendrimers could be combined to increase the signal. If more than one dendrimer cannot bind to the probe, they will not bind to more condensed DNA structures. In order to detect binding, the DNA probes were ^{32}P-end labeled.

The ^{32}P image corresponding to the gel shift assay with specific dendrimers is shown in Figure 3.13. The agarose gel used to run this assay broke when pressed in the exposure cassette, so the SYBR Gold image does not perfectly match the ^{32}P image. Even so, the assay demonstrates that dendrimers were binding to the probe. Lane 2B shows binding of probe 354 to 2-layer dendrimer and when the same probe was incubated with 4-layer and 2-layer dendrimers both of them bound to the probe (lane 3B). It was concluded that both dendrimers hybridized to the same probe

Table 3.7. General information about modified dendrimers.

Code	Gene	Chromosome	Gap* / bases	Tm** / °C
626-943	RARA intron 2	17	350	42
626-1293	RARA intron 2	17	38708	47
354-245	LOC125110-TOP2A	17	183	46
354-428	LOC125110-TOP2A	17	37223	52
737-517	TOP2A exon 22-24	17	420	38
737-937	TOP2A exon 22-24	17	na	34
978-460	PML promoter and exon 1	15	235	46
978-695	PML promoter and exon 1	15	70	44
978-765	PML promoter	15	na	49

* Gap: Number of bases between the oligonucleotide and the closest oligonucleotide designed to bind to the same chromosome

** T_m: Melting temperature calculated for 0.33 M Na^+ and 50 % formamide, where $T_m = 81.5 + 16.6\log[Na+] + 0.41(\%GC) - 0.63(\%formamide) - \{300 + 2000[Na^+]\}/N$, and N is the length in bases of the hybrid.[21, 22] Where %GC is the percentage of guanosines and cytidines in the oligonucleotide or DNA fragment.

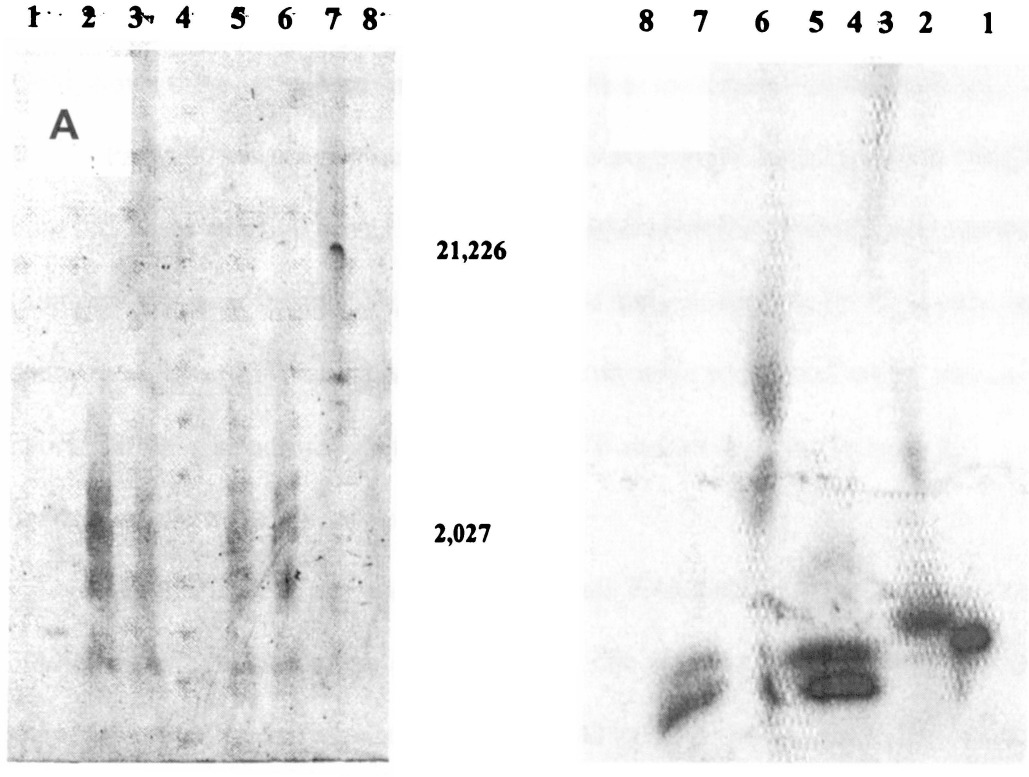


Figure 3.13. Gel shift experiment to determine binding of modified dendrimers to dsDNA probes. Table 3.2 describes the mixtures that were loaded. The gel was run at 110 V for 2 hrs. A: Gel stained with SYBR Gold. B: Scan of a phosphor screen exposed to the same gel as in A.

because there is only one smear close to the well and the same gel stained with SYBR Gold shows the 4-layer dendrimer bands at a lower molecular weight (lane 7A).

Probe 978 was incubated with two different 2-layer dendrimers that would bind 235 bases apart. It seems that both dendrimers hybridized to the same strand (higher MW smear in lane 5B), but there is also some probe hybridized to only one dendrimer (lower MW smear, same lane). The negative control in lane 8B shows no hybridization of dendrimer to probe and lanes7B and 6B are clear because the dendrimers were not labeled with ^{32}P.

Another experiment was done to evaluate if the proximity of the dendrimers' binding site would affect their ability to bind. The distance between the hybridization sites of two modified dendrimers was decreased to 70 bases, and they still hybridized, although some of the probe migrated to lower molecular weight indicating hybridization of only one dendrimer to the probe. The distance from the 5' end of oligonucleotide 2-n 978-765 to the 5' end of oligonucleotide 2-n 978-695 in a double helix is ~ 40 nm (calculated based on 0.34 nm raised per base pair).[23] The diameter of a 2-layer dendrimer is ~ 90 nm, so even though both modified dendrimers were able to hybridize to the denatured probe they most likely would not be able to hybridize to more condensed DNA. Therefore, well separated modified dendrimers in the genome should be used for the FISH experiments.

3.3.2.2.2 Hybridization on interphase chromosomes

Ten experiments were done combining 4-layer dendrimers: **978-765** and **354-245**; the former labeled with Cy-3 and the latter with Alexa-488. The fusion (yellow signal) of both dendrimers, indicating the presence of t(15;17) was detected in 35 out of 44 nuclei. Only 10 nuclei had the expected number of signals: 1 green, 1 red, and 1 yellow (Figure 3.14). The other nuclei had 2-4 red signals, up to 3 green and up to 2 yellow. Figure 3.15 is a good example of two nuclei with multiple signals.

Four 2-layer dendrimers specific for chromosome 17 were evaluated in 6 experiments: **354-245, 354-428, 626-943,** and **626-1293.** A total of 87 nuclei were analyzed; 10 had 3 green signals in the nuclei, 43 had 2 green signals and the rest no signal. When all chromosome-17 specific dendrimers were hybridized in combination with CEP-15 as control; the chromosome-15 satellite probe gave good signal but no dendrimer hybridization was detected.

Although more experiments were done with these types of dendrimers, some slides could not be analyzed either because the background noise was too high or because the morphology indicated that the chromosome-drying process during fixation had not been appropriate.

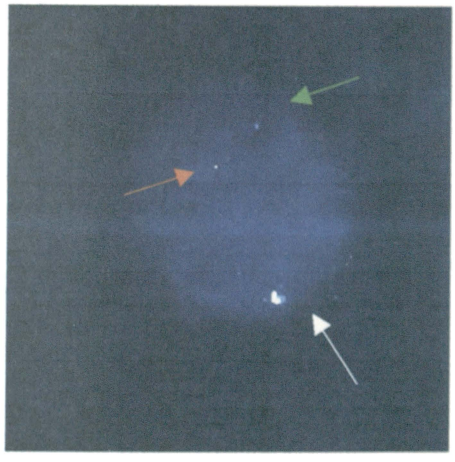


Figure 3.14. HT93A cells hybridized to 10 ng of **978-765** (chr 15, red signal), and 10 ng of **354-245** (chr 17, green signal). Nucleus stained with DAPI, the white arrow points at the fusion of both dendrimers, the red arrow points at the signal from dendrimer **978-765**, and the green arrow points at the signal from dendrimer **354-245**

Figure 3.15. HT93A nuclei hybridized to 10 ng of **978-765** (chromosome 15, red signal), and 10 ng of **354-245** (chromosome 17, green signal). The nucleus to the left shows 2 fusions (white arrows) two green signals and 1 red The nucleus below shows 1 fusion (white arrow), 3 red and 2 green signals.

3.3.2.2.3 Tethered dendrimers

These dendrimers were made based on the hypothesis that the longer arms of the dendrimers would facilitate binding to condensed DNA such as the one on metaphase chromosomes. In general, this type of dendrimer produced higher background. The size of the signal on both nuclei and metaphase chromosomes was considerably larger . Interphase chromosomes show 1 signal of about 10 times the size and greater intensity than other spots on the nuclei. This spot of greater intensity could represent the site of the translocation, in other words, two dendrimers bound in close proximity. Maybe the two dendrimers act as a "nucleus" that causes other dendrimers to bind, especially because of the longer arms. When these slides were submitted to washing in solutions of higher detergent percentage, most of the big spots disappeared, but the background remained high. More stringent washings caused the signal to distribute non-specifically.

3.3.3 Anti-biotin Dendrimers

3.3.3.1 Hybridization on Metaphase Chromosomes

This was the first approach for which signal was consistently observed in both chromatids of a D-group chromosome. Chromosomes 13 through 15 are classified in group D (medium acrocentric) by the International System for Human Genetic Nomenclature.[24] Because chromosome 15 was not identified by a control probe, it can only be concluded based on the morphology that the dendrimers hybridized a D-group chromosome. Figure 3.16 shows the results from this experiment where a

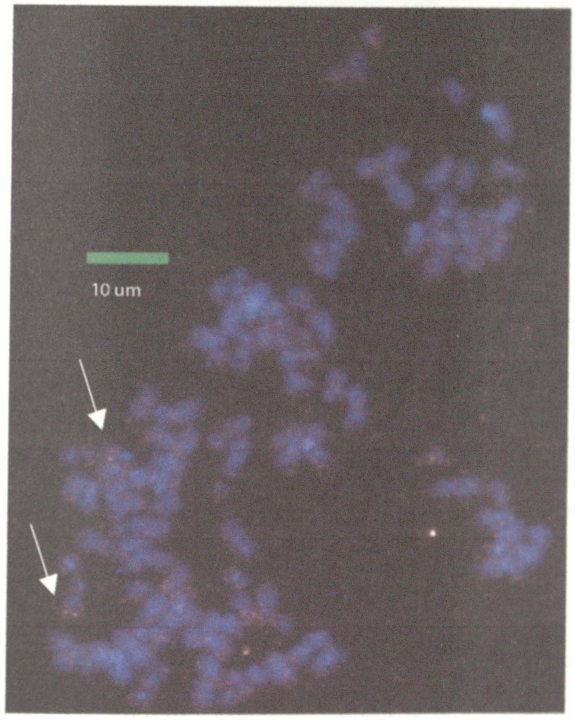


Figure 3.16. 2.4 kb chromosome 15 probe labeled with biotin hybridized to a HT93A metaphase spread. The chromosomes were stained with DAPI (blue signal), and the probe was detected with Cy-3 anti-biotin dendrimers (red signal). Two group-D chromosomes, indicated by white arrows, were detected with anti-biotin 2-dendrimers. One chromosome has with signal in both chromatids and the other in only one chromatid.

2- layer anti-biotin dendrimer was used to detect biotinylated probe **827**. The image shows both chromosomes with signal: one in both chromatids, and the other in one. For this experiment 9 metaphases were analyzed; 55% had signal in at least 1 chromatid of a D-group chromosome. A total of 8 experiments were done with 2-layer anti-biotin dendrimer and probe **827**, and 83 metaphase spreads were analyzed, and the detection efficiency was (40 ± 17)%. The 4-layer anti-biotin dendrimer was used to detect **827** in 4 experiments; 38 metaphase spreads were analyzed, and the detection efficiency was (36 ± 15)%. Both detection efficiencies are very low. This could be caused by: (1) physical constraint (steric hindrance), (2) slow dendrimer binding kinetics, and/or (3) poor labeling of dendrimers with anti-biotin antibodies. Possibilities 1 and 2 are related since the diameter of the dendrimers (90 nm for the 2-layer and 190 nm for the 4-layer) can affect their ability to bind to biotins in close proximity, and their diffusion to the target. The other possibility is related to manufacture of the dendrimer and quality control. Genisphere tests these dendrimers on arrays, but the high number of copies of target sequence on an array might allow binding of some anti-biotins not coupled to dendrimer while the signal on the array remains high. Metaphase analysis is detecting a single target at a time.

