

DIFFERENTIAL LASER-INDUCED PERTURBATION SPECTROSCOPY
FOR ANALYSIS OF BIOLOGICAL AND BIO-RELATED MATERIALS



By

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To my loving family

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Laser-based diagnostic tools have been under research since the use of lasers in clinical medicine, with the main target to find common reliable tools for early diagnosis of degenerative diseases such as cancer. For this reason, a novel laser-based optical sensing scheme, differential-laser induced perturbation spectroscopy (DLIPS), is developed which combines common spectroscopic methods such as Raman spectroscopy and fluorescence spectroscopy with UV laser perturbation via difference spectroscopy. Ultraviolet (UV) laser perturbation is used in this work at very low intensities to induce permanent photochemistry. The novel technique has high potential in detection of abnormalities in tissue in early stages. Additionally, the new method is expected to be a popularly used tool with less patient-to-patient variation and having higher sensitivity and specificity rather than traditional spectroscopic schemes. The research has been designed to develop and maximize the performance of the DLIPS method, and eventually enable it to be used in applications in pathology, including *in vivo* tissue analysis.

The work herein covers the continuation of fundamental research on DLIPS and newer steps to advance its performance on cancer detection to further clinical

applications. Three main studies will be presented including DLIPS realized with a Raman probe, DLIPS realized with a fluorescence probe, and DLIPS used to detect cancer from human skin tissues in comparison with a fluorescence probe. The DLIPS is realized with the Raman probe *in situ* for the first time and its performance is evaluated by classifying the proteins and dipeptides, L-Alanine, Glycine, L-Proline, Ala-Gly, Gly-Gly, Gly-Pro, which are the basic building blocks of biological molecules. Accordingly, a 40% improvement on classification is observed in these Raman studies. The DLIPS is realized with fluorescence probe by exciting the endogenous fluorophore, L-Phenylalanine, L-Tyrosine and L-Tryptophan, mixtures with 193 nm, and perturbing them with 193, 220, 230, and 245 nm wavelengths. Overall a 20% improvement on classification is observed in the fluorescence studies. Finally, DLIPS is compared with a fluorescence probe by exciting/perturbing skin samples with 193 nm. The application was successful in yielding differences in spectral features, but quantitatively did not outperform fluorescence probe for tissue classification.

CHAPTER 1 INTRODUCTION

Motivation

The first step to cure of any disease is to identify its presence inside the body. A curiosity on detection of formation of any disease has initiated many developments on probes for biosensing applications. Being an alternative to their traditional widely accepted similar-biosensing techniques, recently popular accurate and robust diagnostic methods such as laser-based spectroscopic methods have undergone many improvements in order to detect early signs of diseases, especially cancer. But, the drawbacks of these methods still impair the method's detection performance by mostly affecting the selectivity and the sensitivity of the method for clinical applications. Early detection is highly beneficial in treatment of mortal diseases (i.e. cancer), and may prevent their rapid progression. Additionally, the identification of the stage of the cancer is important to cure cancer. Many existing optical diagnostic methods for cancer tend to be tedious and painful, as well as they are not successful enough to precisely detect cancer in its early stages *in vivo*. Because of all these limitations, laser-based diagnostic methods are getting more popular due to their highly informative, non-invasive, and easily applicable nature for *in vivo* detection. Furthermore they are widely open for the new developments rather than their alternative biosensing probes.

Accordingly, in this dissertation a novel laser-based diagnostic method, which may overcome previous complications in traditional laser applications and may yield higher sensitivity and specificity, will be presented in detail. Specifically, the uniqueness of the novel method emerges from the rich interactions of UV laser irradiation with biological samples at low intensity level. In this chapter, a general picture of whole

background relevant to our study will be drawn. First of all, the different impact mechanism of UV light on biological and some synthetic materials will be presented. Then, the general information and the limitations of the pre-existing biosensing methods such as fluorescence spectroscopy, and Raman spectroscopy will be explained. Hopefully, their limitations will motivate the necessity of using novel difference spectroscopy approach. At the end of this chapter, previous studies on our novel difference spectroscopy method will be presented.

Principals of Lasers and Current Laser Technology

Lasers, which can be simply represented by radiative transitions between quantum energy levels, are light sources that emit highly coherent, directional, monochromatic, and intense beams of light. The laser process is originated from stimulated emission of electrons moving from higher energy level to a lower energy level while population inversion is reached. Regardless of the technology where the gain is established, lasers have similar concepts in terms of their operating processes. All lasers have gain medium as an amplification media, highly reflective cavity as a feedback mechanism and pumping mechanism. Lasing starts once threshold population is reached between the laser transition quantum levels.

Classification of lasers can be put in two different groups which are continuous wave (CW) lasers and pulsed lasers. Continuous wave lasers have an output power that stays constant during lasing operation providing that population inversion is established all the time. On the other hand, pulsed lasers have a higher gain than the threshold level for short repetitive times during operation. The pulsed operation can be controlled by pulsed excitation which requires a triggering mechanism. The intensity of the pulses can be amplified by Q switching and mode locking techniques.

Excimer lasers, a key focus of the study presented in this dissertation, having an active medium called exciplex are the rare example of two level lasers. They are categorized into electrically pumped gas lasers. They emit in ultraviolet region of an electromagnetic spectrum and their wavelengths only differ based on the active gas filling the laser medium. Most common excimer lasers are argon fluoride (193 nm), krypton fluoride (248 nm), xenon chloride (308 nm), and xenon fluoride (351 nm). Fluorine laser (157 nm) is also used in addition to these common wavelengths but is requires a special beam path under vacuum because this wavelength is immediately absorbed by air over small distances. Their single pulse energies are up to 1J and their repetition rates can reach to 500 Hz. Excimer lasers have standard applications in biology and industry including eye surgeries, semiconductor fabrication process, and engraving of materials in high precision.

Introduction to Laser and Tissue Interactions

Lasers interact with tissues in a wide variety of ways, which many different parameters can determine the interaction mechanism. For instance, all materials, including tissues, have the optical properties (i.e. reflection, absorption, and scattering) that play an important role on the interaction mechanisms. Such tissues are living matters so their interaction mechanisms and threshold values are also dependent on other properties, for example heat conduction and heat capacity. On the other hand, laser properties are itself important in terms of determining how much energy has to be delivered since optical properties are highly wavelength dependent, notably in the UV range. These radiation properties are explained by exposure time, fluence, irradiance, and intensity.

The general picture of the laser tissue interactions is shown in Figure 1-1, where the interactions are divided into four main groups. The photothermal and photochemical interactions are generally the result of continuous wave irradiation, whereas the electromechanical and photoablative interactions are the result of pulsed irradiances. The coordinates of the map are plotted in logarithmic scale and y-axis of the map shows the irradiance or more commonly power density in W/cm^2 , and x-axis shows the exposure time. The enclosed areas of the groups are rough estimations based on previous works. Couple of deductions can be made from the Figure 1-1. The data aligned on a diagonal show constant fluence at $1 \text{ J}/\text{cm}^2$ and $1000 \text{ J}/\text{cm}^2$. The inverse relation between the power density and time indicates a required standard energy dose to start laser tissue interactions. The dose of energy is a determining parameter in this case. The groups which are aligned on the constant fluence diagonal can be separated by duration of interaction.¹

Three relative main laser tissue interactions will be discussed in this section including photochemical interactions, thermal interactions, and photoablation. Specifically, this study main focus will be dedicated to the region in the vicinity of $1 \text{ mJ}/\text{cm}^2$ diagonal and also adjacent to photoablation region (i.e. nanosecond time region) for the living tissues and simple biological components. Common laser treatment applications in medicine have been established by taking advantage of these effects. Mainly the effects are separated by the laser beam energies (i.e. emission wavelength) and fluence that causes either damage, cutting, vaporization, or chemical effects. Either one of these effects or combination of these effects can be observed in

laser applications used in medicine. Both continuous wave (CW) and pulsed laser applications will be overviewed while mentioning these laser-sample interactions.

Photochemical Interaction

After being irradiated by a light source, a targeted molecule can undergo one of several chemical processes. For example, if the light source delivers a photon with sufficient energy, the molecule can reach an excited state which results in possible chemical reactions. Photochemical interactions occur at relatively low irradiance levels ($\sim 1\text{W}/\text{cm}^2$) and become one of the very advantageous tools in biological sciences. For example operations utilized photochemical interaction mechanism may selectively destroy unhealthy tissues in the body without affecting the healthy ones. Photodynamic therapy (PDT) is a classic example where injected drugs inside the body are selectively uptaken by target tissues (e.g. tumors), and can absorb photons from the light sources. The process generates reactive oxygen species which leads to an inevitable cell necrosis within the target tissue. These injected drugs inside the body are called photosensitizers, which can be activated by exposure of monochromatic light sources to turn into toxic materials. It should be noted that photosensitizers stay inactive before the exposure so they can distribute inside the body till they attach to their targeted unhealthy zones. Photosensitizers can be eventually rejected from the body over time causing the decrease in their concentration inside the body. On the other hand, unhealthy tissues like malignant tumors are likely to store these injected light activated materials longer than healthy tissues. Sometime after injection, PDT starts when the concentration of the photosensitizers inside the healthy cells are sufficiently low. For example Hematoporphyrin, one type of the photosensitizer, was only absorbed by

tumor cells. It was introduced for photodynamic treatments because of its fluorescence detection and light activated destruction.²

The PDT technique has been widely used in treatment of tumors of different parts of the body especially for the neck and head tumors.³ In recent clinical applications of PDT, diode lasers are being taken over dye lasers since they are cheaper, easy to handle, have less complexity. In clinical applications of PDT, tumor area can be illuminated directly via optical fibers especially for thin tumors, if the tumor can easily be seen on the skin surface. On the other hand, fibers equipped with diffuser tips can be implemented into tumors inside the body if tumor is under the skin and relatively huge. Sometimes tumor is removed and treatment is applied to the tumor bed area.

Briefly, low laser energies are preferred for photodynamic therapies (such as output of dye lasers in the range of 1-4 W and supplied irradiance lower than a few hundred mW/cm²). The energy causes destruction of photosensitizers absorbed by malignant tumor cells but no destruction occurs due to the laser energies. Generally since the exposure times are relatively long (around thousand seconds) thermal effects do not occur since there is a time for thermal conduction to the surroundings.

Thermal Interaction

This type of laser-sample interaction is very important in a way that it is a measure of a successful method which is the one staying away from thermal effects as much as possible. On the contrary, thermal effects are actually preferred for some clinical applications because of its advantages. Several different types of destructive impacts are considered via thermal interaction, which are generally happens as a process of changing the physical state of the tissue or somewhat impair its physical and

chemical structure. Coagulation, vaporization, carbonization, and melting processes can be put into this interaction category. Thermal interactions are considered when the local temperature of the tissue exceeds the acceptable levels, and understanding these interactions requires thermal models to be built depending on the tissues.

Thermal interactions start initially as a result of absorption of a beam energy by the irradiated molecules. The absorption of photons increases the kinetic energy of the molecules within penetration depth, which is followed by dissipation of this energy through inelastic collisions with adjacent molecules. This results in an increase in the kinetic energy of the surrounding molecular structures. This process is non-radiative and leads to a rise of the temperature at the irradiated area, and in the extreme, it is useful in tissue cutting with lasers or hemostasis.¹ The summary of the effects of the temperature ranges on the tissues can be seen from the Table 1-1.

Temperature of the tissue increases due to the absorption of the incident light by water molecules or other macromolecules inside the tissue such as pigments, and proteins. Specifically, heat generation is mostly caused by water molecules since their absorption coefficients are very high at the laser emission wavelengths commonly used in these applications related to thermal interactions.⁴ For YAG laser and CO₂ laser emissions, absorption coefficients of water become significant. In addition to the IR regime, water has a notable absorption coefficient corresponding to the emission wavelength of ArF laser. Vaporization effects of the lasers can be realized where the water molecules inside the tissue layers change phase under incident irradiation. During phase change local pressure increases quickly and may cause micro explosions, which are commonly seen in dental operations with lasers, and referred as thermomechanical

effects. For instance, this kind of side effect is observed while assessing the effectiveness of CO₂ laser therapy used in treatment of cervical intraepithelial neoplasia (CIN).⁵ Laser energy is delivered to create 5 to 6 mm of laser vaporization zones, and the effectiveness of the operation was observed 97.6% of 256 cases.

The values of optical properties of thermally impaired tissues are changing while temperature increases at the coagulation process.⁶ In order to show this relation, CW Nd:YAG laser is used to deliver energy to liver, myocardium, and prostate samples to create coagulation zones. Three different outputs (1064 nm, 532 nm, and 355 nm) of Q switched Nd:YAG laser are used at the different sections of experiments. During the energy transfer, some non-invasive probing techniques including optoacoustic and diffuse reflectance are used to monitor real time changes in the value of the optical properties of tissues such as total diffuse reflectance, effective attenuation, absorption, and reduced scattering coefficients. It was observed that the values of all those properties distinctively increase after coagulation. These changes in optical properties start at around 53°C, and then they drastically keep rising through the temperature around 70°C. Clearly, laser irradiation may leave a notable effect on biological tissues which generally seen as a side effect.

Photoablation

Unlike other tissue removal methods, photoablative decomposition or shortly photoablation, which is consistent with the use of excimer lasers at the ultraviolet region, is an etching process which is realized by disrupting chemical bonds, generally without causing a significant thermal damage to the surrounding structure. This phenomenon is first observed with polymer studies.⁷ By giving this introduction, the details of effect of the photoablation on polymers will be discussed in the following

section while in here only the biological applications related with photoablation will be presented. For all these types of samples, the laser interaction is termed photoablation. As mentioned earlier in this chapter, excimer lasers cover most of the ultraviolet region such as argon fluoride (ArF; 193nm/6.4 eV), krypton fluoride (KrF; 248nm/5eV), and xenon chloride (XeCl; 308nm/4eV), where these UV wavelengths are well absorbed by most of the biological samples.¹ Excimer lasers are all pulsed lasers and typically have an average penetration depth of 1μm or less, and are capable of delivering sufficient energy to the macromolecules (such as proteins) to disrupt their molecular bonds. These lasers are popular in tissue cutting operations because of short pulses (10-100 ns), and their high beam energies enable operators to etch biological materials without leaving thermal effects at the surroundings.⁸ These strong advantages of ArF excimer lasers are first realized while testing the possible usage of ArF laser for the eye surgery operations, specifically, ablation of cow eyes with 193 nm.⁹ The results agreed with the expectations that the low pulse rate from 1 to 20 Hz, and pulse energy of 1 J/cm² ablates precisely 1 μm without leaving any thermal effects at the surroundings

The most common biological applications of this process can be found in eye surgeries. Today, there are two common procedures available in the eye surgery operations: photorefractive keratectomy (PRK) and laser *in situ* keratomileusis (LASIK). These procedures are being performed in order to correct one's vision defects. Both procedures exploit excimer lasers to ablate the tissue. In both operations after removal of corneal epithelium layer, 193 nm excimer laser is used to reshape the corneal stroma by removing some tissue due to the ablation, without resulting in any damage at the adjacent stromal tissues. The effectiveness of both LASIK and PRK are comparable

and no major differences have been found of these methods yet. Only significant problem is noted as recovery pain which is longer in PRK operations.¹⁰ The ablation rate, which is a parameter demonstrating the amount of material removed for per pulse, is significantly important in eye surgery operations such as PRK and LASIK operations. Accordingly, the ablation performance of 213 nm and 266 nm radiations are investigated on the porcine cornea to evaluate the performance of using these wavelengths for the eye surgery operations.¹¹ As expected, at these wavelengths, which are longer than 193 nm, leave a thermal damage at the surroundings. But 213 nm left a relatively small damage zone, which the thickness is lower than 1 μm . In addition, ablation rate of 213 nm is found 0.3 μm per pulse in clinical applications which is similar to the ablation rate of the wavelength of 193 nm in eye operations.

Photochemical constants such as photodecomposition and photoionization yields are calculated by using a 193 nm laser beam with low fluence (~ 17 to ~ 50 mJ/cm^2) directed to liquid cells of collagen components.¹² In many cases, photodecomposition rate is found to be higher than the photoionization rate and linear relation is observed between the amount of the incident photons and photoionization rate. At this low fluence range peptide bond cleavage is also reported.

The analytical determination of optical properties such as absorption coefficient of particular tissue can be aided by rigorous modelling. For example, estimation of the absorption coefficient for the wavelength of 193 nm cannot be accomplished precisely only with static absorption model which utilizes static Beer-Lambert law. The Beer-Lambert law is given by Eq. 1-1.

$$I(x) = I_0 \exp(-\alpha x) \quad (1-1)$$

In Eq. 1.1, I_0 is the laser beam intensity incident at the sample surface ($x=0$), $I(x)$ is the beam intensity of the laser beam which is a function of penetration depth x (cm) in the sample. α is the constant with respect to time for a given concentration which is called absorption coefficient (cm^{-1}). The law states that for a given particular wavelength and a solute, molar absorptivity of a substance is constant over time and only proportional with a concentration when the light approaches at right angles. So by measuring the transmission and the light path length through sample, the absorption coefficient can be calculated for a particular substance. However, a static Beer-Lambert law is valid at lower intensities of a light where estimated absorption coefficient from these intensities is referred to as a small-signal absorption coefficient. Instead, a dynamic model is first built which agrees with the published data for the ablation rates and absorption coefficients of cornea and collagen.¹³ This dynamic model is constructed by considering time and location dependency of absorption coefficient composition of collagen, water, a transient absorber, and a stable non-absorber. To build a model, differential form of Beer-Lambert law and rate equations are used. Quantitatively 25% to 50 % improvement is observed compared to the statistic values in absorption coefficient predictions. Moreover, the necessity of using low pulse repetition rates for the eye surgery operations are questioned by comparing the results with the high pulse repetition rates (up to 400 Hz) of 193 nm excimer laser.¹⁴ The detailed analysis on the ablated zone, and plume analysis revealed that there are no significant differences between using low (60 Hz) and high (400 Hz) pulse repetition rates.

In summary, the interactions of ultraviolet laser irradiation of the living tissues falls into photoablation class where the process leads to several chemical effects to be

occurred accompanied with a negligible thermal effect. Noting that, this section and the following section on polymer research are connected to each other but, they are divided in terms of the materials used in research. Generally UV laser-polymer interactions are studied as an early effort to understand the underlying mechanisms of these interactions for the irradiation of living tissues. The discussion is limited to ultraviolet (UV) lasers, since UV laser beam energy, especially 193 nm beam ($h\nu = 6.4\text{eV}$), is sufficient to disrupt the molecular structure of the biological materials effectively and leaving no trace of thermal effects. These lasers primarily cause bond disruption rather than thermal effects which can be utilized for the statistical assessment purposes.

Excimer Laser Interaction Research with Polymers

Polymers, synthesized from smaller molecular unit called monomers as a result of using different types of polymerization techniques, are often used as surrogates for investigation of laser ablation processes and effects. After discovering the clear etching process by using the short pulses (repetition rate: 1 Hz, pulse duration 12 ns) of far-ultraviolet (193 nm) laser radiation, a detailed model is constructed to understand ablation kinetics and crater formation under irradiated area by Garrison and Srinivasan. It is assumed that the splitting products due to the photodecomposition have higher specific volume than the polymer which is not photo-decomposed. So scattered mass transformed from the sample is explained by an explosion model. Because of this reason early research of photodecomposition is performed with polymethyl-metacrylate (PMMA, $(\text{C}_5\text{O}_2\text{H}_8)_n$) since it has a similar behavior of specific volume change during phase change. The polymer is modelled by layers, which are formed by face-centered cubic (fcc) crystalline elements, of structureless monomer components. During

irradiation, small amount of monomer units are assumed to react photochemically and other monomer units are assumed to be excited by the laser irradiation. Since the energy of 193 nm laser beam (6.4 eV) is higher than the energy of the strong attractive monomer bonds, irradiation of these layers initiates break down process by changing the bond potential between these monomer units from attractive state to the repulsive state. The monomer units which chemically interact with the incident beam are freed from the main structure. These units are assumed to be a member of approximately 500 layers per pulse. In the model, kinetics of the photoablation was modelled by Newton's equation of motion. In addition to explosion model, thermal model is built to explain photothermal effects of the 193 nm laser and different lasers with longer wavelengths. It was assumed that, laser energy is absorbed into vibration modes and distributed into kinetic energy to the surroundings. Eventually, it is observed that the photochemical process etches a neat crater, while on the other hand the photothermal process causes melting and distortion due to temperature increase around the irradiated area. The velocity of the ejected material is estimated to be 1000-2000 m/s. Ablative and thermal effects of 193 nm and 532 nm lasers can be seen from the Figure 1-2. The 193 nm laser light leaves no traces of thermal effects while 532 nm laser light does cause thermal effects. The phenomena can be understood in detail by looking at the electronic transitions of organic materials.^{7, 15-17}

Photoablation is defined as a transient process and shown on a profile with three different regions: pre-ablation, ablation and post-ablation with the help of 193 nm laser delivered at 200 mJ/cm².¹⁸ Ablation starts when the incident laser beam intensity reaches the ablation threshold intensity for that particular sample. Here, ablation is

assumed to be a spontaneous etching process where it has a moving interface rather than explosion model. Moving interface speed is calculated from incident laser intensity, threshold intensity, and dynamic absorption coefficient. The ablation process is modelled with transient Beer Lambert law and ablation threshold is calculated from volumetric analysis. By using quartz crystal microbalance technique, etch depth and fluence dependence is obtained for a region of interest by using mean absorption coefficient and rate constant for different polymer types. So by knowing the total energy deposited on the sample, the maximum etch depth can be calculated.

The values of optical properties of the polyimide such as transmission, reflectivity, and scattering changes during ablation hence, the excimer laser pulse induces transient effects on these properties. The changes in optical properties are investigated with a setup containing ArF excimer laser and dye lasers.¹⁹ 461 nm, 520 nm, and 695 nm dye lasers are employed in order to probe optical properties of the samples. Time delay is generated between excimer laser and the probe lasers in order to obtain transient optical property data points up to 600 ns. Significant changes on the properties are observed in first 150 ns after the excimer laser pulse. Later, the properties return to initial values as measured before the excimer laser pulse. For instance, in first 20 ns transmission increases about 8% of its initial value whereas the reflection of the incident beam also decreases about 8%. This similar change is attributed the change in refractive index of the polyimide layer. Monitoring optical changes of the polyimide during ablation is conducted with the laser wavelength of 193 nm.²⁰ Reflectivity of the polyimide layer decreases with increasing fluence even below the ablation threshold. The value of the reflectivity decreased around 20% to 40% of the

initial value (obtained before the irradiation) over the fluence range from 75 to 175 mJ/cm². This also implies the decrease of the absorptivity, since the population of the excited states changes while applied fluence changes unless the saturation limit is not reached. In other words, the responsible mechanism for the change of the reflectivity is attributed to the saturation effects of the polyimide film. Optical transmission behavior of the polyimide is monitored over the fluence range between 10mJ/cm² to 10 J/cm² for the 193 nm, 248 nm, and 355 nm lasers.²¹ In addition to these, a two-level theoretical chromophore model is constructed in a simplest case where all chromophores of the polymer are assumed to be identical in order to seek a theoretical curve for the experimental fluence versus etch depth data. Transmission ratio is obtained by dividing high fluence transmission by the low fluence (low signal) fluence transmission. It was observed that transmission increased 5 fold for 193 nm laser beam where the value is still far away from the theoretical maximum transmission for this wavelength. Transmission at 248 nm is also increased which is 10 fold but the value is very close to the theoretical maximum transmission value at this wavelength. However transmission decreases almost 50% for 355 nm laser wavelength. Theoretical chromophore model is found to be successful for the sufficient prediction of etch depth for the presented fluence region.

Photochemically bond breaking under the irradiation of XeCl Excimer laser is investigated with a Raman microprobe by taking Raman spectrums of thin polyimide films before and after the irradiation.²² The results of irradiations with 2000 pulses at a fluence of 20 and 40 mJ/cm² is showing that the bond at 1783 cm⁻¹ is decreasing after the irradiation which is more distinguishable after the higher fluence irradiation. In

Raman data, unusual broad heads emerged at 1336 and 1593 cm^{-1} which is an indication of graphite crystal formation and characteristics of disordered carbons. This work is a proof that Raman probe is a useful tool in monitoring effects of UV radiation such as photo-induced chemical effects on the polymer samples.

Ultraviolet excimer laser ablation is dependent on the absorption performance of the polymers; consequently the molecular composition of the materials plays an important role on the absorption coefficient. The absorption of poly(tetrafluoroethylene) (PTFE, $(\text{C}_2\text{F}_4)_n$) after doped with polyimide (PI), where the doping process increases the absorption performance of the polymer, is highly dependent on the dopant concentration for the laser wavelengths at 248 nm and 308 nm.²³ This shows that concentration of ultraviolet absorbers inside the materials plays an important role in ultraviolet absorption and etching. As a result, the micrographs clearly indicates temperature effect for both lasers, and the longer wavelength yielded more thermal degradation of the etch sites. Another study shows that laser ablation changes the surface morphology of the irradiated materials. The conic shapes resulting from the irradiation are observed with many different monitoring methods such as Fourier transform infrared spectroscopy, time-resolved emission and X-ray photo-electron spectroscopy, atomic force microscopy, and from the contact angle measurements.²⁴

So far, general picture of the laser-sample interactions are drawn especially for UV lasers. The advantages of these interactions are highly important, so knowledge of these interactions benefits for most of the applications in medicine. Specifically, thermal interactions are mostly avoided in these applications where thermal interactions emerged minimally in photoablation. In the following section, widely popular laser based

bio-sensing methods will be mentioned. These spectroscopic techniques will be explained with their high advantages and their deficiencies.

Traditional Bio-Sensing Methods

Fluorescence

Fluorescence phenomena can be well understood by looking at Jablonski Diagram of a molecule which shows all process of deactivations for an excited molecule. Transitions between ground singlet electronic state, first, and second electronic states (S_1 and S_2) are shown in Figure 1-3. Transition times have crucial role on these deactivations as well as arbitrary quenching. Fluorescence is a traditional transition and always occurs at longer wavelengths than the absorption ²⁵, except in the case of two photon absorption. Fluorescence starts with an excitation of a molecule by absorption of photons. This kind of excitation of a molecule with a light source is termed as photoluminescence and it takes a total time in femtoseconds (10^{-15} seconds). Then the excited state electrons arrive to the lowest energy level of this excited state via vibrational relaxation in picoseconds (10^{-12} seconds). Eventually, molecules return back to arbitrary vibrational levels of ground state by emitting ultraviolet or visible light, which is called fluorescence, within a period in nanoseconds (10^{-9} seconds).

