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TITLE:

Delivery of Antibodies into the Murine Brain Via Convection-Enhanced Delivery

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SUMMARY:

Convection-enhanced delivery (CED) is a method enabling effective delivery of therapeutics into the brain by direct perfusion of large tissue volumes. The procedure requires the use of catheters and an optimized injection procedure. This protocol describes a methodology for CED of an antibody into a mouse brain.

ABSTRACT:

Convection-enhanced delivery (CED) is a neurosurgical technique enabling effective perfusion of large brain volumes using a catheter system. Such an approach provides a safe delivery method by-passing the blood brain barrier (BBB), thus allowing treatment with therapeutics with poor BBB-permeability or those for which systemic exposure is not desired, e.g., due to toxicity. CED requires optimization of the catheter design, injection protocol, and properties of the infusate. With this protocol we describe how to perform CED of a solution containing up to 20 µg of an antibody into the caudate putamen of mice. It describes preparation of step catheters, testing them in vitro and performing the CED in mice using a ramping injection program. The protocol can be readily adjusted for other infusion volumes and can be used for injecting various tracers or pharmacologically active or inactive substances, including chemotherapeutics, cytokines, viral particles, and liposomes.

INTRODUCTION:

The blood brain barrier (BBB) forms a semipermeable border separating the central nervous system (CNS) from the blood circulation. Reaching the CNS with therapeutics is however necessary in context of various diseases, like brain tumors, Alzheimer's disease (AD) or Parkinson's disease (PD) among others[1]. This becomes important in the development of new therapies, especially if the tested drug exhibits poor BBB permeability or its systemic exposure can lead to dangerous toxicity[1,2]. Some of the clinically used antibodies display both of these features. A solution to this problem would be to deliver the therapeutics directly behind the BBB. Convection-enhanced delivery (CED) is a neurosurgical technique enabling effective perfusion of large brain volumes. This is achieved by surgically installing one or more catheters in the target area. During the drug application, a pressure gradient is formed at the opening of the catheter, which becomes the driving force of the infusate dispersion in the tissue[3,4]. It is thus the duration of infusion and not the diffusion coefficients that determine the perfusion range[2,4,5]. This provides uniform delivery of the infusate over a much larger brain volume compared to conventional, diffusion based intracerebral injection methods[2,6]. At the same time, this delivery modality has a lower risk of tissue damage[2]. Accordingly, CED can enable safe and efficacious administration of conventional chemotherapeutics for treatment of CNS tumors, as well as delivery of immunomodulatory agents or agonistic and antagonistic antibodies in a multitude of other CNS disorders[2,7-9]. CED is currently tested in therapies of Parkinson's disease, Alzheimer's disease, as well as high-grade glioma[2,7,8,10,11].

Catheter design and the injection regimen are among the most important factors influencing the outcome of CED [10,12-16]. Furthermore, it requires specific physicochemical properties of the infusate, including moderate size of the particles, an anionic charge, and low tissue affinity [10,17]. Each of these parameters has to be potentially adjusted according to the histological features of the brain region to be targeted[2,10,17].

Here we describe methodology for performing CED of an antibody solution into the caudate putamen (striatum) of mice. Furthermore, the protocol includes preparation of step catheters in a laboratory setup, testing them in vitro and performing the CED.

There are multiple catheter designs available in the literature, differing by the shape of the cannula, the materials used and the number of catheter openings[12,15,18-22]. We are using a step catheter made of a fused silica capillary protruding 1 mm from a blunt end metal needle. This catheter design can be easily manufactured in a research laboratory and reproducibly gives good CED results when tested in vitro with agarose blocks with physical parameters resembling brain parenchyma in vivo[23].

Moreover, we implement a ramping regimen for delivering 5 µL of infusate in vivo. In such a protocol the injection rate is increased from 0.2 µL/min to a maximum of 0.8 µL/min, thus minimizing chances of infusate reflux along the catheter as well as risk of tissue damage[16]. Using this protocol, we have successfully administered mice with up to 20 µg of antibody in 5 µL of PBS over the course of 11 min 30 s.

The protocol can be readily adjusted for other infusion volumes or for injecting various other substances, e.g. chemotherapeutics, cytokines, viral particles or liposomes[2,10,14,18,22]. In case of using infusate with drastically different physicochemical properties compared to a phosphate buffered saline (PBS) or artificial cerebrospinal fluid (aCSF) solution of antibodies, additional validation steps are recommended. For catheter assembly, validation and CED, we describe all steps using a stereotactic robot with a drill and injection unit mounted onto a regular stereotactic frame. This procedure can also be performed with a manual stereotactic frame connected to programmable microinfusion pump that can drive the described glass microsyringes.

PROTOCOL:

All methods described here have been approved by the Swiss Cantonal Veterinary Office under license number ZH246/15.

1. Preparation of the step catheters

1.1. Preparation of a fused silica tubing for the step of the catheter

1.1.1. Cut the fused silica capillary with inner diameter of 0.1 mm and wall thickness of 0.0325 mm tubing to a length of 30 mm.

1.1.2. Examine the tubing for cracks and heat polish the ends using a microforge to ensure the tubing openings have a smooth surface.

1.2. Fixation of the inner tube in a metal needle

1.2.1. Mount a 27 G needle on a syringe and place the syringe in a stereotactic robot.

1.2.2. Using the robot, move the syringe over a hard surface and touch it with the needle tip. This position should be noted or saved in the software because it will serve as a reference surface for setting the length of the catheter step.

1.2.3. Elevate the needle to enable placing of the fused silica capillary inside of the needle

1.2.4. Place the fused silica capillary in the needle such that 20 mm of the capillary is protruding from the needle.

1.2.5. Using a pipette, evenly spread 5 µL of high viscosity cyanoacrylate adhesive over the capillary, starting from the metal needle and finishing 10 mm above the lower end of the capillary, as depicted in **Figure 2**.

1.2.6. Using the stereotactic robot, lower the needle until the tip of the metal needle is 1 mm over the reference surface. This way the fused silica capillary will be fixed in the metal needle and will form a 1 mm step from the tip of the metal needle. Remove any excess of glue forming

at the end of the metal needle to avoid blunting the step. Confirm that at the step all excess glue has been removed by checking the tip of the catheter under a microscope.

1.2.7. Wait 15 min for the glue to harden and remove the syringe with the catheter from the stereotactic robot.

1.3. Testing the step catheter using a block of agarose

1.3.1. Prepare 0.6% agarose solution in PBS in a conventional gel tray and wait until it polymerizes. Cut the agarose in approximately 20 mm x 20 mm blocks. Until use, keep the blocks immersed in PBS.