The first experiment to be performed with the anti-biotin dendrimers had the highest signal to noise ratio of 2.4. The average intensity of the three signals in Figure3.16 was 240 ± 40, and the average of 10 dendrimer-background signal in the surroundings was 100 ± 20. Figure 3.17 shows the intensity profile of the cross

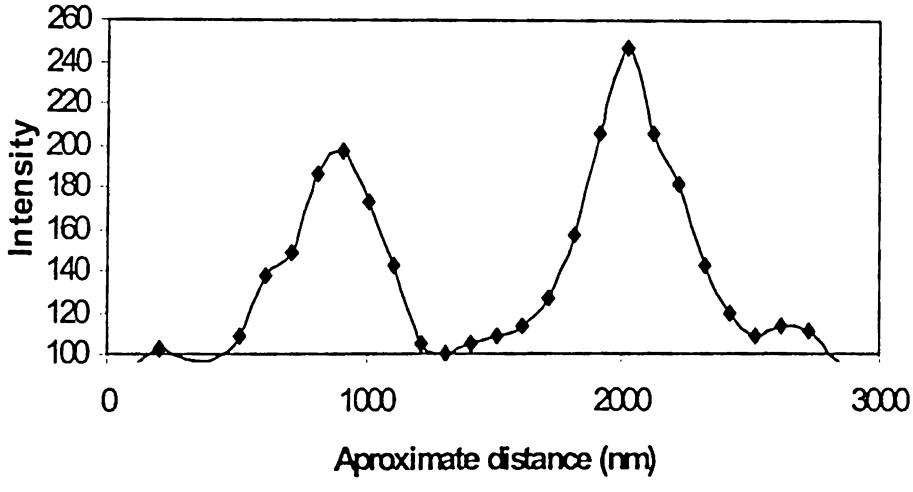


Figure 3.17. Intensity profile from a cross section of a D-group chromosome. The two peaks represent the signal from 2-layer dendrimers on both chromatids of the chromosome.

section of the chromosome with 2-chromatid signal. Both peaks are sharp and easy to differentiate from the background; they are also approximately 1 μm apart in accordance with the distance between 2 chromatids on a metaphase chromosome.[23] The other experiments had an average signal to noise ratio of 1.7 ± 0.2. The S/N for 4-layer dendrimers' signal was only 1.7 compared to 2.4 for the 2-layer. This result indicates that more 2-layer dendrimers are able to bind to the target, in support of the hypothesis that there are physical constraints for multiple dendrimers to bind to target DNA probes.

The great number of fluorophores (375) attached to 4-layer dendrimers should allow detection of biotinylated probes even if only one dendrimer binds. With this in mind two experiments were done in parallel where 2-layer and 4-layer dendrimers were used (separately) for detection of probe **978** (0.9 kb). The probe could not be detected with either 4 or 2-layer dendrimers. There are several possible explanations: (1) the probe did not hybridize, (2) there is not enough biotin incorporated for detection, or (3) biotins are not accessible for binding. This probe has not been characterized with avidin or streptavidin due to its short length, so it is possible that hybridization efficiencies are low and not enough probe-fragments hybridized. Since the percentage of guanosines and cytidines of **978** and **827** are very similar (55.6 compared to 55.3), and both probes were labeled by nick translation with DNA Polymerase from the same batch, it is unlikely that the incorporation of biotin differs significantly between the two probes. Furthermore, since probe **827** has been detected with avidin and streptavidin (see chapter 2), demonstrating good biotin

incorporation, the same could be inferred for 978. The last possibility is related to the 978 length: the probe is 2.5 times shorter than 827, so even if all fragments bind, maybe biotins are not spaced out far enough for dendrimers to bind. In other words there are not enough biotins available for binding. The steric hindrance hypothesis is further studied in chapter 4.

The hypothesis that dendrimers have slower kinetics than molecules such as streptavidin and avidin was confirmed by 6 experiments where a second incubation with fresh dendrimer solution increased detection efficiency from (21 ± 23)% to (47 ± 13)%. The standard deviation for the first incubation is high because for 3 of the experiments no signal was detected. Statistical analysis indicated with 95% confidence that the increment in detection efficiency obtained after the second incubation was significant. Furthermore, the detection efficiency increased from 42% to 67% when a slide previously incubated with 2-layer dendrimer was incubated with streptavidin (labeled with Cy-3). This indicates that there where biotins available for binding even after a 45 minutes incubation with 2-layer dendrimers.

It is interesting to observe that not even after second incubations were the detection efficiencies as high as that of the first experiment. It is possible that the dendrimer anti-biotin complex slowly degrades at 4°C. This would also explain why experiments had poor reproducibility. This suggests the need for a stability test on all reagents.

3.4 CONCLUSIONS

An attempt was made to use DNA dendrimers to amplify the fluorescent signal of shorter scFISH probes. The capture sequence approach was successful in binding specifically to the complementary capture probe in solution, in the absence of dendrimer. Although detection on DNA arrays with dendrimers was successful, specificity was not proven due to the low copy number of target sequence per spot. When tested on more complex samples, such as metaphase chromosomes, high background was observed in most sections of the slide and signal was never detected on both chromatids. These observations discredit the successful identification of specific binding by 2-layer dendrimers to a 2.4 kb PML probe. The non-specific binding to other chromosomes could be related to the spacer arm sequence attached to the scFISH probe or excess ligation mix left in the probe after ligation if such small fragment could non-specifically bind to other chromosomes and leave enough capture sequence for the dendrimer to bind. The identification of specific binding leaves hope for this approach.

Specific dendrimers worked really well in solution, as different dendrimers were able to bind to sequences only 70 bases apart. The results in interphase chromosomes were encouraging (almost 80% identification of t(15;17)), but their specificity needs to be determined in metaphase chromosomes. The close proximity of the specific sequence to the dendrimer was thought to impair binding to highly condensed DNA, such as metaphase chromosomes, but dendrimers with longer spacer arms showed higher non-specificity. A more exhaustive evaluation of different

hybridization conditions could improve the specificity of these dendrimers, another approach would be to use specific sequences longer than 45 bases, but still within single copy regions. If the conditions for specific binding were found, this approach would constitute a big breakthrough in the identification of chromosomal regions of less than 100 bases, where the probes can be synthetically generated, and no enzymatic amplification is required.

The anti-biotin dendrimers were the most successful in terms of specific binding and detection of both chromatids. The downfalls to this approach are the low detection efficiency and the lack of reproducibility. Once conditions are optimized detection of probes of less than 2 kb should be determined, otherwise there would be no significant advantage over the use of streptavidin or avidin.

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4. STERIC HINDRANCE AND EVALUATION OF QUANTUM DOTS AND FLUOSPHERES AS AN ALTERNATIVE TO SIGNAL AMPLIFICATION OF SCFISH PROBES

4.1 INTRODUCTION

The results with anti-biotin dendrimers, presented in chapter 3, generated questions about the feasibility of multiple dendrimer binding to a biotinylated probe. In theory, a 2-layer dendrimer has 6 to 10 times the signal intensity of streptavidin/avidin, but experimentally the intensity from 2-layer dendrimers bound to a 2.4 kb probe had only twice the intensity of the same probe detected with avidin. So, it was hypothesized that steric hindrance was keeping more dendrimers from binding.

4.1.1 Steric Hyndrance Theory

Klevan and Gebeyehu[1] have reported on the physical constraints involved in the biotin-streptavidin detection system. To evaluate this, they incorporated biotin-7-dATP by nick translation into DNA probes and analyzed aliquots at different time intervals by immobilization onto a nitrocellulose membrane and detection by streptavidin and biotinylated alkaline phosphatase. They determined that incorporation of biotin into DNA at levels greater than 7-35 biotins/kb did not increase the sensitivity.

Huang and coworkers[2] evaluated the effects of particle (streptavidin coated polystyrene particles) diameter, and probe length (5'-end biotinylated DNA probe) on the streptavidin-biotin binding constants when the DNA molecules were immobilized. The particle-size studied ranged from 90-944 nm, and the DNA molecules were double stranded and ranged from 100 to 5000 bp. They found that the forward reaction (association between biotin and streptavidin) is diffusion limited and suggested steric hindrance as a possible explanation. Their observations can be explained in terms of the Sherwood number (Sh), a parameter that describes the degree of diffusion limitation in unstirred reactions.

$$Sh = k_f d / D, \qquad [4.1]$$

where k_f is the mass transfer coefficient, d is the diameter of the particle and D the diffusion constant. A high Sh means that the reaction is limited by diffusion. The Sh was greater than 1 even for the reaction between 90 nm streptavidin-coated particles and 5Kb biotinylated DNA, indicating that the reaction was diffusion limited.

Both studies further confirm our hypothesis that dendrimers cannot bind to all biotins incorporated into the probe. If there are physical constraints when the DNA is not wound around histones, more than likely these constraints will increase when DNA is highly condensed, as metaphase chromosomes are. It is also likely that molecules 15 times bigger than streptavidin (~60 KDa), such as 2-layer dendrimers (~900 KDa), would have greater constraints preventing access to the biotin, and that these constraints would have an effect on the binding constant.

It is a goal of this work to evaluate by confocal microscopy the number of dendrimers that are able to bind to a biotinylated DNA probe in solution, without the physical constraints that could be present in metaphase chromosomes. A second important goal is to determine if the size of the dendrimer (or particle) has a detrimental effect on its ability to bind to biotin incorporated into the DNA probe. In order to do this, two different size anti-biotin dendrimers (90nm and 190 nm), as well as polystyrene particles of 40 nm, and quantum dots of 15 nm in diameter were studied.

4.1.2 Polystyrene Particles

The use of spheres (mainly latex and polystyrene) for antigen detection and other biochemical applications goes back to the early 70's. Molday and coworkers[3] used them to visualize antigens on lymphocytes, and Truffa-Bachi and Wofsy[4] used them for affinity purification. Nowadays, polystyrene particles are commercially available (Seradyn, IN; IDEXX Laboratories, ME; Molecular Probes, OR and others), and immunoassays[5-7] remain one of the most popular applications. Other applications include flow cytometry,[8] protein detection on stretched DNA,[9] and amplification of electrochemical detection of DNA hybridization.[10]

4.1.3 Quantum Dots

Quantum dots are semiconductor nanocrystals made of CdSe. They have very narrow emission bands,[11] and have become a good alternative to conventional dyes

which have wide emission bands that limit their use in multi-color analysis. The other advantage of quantum dots is their small size. It is irrelevant how many fluorophores a particle can hold or its brightness if its size impairs it from binding to the target or if the number of particles that can bind does not provide the signal necessary for detection. The immobilization or coating of different molecules (streptavidin, serotonin, transferrin, etc) onto the outer shell of the quantum dot has facilitated their use for the study of cell surface receptors,[12] indirect detection of biotinylated DNA probes,[13] and detection of cell cancer marker Her2,[14] to name a few examples.

4.2 EXPERIMENTAL

4.2.1 Polystyrene Particle Quantum Yield Determination

The polystyrene particles (FluoSpheres®) used were purchased from Molecular Probes (Eugene, OR). They were selected with a 40 nm diameter, labeled with 3.5 x 10^2 Fluorescein equivalents of Yellow-Green dye (proprietary of Molecular Probes), and a coating of NeutrAvidin, a non-glycosylated form of avidin.

The quantum yield was determined by comparison with a reference solution of Fluorescein of known quantum yield, using equation [4.2].

$$Q = Q_R \frac{\int I(\lambda) d\lambda}{\int I(\lambda)_R d\lambda} \frac{A_R}{A} \frac{\eta^2}{\eta_R^2} \qquad [4.2]$$

where Q is the quantum yield, I is the intensity, A is the absorbance and η is the refractive index. The subscript R refers to the reference fluorophore (in this case Fluorescein).

The integral was replaced by the sum of the data points of the emission spectra taken at 1 nm intervals, and because the emission maximum is the same for sample and reference, the refractive index term becomes unity; so equation [4.2] was simplified to

$$Q = Q_R \frac{\sum I(\lambda)}{\sum I_R(\lambda)} \frac{A_R}{A} \qquad [4.3]$$

A QuantaMaster luminescence spectrometer from Photon Technologies Inc (PTI) (Lawrenceville, NJ) was used for the collection of emission spectra, and a Cary 100 Bio UV-VIS spectrometer from Varian (Palo Alto, CA) for the collection of absorbance spectra. A Fluorescein (Aldrich, Milwaukee, WI) working solution was prepared by diluting 1 µL of a 0.01 M stock solution in 5 mL of 100% EtOH. The response of the photomultiplier tube (PMT) and the transmission of the emission monochromator were corrected by comparing the spectrum of the Fluorescein working solution in the PTI fluorimeter to the standard spectrum of the same dye for which the intensities were published.[15] From the comparison a correction factor for each wavelength was calculated. This correction factor was also used for the emission spectra of the FluoSphere solution, prepared by diluting 1 µL of the 1% solid stock solution in 880 µL of 0.01 M HEPES (4-[2-hydroxyethyl]-1-piperazinethane sulfonic acid), pH 8.0. The emission spectra of ethanol and HEPES

buffer were taken and subtracted from the emission spectra of Fluorescein and FluoSpheres respectively. The intensity values at each wavelength were then corrected for detection efficiency and used in equation [4.3] to calculate the quantum yield. The absorbance values at 470 nm (excitation wavelength) were entered into the equation. The emission spectra were taken with a bandpass of 6 nm.

