Nanobiophotonics: Touching Our Daily Life
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Lecture No. 21
Basics of Flow Cytometry - Part 1

Hello and welcome. We will continue our discussion on nanobio-photonics and today we have a very interesting topic on bio-photonics for disease diagnosis. So, I have a question for you. How do you diagnose disease? Like I will give you a simple example. Recently we found out that my daughter is ill and we understood by measuring her temperature. Her body was hot.

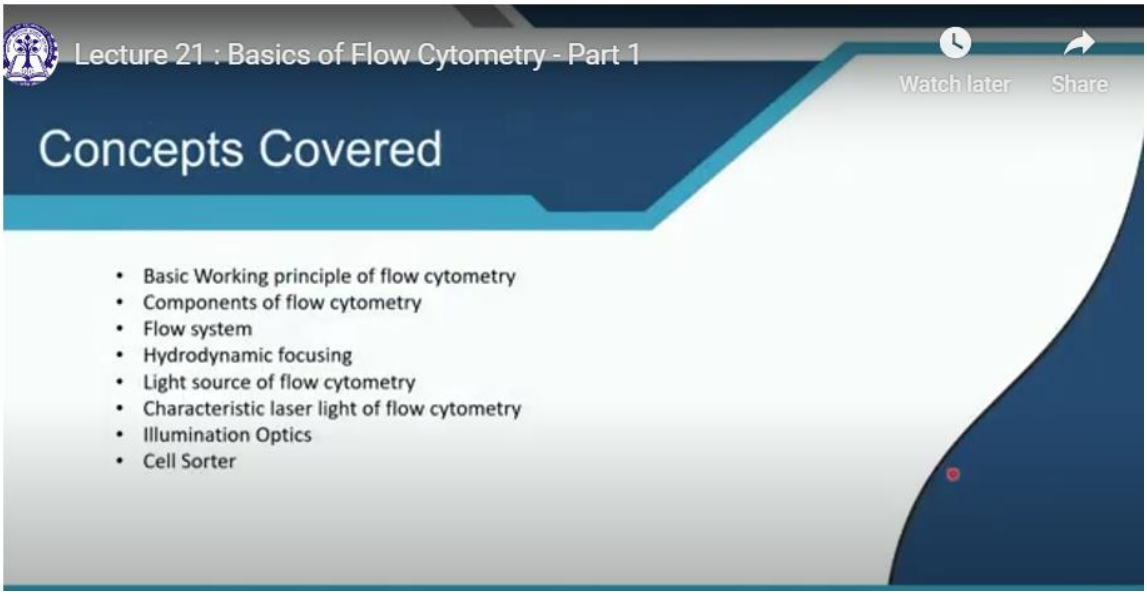
We just felt it with our hands. She was sweating, her feet were quite hot, her forehead was hot and then we took the thermometer to measure her temperature and found out that her body temperature is approximately 100-degree Fahrenheit. So, we immediately gave her some paracetamol that reduced the fever for the time being and then in the morning we refer to a pediatrician. 90% of the time you have done very very similar things for anybody either yourself or anybody that you know of.

For children we have to diagnose but for adults we understand that there is something wrong with us either we are feeling feverish, we are feeling cold though our body temperature has increased, we have a runny nose, headache, cough, similar things. And then based on that either you self-medicate aspirin or paracetamol on the counter medicine and then go to the doctor. The doctor depending on her or his experience uses the stethoscope, maybe use the thermometer and based on that can prescribe you some medicine, antibiotic maybe. If for that several of the diseases are already cured but for the next level the doctor may prescribe you blood test or test of any other blood body fluid like urine, stool, similar things. Based on the nature of your disease not all diseases it has fever associated with it, you can go for various other medical tests like x-ray, CT scan, MRI all of those things.

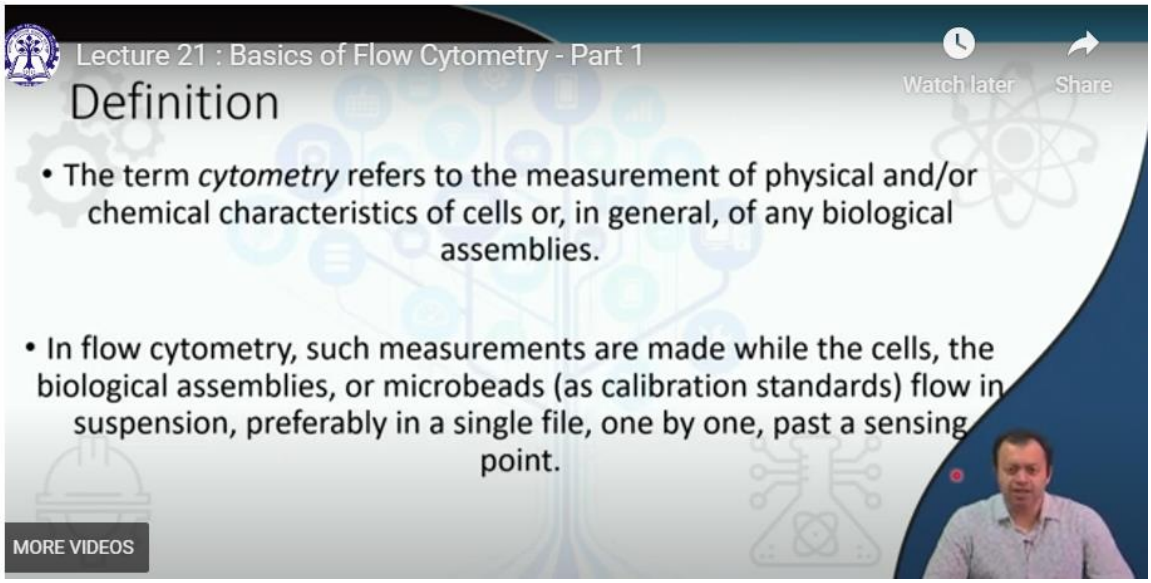
But if you want to understand disease in a cellular level what then? What do you do when you want to know the onset of a disease which has started from individual cells? What do you do then? Well of course there are several other methods you can simply put, extract some cell from certain part of the body, put it in a slide, look it under the microscope and then utilize your own analysis, your own intellect to see if certain cells are as good or as bad. You can simply use a microscope to understand if a cell is going bad i.e. a disease is affecting a particular cell but then you have to extract lots of cells and you need to redo the process again and again. You can also use spectroscopy, we have seen how

