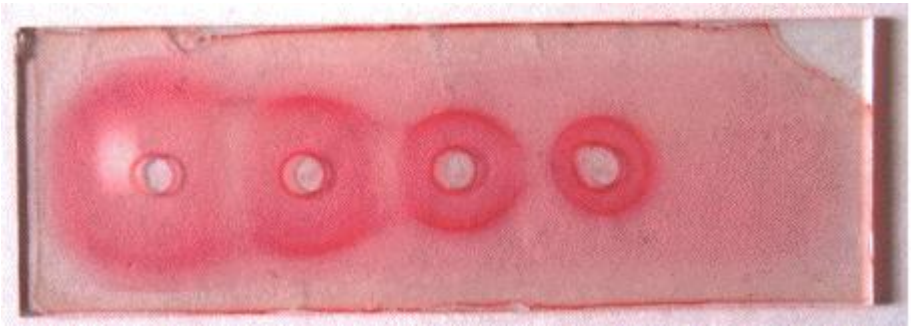
Sheet 9 : Continuation of the serology

Single Radial immunodiffusion

- we said that when we talk about **double diffusion**, it is really a qualitative method used in the lab but does not give us any indication of how much antigen or antibody we are really dealing with, so we can adapt this immune diffusion for the purpose of measuring these elements.

What we do here actually is that we prepare the agar just the same as we did before but before we pour it onto the plate we mix the antibody and then we pour it down onto the glass slide, that means that the antibody will have a uniform concentration all over the agar, and consequently it will not diffuse (because diffusion just occurs when there is difference in the concentration, then the antibodies will move from the high concentration to the low concentration), so in this plate the agar contains the antibody and the antibody does not diffuse because it has an equal concentration all over the agar.

Now we can make holes into the agar and put the antigen in the pores then if we leave the plate over night the antibody will not diffuse but the antigen will actually diffuse radially, and because we have the antibody all over the plate at a certain concentration, we will have a precipitation line as a shape of a circle, at the zone of equivalence we will get a precipitation line which is the circumference of a circle.

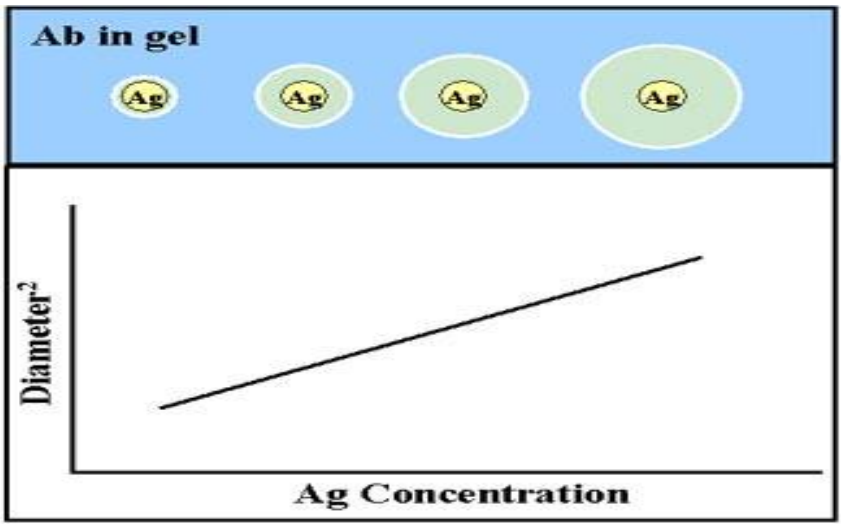


That's why we call it single radial immunodiffusion (single>>>one component is only diffusing, radial>>>radially producing a circle, immunodiffusion).

