A Study of Burst-Mode Ultrafast-Pulse Laser Ablation on Soft Tissues and Tissue-Proxies

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Physics
University of Toronto

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Abstract

This thesis research presents an experimental study of both the physics mechanisms and biological effects of burst-mode ultrafast-pulse laser ablation. A 3D living-cell-culture tissue-proxy based on agar hydrogel was developed, and this tissue-proxy was used to quantify the cellular necrosis range, to identify the types of cellular death, and to measure the volume of material removal post burst-mode laser ablation. The potential hazards of cellular DNA damage were also evaluated.

A time-resolving energy-partition diagnostics system was designed and built for characterizing the dynamic scattering and absorption of pulses during burst-mode ablation. Such characterizations were carried out on soda-lime glass, aluminum, porcine tissues, distilled water, and agar gels using this diagnostic system. Each type of target materials displayed distinct features in their absorption patterns. An array of characteristics of the absorption and their relation to the ablation dynamics were analyzed, and valuable insight about the burst-mode ablation process was gained. The characterization of the dynamic absorptions allowed the evaluation of the roles of different physics mechanisms in the resulting cellular damage and material removal.
Acknowledgments

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<th>Description</th>
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<td>AMP</td>
<td>amplifier</td>
</tr>
<tr>
<td>AOM</td>
<td>acoustic-optical modulator</td>
</tr>
<tr>
<td>BS</td>
<td>beam splitter</td>
</tr>
<tr>
<td>CFLSM</td>
<td>confocal fluorescent laser-scanning microscope</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DRI</td>
<td>diffuse-reflection integrator</td>
</tr>
<tr>
<td>DSB</td>
<td>double strand break</td>
</tr>
<tr>
<td>ETP</td>
<td>equivalent target plane</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FPGA</td>
<td>field-programmable gate array</td>
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<tr>
<td>FWHM</td>
<td>full width half maximum</td>
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<tr>
<td>GVD</td>
<td>group velocity dispersion</td>
</tr>
<tr>
<td>HC-PCF</td>
<td>hollow-core photonic-crystal fibre</td>
</tr>
<tr>
<td>HV FET</td>
<td>high-voltage field-effect transistor</td>
</tr>
<tr>
<td>IEI</td>
<td>incident energy integrator</td>
</tr>
<tr>
<td>IRIS</td>
<td>intratissue refractive index shaping</td>
</tr>
<tr>
<td>LASIK</td>
<td>laser-assisted in situ keratomileusis</td>
</tr>
<tr>
<td>LIOB</td>
<td>laser induced optical breakdown</td>
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<tr>
<td>LS</td>
<td>lower sphere</td>
</tr>
<tr>
<td>NIR</td>
<td>near infrared</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
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<tr>
<td>PULSAR</td>
<td>pulsed laser sequencer</td>
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<tr>
<td>RF</td>
<td>radio frequency</td>
</tr>
<tr>
<td>SRI</td>
<td>specular-reflection integrator</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>US</td>
<td>upper sphere</td>
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Chapter 1

Introduction

Burst-mode ultrafast lasers have had a significant impact on materials processing during the past two decades, and this class of lasers has shown great potential and growing importance in biomedical applications. This dissertation describes my studies of the ways the advantages of burst-mode ultrafast laser translate to the biomedical sphere, especially to future laser surgery procedures.

In the performance of materials processing, burst-mode ultrafast lasers possess all the advantages ultrafast lasers have shown since the 1990s. In addition, burst-mode lasers enable a new kind of control by irradiating targets with, not just one, but a “packet” of pulses in one duty cycle. This new regime of pulse delivery leads to qualitatively new features in materials processing, such as the creation of smooth holes in glass without the occurrence of shattering [1].

For laser physicists, the new modality of pulse delivery brings new physics mechanisms to explore. The pulses in a packet arrive so quickly at the target material that they can create a “memory” in the target. The duration of this “memory” depends on how the time interval between pulses compares to the relaxation time of different characteristic processes (e.g., electron thermalization, ionization and recombination, shock wave propagation,
cavitation). In other words, the burst-mode laser-material interaction has a repetition-rate dependency. This repetition-rate dependency in laser-material interaction has been looked at phenomenologically, but full utilization of it requires systematic study in order to understand the physics principles involved.

At the same time, the application of burst-mode in laser surgery also requires understanding of the biological effects of laser ablation, such as the cell survival rate, the range and type of cellular deaths, the scale of tissue removal, potential DNA damage, etc.

The aim of this thesis research is to understand the dynamic processes of pulsetrain-burst interaction with biotissues. In specific terms, this thesis research uses different models (water, agar gel of different concentrations, gels with cells, ex vivo tissues, glass, etc.) to investigate how the different time scales play into the physical processes of burst-mode ablation. The physical processes of interest include cavitation, shock waves, and laser-induced optical breakdown. In addition, this thesis research quantifies the biological effects, namely, cellular deaths and DNA damage, that result from pulsetrain irradiation.

1.1 A brief history of laser ablation

Shortly after the invention of the laser in the 1960s, attempts were made to explore its potential as a surgical tool. After decades of research and development, surgical laser systems have in many cases surpassed mechanical cutting and drilling tools in the performance of precise and minimally invasive procedures.
In this dissertation, ultrafast lasers refer to lasers with picosecond or shorter pulse width. Prior to the adoption of ultrafast lasers in surgery, tissue ablation by longer-pulse (nanoseconds or milliseconds) lasers or by continuous wave (CW) lasers relied on linear absorption of laser energy through endogenous chromophores. In this regime of fluency delivery, tissue ablation is often accompanied by heat damage outside the treated region. Ablation through a linear absorption mechanism also requires the presence of endogenous chromophores.

Ablation by ultrafast lasers overcomes these two limitations because ultrafast laser ablation is nonlinear and plasma-mediated. An ultrafast pulse, which reaches a material-specific irradiance referred to as the breakdown threshold, will ionize the target material and will form a plasma at the focus. The high pressure-gradient of the plasma rapidly drives away the heated layer of material, while the substrate layer stays cool[1]. This unique mechanism ensures the low collateral damage of ultrafast lasers, as compared to their longer-pulsed counterparts. In addition, absorption through plasma eliminates the limitation that endogenous chromophores produce.

Currently, ultrafast lasers have been successfully incorporated into a number of ophthalmology procedures, and their applications outside ophthalmology are being actively explored (see the reviews by Hoy et al.[2], Mazur et al.[3] ). In ophthalmology, femtosecond lasers have replaced the mechanical cutting tools in LASIK and keratoplasty. Ultrafast lasers create a finer cut than mechanical cutting tools, thus reducing post-surgery complications. In keratoplasty, another advantage of the use of ultrafast lasers for cutting is that laser ablation can easily generate a complex cutting pattern on corneas [2,3]. The use of such a complex pattern provides better results in the process of grafting the donor’s
cornea to the recipient’s eye. Outside the practice of ophthalmology, the use of ultrafast lasers is being investigated in the fields of the microsurgery of vocal folds [4], craniofacial osteotomy [5], stapedotomy [6], cardiology [7,8], dentistry [9], and sub-cellular nanosurgery [10]. Given the strong and ongoing potential that ultrafast lasers have shown, investigation of the ways to make best use of ultrafast lasers in surgeries forms a vibrant area of interdisciplinary research in physics, biology, and medical science.

1.2 New features created by high-repetition-rate/burst-mode ultrafast lasers

In this dissertation, the term “high-repetition-rate lasers” refers to lasers with a pulse-repetition-rate of 100 kHz and above. During the past two decades, the discovery has been made that high-repetition-rate ultrafast lasers (including both burst-mode and continuous-running mode) not only possess the advantages of previous ultrafast-lasers, but also have other promising features.

A straightforward improvement that results from ramping up the repetition rate is the increase in the material removal rate. More interestingly, new physics mechanisms emerge as the pulse repetition-rate increases. When pulses are applied up to tens of kHz, there is little cumulative effect from pulse to pulse: By the time the next pulse arrives, the plasma created by previous pulse has already vanished, and the material has cooled down after the previous pulse. The ablation of each individual pulse at a low repetition rate can be seen as an isolated event. However, when the repetition rate reaches hundreds of kHz or even MHz, a cumulative effect starts to occur.
In the late 1990s and the 2000s, pioneering work showed that, in the ablation of brittle materials such as fused silica, the application of ultrafast pulses in >100 MHz pulsetrain-bursts can create a smooth, deep ablation crater without shock-induced microcracking [9,11,12]. The creation of the crater was thought to result from the heat accumulation between high-repetition-rate pulses, which increased the ductility of the target material and thereby mitigated shock-induced microrcracking in the periphery of the ablation crater [1]. A similar feature was also observed in the laser material processing of metal, when a single high-repetition-rate burst resulted in clean, high-aspect-ratio holes [11,13]. High-repetition-rate burst lasers have shown increased material removal efficiency, while they provide ablation features that are comparable or even superior to those of other, lower repetition-rate ultrafast laser systems [14].

The heat accumulation effects of MHz ultrafast lasers have been used with great effect in laser direct-writing of waveguides [15,16]. For MHz repetition-rate ultrafast lasers, oscillator pulses with ~100 nJ pulse energy are sufficient to induce index-change in glass [15]. This heat accumulation effect has also been used in the introduction of index-change to ophthalmological hydrogel-polymers for manufacturing contact lenses [17-19].

In the wake of the success in direct index-change to hydrogel-polymers, research is under way to apply this technique to the next generation of refraction-correction surgery, which is referred to as intra-tissue refractive index shaping (IRIS) [20]. Instead of modifying the optical power by modifying the figure of the cornea, IRIS modifies the optical power of the cornea by modifying the refractive index of the tissue itself, by using a high-repetition-rate ultrafast laser, thus further reducing the invasiveness, as compared to fs-LASIK. Savage and colleagues [20] reported the first IRIS carried out in vivo on adult cats in 2014.
In this work, the authors changed the optical power of live cats’ corneas, using 400-nm 100-fs pulse at 80 MHz repetition-rate. The induced feature remained stable over a 12 month period, without significant change in the curvature or thickness of the cornea [20].

In addition to residual heat, the other notable cumulative effect is caused by the secondary ionizing radiation, which occurs due to the free electrons generated by ultrafast pulses. Free electrons can damage cellular DNA directly or through the generation of reactive oxygen species and free radicals in an aqueous environment. Mathur and colleagues [21,22] demonstrated in situ DNA strand-breaks for DNA plasmids in an aqueous solution, which occurred through the low density plasma generated using near- to mid-infrared femtosecond pulses running at 1 kHz repetition rate, > 1 TW cm² intensities. In oncology, this in situ ionizing radiation effect can be desirable because it provides a way of delivering ionizing radiation using filamentation, with the benefit of zero entrance dose. Meesat et al. [23] first demonstrated this concept on dosimetry gels and on an animal tumor model, with an 800-nm, 0.3-mJ, 100-fs laser running at a one kHz repetition-rate. In surgical settings, this particular secondary ionization has to be mitigated, because the secondary radiation and the resulting reactive oxygen spices and free radicals expose healthy cells to the risk of DNA damage or even mutation.

1.3 Recent progress on burst-mode ultrafast laser systems

The burst-mode laser system used in this research uses free-space optics, occupies a large space, and requires a 4-min cooling-time between amplified pulsetrains. In practical settings, an ideal surgical-laser platform should be compact and robust. It should also have
a high work-rate and produce stable pulsetrain-bursts. Another important component of a surgical platform is a flexible way of delivering the pulses (fibre optics with a compatible catheter). During the past few years, there have been exciting technological developments that move toward a surgical-laser platform with these desirable features.

In terms of laser systems, a new class of high-power, MHz-repetition-rate (including continuous-running and burst-mode) fiber lasers has emerged [24-27]. These lasers often use the master-oscillator, power-amplifier (MOPA) configuration and Yb-doped clad-tapered optical-fibre [24-27]. Some of these lasers have shown a preliminary capability for shaping a pulsetrain-burst envelope [28].

Discovery of ways to combine ultrafast lasers with existing endoscopic techniques has been a technical challenge in the field. Damage-free delivery of ultrafast pulses at intensities sufficient to introduce optical breakdown to the target material is difficult because of distortion from group velocity dispersion (GVD) and damage resulting from the Kerr effect [29,30]. In recent years, the introduction of hypocycloid-core-shaped kagome hollow-core photonic-crystal-fibre (HC-PCF) has greatly increased the power of the ultrafast pulse that can be delivered through optical fibre [29,30]. In 2014, Debord et al. [30] demonstrated damage-free delivery of ~600-fs, 1-mJ pulse with up to ~650 µJ transmission, using a 3m-long, 3-bar He-filled, 19-cell core, hypocycloid-core shaped kagome HC-PCF. For the same type of fibre, but, in this case, 10m-long and filled with air, under same pulse input, the transmission is ~400 µJ. This is the highest intensity ultrafast pulse delivered damage-free through PCF reported to date. In summary, it may be said that these technical advancements show great promise that they will become part of future burst-mode ultrafast-laser surgery platforms.
1.4 Previous research on ultrafast laser ablation of tissues

Ultrafast-laser ablation of tissues is a still evolving field, thus this section only briefly introduces the topics that have been investigated in the past two decades. Existing literature have covered a range of topics, including the mechanism of laser-induced optical breakdown [31-35], the role of linear and nonlinear absorption [36], the breakdown thresholds of different materials [34], and the dependence of ablation results on wavelength [37] and pulse width [34,38]. In research related to surgical applications, researchers have also examined tissue viability, damage due to heat [39], cavitation[40,41], and shock wave[42] for different tissue types. A comprehensive review on the phenomena and effects of tissue ablation has been done by Vogel and Venugopalan in 2003 [43]. Background research relevant to the present research work will be reviewed in Chapter 2.

Overall, the single-pulse/low-repetition-rate pulses-material interactions are relatively well understood. However, an understanding of how a high-repetition-rate pulsetrain interacts with biotissues, particularly soft-tissues, is still lacking. The aim of this present research is to fill this gap.

1.5 Objectives and approaches of the research

1.5.1 Objectives

The physics mechanisms of high-repetition-rate pulsetrain-burst ablation differ from those of low-repetition-rate laser ablation in the sense that the affected target-material keeps a “memory” of previous pulses due to residual heat and plasma. Therefore, it is essential to
investigate the pulse-to-pulse interaction and the way this pulse-to-pulse interaction affects heat accumulation, material removal, cavitation, and shock wave propagation.

Examination of the resulting biological effects should first: quantify the scale of cellular death, identify of the type of cellular deaths, and evaluate the risk of cellular DNA damage. Then the research should address the role of different physics processes that result in the above damage, so that recommendations can be made about how to mitigate the risks.

1.5.2 Challenges and approaches

The challenge of investigating the physics mechanism is the need to time-resolve the per-pulse dynamics during burst-mode ablation on a nanosecond time scale; the challenge in the examination of the biological effects is that differentiated tissues are heterogeneous in composition and structure, and are often not transparent. These challenges create difficulties in the precise quantification of the damaging effects.

To tackle these two challenges, this research was carried out through two projects: In order to time-resolve the per-pulse dynamics, a time-resolving energy partition diagnostic based on an integrating-sphere principle was designed, built, and tested. This diagnostic was later used in the processes of capturing the dynamic transmission and scattering during the ablation of various types of targets, and then of providing insights about burst-mode ablation dynamics.

In order to examine the biological effects in 3D, a transparent living-cell tissue phantom was developed. The tissue phantom allows the diffusion of a number of different fluorescent biomarkers. By using confocal fluorescent laser-scanning microscopy
(CFLSM), the biological effects that resulted from laser ablation were reconstructed in 3D. Using this tissue phantom, the extent of cellular damage and the damage’s dependence on laser parameters were examined.

1.6 Overview of the Dissertation

This introductory chapter includes a brief summary of the historical development of laser surgery techniques and of the role of ultrafast lasers in laser ablation. This summary is followed by a report about the current state of ultrafast ablation research and about the development of new ultrafast laser systems. The objectives and approaches of the present research are described.

Chapter 2 summarizes the background theories and relevant experimental techniques related to the present research. The topics covered include: laser-induced optical breakdown mechanisms, cavitation and shock wave propagation, and thermal damage and potential risk to DNA.

Chapter 3 describes the pulsetrain burst-mode picosecond-pulse laser system used in this study. The chapter also explains the operation of the oscillator, the amplifiers, and the feedback-control electronics and diagnostics at the target-machining stage.

Chapter 4 describes the 3D living-cell culture tissue-proxy project. The tissue phantom was developed together with a number of different fluorescent assays for tagging different types of cellular damage. The risk of DNA double-strand-breaks after burst-mode laser
irradiation and also the range and distribution of cellular deaths are studied by using the confocal-microscopy technique.

