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DNA-Directed Assembly of Antibody-Fluorophore Conjugates for Quantitative Multiparametric Flow Cytometry

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Abstract

Multiparametric flow cytometry offers a powerful approach to single cell analysis with broad applications in research and diagnostics. Despite advances in instrumentation, progress in methodology has lagged, offering no simple and efficient method for antibody labeling or quantifying antibody binding per cell. Here, we describe a DNA-directed assembly approach to fluorescent labeling that overcomes these barriers. Oligonucleotide-tagged antibodies and microparticles are annealed to complementary oligonucleotides bearing fluorophores to create assay-specific labeling probes and controls, respectively. The relative fluorescent intensity of labeled cells to control particles allows direct conversion of qualitative data to quantitative units of antibody binding per cell. Importantly, a single antibody can be labeled with any fluor using a simple mix-and-match labeling strategy. Thus, any antibody can provide a quantitative probe in any fluorescent channel, overcoming major barriers that limit the value of flow cytometry as a tool for systems biology and clinical diagnostics.

Keywords

antibodies; fluorescent probes; immunology; hydrazones; oligonucleotides

Introduction

Over the last two decades, the use of multiparameter, multicolor flow cytometry to interrogate cells with broad panels of fluorescent antibody conjugates has been established as a powerful approach for cellular analysis,^[1] clinical assays,^[2] and high-content screening.^[3] Though now a relatively mature technology, flow cytometry has yet to achieve levels of quantitation characteristic of many other immunoassays. Beyond intrinsic cell-to-cell variation in antigen expression, a range of factors including sample handling, choice of antibody clones and fluorophores, instrumentation specification and settings, and methods of data acquisition and analysis can all affect results, leading to high sample-to-sample, day-to-day, operator-to-operator, and site-to-site variability.^[4] Recently, the literature has seen increasing calls to action towards improved methods of standardization and quantitation in the field,^[5] yet significant barriers remain.

Benchtop, multiple-laser flow cytometers are widely available, and an ever-increasing number of fluorescent dyes and proteins available on the market has greatly simplified multiparametric target analysis and cell phenotyping.^[6] While the range of commercially available fluorescent antibodies continues to expand, purchasing even commonly used

antibodies in a full spectrum of colors can be cost-prohibitive, and many antibodies may only be offered conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE), hindering their use in multiple-target panels. Indirect detection with fluorescent secondary antibodies allows greater flexibility in antibody/fluorophore pairing, but effectively limits multitarget analysis in a single sample to two or three antigens. Direct fluorescent labeling of primary antibodies at the laboratory bench is time consuming, and remains inefficient, unpredictable and poorly reproducible.

Another limitation of current methods of cytometric analysis is that flow cytometry is not readily applied to quantitative analysis of cellular antigen levels, and thus offers limited potential as a tool for systems biology. Traditionally, multiparameter data are displayed as distributions of cells based on their relative fluorescent intensity in multiple channels, offering only qualitative impressions of patterns of immunoreactivity.

Here, we describe a strategy applying DNA-directed assembly^[7] to overcome each of these barriers. Each primary antibody is labeled with an oligonucleotide (Scheme 1). A complementary oligo'-polyfluorophore construct (Scheme 2) consists of multiple fluorophores conjugated to an oligo-dextran scaffold. The conjugation of multiple fluorophores per oligo-dextran results in increased fluorescence intensity, improving flow cytometric signal detection. The antibody-oligo is then annealed to the desired complementary oligo'-polyfluor (Scheme 3) and the resulting hybrid is used to stain cells for flow cytometry in the conventional manner, much like a commercial fluorescent antibody conjugate.

To enable quantitation of antibody binding per cell (commonly referred to as ABC), microspheres are oligo-modified at the surface, and then annealed to a known number of complementary oligo'-polyfluor molecules (Scheme 4) identical to the oligo'-polyfluor used in the cell labeling assay. Cytometric analysis of the fluorescent microspheres permit ready conversion of measurement of relative cytometer fluorescence intensity to molecules of fluorophore per cell. Where each antibody-polyfluor hybrid is prepared at a ratio of one antibody to one oligo'-polyfluor, comparison of microsphere intensity to fluorescence intensity of antibody-fluorophore labeled cells offers a direct route to quantitative measurement of ABC. ABC measurement standardizes experimental data and allows independence from variability in antibody-fluorophore pairings, instrumentation properties, and operator settings. This simple and flexible methodology has the potential to enable fully automated and quantitative multicolor flow cytometry.

