SUB-SURFACE, FEMTOSECOND LASER INCISIONS AS A THERAPY FOR PARTIAL EPILEPSY

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Abstract

For many patients, focally initiated cortical epilepsy is largely intractable, leaving surgical treatment as a last option. Resection of the seizure focus will stop seizures but frequently leads to other neurological deficits such as stroke. A less invasive technique, multiple subpial transections (MST), uses a mechanical hook to produce a series of incisions in the epileptic tissue, with the goal of disrupting neural connections that facilitate seizure propagation. This procedure is performed manually and, as a result, cuts are difficult to control and can often lead to collateral damage. A more controlled and reliable solution to preventing seizure propagation is desired in order to surgically treat focal epilepsy with few undesired consequences. The development of femtosecond laser technology allows for extremely precise and localized cuts below the cortical surface, all while producing minimal collateral damage. Here, we propose the use of femtosecond laser pulses as a light scalpel to produce incisions around the epileptic focus and stop seizure propagation. We use a rat model of focal epilepsy in which 4-aminopyridine (4-AP), a seizure-inducing drug, is locally injected into the cortical tissue. First, femtosecond laser pulses are tightly focused into the brain to produce multiple 750 by 750 µm box cuts at depths ranging from 200 µm to 800 µm below the brain surface. Through a glass micropipette, 4-AP is injected within the cut box to induce seizures and record local field potential (LFP), while another distant electrode is implanted outside the box to record LFP. Here we show that femtosecond laser incisions are capable of blocking seizure propagation beyond the focus. These cuts also alter other seizure properties, indicating the technique’s additional effect on seizure initiation. Preliminary evidence also suggests that these cuts do not disrupt normal neural functionality. All together, this work provides strong support for femtosecond laser incisions as a precise and controlled method of preventing focal seizure propagation.
Introduction

Epilepsy, one of the most prevalent brain disorders, affects approximately 50 million people worldwide (Kramer 2012). Knowing no boundaries it can affect any age group and arise from a multitude of causes, both genetic and environmental. Epilepsy is characterized by chronic seizure activity due to a disruption of the normal excitation-inhibition balance within the neural circuitry (Ribak et al., 1979; Treiman, 2001). Classified into one of two broad groups, epilepsy is generally defined by the location of seizure onset (Benbadis, 2001). Generalized epilepsy involves seizure onset within large regions of the cortex with no single source of initiation and is most often a result of biochemical irregularities. In contrast, partial (focal) epilepsy initiates from a specific region of the cortex. Focal epilepsy is most commonly a result of malformed brain tissue, often caused by injury and resulting in cortical foci that are superficial. Antiepileptic medications are the primary source of treatment for epilepsy and can be successful, particularly in treating generalized forms (Duncan et al., 2006). Nevertheless, epilepsy is a widespread disease, and the 20% of patients for whom seizures are intractable leaves a large number that continue to suffer (NIH Consensus Development Conference Statement, 1990). For intractable partial epilepsy, surgical treatment is often the only viable option. If the focal region is well localized and far enough from critical cortical regions, resection of the affected tissue is an option. However, this is often not the case, as so many regions of the cortex are essential to critical physiological functions (Romanelli et al., 2012). Multiple subpial transections (MST) is a surgical procedure that can be used as an alternative to resection. This technique relies on the fact that seizure activity propagates along intra-layer horizontal networks in the cortex (Telfian and Conners, 1998). On the other hand, inter-layer, vertical connections are important for movement of information between cortical layers (Mountcastle, 1997; Figure 1C). MST seeks to disrupt the horizontal connections that enable
seizure propagation without interfering with the vertical connections essential to normal behavior (Morrell et al., 1989). To do this, vertical incisions (that span the width of the cerebral cortex) are made and separated by about 5mm (Figure 1A, 1B). The surgical procedure is crude, making use of a small, blunted hook in order to tear the tissue to disrupt connections (Morrell et al., 1989). Extensive acute pyknosis and tissue edema have been found adjacent to transections in histological examinations. Additionally, while studies indicate the potential success of this technique in reducing or eliminating seizures (Sawhney et al., 1995; Blount et al., 2004), there is also significant evidence for both acute and long-term neurological dysfunction as a result of these incisions (Devinsky et al., 1994; Schramm et al, 2002). Because seizures propagate typically in layers II-IV of the cortex (Rheims et al., 2008), MST potentially damages tissue uninvolved in the disease, as incisions must begin at the surface of brain tissue. Therefore, a more precise and controlled means of disrupting neural connections is desired in order to minimize collateral damage and guarantee continued functionality of the targeted region.