Advantages and disadvantages

Fluorescence spectroscopy is one of the useful pathological tools in order to be used in a diagnosis of a disease or imaging instrument in medical applications. It is non-invasive, functional, easily adaptable, highly sensitive tool which can be used to detect early stages of cancer. Both organic and non-organic molecules may exhibit fluorescence, and biological materials which are invisible under regular monitoring applications can be identified with their fluorescence spectrums. Moreover, excitation

and emission spectrums of fluorescent molecules and many different fluorogenic reagents increases the method's selectivity. The method is compatible with many different diagnostic tools so that it can be used for *in vivo* imaging and may accompany finely to well-equipped laboratory environments.

Sensitivity and the specificity are the ratios that give an idea of the statistical method's performance and the accuracy of the application. Sensitivity is referred as a true positive rate and specificity is referred as a true negative rate. Thus, fluorescence detection is preferred for clinical applications because it is highly sensitive tool and a fast way of detection of pre-cancerous tissues.²⁶ In fluorescence related applications, generally sensitivity of the application tends to be higher; however, specificity of the application tends to be lower. So it is desirable for fluorescence data to have sufficient specificity while still maintaining the high sensitivity. For instance, common ways to overcome this problem are either labelling target tissues with fluorogenic dyes or combining different optical acquisition techniques.²⁷⁻³⁰ When recent advanced acquisition techniques including custom-made fiber optical probes, highly efficient microscopes, and samples prepared with elaborate labelling combined with fluorescence spectroscopy, the precision of fluorescence detection can be increased significantly. Nevertheless, the fluctuation in the data may hurt the specificity of the method due to the patient to patient variations, or performance of the operator at the application of the protocols. Even if the dyes still increase the specificity, they might bring other difficulties to the experiments such as they can cause a change in physical properties of the biological materials when they attached to them.

Autofluorescence and exogenous fluorescence detection are the two types of diagnosis method. In autofluorescence detection, signal is originated from endogenous molecules including aromatic amino acids, nicotinamide adenine dinucleotide (NADH), collagen, flavins, chlorins, porphyrins and their derivatives. On the other hand in exogenous fluorescence detection, exogenous fluorophores including chlorins, phthalocyanines, or 5-aminolevulinic acid (ALA), a natural precursor of protoporphyrin IX are injected inside the body to bind target molecules or tissues to acquire a selective signal.^{26, 31} Using direct methods is more preferable; however, fluorescence background is undesirable for most of these applications in biology. Alternatively, some compounds that are of research interest do not fluoresce in their natural state. Because of these problems exogenous fluorophores have been under research for a long period and they promise better specificity and sensitivity. For instance, fluorescent dyes called Alexa are developed and their performance is compared with fluorescein which is a commonly used fluorophore.³² It is observed that, the new set of reagents produced more intensive signals and they are more resistant to photobleaching. Recently popular fluorogenic reagents called quantum dots promise better sensitivities with the help of increasing nanotechnology.³³ Quantum dots are basically nanostructures with great optical properties such as high extinction coefficients. They tend to be much brighter than dyes and they can resist photobleaching for much longer periods. The size of quantum dot determines band gap energy of quantum dot so its emission color which makes them favorable for fluorescent applications. Then can be very sensitive in tracking of the biological molecules inside the body.

Biological applications of fluorescence

Common fluorescence spectroscopy applications in medicine include imaging and diagnosis of cells and tissues both *in vivo* and *in vitro*. Additionally, the optical properties of organic and inorganic substances are well studied with fluorescence microscopes. The early stages of fluorescence applications were initiated in order to track antigens by taking the advantage of marking antibodies with fluorescein isocyanate, which is a common type of exogenous fluorophore, monitored under yellow-green light.^{34, 35} The main reason of using exogenous fluorophore is that antibodies conjugated with this specific fluorophore kept unchanged during antigen-antibody interactions.

After many improvements, the performance of fluorescence spectroscopy has been tested in recent *in vivo* studies to detect cancerous formations in their early stages. *In vivo* fluorescence detection of internal organs is generally accomplished with guided fluorescence by using specific tools such as special custom hoses and fiber optics.³⁶⁻³⁸ Endogenous fluorophores are commonly accompanied to these studies. Approximately, sensitivities between 30% and 60% for the *in vivo* fluorescence detection are reported. The signal quality of the *in vivo* studies are tend to be lower because of the information is highly varied with respect to the chemical composition of the probed area and distorted with broadband fluorescence responses, useless information from unknown sources, noises, and multiple emissions.

In one of landmark study, fluorescent characteristics between malignant and normal tissues are analyzed by taking advantage of natural fluorophores (i.e. flavin and porphyrin) in the living cells to identify health conditions of cells.³⁹ In this study, visible luminescence spectroscopy is performed on the native animal tissues. Tumors are

implanted into anaesthetised experiment animal group, with special interest is given to the rat kidney, bladder, and prostate. The main purpose of the investigation is to identify the characteristic features which have the significant effect on the difference between the normal and abnormal tissue's spectra. As a result, the strong peak of 521 nm is found to be an indication of cancerous tissue whereas the strong peak of 531 nm is an indication of healthy tissue.

In a relevant study, cancer is induced on rats in order to detect colorectal cancer in its early stages.⁴⁰ Urine sample sets in different concentrations are analyzed under different fluorescence excitation wavelengths. Significant difference is observed at the excitation wavelength of 300 nm which is attributed to tryptophan inside the urine samples. Sensitivity and specificity are reported 76% and 92% respectively. In another *in vivo* study, Meth-A fibrosarcoma cells are implanted into laboratory mice in order to induce cancer.⁴¹ The target tissues are excited by using NIR fluorescence (785 nm) light source. Sensitivity and specificity are reported 93.8% and 87.5 % respectively.

Notably, aromatic amino acids inside the body such as phenylalanine, tyrosine, and tryptophan have significant role on cancer studies, are capable of fluorescence in their nature structures. The wavelength of absorption bands ca. 295 nm for tryptophan, 280 nm for tyrosine, and 256 nm for phenylalanine can be used to induce fluorescence to monitor *in vivo* events related with peptides and dyes. The fluorescent peaks are at ca. 350 nm for tryptophan, 303 nm for tyrosine, and 282 nm for phenylalanine, respectively.⁴² Phenylalanine and tyrosine can be resolved in the absence of tryptophan, whereas tryptophan is easy to resolve since it's the dominant in terms of fluorescence among others. These aromatic amino acids are mainly under interest in

auto fluorescence studies and their non-standard levels may be the early sign of abnormal formations inside the body, and may indicate the pre-neoplastic lesions.⁴³⁻⁴⁵ For instance, high levels of aromatic amino acids in gastric juice were an indication of gastric cancer due to excessive production in cancerous tissue.⁴⁶

Raman Spectroscopy

When beam radiation interacts with matter, many further events happen beyond absorption or direct transmission of the beam. The beam can be refracted, reflected, or scattered. Only the last one has a meaning for the analytical technique of Raman. The scattering of the beam causes either the scattered radiation which has a same frequency (i.e. elastic) with the incident radiation or which has a different frequency (i.e. inelastic) with the incident radiation. Elastic scattering is referred to Rayleigh scattering, whereas inelastic scattering may correspond to Raman scattering. In Raman scattering, the changes in the frequency are induced by rotational and vibrational transitions in molecules. The scattered photons can be either initially in the ground vibrational state or in the lowest excited vibrational state. These shifts build a Raman spectrum with several Raman peaks describing molecules. In Figure 1-4 two possible outcomes of Raman scattering are shown. If the emitted photon has a lower energy than the absorbed photon, it is called Stokes Raman scattering, if the emitted photon has a higher energy than the absorbed photon is called anti-Stokes Raman scattering.

The important difference between Raman and IR activity can be stated as; in IR spectroscopy, there is always a change in the dipole moment during vibration, while on the other hand in Raman spectroscopy, there is always a change in the polarizability during vibration. Polarizability of a molecule is a property that dictates how an incident electromagnetic wave can induce a dipole in the molecule.

Advantages and disadvantages

Raman spectroscopy is a popular probing tool in medical applications due to its non-destructive, minimally invasive nature plus it provides deeper information about molecular structures. The technique does not need any injection of materials such as fluorescent dyes for *in vivo* applications. Characteristic Raman peaks in the Raman spectra enable this technique to distinguish different molecules and label them by providing information about their chemical bonds. Application of Raman spectroscopy has a similar approach like fluorescence spectroscopy in a way of detection of signals. Raman spectroscopy is a great candidate in biological applications such as diagnosis of malignancy of the cells, acquiring chemical information of DNA, RNA, proteins, lipids, and several bio-components in tissue, monitoring interactions of these biomolecules, and labelling cells and bacteria.⁴⁷

Raman spectroscopy can be used to diagnose specific abnormalities of the body, but may be accompanied by unwanted fluorescence background. This background is an important issue in Raman studies and may mask the useful information and may impair the quality of the Raman spectroscopy results. Excitation wavelength is considered to be important parameter that can reduce the fluorescence background. To understand the effect of excitation wavelength on fluorescence background, crystals in the synovial fluid of joints, which is called Gout disease, are excited with different wavelengths between 532 and 785 nm.⁴⁸ It can be immediately realized from the confocal microscope images that the fluorescence background is almost vanished at the longer wavelength (785 nm). Both output Raman spectra from the custom and OEM Raman systems in this *ex vivo* study support the idea that the excitation with 785 nm has the

lowest fluorescent background. Overall, fluorescence is greater at shorter wavelengths, notably short visible and UV.

The Raman signal is weak in its nature, in another words Raman spectroscopy has a low signal to noise ratio due to the high fluorescence background compared to Raman signal, so the Raman peaks are hard to resolve especially for biological samples. To overcome this problem several techniques are developed to enhance Raman signals. One way to increase Raman signals is known as Resonance Raman Scattering (RRS) or Resonance Enhanced Raman Scattering (RERS or RR). Enhancement is accomplished by selecting excitation at the center wavelength of an electronic transition of the interested molecule or crystal. By this way enhancement is provided via resonance effect. The technique is initially used to probe pigment molecules in intact plant tissues.⁴⁹ The application of the technique remarkably increased the signal efficiency of several vibrational modes of the pigments despite the fluorescence background and losses.

Surface Enhanced Raman Scattering (SERS) is one of the most powerful method for acquiring better Raman signals where the samples are deposited onto a roughened metal surfaces or absorbed by nanostructures such as silver, copper, gold, and several alkali metals. In this technique Raman signal is can be increased to a higher intensities then its natural intensity. The phenomenon is explained by two globally accepted mechanisms: electronic and chemical enhancement. Electronic enhancement is the main reason for the enhancement process. That is, coherent electron oscillations present at the interface between two materials called surface Plasmon becomes resonant with both laser and Raman fields which amplifies the

Raman signals after surface Plasmon is excited by laser. The laser wavelength should be resonant with the surface Plasmon. On the other hand, the chemical enhancement is an auxiliary process that can explain several low enhanced cases for particular molecules.⁵⁰

Biological applications of Raman

Highly sensitive confocal resonance Raman spectroscopy, where the system has a high numerical aperture microscope objective to avoid sample destruction, used to acquire Raman signals from the living single cells using 660 nm excitation laser with the labelling technique.⁵¹ The study showed that Raman method was a good candidate for monitoring activities inside single cells. In another study, near infrared (NIR) Fourier transform (FT) Raman spectroscopy is used to diagnose the atherosclerosis disease in human aorta with an excitation wavelength of 1064 nm.⁵² It is shown that different peaks that are resolved from different sites of the aorta from various patients mostly emerged from protein vibrations between relative shifts of 1200 cm^{-1} and 1600 cm^{-1} . The differences between Raman spectra of normal and diseased aorta are analyzed by comparing several characteristic peaks of these vibrational modes. Normal aorta shows vibrations including protein vibrations, amide I vibration, and C-H bending vibrations. Various plaques and calcifications are managed to be monitored, as well as elastin, collagen, cholesterol, cholesterol esters, lipids, carotenoids, and calcium apatite deposits are recognized by this technique. Eventually, the significant peaks indicating difference between normal and fibrous plaque specimens are identified as the vibrations of C-H bending band, and elastin.

Single bacteria detection is also accomplished with Raman spectroscopy. Micro-Raman setup with an excitation laser at the wavelength of 532 nm is used to acquire

Raman spectra.⁵³ The statistical classification is handled by method called support vector machine (SVM). SVM is a part of machine learning processes and it has a some kind of intelligent loop system to update probability based on new instances. In this method, problem is divided into two different classes and classes are divided by a model which tries to draw a best line between these two classes. Baseline correction, normalization, first derivative, and median filtering methods are used as a pre-processing step to reshape the data to turn into a more meaningful input data. In this study for nine different bacteria species sensitivities between 89.2% and 93.6% are reported by using SVM algorithm.

Undifferentiated embryonic murine stem cells (mES) is monitored *ex vivo* while they are going through differentiation process.⁵⁴ Three different transition states of these cells (undifferentiated cells, differentiated cells by two different mechanisms) are able to be identified by their *in vitro* Raman spectrums after applying principle component analysis (PCA) and hierarchical cluster analysis (HCA). Common pre-processing methods such as baseline correction by fitting fifth order polynomial, a Savitsky-Golay smoothing filter, and normalization are applied. No single miss-match is observed for three different classes in this study. In other words, all samples pertaining to one of these three categories are also correctly identified with statistical analyses.

By generating biochemical fingerprints from tissue samples, Raman spectroscopy could be a great diagnostic tool for cancer diagnosis. The possible advantage of using Raman spectroscopy to diagnose cancerous cells is tested by generating pseudo-color Raman maps from a hundred principal components of the data acquired from basal cell carcinoma (BCC).⁵⁵ BCC and its adjacent non-cancerous

tissue spectrums are compared based on Raman maps and H&E stained sample images in conjunction with K-means clustering analysis (KCA). As a result, different clusters are identified with high selectivity (93%). Accordingly, guided Raman signal is generally used for *in vivo* studies. Raman endoscopy is used to diagnose gastric cancer.⁵⁶ Ant colony optimization (ACO) technique, which is similar to PCA, is used to build models from Raman spectra from tissues. NIR (785 nm) excitation light is used for this study. Sensitivity and specificity are reported 89.3% and 97.8% respectively.

Microcalcifications can be traced with a Raman spectroscopy as a symptom of breast cancer. Microcalcifications are the aberrant formations within breast indication of cancer. Mammographic methods are not so powerful to distinguish benign and malignant formations. Three different groups including normal tissue, lesions with and without microcalcifications are identified by constructing a linear model in the first step. Additionally, subgroups of lesions such as fibrocystic change (FCC), fibroadenoma, cancer are also identified in the second step with the more detailed linear model.⁵⁷ It should be noted that, this linear model is a combination of spectra of ten different basic breast tissue components where this model is specific to this research. Raman technique is advantageous in this kind of application because its feasibility to be easily applied clinical diagnosis where it can accompany stereotactic needle biopsy in conjunction with compatible tools to facilitate the flexibility of the diagnosis. Raman data is collected with an excitation source wavelength of 830 nm. Single step SVM algorithm is found to be successful in identifying normal tissue and lesions regarding microcalcifications. The results are analyzed based on statistical factors which are resulted for single step as sensitivity (SE) of 62.5%, specificity (SP) of 100%, positive

predictive value (PPV) of 100%, and negative predictive value (NPV) of 95.6% for the diagnosis of breast cancer (AUC 0.92). On the other hand the optimized 2-step algorithm results a SE of 56.3%, SP of 100%, PPV of 100%, and NPV of 94.9% for the diagnosis of breast cancer. Leave-one-site-out cross-validation was applied for both algorithms.

Most of the cancer studies are performed on animal tissues. In a related work, Fourier transform (FT) Raman spectroscopy is used for oral carcinoma diagnosis.⁵⁸ Oral carcinoma is induced chemically on hamsters. And the samples are examined *ex vivo*. PCA is used to classify between two groups such as normal tissue and cancerous tissue. Sensitivity and specificity are reported 100% and 55% respectively. In another similar study, PCA and linear discriminant analysis (LDA) are used to build statistical models by using *in vivo* Raman spectra from chemically cancer induced marine animal tissues.⁴¹ Sensitivity and specificity are reported 81.3% and 100% respectively.

Data processing methods for Raman spectroscopy

The interpretation of Raman signal is not always straightforward process, since Raman data may be collected from many different devices, may have high fluorescence background, or may have irregular baseline shapes. This raw data could affect the result of the statistical analysis and it can mask some important information. After cleaning the data with pre-processing methods, data is ready to be an input for the appropriate statistical analysis. By this way the characteristic pathways inside the data can easily be realized. In this section common statistical methods for vibrational spectroscopy along with pre-processing methods will be discussed.

During acquisition, broad fluorescence signal accompanied to useful information causes an unwanted background in the data. This results in irregular baseline shapes.

The general correction of this problem is to fit a low-order polynomial (up to third order maximum) as a new baseline for the collected the spectra and subtract the area under fitted polynomial from the Raman spectra.⁵⁹ Classical polynomial baseline fitting is reliable and it ensures correction without hurting Raman peak contours. Different fitting techniques can also be used in order to obtain better fit such as least-squares-based polynomial fitting. Other common pre-processing methods including normalization that the method tries to fit all samples on the same scale and smoothing that the method tries to reduce the effect of random noise are used to prepare more meaningful dataset.⁶⁰

Using spectroscopic methods may introduce large datasets acquired from the high number of measurements. Obviously, interpretations of such large datasets are very tedious and time consuming. Instead of analyzing high number of raw data, Principal Component Analysis (PCA) is preferred where the goal is to compress the most of the useful information of the large datasets into few components called factors by explaining the data with new variable space. PCA is a technique that rebuilds new axes and reveals the inter-sample relations in a more effective way by highlighting similar and different patterns inside the data. Perpendicular dimensions of the new axes are called principal components (PCs) or factors. The first PC explains the maximum variation inside the dataset, and then the variation decreases at the following PCs. Then the variables distributes inside the new variable space. The perpendicular distance between a sample and a PC is called a score of that sample. Since the PC is constructed from the contributions of the original datasets, scores are important scales for the interpretation. By this way whole data can be redefined and analyzed easily by

taking advantage of new scores and factors of the method. PCA is typically used for classification purposes in biological sciences. For example distinguishing normal T and B cells from the cancerous ones is accomplished by taking advantage of the usage of principal components.⁶¹

Hierarchical Cluster Analysis (HCA) is used for exploratory analysis of the data. The interrelations of the variable and samples can be more sensitively visualized with HCA's two-dimensional plot called dendrogram. These dendrograms show linkage paths between samples and distinguish analytical clusters within large dataset. For instance the three different groups can be immediately identified of the mES cells from their dendrogram, which is seen from the Figure 1-5, as previously mentioned at the Notingher's work.

In summary, the diagnostic applications of traditional spectroscopic schemes along with common statistical methods have been reviewed. The unique advantages of these laser-based techniques motivate researchers to exploit these techniques for particular clinical applications; however the disadvantages or the limitations trigger them to find new modifications for these techniques. Each of them has found unique areas of usage and they are still under development. However, the results show that none of them have been globally accepted yet. Still, there is a chance for newer laser-based bio sensing sensors in diagnostic applications. In the next section, the background and early studies of recently proposed laser-based technique will be revealed.

Introduction to the Differential Laser-Induced Perturbation Spectroscopy Technique

Evolution of spectroscopic techniques and their feasibility for effective monitoring of cellular activities inside the body, as well as their high resolution capabilities and their

minimally invasive nature, have gained lots of interest in medical applications such as diagnosing of multiple diseases or performing laser surgeries. However current clinical applications of these spectroscopic methods have limitations including patient-to-patient variations, low signal nature, high complexity of the system, rich signal environment and thermal side effects. Specifically, while Raman spectroscopy mainly suffers from low signal-to-noise ratios, fluorescence spectroscopy mainly suffers from photo bleaching cases with *in vivo* applications. Hence, no common laser diagnostic procedure has been established for *in vivo* applications yet. Above mentioned traditional spectroscopic methods are still under development. As a new solution to these drawbacks of others, differential laser-induced perturbation spectroscopy (DLIPS) is developed where interaction of the biological matrix with low intensity ultraviolet radiation, at non-invasive levels, may reveal a novel unique optical sensing scheme. The difference is induced by perturbation of the samples with an excimer laser, and the low intensity radiation allows this method to avoid destructive ablation effects. That is, low fluence irradiation of excimer beam causes preferable bond cleavage without leaving any thermal effects to the surroundings, by this way no ablation is realized. Consequently, the main advantage of DLIPS technique is originated from combination of traditional scattering effects of the excited samples accompanied with diverse UV laser-sample interactions. In this section early studies on the novel optical scheme will be presented in detail.

The Technique

DLIPS is first realized by Smith et.al. with traditional fluorescence spectroscopy by using a Q-switched, frequency-tripled Nd:YAG laser at the wavelength of 355 nm as an excitation source and an ArF excimer laser at the wavelength of 193 nm as a perturbation laser as basically shown in the Figure 1-6. The main idea of this application

is to probe the target area on the sample with 355 nm laser beam and to perturb the same area on the sample with the 193 nm laser beam. In order to accomplish that two lasers are concentrically superimposed at the same spot where the area of the 193 nm beam is set to be larger than the 355 nm. By this way, complete perturbation on the probe area is guaranteed. Initially, the fluorescence signal is collected from the spot area by exciting the sample with 355 nm laser, and it is called “pre-perturbation spectrum”. Then, the same spot area on the sample (without moving it) is irradiated with 193 nm laser beam. The perturbation causes bond cleavage on the sample spot. After certain time is introduced, a dark signal is acquired without delivering any excitation pulses to the sample. At the end, a second fluorescence signal is acquired by exciting the sample with 355 nm laser, and it is called “post-perturbation spectrum”.

In the optical path both laser beams are coaxially aligned by using a dichroic mirror as a beam combiner and superimposed on the thin films of samples on the quartz plates and mice skin. The higher diameter of perturbation beam ensures complete perturbation of the entire fluorescence probe area as well as homogenous perturbation on the target area. The fluorescence emission of the sample is reflected by a pierced mirror positioned at 45° to the target plane and focused on the fiber optic bundle. Before entering the fiber, two filters are used to allow only the light within spectral window between approximately 390-500 nm. Then the light is passed to a 0.3 m Czerny-Turner spectrometer and recorded with an intensified CCD (ICCD) array detector synchronized by delay generator to the laser pulse. By this way difference of the fluorescence signal could be computed.⁶²

Previous Applications

In the first *ex vivo* study, to show the effectiveness of DLIPS method by using low intensity deep-ultraviolet excimer laser at the wavelength of 193 nm as a perturbation laser, several features of the novel method are examined on the thin films of samples deposited on the quartz plates.⁶³ The performance of the DLIPS method is evaluated in four steps:

1) At the initial study, a mixture of Coumarin450 (named shortly C450, $C_{13}H_{16}NO_2$) and BBQ ($C_{48}H_{66}O_2$), which are organic dyes, are used in DLIPS studies. As a procedure of DLIPS, pre-perturbation spectra before perturbation and subsequently post-perturbation spectra after perturbation are acquired and saved. In order to perturb samples, a total of 250 laser pulses of 193 nm beam with the pulse energy of 100 μJ /pulse corresponding fluence of 3 mJ/cm^2 are delivered to the probed area. The results are presented in Figure 1-7. The marked peaks correspond to characteristic fluorescence bands of the two dyes: peak A at 403nm corresponds to BBQ, and peak B at 440 nm corresponds to C450. While the intensity of the peak A corresponding to BBQ shows almost no change after perturbation, the intensity of peak B corresponding to C450 decreases significantly. As a conclusion, the difference in the decrements of the peaks showed the selectivity of the DLIPS method.

2) Another supplementary study is performed by using a solution of dissolved collagen (Type III, Sigma C3511) trapped in a UV-grade transmission cell to quantitatively observe the change in number of peptide bonds in a solution during perturbation. A 193 nm perturbation beam at the fluence of 10 mJ/cm^2 and a 355 nm perturbation beam at the fluence of 9 mJ/cm^2 are delivered to the transmission cell. According to the results, only the cleavage of peptide bonds, which is a decrease in

number of peptide bonds, is realized with the perturbation with 193 nm laser.

Consequently, it is showed that 355 nm laser beam has no effect on the peptide bonds.

Illustration of the setup is shown in Figure 1-8 along with the results. 193 nm laser beam is proved to have a sufficient energy (6.4 eV) to cleave peptide bonds.

3) A trial study of DLIPS method realized with Raman probe is performed at the next step. Pre-perturbation spectrum, which is an initial Raman spectrum acquired before UV perturbation, and post-perturbation spectrum, which is a spectrum acquired after UV perturbation, of the glycine-glycine thin films are acquired with a confocal micro-Raman spectrometer (JY Horiba LabRam) with 632.8 nm excitation wavelength. During experiments, the target area is marked, and samples are carried back and forth between Raman instrument and perturbation setup. At the perturbation step, a total of 700 pulses (100 $\mu\text{J}/\text{pulse}$, 3 mJ/cm^2) of 193 nm laser beam are delivered to thin films. The difference spectrum is obtained after preprocessing is applied to the raw spectra. From the Figure 1-9 several differences at the Raman shifts can be identified. The marked characteristic peaks are reported as A, 588 cm^{-1} amide VI and C=O out-of-plane bending; B, 910 cm^{-1} C—C stretch; C, 1007 cm^{-1} CH₂ rocking; D, 1249 cm^{-1} amide III; E, 1408 cm^{-1} CO₂ symmetric stretch; F, 1447 cm^{-1} CH₂ deformation; and G, 1647 cm^{-1} amide I.⁶⁴

4) The 2D application of DLIPS method is realized with the imaging tools by using beam expander at the wavelength of 355 nm to excite samples. Patterned excimer laser beam at the wavelength of 193 nm (45 $\mu\text{J}/\text{pulse}$, 1.25 mm full-width Gaussian profile, average fluence of 3.6 mJ/cm^2) used to perturb samples. As seen from the Figure 1-10, while no direct difference can be seen by comparing pre and post

perturbation images at the first glance, the difference of these images reveals subtle perturbed pattern.

As a conclusion of the first study, the DLIPS method is very effective and promising diagnostic tool when used with fluorescence and Raman spectroscopy as well as imaging techniques. DLIPS method results in preferable bond cleavage for biological materials while no trace of ablation realized under the microscope. In addition, Raman study reveals that two spectra of DLIPS and traditional Raman differ from each other for some characteristic peaks.

The second study is accomplished *in vivo*, and DLIPS method is studied in order to diagnose skin tumors using a murine animal model.⁶⁵ The main aim of the study is determined to diagnose of the disease in its early stages by classifying the traditional fluorescence and DLIPS spectra of normal and cancerous tissues of the mice skin weekly. A tumor inside the mice skin is stimulated by ejecting a solution of DMBA (7,12-dimethylbenz(a)anthracene, Sigma-Aldrich, St. Louis, MO) dissolved in mineral oil (Fisher Scientific, Pittsburgh, PA) at a concentration of 0.5% w/w, which is applied topically to the dorsal skin of mice. The presence of tumors is proved by the images of H&E stained histology sections of skin. Simultaneously, DLIPS data are collected to compare with the imaging results.

