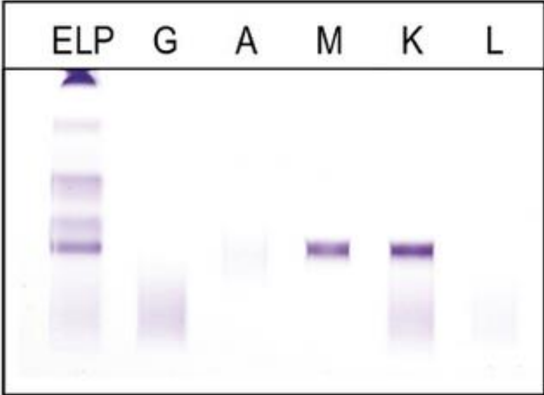
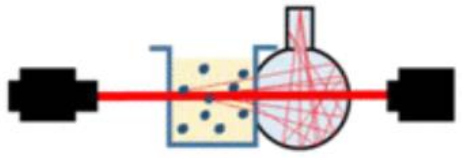
Antibody-based diagnostic tests

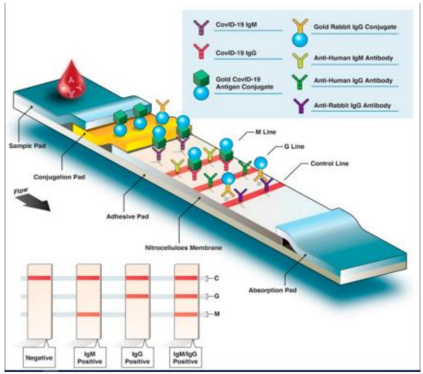
Immunofixation electrophoresis



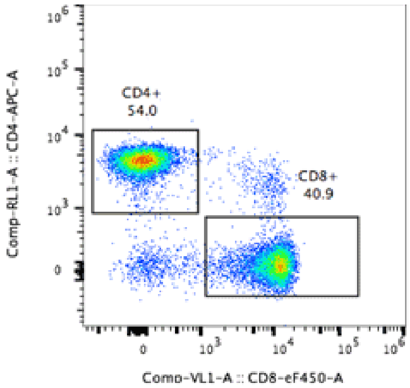
Immunonephelometry



Rapid diagnostic tests

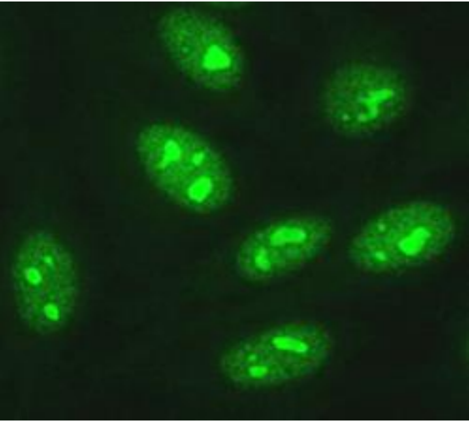


Flow cytometry

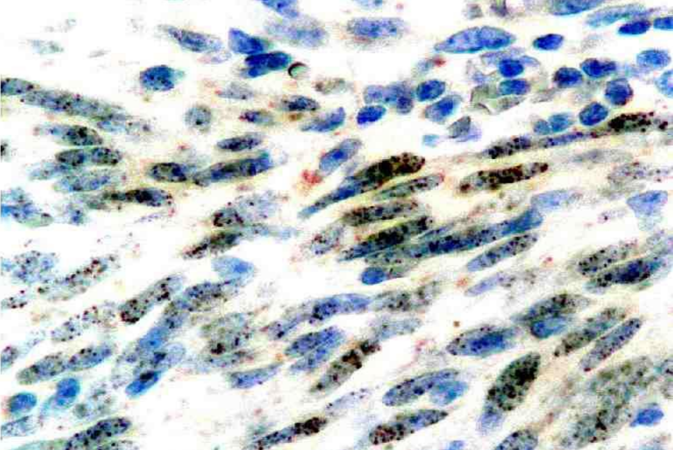


Immunoagglutination

Immunoflourescence

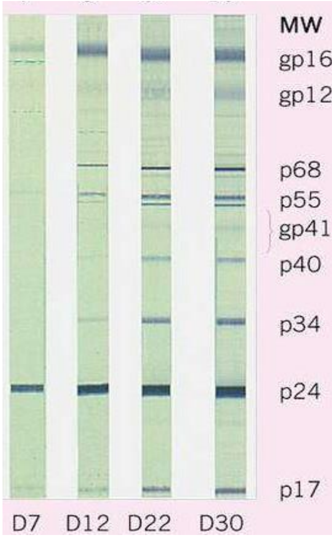


Immunohistology



ELISA test

Immunoblot



Viviana Marin-Esteban

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Antibody-based diagnostic tests:

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   - Immunoprecipitation methods
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   - Immunoagglutination methods

2. Immunolabeling methods
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   - Immunoblot
   - Immunochromatography (rapid diagnostic tests)
   - Immunohistology
   - Immunophenotyping and Cell cytometry

Some history of antibody-related discoveries and techniques

1890s: Emil von Behring and Kitasato Shibasaburo, **serum specific activity** against diphtheria and tetanus toxins, and started the practice of serotherapy to treat these diseases. *

1908: Astrid Fagraeus, **plasma B cells** are specifically involved in **antibody generation**.

1930s: Arne Tiselius, first **serum protein electrophoresis** technique.

1957: Frank Burnet and David Talmage, **clonal selection theory →** a B lymphocyte makes a single specific antibody molecule whose identity/specificity is determined before it encounters an antigen.

1959: Gerald Edelman and Rodney Porter, **molecular structure of antibodies. ***

1953: Grabar and Williams, **immunoelectrophoresis** to identify different serum Ig fractions.

1953: Coulter, **first cytometer device** for **counting and sizing** dilute suspensions of cells

1959 : Rosalyn Sussman Yalow, **radioimmunoassay technique. ***

1964: Wilson, **immunofixation** technique.

1968: Wolfgang Göhde, **first fluorescence-based flow cytometry device.**

1971: Killingsworth and Savory, applied **nephelometry to quantify of immunoglobulin isotypes in serum**.

1975: Georges Köhler and César Milstein, hybridome technique to produce murine **monoclonal antibodies**. *

1988: Greg Winter and his team pioneered the techniques to humanize monoclonal antibodies.

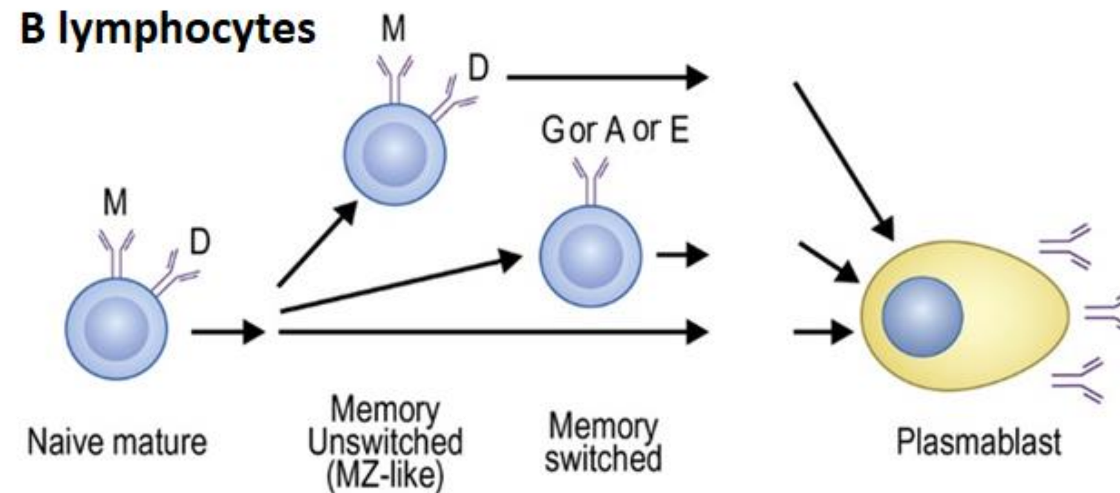
* Nobel prize winners

Serum immunoglobulins (Igs) are produced by plasmablasts/plasma cells (effector B lymphocytes)

-Present at the cell surface of naïve and memory B lymphocytes, **secreted by plasmablasts / plasma cells** → antigen-receptors of the humoral adaptive response.

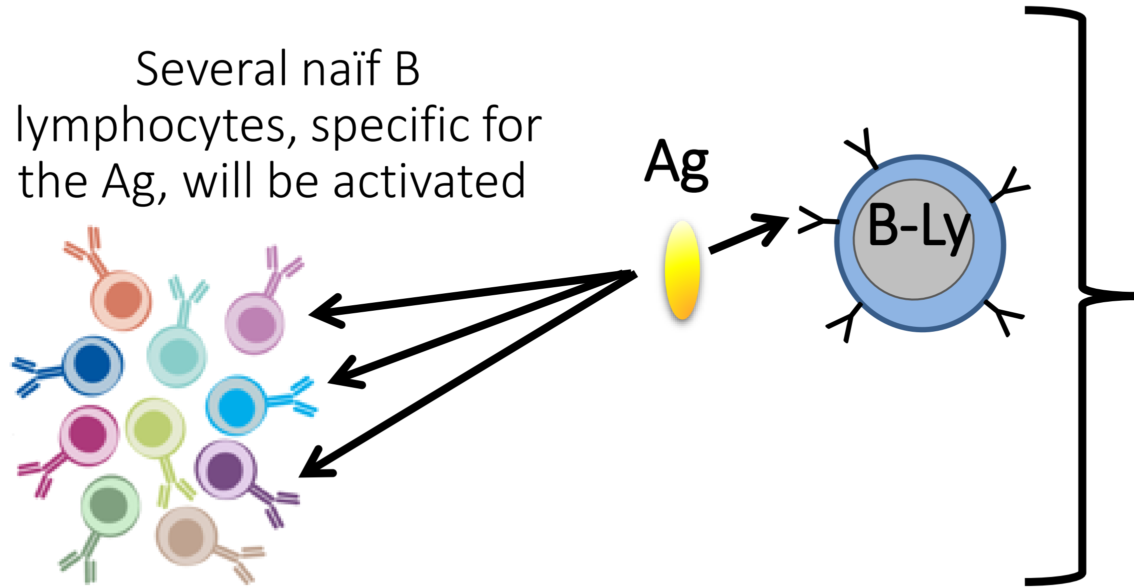
-**Five isotypes**: **IgM, IgD, IgG, IgA, IgE**. IgG further subdivides in 4 (IgG1, IgG2, IgG3, IgG4) and IgA in 2 (IgA1, IgA2).

- IgG, IgA, and IgM levels show an increase with age, from infancy to adulthood.



Schroeder *et al. Clinical Immunology* 2013

Humoral adaptive response (B lymphocytes) – a polyclonal response, polyclonal serum

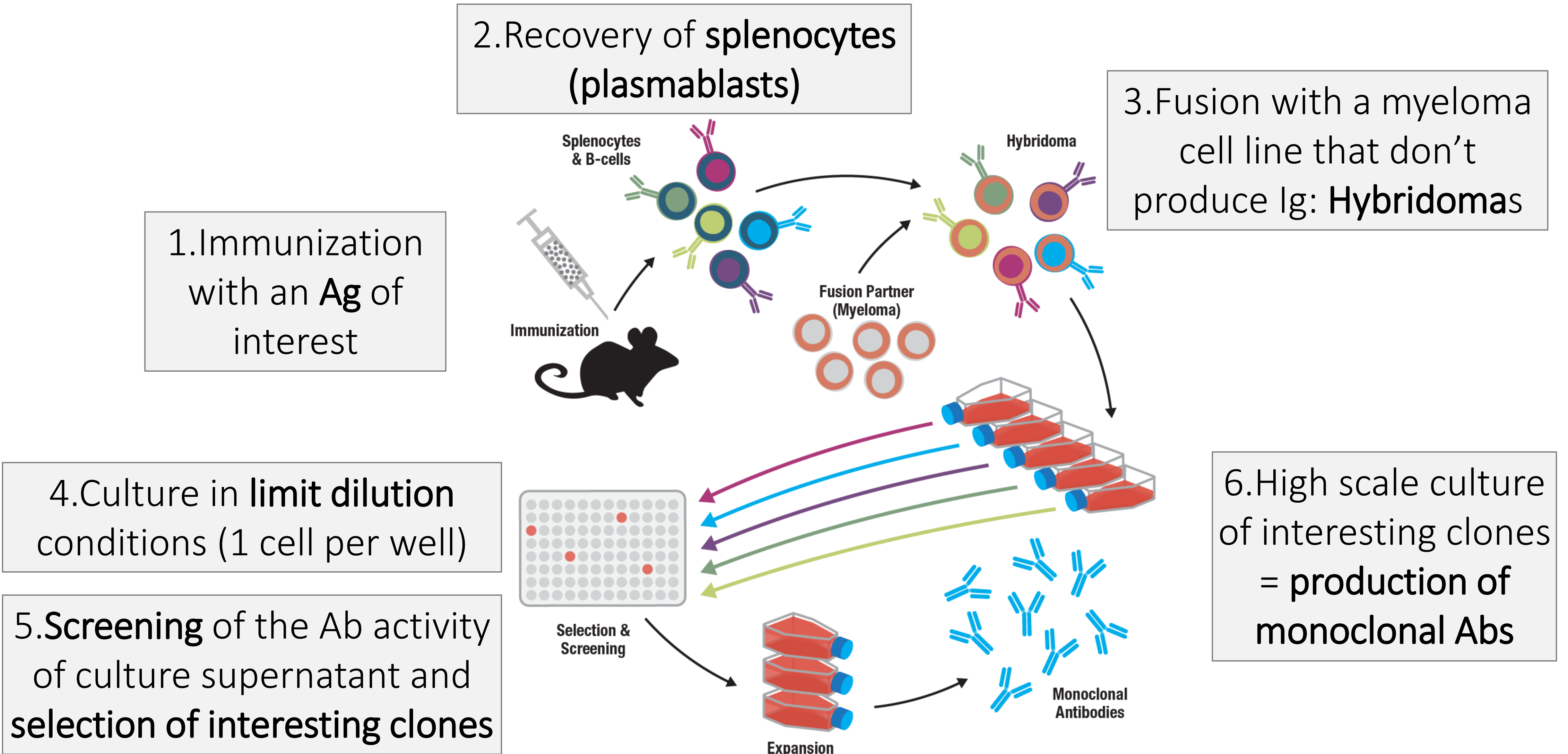


This polyclonal response ends up with the production of **a polyclonal pool of plasma cells and memory B cells specific for the Ag**

Plasma cells secret **soluble immunoglobulins** (… or **antibodies, Abs**) that will enrich the diversity of plasma immunoglobulins.

Adult serum: > 10 million different Ig identities!

Production of monoclonal antibodies for diagnostic or research

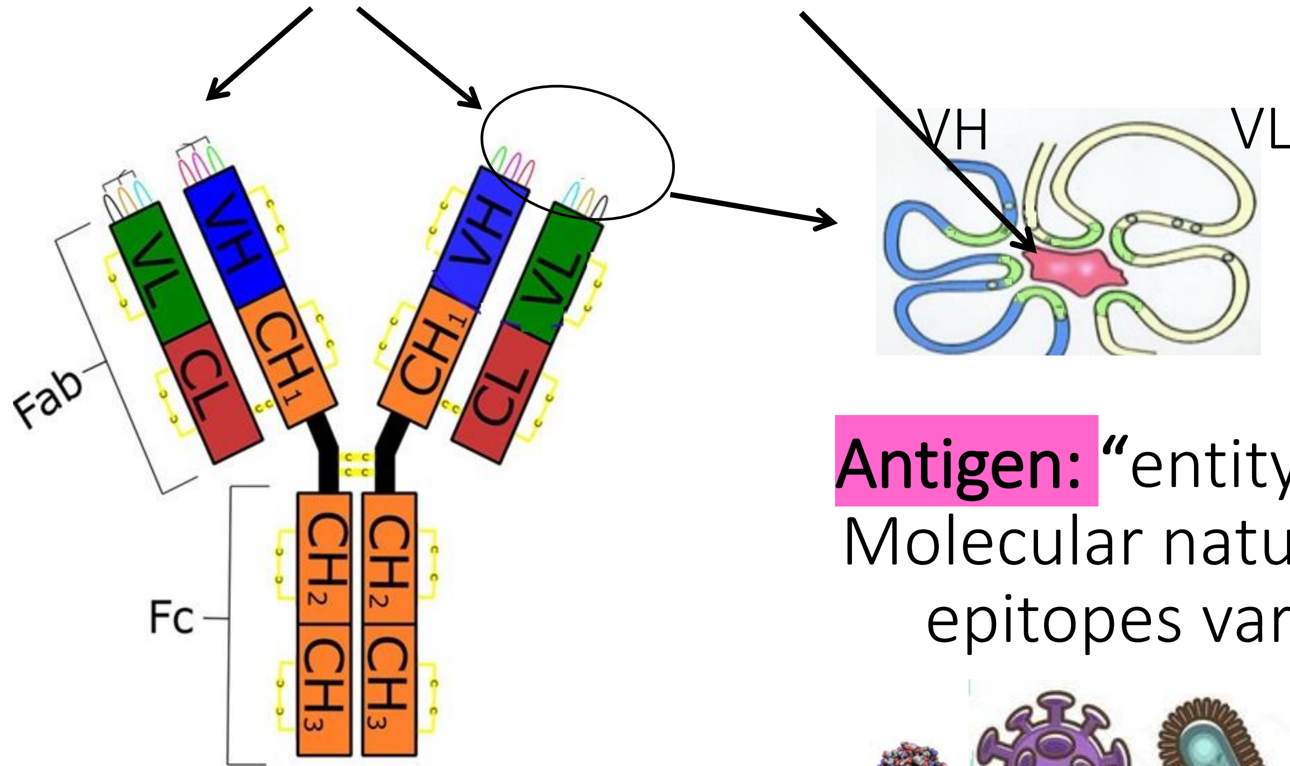


https://blog.cellsignal.com/antibody-essentials-part-4-polyclonal-vs-monoclonal-antibodies

Antibody and antigen structure

Antibody

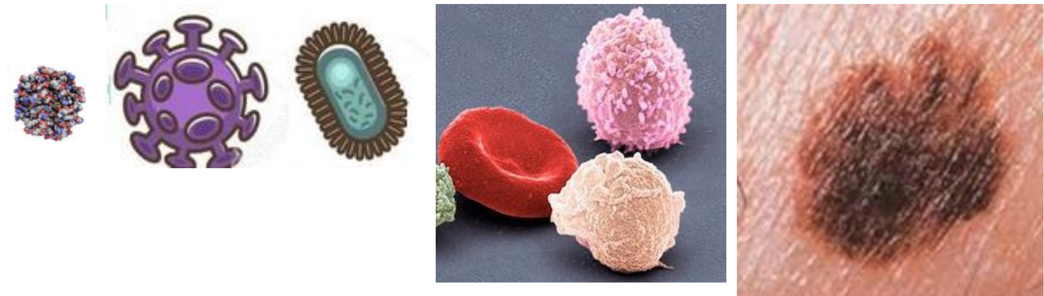
Paratope epitope



Light chains
Isotypes : k, l

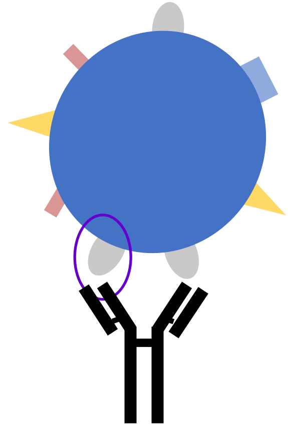
Heavy chains
Isotypes : m, d, g, a, e

Antigen: "entity" recognized by an Ab.
Molecular nature, size and number of epitopes vary among "Antigens"

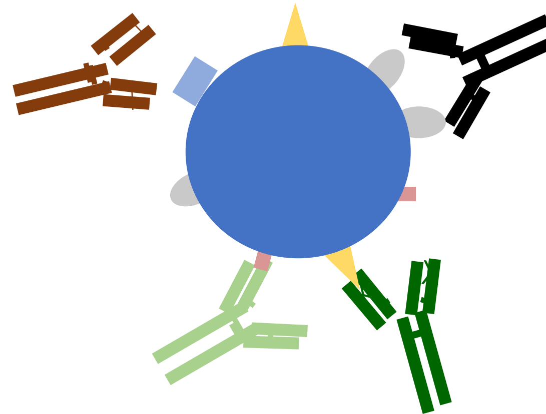


Immune complexes can have different presentations

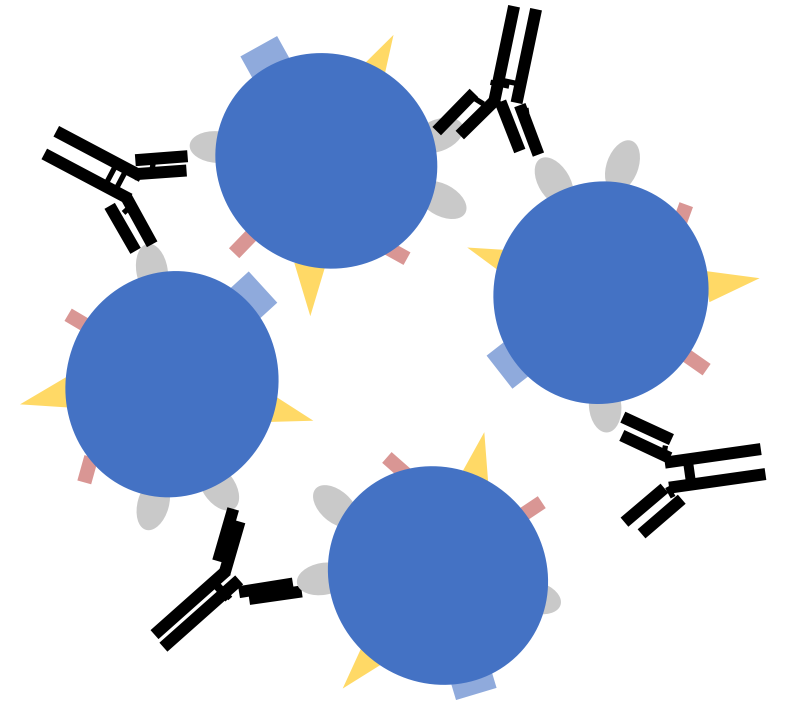
A bimolecular complex
Single Ab + single Ag



Multiple Abs fixed to a single “Ag” (to repetitive identical or different epitopes)



Immune complex lattices
Multiple Ab molecules and Ags bound together



Important point to consider when choosing an Ab-based analytical method

Analytical <u>objective</u> (which is the question)

→ exploring the presence of an Ag
→ or exploring the presence of an Ab in a biological sample

Quantitative or qualitative result

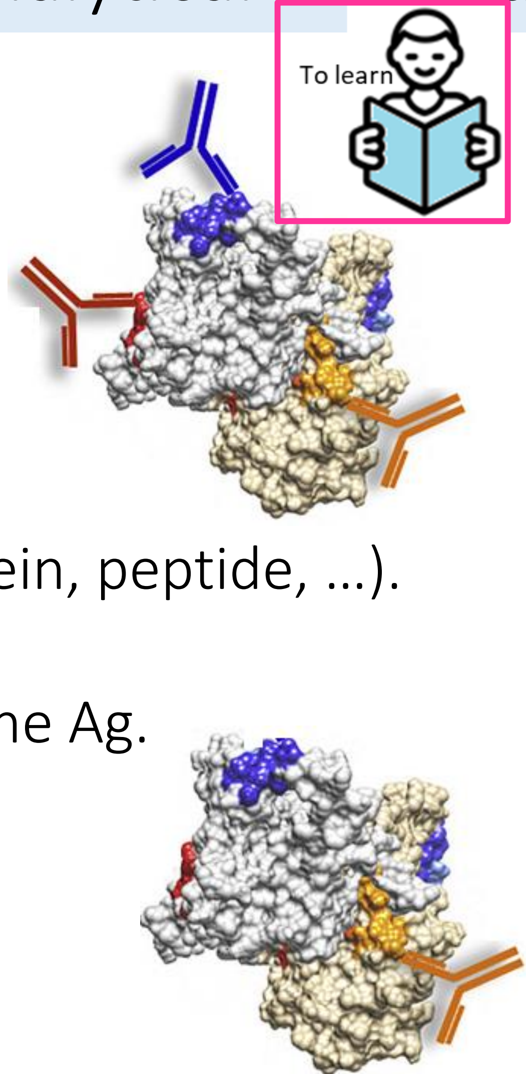
→ qualitative result: yes/no
→ or quantitative result : Ag dose (g/L)
Ab titer (U/L)

Quality of the method:

Specificity: ability to yield a negative result when the analyte of interest is not present in the sample.

