final report

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Evaluation of microwave energy as a humane stunning technique based on electroencephalography (EEG) of anesthetized cattle

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Executive summary:

Humane slaughter implies that an animal experiences minimal pain and distress before it is killed. Stunning is commonly used to induce insensibility. However, current stunning techniques can lead to variable results or are considered unsatisfactory by some religious groups. Alternative stunning techniques that are quick, humane and acceptable by all are needed. Microwave technology can induce loss of consciousness in rats. High power equipment has now been developed that can focus the energy to produce a rapid rise in temperature in cattle brains (Patent number PCT/AU2011/000527). It is expected that rising brain temperature will stop brain function and result in reversible insensibility.

We investigated the effectiveness of different settings for microwave delivery, power and duration, on anesthetized cows to induce insensibility. We used quantitative electroencephalograpic (EEG) analyses under halothane anaesthesia to assess alterations in brain function that would be indicative of loss of consciousness in awake animals. Insensibility was assessed in this study by the appearance of seizure-like complexes in the EEG. Power and duration were tested on a stop-go basis to minimize animal use.

All applications resulted in EEG changes indicative of seizure-like activity, an EEG pattern considered incompatible with awareness. We imposed 5 combinations of 3 different powers and 4 different durations. Shorter duration of application resulted in more rapidly developing EEG changes, with both shorter time of onset of EEG suppression (as soon as 3 sec) and shorter time to nadir of EEG suppression. Higher power resulted in a longer duration of EEG suppression, at least 37 sec and up to 162 sec.

Transient bradychardia (average -29%) was observed between 5 and 30 sec post-delivery.

Post-mortem autopsies revealed that most histological changes occurred adjacent to the zone of application whereas deeper brain regions showed small to no changes. These preliminary findings indicate that, as soon as 3 sec after the end of the microwave application, animals would attain insensibility based on the appearance of seizure-like complexes in the EEG. Recommendations on future research are included after the Discussion section.

Contents

Introduction

Humane slaughter implies minimal pain and distress on an animal before it is killed. Various factors can influence pain, fear and distress in abattoir settings including: the previous experience and breed of the animal; the facilities' design (Grandin, 1990); handling techniques (Hemsworth et al., 2011); and the slaughter method used (Anil, 2012). In Western countries, stunning of the animal is a legislative requirement mandated to induce insensibility and thus ensure that the animal does not feel pain. Different stunning techniques can be used depending on factors such as the species and age of the animal, practicality, animal and worker safety and economical considerations. Some stunning techniques qualify as reversible, with the animal able to recover from the process (e.g. electrical stun), whereas others are irreversible if performed correctly (e.g. captive-bolt stun). However, current stunning techniques can lead to variable results (Gouveia et al., 2009), hence the search for novel stunning techniques or alternatives.

Halal and Kosher meat production requires that animals being processed for human consumption are healthy and uninjured at the time of slaughter. As a result, many of the methods of stunning used in modern commercial abattoirs are not acceptable as the animals are considered injured by the stunning process and the animals cannot recover from stunning. The current standards around slaughter in Australia (AS 4696) require that an animal is rendered unconscious prior to slaughter and remains so until death (Sub-clause 7.10). However, ritual slaughter without prior stunning is enabled under AQIS exemptions where a specific notice for a practice is issued under their licence. This means that for these animals, no prior stunning is required before slaughter.

The pain that an animal experiences at slaughter is central to the legislative requirement to stun in Western countries. Pain is difficult to study because it is an inherently subjective experience. While humans can report pain, only indirect indices of pain are available for use in animals. Furthermore, many of the traditional behavioural and physiological indices that have been used to study pain are also measures of non-painful stressors. For example, measures such as hormone response (e.g. catecholamines, glucocorticoids) and behaviour are not specific to pain. However, neurophysiological tools are now widely used in humans to assess pain. Studies in humans experiencing pain have demonstrated that in contrast to the more traditional physiological measures, electroencephalographic (EEG) data correlate well with subjective evaluations of pain, indicating the value of quantitative EEG analysis as an indicator of the degree of pain perceived by humans. Recently, neurophysiological responses of animals, recorded by EEG have been shown to provide valuable insights into the perception of pain by animals (Murrell and Johnson, 2006), and specifically that slaughter by ventral-neck incision without prior stunning is painful, based on EEG changes (Gibson et al., 2009a). Furthermore, this practice is likely to be of more concern in cattle than other species such as smaller ovine species (sheep, goats), due to the longer bleeding times required in cattle before loss of consciousness occurs (Newhook and Blackmore, 1982; Gregory et al., 2010). Hence, an important welfare consideration in slaughter is that the animal is insensible at the time of the ventral-neck incision. Alternative stunning techniques that are quick, humane and acceptable by all are needed.

Microwave application has been reported to induce loss of consciousness when applied to conscious rats, causing petit or grand mal seizures for 1 min after exposure and an unconscious state for the following 4 to 5 min with the animal ultimately recovering (Guy and Chow, 1982; Lambooy et al., 1989). However, Lambooy et al. (1989) deemed this technique unsuitable for pigs at that time, partly because of the capacity of the microwave generator being too low to deliver sufficient power. In recent years, microwave technology has developed to the point that high power equipment is available that can focus the energy to produce a rapid rise in temperature in cattle brains (Patent number: PCT/AU2011/000527). It is expected that raising the brain temperature will stop brain function and result in insensibility, whilst still allowing the animal to regain consciousness after a period of time. Consequently, this may allow for a recoverable insensibility acceptable for religious slaughter since it would not 'physically injured' the animal (James Ralph, personal communication).

The aim of this project was to investigate the effectiveness of different settings of the microwave technique, power and duration of application, on anesthetized cows to induce insensibility. Insensibility was assessed by the appearance of seizure-like complexes in the electroencephalogram (EEG). All animals in this project were kept under anaesthesia for the whole procedure. The low anesthetic model has been previously validated as a suitable model to investigate the effects of noxious stimuli (Gibson et al., 2007).

Materials and methods

Animal welfare and ethical considerations

The project was approved by the University of Melbourne Ethics Committee (approval number 1212620.1) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All animals used in this project were anaesthetized prior to being subjected to the experimental treatment and kept under anaesthesia for the whole procedure. Hence, it allowed us to study the effects of microwave application without inflicting any pain or distress to the animal. The animals were never given the opportunity to regain consciousness and were ultimately humanely euthanized by lethal barbital overdose. The different settings of microwave delivery, that is, power and duration, were tested based on a stop-go basis to minimize animal use.

Animals

Ten crossbred female cows were used over a total of 6 days. The animals were sourced from the daily intake of the abattoir and originally intended to be slaughtered for meat consumption, with a body weight estimated around 180 kg. Food was withdrawn for 24 hours and water was withdrawn overnight prior to the procedure to avoid regurgitation during induction of anaesthesia. In order to minimize handling stress, no electric prods were utilised when moving the animals.

General handling and anaesthesia procedures

All animals were held in lairage and handled in a similar manner. At the time of treatment, the designated animal was moved from the lairage area through a single chute race and into a restraining box. The head of the animal was restrained and anaesthesia was induced and maintained following a previously validated procedure for cattle (Gibson et al., 2007), by administrating the anaesthetic agent (a mixture of 3.4 mg/kg ketamine and 4.1 mg/kg propofol) into the jugular vein of the animal. Once the animal was anaesthetised, as indicated by loss of posture, the side of the box was opened and the animal rolled into lateral recumbency. Endotracheal intubation was carried out by a veterinarian by advancing the endotracheal tube to the rima glottidis and confirming correct placement by palpation as the tube advanced into the trachea. This is a standard clinical technique for the intubation of adult cattle. Once intubated, the animal was placed in dorsal recumbency onto a specifically designed V-restrainer rolling crate. The palpebral reflex was continuously monitored and additional propofol immediately administered if the animal showed signs of recovery from the anaesthetic. The animal on the rolling crate was finally moved to an adjacent room 15 m away and the endotracheal tube connected to the anaesthetic machine delivering halothane in oxygen via a circle breathing system using standard clinical flow rates and vapouriser settings. The animal was allowed to breathe spontaneously throughout the experiment. End-tidal halothane tension was maintained at 0.9%. Patient stability and depth of anaesthesia was monitored throughout the procedure using an anaesthetic agent monitor (Cardell® Veterinary Monitor Max-12HDim multiparameter monitor), recording the end-tidal carbon dioxide

tension, end-tidal halothane tension, respiratory rate and heart rate every 5 minutes throughout the anaesthetic procedure. The corneal reflex was also monitored every 5 minutes, by touching the side of the eye. Anaesthesia under halothane was maintained for at least 30 min to allow for the effects of the anaesthetic induction agents (ketamine and propofol) to wear off and reach a steady state of halothane anaesthesia.

Electroencaphalography (EEG) placement

The EEG electrodes were placed on the animal's head. Subdermal 27-G stainless-steel needle electrodes were placed in a four-electrode montage to record two channels of EEG. For each channel, the common non-inverting electrode was placed midline between the medial canthi of the eyes, the inverting electrodes placed bilaterally over the mastoid processes, and the ground electrode placed caudal to the poll.

The EEG was amplified using isolated differential signal amplifiers (Iso-Dam isolated physiological signal amplifiers; World Precision Instruments, Sarasota FL, USA), with a gain of 1,000 and pass-band of 0.1 to 500 Hz and digitised at a rate of 1 kHz (Powerlab/4sp; ADInstruments Ltd, Sydney, Australia). Data were analysed off-line after completion of the experiment.

Experimental treatment

The microwave device (Patent number: PCT/AU2011/000527) has been developed by Advanced Microwave Technologies, University of Wollongong (New South Wales, Australia), to deliver quantifiable and targeted microwave emissions specifically to the

head of livestock. The wand of the device was applied in contact with the top of the front head, on a mid-line half way between the medial canthi of the eyes and the poll. The exact location varied slightly depending on the head shape.

The microwave application was imposed at different powers and for different durations. It was repeated 35 min later at an identical dose to give a total of 2 applications per animal (see Figure 1). This second application was used in an attempt to deliver multiple applications to a single animal maintained under anaesthesia, hence allowing the collection of additional data while reducing the number of animals needed for the experiment (see External head temperature result section below). From the time the microwaves were applied, the animal was never given the opportunity to regain consciousness before death ensued. The animal was euthanized 10 min after the last microwave application by administrating a lethal dose of barbital into the jugular vein.

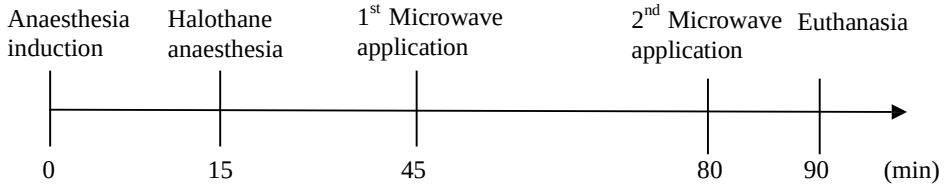

Figure 1. Typical schedule for the experimental treatment on one animal

EEG analysis

EEG epochs contaminated by artefacts, overscale or underscale were manually rejected from analysis of raw EEG data, using Chart 4.2.3 (ADInstruments Ltd).

Refined analyses were then performed by calculating the F50, F95 and Ptot for consecutive non-overlapping 1-sec epochs, using purpose-written software (Spectral Analyser; CB Johnson, Massey University, Palmerston North, NZ, 2002). Data were multiplied using a Welch window. Fast Fourier Transformation was applied to each epoch, generating sequential power spectra with 1-Hz frequency bins and median frequency, 95% spectral edge frequency and total EEG power were derived from the power spectra. Subsequent analysis was performed using Microsoft Excel.

Four variables were derived from combinations of the raw data and/or the variables derived from the frequency spectra: time to onset of EEG suppression (raw data), time to nadir of EEG suppression (95% spectral edge), duration of EEG suppression (combination of raw data, 95% spectral edge and total EEG power), and maximum effect (95% spectral edge) (see Figure 2). Time to onset of EEG suppression was measured from the start of the microwave application until the first appearance of seizure-like complexes in the EEG, hence including the duration of microwave application. Time to nadir of EEG suppression was measured from the start of the microwave application until the maximum depression of 95% spectral edge. Duration of EEG suppression was measured from the time of onset until the re-emergence of a normal EEG pattern similar to that seen prior to the application of the microwaves. The maximum effect was determined by the maximum reduction of the 95% spectral edge frequency.

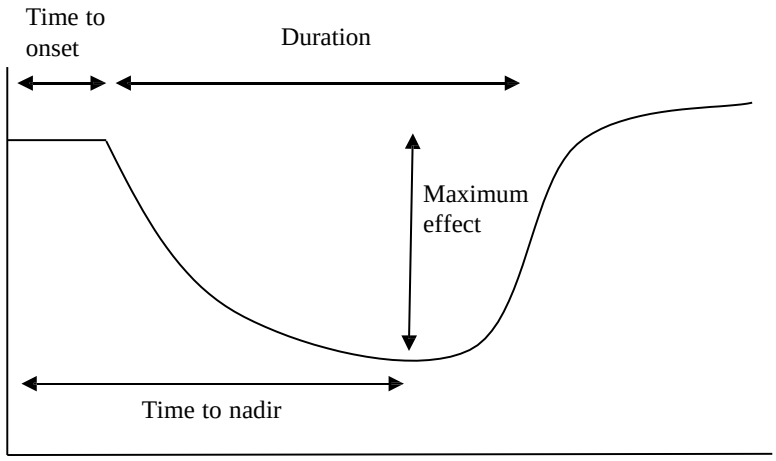

Figure 2. Visual description of the four variables derived from the EEG data: time to onset of EEG suppression (raw data), time to nadir of EEG suppression (95% spectral edge), duration of EEG suppression (combination of raw data, 95% spectral edge and total EEG power), and maximum effect (95% spectral edge).

Electrocardiography (ECG) analysis

Electrocardiogram (ECG) data were recorded by placing three ECG electrodes on the animal's body, for the last 4 animals. The electrodes were placed in a three-electrode montage. Adhesive electrode pads were adhered to the skin; the positive electrode was placed on the chest wall 5 cm behind the left point of the alecranon; the negative electrode was situated and 10cm out from midline of the thoracic back on the right hand side; the ground electrode was placed in the same position as the negative electrode but on the left han2d side. Electrocardiogram recordings were acquired using Powerlab 4/25T (ADInstruments, Castle Hill, Australia) and Chart 5® software (version 5.5.5)

(ADInstruments, Castle Hill, Australia). The ECG tracings were analysed using Chart 5 software to produce continuous heart rate recordings.

External head temperature

The external head temperature was recorded after microwave delivery by using a digital electric probe placed on the front head of the animal approximately 5 cm below the application point on the surface of the skin, for the first 3 animals. The temperature was monitored continuously from 3 min after the first microwave delivery until the second microwave delivery.

Post-mortem head histopathological autopsies

Two animals were given two microwave applications whereas two other animals were only given one microwave application in order to reliably observe the effects of a single microwave application as its intended use in the field. Post-mortem autopsies were performed by a veterinary pathologist on the head of the last four animals to determine histological changes in the skin and brain tissues. The skin was observed after a haematoxylin and eosin staining. The parts of the brains that were examined consisted of the frontal and parietal lobes (meninges, cortex, white matter), the basal nuclei, the thalamus and hypothalamus, and the caudal colliculii. These tissues were assessed for different parameters: tissue necrosis, vascular necrosis, cavitation or rarefaction, vascular haemorrhage, vascular congestion or oedema, and thrombosis using a grading scale from none to mild, moderate or severe.

Statistical analyses

Due to the potential ethical implications of this novel technique, only nine cows were used, hence only allowing for descriptive analyses. Descriptive analyses are commonly used in the scientific literature to report EEG results. Results are reported as average ± standard deviation unless otherwise noted.

Results

The microwave settings used in the first two animals (20 kW for 15 sec) replicated those used in a preliminary experiment (Rault et al., unpublished) to confirm that these microwave settings could alter the EEG signal. We then applied 20 kW for 10 sec on two animals, 30 kW for 10 sec on three animals, 30 kW for 5 sec on one animal, and 12 kW for 25 sec on one animal.

Although we administered two applications per animal for Animals one to seven, only the first application is discussed as the results of the second application were clearly different to the second application based on a much greater penetration of the microwaves as a result of the first application. The use of two applications of microwave was intended to maximize the data generated by the study in order to provide a more accurate indication of the EEG effects of microwave application. Both applications were set at the same power and duration in order to be able to test that there were no order effects due to repeated application. Initial analysis of the efficiency of the microwave applications together with subsequent analysis of the EEG data indicated that there was a

substantial order effect with the second application resulting in more efficient microwave delivery and more profound EEG effects than the first. A consequence of this order effect is that the second application does not accurately reflect field conditions under which each animal would be subjected to a single application. For this reason the data from the second applications for each animal were not included in further analysis.

