Characterization of femtosecond-laser pulse induced cell membrane nanosurgical attachment

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Abstract: This article provides insight into the mechanism of femtosecond laser nanosurgical attachment of cells. We have demonstrated that during the attachment of two retinoblastoma cells using sub-10 femtosecond laser pulses, with 800 nm central wavelength, the phospholipid molecules of both cells hemifuse and form one shared phospholipid bilayer, at the attachment location. In order to verify the hypothesis that hemifusion takes place, transmission electron microscope images of the cell membranes of retinoblastoma cells were taken. It is shown that at the attachment interface, the two cell membranes coalesce and form one single membrane shared by both cells. Thus, further evidence is provided to support the hypothesis that laser-induced ionization process led to an ultrafast reversible destabilization of the phospholipid layer of the cellular membrane, which resulted in cross-linking of the phospholipid molecules in each membrane. This process of hemifusion occurs throughout the entire penetration depth of the femtosecond laser pulse train. Thus, the attachment between the cells takes place across a large surface area, which affirms our findings of strong physical attachment between the cells. The femtosecond laser pulse hemifusion technique can potentially provide a platform for precise molecular manipulation of cellular membranes. Manipulation of the cellular membrane is an important procedure that could aid in studying diseases such as cancer; where the expression level of plasma proteins on the cell membrane is altered.

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1. Introduction

The ability to precisely manipulate the cellular membrane without interfering with the internal structures of the cell has important implications for cellular biology research, tissue engineering, and the creation of cell-based therapeutics. The cellular membrane is made of a phospholipid bilayer, which surrounds the cytoplasm of living cells, thereby holding the contents of the cell and physically separating the intracellular components from the extracellular environment [1]. The cellular membrane is selectively permeable to material and acts as a “gateway” into and out of the cell [1]. It regulates what material can enter and exit the cells, thus facilitating transport of substances essential for the cell’s survival. Transport of material can be either passive, where the cell does not have to use-up energy for transport, or active, where the cell is required to spend energy for transport of material [1]. The cellular membrane maintains a membrane potential, allowing transport of materials with specific charges, thus acting as a filter. Physically, the cell membrane determines the shape of the cells by anchoring the cellular cytoskeleton [1]. It is also responsible for attachment of the cell to neighboring cells and to the extracellular matrix, thereby forming groups of cells which, in turn, form tissue and organs [1]. Finally, the cell membrane binds cellular structures such as the plasma membrane proteins, lipids, carbohydrates, and complex structures such as cilia [1].

The ability to manipulate these membrane structures is extremely important for cancer research. For example, the expression level of plasma proteins is significantly altered in various cancer types [2]. Increasing/decreasing plasma protein levels without modifying the internal parts of the cell would allow researchers to understand how these proteins affect the behavior of these cells, and potentially open up new doorways to the production of patient-specific cancer therapies.

A tool that can precisely manipulate the cellular membrane without destroying the integrity of the internal structure of the cell is key for studying the cellular membrane. In order to perform studies in a controlled environment, one would need to modify the cellular membrane without the introduction of external material into the cell, that is, without porating the cell during the procedure.

In recent years, femtosecond laser pulses have emerged as an enabling tool for non-invasive manipulation of living biological systems and for modifying molecules, by introducing and removing cellular material within the cellular environment. When near-infrared femtosecond laser pulses interact with tissue, multiphoton absorption of the photons takes place in the time scale corresponding to the pulse duration [3,4]. The electron density can grow exponentially leading to avalanche ionization process [3,4]. Tissue manipulation using femtosecond laser pulses takes place due to chemical process (i.e. material ionization) of the material due to the high concentration of free-electron distribution at the focal spot, and not due a thermal breakdown of the material [3,4]. Hence, this makes femtosecond laser pulses an ideal tool to precisely manipulate tissue without causing thermal damage [3,4].
Some of these applications include cell ablation [5], cell isolation [6], cell nanosurgery [6], reversible optical perforation/cell transfection [7–10], and cell-cell fusion [11]. The ability to manipulate such fine structures stems from the non-destructive characteristics of extremely short laser pulses. Femtosecond laser pulses have been shown to generate much smaller cavitation bubbles and induce much lower temperature rise in tissue compared to longer laser pulse duration [4].

