The University of Zambia

School of Health Sciences

Department of Biomedical Sciences

Principles and Concepts of Immunological Methods

S. M. Munsaka, PhD

16th May, 2016

Lecture prepared by: Geoffrey Kwenda, BSc (Hons), MSc, PhD

Definition of Immunology

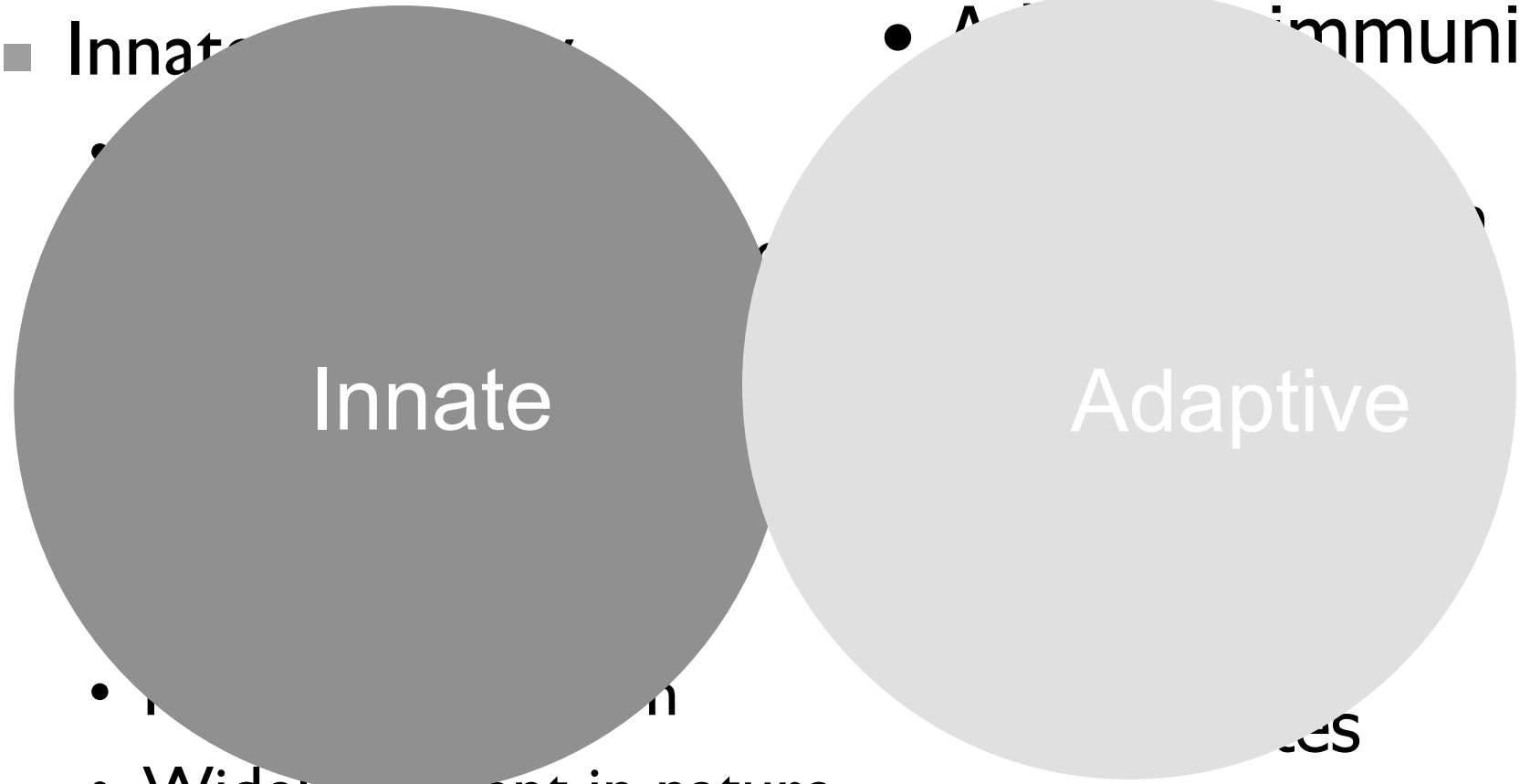
- Immunology is the study of our protection from foreign macromolecules or invading organisms and our responses to them.
  - Latin - *Immunis* = “exempt”
  - English = protection from disease

Immunology

- Recognition of self and non-self
  - —Antigens
- Elimination of non-self
  - —Exogenous targets
    - Microbes
    - Allergens
    - Foreign material
  - —Endogenous targets
    - Tumours

Two Arms of Host Defence

- Innat
  - Widely present in nature



Key Players in Immunology

	Innate	Adaptive

Cells	• Phagocytes
	• Epithelial Cells
	• NK Cells
		• Lymphocytes
		  • B-cells
		  • T-cells

Defence Proteins	• Complement • Antimicrobial (poly)peptides • Interferons	• Antibodies

Overview of Immune System

- Innate Immunity:
  - Fast-acting
  - Less specific recognition
  - Early during evolution
  - e.g. barriers to infection such as skin and mucus surfaces

- Adaptive Immunity:
  - Specificity
  - Distinguish antigens sometimes present from those always present
  - Memory and Recall

- Cells of the immune system
  - Leukocytes originating from bone marrow stem cells

- Communication with other systems
  - Endocrine system
  - Central nervous system
  - Skeletal system

  } Psychoneuroimmunology

- Disruption of the Immune System
  - Hypersensitivity
  - Autoimmunity
  - Immunodeficiency

Manifestations of Infection

- Clinical presentation of infectious disease reflects interaction between host and microorganism
- Interaction affected by host immune status and microbial virulence factors
- Signs and symptoms vary according to site of infection and severity of infection (acute or chronic?)
- Physician may predict course of disease and likely cause by combining epidemiological clues with signs and symptoms

Immunological techniques

Immunological techniques are used to:

- Detect, identify & quantify Ag in clinical samples
- Evaluate Ab response to infection
- Evaluate Person's history of exposure to infectious agents

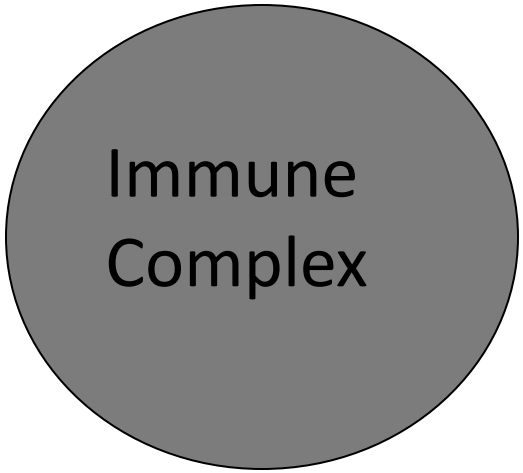
Specificity of Ab-Ag interaction & sensitivity of many immunological techniques make them powerful tools

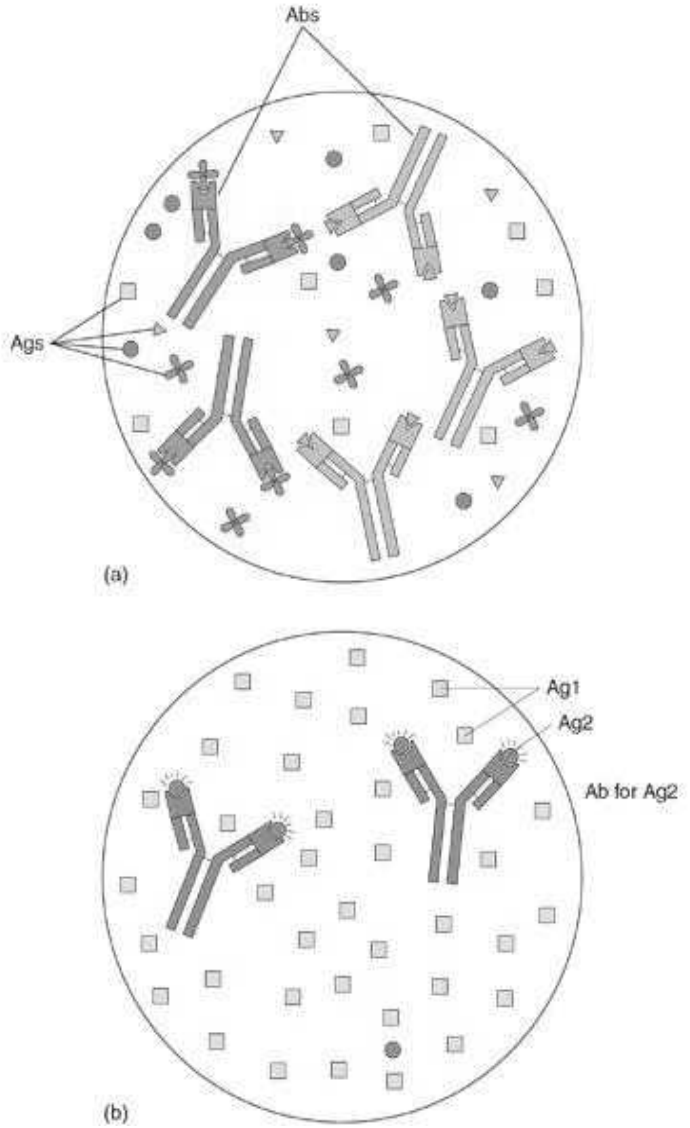
In most cases same technique can be used to evaluate a person's Ag & Ab status

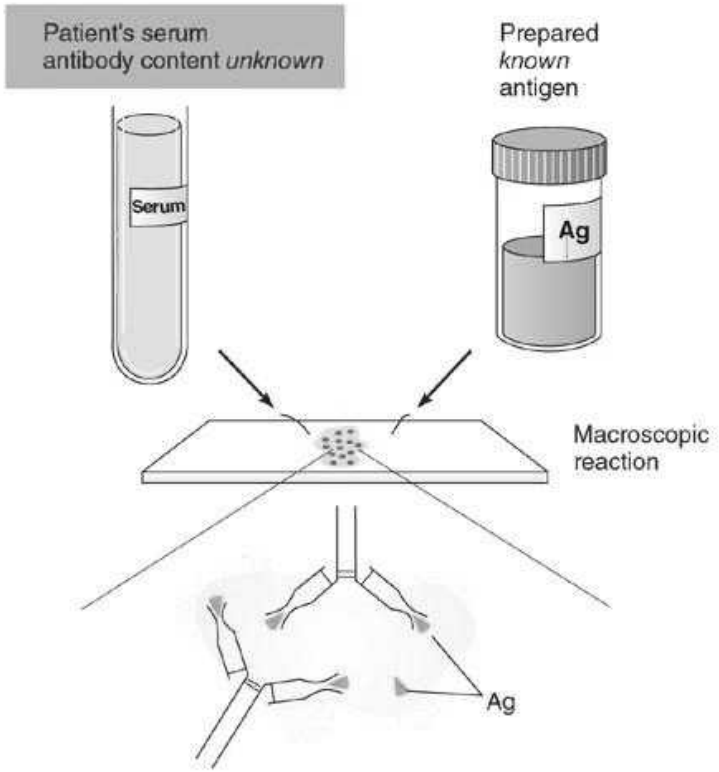
Antigen

+

Antibody

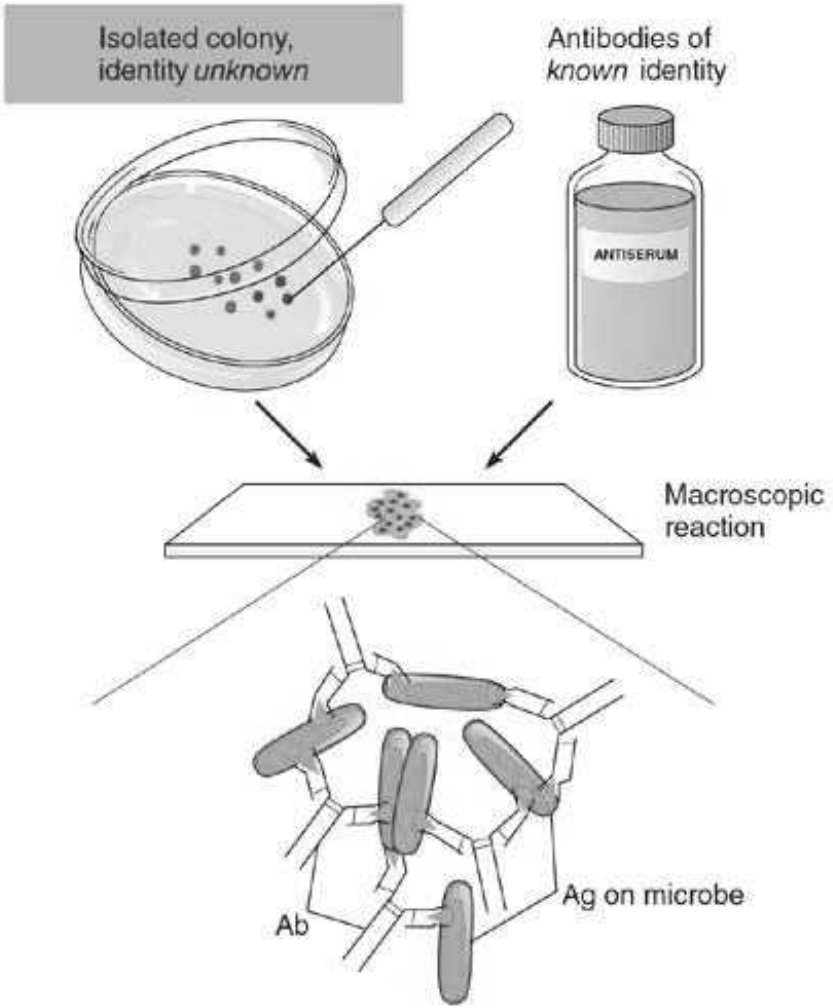






(a) In serological diagnosis of disease, a blood sample is scanned for the presence of antibody using an antigen of known specificity. A positive reaction is usually evident as some visible sign, such as color change or clumping, that indicates a specific interaction between antibody and antigen. (The reaction at the molecular level is rarely observed.)

Copyright © The McGraw-Hill Companies, Inc. Permission required for reproduction or display.



(b) An unknown microbe is mixed with serum containing antibodies of known specificity, a procedure known as serotyping. Microscopically or macroscopically observable reactions indicate a correct match between antibody and antigen and permit identification of the microbe.

Methods of Detection

1. Precipitation of soluble Ag
    - Immunodiffusion
    - Immunoelectrophoresis

2. Agglutination of cell-bound Ag

3. Immunolabelling
    - Radioimmunoassay
    - Enzyme-linked assays
    - Immunohistochemistry
    - Leukocyte assays
    - Flow Cytometric analysis
4. Rosette & plaque formation

1. Precipitation of soluble Ag

- Precipitation techniques are based on
  - ability of most Ab preps to interact with more than one epitope on a protein or infectious agent
  - fact that each Ab molecule interacts with more that one Ag

Ab → diffuse & meet ← Ag

↓

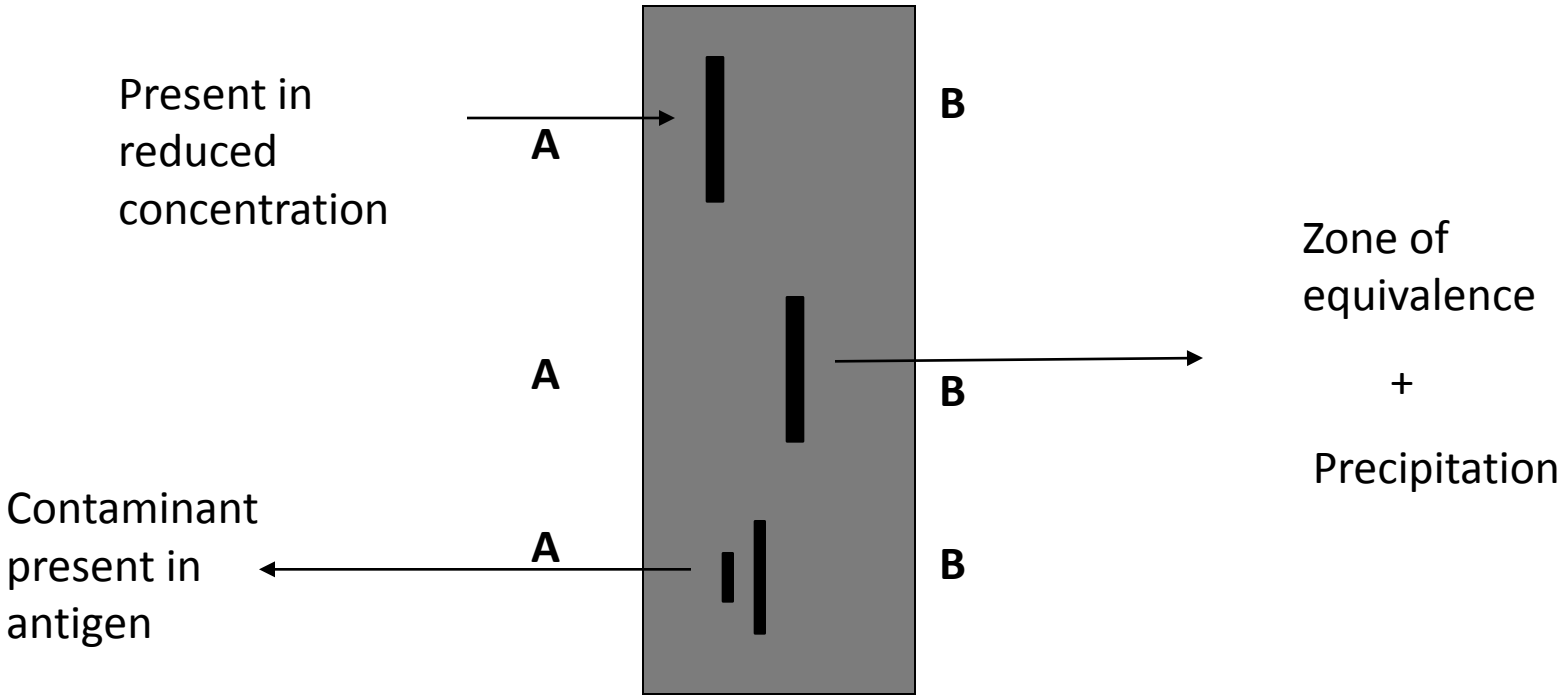
At appropriate conc visible ppte forms

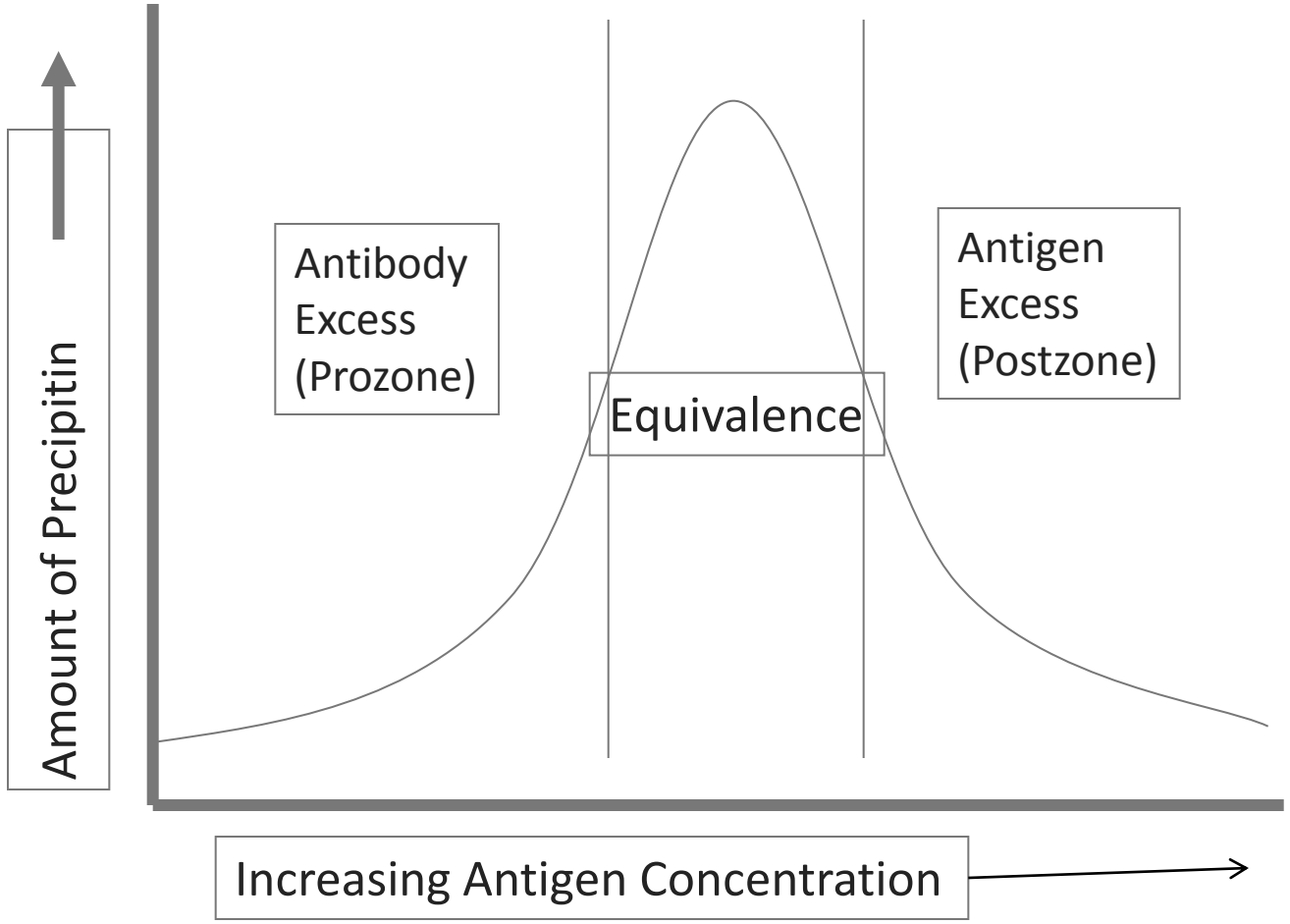
↓

Stain ppte (eg for protein)

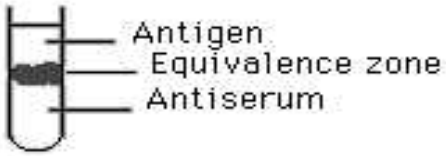
a) Immunodiffusion

Within limited conc^{n} range for both Ag & Ab, termed **EQUIVALENCE ZONE**, Ab cross-links Ag into complex that is too large to stay in sol^{n} & therefore pptes

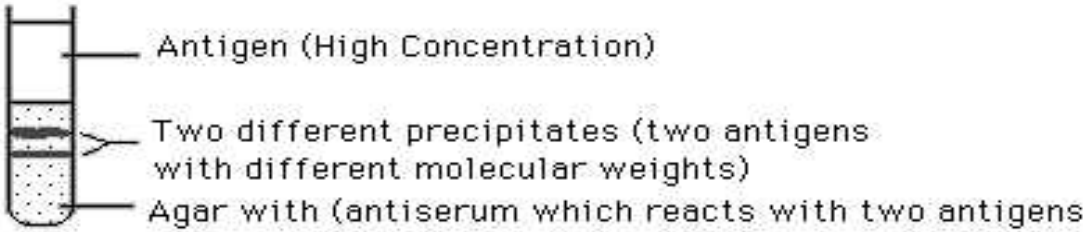



Amount of Precipitin
Antibody Excess (Prozone)
Equivalence
Antigen Excess (Postzone)
Increasing Antigen Concentration

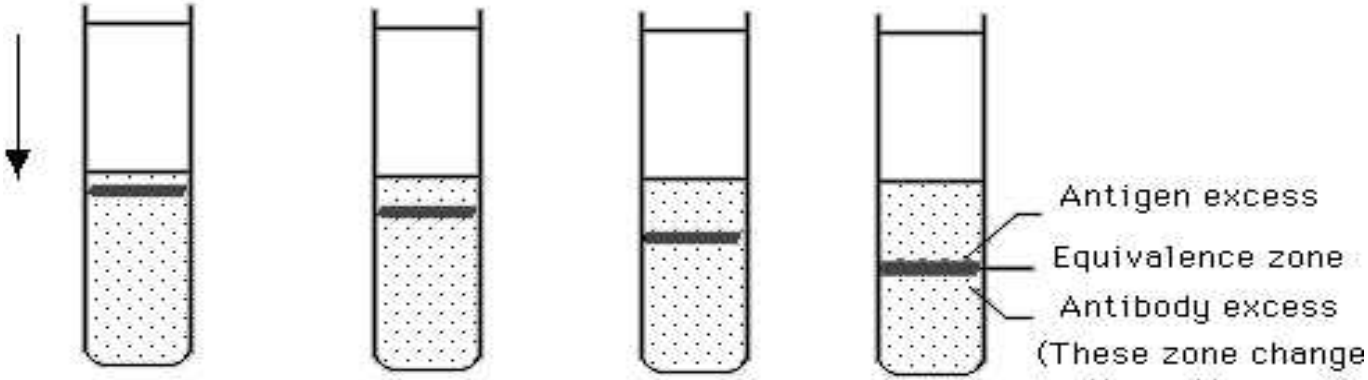
Ring precipitation test



Single dimensions single diffusion



Pseudomigration of the precipitate with time

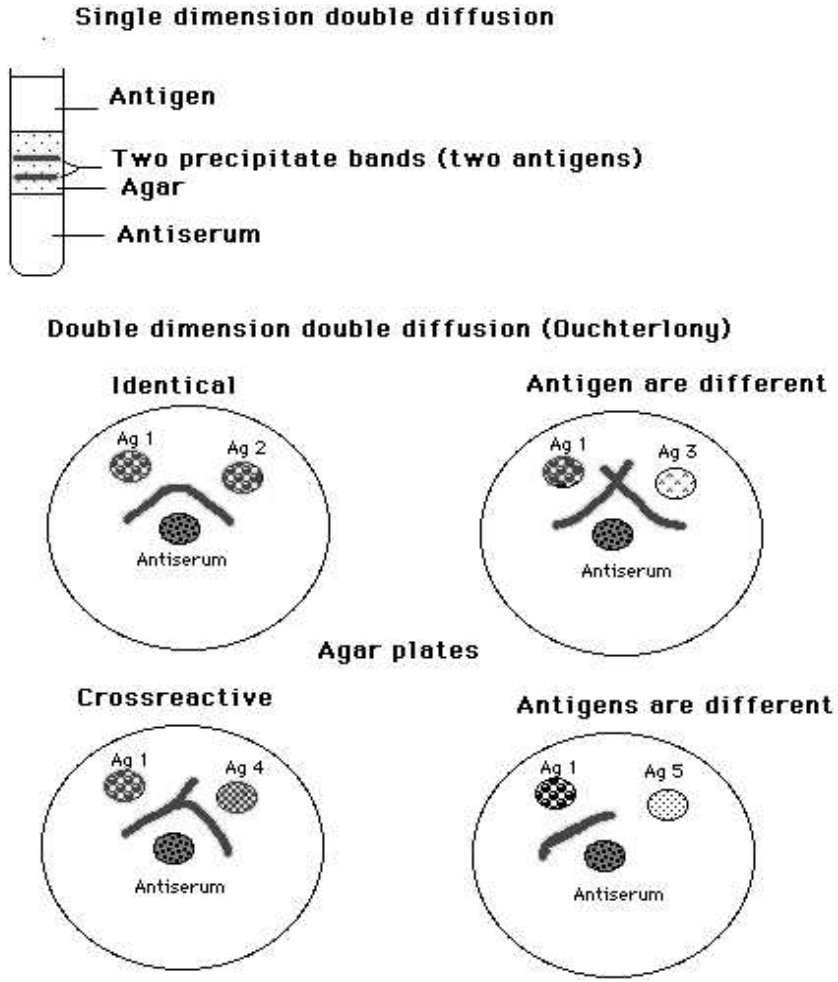


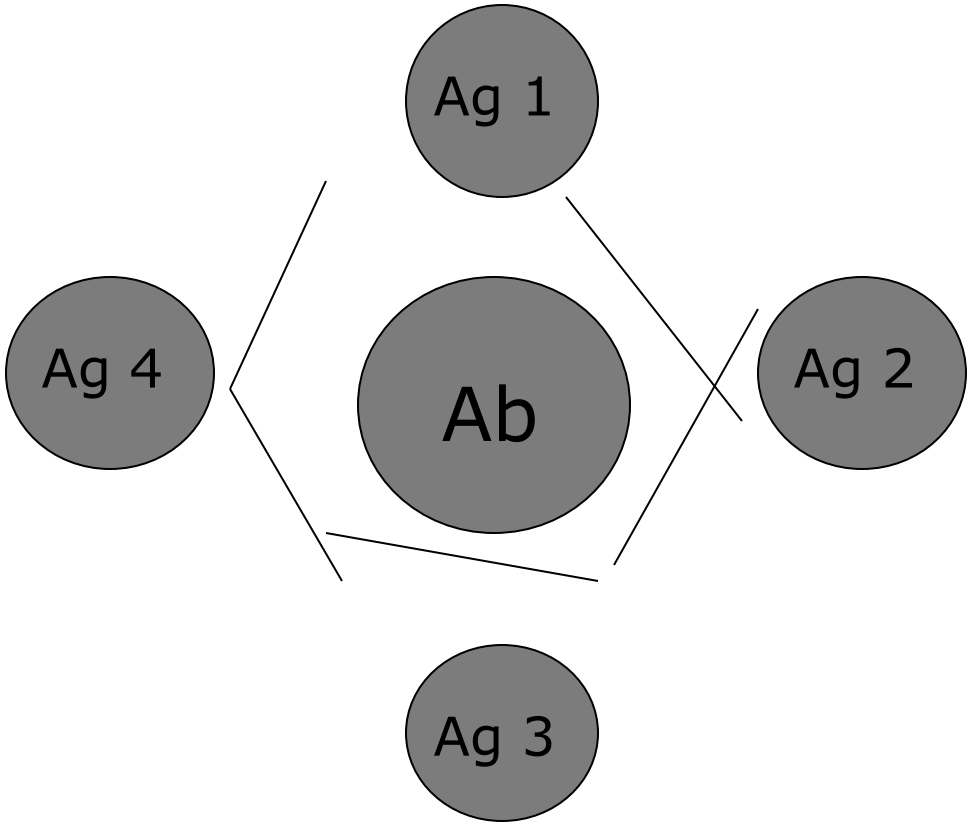
The precipitate is forming and disolving as the antigen migrates.

