

From Ideogram to Spectrogram

Solomon F.D. Paul, Vijayalakshmi J., Koshy T., Kaur H., Venkateswaran N. and Venkatachalam P.

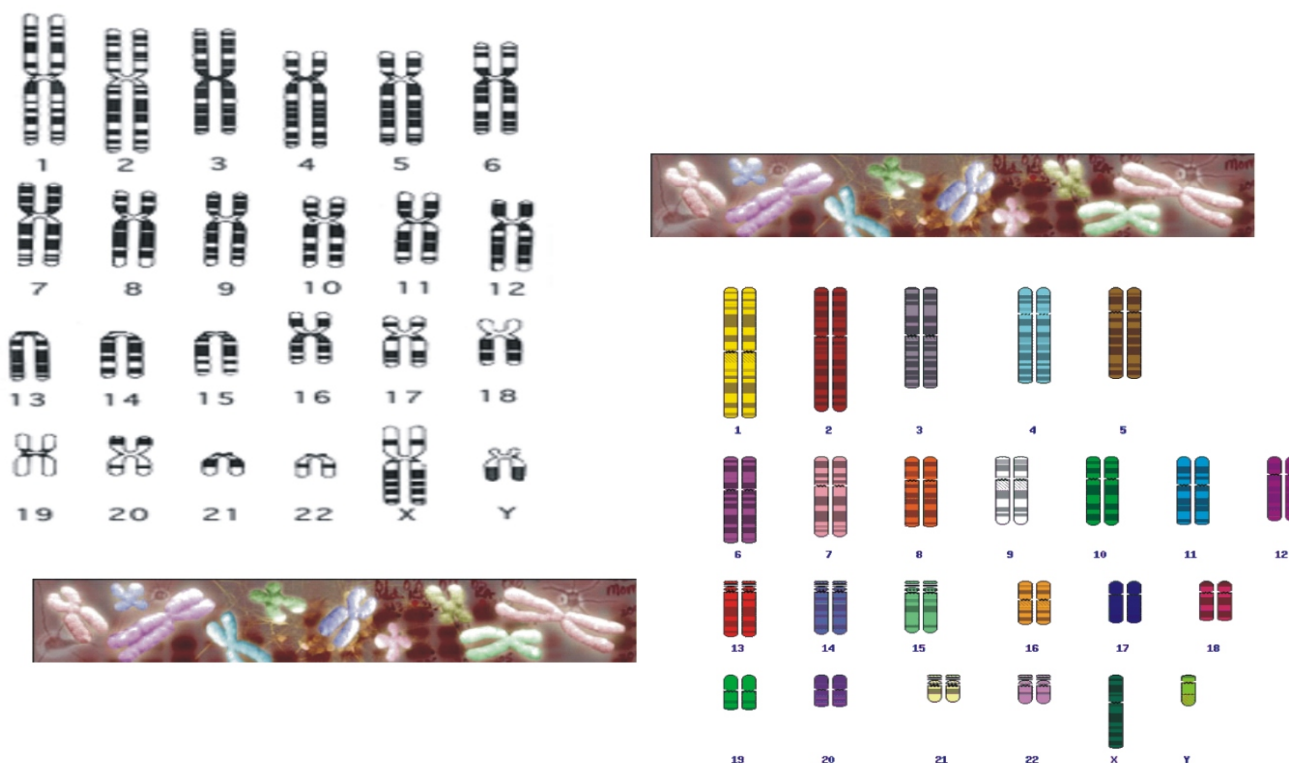
The beginning of human cytogenetics is five decades old, ever since the exact number of human chromosomes in somatic cells was identified as 46 by Tjio and Levan (1956). History extends beyond this and research pertaining to chromosomes has been ongoing for over a century (Waldeyer, 1890). Over the past five decades, the role that chromosomes have played in human diseases and developmental disorders has become a primary concern of many biologists and clinicians. With the advent of various staining methodologies in the early 1970s, a unique sub-specialty of genetics called clinical

cytogenetics emerged. To date, voluminous literature exists on the role of genetics in human diseases aiding in the identification of several new syndromes.

The important role of cytogenetic techniques as a biomarker for mutagenic insult to the cell to various chemical and physical agent which causes random and unpredictable pattern of aberration stressed the need for automation. In the 1980s molecular biologist, coupled with the cytogenetist and programmers, resulted in the emergence of a new discipline, molecular cytogenetics. The combination of chromosomal analysis, automated

karyotyping image analyzers, classifiers and neural network has transformed the karyotype to colorful spectrum of chromosomes. Patenting of novel ideas associated with the molecular cytogenetics and potential market for the probes built competition between companies. This yielded new novel methods with modified probe chemistry, filter chemistry, methodology and application. Further to this, the support from the fast developing image analyzer has completely transformed the black and white ideogram to more meaningful spectrogram (Fig. 1).

