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Quantitative karyotyping of human chromosomes by dual beam flow cytometry

(isolated chromosomes/chromosome polymorphisms)

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ABSTRACT **Dual beam flow cytometry of chromosomes stained with Hoechst 33258 and chromomycin A3 has been proposed as a method for quantitative classification of human chromosomes (bivariate flow karyotyping). In this paper we investigate the sources and magnitudes of variability in the mean fluorescence intensities of each chromosome group resolved in bivariate flow karyotypes and study the impact of this variability on chromosome classification. Replicate bivariate flow karyotypes of chromosomes isolated from lymphocytes from 10 individuals demonstrated that person-to-person variability was significantly greater than run-to-run variability. The total variability was sufficiently small that it did not interfere with classification of normal chromosome types except chromosomes 9 through 12 and chromosomes 14 and 15. Furthermore, the variability was generally smaller than 1/600th of the mitotic genome, so that one-band rearrangements should be detectable in bivariate flow karyotypes.**

Recent advances in the use of flow cytometry for analyzing metaphase chromosomes suggest that this approach may be well suited for quantitative karyotyping of human chromosomes (1, 2). Conventional methods of karyotyping, based on visual analysis of banded metaphase chromosomes, have provided powerful tools for classifying individual chromosome types and for identifying chromosome rearrangements or aneuploidy associated with genetic disorders (3, 4). However, quantitative interpretation of banded karyotypes can be limited by cell-to-cell variability in chromosome condensation and staining characteristics. Thus, it may be difficult to determine whether a band is truly missing or simply not visible in the preparation. The subjective nature of banded karyotype analysis also potentially complicates interlaboratory comparisons of the size or staining characteristics of specific lesions or polymorphisms.

In flow cytometry, isolated chromosomes suspended in a fluorescent stain solution flow one at a time through a laser beam at rates of up to 1,000 chromosomes per sec. The fluorescence signals resulting from laser excitation are measured for the chromosomes yielding a frequency distribution of chromosomal fluorescence. Flow cytometry has a number of advantages over microscopic methods for quantitative analysis of chromosomes. Chromosomes are suspended at thermodynamic equilibrium with the stain, thereby minimizing chromosome-to-chromosome staining variability. Because of the large number of chromosomes analyzed in each experiment, flow analysis provides high-precision population averages that are insensitive to cell-to-cell variations in chromosome condensation. Stain combinations can be utilized to discriminate between chromosome types based on cytochemical staining characteristics and DNA content.

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The utility of flow karyotyping for classifying normal chromosome types, quantitating polymorphisms, and detecting subtle chromosome rearrangments is determined by the measurement variability and by biological variability among normal individuals. In this study we use flow karyotypes of lymphocyte chromosomes from 10 phenotypically normal individuals to determine the magnitude of these sources of variability.

MATERIALS AND METHODS

Chromosome Preparation and Staining. The detailed procedures for cell culture and chromosome isolation from peripheral lymphocytes have been reported (ref. 5, method A). Briefly, lymphocytes were separated from ≈20 ml of peripheral blood with lymphocyte separation medium [although as little as 2 ml will suffice (5)], stimulated with phytohemagglutinin, and cultured for ≈4 days, at which time Colcemid (0.2 μg/ml) was added to the medium for 10 hr. The cells were then swollen in hypotonic solution (75 mM KCl), resuspended in isolation buffer (25 mM Tris, pH 7.5/0.75 M hexylene glycol/0.5 mM $CaCl_2$/1 mM $MgCl_2$), and forced through a 22-gauge needle to release the chromosomes into suspension.

A spectrofluorometric DNA assay (6) was used to adjust the DNA concentration to 200 μM (5×10^6 mitotic cells per ml) prior to staining because the relative staining of individual chromosome types is affected by the dye-to-base-pair ratio in the final stained sample (7). The chromosome suspension was then mixed with a staining solution of Hoechst 33258 and chromomycin A3 in dilute KCl to give a final stained suspension containing 50 μM DNA, 1.3 μM Hoechst 33258, 18 μM chromomycin A3, 6 mM Tris (pH 7.5), 0.19 M hexylene glycol, 0.1 mM $CaCl_2$, 0.25 mM $MgCl_2$, and 19 mM KCl. Flow cytometry was generally performed the day after staining, but stained suspensions can be stored at 4°C for up to 7 days before flow analysis.

