Principles and Concepts of Immunological Methods

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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

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Immunology

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

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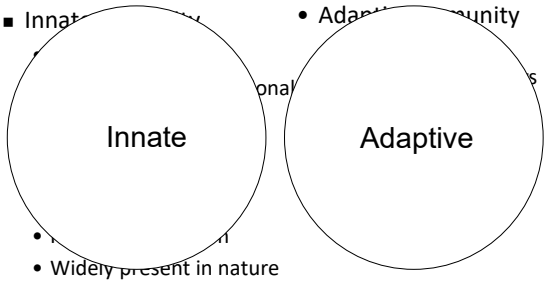
Two Arms of Host Defence

- Innat
- Adap unity
- onal s
- Widely present in nature



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Key Players in Immunology

	Innate	Adaptive
Cells	• Phagocytes • Epithelial Cells • NK Cells	• Lymphocytes (B-cells, T-cells)
Defence Proteins	• Complement • Antimicrobial (poly)peptides	• Antibodies

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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 } Psychoneuroimmunology
  - Skeletal system
- Disruption of the Immune System
  - Hypersensitivity
  - Autoimmunity
  - Immunodeficiency

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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

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Immunological techniques

Immunological techniques are used to:

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

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

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

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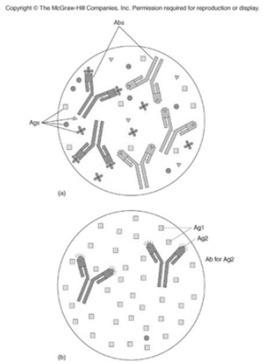
Antigen
+
Antibody
→ Immune Complex



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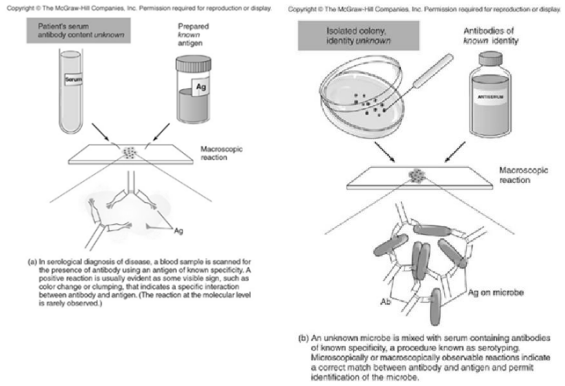

(a) In serological diagnosis of disease, a blood sample is scanned for the presence of antibody using an antigen of known specificity. A positive reaction is usually evident as some visible sign, such as color change or clumping, that indicates a specific interaction between antibody and antigen. (The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

(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.

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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 and Luminex
4. Rosette & plaque formation
5. Immunogenetics
6. Antibody production
7. Evaluation Methods

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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 and meet ← Ag

↓

At appropriate conc visible ppte forms

↓

Stain ppte (eg for protein)

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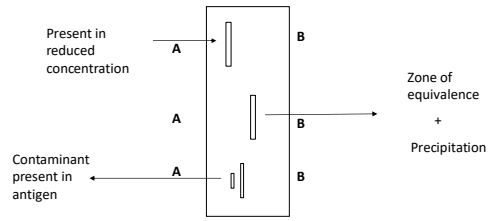
a) Immunodiffusion

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



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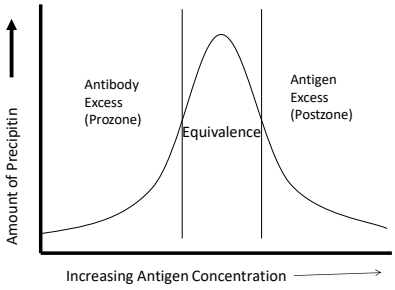

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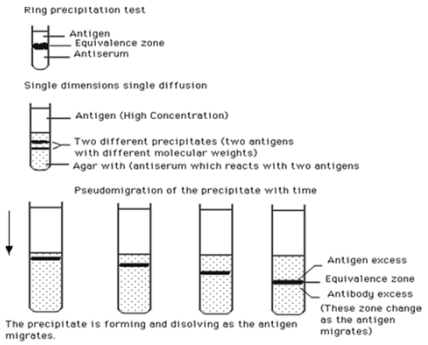

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i) Ouchterlony (Qualitative Double Diffusion)

- 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

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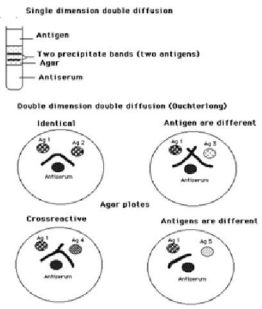
Qualitative (Double Diffusion): Ouchterlony



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

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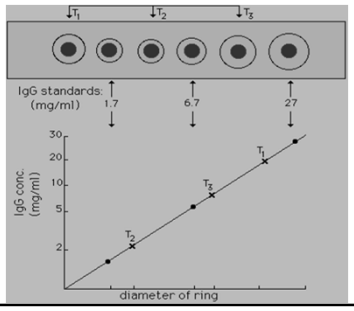
ii. Quantitative: Radial Immunodiffusion

