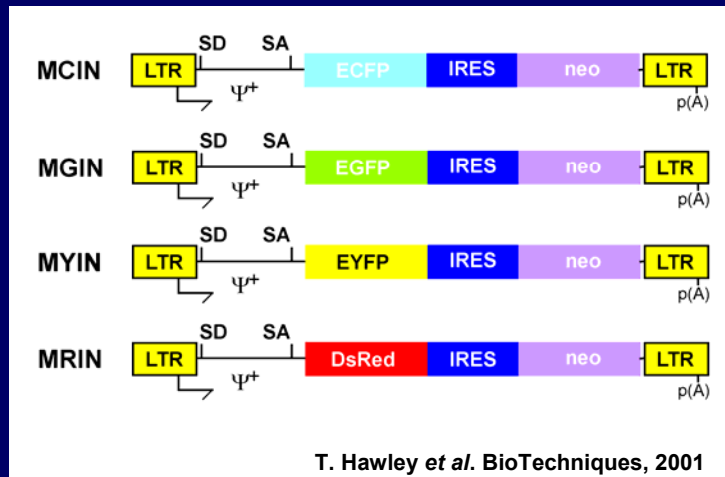


Simultaneous Detection of Multiple Fluorescent Proteins

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Fluorescent proteins are used here as an example of multiparameter flow cytometry. The same principle applies to commonly used fluorochromes (such as FITC, PE, PE-TexasRed, PE-Cy5, PerCP-Cy5.5, PE-Cy7, APC, APC-Cy7, Cascade blue, Cascade yellow, or the Alexa dyes) as well as Qdots.

MSCV Oncoretroviral Vectors Containing Fluorescent Protein Genes



Each of these oncoretroviral vectors carries a fluorescent protein gene, the IRES sequence (which initiates internal translation of a bicistronic mRNA), and the neo (bacterial neomycin phosphotransferase) gene. The neo gene confers resistance to the neomycin analog (G418) in mammalian cells. It facilitates derivation of cells expressing the individual fluorescent protein genes.

MCIN, MGIN, MYIN and MRIN were constructed by Dr. Ali Ramezani. MTIN (containing dTomato), not shown here, was constructed by Sara Karandish. Both Ali and Sara are members of Dr. Robert Hawley's research team.

Derivation of Cells Stably Expressing Individual or Multiple Fluorescent Protein Genes

- Packaging cell line: GP+E-86 containing gag, pol and env
- Transfect GP+E-86 with oncoretroviral vectors containing ECFP, EGFP, EYFP or DsRed cDNAs
- Select in G418 (Geneticin) to derive stable cell lines expressing the individual fluorescent protein genes: 0, C, G, Y, R
- Target cell line: Sp2/0-Ag14
- Transduce Sp2/0-Ag14 with supernatant containing different combinations of fluorescent protein retroviral vector particles
- Select in G418 and/or by FACS to derive stable cell lines expressing individual or multiple fluorescent protein genes: 0, C, G, Y, R, CY, GY, CGY, GYR, CGYR

GP+E-86 is a mouse fibroblast cell line (based on NIH3T3 cells).

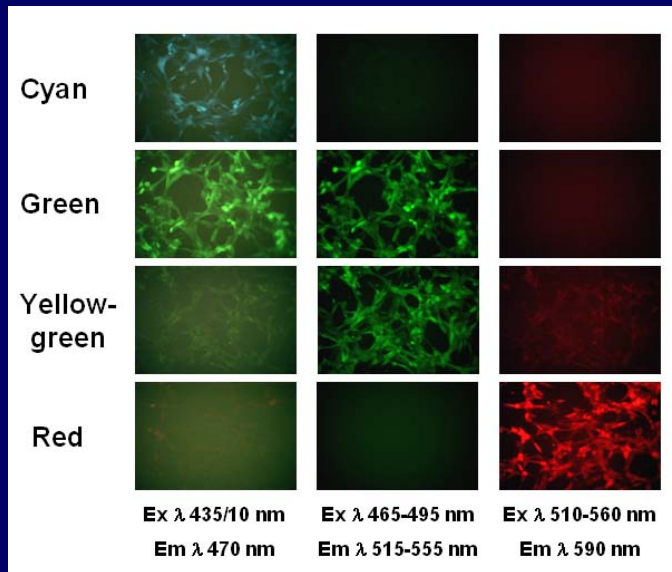
Sp2/0-Ag14 is a mouse hybridoma cell line. These cells grow readily in suspension.

