Published in final edited form as:

Methods Mol Biol. 2018 ; 1678: 49–77. doi:10.1007/978-1-4939-7346-0_4.

RNA Flow Cytometry using the Branched DNA Technique

Kah Teong Soh* and Paul K. Wallace

Roswell Park Cancer Institute, Department of Flow & Image Cytometry, Elm and Carlton Streets, Buffalo, NY 14263

Abstract

The systematic modulation of mRNA and proteins governs the complicated and intermingled biological functions of our cells. Traditionally, transcriptomic technologies such as DNA microarray and RNA-Seq have been used to identify, characterize, and profile gene expression data. These are, however, considered bulk methods as they are unable to measure gene expression at the single cell level, unless the cells are pre-sorted. Branched DNA is a flow cytometry-based detection platform that has been developed recently to measure mRNA at the single cell level. Originally adapted from microscopy, the current system has been modified to achieve compatibility with the detection of surface and intracellular antigens using monoclonal antibodies conjugated to fluorochromes, thus permitting simultaneous detection of mRNAs and proteins. The Branched DNA method offers a variety of advantages when compared to traditional or standard methods used for the quantification of mRNA, such as (a) the detection of specific mRNA on a per cell basis, (b) an alternate detection tool when the measurement of a protein is technically infeasible (i.e. no quality antibody exists) or the epitope is not assessable, and (c) correlate the analysis of mRNA with protein. Compared to earlier attempts at measuring nucleic acid by flow cytometry, the hybridization temperature applied in the Branched DNA assay is much lower, thus preserving the integrity of cellular structures for further characterization. It also has greatly increased specificity and sensitivity. Here we provide detailed instruction for performing the Branched DNA method using it in a simple model system to correlate the expression of CD8 mRNA and CD8 protein by flow cytometry.

Keywords

RNA flow cytometry; Branched DNA; CD8 protein and mRNA correlation; *in-situ* hybridization; peripheral blood mononuclear cells

1. Introduction

The relationship between the expression of mRNAs and proteins during the homeostatic regulation of physiological processes and clinical manifestations of disease, such as cancer and inflammation, cannot be separated. Following the transcription of DNA, the stability and eventual translation of mRNA are regulated through a series of modifications including its

*Corresponding Author: Kah Teong Soh, Roswell Park Cancer Institute, Department of Flow and Image Cytometry, Cancer Cell Center Room 315A, Elm and Carlton Streets, Buffalo, New York 14263, Voice: 716-845-8471, Fax: 716-845-8806, kahteong.soh@roswellpark.org.

exportation to the cytoplasm, splicing, capping, and the addition of poly(A) tails, serving as a switch to control the abundance of protein in cells at any given time [1,2]. Studies by separate investigators have shown that the expression levels of mRNA and protein can be utilized as independent prognostic indicators to unravel interesting and important information associated with malignancy and viral diseases. For instance, Patterson and his group found that while both infected CD4⁺ T lymphocytes and CD14⁺ monocytes sheltered HIV, transcriptional activity was only observed in monocytes, even during late stage disease progression [3,4]. Another example is the detection of IFN- γ mRNA in activated lymphocytes, in which the mRNA expression was found to precede protein expression by approximately 90 minutes [5].

There are several methods to profile gene expression including Next-Generation Sequencing, reverse transcriptase polymerase chain reaction (RT-PCR), Northern blotting, and nuclease protection assays which have been historically employed for mRNA measurements. A significant limitation of these methods lies in the fact that an average value is generated. Measuring gene expression with a multiparametric technology such as flow cytometry is an attractive alternative to bulk methods because it can be used to study the intricacies within the sample. For example in neoplasia, it is extremely challenging if not impossible without cell sorting to determine if the detected mRNA originated from the tumor cells, associated stroma, or from infiltrating leukocytes as soon as the biopsy sample is homogenized. Consequently, not only can the characteristics of collected cells be distinguished on a cell-by-cell basis, but a flow-cytometric approach, being quasi-quantitative, permits the investigator to examine heterogeneity within related and unrelated cells. In addition, large numbers of events can be reliably collected by flow cytometry thus generating the appropriate statistical sampling power which is especially important in rare cell event analysis.

Barbara Trask and her colleagues were the first to detect DNA by flow cytometry [6]. They cross-linked nuclear proteins using dimethylsuberimidate to prevent nuclei disintegration during denaturation and hybridization. These stabilized mouse nuclei were then hybridized with a probe specific for mouse satellite DNA sequences, which comprise approximately 10 percent of the total DNA in mouse nuclei. The probe had been treated with N-acetoxy-2-acetylaminofluorene (AAF) which modified approximately 20% of the guanine bases to N-(guanine-8-yl)-AAF. This was followed by an indirect immunofluorescent labeling procedure (rabbit anti-AAF followed with goat anti-rabbit Ig conjugated with rhodamine) to quantify both the amount of hybridized probe and DNA content (using Hoechst 33258) at the single cell level. Other investigators subsequently integrated PCR with flow cytometry to improve signals by amplifying the target sequence [7,8]. These investigators also developed a biotin-streptavidin detection system to detect surface antigens, labeling before the PCR a cell surface epitope with a biotinylated antibody and then detecting with a fluorescently-labeled streptavidin after the PCR. Early efforts to detect RNA used a fluorescence in situ hybridization (FISH) approach. Patterson et. al. employed a cocktail of different 5-carboxyfluorescein-labeled oligonucleotide probes which in aggregate hybridized to the majority of expressed HIV RNAs [4]. They combined this with the previously mentioned biotin-streptavidin detection system to phenotype cells. Studies from Alan Waggoner's lab [9] were the first to detect multiple RNA species using three different 5' cyanine-labeled

oligodeoxynucleotides probes (e.g. Cy5.18, Cy3.18, and OCy3) to simultaneously detect Histone H4 mRNA, 18S rRNA, and 28S rRNA in HeLa cells.

Unfortunately, these early successes to measure nucleic acid by flow cytometry presented several impediments to progress. First, the success was limited to the detection of nucleic acids that were highly abundant and repetitive in nature. A second major problems with this approach was the increased autofluorescence caused by the high temperatures required for annealing and hybridization and then the subsequent loss of scatter characteristics. Third, the PCR protocol compromised the fluorochromes used for detection of surface antigens. And finally, the use of the biotin-streptavidin detection system restrained the immunophenotyping to just one antigenic determination at a time.

In early 2010, two microscopy-based mRNA detection platforms were adapted to flow cytometry allowing a correlated determination of mRNA and protein. The SmartFlare™ (EMD Millipore) system employs gold nanoparticles conjugated to oligonucleotides with specificity complementary to an mRNA of interest. Bound to the complementary oligonucleotide strand is a short fluorescently-labeled reporter strand whose fluorescence is quenched by the gold nanoparticle. When placed into culture with cells, the gold nanoparticles are taken into the cytoplasm, a process dependent entirely on the endocytic machinery of the cell. If the mRNA of interest is present inside the cell, it will displace the lower-affinity fluorescently-tagged reporter strand from the complementary oligonucleotide. Consequently, the reporter oligonucleotide is no longer quenched and the emitted fluorescence can be detected by the flow cytometer. To date, the SmartFlare™ system is the only methodology known to work with live cells for sorting and subsequent downstream assay but is limited to only Cy3 and Cy5 fluorescent probes.

Alternatively, PrimeFlow™ (Thermo Fisher Scientific), generically referred to here as the 'Branched DNA' method can be used to determine the differential mRNA expression within a heterogeneous population of cells. Originally adapted from microscopy, the current system has been modified to achieve compatibility with the detection of selected antigens using monoclonal antibodies conjugated to fluorochromes, thus permitting simultaneous detection of mRNA and protein at the single cell level. In brief, cells are initially fixed and permeabilized using a proprietary 'Fixation Buffer' to preserve mRNA transcripts (Fig. 1). The sample is then exposed to the first hybridization step, during which a pair of mRNA-specific oligonucleotide Target Probes are annealed to a sequence, approximately 50 base pairs in length, on the target mRNA of interest. An mRNA sequence with 1,000 bases can accommodate up to 20 different pairs of Target Probes, which serve as the foundation for signal amplification. To increase the specificity of the assay, each pair of Target Probes must hybridize in close proximity to each other before signal amplification can occur. The specificity is insured by the addition of 'Pre-Amplifiers', as these molecules will only form a stable complex when both Target Probes have hybridized appropriately. The detection system is further magnified using 'Amplifiers', which hybridize to multiple sites on each 'Pre-Amplifier'. Finally, multiple 'Label Probe' oligonucleotides conjugated to either Alexa Fluor® 488, Alexa Fluor® 647, or Alexa Fluor® 750 are hybridized to multiple sites on each 'Amplifier'. An optimally assembled Branched DNA complex can provide a theoretical 8,000- to 16,000-fold signal amplification of the targeted mRNA.

