ULTRAFAST LASER SAMPLING OF A PLANT TISSUE AND ION CONDUCTIVITY MEASUREMENT FOR INVESTIGATION OF LIGHT STRESS GENERATION MECHANISMS

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In this study we applied ultra-short laser pulses on a biological sample (Arabidopsis), in order to cut it precisely in a square pattern and subsequently use it for studying stress generation mechanisms. For this purpose, we utilized femtosecond laser pulses at 100 fs pulse width and 80 MHz repetition rate. We took two processing parameters into consideration such as laser power, laser exposure time which is related to the stage speed. Therefore, we were able to find the laser optimum conditions for ablation of biological tissues. The mutant and wildtype (control) obtained from laser cutting with a size of 500 µm × 500 µm were directly transferred (*in-situ* with laser cutting) into a microfabricated chamber containing ~500 nanoliters deionized water for measuring ion conductivity. The ion conductivity is a signature of cell-death mechanisms caused by various stresses. A light with intensity of 100 µmol was exposed to the samples for 2 hours and 20 minutes as a source of stress.

A quantitative electrical analysis with high accuracy was assured by utilizing a microchamber, which enables a measurement in nanoliter volume. We measured the impedance which is reciprocal of conductivity using a lock-in amplifier and a precise current source at frequency of 130 Hz. Initially high impedance of mutant sample tended to drop within 2 hours and finally approached the constant value which signified that the cell death mechanism was complete. However, the wildtype sample demonstrated approximately constant impedance (conductivity) during the experiment.
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CHAPTER 1

INTRODUCTION

1.1 About Lasers, Laser Types and Applications

Laser is an acronym for light amplification by stimulated emission of radiation. This means that laser is a device that releases electromagnetic radiation (light) throughout the procedure named stimulated emission. It can be described as an instrument which manages the mode that energized atoms free photons. A more straightforward description is that laser is a machine that intensifies light. In opposition to light from different sources, the laser light is made of one or no more than few wavelengths. Being able to control the wavelength in lasers is a benefit when accurate material processing by using lasers is needed. Moreover, a laser generates a beam of monochromatic light. Monochromatic nature of light is a unique property that is offered by lasers, in which all of the waves are coherent or in phase. Coherency is also known as a property where group of photons have the same comparative phase.

Lasers are significant devices being utilized in diverse fields such as military, medicine, technical research, communications and industry because of their light’s features. In industry, they can be employed for cutting, drilling, and welding having high level of accurateness. In medical fields, lasers are applied in order to take dead body tissue away, causing very little damage to the adjacent
regions. Furthermore, laser eye surgery, LASIK, which is an acronym for laser in situ keratomileusis, is nowadays a very common method to rectify near sightedness problems. In military, lasers can be utilized as weapons’ guiding organism or as a weapon itself. Lasers are widely used in communications industry where for instance, they convey data and voice by the use of optical fibers. They are applied in CD and DVD players as well.

![Fig. 1.1 Femtosecond LASIK surgery of eye (Chung et al., 2009)](image)

A continuous wave (CW) laser is the laser which is pumped incessantly and releases light continuously and the laser’s output is steady with respect to time. It can be employed for transient phenomena recognition throughout material processing (Park et al., 1993). On the other hand, pulsed laser’s output
changes with respect to time. In pulsed lasers, since the laser energy can be put down on the surface within an extremely short interaction period, so the proper localization and elevated heating rates are probable. Therefore, pulsed laser have a preference in the whole kinds of laser processing. In addition, pulse width of the laser can manage heat affected zone (HAZ) and best possible laser fluence.

For accurate microfabrication and machining purposes with several functions in electron microscopy sciences (EMS), medical and biomedical devices, microelectronics and aerospace, now it is confirmed that laser machining is a very profitable implement. Lasers with pulse durations in nanosecond regime are believed to have considerable heat affected zone while femtosecond lasers are typically known as lasers with smallest heat affected zone and that is for the reason that during irradiation, there is no straight coupling of the material’s thermal modes with laser energy. Ultra-short laser pulses in pico- and femtosecond ranges are widely applied in semiconductor materials’ electron dynamics analysis (Goldman et al., 1994) in addition to laser micromachining (Pronko et al., 1995), (Momma et al., 1998) and ultrafast phase alteration in materials (Sokolowski et al., 2000).
1.2 Femtosecond Laser Micromachining

A procedure in which by applying focused optical light beam selected materials are removed from the substrate in order to obtain a preferred characteristic on the substrate is named laser machining. The amount of heat deposition to the target sample is lower if compared to other methods of mechanical machining. In general, this technique which is also called a non-contact machining depends on two mechanisms: linear optical absorption and plasma formation. Moreover, no pre and post processing of material is necessary in laser micromachining and the whole procedure can be computerized. In addition, this technique can be employed for machining of wide range of materials such as fragile, transparent, extremely thin or materials with high reflectivity. With the purpose of fabricating pieces with dimensions in the range of microns, typical continuous wave or pulsed lasers are not applicable for the reason that material's linear optical absorption damages the neighboring regions and causes heat deposition frequently. Conversely, in femtosecond laser machining, since the procedure does not depend on linear absorption at laser's wavelength, hence various materials such as metals, dielectrics, and rigid materials can be processed with an identical laser beam.

Femtosecond lasers are those ultrafast or ultra-short pulsed lasers with pulse duration in the domain of femtoseconds where . Comparing with lasers with longer pulse duration, femtosecond laser reveals several advantages such as supplying particular laser ablation thresholds even if the
density of laser energy is considerably decreased, and it also exposes very high intensities. The laser can be precisely manipulated and controlled in order to diminish the amount of heat that goes through the sample and coming up with a reproducible laser ablation procedure as well. Therefore, very precise and clean cuts with minimal amount of damage to the surroundings and no debris remaining are feasible. In addition, since thermal losses are minimized, the efficiency of the short-pulse process is a lot greater than the efficiency of the long-pulse ablation.