Results and Discussion

Preparation of oligonucleotide conjugates and annealing to form fluorescent antibody probes

We initially selected a panel of four complementary sequence pairs of DNA oligonucleotides, **1/1'** – **4/4'** (Supporting Table 1), from oligonucleotide pairs designed by Feldkamp et al.^[8] for use in DNA-directed assembly experiments and validated to display low cross-annealing at room temperature. A fifth pair of complementary oligonucleotides, **A/A'**, was designed using CANADA design software for DNA directed assembly probes^[9], to serve as an example of a “universal” oligonucleotide tag sequence.

Antibody-oligonucleotide conjugates were prepared using HyNic/4FB bis-aryl hydrazone conjugation as illustrated in Scheme 1. The antibody was modified with a succinimidyl 6-hydrazinonicotinate acetone hydrazone (S-HyNic) crosslinker to incorporate a limited number of HyNic linker moieties on the ϵ -amino groups of lysines. A 5'-amino-modified DNA oligonucleotide was converted to a 4FB-modified oligonucleotide using succinimidyl

4-formylbenzoate (S-4FB). The HyNic-modified antibody and excess 4FB-modified oligonucleotide were combined in the presence of aniline as a catalyst^[10] to yield the desired antibody-oligonucleotide conjugate, which was then purified by size exclusion chromatography. Here, we prepared conjugates for each primary antibody yielding a conjugation efficiency of approximately 2 – 5 oligonucleotides per antibody molecule after purification (for antibody-oligonucleotide labeling data, see Supporting Table 2).

Complementary oligonucleotide-fluorophore labels were assembled stepwise by linking a single oligonucleotide (from hereon, "oligo") to a multivalent amino-dextran scaffold to which multiple fluorophores could be conjugated (Scheme 2). Degree of fluorophore labeling of oligo conjugates could be increased by addition of increasing mole equivalents of fluorophore used in the conjugation reaction (for oligo'-polyfluor labeling data, see Supporting Table 3).

To test the sensitivity and specificity of our approach, we probed normal, viable murine splenocytes to detect expression of well-characterized cell-surface markers. CD4 and CD8 label non-overlapping populations of helper and cytotoxic T-cells, respectively. We also probed for adhesion regulation molecule CD43 (sialophorin) and the adhesion molecule CD62L (L-selectin), which are expressed on both CD4⁺ and CD8⁺ T lymphocytes. As a panel, antibodies against these four cell-surface antigens are sufficient for phenotypic delineation of several subsets of murine T-lymphocytes.

Oligo modification, fluorophore labeling, and annealing were optimized by a series of trials evaluating the influence of experimental conditions on the quality of flow cytometry data (Supporting Figure S1). Antibody-fluorophore hybrids were compared to commercially available fluorescent antibodies (Supporting Figures S2 and S3 and Supporting Table 4), demonstrating comparable performance. Once optimized and validated, antibody-polyfluor hybrids were prepared by annealing stoichiometric amounts of antibody-oligo to complementary oligo'-polyfluor using standard conditions of PBS, and 15 minute incubation at room temperature, and then used directly to label cells for quantitative flow cytometry.

Multiple antigen labeling for multiparameter analysis

Using optimized assay conditions, we formed multiple antibody-polyfluor hybrids and used them one at a time or in cocktails to label splenocytes (Figure 1A). Using CD4, CD8, CD43 and CD62L antibody-oligo conjugates, each was annealed in solution to a complementary oligo'-polyfluor labeled with the Dylight fluorophores Dy490, Dy549, Dy649, and Dy405, respectively.

The hybrids were then used to label viable murine splenocytes and the stained cells were analyzed by flow cytometry. Leukocyte populations were scatter gated according to standard methods.^[13] All antibody-labeled cell samples displayed distinct antigen-positive populations with percentages within expected ranges.^[14] Multi-target staining results were comparable to single-antigen staining.