The advent of these flow cytometry-based mRNA detection platforms allow for many determinations that were previously unachievable. Notably, the ability of the technology to correlate the measurement between mRNA transcripts and antigen expression at the single cell level, as well as to label cell targets for which antibody reagents do not exist. In this chapter, we detail the instruction for performing the Branched DNA method to correlate the expression of CD8 mRNA and protein by flow cytometry as our model system. We discuss extensively within the “Notes” section tips and techniques for a successful experiment, including the basics of sample preparation, quality control, analysis strategies and precautions that must be considered to prevent adverse experimental outcomes.

2. Materials

2.1. Supplies and Reagents

1. Suitable biological specimens such as a cell line, EDTA or sodium heparin anti-coagulated human peripheral blood or bone marrow. Store blood/bone marrow samples at room temperature for no more than 48 hours before use.
2. Unless otherwise specified, all of the following reagents are a component of the PrimeFlow RNA kit (Affymetrix/eBioscience, Division of Thermo Fisher Scientific, Santa Clara, CA).
 - a. 1.5-mL Branched DNA Microcentrifuge Labeling Tubes (see Note 1).
 - b. Branched DNA Fixation Buffer 1A (2X) and Branched DNA Fixation Buffer 1B (2X). Store at 2–8°C until use. Prepare fresh 1X Fixation Buffer by mixing equal volume of Fixation Buffer 1A and Fixation Buffer 1B. Gently mix the solution by inverting the dilution container and avoid vortexing. Diluted buffer solution is stable at 2–8°C for no more than 1 day.
 - c. Molecular Grade DNase-, RNase-, and Protease-Free Water (Corning, Manassas, VA).
 - d. Branched DNA Permeabilization Buffer (10X). Store at 2–8°C until use. Dilute 10X Permeabilization Buffer with Molecular Grade DNase-, RNase-, and Protease-Free Water (Corning, Manassas, VA) to yield a final concentration of 1X Permeabilization buffer. Store diluted buffer at 2–8°C. Diluted buffer solution is stable at 2–8°C for 1 day.
 - e. Branched DNA Wash Buffer.
 - f. Branched DNA Fixation Buffer 2 (8X). Store at 2–8°C until use. Before use, warm 8X Fixation Buffer 2 and Branched DNA Wash Buffer to room temperature. Prepare 1X Fixation Buffer 2 by diluting 8X Fixation Buffer 2 with per-warmed Branched DNA Wash Buffer to

¹Microcentrifuge tubes are provided by Affymetrix/eBioscience in the PrimeFlow RNA kit. The utilization of inappropriate microcentrifuge tube can result in severe cell loss and/or decrease in hybridization efficiency due to inefficient heat transfer, which can lead to low cell yield and poor signal detection. If an investigator wants to replace this component of the assay with alternative source of tubes, the tubes should be thoroughly tested for compatibility with the assay using relevant positive and negative controls.

yield a 1X Fixation Buffer. Diluted buffer is stable at room temperature for up to a day.

- g.** Branched DNA Target Probe Diluent. Store at 2–8°C until use.
 - h.** Branched DNA PreAmp Mix. Store at 2–8°C until use.
 - i.** Branched DNA Amp Mix. Store at 2–8°C until use.
 - j.** Branched DNA Label Probe Diluent. Store at 2–8°C until use.
 - k.** Branched DNA Storage Buffer. Store at 2–8°C until use.
 - l.** RNase Inhibitor 1 (1000X). Stored at –20°C until use. Dilute the stock solution using either 1X Permeabilization Buffer or Wash Buffer to a final concentration of 1X RNase Inhibitor. Keep the diluted RNase Inhibitor at 2–8°C for no longer than 1 day.
 - m.** RNase Inhibitor 2 (100X). Stored at –20°C until use. Dilute the stock solution using either 1X Permeabilization Buffer or Wash Buffer to a final concentration of 1X RNase Inhibitor. Keep the diluted RNase Inhibitor at 2–8°C for no longer than 1 day.
 - n.** Branched DNA Label Probe (100X). Store at –20°C until use and minimize exposure to light (see Note 2 for a discussion on available detection probes).
 - o.** Positive Control Target Probe Sets (20X) for the detection of RPL13A housekeeping gene using AX488, AX647 or AX750 fluorescence probes. Store at –20 °C until use.
 - p.** Target Probe(s) specific to CD8a mRNA (100X) (see Note 3).
- 3.** PrimeFlow™ Compensation Kit (Affymetrix/eBioscience).
 - 4.** 12 × 75 mm Polystyrene Tubes compatible with flow cytometer used for sample acquisition.
 - 5.** Appropriate 15- and 50-mL Conical Centrifuge Tubes as required for the preparation of biological specimens and buffers.

²Currently, the labeling probe formats for this Branched DNA assay are conjugated to either Alexa Fluor® 488, Alexa Fluor® 647, or Alexa Fluor® 750. When acquired on a conventionally configured flow cytometer (i.e., configured with 405 nm, 488 nm, and 640 nm lasers), the fluorescence intensity of Alexa Fluor® 647 labeled probe is the brightest option. Therefore, it is recommended that Alexa Fluor® 647 should be reserved for the detection of mRNA targets with lowest abundances or of highest priority. While optimally excited by a 488 nm laser, Alexa Fluor® 488 exhibits lower fluorescence yield than Alexa Fluor® 647. Also, the detection channel in which Alexa Fluor® 488 is measured is associated with a higher level of background fluorescence. Alexa Fluor® 750 is optimally excited at 750 nm, but on conventional flow cytometers it can be sub-optimally excited by the 640 nm laser. Since it produces the lowest detectable fluorescence, mRNAs which are typically expressed at higher frequencies, such as the control or housekeeping mRNAs are relegated to the Alexa Fluor® 750 probe set. These 3 detection probe sets are commercially referred to as Type 1 (Alexa Fluor® 647), Type 4 (Alexa Fluor® 488), or Type 6 (Alexa Fluor® 750). It is important to note that all of the amplification components found in the Branched DNA kit provided by Affymetrix/eBioscience (i.e., the Pre-Amplifiers, Amplifiers, and Label Probes) consist of an inseparable mixture of all three type-specific probe sets.

³Commercially available Target Probe sets used for the detection of mRNA of interest can be found on Affymetrix's Website using the following link: <media.ebioscience.com/data/pdf/literature/quantigene-flowrna-available-probe-sets.pdf>. Alternatively, a customized Target Probe set can be constructed by Affymetrix for sequences that are not listed or to increase/decrease detection sensitivity.

6. Lymphocyte Separation Medium (LSM)(GE Healthcare, Mississauga, ON) or equivalent Ficoll Hypaque reagent.
7. Hanks' Balanced Salt Solution (HBSS; magnesium, calcium and phenol free).
8. Fluorochrome-conjugated monoclonal antibody (mAb) (BD Biosciences, San Jose, CA; BioLegend, San Diego, CA; eBioscience, San Diego, CA). See Table 1 for a complete list of mAb used in this chapter.
9. Flow Cytometry (FCM) buffer: Phosphate Buffered Saline with 5 g/L Bovine Serum Albumin, 1 g/L sodium azide, and 0.04 g/L disodium ethylenediaminetetraacetate. Store at 2–8°C until use.
10. 10% Formaldehyde, methanol free, Ultra Pure. To prepare 2% formaldehyde, dilute 1 part of 10% formaldehyde with 4 parts of Dulbecco's Phosphate Buffered Saline (calcium and magnesium free).
11. Human IgG Fc Block Solution: 12 mg/mL γ -Globulins from human blood (Sigma Aldrich, St. Louis, MO) diluted in RPMI 1640.