The FluoSpheres were purchased as a 1% solid solution, so the concentration were determined by

$$\frac{\#\,microspheres}{mL} = \frac{6\,C\,10^{12}}{\rho\,\pi\,\phi^{3}} \qquad [4.4]$$

where C is the concentration of suspended beads in g/mL, ρ is the density of polymer in g/mL (1.05 for polystyrene), and ϕ is the diameter of the microspheres in μm. The molar concentration was calculated using Avogadro's Number, and the molar absorptivity was estimated from Beer-Lambert equation.

4.2.2 Primer Design

From the probe **827** sequence (chapter 2) four sets of primers were designed to amplify probes of varying lengths (Table 4.1). The primers were designed with *GeneFisher* – software.[16] The melting temperatures (T_m) were calculated with the program OligoAnalyzer 3.0 (Copyright (c) 2000 Integrated DNA Technologies). The primers designed for **827** and **978** (chapter 2) were also used for probe amplification.

Table 4.1. Primers designed for the generation of DNA probes of different lengths within the same chromosomal region.

Name	Sequence	%GC	T_m*	Modification	Product (bp)
827F-13	TCTAACCCCCACTGAACC	55.6	54.1	5' Alexa 488	na
827R-2373	TCCACAGCATCTACTGCC	55.6	54.8	none	2360
827R-1071	AGCAGGTCATCCTGCAGA	55.6	56.7	none	1058
827R-560	GGGTGATGCAAGAGCTGA	55.6	55.5	none	547
827R-260	CCGCACGTGAGCTACCA	64.7	57.8	none	247

*T_m assumptions:

- Applies to Oligos 8 to 40 bases in length.
- Melting Temperature Accuracy: (Oligo/Template) DNA/DNA is ± 1.4°C
- Applies to buffered solutions (pH 7 - 8) with monovalent cation 0.01 to 1.2 M

4.2.3 Probe Generation

The probes were generated as described in section 2.2.4.2 with the following modifications: the dNTP composition was 0.1 mM in biotin-16-dUTP, and 0.2 mM each of dATP, dCTP, and dGTP. PCR conditions were as follows: 94 °C for 2 minutes and 3 s, followed by 28 cycles of denaturation at 98 °C for 20 s, annealing at the primer optimized temperature for 5 minutes; and extension at 68 °C for 10 minutes. The purification and gel extraction were done as described in section 2.2.4.2.

4.2.3 Probe-Particle Hybridization

Approximately 8.3 x 10^{-16} moles of probe were incubated with 6.7 x 10^{-15} moles of dendrimer in 540 mM NaCl, 54 mM $Na_3Citrate$, and 0.1% Triton X-100, at 37 °C for at least an hour. For biotinylated probes no denaturing was necessary. When specific dendrimers were used (described in section 3.3.2), the probes were not biotinylated and denaturing at 100°C for 5 minutes prior to incubation with the dendrimers was necessary to ensure that the probes were single stranded. For incubation with quantum dots, the stock solution (2 x 10^{-6} M) was diluted to 1 x 10^{-8} M in 0.01 M PBS, and 3 x 10^{-10} moles of quantum dots were incubated with the probe (8.3 x 10^{-16} moles) in 0.01 M PBS, at 37 °C for at least an hour. Incubation with FluoSpheres (2 x 10^{-15} moles) was done under the same conditions as the quantum dots.

4.2.4 Polyacrylamide Immobilization

The dendrimer-probe solution was mixed with an equivalent volume of acrylamide / bis-acrylamide 37.5:1 solution (Amresco, Solon, OH), 1 μL of 10% ammonium persulfate (Fisher Biotech, Pittsburgh, PA), and 1 μL of TEMED (N, N, N', N'-tetramethylethylenediamine, Fisher Biotech). The mixture was pipetted up and down to promote mixing, 20 μL were pipetted onto an acid-cleaned glass coverslip, and a second coverslip was put on top to create a uniform film and prevent drying.

4.2.5 Confocal Spectroscopy Studies

The immobilized samples were imaged with an epifluorescence microscope (Nikon TE2000; Nikon, Melville, NY), previously described by Allen and coworkers.[17] Briefly, the sample was placed on a piezoelectric scanning stage (Nano H100; Mad City Labs, Madison, WI), and a raster scanned above a 1.3 numerical aperture, 100X objective (CFI Superfluor, Nikon). The 488 nm line of an Ar+/Kr+ laser (Lexel/Evergreen Laser Corp., Durham, CT) was used for sample excitation. The line was reflected with a dichroic mirror (QP500LP, Chroma Technologies, Rockingham, VT) and focused on the sample by the objective. The fluorescence emission was collected by the same objective, passed through the dichroic mirror, and directed out the side port of the microscope by a prism. A long pass dichroic mirror (565 DCLP) and a band-pass emission filter (HQ525/50M for detection of quantum dots and FluoSpheres; or HQ620/75M for detection of dendrimers) were used to

collect the fluorescent emission. Single-photon counting avalanche photodiodes (SPCM, ARQ-14, Perkin-Elmer) were used to count the fluorescence photons. It is important to clarify that with the current set up the system is not quite confocal, the pinhole is simply the active area of the avalanche photodiode (180 μm) and this does not discriminate completely against out-of-focus light.

Sections of 20 x 20 μm (200 x 200 pixels) at different heights (usually 4 scans, each 2 μm apart) above the bottom of the cover slip were scanned. The scan rate was set at 1 Hz and the excitation energy at 1 μWatt.

4.2.6 Image Analysis

Jay R. Unruh did the analysis of data collected from samples scanned by confocal microscopy. Briefly, the files were viewed with the software Scion Image (Scion Corporation, Frederick, MD) to obtain the coordinates of the particle of interest. Then, the same file was opened on Microsoft Excel, and the integral of the signal intensities at the coordinates of interest was calculated.

Whenever possible the samples were imaged with a Zeiss Axioplan 2ie upright high-resolution epifluorescence microscope (Carl Zeiss, Thornwood, NY), as described in section 2.2.4.6.2. These images were analyzed with Lispix software,[18] version Lx29P to determine the number of particles with intensities above a set threshold.

4.2.7 Calculations of how many Particles could bind to condensed DNA

Based on the dimensions of higher-order chromatin structures[19] a theoretical calculation was done for the number of particles that would be able to bind a 2.4 kb biotinylated probe bound to condensed DNA.

4.3 RESULTS AND DISCUSSION

4.3.1 FluoSpheres Quantum Yield Determination

The FluoSpheres quantum yield was determined 3 times. The quantum yield of the dendrimers was previously determined in the same way by Jay Unruh, and the quantum yield of the quantum dots was obtained from the manufacturer. Table 4.2 compares the three types of particles based on their quantum yield and brightness. A direct comparison is not possible because each particle has a different size, and different dyes, but it is evident that the FluoSpheres are by far the brightest particles of the four. The quantum yield of the Cy-3 dendrimers might seem low, but it was determined by Jay Unruh that the intensity fluorescence per dye label increases when Cy-3 is attached to a 4-layer dendrimer compared to when it is attached at the 5' end of a 30 base oligonucleotide, and that there is no self-quenching among Cy-3 dyes in the dendrimer. Similar observations were made by Mujumbar and colleagues,[20] who determined that the quantum yield of Cy-3 is highly dependent on the environment. For example, Cy-3 quantum yield in buffer was 0.04, while in glycerol it was 0.52.

Table 4.2. Dendrimers compared with other commercially available nanoparticles, FluoSpheres and Quantum dots.

Particle	Size (nm)	Quantum Yield	Extinction coefficient ($cm^{-1} M^{-1}$)	Brightness*
2-layer Dendrimer	90	0.2	1.5×10^5 (Cy-3)	1.4×10^5
4-layer Dendrimer	190	0.2	1.5×10^5 (Cy-3)	1.0×10^7
FluoSphere	40	0.8 ± 0.1	$(7.7 \pm 1.1) \times 10^7$ (particle)	6.4×10^7
Quantum Dot	15	0.5 ± 0.1	1.3×10^5 (particle)	6.5×10^4

*The brightness of the dendrimers was calculated by multiplying the extinction coefficient times the quantum yield times the number of Cy-3 molecules. The extinction coefficient of the FluoSpheres is of the particle, not the dye, so the brightness was calculated by multiplying the extinction coefficient times the quantum yield. Qdot Corporation provided the quantum dot data.

4.3.2 Probe PCR amplification

The first amplification was accomplished as described in section 2.2.4.2 with an annealing temperature of 58 °C. The forward primer used was **827-F** (Table 2.2), combined with the reversed primers from Table 4.1. A control amplification with primers **827-F** and **827-R** (Table 2.2) was run since it had proven to be succesful in previous runs. The amplifications with the control (**827-R**), and with primer **827R-2373** were successful, but the yield for the latter was approximately half. This result indicated that all reagents were in good condition. The amplifications with the rest of the primers gave smears and in some cases a faint band of the expected size. In order to determine the optimal annealing temperature for each set of primers, the experiment was repeated at 54.6, 61.5, and 65.5 degrees. The best results were obtained at 54.6 °C, but the bands remained faint and embedded among smears. Amplification using an annealing temperature of 54 °C, and primer **827F-13** significantly decreased the amount of smear, but gave products of multiple bands. Modifications in the PCR program were unsuccessful, so it was decided to amplify a long probe with primers **827F-13** and **827-R**, and a smaller one with the **978** primers (Table 2.2). Both amplifications were successful, with yields slightly lower than amplifications where no 16-biotin-dUTP was incorporated. This result was expected, based on reports that DNA in which biotin is incorporated is not a good template for *Taq* polymerase.[21] Furthermore, Richard and coworkers[22] reported a 20% reduction in the total amplification product for biotin incorporation, and up to 50% for digoxigenin incorporation.

The PCR amplification results show that longer primers not only gave higher yields, but were more specific, because the main product consisted of a single band, instead of multiple ones, as observed when both primers were 18 bases long.

4.3.3 Confocal Spectroscopy Studies

4.3.3.1 Dendrimers

Due to the nature of the labeled probe and anti-biotin dendrimers it is important to describe their possible interactions in solution. The drawings in Figure 4.1 show the biotinylated double stranded DNA probe represented by a single blue line and the anti-biotin dendrimers by red circles, and some of the possible interactions between dendrimer and probe are shown in Figure 4.2. Possibility 1 has as many dendrimers bound as possible; although there could be variations in the number of dendrimers bound. Because each dendrimer has approximately 4 anti-biotin antibodies, it is possible that some dendrimers will bind to more than one probe (shown in case 2). Other variations are also expected. Finally, the probe could wrap itself around the dendrimer, as shown in 3. Thus several biotin molecules from the same probe would interact with one dendrimer. The possibilities were considered taking into account that the dendrimers have a diameter of 90 nm and a 2381 bp probe in a double helix conformation will be ~809 nm long.

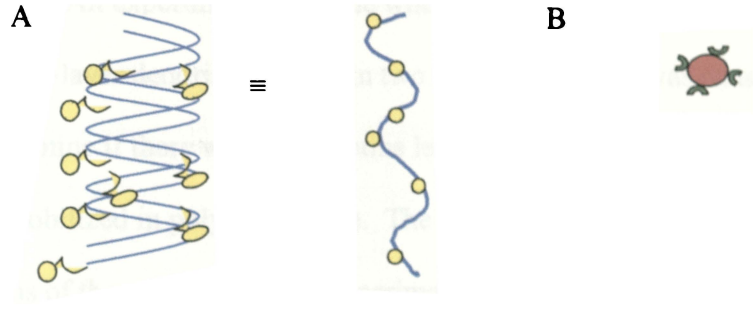

Figure 4.1. Drawing representation of biotinylated double stranded DNA probe and anti-biotin dendrimer. A: The blue lines represent the DNA, and the yellow circles with a connecting-line represent the biotin-16-dUTP. A simplified structure of the biotinylated double stranded DNA probe is shown to the right. B: The red circle represents a dendrimer and the green semicircles the anti-biotin antibodies.