These results allow for phenotypic delineation of the cell population (Figure 1B). Lymphocyte subsets of note in the first row of dot plots include CD4⁺CD8⁻ (30%); CD4⁻CD8⁺ (27%); CD4⁻CD43^{HIGH} and CD4⁺CD43^{LOW} cells (7%, 21%); and CD4⁺CD62L⁺ (20%). The last panel in the first row depicts the CD4⁺ lymphocyte population, of which the majority are CD4⁺CD43⁺CD62L⁺ (64%). In the second row, lymphocyte subsets include CD8⁺CD43^{HIGH} (28%); CD8⁺CD62L⁺ (26%); CD43^{HIGH}CD62L⁺ (33%); and for CD8⁺ lymphocytes, nearly all cells were CD43⁺CD62L⁺ (93%). These results provide substantial evidence that antibody-polyfluor constructs can be used for specific and sensitive multiparameter cellular phenotyping.

“Mix and Match” fluorophore labeling using a universal oligo sequence

For the experiments discussed thus far, the oligo pairs **1/1'** – **4/4'** were used to link antibodies to fluorophores. However, by utilizing the single oligo pair **A** and **A'**, any antibody-**A** may be annealed to any **A'**-polyfluor.

We first validated this approach using CD4 antibody-**A** with four spectrally distinct **A'**-polyfluors (Supporting Figure S4). The percentage of cells positively stained for CD4 was similar regardless of **A'**-polyfluor used, suggesting that consistent single-plex labeling might be generally achievable using mix-and-match via the **A/A'** universal labeling approach.

A potential limitation to applying the universal oligo approach to a multi-targeted staining experiment would be false double-positives caused by dissociation of hybrids, leading to exchange of **A'**-polyfluors between different antibody-**A** conjugates.

As mentioned previously, CD4 and CD8 cellular antigens are considered mutually exclusive, so they provide a model system in which to evaluate false double-labeling. Thus, we annealed CD4 antibody-**A** and CD8 antibody-**A** to pairs of **A'**-polyfluors as shown in Figure 2. For each experiment, the two pairs of antibody-polyfluor hybrids were combined along with an excess of unlabeled **A** as a competitor to block exchange. The blocked hybrid cocktail was then used to stain viable murine splenocytes.

Oligo exchange was evaluated by observing events falling within the upper-right quadrant of the dot plots shown. We observed highly specific labeling of CD4⁺ and CD8⁺ cells, with < 1% CD4⁺CD8⁺ cellular events. Therefore, we conclude that multitarget labeling using **A/A'** as a universal tag is both feasible and highly specific.

Determination of antibody binding per cell (ABC) using oligonucleotide-coated microsphere calibrants

To apply DNA-directed assembly to form sets of quantitative fluorescent calibration particles, oligos were hydrazone-conjugated to the surface of monodisperse microparticles, which were then annealed to known numbers of oligo'-polyfluors to obtain multiple levels of surface fluorescence (low, medium low, medium high, and high). The polyfluor-labeled microparticles were then used to quantify the number of antibodies bound per cell (commonly termed ABC). ABC is an important measurement to standardize and interpret typically subjective cytometric data.

To determine number of oligo'-polyfluors per microsphere, particle fluorescence for each population was analyzed by microplate fluorimetry against a standard curve of oligo'-polyfluor dilutions (Supporting Figure S5). The number of particles per microwell was then determined using a handheld counting device, allowing precise calculation of oligo'-polyfluors per microsphere (Dy490, Figure 3A; Dy549, Dy649, Dy405, Supporting Figure S6).

Next, each population of particles was analyzed individually by flow cytometry (Figure 3B). This enabled a quantitative standard curve to be constructed by plotting cytometer mean fluorescence intensity vs. number of oligo'-polyfluor per microsphere (Figure 3C). This standard curve was used to calculate ABC based on cytometric cellular data.

To determine ABC for each of the antibody-polyfluor hybrids, microsphere calibrants bearing poly-Dy490, poly-Dy549, poly-Dy649, and poly-Dy405 were combined and added to an equal volume of viable murine splenocytes stained with the antibody-polyfluors α CD4-polyDy490, α CD8-polyDy549, α CD43-polyDy649 and α CD62L-polyDy405. The cells mixed with calibrants were analyzed by flow cytometry. Leukocytes and calibrant

particle singlets were gated prior to analysis (Supporting Figure S7). Resulting cytometric data histograms are shown in Figure 4. Filled histograms represent cellular staining distribution, which appeared to be unaffected by the presence of the microsphere calibrants. Open histograms represent microsphere calibrant staining distribution, in four peaks of increasing intensity, in each fluorescent channel.