From IDEOGRAM To SPECTROGRAM



A basic understanding of the molecular cytogenetic techniques has been discussed here.

Physical DNA mapping technique based on complimentary base pairing in homologous sequences compared to non-homologous sequences is the main principle behind in situ hybridization (**ISH**) technique. The concept of probing the sequences on DNA or RNA by isotopic in situ hybridization (**IISH**) has now become outdated. Of late the fluorescent-labeled non-isotopic in situ hybridization (**NIISH**) has gained a lot of importance in identifying different chromosomes, telomeric and centomeric specific sequences and chromosome bands using the unique sequence found on them. This helps in rapid scoring of numerical and structural aberrations on the chromosomes. The application of FISH has gained a lot of importance in almost all the areas of cytogenetic investigation viz. cancer genetics (Philadelphia Chromosomes) and interphase cytogenetics or FISH (study the chromosomal defects on interphase cells, **IFISH**). Of late several single gene disorders, microdeletion syndromes etc. have been diagnosed at chromosomal level using FISH. FISH has paved the way for accurate estimation of absorbed dose in case of occupational exposure to radiation (biodosimetry) and genotoxic agents. An array of FISH applications in Preimplantation genetics (PGD) and prenatal diagnosis (PND) has enabled geneticists to give better counseling to couples.

Using the primary color (Green, Red and Blue) one can generate more than 24 colors using ratio labeling of fluorochromes during probe construction. Simultaneous viewing of all these colors can be effectively obtained using suitable filter combinations and software attached to the image analysis system. Multi FISH (**M-FISH**), Spectral Karyotyping (**SKY FISH**), Combined Binary Ratio Labeling (**COBRA FISH**) techniques were discovered to achieve this goal. Similar to the GTG banding, colorful banding patterns can be obtained by multiple banding (**mBand** or **Bar coding**) techniques. This increases the accuracy of detecting minute anomalies. Reverse Chromosome Painting

(**RCP**) technique allows us to identify and map small fragments like double minutes. Whole chromosome painting (**WCP**) enables us to identify reciprocal translocations quite effectively. Comparative Genomic Hybridization (**CGH**) allows one to study gene expression at chromosome level. Cross Species Hybridization (**Rx FISH**) and Genomic in situ Hybridization (**GISH**) facilitates homology study between different species. Quantitative FISH (**Q-FISH**) is currently being used in identification of DNA repeat sequences found on chromosomes. Extended FISH or Fiber FISH (**F-FISH**) performed on extended DNA fibers for mapping purposes, is also gaining importance.

From the concept of FISH various techniques like automated DNA sequencing, real time PCR and DNA chip technology other wise known as micro array have evolved. These techniques have proved their important role in diagnosis and research. They have enriched the existing techniques and new novel methods of detections have been developed for today's hungry science. They have proved their worth in the diagnosis of new OMIM disorders.

Fluorescence In Situ Hybridisation (FISH)

A set of DNA probes derived from a single chromosome type can be used to delineate an entire region of that chromosome by in situ hybridization. The direct visualization of specific chromosomes by hybridized fluorescent Whole Chromosome Probes has led to the term "Chromosome Painting" where the whole chromosome specific probes are referred to as paints. The advent of Competitive In Situ Suppression hybridization (**CISSH**) allows removal of ubiquitous repeat sequences from Whole Chromosome Probes before their use.

Probes are available in different sizes and complexity. They are chromosome specific and labelled with different colours. By using fluorescent-labeled DNA probes one can selectively paint a chromosome while the non-painted chromosomes are stained with a different color using suitable counter stains. If

the fluorescent-labeled chromosome had undergone any changes a bi-colored chromosome shows up. This helps in rapid scoring of large number of numerical and structural chromosomal aberrations. Of late several single gene disorders have been effectively diagnosed. Genotoxicity and biological dosimetry studies have effectively used whole chromosome probes (WCP) to evaluate the dose concentrations.

FISH has evolved a lot within a short period of time, each technique having advantages over the others in terms of sensitivity and application. This drastic development was due to the increasing competition between companies providing fluorochromes, filters, probes, CCD cameras, hardware and software. The various FISH techniques available for genetic analysis of Interphase cells, Chromosomes and DNA have been discussed below.

