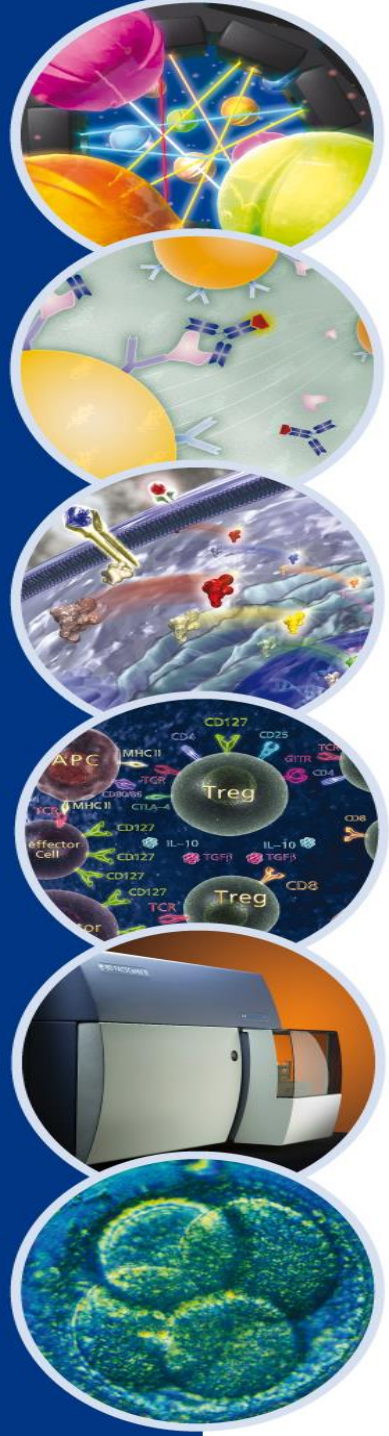


流式基础原理介绍及
流式实验设计

BD生命科学部
胡 启 萍
18030601919
Qiping.hu@bd.com

Maxwell W. Becton Fairlegh S. Dickinson

BD

Advancing the world of health

BD, founded in 1897, is a global medical technology company that is advancing the world of health by improving medical discovery, diagnostics and the delivery of care. BD has more than 45,000 associates across 50 countries who work in close collaboration with customers and partners to help enhance outcomes, lower health care delivery costs, increase efficiencies, improve health care safety and expand access to health.

给药流程解决方案
MPS

标本分析前处理系统
PAS

生物科学
BDB

呼吸解决方案
RS

糖尿病护理
DC

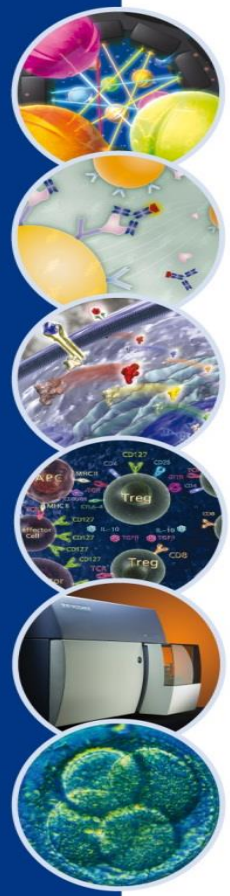
制药系统
PS

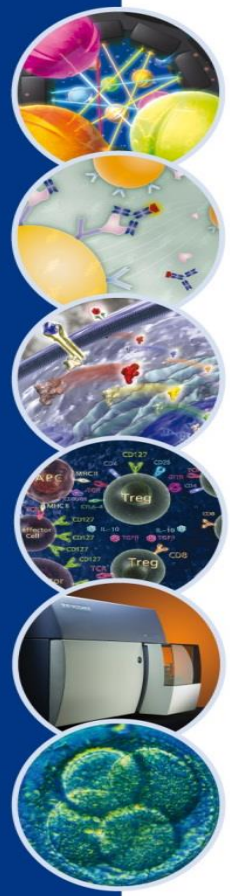
药物制备及分发技术
DPT

诊断系统
DS

内容：

- 1、流式基本原理介绍
  - 1.1、 基本概念
- 2、多色流式实验
  - 2.1、实验设计
  - 2.2、多色流式实验配色
  - 2.3、样本处理



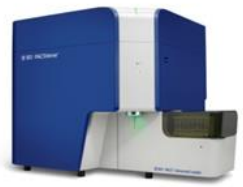


BD FACSCelesta
Research platform
10C 12C 14C solution

FACS AriaIII
Intelligent Sorters

FACSAria Fusion
Intelligent Sorters

Influx
Exploring Sorters



BD FACSVerse
Research platform
4C 6C 8C solution

LSRFortessa
Pioneer grade cytometers
10C - 18C+solution

Accuri C6 (2L4C)
Personalized Cytometer
4C solution

FACSCalibur (2L4C)
Classic Platform
4C solution

FACSCanto (3L 10C)
Professional Clinical platform
10C solution

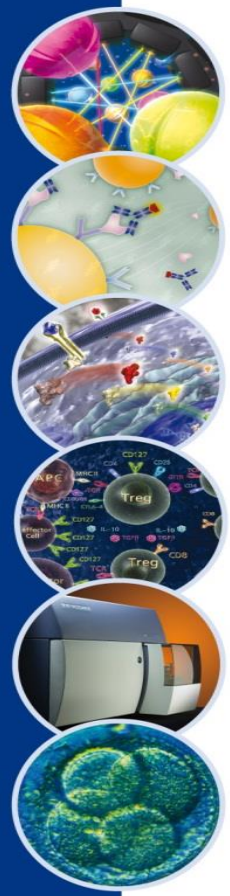
FACS AriaII
Intelligent Clinical Sorters

BD

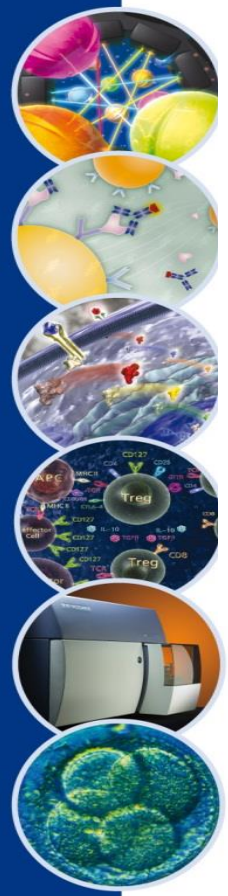
一、流式细胞术的基本概念

- 流式细胞术(Flow Cytometry, 简称FCM)就是利用流式细胞仪对处于快速直线流动状态中得单列细胞或生物颗粒进行逐个、多参数、快速的定性、定量分析的技术。 同时可以对特定群体加以分选。

- 流式细胞仪（Flow Cytometer）集激光技术、电子物理技术、光电测量技术、电子计算机技术、细胞荧光化学技术、单克隆抗体技术为一体的一种新型高科技仪器。

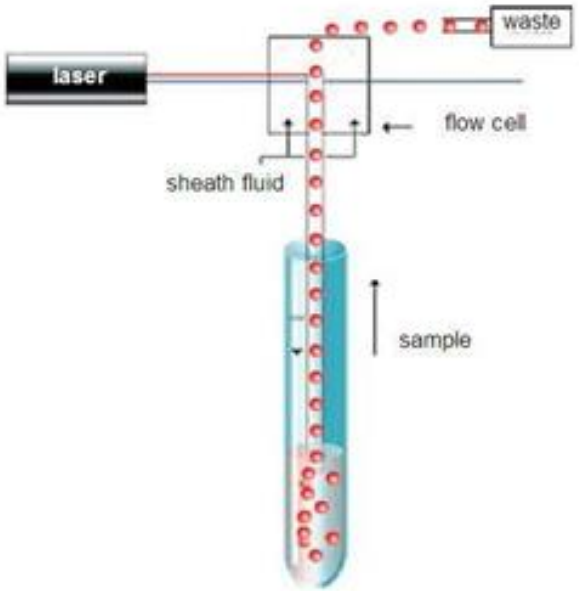


流式细胞仪的结构



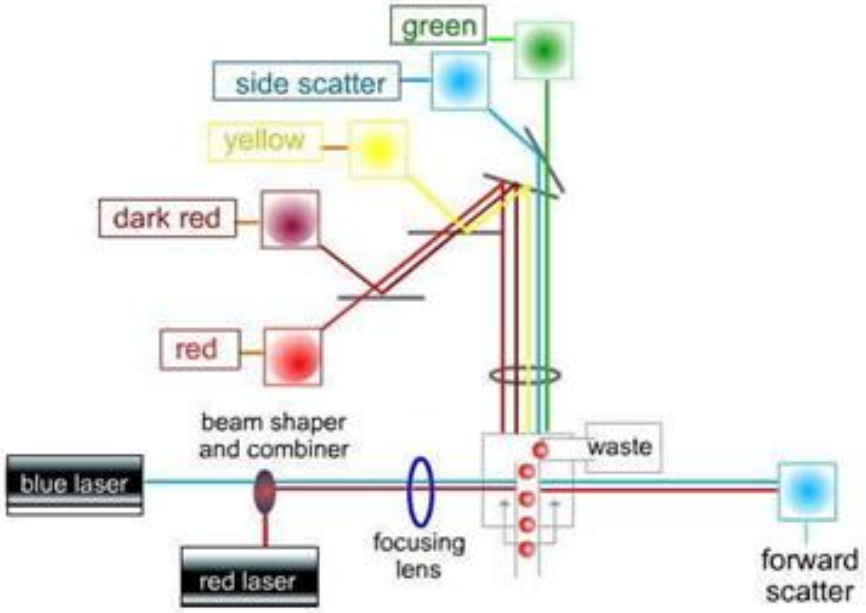
液流系统

聚集细胞



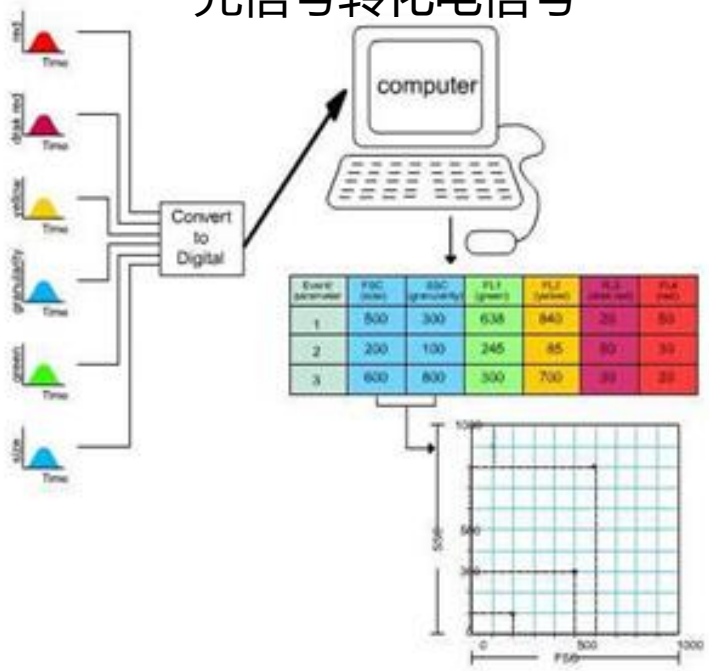
光学系统

激发和收集信号



电子系统

光信号转化电信号



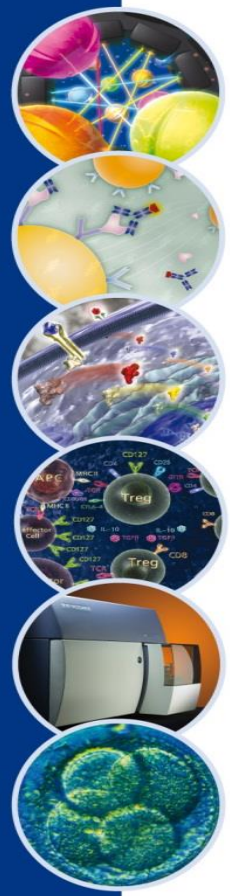
流式细胞仪的检测范围

细胞结构

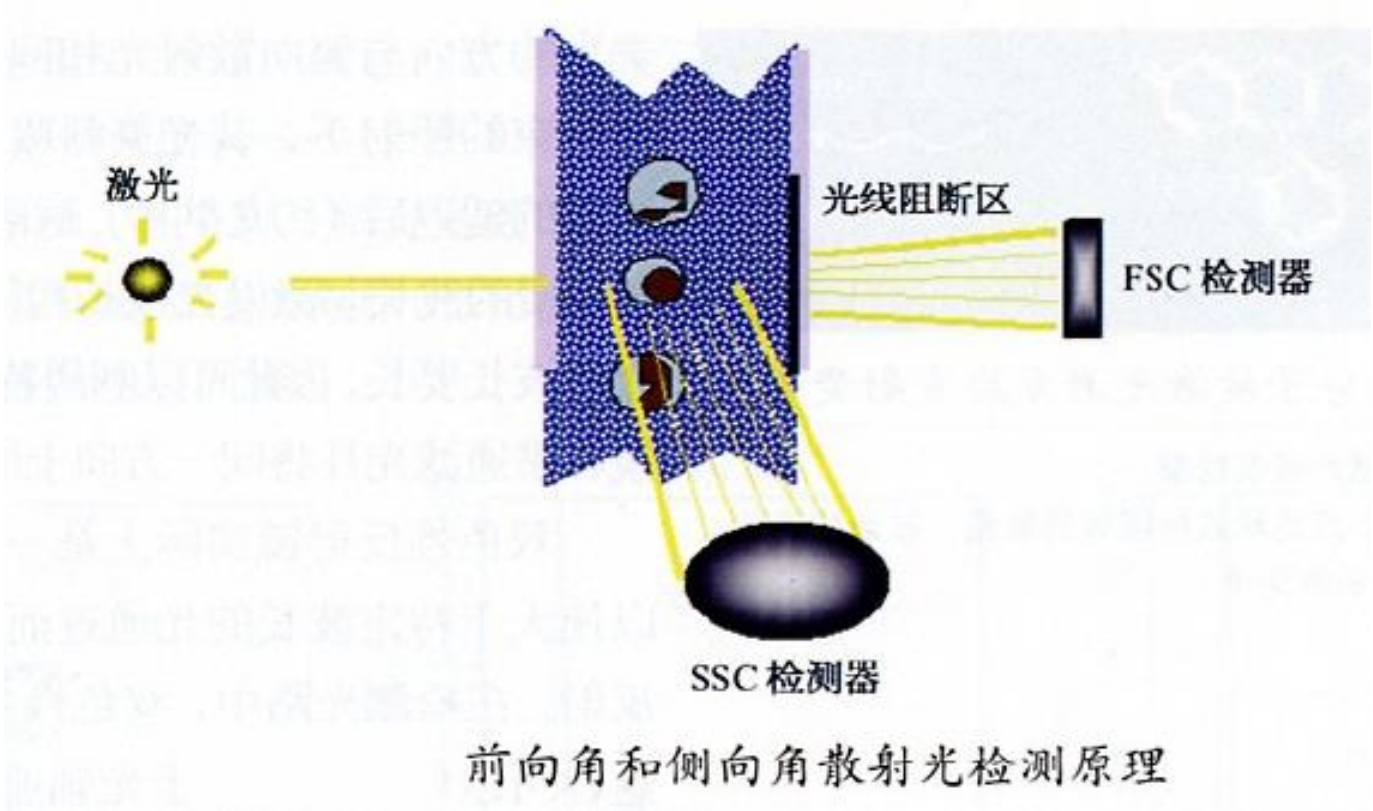
- 细胞大小
- 细胞粒度
- 细胞表面面积
- 核浆比例
- **DNA**含量与细胞周期
- **RNA**含量
- 蛋白质含量