Chapter 5 consists of two parts: Part I describes the design and testing of an energy-partition diagnostic that is based on an integrating-sphere principle for time-resolving the dynamic absorption of burst-mode ablation. Part II describes the test runs of dynamic scattering measurements, which were carried out on soda-lime glass, metal, and some ex vivo animal tissues. Various types of errors are also analyzed.

Chapter 6 presents a systematic study of the dynamic absorption of water and agar gels. An array of the characteristics of the dynamic absorptions is presented in this chapter, including the relation between absorption and irradiance, the way mechanical properties affect the absorption dynamics, and the roles of different mechanisms in material removal and tissue damage. Analysis of these characteristics creates valuable insights about ablation dynamics.

Finally, Chapter 7 reviews the results of dissertation, summarizes the major conclusions, and provides suggestions for future research.
Reference


Chapter 2

Background Theory and Techniques

This chapter provides a summary of the background theory and relevant experimental techniques that relate to the present research. Section 2.1 lists the materials used in the present research and describes some of their basic properties. Section 2.2 describes the mechanisms of laser-induced optical breakdown in dielectrics. Each of the sections 2.3, 2.4, and 2.5 describes one damage mechanism. Section 2.3 describes cavitation and shock wave; Section 2.4 describes thermal effects; Section 2.5 describes potential cellular DNA damage from ultrafast laser irradiation.
2.1 Target materials

In this research, the materials used as targets include aluminum, soda-lime glass, water, agar hydrogel, and sacrificed porcine tissues.

Aluminum was used as a representative of metals as a class of materials in Chapter 5. The process of energy deposition from laser to metals is straightforward. Because free electrons are already present in metals prior to laser irradiation, a laser can directly deposit energy into metal through linear absorption. In contrast with metals, in dielectrics, most of the free electrons present during ultrafast laser ablation are generated from laser-induced optical breakdown.

Glass is a common class of solid dielectric material. The interaction between an ultrafast-laser pulse and glass has been widely researched, and ultrafast-laser processing of glass has achieved significant commercial success. Varieties of glass have different purposes, and their properties differ. The work in Chapter 5 used soda-lime glass microscope slides (Goldline™ microscope slide, VWR, USA) as an example of solid dielectric material.

Water is another type of dielectric material, and it is one of the most abundant substances in the human body. For this reason, water is often used as a “zero-order” approximation for soft tissues. Investigation of ablation-related physics mechanisms in water is also quite valuable for the reason that, in many applications, bio-tissue ablation is performed in aqueous environments.

Hydrogel is a class of materials widely used in tissue engineering to mimic different types of tissues. This research also used agar gels as a proxy for soft tissues in Chapter 4 and
Chapter 6. These agar gels consist of > 95% (w/w) water, so they are similar to water, but have more tensile strength.

Sacrificed *ex vivo* animal tissue is the most complex class of material used in the present research. Differentiated tissues are not homogenous in their properties and contain different types of chromophores (Figure 2.1, reproduced from ref.[1]). Chapter 5 used porcine cornea, liver, and cartilage tissues as examples of differentiated tissues.

Figure 2.1 The absorption coefficient of major chromophores in tissues between 0.1-1.2 µm. The plot is reproduced from ref. [1] by Vogel and Venugopalan.
2.2 Laser-induced optical breakdown (LIOB)

2.2.1 Generation of seed free electrons

The vast majority of seed free electrons in the process of laser-induced optical breakdown of dielectrics are produced by photoionization. For un-irradiated dielectrics, there are electrons in the conduction band due to thermal excitation, and the probability of finding an electron in the conduction band can be described by \( \exp\left(-\frac{\Delta}{K_B T}\right) \) [2], where \( \Delta \) is the band gap energy between the conduction band and the valence band, \( K_B \) is the Boltzmann constant, and \( T \) is the temperature. For instance, distilled water is an amorphous semiconductor with \( \Delta = 6.5\text{eV} \) [3]. At 300K, \( \exp\left(-\frac{\Delta}{K_B T}\right) \) of distilled water gives \( 9.8\times10^{-110} \).

Because the total electron density of water is on the order of \( 10^{23} \text{ cm}^{-3} \), the electron density in the conduction band of un-irradiated distilled water at 300K is negligible, and, therefore, most seed free electrons are provided by photoionization.

Multiphoton ionization and tunneling ionization are two pathways of photoionization that could lead to the generation of free electrons. After seed free electrons are generated, while some seed free electrons will be lost due to either recombination or diffusion out of the focal volume, the remaining seed free electrons will continue to gain energy in the laser field through inverse bremsstrahlung absorption [1,4,5]. Inverse bremsstrahlung absorption is the process through which a free electron gains energy in the laser field during collision with a second heavy particle, such as an ion or a nucleus [1,4,5]. Due to the requirement for the conservation of energy and momentum, the participation of a second particle is essential [1,4,5].
After a series of inverse bremsstrahlung absorption events, a seed free electron gains sufficient kinetic energy to generate another free electron through impact ionization. The new free electrons generated through impact ionization then iterate the process of “inverse bremsstrahlung absorption – impact ionization,” and the result is an avalanche-like generation of free electrons [1,4]. This process is also referred as cascade ionization [1,4]. Due to the requirement for momentum and energy conservation, the kinetic energy required for the resulting impact ionization is higher than the ionization potential [4].

2.2.3 Evolution of free electron density within the focal volume

Considering the processes described above, an equation describing the time evolution of the free electron density within the focal volume can be written as [1,4]

\[
\frac{dn_e}{dt} = (\frac{dn_e}{dt})_{PI} + (\frac{dn_e}{dt})_{cas} + (\frac{dn_e}{dt})_{diff} + (\frac{dn_e}{dt})_{rec}. \tag{2.2.1}
\]

\( (\frac{dn_e}{dt})_{PI} \) is the rate of photon ionization, which is the rate of multiphoton ionization\( (\frac{dn_e}{dt})_{mp} \) and the rate of tunnelling\( (\frac{dn_e}{dt})_{tunnel} \) combined [4]. The rate of multiphoton ionization\( (\frac{dn_e}{dt})_{mp} \) is proportional to \( I^k \), where \( k \) is the number of photons required for multiphoton ionization [4,6]. \( (\frac{dn_e}{dt})_{cas} = \eta_{cas}n_e \) is the rate of cascade ionization. \( (\frac{dn_e}{dt})_{diff} \) is the rate of free electron loss due to diffusion out of the focal volume, and it is proportional to free-electron density [2,7,8], assuming that there is a flat density distribution across the focal spot:

\[
(\frac{dn_e}{dt})_{diff} = -g_n_e = -\frac{\tau E_{avg}}{3mA^2} n_e, \tag{2.2.2}
\]
where \( g = \frac{\tau E_{\text{avg}}}{3m\Lambda^2} \) is the diffusion rate, \( \tau \) is the average time of between collisions, \( E_{\text{avg}} \) is the average kinetic energy of free electrons, and \( \Lambda \) is the characteristic diffusion length; \( m \) is given by \( \frac{1}{m} = \frac{1}{m_c} + \frac{1}{m_v} \), where \( m_c \) is the effective mass of free electrons in the conduction band, and \( m_v \) is the effective mass of the hole in the valence band [6,8].

\[
\left( \frac{d n_e}{dt} \right)_{\text{rec}} = -\eta_{\text{rec}} n_e^2
\]
is the rate of loss due to \textit{in situ} recombination inside focal volume, and it is proportional to the square of electron density [1,9]. Docchio [10] previously measured the value of \( \eta_{\text{rec}} = 2 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1} \) in water by observing the decay of plasma luminescence. The loss of free electrons during interaction with a pulse of < 10-ps pulse width is often negligible, because the pulse width is short, as compared to the time scale of diffusion or recombination [11].

2.2.4 Criteria of breakdown

The plasma frequency \( \omega_p \) increases with the growth of the free electron density \( n_e \):

\[
\omega_p = \sqrt{\frac{e^2 n_e}{m_e \varepsilon_0}}
\]  
(2.2.3)

A critical free electron density \( n_{cr} \) is defined when the resulting plasma frequency is equal to the laser light frequency [11]:

\[
n_{cr} = \frac{\omega^2 m_e \varepsilon_0}{e^2}
\]  
(2.2.4)
The critical free electron density for optical breakdown at a visible and near-infrared wavelength is on the order of $10^{21}$ cm$^{-1}$. For dielectrics, the creation of a critical-density plasma is the theoretical criterion for laser-induced optical breakdown.

In an experimental context, optical breakdown can be recognized by the occurrence of plasma luminescence or the generation of a cavitation bubble [1,8]. Within bulk material, plasma will expand beyond the focal region, and, at above-threshold irradiance, the plasma will grow in the direction of the incoming laser pulse, forming an elongated breakdown-region [8,11]. This formation occurs because, at above-threshold irradiance, the material reaches the breakdown threshold before the pulse peak arrives at the focus. After the breakdown starts, the plasma absorbs energy from the pulse and expands towards the pulse peak, where the irradiance is even higher. At the same time, this absorptive plasma shields the region behind it. On the material surface, the plasma at the beginning of breakdown is only a thin layer ~100 nm thick [12,13].

The threshold irradiance for picosecond and femtosecond pulses to induce breakdown in water is on the order of $10^{12}$ to $10^{13}$ W cm$^{-2}$[4]. Nanosecond pulses with irradiance on the order of $10^{11}$ to $10^{12}$ W cm$^{-2}$ can also induce optical breakdown. In other words, less fluence (measured in J cm$^{-2}$) is required to introduce optical breakdown using picosecond and femtosecond pulses. The breakdown-thresholds of picosecond and femtosecond pulses measured in fluence (in J cm$^{-2}$) are one to two orders of magnitude smaller than the breakdown-thresholds of nanosecond pulses[8].
2.3 Cavitation and Shock Wave

Plasma pressure is proportional to the product of free electron temperature $T_e$ and free electron density $n_e$ [5]. In LIOB, because both the ionization (characterized by $n_e$) and the absorption (characterized by $T_e$) are highly localized, there is a large plasma pressure gradient. This large plasma gradient results in rapid expansion of plasma, which will lead to the formation of a cavitation bubble in soft materials. When the expansion speed exceeds the speed of sound, the plasma expansion will lead to formation of a shock front [14,15].

Experimental investigations of shock and cavitation phenomena in bio-tissues can be challenging, because most bio-tissues are turbid, and such investigations heavily rely on photographic techniques. Fortunately, investigation of shock and cavitation phenomena in water sets out the physics principles of such phenomena in soft bio-tissues. Shock and cavitation phenomena in water have been extensively researched also because of their wide applications in underwater detonation [16], maritime remote sensing [17], and propulsion [18]. The following sections summarize the characteristics of shock wave and cavitation bubble in relation to ultrafast-laser ablation.
2.3.1 Characteristics of shock wave

In water, the energy contained in a spherical shock wave $E_S$ is given by [19]

$$E_S = \frac{4\pi R_s^2}{\rho_0 c_0} \int p(t)^2 \, dt,$$  \hspace{1cm} (2.3.1)

where $R$ is the distance from the launch site to the shock, $\rho_0$ and $c_0$ are the density of the water and the speed of sound in the water, and $p(t)$ is the pressure profile of the shock.

In the region a few millimetres away from the breakdown site, the shock profile of a shock can be measured by using a hydrophone or PVDF sensors. Previous research [20,21] found that, at this point, the shock acquired the typical profile of a steep shock leading edge with an exponential trailing edge [20,21]:

$$p(t) = p_s \cdot e^{-\frac{t}{t_0}},$$  \hspace{1cm} (2.3.2)

where $p_s$ is the peak pressure of the shock, and $t_0$ is the time for the shock pressure to decay to $1/e$ of the peak pressure.

Equation (2.3.1) and (2.3.2) infer that the peak pressure is proportional to $E_S^{\frac{1}{2}}$:

$$p_s = \left(\frac{\rho_0 c_0}{2\pi \rho_0}\right)^{\frac{1}{2}} E_S^{\frac{1}{2}} \left(\frac{1}{R}\right).$$  \hspace{1cm} (2.3.3)

In an experiment, the peak pressure of a shockwave can be determined by measuring the speed of a shock wave, as shown in the following formula [20,21]:

$$p_s = c_1 \rho_0 u_s \left(\frac{u_s - c_0}{c_2}\right) + p_0,$$  \hspace{1cm} (2.3.4)
where $u_s$ is the speed of the shock, $p_0$ is the hydrostatic pressure, $\rho_0$ and $c_0$ are the density and speed of sound in the water under normal conditions, $c_1 = 5190 \text{ m/s}$, and $c_2 = 25306 \text{ m/s}$. The parameters $c_1$ and $c_2$ are determined from Rice and Walsh’s measurements on the Hugoniot curve [22].

In experiments, shock wave propagation induces refractive index change and refractive index gradient change in water via small density changes, and these changes are made visible by shadowgraphy and Schlieren imaging. Shock wave propagation can be captured by continuously using a high-speed framing camera, or by using a regular camera to capture a series of images at different time delays under the same laser parameters. Then the shock speed is calculated based on the distance of propagation and the time interval between two frames.

The speed of the shock in the vicinity of the optical breakdown can reach as high as Mach 3[14,20]. As the energy contained in the shock is also dissipated into heat, due to liquid viscosity, the shock speed slows down to ~ Mach 1 after a few millimetres [17,20]. Just after the formation of the shock front, the shock pressure decays to close to $\sim R^{-2}$. When the shock pressure decays to $\sim 100 \text{ MPa}$, the pressure decays by $\sim R^{-1}$[20].

A shock wave pressure of 50 to 100 MPa can introduce cellular lysis by rupturing cellular membrane [15], but at the tissue level, shock wave is not the primary damage mechanism for extracellular matrix, because the displacement of material due to a shock wave is small. Instead, the expanding and collapsing of the cavitation bubble accounts for most of the tearing of the extracellular matrix [20].

2.3.2 Characteristics of a cavitation bubble
Within bulk water (i.e., away from the free surface), the dynamics of a cavitation bubble can be described by the Rayleigh model [15]. The Rayleigh model neglects surface tension and liquid viscosity, and assumes that the liquid is incompressible. Based on such an assumption, the dynamics of a spherical bubble are described by [15]

\[ R\dddot{R} + \frac{3}{2} \dot{R}^2 = \frac{(p_R - p_\infty)}{\rho}, \]  

where \( R \) is the radius of the bubble, \( \dot{R} \) and \( \dddot{R} \) are the first and second order derivative of \( R \) over time, \( \rho \) is the density of the liquid, \( p_R \) is the pressure at the bubble boundary, and \( p_\infty \) is the liquid pressure far away from the bubble.

An important result provided by the Rayleigh model is that the time from the bubble at maximum size to collapse is [15]

\[ T_c = 0.915 \cdot R_{\text{max}} \sqrt{\frac{\rho}{p_\infty - p_R}}, \]  

where \( R_{\text{max}} \) is the bubble radius at the maximum. Assuming that there is no damping during the expansion and collapse of the cavitation bubble, the time of one cycle of bubble from the start of expansion to the collapse is \( T_B = 2 \ T_c \) [15]. Equation (2.3.6) provides a method of estimating the maximum bubble radius based on acoustic measurement, because \( T_B \) can be measured using a hydrophone. Based on the \( R_{\text{max}} \), the energy contained in a spherical bubble can be obtained, using [21]

\[ E_B = \frac{4\pi}{3} \left( p_\infty - p_R \right) R_{\text{max}}^3. \]
In many bio-tissues, both the expansion and collapse of the cavitation bubble will be strongly suppressed, due to the fact that the mechanical strength of the bio-tissues is greater than that of water [23,24].

The physics picture of cavitation introduced at the air-liquid surface is quite different from the picture within bulk liquid. One major difference is that the cavitation, on the liquid side, is close to being hemispherical instead of spherical, as in bulk liquid [17]; therefore, the collapse of the cavitation at the air-liquid surface is asymmetrical. The collapse of the cavitation bubble close to or at tissue surface can form a jet of tissue or aqueous media that enters into the air side [25,26]. This effect can lead to tissue damage [11].

The partition of absorbed laser energy coupled into cavitation and shock wave is an important measure of the disruptive effects in laser ablation. Vogel et al. characterized the energy contained in the cavitation bubble and shock wave generated from LIOB in water. These researchers showed that, for optical-breakdown within bulk water induced by nanosecond pulses, ~90% of the absorbed energy is ultimately coupled into cavitation and shock wave energy [21]. In comparison, only ~15% of the absorbed energy is coupled into cavitation and shock in breakdown induced by femtosecond pulses [21]. This result shows that ultrafast lasers will introduce mechanical effects that are significantly smaller than those introduced by nanosecond lasers.