Sensitivity: ability to yield a positive result when the analyte of interest is present in the sample.

Important point to consider when choosing an Ab-based analytical method



Antibody reagents

→ Polyclonal serum (usually purified IgGs)

Mix of different IgG against a single Ag. All these different IgGs **fix the Ag with different affinities and at different epitopes**.

Production strategy:

- Immunisation of an animal with the purified Ag (pathogen, protein, peptide, …).
- Recuperation of the serum, fractionation of IgGs
- Purification of specific IgGs by affinity chromatography against the Ag.

→ Monoclonal antibody

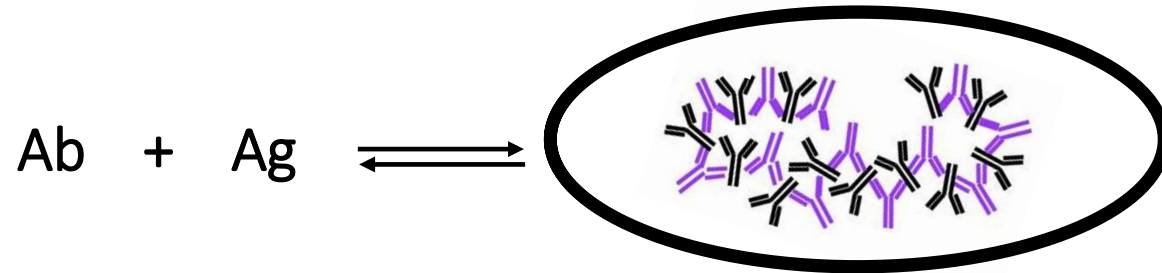
A single Ab identity issue from a single plasmocyte clone → **fix the Ag at a single epitope and with a unique affinity**.

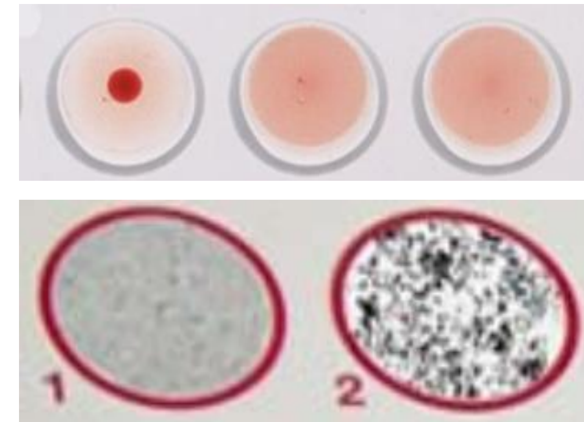
- After immunisation of the animal, recuperation of plasmoblasts, creation of hybridomes and cloning and selection of single hybridomes.

Antibody-based diagnostic methods: **label-free vs immunolabeling**

Label free: Large Immune complex lattices.

Methods with low sensitivity: **g/L, mg/L**





Immunoagglutination

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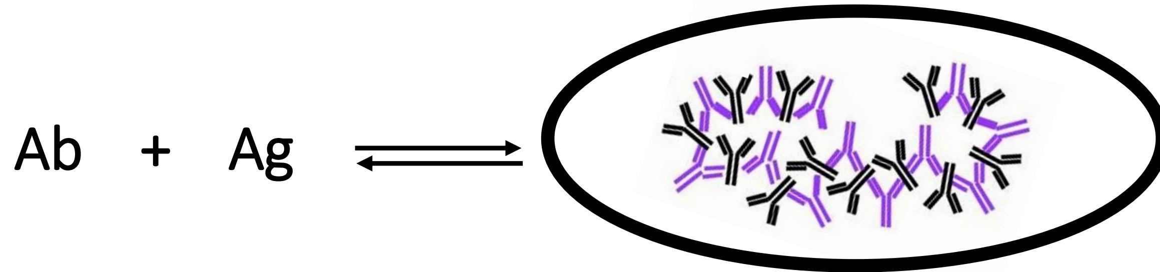
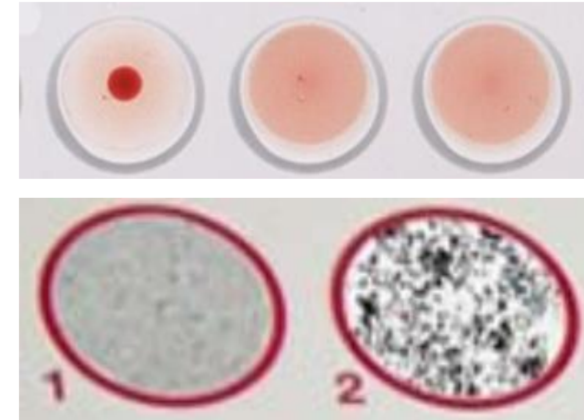
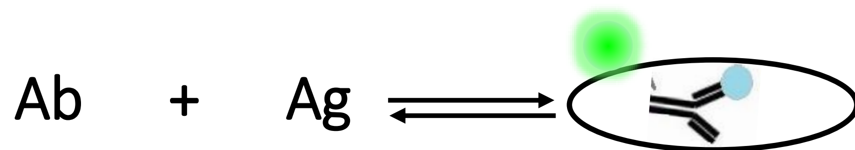
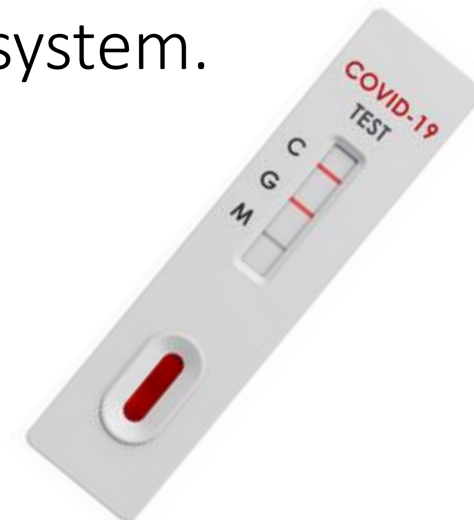




Immunolabeling methods: use Ag or Ab reagents conjugated to a tag, **detectable at tiny amounts** by a sensitive detection system.
Methods with high sensitivity: ng/L



Tag
- Colloidal gold
- Fluorochrome
- Enzyme
- Radioactif tracer



Label free methods for **serum immunoglobulin** assessment

Immunoprecipitation methods

- Nephelometry for quantitation of serum immunoglobulins
- Serum protein electrophoresis (not an Ab-based method but required to start exploring **serum immunoglulins**)
- Immunofixation electrophoresis for typing monoclonal immunoglobulins

Why to assess serum immunoglobulin levels ?

Quantitative immunoglobulin anomalies :

Hypogammaglobulinemia

- certain primary immunodeficiencies
- secondary to protein loss or iatrogenic causes (including medication)

Hypergammaglobulinemia

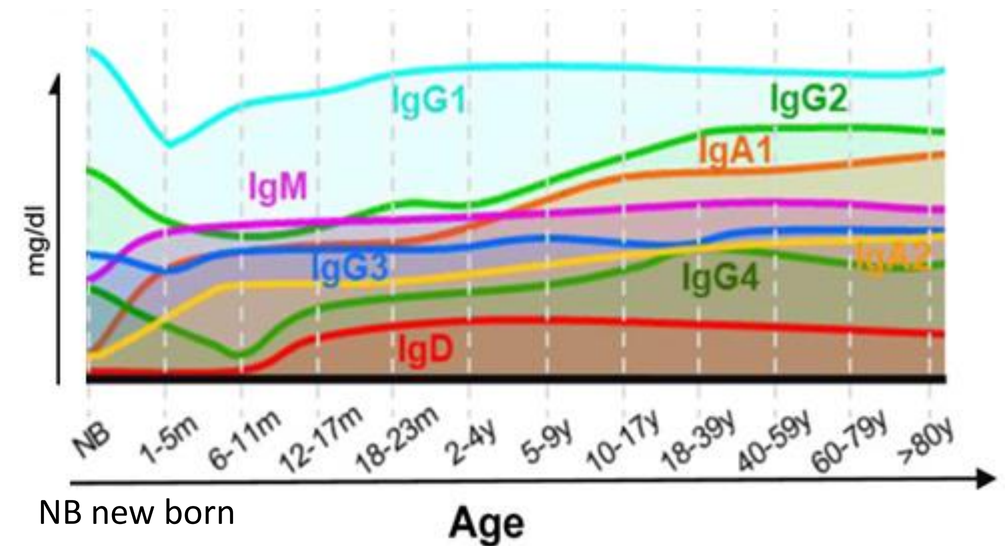
Polyclonal: in some chronic inflammatory diseases

- infectious diseases,
- autoimmune diseases
- chronic liver diseases

Monoclonal band

- Monoclonal gammopathies (malignant or not)

Serum Immunoglobulin levels vs. age



Blanco *et al.* JACI 2018

Concluding on quantitative alterations of serum immunoglobulins (or other fluid) requires **correlation with reference ranges** developed from **age-matched**, healthy individuals, representative of both genders. Each laboratory must generate its own reference values.

Immunonephelometry to quantify total blood immunoglobulins

Immunoprecipitation technique for liquid samples

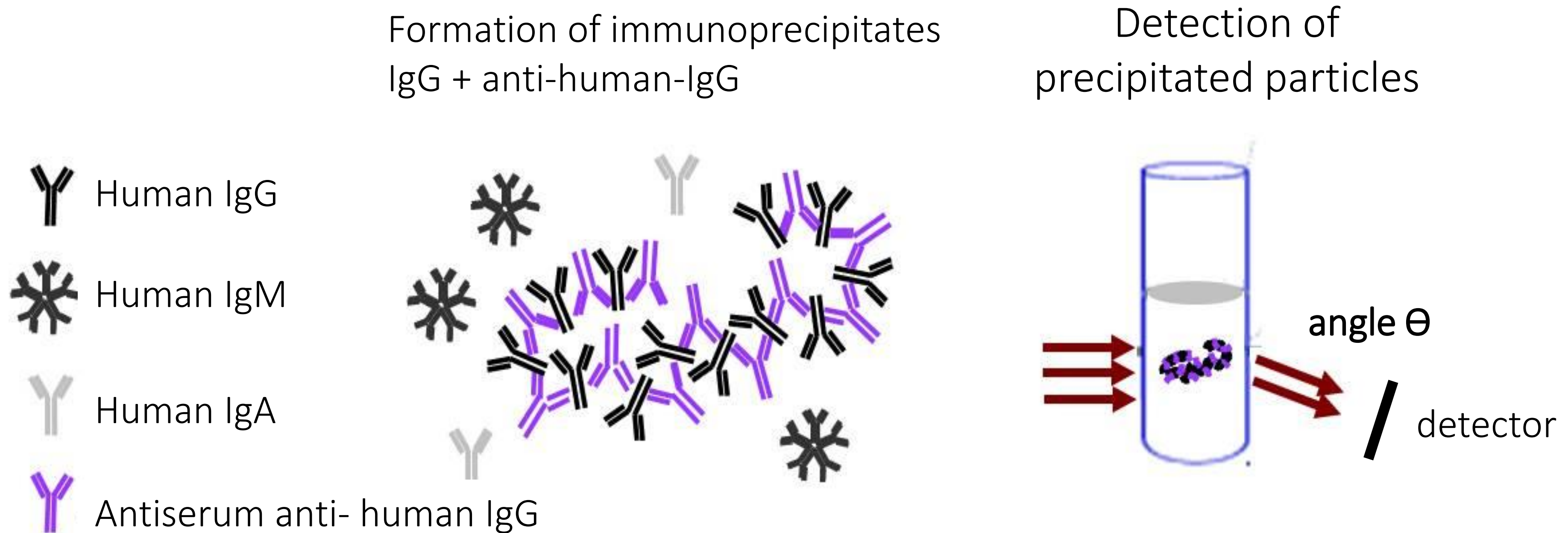
→ Yields quantitative results of

- total immunoglobulins
- Ig isotypes
- Ig isotype subclasses
- free light Ig chains (monoclonal gammopathies)

Quantitation of serum IgG by nephelometry

Steps :

1. Incubation of **serum** with specific **anti-serum anti-heavy chain for human IgG** (anti-gamma).
2. Place sample into the machine for an end-point measure.
3. Measurement of scattered laser light intensity.
4. Calculating IgG concentration according to a **standard curve**. (normal IgG values 6 – 16 g/L)



Measures laser light-scattering by immune complexes in solution. Detector placed at a fix angle. As the concentration of immune complexes increases, scattered light increases

Serum protein electrophoresis (SPEP)

Separation of serum proteins according to their electric charge under the action of an electric field.

Serum is buffered at pH 8.6 so most of the proteins become negatively charged.

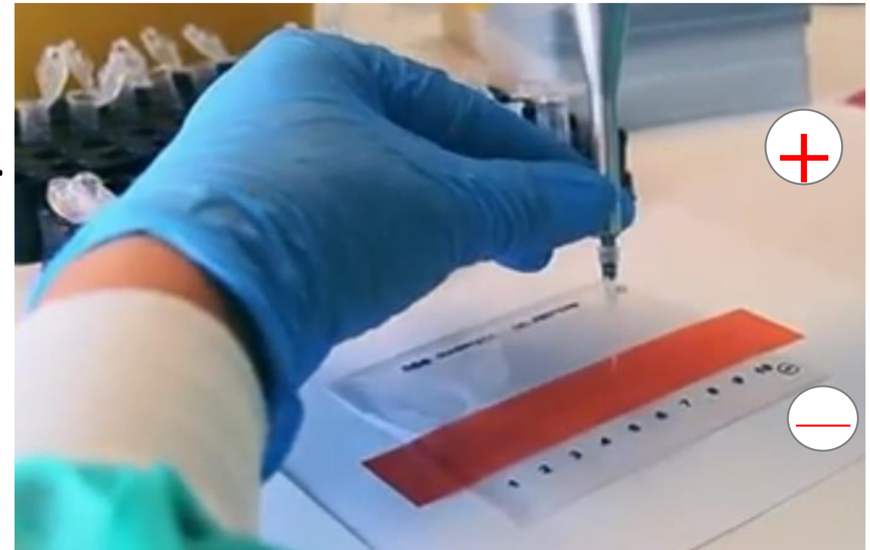
Steps :

1- Centrifugation of coagulated blood sample to recover serum.

2- Set sample pH at 8.6

3- Loading sample on the cellulose acetate gel.

4- Application of an electric field (electrophoretic migration).



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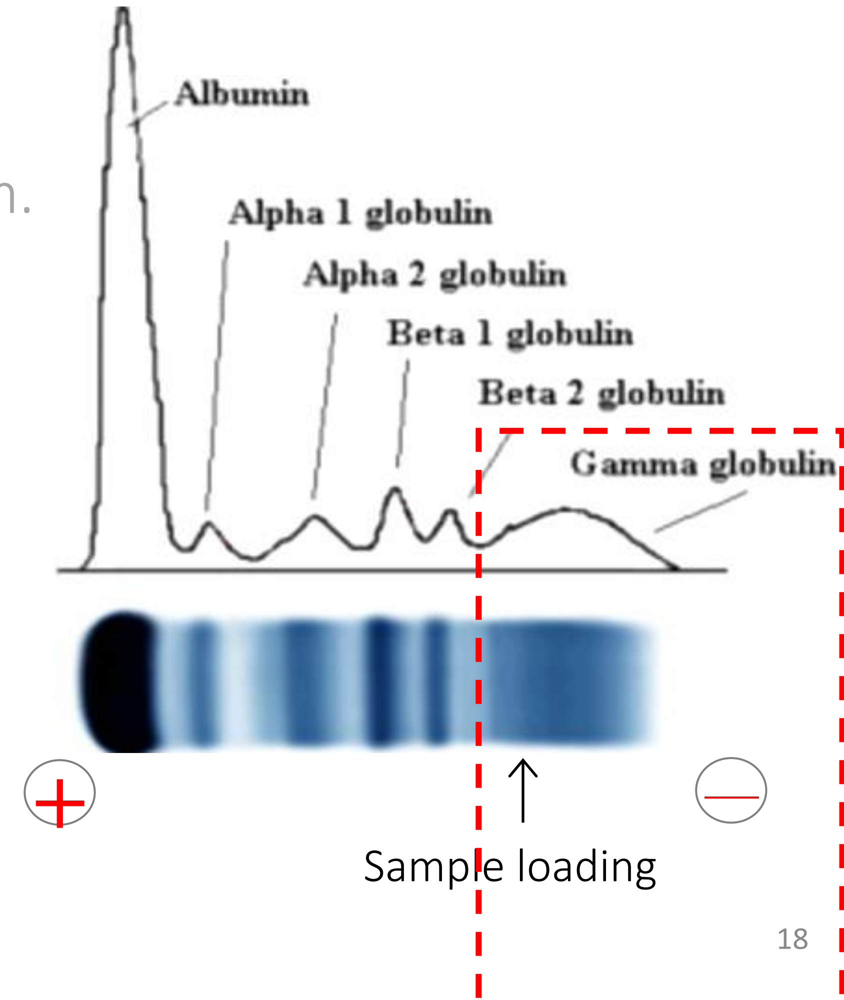
5- Fix proteins (acidic precipitation).

6-Staining proteins (coomassie blue or other dyes).

7- Densitometry scanning.

8- Translating AUC into protein concentration.



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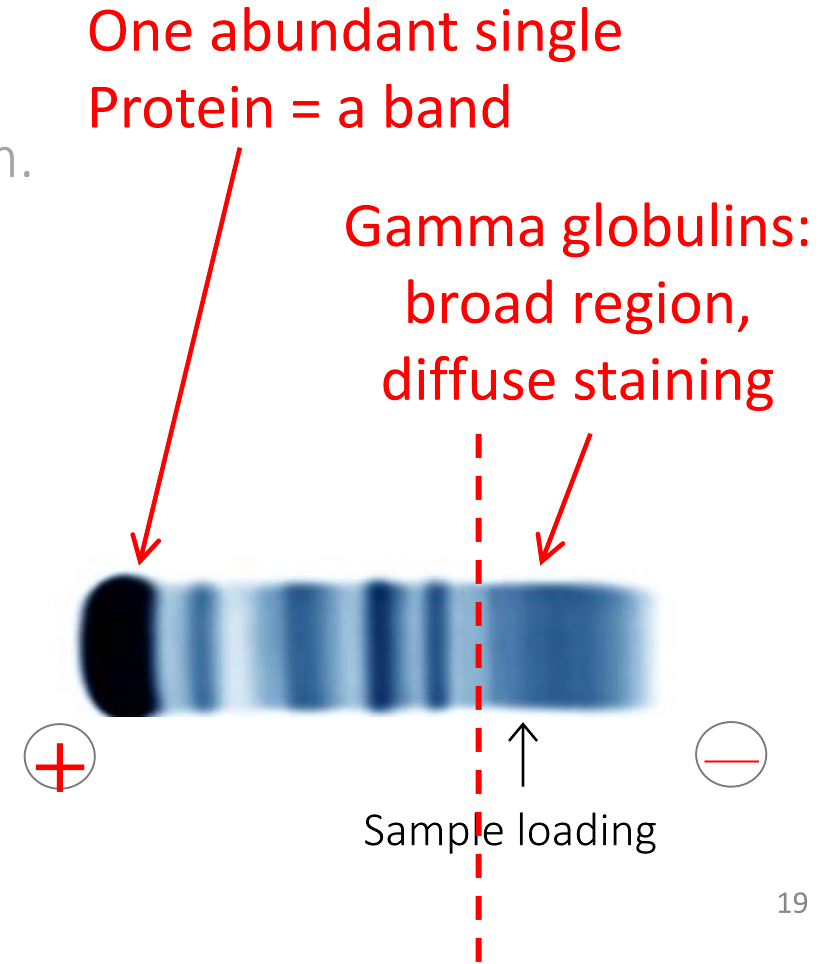
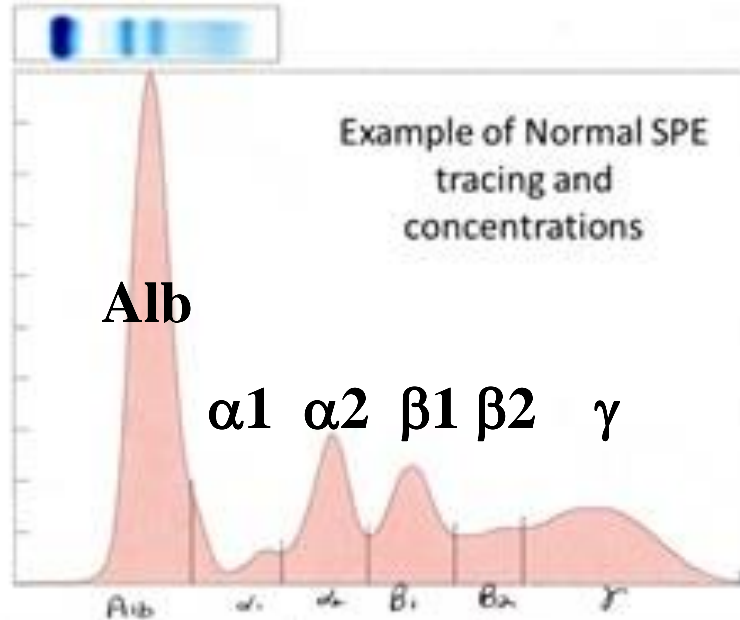


Densitometric tracing of SPEP allows for a quantitative analysis (area under the curve)

