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In Vitro and In Vivo Delivery of Magnetic Nanoparticle using a Custom-Built Hyperthermia Delivery System

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JoVE
Editor -in -Chief

February 29, 2020

Re: Invited video methods manuscript

Dear Editor-in-Chief at JoVE,

Please consider this manuscript; Design and implementation of a robust magnetic nanoparticle hyperthermia delivery system for *in vitro* and/or *in vivo* experiments, for publication in JoVE. The first and corresponding author is Kayla Duval, a senior graduate student in my lab.

Please let me know if additional information would be helpful. Thank you

Sincerely,

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TITLE:

In Vitro and In Vivo Delivery of Magnetic Nanoparticle using a Custom-Built Hyperthermia Delivery System

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KEYWORDS:

hyperthermia, alternating magnetic field, magnetic nanoparticle, murine melanoma, cell culture, thermal dose

SUMMARY:

This protocol presents techniques and methodology necessary for the accurate delivery of magnetic nanoparticle hyperthermia using a sophisticated delivery and monitoring system.

ABSTRACT:

Hyperthermia has long been used in the treatment of cancer, through a variety of modalities, with temperatures ranging from fever-level (39 ˚C) to greater than 1,000 ˚C (electrocautery). The range of temperatures have very different effects, which is directly related to the temperature and treatment time (thermal dose) with high temperatures resulting in the tissue ablation and lower temperatures creating a variety of sublethal effects such as increased blood flow, accumulation of drugs, and immune stimulation. One of the more recent methods for delivering medical hyperthermia is magnetic nanoparticle therapy. This technique involves activating magnetic nanoparticles that can reside inside or outside of cells. The size and construct of the magnetic nanoparticles and the frequency and field strength of the magnetic field are major heating determinants. Using both in vitro and in vivo techniques and instrumentation, we have assembled a sophisticated process for delivering reproducible hyperthermia in large animal, small animal, and cell biology settings. This approach, using continuous, real time temperature monitoring in multiple locations, allows for the delivery of well-defined doses to the target tissue (tumor) while limiting non-target tissue heating. Precise control and monitoring of temperature allows for the accurate determination of the global quantitative hyperthermia standard: cumulative equivalent minutes at 43 ˚C (CEM43). Our system, which allows for a wide variety of temperatures, thermal doses, and biological effects, was developed through a combination of component acquisitions and inhouse engineering and

biology. This system has been optimized in a manner that allows for the rapid conversion between ex vivo, in vitro, and in vivo situations. The goal of this protocol is to demonstrate how to design and implement an effective technique and system for delivering robust and accurate experimental in vitro and in vivo magnetic nanoparticle therapy (mNP) hyperthermia.

INTRODUCTION:

Hyperthermia has historically been used in cancer therapy, either alone or in combination with other treatments. Although it has a long history of use, the most advantageous method for delivering this treatment is still being debated and is dependent on the disease site and location. Methods for hyperthermia delivery include microwave, radiofrequency, focused ultrasound, laser, and metallic nanoparticles (such as gold or iron oxide)[1–4]. These methods of delivery can lead to a range of treatment temperatures from fever-level through to hundreds of degrees C. The biological effect of hyperthermia depends primarily on the temperatures used and the duration of the treatment[5]. For this manuscript and purpose, we are focusing on magnetic nanoparticle hyperthermia (mNPH). This method allows for focused, localized, well monitored, and controlled temperature changes, using non-toxic, FDA approved, iron oxide nanoparticles.

One pitfall of other hyperthermia modalities is a lack of precise cellular targeting; hyperthermia does not a have an inherently high therapeutic ratio, therefore, careful thermometry and targeting is necessary[6]. mNPH allows for systemic or intratumoral injection of mNPs, with heat only being generated where the mNPs are located, thus targeting the treatment to the tumor directly. mNPH can be effective when the magnetic nanoparticles are located inside or outside of the cell. For cancer therapy, the general overview of mNPH is that the magnetic nanoparticles are injected (intratumorally or intravenously), then an alternating magnetic field is applied, causing the nanoparticle magnetic poles to constantly realign, leading to a localized heating of the cells and tissue associated with the nanoparticles[7,8]. By adjusting the volume of nanoparticles and the frequency/strength of the alternating magnetic field (AMF), it is possible to carefully control the temperature generated within the tissue.

This treatment works well in tumors that are near the body surface, as deeper tumors require stronger AMF so the risk of eddy current heating increases[9]. There is evidence of hyperthermia being used clinically as a monotherapy, however, oftentimes hyperthermia is combined with radiation therapy or chemotherapy, leading to a more targeted anti-cancer effect[10–12]. Clinical evidence of hyperthermia working in combination with radiation therapy is reviewed in a previous publication[13]. Our lab has successfully treated a variety of animals, from mice to pigs and spontaneous canine cancers, using the mNPH method[12,14,15]. This protocol is designed for those interested in investigating the effects of localized hyperthermia treatment, either alone or in combination with other therapies.

One of the most important factors in hyperthermia is being able to measure and understand, in real time, the thermal dose being delivered to the target/tumor tissue. A standard way of calculating and comparing dose is through demonstration of the cumulative equivalent minutes of heating at 43 ˚C; this algorithm allows for the comparison of doses independent of the

delivery system, maximum and minimum temperatures (within a specific range) and heat up/cool down parameters[5,16]. The CEM calculation works best for temperatures between 39-57 ˚C[5]. For example, in some of the studies we have performed, we have chosen a thermal dose of CEM43 30 (i.e., 30 min at 43 ˚C). Choosing this dose allowed us to look at a safe, effective, immunogenetic effects in vitro, both alone, and in combination with a single dose of radiation[17].

With magnetic nanoparticle hyperthermia, there are several factors that need to be considered in building an appropriate delivery system. The instrumentation design includes important safety factors, such as the use of a chiller to ensure the magnetic field delivery equipment remains cool even when operated at high power, and fail-safe procedures that prevent the system from being turned on if all temperature, power assessment, and control systems have not been activated. Additionally, there are important biological factors that need to be considered for both in vivo and in vitro situations. When using cultured cells, it is necessary to treat in growth media and maintain at a consistent viable temperature to avoid physiological changes that could affect results. For individual nanoparticle types, it is important to know the specific absorption rate (SAR) when calculating AMF based heating parameters. Similarly, it is important to know the mNP/Fe concentration, in cells and tissues, that is necessary to achieve the desired heating. In vivo methods require even more attention to detail since the animal must be maintained under anesthesia during treatment and the animal's core body temperature maintained at a normal level throughout the treatment. Allowing for the animal's body temperature to drop, as happens under anesthesia, can affect the overall results, with respect to the thermal dose of the tissue being treated.