1.3.2. Manually fill the step catheter syringe with 10 µL of 0.4% solution of filtered trypan blue.

1.3.3. Using the stereotactic robot, dispense 1 µL at 0.2 µL/min in order to assess sealing of the step of the catheter during the fixation procedure. Trypan blue solution should be visible solely on the tip of the catheter. Wipe it off with a paper tissue.

1.3.4. Place the agarose block in the stereotactic robot and calibrate the robot so the tip of the catheter is referenced against the surface of the agarose block.

1.3.5. Program the injection parameters for CED.

1.3.5.1. For the injection volume of 5 µL, use the following steps: 1 µL at 0.2 µL/min, then 2 µL at 0.5 µL/min and 2 µL at 0.8 µL/min. Adjust the final injection volume according to the specific experimental plan by proportionally changing the duration of each of the steps.

1.3.5.2. In order to inject the solution into murine caudate putamen (striatum), perform such injection in a position 1 mm frontal and 1.5–2 mm lateral from bregma at the depth of 3.5 mm.

1.3.5.3. After the injection, leave the catheter in place for 2 min and then retract at 1 mm/min to ensure proper dispersion of the fluid in the brain and sealing of the injection tract during catheter removal.

NOTE: Depending on the specific stereotactic robot used, all the parameters can be programmed into a single script. An example script is available as Supplementary Material.

1.3.6. Start the CED procedure and inject 5 µL of trypan blue solution into the agarose block.

1.3.7. Assess the shape of cloud of trypan blue in the agarose and potential leakage along the catheter tract. Trypan blue should form an ellipsoid or a round cloud with the center around the catheter tip and a diameter of at least 1 mm. No major backflow over the tip of the metal needle should be visible.

1.3.8. Place a new agarose block and start a second injection of 1 µL at 0.2 µL/min in order to assess clogging of the catheter with the agarose. Trypan blue should again start forming a cloud from the tip of the catheter immediately after the start of the injection.

1.3.9. Assess whether the leftover volume in the syringe corresponds to 3 µL. Any variations might point towards a leakage of fluid through the catheter mounting or syringe plunger.

1.3.10. If all the test injections are successful, the catheter is well sealed, straight and no trypan blue solution is observed from other spots than the catheter tip, wash the catheter with deionized H_2O (dH_2O) until no traces of trypan blue are visible and then wash ten times as follows: 70% ethanol and 100% ethanol followed by flushing again with 70% ethanol and clean deionized water.

1.3.11. Store the catheter under dry conditions.

2. Convection-Enhanced Delivery of antibody solution into the murine brain.

NOTE: Depending on local animal welfare regulations, various types of anesthetics, analgesics and antibiotics can be implemented for this procedure. This protocol describes the use of injection anesthesia. Inhalation anesthetics such as isoflurane can also be used by mounting a nose mask on the stereotactic frame. In addition, we recommend adding antibiotics to the drinking water for infection prophylaxis.

2.1. Surgical setup

2.1.1. Prepare anesthetics and antidote solutions. Mice can be safely sedated using a three-component anesthesia containing fentanyl (0.05 mg/kg), midazolam (5 mg/kg) and medetomidine (0.5 mg/kg) diluted in sterile dH_2O. We perform a two-step wake up procedure using two antidote solutions, one containing flumazenil (0.5 mg/kg) and buprenorphine (0.1 mg/kg) in sterile dH_2O (first antidote solution). The second one contains atipamezole (2.5 mg/kg) in sterile dH_2O (second antidote solution).

2.1.2. Prepare analgesia solution containing carprofen (5.667 mg/kg) diluted with sterile dH_2O.

2.1.3. Clean the stereotactic frame, heating pad and elements of the stereotactic robot. Bear in mind that not all the parts of the robot can be cleaned without risk of damage. Refer to the manual of the robot for details on cleaning and preparing for usage.

2.1.4. Assemble the syringe with the step catheter and flush it multiple times with dH_2O, 70% ethanol and 100% ethanol followed by flushing again with 70% ethanol and dH_2O. Finally, flush the syringe with PBS or other buffers to be used for preparation of the solution for intracranial injection, e.g. artificial cerebrospinal fluid. The plunger of the syringe should move smoothly and freely during the whole procedure.

2.1.5. Calibrate the stereotactic robot software with the stereotactic frame.

2.1.6. Test the stereotactic robot software by ensuring that the robot arms move freely and that the injection pump is properly connected and can perform the CED procedure without any disturbances. This includes testing robot movement, ramping injection, checking the 2 min waiting step and the speed of catheter retraction. All the parameters should fit the preprogrammed CED procedure described in point 1.3.5.

2.1.7. Insert the drill bit in the drill. It is recommended to sterilize the drill bits before use.

2.1.8. Prepare antibody solution using PBS or other buffer solutions such as aCSF. 1 to 20 µg of antibody in 5 µL can be injected in a single CED procedure. Other volumes and protein amounts should be tested prior to performing the experiment. Be aware that using high viscosity solutions might lead to catheter clogging.

2.1.9. Manually load the syringe with the diluted antibody.

2.2. Antibody injection by CED into striatum

2.2.1. Weigh the mouse and inject the three-component anesthesia solution into the peritoneum according to the body weight. Note the injection time. Transfer the mouse to a separate cage heated with a heating pad.

2.2.2. Observe the mouse to determine when the sedation starts. As soon as the mouse stops moving, apply ophthalmic ointment on the eyes to protect the cornea from drying out during the surgery. Full sedation usually starts 10–15 min from the injection of the three-component anesthesia solution.

2.2.3. Check pain reactions using the pinch-reflex test to ensure full anesthesia of the animal.

2.2.4. Shave the head using a hair trimmer.

2.2.5. Disinfect the skin with cotton swabs soaked in iodine solution.

2.2.6. Using a scalpel, make a 10 mm skin incision along the cranial midline finishing on the eye level.

2.2.7. Fix the mouse in the stereotactic frame using the nose clamp and ear bars. Ensure that the skull surface is horizontal and tightly secured. Apart from correct anatomical navigation this is also crucial to avoid tilting of the skull during the drilling and the CED procedure.

2.2.8. Place the syringe in the stereotactic robot.

2.2.9. Synchronize the drill bit with the tip of the catheter on a reference point. It is crucial that the relation between the position of the drill and the syringe is precisely determined in the software, so the injection can be performed in the desired anatomical region of the brain.