Recently, we reported the application of femtosecond laser pulses to precisely manipulate the external phospholipid molecules of a cellular membrane in order to perform cell-cell surgical attachment, and neuronal nanosurgical connection [12,13]. It was hypothesized that when a femtosecond laser pulse is delivered to the contact point between the cell membranes, the high laser pulse intensity results in ultrafast reversible destabilization of the phospholipid molecules. The phospholipid molecules quickly reattach to form a single shared membrane between the cells. The proposed cell membrane attachment model is schematically depicted in Fig. 1. When the femtosecond laser pulses are delivered to the contact point between two cell membranes, as shown in Fig. 1(A), a process of multiphoton absorption and avalanche ionization generates a high density of electrons and ions, which cross the center region of the membrane and break the bonds between the fatty acid tails, as shown in Fig. 1(B). Since multiphoton absorption and avalanche ionization are nonlinear light-matter interaction processes, they occur across the location of the peak intensity of the laser focal spot. By precisely tuning the laser intensity, the non-linear interaction area can be made much smaller than the laser diffraction limited FWHM spot (i.e. the non-linear interaction occurs at the peak of the Gaussian profile laser spot which can be smaller than the actual FWHM of the focused laser beam). During the relaxation process, the phospholipid molecules reach an equilibrium state and form bonds with the closest phospholipid molecule. Due to the close proximity to the molecules that belong to the neighboring cellular membrane, some molecules cross-link and form new joint membrane, as shown in Fig. 1(C). This mechanism for cell-cell attachment is known as femtosecond laser pulse-induced hemifusion.

While in the previous work, this mechanism was hypothesized to take place, no direct confirmation was provided. In the following article, we provide further evidence to support our hypothesis and we show, via transmission electron microscopy (TEM) images of the...
contact region between two attached retinoblastoma cells, that femtosecond laser induced surgical attachment of two cell membranes occurs via phospholipid layer hemifusion.

2. Methods

2.1 Cell culture

Y79 retinoblastoma cells grown in suspension were used to confirm the hemifusion hypothesis. The cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, and 100 U/ml penicillin and 100 μg/ml streptomycin. For experimental manipulations, approximately one million cells were used.

2.2 Setup characteristics

The cellular membrane attachment was achieved by using 10 femtosecond laser pulses, at a central wavelength of 800 nm that were delivered from a Ti:Sapphire laser oscillator at a repetition rate of 80 MHz. The pulses are pre-compensated using chirped mirrors, and pulse width delivered from the laser oscillator is transform limited to 10fs as measured via autocorrelation. The ultrashort pulses were coupled to an upright Nikon Eclipse 80i optical microscope and directed towards the Y79 retinoblastoma cells. A 60 × (numerical aperture (NA) = 1) water immersion microscope objective was used to image the cells and to focus the laser beam (see Fig. 2). A laser tweezer was made collinear with the femtosecond laser pulses, in order to move cells inside the dish. The laser spot was 488 nm in radius, with a depth of focus of 2 μm. At the focal point, the laser pulse train average power, energy, and intensity were: 200 mW, 2.5 nJ/pulse, and $1.71 \times 10^{12}$ W/cm$^2$, respectively [12,13]. Irradiation time was 15 ms per pulse train.

2.3 Experimental procedure

Y79 retinoblastoma cells were placed inside a glass-bottom-dish with a 600 μm × 600 μm grid and mounted on a motorized x-y-z nano-translation stage. Individual cells selected for attachment were brought into contact using the optical tweezer beam, and attached using a 15 ms long femtosecond laser pulse train. Once the pair of cells is attached, the attachment was confirmed by inspection of the mechanical integrity of the attached joint (see Katchinskiy et al, 2014). Using the optical tweezer, a single cell was bound to the optical tweezer’s focal spot, and moved in various paths. In order to verify that physical attachment was obtained, three criteria were assessed: (1) movement of all cells in unison, (2) movement of all cells due to trapping of any cell in the group, and (3) no detachment due to twisting, and drag forces. We confirmed the physical cellular attachment by following the translation of the trapped cell as an integral unit together with the other cells without detaching from each other. After the attachment procedure was completed, the cells were fixed for TEM imaging, and the location of the desired cells on the grid was recorded.
2.4 TEM preparation