During the experiments, a ND:YAG laser at the wavelength of 355 nm is used to excite tissues along with the ArF laser at the wavelength of 193 nm as a perturbation laser. 200 hundred shots (which corresponds to collection of 200 images) of low intensity (no ablation) excitation beam and a total of 2500 perturbation pulses (100

$\mu\text{J/pulse}$, 3mJ/cm^2) are delivered to the mice tissues *in vivo*. The illustration of the setup of DLIPS can be seen from the Figure 1-11.

Eventually, the DLIPS spectrum for a particular single spot is obtained by calculating the Eq. 1-2 for each spot. In the Eq. 1-2, $Em_{PRE}(\lambda)$ (absolute pre-perturbation spectrum) is subtracted from the $Em_{POST}(\lambda)$ (absolute post-perturbation spectrum). After that, the difference is dividing by the absolute pre-perturbation spectrum obtained by subtracting dark signal from pre-perturbation spectrum. Then the classification between DLIPS dataset and the fluorescence dataset is evaluated by using various multivariate analyses.

$$DLIPS(\lambda) = \frac{Em_{POST}(\lambda) - Em_{PRE}(\lambda)}{Em_{PRE}(\lambda)} \quad (1-2)$$

DLIPS performance in classification over traditional laser induced fluorescence is proved and illustrated by comparing the principal component analysis (PCA) scores and the receiver operating character (ROC) curves. In PCA, only the first principal component is of the interest since there are not much variation inside the fluorescence and DLIPS datasets. Score plots indicate that the samples collected by traditional fluorescence probe from the eleven week study are gathered closely in the same region, that is, no clear separation between different classes is realized. However, the DLIPS dataset from the eleven week study are distributed separately in the score plot. Only the score plot of the DLIPS dataset indicates an early change inside the probed volume which is proved by the H&E stained images. In addition, the loadings of the DLIPS data reveal different characteristic features between the control group and DMBA treated group. In ROC curves, DLIPS spectra show great predictions over

traditional fluorescence data especially between the 4th and 11th week period. The mouse skin has an important band between 400 and 420 nm which is attributed to collagen cleavage in extracellular matrix (ECM) by Kozikowski et al. The sensitivity of DLIPS (true positive rate) is little lower as compared to traditional fluorescence but its specificity (1-false positive rate) is remarkably much better than raw fluorescence data.

As a conclusion, DLIPS method is a strong candidate to diagnose cancerous formations *in vivo*. In this thesis, recent developments on DLIPS methods will be presented in proceeding sections.

Summary and Conclusions

As seen so far, laser light interacts with biological materials in different ways such that every single interaction mechanism has been used for different applications in medicine. Notably, UV light has a unique ablative effect on biological samples, which makes them highly favorable for clinical applications especially for eye surgeries. Generally, the 193 nm causes only photochemical ablation, however, higher UV wavelengths causes both ablation and thermal effects. The ablation process, such as penetration depth, is highly dependent on the absorption performance of the biological samples and beam wavelength. In the previous applications of our novel differential spectroscopy technique, cleavage effects of 193 nm are used for biological classification for the first time. Supplemental studies with polyester substrates are performed in order to understand UV light effects on the living tissues in detail. Current laser based probes are very good candidates of early detection but none have reached the full capacity yet. Because of these reasons, more about the DLIPS method and its performance on classification of biological materials will be explained in this

dissertation. By this way DLIPS can find its own path to be widely used in clinical diagnostic applications as a reliable stand-alone tool.



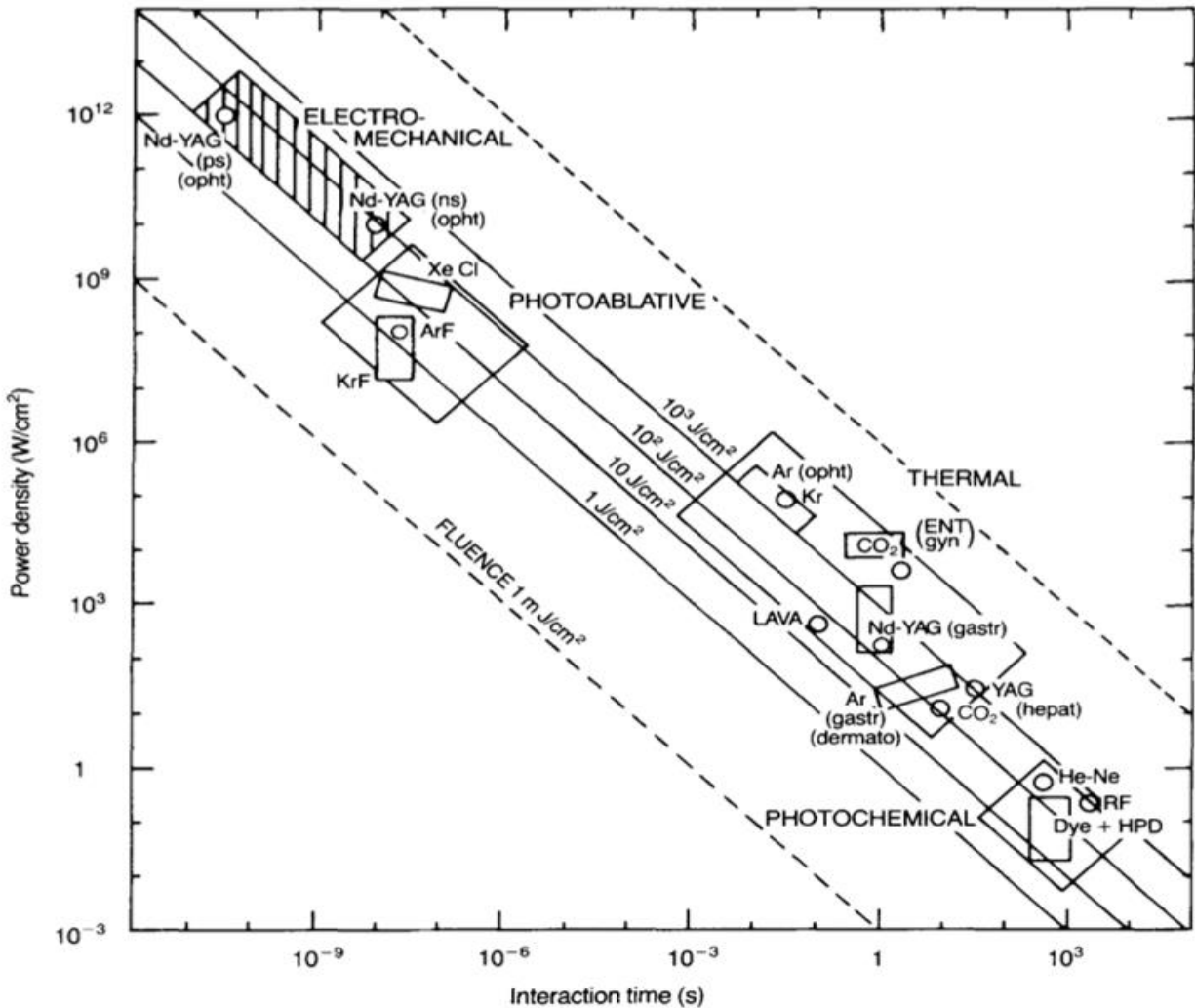


Figure 1-1. Map of laser-tissue interactions. Nd-YAG, neodymium-doped yttrium aluminium garnet laser; XeCl, xenon chloride laser, ArF, argon fluoride laser; KrF, krypton fluoride laser; Ar, argon laser; Kr, krypton laser; CO₂, carbon dioxide laser; Lava, laser-assisted vascular anastomosis; He-Ne, helium-neon laser; HPD, haematoporphyrin derivative. RF, radio frequency; ps, picosecond; ns, nanosecond; opht, ophthalmology; ENT, otorhinolaryngology; gyn, gynaecology; gastr, gastrology; dermat, dermatology; hepat, hepatology. The map was taken from Boulnois.¹

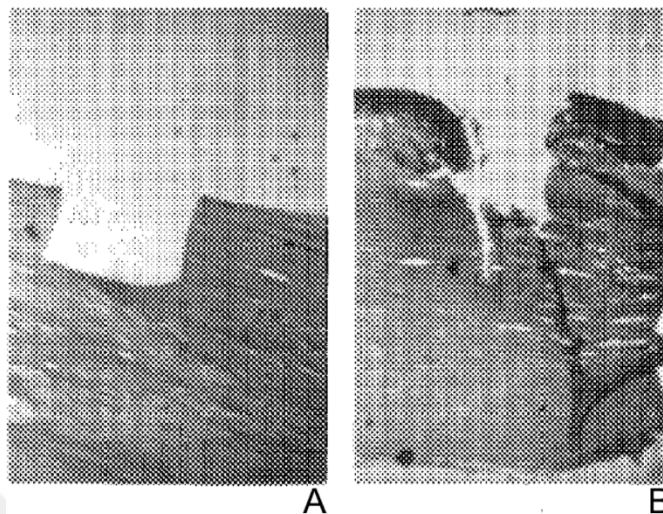


Figure 1-2. Cross section of the luminal side of an aortic wall. A) 0.35 mm trench was produced by far-UV (193 nm) radiation with a pulse duration of 14 ns. A total of 1000 pulses of fluence 2.5 mJ/mm^2 was applied to the sample. Approximately 2.5 J/mm^2 of energy was thus deposited. B) 4 mm crater was produced by visible (532 nm) radiation with a pulse duration of 5 ns. A total of 1800 pulses of fluence 10 mJ/mm^2 was applied to the sample. Approximately 18 J/mm^2 of energy was thus deposited.⁶⁶

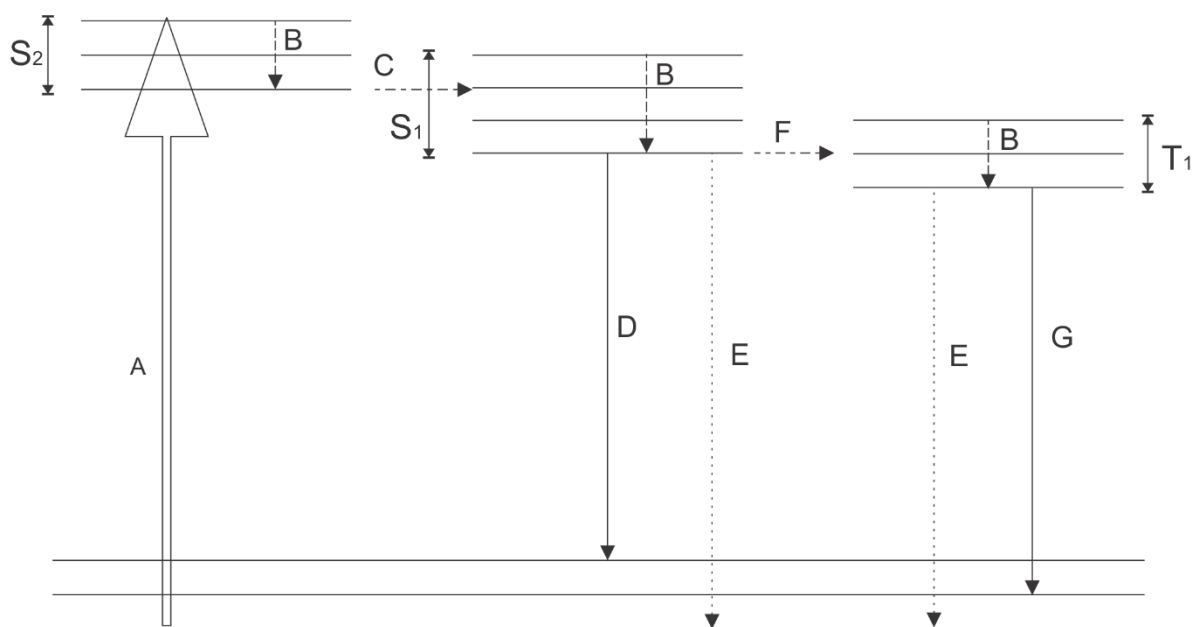


Figure 1-3. Deactivation process for an excited molecule. A) Absorption. B) Vibrational relaxation. C) Internal conversion. D) Fluorescence. E) External conversion. F) Intersystem crossing. G) Phosphorescence. Figure is adapted from the original figure at "Spectrochemical Analyses" book.²⁵

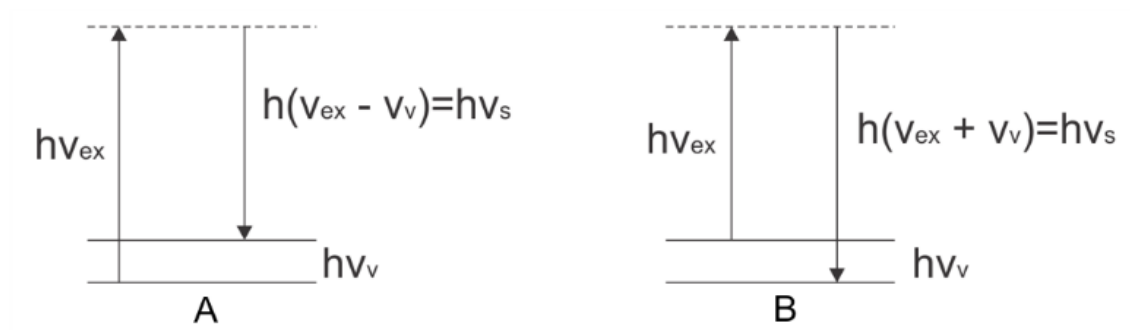


Figure 1-4. Raman scattering illustration. A) Stokes scattering. B) Anti-stokes scattering.

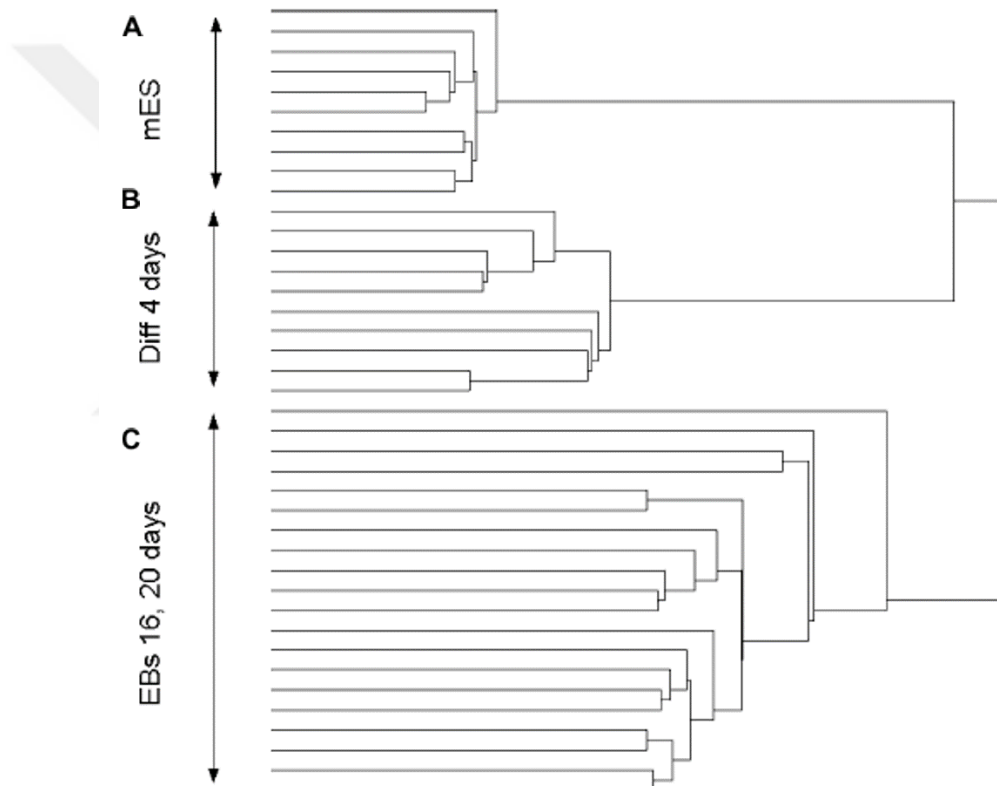


Figure 1-5. Dendrogram of HCA of the Raman spectra. A) Undifferentiated mES cells. B) Spontaneously differentiated cells for 4 days. C) Differentiated cells via EBs. Image is taken from reference.⁵⁴

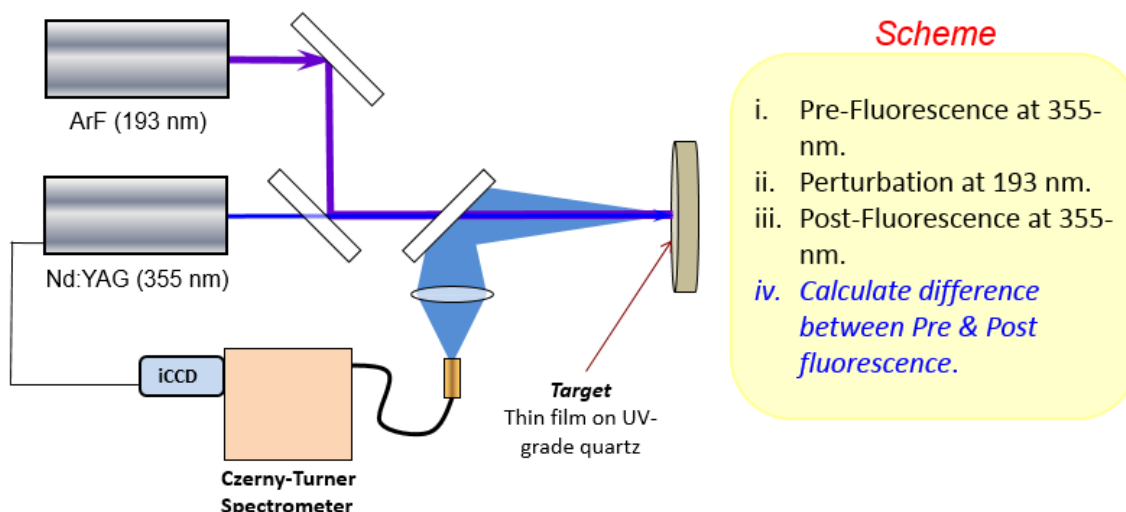


Figure 1-6. General view of the first DLIPS experimental configuration. Experiment includes two UV lasers; a 193 nm ArF laser and frequency-tripled ND:YAG laser.⁶²

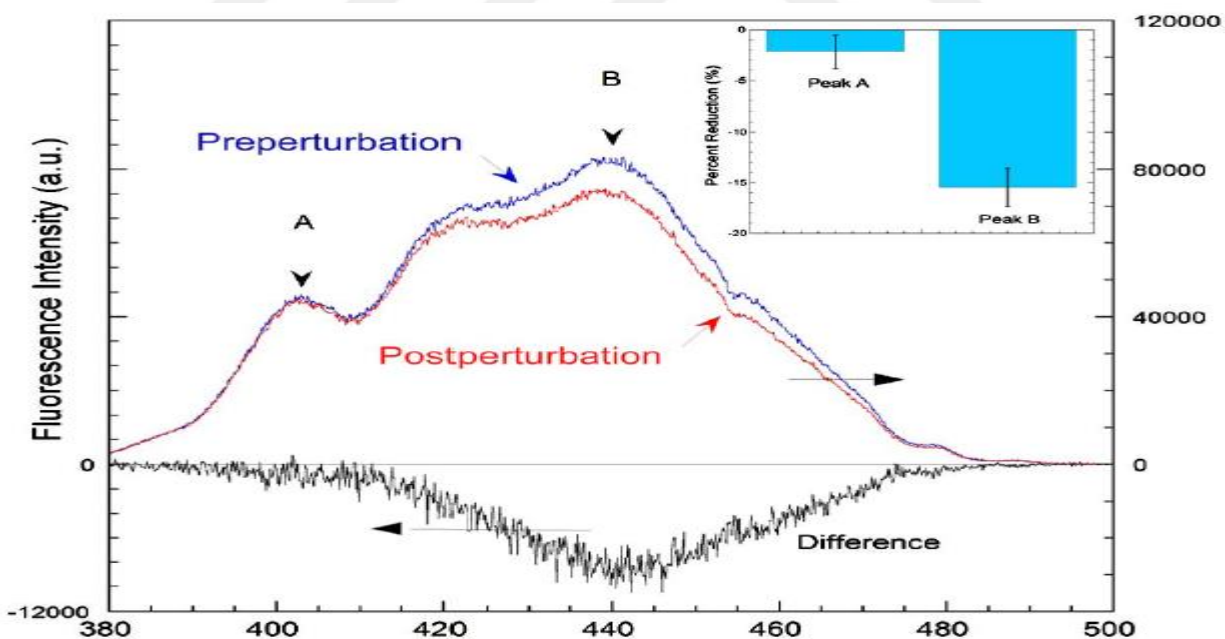


Figure 1-7. (Color online) Fluorescence spectra recorded from C450/BBQ thin films before (Pre) and after (Post) exposure to 250 pulses from the 193nm perturbation laser. Both spectra have the same scale, and are corrected for relative detector response. The lower spectrum corresponds to the difference between the post-perturbation and pre-perturbation spectra.⁶³

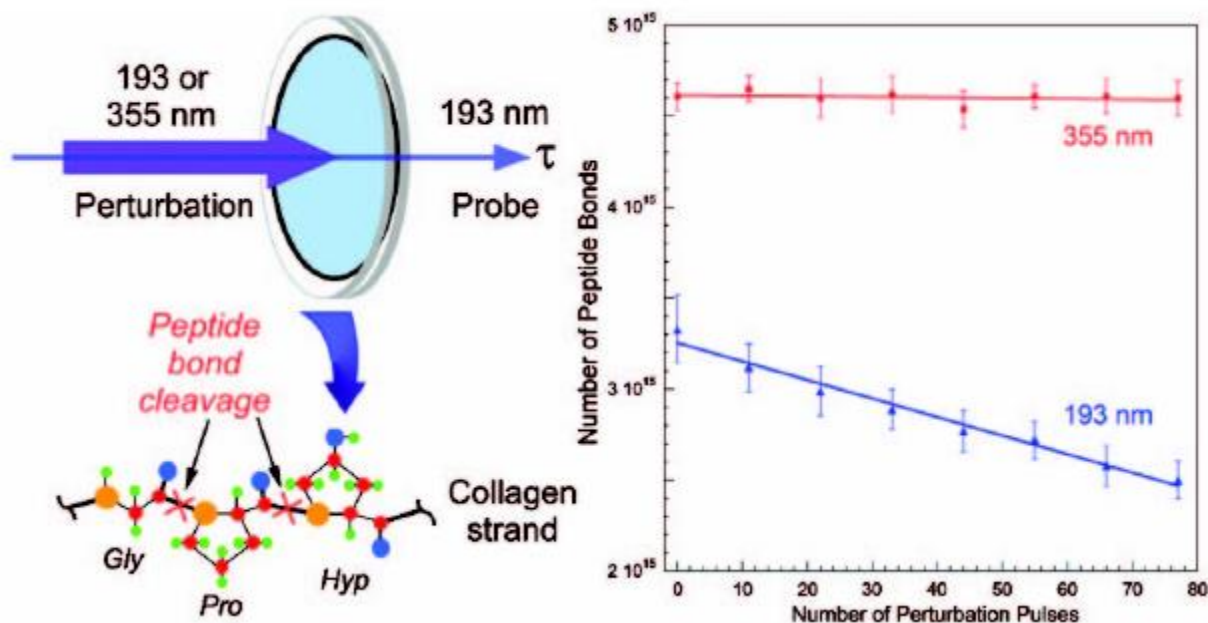


Figure 1-8. Number of peptide bonds in the collagen solution sample volume as a function of incident laser pulses for 193 and 355 nm perturbation laser wavelengths.⁶³

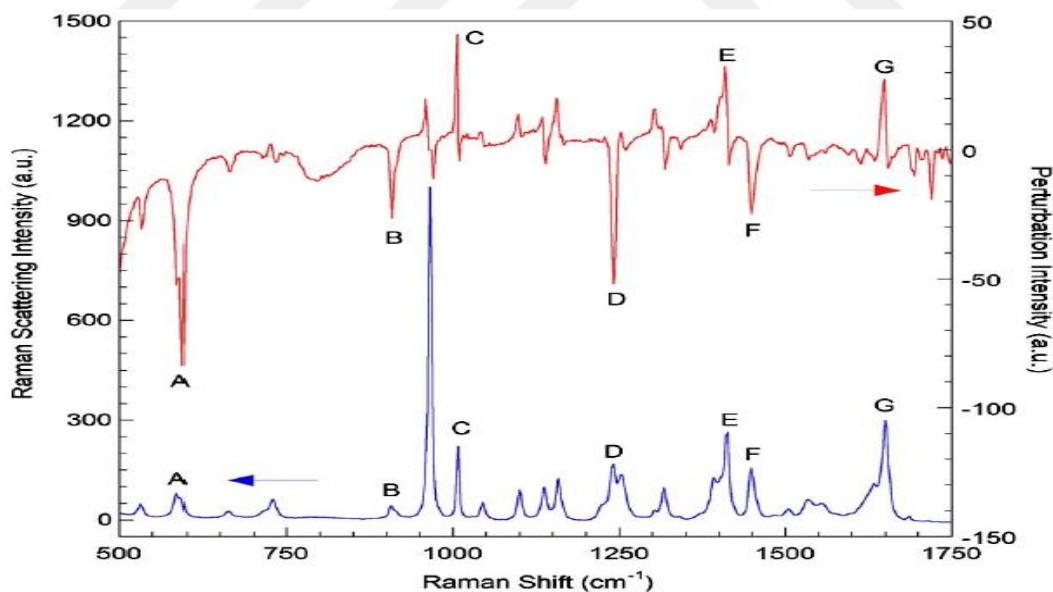


Figure 1-9. Plots of the Raman and corresponding DLIPS spectra of Gly-Gly thin film. (Color online) Upper curve is the DLIPS spectrum of a Gly-Gly thin film corresponding to 700 perturbation pulses from the 193 nm excimer laser. The lower curve corresponds to a traditional Raman spectrum of a Gly-Gly thin film. For calculation of the DLIPS spectrum, the pre-perturbation and post-perturbation Raman spectra were normalized to the 968cm^{-1} C—C Raman band. Peak labels A–G indicate corresponding peak pairs between the Raman and DLIPS spectra, with no shifting of peaks observed between pairs.⁶³

