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AUTHOR(S): L. Scott Cram, John Fawcett, Larry Deaven

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Los Alamos National Laboratory
Los Alamos, New Mexico 87545



FLOW CYTOGENETICS: FUNDAMENTALS AND NEW DEVELOPMENTS

**L. Scott Cram, J. Fawcett, and L. L. Deaven
Life Sciences Division
Center for Human Genome Studies
University of California, Los Alamos National Laboratory
Los Alamos, NM 87545**

I. INTRODUCTION

The ability to rapidly and quantitatively analyze and separate single chromosomes with a flow cytometer has made a significant impact on several biomedical research disciplines. The analysis and sorting of single specific chromosomes in a flow cytometer is called flow cytogenetics. One area where flow cytogenetics has served an enabling role is the human genome program. To reduce the complexity of a genome, chromosome sorting offers the advantage of sorting individual chromosomes for the cloning and mapping of separate distinct components. By dividing up a cell's genetic material into the 24 different types of chromosomes and producing chromosome specific libraries from these sorted populations, the task of mapping the human genome has been greatly simplified.

The advantage of chromosome specific libraries is even more significant now that yeast artificial chromosome (YAC) libraries containing large genomic inserts are being constructed from flow sorted chromosomes (McCormick, 1992). These libraries have an average insert size of 200 kb and are extremely valuable for identifying and constructing contiguous elements derived from smaller cosmid libraries. In addition to sorting, the ability to rapidly and quantitatively analyze thousands of chromosomes per second has proven to be an important complement to cytogenetic analysis. The throughput, high sensitivity, and quantitative aspects of flow cytogenetic analysis has made it possible to process of large numbers of samples for cytogenetic analysis.

Opportunities for further developments and new applications in this field are numerous and will emerge from the challenges involved in overcoming current limitations. For example, intracellular chromosome identity is lost in the present chromosome isolation process. Solutions to this problem have been proposed

(Cram, 1985) and one implemented (Poletaev, 1991). Improved resolution of individual chromosome types, new probes with better specificity, and a basic understanding of the binding properties and characteristics of presently used probes would expand both clinical and research applications of flow cytogenetics.

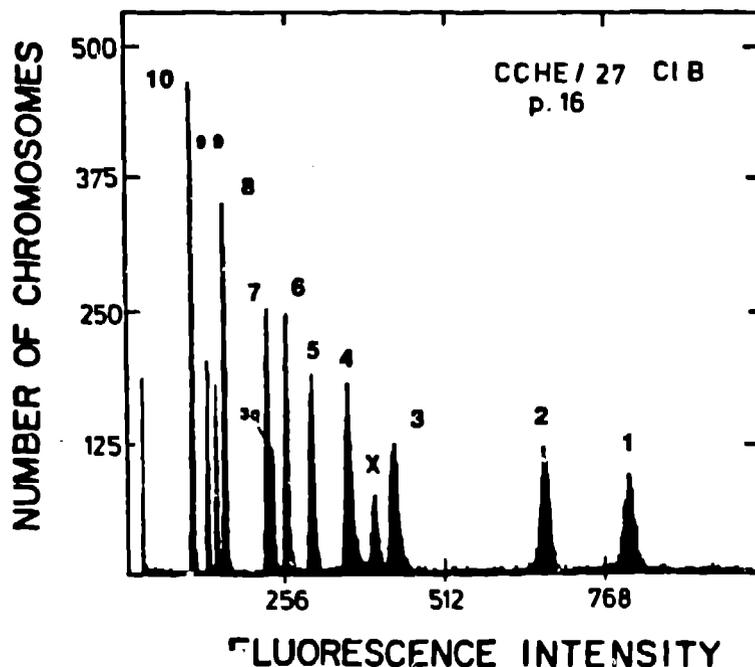
The purpose of this brief chapter is to provide 1) an introduction to flow cytogenetics and 2) resource information for additional and more detailed information for those interested in either using or further developing the technology. Several recent reviews are available and provide in-depth information on all aspects of flow cytogenetics, this information will by and large, not be repeated here (Gray, 1989c, Melamed, 1990, Darzynkiewicz, 1990). Examples of common types of flow cytogenetic data are included along with an explanation of their interpretation. In addition, recent developments are discussed as examples of what one can anticipate for the future. Further developmental needs are discussed in hopes of stimulating new ideas.

The field continues to expand as evidenced by the development of new protocols designed to meet the requirements of new cloning vectors, new chromosome labeling protocols, and chromosomes from new species are being analyzed, identified, and sorted.

