Oral Biology
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Lecture -19
Flow Cytometry in Cell and Molecular Biology

Welcome to today's lecture on another interesting topic Flow Cytometry in Cell and Molecular Biology.

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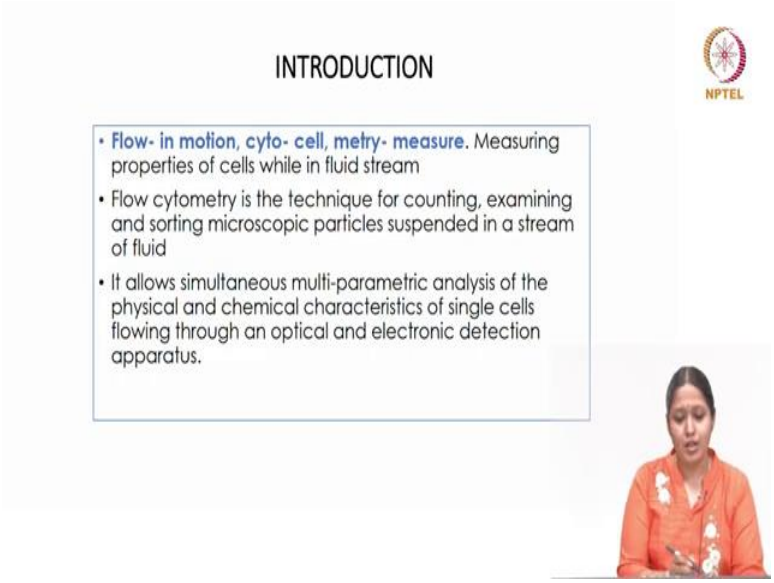

So, flow means in motion, cyto means cell, metry means measure. So, measuring cells that are in motion or that are flowing that is known as flow cytometry. Flow cytometry measures properties of cells while in fluid stream. So, flow cytometry is a technique for counting, examining and sorting microscopic particles suspended in a stream of fluid. It allows simultaneous multi-parametric analysis of the physical as well as the chemical characteristics of single cells flowing through an optical and electron detection apparatus.

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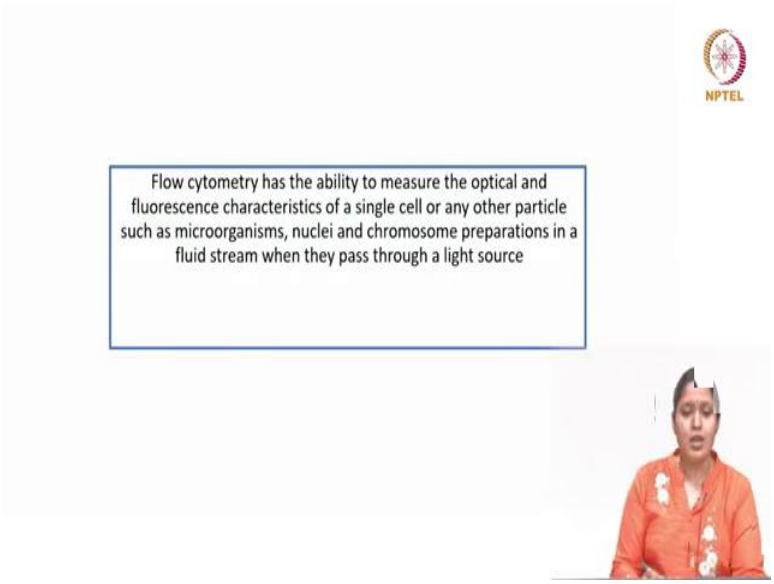

So, this flow cytometry procedure it has got the ability to measure the optical as well as fluorescence characteristics of a single cell or any other particle such as microorganisms, nuclei and chromosome preparations in a fluid stream when they pass through a light source. So, this procedure has got the ability to measure the optical as well as fluorescence characteristics of a single cell or it can also measure other particles such as microorganisms, nuclei as well as chromosomal preparations.

While these particles will be flowing through a fluid stream and light source will pass on to through these particles.

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First impedance based flow cytometry was discovered in the year 1953, it was discovered by Wallace H Coulter, for this reason this flow cytometer is also known as Coulter counter. Mack Fulwyler was the inventor of forerunner to today's flow cytometer that is called as cell sorter. First fluorescence activated cell sorter instrument was introduced from Becton Dickinson in the year 1974. So, this is a brief history about flow cytometry.

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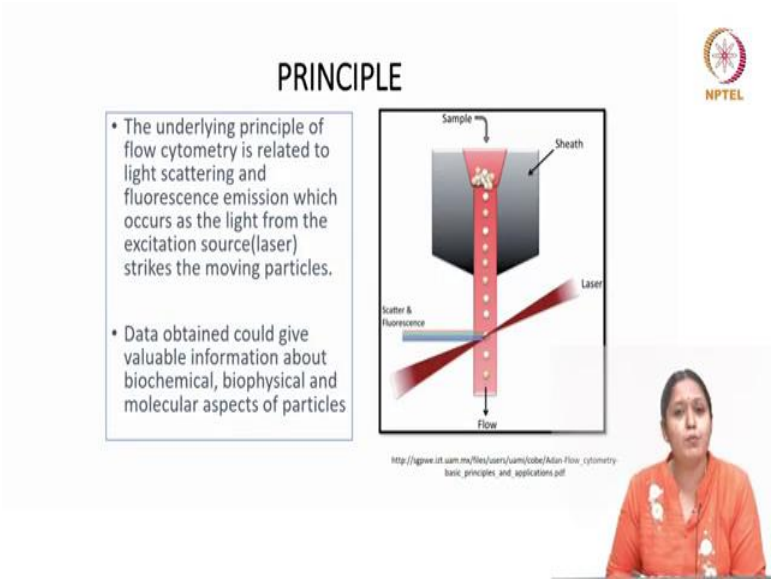

Now, let us come to the principle, the main principle of flow cytometry. The underlying principle of flow cytometry is related to the property of light scattering as well as fluorescence emission. So, you should remember light scattering and fluorescence emission and this occurs as a light from the excitation source. Mostly laser is used, it strikes the moving particles.

So, the main principle of flow cytometer is related to the light scattering as well as fluorescence emission. Data obtained could give valuable information about biochemical, biophysical as well as the molecular aspects of the particles. So, this picture clearly explains the principle of the flow cytometer where you can see, this is the central portion where the sample is sent through inside or, you can see the cells are passing through down and this is the sheath.

And as the cells move down what happens is laser or from the excitation source mostly laser will pass through these cells and it causes scatter and fluorescence and this is measured and thus flow cytometer works. This is a basic working principle.

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So, this light scattering is directly related to the structural as well as morphological properties of the cell. Fluorescence emission is directly proportional to the amount of fluorescent probe that is bound to the cell or cellular component. We use fluorescent probes which are bound to a cell and this helps in the property of fluorescence

, it will emit fluorescence.

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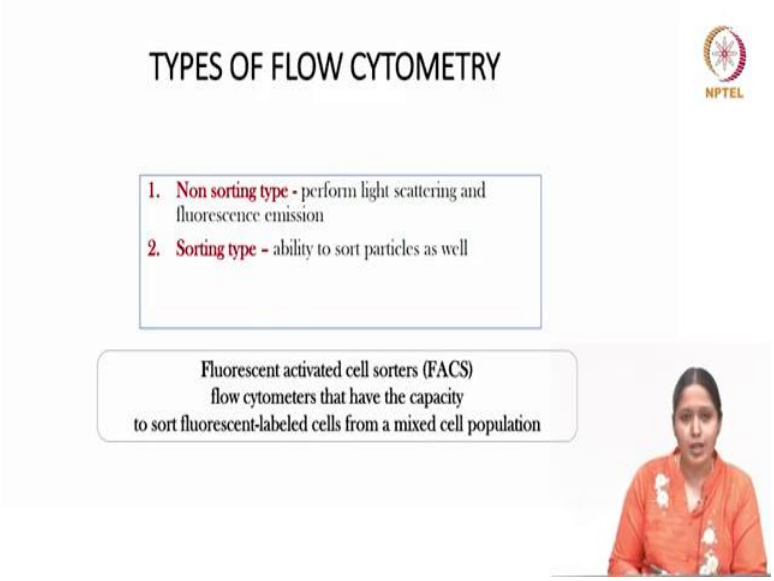

There are different types of flow cytometers or flow cytometry first type is non sorting type and other is the sorting type. In non sorting type if they perform light scattering as well as fluorescence emission, sorting type has the ability to sort particles as well. So, Fluorescent Activated Cell Sorters that is FACS, they have got the ability to sort fluorescent labeled cells from a mixed cell population that is FACS fluorescent activator cell sorters.

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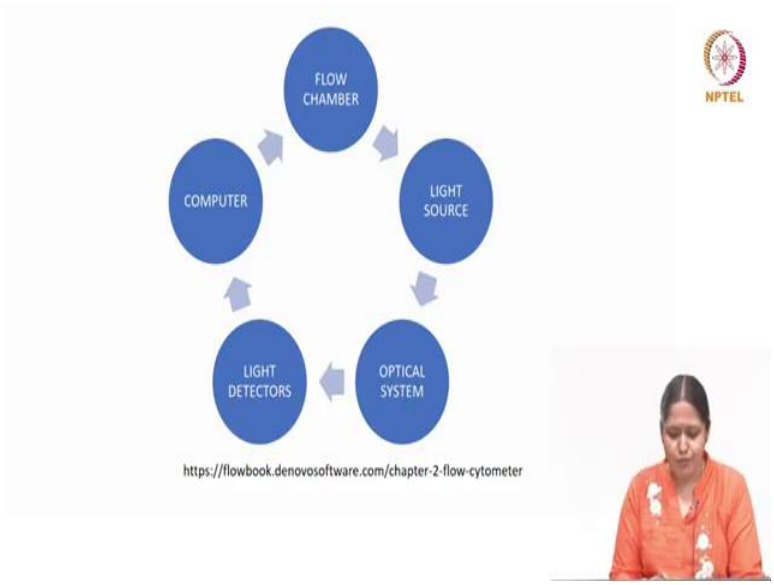

So, brief flow chart which shows what are all the parts or main components involved in the flow cytometer, they include flow chamber, light source, optical system, light detectors and computer. Thus, this picture shows a simple diagrammatical representation of the major components of flow cytometer. Now, we will be describing each and everything in detail.

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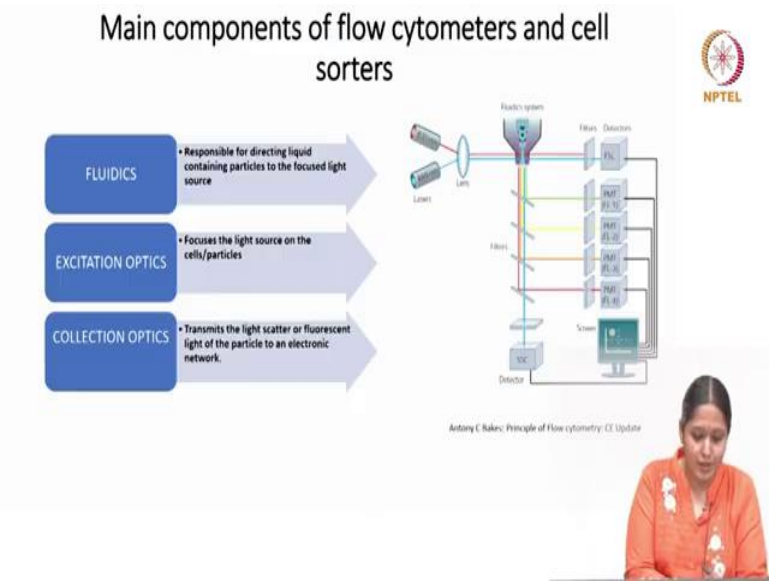

Main components of flow cytometers and cell sorters are first one is fluidics. So, fluidics are responsible for directing liquid containing particles to the focused light source. So, the as I mentioned in the previous picture there will be central fluidic system, where the fluid will be passing through it and the cells which we are going to examine will move through that.

So, the fluidics will be responsible for directing the liquid containing particles to the focused light. Next comes the optic system which includes excitation optics as well as collection optics, excitation optics focuses the light source on the cells or particles. So, mostly excitation optics will be laser which we use in flow cytometric procedure. Collection optics it transmits the light scatter or fluorescent light of the particle to an electronic network, that is the function of collection optics.

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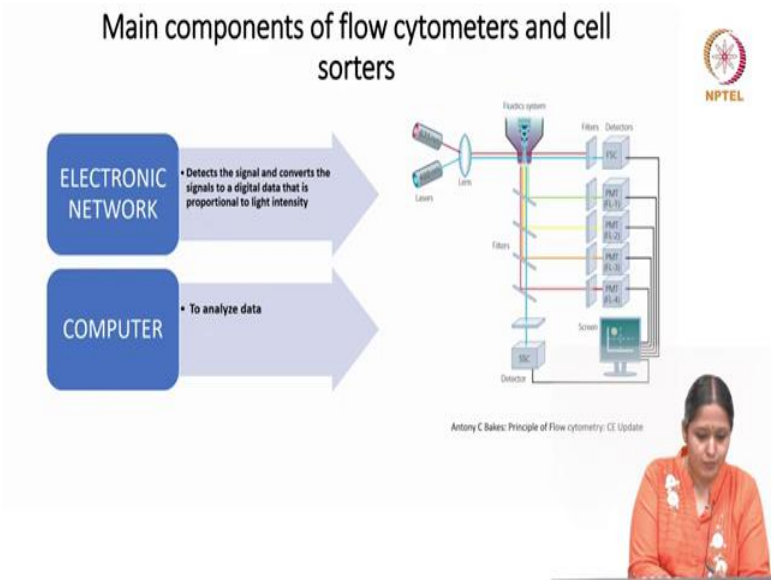

And the next component is electronic network it detects the signal and converts the signal to a digital data that is proportional to the light intensity. So, the signal which is received after light scattering, the it is received by electronic network and that signal is converted to a digital data and this is proportional to the light intensity. And finally, for the data analysis we use computer. These are all the major components of flow cytometer.