spectroscopy can be used.

But there is a far more efficient and simple method by which you can understand defects, anomalies, diseases at a cellular level using biophotonic technologies which we called as flow cytometry. So welcome to today's class. Today we are going to discuss about the basic of flow cytometry.



These are the concepts which I am going to cover today and let us start our lesson. So, what exactly is cytometry? Cyto is cell, right? Metry like you have studied geometry in mathematics in your schools, middle school or high school, I know several of you hated it, I hated it myself.



Geometry measurement of earth, geo is earth, metry is measurement. So, cytometry refers

to as measurement of cell, yeah? Measuring cell. It is part of cytology. Logy is study, biology, geology. So, cytology is study of cell.

So cytometry is measurement of cell. Now I leave it entirely up to your imagination, you want to study cell first and then measure it or measure cell first and then study it. But the term cytometry refers to the measurement of physical and or chemical characteristics of cell. So, your measuring the physical and chemical characteristics of cell or in general any biological assembly, any biological assembly like most of the time when you take out say for example blood, not only it will contain the sera but it will also have red blood cells, white blood cells, platelets, all of those things combined. So usually it is understanding or measuring the physical and chemical characteristics of cell but for a broader sense it could be any biological assembly, for example blood.

So in flow cytometry the measurements are made while the cells and biological assemblies flow in suspension preferably in a single file one by one past a sensing point. So, I will be describing each point mentioned here in detail. You basically have a tube-like structure which contains fluid, liquid media that liquid contains cells. That liquid is made to pass through I will describe you how using certain conditions, certain structures, certain prepositions are put into it. So just like you have an hourglass clock you know where sand flows through first an elongated shape and then a very narrow path.

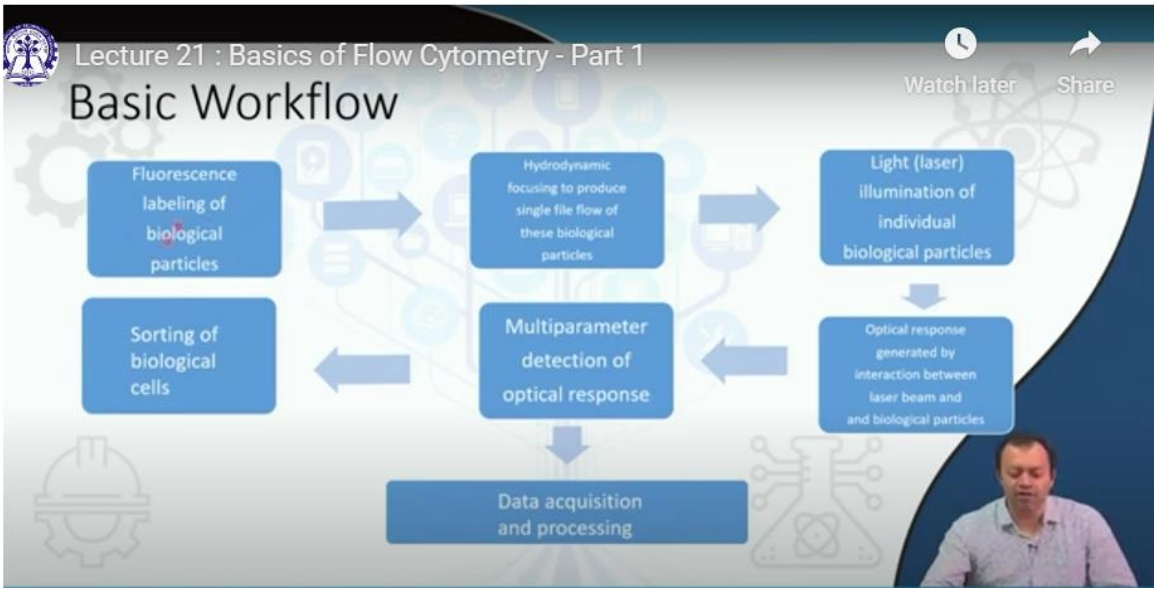
Similarly it uses something very very similar structure in which cells are made to flow through certain areas, certain tubes which are of a slightly smaller diameter along with several other condition associated with it. So, cells basically flow in column one at a time. So, the liquid containing cells, blood containing cells RBC, WBC etc. can be made to flow one cell at a time through a narrow tube, through a narrow tube. I will describe you in detail in coming slides.

