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Simultaneous, Single-Cell Measurement of Messenger RNA, Cell Surface Proteins, and Intracellular Proteins

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Abstract

Nucleic acid content can be quantified by flow cytometry through the use of intercalating compounds, however measuring the presence of specific sequences has hitherto been difficult to achieve by this methodology. The primary obstacle to detecting discrete nucleic acid sequences by flow cytometry is their low quantity and the presence of high background signals, rendering the detection of hybridized fluorescent probes challenging. Amplification of nucleic acid sequences by molecular techniques such as *in situ* PCR have been applied to single cell suspensions, but these approaches have not been easily adapted to conventional flow cytometry. An alternative strategy implements a Branched DNA technique, comprised of target-specific probes and sequentially-hybridized amplification reagents; resulting in a theoretical 8000 – 16,000-fold increase in fluorescence signal amplification. The Branched DNA technique allows for the quantification of native and unmanipulated mRNA content with increased signal detection and reduced background. This procedure utilizes gentle fixation steps with low hybridization temperatures leaving the assayed cells intact, to permit their concomitant immunophenotyping. This technology has the potential to advance scientific discovery by correlating the low abundance of mRNA with many biological measurements at the single-cell level.

Keywords

Flow Cytometry; Branched DNA; mRNA Sequence; Target Probe; *in situ* Hybridization; Leukocytes; Transcription Factors

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KEY REFERENCE

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INTERNET RESOURCES

- <http://www.acdbio.com/products/rnascope-assays>
- <http://www.ebioscience.com/knowledge-center/application/flowrna/technology.htm>
- http://www.ebioscience.com/media/newpdf/PrimeFlowRNAAssay_UM010915.pdf

INTRODUCTION

The genome has become an increasingly accessible repository of information for the academic and clinical study of disease etiology, and its detection and diagnosis. As part of the modern bioinformatics revolution, a vast amount of knowledge has been garnered from transcriptomic technologies such as Microarray, Next Generation Sequencing and Whole Transcriptome Shotgun Sequencing; enabling high resolution and insight into the genome. While these advanced technologies can yield comprehensive gene expression data, their most significant shortcoming lies in the fact that unless a pre-sorted population of cells is obtained in advance (i.e. via cell sorting), the transcriptional analysis of bulk samples will be obscured with large amounts of data generated from irrelevant cell populations.

Integrating the measurement of genomic expression via the Branched DNA assay with a discriminative technology such as flow cytometry represents an elegant solution to the problem of sample heterogeneity, as multiparametric flow cytometry permits the simultaneous evaluation of mRNA and protein expression at the single-cell level (Buckingham and Flaws, 2007; Wang et al., 2012). The advent of Branched DNA technology complements flow cytometry by allowing for many determinations that were previously unachievable. Of significance, is the ability of Branched DNA technology to label cell targets for which antibody reagents do not exist; whether because the determinants are novel, represent alternative splice variants, or would otherwise require complicated and sometimes inconsistent antigen-retrieval techniques.

In its simplest form, the combination of Branched DNA technology with flow cytometry can be employed for qualitative and semi-quantitative determinations such as the characterization of cellular targets of viral infection, with a concomitant quantification of their viral load. This technology is also ideal for correlating mRNA and protein levels, for studying their expression kinetics, their respective half-lives, and also for verifying the effectiveness of mRNA silencing or regulatory interventions. The ability to measure gene expression by flow cytometry creates many opportunities for efficient investigations of nucleic acid expression characteristics in heterogeneous populations.

Capitalizing on the simultaneous and correlated detection capabilities of multiparametric flow cytometry allows for the determination of how much mRNA is being transcribed, and which specific cells are expressing the interrogated mRNA sequences. Data of this nature can be compared individually or in combination with the aforementioned measurements which can already be performed by flow cytometry. In this regard, the expression profiles of multiple mRNA species in distinct and phenotypically-defined cellular subsets can be correlated with metrics such as cell cycle progression, apoptosis, protein phosphorylation state, signaling kinetics, downstream protein expression; and the cellular response to pharmacologic agents, therapeutic interventions, stimulation, suppression, or other environmental conditions. Branched DNA technology represents an opportunity to explore data correlating the potentially small quantities of mRNA with many biological measurements.

Clinically, this technique might be applied to the detection of chimerism in a recipient host from an unsorted sample, or in quantifying viral load in infected cells, or in calculating the fraction of a cell sample that expresses tumor-specific genes, or exhibits other abnormalities that result in aberrant or elevated mRNA expression (Garcia-Morales et al., 1997). Theoretically, no barrier exists to the identification of target mRNAs, provided that their sequences are known.

An important advantage to the use of flow cytometry for the measurement of mRNA species is the fact that many individual cells can be readily interrogated for the expression of a transcript. Modern clinical flow cytometry has become increasingly driven towards improved detection of minimal residual disease, with current detection sensitivities of 0.01% and proposed methodological advances approaching 0.001%; which rivals the assay sensitivity of PCR-based detection (Arroz et al., 2015; Neale et al., 2004; Weng et al., 2013). Accordingly, the flow cytometry-based Branched DNA technique represents a unique approach to the detection of rare events having clinical significance.

The following protocols provide detailed instruction for performing the Branched DNA procedure using human peripheral blood mononuclear cells (PBMCs). Therein, the BASIC PROTOCOL illustrates the requisite steps to quantify mRNA within cells by flow cytometry, using the measurement of CD8 mRNA in PBMCs as an example. To elucidate which cells are producing mRNA, a proof-of-principle experiment is demonstrated in ALTERNATE PROTOCOL 1, which combines cell surface immunophenotyping with mRNA measurement by flow cytometry. ALTERNATE PROTOCOL 2 extends this assay to include the additional measurement of intracellular proteins, and an example is provided which details the correlated analysis of T-bet and EOMES transcription factors with CD8 mRNA in phenotypically defined lymphocyte subsets.

BASIC PROTOCOL. MEASUREMENT OF mRNA TRANSCRIPTS IN PBMCs USING THE BRANCHED DNA ASSAY

In situ hybridization is a widely used technique that employs complementary DNA or RNA probes to detect specific nucleic acid sequences within cells. Historically, two approaches have been utilized to improve the sensitivity of mRNA *in situ* detection; they are (1) the amplification of mRNA sequences prior to their hybridization to reporter molecules (i.e. RT-PCR) or (2) the amplification of signal using secondary detection reagents after target sequences have been hybridized to a reporter probe (i.e. *in situ* hybridization). Unfortunately, amplification bias and also the amplification of background noise have been impediments to the precise and accurate measurement in both methodologies (Wang et al., 2012). Modern Branched DNA technology provides a unique approach to mRNA detection by greatly amplifying the reporter signal instead of the mRNA transcript. In addition to improving assay sensitivity, this novel strategy has been developed to overcome non-specific binding of detection probes.

In brief, cells are fixed and permeabilized using a 'Fixation Buffer' to preserve mRNA sequences of either human or mouse cells in suspension. During the first hybridization step, a pair of mRNA-specific oligonucleotide 'Target Probes' with each contain approximately

20 complementary DNA bases is annealed to the targeted mRNA sequence (Figure 1). These Target Probes are designed to hybridize adjacent to each other on the mRNA sequence, in order to increase the specificity of the detection system, as both probes must be in proximity to each other to permit signal amplification to proceed. An additional layer of specificity is conferred when 'Pre-Amplifier' molecules are added, as these molecules will only form a stable complex in the presence of both hybridized Target Probes. The detection system is further augmented using 'Amplifier' molecules, which hybridize to the branched DNA structure through complimentary pairing to multiple sites on the 'Pre-Amplifier' molecule. Finally, 'Label Probe' oligonucleotides conjugated to Alexa Fluor® 488, Alexa Fluor® 647 or Alexa Fluor® 750 are hybridized to multiple target sites on 'Amplifier' molecules. This comprises one fully-assembled branched DNA structure. To further amplify the signal, the complete Branched DNA complex is composed of approximately 20 such structures; each harboring specificity to a different sequence on the mRNA. The actual number of these structures will depend on the length of the mRNA species being interrogated. Accordingly, the complete Branched DNA complex will theoretically result in an 8,000 to 16,000-fold amplification of signal from a single mRNA transcript. Labeled samples are then acquired by conventional flow cytometry.

Materials

Specimen—Fresh, EDTA or sodium heparin anti-coagulated human blood, bone marrow, or leukoreduction filter retentate specimens may be used in this protocol. Samples should be stored at room temperature for less than 48 hours.

- See Tario et al. (Tario et al., 2011) for a discussion on the preparation of PBMCs from leukoreduction filter retentates.

Although this procedure has been optimized for use with human lymphocytes and monocytes, the technique has also been implemented with mouse splenocytes, thymocytes, bone marrow and secondary lymphoid tissues. Additionally, this protocol has been successfully executed with certain adherent (HeLa, PC9) and suspension (U937, Jurkat) human cell lines. Fresh, stimulated, and cryopreserved cells can also be employed as suitable specimens (Hanley et al., 2013; <http://www.ebioscience.com/resources/faq/flowrna-faq.htm>; Porichis et al., 2014; Van Hoof et al., 2014).

Reagents—Unless otherwise specifically indicated, all reagents are part of a Prime FlowRNA kit that is marketed by Affymetrix (Catalog # 88-18009-210). The use and mention of this product in the following protocols does not represent an exclusive endorsement of this brand. Rather, the aforementioned kit is the only commercially-available product for this application at the time of this protocol's writing. For the purposes of maintaining neutrality, subsequent reference to the kit will generically be termed 'Branched DNA'.

