Optimization in Detection of Antigen-Specific T Cells Through Differentially Labeled MHC Multimers

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● **Abstract**

A large variety of fluorescent molecules are used on a regular basis to tag major histocompatibility complex (MHC) multimers for detection of antigen-specific T cells. We have evaluated the way in which the choice of fluorescent label can impact the detection of MHC multimer binding T cells in an exploratory proficiency panel where detection of MHC multimer binding T cells was assessed across 16 different laboratories. We found that the staining index (SI) of the multimer reagent provided the best direct correlation with the value of a given fluorochrome for T cell detection studies. The SI is dependent on flow cytometer settings and chosen antibody panel; hence, the optimal fluorochrome selection may differ from lab to lab. Consequently, we describe a strategy to evaluate performance of the detection channels and optimize the SI for selected fluorescent molecules. This approach can easily be used to test and optimize fluorescence detection in relation to MHC multimer staining and in general, for antibody-based identification of rare cell populations. © 2019 The Authors. Cytometry Part A published by Wiley Periodicals, Inc. on behalf of International Society for Advancement of Cytometry.

NUMEROUS fluorescent tags are used to detect major histocompatibility complex (MHC) multimer binding T cells, either using a single or combination of colors to enhance the number of T cell populations that can potentially be detected in a given sample.(1,2) Considering that antigen-specific T cells are generally present at very low frequencies in a biological sample, multimer staining needs to be optimized for robust detection of true cell events. Detection of MHC multimer binding T cells may vary substantially across different laboratories as demonstrated by proficiency panels organized by the Cancer Immune Therapy Association Immunoguiding Work Group (CIMT/CIP) and other consortia.(3) In particular, harmonizing and optimizing the staining and gating protocols through proficiency panels that evaluate the detection of antigen-specific T cells using MHC multimers, has helped reach lower interlab variability (http://www.cimt.eu/working-groups-1).(3–5) In addition to variance based on the applied protocol, differences in detection of antigen-specific T cells when using MHC multimers may arise as a consequence of the different fluorochromes used to tag the MHC multimers and the fluorescence detection. This is determined by the performance of the flow cytometry instrument, cytometer settings, and additional fluorescently labeled antibodies used for T cell identification.

In a previous proficiency panel (CIP_ID07_2010_MUL/D), we showed that differentially labeled MHC multimers could be used to detect multiple T cell populations in a single sample with an efficiency that is comparable to separate detection of each population (Supporting Information Fig. S1). However, although no differences in T

cell detection arose from mixing MHC multimer reagents for concurrent detection of T cells, we observed a tendency toward enhanced detection frequencies with bright fluorescent molecules, such as phycoerythrin (PE), compared to low intensive fluorescent molecules, such as Quantum Dot (QD) 705.

In the present study, we investigated whether different fluorescent tags resulted in similar T cell detection when assessed across different laboratories working with various protocols, antibodies, and cytometers. We investigated potential differences in detection of antigen-specific T cells ranging from low-frequency populations (<0.1% of CD8 T cells) to high-frequency T cell populations (1%–2% of CD8 T cells) when using four different MHC-multimer labels: (1) PE, (2) allophycocyanin (APC); (3) QD605; and (4) QD705, representing both bright, medium, and dim, as well as classical and nonclassical fluorochromes. We compared detection rates, frequencies of multimer+ CD8 T cells, and the staining indices (SI) for individual populations across 16 different laboratories participating in this exploratory proficiency panel (CIP_ID13_2012_MUL/D).

Furthermore, we introduced a fast and easy bead-based evaluation of cytometer performance, which correlates with SI of the MHC multimer population, for detecting relevant fluorescent molecules. This bead-based system has broad applications for optimizing fluorescent detection in selected channels.

MATERIALS AND METHODS

PBMC Samples

Leukaphereses and buffy-coats (BC) were obtained by venipuncture from HLA-A*0201 positive healthy donors at the Center for Clinical Transfusion Medicine in Tübingen, Germany or the Central Blood Bank, Rigshospitalet, Denmark after written informed consents according to the declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation on LymphoPrep™ (Axis-Shield PoC, Fisher Scientific, Göteborg, Sweden) within 8 h after collection. Cells were washed twice in RPMI 1640 medium (Gibco, Fisher Scientific, Göteborg, Sweden), centrifuged at 300g *for 5 min, counted, and frozen at 10 to* 20×10^6 *cells/ml in freezing containers with fetal bovine serum (Gibco, Fisher Scientific, Göteborg, Sweden) with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Damstadt, Germany). Cells were then transferred to the gas phase of a liquid nitrogen tank or to* $-150^\circ C$ *freezers for long-term storage. The cells were shipped to the participating labs within a year after freezing.*

Reagents for Flow Cytometry

HLA-peptide multimers

HLA-A*0201-peptide monomers and multimers used for the proficiency panel were produced in-house by the classical refolding method as previously described.(6) For additional fluorochrome detection optimization, MHC multimers were generated either by the classical refolding method or by UV-exchange according to previous description.(7,8) Fluorescent multimers were generated by coincubating monomers with streptavidin-fluorochromes (all from Life Technologies, Darmstadt, Germany), either at a 4:1 monomer/streptavidin ratio (-PE, -APC) or at a 30:1 monomer/quantum dot ratio (QD605 or 705).(9) The following specificities were included: known epitopes derived from the viruses Human cytomegalovirus (HCMV) (pp65 495–503 NLVPMVATV, i.e., CMV), Influenza A (Flu Matrix 58–66 GILGFVFTL, i.e., FLU), and Epstein Barr virus (EBV) (BMLF1 259–267 GLCTLVAML, i.e., EBV1 and EBV BRFL1 109–117 YVLDHLIVV, i.e., EBV2). In addition, a multimer refolded with the HLA-A*0201 UV exchangeable peptide KILGFVFJV (A2*p) was included as negative control. All multimers were frozen after addition of cryoprotectants containing glycerol (FLUKA, Fisher Scientific, Göteborg, Sweden) and bovine serum albumin (BSA) (Sigma-Aldrich, Darmstadt, Germany) at 16% and 0.5%, respectively.(10)

