Nanobiophotonics: Touching Our Daily Life
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Lecture No. 54
Higher Harmonic Generation Imaging for Neuropathology

Welcome back. We are at module 11, the penultimate one of our nanobiophotonics course and we are still discussing the emerging areas, emerging research areas in neuro-photonics. So, today's topic I chose as an extension of the two-photon microscopy which is higher harmonic generation imaging for neuropathology. So, as I said it will be an extension of the two-photon microscopy that we have done. Here not only we will be going for non-linear interaction of light with brain matter, non-linear interaction of light with neurons and different areas of the nervous system. We will go for both the second harmonic as well as the third harmonic generation, right.

So, consider it is an extension of the previous microscopy technique that part is well established people are doing two photons for almost 10 years plus minus and now, but now we are thinking of using third harmonic generation. Two photons are second harmonic generation usually, three photons can be considered as third harmonic generation. It has its own advantage and limitations as you are about to see. So, why do this? Well, obviously, there exist a huge diagnostic gap.

Presently, I told it many times that MRIs, magnetic resonance imaging offers you know preoperative diagnostics and all of that thing, but the resolution as well as the real time accuracy is pretty less. Brain or any other organ for that matter is a dynamic system. It is constantly evolving, constantly changing. There is a dynamic flow of it. With your

magnetic resonance imaging, the magnetic field will not be able to capture what exactly is happening into the brain at a real time plus the resolution.

The resolution is the most important thing. So, very small resolution, very high resolution at a cellular level is a requirement if you want to detect several diseases at the earliest stage. MRI for the time being of course, there has been several instances of development in MRI, but till now MRIs to the best of my knowledge lack the higher resolution, a single neuron level and at the same time real time accuracy i.e. few nanosecond change in individual neurons from resting potential to action potential to back again resting potential using trying to detect it using MRI is going to be difficult right.

Of course, you can do histopathology. You know open up the brain, scoop out some part of the portion, stain it, stain the tissue and look it under the microscope like any other staining. Medical students know biology student know staining and probably several of you who have taken biology in high school also know where you used to peel you know onion skins and then put some amount of you know the reddish ink, the reddish dye. Yeah, remember that and look it under the microscope and then see how the cells are arranged etcetera. It is one thing to look into onion skin under the microscopes, stained onion skin under the microscope.

It is completely different and far more complicated to stain part of tissue and to diagnose if there is any you know cancerous growth. You do know cancer does not spread homogeneously even inside the tumor, even inside you know whole tumor you will have different areas of the tumor displaying different amount of you know metastasis. So, histopathology provides accurate diagnostic. Pathology staining based things are have have the biopsy has improved by you know leaps and bounds. Now, AI is used being used artificial intelligence is being used, but it still depends on the person.

It still depend on the accuracy of the pathologist. It still depends on the staining technique which is technically inaccurate and of course, it is time consuming. It takes up to 24 hours post surgery. Intraoperative techniques such as stained section or smear preparation are limited and time consuming and most importantly these staining or histopathology per say which is still the gold standard for biopsy or histopathology staining a tissue and looking it under the microscope and trying to trying to diagnose. A suffers completely from this intraoperative as well as interoperative variability.

What does that mean? Meaning one single slide of the same tissue that has been stained can be diagnosed different by the different pathologist. Pathologist A will say cancer stage 2, pathologist B will say cancer stage 3. The difference is their experience. The problem is intraoperative variability where the same stained tissue, same fixed tissue you give to

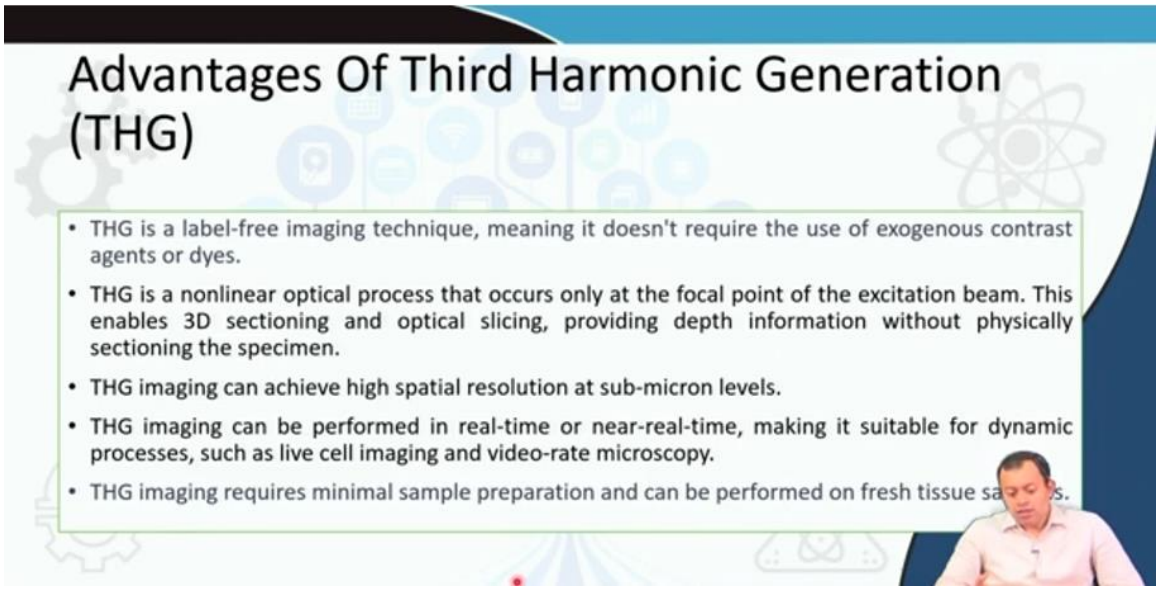
pathologist A today. This will be the diagnosis.