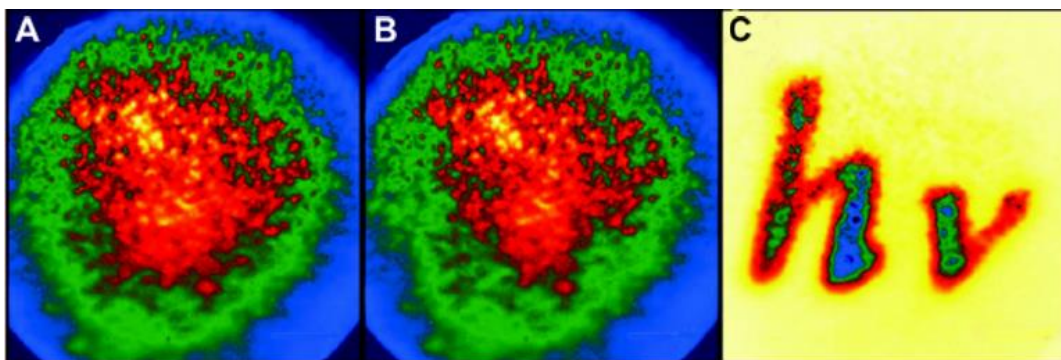


Figure 1-10. Imaging application of DLIPS method. A) 355nm fluorescence image recorded from white card stock prior to laser perturbation. B) 355nm fluorescence image recorded from the same spot as A, following laser perturbation using 25 193nm laser pulses per grid point. C) DLIPS image created by subtracting the pre-perturbation image A directly from the post-perturbation image B. A and B have the identical false-color intensity scale (blue to red indicating increased intensity). C has a different intensity scale, with white (zero counts) to blue indicating decreased intensity counts.⁶³

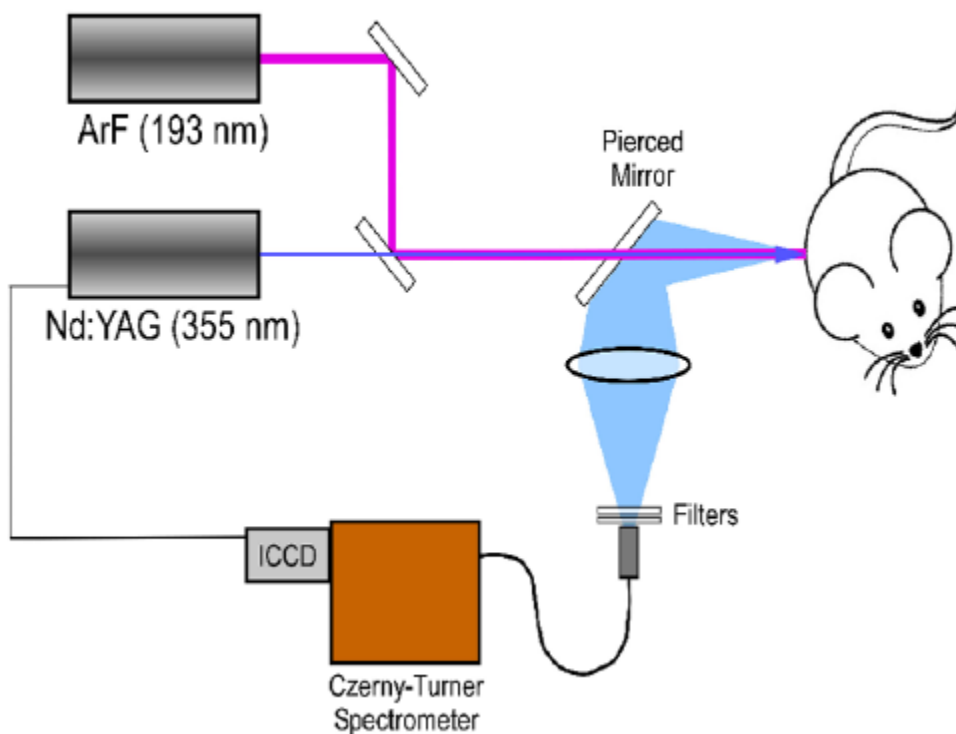


Figure 1-11. Schematic of the DLIPS system for the mice study.⁶⁵

Table 1-1. Physical principles of photothermal processes: Conversion of electromagnetic radiation into heat increases the tissue temperature.¹

Group	Temperature	Effects on Tissue
1	43-45 °C	Conformational changes Retraction Hyperthermia (cell mortality)
2	50 °C	Reduction of enzyme activity
3	60 °C	Protein denaturation Coagulation
4	80 °C	Collagen denaturation Membrane permeabilization Carbonization
5	100 °C	Vaporization and ablation

CHAPTER 2

DLIPS RAMAN SPECTROSCOPY: CLASSIFICATION OF AMINO ACIDS AND PEPTIDES

Motivation

In the previous chapter a general background of our studies is presented including laser-based detection methods, laser-tissue interactions, and previous DLIPS applications. In this chapter, we take a step back from our earlier *in vivo* measurements and focus on the fundamental constitutive materials using a Raman probe to gain additional insight into the DLIPS scheme in the context of classification. Specifically, Raman spectroscopy, a noninvasive, molecular sensitive spectroscopic tool with a significant amount of research done to improve and test its performance for biosensing,⁶⁷⁻⁷⁰ has been widely recognized and assessed, especially for early stage cancer investigation.^{68, 71, 72} The Raman spectrum can identify different biological and tissue components both *in vitro* and *in vivo*⁷³ by assigning specific groups within the molecular structures to their corresponding Raman vibrational bands. Unfortunately, this method often fails inside molecularly rich environments such as tissues consisting of varied components due to overlap of Raman bands that mask the useful information and hinder the ability for accurate and precise characterization.^{52, 74, 75} As noted in a recent review paper, vibrational spectroscopic methods have been widely explored for analysis of various pathologies and organ systems, but as yet, “none have entered routine clinical practice”.⁶⁸ A 2015 review article concludes that if the combination of vibrational spectroscopy and chemometric analysis is to be successfully transferred into clinical practice more extensive studies are needed.⁷⁶ Clearly, new schemes and approaches to vibrational spectroscopy are required for biological and tissue analysis.

We present here a method called differential laser-induced perturbation spectroscopy (DLIPS) which combines low intensity ultraviolet (UV) laser-material interactions (nondestructive) with difference Raman spectroscopy for analysis of thin films of biologically relevant materials, namely amino acids and dipeptides, which are considered basic constituents of collagenous tissues. The analysis of the thin films of these biologically relevant materials is a key step to understanding the optimal use of DLIPS for future *in vivo* diagnostics. The material in this chapter already published and presented in our previous paper.⁷⁷

Materials and Methods

Sample Preparation

The goal of the current study is to investigate basic solutions of molecules representative of collagenous tissues corresponding to the fundamental building-block level; hence solutions of three basic amino acids and their related dipeptides were selected. Amino acid solutions were created by separately dissolving the three amino acids L-Proline (17.3 mM), purchased from Fluka, and Glycine (13.3 mM) and L-Alanine (5.61 mM), purchased from Sigma Aldrich, in ultra-purified deionized (DI) water (Fisher Scientific). Dipeptide solutions were created by separately dissolving (0.5 to 2 mg solute per ml of DI water) the three dipeptides Gly-Gly (7.57 mM), Ala-Gly (3.42 mM) and Gly-Pro (11.6 mM), purchased from Sigma Aldrich, in DI water. To prepare thin films of samples, the solutions were first stirred for 24 hours and deposited onto 50-mm diameter UV-grade quartz flats, which were recrystallized at 35°C, resulting in dry thin films of the desired compounds. Microscopic examination of the resulting films revealed fractal-like structures dispersed over the entire quartz surface. To minimize any background fluorescence from the UV-grade flats, each was thoroughly cleaned in

acetone and photobleached with an intense mercury lamp for a minimum of 40 minutes prior to solution deposition.⁷⁸

Experimental Setup

The DLIPS set-up is realized with two lasers, enabling UV laser perturbation and Raman scattering without repositioning the target, as depicted schematically in Figure 2-1. A 488 nm Ar-ion laser is used as the excitation source for all Raman scattering measurements. A 488 nm laser line filter is placed at the Ar-ion laser output to provide monochromatic output by eliminating all other Ar-ion laser transitions. The Ar-ion laser beam is directed to a 488 nm dichroic Raman beam splitter (Semrock LPD01-488RU) and focused on the sample with a spot size of approximately 2 μm using a microscope objective lens (M Plan Apo 50X/0.55, Mitutoyo) at the working distance of approximately 15 mm. A kinematic mirror is employed to reflect the image directly to a real-time CCD camera to ensure accurate alignment and focus at the desired target spot. The Raman scattered light was collected in backscatter by the same microscope objective lens, collimated and subsequently passed through the dichroic beamsplitter where it was lens-coupled into an optical fiber bundle. A long-pass Raman edge filter (488 nm RazorEdge, Semrock, LP02-488RU) is placed in front of the fiber bundle to reject any 488 nm scattered laser light. The fiber is coupled to a 0.3-m Czerny-Turner spectrometer, dispersed using a 1200 gr/mm grating and recorded with a thermoelectrically cooled CCD array detector (Pixis, Princeton Instruments). Similar custom setups have been reported with various excitation sources.⁷⁹⁻⁸² The Ar-ion laser beam power is controlled to prevent any damage to the target film and was set to approximately ~ 0.6 mW or ~ 1.1 mW, depending on the specific sample. Film stability is assessed by subtracting consecutive Raman spectra acquired for a given film and

power setting, reducing the power as necessary such that a difference of zero was realized repeatedly between any two consecutive recorded spectra.

To create the laser-induced perturbation effect for the DLIPS scheme, a 193 nm ArF laser beam (X5 Excimer laser, GAM Inc., 10.2 ns FWHM pulse width) is directed to the sample holder, focused using a UV-grade plano-convex lens to a diameter size of about 1.4 mm at the target, and projected onto the sample spot surface with 65° degree angle of incidence. The excimer laser is operated at 50 Hz for all experiments, using software control to precisely deliver a preselected number of pulses for each experiment. The centers of the Raman scattering and excimer laser perturbation beams are concentrically superimposed at the same target spot. The large mismatch in Raman and excimer beam focal diameters ensured that the entire Raman probe volume is uniformly exposed to the 193-nm perturbation beam.

The 193-nm excimer laser beam energy is set to 110 $\mu\text{J}/\text{pulse}$, providing the desired fluence of 3 mJ/cm^2 at the target focal spot. This magnitude of excimer fluence is sufficiently low to avoid any direct ablation of the samples, as presented in previous reports of our laboratory^{65, 83}, noting that the typical ablation threshold of tissue and biological materials for the 193-nm excimer laser is on the order of 50 mJ/cm^2 . It was necessary to deliver the excimer laser at near normal incidence rather than through the microscope due to the microscope objective incompatibility with the deep UV wavelength of 193 nm; however, the long working distance of ~15 mm readily allowed beam access of the excimer beam. Because the DLIPS approach is based on difference spectroscopy, it is imperative that the pre-perturbation and post-perturbation Raman spectra be recorded from the exact same location; hence the current static

system with fixed probe and perturbation beams aligned to a common probe volume. As noted above, the zero difference of any two consecutive Raman spectra validates the spectrum-to-spectrum stability of a given sample spot in the absence of any perturbation laser.

Raman data were collected from multiple spots spread over multiple thin film samples and flats. The 488 nm excitation, as described above, was collected and saved with Winspec/32 software (Princeton Instruments). The resulting Raman spectral window ranged from 497 cm^{-1} to 1608 cm^{-1} . Various thin films were analyzed for a particular amino acid or dipeptide, thereby averaging over multiple films and substrates. For a given sample spot, each final spectrum was an accumulation of 40 images, with a per spectrum acquisition time of 3 seconds, for a total integration time of 120 seconds.

Data Interpretation

The acquired Raman spectra before the perturbation step were considered the pre-perturbation data, and the acquired Raman spectra following excimer laser perturbation were considered the post-perturbation data for a given sample spot. Specifically, after the pre-perturbation data was acquired for a given sample site, the shutter of the Raman laser was closed and immediately 800 shots of the 193 nm perturbation beam were delivered to the sample. Following perturbation, a dark signal (i.e. background signal plus dark counts) was then collected while the Raman laser shutter remained closed for the same total accumulation time without moving sample. The 120 seconds of dark signal acquisition following perturbation ensured two items. First, that the dark signal was recorded under identical conditions for each sample spot, thereby accounting for any changes in surface reflectivity or film transmission of ambient light. Secondly, it provided a fixed time period (i.e. repeatable) to ensure that

any transient optical effects immediately following 193 nm UV irradiation were dissipated before then acquiring the post-perturbation Raman signal. Earlier studies of probe beam transmission through collagen solutions following 193 nm excimer laser perturbation revealed both transient perturbation to optical properties as well as permanent bond cleavage, with transient effects decaying on the order of tens of seconds 30. Following dark signal collection, the Raman laser shutter was opened and post-perturbation Raman data was collected and saved using identical signal collection parameters. The DLIPS spectrum was finally obtained by directly calculating,

$$DLIPS(\lambda) = \frac{Em_{POST}(\lambda) - Em_{PRE}(\lambda)}{Em_{PRE}(\lambda) - Em_{DARK}(\lambda)} \quad (2-1)$$

in which the numerator represents the absolute difference in post-perturbation, $Em_{POST}(\lambda)$, and pre-perturbation, $Em_{PRE}(\lambda)$ spectra, noting that a negative signal represents a decrease in signal intensity at a specific wavenumber following laser perturbation, while a positive value likewise represents an increase in signal, and where $Em_{DARK}(\lambda)$ represents the dark Raman signal as described above. The denominator represents the absolute pre-perturbation Raman signal (i.e. dark-count subtracted), which has the effect of normalizing the difference spectrum, thereby generating a DLIPS signal indicative of the fractional change in Raman spectral intensity at each wavenumber (i.e. each pixel). For example, a value of -0.2 for a given wavenumber would correspond to a 20% decrease in Raman intensity following excimer laser perturbation. The DLIPS spectra were then normalized to the largest positive value. It is noted that for the peptide and dipeptide films examined in the current study, the observed pre-perturbation Raman vibrational peaks generally revealed decreases or no

changes, while as discussed below, some new peaks were revealed following laser perturbation. In addition, the observed background signal (i.e. continuum baseline), which is attributed to broadband fluorescence as expected for the current biomolecular samples with 488 nm excitation, was always observed to increase following excimer laser perturbation. As a result, the overall DLIPS spectra were always positive.

In summary, DLIPS data were collected and processed for a total of 45 sample spots for each of the six sample types (L-Alanine, Glycine, L-Proline, Ala-Gly, Gly-Gly, Gly-Pro) in the study. All of the calculations were conducted by Winspec/32 Software as described above prior to using any of the multivariate analysis methods described at the following section.

Data Processing

All of the absolute Raman data, calculated as, and the DLIPS data, per Eq. 2-1, were processed in an identical manner as follows. Whole data were mean-centered, baseline corrected (using a cubic fit), divided by the sample range and normalized to the most intense band. Finally, spectra were smoothed by second-order Savitzky-Golay polynomial filter using 11 points. These pre-processing spectral methods have been widely used.^{53-55, 61, 84} For initial analysis of the data, the Mahalanobis distance⁸² was estimated and the data falling far away from this distance were considered as outliers. Approximately 10% of the whole dataset (from the original 270 Raman spectra and 270 DLIPS spectra) were dropped based on the outlier test, which are attributed to poor Raman signal-to-noise ratios due to thin film regions, instabilities in laser power, film anomalies or impurities, or for the case of DLIPS, slight sample movement/misalignment between the pre-perturbation and post-perturbation Raman

spectra, noting the rather high magnification (i.e. 50x). The remaining data were then used for the multivariate analysis, with no further data omission.

Common multivariate analysis such as Principal Component Analysis (PCA), Hierarchical Component Analysis (HCA), and finally Partial Least Squares (PLS) methods were used to explore the effects of the DLIPS scheme as compared to traditional Raman spectroscopy, and to evaluate the performance of the DLIPS method for spectral classification. Particularly, in HCA data clusters are formed by linking naturally similar samples based on their multivariate distances, and the resulting dendrogram of HCA reveals these groupings visually.⁵⁴ For dendrogram generation, samples were linked and grouped together based on the similarities in their structure, and the resulting tree-shaped structure shows the sample relations where the branch lengths are proportional to cluster distances. The similarity variable in HCA is a scale which is a customary transformation of inter-sample distances into a comprehensive value. It is inversely proportional with the cluster distances. PCA and PLS models, which can boost the performance of the interpretation of the data by magnifying the natural changes between two different groups by highlighting important variations inside the dataset, reduce the dimensionality of the data into a few components covering most of the variation information inside the data.⁶⁰ The principal components in PCA are forced to be orthogonal, whereas in PLS they do not have to be orthogonal.^{61, 79} All the chemometric analysis in the current study was performed using Pirouette (Infometrix, version 4.5).

Results and Discussion

Raman and DLIPS Spectra

The Raman and DLIPS spectra recorded from the amino acids and dipeptides were rich in spectral features between the wavenumber ranges of about 500 to 1,600 cm^{-1} . The Raman shifts of amino acids were compared with the literature, allowing identification of most prominent peaks as discussed below.⁸⁵ For the illustration purposes, three representative spectra from a single sample spot, namely the pre-perturbation and post-perturbation Raman spectra and the corresponding DLIPS spectrum, are shown in Figure 2-2 for an L-proline sample. As noted above, the increase in background fluorescence, which has the effect of a positive offset in the post-perturbation Raman spectra, has the effect of generating overall positive DLIPS spectra. Relative decreases, as compared to the fluorescence background, in the Raman vibrational peaks are therefore manifest as downward peaks (i.e. less change than the baseline), which gives the DLIPS spectra an overall inverse appearance with regard to the traditional Raman spectra. This is readily observed in Figure 2-2, where vibrational bands appearing in the traditional Raman spectra as positive peaks are seen as downward peaks in the DLIPS spectrum.

In general, longer wavelengths (especially near IR) are commonly used to excite molecules for Raman systems for biological applications⁷⁰; however, in order to increase the Raman signal, shorter wavelengths are often selected, given the inverse fourth-order dependence of Raman scattering cross-section on wavelength.²⁵ Since thin films were used in this study (i.e. low concentration of $\sim 0.1 \text{ mg/cm}^2$), 488 nm excitation was selected to successfully resolve sufficient Raman peaks for classification studies. For all six sample types examined in this study, subtraction of two subsequent Raman

spectra in the absence of any excimer laser perturbation revealed no difference (i.e. zero counts), ensuring that the 488 nm beam power was itself non-destructive (i.e. non-perturbative), and thereby promoting stable Raman spectra for the sample films and importantly, that any differences recorded with the DLIPS scheme were a result of only excimer laser perturbation.

Representative traditional Raman spectra (i.e. pre-perturbation Raman spectra) and DLIPS spectra as averaged over all sample spots for each sample type are plotted in Figure 2-3. Because both the DLIPS and Raman spectra are normalized between 0 and 1, noting that the maximum peak is generally different between the two spectral methods, they reveal similar spectral features and appear rather like complementary plots, as described above, although there exists key differences in the relative intensity of similar bands, as readily observed in the figures and discussed in detail below.

For quantitative analysis of the Raman and DLIPS spectra, PCA was employed to detect any differences in relative peak magnitudes by comparing loadings of the sample sets. The three PCA loadings out of the total Raman spectral dataset are shown in Figure 2-4A through 2-4C, and in Figure 2-4D through 2-4F for the Raman alone and DLIPS spectral datasets, respectively. Accordingly, the most significant vibrational bands identified through the loadings of the PCA analysis of Raman and DLIPS datasets for classification, including their relative intensity in the Raman or DLIPS spectra were tabulated in Table 2-1 in detail. The vibrational bands ca. 835, 851, 853, 893, 918, 920, 927, 968, 1323, 1362, 1389, 1397, 1404, and 1471 cm^{-1} were more prominent in the Raman spectra than in the DLIPS spectra, while the bands ca. 641, 651, 652, 682, 725, 920, 981, 995, 1020, 1041, 1045, 1048, 1059, 1076, 1101, 1113,

1133, 1141, 1147, 1166, 1173, 1197, 1278, 1316, 1520, 1526, 1543, and 1553 cm^{-1} were more prominent in their respective DLIPS spectra, noting that the band assignments for these shifts can be readily found in the literature for the amino-acids and dipeptides.⁸⁶⁻⁹³

The relative intensity differences as well as loading differences of the DLIPS spectral bands as compared to the Raman spectral bands are attributed to differences in the coupling of the excimer laser into the various amino acids and dipeptides. It should be highlighted that these intensity differences between DLIPS and Raman spectral bands originated from the perturbative role of the UV light on the molecular bonds with DLIPS, as opposed to traditional vibrational response with Raman. Based on earlier studies, the 193 nm radiation is strongly coupled into and effectively photochemically cleaves C-N peptide bonds.^{63, 83} In general, the high photon energy of 193 nm excimer laser (6.4 eV) is capable of cleaving most bonds in biological molecules; however, the current results (Table 2-1) reveal a preference for C-N bond perturbation over, for example, C-C and C-O bond perturbation. The effectively cleaved molecular peaks observed in this study are dominated by the many stretching or bending C-N vibrational modes.⁸⁹ In fact, it is a selective (i.e. preferential) bond perturbation with the excimer laser that is the key to the DLIPS scheme, providing additional spectral information beyond simple vibrational spectroscopy (e.g. Raman or FTIR).

Additionally as presented in Table 2-1, increased intensity of several vibrational bands distinctive from C-N vibrations for different samples were recognized in their DLIPS spectral data. Two distinct cases were observed for these changes. Firstly, there

were intensity changes at the particular vibrational bands of NH_2^+ , and NH_3^+ groups, as well as some smaller groups, which are attached to the cleaved C-N bonds. It is suggested that once 193 nm light effectively cleaved the C-N bonds, these groups were liberated from their molecules at the sample surface, which results in the recorded difference in their vibrational modes. For the second case, an increase in intensity was observed for different vibrational modes of COO^- ions. This is attributed to the electron deficiencies of carbon atoms inducing hydrogen (H) migration from the carboxyl groups that were previously connected to the nitrogen atoms prior to perturbation. The last effect was seen exclusively in amino acids in this study rather than the dipeptides. Such mechanisms most likely play a role in the overall increase in broadband fluorescence observed in the post-perturbation Raman spectra. In aggregate, such photochemical mechanisms are hypothesized to account for a portion of the additional spectral information realized with DLIPS.

Raman and DLIPS Performance in Classification

The resulting 2D PCA scatter plots of all six samples using three principal components are shown in Figure 2-5, which together account for approximately 70% of the total variance. Visually, it is shown that groups of samples defined by their DLIPS PCA data are more separated than the traditional Raman spectroscopy data. Additionally, comparison of the factors of both DLIPS and Raman datasets reveals that the three PCA factors of the DLIPS dataset are slightly lower than the three factors of the PCA using the Raman dataset, which is another sign of separation of these groups.

To quantify the PCA performance of the DLIPS and Raman spectra for classification of the amino acids and dipeptides, HCA analysis was employed to the six

samples. From Figure 2-6A and 2-6B, the HCA dendrograms of six sample types constructed by the Raman data and the DLIPS data can be seen, respectively. In this case, their features (vibrational bands) in their respective spectra were the recognition elements, and the similarity variable is shown at the top scale. The similarity variable was taken at the significant node of the dendrogram, the first node at which the six different groups can be distinguishable from each other. The similarity variable was 0.315 for samples defined by their traditional Raman scattering data, whereas the similarity variable was 0.201 for samples defined by their DLIPS data, noting that a smaller similarity variable corresponds to a more successful degree of classification. This concludes that the groups of samples defined by their DLIPS data are further away from each other than the groups of samples defined by their Raman data, with the latter data yielding a more than 50% greater similarity variable. In this analysis, the similarity variable is appropriate because all of the samples that are marked with the pre-defined classes fall into the same HCA-defined classes correctly at the point where all six different groups could be realized, (i.e. no single miss matching between pre-defined and HCA-defined classes).

As noted above, PCA and HCA analysis revealed the DLIPS method as a classification scheme for the six biologically relevant samples. In addition, a PLS regression model was developed to further quantify the classification ability of the DLIPS and traditional Raman data sets.^{79, 81, 84} For PLS analysis, 10 factors were used to build a model which together accounted for approximately 99% of the total variance. Since the purpose was to directly compare the two datasets on PLS model quality, rather than dividing the data set into two halves for model development and validation,

respectively, as commonly done ⁸², the entire data sets were used to evaluate the PLS model performance. The PLS models were then used to classify the entire datasets. The predictions of PLS models for both Raman and DLIPS spectral datasets are shown in Figure 2-6C and 2-6D. The solid line represents the $x=y$ values, whereas the nodes are individual prediction of samples, where it is observed that samples described by DLIPS dataset were tighter. For quantification of the two models, the matrix of residuals of the PLS models was used to calculate error sum squares (ESS). The ESS was calculated as 8.03 for the Raman dataset and was 5.33 for DLIPS dataset. Similar to the HCA analysis, the RSS value of the Raman data was slightly more than 50% larger than recorded for the DLIPS data, corroborating the superior classification with the DLIPS approach.

In conclusion, the results sufficiently supported that DLIPS did successfully use the advantage of UV irradiation of biological materials used in this study, by combining the characteristic responses of these materials not only to excitation sources but also the responses to the perturbation. More about DLIPS performance constructed with other probes (i.e. fluorescence probe) and more fundamental information will be presented in the following chapter.