Fluorescent calibration beads

Quantum™ MESF and Quantum™ Simply Cellular® 6–9 μm diameter microspheres (Bangs Laboratories,Inc., Fishers, Indiana) were used to monitor the flow cytometers' performance in the four fluorescence channels also used for the multimer-detection, PE, APC, QD605, and QD705. PE- and APC-beads were obtained from the manufacturer (Quantum™ MESF). For QD605 and QD705, microspheres coupled with anti-mouse capture antibodies (Quantum™ Simply Cellular®) were incubated with the mouse monoclonal antibodies (mAb) S3.5-QD605 or 3B5-QD705 (both from Life Technologies, Darmstadt, Germany) for 30 min at room temperature; Qdot conjugates were centrifuged *5 min at 10,000g and 4°C before use in order to remove aggregates. Beads were then washed three times with phosphate-buffered saline (PBS) (Lonza, Houston, Texas) at 850 g for 5 min, resuspended in PBS with 0.5% BSA, 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, Darmstadt, Germany), and 0.02% azide, aliquoted at* 4×10^4 *beads in 100–200 μl/vial and frozen at* $-80^\circ C$ *until use. For all fluorochromes, unstained (blank) and beads labeled with increasing amounts of fluorescence (dim, mid, and bright) were available. Representative results for the bright-fluorescence beads are reported.*

Proficiency Panel Design

The aim of the proficiency panel was to compare the results obtained by different laboratories when staining PBMC with the same multimers coupled to four different fluorochromes and test the feasibility and utility of cryopreserved calibration beads for controlling flow cytometer performance. Participants were all experienced in multiparametric flow cytometry and for most of them with multimer staining (15/16). All

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received one parcel on dry ice containing three preselected HLA-A*0201 PBMC cryovials (one vial/donor), aliquots of five different multimers (CMV, FLU, EBV1, EBV2, and A2*p) each coupled to the four study fluorochromes (PE, APC, QD605, and QD705), and calibration beads for the same four fluorochromes. According to prescreening experiments, the three PBMC samples contained a total of 11 virus-specific T cell populations (CMV, FLU, EBV1, and EBV2) with high (> 1%, n = 3), intermediate (0.1%–1%, n = 5) and low (<0.1%, n = 3) frequencies of CD3+CD8+ (Table 1). Cell viability after thawing was evaluated by the individual labs, and the median percentage of dead cells in each sample was 10% (ranging from 0% to 44%).

Test conditions

Except for the HLA-multimers and a few requirements for the procedure, all staining reagents and protocols, including the use of dasatinib in addition to flow acquisition and analysis conditions, were free of choice. As in previous proficiency panels organized by CIP,(4,5) some parameters were, however, mandatory: (1) the number of cells per stain (between 1 and 2×10^6); (2) the inclusion of at least a CD3 and a CD8 mAb; (3) the conditions of the multimer staining (4 ug/ml of each multimer at room temperature for 30 min); (4) the acquisition of all cells contained in stain tubes; and (5) the acquisition of the calibration beads (at least 1×10^4) in the same experiment and using the same settings (photomultiplier tube (PMT) voltage and compensation) as used for the PBMC stains. Participants were free to include additional mAbs and/or a dead cell dye (Ab clones and dyes were free of choice) and use local buffers and staining protocols. A panel guideline, cell staining protocol, and detailed instructions on handling, acquiring, and analyzing the beads were provided. An example of the full gating strategy from one lab, donor and response is shown in Supporting Information Figure S2. Each PBMC sample was split into six subsets, and each fraction was stained with the four different virus peptide/multimers labeled with four different fluorochromes. Four out of the six fractions were stained with all of the virus peptide/multimers labeled with different combinations of fluorochromes so that all multimer/fluorochrome combinations were tested. The last two fractions were used for control stains, one with the four different A2*p negative control multimers, and one "fluorescence minus one" (FMO), containing only the antibody mix and no multimer. This was done for all three donors giving a total of 18 stains per participating laboratory. In addition, 16 calibration beads (blank, low, mid, and high intensity for the four different fluorochromes) were acquired together with the cells and analyzed by each lab. The gating strategy for the bead tests was standardized with examples for both cells and beads displayed in the panel guideline.

Data reporting

The number of CD3+CD8+ and CD3+CD8+multimer+ lymphocytes was recorded for each PBMC sample and each multimer specificity. In addition, the median fluorescence of the CD3+CD8+multimer+ and of the CD3+CD8+multimer− subsets

and standard deviation (SD) of the CD3+CD8+multimer− cells was documented for all multimer fluorescence channels (PE, APC, QD605, and QD705). For the beads, median fluorescence and SD were determined. Additional parameters (such as cell recovery after thawing, number of PBMCs per stain, number of CD3+ cells counted, and details on staining reagents and cytometer configuration) were collected for interlaboratory comparisons but are not presented in this article (a detailed report of the panel CIP_ID13_2012_MUL/D is available on request).

Central Data Analysis

Central assessment of the panel data

All dot- or pseudocolor-plots of MHC multimer stains were analyzed and scored by three experienced flow users from three different labs. Each possible MHC multimer binding T cell population was given a "multimer score": not present (score = 0); maybe present (score = 1); or obviously present (score = 2). From the independent evaluation by the three experienced flow users, the maximum summed multimer score was thus six (Fig. 1). Only populations with scores of at least 4 were considered to be true MHC multimer binding T cell populations and hence, was included when the frequency of CD8+multimer+ cells was calculated.