The amount of antigen (the antibody amount is fixed) here or the concentration will be proportional to the area of the circle because the more antigen you have, the more it is going to diffuse, the larger the circle is going to be. For example in the picture above the amount of antigen in the large circle is going to be much more than the that in the small circle, (the antigen is diffusing while the antibody is fixed so if we have more antigen in the hole, then to reach the zone of equivalence it has

to diffuse more and to be diluted more, because the amount of antibody in the agar is the same , so the more antigen you have, the larger circle will result), so the concentration of the antigen is proportional to the area of the circle, $A = \pi r^2$, π is constant, so the concentration of the antigen is directly proportional to the square of the radius, but because the radius might be difficult to measure (because there is a hole and it is hard to determine the center), so we measure the diameter rather than the radius, so we can say it is proportional to the square of the diameter.

So now if we go back to our original plate with the fixed amount of antibody in the agar, and then we put 4 pores and in each one we put a certain amount of a standard (an antigen of known different concentrations) then we let them diffuse over night, then we measure the diameters, we square them and then we plot them against the concentrations which are known, and if you did the experiment properly you should have a straight line as you can see in the picture below, and then if we have an unknown antigen concentration (X) we measure the diameter of the circle produced by (x) we square it and then we can drop a line on the X axis and find the concentration of the unknown.



This method is not very accurate as the previous ways (turbidimetry, precipitation) but it is well known for routine work in the lab.

Rocket electrophoresis

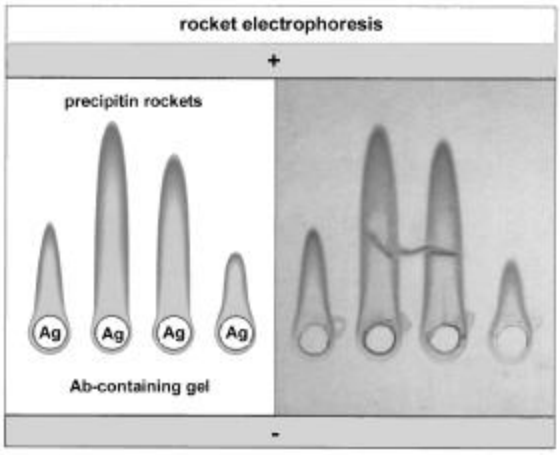
If you want to get the result of the previous experiment more quickly, and you don't want to wait till the following day-although it does not really matter, but for diagnostic purposes it is ok to be in hurry-, you can apply an electrical current.

Here again there is the glass plate, we put the antibody in the agar, then we makes pores there in which we put the antigen, and then we apply an electrical current.

- Note: you have to make sure that the antibody is really neutral, by adjusting the ph so that the antibody is neutral, because you don't want the antibody to move, you want it to stay uniformly distributed all over the agar.

Then you apply the current, so in this case only the antigen is moving , and make sure that the antigen is moving in the right direction not in the wrong direction (again by adjusting the PH before that).

So if you do this instead of getting a circle when diffusion comes it slightly looks like if you have a circle and then you pushed it out, and that is because of the electrical current, this is known as rocket immunoelectrophoresis, because the precipitation line actually looks like a rocket as you can see in the picture below.



In this case the concentration of the antigen is directly proportional to the distance that is travelled, and again you can plot the concentrations of the standard against the distance traveled, and get a graph and then when you come to the unknown you take the distance traveled, you plot it on the graph and then you got the concentration of the antigen of that unknown.

- There are other applications of electrophoresis and precipitation, you can separate the proteins in the plasma or the serum by electrophoresis and then you can have an immunodiffusion to precipitate them.

EXAMPLE: First of all you apply the electrical current and separate the plasma proteins, because they have different charges so they have different mobilities, then you take a part of the gel outside and then we put antibodies against them, then the antibodies can diffuse and produce precipitation lines, so in this case you can determine whether there is a component missing or not.