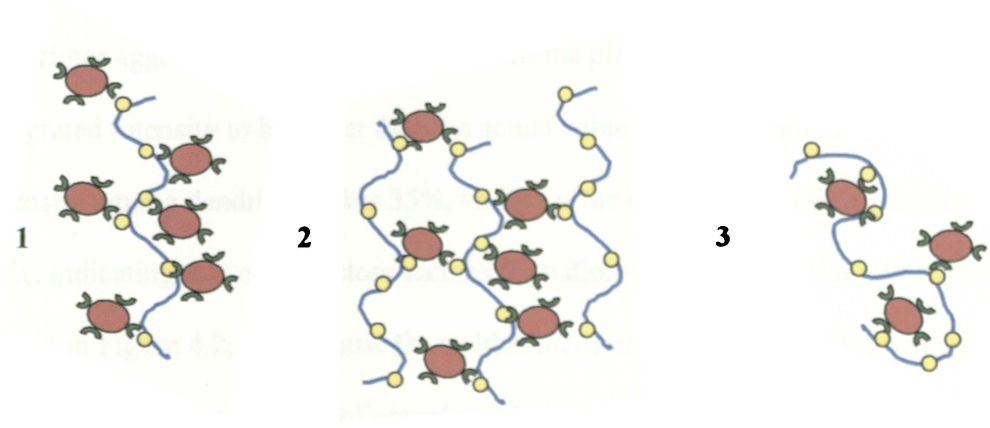

Figure 4.2. Drawing representation of possible interactions between biotinylated double stranded DNA probe and anti-biotin dendrimer. 1: Ideal interaction, one dendrimer per biotin. 2: More than one biotin (from different probes) binds per dendrimer. 3: More than one biotin (from the same probe) binds per dendrimer.

An experiment was done where a ~ 2.4 kb biotinylated probe was incubated with 2-layer dendrimers, split in two samples and one was chased with streptavidin to determine if there were any biotins left available for binding. Both samples were immobilized in polyacrylamide. The streptavidin data were collected from different scans of the same sample (1 experiment), while data from different scans of 3 different experiments were analyzed to calculate the integrated intensities of 2-layer dendrimers and 2-layer dendrimers incubated with a ~ 2.4 kb biotinylated probe. The results are summarized in Table 4.3; the average values for the integrated intensities are: (459 ± 162) for the 2-layer dendrimer, (787 ± 427) for the dendrimer-probe mixture, and (1732 ± 557) for the 2-layer dendrimer-probe-streptavidin mixture. The great variation among the dendrimer-probe mixture integrated intensity values could be explained by the different binding-possibilities between the two molecules, dendrimer aggregation, and molecules out of the plane of focus, causing their integrated intensity to be lower than the actual value. The variation in the integrated intensity among dendrimers was 35%, while for the dendrimer-probe mixture it was 54%, indicating that other factors such as formation of multimolecular complexes, as shown in Figure 4.2, could cause the sudden increase in variability. Because of these factors, quantitative analysis of complexes between dendrimers (or other particles) and biotin labeled DNA probes remains a challenge. Van Rompaey and coworkers[23] reported similar difficulties. They used fluorescence fluctuation analysis for the study

Table 4.3. Average integrated intensity values of different mixtures of anti-biotin dendrimer, biotinylated probe, and streptavidin, immobilized in polyacrylamide.

Integrated Intensity 2n Dendrimer (n = 13)	Integrated Intensity 2n dendrimer + probe (n = 12)	Integrated Intensity 2n dendrimer + probe + streptavidin (n = 5)
459 ± 162	787 ± 427	1732 ± 557

All written as (mean ± sem)

n represents the number of points analyzed

of interactions between oligonucleotides and polycationic polymers, and observed sudden fluorescence bursts that were attributed to the formation of multimolecular complexes.

It is important to note that the dendrimer-probe mixture integrated intensity average value is only 1.6 times greater than the integrated intensity average value of the dendrimer alone, while it was expected that at least 8 dendrimers would bind (assuming there were enough biotins uniformly distributed, Figure 4.2). Besides the factors that cause variability, quenching among dendrimers could cause such a small increment in the integrated intensity. This issue will be further discussed later, for now it is important to point out that the integrated intensity average value of the dendrimer-probe-streptavidin mixture, it was approximately 3 times greater than the values for the dendrimer-probe mixture. This result confirms the hypothesis that dendrimers were not binding to all biotin molecules. Assuming no quenching among dendrimers, and knowing that streptavidin has an average of 7.7 fluorophores compared to 50 fluorophores per dendrimer, this increment would correspond to the binding of 13 streptavidin molecules. This is a reasonable number, assuming that two dendrimers were already bound, 15 would be the total number of biotins in a ~2.4 kb probe, this corresponds to ~ 1 biotin per 160 nucleotides, which is a reasonable incorporation level according to manufacturer of the DNA polymerase used for the PCR amplification (Invitrogen, Rockville, MD).

Another experiment was done where the 2-layer dendrimer and a biotinylated ~0.9 kb probe were incubated. The average values for the integrated intensities were:

(502 ± 197) for the 2- layer dendrimer, and (316 ± 76) for the dendrimer-probe mixture. When the same slides were analyzed with the LixPix program (Table 4.4), 2% of the dendrimer-probe spots are above intensities of 55 pixel units, while 7% of the dendrimer spots are above 60. This indicates that the intensity of the 2-layer dendrimer decreased when incubated with the probe, as observed to a lesser degree with the ~2.4 kb probe (signal was not as high as expected). Another observation consistent with this result is shown in Figure 4.3, where the emission of a 2-layer dendrimer solution in 0.01 M HEPES buffer decreases upon incubation with a ~2.4 kb biotinylated probe, indicating quenching upon binding. It is possible that the quenching effect was more drastic with the 0.9 kb probe if bound dendrimers were spatially closer. The 2.4 kb probe might allow dendrimers to bind farther apart, so that there is less quenching. It is important to note that the same phenomenon was observed with the 4-layer dendrimer, and in NaCl/Na_3Citrate buffer.

A different phenomenon was observed when 2 specific dendrimers (described in chapter 3) where incubated with denatured probes. Figure 4.4 shows the emission spectra of 2 different specific dendrimers (designed to bind 420 bases apart from each other), prior and after incubation with a ~1.3 kb probe, where the dendrimer emission increases upon incubation with the DNA probe. A similar effect was observed when a different set of dendrimers (**354-245** and **354-428**), designed to bind 183 bases

Table 4.4. Number of spots within a set intensity threshold at a set pixel-minimum area.

Dendrimer alone 9-174-6			Dendrimer + 912 bp probe 9-174-5		
Intensity Threshold	# spots with a 3-pixel Area	# spots with a 10-pixel Area	Intensity Threshold	# spots with a 3-pixel Area	# spots with a 10-pixel Area
116-255		1	100-193	2	2
70-255	9	3	80-193	2	2
60-255	48	9	55-193	7	3
48-255	699	357	40-193	347	119

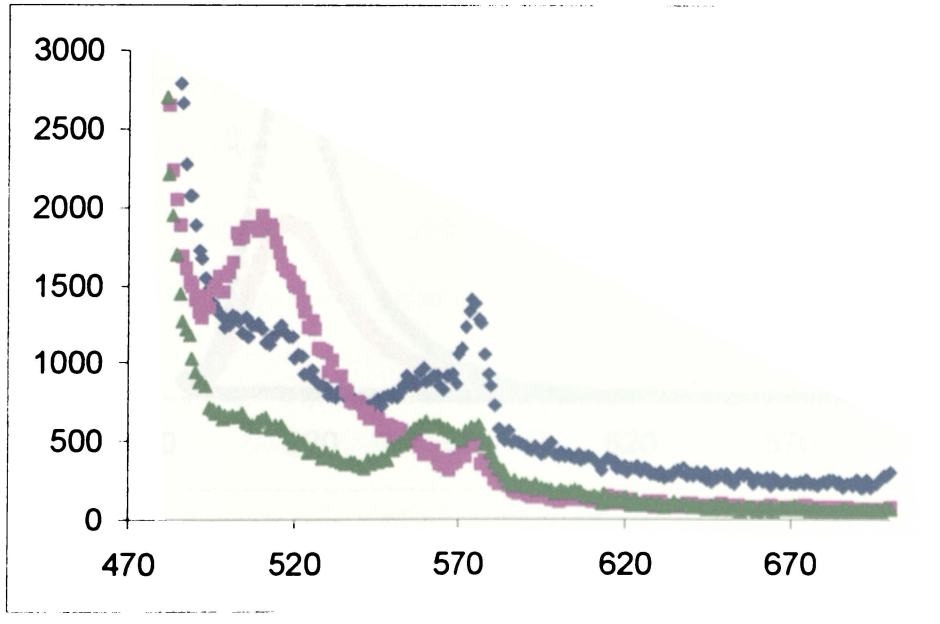

Figure 4.3. Emission spectra of different mixtures of dendrimer, and biotinylated probe. The blue trace shows the emission spectrum of HEPES buffer with 2-layer anti-biotin dendrimers, the pink trace indicates the emission spectrum of buffer with probe **827** (~2.4 kb probe 5' labeled with Alexa 488), and the green trace the emission spectrum of buffer with dendrimers and probe. The fluorescence emission of the 2-layer dendrimer was quenched upon probe incubation.

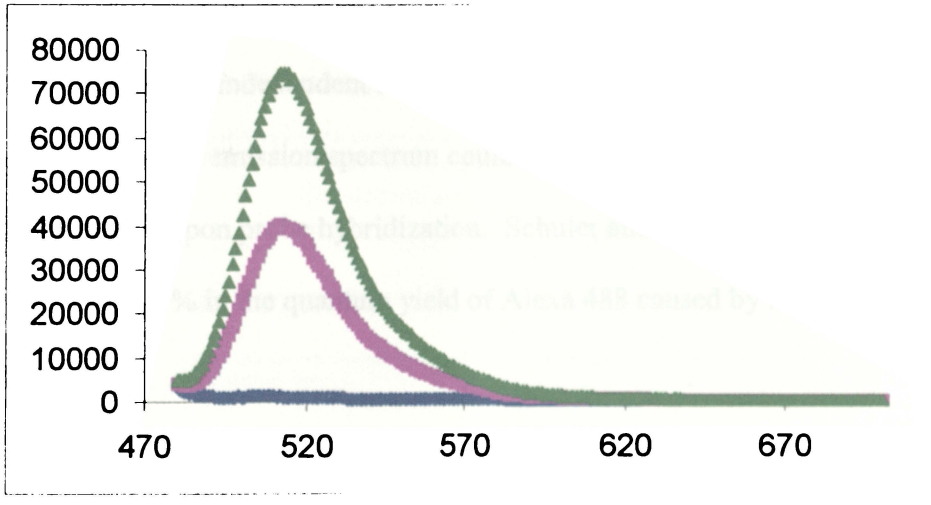

Figure 4.4. Emission spectra of different mixtures of dendrimer, and probe The blue trace indicates the emission spectrum of buffer with probe **737**, the pink trace the emission spectrum of buffer with dendrimers **737-517** and **737-937**, and the green trace the emission spectrum of buffer with dendrimers and probe.

apart) were incubated with the respective probe of~1.2 kb. This phenomenon was reproducible and independent of the type of buffer (HEPES or NaCl/Na_3Citrate). The increment in the emission spectrum could be caused by a change in the quantum yield of Alexa 488 upon probe hybridization. Schuler and coworkers[24] have reported changes of 25% in the quantum yield of Alexa 488 caused by its interactions with a protein.

The results from scanning the specific dendrimer samples immobilized in polyacrylamide (Table 4.5), showed no significant difference between the average intensities when considering the standard deviations. The high intensity of some of the "single-dendrimer" spots analyzed could be due to aggregates. If so, the signal from **354** incubated with dendrimers designed to bind 183 bases apart seems to indicate that no more than one dendrimer was able to bind under the experimental conditions. One possibility is that the number of **354** molecules bound to two dendrimers was very low, therefore not found in the sections that were scanned. The experiments for scanning were done with ~2 x 10^{-15} moles of probe and ~2 x 10^{-14} moles of dendrimer. This corresponds to a probe concentration of probe of ~1 x 10^{8} molecules·cm^{-2}, so in a 20 μm x 20 μm section there would be ~24 when considering the thickness of the scanned section. In the event that the probe concentration was overestimated, as could have happened because ethidium bromide was used in quantitative analysis of nucleotides,[25] there might not be enough molecules per

Table 4.5. Average integrated intensity values of different mixtures of specific dendrimer, and probe, immobilized in polyacrylamide.

Intensity Specific Dendrimers (n = 5)	Intensity Specific Dendrimers + probe **737** (n = 5)	Intensity Specific Dendrimers + probe **354** (n = 5)
1282 ± 786	912 ± 341	693 ± 123

All written as (mean ± sem)

scanned section. Another possibility is that the probe concentration was underestimated, so that there were not enough dendrimers per probe. This last possibility seems less likely because the same dendrimer / probe ratio was used for the collection of emission spectra, where an increment in the emission was observed for probe-dendrimer mixtures respect to dendrimers alone. So perhaps the dendrimers are bound in solution but the hydrogen bonds between dendrimer and probe became disrupted upon polymerization, causing the dendrimers to unbind. The effect on 737 might not have been as drastic since the dendrimers bind farther apart, so the steric hindrance does not contribute to the disruption of the hydrogen bonds. It is important to note that the integral intensities of the specific dendrimers were higher than those of the anti-biotin dendrimers because they were labeled with Alexa-488, rather than Cy-3 and therefore excited at the required wavelength.