Data Analysis and Statistics

Cell samples

Frequencies of multimer+ cells are expressed as percentage of CD3+CD8+ cells and were calculated using the cell numbers reported by the individual labs. In order to compare the brightness of the multimers coupled with the four different fluorochromes (Figs. 1–3) or the staining with single multimers in combinations with various Ab (Fig. 4), SI were calculated using the equation: SI_{cells} = (median fluorescence CD3+CD8+multimer+ subset − median fluorescence CD3+CD8+multimer− subset)/2 × fluorescence SD of CD3+CD8+multimer− subset.(10,11)

The SI calculations are based on Mean Fluorescence Intensity (MFI) values reported by the individual labs, whereas the multimer score is based on expert evaluation, which allows us to evaluate the impact of SI on detection of multimer+ T cells. Therefore, in specific cases a SI is assigned to a multimer+ T cell population, which based on expert evaluation is defined as untrustworthy (score 0–2) (Fig. 2a).

Calibration beads: for each participating lab, we calculated two SIs that were used to evaluate the sensitivity of the flow cytometry instruments and the staining procedure: $SI_{beads + FMO}$ was defined as = (median fluorescence of bright beads − median fluorescence of the CD3+CD8+multimer− cell subset in the FMO cell staining)/2 × fluorescence standard deviation of the CD3+CD8+multimer− cell subset in the FMO cell staining. $SI_{beads + Mult\ irrel.}$ was defined as (median fluorescence of bright beads − median fluorescence of the CD3+CD8+multimer− cell subset of the A2*p multimer cell staining)/2 × fluorescence SD of the CD3+CD8+multimer− cell subset of the A2*p multimer cell staining. $SI_{beads + FMO}$ can be considered as a measure of the accumulated effects of the flow cytometer settings, cell autofluorescence, and Ab/fluorochromes (spreading error) included in the stain. $SI_{beads + Mult\ irrel.}$ can additionally provide a measure of the MHC multimer-related background.

Statistics

Statistical analyses, Kruskal Wallis test (Fig. 1) Two-way analysis of variance (ANOVA) (Fig. 1 and Supporting Information Figure S4), Spearman's rank correlation tests (Figs. 2 and 3), and linear regressions (Fig. 3 and Supporting Information Figure S6) were performed with GraphPad Prism.

BEAD-BASED OPTIMIZATION OF MHC MULTIMER STAINING

Two of the labs involved in the organization of and participating in the proficiency panel performed an extended MHC multimer optimization test. The experiment was conducted as per lab protocol. For lab ID04, prescreened PBMCs from three different donors were thawed, counted, split into four fractions of ~2 × 10^6 cells, and stained as per standard lab procedure. Two fractions from each donor were stained with an antibody mix containing CD8 Ab, live dead stain (Near-IR from Invitrogen, Fisher Scientific, Göteborg, Sweden), and dump channel markers, CD4 (SK3), CD14 (MφP9), CD16 (NKP15), CD19 (4G7), and CD40 (LOB7/6) (fluorescein isothiocyanate [FITC] from BD Biosciences, San Jose, CA) Abs in addition to an APC- and a PE-labeled A2*p multimer at 1 μg/ml (negative control multimer); the two other fractions were stained only with the antibody mix (FMO). In order to determine the impact of different CD8 antibodies on the multimer staining, either CD8-PerCP (3B5, Life Technologies, Darmstadt, Germany) or CD8-Alexa Fluor 700 (YTS156.7.7, BioLegend, San Diego, CA) was used in the Ab mix. For lab ID08, PBMCs from one donor were thawed, counted, and 2 × 10^6 cells were stained with CD3 (OKT3-FITC, in-house labeling) and CD8 antibodies (either SFCI21Thy2D3-PE-Cy7, Beckman Coulter, Indianapolis, IN, 3B5-QD605/705 life Technologies, Darmstadt, Germany, or SK1-PerCP Becton Dickinson, Franklin lakes, NJ).

The staining procedure was performed in two successive steps, essentially following the CIP protocol (http://www.cimt.eu/working-groups-1). At first, the multimer staining was performed either at 37°C for 15 min or at room temperature for 30 min followed by a second staining with the antibody mix for 30 min on ice. Stained cells were acquired on LSR II flow cytometers (BD Biosciences, San Jose, CA) equipped with the FACSDiva software. Each cell fraction was acquired at two different PMT settings before and after flow cytometry, and PMT optimization was performed according to Perfetto et al. (12) In brief, this procedure allows for identification of optimal PMT voltage values for each specific fluorochrome by using cyto-cal (Thermo Scientific, Göteborg, Sweden) and quantum simply calibration beads (Bangs Laboratories Inc., Fishers, Indiana).(12) Before acquiring the cell samples, PMT voltages and compensations were adjusted for each fluorescence channel using unstained cells and compensation beads (BD Biosciences, San Jose, CA or Invitrogen, Fisher Scientific, Göteborg, Sweden) labeled with antibodies or ArC amine reactive compensation beads (Invitrogen, Fisher Scientific, Göteborg, Sweden) suitable for viability dyes. The PMT values were adjusted to fit with the selected antibody panel, ensuring the least possible overlap between fluorochromes while maintained as close to their optimum as possible. Both the MHC multimer staining and the same calibration beads used in the proficiency panel were run at the two different PMT settings (old and new PMT voltage, before and after the optimization).

Data analysis

In order to compare the fluorescence between PMT settings, SIs were calculated as for the proficiency panel data. SI$_{beads + FMO}$ was defined as (median fluorescence of bright beads − median fluorescence of the CD3$^+$CD8$^+$multimer$^−$ cell subset in the FMO cell staining)/2 × fluorescence SD of the CD3$^+$CD8$^+$multimer$^−$ cell subset in the FMO cell staining. SI$_{beads + Mult irrel.}$ was defined as (median fluorescence of bright beads − median fluorescence of the CD3$^+$CD8$^+$multimer$^−$ cell subset of the A2*p multimer cell staining)/2 × fluorescence SD of the CD3$^+$CD8$^+$multimer$^−$ cell subset of the A2*p multimer cell staining.