Flow Cytometry and Data Analysis. Flow analysis of chromosome suspensions was performed with the Lawrence Livermore National Laboratory dual beam flow sorter (8) equipped with two Spectra Physics 171 ion lasers. Chromomycin A3 was excited with the beam from an argon ion laser (458 nm; 1.0 W), and the resulting fluorescence was detected through a spectral filter passing wavelengths longer than 480 nm (Corning 3-71). Hoechst 33258 was excited with the beam from a krypton ion laser (multiline UV excitation, 337–356 nm; 0.8 W) and the resulting fluorescence was detected through a filter passing wavelengths longer than 420 nm (Ditric Optics 420 HP). We have shown (7) that Hoechst is selectively excited by the UV laser beam, but that the fluorescence emission from Hoechst is affected by energy transfer to chromomycin. For simplicity, fluorescence resulting from UV excitation will be called Hoechst fluorescence. Chromosomes were measured at a rate of 1,000 chromosomes per sec, with 10^5–10^6 chromosomes analyzed in each fluorescence distribution.

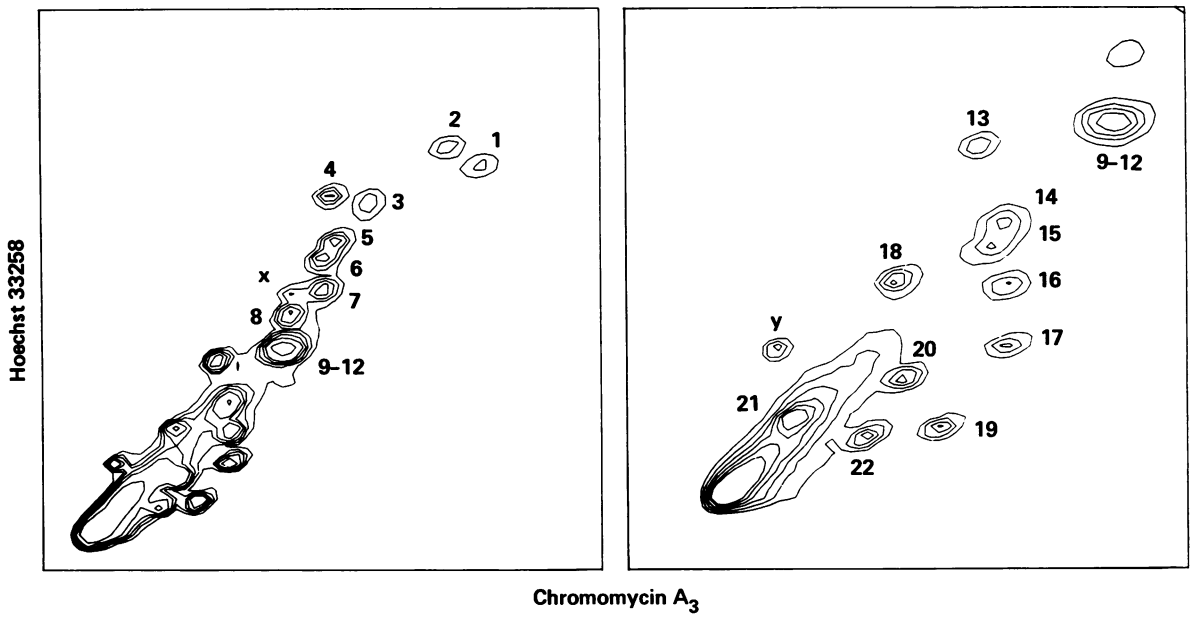

FIG. 1. (*Left*) Bivariate flow karyotype of human lymphocyte chromosomes from individual number 400. The height of each peak in the distribution is indicated by contours. (*Right*) Expanded view of chromosomes smaller than chromosome 8. Peak widths are 2–6% (standard deviation/mean).

Two bivariate distributions (actually 64 × 64 channel arrays) were accumulated during each analysis, one containing all chromosome types and the other containing chromosomes smaller than chromosome 8. Individual chromosome types or groups of chromosomes having the same DNA content and base composition produce peaks in the distribution. Bivariate gaussian distributions were adjusted to fit the peaks to determine the mean intensities and volume (frequency of occurrence) of the peak for each chromosome type. During fitting, the flow karyotype was divided into regions containing only a few peaks to simplify the computational procedure (fitting a normal distribution to one peak requires adjustment of six parameters: mean

Table 1. Fluorescence parameters for each human chromosome type based on the 10 individuals in the study