1. Whole Chromosome Probes (WCP)

Fluorescence In-Situ Hybridisation (FISH) has made significant contributions to Clinical Cytogenetics. Up to the late 1990's there were just three types of fluorochromes available, namely TRITC, FITC and AMCA having emission at the red, green or blue regions of the visible spectrum. Using chromosome-specific sequences labeled with each of these fluorochromes, up to three chromosomes can be painted and visualized simultaneously using multiple band pass filters. However, they fail to allow identification of derivative chromosomes (markers), small translocations, insertions or microdeletions from unstained chromosomes. The limitations of conventional methods are the number of fluorochromes available and quality of the preparations. This poses difficulty in cytogenetic studies of leukemia and other malignancies.

2. Interphase cytogenetics - Interphase FISH

The long time period required by conventional Cytogenetics, poor growth of cells in culture, poor quality spreads and need for rapidity in screening defects has always spurred on the development of new and better

cytogenetic assays. The advent of FISH has given rise to a new era of interphase cytogenetics where single gene mutations, translocations and numerical abnormalities can now be effectively studied using the interphase chromatin.

3. Primed *in situ* Labeling (PRINS), Self Primed *in situ* Labeling (SPRINS) and Multicolour primed *in situ* Labeling (MPIL or Multi PRINS)

The PRINS technique represents a relatively new increasingly popular approach to the detection of specific DNA or RNA sequence *in situ*. The method consists of annealing oligonucleotides to complementary sequences on fixed chromosomes, followed by a DNA polymerase driven extension in the presence of labeled deoxynucleotides. Multi PRINS combines speed with multiple probe analysis. (Volip and Baldini, 1993).

4. Multicolor FISH or M-FISH

One important draw back of WCP is that only a partial genome can be analyzed due to limitations in the available filters and fluorochromes. The obvious next step is to paint all chromosomes, each pair in a different color. This would detect any type of rearrangement, particularly translocations, within the resolution of the technique (1Mb).

Each chromosome "paint" could be a unique combination of the three pre-existing fluorochromes. M-FISH is a gradual capture of the image, though a set of specific filters, where the final image is built up as the sum of the individual images. The images obtained in this way are then captured with a CCD camera system and processed by computer software which can distinguish the difference in color not discernible by the naked eye. This would create pseudo-color images.

5. Spectral Karyotyping (SKY)

Spectral Karyotyping or SKY-FISH is a novel molecular cytogenetic technique, which simultaneously scans and displays each chromosome in a different color, permitting unequivocal discrimination of the entire chromosome complement (Schrock *et al.*, 1996). A 24-colour FISH system (using different labeled chromosome painting

probes) is visualized through a spectral interferogram cube. This serves to measure the entire emission spectrum with a single exposure at all image points. To aid in visual discrimination of the image, an artificial color could be assigned to each homologue. This approach is known as Spectral Karyotyping. SKY has made possible the identification of chromosomal material of unknown origin, complex rearrangements such as those seen in cancer cell lines and subtle translocations not detected on metaphase chromosomes.

6. Combined Binary Ratio Labelling (COBRA) FISH

Multicolour *in situ* hybridisation (mFISH) is increasingly applied for karyotyping and detection of chromosomal abnormalities. The next variation is a 27-colour analysis system using a combinatorial approach. The introduction of Combined Binary Ratio Labelling (COBRA) FISH by Speicher *et al.*, (1998) enables one to generate several colours using limited dyes. The application of the same can be extended in generating more colours in animals/plants having more number of chromosomes. i.e., the mBand FISH or Bar coded FISH.

7. Micro FISH or Micro-dissection FISH or Reverse Chromosome Painting (RCP)

This procedure is invaluable in the detection of "marker chromosomes." Region-specific PCR products incorporating suitable reporter molecules (probes) are used to amplify and tag regions from a single micro-dissected chromosome. This is then 'painted' back onto normal metaphase spreads so that the chromosomal region of the micro-dissected DNA can be identified.

8. Bar-Coding or mBand FISH

The mBand FISH technique evolved after the advent of the laser dissection microscope. Muller *et al.*, (1998) reported another new innovation in FISH for individual chromosome identification. They described a method where human chromosomes if color-banded would allow discrimination of each chromosome in a single FISH reaction with a set of sub-regional DNA probes. Alu/PCR

products of various human/rodent somatic cell hybrids (fragment hybrids) were pooled into two probe sets that were labeled differently and detected by red and green fluorescence. Chromosomes regions hybridized by DNA present in both pools appeared yellow. The result was a multicolor set of 110 distinct signals per haploid chromosome set for the human karyotype. Each individual chromosome showed a unique sequence of signals, termed as the "Chromosome bar code". This method allows rapid identification of chromosomes and chromosomes rearrangement. It is widely used in diagnostic cytogenetics and genome studies.

