Introduction to Complex Biological Systems
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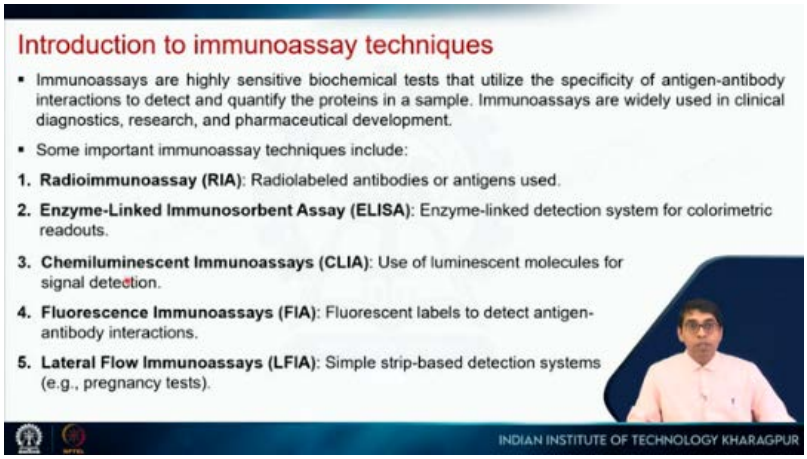
Lecture 59
Immunoassay techniques

Welcome to the fourth lecture of Week 12. So, in this lecture, I am going to discuss immunoassay techniques. So, this is part of our various biophysical and biochemical methods that we are studying. So, today I am going to introduce what immunoassay techniques are.

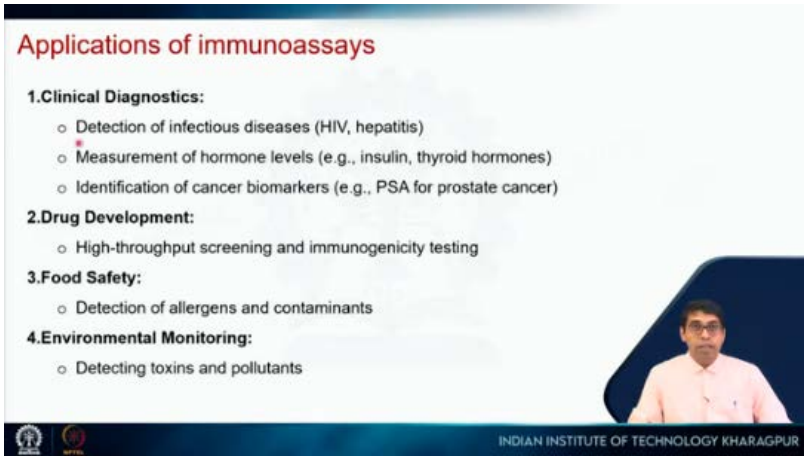
So, I will give you a general overview, then I will discuss specific methodologies: ELISA, Western Blot, Immunoprecipitation or Coimmunoprecipitation, Immunohistochemistry Assay, Radioimmunoassay, and Immunofluorescence Assay. You will see that the basic principle for all these assays is very similar, and apart from this, there are also many other methods, which will be some variations of these methods. What are immunoassay techniques? Immunoassay techniques are highly sensitive biochemical tests that utilize the specificity of antigen-antibody interactions to detect and quantify proteins in a sample. These techniques use the highly specific and tight interaction between an antigen and an antibody. In theory, we can raise very specific antibodies against any antigen, and this quality of antibodies is used in these techniques. So, immunoassay techniques are widely used in clinical diagnostics, research, and pharmaceutical development. We will see examples as we go through.

So, here are the important immunoassay techniques. The first one is radioimmunoassay. In this case, the antibody or the antigen is radiolabeled, and since we are using radioactivity, one has to be very careful in terms of using this assay. However, the advantage of this assay is that it is highly sensitive. Very small amounts of antigens can be detected using radioimmunoassay. The next one is enzyme-linked immunosorbent assay, or ELISA. This is the most popular one. So, in this case, the antibody is linked with an enzyme, and it is the enzyme activity that is used to detect.



Since this is an enzyme activity, it will amplify your signal and also increase the sensitivity. So, these are all variants of these methods. Chemiluminescent assay. In this case, chemiluminescence is used for detection. In fluorescent immunoassays, we use fluorescent labels for detection and then there is the lateral flow immunoassay. In this case, it's a simple strip-based detection system. Examples of this would be a pregnancy test. What are the applications? They have applications in clinical diagnostics, drug development, food safety, and environmental monitoring.