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

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b) Immunoelectrophoresis

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

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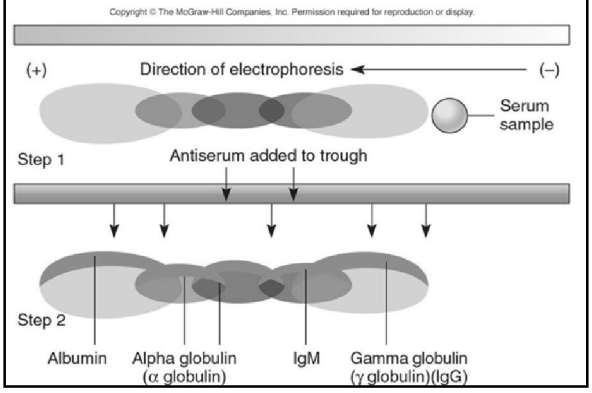
i. Qualitative



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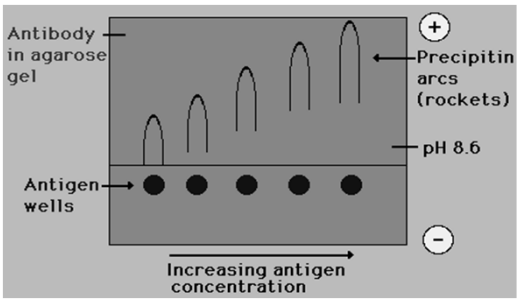
ii. Quantitative: Rocket electrophoresis

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



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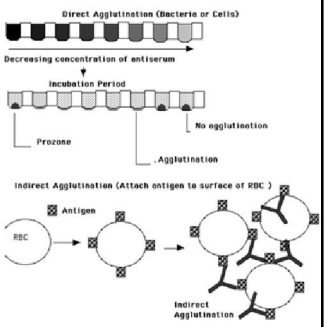
2. Agglutination of cell-bound Ag

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



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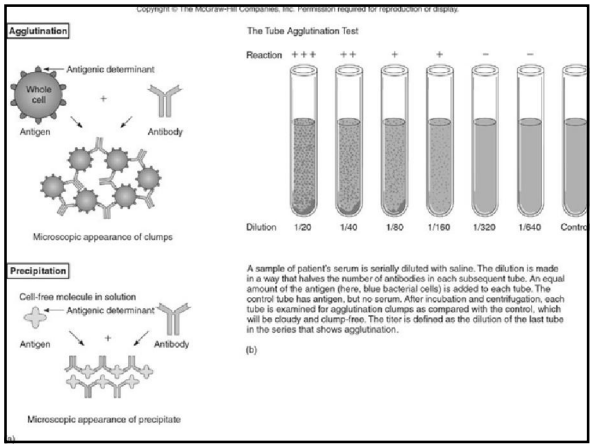

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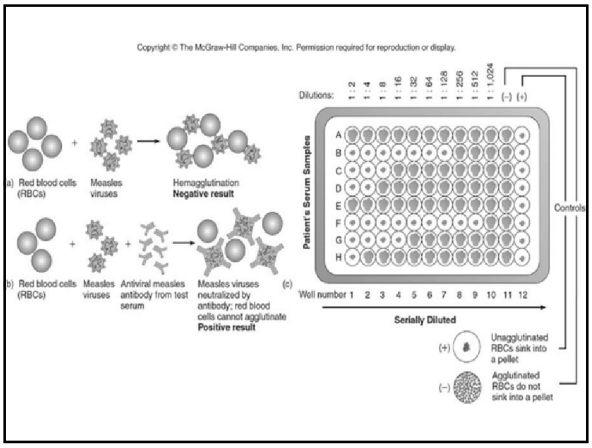

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Complement Fixation Tests

- Complement fixation tests detect lysins- Ab that fix complement and can lyse target cells.
- Involves mixing test Ag and 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.

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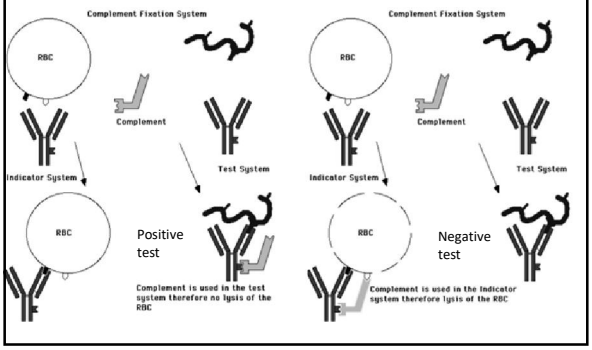
Agglutination: Continuation

eg, Haemagglutination and Complement Fixation



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3. Immunolabelling Methods

- Use detectable label covalently bound to Ab or Ag or second molecule which binds to Fc region of Ab in immune complex
- More sensitive
- Direct and 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

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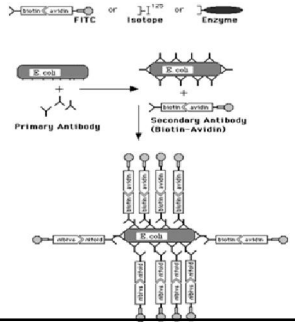
a) Biotin-Avidin Assay

Avidin: glycoprotein derived from egg albumin has very high affinity for vitamin, biotin, and 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 2º Abs are labelled, using the biotin-avidin system, they can be efficiently employed in indirect IF, ELISA and RIA.



