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Cytochemical nucleic acid research during the twentieth century*

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> *In the long course of the cell on this earth it remained for our age, for our generation, to receive the full ownership of our inheritance.*
> *We have entered the cell, the mansion of our birth, and started the inventory of our acquired wealth.*
>
> Albert Claude - 1975

INTRODUCTION

Nucleic acids have the interest of scientists in many basic and applied biomedical disciplines. During this century, investigations have revealed, that nucleic acids are ‘the bearers of life and heredity’.

They contain programs which regulate the many activities of the cells of which organisms are comprised, in the form of a series of discrete units of specific sequences of nucleotide bases. These specific sequences are called ‘genes’ (from pangenes - de Vries, 1889). The genes are arranged linearly on the chromosomes.

Prior to each cell division the complete set of genes of the mother cell is duplicated, so that during the division the two daughter cells each receive a complete set.

In unicellular organisms, all genes are active to control the cell activities from cell division to interactions with the environment. In the cells of multicellular creatures, only those genes necessary to develop and maintain the functions of those organs are active, although - again - all genes are present in each of the cells.

This contribution deals with some early observations which stimulated interest in the smallest units of life, the cells, and from there, the interest in nucleic acids. The results of these and other observations directed a part of nucleic acid research towards a demand for accurate methods for cell analysis which would allow to obtain ever more detailed information, about parts of DNA in a **morphologic** setting. This - among others - led to *in situ* hybridization methods.

After highlighting some important events of the early period, most attention will be given to this **cytochemical** approach, with special emphasis on the most recent developments in that field of science.

Obviously, the choice of topics is largely determined by the author’s bias; there certainly has not been seriously strived to achieve completeness.

THE EARLY YEARS

First, let us dwell a moment on the ‘early years’. Early, not only because the century had just started, but also early in the history of nucleic acid research.

Nucleic acids reside mainly in cell nuclei which can only be observed with optical magnifying instruments like microscopes, which implies (as has been stated often) that: the development of ‘**cell biology**’ as an independent scientific disci-

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**Presented at the 28th National Congress of the Italian Society for Histochemistry June 2-4 1999 Camerino (Italy) as “Maffo Vialli Lecture” while receiving the Maffo Vialli International Award for 1999* pline is closely related to the development of optical lenses and the construction of the microscope.

From the middle of the 17th century scholars had made observations on microscopic objects. A Dutchman, Anthonie Van Leeuwenhoek with his accurately manufactured microscopes was the first to detect unicellular organisms like bacteria in several kinds of material. Van Leeuwenhoek had the advantage that he had invented a rather simple microscope, that due to its simplicity was relatively insensitive to optical aberration. The images obtained with more complicated microscopes of that period (with two or more lenses), at higher magnification often were so blurred by this phenomenon, "*that most details had to be filled in by the imagination*" (de Duve, 1984).

Robert Hooke, a biologist, physicist, meteorologist, architect and, amongst others, an early inventor of microscopes, coined the word '**cell**' in his publication 'Micrographia' (a collection of beautiful drawings of microscopic observations - 1665), designating the regular array of 'microscopic pores' present in a thin slice of cork. 'Cell' stood for a small chamber like e.g. the cell of a prisoner. Later on the word was no longer used for the cavities, but for the cells that in the living tree were present in these cavities. His more complicated microscope, due to the cumulation of spherical and chromatic aberrations, did not allow him to reproduce van Leeuwenhoek's experiments concerning the bacteria. When urged too much by the Royal Society, he seems to have 'used his imagination' and, after 3 experiments, thought he 'was able to confirm van Leeuwenhoek's observations of unicellular organisms' (James, 1994). In December 1677 he wrote to van Leeuwenhoek: "*The President* (of the Royal Society) *and all the members were satisfied & it seems very wonderful that there should be such an infinite number of animals in soe imperciptible quantity of matter"*.

In the early years of the 19th century, the Italian physicist Giovanni Battista Amici (1823) and others, discovered how to correct the major optical aberrations of lenses. The microscope then was ready for **morphological** investigations of cells.

*"The increase in the sharpness of the images was dramatic; so much that only a few years later the generalized theory could be formulated that all plants and animals are made of one or more similar units, the cells. For plants this theory was proposed in 1838 by the German botanist Mathias Schleiden; his friend, the physiologist Theodor Schwann, extended it to animals. It was completed by the pathologist Rudolf Virchow in 1855, when he proclaimed: '**omnis cellula e cellula**'.*

By the middle of the nineteenth century the cell theory was firmly established and the science of cells, or cytology, started to flourish" (de Duve, 1984).

Another scientific milestone of that period is the discovery of the laws of heredity by the Austrian monk Johann Gregor Mendel (1865). He conducted cross-breeding experiments on garden peas and found specific regularities in the pattern of occurrence of different traits. Because he chose an obscure local journal for his publication, hardly anybody paid attention to these 'Mendelian Laws' for about thirty years.

His conclusions had been that heredity in peas is based on individual, defined factors that are independent of each other. Transmission of these factors (like colour and shape) to the next generation will occur in predictable proportions. Each of these factors is responsible for a certain trait.

Expressed in modern phrasing, the genetic program consists of discrete units of information. Each unit controls a defined function. Those units, named 'genes' since 1909, are transmitted from one generation to the next; they exist in pairs (alleles), one provided by the female parent, one of the male parent.

As will be discussed shortly hereafter, genetically determined traits are only independently inherited when they are located on different chromosomes or far enough apart on the same chromosome to be separated each time by recombination (i.e. when there is no genetic linkage).

Then, in 1879 Flemming, introduced the term 'chromatin' for the substance present in the nucleus and also discovered 'structures' (=chromosomes - name coined by Waldeyer, 1888), describing their behaviour during a mitotic division; Strasburger (1888) described their demeanour during meiosis.

- The person believed to have been the first to study chromosomes in pathological material is Arnold, already in 1879. Hansemann (1891) also was active in this field.
- In 1901, De Vries recognized that 'genes' can change and 'mutate' (although it remained unclear what happened to which substance during a mutation). In 1927 Muller discovered that mutations

can be induced by X-rays. Early in the nineteen fourties several independent investigations showed that certain chemical substances also could induce mutations.

- The danger of exposure to 'chemicals' (or the importance of environmental conditions) had already been suspected around 1775 by Sir Percival Pott and John Hunter, who had noticed that young chimney sweepers had a high risk of developing scrotal cancer later in their life. He surmised that the regular exposure to the tar from the smoke channels, was the origin of this complaint and recommended frequent washing and changing of clothing to diminish 'carcinogenic' exposure. Because there was (even in the nineteen fourties) no knowledge about the physical basis for the transfer of genetic information, it remained unclear what a mutation actually was.

Thomas Morgan concluded from studies with Drosophila that shortly before the onset of the pachytene stage, homologous chromosomes come intimately close to each other and form a synaptonemal complex, a combination of two homologs in which the almost identical base sequences of the paternal and maternal DNA are into register with each other. At these chiasmata, breakage of the chromatids can occur, followed by recombination of the remaining parts with each other. When during this last process, exchange of (parts of) chromatids takes place, we speak of 'crossing-over'. As a result of crossing-over, the genetic diversification is strongly enhanced. The haploid genomes carried by each spermatozoon or ovum, are not identical copies of those in the original parental genomes, but each chromosome in the new individual is the result of a number of fragments copied from the male's or the female's contribution to the new genome.

Morgan had started genetic studies with the fruit fly (*Drosophila melanogaster*) in 1910. From these studies (by him and many others over the world) it became clear that genes are arranged linearly over the length of chromosomes (Morgan, 1926). Two genes that are located closely to each other on one chromosome, will (most often) be inherited together; will be 'linked'. (The closer two genes are located on the same chromosome, the smaller the chance that they become unlinked by crossing-over). It will be clear that due to crossing-over, two genes which are located farther away from each other on each of the homologs, have more chance to be crossed-over or recombined. In other words: *"The probability of two genes located on distinct homologous chromosomes to be recombined, is proportional to the distance separating the loci that they occupy on the chromosomes"*. Frequency of recombination can be used as a measure of the physical distance between gene loci (Morgan, 1915).

A gene now can be defined as a sequence of DNA that codes for a certain polypeptide (one gene, one polypeptide). One or more polypeptides form a protein. This implies that several genes can be involved in the production of a protein.

We must, however, realize that at that time, there was not yet any notion of any functional relationship between chromosomes and genes. The nature of genes and how they function was still totally unclear.

There is another scientific result from the nineteenth century that is important for this presentation. The **morphological** investigations of cells, and the growing idea that cells had to do with heredity, had stimulated interest for its nucleus, and it was in 1865 that a Swiss pathologist (Johann-Friedrich Miescher, fascinated by the affinity that the nuclear material showed for the - in that period newly developed - basic anilin dyes, decided to analyse that material. Originally he worked with white blood cells isolated from pus extracted from septic bandages. Later he took salmon sperm cells for his **biochemical** investigations.

In 1868 he succeeded to isolate and analyse the nuclear contents, and discovered that it contained (next to histones and other proteins) an acidic substance which he called "Nuclein". That substance contained not only Hydrogen, Oxygen, Carbon and Nitrogen (present in the proteins as well) but also an amount of Phosphorus (Miescher 1871, 1897).

However, the composition in terms of these chemical elements was the same in human leucocytes, in salmon sperm and cell nuclei of other species and - as Oswald Avery put it in 1943: " ... *the thymus type of nucleic acids which are known to constitute the major part of chromosomes but have been thought to be alike, regardless of origin and species ... how could it possibly be endowed with such biologically active and specific properties as necessary for heredity"* (Stryer, 1981). For that reason, there was not much interest for tis substance for a long time.

Table I
The early years

1900 - rediscovery Mendelian Laws of inheritance - Correns, Tschermak, de Vries

1901 - 'genes' can become altered (mutated) - de Vries

1906 - 'genetics': study of the rules which govern heredity and variation

1909 - some metabolic diseases recognized as being transmitted by autosomal recessive inheritance - Garrod

Which substance is the bearer of heredity?

Although since about 1855 it was accepted that cells were the basis of living organisms, it remained unclear for a long time which substance in the cell was responsible for the transmission of hereditary information from one generation to the next.

Mendelian inheritance was systematically analyzed in plants, animals and also in man, and some human diseases were recognized to have a hereditary cause. But 'prescient' Theodor Boveri, who around 1900 already spoke about the 'genetic individuality of chromosomes', was still an exception (Passarge, 1995; Bovery, 1909).

Actually it had been Rabl who already in 1885 had discussed this topic. Counting individual 'Fäden' (threads = chromosomes) in prophase cell nuclei of the *Salamandra maculata*, he never found more than 24 individual Fäden per nucleus. From this observation he concluded that there existed a **constant** number of **individual** chromosomes per cell in that tissue. Furthermore, he hypothesized that for every type of species, there would exist a species specific number of chromosomes per cell nucleus.

According to his expectations, these chromosomes would remain individual entities during interphase: "*Die Behauptung daß beim Beginn der Knäuelbildung ein einziger kontinuierlich zusammenhängender, windungsreicher Faden den ganzen Kern durchziehe*", in the eyes of Rabl, was: "*eine Angabe, die sich wie eine Erbsünde durch fast alle Arbeiten über Zelltheilung hindurchzieht*". And: "*Niemand wird annehmen wollen, daß die Fäden im Mutterknäuel aufschiessen wie die Kristalle in einer Mutterlauge, oder daß beim Übergang des Tochterknäuels zur Ruhe die Fäden sich vollständig auflösen oder in Stücke zerfallen*".

In 1911, Rous had discovered that a viral **nucleic acid** can cause cancer in the fowl, but for a long time there was no evidence or indication that nucleic acids play a role in human cancer, or that nucleic acids were carriers - even bearers - of heredity. In this period, only the hypothesis of Boveri (1914) that a change in chromosome constitution is a prerequisite for neoplastic growth is one of the very few signs that some scientists considered nucleic acids (or the chromatin proteins) to be of importance in this respect.

As far as inheritance was studied experimentally, in this period, the fruit fly *(Drosophila melanogaster)* proved to be an excellent laboratory animal. Genetic studies by the group of Morgan and many others over the world, during the following 30 to 40 years, established 'genetics' as an independent science and provided many important results.

Among others, genes were recognized to be arranged linearly on chromosomes; chiasmata, crossing-over, linkage and recombination were understood and in 1915 the chromosome theory of inheritance was formulated (Morgan *et al.*, 1915).

For many scientists, even then the chromosomal proteins - because of their complexity - seemed a more likely candidate to be carriers of heredity. It is difficult for us, living in the Watson-Crick era, to imagine that only 60 years ago most scientists believed that DNA was too simple a substance, with too much similarity in different species, to play any but a minor role in heredity. Even when its association with the chromosomes became clear, and the role of chromosomes was seen as bearers of the genes, only the chromosomal **proteins** (perhaps in the form of 'nucleo-proteins') were considered to have enough chemical complexity and inexhaustive possibility of variation, as was required for a genetic material.