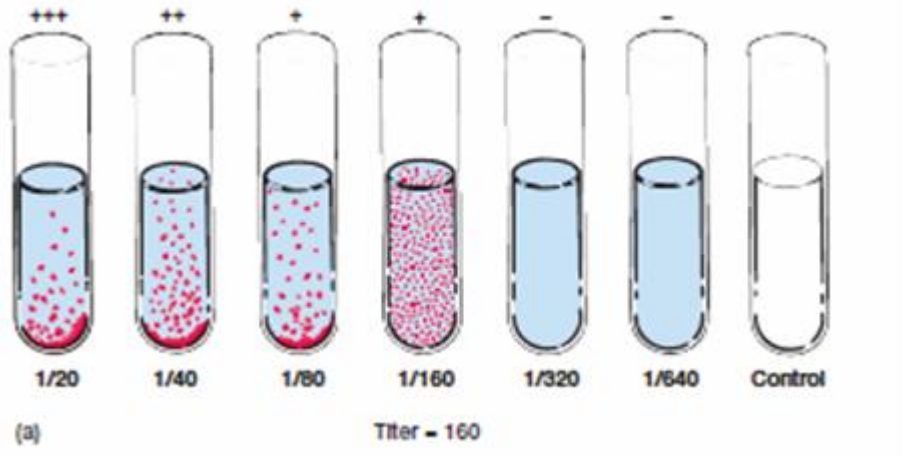
- This actually finishes the immunodiffusion.

Agglutination

Now the next thing we are going to talk about is agglutination, which is something that can be done and seen by the naked eye, because the cells are actually the antigens and indeed when they agglutinate together they actually precipitate.

Now when we come to agglutination, again we have the same problem with the pre-zone and post-zone and the zone of equivalence, so in order to do away with this problem, we actually take serial tubes and we put a fixed amount of antigens (for ex. RBCs) in all of the tubes, so the amount of antigen is fixed then we bring the serum which is usually the antibody, and we will add a certain amount, suppose that you put a neat serum (without dilution) in the first one, then in the next tube we dilute the serum by 2, we add 1 CC of the serum and 1 CC of saline, and then we have ½ concentration of the serum then again we do it in the next tubes like the following ¼, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, the **purpose** of this serum dilution is **to get to the zone of equivalence**.

In the tube that you have too much antibodies you will not have the agglutination, (as you are diluting the serum, and reducing the amount of antibodies), you will get a tube that reaches the zone of equivalence e.g. 1/32 has agglutination, the tubes before it represent the pre-zone and after it the post-zone (no or little agglutination). This means that the titer of the serum is 32 (titer of the serum is the highest dilution at which agglutination occurs). In the picture below the titer is 160.



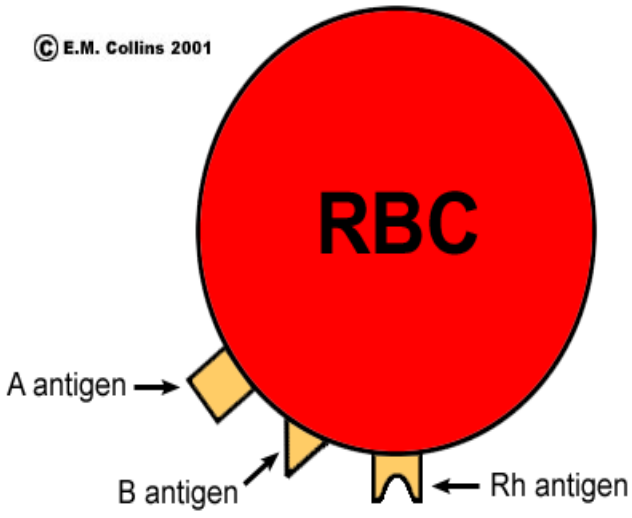
- This is useful in the widal (typhoidal) test or brucella, and since you are measuring the amount of antibodies in the serum so indeed the antigen here will be bacteria (typhoid cells) if we got agglutination this means that we have antibodies against the typhoid organism and this titer is 1/32. Sometimes we are not sure if it is significant or not significant (especially in endemic areas) , so we can wait for 3-4 days and then we repeat the same experiment again using the same amount of antigen but with a fresh serum, but in this case may be we will get

the agglutination at a titer of 1/256, this means that the amount of antibody in the serum has increased which means that there is an ongoing infection.