While it is flowing, while the narrow tube, the cells are flowing through a narrow tube just like sand is flowing through the middle of the hourglass sand clock, you illuminate a specific point which is called the sensing point, you illuminate the specific point of the middle tube of the middle part one cell at a time. The output of such illumination, the output, the light reflected, scattered, transmitted etc. by the cell when illuminated by a laser light is then subjected to some kind of a spectrometer, a photomultiplier tube or some kind of a photo detector that will then analyze the cellular mechanism depending on the reflected light or transmitted light or scattered light or absorbed light. Similarly, spectroscopy but in a dynamic manner. Previously all the spectroscopy that we have done we put it drop in a slide, put the slide inside the microscope, micro spectroscopy measured it.

Here it is a dynamic process. It is a dynamic process in which a cell is made to flow one cell at a time in column in suspension, flow in suspension preferably in single file, preferably in single file one at a time and they are constantly being illuminated, constantly being measured. They are also called another term for understanding a cytometry, flow cytometry is FACS, FACS, Fluorescence Assisted Cell Sorting where you label each cell with a particular fluorophore, particular chromophore, particular fluorophore and illuminate it using an input light. That input light will illuminate the cell which contains fluorophore. The fluorophore will absorb the light and will fluoresce giving a wavelength of a different frequency, giving the wavelength of a different frequency.

The intensity of that frequency, intensity of the fluorescence light is then measured at an output and thereby you try to understand how each cell that is falling through this tube is different from another, is different from another. A bad cell will have different attachment to the fluorophore than a good cell. I told you there will be chemically difference. So, a fluorophore attaches with a cell using either antigen-antibody complex or similar structure. If the cell has gone bad, the attachment will be poor.

If the attachment will be poor, the intensity of the fluorescence light at the output will be different and from that, from a normal cell versus an abnormal cell depending on the intensity of the fluorescence, you can very well detect something is wrong at a cellular level.

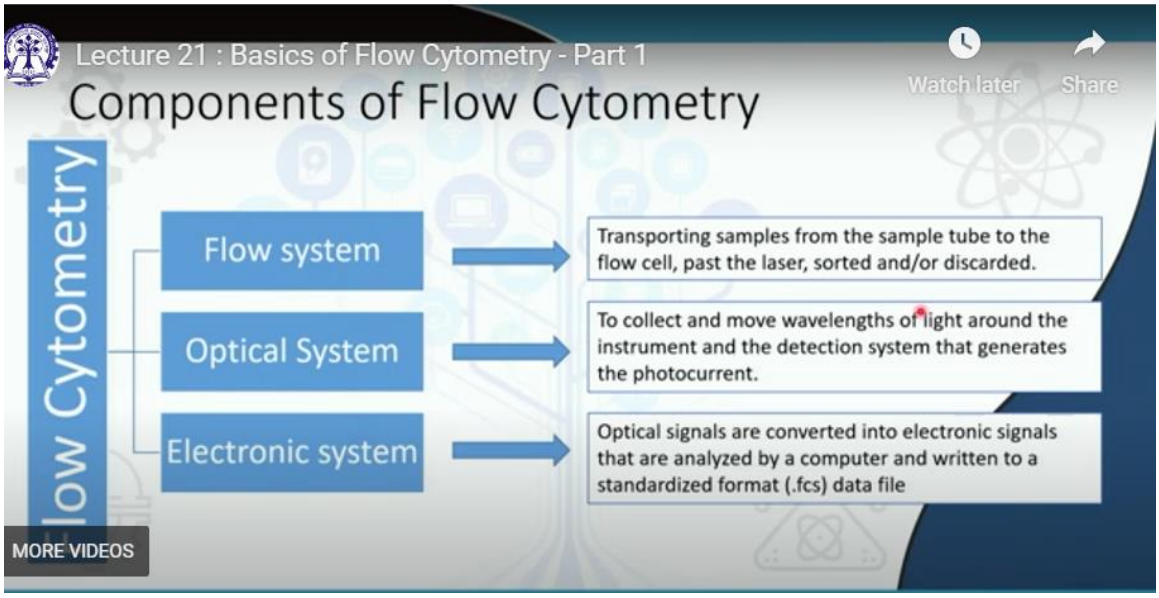


So, the basic workflow is fluorescence levelling of biological particles in this particular case cells which is then made to pass through some hydrodynamic focusing so that a single file flows from these biological particles. The single file is then illuminated with light, basically laser. The optical response generated by the interaction between laser beam and

biological particle is obtained. Multiple detection of optical response, several output detectors are present, photomultiplier tube, photodetector, all of those things.