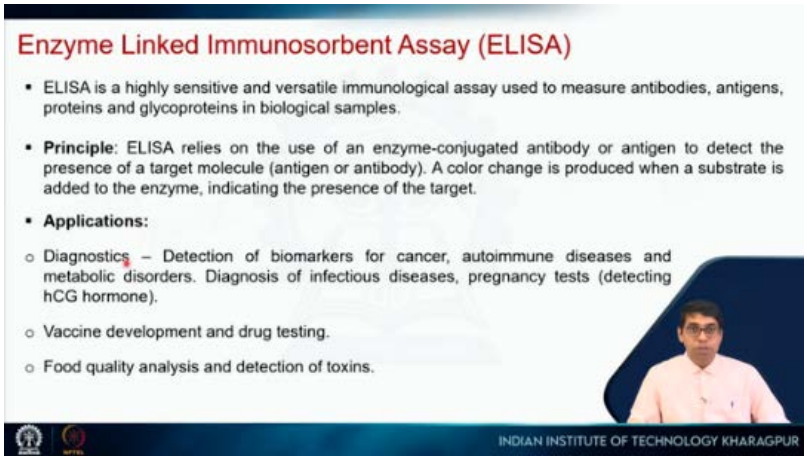
So, in clinical diagnostics, they can be used to detect various infectious diseases such as HIV and hepatitis. They can be used to measure different hormone levels, such as insulin or thyroid hormones, from blood samples. They can be used to identify cancer biomarkers, for example, PSA for prostate cancer. So, there are many examples like this. Drug development, of course, these are very important for drug development, and they are used in basic research in different laboratories.



So, they can be used for high throughput screening of different drug molecules or they can also be used for immunogenicity testing. In the food industry, they are also very useful. They can be used to detect allergens and contaminants that can be present in a food that is mass produced. Environmental monitoring, so it can be used for detecting toxins and pollutants. Let's look at enzyme-linked immunosorbent assay, which is most probably the most popular technique in immuno assay techniques.

ELISA is a highly sensitive and versatile immunological assay, and it is used to measure antibodies, antigens, proteins, and glycoproteins in blood samples. You can measure any of these things using ELISA. What is the principle? It relies on the use of enzyme conjugated antibody which means that the antibody on the heavy chain covalently linked enzyme is present for example let us say HRP or horseradish peroxidase and its reaction will be used for detection.

So this enzyme is linked to the antigen or the antibody or the antigen to detect the presence of a target molecule, which is an antigen or antibody. So typically you will detect antigen using the antibody. What is the readout? The readout is a color change produced when a substrate is added to the enzyme indicating the presence of the target. Again, the applications are in diagnostics, so various biomarkers for cancer, in case of autoimmune disease, so biomarkers for autoimmune disease, biomarkers for metabolic disorders, diagnosis of infectious diseases, pregnancy test, etc.



It can be used for vaccine development, drug development, and drug testing. It can also be used for food quality analysis and the detection of toxins. Now, ELISA comes in different flavors. The first one is ELISA. In this case, it involves the binding of the primary antibody directly to the antigen. This is the antigen. Ag stands for antigen. The antigen is immobilized. Now, you add the antibody, which specifically binds to this antigen. Here, you have some enzyme that is linked. Once this binding happens, you must wash it off so that any non-specific interaction is removed.

Then, you pass the substrate. This substrate will be converted into a product. There will be some color change, which you can detect. This is direct ELISA because your antibody is directly binding to the antigen. The next one is indirect ELISA. So, in this case, the antibody, there are two antibodies. There is a primary antibody and a secondary antibody. So, the primary antibody binds to the antigen coated on the plate. So again, the antigen is here on the plate.

The primary antibody, which is raised against this, binds to it. Now, there is a secondary antibody to which the enzyme is linked, and this antibody binds to the primary antibody. So, this antibody is raised against the primary antibody. So now, you can use this. So then again, you add the substrate, and from the reaction, you can detect it.