You give the same tissue to the same pathologist one month later. There are chances that the pathologist will say a different thing. So, there is a huge variability or deviation both within the same pathologist as well as a different pathologist. Those of us who are working in oral cancer specifically oral cancer as this is very much prevalent in Southeast Asia because of our lifestyle choices of chewing tobacco rather than smoking, interoperative and intraoperative variability is tremendous. But anyway, we are not talking about oral cancer.

Today we are talking about brain. So, moral is that there exists a diagnostic gap. I want to understand what is happening at a cellular level, at a neuronal level. At the same time, I want to be accurate. It is not that 4 people look into the neuron, look into the same neuron and give 4 different diagnosis.

Yes, no, maybe, I do not know. All 4 options should be limited, should be eliminated and only one option should remain when it comes to you know trying to save lives and trying to diagnose because think about it. These days if a person goes who suspects she or he is suffering from oral cancer go to 4 different pathologists in 4 different you know diagnostic centers, there is a pretty good chance that that person will get 3 to 4 different types of diagnostics. Yes, cancer, no, not cancer, maybe cancer and I do not know cannot say. So, how is which one is the person going to accept and based on that where the person is going to go to seek medication because diagnosis is the first very first stage based on which medication is prescribed.

Fluorescence imaging during surgery has limitation like uneven dye distribution and non-specific staining. So, you have opened some persons brain up, you are trying to look into a tumor, trying to remove it, but you also want to know if the tumor is just at a specific position or it has spread and then you have dyed inside the brain if you if you think it is suitable and then you are trying to look through a microscope while the patient is anesthetized and the brain is open and then try to figure out. So, imagine the complexity, imagine the complexity and the limitations it is provide like uneven dye distribution and non-specific staining. So, these are I mean real time challenges that medical personnel face on a day to day basis if the diagnosis is proper, if the biopsy is right, if the staining was even, if the staining was uneven and based on which a person's life will depend. So, we need to utilize some new type of technologies that could have potential that could have potential to do that.

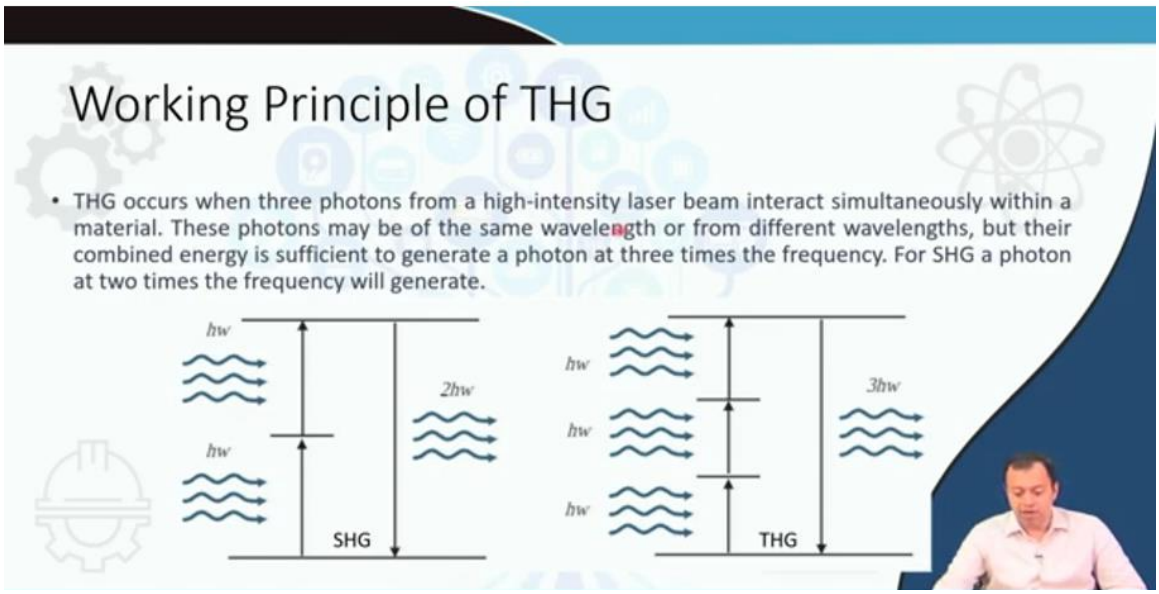


One such potential wise a technology is third harmonic generation, third harmonic generation microscopy. So, third harmonic generation is a label free imaging technique, you do not need to worry about how evenly homogeneously the dye has spread. So, it does not require the use of exogenous contrast agents or dyes. Third harmonic generation is a non-linear optical process that only occur at the focal point of the excitation beam, this enables 3D sectioning. So, you are sending a laser beam containing 3 photon, nu 1, nu 2 and nu 3 and the band gap is nu 1 plus nu 2 plus nu 3.

You know third harmonic generation we have discussed non-linearity, we have discussed non-linearity twice already twice and I am doing it three times. So, the specific area where the laser light is focused depending on the band gap, HOMO versus LUMO, depending on the laser's energy, depending on these photons' omega nu 1, nu 2, nu 3 only that area will absorb. So, meaning this is very very selective, this is very very selective the laser is at a specific area, the laser is focused as a specific area within the brain and you know lasers focal point can be few microns or even less. So, laser is focused on few micron which is basically the size of good enough neurons maybe 1 or 2, you have localized the light, localized the focus on to this 1 or 2 neurons at a time and then you are trying to see if the 3 photons that is coming from the laser light is being absorbed, is being absorbed by those areas those localized area 2 or 3 neurons 1 or 2 neurons and in turn are they emitting, are they emitting 3 nu 1, nu 1 plus nu 1 plus nu 1 or nu 1 plus nu 2 plus nu 3 is equal to 3 whatever. So, you have sent 3 photons nu 1, output is 3 nu 1 or not yeah nu 1 and 3 nu 1 are 2 different numbers, 2 different values even nu 1 plus nu 2 plus nu 3 should be x and that is x is the output.

