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White Paper

Twelve-color Chimerism Analysis after HLA-mismatched Hematopoietic Cell Transplantation Using Anti-HLA Antibody and Spectral Flow Cytometer

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1. Abstract

During the early phase after hematopoietic cell transplantation (HCT) so-called mixed chimerism sometimes occurs in peripheral blood. Donor/recipient chimerism analysis using the HLA-Flow method is a very useful technique for early diagnosis of graft failure and relapse of leukemia and for elucidation of the underlying mechanisms in various pathogenic states after HCT. Reported herein are the results of several twelve-color chimerism analyses using the Spectral flow cytometer developed by Sony Corporation. The performance was equivalent to a common commercial instrument. Moreover, one of the unique features of this instrument is the ability to display each fluorescence spectrum for each cell, demonstrating the accuracy of analysis and guarantying further accurate analysis especially in multi-color analysis.

2. Introduction

Although HCT has been increasingly used to treat hematological malignancies, metabolic disorders and congenital immunodeficiencies, HCT is frequently complicated by graft failure and relapse of primary diseases. Since persistence or increase of recipient-derived hematopoietic or malignant cells has pathogenic import, analysis of donor/recipient chimerism should be useful to make early diagnosis of graft failure and relapse of primary-disease and to understand the mechanisms of these pathogenic conditions. Polymerase chain reaction (PCR)-based short tandem repeat analysis and X/Y chromosome analysis using fluorescence *in situ* hybridization (FISH) after gender-mismatched transplantation are the methods most commonly used for chimerism analysis. PCR- or FISH-based methods, however, are complicated, insensitive, and time-consuming. In addition, we cannot directly analyze lineage-specific mixed chimerism. If donor- and recipient-derived cells have specific surface markers which can be stained by fluorescence-conjugated antibodies, it is possible to perform chimerism analysis by flow cytometry in a rapid, quantitative, and highly sensitive manner. Since the number of HLA-mismatched transplantations, e.g. cord blood transplantation or haplo-identical HCT, has increased recently, we have developed a flow cytometry-based method of chimerism analysis using fluorescence-conjugated anti-HLA mAbs after HLA-mismatched transplantation (Figure 1). Since we can analyze lineage-specific chimerism in a rapid, quantitative, and highly sensitive manner, HLA-Flow is a very useful method compared with present methods. Due to the expansion in the application of HCT, non-remission phase HCT has increased. In such situations, we need to detect leukemia cell in addition to lineage-specific chimerism among normal leukocytes. In our laboratory nine-color analysis is commonly used for monitoring engraftment. If residual leukemia cells are detected during the engraftment phase, we need more fluorescent colors to monitor engraftment and minimal residual disease simultaneously. Because of the limited availability of fluorescence-conjugated antibodies and a complicated compensation technique, analyses with ten or more colors are still very difficult for common flow cytometry users.

In order to solve the problems, we tried to evaluate a new cytometer developed by Sony Corporation (Tokyo, Japan) replaces filter-based optics with a spectral detection system based on a multi-anode spectral PMT.

3. Instrument configuration

The spectral flow cytometer is able to increase the number of analyzed colors and easily separates similar fluorescence spectra via a 32 channel linear array PMT (Figure 2). It is also able to measure and subtract varying autofluorescence, permitting increased signal to noise ratios and improving the resolution of dim signals. To obtain a dynamic range of fluorescence intensity detection, Sony newly developed the independently voltage-controlled 32channel PMT with high-sensitivity to detect from 500n to 800nm by using a series of prisms. The excitation lasers are laser diode (LD) with 488nm wavelength and 60mW power and a 638nm LD with 110mW. Multiple fluorochromes are mathematically unmixed by using component analysis.