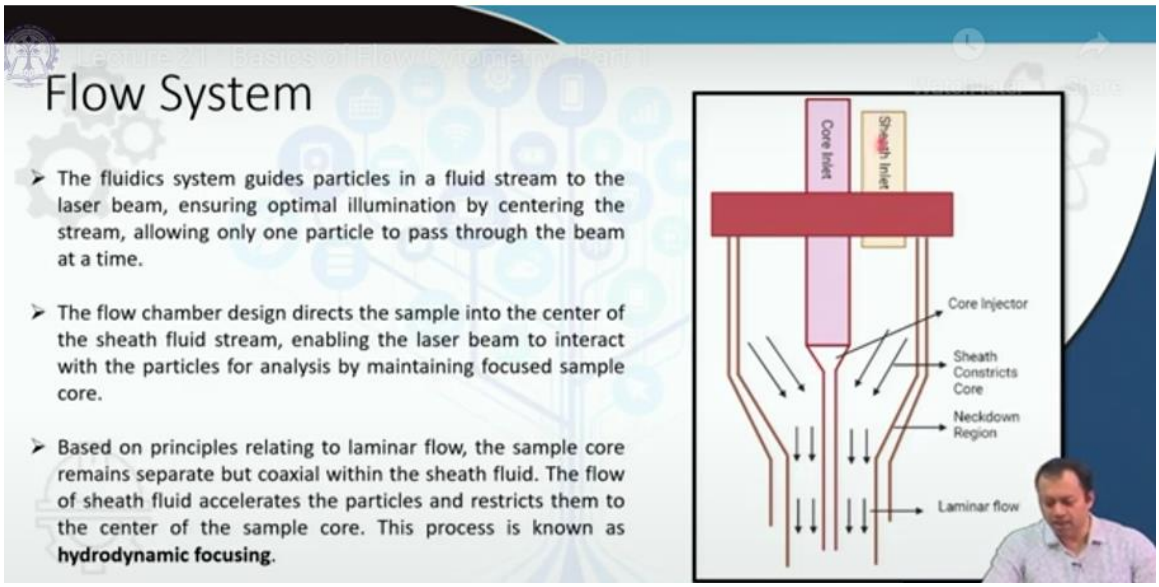
They convert the data analog to digital converter, light to optical converter. You have the data acquisition and processing and from that you can sort out, you can classify all the cells coming into good or bad category, good bad or good bad medium, different stages of medium, etc. So, it is that simple. It is basically either fluorescence or spectroscopy or a combination of both as we will see in a dynamic process, in a liquid flow where cells are constantly flowing one at a time, preferably one at a time. You are illuminating one cell at a time and you are measuring the output so on and so forth and thereby you calculate the disease in a large column of this many number of cells.

So like 80% of cells are good, 20% are bad. You are okay. Some cells will always be bad. But if the reverse is that, then you know that some sort of disease, some sort of malfunction, some sort of anomaly ailment has set in and then you need to start working from a therapeutic or a medicinal point of view.



So, what are the different components of flow cytometry? There are three major components. First is the flow system. This is the tube where transporting samples from sample tube to the flow cell, pass the laser, sorted and discarded. You just have a tube falling through. The cell is made to pass through a particular area, particular tube in a rank and file, pass the laser and then what is the output? It gets discarded or sorted out for later application. Optical system to collect and move different wavelengths of light around the instrument and detection system that generates the photo current. This photo current, this output of light, the output fluorescence light or output spectroscopic light is then optical are converted into electrical signals that are analyzed by computer and it is written into a

standardized format either dot FCS or we have also done excel sheet and then you do the measurement.
So three systems, the tube, the laser and the electronic system, the display that allows us to understand or see it.



So, this is the flow system. Let us go through the flow system as such. So, this is the core inlet. You see this small tube, this is the core inlet through which the cells, the cellular structures are made to pass through.

Then there is the sheath inlet. The sheath inlet contains some kind of a liquid suspension and this is made to flow through surrounding the core inlet. So, the core liquid which contains the cell is at the center, surrounding it, it is a sheet liquid. This does not contain any cell. This is simply some kind of a brine solution, some kind of a salt solution or there are different types of salts, phosphate buffer and what not.

These are maintained. So, the sheath inlet and the core inlet determine the overall particle flow through this tube. What does the sheath inlet do? You have understood that this is where the actual cellular material is made to pass through. But how do you ensure that there will be one cell at a time flowing through this? Well the insurance, how do you make it flow through is made using the sheath inlet. Remember as a high school student you have learnt about Reynolds number, fluid mechanics, Reynolds number that determine whether a fluid is laminar or turbulent. You know low Reynolds number means that the flow of fluids, a liquid in this particular case, a sheet like laminar where different viscosity is never matching to one another, they flow in different planes without mixing with each other.

Whereas very high Reynolds number make it a turbulent flow where everything is mixing and matching. So fluid flow, liquid flow can be divided into, broadly divided into, I know physics student knows much better than this topic, but broadly the flow of fluids can be divided into streamlined or laminar and turbulent flow. So, the sheath inlet and the core inlet have specific Reynolds number. A combination of these two Reynolds number make sure that there is one cell at a time passing through the tube as you have illuminated it, as you have illuminated it using some sort of a laser light. So, the fluidic system guides particles in a fluid stream to the laser beam ensuring optical illumination.

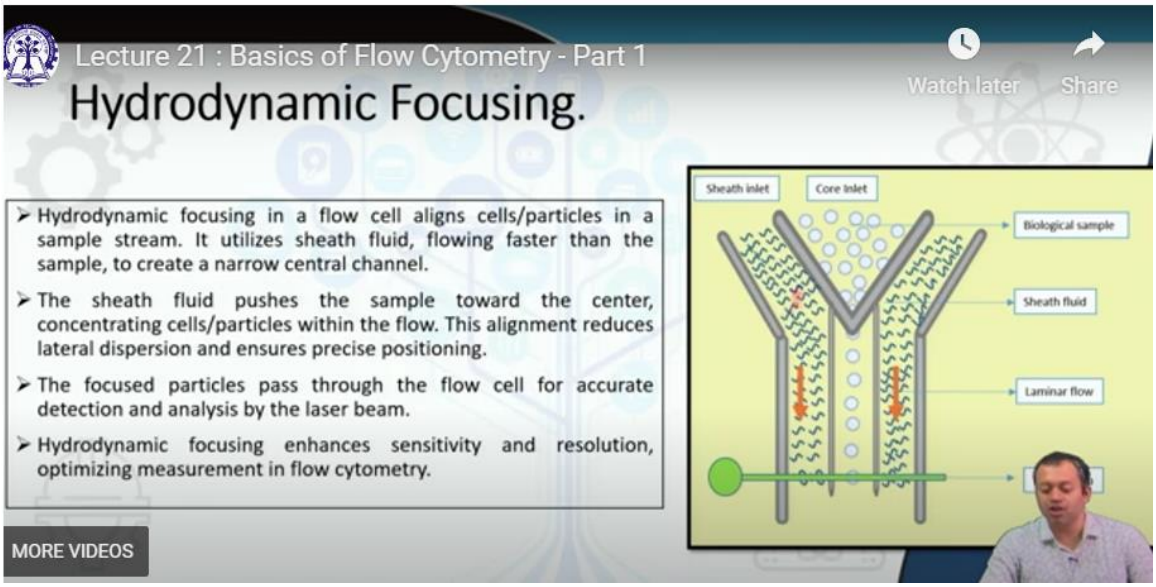
The flow chamber design directs the sample into the center of the sheet fluid seam. So, this is the center of the sheet inlet which is outside enabling the laser beams to interact with the particles. Based on the principle relating to laminar flow, the sample core remains separate but coaxial with the sheath fluid meaning this liquid, the sheath liquid and the core liquid will not mix like water and oil. The center fluid will have a different Reynolds number than the sheet liquid. The flow, now here is the crux of the matter, the flow of this liquid, the flow of this cell in the core, the flow of cellular structure through the core inlet depends on three main factors.