细胞功能

- 细胞表面/胞浆/核的特异性抗原
- 细胞活性
- 细胞内细胞因子
- 酶活性
- 激素结合位点
- 细胞受体
- 细胞内钙离子



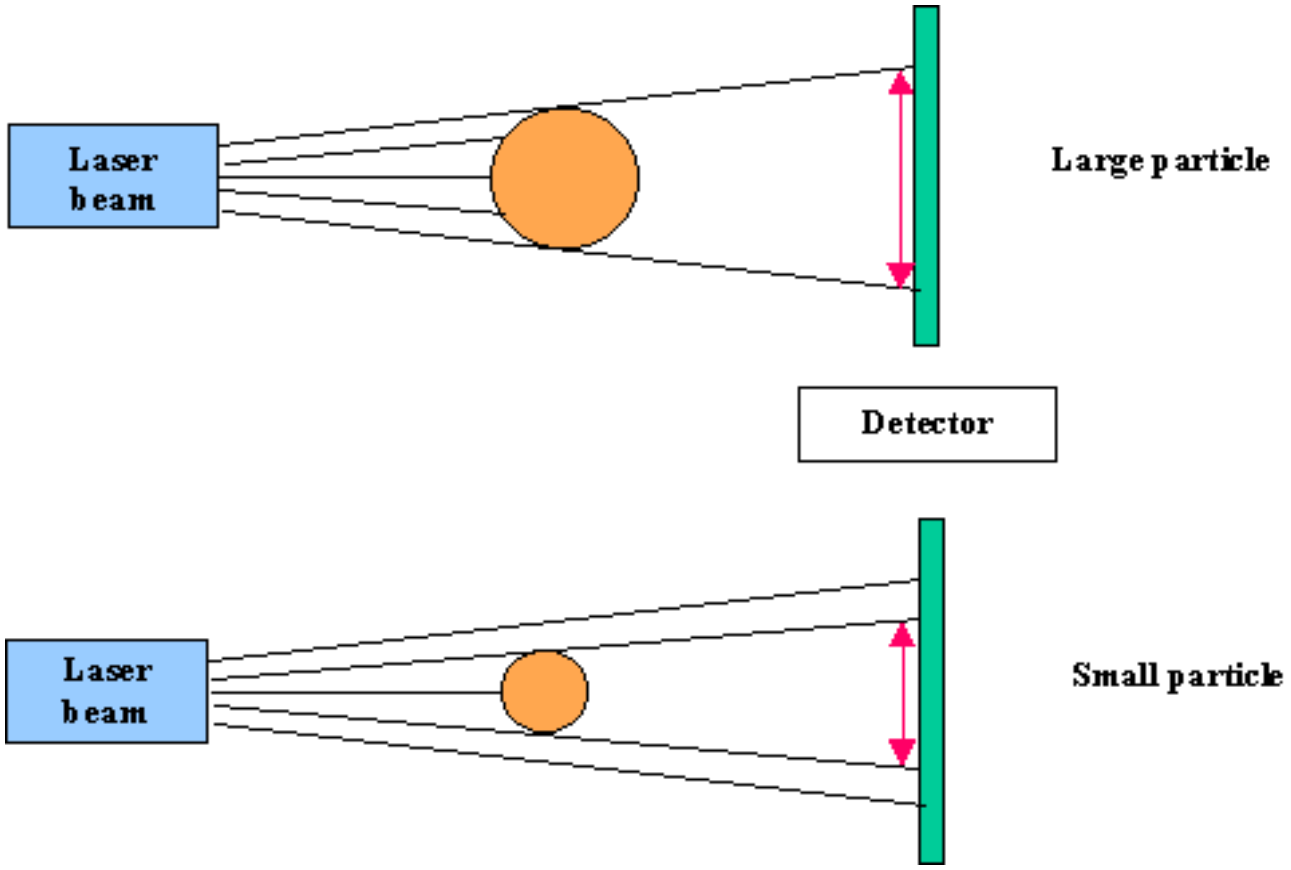
散射光信号



前向角和侧向角散射光检测原理

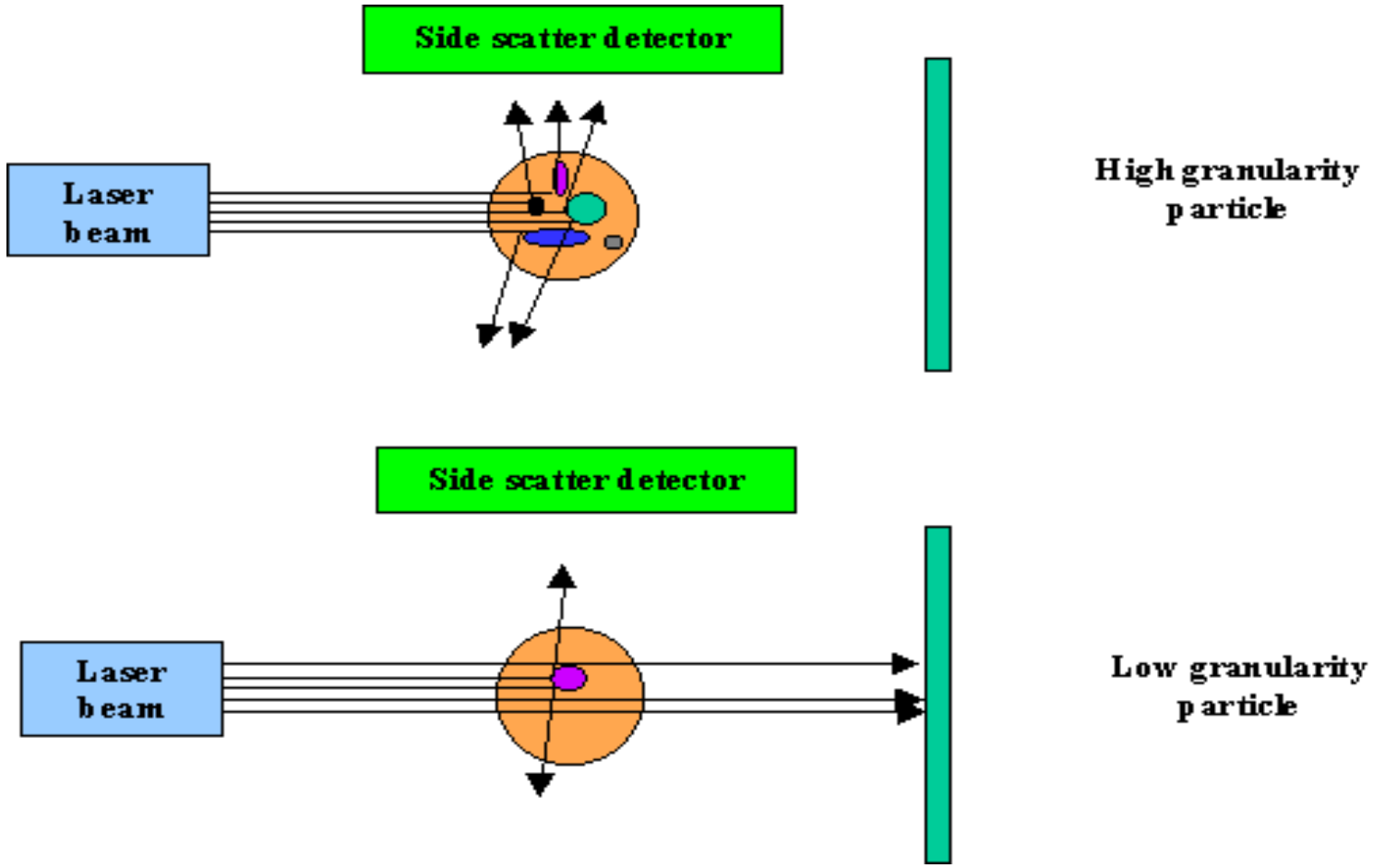
- **当细胞颗粒通过聚集的激光束时，激光向各个方向散射。与激光束方向同轴的称前向角散射光信号（FSC）。与激光束垂直的称为侧向角散射光信号（SSC）**

前向角散射光信号



- **前向角散射光（FSC, Forward Scatter）　细胞相对大小及其表面积**

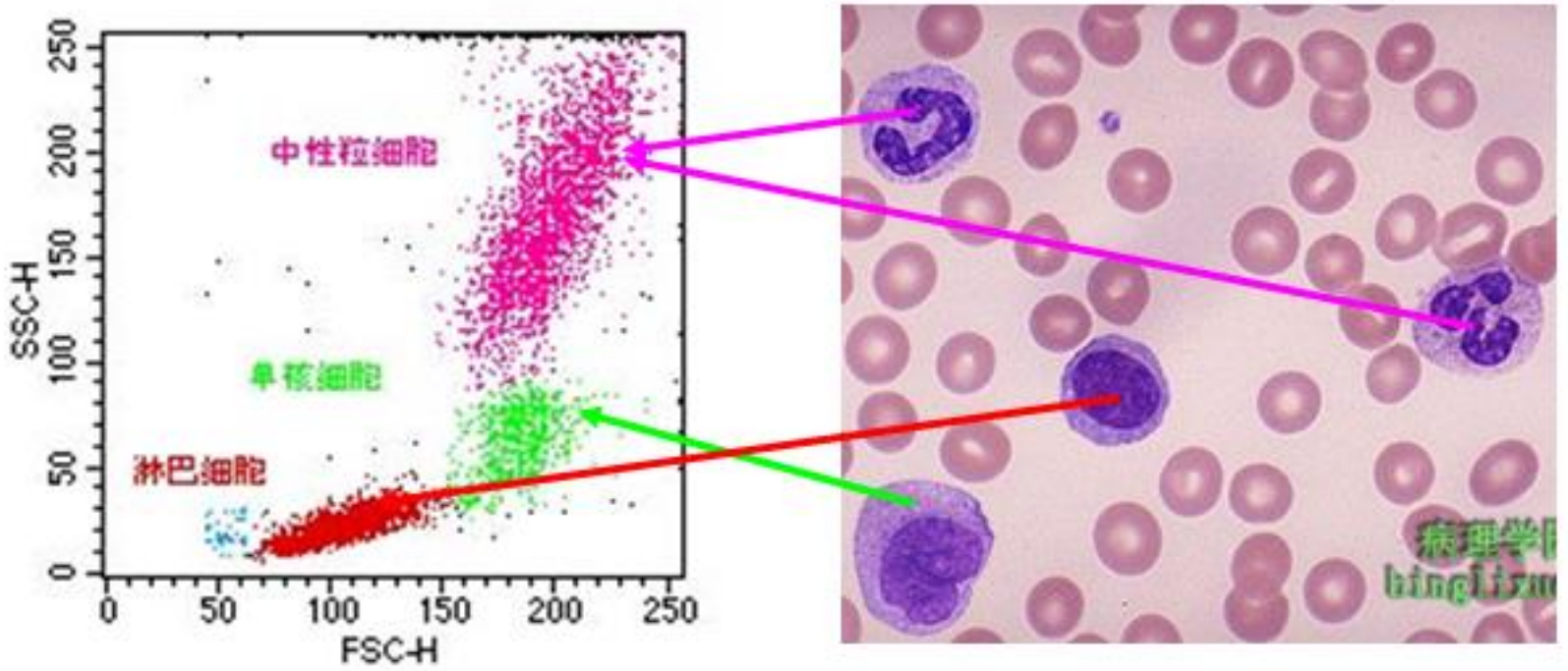
侧向角散射光信号



- **侧向角散射光（SSC, Side Scatter）细胞颗粒度及细胞内细胞器的相对复杂性**

外周全血细胞散射光双参数点图（红细胞溶解后）

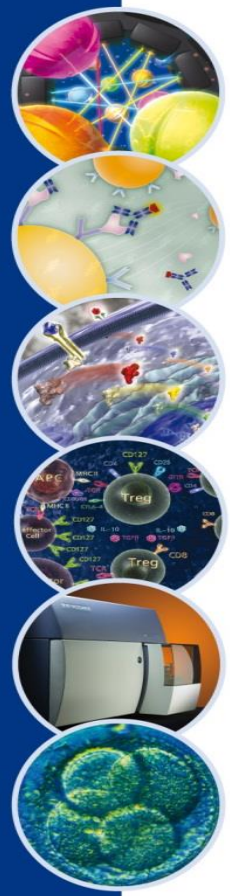
SSC：
区分细胞
粒度



FSC: 区分细胞大小

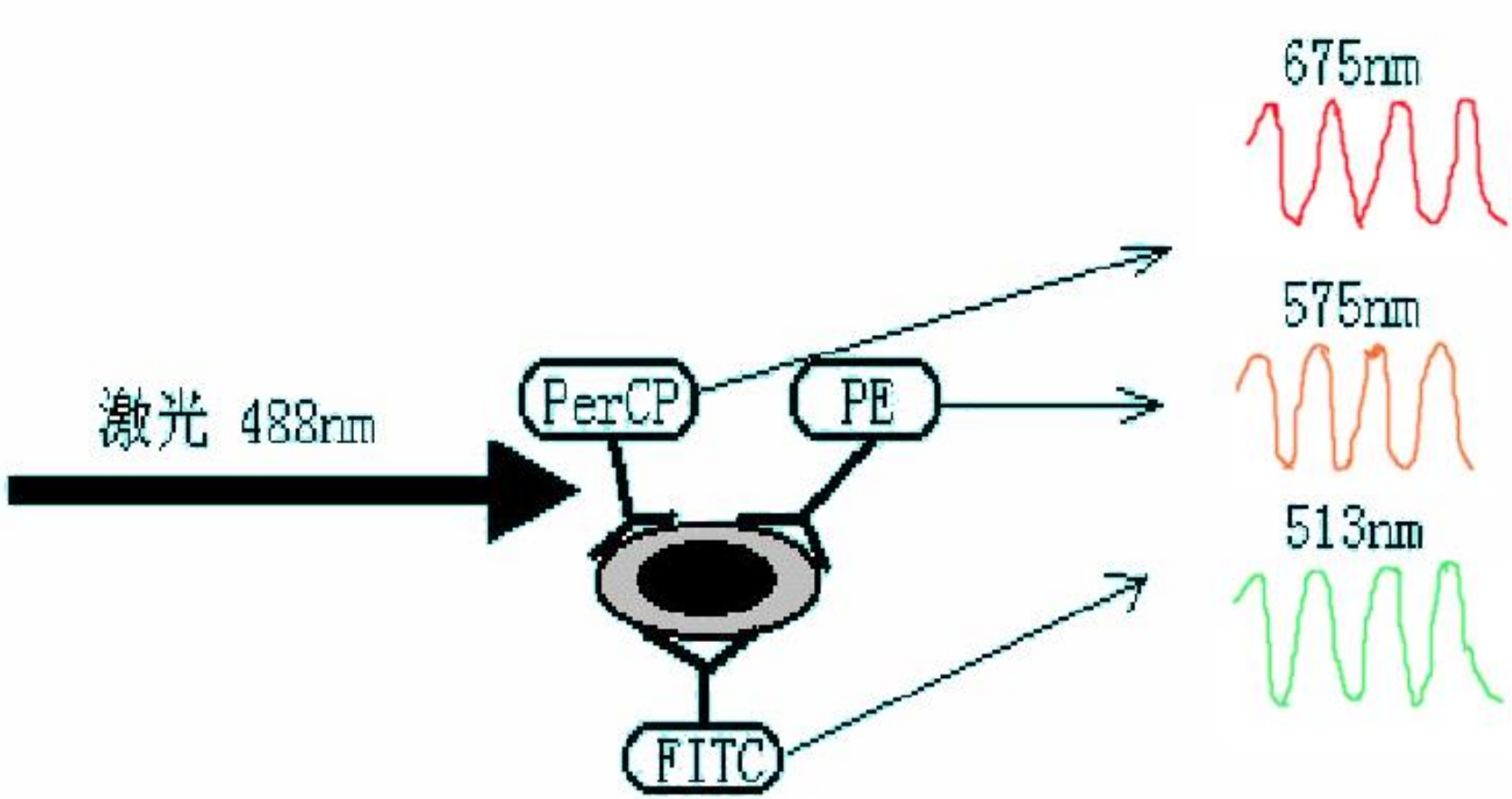
荧光信号-荧光素的激发和发射光谱

- 任何发荧光的物质分子都具有这两个特征光谱(nm)
- 激发光谱(Excitation，Ex)：
  - 是指能特异性地激发某种荧光素的一定波长范围内的光线，也称为吸收光谱。
  - 吸收波峰(最大吸收波长)：Ex-Max
- 发射光谱(Emission，Em)：
  - 是指某一波长激发光引起荧光素发射的一定波长范围内的荧光
  - 发射波峰(最大发射波长)：Em-Max
- 荧光素的使用：
  - 选择正确的激光器
  - 确定所需探测器(PMT)



荧光信号

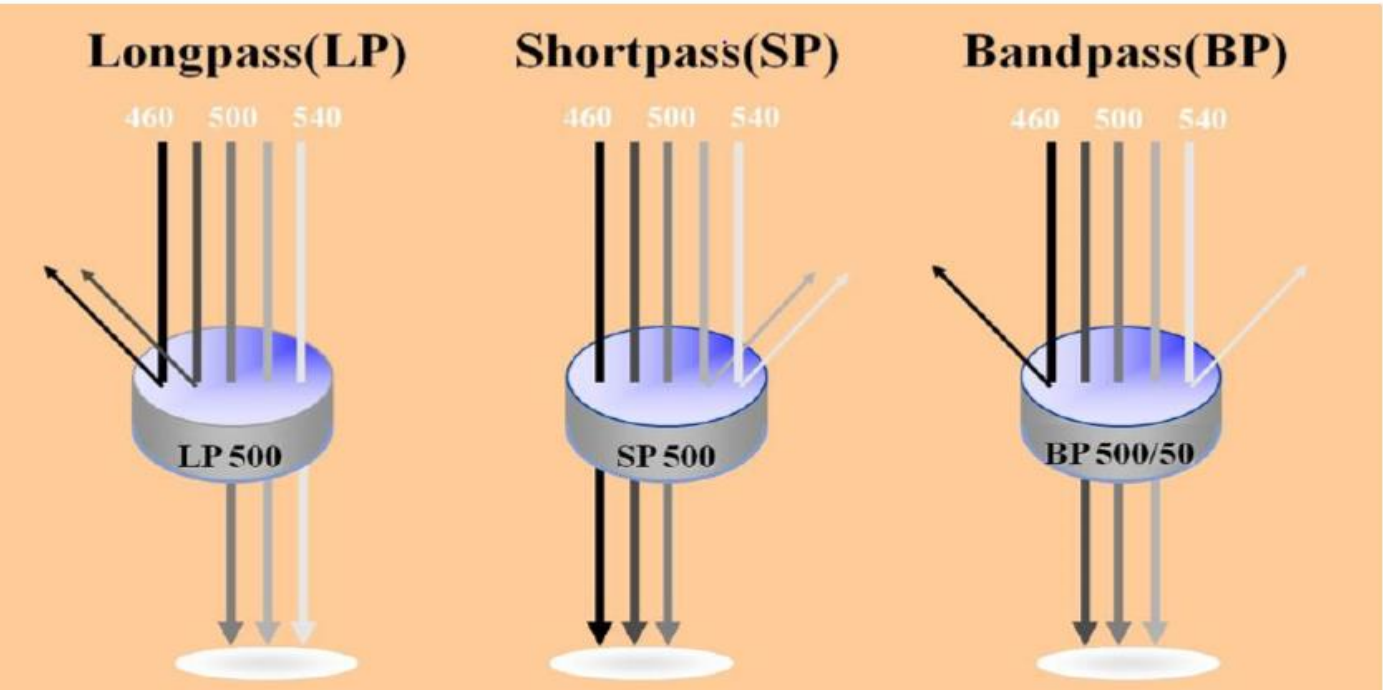
- 使用不同荧光标记的单克隆抗体染色，做多色分析
- 荧光信号的强弱，反映了细胞抗原的表达含量