RESULTS

Detection of MHC Multimer Binding T Cells Using Differentially Labeled MHC Multimers

Across 16 different labs, we tested the capability of identifying MHC multimer binding T cells when using four different fluorescent labels for MHC multimer detection (PE, APC, QD605, and QD705). We used three different donors and in each donor, the detection of four different virus-specific T cell populations CMV- NLVPMVATV, EBV1- GLCTLVAML, EBV2- YVLDHLIVV, and FLU- GILGFVFTL was tested (Table 1). In order to avoid potential bias due to differences in the frequency and pMHC avidity among different populations of antigen specific T cells, each T cell population was detected using MHC multimers tagged with each of the different fluorescent labels. Examples of all staining for one donor is shown in Supporting Information Figure S3. Based on a central evaluation, each possible MHC multimer binding T cell population was given a multimer score: 0 for not present; 1 for maybe present; or 2 for obviously present. From three independent evaluations, the maximum summed multimer score was 6, and any population with a score of at least 4 was considered to be a positive MHC multimer binding T cell population. For one of the three donors (Donor 2), there was a significant difference in the mean multimer score between PE and QD705. For the other two donors, no significant differences were found when comparing the four different fluorescent labels (Fig. 1a). We then investigated the frequency of MHC multimer binding T cells among total

Table 1. Overview and size of the virus specific T cell responses present in each donor. Numbers represent the percentage of virus specific cells out of total CD3$^+$CD8$^+$ T cells

DONOR	CMV	EBV1	EBV2	FLU
1	1.36%	0.18%	0.16%	0.06%
2	1.77%	0.14%	0.47%	1.95%
3	No response	0.08%	0.24%	0.06%

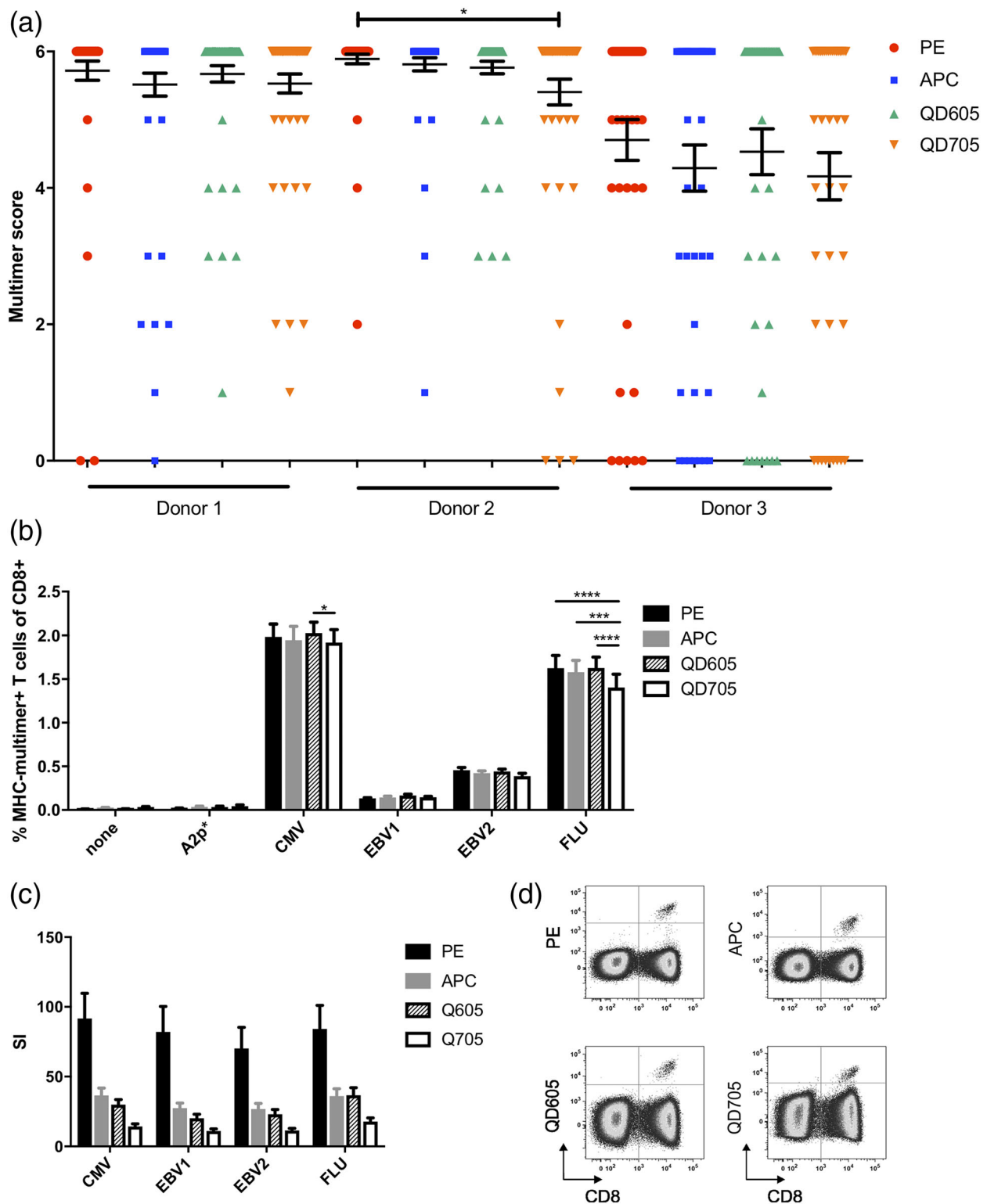*Optimization Detection of MHC Multimer-Binding T Cells*

