Selective Inactivation of Viruses with Femtosecond Laser Pulses and its Potential Use for *in Vitro* Therapy

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ABSTRACT

Introduction: Traditional biochemical and pharmaceutical methods employed today encounter problems of clinical side effects and drug resistance, and their use is becoming limited. Therefore, it has become important and necessary to develop new, alternative strategies to combat viral diseases.

Materials and Method: A variety of viruses including M13 bacetriophage (nonenveloped ssDNA), tobacco mosaic virus (nonenveloped ssRNA), human papillomavirus (nonenveloped dsDNA) and human immunodeficiency virus (enveloped ssRNA), together with human red blood cells, Jurkat T-cells and mouse dendritic cells in their buffer solutions have been irradiated with near-infrared subpicosecond laser pulses *in vitro*.

Results: A window of laser power density, approximately between 1 GW/cm^2 and 10 GW/cm^2, has been observed that allows killing the viral particles while leaving mammalian cells unharmed.

Conclusion: The ultrashort pulsed laser technology may have great potential for disinfection of blood components.

Keywords: Viruses, femtosecond laser, selective inactivation

1. INTRODUCTION

Infectious disease remains a leading cause of suffering and death worldwide. Traditional biochemical and pharmaceutical disinfection methods employed today encounter problems of clinical side effects and drug resistance, making their use becoming more and more limited. As multi-drug resistant viruses and bacteria emerge,

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the risk of uncontrollable outbreaks rises with potentially catastrophic consequences. Several disinfection methods employing electromagnetic radiation have been employed with limited success. The ultraviolet (UV) disinfection method [1, 2] can kill viruses; however, it kills both the viruses and healthy materials such as mammalian cells. Besides, ultraviolet irradiation raises concerns of mutation. The microwave absorption technique is not effective because most of the microwave energy is transferred to the water, not to the target viral particle. A recent photochemical technique developed to disinfect blood supplies sterilizes plasma using ultraviolet A (UVA, electromagnetic radiation with wavelength between 315 nm and 400 nm) light and some psoralens (UV sensitive substance that binds permanently to DNA, therefore preventing DNA replication) [3]. Such technique is currently employed in a small scale in Europe only for non-cellular products such as Fresh Frozen Plasma. Psoralens and UV light has caused platelet activation and destruction. The use of such technology in red blood cells has failed since the penetration of UV light into a bag of red cells is very limited. Furthermore, a procedure that removes unbound psoralens from the product bag is necessary, since psoralen is toxic to the skin (causing severe sunburns and blindness in patients who receive psoralen and are exposed to natural UV light from the sun). Therefore, it has become important and necessary to develop new, alternative strategies to combat viral diseases.

In this paper, an innovative method for the selective inactivation of viruses using near-infrared subpicosecond laser pulses is reviewed [4–9]. By properly manipulating and controlling an ultrashort pulsed laser system, it has been demonstrated that selective inactivation of target viruses without causing cytotoxicity in mammalian cells is possible [7–9]. This strategy targets the mechanical (vibrational) properties of microorganisms, and thus its antimicrobial efficacy is likely unaffected by genetic mutation in the viral particles. Such a method is expected to be effective against a wide variety of viruses including the drug-resistant strains, and has broad implications in the disinfection of the blood supply in blood banks.

2. EXPERIMENTAL SETUP AND ASSAYS

2.1. Experimental Setup

Figure 1 shows the experimental setup employed to inactivate viruses with a near infrared subpicosecond fiber laser. The excitation source was a compact, ultrashort pulsed fiber laser (with seed and amplifier, Raydiance Inc., Petaluma, CA). The fiber laser, which had a wavelength of 1.55 μm, was operated at a repetition rate of 500 kHz and 5 μJ per laser pulse. The output of the second harmonic generation system of the fiber laser was used in the laser-irradiation experiments. It had a wavelength of 776 nm, about 1.4 μJ per laser pulse, a pulse width of full-width-half-maximum about 600 fs and a spectral width of about 70 cm^{-1}. Different laser power density was achieved by varying the average laser power and the size of the laser beam with an achromatic lens of long focal length. A magnetic stirrer (Corning Model PC-420) was used to stir the viral sample in its buffer solution so as to facilitate the interaction of the photons with the viral particles. For M13 bacteriophage and tobacco mosaic virus (TMV), viral particles were suspended in double-distilled water. For human papillomavirus (HPV) and human immunodeficiency virus (HIV), the viral particles were suspended in