No significant difference was determined between the intensity of 4-layer and 2-layer dendrimers by this method. The average values were (428 ± 126) for the 2-layer dendrimer, and (499 ± 168) for the 4-layer dendrimer, both at a concentration of 308 pM in the gel. It is possible that this was mainly caused by aggregation, and further studies by other methods and at lower concentrations will be required to quantitatively determine the number of dendrimers that bind to a biotinylated probe.

4.3.3.2 FluoSpheres

Under the experimental conditions evaluated, the results from incubations of FluoSpheres with biotinylated probes were inconclusive. Table 4.6 summarizes the

results from two different experiments where the FluoSpheres were sonicated prior to incubation. There is great variability among the integrated intensities of different spots, so when considering the standard deviation, there is no significant difference among different samples. The average values for the integrated intensities for day 1 were: (7,914 ± 1,831) for the FluoSpheres, (11,183 ± 4,809) for the FluoSphere-2.4 kb probe mixture, and (7,837 ± 2,369) for the FluoSphere-0.9 kb probe mixture. The integrated intensities for day 2 were significant different from day 1: (16,919 ± 4,001) for the FluoSpheres, and (19,789 ± 7,669) for the FluoSphere-0.9 kb probe mixture. The big variation among intensities within the same sample and between days may be caused by aggregation. The FluoSpheres might need to be sonicated for longer periods of time and different buffers should be evaluated. The values from FluoSpheres incubated with a 2.4 kb probe are approximately 1.4 times higher than the values for FluoSpheres alone. If the two highest values are average, their intensity is about twice that of FluoSpheres alone. This indicates that under these conditions approximately the same number of FluoSpheres bind to a biotinylated 2.4 kb probe compared to the 2-layer dendrimers. This could be possible because the dendrimers are made of DNA, so they are more flexible than polystyrene particles. But as was mentioned before, there is too much variation in the data to make a quantitative interpretation of the data.

Table 4.6. Average integrated intensity values of different mixtures of FluoSpheres, and probe, immobilized in polyacrylamide, prepared and analyzed on different days

Day 1			Day 2	
Intensity FluoSpheres (n = 4)	Intensity FluoSpheres + 2.4 kb probe (n = 4)	Intensity FluoSpheres + 0.9 kb probe (n = 3)	Intensity FluoSpheres (n = 5)	Intensity FluoSpheres + 0.9 kb probe (n = 6)
7,914 ± 1,831	11,183 ± 4,809	7,837 ± 2,369	16,919 ± 4,001	19,789 ± 7,669

All written as (mean ± sem)

4.3.3.3 Quantum Dots

Quantum Dots used were CdSe crystals, coated with a ZnS shell to improve the optical properties of the crystal, followed by a polymer shell directly coupled to streptavidin. According to the User Manual (PN 90-0003, Rev 4), the final product has a diameter of only a few nanometers (10-15 nm).

The results from scanning immobilized samples of quantum dots and quantum dot-probe mixtures are summarized in Table 4.7. The average values for the integrated intensities were: (124 ± 54) for the quantum dots, (198 ± 163) for the quantum dot-0.9 kb probe mixture, and (1039 ± 369) for the quantum dot-2.4 kb probe mixture. This corresponds to ~2 quantum dots binding per 0.9 kb probe and ~8 quantum dots binding per 2.4 kb probe. These data are supportive of the steric hindrance hypothesis because it shows a greater number of quantum dots (8) binding to the 2.4 kb probe, compared to only ~2 dendrimers. Considering that the quantum dots are ~6 times smaller than the dendrimers, it is reasonable to find that ~4 times more quantum dots than dendrimers are binding. The number of quantum dots binding is smaller than the number of streptavidins calculated to bind (19), indicating that there still might be some constraints for 15 nm particles to bind to all biotins.

Xiao and Baker[13] used 15 nm quantum dots conjugated to streptavidin to detect a 168 kb probe. When they labeled total genomic DNA and hybridized it to metaphase chromosomes, no signal was detected in centromeric regions. They

Table 4.7. Average integrated intensity values of different mixtures of streptavidin coated quantum dots, 2.4 kb biotinylated probe, and 0.9 kb biotinylated probe, immobilized in polyacrylamide.

Intensity Qdot (n = 6)	Intensity QDot + 2.4 kb probe (n = 7)	Intensity QDot + 0.9 kb probe (n = 3)
124 ± 54	1,039 ± 369	198 ± 163

All written as (mean ± sem)

suggest that these results were due to the quantum dots blinking signal. Another possibility, not discussed by the authors, is that 15 nm quantum dots were not able to bind to biotinylated probes bound to the highly condensed centromeric regions.

4.3.4 Chromatin Structure Organization Calculations of How Many Particles Could Bind to Condensed DNA

According to the dimensions of high-order chromatin structures described in Figure 4.5 and previously determined by others,[19] 165 base pairs of DNA wrap around the histone complex to form the nucleosome. So, a 2.4 kb probe would be wrapped into 14 –15 nucleosomes. There are several models of how the nucleosomes are further condensed. If we use the solenoid model, also shown in Figure 4.5, the greatest distance between any two points around the solenoid is ~32 nm. Assuming that the particles are rigid structures, and that there will be biotins (from the labeled DNA) on the outside of the structure, the solenoid could only accommodate three 2-layer dendrimers (90 nm diameter), five 40 nm FluoSpheres, or nine 15 nm quantum dots around it.

These calculations agree with our results on less condensed DNA, where 4 times more quantum dots than 2-layer dendrimers were determined to bind to the target.

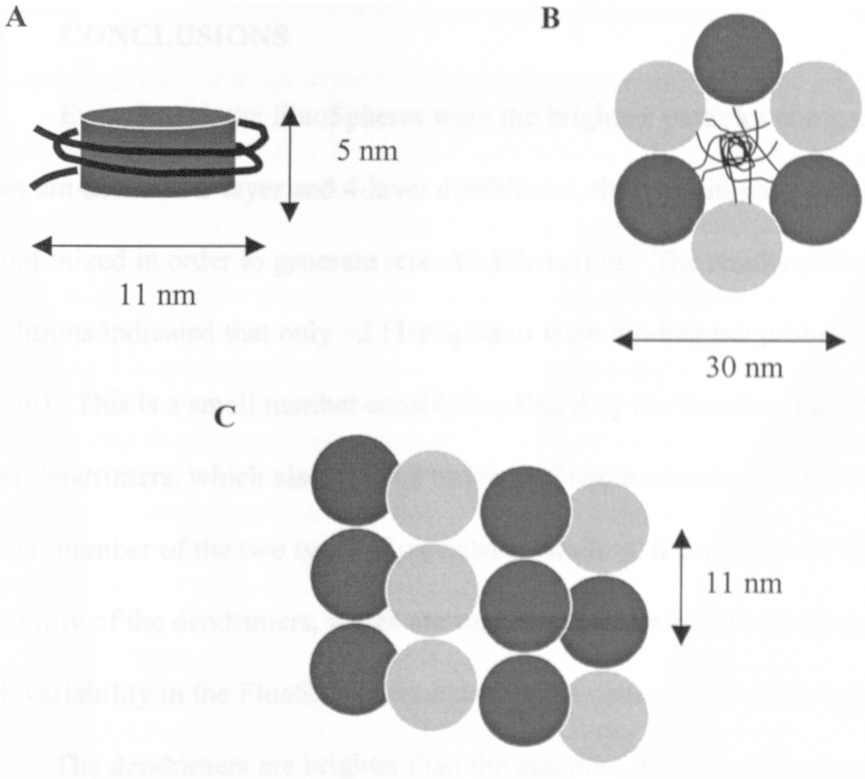

Figure 4.5. Different components of the chromatin structure with their dimensions.

A: Nucleosome, B: Solenoid, and C: Side view of the solenoid.

4.4 CONCLUSIONS

Even though the FluoSpheres were the brightest particles compared to quantum dots and 2-layer and 4-layer dendrimers, the experimental conditions need to be optimized in order to generate reproducible results. The results under the present conditions indicated that only ~2 FluoSpheres were binding per probe (2.4 kb in length). This is a small number considering that they are less than half the size of 2-layer dendrimers, which also showed binding of two molecules per probe. If in fact a similar number of the two types of particles is binding, it could be due to the higher flexibility of the dendrimers, which are made of DNA rather than polystyrene. The high variability in the FluoSphere results could be caused by particle aggregation.

The dendrimers are brighter than the quantum dots even when excited at 488 nm (the ideal excitation wavelength for Cy-3 is 550 nm). Although the intensity of a single dendrimer labeled with Cy-3 excited at 488 nm was more than 3 times that of a quantum dot, the intensities of the dendrimer bound to probe (787 ± 427) were ~ 1.3 times lower than that of quantum dots bound to probe (1039 ± 369). This result seems to be caused by quenching of the anti-biotin dendrimer fluorescence upon probe binding and a greater number of quantum dots binding per probe. Even with quenching, the integrated signal intensity of dendrimer-probe was almost double of that of dendrimer alone, indicating signal amplification. Unfortunately, because of the quenching it cannot be determined how many dendrimers are actually binding.

It was demonstrated that 2-layer dendrimers are not able to bind to all biotins because the integrated signal intensity increased upon incubation with streptavidin.

Furthermore, the number of streptavidins calculated to bind (19) coincides with the predicted number of biotins that could be incorporated into a 2.4 kb probe. These results are also supported by theoretical calculations of the number of particles that would be able to bind to a biotinylated probe condensed into a solenoid. Approximately 3 times more quantum dots were determined to be able to bind to the target than 2-layer dendrimers would. The experimental results for less condensed DNA showed that 4 times more quantum dots were binding to the probe. This number is slightly larger than the theoretical calculation predicted, but could be reduced when considering the quenching of the dendrimer fluorescence upon binding.

The highest signal amplification was observed for the quantum dots, where the quantum dot-probe signal had 8 times the intensity of quantum dots alone. Based on these preliminary results, quantum dots seem like the best alternative for signal amplification, due to their ability to bind more biotins. But before making that a final conclusion more experiments are necessary, the comparison should be done among particles of the same or very similar diameter, and under conditions of none or little particle aggregation. Dendrimers would be a good alternative once the quenching issue is resolved. Because no quenching was observed for the specific dendrimers in the emission spectra, it would be important to study their binding to probes in solution without immobilization in polyacrylamide, in case the polymerization reaction disrupts the hydrogen bonds between probe and dendrimers.

Due to particle aggregation and analysis difficulty caused by molecules out of focus, other methods should be explored for the analysis of the probe-particle

complexes. Van Rompaey and colleagues[23] used the photon counting histogram (PCH) technique to analyze fluorescence fluctuation data of multimolecular complexes of oligonucleotides with polymers. Even though they found the quantitative interpretation of the data from these complexes to remain a challenge, the technique would help gain a better understanding of how many particles bind to the biotinylated DNA probe in solution.

