Novel Compensation Standard for the Violet Laser

Kathryn Turner[1], Sharmila Isaiah[1], Richard Schretzenmair[2], Jamie Tijerina[2], Andrew Bantly[2]

[1] Bangs Laboratories, Inc., 9025 Technology Drive, Fishers, IN 46038; [2] Department of Pathology and Laboratory Medicine and the Flow Cytometry and Cell Sorting Resource Laboratory, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Booth #313

ABSTRACT

Background
Polymer-based calibration beads are commonly used for the routine set-up and QC of flow cytometers. They can be made to approximate the size and scatter characteristics of lymphocytes and other cells, and may be dyed or coated for use with a broad range of reagents and instruments. While these types of standards have almost universal application, there are special cases where a different bead matrix would be advantageous. Specifically, common polymer compositions (polystyrene, etc.) possess a significant absorbance band in the UV/Violet region. This can lead to seemingly higher detection thresholds and complicate bead-based compensation when using 405nm excitation. A novel bead matrix has been developed that exhibits low autofluorescence with violet excitation, and could present advantages in these cases.

Methods
Base beads were coated with Fc-specific Goat anti-Mouse IgG (GAM). Coated beads were labeled with a FITC-conjugated mouse mAb and run on an LSRII to ensure that they would approximate a high positive cell population when stained. Microspheres that passed the routine FITC assay were then evaluated using the violet laser. Coated beads or human leukocytes were labeled with various violet fluorophore-conjugated antibodies (CD3 Pacific Blue or CD3 V450, CD45 V500 and CD11b eF605NC), and they and their unlabeled counterparts were run on an LSRII using 405nm excitation. Compensation matrices were determined by the BD DiVa software and analysis was performed using FlowJo.

Results
The GAM-coated microsphere standard exhibited low autofluorescence with violet excitation, and high antibody binding capacity.

Conclusions
The feasibility of using microspheres of a novel composition was investigated as a means to circumvent the higher autofluorescence that is typically observed of traditional bead matrices with 405nm excitation. Antibody-coated microspheres developed using the novel matrix exhibited both low autosignal and high binding, this allowed for a high signal to noise ratio and demonstrated superior performance as a compensation standard for violet optics.

INTRODUCTION

Autofluorescence is an important consideration in the design and use of microsphere-based calibration standards for the flow cytometer. Historically, common bead matrices (e.g. polystyrene) have been appropriate, as the autofluorescence profiles generated from common excitation lines (488nm, 633nm) often approximate those of lymphocytes and other cells. These same materials, however, exhibit a characteristic absorbance band in the UV/Violet, which results in higher apparent background with excitation in this region. The growing popularity of instruments equipped with violet lasers has evidenced the need for alternative bead matrices that are not only able to fulfill general performance requirements (uniformity, antibody coating capacity), but also demonstrate low autofluorescence with +/- 405nm excitation. The present study introduces a novel bead matrix that fulfills these requirements (Simply Cellular® anti-Mouse for Violet Laser), and functions as an exceptional surrogate for lymphocytes when establishing compensation settings for violet detectors.

Figure 1: *Absorbance of Polystyrene Compared to Simply Cellular® Beads in the UV/Violet Region*

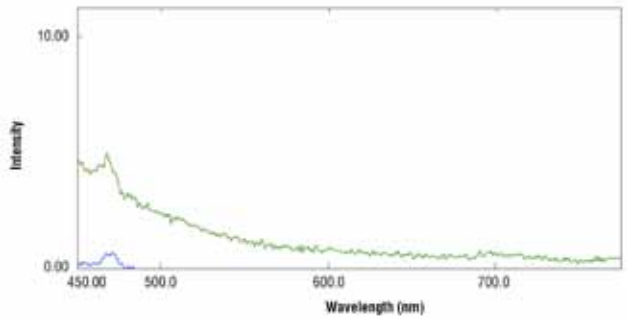

Figure 1: Suspensions of microspheres comprised of either polystyrene or the new matrix were run on the fluorimeter (Shimadzu RF-5301) to elucidate absorbance bands across the UV/Vis spectrum. Polystyrene (green) exhibits notable absorbance in the UV / Violet region of the spectrum compared to the novel matrix (blue).