2.4 Thermal effects

2.4.1 Vaporization and cumulative heating
In laser ablation, targets are irradiated at a rate equal to or higher than LIOB threshold irradiance. As a result, the energy density in the focal volume is sufficient to directly vaporize the material. The energy density required to vaporize material $\varepsilon_v$ is given by [21]:

$$\varepsilon_v = \rho_0 (c_p (T_v - T_0) + L),$$

(2.4.1)

where $\rho_0$, $c_p$, and $T_v$ are the mass density, the specific heat, and the boiling point of the material, $T_0$ is the material temperature prior to irradiation, and $L$ is the specific latent heat [21].

At irradiance below the breakdown threshold, high-repetition-rate ultrafast pulses can result in cumulative heating of target material. Some applications capitalize on this heat accumulation effect. For example, laser waveguide-writing and IRIS surgery use the heat buildup to change the refractive index of materials and the cornea [27-29].

### 2.4.2 Thermal damage to tissues

Heat resulting from laser irradiation could lead to protein denaturation in cells. The thermal damage can be modelled as a rate process using the Arrhenius model[11,30,31]. The Arrhenius model indicates that the thermal damage is not only a function of temperature, but that it also depends on the time of exposure. With reduced exposure time, the tissue could tolerate a much higher temperature. For example, mammalian cells can tolerate ~40 °C for extended periods, ~70 °C for several seconds, and ~370 °C for only ~10 ms [32]. The extracellular matrix can remain intact at a temperature far in excess of 100 °C for nanoseconds or milliseconds of exposure [11].
Using the Arrhenius model, the damage can be quantified by a dimensionless quantity \( \Omega \) [1]:

\[
\Omega = \int_0^{t_{\text{exp}}} A \exp \left( \frac{-E_a}{k_B T(t)} \right) \, dt, \tag{2.4.2}
\]

where \( A \) denotes the frequency of damage, \( E_a \) is the activation energy barrier for the protein denaturation, and \( T(t) \) and \( t_{\text{exp}} \) are the temperature and time of thermal exposure.

2.4.3 Experimental means to identify heat damage

There are varieties of experimental techniques for identification of thermal damage to cells and tissues. In cells, heat insult will induce the expression of Hsp70 protein, which can be identified using bio-luminesce and western blot [30]. In an extracellular matrix, heat damage can be identified using H&E staining in histology. In some of the collagen-rich tissues, heat damage can also be detected by the loss of birefringence through the use of polarization-sensitive optical coherence tomography or nonlinear microscopy[33,34].

2.5 Potential damage to DNA

Beyond mechanical and thermal damage, a third type of biological insult in plasma-mediated ablation comes from the ionizing radiation of free electrons. The ionizing radiation is a potential mutagen because it can cause strand breaks to cellular DNAs, where the genome of a cell is stored. A safe laser surgery procedure should ensure the annihilation
of such cells with DNA damage (especially DNA double-strand breaks) in order to mitigate future risk.

2.5.1 DNA damage and repair

DNA (deoxyribonucleic acid) is a biopolymer existing in both the nucleus and organelles such as the mitochondria. The integrity of nucleic DNA is particularly important to cells because it encodes the genetic information of a cell and regulates the expression of proteins. DNA molecules in mammalian cells consist of two strands of nucleotides that form a double helix structure. Under normal condition, a DNA molecule is extremely stable, but radiation (UV, X-ray, etc.), free electrons, and chemicals (oxidative, alkylating agents, etc.) could cause single- or double-strand breaks to a DNA molecule. DNA double-strand breaks are more lethal to cells than single-strand breaks. This is so because single-strand breaks can be repaired using the undamaged strand as a template. In contrast, neither of the strands can serve as a template in double-strand breaks, which endanger the genetic information.

2.5.2 DNA damage from free electrons

Free electrons can damage DNA via two pathways. One pathway is direct breaking of bonds through resonant electron-molecular scattering; the other is indirect damage produced by generating reactive-oxygen-species in aqueous environments [4].

Direct damage to DNA by low energy (0-100 eV) free electrons was characterized by Sanche and colleagues [35-37]. They reported that free electrons with energy as low as 0 to 4 eV could cause solitary single-strand breaks, but not double-strand breaks [37]. The
first resonant energy for free electron-induced solitary single-strand breaks was ~0.8 eV [37]. At free electron energy > 4 eV, a second resonant window of solitary single-strand breaks showed up between ~7 and 12 eV, and the first resonant energy for solitary double-strand breaks showed up at ~10 eV [35]. At free electron energy from 15 to 100 eV, both solitary single-strand breaks and solitary double-strand breaks increased almost monotonically with the increase of free electron energy. At free electron energy from 30 eV to 100 eV, there is a monotonic increase of multiple double-strand breaks[36].

Strand-breaks induced by electrons with energy < 15 eV is considered to be a two-step process [35,36]. First, a free electron attaches to a molecule RH, forming a transient molecular anion state RH^−. This transient molecular anion state has a repulsive potential along the R-H bond coordinate. Then the transient molecular anion is dissociated along one or multiple bonds such as R' + H^- or R^- + H'[35,36].

The unveiling of a resonant energy window at ~10 eV for free electrons to induce strand breaks has significant implications for plasma-mediated laser surgery in two aspects. First, it demonstrates that free electrons can induce strand breakdown at a significantly lower energy level than the level of other types of radiation sources, such as X-rays or gamma rays. Second, this energy is comparable to the typical free electron energy during laser ablation; the typical free electron energy generated in plasma-mediated ablation would be higher or a few times greater than that of the effective ionization potential, so that free electron energy could cause impact ionization to occur[8]. The band gap energy of water is 6.5 eV[3]. Therefore, there would be abundant free electrons with energy close to the resonant energy of ~ 10 eV that would induce DNA damage.
2.5.3 DNA damage from oxygen species

The other pathway for inducing DNA strand breaks by free electrons is the pathway through the generation reactive-oxygen-species in an aqueous environment. Reactive-oxygen-species such as H$_2$O$_2$ and OH$^*$ are generated through ionizing and dissociation of water molecules [4,38]. Tirlapur et al. demonstrated that the reactive-oxygen-species generated by a 170-fs, 80-Mhz, NIR laser running at 7 mW could induce DNA strand breaks, and introduce apoptosis-like death of a mammalian cell [39].

The relative importance of the direct and the indirect damage pathways was compared by Arthur and colleagues [40,41]. The authors [40,41] compared the extent of DNA strand breaks after the introduction of free-electron- and radical- scavengers, respectively. The results indicated that the radicals were the primary but not the sole cause of DNA damage.

2.5.4 Experimental techniques to detect DNA damage

Experimental methods to identify DNA strand breaks include gel electrophoresis and immune-histological staining. Gel electrophoresis is the most common method for examining DNA strand breaks. DNA fragments carry a net charge, and they migrate under an electric field. Fragments of different sizes are distinguished by their speed of migration in gel; larger fragments will migrate more slowly than smaller fragments. The fragments in electrophoresis are made visible by tagging with ethidium bromide, which fluoresces under UV light.

The other way of identifying double-strand breaks is immune-histological staining of γ-H2AX. H2AX is a histone protein of the H2A family. DNA double-strand breaks cause
phosphorylation on the Serine-139 of H2AX [42]. The phosphorylated H2AX is referred to as the γ-H2AX. Immune-histological staining of γ-H2AX uses antibodies of γ-H2AX that are conjugated with fluorescein to tag the DNA double-strand break site. An advantage of the immune-histological staining method is that it can detect DNA double-strand breaks in situ. This feature is desirable in the process of characterizing the spatial extent of DNA damage in bio-tissues.
References


Chapter 3

The Burst-Mode Laser System

This chapter describes the laser system used in the present research. The laser system consists of a pulsetrain-burst-mode ultrafast-pulse oscillator, two 4-pass amplifiers, and a target translation-stage.

The laser’s oscillator (See Figure 3.1) is a Nd:phosphate-glass, 1053-nm, flashlamp-pumped (MegaWatt Lasers), pulsetrain-burst-mode oscillator purpose-built by Marjoribanks and colleagues at the University of Toronto and described in more detail in ref. [1]. The oscillator is active-passive hybrid mode-locked, a state that is achieved by using a plano-concave resonator and an intra-cavity telescope with a Brewster-angle saturable-absorber flowing-dye cell. The concentration of the saturable-absorber dye is set to optimize stability, so that the oscillator generates pulses of 1.5-ps pulse width (FWHM). The intra-cavity acoustic-optical modulator (AOM) is driven by a digital tunable RF source running at half of the pulse repetition rate, as per standard.

The intensity of the pulses circulating within the cavity is monitored in real time by a photodiode looking at the Fresnel reflection from one face of the laser rod. The photodiode signal is then the input to the fast HV FET-driver negative-feedback controller, which is connected to an intra-cavity Pockels-cell in order to feedback-stabilize pulse intensities
within each pulsetrain-burst. The output of the oscillator is the rejected fraction of the circulating power of the laser.

![Diagram of pulsetrain-burst mode laser oscillator](image)

Figure 3.1 The configuration of the pulsetrain-burst-mode laser oscillator, adapted from ref [1].

The oscillator is capable of generating up to 30 µs of 133 MHz pulsetrains (~7.5-ns pulse-to-pulse separation) at 1 Hz (1 pulsetrain-burst/s). Following the oscillator, a “N-Pulse Selector,” analogous in operation to a pulse-picker (see Figure 3.2), controls the number of pulses from a natural pulsetrain-burst that would be used in a given experiment. This way of operating has the advantage that it makes the onset of each on-target pulsetrain-burst regular, since, in the oscillator, these pulses grow from zero to the control-level over approximately 10 to 100 pulses. The N-Pulse Selector could make any selection between five pulses and the full pulsetrain-burst from the oscillator. The trigger signals provided to the N-Pulse Selector unit for selecting a pulsetrain-burst window were synchronized to the oscillator’s AOM RF signal through a “Pulsed Laser Sequencer” (PULSAR) developed in-house. The PULSAR is an FPGA-based electronic device, which uses the AOM RF signal as a system clock and then digitally generates one or more low-jitter trigger signals (patent pending, Marjoribanks and Stummer).
Figure 3.2 A schematic of a pulsetrain-burst laser system and typical pulsetrain-burst before and after the N-pulse Selector, adapted from ref [2,3]. AMP 1 and AMP 2: the two 4-pass amplifiers, TFP: the thin film polarizer, FR: Faraday rotor, \( \lambda/4 \): quarter waveplates, IEI: incident energy integrator.

After a pulsetrain-burst was selected by the N-Pulse Selector, it was then amplified by two 4-pass flashlamp-pumped Nd:phosphate-glass amplifiers. After amplification, pulsetrains reached per-pulse energy up to 30 \( \mu \)J. Amplified pulsetrain-bursts were focused onto a target to a near-diffraction-limited \(~5-\mu\text{m}-\text{FWHM}\) spot, using an \( f=20 \text{ mm aspherical lens (AL2520-B, Thorlabs, USA)} \). A portion of light back-reflected from the target was split off as it was passing back through the target lens and imaged with 15\( \times \) magnification (\( f=300 \text{ mm imaging lens} \)) onto a CCD camera set at the retro-reflected equivalent-target-plane (ETP) position. This ETP imaging system was used to monitor the size and transverse profile of the focal spot. A calibrated “incident-energy integrator” (IEI), consisting of a
10% splitter, a fast photodiode, and a small integrating-cavity (Kodak high reflectance coating), was used to record the intensity of every pulse in the train incident on the target.

Reference


Chapter 4

Study of the Effects of Burst-Mode Ultrafast-Pulse Laser Ablation, Using a 3D Living-Cell Hydrogel Soft-Tissue Proxy

Quantifying the effects of laser-irradiated tissue can be quite difficult due to the complexity and the lack of homogeneity of naturally differentiated tissues. This chapter describes an alternative approach; a standard hydrogel tissue proxy was developed for investigation of both the physical and the biological effects of burst-mode ultrafast-pulse laser ablation.

Section 4.1 describes the experimental need for developing a standard tissue model. Section 4.2 describes the tissue proxy used in the laser irradiation experiments and also the staining and imaging protocols developed to use with the tissue proxy. Section 4.3 describes the characterization of the tissue proxy and the quantification of the effects of pulsetrain-burst ablation. Section 4.4 summarizes the conclusion of the tissue proxy study and discusses the implications of the results.

The content of this chapter is adapted from a published article; I was the lead author of “Pulsetrain-burst mode, ultrafast-laser interactions with 3D viable cell cultures as a model for soft biological tissues,” Biomedical Optics Express, Vol. 5, no. 1, pp. 208 (2014).
4.1 The experimental need for a living-tissue proxy

One major objective of this research is to determine the biological effects of irradiation and then to identify the corresponding physics mechanisms for these effects. Examination of these effects in vivo will provide results that are closest to that of a real laser surgery procedure, but there are many challenges involved in carrying out these experiments on differentiated tissues. Naturally, differentiated tissues are heterogeneous in structure and composition, and they often contain regions of connective tissue or vascular tissue. Characterizing how different laser parameters affect the results of ablation in differentiated tissues can be quite difficult. Therefore, as a first step, use of a standardized tissue model is a more desirable experimental approach than use of differentiated tissues.

Major damage mechanisms involved in burst-mode plasma-mediated laser ablation include heat accumulation, propagation of shock wave, expansion of cavitation, and secondary ionizing radiation. Heat accumulation, shock wave propagation, and cavitation can cause necrotic and/or apoptotic cell death. Ionizing radiation (extreme ultraviolet photons and Auger electrons), reactive oxygen species, and free radicals generated in the ionization process can lead to single- or double-strand breaks of cellular DNA and then to apoptosis, mutagenesis, or oncogenesis. Necrotic cell death presents immediately after laser irradiation, but apoptotic cell death and the formation of a DNA-repair complex may take several hours or even longer to develop before they can be identified. In order to see the evolution of subcellular damage over time, the tissue model needs to be biologically alive throughout the incubation period.
A standardized tissue model for identification of the physics mechanisms underlying certain biological effects needs to be simple and homogenous. Its thermal properties and mechanical strength should be representative of the thermal properties and mechanical strength of the target tissue, so that the range of damage due to heat accumulation, shock wave, and cavitation would mimic the damage to a real tissue.

The standardized tissue model should also be three-dimensional (3D), because the damage from heat accumulation, shock wave propagation, or the hazards of secondary ionizing radiation can extend beyond the initial interaction volume. A 3D tissue model will allow realistic modelling of not only the laser energy deposition at the focal spot, but also of the effect of the subsequent propagation and dissipation of absorbed energy outside the focal volume. The homogeneity of the tissue model will help to simplify the spatial quantification of these damage mechanisms.

Proxy tissues that adhere to these requirements have several advantages over ex vivo differentiated tissues. Due to large cell densities, simple diffusion (without a functioning vascular system) leads to low oxygen and nutrient delivery; thus, ex vivo tissues have limited cell viability. This limited cell viability makes it difficult to characterize delayed cellular damage-response and cell death post-laser-irradiation.

While plated cell cultures and cell cultures in suspension will provide extended cell viability, neither of these is a suitable candidate for this research. Plated cell cultures are two-dimensional by definition, and this quality precludes study of three-dimensional damaging effects. Cell cultures in suspension are three dimensional, but the mobility of cells in suspension makes it difficult to localize damage effects at the ablation site in situ.
One possible candidate for a 3D tissue model is a matrix of cross-linked polymer chains populated by viable cells. The thermal and mechanical properties of cross-linked polymer chains are homogenous and can mimic a tissue to a certain extent in some ways. Hydrophobic polymers can make a mechanically strong matrix, but they are not suitable for culturing viable cells [1]. In contrast, hydrogels consisting of hydrophilic polymers are frequently used as cell-culturing scaffolds. Many hydrogels are naturally derived from polymers such as agar and collagen, which are found in living organisms. Such hydrogels are non-toxic to cells. They allow the diffusion of oxygen and nutrients, and provide a desirable environment for encapsulated cells. More importantly, these naturally derived hydrogel polymers possess macromolecular properties similar to those of the extra-cellular matrix in living tissues [1,2].