In this manuscript, we discuss the methods used to design and construct a versatile magnetic nanoparticle hyperthermia system, as well as important use factors that need to be considered. The system described allows for the robust, consistent, biologically appropriate, safe, and well-controlled delivery of magnetic nanoparticle hyperthermia. Finally, it should be noted that the mNPH studies we conduct often involve other therapies such as radiation, chemotherapy, and immunotherapy. For these results to be meaningful, it is important to determine how the delivered heat can affect the efficacy and/or safety-toxicity of other modalities (or vice versa) and the well-being of the animal. For this reason and the dosimetry and therapeutic situations previously mentioned, it is essential to pay strict attention to the magnetic nanoparticle hyperthermia dosing accuracy and the continuous core and target temperature measurements. The goal of this protocol is to provide a straightforward, consistent method and description for the delivery of safe and effective magnetic nanoparticle hyperthermia.

PROTOCOL:

The Dartmouth College Animal Care and Use Program is accredited by the American Association for the Accreditation of Laboratory Animal Care (iAAALAC) and adheres to all UDSA and NIH (Office of Laboratory Animal Welfare) guidelines and regulations. All in vivo studies were approved by the Dartmouth College Institutional Animal Care and Use Committee (IACUC). Euthanasia procedure adhere to the 2020 AVMA *Guidelines for the Euthanasia of Animals.*

1. Instrumentation/design of the system

1.1. Design custom AMF antenna (coil) to be a closed loop, choosing shapes to create the desired magnetic field. Use inductance formulas and characteristics from power generator choice to design compatible coils to generate the desired field. Use different designs for in vitro and in vivo experiments.

1.2. Ensure the AMF antenna inductance falls within the acceptable range of the power generator. Add or subtract capacitors to match (tune) the antenna to the power generator.

1.3. For in vitro experiments, design a 14-turn helical coil, inner diameter 2 cm and length 14 cm, that can contain 1.5 mL tubes, allowing for the treatment of multiple samples simultaneously. Insulate the coil with a vinyl polymer and use a polystyrene spacer to separate the coil from the tubes. Details of design specification and considerations are present in the **Supplementary File 1.**

1.4. For in vivo experiments, acquire a custom built whole-body helical coil from a manufacturer with proprietary design information. Use 8 mm square tubing (as it creates a more uniform field within the bore of the coil), and a concentrator at the targeted treatment area. Make the concentrator 5.0 cm long, with a total of 5 turns resulting in a 3.6 cm inner diameter, 5.2 cm outer diameter, and have its location at the targeted treatment area. Surround the coil with a polycarbonate shell.

1.5. Use an AMF generator with adjustable power and frequency, rated at 10 kW or greater as the power source. Inductance match the power source and antennae/coils to a range of 0.62 to 1.18 µHenries (µH), allowing for frequencies ranging between 30-300 kHz. Cool the generator using recycled water through a centrifugal boost pump, pressure regulated to 50 psi.

1.6. Cool the coils with a 5.6 ton cooling capacity chiller that pumps 25% ethylene glycol-based heat transfer fluid diluted with water through the AMF antenna. Set the temperature of the chiller to such that the antenna does not heat or cool the sample.

1.7. For the animal containment, construct a tubular holder that can be suspended in the center of the coil with a 0.5 cm air gap between the holder and the coil surface. Connect an adjustable conditioned air pump that circulates air through the shell around the coil and set it to maintain a normal animal core temperature. Connect the anesthesia machine to the tubular animal holder near the head of the animal to ensure proper delivery of anesthesia.

1.8. For cell containment, create an apparatus that circulates water from a water bath through the spacer where tubes are placed. Set the temperature of this water bath such that the tubes are surrounded by water at 37 °C.

1.9. Use fiber optic probes to monitor temperatures within the tumor, the animal's core, and the animal environment or for in vitro studies, monitoring the temperature of the cell pellet,

and the water surrounding the tubes.

1.10. Use magnetic iron oxide nanoparticles that are 100 nm in size for all experiments.

NOTE: Concentration and specific absorption rate (SAR) are two characteristics that must be considered when choosing nanoparticles, as they directly affect the possible heating and thermal dose[18].

2. Hyperthermia in vitro

2.1. Culture B16F10 murine melanoma cells in RPMI media with 10% FBS and 1% Pen/strep. Plate 150, 000 cells/well in 6-well plates, with 2 mL of complete medium.

2.2. Determine the appropriate treatment for each well, i.e., cells without mNPs and no AMF, cells with mNPs and no AMF, cells without mNPs and AMF, cells with mNPs and AMF.

NOTE: Additionally, ensure there are appropriate controls if combining hyperthermia with another therapy. AMF is performed in a standard research bench laboratory retrofitted with the needed power and cooling capabilities.

2.3. 24 h following plating, add mNPs to the appropriate wells as determined in the previous step. Add mNPs to a concentration of 3 mg iron/mL. Ensure the mNPs are distributed throughout the well, either by creating a stock media/mNP solution (removing old media, adding this solution) or by adding the mNPs directly and gently swirling plates for homogenous distribution.

2.4. Begin the treatment, 48 h after the addition of mNPs, when wells are ~80% confluent, by removing the media and washing wells with fresh media. Remove the media.

2.5. Add 0.5 mL of trypsin to each well being treated, and gently swirl. Use a microscope to check that the cells are detached.

2.6. Add 1 mL of media to each well to collect the cells into 1.5 mL tubes. Collect all the cells from the well (~1 x 10^6 cells). Use a clearly labeled separate tube for each well.

2.7. Spin tubes at 60 *x g* for 2-3 min to allow cells to pellet. Retain the pellet in the media.

2.8. Place tubes in the spacer full of water within the coil. Set the temperature of the water bath such that the media and cell pellet are maintained at 37 °C. Monitor the temperature within the tube and the water bath using separate fiber optic temperature probes.

2.9. Turn on the chiller, check that the coolant is flowing through the coil. Turn on the power source and adjust the percent of maximum to field desired. Operate the 14-turn solenoid coil, powered by 10 kW generator, at 165 kHz and 23.87 kA/m (300 Oe).

2.10. Place a separate fiber optic temperature probe into one of the tubes. Treat the cells until the previously determined protocol thermal dose. An example is 30 min at 43 ˚C (CEM43 of 30).

2.11. Resuspend cells in the media that is in their tubes and re-plate into new 6 well plates. Clearly label the new plates. The goal is to re-plate all the cells collected (~1 x 10^6 cells).

NOTE: New 6 well plates should be used to ensure the cells being cultured received treatment. If the old plates are used there could still be cells left on the plates that were not successfully trypsinized.

2.12. If necessary, for the next experimental procedure, lyse the cells for RNA or protein expression analysis.

3. Hyperthermia in vivo

3.1. Cell culture and inoculation

3.1.1. Culture B16F10 murine melanoma cells in RPMI media with 10% FBS and 1% Pen/strep. Use plates/dishes that will provide enough cells for inoculation of the desired number of animals. For example, 10, 100 mm dishes, plated at 100, 000 cells will be confluent with enough cells for 20 mice injections within 48 h.

3.1.2. Trypsinize cells and collect using pure RPMI media (no FBS or pen/strep).

3.1.3. Count cells and create a solution for the desired concentration of cells, based on the inoculation volume, and mouse numbers.

3.1.4. Anesthetize 6-week old female C57Bl/6 mice using vaporized isoflurane and oxygen. Place animals into a plexiglass box with 5% isoflurane and 95% oxygen until induced. Once induced, remove animal and use a face cone at 2% isoflurane to complete steps 3.1.5-3.1.7 and 3.3.3-3.3.6.