Figure 1. Influence of the fluorochrome coupled to MHC–peptide multimers on the detection of antigen specific T cells. **a)** Multimer score for all virus specific T cell populations in each donor using the multimers coupled to four different fluorochromes (n = 11 cell populations per fluorochrome across the three donors). Each symbol represents an individual lab (n = 16), bars represent mean values and error bars indicate the standard error of the mean (SEM). *P < 0.05 (Kruskal Wallis test with Dunn's multiple comparisons test). **b)** % CD3⁺CD8⁺multimer⁺ and **c)** SI for one exemplary donor out of the three tested (Donor 2, mean from all labs is shown (group analysis)). SEM is indicated. For b) *P < 0.05, ***P < 0.001, ****P < 0.0001 (two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test). **d)** Dot plots from EBV2-multimer staining with each of the four fluorochromes are shown for Donor 2 from one exemplary lab.
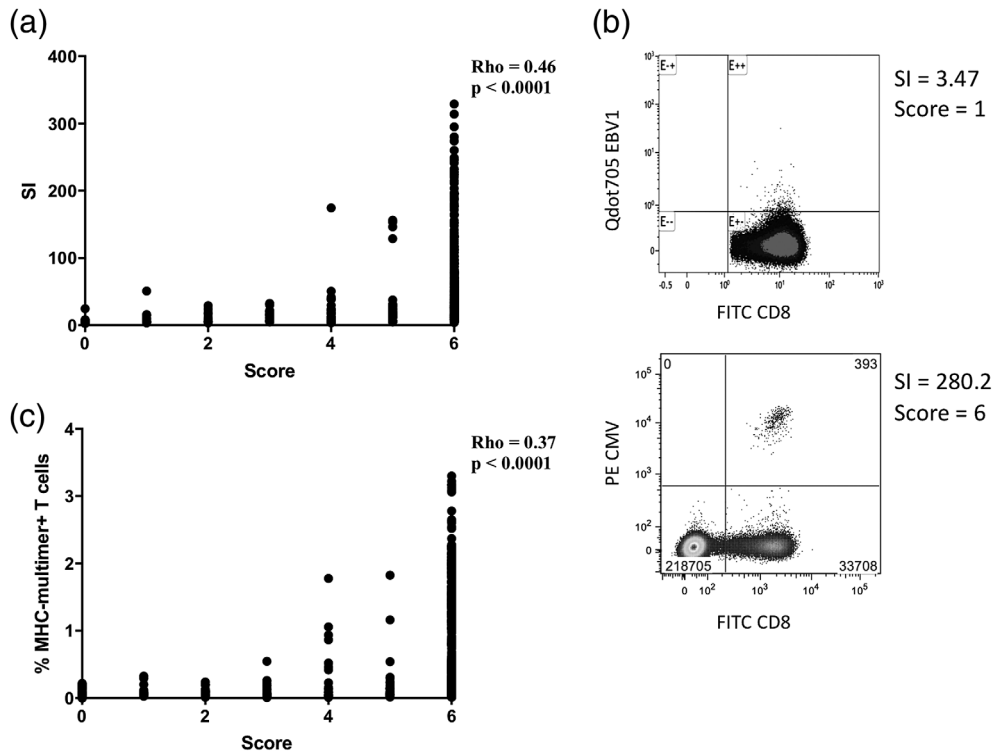
Figure 2. Correlates of multimer⁺ cell detection. The multimer score attributed by the central assessment for each MHC-multimer⁺ T cell population (n = 256 in total) is plotted in relation to *a*) the SI and *c*) the % CD3⁺CD8⁺multimer⁺ of the same population. *b*) Shows two examples of MHC multimer staining performed by two different labs, one with a good SI and multimer score and one with a poor SI and multimer score. ρ and P values are indicated (Spearman's rank correlation).

CD8 T cells detected for a given T cell response when comparing the different labels. We found that FLU and CMV specific T cell populations were detected at a significantly lower frequency when using QD705-labeled MHC multimers. This was evident when compared to PE, APC, and QD605-labeled MHC multimer detection of the CMV response in donor 1 (Supporting Information Fig. S4a) and the FLU response in Donor 2 (Fig. 1b), and also to QD605 detection of the CMV response in Donor 2 (Fig. 1b). Although the T cell detection rates of different MHC multimer-associated labels were comparable, it was also evident that the SI of the particular populations was largely dependent on the fluorescent label used (Fig. 1c and Supporting Information Figure S4b,d). In particular, the SI for QD705 was ~twofold lower than that of QD605 and APC and ~sixfold lower than that of PE. This finding is also evident from Figure 1d in which it can clearly be seen that the separation between positive and negative events depends on the choice of label.

In order to further evaluate if differences in SI could impact T cell detection, we again looked at the multimer scores assigned to each T cell population after expert central review and compared them to the SIs of that particular population. This analysis revealed that even across many different laboratories, the SI is correlated to the capability of detecting a certain MHC multimer binding T cell population (Fig. 2a). In Figure 2b, an example of a good and poor MHC multimer

staining, the relationship between SI and MHC multimer binding T cell detection can be clearly appreciated. Furthermore, it is also evident that larger populations of MHC multimer-binding T cells (>0.6% of CD8 T cells) were always assigned a multimer score of ≥4 and are thus more easily detected than low frequent populations (<0.6% of CD8 T cells) (Fig. 2c).