Gel image

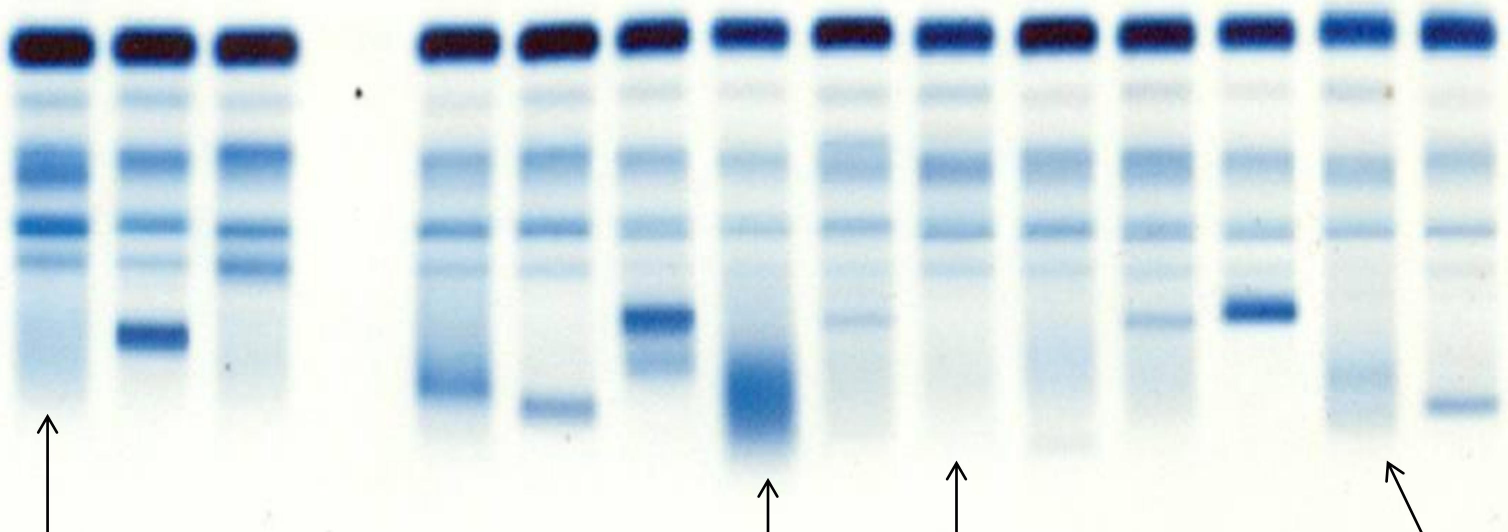
densitometer tracing



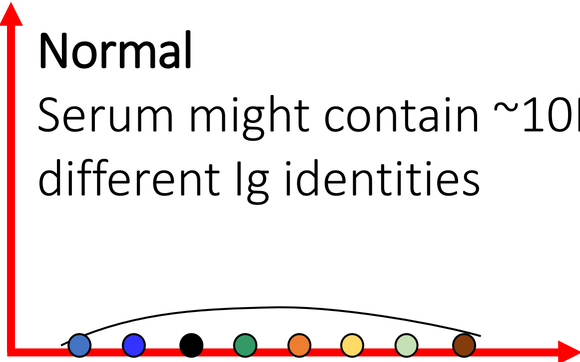
concentrations of protein within each band

Parameter	Results	Reference Interval
Total Protein (g/dL)	6.6	5.3 – 7.0
Albumin (g/dL)	2.91	2.19 – 3.29
α1 (g/dl)	0.11	0.1-0.31
α2 (g/dl)	1.02	0.94-1.63
β1 (g/dL)	0.95	0.34 – 1.01
β2 (g/dL)	0.55	0.21 – 1.03
γ (g/dL)	1.02	0.34 – 1.09
A/G	0.79	0.51 – 1.14

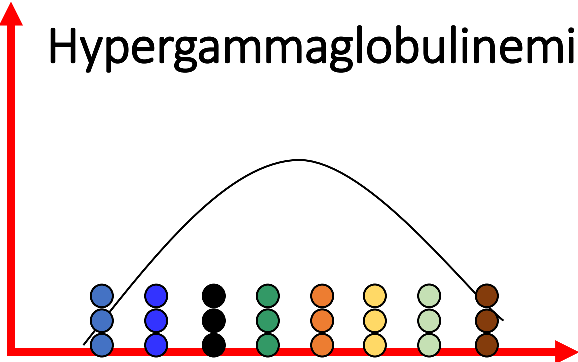
SPEP anomalies in the gamma region



Hypogammaglobulinemia



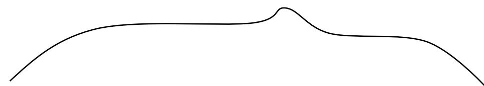
Normal
Serum might contain ~10M different Ig identities



Hypergammaglobulinemia

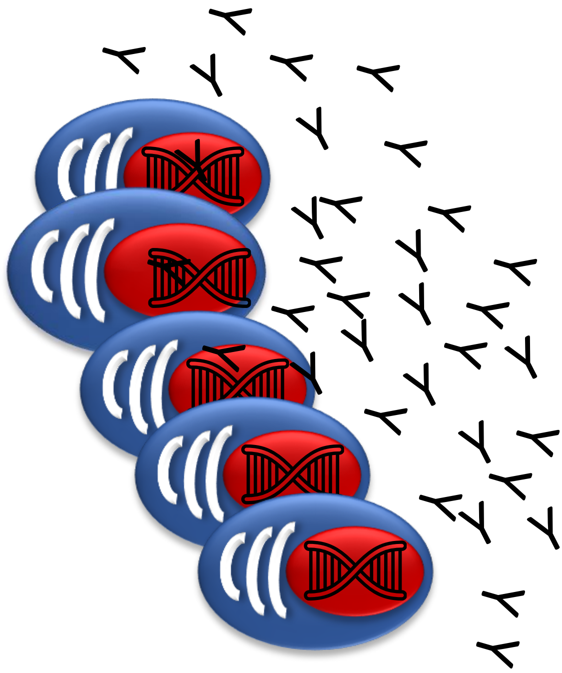
Increased amount of many different Igs = polyclonal response

Low abundance **monoclonal Ig**

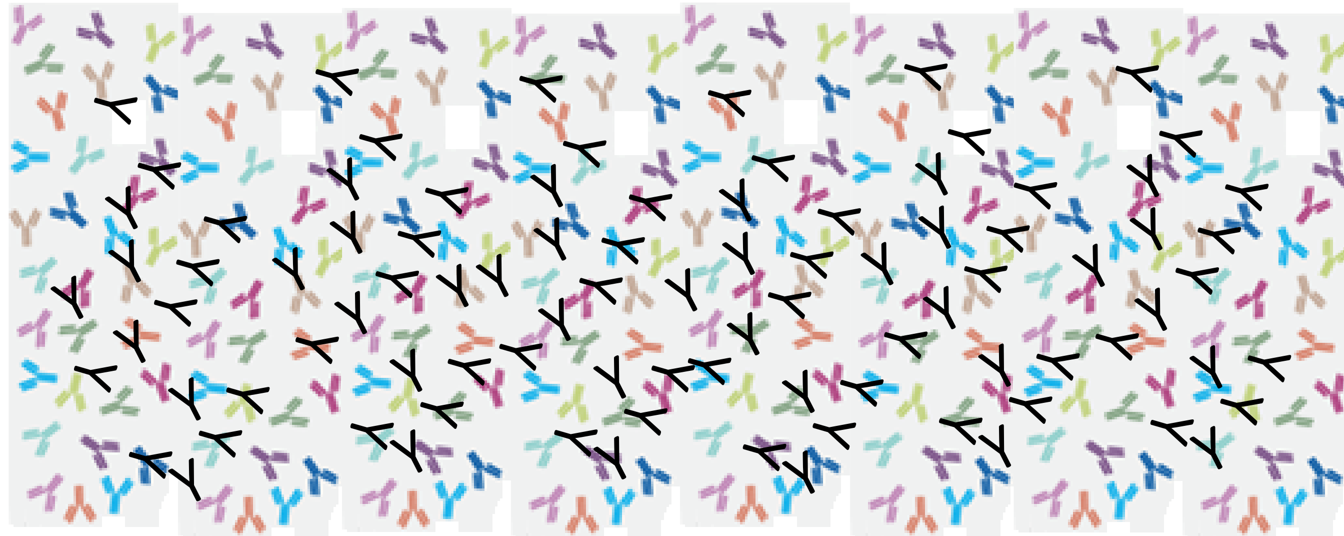


Monoclonal gammopathy

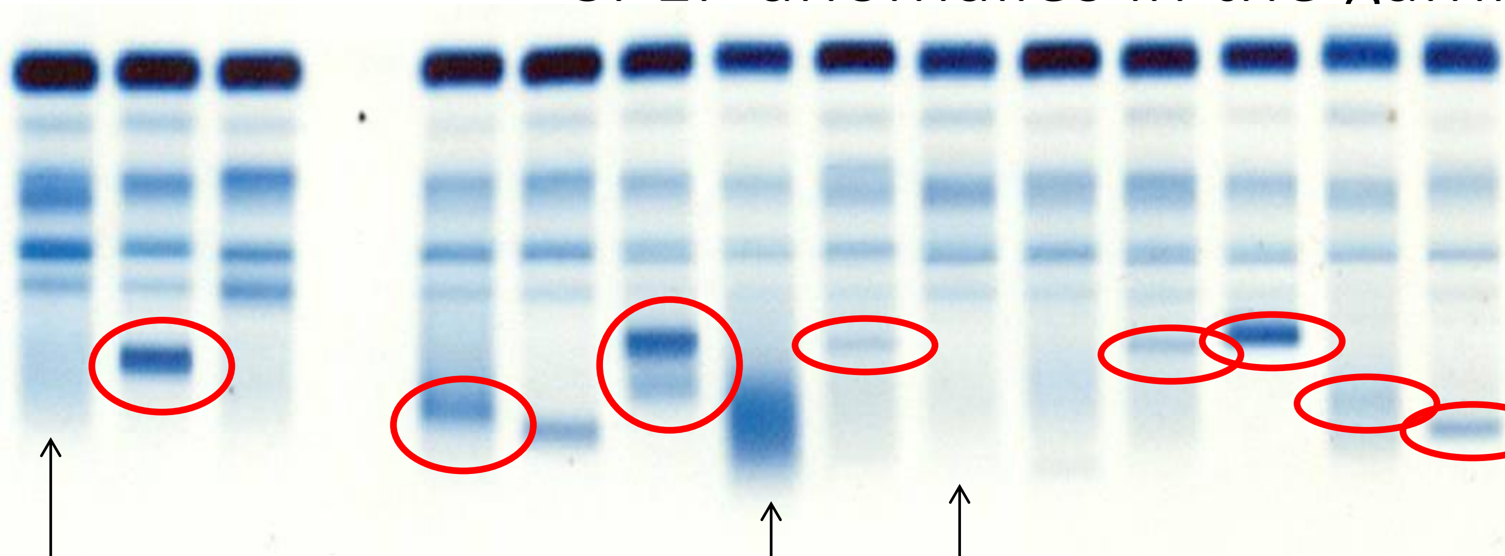
Mutations can occur in a single plasma cell leading to an uncontrolled cell growth
→ **Uncontrolled cell multiplication of a single plasma cell and overproduction of a single Ig identity**



This single Ig will be overrepresented in the serum of the patient.
Production of other Igs (polyclonal pool) might or not be affected.

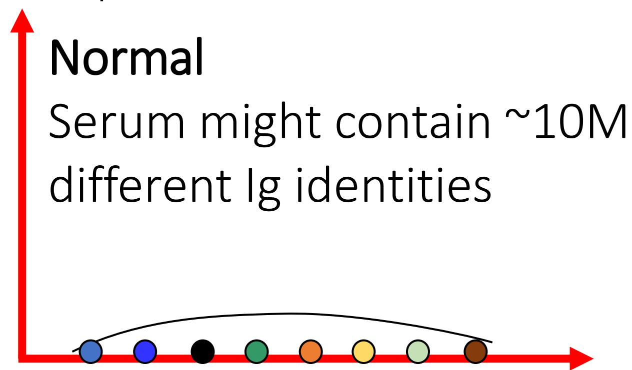


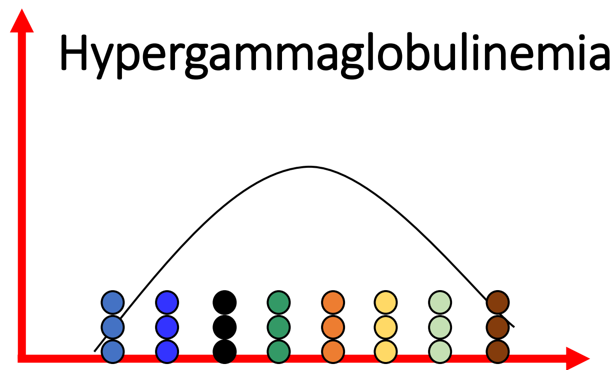
SPEP anomalies in the gamma region



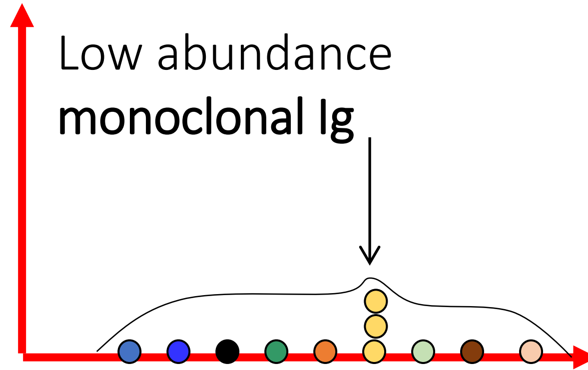
A monoclonal Ig is suspected when **a protein band** can be identified in the region of gamma globulins

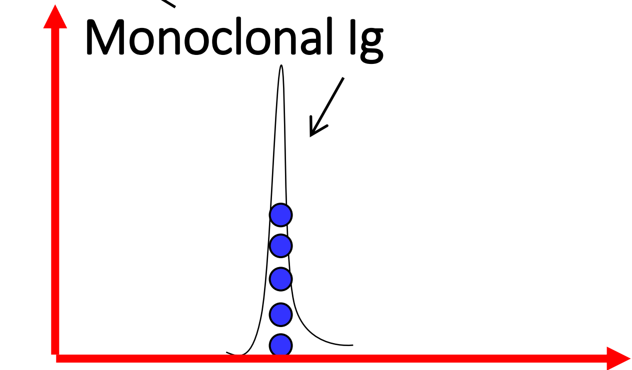
Hypogammaglobulinemia





Increased amount of many different Igs = polyclonal response



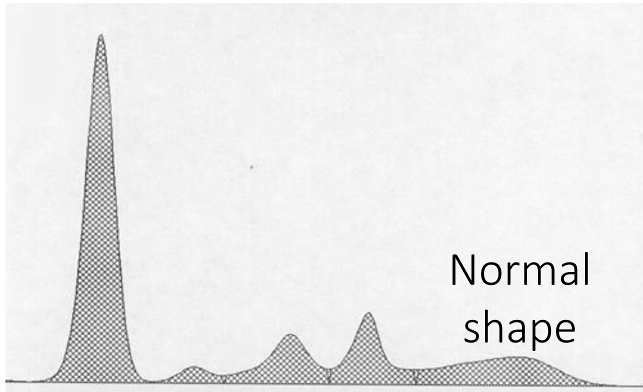


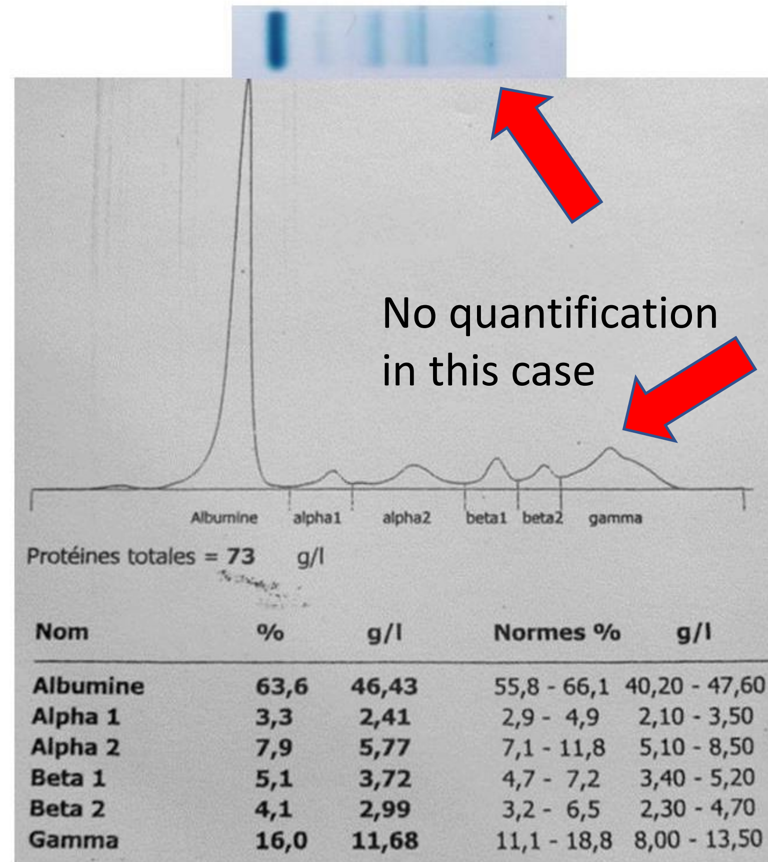
Expansion of a single B lymphocyte clone = monoclonal Ig

SPEP allows the identification of monoclonal immunoglobulins and their **quantitation** if they are abundant (individual peak)

Identification by alteration of the normal densitometry tracing in the gamma region

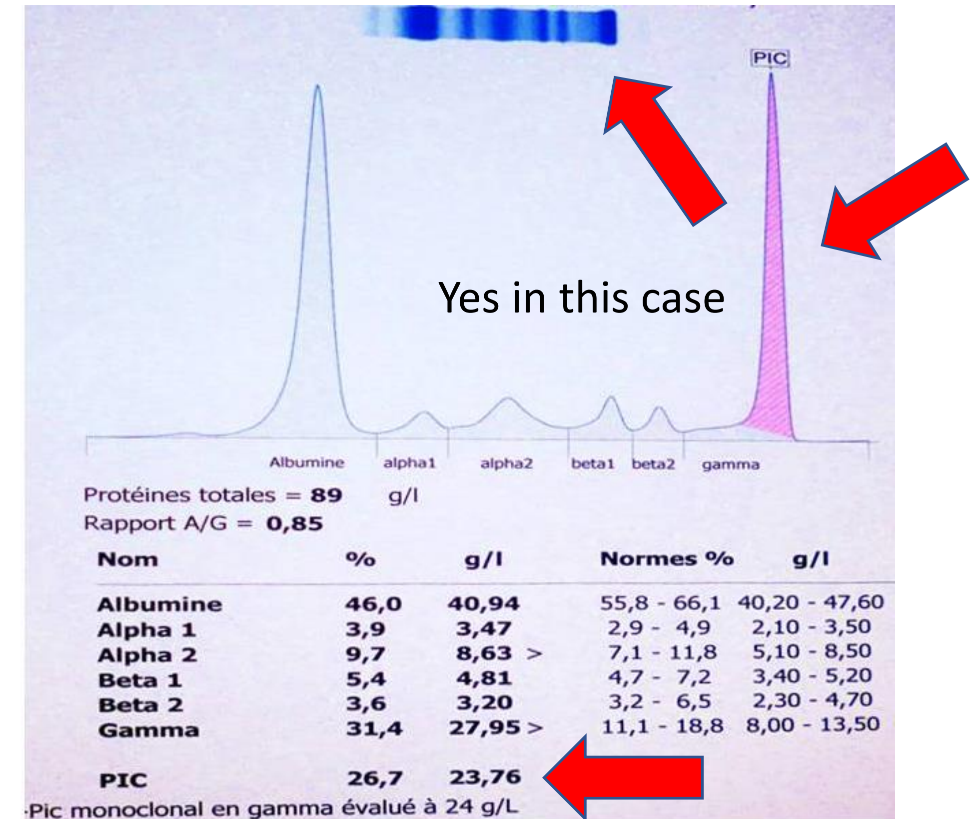
For an abundant single protein a base in the peak is defined in the densitometry tracing, to allow for quantitation





Protéines totales = 73 g/l

Nom	%	g/l	Normes %	g/l
Albumine	63,6	46,43	55,8 - 66,1	40,20 - 47,60
Alpha 1	3,3	2,41	2,9 - 4,9	2,10 - 3,50
Alpha 2	7,9	5,77	7,1 - 11,8	5,10 - 8,50
Beta 1	5,1	3,72	4,7 - 7,2	3,40 - 5,20
Beta 2	4,1	2,99	3,2 - 6,5	2,30 - 4,70
Gamma	16,0	11,68	11,1 - 18,8	8,00 - 13,50



Protéines totales = 89 g/l
Rapport A/G = 0,85

Nom	%	g/l	Normes %	g/l
Albumine	46,0	40,94	55,8 - 66,1	40,20 - 47,60
Alpha 1	3,9	3,47	2,9 - 4,9	2,10 - 3,50
Alpha 2	9,7	8,63 >	7,1 - 11,8	5,10 - 8,50
Beta 1	5,4	4,81	4,7 - 7,2	3,40 - 5,20
Beta 2	3,6	3,20	3,2 - 6,5	2,30 - 4,70
Gamma	31,4	27,95 >	11,1 - 18,8	8,00 - 13,50
PIC	26,7	23,76		

Pic monoclonal en gamma évalué à 24 g/L

Immunofixation electrophoresis

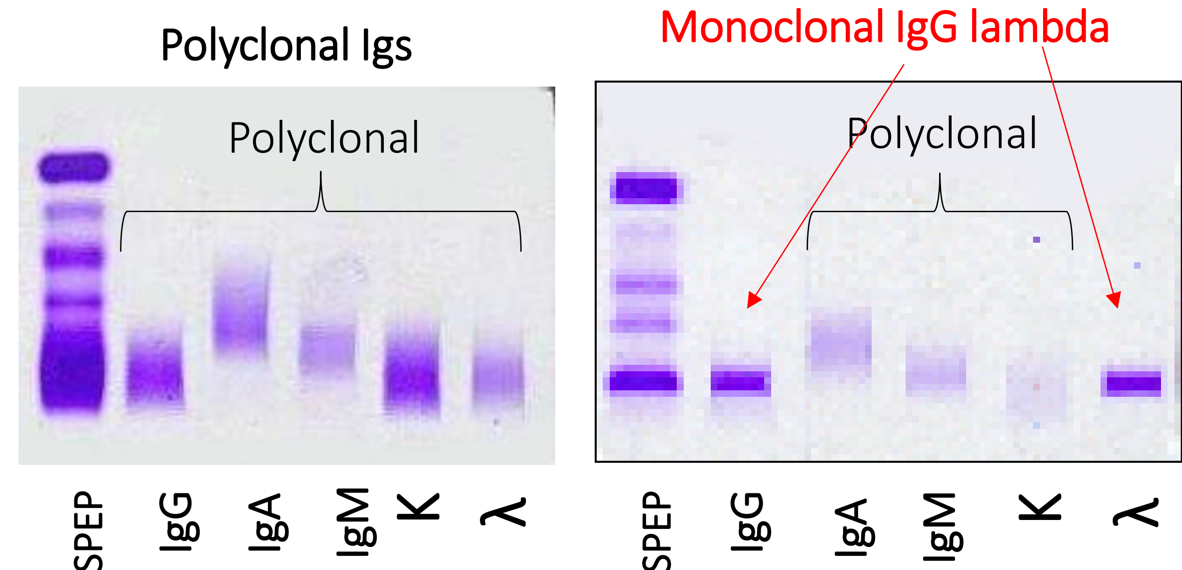
Immunofixation allows detection and typing of monoclonal Ig. Qualitative method.