NOTE: For anesthesia during the treatment use a built-in anesthesia containment. Follow standard institutional protocols for mouse anesthesia. Prior to animal experimentation ensure appropriate IACUC approval. After anesthesia, return the animal to cage and monitor recovery to ensure no complications.

3.1.5. Check for the lack of response to the righting reflexes.

3.1.6. Shave the right flank using an electric shaver.

3.1.7. Clean the injection area with an alcohol wipe. Inject 1-2 x 10^6 cells, using a 100 µL glass syringe with a 28 G needle, dispersed in 50 µL of media intradermally on the shaved right flank

of anesthetized.

3.2. Tumor growth/Nanoparticle injection

3.2.1. Measure tumors in 3 dimensions using calipers (length, width, and depth), and calculate volumes by (length x width x depth x $\boldsymbol{\pi}$)/6.

3.2.2. When tumor volumes reach 120 mm^3 (+/- 20 mm^3), place the animals on study. Design the study, ensuring there are appropriate control and treatment groups including combination therapy cohorts (i.e., control, mNPH, radiation, and the combination).

3.2.3. Anesthetize mice that will be receiving mNPs as described in 3.1.4.

3.2.4. Clean the area with an alcohol wipe. Inject mNPs into the tumor 3 h before AMF treatment. Inject a volume such that the dose is 7.5 mg of iron/cm^3 of tumor.

NOTE: Unpublished data from the lab suggests maximal mNP uptake occurs at 3-6 h.

3.3. AMF treatment

3.3.1. Anesthetize the mouse and place on a heating pad to maintain core temperature.

3.3.2. Check for the lack of response to the righting reflexes. Remove the ear tag or any other metal objects on the mouse.

3.3.3. Gently place a lubed fiber optic temperature probe into the rectum of the mouse.

3.3.4. Place a catheter into the tumor, removing the needle. Cut the catheter such that it does not stick out of the tumor too much.

3.3.5. Insert a 3-sensor fiber optic temperature probe into the catheter. The catheter protects the fiber optic temperature probe sensors.

3.3.6. Tape the rectal and intratumoral probe to the tail of the animal to ensure they remain in place.

3.3.7. Place the mouse into a 50 mL tube, head to the bottom. The tube should have a hole near the head where the anesthesia will be connected and delivered.

3.3.8. Place the tube within the coil set up and reconnect the anesthesia.

3.3.9. Place a fiber optic temperature probe loosely into the tube to measure the environment temperature.

3.3.10. Turn on the chiller, and ensure coolant is being circulated.

3.3.11. Check and ensure the computer software is displaying the various temperatures and begin recording to allow for a CEM43 calculation to be displayed in real time. The required CEM43 is the dose previously determined.

NOTE: Before the magnet is turned on, ensure no metal items are attached to the animal, as these will heat rapidly. Additionally, ensure that everyone in the room does not have a pacemaker and it is safe for them to be there.

3.3.12. Turn on the magnet at a low power percentage.

3.3.13. Ensure that the fiber optic temperature probes are recording temperature changes. Temperatures will increase once the AMF is activated as the field increases. Ensure the core temperature of the animal remains at 38 ˚C. Regulate the core temperature using the conditioned air jacket.

3.3.14. Adjust the strength of the magnetic field by changing the power on the generator, using the built-in control dial, which in turn controls the temperature level in the tumor.

3.3.15. Shut off the AMF once desired dose, as previously determined by the user (for example CEM43 40), is achieved within the tumor.

3.3.16. Once the AMF is shut down, remove the tube from the coil.

3.3.17. Remove the mouse from the tube, extracting the various probes & catheter. If necessary, tag the animal with a new metal ear tag.

3.3.18. Once treatments are completed, shut down the chiller.

3.3.19. Recover the animals from anesthesia ensuring no complications. Monitor their behavior to ensure return to normal.

REPRESENTATIVE RESULTS:

In vitro studies

Cells will only achieve and maintain the desired temperature and thermal dose if the amount and concentration of the magnetic nanoparticles/iron and the AMF are appropriately matched. When using magnetic nanoparticles to heat cells in vitro (and in vivo), it should be noted that to achieve hyperthermia in cells with internalized magnetic nanoparticles, a specific level of intracellular mNP/Fe will be necessary, and number and proximity of mNP loaded cells, to each other, will be necessary. If the level of mNP/Fe in the target cells/tissue is sufficient to achieve a heating effect, the magnetic field frequency and strength can be adjusted to achieve the desired temperature and effects. If plated properly, then further studies looking at genetic and molecular differences between different doses and timings can be pursued[17]. **Figure 1**

represents a schematic of the in vitro methods.

These in vitro methods can be used to investigate cellular mRNA and protein expression change. A recent example from our lab determined immunogenetic differences following CEM43 30 mNPH treatment, an 8 Gy radiation treatment, and the combination. We were able to identify similarities and differences in expression across immune and cytotoxic pathways to gain a better understanding into the mechanism behind the effects, and how they combine synergistically[17]. Every experiment utilizes a variety of environmentally and heated control samples. The controls will have different mRNA and protein expression levels as compared to those receiving hyperthermia treatment.

In vivo studies

In in vivo studies there are additional considerations. Regardless of the target thermal dose it is absolutely essential to maintain a physiologically acceptable core temperature in the animal being treated. This can be challenging with rodents under anesthesia as core temperature can be quickly lost (core temperature modulating techniques such as heating pads are often necessary). Lower than normal body temperatures can necessitate the need to push the AMF-mNPH too far, when trying to achieve a specific thermal dose in the tumor, resulting in unacceptable effects in the non-target tissue (non-target tissue eddy current heating is one such possibility). Even minor deviations in core body temperature can lead to undesirable physiological complications in the tumor or normal tissue. As mentioned previously, however worth repeating, for accurate, reproducible heating, it is essential to achieve a match between the mNP/Fe tissue concentration, AMF frequency, and field strength temperature monitoring parameters and target tissue size and depth. There must be a baseline concentration of mNPs within the tumor to allow for measurable heating. The level/ability of heat depends on not only mNP tissue concentration (mg Fe/g tissue) and their relative distribution within the tumor, but also the frequency of the AMF and subsequent field strength. Changes in any of the above can lead to different ranges of attainable temperatures within the tissue. Through many years of experience, we have optimized the concentration we use for preclinical tumor treatments and the frequency and field strength of the AMF system to allow for safe and effective activation. Because it is impossible to measure the temperature/thermal dose in all tissue sites, it is also essential to place as many fiber optical temperature probes as possible in strategic sites that allow for real-time efficacy and safety assessment, as seen in **Figure 2**. These probes allow for the recording of temperatures throughout the experiment, allowing for accurate dosimetry and thermal history of the experiment. **Figure 3** demonstrates curves generated during an in vivo experiment, highlighting the capability to closely monitor temperature and adjust the system to maintain tumor temperatures within the desired range. **Figure 4** summarizes the in vivo methods.