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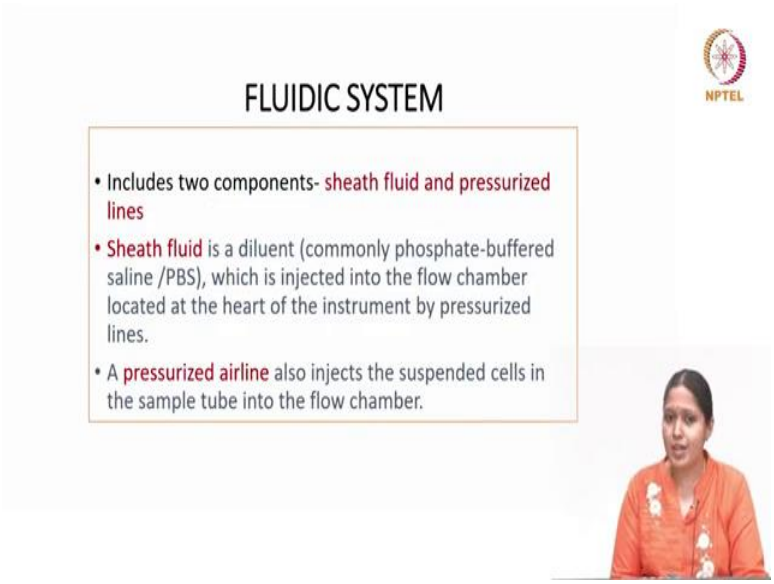

So, this is the flow cytometer which we use in our institution that is BD FACSlyric flow cytometer just a picture to show you. First comes the fluidic system. So, fluidic system it includes two components mainly sheath fluid and pressurized lines. Sheath fluid is nothing but a diluent commonly phosphate buffered saline and it is injected into the flow chamber located at the heart of the instrument by pressurized lines.

A pressurized air line also injects the suspended cells in the sample tube into the flow chamber. So, mainly fluidic system consists of sheath fluid as well as pressurized lines.

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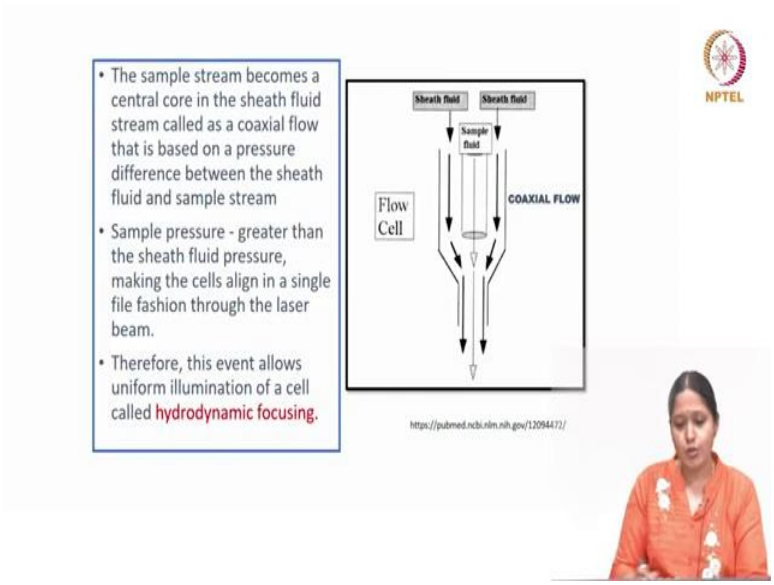

The sample stream, in this picture you can see everything in detail, the sample stream it becomes a central core ok. The sample stream will become the central core in the sheath fluid it will pass through down and that movement is called as coaxial flow and this coaxial flow is based on the pressure difference between the sheath fluid as well as the sample stream.

So, always there exists a pressure difference between this outside sheath fluid which is nothing but phosphate buffered saline as well as the sample fluid and the movement of fluid the sample stream, that movement of fluid is known as coaxial flow. So, the sample pressure is always greater than the sheath fluid pressure.

So, always remember this, the sample pressure is always greater than the sheath fluid pressure this will make the cells align in a single fashion, single lined fashion through the laser beam. So, cells will go in a single lined fashion one by one because of this pressure difference because sample fluid pressure is more as compared to the sheath fluid pressure.

So, this event will allow uniform illumination of the cell. So, as a result of aligning in a single file fashion what happens when the light from the excitation source hits these particles it allows uniform illumination of a cell and this property is known as hydrodynamic focusing. So, this property is known as hydrodynamic focusing. This is about fluidic system.

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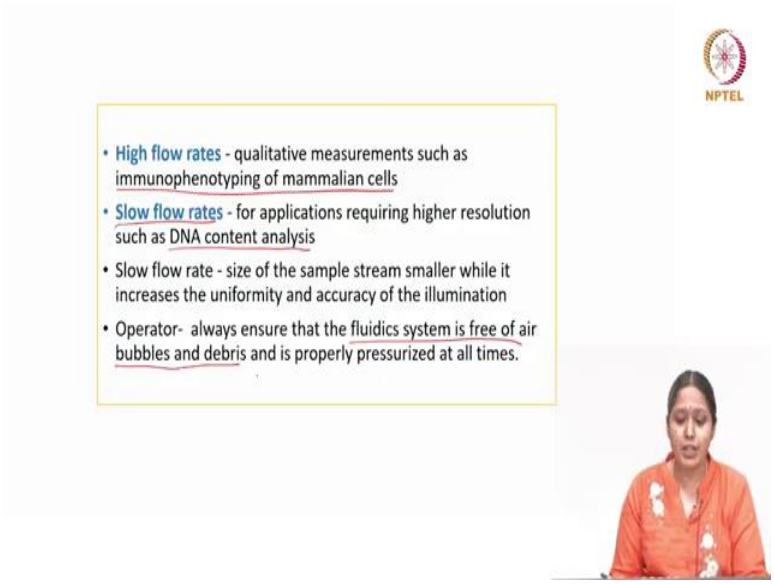

High flow rates are mainly used for the qualitative measurements such as immunophenotyping of mammalian cells. So, qualitative measurements like immunophenotyping of mammalian cells require high flow rate whereas, slow flow rates are used for applications that require higher resolution such as DNA content analysis.

So, that will require slow flow rate whereas, immunophenotyping of mammalian cells it will require high flow rate, slow flow rate depends upon the size of the sample stream. If the size of the sample stream is smaller, it can increase the uniformity as well as accuracy of the illumination. So, slow flow rate if in case, in that case if the size of the sample fluid is smaller it can increase the uniformity as well as the accuracy of the illumination.

So, operator while operating this flow cytometer and mainly while operating this fluidic system, operator should always ensure that the fluidic system is free of air bubbles and debris ok. It should be always free of air bubbles and debris and it should be properly pressurized at all times.

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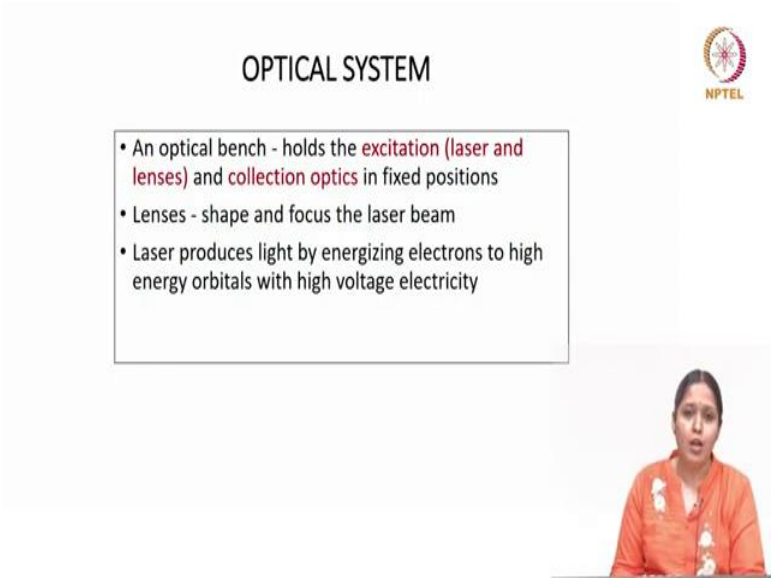

So, just now we have discussed about one of the major components of a flow cytometer that is fluidic system, next moving on to the optical system. So, optical system mainly it consists of an optical bench that holds the excitation optics as well as the collection optics. So, what is the excitation optics that we use in flow cytometer? It includes laser

and lenses. So, laser and lenses are the excitation optics that we use in flow cytometer and then comes the collection optics.

So, these optics are in a fixed position in an optical bench, lenses consist of the shape it will mainly shape and focus the laser beam. So, the main function of the lens is to shape and focus the laser beam. The laser produces light by energizing electrons to higher energy orbitals with high voltage electricity.

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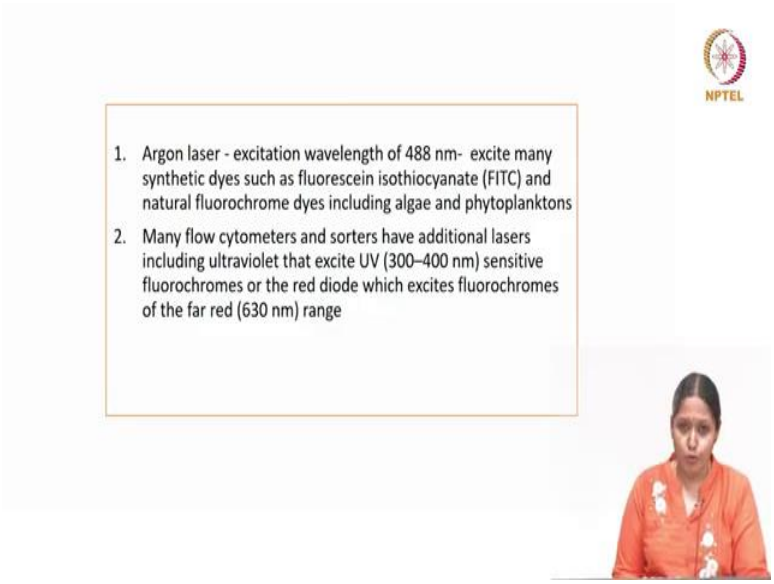

So, we use different type of lasers in flow cytometer, one such laser is Argon laser. Argon laser it has got an excitation wavelength of 488 nanometer, argon laser it will excite many synthetic dyes such as fluorescein isothiocyanate and natural fluorochrome dyes such as algae as well as phytoplanktons. And many flow cytometers and sorters have an additional laser which includes ultraviolet rate that excite UV at the wavelength of 300 to 400 nanometer.

They will excite UV sensitive fluorochromes or the red diode which excites fluorochromes of the far-red range that is 630 nanometer range.

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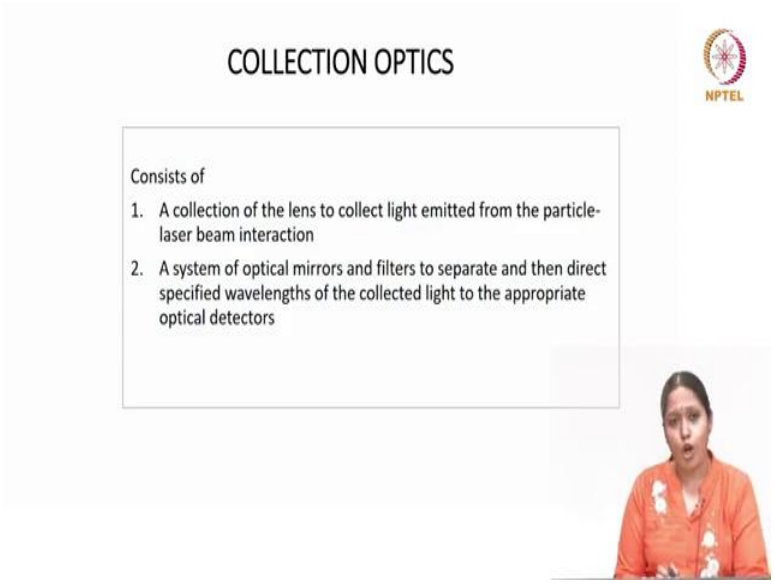

So, as I mentioned optical system consists of excitation optics and collection optics, Excitation optics mainly include lasers as well as lenses. Now, let us see what are the collection optics. So, collection optics consist of collection of lenses that are used to collect the light emitted from the particle laser beam interaction. So, after the particle and the laser beam interaction light will come right, that light will be collected by the collection optics.

So, a system of optical mirrors and filters constitute the collection optics. So, a system of optical mirrors and filters to separate and then direct the specified wavelength of the collected light to the appropriate optical detectors.

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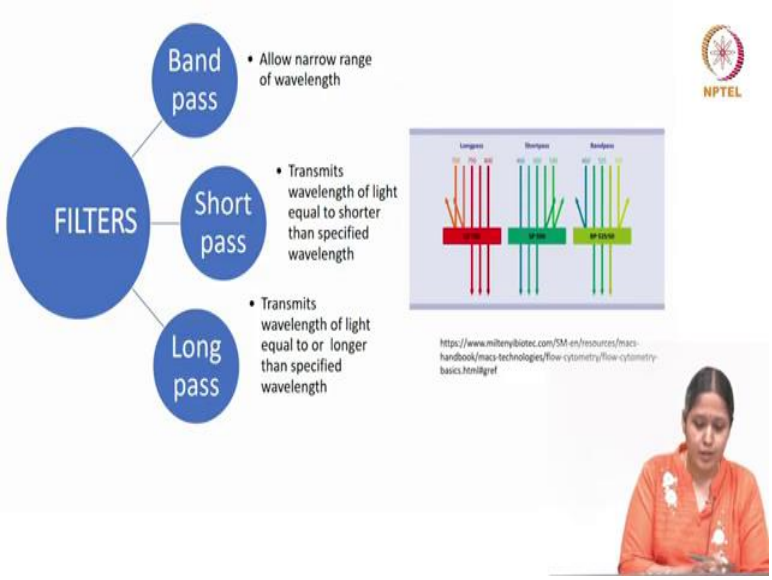

So, what are the different types of filters that we use in flow cytometer. So, the different types of filters are band pass filters, short pass filters and long pass filters. So, the different types of filters are band pass, short pass and long pass filter. So, what is band pass filter? It will allow narrow range of wavelength of light to pass through.