So, you are absolutely 100 percent sure that the output emission that is coming out is from that localized area localized area where laser is focused. No other area nearby or otherwise has the capacity to produce 3 nu 1 or x simple as that simple as that no other area can

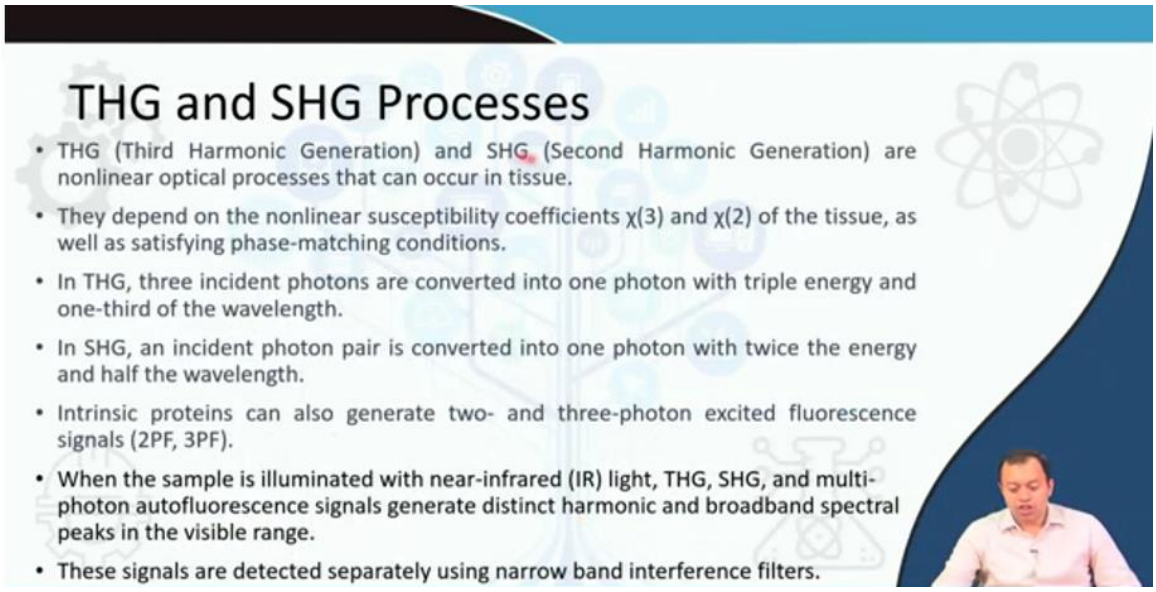
produce nu 1 plus nu 1 plus nu 1 because this is third harmonic generation. So, third harmonic generation imaging can achieve high spatial resolution at submicron level TGH imaging can be performed in real time or almost near real time making it suitable for dynamic processes such as live cell, live neural imaging and TGH imaging require minimal sample preparation and be con performed on fresh tissue samples. You take some part of the tissue out a point y of c just put a needle and take out a point about a portion of the tissue the tissue size could be few micron just a dot put it under glass slide subject it to laser based analysis microscope 10 times 50 times 50 x 10 x 100 x can expand it and you pixel by pixel point the laser see if the third harmonic is generated. Laser there will be a normal response from a normal type of neuron and there will be an abnormal response from an abnormal type of neuron if you have you know a tissue of 5 micron very small and then you are trying to map it different areas using this laser and try to see if the third harmonic is generated at different areas or not depending on what type of output 3 x by sorry 3 new one or x is coming up compare and contrast it with a normal sample that is it right.



So, this is this is again working principle of third harmonic generation when 3 photons from a high intensity laser beam interact simultaneously within a material these photons may be of the same wavelength or for different wavelengths, but their combined energy sufficient to generate a photon 3 times the frequency either 3 h omega it should be omega lot of the w and these are obviously, less. So, they produce the incoming photon is pretty less energy as compared to this. So, they damage less the area for you know second harmonic generation you know both only 2 photons its output is 2 h omega here the output is 3 h omega this is second harmonic generation this is third harmonic generation. Now you will obviously, ask like what is the difference between or why do third harmonic generation when we have to second harmonic generation yeah when you have 2 photon microscopies remember I discussed this in nonlinearity that 2 photons are selective second