Utilizing femtosecond laser pulses is preferred to picoseconds or nanosecond pulses, while small volumes receive laser energy by two mechanisms: nonlinear optical absorption and avalanche ionization afterward. The time required for heat diffusion is in the range of nano to microsecond and the time necessary for electron-phonon combination is in the range of pico to nanosecond. As a result, the process of light and material interaction is basically fixed in time given that the laser energy deposition time is much smaller than the time needed for heat diffusion together with electron-phonon pairing. The region on target which is affected by laser beam is first transformed from solid phase to vapor followed by plasma formation.

In general, the functioning of femtosecond lasers is controlled by three imperative aspects which are pulse duration, pulse average power and energy, and pulse repetition rate. Other parameters that can affect femtosecond laser machining process are wavelength, depth of focus, lens focal length, and beam
shape. Wavelength and lens focal length affect feature size where beam energy and pulse width have an effect on the size of heat affected zone (HAZ). Additionally, beam shape can change the feature shape and depth of focus manipulates the aspect ratio. In Figures 1.2, 1.3, and 1.4 different examples of femtosecond laser micromachining on diverse materials are illustrated.

Fig.1.2 Femtosecond laser micromachining examples, aluminum (left) and steel (right) (N. H. Rizvi, 2003)
Fig. 1.3 Using femtosecond laser for micromachining a glass (N. H. Rizvi, 2003)

Fig. 1.4 Micromachined silica using femtosecond laser (N. H. Rizvi, 2003)
1.3 Goals and Objectives of the Study

In this research, the author focused on femtosecond laser micromachining procedure on biological samples in order to find the optimum laser ablation conditions as the main goal, to prepare the samples (wildtype and mutant Arabidopsis leaves) for further ion conductivity measurements. There are several controlling parameters for femtosecond laser ablation process such as laser pulse power, laser exposure time, pulse duration and repetition rate, laser wavelength, and beam’s shape. However, in this study we worked on laser pulse power and laser exposure time as the two controlling parameters in order to achieve the optimum conditions. The ion conductivity measurement was performed as a signature of cells’ death mechanisms caused by applied stresses where in this case the light is the source of stress. Measuring the ion conductivity of both samples to see the possible changes in the conductivity value and comparing the results for analyzing the cell death mechanisms was defined as other objective of this study. In short, the motivation of this work was to find the optimum conditions for ultrafast laser sampling of biological tissues in order to accomplish precise ion conductivity measurements that can be helpful for further studies on the function and effect of different genes on the biological tissue’s strength against diverse sources of stresses.
2.1 Microdissection of Plant Cells by Using Lasers

Laser pulses are not used only for solid materials’ micromachining. They have a wide range of applications for soft targets such as biological tissues for nanosurgical isolation, nanosurgical manipulation, and cell dissection. Two laser microdissection technologies have been developed: laser capture microdissection and laser cutting microdissection (Murray, 2007).

In laser capture microdissection, the cells of interest are covered by a membrane which is a thermoplastic material and is connected to a plastic cap. When the laser beam with low power is applied, a small section of plastic melts. Subsequently, the cells that lie beneath the molten section are appended to the plastic. After all, when the plastic becomes cold, the cells that are attached to the membrane can be disconnected from the whole tissue (Murray, 2007). The entire process can be completed in few milliseconds; therefore it can be done again and again using the same membrane for several dissections. Both the laser power and diameter of the laser beam can be modified by the experimenter in order to achieve the most favorable parameters. An example of laser capture microdissection on a biological sample is shown in Figure 2.1.
In laser cutting microdissection, the laser beam is applied to cut the cells of interest in any desired pattern. The microdissected cells can be placed in a collection cylinder by three different methods. The first technique is so called pressure catapulting where the laser is applied to shoot the microdissected cells to a collection cylinder (Murray, 2007). In another method, the dissected cells are positioned in a collection cylinder by the assistance of gravitational force. And in a last method, an extremely fine needle which is made from stainless steel is
utilized to put the cells in a collection cylinder. Apart from the last approach, all laser microdissection techniques are thoroughly non-contact systems, therefore the option of contaminating or damaging the sample is significantly eluded. Furthermore, the smallest possible diameter for laser beam is preferred in both microdissection techniques, where in laser cutting microdissection the laser beam diameter is less than 1 µm while in laser capture microdissection 7.5 µm is the narrowest laser beam diameter than is applied (Murray, 2007).

The laser capture microdissection system is simpler and quite large numbers of cells can be microdissected quickly in this system whereas the laser cutting microdissection which is completely a non-contact procedure is preferable when just small number of cells have to be microdissected with more precision. Laser beam diameter is rather smaller in laser cutting systems than laser capture systems (Murray, 2007). Figure 2.2 displays cells' isolation process for further studies.

Fig. 2.2 Clonis™ (Bio-Rad) system created for isolating living cells for investigation (Day et al., 2005)
2.2 Using Femtosecond Laser Pulses for Cell Nanosurgery

In biology, there are two major applications for femtosecond lasers: getting the image of cells’ formation in microscopy and microdissecting and nanoprocessing of very small structures in biological materials. These two applications can be distinguished by laser-pulse energy (Maxwell et al., 2005).