Furthermore, one animal did not respond to the anaesthetic agents and had to be euthanized before any treatment could be applied. Hence, we obtained data from nine animals.

EEG results

The microwave application caused artefacts that rendered the EEG recording useless during and until about 3 sec after the end of the microwave application. Therefore, the duration is based on the time to visible EEG changes, and consequently some animals could have shown EEG changes before the end of the microwave application.

All of the applications resulted in changes in the EEG pattern indicative of seizure-like activity, as seen in Animal six (Figure 3 and Figure 4). The derived variables time to onset of EEG suppression, time to nadir of EEG suppression, duration of EEG suppression, and maximum effect for each individual animal are presented in Table 1.

Table 1. EEG results for each individual animal: time to onset of EEG suppression, time to nadir of EEG suppression, duration of EEG suppression, and maximum effect. Time was measured from the start of the microwave application.

Animal ID	**1**	**2**	**3**	**6**	**4**	**7**	**8**	**5**	**9**
Power (kW)	20	20	20	20	30	30	30	12	30
Duration (sec)	15	15	10	10	10	10	10	25	5
Total Energy (kW × sec)	300	300	200	200	300	300	300	300	150
Time to onset (sec)	24	50	14	12	16	27	14	138	12
Time to nadir effect (sec)	65	52	22	28	24	34	37	142	20
Duration (sec)	129	81	140	78	109	80	215	37	50
Maximum effect (%reduction SE)	25	18	29	21	31	11	59	6	8

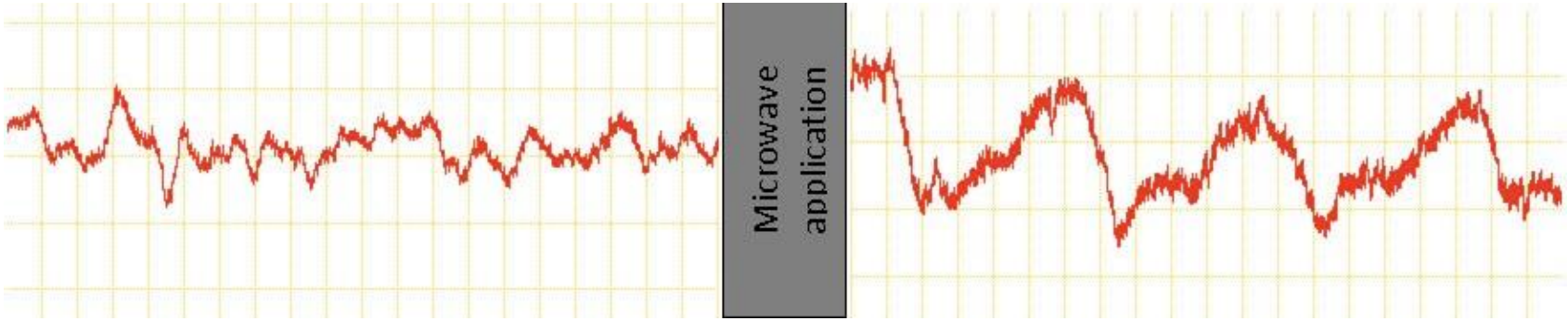

Figure 3. Four-sec sample of EEG data prior to and after microwave application, recorded from Animal six receiving 20 kW for 10 sec. Horizontal divisions represent 200 ms, vertical divisions represent 100 microvolts.

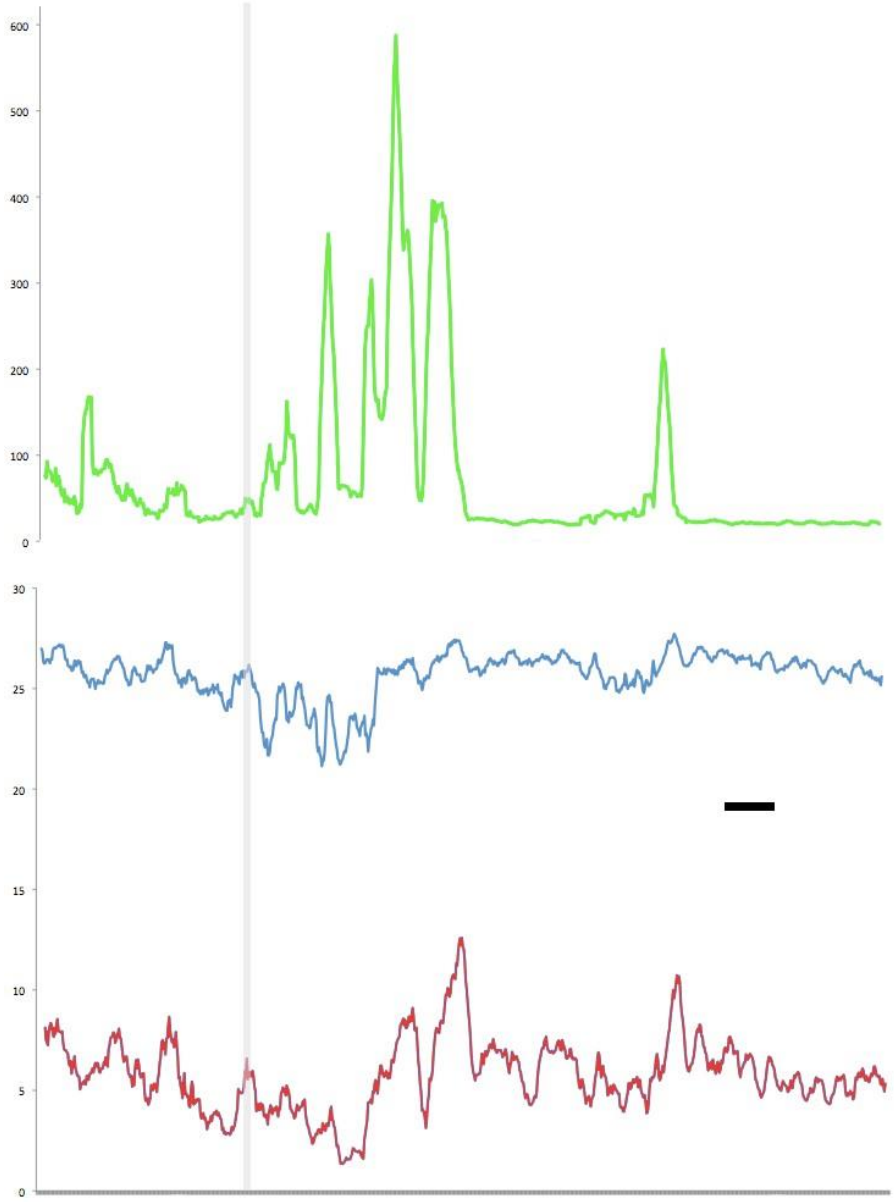

Figure 4. Total EEG power (top; arbitrary units) and 95% spectral edge (middle, Hz units) and median frequencies (bottom; Hz units) derived from the EEG frequency spectra of Animal six, receiving 20 kW for 10 sec. The time of microwave application is indicated by the grey shading, the horizontal bar represents a 1-min duration. A low pass

filter has been applied to the traces (ten point moving average) to make them easier to interpret visually.

In applying 40 kW for 5 sec to Animal 9, the microwave generator reached its limit and shut down shortly after starting. Another application was attempted and also resulted in the equipment shutting down. A third and last attempt was performed on Animal nine with the application of 30 kW for 5 sec. These results have been discarded from the main EEG results as the result obtained after the third application was likely to be somewhat inaccurate because the application was preceded by two aborted applications. It is evident from the EEG results below (Figure 6) that the aborted applications likely still delivered some energy prior to the shutdown. Similarly, the EEG from Animal seven was heavily contaminated by ECG artefacts. Hence, the EEG data from Animal seven have been discarded from further consideration because it was considered to be of insufficient quality to reach sound conclusions. Thus, seven animals' EEG with four different microwave treatments were analysed (Table 2).

Table 2. EEG results pooled by treatment: time to onset of EEG suppression, time to nadir of EEG suppression, duration of EEG suppression, and maximum effect (average and (*value1*,*value2*)). Time is counted from the start of the microwave application.

Treatment	**1** (n=2)	**2** (n=2)	**3** (n=2)	**5** (n=1)
Power (kW)	20	20	30	12
Duration (sec)	15	10	10	25
Total Energy (kW × sec)	300	200	300	300
Time to onset (sec)	37 *(24,50)*	13 *(12,14)*	15 *(14,16)*	136
Time to nadir effect (sec)	59 *(52,65)*	25 *(22,28)*	31 *(24,37)*	142
Duration (sec)	105 *(81, 129)*	109 *(78,140)*	162 *(109,215)*	37
Maximum effect (%reduction SE)	22 *(18,25)*	25 *(21,29)*	45 *(31,59)*	6

The duration of the microwave application seemed to influence the time to onset of EEG suppression, as well as time to nadir of EEG suppression, with shorter duration causing more rapidly developing effects (Table 2: 10 sec < 15 sec < 25 sec). On the other hand, the power of the microwave application seemed to influence the duration of EEG suppression, with higher power resulting in longer duration (Table 2: 30 kW > 20 kW > 12 kW). The factors underlying the maximum effect cannot be explained from the current data. Because time of onset included the duration of application of the microwave, this means that the EEG started changing as soon as 3 sec after the end of the microwave application, and possibly before since the artefact caused by the microwave rendered the EEG unreadable until that time. However, the low power treatment of 12 kW required almost 2 min (113 sec) to take effect. The interval between the time when effects started

appearing ('Time to onset') and the maximum effects were seen ('Time to nadir') was within 4 to 22 sec depending on the treatments. All treatments could suppress the EEG for at least 37 sec and up to more than 2 min (162 sec) ('Duration'). The maximum effect remained very variable even between two animals administered the same treatment.

ECG results

The microwave application had characteristic effects on the heart rate of the last four animals (Figure 5 and Table 3). The heart rate baseline recorded over the five minutes prior to the microwave application was 92.2 (± 8.4) bpm. After the start of the microwave application, the heart rate dropped within 5.0 (± 2.4) sec to 65.8 (± 24.0) bpm, and then rebounded after 23.75 (± 1.9) sec from the start to 82.3 (± 10.3) bpm. It stabilised within 160 (± 37.4) sec to 73.2 (±12.1) bpm, a lower level than the initial baseline obtained prior to the microwave application.

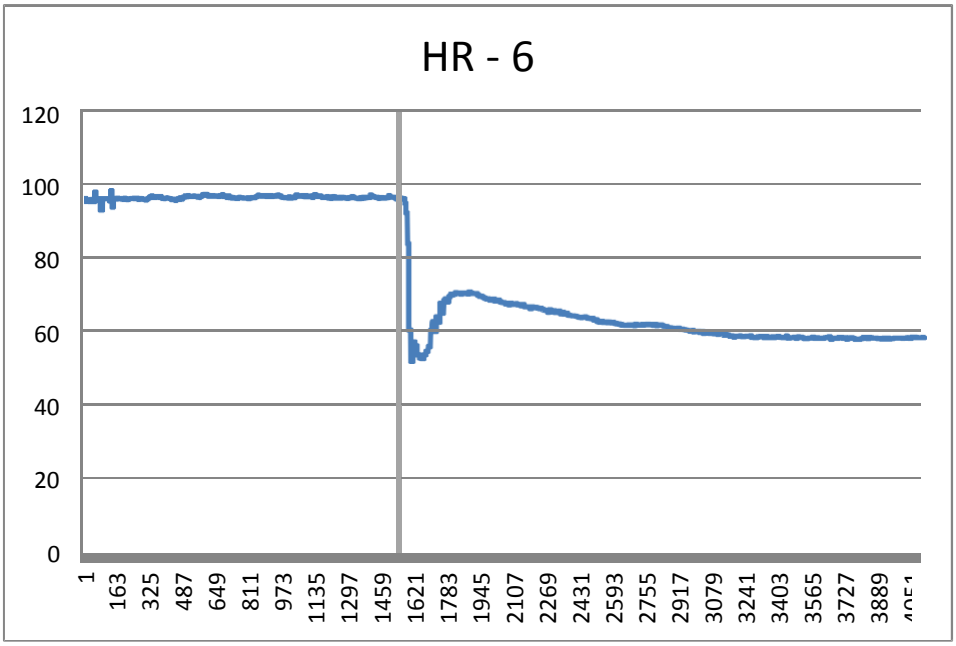

Figure 5. Typical ECG pattern after microwave application, recorded from Animal six receiving 20 kW for 10 sec. The time of microwave application is indicated by the grey shading. Time is counted from the start of the microwave application.

Table 3. ECG results for each individual animal: baseline heart rate (HR; beats per minute, average ± standard deviation), minimum heart rate after the microwave application, heart rate recovery after the microwave application and time to stabilize the heart rate. Time is counted from the start of the microwave application.

Animal ID	**6**	**7**	**8**	**9**[1]	**AVERAGE**
Power (kW)	20	30	30	30	
Duration (sec)	10	10	10	5	
Total Energy (kW × sec)	200	300	300	150	
Baseline HR pre-application (bpm)	97.1 ± 0.6	88.0 ± 0.2	82.6 ± 0.2	101 ± 0.2	**92.2 ± 8.4**
Minimum HR (bpm) and [sec] reached	52.8 [3]	83.6 [6]	38.5 [3]	88.2 [8]	**65.8 [5]**
Rise post-application (bpm) and [sec] reached	71.3 [21]	93.1 [24]	75.9 [25]	88.7 [25]	**82.3 [23.8]**
Baseline HR post-application (bpm) and [sec] reached	59.1 [210]	81.4 [150]	67.2 [160]	85.0 [120]	**73.2 [160]**

[1]The ECG for Animal 9 was collected for the third application, after two failed attempts.

External head temperature observations

For the first three animals, the external head temperature was recorded through an electric probe placed on the surface of the skin, on the front head of the animal approximately 5 cm below the application point. External head temperature increased quickly after microwave application and returned to baseline within 35 min (Table 4), hence the rationale for using a second microwave application 35 min following the first microwave application. Observations by the researchers within 3 min of the microwave application indicated that the external head temperature only increased slightly for Animal 3, submitted to 20 kW for 10 sec, in comparison to Animals 1 and 2, submitted to 20 kW for 15 sec, which showed significant and long-lasting temperature increases (Table 4).

Table 4. External head temperature (°C) recorded overtime after microwave application, on the front head skin surface approximately 5 cm below the application point.

Time post-microwave application (min)	**5**	**10**	**15**	**20**	**25**	**30**	**35**
Animal 1 (20 kW, 15 sec)	44.3	39.7	37.0	35.6	34.8	-	-
Animal 2 (20 kW, 15 sec)	44.8	43.2	40.0	37.6	35.9	34.6	33.4
Animal 3 (20 kW, 10 sec)	33.1	-	-	-	-	-	-

Post-mortem autopsies results (Appendix 1)

The two first animals (Animals six and seven) submitted for post-mortem analyses were given two microwave applications. The subsequent two animals (Animals eight and nine) were only given one microwave application in order to reliably observe the effects of a single microwave application as its intended use in the field. However, it should be noted that Animal nine received two failed attempts before the third and only successful application. Hence, the data from Animal nine should be treated with caution, as it may not be representative of the results of a single application.

Skin results. All animals displayed similar skin lesions (Appendix 1). Centrally over the forehead there was an area of complete skin loss, surrounded by a larger region displaying grey-tan discolouration of the subcutaneous tissue. This skin within this area displayed full-thickness coagulative necrosis extending down to the skull. Beyond this area, there was rapid progressive decrease in the severity of necrosis, with normal unaffected skin present within 0.5cm of the margin.

Brain results. The detailed results of the post-mortem autopsies on the brains for each animal are presented in Appendix 1.

Two consecutive microwave applications. Animals six and seven, submitted to two microwave applications, showed severe lesions at various levels (meninges, cortex and white matter) in the frontal and parietal lobes, with severe tissue and vascular necrosis, cavitation, haemorrhage and vascular congestion. However, only minor if any changes were apparent in the basal nuclei, thalamus, hypothalamus, and the caudal collicus. The

lesions in this more basal region consisted mainly of haemorrhage, vascular congestion and thrombosis.

Single microwave application. The duration of application may underlie the amount of histological changes, as Animals eight submitted once to 30 kW for 10 sec had medium to severe lesions in the frontal and parietal lobes and Animal nine submitted to 30 kW for 5 sec had relatively minor to no lesions in the same regions. For both animals, the basal nuclei, thalamus, hypothalamus, and the caudal collicus were relatively unaltered except for mild to moderate vascular congestion.