First is the differential pressure, i.e. the pressure of the sheath liquid and the pressure of the core liquid, how much it is different. The second thing is the concentration, how concentrated is the core inlet is, how much concentrated is the core inlet is, how much concentrated your sample is. Thirdly is the velocity of the sheet metal, velocity not the metal, velocity of the sheet liquid.

So three factors overall can determine that at a time one cell per unit time can pass through and this per unit time is few milliseconds. I mean people have flow cytometry system where 7500 cells are passing through a point of sensor, point of sensing per second. So how fast they go, but they go one at a time. How do you control that they are going one at a time? By changing the concentration, by changing the velocity and changing the differential pressure between the inner liquid and outer liquid. The idea here is that the inner liquid, the core liquid will not mix with the sheath liquid.

The Reynolds number has to be so much so that they flow in a laminar fashion, they remain separate but coaxial, they have like concentric circle, you know cylinder inside a cylinder. They have the same center, but they are two separate things. So concentric circles, they are like concentric circle having the same center, but they are two separates, they will not mix because the viscosity is different, because the Reynolds number is different, because the concentration of this is different and whether you want to have more than one cell at a time or less than one cell at a time, how do you control how many cells per unit time is passing through? You increase or decrease the output, the sheath liquid's

velocity, you change the concentration, you change the differential gradient, the differential pressure between inner liquid and outer liquid. That is, it. You just control the total number of particles, total number of cells passing through it and you have a laser like structure.



This is the sensing point perpendicular, orthogonal to it. So, this is the cellular structure. This is the sheath inlet that is some kind of a buffer like solution. I have just put this line to show that they are separate, but actually there is no separate line, there is no line per se.

It is liquid inside the liquid. It is like oil inside water or water inside oil. They will not mix. This is just a schematic diagram. So, these are the cells. You make them pass through this so that they are one at a time and this is the laser that is orthogonally, perpendicularly illuminating it as it is getting illuminated.

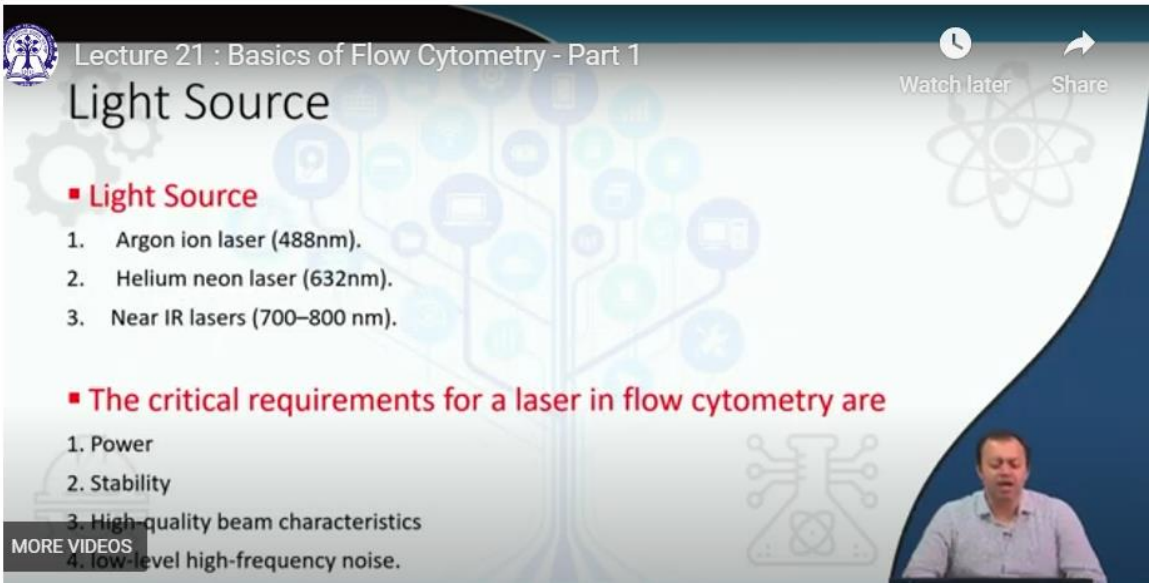
The output of the laser beam is measured using some kind of a detector, transmission scattered, reflected, all these things. We have detectors in several different places. We measure it and we try to understand the property of the individual cell one at a time as it goes through this liquid, this laser light is very fast. It is light at the end of the day and no matter how fast they are going, if they are one at a time, you will be zapping, you will be illuminating every single cell that pass through this point and the laser beam is measuring them and you have to have a fast enough computer to detect every single flow, every single cellular illumination and thereby you get the analysis done. So hydrodynamic focusing in a flow cell aligns cell particle in a sample system.