- a. Store at room temperature:
 - 1.5 mL Branched DNA Microcentrifuge Labeling Tubes (provided with the kit)
 - 12 x 75 mm Polystyrene Tubes (BD Falcon, Catalog # 352052)

- Lymphocyte Separation Medium (Corning, Catalog # 25-072-CV)
 - DNase-, RNase-, and Protease-Free Water (RNase-free Water); Molecular Biology Grade (Corning, Catalog # 46-000-CV)
 - Conical Centrifuge Tube, as Needed
- b.** Store at 2 – 8 °C:
- Branched DNA Fixation Buffer 1A (2X)
 - Branched DNA Fixation Buffer 1B (2X)
 - Branched DNA Permeabilization Buffer (10X)
 - Branched DNA Fixation Buffer 2 (8X)
 - Branched DNA Wash Buffer (1X)
 - Branched DNA Target Probe Diluent
 - Branched DNA PreAmp Mix
 - Branched DNA Amp Mix
 - Branched DNA Label Probe Diluent
 - Branched DNA Storage Buffer
 - Hanks' Balanced Salt Solution (HBSS; Corning, Catalog # 21-023-CV)
- c.** Store at –20 °C
- RNase Inhibitor (1000X)
 - Positive Control Target Probe Sets (20X; one for each mRNA detection channel)
 - Branched DNA Label Probes (100X)
 - Target Probe(s) specific to mRNA sequence(s) of interest - in this example, CD8 mRNA Alexa Fluor® 647 (Type 1, Affymetrix, Catalog # VA1-16494) and RPL13A Alexa Fluor® 750 (Type 6, Affymetrix, Catalog # VA6-13186) probes are employed; custom target probe sets for specific mRNA target sequences of interest can be obtained from the manufacturer (tech@ebioscience.com)
 - Zombie UV™ Fixable Viability Dye (BioLegend, Catalog # 423107)

Equipment

- Flow Cytometer equipped with excitation sources and detector configurations for measuring fluorescence generated from Alexa Fluor® 488, Alexa Fluor® 647 and/or Alexa Fluor® 750.
- Digital NIST-traceable thermometer capable of accurately measuring temperature at 40 °C (Affymetrix, Catalog # QV0523)

- Aluminum heat block that is milled to closely-fit 1.5 mL microcentrifuge tubes (VWR, Catalog # 13259-002)
- Hybridization oven or incubator capable of achieving and maintaining a temperature of 40 ± 1 °C. (Affymetrix, Catalog # QS0704)
- Vacuum Aspiration Device
- Automated Cell Counter or Hemacytometer and Light Microscope

Protocol Steps

Fixation, Permeabilization, Target Probe Hybridization, and Signal Amplification Procedures

1. Separately label as many Branched DNA microcentrifuge tubes as is required for the experiment.
2. Prepare PBMCs using Ficoll density gradient centrifugation or other suitable technique.
 - See Boyum et al. (Boyum, 1968) and Fuss et al. (Fuss et al., 2009) for a discussion on cell preparation using Ficoll density gradient centrifugation or related methodologies. (Graham 2015, CPCB Unit 3.1)
3. Wash the resultant cell suspension twice using HBSS.
4. Perform a cell count and then adjust the cell density to $1 - 5 \times 10^7$ cells / mL.
5. Transfer 100 μ L of cell suspension into each microcentrifuge tube.
 - See Table 1 for a proposed Experimental Design for the BASIC PROTOCOL.
 - For immunophenotyping of cell surface antigens, refer to ALTERNATE PROTOCOL 1.
6. Prepare 1X Fixation Buffer 1 by mixing equal parts of 2X Fixation Buffer 1A and 2X Fixation Buffer 1B into a conical centrifuge tube of suitable size. Mix the combined suspension to homogeneity by gently inverting the tube several times.
 - Avoid vortexing or vigorously shaking this buffer. One mL of this buffer will be required per sample. Prepare this buffer in bulk to accommodate all samples. This buffer should be prepared fresh at the time of use. Properly dispose of any unused buffer according to standard laboratory practice.
7. Add 1 mL of prepared 1X Fixation Buffer 1 to each sample, invert capped tubes several times to mix, and then incubate samples for 30 minutes at $2 - 8$ °C.
8. Centrifuge fixed samples at $800 \times g$ for 5 minutes.
9. Remove the resultant supernatant down to 100 μ L by careful vacuum aspiration and then resuspend cells in the residual buffer volume.

- Use the 100 μ L mark on the microcentrifuge tube for a volume reference.
10. Prepare 1X Permeabilization Buffer with 1000X RNase Inhibitor by first diluting 10X Permeabilization Buffer to 1X with RNase-free water, and then by adding 1000X RNase Inhibitor to the 1X solution at a 1:1000 dilution. Gently mix the solution by inversion and store the Permeabilization Buffer on ice when not in use.
 - Avoid vortexing or vigorously shaking this buffer. For planning purposes, 2 mL of the 1X Permeabilization Buffer is recommended to be used per sample. To ensure consistency amongst determinations, prepare this buffer in bulk to accommodate all samples. Prepare this buffer fresh at the time of use, and dispose of any unused buffer.
 11. Add 1 mL of 1X Permeabilization Buffer with RNase Inhibitor to each sample. Invert tubes to mix, centrifuge at $800 \times g$ for 5 minutes, aspirate supernatant to the 100 μ L mark, and resuspend cells in the residual volume.
 12. Repeat Step 11.
 - For intracellular protein labeling, refer to ALTERNATE PROTOCOL 2.
 13. Prepare 1X Fixation Buffer 2 by combining Fixation Buffer 2 (8X) with the required volume of Wash Buffer. Mix this suspension gently by inverting.
 - Wash Buffer should be pre-warmed to ambient temperature before each use.
 - For accounting purposes, a volume of 1 mL of this buffer is needed per sample. Prepare this buffer in bulk to accommodate all of the samples. For example, for 10 test articles combine 1.25 mL of Fixation Buffer 2 (8X) with 8.75 mL of Wash Buffer to yield 10 mL of 1X Fixation Buffer 2. Prepare this buffer fresh at the time of use, and dispose of any unused buffer.
 14. Add 1 mL of 1X Fixation Buffer 2 to each sample, invert to mix and then incubate for 60 minutes in the dark at room temperature.
 15. Centrifuge fixed samples at $800 \times g$ for 5 minutes, and then aspirate all but 100 μ L of the resultant supernatant. Resuspend cells in the residual volume.
 16. Add 1 mL of Wash Buffer to each sample, invert to mix, and centrifuge at $800 \times g$ for 5 minutes, then aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume.
 - It is critical that the residual volume be as close to 100 μ L as possible. Use the markings on the 1.5 mL tubes provided in the kit to assist with this determination.
 17. Repeat Step 16.

- This represents an optional Stopping Point in the protocol for maintaining a more manageable workflow. Cells may be stored in Wash Buffer with 1X RNase Inhibitor overnight in the dark at 2 – 8 °C. To do so, add RNase Inhibitor to Wash Buffer at a 1:1000 dilution for the last wash in Step 17.
18. Thaw Target Probes, including Positive Control Probes, and maintain at room temperature.
 19. Pre-warm Target Probe Diluent to 40 °C.
 20. Dilute Target Probes 1:20 in Target Probe Diluent. Mix thoroughly by pipetting up and down.
 - A volume of 100 µL of diluted Target Probes will be required for each sample. If more than one Target Probe is employed per sample, adjust the volume of the Target Probe Diluent accordingly; by subtracting the volume of the additional Target Probe(s) from the total volume of 100 µL (i.e. for three target probes add 5 µL of each probe to 85 µL of Target Probe Diluent).
 21. Add 100 µL of diluted Target Probe(s) directly into the cell suspension for the appropriate samples, briefly vortex to mix, and then incubate for 2 hours at 40 ± 1 °C. Invert samples to mix after 1 hour.
 - When adding Diluted Target Probes to processed cell samples, it is critical that the residual sample volume be as close to 100 µL as possible. Diluted Target Probes should then be pipetted directly into the 100 µL of residual volume and samples should be well-mixed before incubating. Be careful not to pipette solutions directly onto the walls of the tubes.
 - Maintaining the 40 ± 1 °C temperature is critical to the success of this procedure. See SUPPORT PROTOCOL 1 and the ‘CRITICAL PARAMETERS AND TROUBLESHOOTING’ Section for additional details.
 22. Add 1 mL of Wash Buffer to each sample, invert to mix, and centrifuge at 800 *x g* for 5 minutes. Aspirate all but 100 µL of supernatant and resuspend cells in the residual volume.
 - This 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.
 23. Repeat Step 22 two more times, for a combined total of three washes.

- If samples were stored overnight at 2 – 8 °C, ensure that samples and Wash Buffer reagents are equilibrated to room temperature prior to executing Step 23.
24. Pre-warm the PreAmp Mix, Amp Mix, and Label Probe Diluent to 40 ± 1 °C.
 25. Add 100 μ L of PreAmp Mix directly into the cell suspension for each sample, briefly vortex to mix, and then incubate for 1.5 hours at 40 ± 1 °C.
 - For all signal amplification steps, it is critical that the residual volume remaining in the sample after all washes are performed be as close to 100 μ L as is possible.
 - PreAmp Mix, Amp Mix, and diluted Label Probes should be pipetted directly into the 100 μ L of residual volume, and samples should be mixed well before incubating. Do not pipette these solutions onto the walls of the tubes.
 26. Add 1 mL of Wash Buffer to each sample, invert to mix, and centrifuge at 800 $\times g$ for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume.
 27. Repeat Step 26 once, for a combined total of two washes.
 28. Thaw Label Probes on ice and in the dark.
 29. Add 100 μ L of Amp Mix directly into the cell suspension for each sample, briefly vortex to mix, and then incubate for 1.5 hours at 40 ± 1 °C.
 30. Add 1 mL of Wash Buffer to each sample, invert to mix, and centrifuge at 800 $\times g$ for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume.
 31. Repeat Step 30.
 32. Dilute 100X Label Probes 1:100 with Label Probe Diluent.
 - A volume of 100 μ L of diluted Label Probes will be necessary for each sample. Prepare diluted Label Probes in bulk to accommodate all samples.
 33. Add 100 μ L of diluted Label Probes directly into the cell suspension for each sample, briefly vortex to mix, and then incubate for 1 hour at 40 ± 1 °C.
 34. Add 1 mL of Wash Buffer to each sample, invert to mix, and centrifuge at 800 $\times g$ for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume.
 35. Repeat Step 34.
 36. Wash samples with 1 mL of Storage Buffer, then transfer samples to 12 x 75 mm polystyrene tubes and acquire samples on a flow cytometer.
 - Samples 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 processed samples should be acquired as soon as possible following the execution of the Branched DNA protocol, in order to preserve optimal fluorescence signal resolution.

