Laser in Cytometry: Applications in Flow Cytogenetics

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Flow cytometry has become an important tool in biomedical research and clinical diagnosis. In particular, its ability to make multiple measurements of individual particles at a rate of up to 20000 particles per second allows the quantitative characterization ("flow karyotyping") and sorting of isolated chromosomes. Major areas of application of "flow cytogenetics" are in molecular genetics, human genetics, and tumor cytogenetics. Several approaches to increase the resolution of one and two parameter flow cytometry of chromosomes will be discussed. On one hand different algorithms of flow cytometric data processing can be applied to achieve a better signal/apparent noise ratio. To obtain a more accurate evaluation of flow karyotypes they may be approximated as a sum of Gaussian distributions. On the other hand the flow cytometric resolution of specific chromosome types can be improved by chemical modifications of the chromosomal DNA, e.g. incorporation of base analogues or in situ hybridization in suspension with specific DNA probes.

Perspectives and Overview

Lasers have found a variety of significant applications in biomedical sciences. They are used in manyfold ways to obtain quantitative information on cells, cell organelles, and parts of cells such as chromosomes. This report will be limited to a special application being of growing importance in many branches of biological and medical sciences, the use of lasers in flow cytometry.

In conventional flow cytometry (also called "zero resolution" flow cytometry because the dimension of the morphological resolution is zero) one or several fluorescence values resulting from excitation with continuous-wave (CW) laser light are measured for each particle stained with a specific fluorochrome. According to the variety of fluorescent staining procedures characterization of biological particles is possible in many different ways, e.g. DNA content, DNA base ratio, internal substrate concentrations, surface antigens etc. In addition, some gross morphologic features may be extracted by evaluation of forward (0.5°–20°) and right angle scattered laser light (e.g. information on size and shape).

The power of flow cytometry (as compared to quantitative fluorescence microscopy) derives from the ability to make multiple measurements of each particle simultaneously at very rapid rates. Presently, a rate of several thousand particles per second may be achieved in commercially available flow cytometers; in specially designed instruments [1], the rate may be increased up to 20000 particles per second.

Another important advantage is that particles may physically be separated due to preselected optical parameters. Under favourable conditions high purities (up to 98%) may be obtained.

Due to its quantitative capacity, versatility, sensitivity, speed, and ability to identify and sort cell subsets in heterogeneous populations, flow cytometry is presently used world wide in hundreds of biological and biophysical re-
search laboratories and medical institutions. For example, flow cytometric measurements of cellular DNA content allow the quantitative characterization of a variety of tumor cell lines and thus have found a vast application in tumor diagnosis [2, 3].

This report will focus on some aspects of the use of flow cytometry for quantitative characterization of chromosomes ("flow cytogenetics"). Since a general review on this field has been published recently [4], this article in particular will discuss in more detail some approaches to increase the resolution of flow cytometric data.

Principles of Laser Fluorescence Activated Analysis and Sorting

The technique of laser fluorescence activated analysis and sorting of cells and chromosomes has mainly been developed in the last two decades [5] and has become important for many applications, both in biomedical research and clinical diagnosis (for general reviews see [6 - 9]).

In most cases argon- and krypton-ion lasers (excitation of dyes in the visible and near UV) are used with total emission power of several watts. For some immunological applications, helium-neon or ion-pumped dye lasers are used, too. A high stability is required for precise measurements because for small particles as chromosomes, any variation in laser power may result in a variation of the emitted fluorescence intensity.

For each particle the fluorescence emission is detected by photomultipliers (PMT) during the few microseconds taken to traverse the focal volume. The PMT signal is either linearly or logarithmically integrated, digitized, and registered by a multi-channel analyzer. The larger for instance the DNA content of a cell nucleus or a chromosome is, the more dye may be bound and consequently the fluorescence intensity increases. Thus it is possible to discriminate different types of particles according to their fluorescence properties.