These in vivo methods, similar to the in vitro methods, can be used to investigate different cancer type, different hyperthermia doses, and with various combination treatments. For example, previous studies in our laboratory have investigated the combination of hyperthermia and chemotherapy[12]. We have also completed numerous hyperthermia and radiation experiments for the determination of efficacy and molecular mechanisms. The control mice for

these experiments undergo all procedures except for the actual generation of hyperthermia. **Figure 5** contains two volcano plots that demonstrate differentially expressed genes following in vitro and in vivo mNP hyperthermia treatment(mNPH). These figures are examples of how we use molecular techniques to monitor the hyperthermia effects.

FIGURE AND TABLE LEGENDS:

Figure 1: In vitro mNP hyperthermia schematic. This schematic demonstrates the method for in vitro magnetic nanoparticle hyperthermia. To ensure heating occurs, cells must be provided enough particles and time for adequate mNP uptake.

Figure 2: Placement of catheters for temperature monitoring. This figure demonstrates the placement of catheters that house the fiber optic temperature probes to record temperatures at different locations in the tumor and/or the tumor region. This figure is adapted from ref.[19].

Figure 3: Real time temperature monitoring during treatment of a mouse tumor. This graph demonstrates the real time temperature readings that allow for monitoring the core body temperature, the environmental temperatures, and multiple temperatures within the tumor, during an in vivo experiment. The control of temperatures within the tumor are demonstrated through the minimal large-scale variations on the zoomed in portion of the figure.

Figure 4: In vivo mNP hyperthermia schematic. This schematic demonstrates the method for *in vivo* magnetic nanoparticle hyperthermia. Injection of sufficient nanoparticles as well as enough time for distribution and absorption, ensures the ability to deliver the desired thermal dose.

Figure 5: Differential gene expression. Differential gene expression following in vitro (**A**) and in vivo (**B**) mNP hyperthermia treatment. These volcano plots represent genetic changes on a log 2 x-axis, with significance on the y-axis, for both in vitro and in vivo mNPH methods. Each circle represents a different gene, with the 20 most significant differentially expressed genes labeled. The further the gene is from zero on the x-axis, the greater the fold change, and the higher the gene is on the y-axis, the lower the p-value. Although both had the same thermal dose, in vivo hyperthermia led to greater gene expression changes than in vitro. These plots are examples of the biological data that can be generated using the protocol described. The in vitro volcano plot has been adapted from ref.[17].

DISCUSSION

The design and implementation of this system provides the ability to conduct accurate and reproducible in vitro and in vivo magnetic nanoparticle hyperthermia experiments. It is critical that the system is designed such that the AMF frequency and field strength are adequately matched to the magnetic nanoparticle type, concentration, and the tissue location and temperature desired. Additionally, the accurate monitoring of the temperature in real time is crucial for safety and the calculation of an accurate thermal dose (cumulative equivalent minutes at 43 °C/ CEM). The placement of probes as demonstrated in **Figure 1**, allows for the real time monitoring of thermal dose and core body temperature as seen in **Figure 2**.

The first step in accurate delivery of magnetic nanoparticle hyperthermia is building a safe system for animals and operators. All components of the system should also be well understood from an operational and delivery standpoint. In this situation, that means understanding the potential for AMF eddy currents and knowing where magnetic particles are located. The antennas, or coils, are a key factor in the shape and strength of the field, and the cooling system used is important to prevent coil overheating[20]. The field strength outside of the conductor is directly proportional to the current strength flowing through the conductor. The magnetic field strength at any point in the space surrounding the conductor is the vector sum of the fields produced by the conductors in the surrounding area. The magnetic field is produced at a right angle to the current flow and the strength decreases exponentially, as a function of the distance from the conductor, as per the Biot-Savart inverse square rule[21]. Thus, square tubing is used for in vivo hyperthermia for a more uniform field within the coil. Creating a magnetic field with the strength and volume needed for a potentially clinically relevant system, requires a high electrical current. Therefore, antenna designs must be able to accommodate significant electrical power levels. Also, AMF antennas must be designed so their inductance falls within the acceptable range of the power generator. At the frequencies typically used, most of the current flow is on the surface of the antenna conductor, meaning the surface affects the resistive heating which can be minimized by eliminating surface defects. This resistive heating also means that a coil cooling system is needed to ensure the coil and environment does not overheat.

A limitation of our system design is that it does not allow for a total range of frequencies and magnetic fields, but it does allow for fields to be generated that are appropriate for cells, rodents and large animals. Specifically, the maximum field strength available from any induction heating system is directly related to the current flow in the antenna (coil). AMF generators are rated in kilowatts, which are calculated by multiplying the available voltage by the available current (amperes). So, a 10kW system with a 500 V limit would have a maximum amperage of 20 A. The coils design will determine which limit is reached first, and thus the systems limit. The magnetic field strength created by any current decreases exponentially as a function of the distance from the conductor. Therefore, a larger diameter coil with the same geometry as a smaller diameter coil, run on the same system, would have a lower field strength at the center of the coil. Thus, the required magnetic field size and strength are limited by the capacity of the AMF generator. Building a larger coil and using more power leads to additional concerns, primarily eddy current heating.

There are several safety concerns that must be addressed when using this system to protect users, animals, and the system itself. First, adequate room ventilation must be maintained during the use of anesthesia. Second, all areas associated with the coil must be clear of metal and or conductors including high saline mixtures. Users must remove rings and other jewelry when working around the AMF, and samples should not contain any type of metal. Of most importance, people with pacemakers or other implanted devices or objects should consult with their doctor before working around the AMF. To protect the system, a fail-safe system should be used that ensures the generator and coil cooling needs are met before power is applied.

Additionally, a thermal camera overview should be used to detect unintended heating.

For in vitro studies, the most important steps to follow are the concentration of iron in cells, the concentration of cells, AMF parameters, and thermal dose assessment. Cells can be treated/heated with magnetic nanoparticle hyperthermia by placing the magnetic nanoparticles in the supernatant, cells, or both. The amount of magnetic nanoparticle heating will depend on the level of magnetic nanoparticles/Fe. If the desire is to treat only cells with internalized iron, our experience is that individual cancer cells will only uptake a limited number of magnetic nanoparticles and that even when the uptake is optimal the cells must be aggregated/pelleted to create cell heating situation, even with optimized AMF. Maintaining the temperature of the media and cells at biologically relevant levels (when not being heated) is also important for accurate measurement of true heating. The 14-turn solenoid coil described here allows for biologically relevant temperatures to be maintained by submerging the samples in a thermally controlled water column.