2.2. Equipment

1. Flow Cytometer equipped with excitation lasers and detector configurations used for collecting fluorescence signals emitted by Alexa Fluor® 488, Alexa Fluor® 647, Alexa Fluor® 750, as well as for the detection of fluorochromes conjugated to the mAb of interest. In our experiments, an LSRFortessa (BD Biosciences) flow cytometer equipped with 355 nm (60 mW) UV laser, 405 nm (50 mW) violet laser, 488 nm (50 mW) blue laser, and 640 nm (40 mW) red laser was utilized for the acquisition of all experiments in this chapter. Performance of the instrument is monitored daily using Cytometer Setup and Tracking Beads (BD Biosciences).
2. Horizontal Air Flow or Hybridization Oven capable of achieving and maintaining a temperature of $40 \pm 1^\circ\text{C}$ (Affymetrix/eBioscience).
3. Digital NIST-traceable thermometer capable of accurately measuring temperatures at 40°C (Affymetrix/eBioscience).
4. Modular Heat Sink for Microcentrifuge Tubes (VWR, Radnor, PA).
5. Vacuum or other aspiration device.
6. Manual or automated cell counting device. In our experiments, an AcT Diff (Beckman Coulter, Miami, FL) was used.
7. Centrifuge with swinging bucket for 1.5-mL microcentrifuge tubes (e.g., Sorvall™ Legend™ XT/XF Centrifuge Series, Thermo Fisher Scientific).

3. Methods

Using the Branched DNA assay, the expression of any mRNA transcript produced by a cell can be detected by conventional flow cytometer. A Branched DNA Target Probe set is

typically designed to detect a 1,000 base pair sequence using at least 20 oligonucleotide pairs that hybridize to the target mRNA transcript. As discussed in the introduction there are currently three Alexa-conjugated fluorochromes available for detection. When a new Target Probe is being designed, it is desirable to use Alexa Fluor® 647 which has the best signal-to-noise resolution on conventionally configured flow cytometers. Temperature optimization is another important step to be considered prior to the initiation of the experiments, as any temperature outside of $40 \pm 1^\circ\text{C}$ will fail. In our experience, a typical full-length Branched DNA assay will require a minimum of 10 hours to complete, and the length will increase as the number of experimental samples and variables are increased. The inclusion of cell surface and intracellular labeling steps will add an additional 1.5 to 2.5 h to the procedure. In this chapter, we describe two steps where the assay can be paused overnight.

3.1. Validation of Hybridization Oven

One of the major advantages of using the Branched DNA assay for the measurement of mRNA is the preservation of cellular architecture. This is accomplished by using a much lower annealing temperature ($40 \pm 1^\circ\text{C}$) than typically required for traditional PCR hybridization methods. In order to achieve an optimal signal-to-noise ratio while reducing non-specific hybridization, it is extremely important to establish and maintain the temperature of the hybridization oven at $40 \pm 1^\circ\text{C}$ (see Note 4). A milled aluminum heat sink should be used to facilitate the heat conduction to the microcentrifuge labeling tube, so that the reaction mixtures can equilibrate to the obligatory temperature as rapidly as possible. A NIST-traceable temperature probe is employed to allow for accurate monitoring of temperature during the calibration procedures.

3.1.1. Temperature Adjustment and Validation

1. Set the hybridization oven to 40°C , and place the milled aluminum heat sink in the chamber center.
2. Allow the temperature to equilibrate overnight (see Note 5).
3. Drill a hole in the lid of a Branched DNA microcentrifuge tube to accommodate the temperature probe and add 200 μL of the Branched DNA Target Probe Diluent to the tube.
4. Insert the temperature probe through the hole and into the diluent sealing the microcentrifuge tube with laboratory parafilm.
5. After overnight temperature equilibration, insert the microcentrifuge tube into the pre-warmed heat sink and immediately close the door of the hybridization oven.
6. Record the temperature every minute for 10 min.

⁴The heat sink should be milled to fit the 1.5-mL microcentrifuge tubes, or any alternative and validated tubes that the investigators desire to use. A rapid, efficient, and consistent heat transfer from the heat sink to the tubes is an important consideration in order to reach and maintain $40 \pm 1^\circ\text{C}$, the optimal temperature for hybridization of Target Probes and amplifiers in the Branched DNA kit. In addition, a small amount of water can be added into each well to facilitate heat transfer.

⁵If the temperature of the hybridization oven fails to achieve $40 \pm 1^\circ\text{C}$ after the overnight equilibration period, adjust the incubator's baseline temperature setting such that a $40 \pm 1^\circ\text{C}$ temperature is achieved, and then repeat Subheadings 3.1.1 and 3.1.2.

7. If the oven has not equilibrated to 40°C, adjust the thermostat control and repeat Steps 5–7.

3.1.2. Temperature Ramp-up Time

1. After the temperature of the oven has been calibrated to maintain $40 \pm 1^\circ\text{C}$, use the 1.5-mL microcentrifuge tube prepared in step 3 of Subheading 3.1.1 to assess the temperature ramp-up time.
2. Open the incubator door for 1 min, then place the 1.5-mL tube containing 200 μL of Target Probe Diluent at room temperature into the heat sink and close the door.
3. Record the temperature every minute for 10 min to determine the time required for the temperature to return and maintain $40 \pm 1^\circ\text{C}$.
4. Repeat this process at least one more time.
5. Do not use the hybridization oven if it takes longer than 5 min to return to 40°C or if it overshoots the set point temperature by more than 2°C during this process.

3.2. Preparation of Mononuclear Cells from Human Blood or Bone Marrow Specimens

Mononuclear cells are prepared by conventional Ficoll Hypaque density gradient centrifugation [10,11]. It is important to keep in mind that the detection of mRNA in cells can be easily compromised if precaution is not taken, as the ubiquitous presence of RNases can readily degrade your target mRNA. For this reason, one should always be wearing gloves along with other suitable Personal Protection Equipment when working with mRNA. Commercially available RNase inactivating agents can be used to treat bench top surfaces and glassware to remove RNases and RNase-free reagents should be used throughout the procedure. When preparing biological specimens for experiments, fresh tissue samples are always preferred and should be kept cold (4–8°C) or on ice to preserve the mRNA integrity.

1. Collect human peripheral blood or bone marrow into suitable blood collection tubes containing either EDTA or sodium heparin.
2. Combine equal volumes of HBSS and blood or bone marrow. Gently mix the tube by inversion.
3. Gradually overlay 2 parts of the diluted sample on top of 1 part of Ficoll. Be careful not to mix the layers (see Note 6).
4. Centrifuge the cells at 400xg for 30 min with no brake applied.
5. Collect the mononuclear cells at the interphase between plasma and Ficoll into a separate conical tube, and add ice-cold HBSS to obtain a 5X final total volume.

⁶For the best isolation of MNCs, the reagent should be equilibrated to and the separation procedure carried out at room temperature (18–22°C) as per manufacturer's recommendation.

6. Centrifuge the cells at 800xg at 4–8°C for 10 min and discard the supernatant. Resuspend the cells in ice-cold HBSS and centrifuge the cells at 800xg at 4–8°C for 5 min.
7. Discard the supernatant. Resuspend the cells to 1 mL with ice-cold HBSS.
8. Perform a cell count and adjust the cell density to $1-5 \times 10^7$ cells/mL by adding an appropriate amount of ice-cold HBSS.
9. Keep the cells on ice until use.

3.3. Measurement of Housekeeping $\beta 2M$ and RPL13A mRNAs in Human Peripheral Blood Mononuclear Cells

Housekeeping genes are essential for the maintenance of basal cellular functions and are therefore expected to be expressed at high levels in cells under normal and pathophysiological conditions. Since their expression is usually in a steady state, housekeeping mRNAs are convenient controls that can be used in the Branched DNA assay to verify if the procedure has been executed correctly. The expression level of a control candidate housekeeping gene should be validated prior to use, as their expression levels can vary (i.e., the expression of mRNA can increase or decrease) depending on tissue types and experimental conditions.