	Hoechst*			Chromomycin*		
Chromosome	Mean	SD† within	SD† among	Mean	SD† within	SD† among
1	4.174	7	64	4.313	17	38
2	4.306	11	26	3.993	21	24
3	3.699	8	34	3.228	10	36
4	3.788	11	26	2.825	16	45
5	3.373	17	25	2.916	16	32
6	3.152	11	24	2.784	11	33
7	2.844	11	23	2.760	10	18
X	2.837	34	43	2.463	30	35
8	2.600	8	18	2.440	10	18
9–12	2.296	3	34	2.375	4	15
13	2.127	5	31	1.720	7	32
14,15	1.705	5	90	1.807	4	56
16	1.401	6	55	1.829	8	30
17	1.168	5	21	1.856	15	35
18	1.471	3	17	1.322	6	19
19	0.774	8	31	1.531	18	44
20	1.011	5	15	1.349	5	20
21	0.836	6	74	0.873	10	39
22	0.707	5	40	1.167	9	37
Y	1.192	14	132	0.750	11	36
Average	2.262	9	41	2.244	12	32

* Calculated peak means for each individual were normalized so that the total fluorescence from all chromosome types except chromosomes X and Y was 100 for each fluorescence parameter (autosomal normalization). Average chromosome means were calculated for males.

† All standard deviations are multiplied by 1,000 in the table.

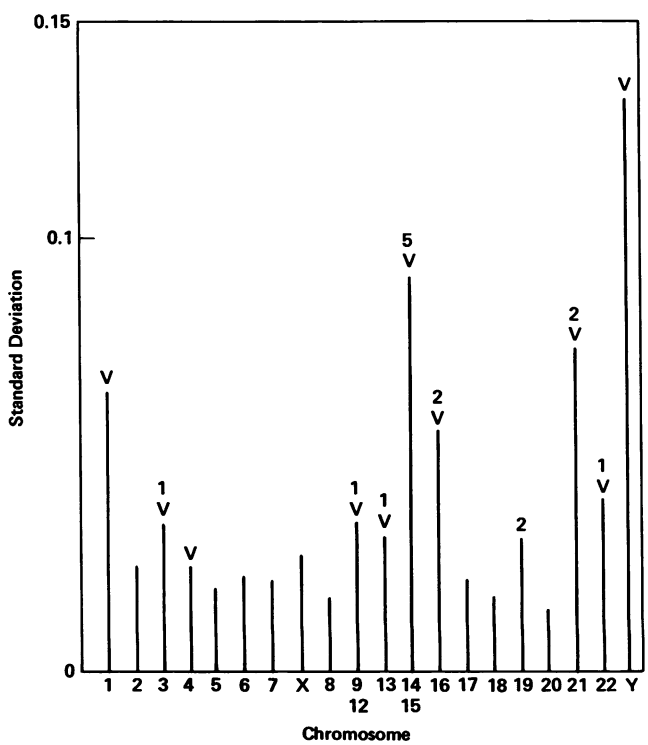

FIG. 2. Biological variability among individuals (ind.) in Hoechst fluorescence. Biological variability was determined from the following expression: standard deviation (SD) = $[(\text{SD among ind.})^2 - (\text{SD within ind.})^2]^{1/2}$. Chromosome types that are known to have variable regions from banding studies are marked with a V. The number of individuals out of 10 analyzed having separately resolved homologs for each chromosome type is also indicated above the lines.