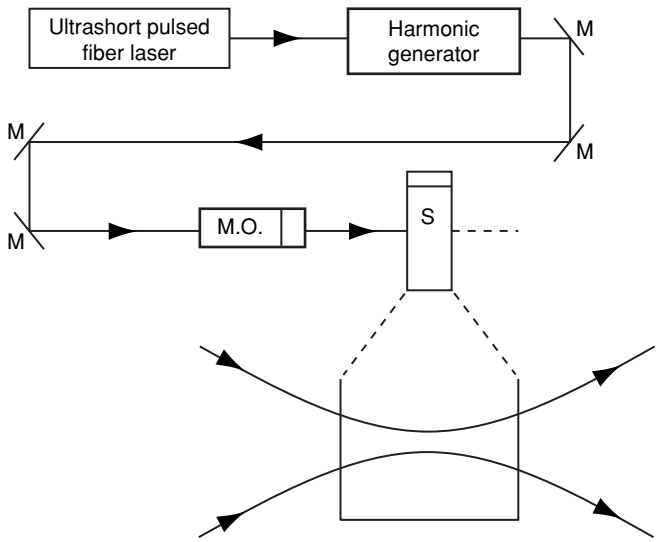

Figure 1. Experimental set up for the inactivation of viral particles with a near infrared subpicosecond fiber laser. M: mirror; M.O.: focusing lens; S: vial containing viruses in buffer solutions.

DMEM (with no phenol red). Typical absorption of the laser beam by either the solution or viruses was very minimal, as demonstrated by the measurements of laser average intensity entering and exiting the glass vial, taking into account the effects of glass vial. The typical viral concentration used was 1×10^8 cm^3. The sample volume was typically 0.1 cm^3. The duration of the laser irradiation was 2 hour in all of our experiments.

2.2. Assay for M13 Bacteriophage

Plaque forming unit (pfu) was used as an indicator for infectivity of M13 bacteriophage [10]. The VCSM13 Interference-Resistant Helper phage was purchased from Stratagene. For determining the infectivity of the helper phages from different batches, the phage was diluted to 10^3 *pfu* in 50 µl of phosphate-buffered saline (PBS) and added the diluted phage to 1 ml of TG-1 E coli growing at an OD 600 of 0.4. The E coli solution was then added into 3 ml of agarose top (10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl, 1 g $MgCl_2 \bullet 6H_2O$, 7 g agarose in 1 liter of water). After brief vortex, the mixture was poured evenly onto TYE plates (15 g Bacto-Agar, 8 g NaCl, 10 g Tryptone, 5 g Yeast Extract in 1 liter of water) and cooled down at room temperature until solidification. The plates were incubated at 37°C overnight and plaques were counted on the next day. Plaque formation assay was performed in triplicate for each batch of phage. On another assay, the phage in serial amount from 10-10^3 pfu in 50 µl of PBS was diluted and added into 1 ml of TG-1 culture followed by plating as described above. After overnight culture of TG-1 E coli with helper phage on the agars plate, the discrete plaques was labeled and counted.