Assessment of Fluorochrome Detection Efficiency

We next asked whether the differences in detection rates and SI of a given MHC multimer binding T cell population correlate to the intrinsic efficacy of detection of the chosen fluorescent label. We calculated the SI of calibration beads labeled with each of the four selected fluorescent markers, which were also analyzed by each participating laboratory. The SIs were calculated for the bead fluorescence versus the negative cell population from both FMO and irrelevant multimer staining (see Material and Methods for details). We found that across different laboratories, the SI for the different fluorescent labels could vary greatly (Fig. 3a–d, and Supporting Information Figure S5a–d). For example, for the PE bright bead+FMO, there was a ~40-fold difference between the highest and lowest SI (Fig. 3a). The difference in SI was observed both when beads were assessed alone without considering the negative cell population (Supporting Information Fig. S5e–h), when the beads were evaluated in combination with cells stained
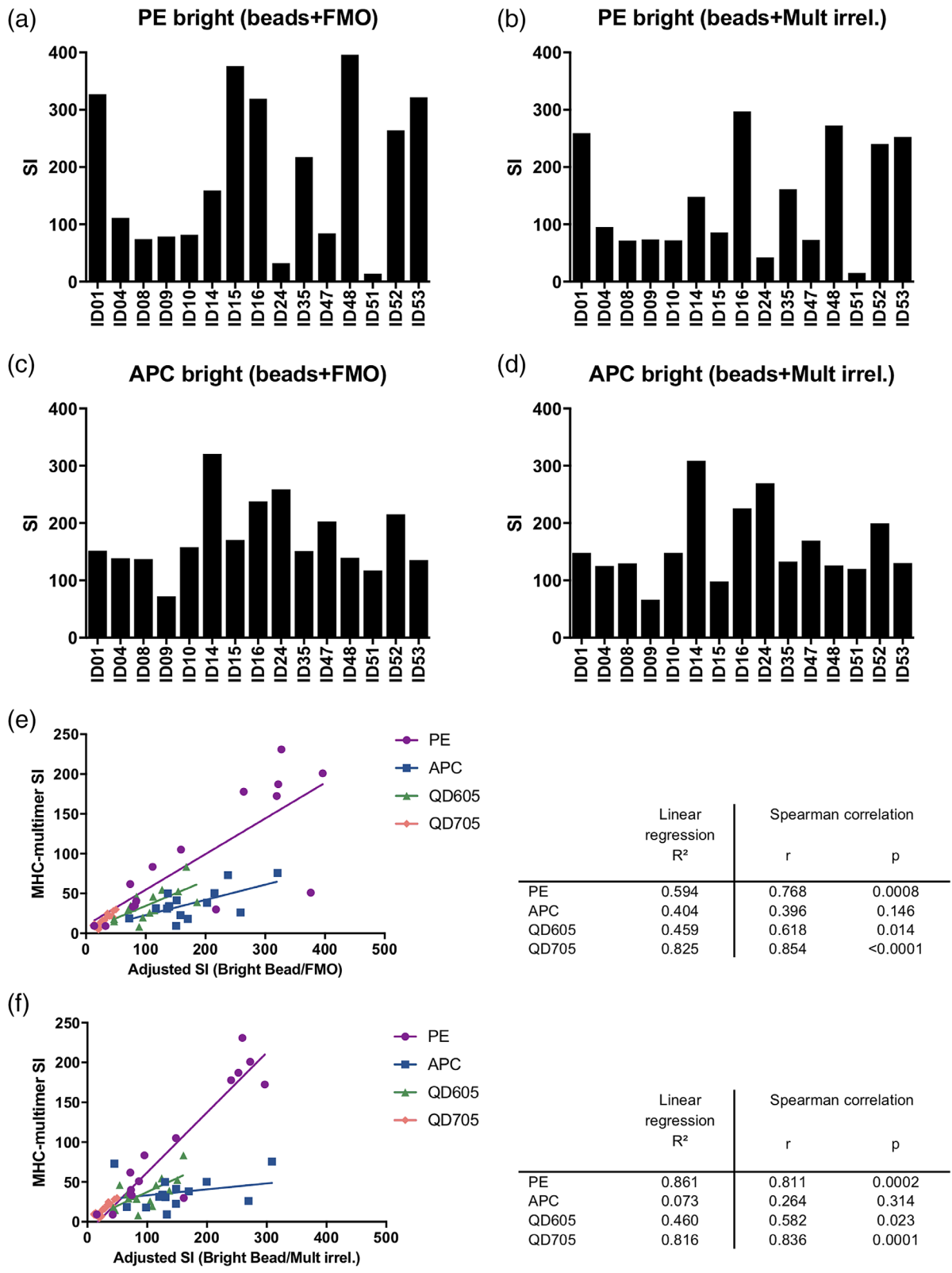
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Figure 3. Interlaboratory performance assessment based on detection of fluorescent calibration beads. SIs were calculated for the bright-fluorescence beads for the PE and APC channels. $SI_{beads + FMO}$ (**a, c**) and $SI_{beads + Mult\ irrel.}$(**b, d**) are shown for each individual lab. **e** and **f)** Correlation analyses between multimer SIs calculated from PBMC stains with each of the four multimer-fluorochromes and $SI_{beads + FMO}$ (e) or $SI_{beads + Mult\ irrel.}$ (f) Statistics are shown next to the plots.
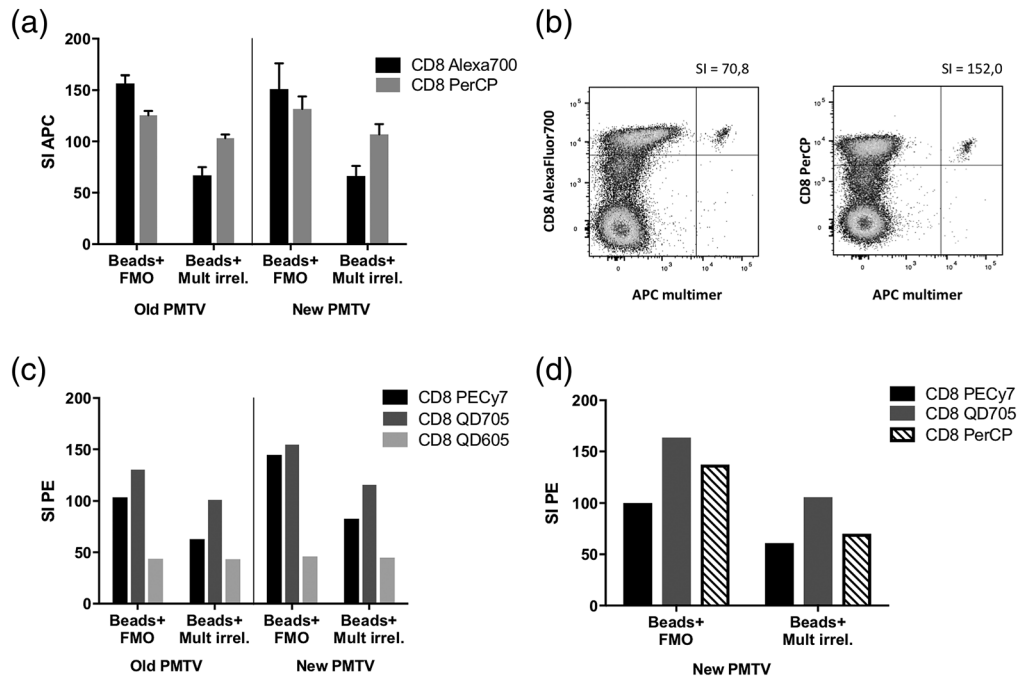
Figure 4. Bead-guided quality control and optimization measures. SIs for APC (**a**) calculated as bright bead+FMO- and bright bead+Mult irrel. -derived SIs are shown for lab ID04. Two different fluorochromes for detection of CD8 T cells (CD8 Alexa700 and PerCP) and PMT voltages (PMTV, old and new) were tested on three different donors. Bars indicate mean values with SD. **b)** Examples of dot plots showing MHC multimer staining using either AlexaFluor700 or PerCP for CD8 detection. **c** and **d)** SIs for PE also calculated as bright bead +FMO- and bright bead+Mult irrel.-derived SIs for lab ID08. c) Three different fluorochromes for detection of CD8 T cells (CD8 PECy7, -QD705, -QD605) and PMTV (old and new) were tested on one donor. In (d) PE SIs were calculated after a second measurement of different fluorochromes for detection of CD8 T cells (CD8 PECy7, QD705, PerCP) at new PMTV settings for one donor.