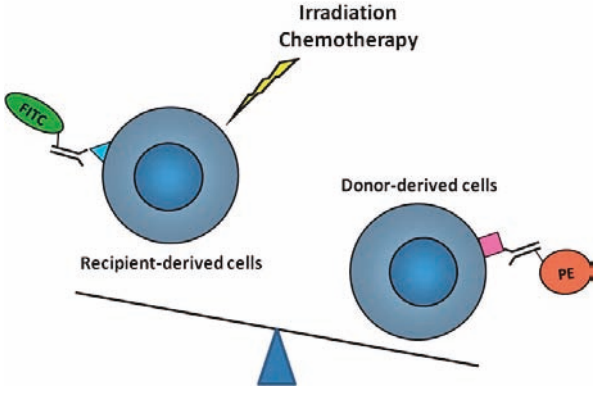


Figure 1. Flow cytometry-based method of chimerism analysis using anti-HLA mAbs

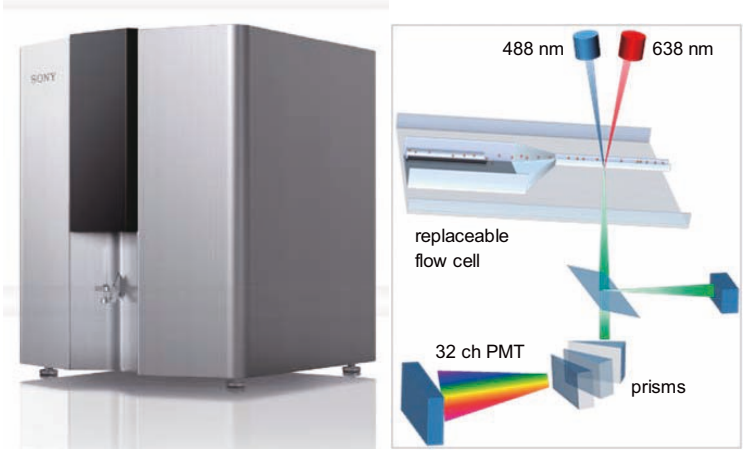


Figure 2. Next-generation spectral flow cytometry

4. Twelve-color chimerism analysis

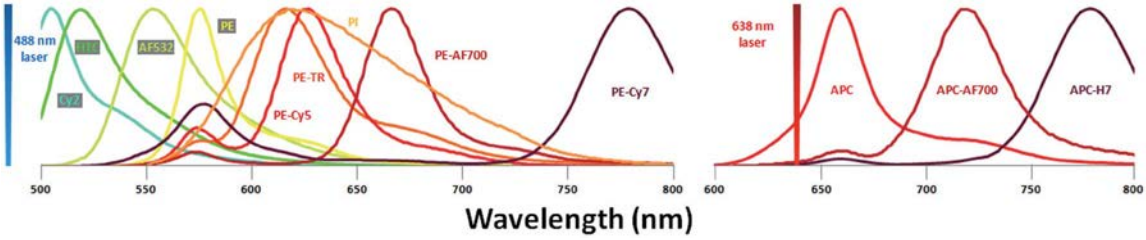
Twelve-color chimerism analyses using artificial chimerism samples composed of cord blood and healthy individual's blood were carried out a total of 3 times to verify the performance and equivalence between the spectral flow cytometer and a conventional flow cytometer. Cord blood was provided from Japanese Research Cord Blood Bank. We determined the type of HLA using SRL, Inc. contract service before this experiment. HLA-A2 positive and HLA-A24 negative cord blood was used for experiments. On the other hand, peripheral blood from healthy individuals with HLA-A2 negative and HLA-A24 positive was used. We selected twelve-color immunostainings to determine the lineage-specific chimerism of hematopoietic cell subsets: HLA-A2/HLA-A24 for chimerism, CD34/CD45 for hematopoietic stem/progenitor cells or leukemia cells, and CD3/CD4/CD8/CD14/CD19/CD56 for normal leukocyte subsets (Figure 3).

Since the spectral flow cytometer is equipped with only two lasers, 488 and 638 nm lasers, it appears to be a little bit harder to analyze twelve-color samples simultaneously. However, because of the combination of prism optics, 32ch array PMT, and Spectral Deconvolution, the spectral flow cytometer could separate twelve-color samples with extraordinary fluorochromes and their combination such as Cy2, PE-Alexa Fluor 700, PE-TR/PI, and PE-Cy5/APC (Figure 4).