In this regard, femtosecond laser pulses are an ideal tool for targeted damage to brain tissue. While near-infrared light can be weakly absorbed by brain tissue (Strangman et al., 2002), the precise focusing of a femtosecond laser pulse can lead to high intensities at the focus that are capable of ablating tissue (Nishimura et al., 2006). Since the process for depositing energy is based on nonlinear absorption, with a tightly-focused femtosecond laser pulse the laser intensity can be high enough to cause damage only at the focus. This trait makes femtosecond laser pulses an extremely powerful tool for making precise, sub-surface incisions in brain tissue that will minimize collateral damage to untargeted areas (Nguyen et al., 2011; Figure 2A). In this study we make use of femtosecond laser pulses as a sophisticated improvement on MST for the surgical treatment of partial epilepsy (Figure 1D). We utilized femtosecond (fs) laser ablation to create a series of sub-
surface box cuts in the rodent cortex in order to disrupt neural connections surrounding a region of cortical tissue. Microelectrodes were then implanted inside and outside of these cuts to record perform electrophysiology while inducing seizure activity inside of the boxed region. Results indicate that femtosecond laser incisions are capable of halting propagation of seizure activity beyond the focal region. Additionally, these cuts can weaken seizures initiating within the focal region. Together, these results indicate that femtosecond laser incisions interfere with both seizure initiation and propagation, providing a potential new therapy for partial epilepsy that improves upon current surgical techniques.

**Materials and Methods**

The Institutional Animal Care and Use Committee of Cornell University approved all animal procedures. Experiments were performed on 15 male Sprague Dawley rats (Harlan Inc., South Easton, MA, USA) ranging in mass from 250g to 400g. Laser cutting experiments were performed on 13 animals and 2 animals were used for sham experiments. Rats were anesthetized using 5% isoflurane (VetOne) and maintained at 1.5-3% isoflurane. Glycopyrrolate (0.5mg/kg rat) was administered intramuscularly to prevent secretions. Body temperature was maintained at 37°C with a heating blanket and rectal thermometer (50-7053; Harvard Apparatus, Holliston, MA, USA). Heart rate and arterial oxygen saturation were monitored at all times using a pulse oximeter (MouseOx; Starr Life Sciences Corp., Oakmont, PA, USA). Subcutaneous injections of 5% glucose in saline (1mL/kg) were administered hourly for to maintain hydration of the animal.
**Surgical preparation of cranial window**

A craniotomy was performed in order to expose the brain for optical and electrophysiological recording access. Animals were restrained in a custom built stereotax. Bupivacaine (0.1mL per incision, 0.125% wt/vol in deionized water) was administered to minimize pain at the site of incision. A \( \sim 4 \times 6 \text{ mm}^2 \) portion of the skull was removed over the parietal cortex. The dura was carefully removed and the brain was kept moist with artificial cerebral spinal fluid (ACSF). An 8-mm diameter glass cover slip (50201; World Precision Instruments, Sarasota, FL, USA) was placed over the top of the craniotomy and kept in place using dental cement (Co-Oral-Ite Dental Mfg Co.). Intravenous injections of 0.3mL of 5% (wt/vol) 2-MDa fluorescein-conjugated dextran (FD2000S; Sigma) in saline were made in order to use two-photon excited fluorescence (2PEF) microscopy to visualize vasculature and determine placement of laser cuts.

**Two-photon excited fluorescence imaging of cortex for placement of femtosecond laser cuts**

Images of the cerebral cortex were obtained *in vivo* using a custom-designed 2PEF microscope utilizing a low-energy, 100 fs, 800-nm, 76-MHz repetition rate pulse train generated by a Ti:sapphire oscillator (Mira-HP; Coherent Inc., Santa Clara, CA, USA) pumped by a continuous wave diode-pumped solid state laser (Veri-V18; Coherent Inc.). Data acquisition and laser scanning were controlled using MPSCOPE software (Nguyen et al., 2006). A 0.28 numerical aperture, 4X magnification air objected (Olympus, Center Valley, PA, USA) was used in order to determine a general region for laser cuts by observing the full cranial window region. A 0.95 numerical aperture, 20X magnification, water-immersion objective (Olympus, Center Valley, PA, USA) was used in order to precisely position the animal for cuts. Using 2PEF image maps, incision locations were
determined in order to minimize interference of large surface vessels that would attenuate laser power and weaken cuts (Figure 2C, 2D).