Using a single laser beam device, two fluorescence parameters (following simultaneous staining with two fluorochromes of similar absorption spectra) may be measured if the fluorescence emission spectra of the two dyes do not overlap. In this case e.g. green and red fluorescence are separated by appropriate edge or band pass filters. In addition, the laser light scattered by a particle may also be measured and used for further discrimination. For this, forward and right angle light scatter is detectable. Nowadays many commercial machines are equipped with more than four detectors and two lasers for two, three, or multicolor fluorescence measurements.

For physical separation of specific types of particles ("sorting") a hydrodynamic electronic procedure is applied which allows to sort them into collection vessels according to preselected optical properties measured as described above. To do this, the fluid jet in air is disrupted into single droplets (diameter about 1.8 of the jet diameter) after having passed the laser intersection point. This is realized by mechanical vibrations of the flow cell up to 40 kHz in vertical direction parallel to the streaming jet. Those droplets containing particles to be sorted are charged electrically before separation from the jet and deflected into recipient vials by an electrostatic field of a few kV/cm. As mentioned above, the method presently allows analysis and sorting of particles between several hundreds and 20000 per second.

Flow Cytometry of Chromosomes: Univariate Distributions

In this report, we shall focus on a special feature of laser fluorescence activated analysis and sorting, i.e. flow cytometry of isolated metaphase chromosomes in suspension (also called "flow cytogenetics") ([10]; for review see [4]). This has found important applications in molecular biology, human genetics, and clinical diagnostics of genetic diseases. Presently, one of the main applications of chromosome sorting is the construction of chromosome specific DNA libraries [11, 12].

Fig. 2 gives an example of a so called univariate "flow karyotype" using one fluorescence parameter only: On the ordinate the number of the fluorescence signals (particles) is given; on the abscissa the logarithm of the fluorescence intensity is plotted. Ideally, each fluorescence signal represents...
Univariate flow karyotype of chromosomes from the Chinese hamster cell line CHL (DAPI staining, laser excitation in the near UV); ordinate: Number of particles; abscissa: Channel number (in this case corresponding to the logarithm of the fluorescence intensity). The peak designation (No. 1 – 9) was made according to decreasing fluorescence intensity (correlated with the chromosome length in case of chromosomes of the Chinese hamster). The flow karyotype shown has been obtained without further processing of the data one chromosome with a specific DNA content. In the case shown in Fig. 2, chromosomes isolated from a Chinese hamster cell line were used. The chromosome suspension was stained with the fluorochrome DAPI (5 μM) specific for doublestranded DNA. Excitation was done by an argon-ion laser (UV multiline 351.1 – 363.8 nm) with 50 mW. Under favourable circumstances and optimum resolution each chromosome type of a cell line is represented by one individual peak. One possibility to assign the peaks to individual chromosome types is to sort the peak contents and to identify the chromosomes by conventional cytogenetic methods [10, 13]. Another preliminary assignment is usually possible by comparison of the flow cytogenetic data with light microscopic observations. Under appropriate conditions, i.e. that the AT/GC correlation is about the same for each chromosome type, an excellent correlation may be obtained between chromosome length and relative fluorescence intensity of the flow karyotype.

To achieve the flow karyotype shown in Fig. 2, a chromosome isolation method [14, 15] was applied using a hexandiol isolation buffer to stabilize the chromosomes. This procedure has the particular advantage that chromosomes in suspension may be stored at 4°C for long times (in our experience up to 5 years) without a significant deterioration of the resolution of the flow karyotype [16].

Data Evaluation

In many cases the flow karyotypes obtained under various experimental circumstances are not as well resolved as shown in Fig. 2. This may be caused by different preparation conditions; one of them is the buffer system of the chromosome suspension which may not always be optimal for flow karyotyping but necessary with respect to other experimental studies done with the chromosomes.

For example, free flow electrophoresis of isolated metaphase chromosomes requires a buffer system of low ionic strength [48]. Flow cytometric characterization may lead to poorly resolved flow karyotypes because of morphological instabilities of the chromosomes in this buffer.

