Cytometry and Genetics

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FLOW CYTOGENETICS

Flow cytometric analyses of individual chromosomes were made possible by the development of techniques for isolating intact chromosomes (1) and by procedures for staining chromosomes with DNA-specific fluorescent dyes. The term flow cytogenetics (2) was coined in the early 1980s to describe the application of flow cytometry and sorting to chromosome classification and purification. Two publications that appeared in 1975 described high-resolution measurements of single mammalian chromosomes in a flow cytometer (3,4) and illustrated the use of sorting for chromosome purification (5). Although single-color measurements were an exciting development and a portent of what was to come, the development of two-color staining provided the resolution needed to separate most of the human chromosomes. The introduction of Hoechst and chromomycin staining of mammalian chromosomes in 1979 (5) provided the breakthrough that allowed flow cytogenetics to achieve the successes described below. The ability to distinguish among individual chromosome types provided the first opportunities to distinguish and purify them in quantities suitable for visual, chemical, and molecular analyses. Initial sorting studies allowed chromosome types to be associated with flow karyotype peaks and provided chromosomes in a form that could be used for multiple purposes.

DNA PARADOX

An early contribution, now being referred to as the DNA paradox, illustrated the fact that DNA content does not necessarily track with chromosome number (6). That is, the number of chromosomes in a cell can vary widely from cell to cell (HeLa cells), whereas the intercellular DNA content remains the same. When the resolution of cells in G1 phase of the life cycle (G1 peak coefficient of variation) was compared across a large number of cell strains and cell lines, mammalian cells with a large variation in their chromosome number (numerical and structural aberrations) had the same DNA content and same G1 resolution as did cells with a euploid or normal number of chromosomes. This finding demonstrated that a cell could become highly aneuploid and retain normal DNA content and that the variation in DNA content between cells does not increase, although the chromosome number varies significantly. This finding and the vast amount of cytogenetic literature provided incentives to develop accurate, statistically precise chromosome classification methods using the capabilities of flow cytometry. However, at that time, it was not obvious that suitable single-chromosome suspensions could be prepared and, if so, whether flow cytometers had the needed sensitivity.

CHROMOSOME-SPECIFIC LIBRARIES

Early discussions about DNA sequencing the entire human genome were considered credible in large part due to the ability to flow sort, with high purity, each of the human chromosomes. High-purity sorting made it possible to clone and produce chromosome-specific libraries suitable for sequencing. The first chromosome-specific library was produced for the X chromosome (7). Shortly thereafter, the Department of Energy through its Office of Health and Environmental Research provided initial funding (1983) for what became the National Laboratory Gene Library Project at the Los Alamos and Lawrence Livermore National Laboratories. The success of the project required the development of numerous procedures, e.g., efficient chromosome isolation; high-purity sorting, throughput, and recovery, sorting procedures compatible with cloning requirements, and library characterization. Initially small-insert libraries such as Charon 21A were constructed and distributed to the international community. Later, large insert libraries such as Charon 40 were produced, as were many others including YAC and BAC libraries. In the end, libraries were generated for all human chromosomes.

The availability of flow-sorted chromosome-specific libraries to the international scientific community rapidly facilitated the construction of physical maps of each human chromosome. These maps were particularly valuable in early stages of the Human Genome Project. Numerous references will lead the reader to complete and detailed information on this topic (8–11).

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Contract grant sponsor: U.S. Department of Energy, Office of Health and Environmental Research; Contract grant sponsor: National Institutes of Health; Contract grant number: RR01315; Contract grant sponsor: Wellcome Trust; Contract grant sponsor: NIH/NCI Bay Area Breast Cancer Translational Research Program (SPORE); Contract grant number: CA-58207.

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Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/cyto.a.20008
DISCOVERY OF THE TELOMERE SEQUENCE
The ability to sort human chromosomes with high purity and hybridize genomic sequences to those chromosomes validated the identification of a highly conserved, functional, repetitive DNA sequence \((TTAGGG)_n\), present at the telomeres of mammalian chromosomes (12). Quantitative slot blot analysis using flow-sorted human chromosomes was used to demonstrate that similar amounts of this repetitive DNA sequence is present on each chromosome, regardless of absolute chromosome length. This sequence pattern of hybridization was shown to be in contrast to what is observed with other tandem repetitive DNA sequences that are localized to distinct chromosomes or interspersed repeat families such as \(Alu\) sequences. Subsequent studies have led to an understanding of the importance of how this repetitive sequence at the end of each chromosome arm is maintained or eroded with the processes of aging and tumorigenicity.

KARYOTYPE INSTABILITY AND TUMORIGENESIS
High-resolution flow karyotype analysis has been used to analyze the progressive stages of karyotype instability that occur as a population of cells spontaneously progresses from normal to euploid to aneuploid and then to neoplastic (13). The ability to detect and analyze early karyotype changes has been shown to be critical for understanding the neoplastic process from a cytogenetic standpoint. In a similar fashion, marker chromosomes have been readily identified using flow karyotype analysis (14). The differential identification of true marker chromosomes from the extreme heterogeneity of chromosome types in tumor cells is critical for the field of clinical cytogenetics because true marker chromosomes replicate and expand with a clonal population, as opposed to the large numbers of rearranged chromosomes that are heterogeneous in structure and DNA content (15).