So, in this picture you can see band pass filter. So, in this band pass filter, what type of light is allowed/it will allow? It will allow narrow range of wavelength, narrow range of wavelength of light is passed through band pass filter. Next comes the short pass filter. So, short pass filter transmits wavelength of light that is equal to or shorter than the specified wavelength.

So, here in short pass filter you can see it will transmit wavelength of light that is equal to or shorter than the specified wavelength. So, what is the specified wavelength here in short pass? It is 500 see it its passing wavelength of light which is less than 500 nanometers, that is 460 nanometers. So, those filters which allow or transmits wavelength of light which is less than equal to or less than the specified wavelength that is known as or those types of filters are called short pass filters.

Next type of filter is long pass filter: so, long pass filter will transmit wavelength of light that is equal to or greater than the specified wavelength. So, in this picture you can see long pass filter, in long pass filter the specified wavelength is 750 nanometers, but see

what this long pass filter will do it will transmit light that is greater than the 750 nanometers, see its passing greater than 750 nanometer wavelengths of light.

So, this is known as long pass filter. So, filters which we used in flow cytometers are band pass filter, short pass and long pass filter.

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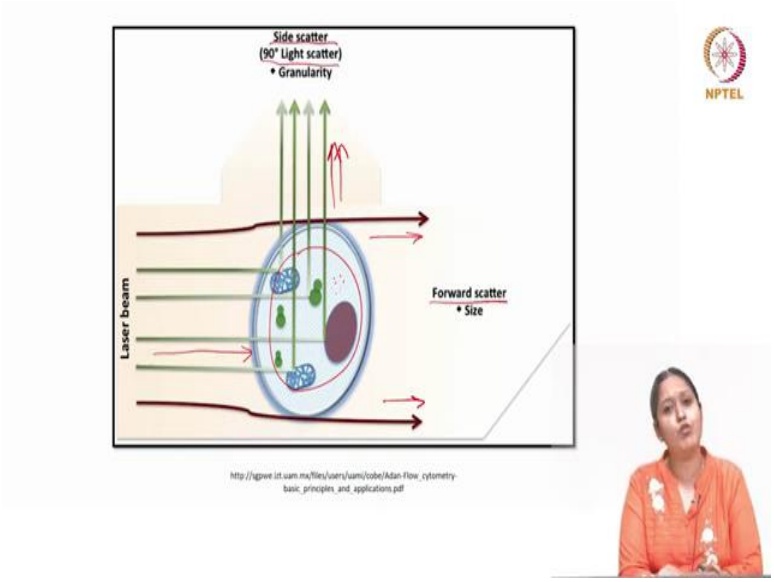

So, before going to the next component we should know about a small principle that is the principle which is used in the main principle which is used in the flow cytometry you should know about two types of scattering as I explained earlier, through the central fluidic stream, cells will pass through.

It will form a coaxial flow as a result of the fluid pressure cells will go inside and as a result of fluid pressure each and every cell will go in a single lined fashion it will undergo hydrodynamic focusing a light will pass through the from the excitation optics mostly the laser, light will pass through the cell. And what happens while passing through the cell?

While passing through the cell light will scatter right. So, there are two types of scattering, forward scatter and side scatter ok. So, as from the excitation optics as the laser beam excite from the any other light from the excitation optics, strikes the cell of interest which you are going to examine in flow cytometer it will undergo scattering.

There are two types of scattering forward scatter as well as side scatter. So, this forward scatter here you can see this is a forward scatter it depends upon the size of the cell which you are going to examine, smaller the size of the cell, lesser will be the forward scatter, if larger the size of the cell, then forward scatter will be more ok. So, forward scatter depends upon the size of the cell.

But, see the side scatter side scatter is nothing but 90-degree light scatter ok. So, 90-degree light scatter. So, side scatter, it depends upon structural complexity of the cell as well as the granularity inside the cell ok. So, side scatter as the laser beam passes, it enters strikes the cell the structural complexity inside the cell as well as the granules will determine how much will be the side scatter, how much the light will scatter from the cell towards side at 90-degree angle ok.

So, you should always remember forward scatter and side scatter which is an important parameter these both are the important parameters for analyzing the data which we are going to obtain after the flow cytometric procedure.

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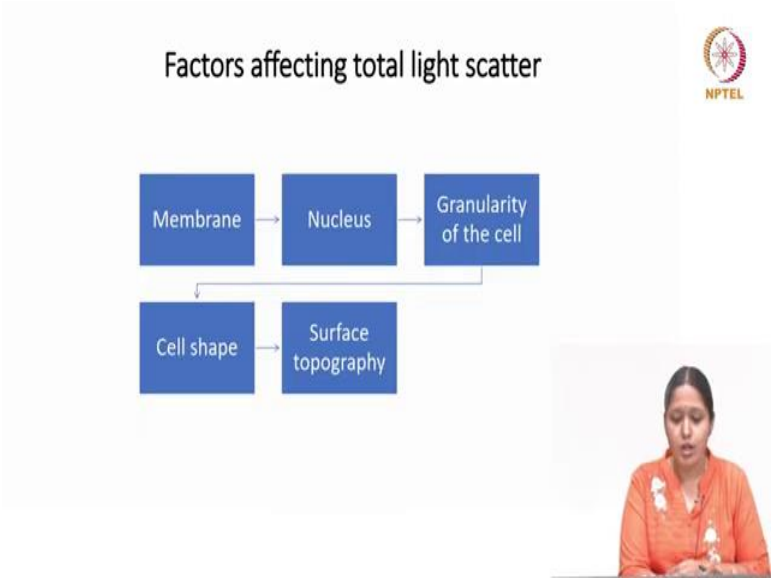

So, what are the factors that affect the total light scatter? So, total light scatter is affected by various factors like membrane, nucleus of the cell, granularity of the cell, surface topography as well as the cell shape. So, these five factors will affect the total light scatter.

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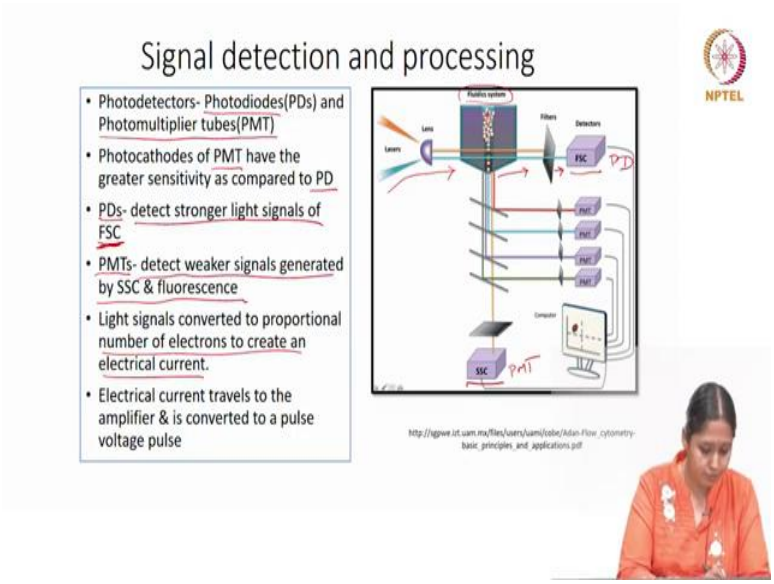

So, we have seen various types of components of the flow cytometer, we have seen the fluidic system we have seen the excitation optics, collection optics, various types of lenses and filters we have seen now we are going to see that signal detection and processing. So, after the light from the excitation optics strike the cell what happens you will get a forward and side scattering.

So, next step is signal detection and processing. So, signal detection and processing are done by means of photo detectors. So, photo detectors they are of two types, photo diodes and photo multiplier tubes. So, these photo diodes and photo multiplier tubes plays an important role in signal detection and processing. Photocathodes of photomultiplier tube have a greater sensitivity as compared to photodiode.

So, photo multiplier tubes, has got greater sensitivity when compared to photodiode. Photodiode has got an ability to detect stronger light signals of forward scatter ok. So, the forward scatter stronger light signals are mainly detected by photodiodes whereas, the photomultiplier tubes mainly detect weaker signals that are generated by side scatter and fluorescence.

So, you should always remember this point photodiode helps in identifying stronger intensity of light signals that is formed because of the forward scatter whereas, the photo multiplier tube has the ability to detect weaker signals generated by side scatter and

fluorescence. So, what happens these light signals are converted to proportional number of electrons to create an electrical current.

So, the light signals are converted to electrical current, this electrical current will travel through the amplifier and it is converted to a pulse voltage pulse. So, this picture, just this picture shows everything in nutshell you can see fluidic system, cells passing through it then it will align a single line fashion from laser passing through lens. Lens helps in focusing the light, it will hit the cell after hitting the cell it will undergo scattering.

It will go through the filters after the scattering it will go through the filters and what happens? It will be detected by signal detection and processing has to be done right it will go to photo detector and photo multiplier tube. So, those detected by forward scatter will be detected by photodiode and those detected by side scatter will be detected by photo multiplier tube ok. So, forward scattered by photodiode and side scatter by photo multiplier tube.

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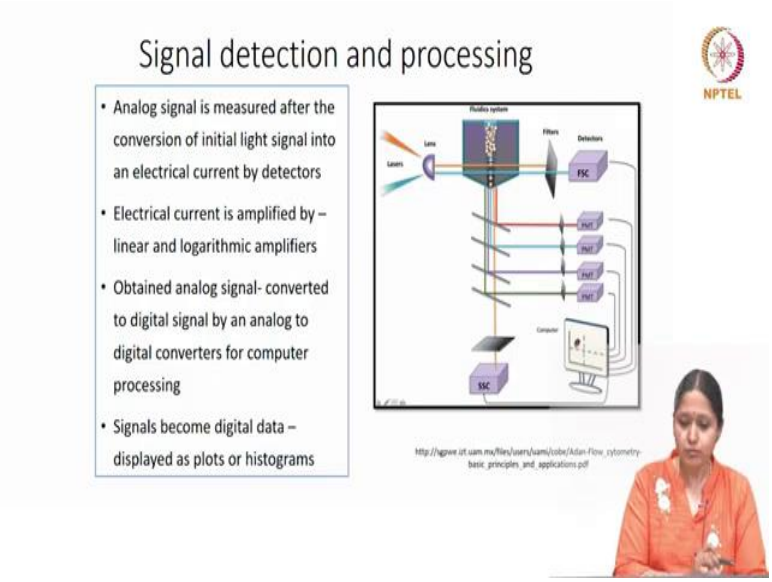

So, after the signal detection it has to be processed right. So, analog signal is measured after the conversion of initial light signal into an electrical current by detectors. So, electrical current is amplified by amplifiers, there will be amplifiers linear as well as logarithmic amplifiers. So, the obtained analog signal will be converted to digital signal

by an analog to digital converters. So, we will receive an analog signal it has to be converted to a digital signal.

So, this analog signal is converted to digital signal by means of analog to digital converters. So, only if it is converted to digital signal, it can be processed by means of by using computer signals will become digital data ok. So, after converting the analog to digital signal it will become a digital data and we will see the result and computer has plots or histograms which will be discussing later ok. So, this is about signal detection and processing.

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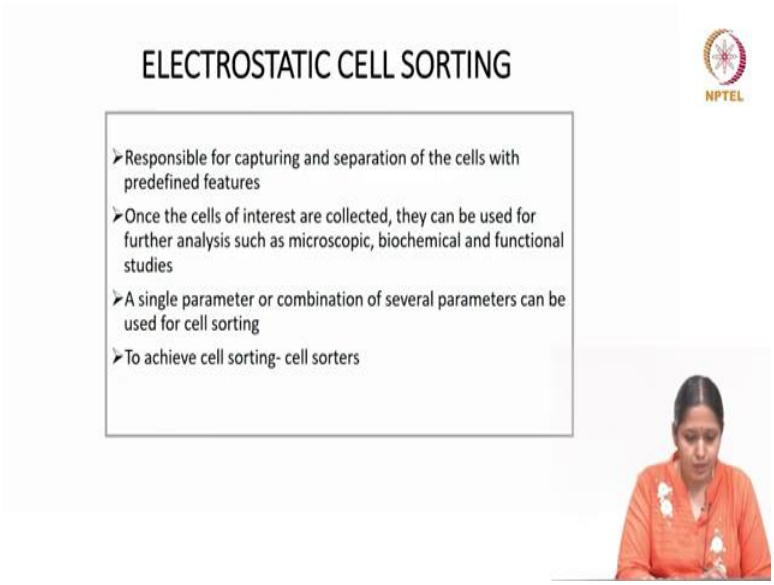

Now, another important thing which you should know about cell sorters is electrostatic cell sorting. So, what do you mean by electrostatic cell sorting? They are responsible for capturing and separation of cells with the predefined features. Once the cells of interest are collected, they can be used for further analysis such as microscopic, biochemical as well as functional studies. So, this electrostatic cell sorting is it mainly helps in a single parameter or a combination of several parameters ok.

So, a single parameter or a combination of several parameters can be used for electrostatic cell sorting. So, for doing this electrostatic cell sorting we have cell sorters. So, to achieve this cell sorting we have cell sorters.

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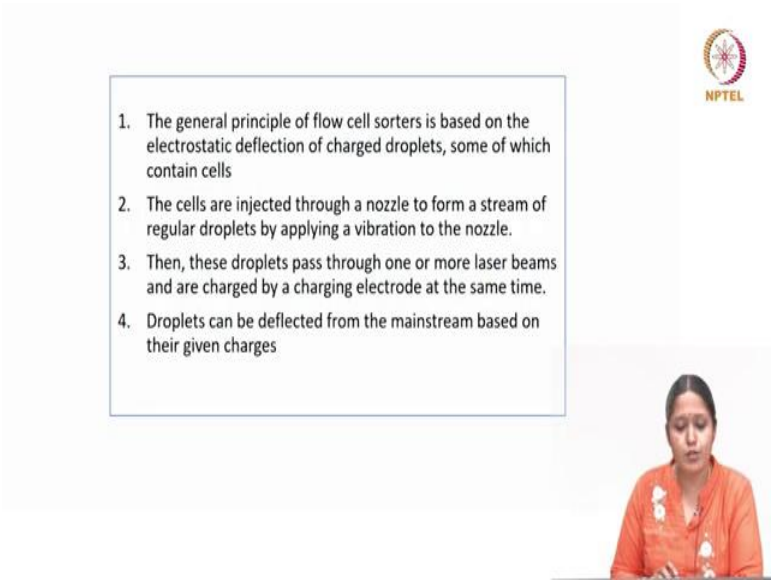

The general principle of flow cell sorters is based on the electrostatic deflection of charged droplets some of which contain cells ok. So, always it depends upon electrostatic deflection of the charged droplets. What happens is the cells are injected through a nozzle to form a stream of regular droplets by applying a vibration to the nozzle, cells are injected through a nozzle.