Examples of failed microwave applications: Animal nine

Animal nine was first subjected to two attempts with the application of 40 kW for 5 sec, which resulted in the microwave generator shutting down. The microwave engineers verbally reported at that time that, on the first attempt, no microwaves have been delivered based on the feedback obtained from the wave tuner. Following the second attempt, the microwave engineers verbally reported that the wave tuner indicated a delivery of about 5 kW. However, the ECG results on Animal nine show that the first and second delivery led to heart rate changes for 3 sec and 4 sec respectively (Figure 6). Hence, the EEG recorded from the third application could not be considered as an application on an intact brain and had to be discarded in the same way as we discarded the data from the second application on all other animals.

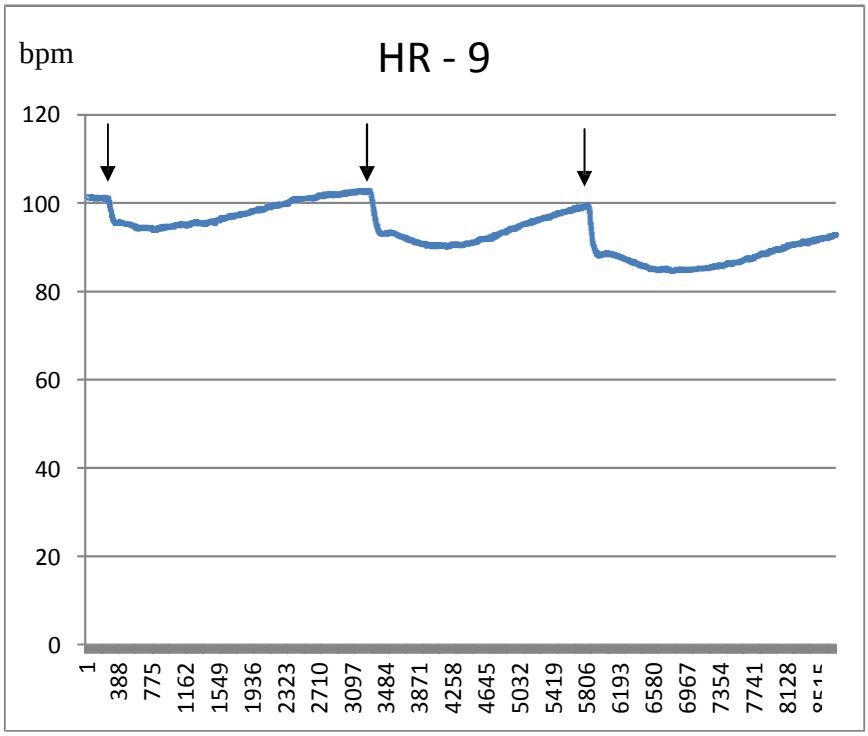

Figure 6. ECG (beats per min) recording for Animal nine, first subjected to two failed attempts at 40 kW for 5 sec before a third application of 30 kW for 5 sec. The 'missed' applications still had observable effects on the animal as the three applications are clearly visible on the ECG pattern.

Discussion

The microwave application induced EEG changes in all nine animals studied. Microwave application induced a pattern of seizure-like activity in the EEG, a pattern that is not considered to be compatible with continued awareness (Devine et al., 1986; Coenen, 1998; Velarde et al., 2002). Hence, this pattern of seizure-like activity in the EEG is interpreted as an assessment of insensibility in this study. The anaesthetic agent used (halothane) would have prevented most of the motor activity associated with these seizures, but motor effects such as limb rigidity and possibly tonic or clonic movements may be seen in awake animals, similar to what has been reported with rats (Guy and Chow, 1982: irradiating heads and shoulders; Lambooy et al., 1989: irradiating heads only). The EEG changes observed in the present study following microwave application occurred rapidly, as soon as 3 sec after the end of the microwave application. Unfortunately, the microwave application caused artefacts that rendered the EEG unreadable until about 3 sec after the end of treatment. Therefore, the duration was based on the time to certain EEG changes. Consequently, some animals could have shown EEG changes before the end of the microwave application.

For any stunning technique, the search for the minimum duration of application necessary to induce insensibility is crucial. Reducing that interval is likely to improve animal welfare by reducing the time that the animals may experience pain and distress. Our results indicate that an application for 10 sec resulted in insensibility within 3 sec later. In comparison, non-penetrative captive-bolt stunning induced EEG changes in about 8 sec in calves, in a study which also used the low anaesthesia model (Gibson et al.,

2009b). These results confirm findings on rodents (AVMA, 2013) that microwave irradiation is a relatively quick process in comparison to other reversible stunning procedures such as cattle electrical stunning, for which applications of up to 15 sec can be performed in order to depolarize the spine and reduce kicking (AMI, 2010), or carbon dioxide stunning in pigs, with latency of about 25 sec to the loss of posture (Velarde et al., 2007). Furthermore, our results indicated that a shorter duration of application induced more rapidly developing EEG changes, in the range of duration tested (10-25 sec), as evidenced by shorter 'time to onset' and 'time to nadir'.

Another consideration for reversible stunning techniques is the duration of insensibility, or the time before the animal regains consciousness. Insensibility should last until death ensues through exsanguination. Following microwave application, EEG suppression lasted for at least 37 sec and up to more than 2 min. Our results also indicated that applying higher power extended the duration of insensibility. The search for a long period of insensibility is useful for cattle because consciousness can last from 1 to 2 min after exsanguination (Newhook and Blackmore, 1982; Gregory et al., 2010).

Unfortunately, the microwave application caused artefacts that rendered the EEG unreadable until after the end of treatment. This is an inherent limitation of using electroencephalographic technique to assess the microwave technique since both techniques interact with electric activity. This leaves a window of uncertainty regarding the aversiveness of the microwave technique during its application and the experience of the animal during that short period of time. The animal's perception of the procedure up to the induction of insensibility (in the order of 10-15 sec) should be investigated with

alternative scientific methods that allow for data collection during microwave application.

The abrupt bradycardia (i.e. drop in heart rate) observed following microwave application is in agreement with the literature on the heart rate response to noxious stimuli (Woodbury et al., 2005; Johnson et al., 2005; Gibson et al., 2007). The magnitude of that drop differed between animals, but the heart rate rebounded within 24 sec, irrespective of the treatment applied. Interestingly, the heart rate stabilised to a different, lower level, following microwave application. The most plausible explanation is that this may be the result of temporary and longer-persisting effects on the brain-stem or thalamus (Benarroch, 2001).

Based on the post-mortem autopsies, most histological changes appeared in the upper regions of the brain, with the frontal lobe, adjacent to the zone of application of the microwave, being the most affected, closely followed by the parietal lobes which are located on the sides of the animal's brain. However, the regions of highest interest in regards to consciousness, the deeper regions of the brain, namely the basal nuclei, thalamus, hypothalamus, and the caudal collicus, appeared to be relatively unaffected by microwave application, even following two microwave applications. Further research is warranted regarding the dissipation of energy throughout the brain and whether this is a homogeneous process or not. The lesions observed would suggest that animals may be able to regain consciousness following microwave application, although the frontal regions of the brain would unlikely be intact.

Various questions arose during this experiment. For example, it would be useful to know whether differences between animals in characteristics such as head shape, age or gender influence the efficiency of the microwave technique. Such characteristics can affect stunning efficiency for other widely-used techniques such as captive-bolt gun (Gouveia et al., 2009). As an anecdotal observation, one animal (animal seven) died following the second microwave application which consisted of 30 kW for 10 sec. Hence, it is possible to obtain non-reversible insensibility (i.e. kill) using the microwave technique. It appeared that the effects of microwave application on EEG changes did not follow a simple equation such as Total Energy = Power × Duration delivered. The parameters Power and Duration influenced different aspects of the EEG changes. The duration of application influenced the onset of insensibility whereas the power delivered influenced the length of time during which the animal was insensible.

Practical implications for the use of microwaves as a stunning technique

Three parameters can be considered essential for the welfare implications of a stunning technique: the time to onset of insensibility (‘how quickly the stun acts’, the shorter the better), the duration of insensibility (underlying the ‘stun-stick interval’, the longer the better), and the variability between animals (the lower the better). Among the treatments that have been tested within the present experiment, 20 kW for 10 sec yielded the best outcomes: these two animals were judged insensible in less than 14 sec from the start of the microwave application and remained insensible for at least 78 sec. These outcomes are promising. A microwave application of less than 10 sec may be sufficient,

although this requires further rigorous testing. The animal's perception of the procedure up to the induction of insensibility (the first 14 sec) should be investigated with alternative scientific methods that allow for data collection during microwave application

Variability in stunning efficiency

A good stunning technique should lead to consistent results with no return to consciousness, both to ensure animal welfare and worker safety. Based on animal ethical consideration, the variability in the efficiency of microwave application ("what makes for a good shot") should be validated first from an engineering perspective. There is a need for scientific data demonstrating the level of accuracy and consistency of the microwave delivery technology. As an example, although the wave tuner (used to judge of the quality of the delivery process by the microwave engineers) suggested that Animal nine had not received any microwaves following the first failed application, the ECG changes observed on the animal itself (Figure 4) refute this idea. A thorough knowledge of the microwave delivery technology and the way it acts on an animal is essential to ensure that the technique can be developed to reliably deliver consistent applications before animals are used to test biological variability. Clearly, this experiment conducted on a small number of animals represents preliminary results and more animals are needed to determine the variability that exists between animals in response to microwave application.

Future research directions

This experiment provided novel and crucial knowledge regarding the effects of different power and duration of microwave applications on anesthetized cattle. However, only a small number of animals, hence a small number of settings, could be tested. A possibility for future research is to perform further anaesthesia trials to refine the duration of application (< 10 sec), the potential for higher power to lengthen the duration of insensibility, and the variation between animals.

This experiment also raised questions regarding the mechanisms by which microwaves induce insensibility. A more thorough understanding of the specific brain regions affected, and of the dissipation of energy throughout the brain (homogenous distribution or not), is critical to assess the implications in terms of consciousness and to assess the animal's perception of the procedure. Clarifying the mechanisms does not only contribute to scientific knowledge; it will also provide practical knowledge to optimize the microwave procedure once the mechanisms are more thoroughly understood. It would be both prudent and more ethical to do this work first on carcasses and then using a smaller animal model, such as the rat or sheep, rather than using cattle as it may require a large number of samples and a more uniform source of animals. This work could also address the reversibility of the microwave technique.

Finally, there is the possibility of returning to a live trial on conscious, non-anesthetised, animals. In a previous experiment, two non-anesthetised cows were subjected to a microwave treatment of 20 kW for 10 and 15 sec. These animals did not

collapse within 20 sec following the end of microwave application, nor did they show spasms, jerks or jumping as reported by with rats (Guy and Chow, 1982; Lambooy et al., 1989). Theorically, behavioural response such as loss of posture should occur at the same time or before loss of consciousness occurs, as indicated by the electrophysiological method (3 sec after the end of microwave application at 20 kW for 10 sec). It is possible that the microwave application resulted in a transient rigid posture, which was difficult to observe in our live trial due to the necessity to restrain the animals within a supporting sling and a head restraint for a correct microwave application (another limitation of using scientific methods in the current microwave setting). In any case, a live trial is inevitable at some point to validate the humaneness of that technique and because the EEG cannot tell us about the experience of the animal until insensibility occurs due to technical limitations associated with obtaining EEG recordings during microwave application. Furthermore, the identification and validation of appropriate signs of insensibility following microwave application (e.g., behavioural response to a corneal reflex, papillary light reflex, others?) would be valuable for use in both research and industry settings to assess insensibility. Returning to a live animal trial is a very sensitive decision, especially considering the current pressure from animal activists targeting Animal Ethics Committee's judgements across Australia.

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Appendix 1: Brain histopathology report

ANATOMIC PATHOLOGY

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Submission Date: 26/11/12
Pathology Number: 1021-12

Four fresh cattle heads submitted. Cows A and B submitted on 26/11/12 and cows C and D submitted on 27/11/12. Cow A had no ear tag. Cow B had a right ear tag marked SWS D273. Cow C had a left ear tag marked F17. Cow D had a left ear tag marked SWS B236.

Pathology findings

Skin: All animals displayed similar lesions. Centrally over the forehead there was an area of complete skin loss, surrounded by a larger region displaying grey-tan discolouration of the subcutaneous tissue. This skin within this area displayed full-thickness coagulative necrosis extending down to the skull. Beyond this area, there was rapid progressive decrease in the severity of necrosis, with normal unaffected skin present within 0.5cm of the margin.

The dimensions of the affected regions in each cow are as follows:

	Central skin loss	Marginal necrosis
Cow A	5 cm x 10 cm	14 cm x 17 cm
Cow B	8 cm x 12 cm	17 cm x 20 cm
Cow C	3 cm x 9 cm	14 cm x 16 cm
Cow D	3 cm x 5 cm	14 cm x 16 cm

Brain: All cattle displayed a variable degree of grey brown discolouration affecting the frontal and parietal lobes, with diffuse engorgement of meningeal vasculature. In order of extent of discolouration, the most affected cow was B, followed by C, A, and D. Cow B displayed herniation of friable cerebral tissue through the meninges rostro-dorsally, as well as severe ventral haemorrhage extending dorsally rostral to the cerebellum. There was also moderate cerebellar herniation through the foramen magnum. Cow C displayed a small focus of friable pale cerebral tissue over the left frontal lobe. Histological lesion scores are presented separately.

Date: 13/12/12

Pathologist: Andrew Stent, Tutor in Veterinary Pathology

	Tissue necrosis	Vascular necrosis	Cavitation/rarefaction	Haemorrhage	Vascular congestion/	Thrombosis
Animal 6: microwave 2 applications, 20 kW for 10 sec						
Frontal lobe						
Meninges	superficial		X			N
Cortex				N		
White matter						N
Parietal lobe						
Meninges			X			
Cortex	superficial					
White matter						
Basal nuclei	N		N	N		
Thalamus/Hypoth	N	N	N		N	
Caudal colliculus	N	N	N		N	
Animal 7: 2 microwave applications, 30 kW for 10 sec						
Frontal lobe						
Meninges			X			
Cortex	superficial			N		
White matter				N		
Parietal lobe						
Meninges			X	N		
Cortex	superficial			N		
White matter				N		
Basal nuclei		N	N	N		
Thalamus/Hypoth	N	N	N	N		
Caudal colliculus	N	N	N	N		
Animal 8: 1 microwave application, 30 kW for 10 sec						
Frontal lobe						
Meninges			X	N	N	
Cortex		N		N	N	
White matter		N				
Parietal lobe						
Meninges			X	N		
Cortex	N	N		N		
White matter	N	N				
Basal nuclei	N	N	N	N	N	
Thalamus/Hypoth	N	N	N	N	N	
Caudal colliculus	N	N	N	N	N	
Animal 9: 1 microwave application, 30 kW for 5 sec						
Frontal lobe						
Meninges			X	N	N	
Cortex	N	N	N	N	N	
White matter	N	N		N	N	
Parietal lobe						
Meninges	N	N	X	N	N	
Cortex	N	N	N	N	N	
White matter	N	N		N	N	
Basal nuclei	N	N	N	N	N	
Thalamus/Hypoth	N	N	N	N	N	
Caudal colliculus	N	N	N	N	N	

Brain histopathology grading criteria

On the table of Appendix 1, lesions qualified as 'mild' are symbolized by a green colour,' moderate' by a orange colour and 'severe' by a red colour.