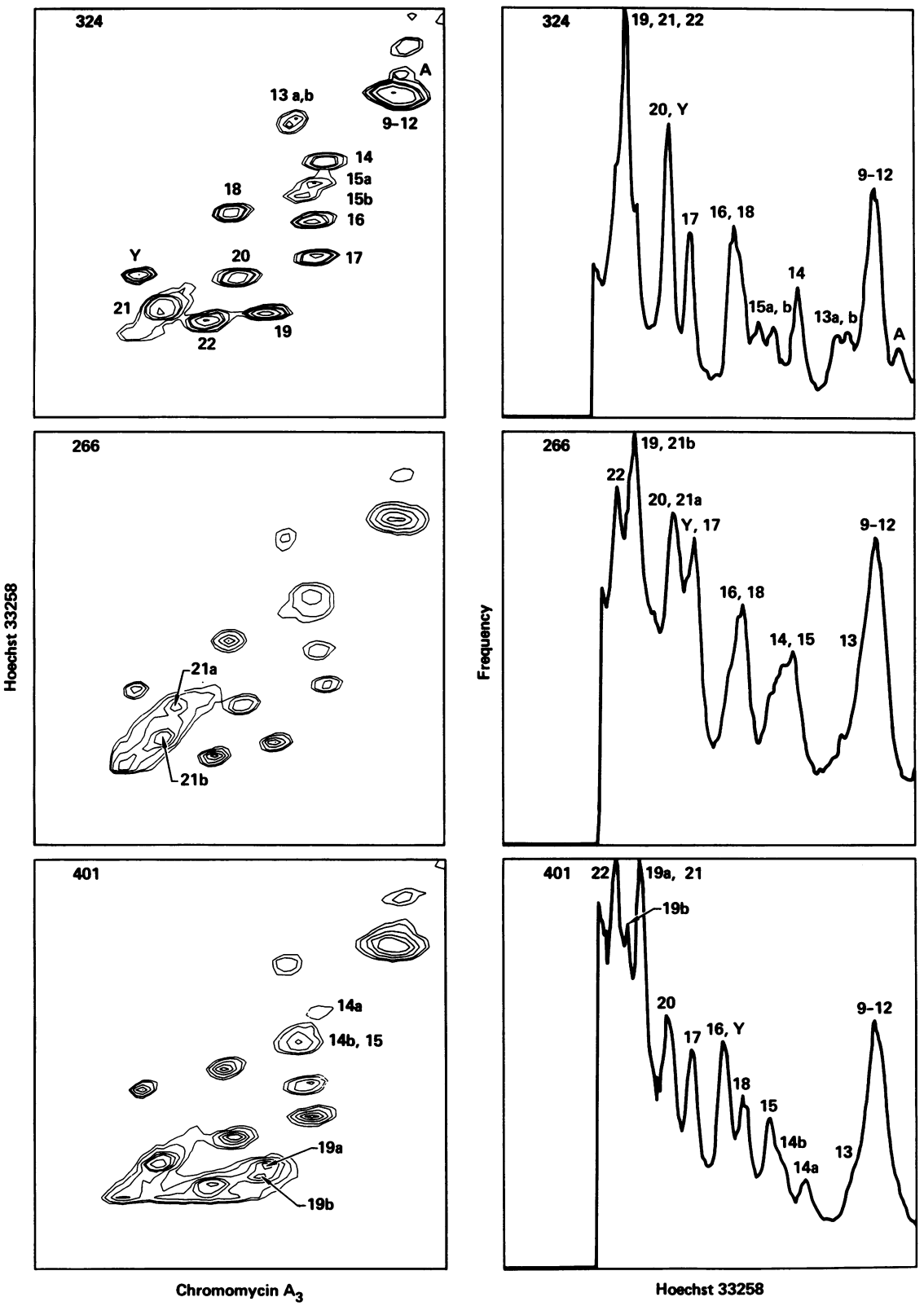

FIG. 3. Flow distributions of the smaller chromosome types from three different individuals (numbers 324, 266, and 401) in the study. The separately resolved homologs in the bivariate distributions (*Left*) illustrate the magnitude of polymorphic variability among individuals. The peak marked A is a single homolog of the 9–12 chromosome group. C-band differences between the homologs of chromosome 9 for this individual suggest that this peak corresponds to one homolog of chromosome 9. Univariate distributions (*Right*) are from the same individuals.

and variance for each of the two variables, frequency of occurrence, and correlation between the two variables). One limitation of our current fitting procedure is that it does not contain a function for the debris continuum produced by fragmented chromosomes, which predominates at low fluorescence values (1). This limitation has little effect on estimation of peak means

but does lead to errors in estimation of the frequency of occurrence of some chromosome types. Fitting procedures that contain a function for the debris continuum should provide reliable estimates of the frequency of occurrence required for the detection of numerical abnormalities (aneuploidy).

Two to four flow karyotypes were measured for each of 10 individuals (8 males and 2 females) to permit evaluation of the variability in bivariate peak means within an individual and among individuals. The standard deviation of peak means within an individual was determined by calculating the variance among replicate experiments for each individual and then averaging these variances from all individuals. The standard deviation among individuals was calculated from the variance of all experiments on all individuals. When homologs of a chromosome type were separately resolved, peak means for each homolog were used for statistical analysis so that the overall estimates of variability in peak means include variability caused by homolog differences. Standard deviations calculated separately for Hoechst fluorescence and chromomycin fluorescence are used in Table 1, whereas the ellipses in Fig. 4 also include the correlation between these two variables.