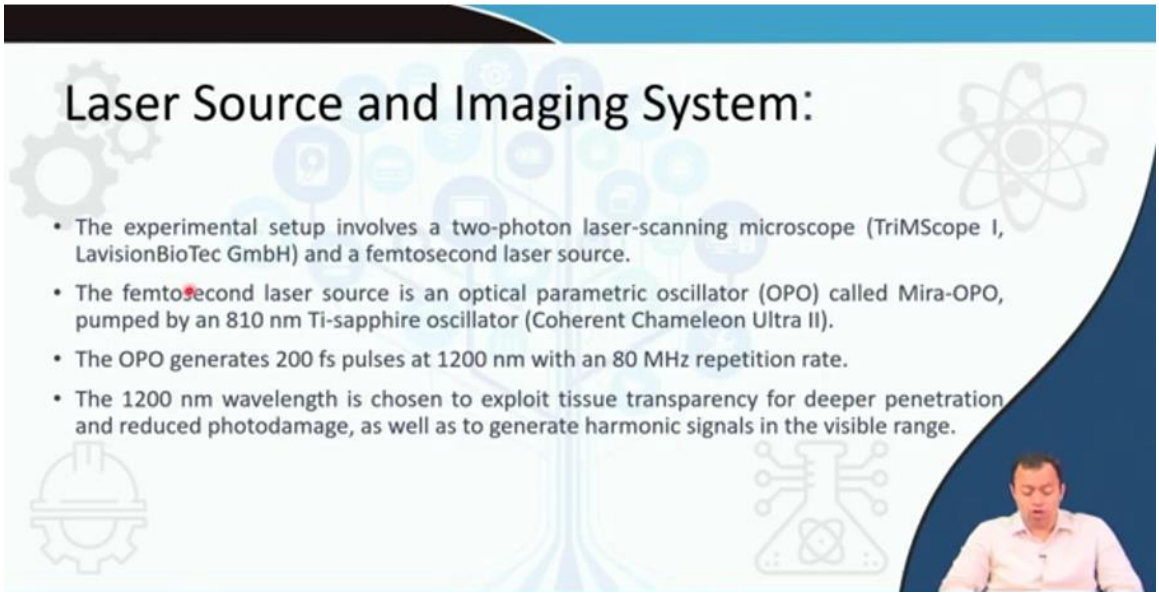
harmonic generation only few areas collagen etcetera shows this 2-photon nonlinearity second harmonic generation. But more or less third harmonic generation could be shown its very very weak, but could still be detected from almost all materials almost all with a underlined of the term almost yeah only few biological material exhibit second harmonic generation whereas, a large number of biological materials generate third harmonic generation.

So, even if you have a complicated structure like the brain you are not only detecting collagen which can be given by second harmonic generation, but you are trying to detect everything collagen, proteins, this, that, neurotransmitter, blood vessels etcetera for non non non brain and you want to see weak effect second harmonic generation by definition is weak effect, but now a days we have sensitive enough detectors to measure this 3 omega 3 times this omega h omega 1 plus h omega 2 plus h omega 3 and try to map that entire area and the thing is that this 3 omega will only be generated if omega 1 omega 2 and omega 3 are simultaneously there otherwise it will not work and you are ensuring that it is coming from a specific area because you have focused the light on that specific area itself of the tissue. So, that is it if you have understood this then you understand the overall process of third harmonic generation.

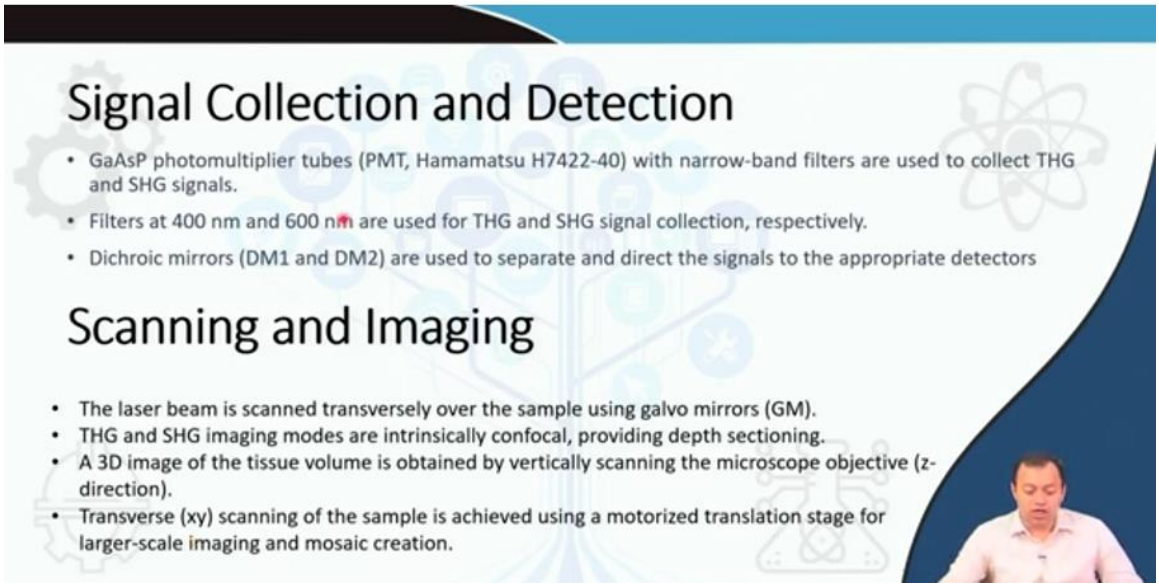


So, third harmonic generation and second harmonic generation are both non-linear optical processes they depend on non-linearity coefficient chi 3 and chi 2 of the tissue third harmonic generation 3 incident photons are converted in second harmonic generation and incident photon pair meaning 2 are converted intrinsic proteins can also generate 2 and 3 photon excited fluorescence signal when a sample is illuminated with near infrared light third harmonic generation second harmonic generation and multi photon auto fluorescent signal generate distinct harmonic and broadband you can depending on your money you