After that these droplets pass through one or more laser beam, after passing through the laser beam they are charged by charging electrode at the same time ok. So, laser beam will strike those particles as well as they are also charged by charging electrode. Droplets can be deflected from the mainstream based on the given charges.

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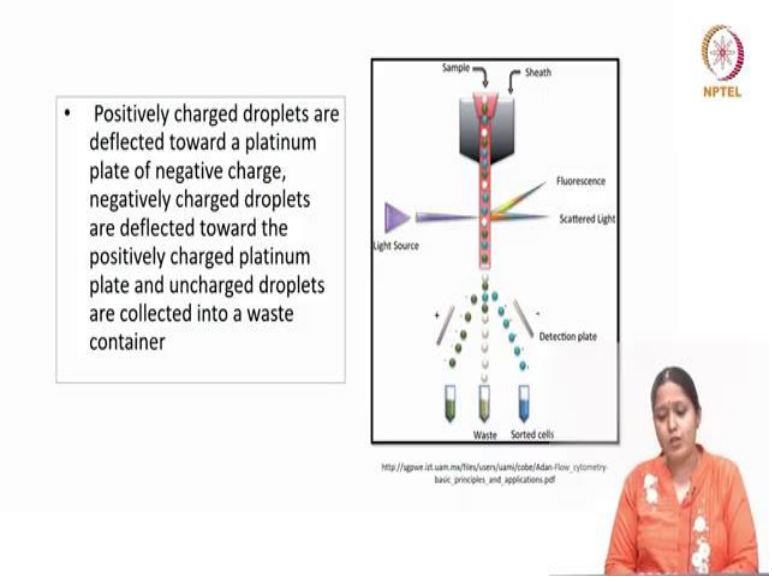

So, this can be better explained from this picture. So, electrostatic cell sorting based on the charges there we are sorting the cells right. So, what happens is positively charged droplets they are attracted towards the negatively charged plate, a platinum plate. So, you can see positively charged droplets they are deflected towards negatively charged plate and thus they are sorted out what happens to negatively charged droplets.

So, negatively charged droplets they are attracted towards positively charged platinum plate and thus they are sorted. So, positively charged droplets are attracted towards negatively charged platinum plate and negatively charged droplets are attracted towards positively charged platinum plate. And what happens to those cells or droplets which have no charge they are collected as a separate container, they are collected in a separate container as a waste ok.

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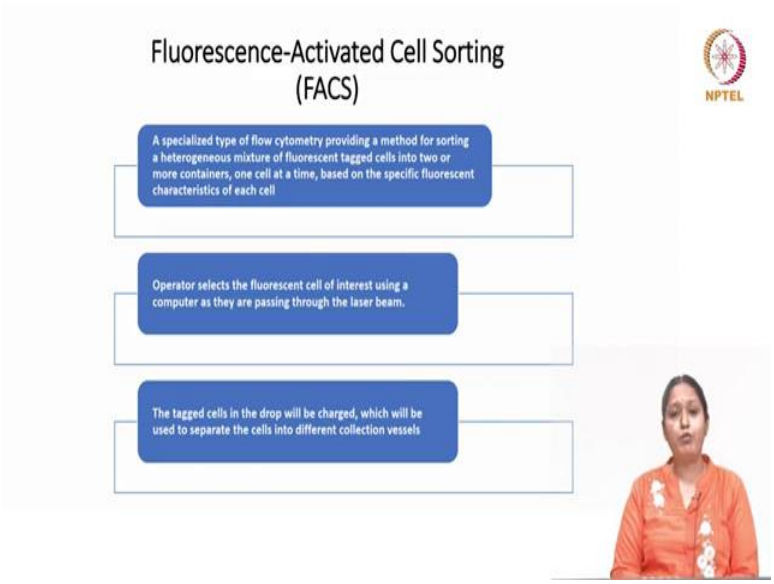

Next is the Fluorescence Activated Cell Sorting, FACS. So, it is a specialized type of flow cytometry providing a method for sorting a heterogeneous mixture of fluorescent tagged cells into two or more containers, one cell at a time, based on the specific fluorescent characteristics of a cell. Operator will select the fluorescent cell of interest using the computer as the they will pass through the laser beam ok.

The tagged cells in the drop will be charged which will be used to separate the cells into different collection vessel. So, the main function, the main advantage of this fluorescent activated cell sorter is; so, any cell which is attached to the fluorescent probe. So, we can any cell of interest which you are going to examine, the operator can select that fluorescent cell ok, as it passes through the light laser beam later on the tagged cells in the drop will be charged, charge will be given as a result there we can easily separate the cells into different collection vessels.

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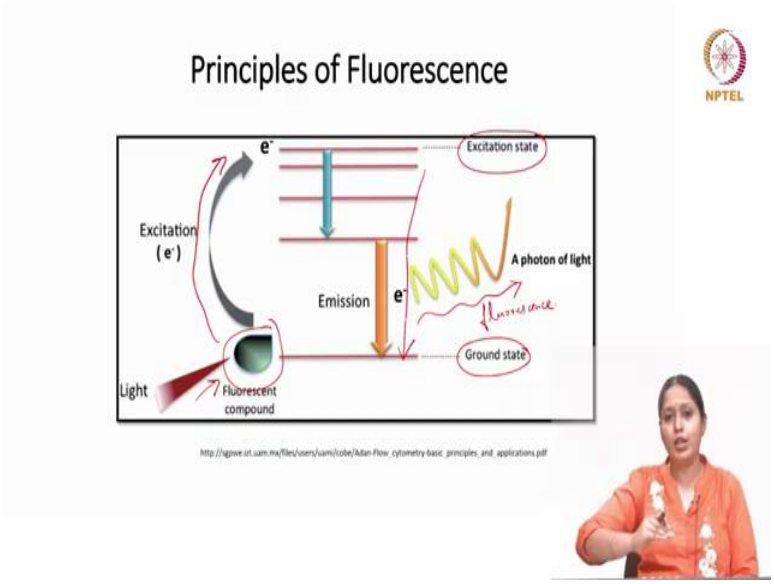

Now, what is the basic principle of fluorescence ok, fluorescence means what? Usually, a fluorescent compound it will absorb energy of any wavelength as a result the electrons in the fluorescent compound will get excited as a result of the light source it will get excited and it will go from the lower energy level or ground state to the higher energy level excitation state.

So, after the fluorescent compound get excited then electrons will excited, it will go from the ground state to the higher energy level, but what happens is those electrons they will not stay at the higher energy level that is excitation state for a longer period of time ok. They cannot stay at that stage for a longer period of time, they will immediately come back to the ground state.

So, they will not stay at the upper excited state they will immediately come back to the ground state, but while coming back to the ground state they will emit a photon of light that is called as fluorescence . So, this is the basic principle of fluorescence. So, as the fluorescent compound gets hit by a light or gets excited what happens is the electrons in the fluorescent compound will jump from the lower energy level to the higher energy level and those electrons will not stay at the higher energy level for a longer period of time, they will immediately come back to the lower energy level.

While coming back they will emit a photon of light and that is known as fluorescence. This basic principle of fluorescence is also applied in this flow cytometer.

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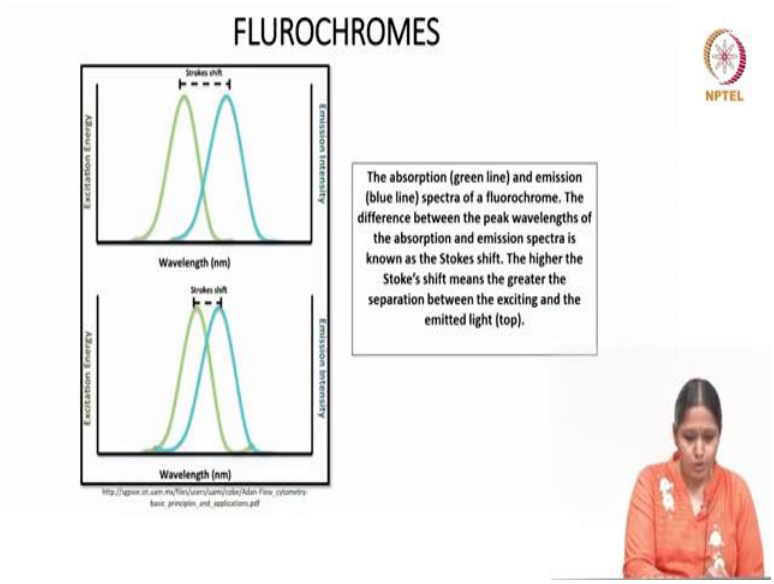

So, what are fluorochromes? So, fluorochromes are the dyes or the chemicals which we use in flow cytometer. So, what happens is the fluorochromes have two types of emission ok, it has got two types of spectra. It has got absorption spectra as well as emission spectra ok, the one which you see in the green color is called as absorption spectra and one you see in the blue color is called as emission spectra.

So, what happens is the difference between the peak wavelength between the absorption spectra and emission spectra, that difference is known as stokes shift. So, strokes shift means the difference between the peak wavelength between absorption spectra and emission spectra that is known as stokes shift.

Higher the stokes shift means greater the separation between the exciting and the emitted light ok. So, greater the stokes shift, greater will be the separation between the exciting light as well as the emitted light. So, this is a small description about stokes shift.

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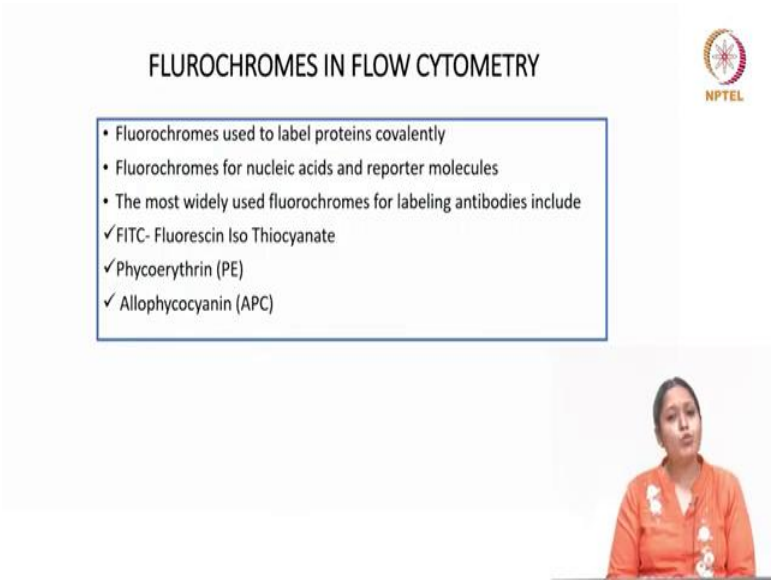

So, what are the fluorochromes that we use in flow cytometry? So, this fluorochrome has an absorption spectra and emission spectra and the difference in the peak wavelength is known as stokes shift all this I have mentioned now. So, what are the different types of fluorochromes that we use in flow cytometry? So, fluorochromes that we use in flow cytometry they are either used to label proteins covalently or they are also used for nucleic acids as well as reporter molecules.

The most widely used fluorochromes for labeling antibodies are fluorescin iso thiocyanate, phycoerythrin, allophycocyanin. So, FITC, that is Fluorescin Iso Thiocyanate, APC that is Allophycocyanin as well as PE that is Phycoerythrin, these are the different types of fluorochromes that we use in flow cytometry procedure.

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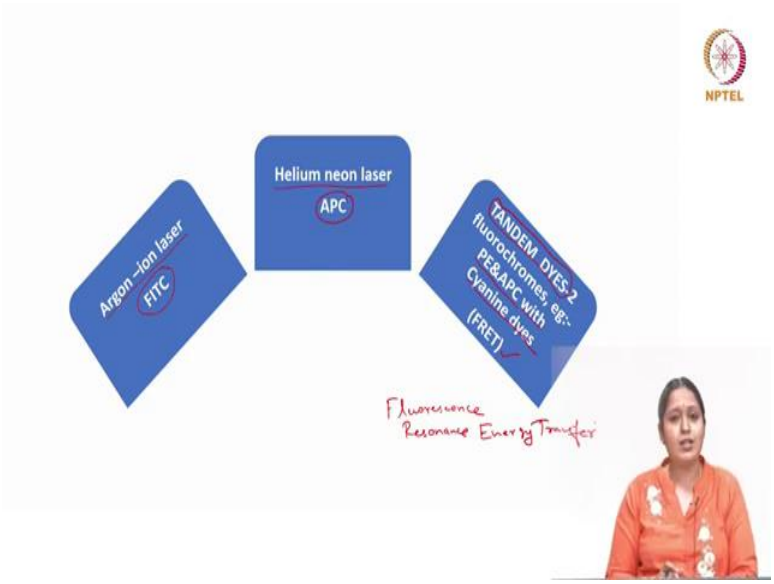

And another important thing or the important parameter that we all should remember is the laser as I mentioned before the excitation optics mostly consist of laser ok. So, what type of laser which you are going to use, it depends upon the type of fluorochrome which you are using ok.

For example, Argon-ion laser if you are using; that means, then the FITC should be the fluorochrome, fluorescin isothiocyanate should be the fluorochrome. And for allophycocyanin we use helium neon laser, and there is something called as Tandem Dyes, tandem dyes.

So, tandem dyes are nothing but we use two fluorochromes. So, two fluorochromes are used, for example, we use both phycoerythrin as well as allophycocyanin PE and APC with cyanine dyes. So, tandem dyes right, so we are using two fluorochromes along with dyes for example, phycoerythrin as well as allophycocyanin.