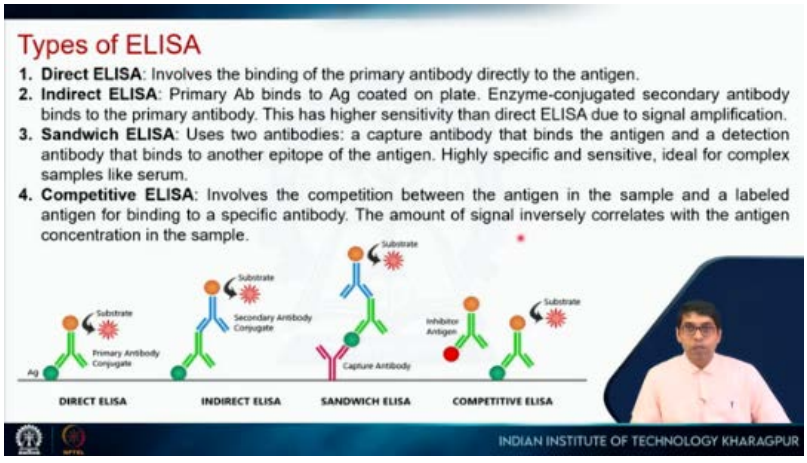
Now, the advantage of this ELISA over the other ELISA is that here, the enzyme-linked secondary antibody can be constant, and this can vary. So, you can raise different primary antibodies against different antigens and use the same enzyme-linked secondary antibody,

which will detect this, and you can have your ELISA. In this case, you have to link your enzyme to the primary antibody every time. So, this turns out to be cheaper than the other.

The third one is sandwich ELISA. So in this case, it uses two antibodies for the same antigen, the first one is a capture antibody. So here the antigen is not on the surface. It is the capture antibody that is on the surface and your antigen, it is most probably in some liquid sample, let us say.

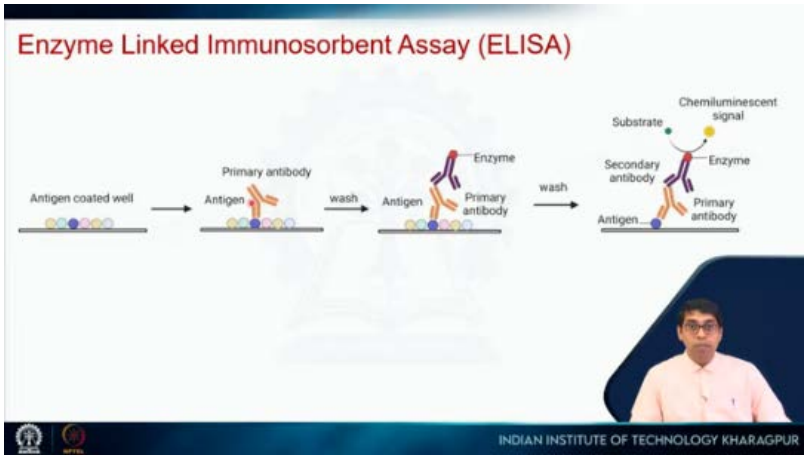
So you flow it, your antigen will bind to this capture antibody. So, this is practically more useful and then you use a detection antibody which is this green one. So, this will bind to the antigen. The only catch here is that in this case these two antibodies should identify two different epitopes of this antigen. So, the capture antibody will identify an epitope which is on this side and this antibody will identify an epitope on the other side.

Now once this antibody is there the secondary antibody will bind to this antibody, the detection antibody and then you can do your reaction to detect and finally, the last one is competitive ELISA. It involves the competition between the antigen in the sample and a labeled antigen for binding to a specific antibody. The amount of signal inversely correlates with the antigen concentration in the sample. So this is more for a quantitative determination of how much antigen is present in a particular sample. In this case, you have the antigen from the sample which is there, and you have this other antigen. You will compete this off and when this competes this off your antibody comes off and then basically the signal, more signal that you get means more of this is coming off. So, that is why it is inversely correlated to the antigen concentration in the sample.



So, this is the basic process. So, that antigen is coated on the well. In the second one we saw that there will be the capture antibody which will be coated on this well. Now, you have to add some sort of blocking agent so that you can reduce non-specific interaction. So, some people use BSA.

So, there are different mixtures that people use and then you add your primary antibody Which will bind to this antigen very specifically, then again you have to wash with different buffer conditions so that you remove anything that is non-specifically bound, and then you go for the detection, so you add the secondary anti antibody, again wash off anything that is non-specifically bound, and then you add your substrate to get your chemiluminescent signal, and then you can detect it using some detector. So, another very important and very robust and highly used experiment is western blotting. So, what is western blotting?