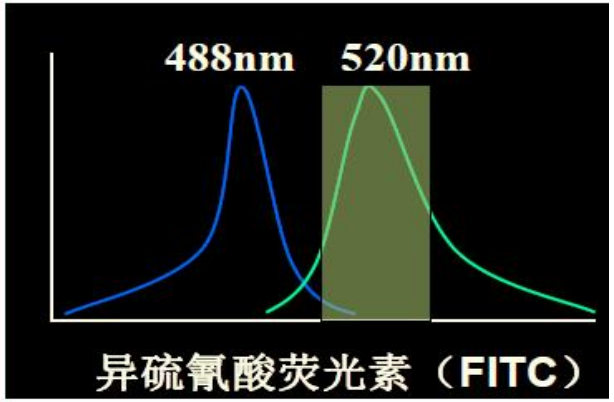


滤光片

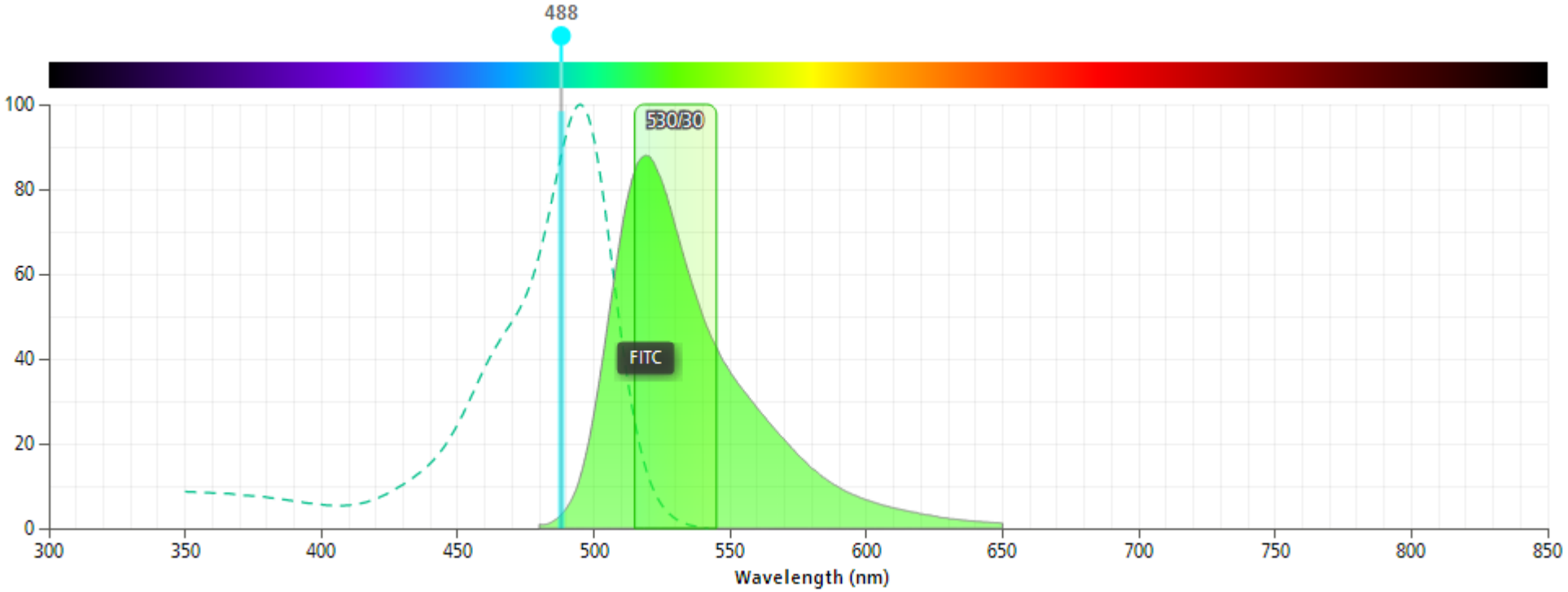
滤光片

- 如何将一束混合的荧光区分开来，分别进行收集呢？
- 滤光片置于荧光探测器前，限定其接收的荧光的波长范围
- 长通（LP）、短通（SP）、带通（BP）





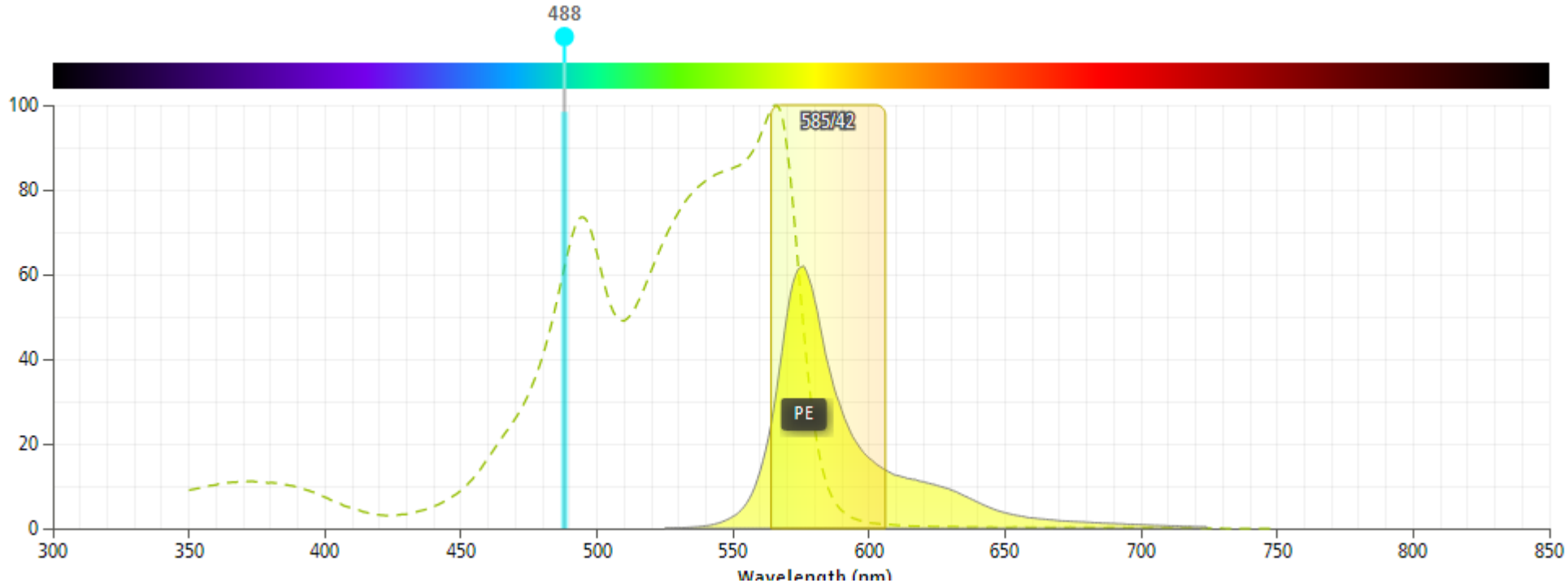
FITC的激发与发射光谱



常用染料FITC可被488nm激光激发，Em-Max：525nm，

可以用530/30接收通道来接受荧光信号。

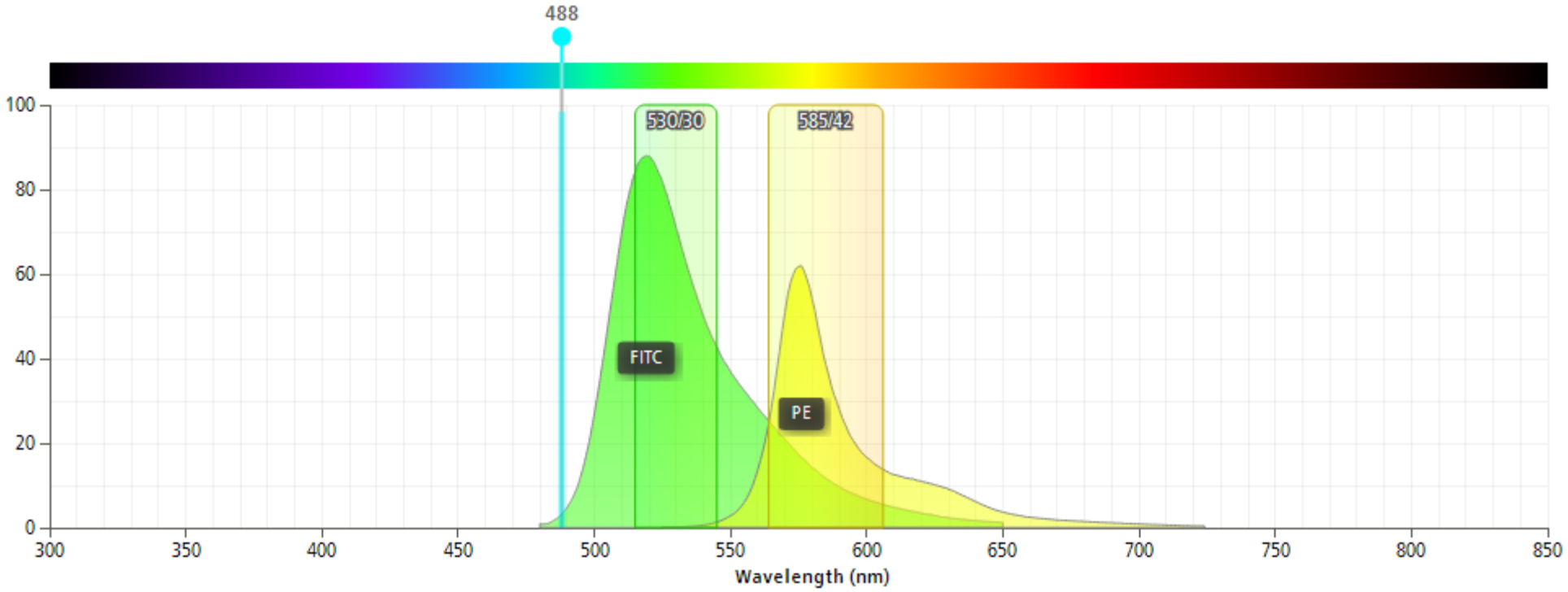
PE的激发与发射光谱

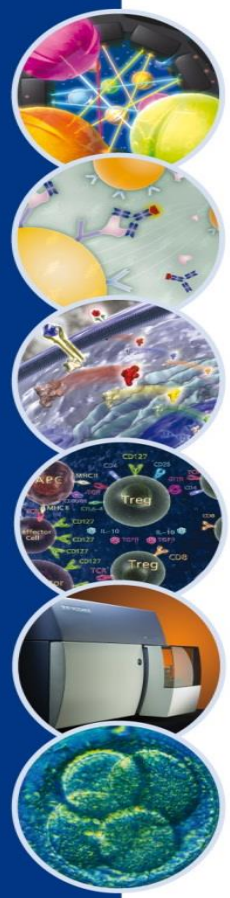


常用染料PE可被488nm激光激发，Em-Max：590nm，

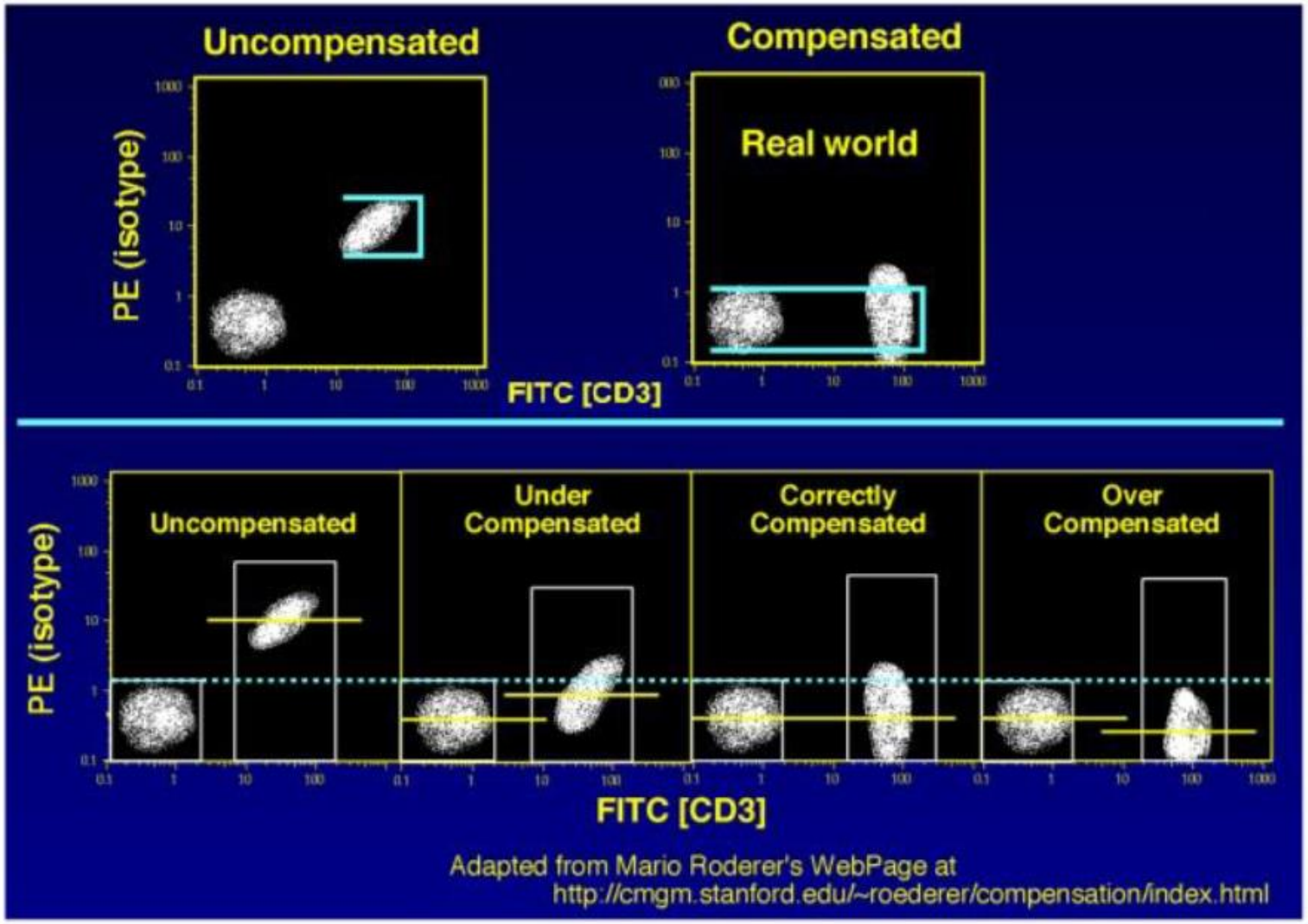
可以用585/42接收通道来接受荧光信号。

荧光信号—荧光溢漏


488
100
80
60
40
20
0
530/30
585/42
FITC
PE
300
350
400
450
500
550
600
650
700
750
800
850
Wavelength (nm)

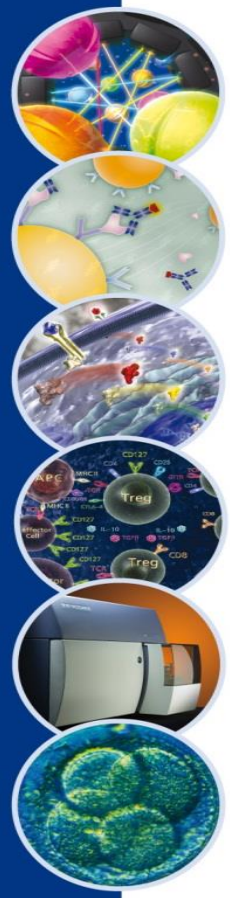

Treg
Treg

荧光信号--补偿


Uncompensated
Compensated
Real world
PE (isotype)
FITC [CD3]
Uncompensated
Under Compensated
Correctly Compensated
Over Compensated
PE (isotype)
FITC [CD3]
Adapted from Mario Roderer's WebPage at
http://cmgm.stanford.edu/~roederer/compensation/index.html

