

## Cytometry and Fluorescence Activated Cell Sorter (Lecture in English)

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フローサイトメーターは個々の細胞の分子の発現を半定量的に測定できる装置である。装置は解析専用のアナライザーと解析分取が可能なセルソーターの2機種があり、分子の発現の同定には蛍光色素で認識された抗体がよく用いられる。更に標識抗体以外の蛍光物質を測定する事も試みられている。

今回は実習として、機器更正用ビーズを用いて、FACSにて検出する。

### ◎講義内容

FACSの基本原理を紹介し、FACSを用いた実験手技については具体例を挙げながら説明します。

Flow cytometer is a device that can semi-quantitatively measure the expression of individual cell molecules. There are two types of devices, an analyzer and a cell sorter. Antibodies recognized by fluorescent dyes are often used to identify the expression of molecules. It has also been attempted to measure fluorescent substances other than labeled antibodies.

As a practical training, we detected with FACS using beads for flow cytometer setup.

\* FACS (fluorescence activated cell sorter) is a trademark of Becton, Dickinson and company.

### ◎Lecture content

I will introduce the basic principle of FACS and explain the experimental technique using FACS with concrete examples.



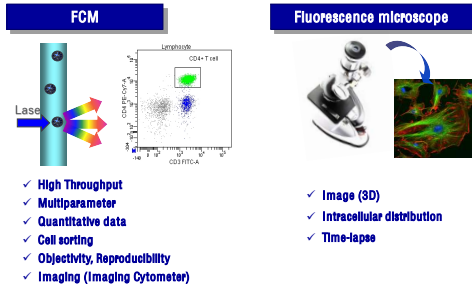
## BD FACS™ series



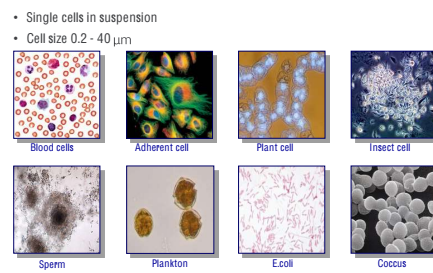
## Central Research Laboratory : Shiga University of Medical Science