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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 partly bind to antibody as a complex is precipitated either by ammonium sulphate (Farr) or by an antiglobulin (antiglobulin coprecipitation.)

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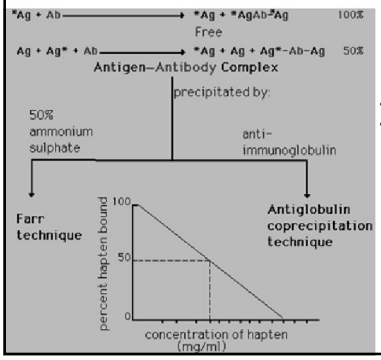
b) Radioimmunossays (RIA)



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

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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.

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Direct ELISA – Detect antigen

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

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

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

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Indirect ELISA – look for antibody

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

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

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

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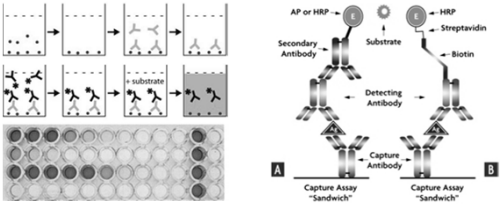
Enzyme-Linked ImmunoSorbent Assay (ELISA)



Indirect ELISA Sandwich ELISA

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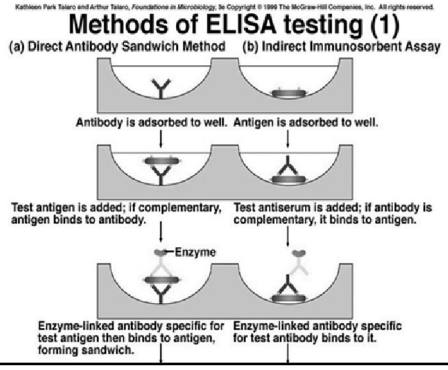
Use β-galactosidase, Alkaline phosphatase or Horseradish peroxidase (HRP) linked to Ab, Ag or other molecule



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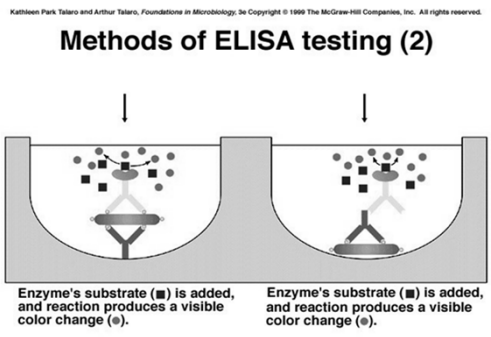
Visualization of Reaction



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

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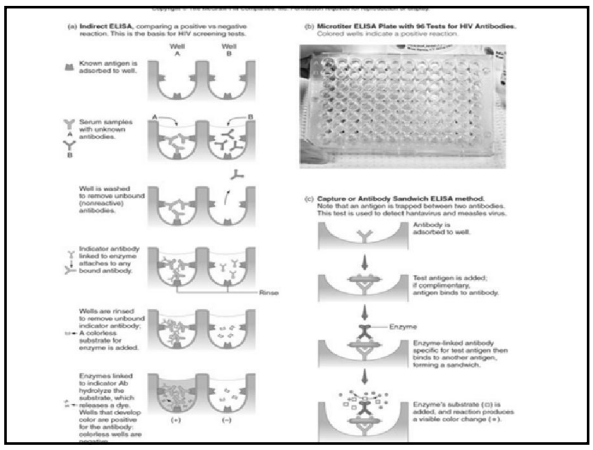

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d) Immunoblotting (Western Blotting)

- 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

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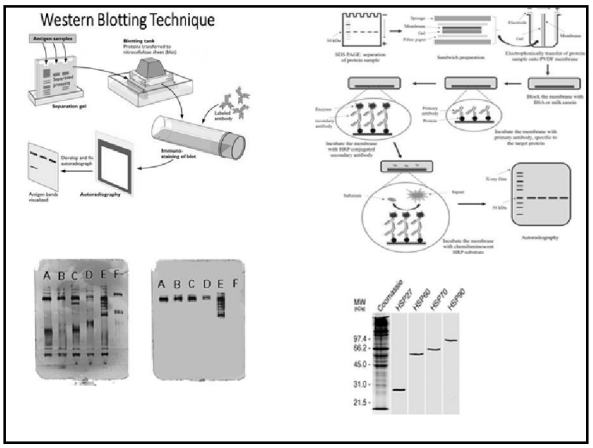