2.2.10. Retract the skin using forceps and localize bregma on the skull surface.

2.2.11. Reference bregma in the software using the tip of the drill bit.

2.2.12. Move the drill to a position 1 mm frontal and 2 mm lateral from bregma and drill a burr hole. Be careful not to damage the dura mater.

2.2.13. Move the syringe over the burr hole.

2.2.14. Dispense 0.5–1 µL from the syringe to ensure that no air bubbles are left in the catheter.

2.2.15. Start the CED program described in point 1.3.5. Observe the skull surface for any traces of fluid backflow from the injection spot. Monitor the breathing rate of the animal.

2.2.16. Once the CED program is over and the catheter is withdrawn from the brain, start the injection pump at 0.2 µL/min in order to check for catheter clogging during the CED. If no clogging occurred, you should immediately see a droplet of injection mix coming from the catheter tip.

2.2.17. Prior to reusing or storing the catheter, visually examine the catheter step for any signs of damage or wear under a microscope and clean it as in step 1.3.10.

2.3. Waking up procedure

2.3.1. Gently remove the mouse from the stereotactic frame.

2.3.2. Wash the surgery site with sterile saline solution.

2.3.3. Using forceps, fill the burr hole with bone wax.

2.3.4. Close the skin with thin-tipped forceps and apply surgical glue with a 10 µL pipette over the cut. Wait 15–30 seconds for the glue to polymerize.

2.3.5. Apply analgesia solution by subcutaneous injection. Note the time of injection.

2.3.6. Apply the first antidote solution. Note the time of injection.

2.3.7. Transfer the mouse to a separate cage with a heating pad and monitor the animal for startle reflexes.

2.3.8. If mouse has not gained full consciousness 15 min after administration of the first antidote solution, apply the second antidote solution by subcutaneous injection.

2.3.9. Monitor animals during recovery phase.

2.3.10. Check 1–2 h later as well as the next day for post-operative complications. If necessary, re-apply the analgesia.

2.3.11. For infection prophylaxis, add sulfadoxin (final concentration 0.08% w/v) and trimethoprim (final concentration 0.016% w/v) to drinking water to which the animals have access ad libitum for 1 week after the surgery.

REPRESENTATIVE RESULTS:

This protocol enables preparation of step catheters (**Figure 1**) for use in the CED procedure in a laboratory environment. In order to control the catheters for leakage, reflux along the needle tract and clogging, we recommend performing injections of a dye, e.g., trypan blue solution, into an agarose block. **Figure 3** depicts a cloud of trypan blue forming after injection of 1 µL at 0.5 µL/minute using a CED catheter (**Figure 3A**). No reflux along the needle tract was visible over the beginning of the catheter step. Furthermore, the dispersed cloud formed a desired spherical shape. This is in contrast with the results obtained using a conventional 27 G blunt end needle (**Figure 3B**), where significant reflux could be observed.

Moreover, CED requires an optimized injection procedure. **Figure 4** shows the results of injecting 2 µL of trypan blue into an agarose block using the ramping procedure described in the protocol (A) compared to an injection at a steady rate of 2 µL/minute (B). High injection speed forced the reflux along the catheter even when a CED catheter was being used.

Finally, as shown in **Figure 5**, CED enables perfusion of large volumes of the murine brain. Mice were injected with a rat anti-mouse TNFα antibody combined with FITC-dextran in 5 µL of PBS by CED (upper panel) or by a conventional bolus injection (bottom panel). The perfusion profile of CED was more uniform than of conventional injection and less tissue damage could be observed. In both cases there was a typical distribution profile of antibody and dextran particles over the corpus callosum. However, the dispersion profile of the injected antibody was more diffuse than of the high molecular weight dextran, exemplifying differences in distribution between different infusates.

FIGURE AND TABLE LEGENDS:

Figure 1. A schematic drawing showing the CED step catheter tip. Frontal (**A**) and side (**B**) views.

Figure 2. A schematic drawing depicting the application area of the adhesive. The upper 10 mm of the fused silica tubing are inserted in the metal needle. Apply the adhesive on the 10 mm of tubing starting from the tip of the metal needle.

Figure 3. Comparison of infusion results using CED catheter or a blunt-end needle. Injection of 1 µL of 0.4% trypan blue into an 0.6% agarose block at 0.5 µL/minute using a CED catheter (A) and a 27G blunt-end needle (B). Pictures taken immediately after the catheter or needle withdrawal. Cross marks the tip of the catheter or needle. Scale bar 5 mm.

Figure 4. Comparison of infusion results of ramping CED protocol with steady rate protocol. Injection of 2 µL of 0.4% trypan blue into 0.6% agarose block using a ramping CED protocol (0.4 µL at 0.2 µL/min, then 0.8 µL at 0.5 µL/min and 0.8 µL at 0.8 µL/min (**A**) or a 2 µL/min steady rate injection protocol (**B**). In both cases a CED catheter was used. Pictures taken immediately after the catheter withdrawal. Cross marks the tip of the catheter. Scale bar 5 mm.

Figure 5. Representative results of murine striatum perfusion by CED or by conventional bolus injection. Mice were injected into the striatum (position 1 mm frontal and 2 mm lateral from bregma, depth of 3.5 mm) with 1 µg of rat anti-mouse TNFα combined with 1 µg of FITC-Dextran with the molecular weight 2000 kDa in 5 µL of PBS. CED protocol (upper panel) or a conventional bolus injection (27 G needle, injection rate 1 µL/minute) was performed (bottom). Mice were sacrificed immediately after the CED procedure by controlled CO_2 asphyxiation and perfused with 4% formaldehyde in PBS. Brains were dissected and additionally fixed with 4% formaldehyde in PBS at 4 °C for 24 h. Subsequently, brains were washed with 15% sucrose for 60 minutes and transferred to 30% sucrose at 4 °C. After 24 h, brains were frozen on dry ice. Free-floating sections (25 µm) were stained using polyclonal goat anti-rat IgG (H+L) antibody coupled with Alexa Fluor 647 and counterstained with DAPI. Images were processed using the Fiji distribution of ImageJ. 10x magnification, scale bar 5 mm. 4 mice per group; a representative picture is shown.