Flow Cytometric Acquisition

1. For sample acquisition, excite the fluorochrome Alexa Fluor® 488 using a 488 nm laser and collect its peak emission of approximately 519 nm using an appropriate bandpass filter (e.g. 530/30 nm or equivalent).
2. Excite the fluorochrome Alexa Fluor® 647 using a conventionally-equipped red laser (e.g. 633 nm or 640 nm), and collect its peak emission of approximately 668 nm using an appropriate bandpass filter (e.g. 670/14 nm or equivalent).
3. The fluorochrome Alexa Fluor® 750 is sub-optimally excited using a conventionally-equipped red laser. Nevertheless, the red laser should be used to excite this fluorochrome unless an alternative excitation wavelength is available (excitation maximum = 749 nm). Detect the peak emission of Alexa Fluor® 750 at approximately 775 nm using a 780/60 or equivalent bandpass filter.
4. The voltages for each detected parameter should be established based upon the fluorescence distribution of unlabeled cells that have been subjected to the entire Branched DNA assay.
5. Fluorescence emissions detected in these channels are routinely measured in logarithmic scale. Additionally, forward scatter and side scatter characteristics (including signal pulse area and signal pulse height for both) are collected in linear scale. Apply an appropriate threshold set on FSC-A to exclude debris.
6. For proper spectral compensation calculations, the Positive Control Target Probe Sets provided with the kit must be used. Do not perform compensation for mRNA signal(s) using corresponding fluorochrome-conjugated monoclonal antibodies (mAbs). According to the manufacturer, the Label Probe Sets have different fluorescence characteristics than conventional fluorochrome-conjugated mAbs, and the substitution of mAbs for Label Probes will result in different spillover values in the calculated compensation matrix.
 - Alternatively, the PrimeFlow™ Compensation Kit (Catalog #88-17001-42) can be employed to compensate for RNA signals as per manufacturer's recommendations.

Analysis and Gating Strategy

1. Using a flow cytometry data analysis software package (e.g. WinList, Verity Software House; FlowJo, FlowJo, LLC; FACSDiVa, BD Biosciences; or Kaluza, Beckman Coulter), generate the following bivariate plots and a single parameter histogram as per Figure 2:
 - A. Time vs. FSC-A
 - B. FSC-A vs. FSC-H

- C. FSC-A vs. SSC-A
 - D. SSC-A vs. control mRNA (RPL13A mRNA Alexa Fluor® 750)
 - E. Fluorescence intensity of test article mRNA (e.g. CD8 mRNA Alexa Fluor® 647)
2. Place a rectangular region (R1) on the bivariate plot of Time vs. FSC-A to circumscribe the events acquired in continuity.
 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 a region (R3) to circumscribe the cell population of interest. Gate this bivariate histogram on (R1 and R2).
 5. On the bivariate plot of SSC-A vs. control mRNA (RPL13A mRNA Alexa Fluor® 750), create a region (R4) to include the cell population that is positive for control mRNA expression, excluding cells that did not successfully amplify the control mRNA signal. Gate this bivariate histogram on (R1 - R3).
 - *The boundary of all positive events should be established based upon 'Fluorescence Minus One' controls, in which the panels would be identical to the experimental tubes; except for the exclusion of the fluorochrome that is being controlled (Roederer, 2001).*
 6. The expression level of the mRNA transcript of interest is then evaluated using a single parameter histogram that is serially-gated on (R1-R4). A region (R5) on this histogram is used to discriminate between positive and negative events.

SUPPORT PROTOCOL 1. TEMPERATURE VALIDATION FOR THE HYBRIDIZATION OVEN TO ACHIEVE AND MAINTAIN 40 ± 1 °C

It is extremely critical to establish and maintain a temperature of 40 ± 1 °C in the hybridization oven, in order to obtain successful annealing of nucleic acid components of the Branched DNA complex. A milled aluminum block is employed to facilitate the conduction of heat to the microcentrifuge reaction tube, which also assures rapid equilibration of the reaction mixture to the necessary temperature. If desired, a small amount of water can be added to the well of the milled aluminum block to enhance heat transfer from the block to the reaction tube. It is important to utilize equipment that can re-establish proper temperature specifications within 5 minutes after opening the chamber door to accommodate the placement of samples. The use of a NIST-traceable temperature probe allows for the accurate monitoring and calibration of temperature during the re-equilibration of the chamber.

Materials

Specimen—The specimen requirements for ALTERNATE PROTOCOL 1 are the same as those for the BASIC PROTOCOL

Reagents

- Refer to the BASIC PROTOCOL

Equipment

- Refer to the BASIC PROTOCOL

Protocol Steps

1. Set the hybridization oven to 40 °C, and place the milled aluminum heating block at the center of the chamber.
2. Allow the temperature to equilibrate overnight.
 - *If 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 as is appropriate such that a $40 \pm 1^\circ\text{C}$ temperature is achieved, and then repeat Steps 1 and 2.*
3. Drill a hole in the lid of a Branched DNA microcentrifuge tube to accommodate the temperature probe, and then add 100 μL of Branched DNA Target Probe Diluent into the tube.
4. Insert the temperature probe through the hole and into the diluent, and seal the microcentrifuge tube with laboratory parafilm.
5. Insert the microcentrifuge tube into the pre-warmed heating block and close the door of the hybridization oven.
6. Record the temperature every minute.
 - *Do not use the hybridization oven if it takes longer than 5 minutes to return to 40°C or if it overshoots the set point temperature by more than 2°C during this process.*

ALTERNATE PROTOCOL 1. SIMULTANEOUS MEASUREMENT OF mRNA TRANSCRIPTS IN, AND CELL SURFACE PROTEINS ON PBMCs USING THE BRANCHED DNA ASSAY

The Branched DNA methodology allows for measuring a correlation between the product of nucleic acid transcription and translation on unsorted samples. ALTERNATE PROTOCOL 1 describes the simultaneous measurement of mRNA transcripts and cell surface antigens via detection by flow cytometry. Correlated measurement of CD8 protein and message on lymphocytes are presented as an example. Before starting an experiment, care should be taken to ensure that the phenotyping reagents and antibodies are compatible with the Branched DNA procedure. Refer to SUPPORT PROTOCOL 2 for more details. As an extension of ALTERNATE PROTOCOL 1, a kinetic study of CD8 mRNA and CD8 protein expression was evaluated in stimulated PBMC subsets (see SUPPORT PROTOCOL 3). In this experiment, it was observed that high expression of CD8 mRNA correlated with high CD8 protein expression in lymphocyte subsets.

Materials

Specimen—The specimen requirements for ALTERNATE PROTOCOL 1 are the same as those for BASIC PROTOCOL

Reagents—Refer to BASIC PROTOCOL

Additional Reagents

- Pre-Titrated, fluorescently-labeled mAbs with specificity to targets of interest - in this example, CD8 BV421 (Biolegend, Catalog # 301035, Clone RPA-T8), CD14 BV510 (Biolegend, Catalog # 301841, Clone M5E2), and CD56 PECy7 (BD Bioscience, Catalog # 335809, Clone NCAM16.2) were used
- Human IgG Fc Block Solution, 12 mg / mL (Sigma Aldrich, Catalog # I2511) diluted in RPMI 1640 (Corning, Catalog # 10-040-CV)
- Flow Cytometry Staining (FCM) Buffer (see recipe in the 'REAGENTS AND SOLUTIONS' Section)

Additional Equipment—Refer to the BASIC PROTOCOL, and ensure that the cytometer is capable of measuring fluorescence emission from cells labeled with these additional fluorochrome-conjugated mAbs

Protocol Steps

Surface Labeling

1. Separately label as many Branched DNA microcentrifuge tubes as is required for the experiment.
2. Prepare PBMCs using Ficoll density gradient centrifugation or other suitable technique.
3. Wash the resultant cell suspension twice using FCM Buffer.
4. Perform a cell count, and then adjust the cell density to $1 - 5 \times 10^7$ cells / mL.
5. Transfer 100 μ L of cell suspension into each microcentrifuge tube.
 - See Table 2 for a proposed Experimental Design for ALTERNATE PROTOCOL 1.
6. Prior to cell surface labeling, block unwanted binding of mAbs to Fc receptors by adding 10 μ L of Human IgG Fc Block per 100 μ L of sample volume. Incubate blocked cells on ice for 10 minutes.
7. Transfer laboratory-optimized volume(s) of employed mAb(s) to each sample tube and incubate on ice for 60 minutes. In this example, CD8 BV421, CD14 BV510, and CD56 PECy7 were employed.
 - From this step onwards, protect the samples from exposure to light to minimize the potential effect of photo-bleaching.

8. Add 1 mL of FCM Buffer to each sample, invert to mix, centrifuge at $800 \times g$ for 5 minutes, aspirate supernatant, and then resuspend the washed cells in the residual buffer.
9. From this point onwards, execute Steps 6 – 36 of the BASIC PROTOCOL.

Flow Cytometric Acquisition

1. In addition to the considerations discussed in the BASIC PROTOCOL 'Sample Acquisition' Section, optimize the instrument for the excitation and emission of relevant fluorescently-conjugated mAbs as per standard laboratory protocol.
 - For proper spectral compensation calculations of the fluorescence emission associated with the detection of mRNA, the Positive Control Target Probe Sets provided with the kit must be used as described in the BASIC PROTOCOL. Do not perform compensation calculations for mRNA signal(s) using fluorochrome-conjugated mAbs.
 - For proper spectral compensation of the fluorescence emission associated with protein measurement, the use of single-color mAb stained cells is recommended.

