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High Resolution Chromosome Analysis: One and Two Parameter Flow Cytometry

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Abstract. Isolated mammalian chromosomes have been quantitatively classified by high resolution flow cytometry. Chinese hamster chromosomes stained with 33258 Hoechst and excited in the UV showed a fluorescence distribution in which the 14 types of Chinese hamster chromosomes were resolved into 16 groups seen as distinct peaks in the distributions. Chinese hamster chromosomes were also stained with both 33258 Hoechst (HO) and chromomycin A3 (CA3); the two dye contents were measured by selective excitation in the UV and at 458 nm in a dual beam flow cytometer. The resulting two parameter distribution (HO versus CA3) showed 10 chromosome groups[1]. Human strain LLL 761 chromosomes stained with HO and excited in the UV showed a fluorescence distribution in which the 23 types of human chromosomes were resolved into 12 groups. Human chromosomes stained with both HO and CA3 and measured in the dual beam flow cytometer produced two parameter fluorescence distributions which showed 20 groups. The chromosomes associated with each group were determined by quinacrine banding analysis of sorted chromosomes and by DNA cytophotometry of preidentified metaphase chromosomes. The relative HO and CA3 stain content and frequency of occurrence of chromosomes in each group were determined from the fluorescence distributions and compared to the results from DNA cytophotometry. The chromosome to chromosome variations in HO and CA3 staining are attributed to variations in chromosomal base composition.

Introduction

The quantitative classification and purification of isolated mammalian chromosomes is facilitated by the application of flow cytometry and sorting (Gray et al., 1975a; Stubblefield et al., 1975; Carrano et al., 1976; Otto et al., 1978; Cram

[1] A chromosome group is a unique chromosome class determined from the fluorescence distributions. It may be produced by one or more chromosomes as determined by banding analysis

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et al., 1978). In this approach, individual chromosomes are released from metaphase cells into an isolation buffer to which is added a DNA specific fluorochrome. The stained chromosomes are then forced to flow one-by-one through the illumination beam of a flow cytometer (Van Dilla et al., 1974; Gohde, 1970); the resulting fluorescence is measured and used to quantitatively classify each chromosome. Thousands of chromosomes can be measured each second so that a statistically precise fluorescence frequency distribution, representative of the total chromosome population, can be accumulated in a few minutes. Typically, these fluorescence distributions have several peaks superimposed on a low continuum. Each peak represents a distinct group of chromosomes (ideally one peak for each chromosome type); its mean is proportional to the chromosomal group fluorescence and its area is proportional to the frequency of occurrence of chromosomes within that group. The continuum is produced by chromosomal debris, clumps and other fluorescent cellular debris.

The information contained in a fluorescence distribution, namely chromosome group mean and frequency of occurrence, is similar to that obtained by conventional karyotyping so that the fluorescence distributions can be used as karyotypes (Gray et al., 1975b; Carrano et al., 1978b). These flow karyotypes are particularly sensitive to aberrations which occur homogeneously in all cells and which cause changes in chromosomal fluorescence (seen as a change in peak mean) or in chromosome loss or gain (seen as a change in peak area). Random aberrations which occur only in one or a few cells contribute to the continuum in the fluorescence distribution. The height of the continuum has been used as a semiquantitative estimate of the frequency of these heterogeneously ocurring aberrant chromosomes (Carrano et al., 1978a). A more direct approach to the estimation of the frequency of occurrence of certain classes of heterogeneous aberrant chromosomes like dicentrics, would be to measure the fluorescence profile of each flowing chromosome (Van Dilla et al., 1976). Initial experiments show that dicentric chromosomes can be recognized on the basis of their abnormal profiles and their frequency of occurrence can be recorded (Gray et al., 1979).

Flow sorting (Carrano et al., 1976) introduces an additional feature; chromosomes within each group can be collected into aliquots of unprecedented homogeneity. Sorting also has been used to determine the identity of chromosomes producing the peaks in the fluorescence distribution (Carrano et al., 1976, 1979) and to produce the material for biochemical investigations of the structure and function of individual chromosome types (Sawin et al., 1977, 1978).

The effectiveness of flow karyotyping, aberration detection and chromosome purification depends to a large degree on the number of groups into which the isolated chromosomes can be resolved. In this paper, we describe the use of the DNA specific dyes 33258 Hoechst (HO) and Chromomycin A3 (CA3) and new flow cytometric techniques which allow every chromosome from the Chinese hamster M3-1 line cell to be resolved and chromosomes from human cell strain LLL (Lawrence Livermore Laboratory) 761 to be resolved into 20 groups. The identification of the chromosomes in each group was made by banding analysis of sorted chromosomes. The implications of chromosome to chromosome differences in HO and CA3 staining are also discussed.

Materials and Methods

Chromosome Preparation and Staining. Chromosomes were isolated from Chinese hamster M3-1 cell line and human foreskin strain LLL 761 using a modification of the procedure of Wray and Stubblefield (1970) which have reported previously (Carrano et al., 1979). Briefly cells growing in roller cultures were arrested in mitosis by treatment with colcemid (0.32 μg/ml for strain LLL 761 and 0.032 μg/ml for the M3-1 cell line) for a time equal to about one half the cell cycle duration. The mitotic cells were selectively removed by rapid rotation of the roller bottles, resuspended in hypotonic KCl (0.075 M), and then resuspended in a Tris buffered isolation medium (25 mM Tris-HCl, pH 7.5, 0.75 M hexylene glycol, 0.5 mM $CaCl_2$, and 1 mM $MgCl_2$) at about 5×10^6 cells/ml. The cells were mechanically ruptured in a Virtis "45" homogenizer to release the chromosomes into the isolation buffer.

The DNA specific dyes HO and CA3 were added, independently or together, to about 10^8 chromosomes in 1 ml isolation buffer; final concentrations of HO and CA3 were 2 μg/ml and 80 μg/ml respectively except for the HO stained M3-1 line chromosomes where the concentration was 16 μg/ml. Both dyes increase in quantum efficiency upon binding to DNA so that the chromosomal fluorescence could be measured while the chromosomes remained in the dye solution. HO binds preferentially to AT rich DNA (Latt et al., 1975), is excited with high efficiency in the UV at 351 nm + 364 nm ($\varepsilon_{UV} = 3.3 \times 10^{+4}$) and minimally at 458 nm (ε_{458} 10^2), and emits maximally at 460 nm[2]. CA3 binds preferentially to GC rich DNA (Latt et al., 1977), is excited with high efficiency at 458 nm ($\varepsilon_{458} = 6.2 \times 10^3$), and minimally at 351 + 364 nm ($\varepsilon_{UV} = 7 \times 10^2$), and emits maximally at 585 nm. HO and CA3 when used together to stain DNA, exhibit efficient energy transfer from HO to CA3 and little competitive binding (Langlois and Jensen, 1978).

Flow Cytometry. A newly developed high precision, dual beam flow cytometer (Dean and Pinkel, 1978) was used for the chromosome measurements reported herein. The machine operation is illustrated schematically in Figure 1. During operation, chromosomes are forced to flow one-by-one across a water filled measuring region where they are illuminated sequentially with two laser beams. The two fluorescence emissions resulting from the chromosome stream-laser beam intersection points are collected by a f = 0.95 lens and independently projected onto separate photomultipliers (EMI9798B).

