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# Osmotic pump-based drug-delivery for in vivo remyelination research on the central nervous system

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**TITLE:**

# Osmotic Pump-based Drug-delivery for *In Vivo* Remyelination Research on the Central Nervous System

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**SUMMARY:**

Demyelination takes place in multiple central nervous system diseases. A reliable *in vivo* drug delivery technique is necessary for remyelinating drug testing. This protocol describes an osmotic pump-based method that allows long-term drug delivery directly into the brain parenchyma and improves the drug bioavailability, with broad application in remyelination research.

**ABSTRACT:**

Demyelination has been identified in not only multiple sclerosis (MS), but also other central nervous system diseases such as Alzheimer's disease and autism. As evidence suggests that remyelination can effectively ameliorate the disease symptoms, there is an increasing focus on drug development to promote the myelin regeneration process. Thus, a region-selectable and result-reliable drug delivery technique is required to test the efficiency and specificity of these drugs *in vivo*. This protocol introduces the osmotic pump implant as a new drug delivery approach in the lysolecithin-induced demyelination mouse model. The osmotic pump is a small implantable device that can bypass the blood-brain barrier (BBB) and deliver drugs steadily and directly to specific areas of the mouse brain. It can also effectively improve the bioavailability of drugs such as peptides and proteins with a short half-life. Therefore, this method is of great value to the field of central nervous system myelin regeneration research.

**INTRODUCTION:**

The osmotic pump is a small implantable solution-releasing device. It can be used for systemic delivery when implanted subcutaneously or in the abdominal cavity. The surface of the osmotic

pump is a semi-permeable membrane, and its inner side is a permeable layer. The osmotic pump operates by using the osmotic pressure difference between the osmotic layer and the tissue environment where the pump is implanted. The high osmolality of the osmotic layer makes the water in the tissue flow into the osmotic layer through the semi-permeable membrane on the pump surface. The osmotic layer expands and compresses the flexible reservoir inside the pump, thereby displacing the solution from the flexible reservoir at a certain rate for a long duration[1]. The pump has three different reservoir volumes, 100 µL, 200 µL, and 2 mL, with their delivery rates varying from 0.11 µL/h to 10 µL/h. Depending on the selected pump type, the device can operate from 1 day to 6 weeks[2]. In this protocol, a 100 µL osmotic pump with a transfer rate of 0.25 µL/h that can operate for 14 days is used.

Back in the 1970s, the osmotic pump had been used in neuroscience research[3,4]. For instance, Wei et al. adopted the osmotic pump approach to inject opioid peptides into the ventricle in a study of drug addiction[3]. After continuous improvement, the osmotic pump has now been used in the study of the controlled delivery of thousands of drugs, including peptides, growth factors, addictive drugs, hormones, steroids, antibodies, and so on. In addition, with special catheters (Brain Infusion Kits) attached, it can be used for targeted infusion to specific tissues or organs, including the spinal cord, brain, spleen, and liver[5-7].

In the study of remyelination, many drugs have been shown to promote myelin regeneration *in vitro*, but most of them have not achieved significant effects *in vivo*, possibly due to the lack of an appropriate administration method. Traditional administration methods such as intraperitoneal injection, subcutaneous injection, and intragastric administration have limitations in the bioavailability of the drugs. In addition, some drugs have poor blood-brain barrier permeability, which undermines their access to the brain parenchyma. Together, these limitations call for a novel efficient delivery method. In combination with the brain infusion kits, osmotic pumps can bypass the blood-brain barrier and deliver drugs directly to the corpus callosum, which effectively improves the bioavailability of drugs, especially for some polypeptide and protein drugs with a short half-life. Therefore, the osmotic pump as a new drug delivery technique is of great value to the field of central nervous system myelin regeneration research. The application of this technique will be introduced in detail below.

**PROTOCOL:**

All animal procedures were conducted under institutional guidelines and protocols approved by the animal welfare and ethics committee of the Third Military Medical University.

## 1. Establishment of the lysolecithin-induced demyelination mouse model

1.1. Prepare 1% lysolecithin (also called L-α-Lysophosphatidylcholine) solution with sterile PBS.

1.2. Turn on the small animal anesthesia machine, anesthesia air pump, gas evacuation apparatus, and homeothermic monitoring system. Sterilize scissors, forceps, curved hemostat, and other surgical instruments and soak them in 75% alcohol solution until use. Lay sterile sheets in the surgical area.

1.3. Anesthetize a postnatal day 56 (P56) C57BL6 mouse as follows.

1.3.1. Place the mouse in the isoflurane chamber of the small animal anesthesia machine. Adjust the $O_2$ flow to 300–500 mL/min and isoflurane to 3%–4%. After sufficient anesthesia, when the mouse becomes immobile with a slow and stable breath, transfer the mouse to the stereotaxic apparatus with a heating pad.