Analysis and Gating Strategy

1. Using a flow cytometry data analysis software package, generate the following bivariate plots, as per Figure 2:
 - (A') Time vs. FSC-A
 - (B') FSC-A vs. FSC-H
2. Using a flow cytometry data analysis software package generate the following bivariate plots, as per Figure 3:
 - (A) FSC-A vs. SSC-A
 - (B) SSC-A vs. RPL13A mRNA Alexa Fluor® 750
 - (C) SSC-A vs. CD8 protein BV421
 - (D) SSC-A vs. CD56 PECy7
 - (E) SSC-A vs. CD14 BV510
 - (F–H) Three separate bivariate plots (Plots (F), (G), and (H)) of CD8 protein BV421 vs. CD8 mRNA Alexa Fluor® 647
3. Perform analysis and gating of Plots (A') – (B') as per Figure 2, (see BASIC PROTOCOL); and (A) – (B) as per Figure 3, adjusting (R3) on Plot (A), to circumscribe the PBMCs.
4. Create an irregular region on Plot (C) to discriminate events that are positive from events that are negative for CD8 protein BV421. Similar regions should be created on Plots (D) and (E) to discriminate respectively, CD56 and CD14

antigen-positive events from antigen-negative events. Serially gate these bivariate histograms on (R1 – R4).

5. Create a 'Quadstat' region on the three 'CD8 protein BV421 vs. CD8 mRNA Alexa Fluor® 647' plots (Plots F – H). These plots should be serially gated on (R1 - R4), and either: (not R7 and not R8), or (R7 and not R8), or (R8 and not R7), respectively.
 - The boundary of all positive events should be established based upon 'Fluorescence Minus One' controls, in which the panels would be identical to the experimental tubes; except for the exclusion of the fluorochrome that is being controlled.

SUPPORT PROTOCOL 2. EFFECT OF THE BRANCHED DNA TECHNIQUE ON THE MEASUREMENT OF DIFFERENT CD ANTIGEN CLONES AND FLUOROCHROMES

The temperature of *in situ* flow cytometry techniques has previously been reported to adversely contribute to increased autofluorescence in acquired cell populations (Muty et al., 1999). Environmental conditions such as the fixation reagents that are associated with the protocol have also been implicated in the attenuation of measured signal from fluorochrome-conjugated antibody reagents. SUPPORT PROTOCOL 2 describes an experiment that should be performed to evaluate the effect of the Branched DNA process on the levels of autofluorescence in measured detection channels, as well as the fluorescence intensities of all utilized mAbs.

Materials

Specimen—The specimen requirements for SUPPORT PROTOCOL 2 are the same as those for ALTERNATE PROTOCOL 1

Reagents—Refer to ALTERNATE PROTOCOL 1

Additional Reagents—Refer to Table 3 for a comprehensive list of fluorochrome-conjugated mAbs that were tested

Equipment—Refer to ALTERNATE PROTOCOL 1

Protocol Steps

1. Using pre-titrated and fluorescently-labeled mAbs with specificity to targets of interest; process samples according to Steps 1 – 9 of ALTERNATE PROTOCOL 1.
 - Expose these samples to the complete Branched DNA procedure, excluding the addition of Target Probes (Step 20 of BASIC PROTOCOL).

2. Process a parallel set of samples according to Steps 1 – 7 of ALTERNATE PROTOCOL 1.
3. For this parallel set of samples only, add 1 mL of FCM Buffer to each sample. Invert to mix, centrifuge at $800 \times g$ for 5 minutes, aspirate supernatant and then resuspend the washed cells in the residual buffer.
4. Repeat Step 3.
5. Add 500 μ L of FCM buffer to each sample and mix to homogeneity. Transfer samples to appropriately labeled 12 x 75 mm polystyrene tubes.

Flow Cytometry Acquisition

1. Acquire samples by flow cytometry as previously described in Step 1 of ALTERNATE PROTOCOL 1, except that the voltages for each relevant detection channel should be established using unlabeled cells which have not been processed according to the Branched DNA assay.

Analysis and Gating Strategy

1. Using a flow cytometry data analysis software package, generate the following bivariate plots:
 - a. Time vs. FSC-A
 - b. FSC-A vs. FSC-H
 - c. FSC-A vs. SSC-A
 - d. Intensity of measured antigen (e.g. CD3 PE or CD4 APCH7) vs. SSC-A
2. Place a rectangular region (R1) on the bivariate plot of Time vs. FSC-A to circumscribe the events acquired in continuity.
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. Create an elliptical region (R3) on FSC-A vs. SSC-A to circumscribe the cellular events having lymphocyte scatter properties or mononuclear cells scatter properties, dependent upon the antigen being studied. Gate this bivariate histogram on (R1 and R2).
 - Use a lymphocyte scatter region for the measurement of fluorescence intensities from cells labeled with mAbs harboring specificity for CD3, CD4, CD8, CD16, CD20, CD45RO, or CD56.
 - Use a mononuclear cell scatter region for the determination of fluorescence intensities from cells labeled with mAbs harboring specificity for CD13, CD14, CD33, or CD64.
5. On the bivariate plot displaying data for the intensity of a measured antigen vs. SSC-A, create regions (R4) and (R5) to circumscribe the negative and positive cell populations, respectively. Gate this bivariate histogram on (R1 - R3).

- The boundaries which discriminate negative from positive events should be established based upon the intensity levels of the corresponding unstained controls.
6. An appropriate method for comparing the data from conventionally-labeled, vs. Branched DNA-processed samples is to calculate a Stain Index for each sample using the formula:

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

The results of these example experiments (Figures 4 – 6) demonstrated that the autofluorescence of interrogated cell samples increased in certain detection channels; and that particular clones, antigens or fluorochromes are more susceptible to the Branched DNA procedure; resulting in an overall decrease in the ability to resolve positive from negative populations (i.e. a decreased Stain Index). Investigators should perform related evaluations to ascertain if their reagents experience similar effects.

SUPPORT PROTOCOL 3. KINETICS OF CD8 mRNA IN, AND CD8 PROTEIN EXPRESSION ON PBMCs AFTER α -CD3 AND α -CD28 ACTIVATION

An advantage of Branched DNA technology is that it permits the simultaneous measurement of mRNA and protein expression dynamics, which can change considerably following cell stimulation. Prior work for example, has elegantly demonstrated the kinetics of IFN γ upregulation in CD4⁺ T cells following stimulation with PMA and ionomycin (Van Hoof et al., 2014). T cells can also be activated with mitogens, calcium ionophores and presented antigens. A convenient method of stimulation is to cross-link the T cell receptor using anti-CD3 and anti-CD28 mAbs (Tario et al., 2011). In this SUPPORT PROTOCOL, the kinetics of CD8 mRNA expression in stimulated PBMCs is correlated with CD8 protein expression on CD8 T cells, NK cells, and monocytes.

As shown in Figure 7, CD8 mRNA was generally observed to be expressed to varying degrees in lymphocytes, NK cells, and monocytes. As expected for lymphocytes (CD14⁻ CD56⁻), the expression of CD8 mRNA was observed to positively correlate with the expression of CD8 protein at all time points in the study. A similar observation was noted for NK cells (CD14⁻ CD56⁺). CD8 protein was also detected in monocytes (CD14⁺ CD56⁻), and this finding is consistent with another published report (Gibbins et al., 2007). The presence of CD8 mRNA in monocytes corroborated the measurement of CD8 protein.

To characterize the cellular localization pattern of CD8 mRNA in all 3 leukocyte subsets, labeled samples were acquired using ImageStream cytometry. The intracellular staining of CD8 mRNA was represented by the punctate and well-segregated red fluorescence signal shown in Figure 8. A spot count of mRNA signal was performed for each acquired cell and the cumulative fluorescence intensity for these spots was subsequently calculated. A linear regression analysis ($R^2 = 0.9879$) demonstrated that a higher cumulative fluorescence

intensity of mRNA could be explained by the proportionate increase in the overall number of detected spots, and was not due to an elevated intensity of a single spot.

Materials

Specimen—The specimen requirements for SUPPORT PROTOCOL 3 are the same as those for ALTERNATE PROTOCOL 1

Reagents—Refer to ALTERNATE PROTOCOL 1

Additional Reagents

- Unconjugated azide-free anti-CD3 (eBioscience, Catalog # 16-0037, Clone OKT3)
- Unconjugated azide-free anti-CD28 (eBioscience, Catalog # 16-0289, Clone CD28.2)
- 6 Peak Ultra Rainbow Fluorescent Calibration Beads, (Spherotech Inc., Catalog # URCP-50-2K)

Equipment—Refer to ALTERNATE PROTOCOL 1

Additional Equipment

- 96-well, round bottom polypropylene plates (Corning, Catalog # 3365)
- Tissue culture incubator capable of achieving and maintaining 37 °C, 5 % CO₂, and 100 % humidity
- ImageStream^X Mark II imaging cytometer (EMD Milipore)

Protocol Steps

1. Generate PBMCs as described in Step 2 of the BASIC PROTOCOL.
2. Incubate 1.5×10^6 PBMCs / mL at 37 °C, 5 % CO₂, and 100 % humidity for up to 36 hours in 96-well, round bottom polypropylene plates with 1 µg / mL azide-free anti-CD3, and 0.5 µg / mL azide-free anti-CD28 in Complete Media (refer to recipe in the REAGENTS AND SOLUTIONS Section).
 - *Details of this procedure are described in Tario et al., 2011 (Tario et al., 2011)*
3. At the indicated times, harvest and process the cells according to ALTERNATE PROTOCOL 1.

Flow Cytometric Acquisition

1. Configure cytometer settings as discussed in ALTERNATE PROTOCOL 1.
2. Prior to performing spectral compensation calculations at Day 0 of the assay, collect a minimum of 10,000 singlet bead events using Ultra Rainbow 6 Peak Fluorescent Calibration Beads, and record the fluorescence intensities of

resolved peaks in all relevant detection channels. Fix and tightly restrict separate linear regions to span resolved peaks on the histograms for each detected parameter. Use these regions as reference points for properly setting detector voltages on each subsequent acquisition. If instrument variability causes the fluorescence peaks of acquired beads to fall outside of these pre-established regions, adjust the detector voltages appropriately prior to sample acquisition.