DISCUSSION:

Convection-enhanced delivery, or pressure-mediated drug infusion into the brain, was first proposed in the early 1990[3]. This approach promises perfusion of large brain volumes behind the blood brain barrier in a controlled manner[2]. However, so far, only a few clinical trials have been performed using this approach, partially because CED in a clinical setup has shown to be technically demanding[24,25]. Recent developments in the catheter design and infusion programs seem to have overcome these technical difficulties[8,19]. Progress made in clinical implementation of therapeutic antibodies, including the advent of immunomodulatory checkpoint blocking agents, awaits application in the treatment of CNS disorders[10]. This development can be greatly augmented by employing CED in the experimental setup, such as using small rodent models.

Various CNS disease models are available in mice. These include experimental autoimmune encephalomyelitis (EAE) for multiple sclerosis (MS) and genetically engineered models for Alzheimer's disease (AD), Parkinson’s disease (PD), or for brain cancer. Many brain tumor models also rely on orthotopic tumor inoculation of murine glioma cell lines or implantation of patient-derived xenografts. This protocol enables delivery of antibody solutions directly into specific anatomic locations, thus resembling therapeutic procedures. It can be implemented in various experimental layouts where delivery of antibody into a precise brain region plays a pivotal role.

The critical factor in performing CED in mice is the availability of catheters. This protocol contains a precise description how to assemble a step catheter and test it in a series of in vitro experiments. One should bear in mind that the fused silica of which the step tubing is made is a brittle material and the quality of CED with a given catheter might decline over time. It is recommended to control the parameters of the step catheters in between the in vivo experiments by repeating the in vitro tests described in the protocol section 1.3.

The protocol can be adjusted for different injection volumes, types of infusate and brain regions. The injection volume can be manipulated by proportionally changing the duration of the injection steps. Here we describe infusion of 5 µL, but CED with 10 µL of antibody solution has been reported in the literature using a similar approach in murine brain tumor models, achieving excellent tissue distribution and perfusion volumes vastly exceeding bolus injection[7]. Furthermore, up to 28 µL infusate volumes have been reported using CED for application of liquids into the rat brain [22,26]. Non-proteinaceous substances can also be injected by CED, keeping in mind that the infusate should not be of high viscosity to avoid clogging of the narrow catheter tip. Using liposomes, it has been demonstrated that the charge of the infused molecules can vastly influence the tissue penetration, with neutral or negatively charged particles being able to be distributed over the largest volumes[22]. As depicted in **Figure 5**, FITC-dextran and antibody disperse differently: although both antibody and FITC-dextran distribute similarly along the corpus callosum, the antibody penetration of brain parenchyma is more diffuse than for FITC-dextran, which shows a smaller radius and a more spotty distribution pattern. This underlines the differences in CED profile between infusates with varying physicochemical properties.

Furthermore, the CED experiment described here and shown in **Figure 5** was performed injecting an anti-mouse TNFα antibody into healthy mice, so assuming minimal target amount in the striatum. Presence of cognate antigen will change the tissue distribution pattern. It can be further affected by inhomogeneous tissue at an anatomical site, as depicted in **Figure 5** by distribution of the infusate along the corpus callosum.

Finally, CED is affected by the flow of interstitial fluid, which in the case of striatum injection, can flush the infusate towards the lateral ventricles[27]. Indeed, even when the tissue is fixed immediately after finishing CED, we can observe a marked adhesion of the injected antibody to the ventricle wall (**Figure 5**). This can be further affected by pathological conditions of the CNS,e.g. in context of brain tumors. Focal necrosis, often observed in high grade brain tumors[28], can affect the flow of interstitial fluid and thus alter the distribution pattern of the infusate[29]. Other pathological conditions that can lead to changed tissue distribution of infusate as compared to healthy parenchyma include stroke or traumatic brain injury[30]. To sum up, every series of CED experiments has to be carefully validated to ensure successful perfusion of the target brain region.

Currently, researchers frequently use implantable osmotic pumps to deliver substances into the CSF or brain (tumor) parenchyma[31-33]. In certain cases CED as described here can be used as an alternative. It can be performed multiple times with frequencies depending on the brain region, type of infusate, volume and anesthesia protocol used. Intermittent drug delivery can be

particularly relevant when an extended exposure to the infusate leads to tolerance or systemic side effects. It is conceivable that in cases where high retention and half-life infusates are being delivered, this approach would represent a refinement according to the 3R principle since no pump implantation would be necessary. In conclusion, this protocol describes an efficient way of infusing large volumes of antibody solution into the murine striatum and can be adjusted for other brain regions and types of infusate.

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DISCLOSURES:

Johannes vom Berg is mentioned as an inventor on a patent regarding immunotherapy of glioblastoma PCT/EP2012/070088. The authors have no additional financial interests.

REFERENCES:

1 Scherrmann, J. M. Drug delivery via the blood-brain barrier. *Vascular Pharmacology.* **38** (6), 349-354 (2002).

2 Barua, N. U., Gill, S. S. Convection-enhanced drug delivery: prospects for glioblastoma treatment. *CNS Oncology.* **3** (5), 313-316 (2014).

3 Bobo RH et al. Convection-enhanced delivery of macromolecules in the brain. *Proceedings of the National Academy of Sciences of the United States of America.* **91** (6), 2076-2080 (1994).

4 Morrison, P. F., Laske, D. W., Bobo, H., Oldfield, E. H., Dedrick, R. L. High-flow microinfusion: tissue penetration and pharmacodynamics. *American Journal of Physiology.* **266** (1 Pt 2), R292-305 (1994).

5 Zhou, Z., Singh, R., Souweidane, M. M. Convection-Enhanced Delivery for diffuse intrinsic pontine glioma treatment. *Current Neuropharmacology.* **15** (1), 116-128 (2017).

6 Barua, N. U. et al. Intrastriatal convection-enhanced delivery results in widespread perivascular distribution in a pre-clinical model. *Fluids and Barriers of the CNS.* **9** (1), 2 (2012).

7 Shoji, T. et al. Local convection-enhanced delivery of an anti-CD40 agonistic monoclonal antibody induces antitumor effects in mouse glioma models. *Neuro-Oncology.* **18** (8), 1120-1128 (2016).

8 Souweidane, M. M. et al. Convection-enhanced delivery for diffuse intrinsic pontine glioma: a single-centre, dose-escalation, phase 1 trial. *The Lancet Oncology.* 10.1016/S1470-2045(18)30322-X (2018).

9 Zhang, X. et al. Targeting immune checkpoints in malignant glioma. *Oncotarget.* **8** (4), 7157-7174 (2017).

10 Barua, N. U., Gill, S. S., Love, S. Convection-enhanced drug delivery to the brain: therapeutic potential and neuropathological considerations. *Brain Pathology.* **24** (2), 117-127 (2014).