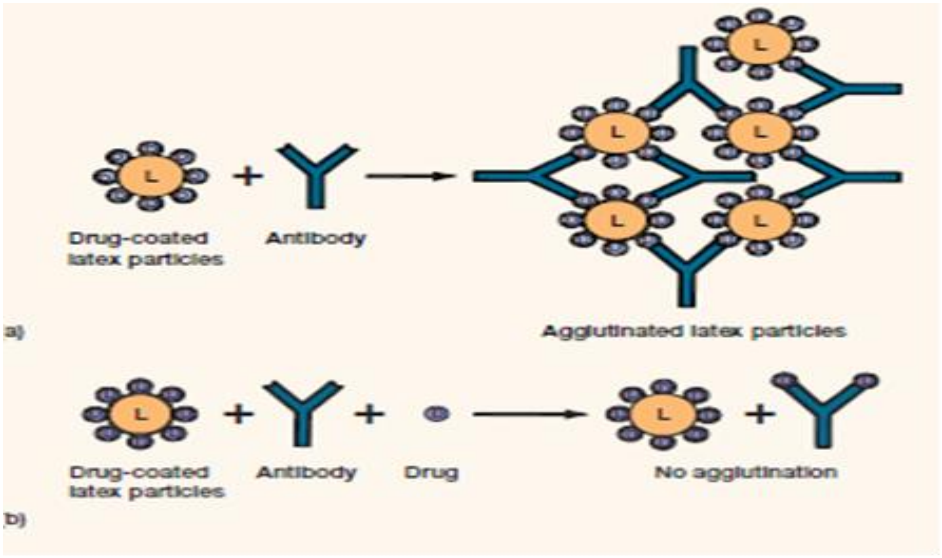
- That's what we mean by a rising titer, it means that there is an increase in the amount of antibody which means there is an ongoing infection, this can be done for viruses, brucella and so on. If it was the opposite after 3-4 weeks (the titer is going down) it means that the patient is getting better and he is responding to the treatment and the amount of antibodies is getting less. To be significant, a rise in titer has to be 4 fold, it has to be increased by more than two tubes, if less than 4 folds it is not significant because it is within the experimental error (for example if it was 1/32 the first time, the next time it should be at least 1/128)

Now when we talk about agglutination it could be **direct** or **passive**.

Direct agglutination: it means that the antigen or the epitope is really an original part of the particle, for example blood grouping (we actually take IgM antibodies anti A, or anti B mixing them with the RBCs and they agglutinate, the antigen A or B is actually a part of the red blood cell, also like the bacterial test (typhoidal/widal) the antigen is part of the particle, so this is known as direct agglutination, as you can see in the picture.



Passive agglutination: in this case we can attach either an antigen or an antibody to a **particle**, you can look for the antibody using the antigen or for the antigen using the antibody, this is known as passive agglutination, **you artificially put an antigenic determinant on a particle**, the particle could be a RBC or **latex** (small pieces of rubber that can be seen when they agglutinate, it is used instead of the red blood cells which have a short life (they eventually disintegrate), while the latex can stay for long time) an example is **pregnancy testing**, it actually has an antibody against HCG Human Chorionic Gonadotropin, so you have antibody against it fixed to the particle, we put a sample of urine on it, if there is agglutination then the urine must have HCG in it and that means that the woman is pregnant, if there is no HCG then there will be no agglutination and the woman is not pregnant.