内容：

- 1、流式基本原理介绍
  - 1.1、 基本概念
- 2、多色流式实验
  - 2.1、实验设计
  - 2.2、多色流式实验配色
  - 2.3、样本处理

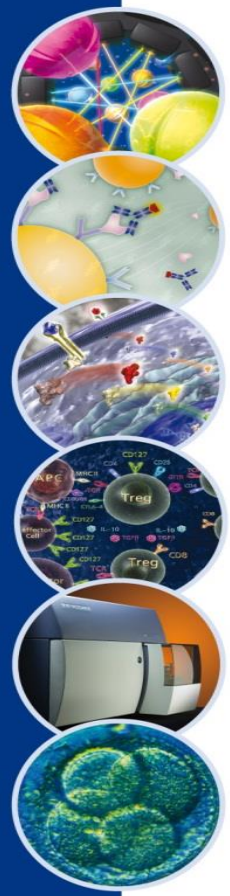


二、多色实验设计

样品荧光组成=自发荧光+特异荧光+非特异荧光

- 自发荧光，即不经荧光染色细胞内部荧光分子经光照发出的荧光。自发荧光信号为噪声信号。

一般说来，细胞成分中能产生自发荧光的分子（核黄素、细胞色素等）的含量越高，自发荧光越强，如肿瘤细胞、粒细胞等；样本死细胞比例越高自发荧光越强

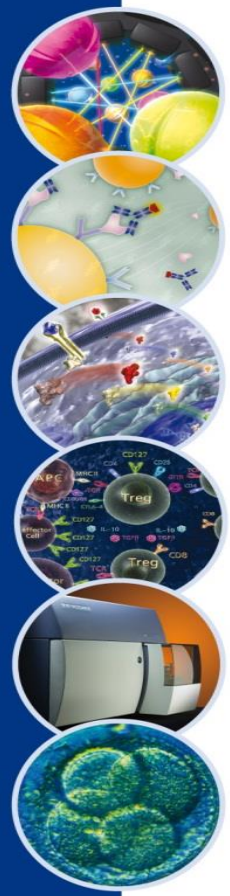


- 特异荧光，即抗体F(ab')2段与细胞抗原特异结合上的荧光染料受光照发出的荧光
- 非特异荧光，即抗体Fc段与细胞表面的Fc受体非特异结合上的荧光染料受光照发出的荧光

BD

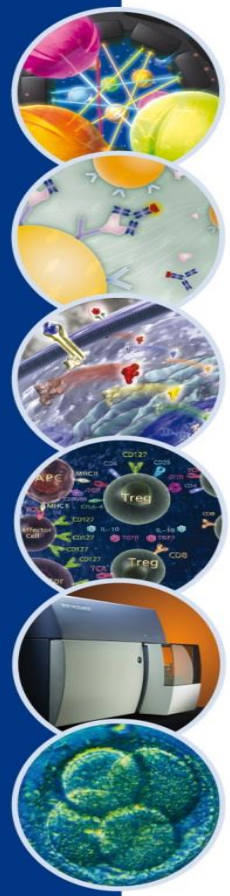
流式对照设置

- 空白对照
  - 自发荧光
- 实验样本
  - 自发荧光
  - 非特异荧光
  - 特异荧光
- 同型对照（自发荧光＋非特异荧光）



对照设置：同型对照（Isotype Control）

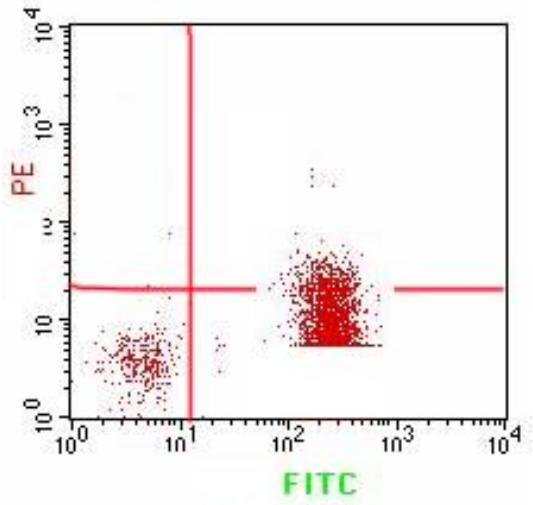
- 与实验染色的单克隆抗体特异性无关的免疫球蛋白亚型
  （即Fc段相同， F(ab')2段不同）
- 与染色的单克隆抗体
  ①相同种属来源
  ②相同免疫球蛋白及亚型
  ③相同荧光素标记
  ④相同剂量和浓度
  ⑤但由未免疫动物血清纯化而来
- 用于消除由于抗体非特异性结合到细胞表面的Fc受体而产生的背景染色
- 例如，标记FITC的单克隆抗体为小鼠IgG1亚类抗体，标记PE的单克隆抗体为小鼠IgG2a亚类抗体，同型对照应用相同浓度和剂量的未免疫小鼠血清的纯化IgG1（γ1）和IgG2a（γ2a），并分别标记FITC和PE



对照设置：补偿设置

- 补偿对照（双色或多色分析中，荧光素发射光谱重叠时）
  - 荧光补偿（**compensation**）
  - 使用单种荧光素标记的单克隆抗体和其余荧光素对应的同型对照抗体分别进行染色

    ※几色分析需要制备几个补偿对照管

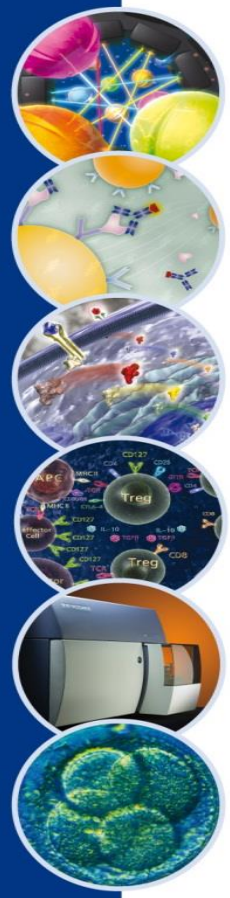


真双阳？假双阳？

对照设置

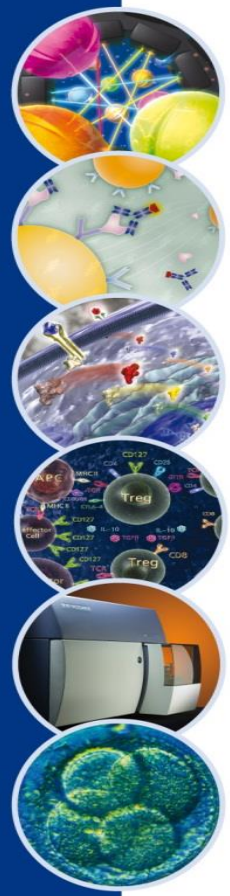
No.	Antibody	Isotype
	Mouse anti-human	未免疫Mouse 血清纯化而来
1	A-FITC（γ1）	γ1-FITC
2	B-PE（γ2a）	γ2a-PE

分析类型	管	功能	加入的抗体	
双色分析	1	Isotype	γ1-FITC	γ2a-PE
	2	Compensation1	A-FITC	γ2a-PE
	3	Compensation2	γ1-FITC	B-PE
	4	Sample1,2……	A-FITC	B-PE



多色实验—数据处理

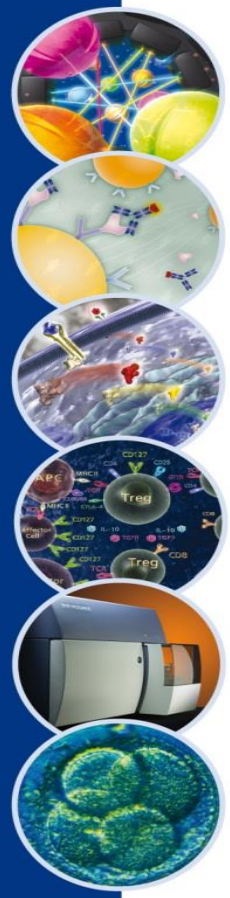
- **FCS 2.0格式**
- **常以列表模式（LIST MODE)：将每个细胞的每个检测参量依次排列顺序存储**



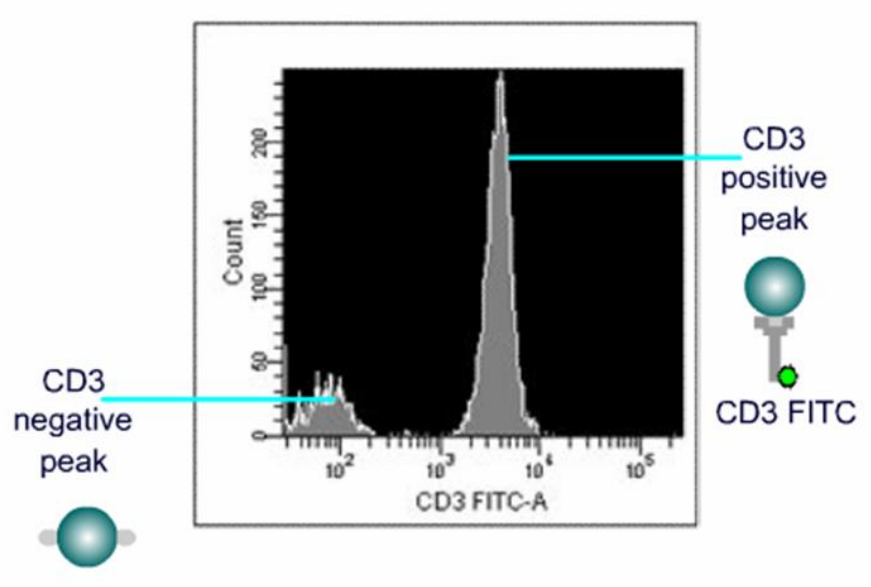
	FSC	SSC	FL1	FL2
Event 1	30	60	638	840
Event 2	100	160	245	85
Event 3	300	650	160	720

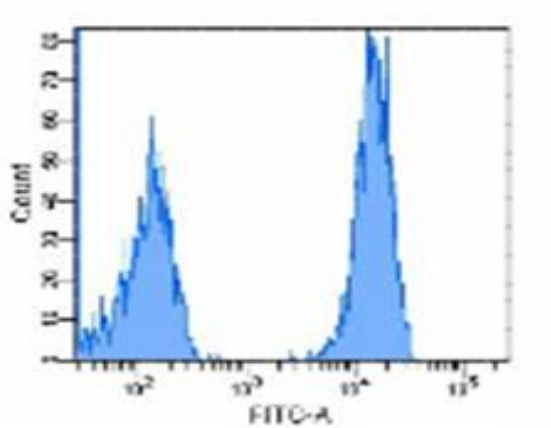
多色实验—数据显示

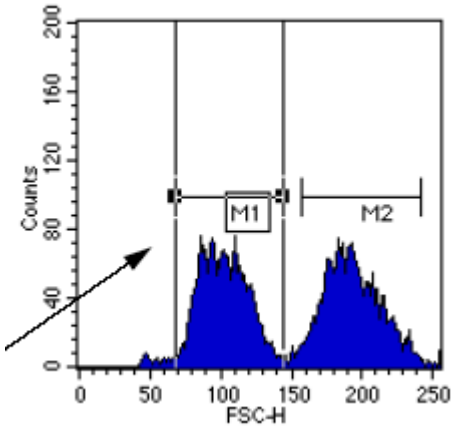
- **直方图（Histogram）**
- **二维点图（Dot Plot）**
- **等高线图（Contour Plot）**
- **密度图（Density）**
- **三维图（3D Plot）等**



单参数直方图分析

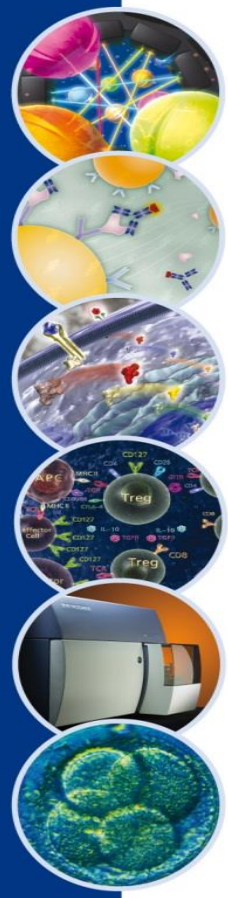


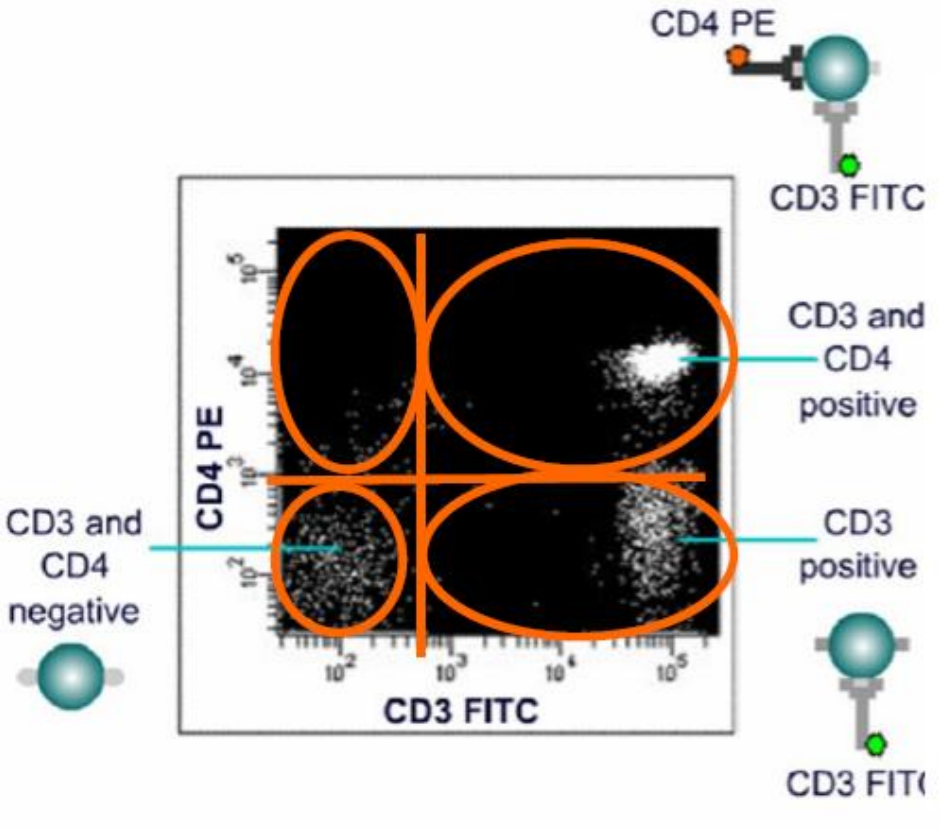


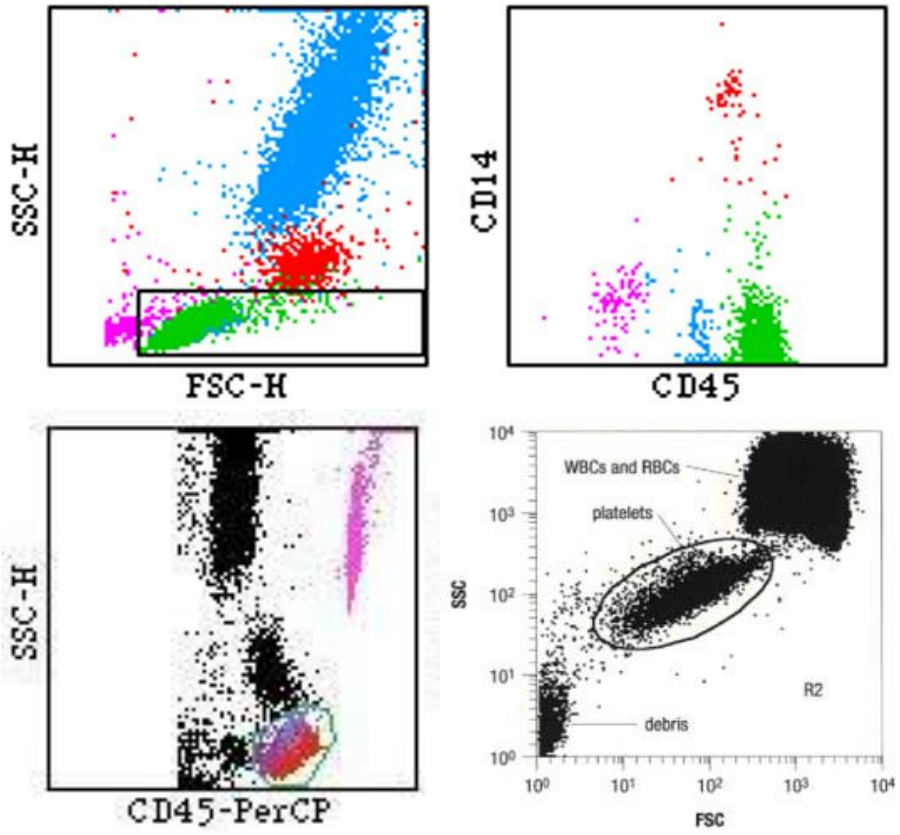


- 荧光峰的高低即在纵轴对应的高度反映了具有此种荧光强度的细胞在样本中所占比例的相对多少，而非绝对数目

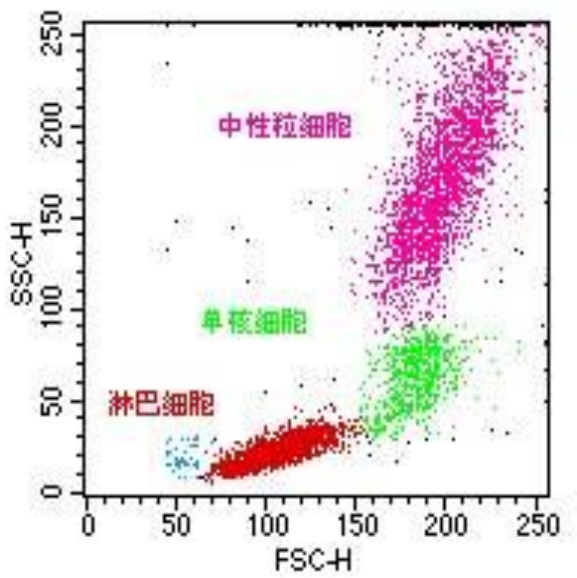
双参数散点图分析

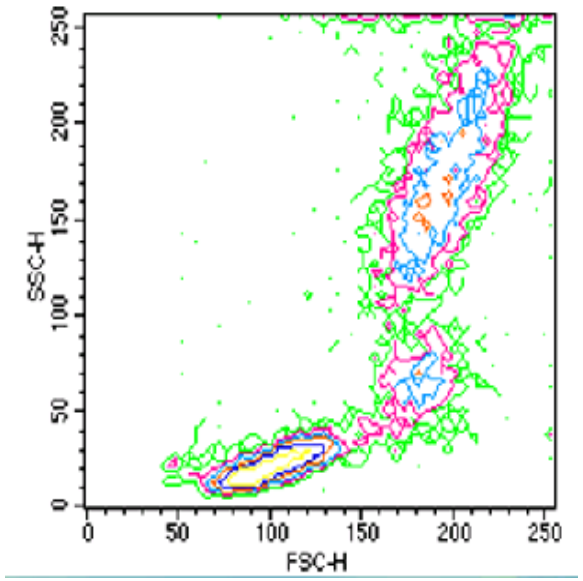


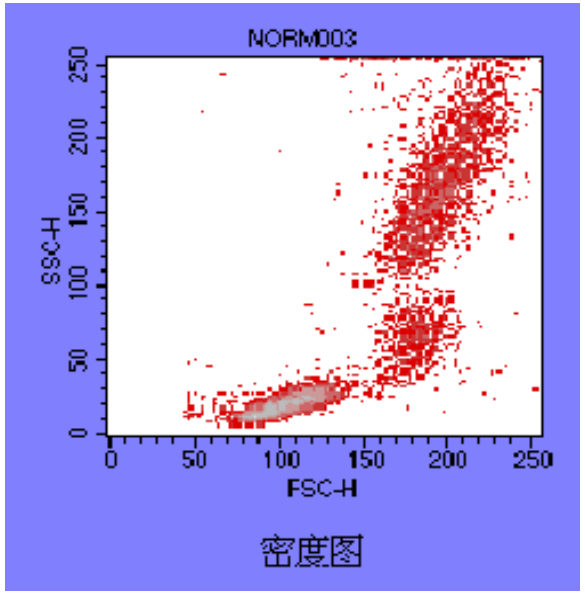

CD4 PE
CD3 FITC
CD3 and
CD4
positive
CD3 and
CD4
negative
CD3
positive
CD4 PE
CD3 FITC
CD3 FITC