with the full antibody panel used for T cell detection in the individual participating laboratories (Fig. 3a,c), and with additional inclusion of an irrelevant MHC multimer (Fig. 3b,d). Furthermore, the same was observed when using mid and dim intensity beads (data not shown).

We further analyzed the correlation between the bead-determined SI for each fluorescent label and the SI of the MHC multimer binding T cell population. For three out of four fluorescent labels (all except APC), we observed a correlation between the bead-based determined SI and the MHC multimer SI (Fig. 3e,f, Supporting Information Figure S6). We therefore conclude that fluorescent beads can be used to evaluate and optimize the SI for the majority of MHC multimer-associated fluorescent labels.

Optimization of Fluorescence Detection at Single Centers

We next initiated an optimization procedure at two different laboratories, ID04 and ID08, with the aim of enhancing the SI for APC and PE labeled multimers, respectively (Fig. 3a–d). We attempted to optimize two basic parameters to potentially enhance the SI: (1) the cytometer PMT voltage values, according to the PMT performance evaluation procedure published by Perfetto et al.(12) and (2) the fluorescent labels selected for the antibody panel in order to identify the CD8 T cells. Evaluation and adjustment of the PMT values was performed, and the new and old voltage values were compared

by running bright PE- and APC-labeled beads as well as a number of multimer staining at both settings.

For lab ID04, the performance was evaluated on three donors. PBMCs from each donor were divided into four fractions. Two fractions were stained with an antibody panel (Ab Set) containing anti-CD8 labeled either with Alexa Fluor 700 or PerCP to determine the impact of the CD8 fluorescent label on APC detection. Furthermore, each of these fractions was further divided in two and stained either using FMO (no MHC multimer) or using irrelevant multimer (negative multimer). Combined with these PBMC samples, the SI for APC was determined using bright beads. The SI was compared for each Ab Set at two different cytometer settings (old and new PMT voltages) using the bright calibration beads. Beads combined with FMO were used to estimate the effect of the Ab combination, and beads combined with the irrelevant multimer were used to determine additional unspecific effects of adding an MHC multimer. Indeed, we found that multimers had an impact on the relevant flurorochome detection (APC and PE) as the beads+mult irrel. SIs were generally lower than the beads+FMO SIs. When comparing the APC SI between new and old PMT settings, no dramatic differences were visible (Fig. 4a), suggesting that PMT voltages were already optimal in the old setting at lab ID04. However, changing the CD8 antibody from Alexa Fluor 700 to PerCP led to a twofold increase in APC SI when calculated in relation to the irrelevant MHC multimer staining.

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Lab ID08 made additional observations when attempting to optimize detection of PE-labeled multimers. This lab determined the performance of the multimer stainings when conducted in combination with a CD8 Ab coupled to PE-Cy7, QD705, or QD605. Optimization of the PMT voltages was performed following the same procedure as at lab ID04. For lab ID08, the optimization of PMT voltage did increase SI of PE, both in relation to the FMO and the irrelevant multimer control, indicating that nonoptimized PMT settings had influenced the PE fluorescence detection. In addition, a change of CD8 Ab fluorescence from PE-Cy7 to QD705 enhanced the PE SI by up to approximately 50%, both for new and old PMT settings and for the FMO and the irrelevant multimer samples (Fig. 4c,d). In contrast, CD8-QD605 reduced the SI of PE compared to the CD8-PE-Cy7 (Fig. 4c). CD8-PerCP was evaluated using the new PMTV settings but showed only marginal improvement of the PE SI compared to PE-Cy7 (Fig. 4d). In conclusion, for this laboratory, the PMT voltage optimization and the careful selection of Ab fluorescence could improve the SI for PE, which consequently enhanced performance for the PE-labeled MHC multimer staining.

