Comparison of CFSE and PKH26 with CellVue™ Claret, A New 675nm-emitting Proliferation Dye

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GOAL

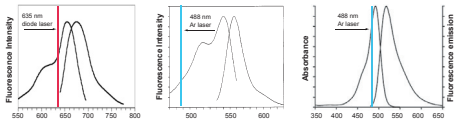
Cell tracking dyes such as PKH26 and CFSE have proven useful in numerous applications including assessment of cell proliferation. We sought to determine whether CellVue™ Claret (formerly PTIR289), a far-red fluorescent membrane intercalating dye, could be used as an alternative to PKH26 and CFSE in multicolor proliferation studies on a standard 2 laser/4 color BD FACSCalibur.

DYE CHARACTERISTICS

CellVue Claret
- Ex (max): 654 nm
- Em (max): 677 nm
- Labeling Mechanism: Lipophilic dye, partitions non-covalently into cell membranes

PKH26
- Ex (max): 551 nm
- Em (max): 567 nm
- Labeling Mechanism: Lipophilic dye, partitions non-covalently into cell membranes

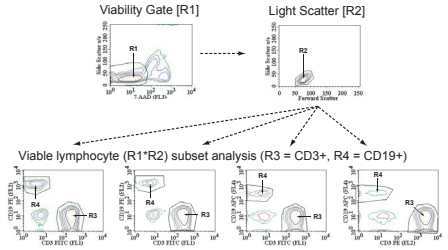
CFSE
- Ex (max): 494 nm
- Em (max): 515 nm
- Labeling Mechanism: Reactive dye, binds covalently to cytosolic and membrane proteins



Spectra courtesy of PTI Research (www.ptiresearch.com) | Spectra courtesy of Sigma Chemical Co., Inc. (www.sigma.com) | Spectra courtesy of Invitrogen (http//:.probes.invitrogen.com)

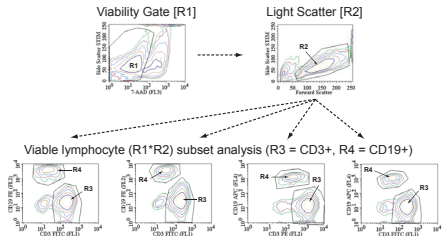
Figure 1

A Viability Gating and Two-Color Lymphocyte Immunophenotyping in UNSTIMULATED 96 hour Cultures with and without Proliferation Dyes



NO DYE	4.0 µM CellVue Claret	10.0 µM PKH26	0.5 µM CFSE
% CD3+: 84 ± 2.6	82% CD3+	82% CD3+	84% CD3+
% CD19+: 4.9 ± 0.9	6.6% CD19+	4.2% CD19+	3.8% CD19+

B Viability Gating and Two-Color Lymphocyte Immunophenotyping in STIMULATED 96 hour Cultures with and without Proliferation Dyes



NO DYE	4.0 µM CellVue Claret	10.0 µM PKH26	0.5 µM CFSE
% CD3+: 74 ± 2.0	78% CD3+	75% CD3+	77% CD3+
% CD19+: 9.2 ± 0.3	9.2% CD19+	8.4% CD19+	8.0% CD19+

EXPERIMENTAL STRATEGY

1) Identify optimized staining conditions for CellVue Claret (not shown). Concentrations selected for this study:
   a) Gave 3-4 log resolution between stained and unstained cells at T0.
   b) Did not alter viability or response kinetics of lymphocyte subsets in stained vs. unstained cells.
2) Optimize gating strategy for lymphocyte subset analysis by incorporating a viability dye in addition to light scatter gating (Figure 1A and 1B).

   NOTE: Previous studies in our laboratory found that use of light scatter gating alone resulted in inclusion of some dead cells, which exhibited reduced proliferation dye intensity due to loss of membrane and/or cytoplasm. *This led to overestimation of extent of cell proliferation, with the effect being most pronounced in lymphocyte subsets present at low frequency.*
3) Compare ***lymphocyte viability*** (Figures 2A and 3A), ***proliferative fractions*** (Figures 2B and 3B), ***precursor frequencies*** (Figures 2C and 3C), and ***T cell proliferation profiles*** (Figures 4 and 5) obtained using CellVue Claret, PKH26 or CFSE dye dilution to monitor proliferation.

   NOTE: 3-way dye comparisons were carried out in 6 independent studies using 5 different donors, three of whom exhibited strong proliferative responses to α-CD3+IL-2 and two of whom exhibited moderate proliferative responses. Data shown (Donors 4 and 5) are representative of results for strong responders.

MATERIALS & METHODS

CellVue Claret, CFSE and PKH26 LABELING*
Freshly isolated PBMCs from healthy donors were stained according to the manufacturer's instructions with 4.0 µM CellVue Claret (previously PTIR289; *PTI Research*, Exton, PA), 10.0 µM PKH26 (*Sigma-Aldrich,* St Louis, MO), or 0.5 µM CFSE (*Invitrogen/Molecular Probes,* Eugene, OR).

*Staining protocols available on request (abantly@mail.med.upenn.edu).

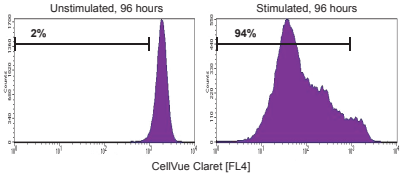
In Vitro STIMULATION
After labeling with proliferation dyes, PBMCs were cultured in AIM-V medium supplemented with 1% L-glutamine. Cells were incubated with or without 100 ng/mL anti-CD3 (*Roche Diagnostics*, Indianapolis, IN) and 200U/mL IL-2 (*Beckman Coulter,* Miami, FL) for 24, 72 or 96 hours.

