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Flow Analysis and sorting of microchromosomes (<3Mb)

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Abstract

Background—The analysis and isolation of high numbers of chromosomes smaller than 3 Mb in size (microchromosomes) with good purity is dependent primarily on the detection sensitivity of the flow cytometer and the precision of the sort unit. The aim of this study was to investigate the capability of using a conventional flow cytometer for the detection and sorting at high purity microchromosomes with an estimated size of 2.7Mb.

Methods—Chromosomes were isolated from a human cell line containing a pair of X-derived microchromosomes using a modified polyamine isolation buffer. The chromosome preparation was labelled with Hoechst and Chromomycin and analysed and purified using a MoFlo sorter (DAKO) configured for high speed sorting. The purity of the flow sorted microchromosomes was assessed by reverse chromosome painting.

Results—Improved resolution of the peak of microchromosomes in a bivariate plot of Hoechst versus Chromomycin fluorescence was obtainable after discriminating clumps and debris based on gating data within a FSC versus pulse width plot.

Conclusions—Chromosomes of smaller size, less than 3Mb, can be detected with high resolution and flow sorted with high purity using a conventional flow sorter.

Keywords

microchromosomes; mammalian artificial chromosomes; metaphase; bivariate; univariate, flow karyotype; resolution; sorting; FISH; DOP-PCR

Chromosomes smaller than 20 Mb have been reported previously to occur naturally as chromosome aberrations (1,2) and in animal species such as birds (3) and in certain species of turtles (4). Previously, flow cytometry has been applied successfully to the isolation of microchromosomes greater than 20Mb in size (5-8). To date, we are not aware of any reports on the use of flow cytometry to isolate small (< 3Mb in size), experimentally generated, microchromosomes or mammalian artificial chromosomes, MACs (9-12), which are a useful research tool for the functional characterization of genes as well as potential gene carriers for somatic gene therapy (12-14).

Here we report the first successful application of a conventional flow cytometer for the detection and sorting of microchromosomes smaller than 3Mb using chromosomes prepared from a human cell line using a modified polyamine isolation buffer (15).

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MATERIALS AND METHODS

Cell Culture

Chromosomes were prepared from a cell line (B5-3) derived from human fibrosarcoma cell line, HT1080. B5-3 contains a pair of ~2.7Mb X-centromere based microchromosomes generated using an approach involving telomere-associated chromosome fragmentation (10,16,17).

The cell line was cultured in DMEM (Gibco) medium supplemented with 15% fetal bovine serum (FBS, Gibco) and 500μg/ml of Geneticin (Invitrogen). The cell line was treated with demecolcine (0.1μg /ml) for 6hr after subculturing for 24hr.

Chromosome Preparation and Staining

Chromosomes were prepared as described previously (15) and stained overnight with Hoechst 33258 (Sigma) and Chromomycin A3 (Sigma). The stained chromosomes were treated with 25mM of sodium sulphite an hour before flow analysis.

Flow Cytometric Analysis and sorting

Stained chromosome suspensions were analysed on a flow cytometer (MoFlo®, DAKO) as described previously (15). In addition to Hoechst and Chromomycin fluorescence, forward scatter and pulse width parameters were collected. A region (R1, Figure 1D) was created on the plot of linear Forward Scatter (FSC) versus linear Pulse Width to exclude clumps and debris and bivariate plots of Hoechst versus Chromomycin fluorescence were gated on this region. A total of 100,000 events were acquired for the cell line at a data rate of 1000 events per second. Data collected from the experiments were analysed using Summit V3.1 (analysis software from DAKO).

The stained chromosome suspension was flow sorted at a data rate of 10,000-15,000 events per second with an optimal setting of the sheath pressure of ~60 psi and drop drive frequency to ~95 KHz using a 70μm Cytonozzle tip on a high purity sort option of single mode per single drop envelope. The microchromosomes were flow sorted into sterile 500μl Eppendorf tubes containing 33μl of sterile UV treated distilled water.

Verification of microchromosome peak

The purity of the flow sorted microchromosome peak was assessed by preparing a chromosome paint as described previously (18,19) from 500 sorted microchromosomes amplified using partially degenerate primers (DOP-PCR). The chromosome paint was directly labelled with Cy3 and reverse painted onto metaphase spreads of the human cell line containing the 2.7 Mb microchromosomes.