RESULTS AND DISCUSSION

A typical bivariate flow karyotype of human lymphocyte chromosomes stained with Hoechst 33258/chromomycin A3 is shown in Fig. 1. We have shown (7) that fluorescence differences between A·T-specific Hoechst and G·C-specific chromomycin are primarily determined by differences in base composition among chromosome types. Thus, in bivariate flow karyotypes, chromosome types are separately resolved based on differences in base composition and DNA content. The results in Fig. 1 demonstrate that all human chromosome types except chromosomes 9–12 and chromosomes 14 and 15 can be separately resolved in the bivariate flow karyotype. Chromosomes 9–12 are sufficiently similar in DNA content and base composition that they cannot be separately resolved by this approach. Chromosomes 14 and 15 are separately resolved for many individuals (e.g., Fig. 1), but their staining properties are sufficiently variable among individuals that they are not clearly resolved for all individuals studied. Assignments of the chromosome types responsible for each peak in the flow karyotype based on flow sorting of fibroblast chromosomes (1) were confirmed by sorts of lymphocyte chromosomes from one of the individuals in this study (5).

The precision of peak means determined from replicate experiments of 10 individuals analyzed in this study are reported in Table 1. The average standard deviations within individuals (0.009 for Hoechst and 0.012 for chromomycin) calculated with data from all 10 individuals are comparable to the values obtained from four different preparations of the same individual analyzed at different times in the study (0.015 for Hoechst and 0.012 for chromomycin). Thus, the precision of peak means in the flow karyotype is on average 0.5% (standard deviation/mean, from Table 1), which is substantially better than the precision obtained by other methods for measurements on human chromosomes (9, 10).

The standard deviation among individuals reported in Table 1 demonstrates that, for both fluorescence parameters, the variability among individuals is larger than the variability among replicate measurements on the same individual. Thus, the total variability in peak position is determined mostly by biological variability among individuals. Banding studies have demonstrated that some human chromosome types contain polymorphic regions that vary in size among individuals and vary between homologous chromosomes within an individual (10, 11). Fig. 2 shows that chromosome types known to contain major polymorphic regions show larger biological variability in Hoechst fluorescence than do other chromosome types. The largest variability in Hoechst fluorescence was seen in the Y chromosome, where the largest Y had 37% more Hoechst fluorescence than the smallest Y chromosome. The flow karyotypes in Fig. 3 show that individual homologs of polymorphic chromosomes can differ significantly in staining properties. Homolog differences are commonly observed (15 separately resolved homologs were observed in the 10 individuals studied), and the staining differences between homologs can be large (the two homologs of chromosome 21 in Fig. 3 differ in Hoechst intensity by 30%). The distribution of homolog differences among chromosome types reported in Fig. 2 is closely correlated with the distribution of polymorphic regions. Thus, flow karyotyping may facilitate quantitative studies of the distribution and heritability of polymorphic regions.

Polymorphic variations can introduce ambiguities in the classification of normal chromosome types. The univariate flow karyotypes in Fig. 3 demonstrate that Hoechst fluorescence alone does not provide sufficient information to determine the chromosome types responsible for each peak in the fluorescence distribution. Fig. 4 presents a statistical summary of the variability of peak means in the bivariate flow karyotype based on all analysis from the 10 individuals in the study. Each chromosome type is represented by a 95% tolerance ellipse that is expected (with probability 0.90) to contain 95% of the peak means for normal persons. There were no experiments where the peak position of one chromosome type occurred in regions of the distribution occupied by other chromosome types. Thus, with bivariate karyotyping, all normal chromosome types (or groups for chromosomes 9–12 and 14 and 15) can be unambiguously classified solely on the basis of peak position in the distribution.

The sensitivity of quantitative karyotyping for detecting small chromosome aberrations also is determined by the nor-