Applying ultrashort laser pulses for biological materials ablation is a convenient technique since living cells can be disrupted in submicron sizes without having an effect on adjacent cells. This is because that the laser energy is set down by nonlinear absorption and ionization when femtosecond laser pulses are firmly focused on the biological sample resulting in multiphoton electronic excitation, therefore the tissue is disrupted in the focal volume. Objectives with high numerical apertures (NA>1) are employed in order to have the laser emission restricted in an extremely small focal volume, increasing the photon density to generate multiphoton absorption (Maxwell et al., 2005). In Figure 2.3 the temperature distribution of water as a sample is illustrated where different laser pulses are applied with 800 nm wavelength and 80 MHz repetition rate and numerical aperture, NA=1.3. As it is clear from the figure, for pulses below the micrometer scale, the temperature distribution width is much smaller than for pulses in micrometer range and above (Vogel et al., 2007). Therefore,
for laser pulses in sub-micron range, such as picoseconds or femtosecond, thermal impacts are confined to a very small volume.

![Diagram of temperature distribution](image)

**Fig. 2.3** Temperature distribution in water, applying different laser pulses, 800 nm wavelength and 80 MHz repetition rate (Vogel et al., 2007).

The thermal diffusion time is in the range of picoseconds to nanoseconds and femtosecond pulses are quite shorter than this time, therefore the following heat shock cannot damage the biological tissue due to heat transfer reduction. This can turn the cell nanosurgery procedure into a non-thermal process. Thus, the heat effects and damages on the biological sample are insignificant and ultrashort laser pulses are able to dissect the cells in small sizes inside the focal volume (Kohli et al., 2005). In other words, neighboring cells are not affected and
damaged because the laser energy is only absorbed in the tiny focal volume, so the precise and clean disruption of biological sample in micron scale is feasible.

2.3 Mechanisms of Femtosecond Laser Nanosurgery of Biological Cells

As it was explained in previous section, in order to have nonlinear absorption of femtosecond laser pulses which results in a very precise ablation with laser effect that is confined to a small area, objectives with high numerical aperture (NA) should be utilized. In other words, to decrease the diffraction restricted focus diameter and also to keep away from filamentation and self focusing, quite big numerical apertures are needed (Vogel et al., 2007).

To fully take advantage of femtosecond laser pulses for perfect and very precise nanoprocessing of biological samples, it is necessary to comprehend all fundamental mechanisms. To do so, mechanisms such as development of plasma formation and thermomechanical, thermal, and chemical impacts from laser irradiance on biological tissue that are caused by femtosecond optical breakdown should be considered (Vogel et al., 2007).

In general, optical breakdown is a type of spark which is generated by powerful laser radiance. It can be controlled by modifying the laser light intensity. Moreover, pulse duration is the parameter that laser intensity which is necessary for optical breakdown is depended on. For instance, for 1 ps pulse duration the required optical intensity is in the range of . In laser machining of transparent materials, breakdown can be utilized for changing the material's
construction and formation by altering the material’s refractive index. As a result, very small and fine pieces can be cut and shaped in the material where the intensity of laser beam is concentrated whereas the other sections of the material remain unchanged.

Comparing optical breakdown in nanosecond and femtosecond laser pulses for material processing purposes, we see that unlike nanosecond breakdown which is extremely immediate, in femtosecond breakdown, the creation of electrons is in a rather big irradiance range which is lower than optical breakdown threshold and there is an established correlation between density of electrons and irradiance. For this reason all of mechanical, chemical, and thermal effects caused by femtosecond optical breakdown are restricted in a very small volume on biological sample, making femtosecond pulses more interesting for laser nanoprocessing and cell surgeries. In Figure 2.4 development of electron density at the optical breakdown threshold in nanosecond and femtosecond range with different wavelengths for water are demonstrated. is the laser pulse duration. Also, a dotted line exhibits the contribution of multiphoton ionization to the entire electron density. In Figure 2.5 the maximum electron density versus irradiance is illustrated. A dotted line presents the threshold irradiance ( ) and a related maximum free electron density ( ) (Vogel et al., 2007).
Fig. 2.4 Development of electron density at optical breakdown threshold as a function of time for 6 ns pulse widths and 1064 nm wavelength (a), and 100 fs pulse width and 800 nm wavelength (b) (Vogel et al., 2007).

Fig. 2.5 Development of electron density at optical breakdown threshold as a function of irradiance for 6 ns pulse widths and 1064 nm wavelength (c), and 100 fs pulse width and 800 nm wavelength (d) (Vogel et al., 2007).
In order to verify the temperature increase caused by femtosecond laser pulses, it is essential to compute the amount of volumetric energy density collected by the plasma for the period of pulsation. Volumetric energy density can be estimated by multiplication of total density of free electrons (\(n_e\)) by mean energy acquired by every electron. The electron’s acquired mean energy can be found as a summation of mean kinetic energy and ionization potential (Vogel et al., 2007). In addition, the thermal constants of the biological tissue determine the plasma energy which is correlated to temperature increase.

Figure 2.6 displays the thermal effects of different laser pulses on a biological sample which in this case is water, for 80 MHz repetition rate, 800 nm wavelength, and diverse numerical apertures. The red line represents the temperature development during continuous wave laser radiation. The dotted lines correspond to the temperature drop following the single pulse.

Fig. 2.6 Temperature change at the spot where the laser light is focused, 80 MHz repetition rate and NA=1.3 (a) and NA=0.6 (b) (Vogel et al., 2007)
As it is noticeable from the Figure 2.6, for the smaller numerical aperture (NA=0.6), the maximum temperature is much greater after several pulses than following the single pulse, while for the bigger numerical aperture (NA=1.3), the difference between temperatures after single and several pulses is smaller. This implies that with objectives with smaller numerical aperture we can have a more powerful laser irradiation. However, when the repetition rate is reduced, even with the smaller numerical aperture, the temperature increase is not evident. This is due to the limited number of free electrons which are the resources of heat generation; therefore the heat distribution became very quick. Figure 2.7 presents the temperature evolution for 1 MHz repetition rate.