SSC-H
FSC-H
CD14
CD45
SSC-H
CD45-PerCP
WBCs and RBCs
platelets
debris
R2
SSC
FSC

等高图和密度图

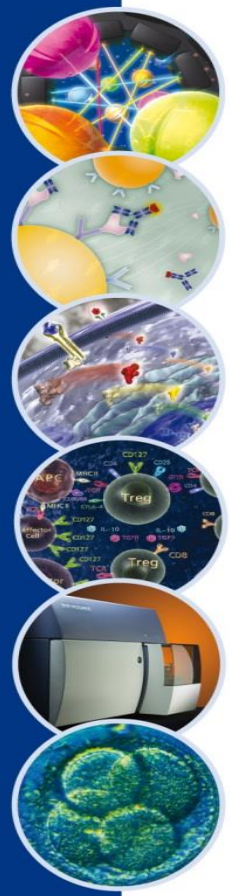

中性粒细胞
单核细胞
淋巴细胞
SSC-H
FSC-H
0
50
100
150
200
250


SSC-H
FSC-H
0
50
100
150
200
250


NORM003
SSC-H
FSC-H
0
50
100
150
200
250
密度图

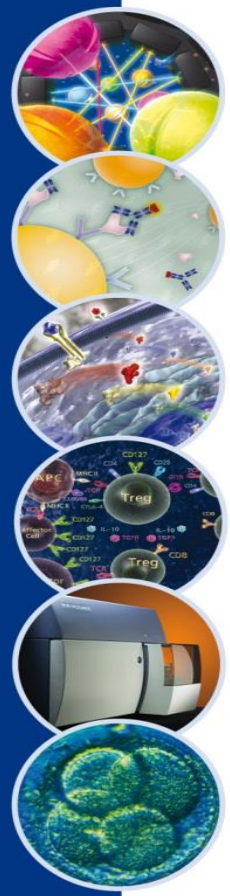
内容：

- 1、流式基本原理介绍
  - 1.1、 基本概念
- 2、多色流式实验
  - 2.1、实验设计
  - 2.2、多色流式实验配色
  - 2.3、样本处理



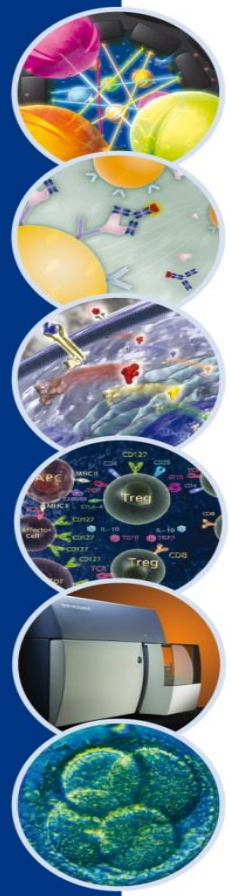
多色组合原则

- 按照机器设置
- 染料的亮度与抗原表达相匹配
- 同种细胞的Marker染料重叠最小化
- 尽量避免偶联染料使用带来的假阳性
- 如果可能，用红激光做自发荧光高的样本。



染料选择-按照机器设置

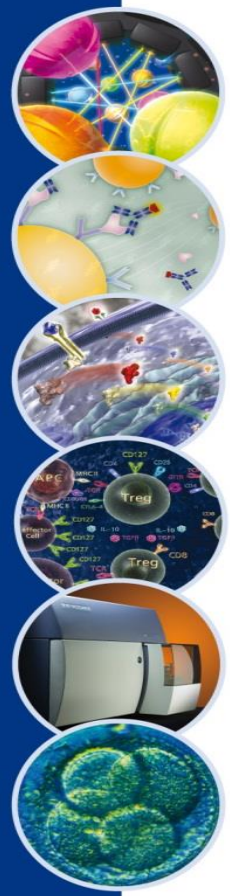
6-color	8-color	Additional
FITC or Alexa 488	FITC or Alexa 488	FITC or Alexa 488
PE	PE	PE
	PE-CF594 or PE-Texas Red or PE-Alexa 610/594	PE-CF594 or PE-Texas Red or PE-Alexa 610/594
PerCP/PE-Cy5/PerCP-Cy5.5	PerCP/PE-Cy5/PerCP-Cy5.5	PerCP/PerCP-Cy5.5/PE-Cy5/PE-Cy5.5
PE-Cy7	PE-Cy7	PE-Cy7
APC or Alexa 647	APC or Alexa 647	APC or Alexa 647
	APC-Cy5.5/Alexa 680 or Alexa 700	APC-Cy5.5/Alexa 680 or Alexa 700
APC-H7/APC-Cy7	APC-H7/APC-Cy7	APC-H7/APC-Cy7
		BV 421
		Horizon V450/V500
		Pacific Orange, Q-dots



2

染料选择

表达强弱-
Antigen Density



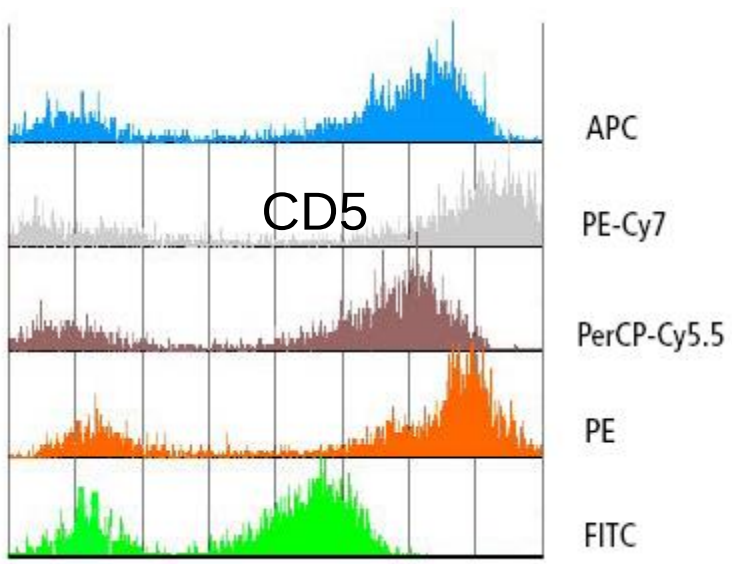
Cell	Antigen	Molecules per Cell
T cell	TCR	100,000
	CD2	55,000
	CD3	124,000
	CD5	90,000
	CD7	20,000
	CD45	>200,000
CD4+ T cell	CD4	100,000
	CD28	20,000
	CCR5	4,000-24,000
CD8+ T cell	CD8	90,000
	CD28	15,000
B cell	CD19	18,000
	CD20	109,000
	CD21	210,000
	CD22	14,000
	HLA-DR	85,000
	CD11a	10,000
	CD40	2,000
	CD86	16,000
	CD80	2,000
Dendritic cell	CD11a	27,000
	CD40	17,000
	CD80	132,000
	CD86	208,000
Monocyte	CD14	110,000
	CD32	21,000
	CD64	13,000
Neutrophil	CD14	3,500
	CD16	225,000
NK cell	CD56	10,000
Red Blood Cell	Glycophorin A	340,000
Basophil	CD23	15,000

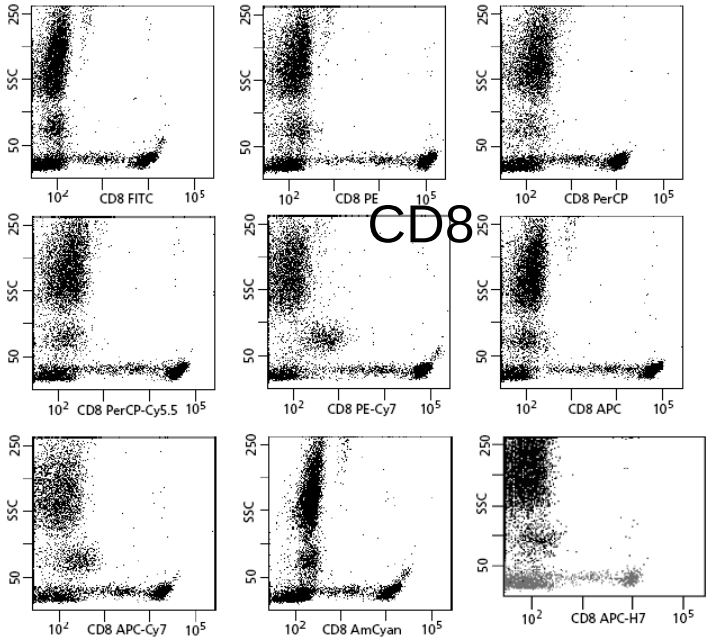
BD

2

染料选择

- 荧光染料的强度

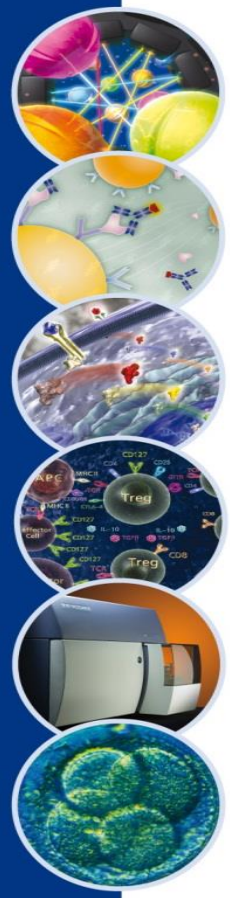




2

Example

高表达的抗体可用不太亮的染料，低/弱表达的Marker用亮的染料



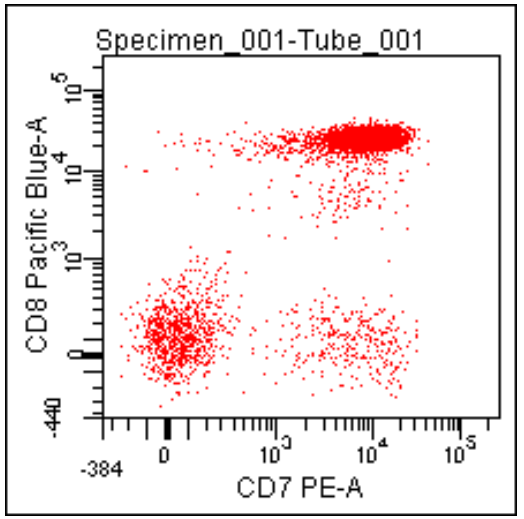
Example:

CD8 “bright” ⟶ Pacific Blue

CD7 “less bright” ⟶ PE

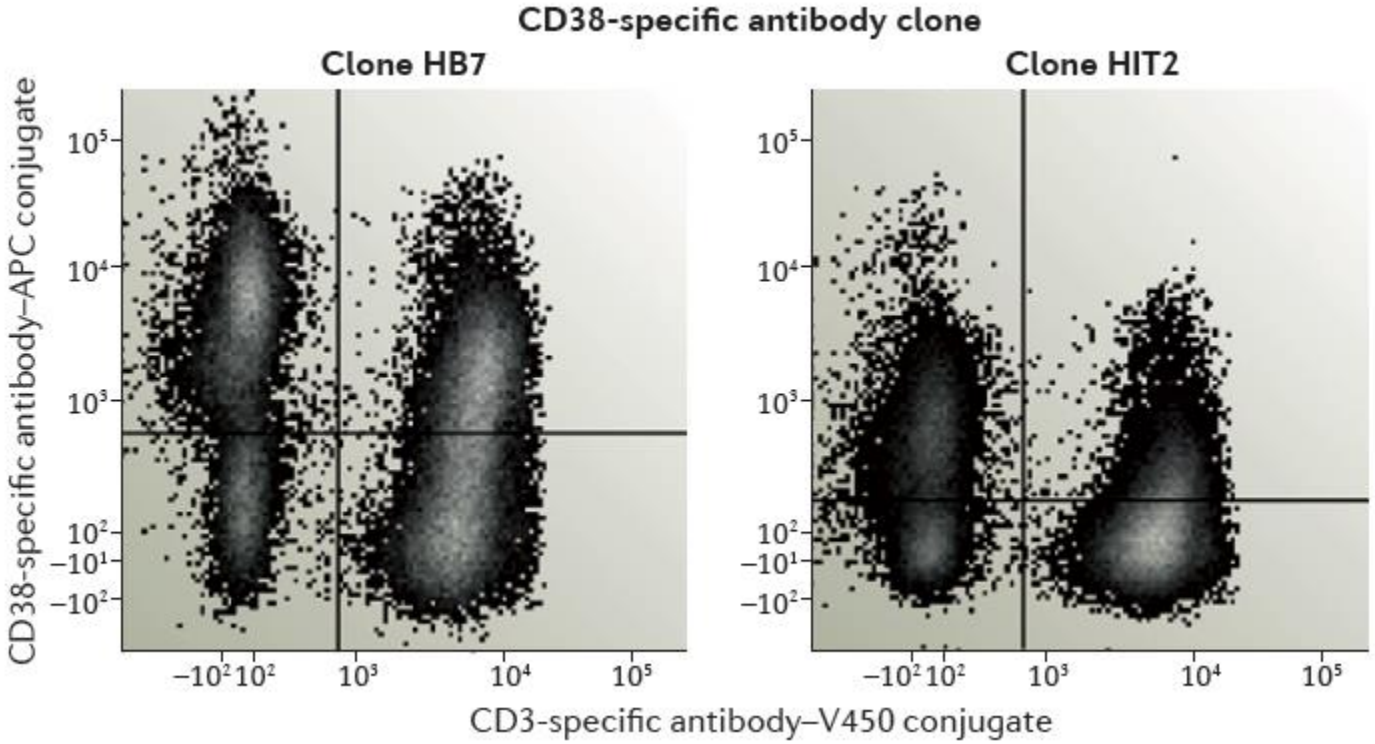
CD8 = 90K molecules/cell

CD7 = 20K molecules/cell



BD

The importance of antibody choice



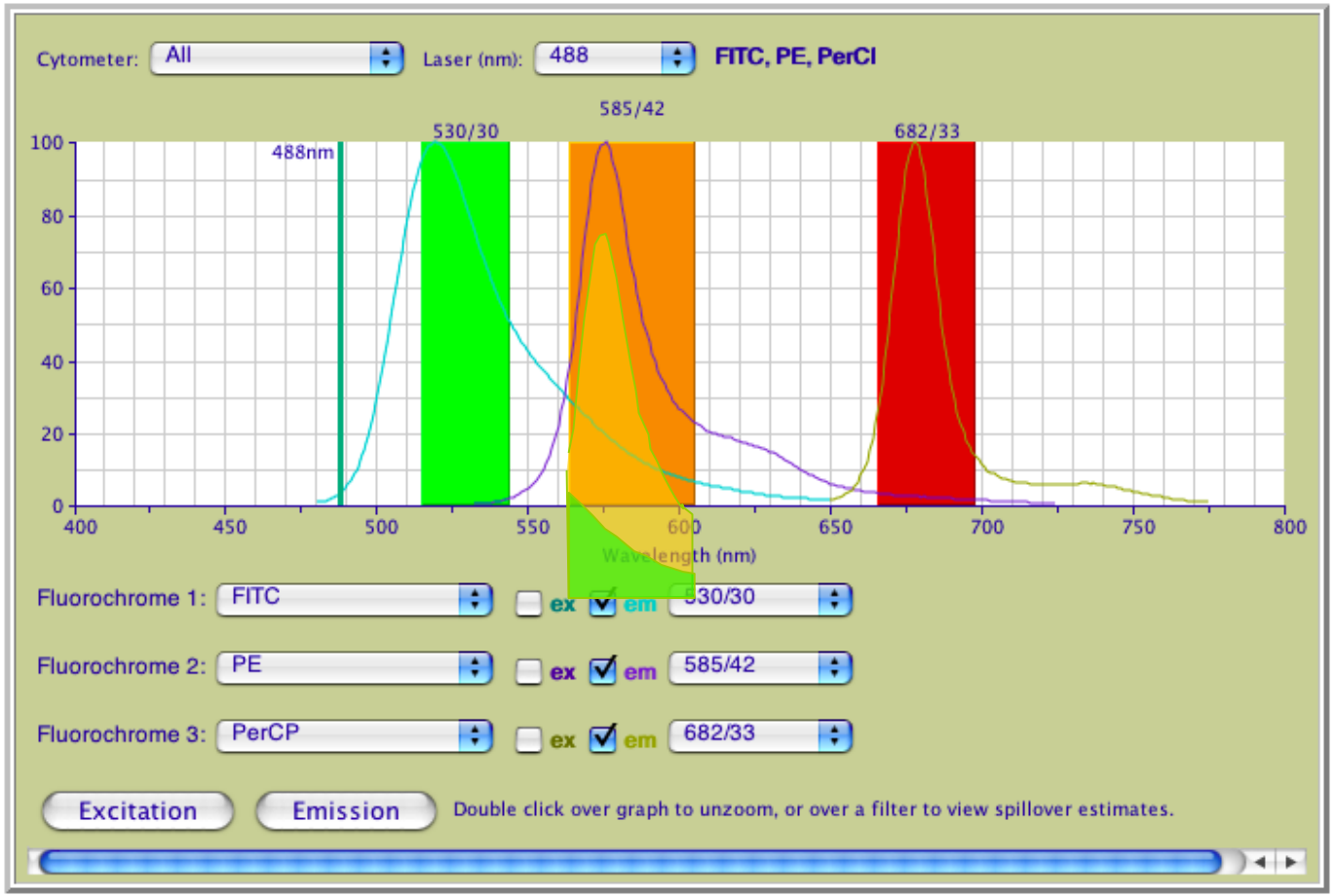
The staining patterns of two commercially available clones of human CD38-specific antibody are very different, despite the fact that both antibodies were conjugated to allophycocyanin (APC) by the same vendor, and were used to stain peripheral blood mononuclear cells (PBMCs) from the same healthy subject under identical conditions. V450, violet 450. Data courtesy of Angelique Biancotto, National Heart, Lung and Blood Institute, USA.

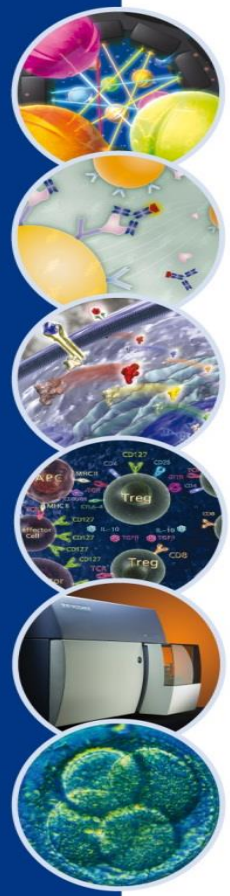
Nat Rev Immunol, 2012

3

荧光染料光谱重叠最小化

spectra viewer

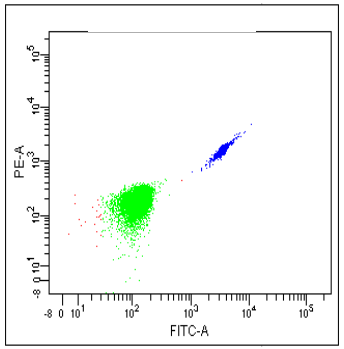




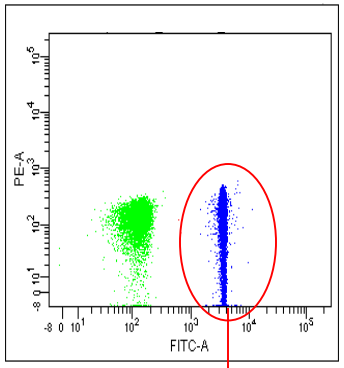
BD

光谱重叠会导致数据丢失

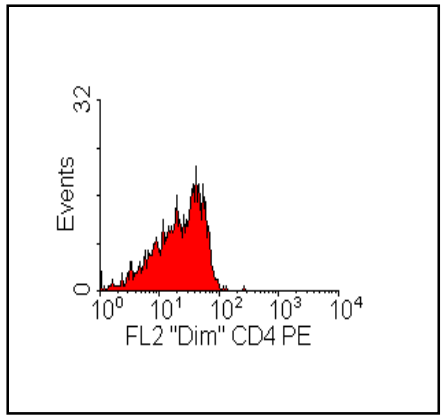
Uncompensated

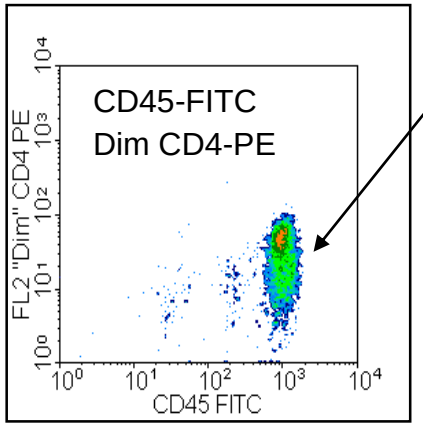


Compensated

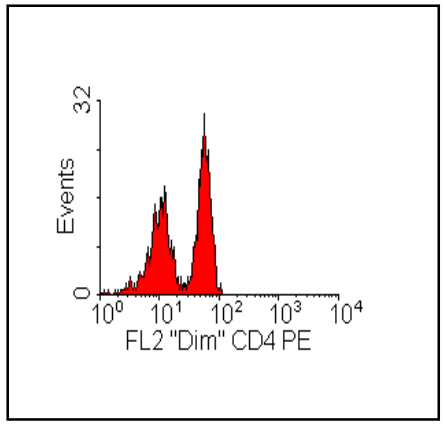


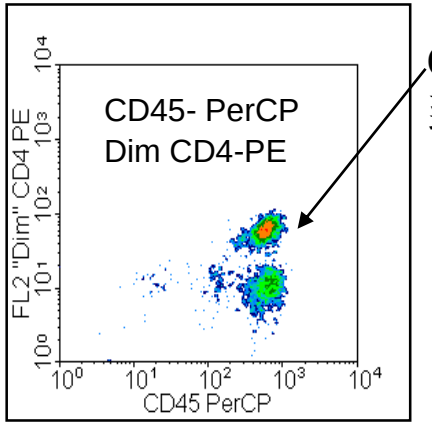
data spread due to spillover





CD45 FITC 漏到 PE， CD4 PE 弱信号分不开



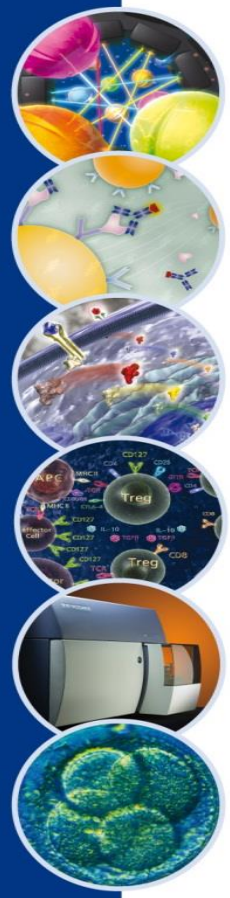


CD45 PerCP 不漏到 PE， 弱 CD4 可以分开

3

采用多激光激发减少重叠

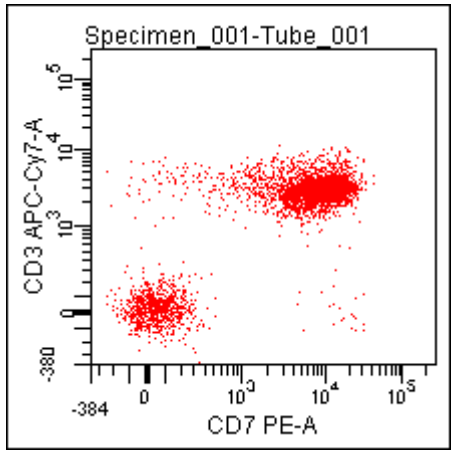
同一细胞的抗原, 用不同激光激发减少重叠



Example:
CD3 “bright” APC-Cy7
CD7 “less bright” PE

Both antigens expressed on same cell, low spillover of CD3 into CD7 and vice versa.

CD3 = 124K molecules/cell
CD7 = 20K molecules/cell



BD

4

荧光染料选择

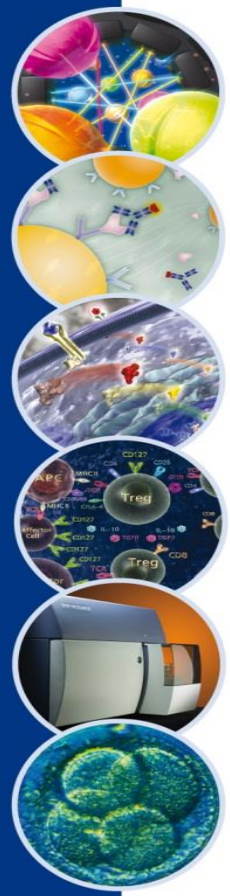
- 避免染料和其衍生物（ tandem-dye）在同一细胞上标记，或者选用更稳定的 tandem-dye

Antigen	Fluorochrome
CD80	PE
CD56	PE-Cy™7
CD19	APC
CD3	BD Horizon™ V450
CD4	PerCP-Cy™5.5
CD8	FITC
CD14	BD™ APC-cy7
CD45	BD Horizon™V500

不同细胞

Antigen	Fluorochrome
CD80	PE
CD56	PE-Cy™7
CD19	APC
CD3	BD Horizon™ V450
CD4	PerCP-Cy™5.5
CD8	FITC
CD14	BD™ APC-cy7
CD45	BD Horizon™ V500