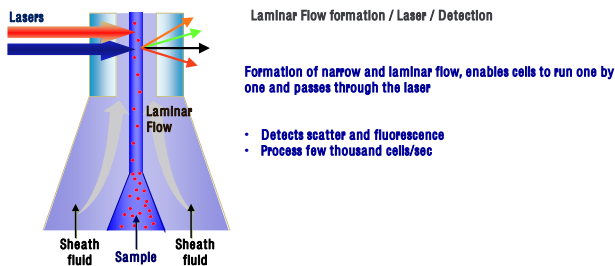
## Character of FCM



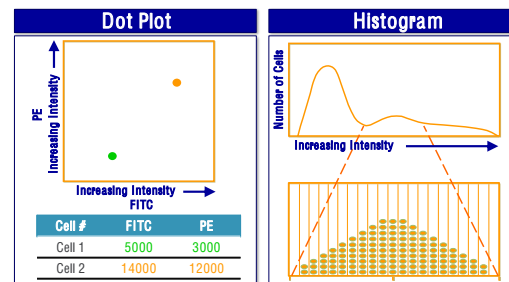
## Measurable sample with FCM



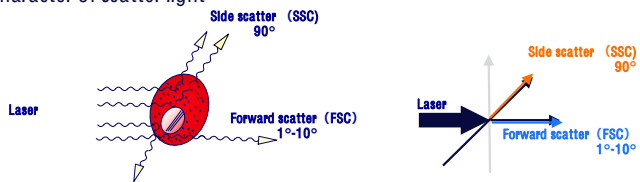
## Detection of FCM



## Data Display : Dot Plot and Histogram

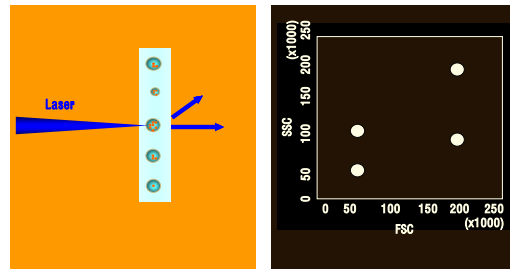


### Character of scatter light

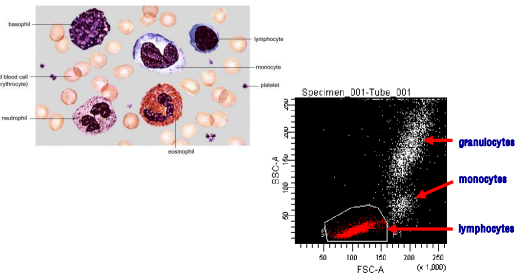


- **Forward Scatter (FSC)**  
Indicates the size of cells, surface area
- **Side Scatter (SSC)**  
Indicates the complexity of internal structure, cellular granule

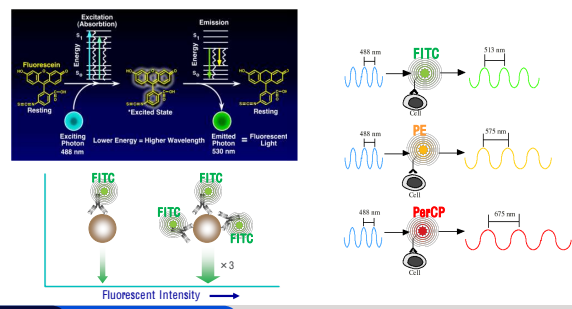
### Scatter light signal



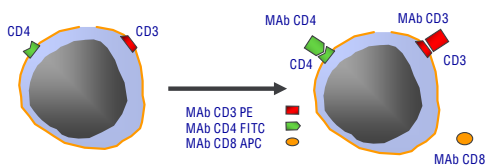
### Scatter light analysis of blood cells



### Character of fluorescence

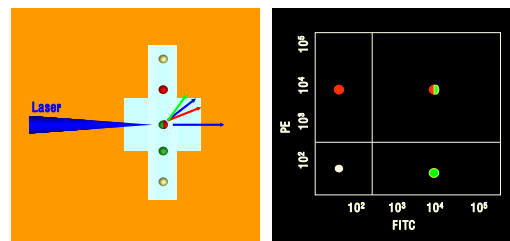


### Fluorescent staining with monoclonal antibody

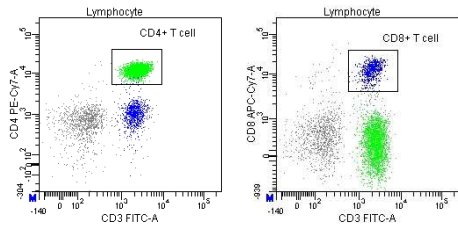


CD # = cluster of differentiation  
MAB = monoclonal antibody

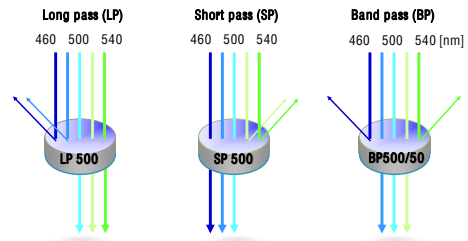
### Fluorescent signal



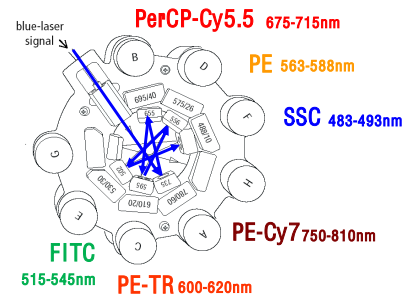
## Fluorescent signal analysis



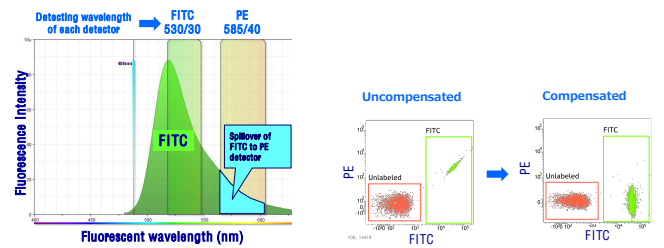
## Mechanism of fluorescence detector



## Fluorescence detector of FCM



## Requires compensation in multi-color analysis



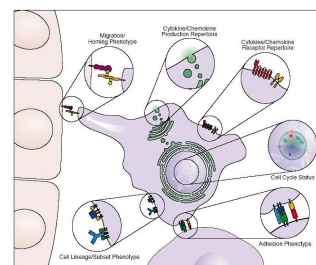
## Today's Contents

### Introduction to Flow Cytometry (FCM)

1. Basic of FCM
2. Application for FCM

## Applications for FCM

- ① Cell surface antigen
- ② Intracellular antigen
- ③ Fluorescent protein
- ④ Cell cycle
- ⑤ Cell proliferation
- ⑥ Apoptosis
- ⑦ Cytokine