Fig. 2.7 Temperature change at the spot where the laser light is focused, 1 MHz repetition rate and NA=0.6 (Vogel et al., 2007).
2.4 Ion Conductivity Measurement for Investigating Cell Death Mechanisms

Various stresses applied to a biological sample can be a cause of cell death, and ion conductivity is recognized as a signature of cell death mechanisms. When the cell is subjected to different types of biotic stresses (damages caused by living factors) and abiotic stresses (damages produced by non-living factors) such as light, heat, and toxic chemicals, it reaches beyond the critical level of toxic reactive oxygen species (ROS). Consequently the cell rips apart and ionic species are released all the way through the cell membrane. Therefore the cell death mechanism can be quantified as ion leakage. Previous to the initiation of the cell death mechanism, the conductivity which is reciprocal of resistivity has a small value. However, when the plant cell starts dying caused by different applied stresses, the ion conductivity starts increasing until it reaches the constant value at last which signifies that cell death mechanism is complete.

The two samples that were considered for conductivity measurement in this study are mutant and wildtype Arabidopsis leaves where the wildtype is used for a control experiment. The mutant sample was genetically manipulated (Wagner et al., 2004) and was supposed to be susceptible to the applied light stress. The wild type sample was assumed to show insignificant response to the applied stress. The experimental results are discussed in Chapter 5.
2.5 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are radicals (i.e. atoms containing unpaired electron in their outermost electron shell), carrying the oxygen atoms. This configuration is very unstable and radicals are expected to react with other radicals or molecules in order to achieve the stable configuration, therefore they are identified as extremely reactive atoms. These reactive chemical radicals are created as oxygen degradation outcomes. They can be inorganic or organic molecules that contain oxygen ions or peroxides. A singlet oxygen ( ), hydrogen peroxide ( ), superoxide anion ( ), and hydroxyl radical ( ) are known as oxygen reactive species.

Fig. 2.8 Reactive Oxygen Species (ROS). Adapted from www.cellscience.com/Reviews2/Hydrogen_Peroxide_Redox_Regulator.html
An oxidative stress is applied to the cells when a number of reactive oxygen species are accumulated; therefore the cells’ reaction is by triggering different protection mechanisms in order to defend themselves against an undesired impact, or by dying. It is obvious that the level of stable ROS in cells should be controlled firmly. For instance, there are approximately 150 genes in Arabidopsis that are engaged in the regulation process of ROS level.

In general, cells are capable of protecting themselves against ROS by employing different kinds of enzymes and antioxidants. Since dissimilar ROS are created at the same time, verification of each ROS action mode and also biological activity is rather complicated. So as to deal with this problem, it is essential to know circumstances where in a specific time, only one particular ROS is created inside a sub-cellular section and conditions under which that specific ROS initiates a response to the stress that can be easily recorded (Wagner et al., 2004).

The conditional fluorescent (flu) mutant of Arabidopsis thaliana satisfies the aforementioned conditions. It is capable of producing singlet oxygen in plastids in a way which is completely controlled. Right after the singlet oxygen generation, the growing process of flu mutant plant is brought to an end and seedlings start losing their color and dying (Op den Camp et al., 2003).
2.6 Arabidopsis thaliana

Arabidopsis thaliana or simply Arabidopsis is a small plant from the mustard family (Brassicaceae) with short life cycle (about 6 weeks) which produces flowers and is originally from Asia, Europe, and northwestern parts of Africa. Arabidopsis has not a great importance in agricultural area; however it is recognized as an accepted model organism in genetics and biology fields.

Fig. 2.9 Arabidopsis thaliana (Wild type). Adapted from http://www.arabidopsis.org/portals/education/aboutarabidopsis.jsp

Its small size and fast cycle of life makes it an interesting plant for research activities. It is capable of producing large number of seeds and due to its small size; it can be cultivated in a quite small area. Moreover, it has a small and uncomplicated genome and offers several available mutations. With the purpose of comprehending the molecular biology of different types of plants with dissimilar characteristics, Arabidopsis is employed as a standard tool. It plays the
same role in plant biology as mice play in animal biology. It can be grown in the lab in petri dishes under fluorescent lights.

Fig. 2.10 demonstrates three different Arabidopsis seedlings grown in different conditions. The one on the left is a wild type which is grown in the light. The middle one is a wild type grown in the dark, and the last one in the mutant type which is grown in the dark. It is conspicuous that the light-grown wild type is similar to the mutant type grown in the dark.

Fig. 2.10 Light-grown wild type (left), dark-grown wild type (middle), and dark-grown mutant type (right) of Arabidopsis seedlings. Adapted from http://www.yale.edu/denglab/What%27s_Arabidopsis.html
3.1 Rate Equations

Femtosecond laser has a very high intensity which is high enough to cause the multiphoton ionization. Therefore, in femtosecond laser processing the excitation phenomena of electrons which is expressed by the rate equations for electrons and ions should be taken into consideration. For metals, this process is usually neglected since there is large number of free electrons. In general, the rate equation should be taken into account for the bandgap materials such as semiconductors and insulators. The rate equation of electron density in general form is as follows:

\[
\frac{\partial n_e}{\partial t} = \frac{1}{e} \left( \frac{\partial}{\partial x} \left( D_e \frac{\partial n_e}{\partial x} \right) + \nu_e - n_e \right)
\]

where \( n_e \) is free electron density as a function of space and time, \( \nu_e \) corresponds to electron generation via multiphoton absorption and represents electron generation through cascade (or avalanche) ionization (Zhou et al., 2007). \( D_e \) stands for electrons diffusion outside focal volume and indicates the recombination loss. The constant in diffusion loss is:
where \( I \) is ionization energy, \( t \) is the time between collisions of electrons and particles, and \( m \) is electron’s mass. Also, \( L_0 \) is the Rayleigh length and \( w_0 \) is the laser beam radius at the focus spot (\( z=0 \)) (Zhou et al., 2007). The equations for Rayleigh number and radius of laser beam are as follows in equations 3.3 and 3.4 respectively:

\[
R = \frac{I}{n^2} \frac{t}{m} \quad \text{and} \quad w_0 = \frac{2}{n^2} \frac{t}{m} L_0
\]

where in (3.3), \( n \) corresponds to the medium’s refractive index and \( \lambda \) represents the laser’s wavelength (Zhou et al., 2007), (Fan et al., 2001). The constant in recombination rate is an empirical value which is equal to \( A = 1 \times 10^{16} \). The Gaussian profile is presumed for the laser beam before its interaction with the material. The theoretical expression for the Gaussian beam profile is shown in equation 3.5:
where power of the laser pulse is expressed by $P(t)$, time is denoted by $t$ and at maximum power ($t=0$), is the pulse's full width at half maximum (FWHM). Also, the maximum power of the laser, $P_{\text{max}}$ has correlation with which is the measured pulse energy and their relation is expressed in equation 3.6:

\[
\frac{\lambda}{\omega_0^2}.
\]

Hence, the laser irradiance, $I$, can be derived by dividing laser pulse power, $P(t)$ by where $\omega_0$ is the radius of the beam at point $z$ (Fan et al., 2001):

\[
\frac{\lambda}{\omega_0^2}.
\]

In lasers with intensities in the range of , there are two different mechanisms competing with each other for free electron generation: (1) avalanche (cascade) ionization and (2) multiphoton ionization. In multiphoton ionization, when the molecule or atom to be ionized has a bigger ionization energy ($E_i$) than the photon energy, the amount of needed electrons is $k$, which is the smallest number where . In this inequality, is identified as the
Planck constant (Zhou et al., 2007). The following is the form of multiphoton absorption rate:

\[ \text{rate} \propto \frac{1}{\lambda^2} \frac{1}{I} \frac{1}{c} \]

where \( \nu \) is laser frequency, \( I \) is laser intensity, \( c \) is the speed of light and \( \lambda \) is laser's wavelength. \( m \) is electron's mass, \( e \) is electron's charge and \( \varepsilon_0 \) is free space permittivity (Zhou et al., 2007).

3.2 Source Terms

In the next step, the laser energy has to be absorbed. Excited electrons and ions can interact with electromagnetic wave (generated by the laser) through the inverse bremsstrahlung (i.e. breaking radiation) process. Consequently the laser energy is absorbed by electrons and depending on laser energy, the electrons’ temperature increases up to 10,000 \( \cdots \) 100,000 K. The whole absorption procedure can be expressed by Beer-Lambert law which discusses the relationship between light absorption and properties of the material that light goes all through it and it is written in the following form:
where $T$ is transmissivity of light all the way through a material, $I_0$ and $I$ are light intensity before and after entering the material, $\alpha$ is the material’s coefficient of absorption, $L$ is the path length through which light travels inside the material, $\sigma$ is the absorption cross section, and $N$ is the number of absorbers.

In cascade or avalanche ionization, after the generation of free electrons by multiphoton ionization mechanism, the electric field energy is achieved by electrons via inverse bremsstrahlung absorption (IBA) (Zhou et al., 2007). When the impact takes place between free electrons and neighboring molecules, conservations of momentum is necessary, thus the absorption of photons that generated from laser pulses occurs. Following the repetition of IBA, the free electron’s energy goes beyond the $\Delta E$ which is an ionization energy (Zhou et al., 2007). After that, there will be a collision between the electron with high energy and other molecules, resulting in ionizing those molecules and followed by production of new free electrons with low energy. Once again, the IBA process repeats between numbers of free electrons as well as the one with the smallest kinetic energy and electrons that have been recently created, and impact ionization afterwards. As long as the laser pulse is on, the aforementioned procedure is repeated (Zhou et al., 2007). The constant in cascade ionization is written as follows:
where $M$ is medium molecule’s mass (for water), $\tau$ is the time between collisions of electrons and particles. The first expression inside the brackets represents the energy which is obtained from the electric field and the second expression stands for the amount of energy which is transferred from electrons to molecules for the period of collisions (Zhou et al., 2007).

3.3 Internal Energy Transfer

The heat from high temperature electrons is transferred to the other carriers such as ions or atoms via collision. As a result, the temperature difference between electrons and ions decreases. This relaxation time scale to reach the equilibrium condition is in the range of 10 to 100 ps for different materials. The transportation of energy during the laser ablation procedure is taking place in two phases: (a) absorption of photon energy by means of creation of free electrons and heating up (b) ablation and material removal when this absorbed energy is delivered to the lattice (Jiang et al., 2004).

For internal energy transfer considerations, it is essential to figure out whether the two-temperature model (i.e. electron temperature and lattice temperature) is applicable or not.
where sink term signifies transferring energy to lattice vibration, and in lattice, source term will be a sink term.

In order to consider the heat transfer process in mammalian tissues, the bio-heat transfer equation is employed. Since these kinds of tissues have a complicated structure, for simplifying the problem in theoretical analysis and mathematical modeling procedures, they are usually supposed to have a homogeneous arrangement (Zhou et al., 2007). Furthermore, because of Gaussian distribution of the laser beam in the radial direction, a cylindrical coordinate system which is axisymmetric is applied to express the geometrical model as it is depicted in Figure 3.1.