Analysis and Gating Strategy

1. Perform gating strategy and data analysis as described in Steps 1 – 5 in ALTERNATE PROTOCOL 1.

ALTERNATE PROTOCOL 2. SIMULTANEOUS MEASUREMENT OF mRNA TRANSCRIPTS AND INTRACELLULAR PROTEINS IN PBMCs USING THE BRANCHED DNA ASSAY

Transcription factors are proteins that localize in the cytoplasmic and nuclear compartments. They play a major regulatory role by activating or repressing the rate at which genetic information is transcribed from DNA into mRNA, therefore they can be regarded as important modulators controlling the expression profile of cells. Given this important regulatory function, interest in deciphering the expression patterns of transcription factors in immune cells has grown tremendously, and should provide novel opportunities to better understand the immune system.

Traditionally, bulk analytical methods such as western blot have been employed to measure transcription factors, but the inability to resolve data due to leukocyte heterogeneity, and the modest sensitivity and specificity of such methods have hampered the application of these techniques. An example of this is illustrated by the study of Forkhead box P3 (FOXP3) which was initially believed to be expressed by CD4⁺ T regulatory cells. However, using the discriminating power of multiparametric flow cytometry to elucidate the expression of FOXP3, it became apparent that FOXP3 was not restricted to T regulatory cells, but could also be found in other leukocyte subsets. It was determined that the expression of FOXP3 could instead be the consequence of cellular activation, and was even found to be expressed in many tumors (Liang et al., 2015).

The measurement of transcription factors by flow cytometry has typically required specialized buffer systems. The current version of the Branched DNA assay enables the detection of cell surface proteins and intracellular proteins in conjunction with the measurement of mRNA. ALTERNATE PROTOCOL 2 describes the use of this methodology to detect cell surface expression of CD8 and CD56 with intracellular T-bet and EOMES proteins and also CD8 mRNA. These transcription factors were investigated because studies have shown that T-bet and EOMES play regulatory roles in T cell and NK cell differentiation (Buggert et al., 2014; Knox et al., 2014; McLane et al., 2013).

Results from this experiment are presented in Figure 9 and are consistent with prior observations, demonstrating that the expression levels of CD8 protein and CD8 mRNA were

positively correlated with each other ($r^2 = 0.770$). Two distinct populations were observed in the CD8^{Bright} CD56⁻ lymphocyte subpopulation (i.e. CD8⁺ T cells); one population was EOMES⁻ and T-bet^{-dim}, while the other population was EOMES⁺ and T-bet⁺. The CD8⁻ and CD8^{dim} NK cell populations were heterogeneous for EOMES expression, and exhibit the highest T-bet expression level of all CD56⁺ NK cells. Only the CD8^{Bright} NK cells were positive for EOMES, whilst exhibiting attenuated T-bet expression. The expression of EOMES and T-bet in CD8^{Bright} NK cells corresponded with the transcription factor expression profile in the CD8⁺ T cell population. This observation is consistent with immunologic dogma, as these transcription factors have been reported to play a role in the cytolytic maturation of both effector CD8 T cells and NK cells (Intlekofer et al., 2008).

Materials

Specimen—The specimen requirements for this ALTERNATE PROTOCOL 2 are the same as those for the BASIC PROTOCOL.

Reagents—Refer to ALTERNATE PROTOCOL 1.

Additional Reagents

- Pre-Titrated, fluorescently-labeled mAbs with specificity to targets of interest - in this example, anti-human T-bet PE (eBioscience, Catalog, # 12-5825, Clone eBio4B10) and anti-human EOMES PE-eFluor610 (eBioscience, Catalog # 61-4877, Clone WD1928) were used

Additional Equipment—Refer to the BASIC PROTOCOL, and ensure that the instrument is capable of measuring fluorescence emission from cells labeled with these additional fluorochrome-conjugated mAbs

Protocol Steps

1. Perform Steps 1 to 8 as described in ALTERNATE PROTOCOL 1.
2. Perform Steps 6 to 12 of the BASIC PROTOCOL.
3. Prior to intracellular labeling, block nonspecific binding of mAbs by adding 10 μ L of Human IgG Fc Block per 100 μ L of sample volume. Incubate blocked cells at ambient temperature for 10 minutes.
4. Transfer laboratory-optimized volume(s) of mAb(s) to each sample tube, and incubate at ambient temperature for 30 minutes. In this example, T-bet PE and EOMES PE-eFluor610 were used.
5. Prepare 1X Permeabilization Buffer with RNase Inhibitor by first diluting 10X Permeabilization Buffer to 1X with RNase-free water, and then by adding 1000X RNase Inhibitor to the 1X solution at a 1:1000 dilution. Gently mix the solution by inversion and store the Permeabilization Buffer on ice when not in use.
 - Avoid vortexing or vigorously shaking this buffer. For planning purposes, approximately 1 mL of the 1X Permeabilization Buffer is recommended to be used per sample. To ensure consistency amongst

determinations, prepare fresh buffer in bulk to accommodate all samples. Dispose of any unused buffer.

6. Add 1 mL of 1X Permeabilization Buffer with RNase Inhibitor to each sample, invert to mix, and centrifuge at $800 \times g$ for 5 minutes, then aspirate supernatant to the 100 μ L mark and resuspend cells in the residual volume.
7. From this point onwards, execute Steps 13 – 36 of the BASIC PROTOCOL.

Flow Cytometric Acquisition

1. In addition to the considerations discussed in the ALTERNATE PROTOCOL 1 'Sample Acquisition' Section, optimize the excitation and emission of relevant fluorescently-conjugated mAbs as per standard laboratory practice.

Analysis and Gating Strategy

1. Using a flow cytometry data analysis software package, generate the following bivariate plots as per Figure 2:
 - (A') Time vs. FSC-A
 - (B') FSC-A vs. FSC-H
 - (C') FSC-A vs. SSC-A
 - (D') SSC-A vs. RPL13A mRNA Alexa Fluor® 750
2. Generate the following bivariate plots as per Figure 9:
 - A. A bivariate plot of CD56 protein PECy7 vs. CD8 protein BV421
 - B. A bivariate plot of CD8 protein BV421 vs. CD8 mRNA Alexa Fluor® 647
 - C. Four separate bivariate plots of EOMES protein PE-eFluor610 vs. T-bet protein PE
3. Perform analysis and gating for Plots (A') to (D'), as per the BASIC PROTOCOL
4. Create (R9-R12) on Plot (A), to circumscribe the following populations: R9: CD56 protein PECy7⁻ CD8 protein BV421⁺; R10: CD56⁺ CD8^{bright}; R11: CD56⁺ CD8^{dim}; R12: CD56⁺ CD8⁻. Serially gate this plot on (R1 – R4).
5. Create a 'Quadstat' region on each of the four bivariate plots of EOMES protein PE-eFluor610 vs. T-bet protein PE. These plots should be serially gated as follows: (B) (R1-R4); (C) (R1 - R4 and R9); (D) (R1 - R4 and R12); (E) (R1 - R4 and R11); (F) (R1 - R4 and R10)
 - The boundary of all positive events should be based upon 'Fluorescence Minus One' controls, in which the panels would be identical to the experimental tubes; except for the exclusion of the fluorochrome that is being controlled.

REAGENTS AND SOLUTIONS

1. Flow Cytometry (FCM) Buffer

- Bovine Serum Albumin (0.5 %), Sodium Azide (0.1 %), and Sodium EDTA (0.04 g / L) in Phosphate Buffered Saline. Purchased from Leinco Technologies as 1 liter of a 10X concentrate (Catalog # S622). Reconstitute to 1X with 18.2 MΩ diH₂O. Store at 2 – 8 °C for no longer than 30 days.

2. Complete Media

- RPMI 1640 supplemented with 10 % heat-inactivated fetal bovine serum, 25 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM fresh glutamine, 50 µg / mL gentamicin sulfate (all preceding reagents from Corning) and 5×10^{-5} M β-mercaptoethanol (Sigma Aldrich).

COMMENTARY

RNA represents one of the cell's most important messaging networks, and the transcriptome is believed to reflect the condition of a cell's active functional state at any given point in time (Cox and Mann, 2007; Tyakht et al., 2014). The levels of different mRNA molecules within a cell are dynamic, and are more quickly responsive to external stimuli than downstream protein expression. For example, the detection of IFN γ mRNA in activated lymphocytes has been measured using the Branched DNA assay to precede the expression of protein by 90 minutes (Van Hoof et al., 2014). It is noteworthy that the transcriptome is subjected to post-transcriptional modification and for this reason, complementarity and not necessarily congruence, between transcriptome and proteomic profiles could yield important information related to protein synthesis and regulation pathways; particularly if this data can be measured in a correlated manner (Hanley et al., 2013; Unwin and Whetton, 2006). Studies have shown that both mRNA and protein expression levels can serve as independent prognostic indicators of outcome in both malignant and viral disease states, however the generation of such data through traditional mRNA analysis techniques has hitherto been laborious and time-consuming (Bishop et al., 2012; Rondeau et al., 2015; Ukpo et al., 2011; Walter et al., 2015).

Historically, the measurement of mRNA from biological samples has required several different steps, including tissue homogenization, nucleic acid extraction and purification; followed by Northern blotting or PCR analysis after an additional reverse transcription step. Owing to the fact that large amounts of nucleic acids are required for such analyses, these uniparametric approaches are generally compromised by a high degree of sample heterogeneity from cells of disinterest that invariably contaminate bulk material. Further, processing also disrupts the native tissue architecture, therefore spatial information related to transcript expression patterns is necessarily lost.

To preserve spatial information related to nucleic acid expression, and also to minimize potential sample contamination requires an *in situ* measurement within individual cells. This approach also minimizes the risk of RNase-mediated sample degradation, which is a bane of bulk nucleic acid detection methodologies (Klemm et al., 2014). The first investigations of

this nature were reported in 1969, when Gall and Pardue described the use of RNA probes to target specific DNA sequences through a process termed *in situ* hybridization (Gall and Pardue, 1969). At this time, the probes were detected with radiolabels, which was a lengthy and labor-intensive procedure (Koopman, 2001). Advancements in radiolabeling techniques eventually permitted the detection of only a few dozen mRNA molecules per cell (Harper et al., 1986).