For these experiments the system was equipped with two high power argon ion lasers (Spectra Physics Model 171). The laser producing the beam crossed first by the chromosomes was operated in the ultraviolet (351 nm + 364 nm) at 0.8 W to preferentially excite HO. The other laser was operated at 458 nm at 1.0 W to preferentially excite CA3. The fluorescence from the chromosome-UV beam intersection was projected through a filter (transmission greater than 50% above 450 nm) to exclude scattered UV light and then onto a photomultiplier which produced an electrical pulse proportional to the fluorescence intensity. The fluorescence from the chromosome-458 nm beam intersection point was projected through a Corning 3–71 filter (transmission greater than 50% above 520 nm) to exclude scattered 458 nm light and onto a second photomultiplier. The signals from both photomultipliers were integrated and amplified to produce voltage pulses proportional in amplitude to the incident fluorescence pulses. Each pulse from the UV beam crossing was delayed about 20 μ sec to bring it into temporal coincidence with the pulse from the 458 nm beam crossing; this was necessary because the chromosome transit time between beams was about 20 μ sec. Each signal of the pair produced for each chromosome was digitized into an integer between 0 and 65; this pair of integers was stored in the memory of a pulse height analyzer in a 64×64 array. The result of processing a large number of chromosomes (usually about 10^6) was a two parameter frequency distribution whose axes were fluorescence due to UV excitation, (X axis) and fluorescence due to 458 nm excitation (Y axis).

As shown later, to a first approximation the fluorescence due to UV excitation is proportional to the chromosomal HO content, and the fluorescence due to 458 nm excitation is proportional to the chromosomal CA3 content. These simple relations are not strictly accurate because of the effect of the molecular environment on fluorescence emission and energy transfer efficiency from HO to CA3. However, to simplify subsequent discussions we will refer to the fluorescence due to excitation in the UV as HO content and fluorescence due to excitation at 458 nm as CA3 content.

[2] ε = molar absorptivity of bound dye ($M^{-1}cm^{-1}$)

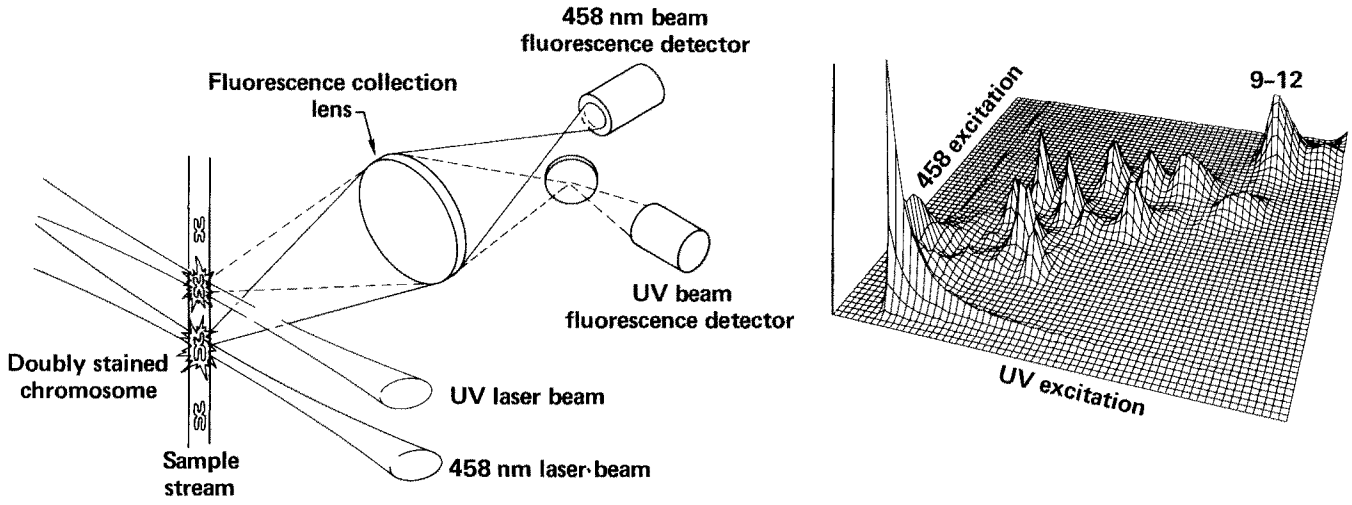

Fig. 1. A schematic representation of the dual beam flow cytometer. The chromosomes flow in single file sequentially through two laser beams. The fluorescence from each of the two laser beam-chromosome intersection points is collected by separate photomultipliers which convert the two fluorescent light flashes into electrical pulses proportional in amplitude to the fluorescence intensities. Each of the pair of pulses is subsequently processed, digitized and stored to form a two parameter frequency distribution. The frequency distribution shown is for HO and CA3 stained human chromosomes smaller than chromosome 8 and excited in the UV and at 458 nm

The dual beam flow cytometer was also used for single parameter chromosome measurements. During single parameter operation, one laser was used and operated either in the UV at 1.0 W for analysis of HO stained chromosomes or at 458 nm at 1.0 W for analysis of CA3 stained chromosomes. The electrical pulses resulting from the fluorescence pulses were digitized into integers between 0 and 513; integers for about 10^6 chromosomes were stored in the memory of the pulse height analyzer to form a one parameter frequency distribution.

Flow Sorting. The identification of the chromosomes associated with the peaks in the one parameter fluorescence distributions was accomplished by banding analysis of sorted chromosomes as previously described (Carrano et al., 1979). The LLL flow sorter (Van Dilla et al., 1974) was used for the chromosome sorting. The chromosomes responsible for the peaks in the one parameter fluorescence distribution were identified by sorting about 20,000 chromosomes from each peak. Each aliquot of sorted chromosomes was collected in a frozen pellet, fixed, centrifuged onto a glass slide and the chromosomes were banded with quinacrine dihydrochloride. As described by Carrano et al. (1978c), only about 10–15% of the sorted chromosomes appear banded. Chromosome assignments were made assuming that the banded chromosomes were representative of the entire population.

One parameter sorting was also used to aid in the identification of the peaks in the two parameter (HO versus CA3) fluorescence distributions since the dual laser flow cytometer lacked sorting capability. Chromosomes were sorted from several regions of the CA3 and HO one parameter distributions, banded with quinacrine and classified. Chromosomes were assigned to peaks on the basis of frequency of occurrence in the sort regions and peak area. In this way, the identity of the peaks in the two parameter distributions was inferred from the single parameter distributions.

Data Display and Analysis. Single parameter fluorescence distributions were analyzed as previously described (Gray et al., 1975a) to determine the mean fluorescence and frequency of occurrence of chromosomes of each group. Briefly, a least squares best-fit technique was used to fit the fluorescence distribution with the sum of several normal distributions plus a smooth function, f(N), representing the continuum produced by fluorescent debris. Here $f(N)=A+BN^{-C}$ where N is the distribution channel[3] number and A, B, and C are free parameters. Each peak was fitted by a single normal distribution whose mean characterized the fluorescence of the chromosomes in that peak, whose area approximated the frequency of occurrence of chromosomes in that peak, and whose coefficient of variation, CV, determined the measurement precision.