For quantification of ABC, log cytometer intensity of the calibrants was plotted against log oligo'-polyfluor per microsphere (Figure 5; log 10 geometric means were used). A best fit trendline was generated for each plot. The trendline was used to convert the mean fluorescence intensity for CD4⁺, CD8⁺, CD43^{LO/HL} and CD62L⁺ populations to oligo'-polyfluor per cell; which, given the 1:1 stoichiometry of antibody-oligo to oligo'-polyfluor, serves as a direct measure of cellular ABC. The data would be consistent with a mean antigen density of ~30,000 CD4 molecules per CD4⁺ cell and ~8000 CD8 molecules per CD8⁺ cell, were the antibodies saturating binding sites in this experiment.

We then examined applying this approach on a cell-by-cell basis by analyzing single cell events from the above flow cytometry analysis (Figure 6). Here, using the trendlines derived in Figure 5, 1000 gated leukocyte events were directly converted to quantitative ABC for each probe on a cell-by-cell basis. Direct comparison of ABC for pairs of probes via dot plots as in Figure 1 revealed the familiar patterns of antigen expression observed with conventional analysis, but with quantitative significance regarding the characteristic antigen density for populations of cells considered CD4⁺, CD8⁺, etc.

To validate our approach to quantitative flow cytometry, we used commercial quantitative fluorescent microspheres (BD QuantiBrite PE) to quantify ABC_{CD4} using similar methodology and the same monoclonal antibody used for ABC_{CD4} quantitation in our novel system (Supporting Figure S8). ABC_{CD4} data were very similar for commercial vs novel method (29.7×10^3 vs 28.6×10^3 CD4 antibody per cell, 4.2% variance).

Conclusions

Here, we have shown that DNA-directed assembly offers a powerful tool to enhance multiparametric fluorescent detection in flow cytometric analysis of viable cells, and importantly, enables a new and robust strategy for quantitative flow cytometry. By taking advantage of chemistries for oligonucleotide conjugation, we found that annealing-mediated fluorescent labeling of antibodies is simple and reproducible. Our flexible labeling approach allows an alternative to the often cumbersome current methods of cytometric assay panel design, which are generally limited by commercially available antibody-fluorophore options. A practical advantage of applying DNA-directed assembly to fluorescent antibody labeling is the simple path to commercialization. Indeed, each antibody in a catalog could be made available as an oligonucleotide conjugate at nominal cost, and mix-and-match labeling enabled by providing a range of fluorescent probes conjugated to the complementary oligonucleotide.

Due to the current lack of a robust, efficient, and straightforward strategy for quantitative analysis (see Supporting Discussion), practical flow cytometry remains a largely qualitative approach. Here we show the use of microsphere calibrants formed by DNA directed assembly to enable quantitative determination of antibody binding offers an elegant new approach to quantitative cytometry, resolving many of the challenges presented by currently available technologies. The microsphere calibrants can be prepared quickly and easily and do not require antibody to prepare. Conjugation of oligonucleotides to surfaces is readily controlled and the density of oligonucleotides tethered to a suspension of particles can be determined with high confidence.

Fully automated quantitation would require only minor adaptation of the computation methodology described here, by (e.g.) implementation of a plug-in wizard for commercial post-acquisition analysis software. Alternatively, modifying the system software installed on commercial flow cytometers would enable calculation of ABC during real-time analysis. We see a straightforward application for our technology in making quantitative, automated multiparametric single-cell immunoanalysis widely available. In turn, the ability to directly determine antigen levels via internal controls might help overcome current barriers slowing FDA approval of flow-cytometry based cellular diagnostics.

Whether used to enable quantitative analysis, or simply as an alternative to conventional antibody-fluorophore conjugates for qualitative analysis, our DNA-directed assembly approach offers significant advantages over prior methods. We believe that adopting DNA-directed assembly as a core approach for antibody labeling will bring enhanced efficiency, added flexibility and improved quantitation to the ever-growing community of scientists who exploit flow cytometry in their research, as well as physicians and their patients who stand to benefit from broader use of flow cytometry as a diagnostic tool.