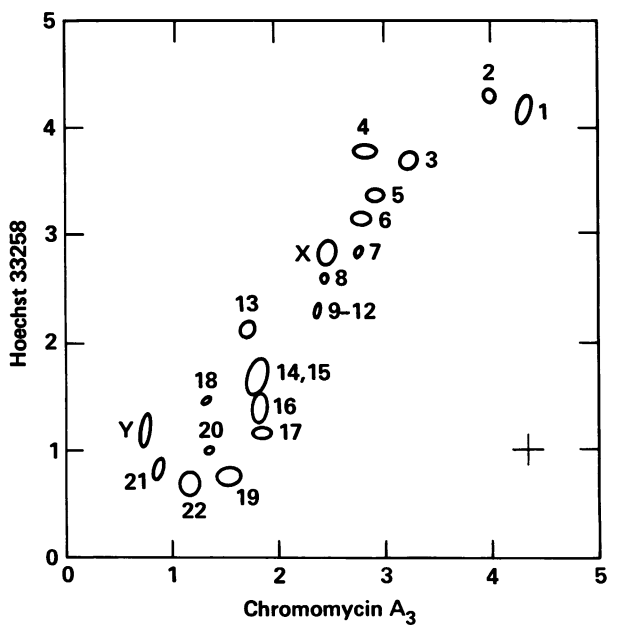

FIG. 4. Statistical summary of the total variability in peak means for all individuals in the study. Each chromosome type is shown as an ellipse that is expected to contain 95% of the peak means for that chromosome type (assuming both variables are normally distributed and including the correlation between the two variables). The cross shows the expected change in peak mean for the addition or deletion of fluorescence corresponding to one band from a chromosome (see text).

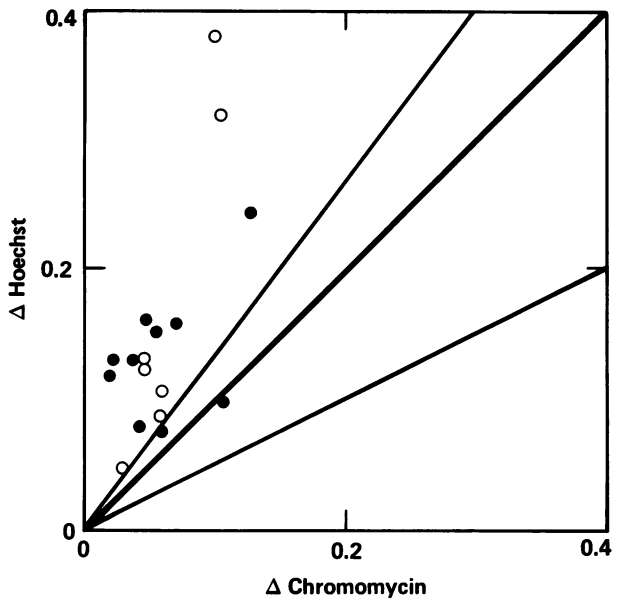

FIG. 5. Staining characteristics of polymorphic regions. ●, Differences in Hoechst and chromomycin fluorescence between separately resolved homologs; ○, fluorescence differences between the smallest Y chromosome and other Y chromosomes. The diagonal lines show the fluorescence changes expected from addition or deletion of material with a Hoechst-to-chromomycin (H/C) ratio of chromosome 19 (the chromosome with the lowest H/C ratio) (bottom line), the whole cell average (middle line), or chromosome 4 (the chromosome with the highest H/C ratio when the polymorphic Y chromosome is not included) (top line).

mally occurring polymorphic variability. The ellipses in Fig. 4 provide an estimate of the minimum change in Hoechst or chromomycin fluorescence caused by a structural rearrangement that can be reliably distinguished from normal polymorphic variability. The cross on Fig. 4 shows the range over which a peak mean might be expected to vary if a chromosome were to increase or decrease in Hoechst or chromomycin fluorescence by the average fluorescence of one band [the cross corresponds to ± 1/600th of the mitotic genome (10)]. The observed widths of the ellipses are generally smaller than the width of the cross, indicating that a one-band change (loss or gain) could be detected. Although polymorphic variability for some chromosome types (i.e., chromosomes 21 and Y) can be larger than the loss or gain of one band, the unique cytochemical characteristics of polymorphic regions known from Q banding and C banding (11) may facilitate detection of aberrations involving these chromosome types. Intensity differences between separately resolved homologs were used to determine the staining characteristics of polymorphic regions with Hoechst and chromomycin. The results in Fig. 5 show that the Hoechst-to-chromomycin ratio is larger for most polymorphic regions than would be expected from chromosome rearrangements. Thus, the Hoechst-to-chromomycin ratio of variant chromosomes may assist in differentiating between chromosome rearrangements and polymorphisms.

In summary, flow karyotyping provides an objective and quantitative method for classifying human chromosomes and characterizing chromosome polymorphisms. The total variability in peak means is sufficiently small that homogeneously occurring chromosome rearrangements that result in a net change in fluorescence of loss or gain of one band should be detectable. It is not possible to evaluate the sensitivity of flow karyotyping for detecting numerical abnormalities (aneuploidy) or nonhomogeneous abnormalities (mosaicism) by using results from the current fitting procedure, which neglects debris. However, improved fitting procedures should allow proper analysis of the debris continuum so that both numerical abnormalities and structural rearrangements can be detected.

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