1.3.2. Switch the gas output from the chamber to the anesthesia mask and adjust isoflurane to 1%–1.5% to maintain the mouse in the anesthesia state. Before the operation, pinch the toes of the mouse and check its reaction to confirm successful anesthesia[8].

1.3.3. To keep the mouse's eyeballs moist while under anesthesia, cover the surface of the eyeballs with the right amount of an erythromycin eye ointment.

1.4. Secure the mouse head in the stereotaxic apparatus with tooth bar and ear bars. (**Figure 1A**).

1.5. Trim the hair on the mouse skull and disinfect the skin with 75% alcohol. For ethical concerns, cover the animal body except for the surgery site. Using a scalpel, make a 1 cm long mid-sagittal incision of the skin from the base of the neck to in between the eyes to expose the skull (**Figure 1B**).

1.6. Gently wipe the surface of the skull with a cotton swab containing 30% hydrogen peroxide to visualize the cranial sutures (**Figure 1C**). Adjust the height of the tooth bar and ear bars to place the lambda point and bregma point at the same height (i.e., with the same z-axis coordinates when the needle tip touches the points), so that the sagittal suture is horizontal.

1.7. Gently place the tip of the microliter syringe needle (10 µL, 33 G) at the bregma point and reset the x, y, and z coordinates to 0 (**Figure 1D**). Move the syringe to the injection site (x: 1.04; y: 1.0, i.e., 1.04 mm lateral to the midline and 1.0 mm posterior to the bregma point) according to the prompt of the digital readout (**Figure 1E**).

1.8. Slowly drill a small burr hole through the skull at the injection site without penetrating the dura with a 1 mL syringe needle (26 G, 0.45 mm) (**Figure 1F**). Slowly insert the microliter syringe needle into the brain tissue through the hole until a certain depth is reached (z = -1.62 mm for most P56 mice) (**Figure 1G**).

NOTE: Empirically, the insertion depth of -1.62 mm allows the needle tip to reach the middle of the corpus callosum of most P56 mice so that the lysolecithin could be directly delivered into the corpus callosum to induce demyelination.

1.9. Inject 1.5 µL of 1% lysolecithin at a speed of 0.3 µL/min. After the injection, wait for 5 min before slowly pulling out the microliter syringe to prevent the leakage of liquid along the injection needle path.

1.10. Stitch the skin with 5-0 surgical sutures (**Figure 1H**).

1.11. Place the mouse that has undergone surgery in a cage alone and feed with moist food until fully recovered. Monitor the mouse daily after the operation.

NOTE: If the mouse shows signs of pain or discomfort, such as abnormal posture, abnormal breathing, salivation, tremor, etc., administer the mouse a subcutaneous injection of 5 mg/kg carprofen every 24 h to relieve the pain. If an infection occurs, apply erythromycin ointment to the infected area every day to ensure that the wound heals properly.

[Place **Figure 1** here]

## 2. Preparation of the osmotic pump

NOTE: Key components of the pump are shown in **Figure 2A**.

2.1. Determine the depth of insertion of the brain infusion cannula into the brain. Ensure that the needle of the brain infusion cannula used is 3 mm long and each depth-adjustment spacer is 0.5 mm. To achieve an injection depth of 1.5 mm (close to the callosum), attach three depth-adjustment spacers to the needle of the brain infusion cannula with tissue adhesive (**Figure 2B, C**).

2.2. To fill the osmotic pump, attach the syringe needle that comes with the pump package to a 1 mL syringe and aspirate the drug. Hold the pump upright, insert the syringe into the opening at the top of the pump, and slowly inject the drug, being careful not to create bubbles[9] (see **Figure 2D**). When the liquid flows out of the opening, slowly pull out the syringe.

2.3. Remove the white flange from the flow regulator with scissors or pliers being careful not to bend or crush the flow moderator. Then, insert the flow moderator into the pump (**Figure 2E**). To determine whether there are bubbles in the osmotic pump, weigh the osmotic pump separately before and after filling.

2.4. Trim the catheter to a certain length according to the size of the animal (20–25 mm catheters for P56 mice that weigh about 25 g). Attach the catheter to the brain infusion cannula.

2.5. Fill the catheter with drugs using the syringe without introducing air (**Figure 2F**).

2.6. Connect the catheter to the flow moderator. After attachment, ensure that the catheter covers about 4 mm of the exposed flow moderator (**Figure 2G**).

2.7. To ensure that the osmotic pump can work instantly after implantation, immerse the filled pumps in sterile 0.9% saline or PBS at 37 °C for at least 4 to 6 h (preferably extend to overnight) to pre-wet the semi-permeable membrane on the pump surface with solutions that have the

same osmotic pressure as the tissue environment (**Figure 2H**).

[Place **Figure 2** here]

## 3. Implantation of the osmotic pump

3.1. Wait for 3 days after the establishment of the corpus callosum demyelination model. Turn on the small animal anesthesia system. Disinfect scissors, tweezers, and hemostatic pliers and soak them in 75% alcohol solution. Lay sterile sheets in the surgical area.