2.4.1 Reagents

1) Fixative: 2% Paraformaldehyde (Sigma) and 2.5% Glutaraldehyde (Electron Microscopy Sciences) in Phosphate Buffer (PB) for routine TEM

2) PB: 0.1 M phosphate buffer pH 7.3

3) 1% OsO₄ /0.1 M phosphate buffer

4) 1% carbohydrazide (Sigma) in ddH₂O

5) ddH₂O

6) Graded series of ethanol 30% 50% 70% 95% and 100%

2.4.2 Fixation

The culture medium was carefully removed from the culture dish, and fixative solution was added at 37°C. The cells were left at 37°C for 30 min in the fixative and then left at room temperature for an additional 40 min. The cells were rinsed with 0.1 M PB 3 times for 5 min at room temperature. Post-fixation was done in 1% OsO₄/0.1 M PB for 15 min, and then rinsed with PB 5 times for 5 min. The cells were incubated in 1% carbohydrazide solution (in H₂O) at room temperature for 10 minutes, then rinsed with ddH₂O 5 times for 5 min. The cells were incubated again in 1% OsO₄/0.1 M PB for 1 hr, then rinsed with ddH₂O 3 times for 5 min.
2.4.3 Dehydration

The cells were dehydrated with 30%, 50%, 70% EtOH for 10 min each and block stained with 1% Uranyl acetate in 70% EtOH for 30 min at room temperature. Finally, the cells were dehydrated with 95% EtOH for 5 min, and 3 times in 100% EtOH for 5 min.

2.4.4 Resin infiltration

The sample was infiltrated with Spurr’s: 100% EtOH with a ratio of 1:1 for 1 hr, then infiltrated with Spurr’s: 100% EtOH with a ratio of 3:1 for 1 hr. Finally, the sample was infiltrated with 100% Spurr’s for 1 hr. The infiltrated cells were polymerized at 65°-70°C.

2.4.5 Sectioning

The location of the cells was found based on the recorded location on the grid. The perimeter around the required cells was cut, and sectioned to 70 nm thick slices (z-slices), and mounted on TEM grid for imaging. The z-slices were taken parallel to the original plane of observation.

3. Results

TEM images of cellular membranes in a group of retinoblastoma cells that were left to naturally attach are depicted in Fig. 3. Under low magnification (Figs. 3(A), 3(B)), the outline of each cell is clearly visible. Under high magnification, as shown in Figs. 3(C) and 3(D), the membranes are easily distinguishable and separated by a 10-20 nm gap. These findings affirm that for retinoblastoma cells, at a location where the cells are left to naturally attach, the cell membranes are situated 10-20 nm apart throughout the entire length of attachment. This gap consists of the extracellular matrix which contains a cluster of molecules secreted by the cells, which provide chemical and structural support for the cells [14].

![TEM images of cellular membranes](image)

Fig. 3. TEM images of cellular membranes of a group of retinoblastoma cells that were left to naturally attach, in order of increasing magnification from A-D. A small gap of 10 to 20 nm separates between the two phospholipid cell membranes throughout the entire attachment region.

In order to demonstrate the differences in the structure of naturally attached cell membranes and the femtosecond laser-hemifused membranes, three retinoblastoma cells were brought into close proximity to each other. One cell was selected for targeting where one end of its membrane was femtosecond laser pulse hemifused to one cell and the other end was allowed to naturally attach to a different cell. These three cells are depicted in Fig. 4 under light microscopy, after the femtosecond laser pulse hemifusion procedure was completed. As...
shown in Fig. 4, cells (i) and (ii) were femtosecond laser pulse hemifused to each other, and cells (ii) and (iii) were naturally attached to each other. Note that the out of focus cell (iv) is not attached to any of the cells and is situated underneath. By performing natural attachment and femtosecond laser pulse hemifusion on the same cell, we were able to contrast the differences between the structure of naturally attached cell membranes and the femtosecond laser pulse hemifused membranes on the same cell. A key advantage of using the same cell for both natural attachment and femtosecond laser pulse hemifusion is the elimination of any structural and behavioral variations between cells. Additionally, both sites of attachments can be imaged in the same TEM slice, which guarantees the same imaging, staining and handling conditions; thus, eliminating any discrepancies that may arise from using different TEM slices or imaging conditions.