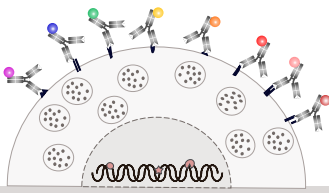
## ① Analysis of cell surface antigen

### Major cell surface antigen

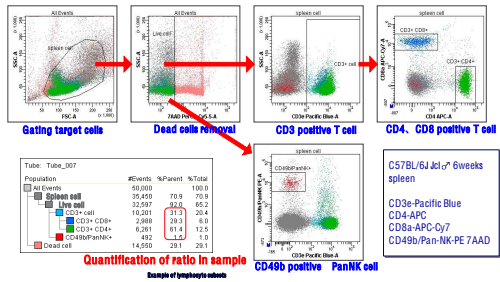
- ✓ Cellular membrane composing protein
- ✓ receptors
- ✓ Adhesion molecules
- ✓ Transporters

### Cluster of Differentiation (CD classify)

International classification of monoclonal antibodies used for cell surface antigen



## Analysis of cell surface antigen



## BD Lyoplate™ Screening Panels

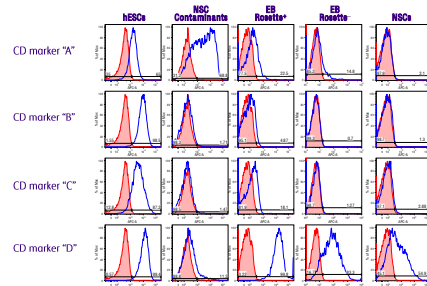
- Multiple (242 human CD markers or 176 mouse CD markers) antibodies are dispensed in 96 well plate
- Profiling cell surface marker in low cost\*
- Antibody is labeled with Alexa Fluor® 647, able to use with GFP expressing cells



\*for FACS analysis, 96well plate auto sampler is required

Product	Contents	Size
BD Lyoplate™ Human Cell Surface Marker Screening Panel Cat. No. 560747	<ul style="list-style-type: none"> <li>• 242 CD markers</li> <li>• Isotype controls</li> <li>• Alexa Fluor® 647 second step</li> </ul>	5 tests
BD Lyoplate™ Mouse Cell Surface Marker Screening Panel Cat. No. 562209	<ul style="list-style-type: none"> <li>• 176 CD markers</li> <li>• Isotype controls</li> <li>• Biotin second step</li> <li>• Alexa Fluor® 647 streptavidin third step</li> </ul>	5 tests

## BD Lyoplate™ Screening Panels



## ② Analysis of intracellular antigen

### Intracellular antigens

- Cytokine
- Transcription factor
- cytoskeleton

Cells fixed and permeabilized makes antibody and reagents possible to access intracellular

### Fixation reagent

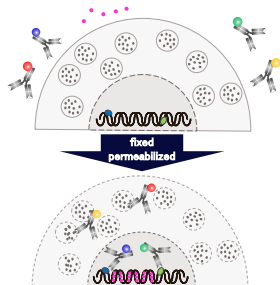
- Ethanol
- Methanol
- Paraformaldehyde

### Permeabilization reagent

- Saponin
- Triton-X

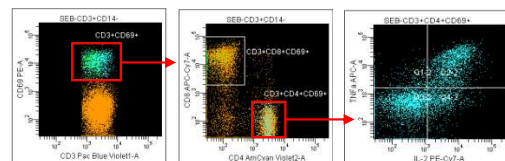
### Nucleole acid staining reagent

- PI
- 7AAD



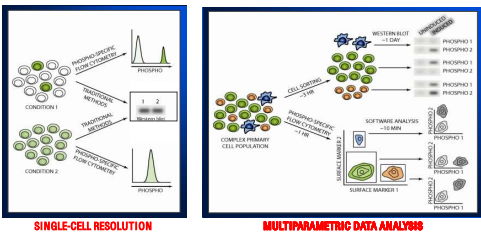
## Cytokine producing cells analysis

- Staining with cell surface antigen, enables phenotype analysis of cytokine producing cells
- Possible to detect cytokine production even from rare cells