Steps:

1. Serum from a single patient is loaded in every track of a 6-track electrophoresis gel
2. SPEP is performed
3. In line 1, as for SPEP, all proteins are fixed by acidic precipitation
4. In lines 2 to 4 add a monospecific antiserum per line: anti-heavy chain (HC) of IgG, IgA and IgM
   In lines 5 to 6 add a monospecific antiserum per line anti-light chain (LC) anti-kappa or anti-lambda
   → **immunoprecipitation takes place in the gel matrix**
4. Wash out non precipitated proteins from gel
5. Stain precipitated proteins

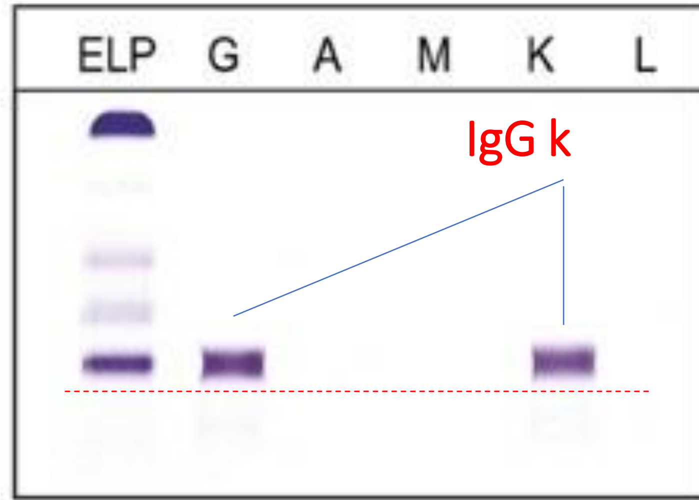
Polyclonal immunoglobulins are evidenced as **large regions** with diffuse coloration.

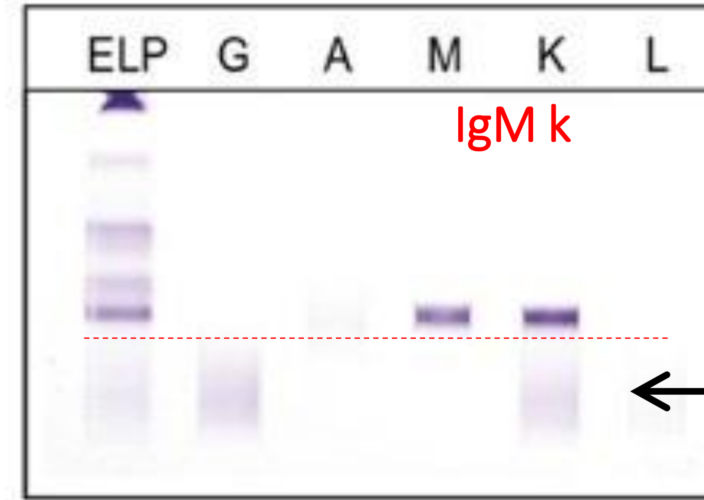
A monoclonal Ig is evidenced by the presence of **a colored band** at the same level of migration in SPEP tracks in **one HC class** track and in **one LC isotype** track.



Immunofixation electrophoresis, more examples

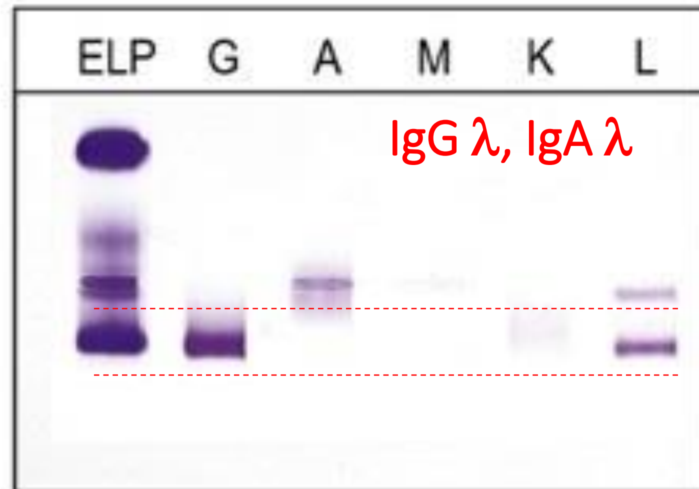
Monoclonal Immunoglobulins:

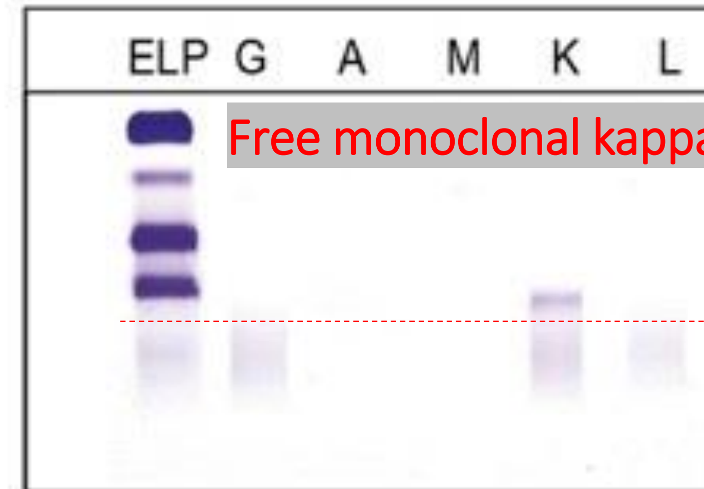




Polyclonal Igs

Oligoclonal gammopathy





Free monoclonal kappa Light Chain

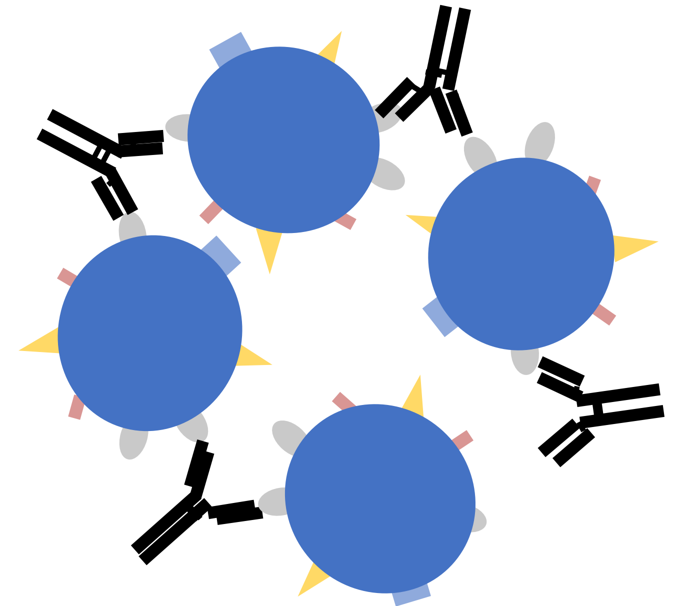
The following slides were not covered in the lecture.
The information they have will not be on the final exam except if treated in another lecture.

Label free methods based on immunoagglutination

Reaction between insoluble particles and its partner (Ag or Ab) in solution.

Usually, **antigen is presented as insoluble particles.** Antigens are naturally present on the surface of these particles (bacteria, red blood cells, …) or are attached by chemical coupling (antigen-coated latex beads).

The presence of **antibodies directed against one or several of the exposed epitopes** leads to the formation of large particle lattices, eventually visible to the naked eye.



Agglutination methods for detection of antigens

Blood typing: ABO, Rh, Duffy, …

By incubation of mixture of a drop of blood plus a commercial Ab specific against a given blood type marker, e.g. anti-B, anti-Duffy, …

Bacterial sero-typing and sero-grouping (*Vibrio cholerae, Salmonella spp*)

Positive Negative

Identification and quantitation of antigen-specific antibodies and auto-antibodies

Testing for specific antibodies and autoantibodies is clinically relevant
- **to screen for disease** (allergies, infectious diseases),
- to establish a **specific diagnosis** (autoimmune diseases, HIV), and
- to monitor the **clinical course of a disease**.

Different immunoassay methods :
- Agglutination
- Elisa
- Immunofluorescence
- Immunochromatography

Analytical objective = to assess the presence of a specific **Ab** in a biological sample,
These methods rely on the **use of antigens as analytical reagents :**
- **crude or complex antigen preparations** used primarily for screening tests (e.g. IgG anti **coronavirus**)
- **purified or recombinant antigens** useful to identify disease-specific markers (igG against the **spike protein** of coronavirus, or IgG against the **RBD domain in the spike protein.**

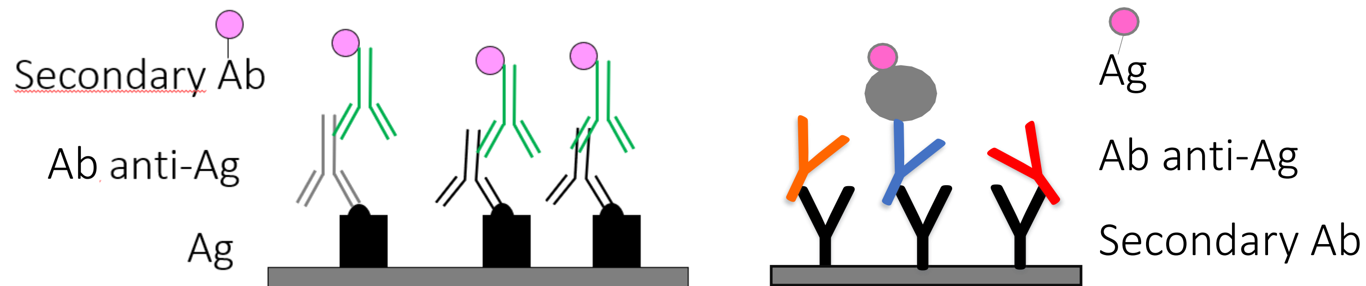
Immunolabeling methods for **antigen-specific antibodies** assessment and also for **antigen** detection

Immunolabeling strategies in solid phase reactions

- Immunometric methods : ELISA test
- Immunoblot
- Immunochromatography
- Immunoflurescence:
  - Immunohistology
  - Immunophenotyping of blood lymphocytes and cell cytometry

Immunolabeling **strategies** in solid phase reactions

Indirect reaction: detection of an Ag-specific antibody using a secondary labeled antibody

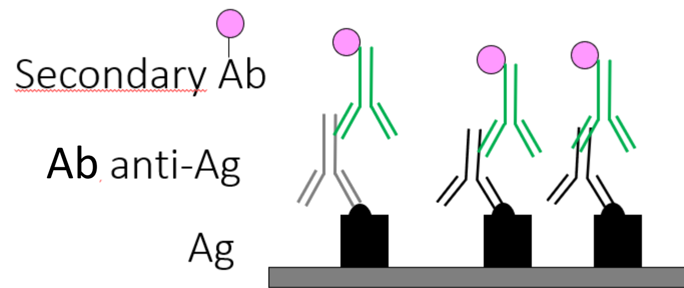


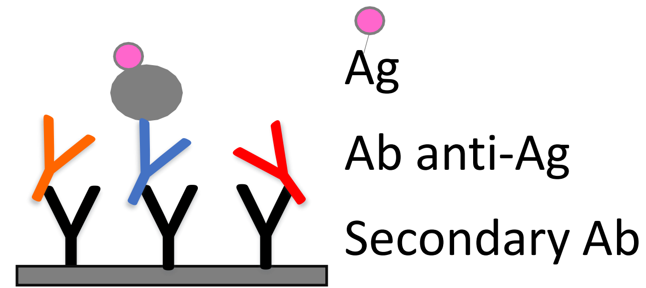
The first immune reagent is fixed on the solid phase: **usually the Ag**, can be the secondary Ab.

- Incubation with the **serum from patient**. Antigen specific Abs or « whole » IgG are captured
- Washing step
- Incubation with a **second immunological reagent (Ag or Ab), labelled**: secondary Ab or Ag
- Washing step
- Detection of the labelling tag

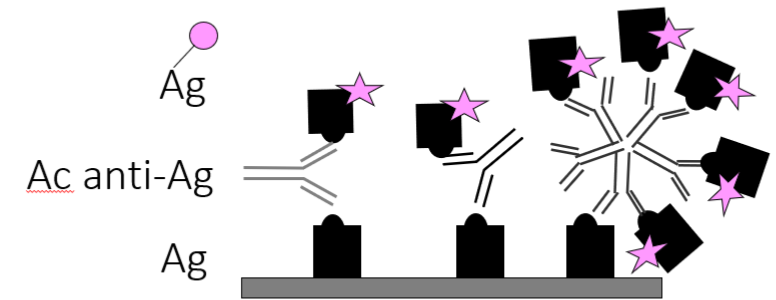
Immunolabeling strategies in solid phase reactions

Indirect reaction





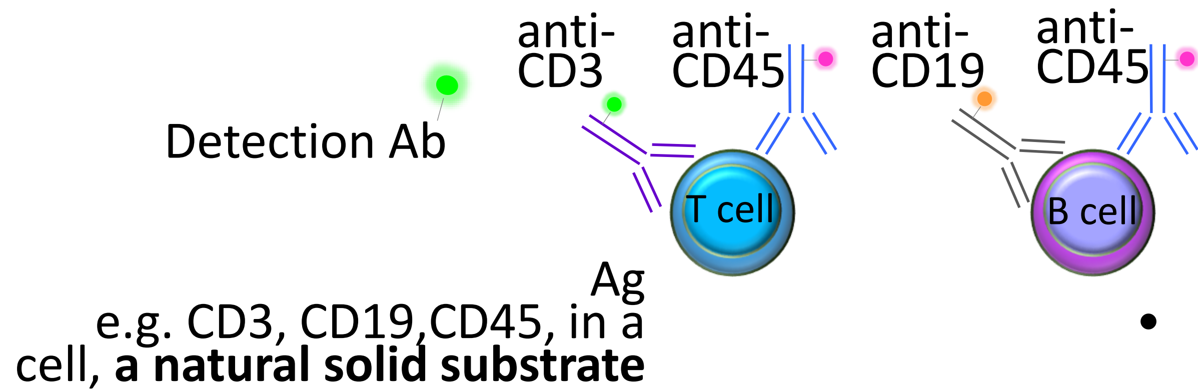
Sandwich reaction for Ag specific Ab detection



Immunolabeling strategies in solid phase reactions

Direct reaction : one step reaction. For Ag detection.

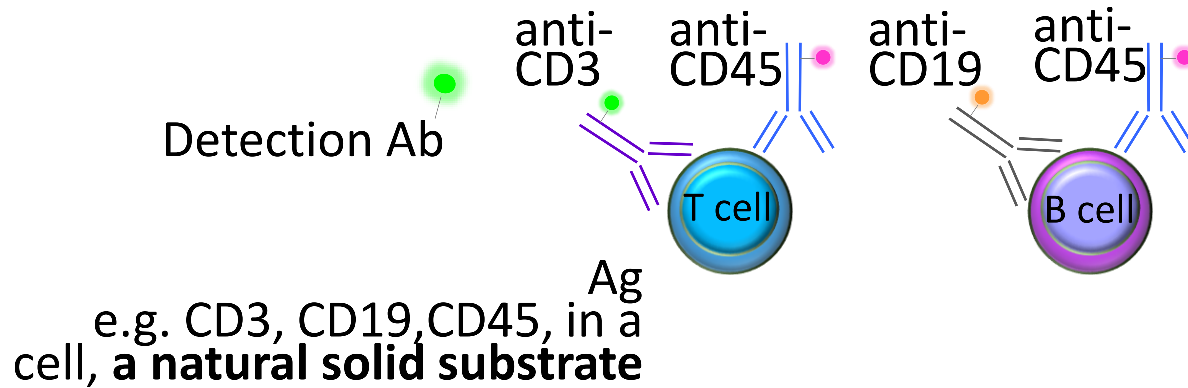
Solid phase + 1st immune parter is naturally occuring (cell, tissu)



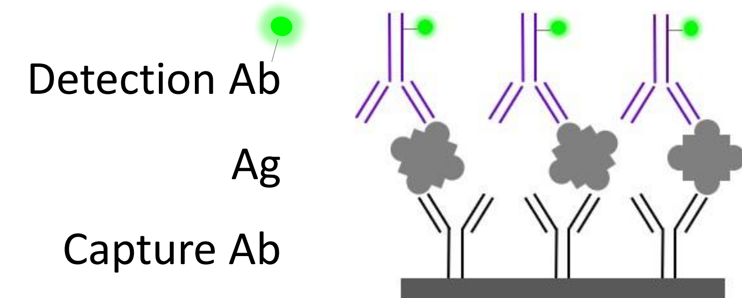
- Direct incubation with a **labelled specific Ab**
- Detection of the labelling tag

Immunolabeling strategies in solid phase reactions

Direct reaction for Ag detection



Sandwich reaction for Ag detection

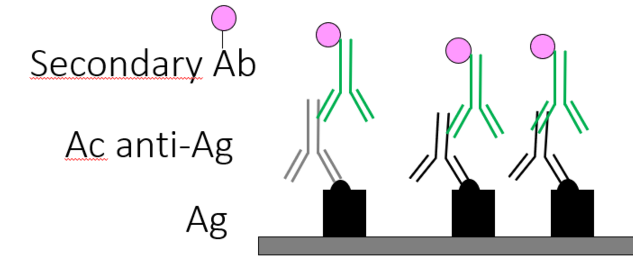


Competition reaction not shown

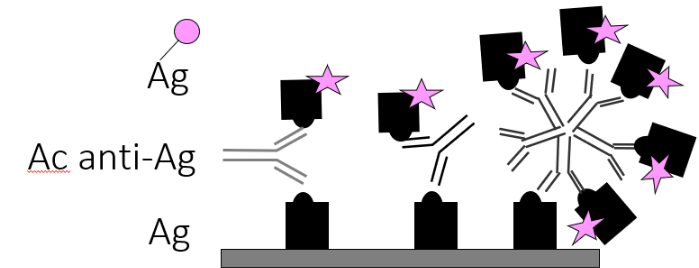
Immunometric methods : Enzyme-linked immunosorbent assay (ELISA)

- To detect / quantitate Ag or Abs in liquid samples
- Excellent sensitivity, up to ng/L or higher (modern elisa-like techniques)
- **Quantitative** measurements if calibrated standard curve

Indirect reaction



Sandwich reaction (for Abs or Ags)



Applications:

Quantitation of serum proteins present at very low concentration: total IgE, antigen-specific IgGs (auto-antibodies, Ab anti-HIV, anti-vaccines), allergen specific IgE, ...

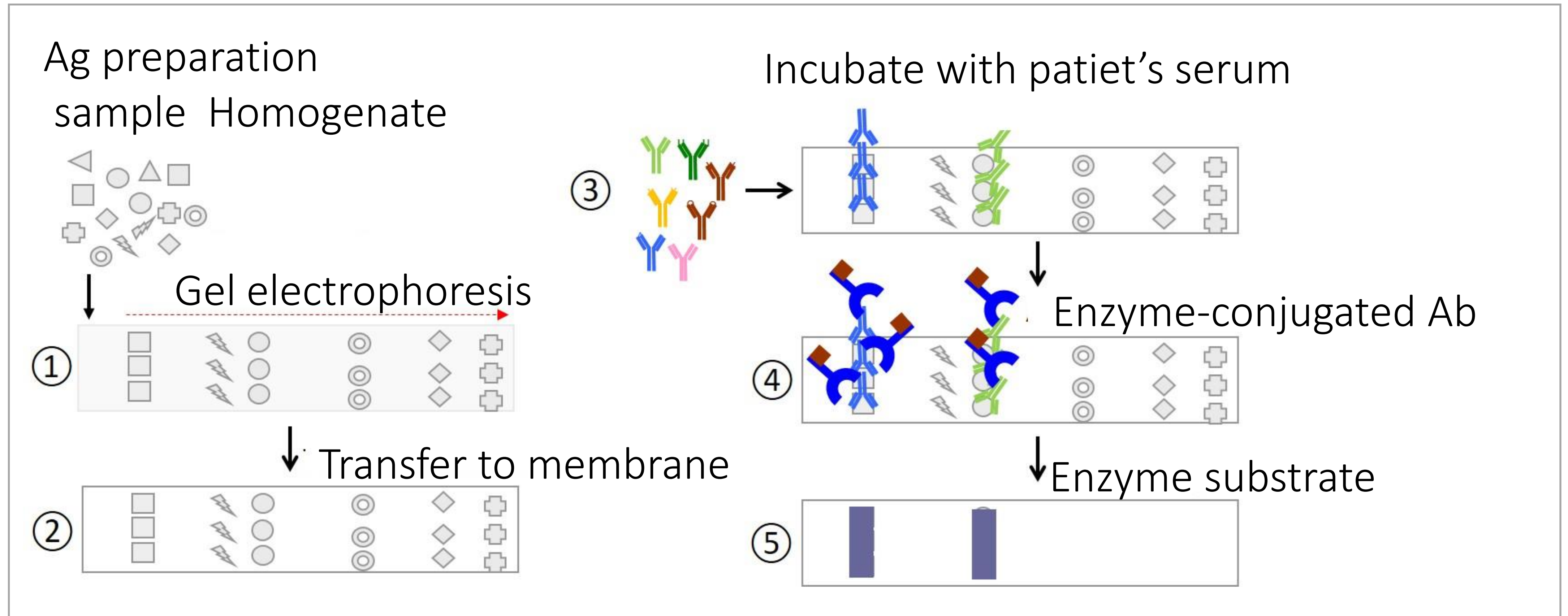
Steps for detection of specific Abs:

1. Plastic wells coated with the **Ag**.
2. Blocking non-specific binding sites with non relevant protein, at [high].
3. Incubation of **patient's serum** (and controls: blank, negative, standard)
4. Immunolabeling: Incubation with **second immunological reagent** (Ag or Ab) conjugated with an **enzyme** (e.g. peroxidase).
5. Add **substrate** (e.g. chromogenic). Amount of product generated is proportional to the amount of searched Ab in the sample.

Immunoprinting / Immunoblot / westernblot

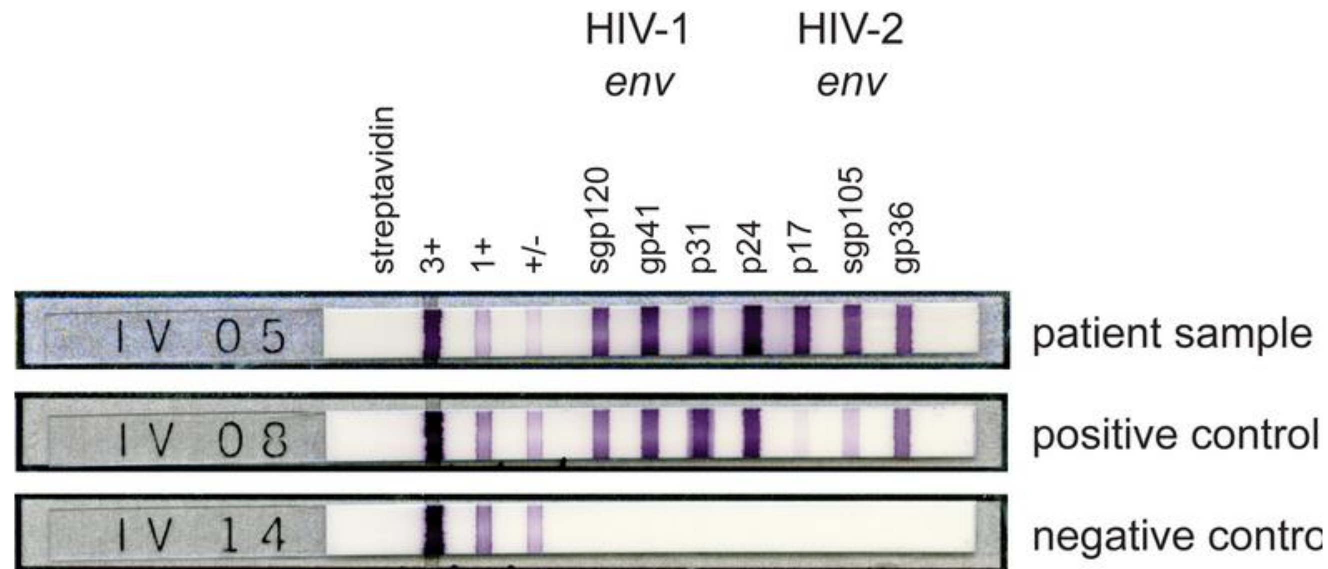
Intended for the detection of either specific Acs in patients' sera or a given protein (Ag) in complex biological preparation.