In later years, bacteria and viruses with a much simpler life cycle, proved to be of great practical importance as laboratory research organisms. Bacteria have no nucleus; may start duplicating their DNA even before separating from their twins; their

Table II
From hypothesis to knowledge

1909 - genetic individuality of chromosomes - Boveri

1911 - a viral nucleic acid can cause cancer in the fowl - Rous

1914 - change in chromosomes is prerequisite for cancer - Boveri

1915 - theory of inheritance - Laws of heredity - Morgan *et al.*

1924 - chromosomes do contain DNA - Feulgen & Rossenbeck

doubling time can be as short as 30 minutes, while the length of the complete cycle of the cells of higher animals may last 20 to 24 hours.

Following an earlier observation of Griffith, it was during a study with *Pneumococcus* bacteria that Avery in 1944 was able to demonstrate that DNA was *"the fundamental unit of the transforming principle of Pneumococcus"*.

During the fifties, after it had become clear that DNA was the bearer of heredity and cytometrical measurements had confirmed the DNA constancy hypothesis, DNA content and concentration determinations became an important issue in biology. Amongst others, Vialli contributed to this topic by elaborate studies in a variety of species. Especially in hybrid cells of lower vertebrates he could demostrate remarkable differences in DNA concentrations (1957).

Long-term Cell and Tissue cultures

Already early during this century, Ross Harrison and Alexis Carrel had shown that animal cells could be cultured *in vitro* like unicellular microorganisms. They thereby demonstrated the cells' capacity for independent life and set up a technique that is still rendering major services today although it is not an easy procedure to accomplish and connected with a number of pitfalls (De Duve, 1984).

During the early nineteen fifties, when methods to obtain good quality metaphase chromosome preparations had been developed (see hereafter), chromosome analysis of long-term cell lines was started. One of the conclusions obtained from many studies was, that the cells in the rodent lines (even those in lines established from normal tissues) *"invariably change their chromosome constitution after growing in culture for a period of time"* (Hsu, 1979).

This fact a.o. hampered research to check the Boveri hypothesis of 1914, that a change in chromosome constitution is a **prerequisite** for neoplasia.

Trying to produce and maintain long-term cell cultures from normal human tissues, originally proved to be difficult. Hayflick and Moorhead (1961) concluded from extensive studies on a variety of human tissues, that for such cultures several phases of growth can be discerned.

After a slow growth rate in the period that the cells have to adapt to the *in vitro* environment, a period of exponential growth is observed, that lasts till the twentieth or early thirtieth passage. Then the growth slows down and during the fourtieth to fiftieth passages, the cell lines completely cease to proliferate; the cells become larger and granular and eventually degenerate.

The chromosomal constitution was found to remain 'predominantly' diploid, even towards the end of the cultures' lifes. Freeze-storage at various passages is possible but/and will not change the trend towards 'senescence' of the culture. One of the developments in long-term human cell cultures methodology was the discovery of permanent lymphoid cell lines. As we will discuss hereafter, from 1960 on cytogeneticists have been using human peripheral blood or lymphocytes for short-term cultures as a routine procedure for chromosome analysis. These cultures only will survive for a few cell generations.

After an indication that bone marrow cells from patients suffering from infectious mononucleosis, might transform in culture and become permanent lines, Glade *et al.* (1968) tested peripheral blood lymphocytes of such patients and discovered that those cultures can be maintained *in vitro* as permanent suspension cultures. Such human lymphoid cell lines had several advantages in comparison with fibroblast cultures. They not only can be continuously propagated without signs of reaching a senescence stage; they grow fast and grow in suspension and therefore are easy to handle (no scraping or trypsinization required) (Hsu, 1979).

Table III

Which substance is the bearer of hereditary information?

1915 -	genes are arranged linearly on chromosomes - Morgan
1928 -	genetic changes can be induced by cell-free extracts - Griffith
1940 -	'one gene - one enzyme'
1944 -	biochemical evidence that DNA transmits genetic information - Avery *et al.*
1950 -	in DNA, number of purine bases is equal to number of pyrimidine bases - Chargaff
1950-1955 -	cytometrical evidence that DNA contents of neoplastic cells differs from that of normal cells - Caspersson, Leuchtenberger *et al.*, Mellors, Pollister, Ris & Mirsky, Swift, Thomson & Frazer, Zetterberg & Eposti
1952 -	genetic information is transferred by DNA alone - Hershey & Chase
1953 -	'the double helix' - Watson & Crick
1961-1965 -	genetic code in triplets - Crick *et al.* - deciphering of code - Nirenberg

NEW DEVELOPMENTS IN MOLECULAR BIOLOGY (see Cairns *et al.*, 1966)

"*During the second half of this century, several independent research activities resulted in procedures which, directly or indirectly, contributed to the field of mammalian and human cytogenetics*" (Hsu, 1979). The most spectacular progress was achieved in the area of recombinant DNA technology. This technology allows the formation of new DNA molecules by the breakage and reunion of DNA strands (Maniatis *et al.*, 1989). As Motulsky phrased it: "*It is likely that we are living in a golden age of the biomedical sciences. The achievements of these sciences are likely to be long remembered as high points of Western civilization in the last part of the 20th century*" (Motulsky, 1986).

"*Genetic engineering was born, perhaps to become one of the most powerful techniques ever developed by mankind*" (De Duve, 1984). Molecular biology in combination with cytogenetics, resulted in a new discipline: 'molecular cytogenetics' (Landegren *et al.*, 1988).

Restriction endonucleases are able to recognize specific nucleotide sequences in double-stranded DNA, and to cleave/cut the double helix on this 'recognition site'.

A 'recognition' or 'cleavage' site is a specific sequence of four to eight base pairs (Hamilton, 1979; Nathans, 1979; Smith, 1979). More than 400 different types of these enzymes have been isolated from bacteria, which produce them as a protection from foreign DNAs. Three to five of these endonucleases are frequently applied for analytical purposes.

Some restriction enzymes have a small recognition site (with limited specificity), which implies that they can cleave the DNA often, and thus produce many relatively small fragments. Enzymes with larger recognition sites, find less sites to cut, which results in less but larger fragments. So, the resulting DNA fragments have various lengths, depending on the position of the cleavage sites in the original duplex molecule and on the type of enzyme applied. Gel electrophoresis will sort the fragments by size: smaller fragments will migrate faster.

Another difference in size can arise when the nucleotide sequence in a segment of e.g. two homologous chromosomes differs in such a way (DNA polymorphism - happens relatively often) that one or more recognition site(s) for a given restriction enzyme, present on one chromosome, is/are not present on the other (Restriction Fragment Length Polymorphism: RFLP). Gel electrophoresis after treatment with a specific restriction enzyme, followed by Southern blotting (1975) will visualize such differences (DNA fingerprinting). Even small differences in fragment size of related DNA molecules can be readily detected by such an analysis.

The resulting DNA fragments possess a short, single-stranded segment on each side; these two ends are complementary and so can be fused together by a **DNA ligase** (Lobban & Kaiser, 1973). In this way, DNA fragments of different origin (even of unrelated species) can be joined (if they have originated from cleaving by the same restriction enzyme): resulting in a new genome (Spiegelman, 1964). These new genomes can be inserted covalently in suitable host cells (Campbell, 1976) by means of a **vector**, and amplified many times by the DNA machinery of that host cell *in vitro*. Specific eukaryotic genes can e.g. be **cloned** in *E. coli* starting with a digest of genomic DNA.

Plasmids (Cohen *et al.*, 1973; Helinsky, 1978) and **cosmids** (Collins & Hohn, 1978) have become important classes of mobile genetic elements. These small circular duplex DNA molecules can be used as vectors to clone DNA fragments, the DNA sequence of interest being covalently linked to the vector DNA. By cloning, multiple copies of a specific DNA fragment (e.g. a gene) can be easily and rapidly produced *in vitro*.

Plasmids, inserted in bacteria, can replicate autonomously of the host chromosome. At present, a whole battery of such vectors or 'translocatable elements' is available; Table IV shows some 'choice vectors'.

- The discovery of restriction endonucleases had an enormous impact and facilitated DNA sequencing tremendously. When several of these nucleases are combined, they form an indispensable tool for the analysis of chromosome structures (chromosome mapping purposes).
- Restriction mapping is of considerable importance in medical genetics and evolutionary research.
- Cloning is the production of multiple copies of a specific DNA fragment *in vitro*. Since bacteria grow fast, the DNA sequence of interest can be reproduced in large amounts in a relatively short period of time.

Methods to synhesize DNA enzymatically *in vitro* (see Kornberg, 1980).

In 1955, Arthur Kornberg *et al.* started investigations for the enzyme that was responsible for the synthesis of DNA. Because pathways of purine and pyrimidine biosynthesis lead to 5'-phosphates, they correctly 'deduced' that deoxyribonucleoside 5'-triphosphates were most probably the activated intermediates for this synthesis (Kornberg, 1960). They also expected that during the initial *in vitro* experiments, where nucleases are prevalent, the amount of DNA synthesis would be very small, so they started with a very sensitive (radio-isotope-labelled) assay.

Incorporation of the precursors into DNA was checked by measuring the radioactivity of an acid-precipitate of the incubation mixture - DNA being precipitated, whereas precursor nucleotides stay in solution. An incubation of an extract of *E.coli* bacteria with highly radioactive deoxythymidine 5'-triphosphate, resulted in the synthesis of a few picomoles of DNA. Kornberg wrote: *"Although the amount of nucleotide incorporated into nucleic acid was miniscule, it was nonetheless significantly above the level of background noise. Through this tiny track we tried to drive a wedge. The hammer was* ***enzyme purification,*** *a technique that we had matured during the elucidation of alcoholic fermentation"* (Stryer, 1981). The enzyme they found, is now called Polymerase I, because more DNA polymerases have been discovered in the mean time. It took a decade of effort and one hundred kilograms of *E.coli* cells, to produce five hundred milligrams of pure enzyme (Stryer, 1981).

Methods to fuse cells of different species in culture (**somatic cell hybridization** - Barski *et al.*, 1960) and **somatic cell genetics** (Pontecorvo, 1958).

As a result of these methods, the genetic approach (so far quite successfully in bacteria and viruses) now could be applied in higher organisms, without the drawbacks of breeding experiments and longer generation times.

Table IV
Favourite Cloning Vectors

plasmids can contain about	01 - 10	kb of inserted DNA
phages	15	
cosmids	40	
P1s	100	
BACs	100 - 200	
YACS	100 - 1000	

- Special (conditioned) cell culture media were designed to select mutants from other cultured cells (**HAT medium** - Littlefield, 1964).

Polymerase Chain Reaction - a procedure to amplify a specific sequence of DNA *in vitro* (PCR). The method does not require to apply cultures of living cells like bacteria. Starting from a single copy, a DNA segment can easily be reproduced a millionfold, in order to obtain sufficient amounts for a molecular-biological investigation, e.g. demonstration of a mutation.

The resulting endproduct can of course also be used as a probe for an *in situ* hybridization (ISH) analysis.

Mullis, who invented this principle, wrote about the origin of his ideas: *“Sometimes a good idea comes to you when you are not looking for it. Through an improbable combination of coincidences, naïveté and lucky mistakes, such a revelation came to me one Friday night in April 1983 as I gripped the steering wheel of my car and snaked along a moonlit mountain road into Northern California’s red wood country. That was how I stumbled across a process that could make unlimited numbers of copies of genes, a process now known as the polymerase chain reaction (PCR)”* (Mullis, 1991).

This PCR reaction is one of the procedures that makes life much easier for molecular biologists and cytogeneticists. For instance: the PCR amplification of human DNA sequences from chromosomes obtained by flow sorting or by micromanipulation, allows generation of probe sets which specifically ‘paint’ one type of chromosome or a part thereof.

The method requires only small amounts of duplex DNA that contains (at least one copy of) the specific sequence of interest, a test tube (Eppendorff tube), a source for heating and cooling, a few simple reagents, among which two synthetic oligonucleotides to be used as primers, enough of the four deoxyribonucleotide triphosphates to be incorporated in the DNA copies to be produced, and: **Taq** (DNA) **polymerase**.

The procedure consists in denaturation of the genomic DNA sequence, annealing the synthetic primers, and replication of the DNA segments which are of interest, by extension.

After the first duplication, this procedure is repeated as many times as necessary, each time all the resulting DNA, serving as a template during the next cycle. During each cycle the amount of specific nucleic acid will double, and since each duplication cycle takes only a few minutes, after one hour already more than a million specific copies will have been synthesized.

- Very convenient in this process is the application of Taq polymerase, an enzyme that was extracted from the bacterium *Thermus aquaticus*, which is present in hot springs.

Table V

Developments in molecular biology

1952 - Plasmids - Lederberg (see Broda, 1979; Cohen *et al.*, 1973)

1956 - cloning of cells *in vitro* - Puck methods to synthesize DNA *in vitro* - Kornberg

1958 - somatic cell genetics - Pontecorvo

1960 - cell fusion - Barski *et al.*; Ephrussi & Weiss - a human metabolic defect is demonstrated for the first time in cultured human cells

1964 - HAT medium - Littlefield

1972 - inserting genetic information into DNA of SV40 - Jackson *et al.*

1977 - sequencing of DNA - Maxam & Gilbert; Sanger *et al.* - first recombinant molecule containing mammalian DNA

1979 - restriction enzymes - Arber; Hamilton; Nathans; Smith - synthesis of a total gene *in vitro* - Crea *et al.*; Khorana

1985 - Polymerase Chain Reaction - Mullis

1989 - cloning of defined segments of chromosomes obtained by micro-dissection

This enzyme is stable and active at high temperatures, which implies that it only needs to be added once, at the start of the reaction procedure. Taq polymerase is now produced by genetically engineered bacteria.