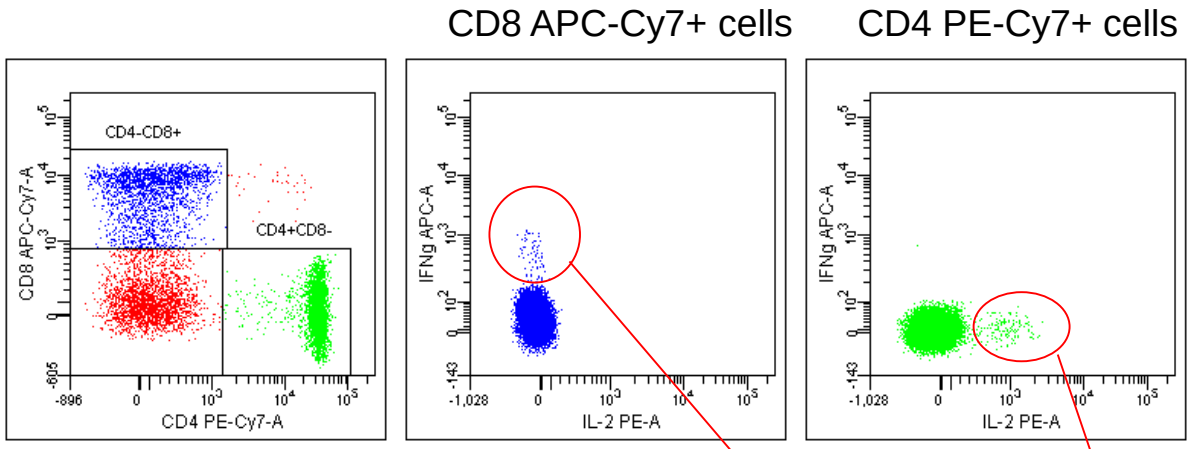
不同细胞



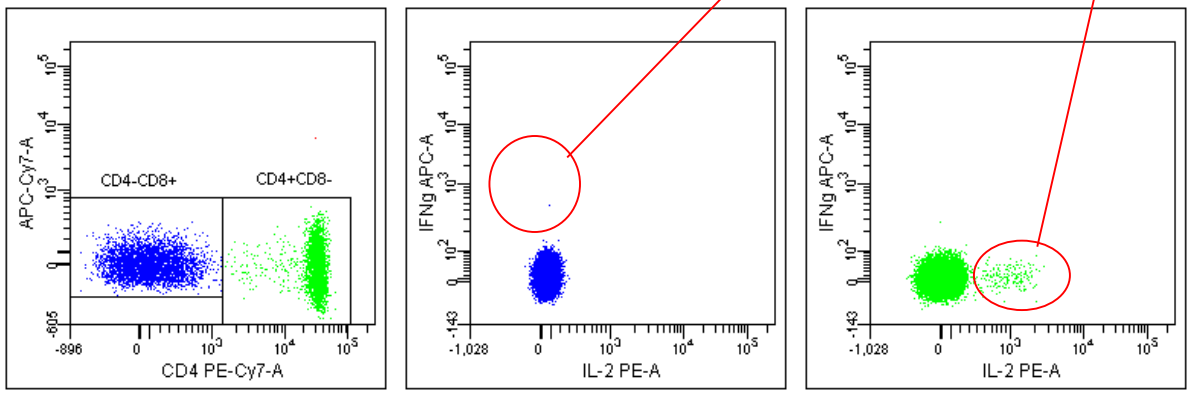
BD

由于 tandem-dye 降解会产生加阳性

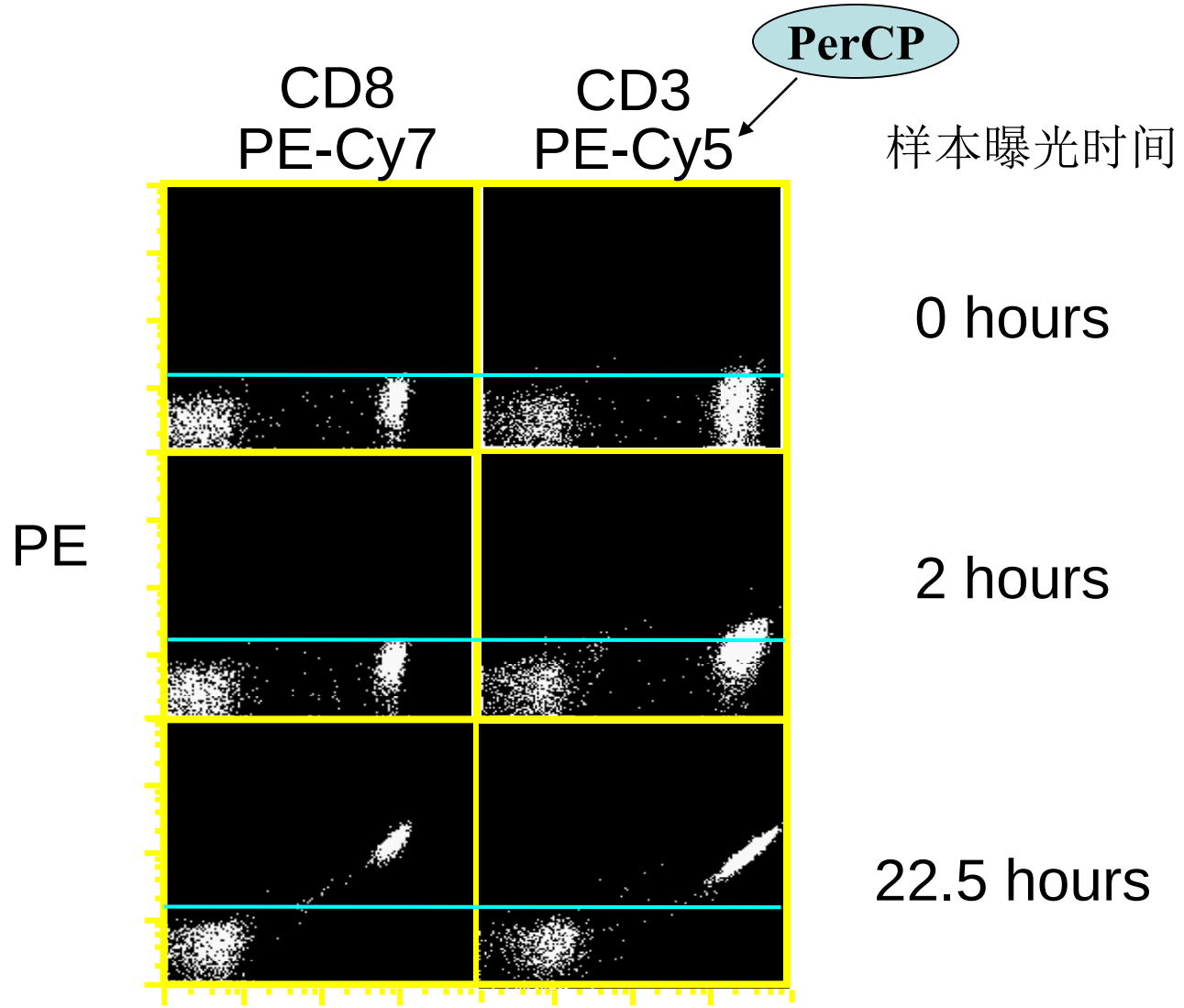
A. With CD8 APC-Cy7 and CD4 PE-Cy7



B. Without CD8 APC-Cy7



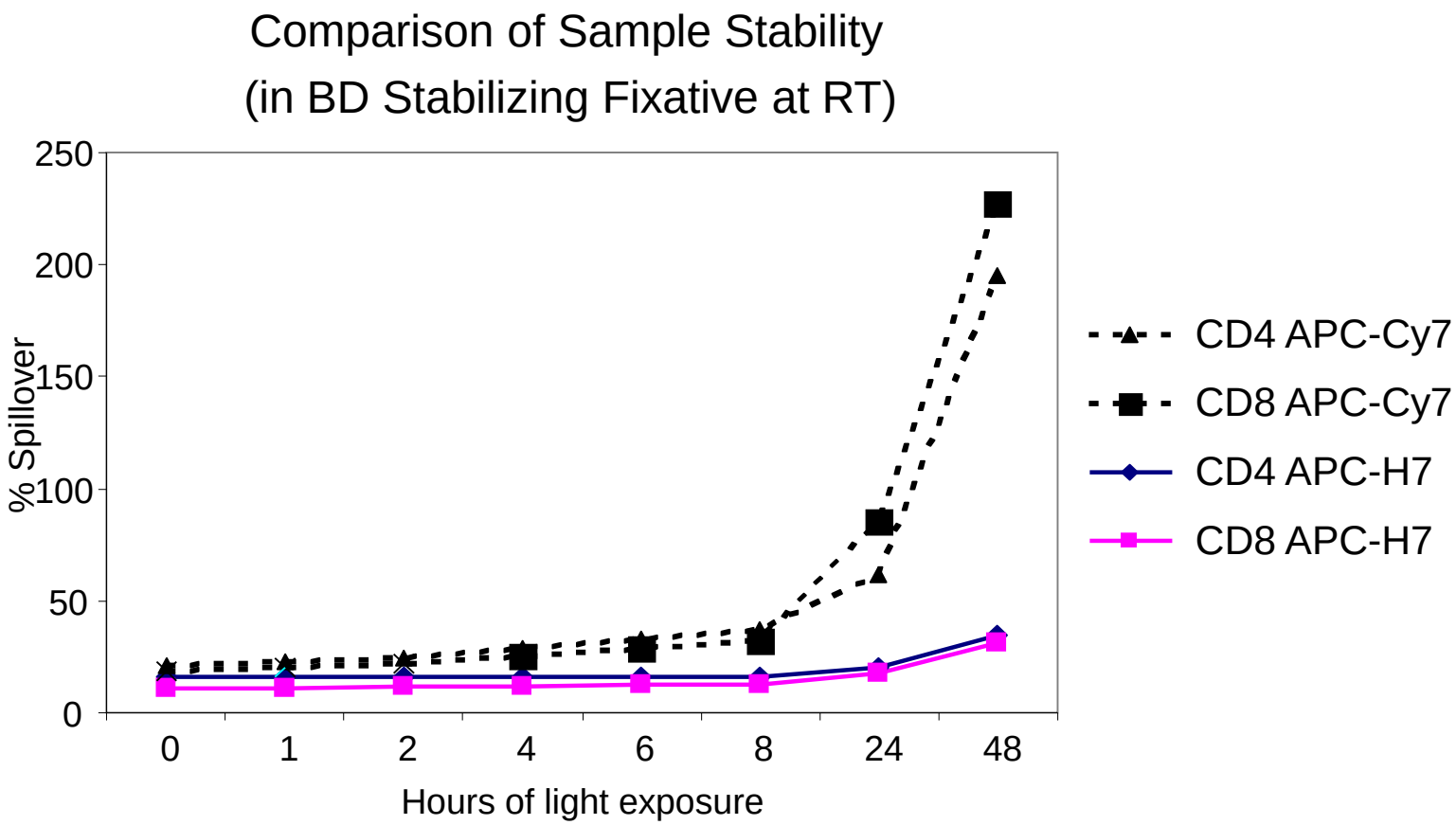
补偿: Tandems

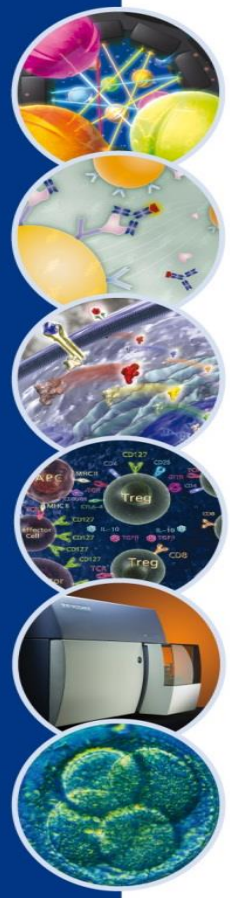


4

New Tandems Are More Stable

APC-H7 to replace APC-Cy7:





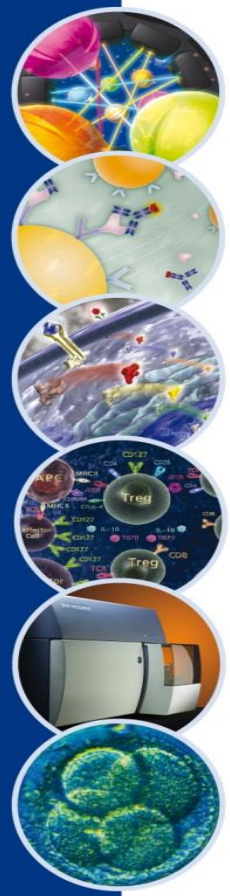
BD

5

染料选择

- 自发荧光多的选择用红激光

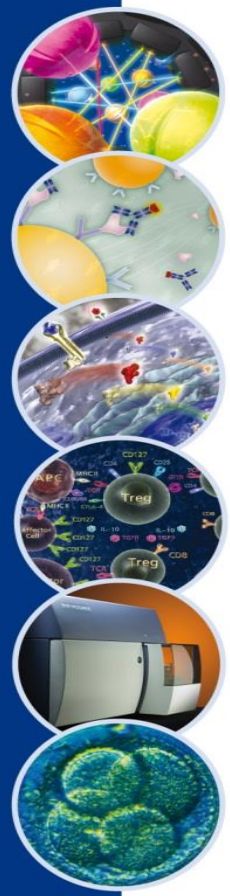
Antigen	Fluorochrome
CD80	PE
CD56	PE-Cy™7
CD19	APC
CD3	BD Horizon™ V450
CD4	PerCP-Cy™5.5
CD8	FITC
CD14	BD™ APC-H7 → Perfect!
CD45	BD Horizon™V500



BD

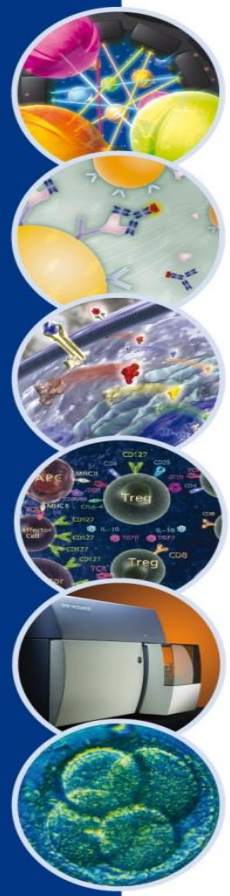
内容：

- 1、流式基本原理介绍
  - 1.1、 基本概念
- 2、多色流式实验
  - 2.1、实验设计
  - 2.2、多色流式实验配色
  - 2.3、样本处理



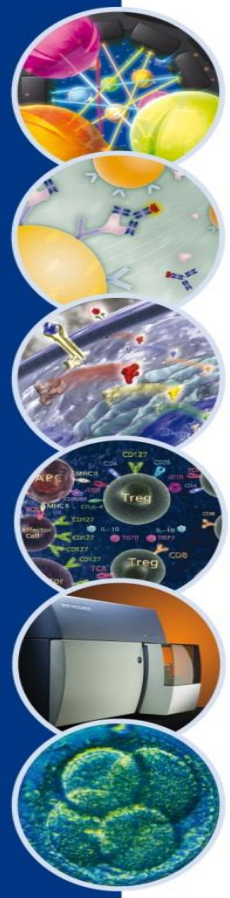
流式检测的样本

- 可检测的样本种类多样：
  - 各种细胞（如外周血，骨髓，细针穿刺，灌洗液，实体组织，悬浮或贴壁培养的细胞），微生物，人工合成微球等
  - New!血清、血浆、培养上清、细胞裂解液
- 样本制备：
  - 单细胞悬液（天然，机械研磨/消化）
  - **5×10^5～1×10^{6}cells/ml，约0.5～1ml，300目筛网过滤**

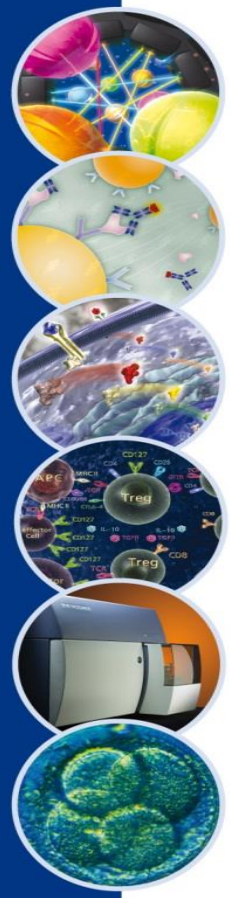


样本处理

- 单细胞悬液的制备
  - 血液样本
  - 组织



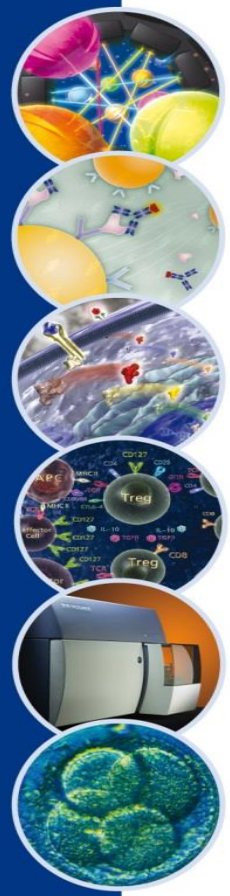
血液样本



- 处理过程可能导致目的细胞的丢失，推荐流式分析样本尽可能与处理前的样本接近
- 一、溶解红细胞：对标本影响最小的方法
  - 可在单抗标记之前、之后进行
  - 如果先溶血再标记，需要注意的是
    - 抗原不受溶血素的影响
    - 彻底去除溶血素，细胞充分洗涤，抗体结合不受影响
    - 所用的溶血素不含固定剂，因为固定剂会改变细胞的活力，从而影响表面标记的结果
  - 溶血素选择标准
    - 仅溶解成熟红细胞
    - 对标本中其它细胞影响最低

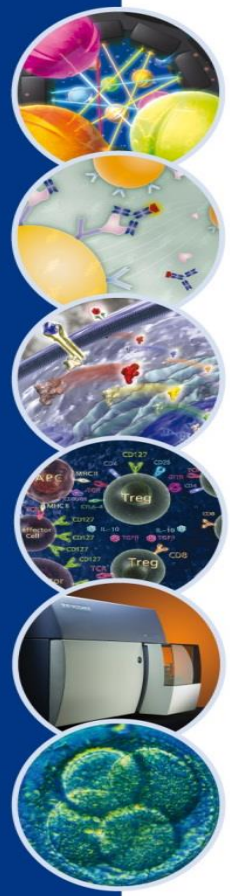
血液样本

- 二、PBMC
  - 密度梯度离心
    - 可去除标本中的死细胞
    - 可能造成重要细胞群体的选择性丢失



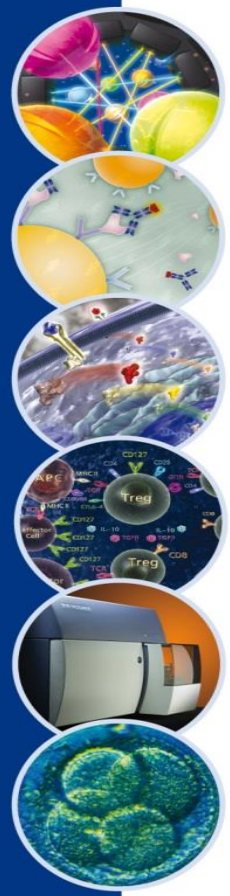
组织

- 获得单细胞并保证得率及细胞结果和抗原的完整性
- 机械法：温和
- 酶消化法：细胞之间粘附牢固或组织干扰细胞分离
  - 避免破坏抗原分子及细胞活力



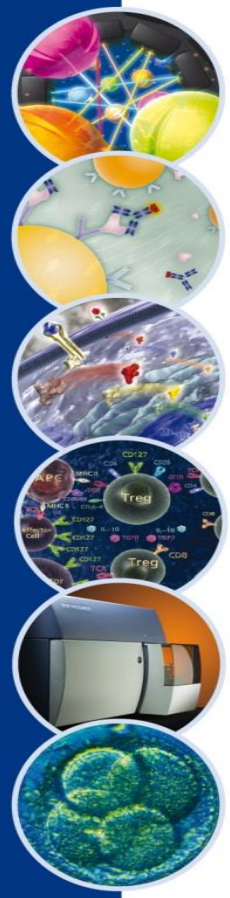
样本保存

- 短期保存（＜1小时），所有样本放于室温（16-28ºC）
- 非液体的样本，要加足量的无菌等渗液，以免样本脱水
- 长期保存，血液和骨髓样本应放于室温，如果可能，严格控制在16ºC 。一般不需要加培养基，有时候，加培养基可能会提高样本的稳定性。
- 样本保存的时间，与样本的性质及保存条件有关。
- 肝素钠抗凝的外周血和骨髓，可保存48-72小时
- EDTA抗凝的外周血和骨髓，可保存12-24小时（对髓系细胞的影响）。
- 单纯标记胞内抗原，固定细胞后可长期保存。但与检测的抗原及染色方法有极大关系。所以需要新鲜样本验证。



补偿调节- BD CompBeads

- 微球上包被有抗Ig的抗体（**Anti-mouse-552843, rat-552844, anti-rat/hamster-552845**）
- 与荧光抗体结合后，有恒定的荧光强度
- 自动/手动形成补偿条件



· 340 · 免 疫 学 杂 志 第 24 卷 第 3 期 2008 年 5 月 IMMUNOLOGICAL JOURNAL Vol. 24 No. 3 May. 2008

[文章编号]1000-8861(2008)03-0340-06

应用 CompBeads 建立流式多色分析补偿的方法

傅晓岚，周 伟，王 晴，杨 翌，谢谆怡，吴玉章*（第三军医大学全军免疫学研究所，重庆 400038）

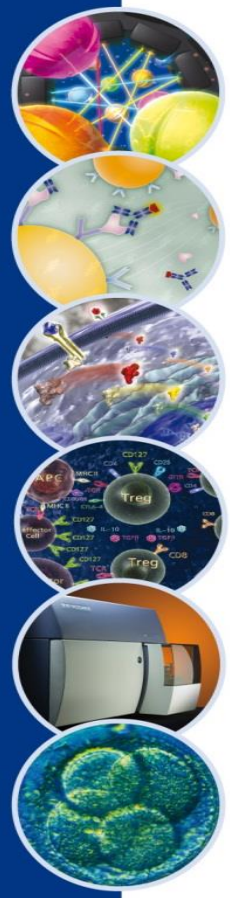
[摘 要] **目的** 探讨一种快速简便的流式细胞仪多色分析时的关键参数（荧光补偿）设置方法。**方法** 以 BD CompBeads、实验样品（本实验为慢性乙型肝炎患者外周全血）分别与单克隆荧光抗体染色，结合流式细胞仪 Diva 软件调节细胞仪光电倍增管（PMT）电压，计算建立荧光补偿矩阵。同时以获得的参数设置仪器，运行慢性乙型肝炎患者外周全血 6 色荧光标记实验样品。**结果** 用 CompBeads 建立的补偿参数稳定可靠，能有效消除荧光光谱之间的交叉重叠。6 色荧光标记实验样品检测的结果，可见细胞亚群分布清晰，荧光强度比例表达适当，与通常采用的实验样品单染色方法补偿结果一致（$P > 0.05$）。**结论** 该方法能快速方便进行流式细胞仪检测时仪器参数最佳化设置调整，在多色免疫标记流式检测中有较大的应用价值。