I) Ouchterlony (Qualitative Double Diffusion)

- Double diffusion precipitation tests involve the diffusion of Ags and Abs in a soft agar gel, forming zones of precipitation where they meet
- Characterise relationship between 2 antigens
- If antigens are in the same precipitation line ,they fail to cross

Qualitative (Double Diffusion): Ouchterlony

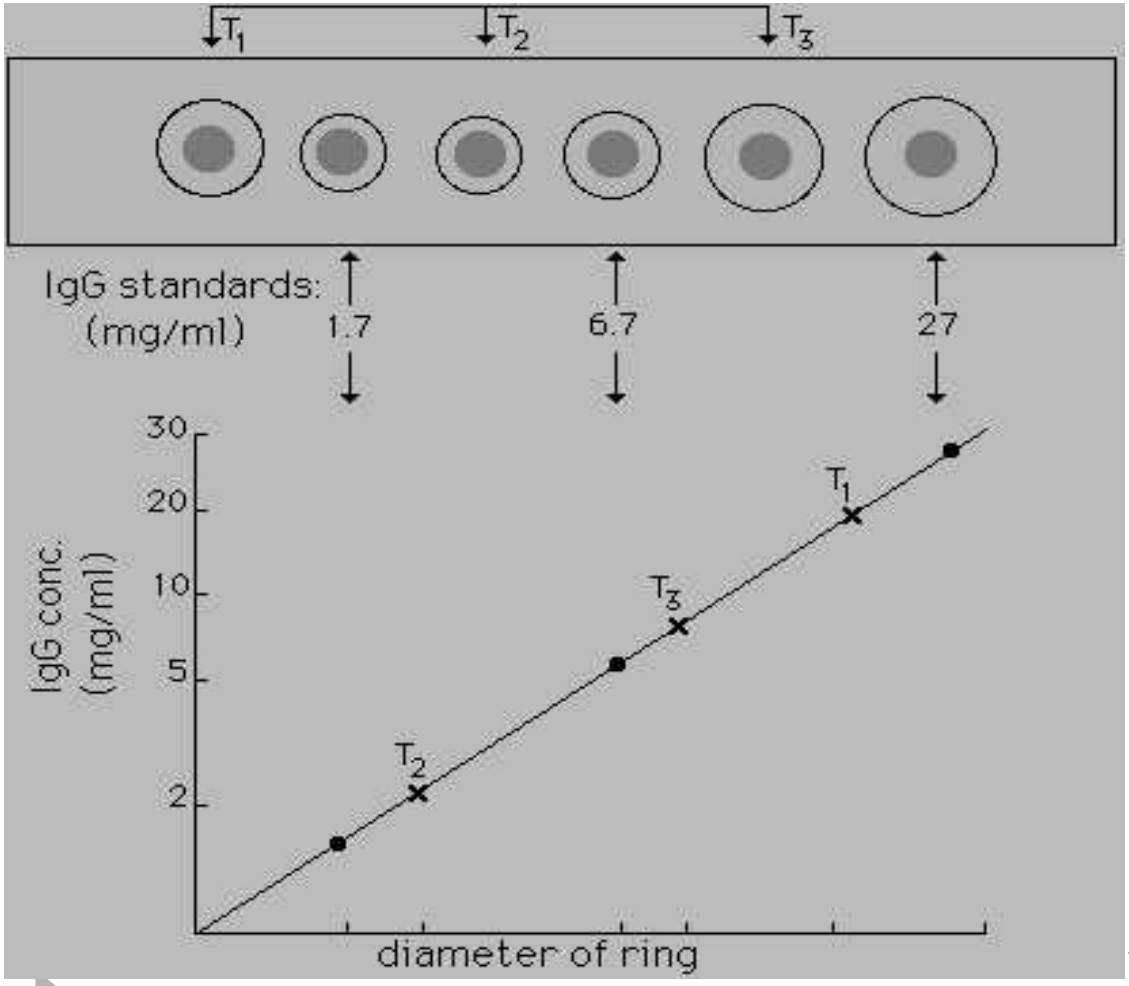




Limitations :
Insensitive
Needs large unit of Ab & Ag
Resolve by CIE – placing agar in electric field

ii. Quantitative: Radial Immunodiffusion

Measurement of IgG concentration in serum by single radial immunodiffusion. The diameter of the standards (0) enables a calibration curve to be drawn and the concentration of IgG in the sera under test can be read off: T1-serum from patient with IgG myeloma; 15mg/ml, T2-serum from patient with hypogammaglobulinaemia; 2.6 mg/ml, T3-normal serum; 9.6 mg/ml



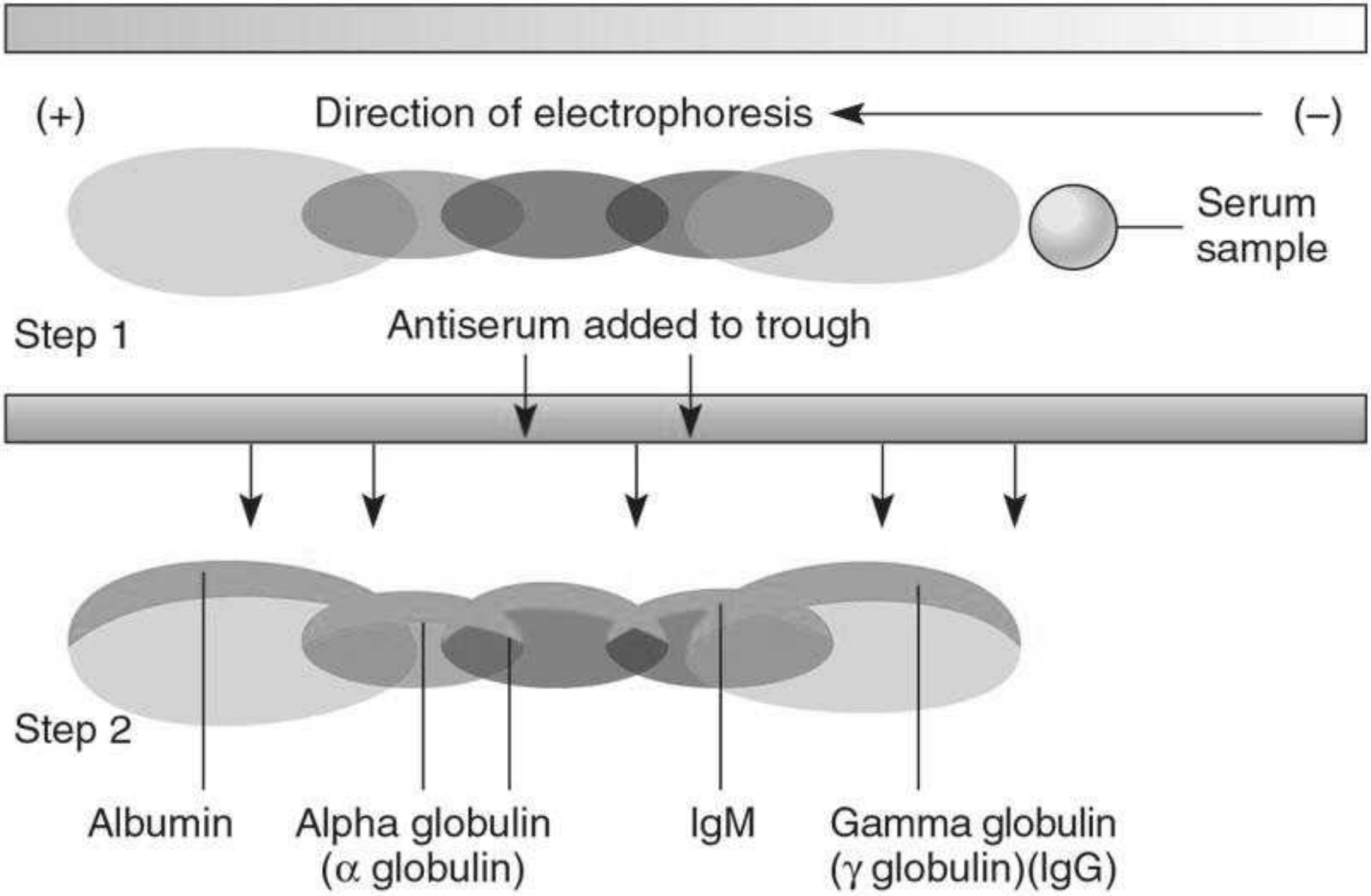
Demerits

- Only 1 Ag can be tested at a time
- Method slow

b) Immunoelectrophoresis

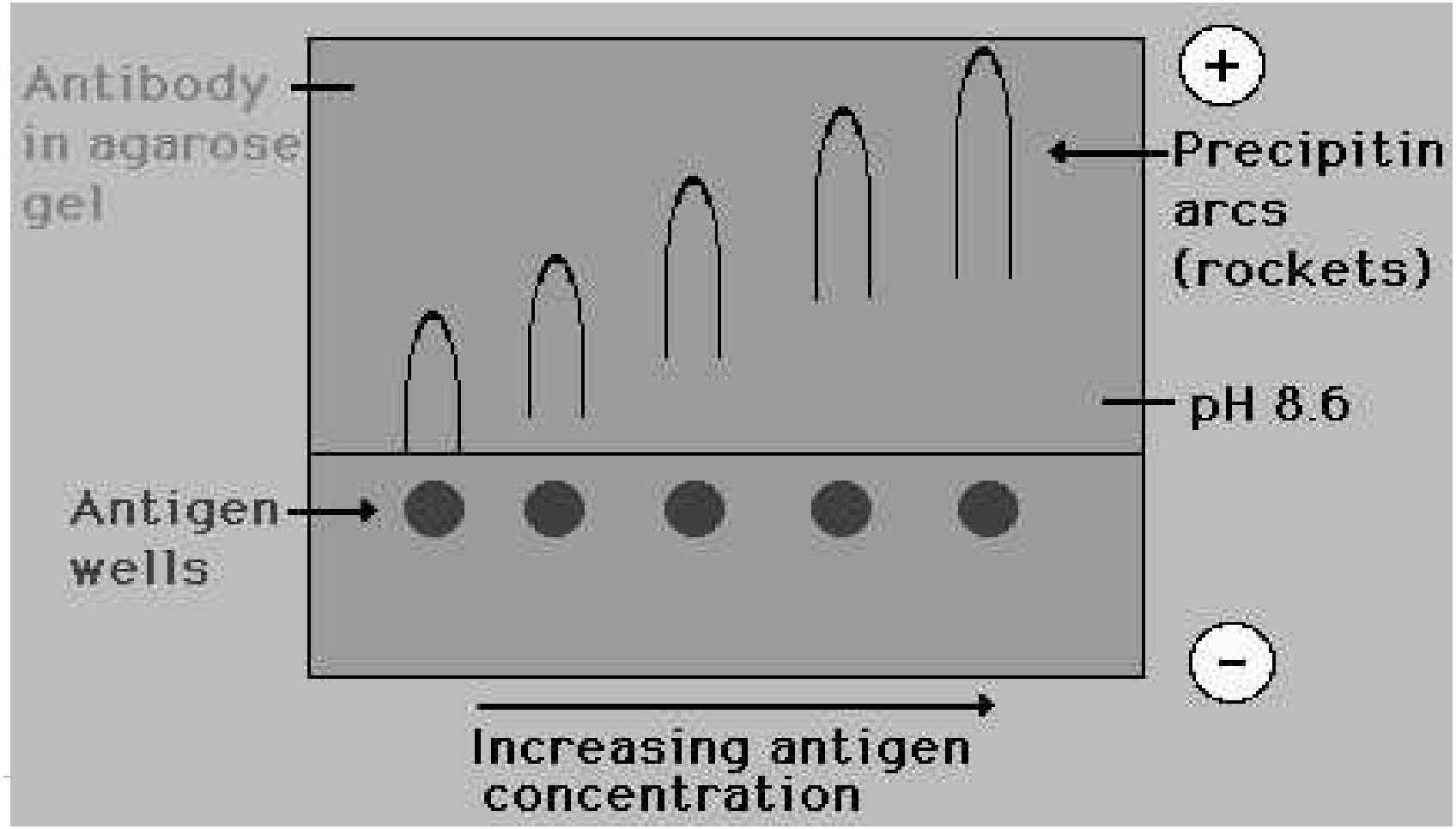
- Migration of serum proteins in gel is combined with precipitation by Ab
- Used to study complicated Ag mixtures
- Ag separated by electrophoresis

i. Qualitative



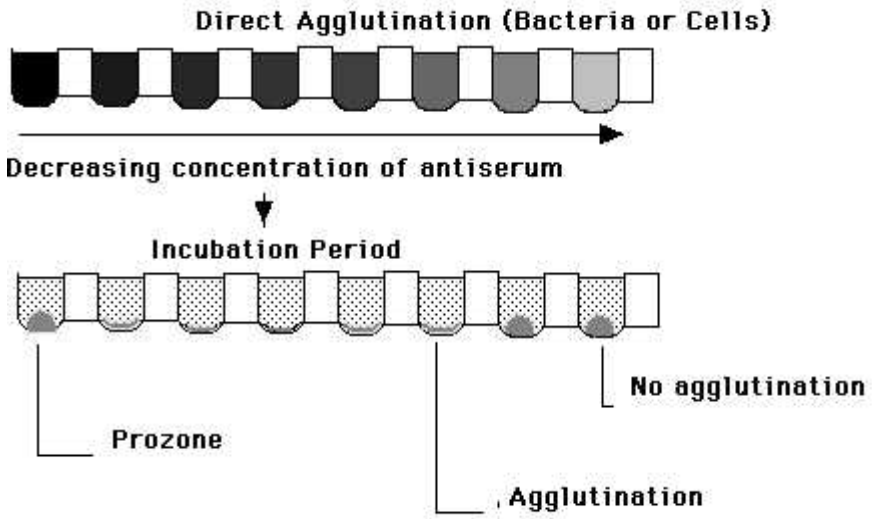
ii. Quantitative: Rocket electrophoresis

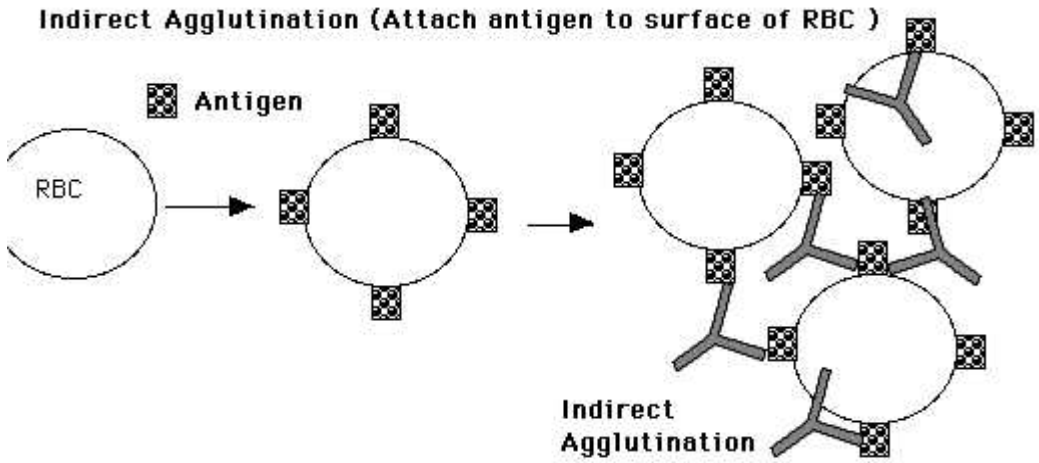
Ag is electrophoresed into gel containing Ab. Distance from starting well to the front of rocket shaped arc is related to Ag concentration.



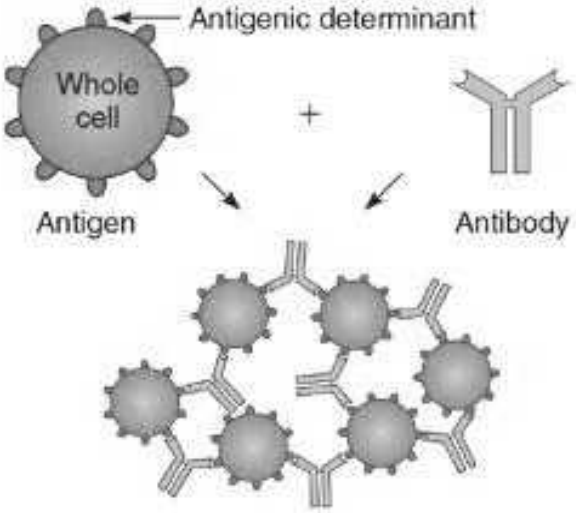
2. Agglutination of cell-bound Ag

- Agglutination tests – Ab cross-links whole cell Ag, forming complexes that settle out and form visible clumps in the test chamber
  - blood typing
  - bacterial & viral disease detection



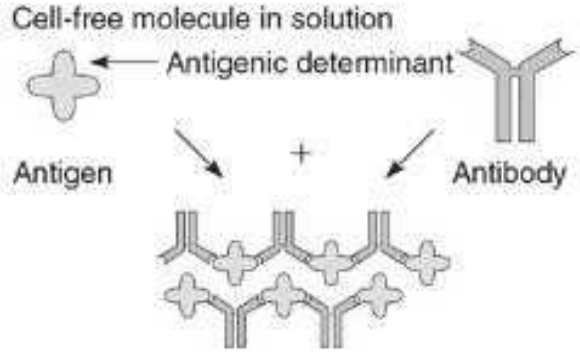


Agglutination



Microscopic appearance of clumps

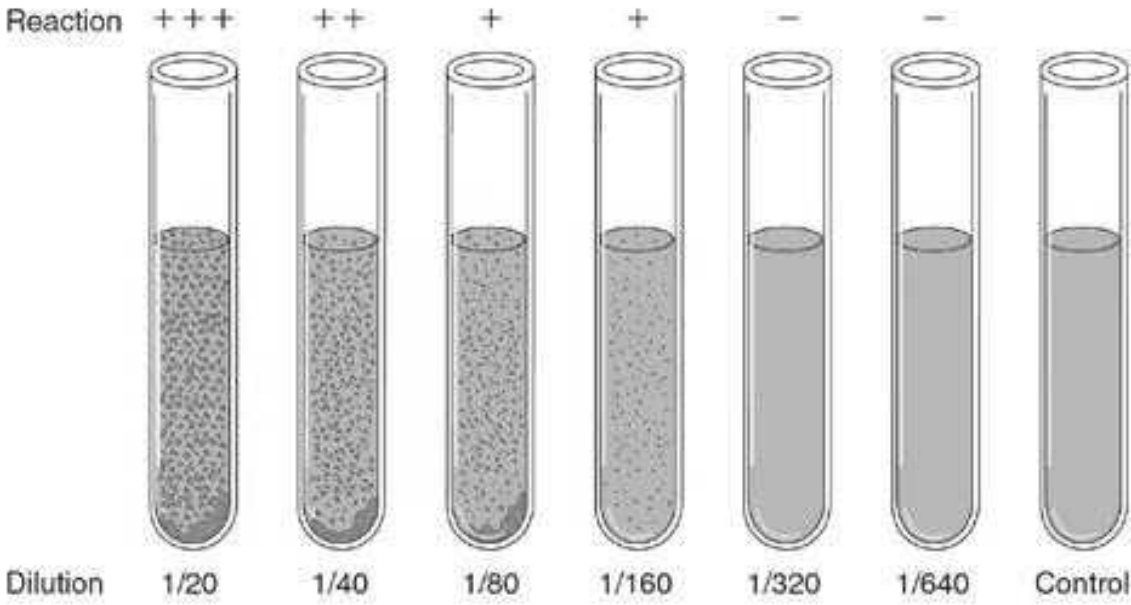
Precipitation



Microscopic appearance of precipitate

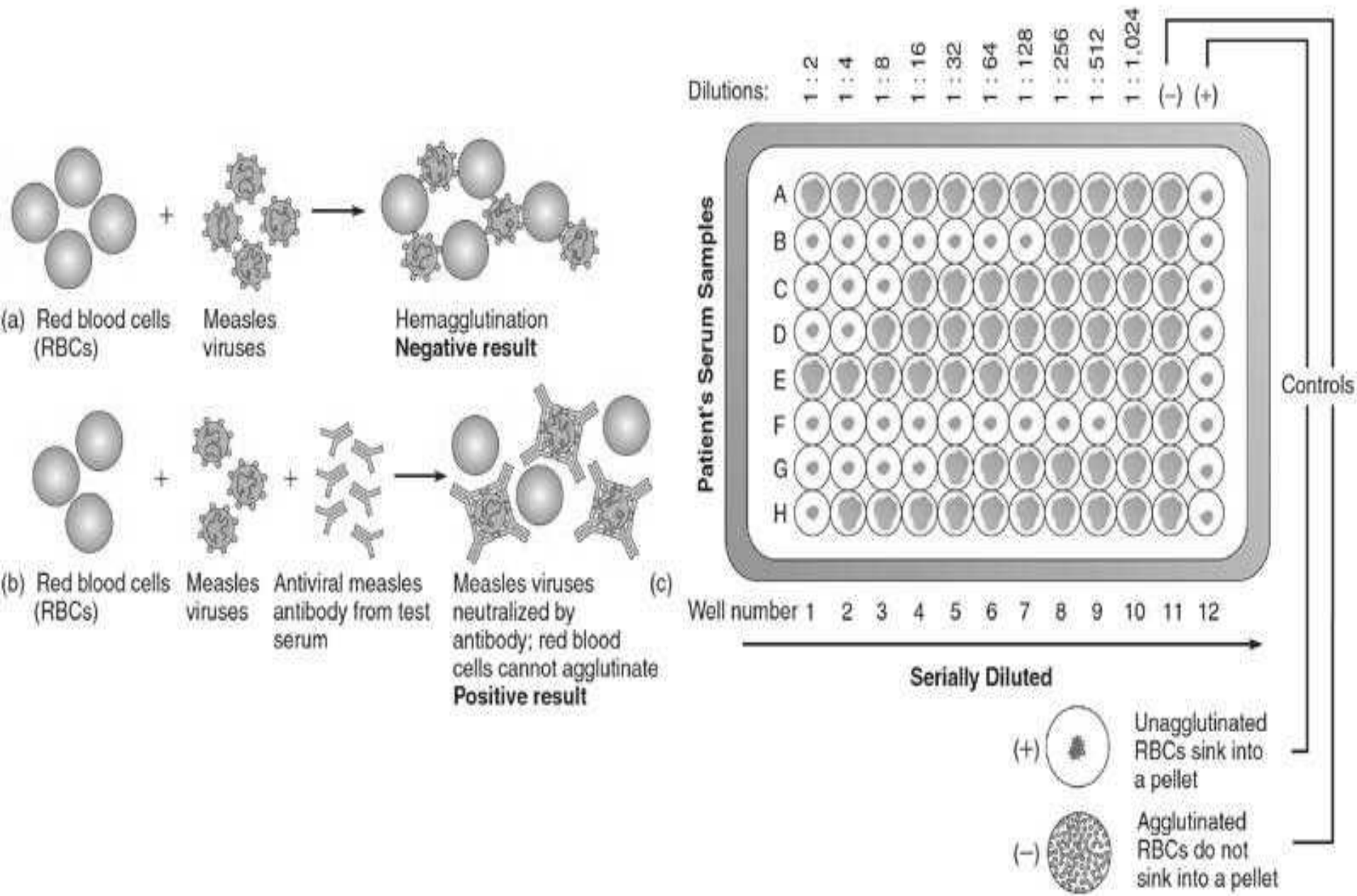
(a)

The Tube Agglutination Test



A sample of patient's serum is serially diluted with saline. The dilution is made in a way that halves the number of antibodies in each subsequent tube. An equal amount of the antigen (here, blue bacterial cells) is added to each tube. The control tube has antigen, but no serum. After incubation and centrifugation, each tube is examined for agglutination clumps as compared with the control, which will be cloudy and clump-free. The titer is defined as the dilution of the last tube in the series that shows agglutination.

(b)

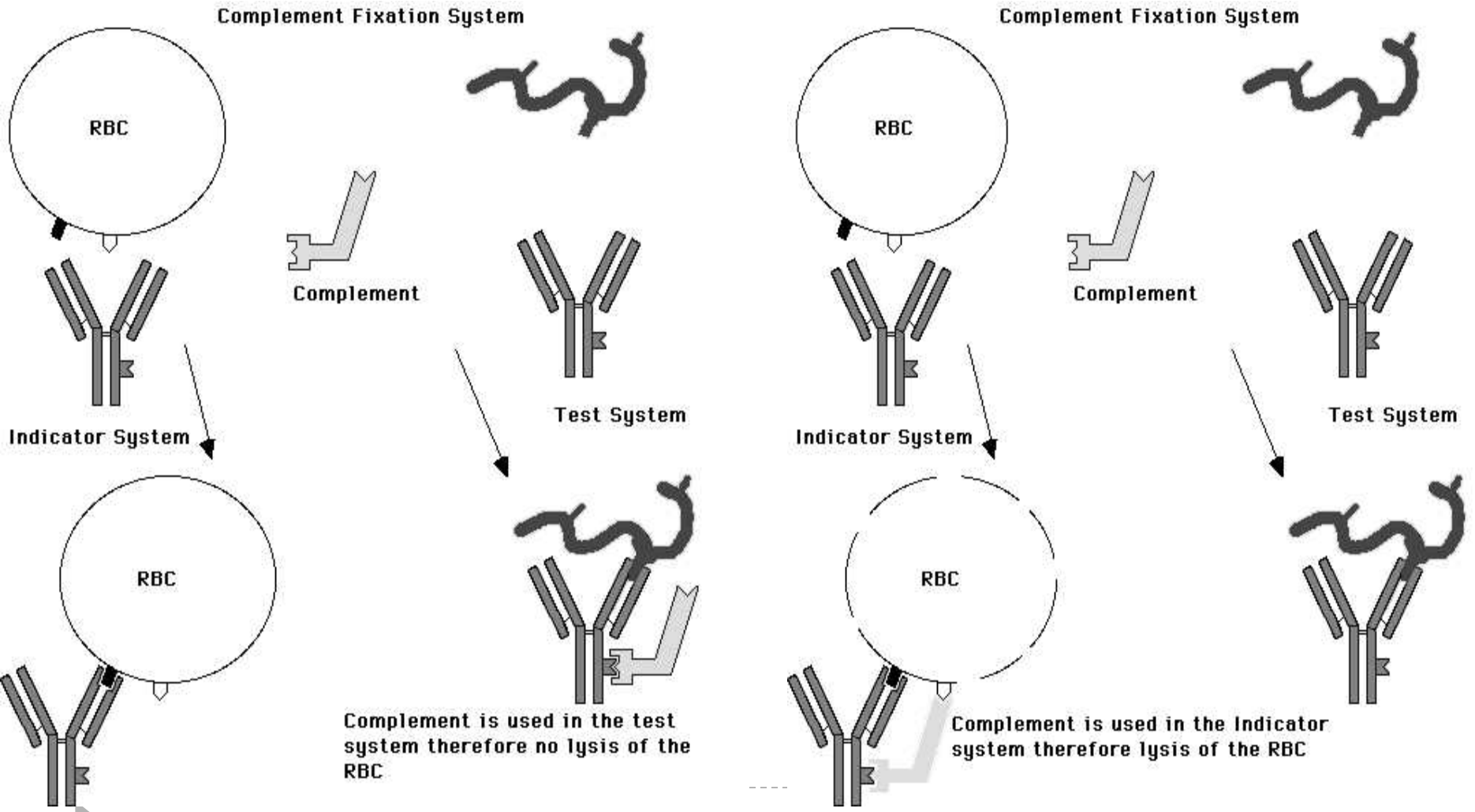


Complement Fixation Tests

- Complement fixation tests detect lysins- Ab that fix complement and can lyse target cells.
- Involves mixing test Ag & Ab with complement and then with sensitised sheep RBCs.
- If complement is fixed by the Ag-Ab, the RBCs remain intact and the test is positive.
- If RBCs are haemolysed, specific Ab are lacking and the test is negative.

Agglutination: Continuation……

eg, Haemagglutination & Complement Fixation

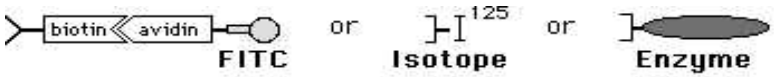


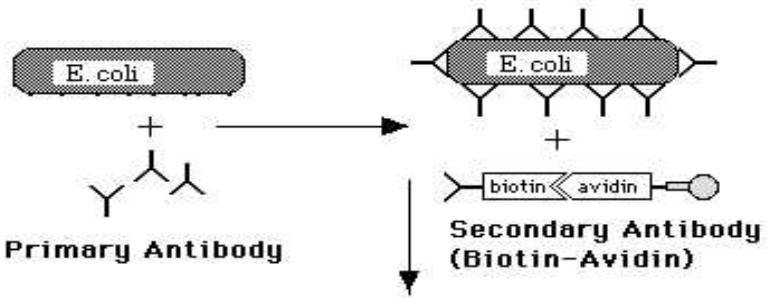
3. Immunolabelling Methods

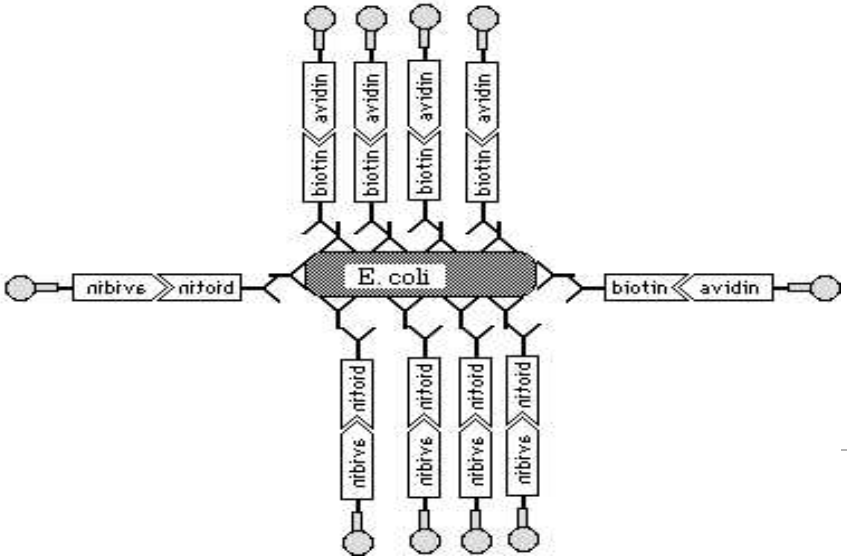
- Uses detectable label covalently bound to Ab or Ag or second molecule which binds to Fc region of Ab in immune complex
- More sensitive
- Direct & indirect methods
- One of immune complex partners is fixed to solid support, ie Ab or Ag
- Label always linked to soluble partner or 2nd molecule
- Assays depend on detecting or quantifying label bound to immune complex

a) Biotin-Avidin Assay

Avidin: glycoprotein derived from egg albumin, has very high affinity for vitamin, biotin, & does not bind to other substrates. Biotin can be easily coupled to IgG. Avidin can be labelled with fluorochromes, enzymes, or radioactive chemicals. If the 2^{o} Abs are labelled, using the biotin-avidin system, they can be efficiently employed in indirect IF, ELISA and RIA.



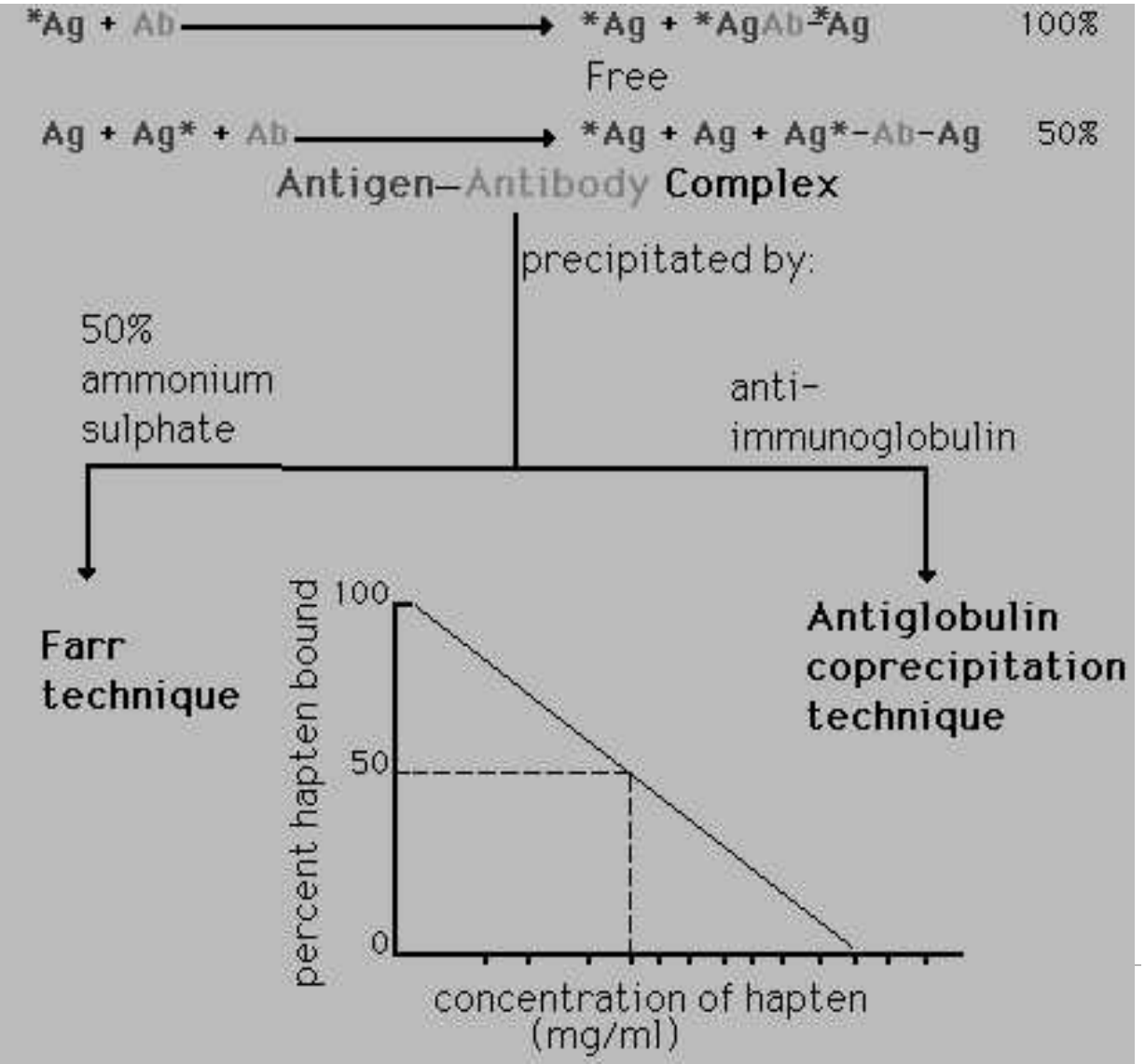




b) Radioimmunossays (RIA)

- Radioimmunoassay –Ag or Abs are labelled with radioactive isotopes and traced
- Determination of Ag-binding capacity.
- After addition of excess radioactive antigen (*Ag), that part bound to antibody as a complex is precipitated either by ammonium sulphate (Farr) or by an antiglobulin (antiglobulin coprecipitation.)

b) Radioimmunossays (RIA)



- Very sensitive: 10^{-12}g/ml
- Used to detect:
  - Cancer associated proteins
  - Drugs
  - Hormone levels

c) Enzyme-Linked ImmunoSorbent Assay (ELISA)

- ELISA can detect unknown Ag or Ab by direct or indirect means.

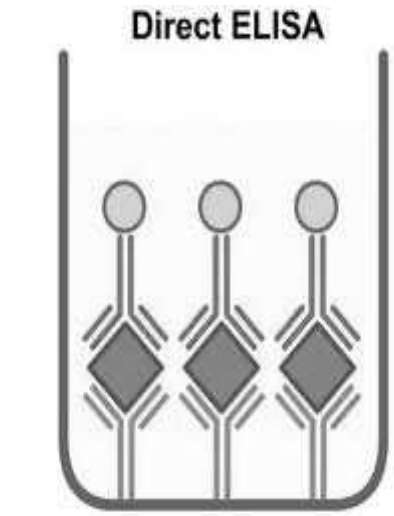
- A positive result is visualised when a coloured product is released by an enzyme-substrate reaction.