9. Rx-FISH or Cross Species Hybridization

Yet another approach to the individual classification of each of the 24 chromosomes is Rx-FISH, in which chromosome-specific probes developed for one species of primate are hybridized to the chromosomes of another species (Humans). The Banded chromosome pattern achieved by translocations and inversions reflects on the evolutionary relationship between both species. Rx-FISH is at present a research tool and is used alongside G-band analysis as a complementary approach. It is useful in identifying cryptic rearrangements common in leukemia, lymphoma, and myelodysplastic syndromes. It is, however, expensive and requires special software, and is therefore not used widely.

10. Quantitative FISH (QFISH)

These are method by which the number of repeats on the telomeric region of chromosome is quantified by suitable sensors fitted on the image analyzers. In humans, the telomeres consist of a large number (on average, 2000) of $(T_2AG_3)_n$ repeats. Since DNA polymerase that replicates DNA cannot copy chromosomes all the way to very end, the number of telomere repeats decreases with the number of cell divisions and with age. Alternative Lengthening of Telomeres (ALT) has proved to play an important role in cancer research. An accurate, efficient way to measure telomere length will be best studied

the brightness of the FISH spot - the longer the telomere sequence, the brighter the signal.

11. Genomic *in situ* hybridisation (GISH)

Genomic *in situ* hybridisation (GISH) is used to verify inter-specific hybrids or introgressions within various plant and animal families. This technique allows discrimination between chromosomes originating from different species. GISH can also be used to study meiosis of somatic and back cross hybrids.

12. CGH - Comparative Genomic Hybridisation

CGH is a method to detect global loss or gain of material from the whole genome. It involves the extraction and labeling of DNA from reference and tumor cells with different fluorochromes. Both the reference and tumor DNA is then hybridized to normal human metaphase chromosomes. The relative intensities of fluorochromes along the length of target chromosomes are compared. Alterations are visualized as color shifts i.e., gene amplification or reduction (Kallioniemi *et al.*, 1994). This method has been the fore runner for the development of the CGH microarray technology.

13. Extended Chromatin Fiber FISH (ECF-FISH)

FISH with metaphase chromosomes or interphase nuclei cannot reveal small changes like micro-deletion syndromes. In ECF-FISH the chromatin is released from the cell by

detergent lysis, the DNA is allowed to run down a slide and hybridisation is carried out as usual. ECF- FISH permits mapping in the order of 5-700 Kb using metaphase chromosomes.

14. Microarray Technology - Gene Chips

DNA microarray, technology promises to monitor the whole genome on a single chip so that researchers can have a better picture of the interactions among thousands of genes simultaneously. An array is an orderly arrangement of samples. It provides a medium for matching known and unknown DNA samples based on base-pairing rules and automating the process of identifying the unknowns. An array experiment can make use of common assay systems such as microplates or standard blotting membranes, and can be created by hand or make use of robotics to deposit the sample.

There are two major application for the DNA microarray technology: Identification of sequence (gene / gene mutation); and Determination of expression level (abundance) of genes. This technology is having a significant impact on genomics study. Many fields, including drug discovery and toxicological research, will certainly benefit from the use of DNA microarray technology.

15. Real Time Quantitative PCR

Quantitative RT-PCR is an important step for the validation of expression data generated by microarray analysis and other genomic techniques. This has been facilitated by the

development of instruments that measure the amount of PCR product produced during each PCR cycle (ie, in real time) as opposed to the endpoint detection. The real-time PCR system is based on the detection and quantitation of a fluorescent reporter.

This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during the 3-15 cycles indicates the detection of accumulated PCR product.

Editors Note: As this article encompasses a complete spectrum of an important technique, a second part as a continuation to this part-1 will be featured in the next issue of Advanced Biotech.

About the Authors

**Solomon F.D. Paul,
Vijayalakshmi J.,
Koshy T., Kaur H.,
Venkateswaran N. &
Venkatachalam P.**
Department of Human Genetics,
Sri Ramachandra University,
Porur, Chennai 600 116 (TN). India.
Email: wise_soly@yahoo.com

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