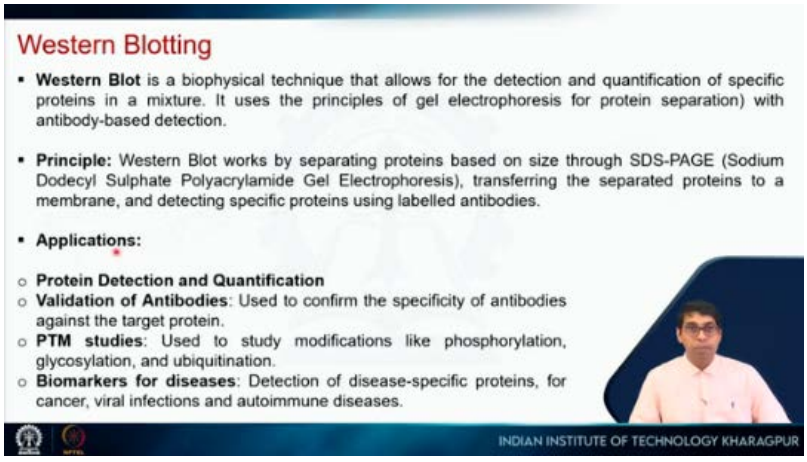
It is a biophysical technique that allows for the detection and quantification of specific proteins in a mixture. So, it uses the principles of gel electrophoresis for protein separation with antibody based detection. So let's see. Western blot works by separating proteins based on size through SDS PAGE. We have already seen SDS PAGE.

Let's see you have a cell lysate. So you have a mixture of proteins. You can separate them out through SDS PAGE. But the problem is that you don't know the protein that you are looking for whether it is present in this mixture of proteins or not. If you have purified your protein and there is only one band, then that is not a problem, but if it is still a cell lysate, it is a mixture of, let us say, several thousand proteins, then you do not know whether your protein is present, and even if you know the mass of your protein, let us say it is 25

kilodaltons, there might be multiple proteins which are present at that position. So, you do not know for sure whether your protein is present in that particular band which is near 25 kilodaltons. So that is where we use detection using an antibody. To do that what is done is that all these proteins are transferred. So the separated proteins are transferred to a membrane and then the specific protein is detected using labeled antibodies.

So applications, the protein detection and quantification, you can detect and also quantify using densitometric analysis. So validation of antibodies can be used to confirm the specificity of antibodies against the target protein, so if you are developing some antibodies, you can use western blot to validate that or you can use it for actual research purposes.

So, post-translational modifications, for example phosphylation, glycosylation and ubiquitination so one can raise antibodies against these post-translational modifications of a particular protein and then use that to detect how much of the protein in a cell in a particular condition is phosphorylated or glycosylated or ubiquitinated and it can be also used for detection of biomarkers for various diseases.



So this is the basic principle. So you have a cell lysate. It can be from bacteria. It can be from mammalian cells and then we run an SDS-PAGE gel electrophoresis.

So you have these separated proteins. Now these proteins will be transferred onto a membrane. Typically, a PVDF membrane is used. So for that, we will use electrotransfer again. We will use a gradient of the electric field and transfer it.

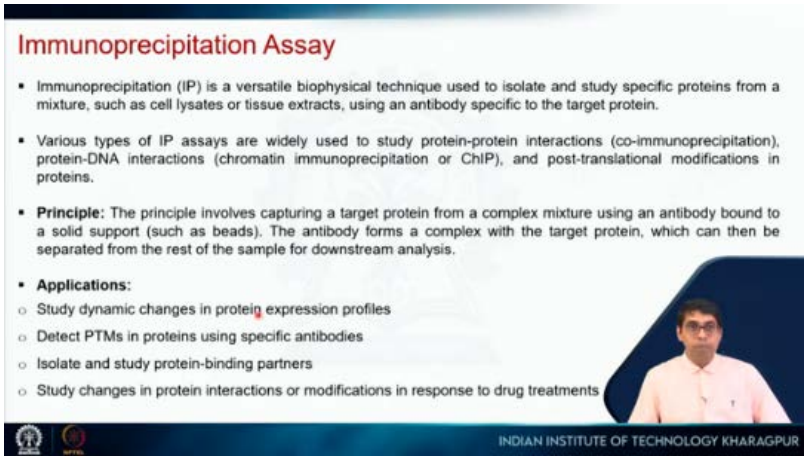
Now, one thing you have to be careful about is that the transfer of these proteins is not exactly the same. Larger proteins will transfer at a different rate than smaller proteins. So one has to be aware of the mass of the protein you want to look at and then adjust your transfer conditions accordingly. Once it is transferred to this membrane, you have your protein on the membrane. So you add the specific antibody, which will bind to that and for detection, you add the secondary antibody, which will bind to the specific or primary antibody, and this will have some enzyme link.