Direct ELISA – Detect antigen

Plate with Known Ab ------ put serum ?Ag

-------wash--------add enzyme-labelled Ab

-------wash--------read in spectrophotometer



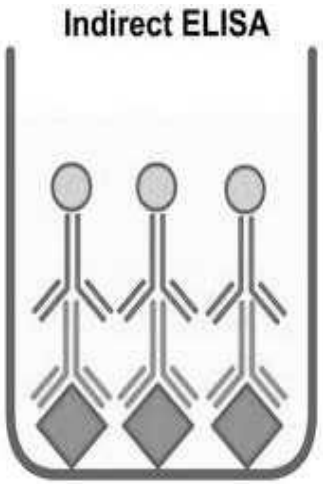
The secondary antibody attaches to the bound protein from the sample, forming a complex consisting of immobilised antibody, protein and labelled secondary antibody.

Indirect ELISA – look for antibody

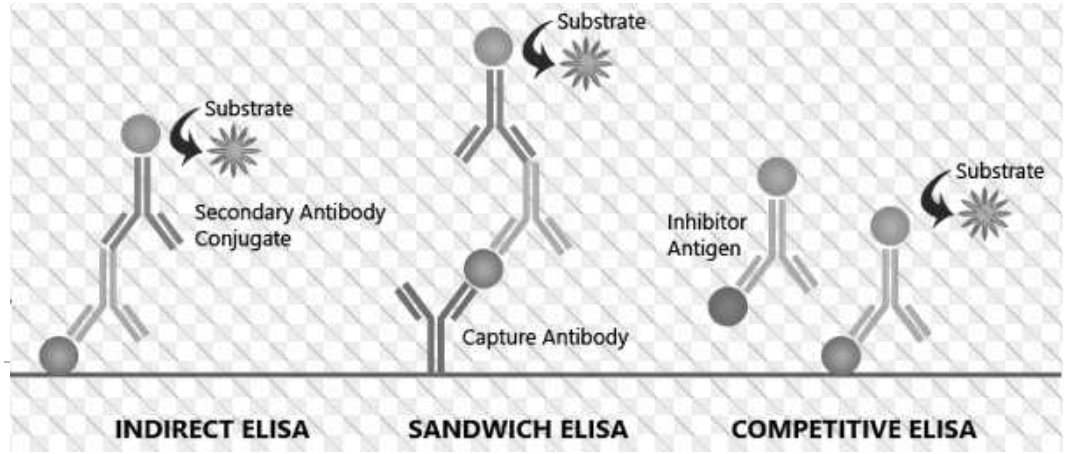
Plate with Antigen --------+ put serum**? Ab**

-----wash--------- + antihuman globulin

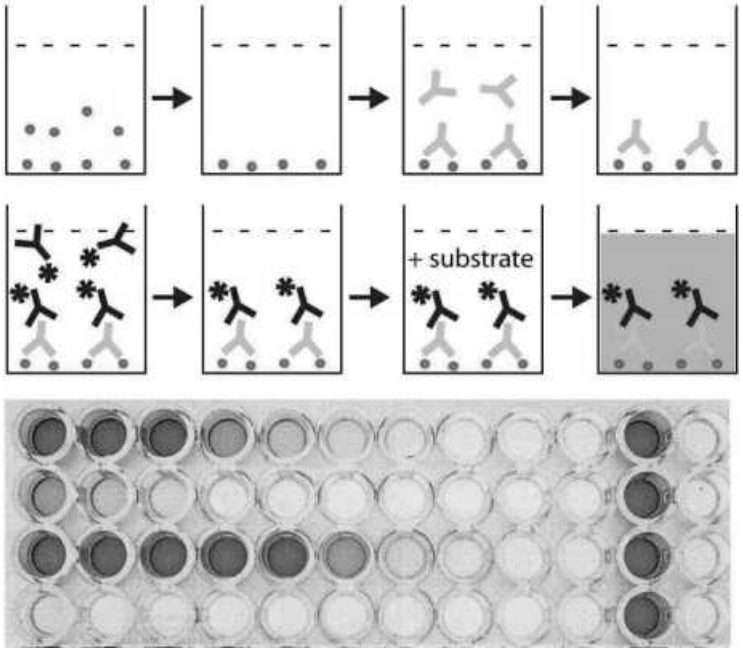
-----wash-------- read in spectrophotomete



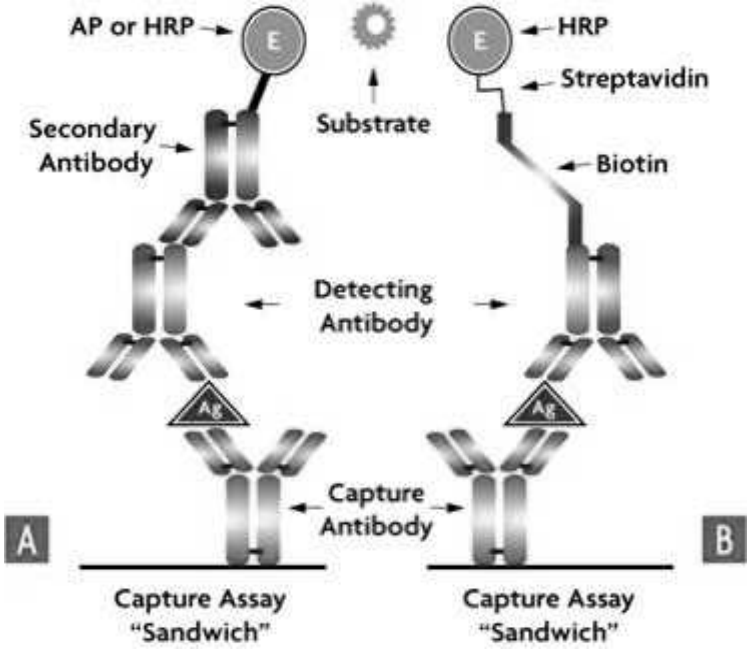
These reporter antibodies bind to the bound autoantibodies from the sample forming a complex consisting of immobilised antigen, autoantibody and abelled reporter antibody. The wells are again rinsed to remove any unbound antibodies.



Enzyme-Linked ImmunoSorbent Assay (ELISA)

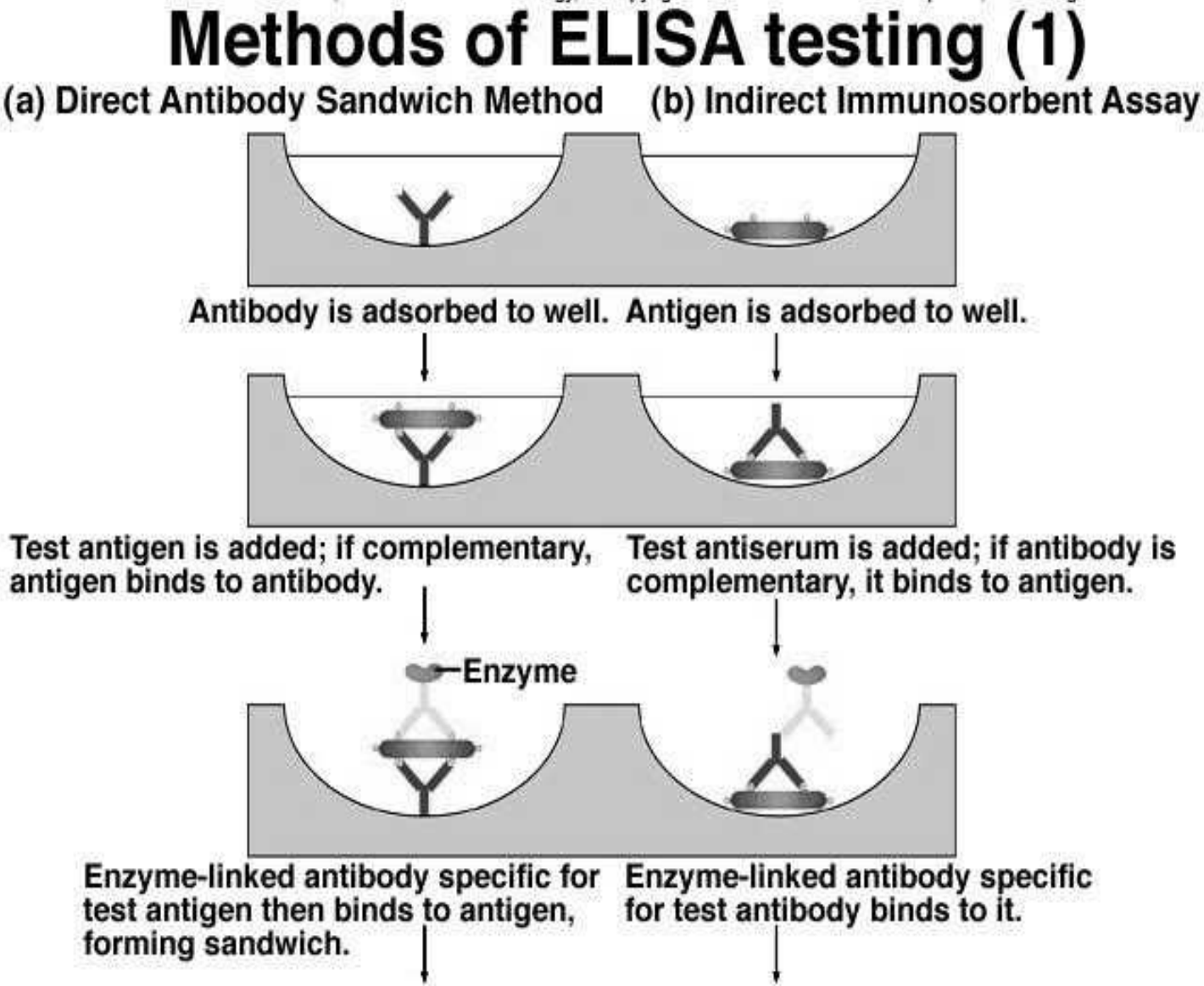


Indirect ELISA



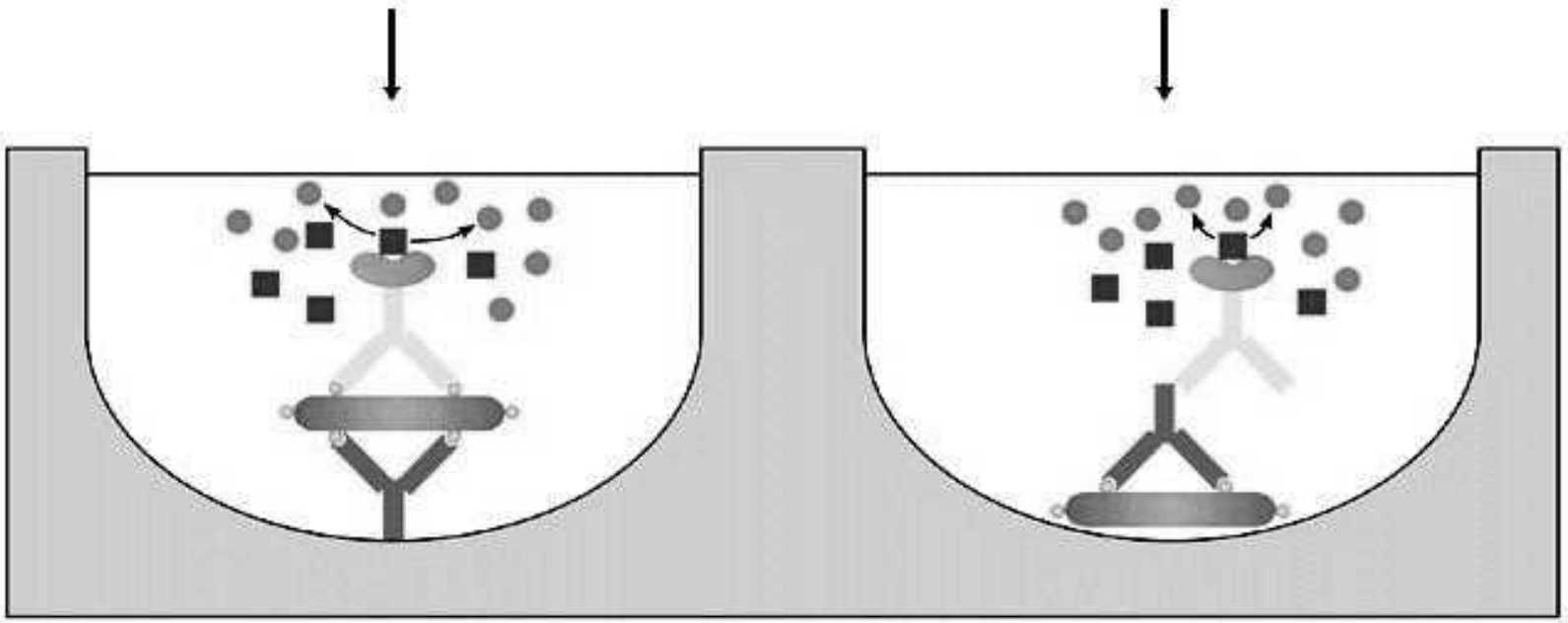
Sandwich ELISA

Use β-galactosidase, Alkaline phosphatase or Horseradish Peroxidase (HRP) linked to Ab, Ag or other molecule



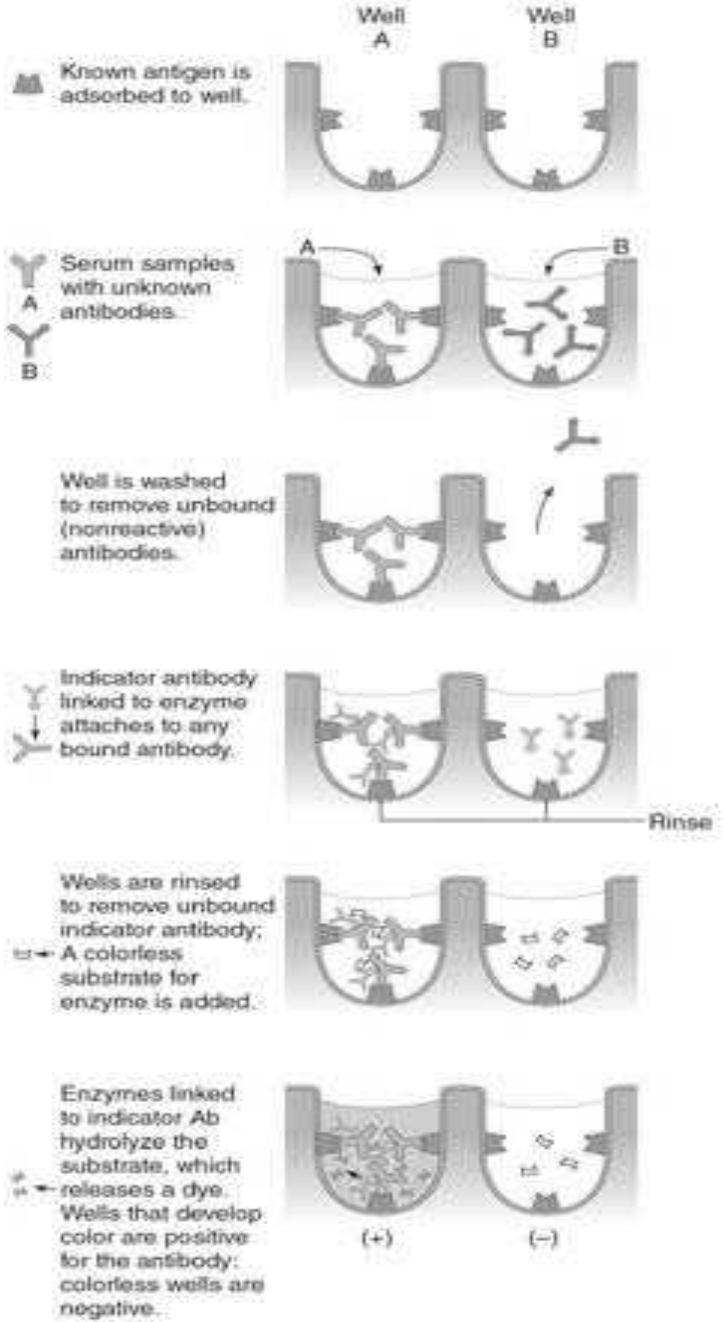
Visualization of Reaction

Methods of ELISA testing (2)

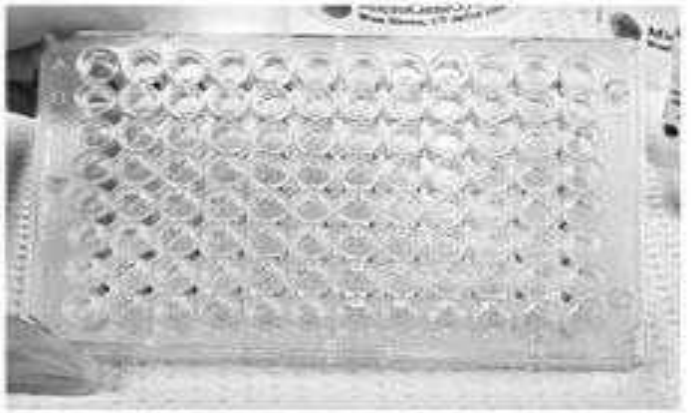


- Sensitive: 10^{-9}gml, simple, cheap & safe

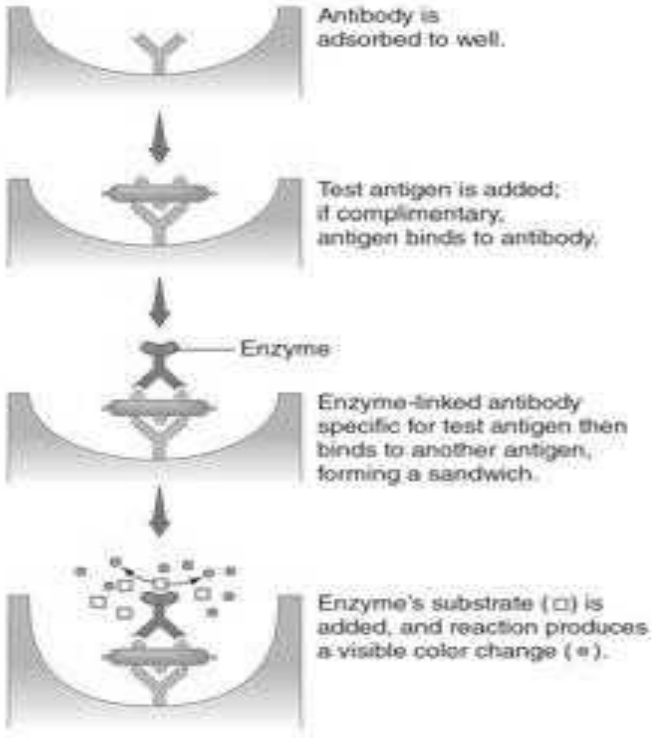
(a) **Indirect ELISA,** comparing a positive vs negative reaction. This is the basis for HIV screening tests.



(b) **Microtiter ELISA Plate with 96 Tests for HIV Antibodies.** Colored wells indicate a positive reaction.



(c) **Capture or Antibody Sandwich ELISA method.** Note that an antigen is trapped between two antibodies. This test is used to detect hantavirus and measles virus.

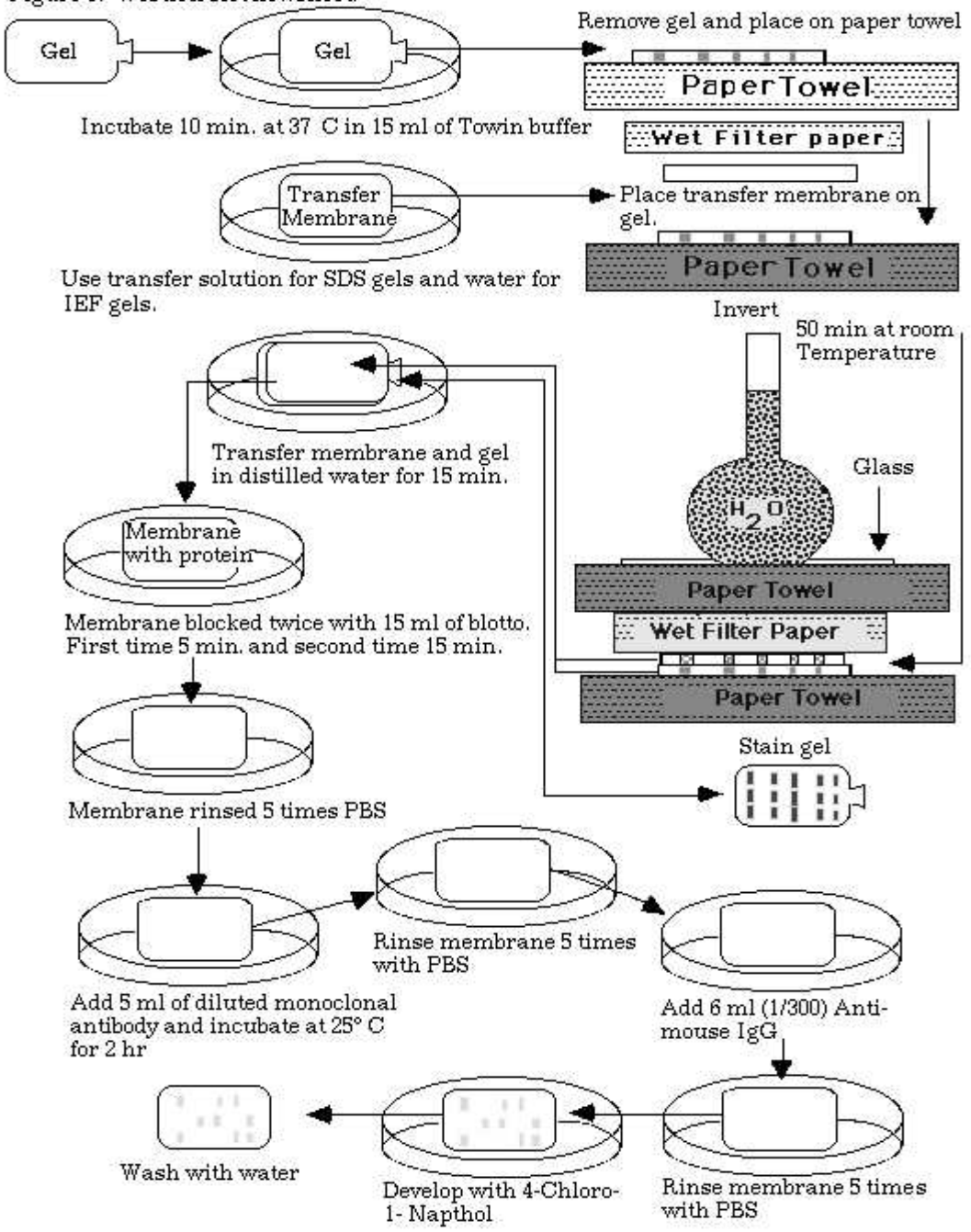


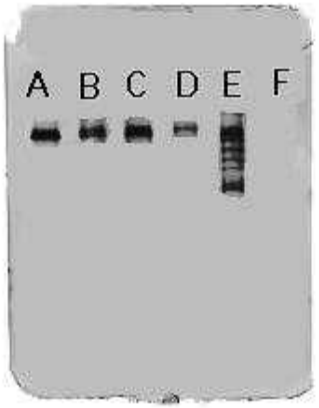
d) Immunoblotting (Western Blotting)

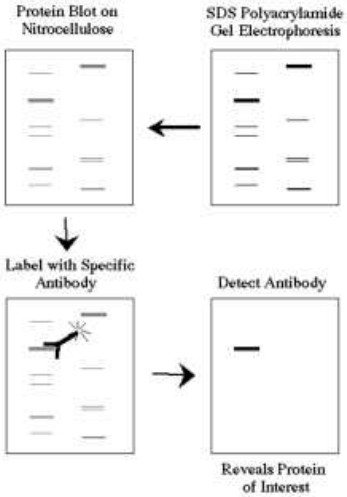
- Western blotting: Proteins
- Southern blotting: DNA
- Northern blotting: RNA

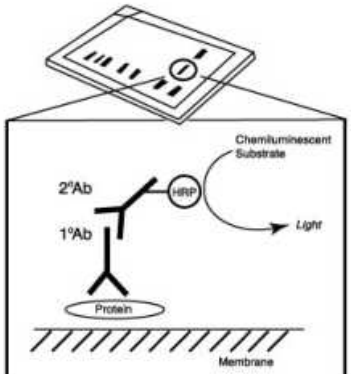
- Utilises radio/enzyme labelled Ab to identify Ag separated by gel electrophoresis
- Can utilise mAbs (poor precipitators)
- More sensitive than precipitation in gel

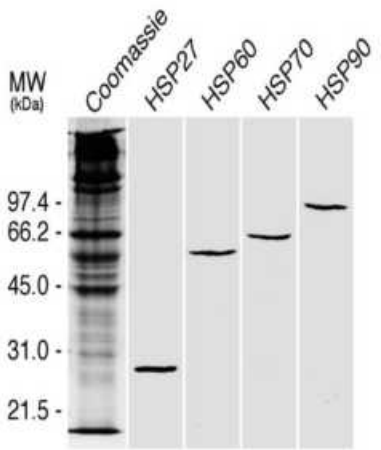
Figure 1: Western blot flowsheet.







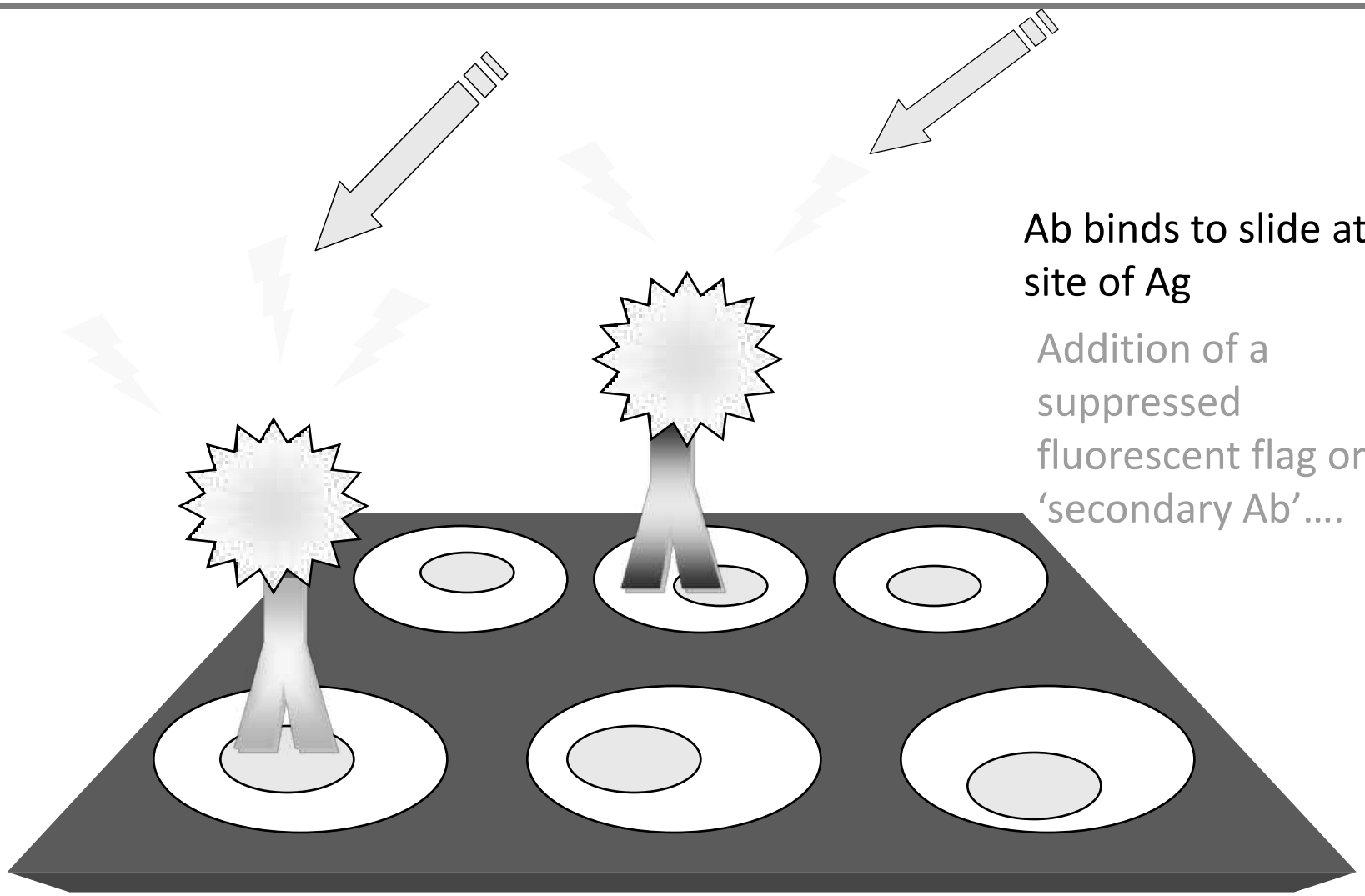




e) Immunofluorescence

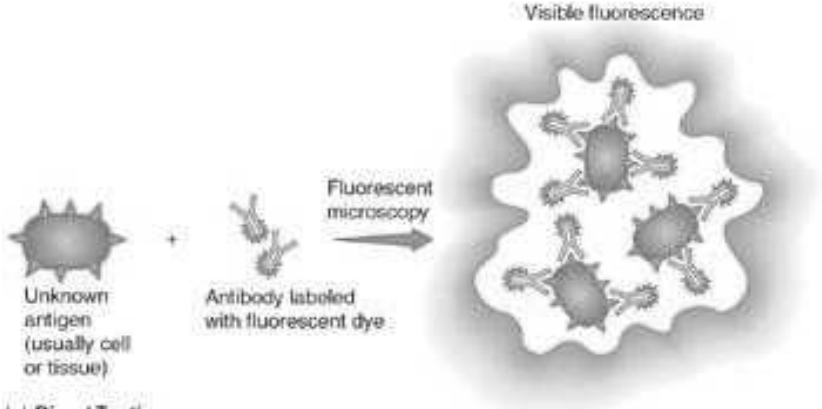
- Uses fluorescent Ab either directly or indirectly to visualise cells or cell aggregates that have reacted with the Abs
- Labels: radio or enzyme labels but more commonly immunofluorescence
- Examine under UV microscope

Immunohistochemistry

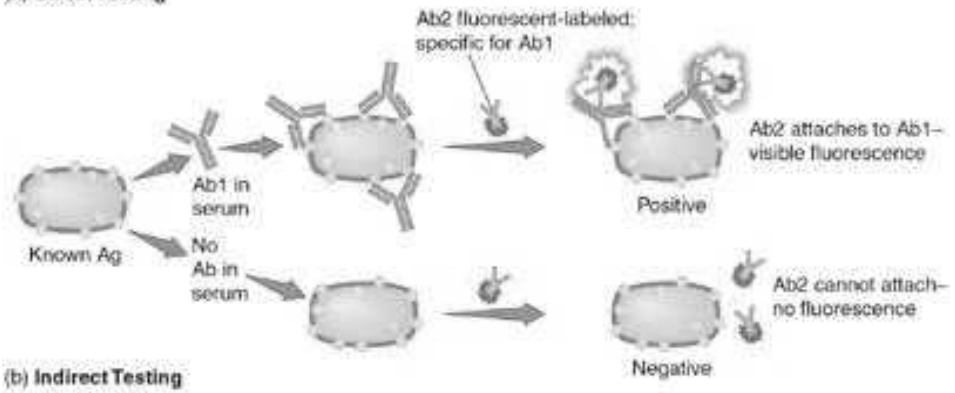


Ab binds to slide at site of Ag

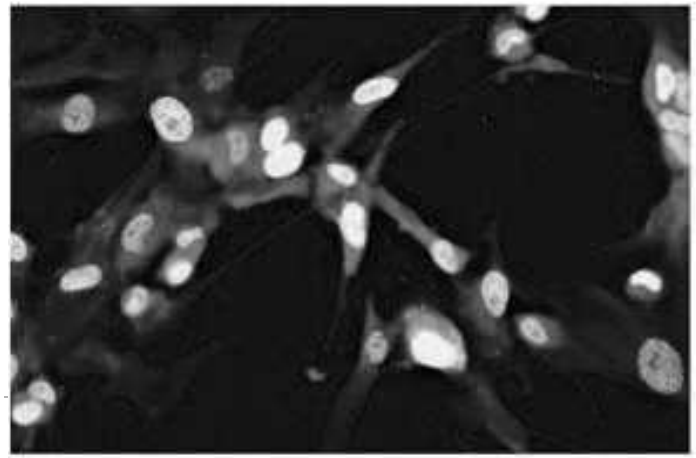
Addition of a suppressed fluorescent flag or ‘secondary Ab’….