In Fig. 3a the experimental flow karyotype of chromosomes of a Chinese hamster cell line in free flow electrophoresis buffer prior to data processing is presented. The low resolution of the peaks is caused by a large amount of “noise”. Furthermore, randomly broken chromosomes and nuclear fragments form a broad underlying “background continuum” [4] which may be increased considerably following a treatment with environmental mutagens prior to chromosome isolation [17]. Therefore, it is desirable to reduce the noise fluctuations and to eliminate (or to quantify) the amount of background, present in the histogram, by computer analysis of the stored multichannel data.

For this purpose, a variety of mathematical procedures are available. With smoothing routines the measured signal may be transformed into a signal with a better signal/apparent noise ratio. According to our experience, the so called “k-nearest-neighbours” method with an appropriately sized
interval of abscissa values \((2k + 1)\) seemed to fulfill this transformation quite well for flow karyotypes. Here, it is assumed that the measured curve consists of two parts, a "true" smooth curve and the noise values \([18]\). Within the chosen interval the latter are supposed nearly to sum up to zero. Then each ordinate value of the new transformed "smooth" flow histogram is computed by a weighted sum of the \(k\) nearest neighbor ordinate values of the original curve. The used weighting factors have the form of binominal factors

\[
a_j = \frac{(2k + 1)!}{j!(k + 1 - j)!} \quad (\text{with } j = -k, \ldots, +k).
\]

Under the conditions chosen the area of the total flow karyotype changes only less than 0.1% compared to the original measured histogram.

In Fig. 3b the result of such a smoothing protocol is shown (applied to the data of Fig. 3a). Reducing the apparent noise, a number of "true" chromosome peaks is highlighted.

In addition, the background was quantified and subtracted. In this case, the assumption of a Chi²-distribution function resulted in a better background subtraction than the exponential assumption often used (J. Dölle et al., manuscript in preparation).

Flow histograms thus obtained may be analyzed further. According to theory, an ideal linear flow karyotype (without noise and background) is composed of Gaussian distributions, one for the fluorescence values of each different chromosome type \([4]\). The areas of these Gaussians correspond to the relative frequencies of the respective chromosome types in the suspension. Peak means and coefficients of variation (CV: = standard deviation of the peak divided by peak mean) give precise quantitative information on the composition of the flow karyotype. To obtain these data, i.e. composition of the single peaks, relative and absolute peak area, CV etc., flow karyotypes may be approximated as a sum of Gaussian distributions.

Considering the rise of the logarithmic amplification used in most flow cytometers it is, according to our experience, also possible to use the same fit procedure for flow karyotypes with a logarithmic distribution along the fluorescence axis. In this case, the area of a peak typical for a chromosome in a logarithmic flow karyotype usually differs less than 1% from the area of the theoretical peak resulting from a Gaussian fit. Fig. 4 gives an example for this kind of evaluation. In Fig. 4a, a flow karyotype of the Chinese hamster cell line CHL is presented after application of the smoothing protocol and background subtraction as described above. In Fig. 4b a theoretical flow karyotype is shown, composed of 10 single Gaussian curves by an interactive PC program. Peaks consisting of several chromosome types are separated into single overlapping peaks. The resulting curve summing up those peaks fits the measured flow karyotype quite well (difference in peak area less than 2%).

Data evaluation procedures, such as those described above, may contribute considerably to improve the analysis of experimental flow karyotypes. However, the decomposition into Gaussian distributions requires low background levels and sufficiently separated peaks to be reliable.

Since noise and background levels critically depend on the experimental conditions (e.g. chromosome isolation procedures, number of particles measured, laser light intensity and stability, stability of the fluid flow system, electronic setup), a careful adjustment of these parameters may be critical for sufficiently well resolved flow karyotypes.

Under the conditions described so far, the peak separation, however, may not be improved beyond a certain limit, i.e. if two chromosome types have the same total DNA content, they cannot be separated by univariate flow cytometry alone, using DNA stains, which do not prefer either AT or GC base pairs (for instance intercalators like Pro- pidium Iodide or Ethidium Bromide). Therefore, a significant progress in this context was achieved by using AT, GC specific DNA stains (e.g. Hoechst 33258, Hoechst 33342, Chromomycin A3, Mithramycin etc.) together with dual laser flow cytometry.

