Application Note 4

Detection of X and Y Chromosomes by Fluorescence In Situ Hybridization (FISH) in Whole Cells Sorted on the Basis of Surface Immunophenotype

Introduction

Many new technologies, such as the polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH), have been developed to study cell structure, genomes, and gene expression. Application of these techniques to study enriched or purified cell populations is useful in many areas of basic and clinical research. Enrichment or purification can be especially useful in experiments in which the frequency of targets is very low, or in which the background is too high. Enrichment also allows correlation of the properties used to enrich (eg, surface phenotype) with the property being studied (eg, chromosome presence). One very powerful enrichment or purification technique is cell sorting.

High-purity particle or cell sorting is useful in many fields of research. One of the most frequently used sorting criteria is the surface immunophenotype of whole cells. In this note, we demonstrate that purified cell populations, sorted on the basis of surface immunophenotype using the FACSort™, can be analyzed successfully by FISH.* The use of a two-color X and Y probe mixture is a good model for many FISH applications that require a count of whole chromosomes or specific chromosome segments of interest.

* For research use only. Not for use in diagnostic or therapeutic procedures.

Materials and Methods

Cell Preparation and Sorting

A mixture of two cell types was prepared for sorting: peripheral blood mononuclear cells (PBMCs) from a female donor isolated using Ficoll-Paque (Pharmacia, Piscataway, NJ) and cells from the male B lymphoblast Daudi[1] cell line (ATCC #CCl 213). Approximately 2 x 10^6 PBMCs and 10^6 Daudi cells were mixed and stained with CD20 (Leu™-16) peridinin chlorophyll protein (PerCP), CD3 (Leu-4) phycoerythrin (PE), and CD8 (Leu-2a) PerCP. Preparations were made in triplicate, then pooled and resuspended in phosphate-buffered saline (PBS; BioWhittaker Products, Walkersville, MD) to give a final concentration of 4 x 10^6 cells/mL.

The FACSort amplifiers were set with forward scatter and side scatter in linear mode and fluorescence (FL)2 and FL3 in log mode. The spectral overlap of PE into the FL3 channel was subtracted by setting the FL3-%FL2 compensation. No FL2-%FL3 compensation was needed, because PerCP has virtually no emission in the FL2 channel. Two sorts were performed, one to isolate the $CD3^+CD8^-$ PBMCs and one to isolate the $CD20^+$ Daudi cells (see Figure 1). The PBMCs were sorted using a gate that was the logical combination (R1 and R2) of a lymphocyte scatter region (R1) and a $CD3^+$, $CD8^-$, and $CD20^-$ region (R2). Daudi cells were sorted using a combination (R3 and R4) of a scatter region (R3) and a $CD20^+/CD3^-$ region (R4).

Cells were sorted in the Single Cell sort mode on the LO flow rate into polypropylene 50-mL conical tubes coated with 4% bovine serum albumin (see *FACSort User's Guide,* Chapter 4). Approximately 2 x 10^5 $CD3^+CD8^-$ lymphocytes were sorted at a rate of 80 cells/s. Approximately 4 x 10^5 Daudi cells were sorted at a rate of 100 cells/s. Filled tubes were stored on ice until the sort was complete. Cells were recovered by centrifugation at 300 x *g* for 5 minutes. The cell pellets were resuspended in PBS, then pooled and counted. No measurable decrease in viability was seen using trypan blue exclusion. Each sorted cell type was reanalyzed on the FACSort.

Slide preparations were made of the unsorted stained cell mixture, the sorted lymphocytes, and the sorted Daudi cells. Sorted lymphocytes were at 2.3 x 10^5 cells/mL, Daudi at 2.5 x 10^5 cells/mL, and the unsorted cell mixture was diluted in PBS to 5 x 10^4 cells/mL. Cytocentrifuge preparations (100 µL each) were prepared as described in *Sorting Application Note 1*. Slides were air-dried for 15 minutes, fixed in the appropriate paraformaldehyde (PFA) concentration for 1 minute at room temperature, then washed twice in PBS for 1 minute and fixed in 70% ethanol.

For successful FISH results, optimization of the specimen preparation was important. The best fixation condition for PBMCs was not the best for Daudi cells. The best compromise for preparation of the unsorted cell mixture was to fix in 2% PFA (prepared fresh on the day of the sort) for 1 minute.