**Femtosecond laser ablation to produce sub-surface, layer spanning cuts.**

Ablation was performed using a 50-fs, 800nm, 1-kHz pulse train produced by a Ti:sapphire regenerative amplifier (Legend 1k USP; Coherent Inc.) pumped by a Q-switched laser (Evolution 15; Coherent Inc.) and seeded by a Ti:sapphire oscillator (Chinhook Ti:sapphire laser; Kapteyn-Murnane Laboratories Inc., Boulder, CO, USA, pumped by Verdi-V6; Coherent Inc.). Animals were positioned under the laser light and moved smoothly for cutting using a translation stage. Once correctly positioned, we produced multiple 750 µm x 750 µm enclosed boxes at depths ranging from 800 µm up to 150 µm below the surface of the brain (cortical layers II-IV). Each planar box cut was separated by 10 µm in depth (Figure 2B). The tissue damage from each planar cut is three-dimensional and so the cut damage overlaps along the z plane, forming an enclosed box of cuts. In order to automate this procedure, custom Matlab software was written that coordinated the translation stages, a mechanical shutter (SH-10, Electro-Optical Products Corp.) and waveplate (05RP02-46, Newport Corp.). This software allowed the use of 2PEF images to indicate precise coordinates for cuts. The stage was translated at a speed of 50 µm/s along each side of a box, with the shutter opening during translation and closing during pauses at the corners of each box. After the completion of a full planar box, the stage was translated along the z-axis 10 µm to begin the next box. At increasing depths, with constant laser energy, the focal intensity decreases. Since we wished to maintain constant intensity across all cut depths, a rotating wave plate was correlated with the current z-axis position of the focus in order to alter incidence laser energy and maintain intensity. Laser energy during cuts was ~450 nJ/pulse at the laser focus for all experiments.
Epileptogenesis

Seizures were initiated with an injection of 4-Aminopyridine (4-AP), a potassium channel blocker, using a glass microelectrode (De la Cruz et al., 2011). At a single location, approximately 0.5 µl of 25 mM concentration was injected into the cortex using a Nanoject II (3-000-204, Drummond Scientific). These injections were performed with the electrode tip at a depth of ~350 µm below the surface.

Electrophysiological recording of seizure propagation

When cuts were finished, animals were relocated for electrophysiological recording. The cover slip was removed allowing for electrode access to the brain, which was maintained with ASCF during recording. Micromanipulators were used to hold and position two glass microelectrodes (MLN-33 and MMN-33, Narishige). One electrode was back-filled with 4-AP and implanted within the center of the box cuts to initiate focal seizures within the cut region. The second electrode was back-filled with saline and placed outside the box, ~1-2 mm away from the edge of the box cuts. Both electrodes were implanted to a depth of ~350 µm and were used to record local field potential (LFP) at the two locations (Figure 2B). With the electrodes implanted, a custom-built faraday cage was placed over the setup in order to eliminate any interfering signals during recording. Local field potentials were amplified by 1000 and signals were filtered with a low pass of 10 Hz and high pass of 1 kHz (ISO-80, World Precision Instruments). Signals were acquired at a rate of 2 kHz using an A/D DAQ board (DT9834 16-4-16, Data Translation). Custom Matlab (Mathworks) software was used to interactively display data and record signals to a text file. Typically, signal recordings were taken for approximately one hour after initial injection of 4-AP, as seizure frequency declined significantly by this time.
Electrophysiological recording of resultant hind paw stimulation

In some animals, cuts were performed in the primary somatosensory cortex (S1), specifically the hind paw region of S1. This region was roughly determined through distance measurements and controls verified our typical region identification. The procedure above was followed; however, prior to injection of 4-AP, an electrical stimulator (Model 2100, A-M Systems) was set up to apply current to the animal’s hind paw. Custom Matlab (Mathworks) code was written to control the stimulator and apply three evenly spaced bursts of 1mA current over a one second duration. During stimulation, the local field potential inside of the box region was recorded in order to determine if a neural response to stimulation occurred. Typically, ~10 individual stimuli were applied per animal, with at least a minute between each.