Tissue necrosis
Mild – Scattered individual cell necrosis
Moderate – Occasional areas of necrotic tissue
Severe – Widespread or extensive multifocal coagulative or liquefactive necrosis

Vascular necrosis
Mild – Rare coagulative necrosis of vessel walls, typically only partial thickness
Moderate – Multifocal coagulative necrosis of vessel walls, may be full thickness
Severe – Diffuse necrosis of vessel walls, often accompanied by vascular disruption

Cavitation/rarefaction
Mild – Scattered areas of tissue disruption or mild white matter rarefaction
Moderate – Occasional foci of tissue cavitation and spongiosis of tissue
Severe – Widespread areas of cavitation, spongiosis and disruption of tissue architecture

Vascular haemorrhage
Mild –Occasional haemorrhage confined to the perivascular space
Moderate – Widespread haemorrhage occasionally extending into the parenchyma
Severe – Large areas of haemorrhagic disruption of normal tissue

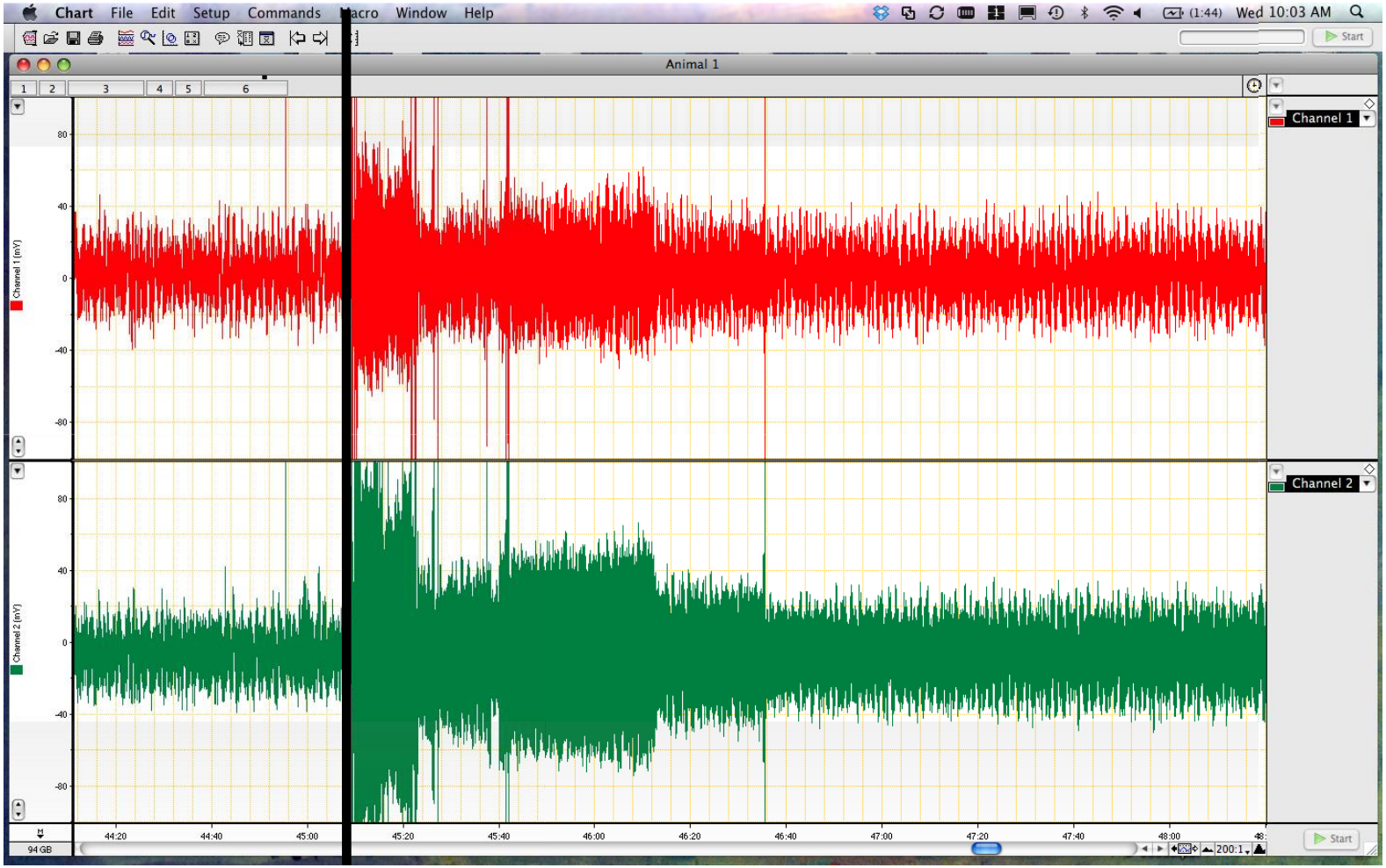
Vascular congestion/oedema
Mild – Vessels mildly engorged with blood
Moderate – Vessels diffusely engorged with mild perivascular clearing
Severe – Severe engorgement of vessels with large cleared spaces (oedema) around vessels.

Thrombosis
Mild – Occasional scattered microthrombi observed within vessels
Moderate – Numerous microthrombi or rare large thrombi present within vessels
Severe – Widespread complete vascular occlusion by thrombi

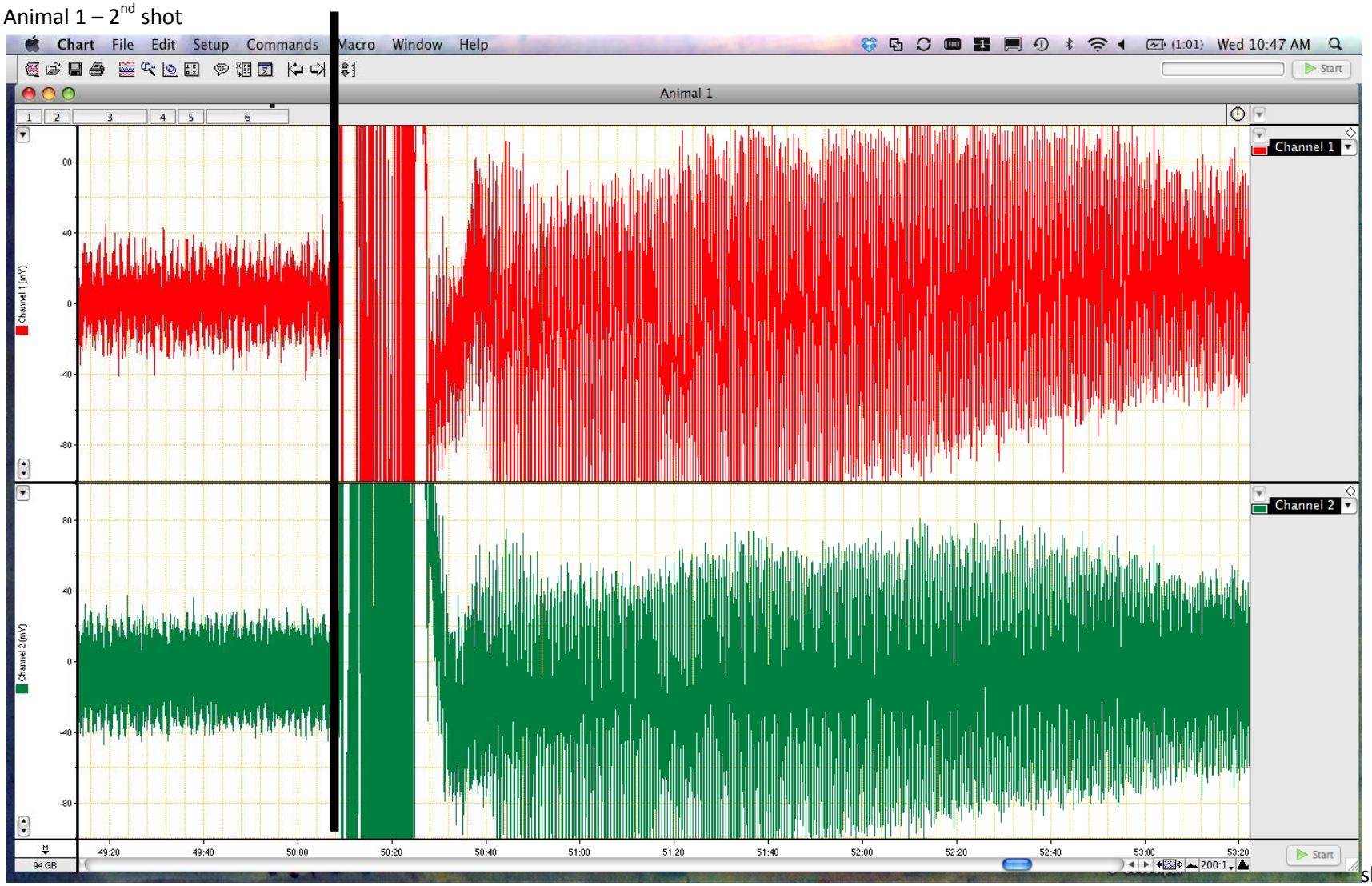
Appendix 2: Raw EEG recordings for each animal

On each graph, the black line indicates the start of microwave application.