In the Branched DNA assay, the expression of housekeeping mRNAs can be used as an internal control to circumscribe cells that have undergone successful hybridization. The specific staining of a housekeeping gene or any mRNAs is differentiated from background by establishing the cells autofluorescence using a No Target Probe Control, in which cells are treated with all Branched DNA reagents with the exclusion of Target Probes.

In the protocol below, we described a complete Branched DNA procedure to label the expression of housekeeping $\beta 2M$ and RPL13A mRNAs in human peripheral blood, as a model system and to contrast the Probe Sets. The experimental design can be found in Table 2. The results shown in Fig. 2 illustrate the Type 1 AX647 Probe Set intensity is brighter than either Type 4 AX488 or Type 6 AX750 Probe Set for $\beta 2M$ and RPL13A mRNAs.

3.3.1. Fixation, Permeabilization, Target Probes Hybridization and Signal Amplification

1. Transfer 100 μ L ($1-5 \times 10^6$ cells) of the prepared cell suspension into pre-labeled microcentrifuge tubes.
2. Prepare 1X Fixation Buffer 1 by mixing equal volumes of 2X Fixation Buffer 1A with 2X Fixation Buffer 1B. Gently invert the suspension several times (see Note 7).
3. Add 1 mL of 1X Fixation Buffer 1 to each microcentrifuge tube, cap the tubes and invert several times to mix. Incubate samples for 30 min on ice.

⁷One mL of this buffer will be required per sample. Prepare this buffer in bulk to accommodate all samples. To prevent potential denaturation and degradation of the reagents, do not vortex or vigorously shake this buffer after mixing. This buffer should be prepared fresh at the time of use. Properly dispose of any unused buffer according to standard laboratory practice.

4. Centrifuge fixed samples at 800xg for 5 min.
5. Remove the supernatant by careful vacuum aspiration leaving 100 µL of buffer. Resuspend the cells in residual buffer (see Note 8).
6. Prepare 1X Permeabilization Buffer with RNase Inhibitors. First dilute 10X Permeabilization Buffer to 1X with RNase-free water. Then add RNase Inhibitor 1 (1,000X) at a 1:1,000 dilution and RNase Inhibitor 2 (100X) at a 1:100 dilution. For example, to make 50 mL of 1X Permeabilization Buffer add: 5 mL of 10X Permeabilization Buffer, 45mL of RNase-free water, 50 µL of 1,000X RNase Inhibitor 1, and 500 µL of 100X RNase Inhibitor 2. Gently mix the solution by inversion and store the Permeabilization Buffer on ice when not in use (see Note 9).
7. Add 1 mL of 1X Permeabilization Buffer with RNase Inhibitors to each sample. Cap and invert tubes several times to mix, centrifuge at 800xg for 5 min, aspirate supernatant to the 100 µL mark, and resuspend cells in the residual fluid.
8. Repeat step 7 for a total of 2 washes.
9. Prepare 1X Fixation Buffer 2 by combining 1 part of 8X Fixation Buffer 2 with 7 parts of Wash Buffer. Mix this suspension gently by inversion (see Note 10).
10. Add 1 mL of 1X Fixation Buffer 2 to each sample. Cap the tubes and invert several times to mix. Incubate the samples for 60 min in the dark at room temperature.
11. Centrifuge fixed samples at 800xg for 5 min, and then aspirate all but 100 µL of the supernatant. Resuspend cells in the residual fluid.
12. Add 1 mL of Wash Buffer to each sample. Cap, invert to mix, centrifuge at 800xg for 5 min, and aspirate all but 100 µL of supernatant and resuspend cells in the residual fluid.
13. Repeat step 12 (Optional stopping point; see Note 11).
14. Thaw Target Probes, including Positive Control Probe, and maintain at room temperature.

⁸Due to the high number of washing steps involved in the Branched DNA assay, removing supernatant after centrifugation through the use of vacuum aspiration device is highly recommended to prevent or minimize cell loss. Use the 100 µL mark imprinted on the microcentrifuge tube as a volume reference each time a wash step is performed.

⁹Two mL of the 1X Permeabilization Buffer is required for each sample. If intracellular staining is performed, an additional 1 mL is needed. For example, to make 50 mL of 1X Permeabilization Buffer add: 5 mL of 10X Permeabilization Buffer, 45mL of RNase-free water, 50 µL of 1,000X RNase Inhibitor 1, and 500 µL of 100X RNase Inhibitor 2. Do not vortex or vigorously shake this buffer. To ensure consistency, prepare this buffer fresh at the time of each use and in sufficient quantity to accommodate all samples. Dispose of any unused buffer.

¹⁰Wash Buffer should be equilibrated to ambient temperature prior to use. According to our experience, purifying the Wash Buffer by running it through a 0.8-µm filtration unit can help reduce some non-specific or background noises, and this will not adversely affect subsequent fixation and hybridization steps. For planning purposes, 1 mL of fresh Wash Buffer is needed per sample and the buffer should be prepared in bulk to accommodate all of the samples. Any unused buffer should be discarded properly at the end of each experiment.

¹¹This depicts an optional 'Stopping Point' in the protocol for maintaining a more manageable workflow. Cells may be stored in Wash Buffer with 1X RNase Inhibitor 1 overnight and in the dark at 2–8°C. To do this, add RNase Inhibitor 1 to Wash Buffer at a 1:1,000 dilution during the second wash step. For the best detection, it is recommended that first time users do not stop at this point, in order to avoid the possibility of mRNA degradation.

15. Pre-warm Target Probe Diluent to 40°C.
16. Dilute Target Probes 1:20 in Target Probe Diluent. Mix thoroughly by pipetting several times (see Note 12).
17. Add 100 µL of diluted Target Probe directly to the cell suspension for the appropriate samples and vortex briefly to mix. Incubate samples for 2 h at 40 ± 1°C, inverting samples to mix after 1 h (see Note 13).
18. Add 1 mL of Wash Buffer to each sample. Cap, invert to mix, and centrifuge at 800xg for 5 min. Aspirate all but 100 µL of supernatant and resuspend cells in the residual fluid (Optional stopping point; see Note 14).
19. Repeat step 18 twice, for a total of three washes (see Note 15).
20. Pre-warm the PreAmp Mix, Amp Mix, and Label Probe Diluent to 40 ± 1°C.
21. Add 100 µL of PreAmp Mix directly to the cell suspension in each tube, briefly vortex to mix, and incubate for 1.5 h at 40 ± 1°C (see Note 16).
22. Add 1 mL of Wash Buffer to each sample. Cap, invert to mix, and centrifuge at 800xg for 5 min. Aspirate all but 100 µL of supernatant and resuspend cells in the residual fluid.
23. Repeat step 22 for a total of 2 washes.
24. Thaw Label Probes on ice and in the dark.
25. Add 100 µL of Amp Mix directly to the cell suspension in each tube, briefly vortex to mix, and then incubate for 1.5 h at 40 ± 1°C (see Note 16).
26. Add 1 mL of Wash Buffer to each sample, invert to mix, and centrifuge at 800xg for 5 min. Aspirate all but 100 µL of supernatant and resuspend cells in the residual fluid.
27. Repeat step 26 for a total of 2 washes.
28. Dilute 1 part of 100X Label Probe (100X) with 99 parts of Label Probe Diluent.
29. Add 100 µL of diluted Label Probe directly to the cell suspension for each sample. Cap, briefly vortex to mix, and then incubate for 1 h at 40 ± 1°C.

¹²The probes are incubated with the cells in a final volume of 200 µL. A volume of 100 µL should already be present in the tube from the previous washing step, leaving 100 µL for the probe dilution. If one Target Probe is employed per sample, combine 5 µL of Target Probe with 95 µL of Target Probe Diluent. If more than one Target Probe is employed per sample, adjust the volume of the Target Probe Diluent accordingly by subtracting the total volume of the Target Probe(s) from 100 µL to calculate the volume of Target Probe Diluent required.

¹³When adding diluted Target Probes to each sample tube, it is important to ensure that the residual buffer be as close to 100 µL as possible. Diluted Target Probes should be pipetted directly into the 100 µL of residual buffer and then samples should be briefly vortexed before incubating at 40 ± 1°C. Avoid pipetting the solutions onto the walls of the tubes. Maintaining the 40 ± 1°C temperature is critical to the success of this procedure (see Subheading 3.1).