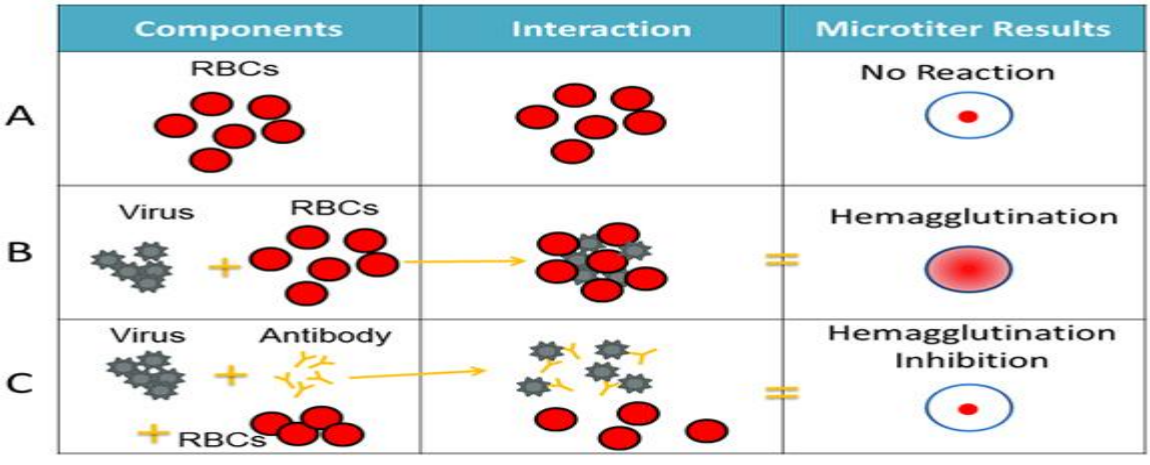
Hemagglutination inhibition test

Some viruses are known to be able to agglutinate RBCs because they have like receptors on them that could attach to RBCs, like the influenza virus, Mumps and so on

So if we mix the virus with the RBCs it will agglutinate, before we use the virus and mix it with RBCs we first mix it-the virus- with the serum from a Patient and after that we mix it with RBCs, either you will get agglutination or you will not get agglutination

If you get it, this means that the virus is still there as it was, with its receptors, and it agglutinates with RBCs, but if there was no agglutination this means that you have hemagglutination inhibition, which means that the receptors on the virus have been occupied or coupled, it means there must be an antibody in the serum to block these receptors, and prevent it from binding to RBCs.

So this is hemagglutination inhibition, if there is antibody in the serum then there is no agglutination, and if there is no antibody there will be an agglutination (if it is positive -there is agglutination- it is a negative result -the patient doesn't have the antibody- and vice versa)



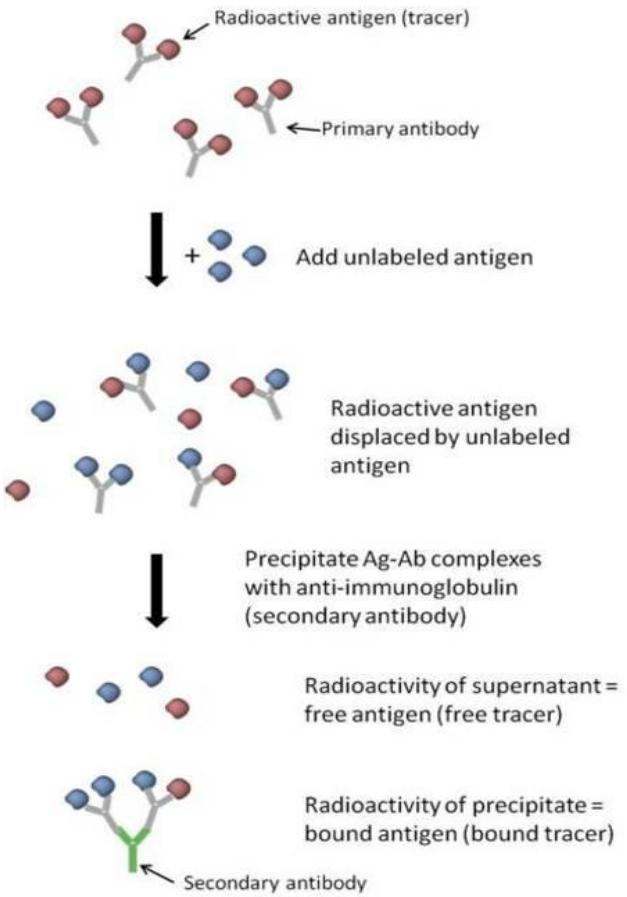
- Now we are going to talk briefly about the other methods, which are: Radioimmunoassay, ELISA and immunofluorescence, these are really three ways in which we can detect and measure the amounts of antibody or antigen or anything using these methods and these methods usually are **very sensitive** and used for **measuring components or proteins that are very very small in their concentration,** for example IgE because it is so little in the serum and it is not sensitive to other ways, so in this case we will use radioimmunoassay.