Steps :



Immunoprinting / Immunoblot / westernblot example case

Western blotting assay is required to confirm diagnostic after a positive anti-HIV IgG ELISA test



Zbinden et al. 2016

For diagnostic purposes "blotted" membranes ready for testing patients' sera are sold by biomedical companies. Native protein homogenates can be substituted by **recombinant antigens**. This allows omitting electrophoresis and transfer steps. **Antigens are directly spotted by a robot** in a region of the reaction membrane (**immunoprinting**).

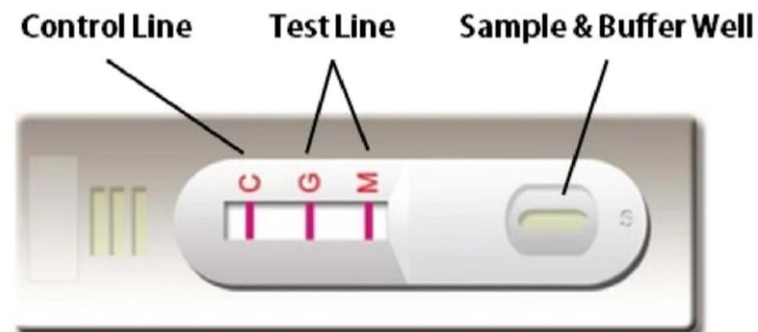
Immunochromatography, rapid diagnostic tests, lateral flow reaction tests

Allows for the rapid detection of specific Ab (anti-pathogens, anti-allergens, etc.) or Ags (hormones, drugs, etc.) in biological fluids. (liquid samples)

- Yield results in few minutes
- Highly sensitive (of the order of ng / L),
- Tag for immunodetection = nanoparticles of colloidal gold, colloidal selenium, …

Detection of Ab anti-HIV

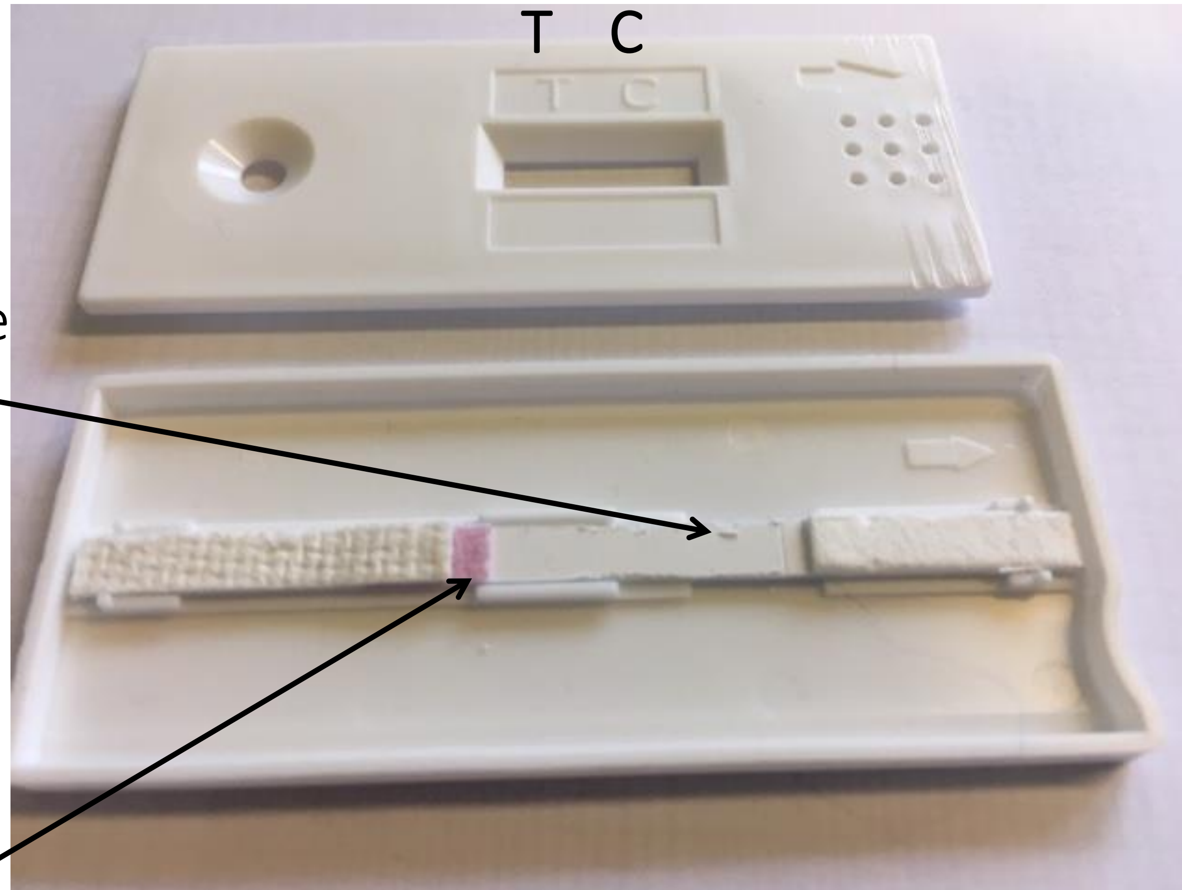
Detection of Ig or IgM anti-coronavirus



Pregnancy test, detection of hCG

Immunochromatograpy test : device and reagents

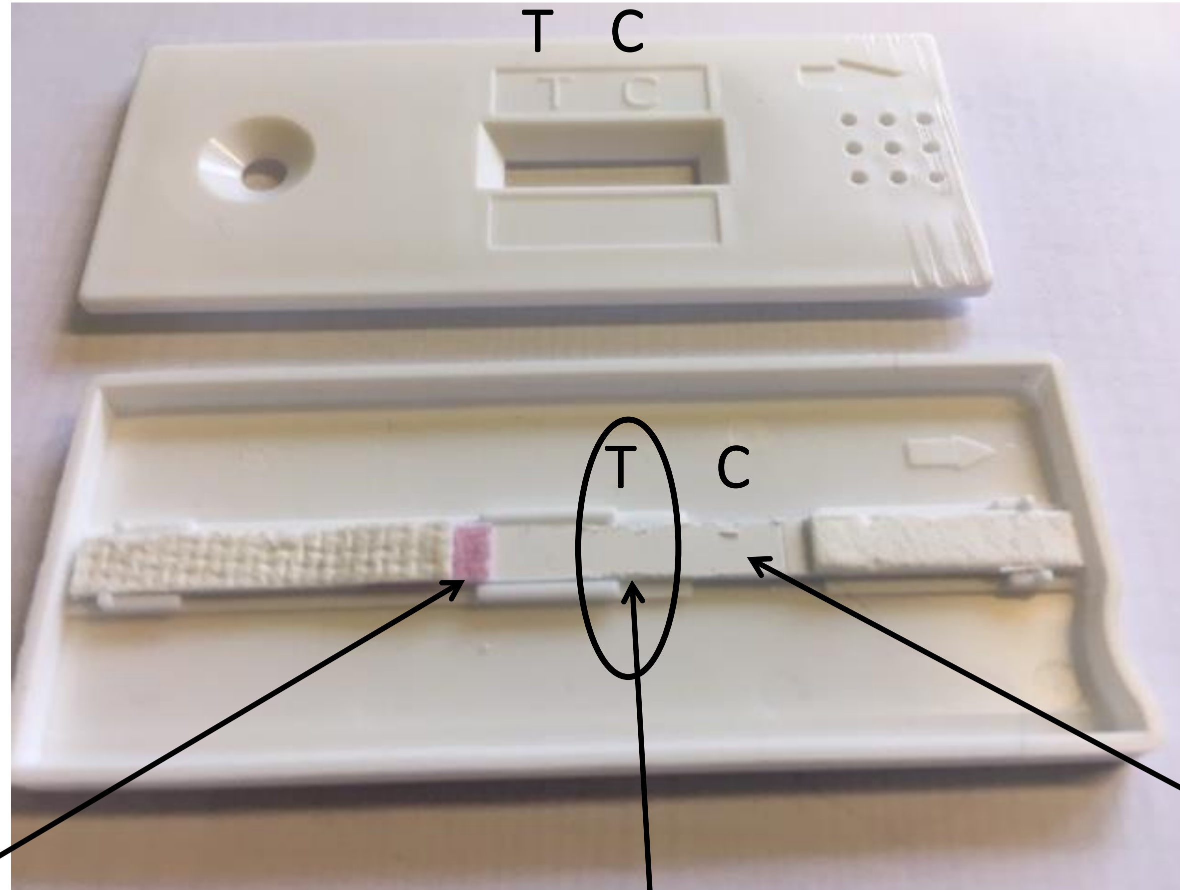
Solid phase:
nitrocellulose membrane



colloidal gold

Labeled immunologic reagent
Lyophilised and « free »
Will run with liquide sample

Immunochromatograpy test : device and reagents



colloidal gold

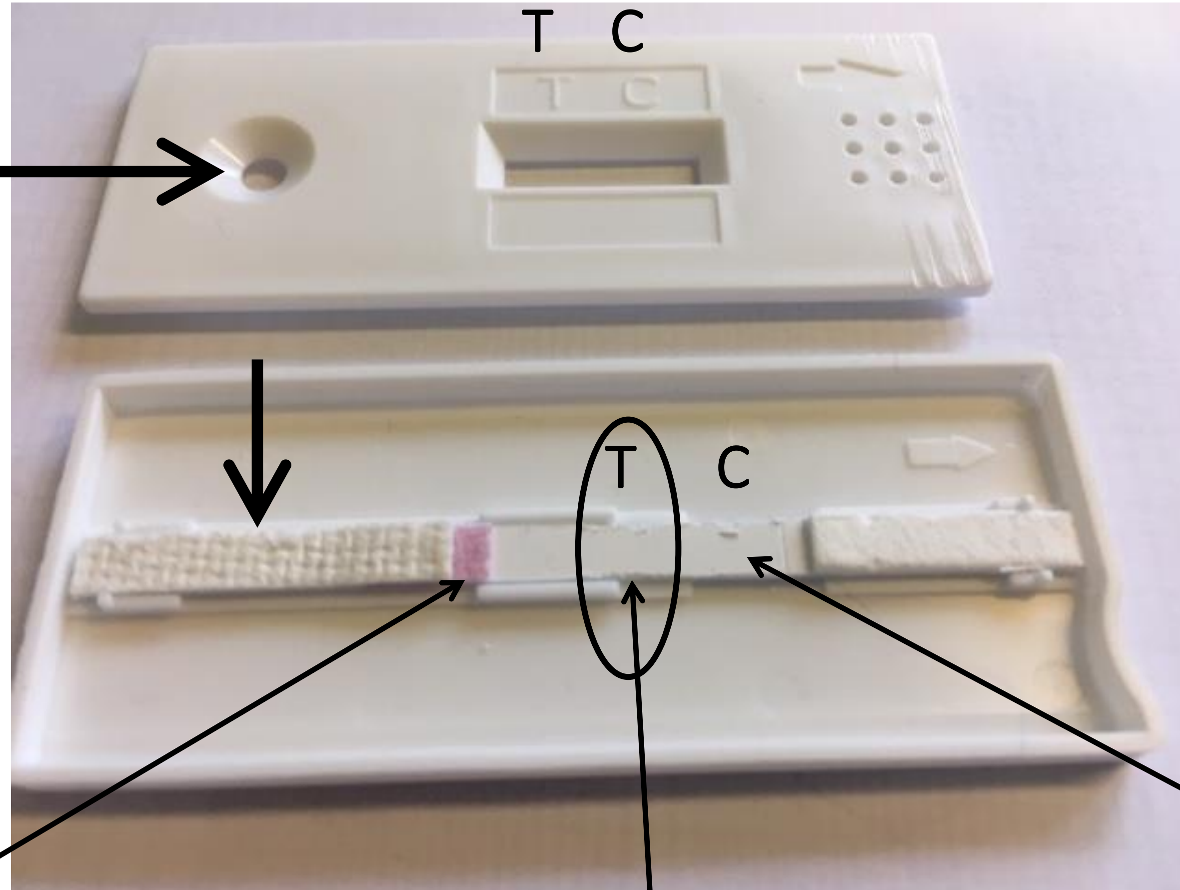
Labeled immunologic reagent
Lyophilised and « free »
Will run with liquide sample

Non labeled and immobilized immunologic reagent Capture Ag or Ab)

Control: Ab to trap labeled immunological reagent

Immunochromatography test : detection a specific Ab

Sérum from patient



- Solubilsation of Ag
- Cromatographic migration starts
- Reaction of sérum Abs with Ag → Immune complexes

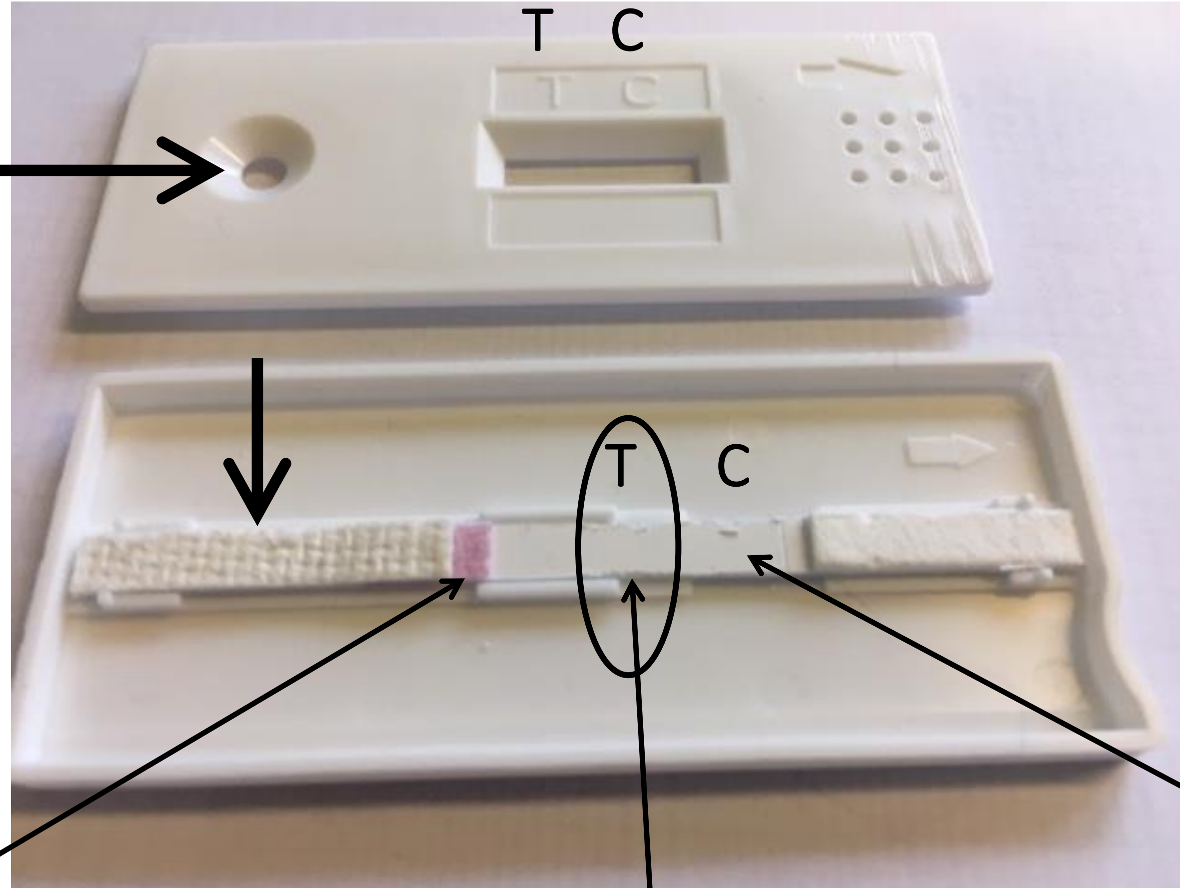
Labeled Ag

Non labeled and immobilized immunologic reagent (Capture Ab or Ag)

Control: Ab to trap labeled immunological reagent

Immunochromatography test : detection a specific Ab

Sérum from patient



- -Solubilsation of Ag
- -Cromatographic migration starts
- -Reaction of sérum Abs with Ag → Immune complexes
- -Reaction of immune complexes with « Test region » reagent
- -Excès of free Ag arrives to « C region »

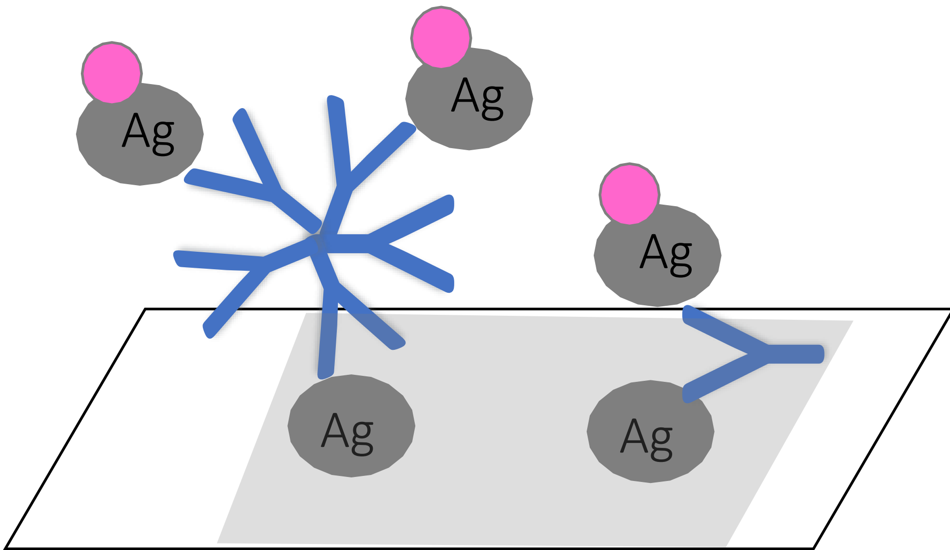
Labeled Ag

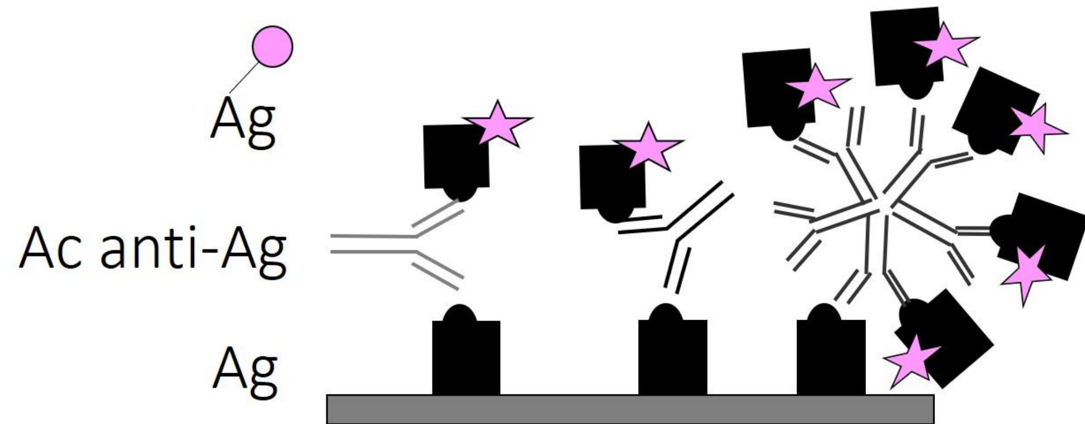
Non labeled and immobilized immunologic reagent (Capture Ab or Ag)

Control: Ab to trap labeled immunological reagent

Immunochromatography for rapid detection of anti-HIV antibodies (any isotype), sandwich reaction

Sandwich reaction in Test region

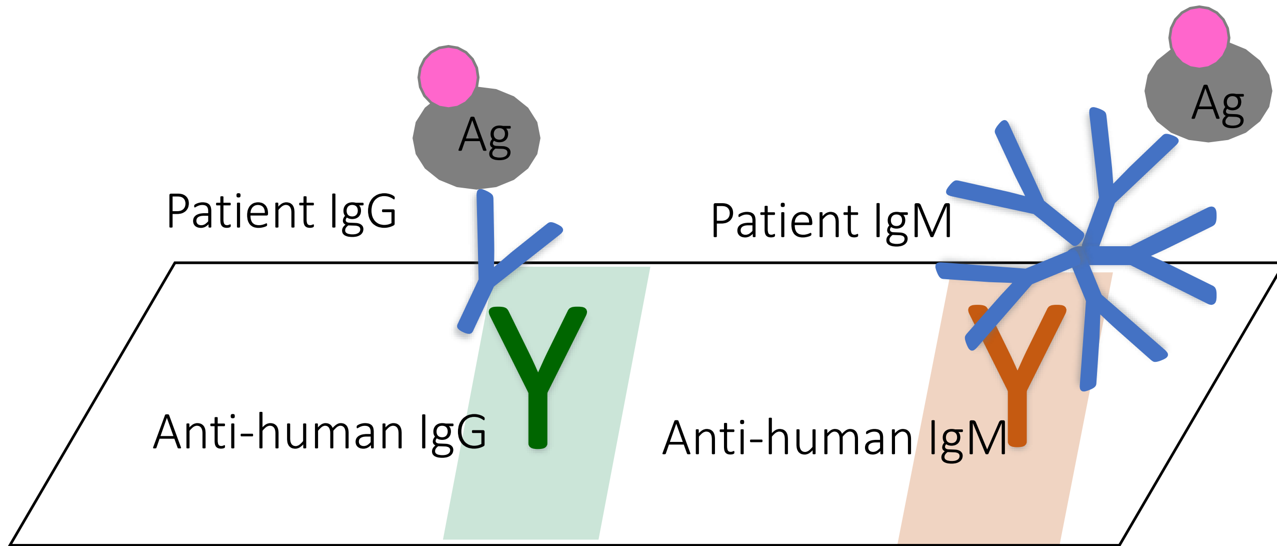


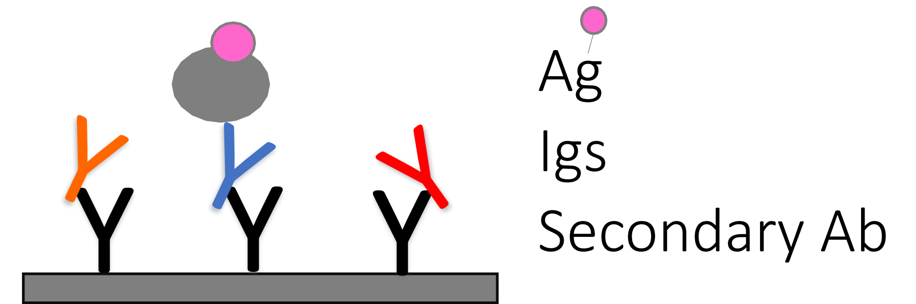


Detected Ab isotype: All Ab classes

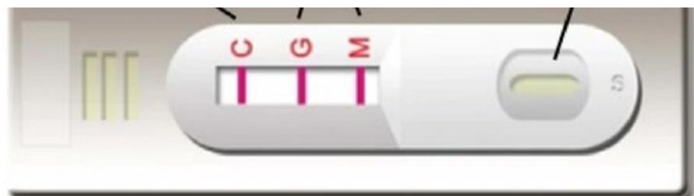
Immunochromatography for rapid detection of anti-coronavirus IgGs and IgMs antibodies, indirect reaction

Indirect reaction in Test region



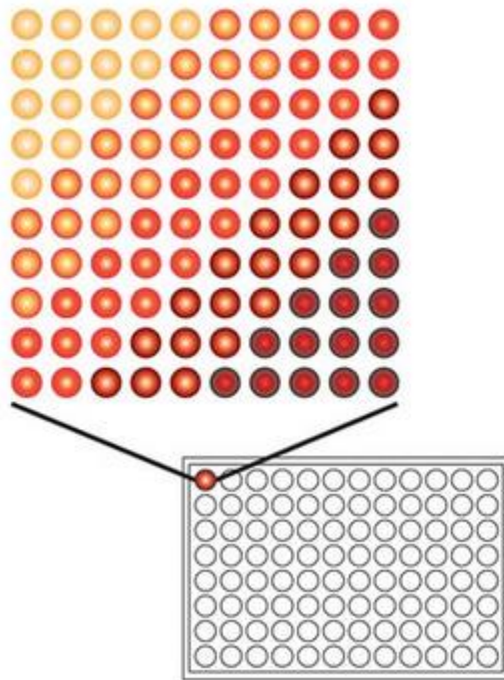


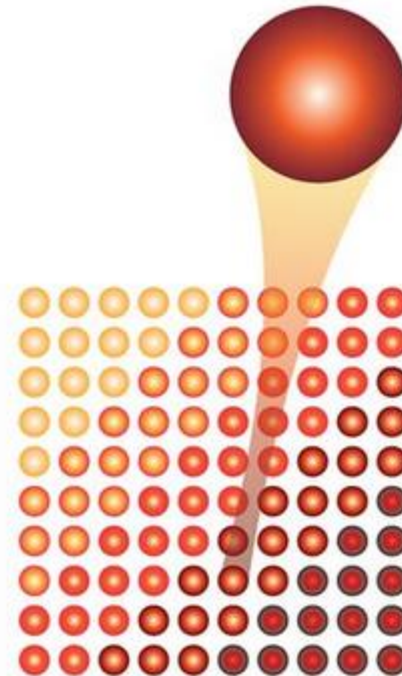
Detected Ab: Precise isotypes. IgG if anti-human gamma Abs, IgM if anti-human mu Abs.