- Recombinant nucleic acid technology and the ability to clone and amplify genes e.g. in microorganisms or by PCR, have been of extreme importance for the expansion of human genetics, and for diagnostic purposes as well (Belgrader *et al.*, 1999).
- Since early 1960, knowledge about genetics in general has been obtained, often for the first time, by studies in man.
- Analysis of genetically determined diseases in man has yielded important insights into the normal function of genes in other organsims as well, and today, *"more is known about the general genetics of man than about that of any other species* (Passarge, 1995).

HOW TO OBTAIN GOOD METAPHASE PREPARATIONS?

As stated already, the progress obtained by cytogeneticists during the first half of our century was mainly the result of studies on plant or insect chromosomes. From these organisms it was much easier to obtain appropriate material and preparations suited for microscopic studies than it was from man or other mammals.

The studies which were started after human chromosomes had been observed in mitoses (Flemming, 1879) mainly *"related to the behaviour of chromosomes rather than to their number, morphology and relation to cell lineage"* (Hsu, 1979).

All these years, however, it was very difficult to produce preparations in which the chromosomes of a metaphase could be clearly distinguished. In retrospect *"…when browsing through the literature of the early years, we find that most of the efforts on mammalian cytogenetics were really wasted"* and: *"One finds great discrepancies in chromosome counts of the same species, and, after comparing these numbers with data obtained by modern methods, practically no useful information"* (Hsu, 1979).

Till 1920 a human metaphase could contain from 8 up to 50 chromosomes - the most likely number for a diploid cell was 24. Painter (1922) came to the conclusion that the number for a diploid cell was somewhere in the forties. He hesitated between 46 and 48: *"In the clearest equatorial plates so far studied only 46 chromosomes have been found"* (Hsu, 1979), but because Von Winiwater - an authorative cytologist at that time - had postulated 48, Painter accepted that number as correct. This remained so till 1956.

Originally paraffin sections were used for these studies. This had the disadvantage that there was never certainty that the sections contained complete cells.

In 1921 Belling introduced the squashing method, which had several advantages. Due to the pressure that is exercised, in most cases the (complete) cells will be flattened, so that the material of interest more likely will be brought in one plane of focus. The flattening will also enlarge the area taken by the cell which will promote spreading of the chromosomes.

During the nineteen thirties, the introduction of colchicine to tissues or cells growing in culture, was another improvement (Blakeslee & Avery, 1937; Levan, 1938).

Colchicine (an alkaloid from the bulbs of a plant Colchicum) in growing tissues interferes with the formation of the mitotic spindle, and the cell is therefore arrested at metaphase. Colchicine treatment increased the number of mitotic cells in the preparation under investigation and allowed the chromosomes to condense further, reducing the number of overlapping chromosomes. The method was first applied in plant cytology, but was readily accepted in mammalian cytogenetics.

The real breakthrough came in 1952. In that year three independent papers announced the (re)discovery of the effect of hypotonicity on chromosomes (Hughes, 1952; Hsu, 1952; Makino & Nishimura, 1952).

The effect had already been observed by Eleanor Slifer in 1934, but then as a by-product of investigations on locust embryos, and so the news had not spread to scientists interested in human metaphase chromosomes.

As Hsu describes his observation, it was a purely serendipitous discovery (Hsu, 1961). A technician who had to prepare a balanced salt solution, most probably misread the scale and the result had been a hypotonic solution. Not aware of this fact, Hsu - who had set up some cultures of material obtained from therapeutic abortions - using this 'balanced' salt solution, fixed some of the cultures and stained them with hematoxylin. He writes down, that when he started to inspect the preparations under the microscope, he was not looking for anything in particular, but thought that he might be able to see lymphopoiesis *in vitro*.

But then: *"I could not believe my eyes when I saw some beautifully scattered chromosomes in these cells. I did not tell anyone, took a walk around the building, went to the coffee shop, and then returned to the laboratory. The beautiful chromosomes in those splenic cultures were still there; I knew they were real*" (Hsu, 1979).

There followed 3 months of checking every factor that might have caused the metaphases to spread so miraculously, but nothing worked until he changed the tonicity of the balanced salt solution, used by everyone in the lab to rinse cultures before fixation. The miracle reappeared and the mystery was solved. This rediscovery was most important and still is today, because 'the miracle' worked on cell cultures of all species.

As a result of the better spreading it finally became possible to determine the exact number of chromosomes in a metaphase cell of healthy humans (man): the human diploid number proved to be 46 (Tjio & Levan, 1956; Ford & Hamerton, 1956).

This discovery initiated the development of medical genetics: Lejeune *et al.* (1959), investigating fibroblast cultures from biopsy tissue of three young Down's syndrome patients, observed that their cell nuclei contained 47 chromosomes: there was one small chromosome extra in each of the metaphases. Originally Lejeune did not dare to believe that this specific trisomy was a common feature in 'all' Down's patients, but soon his observation was confirmed by others, which brought him to the exclamation: *"Mais c'est vrai!"*.

Waardenburg in 1932 already had made a speculation that congenital anomalies in man, like mongolism, might be caused by chromosomal abnormalities: *"I would like to urge cytologists to consider the possibility that in mongolism lies an example of specific chromosome aberration in man …perhaps a chromosome deficiency through nondisjunction or the reverse, a chromosome duplication"* (Hsu, 1979).

As Hsu states: *"If it were not for Jérôme Lejeune's discovery of a trisomic condition associated with mongolism, human cytogenetics would probably have died soon after the correct diploid number was determined and the chromosomes characterized"* (Hsu 1979).

Shortly thereafter, more chromosome aberrations were discovered in some other well-known human disorders: a missing X chromosome in Turner syndrome (45,X0) (Ford *et al.*, 1959), an extra X chromosome in Klinefelter syndrome (47,XXY) (Jacobs & Strong, 1959 - this publication also brought the first evidence that genetic factors on the Y chromosomes of mammals are important in determining male sex - de Grouchy & Turleau, 1984), Trisomy of chromosome 13 (Patau *et al.*, 1960) and trisomy of 18 (Edwards *et al.*, 1960); and the Philadelphia chromosome (Nowell & Hungerford, 1960), which is present in bone marrow cells of most patients with the disease chronic myeloid leukemia (CML) and in some acute leukemias. The 'Philadelphia' (Ph1) chromosome - the name is derived from the fact that is was observed for the first time in Philadelphia - is the product of a reciprocal translocation between a No. 22 and a No. 9 chromosome (Janet Rowley, 1973).

Subsequent analysis of tumour karyotypes - originally hampered by problems - supported the early hypothesis of Boveri (1914), that chromosome abnormalities are among the cellular changes that cause the transformation from normal to malignant

Table VI
How many chromosomes in a human diploid cell?

1890-1920 -	from 8 till 50 chromosomes per human metaphase, 'best' value for a diploid cell: 24 chromosomes
1912 -	48; 'pairing' of chromosomes for classification purposes - Von Winiwater
1922 -	diploid human metaphases contain 46/48 chromosomes - Painter
1956 -	human normal diploid number of chromosomes is 46 - Tjio & Levan; Ford & Hamerton

proliferation. Some of these chromosomal changes were found to be tumour specific, like the small aberrant Philadelphia chromosomes in patients with chronic myeloid leukemia are proverbial in this respect (Nowell & Hungerford, 1960).

Cell culture procedures for chromosome analysis

As stated already, the cell culture method introduced by Harrison & Carrel is not an easy to accomplish procedure, relatively expensive and connected with a number of pitfalls (De Duve, 1984). It was not practical to set up cell culture facilities solely for the study of chromosomes.

So, 'the lymphocyte (short) culture method discovered by Peter Nowell (1960 - see also Moorhead *et al.*, 1960) … was one of the most timely and welcomed contributions to human cytogenetics' (Hsu, 1979). The method has no need for biopsy specimens to start with, but can be performed with only a small quantity of peripheral blood.

This discovery too was unexpected, because in those days, peripheral blood lymphocytes were thought to be end-products of cellular differentiation, not able to undergo mitosis. Nowell's cultures, however, after some days of incubation showed a large number of lymphoblasts. When investigating which factor initiated this unexpected phenomenon, he discovered that the phytohemagglutinin (PHA) which he had used prior to the culturing procedure in order to separate erythrocytes and leukocytes by agglutination, not only facilitated the separation, but also possessed this stimulating potential.

When Nowell submitted his manuscript to 'Cancer Research' one of the reviewers' comments was: "*It is an interesting observation but of no conceivable significance to science*". Twenty years, later a compilation made by 'Current Contents' over the years 1954-1974, showed that this paper (1960) had become the most widely cited paper in that Journal during those years (Hsu, 1979).

MORE DETAILED INFORMATION FROM METAPHASE CHROMOSOMES AND CELL NUCLEI

Although methods to prepare better-spread human metaphase preparations in larger numbers, now were available, it often remained an arduous task to obtain a good karyogram (especially from clinical material) in which every chromosome was impeccably identified.

The overall staining techniques applied in those days, at best allowed a classification of human metaphase chromosomes in seven groups, based on sizes and centromere positions. Morphology-based recognition, especially when chromosomes had been altered by translocations or small deletions, in most cases remained very unreliable.

The same difficulties were encountered by cytogeneticists who were working with other mammals. Several approaches to improve this situation (a.o. by image analysis of chromosomes with compterized microscopy (Hilditch & Rutovitz, 1969) and measurements of DNA contents and DNA-based centromere indices (Mendelsohn *et al.*, 1966, 1969; Bosman *et al.*, 1977) did not really offer much relief. *"If it were not for several important developments made during the very late 1960s and early 1970s, many cytogeneticists probably would have abandoned the field altogether* (Hsu, 1979).

It was in these days that Caspersson from the Karolinska Institute in Stockholm - who had been appointed as a consultant by the Children's Cancer Research Foundation in Boston - launched the following brilliant idea.

Chromosome banding

Caspersson, hoping for a nonrandom distribution of base pairs along the length of the chromosomes, suggested to try to induce specific differential staining patterns of parts of metaphase chromosomes.

Table VII
How to obtain good metaphase preparations?

1952 - hypotonic salt solution (rediscovered) - Hsu
1956 - colchicine - introduced for mammals by Tjio & Levan
1960 - cell culture methods for peripheral blood lymphocytes using phytohemagglutinin - Nowell

If the distribution of base pairs along chromosomes would be nonrandom, theoretically there could exist guanine-cytosine-rich (GC-rich) areas and adenine-thymine-rich (AT-rich) areas over the length of metaphase chromosomes. When fluorescing compounds could be attached chemically to an alkylating agent that will crosslink guanines of the DNA, the fluorophore would give stronger fluorescence emission in GC-rich areas than in AT-rich.

If this worked, one could expect a chromosome to show differential fluorescence along its length into bright and dim zones. And if this proved to be the case, one should be able to differentiate chromosomes of similar gross morphology, by their (hopefully differing) fluorescence patterns.

Ed Modest, an organic chemist at the Children's Cancer Foundation, synthesized quinacrine mustard (QM) which was tried on chromosome preparations in Stockholm by Lore Zech *et al.*, (Caspersson *et al.*, 1968, 1969, 1970).

It worked: Metaphase chromosomes of Vicia, Scilla, ànd man, showed differential fluorescence along each chromosome.

When finally the experimental details had been disclosed, numerous problems so far unresolved could be solved, and: *"The resulting advance caused the till then existing knowledge in the field of human cytogenetics to become obsolete almost overnight"* (Hsu, 1979).

The method also was very important to chromosome studies in the laboratory mouse *Mus musculus*.

One detail differed from Caspersson's original idea. He had suspected that the GC-rich zones would become the bright zones. However, analysis of the composition of bright and dim fluorescing chromosome parts led to the conclusion that bright emission occurs in the AT-rich DNA. It is apparently not the alkylating mustard moiety of QM that gives rise to the stronger fluorescence, since Atebrin (quinacrine dihydrochloride) that does not contain an alkylating agent, gives exactly the same fluorescence pattern (Pearson *et al.*, 1970). It appears that Atebrin has no preferential chemical affinity for AT base pairs, but that its fluorescence quantum yield depends largely on the base composition.

Soon therafter several other banding techniques were developed, of which the Giemsa-induced G banding and the R (or reverse) banding are most widely used (Arrighi & Hsu, 1971; Drets & Shaw, 1971; Dutrillaux & Lejeune, 1971; Eiberg, 1974; Patil *et al.*, 1971; Seabright, 1971; Sumner *et al.*, 1971; Latt, 1973 and others - for a survey see Sumner (1982).