Two methods of depicting two parameter data are used in this paper, isometric and contour. The isometric plot gives the viewer a three-dimensional view of the data, the dimensions being HO content, CA3 content and number of chromosomes. The isometric presentation emphasizes the dynamic range of the distribution, i.e., the peak to peak variation in the number of chromosomes. However, it provides little quantitative information about peak means. The contour plots, whose axes are HO content and CA3 content, complement the isometric plots since they allow accurate estimates of peak means. In each contour plot we also show the projection of the two parameter data onto both the CA3 and the HO axes. These collapsed, one parameter plots are those that would be obtained from one-parameter measurements.

The analysis of the two parameter fluorescence distributions is less sophisticated than that for single parameter distributions since computer programs to fit the two parameter distributions have not yet been produced. For this paper, the bivariate mean of each peak (one value for HO fluorescence and one for CA3 fluorescence) was assumed to be the same as the peak mode (highest point in the peak). This method is not completely satisfactory for the 64×64 distributions since its accuracy is limited by the coarse grid size of the distributions. Thus, errors in peak locations as large as 10% may occur for weakly fluorescent chromosomes. The frequency of occurrence of chromosomes in each two parameter distribution group is proportional to peak volume. Each peak volume was estimated by summing the values of each channel[3] falling within a contour defining the base of the peak. The base of each peak was taken to be at 10% of the highest peak in the distribution so that within one distribution all peak defining contours were taken at the same "elevation".

[3] Each distribution element in a one or two parameter fluorescence distribution is referred to as a channel

For one or two parameter distributions, the peak areas or volumes are normalized to sum to 23 for Chinese hamster M3-1 line chromosomes and to 46 for human chromosomes. The peak means are normalized so that the sum of the peak means times the *expected* number of chromosomes of that group (an integer) equals to 100. This normalization expresses the fluorescence of each chromosome as a percent of the total chromosomal fluorescence of one cell.

A fractional difference (FD) test was used to quantify chromosome to chromosome differences in staining for any pair of stains. The net FD is defined to be

$$FD = \frac{2}{K} \times \sum_{i=1}^{K} [|S1_i - S2_i|/(S1_i + S2_i)]$$

where $S1_i$ and $S2_i$ are the normalized stain contents of the ith chromosome for stains 1 and 2 respectively and K is the total number of chromosomes. When applied to chromosome peak parameters normalized as described above, the FD values tend to zero as the normalized stain contents became equal for all chromosomes. Thus, two stains for which the FD value is small can be expected to have similar binding characteristics. In addition, since the FD values are normalized by the number of chromosomes per cell, they can be compared for different cell types. The biochemical heterogeneity of chromosomes for two cell types can thus be compared after staining with a pair of dyes like CA3 and HO with different binding characteristics. The cell type with the largest FD value is the most heterogeneous.

Scanning Cytophotometry. Chromosomal DNA content values were determined independently by scanning cytophotometry of metaphase spreads stained with gallocyanin chrome alum under conditions where the dye was specific for DNA (Mendelsohn et al., 1973; Mendelsohn and Mayall, 1974). The chromosomes in each spread were preidentified by quinacrine banding so that the DNA content of each chromosome would be determined. The DNA values were normalized so that the average DNA content values for all chromosome types sums to 100. All homolog pairs were examined statistically (Moore II, 1973), to detect significant ($p \leqq 0.01$) homolog differences in total DNA content and centromeric index.

Results

M3-1 Chromosomes

The single parameter fluorescence distribution in Figure 2 for HO stained Chinese hamster M3-1 chromosomes shows the 14 chromosome types resolved into 16 groups. The peak identification, means, areas and CVs are shown in Table 1. Figure 3a is a photomicrograph of banded chromosomes sorted from the region of the HO fluorescence distribution that we determined was produced by the number 1 chromosome. This photomicrograph is representative of those used to identify the chromosomes producing the other peaks in the distribution. Since the resolution during sorting was not as high as during flow cytometry the peaks N, O, P shown in Figure 2 could not be resolved during sorting. The identification of these peaks was made by assuming that the chromosome ranking by HO fluorescence was the same as the DNA ranking determined by scanning cytophotometry. Thus, chromosomes 10, 11, M2 had decreasing DNA contents and were assigned to peaks N, O, P respectively. Chromosome types 1, 7 and 9 were each resolved into two groups indicating homolog differences detected by flow cytometry. Homologs of chromosomes 7 and 9 were indistinguishable by banding analysis and by scanning cytophotometry. The average fluorescence CV for the 16 groups in the distribution was 1.8% making