(a) **Direct Testing**



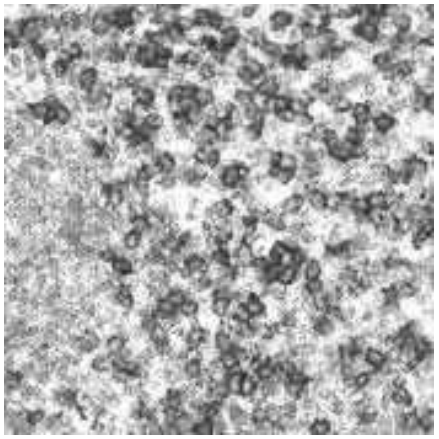
(b) **Indirect Testing**



(c) **Indirect Immunofluorescence Testing**

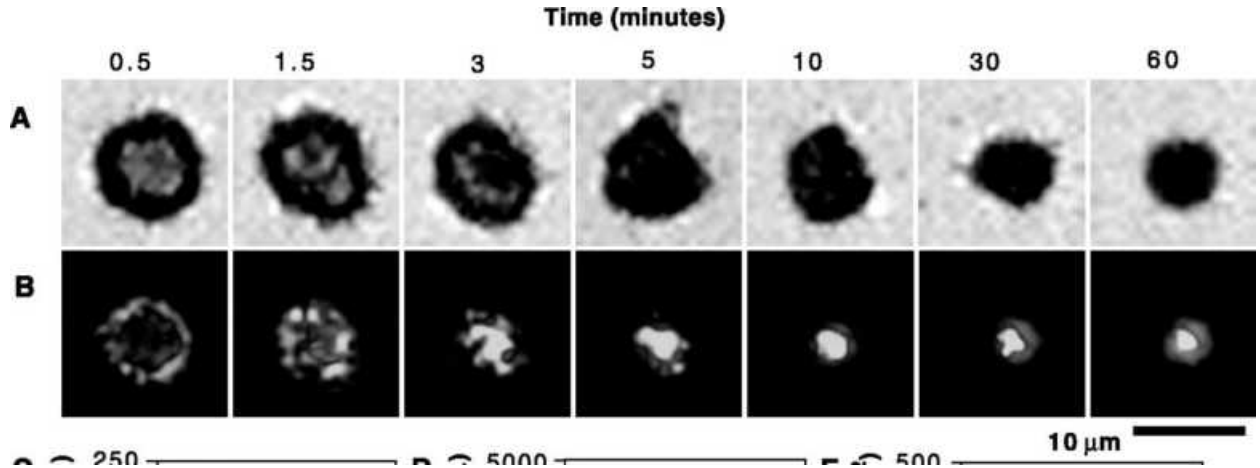
Immunohistochemistry

- Stain for specific markers in a tissue of interest (intracellular and cell surface)
- Preserves the anatomy of the tissue

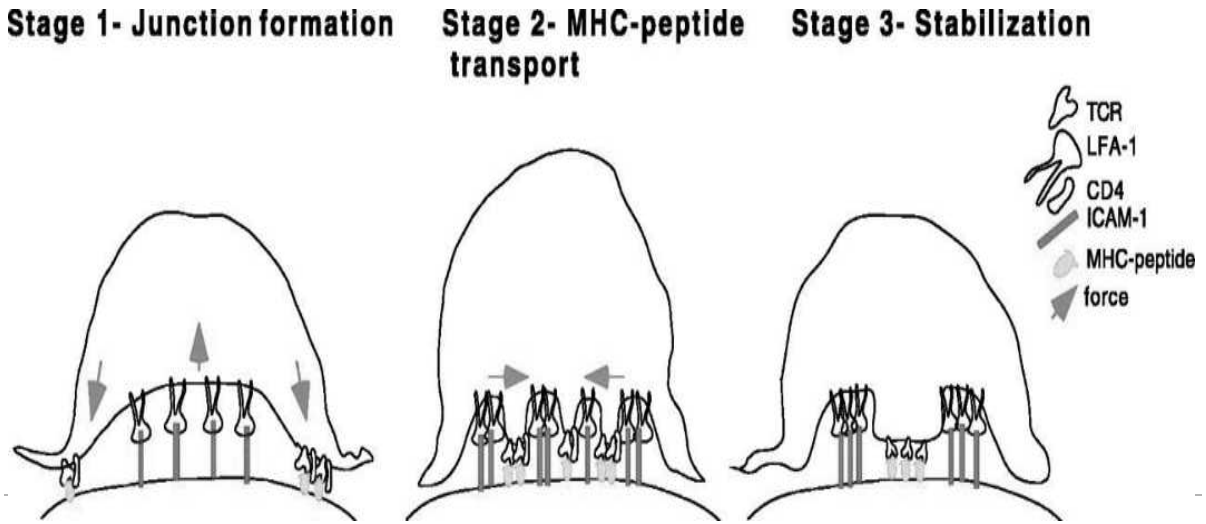


T cells – CD3 staining

Immunofluorescence: *In vitro* imaging

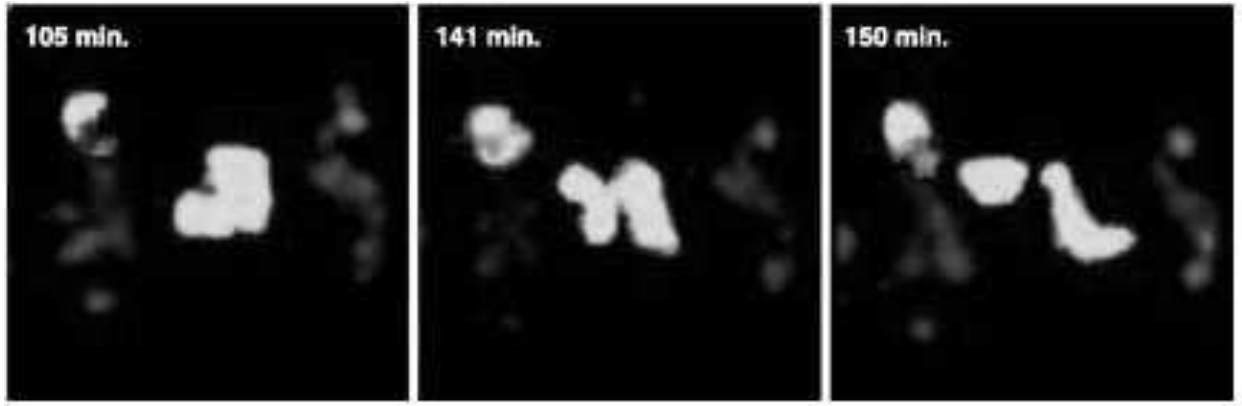


- Immunological synapse imaging after contact between a T cell and a Antigen Presenting Cell (APC)
  - Green: MHC-peptide
  - Red: ICAM-1



Grakoui *et al*., Science 1999

Immunofluorescence: *In vivo* Imaging



- **T cell division**
  - **Green: T cell**
  - **Red: Dendritic cell**

Delon *et al.*, Immunol Rev 2002

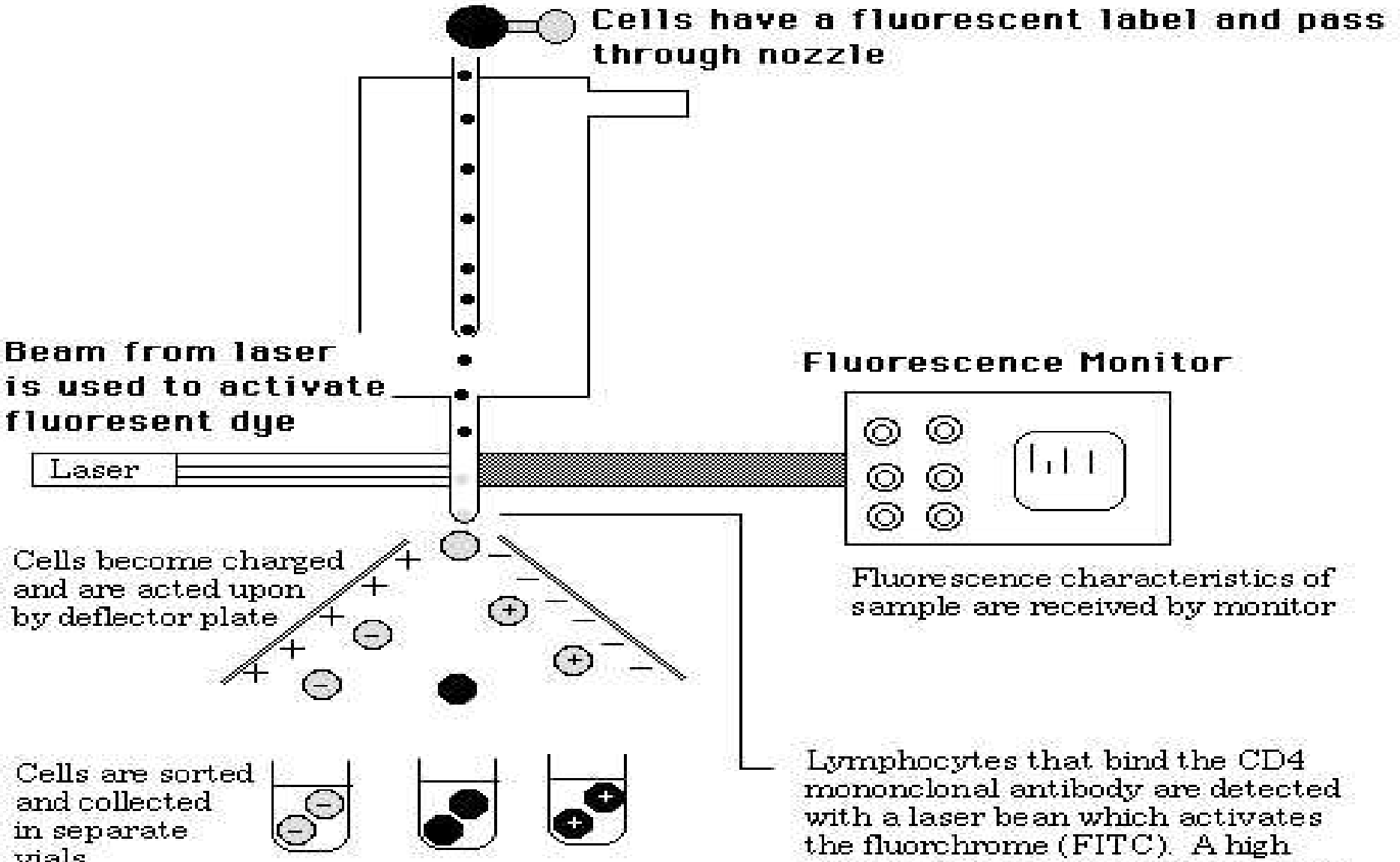
f) Leukocyte Assays using labelled Abs

- Cells can be analysed & isolated on basis of their distinct surface Ags, size, or both by Flow Cytometry.

- Flow Cytometers are instruments that can analyse properties of single cells as they pass through an orifice at high velocity. These instruments measure light scatter, volume, & fluorescence.

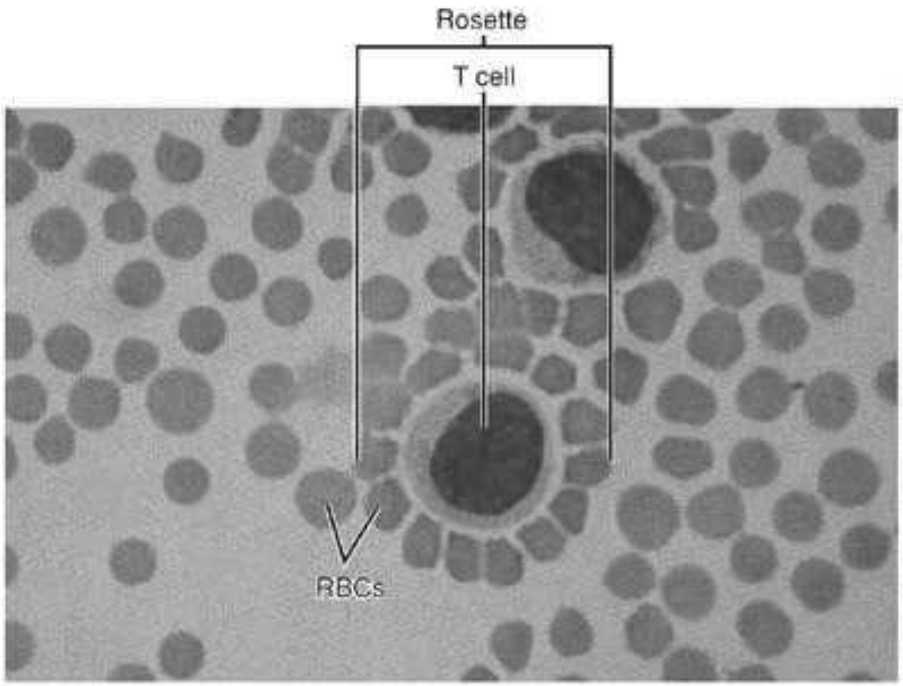
- FACS can analyse & sort lymphocyte sub populations, as identified by fluorescein-labelled mAb

Fluorescence-Activated Cell Sorter (FACS)

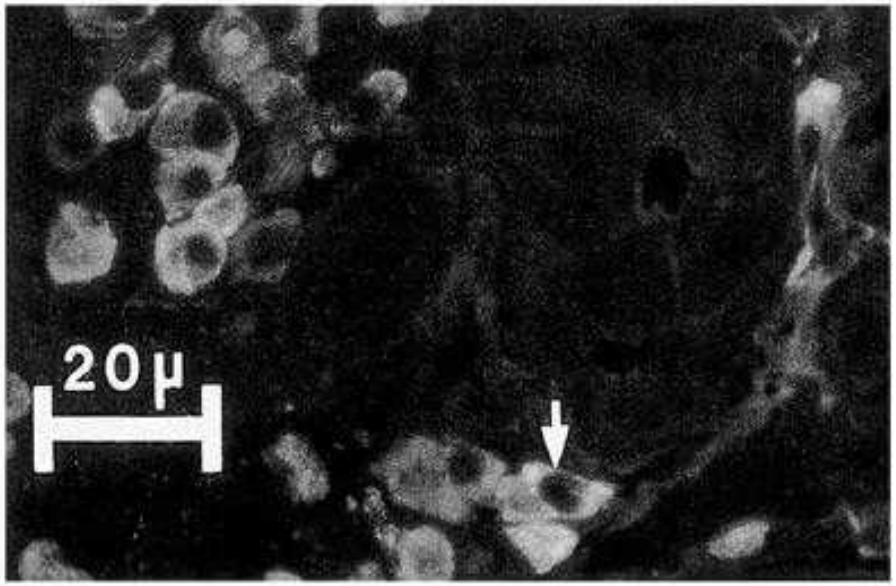


Rosette & Plaque Formation

- Tests can differentiate B cells from T cells and their subtypes



(a)



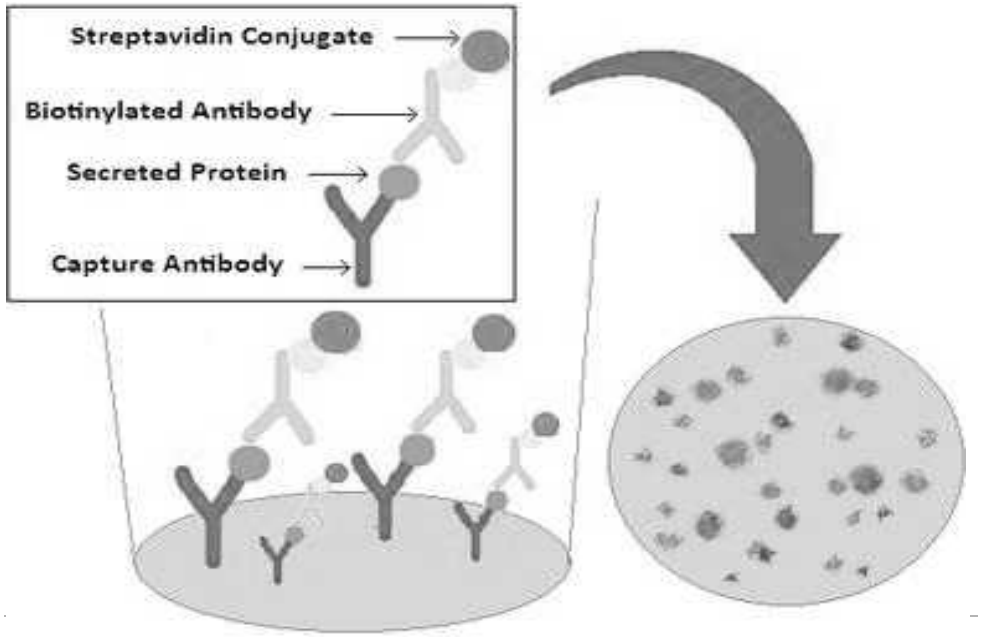
(b)

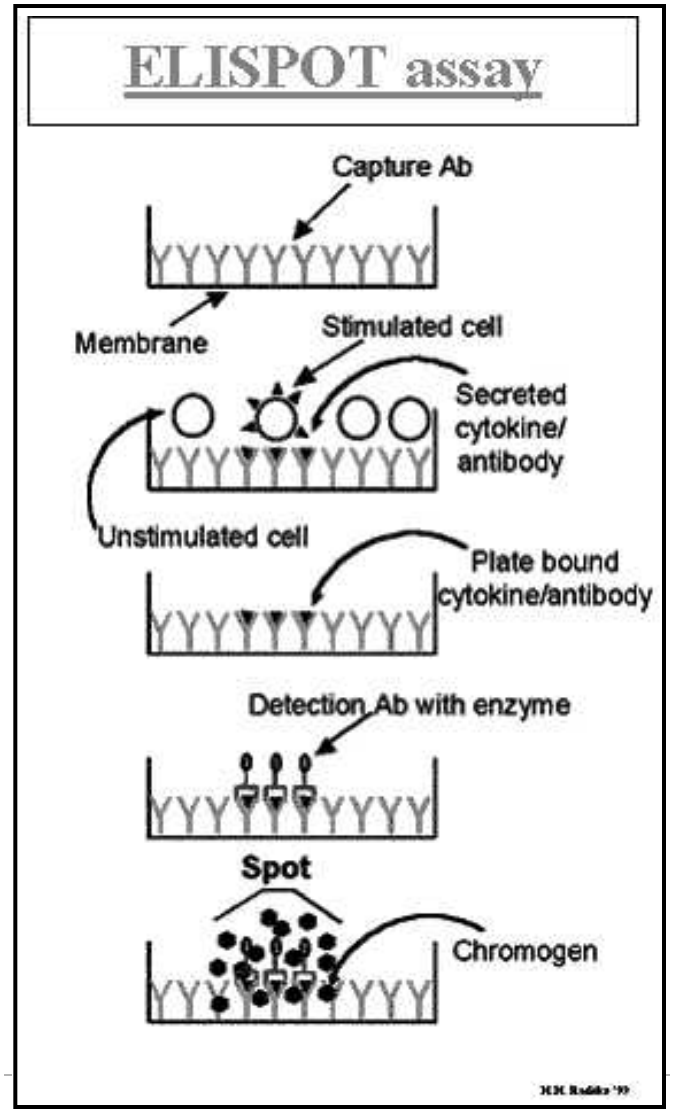
ELISPOT Assays

- PBMC are plated on a filter-bottom 96-well plate coated with anti-cytokine antibody.
- The plate is cultured for 24-48 hours to allow cytokine secretion and capture on the plate.
- Cells are washed off and detector antibody is added, followed by enzyme substrate.
- Cytokine-secreting cells are identified as spots of secreted cytokine.

Enzyme-Linked Immunospot (ELISPOT)

- Detect cells secreting a specific antigen
- Most commonly used to detect cytokine secretion by T cells upon stimulation (IFNγ, IL2, IL4, etc.)
- Can detect cytotoxic activity (perforin)



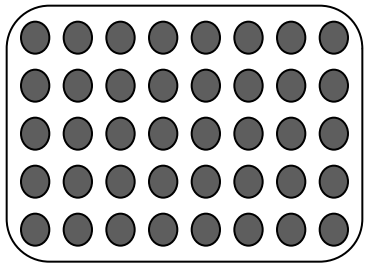


ELISPOT Assay Principle

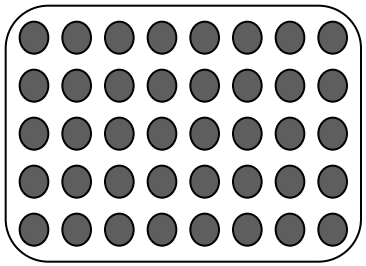
Prepare PBMC and count

Add PBMC

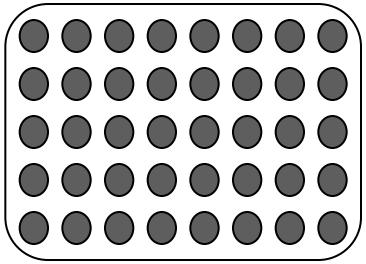
Wash out cells, add detector Ab



wash



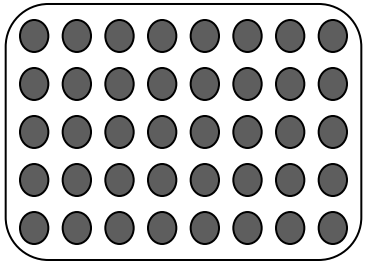
24 h



Coat plate with anti-cytokine Ab

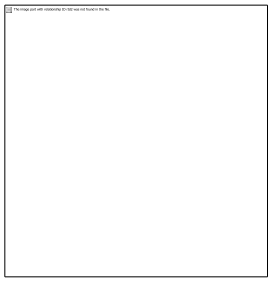
Add Ag

1 h



15 min

Wash, add substrate

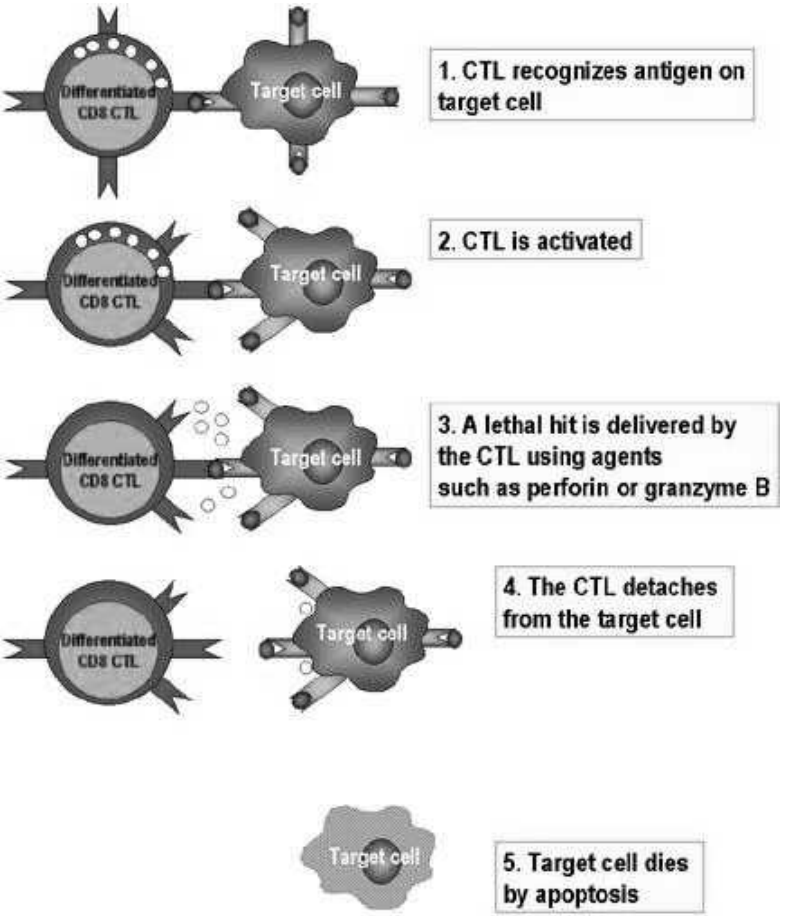


Count on dissecting microscope

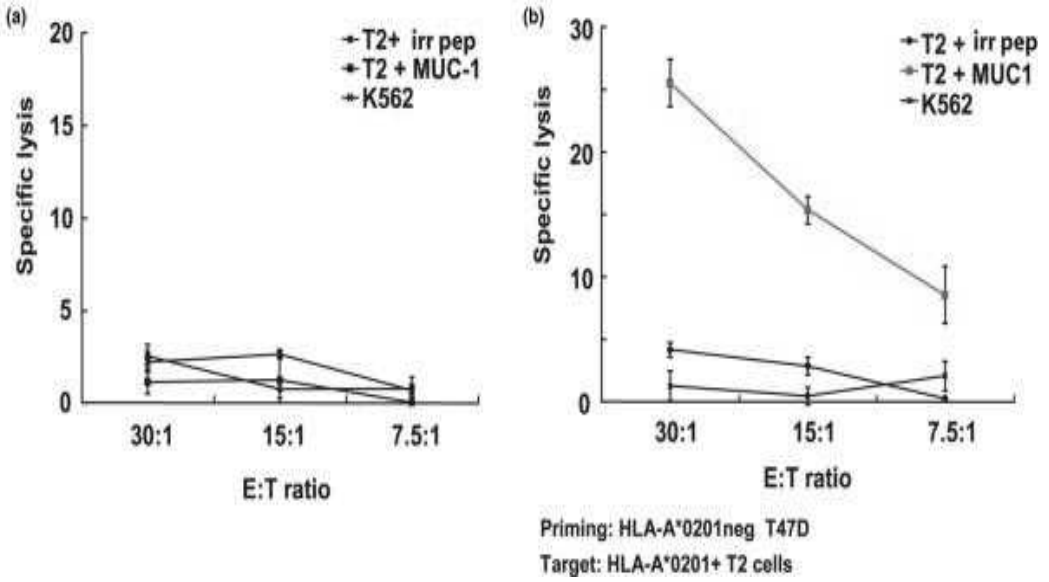
or

Analyse on automated reader

Cytotoxic T Lymphocyte (CTL) Assay



- Evaluate cell cytotoxicity against a specific target cell
- Target cells are labelled with radioactive compounds (Cr_{51}) or non-radioactive compounds (Europium)
- After being killed by the CTLs, target cells release the labelling compound, which is detected



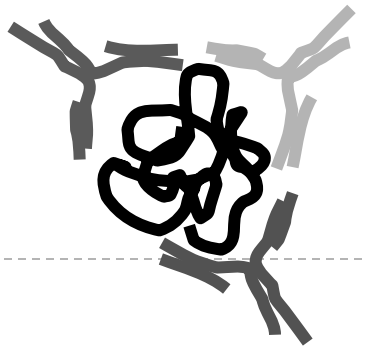
Diagnostic and Therapeutic use of Antibodies

Antibodies

Polyclonal

Antibodies that are collected from sera of exposed animal

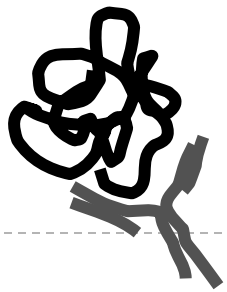
recognize multiple antigenic sites of injected biochemical.



Monoclonal

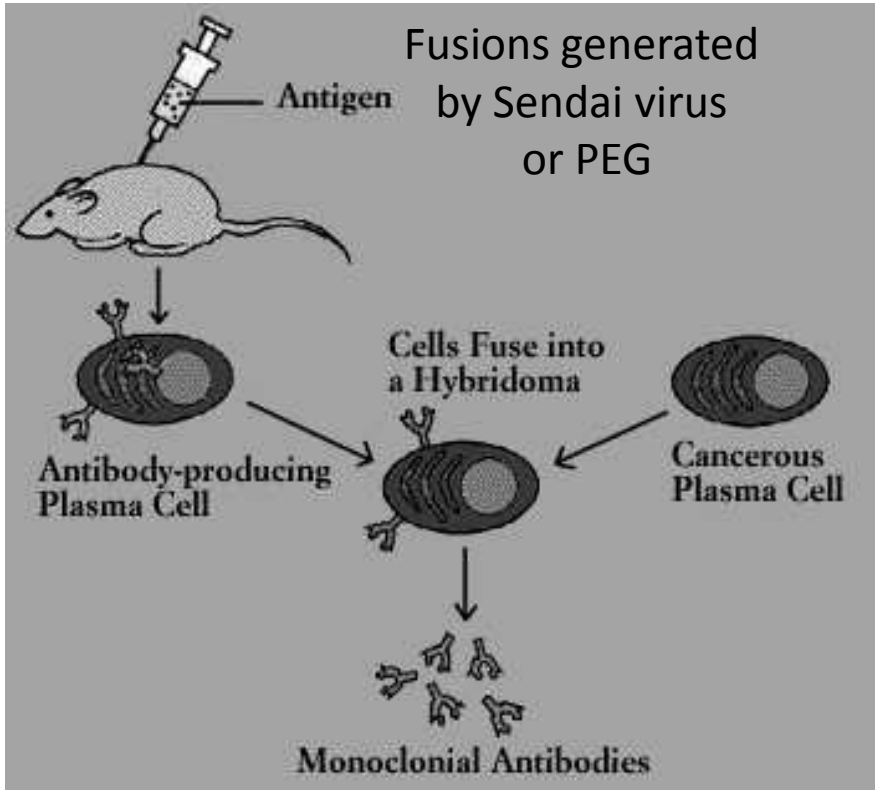
Individual B lymphocyte hybridoma is cloned and cultured.
Secreted antibodies are collected from culture media

recognize *ONE* antigenic site of injected biochemical

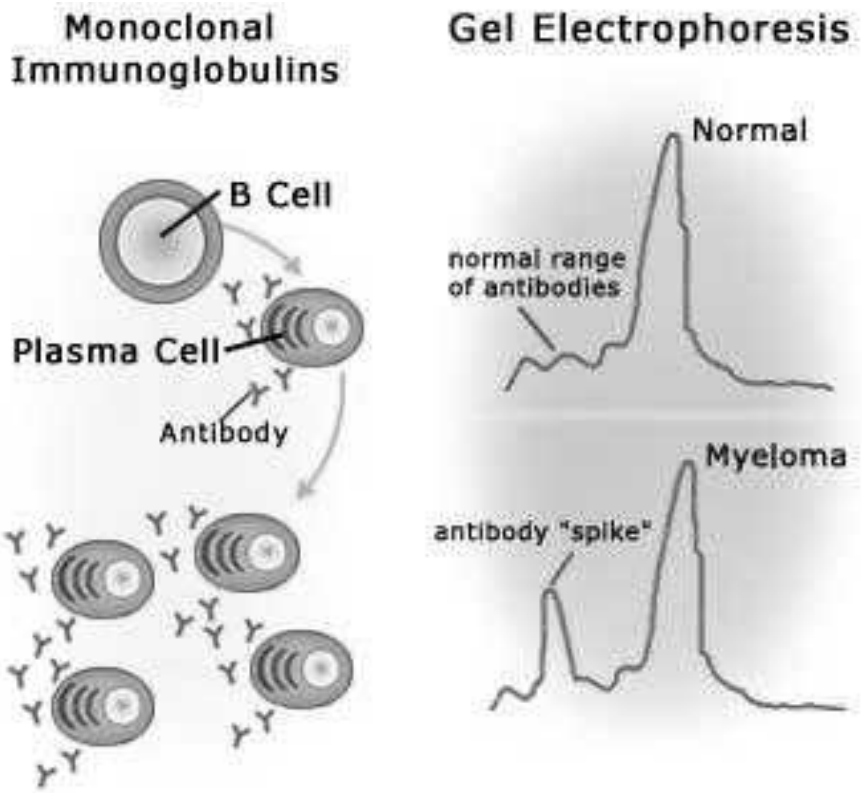


Hybridoma Technology

1975 Kohler and Milstein



Multiple myeloma (human disease)



Bence-Jones proteins in urine

http://www.immunecentral.com/images/immune_series/immune29.gif

Hybridoma Selection (HAT Media)

The de novo pathway can be inhibited using aminopterin,
which inhibits the transfer of methyl groups from activated dihydrofolic acid.

cells need Hypoxanthine and Thymine as sources of purines and pyrimidines for the salvage pathway.

The enzyme hypoxanthine-guanine phosphoribosyltransferase (*HGPRT*) is one of the central enzymes that recycle the building blocks of RNA and DNA

Myeloma cells are HGPRT- and cannot create nucleotides in the salvage pathway.

Plasma cells are HGPRT+ and can utilize hypoxanthine in the salvage pathway.

