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Notes & Tips

Multiplexed labeling system for high-throughput cell sorting

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ABSTRACT

Flow cytometry and fluorescence activated cell sorting techniques were designed to realize configurable classification and separation of target cells. A number of cell phenotypes with different functionalities have recently been revealed. Before simultaneous selective capture of cells, it is desirable to label different samples with the corresponding dyes in a multiplexing manner to allow for a single analysis. However, few methods to obtain multiple fluorescent colors for various cell types have been developed. Even when restricted laser sources are employed, a small number of color codes can be expressed simultaneously. In this study, we demonstrate the ability to manifest DNA nanostructure-based multi-fluorescent colors formed by a complex of dyes. Highly precise self-assembly of fluorescent dye-conjugated oligonucleotides gives anisotropic DNA nanostructures, Y- and tree-shaped DNA (Y-DNA and T-DNA, respectively), which may be used as platforms for fluorescent codes. As a proof of concept, we have demonstrated seven different fluorescent codes with only two different fluorescent dyes using T-DNA. This method provides maximum efficiency for current flow cytometry. We are confident that this system will provide highly efficient multiplexed fluorescent detection for bioanalysis compared with one-to-one fluorescent correspondence for specific marker detection.

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Flow cytometry has been used for effective analysis of analogous biological markers that represent the lineage or state of target cells [1,2]. Biological markers are labeled with specific targeting molecules (also referred to as probing molecules). Targeting molecules (e.g., monoclonal antibodies, aptamers) are tagged with fluorescent dyes for facile detection [3,4]. As cells flow within a narrow orifice tube in the machine, fluorescence signals are very precisely detected. Cells in various states can be distinguished based on the fluorescent dye tags attached to each cell. Such technology is often applied in bioresearch fields, including cancer diagnosis, immune activation, and stem cell differentiation analysis [5–9]. Fluorescence activated cell sorting (FACS) was developed as a combination of the analysis tools of flow cytometry and cell sorting to facilitate the separation of specific cells [10]. By following the biological markers of target cells distinguished by flow cytometry, a user can designate the separation of targets with a specific phenotype [11,12]. Recent progress in evolutionary biology has revealed a number of cell phenotypes, and the ability to distinguish each type is technically critical for advanced cell biology [13,14]. Thus, there is a growing demand to introduce multiple sorting techniques.

To make this method very effective, the emission signals of a variety of fluorescent dyes could be adopted, and these dyes should be differentiated using a photomultiplier tube (PMT) in a FACS machine. However, the few fluorescent dyes available prohibit multiplexed detection of several markers, which could enable disease targeting or multiplexed cell sorting. This may be due to broader overlap of the wavelengths of several commonly used dyes. A few fluorescent dyes can be excited using the same laser source, whereas a single PMT detects many fluorescent emission signals at the same time. However, multiplexed detection is hindered due to limits in the number of dyes that can be used simultaneously because multiple dyes make it difficult to identify specific signals.

To overcome the shortcomings associated with adopting multiple fluorescent dyes through FACS operation, several methodologies have been developed [15]. First, multiple laser sources have

Abbreviations used: FACS, fluorescence activated cell sorting; PMT, photomultiplier tube; PE/Cy5, phycoerythrin and Cy5; Y-DNA, Y-shaped DNA nanostructure; T-DNA, tree-shaped DNA; GEMSA, gel electrophoretic mobility shift assay; UV, ultraviolet.

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been installed, enabling a broader excitation range in the FACS machine. Second, emission spectra originating from different fluorescent dyes have been measured and input into the FACS software such that overlapping signals can be subtracted from one another, leaving only one signal source. This compensation (by signal subtraction) may enable the differentiation of two fluorescence signals even though a portion of the emission spectra overlaps. Third, in addition to these advancements in both hardware and software, there have been attempts to overcome the shortcomings caused solely by the inherent characteristics of fluorescent dyes (e.g., overlapping colors). For example, tandem dyes that contain two excitable molecules have been developed [16,17]. By attaching some functional molecules to a fluorescent dye, it is possible to influence the emission and excitation spectra. However, previous researchers have speculated that the emission spectrum of one single molecule in a tandem dye can interfere with that of an excitation spectrum of another molecule through a Förster resonance energy transfer [18]. A combination of phycoerythrin and Cy5 (PE/Cy5) is a representative tandem dye that shares one excitation wavelength at 496 nm, whereas Cy5 itself has an excitation wavelength of 648 nm. These kinds of tandem dyes may allow for potential combinations of fluorescent dyes that operate by shifting their emission spectra at a constant excitation wavelength. However, employing a number of laser sources in a single machine results in increased cost. In addition, when the spectra of two dyes broadly overlap, compensated signals may be too weak. Therefore, to achieve simultaneous detection, samples must be sorted in various steps. This is usually time-consuming and requires additional purifications. In this study, we present the development of a DNA nanostructure-based fluorescence color-coding system capable of multiplexed FACS analysis. Three oligonucleotides can be designed to have complementary base sequences to each other, self-assembling into a Y-shaped DNA nanostructure (Y-DNA), which can be further built up into tree-shaped DNA (T-DNA). This way, by using oligonucleotides whose 5′ termini are conjugated with fluorescent dyes, these DNA nanostructures can be programmed as a fluorescent-coding system at the molecular level [19–21]. Using a model system, we showed that several different fluorescent codes on DNA nanostructures are distinctly separated by FACS. Such a detection system could be employed for fluorescent dyes in FACS analysis.