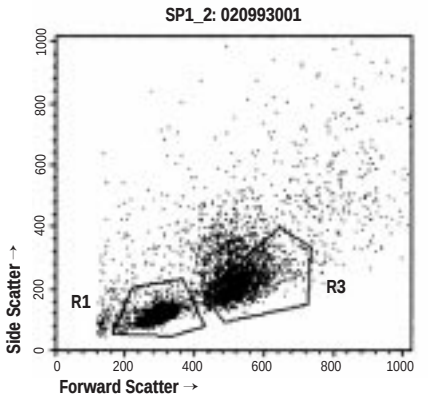

Figure 1a

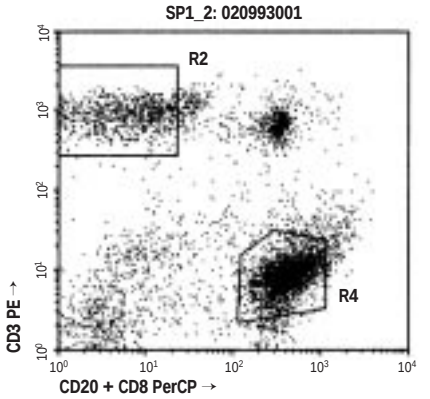

Figure 1b

Figure 1 *Dot plots of forward scatter versus side scatter (1a) and PE fluorescence (FL2) versus PerCP fluorescence (FL3) (1b) for a mixture of Daudi cells and female PBMCs. The mixture was stained with CD20 (Leu-16) PerCP, CD3 (Leu-4) PE, and CD8 (Leu-2a) PerCP. $CD8^-$ T cells were sorted using G1=R1 and R2, Daudi cells were sorted using G2=R3 and R4.*

Probe

Chromosome X^2 and Y^3 probes were derived from an alphoid repetitive sequence mapping to the centromere of each chromosome. The X probe was labeled with digoxigenin-11-dUTP and the Y probe with biotin-16-dUTP (Boehringer-Mannheim, Indianapolis, IN) by PCR incorporation.

Hybridization

Slides were dehydrated through 90% and 100% ethanol baths for 3 minutes and air-dried. Prewarmed slides were immersed for exactly 2 minutes in denaturing solution (70% formamide, 2X saline sodium citrate [SSC]) at 70°C. Slides were transferred to 70% ethanol for 3 minutes, followed by 90% and 100% ethanol for 3 minutes each, then air-dried.

An aliquot of probe mixture was lyophilized in a speed-vac and resuspended in 3 µL of deionized water to which 7 µL of hybridization mix (1 g dextran sulfate, 5 mL formamide, 1 mL 20X SSC [pH 7.0], adjusted to 7 mL) was added. The probe solution was vortexed, centrifuged at top speed in a microfuge, denatured by heating for 5 minutes in a 70°C water bath, and transferred to 37°C for 15 minutes.

Slides were placed on a slide warmer (~40°C) and 10 µL of denatured DNA probe mix was applied. The probe was immediately covered with a 22-mm square glass coverslip and sealed with rubber cement. Slides were placed in a humidified chamber and incubated overnight at 37°C. After the rubber cement was removed, coverslips were loosened in post-hybridization wash (50% formamide, 2X SSC) and removed. Slides were washed three times for 5 minutes in post-hybridization wash at 50°C, with mild agitation. Slides were washed at room temperature for 5 minutes each in 2X SSC, then in 0.1 M phosphate buffer, pH 8.0, with 0.1% NP-40, abbreviated here as PN.

A 1.5-mL aliquot of blocking solution (5% nonfat dry milk in PN with 0.05% sodium azide) was centrifuged in a microfuge tube at 15,000 rpm for 10 minutes, and 50 µL of supernatant was applied to each hybridization region. This region was then carefully covered (no air bubbles) with a parafilm coverslip. The slides were incubated for 5 minutes at room temperature.

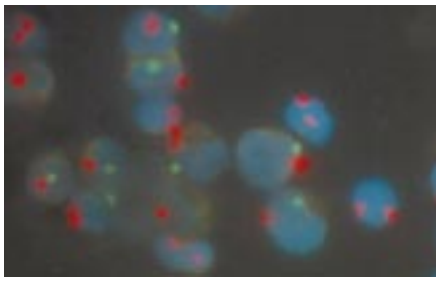

Figure 2 *Mixture of female PBMCs and male Daudi cells cytocentrifuged, fixed as whole cells, and hybridized with chromosome X (red) and chromosome Y (green) probes. Cells were counterstained with DAPI (blue). Photomicrograph was taken at 63X magnification through a triple band–pass filter for concurrent visualization of the three fluorescent dyes.*