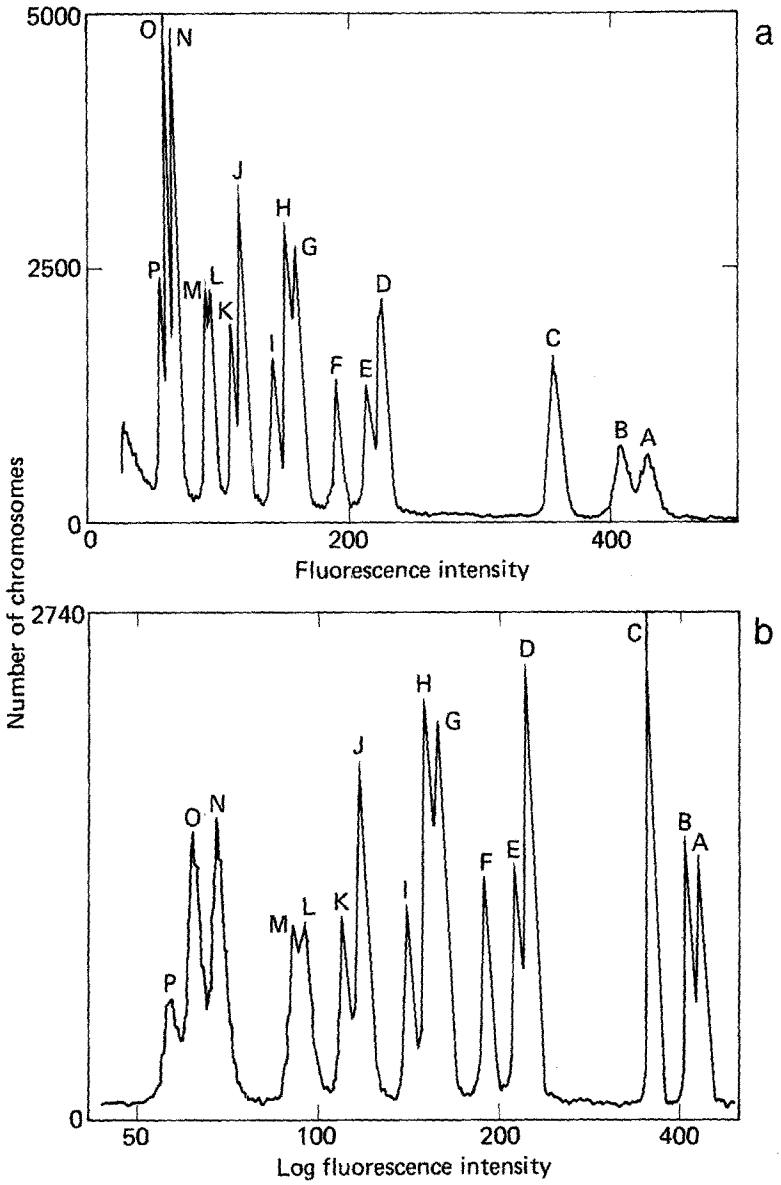

Fig. 2. a A one parameter frequency distribution for Chinese hamster M3-1 chromosomes stained with 16 μg/ml HO and excited in the UV. The frequency of chromosome occurrence is the ordinate and chromosomal fluorescence is the abcissa. **b** The same data as in a but with the logarithm of chromosome fluorescence as the abcissa. In this representation, chromosomes occurring with equal frequency and having the same fluorescence coefficient of variation will produce peaks of equal height.

Table 1. Peak identification and parameters determined from the single parameter HO fluorescence distribution (Fig. 2) for Chinese hamster M3-1 chromosomes

Peak	Chromosome ID	Peak parameters			Scanning cytophotometry DNA[b]
		Area[a]	Mean[b]	CV	
A	1	1.0	10.56	1.3	10.70
B	1	1.0	10.03	1.2	10.70
C	2,2	1.9	8.87	1.3	9.08
D	4,4	2.1	5.60	1.5	5.76
E	t(X, 5)	1.0	5.60	1.4	5.37
F	5	1.0	4.75	1.4	4.58
G	6,6	2.2	4.01	1.8	3.92
H	7,Y	2.0	3.80	1.6	3.57
I	7	1.0	3.48	1.7	3.57
J	8,8	2.1	2.96	1.9	2.99
K	M1	1.0	2.75	1.7	2.39
L	9	1.1	2.32	2.0	2.34
M	9	0.9	2.22	1.8	2.34
N[c]	10,10	2.1	1.69	2.3	1.54
O[c]	11,11[d]	2.0	1.58	2.3	1.49
P[c]	M2	0.8	1.37	2.5	1.30

[a] Normalized so that the sum equals 23

[b] Normalized so that the sum of the means times the expected number of chromosomes equals 100

[c] These assignments were made on the basis of the total DNA content determined by scanning cytophotometry since these groups were not resolved during sorting

[d] Homolog difference in centromeric index detected by scanning cytophotometry, $p \leqq 0.01$

this the most precise measurement of chromosomal fluorescence intensity ever achieved. The high resolution is probably the result of improvements in cytochemistry (HO is a highly DNA specific stain) and in flow cytometry (more intense illumination and more efficient light collection). The improved resolution also improves the estimates for the frequency of occurrence of chromosomes. For the 16 resolved groups, the frequencies of occurrence calculated from the distribution deviate from the expected frequencies, on average, by about 5%.

Table 1 also shows that the peak CVs increase with a decrease in chromosomal fluorescence. For example, the CV of peak A is 1.3% while that for peak P is 2.5%. This is shown more clearly in Figure 2b where the abcissa is log fluorescence intensity; in this representation, peaks of constant coefficient of variation and area should have equal height. The decrease in peak height for peaks A, B, E, F, I, K, L, M and P, each produced by a single chromosome and thus of equal area, illustrates the increase in coefficient of variation with decreasing fluorescence intensity. This increase probably is due to statistical fluctuations in the number of detected photons for each chromosome so that the square of the CV is inversely proportional to the fluorescence intensity plus an instrumental constant (Holm and Cram, 1973); as the fluorescence intensity decreases, the CV increases. In spite of the high numerical aperture collection optics and the intense chromosome excitation used in these measurements, the chromosome measurement precision is still limited by statistical fluctuations.

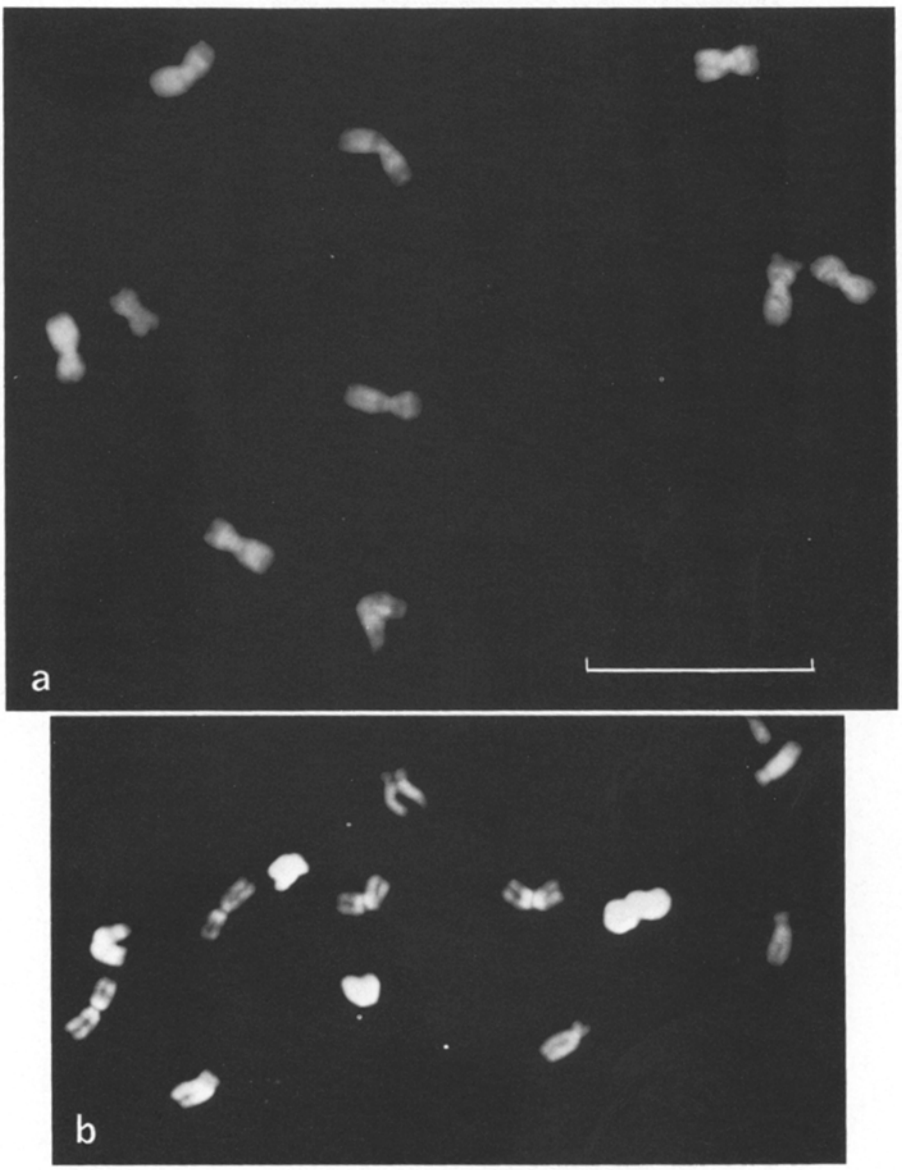

Fig. 3. **a** A representative photomicrograph of quinacrine banded Chinese hamster M3-1 chromosomes sorted from the region of the HO fluorescence distribution determined to be produced by the number one chromosome. **b** A representative photomicrograph of quinacrine banded human strain 761 chromosomes sorted from the region of the HO fluorescence distribution determined to be produced by the number 3 and 4 chromosomes. The bar represents 10 μm