The frequencies of each cell population by the number of CD45$^+$ population were calculated and compared between both instruments. Acquired by the spectral flow cytometer, hematopoietic stem/progenitor cell population and white blood cell subsets were clearly determined and equivalent to data from the common commercial instrument. Figure 4B&C shows spectra charts of the measurement results of twelve-color chimerism analysis excited with 488 and 638 nm lasers, respectively. We can see many lines derived from each fluorescent reagent. We can also confirm each spectrum shape derived from each cell one by one. This unique and useful feature enables us to make more accurate judgment, which is especially important for both multi-color analysis and rare cell analysis.

5. Conclusion

Twelve-color chimerism analyses using 3 artificial chimerism samples composed of cord blood and healthy individual's blood were carried out to verify the performance of the spectral flow cytometer. The frequencies of each subpopulation of hematopoietic stem/progenitor cells, mature white blood cells, and lineage-specific chimerism acquired by the spectral flow cytometer were clearly determined and were equivalent of data from a common commercial instrument. Moreover, one of the unique feature of the spectral flow cytometer, display of each spectrum derived from the fluorescence of each cell, demonstrated the accuracy of analysis and guarantee for further accurate analysis especially in multi-color analysis.



Fluorochrome	FITC	PE	PI	APC-AF700	APC	APC-H7	AF532	PE-AF700	PE-TR	PE-Cy5	Cy2	PE-Cy7
Stain	HLA-A24	HLA-A2	Dead cell	CD45	CD34	CD14	CD3	CD4	CD8	CD19	CD16	CD56

Figure 3. Spectra of fluorochromes: excited with 488 nm laser (A), 638 nm laser (B)
Table. Fluorochrome panel for twelve-color chimerism analysis

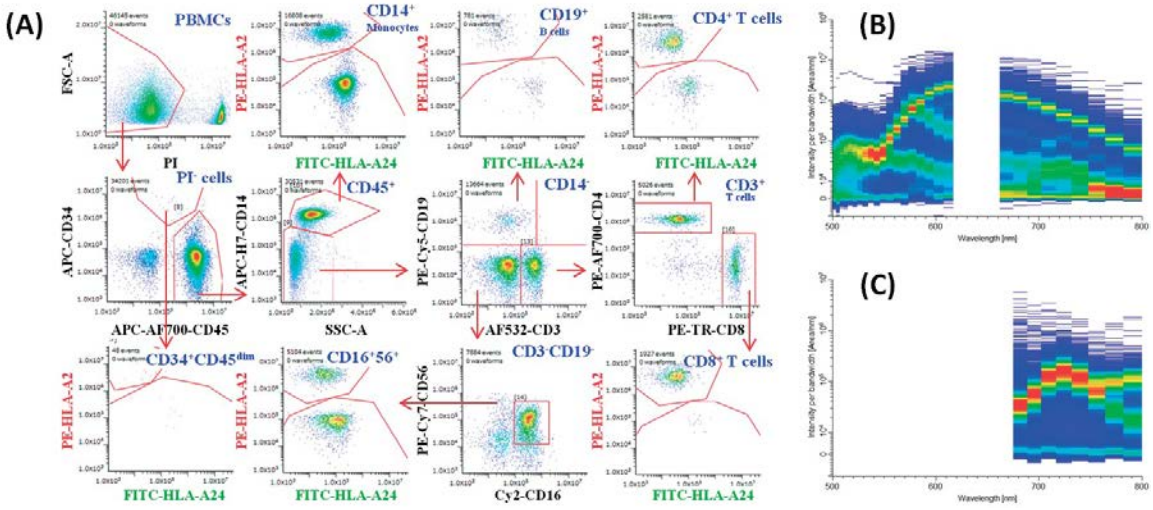


Figure 4. Result of twelve-color chimerism analysis (A),
Spectra chart of sample stained with twelve-color fluorochromes excited with 488 nm laser (B),
638 nm laser (C)

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