For the in vivo studies, maintaining the animal core temperature and accurately measuring the temperature within the tumor are key factors. This animal containment system and design of the coil eliminates thermal drift in the animal's environment due to coil/power settings and helps to maintain normal core body temperature. Maintaining the body core temperature is critical for meaningful experiment results. The rectal probe allows for real time monitoring of the animal's core temperature. When under anesthesia, an animal's core temperature inherently decreases. To address this situation, we developed an environmental heating system that delivers warm air around the animal containment vessel, allowing the core temperature to stay in the normal range. Maintaining normal core temperature is essential for ensuring accurate interpretation of hyperthermia treatment results, and the elimination of environmental factors. The placement of the temperature monitoring probes in multiple sites in the target tissue/tumor is important to get an accurate assessment of the temperature and thermal dose achieved. Because it is extremely difficult if not impossible to distribute magnetic nanoparticles homogeneously within a tumor, knowing the heating parameters in multiple sites is essential at achieving a consistent and accurate tissue/tumor thermal dose. It is important to note that the concentration for in vitro and in vivo studies is variable. This variation is because there are fewer boundaries in cell culture with cells having more access to the mNPs, so a lower concentration can be used. In vivo, a higher concentration is necessary due to the heterogenous nature of tumors and the complicated 3D morphology. Therefore, using the same concentration of particles in vivo and in vitro would lead to far fewer being taken up by cells.

This manuscript describes the parameters and instrumentation necessary to develop an effective and flexible alternating magnetic field generator and coil system for magnetic nanoparticle hyperthermia treatments. This system can be used for both in vitro and in vivo studies. The system is effective for localized/targeted hyperthermia and the sparing of normal tissue making it appealing, compared to other AMF-mNP hyperthermia systems. These hyperthermia treatments can be altered to investigate the effects of different doses, with a variety of nanoparticles or nanocarriers and adjunct treatments. Since tissue heating, especially magnetic nanoparticle heating, can be affected by so many variables, it is essential to

understand the parameters in an investigation. If these criteria are met magnetic nanoparticle hyperthermia can address many molecular, cellular, and clinical situations, including independent and adjuvant tumor control. Although the methods described here require significant effort, if the guidelines are followed, the full potential of mNP hyperthermia can be realized.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