Figure 5 depicts the TEM images of the cells presented in Fig. 4. Note that only three cells are seen in Fig. 5, since cell (iv) (in Fig. 4) is located underneath the three manipulated cells and, thus, is not in the 70 nm thick TEM slice plane. A low magnification TEM image of the cells is shown in Fig. 5(A), where the two separate membranes are apparent at the site of natural attachment. The membrane structure at the natural attachment region is acutely different compared to the membrane structure of the femtosecond laser pulse hemifused region. Shown in Figs. 5(B) and 5(C) are higher magnification TEM images of two different areas of the naturally attached membrane regions, with membranes highlighted with a transparent blue line to assist the reader with locating the cell membranes. Clearly, these images reveal that the naturally attached membranes are separated by a 10-20 nm gap throughout the entire length of the contact region, indicating that each cell’s membrane remained intact and separate with its own phospholipid bilayer, as expected. However, high magnification images of the femtosecond laser pulse hemifusion site reveal that the two membranes converge to one single phospholipid bilayer. The entire region of the femtosecond laser pulse hemifusion was examined under high magnification, and the top, middle, and bottom sections are shown in Figs. 5(D), 5(E), and 5(F), respectively. In Figs. 5(D) and 5(F), the top and bottom edges of the laser induced hemifusion region are shown where the two cell membranes coalesce into a single cell membrane interface. At the central region of the hemifused membrane, hemifusion took place along most of the interface, as shown in Fig. 5(E). In some regions hemifusion did not take place (as seen in Fig. 5(E)), where gaps remain in between the two cells. This non-uniformity in hemifusion stems from the natural non-uniformity of the cellular membrane. The cell membranes are uneven surfaces that contain proteins and saccharides which prevent the cells from coming into close contact. Thus, it is possible that these sections of the cell membranes are too far apart to fuse during the application of the femtosecond laser pulses. Proteins, saccharides, or water molecules might also interfere with the fusion process by accumulating in certain sections, thereby separating
between the two membranes and not allowing the process of hemifusion to take place. Additionally, the laser intensity across the region of attachment is non-uniform due to random events such as diffraction, reflection, and varying multiphoton absorption processes. Therefore, some phospholipid molecules might not be ionized, and remain in their original bound formation. At regions where the cells were in close proximity to each other, on the other hand, the phospholipid membranes hemifuse to form a single membrane with a joint phospholipid bilayer.

While we demonstrated that hemifusion takes place during attachment of cell membranes using femtosecond laser pulses, it is also important to confirm that this process takes place throughout the entire penetration depth of the femtosecond laser pulses. In order to obtain strong adhesion between the cells, it is ideal to maintain hemifusion across a large surface area of the membrane. Therefore, we examined other TEM z-slices located along the entire penetration depth of the femtosecond laser pulses. Figure 6 depicts an exemplary z-slice of the same group of cells. Similar to Fig. 5(A), the naturally attached membranes and the laser-induced hemifused membranes are clearly identifiable in Fig. 6(A). High magnification TEM images of the edge of the femtosecond laser pulse induced hemifusion region are provided in Figs. 6(B)–6(D). Again, at the area where the cells were in close proximity to each other, the phospholipid membranes hemifused to form a single membrane with one shared phospholipid bilayer. The hemifusion process takes place at the entire contact surface along 2μm depth, supporting our previous findings that the attachment formed between the two cells is strong and is resistant to twisting and pulling [12].
4. Discussion