II. CURRENT TECHNOLOGIES

Univariate Analysis. Remarkable resolution of distinct chromosome types can be achieved with univariate (single parameter) analysis. For some applications the use of a single "DNA specific" fluorochrome resolves most if not all chromosome types. For species such as Chinese hamster, propidium iodide stained chromosomes can be completely resolved into twelve populations, one peak in the flow karyotype for each chromosome type. Figure 1 illustrates typical results obtained with a commercial flow cytometer. Procedures used for obtaining these results are available from several references; cell culture (Lozes, 1989), chromosome isolation and staining (Cram, 1990a, Cram, 1990b, Trask, 1989, Trask 1990), sample handling (Gray, 1989c, van den Engh, 1985), and instrument modifications and alignment (Bartholdi, 1987a, Gray, 1989a).

Figure 1. Chromosomes isolated from cloned Chinese hamster embryo cell sat passage 16 were stained with propidium iodide and analyzed on a flow cytometer. Each peak is identified by its chromosome assignment.



Each peak in figure 1 is a single chromosome type. Chinese hamster chromosome 1 contains the most DNA and is therefore the brightest and is in the most fluorescent peak to the far right. Peak area is proportional to the number of chromosomes of that type and peak position is proportional to DNA content. In the distribution shown, the integrated number of events in chromosome peaks 1, 2, 3, and 4, for example, are the same because the cell line is near diploid (two homologues of each chromosome type). The two number 9 chromosomes (the two homologues) have a slightly different DNA content, one homologue having been inherited from each parent (Gray, 1989b). Peak height and peak width changes across the distribution as the result of the data recording technique. There is no biological significance associated with this change in shape.

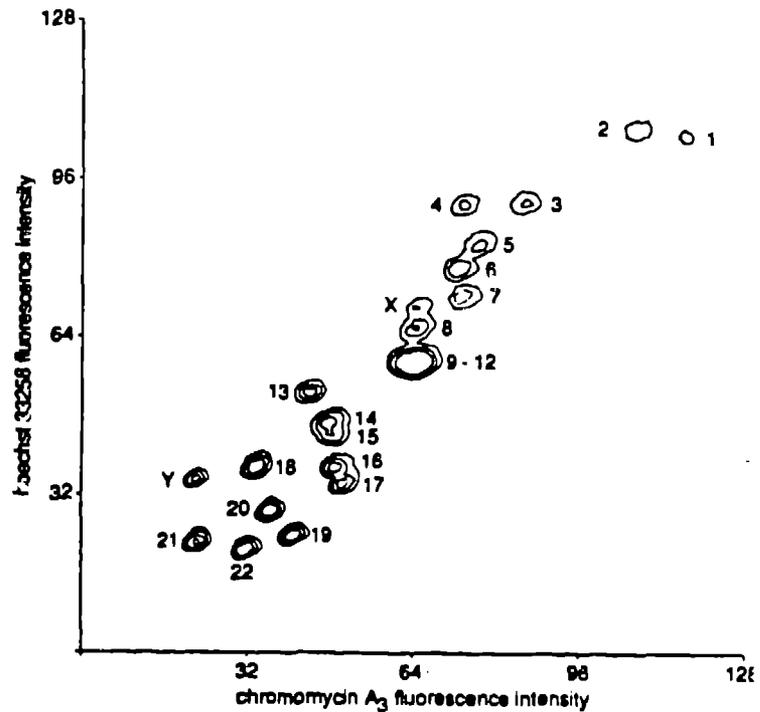
Numerical chromosome aberrations (trisomy for example) are reflected in peak area and structural aberrations (non-reciprocal translocations, deletions, etc.) can result in a variety of changes in the flow karyotype. Any net change in DNA content by more than a couple of percent will in principle, result in a new peak. In the case of a marker chromosome which ends up with the same DNA content as another chromosome, a new peak would not appear. In the initial stages of karyotype instability only a fraction of the cells will typically contain a marker

chromosome. A flow karyotype in which only a fraction of the cells contain a marker chromosome (chromosomes with a net gain or loss of DNA) will exhibit a new peak with an area proportional to the number of cells containing the marker. A new peak will also be present with an increase or loss of fluorescence in proportion to the marker chromosomes gain or loss of DNA.

Univariate flow karyotypes have been used for the analysis of chromosomes isolated from a broad range of species and cell types including fibroblasts, peripheral lymphocytes, cultured tumor cells, and primary cells (Lozes, 1989). Applications have included the analysis of karyotype instability associated with spontaneous neoplastic progression (Bartholdi, 1987b), detection of chromosome polymorphisms (Ray, 1984), analysis of radiation damage to chromosomes (Aten, 1989), and the detection of random chromosome changes as a measure of genetic change (Green, 1989). Chromosome sorting from univariate flow karyotypes has also been used for identifying peaks, gene mapping and for all the applications described below.