Data Analysis

Ictal (seizure event) onset is typically identified by the appearance of a large initial spike (Bahar et al., 2006). However, we did not consistently see this initial spike across all seizure events and therefore a different standard for determining onset times was necessary. Seizure onset was defined as the first sustained spiking that did not return to pre-ictal baseline. Similarly, seizure termination was classified as the time at which activity returned to baseline levels after onset. In both cases, baseline was defined as the typical spiking activity seen in the seconds prior to the event. Examples of determined onset and termination are illustrated in Figure 3. All seizure times were determined visually using these criteria. A seizure propagated if the same event was seen in both the focal microelectrode local field potential (LFP1) and the distant microelectrode local field potential (LFP2). A seizure did not propagate if an event seen in LFP1 was not seen in LFP2. Seizure propagation delay was determined as the difference in onset time between a seizure event in LFP1
and the corresponding event in LFP2. Seizure duration is the difference in time between seizure onset and termination. To determine the maximum amplitude spike and power of a seizure, signals were squared over the duration of the seizure and maximum amplitude found by searching through the signal for the largest individual spike. Total LFP power was calculated by integrating the area 2 standard deviations above baseline activity under the squared signal for the duration of the seizure. All comparisons between groups were performed using 2-tailed t tests. All values larger than 2 standard deviations above the mean were considered outliers and were removed for statistical comparison of groups. Differences were considered significant for p < 0.05.

**Post mortem histology**

At the conclusion of each experiment, animals were euthanized and perfused with 150 ml Phosphate Buffered Saline (PBS) followed by 150 ml 4% (wt/vol) paraformaldehyde (PFA) in PBS. Brains were extracted and post-fixed in 4% PFA for at least 24 hours. Brains were then cryoprotected using 30% (wt/vol) sucrose in PBS followed by 60% sucrose in PBS with 1% Triton X-100 (wt/vol) (T8787, Sigma), embedded in a cryomold with Optimal Cutting Temperature Compound (O.C.T. Compound, Tissue-Tek) and frozen in the cryostat’s peltier. Brains were either cut coronally or transversely in to 30-µm thick sections with a cryostat (HM 550 Microm, Thermo Scientific). Slices were then stained with 3,30- diaminobenzidine (DAB) (SK-4100, Vector Labs) to detect red blood cells and hematoxylin and eosin (H&E) to analyze tissue structure. Slides were imaged and examined using an upright microscope (BX41 with DP70 camera, Olympus).
Results

We used 2PEF microscopy to visualize the surface vasculature of the cerebral cortex in order to properly place sub-surface femtosecond laser pulses to create a series of box cuts that would neurologically isolate a region of cortical tissue. These cuts spanned layers II-IV of the cortex. These layers have been shown to be involved in seizure propagation (Telfian and Conners, 1998). Microelectrodes were implanted both within the cut region and outside of the box cuts in order to record LFP at these two sites. A potassium channel blocker, 4-AP, was injected into the cut region below the surface to act as a focal point for seizure initiation. Electrophysiological activity was recorded for both the focal and distant electrode to examine the effect of box cuts on seizure formation and propagation.

Box cuts can halt seizure propagation

We investigated the ability of femtosecond laser cuts to completely eliminate seizure propagation. We monitored the occurrence of seizure activity inside of the box cuts and whether this activity was able to spread beyond the cuts and appear at LFP2 of the distant electrode. In sham experiments where no box cuts were made, seizures were seen to propagate to the distant electrode 100% of the time (n = 2 rats; 18 seizures). When box cuts were produced, the number of seizures seen to propagate was significantly reduced, with 53.76% of seizures reaching the distant electrode (n = 13 rats; 90 seizures; p < 0.0005; Figure 4). We encountered a large degree of variability in propagation on a per animal basis. In some animals there was no propagation for all seizures seen in that animal while in other animals there was propagation seen for all seizures. Additionally, there were also many animals in which some seizures were blocked and others propagated.
**Box cuts delay propagation of seizures but do not reduce seizure power**

We further determined if laser cuts had any effect on other aspects of seizure properties for those seizures that were able spread outside of the focal region. We compared ictal onset times of corresponding seizures in LFP1 and LFP2 for shams and for those instances where seizures propagated after laser cuts in order to determine if cuts delayed arrival of seizure activity at LFP2. For sham experiments, mean seizure delay was found to be 0.34 ± 0.09 seconds (n = 15 seizures). Cuts significantly increased the mean delay of seizure onset to 9.5 ± 1.99 seconds (n = 49 seizures; p < 0.02; Figure 5A). To determine if the box cuts decreased the power of the seizure when it reached LFP2, we calculated the normalized power difference between LFP1 and LFP2:

\[
\frac{\sum LFP1 - \sum LFP2}{\sum LFP2}
\]

The mean normalized power difference in controls was 8.39 ± 3.49 (n = 17 seizures). If cuts were limiting seizure power, we would expect this value to be significantly larger when box cuts were made. However, we found that the mean power was significantly lower for seizures that propagated outside of cuts, 2.50 ± 0.59 (n = 47 seizures).