11 Mehta, A. M., Sonabend, A. M., Bruce, J. N. Convection-Enhanced Delivery. *Neurotherapeutics.* **14** (2), 358-371 (2017).

12 Krauze, M. T. et al. Reflux-free cannula for convection-enhanced high-speed delivery of therapeutic agents. *Journal of Neurosurgery.* **103** (5), 923-929 (2005).

13 Nash, K. R., Gordon, M. N. Convection Enhanced Delivery of Recombinant Adeno-associated Virus into the Mouse Brain. *Methods in Molecular Biology.* **1382** 285-295 (2016).

14 Ohlfest, J. R. et al. Combinatorial antiangiogenic gene therapy by nonviral gene transfer using the sleeping beauty transposon causes tumor regression and improves survival in mice bearing intracranial human glioblastoma. *Molecular Therapy.* **12** (5), 778-788 (2005).

15 Yin, D., Forsayeth, J., Bankiewicz, K. S. Optimized cannula design and placement for convection-enhanced delivery in rat striatum. *Journal of Neuroscience Methods.* **187** (1), 46-51 (2010).

16 Mamot, C. et al. Extensive distribution of liposomes in rodent brains and brain tumors following convection-enhanced delivery. *Journal of Neuro-Oncology.* **68** (1), 1-9 (2004).

17 Saito, R. et al. Tissue affinity of the infusate affects the distribution volume during convection-enhanced delivery into rodent brains: implications for local drug delivery. *Journal of Neuroscience Methods.* **154** (1-2), 225-232 (2006).

18 Oh, S. et al. Improved distribution of small molecules and viral vectors in the murine brain using a hollow fiber catheter. *Journal of Neurosurgery.* **107** (3), 568-577 (2007).

19 Barua, N. U. et al. A novel implantable catheter system with transcutaneous port for intermittent convection-enhanced delivery of carboplatin for recurrent glioblastoma. *Drug Delivery.* **23** (1), 167-173 (2016).

20 Rosenbluth, K. H. et al. Design of an in-dwelling cannula for convection-enhanced delivery. *Journal of Neuroscience Methods.* **196** (1), 118-123 (2011).

21 Debinski, W., Tatter, S. B. Convection-enhanced delivery for the treatment of brain tumors. *Expert Review of Neurotherapeutics.* **9** (10), 1519-1527 (2009).

22 MacKay, J. A., Deen, D. F., Szoka, F. C., Jr. Distribution in brain of liposomes after convection enhanced delivery; modulation by particle charge, particle diameter, and presence of steric coating. *Brain Research.* **1035** (2), 139-153 (2005).

23 Chen, Z. J. et al. A realistic brain tissue phantom for intraparenchymal infusion studies. *Journal of Neurosurgery.* **101** (2), 314-322 (2004).

24 Sampson, J. H. et al. Poor drug distribution as a possible explanation for the results of the PRECISE trial. *Journal of Neurosurgery.* **113** (2), 301-309 (2010).

25 Wick, W., Weller, M. Trabedersen to target transforming growth factor-beta: when the journey is not the reward, in reference to Bogdahn et al. (Neuro-Oncology 2011;13:132-142). *Neuro-Oncology.* **13** (5), 559-560; author reply 561-552 (2011).

26 Saito, R., Tominaga, T. Convection-enhanced delivery of therapeutics for malignant gliomas. *Neurologia Medico-Chirurgica.* **57** (1), 8-16 (2017).

27 Bedussi, B. et al. Clearance from the mouse brain by convection of interstitial fluid towards the ventricular system. *Fluids Barriers CNS.* **12** 23 (2015).

28 Noroxe, D. S., Poulsen, H. S., Lassen, U. Hallmarks of glioblastoma: a systematic review. *ESMO Open.* **1** (6), e000144 (2016).

29 Boucher, Y., Salehi, H., Witwer, B., Harsh, G. R. t., Jain, R. K. Interstitial fluid pressure in intracranial tumours in patients and in rodents. *British Journal of Cancer.* **75** (6), 829-836 (1997).
30 Glushakova, O. Y. et al. Prospective clinical biomarkers of caspase-mediated apoptosis associated with neuronal and neurovascular damage following stroke and other severe brain injuries: Implications for chronic neurodegeneration. *Brain Circulation.* **3** (2), 87-108 (2017).
31 Vom Berg, J. et al. Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease-like pathology and cognitive decline. *Nature Medicine.* **18** (12), 1812-1819 (2012).
32 Vom Berg, J. et al. Intratumoral IL-12 combined with CTLA-4 blockade elicits T cell-mediated glioma rejection. *Journal of Experimental Medicine.* **210** (13), 2803-2811 (2013).
33 Kurdi, A. et al. Continuous administration of the mTORC1 inhibitor everolimus induces tolerance and decreases autophagy in mice. *British Journal of Pharmacology.* **173** (23), 3359-3371 (2016).