Creation of Monoclonal Antibodies (mAbs)

1. Hyperimmunise mouse with Ag

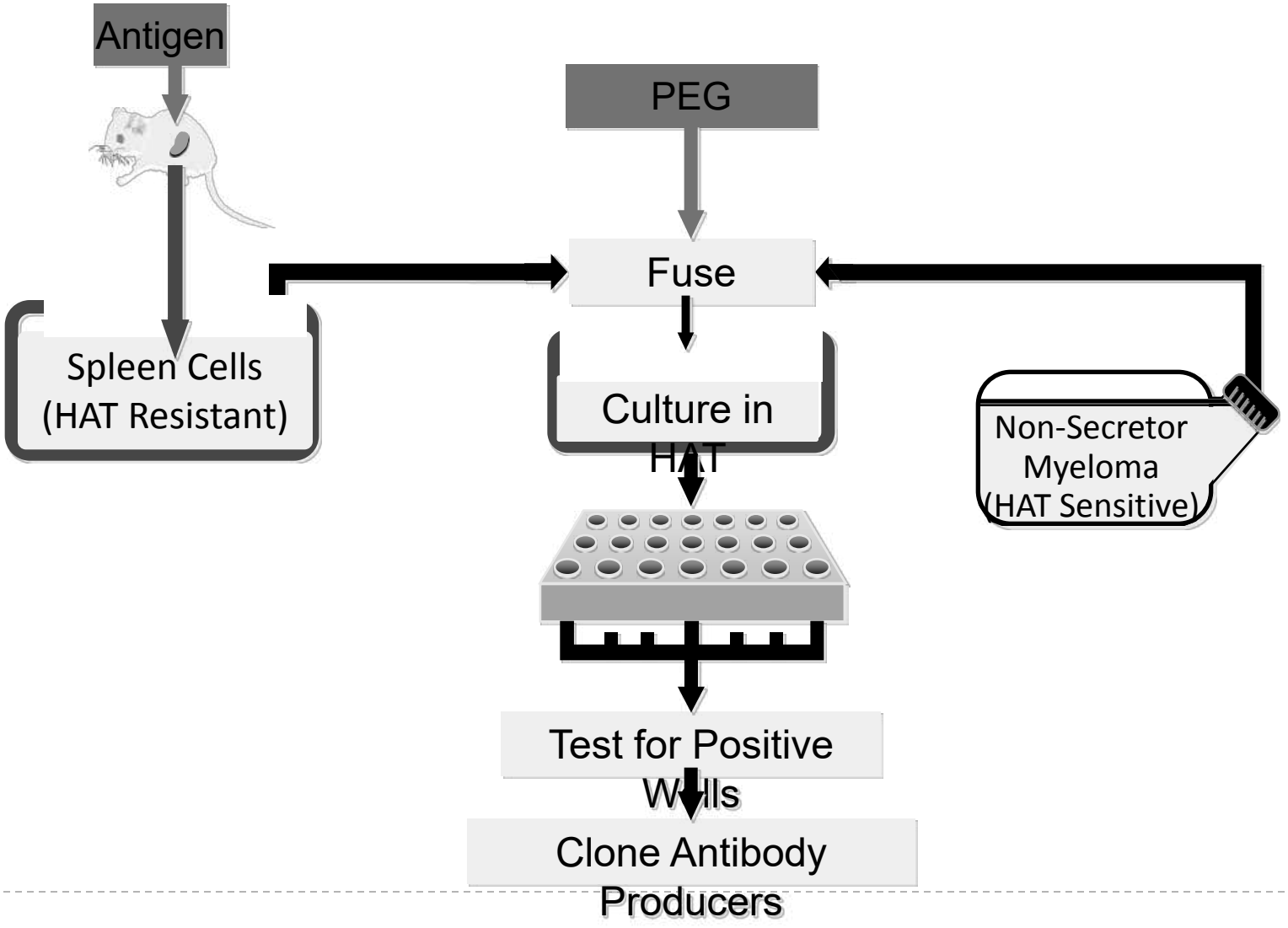
2. Fuse B cells with tumour fusion partner (+ PEG)

3. Limiting dilution (96 wells)
to fractionate fused cells
in HAT media selection

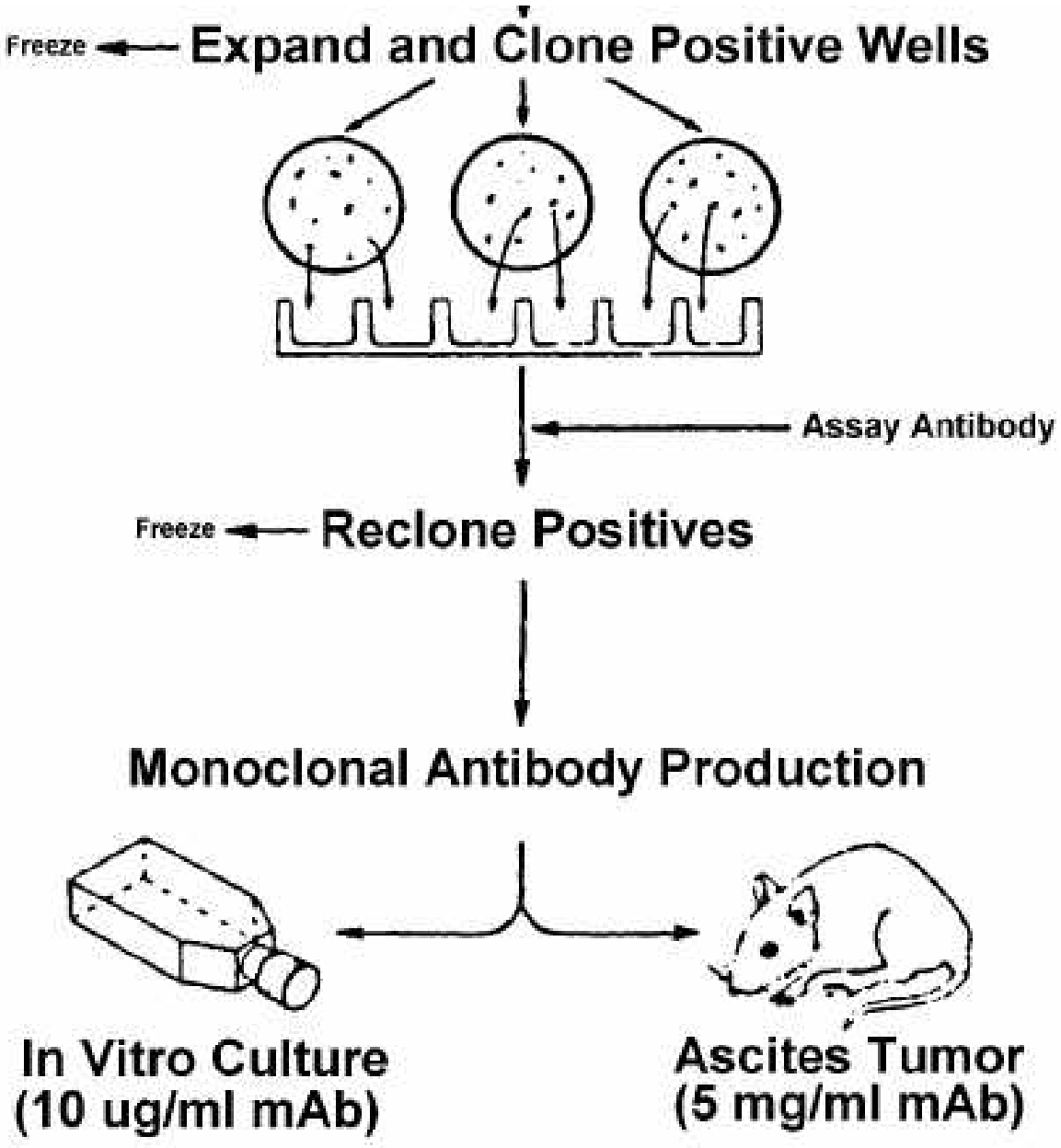
HAT media (hypoxanthine, aminopterin, thymidine).

B cells die (mortal, HGPRT-positive)
Tumour cells die (HGPRT deficient and cannot utilise salvage pathway)
Fusions live (HAT Resistant and immortal)

Monoclonal Antibody Production



Expand in ice or expand *in vitro*

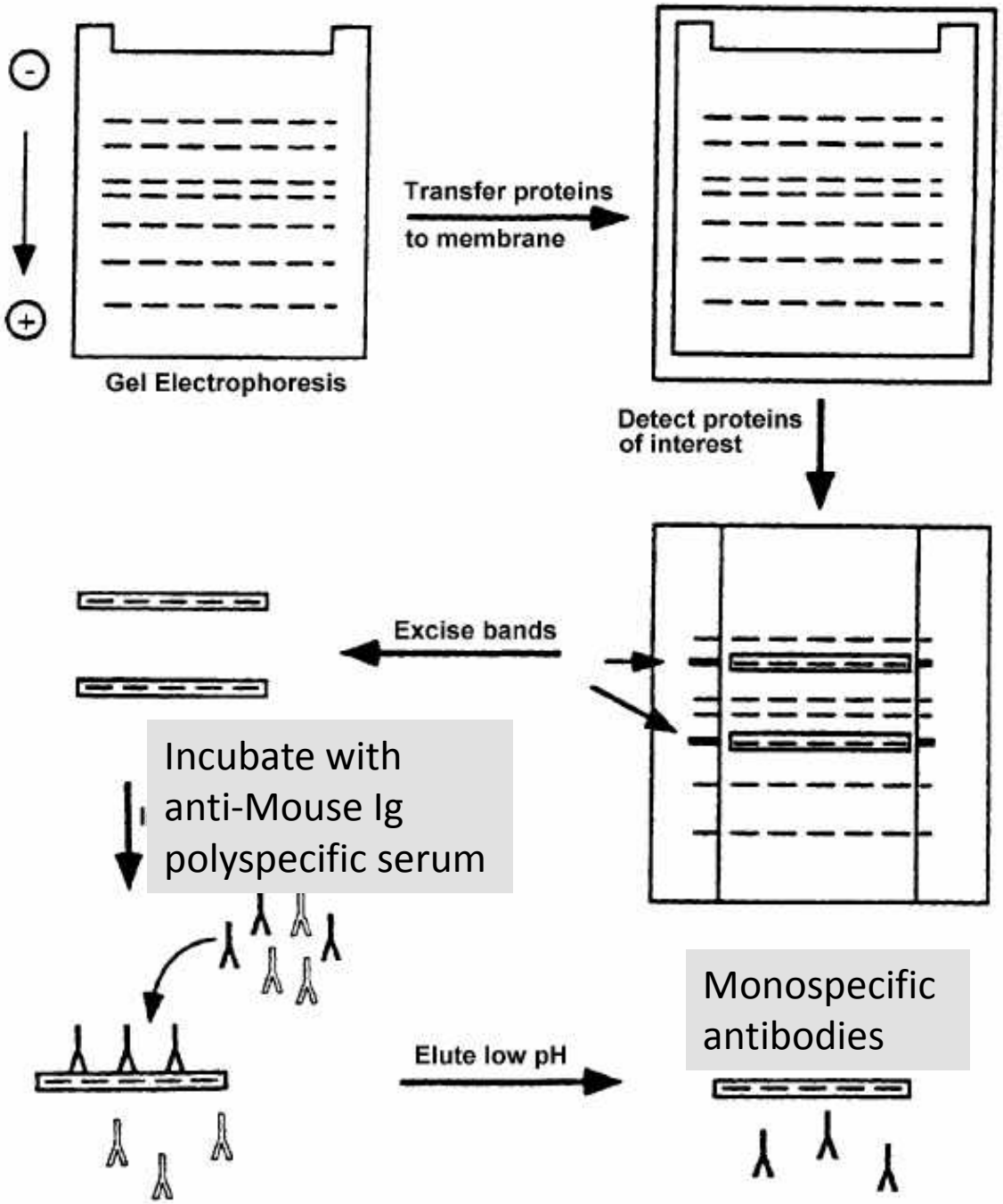


***in vitro* material is less concentrated and contains bovine serum**

ascites fluid contain high [mAb] and minor contamination with mouse Ig

http://www.tulane.edu/~wiser/methods/handouts/pwpt/17_mab.pdf

How to purify your mAb



Affinity Purification

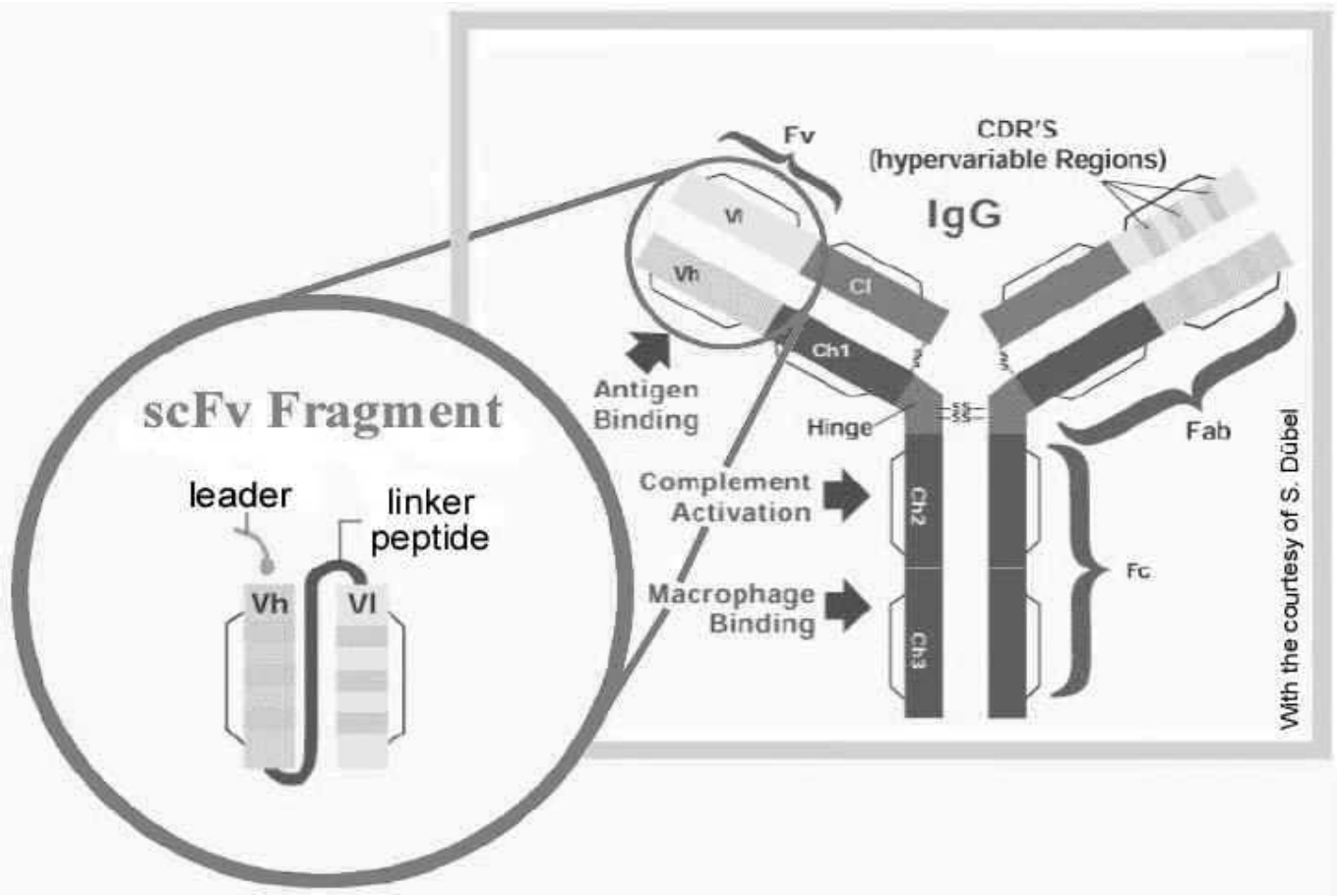
Or use Ag or epitope
to make
affinity column

http://www.tulane.edu/~wiser/methods/handouts/pwpt/17_mab.pdf

Uses of Monoclonal Antibodies

- Protein purification
- Identification and isolation of cell sub-populations using fluorescence cell sorting.
- Tumour detection and imaging
- Tumour killing
- Diagnostic reagents.
- Drug Detoxification
- Catalytic antibodies

Single chain Fv fragments



The size and specificity of the ScFv fragment may allow for attachment to cryptic sites

http://www.gene-therapeutics.com/Service/custom%20scFv%20antibodies/Abb-scFv.gif

Introduction to Flow Cytometry

What Is Flow Cytometry?

- Flow Cytometry is a rapid, accurate, and objective way to simultaneously measure multiple characteristics of a single particle, usually a cell.
- FACS – **F**luorescence **A**ctivated **C**ell **S**orter is the generic term used for Flow Cytometry (even without sorting)
- Measurements are made on a per-cell basis at routine rates of 500 to 4000 cells per second

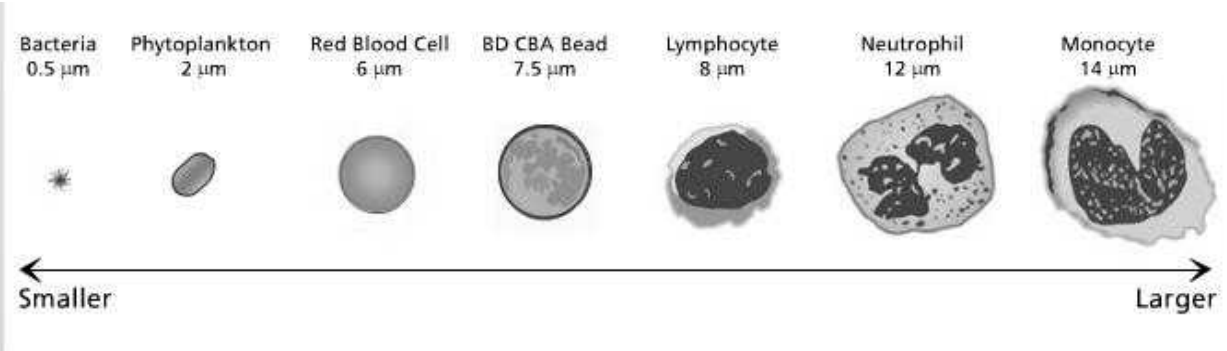
Flow Cytometry

- Web course: http://www.bdbiosciences.com/us/support/s/itf_launch

- Flow: fluid
- Cyto: cells
- Metry: measurement
  - Cells
  - Chromosomes
  - Bacteria
  - Beads

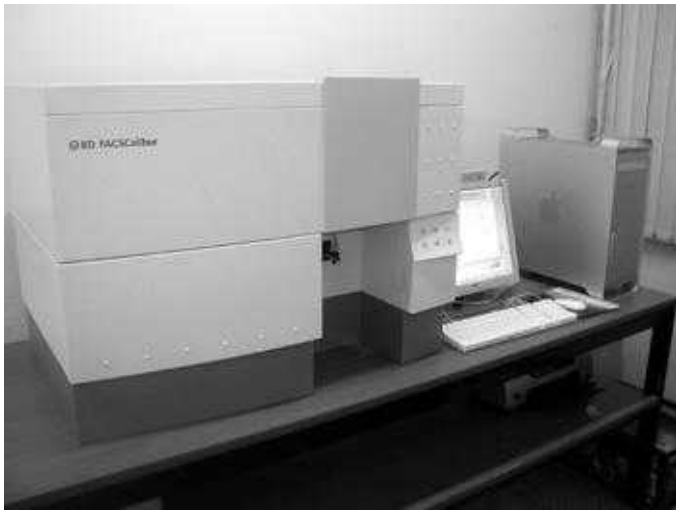
Flow cytometer comprise

- Fluidics
- Optics (lasers and filters)
- Electronics



Flow Cytometry

- Simultaneous analysis of different physical parameters in a single cell
- Can analyse up to several thousands of cells per second
- Versatile, sensitive



What is in a Flow Cytometer?

- Fluidics
  - To introduce and focus the cells for interrogation by a laser
- Optics
  - To generate and collect the light signals (scatter and fluorescence)
- Electronics
  - To convert the optical signals to proportional electronic signals and digitise them for computer analysis (PMTs)

PMTs = photomultiplier tubes

Three Subsystems

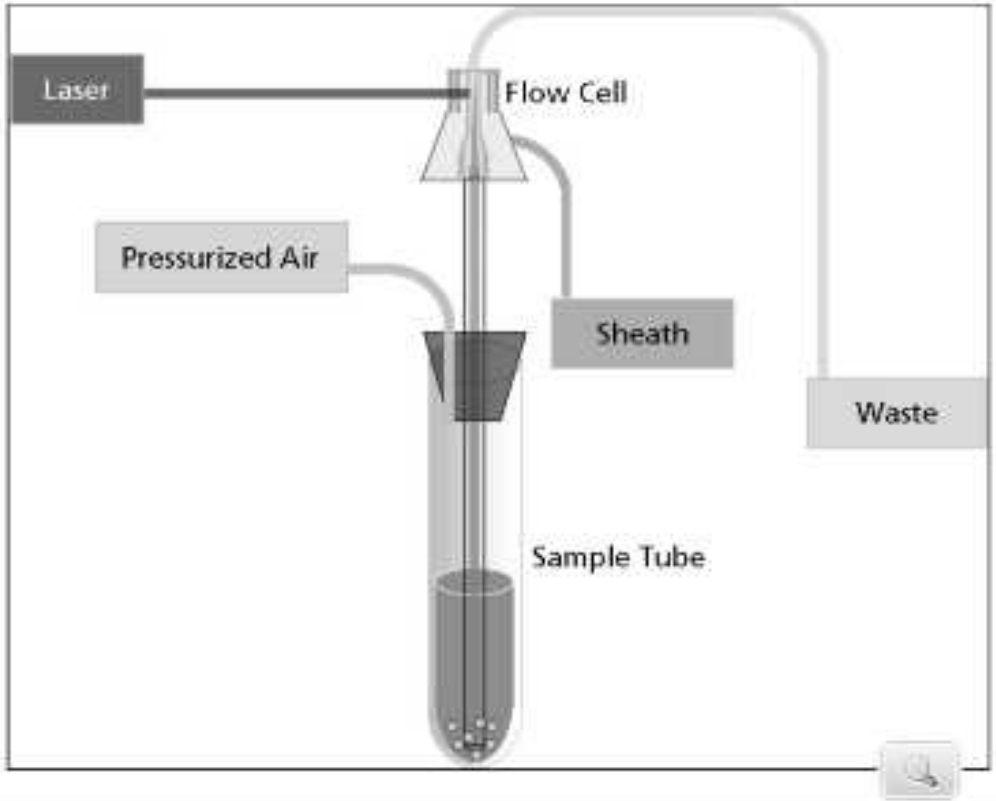
Fluidics

Optics

Electronics

Fluidics

The fluidics subsystem brings the particles of interest to the interrogation point where they interact with the excitation



Three Subsystems

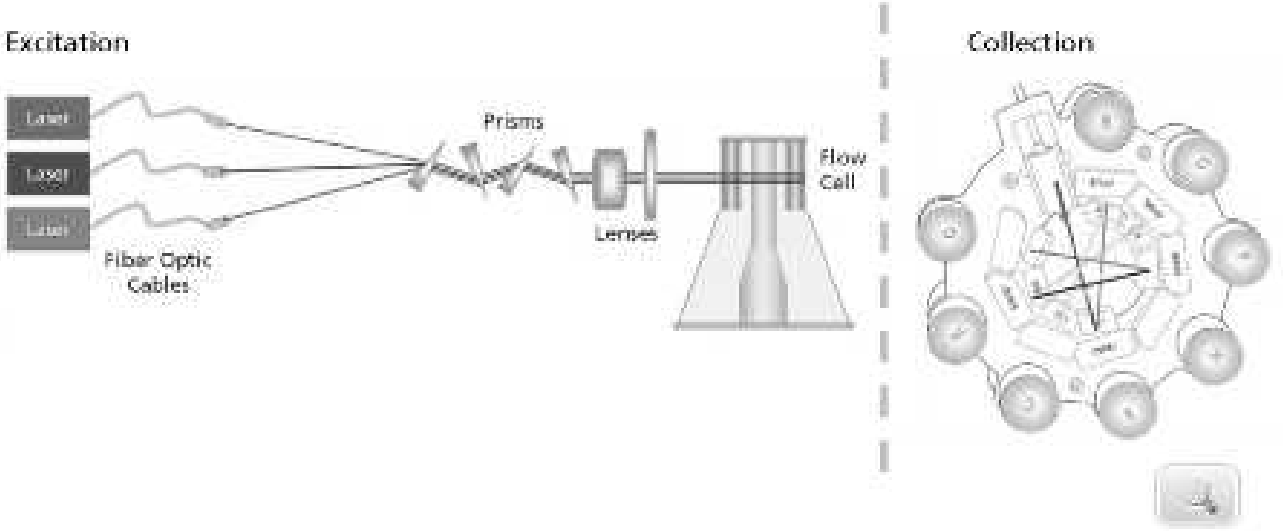
Fluidics

Optics

Electronics

Optics

The optics subsystem provides the excitation sources and the components to collect light signals and route them to the appropriate detectors.



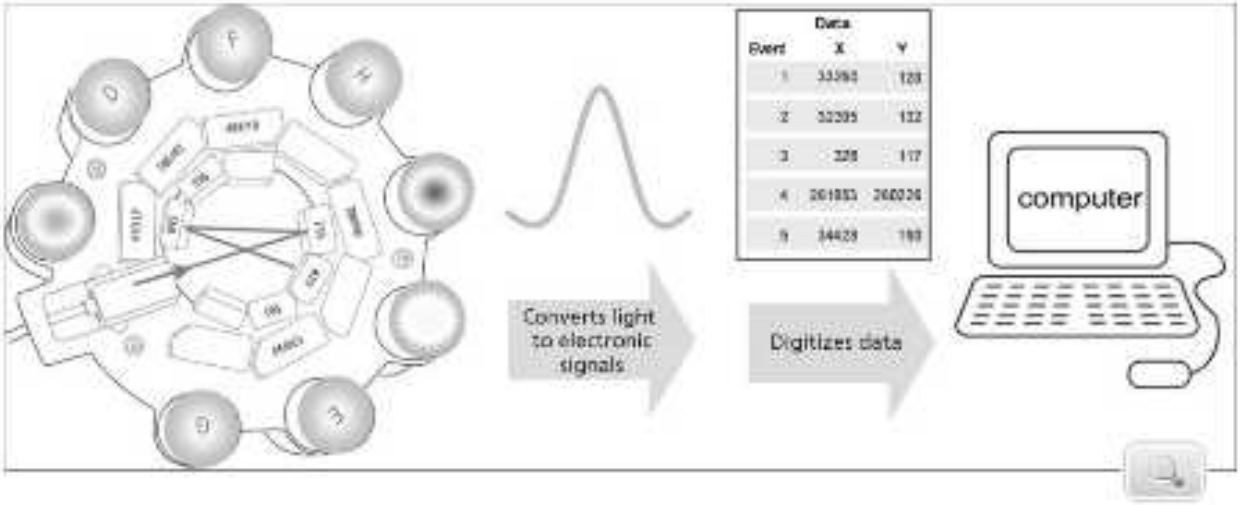
Three Subsystems

Fluidics

Optics

Electronics

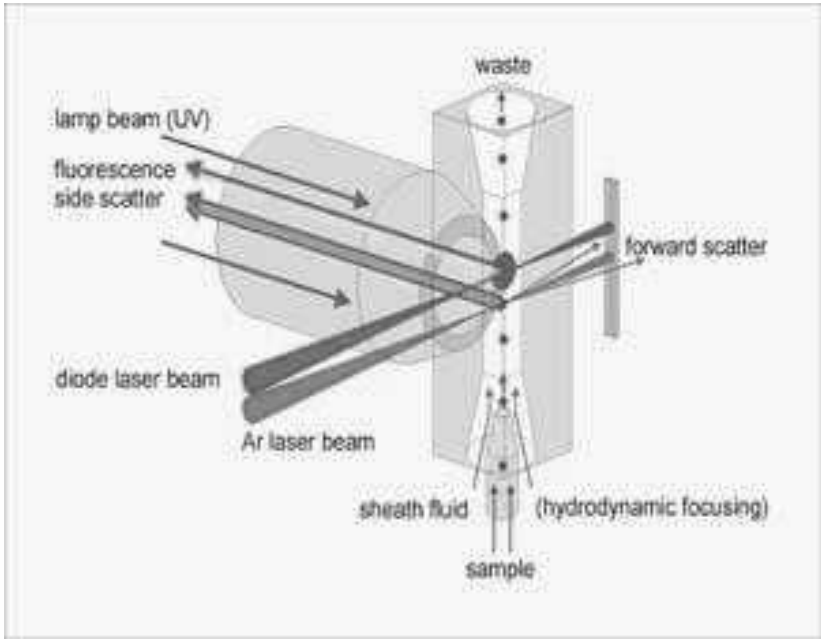
The electronics subsystem converts light signals to equivalent electronic signals.

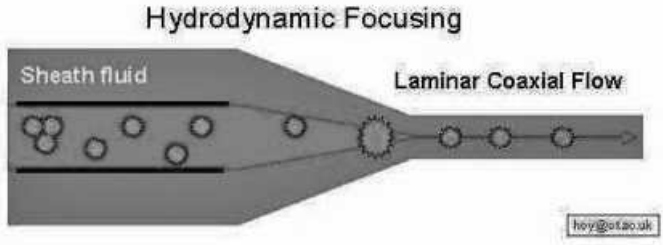


Electronics

Principle of Flow Cytometry

- Cell sample labelled with appropriate fluorescent Abs
- Cells in suspension are passed through machine in single file in a stream of fluid
- Stream is focused through one or more laser beams, measuring light scatter and fluorescence characteristics
- Fluorescence detected by photomultiplier tubes (PMTs)
- Signals sent to computer for analysis

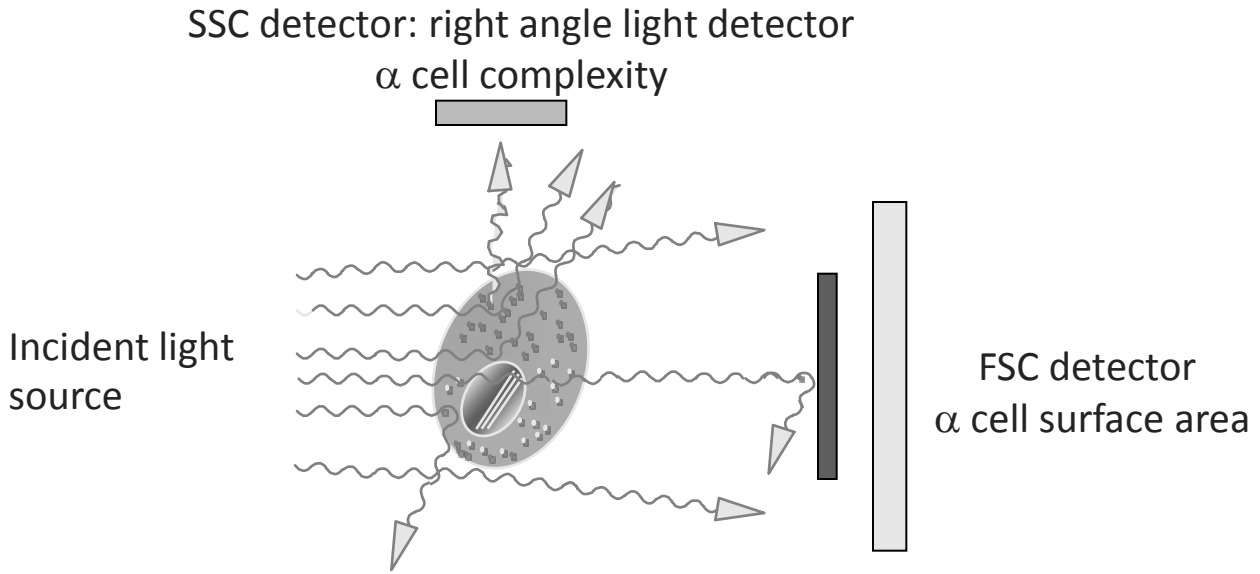




What Can a Flow Cytometer Tell Us About a Cell?

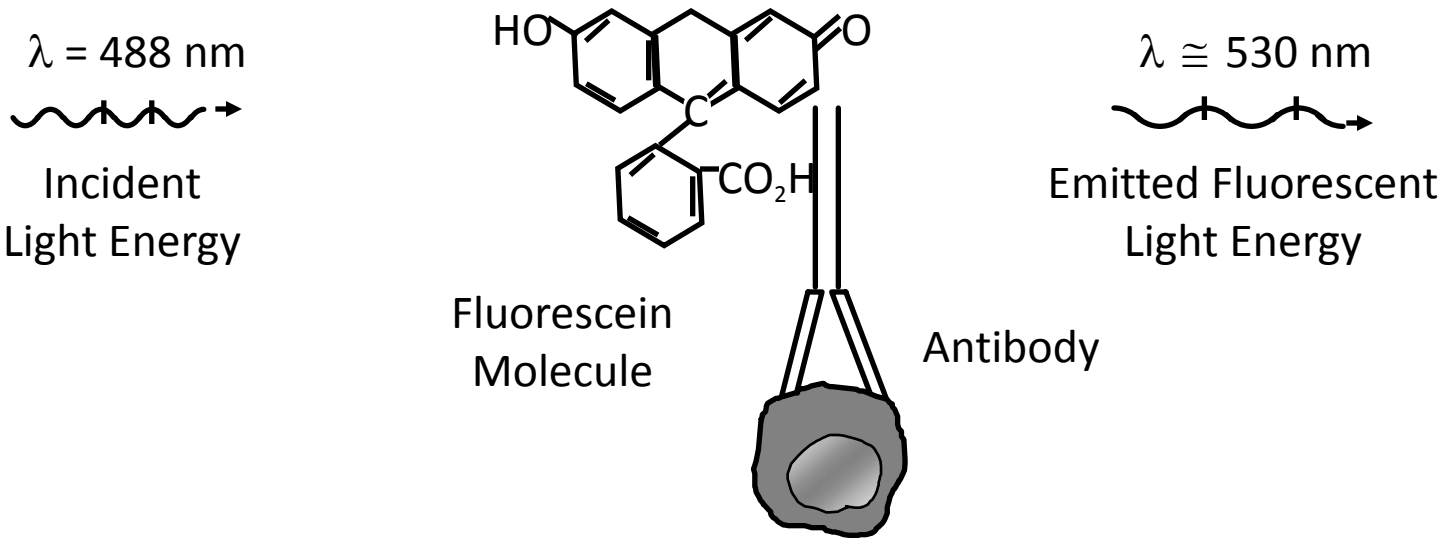
- Forward Scatter (FSC): Its relative size
- Side Scatter (SSC): cell internal complexity (relative granularity)
- Fluorescent labelling of cell surface or intracellular structures using fluorescent antibodies: allows investigation of cell molecules and function (Its relative fluorescence intensity) (FLI, FL2, FL3, FL4, etc..)