![Fig. 3.1 Axisymmetric cylindrical coordinate system (Zhou et al., 2007)]
Penn’s bio-heat equation for mammalian tissues (3.14), is the most frequently used equation for heat transfer in tissues which includes the Fourier heat conduction equation (3.12).

where $q$ is the heat flux, $r$ and $z$ are spatial coordinates, $t$ is time, $\kappa$ is thermal conductivity and $\nabla$ is the gradient and $T$ is the temperature. In general, the equation of energy is written as follows:

$$
\rho c \frac{\partial T}{\partial t} = \nabla \cdot (\kappa \nabla T) + S
$$

in which mass density of the tissue is $\rho$, specific heat of the tissue is shown by $c$, and $S$ is laser source term. Penn’s bio-heat transfer equation for mammalian tissues is expressed as:

$$
\rho_b c_b \frac{\partial T_b}{\partial t} + \rho_c c_c \frac{\partial T_c}{\partial t} = \nabla \cdot (\kappa_c \nabla T_c) + S
$$

where mass density of blood is $\rho_b$, $c_b$ and $c_c$ is blood’s specific heat as well (Zhou et al., 2007). The tissue and blood temperatures are indicated by $T_c$ and $T_b$, respectively. $\frac{\partial T_b}{\partial t}$ is the perfusion rate of blood, $\kappa_c$ is tissue’s thermal conductivity, and the last term is metabolic heat generation, $S$. 

When the laser beam interacts with a biological tissue, the heat transfer can be expressed using the following bio-heat transfer equation:

\[
\text{where } \ \text{stands for volumetric heat source of the laser (Zhou et al., 2007).}
\]

As it was mentioned in previous sections, there are two main mechanisms for free electron generation in lasers with intensities in the range of \( \text{, which are multiphoton ionization and avalanche or cascade ionization. Using the Fokker-Planck equation, standard mathematical model for these two mechanisms has been created and confirmed by different tests. But this free electron generation model is applicable for the situation where the optical properties are constant and free electron’s temperature rise and its quantum influences are not mentioned as well (Jiang et al., 2004).}

With the intention of starting the laser ablation procedure for dielectric materials, creating couple of free electrons is required. When the free electrons density in femtosecond lasers reaches the value where the laser frequency and plasma oscillation frequency are identical, that density is called critical density at which the laser ablation initiates (Jiang et al., 2004). The critical density can be determined by the following equation:
where \( \lambda \) is the laser wavelength, \( m \) is the electron's mass, \( e \) is the electron charge, and \( \varepsilon_0 \) is the permittivity.

In order to compute the free electron generation, the Fokker-Planck equation is utilized as follows:

\[
\text{equation}
\]

where time is denoted by \( t \), the distance to the axis of Gaussian beam is indicated by \( r \), and \( z \) is the depth from the bulk material's face. \( n \) is density of free electron and \( \alpha \) is the constant for avalanche or cascade ionization. \( I \) is the laser intensity and finally \( \sigma_{N\text{ph}} \) represents the N-photon (multiphoton) absorption cross section (Jiang et al., 2004).

The laser intensity within the materials can be stated as follows subsequent to study the optical properties which are dependent upon space and time:

\[
\text{equation}
\]
where $F$ represents the laser fluence, $\tau$ denotes the pulse duration, $r$ stands for the laser beam radius, $R$ is reflectivity, and $\alpha$ corresponds to the coefficient of absorption and can be calculated by equation 3.19 as follows:

$$\alpha = \frac{1}{3000} \frac{\epsilon^2}{\lambda^2} \frac{F}{r^2} \frac{\tau}{R} \frac{\mu}{\sigma} \frac{1}{\sigma^2}$$

where $\mu$ is free electrons mean kinetic energy, and $\sigma$ is the materials ionization potential. This total coefficient of absorption, $\alpha$, provides an explanation for free electron heating absorption and cascade or avalanche ionization and multiphoton ionization absorption (Jiang et al., 2004).

In order to verify the optical properties of dielectric materials that have been greatly ionized with femtosecond laser pulses, consideration of plasma properties would be helpful. Therefore, plasma’s dielectric function which is dependent on time and space is stated as:

$$\epsilon(t, \mathbf{r}) = \epsilon_0 \left( 1 - \frac{\omega_p^2}{\omega^2} \right)$$

where $\epsilon_0$ is the electron’s mass, $e$ is the electron charge, $\epsilon$ is free space electrical permittivity, the laser frequency is expressed by $\omega$ (Jiang et al., 2004).

Finally, $\tau$ is relaxation time for the free electron and it can be computed as follows:
In equation 3.21, \( k \) is the Boltzmann constant, the electron’s temperature is expressed by \( T \), \( n \) is the state of ionization, and chemical potential is indicated by \( \mu \). \( \ln \Omega \) is known as the Coulomb logarithm and it is written as:

\[
\ln \Omega = \ln \frac{\Omega_{\text{max}}}{\Omega_{\text{min}}}
\]

where \( \Omega_{\text{max}} \) and \( \Omega_{\text{min}} \) are maximum and minimum collision parameters respectively. Moreover, \( \zeta \) is the Fermi-Dirac integral which in general has the following form in equation 3.23 and for \( j=0 \) the equation 3.24 is formed:

\[
\zeta_j(x) = \int_0^\infty \frac{e^{-xt}}{1 + e^{-t}} dt
\]

Also the chemical potential is computed using the following equation:

\[
\mu = \frac{e^2}{\hbar c} \zeta_0(x)
\]

where \( \hbar \) is the Fermi energy defined by equation 3.26 in which letter \( c \) represents the scalar light speed in vacuum (Jiang et al., 2004).
At last, in order to calculate the temperature of the electron the following equation can be utilized:

where \( C \) is the free electrons specific heat and \( \alpha \) is the coefficient of absorption in free electron heating (Jiang et al., 2004).

in 3.28 is the mean kinetic energy which is verified by Fermi-Dirac distribution as follows:

where \( \beta \) and states density is \( \frac{\text{f}(E)}{\beta} \).