FLUORESCENCE IN SITU HYBRIDIZATION
Flow cytogenetic analyses were limited by the need to prepare isolated chromosomes. This was possible only for living cells that could be cultured and blocked in mitosis. As a consequence, cells from most normal and disease tissues could not be analyzed. This limitation was removed in part by the development of fluorescence in situ hybridization (FISH) (16,17). In this approach, specific DNA segments of interphase nuclei and mammalian chromosomes were “stained” by hybridizing to them, labeled, complementary DNA fragments. These applications required suitable specific probes. The first generally useful chromosome-specific probes that produced signals sufficiently intense for interphase analyses were homologous to repeated sequences uniquely found at the chromosome centromeres (18). The use of chemical amplification strategies resulted in bright hybridization signals that could be scored accurately in interphase nuclei from cultured cells and normal and diseased tissues (19). These probes are now used in assays approved by the U.S. Food and Drug Administration to detect aneuploidies associated with bladder and other cancers (20). At this same time, work was progressing on the use of FISH for visualization and chromosomal localization of unique DNA sequences (21,22) This technology was critical in assembling the physical maps needed early in the Human Genome Project (23,24).

CHROMOSOME PAINTING AND REVERSE PAINTING
Flow-sorted chromosome-specific DNA libraries constructed by the National Laboratory Gene Library Project found an arguably wider ranging use in the form of chromosome paints (19,25–27). The libraries, fluorescently labeled and hybridized to metaphase spreads, allowed the whole chromosome corresponding to the original sorted chromosome to be directly visualized. Key to this application was the inclusion of unlabeled, repeat-rich DNA sequences to block the hybridization of labeled interspersed repeated sequences (19). When hybridized to the chromosomes of patients, the paints allowed chromosome rearrangements to be analyzed in detail. The first widely distributed libraries were prepared by subcloning the Charon 21A libraries into plasmids to facilitate preparation of painting probes (28). Subsequently, methods based on polymerase chain reaction of chromosome amplification were developed (29), which allowed chromosome paints to be generated rapidly from only a small number of sorted or microdissected chromosomes (30). This in turn led to the development of reverse chromosome painting, where the paint used was constructed from a patient’s rearranged chromosomes and hybridized back onto normal metaphase spreads (31). The pattern of hybridization on the normal chromosomes identifies not only the composition of the aberrant chromosome but also the position of any chromosome breakpoints. More recently, reverse chromosome painting has benefited from the application to DNA microarrays, where much higher resolution analyses of aberrant chromosomes can be obtained (32). The power of chromosome painting has been extended by the application of advanced fluorescence labeling and imaging techniques. By combining five or more fluorochromes in a combinatorial approach, 24 different human chromosome paints could be hybridized to the same metaphase spread to identify each chromosome type in a unique color. Analysis of images produced with these complete painting sets could be achieved by using conventional fluorescence microscopy (33) or interferometry (34). This technology has allowed automated karyotype analysis and has been further applied to the chromosome analysis of other species.

The power of FISH was further increased by development of libraries of large-insert probes (coming from genome programs) that produced bright hybridization signals. Initial probes were made from YACs, cosmids, and collections of phage and plasmids. However, BACs eventually became the currency of mammalian genome-sequencing programs. The inserts in these probes are sufficiently large to produce intense hybridization signals and are now being applied in numerous clinical studies to
allow detection of changes in genome copy number and structure. Some of the earliest clinical applications demonstrated detection of ERBB2 amplification in breast cancer (35) and the presence of the t(9;22) translocation in chronic myelogenous leukemia (36).

FISH using whole chromosomes and regional probes also allowed investigation of the genomic organization of the interphase nucleus for the first time. Early studies demonstrated that chromosomes are organized into discrete domains (19,27). These studies eventually led to detailed analyses of chromosomal structures and nuclear function (37,38) and to insights into the details of DNA packaging (39).

KARYOTYPE EVOLUTION VIA CROSS-SPECIES PAINTING

Although the chromosome painting described above has allowed the analysis of chromosome rearrangements in patients and experimental animals, the field of karyotype evolution has been revolutionized by this methodology. Although different mammals display very different karyotype organizations in the size and shape of their chromosomes, large blocks of DNA retain considerable sequence homology across species. With some minor experimental modification, chromosome paints from one species can be used to highlight homologous blocks of DNA in a second species and vice versa (40,41). Reciprocal cross-species painting experiments have allowed the major chromosome rearrangements that have occurred during speciation to be directly visualized and the karyotype of common ancestral karyotypes to be inferred (42).

COMPARATIVE GENOMIC HYBRIDIZATION

Another advance in molecular cytogenetics came from the discovery that changes in genome copy number could be mapped onto representations of the normal genome using FISH (35). This technology, called comparative genomic hybridization (CGH), proved to be particularly useful for analysis of genomic aberrations in solid tumors, which before CGH had been difficult to analyze because of the remarkable genome complexity. Initial CGH analyses were carried out by hybridizing differentially labeled tests (e.g., green fluorescing tumor DNA) and references (e.g., red fluorescing normal DNA) and excess unlabeled repeat-rich DNA to normal metaphase chromosomes. Rates of hybridization were concentration dependent so that regions of increased and decreased copy number in the tumor appeared as regions of altered relative fluorescence (e.g., altered red:green fluorescence ratios) on the normal metaphase chromosome targets. This approach has been applied to thousands of tumor samples and provided a clear picture of their remarkable genomic complexity and regions of recurrent abnormality. These data are clearly summarized in an on-line database by Knuttila et al. (43). More recently, metaphase chromosomes have been replaced by arrays of BACs or other cloned probes as the representation onto which aberrations are mapped (44,45).

A MULTITUDE OF CONTRIBUTIONS

These examples are but a small collection of how cytometry has contributed to the field of genetics. Additional applications stretch from the genetic analysis of plant chromosomes (46,47) to interspecies genetic comparisons (48), gene mapping (49), gene library construction (12), and cytogenetic analysis of patients with difficult-to-diagnose neoplastic diseases (50,51). Collectively these contributions from laboratories around the world have had a highly significant impact on the discipline of genetics.

LITERATURE CITED