2.3. Assay for HPV

HPV16 SEAP assay was performed as described in [11]. Tissue culture infective dose (TCID) was used as an indicator. Neutralization buffer was prepared by mixing DMEM without phenol red, 10% heat-inactivated FBS, 1% nonessential amino acids, and 1% penicillin–streptomycin. 293TT cells were plated 3–4 h before treatment in 96-well tissue culture-treated flat bottom plates at 30000 cells/well in 100 µl neutralization buffer. Optiprep-purified HPV16 PV having SEAP as a reporter was diluted 1000 fold. At these dilutions, target cells typically generated enough secreted alkaline phosphatase (SEAP) for an output reading. The 100-µl pseudovirus treated with the laser was transferred onto the preplated cells and incubated for 68–72 h. At the end of the incubation, 40 µl of supernatant was harvested and clarified at 1500 X *g* for 5 min. The secreted alkaline phosphotase content in the clarified supernatant was determined using *p*-nitrophenyl phosphate tablets (Sigma, St. Louis, MO) dissolved in diethanolamine, and absorbance was measured at 405 nm.

2.4. Assay for HIV

Tissue culture infective dose was used as an indicator for infectivity of HIV. HIV stocks (NL4-3, provided by National Institute of Health) were diluted to 1×10^5 cpm/ml in DMEM (with no phenol red) for the laser-irradiation experiments. U373-MAGI-$CXCR4_{CEM}$ cells (NIH AIDS Research & Reference Reagent Program) were seeded at 6×10^4 cells/well in 24-well plates. To assay the infectivity of HIV, these cells were infected with samples, either laser-irradiated or not irradiated (control), at the indicated viral concentrations. Following a 48-hour incubation, cells were fixed with a 1% paraformaldeyhde, 0.2% gluteraldehyde solution prepared in PBS. Fixed cells were washed 2X with PBS and stained with a solution containing 0.4 mM potassium ferrocyanide, 2.0 mM $MgCl_2$, and 0.4 mg/ml X-gal. Cells positive for β-galactosidase activity were counted manually (sum of 10 fields, duplicate samples).

2.5. TMV

Tobacco mosaic viruses from the infected tobacco leaves were extracted and used as the source of virus. No biological assay was performed. The atomic force microscope (AFM) images showing the release of single-stranded RNA from TMV were used as an indication of the inactivation. Once the single-stranded RNA of a TMV was released from the capsid, the virus becomes non-infectious; on the other hand, the TMV might be inactivated (due to damage caused by the laser irradiation) while the single-stranded RNA was still within the capsid. The log-kill for TMV was obtained by taking the ratio of the total number of TMV in the control to the total number of TMV subtracted by the number of single-stranded RNA observed in the laser-irradiated sample. Therefore, our log-kill result for TMV represented a lower bound for the inactivation of the virus.

2.6. Assay for Human Jurkat T-Cells and Mouse Dendritic Cells

The survival of laser-treated mammalian cells such as Jurkat T-cells and mouse dendritic cells in serum solution was measured by MTT assay [12]. The optical density (OD) was measured at 570 nm.

2.7. Survival of Red Blood Cells

In this study, the survival of laser-treated red blood cells was measured by hemolysis. Although more elaborate tests for the functionality of red blood cells are needed, as a preliminary indicator of damage, the levels of released hemoglobin from red blood cells were used. Hemolysis was determined by measuring the absorbance of hemoglobin at 413 nm in the supernatant obtained by centrifuging the suspensions.

3. EXPERIMENTAL RESULTS

3.1. Inactivation of Viruses

The results of inactivation of viruses ranging from non-pathogenic viruses such as M13 bacteriophage, tobacco mosaic virus (TMV) to pathogenic viruses like human papillomavirus (HPV) and human immunodeficiency virus (HIV) are summarized in Table 1. It has been found that the log-kill of viruses by the irradiation of the subpicosecond near-infrared fiber laser are 10^3, $10^{0.4}$, 10^5 and 10^3 for M13 bacteriophage, TMV, HPV and HIV, respectively. The laser power density used was 0.12, 1.4, 1.5 and 1.6 GW/cm^2 for M13, TMV, HPV and HIV, respectively.

