Krome Orange™: A Novel Orange-emitting Dye for Use in Multicolor Flow Cytometry Panels Using Violet Diode Laser Excitation

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Abstract

Background. Violet diode lasers have become common excitation sources on flow cytometers, but the advantages of this laser are constrained by limited choices for organic, violet-excited fluorochromes. These fluorochromes include several dyes emitting around 450 nm, and two (Horizon™ V500 and Pacific Orange™ dyes) emitting at longer wavelengths. These longer wavelength fluorochromes are plagued by relatively low fluorescence signals in relation to background, and generally must be used with densely expressed antigens. We have developed a novel violet-excited organic dye, Krome Orange, which has an emission spectrum closely matching that of Pacific Orange dye. It has excitation and emission maxima of 398 nm and 528 nm, respectively.

Results. The Krome Orange fluorochrome shows optimal performance with violet laser excitation and a 550/40 bandpass filter (the standard FL10 channel on Gallios™ and Navios™ flow cytometer systems), while no excitation is detected using a 488 nm laser. Krome Orange dye is at least as bright as V500 dye and can provide more than twice the population separation obtained with Pacific Orange dye conjugates, with little compensation versus Pacific Blue™ dye. Therefore, common gating markers can be easily transferred to this fluorochrome, freeing other valuable fluorochromes for use with additional markers.

Conclusions. Krome Orange dye conjugates can be used on any flow cytometer equipped with violet excitation (approximate wavelength range 400 to 410 nm) and appropriate emission filters. Data is shown using Krome Orange conjugates of anti-human CD4, CD19 and CD45 in multicolor applications, including side scatter/CD45 gating and a 6-plus color stain. As a result, Krome Orange dye provides an optimized second organic fluorochrome for violet excitation, enabling 10-color applications on the Gallios / Navios flow cytometer systems.

Materials and Methods

Antibodies were conjugated with Krome Orange dye via N-hydroxysuccinimide ester. Final fluorochrome:protein molar ratio (F:P) was optimized for each antibody, but ratios were generally optimal in the range of 9 to 14. Other antibody conjugates were acquired from Beckman Coulter (Brea, CA), BD Biosciences (San Jose, CA), or Invitrogen (Carlsbad, CA).

100 μL of normal blood was stained with conjugates for 20 minutes at room temperature, then lysed with VersaLyse™ lysing solution and washed before analysis. Samples were resuspended in buffer + formaldehyde unless otherwise noted in figure legends.

Samples were evaluated on Gallios™ cytometers (Beckman Coulter) equipped with violet, blue and red lasers and with detectors for 10 channels. Standard filter configurations were used except as noted. Data were analyzed with CXP™ or Kaluza™ software (Beckman Coulter).

Staining index (SI) was calculated as: $(median_{positive} - median_{negative})/(2 \times StDev_{negative})$.

Table 1. Antibody conjugates used in the study.

Target	Fluorophore	Clone
CD3	Krome Orange Dye	UCHT1
CD4	Krome Orange Dye	13B8.2
CD8	Krome Orange Dye	B9.11
CD14	Krome Orange Dye	RMO52
CD16	Krome Orange Dye	3G8
CD19	Krome Orange Dye	J4.119
CD20	Krome Orange Dye	B9E9
CD45	Krome Orange Dye	J.33
CD3	Pacific Orange Dye	UCHT1
CD4	Pacific Orange Dye	S3.5
CD8	Pacific Orange Dye	3B5

Target	Fluorophore	Clone
CD45	Pacific Orange Dye	HI30
CD45	Pacific Blue™ Dye	J.33
CD8	V500 Dye	RPA-T8
CD45	V500 Dye	HI30
CD3	AmCyan	SK7
CD4	AmCyan	SK3
CD8	AmCyan	SK1
CD45	AmCyan	2D1
CD45	ECD	J.33
CD8	Fluorescein	B9.11
CD19	R-PE	J4.119

Results

Table 2. Physical properties of fluorophores. Although the photon absorbing capacity (molar extinction coefficient) for Krome Orange is lower than the value for Pacific Orange dye, Krome Orange proves to be brighter in application. Brightness in conjugates is also related to quantum yield and environmental effects on the antibody protein as dyes are conjugated.