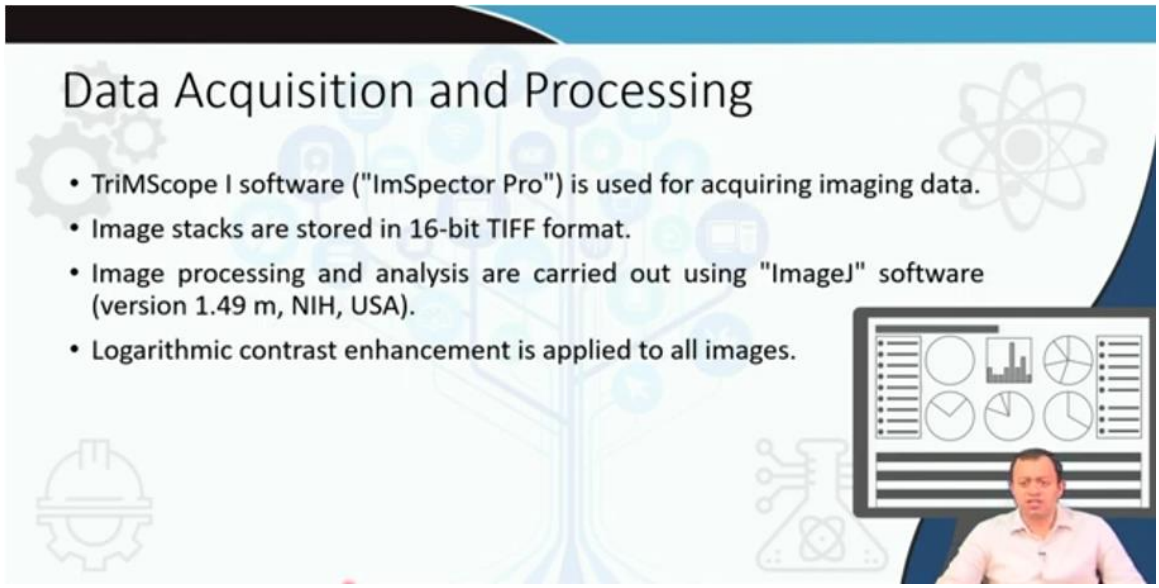
can simultaneously give 2 photon generation 3 photon generation fluorescence infrared whatever and then try to map the entire brain something or other will work these signals are detected separately using narrow band interference filters provided you have enough money to generate 3 photons accurately 2 photons accurately both together this 2 photon laser and this 3 photon lasers are different from one another and they do not have an overlap and then you have fluorescence etcetera. So, you need to have 2 photon laser scanning microscope a femtosecond laser is an optical parametric oscillator this is this is something that we use. So, the pulse exist for few femtoseconds only.



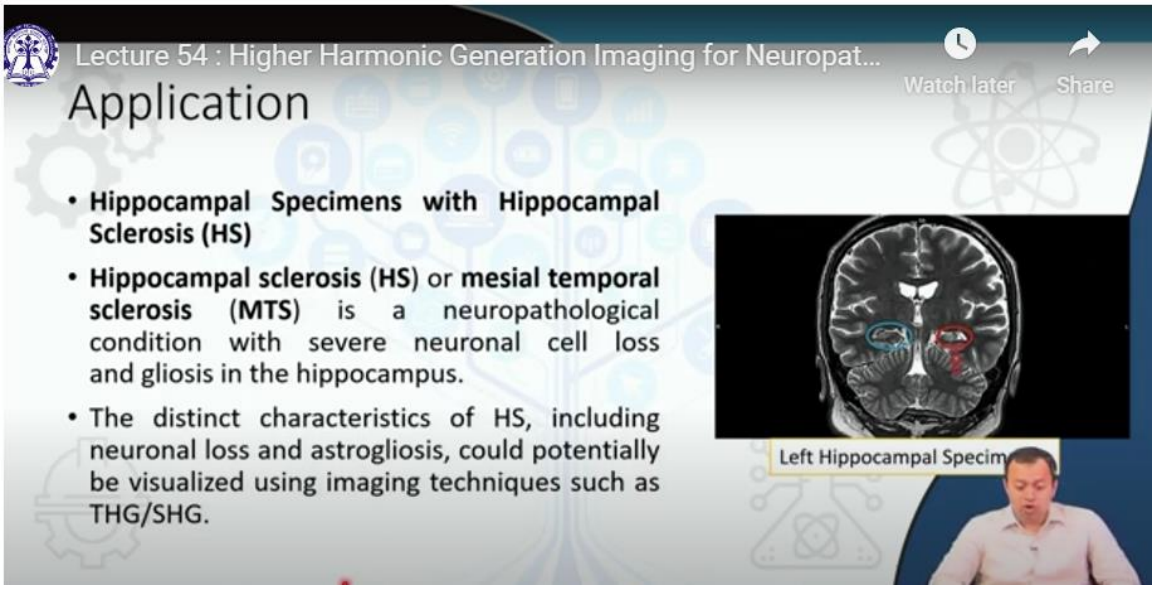
So, that you ensure that you are not damaging the entire cellular mechanism entire cellular state the optical parametrical oscillates you know 200 femtosecond pulse at around 1.2 micron with 80 megahertz repetition rate and that 1.2 micron 1200 nanometer wavelength is chosen to exploit I told you the tissue transparency for deeper penetration and reduce photo damage as well as generate harmonic signals in the visible range right.



So, pulses such as these can be utilized for third harmonic generation second harmonic generation. We can have a photomultiplier tube we can have filters of 400 nanometer and 600 nanometer for you know TGH and SGH mirrors will be required and you need a scanning and imaging system it is scanned transversely over the sample using mirrors TGH and SGH imaging modes are intrinsically confocal providing depth sectioning I told you about confocal the out of focus areas can be removed out of focus beams can be removed the 3D image of tissue volume is obtained by vertically scanning the microscope in z direction transverse scanning of the sample is achieved using motorized translation stress.