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e) Immunofluorescence

- 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

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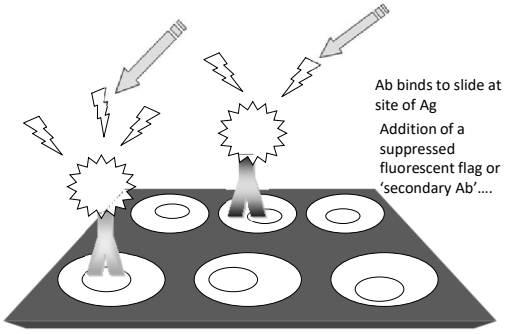
Immunohistochemistry



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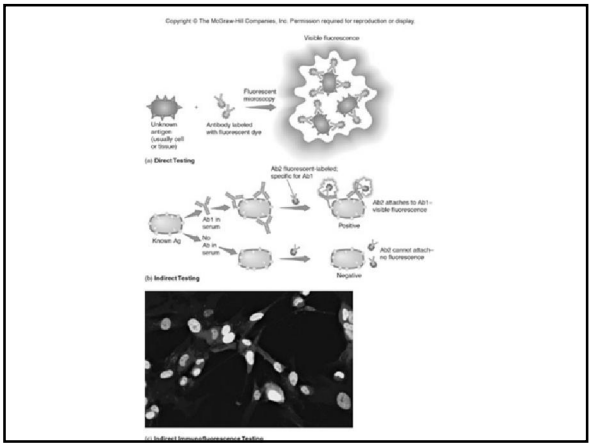

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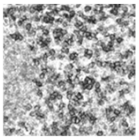
Immunohistochemistry

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



T cells – CD3 staining

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f) Leukocyte Assays using labelled Abs

- Cells can be analysed and isolated on basis of their distinct surface Ags, size, or both by Flow Cytometry.
- Flow Cytometres are instruments that can analyse properties of single cells as they pass through an orifice at high velocity. These instruments measure light scatter, volume, and fluorescence.
- FACS can analyse and sort lymphocyte sub populations, as identified by fluorescein-labelled mAb

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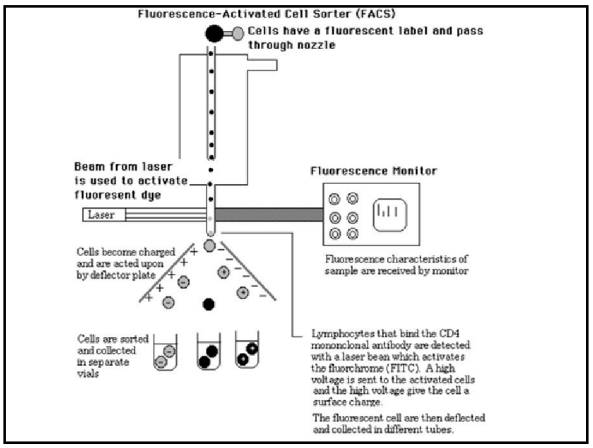

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Rosette and Plaque Formation

Separation of T and Non - T Cells from Monounclear Cells (MNCs)

- The E-rosetting technique describes a procedure for separating T cells and non -T cells from a population of MNCs.
- This method is based on the ability of human T cells to bind to sheep RBCs via their CD2 molecule.

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Rosette and Plaque Formation

- Neuraminidase treatment of sheep red blood cells (SRBCs) enhances the binding of SRBCs to T lymphocytes:
  - First neuraminidase treated SRBCs are prepared.
  - Secondly, SRBCs and MNCs are mixed to form rosettes (E+, which are then isolated from the non - rosetting population (E-, i.e., B cells and monocytes) by Ficoll gradient centrifugation.
  - In the last step, bound SRBCs are separated from the rosetted T cells by hypotonic lysis (hypotonic buffer capable of lysing red cells, but keeping white cells (or at least nuclei) intact

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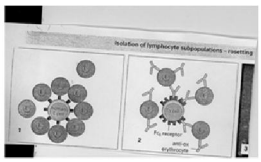
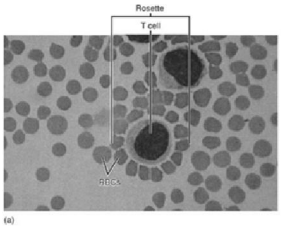
Rosette and Plaque Formation



E-rosetting



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ELISPOT Assays

- PBMCs 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.