Figs. 1/6 - 1) FISH on fibers of a tetraploid nucleus, with a probe specific for the centromere region of chromosome 17, visualises parts of four DNA fibers in green.
(by kind permission of J. Wiegant)

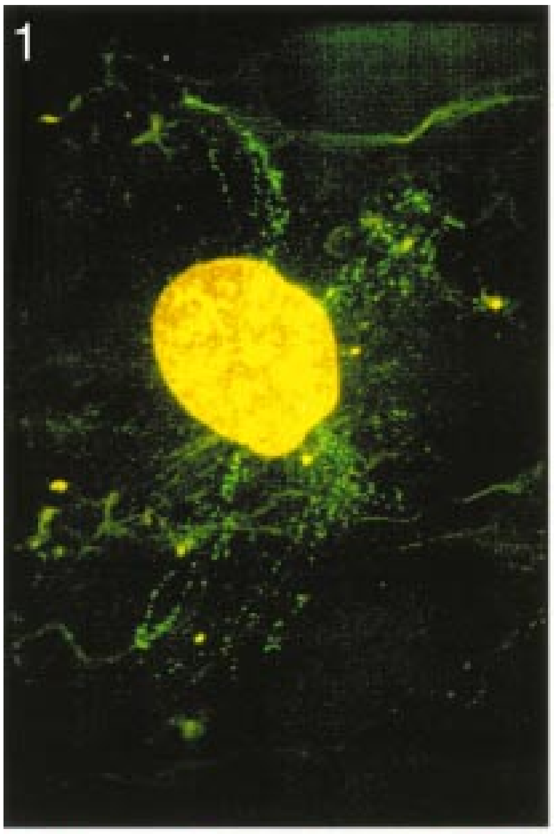
2) - FISH resolution in DNA fibers.
Five targets on a DNA fiber were visualized with five single copy thyroglobulin plasmids producing alternately a red or a green signal. The signals of the two neigbouring targets at right (respectively 1.8 and 2.7 kb) are visually resolved because the degree of (de)condensation in the fibers is such, that only 1 kb of DNA is present per 0.34 µm of fiber. This follows from digital imaging and analysis experimants, which showed that the midpoint-to-midpoint distance of those neighbouring hybridization signals is 0.74 +/- 0.16 µm (n=65) for 2.25 kb of DNA (0.5 x [1.8+2.7] kb), or 1 kb per 0.33 µm of fiber.
(see Florijn *et al.*, 1995 for more details. Reproduced by kind permission of the Oxford University Press - Human Molecular Genetics, Vol. 5, 831-836, 1995, "High-resolution DNA fiber-FISH for genomic DNA mapping and colour bar-coding of large genes" Florijn R.J., den Dunnen J.T., Tanke H.J., van Ommen G.J.B., Raap A.K., figure 2 only).

3) - Indirect visualization of mRNA coding for a ovulation hormone (CDCH) in caudodorsal cells of *Lymnaea stagnalis* by FISH with oligonucleotide CDCH-1 3' (bio)n. Hybrids were detected with a TRITC-avidin conjugate; DAPI (blue fluorescence) was used as a general DNA counterstain.
(see Dirks *et al.*, 1990 for more details)

4) - Sensitivity of Fiber-FISH: a biotinylated PCR fragment of 202 bp(red) from a DMD exon, was co-hybridized with its cognate 40 kb cosmid (green), on a DNA fiber preparation.
(see Florijn *et al.*, 1996 for more details)

5) - A (single copy) 2.8 kb fragment of the human thyroglobulin gene visualized by FISH in metaphase chromosomes of two metaphases.
(by kind permission of J. Wiegant)

6) - Bi-colour FISH on Astrocytoma cell nuclei visualizes an extra chromosome 7 centromere in red, and the loss of one chromosome 10 (centromere) in 'green'. Counterstaining with DAPI visualizes the DNA of the cell nuclei; triple-exposure microphotograph.
(see Arnoldus *et al.*, 1991 for more details)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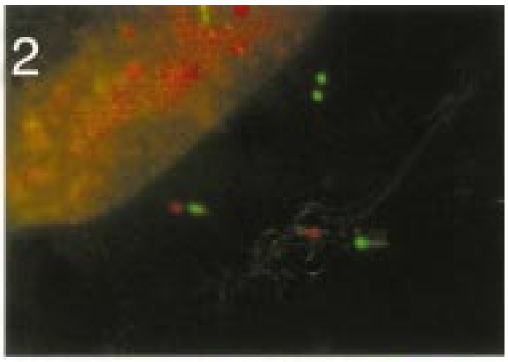
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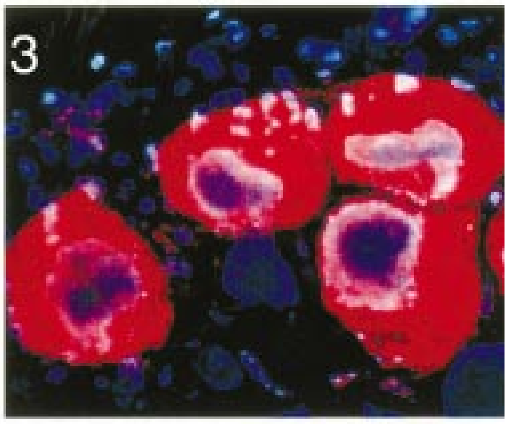
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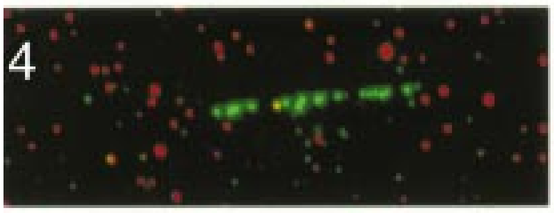
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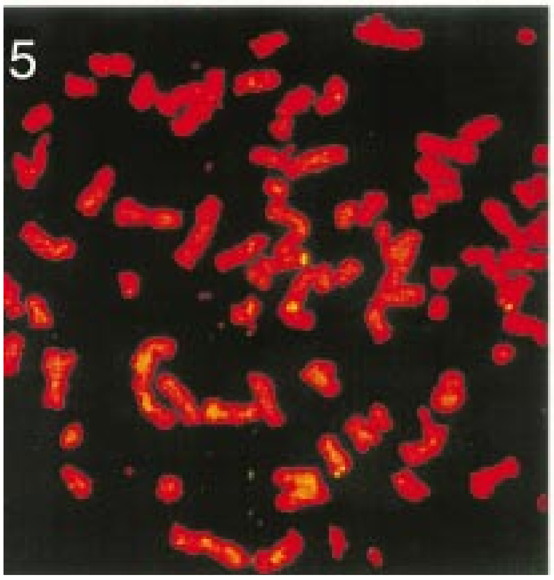
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This type of staining allowed identification of all individual (normal) chromosomes. It also enabled to identify numerical and relatively large structural chromosome aberrations in fresh or short term culture preparations of tumour material, including homogeneous staining regions (HSRs) and small extrachromsomal elements (double minutes). One of the early results of the banding procedures was the possibility to distinguish between the chromosomes No. 21 and No. 22. Also the fact that in CML, the Philadelphia (Ph1) chromosome is not just a terminal deletion of chromosome 22, but the result of a reciprocal translocation between a chromosome 22 and a chromosome 9 was detected (Rowley, 1973) by analysing banded metaphases (not detectable with the - at that time - ‘standard’ karyotyping procedure).

The breakpoints are in 9q34 and in 22q11. In the Ph1 chromosome, the *bcr* gene of chromosome 22 has fused with the *abl* gene coming from chromosome 9. The resulting fusion gene is responsible for the production of a protein with a high tyrosinase activity that causes uncontrolled cell division and tumour growth. With an *in situ* hybridization procedure (see hereafter), applying a cosmid probe specific for the *abl* gen, in combination with a probe for the *bcr* target, with different fluorescent labels, the translocation can be visualized in both metaphases and interphase nuclei (Fig. 13).

A limitation of the banding procedures is the **resolution**, which is insufficient to allow detection of more detailed chromosome changes like aberrations which involve chromosome fragments smaller than the size of one band.

The haploid human genome is 3×10^9 base pairs; it is thought to contain approximately 100,000 structural genes. By QM or G banding, 500-1000 bands can be obtained and recognized at best (this number is smaller in cancer cytogenetics, where the quality of the mitoses is less optimal). Assuming that size and the degree of condensation of chromosome bands is equal over the whole genome, a band contains approximately 3×10^6 base pairs (3,000 kbp) or 100 genes. From molecular and cytogenetic points of view, these are enormous numbers. Furthermore, none of the banding techniques is capable of providing specific information about the nucleotide sequence of the DNA present in an individual chromosome band.

In situ hybridization procedures (to be discussed hereafter) visualize strands of nucleic acids with a specific sequence. Above this, their physical resolving power is at least a hundred times better than the chromosome banding procedures.

In situ hybridization

In situ hybridization (ISH) is a cytochemical procedure that allows the sensitive detection and localization of specific nucleic acid sequences in morphologically preserved biological structures (directly in microscopical preparations).

It was developed in the same year (1969) as was the first chromosome banding technique. This procedure is based on the specific annealing possibilities of two complementary nucleic acid sequences, which is one of the important reaction principles in molecular genetics. Shortly after the discovery that RNA molecules can anneal with complementary sequences in DNA to form DNA-RNA hybrids, biologists started to consider the possibility of DNA-RNA hybridization in cytological preparations.

In this cytochemical procedure, one distinguishes the ‘**target**’ which is the sequence that is to be localized in the cell or chromosome preparation and the ‘**probe**’ which is a specific complementary sequence, that has been labelled with a ‘**reporter**’ group or molecule, to visualize the probe-target hybrid.

During the first decade, the probes were labelled with radio-isotopes. The experimental procedures were relatively hazardous, slow, and resulted in

Table VIII

How to obtain more detailed information from (pro)metaphase preparations?

1969 - chromosome banding - Caspersson & Zech; Seabright; Pearson; Sumner; Dutrillaux; and others

1969 - *in situ* hybridization

1973 - BrdU incorporation, sister chromatid exchanges - Latt

Table IX
Early publications on (radio-isotope-labelled) in situ *hybridization*

1969 - Gall & Pardue; Buorngiorno-Nardelli & Amaldi; John, Birnstiel & Jones	
1981 - Harper & Saunders - detection of unique sequences	

labelled targets/hybrids, which - after autoradiography - could be observed with a relatively good sensitivity, but with limited spatial resolution.

The introduction of **fluorescence** *in situ* hybridization has significantly changed the interest for ISH and led to the rapid development of sensitive, specific, multicolour FISH, which very fast has become a widespread and diversely applied technology in both biology and cytogenetics.

Non-radioactive ISH

After initial application of anti-RNA.DNA antibodies to detect target-probe hybrids, probes with broader application potentials were independently introduced by the groups of Pieter van Duijn and that of David Ward, applying respectively 3'-end chemically labelling of RNA probes and biotin-labelling by application of enzymatic procedures. Both procedures have the inherent advantage that more than one target sequence can be visualized simultaneously in the same preparation.

For non-radioactive *in situ* hybridization procedures, two types of approaches are discerned, according to (their) probe modifications. For the **direct** detection methods, the nucleic acid probe is modified with a reporter molecule that is directly detectable after hybridization. Among others, Fluorescein, Rhodamine, Cyanin and Alexa dyes, are optimal for such procedures.

In the **indirect** methods, the probe is haptenized (with biotin, digoxigenin, fluorescein, etc.), which allows the detection of the probe-target hybrid with a subsequent immunocytochemical reaction (possibly followed by amplification of the signal). For both approaches, probes can be labelled, applying either enzymatic or chemical methods. Visualization of the endproduct can be obtained by use of fluorophores, colloidal gold, or by deposition of endproducts of chromogenic enzyme- (Mullink *et al.*, 1989) or immunocytochemical procedures.

A recently developed signal amplification procedure in which enzyme cytochemistry is combined with immunofluorescent visualization (Raap *et al.*, 1995) is discussed hereafter.

Major advantages of fluorescence *in situ* hybridization (FISH) over bright-field procedures are the facts that fluorescence in general can be detected with higher sensitivity and resolution, and that there are more fluorophores to identify simultaneously individual target-probe combinations. Furthermore, a fluorescent banding procedure (e.g. with DAPI) can be used to recognize the individual chromosomes (Lichter *et al.*, 1990; Lengauer *et al.*, 1993; Mueller *et al.*, 1996).

The advantage of the probe-labelling with an allylamine derivative of (d)UTP (e.g. biotin-dUTP), introduced by the group of Ward (Langer *et al.*, 1981) was its compatibility with enzymatic procedures, which also were applied in nucleic acid labelling formats for well-established radioactive hybridization procedures. This fact has contributed significantly to the relatively fast acceptance of this method of probe production for non-radioactive ISH.

Non-radioactive ISH has particularly benefitted from methods to couple/conjugate nucleotides with a label in such a way that the modified nucleotides still could be incorporated into DNA or RNA. The first of such methods - rather soon widely accepted - was the conjugation of biotin to nucleotides (Langer *et al.*, 1981).

Rigby *et al.* (1977) had introduced nick translation with a hapten, or fluorophore-modified deoxynucleoside triphosphates for probes present in amounts of at least micrograms. Nick-translation is still the most widely used method for probe labelling. Random priming (Feinberg & Vogelstein, 1984) is a procedure that is less commonly applied for smaller quantities of smaller fragments. In later years Boehringer introduced digoxigenin as a hapten (Kessler, 1990).