3.4 Stress Field

Since temperature gradient causes thermal stress, therefore the thermoelastic stress analysis can be taken into consideration by combining the
elastic wave propagation equation with other equations explained in steps 3.1, 3.2, and 3.3.
CHAPTER 4

EXPERIMENTAL DESCRIPTION

4.1 About the Femtosecond Laser Used in the Experiment

Spectra-Physics® Mai Tai® SP ultrafast laser is the laser used for biological tissues micromachining procedure in this project (Figure 4.1). It can generate wide range of bandwidths (from less than 12 nm to greater than 60 nm) which can be modified by computer by using the graphical user interface (GUI) and has a pulse width in the series of 25 to 100 fs.

Fig. 4.1 Mai Tai® Femtosecond laser adjustable with computer
Also, the wavelength can be adjusted by the GUI and it is in the range of 780 nm to 820 nm. The wavelength applied in this experiment is 800 nm and femtosecond laser pulses have the pulse width of 100 fs and 80 MHz repetition rate. Other noticeable characteristics of Mai Tai® laser are its long-lasting stability in different environmental circumstances and no requirement for realignments as well. Moreover, the specific type of diodes used in this laser makes generating pulses without interruption possible for maximum 10,000 hours.

4.2 Optical Setup

The optical setup for femtosecond laser ablation of biological sample is shown in Figure 4.2. As it can be observed from the figure, the laser beam goes through the beam splitter and deflected by 90 degrees in order to pass through the objective lens. The objective lens is the Nikon achromatic finite conjugate objective with 10X magnification and effective focal length (EFL) of 16.60 mm, the working distance of 5.60 mm, and numerical aperture, NA = 0.25. This type of objective lens is very appropriate for high power imaging systems. It is called achromatic lens, because it is capable of preventing the chromatic aberration. When the lens is not able to have all different colors focused at the same spot, it is called chromatic aberration. On the other hand, achromatic lens which is utilized in this experiment restricts the consequences of chromatic aberration and takes lights with dissimilar wavelengths to a common focus.
Fig. 4.2 Femtosecond laser ablation optical setup. Two beam splitters are used to direct the laser beam and white light towards the sample. The CCD is placed on top for proper imaging process.

The light for illumination comes from the light source and passes through the collimator and is deflected by the beam splitter to be directed to the sample. A motorized x-y-z translation stage controlled by LabVIEW program is used for precise laser cutting procedure where by modifying the stage speed the laser exposure time is changed as well, results in obtaining optimum conditions for laser ablation process. In addition to the laser exposure time, laser power is also considered as one of the controlling parameter in order to achieve the laser
ablation optimum conditions and reproducibility. By increasing the power, the laser cutting size is increased as well and that is because of the Gaussian nature of the laser beam where by increasing the power the spatial extent of the laser intensity expands too.

4.3 Acquiring Optimum Conditions for Laser Ablation

For this purpose, several cuts with different ablation parameters were created on the clover sample which is quite similar to Arabidopsis as a main sample in this experiment. Considering the SEM images of different cuts and measuring the cut width helped to choose the most appropriate laser power and laser exposure time for the main experiment on Arabidopsis. In Figures 4.3, 4.4 and 4.5 different cuts at the power of 0.3 W and diverse stage speeds are shown.

Fig. 4.3 SEM image of cut profiles made on sample at power of 0.3 W and stage speeds of 0.15, 0.20 and 0.25 mm/s (a), and 0.35 and 0.40 mm/s (b)
Fig. 4.4 SEM images of cut profiles made on clover sample at power of 0.3 W and stage speed of 0.15 (a), 0.20 (b), 0.25 (c), and 0.30 mm/s (d)

Fig. 4.5 SEM images of cut profiles made on clover at power of 0.3 W and stage speed of 0.35 (a), and 0.40 mm/s (b)
It can be observed from Figures 4.4 and 4.5 that the cut width in most cases is less than 100 µm where the laser power was set to 0.3 W. Changing the laser power to 0.4 W, the author made some optical images of the cuts made on the clover at different speeds (Figure 4.6) in order to compare the cut width with the cuts made at the power of 0.3 W.

Fig. 4.6 Optical images of cut profiles made on clover at the power of 0.4 W and stage speed of 0.30 (a), 0.35 (b), and 0.40 mm/s (c)
It is noticeable from the images that at the higher power (0.4 W) the cut width is noticeably greater (>100 µm) than in the cuts created at 0.3 W (<100 µm). Therefore we decided to choose 0.3 W as an optimum laser power for the main experiment on Arabidopsis. In addition, the optimum stage speed was decided to be in the range of 0.25~0.35 mm/s.

4.4 Ion Conductivity Measurement

The optimum conditions for laser ablation were used to cut two wildtype (control) and mutant Arabidopsis samples in a square pattern with 500 µm x 500 µm dimensions. Both leaves cut by femtosecond laser were transferred directly to the microfabricated chamber in situ with laser ablation as shown in Figure 4.7.

Fig. 4.7 Square leaf sample cut by femtosecond laser in 500x500 micron dimension. The cut width is less than 100 microns (a). Microfluidic chamber made on glass with gold electrodes used for ion conductivity measurements (b).
A microchamber with gold microelectrodes was fabricated by using the standard microfabrication technology. This structure was manufactured by light lithography after which the metal pattern (gold electrodes with ~100 nm thickness) is defined on a glass wafer by physical vapour deposition and lift-off process. The chamber was made with an SU-8 photoresist. SU-8 is widely used for a structural material in micro electro mechanical system (MEMS) devices owing to its robustness and strong bonding characteristics. The chamber contains ~ 500 nanoliters of deionized water and the gold electrodes shown in Figure 4.7b are utilized for ion conductivity measurement. In order to avoid water evaporation, each well was covered with a PDMS cap during the experiment. By doing the experiment on water only and having no change in conductivity, we made sure that there was no substantial water evaporation for the duration of our experiment.