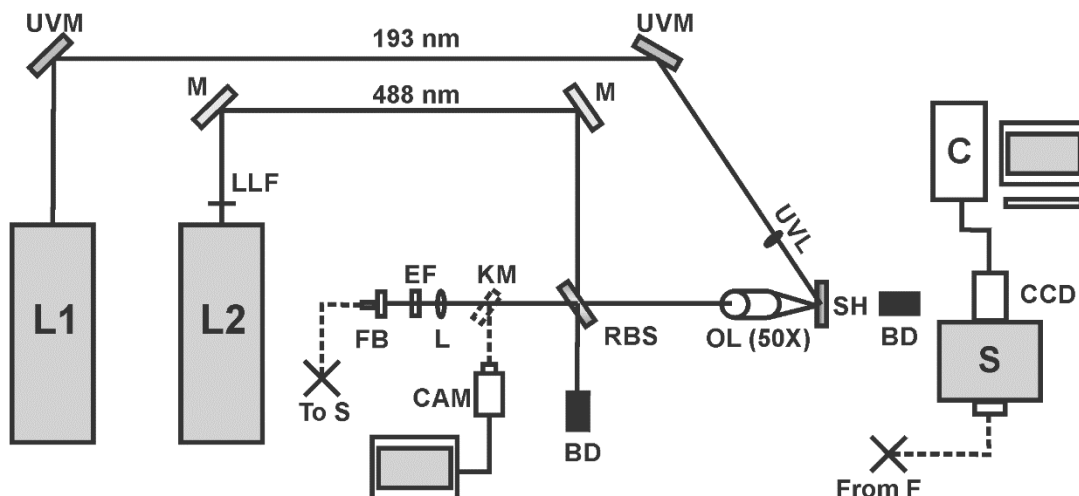


Figure 2-1. Schematic of the experimental setup. L1: 193-nm excimer laser; L2: 488-nm Ar-Ion laser; UVM: ultraviolet mirror; M: mirror; LLF: laser line filter; FB: fiber bundle; F: fiber; EF: edge filter; L: lens; KM: kinematic mirror; CAM: camera; RBS: Raman beam splitter; BD: beam dump; OL: objective lens; S: Czerny–Turner spectrometer; UVL: ultraviolet lens; SH: sample holder, and C: computer.⁷⁷

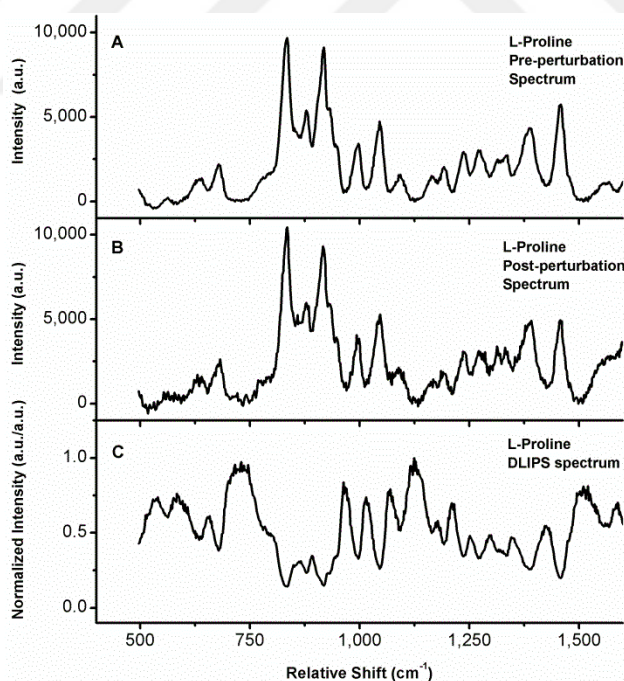


Figure 2-2. Representative Raman and DLIPS spectra of a single L-Proline sample spot. A) Raman spectrum of L-Proline acquired before perturbation. B) Raman spectrum of L-Proline acquired after perturbation. C) Calculated DLIPS spectrum of L-Proline based on the Eq. (2-1). The Raman spectra have been baseline corrected.⁷⁷

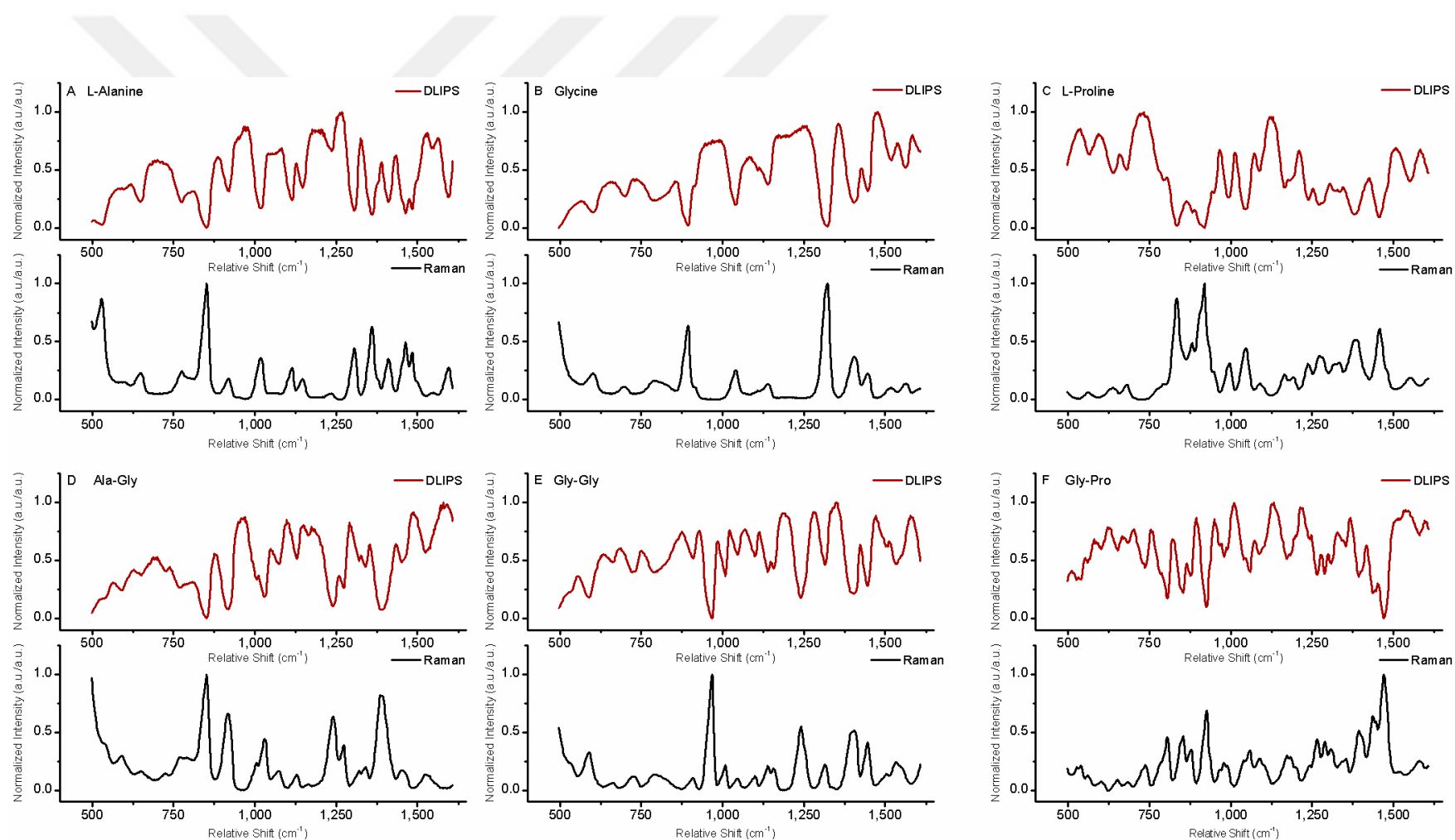


Figure 2-3. Average Raman and DLIPS spectra of amino acids and dipeptides. A) L- Alanine. B) Glycine. C) L- Proline. D) AlaGly. E) GlyGly. F) GlyPro. Black (lower) profiles denote average Raman spectra and red (upper) profiles denote average DLIPS spectra for each sample.⁷⁷

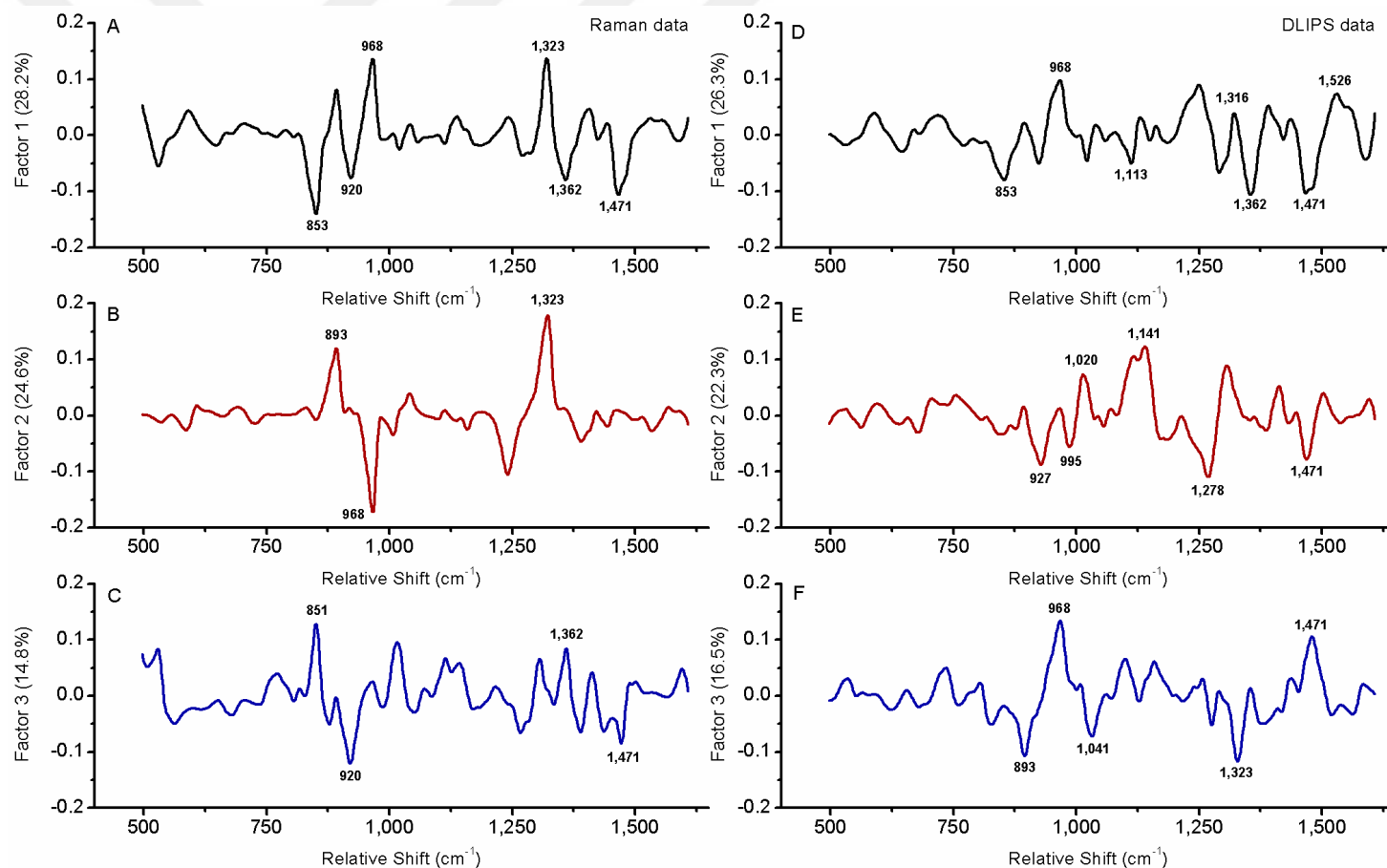


Figure 2-4. PCA loadings for the various datasets. A) through C) are the three loadings of traditional Raman dataset. D) through F) three loadings of DLIPS dataset. A total of approximately 70% of the variation in the data were explained by three loading factors. Prominent loading wavenumbers are labeled for both the Raman and DLIPS data sets, which correspond to vibrational peaks.⁷⁷

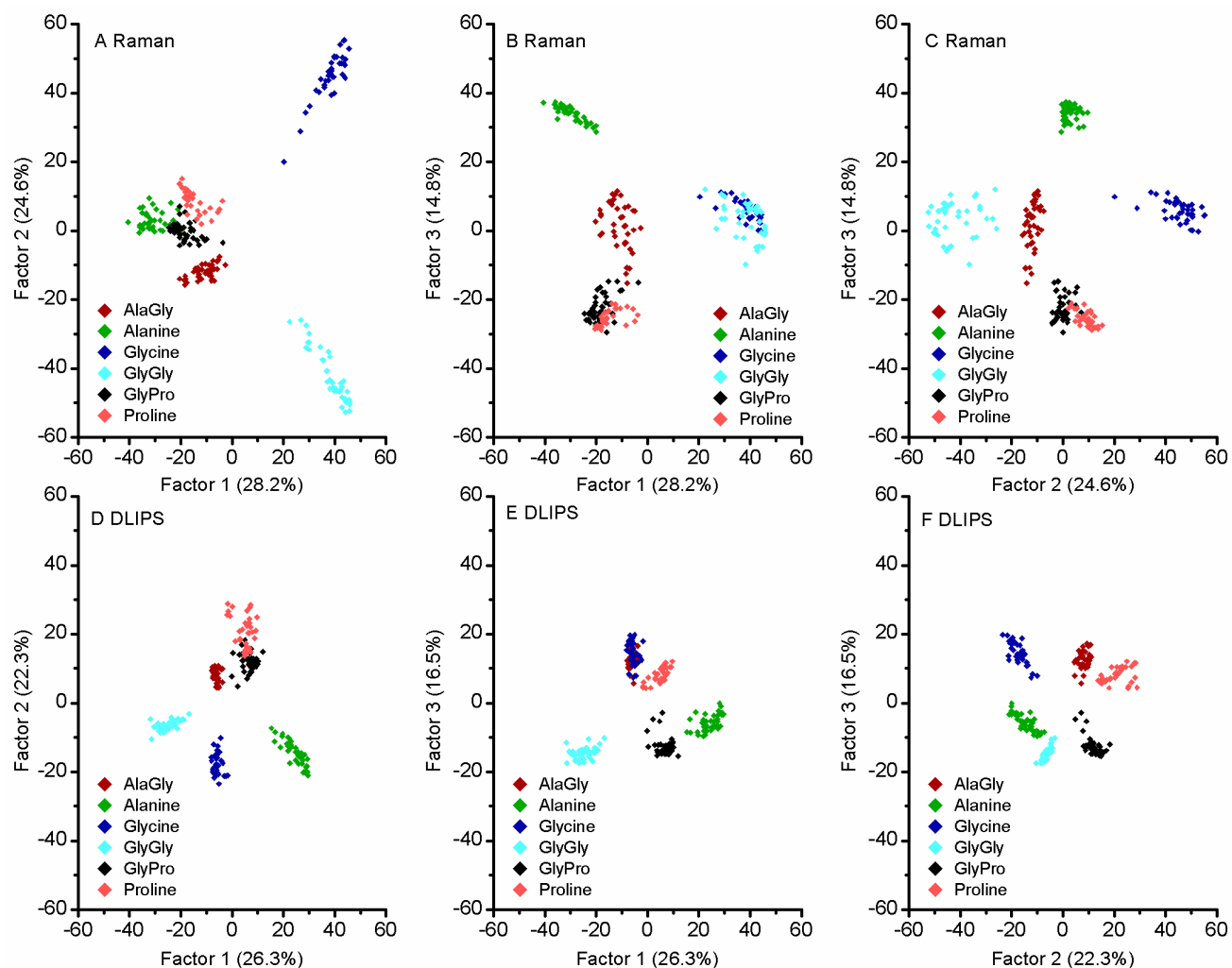


Figure 2-5. The 2D score plots of whole dataset: A) through C) are the Raman only dataset. D) through F) are the DLIPS dataset.⁷⁷

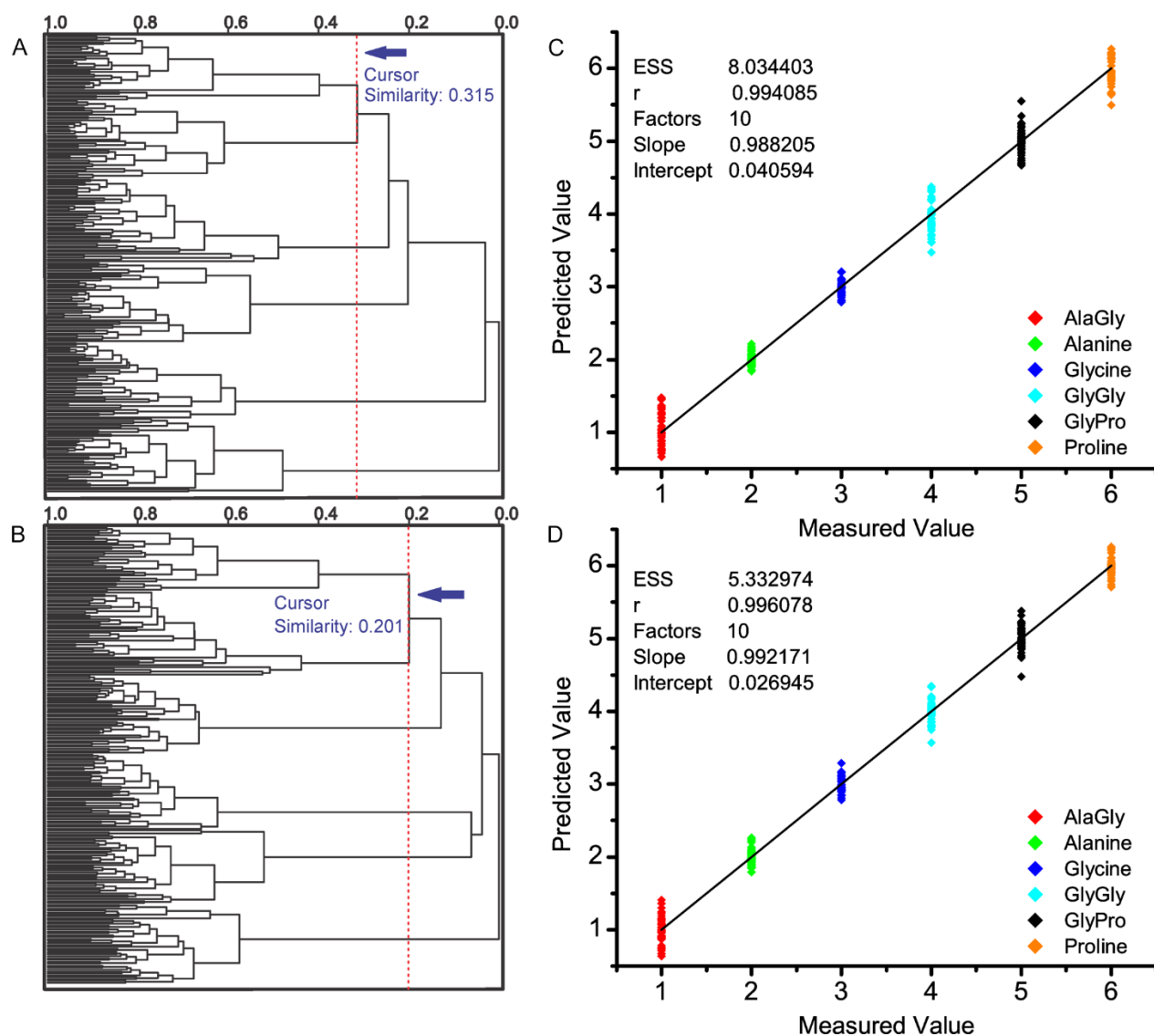


Figure 2-6. Distributions of the samples and modelling quality. A) HCA dendrogram of the Raman dataset. B) HCA dendrogram of the DLIPS dataset. Cursor of the similarity variable is placed at the node where all six different groups are first recognized. C) PLS model of the Raman dataset. D) PLS model of the DLIPS dataset. Approximately total of 99% variation in the data were explained by ten PLS factors.⁷⁷

Table 2-1. Significant Raman bands of amino acids (L-alanine, glycine, L-proline) and dipeptides (glycine-glycine, glycine-proline, glycine-alanine) that are affected by 193 nm irradiation.⁷⁷

Relative Shift (cm ⁻¹)	Biological Molecule	Band Assignment	Magnitude in DLIPS compared to magnitude in Raman
652	AlaGly	C-O out-of-plane bending	More
851	AlaGly	C-C stretching	Less
920	AlaGly	C-C stretching	Less
1076	AlaGly	C-N stretching	More
1133	AlaGly	C-N stretching	More
1166	AlaGly	CH ₂ torsion	More
1278	AlaGly	C-N stretching	More
1389	AlaGly	C-C stretching	Less
1526	AlaGly	C-N stretching	More
651	Alanine	COO ⁻ deformation	More
853	Alanine	C-C stretching	Less
920	Alanine	C-COO ⁻ stretching	More
1020	Alanine	C-N stretching	More
1113	Alanine	NH ₃ ⁺ deformation	More
1147	Alanine	NH ₃ ⁺ deformation	More
1362	Alanine	CH ₃ sym. deformation	Less
1543	Alanine	NH ₃ ⁺ deformation	More
893	Glycine	C-C stretching	Less
1041	Glycine	C-N stretching	More
1141	Glycine	NH ₃ ⁺ deformation	More
1323	Glycine	CH ₂ wagging	Less

Table 2-1. Continued.

Relative Shift (cm ⁻¹)	Biological Molecule	Band Assignment	Magnitude in DLIPS compared to magnitude in Raman
1520	Glycine	NH ₃ ⁺ bending	More
725	GlyGly	C-N stretching	More
968	GlyGly	CH ₂ rocking	Less
1045	GlyGly	C-N stretching	More
1101	GlyGly	C-N stretching	More
1316	GlyGly	C-N stretching	More
1404	GlyGly	CH ₂ deformation	Less
927	GlyPro	C-C stretching	Less
981	GlyPro	C-N stretching	More
1059	GlyPro	C-N stretching	More
1173	GlyPro	NH ₃ ⁺ rocking	More
1397	GlyPro	COO ⁻ sym. stretching	Less
1471	GlyPro	CH ₂ deformation	Less
641	Proline	COO ⁻ wagging	More
682	Proline	COO ⁻ deformation	More
835	Proline	C-C stretching	Less
918	Proline	C-C stretching	Less
995	Proline	C-N stretching	More
1048	Proline	C-N stretching	More
1197	Proline	NH ₂ ⁺ deformation	More
1553	Proline	NH ₂ ⁺ bending	More

CHAPTER 3 DLIPS FLUORESCENCE SPECTROSCOPY

Motivation

In the previous chapter, the use of *in situ* DLIPS method using a Raman probe was presented. Subsequently in this chapter, performance data using differential laser-induced perturbation spectroscopy (DLIPS) will be presented as compared to a traditional fluorescence probe, which will be shown to be sensitive to the slight changes in aromatic amino acid concentrations, by classifying aromatic amino acid analyte sets prepared in differing mass concentrations. The DLIPS method is an alternative laser-based diagnosis tool, which is established here by combining difference fluorescence spectroscopy and low intensity ultraviolet (UV) laser perturbation (i.e. non-destructive interactions) for analysis of thin films of aromatic amino acids at the deep UV wavelength region. Fluorescence spectroscopy is a widely used multi-purpose biosensing tool, including use in detection of abnormal changes inside the body which are indicative of precancerous or cancerous formations.^{26, 31} Motivating such use is the fact that early cancer diagnosis has a great significance in treatment.⁹⁴ By this way, survival rates may be increased as the prognosis can be determined in advance and neoplastic tissues may be removed quickly, notably so for cancer treatment.^{26, 95-98}

Since the fluorescence spectroscopy method is functional, non-invasive, highly selective and potentially a sensitive tool, it has inherent potential for cancer diagnosis in early stages; preferably to be used in early diagnosis rather than histopathological examination of biopsies.^{36-38, 40} However, often fluorescence information is acquired from complex mixtures of biological samples and tissues from the body. Since this spectral information is highly rich in terms of the chemical composition of the probed

area, it is potentially distorted by broadband fluorescence responses (e.g. background or confounding signals), confounding information from unknown sources, spectral noises and multiple emissions.⁹⁹ Such phenomena results in impaired signal quality (i.e. low signal-to-noise ratio) and often masks useful information. Hence, the specificity and the sensitivity of the fluorescence probes tend to be lower for *in vivo* applications.^{30, 36,}
¹⁰⁰ On the other hand, the sensitivity and specificity values have been reported sufficiently for *ex vivo* studies, where the studies generally aim to extract as much information as they can (i.e. scanning the target area with broad excitation wavelengths) from the probed regions; however, notable successes for *in vivo* applications remain lacking.^{40, 41, 101, 102} In addition, the results of *in vivo* analyses often are limited by observed patient-to-patient variations.¹⁰³

Aromatic amino acids including phenylalanine, tyrosine, and tryptophan are the endogenous fluorophores inside the body, and their aromatic side chains are responsible for their emission when they absorb incident light at the ultraviolet (UV) region.^{42, 104} The effects of coupling of different UV laser wavelengths such as 193, 248, 254 and 266 nm into collagenous materials have been examined, especially in terms of photo-products resulting from photoionization and photodecomposition.^{105, 106} Of significance, aromatic amino acids are shown to have relatively high extinction coefficients in the UV range, notably up to 248 nm¹⁰⁶⁻¹⁰⁸, making them ideal targets for DLIPS analysis.

Materials and Methods

Sample Preparation

Aromatic amino acids L-Phenylalanine, L-Tyrosine and L-Tryptophan are used in this study as representative of endogenous fluorophores inside the body, noting that

their excessive levels are potential indicators of pre-cancerous formations. All samples are obtained as dry powders from Sigma-Aldrich and are dissolved in ultra-purified deionized (DI) water (Fischer Scientific). In order to evaluate the performance of DLIPS for quantitative analyses of relative changes in concentrations of aromatic amino acids, four analyte sets with different mass ratios of phenylalanine, tyrosine and tryptophan are prepared, specifically four unique ratios, namely 1-0.5-1, 1-0.5-2, 1-1-1 and 2-1-0.5 of phenylalanine-tyrosine-tryptophan in mg solute/ml of DI water, respectively. The tyrosine concentration ratio is adjusted lower than the others because of the low solubility of tyrosine in water ¹⁰⁹, as the complete dissolution of all three components was thereby ensured. Freshly prepared liquid mixtures are stirred and heated at 50°C for one hour, and the mixtures were then precipitated on quartz plates at 35°C, resulting in thin films of the various mixtures.

Experimental Setup

Throughout the experiments, fluorescence excitation of all four analyte sets is accomplished by using 193 nm ArF excimer laser light with 10 ns FWHM pulse length and 10 Hz repetition rate (X5, GAM Laser), suitably reduced in intensity using UV-grade ND filters as described below. A switch-controlled external shutter is placed in front of the 193 nm laser output in order to precisely limit the excitation duration, while allowing the laser to stabilize between exposures. The shutter allows precise delivery of the 193 nm excimer laser pulses as either a fluorescence probe or a perturbation laser, thereby preventing delivering any extra pulses to the target sample. Neutral density (ND) filters are placed in the optical path using a kinematic holder with total optical density of 1.6, which is enabled/disabled during fluorescence excitation and perturbation, respectively. Specifically, for perturbation, the 193 nm laser beam energy is adjusted to 50 μ J/pulse

with no ND filters, providing a final fluence at the target spot 2 to 3 mJ/cm², well below the ablation threshold of biological materials, typically in the range of 50 mJ/cm².¹ When the 193 nm laser is used as the fluorescence probe, the ND filters are enabled, reducing the excimer pulse energy about 40 fold, to about 1 μ J/pulse. Following the ND filters, the 193 nm beam is directed to the sample holder by passing through a pierced mirror placed at a 45 degree angle to the sample holder plane normal. The pierced mirror is coupled with two 50-mm diameter UV-grade lenses onto a fiber bundle, thereby reflecting, collimating and focusing the fluorescence emission. Before entering the bundle, a dielectric coated 193-nm laser maxline mirror is placed at the maximum reflection angle in order to reject the majority of the 193 nm excitation pulse back-reflected from the sample, noting that the UV-grade mirror substrate allows the fluorescence emission to pass to the fiber. The filtered fluorescence signal is subsequently dispersed (600 gr/mm grating) onto an array detector by 0.3 m Czerny-Turner spectrometer (SpectraPro-300i, Acton) equipped with intensified CCD (ICCD) camera. A delay generator is employed in order to synchronize camera shutter and excitation pulse triggers. The ICCD gate width is set to acquire the entire fluorescence signal temporally using a 800-ns gate coincident on the excitation laser pulse.