- The polymerase chain reaction procedure is particularly useful for more complex probes such as flow-sorted chromosomes or somatic cell hybrids. It is able to generate large amounts of probe and needs very small amounts of source DNA to start with. There is also the possibility to produce haptenized probes, by application of one or more haptenized nucleotides for the amplification procedure (Boehringer, 1995).

- Due to the fact that larger insert clones usually contain repeat sequences, their application initially was hampered by high ‘background’ signals. Landegent *et al.* (1987) solved this problem by introduction of *in situ* suppression hybridization, saturating the repetitive sequences of the probe during a pre-incubation with Cot-1 DNA.

- Recombinant (nucleic acid) technology and the ability to clone and amplify DNA sequences in micro-organisms has provided rich sources of probes for FISH.

- FISH karyotyping with ‘whole chromosome’ probes, is successfully applied to elucidate complex chromosome rearrangements (Speicher *et al.*, 1996A, 1996B; Schröck *et al.*, 1996; Tanke *et al.*, 1999). Such cytogenetic investigations are also of great value for clinical cytogenetics (Rowley, see Veldman *et al.*, 1997).

- FISH can contribute to positional cloning of onco- and tumour suppressor genes because it allows rapid mapping of disease regions delineated by genetic markers and cytogenetic observations.

- Next to the flow-sorted ‘whole chromosome’ probes, more and more painting probes for subregions of chromosomes are obtained by amplification of micro-dissected material.

- Probes that recognize (sub)telomeric regions of particular chromosomes are applied in multicolour FISH approaches to detect cryptic translocations that frequently occur in patients with mental retardation (Ning *et al.*, 1996).

- Important features of the non-radoactive *in situ* hybridization procedures are the possibility of:

a) metaphase and interphase cytogenetics;
b) unambiguous identification of marker chromosomes;
c) detection of numerical and structural chromosome aberrations;
d) analysis of archival material.

Oligonucleotide probes, applied directly for hybridization to DNA or RNA, are usually labelled either by the use of modified bases during synthesis, or by end-labelling.

They are also used in PRimed *IN Situ* (PRINS) labelling procedures (Koch *et al.*, 1991). Hybridization to the target is first achieved with a specific unlabelled oligonucleotide, which is then used to prime chain elongation by a polymerase (for DNA) or a reverse transcriptase (for RNA).

Chromogenic cytochemical enzyme deposits as endproducts of ISH are mainly used for clinical applications (Netten *et al.*, 1996).

Multicolour FISH procedures

The number of target sequences that can be detected simultaneously by a multicolour FISH, is mainly limited by the number of spectrally separable fluorophores which are available.

By mixing probes (which are differently labelled) in a hybridization mixture, more than one target can be visualized simultaneously, provided that hybridization conditions like e.g. stringency, will match.

For *in situ* hybridization, originally each specific probe was haptenized or labelled to carry one type of fluorophore in the resulting endproduct.

Nederlof *et al.* (1989B) introduced the simultaneous *in situ* detection of three targets in one preparation. Three individual chromosome-specific probes were labelled to result in green(FITC), red(TRITC) and blue(AMCA - Khalfan *et al.*, 1986) images in the preparation. With these three fluorophores, it is also possible to visualize seven targets simultaneously *in situ*, when three probes

Table X
Early approaches in non-radioactive in situ hybridization

1977 - Rudkin & Stollar: anti-RNA.DNA antibodies as specific detection reagents

1980 - Bauman, Wiegant & van Duijn: RNA probes 3’-labelled with a fluorophore

1981 - Ward *et al.*: biotin-labelled probes (Langer *et al.*)

1986 - Pinkel *et al.*: FITC-conjugated avidin

1987 - Landegent *et al.*: *in situ* suppression hybridization are each labelled with one fluorophore, three with combinations of two, and one probe with three fluorophores (Nederlof *et al.*, 1990). This type of labelling is called **combinatorial labelling**.

Shortly thereafter real multicolour FISH detection could be obtained by using different **ratios** of two or more fluorophores (**ratio-labelling**) to label the individual probes (Nederlof *et al.*, 1992). The resulting fluorescence hybridization signals were analysed using an epi-fluorescence microscope equipped with a multi-wavelength illuminator and a cooled CCD camera for digital imaging. The analysis is performed on basis of the different colour intensity ratios and not on colour composition alone. The ISH results can then be displayed as pseudo-colours on a monitor. This ratio-labelling has great potentials. Dauwerse *et al.* (1992) obtained twelve colours for a 'molecular karyotype', using chromosome libraries labelled with combinations of three fluorophores in different ratios. Yet, till now, most often only binary combinations of fluorophores have been applied to enhance the number of different fluorescent signal that can be detected simultaneously. Ratio-labelling was used far less.

In principle, more (different) fluorescence signals can be obtained by use of more fluorophores. When combinatorial labelling is applied, the number of distinguishable emissions [**n**], for [**k**] different fluorophores, is:

$$n = 2^K - 1$$

The combinatorial FISH approach has been developed during the nineteen nineties to the point that the 24 (different) human chromosomes can be differentiated on basis of FISH color combinations of five fluorophores.

Two imaging principles have been applied for these approaches.

Multi-FISH (M-FISH) - Speicher *et al.* (1996A, 1996B), using chromosome painting probes, achieved their results by applying optimized narrow band pass **filters** (typically 5 to 15 nm bandwidth) to minimize the chance of spectral overlap and undesirable cross talk between optical detection channels.

Spectral Karyotyping (SKY) - Schröck *et al.* (1996) used a different approach. They did not discriminate the 'colours' on basis of single intensity measurements through fluorochrome-specific filters, but combined Fourier spectroscopy, charge-coupled device imaging, and optical microscopy to measure emission spectra, simultaneously in every pixel of the sample. This allowed the application of spectrally overlapping probes, obtained by labelling chromosome-specific composite libraries with nucleotides conjugated to 5 different fluorophores or combinations thereof. Fourier transformation was used to resolve the spectrally overlapping emissions, allowing to identify all individual (human) chromosomes.

COBRA - Tanke *et al.* (1999). The most recent extension in the field of multicolour FISH (at this moment) is the **CO**mbined **B**inary **RA**tio labelling.

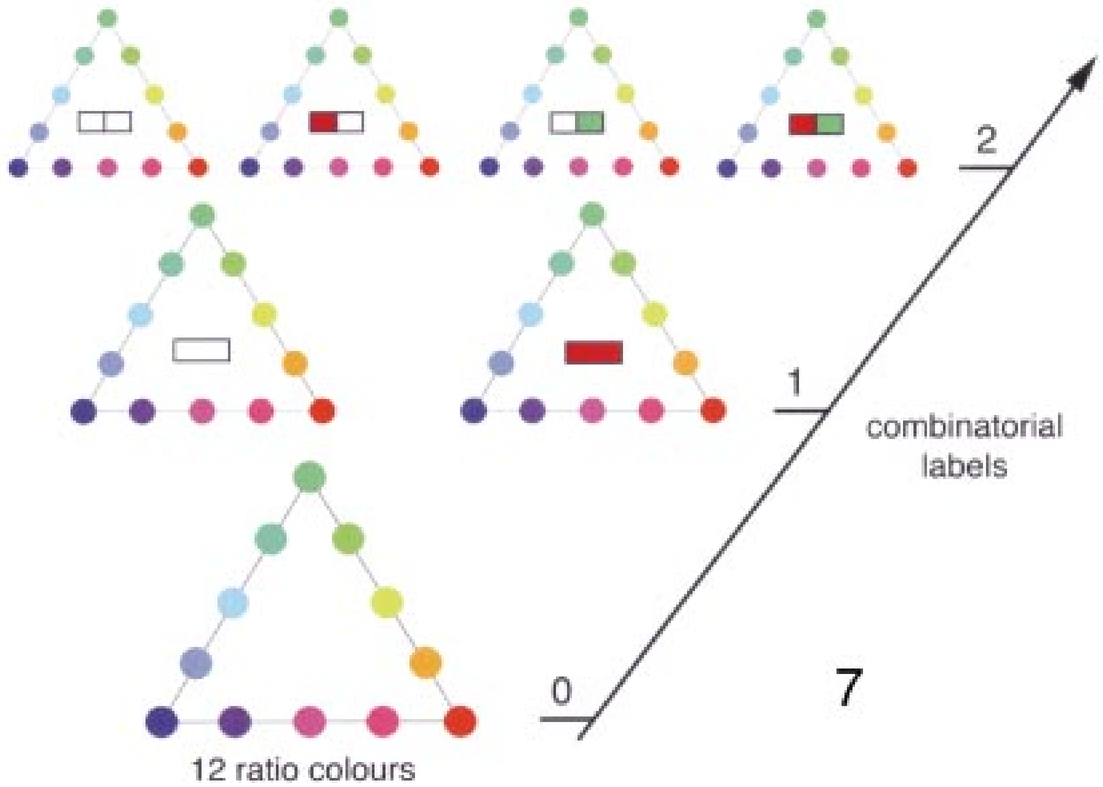
In the COBRA approach, ratio-labelling is combined with combinatorial labelling. In this way five fluorophores suffice to recognize simultaneously 48 different target sequences in metaphase spreads.

The method can be performed with existing technology and requires, apart from accurate measurement facilities for fluorescence intensities, no major investment in microscope hardware other than a good digital fluorescence microscope and a CCD camera.

Figure 7 shows the principle of COBRA. Three fluorophores are applied in pairs for ratio-labelling a set of twelve chromosome painting probes. A second set of twelve probes is labelled identically, but also receives a binary label with the fourth fluorophore. In this way the number of specific emissions is doubled and becomes 24. Introduction of another independent binary label by use of a second hapten, will again double the number of distinguishable 'colours' up to 48 (Fig. 8).

Two strategies have been demonstrated to be applicable in the COBRA approach. For the first, probes were enzymatically labelled with Fluorescein, Lissamine and Cy5 as primary fluorophores. The blue fluorescing diethylaminocoumarin (DEAC) was applied as the combinatorial fourth label to demonstrate incorporated digoxigenin.

The principle was also tested using chemical labelling. Twelve painting probes were labelled by ULS (Universal Linkage System), with DEAC, Cy3 and Cy5 as primary labels. The second set, being labelled similarly, contained also a digoxigenin-ULS label, that was stained indirectly with fluorescein.



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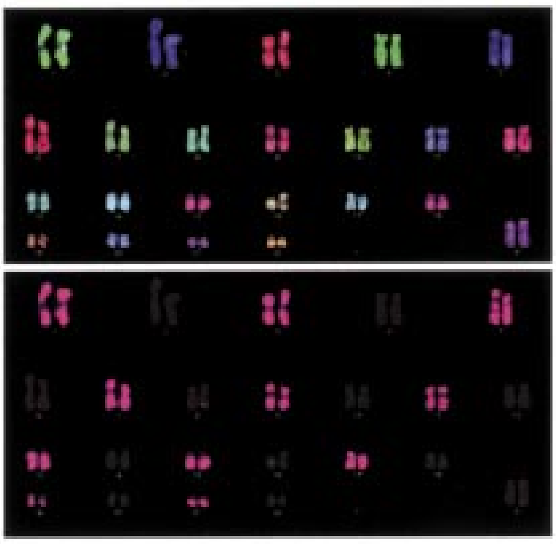

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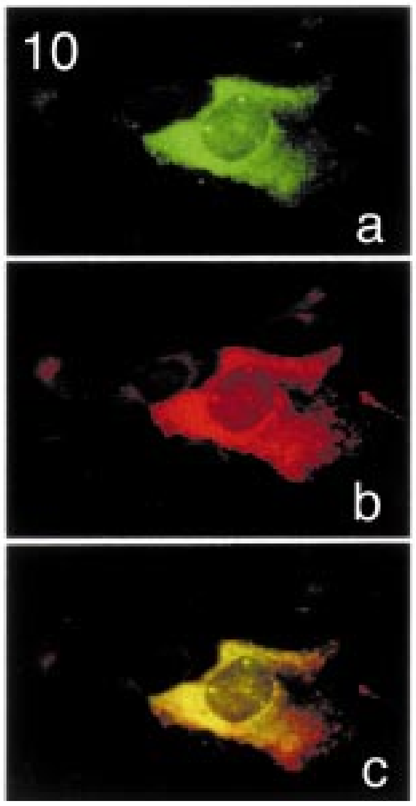


Table XI
Early publications on Bi- and Multicolour FISH

1986B - Bi-colour detection - Hopman *et al.*

1989B - Three-colour FISH; introducing AMCA - Nederlof *et al.*

1990 - Combinatorial labelling - Nederlof *et al.*

1991 - Multiple simultaneous RNA detection - Dirks *et al.*

1992 - Fluorescence ratio measurements - Nederlof *et al.*

1992 - A molecular karyotype with ratio-labelled probes - Dauwerse *et al.*

1992 - Fiber FISH - Wiegant *et al.*; Heiskanen *et al.*, 1995

1993 - Bar-coding of chromosomes - Lengauer *et al.*; Wiegant *et al.*; Mueller *et al.* (1996)

1996A - Combinatorial multi-FISH (M-FISH): painting all human chromosomes simultaneously - Speicher/Ward *et al.*, 27 targets simultaneously with 5 fluorophores

1996 - Spectral karyotyping (SKY): all human chromosomes simultaneously - Schröck/Ried *et al.*

1999 - COBRA: 48 different simultaneous fluorescence signals with 5 fluorophores - Tanke *et al.*

Interphase cytogenetics with FISH

One of the attractive features of FISH procedures, next to their high sensitivity and the fact that several targets can be detected simultaneously in the same preparation, is the fact that FISH also can provide cytogenetic information when performed on interphase nuclei.