RESULTS

Data analysis and gating

Typical bivariate and univariate flow karyograms are shown in Figure 1. The modified polyamine isolation buffer produced ungated flow karyotypes in which all but the microchromosome clusters were well separated and resolved (Figure 1A). The microchromosome cluster (M) was buried in the debris (insert figure 1A and figures 1B and 1C). Back gating of the pulse width plot using regions of the bivariate flow karyogram revealed that chromosomes (and microchromosomes) were contained within the major peak of pulse width measurements with a non-linear correlation between chromosome size and pulse-width length (data not shown). The majority of the debris demonstrated shorter pulse width and aggregates greater pulse width. To discriminate clumps and debris, we applied a

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gate on the FSC versus pulse width plot (region gate R1, Figure 1D). This additional gating led to an improvement in the resolution of microchromosomes such that, after gating, the microchromosome peak could be identified as a clear and distinct cluster (inset Figure 1E) or as a single individual peak in univariate plots (Figures 1F, 1G).

Peak verification

Verification of the microchromosome peak (M) was carried out by painting back the probes derived from the flow sorted microchromosome onto metaphase spreads of the B5-3 cell line. The painting probe from the sorted microchromosome peak hybridized to the two microchromosomes and centromeres of the X chromosomes as expected with no discernable additional signals.

DISCUSSION AND CONCLUSIONS

The sorting of microchromosomes has been reported previously only for microchromosomes larger than 20 Mb in size (5-8,20). The potential of large scale purification of mammalian artificial chromosomes for gene therapy applications (8,13,14,20-23) has generated the need for a better method for the preparation and isolation of chromosomes of smaller microchromosomes with improved purity.

The use of a modified PAB buffer with exclusion of NaCl as previously described (15) improves the resolution of flow karyotypes and facilitates the separation of microchromosomes. The amount of debris produced in chromosome preparations was reduced in the absence of NaCl in the PAB buffer and a further improvement in resolution was achieved by removal of sodium citrate in the staining step (data not shown). More importantly, we found that the separation of the microchromosomes from the debris region was improved significantly upon application of a logic gate based on FSC versus Pulse Width (see figure 1E, 1F and 1G) rather than our usual gating based on low forward scatter and high Hoechst fluorescence (data not shown). Using this region gating, we were able to flow sort microchromosomes smaller than 3 Mb with high purity and yield. The purity of the sorted microchromosomes was confirmed by hybridisation onto B5-3 metaphases using a chromosome paint prepared from the flow sorted microchromosomes.

Using this chromosome preparation and gating strategy, we were able to flow sort approximately 100,000 microchromosomes with high purity in an hour at a data flow rate of more than 10,000 events/sec from a population of microchromosomes which made up on average just 0.6% of the total events.