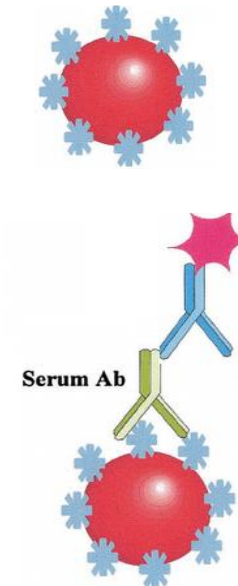
Multiplex methods for Antibody and protein detection

Multiple analytes are detected (quantified) in a single test, in a small sample (e.g. 50 µL) .
Polystyrene micro-beads are used as solid phase to immobilize the first immunologic reagent.
Each bead population has a fluorescence ‘signature’ to be identified, and is coated with a single Ag or Ab identity, to allow identification of either specific Abs or Ag, correspondingly
Different bead populations are pooled together and used for exploring a biological sample.





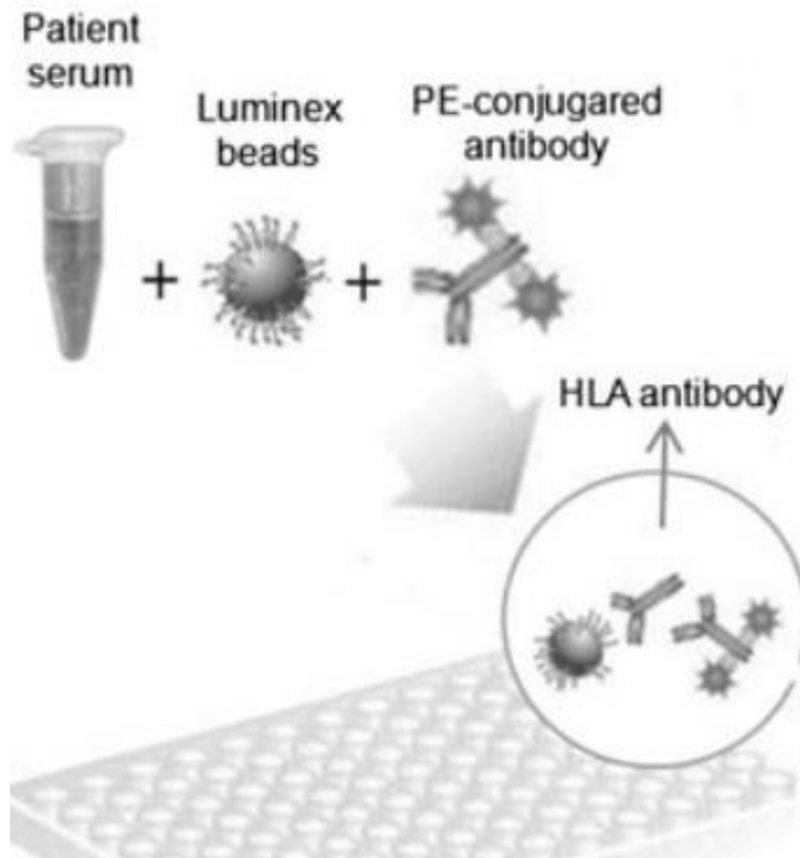
For **Ab** detection
beads+Ag

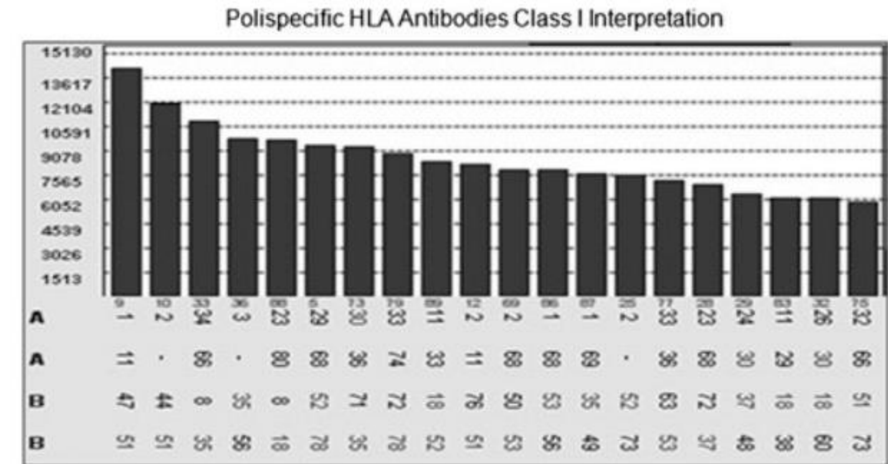


For **Ag** detection
Beads+Abs

Multiplex methods for detection of anti-HLA antibodies

Clinical applications :
Detection of anti-HLA Abs in patient before solid-organ transplantation → multiple bead populations, each one coated with a particular recombinant HLA variant. Possibility of assessing ~90 specificities of Abs anti-HLA in one single tube.





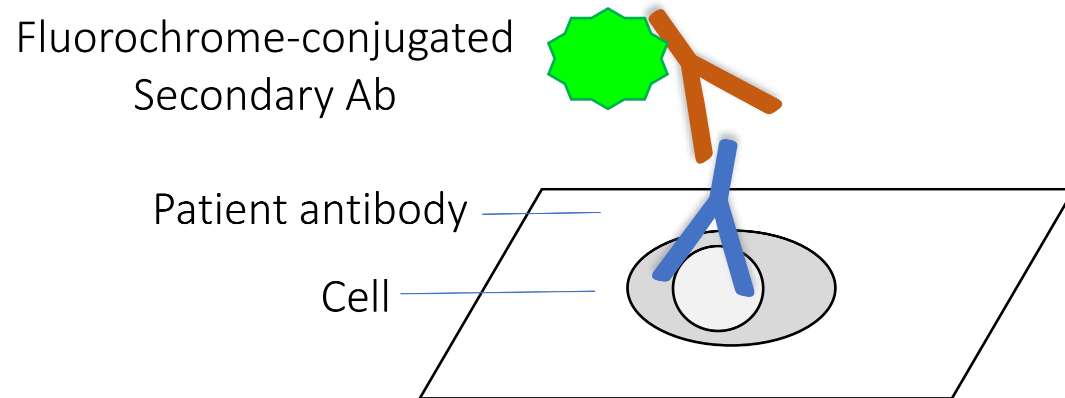
Picascia at al. 2012

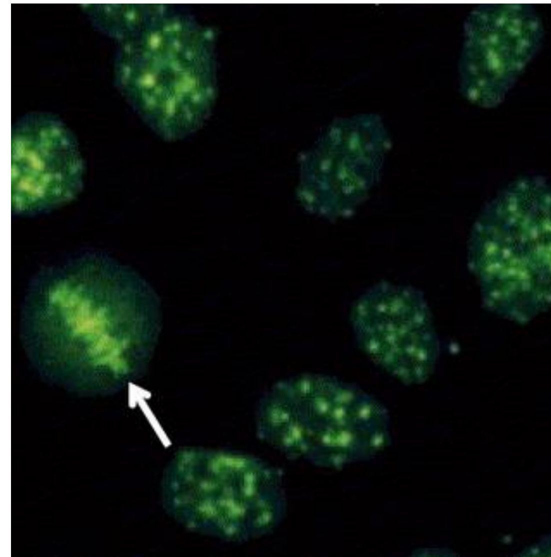
Immunofluorescence methods for detection of specific Abs

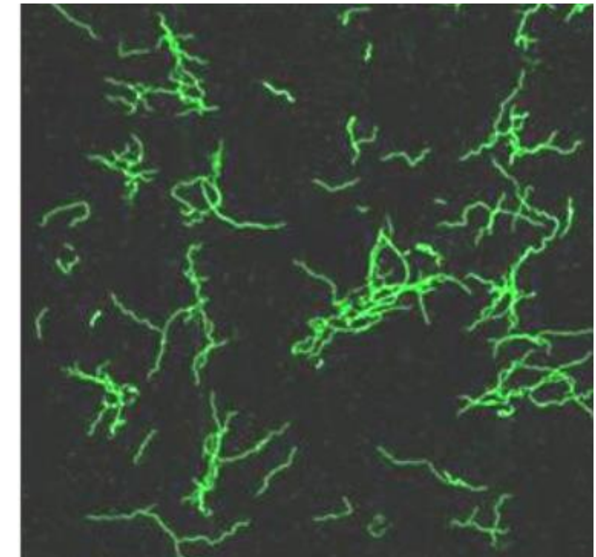
Indirect Immunofluorescence

Routinely used in clinical laboratories to **detect and titrate autoantibodies** associated with autoimmune diseases (ANA, ANCA, …) and **antibodies to some bacteria and spirochetes in infectious diseases** (*Borrelia burgdorferi* – Lyme disease)

Indirect Immunofluorescence





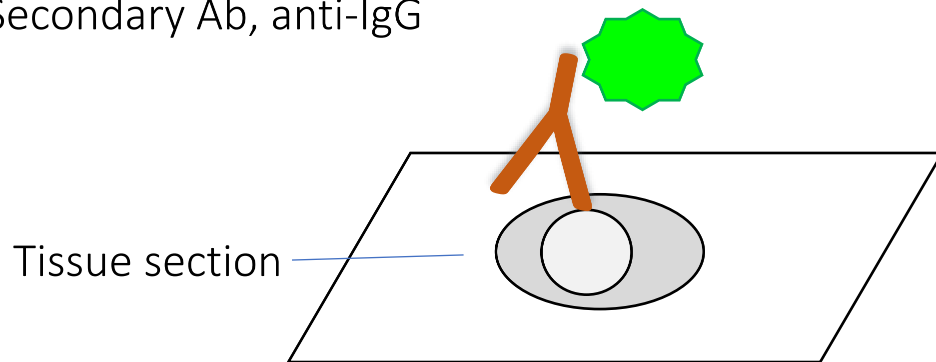


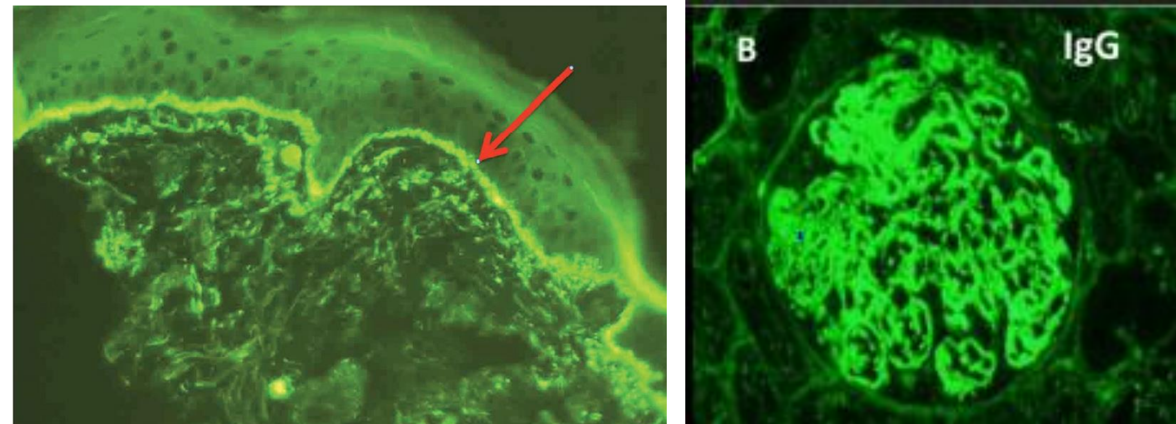
Hachulla et Dubucquoi, 2004 Jedo et al 2014

Immunohistology in clinical diagnosis
Example: direct Immunofluorescence

Used in clinical laboratories to detect **immune complex deposits** in different pathological conditions : autoimmune diseases, graft rejection, amyloid disease

Direct Immunofluorescence

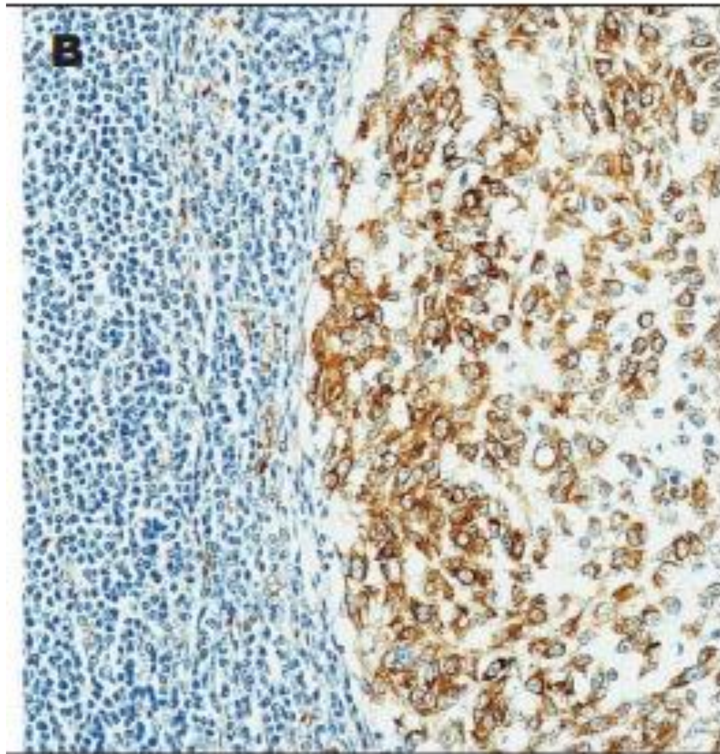




IgG deposits in **lupus nephritis** at the skin dermal-epidermal junction (lupus band test) and in kidneys (whole glomerule view). Cuen M 2018, Parikh et al. 2020.

Immunohistology in clinical diagnosis

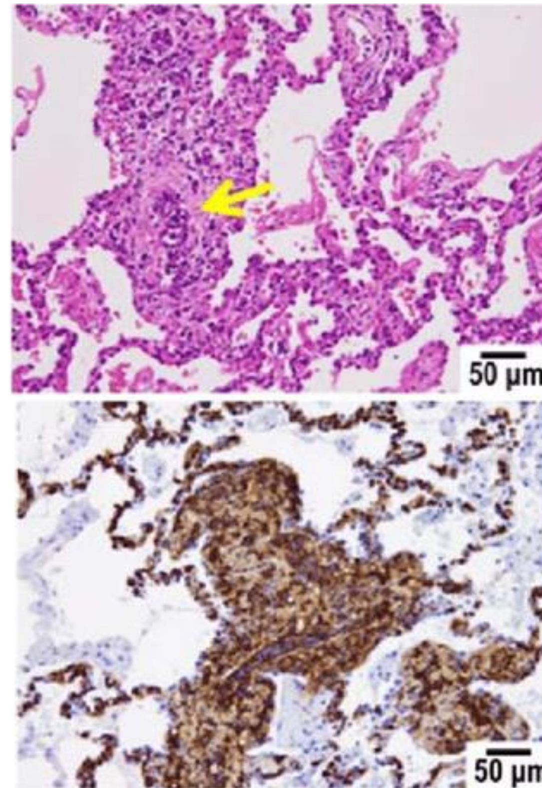
Metastatic melanoma in lymph nodes



Anti-MART-1 + anti-tyrosinase

Tacha 2003

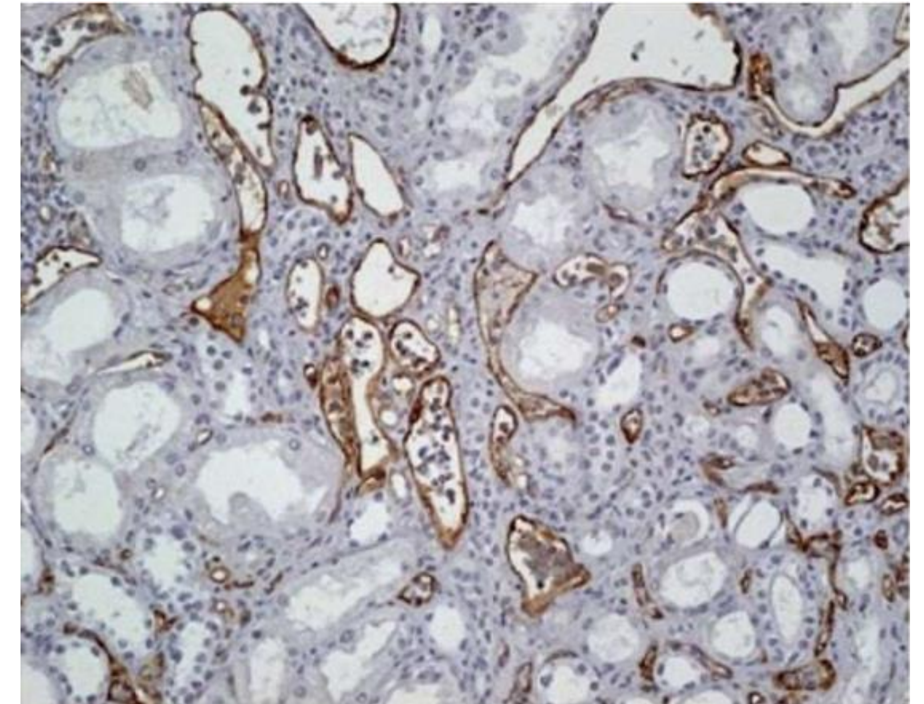
Intravascular Large B-cell Lymphoma



H/E upper panel
Anti-CD20 lower panel

Nishii-Ito et al. 2016

Humoral rejection response in a transplanted kidney



Anti-C4d complement fraction

Yan et al. 2012

Cell cytometry

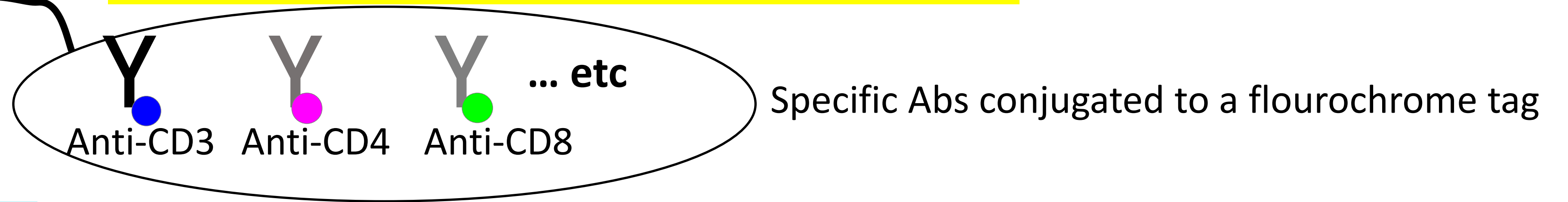
Essential tool in the evaluation of hematopoietic cells (immunophenotyping tests allowing to identify lymphocyte, monocyte, DCs sub-populations, ...

Routine applications of flow cytometry include:

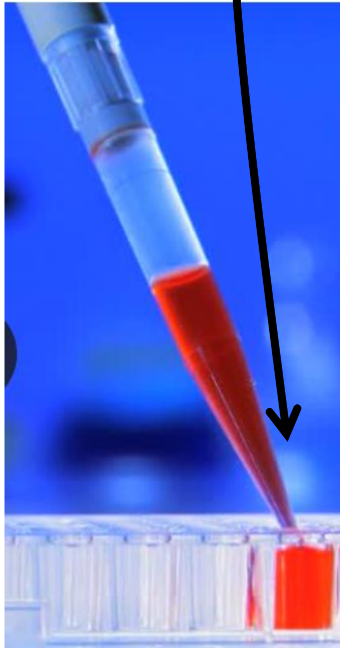
- Identifying and counting **CD4 T-cell in patients infected with the HIV**
- Diagnosis and follow up **of lymphoproliferative disorders**,
- **Characterisation of immune cells in immunodeficiency disorders** and other immune-mediated diseases.

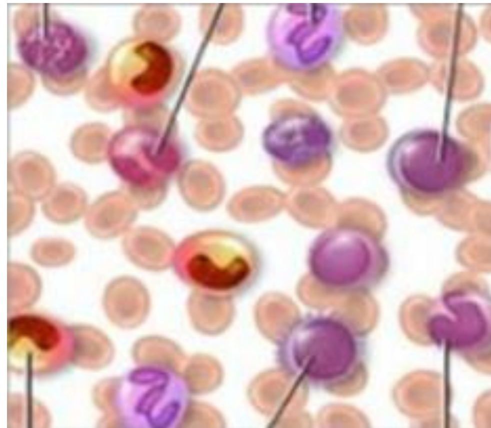
Blood cells are assessed by **direct immunofluorescence labelling of cell surface and intracellular molecules** with fluorochrome-conjugated antibodies.