Bivariate Analysis. The addition of a second fluorochrome with different binding specificity adds an independent variable and results in increased resolution. Bivariate or two fluorochrome staining of human chromosomes, and to a lesser degree other species as well, greatly enhances the resolving power of flow cytogenetics. Figure 2 illustrates the resolution of normal human chromosomes stained with Hoechst 33258 (Ho258) and Chromomycin A3 (CA3). Ho258 preferentially binds to adenine-thymine (AT) rich regions of DNA and CA3 preferentially binds to guanine-cytosine (GC) rich regions (Langlois, 1989). The amount of binding of each fluorochrome is measured for each chromosome as the chromosome is sequentially excited by two laser beams, the first tuned to excite Ho258 (ultraviolet) and the second to excite CA3 (457 nm). The correlated value from each of the two signals is plotted as a bivariate distribution. The number of accumulated events is recorded as an isocontour where a contour represents events of equal number (Figure 2). The interpretation of numerical and structural changes is analogous to the situation with univariate analysis except now the one dimensional peaks are two dimensional isocontours. Alternatively, the data could be displayed as three dimensional distributions with the number of events plotted on the Z-axis, the plane perpendicular to the plane of the paper.

Figure 2. Human chromosomes isolated from a normal, euploid tissue culture cells and stained with Hoechst 33258 and Chromomycin A3 and analyzed on an EPICS V flow cytometer.



Fluorescent debris and chromosome fragments combine to form a underlying continuum on top of which resides individual chromosome peaks. Improved buffers and isolation procedures have largely eliminated the relatively small amount of contamination due to these background counts. Bivariate distributions provide greatly improved resolution for human chromosomes but the advantages are less pronounced in other species. Some human chromosome types have very nearly the same (AT)/(GC) ratio (chromosomes 9,10,11,12) and fall within the same peak. A large variation in Hoechst binding alone separates chromosomes 13 through 17. Human heterogeneity and chromosome polymorphisms limit the clinical utility of bivariate chromosome analysis. For example, skewed peak shape can be the result of variation in homologue DNA content or indicative of a marker chromosome (Moore, 1989, Gray, 1989b).

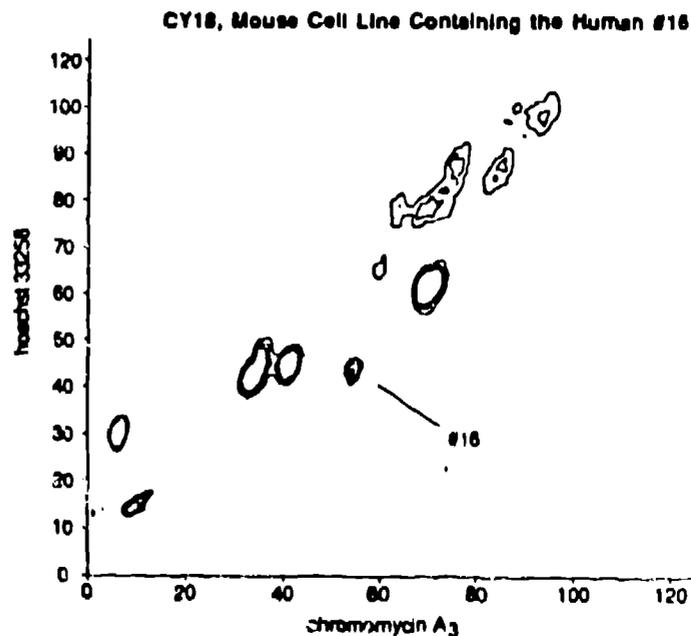
Chromosome Sorting. This is one of the few areas in which the sorting capabilities of flow cytometers are heavily exercised. Sorting is used to identify peaks by banding of the sorted material (Young, 1989), for construction of chromosome specific libraries (Van Dilla, 1989), and for gene mapping (Lebo, 1989). Sorting rates are a function of analysis rate and the fractional

representation of the population being sorted. Sorting a single human chromosome from a euploid cell line [22 (X,Y)] consists of sorting one chromosome out of every 24 chromosomes. Typical chromosome analysis rates are 1,200 chromosomes per second, which corresponds to a sort rate of about 50 chromosomes per second. A useful benchmark for sorting is the recovery of one million chromosomes per day when sorting from a normal human cell line (Albright, 1991).

Chromosome sorting purity can be determined by parametric analysis of one-dimensional projections of the bivariate flow karyotype and/or by fluorescence in-situ hybridization. Hybridization procedures are preferred since sorted chromosomes are counted directly. The procedure is straightforward when analyzing chromosomes sorted from somatic cell hybrids (REFERENCE). Typical purity values range between 85 to 95% when sorting human chromosomes from somatic cell hybrids. Chromosome recovery, the number of chromosomes actually recovered versus the number of chromosomes sorted by the instrument, is typically 80% to 90%.

Figure 3 illustrates the resolution one can achieve using a somatic cell hybrid as a source of a single human chromosome. CY-18 is a mouse cell line containing a single human chromosome (REFERENCE). Chromosome 16 was sorted from this cell line with a purity of 96% as determined by fluorescence in-situ hybridization and 97% as determined by parametric data analysis of the bivariate data.