The two parameter fluorescence distribution for Chinese hamster M3-1 chromosomes stained with both HO and CA3 and sequentially excited in the UV and at 458 nm is shown isometrically in Figure 4a and as a contour plot in Figure 4b. Single parameter views of the data are shown collapsed on the axes of Figures 4b. These figures show the M3-1 chromosomes resolved into

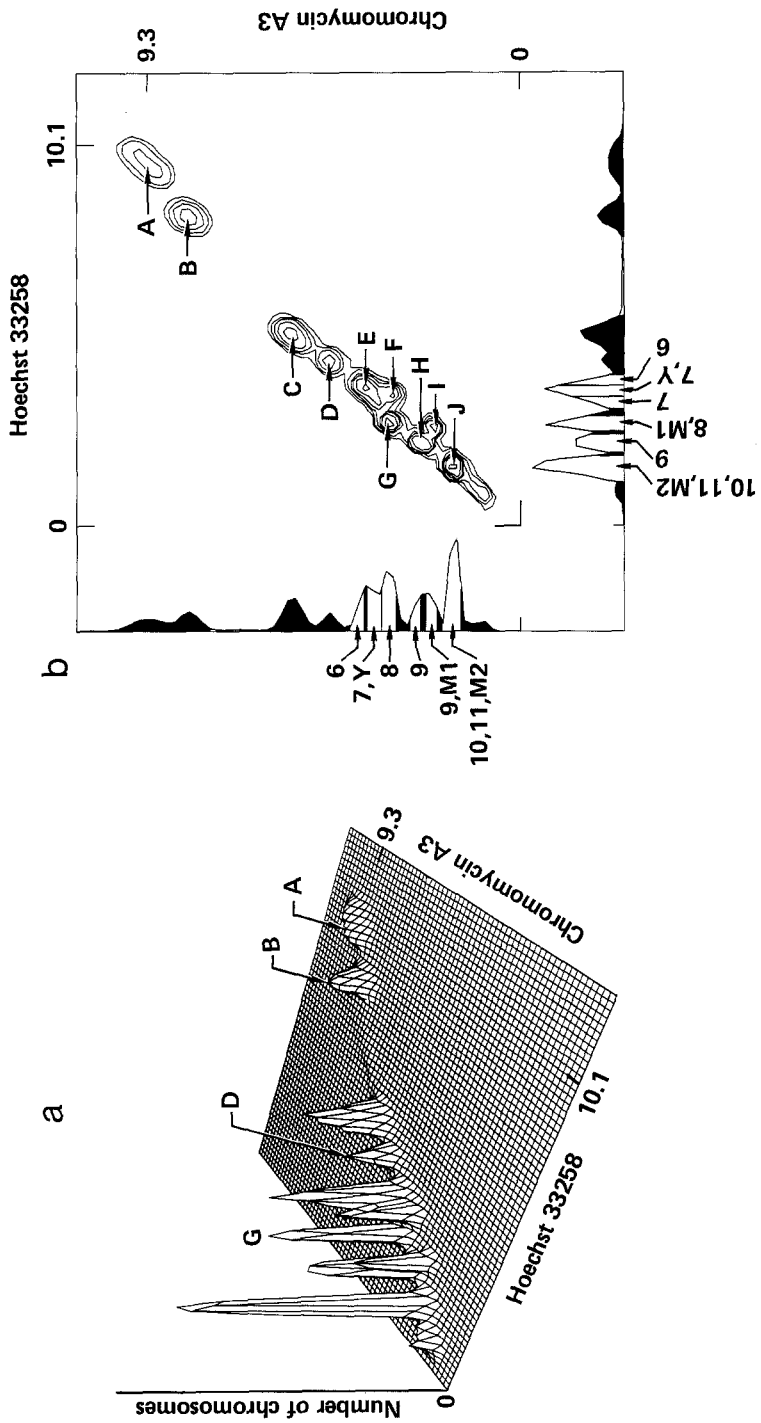

Fig. 4. a An isometric display of the two parameter frequency distribution of fluorescence from HO+CA3 stained Chinese hamster M3-1 chromosomes excited in the dual laser flow cytometer at 458 nm in the UV. **b** A contour display of the same two parameter data shown in a. Also shown are the one parameter distributions which result from collapsing the two parameter data onto the HO and CA3 axes. The assignments of chromosomes to the white regions of the one parameter distributions are indicated. The assignments of chromosomes to the peaks in the two parameter distribution were inferred from the one parameter assignments.

Table 2. Peak identification and parameters determined from the two parameter HO vs CA3 fluorescence distributions (Figure 4) for Chinese hamster M3-1 chromosomes

A	B	C	D	E	F	G	H
Peak	Chromosome ID	Peak volume[a]	Relative fluorescence values				Scanning cytophotometry DNA[b]
			HO[b]	CA3[b]	TDNA[b]	HO/CA3	
A	1	1.9	10.08	9.26	9.8	1.1	10.71
B	2	2.0	8.65	8.23	8.5	1.0	9.06
C	4, t(x,5)	3.1	5.40	5.83	5.6	0.9	5.62
D	5	1.1	4.62	4.80	4.7	1.0	4.69
E	6,7	4.1	3.93	3.95	3.9	1.0	3.73
F	Y	0.9	3.73	3.25	3.5	1.2	3.59
G	8	2.2	2.59	3.43	3.1	0.8	2.99
H	9	2.0	2.37	2.58	2.4	0.9	2.31
I	M1	0.9	2.85	2.23	2.6	1.31	2.39
J	10, 11[c], M2	4.8	1.77	1.88	0.9	1.5	1.48

[a] Normalized so that sum equals 23
[b] Normalized so that sum of the means times the expected number of chromosomes equals 100
[c] Homolog difference in Centromeric Index detected by scanning cytophotometry; $p \leqq 0.01$

10 distinct groups in two parameters but into only 8 groups by either CA3 or HO alone. The decrease in one parameter resolution relative to that shown in Figure 2 is due to the increased difficulty in instrumental adjustment for two parameter analysis and to the relatively few channels (64) into which the one parameter data can be distributed and also perhaps to stain concentration differences. Langlois et al. (manuscript in preparation) have shown that the one parameter distribution for the doubly stained chromosomes excited at 458 nm are almost identical to the one parameter distribution of CA3 stained chromosomes excited at 458 nm. Similarly, the doubly stained chromosomes excited in the UV yield a distribution almost identical to that of chromosomes stained with HO alone. Therefore, the chromosome assignments of the peaks in the two parameter distribution were inferred from the assignments of peaks in the one parameter distribution for singly stained chromosomes. The regions of the one parameter distributions from which chromosomes were sorted and identified are shown as white regions in Figure 4b. The chromosome assignments for the peaks not specifically identified in Figure 4b, were inferred from one parameter sorts of ethidium bromide (EB) stained chromosomes (Carrano et al., 1978b) since the CA3, HO, and EB distributions were similar in these regions. The identification. HO and CA3 contents and volumes of the 10 peaks are listed in Table 2. Also shown is the relative chromosomal DNA content determined by scanning cytophotometry.