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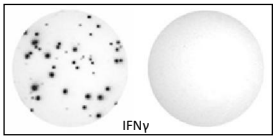
Enzyme-Linked Immunospot (ELISPOT)

- Detect cells secreting a specific antigen
- Most commonly used to detect cytokine secretion by T cells upon stimulation (IFN-γ, IL-2, IL-4, etc.)
- Can detect cytotoxic activity (perforin)



http://www.protocol-online.org/

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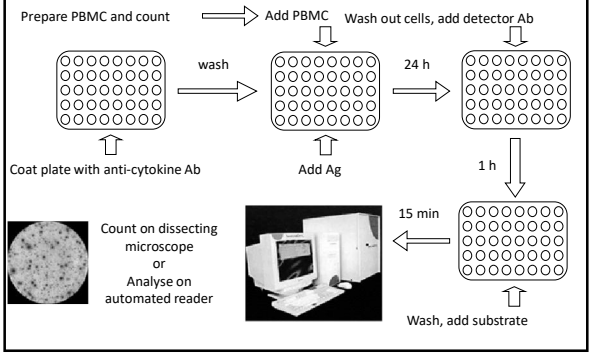
ELISPOT Assay Principle



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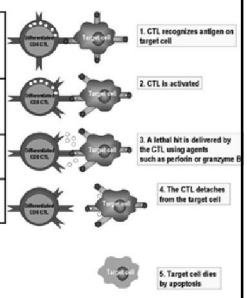
Cytotoxic T Lymphocyte (CTL) Assay

Cell	Function	Released Effector Molecules	Membrane Effector Molecules
Th1	Cytokines to activate CTLs and macrophages	IL-2, IFNγ, TNFβ, GM-CSF	TNFβ
Th2	Activate B cells	IL-4, IL-10,	CD40L
CTL	Kill Target cells	Perforin, Granzymes	Fas Ligand



- Perforin: pore-forming protein
  pokes holes in target membrane
- Granzyme = **Gran**ules en**zymes**
- Granzyme: protease family involved in induction of apoptosis in target cells after entry through perforin pores

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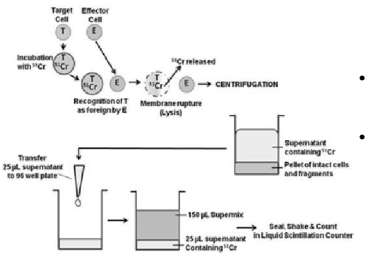
Cytotoxic T Lymphocyte (CTL) Assay



- Target cells mixed with effector cells at various ratios
- Measure release of ^{51}Cr into medium
- Expression as percentage lysis relative to no lysis non-specific effector cells

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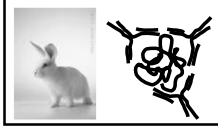
Diagnostic and Therapeutic Use of Antibodies

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

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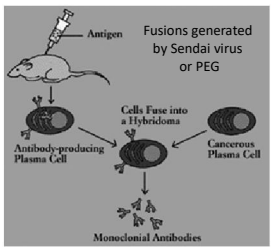
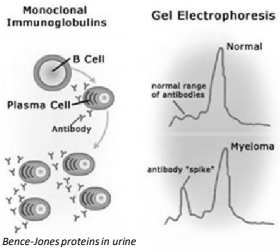
Hybridoma Technology

- George Kohler and Cesar Milstein - 1975
- Shared Nobel Prize – 1984
- Term "Hybridoma" coined by Leonard Herzenberg - 1975



Multiple Myeloma (human disease)



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

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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 = Hypoxanthine, Aminopterin, Thymidine
PEG = Poly Ethylene Glycol

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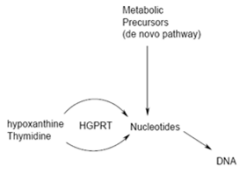
Hybridoma Selection (HAT Medium)



- HAT medium used for selection of hybrid cells
- Nucleotide synthesis essential for cell survival
- In HAT medium **Aminopterin** blocks cellular synthesis of purine and pyrimidines from simple sugars (*de novo* pathway)

- But cells can thrive by using hypoxanthine and thymidine present in medium by a salvage pathway using enzyme **Hypoxanthine-Guanine Phosphoribosyltransferase (HGPRT)**
- HGPRT is one of the central enzymes that recycle the building blocks of RNA and DNA

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How HAT Medium Works in Selection of Hybrid Cells

- B cells are HGPRT-positive and can survive in HAT medium but they undergo normal cell death and some divisions
- In Hybridoma Technology, myeloma cells used are HGPRT-deficient
  - So these cells cannot survive in HAT medium as Aminopterin blocks the *de novo* pathway
- Hybrid cells have HGPRT enzyme from B cells, as well as the property of immortality from myeloma cells
  - So only hybrid cells survive in HAT medium