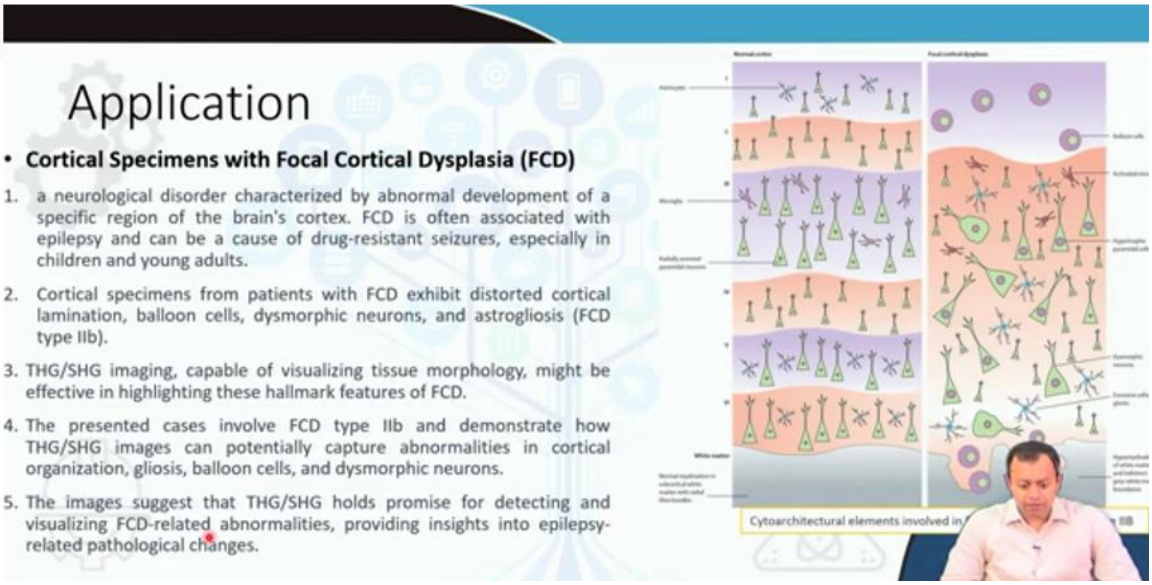
So, these are these are these are the standard procedure data acquisition is through usual software logarithmic contrast enhancement is applied to all images this is standard microscopy enhancement techniques. So, where do you use it? Hippocampal Sclerosis,



Hippocampal Sclerosis or Messier Temporal Sclerosis again I have absolutely no idea what that disease particularly is I assume this is bad Sclerosis is something of an I think tumor and Hippocampal probably is at the hippocampus this is a specific area of the brain the distinct characterization of Sclerosis including neuronal loss and astrogliosis could potentially be visualized using TGH and SGH. Yeah so, a loss of neurons I forgot to tell you a loss of neurons or you know presence of misfolded proteins around the neurons which are characteristic feature of dementia neurodegenerative disease such as dementia Alzheimer's Parkinson's. So, you either have you know loss of neurons a smaller number of cells less amount of nerve cells or the neurons are producing misfolded proteins, proteins whose confirmation has severely changed. So, imagine if a protein has misfolded so, neurodegenerative disease mostly like Alzheimer Parkinson's are characterized because of the presence of misfolded proteins.

Again we still have not yet been confirmed that whether this misfolded protein is the reason of the disease or because of the disease the protein has misfolded we do not know is it a cause or it is the effect, but nevertheless a normal folded protein and know with a normal confirmation will have different third harmonic generation than a misfolded protein why because the structure has changed. The structure has changed just like your allotropy the atomic arrangement has changed the electron cloud surrounding the atoms the complicated atoms of the proteins have now redistributed themselves differently and when they are subjected to intense laser light they will go and they will form different virtual states yeah that is how it works that is how the entire process of non-linearity works. They will perform they will go at different stages and thereby they will produce different 3 omega third harmonic generation or second harmonic generation. So, this you contrast with a normal protein. So, the electron cloud of a normal neuron normal protein versus the electron cloud of the same protein that has misfolded whose atomic structures whose

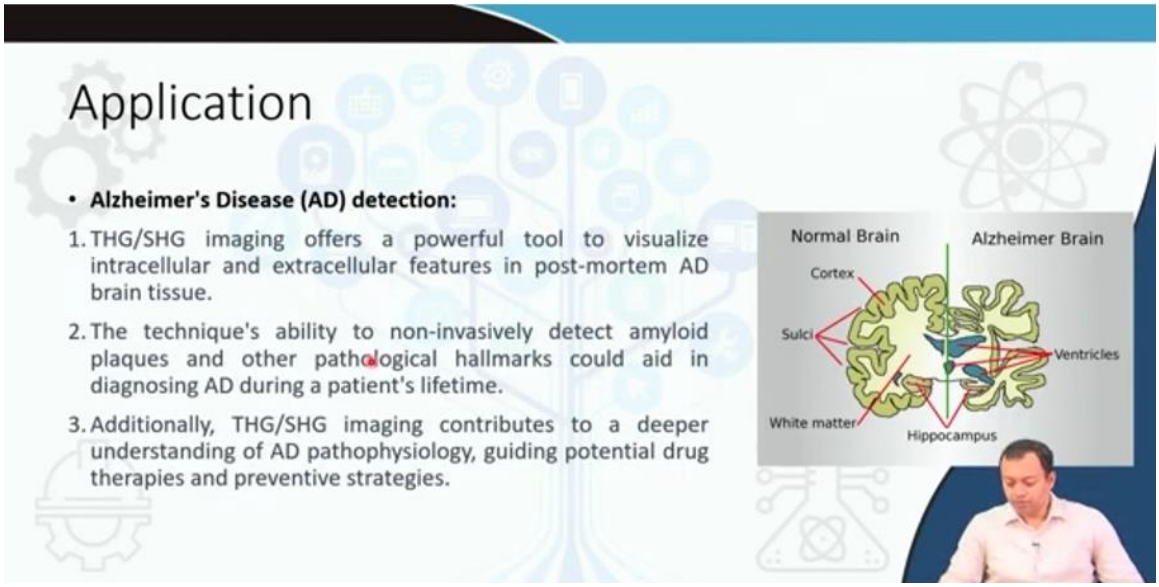
molecular structure whose bending all of those things the lattice if you use the term although sometimes proteins crystallizes, but lattice may or may not be the correct term yeah, but nevertheless you get the point that molecular arrangement is changed.