¹⁴This represents an optional 'Stopping Point' in the protocol for maintaining a more manageable workflow. For overnight storage, cells should be washed once in Wash Buffer with 1X RNase Inhibitor. Resuspend samples in 100 µL of residual volume, and store in the dark at 2–8°C. As per the previous suggestion (see Note 11), in order to avoid the possibility of mRNA degradation, it is recommended that first time users consider not stopping at this point.

¹⁵If samples were stored overnight at 2–8°C, ensure that samples and Wash Buffer reagents are equilibrated to room temperature prior to next step.

¹⁶PreAmp Mix and Amp Mix should be pipetted directly into the 100 µL of residual buffer, and samples should be mixed well by vortexing before incubating at 40 ± 1°C.

30. Add 1 mL of Wash Buffer to each sample. Cap, invert to mix, and centrifuge at 800xg for 5 min. Aspirate all but 100 µL of supernatant and resuspend cells in the residual fluid.
31. Repeat step 30 for a total of 2 washes.
32. Add 1 mL of FCM buffer or if storing overnight Branched DNA Storage Buffer to each sample. Cap, invert to mix, and centrifuge at 800xg for 5 min. Aspirate all but 100 µL of supernatant.
33. Resuspend cells in 500 µL of FCM buffer or Branched DNA Storage Buffer, transfer samples to 12 x 75 mm polystyrene tubes, and acquire samples on the flow cytometer (see Note 17).

3.3.2. Instrument Setup and Data Acquisition

1. Excite the fluorochrome Alexa Fluor® 488 using a 488 nm laser. Collect its emission using a suitable bandpass filter (e.g., 530/30 nm or equivalent). The peak emission of Alexa Fluor® 488 is at approximately 519 nm.
2. Excite the fluorochrome Alexa Fluor® 647 using a 633 nm (or 640 nm) laser. Collect its emission using a suitable bandpass filter (e.g., 670/14 nm or equivalent). The peak emission of Alexa Fluor® 647 is at approximately 668 nm.
3. The fluorochrome Alexa Fluor® 750 can be excited sub-optimally when using a conventionally-equipped red laser (e.g., 633 nm or 640 nm lasers). Collect its emission using a suitable bandpass filter (e.g., 780/60 nm or equivalent). For optimal excitation, a far-red laser with approximately 750 nm excitation is required.
4. For each detection channel, set the voltage such that the auto-fluorescence of the No Target Probe Control (e.g., cells that had been exposed to a complete Branched DNA assay, except no Target Probes are added to this control tube) should accumulate beyond the origin and within the first decade of the histogram.
5. Manually adjust the threshold value for forward scatter area prior to the acquisition of each sample to minimize the collection of debris and dead cells. For each sample, collect a minimum of 50,000 events. When setting the number of events to be recorded, consider the frequency of cellular subpopulation(s) of interest and adjust upwards to ensure a meaningful statistical analysis (see Note 18).

¹⁷Samples may be stored for no more than 3 days at 2–8°C in the dark before flow cytometric acquisition. For best results, it is recommended that samples be acquired as soon as possible following the execution of the Branched DNA protocol, in order to preserve fluorescence and signal resolution.

¹⁸The minimum number of events that should be acquired will depend on the precision required, as well as the frequency of targeted cell population in the sample. First, consider the amount of counting error that is deemed acceptable. As a rule of thumb, it is desirable to count a minimum of 100 target cell events. Counting 100 events will yield a counting error CV of 10%, which is likely the highest amount of error most investigators would accept. Counting error CV is determined by the following formula, where n represents the total number of acquired events for the population in question. For example, if a counting error of 1% is desired, then 10,000 target cell events must be counted.

3.3.3. Gating Strategy and Analysis

1. Generate the following bivariate plots and single parameter histograms as per Fig. 2 using a flow cytometry data analysis software package such as WinList (Verity Software House, Topsham, ME), FlowJo (FlowJo, LLC, Ashland, OR), and FCS Express (De Novo Software, Glendale, CA):

Fig. 2A (1) Time vs. SSC-A

Fig. 2A (2) FSC-A vs. FSC-H

Fig. 2A (3) FSC-A vs. SSC-A

Fig. 2B (4) Single Parameter Histograms for AX488, AX647, and AX750

2. To ascertain the quality of the data acquisition, place a rectangular region (R1) on the bivariate plot of Time vs. SSC-A to circumscribe the events collected in continuity. This dot plot can be used to eliminate any air bubbles or problems which occur during the run.
3. On the bivariate plot of FSC-A vs. FSC-H, create a rectangular region (R2) to include the singlet cell population. Gate this bivariate histogram on (R1).
4. On the bivariate plot of FSC-A vs. SSC-A, create an elliptical region (R3) to circumscribe the cell population of interest and exclude doublets, debris, and apoptotic events. Gate this bivariate histogram on (R1 and R2). For this example, a lymphocyte region is used.
5. Analyze the expression of B2M mRNA and RPL13A mRNA for each detection channel (e.g., Alexa Fluor® 488, Alexa Fluor® 647, and Alexa Fluor® 750) using single parameter histograms that are gated on (R1 – R3).

3.3.4. Compensation of Fluorescence Signals using PrimeFlow Compensation Kit

A proper compensation of fluorescence signal is an important aspect to be considered in virtually any multi-color flow cytometry experiment. Properly compensated data is important when determining the frequency of a cell population and is critical for the accurate measurement of antigen and mRNA densities (e.g., geometric mean fluorescence intensity). The compensation setup described below utilizes the bead-based PrimeFlow Compensation

$$\text{Counting Error CV} = \frac{\text{Sqrt}(n)}{n} * 100\%$$

Consideration is next given to the percentage of targeted cells in the sample. If the target cell frequency (f) is 5% and a 1% counting error is desired, then 200,000 total events must be collected.

$$\text{Number of Events to Collect} = \frac{n}{f}$$

There are no hard and fast rules, but by counting a minimum of 2,500 target cell events, which can be easily done by drawing a stopping region around this population, will yield a 2% Counting Error CV.

Kit (see Note 19). For a comparison of compensation matrices generated using different compensation strategies, see Tables 3 and 4.

1. Separately label as many 12 x 75 mm polystyrene tubes as required for the compensation of each fluorochrome used in the experiment, for the detection of either mRNA or protein targets (see Note 20).
2. Vigorously mix the beads by vortexing for a few seconds.
3. Dispense 1 drop of the beads to each labeled tube.
4. For each mRNA target, add 5 μ L of the appropriate RNA Compensation Control (as provided by the kit) to the appropriate tube. For each fluorochrome-conjugated mAb, add a saturating amount of mAb to the corresponding tube.
5. Vortex the tubes briefly to mix and incubate at 2–8°C for 20 min. Protect the beads from prolonged exposure to light.
6. Add 2 mL of FCM buffer to each tube and centrifuge at 800 $\times$ *g* for 5 min.
7. Discard the supernatant and vortex briefly to resuspend beads in residual buffer.
8. Add 100 μ L of fixation reagent to each tube and incubate at room temperature for 15 min. If desired, beads may be stored in fixation buffer at 2–8°C for a maximum of 3 days.
9. Add 2 mL of FCM buffer to each tube and centrifuge at 800 $\times$ *g* for 5 min.
10. Discard the supernatant and resuspend the beads with 500 μ L of FCM buffer.
11. After optimizing the flow cytometer's voltages (see Subheading 3.3.2), individually acquire each tube and create a compensation matrix using the software embedded in the acquisition program. See Table 4B for an example of a PrimeFlow Compensation Kit compensation matrix.

3.4. Simultaneous Measurement of mRNA and Protein using the Branched DNA Method

Traditional strategies employed for the measurement of mRNA such as PCR will generate an average value because they are bulk methods unless the cells are pre-sorted. As a result, a direct correlation between mRNA and protein can be technically challenging, and the actual expression levels may be underestimated or obscured by contaminating and irrelevant cells. Originally adapted from microscopy, the current version of the Branched DNA assay has been modified to achieve compatibility with the detection of surface and intracellular

¹⁹Modifications are required when using cells to establish a compensation matrix, ensuring any channel used to detect an mRNA target should be compensated with cells labeled with a bright mRNA control. We recommend using the PrimeFlow Compensation kit because compensation using cells labeled with many mRNA detection probe sets (AX488, AX647, and AX750) have dim signals. Regardless of the strategies used for the compensation calculation, it is crucial to emphasize that an appropriate negative population should be used. The beads or cells should possess similar autofluorescence properties as the test article's negative events (e.g., cells that were exposed to all Branched DNA reagents excluding the Target Probe).