[关键词] 流式细胞仪；多色；补偿

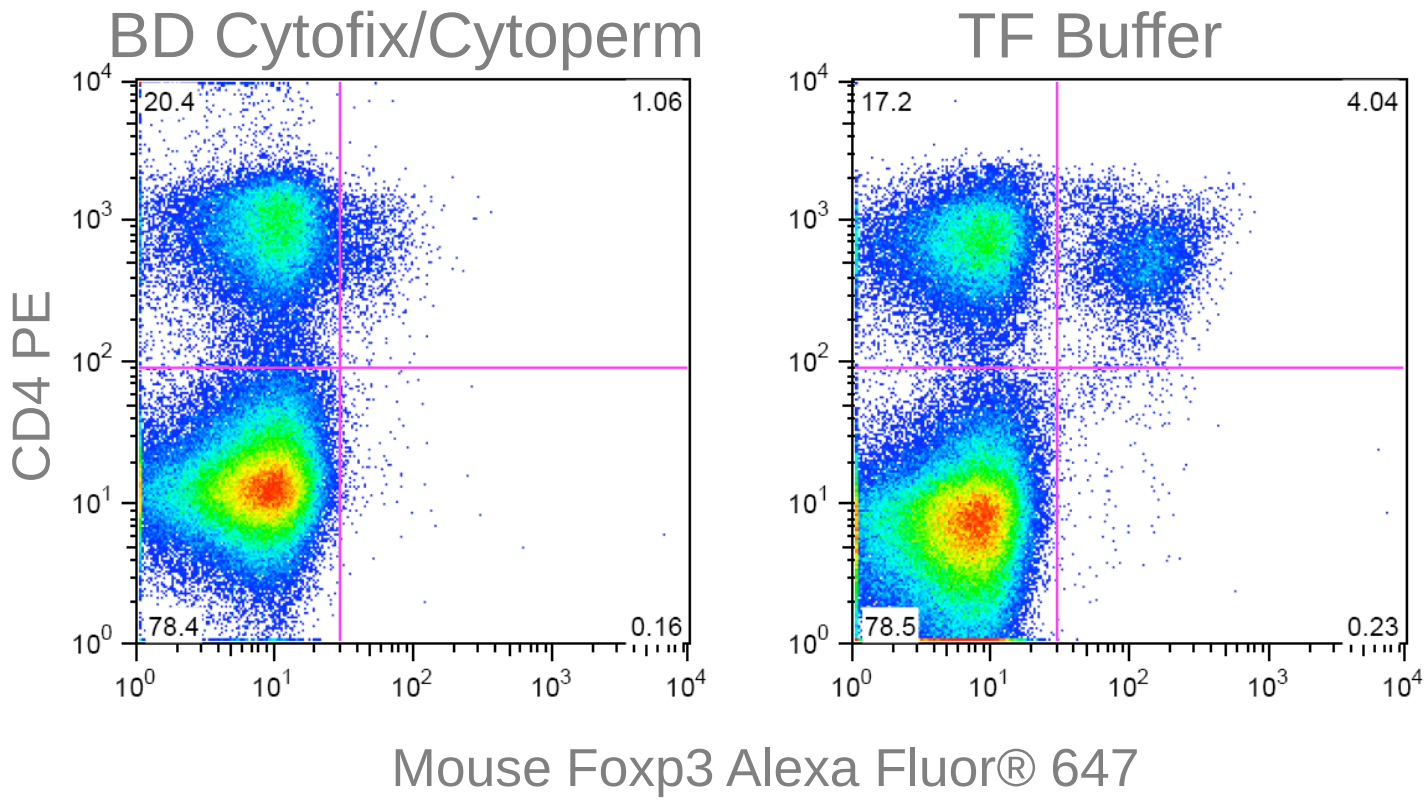
[中图分类号] R392－33 [文献标识码] A

Buffer 选择

- Fixation buffer
- BD Cytofix/Cytoperm™ and BD™ Perm/Wash buffer
- BD Pharmingen™ TF buffer set
- BD™ Phosflow Perm Buffer II
- BD™ Phosflow Perm Buffer III

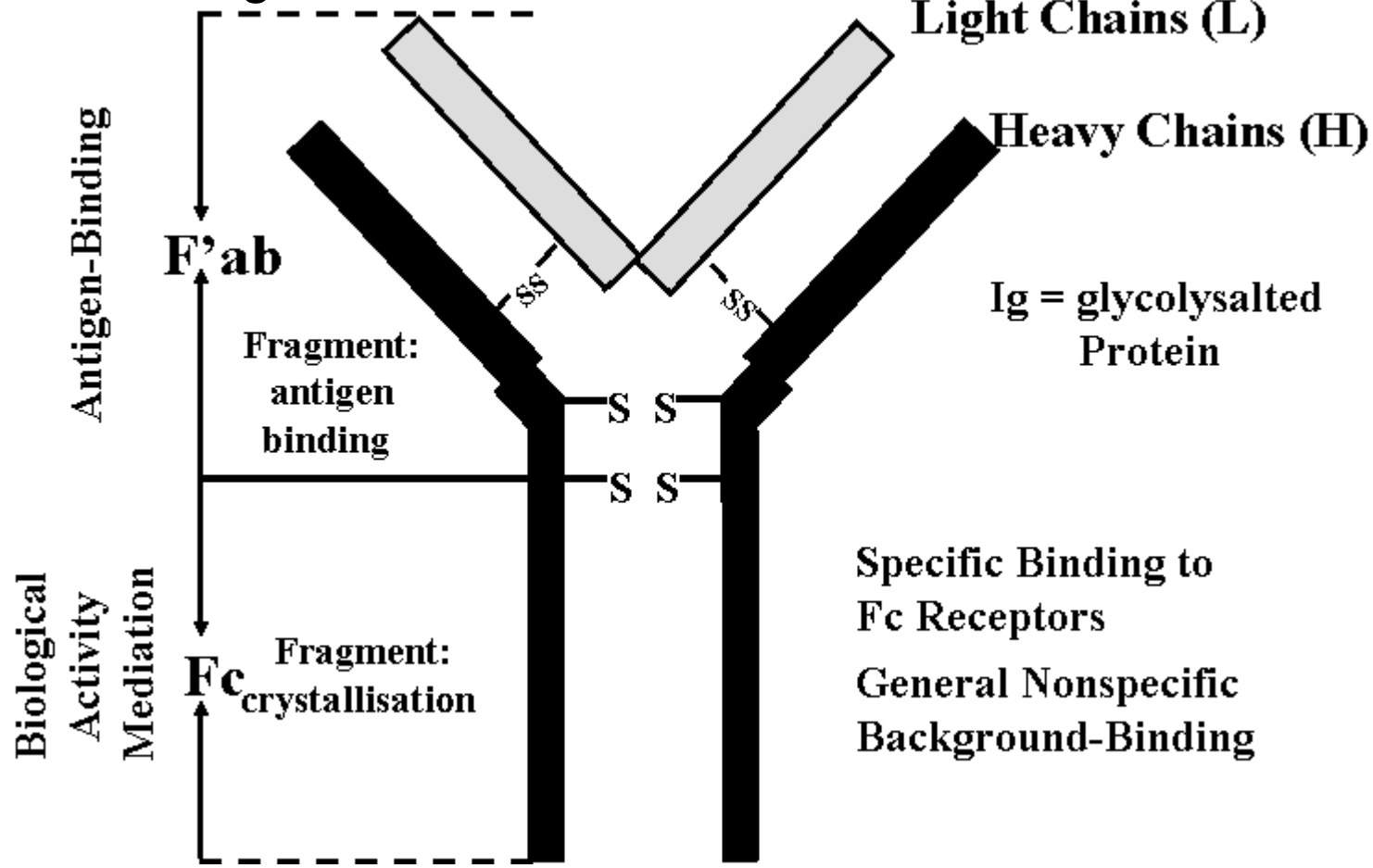


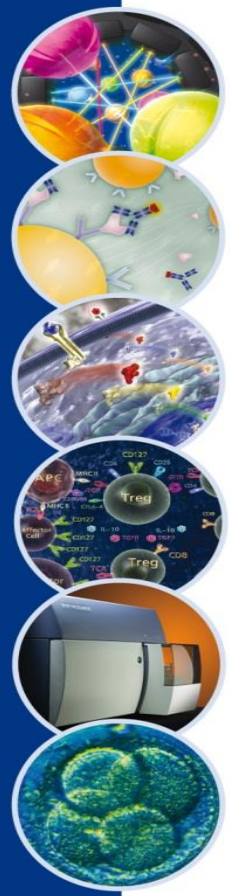
Effect of BD Cytofix/Cytoperm Buffer on Mouse Foxp3 Staining



背景处理

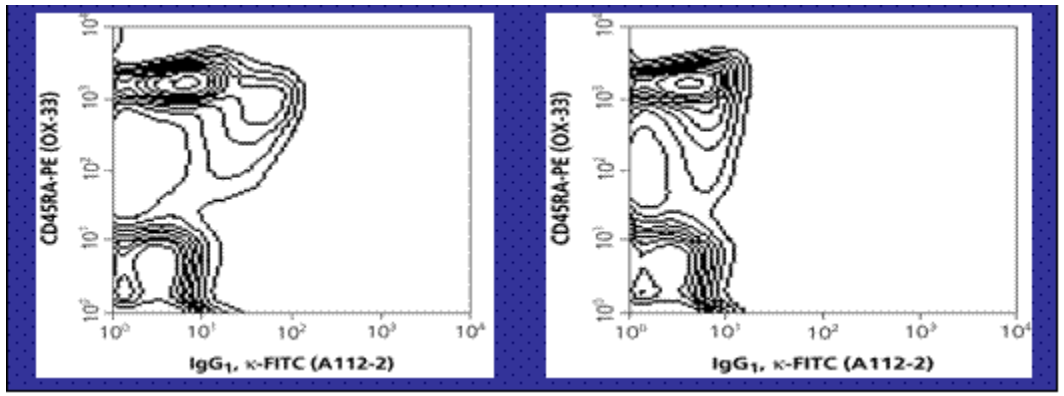
The Immunoglobulin





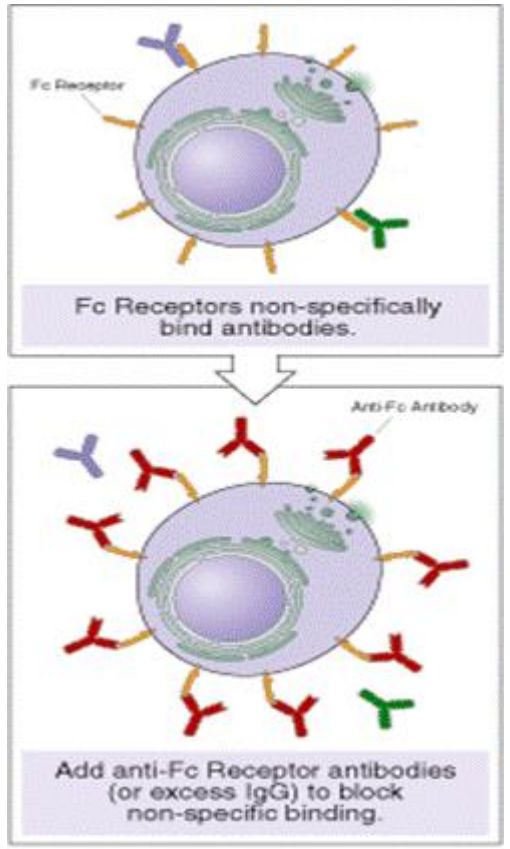
背景处理-Blocking

- FcBlock
  - Mouse FcBlock, purified CD16/32 cat # 553141/553142
    - **Reduces Background Staining**

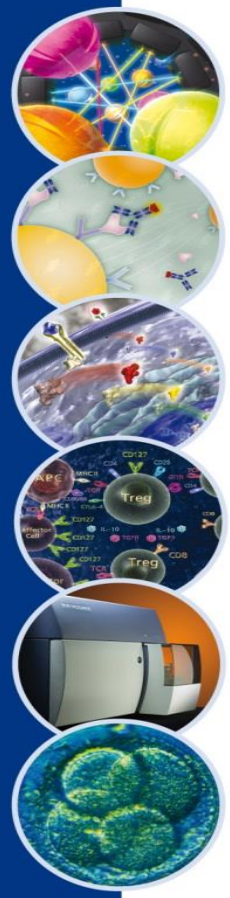


No pre-inc. FcBlock

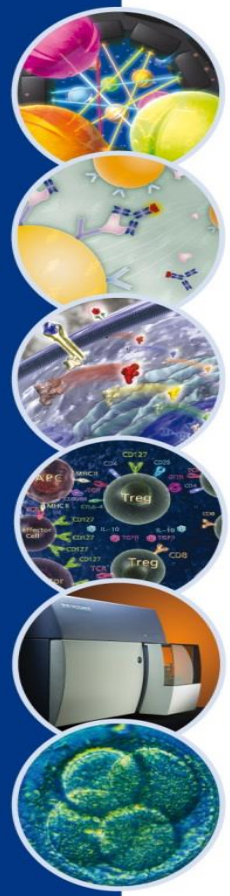
Pre-inc. FcBlock



网上应用工具：http://www.bdbiosciences.com



流式细胞术工具



Selection

We've put together a variety of tools and information to help you design your next multicolor assay.

Fluorescence Spectrum Viewer

Find fluorochromes for a multicolor experiment based on flow cytometer and compensation trade-offs.

Launch the Spectrum Viewer »

Absorption and Emission Spectra

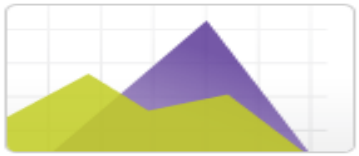
View the range of emission and learn more about each fluorochrome in the BD product line.

See Absorption & Emission Spectra »

BD FACSelect™ Buffer Compatibility Resource

Navigate buffer choices to select the right combination for your intracellular and surface marker experiments.

Use Buffer Compatibility Resource »



Fluorochrome Specifications Chart and Poster

Fluorochrome Specifications Chart »

Fluorochrome/Laser Reference Poster »

BD FACSelect™ Multicolor Panel Designer

Build a panel of reagents based on the species, specificities or clones, and colors of interest.

Use the Panel Designer »

Multicolor Antibody Reagents Catalog

Choose from our extensive portfolio of high-quality fluorescent-conjugated reagents to build your multicolor panels.

Download the Multicolor Catalog »

Example Human and Mouse Panels

Human Panels »

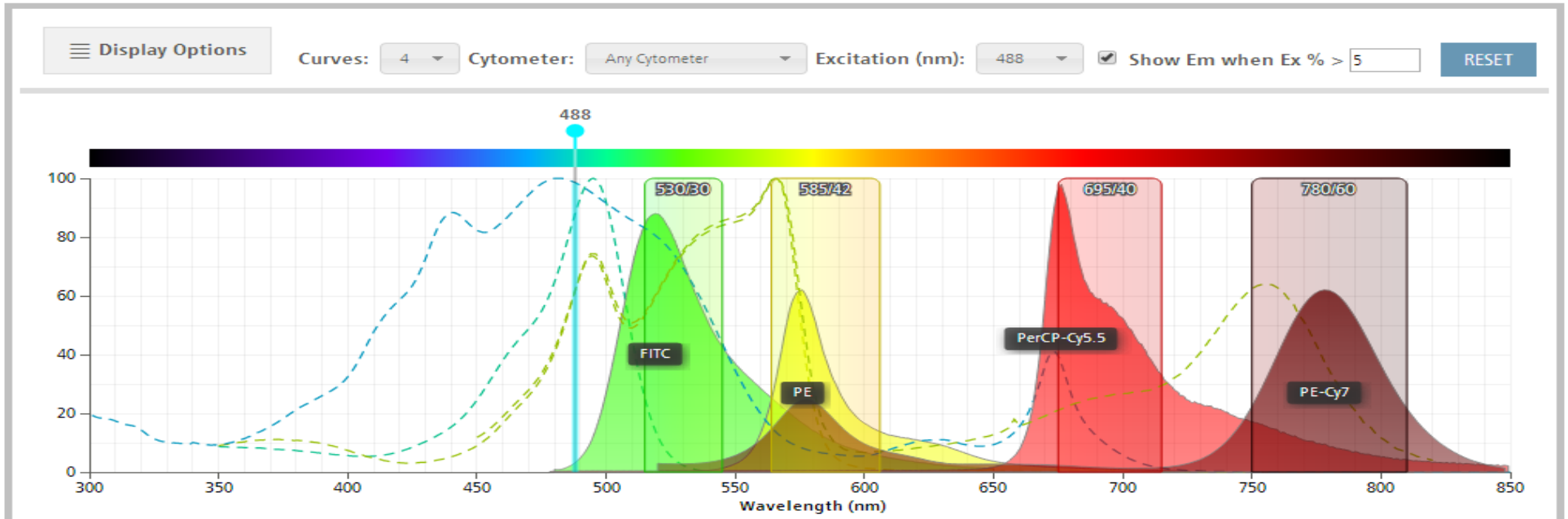
Human Small Size Panels »

Mouse Panels »

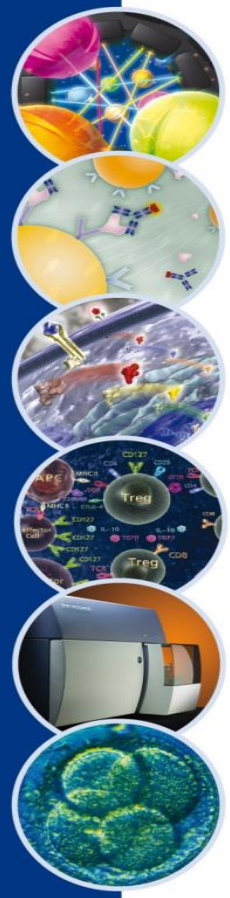
Mouse Small Size Panels »

光谱查看

BD FLUORESCENCE SPECTRUM VIEWER



	Fluorochrome	%Max	Ex	Em	Filters	FITC	PE	PerCP...	PE-Cy7
1	FITC	88.0	✓	✓	530/30	47.4%	0.4%	0.3%	1.8%
2	PE	61.6	✓	✓	585/42	12.5%	70.4%	0.5%	16.4%
3	PerCP-Cy5.5	98.4	✓	✓	695/40	x	2.0%	48.4%	1.4%
4	PE-Cy7	61.8	✓	✓	780/60	x	x	11.9%	60.5%



Thanks!

胡启萍

18030601919

Qiping.hu@bd.com