So, what happens is as a result of excitation one of the fluorochrome will be excited it will get excited. It will transfer the energy from the that particular fluorochrome to the adjacent fluorochrome. For example, if phycoerythrin is excited what happens is the light from the energy from the phycoerythrin will be transferred to the adjacent fluorochrome that is here what we are saying allophycocyanin. So, phycoerythrin, allophycocyanin.

So, if phycoerythrin is excited, if the allophycocyanin is adjacent to it will transfer after excitation it will transfer the energy to the adjacent allophycocyanin. So, the adjacent molecule will also get excited ok. So, this mechanism is known as FRET, FRET is also known as Fluorescence Resonance Energy Transfer. So, this is called as fluorescence resonance energy transfer.

When two fluorochromes are used with cyanine dyes or any other dye tandem dye one gets excited the other one adjacent to it the excited energy from one will be transferred to the another. This mechanism is known as fluorescence resonance energy transfer.

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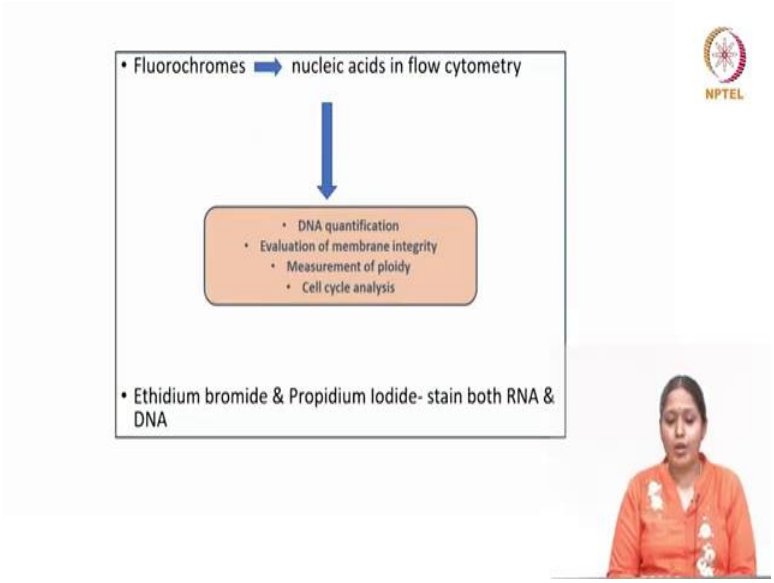

So, fluorochromes are also used to analyze nucleic acids in flow cytometry and this can be used for various applications like DNA quantification, ploidy analysis, cell cycle analysis as well as evaluation of membrane integrity. There are various chains which has got the property to stain both RNA as well as DNA and such stains are ethidium bromide and propidium bromide. So, ethidium bromide and propidium bromide has got the ability to stain both RNA and DNA and if you want to check DNA alone you have to remove RNA by means of RNase enzyme.

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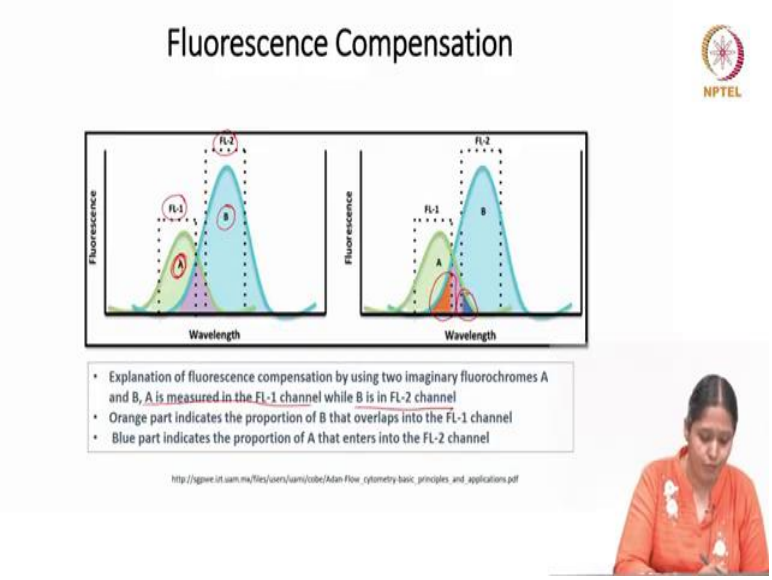

Another important property of fluorescence I mean fluorochromes that we use in flow cytometry is the fluorescence compensation. So, what happens is for example, let us see this picture there are two types of fluorochromes that we use, fluorochrome A and fluorochrome B, it has got an emission a wavelength and all. So, what happens is this fluorochrome A is detected by filter 1, fluorochrome B is detected by FL-2 or filter 2.

So, what happens is whenever some of the wavelength of light or what happens some of the wavelength of light from fluorochrome A will be detected by a filter 2, adjacent filter 2. And some from B will be detected by filter 1 and this is called as what happens is there can be an overlap this is called as a spectral overlap. So, fluorescence compensation, just see the imaginary fluorochromes A and B, A is measured in FL-1 channel and B is measured in FL-2 channel. So, what happens is in this picture you can see the orange portion.

So, orange portion it indicates the proportion of B that overlaps into filter 1. So, some portion of B, proportion of B wavelength from B will overlap or it will be detected by filter 1 and blue part here. So, this blue part, it indicates the proportion of A that enters into the FL-2 channel. So, this is called as spectral overlap. This is mainly known as fluorescence compensation and by subtracting these two values it helps in compensating this, this can be rectified by subtracting those two values.

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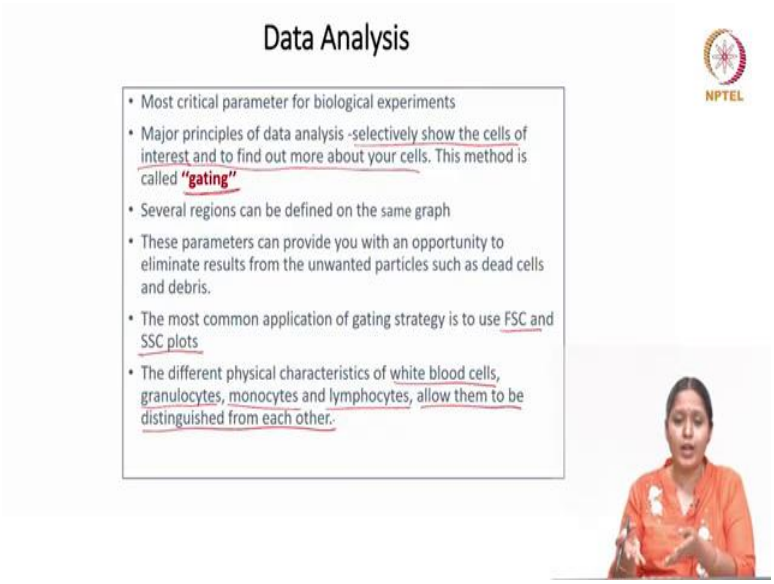

So, we have mentioned, we have seen all the basic structural main structural features of flow cytometer, fluidic system, excitation optics, collection optics, signal detection and processing then we have seen about the fluorochrome that we have used in flow cytometer and different properties of fluorochrome.

We have seen the stokes shift, fluorescence resonance energy transfer and we have seen the different types of lasers used in flow cytometer and also fluorescent compensation and spectral overlap. Now, moving on to the important part of this topic that is data analysis, it is a most critical parameter for the biological specimens. So, the major principle of data analysis is to selectively show the cells of interest because you have to know more about what type of cell you want to see right.

So, it helps to selectively show the cells of interest and to find out more about your cells. This procedure is called as gating. So, you are passing so much of cells and you will be interested in only one type of cell. So, you are going to see that one type of cell and what type of property which you are going to see. So, that procedure is called as gating.

Several regions can be defined on the same graph, these parameters can provide you with an opportunity to eliminate results from the unwanted particles such as dead cells and debris. So, only we are going to see the type the cell which you are interested or which you are going to focus. The most common application of gating strategy is to use FSC and SSC plots.

So, earlier when I mentioned about the forward scatter and side scatter, I have told you about the forward as well as the side scatter are used for the data analysis, it plays an important role in data analysis. So, for this gating strategy you are going to compare only these two parameters in the graph as forward scatter and side scatter plots. The different physical characteristics of white blood cells, granulocytes, monocytes and lymphocytes allow them to be distinguished from each other ok.

So, the different physical characteristics if you want to see a lymphocyte, you can see the lymphocyte you can see what type of property is you are going which you are interested you can see by means of this gating.

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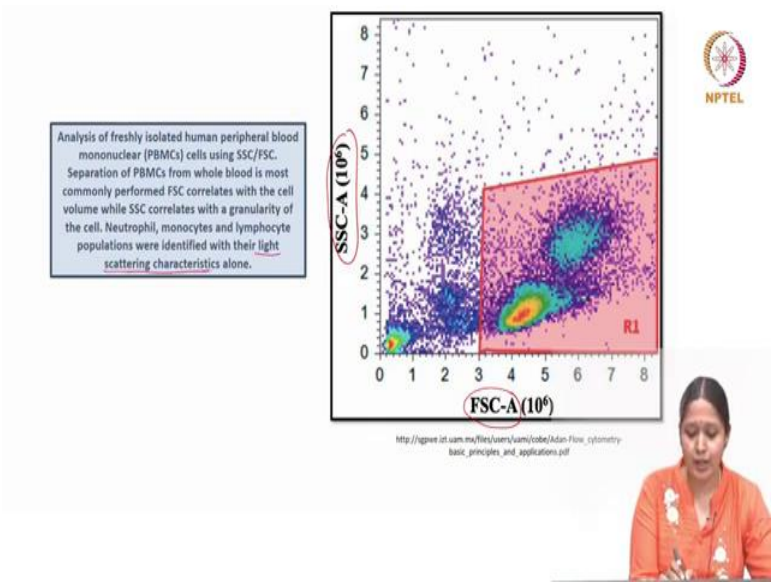

This is one picture which I have got from one article. So, in this picture just for you all to get an idea that how in a plot you are using forward scatter. So, forward scatter is there in the x axis and side scatter is there in the y axis. So, this picture it shows the analysis of freshly isolated human peripheral mononuclear cells, using the properties of forward scatter and side scatter.

So, separation of peripheral blood mononuclear cells from the whole blood is commonly performed. So, forward scatter will correlate with the cell volume and the side scatter will correlate with the granularity of the cell. So, neutrophils monocytes as well as lymphocyte populations were identified with their light scattering characteristics alone.

So, mainly these granulocytes are identified with their light scattering characteristics alone.

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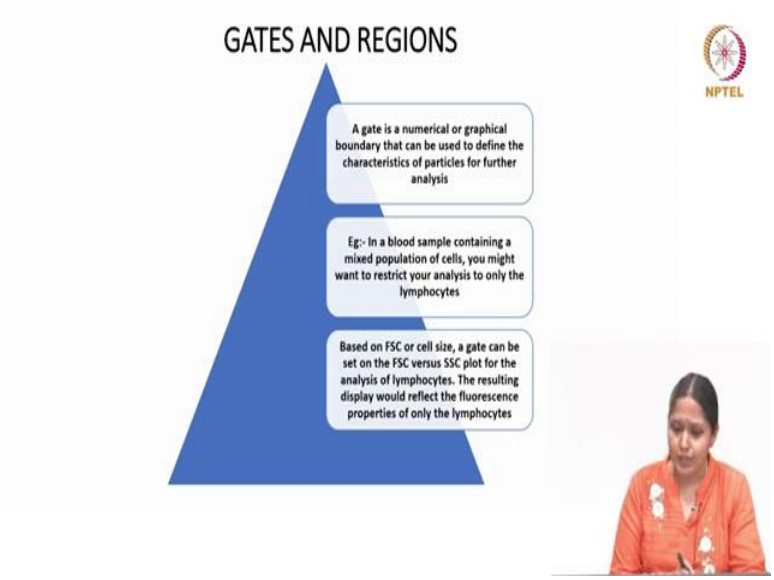

So, gates and region: what do you mean by a gate? A gate is a numerical or graphical boundary that are used to define a characteristic of particles for further analysis. For example: in a blood sample containing a mixed population of cells you want to restrict your analysis to only lymphocytes you can go ahead with that, based on the forward scatter or the cell size a gate can be set on the forward scatter as versus the side scatter plot for the analysis of lymphocyte.

So, the resulting display would reflect the fluorescent properties of only lymphocyte, only that particular region you are going to examine. If you want to see only the lymphocyte you are going to based on the forward scatter, based on the size of the lymphocyte you will adjust the forward scatter the gate can be set on the forward scatter versus the side scatter plot for the analysis of lymphocyte and you will be able to see the fluorescent properties of only lymphocytes.

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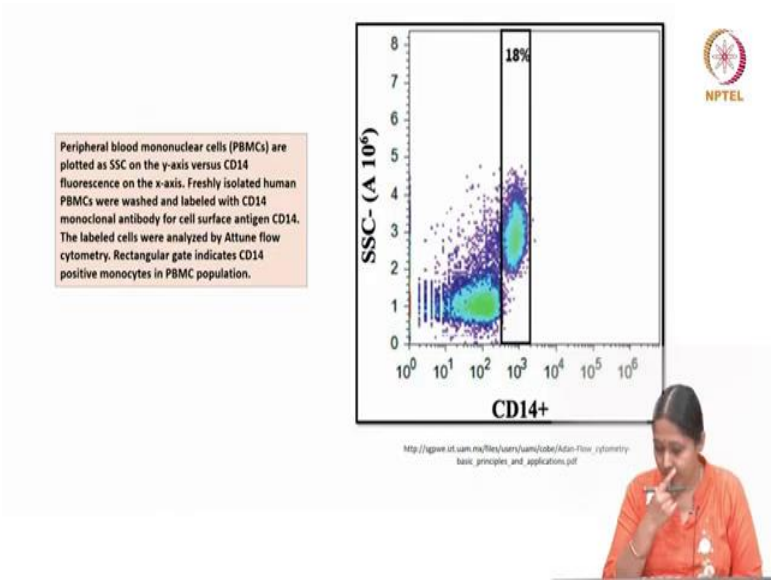

So, this is another picture from the article which for to explain the gate. So, here same similarly peripheral blood mononuclear cells are plotted on the side scatter on the y axis versus CD14, Cluster of Differentiation 14 fluorescence on the x axis. Freshly isolated human peripheral blood mononuclear cells were washed and labeled with CD14 antibodies monoclonal antibody to identify the cell surface antigen CD14 the labeled cells were analyzed by means of attune flow cytometry you can see here right.