It uses sheath fluid flowing faster than the sample to create a narrow central channel. As I said, you need the velocity of the sheath fluid to make this kind of a channel. The sheath fluid pushes the sample towards the center, concentrating cell particles within the flow. The alignment reduces lateral dispersion. The focused particle passes through the flow cell

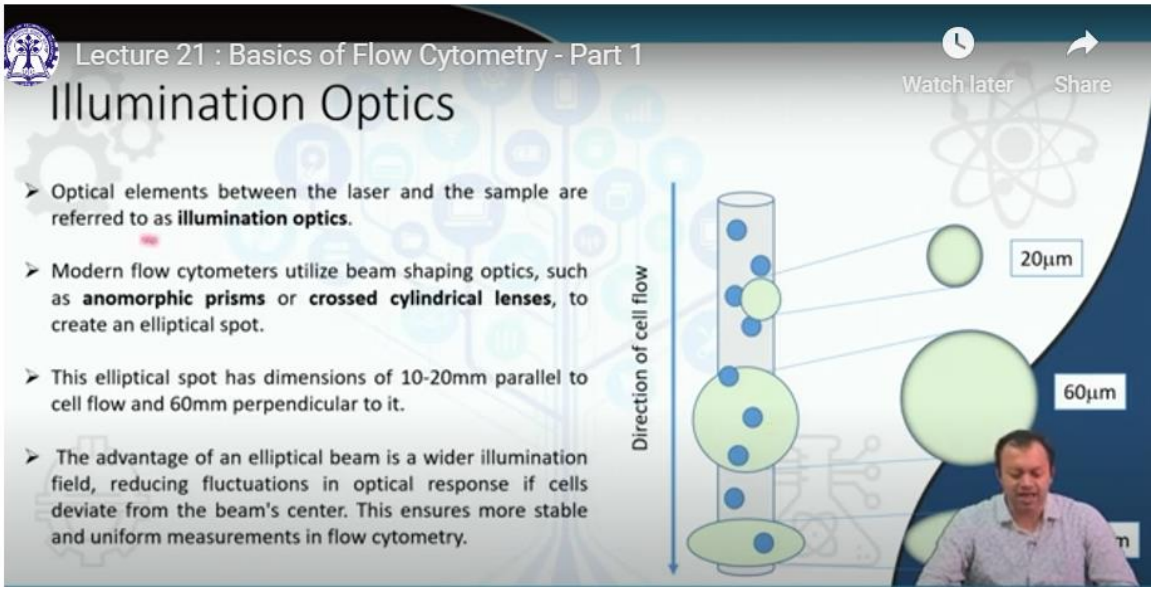
for accurate detection and analysis.

Hydrodynamic focusing enhances sensitivity and resolution, optimizing measurement in flow cytometry. So, with a basic knowledge on fluid mechanics, you can make a dynamic sample structure where samples are not static, they are simply passing through a particular area like a conveyor belt. You have done marketing in a supermarket, right. It is a conveyor belt, the person puts it inside the tail, computer based, laser-based scanning and the price gets up into the computer and finally the total thing is made and this item has this much price and you then pay it. Any supermarket you have seen, conveyor belt and everything.

This is exactly that. This is the conveyor belt. Each item is passing through, they are being scanned at a time and the output is getting in measured into some sort of a computer-based system and it is measuring instead of the price, the property. That is, it. That is exactly it. Supermarket based scanning system, you know, grocery store or a big supermarket, you must have gone to them.