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5. DETECTION OF ACETYLATED AND NON-ACETYLATED HISTONE H4 ON EXTENDED CHROMATIN FIBERS

5.1 INTRODUCTION

As mentioned in the introductory chapter, DNA associates with histones to form nucleosomes and other higher-order structures in order to facilitate its packing into the nucleus. Figure 5.1 shows the X-ray crystal structure of the nucleosome, where the association of DNA with the histone core can be seen and the interactions among histones within the core. For a long time it was thought that the only function of the histones was structural, but now there is considerable evidence indicating that post-translational modifications of the histone tails could play an important role in cellular processes such as transcriptional activation and repression, gene silencing, histone deposition, and mitosis.[1]

5.1.1 Antibody Generation

Antibodies have been used for decades in the study of histones. During the late-seventies antibodies were generated using purified fractions of the different kinds of histones as immunogens.[2] This was troublesome because the histones' native structure was easily altered during purification. The identification of some antigenic regions by several groups[3, 4] facilitated the production of polyclonal and monoclonal[5] antibodies to these epitopes, but the generation of histone antibodies to site-specific

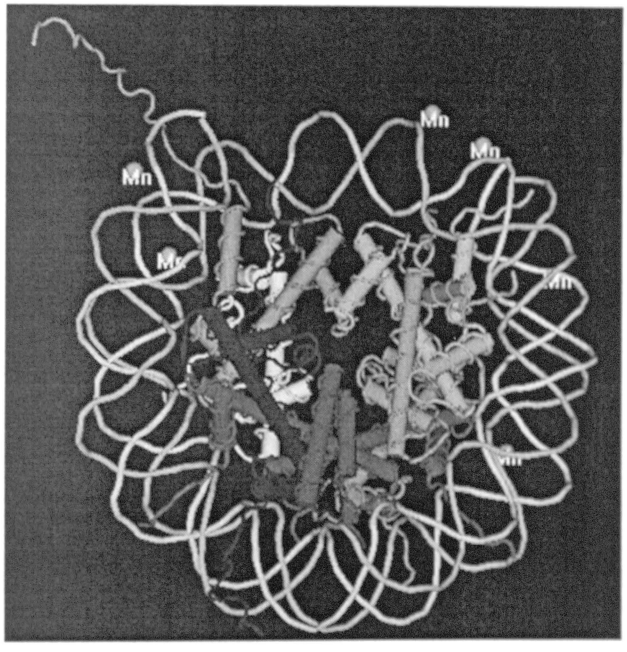

Figure 5.1. X-ray crystal structure of the nucleosome at 2.8 Å resolution determined by Luger *et al.*,[6] and available from the National Center for Biotechnology protein database.

acetylations remained difficult.[7] It was in the mid and late nineties that Boggs *et al.*[8] and White *et al.*[9] independently reported the development of antibodies specific for histones acetylated at defined lysine residues. Histone antibodies have been used to study histone organization in chromatin,[3, 10] localized histones in heavily transcribed regions,[11] and to study histone post-translational modifications.[4, 12]

5.1.2 Histone Modifications

Part of the interest in the study of histone post-translational modifications arised from the proposal[1] that distintict histone modifications form a "histone code", read by other proteins to cause different cellular events. Acetylation has been one of the most studied modifications, since first linked to gene activation by Allfrey.[13] Table 5.1 gives a summary of different types of acetylation, and the reaction is shown in Figure 5.2, at the ε-amino groups of specific lysines in the amino-terminal domains (tails) of the histones. There has also been increasing evidence linking cancer and the histone-modifying enzymes HATs and HDACs[14, 15] (described in chapter 1).

5.1.3 Approaches to Correlate Histone Acetylation to a Particular Chromosome or Chromosomal Region

One of the approaches used to link histone acetylation to a particular gene (known to be active or inactive) has been to immunoprecipitate all acetylated histones and then determine by PCR if the gene of interest is present or not. This assay is

Table 5.1. Summary of acetylation turnover, probable function, and number of acetates per molecule, classified according to histone types. Modified from "Chromosomes and Chromatin".[16]

Histones Involved	Acetates per Molecule	Turnover Rate*	Probable Function
H3, H4	3-4	Fast	Transcription
H2A, H2B, H3, H4	1-2	Moderate	Replication
H2A, H2B, H3, H4	3-4	Moderate	Replacement

*Fast is on the order of 0.2 to 0.5 per minute, Moderate is about 10 times slower.

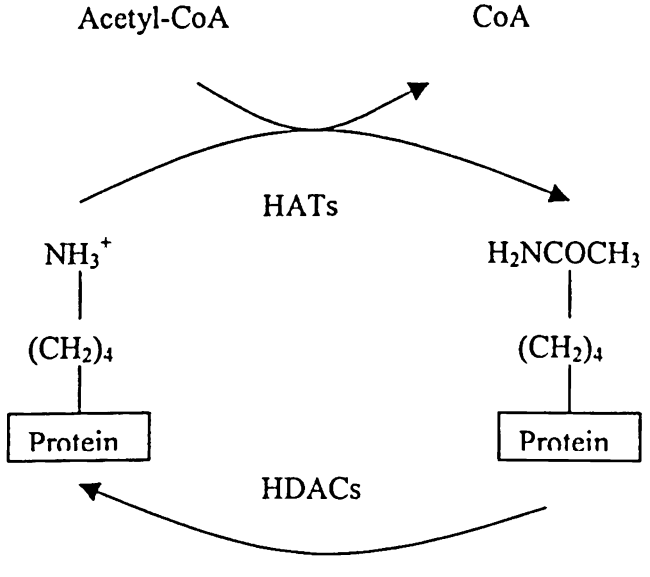

Figure 5.2. Histone acetylation-deacetylation cycle

known as ChIP (chromatin immunoprecipitation), and is a good tool to distinguish overall histone acetylation in active genes from silent ones, but is difficult to control and interpret because the measurements are done in bulk chromatin.

Boggs and coworkers[17] reported reduced levels of histone H3 acetylation in the inactive X chromosome in human females. The determination was done on metaphase chromosomes by immunofluorescence (IF), but preliminary experiments done in the Wilson Laboratory indicated that the resolution that can be obtained from histone antibodies bound to metaphase chromosomes is not sufficient to correlate the histone modification to a particular genomic site.

Collas and coworkers[18] linked histone H4 acetylation to active transgenes in zebrafish by two methods: immunoprecipitation followed by DNA extraction and examination by dot blot hybridization, and IF and fluorescence in situ hybridization (FISH) on interphase nuclei and chromatin extended fibers. The latter had the advantage that the histone-DNA interactions are maintained on extended chromatin fibers, so that signal from the antibody and the DNA probe can be colocalized. Another great advantage is the resolution attained with extended chromatin fibers. Chromosome-based FISH mapping is limited to 1-3 megabases (Mb), while fiber-FISH is able to distinguish two probes separated by 1 kb on a DNA fiber.[19]

The objective of the work presented in this chapter is to generate antibodies to acetylated and non-acetylated histone H4 and evaluate their use in immunofluorescent assays on chromatin extended fibers. This preliminary work was done as a first step in the development of an assay were the scFISH probes described in chapter 2 would

be combined with these antibodies to determine the effect of acetylation levels of histone H4 at the promoter region of genes PML (chromosome 15) and RARA (chromosome 17) in HT93A cells, on transcription of those genes. As indicated in Table 5.1, acetylation of histones H3 and H4 has been linked to transcription, hence H4 being the focus of this work.

5.2 EXPERIMENTAL METHODS

5.2.1 Preparation, Isolation, and Purification of Polyclonal Antibodies

Antibodies were generated against the antigenic peptides H4(K)nonAc and H4(K16)Ac (see Table 5.2) as described by Vitharana.[20] Briefly, purified protein derivative (PPD) was used as a carrier;[21] PPD was activated using sulfo-SMCC (Pierce, Rockford, IL), separated by dialysis in 0.01 M PBS, pH 7.2. The peptide was conjugated to activated-PPD, and separated from non-conjugated peptide by dialysis. Two New Zealand white rabbits were immunized (one for each peptide-PPD antigen), and boosted every two weeks to keep the titer high. Blood was collected every two weeks and the antibodies separated, first by fractionation of the serum from whole blood and then isolation of the γ-globulin fraction (known to contain the antibodies).[22] Antibodies were further purified by affinity chromatography, where the peptide was immobilized into an Ultralink™ iodoacetyl column (Pierce) and the γ-globulin fraction was passed through to isolate only those antibodies against the immobilized peptide. The titer was determined by an enzyme-linked immunosorbent assay

Table 5.2. Amino acid sequence and acetylated positions of histone 4 peptides reported by White *et al*[9] in the production of site-specific antibodies.

Histone	Modification	Peptide*, **
H4	(K)non Ac	RGKGGJGLGKC
	(K5)Ac	SGRGK̲GGKGLYC
	(K8)Ac	SGRGKGGK̲GLGKYC
	(K12)Ac	GKGLGK̲GGALYC
	(K16)Ac	GLGKGGAK̲RHYC
	(K5/8/12/16)Ac	SGRGK̲GGK̲GKGK̲GGAK̲RHYC

*Single letter amino acid code; J, D-lysine isomer; K̲, ∈-acetylated lysine

**Polyclonal antibodies to these peptides are commercially available.

(ELISA) where the wells of a microtiter plate were dry coated with the peptide, no peptide as negative control, and non-antigenic peptide to check for cross reactivity. The serum (or purified fraction) was incubated in all wells at different dilutions and allowed to react, and free serum was washed off. The presence of antibody bound to the peptide was determined with a secondary antibody (anti-rabbit labeled with peroxidase). Unreacted secondary antibody was washed off, and the bound component was detected by adding a peroxidase substrate (TMB:peroxidase B) and quenching the reaction with HCl after a few minutes.

5.2.2 Preparation of chromatin extended fibers

Prior to the preparation of the fibers the glass slides were coated with poly-L-lysine according to Heiskanen *et al.*[23] Briefly, the slides were dipped for 30 seconds in each of the following solutions: 0.2 N HCl, water, and acetone; and then dried at room temperature. The slide was dipped in 0.15% gelatin/0.03% sodium azide for 5 minutes, air dried at room temperature, dipped twice for 10 minutes in 0.2% poly-L-lysine of molecular weight higher than 300,000 g/mol (Sigma, St Louis, MO), rinsed in water, dried overnight and stored at 4 °C.

The preparation of extended chromatin fibers was modified from Schwarzacher and Heslop-Harrison [24] and Collas *et al.*[18] Briefly, 1 mL of cell suspension was centrifuged at 3600 rpm for 5 minutes and gently resuspended in the same volume of 0.01 M PBS, pH 7.2. Approximately 1 or 2 μL of the cell suspension were deposited onto one end of a poly-L-lysine coated slide, and let air-dry. The cell

suspension was then incubated for 5 minutes with 10 µL of STE lysis buffer (0.5% sodium dodecyl sulfate, 100 mM Tris-HCl, pH 7, 50 mM ethylene diamine tretraacetate) at room temperature. After the 5 minutes the slide was tilted at a 45-degree angle and let air-dry. It was then
submerged in 4% paraformaldehyde in 0.01 M PBS for 5 minutes, and 0.1% Triton X-100 in 0.01 M PBS for another 5 minutes, then air dried, and stored with desiccant at 4 °C.

5.2.3 Immunofluorescence on Chromatin Extended Fibers

Chromatin fibers were treated according to Collas and coworkers[25] with a few modifications. Briefly, the fibers were fixed in 4% paraformaldehyde for 10 minutes and washed in 4 changes of 0.01 M PBS/0.01% Tween 20 (PBST) for 1 hour. Then the fibers were incubated in a humidity chamber at 37 °C with ~100 µL of the anti-H4 antibody of choice, at a 1:400 dilution in PBST, for 8 hours. Incubation was followed by washing for at least 2.5 hours, with 6 changes of PBST. In order to detect the antibody, the fibers were incubated with ~30 µL of a secondary antibody (FITC conjugated anti-rabbit IgG, Sigma, St Louis, MO) in a 1:300 dilution, for 6 hours, followed by washing with 4 changes of PBST for 3 hours. Finally fibers were stained and mounted as described in section 2.2.4.6. The experiments were performed with commercial antibodies (Serotec, Oxford, UK), and antibodies generated in the Wilson laboratory.

5.3 RESULTS AND DISCUSSION

5.3.1 Preparation, Isolation, and Purification of Polyclonal Antibodies

Generation of antibodies from natural histones is cumbersome, mainly because of the need to extract and purify the histones, maintain their native conformation, and even the need to conjugate the histones to carriers because they are poor immunogens by themselves.[12] Since the peptide sequence and the lysine residues known to be acetylated are published, the best alternative was to synthetically generate the peptide with the modification of interest to be used as an antigen.