Subsequently, a number of technological and methodological advancements in nucleic acid detection strategies have propelled the field to its current state. A comprehensive review of these developments is beyond the scope of this discussion, but improvements were generally aimed at increasing assay sensitivity, whilst reducing the measured background of alternatives (i.e. fluorescence) to radioactive detection modalities (Goolsby et al., 2000; Holtke and Kessler, 1990; Wiegant et al., 1991; Yang et al., 1994; Yang et al., 1995).

A flow cytometric approach offers a number of logistical improvements over traditionally-employed methodologies, such as faster sampling rates, and improved detection of rare events; with the ability to statistically characterize acquired populations. Events are also interrogated at the single-cell level, which allows for evaluation of the heterogeneity of the measured cell populations. The first report of this nature employed a modified fixation reagent (dimethylsuberimidate), which allowed for nucleic acids to be probed and measured without nuclear disintegration (Trask et al., 1985). Cells processed under these conditions were insensitive to the elevated temperatures and high salt concentrations required for probe hybridization. Detection of nucleic acid sequences was facilitated by the use of a 3-step amplification system that targeted abundant, repetitive DNA; a strategy that was the hallmark of other successful studies (Borzi et al., 1996; Trask et al., 1985; van Dekken et al., 1990).

The detection of mRNA by flow cytometry was first described with single-color experiments which quickly and predictably evolved into the multiplexed detection of mRNA; where 3 separate message species could be reliably detected, and their expression levels could be correlated in parallel samples that were evaluated for DNA content (Bauman and Bentvelzen, 1988; Bayer and Bauman, 1990; Yu et al., 1992). This represented a tremendous achievement, as fluorescence detection signals from mRNA were historically dim, even in specimens with high copy number viral infections (Gibellini et al., 1997; Li et al., 1994; Yu et al., 1992).

A notable advancement contributing to the success of the multiplexed adaptation was the utilization of directly-conjugated fluorescent probes, a strategy that was effective for highly-expressed message. For transcripts expressed at low levels, the use of several separate directly-conjugated probes that bound different regions of the same target was found to increase detection signal as compared to the use of a secondary detection reagent (Koopman, 2001; Tsukamoto et al., 1991; Yu et al., 1992). Methodologically, this is of particular importance as most mRNA species are present in cells at fewer than 50 copies each (Hanley et al., 2013; Levsky and Singer, 2003; Zhang et al., 1997).

Another important strategic development in measuring DNA and mRNA by flow cytometry is represented by the *in situ* PCR amplification of targeted nucleic acid sequences (Chen and Fuggle, 1993; Patterson et al., 1993). A landmark report demonstrated that single-copy HIV DNA target could be suitably amplified, and detected in a highly-specific assay, where cells containing the gene could be readily-discriminated from negative events (Patterson et al., 1993). Separately, the study proved that low-copy number mRNA sequences could also be similarly-amplified *in situ*, and that data from these two measurements could be combined to discriminate populations of productively infected, versus transcriptionally quiescent infected cells. The incorporation of cytometry-based *in situ* PCR with concurrent immunophenotyping was met with limited success as the high temperatures achieved during thermal cycling impart a deleterious effect on fluorochrome and protein stability (Muty et al., 1999). A clever solution to this problem was to employ a biotin – streptavidin detection system, which allowed for the initial labeling of cell surface targets using a biotinylated antibody. After the PCR reaction, a fluorescent streptavidin reagent could be safely added; however, this approach limited flow cytometric immunophenotyping to just one antigenic determination (Patterson et al., 1995).

Measuring low-count mRNA transcripts concomitant with multiparametric cell surface epitope detection could alternatively be accomplished at lower temperatures, and without amplification through the use of a pool of fluorescently-labeled mRNA probes that target multiple mRNAs with several probes for each mRNA. In a striking example of the power of this approach, investigators demonstrated that while infected CD4+ T cells and CD14+ monocytes both harbored HIV DNA, only the monocytes were transcriptionally active, even during late stage disease progression (Patterson et al., 1998). These observations identified an unappreciated reservoir of replicating HIV virus, and clarified persistent questions surrounding the role of leukocyte subsets at different stages of infection.

Recently, efforts have refocused on amplification strategies, however the target of this amplification has become the probe detection system, instead of the mRNA sequence, itself. This procedural modification is known as the ‘Branched DNA’ assay, which utilizes a series of amplification steps for achieving high sensitivity and specificity (Collins et al., 1997; Kapke et al., 1997; Player et al., 2001; Wang et al., 2012). This refinement resulted in the profound amplification of specific mRNA signals, while considerably suppressing the non-specific background noise that has otherwise plagued prior methodologies (Porichis et al., 2014). Interestingly, this approach also allowed for the use of far lower nucleic acid annealing temperatures (40°C), than those that are utilized with conventional *in situ* PCR protocols, thus permitting the simultaneous and correlated measurements of multiple mRNA species and antigens.

The improved sensitivity and specificity of the Branched DNA technique has resurrected interest in the detection, quantification, and visualization of mRNA molecules in single cell suspension by flow and imaging cytometry (Hanley et al., 2013; Porichis et al., 2014). The ability to multiplex the detection of mRNA with pre-existing flow cytometric methodologies represents a major development in the ability to characterize cellular responses to environment, disease and therapeutic intervention.

CRITICAL PARAMETERS AND TROUBLESHOOTING

The implementation of Branched DNA assay for flow cytometry is a relatively new technology that was adapted from microscopy. Certain precautions must be taken to prevent adverse experimental outcomes. This section describes critical parameters that should be optimized individually prior to the execution of a Branched DNA experiment in order to generate quality results that are neither skewed nor distorted due non-specific or weak-to-negative mRNA signal. Refer to Internet Resources Section for a link to a detailed troubleshooting guide. The points below are the most important to consider:

1. Hybridization Temperature is Critical to the Success of an Experiment
 - The overall ability of the hybridization oven and heating block to achieve and consistently maintain at 40 ± 1 °C is crucial to ensure a successful outcome for the Branched DNA assay. This is because hybridization of the Target Probes, Pre-amplifier, Amplifier, and Label Probes will occur only within a narrow temperature range. Deviation from this prescribed range will otherwise retard the annealing process or will inhibit hybridization altogether. For detailed information on the best practices to calibrate the hybridization equipment, refer to SUPPORT PROTOCOL 1. Care should also be taken to minimize entry to the hybridization oven in order to ensure that consistent temperature is maintained.
2. Assignment of mRNA Detection Probes
 - The three Labeling Probe formats currently available for this assay are conjugated to either Alexa Fluor® 488, Alexa Fluor® 647, and Alexa Fluor® 750. Of these, the fluorescence emission of Alexa Fluor® 647 is generally the brightest on conventionally configured instruments. For this reason it should be reserved for mRNA targets of lowest abundance or of highest priority. Since Alexa Fluor® 750 is sub-optimally excited on most standard flow cytometers, mRNAs which are typically expressed at higher frequencies such as the control mRNAs should be detected with the Alexa Fluor® 750 probe set. Alexa Fluor® 488, while optimally excited by 488 nm lasers, has a lower fluorescence yield than Alexa Fluor® 647. Additionally, the detection channel where Alexa Fluor® 488 is measured has been associated with increased levels of background fluorescence.
 - From a nomenclature perspective, the 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). Only the Target Probe type needs to be matched to either of these indicated probe types, depending upon the channel in which mRNA signals are to be detected. All of the other amplification components in the Branched DNA kit provided by Affymetrix (i.e. the Pre-Amplifier, Amplifier, and Label Probes) represent an inseparable mixture of all three amplification probe sets.

3. Reduce Factors which Contribute to High Background
 - Certain variables can lead to increased autofluorescence levels and high background in negative cell populations. Amongst these are excessive fixation, insufficient washing, and the use of improper Target Probe volumes. Ensure that protocol steps are strictly adhered to in this regard in order to obtain the best results possible.
4. Ensure Proper Compensation of mRNA- and Protein-Conjugated Fluorochromes
 - Compensation settings are established for each experiment to subtract the spectral overlap of different fluorochromes employed in the assay. For the proper compensation of mRNA probe signals using the Branched DNA assay, the use of Positive Control mRNA Target Probe Sets is required. Alternatively, the PrimeFlow™ Compensation Kit can be employed to compensate for RNA signals (see BASIC PROTOCOL). For proper spectral compensation of fluorochrome-labeled proteins, single-color mAb stained cells or beads is recommended. These controls should be exposed to the entire procedure in parallel with the test articles. It is appropriate to substitute a brightly-detected fluorochrome-conjugated mAb as a compensation control to replace a similar dimly-detected fluorochrome-conjugated mAb (i.e. CD8 PE is used as a compensation control for T-bet PE). An exception is made for all tandem dyes, such as PECy7 or PE-eFluor610, where the actual mAb-fluorochrome conjugate employed in the experiment should be used to establish the compensation matrix.
5. Assure Accurate Discrimination between Positive and Negative Events
 - The use of FMO controls, which are processed in parallel with test articles, is highly encouraged for the detection channels where mRNA probes are to be measured. This practice will facilitate the proper placement of analysis regions that are used in discriminating mRNA-positive events from mRNA-negative events.
6. Use Appropriate Branched DNA Microcentrifuge Labeling Tubes
 - The tubes included in the Branched DNA kit have been validated for this procedure. If an investigator desires to substitute this component of the assay, the tubes should be thoroughly tested for compatibility with this technique using relevant positive and negative controls. Substituted tubes may have been contaminated with nucleases, might not provide for proper temperature transfer during hybridization steps, and/or may contain wetting agents that interfere with the assay.
7. Considerations for minimizing cell loss
 - The Branched DNA assay involves a series of sequential labeling and amplification steps with intervening wash steps that can result in cell losses. Consider the following points when performing the Branched

DNA assay in order to retain as many cells as possible for flow cytometric acquisition:

- a. Use the recommended Branched DNA Microcentrifuge Labeling Tubes.
- b. Use a centrifuge with a swinging bucket rotor.
- c. Use a vacuum aspirator to remove the supernatant from cell washes.
- d. Ideally process at least 5×10^6 (and a minimum of 1×10^6) cells per experimental tube.