**Dual Laser Flow Cytometry**

To perform dual beam (laser) flow cytometry \([19]\) the isolated chromosomes are stained with two fluorescent dyes instead of one only. These two dyes have different base spe-
cificity and different excitation and emission wavelengths. An intensively used dye combination is Hoechst 33258 (HO), which is AT specific, and Chromomycin A3 (CA3), which is GC specific.

In a dual laser flow cytometer the stained chromosomes pass one after another two slightly elliptically focused CW laser beams of different wavelengths. In case of the fluorochromes named above, UV multline excitation is used for HO; blue laser light (mainly 458 nm) is used to excite CA3 fluorescence. The fluorescence emissions are collected independently from each other. This is possible due to the spatial distance of the two laser intersection points. Thus, information may be obtained not only on the relative DNA content \((0.6 \times HO + 0.4 \times CA3 \text{ fluorescence intensity})\) [26], but on the AT (HO fluorescence)/GC (CA3 fluorescence) ratio as well.

In Fig. 5 shows an application of dual laser flow cytometry in the quantitative analysis of human chromosomes. Here, chromosomes were isolated [20] from a short term culture (72 hours) of female peripheral blood (normal donor). The ordinate of this "bivariate" plot shows the fluorescence of the AT specific dye HO; the abscissa presents the fluorescence of the GC specific dye CA3. The contour curves indicate the lines of equal particle frequency. Therefore, closed contour lines represent a well separated chromosome peak. All of the 20 different chromosome types measured here (No. 4-22; X), may be assigned [20]. In this example, eleven of them (chromosome types No. 4, 5, 6, 13, 16, 17, 18, 19, 20, 21, 22) are represented by individual chromosome peaks. Using dual laser flow cytometry together with the fluorochromes HO and CA3 it is possible to discriminate all human chromosomes [20], except chromosomes No. 9-12, which are too similar both in total DNA content and in AT/GC ratio. Some further progress in the resolution of the 9-12 peak is possible using the fluorescence dye DIPI (preferring AT base pairs [21]) instead of HO.

Using the sorting procedure described above, individual human chromosome types have been purified by dual beam sorting with efficiencies up to 97% [21]. Instead of sorting chromosomes isolated from human cells, it is also possible to sort human chromosomes isolated from Chinese hamster \(\times\) human hybrid cells which contain a reduced number of human chromosome types [12, 22, 23]. In a number of cases, this allows an increased purification.

**Improvement of Flow Cytometric Resolution of Specific Chromosome Types by Chemical Modification of DNA**

Under favourable circumstances, it is nowadays possible to detect quantitatively changes in DNA content as small as \(10^{-15}\) g [4].

However, there are problems which may require additional discrimination. For example, it may be interesting to analyze the chromosomes of other mammals; or to improve the flow cytometric analysis of chromosomes isolated from tumor cells, which often show a variety of complex rearrangements.

**A) Incorporation of Base Analogue**

One possibility to obtain an additional discrimination of specific chromosome types [24, 25] is to use the fact that the chromosomal DNA may be replicated at different times of the cell cycle.

The Chinese hamster Y chromosome, for example, is the last to be replicated. If the base analogue 5-bromo-2-deoxyuridine (BrdUrd) is incorporated instead of thymidine during the last hours prior to mitosis, then the AT-specific fluorescence of the dye Hoechst 33258 is quenched.