Immunophenotyping Staining Strategy:

DYE	FL1	FL2	FL3	FL4
CellVue Claret	CD3 FITC *(BD PharMingen)*	CD19 PE *(Caltag)*	7-AAD *(Invitrogen)*	CellVue Claret ***(PTI Research)***
PKH26	CD3 FITC *(BD PharMingen)*	PKH26 ***(Sigma)***	7-AAD *(Invitrogen)*	CD19 APC *(Caltag)*
CFSE	CFSE ***(Invitrogen)***	CD3 PE *(BD PharMingen)*	7-AAD *(Invitrogen)*	CD19 APC *(Caltag)*

INSTRUMENT SET-UP
8-Peak Beads (***Spherotech Inc. catalog # RCP-30-5A***) were used to standardize fluorescence intensities across days. Target MFI's were established at instrument settings used to run t=0 samples (for CellVue Claret and PKH26) or t=24 samples (for CFSE) and reproduced by adjusting PMT settings as needed at subsequent time points.

DATA ACQUISITION AND ANALYSIS
1) Gate on 7-AAD negative + light scatter gates.
2) Run single color dye and antibody controls and set compensation.
3) Collect proliferation profile (dye distribution), gating on viable CD3+ cells.
4) Determine Proliferative Fraction by setting an inclusive marker encompassing all channels below the the 2nd percentile of the unstimulated population (below, left) and reporting the frequency of events in the same marker region for the stimulated population (below, right).



Proliferative Fraction = 94% (Donor 4)

5) Determine Precursor Frequency using Proliferation Wizard module in ModFit LT (*Verity Software House*, Topsham, ME).

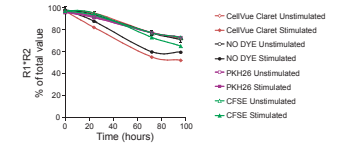
CONCLUSIONS

- CellVue Claret does not appear to alter lymphocyte subset frequencies (Figure 1), viabilities (Figures 2A and 3A), or kinetics of proliferative responses to anti-CD3+IL-2 as compared to those seen with PKH26 or CFSE (Figures 2B, 2C, 3B and 3C).
- CellVue Claret, PKH26 and CFSE give comparable results when used to monitor extent of T cell proliferative responses to anti-CD3+IL-2 stimulation (Figures 2B, 2C, 3B, 3C, 4 and 5), whether a donor was a strong or moderate responder (data not shown).
- The ability to see discrete peaks in the proliferation profile does not appear to be required for accurate proliferation analysis. Proliferative fractions and precursor frequencies found using CellVue Claret are very similar to those for CFSE and PKH26 despite its somewhat broader staining CVs (Figures 4 and 5).
- CellVue Claret thus offers a useful alternative to CFSE and PKH26 for multicolor cell tracking and/or proliferation protocols, allowing broader range of choices for antibodies and/or genetic markers (e.g. GFP and dsRed).

DONOR 4

Figure 2. Comparative Results

A. Lymphocyte Viability*



*Values for stimulated NO DYE or CellVue Claret samples fell within normal sample-to-sample variability as estimated from values for triplicate unstained but stimulated controls evaluated at each time point.

B. Proliferative Fraction **C. Precursor Frequency**

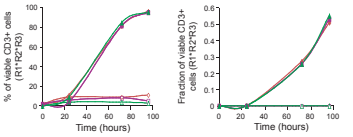
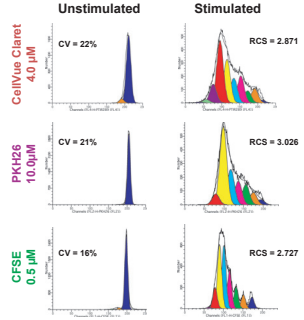


Figure 4. Proliferation Profiles (96 h)

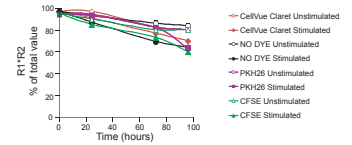


Data were accumulated for at least 30,000 viable CD3+ lymphocytes, using the gating strategy shown in Figure 1. Precursor frequency was determined using the Proliferation Wizard module in ModFit *LT (Verity Software House, Topsham, ME)*, using the unstimulated sample to establish the position of the parent population in the stimulated sample and the floating standard deviation option. RCS = reduced χ2.

DONOR 5

Figure 3. Comparative Results

A. Lymphocyte Viability



B. Proliferative Fraction **C. Precursor Frequency**

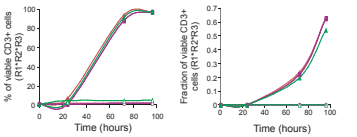
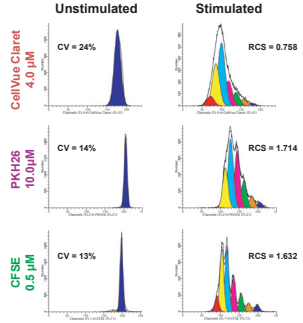


Figure 5. Proliferation Profiles (96h)



Using the "floating" standard deviation option significantly improved goodness of fit (reduced χ2 values for CFSE profiles compared with using a fixed standard deviation, but had minimal effects on goodness of fit for PKH26 or CellVue Claret. This difference may be due to the 2nd (slow) phase of CFSE efflux described by Matera et al. (*Cytometry* **62A**:118-128, 2004) .

FUTURE PLANS

Combine CellVue Claret with a 2nd cell tracking dye to resolve proliferative responses in donor and responder lymphocyte subpopulations present in a two way mixed lymphocyte reaction.