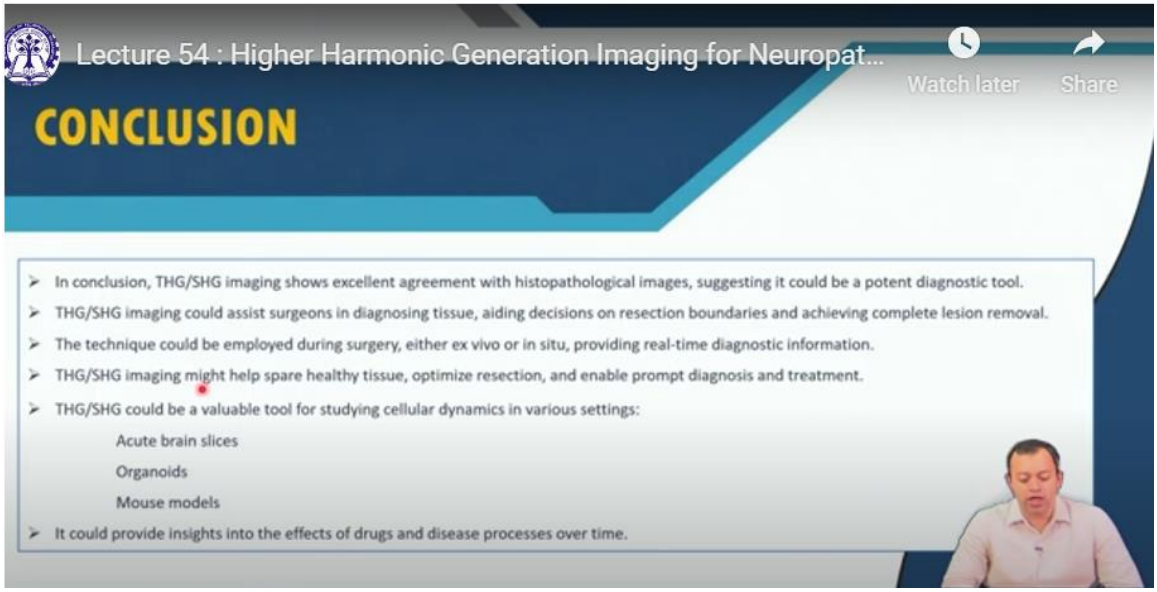
So, now you are able to differentiate using non-linearity right. There are 1001 applications again go through this particular reference Lancet is a very very prestigious journal for in the field of medicine go through this and you will get the you know total application I cannot pretend to tell you that I understand all of it focal cortical dysplasia. Dysplasia is type of cancer, but it is actually the cells becoming you know elongated and like some sort of a spindle formation I see this in oral cancer, but again I am not an expert into brain tumor or brain cancer, but dysplasia is some of the earliest stages of cancer. A neurological disorder characterized by abnormal development of specific region of the brain cortex. FCD is often associated with epilepsy and can be cause of drug resistant seizures especially in children and young adults.

Cortical specimen from patients with FCD exhibit distorted cortical lamination, balloon cells as I said spindle or balloon type cell. Dysmorphic neurons the structural integrity of the neurons have been compromised and I do not know what this means. Astrogliosis, astro is star. So, maybe they have astral is the term from which star or tar, tara etcetera comes.

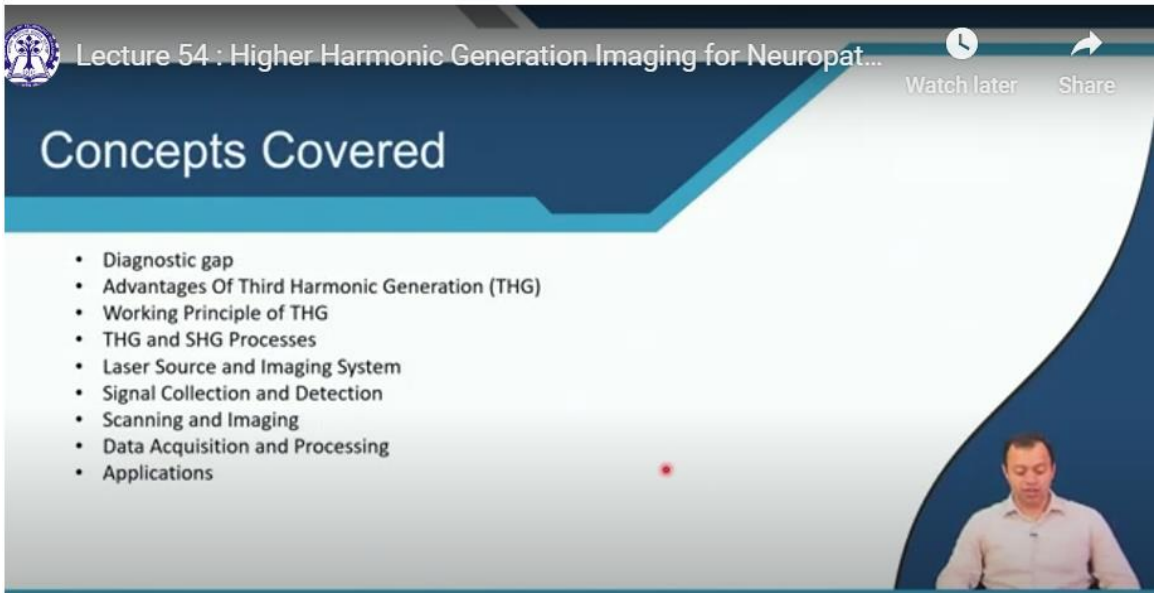
So, so it is like spreads out. So, like this yeah like starring of cells, but this is only coming from my linguistic knowledge rather than my medical knowledge. So, THG SHG imaging capable of visualizing tissue morphology might be effective in highlighting these hallmark features. The present case involves FCD type and demonstrate how third harmonic, second harmonic can be potentially capture abnormalities and therefore, it holds promise for detecting. Please please go through this paper it is quite fascinating right.