Figure 3. Bivariate flow karyotype of the CY-18 somatic cell containing the human 16 chromosome. Chromosomes were stained with Hoechst 33258 and Chromomycin A3 and analyzed on an EPICS V flow cytometer.



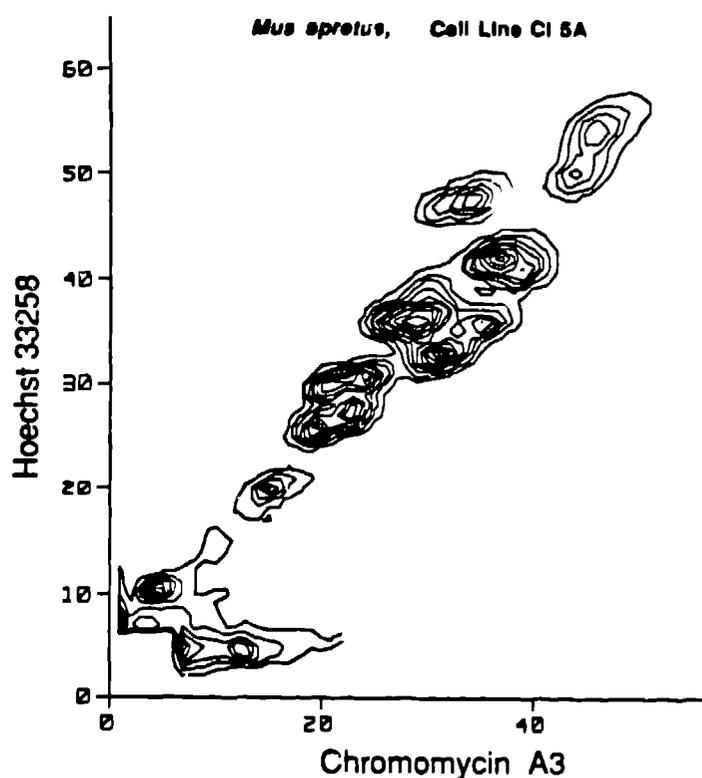
Sorting human chromosomes from somatic cell hybrids offers several advantages. The fractional representation of a human chromosome relative to Chinese hamster chromosomes is higher, a single homologue is sorted, and contamination in the final product is from a different species and can be easily distinguished from material of human origin. Somatic cell hybrids which contain intact, normal human chromosomes in a high percentage of cells (>90%) are required for most applications. Extensive screening of cell lines reported to contain single or a low number of human chromosomes is often required to find a suitable somatic cell hybrid for a particular chromosome.

III. NEW DEVELOPMENTS AND PROCEDURES

Additional species are being added to the list of those from which chromosomes have been isolated for flow cytometric analysis and sorting. Two recent examples are mouse and porcine; both species are of importance for different reasons. Mouse univariate flow karyotypes consist of five peaks which contain the 21 different types of chromosomes [19(X,Y)] (Cram, 1989). Recent bivariate analysis indicates many more peaks can be resolved and sorted, see Figure 4. Thirteen populations have been resolved and sorting for confirmation of peak identity is underway (Cram, 1992). Alternative approaches for resolving the mouse karyotype have been demonstrated using mouse cell lines with multiple Robertsonian translocations (Baron, 1984, Baron, 1986). Several groups are involved in porcine chromosome sorting for the Pig Gene Mapping Project (Haley, 1990). The pig karyotype consists of 38 chromosomes; 18 autosomal pairs, X and Y. Roughly 17 peaks plus the Y chromosome are resolved using Ho258 and CA3 for bivariate flow karyotype analysis. Several of the peaks have been identified and confirmed (Schmitz, 1991).

A new and improved procedure for labeling chromosomes in suspension with antibodies has recently been described. Levy and colleagues report the use of anti-kinetochore staining to improve the resolution of Indian muntjac chromosomes (Levy, 1991). Extension of this technique using antibodies specific for a limited number of chromosomes in combination with other fluorochrome stains will offer new approaches to resolving additional chromosomes and for rapid screening of specific aberrations.

Figure 4. Bivariate flow karyotype of chromosomes isolated from the *Mus spretus* cell line CL 5A. Positive identification of the individual peaks is in progress.



Prospects for flow cytogenetic research applications include a wide variety of requirements for sorted chromosomes not only extending current procedures but the sorting of break point regions and other specialized requirements. Clinical applications are presently limited to confirmatory work (McConnell, 1991), however with specific DNA probes and the means to accomplish in-situ hybridization on chromosomes in suspension, rapid rare event flow karyotype analysis would become a significant technology for clinical analysis - just as flow karyotype sorting has for the research community.

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