Table 1. Inactivation of viruses by subpicosecond near-infrared fiber laser. For each virus, the titer measurement is indicated in the parentheses

	Log-kill of virus by the ultrashort pulesed leaser
M13 Phage (Plaque forming units)	10^3
TMV(AFM)	$10^{0.4}$
HPV(TCID)	10^5
HIV (TCID)	10^3

3.2. Atomic Force Microscope Images

Figures 2(a) and 2(b) show AFM images of M13 phage without and with laser irradiation, respectively. M13 bacteriophage is a rod-shape virus with a diameter of about 6 nm and a length of about 850 nm. Its capsid is made up of α–helix proteins assembled in a helical shape and wrapped around a single-stranded DNA. The laser power density used was 200 ± 20 MW/cm^2. The worm-like features with lighter blue color having a diameter of about 6 nm and about 850 nm in length in Fig. 2(a) reveal the presence of M13 bacteriophages in the control. Figure 2(b) shows that nearly all the worm-like features disappear and are replaced by mucus-like structures (lighter color continuous film) after laser irradiation, indicative of the destruction of the capsid of M13 bacteriophages. The intriguing implication of these AFM images is that the ultrashort laser pulse can affect the structural integrity of protein shell of a virus such as M13 bacteriophage. This result reveals valuable information on the possible mechanism(s) involved in the inactivation of viral particles by the ultrashort laser pulses to be discussed in Section 4.

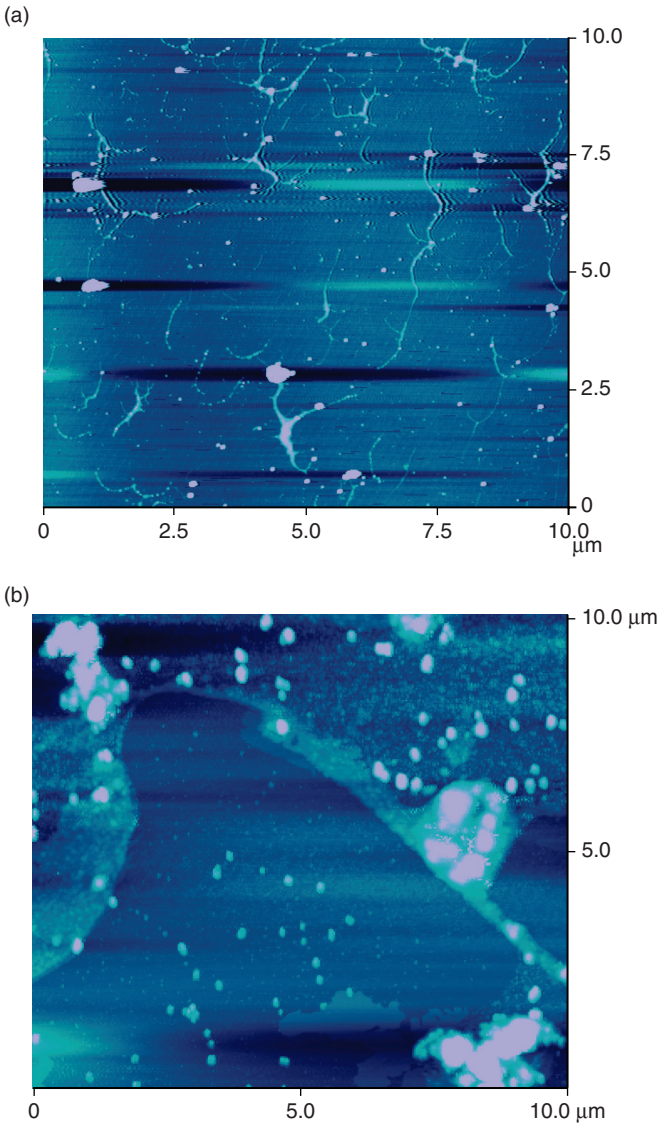

Figure 2. (a) Atomic Force Microscope picture of M13 bacterioaphage without laser irradiation (control). (b) Atomic Force Microscope picture of M13 bacterioaphage with laser irradiation by a subpicosecond near-infrared fiber laser. The worm-like features (with lighter blue color in Fig. 2(a)) having a diameter of about 6 nm and about 850 nm in length reveal the presence of M13 bacteriophages in the control. Fig. 2(b) shows that nearly all the worm-like features disappear and are replaced by mucus-like structures after laser irradiation, indicative of destruction of the capsid of M13 bacteriophages.