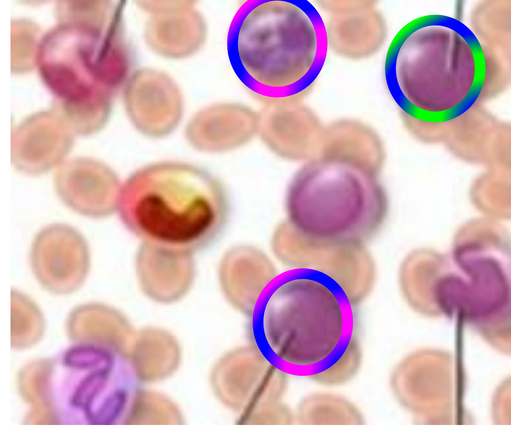
Immunophenotyping blood lymphocyte subpopulations by direct immunofluorescence



Specific Abs conjugated to a flourochrome tag







Blood aliquote

Add Abs

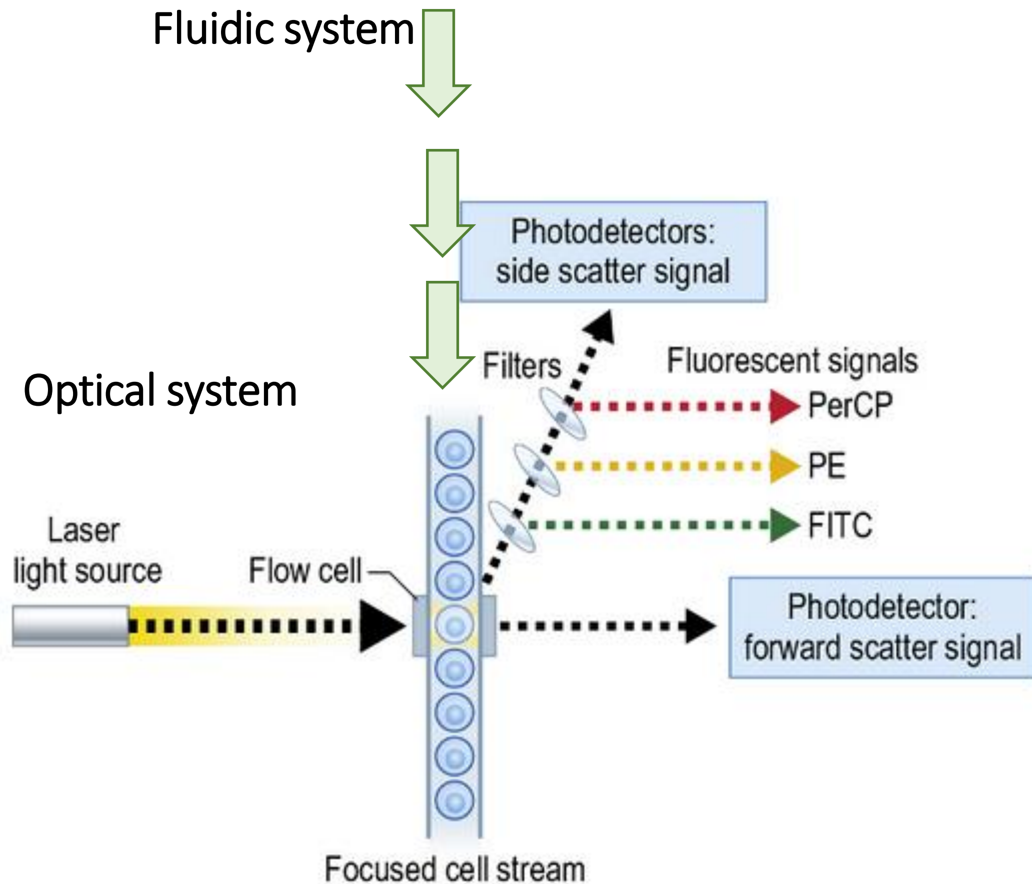
Immuno-labeling

Red blood cells lysis

Sample acquisition/ analysis in cytomètre

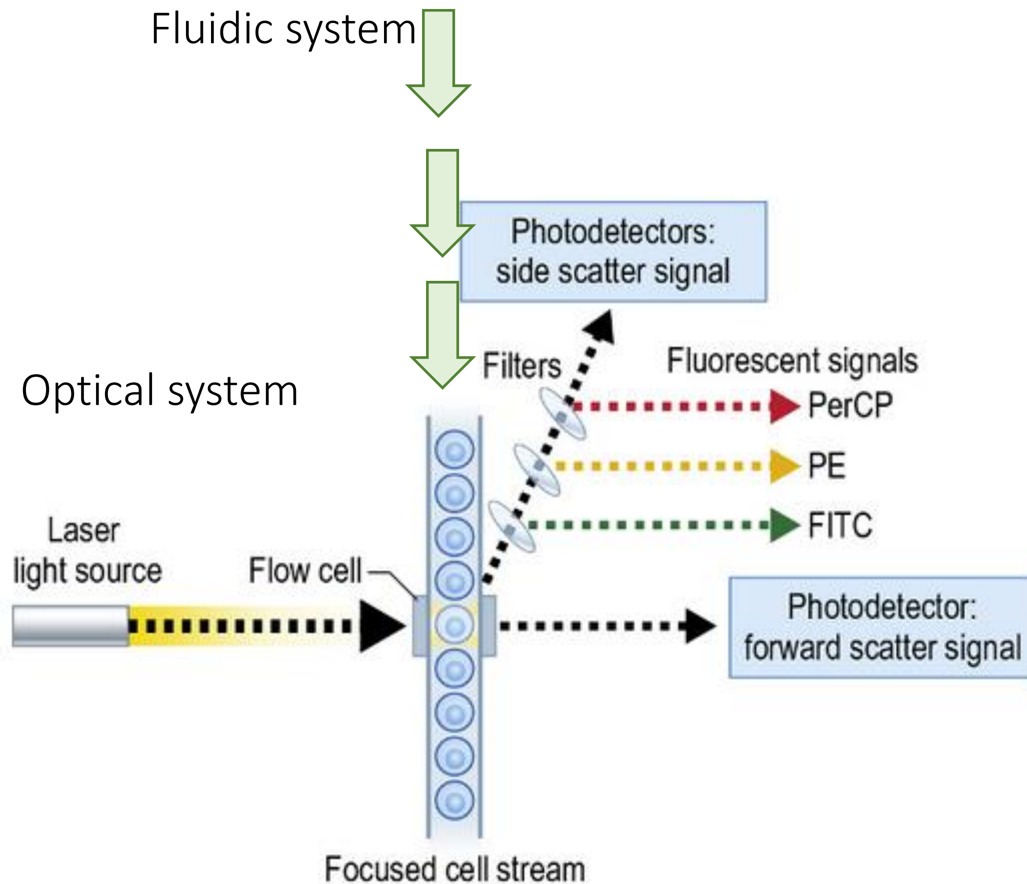
Commonly used fluorochromes in clinical immunophenotyping include organic dyes like -fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin chlorophyll protein (PerCP), allophycocyanin (APC), … Tandem dyes conjugate of PE and APC to cyanines (Cy5, Cy5.5, and Cy7) or to alexa dyes

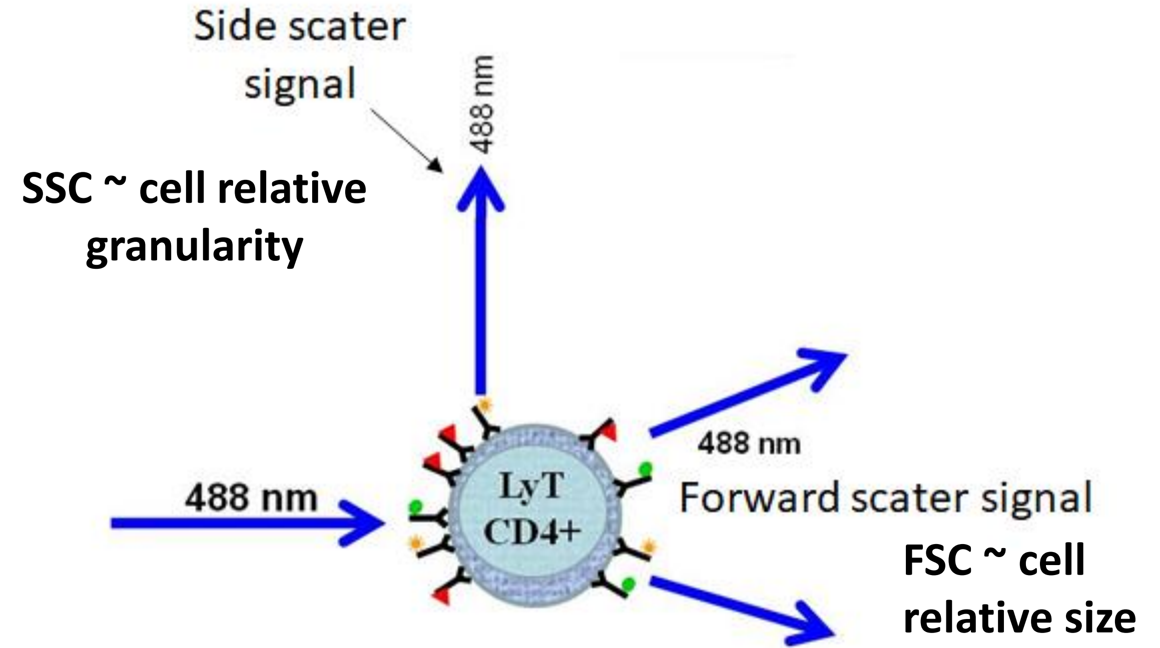
Cell cytometry instrumentation and cell assessment



McKinon et al. 2019; Rich 2013 Clinical Immunology

Cell cytometry instrumentation and cell assessment





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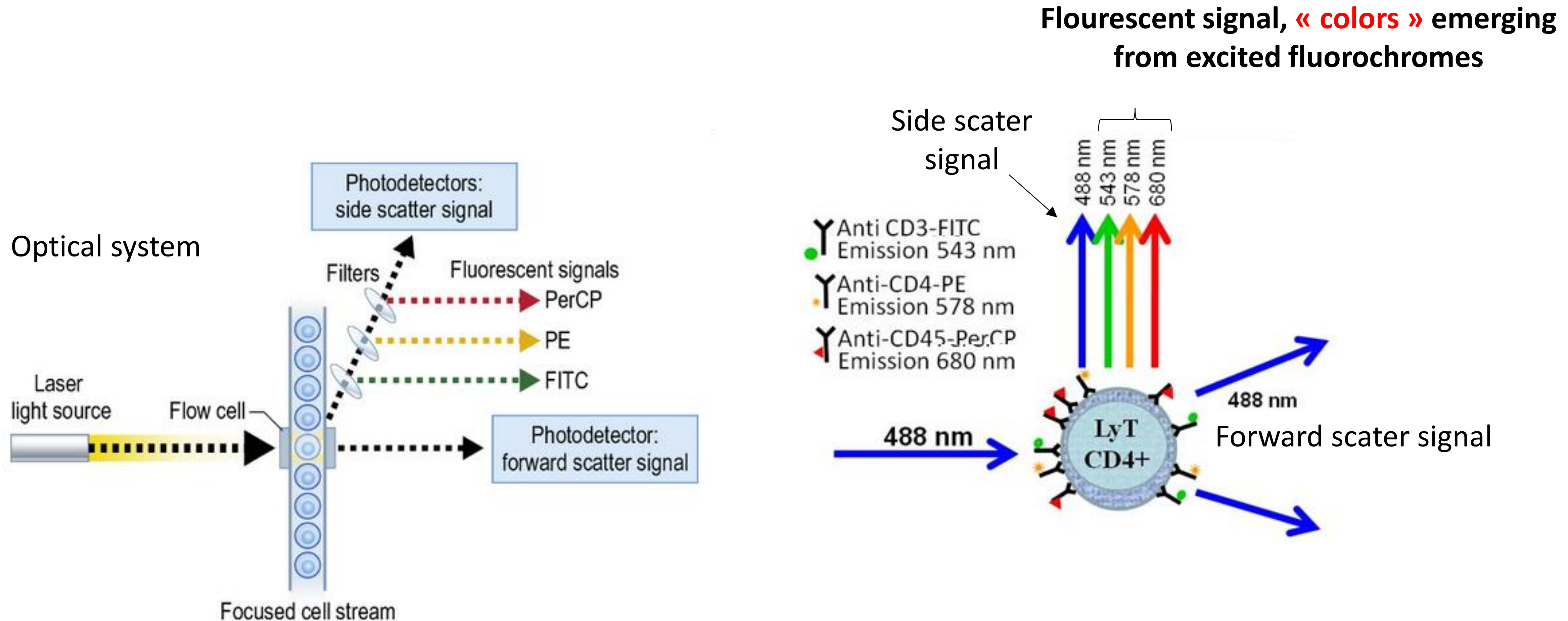
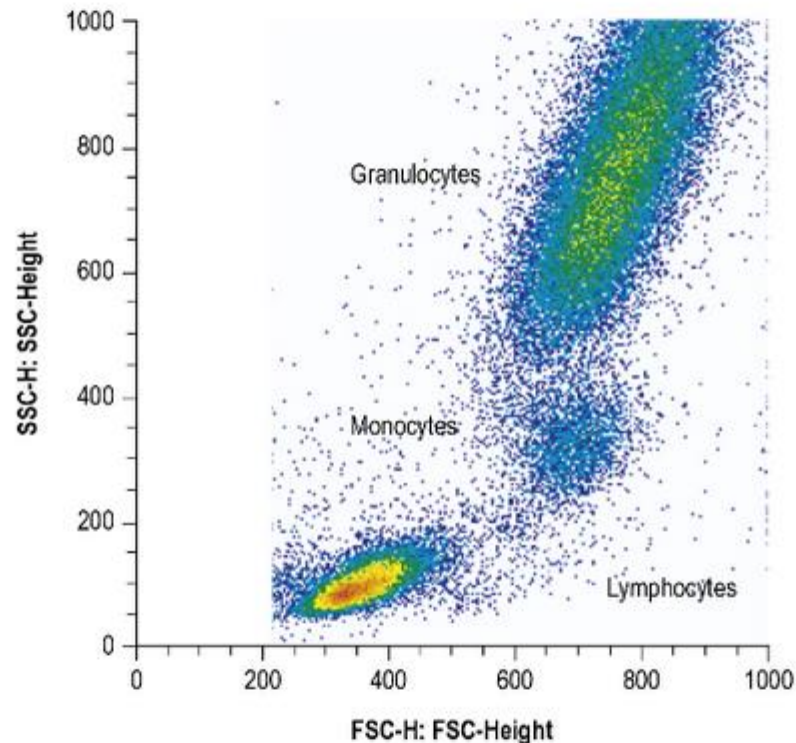


McKinon et al. 2019; Rich 2013 Clinical Immunology

Data representation / exploration

Dot plot or histograme. Cell population can be selected according to **morphology** or **phenotype**

Dot plot : one point represents one cell
Cell morphology: rel. granularity vs. Rel. size



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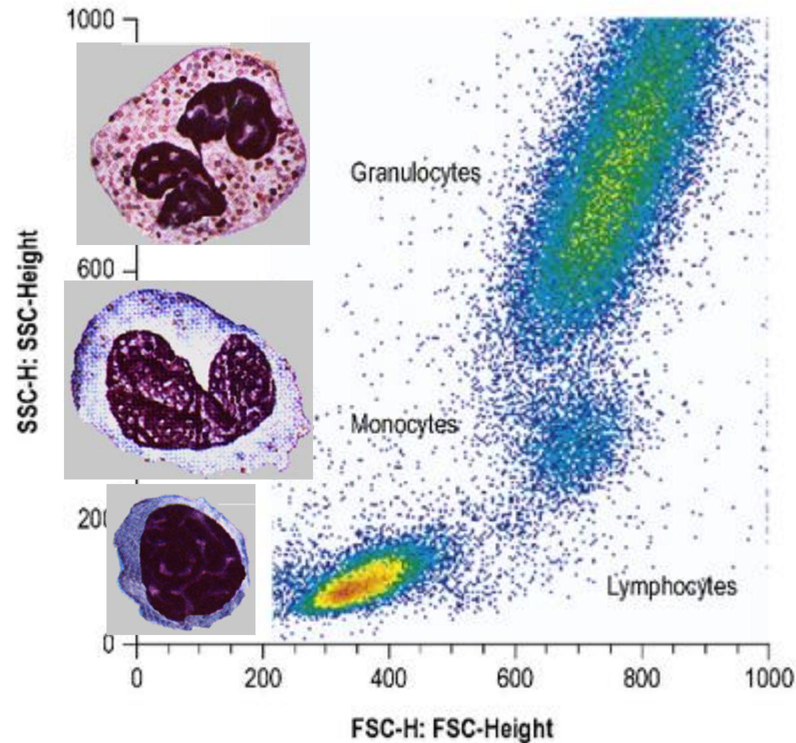

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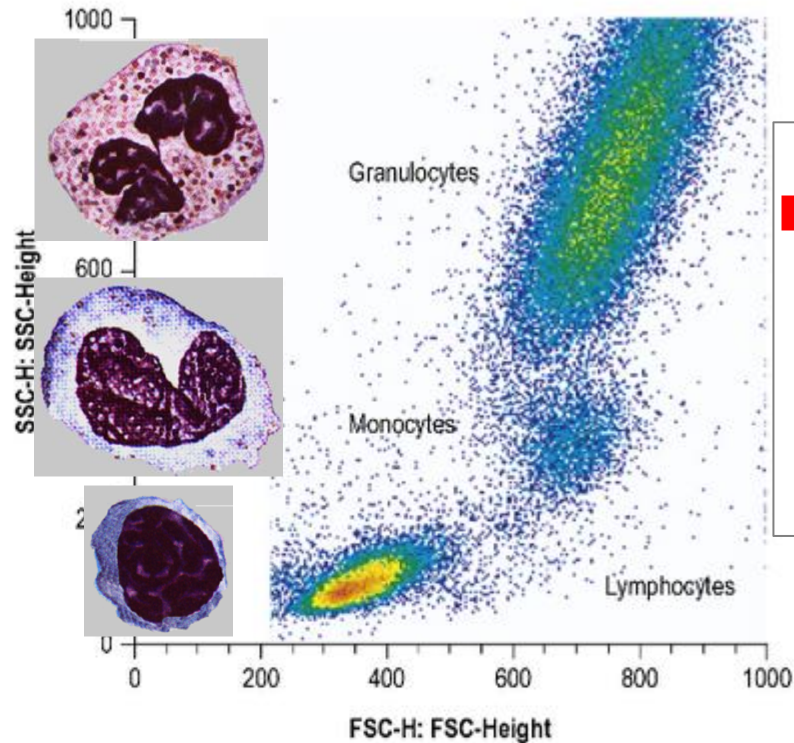
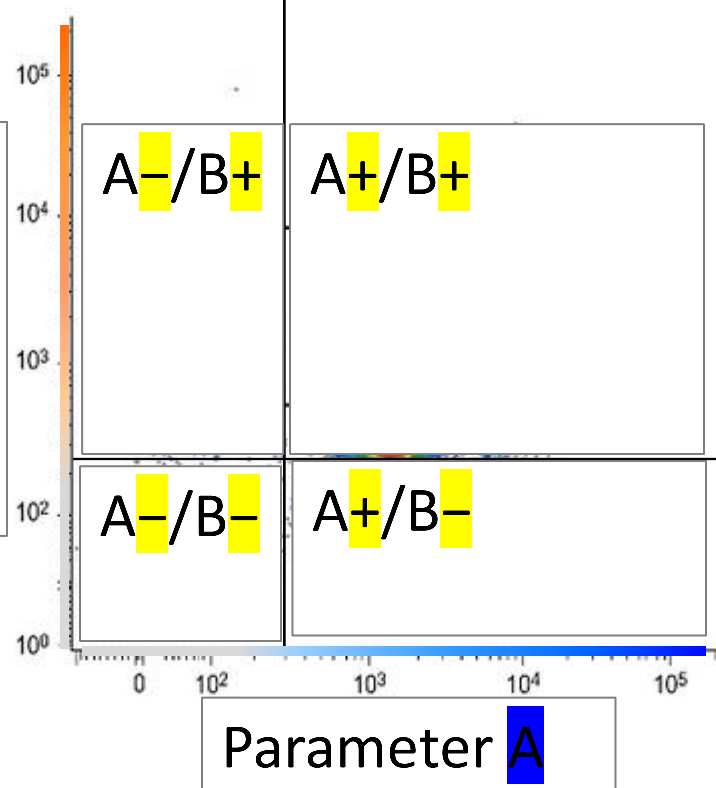
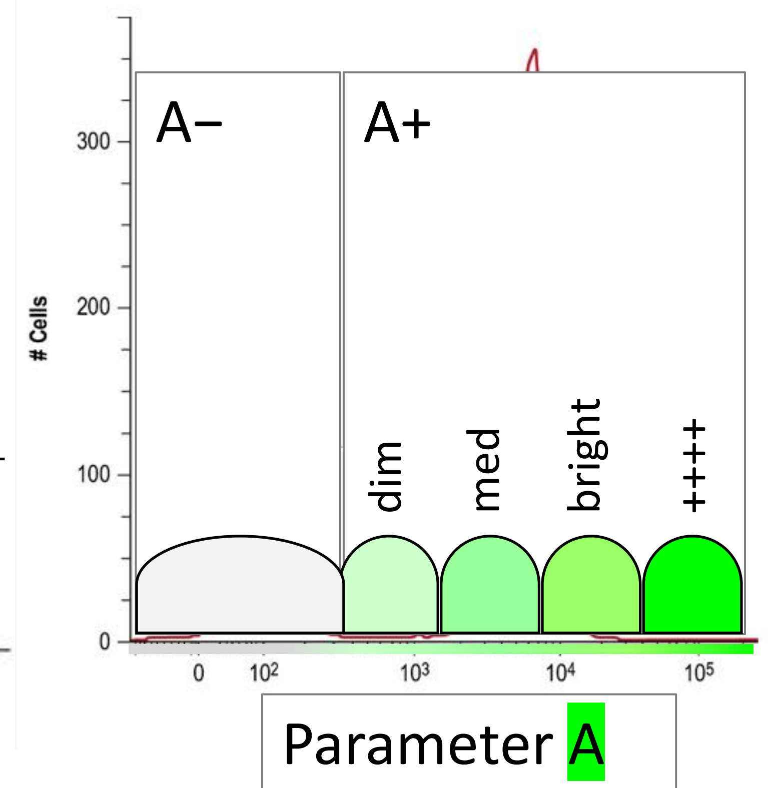


Dot plot
Phenotype: CD14 vs. CD45 (markers of monocytes and leukocytes)