It is clear from Figure 4 and Table 2 that there is some variability among chromosome types in the HO to CA3 fluorescence ratio. For example, the M1 chromosome (peak I) has a HO to CA3 ratio of 1.3 while chromosome 8 (peak G) has a HO to CA3 ratio of 0.8. This can be seen in Figure 4b where peak I lies below the diagonal defined by the various chromosome types and where peak G lies above the diagonal. This variation allow chromosome

types to be resolved in two parameters which can not be resolved in one parameter. In the case of M3-1 chromosomes, the two parameter analysis does not aid in the identification of more chromosome groups (actually fewer are resolved) since the fractional difference (FD = 0.07) between HO and CA3 is small for Chinese hamster M3-1 chromosomes. This can also be seen in Table 2 by examining correspondence between HO fluorescence and CA3 fluorescence.

Because of the AT and GC binding preferences of HO and CA3 respectively, the HO and CA3 coordinates for each peak were combined according to the relation TDNA = 0.6 HO + 0.4 CA3 to approximate total DNA content. We reason that if HO is proportional to AT content, CA3 fluorescence is proportional to GC content, and the AT fraction in mammalian cells is about 0.6; then 0.6 HO + 0.4 CA3 should approximate total DNA content. The FD values for HO, CA3 and TDNA compared to scanning cytophotometric measurements of total DNA content are 0.09, 0.11, and 0.07 respectively. These results suggest that, of the three, the TDNA values are the best approximations of total chromosomal DNA content; however, a more rigorous relation of HO and CA3 fluorescence to base composition will be presented in a subsequent paper (Langlois et al., in preparation).

The two parameter distributions seem to be particularly suited to the determination of the frequency occurrence (peak volume) of the various chromosome groups. As can be determined from Table 2, the average variation between the expected and the flow cytometrically estimated frequencies of occurrrence for the resolved groups is only 4 percent. This is about the same as the variation that is usual for one parameter analyses of comparable resolution (Gray et al., 1975).

Human Chromosomes

The one parameter fluorescence distribution for human cell strain 761 LL stained with HO and excited in the UV shows these chromosomes to be resolved into 12 groups (Fig. 5d). Another strain, LLL 592, has been resolved into 15 groups (Carrano et al., 1978b) by the same method. The one parameter fluorescence distribution for human strain LLL 761, chromosomes stained with CA3 and excited at 458 nm shows the chromosomes to be resolved into 9 groups (Fig. 5a). Unlike the single parameter distributions for Chinese hamster M3-1 chromosomes, the human HO and CA3 distributions are remarkably different suggesting large differences between HO and CA3 staining among the several chromosome types. Thus, two parameter analysis should allow an increased number of resolved groups in this case if HO and CA3 fluorescence can be measured independently; Figure 5 shows that this is approximately true. The one parameter fluorescence distribution for HO + CA3 stained chromosomes excited at 458 nm (Fig. 5b) is similar to the one parameter distribution of CA3 stained chromosomes excited at 458 nm (Fig. 5a). In addition, the one parameter fluorescence distribution for HO + CA3 stained chromosomes excited in the UV (Fig. 5c) is similar to, the one parameter distribution of HO stained chromosomes excited in the UV (Fig. 5d).

The two parameter distribution of HO + CA3 stained human chromosomes in Figure 6a (isometric) and Figure 6b (contour) shows the chromosomes

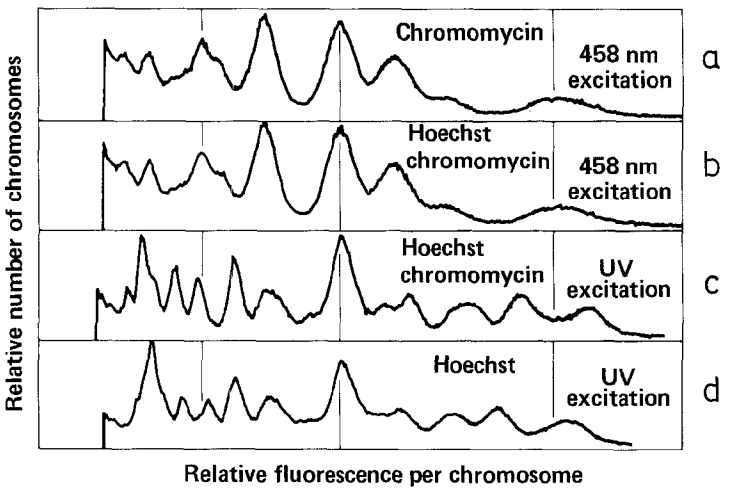

Fig. 5 a–d. Frequency distribution of fluorescence of **a** CA3 stained human strain 761 chromosomes excited at 458 nm, **b** CA3+HO stained human strain 761 chromosomes excited at 458 nm, **c** HO+CA3 stained human strain LLL 761 chromosomes excited in the UV, and **d** HO stained human LLL 761 chromosomes excited in the UV. Operating conditions were adjusted such that the mode of the peak due to the number 9–12 chromosomes fell in the same channel

resolved into 20 peaks, a substantial improvement over the single parameter analyses. This increased number of resolved peaks is related to the large fractional difference (FD=0.15) for HO and CA3 fluorescence. The large FD is reflected in the HO/CA3 ratios listed in Table 3, which range from 0.6 for chromosome no. 19 (peak N) to 1.5 for the Y chromosome (peak R).

The chromosome assignments of the peaks in the two parameter distribution were inferred from the chromosome assignments of the peaks in the single parameter CA3 and HO distributions. The single parameter assignments which were determined by banding analysis of sorted chromosomes are shown in Figure 6b. The regions of the single parameter distributions from which chromosomes were sorted are shown in white. Figure 3b is a photomicrograph of banded chromosome sorted from the region of the HO fluorescence distribution that we determined was produced by the number 3 and 4 chromosomes. This photomicrograph is representative of those used to identify the chromosomes producing the other peaks in the distribution.

Table 3, which summarizes the two parameter data, lists the peak identifications, volumes (frequencies of occurrence), HO, CA3, and TDNA fluorescence means. Thirteen chromosome types are uniquely resolved (numbers 1, 2, 3, 4, 8, 13, 16, 17, 18, 19, 20, 21, 22, Y) and differences between homologs are detected for chromosomes 13 and 22. As for the M3-1 analysis, the estimates of chromosome frequencies of occurrence are quite good. The average difference between expected and flow cytometrically determined frequencies of occurrence is about 8% compared to 11% for high resolution single parameter analysis (Carrano et al., 1978c).