As it is shown in Figure 4.8, the precise current source and lock-in amplifier were connected to the electrodes. The lock-in amplifier permits low-noise, high-resolution ac impedance measurement. The alternate current-excited impedance is reciprocal of conductivity. As a source of stress, a light with intensity of 100 µmol (or µEinstein) was introduced to both wildtype and mutant Arabidopsis leaves. Our measurement was at frequency of 130 Hz and ion conductivity measurement was executed. Also, the switching circuit shown in Figure 4.8 and 4.9 was employed for swapping the measurement between both samples and the whole experiment is controlled by the LabVIEW program.
Fig. 4.8 Conductivity measurement setup. Each well is connected to the switch circuit for substituting measurement between two samples. AC current-excited impedance is measured by lock-in amplifier.

Fig. 4.9 The switching circuit connected to the microchamber.
The biological tissue that is cut by femtosecond laser pulses should be thoroughly flat during the ablation, therefore the laser beam can be focused on the entire surface of the sample. Since biological tissues are not flat naturally, we placed an aluminum plate with the same number of holes as number of wells on the microfabricated chamber and the sample is located between the wells and aluminum plate and is flattened. The device is shown in Figure 4.10, where the gold electrodes on the chamber are connected to the PCB by wire bonding and the PCB is attached to the switching circuit and from there it is linked to the current source and lock-in amplifier for ion conductivity measurement.

![Fig. 4.10 Microfluidic chamber and aluminum plate on top of it for making the biological sample flat for proper laser ablation procedure](image)
5.1 Experimental Results

The whole conductivity measurement was performed for 2 hours and 20 minutes time interval at the same circumstance for both wildtype and mutant Arabidopsis samples in order to check the change in AC impedance which is reciprocal of conductivity, and compare the signals. As is seen from Figure 5.1, essentially no impedance change in wildtype sample is detected. On the other hand, mutant sample starts with high resistance at the beginning of the process which gradually decreases and reaches the constant value at the end after about 100 minutes. The reduction in impedance signifies that the ion conductivity increased as the cell’s response to the applied stress and reaching the constant value at the end of the experiment can assure that the cell death mechanism is complete for the mutant sample, satisfying our expectations. The percent change in impedance for wild sample is 0.83% which can be considered as a negligible change and as it can be seen in figure 5.1, the graph is approximately a straight line.
Fig. 5.1 Normalized impedance vs. time in ion conductivity measurement for wild and mutant samples for 140 minutes duration of experiment.

However, there is 5.6% change in the impedance value for the mutant sample which implies that the two samples’ responses to the stress are not identical. Less change in impedance means less change in ion conductivity for the wild sample, and this confirms that wild sample has more endurance against the light as an applied stress since its ion conductivity did not change significantly due to the exposed light. On the other hand, bigger change in impedance value for mutant sample and reaching the constant conductivity at the end denotes that
it is more vulnerable against the applied light and the ion conductivity does not remain the same. In Figures 5.2 and 5.3, the non-normalized impedance vs. time is depicted for mutant and wild samples respectively, together with error bars indicating the standard deviation of each point that was measured for verification of statistical variations. As it is obvious for mutant sample, the impedance value (reciprocal of conductivity) starts with a high number and gradually decreases for more than 100 minutes and reaches the constant amount after about 2 hours.

![Impedance vs. time](image)

Fig. 5.2 Impedance vs. time in ion conductivity measurement for mutant sample. The total amount of change is 5.6%. Error bars shown in the graph indicate the standard deviation of each measured point as statistical variation
On the other hand, in case of wildtype sample some fluctuations in impedance value are observed (Figure 5.3), and the total percent change which is 0.83% is negligible, signifying that wildtype sample is less affected by the applied light stress since the change in its ion conductivity is insignificant.

Fig. 5.3 Impedance vs. time in ion conductivity measurement for wildtype sample. The total amount of change is 0.83%. Error bars shown in the graph indicate the standard deviation of each measured point as statistical variation.
In this research, the mutant sample was genetically manipulated in a way that the cell’s reaction to the applied stresses was modified (it became more vulnerable against the exposed light as a source of stress) and this change was monitored through accurate conductivity measurements made on both samples in the same conditions. Therefore, the effect of that particular gene on cell’s strength opposed to stresses such as light in this case can be determined precisely through further considerations.
CHAPTER 6

CONCLUSIONS

In summary, the author utilized femtosecond laser pulses for sampling the biological tissues (wild and mutant type Arabidopsis) for further ion conductivity measurements in order to analyze the stress generation mechanisms. For this purpose, different controlling parameters were considered to obtain the optimum laser ablation conditions. Therefore, laser power and translation stage’s speed were changed frequently in order to find the most proper combination for the main experiment. SEM images of different cuts created by different ablation parameters were investigated to choose the parameters that formed the smallest cut width which in our case was less than 100 µm. After finding the optimum ablation conditions, two samples were cut in a square pattern with 500x500 µm size and directly inserted into the microfabricated chamber filled with DI water for ion conductivity measurements. As a source of stress, 100 µmole light was exposed to the sample during the entire experiment. The current-excited impedance (reciprocal of conductivity) was measured precisely using the lock-in amplifier in order to observe the signal change for each sample. The wild sample did not reveal significant response (0.83% change) to the applied stress which in our case is light; however, the mutant sample exposed considerable response
(5.6% change) to the stress as a large drop in its conductivity and reaching the constant value at the end guaranteed that the cell’s death mechanism caused by the applied stress was finalized. The mutant sample was genetically manipulated and the wildtype sample was used as a control sample in this experiment. By considering the results from this experiment, the effect of different genes on the kind of reactions that biological cells make when encountering diverse stresses, can be analyzed through further investigations.
REFERENCES


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