In order to make a suitable tissue proxy, the hydrogel matrix should replicate or be similar to the thermal and mechanical properties of biological tissues, because these properties are important factors in determining both the range of collateral cellular damage and damage mechanisms. The thermal diffusivity of agar hydrogel is comparable to that of water and animal tissues such as muscle, fat, and skin [3]. Generally, hydrogels have a limited ultimate tensile strength (UTS) and rupture easily because they lack a connective scaffold. The UTS of agar-based hydrogels is ~0.05 Mpa [4], which is comparable to low tensile strength, high-water-content tissues, such as liver tissue [5]. Some other hydrogels have been engineered with fracture toughness similar to that of cartilage [1,6,7]. Previously, hydrogels and hydrogel tissue cultures have been used as tissue proxies for laser ablation research [1,2,8,9], and they are also used in studying cellular response to drug delivery and radiation treatments [3,10-12].
I have developed a 3D cell-culture model embedded in agar hydrogel as a proxy for low tensile strength tissue. The tissue proxy enables the examination of the effects of burst-mode, high-repetition-rate, ultrafast laser ablation and the quantification of the extent of tissue damage under different laser parameters. The 3D living-cell tissue proxy has good cell-viability over long periods (~24 hours). Different types of cellular insult, including necrosis, apoptosis, and DNA double-strand breaks can be identified and quantified using commercial fluorescent-biomarker assays, followed by confocal fluorescence laser-scanning microscopy (CFLSM). The tissue proxy is permeable to these small labelling fluorophores and can be virtually sectioned using CFLSM. Combining the tissue proxy with the CFLSM technique allows the quantification of the extent of different types of cellular damages and also the determination of ablated volume under different laser parameter.
4.2 Materials and methods

4.2.1 Making of the tissue proxy

F98 rat glioma cells acting as damage sensors were first cultured in a flask in Dulbecco’s medium (DMEM-H21, GIMCO) and supplemented with fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). Upon reaching ~80% confluency, cells were ready to be passaged from the flask. After first removing the culturing medium and then incubating with trypsin at 37°C for four minutes, cells detached from the bottom surface of the flask. Then Dulbecco’s medium was introduced back into the flask to re-suspend the detached cells. This cell solution was centrifuged at 1,500 RPM for five minutes. The resulting cell pellet was re-suspended in 6 mL of Alpha MEM medium (GIBCO, without phenol red) supplemented with FBS and antibiotics. Phenol red is a broadband fluorophore and would contribute background noise in CFLSM images.

The hydrogels were prepared by dissolving solid agar (Agar Bacteriological [Agar No.1], OXOID, Nepean, ON) in distilled water to 25 µg/mL at 125 ºC in an autoclave for one hour. Afterwards, the agar solution was brought to a temperature of 55 to 60 ºC. Then 4 mL of agar-water was mixed thoroughly with 6 mL of cell solution at room temperature and poured into 35-mm petri dishes for a final cell density from 1×10^6 to 3×10^6 cells/mL (See Figure 4.1). This final cell concentration corresponds to a mean cell-to-cell separation of ~50 µm and provides both adequate spatial resolution between cells, using CFLSM, and sufficient diffusion of oxygen and nutrients throughout the gel to prevent cell starvation. The hydrogel-cell mixture was left to solidify at room temperature for two to three minutes, forming a gel ~3-mm thick in the petri dish. An assay showed that, immediately post-
production, the cell viability was maintained for > 90% of all gel-imbedded cells after solidification of the hydrogel-cell mixture. Punch-hole biopsies, 6 mm in diameter and three mm in thickness, were extracted from the hydrogel for single pulsetrain-burst laser irradiation experiments. Control hydrogels were also prepared and handled identically to experimental gels, but not laser-irradiated. On average, three hydrogels were used on each day of the experiments, and three biopsies were exacted from each gel.

![Diagram](image)

Figure 4.1 The process of making the 3D living-cell tissue proxy

4.2.2 Laser Irradiation

Within one hour of preparation, the hydrogel biopsies were irradiated with a single pulsetrain-burst from the burst-mode picosecond-pulse laser. The amplified pulsetrain-burst was focused onto the gel-sample surface, using a 20-mm focal-length aspherical lens
(AL2520-B, Thorlabs, USA), to a near-diffraction-limited ~5-µm-FWHM spot. The peak intensity at focus was close to \(1 \times 10^{14}\) W/cm\(^2\). Laser light back-reflected from the gel surface was imaged with 15× magnification onto a CCD camera, using a 300–mm focal-length lens, to monitor the size and transverse profile of the focal spot. Each hydrogel biopsy was irradiated with just one pulsetrain-burst (shot) for characterization of the cellular response under a particular irradiation scheme.

4.2.3 Staining and Confocal Microscopy

After laser irradiation, the gel-biopsies were stained with fluorescent marker-dyes to tag different cells for examination under CFLSM. Hoechst-33342 (Invitrogen, Carlsbad, CA) was selected as a marker for all (viable, early-stage necrotic and apoptotic) cells because it can permeate both intact and compromised cellular membrane [4,13], and intercalate with the DNA. Propidium iodide (PI; Invitrogen) was selected to mark only necrotic cells since it cannot penetrate intact cellular membrane [5,14]. Similarly, Annexin-V (conjugated with fluorescein isothiocyanate [FITC]; PHN1010, Invitrogen, Carlsbad, CA) was selected as a biomarker for cells undergoing apoptosis. Annexin-V binds to phosphatidylserine localized on the cytosolic side of the plasma membrane, if this membrane is still intact. When cells undergo apoptosis, phosphatidylserine distributes across the inner and outer membranes, and becomes accessible to Annexin-V. A mixture of 5-µg/mL Hoechst-33342 (0.616 kDa), 5-µg/mL propidium iodide (0.668 kDa), and 100-µL/mL Annexin-V (40 kDa) in binding buffer (Invitrogen, Carlsbad, CA) was added to the gel samples, typically four to five hours after laser exposure. The hydrogels were stained at 5% CO\(_2\) and 37°C for one hour, and afterwards washed with phosphate buffer solution (PBS).
An antibody staining method, γ-H2AX antibody (FITC conjugated; EMD Millipore, Billerica, MA), was used to tag DNA DSBs, since DSBs lead to Serine-139 phosphorylation on histone H2AX[15]. These hydrogel samples were first fixed in 4% paraformaldehyde at 4°C for ~12 hours, and cells permeabilized using 0.2% Triton X-100 (Sigma-Aldrich). After they were washed with 0.5% NP-40 (Sigma-Aldrich, St Louis, MO) and PBS, the fixed samples were each stained with 1 mL of 2-μg/mL γ-H2AX antibody (17 kDa) in a blocking solution of 4% bovine serum albumin and 4% goat serum in PBS. Subsequently, the samples were incubated at 4°C for 12 hours and afterwards washed with PBS.

The distribution of fluorescently tagged cells was mapped in 3D using a confocal laser-scanning microscope (LSM 510, Zeiss, Jena, Germany) with an objective (10×/0.5 N.A., FLUAR, Zeiss, Jena, Germany) that has a 1.9-mm working distance, which is sufficient to access fluorophores 1.5-mm-deep within the hydrogel matrix. The fluorescence excitation ($\lambda_{ex}$) and emission ($\lambda_{em}$) wavelengths for each assay applied standard values: Hoechst-33342 ($\lambda_{ex} = 400$ nm, $\lambda_{em} = 415$ to 735 nm), PI ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 566$ to 1000 nm), FITC-conjugated Annexin-V ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 493$ to 1000 nm), and FITC-conjugated γ-H2AX antibody ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 493$ to 1000 nm). The typical volume scanned within the gel was 1 mm × 1 mm × 0.3 mm with ~1-μm-lateral and ~10-μm-depth increments. The lateral and axial resolution of the confocal fluorescence microscope at 700 nm, for example, was 0.6 μm (0.4$\lambda_{em}$/N.A.) and 5.1 μm (1.4n$\lambda_{em}$/N.A.2), respectively, where n is the refractive index of the hydrogel (n≈1.3).
4.3 Results

The viability of cells was tested in control hydrogels for times up to 24 hours, corresponding to a time greater than the entire sequence of gel preparation, laser irradiation, staining, and CFLSM analysis. Cells can lyse without laser irradiation due to extreme temperatures during preparation or handling, desiccation, or starvation from lack of oxygen and nutrients. These “incidentally necrotic” cells will add to the measured signal from PI staining of the laser-affected cells, possibly depending on the depth below the surface. Punch-hole biopsy samples were extracted, in parallel with experimental samples, from hydrogels having cell densities of 1×10^6 and 3×10^6 cells/mL. These control samples were stained with Hoechst 33342 and PI at one, six, and 24 hours after initial gel preparation and analyzed by CFLSM. Irrespective of the cell densities prepared and the imaging depth, more than 90% of the embedded cells remained viable after six hours, and more than 85% of the cells remained viable after 24 hours. A similar viable fraction (tagged only by Hoechst-33342) was also found in irradiated samples when scanning ~2 mm away from a laser-irradiated spot. These results demonstrate that a high fraction of cells remain viable within the time frame of laser-irradiation experiments and that the supply of oxygen and nutrients is sufficient.
Figure 4.2 A comparison of the number of intentionally insulted cells within the hydrogel to the number of those in a naïve control hydrogel: (a) Cellular necrosis induced by heating with a hot water bath, (b) Cellular apoptosis induced by cis-platin, (c) DNA double-stranded breaks (DSBs) induced by an X-ray source at various dosages. The dimension listed near the top of each plot is the volume scanned by the confocal microscope.

Figure 4.2 demonstrates the feasibility of staining necrotic cells, apoptotic cells, and DNA DSBs within the 3D hydrogel cell culture. For each plot in Figure 4.2, cells in hydrogel biopsies were intentionally insulted (i.e., thermal, chemical, and ionizing radiation), tagged by the appropriate biomarker, and compared to those cells tagged in a naïve control hydrogel. Cells were counted in the 3D CFLSM image by using a 3D cell-counting macro in ImageJ (NIH, Bethesda, Maryland). Cells were counted in CFLSM images by, first, thresholding the measured fluorescence intensity per pixel at a minimum value, which rejected background noise without significantly rejecting fluorescence from cells, and, second, converting the image into a binary image. A median filter and a filter on the minimum cell size were also used to filter out shot noise.

Heating the hydrogel cell culture in a water bath at 65°C for ~10 min induced cellular necrosis in Figure 4.2(a). Cellular apoptosis in Figure 4.2(b) was induced by incubating the cells in 20 mL of a 0.8-mM cis-platin solution in DMEM for five hours, prior to seeding the cells into the hydrogel. Irradiating hydrogel cell cultures with a standard X-ray source
(X-RAD 225Cx Micro IGRT, Precision X-ray, North Branford, CT) at 225 kVp, 13 mA, and ionizing doses from 5 Gy to 20 Gy induced DNA DSBs in Figure 4.2(c). For all cases in Figure 4.2, the intentionally insulted cells are clearly distinguished from those in the control hydrogel, a method that indicates the suitability of CFLSM and these biomarkers to detect the insults within this living cell culture in hydrogel.

Figure 4.3 The distribution of cells as a function of depth into the hydrogel, averaged over four field-of-views of 320µm×320µm: (a) Cells tagged with Hoechst 33324 prior to seeding into the hydrogel, (b) Cells seeded into the hydrogel, then tagged post facto with Hoechst 33342, (c) Necrotic cells within the hydrogel tagged post facto with PI. The cell count is relatively constant up to a depth of ~700 µm from the hydrogel surface.

The maximum depth of cells detected under CFLSM is shown in Figure 4.3 for cells tagged by Hoechst-33342 in Figures 4.3(a) and 4.3(b), and cells tagged by PI in Figure 4.3(c). In Figure 4.3(a), Hoechst-33342 tagged the cells in vitro prior to mixing into the hydrogel, whereas the cells in Figures 4.3(b) and 4.3(c) were tagged in situ in the hydrogel, as described previously. Necrotic cells in Figure 4.3(c) were intentionally insulted in the manner of the necrotic cells in Figure 4.2(a). For all cases in Figure 3, the cell count is relatively constant and independent of depth up to a maximum detectable depth of ~700
μm. Similar results were found for Annexin-V when tagging apoptotic cells in hydrogel. For the laser ablation experiments, the maximum depths scanned were 500 μm.

In principle, the maximum depth for detection of cells under CFLSM could be limited by optical scattering in the gel and by the diffusion rate of fluorescent biomarkers, which in turn depends on their molecular weight. This maximum detectable depth depends upon the detectable fluorescence at deeper depths, which is shown for several biomarkers in Figure 4.4. Cells marked by PI, Annexin-V, and γ-H2AX were intentionally insulted as they were for Figure 4.2. As expected, the fluorescence intensity decreases with depth for all biomarkers as a result of optical scattering of the excitation and fluorescence within the hydrogel cell culture. The fluorescence intensity of dyes premixed into the hydrogel (Rhodamine-123 and Hoechst-33342) is slightly higher than the intensity of those that permeated into the hydrogel, a difference that indicates that biomarker diffusion into the gel biopsies only slightly reduces the detectable fluorescence. This evidence is supported by the similarity of the plots in Figures 4.3(a) and 4.3(b), which also indicates that the diffusion rate of the biomarker is not limiting the maximum detectable depth.
Figure 4.4 The normalized fluorescence intensity detected from various biomarkers as a function of depth into the hydrogel. Each set of data traces is normalized to the maximum intensity of each trace. The fluorescence data for cells tagged by PI, Annexin-V, and γH2AX is from the controlled insult experiments found in Figure 4.2. Fluctuations in the fluorescence intensity with depth may reflect the lack of homogeneity of marked cells within a given hydrogel sample.

At greater depths, cell hypoxia and anoxia can result in widespread cellular apoptosis, inhibiting cellular DNA repair mechanisms, and thus limit the maximum depth detected under γ-H2AX antibody staining. However, there has not been any noticeable increase of apoptotic cells up to the maximum detectable depth of ~700 μm in control hydrogels for up to 24 hours, and this possibility is thus ruled out.

Figure 4.5: (a) A lateral slice through an ablation crater in hydrogel, as viewed under CFLSM. The voids at the crater edges are image artifacts, which develop due to the steep edges of the crater. (b) The volume of the ablation crater in hydrogel as a function of per-pulse laser intensity at several pulsetrain burst durations.

The mechanical impact on the hydrogels after pulsetrain-burst mode laser ablation was investigated by measuring the dimensions of the ablation crater. In order to simplify the confocal measurement, hydrogels prepared with Rhodamine-123, but without cells, were
used. The crater dimensions were determined from CFLSM virtual sectioning. The shape of the ablation crater was an oblate hemispheroid (Figure 4.5(a)), where the crater volume (Figure 4.5(b)) scaled nearly linearly with the per-pulse laser intensity over the range 0.05 – 1.0×10^{14} W/cm^2, but did not depend significantly on the pulsetrain-burst duration between 0.5 μs and 10 μs. Ablation characteristics were found to be reproducible, with the data for Figure 4.5 taken during experiments of four days, using 24 gel biopsies.

The ablation crater volume was expected to increase with the pulsetrain-burst duration, but the results in Figure 4.5 suggest that ablation occurred only for the first handful of pulses in the pulsetrain. Based on the expectation that ablation is plasma-mediated, the plasma self-emission was measured using a 1-ns-risetime photodiode with two short-pass filters (BG39, Schott Glass) to attenuate the reflected 1053-nm laser light at an optical density of ~24. Consistently, the plasma self-emission in the visible range of the spectrum was observed to last for ~100 ns, regardless of the pulsetrain-burst duration used, down to the minimum achievable burst duration of 0.1 μs. This observation indicates that only the leading 10 to 13 pulses contribute to plasma-mediated ablation of the hydrogel. This observation can be explained if the first 10 to 13 pulses vaporize sufficient water to explode in a bubble (i.e., explosive boiling) and eventually eject material. This occurrence leaves a void in the gel extending over the focal volume, does not permit further absorption of laser radiation, and leads to termination of the laser-plasma interaction. It has been shown elsewhere [16] that bubble formation stops absorption of successive laser pulses and subsequent ablation of water when femtosecond-laser-pulses with repetition rates > 1MHz are used.
Bubble formation in hydrogels during laser ablation follows explosive boiling of water. Rupture of the hydrogel is facilitated by its limited tensile strength. Higher tensile strength (e.g., in differentiated bio-tissue with fibrous connective tissue) would resist cavitation, thereby permitting more pulses in a pulsetrain to interact with dense tissue. Irradiating solid materials (e.g., glass or dental hard tissue) with pulsetrain bursts is seen to result in greater material removal with increasing burst duration [17] and to produce plasma self-emission throughout the entire burst.