Properties of FSC and SSC



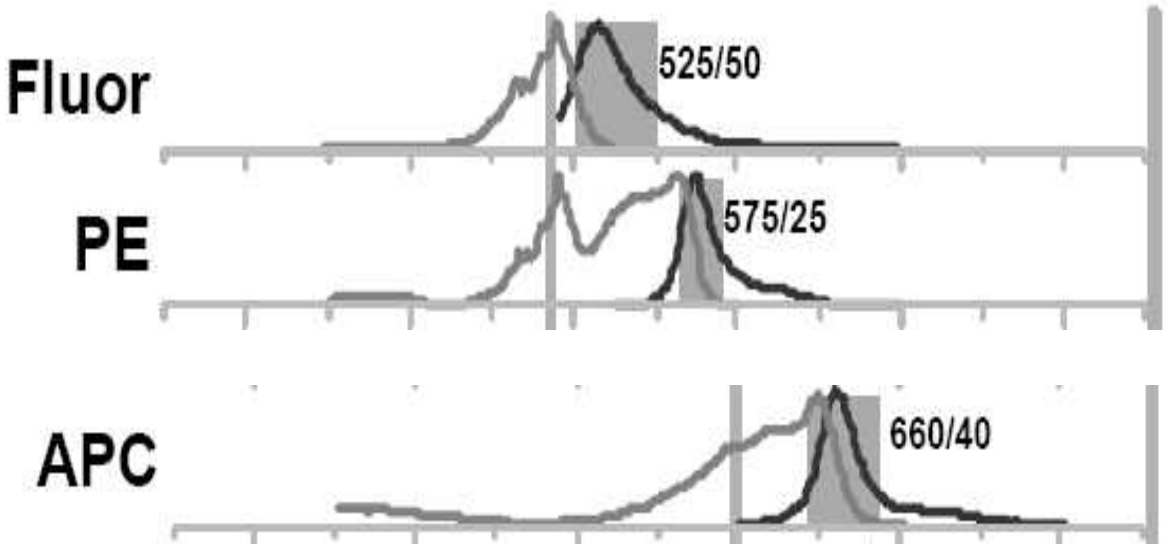
- Forward Scatter
  - Diffracted light
  - Size of the cell
  - Related to cell surface area
- Side Scatter
  - Reflected light
  - Light reflecting from cellular components
  - Related to cell granularity and complexity
  - Detected at 90° to the laser beam

What is Fluorescent Light?



- Antibodies can be conjugated to fluorochromes
- The fluorochrome absorbs energy from the laser
- The fluorochrome releases the absorbed energy by:
  - Vibration and heat dissipation
  - Emission of photons of a longer wavelength
- The amount of fluorescent signal detected is proportional to the number of fluorochrome molecules on the particle

Fluorochromes: Excitation and Emission Spectra

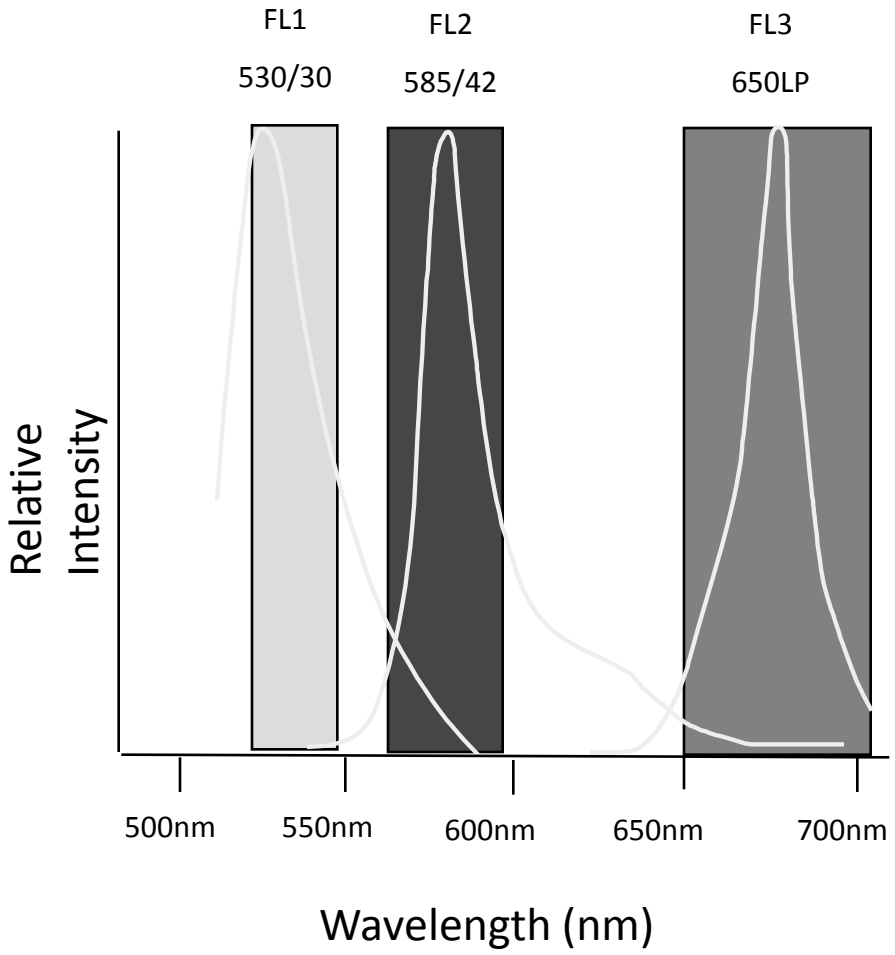


Excitation spectrum

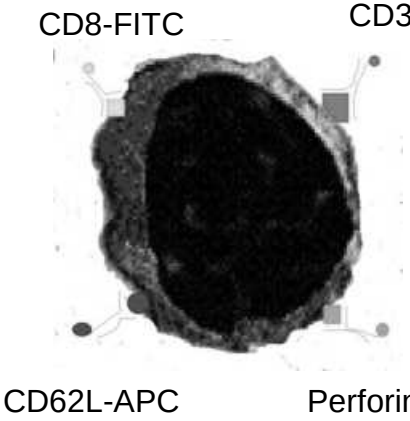
Emission spectrum

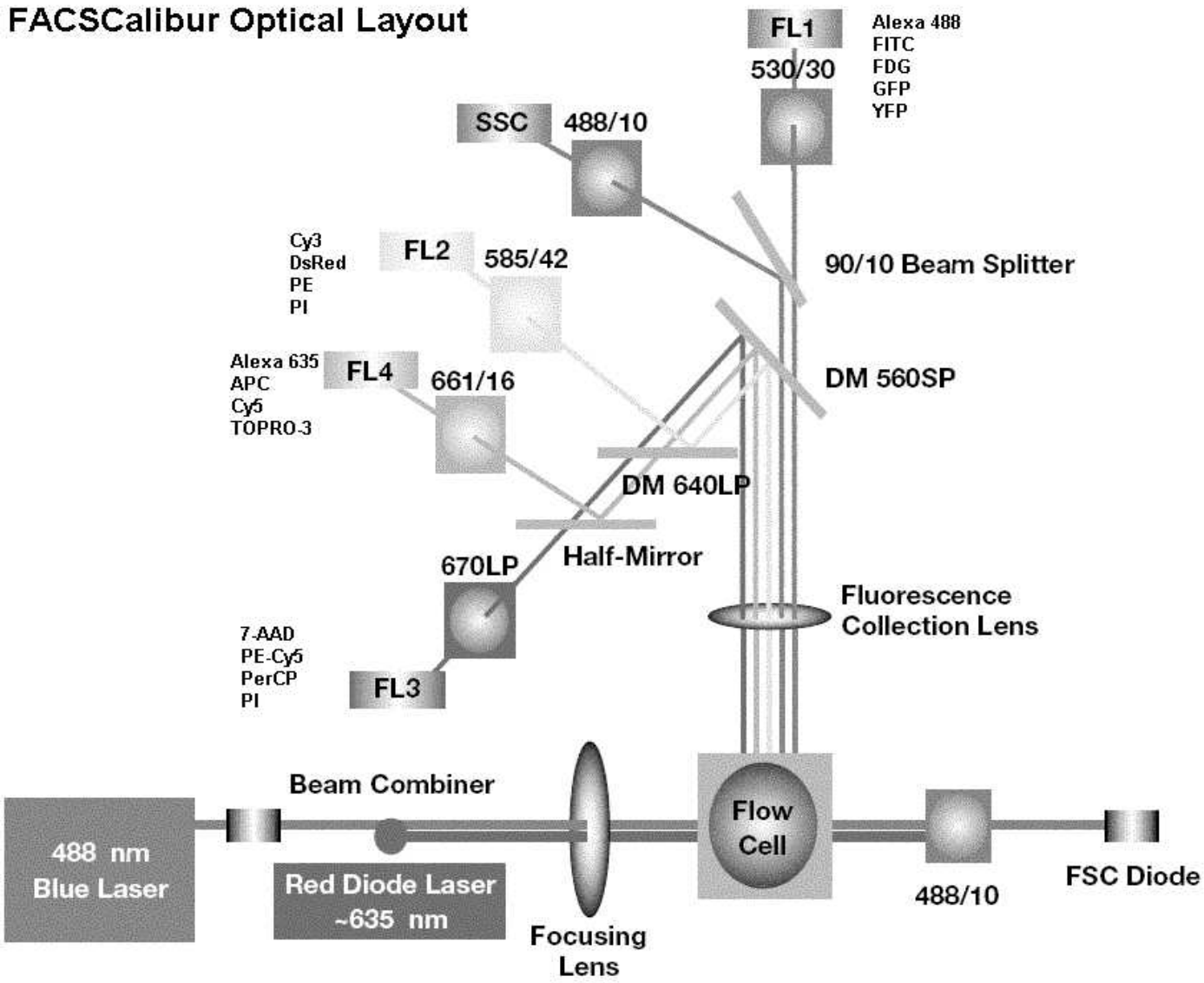
Collection Filter (wavelength/bandpass)

Emission Spectra



FITC PE PerCP


CD8-FITC
CD3-PerCP
CD62L-APC
Perforin-PE

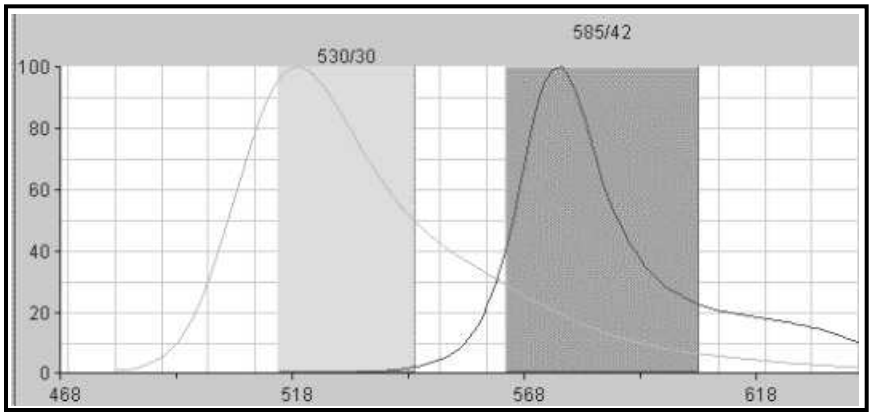

FACSCalibur Optical Layout
FL1
Alexa 488
FITC
FDG
GFP
YFP
530/30
SSC
488/10
Cy3
DsRed
PE
PI
FL2
585/42
90/10 Beam Splitter
Alexa 635
APC
Cy5
TOPRO-3
FL4
661/16
DM 560SP
DM 640LP
Half-Mirror
670LP
Fluorescence
Collection Lens
7-AAD
PE-Cy5
PerCP
PI
FL3
Beam Combiner
488 nm
Blue Laser
Red Diode Laser
~635 nm
Focusing
Lens
Flow
Cell
488/10
FSC Diode

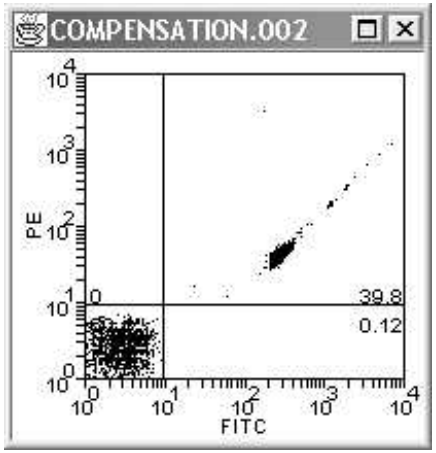
Fluorescence Channels

Channel	Fluorochromes
FL1	FITC, Alexa 488, GFP, CFSE
FL2	PE, DsRed, Alexa 594, PI (DNA)
FL3	PE-Cy5, PE-Cy7 tandem conjugates, PerCP, PerCP-Cy5 PI, 7-AAD (DNA)
FL4	APC, Cy5, DDAO-SE, ToPro-3 (DNA)

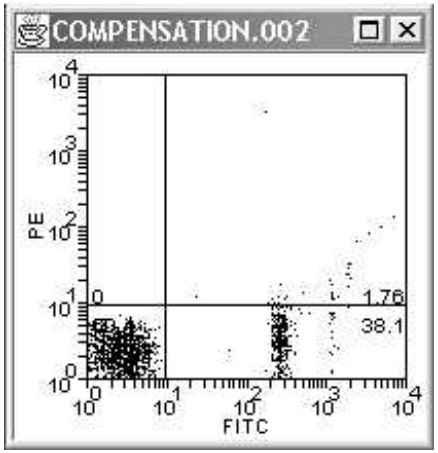
Compensation

- Overlapping emission spectra = Compensation needed
- Method for correcting for spectral overlap is known as compensation
- Essential for data analysis
- Example: estimate the FITC (FL1) contribution to the PE signal (FL2) and subtract it out from the gross detected signal



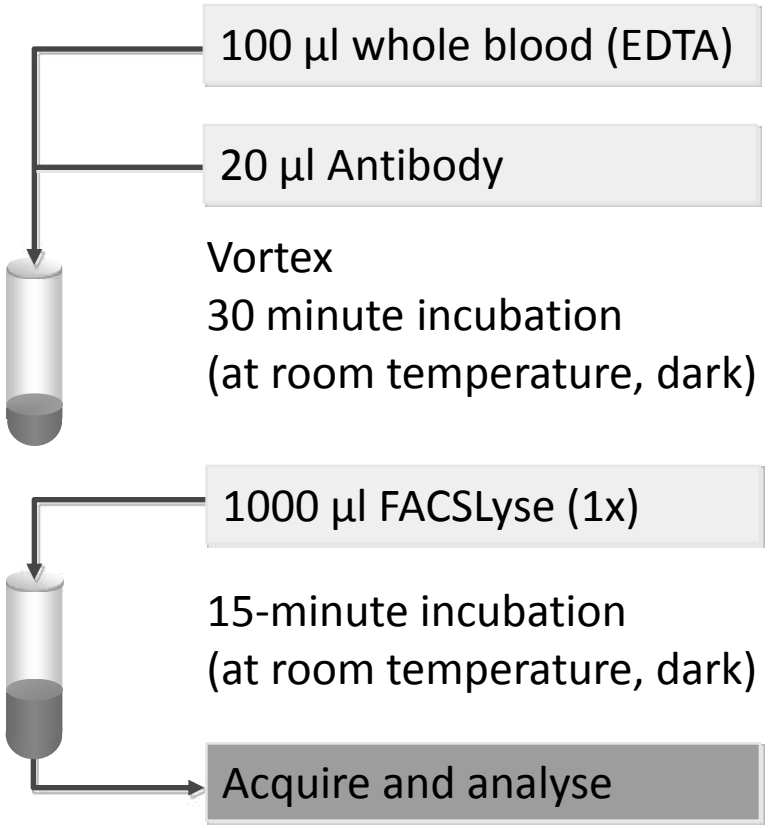


Uncompensated

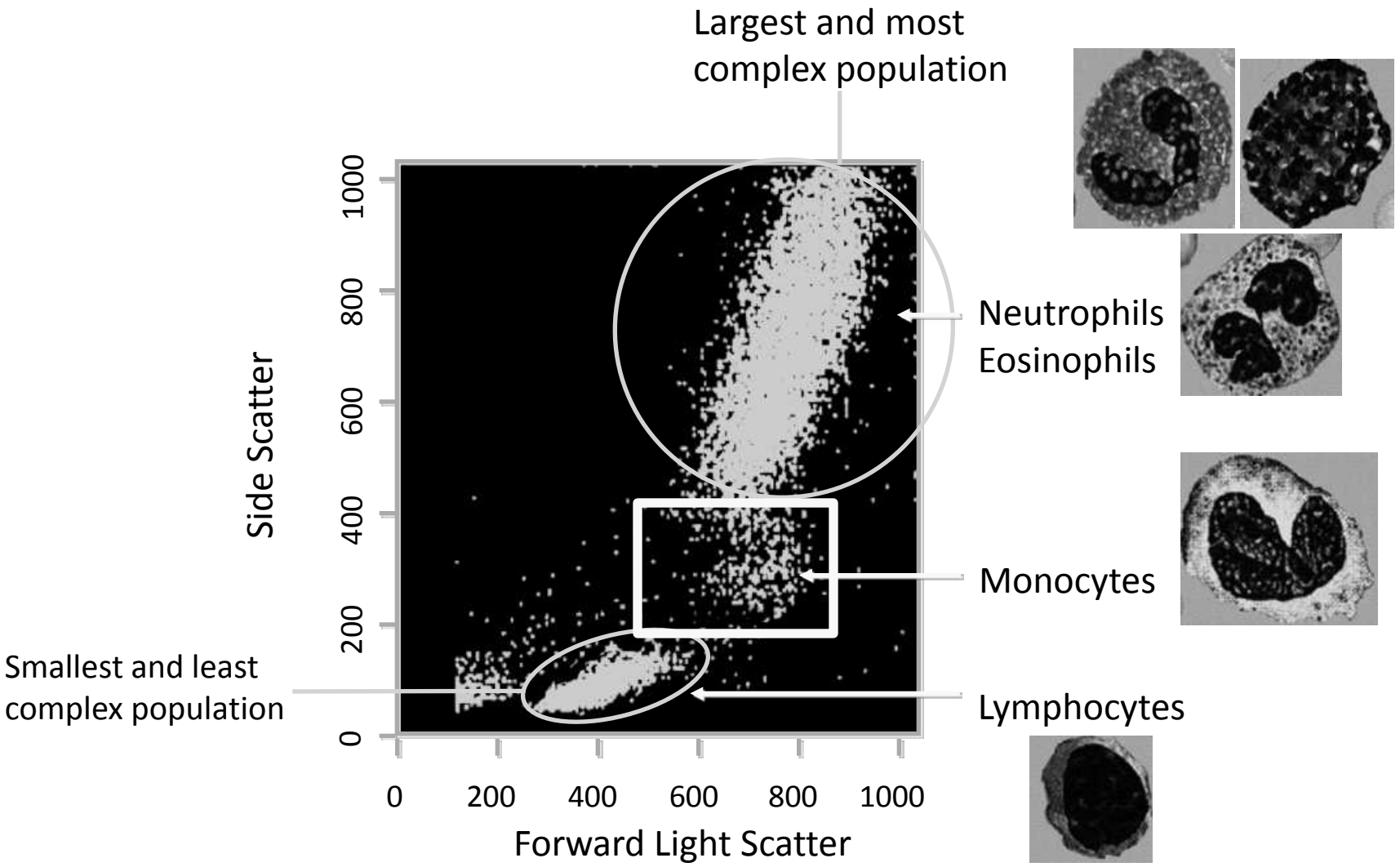


FL2-%FL1

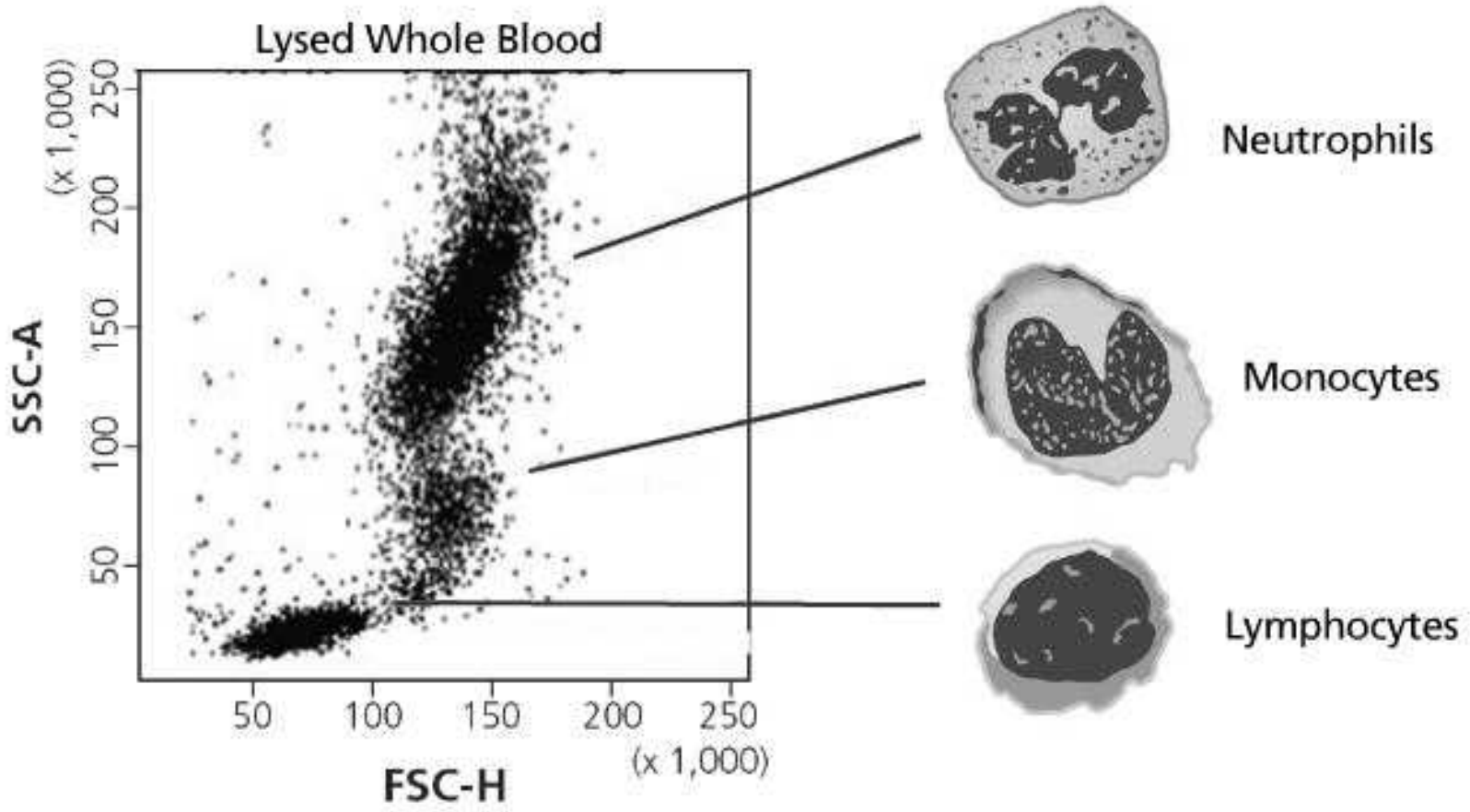
Typical Assay



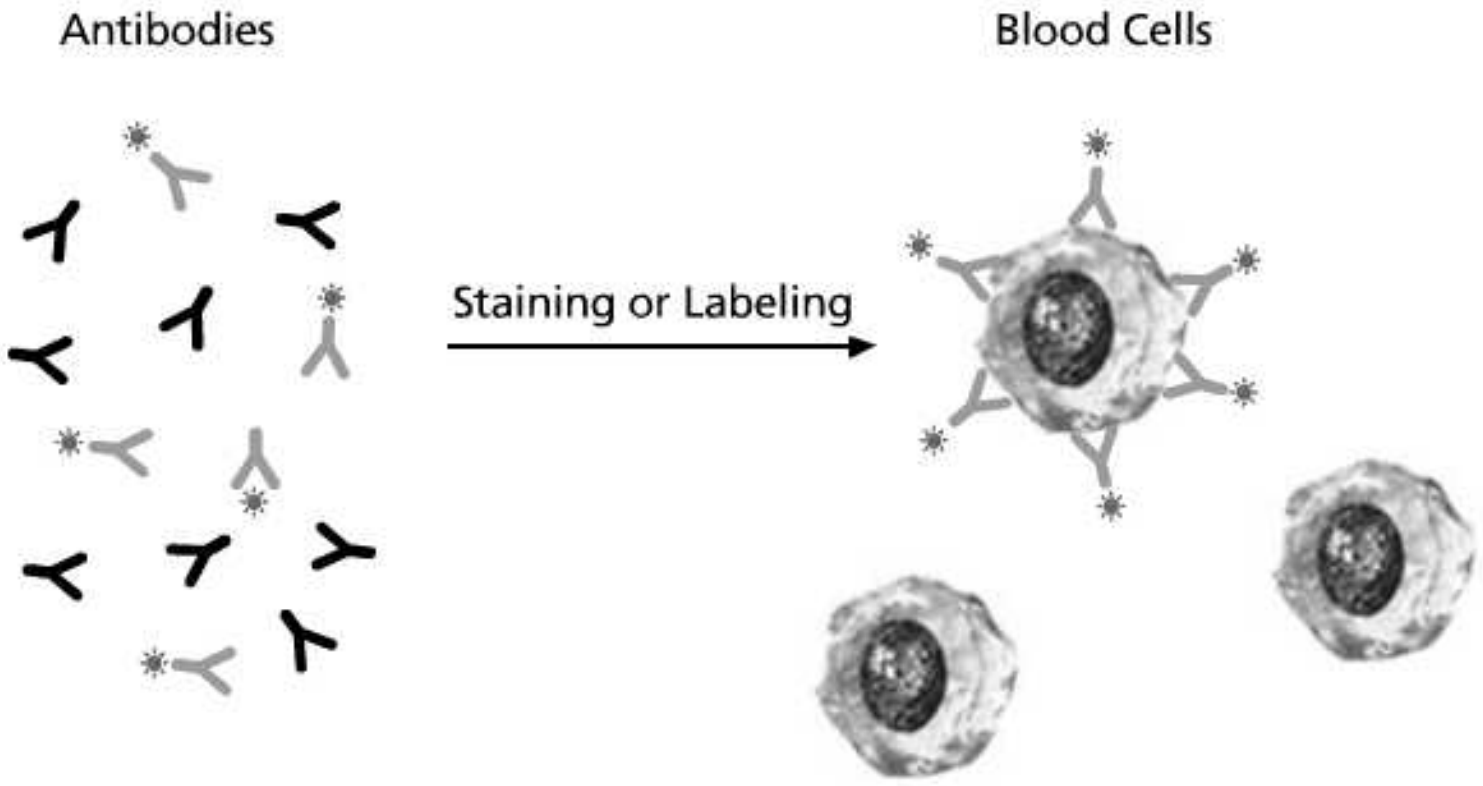
Whole Blood-RBCs lysed



Identifying Cells By Scatter Light Analysis



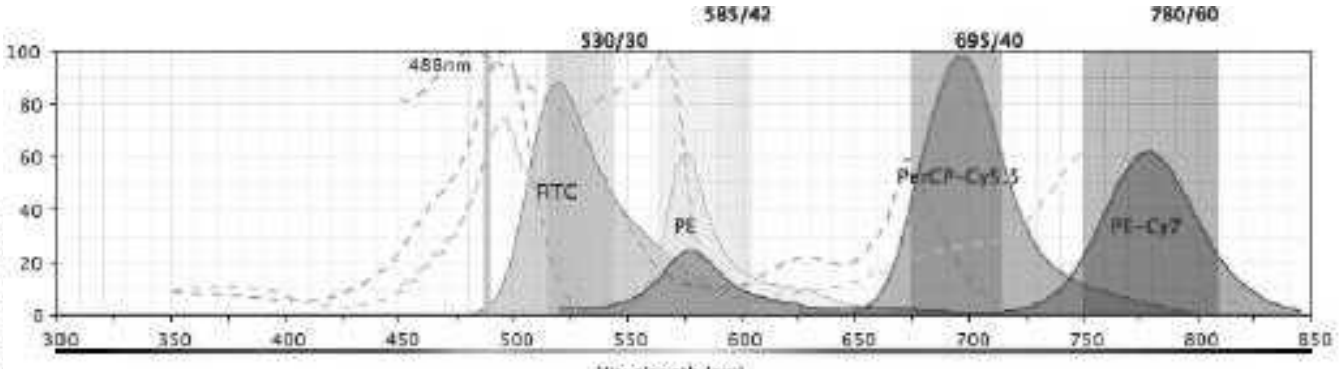
Cellular Markers and Monoclonal Antibodies



Common Fluorochromes

Click each fluorochrome to view its excitation and emission spectra.

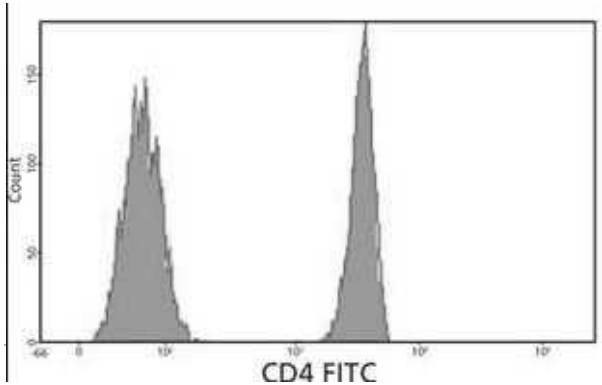
- FITC
- PE
- PerCP-Cy5.5
- PE-Cy7
- APC

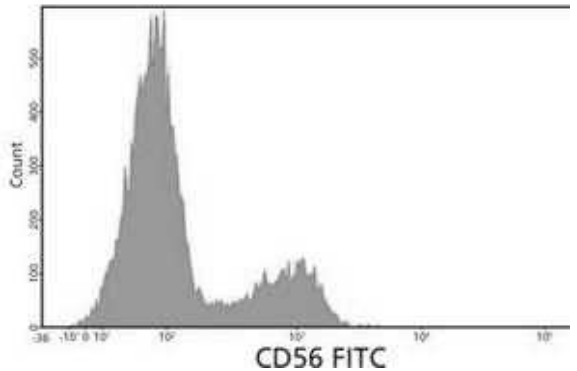


FL1	FITC, GFP, YFP
FL2	DsRed, PE, PI*
FL3	PE-Cy5, PE-Cy7 tandem conjugates, PI*, 7-AAD, PerCP
FL4	APC, Cy5, TOPRO-3

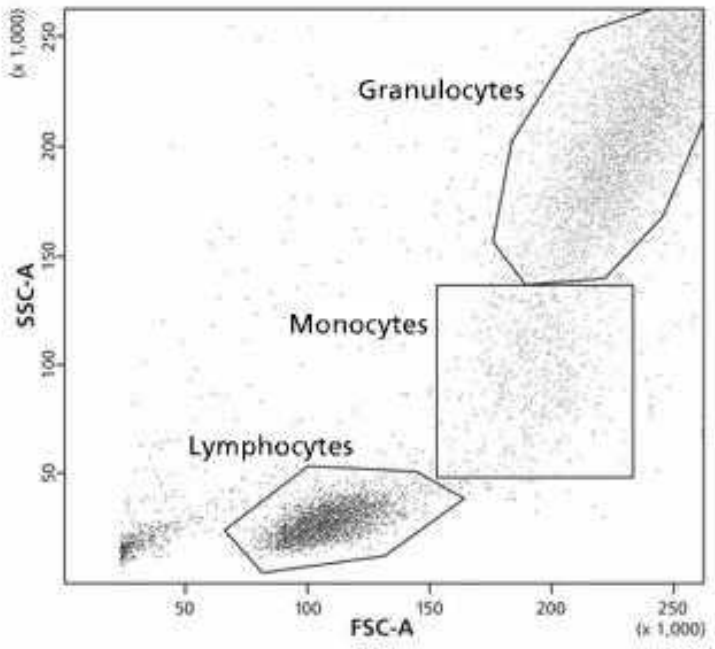
Available Laser Lines at IGC's Flow Cytometry Lab

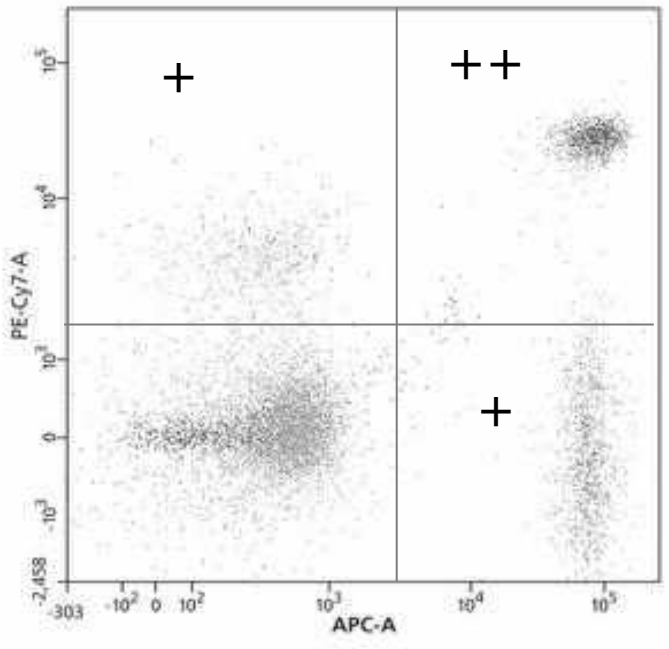
	Bench Top Analyzers				Cell Sorters	
	FACScan	FACScalibur	CyAn ADP	LSR Fortessa	MoFlo	FACSAria
Multiline UV (330-360 nm)					✓	
Violet (407 nm)			✓			✓
Blue Violet (442 nm)				✓		
Cyan (457 nm)					✓	
Blue (488 nm)	✓	✓	✓	✓	✓	✓
Green (514 nm)					✓	
Yellow(561 nm)				✓	✓	
Red (640 nm)		✓	✓		✓	✓