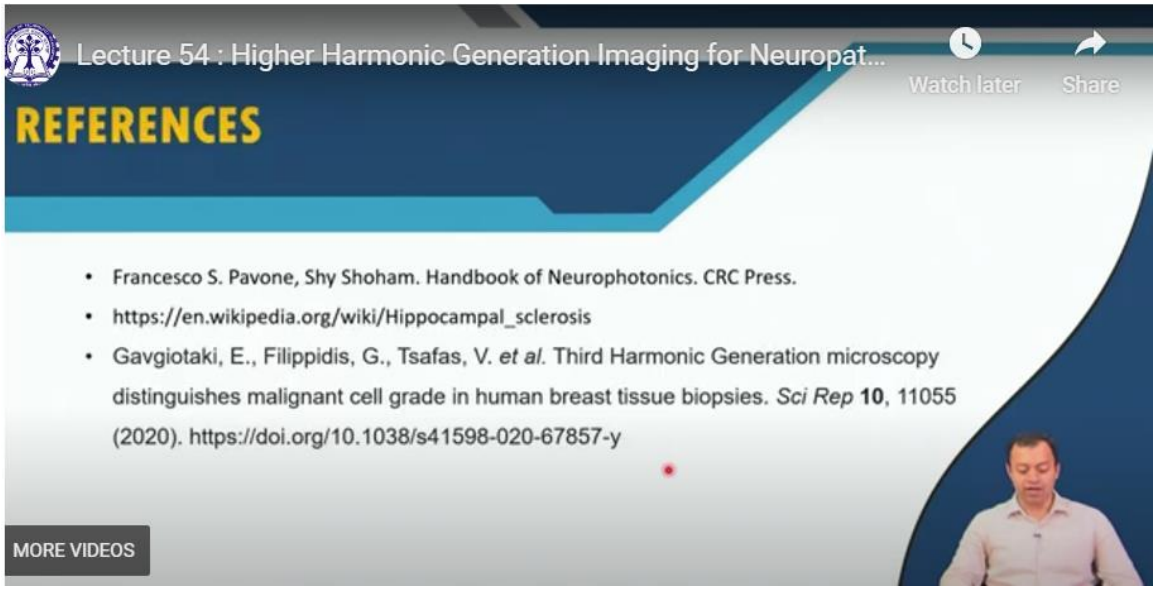
Of course, if it is Alzheimer disease where you see significant amount of misfolded proteins as compared to a normal brain it can noninvasively detect amyloid plaques you know formation of amyloids.
Amyloids are this mis formed misfolded proteins and other pathological hallmarks could aid in diagnosis of Alzheimer disease during patient's lifetime. Similarly, third harmonic generation, second harmonic generation image contributes to a deeper understanding of AD pathophysiology. So, imagine just imagine patient has open patients brain has been opened up or tumor has been detected they are trying to see it, but at the same time you are sending laser light at different areas of the brain opened up brain when the patient is in the bed stretcher under anesthesia under strict medical observation and not only you are able to see the tumors and anything else you are also able to see if the different areas of the brain have started developing this amyloid plaques right. So, along with tumor while the surgery is been going on using laser light focused at different areas and this is very fast laser light is very fast within you know few minutes few hours you can have the potential to map the entire brain and come to some sort of a better understanding that it is not just tumor that is wrong with this patient there are so many other things that is equally wrong with this patients right.



So, the conclusion is third harmonic generation second harmonic imaging shows excellent agreement with histopathological images they could assist surgeons in diagnosing issues imaging might help spare healthy tissues optimize resection and enable prom diagnosis and treatment presently we need to utilize it on mouse models and on organoids how many of you know what organoids are this is something fascinating please please read about organoids they are artificial as well as miniaturized organs that we can develop in a petri dish and know they are they are living I mean they are functioning organs it is not a replica it is not a model it is not made up of clay we can create miniaturized version of organs most organs in a petri dish they are called organoids read about organoids this is not part of nano biophotonics, but organoids is something that is going to change your entire medical field pretty soon you develop individual organs in a petri dish miniaturized living functioning organs in a petri dish and work on it put medicine put laser light and try to see it and then when this simulated this this this artificial organoid thing has worked you can then you know try to utilize similar techniques on to living person previously we used to sacrifice mouses for that, but now you can have a specific organ in a petri dish and just that organ along with you know blood vessels etcetera with proper media and culture and all of that thing you can utilize it is for it is property modification it could provide insight into the effect of drugs and disease processes over time.



So, these are the concepts that we discussed today and these are my references please go



through the lancet paper that I discussed and I will see you in next class. Thank you very much.