²⁰The beads are designed to be compatible with mouse, rat or hamster fluorochrome-conjugated mAbs used for either surface or intracellular staining. Each drop of beads contains 2 separate populations; a positive population that will bind both mAb and the RNA Compensation Control (a product designed to mimic the spectral characteristic of the Label Probes used in the PrimeFlow RNA assay) and a negative population that will not bind either the mAb or the RNA Compensation Control. It is important to note that when these beads are being used for compensation, the negative population associated with each single color control be used to set the background fluorescence for that fluorochrome.

proteins. This enables the correlation of mRNA and protein at the single cell level. Before starting an experiment, caution should be taken to ensure that the phenotyping reagents and antibodies are compatible with the Branched DNA assay (see Subheading 3.5 for more details on the compatibility of fluorochrome choices with the Branched DNA assay).

3.4.1. Labeling of Surface Antigen using the Branched DNA Assay

1. Prepare PBMCs using Ficoll Hypaque density gradient centrifugation or other suitable technique as discussed in Subheading 3.2.
2. After adjusting the cell density to $1-5 \times 10^7$ cells/mL, transfer 100 μ L of cell suspension into each microcentrifuge tube.
3. Add 10 μ L of Human IgG Fc Block per 100 μ L of sample volume to block unwanted binding of mAbs to Fc receptors and incubate cells on ice for 10 min.
4. Transfer saturating amounts of mAb to each sample tube and incubate on ice for 60 min. Protect the samples from exposure to light to minimize the potential effect of photo-bleaching.
5. Add 1 mL of FCM buffer to each microcentrifuge tube, cap the tubes and invert several times to mix. Centrifuge the samples at 800xg for 5 min. Carefully remove supernatant to the 100 μ L mark using a vacuum aspiration device. Resuspend the cell pellet in residue buffer.
6. Repeat step 5.
7. From this point onwards, execute steps 2–33 of Subheading 3.3.1.

3.4.2. Labeling of Intracellular Protein using the Branched DNA Assay

1. Prepare PBMCs using Ficoll Hypaque density gradient centrifugation or other suitable technique as described in Subheading 3.2. If desired, surface staining can be performed according to Subheading 3.4.1.
2. Perform Subheading 3.3.1, steps 1–8 to fix and permeabilize the cells.
3. Add 10 μ L of Human IgG Fc Block per 100 μ L of sample volume to block unwanted binding of mAbs to Fc receptors and incubate cells at ambient temperature for 10 min.
4. Pipet saturating amounts of mAb to each sample tube, and incubate at ambient temperature for 30 min.
5. Prepare 1X Permeabilization Buffer with RNase Inhibitor. First dilute 10X Permeabilization Buffer to 1X with RNase-free water. Then add RNase Inhibitor 1 (1,000X) at a 1:1,000 dilution and RNase Inhibitor 2 (100X) at a 1:100 dilution. Gently mix the solution by inversion and store the Permeabilization Buffer on ice when not in use (see Note 9).
6. Add 1 mL of 1X Permeabilization Buffer with RNase Inhibitor to each sample, cap the tubes, invert several times to mix, and centrifuge at 800xg for 5 min.

Aspirate the supernatant to the 100 μ L mark and resuspend cells in the residual buffer.

7. Repeat step 6 for a total of 2 washes.
8. From this point onwards, execute steps 9–33 of Subheading 3.3.1.

3.4.3. Instrument Setup and Data Acquisition

1. Configure flow cytometer according to the recommendation suggested in steps 1–3 of Subheading 3.3.2 for the detection of mRNA in each channel (e.g., Alexa Fluor® 488, Alexa Fluor® 647, and Alexa Fluor® 750).
2. Set the voltages for each detection channels and acquisition parameters according to steps 4–5 of Subheading 3.3.2.

3.4.4. Gating Strategy and Analysis—To demonstrate the simultaneous measurement of mRNA and protein using the Branched DNA assay, the correlated expression pattern of CD8 mRNA and CD8 protein using human PBMCs was examined (Fig. 3). The experimental design can be found in Table 5. Briefly, Ficoll PBMCs were isolated as per Subheading 3.2, surface-labeled with an antibody to CD8 PE protein (p) as per Subheading 3.4.1, and hybridized with an AX647 Target Probe to CD8 mRNA (m) using the Branched DNA technique as per Subheading 3.3.1. The data were acquired on an LSRFortessa according to the procedure described in Subheading 3.4.3 and analyzed in WinList version 8.1 (Verity Software House) as follows:

1. Generate the following bivariate plots as per Fig. 2A using a flow cytometry data analysis software package such as WinList, FlowJo, and FCS Express:

Fig. 2A (1) Time vs. SSC-A

Fig. 2A (2) FSC-A vs. FSC-H

Fig. 2A (3) FSC-A vs. SSC-A

2. To ascertain the quality of the data acquisition, place a rectangular region (R1) on the bivariate plot of Time vs. SSC-A to circumscribe the events collected in continuity. This dot plot can be used to eliminate any air bubbles or problems which occur during sample collection.
3. On the bivariate plot of FSC-A vs. FSC-H, create a rectangular region (R2) to include the singlet cell population. Gate this bivariate histogram on (R1).
4. On the bivariate plot of FSC-A vs. SSC-A, create an elliptical region (R3) to circumscribe the cell population of interest and exclude doublets, debris and apoptotic events. Gate this bivariate histogram on (R1 and R2). In this example, a lymphocyte region is used.
5. As per Fig. 3, create a quadrant region on a bivariate plot of CD8 protein [CD8 (p)] PE vs. CD8 mRNA [CD8 (m)] AX647. This plot should be gated on (R1 – R3). The boundary of the quadrant region should be constructed based upon ‘Fluorescence Minus One’ controls, in which the FMO tubes would be identical

to the experimental tube; except the mAb/mRNA label probe is excluded in the fluorochrome channel that is being controlled (see Note 21).

3.5. Validation of Surface and Intracellular Labeling Reagent Compatibility

Early investigators who combined cell phenotyping via antibody labeling with PCR encountered poorly detectable antibody signals because fluorochromes exposed to the high thermal cycling temperatures required for nucleic acids amplification (e.g., in excess of 90°C) were partially disintegrated and the cells became highly autofluorescent [7]. The temperatures employed in the Branched DNA assay are much lower ($40 \pm 1^\circ\text{C}$) than those used in PCR, making it possible to detect most fluorochromes. Nevertheless, decreases in fluorochrome intensity and increases in autofluorescence remain as issues that need to be addressed when planning experiments. Fixative and permeabilization reagents used in the procedure may also have adverse effects. The following protocol describes an experimental design that should be carried out to assess the effect of the Branched DNA procedure on the autofluorescence of unlabeled cells and fluorochrome intensity.

3.5.1. Sample Preparation

1. Label as many 12×75 mm polystyrene tubes and Branched DNA microcentrifuge tubes as is required for the experiment.
2. Prepare PBMCs using Ficoll Hypaque density gradient centrifugation technique or other suitable methods. See Subheading 3.2 for more information.
3. Wash the resultant cell suspension 2 times using FCM buffer.
4. Perform a cell count to adjust the cell density to $1\text{--}5 \times 10^7$ cells/mL.
5. Transfer 100 μL of cell suspension into each of the polystyrene tubes.
6. Add 10 μL of Human IgG Fc Block per 100 μL of sample volume to block binding of mAbs to Fc receptors. Incubate the cells on ice for 10 min.
7. Transfer a saturating amount of mAb to each of the sample tube and incubate on ice for 60 min. From this step onwards, protect the samples from exposure to light to minimize the potential effect of photo-bleaching.
8. Add 3 mL of FCM buffer to each sample, centrifuge at $800\times g$ for 5 min and discard the supernatant to remove unbound mAb.
9. Resuspend the polystyrene tube cell pellet in 1 mL of FCM buffer. Transfer 500 μL of this suspension to a microcentrifuge tube, which will be exposed to the entire Branched DNA assay. The polystyrene tube, which will be used for comparison, should be stored at $2\text{--}8^\circ\text{C}$ until acquisition.