Animal 1 – 1ST shot

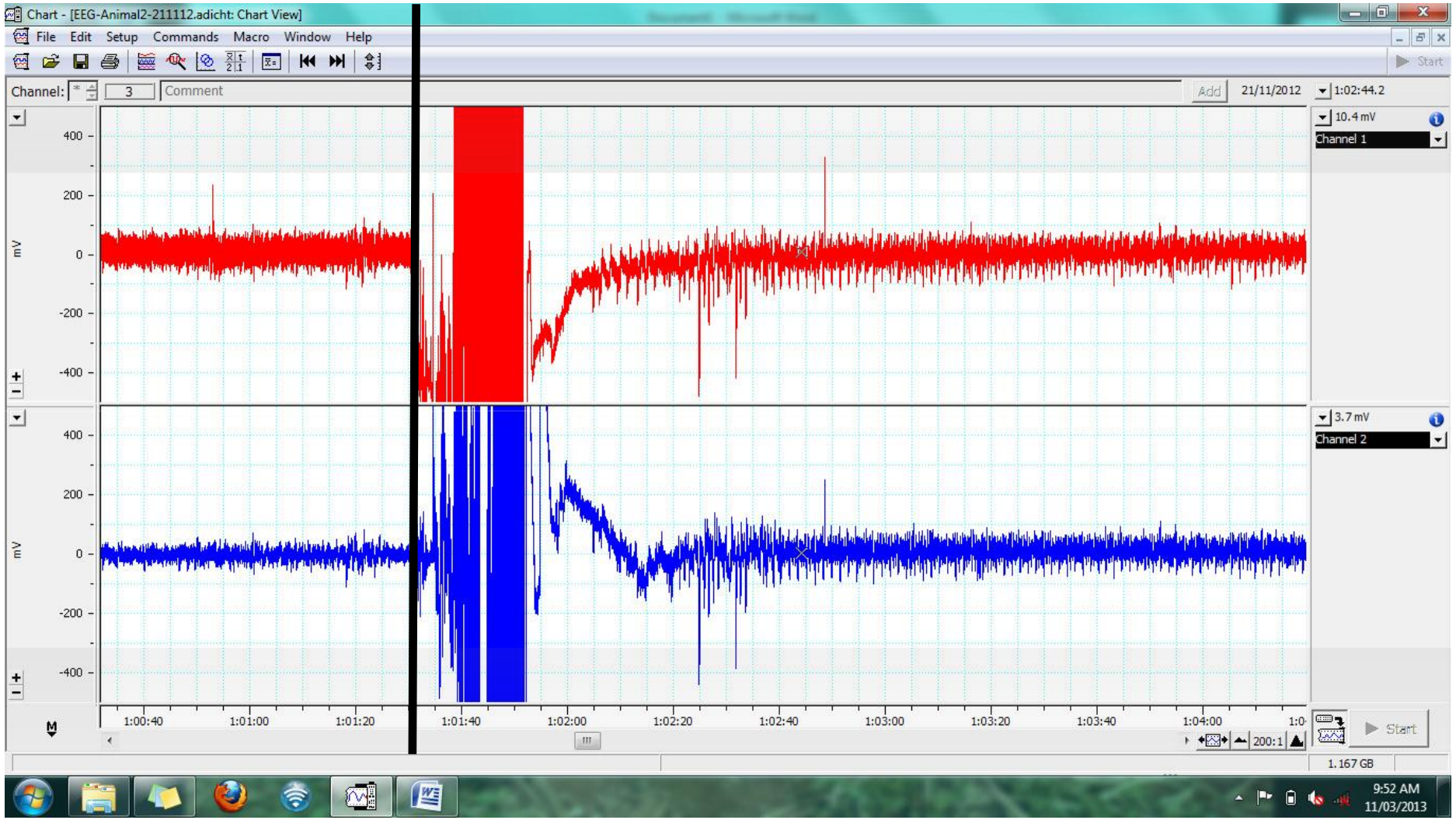


Animal 1 – 2nd shot

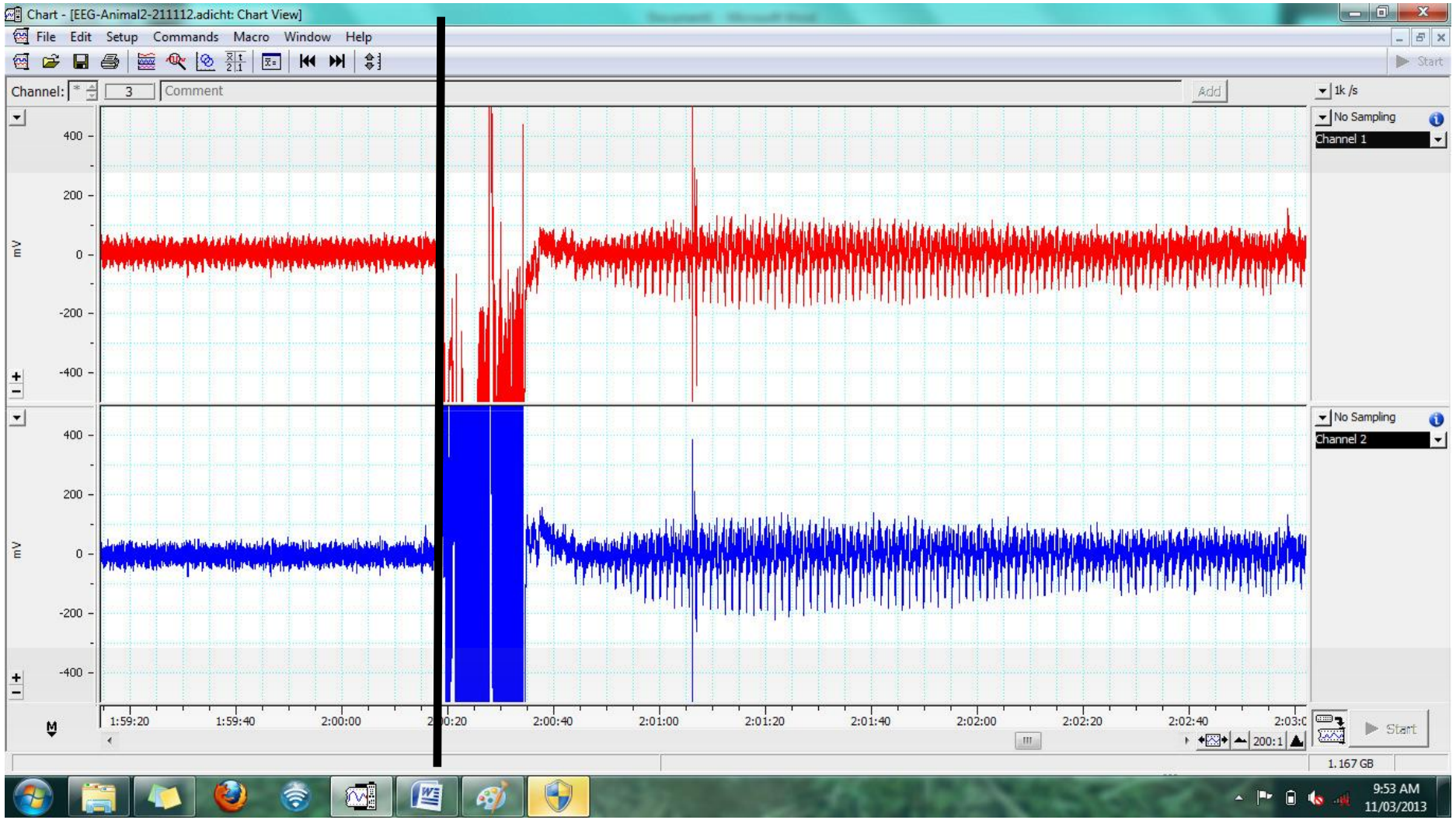


shot

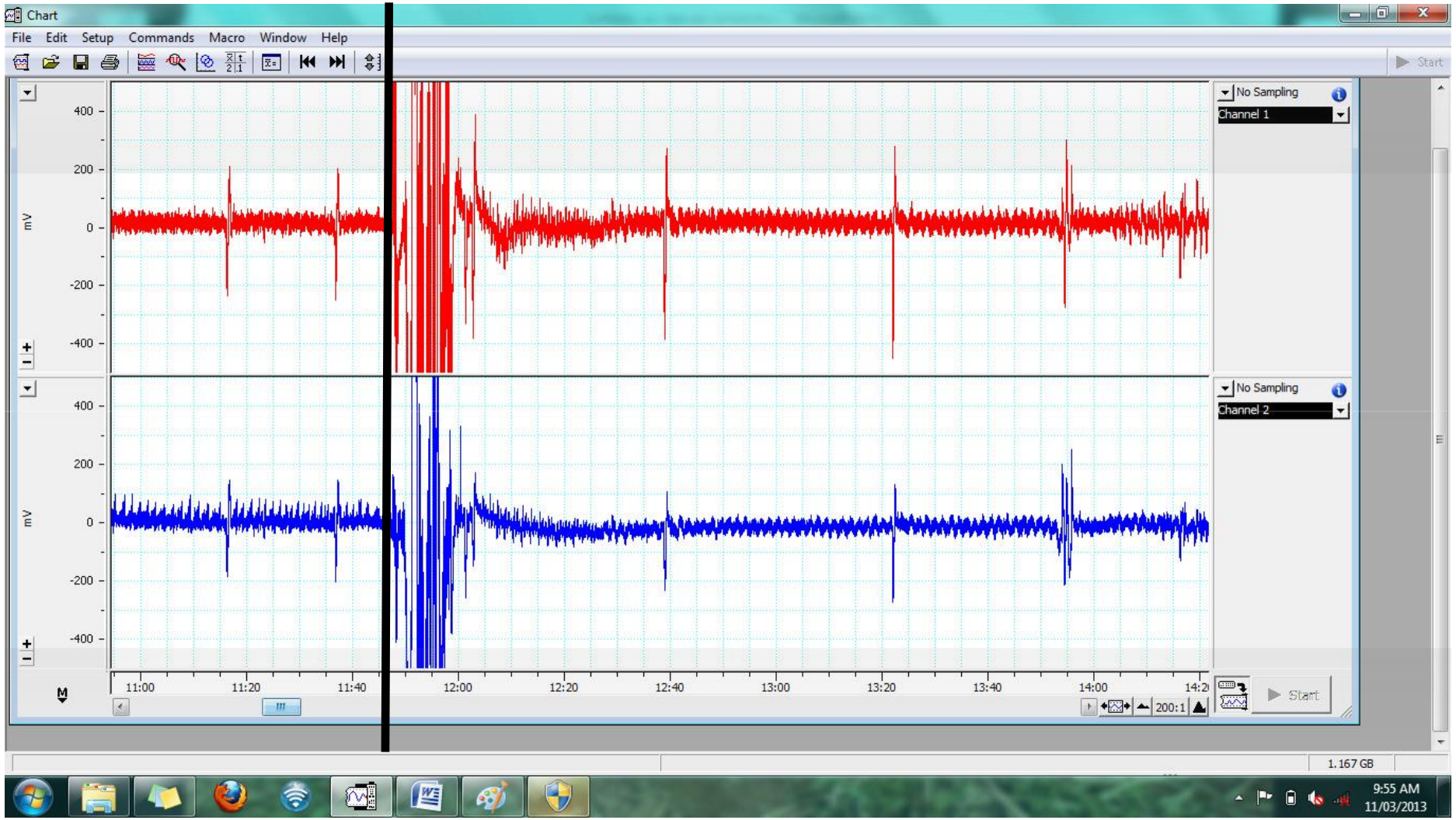
Animal 2 - 1st shot



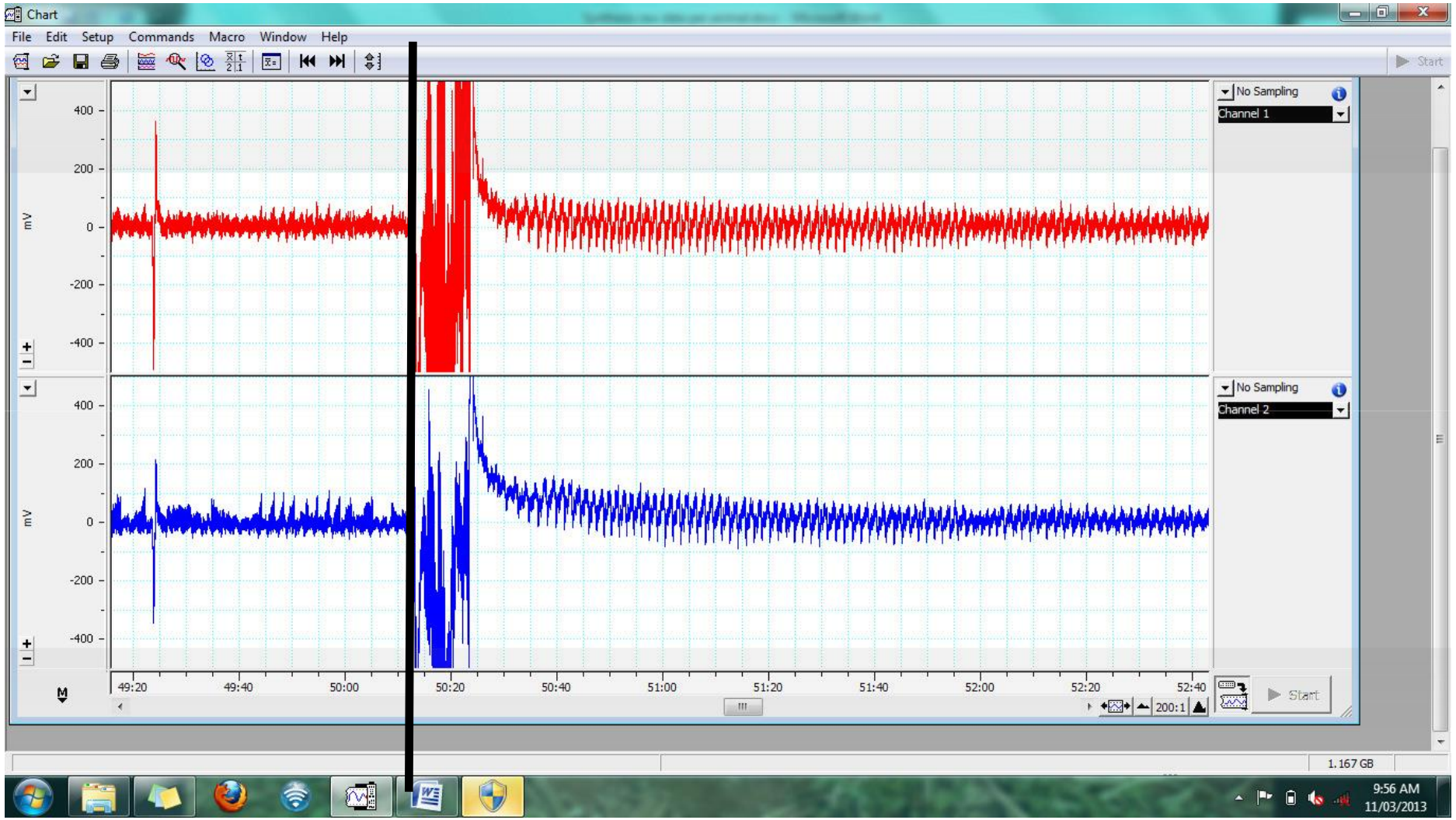
Animal 2 – 2nd shot



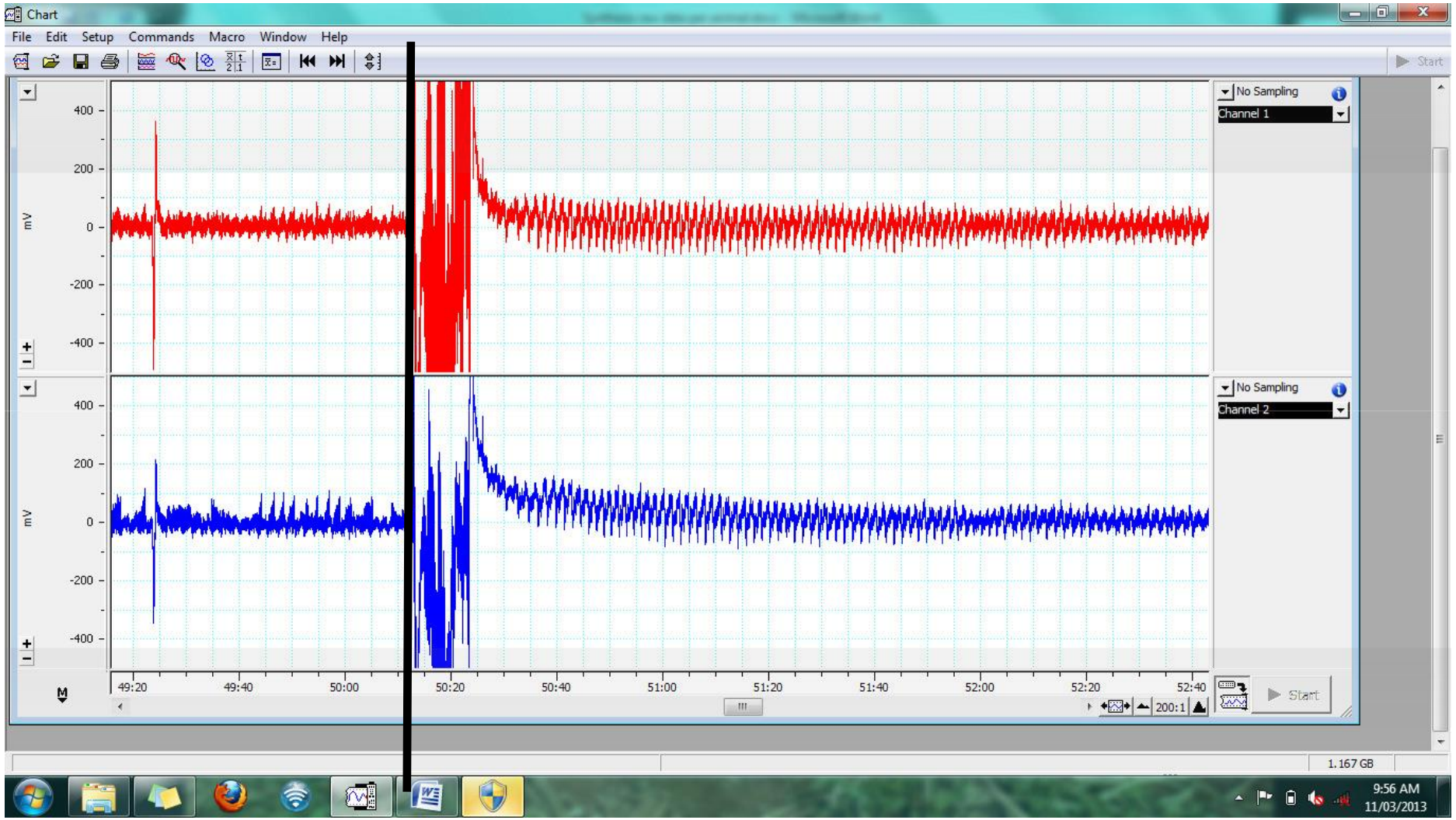
Animal 3 – 1 st shot



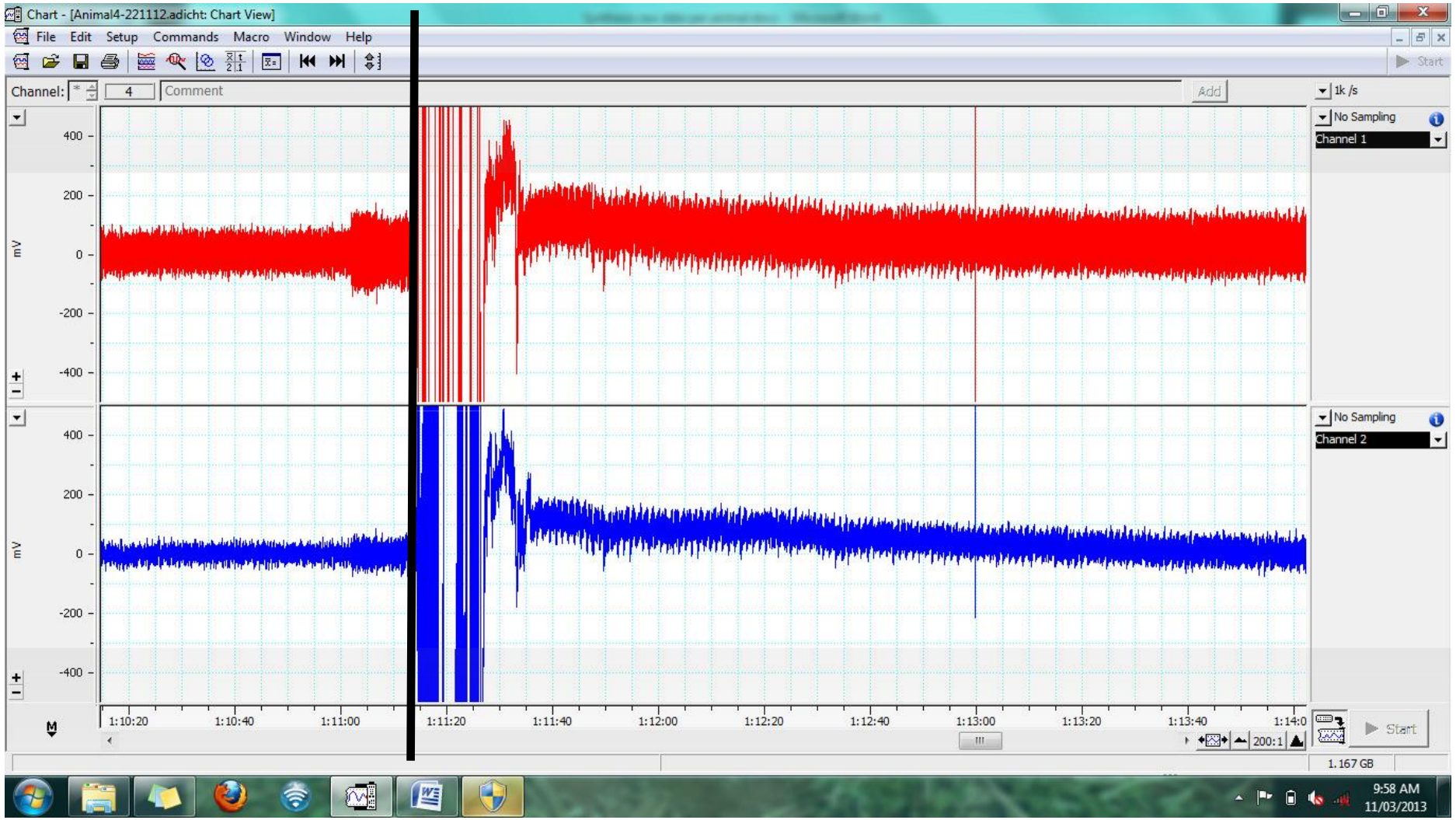
Animal 3 - 2nd shot



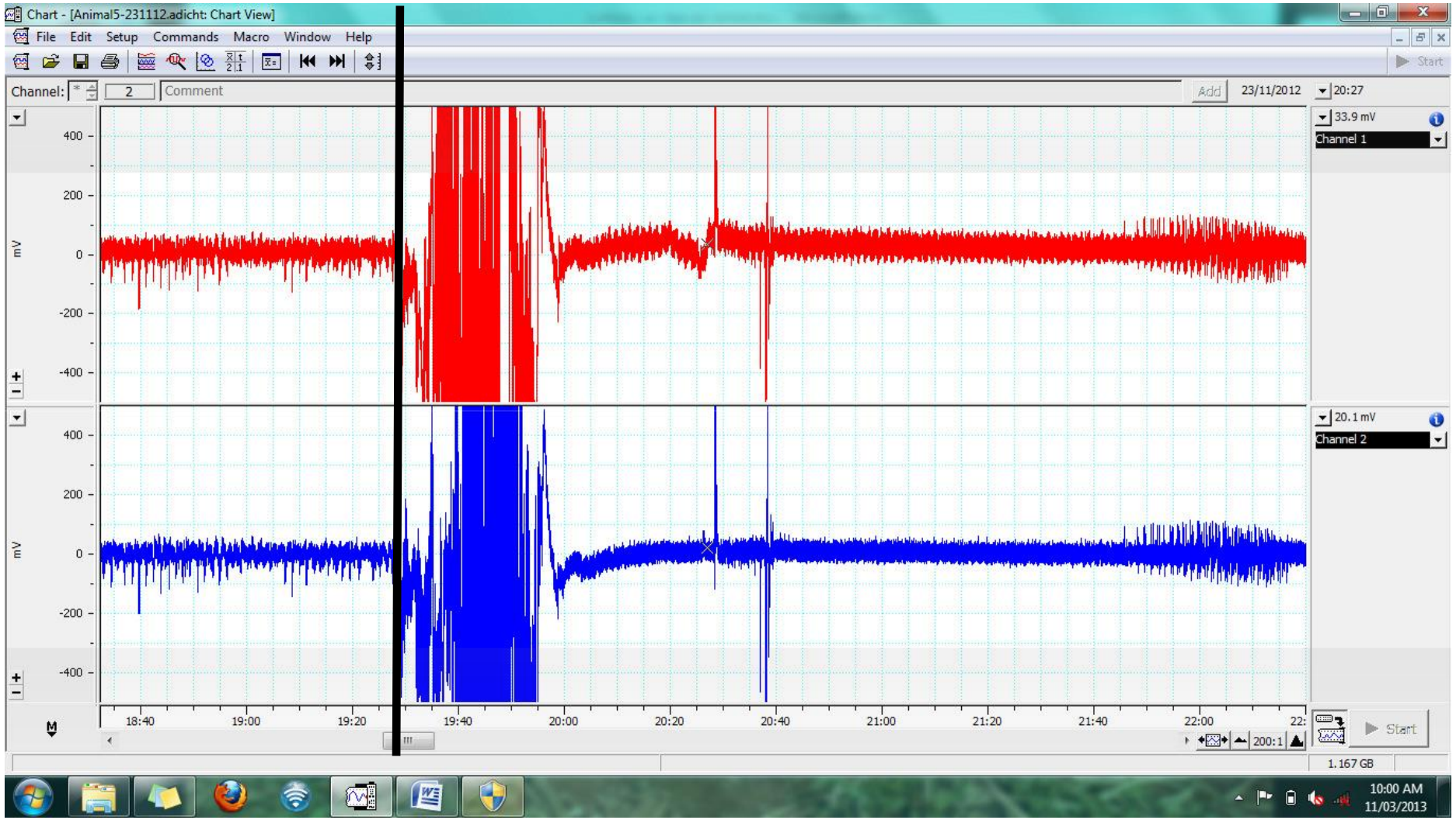
Animal 4 – 1st shot



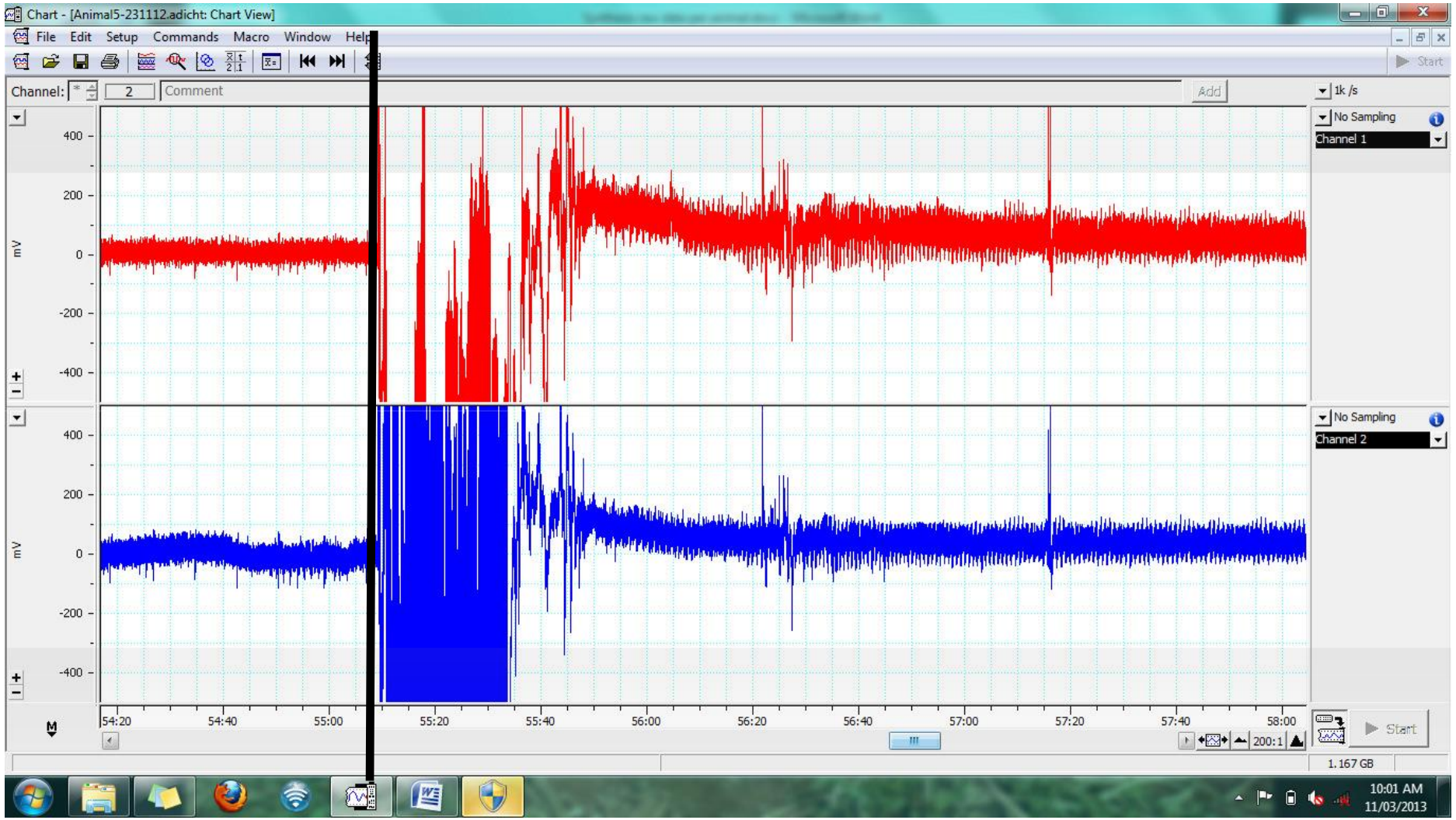