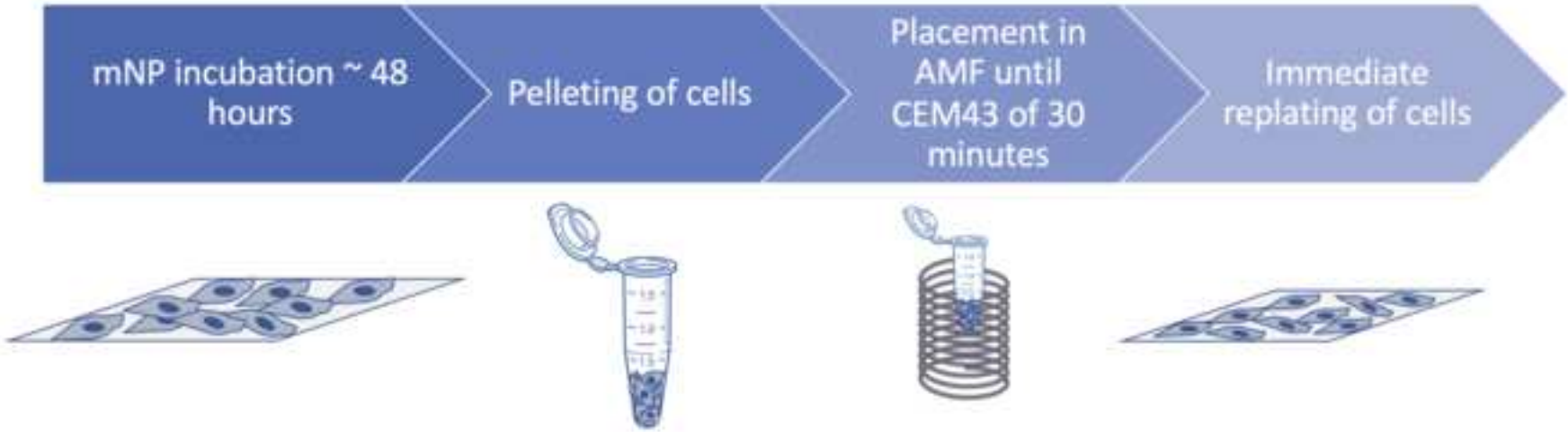

Figure 2

Click here to access/download;Figure;figure 2 updated.tiff

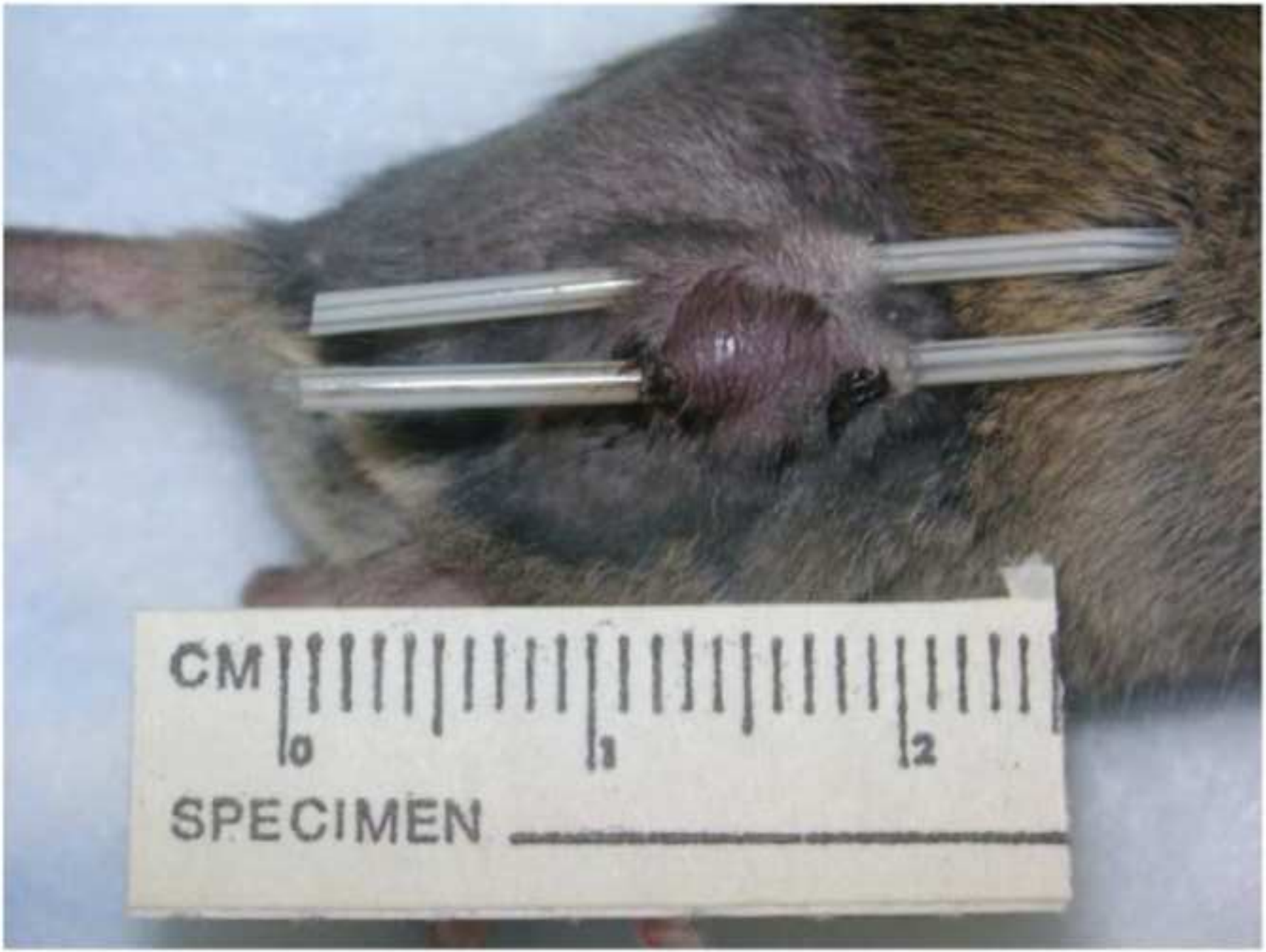

Figure 3

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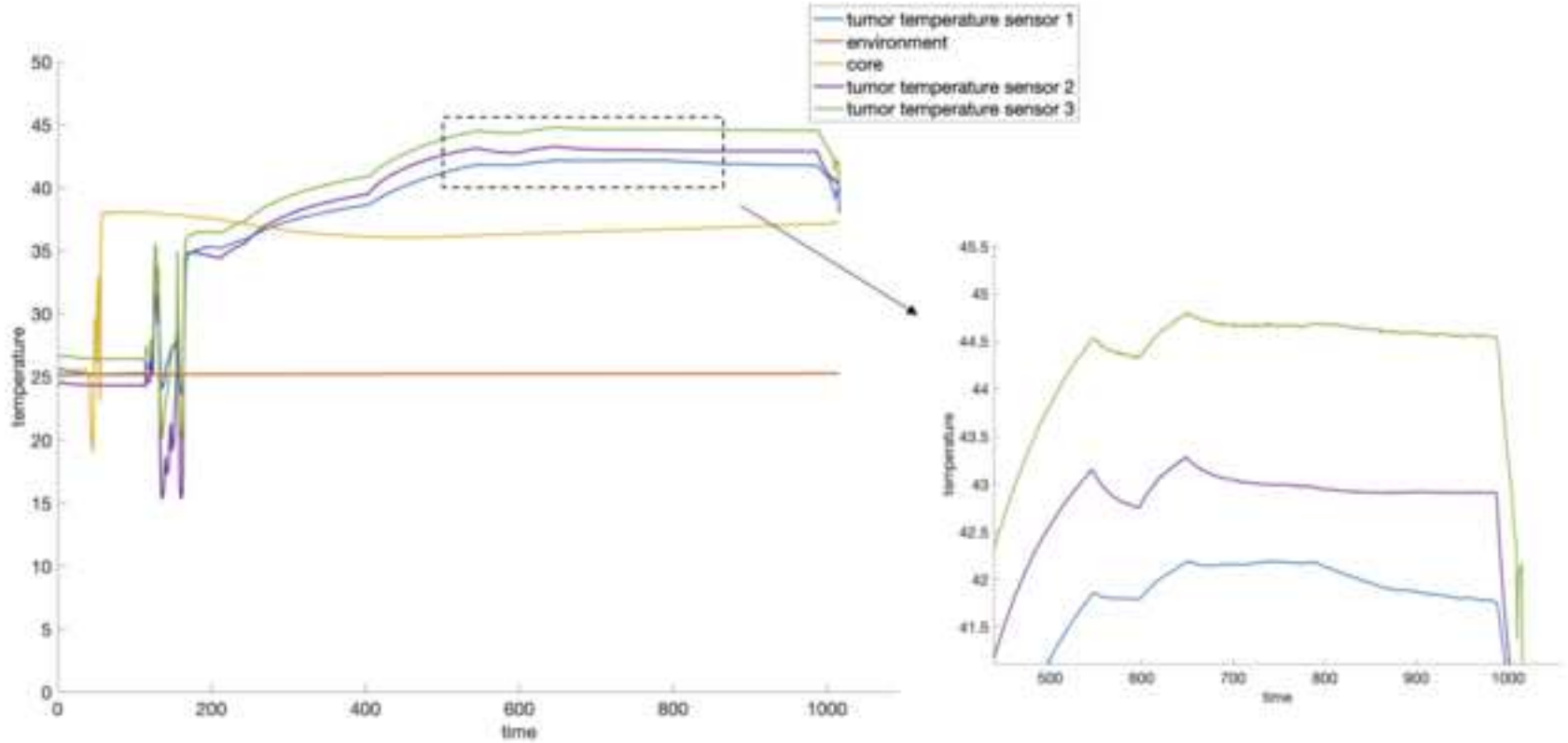

Figure 4

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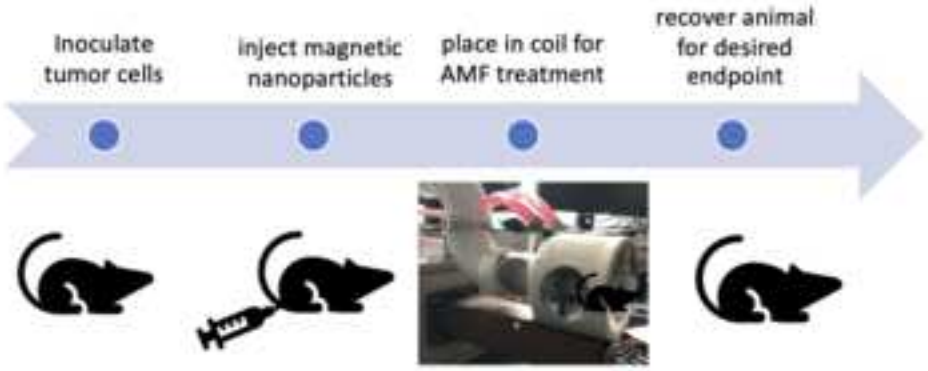