3.2. Anesthetize and secure the mice on the stereotaxic apparatus again. Cover the surface of the eyeballs with an eye ointment to prevent dryness.

3.3. Disinfect the original wound with 75% alcohol. Open the surgical incision that was previously stitched (**Figure 3A**) and expand the incision to the shoulder blades (**Figure 3B**).

3.4. Separate the skin from the subcutaneous connective tissue with hemostatic pliers or tweezers at the scapula to open a cavity (**Figure 3C**). Place the osmotic pump into the cavity (**Figure 3D,E**).

3.5. With a cotton swab, gently wipe and expose the pinhole on the surface of the skull created when establishing the demyelination model (see step 1.8). Insert the brain infusion cannula through this pinhole perpendicularly and secure it on the skull quickly with tissue adhesive (**Figure 3F**).

3.6. Remove the removable tab above the brain infusion cannula with a pair of scissors (**Figure 3G, H**). Alternatively, remove the tab first before inserting the cannula to avoid shaking in this process.

3.7. Stitch the incision or attach it with tissue adhesive (**Figure 3I**).

3.8. After surgery, place the animal in a cage alone and feed with moist food until fully recovered. Monitor the mice and check whether the brain infusion cannula was firmly attached every day.

NOTE: If the mouse shows signs of pain or discomfort, give the mouse a subcutaneous injection of 5 mg/kg carprofen every 24 h to relieve the pain. If an infection occurs, apply antibiotic ointment to the area every day to ensure that the wound heals properly.

3.9. Euthanize the mouse 11 days after the surgery by injecting 150–200 mg/kg Pentobarbital sodium intraperitoneally followed by perfusing transcardially with 4% formaldehyde.

3.10. To verify that the solution is delivered normally, carefully remove the osmotic pump and measure the residual volume in the pump reservoir before brain dissection.

3.10.1. To measure the residual volume, remove the brain infusion cannula, attach a 1 mL syringe to the catheter, and then aspirate the remaining solution to determine its volume. Compare the actual residual volume to the theoretical residual volume (initial volume - mean pumping rate * infusion duration).

NOTE: Excessive residual volume indicates unsuccessful infusion, which might be due to catheter occlusion or pump malfunction.

[Place **Figure 3** here]

**REPRESENTATIVE RESULTS:**

To verify the effect of the osmotic pump in myelin regeneration research, a lysolecithin-induced demyelination model was created in P56 mice, followed by implantation of osmotic pumps containing UM206 (1 mg in 1.5 mL 0.9% saline), a peptide with a short half-life and poor BBB permeability that has been recently reported to promote remyelination[10]. 0.9% saline was used as the control. Fourteen days after the model establishment, mice were transcardially perfused with 4% formaldehyde to isolate the brains for sectioning, followed by *in situ* hybridization and transmission electron microscopy to evaluate the remyelination level.

Staining of DAPI revealed the pinhole in the brain tissue just above the white matter, indicating successful implantation of the brain infusion cannula of the osmotic pump (**Figure 4A**). In the *in-situ* hybridization experiment, the mature oligodendrocyte marker MAG probe was used to label newly differentiated oligodendrocytes as shown in previous studies[10-12]. The results showed that the UM206 treatment yielded more MAG-positive cells in the demyelinated region than the control group (**Figure 4B**). Transmission electron microscopy of the demyelinated region also showed that the number of myelinated axons was increased in the UM206 treatment group compared to the control group (**Figure 4C**), suggesting that UM206 induced a higher level of remyelination. These results show that the osmotic pump can efficiently deliver drugs to the corpus callosum in the remyelination research.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Establishment of the lysolecithin-induced demyelination mouse model.** (**A**) Secure the mouse in the Stereotaxic Apparatus. (**B**) Open a 1 cm mid-sagittal incision to expose the skull. (**C**) Visualize the cranial sutures. (**D**) Reset the x, y, and z coordinates to 0 on the Bregma point. (**E**) Move the syringe to the injection site. (**F**) Drill a hole in the skull at the injection site. (**G**) Insert the needle into brain tissue slowly and inject lysolecithin. (**H**) Stitch the skin.

**Figure 2: Preparation of the osmotic pump.** (**A**) Key components of the osmotic pump. (**B,C**) Attach depth-adjustment spacers to the needle of the brain infusion cannula. (**D**) Fill the osmotic pump using a 1 ml syringe. (**E**) Insert the flow moderator into the pump. (**F**) Fill the catheter using the syringe. (**G**) Connect the catheter to the Flow moderator. (**H**) Immerse the filled pumps in sterile 0.9% saline or PBS at 37 °C.

**Figure 3: Implantation of the osmotic pump.** (**A**) Open the surgical incision. (**B**) Expand the

incision to the shoulder blades. (**C**) Separate the skin from subcutaneous connective tissue to make a cavity. (**D,E**) Place the osmotic pump into the cavity. (**F**) Insert the brain infusion cannula in the pinhole on the surface of the skull and firmly secure it on the skull. (**G,H**) Remove the removable tab from the cannula. (**I**) Stitch the incision.