**Box cuts result in modified seizure attributes**

In quantifying seizure propagation between LFP1 and LFP2, distinct differences in seizure form were observed at LFP1 for those seizures that propagated outside of cuts versus those that did not propagate. In particular, propagating seizures appeared to be similar in strength to seizures observed during control studies, while non-propagating seizures were often much weaker, taking the form of “small-scale” seizures (Figure 3). These observations were quantified through analysis of power, maximum spike amplitude and duration for seizures at LFP1. We separated seizures into three
groups, seizures when no cuts were made (control), seizures that propagated after cuts were made and seizures that did not propagate after cuts. The mean power of seizures at LFP1 for control and propagating seizures did not differ significantly with means of $9.48 \pm 0.981 \text{ mV}^2\text{s}$ ($n = 18$ seizures) and $8.75 \pm 0.675 \text{ mV}^2\text{s}$ ($n= 48$ seizures) respectively. The power of non-propagating seizures differed significantly from both control seizures ($p < 1.0E-11$) and propagating seizures ($p < 1.0E-13$) having a much weaker mean power of $1.54 \pm 0.409 \text{ mV}^2\text{s}$ ($n = 42$; Figure 5B). We found the maximum amplitude spikes for each seizure and used this as an indication of how strong individual spiking was in each ictal event. The mean maximum amplitude was $14.50 \pm 1.492 \text{ mV}^2$ ($n = 17$ seizures) for controls, $4.37 \pm 0.260 \text{ mV}^2$ ($n = 48$ seizures) for propagating seizures and $1.25 \pm 0.235 \text{ mV}^2$ ($n = 41$ seizures) for non-propagating seizures (Figure 5C). We found all groups differed significantly from each other. Both propagating and non-propagating seizures involved spike amplitudes significantly smaller than those seen in control seizures ($p < 1.0E-14$ and $p < 1.0E-17$ respectively), indicating that box cuts are correlated with the reduction in spike amplitudes. Analysis of spike amplitude also emphasized a difference between propagating and non-propagating seizures in which non-propagating events had significantly lower amplitude spiking ($p < 1.0E-12$). Finally, we looked at seizure duration for comparison between groups. As expected, the “small-scale” non-propagating seizures had significantly shorter durations ($36.13 \pm 3.058 \text{ s}$) than both control ($48.02 \pm 3.383 \text{ s}; p < 0.05$) and propagating ($100.1 \pm 5.198 \text{ s}; p < 1.0E-15$) seizures (Figure 5D). Unexpectedly, seizures that propagated beyond the cuts were of a significantly longer duration than seizures seen during control studies ($p < 1.0E-6$).
**Histological Verification of Box Cuts**

Histology was performed in order to verify that full, sub-surface box cuts were made using the cutting procedure. Transverse sections illustrate that the intended box cuts were produced and coronal sections illustrate the continuous vertical nature of the series of box cuts (Figure 6A, 6B). The coronal slice shows cell density near to the cuts is high relative to tissue farther from the cuts, indicating that tissue is being compressed near the incisions.

**Sensory signal arrival is preserved after cuts**

Preliminary results using rat hind paw stimulation indicate that box cuts do not hinder standard neural function in the primary somatosensory cortex with regards to sensory signal arrival, a motivation for using the precision of femtosecond laser pulses to make these cuts. After laser incisions were made in the hind paw region of S1, sets of electrical current were applied to the animal’s hind paw while recording neural activity within the cut region. The LFP during stimulation was examined visually for a typical neural spike response to each application of current (Figure 7A). For each set of stimulation, we classified whether a neural spike occurred or did not occur in response to the first application of current in that set. In control studies where LFP was recorded from the hind paw S1 region without making box cuts, we saw a neural response to 96% of stimulations (n = 26 stimulation sets). This verified the determined location of the hind paw region. In recordings after making box cuts within the hind paw region, we saw 100% neural response to stimulations (n = 20 stimulation sets; Figure 7B).
**Discussion**