Experimental Section

Oligonucleotides were purchased from Eurogentec. Antibodies were either obtained from University of Chicago Fitch Monoclonal Antibody Bank (α CD4, clone GK1.5; α CD8, clone 2.43.1, α CD62L, clone MEL-14, and α Fc γ III/II, clone 2.4G2) or were a gift of J. Kemp at University of Iowa (α CD43, clone S7). NHS-ester DyLight fluorophores were purchased from Dyomics. Amino-dextran was purchased from Invitrogen (Life Technologies). S-HyNic, S-4FB, aniline, 2-SB, and 4FB-surfaced microspheres were obtained from Solulink Biosciences. Chemicals were commercially sourced and molecular biology grade.

Antibody-oligo conjugation

DNA oligos synthesized with a 5' amino-C6 group were dissolved in modification buffer (100 mM NaH₂PO₄, 150 mM NaCl; pH 7.4) and desalted by 4 cycles of concentration and ten-fold dilution in 3kDa MWCO spin diafiltration devices (VivaSpin 500, Sartorius Stedim). Oligo at 0.5 OD₂₆₀/μL was combined with 20-fold molar excess of sulfo-S-4FB, reacted 2 h at room temperature, diluted in conjugation buffer (100 mM NaH₂PO₄, 150 mM NaCl; pH 6.0) and desalted by 4 cycles of diafiltration. The final 4FB-modified oligo concentration was 0.5 OD₂₆₀/μL.

Protein A purified monoclonal antibodies were concentrated to ~ 1 mg/mL in 30 kDa MWCO spin diafiltration devices, buffer exchanged into modification buffer via 2 mL desalting columns (Zeba, Pierce/Thermo Scientific), combined with 20-fold molar excess of S-HyNic, incubated 2 h at room temperature, and desalted into conjugation buffer. Modification efficiency ranged from 6 to 8 HyNic per antibody molecule as determined by 2-sulfobenzaldehyde (2-SB) assay using a molar extinction coefficient at 350 nm of 28,500 L mol⁻¹ cm⁻¹. The HyNic-modified antibody was combined with 4 equivalents of 4FB-modified oligo in 10% (v/v) catalyst solution (100 mM aniline, 100 mM NaH₂PO₄, 150 mM NaCl; pH 6.0), reacted overnight at 4 °C, and purified on a gel filtration column (HR-10/30 Superdex PG, GE Healthcare Life Sciences), yielding baseline resolution of conjugate and unincorporated 4FB-oligo, based on OD₂₆₀. Conjugation efficiency of the resulting antibody-oligos was determined by the ratio of areas of free vs. conjugated oligo chromatographic peaks (OD₂₆₀) and protein concentration was determined by protein assay (BCA, Pierce/Thermo Scientific).

Oligonucleotide-fluorescent dextran (oligo'-polyfluor) conjugation

To form a 1:1 oligo:dextran conjugate, 70 kDa amino-dextran was dissolved in modification buffer, column desalted, combined with 5-fold molar excess of S-HyNic for 2.5 h at room temperature, and desalted into conjugation buffer. The HyNic-modified amino-dextran was combined with a reaction-limiting amount of 4FB-modified oligo (0.5 mole equivalents), reacted overnight at 4 °C and purified via gel filtration into loading buffer (20 mM HEPES, 25 mM NaCl; pH 7.0). The mixture of free dextran and dextran-oligo conjugates was loaded onto an ion-exchange spin column (Vivapure Q Mini-H, Sartorius Stedim), washed with loading buffer and eluted with increasing concentrations of NaCl (90, 450, and 750 mM) in loading buffer. Conjugate eluting in the 450 mM and 750 mM fractions was pooled and buffer exchanged into modification buffer on a desalting column and combined with an excess of NHS-ester DyLight fluorophores Dy490 ($\lambda_{ex/em}$ 490/516 nm), Dy549 (560/575 nm), Dy649 (655/676 nm), or Dy405 (400/420 nm) for 3 h at room temperature. After dialysis against PBS, fluorophore degree of labeling (DOL) of the oligo'-polyfluor conjugates was determined spectrophotometrically.