In general substances with molecular weight of less than 5 – 10 KDa are poor immunogens and require conjugation to "carrier" molecules to be able to elicit an antibody response.[22] That is the case for the acetylated (1042 g/mol) and non-acetylated (1200 g/mol) peptides, used for this work. Purified protein derivative (PPD), with a molecular weight of ~1 x 10^4 g/mol, has proven to be a good immunogen carrier. In a study by De Silva and coworkers,[21] antigens coupled to PPD generated antibodies with higher specificity than those generated when the antigen was conjugated to ovalbumin (OVA), or Keyhole limpet hemocyanin (KLH). When PPD was used as a carrier, few antibodies were generated to the carrier. Thus the majority of antibodies induced are specific to the antigen of interest. The antigenic peptides H4(K)nonAc and H4(K16)Ac elicited a good antibody response when conjugated to PPD, the titers (a measure of the presence of antibody in the blood)

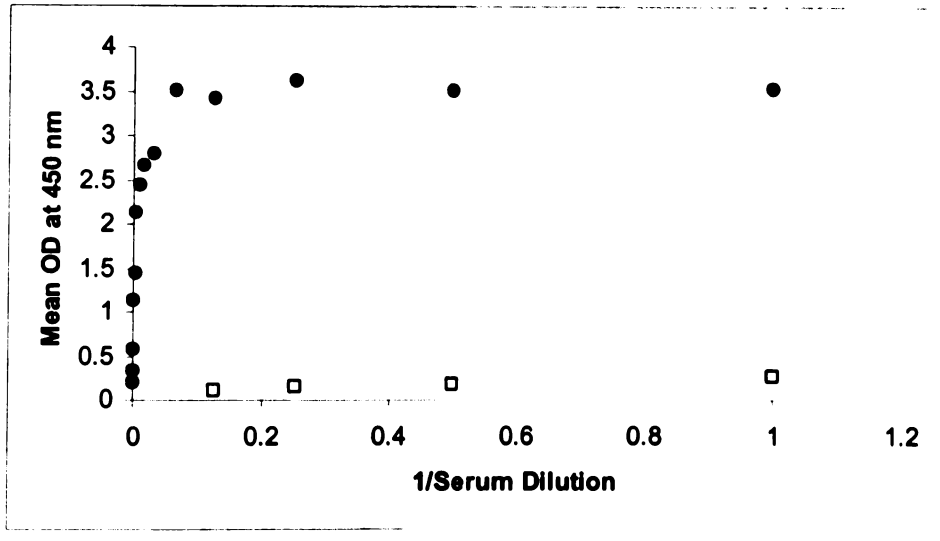

Figure 5.3. Titer curves for anti H4(K)nonAc, approximately 3 weeks after the second boost (●), and prior to immunization (□). The calculated titer for this serum was 2^8.

were 2^9 and 2^7, respectively; while the titer in blood taken prior to immunization was 2^2 and 2^4 , in the same order. Figure 5.3 shows an example of a titer curve determined ~3 weeks after the second boost with H4(K)nonAc. It was found that the titer slightly decreased to 2^8. This slight decrease was expected because when animals are injected with an antigen, the primary response is short and low in magnitude; the secondary response (after the boost) is higher and lasts longer but does not remain high.[22] It was necessary to boost the rabbits every two weeks to keep the titer high, when the boosts were stopped the titer dropped to 2^4.

Immunoglobulin G (IgG) is the main class of antibody molecules and is mostly found in the γ-globulin fraction of serum. It can be isolated by electrophoresis or by slow addition of ammonium sulfate, which causes mostly IgG to precipitate. After this process further purification by affinity chromatography is necessary to ensure isolation of specific antibodies. Stringency of the affinity purification could be detrimental to the antibodies, so the antibody bound fraction was collected into a buffer of pH 8.0 in order to adjust the low pH of the eluting buffer, which can cause denaturation.

The crossreactivity of each antibody to the non-antigenic peptide was determined to be less than 8% and comparable to the response to BSA, but is important to keep in mind that crossreactivity can be a bigger problem when testing antibodies on cells where other antigens could be highly concentrated on a particular organelle. Especially in this case it was important to determine cross reactiviy to peptides acetylated at positions other than lysine 16. Vitharana[20] did the binding

constant determination and found them to be 0.14 x 10^9 M^{-1} for the antibody against H4(K16)Ac, and 0.2 x 10^7 M^{-1} for the antibody against H4(K)nonAc. These numbers represent the avidity of a heterogenous population of antibodies, rather than the affinity of an antibody.

5.3.2 Preparation of Chromatin Extended Fibers

5.3.2.1 Slide Coating

Coating with 3-aminopropyltriethoxysilane (APES) was evaluated as an alternative to coated slides, but results were more reproducible on poly-L-lysine coated slides. Figure 5.4 compares the quality of the fibers immobilized on APES versus poly-L-lysine coated slides. Even though both slides were prepared from the same sample, the poly-L-lysine coated slide consistently produced nicely stretched fibers, and the ones coated with APES showed irregularities as the spots show in Figure 5.4. Poly-L-lysine coated slides tested 1-2 months after preparation kept their quality when stored with desiccant at 4 °C.

5.3.2.2 Extension of Chromatin Fibers from HT93A Nuclear Suspensions

Variations on the inclination angle were necessary to cause the nuclear suspension to slide down, and it was important to check the quality of the fibers prior to any experiments by DAPI staining. Unfortunately unlike metaphase chromosomes, fibers cannot be analyzed with a light-contrast microscope. Fibers were also extended mechanically, with the aid of a coverslip or another slide, but many

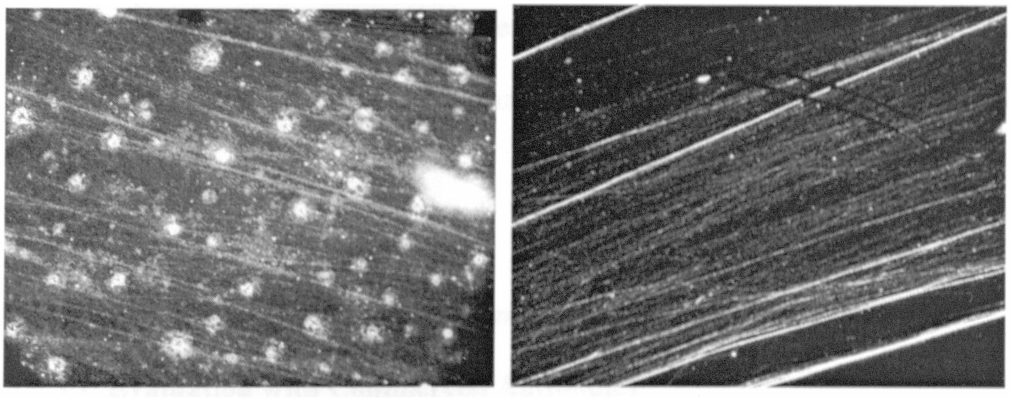

Figure 5.4. HT93A extended chromatin fibers on slides with differently modified surfaces. The image on the left frame corresponds to a slide covered with 3-aminopropyl (trimethoxy) silane, there are spots all over slide, otherwise the fibers are nicely stretched. The right frame shows a slide covered by poly-L-lysine (>300000 MW) dipping, with very good fibers throughout the slide.

fragments collected at the bottom of the slide, and a high concentration of fibers remained on the top. The "inclination-angle" technique was easier to master and gave more reproducible results.

5.3.3 Immunofluorescence on Chromatin Extended Fibers

5.3.3.1 Evaluation with Commercial Antibodies

The acetylation of H4 in mammals is non-random. In humans lysine 16 is the first to be acetylated, followed by lysine 8, lysine 12 and finally lysine 5, but there is a degree of flexibility,[26] Table 5.3 shows the lysines acetylated according to the number of acetates present per H4. When HT93A extended chromatin fibers were incubated with antibodies to H4 nonAc, H4(K5)Ac, H4(K8)Ac, and H4(K12)Ac (purchased from Serotec, Oxford, UK), and treated as described in section 5.2.3, the predicted pattern can be seen in the results shown in Figure 5.4, where the signal from antibodies to H4-Ac12, and H4-Ac8 was present in most of the fibers examined across the slide, and was more contiguous than the signal from anti H4-Ac5, which was segmented and present in less fibers. Those images also indicate that lysine 12 acetylation is higher than lysine 8, which has a more segmented signal.

5.3.3.2 Immunofluorescence with antibodies generated in the Wilson Lab

The antibodies used in these experiments were affinity purified, and dialyzed in 0.01 M PBS. Their concentration was estimated from the sample absorbance at 280 nm,[27] using a = 1.42 mL mg^{-1} cm^{-1}. The concentration for anti acetylated H4

Table 5.3. Order of lysine acetylation in H4

Number of Acetates	Lysines Acetylated
1	16
2	16, 12 (or 8)
3	16, 12, 8 (or 5)
4	16, 12, 8, 5

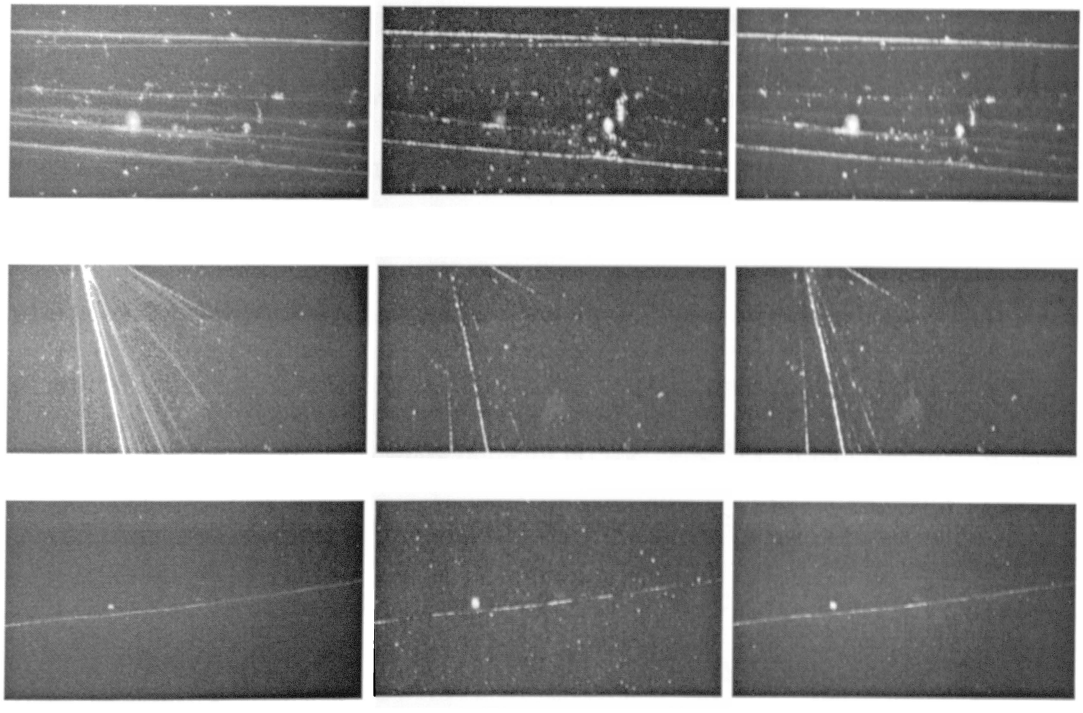

Figure 5.4. The first column shows HT93A extended chromatin fibers, detected by DAPI staining. The second shows signals from anti H4 acetylated at lysine residues12, 8 and 5 (from row 1 to 3), detected with a secondary anti-rabbit antibody labeled with FITC. The third column is the overlay of columns 1 and 2.

was 0.029 mg/mL and 0.032 mg/mL for the antibody against non-acetylated H4. After a series of dilutions of the primary antibody, it was determined that the dilution at which background was lowest but the signal that remained was 1:200. Figure 5.5 shows a fiber with signal from non-acetylated H4 antibody on a HT93A extended chromatin fiber. Almost all fibers analyzed show signal from this antibody as well, while the signal from antibodies against acetylated lysine-16 was present in only some of the fibers. The high number of fibers indicating non-acetylation of H4 is consistent with an acetylation study done on HL-60 cells[28] (a human cell line) where purified mono-nucleosomes were immunoprecipitated with antibodies against H4 acetylated at different positions, and the fraction of non-acetylated mono-nucleosomes was high. This observation is also supported by a study from Desai and colleagues.[29] They showed that the percentage of histones acetylated (by exposure to acetic anhydride) was particularly low in cell populations derived from leukemic cells, less than 40% compared to almost 60% in normal cells.

Compared to the results with commercial antibodies, the background signal is higher, so further optimization of the hybridization and binding conditions is required. Additional experiments are necessary to determine crossreactivity in this type of samples.

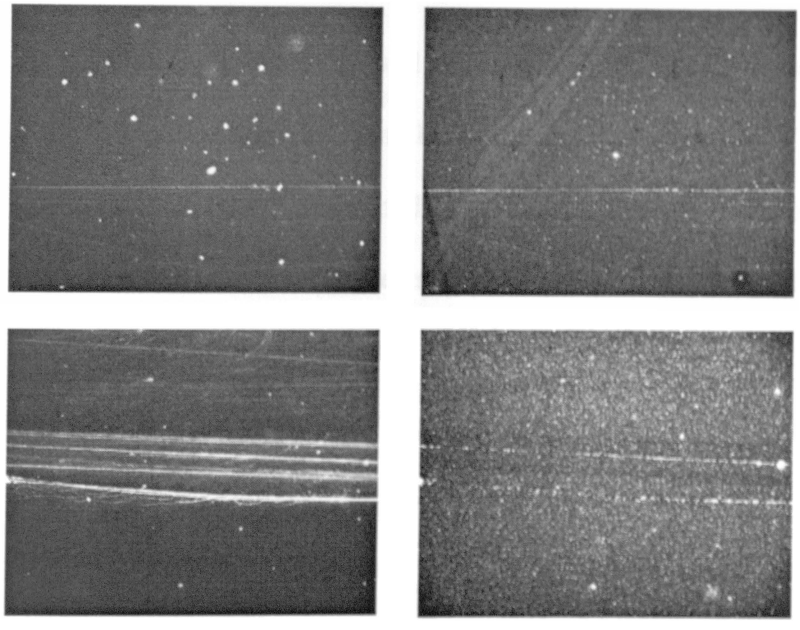

Figure 5.5. The first column shows HT93A extended chromatin fibers, detected by DAPI staining. The second shows signals from anti H4 non-acetylated, and acetylated at lysine residue 16 (from row 1 to 2) detected with a secondary anti-rabbit antibody labeled with FITC.