Displaying Gated Events in Color

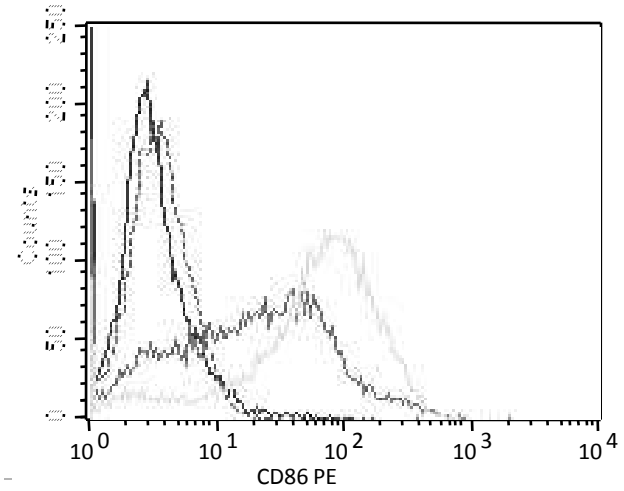




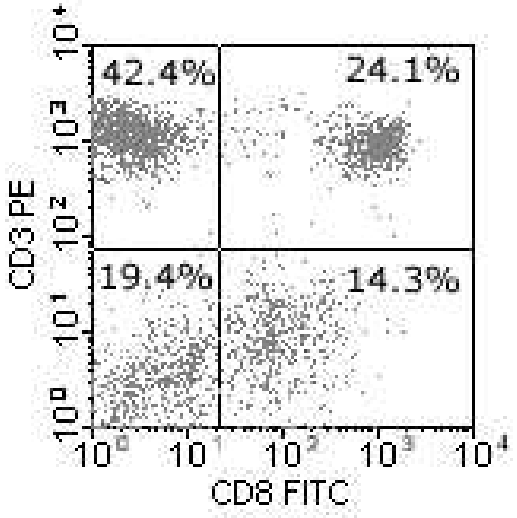
Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Lymphocytes	2,777	27.8	27.8
Monocytes	577	5.8	5.8
Granulocytes	5,792	57.9	57.9

Flow Cytometry Analysis

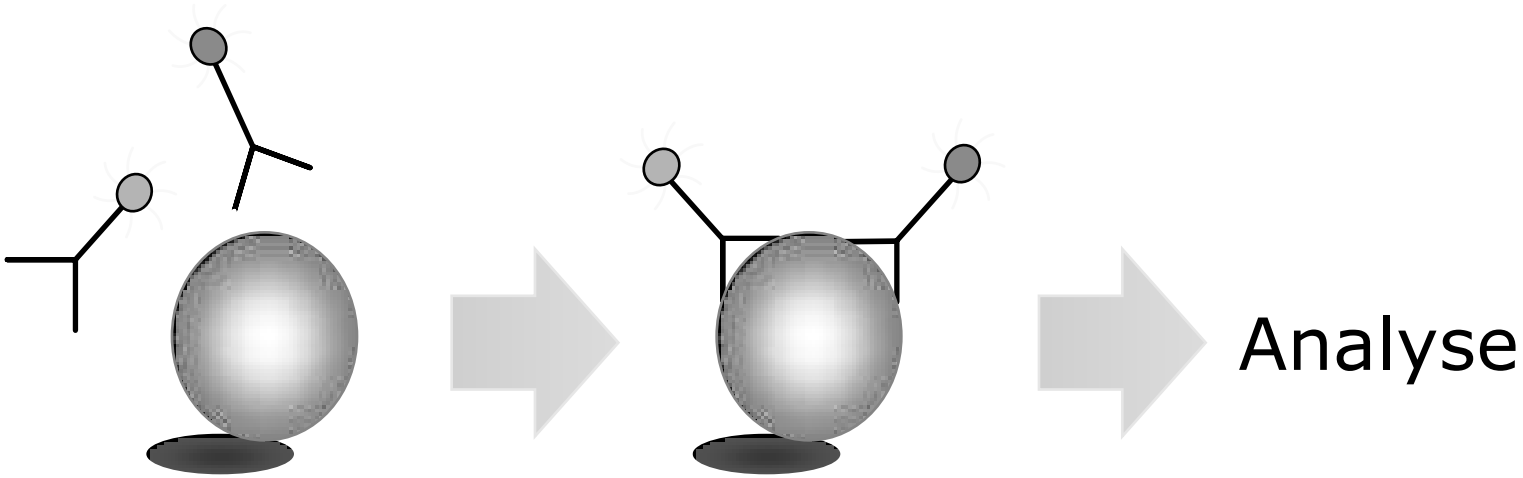
- Single parameter analysis:
  - Histogram plot
  - Horizontal axis: level of fluorescence - brighter cells further right
  - Vertical axis: number of events per channel number
  - Analyze level of expression of marker



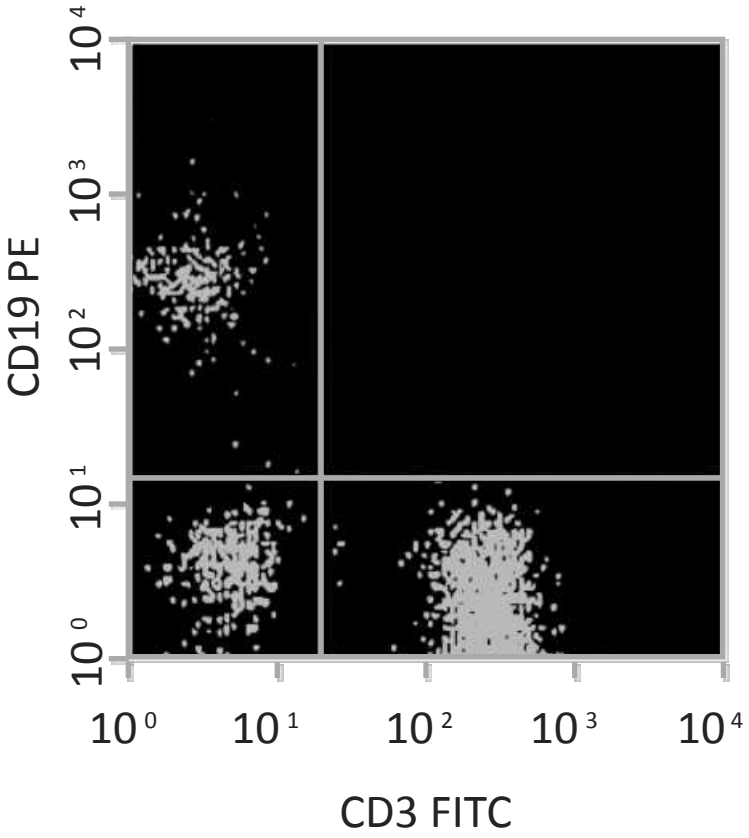
- Two parameter analysis:
  - Dot-plot
  - One axis shows first colour
  - Second axis shows second colour
  - Analysis of individual populations of cells



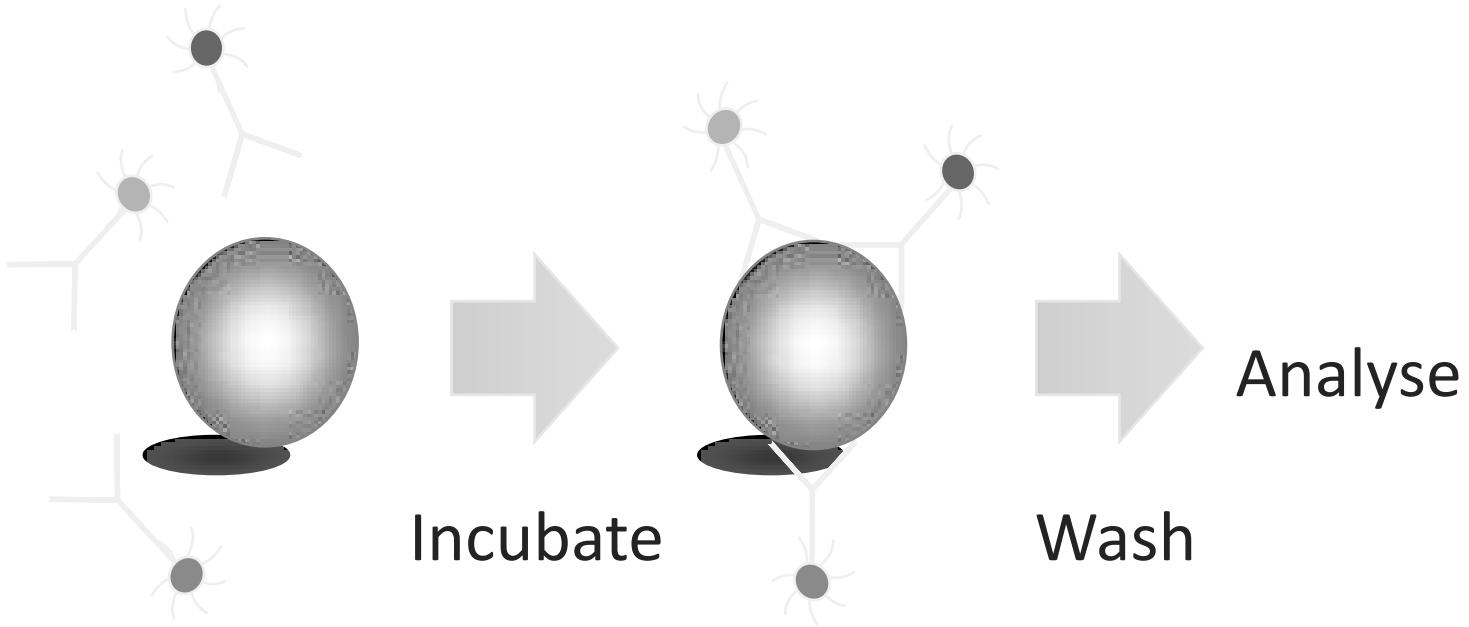
Two-Colour Direct Staining



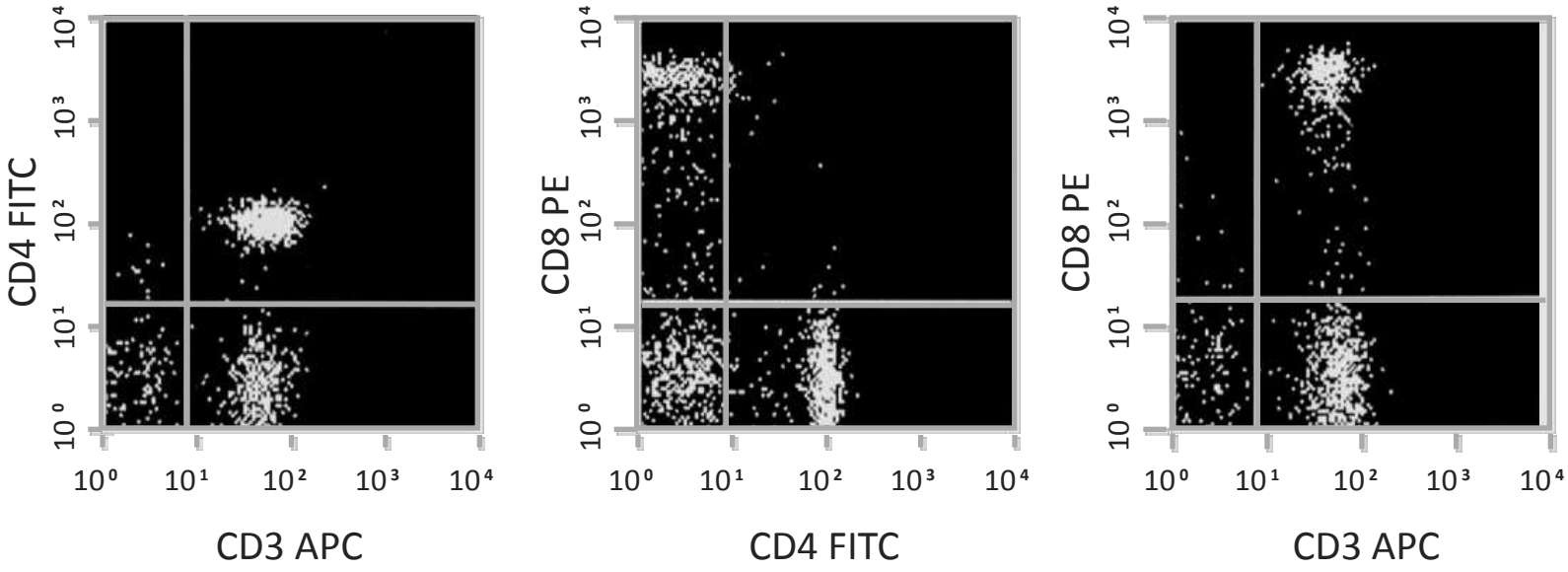
Two-Colour Cell Analysis



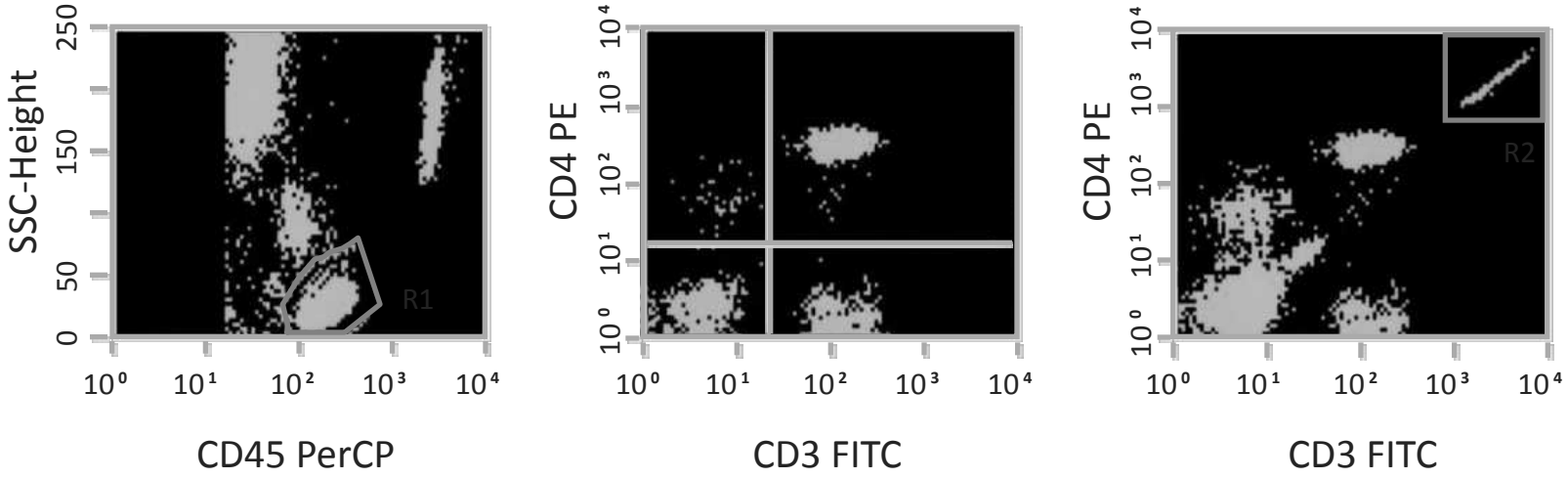
Three-Colour Direct Staining



Three-Colour Cell Analysis



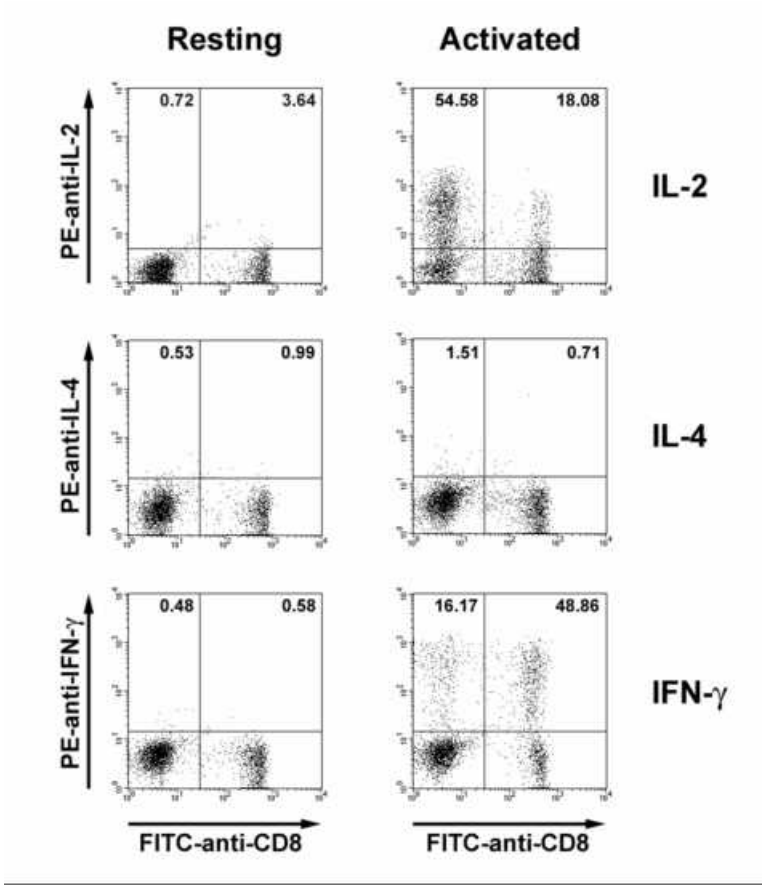
Absolute Counts Using TriTEST™/TruCOUNT™



$$\frac{\text{\# of events in quadrant containing cell population}}{\text{\# of events in Absolute Count bead region}} \times \frac{\text{total \# of beads per test}}{\text{test volume}} = \text{\# CD4 cells/µL}$$

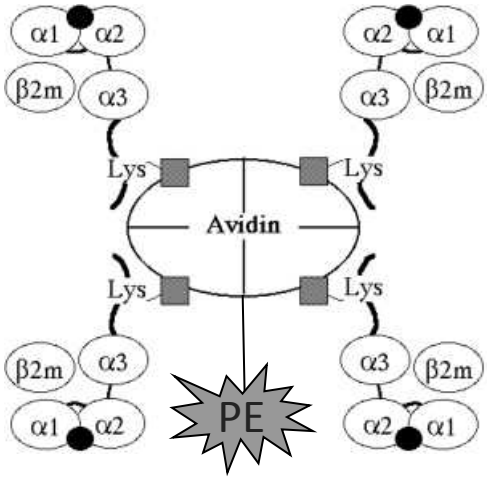
Intracellular Cytokine Staining

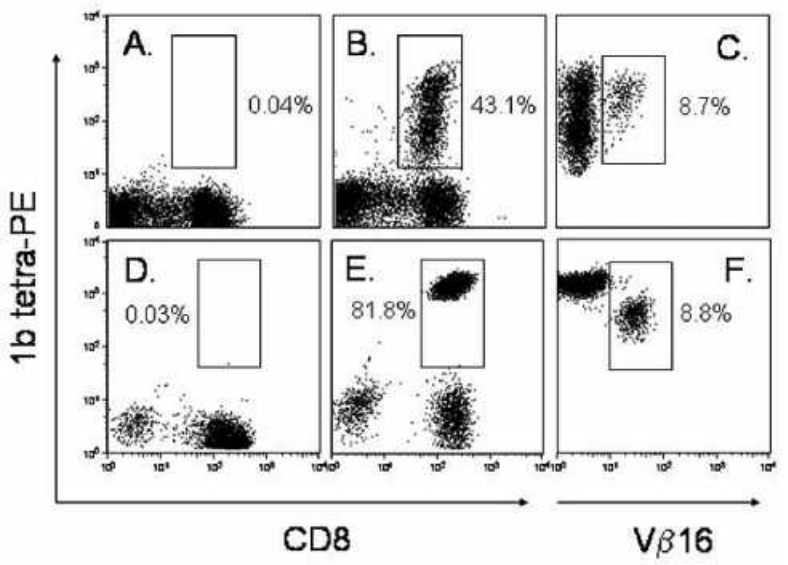
- To detect cytokine production by a specific cell upon stimulation
- Used to define T cell activation by epitope recognition and the T cell polarisation

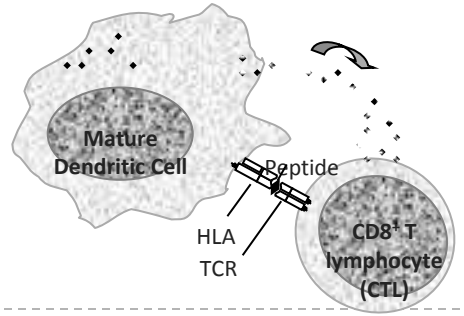


MHC Tetramer Staining

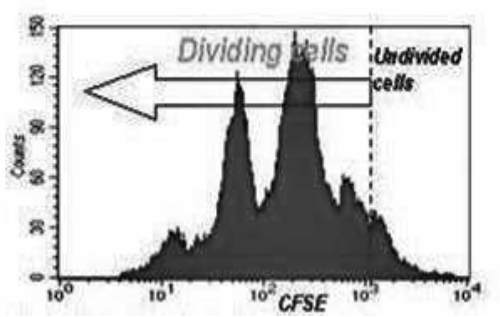
- Identify T cells specific for a certain MHC-peptide complex



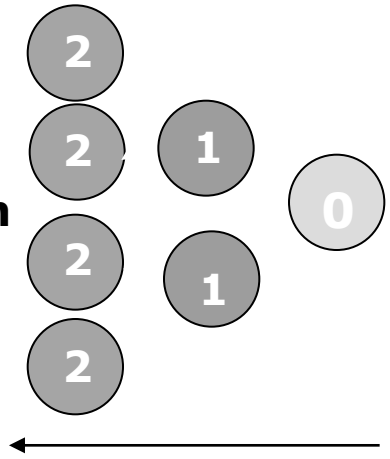




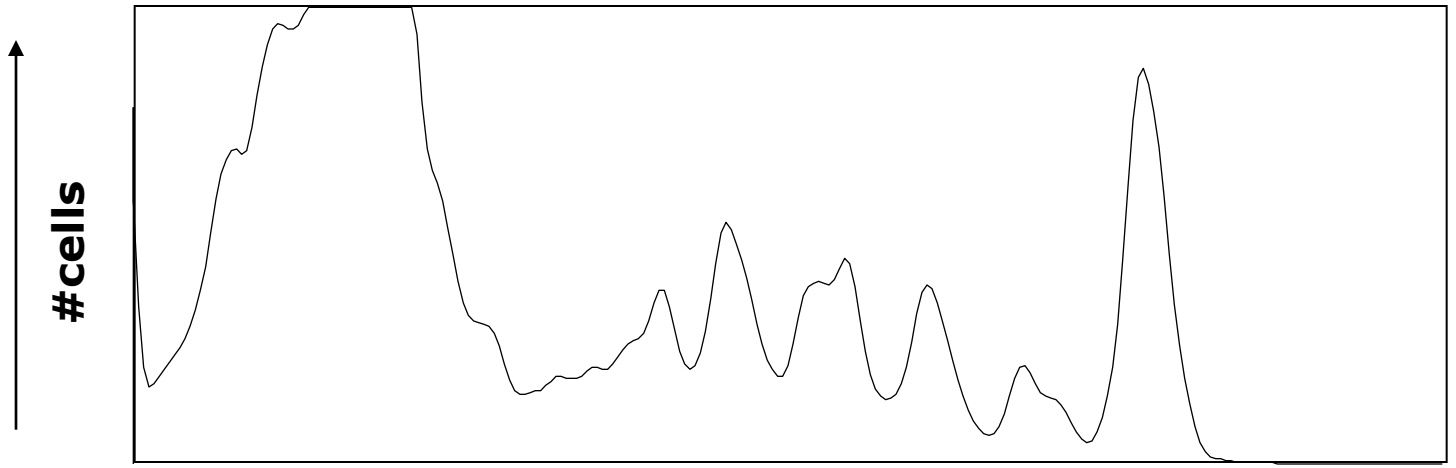
Cell Proliferation Analysis Using CFSE Dye Dilution



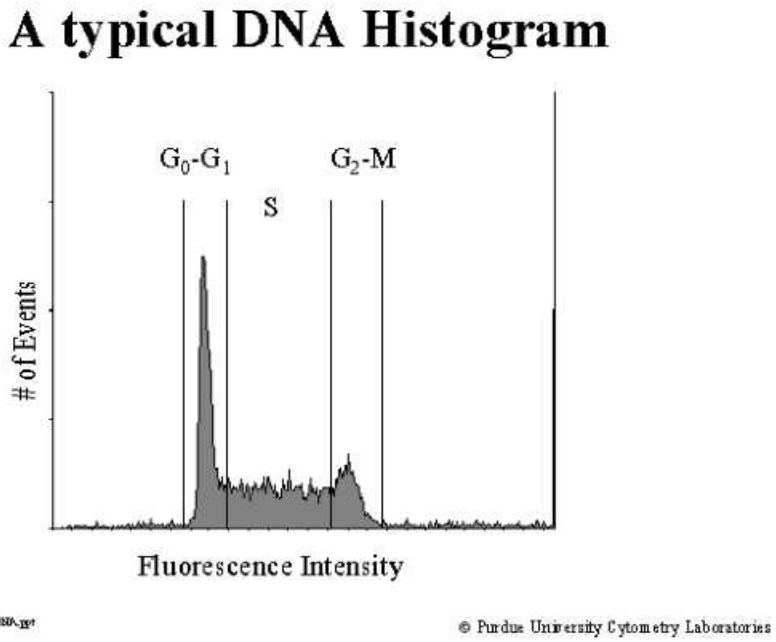
CFSE dilution

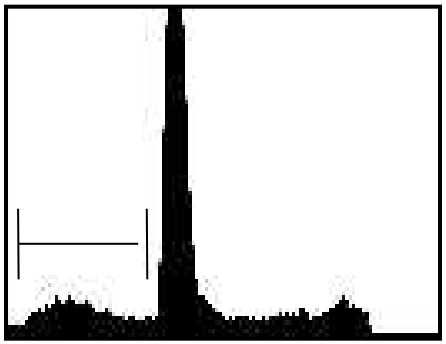


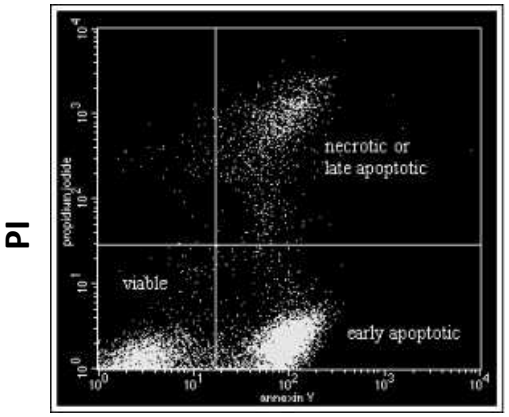
Number of divisions 5 4 3 2 1 0



DNA Analysis / Apoptosis



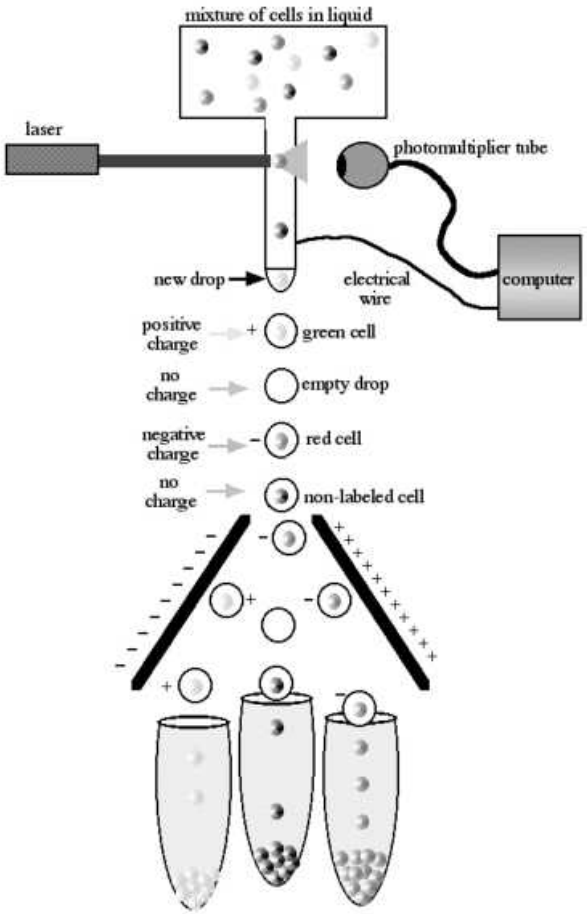


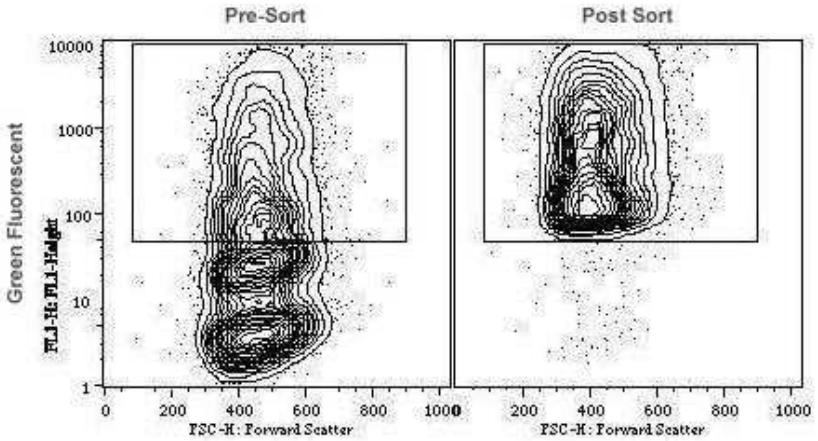


Annexin V

- Ploidy determination, detection of abnormal clones.
- Cell cycle analysis.
- Apoptosis.
- Flow karyotyping (chromosome analysis).