DISCUSSION

In this study, we investigated the impact of the choice of fluorescent label on the detection of MHC multimer-binding T cells. In a proficiency panel involving 16 centers, we found that the overall detection of the antigen-specific cells was affected by the choice of fluorochrome coupled to the MHC multimer as both the mean multimer score (Donor 2 [Fig. 1a]), and the frequency of antigen-specific T cells determined using a QD705 labeled MHC multimer was significantly lower than for the other fluorescently labeled MHC multimers (Donor 2, FLU, and Donor 1, CMV, Fig. 1b and Supporting Information Figure S4a, respectively). General information on the intrinsic brightness of fluorochromes can easily be found in specialized publications and online tutorials from Ab manufacturers. For example, PE is a bright fluorochrome, whereas APC, QD605, and QD705 are weaker. However, in the case of multiparametric staining, numerous parameters influence the capability of detecting T cell populations marked with MHC multimers carrying different fluorochromes. Several different strategies for improving detection of MHC multimer-binding T cells exist. Incubating target cells with the protein kinase inhibitor, dasatinib, prevents down-regulation of the T cell receptor (TCR) on the cell surface following TCR engagement and can thus increase the intensity of the MHC multimer staining.[13] Furthermore, using combinatorial approaches can help reduce the background from unspecific staining due to the gating strategy applied in these approaches,[1,2] and can thus also assist in more accurate detection of MHC multimer-binding T cells. However, these approaches are still dependent on the properties of the selected individual fluorochromes and the capacity for separation of signal to noise (measures by SI) in the given flow cytometry setting. For determining the performance of a given

fluorescence in a complex antibody panel, the SI is a good measure of the separation of the positive signal from the background events since the SI takes the spread of the negative population into account. A low SI indicates a population with poor separation from the background,[11] and MHC multimers labeled with fluorochromes having a low SI might lead to suboptimal T cell detection. This is especially relevant for T cell populations of low frequency, such as single antigen specific T cell populations in the peripheral blood. We found that indeed the SI varied greatly between the selected fluorochromes (Fig. 1c and Supporting Information Figure S4b,c), and this variation translated into different detection rates as very clearly reflected by the correlation between the SI and the positivity scoring of each population in Figure 2a. Thus, in order to gain the most accurate and precise results from MHC multimer staining, it is crucial to optimize the experiment and take into consideration the brightness of the selected fluorochrome in addition to the potential background and signal spreading induced by other fluorochromes that are used in combination for a given experiment. The advantage of the dim fluorochromes (Qdots) used in this study is their very narrow emission spectrum, which may make these fluorochromes useful in a complex experiment with many parameters because they are less likely to cause spillover into other channels than fluorochromes with wide emission spectra. Thus, choosing the best fluorescent labels for a specific experiment is often a compromise between complexity and sensitivity.[14] With the recent developments in flow cytometry, especially the emergence of a new generation of bright fluorochromes with low spillover, it is easier to maintain experimental complexity without inclusion of weak intensity fluorochromes.

In this study, we proposed a bead-based strategy to identify channels in which fluorescence detection might be suboptimal and optimize the SI for a given MHC multimer-associated fluorochrome in the context of the specific selected antibody panel. This strategy serves as a fast and easy alternative to the laborious task of generating multimers labeled with the different fluorochromes intended to be included in the experiment followed by test staining on donor material and calculation of the SI from each fluorochrome. As we observed a correlation between the SI of multimer staining and that of the corresponding premade beads (Fig. 3e,f), we suggest using such beads to optimize the MHC multimer experiment. Additionally, these beads can be used to optimize fluorescence detection in relation to any antibody panel, including dim markers or intended for detection of low-frequency cell populations. The beads can be prepared and cryopreserved in advance. They can be acquired on request to determine the flow cytometer performance, and/or to optimize antibody panels to enhance fluorescence separation. In our study, the same beads run at 16 different laboratories showed great variance in SI between laboratories (Fig. 3a–d). This is most likely a consequence of different detection channels and configurations on the various flow cytometers and highlights the fact that it is crucial to optimize each experiment on the relevant

instrument. Additionally, the different handling and staining protocols at the 16 different labs may also impact the SIs.

Optimization of PMT is one parameter that can be adjusted to improve fitness of the flow instrument for detection of a given fluorochrome A detailed step-by-step procedure has been published previously,(12) and was used here to improve detection in one out of the two testing labs (Fig. 4c). In addition, we observed that a major contributor to the MHC multimer channel SI was the different fluorescent labels used for the CD8 Ab. We observed up to a threefold enhancement of SI after adjustment of fluorescence combinations (Fig. 4c), emphasizing the importance of such optimization for individual flow cytometers. The use of fluorescence-labeled beads, which can be easily prepared on demand by users, as a simple and easy quality control/quality assurance tool to control fluorescence performance over time, identifies suboptimal cytometer or Ab combinations and helps to establish and optimize new Ab panels.

AUTHOR CONTRIBUTIONS
NWP performed experiments, analyzed data, generated figures, and wrote the manuscript. KL performed experiments, analyzed data, generated figures, and revised the manuscript. DM conceived the concept, performed experiments, and analyzed data. SW conceived the study, organized the panel, performed expert gating, and analyzed data. CG conceived the study, performed expert gating, discussed data, and revised the manuscript. SRH conceived the study, performed expert gating, analyzed data, generated figures, and wrote the manuscript.

CONFLICT OF INTEREST
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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