Figure 1

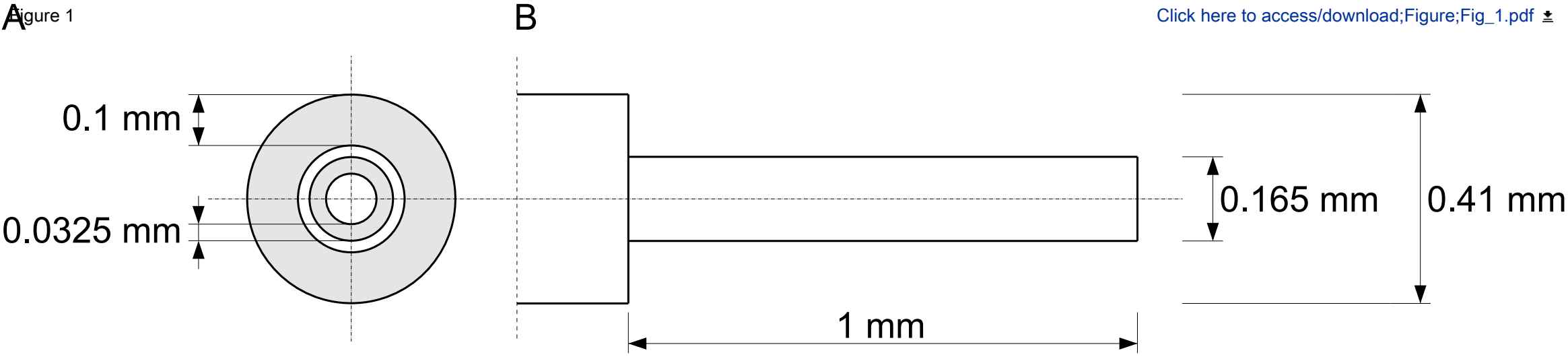


Figure 2

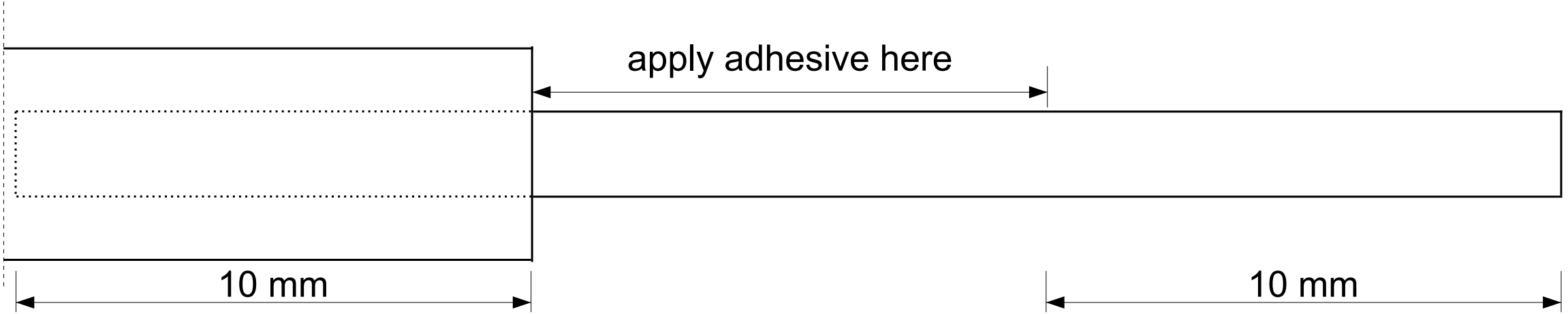
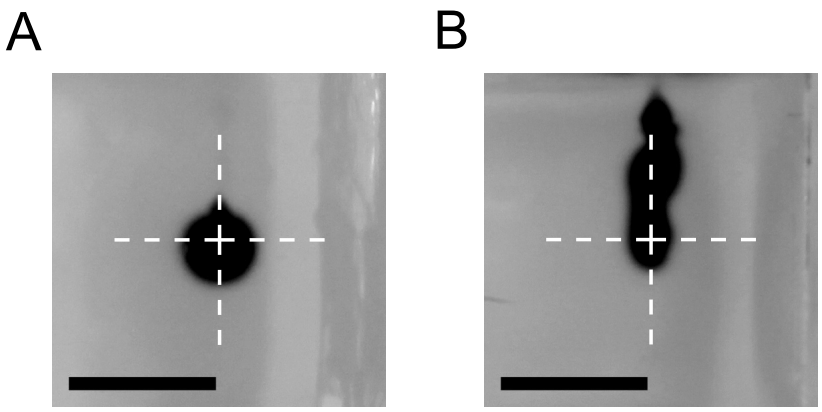


Figure 3



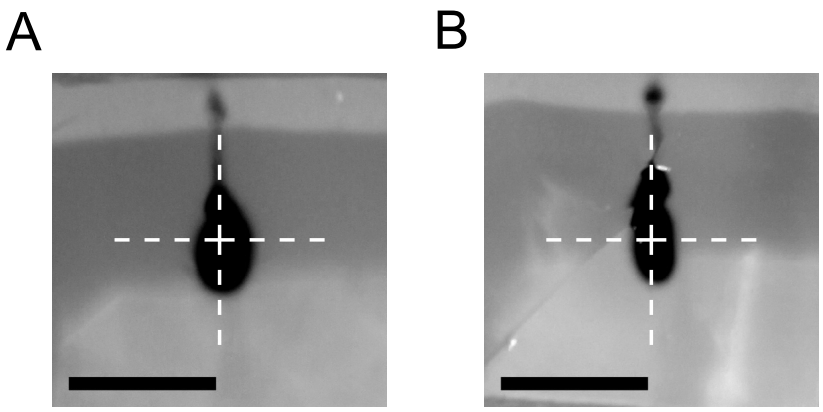


Figure 5

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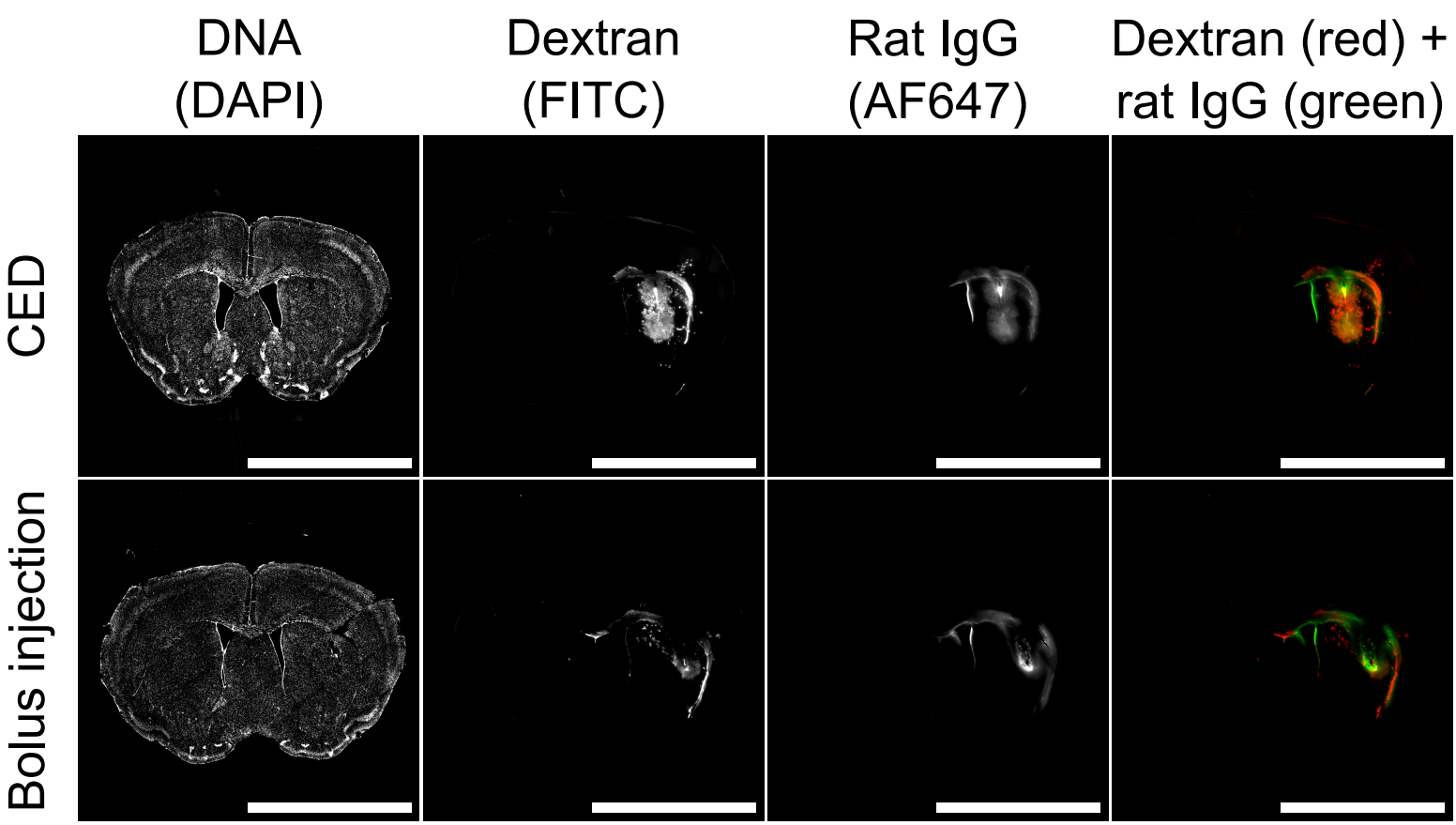