Cell Sorting - FACS





Cytometric Bead Arrays in Flow Cytometry

Cytometric Bead Array (CBA)

Multiplexed solid phase particle based immunoassay to measure multiple (currently up to nine) soluble analytes simultaneously

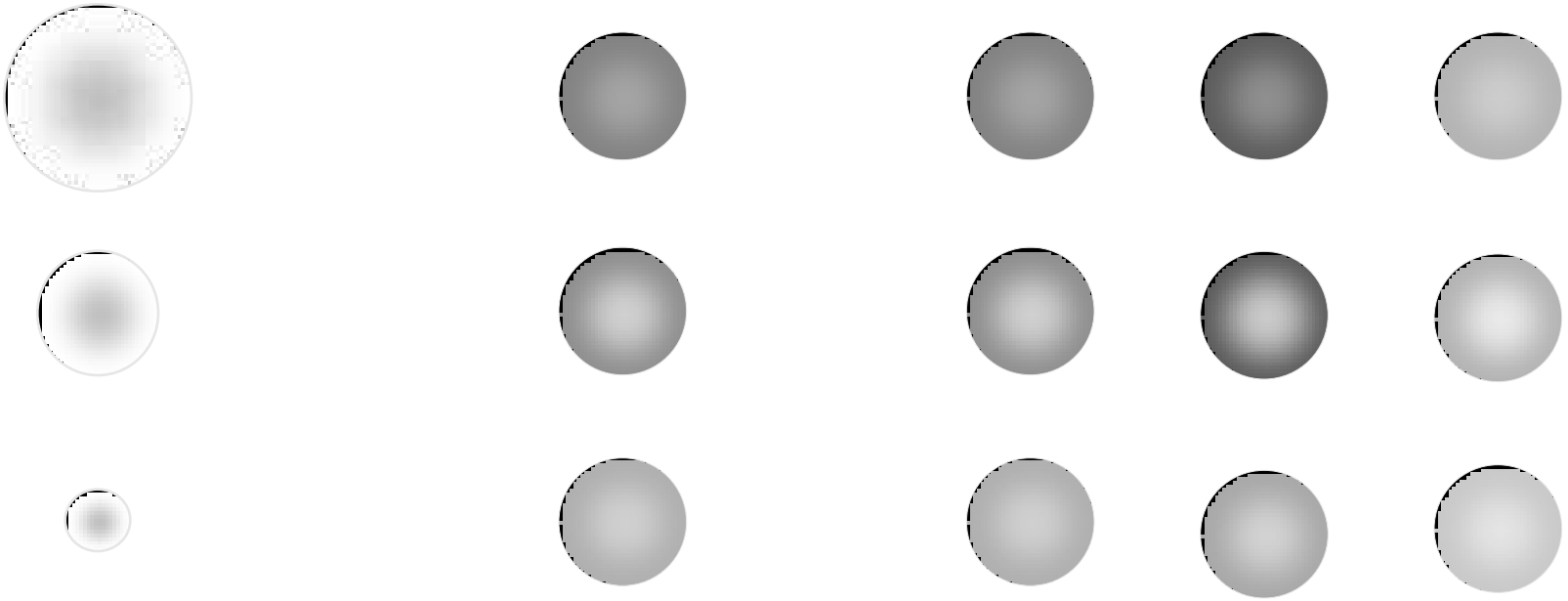
All systems use Flow Cytometry as analysis platform

Currently there are 3 areas using this technology

- Transplantation – Standard Flow Cytometers
- Autoimmune – dedicated Luminex cytometer/ automated/ black box
- Cytokine quantitation - Standard Flow Cytometers

Beads Provide a Flexible Platform

Beads provide an expandable assay platform for use with a flow cytometer



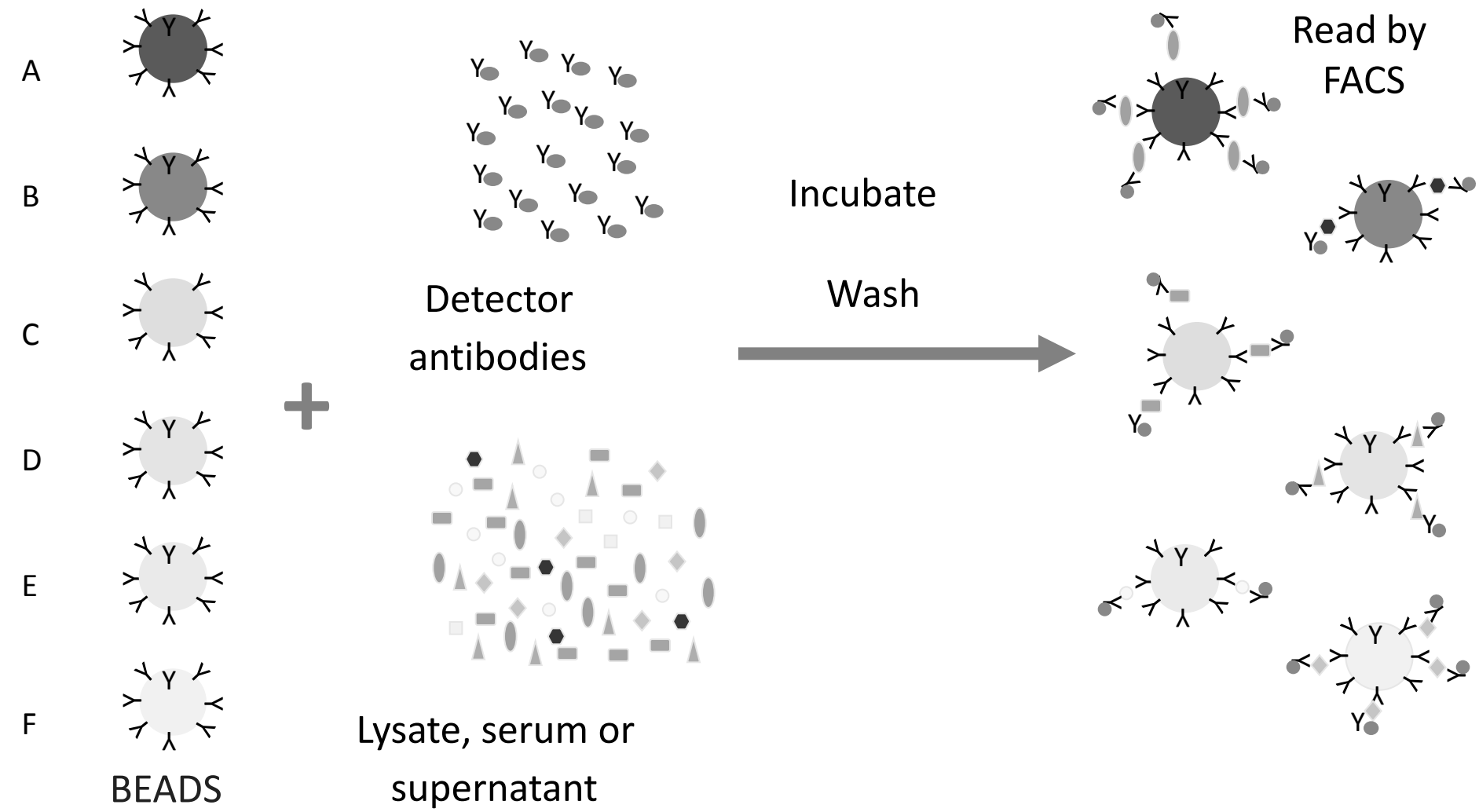
Multiple sizes

Different intensities

Different colours with different intensities

From BD Biosciences

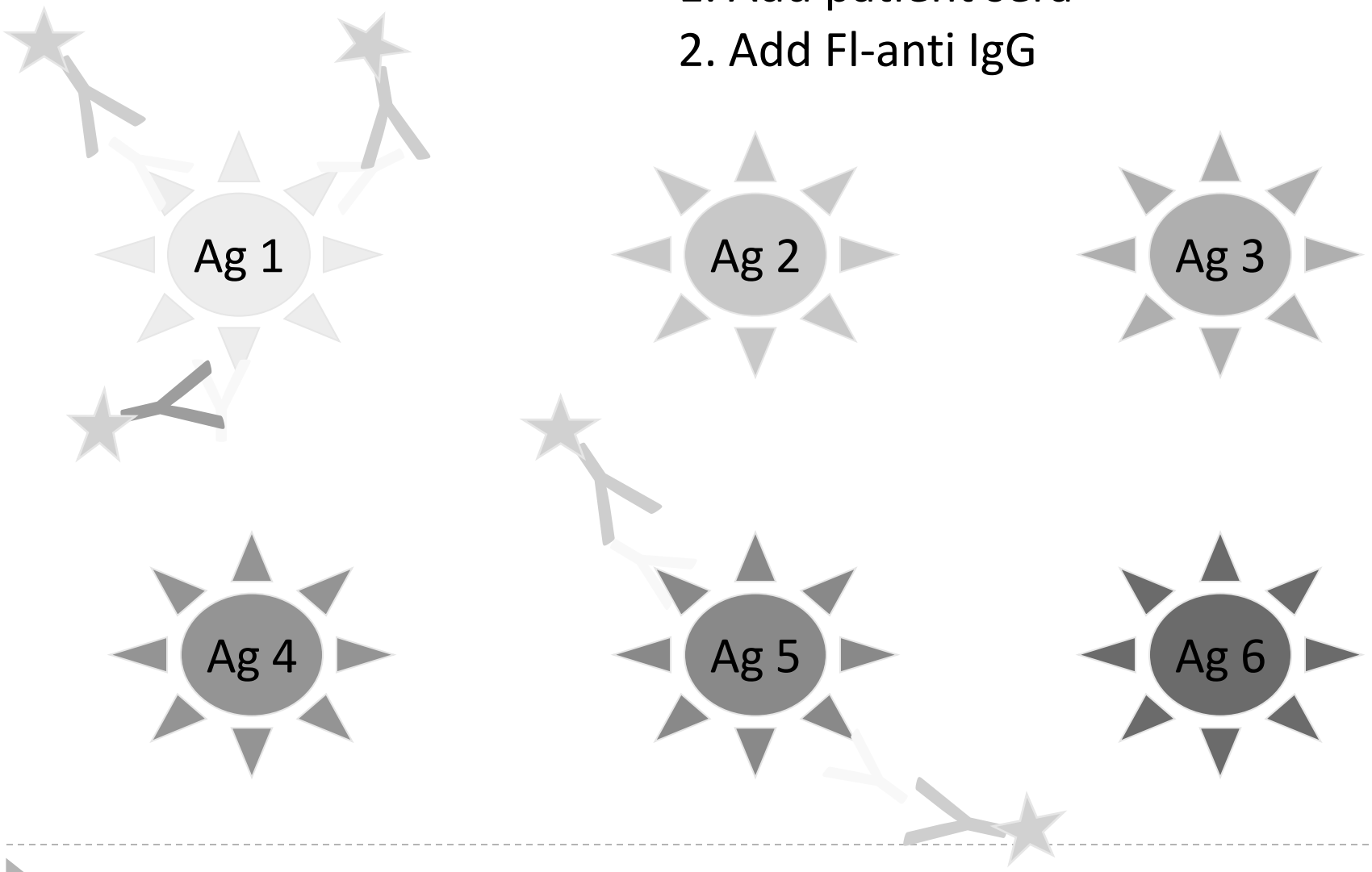
Bead Assay Basics



From BD Biosciences

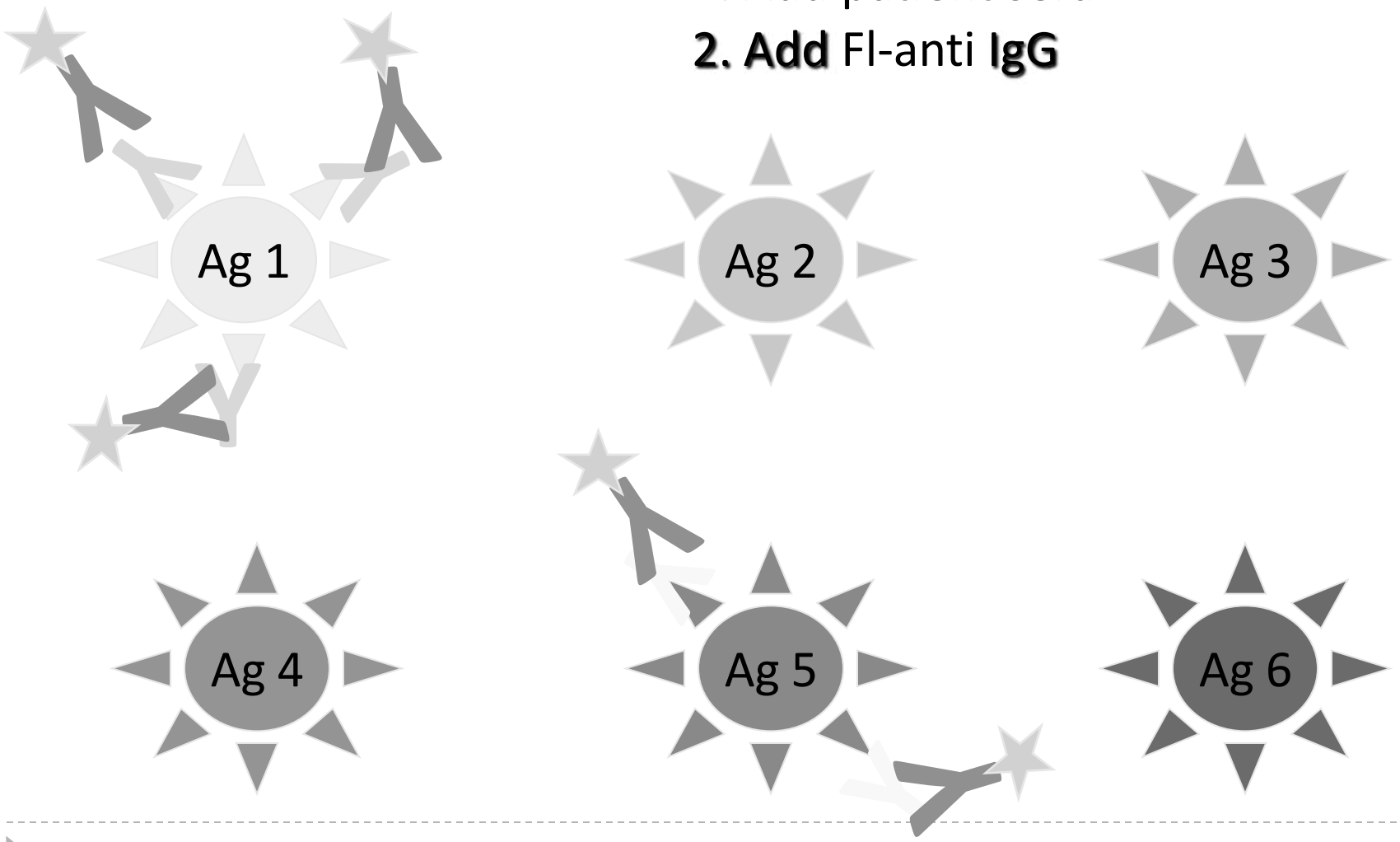
The Technology (Ab detection)

1. Add patient sera
2. Add Fl-anti IgG



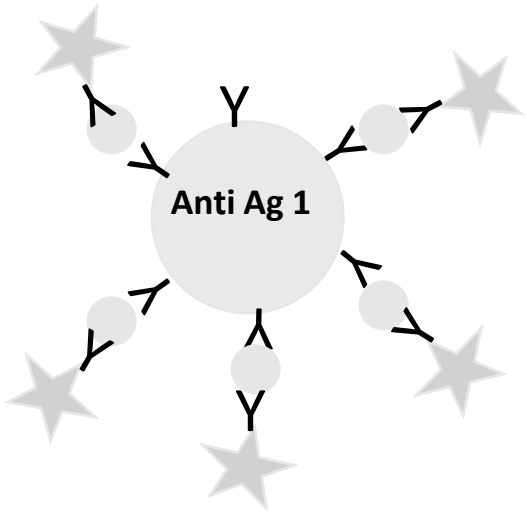
Ab Detection

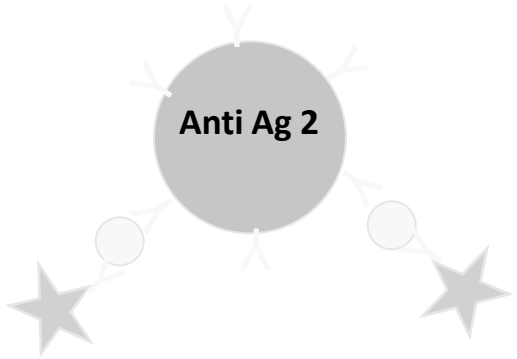
1. Add patient sera
2. **Add** Fl-anti **IgG**

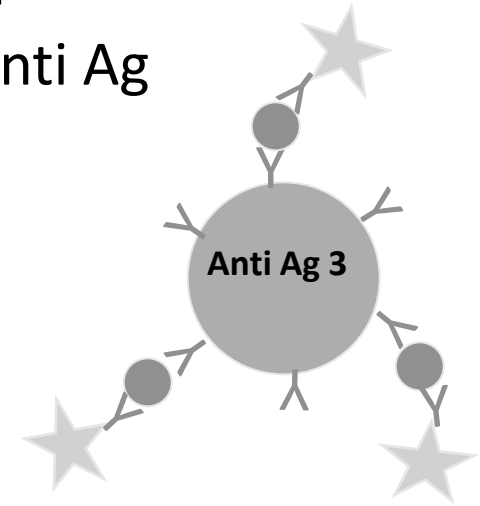


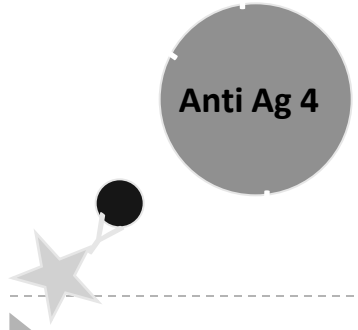
The Technology (Ag Detection)

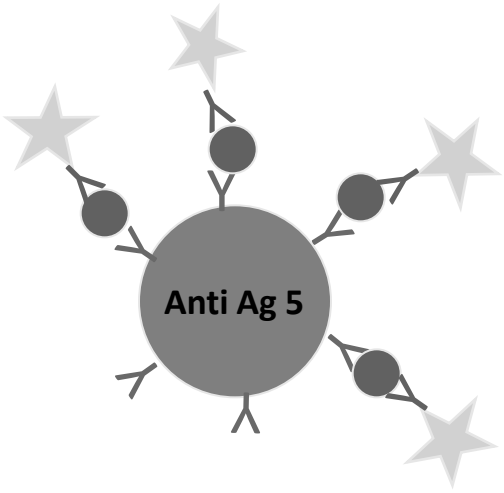
1. Add patient sera
2. Add specific Fl-anti Ag

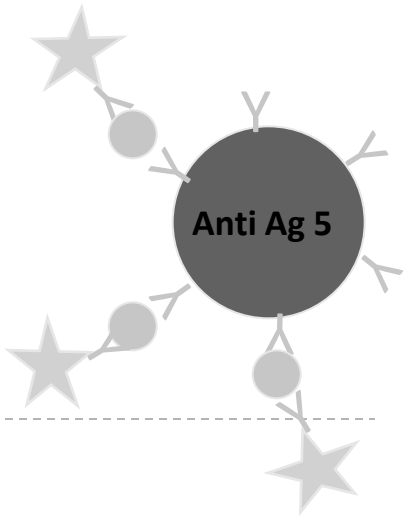




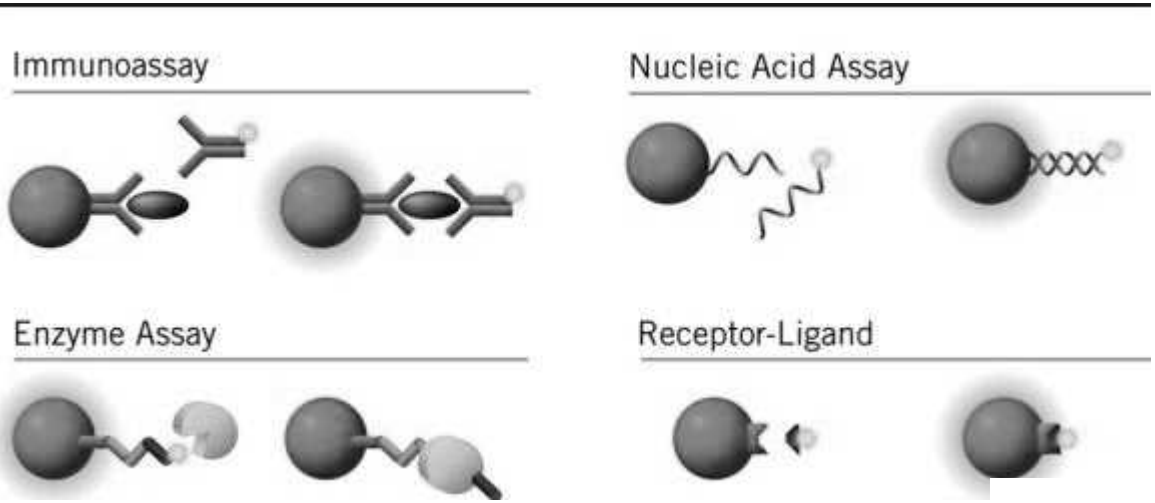


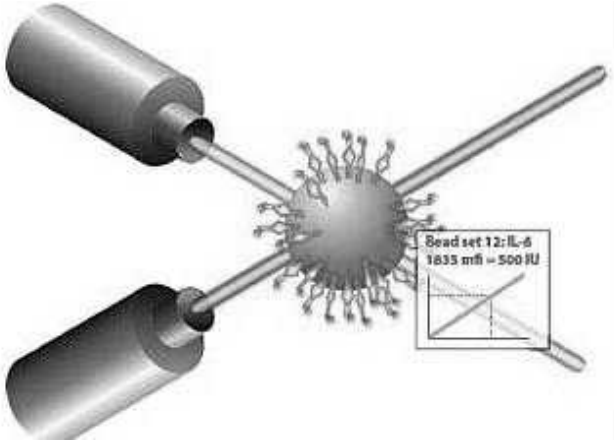


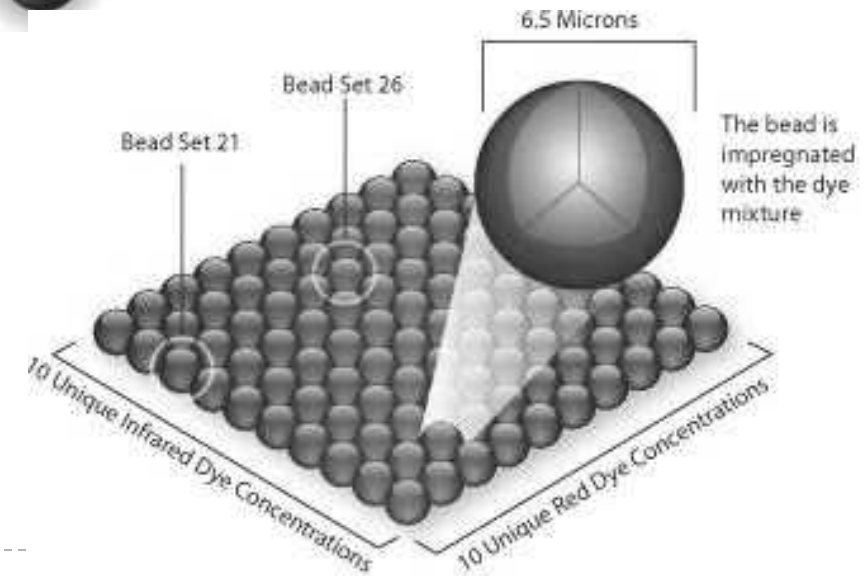




Multi-plex ELISA (LUMINEX)





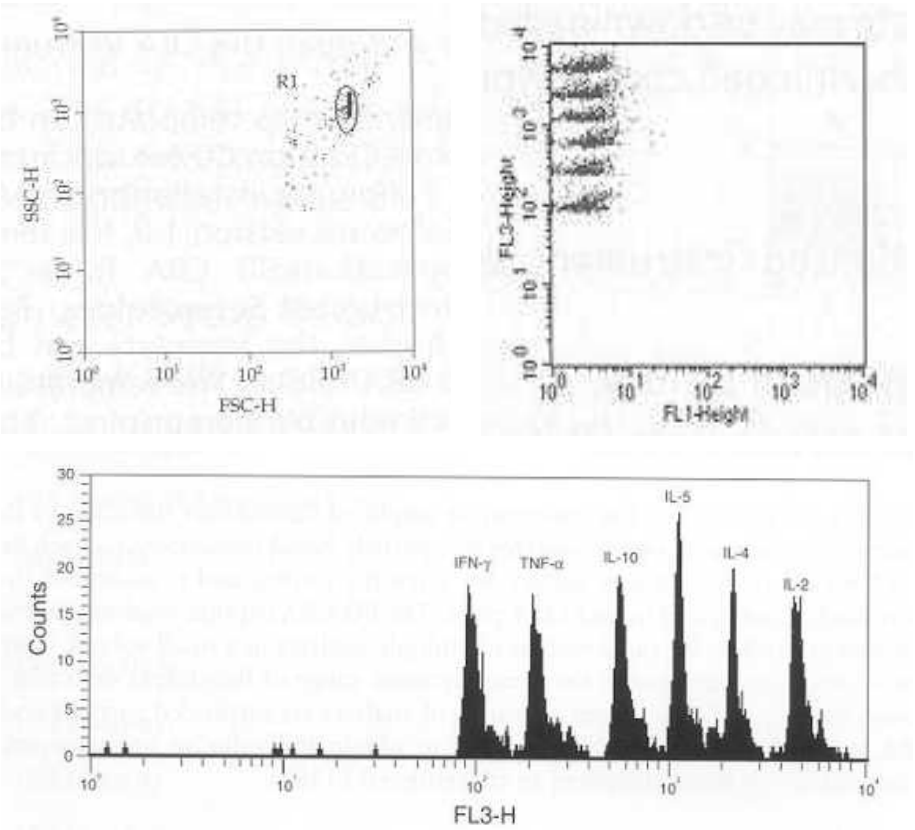


Cytokine Bead Array

Light scatter of beads

- Theoretically – limitless possiblities
- Same size beads – simplifies

Red Fluorescence of negative beads

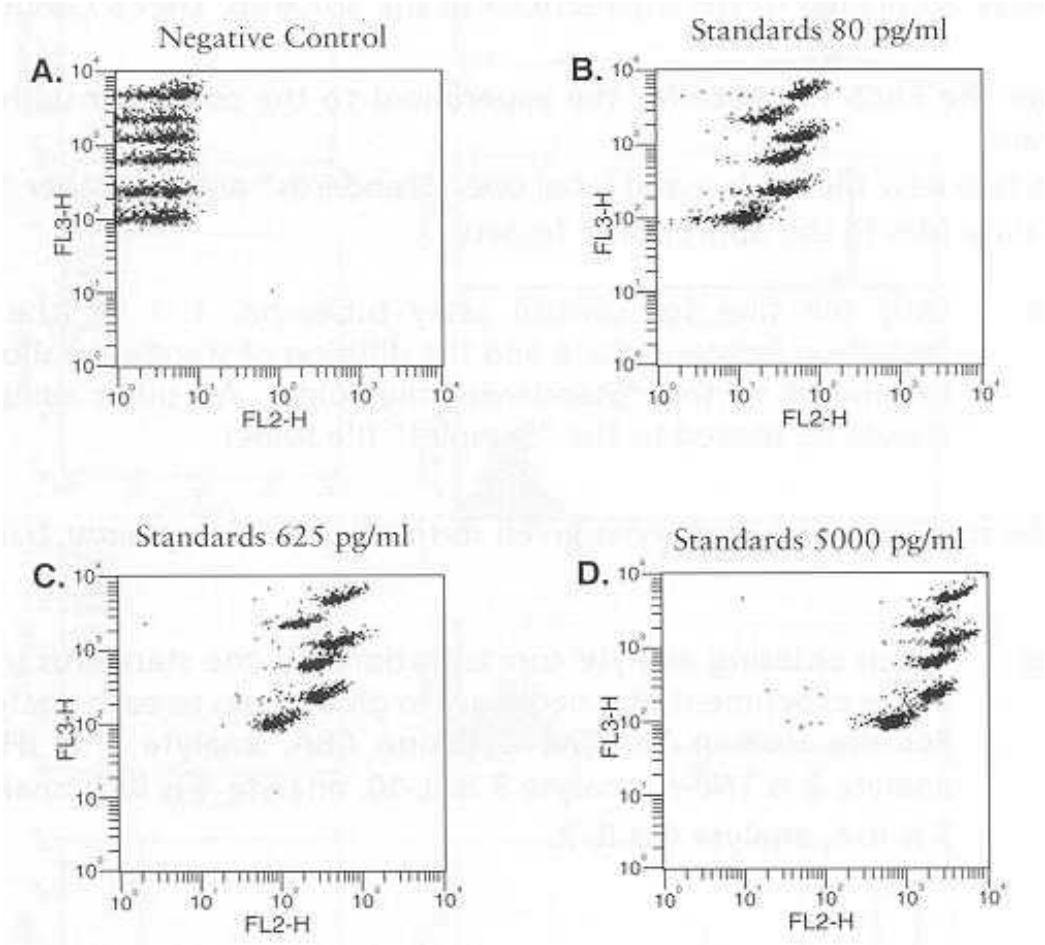


Cytokine Bead Array

9 standards for calibration

(20-5000 pg/ml)

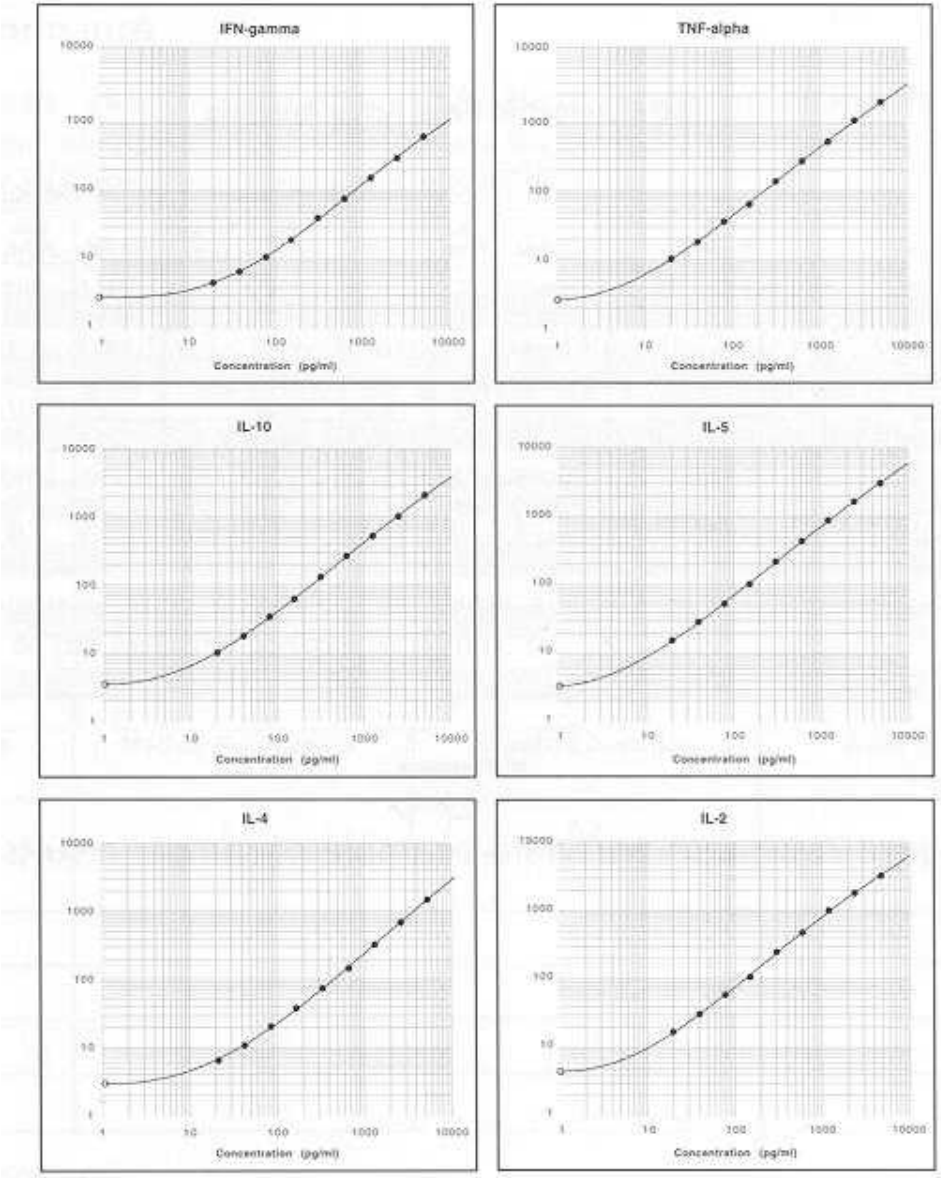
By computer extrapolation of software standard curves down to 2.4 pg/ml



From BD Biosciences

Cytokine Bead Array

Example of software produced standard curves

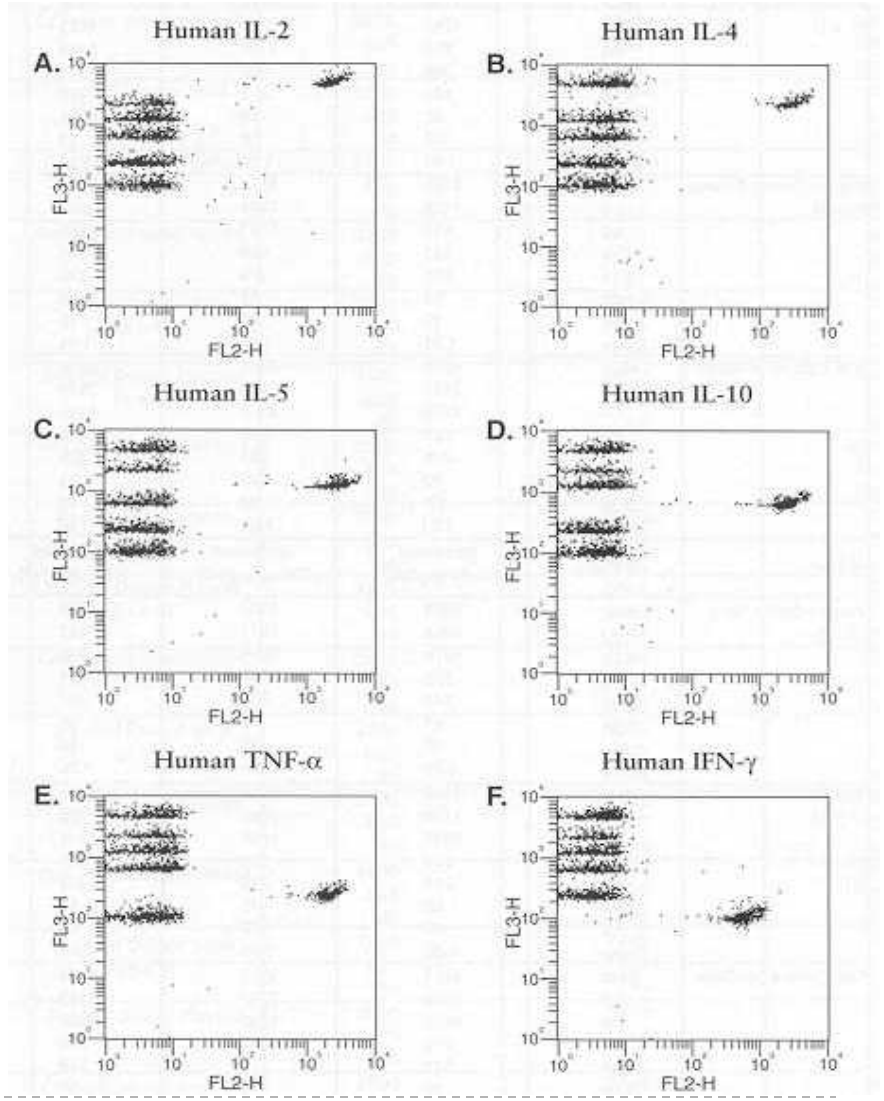


From BD Biosciences

Cytokine Bead Array

Specificity – little to no cross reactivity

Cross reactivity detection important in transplant HLA typing



From BD Biosciences

Assay Range & Analytical Sensitivity

Analyte	Assay Range	Analytical sensitivity
IFN γ	0 - 5000 pg/ml	7.1 pg/ml
TNFα	0 - 5000	2.8
IL 10	0 - 5000	2.8
IL 5	0 - 5000	2.4
IL 4	0 - 5000	2.6
IL 2	0 - 5000	2.6

Sensitivity: Mean (N=6) + 2 S.D. of zero calibrator
Human TH1/TH2 CBA Kit

From BD Biosciences

Common Applications of Flow Cytometry

- Phenotype of cell, surface molecules
- Flow crossmatching
- Intracellular cytokine staining
- Antigen specificity
- Cell proliferation (e.g. CFSE, BrdU incorporation)
- Cell sorting
- Apoptosis analysis
- Cytotoxicity assays
- Phagocytosis assays
- Cell cycle analysis (DNA content analysis)
- Cell signalling molecules, Calcium flux assays
- Organelle-specific studies (e.g. lysosome)
- Cellular transport assays
- Transfection efficiencies