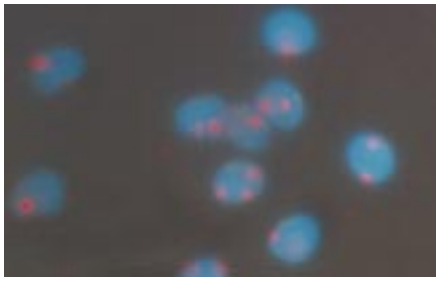

Figure 3 *Female PBMCs (T-cell subset) sorted from the PBMCs/Daudi cell mixture, prepared, hybridized, and photographed as in Figure 2. The cells have two red X signals, and are negative for the green Y signal, as expected.*

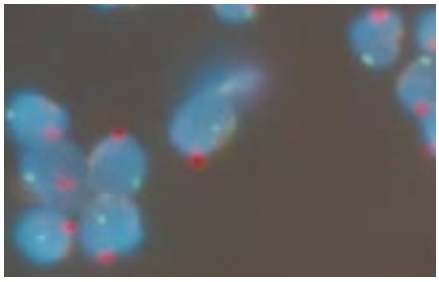

Figure 4 *Male Daudi cells sorted from the PBMC/Daudi cell mixture, prepared, hybridized, and photographed as in Figure 2. As expected, the male Daudi cells have one red X signal and one green Y signal per cell.*

The following steps were performed in a minimal light environment. The blocking solution was drained, 50 µL of 5 µg/mL fluorescein isothiocyanate (FITC)-avidin (Boehringer-Mannheim) was applied, and the hybridization region was covered with a parafilm coverslip. The slide was placed in a moist, dark chamber and incubated for 20 minutes at room temperature. Each slide was washed in PN at room temperature for 3 minutes, then 50 µL of diluted (5 µg/mL) rhodamine-anti-digoxigenin Fab (Boehringer-Mannheim) was added as above. Each slide was washed at room temperature: PN for 3 minutes, 4X SSC for 10 minutes, 4X SSC + 0.1% Triton X-100 for 10 minutes, 4X SSC for 10 minutes, and then 1X SSC for 1 minute. The slides were incubated with 4',6-diamidino-2-phenylindole (DAPI) (20 ng/mL in 1X SSC) for 30 seconds at room temperature and rinsed in 1X SSC for 1 minute at room temperature. About 20 µL of antifade mounting solution (1 mg/mL p-phenylenediamine dihydrochloride in glycerol, 0.1% ascorbic acid, pH 8.0) was added to each hybridization area, and the area was covered with a glass coverslip.

Results

Samples were hybridized with a probe mixture for the simultaneous detection of chromosomes X and Y in two colors. Slides were scored by counting a minimum of 100 cells at the microscope. Figure 2 is a photomicrograph of the unsorted cell mixture hybridized with the probe combination. The smaller PBMCs have two red dots from the X probe, and the larger Daudi cells have one red and one green signal. Figure 3 shows the FISH results of the population sorted for $CD8^{-}CD3^{+}$ and lymphocyte light-scattering properties. For this sample, 98 of 100 cells scored were female PBMCs. Figure 4 shows the FISH results of the cell population sorted for $CD20^{+}CD3^{-}$, high forward and low side scattering characteristics. Photomicrographs are a record of the image at one focal plane, so many of the signals will appear to be missing or large and out of focus; however, every cell showed the expected hybridization signals when viewed at the microscope. The sample contained virtually pure (100 of 100 counted cells) male Daudi cells, each with one red X signal and one green Y signal. The FISH results concurred with the purity estimates from reanalysis of the two sorted populations: greater than 97% purity for the sorted PBMCs and about 97% $CD20^{+}$ for the sorted Daudi sample.

Discussion

We have shown that sorted cell samples recovered from the FACSort are suitable for FISH analysis. Successful FISH requires optimization of the sample preparation and hybridization conditions, depending on the cell types sorted and the probes used for studies. However, the capability of applying new technologies to study samples sorted at high purity will enhance the progress of research and the development of novel assays.

Hints

- Different cell types require different fixation conditions for FISH staining. It is important to establish the appropriate conditions for your sample, usually by varying the paraformaldehyde concentration, fixative, and/or fixation time. We have found that the optimum fixation conditions for sorted cells are frequently less vigorous (that is, shorter time or lower paraformaldehyde concentration) than for the same cells when they have not been sorted.
- Slides should not be allowed to dry during the detection procedure, because the resulting salt crystals cause nonspecific binding of the detection reagents.
- Optimal concentrations of each probe were determined empirically. Each lot of the second step development reagents should be titered for optimal results.

BDIS publishes this method as a service to investigators. Detailed support for non–flow cytometric aspects of this procedure may not be available from BDIS.

References

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2. Waye J, Willard H. Human X-linked alpha satellite repetitive DNA 2.0 kb repeat. *Nucleic Acids Res.* 1985;13:2731–2743.

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