The cellular membrane is made of a phospholipid bilayer, which surrounds the cytoplasm of living cells. It also serves as a scaffold that houses cellular structures such as channels, proteins, and saccharides. The cellular membrane acts as the “gateway” to the cell and is extremely important for the proper functionality and behavior of the cell. Biologists and engineers could greatly benefit from a device that could precisely modify the cellular membrane without affecting the viability of the cell, in order to study the effects of such modifications on the behavior and survival of the cell. It is well-known that imaging the molecular structure of the cell membrane is challenging; meanwhile, functional and structural changes of the cellular membrane are good indicators of precise alterations done to the molecular structure of the cell membrane. In this article we focused on verifying the hypothesis that femtosecond laser–induced cell-cell surgical attachment of cell membranes occurs through the process of hemifusion. This verification indicates that using femtosecond laser pulses, one could modify the structure of the cellular membrane without affecting the cell’s viability. Our results show that cells adhere at the surface level of the cell membranes, where this attachment is molecular in its nature. It was previously reported [6–8,12,13] that at the laser intensity used to perform the femtosecond laser pulse induced membrane hemifusion, the viability of the cells is preserved.

In order to obtain TEM images of a hemifusion location it is critical to preserve the delicate cell structure during the harsh sample preparation procedure. A gridded dish was used in this experiment in order to locate the cells which the attachment procedure was performed on. The attachment is performed on one pair of cells in a dish that contains a few thousand cells. The key is to make the cells adhere strongly to the plate after the attachment is completed, and to perform the cell preparation without detaching the cells from the dish.

The hemifusion of retinoblastoma cells was induced via femtosecond laser pulses with an intensity of $1.7 \times 10^{12}$ W/cm². For retinoblastoma cells, ablation only takes place at a threshold intensity of $2.6 \times 10^{12}$ W/cm², hence, material removal does not take place at the
intensity needed for hemifusion. For femtosecond laser-induced hemifusion, operating at a regime near the threshold level for the multiphoton ionization process is crucial. Precise control of the laser intensity and aiming accuracy of $1.7 \pm 0.08 \times 10^{12}$ W/cm$^2$, and $\pm 0.5$ μm within the cell contact point are required to achieve optimum cell-cell hemifusion [12]. The error $\pm 0.08 \frac{W}{cm^2}$ is obtained from the error of the power meter and fluctuation of our neutral density filter. The error in the aiming accuracy is obtained from the error in the translation stage, and the error in the home built pulse blocker.

For femtosecond laser pulses, the exposure time is much shorter than the thermal diffusion time. Therefore, the thermal relaxation process is decoupled from the electronic excitation where energetic electrons are created locally before they can transfer their energy to the surrounding [15,16]. This physical phenomenon also explains that the process of attachment takes place due to reversible destabilization of the phospholipid molecules, and not due to thermal melting of the cell membranes.

5. Conclusion

We have provided further evidence that the mechanism behind cell-cell surgical attachment is femtosecond laser-induced hemifusion of the cellular membranes. In this manuscript we are providing a visual insight as to how does a laser-induced attached membrane appears after treatment. The images provided demonstrate that the structure of laser-induced attached membranes look substantially different than naturally attached membranes, and seem to agree with the hypothesized hemifused model. However, further evidence could shed more light on the laser-induced attachment process in the future. The laser pulse induced hemifusion takes place along the entire penetration depth of the laser pulses, thus resulting in strong physical cell attachment across a large surface area. Laser-induced avalanche ionization process leads to an ultrafast reversible destabilization of the phospholipid bilayers. During relaxation of the phospholipid molecules, the molecules seek equilibrium state and bind to the nearest free phospholipid molecule, thereby forming a joint membrane at the contact region. The procedure of laser-induced hemifusion is essentially a form of molecular surgery performed on the surface of a living cell. We envisage that other forms of femtosecond laser-induced molecular surgery could potentially serve as a tool for researchers to study and manipulate cellular membrane structures. This innovative procedure can further our knowledge on the key roles of the cellular membrane and allow scientists to develop new cell-membrane targeting drugs and treatment for, currently, incurable diseases.

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