So, this is a rectangular plot. So, this rectangular plot will indicate CD14 positive monocytes in the cell population. So, there will be various types of cells, you want to see monocytes right. So, CD14 positive monocytes are mainly, you can it can be appreciated by means of this rectangular gate. 18 percentage of cells, they mainly represent CD14 positive monocytes.

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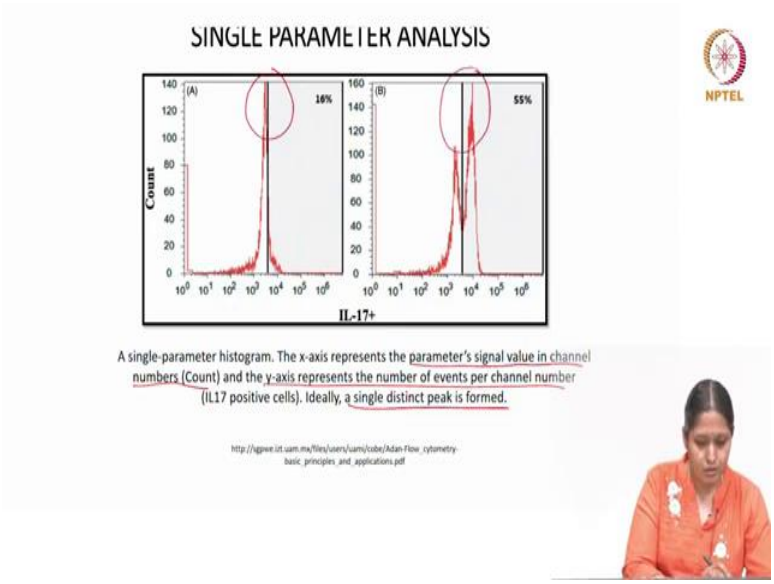

So, the graph can be either a single-parameter or two-parameter. So, single-parameter analysis or two-parameter analysis. So, this is a single parameter histogram where the x axis will represent the parameters signal value in channel number that is count and y axis will represent the number of events per channel number ok. So, here you can see the single parameter analysis what happens is a single distinct peak is formed. So, only a single distinct peak is formed in single parameter analysis.

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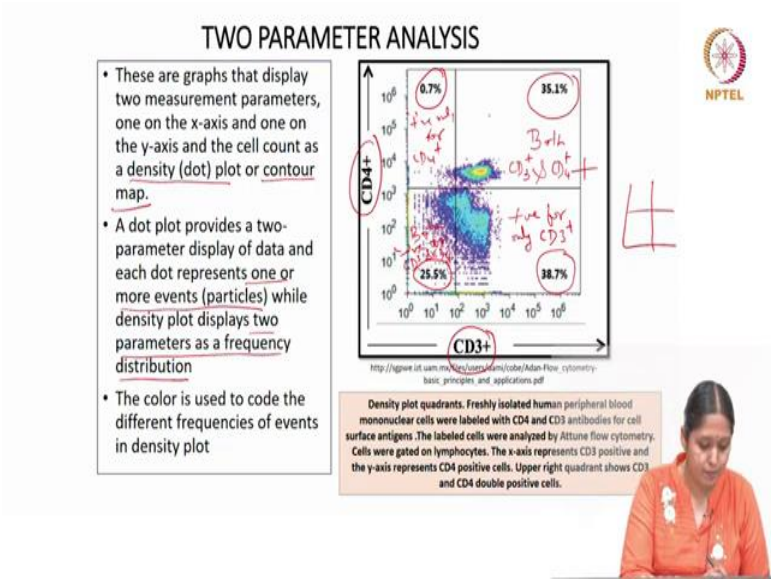

So, what do you mean by two-parameter analysis? So, two-parameter analysis is nothing, but these are the graph that display two measurement parameters, one on the x axis, one on the y axis and the count cell count will be seen as a density plot or dot plot or contour map.

So, either in single parameter analysis it will be seen as a single distinct peak as in histogram whereas, in two-parameter analysis it takes into consideration two measurement parameters one will be on the x axis, one will be on the y axis and the cell count will be seen as what you see here is called as a you call this as density plot or dot plot or contour map a dot plot will provide a two-parameter display of data ok.

So, each dot will represent what each dot will represent one or more events that is particles, while density plot will display two-parameters as a frequency distribution a color, color will be used to color code the different frequencies of events in the density plot. So, here density plot this graph mainly so it makes use of two measurement parameters one in x axis and one in y axis.

In this picture just see you can see CD3 plus and CD4 plus freshly isolated human peripheral blood mononuclear cells are stained with or labeled with cluster of differentiation 3 as well as 4; CD3 and CD4. So, what mainly they are doing is they are dividing this graph into 4 quadrants.

So, graph is there they are dividing it into 4 quadrant and important thing is you can see the various types of cells as a density plot or dot plot or contour map what you see in the upper right portion. So, here 35.1 percentage of cells are in the upper right quadrant. So, those cells are in the upper right quadrant are positive for both CD3 and CD4. So, they are positive for both CD3 plus and CD4 plus.

So, both CD3 and CD4 are positive in the upper right quadrant. Similarly, lower left quadrant, so 25.5 percentage of cells are there in the lower left quadrant. So, those cells which are in the lower left quadrant indicates these cells are negative for both CD3 and CD4. So, 25.5 percentage of cells are both are negative both are negative for CD3 and CD4 ok.

So, upper right quadrant is positive for both x axis and y axis parameter, that is here in this case CD3 plus and CD4 plus and lower left is negative for both CD3 plus and CD4

plus ok, then what about lower right and upper left. So, this lower right 38.7 percentage of cells they will be positive only for CD3 plus they will be negative for CD4 plus.

So, this 38.7 percentage that is lower right quadrant will be positive for only CD3 plus and upper left 0.7 percentage they are positive only for CD4 plus. So, here lower right will be positive only for the x axis parameter that is CD3 plus and upper left will be positive only for the y axis parameter that is CD4 plus.

So, you should always remember the two-parameter analysis, it includes mainly the graph two-parameters are used on the x axis and y axis it is divided into quadrants, four quadrants are there, upper right will be positive for both parameters lower left will be negative for both parameters.

Lower right lower right will be it will be positive only for parameter which is seen in the x axis and upper left will be positive only for the parameter which is seen in the y axis. So, upper left will be positive for the y axis parameter and negative for the x axis parameter, this is the basic principle for the identifying the two-parameter analysis.

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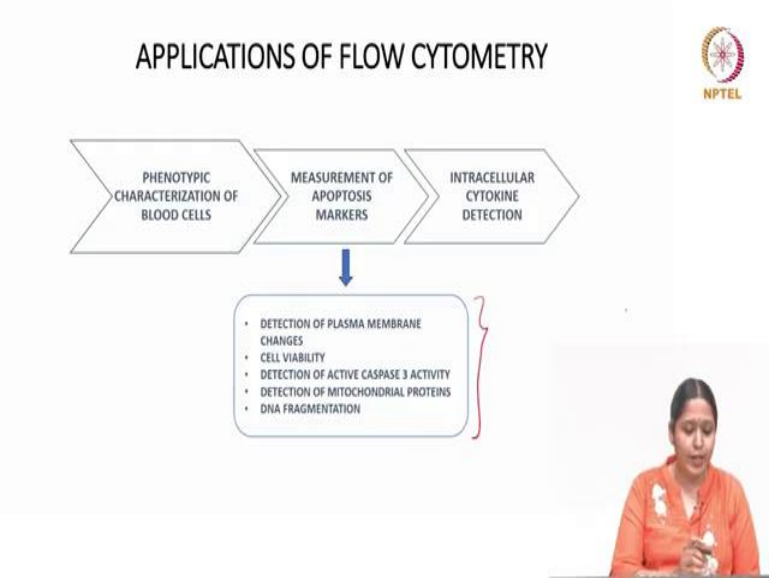

So, till now we have seen that how to interpret data by single peak, single parameter and two-parameter analysis and all. So, what are all the applications of flow cytometry? Where and all you use flow cytometry how it is helpful? So, it helps in phenotypic characterization of blood cells and another important function or application of flow

cytometry is to measure apoptosis that is programmed cell death, it helps for measuring apoptotic markers.

So, it also helps in intracellular cytokine detection. Under measurement of apoptotic markers, they are used for the detection of plasma membrane changes, cell viability, detection of active caspase 3 activity, detection of mitochondrial proteins and DNA fragmentation. So, apoptosis is mainly discussed under all these three headings which I will be discussing now. So, one of the important applications is phenotypic characterization of blood cells, measurement of apoptotic markers. It also helps in intracellular cytokine detection.

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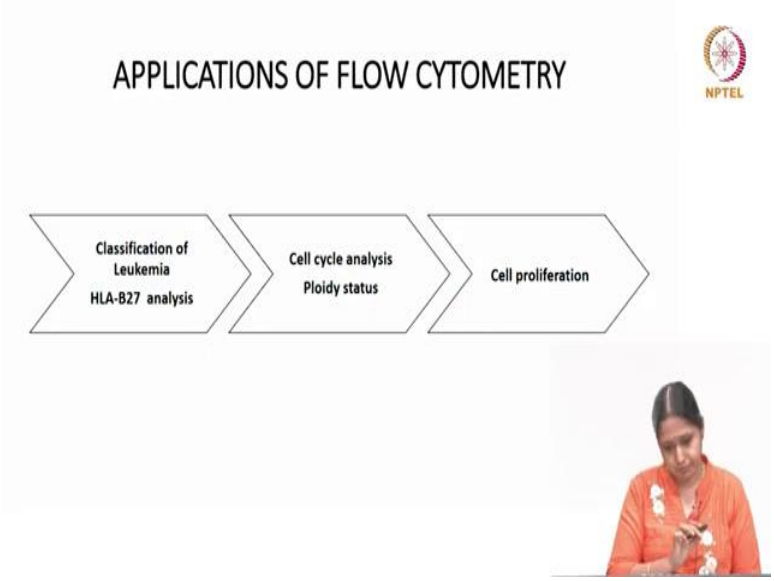

Classification of leukemia, it helps in HLA-B27 analysis, cell cycle analysis, ploidy status and also it helps in cell proliferation. Now, let us see each and everything in detail.

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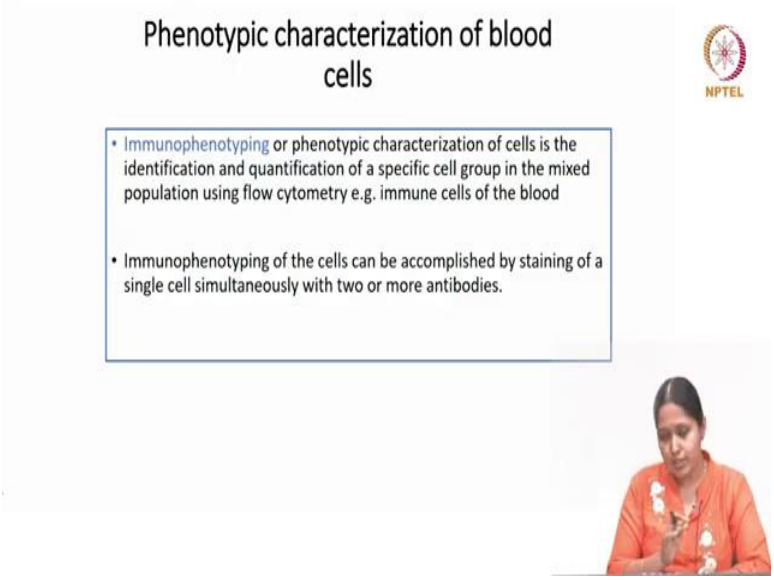

So, the first important function is the phenotypic characterization of blood cells. Immunophenotyping it is a process of identification and quantification of a specific cell group in a mixed population using flow cytometry for example, immune cells of blood. So, you are identifying and quantifying a specific cell group in a mixed population and this can be done by means of flow cytometry immunophenotyping of cells can be accomplished by staining of a single cell, single cell can be stained simultaneously with two or more antibodies.

So, immunophenotyping of the cells mainly it can be done by staining a single cell with two or more antibodies.

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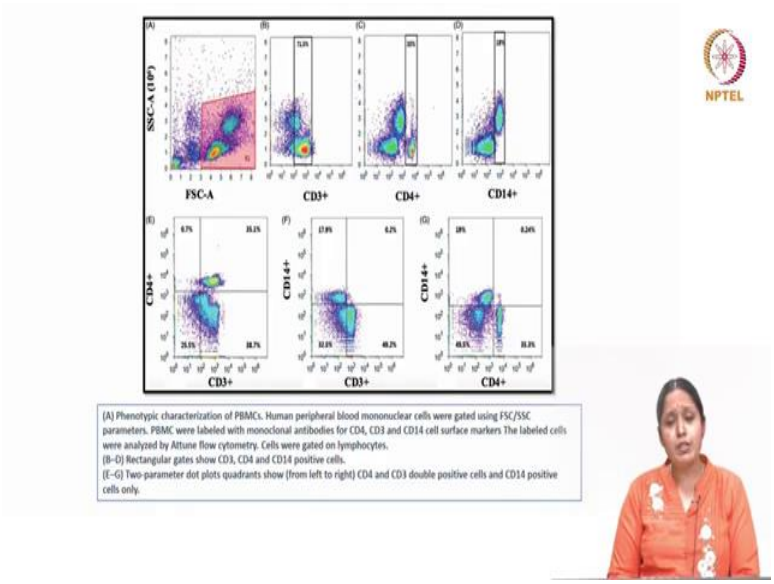

So, this picture it helps in explaining the phenotypic characterization of peripheral blood mononuclear cells. So, what here you are staining the human peripheral blood mononuclear cells are gated using forward scatter and side scatter parameters and here you are using two or more antibodies. You are labeling a single cell with two or more antibodies. What are the antibodies that we use here? CD3 plus, CD4 plus and CD14 plus.