The Fractional Differences between HO, CA3 and TDNA fluorescences and scanning cytophotometric total DNA contents are 0.08, 0.09 and 0.02

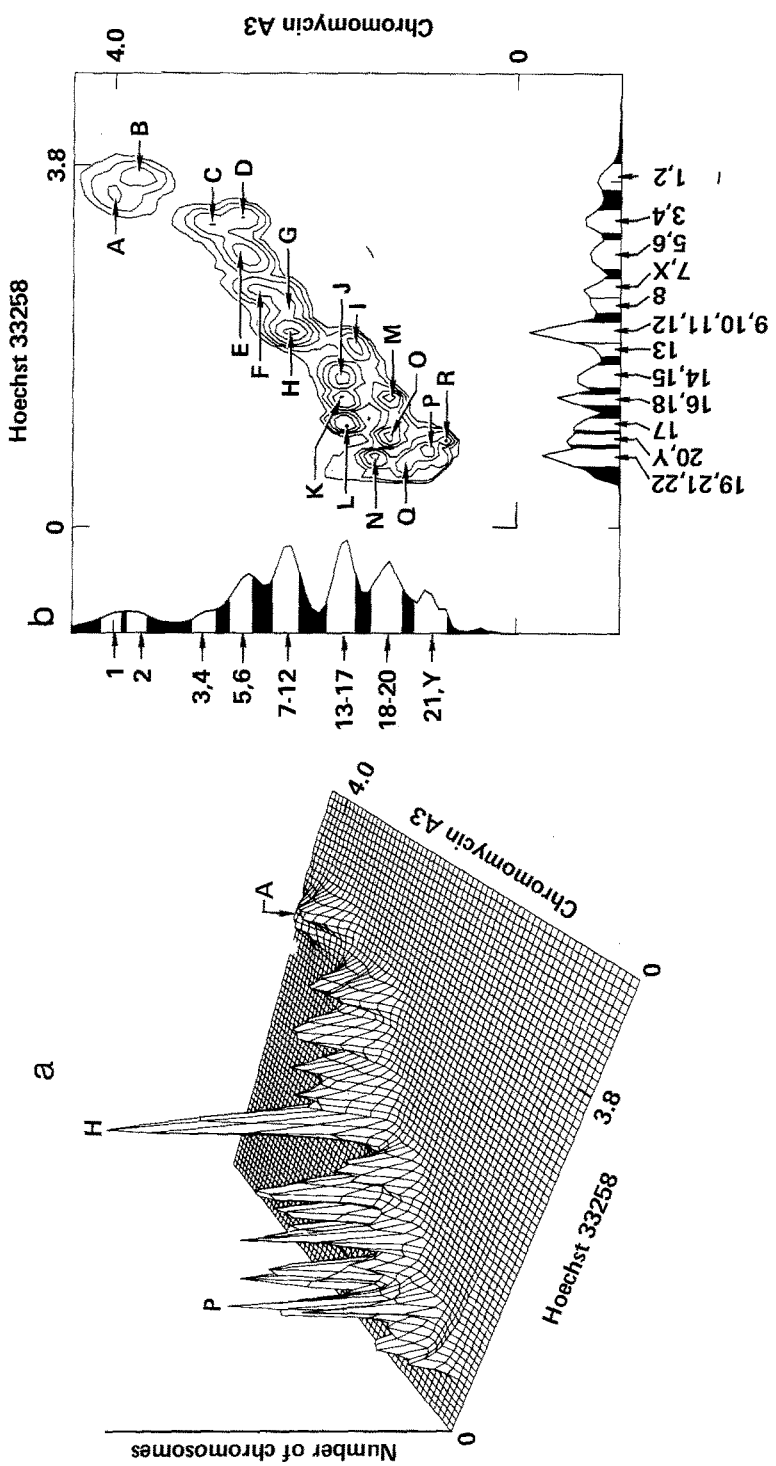

Fig. 6 a An isometric display of the two parameter frequency distribution of fluorescence from HO+CA3 stained human strain 761 chromosomes excited in the dual laser flow cytometer at 458 nm and in the UV. **b** A contour display of the same two parameter data shown in a. Also shown are the one parameter distributions which result from collapsing the two parameter data onto the HO and CA3 axes and can be compared to those in Figure 4b and c respectively. The assignment of chromosomes to the white regions in the one dimensional distributions are indicated. The assignments of chromosomes to the peaks in the two parameter distribution were inferred from the one parameter assignments

Table 3. Peak identification and parameters determined from the two parameter HO vs CA3 fluorescence distribution (Fig. 6) for human chromosomes

A	B	C	D	E	F	G	H
Peak	Chromosome ID	Peak volume[a]	Relative fluorescence				Scanning cytophotometry DNA[b]
			HO[b]	CA3[b]	TDNA[b]	HO/CA3	
A	1[c]	4.2	3.75	4.05	3.87	0.9	4.12
B	2		3.99	3.84	3.93	1.0	4.04
C	3	3.6	3.43	3.07	3.29	1.1	3.35
D	4		3.51	3.07	3.22	1.1	3.20
E	5,6	3.8	3.11	2.65	2.98	1.2	2.99
F	7,X	2.8	2.71	2.65	2.69	1.0	2.66
G	8	2.2	2.55	2.38	2.48	1.1	2.46
H	9–12	7.6	2.23	2.31	2.26	1.0	2.27
I	13[d]	1.8	2.07	1.66	1.90	1.2	1.80
J	14,15	4.1	1.70	1.76	1.72	1.0	1.71
K	16	1.8	1.45	1.76	1.58	0.8	1.59
L	17	1.7	1.20	1.76	1.42	0.7	1.41
M	18	2.3	1.49	1.30	1.41	1.2	1.34
N	19	2.0	0.78	1.43	1.04	0.6	1.03
O	20	2.4	1.04	1.30	1.14	0.8	1.13
P	21	2.0	0.87	0.88	0.87	1.0	0.80
Q	22[e]	2.5	0.74	1.11	0.89	0.7	0.86
R	Y	1.1	1.04	0.71	0.91	1.5	0.82

[a] Normalized so that the sum equals 46

[b] Normalized so that the sum of the means times the expected number of chromosomes equals 100

[c] Homolog difference in centromeric index detected by scanning cytophotometry; $p \leqq 0.01$

[d] Homolog difference in DNA content detected by scanning cytophotometry ($p \leqq 0.01$) and resolved into 2 groups in the 2 parameter distribution

[e] One homolog has a quinacrine bright satellite; homologs resolved into two groups in the two parameter distribution

respectively. In this case, the TDNA fluorescence values are excellent approximations to total DNA contents, with large differences only for chromosomes nos. 13, 18, 21, and Y.