References:

Markowitz, D., Goff, S., and Bank, A. 1988. A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virol.* 62, 1120-1124.

Shulman, M., Wilde, C. D., and Kohler, G. 1978. A better cell line for making hybridomas secreting specific antibodies. *Nature* 276, 269-270.

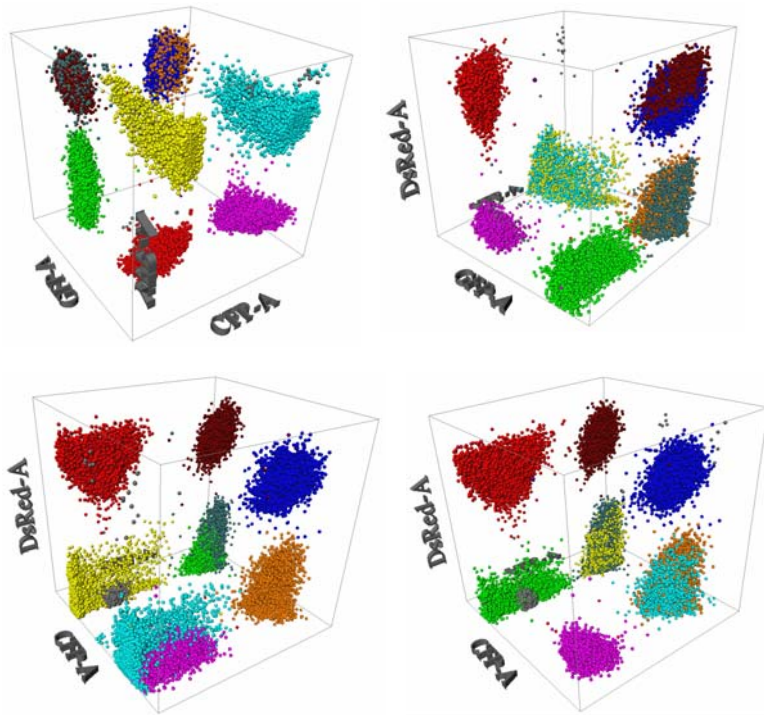
Expression of Fluorescent Protein Variants



T. Hawley *et al.*, *BioTechniques*, 2001

Fluorescence microscopic images of GP+E-86 cells expressing the individual fluorescent protein genes. Note the high degrees of spectral overlap between fluorescent proteins.

**Simultaneous
Detection of
Four
Fluorescent
Proteins**



3D views (WinList 3D v5.0, Verity Software House) of a mixture of ten populations of Sp2/0 cells expressing the individual or multiple fluorescent proteins genes. The populations are denoted as: 0, C, G, Y, R, CY, GY, CGY, GYR and CGYR.

0 (Negative)	= Sp2/0 cells	(gray)
C	= cells expressing ECFP	(purple)
G	= cells expressing EGFP	(green)
Y	= cells expressing EYFP	(yellow)
R	= cells expressing DsRed	(red)
CY	= cells expressing ECFP/EYFP	(light blue)
GY	= cells expressing EGFP/EYFP	(turquoise)
CGY	= cells expressing ECFP/EGFP/EYFP	(orange)
GYR	= cells expressing EGFP/EYFP/DsRed	(brown)
CGYR	= cells expressing ECFP/EGFP/EYFP/DsRed	(dark blue)

Top left view: CFP vs. GFP vs. YFP

Top right view: GFP vs. YFP vs. DsRed

Bottom left view: CFP vs. GFP vs. DsRed

Bottom right view: CFP vs. YFP vs. DsRed

FCOM region list for 4 Fluorescent Proteins

Region	ECFP	EGFP	EYFP	DsRed	Alias	Color
12	-	-	-	-	Negative	Gray
13	+	-	-	-	C	Purple
14	-	+	-	-	G	Green
15	+	+	-	-	CG	
16	-	-	+	-	Y	Yellow
17	+	-	+	-	CY	Light blue
18	-	+	+	-	GY	Turquoise
19	+	+	+	-	CGY	Orange
20	-	-	-	+	R	Red
21	+	-	-	+	CR	
22	-	+	-	+	GR	
23	+	+	-	+	CGR	
24	-	-	+	+	YR	
25	+	-	+	+	CYR	
26	-	+	+	+	GYR	Brown
27	+	+	+	+	CGYR	Dark blue

Presenting the previous display on 2D bivariate histograms makes it difficult to discriminate populations occupying the same 2D space. The FCOM feature in WinList is a useful tool to identify multiple populations within a mixture. FCOM stands for “Combinations Function”. It is a calculated parameter that can be used to classify events based on combinations of selected gates.