Figure 2: *Autofluorescence With 405nm Excitation*

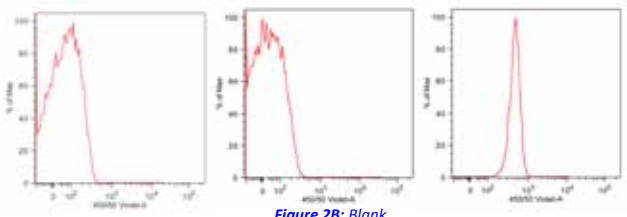

Figure 2A: *Unstained Cells*

Figure 2B: *Blank Simply Cellular® Violet Microspheres*

Figure 2C: *Blank Polymer Microspheres*

Figure 2A, 2B, 2C: With 405nm excitation, unlabeled Simply Cellular for Violet beads demonstrate autofluorescence that is equivalent to that of unstained cells. Unlabeled polymer beads demonstrate greater autofluorescence with violet excitation.

MATERIALS AND METHODS

Figure 3: *Instrument Violet Laser Configuration*

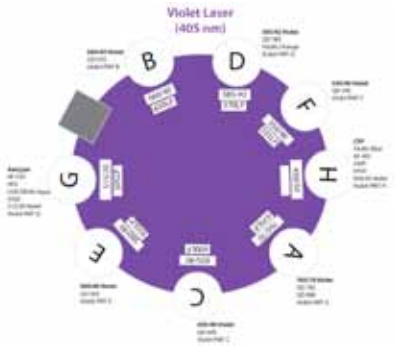

Figure 4: *Channel Spillover of Labeled Simply Cellular® Bead and Lymphocytes with CD3 Pacific Blue*

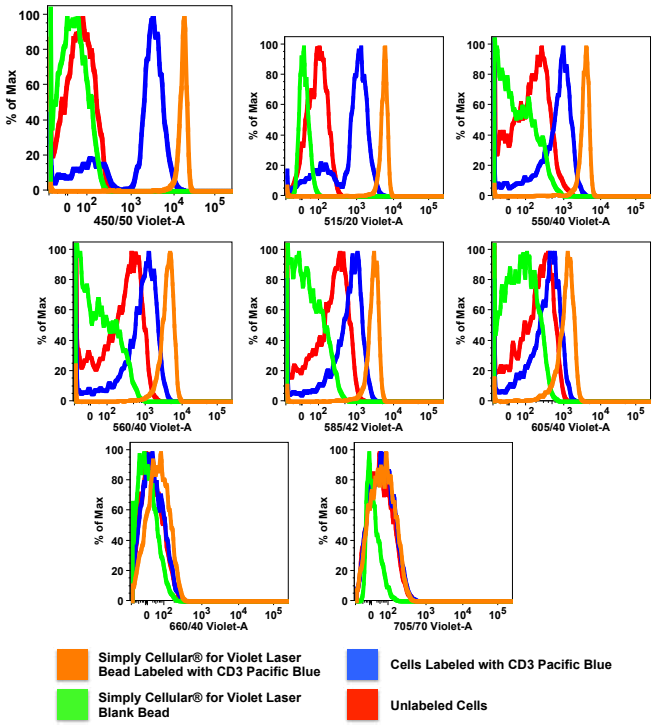

Figure 4: Simply Cellular® anti-Mouse IgG for Violet Laser Bead and healthy donor PBMCs were labeled with anti-human CD3 Pacific Blue™. The Pacific Blue™ labeled beads and cells were run on a BD LSRII (405nm, 50mW) with the configuration illustrated in Figure 3; the PMT voltages chosen were based on the instrument's CS&T setting used for daily Quality Assurance in the fluorochrome parameters. Data were recorded using BD DiVa 6.1.2 and analyzed using FlowJo 9.2.