In situ hybridization on interphase cell nuclei (‘Interphase Cytogenetics’ - Cremer *et al.*, 1986) has powerful potentials for biological and clinical applications, especially when a relatively small number of aberrant cells has to be identified in a larger cell population.

Tumour cells often show numerical and/or structural chromosomal aberrations (Fig. 6). The types of aberrations may, in some cases, provide information about the changes which initiated the tumour. Such aberrations may also correlate with

Figs. 7/10 - 7) - Principle of COBRA. The primary set of 12 ratio colours is doubled each time an independent binary label is introduced, resulting in 24 colours after one hapten, and in 48 colours for two haptens.
(see Tanke *et al.*, 1999 for more details - Reproduced by kind permission of Stockton Press - European Journal of Human Genetics, Vol. 7, 2-11, 1999, “COBRA combinatorial binary ratio labelling: new strategy for multi-colour fluorescence *in situ* hybridization: COBRA combined binary ratio labelling” Tanke H.J., Wiegant J., van Gijlswijk R.P.M., Bezrookove V., Pattenier H., Heetebrij R.J., Talman, E.G., Raap, A.K., Vrolijk H., scheme of COBRA).

8) - 8A - Karyotype after FISH with 12 ratio-labeled probes. Differentiation between e.g. chromosomes 3 and 5 is not yet possible. 8B - The same metaphase after the first combinatorial staining: the pair of chromosomes 3 clearly differs from that of 5.
(see Tanke *et al.*, 1999 for more details)

9) - Cytomegalo virus (CMV)-infected cell in a microscopic preparation made from liquor cerebrospinalis. HCMV detected with ISH and Peroxidase-DAB visualization. Other cells visualized with interference microscopy.
(by kind permission of Frank van de Berg)

10) - HCMV-IE mRNA detection in cycloheximide-treated rat 9G cells. When rat 9G cells are hybridized with digoxigenin-labelled pSS plasmid probe, the results subsequently being visualized with conventional immunocytochemistry, the HCMV-IE integration sites are visible in each nucleus as a bright fluorescent spot, while mRNA fluorescent signals are present in the cytoplasm of about 30% of the cells.
Conventionally visualized hybridization of pSS plasmid (green fluorescence - 10A) and a single HRP-ODN procedure detected by one DNP-tyramide and rat anti-DNP-Rhodamine Red (red fluorescence - 10B) reveals co-localization of the two different probes (double exposure - 10C).
(see van de Corput *et al.*, 1998A for more details - Reproduced by kind permission of Springer-Verlag - Histochemistry and Cell Biology, Vol. 110, 431-437, 1998, “Fluorescence *in situ* hybridization using horse-radish peroxidase labelled oligodeoxynucleotides and tyramide signal amplification for sensitive DNA and mRNA detection” van de Corput M.P.C., Dirks R.W., van Gijlswijk R.P.M., van de Rijke F.M., Raap A.K., Figure 3c, d and e).

the evolution of the tumour and have prognostic value, in which case the results of the analysis can be applied to determine clinical protocols. Therefore, much effort has been invested in the cytogenetic investigation of metaphases obtained from malignant material.

For solid tumours, however, several disadvantages hamper analysis of metaphase chromosomes (Teyssier, 1989). Karyotyping of solid tumors is difficult due to the fact that the mitotic index of most tumours is low, which implies that in order to obtain enough metaphases, extensive culturing is often necessary. Culturing of such material may lead to selective (over)growth by subpopulations of cells among which might be non-neoplastic cells. Moreover - partly depending on the culture medium - the culturing procedure itself may induce changes in karyotype, such as tetraploidization. So, it can be that the karyotypes of the cultured cells do not reflect the original chromosome composition (see Arnoldus *et al.*, 1991; and Heim *et al.*, 1989).

Furthermore, metaphase spreads from solid tumours are often of inferior quality, consisting of fuzzy chromosomes with poor banding patterns, which make correct karyotyping extremely difficult.

Checking for chromosomal aberrations in solid tumours was also tried by measuring the DNA contents of cells in suspension by flow cytometry (Gray *et al.*, 1975; Trask *et al.*, 1990). Because of the relative fast and representative results, many investigators and clinicians had high expectations and have worked at this form of analysis. A drawback of the method is the 'limited cytogenetic sensitivity': the loss of one complete chromosome, often is too small a difference to be detected (Cornelisse & Tanke, 1991). Furthermore, when two nuclei possess the same DNA content, it does not necessarily imply that the same chromosome complement is present in the two cells.

Contrary to chromosome banding procedures, FISH has the advantage that it can also be applied on interphase nuclei, and DNA-fiber preparations (see hereafter).

When originally, scientists tried to convince cytologists and pathologists that FISH on interphase nuclei would provide a good diagnostic tool, a lot of discussion and investigation was started to control the reliability of the results. ("Is one instead of two signals in an interphase nucleus, the result of a monosomy, or is it a failure in the procedure?"). At this moment, nobody doubts that interphase cytogenetics has quite some advantages in cases of e.g. minimal residual disease and for studies on solid tumours.

"The feasibility of evaluating individual interphase cells opens the way to detect and quantitate aberrant cell populations. The potential clinical applications are the detection of minimal residual disease and early relapse. Quantitative interphase cytogenetics can also provide more insight into the evolution of tumour clones, and thus into the biologic significance of the genetic aberrations. This is especially apparent if these clones can be identified immunopathologically" (Kibbelaar, 1994).

One should keep in mind that an *in situ* hybridization only can provide information about the presence or absence of (that part of) the target sequence, that can be discovered by the probe. When FISH with a **centromere**-specific probe gives negative results, the conclusion that the **whole chromosome** is missing, is not justified. In order to allow such a conclusion, a chromosome-specific library probe (that will detect each part of that chromosome if present) should be applied.

Interphase cytogenetics can best be applied when *a priori* information is present about the optimal probe type and size.

Table XII
Early publications on Interphase cytogenetics

1986 - radioactive & non-radioactive ISH - Cremer *et al.* - coined the term 'interphase cytogenetics'

1988 - on solid bladder tumours - Hopman *et al.*

1989A - on bone marrow & colon tumor derived cell lines - Nederlof *et al.*

1989 - on paraffin-embedded sections - Emmerich *et al.*

1989 - on cerebellum - Arnoldus *et al.*

Fiber-FISH for genomic mapping

Multicolour-FISH procedures have been applied for physical DNA mapping and ordering. When considering physical DNA mapping experiments, one has to realize that the spatial resolution in metaphase chromosomes (as a consequence of the highly 'coiled' nature of these entities) in terms of DNA is restricted to about 3 Mb. Regional localization can be achieved either by combining *in situ* hybridization with chromosome banding methods (Takahashi *et al.*, 1990; Wiegant *et al.*, 1991) or fractional length mesurements (Lichter *et al.*, 1990).

In interphase nuclei, the more relaxed condensation state of the chromatin allows a spatial resolution between 100 kb and 1 Mb (Trask *et al.*, 1991; Florijn *et al.*, 1995), but even then DNA sequences which are closer than 100 kb on the physical map, can not be recognized as two separate, individual, spots in a FISH-stained preparation.

The spatial resolution of a light microscope (depending on the magnification and numerical aperture of the objective applied) is theoretically limited to 0.2-0.3 µm in the field of focus. In Giemsa-banded metaphase chromosomes, 0.3 µm corresponds with the size of a smaller chromosome band, which in terms of DNA is still about 3 Mb. The length of 1 kb of relaxed/linear DNA measures 0.34 µm.

For resolutions in the order of 50 to 1000 kb, G_0-interphase nuclei of fibroblasts have been applied successfully (Lawrence *et al.*, 1990; Trask *et al.*, 1989 & 1990). The order of DNA targets can be derived by performing distance measurements in a number of nuclei. With multicolour FISH, DNA sequences with the appropriate physical distance may sometimes also be ordered directly under the fluorescence microscope, provided it is equipped with a high transmission double band-pass filter set for perfect geometric registration of the colour images. Furthermore, there must not exist a specific chromatin configuration in the region of interest (den Dunnen *et al.*, 1992; van den Engh *et al.*, 1992).

In one of the approaches used to obtain higher genomic resolution, pronuclei of human sperm are introduced into hamster oocytes, resulting in chromatin decondensation (Brandriff *et al.*, 1991). This methodology, however, is laborious, requires very specific expertise, and often results in only a few useful cells per fusion experiment.

To improve and facilitate possibilities for physical mapping of DNA clones in microscopic preparations, recently several other approaches have been developed (Raap, 1998).

Wiegant *et al.* (1992) submitted fibroblasts, grown on microscopic slides, to a mild nuclear extraction - adapted from the halo-procedure developed by Vogelstein c.s. (1980) - with detergent, high salt, and an intercalating dye treatment and UV-light. In this way they obtained highly extended DNA loops arranged around the nuclear matrices (Fig. 1). Several other approaches which result in similar decondensation of the chromatin, have been published since then, like that of Parra & Windle (1993), applying SDS extraction on mammalian cells to obtain naked DNA fibers immobilized on glass slides, and that of Haaf & Ward (1994).

The loops consist of stretches of naked DNA that is in such a relaxed state that a resolution of 1 kb per 0.3 µm in practice is achieved. Linear (or near-linear) stretches (with minimal breakage) of several hundreds of kilobases up to over 1 Mb of intact genomic DNA can be easily produced at sufficient density, even from small numbers of cells.

Florijn *et al.* (1995) measured the signals obtained on DNA fibers by hybridization of two neighbouring targets (with two repeat-free single copy plasmids) of which the sizes (determined by restriction analysis) were respectively 1.8 and 2.7 kb (Fig. 2). The midpoint-to-midpoint distance between the signals (determined by digital image analysis) was measured (n=65) to be 0.74 +/- 0.16 µm for the 2.25 kb (half of the 1.8 + 2.7 kb), which comes to 0.33 µm/kb. These results show that the theoretical resolution of 1 kb was obtained in practice.

The lower sensitivity limit of Fiber-FISH is about 200 bp (Fig. 4).

Fiber-FISH in combination with digital imaging microscopy enables DNA mapping ina range of 1 to 500 kb, thus connecting and overlapping interphase FISH mapping, conventional, and pulsed-field gel electrophoresis. This approach contributes to the resolution of gap sizing problems which are inherent to restriction mapping of regions that are only partly covered by cosmid clones, as is often the case in genomic contig building. The potential of this approach for direct genomic DNA mapping and for the identification of gene rearrangements is clearly demonstrated in the publication of Florijn *et al.* (1995). These authors describe the high-resolution mapping of deletions in two patients with Duchenne muscular dystrophy (DMD). First, the order of a twelve cosmid contig isolated from a YAC containing part of the DMD gene (YAC yDMD4) was determined by two-colour Fiber-FISH with various combinations of 2 or 3 cosmids, interpreting the results directly under the microscope.

Then, by use of three subsets of 5 to 7 cosmids (spanning the full 400 kb of the contig), quantitative length determinations were performed by digital imaging microscopy. The deletion breakpoints could be identified by comparison of patients' imaged fiber-FISH tracks with those of a control. In this way, accurate measurements of the deletion sizes in the patients' material could be performed. The authors estimate, that with this approach, it will be possible to identify deletions or short range duplications of 5 to 10 kb.

They also state that 'in the hands of a person with reasonable experience in both techniques, Fiber-FISH mapping is far less labour-intensive than restriction analysis'. Other interesting examples of Fiber-FISH mapping are presented by a.o. Heiskanen *et al.* (1995); Klockars *et al.* (1996) and Lestou *et al.* (1996).

Detection of specific messenger RNAs by use of FISH

Correct and optimal *in situ* detection of mRNAs requires - even more than DNA hybridization - the establishment of optimal conditions for fixation, pre-hybridization incubations, and hybridization. Because most of these nucleic acid molecules are easily degraded by RNases and because they 'radiate freely' from gene to cytoplasm, extra precautions are necessary to allow correct localization of mRNAs (Dirks *et al.*, 1995).

Fixation of the specimen is necessary to retain (as much as possible of) the cellular RNA and to maintain tissue and cell morphology throughout the hybridization and visualization procedures. Too extensive cross-linking by the fixative, however, will decrease the accessibility of target sequences by the probe and detection systems.