For DLIPS, perturbation with the 193 nm beam is performed with the same ArF excimer laser while the ND filters are disabled, as described above. DLIPS perturbation with other wavelengths, namely 220, 230 and 245 nm, is performed using an injection-seeded 355-nm Nd:YAG-pumped (Precision II Series 8000, Continuum) tunable OPO with doubling optics (Panther Ex HEO, Continuum) with a pulse width of about 6 ns FWHM. All beams are delivered at 10 Hz. An external laser shutter is also placed after

the OPO output to control pulse exposures. A variable aperture is placed behind the pierced mirror in order to set the diameter of the irradiated area on the sample to ~ 4 mm for all perturbation wavelengths. With this approach, identical sample areas are ensured for excitation and perturbation pulses. For the OPO laser, the pulse energies of the 220, 230 and 245 nm beams were set to 100, 150 and 150 μJ respectively. For DLIPS perturbation, all laser energies correspond to a fluence which is near or slightly below the desired value of $3 \text{ mJ}/\text{cm}^2$ at the target. All considerations related to the perturbation fluence limitations have been previously discussed in greater detail.^{65, 77}

Data Acquisition and Manipulation

Each final fluorescence spectrum for a specific sample spot is an accumulation of 40 images in a spectral window that ranged from c.a. 294 nm to 413 nm, and saved using Winspec/32 software (Princeton Instruments). For all DLIPS measurements for a particular target spot, the first fluorescence spectrum is collected and saved as the “pre-perturbation” spectral data. Immediately following this fluorescence collection, the external laser shutter is closed to avoid any further irradiation to the target spot. In order to perturb the samples, the external laser shutter (to deliver 193, 220, 230 or 245 nm) is then opened, the ND filters are disabled and perturbation pulses are delivered for a total exposure of 5 to 15 seconds at 10 Hz pulse rate, based on the desired experiment. Total number of photons delivered to the samples are given in Table 3-1. Following laser perturbation at a given wavelength, the external laser shutter is again closed, and a dark signal is collected, noting that the excitation laser shutter also remained closed for dark signal accumulation (about 4 seconds). Following dark signal collection, an additional time lag of 30 seconds is introduced. The total time lag following perturbation ensured that any transient optical effects immediately following any UV irradiation are

dissipated.¹¹⁰ At the end of dark signal collection, ND filters are enabled as appropriate, and the fluorescence probe laser shutter is opened again to collect a second fluorescence signal, which is saved as the “post-perturbation” spectrum. We note that only a single perturbation wavelength is used for a given experimental cycle, where a cycle contains fluorescence/perturbation/fluorescence of all four analyte sets. The overall DLIPS spectrum is formed by calculating Eq. 3-1 for each spot,

$$DLIPS(\lambda) = \frac{Em_{POST}(\lambda) - Em_{PRE}(\lambda)}{Em_{PRE}(\lambda) - Em_{DARK}(\lambda)} \quad (3-1)$$

where $Em_{PRE}(\lambda)$, $Em_{DARK}(\lambda)$, $Em_{POST}(\lambda)$ represent the pre-perturbation spectrum, dark signal spectrum and post-perturbation spectrum intensity, respectively, at each wavelength value (i.e. each pixel value). For each analyte sample and DLIPS combination, 30 separate target spots were analyzed for each of the four analyte targets, using multiple analyte films of each type, generating 120 spectra each for the pre-perturbation fluorescence, post-perturbation fluorescence and resulting DLIPS calculation.

Several multivariate analyses, including Principal Component Analysis (PCA) and Partial Least Squares (PLS) methods, are used to visualize and quantify the DLIPS performance as compared to traditional fluorescence spectroscopy (as assessed using only the absolute pre-fluorescence spectra) in classification. The K-nearest neighbors analysis (KNN) is used to evaluate the advantage of using DLIPS dataset in prediction performance. Details of using PCA and PLS for DLIPS study are discussed previously.⁷⁷ Two dimensional PCA scores are very useful to visualize inter-sample relations and recognizing the natural patterns about the statistical distribution of samples. The PLS

algorithm helps to quantify and compare the quality of models generated from the different datasets (i.e. traditional fluorescence and DLIPS). On the other hand, the KNN algorithm attempts to place unknown samples into pre-defined categories based on its proximity to samples in those categories. In order to accomplish that, k-number of neighbors vote for each unknown sample based on their multivariate distances, i.e. Euclidean distance, which is analogous to polling.¹¹¹ All the chemometric analyses in this study are performed using Pirouette (Infometrix, version 4.5).

Results and Discussion

Fluorescence Data and the Effect of Different Perturbation Wavelengths

Molecular structures of individual samples (L-phenylalanine, L-tyrosine and L-tryptophan) can be seen in Figure 3-1 along with their representative fluorescence emission spectra while excited with 193 nm light. Among the three aromatic amino acids, tyrosine has the lowest fluorescence emission which can be recognized by comparing the signal-to-noise ratios as observed. Four analyte sets with different concentrations of aromatic amino acids were prepared as described above and subsequently analyzed in each DLIPS experiment using 193, 220, 230 and 245 nm perturbation wavelengths and for a fixed 193-nm fluorescence excitation probe wavelength. Representative 193-nm excitation fluorescence emission spectra (i.e. pre-perturbation fluorescence spectra) of the four analyte sample film types are shown in Figure 3-2A. As noted above, ND filters were added to the 193-nm excitation beam path to ensure no perturbation was realized with the fluorescence probe beam alone. This was verified such that subtraction of two subsequent 193-nm excitation fluorescence spectra (i.e. in the absence of any perturbation) revealed no significant difference, ensuring that the 193 nm excitation beam is itself non-destructive for the probe beam

exposure level. The corresponding ensemble-averaged (i.e. all spots) DLIPS spectra of the four analyte sets, for the four different perturbation wavelengths of 193, 220, 230 and 245 nm are shown in Figure 3-2B through 3-2E, respectively. All DLIPS spectra in Figure 3-2 corresponds to 193 nm fluorescence excitation, hence Figure 3-2B corresponds to the DLIPS 193/193/193 scheme, while Figure 3-2C corresponds to the DLIPS 193/220/193 scheme and so forth. As seen from Figure 3-2B through 3-2E, the structure of DLIPS signal reveals a new spectral signature as compared to traditional fluorescence emission of Figure 3-2A, notably in the low wavelength region corresponding to the primary features of phenylalanine and tyrosine (Figure 3-1). Consistent with previous findings with DLIPS in *in vivo* tissue analysis, the shape of the DLIPS spectrum arises from the difference of two relatively broadband fluorescence signals but reveals a significantly different shape.⁶⁵ It is important to note, given the denominator of Eq. 3-1, that the DLIPS spectra are self-normalized; hence all four samples fall on the same relative scale, which is in contrast to the rather broad range of signal intensity in the absolute fluorescence spectra of Figure 3-2A, which are dominated by tryptophan. There are negligible well-defined peaks at about 386 nm, due to the second order of 193 nm reflection from the excitation pulse, on some of curves in Figure 3-2 spectra, but they have no significant effect in statistical analysis, as based on examination of the PCA loadings.

A few qualitative observations are made with regard to the Figure 3-2 spectra. Notably, the black curve representing the 1-0.5-1 analyte set is shifted vertically in the 230 and 245-nm perturbation experiments as compared with the 193 and 220 nm perturbation results. The variations in relative DLIPS curve positions of these analyte

sets under different perturbation wavelengths can be attributed to the different couplings of these molecular structures with different UV wavelengths. For instance, irradiation of biological samples such as proteins and protein-based structures (i.e. DNA) with different UV wavelengths at low intensities results in photoionization of biological samples, which leads to a strand breakage.¹¹² These photo-products of the photoionization processes include, for example, the number of hydrated electrons in aqueous solutions, as measured by Gorner et.al.^{105, 107} According to these studies, linearity between laser pulse intensity versus photo-induced activities (i.e. hydrated electron production) was reported for 193 nm and the wavelengths under approximately 210 nm. Such behavior is also observed during our initial DLIPS studies, which includes irradiation of peptide bonds under low intensities of 193-nm perturbation.⁶³ However, nonlinearity (square dependence to the laser pulse intensity) between laser pulse intensity versus photo-induced activities was observed for 248 nm and 266 nm at low intensities for most cases in Gorner's studies. Such phenomena may also be explained with changing saturation limits at different wavelengths, or multi-photon processes. For our classification cases, these results may support to fundamentally explain the variations in DLIPS curve patterns as a result of different perturbation UV wavelengths. Another point is made by considering the absorption coefficient curves of aromatic amino acids at different UV wavelengths.¹⁰⁸ The values of absorption coefficients differ significantly for each amino acid at selected perturbation wavelengths. If only the absorption coefficients were affecting the results, the DLIPS curves would be expected to shift much more than findings presented in Figure 3-2B through 3-2E.

As noted above, the DLIPS signal is an inherently normalized spectrum; therefore it indicates fractional changes in fluorescence intensity with regard to the baseline pre-perturbation fluorescence spectra. In other words, the value of the DLIPS signal changes between 0 and -1, which can eliminate the effects of absolute fluorescence intensity, for example, due to acquisition system changes or actual sample differences (e.g. thin film spots in the present study or true fluorophore deficiency in an actual sample) since the difference between post-perturbation spectrum and pre-perturbation spectrum is divided by the absolute value of the pre-perturbation spectrum.^{65, 77}

As seen from the Figure 3-2B through 3-2E, the value of the DLIPS signal, which can be considered as a reduction percentage with respect to the pre-perturbation fluorescence signal, indicates the change in the fluorophore response at the target spot. The reduction value is primarily a function of the perturbation wavelength (accordingly the absorption coefficient of the biological sample at this wavelength), beam energy, number of perturbation shots and the duration of the perturbation. Hence, DLIPS affords the opportunity to further optimize the total number of perturbation laser shots for a given photon energy in order to achieve the most discriminating DLIPS signal. In summary, the characteristics of these DLIPS analyte curves such as curve positions with respect to each other is an important factor for the classification performance and will be discussed below quantitatively based on multivariate statistics.

Performance of DLIPS Compared to Traditional Fluorescence on the Classification

Two-dimensional (2D) PCA scores of DLIPS and traditional fluorescence dataset pairs for each UV perturbation wavelength are given in Figure 3-3. At the pre-

processing step, all spectra were smoothed by second-order Savitzky–Golay polynomial filter using 15 points. The PCA model is built with three factors for each pair, however, only the first two factors (principal components) are presented on score plots since the variation inside the PCA factors is found to significantly decrease after the second factor, with the first two factors encompassing approximately 99% variation inside the datasets. Additionally, the fluorescence signal alone (i.e. traditional fluorescence) does not carry a lot of variation due to its nature (Figure 3-2A), namely, a broad featureless signal, so generally the first two principal components are sufficient to visualize 2D PCA scores in both the DLIPS and fluorescence studies.⁶⁵

The overall success of DLIPS scores over fluorescence alone scores for each perturbation experiment can be seen at the first glance by noticing decent clustering of DLIPS scores of analyte sets for all different perturbation wavelengths, as seen in Figure 3-3. In contrast, the fluorescence scores of the analyte sets tend to be randomly distributed in such a way that the distribution and position of scores representing each analyte set change significantly from one fluorescence experiment to another for each perturbation wavelength. For the DLIPS scores, the clusters occupy distinct spaces and the borders of the different clusters do not overlap. From Figure 3-3, it can also be concluded that the DLIPS scores of the four analyte sets acquired during 230 nm perturbation experiment show slightly better clustering than other three perturbation wavelengths and experiments.

Subsequently, the success of DLIPS in classification over traditional fluorescence spectroscopy is quantified with PLS analysis. The PLS model is built for all perturbation experiments with three factors, and a residual sum of squares (RSS) is calculated for

each of them. The results of RSS of all perturbation datasets can be seen in Figure 3-4. The y-axis shows the value of the RSS and the x-axis shows the perturbation wavelength for each traditional fluorescence and DLIPS pair. PLS models are also built with three factors for both fluorescence and DLIPS datasets. All samples (i.e. 120 participants for both fluorescence and DLIPS) in each perturbation experiment are employed for analyses to quantify the performance of fluorescence and DLIPS methods, rather than simply dividing the samples into two halves as training and prediction classes. As analogous to the PCA results, the value of the RSS of the fluorescence sample sets are fluctuating with respect to perturbation wavelength; however, the RSS in the DLIPS sample sets show constant behavior.

Overall, all four perturbation wavelengths revealed a similar significant success in classification of aromatic amino acids for the DLIPS method, and outperformed the fluorescence only analyses considerably, with an average PLS error of 49.5 over the four perturbation wavelengths as compared to a average PLS error of 71 for traditional fluorescence alone. To further assess the robustness of the analysis and experimental data, this result was also validated with several outlier analyses (i.e. outlier rejection) to eliminate the effects of possible outliers. Since the datasets have high number of participants, the changes in RSS values are found to be minor during rejection of possible outliers; hence all data was included as reported above.

According to the multivariate analyses performed, the absorption parameter of sample did affect the classification, but also the molecular structure of sample affected the classification. This also supports the previous findings that the power of the DLIPS method in classification arises from the selective perturbative effects of the UV

wavelength on the chemical structure of the target, such as C-N bond cleavage preference at 193 nm irradiation over other bonds.^{63, 77} Moreover, the DLIPS results in Figure 3-2B through 3-2E demonstrate that the destruction of molecules at the perturbation wavelength of 245 nm is diminished. Note, however, that the photon energy of the 245-nm perturbation beam (5.06 eV) is still enough to disrupt the chemical bonds inside the molecular structure, notably C-N bonds (3.1 eV). Hence, the relatively reduced perturbative behavior at the perturbation wavelength 245 nm may be attributed to low absorption of aromatic amino acids at this wavelength.¹⁰⁸ There is a limit on a perturbation wavelength selection, especially for the classification purposes, since longer UV wavelengths have less photon energies and become increasingly incapable of disrupting molecular bonds of biological structures, noting that earlier work revealed that 355 nm perturbation is ineffective in disrupting peptide bonds.⁶³

At the end of the study, KNN analysis was performed to assess the performance of using DLIPS dataset for labelling unidentified spots. KNN predictions on samples for only the 230 nm perturbation experiment, the optimal wavelength based on Figure 3-3 data, are shown in Table 3-2. In KNN analysis, the fluorescence dataset (120 participants) and the DLIPS dataset (120 participants) are divided into two equal classes (60 participants) as training and prediction sets for both groups. Training sets are used to build KNN models and three neighbors are assigned as voters. The KNN model distributes freshly introduced participants from prediction groups into pre-defined analyte sets. The diagonals in Table 3-2 show the correct assignments to the pre-defined analyte sets. The DLIPS method eventually yields sufficiently better performance (6.69% error) over traditional fluorescence (13.39% error) which can be

seen from Table 3-2 by labelling samples much more correctly. This again demonstrates that the DLIPS method is twice as sensitive to variation of relative aromatic acid quantities. That is, participants misclassified by using fluorescence dataset can be correctly classified by using DLIPS dataset under the same exact conditions in multivariate analysis.



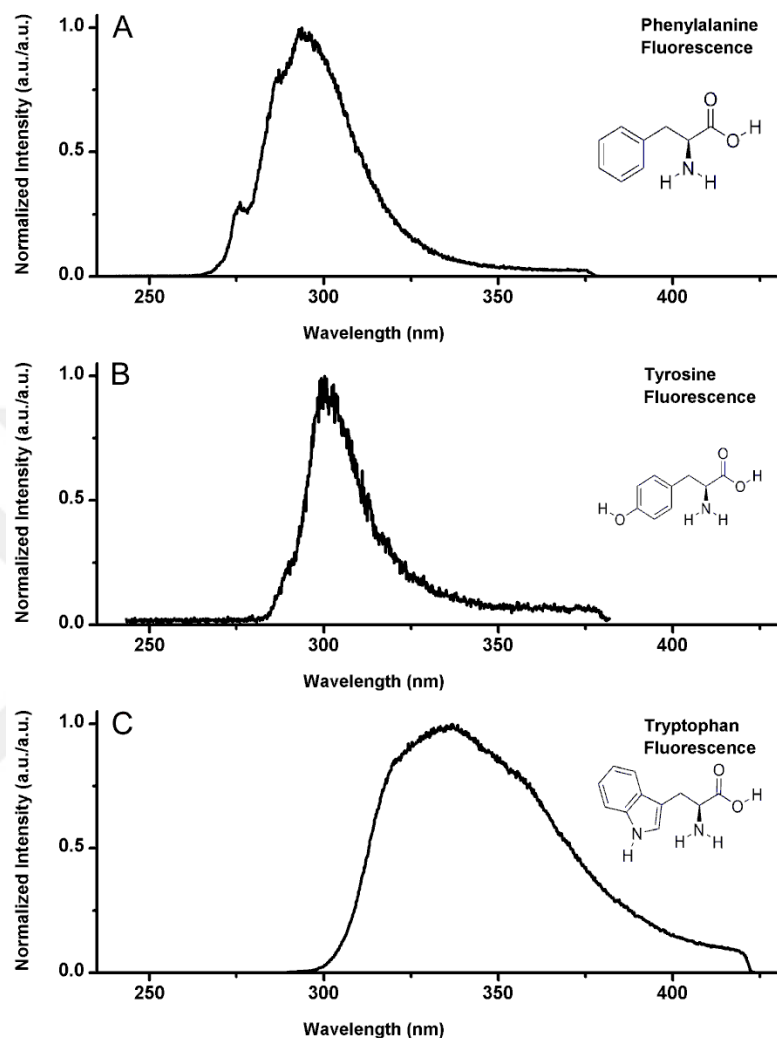


Figure 3-1. Fluorescence spectra and molecular structures of endogenous fluorophores used in the study. A) Phenylalanine. B) Tyrosine. C) Tryptophan. All samples are excited with 193 nm beam.

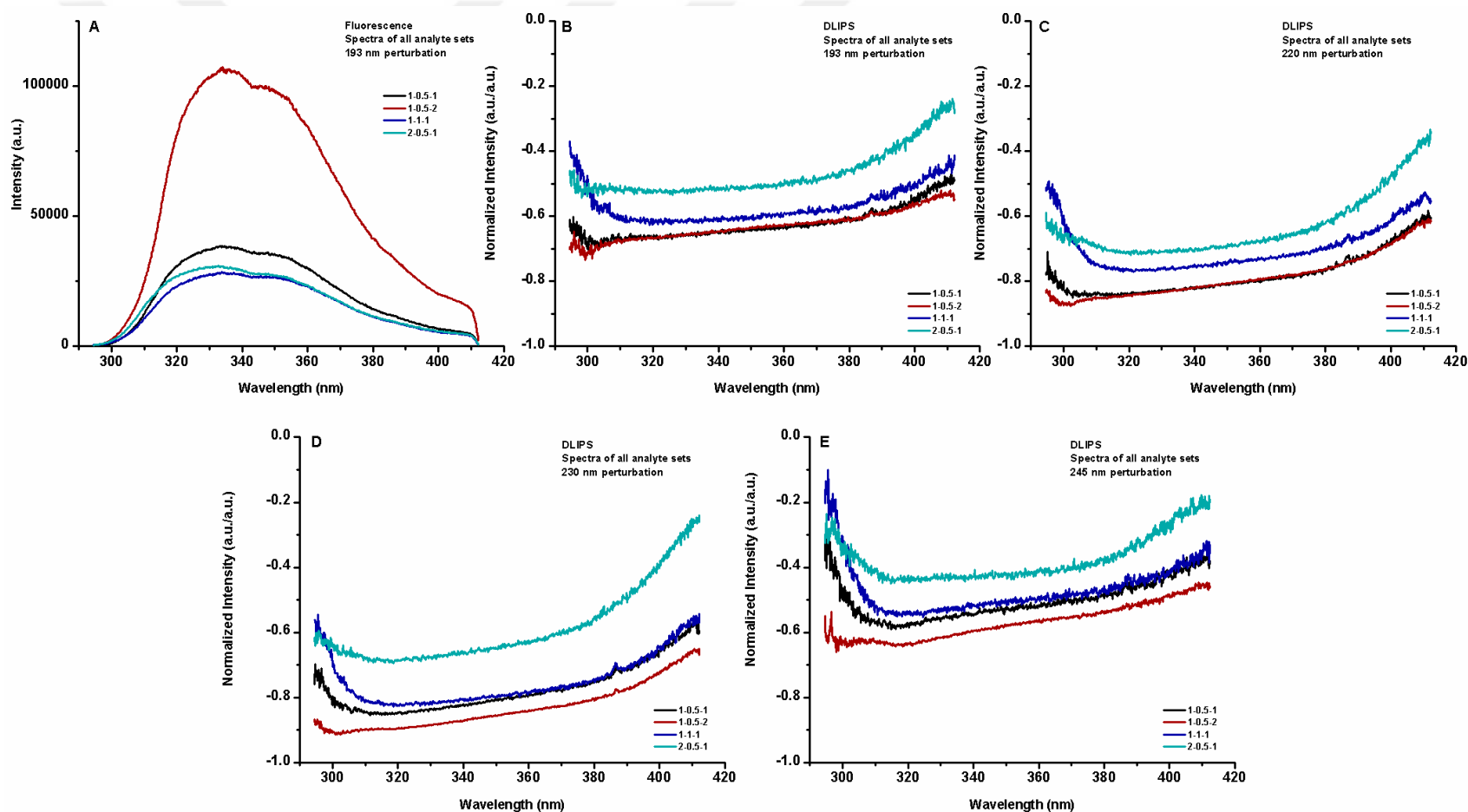


Figure 3-2. Mean spectra of analyte sets for different perturbation wavelengths. A single set of fluorescence spectra is presented as representation. A) Fluorescence spectra of analyte set used in 193 nm perturbation experiment. B) DLIPS spectra of analyte set used in 193 nm perturbation experiment. C) DLIPS spectra of analyte set used in 220 nm perturbation experiment. D) DLIPS spectra of analyte set used in 230 nm perturbation experiment. E) DLIPS spectra of analyte set used in 245 nm perturbation experiment.

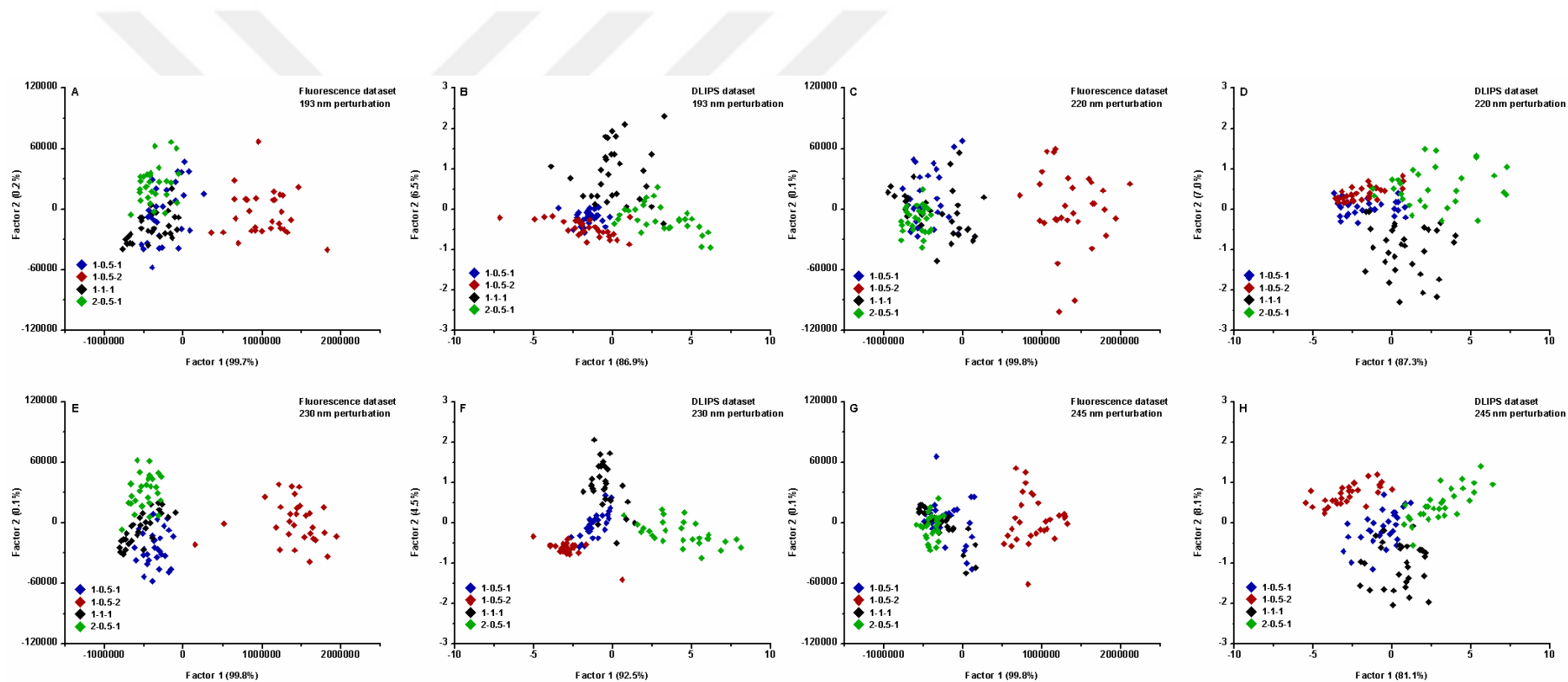


Figure 3-3. 2D PCA score plots of analyte sets. A) Fluorescence dataset of 193 nm perturbation. B) DLIPS dataset of 193 nm perturbation. C) Fluorescence dataset of 220 nm perturbation. D) DLIPS dataset of 220 nm perturbation. E) Fluorescence dataset of 230 nm perturbation. F) DLIPS dataset of 230 nm perturbation. G) Fluorescence dataset of 245 nm perturbation. H) DLIPS dataset of 245 nm perturbation.