So, again, this is similar to ELISA, and then you add your substrate, you get a chemiluminescent signal, and then you use some instrument to capture that signal. So, you use chemiluminescence imaging. So, standard gel dock apparatus are present and available to do this. Next is the immunoprecipitation assay. This is a versatile biophysical technique used to isolate and study specific proteins from a mixture, such as cell lysates or tissue extracts, using an antibody specific to the target protein.

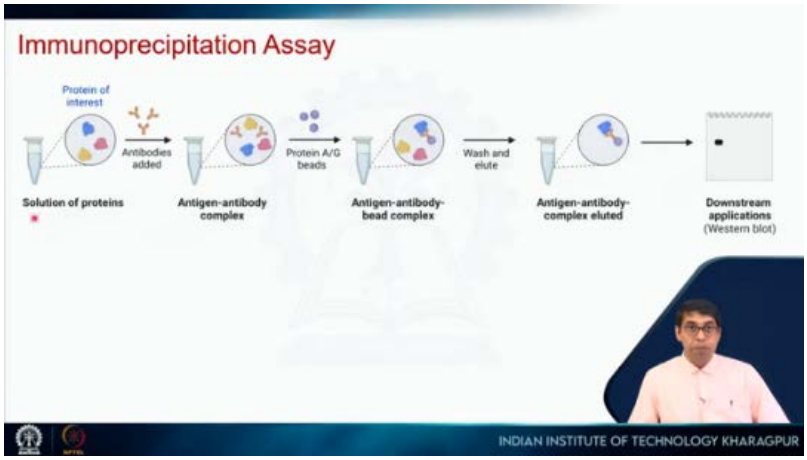
So, for example, there is a specific protein, and you want to know whether another protein, Protein A, is there. You want to know whether Protein B binds to it or not, or in some cases, you might not know what the other proteins that bind to Protein A are, so you want to identify those proteins. So, in that case, the immunoprecipitation assay is very useful. There are various types of immunoprecipitation assays to study protein-protein interactions, so this will be called co-immunoprecipitation or protein-DNA interactions, and this will be called chromatin immunoprecipitation, or ChIP, and then you can also use it to detect post-translational modifications in proteins. So, the principle involves capturing

a target protein. So, let's say protein A, which binds to other proteins and forms a complex, so we will have an antibody which will bind to target A and then that antibody is actually conjugated to a bead so that we can then spin down that bead. So, the bead will precipitate, the antibody will precipitate, and the antibody will bind to your target protein A, which will also precipitate and if the target protein A binds to other proteins, they will also precipitate.

So, you can isolate all of this together and then run a gel, use Western blot analysis, or use other techniques to identify which other proteins are binding to the target protein A. Applications: study dynamic changes in protein expression profiles. You can detect post-translational modifications. You can isolate and study protein binding partners, and you can study changes in protein interactions or modifications in response to drug treatments or in response to different disease conditions. So, you can look at control cells versus disease cells and see how these interactions change.



So, this is the schematic of the experiment. Let us say we have a cell lysate, we have protein of interest and now we add our antibody. So, this antibody will bind specifically to our protein; let us say that is protein B and then we add these beads. These beads will bind to the antibodies and then we can spin it down and we will isolate our protein, this blue from the rest and then we can run it on a gel and do western blot and other methods to detect it specifically.