Fig. 6 gives an example [25]. Here, bivariate contour plots are shown as indicated in Fig. 5. The only difference is that in this case chromosomes of a Chinese hamster cell line (M3-1) [10, 26] were used instead of human chromosomes. Fig. 6a presents the dual parameter flow karyotype of these Chinese hamster chromosomes after HO/CA3 staining but without any other modification. Here, the peak of the Y chromosome specific DNA library [12, 23, 27] or to perform other studies, e.g. gene assignment [21]. In the two contour plots shown in Fig. 6b, c, BrdUrd was added to the culture medium 2.5 hrs (Fig. 6b) and 3.5 hrs (Fig. 6c) prior to chromosome isolation (90 min Colcemid treatment for collection of mitotic cells). In this case, the HO fluorescence of the Y chromosome \(Y_a\) was increasingly quenched while the CA3 fluorescence remained about the same. Due to this it became
Fig. 6
Effect of a terminal label with BrdUrd on the bivariate flow karyotype of HO-CA3 stained chromosomes. M3-1 cells were incubated for 0 (a), 2.5 hrs (b), and 3.5 hrs (c) with BrdUrd before isolation. Chromosome assignment was done according to [26] except for the Y which was identified by dual parameter sorting on microscope slides followed by Q-banding analysis. Experimental settings were chosen to highlight the chromosome regions of interest (Y; 10, 11, M2). This resulted in chromosomes 1 and 2 being eliminated from the display. Experimental conditions were held constant for all measurements (from [25]).

possible to clearly separate the position of the Y, peak from all other chromosomes. In case of the condition shown in Fig. 6c, the Y chromosome was sorted with relatively high purity (75%).

B. Flow Cytometric Detection of Chromosomes Following In situ Hybridization in Suspension

The BrdUrd approach discussed above is one possibility how a specific physico-chemical DNA modification may be introduced to improve the flow cytometric discrimination of certain chromosome types. In practice, the BrdUrd method appears to be useful for chromosome types with grossly different replication kinetics [25].

In this section, a new approach will be described which, in principle, may be applied to any chromosome type. This new technique is based on the modification of the DNA of specific chromosomes by in situ hybridization with specific DNA probes. These probes have been chemically modified, e.g. biotinylated by nick translation; then they are hybridized to the chromosomes. This technique requires denaturation (usually done by thermal treatment) of the chromosomal DNA prior to hybridization (denaturation temperature is typically about 73°C), i.e. the two native DNA strands are physically separated from each other. After renaturation a new double stranded DNA is formed with the complementary sequence of the DNA probe.

The biotinylated DNA strands hybridized to the chromosomal DNA sequences can be detected, e.g. by a specific fluorescing antibody detection system ("fluorescence hybridization", "chromosome painting") or an enzyme detection reaction.

While this technique has already been successfully developed for the analysis of chromosomes fixed on slides [28-35], its application to flow cytometry of chromosomes is still in the very beginning.

Since in this case a suspension of isolated chromosomes is required, the entire fluorescence hybridization protocol has to be performed in suspension, maintaining the morphological stability of the chromosomes. A critical step of this method is the denaturation and renaturation of the chromosomes in suspension. This process may be monitored by flow cytometry. In the results presented in Fig. 7, flow karyotypes of the chromosomes of a Chinese hamster cell line are shown shortly before thermal denaturation (Fig. 7a) and at different times afterwards (Figs. 7b, c, d). Immediately following denaturation, the temperature was lowered (room temperature) so that renaturation of the two strands was possible. For flow cytometry the chromosomes were stained with DAPI, a fluorescent dye preferably binding to double stranded (i.e. renatured) DNA.

All flow cytometric measurements presented in Fig. 7 were performed under the identical instrumental set up (fluorescence gain etc.); aliquots from the same DAPI stained chromosome suspension were used. The chromosomes of the flow karyotypes in Fig. 7b, c, d were additionally stabilized with propidium iodide (PI). This led to quenching of the DAPI fluorescence (quantum yield about 50%). Thus, the flow distributions obtained may be compared with each other.

Two minutes after denaturation (Fig. 7b), the flow distribution was very poorly resolved. The DAPI fluorescence of aB chromosomes was found to be significantly reduced. This suggests that under the conditions used, a considerable amount of denaturation took place. With increasing time, however, the original flow karyotype (Fig. 7a) gradually reappeared (Figs. 7c, d).
Flow cytometric monitoring of the denaturation/reannealing of chromosomes in suspension (univariate measurements)

Fig. 7

- a) flow distribution prior to thermal denaturation (DAPI staining)
- b)–d) flow distributions (DAPI staining) of the same chromosome suspension as shown in a) but additionally stabilized with propidium iodide, measured at different reannealing times performed at 2 min (b); 15 min (c); 25 min (d) following denaturation. In all cases, the same instrumental set up was used.