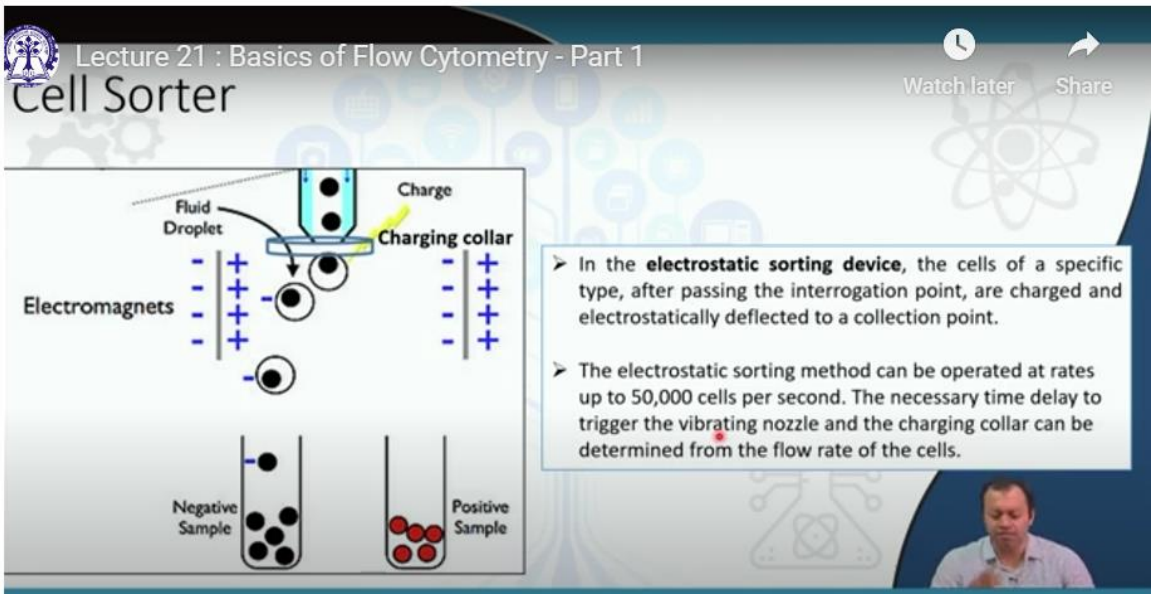


Cell based measurement. So, what are the light source we require? We go for either argon ion laser or helium ion laser and the critical requirements that the system has to have a sufficient enough power without damaging the stability, high quality beam characteristics, low level frequency noises.



So, the illumination optics, the way we illuminate individual cells is using like anamorphic prism or cross cylinder lenses so that the cells are illuminated in some sort of an elliptical spot. They are measured in some sort of an elliptical spot rather than a circular spot. Elliptical spot, so the spot of the laser light.

So my pointer has a circular spot. The illumination in flow cytometer is usually in elliptical spot with 60 millimeter perpendicular to it, has a dimension of 10 to 20 millimeter and there is a requirement, there is an advantage of an elliptical beam is that it has wider illumination. You are illuminating the entire tube, this entire flow, entire core liquid flow so that if by any chance more than one cell comes in, it is getting illuminated properly. Reducing fluctuations in optical response, if cell deviate from beam center, they are not all supposed to come in a despite the best of our attempt, they were all not supposed to come in one single line, they can deviate from the center. This ensures more stable and uniform measurement in flow cytometry. So, the illumination optics is more or less elliptical rather than spherical or circular.



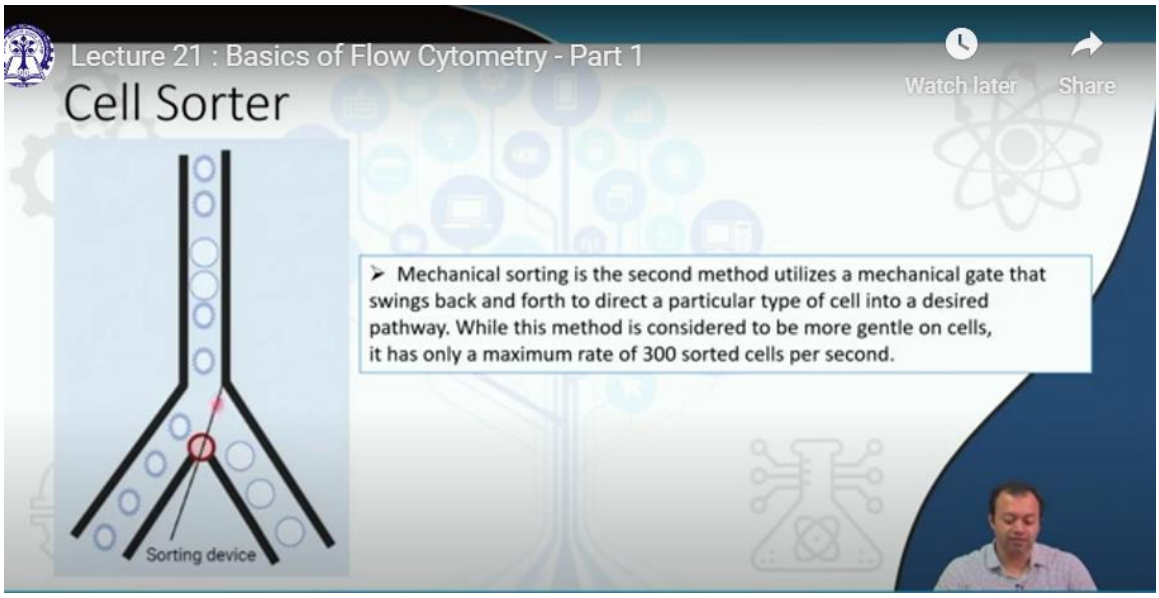
How do you short them? So, you have figured out that one is good, one is bad. Then what? Well, what we can do, we can short them, we can classify them. There is a very easy way to classify them. There are several easy ways to classify them. The one is using electrostatic sorting device and this is very clever.

Think about it. There is a ring through which the cells will pass through. You have measured this. You have measured this beforehand and you have figured out this cell is good; this cell is bad. You have figured it out. So below this, below all of this, below all of this, so the measurement has been done but now you want to differentiate, classify.

So what you do is that this collar, this ring sprinkles some kind of ionized molecules onto the cells. You control it, computer controlled. If it is a bad cell, it will send negative charges. If it is a good cell, it will send positive charges.