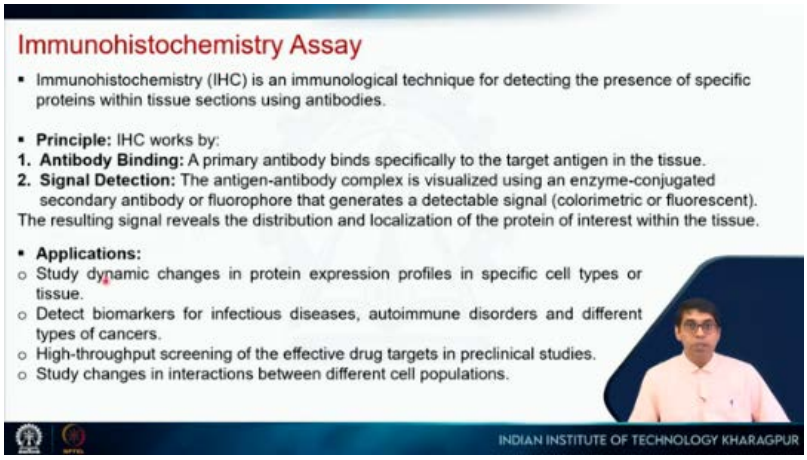
So to do coimmunoprecipitation, let us say there is this mixture of proteins and this blue protein binds to this green protein. But there is this yellow and red, all other proteins are there. We do not know that this green protein binds to this blue protein. So we want to see whether something binds to this target protein and if it is, what is that other protein. So you have the cell lysate, you add your antibody, it binds to it like so, and then you add your beads, so you have this capture complex. So the beads antibody protein blue and then this green protein is bound to this blue protein. So now you can wash and inute or you can spin and wash. You can use all these different methods so that the rest of the proteins are gone and you are left with this complex and then you can run it. So denature everything. If you denature it, this will all come off and you can run it on a gel and then you can detect this protein blue and this protein green using specific antibodies raised against them.

If you do not know what protein green is, you can cut out the band that is there and send it for mass spec analysis. There are various methods by which you can identify the proteins that are binding to your target protein, which is this protein blue. Immunohistochemistry assay, so it is an immunological technique for detecting the presence of specific proteins within tissue sections using antibodies. So far, what we have done is we are looking at cells, breaking them up, and examining the cell lysate. These are more in vitro experiments, but what if you want to see things in a more biological environment, for example, a tissue section.

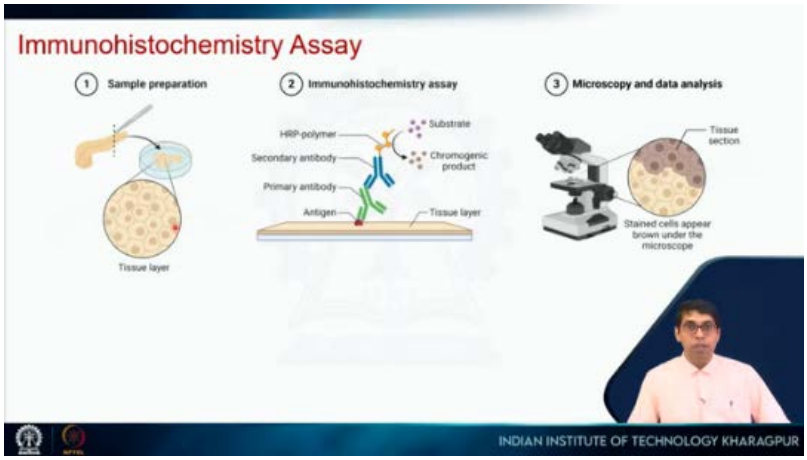
In that case, immunohistochemical assays are very useful. The principle is, again, a primary antibody binds specifically to a target antigen inside the tissue. The antigen-antibody

complex is visualized using an enzyme-conjugated secondary antibody or a fluorophore that generates a detectable signal. So your detection can be colorimetric or it can be a fluorescent signal. The resulting signal reveals the distribution and localization of the protein of interest within the tissue.

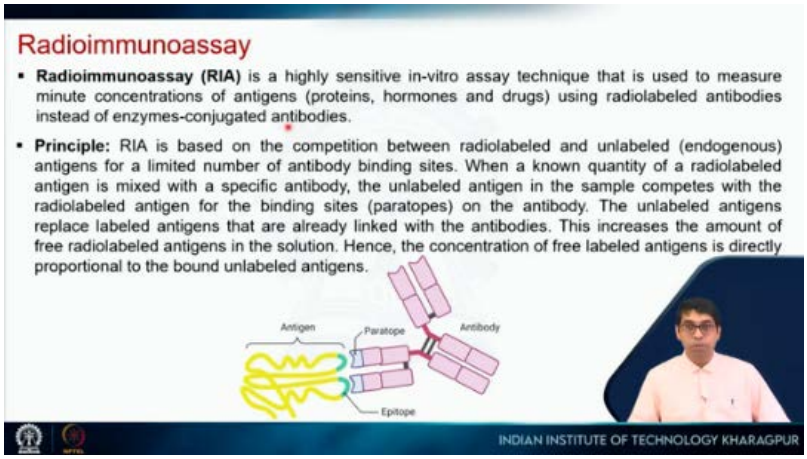
If you have a mixture of cells, you can see which cell types contain your protein of interest, or within a cell, you can use confocal microscopy to see whether it is localized in a particular organelle inside the cell. For example, whether it is in the nucleus, the cytoplasm, or the mitochondria, you can do that. So again, the applications are very similar. You can study dynamic changes in protein expression profiles.