Table XIII
Current status of FISH

- Detection sensitivity	:	0.2 - 1 kb
- Resolution in DNA fibers	:	1 kb
- Multiplicity	:	48 'colours'/targets

Based on the fact that some RNA molecules possess self-splicing properties, RNA molecules have been believed to have been the earliest complex bio-organic molecules on earth, with a primary role in information processing (Darnell & Doolittle, 1986). The role of RNA as a basic carrier of genetic information would have been taken over by DNA during evolution. There exist several types of RNA molecules, of which the messenger RNAs are important because they are involved in the translation of genetic information.

The participation of RNA in protein synthesis was already suspected in 1940 by Caspersson when cytometrical analysis of unstained cells (UV absorbance by all nucleic acids at 260 nm) and subsequent Feulgen-Schiff staining to distinguish the DNA, revealed that most of the RNA is present in the cytoplasm, whereas all DNA proved to be in the nucleus. Biochemical analysis by Brachet on a nuclear and a cytoplasmic fraction obtained from cells, led to the same conclusion. Brachet also showed that cytoplasmic RNA is located in small particles, the ribosomes, which were demonstrated to be the sites of protein synthesis. The concept of mRNA was formulated by Jacob & Monod in a 'classic' paper published in 1961 (Jacob & Monod, 1961; Stryer, 1981). In eukaryotic cells, messenger RNA is synthesized in the nucleus, is processed, and will be transported then to the cytoplasm, where it serves as a template for protein synthesis.

During the past decades, cytochemical procedures have been developed to visualize biomolecules involved in the pathway from gene to protein, which resulted in a better understanding of the interactions between these biomolecules in a structural context.

Among the cytochemical methods to demonstrate RNA in morphologically preserved biological material, the methyl green-pyronin Y staining method (Rothbarth *et al.*, 1976) and the acridine orange method are 'specific'. These methods, however, do not allow to discriminate between different types of mRNA. Gene expression is often studied by use of antibodies specifically raised against the protein that is encoded by the expressing gene.

Because most often, antibodies specific for different types of mRNA are not available, and protein levels do not necessarily reflect the transcriptional activity of cells, ISH procedures are more frequently applied to study gene expression.

These procedures also allow simultaneous localization of different types of RNA at the individual cell level.

Originally, localization of mRNAs by use of non-radioactive *in situ* hybridization could only be performed with a limited sensitivity. During the last decade RNA *in situ* hybridization procedures have been optimized, e.g. on models like the neuropeptidergic systems of the pond snail *Lymnaea stagnalis* and a rat cell line that was transfected with human cytomegalovirus immediate early antigen DNA (Dirks *et al.*, 1989, 1990, 1992). At present reliable and sensitive localization of mRNAs in combination with their cognate proteins (immunocytochemically), is feasible with visualization options under the light and the fluorescence microscope (Fig. 3), as well at the ultrastructural level.

In general, to achieve sufficient sensitivity, relative large probe sequences, which will cover the whole RNA targets, are necessary, the immunocytochemical visualization being obtained with several amplification steps (Dirks *et al.*, 1993).

With the horse radish-tyramide amplification procedure (to be discussed hereafter), an increased detection sensiti-vity has been obtained (van de Corput *et al.* 1998A, 1998B).

In another approach to increase sensitivity, sophisticated image processing applying a deconvolution algorithm, was used after hybridization with a number of oligonucleotide probe sequences that carried several fluorochromic labels (Femino *et al.*, 1998). The authors claim that this procedure allowed them to detect even a single specific RNA molecule, and in this way to quantitate the kinetics of the β-actin mRNA gene expression.

A special field of science where RNA-FISH is applied, is the study of gene activities taking place in the cell nucleus. Since a number of investigations in recent years has shown that cell nuclei contain compartments in which molecular processes take place (Cremer *et al.*, 1993; van Driel et al, 1995; Singer & Green, 1997), their structural and functional architecture has become a topic of great interest.

As already predicted by Rabl (1885), in non-cycling interphase cell nuclei chromosomes were found to occupy distinct territories, as do some nuclear proteins and RNAs (Wansink *et al.* 1994).

The analysis of gene dynamics necessitates the localization of nuclear components in space and time within the three-dimensional context of the (living) cell nucleus (Robinett et al, 1996; Tvaruskó *et al.*, 1999). Proteins can be labelled with an autofluorescent protein (like green fluorescent protein) by means of molecular cloning (Marshall *et al.*, 1997; Misteli *et al.*, 1998) or antibodies (Buchenau *et al.*, 1997). With this procedure also single chromosome loci or artificially introduced subregions can be visualized, by detecting the proteins which bind to specific loci or subregions. The group of Cremer (Zink *et al.*, 1998A, 1998B - see also Manders *et al.*, 1999) introduced a fluorescent thymidine analog (Cy3-AP3-dUTP) by microinjection in cells which were in culture. Integration of the probe during further culturing, resulted in daughter cell nuclei with individual labelled chromatid territories composed of subchromosomal foci with a diameter of about 400-800 nm.

- The localization of specific genes and gene transcripts in living cells is possible by FISH with fluorophore-labelled probes which have to be introduced in the cell, e.g. by microinjection.

- Several types of probes have been explored and are being applied, like oligoribonucleotides, peptide nucleic acids (PNAs) and molecular beacons. Theoretically, molecular beacons are optimally suited for living cell analysis (Dirks *et al.*, 1999). These probes have a stemloop strucutre with a fluorophore at one end of the stem and a quencher of fluoresecence at the other, the loop consisting of a sequence complementary to the target. In the closed confirmation, the probe will not fluoresce due to the fact that fluorophore and quencher are in close proximity. Upon hybridization, the beacon will open-up and fluoresce.

Early studies concerning transcript transport from the nucleus to the cytoplasm (Dirks *et al.*, 1995) seemed to indicate that transcripts of a given gene find their way to the cytoplasm through a defined pore complex (‘gene gating’- Blobel, 1985). Human cytomegalovirus immediate early antigen (HCMV-IEA RNA) transcripts in rat 9G fibroblast cells were detected in track-like configurations, suggesting that these represented RNA transport routes. However, more detailed analysis revealed that HCMV-IEA mRNAs were not only present in the track-like domains, but also throughout the nucleoplasm of these cells excluding nucleoli, with

Table XIV

Early publications on mRNA detection using non-radioactive ISH procedures

1982 -	Singer & Ward
1985, 1986 -	Lawrence & Singer
1989 -	32P, 35S, and peroxidase-DAB visualization - Dirks *et al.*
1989 -	Lawrence *et al.*
1990 -	two-colour FISH detection - Dirks *et al.*
1991 -	multicolour FISH detection - Dirks *et al.*
1992 -	colloidal gold EM detection of mRNAs - Dirks *et al.*
1998B -	tyramide-amplified mRNA detection - van de Corput *et al.*

the highest amount occurring near the transcribing gene cluster (Dirks *et al.*, 1995; Macville *et al.*, 1995). From these observations it seems highly probable that HCMV mRNAs are not transported along one specific track, but travel in a diffuse way throughout the nuclear interior to the cytoplasm.

Politz *et al.* (1999) recently applied a caged fluorescein-labelled (nonfluorescent) oligo (dT) probe, that in living cells hybridizes to nuclear poly(A)-RNA. Upon photoactivation of the fluorescein, it is possible to follow the movements of the hybrids in 2 μm nuclear spots. The diffusion coefficient of the hybridized oligo (dT), that appeared to move in all directions, was found to be the same at 23°C and at 37°C, which suggests that the transport process is energy independent.

Recent studies have provided evidence that the positioning of gene sequences within the cell nucleus is important for the control of gene expression (Cremer *et al.*, 1993; Stein *et al.*, 1998; Cockell & Gasser, 1999). RNA processing factors and a number of transcription factors are highly compartmentalized in the cell nucleus. They are found to be enriched in so-called ‘speckle domains’. It is rather probable that the dynamic position of genes, relative to these nuclear domains, plays a role in the regulation of gene transcription and RNA processing.

Comparative Genomic Hybridization

As just described, FISH is well suited to detect and characterize structural and numerical changes in chromosome make-up of interphase and metaphase cells.

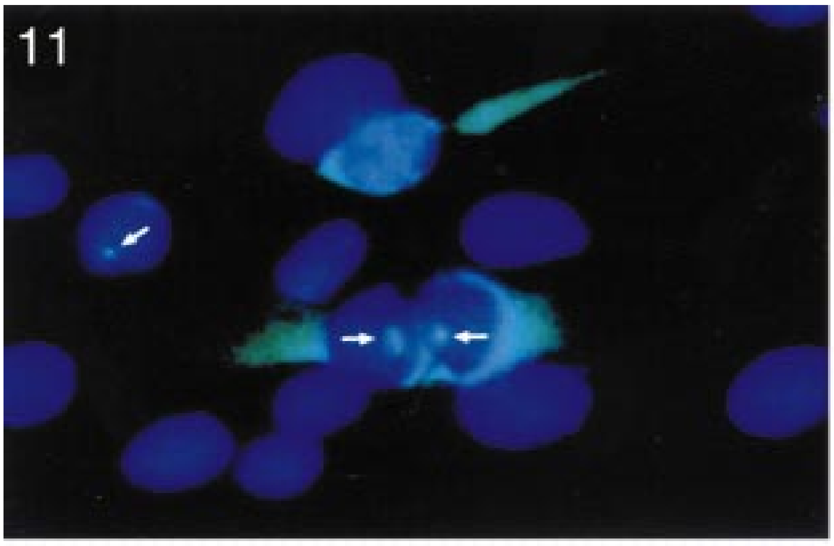
Consequently, it is used diagnostically and prognostically, especially on nuclei or chromosomes when ***a priori*** knowledge is available about the

Figs. 11/13 - 11) Visualization of mRNA expression of human cytomegalovirus (HCMV) immediate early antigen (IEA) by *in situ* hybridization with fluorescein-pSS DNA to cycloheximide-treated rat 9G fibroblast cells.
Induction of the expression of the transfected HCMV-IE DNA in these cells is S-phase dependent. The preparation had not been thermally denatured, so only the positive cells show nuclear (arrow heads) and cytoplasmic IE RNA (green signals). DAPI counterstaining.
(see Raap *et al.*, 1991 for more details)

12) - FISH-bar-coded human chromosome 11
Monitor image in pseudocolours obtained after processing the fluorescence (recorded with a cooled CCD camera) of a chromosome 11, that was hybridized with six different probes which were visualized with combinations of fluorescein, rhodamine and coumarin fluorophores.
(see Wiegant *et al.*, 1993 for more details)

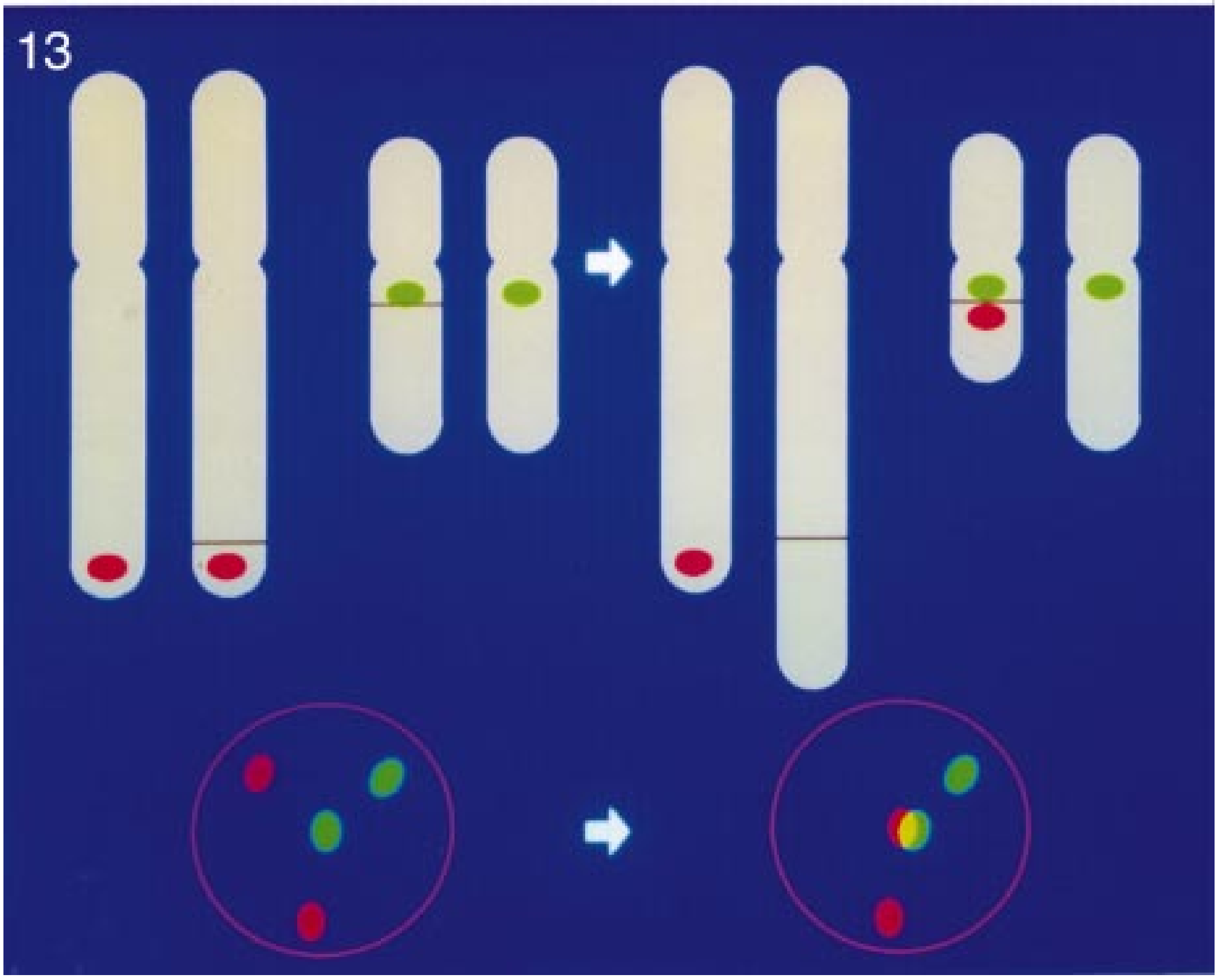
13) - Schematic representation of the bi-colour FISH detection of a reciprocal translocation between a chromosome 22 and a chromosome 9. Part of the long arm of chromosome 22 is translocated to the long arm of a chromosome 9, and a small part of that chromosome 9 has fused with the remaining part of the chromosome 22, resulting in the formation of a Philadelphia (Ph1) chromosome. In a normal cell nucleus two red and two green spots will be visible. If a Ph1 chromosome is present, one red and one green spot will be seen as well as za (partly) co-localising red-and-green spot.
(see Arnoldus *et al.*, 1990 for more details)

11



12

13



genetic changes that could be present in the sample under investigation.