Radioimmunoassay

Radioimmunoassay is simple it's that we actually label usually with *Iodine 132* (labeling it with gamma-radioactive isotopes of iodine, such as 132-I) [1] either the antibody or the antigen , then we mix the labeled agent with the suspected other component, then we get immune-complexes forming, after you get rid of the excess radioimmuno-reactant you can measure the amount of reactivity.

Suppose we have antibodies that are radioactive and then we have antigens, you actually can react them together. And of course the more antibodies linking to the antigens, the more radioactivity there is going to be, so when you measure that, you can come up with the amount of antigens that are present. Again you can have standards and you plot them against the graph, the graph here is a little bit more complicated, because it is log antilog graph, it is not really a simple graph like the one we have measured in the single radial immunodiffusion.

So here we label with the radiation like Iodine 132, and then the amount of radiation bound onto the component that we are measuring will indicate how much there is of that component.

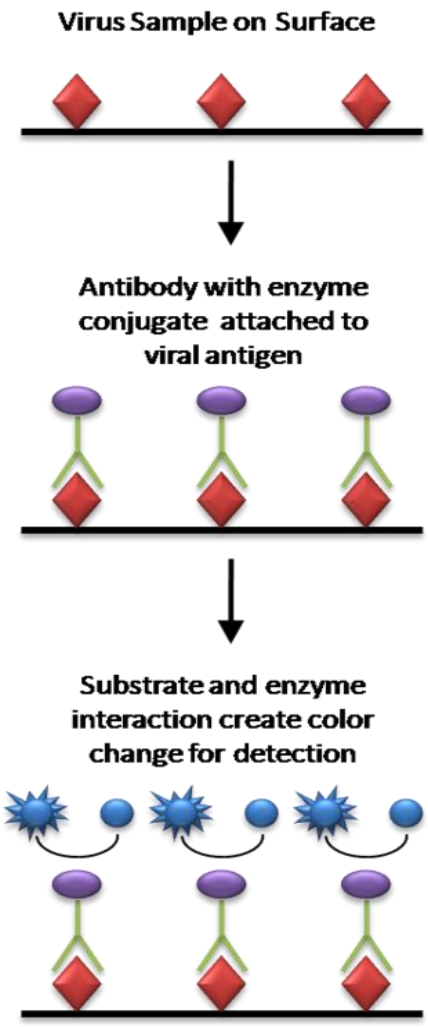


[1] http://en.wikipedia.org/wiki/Radioimmunoassay

So this is the basis of radioimmunoassay, it is **very accurate**, but there are disadvantages of course because radiation is always a little bit hazard to work with, also you find that it is difficult to get rid of it as a waste, you can't do the experiment and then throw it in the sink because you will contaminate your environment, also it is expensive and also the half-life of radioactivity (shelf-life) is usually short, a couple of months then the kit will be no more used because the radiation have gone.

ELISA

ELISA which is enzyme-linked immunosorbent assay, here we are still using labeling but instead of using radioactive iodine we use an enzyme that can be Alkaline phosphatase, Horseradish peroxidase.
Then you react the two components together the labeled with the one unlabeled just like radioimmunoassay, and then you will detect the activity of the enzyme, (the more antigen there is, the more enzyme has been attached) by adding the substrate to the mixture, after that we mix them together, and this will give you a color that might be green, orange, etc.. That result from binding the enzyme to the substrate, the intensity of the color on the substrate will indicate how much antigen there is; again you can plot a graph and work out the concentrations of the antigens that you are looking for. It is a cheaper method, with the same sensitivity as radioimmunoassay, safer and also has a long shelf life.