Multiple subpial transections have been shown to be capable of reducing and preventing seizures in a reasonable percentage of patients. Success rates for the technique range from 50-86%, with typically around 50% of patients becoming completely seizure free (Schramm et al., 2002; Morrell et al., 1989; Vaz et al., 2008; Blount et al., 2004). While the technique is effective, it has the potential to cause tissue damage to untargeted areas, such as ~2-3mm of tissue along the sides of the transections. Additionally, all incisions must begin at the brain surface, despite the location of targeted tissue in sub-surface cortical layers.

Femtosecond lasers are already being utilized in a surgical setting. In particular, these lasers are beginning to gain popularity in the field of ophthalmology for corneal surgery and have led to improvement in traditional outcomes (Farid and Steinert, 2010). This work utilizes femtosecond lasers as an improvement over current techniques for surgical treatment of partial epilepsy and shows that using femtosecond laser pulses as a “laser scalpel” are capable of preventing seizure propagation beyond a focal region. Femtosecond incisions provide a level of precision not obtainable from a manual technique, as well as the ability to selectively target sub-surface tissue with no damage to overlying regions (Nguyen et al., 2011). Cuts involved using laser energies approximately four times greater than what is needed to cause optical breakdown of brain tissue. This was done in order to ensure overlap of each planar box cut in the z plane, as lower energies may have left vertical gaps in the cuts as a result of the 10 µm step size between cutting planes. Increasing laser energy rather than decreasing step size was preferred because a decrease in step size would result in a longer cutting period. Cutting time with the parameters used for these studies was already ~65 minutes and we did not wish to prolong the period that the rodent was under anesthesia. Additionally, higher energies better compensated for laser attenuation due to large surface vessels.
Absorption and light scattering by blood vessels within the path of cuts can attenuate laser energy (Horecker, 1943) and a large enough vessel may disrupt the success of deeper cuts. Both increasing laser energy and actively avoiding large vessels when choosing a cut location prevented this issue. Use of such high energies may other cause complications, such as the formation of a cavitation bubble that is capable of disrupting tissues outside of the focal region (Vogel et al., 2005). In our post-mortem histology we observed a compression of tissue nearby the cuts, perhaps as a result of the higher energies that were used. Histological slices also show an increase in cut width towards the surface of the tissue, likely due to both imperfect calibration of the waveplate and z-stage as well as the use of high laser energy. In an effort to avoid issues related to the use of high energy, future work should investigate the minimum laser energies necessary for cuts that still result in seizure disruption.

Femtosecond laser incisions were able to prevent seizure propagation beyond a focal region in almost 50% of occurring ictal events. This success rate is extremely promising for the future of this optically based surgical method for partial epilepsy, as MST itself does not perfectly disrupt seizures in all patients. Nevertheless, we must question why we were unsuccessful in preventing seizure propagation in all cases. While most seizures that were blocked from propagating were “small-scale” in nature compared with control seizures, the box cuts were also able to stop seizures with powers more comparable to controls (Figure 5b). This suggests that the ability of cuts to block propagation does not solely rely on a seizure being weak. Instead, it seems likely that successful cuts limited the ability of the focal region to produce normal seizures; the cuts to some degree disrupt seizure initiation. This disruption is not necessarily reducing the occurrence of seizures but rather limiting the maximum strength of a seizure forming within the focus. Given that these cuts were associated with limited seizure power, it follows that they were also able to contribute towards
preventing seizure propagation as observed. In instances where propagation occurred, seizure power was comparable to controls, indicating that cuts were both unable to limit seizure power and unable to stop propagation. Despite no change in power, the maximum spike amplitudes and durations were significantly altered from controls even in unsuccessful cuts. Thus, even cuts that are unable to stop propagation still have some effect on seizure attributes. In these cases, cuts may have been incomplete or weaker than necessary to completely treat focal seizures. The lack of a 100% success rate may not be an indication of a limitation of the method itself but may rather be the result of procedural issues encountered during experiments. The complex nature of these experiments left room for many unintended issues that may ultimately have affected success of cutting. A number of issues may have disrupted incisions such as laser stability, unavoidable vessels in the path of cuts, variations in laser energy or unexpected animal movement during cutting. Future work should be aimed at optimizing these methods in order to achieve a higher success rate for preventing the propagation of seizure activity. Despite apparent inconsistencies in laser cutting, we were still able to achieve prevention of seizure propagation that rivals the success of MST.