**Figure 4: Representative results.** (**A**) Representative image of DAPI-stained slice showing the pinhole in the brain tissue. Scale bar: 1,000 µm. (**B**) Representative images showing *in situ* hybridization of MAG in the demyelinated region as shown by DAPI staining. UM206 treatment increased the number of MAG-labeled oligodendrocytes. Scale bar: 100 µm. (**C**) Representative transmission electron microscopy images of the demyelinated region. UM206 treatment increased the number of myelinated axons. Scale bar: 10 µm.

**DISCUSSION:**

This protocol describes the osmotic pump as a novel drug delivery technique for myelin regeneration research, which can deliver drugs directly to the treatment site and allow consistent drug delivery for a prolonged period, creating a stable drug concentration in the micro-environment of the central nervous system in the whole experimental duration. Compared with other drug delivery methods, the osmotic pump is more conducive to maintaining drug concentration in the demyelination lesion[13]. For example, for certain neurotrophic factors, systemic medication cannot achieve any effect because of the low concentration of the drug at the lesion site. But if the dosage is increased, the side effects will be more significant[14]. In such cases, administering to a specific site through an osmotic pump can reduce peripheral side effects effectively[15]. In addition, many myelin regeneration-related drugs have poor blood-brain barrier (BBB) permeability or display a short *in vivo* half-life due to susceptibility to proteolytic degradation. These problems could be well addressed by osmotic pumps.

However, the osmotic pump method is not without caveats and limitations. First, being an invasive drug delivery system, it inevitably causes brain tissue damage and neuroinflammation at the brain infusion cannula insertion site, which might obscure the drugs' effect. Thus, a proper solvent-only control group must be set up. Second, some drugs require solvents like dimethyl sulfoxide (DMSO), N-methyl-2-pyrrolidone (NMP) to dissolve, but these solvents are incompatible with the reservoir material and can cause a significant failure of the pumps. For example, high concentrations of dimethyl sulfoxide (DMSO) and PEG400 have been shown to adversely affect pump release and may not be suitable for use in osmotic pumps[16-18]. Third, drugs that are unstable at 37 °C might not be suitable for long-term infusion using the osmotic pump. All these issues are worthy of attention if planning to apply the osmotic pump.

Several steps in this protocol require extra attention during the experiments. For the normal operation of the osmotic pumps, researchers must ensure that the osmotic pump is assembled correctly and that no bubble is introduced into the pump, which will otherwise greatly undermine the infusion efficiency. In addition, catheter occlusion or osmotic pump malfunction may cause infusion failure[19], which could be determined by the measurement of the residual volume in the pump reservoir after the experiment. For the application of the osmotic pump in younger mice with smaller brain sizes, a trial experiment is recommended to ensure a suitable depth of

insertion. Furthermore, the brain infusion cannula must be firmly secured on the skull to minimize its movement during infusion.

At present, many *in vitro* studies have found a variety of drugs that can promote myelin regeneration, but due to poor BBB permeability, short half-life, and other problems, these drugs are difficult to be successfully validated *in vivo*. Therefore, the osmotic pump is of great value to the field of central nervous system myelin regeneration research, especially relevant for those drugs with a short half-life, poor BBB permeability, and obvious peripheral side effects.

**ACKNOWLEDGMENTS:**

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**DISCLOSURES:**

The authors declare no conflicts of interest.