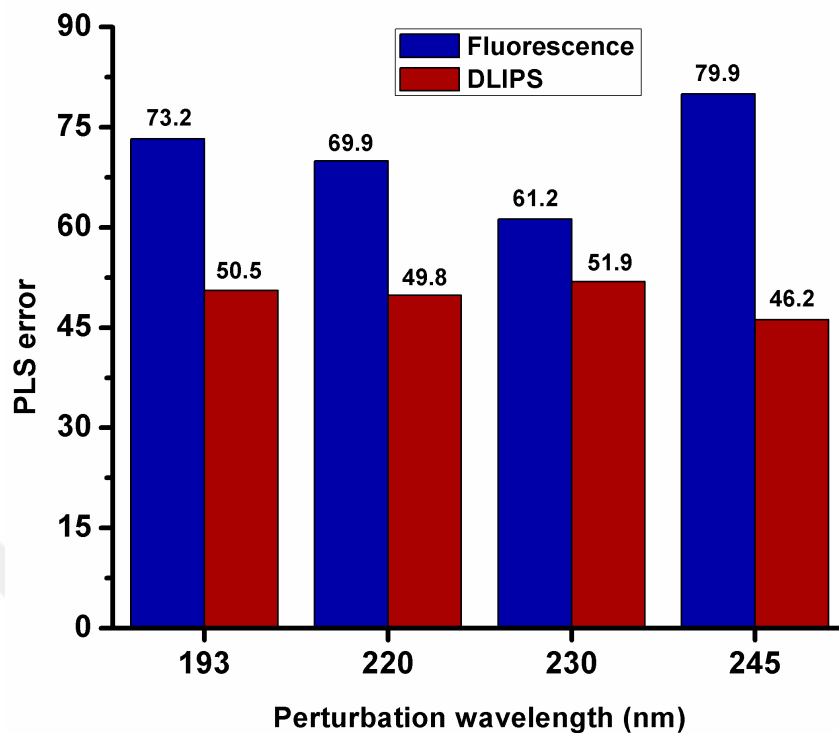


Figure 3-4. Bar plot of PLS error of fluorescence and DLIPS pair for each perturbation experiment for classification of the four fluorophore samples sets. PLS model is built with three factors.

Table 3-1. Detailed information on perturbation pulses delivered to samples. Different total number of photons are delivered for each perturbation wavelength.

Perturbation Wavelength (nm)	193	220	230	245
Energy/pulse ($\mu\text{J}/\text{pulse}$)	50	100	150	150
Duration (sec)	5	10	10	15
Photon energy (eV)	6.4	5.6	5.4	5.1
Total photons ($\times 10^{14}$)	24.3	110.6	173.4	275.2

Table 3-2. KNN predictions of samples perturbed with 230 nm. A total of 120 samples divided into two equal groups to create training and prediction sets for both fluorescence dataset and DLIPS dataset. Classes 1,2,3,4 are corresponds to 1-1-1, 1-1-2, 1-2-1, and 2-1-1 concentration sets respectively. Diagonal shows correctly predicted classes.

Fluorescence dataset					DLIPS dataset				
	Pred. CS1	Pred. CS2	Pred. CS3	Pred. CS4		Pred. CS1	Pred. CS2	Pred. CS3	Pred. CS4
Actual CS1	12 (/15)	0 (/15)	3 (/15)	0 (/15)	Actual CS1	14 (/15)	0 (/15)	1 (/15)	0 (/15)
Actual CS2	0 (/15)	15 (/15)	0 (/15)	0 (/15)	Actual CS2	0 (/15)	15 (/15)	0 (/15)	0 (/15)
Actual CS3	3 (/15)	0 (/15)	12 (/15)	0 (/15)	Actual CS3	2 (/15)	0 (/15)	13 (/15)	0 (/15)
Actual CS4	0 (/15)	0 (/15)	2 (/15)	13 (/15)	Actual CS4	0 (/15)	0 (/15)	1 (/15)	14 (/15)

CHAPTER 4

CLINICAL STUDY: CANCER DETECTION ON HUMAN SKIN SAMPLES

Motivation

In order to bring a conclusion to a long term study presented in chapters 1, 2 and 3, herein skin samples collected from several cancer patients are probed and classified by both fluorescence spectroscopy and DLIPS method. Our initial studies using skin samples indicated that the fluorescence probe is the most convenient tool to acquire signal from skin samples rather than Raman method based on the improved signal-to-noise. Mainly, skin has three basic layers including epidermis, dermis, and hypodermis arranged from outermost layer to inner layer, respectively. The thickness of the epidermis varies in different types of skin, such as it is 50 μm thick on the eyelids, and is 1.5 mm thick on the palms and the soles of the feet. The dermis is the thickest of the three layers of the skin, and its thickness ranges from 1.5 to 4 mm. In general, these layers accommodate many endogenous fluorophores in their building cells. The information obtained from these fluorophores may be helpful to monitor and diagnose early formation of cancer in skin tissues. The DLIPS method has potential to detect cancer formation sensitively and may eliminate the need of biopsy which is difficult, time consuming, and troublesome as a diagnosis method, but remains the goal standard in clinical practice.

Materials and Methods

Experimental Setup

The DLIPS setup is realized as similar to the one presented in chapter 3, where only the 193 nm ArF laser (a single laser) is used throughout the experiment, in order to excite and perturb skin samples. A kinematic filter holder holding two ND filters (a total

optical density of 1.6) is used to bring the intensity of the 193 nm beam to the ineffective level during excitation. By this way, no additional perturbation is generated during excitation. It should be noted that a 193 nm beam has ~6.4 eV photon energy and can easily cause bond cleavage. A switch controlled laser shutter is placed in front of the ArF laser to prevent delivery of any extra laser pulses at the target spot and allow us to keep the laser running so as to provide stable energy throughout the experiment.

The 193 nm laser beam is directed horizontally to a sample holder, later elevated and turned 270 degrees with respect to the incidence plane using of three additional UV grade excimer laser mirrors so that the beam can approach the sample vertically. While 193 nm beam approaches the sample holder, the beam is passed through a pierced mirror which has an angle of 45° degree to the sample holder plane. In order to easily target spots on the samples, a green diode laser is concentrically superimposed to the vertical UV path to help guide the operator. Fluorescence emission from the skin samples is reflected by the pierced mirror and focused on the fiber optic bundle by using two UV-grade lenses. Before entering the bundle, a dielectric coated 193-nm laser maxline mirror is placed at the maximum reflection angle in order to reject the majority of the 193 nm excitation pulse back-reflected from the sample, noting that the UV-grade mirror substrate allows the fluorescence emission to pass to the fiber. The fluorescence signal is dispersed (600 gr/mm grating) onto an array detector by 0.3 m Czerny-Turner spectrometer (SpectraPro-300i, Acton) equipped with intensified CCD (ICCD) camera. A delay generator is employed in order to synchronize camera shutter and excitation pulse triggers. The ICCD gate width is temporally set to 350 ns to acquire the entire fluorescence signal.

A variable aperture is placed just above the pierced mirror perpendicular to the normal direction of sample plane in order to set the diameter of the irradiated area on the sample to ~ 2 mm. By this way identically same areas are ensured for excitation and perturbation pulses. The 193 nm laser beam energy is adjusted to 60 μ J per pulse while ND filters are disabled for perturbation purposes. Noting this energy yields the fluence around 2 mJ/cm².

Data Acquisition and Manipulation

In all fluorescence and DLIPS measurements, freshly excised skin samples are probed at the same day. Skin samples were collected at the UF Dermatology Springhill Clinic's Surgical Dermatology Department (IRB project number: 201500567). They are brought back to hospital the next day in order to maintain patient care. After samples are introduced into our laboratory, they are taken out from their saline solution and gently wiped with Kimwipe tissues. Relatively dried samples are simply placed on the glass microscope slides (Fisher Scientific, Cat. No: 22-038-103). Representative final appearance of the sample can be seen from the Figure 4-1.

No further preparation is applied. By this way real conditions are created for cancer detection experiments as much as possible. A total of 89 spots collected on 26 skin sites from 22 cancer patients are probed and analyzed in this study. Skin samples are generally cut as a football shape. For this study, non-cancerous, healthy skin signal (either fluorescence or DLIPS) is referred as the “control signal” and it is collected from the edges of the skin samples which are far away from the cancer region. And the “cancer signal” is collected from the cancer region within margin boundaries in most cases resides in the middle of the samples; noting Figure 4-1 for marked target spots.

Fluorescence and DLIPS spectra from samples are finally obtained by merging two acquisition windows and each final spectrum for a specific sample spot is an accumulation of 150 images. The first spectral window ranges approximately from 280 to 418 nm centered at 350 nm, and second window ranges approximately from 391 to 527 nm centered at 460 nm. All spectra are saved by using Winspec/32 software (Princeton Instruments). All acquisitions are performed while the room lights are turned off.

Samples are horizontally introduced to a system on microscope slides. A green diode laser is used to find the best target spot on the sample (far from the hair, stains, blood, etc.). Later the green laser is closed until the next spot. Initial fluorescence spectra for both windows are collected at 2 Hz from a selected spot at the lowest intensity (i.e. ND filters are present at the beam path) using 193 nm excitation. Initial fluorescence spectra are collected and saved as “pre-perturbation” spectra for each window. Noting that at any fluorescence acquisition, the laser shutter is opened/closed while the laser is running and precisely at the time duration while the camera is activated so that no extra pulses are delivered to the sample spot. In each acquisition when the acquisition of the first window is finished, grating is moved to another window (its center wavelength) and second acquisition is completed at the second window at the same conditions. After initial fluorescence acquisitions are done for each window, the ArF laser is stopped, ND filters are moved from the beam path, and laser shutter is temporally opened. The frequency of the same delay generator is set to 50 Hz and a total of 2500 laser pulses are delivered to a same sample spot. At the end, the ND filters are brought back to the beam path, the delay generator frequency set back to 2 Hz, and

laser shutter is closed. Laser is continuously turned on again and dark signals are collected for each window while the shutter is remained closed. Finally, the shutter is opened/closed while the camera is activated again and the final fluorescence spectra are collected for the two windows. These fluorescence spectra are saved as “post-perturbation” spectra for each window.

In order to obtain final absolute fluorescence and DLIPS spectra several steps are applied before analyses. Dark signals are subtracted from both pre and post perturbation spectra for each relevant windows (i.e. dark signal acquired at 350 nm window is subtracted from pre signal acquired at 350 nm window and so forth.). By this way, all spectra are converted to an absolute spectrum. The edges of all spectra (approximately 50 pixels for each side) are cropped from two windows and these windows are merged by averaging ten overlapped pixels. At the end, all data points are multiplied by a correction factor, where the correction factor is derived from a calibrated tungsten lamp acquisition by the same setup and compared with the calibrated curve of this lamp; thereby provide a relative spectral response curve. Each final DLIPS spectrum is obtained by calculating;

$$DLIPS(\lambda) = \frac{Em_{POST}(\lambda) - Em_{PRE}(\lambda)}{Em_{PRE}(\lambda)} \quad (4-1)$$

In Eq. 4-1 $Em_{PRE}(\lambda)$, and $Em_{POST}(\lambda)$ represent absolute pre-perturbation and absolute post-perturbation respectively. When absolute fluorescence and DLIPS datasets are formed, PCA analysis is used to explore sample distributions. PCA analyses in this study are performed using Pirouette (Infometrix, version 4.5).

Results and Discussion

Three different characteristic signals (i.e. pre-perturbation, post-perturbation, and DLIPS) acquired from a single patient (i.e. patient number 3) are shown in Figure 4-2 for illustration purposes. The spectra are probed from three distinctive spots such as one cancer and two different control spots. At the first glance, the intensity and the features of curves of fluorescence and DLIPS signals are seem to be different between control and cancer spots. The difference in spectral features is obvious for fluorescence spectra which can be seen from Figure 4-2A. However the differences in features between cancer and control spots are subtle for DLIPS spectra, so specific region of DLIPS spectra is additionally magnified in Figure 4-2D. After perturbation, a prominent peak around 350 nm is destroyed and a new peak is formed around 455 nm. It can be concluded that when the sample is irradiated, natural fluorophores which emit around 350 nm are destroyed while some fluorophores emitting around 455 nm are excited or relevant quenchers are destroyed. Since their fluorescence peaked around 350 nm region, tryptophan may be the one (fluorophore) that is destroyed at the top layer of the skin ⁴² noting that penetration depth of 193 nm is short inside the skin, on order of a micron or less, as mentioned in chapter 3.

The fluorescence spectra along with the 2D score plots of entire pre-perturbation dataset (i.e. traditional fluorescence) are shown in Figure 4-3. Red color indicates cancer signal while black color indicates control signal (i.e. normal skin). There is a significant overlap region between cancer and control signals in fluorescence dataset which can be seen from Figure 4-3A. The fluorescence intensities of the cancer signals tend to be much higher than the control signals. By comparing 2D PCA score plots of pre-perturbation dataset given in Figure 4-3B, it can be said that control and cancer

signals are fairly well grouped together. So, the overlapped region seem less effective in score plots which tells us the difference in features between control and cancer signals have an influence upon the classification.

Accordingly, fluorescence spectra and 2D score plots of entire post-perturbation dataset are shown in Figure 4-4. As mentioned before, characteristic peaks of the fluorescence curves are shifted from left side to right side of the window in their post-perturbation spectra which can be realized by comparing Figure 4-3A and Figure 4-4A. This means that while some fluorophores are destroyed, the other fluorophores are excited or relevant quenchers are destroyed during perturbation. Occasionally, unusual patterns (i.e. additional peaks and shoulders) are realized in pre- and post-perturbation datasets for a couple of cancerous spots. One of them can be identified in the post-perturbation dataset which is the remainder peak at 350 nm, which was not fully destroyed during perturbation. In addition to that, slight differences in signal contours acquired from cancer spots are observed from time to time. These are attributed to patient-to-patient variation, but no single definitive source is identified. Several reasons can be offered to explain these variations. For example, some harmful substances accumulated inside the body during lifetime can undergo fluorescence. These substances are called advanced glycation end products (AGEs) and can cause the development or worsening of many degenerative diseases such as cancer.¹¹³ AGEs can be found both in serum and skin and are measured by fluorescence techniques. Smoking and diet of the person change their levels inside the body and it is expected some of the patients used for this study may show higher levels of AGEs based on their age and lifestyle. Another reason for this variation can be suggested as the pH change

inside the skin sample after perturbation. Local parameters such as pH change can affect the fluorescence emission^{114, 115}. In fact, this phenomenon has a great significance in explaining unusual contours in the post perturbation spectrum. Because during perturbation bond cleavage occurs and H^+ may be formed since the water molecules or the target molecules in the skin may lose H atoms during UV irradiation. The only difference between our skin sample study and the previous studies is that skin samples are not dry, so pH change may be related to important. H^+ ion migration as noted after C-N perturbation in an earlier study.⁷⁷

It is noted that the peak intensities of the pre- and post-perturbation signals seem to be relatively low with 193 nm excitation. That is, peak of the pre-perturbation signal has approximately 1500 arbitrary counts in average, where the peak of the post-perturbation signal has approximately 150 arbitrary counts in average. This result corresponds to 90 percent decrease in pre-perturbation intensity. The arbitrary intensity count of particular fluorescence signal is a function of penetration depth of UV light, i.e. 193 nm, inside the skin. The signal is acquired only from the surface of the skin for 193 nm excitation. A first-order calculation can give an initial estimation, by considering that the average depth of the epidermis is ~50 micron, which consists of 50 to 100 cell layers, whereas average penetration depth of 193 nm is ~1 micron. Therefore, approximately the information in the 2% of the total skin volume can be acquired per the 193-nm excitation pulse. Extracting information from this small volume may explain the sensitivity of 193 nm excitation to the local changes; hence local changes especially become important for the post-perturbation dataset. However, it is also noted that 193 nm excitation is a very effective wavelength to excite tryptophan molecules.

Visually from the 2D score plots, post-perturbation data is not well grouped as much as the pre-perturbation data. That is, the border between the two different groups is less clear in post-perturbation scores. This theory is validated with PLS algorithm. The residual sum squares errors (RSS) are calculated in order to quantitatively compare the performance of pre and post-perturbation datasets and support the theory. The RSS value calculated for pre-perturbation dataset is found to be 11.8, while it is 14.6 for the post-perturbation dataset. Maximum three factors are used to build PLS model.

Finally, DLIPS spectra and 2D score plots of the whole DLIPS dataset are shown in Figure 4-5. There is a significant overlapped region between control and cancer signals for DLIPS dataset which can be seen from Figure 4-5A. Accordingly, the result of overlapped regime reflects to the 2D score plot which can be seen from Figure 4-5B. The scores of DLIPS dataset are not distributed as clear as the pre-perturbation dataset. This worsening of the score distributions of DLIPS dataset indicates that the advantage of using DLIPS method for single patient (such as crossing of control and cancer curves, i.e. indication of inverse relation between each other, which can be seen from Fig 4.2D), becomes less important in the whole DLIPS data pool. To explore further this issue, PLS algorithm is applied only for the patient number 3 where its data represented in Figure 4-2. The RSS values are found to be 0.1159, 0.1394, 0.0057 for pre-perturbation, post-perturbation, and DLIPS datasets, respectively, noting that DLIPS data has the least modelling error for single patient. Also, it is clear that DLIPS results may be affected by local changes such as pH change as mentioned before.

In Table 4-1, additional statistical analyses are presented for pre-perturbation, post-perturbation, and DLIPS datasets. All curves are integrated over the entire

wavelength region and statistical analyses are performed on the sums of arrays of each signal curve. Maximum, minimum and average values along with the standard deviations of these values for each dataset are calculated. The curves of the DLIPS dataset tend to be tightly distributed, thus the percentage values of the standard deviations of control samples suggest that the variation inside the DLIPS dataset is as low as the pre-perturbation dataset. Additionally, according to the percentage values of the standard deviations of cancer samples, the deviation inside the DLIPS dataset is less than pre-perturbation dataset, which can be directly realized by comparing the Figure 4-3A and Figure 4-5A. Once again these results show the robustness of DLIPS method. The value of the percentage standard deviation of post-perturbation dataset suggests that the variation of the curves inside the dataset is not significant.

Eventually, representative post-histopathology images (i.e. monitored after biopsy) is presented in Figure 4-6. It can be seen that not always is there a significant tumor that remains after biopsy. In some cases, it is found that significant amount of tumor may be swept from the probed region during initial biopsy (i.e. a scrape biopsy). Still non-visible tumor growth and scar tissue will be assumed to be different than control tissue because the pre-perturbation dataset shows distinct classification between pre-defined groups; however the effects of ambiguity in the excision method on DLIPS dataset should be considered. Accordingly, PCA scores of DLIPS dataset is plotted in Fig 4-7 based on the new information. This time DLIPS dataset is divided into three groups based on the post-histopathology information. Initial cancer dataset is divided into two subsets, including real cancer group which has significant amount of tumor remaining after biopsy as verified by post-excision pathology, and scar tissue

which has no significant tumor leftover per pathology. The new model suggests that DLIPS is actually more successful in clustering cancer tissue than clustering scar tissue with less or no tumor reminder, since cancer samples are more tightly grouped in the score plot.

In summary, results imply that DLIPS technique is feasible to be used for human skin samples, and can be performed with the 193 nm excitation and perturbation. The changes induced by perturbation could be monitored and the technique may be still successful for a specific patient by calibrating the model every time by collecting control signal for that specific patient . However, ongoing research on DLIPS technique should continue to optimize the performance of DLIPS and make it a robust clinical diagnostic tool. All these findings suggest that the fluorescence acquisition with 193 nm from the skin samples is significantly dependent on the local parameters of the skin. Further suggestions will be presented in the following section.

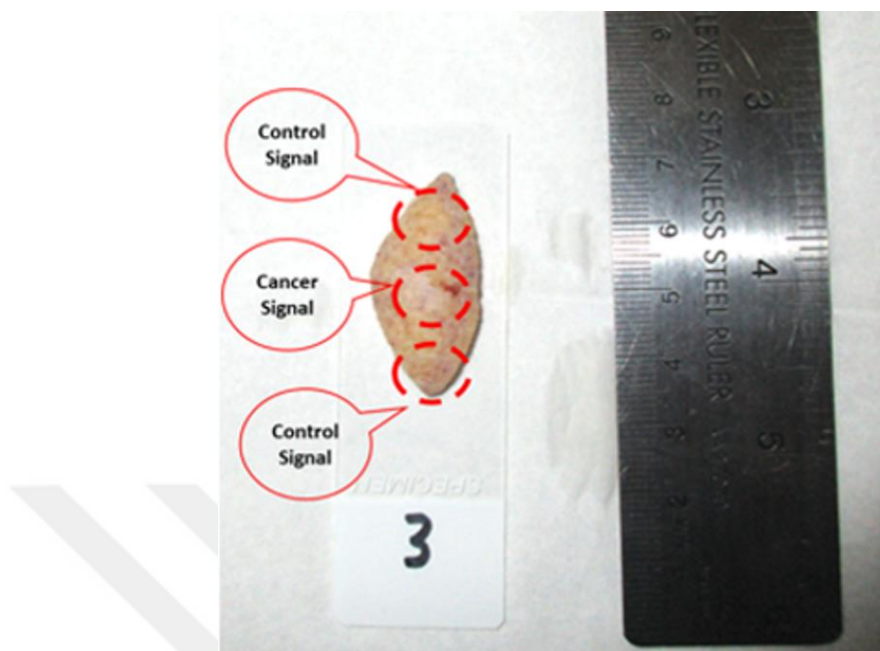


Figure 4-1. Football shaped cut skin sample used in fluorescence and DLIPS experiments. Tissues are dried gently and placed onto microscope slides. Number three on the microscope slide represents patient 3. The numbers on the ruler have a unit of cm.

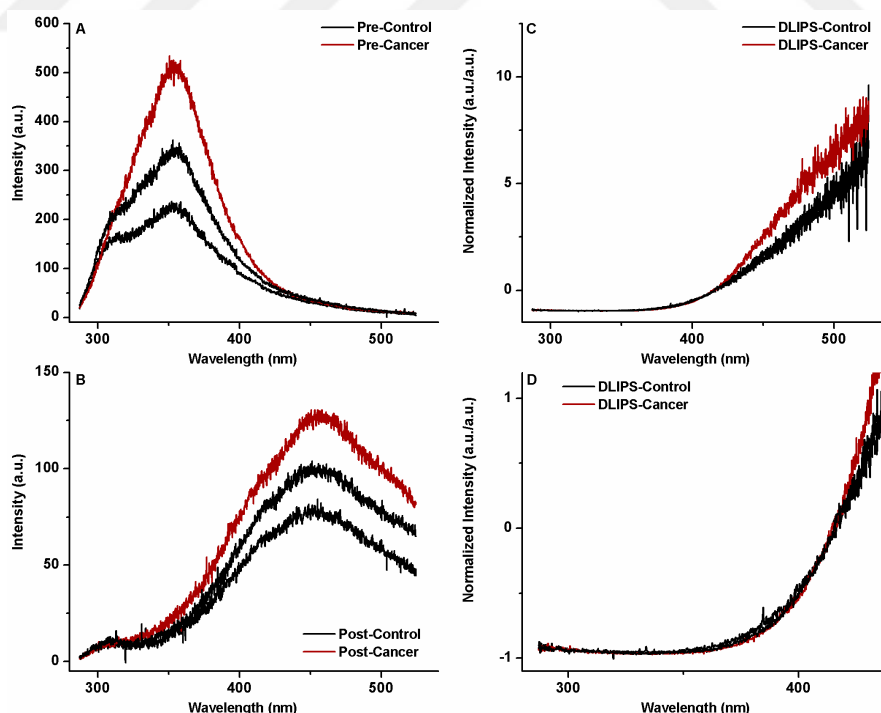


Figure 4-2. General control and cancer signal features from 3 spots (one cancer and two controls) probed on specific patient (patient number 3). A) Absolute pre-perturbation spectra. B) Absolute post-perturbation spectra. C) DLIPS spectra. D) Specific region of the DLIPS spectra. Black graphs illustrate control signals whereas red graphs illustrate cancer signals.

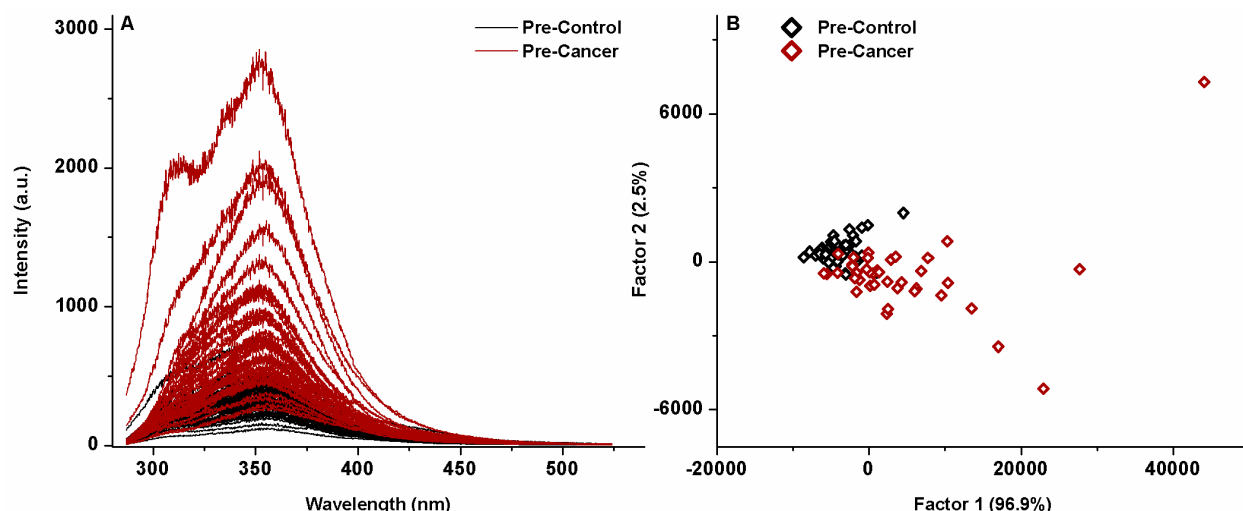


Figure 4-3. Pre-perturbation dataset acquired from cancerous and non-cancerous spots. Red color indicates cancer signal, whereas black color indicates control signal. A) Fluorescence spectra. B) 2D PCA scores. Only two factors are used for the calculations.

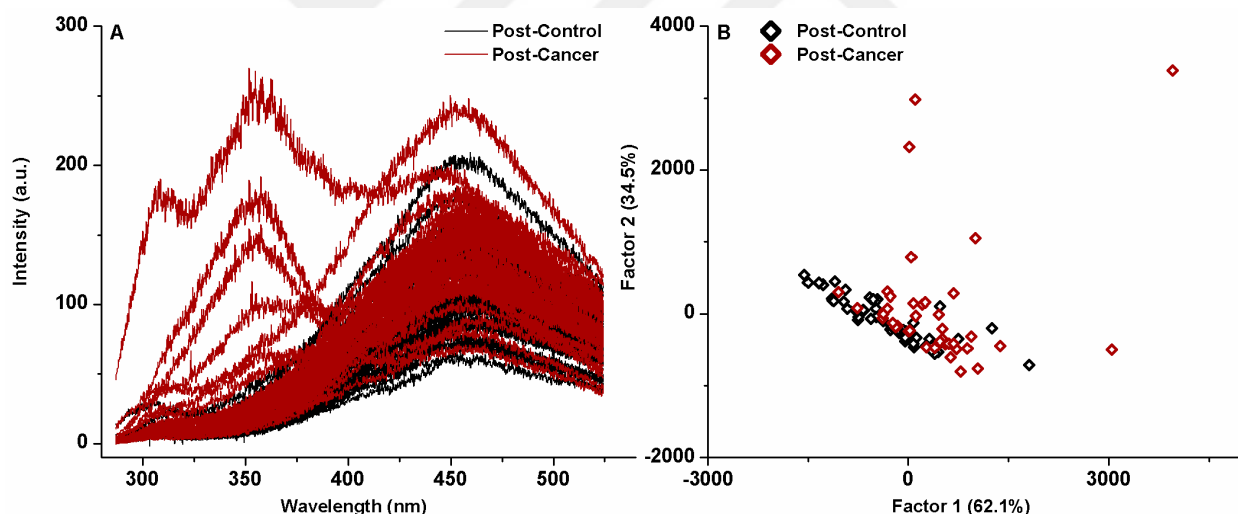


Figure 4-4. Post-perturbation dataset acquired from cancerous and non-cancerous spots. Red color indicates cancer signal, whereas black color indicates control signal. A) Fluorescence spectra. B) 2D PCA scores. Only two factors are used for the calculations.