The impact on cells following pulsetrain-burst mode laser ablation was determined by measuring the extent of cellular necrosis surrounding ablation craters, using the assays combining Hoechst-33342 and PI in combination. The relative locations of both viable and necrotic cells in the CFLSM images were determined using a 3D cell counter macro in ImageJ. Following laser irradiation, the distribution of necrotic cells was roughly a hemisphere, approximately 100 to 250 μm in radius, depending on pulse intensity. Considering the origin to be the point on the gel surface at the centre of this hemisphere, cells were binned by radius into equal-volume, hemispherical shells and counted (MATLAB [MathWorks]) (Figure 4.6(a)). Within each of these bins, the number of viable and necrotic cells provided the necrosis fraction (i.e., the percentage of necrotic cells). To quantify the range of necrosis, this fraction was plotted as a function of distance from the origin and fitted with a smooth curve — a gaussian function, as a smooth few-parameter fit relevant to thermal diffusion, was used. The necrosis range was then taken to be the half-width at half-maximum of this distribution.
Figure 4.6: (a) The number of viable and necrotic cells in hydrogel irradiated at a $4.6 \times 10^{13}$-W/cm$^2$ intensity and 1-μs-duration pulsetrain-burst, as a function of distance from the centroid of the distribution of necrotic cells, but at the gel surface. The cells are binned in equal-volume, hemispherical shells. (b) Cylindrical projection of viable and necrotic cells, with hemispherical bins used for the analysis overlaid. The red hemisphere-line marks the necrosis range, according to gaussian fit. (c) The necrosis range as a function of the per-pulse laser intensity for a 1-μs-duration pulsetrain-burst. The line through the data points is a power-law fit, with the equation shown in the figure, where $I_0 = 1.0 \times 10^{13}$ W/cm$^2$, and $C = 138 \pm 28$ μm. Error bars on data points are standard deviations multiple of gaussian fits using a different total number of hemispherical shells. The data shown was taken over five days of experiments from five separately produced gels providing 21 punch-hole gel biopsies.
Figure 4.6(b) presents ranges of viable and necrotic cells with respect to the ablation centre, employing the same hemispherical shells used for the analysis. A few cells can be seen in the region where a crater is expected. One possible cause is the debris of necrotic cells floating from the surface of the crater into the liquid used for the assay. Another possible cause is that, in the hydrogel, as in a soft tissue, the crater surface over several hours may slowly slump during staining and imaging. Rhodamine-123 allows for 3D measurement of the crater when using CFLSM (e.g., Figure 4.5), but Rhodamine-123 cannot be combined with the living cell cultures needed to measure the necrosis range, since the dye itself is toxic. The live-dead assay liquid index-matches the hydrogel very well, and prevents characterization of the crater shape during scanning, from the Fresnel reflectivity of the hydrogel free surface. Other ways to directly compare necrosis range and crater shape, in the same sample and at the same point in time, are being assessed.

The dependence of necrosis range on peak laser intensity, between $0.8 \times 10^{13}$ and $4.6 \times 10^{13}$ W/cm$^2$ for 1-$\mu$s-duration pulsetrain-bursts, is shown in Figure 4.6(c). The necrosis range scales closely as $I^{1/2}$, the square root of the intensity. The extent of cellular apoptosis surrounding ablation craters following laser ablation was also examined by an assay combining PI and Annexin-V. Three biopsies were irradiated at the highest laser intensity ($1.5 \times 10^{14}$ W/cm$^2$) over three separate days. Hydrogel cell cultures were investigated six to eight hours following laser irradiation, since the collateral physical effects of ablation from pulsetrain bursts would most likely result in pre-programmed cell death. However, no apparent difference in cellular apoptosis was detected between irradiated and control hydrogels.
The feasibility of measuring DNA double-strand breaks in this hydrogel-culture proxy was evaluated by first irradiating viable-cell gels as control samples, using the commercial X-ray source described in connection with Figure 4.2(c) and staining with a γ-H2AX antibody assay. In these control samples, the finding was that DNA double-strand breaks above background could be detected only for ionizing radiation doses of about 5 Gy or greater. (This dose is for water, which has a density close to that of hydrogel [>95% water], but does not include absorption by the cells.) In the case of laser-irradiation at the highest-available peak-intensity (1.5×10^{14} W/cm^2), DNA double-strand breaks were not detectable above background. The γ-H2AX antibody assay depends on detection of the repair-complex formation in living cells. Thus, I conclude that, if any cells received an ionizing-radiation dose of~5 Gy or greater, they were within the population of cells killed promptly or soon after irradiation. No viable cell with DNA double-strand breaks due to pulsed laser ablation was detected; this finding corresponds to the detection threshold of 5 Gy ionizing radiation dose.
4.4 Discussion and conclusion

Though studies on these viable hydrogel cell-cultures certainly do not replace studies on \textit{ex vivo} and \textit{in vivo} tissues, hydrogel cell-cultures do offer clear advantages as a standardized tissue model to study the biophysics of thermal, radiative, and shock wave phenomena in bio-tissue under ultrafast-laser ablation.

While most real tissues contain differentiated structures for support and transport, the homogeneity of hydrogels is an advantage when seeking to directly compare biophysics effects; the homogeneity of live-cell hydrogel proxies permits greater reproducibility of results. The hydrogel cell cultures in this study are also more permeable and less densely populated with cells, as compared to excised tissue. Thus, cells located deep in a hydrogel remain viable over a longer period of time due to better gas and nutrient diffusion. This results in a low count of incidentally necrotic cells causing noise. Statistics in measurements of cellular damage from laser irradiation, as compared to those involving ex \textit{vivo} tissue, are thus improved.

The permeability of hydrogel also permits fluorescent biomarkers to penetrate more easily into the hydrogel than into differentiated tissues. As compared to excised natural tissues, this easy penetration permits more rapid tagging of different cellular damage types (Figure 4.2). The results in Figures 4.2 and 4.3 demonstrate that fluorescent biomarkers can be used successfully for quantitative analysis of these cellular deaths mechanisms in this standardized tissue model.

Hydrogel also has negligible optical absorption and little scattering in the visible wavelengths, qualities that make it well suited for optical virtual-sectioning methods like
CFLSM. Both this minimal attenuation of visible light and the rapid diffusion of fluorescent biomarkers within the hydrogel come together to permit 3D imaging of laser-induced cellular damage deep within the sample (Figures 4.3 and 4.4). To obtain similar 3D measurements of cellular damage in ex vivo tissue, microtome sectioning would be required. However, image-registration errors between slices are considerable, as thin slices have little structural integrity and may stretch or tear. Image-registration error is not an issue when using CFLSM to virtually section and image cellular insult in hydrogel cell cultures.

The hydrogel tissue model, at present, does not reproduce the mechanical or dynamic characteristics of connective tissues (e.g., see a comparison of UTS in Table 4.1), but different approaches are available, which attempt to duplicate in hydrogels the mechanical properties of tissues. One method is to increase the agar concentration, since it is generally proportional to the UTS [4]. Another technique, used to replicate cartilage tissue, is to embed the viable-cell hydrogel within a porous and mechanically strong scaffold (e.g., poly-L-lactide) [18]. Further, synthetic hydrogels containing double networks of long and short cross-linked polymers have been shown [6,7,19] to have high fracture toughness similar to that of cartilage [20]. Though proxy tissues are not hydrogels, proxy tissues engineered by self-assembly and mechanically stimulated in a bioreactor have been developed with a UTS > 2MPa [21].

The above methods can approximate the mechanical characteristics of connective tissues in hydrogels, but other useful properties, such as optical transparency, cell biocompatibility, and biomarker permeability are compromised. For example, increasing
the agar concentration of this hydrogel model also decreases the optical transparency and biomarker permeability. This study opted for the diagnostic advantages of this model.

Table 4.1 The fracture stress and strain of 1% agarose hydrogel and various human bio-tissues

<table>
<thead>
<tr>
<th></th>
<th>Fracture Stress (Tension, kPa)</th>
<th>Fracture Strain (Tension)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose (1% w/w\textsuperscript{a})</td>
<td>50</td>
<td>0.2</td>
</tr>
<tr>
<td>Tendon\textsuperscript{b}</td>
<td>60,000</td>
<td>0.1</td>
</tr>
<tr>
<td>Cornea\textsuperscript{b}</td>
<td>3,300</td>
<td>0.13</td>
</tr>
<tr>
<td>Skin\textsuperscript{b}</td>
<td>13,000</td>
<td>0.6</td>
</tr>
<tr>
<td>Artery\textsuperscript{b}</td>
<td>2,000</td>
<td>0.78</td>
</tr>
<tr>
<td>Liver\textsuperscript{b}</td>
<td>29</td>
<td>0.44</td>
</tr>
</tbody>
</table>

\textsuperscript{a}From [4]

\textsuperscript{b}From [22]

For single-pulse or few-pulse ultrafast-laser interaction, the distinction between different tensile strengths may be unimportant. On such short timescales, inertial forces rather than the tissue’s structural integrity may dominate mechanical dynamics. For ultrafast pulsetrain-burst interaction, however, this study shows here that, in the case of surface ablation, 10 to 13 pulses open a vapor bubble in the hydrogel around the focal location; this opening does not happen in hard tissues [17]. Tissues with a collagen scaffold are expected to be an intermediate between hydrogels and hard tissues.
In surgical applications, ultrafast pulsetrain-burst treatments are thought to offer control over the extent of the eschar zone around the laser-incision in tissue. By controlling the pulse-intensity envelope or the duration of the pulsetrain burst, one can affect the surrounding tissue minimally (cf. single ultrafast pulses) or extensively (cf. long-pulses). Thus one can produce results using pulsetrain bursts that are intermediate between those produced from ultrafast and long laser pulses, or instead exploit the individual advantages of each, as has been shown in solid-materials processing [17]. In hydrogels, explosive boiling and cavitation set a limit on the number of pulses that can be usefully applied in a pulsetrain-burst (Figure 4.5), though the necrosis range can still be controlled through laser pulse intensity (Figure 4.6(b)).

One of the principal results of this study relates to the extent and nature of collateral damage caused by ultrafast-laser pulsetrain-burst interaction with live-culture hydrogels, for different parameters of the pulsetrain-burst. Cellular necrosis in bio-tissues occurs due to a combination of thermal diffusion and shock wave propagation. High repetition-rates lead to more rapid thermal accumulation and plasma-plume formation that may scald nearby cells, while shock waves may create mechanical strain sufficient to rupture cellular membranes. For both mechanisms, the amount of damage is expected to increase with the temperature of the mediating plasma and the strength of the shock wave, which in turn increase with the per-pulse intensity. The increased extent of cellular necrosis with pulse intensity shown in Figure 4.6 supports this expected scaling.

Principal damage mechanisms may change when irradiation is done with either a single ultrafast-laser pulse or a train of ultrafast-laser pulses. For single ultrafast-laser pulses, thermal damage should not play a large role in cellular necrosis. For a long train of closely
spaced ultrafast-laser pulses, thermal accumulation can be a damage mechanism for the surrounding tissue [17]. However, it is unclear for viable-cell hydrogels whether the absorption of 10 to 13 ultrafast pulses results in significant thermal accumulation and causes the range of cellular necrosis seen in Figure 4.6. Still, the first 10 pulses could each generate its own shock wave. Among these shock waves, because of the short pulse-to-pulse separation, stronger shock waves could catch up with weaker shock waves to create one large shock wave. Stronger shock wave could also result from material ejection preceded by bubble formation.

For cells in a hydrogel matrix, irradiation with pulsetrain-bursts resulted in cellular necrosis from tens to hundreds of microns away from the ablation crater, but irradiation did not appear to result in cellular apoptosis in the same region. For pulsetrain-burst ultrafast-laser ablation, cellular apoptosis would probably result from the collateral physical impact of plasma-mediated ablation, such as heat and shock waves, which would not activate death receptors and death signalling pathways through de novo protein synthesis (i.e., programmed cell death). Shock waves might rupture the mitochondrial membrane, lead to immediate release of cytochrome C, and trigger a caspase cascade. The result could be pre-programmed cell death six to eight hours after laser irradiation. However, for hydrogels irradiated with the laser in this study, the immediate physical impact from ablation was strong enough to rupture the cellular membrane directly and cause cellular necrosis. If the cellular membrane remained intact after irradiation, the cells survived.

For cells in a hydrogel matrix, the preliminary results here indicate that ultrafast laser pulses delivered in pulsetrain bursts do not result in gross DNA double-strand breaking equivalent to 5 Gy of the absorbed dose, at least not in cells surviving long enough to
activate the repair-complex mechanism. Therefore, in the experiment described, surviving
cells did not suffer severe DNA damage. Possibly, this result may not carry over to \textit{in vivo}
tissues; for instance, even the relatively low concentration of metallic salts in hydrogels, as
compared to live tissue, may skew the result to lower doses of secondary radiation, since
the flux and spectrum of XUV and X-ray photons depend sensitively on atomic number
(i.e., the power spectral density of bremsstrahlung radiation has a $Z^2$ dependence). It may
also be that absorption of only 10 to 13 pulses in hydrogels produces plasma that is not a
sufficient dose to produce appreciable DNA double-strand breaks, while much longer
puiletain bursts may have a greater effect. However, it is clear that more sensitive
measurements are needed, measurements that are capable of detecting lower densities of
DNA double-strand breaks at lower doses ($<5$ Gy) of ionizing radiation. Direct
femtosecond-laser irradiation of DNA in aqueous solution at $12$ TW/cm$^2$ (below the optical
breakdown threshold of water) has been shown to result in DNA single-stranded breaks by
D’Souza \textit{et al.} \cite{23}, but the results are likely to differ when DNA is located naturally within
organelles inside cells that are embedded in a hydrogel matrix. In this case, irradiation was
also done at above breakdown threshold, whereas D’Souza \textit{et al.} irradiated at below
breakdown threshold. Therefore, in this study, even if irradiation resulted in DNA damage
near the focal spot, the cells close to the focal spot were either ejected during ablation or
were necrotic afterwards because of disruptive effects resulting from shock wave.

For future investigations in determining cellular DNA damage in the tissue proxy, both
positive and negative control group should be included in the comparison with the treated
group for better determination of the DNA DSB detection threshold in the tissue proxy.
Also, it should be noted that in future investigations where direct \textit{in situ} detection of DNA
DSB site is needed, an alternative method to the γ-H2AX antibody assay is the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling technique (also known as the TUNEL assay). In the TUNEL assay, a nick-end of fragmented DNA first binds to a dUTP through the catalyzation of terminal deoxynucleotidyl transferase, and the binded dUTP is subsequently labeled with a biomarker [24]. The TUNEL assay does not require the presence of DNA DSB repair mechanism, and the TUNEL assay is also expected to provide a better diffusion performance in tissue proxies because of the smaller molecules used compared to the γ-H2AX antibody method.

In conclusion, it may be said that a 3D living cell culture was developed and shown to be useful as a proxy for low tensile-strength tissues in order to study cellular response in biological tissues following ultrafast-laser ablation. Cells imbedded in gels are viable for extended times (> 85% viable after 24 hours), and this viability allows time for biological response, cellular expression, and diffusion of a range of fluorescent cell markers. Tagged cells were found to be successfully imaged up to ~700-μm depth below the hydrogel surface, through the use of virtual sectioning via confocal fluorescence laser-scanning microscopy. In this application, the cell necrosis and apoptosis insult that followed pulsetrain-burst mode ultrafast laser ablation were characterized as a function of incident laser parameters. This living tissue proxy is expected to be well suited to fundamental studies of other therapeutic applications, such as photodynamic therapy, proton cancer therapy, and X-ray irradiation.
Reference


Chapter 5, Part I

An Energy-Partition Diagnostic for Characterizing Dynamic Absorption During Burst-Mode Plasma-Mediated Ablation

Chapter 5, Part I describes an energy-partition diagnostic purpose-designed for measuring absorption and scattering in plasma-mediated ablation by a high-repetition-rate (133 MHz), pulsetrain-burst ultrafast-pulse laser. The system time-resolves the partition of elastically scattered laser light into specular reflection, diffuse reflection, and transmission, and gives access to per-pulse absorption dynamics. Section 5.1 explains the experimental need for such a device. Section 5.2 describes the design considerations and the configuration of the diagnostic. Section 5.3 describes the calibration and characterization of the diagnostic. Chapter 5, Part II describes test runs of the dynamic absorption measurements.

Chapter 5 adapts the content from an article published in the Review of Scientific Instruments, Vol. 85, 033101 (2014). I was the lead author of the article, which is titled “Energy-partition diagnostic for measuring time-resolved scattering and absorption in burst-mode laser ablation.”
5.1 Need for time-resolving the dynamic absorption

Pulsetrain-burst mode lasers deliver the pulses in bursts (i.e., packets of pulses) with a fixed, short inter-pulse separation, thus offering new control options for the repetition-rate and the pulsetrain length, and also for enabling new features in material processing [1]. Optimization of this expanded parameter space for burst-mode ultrafast lasers relies on detailed investigation of the physical mechanisms for ablation (e.g., optical breakdown, cavitation, shock wave), which depend on the absorption of laser-pulse energy [2-7]. Hence, studying absorption provides guidance about how to maximize ablation rates and at the same time minimize collateral damage.