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Figure 1

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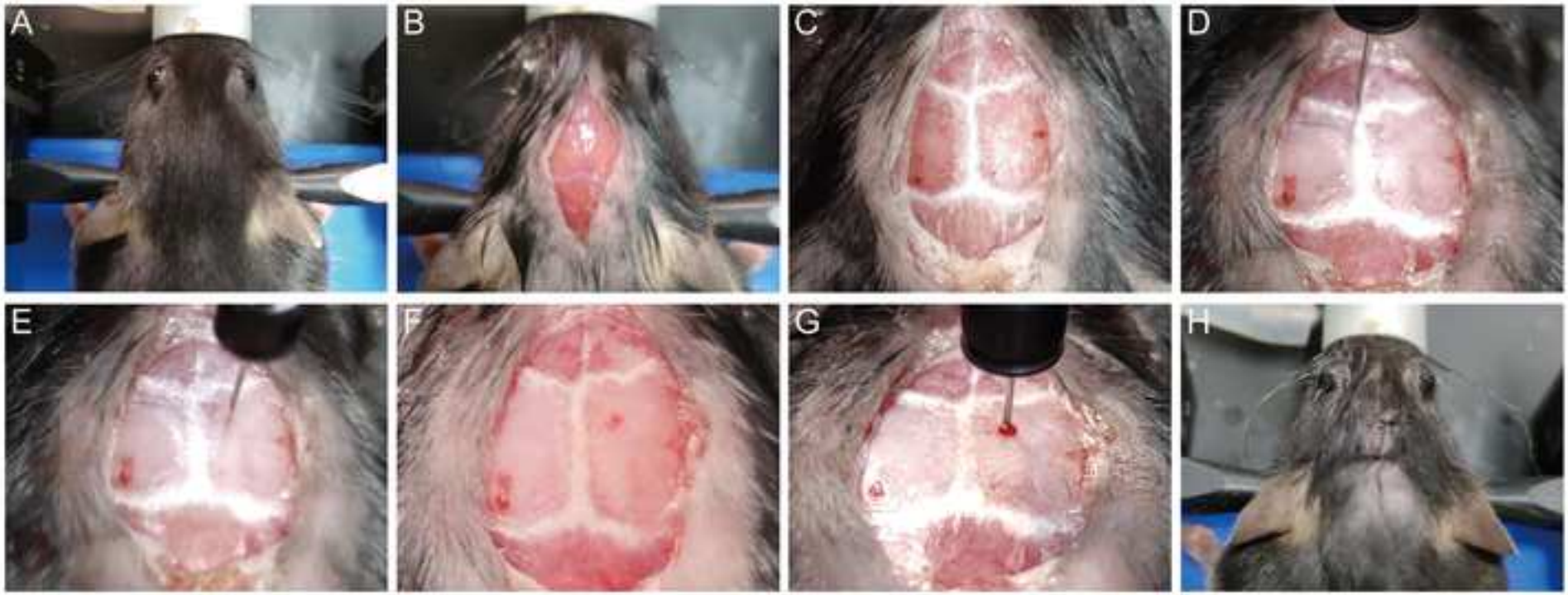
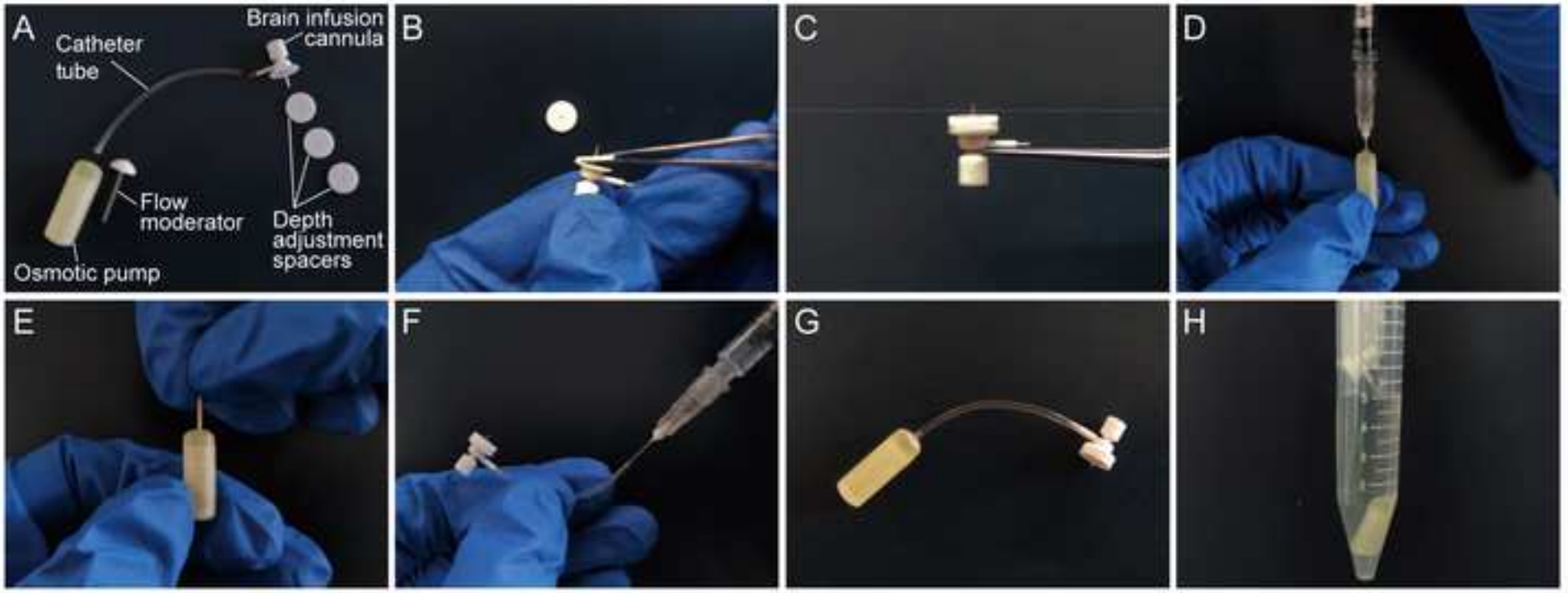