We observed a significant delay in seizure propagation for seizures that were able to escape the box cuts and reach the distant electrode compared with controls. This demonstrates that even unsuccessful cuts were at least partially effective in disrupting propagation. As opposed to propagating along many paths to reach distant tissue, with cuts, seizures likely were forced to follow a subset of these paths dependent upon where cuts were less effective. This possibility explains the propagation delays seen while performing electrophysiology, where seizures could not immediately spread to the distant electrode but were either held up as they were “escaping” through the box or escaped but required time to propagate around the box to the side with the second electrode. While this is a straightforward explanation, our preliminary work had already indicated the ability of these
cuts to delay seizures as they travel around the damage. In future work, it may be possible to “plug” these holes in the cuts by creating two box cuts around the focus, one enclosed within the other. This would introduce redundancy in order to compensate for any ineffective regions in one of the cuts. Unexpected results were obtained with regards to the duration of seizures that eventually propagated beyond the box. In these instances, seizure durations were longer than controls. We expected these durations to either remain the same (as seizure power did) or to decrease (as spike amplitude did). This finding may be indicative of a mechanism through which box cuts are contributing to an increase in seizure duration. This also explains why we observed decreases in maximum spike amplitudes between controls and propagating seizures but these two groups showed similar seizure powers; the differences in seizure duration made up for the differences in amplitude. We can speculate that the cuts are working to contain the seizures within the focal region and in doing so, aid in the continued perpetuation of the seizure activity. Without box cuts, the seizure activity is more easily able to spread outward and ultimately dissipate; but with cuts, this is no longer possible and the activity is mostly trapped within the box. While the seizures ultimately still propagate, a large portion of the seizure focus is isolated and this helps to perpetuate the seizure, leading to longer durations within the box. This result may have implications for studying seizure dynamics and it would be interesting in future work to focus studies on better understanding this result.

Importantly, preliminary evidence suggests that these cuts preserve tested aspects of normal neural functionality at the focal region. As discussed, the essential feature of both MST and this work is that vertical incisions leave important functional connections intact within the cortex. Our results from hind paw stimulation indicate that femtosecond laser incisions can preserve
functionality, as we would expect given the increased precision of the technique in comparison with MST.

It is necessary that future work investigate the effectiveness of femtosecond laser incisions in a chronic setting. We show these cuts are effective acutely for interrupting seizure activity but chronic effects may vary from our observations. The effects of these cuts, including disruption of the blood brain barrier and parenchymal hemorrhage, have been implicated in the acute development of seizures (Herman, 2002; Faught et al., 1989). Additionally, late development of seizures after brain injury has been observed and attributed to remodeling of neural connections (Dichter, 1997). On the other hand, inflammatory responses to injury can result in a glial scar which is capable of preventing formation of new networks, in effect disrupting the mechanism above for late term seizure development (Silver and Miller, 2004). This was likely the case in an analysis of patients who specifically formed glial scars in tissue after MST, where all were either seizure free or had a 95% reduction in seizure frequency (Kaufmann et al., 1996). Ultimately, chronic studies will play an important role in determining the applicability of this technique for the clinical setting.

Should this method continue to prove successful in chronic work, its implications for future clinical treatment of partial epilepsy would be significant. The mechanically based MST faces significant disadvantages that a femtosecond laser based surgical method can circumvent. Particularly, sub-surface foci can be targeted without doing any damage to overlying tissue. The precision of laser-based cuts would minimize damage to untargeted tissue and allow for access to areas too delicate to disrupt manually. One of the most difficult challenges facing an optical therapy is a limitation on penetration depth of the beam. Brain tissue scatters light and this results in an exponential decrease in focal energy with increasing tissue depths (Helmchen and Denk, 2005). Using 800 nm light, as in this study, cutting depth could potentially reach ~2 mm. Longer
wavelengths would allow for less scattering and deeper cuts. A 1300 nm light source can theoretically allow for cuts up to 4.8 mm in depth (Nguyen et al., 2011). The use of such a high wavelength has the potential to create additional issues such as excessive heat deposit from laser energy on non-targeted tissue. Furthermore, techniques for altering power based on vasculature may be necessary to ensure constant cutting power at the focus. It is important to remember that this technique has cortical foci in mind and thus would not be required to reach tissue more than a few millimeters below the surface. Ultimately, a clinical implementation of this technique would require technological developments that increase laser stability while allowing for constant high-energy deposit deep within brain tissue. Although there is much work to be done before reaching a clinical setting, the data presented here provides very strong evidence for the use of femtosecond laser incisions as a therapy for partial epilepsy.
Figure 1 Multiple subpial transections (MST) as a current therapy for partial epilepsy takes advantage of seizure propagation mechanics but is an imprecise technique. 