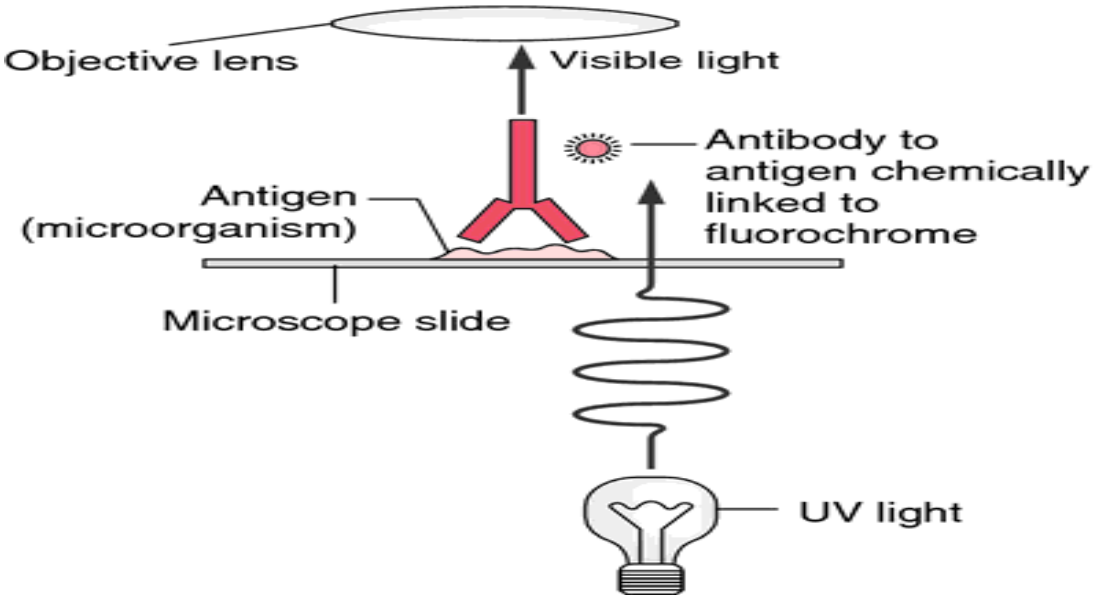
Immunofluorescence

The last one is immunofluorescence, actually it is often used for looking for antigens in the tissues, you can use it if you expect an antigen in a tissue and you have to find out whether it is there or not.

Let's say that we have a kidney section and we want to look for C3 if it is present in the tissue or not, you bring anti-C3 immunoglobulins and then you label them with some fluorescent, here the label is on the antibody, then we mix it with the tissue.

If the C3 is there, the antibody will stick to C3 on the section, and after washing it (to get rid of excess antibody that is not bound to the antigen) you put it under an immunofluorescence microscope and then you can see things fluoresce, it might be red or any color according to the fluorescent agent that you are using, the presence of the fluorescence will indicate that the agent that you are looking for is there.

Another example if you think of the basement membrane of the glomeruli, sometimes it has antibodies against DNA, when you look under the fluorescence microscope you find that the whole basement membrane is actually lit, because of the C3, so really if you want to look for a something in a tissue you use immunofluorescence.



Flow cytometry

The last application is flow cytometry, by this method we can count RBCs and WBCs in the lab. The sample that contains the cells is put in a fluid then it is squeezed in a nozzle, one by one as it goes down it is hit by a laser beam which will give a measure of the size and granularity, so by size and granularity it can sort the cells into lymphocytes, neutrophils, etc.. and then you can count the cells of each type.