Figure 2





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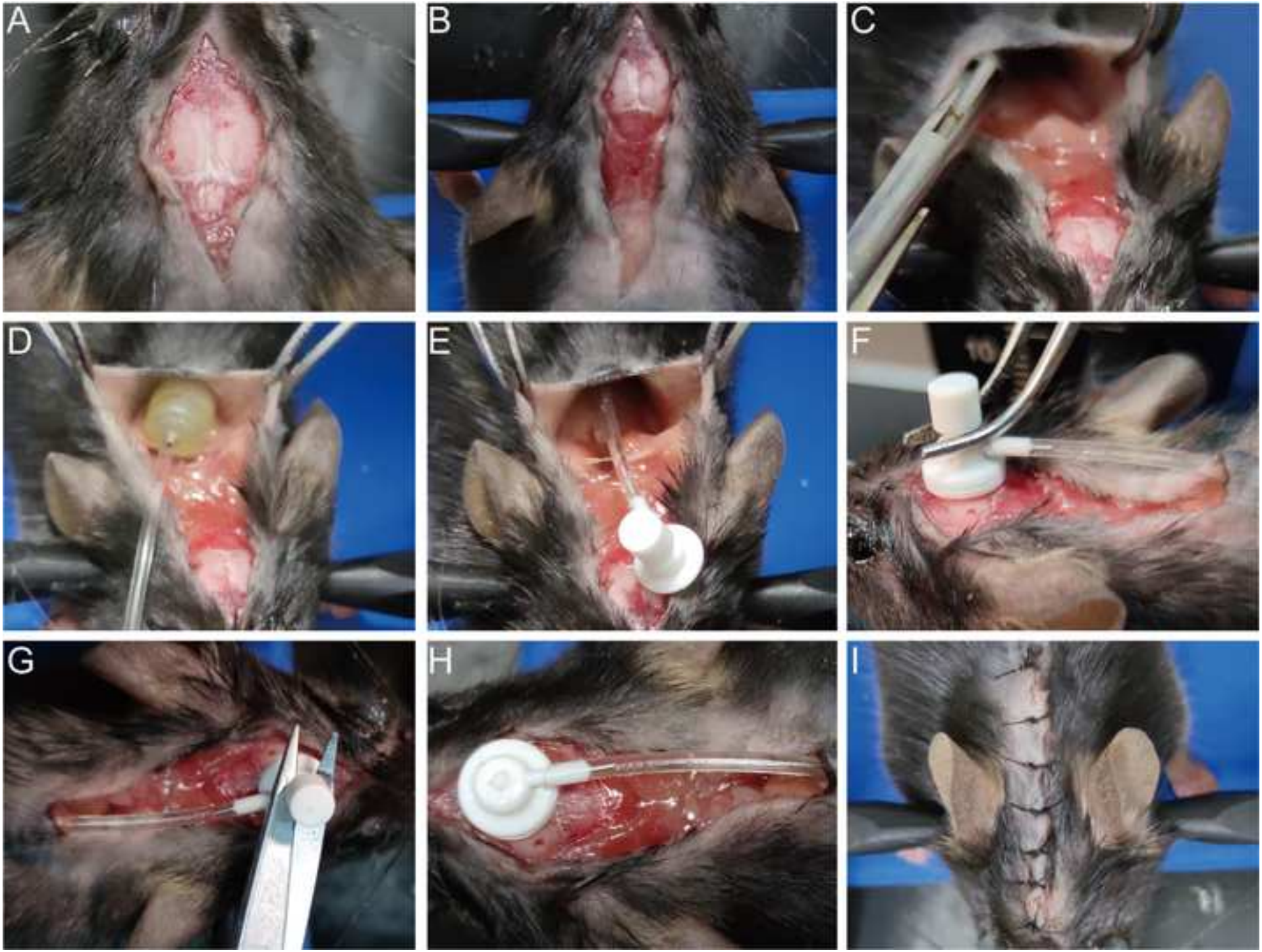