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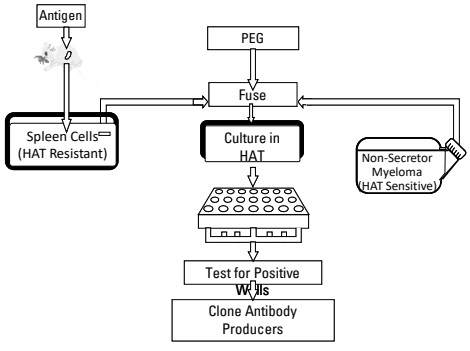
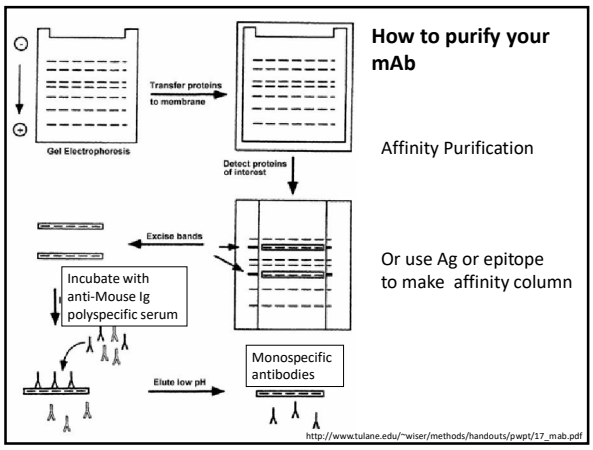
Monoclonal Antibody Production



60



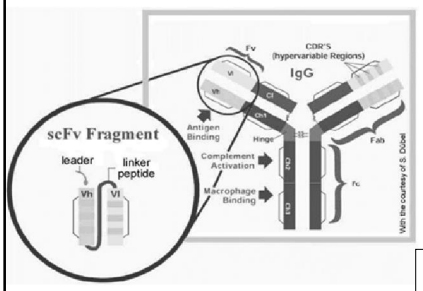
How to purify your mAb

Affinity Purification

Or use Ag or epitope to make affinity column

61

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

62

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

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Introduction to Flow Cytometry

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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

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Flow Cytometry

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

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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

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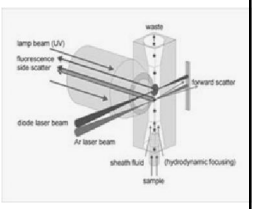
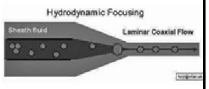
Principle of Flow Cytometry

- 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





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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..)

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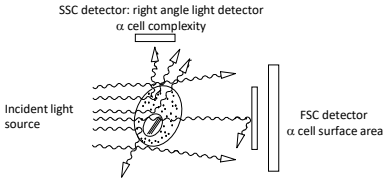
Properties of FSC and SSC



- 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

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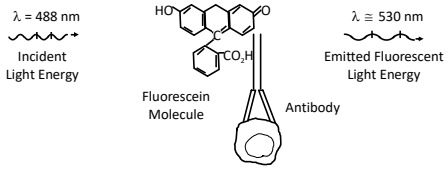
What is Fluorescent Light



- 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

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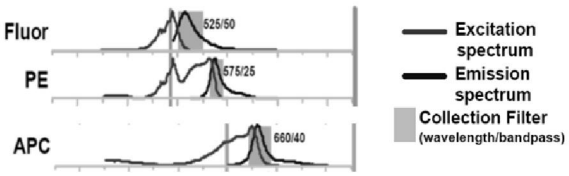
Fluorochromes: Excitation and Emission Spectra



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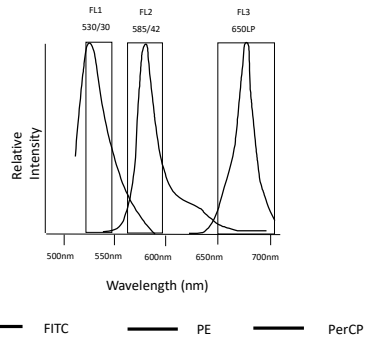
Emission Spectra



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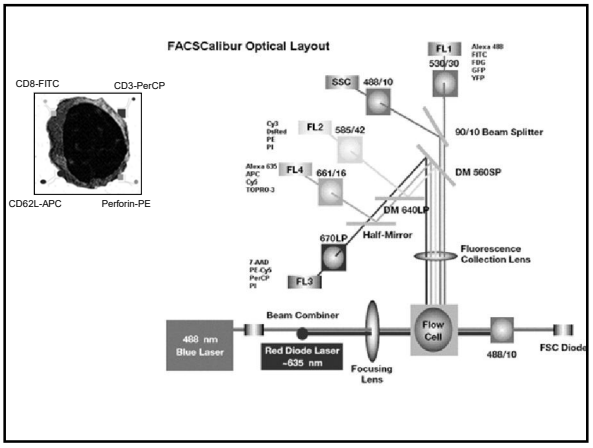

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Fluorescence Channels

Channel	Fluorochrome
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)

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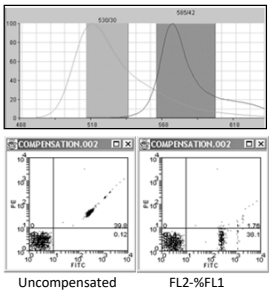
Compensation

- 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



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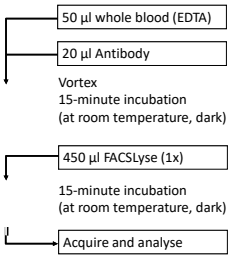
Typical Assay