Animal 4 – 2nd shot



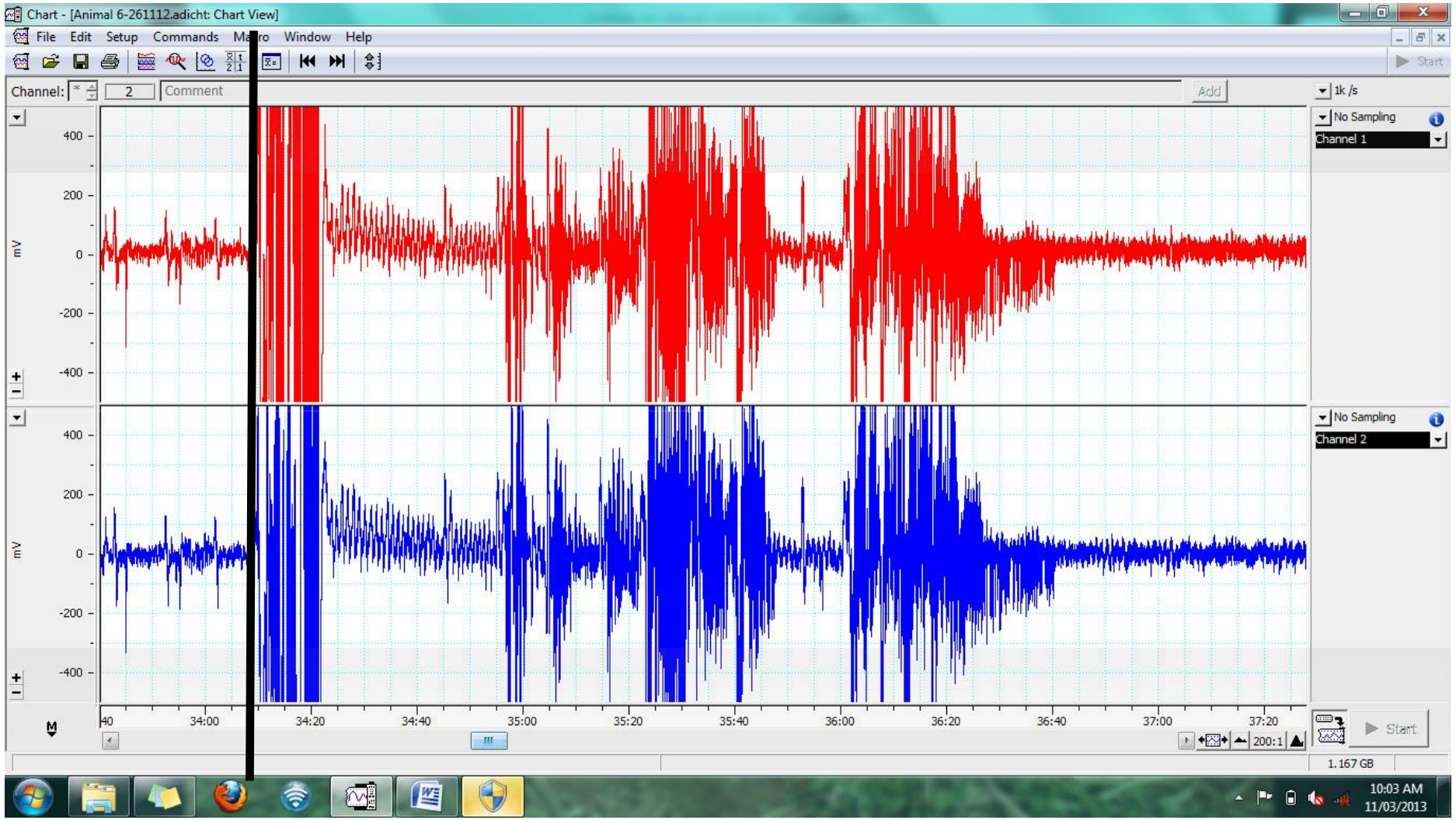
Animal 5 – 1st shot



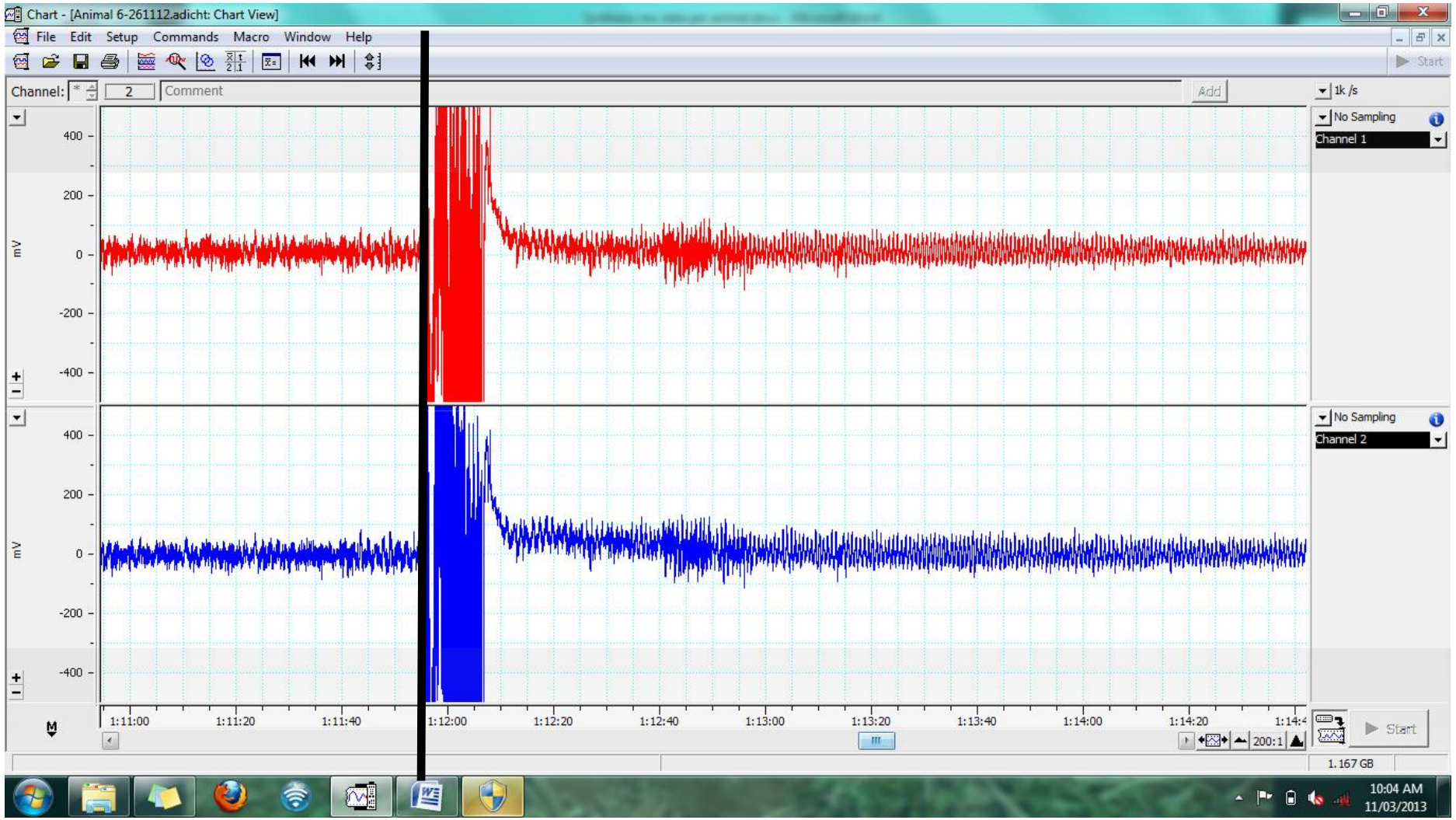
Animal 5 -2nd shot



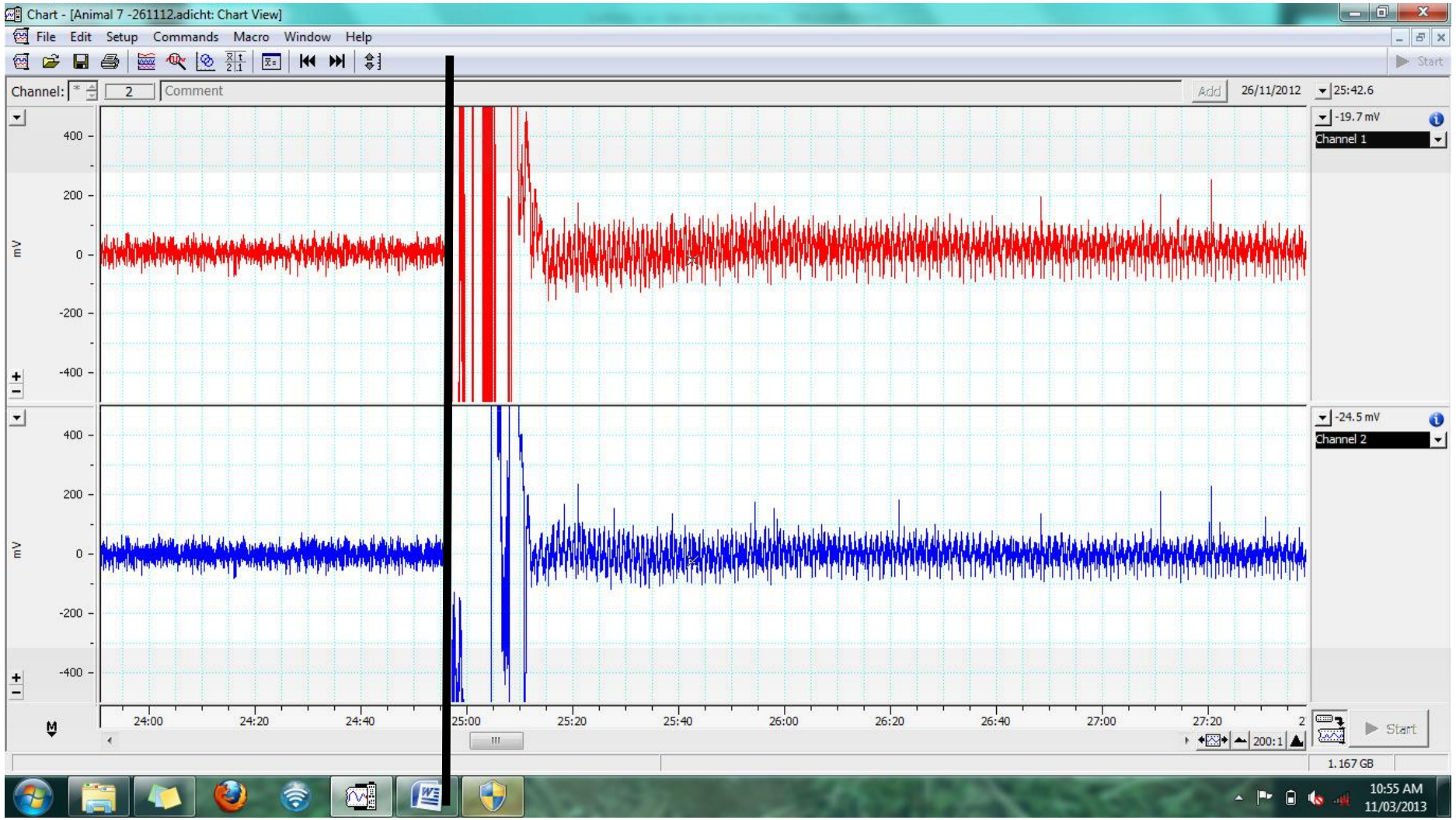
Animal 6 – 1st shot



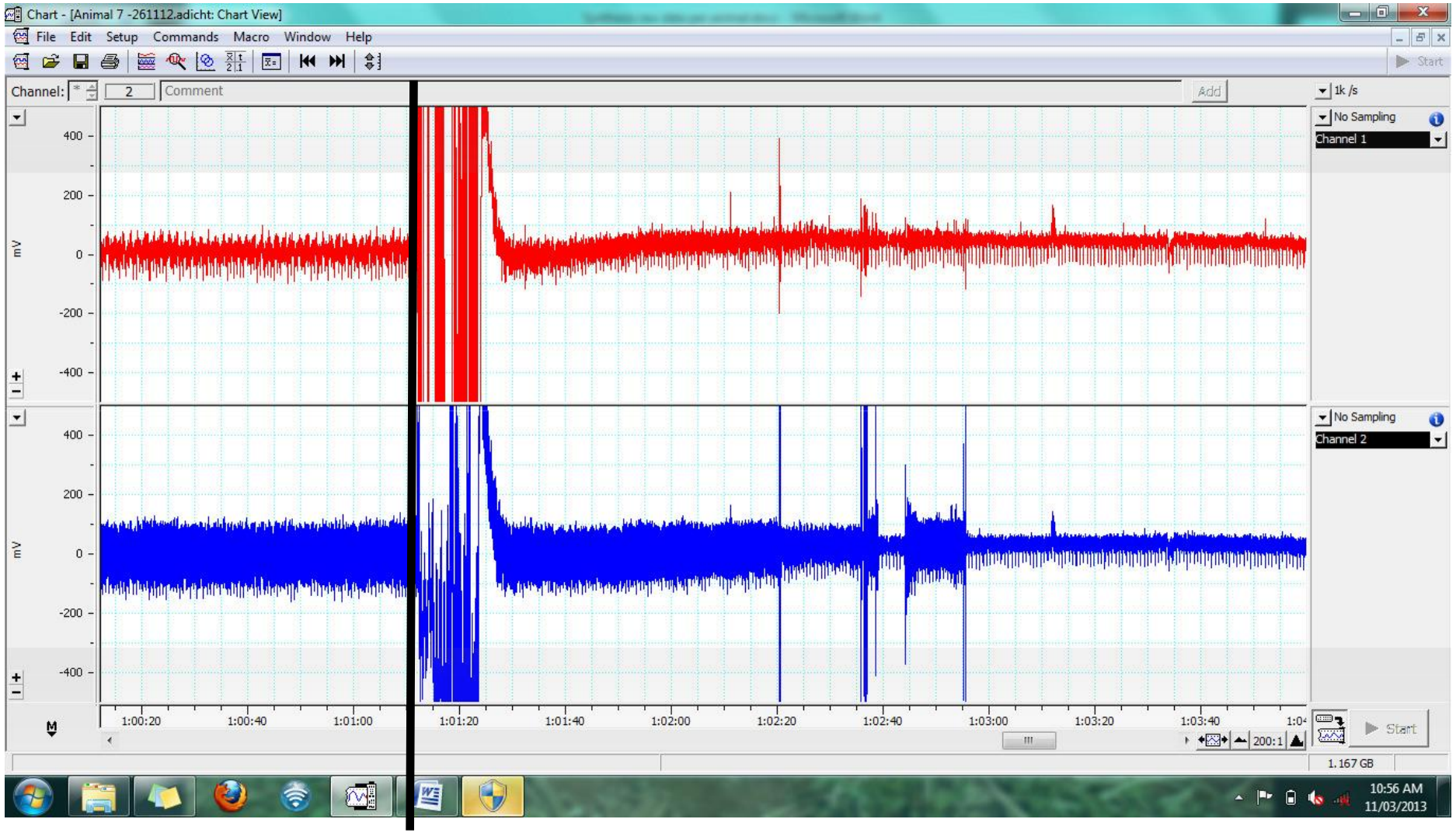
Animal 6 – 2nd shot



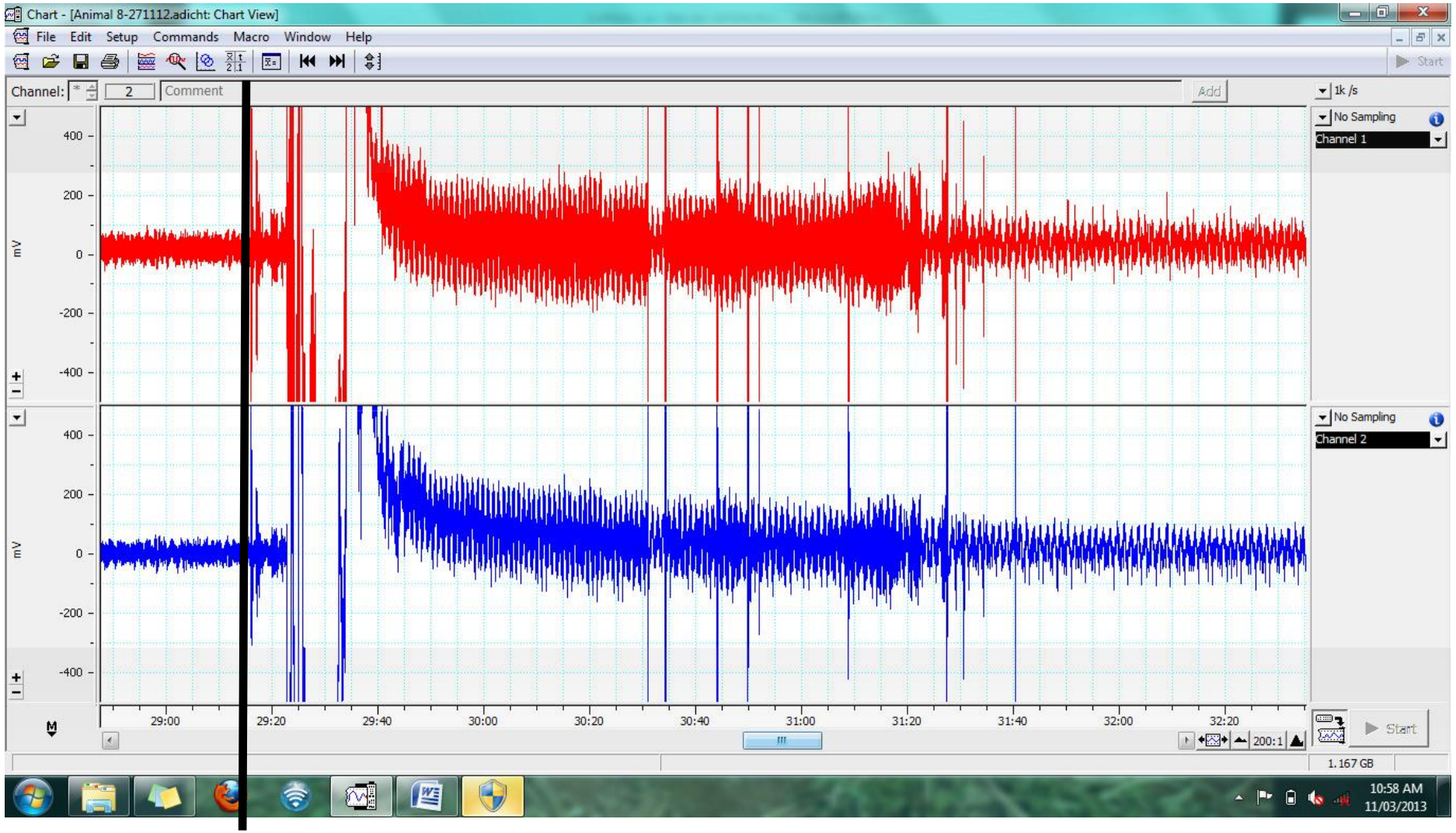
Animal 7 – 1st shot



Animal 7 – 2nd shot

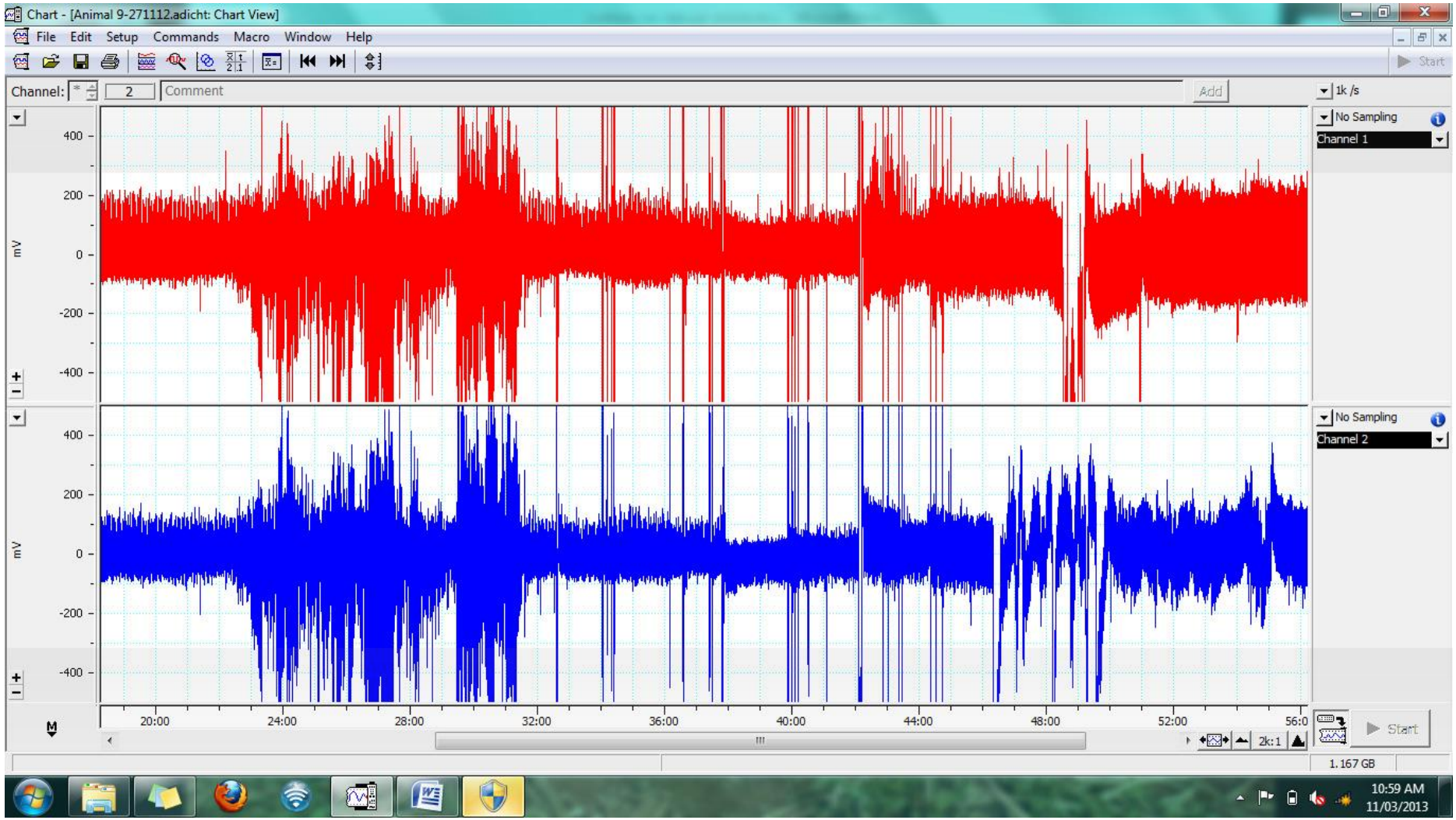


Animal 8 – 1st shot

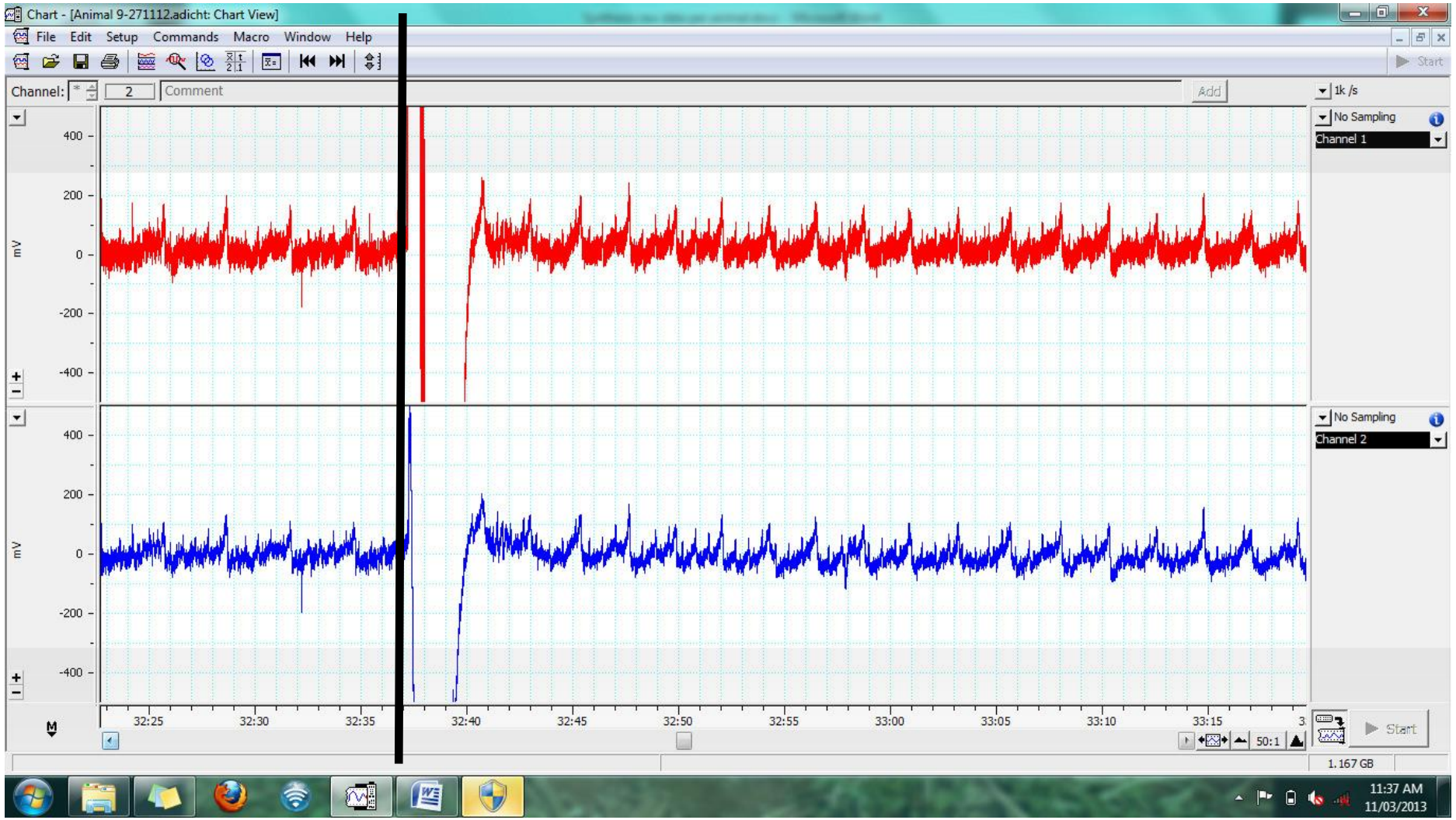