You can detect biomarkers for various diseases and disorders. You can use it for high-throughput screening of drugs, and you can study interactions between different cell populations. So, this is how it looks. So, let us see: you have this organ, and then there are instruments like a microtome, which can be used to create very thin slices of this tissue. So, this is your tissue layer, and then you go through the same steps: add primary antibody, there is a secondary antibody which binds to the primary antibody, and then you can use chemiluminescence or this secondary antibody can have a fluorescent molecule attached to it, so you can see it as fluorescence or you can see it as chemiluminescence and then you see it under a microscope.

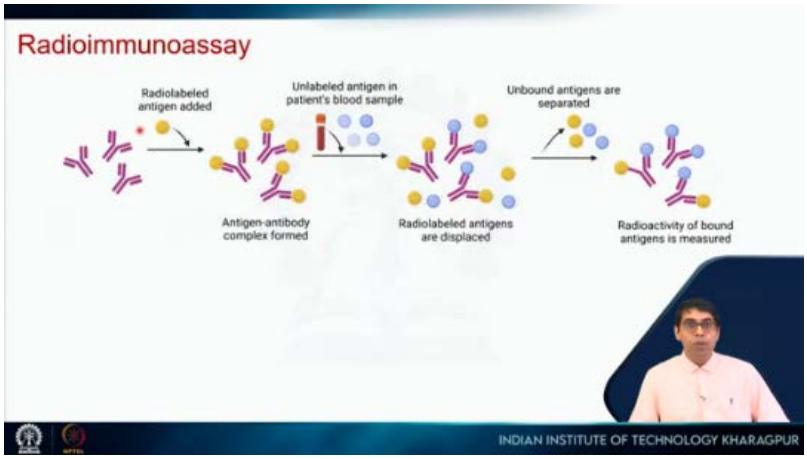


So, there is something that is very similar to this also. In this case, radioimmunoassay or RIA is again a highly sensitive in vitro assay technique that is used to measure minute concentrations of antigen. So, in this case, we are using radiolabeled antibodies instead of enzyme-conjugated antibodies, and since we are using radiolabeled antibodies, the signal you can detect is very low. So, the sensitivity is very high. So, it is based on the competition between radiolabeled and unlabeled antigens for a limited number of antibody binding sites.



So, when a known quantity of radiolabeled antigen is mixed with a specific antibody, the unlabeled antigen in the sample competes with the radiolabeled antigen for the binding sites or the paratopes. So, the paratopes are shown here on the antibody. The unlabeled antigens replace the labeled antigens that are already linked with the antibodies. This increases the amount of free radiolabeled antigens in the solution, and you measure those free radiolabeled antigens, and from there, you get some amount so you can quantify it.

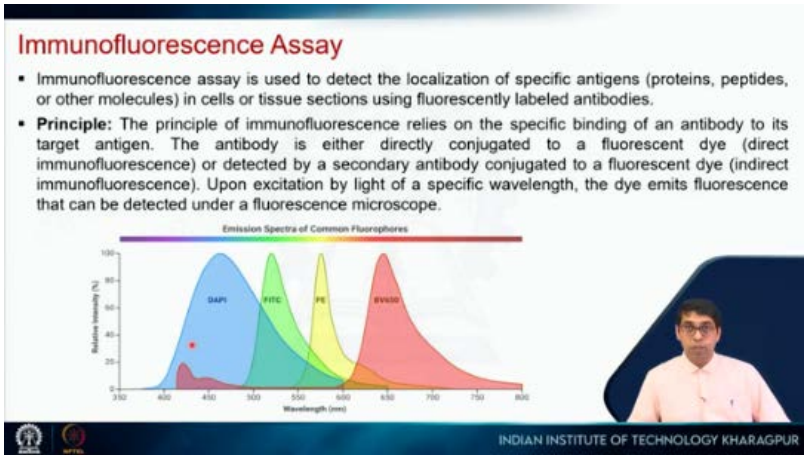
Here is your antibody; radio-labeled antigens are added, and you have the complex. Now, you add your sample, which has the unlabeled antigen. So, this unlabeled antigen will replace these radio-labeled antigens. Now, you have these free radio-labeled antigens.