The Pacific Blue™ signal was read in the 450/50 channel and the remaining seven parameters of the violet laser configuration are shown to illustrate:

- Signal of the CD3(+) cells to Simply Cellular® Bead labeled with Pacific Blue in the 450/50 parameter.
- Spillover of the signal to all other channels off the violet laser.
- Signal to noise ratio between the Blank Bead to the labeled Simply Cellular® Bead and unlabeled cells to labeled cells.
- Compare the Blank Bead to Unlabeled cells.

The results demonstrate that the Simply Cellular® anti-mouse IgG for Violet Laser Bead has comparable staining patterns as labeled CD3(+) lymphocytes and the Blank Beads background signal is similar to cells in the 450/50 channel.

Figure 5: *Simply Cellular® Beads for Violet Laser Can Be Labeled With Many Different Fluorochromes*

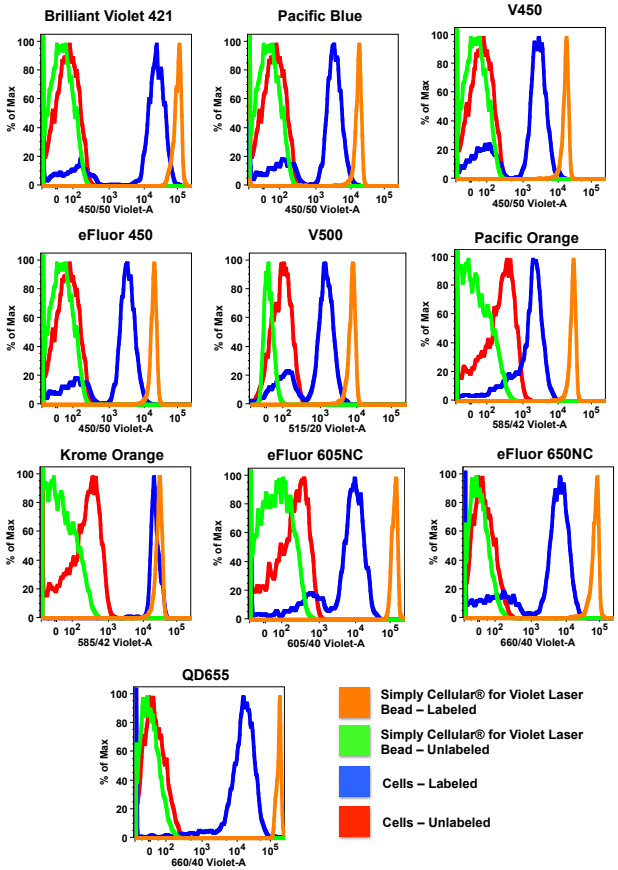

Figure 5: Simply Cellular® anti-Mouse IgG for Violet Laser Bead and healthy donor PBMCs were labeled with one of the fluorochromes in Table 1 and read in the appropriate channel on a BD LSRII with the violet laser (405nm, 50mW; Figure 3 is the violet laser configuration). CS&T voltages were used for all fluorescence channels. Data were recorded using BD DiVa 6.1.2 and analyzed using FlowJo 9.2.

- Labeling on the Simply Cellular® Bead was always at least as bright or brighter than the cells labeled with the same fluorochrome.
- Signal to noise ration between the Blank Bead and Simply Cellular® Bead were always greater than the ratio between labeled and unlabeled cell.
- Blank Bead had either similar or lower background intensity versus cells.
- The Simply Cellular® Beads can be labeled with different types of fluorochromes (small organic or semiconductors).