²¹The Fluorescence Minus One control (FMO) is a type of control commonly included in a multi-color flow cytometry analysis to set boundaries between negative and positive populations. An FMO control contains all the fluorochromes used in a tube, except for one being controlled. It is used to control for fluorescence spreading from one channel into another. This is especially important when the accurate separation of dimly expressed antigens from negative is required [12].

10. Centrifuge the microcentrifuge tube prepared in step 9 at 800xg for 5 min. Aspirate all but 100 µL of supernatant and resuspend the pellet in residue buffer. From this step onwards, execute steps 2–33 of Subheading 3.3.1.

3.5.2. Instrument Setup, Data Acquisition, and Analysis

1. Set the voltages for each detection channels and acquisition parameters according to steps 4–5 of Subheading 3.3.2.
2. Create the bivariate plots as described in Subheading 3.4.4.
3. In addition to the bivariate plots created in step 2, add a single parameter histogram gated on (R1 – R3) for each surface staining mAb. Create regions (R4) and (R5) to circumscribe the negative and positive cell populations, respectively, on the histogram.
4. Calculate the Stain Index for each mAb using the following formula:

$$\text{Stain Index} = \frac{(\text{gMFI of Positive Population}) - (\text{gMFI of Negative Population})}{2 \times (\text{Negative Population Robust Standard Deviation})}$$

5. For each mAb, compare its Stain Index using the standard immunophenotyping technique with its Stain Index measured using the complete Branched DNA assay (see Note 22 for a discussion on the results comparing the effect of Branched DNA on fluorochrome selection).

Acknowledgments

The authors acknowledge Dylan Malayter and Castle Funatake (both from Affymetrix/eBioscience) for their contributions to this chapter. Paul K. Wallace is partially supported by the Roswell Park Cancer Institute Ovarian SPORC NIH Grant 1P50CA159981-01A1. Flow cytometry was performed at Roswell Park Cancer Institute's Department of Flow and Image Cytometry, which was established in part by equipment grants from the NIH Shared Instrument Program, and receives support from the Core Grant (5 P30 CA016056-29) from the National Cancer Institute to the Roswell Park Cancer Institute.

References

1. Bentley DL. Coupling mRNA processing with transcription in time and space. *Nat Rev Genet.* 2014; 15(3):163–175. DOI: 10.1038/nrg3662 [PubMed: 24514444]
2. Hocine S, Singer RH, Grunwald D. RNA processing and export. *Cold Spring Harb Perspect Biol.* 2010; 2(12):a000752.doi: 10.1101/cshperspect.a000752 [PubMed: 20961978]
3. Patterson BK, Till M, Otto P, Goolsby C, Furtado MR, McBride LJ, Wolinsky SM. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. *Science.* 1993; 260(5110):976–979. [PubMed: 8493534]
4. Patterson BK, Mosiman VL, Cantarero L, Furtado M, Bhattacharya M, Goolsby C. Detection of HIV-RNA-positive monocytes in peripheral blood of HIV-positive patients by simultaneous flow

²²The data shown in Fig. 4 indicated cells processed through the Branched DNA assay generally have higher autofluorescence and decreased staining intensity than those labeled using standard immunophenotyping procedure and not processed through the Branched DNA assay. This study emphasizes the importance of testing the suitability of fluorochrome choices prior to starting experiments with Branched DNA assay, as certain clones, antigens or fluorochromes may be more susceptible; resulting in an overall decrease in the ability to resolve positive from negative populations [13].

- cytometric analysis of intracellular HIV RNA and cellular immunophenotype. *Cytometry*. 1998; 31(4):265–274. [PubMed: 9551602]
5. Van Hoof D, Lomas W, Hanley MB, Park E. Simultaneous flow cytometric analysis of IFN-gamma and CD4 mRNA and protein expression kinetics in human peripheral blood mononuclear cells during activation. *Cytometry A*. 2014; 85(10):894–900. DOI: 10.1002/cyto.a.22521 [PubMed: 25123411]
 6. Trask B, van den Engh G, Landegent J, in de Wal NJ, van der Ploeg M. Detection of DNA sequences in nuclei in suspension by in situ hybridization and dual beam flow cytometry. *Science*. 1985; 230(4732):1401–1403. [PubMed: 2416058]
 7. Mutty CE, Timm EA Jr, Stewart CC. Effects of thermal exposure on immunophenotyping combined with in situ PCR, measured by flow cytometry. *Cytometry*. 1999; 36(4):303–311. [PubMed: 10404145]
 8. Patterson BK, Goolsby C, Hodara V, Lohman KL, Wolinsky SM. Detection Of CD4(+) T-cells harboring human-immunodeficiency-virus type-1 DNA by flow cytometry using simultaneous immunophenotyping and PCR-driven in-situ hybridization - evidence of epitope masking of the CD4 cell surface molecule in-vivo. *Journal of Virology*. 1995; 69(7):4316–4322. [PubMed: 7539507]
 9. Yu H, Ernst L, Wagner M, Waggoner A. Sensitive detection of RNAs in single cells by flow cytometry. *Nucleic Acids Res*. 1992; 20(1):83–88. [PubMed: 1738608]
 10. Boyum A. Isolation of leucocytes from human blood. Further observations. Methylcellulose, dextran, and ficoll as erythrocyteaggregating agents. *Scand J Clin Lab Invest Suppl*. 1968; 97:31–50. [PubMed: 4179066]
 11. Fuss IJ, Kanof ME, Smith PD, Zola H. Isolation of whole mononuclear cells from peripheral blood and cord blood. *Curr Protoc Immunol*. 2009; Chapter 7(Unit7):1.doi: 10.1002/0471142735.im0701s85
 12. Roederer M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry*. 2001; 45(3):194–205. [PubMed: 11746088]
 13. Soh KT, Tario JD Jr, Colligan S, Maguire O, Pan D, Minderman H, Wallace PK. Simultaneous, Single-Cell Measurement of Messenger RNA, Cell Surface Proteins, and Intracellular Proteins. *Curr Protoc Cytom*. 2016; 75:7.45:1–7.45.33. DOI: 10.1002/0471142956.cy0745s75 [PubMed: 26742656]

Fig. 1. Target Probes hybridization and signal amplification of mRNA using the Branched DNA assay

Fixed and permeabilized cells are sequentially exposed to and hybridized with Target Probe Pairs, Pre-Amplifiers, Amplifiers, and Labeling Probes at $40 \pm 1^\circ\text{C}$. Washing steps are performed in between each of the hybridization steps to remove un-hybridized material. In order for signal amplification to occur, each pair of Target Probes must first hybridize adjacent to one another on the mRNA sequence, so that the Pre-Amplifier can anneal stably. Each individual Target Probe consists of approximately 18–25 DNA bases with unique specificity for the mRNA sequence to be detected. A targeted mRNA sequence with 1,000 bases can accommodate up to 20 different pairs of Target Probes, thus serving as the foundation for the initial signal amplification. The un-hybridized sequence of each Target Probe Pair is complementary to the Pre-Amplifier sequence, adding another layer of specificity. Next, approximately 20 Amplifier sequences hybridize to each Pre-amplifier sequence, followed by the hybridization of multiple fluorescent Label Probes to each Amplifier molecule. An optimally assembled Branched DNA complex provides a theoretical 8,000- to 16,000-fold signal amplification of the targeted mRNA.

Fig. 2. Measurement of housekeeping B2M and RPL13A mRNAs in human lymphocytes comparing AX488, AX647, and AX750 detection systems

(A) Gating strategy used to identify lymphocytes. *A1*. A rectangular region (R1) is created on a bivariate plot of FTM (event chronology) vs. SSC-A to review acquisition uniformity and to exclude air bubbles, clogs, etc. *A2*. A rectangular region (R2) is created on a gated (R1) bivariate plot of FSC-A vs. FSC-H to circumscribe the singlet cell population. *A3*. An elliptical region (R3) is created on a gated (R1 and R2) bivariate plot of FSC-A vs. SSC-A to circumscribe cellular events with lymphocyte scatter characteristics. (B) Measurement of β 2M and RPL13A mRNAs using AX488, AX647, or AX750 as the detection system. *B4*. Single parameter histograms are gated on R1, R2, and R3. The positive expression (black histograms) and autofluorescence (grey histograms) of β 2M and RPL13A are detected using AX488 (Type 4 probe set), AX647 (Type 1 probe set), or AX750 (Type 6 probe set). The test articles used for the generation of autofluorescent signals were processed by exposing the cells to all Branched DNA reagents except the Target Probes.