Figure 4

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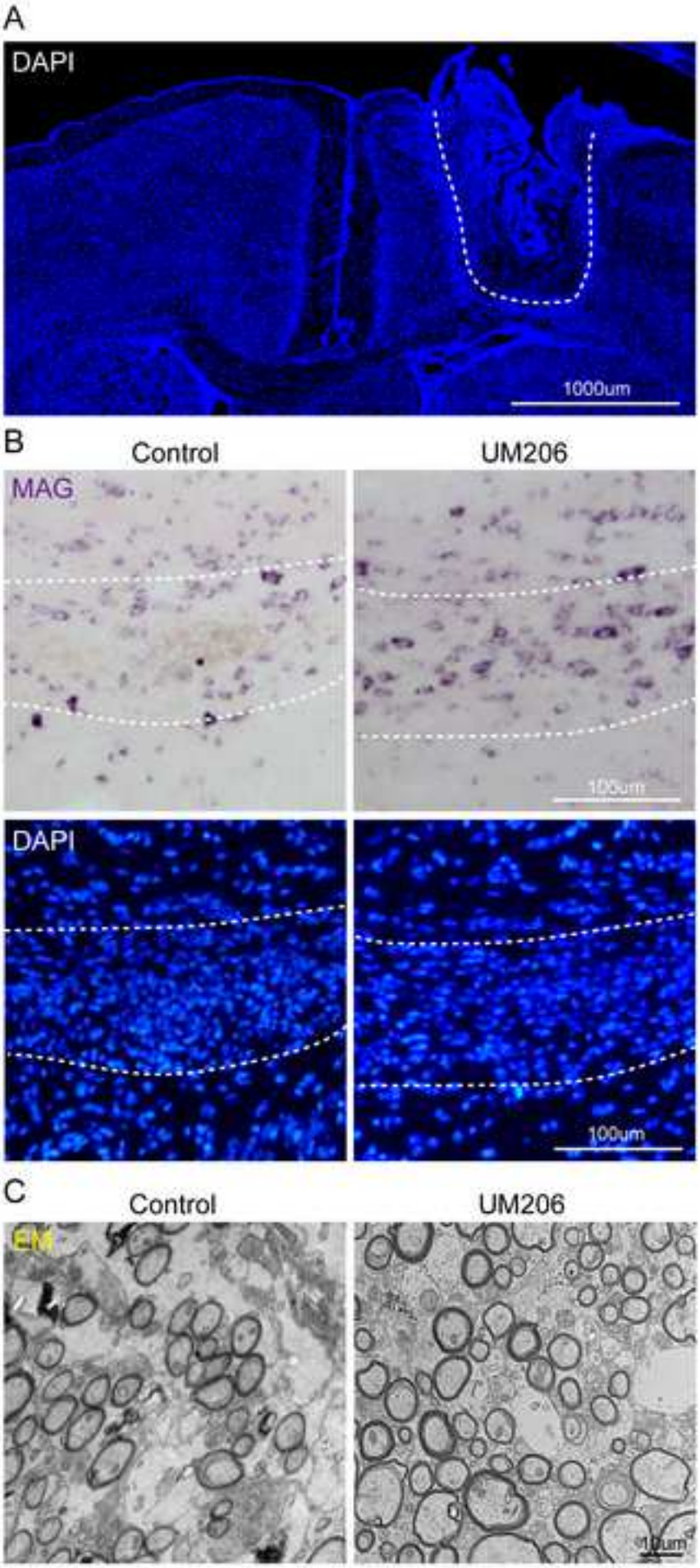

Dear Dr. Manoj Kumar Jana,

We thank the editor and reviewers for their comments, which will help us to improve this manuscript. We also appreciate that they all found this manuscript is very useful for the neuroscience field. In the resubmitted manuscript, we have highlighted the altered text in red, and the essential steps for the video with a yellow background. Please find below a point-by-point response to their comments. We believe that we have addressed and explained clearly all these points.

**Reviewer 1**

Major Concerns:

The following details are needed to enable the readers to perform the techniques.

1. Source of lysolecithin used in 1.1

Response: We thank the reviewer for this comment. According to the requirements of the manuscript format, we have put this part of the information in the table of materials: "L-α-Lysophosphatidylcholine, Sigma, L0906, 1% with sterile PBS"

2. Details on the induction phase and maintenance phase of isoflurane-induced anesthesia, including oxygen percentage in 1.2

Response: We thank the reviewer for this comment. We have added this part of the content in the manuscript: "Adjust the O2 to 300-500 mL/min and isoflurane to 3-4 %. After sufficient anesthesia, when the breath of mouse is slow and stable, transfer the mouse to the stereotaxic apparatus with a heating pad and adjust isoflurane to 1-1.5 % to maintain anesthesia through the anesthesia mask."

3. Type and source of stereotaxic apparatus in 1.2

Response: We thank the reviewer for this comment. According to the requirements of the manuscript format, we have put this part of the information in the table of materials.

4. Indicators of anesthesia in mice in 1.2

Response: We thank the reviewer for this comment. We have added this part of the content in the manuscript: "Before the operation, pinch the toes of the mouse and check its reaction to confirm successful anesthesia"

5. Source and type of eye cream in 1.3

Response: We thank the reviewer for this comment. According to the requirements of the manuscript format, we have put this part of the information in the table of materials: "Erythromycin eye ointment, Along technology, YCKJ-RJ-024780, Cover the surface of the eyeballs during anesthesia"

6. Landmarks for the incision in 1.4.

Response: We thank the reviewer for this comment. We have added this part of the content in the manuscript: "Open a 1 cm mid-sagittal incision of the skin from the base of the neck to in between the eyes with a scalpel to expose the skull"

7. Description of the microliter syringe needle- gauge, volume, type and source in 1.6.

Response: We thank the reviewer for this comment. According to the requirements of the manuscript format, we have put this part of the information in the table of materials: "Microliter Syringe, Hamilton, 65460-05, Syringe Series:1700, 10 uL, 33 gauge"

8. Explain the stereotaxic coordinates in terms of antero-posterior, medio-lateral and dorso-ventral to the bregma in 1.6.