Table 1: *Fluorochromes Used to Label Simply Cellular® Beads for Violet Laser and Cells*

Fluorochrome Tested	Marker	Company	Clone	Catalog Number
Brilliant Violet 421	CD3	Biolegend	UCHT1	300433
Pacific Blue	CD3	Life Technologies	UCHT1	558117
V450	CD3	BD Biosciences	UCHT1	560366
eFluor 450	CD3	eBioscience	17A2	48-0032-80
V500	CD3	BD Biosciences	UCHT1	561417
Pacific Orange	CD3	Life Technologies	UCHT1	CD0330
Krome Orange	CD45	Beckman Coulter	J.33	A96416
eFluor 605NC	CD3	eBioscience	OKT3	93-0037-41
eFluor 655NC	CD3	eBioscience	OKT3	95-0037-41
QD655	CD3	Life Technologies	S4.1	Q10012

Figure 6: *Creating a Compensation Matrix Using the Simply Cellular® anti-Mouse IgG Bead for Violet Laser Produces Similar Data When Compared to Cells*

Figure 6A: *Compensated With Single Stained Violet Beads*

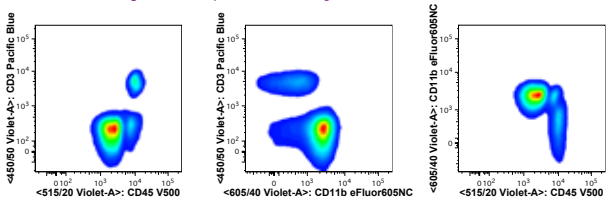

Figure 6B: *Compensated With Single Stained Cells*

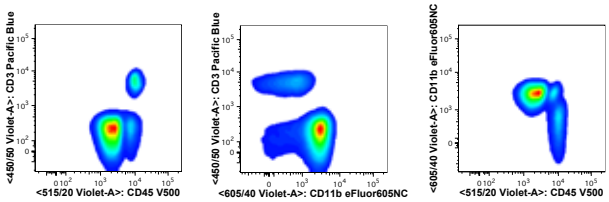

Figure 6C: *Values For Matrices*

Detectors	Value (%)
515/20 Violet – 450/50 Violet	32.6
605/40 Violet – 450/50 Violet	3.06
450/50 Violet – 515/20 Violet	7.42
605/40 Violet – 515/20 Violet	33.51
450/50 Violet – 605/40 Violet	0.05
515-20 Violet – 605/40 Violet	0.05

Compensation Values When Using Single Stained Beads

Detectors	Value (%)
515/20 Violet – 450/50 Violet	31.93
605/40 Violet – 450/50 Violet	0.00
450/50 Violet – 515/20 Violet	6.86
605/40 Violet – 515/20 Violet	32.06
450/50 Violet – 605/40 Violet	0.16
515-20 Violet – 605/40 Violet	0.09

Compensation Values When Using Single Stained Cells

Figure 6: A sample of healthy donor whole blood was ammonium chloride lysed and labeled with CD3 Pacific Blue™ (BD), CD45 V500 (BD) and CD11b eFluor®605NC (eBioscience). Two compensation matrices were created, one with single labeled cells and the other with single labeled Simply Cellular® Beads using the sample reagents. Figure 6A shows the analysis gated on leukocytes using the compensation matrix created using Simply Cellular® Beads, and Figure 6B illustrates the analysis using single stained cells to create the matrix. Values for the matrices are shown in Figure 6C. Data using V450 instead of Pacific Blue™ created similar results (data not shown). **Using the Simply Cellular® anti-Mouse IgG Bead for Violet Laser in place of cells yields highly comparable results.**

CONCLUSIONS

The Simply Cellular compensation standard exhibits autofluorescence and staining characteristics that are highly comparable to lymphocyte populations; notably, this includes low autofluorescence with 405nm (as well as 488nm and 633nm) excitation. The microspheres were shown to serve as suitable surrogates for labeled cells when stained with a variety of mouse monoclonal antibody conjugates. Compensation matrices developed using the new standard were found to be comparable to those developed using stained cells, demonstrating the utility of the new microspheres as a bead-based standard for establishing compensation settings for violet detectors.