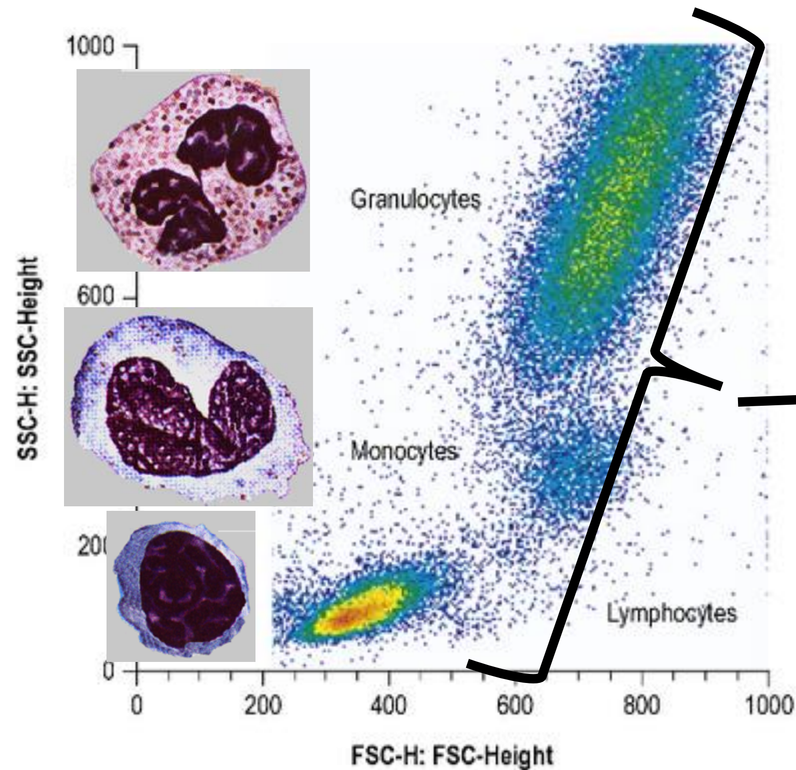
Histograme
Phenotype: CD3 (marker of T lymphocytes)



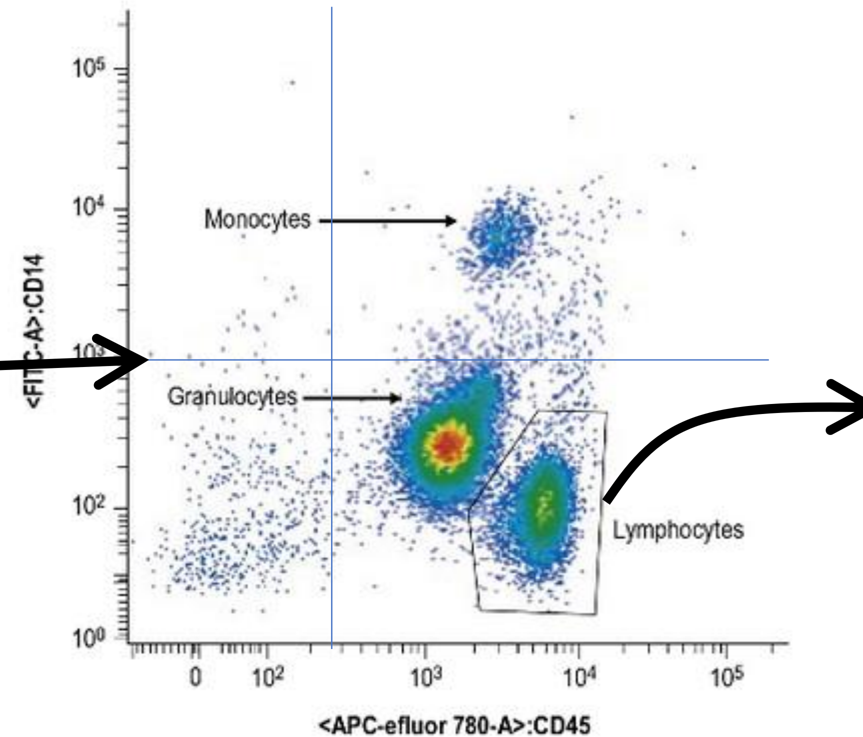
Data representation / exploration

Dot plot or histograme. Cell population can be selected according to **morphology** or **phenotype**

Dot plot : one point represents one cell
Cell morphology: rel. granularity vs. Rel. size



Dot plot – total leukocytes
Phenotype: CD14 vs. CD45 (markers of monocytes and leukocytes)



Data representation / exploration

Dot plot or histograme. Cell population can be selected according to **morphology** or **phenotype**

Dot plot : one point represents one cell
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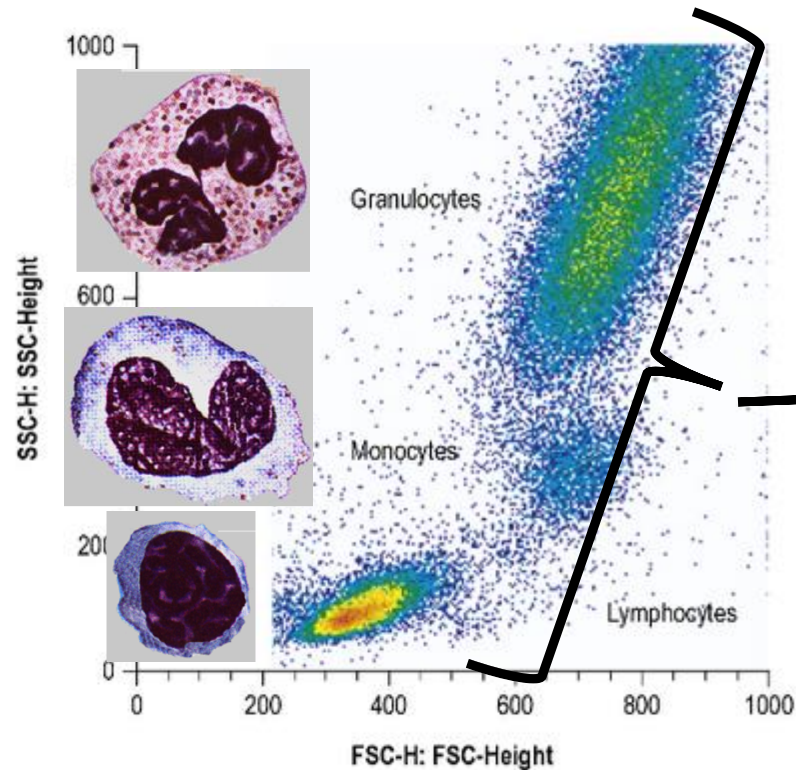
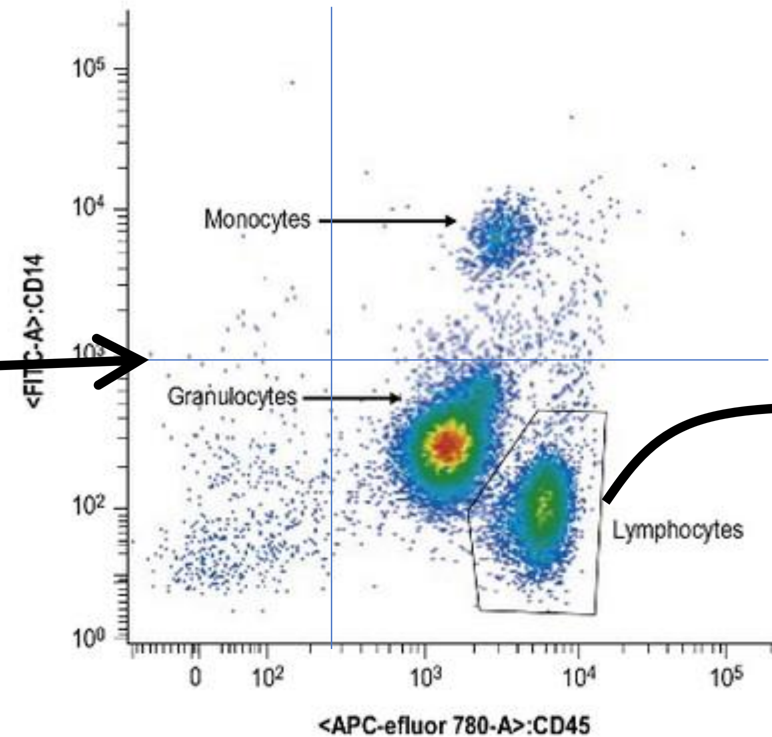
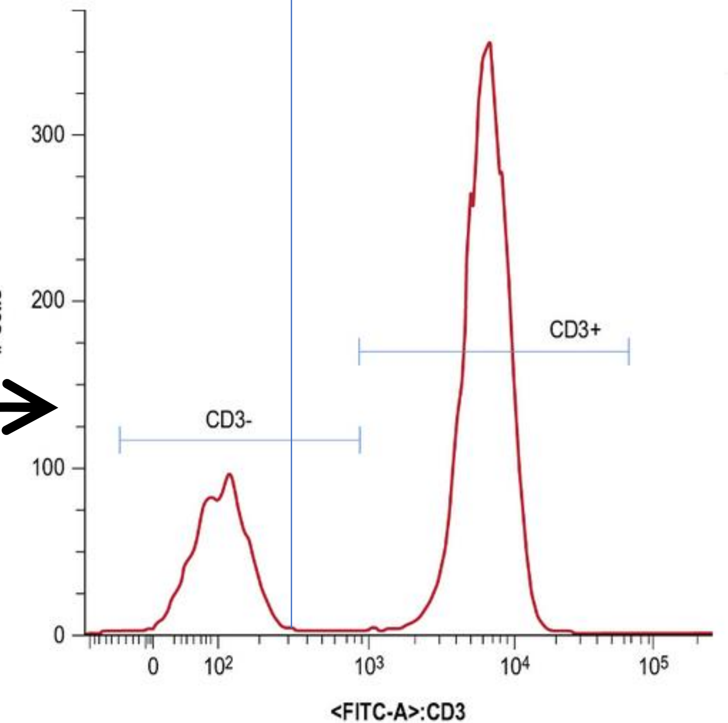


Dot plot – total leukocytes
Phenotype: CD14 vs. CD45 (markers of monocytes and leukocytes)



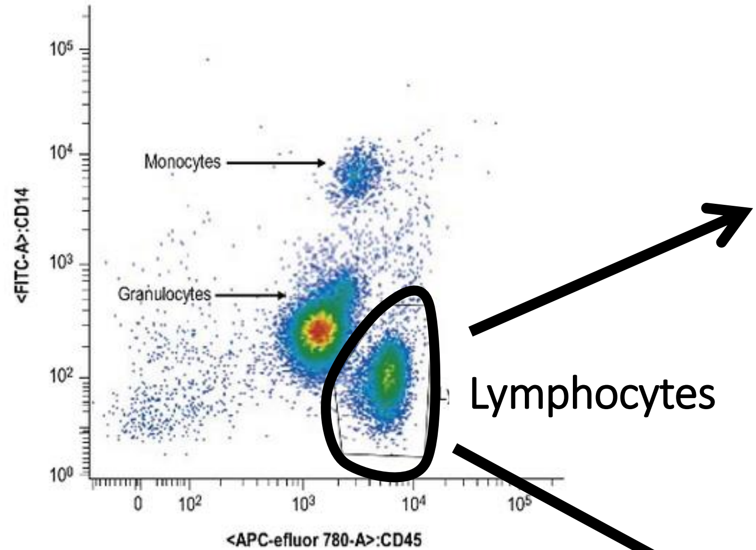
Histograme – Lymphocyte population
Phenotype: CD3 (marker of T lymphocytes)



Blood lymphocytes immunophenotyping
Gating on populations of interest

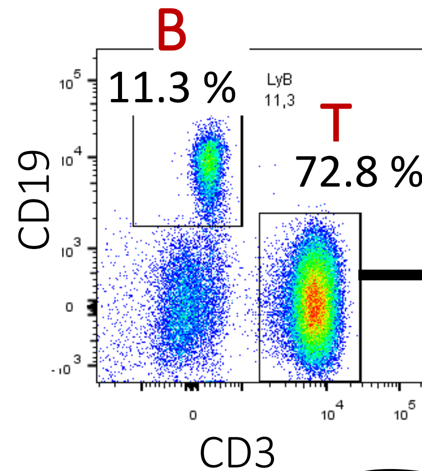
Example 1

Total leukocytes

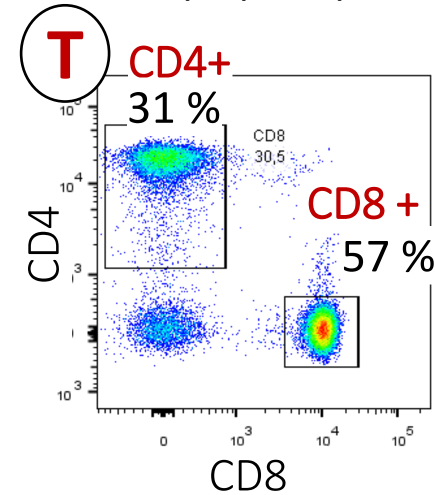


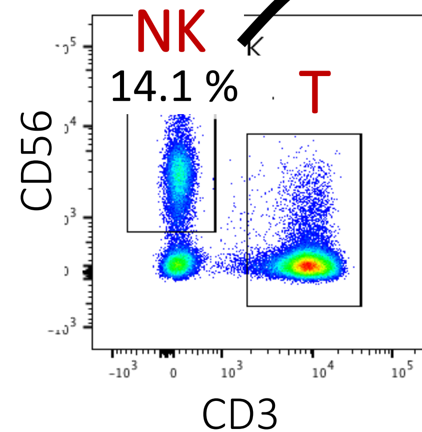
Lymphocytes

Total lymphocytes

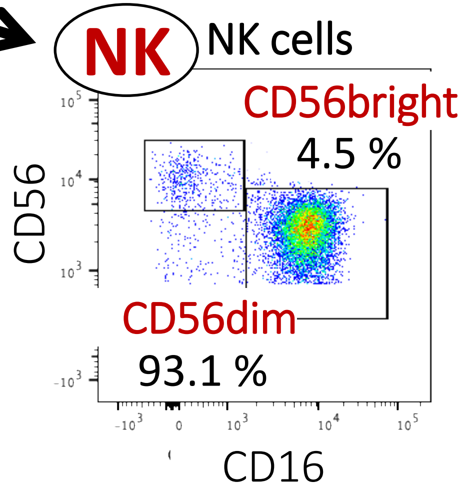


T Lymphocytes



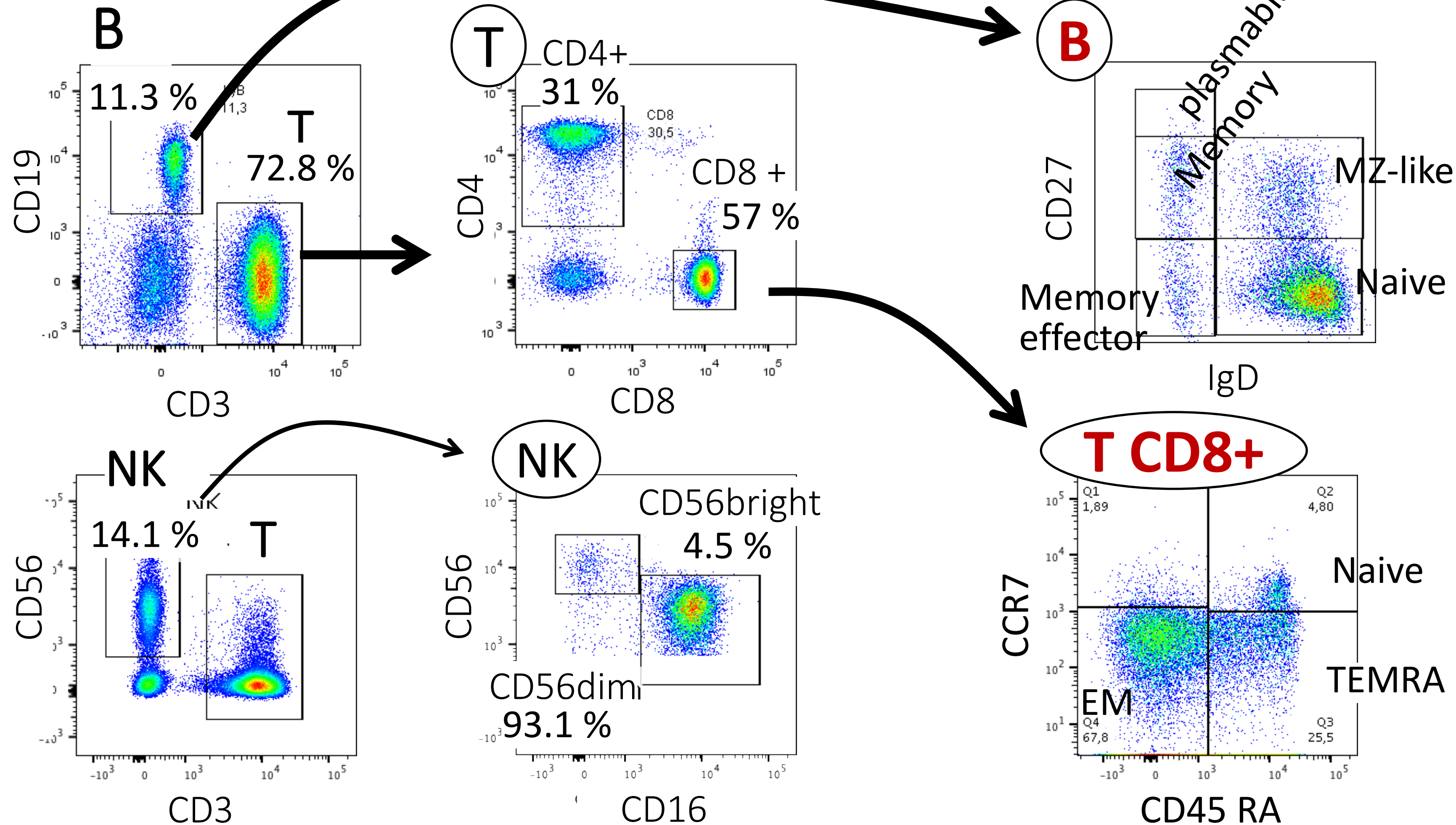


NK cells



Blood lymphocytes immunophenotyping
Gate of lymphocytes

Example 1



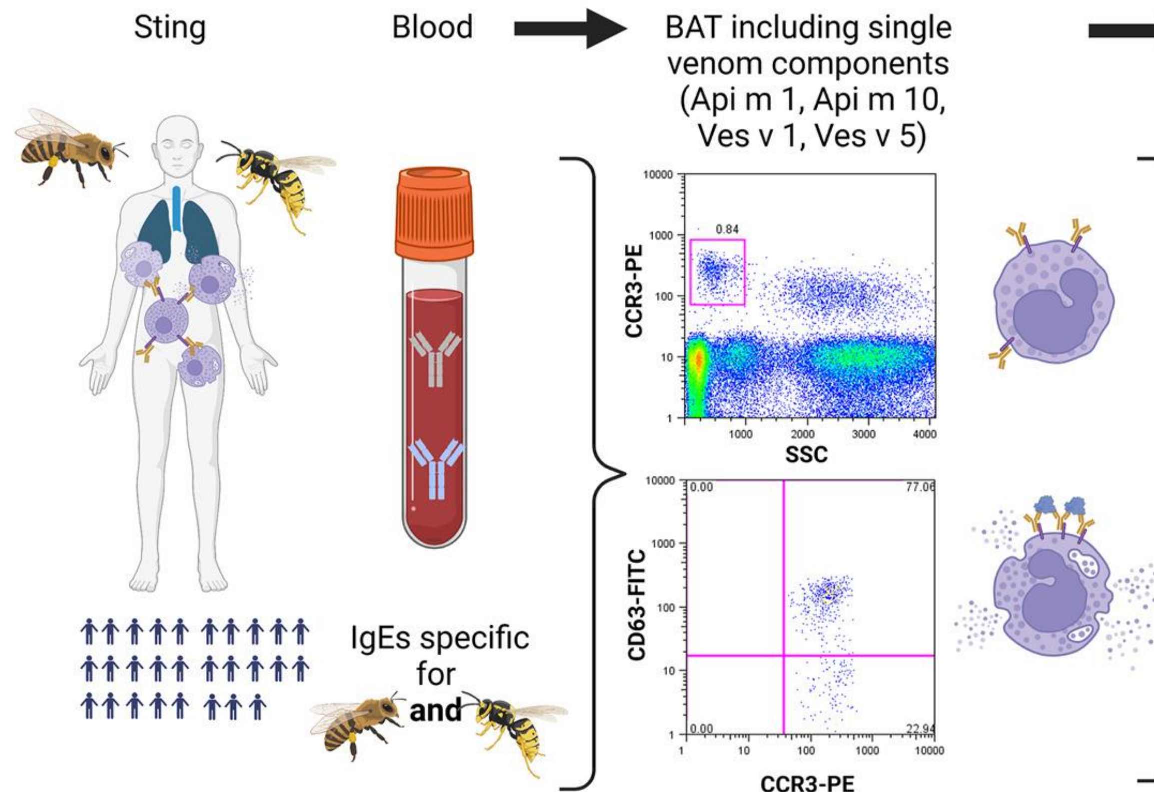
Lots of different diagnostic tests with immunophenotyping analysed by cell cytometry

Function of immune cells:
Incubation of whole blood samples + activation molecule → Fixation, immunostaining (for identification of cell populations and activation markers) → cytometry analysis

-Test of basophil activation (cell surface upregulation of CD63 upon incubation with an allergen)
-Test of neutrophil activation (cell surface upregulation of CD11b, CD66b3, etc upon incubation with LPS; oxydative burst followed by flourescent molecules added befor activation test)
-...

Lots of different diagnostic tests with immunophenotyping analysed by cell cytometry

Function of immune cells:
-Test of basophil activation (cell surface upregulation of CD63 upon degranulation of histamin rich granules in presence of a IgE-specific allergen)



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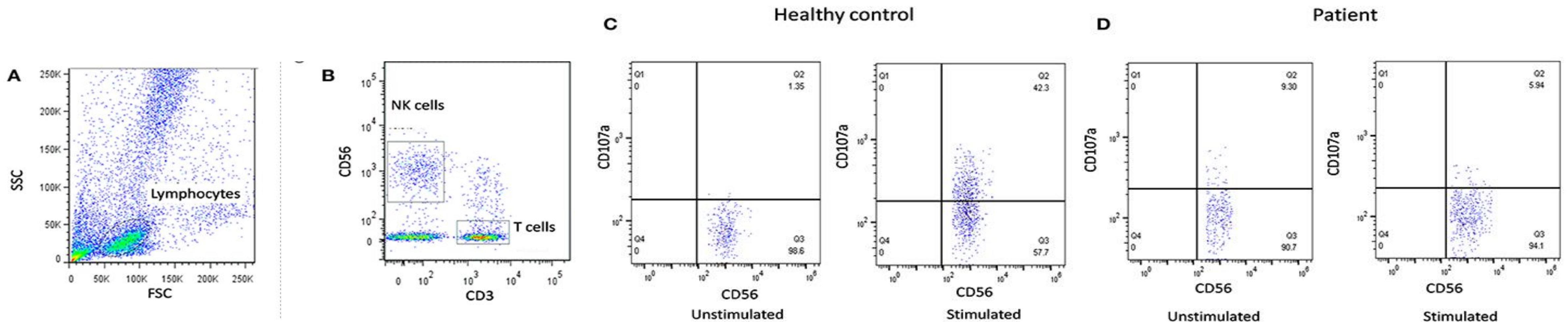
Lots of different diagnostic tests with immunophenotyping analysed by cell cytometry

Function of immune cells:

-Test of NK activation (cell surface upregulation of CD107a upon degranulation of perforin/granzyme granules)



Madkaikar et al Front Immunol 2019

Thank you for your attention

Some references

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