The fact that for many cancers knowledge about the specific genes which are causally related with the disease, is still scarce or missing, has inspired scientists (Kallioniemi *et al.*, 1992) to develop a new FISH methodology: **C**omparative **G**enomic **H**ybridization (CGH).

Kallioniemi and coworkers (1992) and the group of Cremer (Du Manoir *et al.*, 1993) developed a method that enables to obtain insight in the amplification and/or deletions present in the nucleic acids of a neoplastic sample.

The principle of this method is a simultaneous *in situ* hybridization (using normal metaphase chromosome preparations), with DNA isolated from a tumour sample and a reference DNA, under conditions of repeat suppression. The two DNA samples are labelled differently, so that - under the fluorescence microscope - they will show up with different emission colours (e.g. green and red).

If an under- or over-representation of specific genes in the DNA under investigation is present, an imbalance in the red-over-green fluorescence intensity ratio will show up at the cytogenetic location where the over- or under-represented DNA is located. To identify the individual chromosomes, a blue fluorescent banding counterstain is applied. At amplified sites, oncogenes may reside and at sites with less than the normal amount of nucleic acid, tumour suppressor genes.

These sites are then mapped. Subsequently, the proteins involved are to be identified, as is thereafter, the role of these proteins in the tumour genesis.

The CGH procedure has its limitations when it comes to sensitivity as well as to resolution. Detection of extra material is only possible when the product of copy number and amplicon size is 100 to 200 kb; deletions have to be at least 5 to 20 Mb (circa 2 to 7 ‘bands’) in size to be detectable with this approach. Depending on the amount of amplification, visual inspection of CGH images will give clues or - when the changes are more subtle - quantitative fluorescence with digital image analysis of (a number of) metaphases will be necessary before a conclusion can be drawn.

Since CGH software packages are now commercially available, this approach has rapidly been accepted as a tool in cancer diagnostics and oncology research. As a result, many new data are obtained for quite a number of tumour types, allowing correlation of histo- and cytopathological features with specific molecular changes that occur during development of the cancer, or are its initiator.

THREE RECENT APPROACHES IN FISH

Tyramide signal amplification of FISH signals

For the cytochemical localization of a number of different cellular components, reliable cytochemical methods are available which deposit as an endproduct for a light microscopical detection, well-localized, chromogenic or fluorescing endproducts. Especially some of the cytochemical **enzyme** procedures have a high specificity in combination with a low background (Fig. 9).

A drawback of most cytochemical enzyme procedures, however, is the fact that the end-products are not fluorescing, since the detection sensitivity for chromogenic deposits is fundamentally smaller than that of fluorescent reaction products.

With the recent introduction of a method that had been developed for the amplification of a cytochemical horse radish peroxidase (HRP) procedure, a combination of the advantages of cytochemical enzyme methods and fluorescence visualization could be obtained. This method can also be used to enhance fluorescent signals resulting with FISH procedures.

In 1989 Bobrow and co-workers had developed an amplification procedure for a cytochemical peroxidase method that can result in a fluorescent endproduct. In this approach, tyramine (labelled with a fluorochrome or a hapten), is employed as one of the substrates. The enzyme mediates a reaction between hydrogen peroxide and the phenolic part of tyramine, to produce a quinone-like structure bearing a radical on the C2 group. This ‘activated’ tyramide is able to react covalently with tyrosine residues which are in close vicinity of the HRP, resulting in the production of a deposit of labelled tyramides in the direct surrounding of the enzyme molecule.

Adams (1992) was the first to apply this amplification in immunocytochemistry and then, in 1995, both Kersten *et al.* and Raap *et al.* used this principle to enhance ISH signals and obtained remarkable increases in FISH signal intensities. Especially after the detection protocols had been modified (van Gijlswijk *et al.*, 1996B), the approach combines the sharp localization aspects (with low background) of cytochemical HRP methods, with the fact that the procedure results in a fluorescent endproduct, which increases the detection sensitivity considerably.

Recently research was performed to investigate whether it really will be possible to detect small unique DNA sequences and mRNA targets which, because of their low abundance, so far could not be visualized with 'conventional' procedures (van de Corput *et al.*, 1998A, 1998B). Generally speaking the results are, that tyramide signal amplification in its present form, when performed with care, can result in a strong enhancement of weak signals which may be advantageous for diagnostic application in pathology.

Van de Corput *et al.* (1998A & 1998B) applied peroxidase-labelled 40 mer oligodeoxynucleotides (HRP-ODNs), specific for the human cytomegalovirus immediate early gene (HCMV-IE).

When hybridized to metaphase chromosomes of rat 9G cells (which carry an integrated tandem repeat of 50-60 copies of that gene) these could be visualized "with ease". For RNA FISH, the application of synthetic ODNs as probes proved to be advantageous because of their single-strandedness. The fact that only limited numbers of haptens or fluorochromes can be coupled to ODNs was a drawback, because it decreases the detection capabilities. When HRP is introduced in the ODN, many hapten- or fluorochrome-labelled tyramides can be deposited at he hybridization site (van Gijlswijk *et al.*, 1996A). By use of the tyramide signal amplification (TSA) method, the RNA production after cycloheximide initiation of the HCMV-IE gene in rat 9G cells, resulted in an optimal visualization with strong, well-localized fluorescent signals. The conclusions of these studies are, that the HRP-ODN/TSA approach provides a methodological basis for low-abundance mRNA detection by FISH (Fig. 10).

Polypeptide nucleic acid probes

Another recent development that can enhance results of FISH procedures, is the introduction of labelled polypeptide nucleic acid (PNA) oligomers as FISH probes.

These DNA analogues which possess a (2-aminoethyl)-glycine backbone instead of the deoxyribose-phosphate, can readily form stable duplexes with complementary DNA targets. The rapid formation kinetics and the high thermal stability of PNA-DNA duplexes are thought to be the consequence of the fact that there is no electrostatic repulsion between the DNA-phosphate groups and the neutral peptide backbone of the PNA oligomers (Corey, 1997).

Lansdorp *et al.* (1996) were able to visualize all 96 telomeres in human metaphases using fluorescein- or Cy-3 labelled PNA probes ([C3TA2]3PNA), a result that so far had not been achieved with telomeric DNA probes.

It is expected that the high hybridization efficiency of this PNA probe will contribute to a better FISH stoichiometry, providing a reliable basis for determining the (relative) lengths of telomeres on basis of digital fluorescence intensity measurements (Lansdorp *et al.*, 1996).

The approach is also interesting for other repeat sequences such as centromere-specific alphoid DNAs, simple satellite DNAs and Alu-repeats. It is too early yet, to speculate about the potentialities of this kind of probes for the detection of low level repeat and unique nucleic acid sequences.

Padlock probe methodology

Padlock probes can also be mentioned as an example of recent innovations in FISH methodology. They have been introduced by Nilsson *et al.* (1994) to be applied for the *in situ* detection of single nucleotide differences in metaphase chromosomes.

A padlock probe is a linear oligonucleotide with two target-complementary sequences and a non-complementary linking segment in between (Fig. 14). The non-target spacer of about 40 nucleotides long, is carrying hapten groups like biotin or digoxigenin for detection and visualization purposes.

The target-complementary sequences of about 20 nucleotides, are chosen in such a way that upon correct hybridization, they become juxtaposed. After the hybridization, a DNA ligase will be used to close the padlock probe circle enzymatically. Due to the helix nature of double-stranded DNA, it will encircle the target strand and resist strong denaturing procedures like very stringent washing.

Using *a priori* information regarding a genetic polymorphism or deletion to be investigated, the diagnostic nucleotide is incorporated at the end of one of the probe sequences. When the diagnostic base does not fit the target, there will remain a

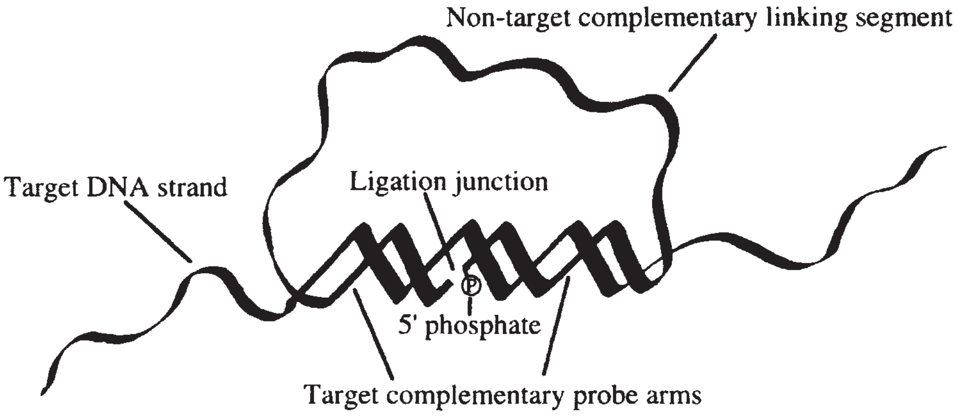


Fig. 14 - Padlock probe. See text and Nilsson (1994) for details.
(by kind permission of M. Nilsson)

loose end where the two probe parts were supposed to meet in the hybrid. The ability to recognize single nucleotide polymorphisms is based upon the fact that the ligase will not be able to ‘thread’ the two probe parts in the hybrid together when, due to a mismatch at the diagnostic site, such a loose end exists in the center of the hybridization complex.

The advantages of this approach are several. Due to the strict requirement of coincident hybridization to the two parts of the target sequence, there will be obtained a higher specificity. Also the formation of a circular molecule (as a result of the correct hybridization and the ligation afterwards) allows for extreme stringent conditions in the washing procedure - loose ends of any aspecific or mismatched hybridization will then be washed away. The methodology so far has been used successfully on repeat sequences in Southern (1975) blots and to investigate the presence and distribution of two closely similar alpha satellite repeat sequences present in the centromeres of human chromosomes 13 and 21. The two sequences differ at two nucleotide positions T38 and C101 in one of the repeats and C38 and G101 in the other. Nilsson *et al.* used two padlock probes to analyse the distribution of the diagnostic nucleotide variants T38 and C38 respectively (1997). In that article the authors also claim to have obtained results sensitive enough to detect single-copy gene sequences in complex genomic DNA.

They further suggested a ‘rolling circle’ amplification principle (by a phage polymerase) to enhance the sensitivity. Recently Amersham Pharmacia Biotec obtained the rights to develop this ampification mechanism.

In conclusion, padlock probes seem to offer a unique possibility to detect DNA sequences with high specificity and to distinguish among sequence variants *in situ* when *a priori* information is available. Single nucleotide differences can be monitored in order to follow the segregation of chromosomes over generations and to probe the structures of centromeres.

EPILOGUE

The opening sentence of this contribution has been taken from a article in which Albert Claude (amongst others) looks back upon his scientific activities.

Another sentence of that publication, describes the position in which Claude found himself in the nineteen thirties, where he states: *“biologists were in the same situation as astronomers and astrophysicists, who were permitted to see the objects of their interest, but not to touch them; the cell was as distant from us as the stars and galaxies were from them”.*

Since then, astronomers, astrophysicists and all of us, have witnessed quite some developments, which changed the limits of our macroscopical and microscopical outlooks.

As has been tried to illustrate here above, for us, cytochemists, the epi-illumination fluorescence microscope, the introduction of confocal laser scanning microscopy, and other progress in the science and technology of microscopy and digital imaging, in combination with the lustrous development of molecular tools, really enabled us to enter the cell and its nucleus in three spatial dimensions. A wonderful world in which, again and again, highly sophisticated structures, molecular processes and functions are being discovered.

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