Figure 5

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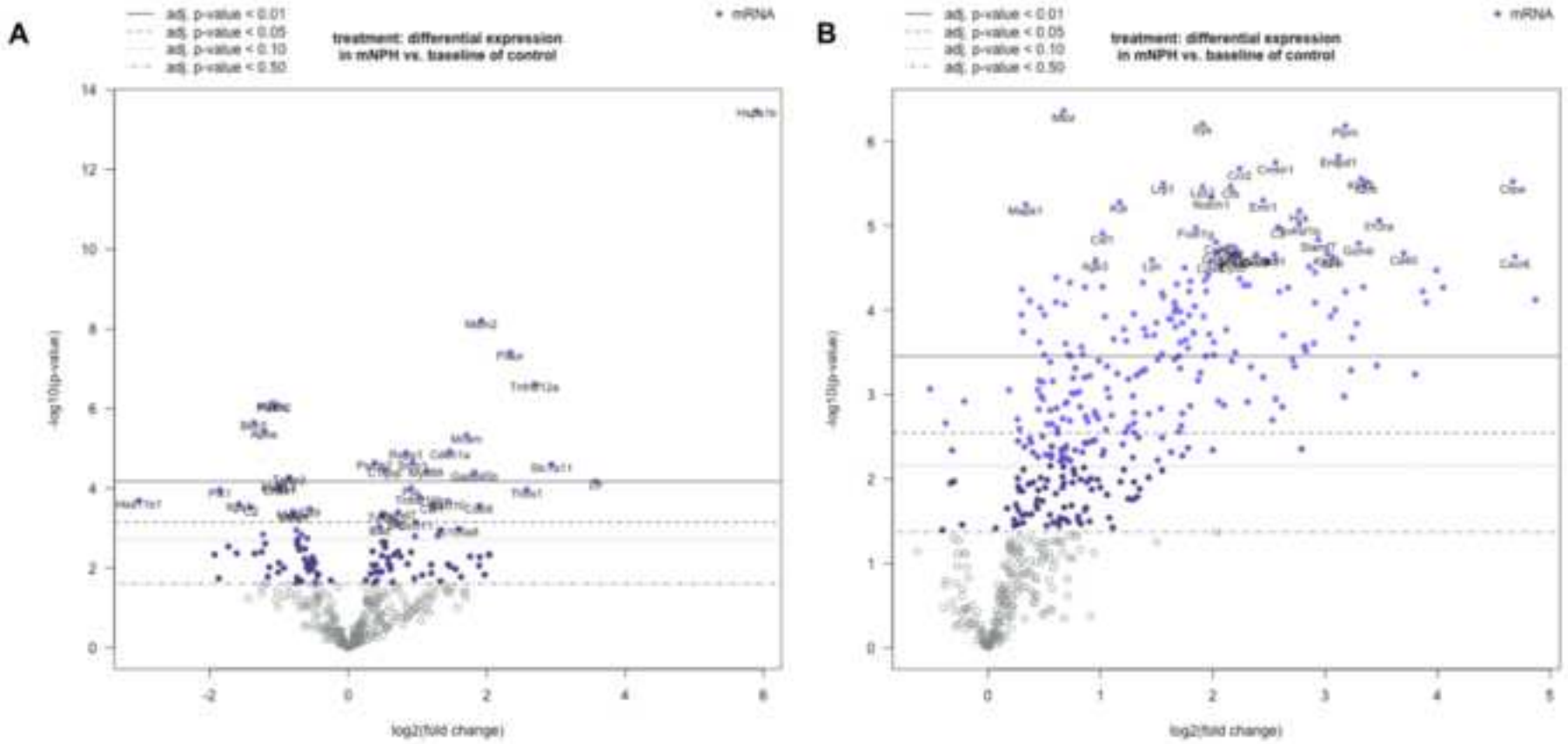

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
.25% Trypsin	Corning	45000-664	available from many companies
1.5 mL tubes	Eppendorf	Eppendorf 22363204	available from many companies
B16F10 murine melanoma cells	American Type Culture Collection	CRL-6475	
C57/Bl6 mice	Charles river	027C57BL/6	6-week-old female mice
Chiller	Thermal Care	NQ 5 series	chiller that cools the coil
Coolant fluid	Dow Chemical Company	Dowtherm SR-1	antenna cooling fluid
Fetal Bovine serum	Hyclone	SH30071	available from many companies
fiber optic probes, software and chassis	FISO		FISO evolution software used to read the temperat
IR camera	Flir		infrared camera to monitor unintentional heating
iron oxide nanoparticles	micromod Partikeltechnologie GmbH	Bionized NanoFerrite	dextran coated iron oxide nanoparticles
mouse coil, solenoid	Fluxtrol	custom built	
penicillin/streptomycin	Corning	45000-652	available from many companies
RF generator	Huttinger	TIG 10/300	power source
RPMI media	Corning	45000-396	available from many companies

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April 28, 2020

Dear Jove editors
Below please find our response to the most recent editorial comments. Please let us know if we have failed to address any of the issues satisfactorily.

Thank you

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

- We have attempted to maintain the journal style in our revisions.

2. Please address all the specific minor comments marked in the manuscript.

- **Please proofread the manuscript well. (Title)**
  - We have proofread the manuscript, hopefully catching grammatical, spelling errors etc.
- **Please reword as: This protocol presents … (Summary)**
  - We have reworded the summary using this introductory phrase. Additional edits have also been made.
- **Please ensure you answer the how question, how is this step performed (Protocol)**
  - We have revised the protocol to address how various steps are performed.
- **Please check the highlight to fit 2.75 pages including headings and spacings. The highlights should be in line with the title. (Protocol)**
  - We have tried to ensure that the highlight fits 2.75 pages and represents the title.
- **Please upload the designs as a figure or supplementary file. (1.1)**
  - Our equipment was custom built following guidelines from references and the required physics and biology. We have added supplementary information to address how to design the coils in more depth.

- **How is this done? Maybe including a figure will be helpful. (1.1.2)**

    - We have added and referenced additional design information (included in the supplementary section)

    .

- **What is the desired location? (1.1.3)**

    - We have enhanced and clarified location in the text.

- **Highlighted this section as well to match with the title of the manuscript. This will be included in the video. Please check. (2)**

    - Thank you, we have tried to highlight 2.75 pages that encompass the entire title

- **Where is AMF treatment being performed-2.8 to 2.10? Please bring out clarity. (2.2)**

    - We have added a statement about where the AMF system is located.

- **What and how? (2.4)**

    - We have expanded on this step and addressed "how" the treatment is performed

- **Please check. (2.6)**

    - We have checked our numbers and reported a single number instead of a range.

- **Do you discard the medium? (2.7)**

    - We do not discard the media and have added that information to the step.

- **How is this done- By pressing the Start button? (2.9)**

    - We have expanded this step.

- **How do you check this -any significance of using this dose? (2.9)**

    - Thermal dose is time and temperature required for a specific application (what the user desires). We have attempted to clarify this situation by providing an example.

- **With fresh medium? (2.11)**
    - We have clarified this step. Media remains in the tube the entire time.
- **Plate used, cell number etc? if same as step 2.1, please mention. (3.1.1)**
    - The cell type and seeding number is variable depending on the experimental design. We have included an example in hopes of clarifying.
- **Volume of isoflurane used? (3.1.4)**
    - Isoflurane is a vaporized inhaled anesthetic gas that varies with animal weight and metabolism and the length and depth of the anesthesia. Therefore, a precise volume is not given.
- **Placed the steps in order. Please check. Do you also clean the area prior to injection? Please include all the steps. (3.1.7)**
    - We have added the step for cleaning the injection area.
- **Size of the needle, syringe? (3.1.7)**
    - We have added the size of the needle and syringe.
- **of medium? (3.1.7)**
    - We have clarified this
- **How is this done? (3.2.1)**
    - We have included information on how the tumor is measured.
- **Please expand? (3.2.1)**
    - We expanded the information regarding on how calipers are used measure the tumor and how tumor volume is calculated.
- **Anesthesia steps cannot be filmed. Please include details of anesthesia or refer to the step above mentioning the step number. Also include the recovery from anesthesia step as well. Do you place these in separate cages? (3.2.3)**

- We updated the anesthesia information and recovery information and refer to the step where it is clarified.

- **Citation? (3.2.4)**

  - This is unpublished data from our lab, we have clarified this situation in the text.