Fluorophore	Molar Extinction Coefficient ($M^{-1}cm^{-1}$)	Absorbance Maximum (nm)	Emission Maximum (nm)
Krome Orange Dye	17,665	398	528
Pacific Blue Dye	46,000	410	455
Pacific Orange Dye	24,500	400	551
AmCyan	NA	458	489
V500 Dye	NA	415	500

Figure 1. Krome Orange dye excitation curve (dashed black line); emission curves for Krome Orange dye, Pacific Orange dye, AmCyan, and V500 dye. The shaded region represents the standard 550/40 band pass filter installed into FL10 on a Gallios cytometers.

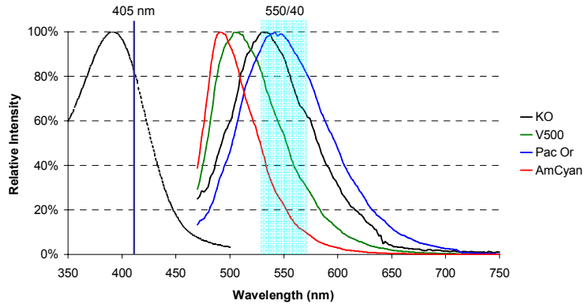


Figure 3. Normal human blood stained with CD3, CD4, CD8, or CD45 conjugates of Krome Orange dye, Pacific Orange dye, AmCyan, or V500 dye. Samples were fixed with formaldehyde before being analyzed on a Gallios™ cytometer. Fluorescence was detected in the FL10 channel using bandpass filters as noted. Positive peaks on histograms are labelled with median fluorescence. F:P values for Krome Orange conjugates: CD3 5.5, CD4 9.8, CD8 12.9, CD45 10.7. Histograms are labelled with staining index (SI) values. Compensation values versus the fluorescein, PE and Pacific Blue channels are shown in the adjacent table.

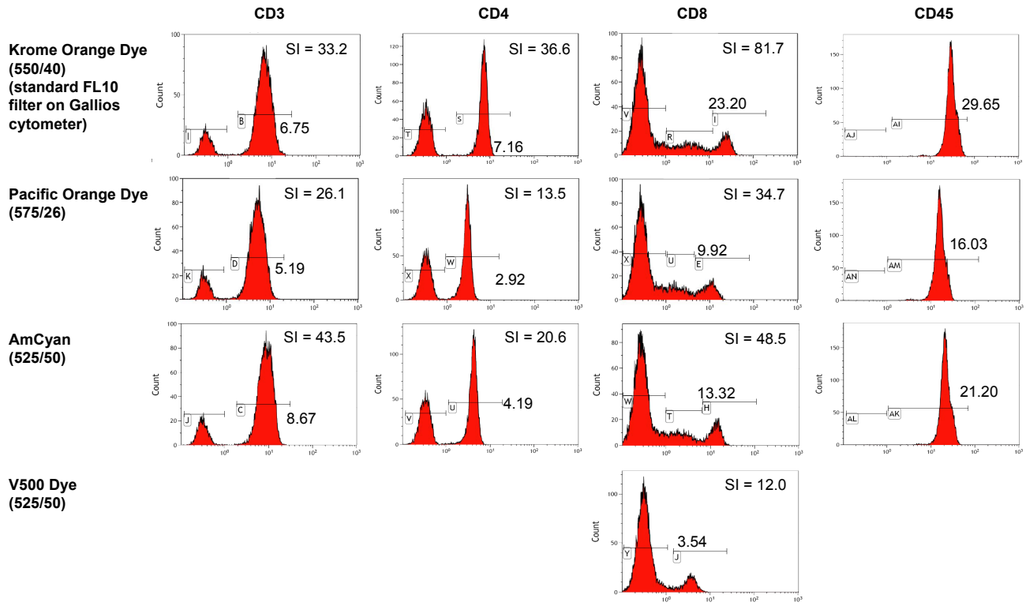
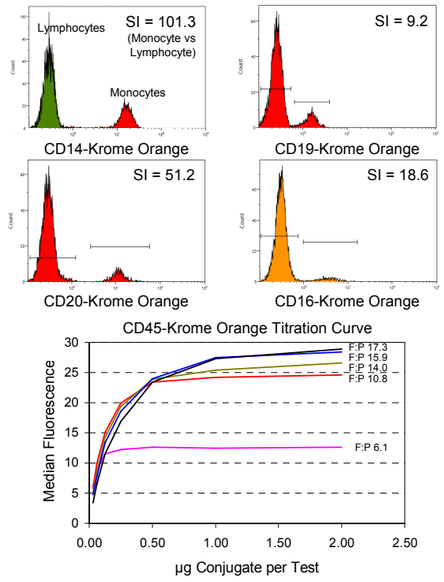


Figure 2. Example histograms showing conjugate staining patterns on gated on lymphocytes, except for the CD14 overlay showing lymphocytes and monocytes, and a titration curve showing the effect of F:P on conjugate brightness. Histograms are labelled with staining index (SI) values.