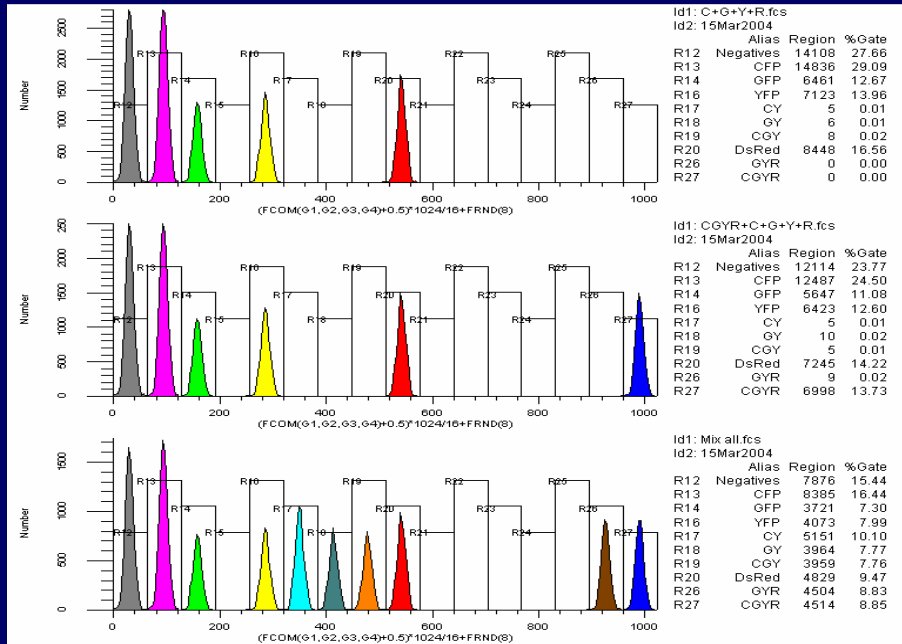
Based on gates drawn around the single color populations, a FCOM histogram displays populations corresponding to all of the possible combinations. For example, for 4 fluorescent proteins, FCOM displays 16 combinations of fluorescent protein gene expression (Regions 12-27 in the above example). Pseudocolors assigned to the populations aid in visualization.

FCOM Histograms Displaying All Combinations of Fluorescent Protein (FP) Expression

C/G/Y/R/0

CGYR/C/G/Y/R/0

CY/YG/CY/YR/
CGYR/C/G/Y/R/0



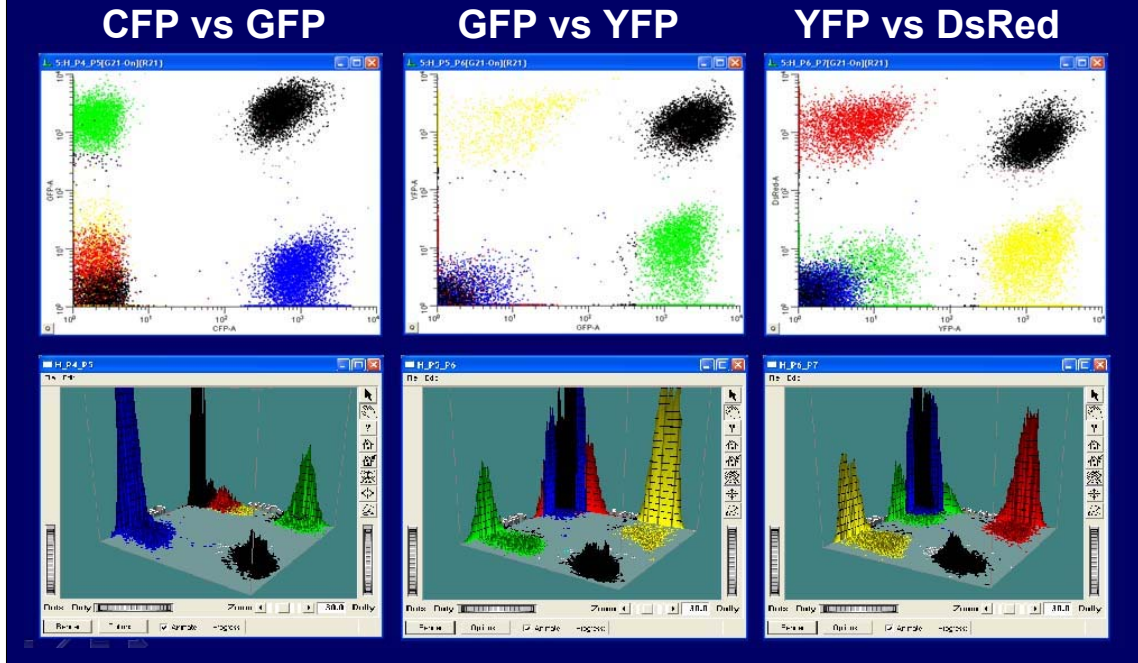
Mixtures of Sp2/0 cells expressing the individual or multiple fluorescent protein genes, displayed on FCOM histograms.