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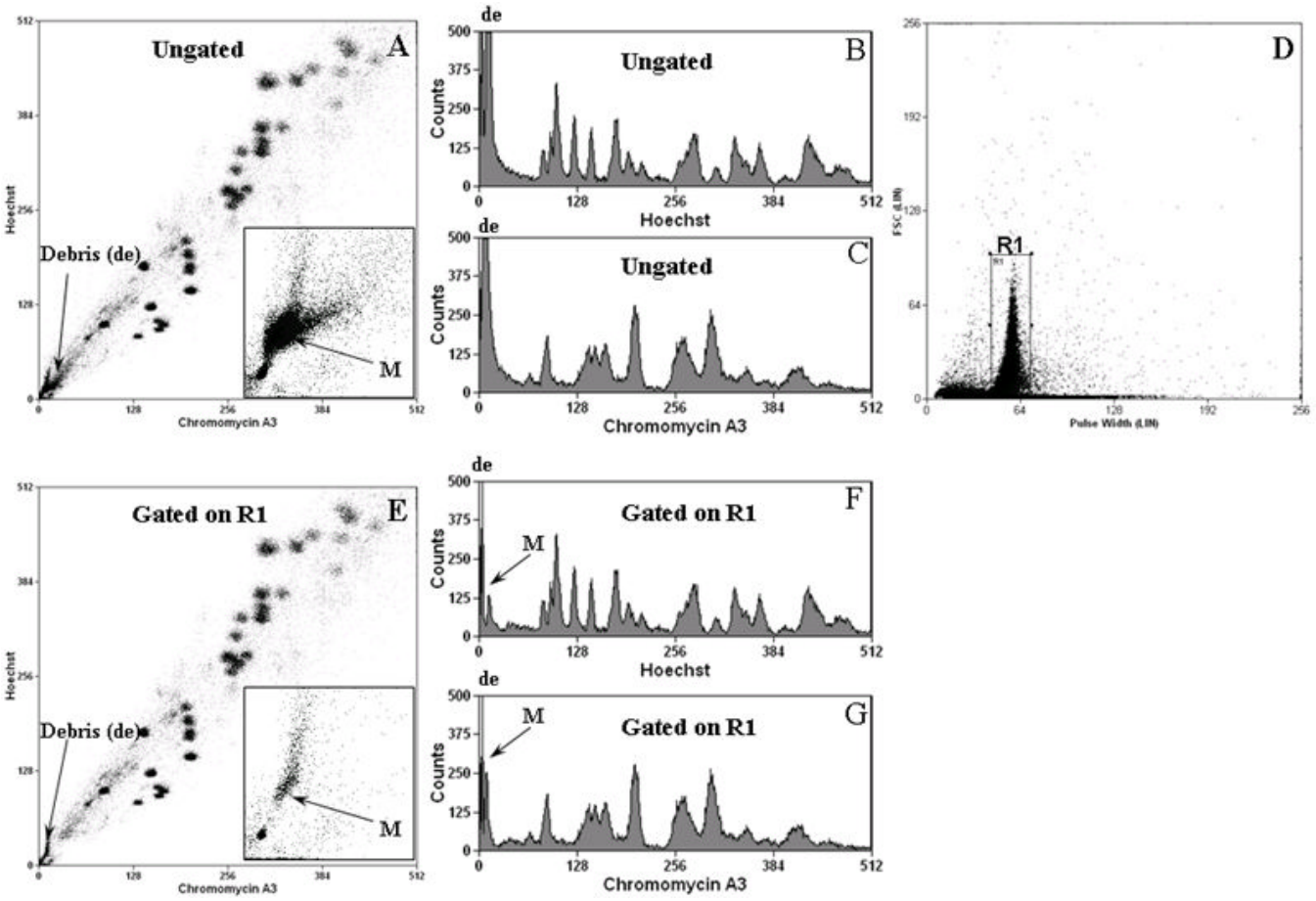

Fig.1.

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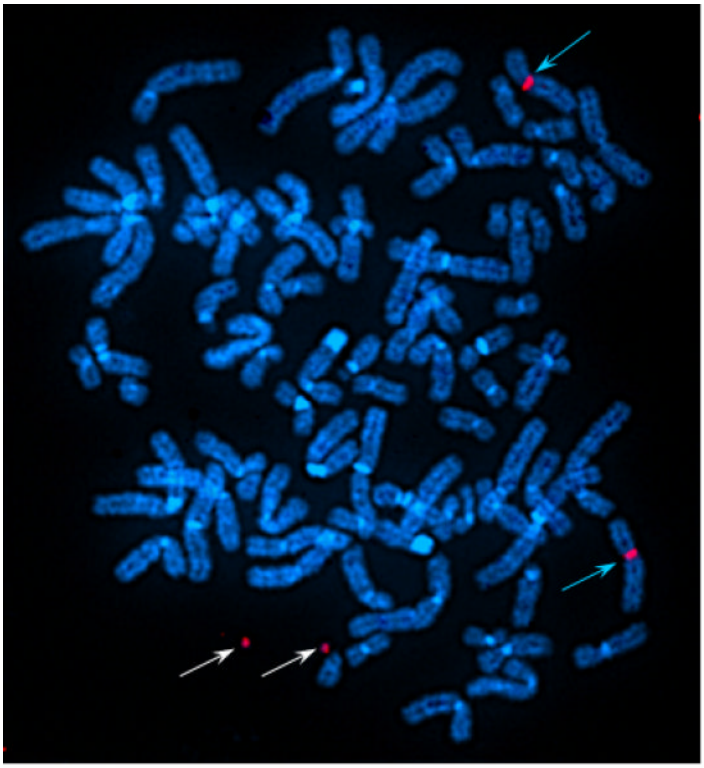

Fig.2.

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