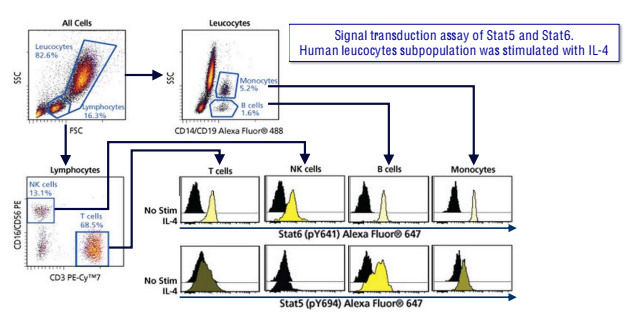


### Protein phosphorylation analysis in single cell

- Protein phosphorylation in single cell level
- Combining the staining of cell membrane markers, allows protein phosphorylation analysis of specific cells in heterogeneous cell population
- Easy, short time and less cell sample compared to Western Blot analysis

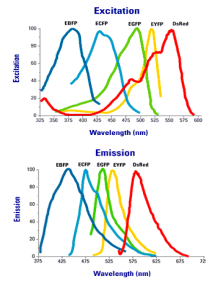


### Protein phosphorylation analysis in single cell

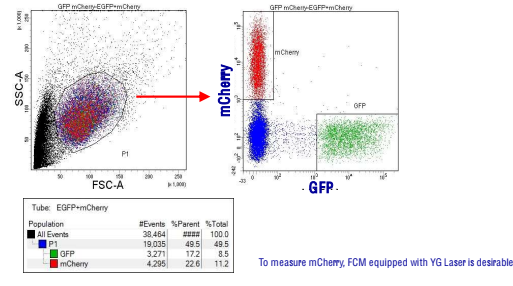


### ③ Expression of fluorescent protein

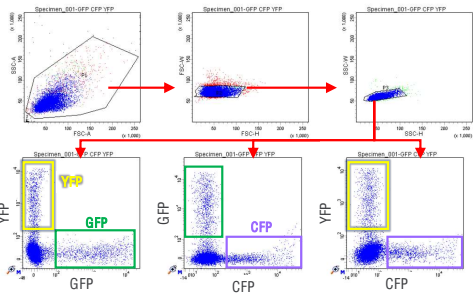
- Purpose**
  - Transgene expression
  - Transplanted cells survival
  - Interaction between transgene
- Fluorescent proteins**
  - GFP series fluorescent protein**
    - GFP, YFP, CFP, RFP, BFP etc.
  - Fruits series fluorescent protein**
    - mCherry, mPlum, mStrawberry, mBanana etc.
  - CoralHue series fluorescent protein**
    - Midonishi-Cyan, Kusabira-Orange, Azami-Green, Keima-Red, Kaede, Doronpa-Green etc.



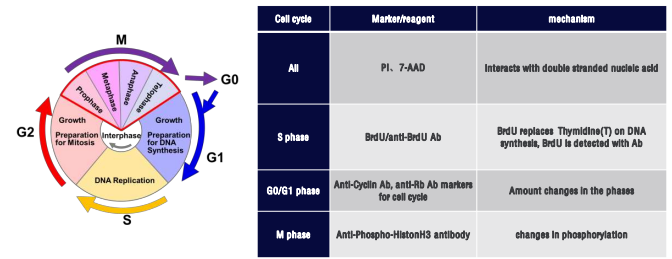
### GFP / mCherry Expressing cell analysis