Absorption of high repetition-rate pulsetrain-bursts, which is different from low repetition-rate laser ablation, is a dynamic process not only across short time scales (femtosecond to picosecond pulse widths and nanoseconds of inter-pulse separation), but also across long (microseconds of pulsetrain length) time scales. In a pulsetrain where the inter-pulse separation is several nanoseconds, any pulse can interact with residual plasma created or sustained by previous pulses. Besides critical-density plasma near the solid surface, a plume or ejected material persists. This plume or ejected material consists of plasma and potentially of nanoparticles, which will absorb, scatter, and reflect laser light, and thus prevent some fraction of laser energy from reaching the target [8,9]. Thus, absorption of a given pulse depends on the history of previous pulses. Moreover, development of an ablation crater or the expansion/collapse of a cavitation bubble in a soft material also contributes to the dynamics of absorption throughout a pulsetrain. Therefore, characterization of the absorption of pulsetrain-bursts by a solid target material and its plume requires a diagnostic device that times-resolves the absorption of each pulse.
Researchers have previously measured absorption of single-pulse or low repetition-rate (kHz) pulses, using approaches including direct measurement by calorimetry [10,11] and indirect measurement through inference of the absorption from the difference between the incident energy, on one hand, and the scattered and reflected energy combined, on the other hand [4-7,12-14]. However, in the process of characterizing high-repetition pulsetrain absorption, the calorimetry method does not offer sufficient temporal resolution to time-resolve the absorption of each pulse. In addition, previous indirect measurements were not capable of making a full energy inventory over all solid angles for a sufficient time and with sufficient resolution to study burst-mode laser ablation.

The integrating sphere or cavity, which was developed in the 19th century [15,16], is an established device used in a variety of optical measurements [17-21]. It offers an indirect measurement of absorption by collecting all of the scattered light and inferring plasma absorption as the difference between the incident energy and the elastic scattered energy [19].

Thus, I designed and built a diagnostic tool, based on integrating-sphere principles, which collects the laser light scattered in plasma-mediated ablation into four different spatial components, thereby allowing indirect measurement of the absorption of each pulse in a 133-MHz repetition rate pulsetrain.
5.2 Design considerations

In plasma-mediated ablation, light scatters or reflects anisotropically from the plasma, so the energy-partition diagnostic collected specular reflection, diffuse reflection, and transmission in four different spatial compartments, using a variety of integrating cavities.

Due to the high reflectivity of a dense plasma, a significant fraction of the incident light was specular- or diffuse-reflected, so a specular-reflection integrator (SRI) was placed onto the retro-reflected equivalent-target-plane (ETP) path (see Figure 5.1). At the same time, a diffuse-reflection integrator (DRI) quantified the back-reflection at angles close to the incoming laser axis, at angles between 4º and 32º. In addition, an upper sphere (US) measured the remaining diffuse reflection in the upper hemisphere, and a lower sphere (LS) measured the transmission of angles from 90º to 180º. Each component was created either out of a sphere or a tube, with its interior painted with a high-reflectance barium sulphate coating (Avian-B™, Avian Technologies, LLC). The reflectivity of the coating at 1,053 nm was 97.8%.

All components were equipped with 1-ns-rise-time photodiodes, and signals were recorded using GS/s sampling rate oscilloscopes (TDS3044B, 5GS/s, 400 MHz, Tektronix, and WaveSurfer 454, 2GS/s, 500MHz, LeCroy). A 1,050-nm bandpass filter with 10-nm bandwidth (Stock # 65-769, Edmund Optics, OD≥4) and a FGL1000 (Thorlabs, OD≥3) long pass filter were installed on each detector port.
Figure 5.1 A schematic of the time-resolving energy-partition diagnostic. SRI: 1-inch long, 1/2-inch-diameter integrating tube at the ETP, DRI: 1-inch diameter, 3-inch-long integrating tube with a 0.5-inch aperture on each end, US: 2-inch-diameter integrating sphere, LS: 1.5-inch diameter integrating sphere, IEI: 2-inch long, 1-inch diameter integrating tube, BS: 90/10 beam-splitter.
5.3 Calibration and characterization of the diagnostic

Calibration of collection efficiency was achieved by sending a known fraction of the total incident energy sequentially to each “to be calibrated” component (See Figure 5.2 for steps).

Figure 5.2 Steps in calibration of each component: (a) SRI: The US and the DRI were removed. A mirror (BB1-E03P, Thorlabs) resulted in 99% specular reflection of the incident energy. (b) DRI: A disc with high reflectance coating sealed the lower aperture of the DRI, resulting in a 97.8% diffuse reflection. (c) US: The disc with high reflectance coating sealed the lower aperture of the US, resulting in a 97.8% diffuse reflection. (d) LS: All components are installed; no target was placed in the target-translation stage.
Each component’s calibration factor was calculated based on more than 1,000 pulses at different intensities. The responsiveness of each component followed a linear fit (Figure 5.3).

Figure 5.3. Responsiveness of: (up) IEI, and (down) SRI, DRI, US, and LS.
It is convenient to define responsiveness as the ratio between the signal peak and the pulse energy, which is shown in Table 5.1. The detection limit of each component was defined as the energy corresponding to the minimum detectable signal peak (1-mV pulse) of the photodiodes.

Table 5.1 Characterization of the double-integrating-sphere system

<table>
<thead>
<tr>
<th></th>
<th>IEI</th>
<th>SRI</th>
<th>DRI</th>
<th>US</th>
<th>LS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Responsivity (mV/μJ)</strong></td>
<td>63±4</td>
<td>13±1</td>
<td>21±1</td>
<td>10±1</td>
<td>25±1</td>
</tr>
<tr>
<td><strong>Detection Limit (nJ)</strong></td>
<td>15±1</td>
<td>74±3</td>
<td>49±1</td>
<td>102±4</td>
<td>39±1</td>
</tr>
<tr>
<td><strong>1/e Rise Time (ns)</strong></td>
<td>0.8±0.7</td>
<td>0.6±0.1</td>
<td>0.9±0.1</td>
<td>1±0.1</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td><strong>1/e Fall Time (ns)</strong></td>
<td>3.1±0.2</td>
<td>1.5±0.1</td>
<td>1.9±0.1</td>
<td>2.0±0.1</td>
<td>1.7±0.1</td>
</tr>
</tbody>
</table>

The temporal response of an integrating cavity depends on the size of the cavity and on the ratio between the area of coated surface and the area of the port. In this experiment, the temporal response of each integrating component was determined from the widths of the response signal following exposure to a 1.5-ps laser impulse (Table 5.1). The FWHM of signals ranges from 1.6 to 2.6 ns, which is sufficient to time-resolve pulses of 7.5 ns interpulse separation. However, the temporal evolution of absorption during each laser pulse (1.5-ps FWHM pulse width), such as the initiation of a plasma, cannot be resolved. The
timebase registration or synchronization of all channels was established using a single-pulse dataset.

Unintended reflection from optics created artifacts within the system, and these artifacts had to be characterized for accurate interpretation of the absorption data. Several sources of these artifacts within the system were discovered and corrected: i.e., reflection from the glass sample holder between the two spheres into the DRI and the US, reflection from the lens of the IEI into the SRI, and the reflection of the aspherical lens into the DRI. Corrections made for these artifacts include the following steps: switching from an uncoated lens to a lens with antireflection coating reduced the reflection from the lens of the IEI into the SRI; reflections from the glass sample holder and the aspherical lens inside the DRI were characterized so that reflections from targets were corrected for this effect.

Use of digital oscilloscopes can also introduce artifacts (i.e., under-sampling, aliasing, etc.). The artifacts associated with digital oscilloscopes will be discussed in Part II of Chapter 5.
Reference


Chapter 5 Part II

Benchmarking the Energy-Partition Diagnostic System

Chapter 5 Part II describes test-runs of dynamic scattering measurements carried out using the energy-partition diagnostic system. These test-runs were first carried out on aluminum and soda-lime glass. Section 5.4 and Section 5.5 summarize the test-run results of the two targets respectively. These two sections are adapted from my previous article as the lead author in the Review of Scientific Instruments, Vol. 85, 033101 (2014) [1]. Section 5.6 discusses errors associated with using digital oscilloscopes in this system. Section 5.7 describes the effort to measure time-resolved scattering and to locate damage spots on \textit{ex vivo} porcine tissues, and the way this effort motivated the work in Chapter 6. Finally, Section 5.8 summarizes the chapter.
5.4 Characterizing dynamic absorption and scattering of aluminum

In contrast to dielectrics, free electrons exist in metals prior to laser irradiation. Free electrons in metals allow absorption of laser energy through linear absorption. This section provide an example of dynamic scattering measurement during burst-mode laser ablation on aluminum foil, using the diagnostics system described in Chapter 5 Part I. Here, aluminum represents metals as a class of materials.

Four shots were fired at a 53-µm-thick aluminum foil at the average pulsetrain irradiance of \( \sim 3 \times 10^{13} \text{ W cm}^{-2} \). Each shot used a single 10-µs pulsetrain consisting of 1,333 separate 1.5-ps pulses. Only the first 230 pulses were recorded because the process was limited by the record-length of the oscilloscope. Figure 5.4 shows an example of measured scattering fractions and the inferred absorption fraction from one of the shots.

Figure 5.4(a) shows the net measured reflection fraction from all reflection and backscattering channels summed (blue trace), and also the transmission fraction (red trace). From the accounting of incident energy and from the measured reflection and transmission, the total absorption is inferred (green trace). Figure 5.4(b) shows a breakdown of the reflection fraction into specular reflection fraction (red trace) captured by the specular reflection integrator (SRI), and also the diffuse reflection fraction (blue trace) captured by both the upper sphere (US) and the diffuse reflection integrator (DRI). Figure 5.4(c) shows the pulsetrain envelope.

In each case for aluminum, there is a systematic dynamic, though details change from shot to shot. The rapid rising of the transmission fraction (at \( \sim 450 \text{ ns} \) in Figure 5.4) indicated that the pulsetrain perforated the aluminum foil. After that, enlargement of the perforated
hole resulted in the increase of the transmission fraction. Most of the laser energy was thereafter transmitted into the lower sphere, using a thin foil target that can be perforated within the record-length permitted testing of every integrating cavity of the system (especially the lower sphere, for recording the transmission signal).

Pulses propagating through an etched channel yield an interesting physics phenomenon; plasma at the wall of the etched channel acts as a wave guide for incident light. The irregularity of the etched channel wall and plasma absorption degrades the transverse coherence of the incident light as it propagates through the channel. Dean et al. [2] characterized this effect by using an earlier version of the burst-mode laser system to drill through aluminum targets of different thickness. They demonstrated that the degradation of the transverse coherence increases the transverse spreading of incident light, as compared to an idealized gaussian profile, which increases the energy coupling into the wall of the etched channel and thus reduces the intensity available at the centre of the channel. Therefore, this degradation of transverse coherence will limit the efficacy of ablation as the pulses drill deeper into the material [2].
Figure 5.4 A 53µm-thick aluminum foil ablated with 10-µs pulsetrain (1,333 pulses in total) of 1.5-ps pulses at an average irradiance of 3×10^{13} W cm^{-2}: (a) Time-resolved total reflection (R), transmission (T), and absorption (A), (b) Time-resolved specular and diffuse reflection. The insert shows the specular and diffuse reflection in the first 0.1 µs of the pulsetrain. (c) The Pulsetrain envelope. (Only the first 230 pulses were recorded; the process was limited by the record-length of the oscilloscope.)
5.5 Dynamic scattering and absorption of glass

Dielectrics (e.g., glass) are distinct with respect to metals in the sense that they have virtually no free electrons to mediate absorption prior to laser irradiation. High-irradiance ultrafast laser pulses produce multi-photon absorption or tunnel ionization, which is immediately followed by avalanche ionization; the process eventually leads to laser-induced optical breakdown in dielectrics [3] (see summary in Chapter 2).

This section presents time-resolved scattering measurement that is similar to the process presented in the last section, but in this case done on soda-lime glass (Goldline™ Extra White [clear, low-iron, soda-lime glass] microscope slides, VWR LLC). (Fused silica is the ideal type of glass to represent dielectrics because of its purity. Compared to fused silica, soda-lime glass could have a different breakdown threshold. However, for the purpose of testing/demonstrating the apparatus, soda-lime glass still suffices.)

A total of three shots were fired at a 1-mm-thick glass microscope slide with a single 10-µs pulsetrain (1,333 pulses), with an average pulsetrain irradiance of 1.0×10^{13} W cm^{-2} (Figure 5.5), 1.3×10^{13} W cm^{-2} (Figure 5.6), and 1.9×10^{13} W cm^{-2} (Figure 5.7).
Figure 5.5 A 1-mm-thick, low-iron, soda-lime glass microscope slide ablated with a single 10-µs pulsetrain (1,333 pulses) at an average irradiance of $1.0 \times 10^{13}$ W cm$^{-2}$: (a) Time-resolved total reflection (R), transmission (T), and inferred absorption (A), (b) Time-resolved specular and diffuse reflection, (c) pulsetrain envelope. (Only the first 230 pulses are recorded; the process was limited by the record-length of the oscilloscope.)
Figure 5.6 A 1-mm-thick, low-iron, soda-lime glass microscope slide ablated with a single 10-µs pulsetrain (1,333 pulses) at an average irradiance of $1.3 \times 10^{13}$ W cm$^{-2}$: (a) Time-resolved total reflection (R), transmission (T), and inferred absorption (A), (b) Time-resolved specular and diffuse reflection, (c) The pulsetrain envelope. (Only the first 230 pulses are recorded; the process was limited by the record-length of the oscilloscope.)
Figure 5.7 A 1-mm-thick, low-iron, soda-lime glass microscope slide ablated with a single 10-µs pulsetrain (1,333 pulses) at an average irradiance of $1.9 \times 10^{13}$ W cm$^{-2}$: (a) Time-resolved total reflection ($R$), transmission ($T$), and inferred absorption ($A$), (b) Time-resolved specular and diffuse reflection, (c) The pulsetrain envelope. (Only the first 230 pulses are recorded; the process was limited by the record-length of the oscilloscope.)
One similarity among the three cases presented (Figure 5.5, 5.6, and 5.7) is that the specular reflection fractions all commenced at ~9%. More precisely, the specular reflection fraction recorded by SRI was in fact “retro-collimated” light, primarily from the upper surface of the microscope slide, where the incident beam was focused. While both surfaces of a glass microscope slide can make specular reflection, because the lower surface is 1 mm beyond the beam focus (much longer than the Rayleigh range), much of the specular reflection from the lower surface of a defocussed gaussian beam will not be re-collimated by SRI, but instead will be collected by US and DRI. This fraction captured by SRI presumably comes partially from the contribution of a reflective plasma, because, without plasma, reflection at an air-glass interface is expected to be ~4%.

The three cases then differ in the subsequent dynamics displayed. The transmission and reflection fractions in Figure 5.5 stayed mostly constant within the record-length. In contrast, in both Figures 5.6 and 5.7, at a later point in the pulsetrain, a decrease of retro-collimated fraction was followed by an increase in the diffuse reflection. At the same time, the transmission started to increase drastically. In Figure 5.6, this transition occurred at ~500 ns after the start of the pulsetrain, whereas, in Figure 5.7, this transition took place at ~30 ns after the start of the pulsetrain.

This transition in the retro-collimated fraction and in the diffuse reflection fraction could result from a developing ablation crater. The concave surface of the crater could direct the reflection into the DRI and the US instead of the SRI, thus decreasing the retro-collimated fraction and increasing the diffuse reflection fraction detected. The drastic decrease in the transmission fraction in Figures 5.7 and 5.8 could have two possible causes. One is that the plasma created was highly absorptive; the other is that, after being scattered by the wall
of the ablation crater, part of the incident light was trapped in the microscope slide due to total internal reflection and consequently escaped from the side of the glass microscope-slide. The fact that the transition observed in Figures 5.6 and 5.7 did not occur within the record-length in Figure 5.5 could result from the ablation of the target at a lower average irradiance, so that the development of a crater was slower, as compared to the other two cases.

As demonstrated by the above test-runs, soda-lime glass and aluminum foil have distinctive ablation dynamics. Because of the time-resolving capability of the diagnostic system, rapid transitions during burst-mode ablation were captured, for example, the perforation of the foil and the development of crater on glass. Such capability is of vital importance to the investigation of burst-mode ablation.
5.6 Errors associated with using digital oscilloscopes

This section provides an analysis of the random, systematic errors introduced through the use of digital oscilloscopes in the dynamic scattering and absorption measurements.

Digital oscilloscopes have finite resolution. For a single pulse, an error associated with converting a signal from analog to digital is random, and such an error is an error of precision. One can reduce this type of error by using oscilloscopes with greater A/D converting precision and by making full use of an oscilloscope’s resolution in measurements.

In addition, digital oscilloscopes have a finite sampling rate. For measuring the peak value of a single pulse, a digital oscilloscope is expected to obtain an equal or smaller value than the actual peak value, because it is unlikely that a scope samples right at the exact location of the peak. When a train of pulses is recording, because the pulse-to-pulse separation is usually not a multiple of the sampling period, the measured peak values may have aliasing. The aliasing is an error of accuracy.