Figure 1 in svg format

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Figure 5 in svg format

Table of Materials

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Name of Material/ Equipment	Company
10 µL syringe	Hamilton
27 G blunt end needle	Hamilton
Agarose	Promega
Atipamezol	Janssen
Bone wax	Braun
Buprenorphine	Indivior Schweiz AG
Carprofen	Pfizer AG
Dental drill bits, steel, size ISO 009	Hager & Meisinger
Ethanol 100%	Reuss-Chemie AG
Fentanyl	Helvepharm AG
FITC-Dextran, 2000 kDa	Sigma Aldrich
Flumazenil	Labatec Pharma AG
Formaldehyde	Sigma Aldrich
High viscosity cyanoacrylate glue	Migros
Iodine solution	Mundipharma
Medetomidin	Orion Pharma AG
Microforge	Narishige
Midazolam	Roche Pharma AG
Ophthalmic ointment	Bausch + Lomb
PBS	ThermoFischer Scie
Polyclonal goat anti-rat IgG (H+L) antibody coupled	Jackson Immuno
Scalpels	Braun
Silica tubing internal diameter 0.1 mm, wall thickn	Postnova
Stereotactic frame for mice	Stoelting
Stereotactic robot	Neurostar
Succrose	Sigma Aldrich
Topical tissue adhesive	Zoetis
Trypan blue	ThermoFischer Scie
Water	Bichsel

Catalog Number	Comments/Description
7635-01	
7762-01	
V3121	
1029754	
1RF009	
179-VL03K-/1	
FD2000S	
F8775-500ML	
MF-900	
Vitamin A Blache	
10010023	
BB518	
Z-FSS-100165	
51615	
Drill and Injection Robot	
S0389-500G	
GLUture	
15250061	
1000004	

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Title of Article: LOCAL DELIVERY OF ANTIBODIES INTO MURINE BRAIN VIA CONVECTION-ENHANCED DELIVERY

Author(s): MICHAL BEFFINGER, LINDA SCHELLHAMMER, STANISLAV PANTELYUSHIN, THORSTEN BUCH, JOHANNES VOM BERG

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Zurich, 08. February 2019

Submission of a revised manuscript for consideration as a protocol article in the Journal of Visualized Experiments

Dear members of the editorial board, Dear Dr. Steindel,

Thank you very much for the opportunity to submit our revised manuscript (in track change mode for most relevant adjustments) entitled "Delivery of antibodies into murine brain via Convection-Enhanced Delivery" for consideration in the Journal of Visualized Experiments. We also would like to thank the reviewers for their helpful comments and considerations which have significantly helped to enhance the quality of our manuscript. Please find below a detailed point by point reply on the following pages.

Thank you very much for your consideration,

Yours sincerely,

Johannes vom Berg

Editorial comments:
General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have gone over the manuscript and have fixed spelling and grammar issues.

2. Please include all authors' email addresses in the manuscript.

All the authors' email addresses have been added to the manuscript below the Authors & Affiliations section

Protocol:

1. Please use the imperative tense throughout the protocol–every step/substep should have at least one imperative command

We have corrected phrasing in point 1.3.9.

2. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have rephrased the protocol steps in a way that new substeps have been created. Please refer to the updated manuscript.

Specific Protocol steps:

1. 1.2.2: Please explain how to calibrate the robot in more detail.

Step 1.2.2 has been rephrased and an explanatory sentence has been added.

2. 1.2.4: How exactly is the drop of adhesive applied?

A new step containing more detailed explanation has been added. Additionally, we have added a schematic drawing to further explain this crucial step (Figure 2).

3. 1.3.5: Can you, e.g., provide an example script (as supplemental material), or otherwise demonstrate how this is actually programmed?

Thank you for this comment. The programming of the injection robot is highly dependable on the precise type of the injection unit and the provider of the software. Furthermore, as

pointed out by the Reviewer #2, the protocol can be also performed using a microinjection pump, although programming the pump might differ from programming injection robot. Nevertheless, we provide the script used for the Drill and Injection Robot Stereotaxic controlled using Stereodrive version 4.4.1 (Neurostar) as supplemental information.

4. 2.2.1–2.2.3: Please do not highlight anesthesia steps.

Highlighted text has been adjusted.

References:

1. Please do not abbreviate journal titles in the references.

References have been corrected.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

The Table of Materials has been proofread and additional information has been added.

Reviewers' comments:
To Reviewer #1:

Major Concerns:
Overall this is a well presented technical manuscript that provides a clear step-by-step guide for individuals hoping to perform convection-enhanced delivery in murine tumor models. One major issue relates to the in-vivo validation of the CED protocol. The figures and the results seem to reference a single CED mouse case describing qualitative differences between bolus and CED infusion. A more thorough experiment with a larger number of mice would allow for quantitative analysis of infusate distribution, tissue damage and other outcomes. Although some may not deem this necessary, this would help validate the researchers findings.