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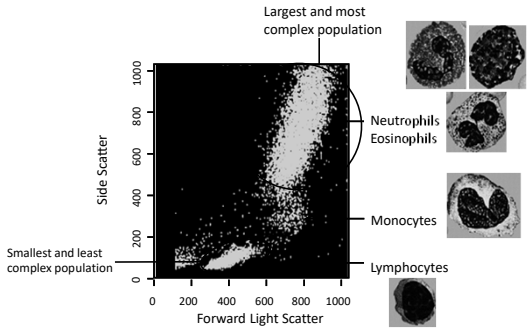
Whole Blood-RBCs lysed



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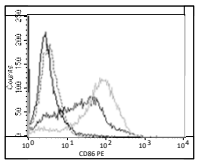
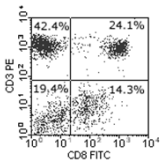
Flow Cytometry Analysis

- 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



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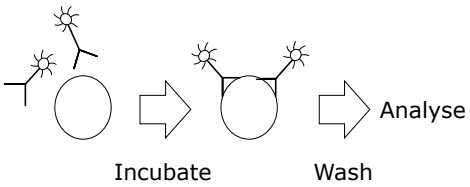
Two-Colour Direct Staining



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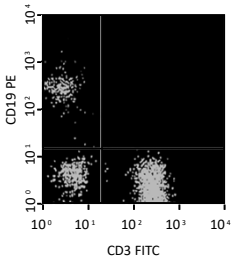
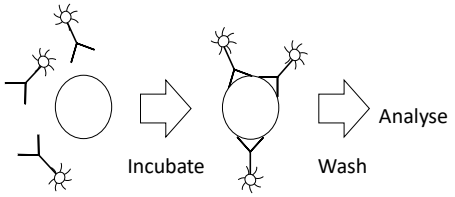
Two-Colour Cell Analysis



81

Three-Colour Direct Staining



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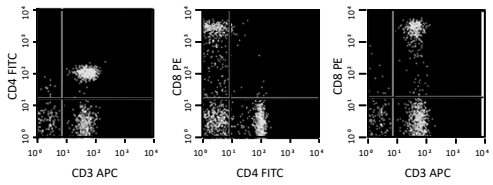
Three-Colour Cell Analysis



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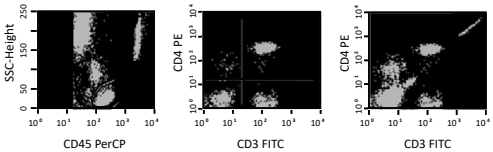
Absolute Counts Using TriTEST™ / TruCOUNT™

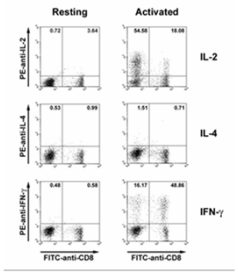


$$\frac{\text{\# of events in quadrant containing cell population}}{\text{\# of events in Absolute Count bead region}} \times \frac{\text{total \# of beads per test}}{\text{test volume}} = \text{\# CD4 cells/µL}$$

84

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



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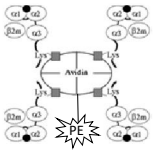
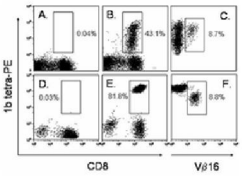
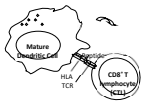
MHC Tetramer Staining

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







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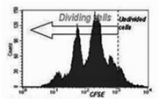
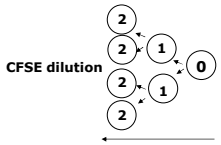
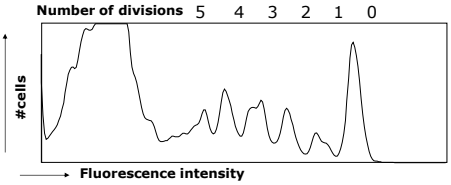
Cell Proliferation Analysis Using CFSE Dye Dilution







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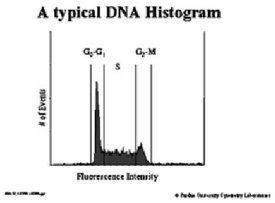
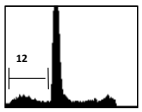
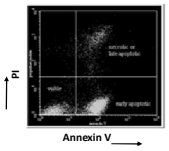
DNA Analysis / Apoptosis







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

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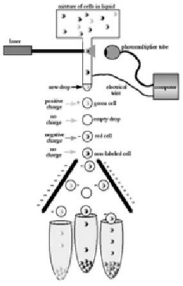
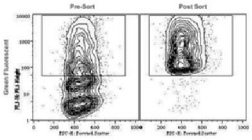
Cell Sorting - FACS





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Cytometric Bead Arrays in Flow Cytometry

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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

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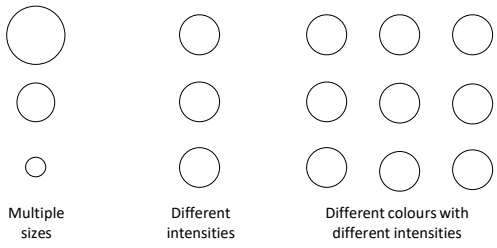
Beads Provide a Flexible Platform