We created three different Y-DNAs in which each single Y-DNA was simply composed of three different oligonucleotides. Bare oligonucleotides were intentionally modified via a 5-primed conjugation of some functional modules such as chromophores and bioprobes (e.g., biotin). As model fluorescent dyes for flow cytometric analysis, Alexa 488 and Cy5 were selected. These were preconjugated to the 5′ termini of oligonucleotides during oligonucleotide synthesis by the manufacturer. Y-DNAs were designed to possess different moieties at each end in order to provide anisotropicity in the DNA nanostructure. In addition, Y-DNAs were used to build T-DNA using the ligase enzymatic activity of overhangs (Fig. 1A). The sequences of each oligonucleotide are shown in Table S1 of the online supplementary material. The formation of T-DNAs was confirmed through a gel electrophoretic mobility shift assay (GEMSA) in which the band indicating T-DNAs was retarded slowly because of their higher molecular weights (Fig. 1B). Band shifting toward the upper position was clearly observed in the gel image based on the growth of T-DNA.

In a conservative FACS analysis, the fluorescent intensity from each dye is usually recorded as a single parameter for the corresponding molecule in the system. This intensity indicates the content of target samples, which is distinguished by an arbitrary signal intensity difference. However, it is possible to realize a multiplexed labeling system via anisotropic characteristics of assembled Y-DNA and T-DNA for FACS analysis. Along with end modification of Y-DNAs, it is possible to code the fluorescence color spectrum. Although the self-assembly of Y-DNA is highly specific and precise, it is possible to combine fluorescent dyes at the molecular level [22]. Thus, it is possible to distinguish fluorescent color-coded DNA nanostructures by specific fluorescence intensity ratio. Alexa 488 and Cy5 were used as model fluorescent dyes. These two fluorescents have individual excitation and emission peaks (excitation/emission: 496/519 nm [Alexa 488]; 648/670 nm [Cy5]) and are commonly used in FACS analytic tests. To expand the number of color patterns and decode the colors, we investigated the use of fluorescent Y-DNAs to construct T-DNAs as a multiplexed color-labeling platform. These T-DNAs were attached onto 5.2-μm silica beads via avidin–biotin chemistry. The bead was designated as a target cell, and the avidin–biotin was a model of a pair of target and probe. The fluorescence-tagged Y-DNAs and T-DNAs were visualized using GEMSA. The fluorescence color patterns were transilluminated under ultraviolet (UV) exposure. As a test model, either Cy5- or Alexa 488-labeled Y-DNAs and synthesized T-DNAs were evaluated (Fig. 1C). Under UV exposure, Cy5 and Alexa 488 demonstrated strong emission of red and green colors, respectively. In the case of Y-DNA, three different color codes were displayed: R, G, and RG. In GEMSA, the Cy5 (R) and Alexa 488 (G) combination, RG, appears as a yellowish (in the web version) color. With the T-DNA, a total of seven color combinations were displayed: RRRR, RRRG, RRG, RRGG, RGG, RGGG, and GGGG. Red and green colored fluorescence and their combinations were observed from each band, corresponding to the designated color emission patterns. The silica beads labeled with combined fluorescence on DNA

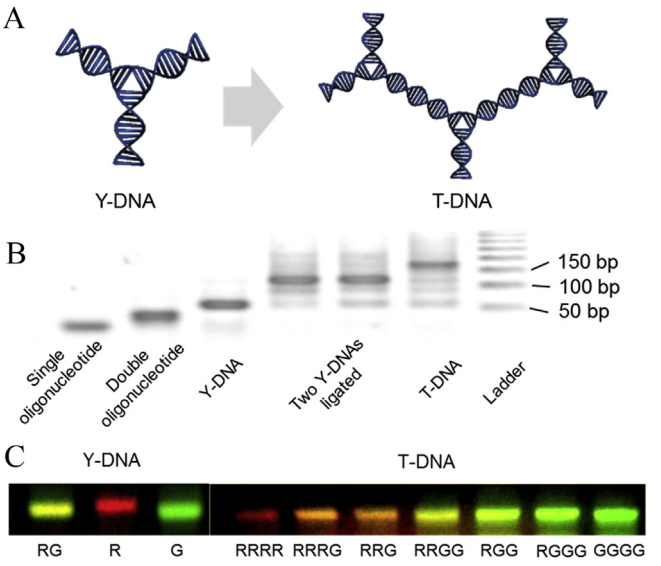

Fig. 1. Schematics of Y-DNAs and T-DNAs, which were confirmed by agarose gel electrophoresis. (A) Y-DNAs are composed of three different oligonucleotides, each having complementary sequences. T-DNA was built from Y-DNAs through enzymatic ligation of overhang sequences in each Y-DNA. (B) Stepwise increases in bands on the gel indicated the formation of DNA materials. The first three lanes from the left demonstrate the formation of Y-DNA (first and second lanes: incomplete Y-DNA; third lane: complete Y-DNA). The next three lanes indicate the formation of T-DNA, where the first two lanes are the cases where two Y-DNAs are attached and the third lane is the case where a complete T-DNA is formed. The last lane indicates the 50-bp ladder. (C) Color images were taken after gel electrophoresis; fluorescent color emissions were observed after UV exposure. Different colors were observed according to the ratio of fluorescent dyes on the DNA platform. From left to right: RG, RR, GG, RRRR, RRRG, RRG, RRGG, RGG, RGGG, and GGGG, where R and G indicate Cy5 and 6-FAM, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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