Splenocyte preparation and staining with antibody-polyfluor hybrids

Spleens obtained from C57BL/6 mice were minced and pressed through a fine mesh nylon filter. Cells were washed in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies). Erythrocytes were lysed by brief incubation in buffered ammonium-chloride-potassium solution, and leukocytes suspended in DMEM with 5% fetal calf serum. For staining, 10^6 cells per sample were suspended in Dulbecco's PBS (Corning) with 1% Bovine Serum Albumin (1% BSA-DPBS) and the lymphocyte Fc γ receptor was blocked by incubation with α Fc γ III/II hybridoma supernatant for 20 min at room temperature. For staining, antibody-oligo was combined with an amount of oligo'-polyfluor sufficient to provide one oligo'-polyfluor per antibody-oligo. Annealing was conducted in 50 μ L 1% BSA-DPBS for 15 min at room temperature and the resulting antibody-polyfluor hybrids were added to leukocytes. Cells were stained for 30 min at 4 °C, washed, and resuspended in 500 μ L 1% BSA-DPBS for analysis by flow cytometry.

Fluorophore-labeled microspheres

Amino-C6-oligo was dissolved in reaction buffer (100 mM NaH₂PO₄, 150 mM NaCl, 100 mM NaSO₄, 10 mM EDTA; pH 7.4), desalted by four cycles of diafiltration, reacted with 25 equivalents of S-HyNic for 2 h at room temperature, and desalted into conjugation buffer (100 mM NaPO₄, 150 mM NaCl, 100 mM NaSO₄, 10 mM EDTA; pH 6.0). Then, 4FB-modified, 2.8 μ m monodisperse superparamagnetic microspheres were combined with HyNic-modified oligonucleotide in conjugation buffer with catalyst buffer to saturate the particle surface. The oligo-labeled microspheres were washed and stored in DPBS.

To prepare fluorophore-labeled microspheres, aliquots of oligo'-polyfluor were added to oligo-labeled microspheres (50 μ g, $\sim 1.25 \times 10^5$ particles) and incubated 15 min at room temperature with gentle agitation. The particles were washed free of unbound oligo'-polyfluor, analyzed in a multiwell fluorimeter (Safire 2, Tecan) and compared to standard curves of fluorescence intensity vs. pmol oligo'-polyfluor for each reference standard to calculate pmol oligo'-polyfluor per microsphere sample. A handheld particle counter (Scepter, Millipore) was then used to determine the number of particles per sample allowing calculation of oligo'-polyfluor molecules per microsphere. Fluorophore-labeled microspheres were analyzed separately prior to combining into a single population for analysis with cell samples to confirm adequate peak separation.

Flow cytometry and data analysis

Flow cytometers (Beckton Dickinson LSRII) equipped with laser lines and fluorescent detectors compatible with DyLight fluorophores were used with voltage settings in each fluorescent channel adjusted to maximize signal separation between positive and negative cell populations and limit median signal for unstained cells to $\sim 10^2$ RFU. Raw cytometric data files were exported in .fcs listmode (3.0 logarithmic format) and analyzed using FlowJo software (TreeStar). Leukocytes were scatter-gated according to standard methods and samples containing microsphere calibrants were scatter-gated for singlets. For samples containing both leukocytes and microspheres, particle singlets represented $\sim 10\%$ of 50,000 total events.

To perform quantitative analysis, a reference standard curve was constructed using microsphere data. Geometric Mean Fluorescence Intensity (GMFI) was determined by gating each reference peak in each channel and calculating GMFI using analysis software. Log GMFI for each peak was plotted vs. log molecules oligo'-polyfluor per microsphere. A trendline was then derived for each probe and used to calculate mean ABC and single-cell ABC. For single-cell analysis, 1,000 sequential cell events were exported in log-scale format from FlowJo software as a text file (.txt) and ABC trendlines were applied to each single cell to convert GMFI to ABC in each channel.