Row 1 = mixture of 0, C, G, Y and R

Row 2 = mixture of 0, C, G, Y, R and CGYR

Row 3 = mixture of 0, C, G, Y, R, CY, GY, CGY, GYR and CGYR

Log Transform



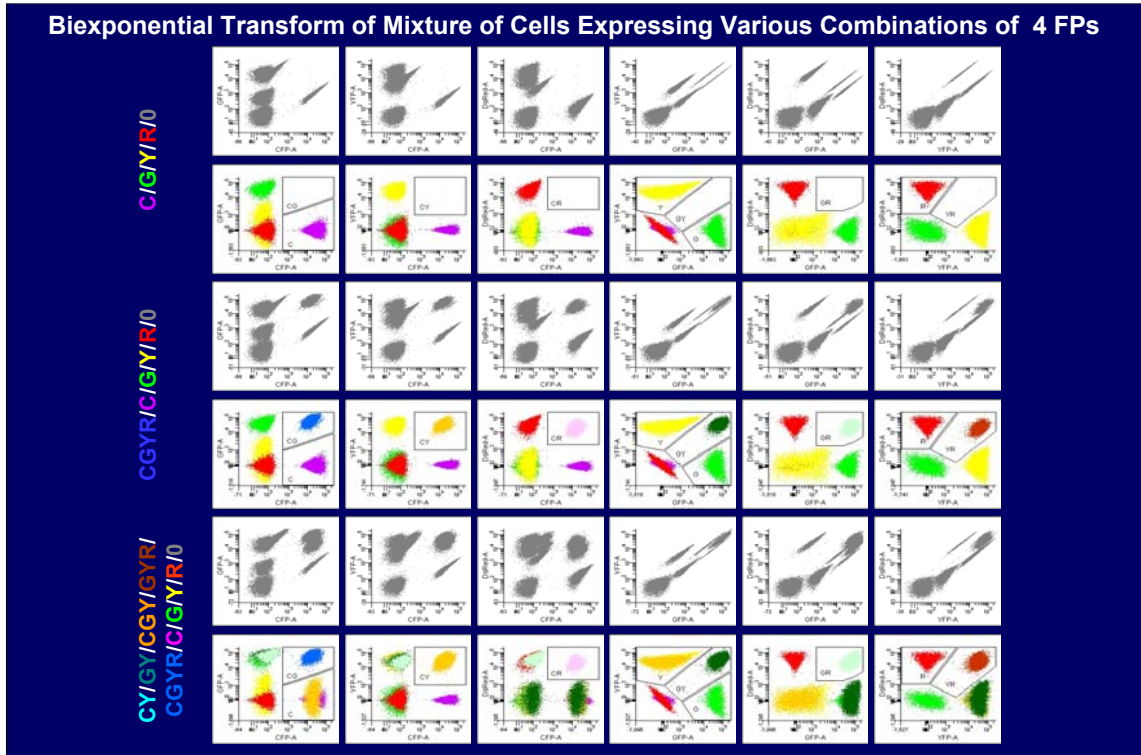
This represents a mixture of six populations: 0, C, G, Y, R and CGYR (= Row 4 on the previous slide).

Top row shows 2D plots of compensated data displayed with the log transform. Bottom row shows the corresponding isometric 3D plots. The third dimension represents the number of events.

The isometric plots clearly illustrate how many events are piled up on the axes when properly compensated data are displayed with the log transform.

These slides and video clips were kindly prepared by Don Herbert at Verity Software House.

Biexponential Transform of Mixture of Cells Expressing Various Combinations of 4 FPs



Unlike the traditional log transform, two recently developed transforms can display events off the axes, making it far easier to visualize properly compensated data.

The Biexponential transform was developed by Wayne Moore and Dr. Dave Parks at Stanford University. It has been implemented in FACSDiva and FlowJo (Treestar).