5.4 CONCLUSIONS

This work was successful concerning the production, and purification of antibodies against H4-Ac16 and H4-nonAc. Cross-reactivity of histone antibodies was evaluated among the antibodies generated, but further characterization of these antibodies has to be done. They should be tested for cross-reactivity to peptides acetylated at different positions. Another concern is the impact 8% crossreactivity might have on the results from an IF determination on extended chromatin fibers, where non-acetylated histones are concentrated in small areas. Micropatterning[30] of peptides of known modifications at a distance and concentration mimicking the fiber environment could be a good control for determination of false positives.

The results with the commercial and in-house antibodies were encouraging because the colocalization of DAPI signal (from the DNA) and the antibody signal (from the histone) demonstrated that the histone-DNA interaction remained after the treatment with lysis buffer used to prepare the fibers. The results also show the expected speckled signal for H4-Ac5 and a more contiguous signal for H4-Ac12, and 8, and present in more fibers.

The hybridization conditions for the antibodies generated in the laboratory, still need to be optimized. A higher dilution of the secondary antibody might be enough to decrease the background signal. As observed in the ELISA, the addition of blocking agents such as BSA can reduce the background as well.

Because the ultimate goal is to perform FISH and IF on the same extended chromatin fibers to determine if there are acetylation changes at the promoter regions

of PML and RARA, it is important to do some calculations regarding the number of molecules that would need to be detected.

As mentioned previously, acetylation in the tails of H3 and H4 is thought to play a role in transcription. Even though H3 has the longest tails, it can only accommodate one antibody per tail. Jones and Thornton[31] found that the antibody-binding site has an area of 500-1000 $Å^2$ compared to the nucleosome's cross section area of 8000 $Å^2$. So, only one antibody per histone could be used for the determination.

The PML probe consists of 2386 bp, and there are 165 bp/nucleosome, thus the probe will bind to 14–15 nucleosomes in that region. With two histones H4 per nucleosome and 1 tail per histone, 28-30 molecules would have to be detected. If the region of interest is smaller (less than 200bp), modifications in one nucleosome will have to be differentiated. This means detecting changes in 2-3 molecules. As mentioned in the introduction, fiber-FISH mapping is able to distinguish two probes separated by 1 kb on a DNA fiber, so the combination of IF and FISH on extended chromatin fibers would only be able to assess acetylation changes in the proximity of the promoter region. The results would have to be supported by other types of assays such as ChIP.

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6. CONCLUSIONS AND FUTURE WORK

The understanding of a disease, in terms of the cellular processes that lead to its cause, is essential for the development of therapeutic agents. That is why there has been so much interest in the study of the effect that histone acetylation may have on the transcription of a particular gene. The analytical challenge faced was to develop a method for the determination of histone acetylation at particular loci, more specifically at chromosomal regions of less than 2 kb, and where DNA condensation directly influences the resolution attainable. Throughout the course of this investigation it was realized that: (1) the study of such chromosomal regions would require the development of short and specific DNA probes, (2) the fluorescent signal from probes shorter than 2 kb was difficult to detect, so different methods for signal amplification needed to be evaluated, (3) there are many particles/molecules available for fluorescent signal amplification and preliminary experiments with 90 nm 3D DNA structures (dendrimers) suggested that the size of a molecular complex could affect its ability to bind to condensed DNA due to steric hindrance, (4) antibody cross-reactivity could become an issue in the determination of histone acetylation, (5) the resolution provided on metaphase chromosomes is insufficient for determining differences at small chromosomal regions, (6) due to the antibody size, determination of more than one type of modification is probably not possible by immunofluorescence. More elaborate comments on each issue are described below, as well as future work required.

6.1. SCFISH PROBE DESIGN

The design of scFISH probes is straightforward, their amplification by PCR is not difficult, but attention needs to be given to the quality of the human genomic DNA. The characterization on metaphase spreads needs to be done with the proper controls, because the quality of the spreads has a big impact on the ability of the probe to hybridize to the target. In some cases detection efficiencies of the probes were >90%, but in others >50%. Lower detection efficiencies could also reflect poor incorporation of the labeled nucleotides, therefore is very important to perform more quality control experiments to determine if the incorporation efficiency of the labeled nucleotides (biotin or digoxigenin) is constant among different batches. A probe of known incorporation efficiency could be use as standard on an enzymatic assay where the labeled nucleotide is detected by color development. The incorporation efficiency of the standard probe could be determined by confocal microscopy where the number of labeled nucleotides are indirectly detected by a fluorophore labeled antibody. If quenching of the antibody signal is suspected, the antibody could be labeled with gold and the determination done by scanning electron microscopy.

It is also important to determine the effect that differences in the melting temperature among a set of probes has on their detection efficiency when they are co-hybridized. Sets of at least three probes with different labels (to detect individually) and with melting temperature differences of: (1) 1-5 °C, (2) 5-10 °C, and (3) 10-15 °C should be tested in parallel.

The probe concentration prior to nick translation could have been under or over-estimated. Therefore, it is important that in future experiments enough probe is amplified to determine the concentration by absorbance readings at 260 nm, or that another dye such as SYBR Gold, which is more sensitive than ethidium bromide, is used for the determination.

The use of monoclonal anti-avidin FITC conjugate generated an insignificant increment in the S/N from 1.8 to 2.4. The same experiment should be done with standard chromosome preparations and probes, if the increment remains insignificant, a second study on less condensed DNA should be done to determine if steric hindrance was keeping more monoclonal anti-avidin antibodies from binding.

6.2. SIGNAL AMPLIFICATION BY DENDRIMERS

Quality control experiments demonstrated that the capture sequence attached to scFISH probes bound specifically to complementary capture probe in solution, in the absence of dendrimer, and that detection on DNA arrays with dendrimers was successful, but specificity was not proven due to the low copy number of target sequence per spot. Detection of scFISH probes on metaphase chromosomes by this approach was not as successful due to high background and lack of signal on both chromatids at the expected chromosomal region. More thorough experiments with blockers to the spacer arm sequence (attached to the scFISH probe) should be done to determine if the spacer arm is the source of the background signal. Lower amounts of ligation mix should be used for the probe labeling with capture sequence and run a

hybridization experiment in parallel with probes labeled with excess ligation mix to determine if that is the source of background. There is no explanation for the consistent lack of signal on both chromatids, but hybridization efficiency of the probe prior to dendrimer detection should be determined with long probes directly labeled with fluorophores to establish the influence of poor probe hybridization. The two chromatids are ~1 μm apart, which rules out the possibility of steric hindrance as an explanation for lack of binding to the other chromatid.

Specific dendrimers proved to bind specifically in non-complex solutions (buffer and probe), and some of results in interphase chromosomes were encouraging (almost 80% identification of t(15;17)), but their specificity needs to be determined in metaphase chromosomes. It was determined that longer spacer arms between the specific sequence and the dendrimer produced higher non-specific binding, but the same dendrimer should be tested at greater ranges of hybridization temperatures. Another important experiment would be to use double stranded DNA for spacer arms to impart some structure to the molecule, in case the arms are collapsing or interacting among them, thus affecting availability of the specific sequences. A very important experiment is that with specific sequences longer than 45 bases, but still within single copy regions. Longer specific sequences would allow raising hybridization temperatures to increase the binding specificity. If the conditions for specific binding were found, this approach would constitute a big breakthrough in the identification of chromosomal regions of less than 100 bases, where the probes can be synthetically generated, so that no enzymatic amplification is required.

The anti-biotin dendrimer approach was the first to produce specific binding and detection of both chromatids. But the detection efficiency was low and results were difficult to reproduce. More experiments need to be done to determine the stability of this type of dendrimer, to ensure that the poor reproducibility is not caused by instability of the dendrimer. Also, these experiments should be done with metaphase spreads of optimal quality to make sure that the hybridization efficiency of the probe does not affect the detection of signal. A very important experiment was done where dendrimer was chased with streptavidin and the detection efficiency increased in 25%. This experiment established that the probe had hybridized, but indicated that may be dendrimers were not able to bind to all the biotins in the probe.

6.3. STERIC HINDRANCE

All the results from these experiments are qualitative. There was great variation among samples, probably due to particle aggregation (causing intensities to be higher than the actual value) and determinations of molecules on a different plane of focus (causing intensities to be lower than the actual value). That said, the most significant finding from these experiments was that the 2-layer dendrimers (90 nm diameter) were not able to bind to all the biotin-labeled nucleotides incorporated into a ~2.4 kb probe. The hypothesis that steric hindrance was keeping the dendrimers from binding was demonstrated by a experiment in solution, where a dsDNA probe (less condensed than the experiments done on metaphase spreads)was first incubated with dendrimer and then chased with streptavidin, after which an increment in the

signal was observed, even though there are fewer fluorophores per streptavin than per dendrimer. These results are also supported by theoretical calculations of the number of particles that would be able to bind to a biotinylated probe condensed into a solenoid. Another piece of evidence that supports this hypothesis was that ~3 times more quantum dots (15 nm diameter) than 2-layer dendrimers were determined able to bind to the target. The experimental results for less condensed DNA showed that 4 times more quantum dots were binding to the probe. This number could be slightly smaller, as the theoretical calculation predicted, when considering the possibility of quenching of the dendrimer fluorescence upon binding.

More experiments need to be done to determine the source of quenching for the dendrimers. Probes with modified nucleotides incorporated farther apart from each other need to be synthesized and incubated with the dendrimers to determine if close proximity among dendrimers is the source of quenching. The amplification in the emission signal from specific dendrimers upon incubation with target-DNA probes was another interesting phenomenon, probably caused by a change in the Alexa-488 environment that results in a higher quantum yield. The binding of these dendrimers to the specific probes needs to be studied in solution by other techniques such as fluorescence fluctuation profiles, because the immobilization in polyacrylamide gels might have disrupted the interactions between the 45 specific-bases and the target probe.

Even though the FluoSpheres were the brightest particles compared to quantum dots and 2-layer and 4-layer dendrimers, the results under experimental

conditions tested were not reproducible. The high variability in the FluoSphere results could be caused by particle aggregation, so different buffers should be tested for the hybridization conditions and longer sonication periods prior to hybridization as well.

6.4. IMMUNOFLUORESCENCE ON CHROMATIN EXTENDED FIBERS

The conjugation of peptide to PPD was successful in eliciting an antibody response against H4-Ac16 and H4-nonAc. Because cross-reactivity of histone antibodies is a big concern, more experiments need to be done where these antibodies are tested for cross-reactivity to peptides acetylated at different positions. The results with the commercial and in-house antibodies were encouraging because the colocalization of DAPI signal (from the DNA) and the antibody signal (from the histone) demonstrated that the histone-DNA interaction remained after the treatment with lysis buffer to prepare the fibers. The results (speckled signal for H4-Ac5 and a more contiguous signal for H4-Ac12, and H4-Ac8) also agreed with the "zip" model of acetylation for H4,[1] where the order of acetylation is from lysine 16 to lysine 5.

Theoretical calculations indicated that study of acetylation changes at promoter regions would require detection of modifications in 1-3 histone molecules. Because fiber-FISH mapping is able to distinguish two probes separated by 1 kb on a DNA fiber,[2] it is inferred that the combination of IF and FISH on extended chromatin fibers would only be able to assess acetylation changes in the proximity of the promoter region, and that the results would have to be supported by other types of

assays such as chromatin immunoprecipitation (ChIP).[3] Another big issue is that in optical microscopy, spatial resolution is limited to about half the wavelength of the incident refraction, so for work done in the visible part of the spectrum that will correspond to 250 - 300 nm.[4] This will make it impossible to quantitatively assess the levels of histone acetylation at regions associated with less than 2 nucleosomes, each of 11 nm diameter. Thus, the determination would have to be done by electron microscopy.

Ideally these determinations would be done by mass spectrometry (MS), which does not have the specificity problems that antibodies may have. MS also has the capability of determining more than one post-translational modification. The current challenges consist in isolation of the chromosomal region of interest and amount of sample required. Cocklin and Wang[5] estimated that 10^{10} cells would be required to determine the acetylation sites. Assuming that 10^{10} cells are required when studying acetylation of the histones associated with the entire Human Genome (3.3 Gbp),[6] and that the region of interest has ~ 2 kb. The amount of cells required in order to obtain the same amount of histones after the isolation of such small region would be ~2 x 10^{16} cells. Since the cells are grown at a density of ~2 x 10^{6} cells/mL, that would require 10^{7} liters of cells, assuming perfect yields after the isolation. So even though MS looks like the optimal technique for the determination, lower detection limits are required.

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