No 2nd shot for that animal

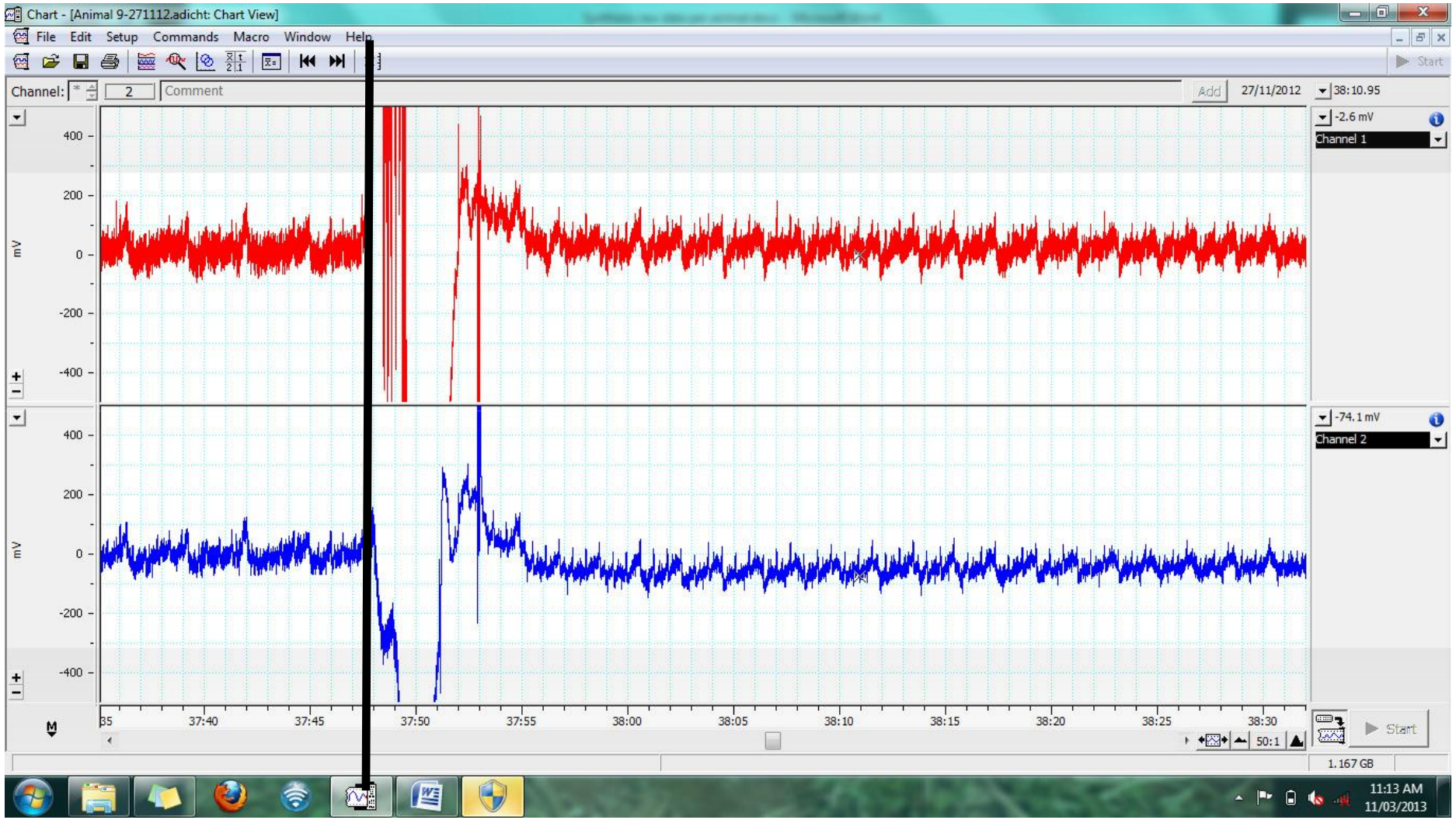
Animal 9 – 1st shot (different X-scale to overview full process with multiple shot attempts)



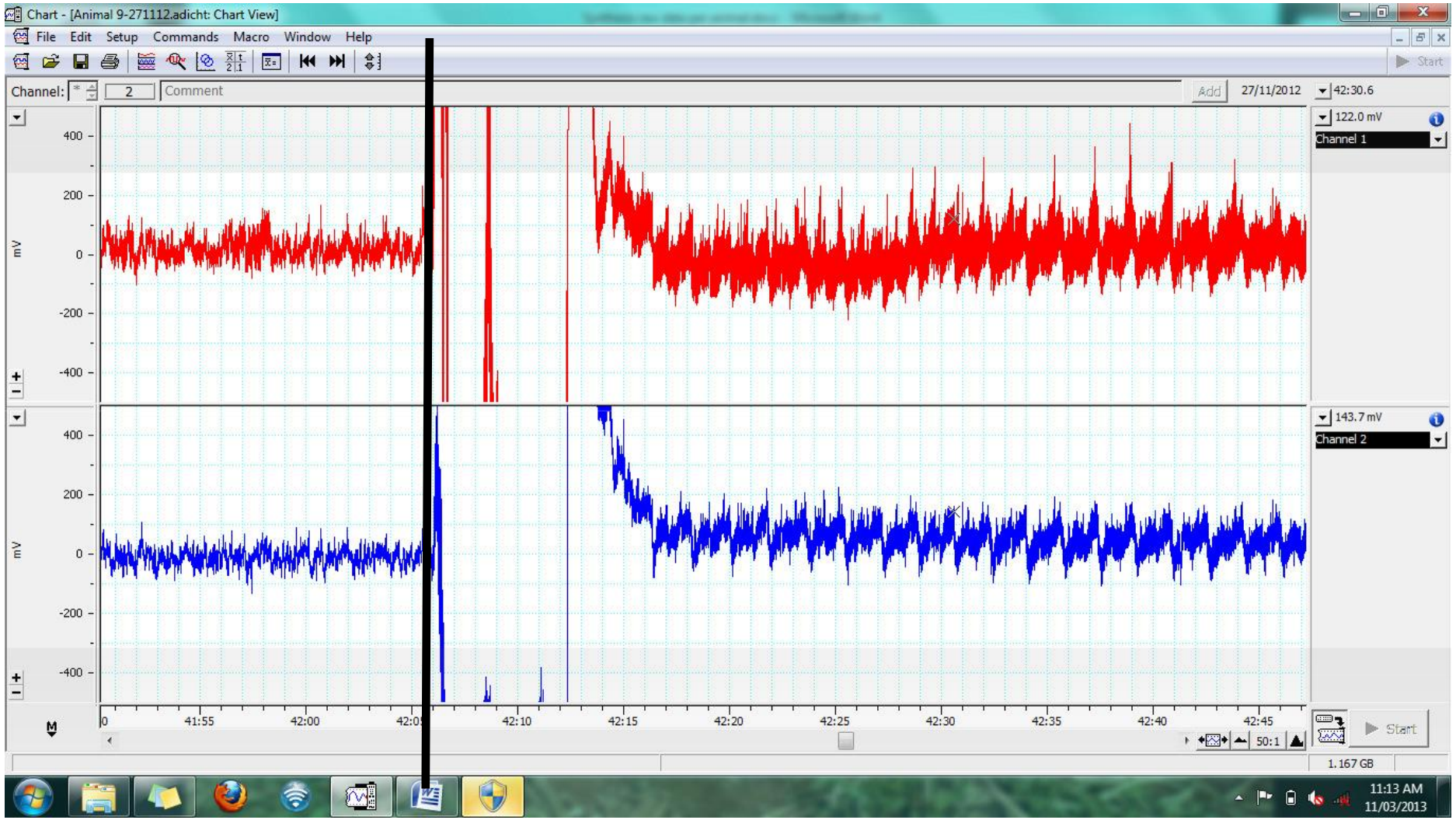
Animal 9 – detailed view 1st (aborted) shot (40 kW for 5 sec)



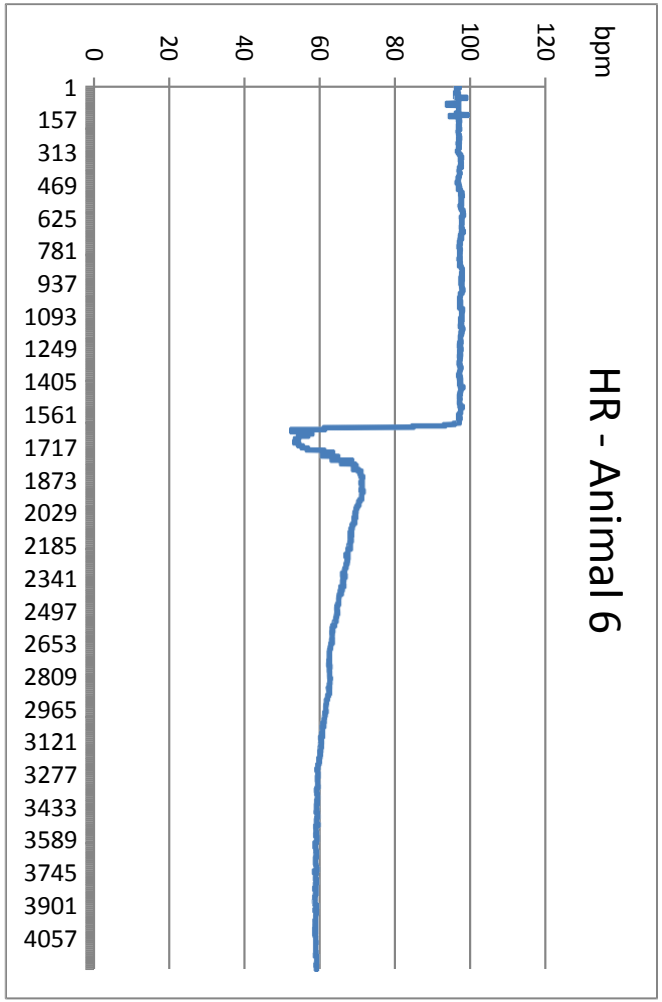
Animal 9 – detailed view 2nd (aborted) shot (40 kW for 5 sec)

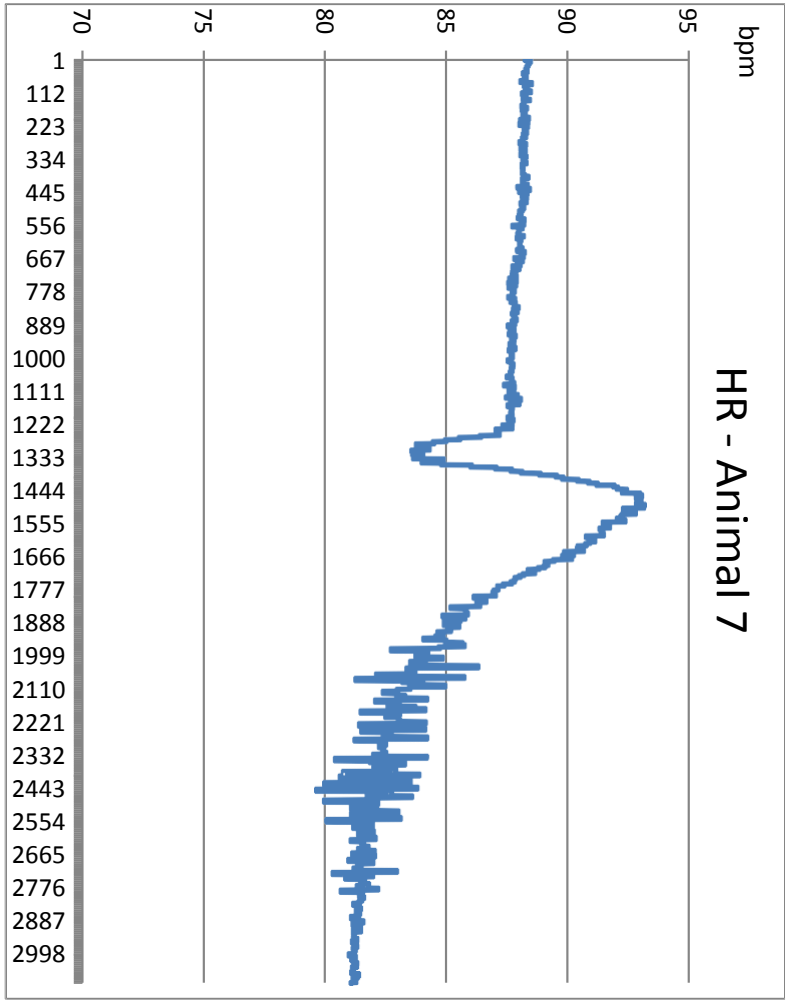


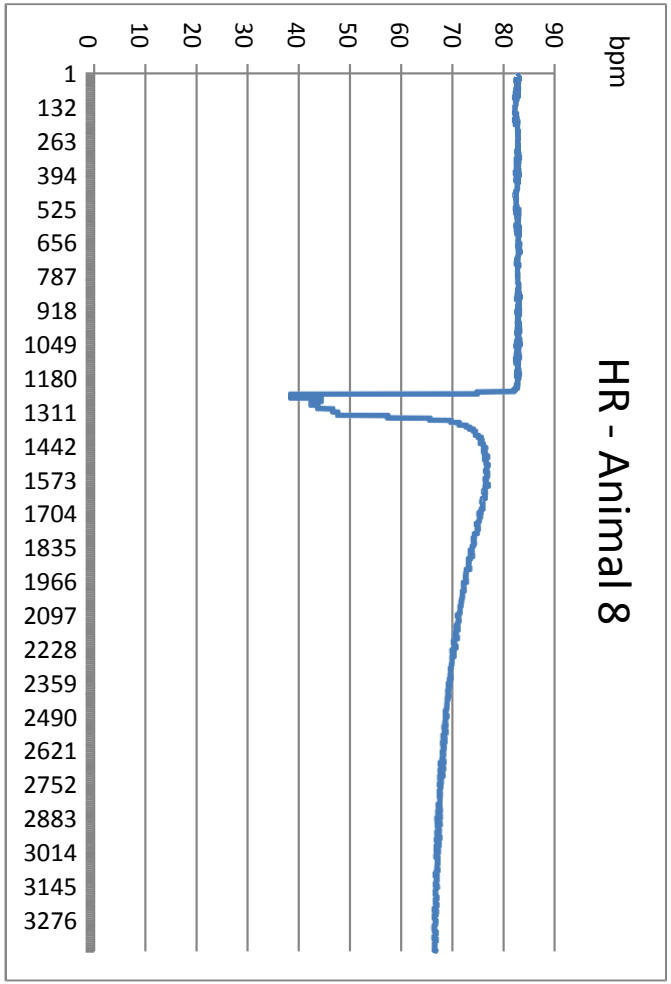