Discussion

We have demonstrated substantial improvements in flow cytometric chromosome analysis following improvements in flow cytometry and chromosome cytochemistry. HO is a particularly good stain because of its 60 fold increase in quantum efficiency upon binding to DNA and because of its high DNA specificity. The use of HO as a DNA specific dye, plus intense dye excitation and efficient fluorescence collection during flow cytometry has allowed complete resolution of the 14 Chinese hamster M3-1 chromosomes classified by standard cytogenetic banding methods. Homolog differences between the chromosomes no. 1 were detected by C-banding, and by both flow cytometry and scanning

cytophotometry. Interestingly, differences detected between homologs for the chromosomes nos. 7 and 9 by flow cytometry could not be detected by quinacrine or Giemsa banding methods or scanning cytophotometry. HO staining also allowed resolution of the 24 human chromosomes from the foreskin fibroblast strain LLL 761 into 12 groups. Four of the groups were due to single chromosomes (nos. 5, 6, 13, and 17). The quantum efficiency increase maximizes the ratio of fluorescence from DNA bound dye to fluorescence from free dye, which is important since the chromosomes are measured while suspended in the stain solution, and the high DNA specificity minimizes the staining of cytoplasmic fragments which pass through the flow cytometer along with the chromosomes.

CA3 complements HO as a chromosome stain since it binds preferentially to GC rich DNA while HO binds preferentially to AT rich DNA. The single parameter HO and CA3 distributions for Chinese hamster M3-1 chromosomes were similar (Langlois et al., manuscript in preparation) but were quite different for human chromosomes (Fig. 5). We have demonstrated that the HO and CA3 content in chromosomes stained with both, can be measured almost independently by selectively exciting the HO in the UV or the CA3 at 458 nm. The fluorescence distributions for CA3+HO (Fig. 5b) and CA3 (Fig. 5a) stained human chromosomes excited at 458 nm are virtually identical suggesting that the HO has no effect on the measurement of CA3 content. This is to be expected since the dyes apparently do not compete for binding sites (Langlois and Jensen, 1978), and the HO is not excited by 458 nm excitation. The fluorescence distributions for CA3+HO (Fig. 5c) and HO (Fig. 5d) stained chromosomes excited in the UV are very similar but not identical. The slight differences may be due to chromosome to chromosome variation in the interactions between bound stains. Such variations might arise because of differences in the distribution of AT and GC base pairs among different chromosomes thus affecting the proximity of the bound HO and CA3 molecules. In any case, the differences between Figure 5c and d are slight and to first approximation, the fluorescence of HO+CA3 stained chromosomes excited in the UV is a measure of the amount of HO bound to the chromosomes. This double staining technique applied to Chinese hamster M3-1 chromosomes yielded a two parameter distribution with 10 peaks, fewer than the 16 seen for the higher resolution single parameter analyses. This decrease in resolution was to be expected since the chromosome to chromosome differences in HO and CA3 staining were slight and the measurement resolution was lower. The two parameter distribution for human chromosomes showed 20 peaks; substantially more than seen in the best single parameter analysis. This improvement in resolution was also expected because of the large chromosome to chromosome differences in the HO and CA3 staining.

Of the human chromosome types not uniquely resolved, 5 and 6; 7 and X; and 14 and 15 are paired in single groups and chromosomes 9 to 12 are grouped together. The measurement of a third parameter like centromeric index (Gray et al., 1979; Van Dilla et al., 1976) may allow discrimination between the chromosomes nos. 5 and 6 and may allow the 9 to 12 group to be resolved into three groups. Thus with the addition of a third parameter virtually all of the human chromosomes might be quantitatively classifiable by flow cytome try.

One parameter flow karyotypes also have been shown to be useful in the detection of homogenous chromosome aberrations. Aberrations involving changes in total DNA content cause a change in peak mean or area. Aberrations which result in a loss or gain of a chromosome cause a change in peak area or volume (Gray et al., 1975b; Carrano et al., 1978a). The use of dyes HO and CA3 with their base sequence dependent binding characteristics offers the additional possibility of detecting reciprocal translocations when the total DNA exchanged is equal but where the base composition of the exchanged chromosome segments is different.

Chromosome classification by flow cytometry thus seems especially applicable where the cells to be analyzed are relatively homogeneous karyotypically, and where they can be cultured to provide the large number of mitotic cells necessary for the chromosome isolation procedure. The chromosomes are classified objectively and reproducibly, thus eliminating the subjective decisions that must be made during banding analysis. The precision of the measurements allows detection of some chromosomal changes which are not detectable by quinacrine or Giemsa banding analysis. Furthermore, the procedure can be accomplished rapidly by machine, thus offering the possibility that it could be completely automated; commercial flow cytometers already exist which automatically classify leukocytes (Mansberg et al., 1974).

The study of chromosome structure and the nature of chromosome staining using fluorescent dyes like HO and CA3 with selective base specificity has already begun (Langlois et al., manuscript in preparation). These studies are greatly facilitated by dual parameter flow cytometry. For example, the relative HO and CA3 staining can be immediately determined from the two parameter distributions and provide information about chromosome composition. The fractional differences of 0.07 and 0.15 for Chinese hamster and human chromosomes respectively, suggest that the human chromosomes are more heterogeneous than the Chinese hamster chromosomes in base composition. The quantitative information of the two parameter distributions may also provide insight into the mechanisms of conventional chromosome staining techniques. As might be supposed, chromosomes with high HO/CA3 ratios and presumably high AT/GC ratios such as the human Y, 18, and 13 also appear bright by quinacrine staining (quinacrine fluorescence is quenched by GC rich DNA so that AT rich chromosomes appear bright, Latt et al., 1974) while chromosomes with low HO/CA3 ratios like chromosome 19 appear dim after quinacrine staining. In addition, chromosomes with quinacrine banding polymorphisms may appear different during two parameter analysis. For example, the two human chromosomes no. 22, one of which has a quinacrine bright satellite, are separately resolved during two parameter analysis even though they have similar total DNA content. Taken as a whole, the HO/CA3 ratios correlate ($r = 0.84$) very well with the quinacrine brightness values reported by Kuhn (1976). In addition, the HO/CA3 ratios also correlate ($r = 0.54$) with the AT/GC ratios measured by Kornberg and Engels (1978) thus supporting the hypothesis that chromosome to chromosome variations in HO/CA3 ratios and quinacrine brightness are due to chromosome to chromosome differences in AT/GC ratios.

The extension of two parameter flow cytometry to flow sorting may further the understanding of chromosome base composition and dye binding characteris-

tics by providing pure aliquots of chromosomes with similar dye binding characteristics for biochemical analysis (e.g., base composition determination). Such sorting capability should facilitate other biochemical chromosome studies as well (Sawin et al., 1977, 1979).

In summary we feel that the measurements presented in this paper suggest that flow karyotyping may become an important adjunct to standard karyotyping by banding analysis because it is capable of providing quantitation not available by banding, and capable of detecting chromosomal differences which are invisible to banding. Furthermore, the two parameter measurements allow unique, quantitative studies of chromosome structure and dye binding, and the extension of the two parameter flow cytometry to sorting should permit collection of aliquots of chromosomes of a single type for biochemical or biological analysis.

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