The first part of Chapter 5 characterized 1/e rise time and 1/e fall time of each integrating cavity (Table 5.1). This information permits estimation of the scale of error in measuring peak values as well as of the scale of the aliasing effect.
Figure 5.8 Illustration of how a digital oscilloscope could miss the actual peak value of a single pulse. In the worst-case scenario, the samples just before and just after the actual peak have equal value. The 1/e rise and fall time is based on the measured value of SRL, which has the shortest 1/e fall time among all integrating cavities. Figures 5.8(a) and 5.8(b) compare two sampling rates: (a) 5 GS/s, (b) 2 GS/s.
First, for each integrating cavity, one can approximate an incoming pulse by combining a gaussian type of rising edge and a gaussian type of falling edge that each match the 1/e rise and 1/e fall time, as measured in Chapter 5. The worst-case scenario, or the largest difference between measured peak value and actual peak value, happens when the sample occurring just before and the next sample just after the actual peak have the same value (see Figure 5.8). Under this approximation, I calculated the largest possible error when I was measuring the peak of a single pulse for each integrating cavity, at 2 GS/s and 5 GS/s sampling-rate respectively (Table 5.2).

Table 5.2 Accuracy of peak measurement using digital oscilloscope

(Calculated on the basis of the worst-case scenario)

<table>
<thead>
<tr>
<th></th>
<th>IEI</th>
<th>SRI</th>
<th>DRI</th>
<th>US</th>
<th>LS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/e Rise Time (ns)</td>
<td>0.8±0.7</td>
<td>0.6±0.1</td>
<td>0.9±0.1</td>
<td>1±0.1</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>1/e Fall Time (ns)</td>
<td>3.1±0.2</td>
<td>1.5±0.1</td>
<td>1.9±0.1</td>
<td>2.0±0.1</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td>Sampled/Actual Peak at 2 GS/s</td>
<td>≥ 0.984</td>
<td>≥ 0.945</td>
<td>≥ 0.969</td>
<td>≥ 0.973</td>
<td>≥ 0.964</td>
</tr>
<tr>
<td>Sampled/Actual Peak at 5 GS/s</td>
<td>≥ 0.997</td>
<td>≥ 0.991</td>
<td>≥ 0.995</td>
<td>≥ 0.996</td>
<td>≥ 0.994</td>
</tr>
</tbody>
</table>

Table 5.2 shows that a higher sampling rate results in reduction in the largest possible error in measuring peak value. At 2 GS/s, the largest possible error in measuring peak value among all channels < 6%, whereas, at 5 GS/s, the largest possible error in measuring peak value among all channels < 1%. The integration of cavities with faster temporal response is also expected to result in a larger possible error in peak value measured. Typically, the IEI, the SRI, the DRI, and the LS sampled at 5 GS/s, and the US sampled at 2 GS/s.
Figure 5.9 and Figure 5.10 show simulations of aliasing when a 100-pulse, 133-MHz repetition rate pulsetrain is recorded through the IEI at 5 GS/s (Figure 5.9) and at 2 GS/s (Figure 5.10). The simulation is set up in such a way that the first pulse of the simulated pulsetrain has the largest possible error. As shown in these two figures, when there is aliasing, the measured peak values will oscillate between the worst-case value and the actual value, due to aliasing. The pattern of aliasing is determined by a number of factors, including the sampling rate, the repetition rate of the pulsetrain, relative delays of samples with respect to the pulsetrain peaks, and the temporal responsiveness of the integrating cavity. In these measurements, the quantity of interest is the ratio between scattered/transmitted energy and incident energy. Because different channels will probably have different aliasing patterns, the ratios of interest will most likely have beating patterns (see Figure 5.11). The scale of this error resulting from the beating can also be estimated from the worst-case scenario. Typically, the IEI, the SRI, the DRI, and the LS are sampled at 5 GS/s, and the US is sampled at 2 GS/s. The relative errors of the integrating cavities sampled at 5 GS/s are in the order of 1%, and the relative error for the US sampled at 2 GS/s is in the order of 3%. In both cases, these relative errors are quite small.
Figure 5.9 Simulation of aliasing when a 100-pulse, 133-MHz pulsetrain is recorded through I EI at 5 GS/s: (a) Timing error relative to the actual peak on both the rising and falling edge of the signal, (b) Sampled/actual peak value.
Figure 5.10 Simulation of aliasing when a 100-pulse, 133-MHz pulsetrain is recorded through I/E I at 2 GS/s:
(a) Timing error relative to the actual peak on both rising and falling edge of the signal, (b) Sampled/actual peak value.
Figure 5.11 Simulation of the beating between channels. Channel A in both figures simulates the IEI sampled at 5 GS/s, and Channel B simulates (a) The SRI sampled at 5 GS/s, and (b) The US sampled at 2 GS/s, which reflects the typical setup in experiments. The initial condition used in both simulations is that the Channel A will begin with the largest possible error, and the Channel B will begin with a perfectly sampled first peak (sampled/actual peak = 1).
5.7 Attempts to measure dynamic scattering and locating damage spots on porcine tissues

In addition to glass and metal targets, dynamic scattering measurements were performed on porcine tissues. A total of 17 or 18 shots were fired on each one of three types of porcine tissues, namely, cartilage, cornea, and liver. All shots used a single 20-µs pulsetrain (2,666 pulses). Figure 5.12 shows the average absorption per pulse plotted against the average irradiance for each shot fired, and Figure 5.13 presents a few cases of these measurements. For a whole pulsetrain, the average absorption per pulse ranges from 67% to 81% (Figure 5.12). The average absorption per pulse shows no apparent difference among the three types of tissues, and there is also no apparent dependence on irradiance. Within a pulsetrain, a common feature among all the shots was that the greatest absorption within the recording length (230 pulses) typically occurred within the first 20 pulses. Because different types of tissues differ in many aspects, it is difficult to ascribe the dynamic of the absorption pattern within a pulsetrain to any particular property of the tissue. Such complication was the motivation for carrying out the work described in next chapter on simple, homogenous tissue-proxies. Doing so made it possible to characterize systematically the dynamics of absorption in a controlled environment.

Attempts were also made to perform histology on tissues with the hope of associating the absorption with the damage to tissues (porcine cartilage, in particular). However, no damage spot could be located on histological sections. It could be that the damage spots were too small to be identified or distinguished from other irregularities on the sample surface. Histological sections of cartilage in these attempts were 5-µm thick, and irregularities on the cartilage surface were of a similar scale. Thus, a crater that can be
located with certainty should exceed 15 to 20 µm in its dimensions (depth and/or diameter). The other limiting factor is that the amplifiers of the laser system require no less than four minutes of cooling time between two firings. At such a low pulsetrain-rate, it was difficult to cut tissues continuously, and thus it was possible that the histological sections could have missed the individual damage sites. For future experiments, access to a higher pulsetrain-rate laser will be desirable for the creation of identifiable damage patterns.

Figure 5.12 Average absorption per pulse plotted against average pulsetrain irradiance for each type of tissue
Figure 5.13 1- to 2-mm thick porcine tissue slice ablated with a single 20-µs pulsetrain (2,666 pulses) on the natural exterior surface. Only the first 230 pulses were recorded; the process was limited by the record-length of the oscilloscope: (a) Cartilage ablated at the average irradiance of $4.9 \times 10^{13} \text{ W cm}^{-2}$, (b) Cornea ablated at the average irradiance of $6.8 \times 10^{13} \text{ W cm}^{-2}$, (c) Liver ablated at $5.3 \times 10^{13} \text{ W cm}^{-2}$. 
5.8 Discussion and conclusion of the chapter

The energy-partition diagnostic was purpose-built to time-resolve absorption during burst-mode ultrafast-laser ablation, and this chapter illustrated its capacities. Detailed calibration showed that the diagnostic had sufficient sensitivity and temporal resolution for time-resolving pulsetrains that operated at a 133-MHz repetition rate.

As demonstrated in test-runs in Sections 5.4, 5.5, and 5.7, burst-mode ablation was a dynamic process. Reflection, transmission, and absorption underwent drastic change during the pulsetrain on a nanosecond timescale. The process of capturing such rapid change raises challenges to the previous methods of absorption measurement. While the calorimetry method used by Vorobyev and Guo [4,5] reliably provides the total (net) energy absorption of ablation by measuring the temperature of the target before and after the entire ablation process, the method does not provide time-resolved information about how absorption varies throughout the pulsetrain. Still, it should be noted that, to time-resolve the pulsetrain ablation using the pump-probe method, one has to carry out repeated trials at different time-delays between the pump and the probe pulse, assuming that the laser parameters remain identical in these different trials. This energy-partition diagnostic complements the previous methods with its capability of continuous recording at high temporal resolution, so that the dynamic reflection, scattering, and absorption can be captured. Although the device is specifically designed for pulsetrain-burst mode ultrafast lasers, it can also be applied to fuller study of the dynamics of plasma-mediated ablation in general.
Reference


Chapter 6
Dynamic Absorption and Scattering of Water and Hydrogel

This chapter reports findings using the energy-partition diagnostic to characterize the absorption dynamics of water and hydrogel during high-repetition-rate burst-mode ablation. Distilled water and different concentrations of hydrogels were used as a model for soft tissues with weak tensile strengths.

Results of the work described in Chapter 4 suggested that the tensile strength of the agar gel tissue proxy could affect characteristic physical timescales in the material, and thereby alter the dynamics of burst-mode absorption. Therefore, in this Chapter’s study, pure water and hydrogels with different tensile strengths were irradiated over a range of irradiances, and the dynamic absorption and scattering throughout the pulsetrain were determined, in order to elucidate potential relationships between the tensile strength, laser irradiance, and absorption. From these results, the roles that heat diffusion, shock wave propagation, and cavitation dynamics may play in material removal and cellular insult were evaluated.
6.1 Materials and methods

Target materials used in this Chapter’s study were distilled water, and agar gel of 1% to 4% agar solid concentration, and they were ablated at the free surface. Agar gels of different concentrations (namely 1%, 2%, 3% and 4%) were prepared by first dissolving agar powder (AGR001.500, BioShop, Burlington, Canada) in distilled water at 80°C. After the agar powder was fully dissolved, the agar solution was first left to cool to 55°C, and was then poured onto a glass microscope slide to form a 2-mm-thick slab of gel.

The targets were ablated using the burst-mode system described in Chapter 3. Non-absorbed energy fractions of the pulses were captured using the energy-partition diagnostic system described in Chapter 5. One small modification was made to the diagnostic system for the work described in this chapter; the Tektronix TDS3044B oscilloscope was replaced with a Tektronix TDS7404 oscilloscope. This change extended the maximum record-length from 2 µs to 10 µs, while the sampling rate was kept the same, at 5 GS/s. In this series, a single 10 µs pulsetrain-burst (1,333 pulses) was used for every shot, and the first 1,250 pulses were recorded.

A total of 68 shots were fired on distilled water and agar gel targets combined. For each different type of target, at least 10 shots were collected.
6.2 Experimental results

Among all the shots recorded, regardless of the target type, the total reflection fractions were comparable to the reflection at the water-air interface at low intensity (~3%), and the total reflection fraction showed little variation throughout a pulsetrain (see Figure 6.1(a) for an example). The variation in the inferred absorption throughout a pulsetrain came predominantly from the variation in the transmission fraction. As a result, the transmission fraction and the inferred absorption almost mirrored each other.

*Characteristics of absorption at the beginning of the pulsetrain*

This section considers the first 200 pulses of every shot only. The absorption at the beginning of the pulsetrain is characterized by a rapid increase of absorption within the first 20 pulses. Over 80% of all the shots fired reached the greatest absorption level within the first 20 pulses (Figure 6.2(a)). Moreover, over 80% of all shots already reached ≥ 90% of the greatest absorption level within the first eight pulses of a pulsetrain (Figure 6.2(b)); over 90% of all shots already reached ≥ 90% of the greatest absorption level within the first 16 pulses (Figure 6.2(b)).

For irradiances less than $3.0 \times 10^{12}$ W cm$^{-2}$, the peak absorption seen among the first 200 pulses absorption was a sensitive function of the irradiance, increasing sharply (Figure 6.3(a)); beyond irradiance $3.0 \times 10^{12}$ W cm$^{-2}$, peak absorption saturated, gradually increasing to ~80% at irradiance $1.5 \times 10^{13}$ W cm$^{-2}$. Type of target (pure water, or hydrogel by concentration) made no evident difference. For a comparison, the nominal breakdown thresholds of water are on the order of $10^{11}$ W cm$^{-2}$ for nanosecond pulses, on the order of
$10^{11}$ to $10^{12}$ W cm$^{-2}$ for picosecond pulses, and on the order of $10^{12}$ to $10^{13}$ W cm$^{-2}$ for femtosecond pulses [1].

To characterize the absorption during the whole pulsetrain, we first calculated the average absorption per pulse for all the shots (Figure 6.3(b)). Similar to Figure 6.3(a), average absorption per pulse throughout the whole pulsetrain increased rapidly in the regime less than $3.0 \times 10^{12}$ W cm$^{-2}$. At irradiance greater than $3.0 \times 10^{12}$ W cm$^{-2}$, however, the average absorption throughout a pulsetrain showed a large variation between shots fired at comparable average irradiance (Figure 6.3(b)), and this variation does not particularly depend on agar solid concentration.

The initial rapid increase of absorption at the beginning of the pulsetrain is often followed by complex fluctuations (see, e.g., Figure 6.1(a)). To evaluate the possible contribution of pulsetrain envelope variation in the oscillation of absorption, I calculated the correlation coefficient between the two (Figure 6.4(a)), which is defined by $\frac{\sum(x-x) (y-y)}{\sigma_x \sigma_y}$. The mean and the standard deviation of all correlation coefficients are -0.1 and 0.3, respectively (see Figure 6.4(a)).

The variation within the pulsetrain envelopes was as a result of active feedback-stabilization within the oscillator. The variations of intensities within one pulsetrain used, measured by coefficient of variation (the ratio of standard deviation and the mean), are within 13% (Figure 6.4(b)).

*Periodicity of oscillations in absorption patterns*
The pronounced fluctuations in absorption, over roughly 1–3 µs, were analyzed to identify any periodicity, any regular oscillation. Such oscillation could result, for instance, if a cavitation bubble were to be created with the initial breakdown: the range of expansion and collapse of a cavitation bubble could be significant compared to the Rayleigh range around focus (~54 µm) and therefore absorption would fluctuate as the laser was focussed into void or solid, alternating.

To characterize any patterns of oscillation in the absorptions, I calculated the autocorrelation of the time-dependent absorption (e.g., Figure 6.5(a)) for bursts shot at irradiance greater than $3.0 \times 10^{12}$ W cm$^{-2}$, where peak absorption was saturated, and absorption behaviour was most reproducible. The autocorrelation trace makes apparent a longer-time order in the absorption, a recurrence that suggests ‘ringing’. The recurrence time provides a metric for the periodicity in the absorption pattern, and the recurrence amplitude characterizes the coherence of the oscillation (Figure 6.5(a)). From such traces for a number of shots, I calculated mean periods for all shots that evidenced definite ringing, defined as more than three recurrences (Figure 6.5(b)). Notably, these appear only in the higher-tensile strength hydrogels — none of the distilled water or 1% agar gel shots show three or more cycles of oscillation within the 10-µs recording length. However, there is no clear distinction between 2% to 4% agar gels in their periods of oscillation.
Figure 6.1 Burst-mode irradiation of a 4% agar gel (single 10-µs burst, 133-MHz pulse repetition-rate, $I_{\text{avg}} = 5.0 \times 10^{12}$ W cm$^{-2}$. A total of 1,250 pulses were recorded, limited by the record-length of the oscilloscope: (a) The time-resolved total reflection ($R$), transmission ($T$), and net absorption ($A$), (b) Input pulsetrain envelope. (c) and (d) each shows the first 3 µs and 1 µs of subplot (a), respectively.
Figure 6.2 Considering only the first 200 pulses: (a) distribution by pulse number $N$ of which pulse in the burst experiences the greatest absorption, (b) distribution by pulse number $N$ of which laser pulse first surpasses 90% of the peak absorption.
Figure 6.3 (a) Peak per-pulse absorption (of first 200 pulses in the burst) as a function of irradiance (b) Average per-pulse absorption across the whole burst, as a function of irradiance. (All samples: distilled water and agar gels of different concentrations; single 10-μs burst, 133-MHz pulsetrain.) The per-pulse peak absorption reflects optical breakdown physics; the per-pulse averaged absorption reflects optical breakdown combined with subsequent ionization dynamics and hydrodynamics.
Figure 6.4 (a) the distribution of coefficients of correlation comparing the intensity of incident pulses and their absorption, for 68 burst-shots. The mean and the standard deviation of all correlation coefficients are −0.1 and 0.3, respectively. (b) stability of input pulsetrain-bursts, from the distribution of coefficients of variance of pulse irradiances. The coefficient of variance is calculated as the ratio between the standard deviation and the mean of the pulsetrain irradiance.
Figure 6.5 (a) The autocorrelation of the absorption corresponding to Figure 6.1(a). (b) Mean periods of oscillation, identified from the autocorrelation of absorption, for shots with $I_{\text{avg}} \geq 3.0 \times 10^{12} \text{ W cm}^{-2}$ and which exhibited three or more cycles of oscillation.
6.3 Discussion

*Ablation dynamics*

The rapid increase of absorption at the beginning of pulsetrains (Figures 6.1 and 6.2) could be a result of a subsequent pulse interacting with the plasma created by preceding pulses. Figure 6.3(a) implies that the absorption at the beginning of the pulsetrain sensitively depend on pulse irradiance, which reflects the nonlinear nature of LIOB. The variations from shot-to-shot in the absorption averaged over the whole burst at comparable laser irradiance (Figure 6.3(b)) could result from variations between the pulsetrain envelopes. However, the correlation coefficients between pulsetrain envelopes and corresponding absorptions only showed a weakly negative correlation between the two (Figure 6.4(a)).