Fig. 3. Correlated measurement of CD8 protein and CD8 mRNA in human lymphocytes using the Branched DNA assay

Human PBMCs with lymphocytic scatter characteristic were identified using the gating strategy described in Figure 2. **(A)** mRNA AX647 FMO control. The expression level of CD8 protein was measured using PE as the detection system. The cells were exposed to a complete Branched DNA procedure except Target Probes for CD8 mRNA were not added. **(B)** Protein PE FMO control. The expression level of CD8 mRNA was measured using AX647 as the detection fluorochrome. **(C)** The correlated expression levels of CD8 protein and CD8 mRNA were measured using PE and AX647 as the detection systems, respectively. Quadrant regions were set using the FMO controls.

Fig. 4. Signal resolution for the detection and measurement of protein expression using the Branched DNA Assay is dependent upon the antigen and fluorochrome being evaluated

To assess the effects of the Branched DNA assay on fluorochromes (rows 1 and 2), CD8 mAbs labeled with eight different fluorochromes were processed through the complete protocol (grey histograms). The geometric mean fluorescence intensities (data not shown) and staining patterns for each of the fluorochromes were compared to a set of parallel samples that were stained directly with mAbs based on standard immunophenotyping procedures (black histograms). The ability to resolve the staining for each of the surface antigens (i.e., CD3, CD28, CD45RA and CD197) and intracellular marker (i.e., T-bet) were compared between samples that have been treated using the Branched DNA reagents and standard immunophenotyping procedure. All utilized PE as the detection fluorochrome (rows 3 and 4). In all cases, the voltage gains applied in each of the detection channel was

set based upon unstained samples processed using the standard immunophenotyping procedure.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Fluorochrome-conjugated mAbs utilized in this study

Antibody Specificity	Murine Isotype	Format	Clone	Source
CD3	IgG _{1,k}	PE	SK7	BD Biosciences
CD8	IgG _{1,k}	BUV395	RPA-T8	BD Biosciences
CD8	IgG _{1,k}	BV421	RPA-T8	BioLegend
CD8	IgG _{1,k}	BV510	RPA-T8	BioLegend
CD8	IgG _{1,k}	BV605	RPA-T8	BioLegend
CD8	IgG _{1,k}	AX488	SK1	BioLegend
CD8	IgG _{1,k}	PE	RPA-T8	BioLegend
CD8	IgG _{1,k}	PECy5	RPA-T8	BD Biosciences
CD8	IgG _{1,k}	PECy7	RPA-T8	BioLegend
CD8	IgG _{1,k}	AX647	SK1	BioLegend
CD8	IgG _{1,k}	APCH7	SK1	BD Biosciences
CD28	IgG _{1,k}	PE	CD28.2	BD Biosciences
CD45RA	IgG _{2b,k}	PE	HI100	BioLegend
CD197	IgG _{2a,k}	PE	G043H7	BioLegend
T-bet	IgG _{1,k}	PE	eBio4B10	eBioscience

Table 2

Experimental layout for the measurement of housekeeping $\beta 2M$ and RPL13A in human peripheral blood mononuclear cells

Tube Number	Tube Description	488 nm Laser	640 nm Laser	640 nm Laser
		AX488 B530/30	AX647 R670/14	AX750 R780/60
1	No Target Probe Control ^a	-	-	-
Experimental Tube				
2	Test articles	$\beta 2M$ (m) ^b	-	-
3		-	$\beta 2M$ (m)	-
4		-	-	$\beta 2M$ (m)
5		RPL13A (m)	-	-
6		-	RPL13A (m)	-
7		-	-	RPL13A (m)

^aSamples are processed through the Branched DNA procedure without addition of mAbs or target probes

^b(m): Denotes the detection of protein

- Denotes either mRNA target probes or fluorochrome-conjugated antibodies were not added

Table 3

Experimental design testing different labeling strategies to create the Branched DNA compensation matrix

	Compensation Strategy	Single Color	AX488	AX647	AX750
A.	mRNA labeled by Branched DNA procedure	Unlabeled	-	-	-
		AX488	RPL13A (m) ^a	-	-
		AX647	-	RPL13A (m)	-
		AX750	-	-	RPL13A (m)
B.	PrimeFlow™ Compensation Kit	Unlabeled	-	-	-
		AX488	AX488 Beads	-	-
		AX647	-	AX647 Beads	-
		AX750	-	-	AX750 Beads
C.	mAbs and mRNA labeled by Branched DNA procedure	Unlabeled	-	-	-
		AX488	CD8 (p) ^b	-	-
		AX647	-	CD8 (p)	-
		AX750	-	-	RPL13A (m)
D.	mAbs labeled by standard immunophenotyping procedure & mRNA labeled by Branched DNA procedure	Unlabeled	-	-	-
		AX488	CD8 (p)	-	-
		AX647	-	CD8 (p)	-
		AX750	-	-	RPL13A (m)

^a (m): Denotes the detection of mRNA

^b (p): Denotes the detection of protein

- Denotes either the mRNA target probes or fluorochrome-conjugated antibodies were not added

Table 4

Compensation matrices values for the different compensation strategies tested in Table 3

A. mRNA labeled by Branched DNA procedure						
Signal	Into					
	B530/30	B575/26	R670/14	R730/45	R780/60	
RPL13A (m) AX488	100.00	21.18	15.87	7.09	10.01	
RPL13A (m) AX647	1.69	0.12	100.00	44.11	42.21	
RPL13A (m) AX750	3.86	1.40	13.31	11.33	100.00	

B. PrimeFlow™ Compensation Kit						
Signal	Into					
	B530/30	B575/26	R670/14	R730/45	R780/60	
Beads AX488	100.00	21.17	0.01	0.00	0.02	
Beads AX647	0.00	0.01	100.00	45.30	41.13	
Beads AX750	0.02	0.08	0.49	2.85	100.00	

C. mAbs and mRNA labeled by Branched DNA procedure						
Signal	Into					
	B530/30	B575/26	R670/14	R730/45	R780/60	
CD8 (p) AX488	100.00	22.82	5.61	3.29	5.61	
CD8 (p) AX647	0.49	0.53	100.00	42.70	38.36	
RPL13A (m) AX750	0.28	0.00	8.69	6.24	100.00	

D. mAbs labeled by standard immunophenotyping procedure & mRNA labeled by Branched DNA procedure						
Signal	Into					
	B530/30	B575/26	R670/14	R730/45	R780/60	
CD8 (p) AX488	100.00	21.54	0.10	0.03	0.13	
CD8 (p) AX647	0.11	0.07	100.00	42.21	38.33	
RPL13A (m) AX750	0.00	0.00	8.29	7.23	100.00	

Table 5

Experimental layout for the simultaneous measurement of mRNA transcripts and cell surface proteins in PBMCs using the Branched DNA assay

Tube Number	Tube Description	488 nm Laser	640 nm Laser
		PE B575/26	AX647 R670/14
1.	No Target Probe Control ^a	-	-
Compensation Tubes (PrimeFlow™ Compensation Kit)			
2.	PE labeled beads	Beads with PE	-
3.	AX647 labeled beads	-	Beads with AX647
FMO^b Controls			
4.	Cells without PE label	-	CD8 (m) ^c
5.	Cells without AX647 label	CD8 (p) ^d	-
Experimental Tube			
6.	Test article	CD8 (p)	CD8 (m)

^a Samples are processed through the Branched DNA procedure without mAbs or target probes

^b FMO: Fluorescence minus one

^c (p): Denotes the detection of mRNA

^d (m): Denotes the detection of protein

- Denotes either mRNA target probes or fluorochrome-conjugated antibodies were not added