ANTICIPATED RESULTS

Typical results for the measurement of mRNA, surface proteins, and intracellular proteins when samples have been labeled using the Branched DNA technique are presented in Figures 2, 3, 7, and 9. In practice, the results obtained from separate experiments may differ considerably from these examples, depending upon the nature of the experiment that is performed (e.g. the mAbs used, the biological specimens tested, and the experimental conditions employed). For any experiment that employs the Branched DNA labeling technique, it is recommended to include FMO controls as the standard reference for region placement; in order to facilitate the discrimination of positive events from negative events. As demonstrated in the example experiment described in SUPPORT PROTOCOL 2, exposure of labeled samples to the Branched DNA assay may adversely affect the detection of fluorescently-conjugated mAb, by either increasing background levels, decreasing measured fluorescence signals, or both. The results associated with these observations are presented in Figures 4 – 6.

TIME CONSIDERATIONS

The number of experimental samples included in the investigation will influence the actual length of the experiment. A typical full-length Branched DNA procedure using the BASIC PROTOCOL will require a minimum of 10 hours to complete. Inclusion of the cell surface labeling steps (ALTERNATE PROTOCOL 1) will add an additional 1.5 hours to the procedure. Execution of the intracellular labeling process (ALTERNATE PROTOCOL 2) will add another hour to the assay.

As described above, the Branched DNA assay has several Stopping Points when samples can be stored and processing can subsequently be resumed at a later time. For potential Stopping Points, see Steps 17 and 22 of the BASIC PROTOCOL.

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Figure 1. Signal Amplification of mRNA using the Branched DNA Assay

Fixed and permeabilized cells were sequentially hybridized at $40 \pm 1^\circ\text{C}$ with Target Probes, Pre-amplifiers, Amplifiers, and Labeling Probes with washing steps performed in between. In order for signal amplification to proceed, a pair of Target Probes must hybridize adjacent to each other, so that the 'Pre-Amplifier' molecules can anneal stably to both Target Probes. Each individual Target Probe is comprised of approximately 18 – 25 DNA bases with specificity for a sequence of interest. A targeted mRNA sequence containing an average of 1,000 bases can accommodate up to 20 different pairs of Target Probes, thus providing the foundation for the initial signal amplification. The portion of the Target Probe that does not bind to the mRNA sequence exhibits specificity for a defined region on one of the three Pre-amplifier sequences. This complementarity assures the specificity of the remaining signal amplification reagents. Thereafter, 'Amplifier' molecules hybridize to multiple sites on the 'Pre-amplifier' molecules, followed by the hybridization of fluorescent Label Probes to multiple sites on the 'Amplifier' molecules. A fully assembled Branched DNA complex can result in approximately an 8,000 to 16,000-fold signal amplification.

Figure 2. Measurement of mRNA Transcripts in PBMCs using the Branched DNA Assay
 (A) A rectangular region (R1) is created on a bivariate plot of Time (event chronology) vs. FSC-A to assess the compositional homogeneity of collected events, in order to exclude events of disinterest from the sample acquisition. (B) A rectangular region (R2) is created on a gated (R1) bivariate plot of FSC-A vs. FSC-H to circumscribe the singlet cell population. (C) An elliptical region (R3) is created on a gated (R1 and R2) bivariate plot of FSC-A vs. SSC-A to circumscribe cellular events having lymphocyte scatter characteristics. (D) A rectangular region (R4) is created on a gated (R1 - R3) bivariate plot of SSC-A vs. RPL13A mRNA Alexa Fluor® 750 (AX750) to circumscribe cellular events that have successfully hybridized with the RPL13A mRNA Target Probe. (E) Finally, a linear region (R5) is created on a gated (R1 - R4) single parameter histogram of CD8 mRNA Alexa Fluor® 647 (AX647) to measure the fluorescence intensity of CD8 mRNA. The placement of (R4) and (R5) are based upon the intensity levels of the corresponding FMO controls (data not shown).

Figure 3. Simultaneous Measurement of mRNA Transcripts in, and Cell Surface Proteins on PBMCs using the Branched DNA Assay

Gating is initially performed using regions similar to (R1) and (R2) from Figure 2.

Thereafter in this Figure, (A) an elliptical region (R3) is created on a gated (R1 and R2) bivariate plot of FSC-A vs. SSC-A to circumscribe cellular events having mononuclear cell scatter characteristics. (B) A rectangular region (R4) is created on a gated (R1 - R3) bivariate plot of SSC-A vs. RPL13A mRNA Alexa Fluor® 750 (AX750) to circumscribe cellular events that have successfully hybridized with the RPL13A mRNA Target Probe. (C) For gating purposes, an irregular region (R6) is created on a gated (R1 - R4) bivariate plot of SSC-A vs. CD8 protein BV421 to circumscribe cellular events that are positive for CD8 protein. (D) An irregular region (R7) is created on a gated (R1 - R4) bivariate plot of SSC-A vs. CD56 protein PECy7 to circumscribe cellular events that are positive for CD56 protein. (E) An irregular region (R8) is created on a gated (R1 - R4) bivariate plot of SSC-A vs. CD14 protein BV510 to circumscribe cellular events that are positive for CD14 protein. (F) Thereafter, three separate bivariate plots of CD8 protein BV421 vs. CD8 mRNA Alexa Fluor® 647 (AX647): (F) gated on (R1 - R4 and not R7 and not R8); or (G) gated on (R1 - R4 and R7 and not R8); or (H) gated on (R1 - R4 and R8 and not R7). The placement of R4, R6, R7, R8, and the Quadstat regions on (F), (G), and (H) are based upon the intensity levels of the corresponding FMO controls (data not shown).

Figure 4. Effect of the Branched DNA Assay on the Measurement of Cell Surface Antigen Fluorescence Intensities, and Autofluorescence Background Levels when Compared to a Standard Immunophenotyping Procedure

Twelve different cell surface epitopes were measured using PE-conjugated mAbs when processed according to the Branched DNA methodology (grey bars). These measurements were compared to parallel samples that were processed using a standard immunophenotyping procedure (black bars). Measurements were performed on bivariate histograms of PE antigen intensity vs. SSC-A which were gated using a lymphocyte scatter region, except for the determinations of CD13, CD14, CD33, and CD64; which were gated using a mononuclear cell scatter region. In all cases, the placement of regions discriminating positive events from negative events were based upon the intensity levels of the corresponding unstained controls. The gMFI of (A) antigen-positive events and (B) antigen-

negative events are graphed. (C) Stain Indices were calculated using the formula from SUPPORT PROTOCOL 2, and these values are graphed. Data shown represent the calculated average of triplicate measurements for each sample, and the error bars depict their standard deviations.

Figure 5. The Effect of the Branched DNA Assay on Signal Resolution Differs Dependent upon the Clone, Antigen, or the Fluorochrome that is Being Measured

Representative histograms comparing the fluorescence intensity distributions of lymphocytes that are labeled with (A) CD8 PE or (B) CD45RO PE when processed in parallel using the Branched DNA assay (grey histograms) or a standard immunophenotyping protocol (black histograms). Fluorescence intensity distributions of lymphocytes that were labeled with (C) CD56 FITC (Clone NCAM16.2), (D) CD56 PECy7 (Clone CMSSB), or (E) CD56 PECy7 (Clone NCAM16.2). In all cases, the placement of regions discriminating CD56 positive events from CD56 negative events was based upon the intensity levels of the corresponding unstained controls (data not shown). Stain Indices were calculated using the formula from SUPPORT PROTOCOL 2, and these values are presented in each histogram.

Figure 6. Differential Effect of the Branched DNA Assay on the Measurement of Fluorescence Intensities from Selected Fluorochromes when Compared to Fluorescence Intensities Measured using a Standard Immunophenotyping Procedure

Eight different fluorochromes were used to measure (A) CD3 (Clone SK7) or (B) CD4 (Clone SK3) when processed according to the Branched DNA methodology (grey bars). These measurements were compared to parallel samples that were processed using a standard immunophenotyping procedure (black bars). Measurements were performed on bivariate histograms of fluorochrome antigen intensity vs. SSC-A, which were gated using a lymphocyte scatter region. In all cases, the placement of regions discriminating positive events from negative events were based upon the intensity levels of the corresponding unstained controls. Stain Indices were calculated from these gMFI measurements using the formula from SUPPORT PROTOCOL 2, and these values are graphed. Data shown represent the calculated average of triplicate measurements for each sample, and the error bars depict their standard deviations.

Figure 7. Kinetic Study Utilizing the Simultaneous Measurement of mRNA Transcripts in, and Cell Surface Proteins on PBMCs using the Branched DNA Assay

PMBCs were isolated from fresh whole blood by Ficoll density gradient separation. These cells were stimulated according to SUPPORT PROTOCOL 3, harvested at indicated time points, and processed according to ALTERNATE PROTOCOL 1. CD8 protein and mRNA levels were evaluated for lymphocytes (excluding CD56⁺ events; top row), NK cells (middle row), and monocytes (bottom row). Each column represents a separate time point. The gating of leukocyte subsets is performed as described in Figure 3 to obtain bivariate plots of CD8 protein BV421 vs. CD8 mRNA Alexa Fluor® 647 (AX647) for the analysis. The placement of Quadstat regions on all plots are based upon the intensity levels of the corresponding FMO controls (data not shown).

Figure 8. Simultaneous Measurement by ImageStream Cytometry of mRNA Transcripts in, and Cell Surface Proteins on PBMCs using the Branched DNA Assay

Samples described in Figure 7 were acquired by ImageStream cytometry to confirm the measurement of CD8 mRNA in leukocyte subsets. Due to this instrument's configuration, CD8 PE (Thermo Fisher Scientific, Catalog # MHCD0804-4, Clone 3B5) replaced CD8 BV421 and Fixable Live/Dead Violet (Thermo Fisher Scientific, Catalog # L34955) was employed in lieu of Fixable Zombie UVTM.