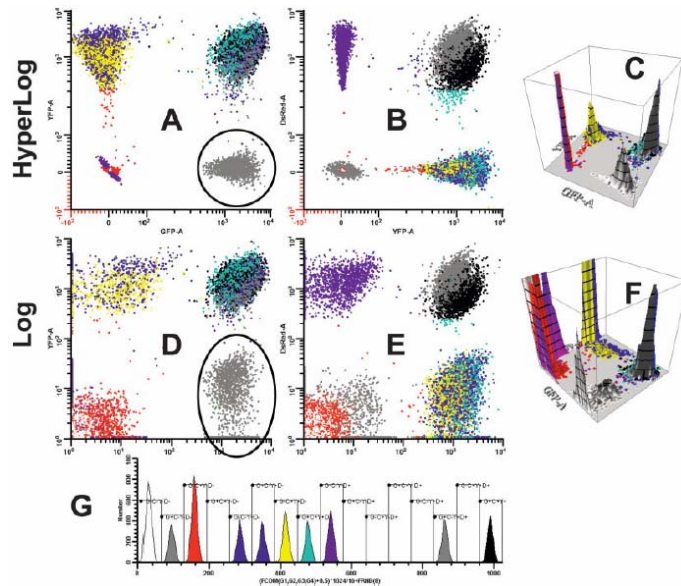
Mixture of Sp2/0 cells expressing the individual or multiple FP genes, displayed with the Biexponential transform (FACSDiva v4.1).

Rows 1 & 2 = mixture of 0, C, G, Y and R, without or with compensation for spectral overlap, respectively

Rows 3 & 4 = mixture of 0, C, G, Y, R and CGYR, without or with compensation for spectral overlap, respectively

Rows 5 & 6 = mixture of 0, C, G, Y, R, CY, GY, CGY, GYR and CGYR, without or with compensation for spectral overlap, respectively

HyperLog—A Flexible Log-like Transform for Negative, Zero, and Positive Valued Data



C. Bruce Bagwell, *Cytometry*, 2005

The HyperLog transform was developed by Dr. C. Bruce Bagwell at Verity Software House. It has been implemented in WinList.

Reference:

Bagwell, C. B. 2005. HyperLog—a flexible Log-like transform for negative, zero and positive valued data. *Cytometry* 64A, 34-42.

Optical Arrangement on FACSaria (BD Biosciences)

Detector B = CFP

470/15 BP

Detector F = SSC

488/10 BP

Detector E = GFP

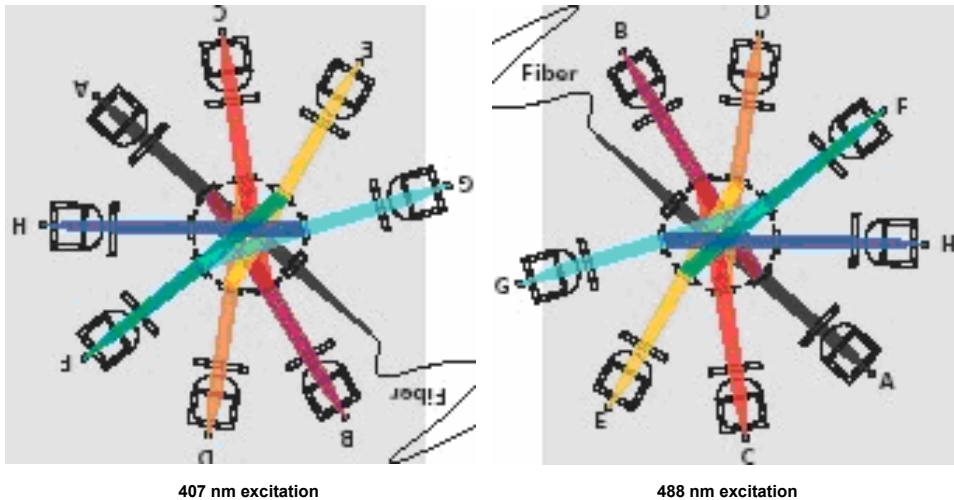
495 LP, 510/20 BP

Detector D = YFP

527 LP, 550/30 BP

Detector C = DsRed or dTomato

595 LP, 610/20 BP



Adapted from FACSaria brochure

This is the configuration for simultaneous detection of four fluorescent proteins on the FACSaria.

407 nm is used to excite ECFP

488 nm is used to excite EGFP, EYFP and DsRed (or dTomato)

Reference:

28th Annual Course in Flow Cytometry, Los Alamos, New Mexico, June 11-17, 2005.