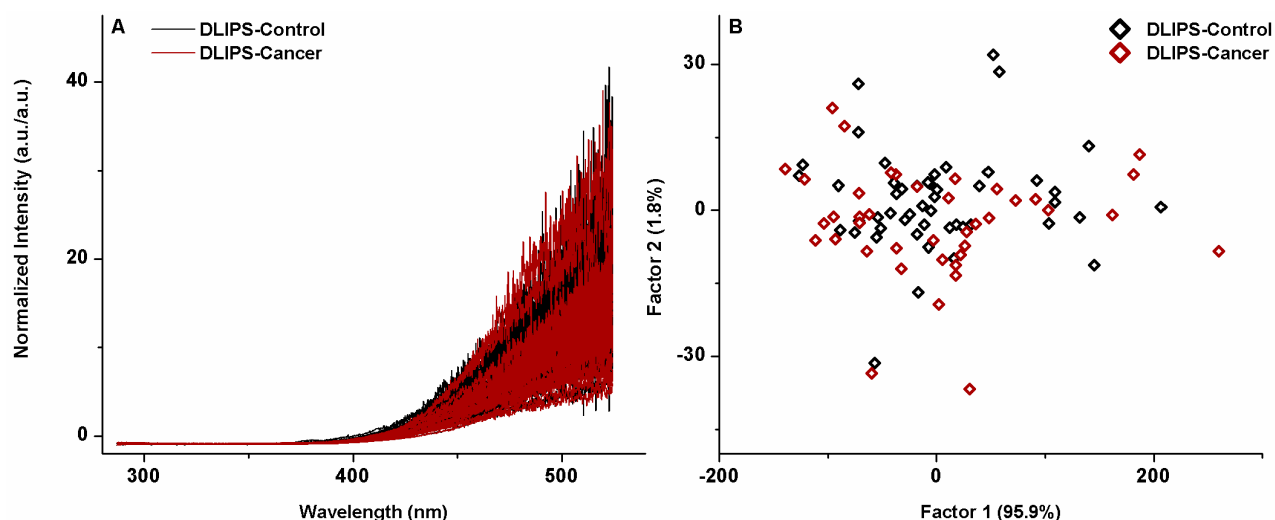


Figure 4-5. DLIPS dataset acquired from cancerous and non-cancerous spots. Red color indicates cancer signal, whereas black color indicates control signal A) Fluorescence spectra. B) 2D PCA scores. Only two factors are used for the calculations.

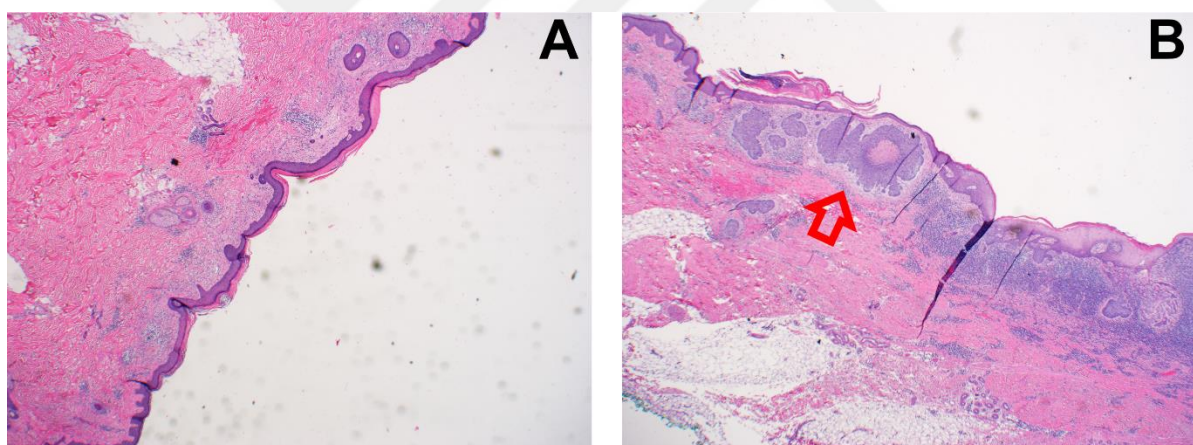


Figure 4-6. Representative histopathology images of samples. Samples were paraffin-stained with hematoxylin and eosin (H&E). A) Section with no significant tumor leftover after biopsy. B) Section left with significant tumor leftover after biopsy. Red arrow points tumor island under surface.

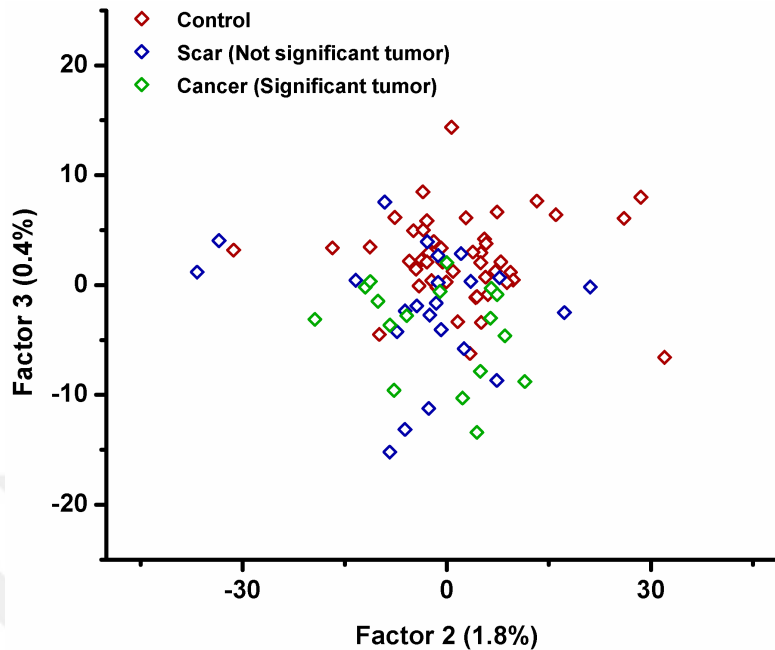


Figure 4-7. PCA scores of DLIPS dataset. Three classes shown including signal acquired from control (red), scar (blue), and cancer (green) tissues. Significant reminder tumor tissue is observed for cancer tissues. Scar tissue may have tumor cells but not significant.

Table 4-1. Additional statistical analyses for pre-perturbation, post-perturbation and DLIPS datasets presented in Figure 4-3, 4-4 and 4-5. Max: maximum value, Min: minimum value, Stdev: standard deviation, Stdev(%): standard deviation of the mean value in percentage. Absolute values are used in calculations for DLIPS dataset.

	Max	Min	Mean	Stdev	Stdev (%)
Pre Control	462581	79904	224312	68714	30
Pre Cancer	1594365	176094	452297	269208	59
Post Control	183292	57593	103592	26411	25
Post Cancer	302892	77077	133020	37928	28
DLIPS Control	11022	3029	6069	1731	28
DLIPS Cancer	12035	2755	5965	2153	36

CHAPTER 5 CONCLUSIONS AND FUTURE WORK

Final Conclusions

At the beginning of the study presented in this dissertation, comparison between the differential laser-induced perturbation spectroscopy (DLIPS) method and the traditional Raman spectroscopy method was demonstrated using several common chemometric data analysis routines. A Raman based *in situ* DLIPS setup was realized with that study for common biological building blocks. It is shown that the use of low intensity UV laser light for perturbation of the amino acid and dipeptide molecular structures, as measured with a Raman probe, provides a new and superior spectroscopy-based classification tool, as rooted in the observed permanent UV-induced photochemistry, notably C-N chemistry. It was observed during calculations that simple subtraction of pre-perturbation signal to post-perturbation signal yields a significant fluorescence background signal increase, thereby all spectra were individually baseline corrected and normalized every time at the pre-processing step. The increase in fluorescence can be explained by the change in the absorption cross section after the molecule is disrupted.⁸³ The cross section change does not have an effect on the Raman peaks; hence it only changes the arbitrary DLIPS baseline. Noting that absorbance can be stated as;

$$A = 0.434\sigma bn \quad (5-1)$$

where σ is the absorption cross section (cm^2), b is in cm , and n is the concentration of the species in atoms or molecules per cm^3 . Thus since the absorption cross-section of biological molecules increases, their absorption performance at 193 nm will not be static and will increase during perturbation.

In the next study, the effect of using different UV perturbation wavelengths for the DLIPS method coupled with a 193-nm fluorescence probe is analyzed and quantified with several statistical analyses. The potential of using the DLIPS method coupled with a fluorescence probe for cancer detection was shown before with an *in vivo* on animal murine model.⁶⁵ The main purpose was to show the DLIPS method's performance to detect the relative changes of the aromatic amino acid quantities and to find the best optimization for DLIPS method by seeking the potential advancement of using different perturbation wavelengths in statistical performance. Also deep UV excitation is used the first time in this study, which allows one to monitor a broader wavelength range. It is noted that, the rather difficult challenge of sample discrimination is posed in the current study; namely, classifying 4 sample sets each comprised of the same 3 fluorophores, with the difference being slight changes in relative concentration. This is a much more difficult classification problem than simple sorting of neat samples, and is believed to be much more relevant with regard to taking the next step to *in vivo* tissue analysis, where normal or abnormal tissue types may have similar sets of fluorophores but at different relative concentrations.

At the first part of this study, the excitation and the perturbation is accomplished only with a single wavelength i.e. 193 nm, which is a deep UV wavelength that leaves no trace of thermal effects during ablation studies.¹ The other longer UV wavelengths used here as perturbation sources are also of interest for their possible advantages, since different wavelength-molecule interactions may increase the detection sensitivity. Fundamentally, different absorption coefficients associated with various UV wavelengths and the linear and nonlinear interactions of various UV wavelengths with

biological samples may lead to a better classification performance. Even if these longer wavelengths may be associated with some concomitant thermal effects at high intensities, DLIPS exploits low intensity perturbation in which the intensity is significantly lower than the ablation thresholds of most biological samples.¹

Furthermore, the DLIPS method with single or dual wavelengths (excitation and perturbation with the same or different UV wavelengths) can easily be adapted to fiber optic use. According to multivariate analyses, all perturbation wavelengths used in this study yield superior quantitative results as compared to traditional fluorescence. The PLS error analyses indicates a limitation on performance of DLIPS method in terms of the perturbation wavelength used, but also shows robustness of DLIPS datasets. The low fluctuations in DLIPS datasets may be very useful to eliminate patient-to-patient variations with *in vivo* analysis. By looking at the PCA scores, 230 nm DLIPS perturbation can be considered as a best case for DLIPS method in order to classify the current aromatic amino acids.

It is clear that further assessment is required for DLIPS method in rich biological environments. In fact, the variation inside the dataset may be much higher in rich environments, which may lead to a better classification, since the perturbation may affect each component inside the optical perturbation volume, resulting in additional probe-target coupling, which is then manifest in the DLIPS signal. Thus, at the last study DLIPS method is compared with traditional fluorescence spectroscopy to evaluate their performance in classification of skin samples collected from various cancer patients. The feasibility of application of DLIPS method using 193 nm excitation is shown for skin samples. Accordingly, the DLIPS method successfully showed different spectral

features between DLIPS curves of cancer and control signal (i.e. crossing of control and cancer signal curves) for a single patient. At the end, DLIPS successfully classifies control and cancer samples. However, DLIPS did not yield significantly better quantitative classification results over traditional fluorescence with the 193/193/193 nm study.

The use of the DLIPS approach in such an orthogonal sensing scheme is advantageous, as the use of the pre-perturbation spectra (e.g. the pre-perturbation Raman spectra or fluorescence spectra) yield the traditional spectral data at no additional cost. Moreover, the nature of the DLIPS method may provide convenience for clinical applications *in vivo* such as enabling a single fiber probe, useful to mitigate target movement via contact, to monitor abnormalities. Higher excimer laser repetition rates (e.g. 400 to 500 Hz) can also greatly increase the speed of such clinical applications. Finally, we note that one is not limited to the 193 nm perturbation wavelength exclusively; hence the opportunity exists for optimization of both perturbation wavelengths as well as resonance probe wavelengths

In summary, the DLIPS method may be advantageous for suppressing patient-to-patient variations, and increasing the sensitivity of the detection by correctly classifying complex biologically relevant samples. It is expected that DLIPS method may overcome drawbacks of traditional spectral emission applications including saturation, patient to patient variation, and background signal problems in clinical applications.

Future Work

Future research should continue exploring complex biological samples, including additional animal models, and skin samples to further validate the DLIPS approach, either as a stand-alone spectroscopy technique, or in conjunction with Raman and

fluorescence spectroscopy. The advantage of using Raman spectroscopy for the differential spectroscopy is significant due to high penetration depth of Raman excitation. Light in visible spectrum or near infrared spectrum can penetrate inside the skin through dermis with approximately penetration depth of 80-120 microns. Different Raman excitation lights such as 532, 633, and 785 nm should be evaluated.

Different excitation/perturbation wavelengths should be included in DLIPS analyses, including the 355/193/355 scheme previously explored for the mice study. 355 nm can penetrate deeper inside the skin allowing larger volume to be probed. As seen from the Figure 5-1, the peak intensity of the 355 nm excitation is much higher than the 193 nm excitation, which shows that 355 nm can probe a larger volume and can be advantageous for DLIPS analysis. That is, the local effects may not be as strong as it is in 193 nm excitation. Of course, the drawback of the configuration is that the fluorophores under 355 nm would not be realized such as tryptophan emission.

The calculation of the DLIPS spectrum should be re-examined to explore new ways to normalize the DLIPS data in order to avoid the potential division by small (near-zero) numbers, e.g. normalize by some predefined average spectrum or add a small offset. Moreover, real-time perturbation should be explored to constrain the number of shots delivered to the tissue by providing decay around 50 percent at 350 nm fluorescent peak in order to keep possible a useful signal level. Finally, fiber optic delivery should be included once the optimal DLIPS setup is established with Raman and fluorescence probes. In addition to these, the tissue sample excision method should be reconsidered and the technique may be modified to ensure more tumor exists on the skin surface.

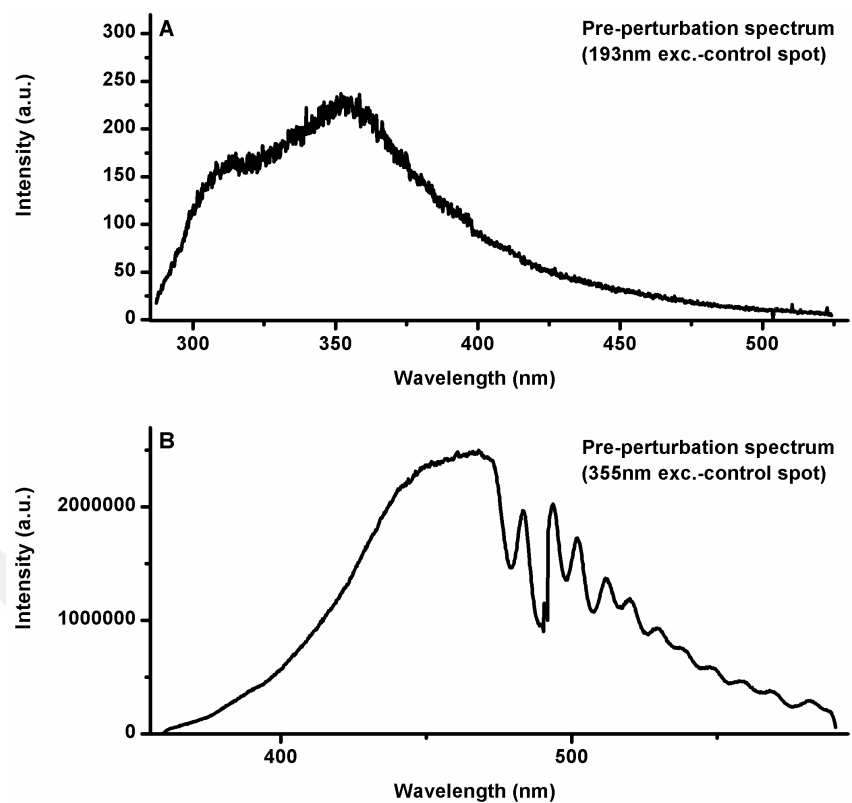


Figure 5-1. Specific pre-perturbation curves from control spots for illustration purposes. A) Fluorescence signal excited by 193 nm light. B) Fluorescence signal excited by 355 nm light. The fluorescence curve is not corrected for 355nm excitation.

APPENDIX MATHEMATICAL BACKGROUD OF STATISTICAL ANALYSES

Introduction

The summary of mathematical background of statistical analyses used in this study will be presented in this section. Principal components are used for exploratory analysis in statistics, because of its superior visualization capabilities showing graphically intersample relationships. The main use of the PCA is to analyze large datasets at the onset of the other complex statistical operations and reduce the effective dimensionality of the large dataset by using vector space transformation.

Common Statistical Concepts

Statistics is a unique discipline which mostly tries to express relations between the sample set that is arbitrarily taken from the population and the entire population. For a vector x consisting of n number of samples; the mean, the variation and the standard deviation which shows how spread out the data can be formulated respectively as follows respectively.

$$\bar{x} = \frac{\sum_{i=1}^n x_i}{n} \quad (A-1)$$

$$\sum_{i=1}^n x_i^2 \quad (A-2)$$

$$s = \left(\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{(n-1)} \right)^{1/2} \quad (\text{A-3})$$

Variance (s^2) is the square of standard derivation. Both terms are the measures of the spread of the data. In general, datasets have more than one dimension. Standard deviation and variance is valid only in one dimensional data. But they can be still calculated in one direction which is independent of other dimensions. Covariance is a measure which is calculated between two dimensions. If covariance is calculated between one dimension and itself then it turns to be variance. The formula of variance and covariance is given in Eq. A-4 and Eq. A-5 respectively.

$$\text{var}(X) = \frac{\sum_{i=1}^n (X_i - \bar{X})(X_i - \bar{X})}{(n-1)} \quad (\text{A-4})$$

$$\text{cov}(X) = \frac{\sum_{i=1}^n (X_i - \bar{X})(Y_i - \bar{Y})}{(n-1)} \quad (\text{A-5})$$

Datasets usually reserve information from many non-specific sensors. However some of the information collected from different sensors is redundant. Thus this redundant information mostly correlates with the rest of the useful information. This is the main reason PCA to be used to identify these patterns in the data and eliminate redundant information in the dataset.

Derivation Steps of PCA

Let's consider $(m \times n)$ matrix 'X' which represents the dataset. The matrix contains m rows which are the independent variables and n columns which are the number of samples (e.g. observations). If X is transformed to another matrix Y, the transformation matrix T have a dimension of $(m \times m)$.

$$Y = TX \quad (A-6)$$

$$TX = (Tx_1 \ Tx_2 \ \dots \ Tx_n) = \begin{pmatrix} t_1x_1 & t_1x_2 & \dots & t_1x_n \\ t_2x_1 & t_2x_2 & \dots & t_2x_n \\ \vdots & \vdots & \ddots & \vdots \\ t_mx_1 & t_mx_2 & \dots & t_mx_n \end{pmatrix} = T \quad (A-7)$$

In Eq. A-7; $t_1, t_2, \dots, t_m$ are the row vectors of T, and $x_1, x_2, \dots, x_n$ are the column vectors of X. It can be noted that the rows of T are the new basis for representing the columns of X. PCA seeks to find new directions in which variance is maximized and then use these directions to define new basis. For n discrete measurements (i.e. samples), we can think X matrix in terms of m row vectors, each of length n .

$$X = \begin{pmatrix} x_{1,1} & x_{1,2} & \dots & x_{1,n} \\ x_{2,1} & x_{2,2} & \dots & x_{2,n} \\ \vdots & \vdots & \ddots & \vdots \\ x_{m,1} & x_{m,2} & \dots & x_{m,n} \end{pmatrix} = \begin{pmatrix} x_1 \\ x_2 \\ \vdots \\ x_m \end{pmatrix} \in \mathbb{R}^{m \times n} \quad (A-8)$$

Then covariance matrix of X is shown in Eq. A.9. Noting that covariance is a measure of how well correlated two variables are.

$$C_X = \frac{1}{n-1} XX^T = \frac{1}{n-1} \begin{pmatrix} x_1 x_1^T & x_1 x_2^T & \dots & x_1 x_m^T \\ x_2 x_1^T & x_2 x_2^T & \dots & x_2 x_m^T \\ \vdots & \vdots & \ddots & \vdots \\ x_m x_1^T & x_m x_2^T & \dots & x_m x_m^T \end{pmatrix} \in \mathbb{R}^{m \times m} \quad (\text{A-9})$$

The fundamental assumption of PCA is to get transformed matrix with highly uncorrelated variables after linear transformation of original data. This is similar to say that the non-diagonal elements of the matrix C_Y should be close to zero as much as possible. At this point $t_1, t_2, \dots, t_m$ are assumed to be orthogonal to find the solution to this problem.

$$C_Y = \frac{1}{n-1} YY^T = \frac{1}{n-1} (TX)(TX)^T = \frac{1}{n-1} T(XX^T)T^T \quad (\text{A-10})$$

$$C_Y = \frac{1}{n-1} TST^T \text{ where } S = XX^T \quad (\text{A-11})$$

S is symmetric matrix. This can be formulated as follows;

$$S = EDE^T \quad (\text{A-12})$$

E is a $(m \times m)$ orthonormal matrix who has the orthonormal eigenvectors (columns) of S , and D is a diagonal matrix who has the eigenvalues (diagonal elements) of S . By choosing the rows of T to be the eigenvectors of S , it can be ensured that $T=E^T$.

$$C_Y = \frac{1}{n-1} TST^T = \frac{1}{n-1} E^T (EDE^T) E = \frac{1}{n-1} D \quad (\text{A-13})$$

The largest variance is called as the *first principal component*, the second largest to the *second principal component*, and so on. Once the eigenvalues and eigenvectors of S are computed, eigenvalues should be sorted in descending order on the diagonal of matrix D. Then orthonormal E matrix can be constructed by placing corresponding eigenvectors to the columns.

According to the singular value decomposition (SVD) principals, the principal components of X (which is tried to be identified) are the eigenvectors of C_X . SVD of any matrix B (n x m) is given in Eq. A-14.

$$B = UH V^T \quad (A-14)$$

where $U(n \times n)$ orthonormal
 $H(n \times m)$ diagonal
 $V(m \times m)$ orthonormal

Thus the principal components should be the columns of the orthogonal matrix, V.

$$Y = V^T X \quad (A-15)$$

$$X = VY \quad (A-16)$$

Preprocessing Operations

Mean Center

Mean center operation is preferred especially for spectral datasets. Relationships between samples can be more conveniently visualized and statistical operations can be performed easily after this operation. Mean of the vector dataset can be computed as follows;

$$\bar{x}_j = \frac{1}{n} \sum_{i=1}^n x_{ij} \quad (\text{A-17})$$

And the centered data can be calculated by subtraction of this mean from the original data.

$$x_{ij(mc)} = x_{ij} - \bar{x}_j \quad (\text{A-18})$$

Divide by Sample Range

Since the data is generally acquired from different instruments, data units might not be the same every time. This transformation provides comparable scale for all measurements.

$$x(norm)_{ij} = \frac{x_{ij} - \min(x_i)}{\max(x_i) - \min(x_i)} \quad (\text{A-19})$$

Baseline Correction

Baseline correction operation corrects offsets by subtracting a fitted profile from the spectral data. The success depends on the determining the degree of the polynomial to be fit. For spectral data generally this degree goes up to third degree maximum. By this way no peak contours of the spectral data is affected. The knowledge of the data is crucial. So the data should be visualized before degree selection. Linear fit section fits the simple linear model to the data.

$$y = \beta_0 + \beta_1 x \quad (\text{A-20})$$

Polynomial fit section fits polynomial with a higher degree to the data.

$$y = \beta_0 + \beta_1 x + \beta_2 x^2 + \dots + \beta_n x^n \quad (\text{A-21})$$

Hierarchical Cluster Analysis

Hierarchical Cluster Analysis (HCA) groups pairs of samples based on their distances (i.e. Euclidean) to each other. HCA highlights natural groupings inside the data. The multivariate distance d_{ab} between two sample vectors a and b, is computed by accounting all m variable distances for these samples.

$$d_{ab} = \left[\sum_{i=1}^m (x_{ai} - x_{bi})^M \right]^{1/M} \quad (\text{A-22})$$

M is the order of the distance. M=2 is the Euclidean distance where this is the most common in multivariate analysis. The scale for the inter-sample distances is similarity variable.

$$\text{similarity}_{ab} = 1 - \frac{d_{ab}}{d_{\max}} \quad (\text{A-23})$$

Samples are linked after distances are calculated between pairs. After first clusters are formed, these clusters are linked to another cluster. And this process continues until all clusters are linked. Newly formed clusters A and B is linked to another cluster C via couple of formulas where n_i is the number of samples in cluster i.

Single link;

$$d_{ABC} = 0.5d_{AC} + 0.5d_{BC} - 0.5|d_{AC} - d_{BC}| \quad (\text{A-24})$$

Median link;

$$d_{ABC} = (0.5d_{AC}^2 + 0.5d_{BC}^2 - 0.25d_{AB}^2)^{1/2} \quad (\text{A-25})$$

Centroid Link;

$$d_{ABC} = \left(\frac{n_A d_{AC}^2}{n_A + n_B} + \frac{n_B d_{BC}^2}{n_A + n_B} - \frac{n_A n_B d_{AB}^2}{(n_A + n_B)^2} \right)^{1/2} \quad (\text{A-26})$$

Group average link;

$$d_{ABC} = \frac{n_A d_{AC}}{n_A + n_B} + \frac{n_B d_{BC}}{n_A + n_B} \quad (\text{A-27})$$



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BIOGRAPHICAL SKETCH

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