### GFP / YFP / CFP Expressing cell analysis

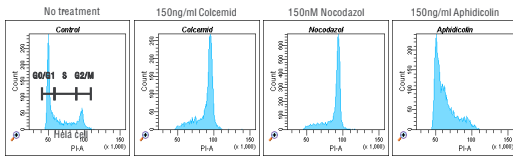


### ④ Cell cycle analysis



#### ④ Cell cycle analysis : PI analysis

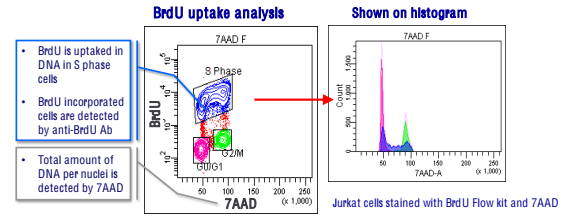
• PI staining cell cycle analysis  
CycleTEST™ PLUS DNA Reagent Kit (Cat#340242)



Hela cells cultured 48hr in DMEM with 0.5% FBS were treated with reagents. After 16hr culture, cell cycle analysis was performed

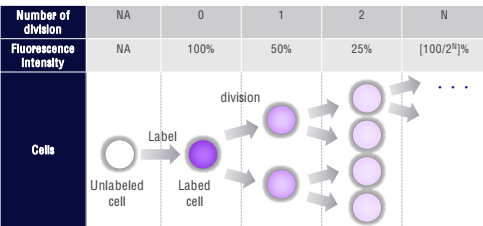
#### ④ Cell cycle analysis : BrdU / 7AAD analysis

BD Pharmingen™ FITC BrdU Flow Kit (Cat#559619)

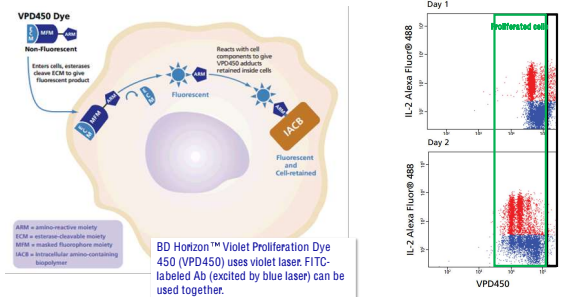


#### ⑤ Cell proliferation assay

BD Biosciences offers BD Horizon™ Violet Proliferation Dye 450 (VPD450) and BD Horizon™ CFSE for the detection of cell proliferation. Once the dye is inside the cell, esterases cleave off the ester group to convert the dye into a fluorescent product and trap it inside the cell. With each replication event the amount of dye in the cell is decreased, leading to a characteristic pattern.



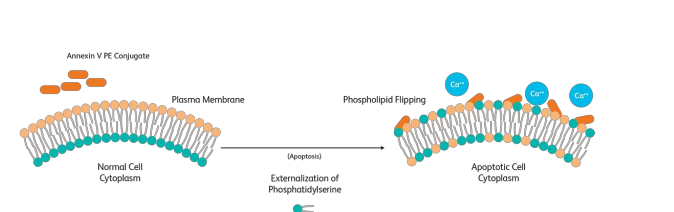
#### ⑤ Cell proliferation assay: VPD450 assay



#### ⑥ Apoptosis analysis

Apoptosis stages	Feature Measured	Assays	Key Features
Apoptosis stages	Plasma Membrane Alterations (Phosphatidylserine Exposure)	<ul style="list-style-type: none"> <li>Annexin V binding assay</li> <li>Single conjugates</li> <li>Annexin V Kits</li> </ul>	<ul style="list-style-type: none"> <li>Detects early apoptosis markers</li> <li>Quick and easy</li> <li>Flow cytometry or immunofluorescence application</li> </ul>
	Mitochondrial Changes	<ul style="list-style-type: none"> <li>BD MitoScreen Kit</li> </ul>	<ul style="list-style-type: none"> <li>Fast, easy, single cell resolution by flow cytometry or fluorescent microscopy</li> </ul>
	Caspase Activation	<ul style="list-style-type: none"> <li>Caspase Activity Assay Kits and Reagents</li> <li>Active Caspase-3 immunoassays (ELISA)</li> </ul>	<ul style="list-style-type: none"> <li>Quick and easy, uses spectrofluorometry</li> <li>ELISA application</li> </ul>
	DNA Fragmentation	<ul style="list-style-type: none"> <li>APO-BrdU TUNEL Assay</li> <li>APO-DIRECT TUNEL Assay</li> </ul>	<ul style="list-style-type: none"> <li>Works adherent cells, single cell resolution in conjunction with cell cycle analysis by flow cytometry</li> </ul>