So, these are the different types of antibodies that we use here you can see here a rectangular gate is there, this rectangular gate it shows CD3 positive this rectangular gate shows CD3 positive cells, CD4 positive cells identified in this rectangular gate and in this gate shows CD14 positive cells.

And lower you can see this 35.1 percentage of cells, here they have done the two-parameter analysis. 35.1 percentage of cells positive for both CD3 and CD4, 0.2 percentage of cells positive for both CD3 and CD14 and 0.24 percentage are positive for both CD4 and CD14. As I mentioned, earlier I described know the different how you will see the contour mapping, I mean in two parameter analysis those things and all that I have done in this, this is an example.

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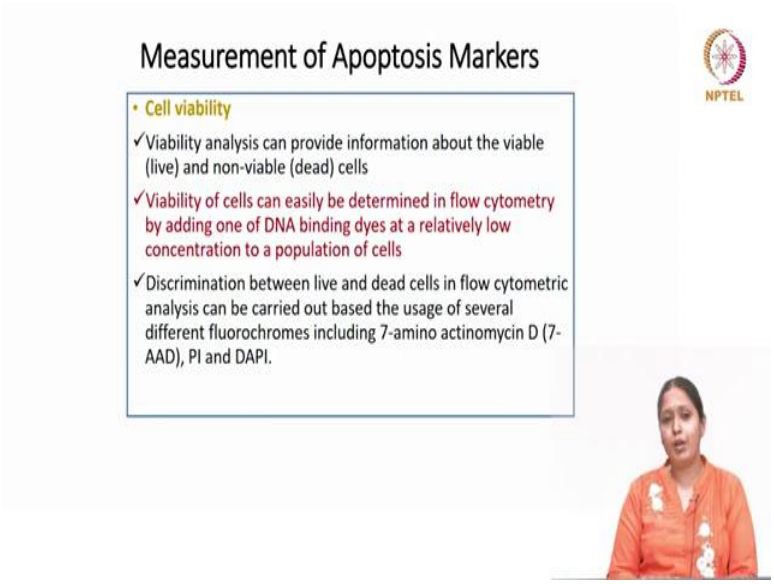

So, one important function that is immunophenotyping or phenotypic characterization of blood cell, coming to the next another important function that is measurement of apoptotic markers. So, under apoptosis, apoptosis is nothing but programmed cell death. So, under that we have seen the various sub heading, the first sub heading is the cell viability ok. So, the viability analysis can provide information about the viable and non-viable cells.

So, cells are either live or dead cells, viability of cells can be easily determined in flow cytometry by adding one of the DNA binding dyes at a relatively low concentration. So, one of the DNA binding dyes can be incorporated under low concentration to study the viability of cell and the discrimination between the live and dead cells in flow cytometric analysis can be carried out based on the usage of several different fluorochromes like 7-amino actinomycin D, propidium iodide and another fluorochrome that is DAPI.

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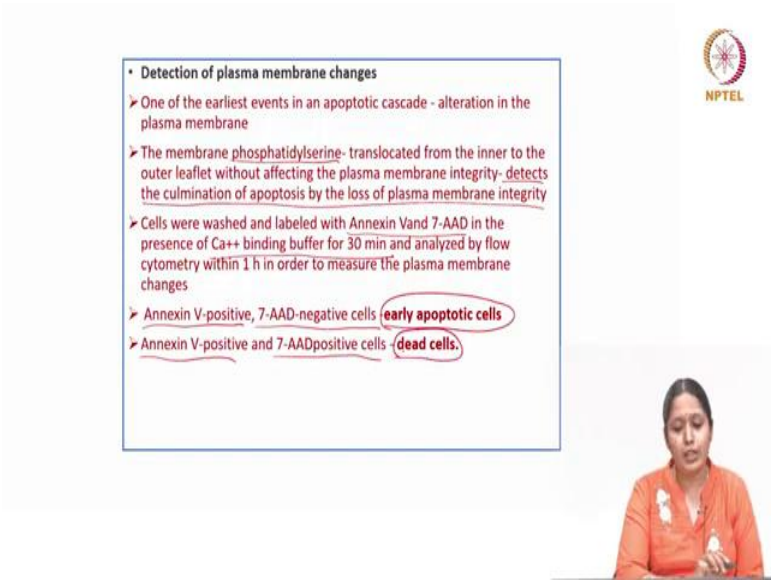

Next parameter is detection of plasma membrane changes. So, what happens is one of the earliest change of apoptotic cascade there will be alteration in the plasma membrane. The membrane phosphatidylserine is translocated from the inner leaflet to the outer leaflet without affecting the plasma membrane integrity. As a result of early change the membrane phosphatidylserine will be translocated from the inner to the outer leaflet and it will detect the culmination of apoptosis by the loss of plasma membrane integrity ok.

So, this is the main thing that happens as a result of early change in apoptosis, cells will be washed and they are labeled with two markers which are important for detecting apoptosis, that is annexin-V and 7-aminoactinomycin D ok. After washing the cells, they are labeled with these two markers annexin-V and 7-AAD in the presence of calcium binding buffer for 30 minutes and they are analyzed by flow cytometry.

And within 1 hour in order to measure the plasma membrane changes ok. So, after staining if, annexin-V is positive, 7-aminoactinomycin D are negative then you can say cells are early apoptotic cells ok, they are under going to undergo their apoptosis has started.

So, if the cells are annexin-V positive and 7-aminoactinomycin D-negative they are early apoptotic cells. Annexin-V positive as well as 7-aminoactinomycin D-positive cells if the cells are positive for both markers then you can say they are dead cells ok, this is about detection of plasma membrane changes.

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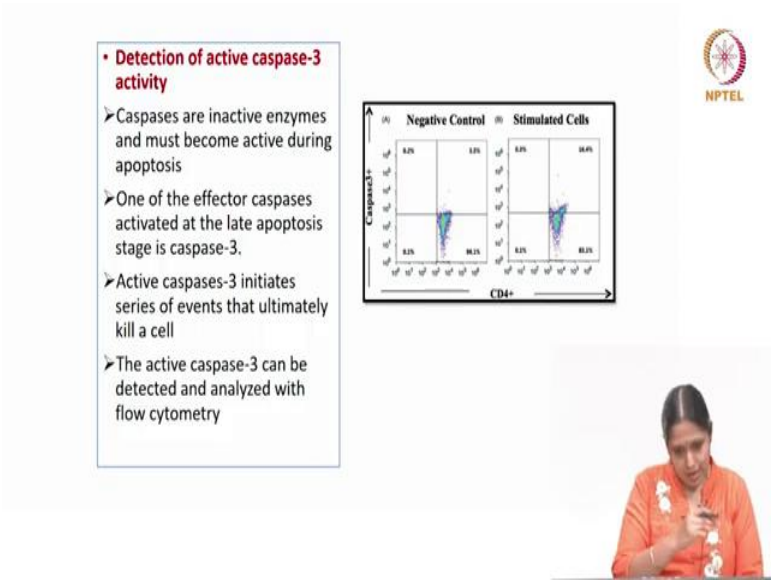

Another feature is a detection of active caspase-3 activity. So, caspases are inactive enzymes and they become active during apoptosis. One of the effector caspases that are involved in this late apoptotic stage is caspase-3. Active caspase-3 will initiate series of events that ultimately kill a cell, the active caspase-3 can be detected and they can be analyzed by means of flow cytometry as shown in this picture.

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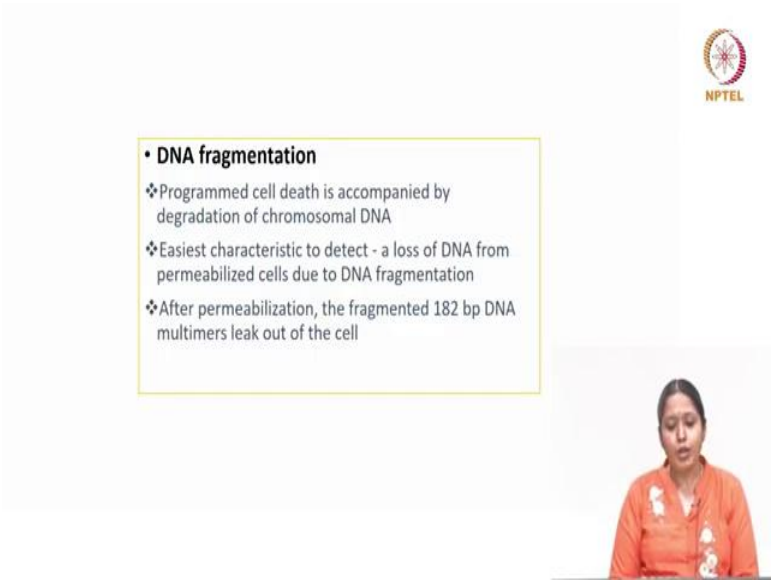

Next function is a DNA fragmentation. Programmed cell death is accompanied by degradation of chromosomal DNA. So, as a result of apoptosis or programmed cell death

there will be degradation of chromosomal DNA. Earliest characteristic to detect a loss of DNA from permeabilized cells due to DNA fragmentation is it is an easiest method, after permeabilization the fragmented 182 base pair DNA multimers will leak out of the cell ok.

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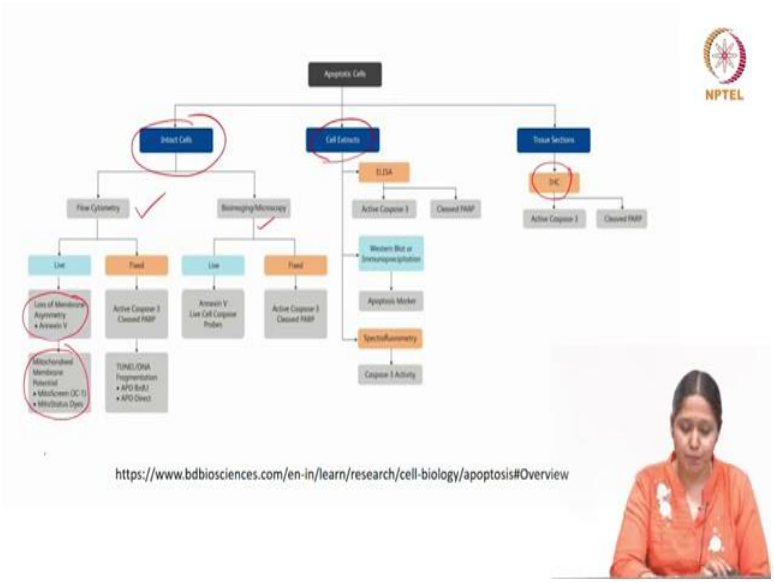

And thus, they can be appreciated. This is another flow chart which shows apoptotic cells there is intact cells cell extracts as well as tissue sections how they can be examined. As a means of intact cells, they can be examined by means of flow cytometric procedure and other procedures like bio imaging and microscopy.

ELISA procedure will help mainly makes use of cell extract and tissue sections can be done by to identify apoptotic cells by means of immunohistochemistry procedure. So, what flow cytometry uses is intact cells to detect apoptotic cells and if you want to see live cells the loss of membrane asymmetry and it can be detected by annexin-V and 7-amino actinomycin D. And mitochondrial membrane changes can be detected by MitoScreen MitoStatus Dyes and all just a flow chart, just a picture or flow charge which explains the how apoptotic cells can be detected.

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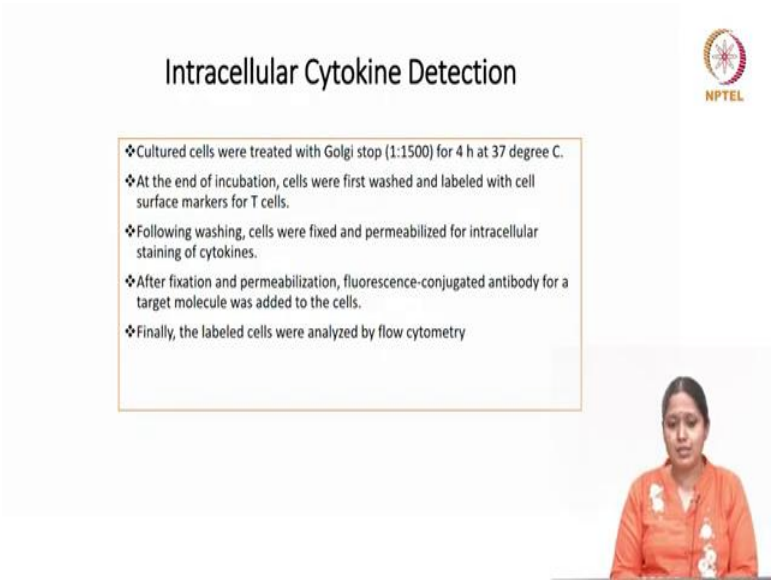

Another function of flow cytometer is intracellular cytokine detection. Cultured cells were washed with the Golgi stop at the ratio of 1 is to 1500 for 4 hours at 37 degrees Celsius. And at the end of incubation cells were first washed and labeled with cell surface markers for T cells. Following washing, cells were fixed and permeabilized for intracellular cytokine detection.

And after fixation and permeabilization fluorescence conjugated antibody for a target molecule was added to the cell. Finally, the labeled cells were analyzed by flow cytometry.

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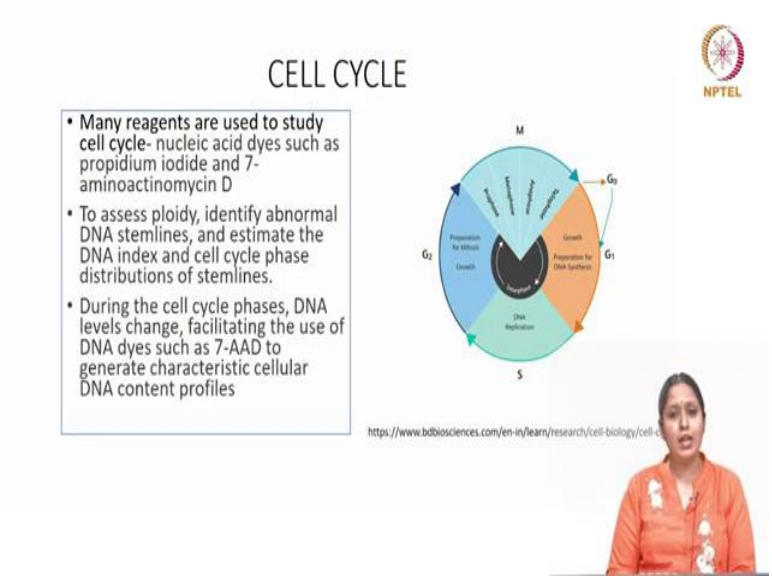

So, another thing is it also helps in identifying where the various stages of the cell cycle. Many reagents are used to study the cell cycle like nucleic acid dyes such as propidium iodide and 7-aminoactinomycin D. In order to assess the ploidy status to identify the abnormal DNA stem cell stem lines and estimate the DNA index and cell cycle phase distribution of stem lines a flow cytometry plays an important role.