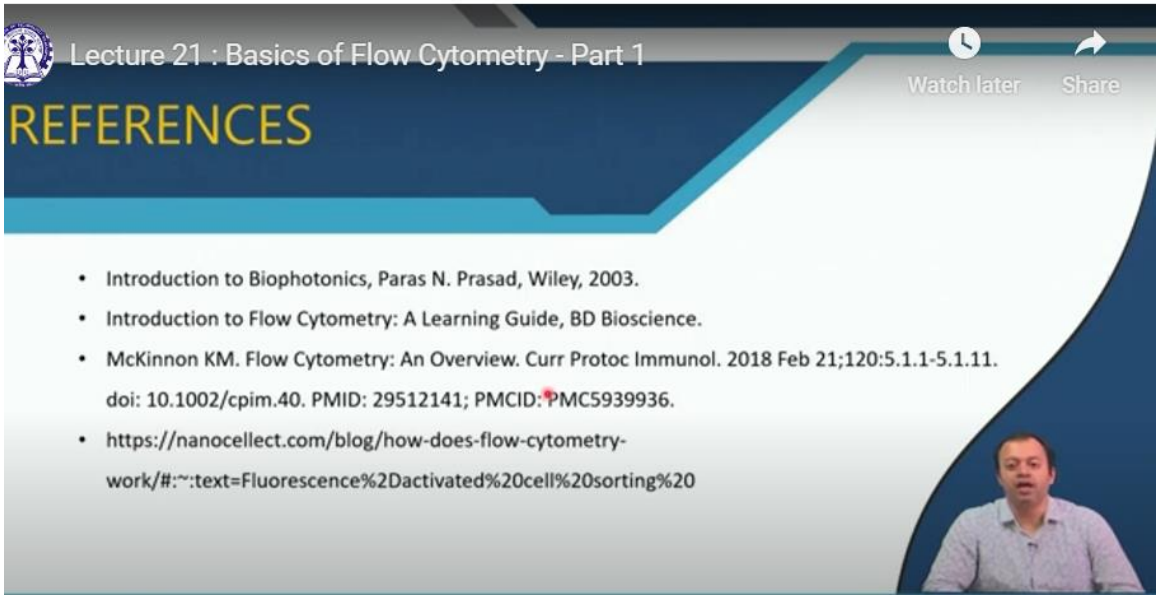
As it is passing through, you have two electromagnets. So, if the negative cells, bad cells will be attracted to the positive part, the anode and the good one will be dividing into the positive circle, the positive side and thereby you can simply differentiate the total number of negative samples versus total number of positive samples. You can then utilize those positive samples for applications or you can look to the negative samples of a group of cells for further classification, for further detection, for further measurement. So, you are not simply measuring in this particular case in flow cytometry, you are sorting them you are distinguishing them, you are classifying them, you are sorting them out, you are choosing them out. I think choice is the better term here. You are cell sorter or cell chooser, you are choosing and you are separating, cell separator can also be a good word, cell sorter or cell separator, you are separating those out.

The electrostatic sorting method can be operated at rates of see how many, 50,000 cells per second, the necessary time delay to trigger the vibrating nozzle and the charging collar can be determined from the flow rate of the cells.

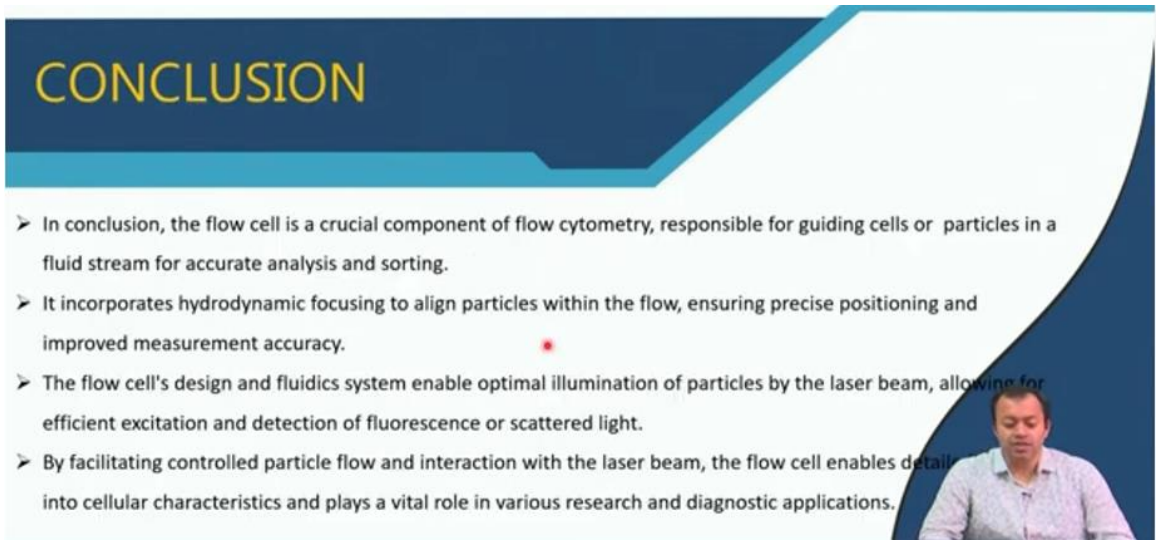


The easiest way is the mechanical sorter. You have a switch, you have a toggle, a mechanical sorting is the second method. If you do not have that much money to have a charging collar which sprinkles positive or negative molecules, positive or negative charges, you have a Y like structure, cells are coming through, you have understood this is good, this is bad. When the good thing needs to come, you close the door, it passes through.

When the bad one is going to come, you open the door, so you close it like this and they can come out. So, it is like a door, it is like a flap, it is like a hinge. In the Y shape flap, you either close it like this or close it like this, but this has to operate very fast. It is a mechanical sorting device and sometimes get bad and as you can see, it is 300 sorted cells per second as compared to 50,000 cells sorted per second. So, another advantage of this is that it is not only measuring, it is also separating good from the bad.



So with this, I come to the end. These are my references. Please go through, there are beautiful videos available in YouTube.



Look for cell cytometry and these are my conclusions which you can read at your own leisure and I will see you in the next class. Thank you. Thank you very much.