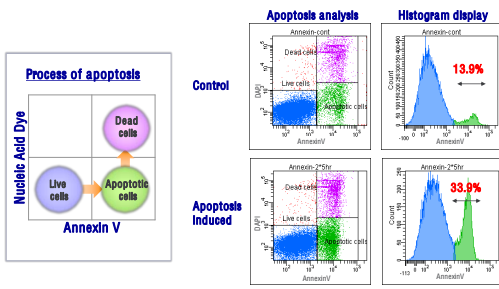
#### Apoptosis analysis: Annexin V assay for Surface Phosphatidylserine detection



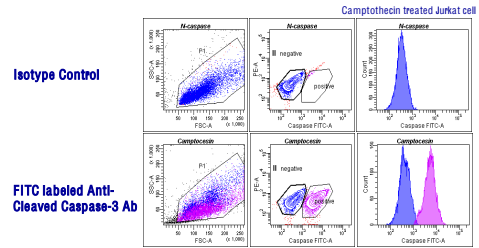
Phosphatidylserine (PS), which is normally located on the cytoplasmic face of the plasma membrane, translocates to the outer leaflet of the plasma membrane during apoptosis and can be detected by flow cytometry and cell imaging through binding to fluorochrome-labeled annexin V when calcium is present.



### Apoptosis analysis: Annexin V assay

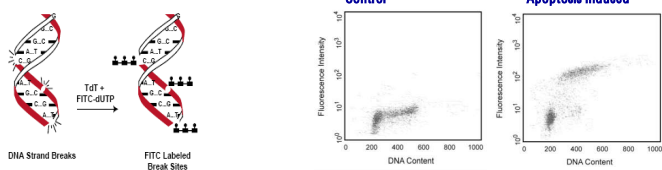


### Apoptosis analysis: Active Cleaved Caspases and Cleaved poly-ADP ribose polymerase (PARP) detection



Caspases are activated upon during the earliest stages of apoptosis. Active caspases can then cleave many proteins, including PARP. This leads to the loss of cellular structure and function, and ultimately results in cell death.

### Apoptosis analysis: Apo-Direct to detect DNA damage



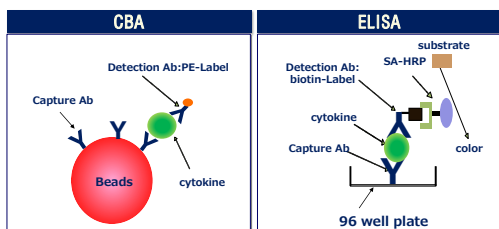
One of the later steps in apoptosis is DNA fragmentation. A method which is often used to detect fragmented DNA utilizes a reaction catalyzed by exogenous TdT, often referred to as "end-labeling" or "TUNEL" (terminal deoxynucleotidyltransferase dUTP nick end labeling).

The APO-DIRECT™ assay is a single-step method for labeling DNA breaks with FITC or APC-dUTP.

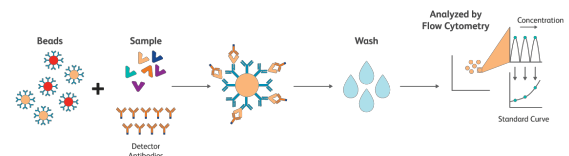
### 7 Cytokine measurement

method	object	measurement	Instrument
ELISA (OptEIA)	Cytokine concentration in solution	Antibody and enzyme reaction in 96 well plate to color	Plate reader
Intracellular Cytokine	Cells producing cytokine	Stain cytokine with Ab within cells	FCM
ELISPOT	Cells secreting cytokine	Antibody and enzyme reaction in 96 well plate to color	Microscope
Cytometric Beads Array (CBA)	Cytokine concentration in solution	Use special beads to measure cytokine	FCM

### Cytometric Beads Array (BD™ CBA)



### Cytometric Beads Array (BD™ CBA)



	BD™ CBA	ELISA
Multiplexing Capability	Up to 30 proteins /sample	one protein /sample
Simple procedure and Time Efficiency	Wash once	Wash more than 10 times
Sample amount	50 uL or less	About 100 uL
Format	From single tube	96 well plate
Required Instrument	Flow cytometer	Plate reader