- **What is the required CEM43 in this case? (3.3.11)**

  - We have clarified the CEM43 thermal dose information in the text. The required CEM43 thermal dose varies by the desired effect/user.

- **How? (3.3.14)**

  - We have clarified how to adjust the strength of the AMF.

- **What is the desired dose? (3.3.15)**

  - We have clarified how thermal doses is determined and adjusted by individual users (applications).

- **After this please include steps to show how the animal is let to recover from anesthesia? Complications if any? These steps need not be highlighted. (3.3.18)**

  - We have included anesthesia recovery details. Anesthesia safety and success is largely determined by the person delivering and a managing the anesthesia. We have included a step on recovering the animal. We have not encountered complications.

- **Some of the considerations can be moved to the discussion. (Results)**

  - We have reorganized some of this section to move specific considerations to the discussion section.

- **Please mark the panels as A and B to show which plot belong to in vitro and in vivo. We need representative result for each section of the protocol (Figure 5)**

  - We have added these letters to the figure.

- **Citation? (Discussion)**

  - We have added a citation

- **Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al. (References)**

    - We have attempted to properly format all references.

3. Once done please ensure that the highlight is no more than 2.75 pages including headings and spacings.

We believe we have highlighted only 2.75 pages.

Gmail

Kayla Duval <kaeduval@gmail.com>

Figure permission request

Katie Sinclair <katies@spie.org> Mon, Mar 2, 2020 at 1:42 PM
To: Kayla Duval <kayla.duval.th@dartmouth.edu>

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Best wishes on your dissertation. Please let me know if I may be of further assistance.

Best,

Katie Sinclair

Editorial Assistant, Publications

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figure permission for figure 5

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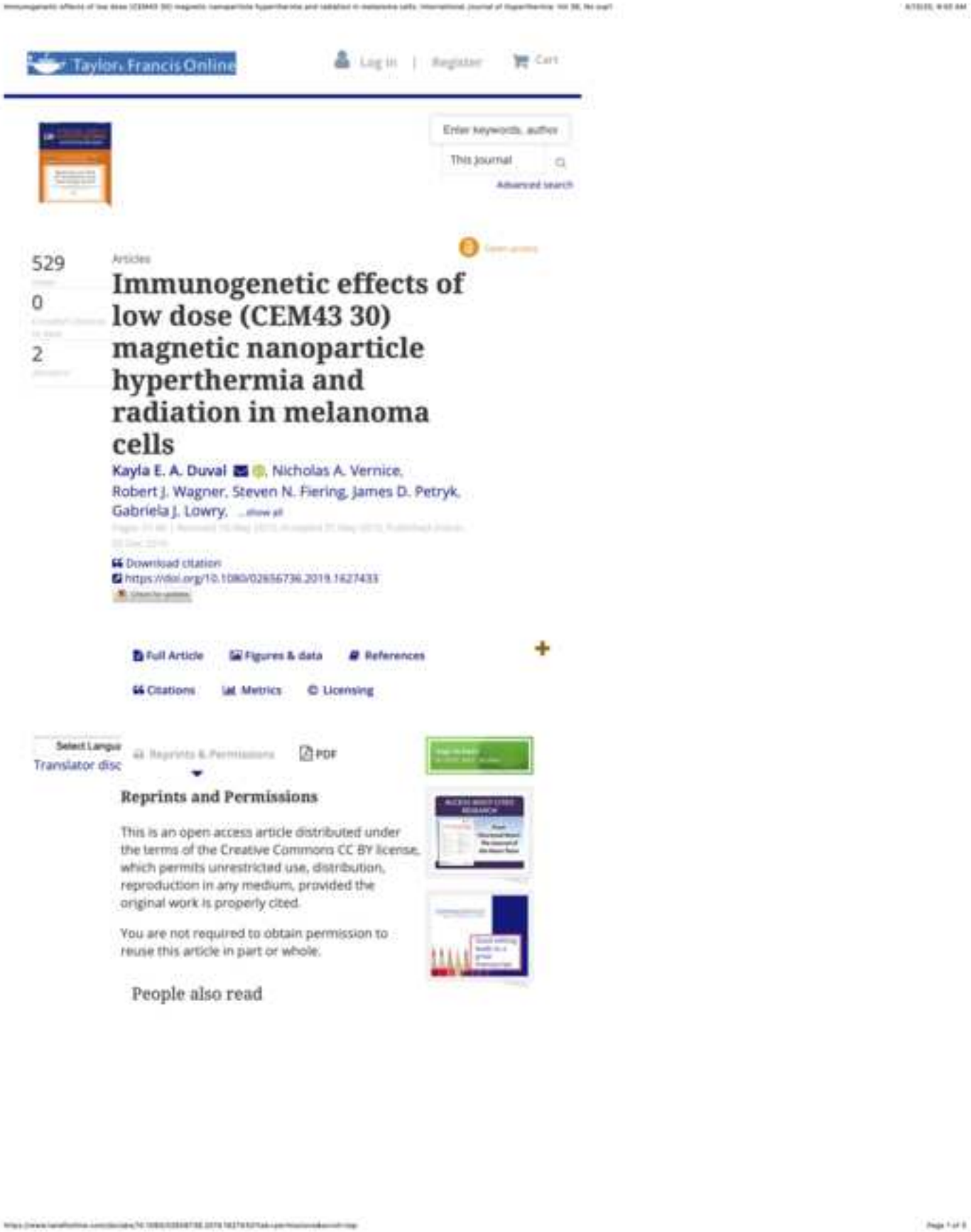

SUPPLEMENTARY DETAILS

The design of a purpose-built induction coil depends on many factors. These include the size and shape of the magnetic field that is needed, as well as the required strength of that field. Induction heating is an old technology which is still used in many industrial settings and there are many available sources covering induction coil design. One of these sources is a paper by Stanley Zinn and S.L Semiatian, **Coil and fabrication design: basic design and modifications**[20]. This paper gives basic information on induction heat coil design, which includes optional shapes and materials. Also, Georgia State University offers online calculators to determine the inductance of a solenoid coil design (http://hyperphysics.phy-astr.gsu.edu/hbase/electric/indsol.html) and expected solenoid magnetic field strengths for solenoid coil designs (http://hyperphysics.phy-astr.gsu.edu/hbase/magnetic/solenoid.html#c3).

To design the 14-turn solenoid coil, we first determined the size of the solenoid that would be needed to hold our samples. After determining the size, we used the Georgia State calculators to ensure that the inductance of the coil was within a range that was compatible with our generator and that we would have sufficient power to provide the field strengths that we would need. Once the coil was built, it was mounted on the signal generator and the system was tuned to the desired resonant frequency by adjusting the number of capacitors in the generators resonant circuit. Fine tuning was accomplished by adjusting the generators internal inductance coil.

To operate the system, the coiling systems are activated, and the main power supply is turned on. When the system is fully powered up, the generator is turned on and the signal pulses are sent to the resonant circuit which creates the magnetic field within the solenoid coil. Varying the voltage to the coil increases the current flow and increases the field strength in the coil. Typically, the voltage and/or current limits of the generator determine the maximum field strengths that can be produced by any specific coil.