During the cell cycle phases, DNA levels will change facilitating the use of DNA dyes such as 7-aminoactinomycin D to generate characteristic cellular DNA content profiles.

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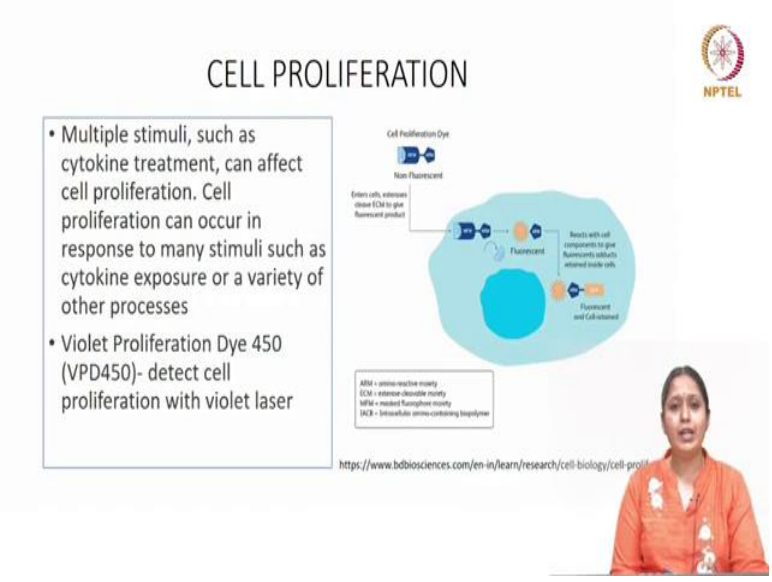

Another function of flow cytometer is to analyze cell proliferation multiple stimuli such as cytokine treatment can affect cell proliferation. Cell proliferation can occur in response to many stimuli such as cytokine exposure or a variety of other processes. Violet Proliferation Dye that is VPD450 it has got an ability to detect cell proliferation with violet laser.

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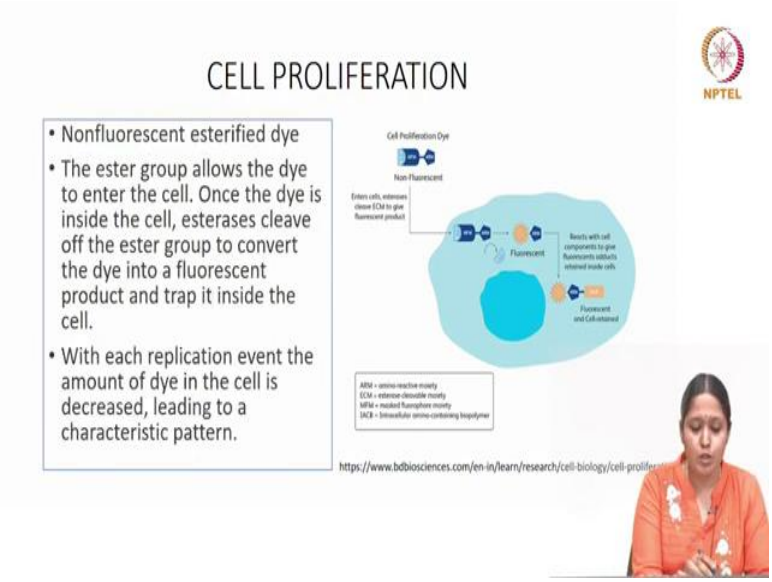

So, this VPD450, it is a non fluorescent esterified dye. So, what happens is the ester group allows the dye to enter the cell. So, the ester group will allow the dye to enter into the cell, this dye can enter into the cell and once the dye is inside the cell esterase will cleave off the ester group to convert the dye into the fluorescent product and trap it inside the cell.

So, the dye is a non fluorescent esterified dye. So, the ester group present it will allow the dye to enter into the cell, once the dye enters into the cell, the esterase will cleave off the ester group to convert the dye into a fluorescent product and it will be trapped inside the cell. With each replication event the amount of dye in the cell is decreased leading to a characteristic pattern thus cell proliferation can be detected.

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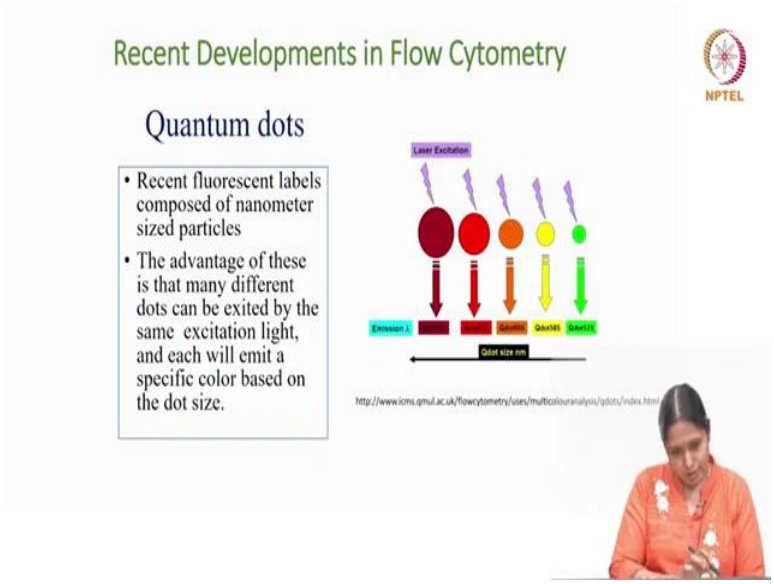

Now, let us see we have seen the various applications of flow cytometry. Now let us see what are the recent developments in flow cytometry. So, one of the recent developments is the quantum dots, it is a recent fluorescent label that are composed of nanometer sized particles.

The advantage of these is that it that many different dots can be excited by the same excitation light and each will emit a specific color based on the dot size. So, you can see that it can be it each will emit a specific color based on the dot size.

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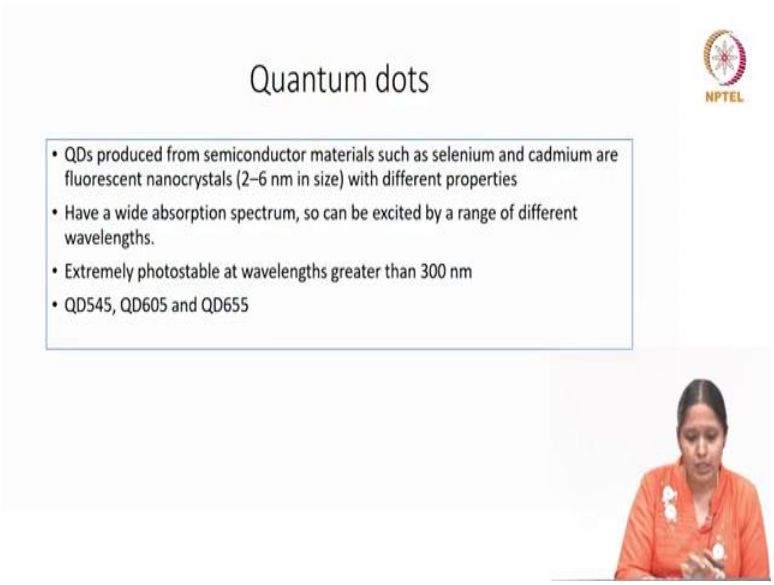

Quantum dots are produced from semiconductor material such as selenium and cadmium that are fluorescent nano crystals 2 to 6 nanometer in size with different properties, they have got a wide absorption spectrum. So, they can be excited by a range of different wavelength. They are extremely photo stable; this quantum dots are extremely photo stable at wavelengths greater than 300 nanometers. And the various types of quantum dots that are available in the market are QD545, QD605 and QD655.

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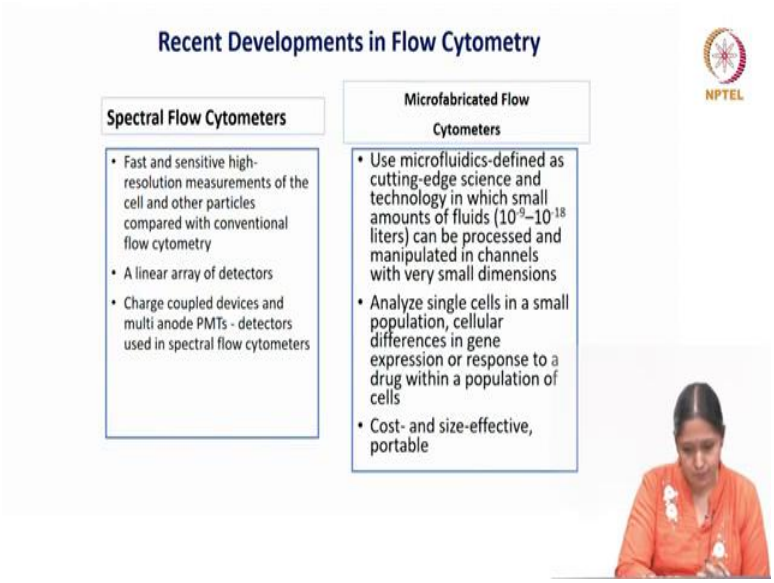

So, after quantum dot another recent development is spectral flow cytometers. So, spectral flow cytometers are fast and highly sensitive and high resolution measurements of the cell as well as other particles can be measured by means of spectral flow cytometers as compared to conventional flow cytometry. It consists of a linear array of detectors and the charge coupled devices and multi anode photo multiplier tubes are detectors which are used in spectral flow cytometers.

So, it is fast and it is sensitive and it has got high resolution measurements when compared to the conventional flow cytometer. After that another recent development is the micro fabricated flow cytometers, this micro fabricated flow cytometer it uses microfluidics which is defined as a cutting-edge science and technology in which small amount of fluids can be processed and manipulated.

Even a very small amount of fluid like 10 to the power minus 9 to the 10 to the power minus 18 liters can be processed and they are manipulated in channels with the very

small dimensions. They are used to analyze single cells in a small population. Cellular difference in gene difference expression or response to a drug within a population of cell. Even single cell in a small population can be analyzed by means of micro fabricated flow cytometers, they are very cost and size effective and another advantage is they are portable.

So, these are the various recent developments quantum dot, spectral flow cytometry and microfabricated flow cytometers.

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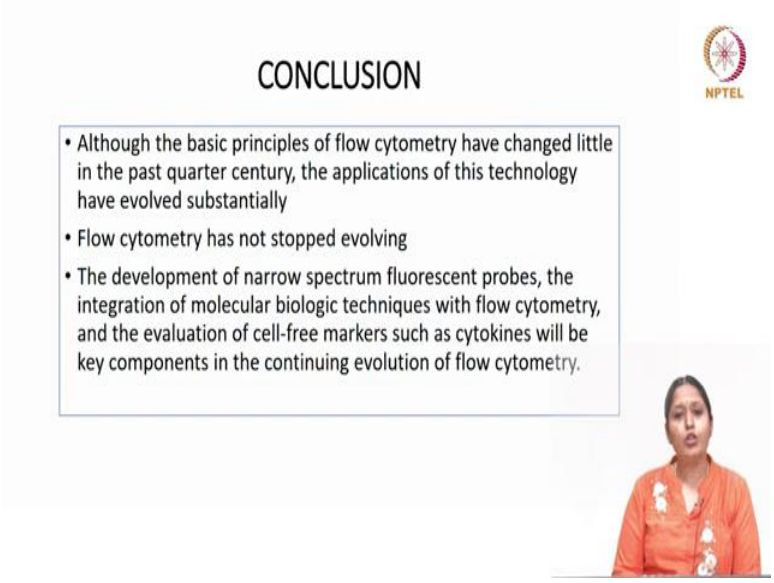

So, to conclude although the basic principles of flow cytometry have changed little in past few years, the application of this technology has evolved substantially and flow cytometer has not stopped evolving. The development of narrow spectrum fluorescent probes, the integration of molecular biologic techniques with flow cytometry and evaluation of cell-free markers such as cytokines will be the key components and continuing evolution in the flow cytometry.

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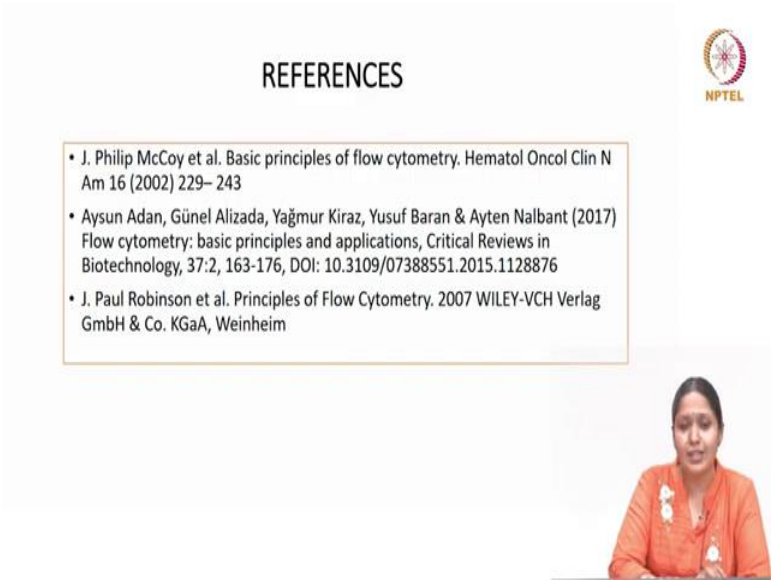

These are the references and.

Thank you.