3.3. Selective Inactivation

Table 2 summarizes the threshold laser power density for inactivation of a variety of microorganisms including viruses, human red blood cells, human Jurkat T-cells and mouse dendritic cells. In each case, the threshold laser power density for killing of microorganisms refers to the value of laser power density at median lethal dose or LD50. Data in Table 2 show that M13 bacteriophage has an inactivation threshold laser power density of the order of 0.1 GW/cm^2, and TMV, HPV and HIV are inactivated at about 1 GW/cm^2, while much higher laser power intensities of the order of 10 GW/cm^2 are necessary to damage the mammalian cells such as human Jurkat T-cells, human red blood cells and mouse dendritic cells. These results suggest a window of laser intensity approximately between 1 GW/cm^2 and 10 GW/cm^2 that allows inactivation of unwanted microorganisms such as viruses while leaving useful materials like mammalian cells unharmed. The present experiment demonstrated that ultrashort pulsed laser may be able to selectively kill pathogens with minimal damage to sensitive materials. It is the selectivity that distinguishes our approach from currently available methods.

Table 2. Threshold laser power density for inactivation of viruses and cells

	Viruses and cells						
	M13	**TMV**	**HPV**	**HIV**	**Human Red Blood Cell**	**Human Jurkat T-cell**	**Mouse Dendritic Cell**
Threshold Laser Power Density for Inactivation (GW/cm^2)	0.06	0.85	1.0	1.1	15	22	12

3.4. Inactivation of HPV in the Whole Blood

In order to quantify the selectivity of our ultrashort pulsed laser method, experiments in which HPV was spiked in the whole blood were performed. The ratio of the inactivation rate of HPV and damage rate of red blood cells was evaluated. Under this circumstance, the selectivity has been found to be about 10^5.

3.5. Inactivation of Wild and Mutated Strains of M13 Bacteriophages

Laser experiments on wild-type M13 bacteriophages in addition to the M13 interference-resistant helper phages were also carried out. The results indicate that the threshold laser power intensities for inactivation of M13 bacteriophage and M13 interference-resistant helper phage are the same (within the experimental uncertainty). In other words, our method can not only kill the wild M13 phage but also the genetically mutated M13 phage. Such results suggest that our laser technology can overcome limitations with current therapeutics due to mutations. The novelty of our method is due to the fact that the excited coherent acoustic vibrations induced in the capsids of M13

phages by the ultrashort pulsed laser are usually of long wavelength. As a result, these excited coherent acoustic vibrations are relatively insensitive to the minor local changes such as those due to mutations.

3.6. Thermal Response

To investigate possible heating effect due to laser exposure, we measured using a thermocouple the temperature of the solution in the glass vial that contains a mixture of HPV and the whole blood before and after the laser irradiation. The results indicated that under our experimental conditions, the temperature of the solution rose no more than 2°C, suggesting that the present ultrashort pulsed laser treatment would not damage the cells and proteins by heating effects.

4. DISCUSSIONS

The specific mechanism of inactivating viral particles with ultrashort pulsed lasers is still not clear at this time. More work is required to better understand the mechanism(s) of such viral inactivation method. Nevertheless, a valuable hint is given by the AFM images for M13 bacteriophages. The AFM images in Figures 2(a) and 2(b) strongly suggest that the possible mechanism for inactivation of viruses by the near infrared subpicosecond fiber laser is concomitant with laser-induced damage of the capsid.