A) Volumetric representation of MST extending from the cortical surface into the tissue. 

B) A cross-sectional representation. Cuts are spaced approximately 5 mm apart. Images from Kauffman et. al, 1996. 

C) While normal neural activity important for function primarily travels via vertical pathways, seizure propagation is mostly horizontal, providing a means of disrupting propagation without harming functionality. 

D) Coronal brain slices showing the capability of sub-surface femtosecond laser ablation. Cuts were produced ~300 μm below brain surface with a single cut made at ~700 μm depth. Slices show clear ablation damage in regions targeted by the laser focus with no effect on surrounding and overlying tissue.
Figure 2 Femtosecond lasers are used to create three-dimensional box cuts within the cortex and electrophysiology is performed using two microelectrodes. A) Low magnification two-photon image projections of surface vasculature. The purple box represents the location of the box cuts while the red and blue dots represent location of electrodes for electrophysiology. B) High magnification of the same cut region. C) Planar box cuts spaced 10 μm apart, each 750 x 750 μm, span cortical depths from 150 μm to 800 μm. D) Electrodes are implanted into the cortex 350 μm deep. An electrode filled with 4-AP is placed inside of the box cuts while a distant electrode is placed outside of the cuts.
**Figure 3** LFP recording examples from different groups.  
**A** A control animal in which no box cuts were made.  
**B** An animal in which box cuts were made but seizures propagated. Note that we see no clear pre-ictal spike events here.  
**C** An animal in which box cuts prevented seizure propagation. Red boxes indicate the region that is magnified in the right column for comparison of seizure forms and determination of onset and termination. Within the magnified regions, purple arrows indicate the time that was considered seizure onset while blue arrows indicate seizure termination.
Figure 4 Laser cuts are able to halt seizure propagation. In controls, all seizures propagated to the distant electrode (n=18 seizures). When cuts were produced, 46.24% of seizures did not propagate to the distant electrode (n=90). Cuts significantly reduce the ability of seizures to propagate beyond the focal region (p < 0.0005). Error bars were calculated using binary response statistics.
Figure 5 Laser cuts are able to delay seizure propagation and alter the formation of seizures at the focus. A) The delay in seizure arrival at the distant electrode is significantly longer for seizures that propagate after cuts than for control seizures. B) Seizure power at LFP 1 is similar for control seizures and seizures which propagate after cuts; however, seizures which did not propagate after cuts show a significant reduction in seizure power at the focus. C) Maximum spike amplitude during seizures was significantly reduced by laser cuts for all seizures. Additionally, non-propagating seizures after laser cuts show a significant reduction in amplitude compared to seizures that did propagate after cuts. D) Seizures that propagated after cuts unexpectedly showed a significant increase in duration at the focus compared with controls. Non-propagating seizures were significantly shorter in duration than controls. For all box plots, the red and black lines indicate the median and mean respectively. Black circles show individual data points and cross hairs show statistical outliers that were excluded in calculation of the mean. (*p < 0.05; #p < 1.0E-11; ##p < 1.0E-7).
Figure 6 Histology verifies the formation of box cuts using laser ablation. A) Coronal section showing the full vertical cuts produced. Note the increase in cut width towards the surface as discussed in the results section. B) Transverse section showing planar box cut. Variations in cut width within the plane were most likely the result of differing vascular topologies in the regions above the plane, altering laser energies due to attenuation. Slices were stained with H&E and DAB.
Figure 7 Hind paw stimulation indicates that normal function is preserved within the region of the box cuts. A) The electrophysiology of a single stimulation set occurring over the duration of one second. Neural response can be seen for each round of 1mA current applied to the rodent’s hind paw. The decrease in spike amplitude is a result of the rapid succession of stimulations. B) Animals with and without cuts did not differ significantly in their neural response to hind paw stimulation, indicating that cuts do not affect function.
References


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