Compensation values for the conjugates in Figure 3 versus FL1 (fluorescein), FL2 (PE), and FL9 (Pacific Blue) channels.

Violet Dye	Target	Pacific Blue - % Violet Dye (FL9 - %FL10)	Fluorescein - % Violet Dye (FL1 - %FL10)	PE - % Violet Dye (FL2 - %FL10)
Krome Orange dye	CD3	1.7	0.6	0.6
	CD4	1.9	0.0	0.0
	CD8	3.3	0.5	0.8
Pacific Orange dye	CD3	1.1	1.0	1.0
	CD4	0.0	0.0	0.0
	CD8	1.8	0.6	0.6
AmCyan	CD3	22.6	>100	>100
	CD4	21.7	>100	18.5
	CD8	29.5	>100	19.3
V500	CD8	15.1	8.5	2.8

Figure 4. Human blood stained with either CD4-Krome Orange or CD4-Pacific Orange in combination with CD8-fluorescein, CD19-PE, and CD45-Pacific Blue. Lymphocytes were gated on CD45 and side scatter. The arrow marks the position of the CD4+ population when labelled with Krome Orange dye. Compensaton values were similar to values listed in Figure 3. Samples were run on a Gallios cytometer; Krome Orange and Pacific Orange dyes were both detected in FL10 with a 550/40 band pass filter.

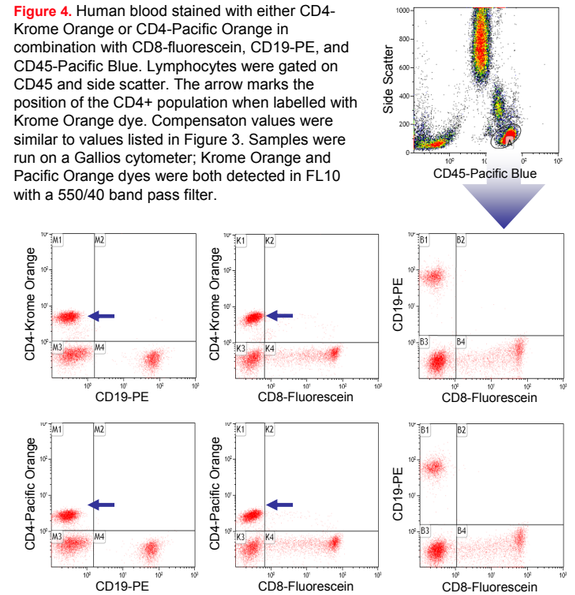
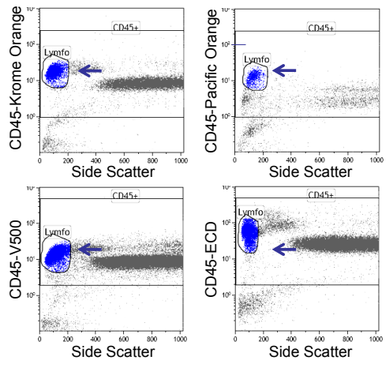


Figure 5. CD45 /side scatter gating of lymphocytes using CD45 conjugates of Krome Orange, Pacific Orange, V500, and ECD. Samples were run without fixation. The arrow marks the position of the CD45+ lymphocyte population when labelled with Krome Orange dye. Data courtesy of F. Preijers, Radboud University Nijmegen Medical Center, The Netherlands. Gallios.



Summary

- Krome Orange dye conjugates can be used on any flow cytometer equipped with violet excitation and appropriate emission filter (550/40 band pass filter recommended).
- Krome Orange dye provides a brighter signal than achieved with Pacific Orange conjugates with equivalent spectral overlaps into adjacent channels
- Depending on the antibody, Krome Orange conjugates can provide brighter signals than AmCyan and V500 conjugates, with much lower compensation values
- Krome Orange dye provides an optimal second organic fluorochrome for violet excitation, enabling 10-color applications on the Gallios flow cytometer system