To our knowledge, there are three possible mechanisms in which an ultrashort pulsed laser can produce vibrational motions on either a solid state system or a biological molecular system. One possible physical mechanism of inactivation of a virus is direct excitation of vibrational Raman-active modes on the capsid of a virus by a near infrared subpicosecond fiber laser through impulsively stimulated Raman scattering (ISRS) process [4–9, 13–20]. If the amplitude of the vibrations is large enough to break the weak links (presumably the hydrogen bonds and the hydrophobic interactions) between the proteins, damage to the capsid of the virus occurs, leading to viral inactivation. In fact, in small molecules with a moderate Raman scattering cross section and sufficiently low vibrational frequency, amplitudes of atomic motion of 0.01 to 1Å can be achieved during the vibrational excitation by ISRS at an appropriate excitation power density.

Another possible mechanism is the indirect generation of vibrations on the capsid of a virus through electronic absorption of the laser radiation. Photons are first absorbed by the viral particles, causing electronic excitations to higher electronic states. The energetic electronic states relax toward the ground state by releasing energy to vibrational states on the capsid of the viral particles, damaging the capsid and leading to the inactivation.

The third possible mechanism is the generation of shock wave by the ultrashort pulsed laser. An ultrashort pulsed laser has been known [21] to produce shock wave when interacting with materials including biological molecules.

There were a few theoretical simulations of ISRS process; however, they failed to explain our experimental results on M13 bacteriophage. First of all, these calculations did not take into account the effect of wavelength on the inactivation of M13 bacteriophage. The amount of energy deposited on the M13 bacteriophage is proportional to the Raman scattering cross section of the incident laser. As a result, a possible resonance Raman effect will change the Raman scattering cross section

drastically, leading to enhanced inactivation of M13 bacteriophage through ISRS process. Secondly, these calculations only took into account the effects of a single ultrashort laser pulse. A single laser pulse from the ultrashort pulsed laser used in our experiment probably cannot generate large enough vibrational amplitude on the protein coat of a viral particle through ISRS to break the weak hydrogen bonds/hydrophobic interactions (For example, if we use Raman scattering cross section for a typical molecule and assume that the vibrational frequency of the capsid is 1GHz, the amplitude of the excitation is estimated to be on the order of 10^{-1}Å); rather, multiple ultrashort laser pulses are required to damage the capsid and to lead to the inactivation. Consider the links between the proteins of a capsid as springs of certain spring constants. The first laser pulse interacting with the capsid excites a small amplitude of vibration on the capsid. Because of the polar nature of water surrounding the capsid, the rearrangement of the polar water molecules effectively screens the hydrogen bonds and as a result, the new arrangement of surrounding water molecules slightly softens the spring constants. Therefore, the second laser pulse will increase the amplitude of the vibration on the excited capsid. The amplitude of capsid's vibration is thus further boosted for hundreds, thousands or millions of times by subsequent laser pulses depending upon the experimental conditions. In support of this mechanism, we have found that the longer the laser exposure time, the greater the log-kill of the virus. The 2-hour laser irradiation time in the present laser-irradiation experiments was chosen merely for the purpose of standardization in demonstrating our laser technology.

5. ADVANTAGES OF FEMTOSECOND LASER TECHNIQUE

5.1. Specificity

An ideal antiviral treatment should be able to selectively inactivate target viruses without cytotoxicity to "friendly" materials, such as human cells and tissues. In this regard, the ultrashort pulsed laser technique presented here is a promising approach for antiviral treatment. As presented in section 3.3, based on data in Table 2 for the threshold laser power density for inactivation of a variety of microorganisms, we have found a window of laser power density approximately between 1 GW/cm^2 and 10 GW/cm^2 in which viruses can be inactivated without causing death to mammalian cells.