Animal 9 – detailed view 3rd shot (30 kW for 5 sec)

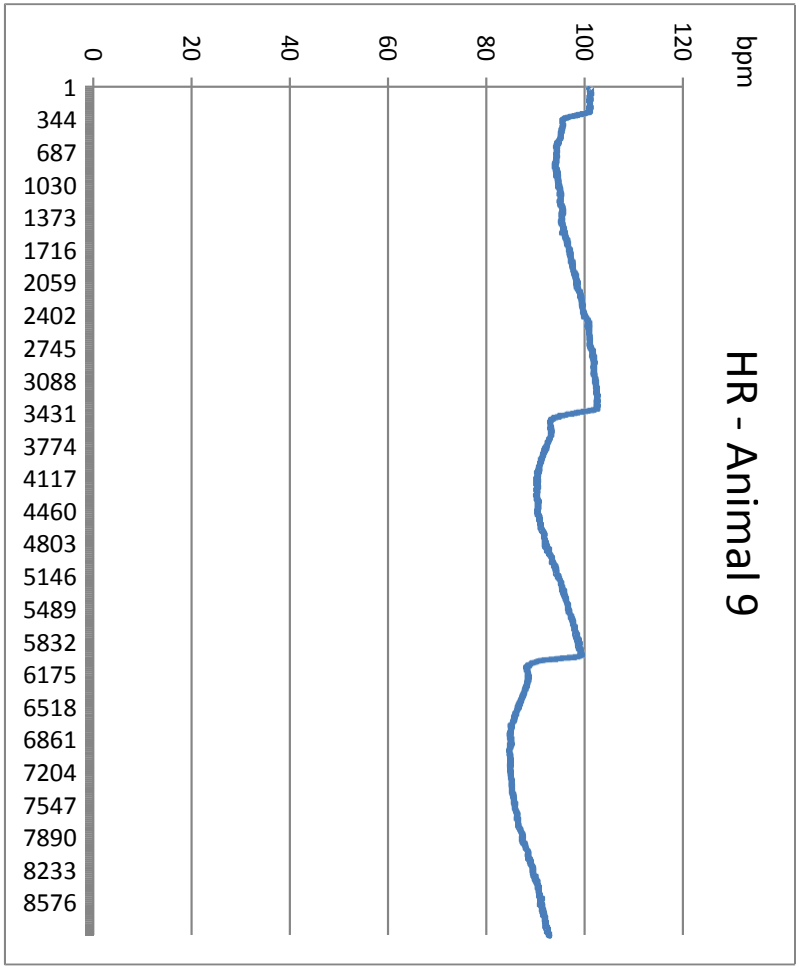


Appendix 3: Raw ECG recordings for each animal






HR - Animal 8
bpm
0
10
20
30
40
50
60
70
80
90
1
132
263
394
525
656
787
918
1049
1180
1311
1442
1573
1704
1835
1966
2097
2228
2359
2490
2621
2752
2883
3014
3145
3276


HR - Animal 9
bpm
0
20
40
60
80
100
120
1
344
687
1030
1373
1716
2059
2402
2745
3088
3431
3774
4117
4460
4803
5146
5489
5832
6175
6518
6861
7204
7547
7890
8233
8576