Fig. 8

Flow karyotypes of a Chinese hamster x human hybrid cell line are shown. The chromosomes were isolated according to a modified hexandiol method [15], hybridized in suspension with total genomic human DNA, and counterstained with DAPI. Fig. 8a shows the DAPI flow karyotype of this cell line prior to the hybridization procedure while in Fig. 8b the karyotype is shown afterwards. Compared to Fig. 8a, the number of events in Fig. 8b decreased from small to large chromosomes. This indicates that the percentage of morphologically intact chromosomes measured by flow cytometry after the hybridization procedure was reduced with increasing chromosomal length. However, the overall structure of the flow karyotype is still recognizable. The comparison of the peak position of the different chromosome types indicates that a considerable percentage of the chromosomes has still about the same DAPI fluorescence before and after the hybridization treatment. This means that renaturation was complete as far as it was measureable by flow cytometry, and that the DNA loss was low.

Future Directions

Slit scan flow cytometry allows to analyze morphologically well preserved normal and aberrant chromosomes like dicentrics [42] or translocations [39,40]. Nonetheless, an improved application of zero-resolution flow cytometry...
of particular interest in this context is the zero-resolution detection of chromosomes following fluorescence hybridization in suspension. If the technical problems of this new method (e.g. hybridization chemistry, morphological stability of chromosomes) can be solved, the rapid characterization (up to several thousand particles per second) of the chromosomal constitution of a variety of cell lines might be improved considerably by means of dual parameter zero-resolution flow cytometry. It is important to note that the range of potential flow applications (both zero-resolution and one dimensional, i.e. slit scan flow cytometry) will critically depend on the DNA probes available for fluorescence hybridization. Ideally, DNA probes are required to highlight specifically any chromosome or chromosome region, especially of the human complement. To realize this goal, recently significant progress has been achieved to delineate chromosomes in human cells by fluorescence hybridization with high specificity [32, 35, 43, 44]. These methods are mainly based on the use of chromosome specific DNA libraries established from flow sorted chromosomes [11, 12, 23, 27]. So far, it has been shown that these delineation procedures allow a considerable improvement of the cytological analysis of cells including tumor cells [32, 44]. With further developments of multi-probe hybridization and detection procedures [45, 46] these methods might, eventually, also be applied in flow cytometry to a rapid screening of tumor cell populations. For instance, using two colour fluorescence hybridization [31–33] it might become possible to realize the following approach:

Chromosomes (e.g. from a tumor cell population) are isolated; chromosomes of type A are hybridized and labelled for green fluorescence, while chromosomes of type B are hybridized and labelled for red fluorescence. In a bivariate contour plot (e.g. abscissa: green fluorescence intensity; ordinate: red fluorescence intensity), chromosomes of type A should form a peak near the abscissa; chromosomes of type B should form a peak near the ordinate, while translocation chromosomes (exchange of material between chromosome types A and B) should form peaks in between.

As compared to the flow cytometric analysis of chromosome translocations with conventional DNA stains [4], such a method should allow to highlight specifically the flow distributions of the chromosomes of interest in the particular case (e.g. in leukemias).

Fig. 1 was obtained by courtesy of the Coulter Corporation. The experimental flow distributions for Figs. 3, 4, 8 were measured by M. H. using the flow cytometer at the University of Tübingen (Dr. H.-J. Bühring). The data presented in Fig. 5 were measured by C. C. during his stay at the Lawrence Livermore National Laboratory (Dr. J. W. Gray). Furthermore we thank Dr. T. Cremer (Yale University/Heidelberg University) for the communication of unpublished manuscripts concerning the specific delineation of human chromosomes.

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