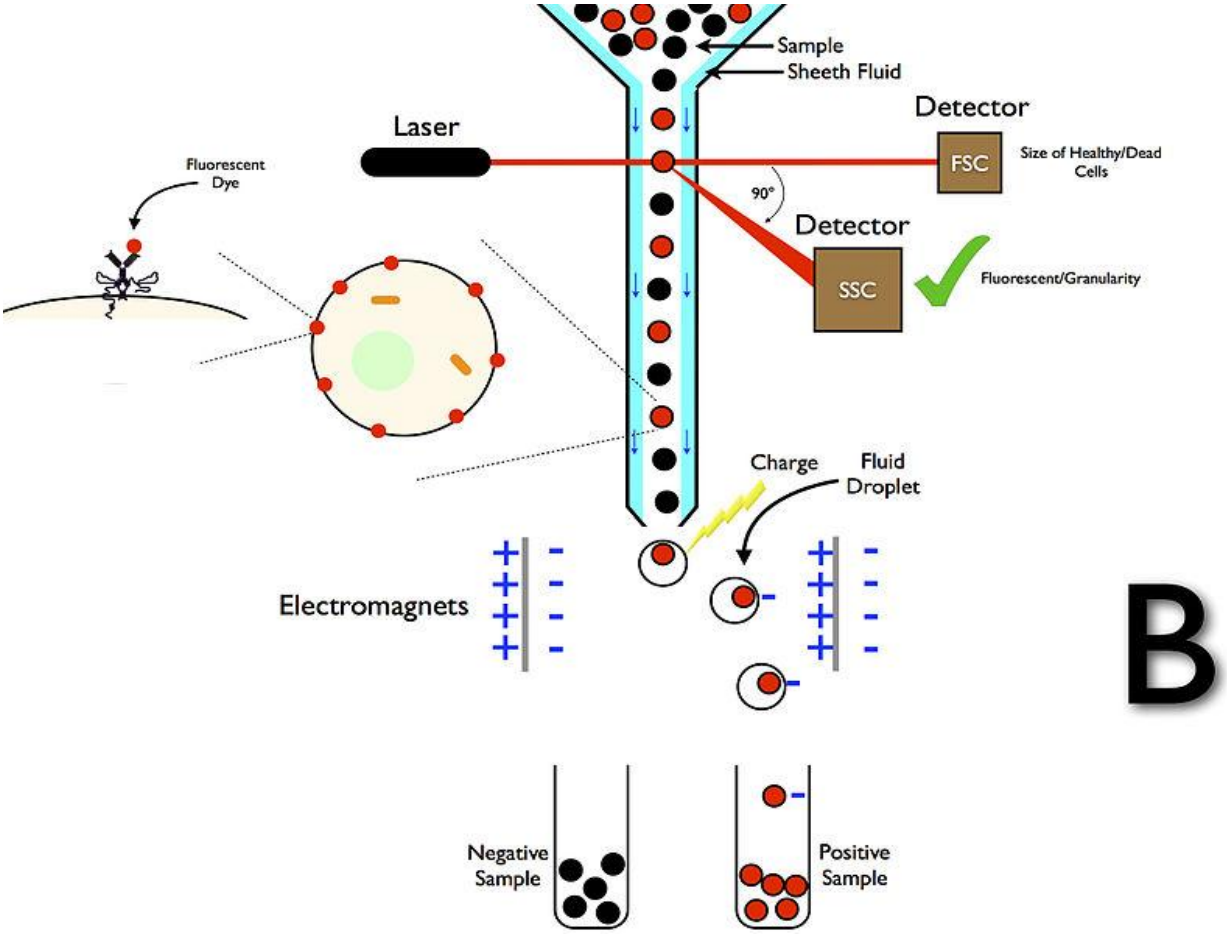
In addition, you can adapt this system using immune-fluorescence to actually sort the cells, if we have so many lymphocytes in the sample and we want to know how many B- lymphocytes and how

many T-lymphocytes, also how many of the T-lymphocytes are T-helper and how many are T-cytotoxic, in this case we have to pick a marker which is really special for that cell, for ex. In T-helper cells the marker is CD4, t-cytotoxic cells the marker is CD8, B-cells the marker is CD19.

Then we can bring anti-CD4 or anti-CD8 or anti-CD19 and these antibodies are labeled with different fluorescent agents in different colors, then we push them through the funnel and as they flow they get hit by the laser, and when the cells get hit by the laser they will shine because they have the fluorescent antibody bound to them.

Then there is an electric charge beam which can actually deflect the cell to wherever you want, for example if it is shines red it can be diverted to the right, if green it will be diverted to the left, so they will be sorted in different tubes, so this is really sorting cells and separating them, and this is known as FACS fluorescent activated cell sorting, because if we have activated fluorescent we can sort them accordingly.

The following diagram illustrates this method :



Done by: Hala Makahleh.

Sorry for any mistakes.