5.2. Long-Term Efficacy

The ability to remain effective in the face of viral evolution is one of the most important advantages of the present ultrashort pulsed laser treatment. The vast majority of existing antiviral drugs target the biological characteristics of a specific virus. However, mutations in viral genes can profoundly affect the efficacy of these drugs. The ultrashort pulsed laser method, on the other hand, targets the intrinsic mechanical properties of viral protein capsids, and may thus be relatively insensitive or perhaps even immune to genetic mutation in the target virus. This is because low-frequency vibrational modes on the capsid of a virus excited by the laser have characteristically long wavelengths and are mostly influenced by global rather than local structure. As a result, changes in viral genetic materials are unlikely to affect the acoustic vibrational properties of the virus. As presented in section 3.5, we have demonstrated inactivation of both M13

helper phages and M13 lytic phages using the same femtosecond laser setup. This strongly suggests that different strains of the same virus are equally susceptible to ultrashort pulsed-laser-mediated inactivation. Therefore, the present method may remain effective in the face of viral evolution.

5.3. Potential of Wide Applicability

The ultrashort pulsed laser technology represents a general method that can potentially be employed to inactivate a wide range of viruses. Our data in Table 2 show that, in addition to M13 phage (nonenveloped ssDNA), ultrashort pulsed laser can inactivate TMV (nonenveloped ssRNA), HPV (nonenveloped dsDNA) and HIV (enveloped ssRNA). These encouraging results underscore the potential of wide applicability of the present technique to be effective in inactivating various types of viruses.

6. FUTURE WORKS

In the future, we plan to perform laser experiments to demonstrate a broad spectrum of high-level inactivation of other types of viruses such as nonenveloped dsRNA, enveloped dsRNA, and enveloped dsDNA, as well as viruses that have integrated into host cellular DNA/RNA. In addition, we plan to carry out laser experiments to inactivate viruses in complex biological fluids (plasma) and in the presence of cells.

7. FUTURE PERSPECTIVE

An immediate conceivable application of the femtosecond ISRS laser technique is in the disinfection of blood for transfusions. Blood safety remains a concern, especially in light of emerging pathogens such as West Nile Virus (for a review, see [22]). The ultrashort pulsed laser technique represents a potential general and non-invasive technology for pathogen inactivation, and can be useful in reducing the risk of transfusion-transmitted infection. One possible approach for the treatment of blood supply is to channel the blood through narrow tubing for laser irradiation (see Fig. 3). Alternatively, the experimental setup in Fig. 1 can be applicable if the laser intensity is substantially greater than that currently available so that the laser spot can cover the size of about 10 cm with the required laser power density to inactivate the viral particles.

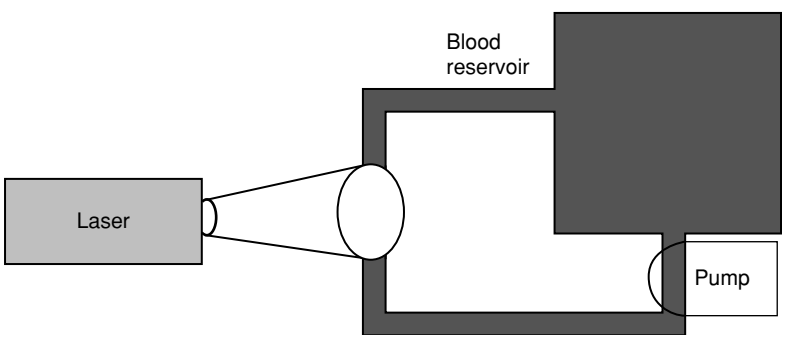

Figure 3. Potential use of femtosecond laser technique in disinfecting blood supply.

8. CONCLUSION

An innovative and revolutionary technique for selectively inactivating viruses using femtosecond laser pulses has been developed. A window of laser power density, approximately between 1 GW/cm^2 and 10 GW/cm^2, has been observed that allows killing the viral particles while leaving mammalian cells unharmed. Our technique inactivates viruses by forced mechanical vibration, and may potentially circumvent problems of pathogen resistance and clinical side effects. This new strategy may have broad implications in disinfection, and has the potential to revolutionize clinical antiviral and antibacterial treatments in the future.

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