Calculation of ABC_{CD4} by commercial method

Viable murine splenocytes were harvested and Fc-receptor blocked as described above. Phycoerythrin-conjugated anti-CD4 antibody (anti-CD4-PE, Beckton Dickinson) was applied at manufacturer recommended titration for 30 min at 4 °C. Cells were washed and analyzed in an LSRII as above. Commercial PE calibration microspheres (QuantiBrite, Becton Dickinson) were analyzed with the same cytometer settings used to acquire cellular CD4 data. Data were exported as .fcs listmode files to FlowJo analysis software. Leukocytes and microsphere singlets were gated according to conventional methods. Microsphere populations and CD4⁺ cell populations were gated and GMFI was determined as described above using analysis software. Log GMFI values were plotted vs. log PE molecules per microsphere using lot-specific data provided by manufacturer. The trendline equation derived from the microsphere standard curve was then used to calculate geometric mean ABC_{CD4}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Antibody-polyfluor hybrids used to label viable murine splenocytes. A) Histograms showing CD4, CD8, CD43, and CD62L labeling of cells. B) 2D dot plots showing phenotypic distribution of splenocytes labeled with four antibody-polyfluor hybrids in a multi-target stain.

Figure 2.

“Mix and Match” oligo'-polyfluor annealing to antibody-oligos. CD4 and CD8 antibodies annealed in two combinations of oligo'-polyfluor (left, Dy490, green vs Dy649, red; right, Dy405, blue vs. Dy549, orange). CD4⁺ and CD8⁺ distributions are distinct and comparable regardless of oligo'-polyfluors annealed. Minimal crosstalk is observed.

Figure 3.

Quantitative fluorophore-labeled microspheres. **A)** Four populations of microspheres are analyzed fluorimetrically to determine precise number of oligo'-polyfluor per microsphere. **B)** The microspheres are then analyzed cytometrically. **C)** Mean cytometric intensity in relative fluorescence units (RFU) vs. number of oligo'-polyfluor per microsphere. The trendline is used to determine antibody per cell, ABC, given $ABC = \text{number of oligo'-polyfluor per cell}$.

Figure 4.

Flow cytometry of cells labeled with antibody-polyfluor hybrids (filled histograms) along with fluorophore-annealed microsphere calibrants (open histograms) that enable ABC quantitation. **A)** CD4-Dy490; **B)** CD8-Dy549; **C)** CD43-Dy649; **D)** CD62L-Dy405.

Figure 5.

Determination of mean ABC using microsphere calibrants. Mean fluorescence intensity was determined for microsphere populations and cellular populations of interest. Microsphere trend lines were used to determine ABC per cell, given $ABC = \# \text{ oligo-polyfluor per cell}$. **A)** CD4-Dy490; **B)** CD8-Dy549; **C)** CD43-Dy649; **D)** CD62L-Dy405.

Figure 6.

Quantitative cytometric data. 1000 events recorded from multiparameter flow cytometry as shown in Figure 1 are plotted as number of antibodies bound per cell (ABC) for each event as shown, after converting fluorescence intensity in each channel to ABC for each antibody, using microsphere-based standard curves. Quantitative data represent a subset of the data shown in Figure 1B, yet offer greater insight into cellular antigen density.

Scheme 1.

Antibody-oligonucleotide conjugation by hydrazone chemistry. A) S-HyNic linker is used to modify antibody and S-4FB linker is used to modify amino-oligonucleotide. B) Modified components are combined in the presence of aniline catalyst and react to form C) hydrazone conjugated antibody-oligonucleotide.

Scheme 2.

Formation of oligo-polyfluor conjugate. A) Oligonucleotide is hydrazone-conjugated to amino-dextran scaffold at one oligonucleotide per dextran. B) NHS-ester fluorophore is added in mole excess to oligo-amino-dextran. Following reaction, the product is purified, yielding C) oligo-polyfluor conjugate suitable for annealing with antibody-oligos.

Scheme 3.

Annealing of antibody-oligo with complementary oligo'-polyfluor yields antibody-polyfluor hybrids in a variety of colors.

Scheme 4.

Fluorophore-labeled quantitative microspheres are prepared by annealing A) oligo-labeled microspheres with complementary oligo-polyfluors to B) increasing amounts of oligo'-polyfluor, resulting in four microsphere populations of increasing fluorescent intensity (low, med low, med high, high). The microspheres are then used as quantitative standards for flow cytometry.