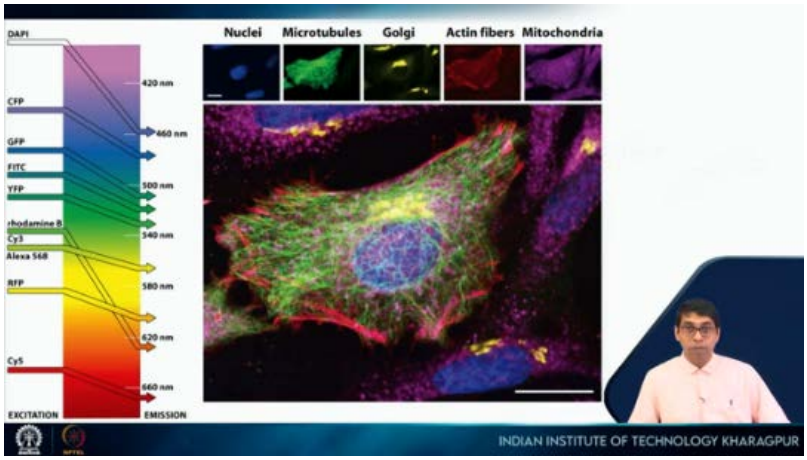
So, you quantify those, and from there, you get a measure of how much unlabeled antigen is present because this free antigen will be directly proportional to the unlabeled antigen that is present in the actual patient sample. So again, it can be used to study protein localization and trafficking, visualize cellular organelles such as mitochondria and nuclei, detect immune markers on cells, diagnose different diseases like autoimmune diseases, identify cancer-specific markers in tissues or cells, study the expression and localization of tumors, etc.

So, the last one is the immunofluorescence assay, and this is something that we have already seen. So, in this case, the antibody is conjugated to the specific fluorescent label and there are all these different fluorescent labels like DAPI, FITC, PE, and this one. You

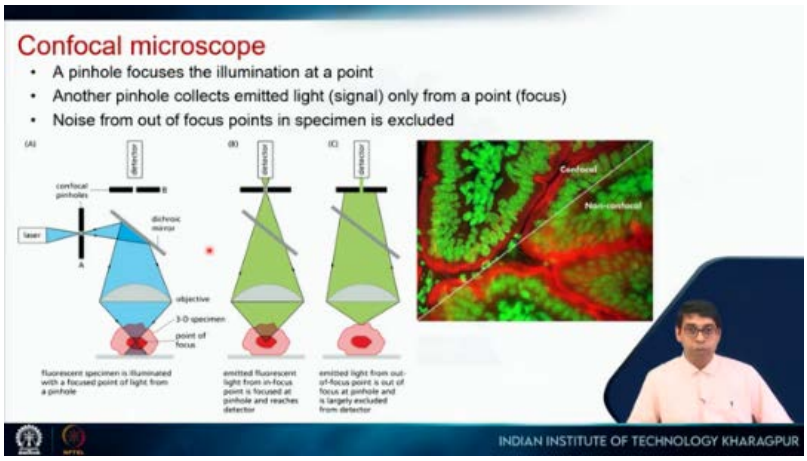
can see they are different colors, which means that they fluoresce at different wavelengths. So, we can use a combination of these different markers to look at different things at the same time. So, that is the advantage of the immunofluorescence assay. Again, you will have your cell or tissue samples, and then you will just add your antibody and look at it under a microscope. So, we have already seen that we are using this antigen-antibody-specific interaction. There are all these different fluorescent molecules, so the excitation wavelengths are shown on the left, and the detection emission wavelengths are shown on the right.



So, for the same sample, we can use different detections. So, something that looks at the nuclei, something that binds to the microtubules, which are then seen using green fluorescence; Golgi seen using yellow, actin fibers seen using red, and mitochondria seen using magenta. So, the same cell you are looking at has these different components, using different antibodies conjugated to different fluorescent dyes and you can combine these images to get an image like this, which shows the localization of all these different molecules inside the cell. You can see the actin is mostly on the cell membranes, microtubules are spread throughout, that's the nucleus, and mitochondria are also distributed throughout the cell.



So, to get an image like this, of course, you have to use confocal microscopy. So, this is something I have already discussed in an earlier lecture.



So, you can go through these books and use them as a reference for the topics I have covered in this lecture. Thank you.