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



Multiple sizes | Different intensities | Different colours with different intensities

From BD Biosciences

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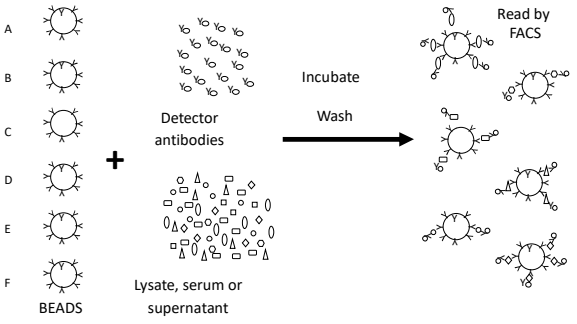
Bead Assay Basics



From BD Biosciences

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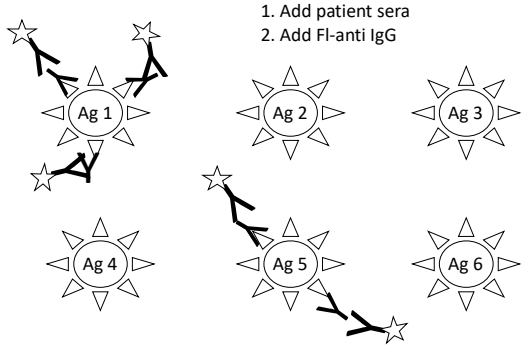
The Technology (Ab detection)

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



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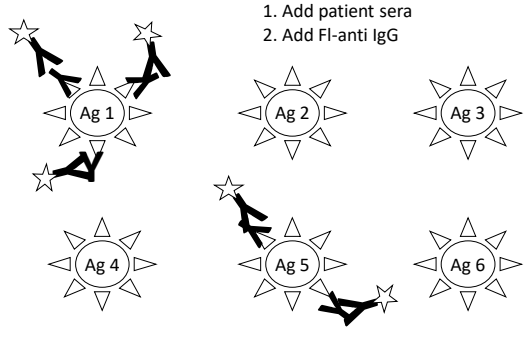
Ab Detection

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



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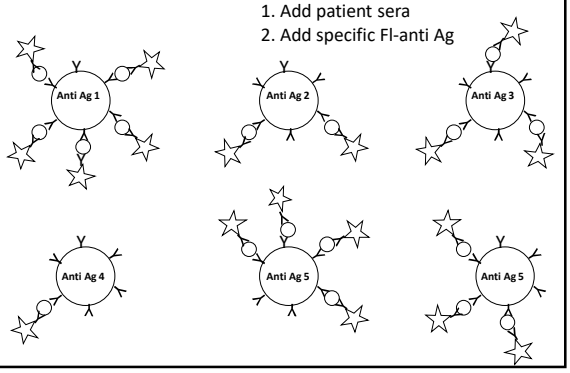
The Technology (Ag Detection)

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



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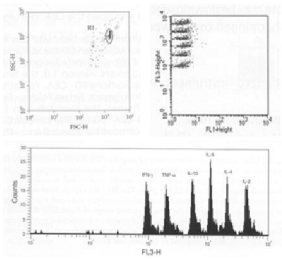
Cytokine Bead Array

Light scatter of beads
- Theoretically – limitless possiblities
- Same size beads – simplifies

Red Fluorescence of negative beads



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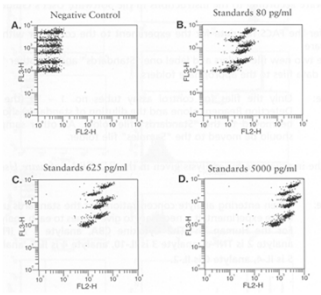
Cytokine Bead Array

9 standards for calibration
(20-5000 pg/ml)

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



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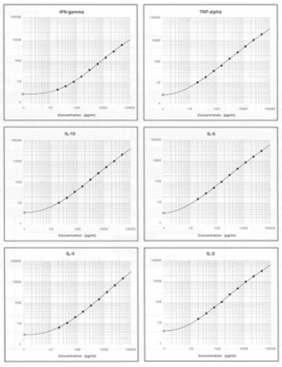
Cytokine Bead Array

Example of software produced standard curves



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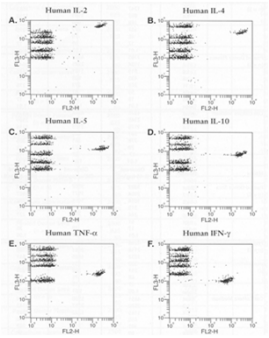
Cytokine Bead Array

Specificity – little to no cross reactivity

Cross reactivity detection important in transplant HLA typing



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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

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Common Applications of Flow Cytometry

- Phenotype of cell, surface molecules
- Flow cross-matching
- 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

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