Previously, Chapter 4’s work showed that the self-emission during burst-mode laser ablation from 1% agar gel targets lasted no more than 10-13 pulses [2], and the hypothesis then [2] was that a cavitation bubble formed after the initial dozen pulses of a pulsetrain, resulting in subsequent pulses focusing into a void. The present series of experiments added further evidence to this hypothesis, because the increase of transmission and the decrease of absorption after the initial 20 pulses (Figures. 6.1 and 6.2) could be explained by subsequent pulses focused into a cavitation instead of a plasma, and absorption would increase again once the cavitation bubble collapsed.

Periods of oscillation have been able to be identified among some of the shots on 2% to 4% gels (Figure 6.5). A recording length longer than the 10-μs used in the present study is required in future investigations for studying the oscillations in weaker targets such as distilled water, and 1% agar gel.
In water, the relation between maximum radius of a cavitation bubble and its oscillating period can be described by the Rayleigh model [3,4]:

$$R_{\text{max}} = \frac{T_B}{2 \times 0.915 \sqrt[3]{\frac{\rho_0}{\rho_0 - p_v}}}$$

(6.3.1)

where $R_{\text{max}}$ is the maximum radius of the bubble, $T_B$ is the bubble oscillation period, $\rho_0$ is the density of water, $p_0$ is the hydrostatic pressure, and $p_v$ is the vapor pressure inside the bubble [3]. The Rayleigh model assumes that the liquid is incompressible, and neglects viscosity and surface tension [4]. According to the Rayleigh model, a 100-µm radius cavitation bubble in water, for an example, would have an oscillating period of ~18 µs. For the cavitation bubbles inside agar gels, the oscillation period would be expected to be shorter due to the higher tensile strength than that of distilled water [5]. The collapse of the cavitation bubbles in this study would also expected to be asymmetric because the bubbles were close to the water-air interface. The collapse of a cavitation bubble near such boundary typically results in the formation of a jet of ejected material [6,7]. For future experiments, shadowgraphy or Schlieren photography should provide more details about the evolution of cavitation bubbles during ablation. Nonetheless, dynamic scattering and absorption measurements are still valuable, because these measurements point to the characteristics to look at in future investigations.

The agar gels and distilled water used in this series of experiments represent a simple model for cavitation dynamics where the elastic modulus is the only variable. Thus, so far the discussion has neglected the effect of viscosity in the cavitation dynamics. It should be noted that actual biotissues possesses both elastic and plastic properties, and the viscosity...
of the tissue often cannot be ignored in the discussion of cavitation dynamics. In biotissues, the viscous damping during cavitation bubble expansion and collapse could lead to longer oscillation period and reduced cavitation bubble size [5]. In this case, the modeling of the cavitation behaviour should consider both the elastic and the plastic properties of the biotissue used.

**Mechanisms in material removal and cellular death**

While dynamic absorption measurements alone, in this work, cannot determine the precise scale of different effects in ablation, knowledge of the absorption nonetheless allows estimation of the relative significance of different mechanisms in producing cellular damage and/or material removal.

Vogel *et al.* [3] measured how energy absorbed from a laser pulse is ultimately partitioned over different physics phenomena, following breakdown within bulk water, using single ultrafast pulses of different pulse widths and pulse energies. In the case that most closely resembles this research, when a 30-ps, 50-µJ pulse induced optical breakdown within water, 58.7% of the pulse energy was absorbed. Out of this absorbed energy, an induced shock wave subsequently accounted for 10.4% to 23.3%, and a cavitation bubble for 11.2% [3]. Vaporization accounted for 15.8%, and 14.8% of the absorbed energy was ultimately unattributed [3]. The following calculation assumes that roughly the same energy partition between different physics mechanisms applies to this chapter’s experiments, except that the cavitation bubble energy in Vogel’s work should in this case be considered as the kinetic energy coupled into both the substrate material and the ejected material, because ablation in this work started at the material surface. The total kinetic energy then accounted
for ~19% of absorbed energy. Because net momentum was zero over the ejected material and substrate material, and the mass of ejected material was much smaller than the substrate, therefore most of this kinetic energy went to the kinetic energy of ejected material. The laser operated on the order of ~10 µJ per pulse in this series of experiments. Chapter 4’s work previously determined that at this per-pulse energy, the material removal in a 1% agar gel is on the order of 10^{-3} \text{mm}^3 [2], and it was likely that the first 10-13 pulses accounted for most of the ablation [2]. Thus if the average absorption for the first 10 pulses is ~ 70%, the average velocity of 10^{-3} \text{mm}^3 ejected material would be ~170 m/s as a result.

In comparison, completely vaporizing 10^{-3} \text{mm}^3 of 1% agar gel at 20°C requires 2.6 mJ of energy, an amount that exceeds the total energy of the whole pulsetrain. Therefore, vaporization cannot be the main contributor of material removal, and much of the removed material was not vaporized.

In Vogel’s work [3], shock wave accounted for up to ~40% of absorbed energy. To estimate the shock wave pressure, I consider the case where a water or agar gel target was irradiated with a single pulsetrain-burst with a flat pulsetrain envelope, and 10-µJ per-pulse energy, and as above, assuming that absorption reached for the early pulses of a pulsetrain is ~70% (estimated from Figure 6.3(a)), then the strongest shock wave generated by the single pulse exhibiting peak absorption should contain ~2.8 µJ energy. Assuming that the pulse will generate a spherical shock wave with an exponential pressure profile [8,9]:

\[ p(t) = p_s \cdot e^{\frac{t}{t_0}}, \tag{6.3.2} \]

and the energy contained in a spherical shock wave E_s is [8,9]
\[ E_S = \frac{4\pi R^2}{\rho_0 c_0} p_s^2 \left( \frac{t_0}{2} \right), \] (6.3.3)

where \( \rho_0, c_0 \) are the density and sound speed in water, \( R \) is the distance from the irradiation spot, and \( t_0 \) is is the characteristic time for shock pressure to decay to \( 1/e \) of peak pressure. For \( t_0 \), I adopt the value estimated by Vogel et al. using the Gilmore model, that \( t_0 \) for a 30-ps, 50-\( \mu \)J pulse induced shock wave within bulk water is 20 ns [9]. It is considered that 50–100 MPa of shock wave pressure are likely to result in cellular damage [4]. From Eq. 6.3.3, one can obtain an upper bound for the shock wave damage. Assuming that there is a shock wave propagating without dissipation in agar gel or water, for a shock wave containing 2.8 \( \mu \)J energy, the damage range at 50-MPa threshold peak shock pressure would be smaller than 110 \( \mu \)m, and the damage range at 100-MPa threshold peak shock pressure would be smaller than 60 \( \mu \)m. This damage range appears to be close to the order of magnitude of cellular necrosis range previously measured in the 1% agar gel tissue proxy under similar laser conditions in Chapter 4 [2]. Therefore, the shock wave was a highly probable cause of cellular necrosis beyond focal spot during ablation.

### 6.4 Conclusion

This chapter has described a series of experiments that measured dynamic scattering during burst-mode ultra-fast laser ablation of distilled water and agar gel targets. Many features of the deduced absorptions are characterized, creating valuable insights about the dynamics of burst-mode ablation.
The experiments revealed that absorptions rapidly increased at the beginning of the pulsetrain. In over 80% of the shots in this series, the pulse within the first 200 pulses that reached the greatest absorption was within the first 20 pulses. This initial rising of absorption was followed by irregular fluctuations. The fluctuations of absorption showed a weakly negative correlation with the pulsetrain envelope. Presumably, these fluctuations were driven by the hydrodynamics of a cavitation bubble created by the initial pulses of a pulsetrain. None of the shots on distilled water or 1% agar gels showed as many as three cycles of oscillation within the 10-µs recording window. A total of eleven shots on 2% to 4% agar gels showed three or more cycles of oscillation. Such difference could be a result of the stronger tensile strengths of higher-agar-concentration gels. However, there was no clear distinction between the oscillations of those eleven 2% to 4% agar gel targets. A longer recording window than the 10-µs one used in this series of experiments would be recommended for future investigations.

From the absorption data and from existing literatures, this chapter’s work resulted in the inference that, in the burst-mode ablations of agar-gel tissue-proxy, vaporization was not the main material removal mechanism, while shock wave could be a primary cause of cellular necrosis resulting from laser irradiation.
Reference


Chapter 7

Conclusion

This thesis research project started when burst-mode ultrafast lasers were emerging as suitable candidates for the next-generation of laser surgery. By that time, in material processing, burst-mode lasers had been shown to possess all the benefits of ultrafast lasers while they also added new controls. However, knowledge was then lacking regarding how these benefits would translate to the laser surgery sphere.

This thesis research therefore set out to investigate the physics mechanisms of pulsetrain-tissue interaction and their biological effects, with a focus on soft tissues. The thesis research was carried out in two projects. One was the hydrogel tissue-proxy project, in which an agar-gel-based, 3D living-cell culture was developed as a proxy for soft-tissues, and different types of damages after pulsetrain-burst irradiation were quantified. The other project was the dynamic scattering measurements project, in which a diagnostics system was purpose-built to capture different partitions of scattered light. The diagnostics system first showcased its capacity in test-run measurements carried out on soda-lime glass, aluminum foil, and porcine tissues. Then, in a series of more systematic measurements carried on water and hydrogels, various aspects of the dynamic scattering measurements were examined, and their implications were discussed.
The following sections first summarize the technical contributions and the scientific findings of both projects, and then make recommendations about future research.

7.1 Conclusion of the hydrogel tissue-proxy project

7.1.1 Technical contributions

The hydrogel tissue-proxy was developed to tackle the technical difficulties of working with ex vivo tissues. The lack of homogeneity of differentiated tissues properties makes it difficult to quantify damages, and examination of damage often requires histological sectioning, which can be both labour intensive and costly.

The hydrogel tissue-proxy provided a solution to the above difficulties. The principles behind this tissue proxy were quite simple. The hydrophilic agar polymers formed the scaffold of the tissue proxy, providing it with structural integrity. The growth medium was mixed into the agar gel to keep the cells inside viable over the experimental period, and cells were distributed in 3D inside this tissue-proxy; they acted as damaging sensors for different damage mechanisms.

Cells inside the tissue proxy showed good viability over time. A total of 90% of the cells remained viable after six hours, and 85% of the cells remained viable after 24 hours. Protocols of necrosis/apoptosis assay and γ-H2AX antibody assay (for detecting DNA double strand breaks) were developed for this tissue proxy to label different types of damages after laser irradiation. The transparent tissue proxy allowed virtual sectioning using CFLSM, so that the damages were measured and quantified in 3D.

7.1.2 Scientific findings
The tissue-proxy was ablated with pulsetrains from 0.5-µs to 10-µs long at various irradiances. Experiments showed that the first 10 to 13 pulses accounted for most of the material removal. When irradiated with single 1-µs pulsetrains at an average pulsetrain irradiance between $0.8 \times 10^{13}$ W cm$^{-2}$ and $4.6 \times 10^{13}$ W cm$^{-2}$, the cellular necrosis range extended from 100 to 250 µm in radius, and the necrosis range scaled closely as $I^{1/2}$. The extent of cellular apoptosis was also examined six to eight hours after laser irradiation. No apparent difference in cellular apoptosis was found between the irradiated and the control group. After being irradiated with the highest-available peak-irradiance of the laser system ($1.5 \times 10^{14}$ W cm$^{-2}$), DNA double-strand breaks were not detectable above the background level. Because the γ-H2AX antibody assay relied on detection of DNA-repairing complex forming in living-cells to indicate DNA double-strand breaks, and because the minimum dosage that could be detected by this assay in the tissue-proxy was ~5Gy, the conclusion was therefore that no viable cell was detected with DNA double-strand breaks. The conclusion was also drawn that, if there were cells that received ionizing radiation of 5 Gy or greater dose during ablation, these cells did not survive the ablation.
7.2 Conclusion of the dynamic scattering measurements project

7.2.1 Technical contributions

The energy-partition diagnostic system is an adaptation of the classic double-integrating-sphere setup. The system time-resolves the scattering light during burst-mode ablation. Integrating spheres are not usually used in time-resolved measurements. With carefully designed details, the system is able to achieve sufficient temporal resolution, while it is also compatible with the ablation lens, the target translational stage, and all the previous target-translational-stage diagnostics. In this regard, the adaptation is successful. In those test-runs carried out on aluminum, soda-lime glass, and porcine tissues, the diagnostic system demonstrated that it was capable of capturing the rapid transitions in the ablation dynamics. This capability proved to be essential in investigation of burst-mode laser-material interaction.

7.2.2 Scientific findings

Systematic characterization of dynamic absorption was carried out on distilled water and agar hydrogels. This project established that, at a 133-MHz repetition-rate, the absorption first increased rapidly within the initial 20 pulses, followed by fluctuations. The greatest absorption reached within the first 200 pulses in a pulsetrain sensitively depended on pulse irradiance. In contrast, the absorptions of later pulses only had a weak negative correlation to the pulsetrain envelope. It was likely that absorptions of the later pulses were affected by the expansion and collapse of a cavitation bubble created by the first ~20 pulses. The project characterized the periodicity in the oscillation of absorptions. In general, the distilled water and 1% agar gel targets displayed longer periods of oscillation in their
absorptions compared to that of the 2% to 4% gels, but no evident difference were shown between 2% to 4% gels in their oscillation periods.

Based on the measurements of absorption, shock wave was estimated to be the primary cellular damage mechanism, while vaporization was excluded as a major material removal mechanism in the hydrogel tissue-proxy.

7.3 Recommendations regarding future research

7.3.1 Imaging cavitation, shock wave, and plume using shadowgraphy/schlieren photography

Cavitation, shock wave, and plume are three important phenomena in pulsetrain ablation dynamics. Cavitation and shock wave are two major tissue-damaging mechanisms in soft-tissue ablation, while cavitation and plume probably affect the energy deposition from a pulsetrain to a tissue. Imaging techniques, such as shadowgraphy and Schlieren photography, will provide information about how these processes evolve during and after a pulsetrain. This information will deepen the understanding of pulsetrain-ablation dynamics and cannot otherwise be obtained from the current diagnostics. Therefore, construction of a shadowgraphy/schlieren setup could be a valuable next-step for this research.

7.3.2 Tissue proxies with other scaffolding materials

Agar was the only type of scaffolding material used in tissue-proxies in the present research. One limitation of the agar-based hydrogels used in this work was that these hydrogels had limited tensile strength, and therefore they were limited to mimicking tissues
with low tensile strength. However, there are numerous other types of scaffolding materials, and certain hydrogel is even stiff enough to mimic cartilage tissue [1]. Use of stronger scaffolding material would provide insight about the difference in ablation dynamics between tissues with low and high tensile strength.

7.3.3 Desirable features in a future burst-mode laser system

The burst-mode laser system used in this thesis research has two major shortcomings. The first shortcoming is that the amplifier cooling time is too long to allow continuous tissue cutting; one of the two amplifiers requires four minutes to cool down between two shots. The other shortcoming is that there are sizable variations in the pulse irradiance envelope within a pulsetrain, and there can be sizable variations of a pulsetrain envelope from shot to shot. These factors could limit the reproducibility of results. Therefore, a high pulsetrain-rate burst-mode laser with a reproducible pulsetrain envelope is very desirable for future experiments. Other desirable features of a future burst-mode laser system may include a programmable pulsetrain envelope or real-time pulsetrain envelope feedback control (e.g., based on real-time measurement of a target’s reflectivity). These features will provide users greater control of ablation results.

Reference