Ten thousand objects per file were acquired at a low flow rate, with the Extended Depth of Field option and 60X objective employed on an ImageStream^X-Mark II cytometer. The 405 nm, 488 nm and 642 nm excitation laser intensities were set at 100, 200, and 100 mW respectively; and the PE, PECy7, Fixable Live/Dead Violet, BV510 and Alexa Fluor® 647 fluorescence emissions were captured in detector channels 3 (560 – 595 nm), 6 (745 – 800 nm), 7 (435 – 505 nm), 8 (505 – 570 nm), and 11 (642 – 745 nm), respectively.

The raw image files were compensated using IDEAS v6.1 software (EMD Millipore). Compensation matrices were constructed based on single-color controls that were acquired with brightfield illumination and the 785 nm scatter laser turned off. Objects were hierarchically gated for single cells (Brightfield Area vs. Aspect Ratio), focus (Gradient RMS of channel 11), and viability (Fixable Live/Dead Violet negative). Bivariate plots were constructed of the relevant parameters, and gated for CD8⁺ lymphocytes, CD14⁺ monocytes, or CD56⁺ CD14⁻ NK cells, with region placements based on internally present negative cell populations. Representative images of hybridization signals obtained from

immunophenotyped subpopulations are depicted in (A) CD8⁺ lymphocytes (B) CD56⁺ NK cells, and (C) CD14⁺ monocytes. In all cases, CD8 mRNA hybridization is represented by red pseudocolor; and CD8, CD14, or CD56 proteins are represented by green pseudocolor. For the spot count determination (D), hybridization signals were segmented by optimizing the signal-to-background ratio as previously described (Minderman et al., 2012) and the spot counts per cell were determined using the 'Spot Count' feature of IDEAS software. The corresponding cumulative spot intensity per cell is a function of the collective hybridization signal intensity for each individual cell. This data is a measurement of fluorescence from only those areas which were identified as spots by segmentation analysis via the software. The graph illustrates the correlation between the number of hybridized spots that were enumerated per CD8⁺ lymphocyte and the total cumulative spot intensity per cell. Error bars represent the standard deviations of mean cumulative spot intensities.

Figure 9. Simultaneous Measurement of mRNA Transcripts and Intracellular Proteins in, and Cell Surface Proteins on, PBMCs using the Branched DNA Assay

Gating was initially performed using regions similar to (R1 – R4) from Figure 2. In this Figure, (A) rectangular regions were created on a bivariate plot of CD56 protein PECy7 vs. CD8 protein BV421 to circumscribe the following populations: (R9): CD56 protein PECy7⁺ CD8 protein BV421⁺; (R10): CD56⁺ CD8^{bright}; (R11): CD56⁺ CD8^{dim}; (R12): CD56⁺ CD8⁻. (B) A gated (R1 – R4) bivariate plot of CD8 protein BV421 vs. CD8 mRNA Alexa Fluor® 647 (AX647) was created to measure the correlation between CD8 protein and mRNA expression levels in lymphocytes. Four gated bivariate plots of EOMES protein PE-eFluor610 vs. Tbet protein PE were created to evaluate the intracellular protein expression profile of lymphocyte populations. Histogram gates were defined as: (C) (R1 – R4 and R9); (D) (R1 – R4 and R12); (E) (R1 – R4 and R11); (F) (R1 – R4 and R10) to display the EOMES vs. Tbet fluorescence data for the populations defined in (A). The placement of the Quadstat regions on (B – F) were based upon the intensity levels of the corresponding FMO controls (data not shown).

Table 1

Experimental Layout for the Measurement of mRNA Transcripts in PBMCs using the Branched DNA Assay.

Tube Number	Tube Description		640 nm Laser	
			AX647 ^a	AX750 ^b
			R670/14	R780/60
1	Unlabeled ^c		-	-
2	Single Color Controls	AX647	RPL13A (m) ^d	-
3		AX750	-	RPL13A (m)
4	Fluorescence Minus One Controls	No AX647	-	RPL13A (m)
5		No AX750	CD8 (m)	-
6	Experimental Tube		CD8 (m)	RPL13A (m)

^a AX647: Alexa Fluor® 647

^b AX750: Alexa Fluor® 750

^c Samples are exposed to all Branched DNA reagents, except for Target Probes

^d Denotes the measurement of mRNA for the associated target sequence

- Denotes no Target Probes are added

Table 2

Experimental Layout for the Simultaneous Measurement of mRNA Transcripts and Cell Surface Proteins in PBMCs Using the Branched DNA Assay.

Tube Number	Tube Description	405 nm Laser		488 nm Laser	640 nm Laser	
		BV421	BV510	PECy7	AX647 ^a	AX750 ^b
1	Unlabeled ^c	-	-	-	-	-
2	Single Color Controls	CD8 (p) ^d	-	-	-	-
3		-	CD14 (p)	-	-	-
4		-	-	CD56 (p)	-	-
5		-	-	-	RPL13A (m) ^e	-
6		-	-	-	-	RPL13A (m)
7		No BV421	CD14 (p)	CD56 (p)	CD8 (m)	RPL13A (m)
8	Fluorescence Minus One Controls	No BV510	-	CD56 (p)	CD8 (m)	RPL13A (m)
9		No PECy7	CD14 (p)	-	CD8 (m)	RPL13A (m)
10		No AX647	CD14 (p)	CD56 (P)	-	RPL13A (m)
11		No AX750	CD14 (p)	CD56 (P)	CD8 (m)	-
12	Experimental Tube	CD8 (p)	CD14 (p)	CD56 (P)	CD8 (m)	RPL13A (m)

^a AX647: Alexa Fluor® 647

^b AX750: Alexa Fluor® 750

^c Samples are exposed to all Branched DNA reagents, except for Target Probes

^d Denotes the measurement of protein expression

^e Denotes the measurement of mRNA for the associated target sequence

- Denotes no Target Probes or fluorochrome-conjugated mAbs are added

Table 3

Fluorochrome-conjugated mAbs used in the Study.

Antibody Specificity	Format	Catalog Number	Clone	Vendor
CD3	PE	347347	SK7	BD Biosciences
CD3	V450	652356	SK7	BD Biosciences
CD3	BV510	344827	SK7	Biolegend
CD3	FITC	349201	SK7	BD Biosciences
CD3	PE	347347	SK7	BD Biosciences
CD3	PerCPCy5.5	340948	SK7	BD Biosciences
CD3	PECy7	341101	SK7	BD Biosciences
CD3	APC	340661	SK7	BD Biosciences
CD3	APCH7	641406	SK7	BD Biosciences
CD4	PE	340670	SK3	BD Biosciences
CD4	V450	651850	SK3	BD Biosciences
CD4	BV510	562970	SK3	BD Biosciences
CD4	FITC	340133	SK3	BD Biosciences
CD4	PE	340670	SK3	BD Biosciences
CD4	PerCPCy5.5	341653	SK3	BD Biosciences
CD4	PECy7	348799	SK3	BD Biosciences
CD4	APC	340672	SK3	BD Biosciences
CD4	APCH7	641407	SK3	BD Biosciences
CD8	PE	MHCD0804-4	3B5	Thermo Fisher Scientific
CD10	PE	IM1915U	ALB1	Beckman Coulter
CD13	PE	340686	L138	BD Biosciences
CD14	PE	340683	MφP9	BD Biosciences
CD16	PE	MHCD1604-4	3G8	Thermo Fisher Scientific
CD20	PE	346595	L27	BD Biosciences
CD25	PE	341010	2A3	BD Biosciences
CD33	PE	IM1179U	D3HL60.251	Beckman Coulter
CD45RO	PE	IM1307	UCHL 1	Beckman Coulter
CD56	PE	6603067	NKH-1	Beckman Coulter
CD56	PE	12-0567-41	CMSSB	eBioscience
CD56	PECy7	25-0567-41	CMSSB	eBioscience
CD56	PECy7	335809	NCAM16.2	BD Biosciences
CD56	FITC	340410	NCAM16.2	BD Biosciences
CD64	PE	CD64-322P	32.2	Trillium Diagnostic
CD69	PE	341652	L78	BD Biosciences

Experimental Layout for the Simultaneous Measurement of Cell Surface Proteins, Intracellular Proteins, and CD8 mRNA in PBMCs using the Branched DNA Assay.

Table 4

Tube Number	Tube Description	405 nm Laser		488 nm Laser		640 nm Laser	
		BV421	BV510	PE	PE-eFluor610	PECy7	AX647 ^a AX750 ^b
1	Unlabeled ^c	-	-	-	-	-	-
2	Single Color Controls	CD8 (p) ^d		-	-	-	-
3		BV510	CD14 (p)	-	-	-	-
4		PE	-	CD8 (p)	-	-	-
5		PE-eFluor610	-	-	EOMES (p)	-	-
6		PECy7	-	-	-	CD56 (p)	-
7		AX647	-	-	-	RPL13A (m) ^e	-
8		AX750	-	-	-	-	RPL13A (m)
9		No BV421	CD14 (p)	T-bet (p)	EOMES (p)	CD56 (p)	CD8 (m) RPL13A (m)
10	Fluorescence Minus One Controls	No BV510	CD8 (p)	T-bet (p)	EOMES (p)	CD56 (p)	CD8 (m) RPL13A (m)
11		No PE	CD8 (p)	-	EOMES (p)	CD56 (p)	CD8 (m) RPL13A (m)
12		No PE-eFluor610	CD8 (p)	T-bet (p)	-	CD56 (p)	CD8 (m) RPL13A (m)
13		No PECy7	CD8 (p)	CD14 (p)	EOMES (p)	-	CD8 (m) RPL13A (m)
14		No AX647	CD8 (p)	CD14 (p)	EOMES (p)	CD56 (p)	- RPL13A (m)
15		No AX750	CD8 (p)	CD14 (p)	EOMES (p)	CD56 (p)	CD8 (m) - RPL13A (m)
16		Experimental Tube	CD8 (p)	CD14 (p)	EOMES (p)	CD56 (p)	CD8 (m) RPL13A (m)

^a AX647: Alexa Fluor® 647

^b AX750: Alexa Fluor® 750

^c Samples are exposed to all Branched DNA reagents, except for Target Probes

^d Denotes the measurement of protein expression

^e Denotes the measurement of mRNA for the associated target sequence

- Denotes no Target Probes are added