Response: We thank the reviewer for this comment. We have added this part of the content in the manuscript: "Move the syringe to the injection site ( x: 1.04; y: 1.0, i.e., 1.04 mm lateral to the midline; 1.0 mm posterior to the bregma ) according to the prompt of the digital readout. "

9. Describe the drill and the drill bit used for drilling the skull in 1.7.

Response: We thank the reviewer for this comment. According to the requirements of the manuscript format, we have put this part of the information in the table of materials: "Syringe needle (1 mL), Shanghai KDL, 6930197811018, 26 gauge (0.45 mm * 16 mm)"

10. Source and type of surgical suture in 1.9.

Response: We thank the reviewer for this comment. According to the requirements of the manuscript format, we have put this part of the information in the table of materials: "Surgical sutures, Shanghai jinhuan, F504, 5-0 "

11. Details on post-surgical management in 1.10 such as- where is the mouse housed, how is the mouse monitored for pain, is the mouse provided with moist food, what is the generic name for carprofen and how is the drug administered, what are the signs of infection and which antibiotic ointment is used?

Response: We thank the reviewer for this comment. We have added this part of the content in the manuscript. "Place the animal that has undergone surgery in a cage alone and feed with moist food until fully recovered. Monitor the mouse daily after the operation. If the mouse shows signs of pain or discomfort, such as abnormal posture, abnormal breathing, salivation, tremor, etc, give it a subcutaneous injection of 5 mg/kg carprofen every 24 hours to relieve the pain. If an infection occurs, apply erythromycin ointment to the infected area every day to ensure that the wound heals properly."

12. What is the type and source of tissue glue in 3.5?

Response: We thank the reviewer for this comment. According to the requirements of the manuscript format, we have put this part of the information in the table of materials: "Tissue adhesive, 3M Vetbond, 1469SB"

13. Negative control for the MAG ISH should be shown even though this is not about the data.

Response: We thank the reviewer for this comment. However, the MAG in situ hybridization experiment has been one of the routine experiments for the remyelination experiment,

which has been used widely in our and other's previous experiments (Gerald A. Higgins et al., 1988; Helene Breitschopf et al., 1992; Bruce A. C. Cree et al., 2018; Jianqin Niu et al., 2021). Thus, a negative control was not included in this manuscript. The enriched signals in the corpus callosum area, the morphology of $MAG^+$ cells, and the low background together indicate successful MAG in situ hybridization experiments.

**Reviewer 2**

minor concerns

Introduction:

1. It is highly suggested to discuss the concerns and issues to test the efficiency and specificity of some drugs in remyelination research first and then explain how the osmotic pump can solve this issue.

Response: We are very grateful to the reviewer for this suggestion. In order to give readers a preliminary understanding of the Osmotic pump, we briefly introduce the principle and development of the Osmotic pump in the first two paragraphs of the introduction. After that, in the third paragraph, we discussed the disadvantages of the current traditional drug delivery methods, before we introduced the advantages of Osmotic pumps in this application.

Methods:

2. In part, 1.1 the concentration of LPC is mentioned 0.1 %. However, it seems that the author used LPC at 1% concentration (part 1.8). Please re-check it.

Response: We thank the reviewer for this comment. Under the suggestion of the reviewer, we have found and corrected this mistake.

3. How to make sure that the tube is not blocked specially for long-term injection?

Response: We thank the reviewer for this comment. As we mentioned in the discussion section, this is indeed an issue that deserves our attention. We think that when the equipment is properly assembled, there will be very few cases of blocking. But if a blockage does occur, we can eliminate the failure group by measurement of the residual volume in the pump reservoir after explantation. We have added this point in our manuscript.

4. What is the rational for immersing the filled pumps in sterile 0.9% saline or PBS before implantation?

Response: We thank the reviewer for this comment. According to the product instructions, immersing the filled pumps in sterile 0.9% saline or PBS at 37℃ for at least four to six hours before implantation is to prewet the semi-permeable membrane on the pump surface with solutions with the same osmotic pressure as the tissue environment, so that the Osmotic pump can work instantly after implantation. This is an indispensable and important step.

5. Some picture are taken from very small equipments, so higher magnification photographs are needed.

Response: We thank the reviewer for this comment. We have replaced them with higher-

magnification pictures according to the reviewer’ s suggestion.

6. For some ethical concerns, covering the animal body except the surgery site is recommended, in addition for this type of operations on anesthetized animals it is recommended to cover the eyes with ointment to protect eye against consequent ulcers.

Response: We thank the reviewer for this comment. Under the suggestion of the reviewer, we have added this point in our manuscript and replaced the picture showing only the surgery site, whilst we have added the information of eyes ointment in the table of materials.