Thank you very much for this comment. We fully agree that a quantitative analysis of infusate distribution and assessment of tissue damage would contribute further to the description of this method for delivery of antibodies in a precise brain region of a healthy murine brain. Since in many cases this method will be used to infuse antibodies into diseased brains, we feel that a thorough analysis in healthy and diseased (e.g. brain tumor bearing) animals would be even better. Such data exists. For example, in context of a brain tumor, the same injection ramping-up regimen that we describe has been used by Shoji *et al*. for CED of 10 µg of anti-CD40 in 10 µL of PBS into a bRiTs-G3 tumor lesion. The authors have quantitatively assessed the distribution profile and report over 7x increased distribution volume as compared to bolus injection (Neuro Oncol. 2016 Aug;18(8):1120-8. doi:

10.1093/neuonc/now023). We now explicitly refer to this study in the introduction and in the discussion paragraphs of the revised version of the manuscript. Nevertheless, we hope that we have sufficiently underlined in the manuscript, that such evaluation should be performed every time a new infusate and a new target brain region are chosen. We believe that a repetition of the experiment with a larger cohort of mice (ideally with and without brain tumors) for assessing the tissue damage and infusate distribution is exceeding the scope of the current manuscript which is solely focused on the generation, validation and use of the CED step catheter. The shown *in vivo* data serves only as an example.

Minor Concerns:
Pg. 2 Short Abstract: "(CED) is a method allowing perfusion of large tissue volumes without infusate reflux". CED can reflux in cases of local tissue disruption/excess shear force. This sentence should focus on the benefits of CED such as the use of bulk flow to attain high-local concentrations of infusate with minimal systemic leakage.

Thank you for these suggestions. The sentence has been rephrased within the allowed space limit of 50 words.

Pg. 3 Introduction Line 78: "administered up to 20 µg of antibody in 5 µl of PBS in mice." Would also add time period infusion occurred over ie. "up to 20 µg of antibody in 5 µl of PBS over 3 minutes in mice"

The required additional information has been added.

Protocol:
pg.4 line 98 "1.2.4" - This description of where to put the super glue is difficult to visualize. If the language could be made clearer or if a pictoral representation could be provided that would be helpful.

Step 1.2.4 has been divided into two steps and rephrased. We have also added a new figure (Figure 2) describing where to apply the adhesive.

pg. 4 line 101 "1.2.5." - What is the stereotactic robot? Company or make/model would be helpful for reproducibility.

Text corrected, more information has been added to the Material Table and a short introduction to point 1 of the protocol has been added.

pg. 7 line 186 "2.2.6" - Should include landmarks for start and finish of the incision.

Landmarks have been added.

Discussion:

pg. 10 line 306 - The authors state "there is a marked difference between the dispersion of the antibody and the FITC-dextran". When looking at Figure 4 minimal difference seems apparent between CED of these two infusates. Can the authors clarify this finding either in the text or in the figure (ie. by removing the DAPI in the composite to highlight differences between dextran and antibody).

Thank you very much for this thoughtful remark. Both FITC-dextran and antibody seem to be distributed along the corpus callosum, however we have repetitively seen that the antibodies seem to be more diffusely distributed in the parenchyma than the FITC-dextran. The FITC dextran signal pattern seems to be 'more spotty' and sharply confined compared to the antibody signal, which we believe is due to the difference in molecular weight (2000kDa vs 150kDa). Antibodies also seem to have a higher tendency to shift towards the next fluid filled space, which can be seen here as the strong signal staining at the wall of the neighbouring ventricle next the injection side, even though samples were taken immediately after the CED procedure. This becomes particularly prominent if samples are taken 30-60 minutes post CED, this might be explained by the flow of interstitial fluid towards ventricles (Bedussini *et al*. Fluids Barriers CNS. 2015 Oct 5;12:23. doi: 10.1186/s12987-015-0019-5). This point has been now raised in the discussion as well. Furthermore, we have corrected the contrast on the Figure 4 (now Figure 5).

pg. 10 line 317 - A brief discussion of how different pathological states, ie. tumor, can affect CED and infusate delivery should be undertaken here.

More information on the potential hurdles of CED in context of brain tumors has been added to the discussion. Thank you for the comment.

Reviewer #2:

Major Concerns:
no major concerns, the protocol is well documented, and differences between bolus injection and CED seem to be drastic in terms of target distribution of the injected agents.

Minor Concerns:
The manufacturing of the step catheters is well described. Re-assembling and testing time before each use might be time consuming and prone to variability though. Can the assembly be standardized in a way that repetitive experiments with identical outcomes are achieved?

Thank you for this remark. We have used the catheters prepared according to the presented protocol multiple times and achieved reproducible results. The most fragile part of the catheter is the step made of fused-silica, and this can be easily examined for any signs of cracks or damage. A new point 2.2.17 has been added to underline the possibility to reuse these catheters.

I would suggest to reformulate the part that describes the use of a stereotactic robot. Not many labs have access to this device. To reach a larger audience, I would state that the

procedure is also feasible with a simple stereotactic frame and microinfusion pump that can be connected to respective hamilton syringes

This is a very helpful comment to make sure the protocol is applicable to a wide range of laboratories. We have added an introduction to point 1 of the protocol describing the possibility to use different stereotactic setups.

2.1.1: Anesthesia methods are variable between labs and animal care/use commissions, a sentence for other anesthesia modalities (e.g. isoflurane) might be inserted.

Great suggestion. We are now discussing the use of different anesthesia and analgesia in introduction to point 2 of the protocol.

2.1.4: maybe it would be worth to autoclave the syringes before use. Is this possible with a silica tube or do they disintegrate upon the sterilization process?

The fused silica tubing is a chemically inert material, but we would not recommend autoclaving or any other heat- and pressure-based sterilization procedures due to a possibility of compromising the seal between the fused silica tubing and the external metal needle. Furthermore, according to the guidelines for the syringes used in our laboratory (10 µl with removable needle, model 701, Hamilton), repeated autoclaving might have a deteriorative effect on the syringe performance. We suggest using chemical sterilization methods and we have described in the protocol cleaning with ethanol solution.

2.1.7.: I would mention that the drill bit is autoclaved/desinfected before every use.

Thank you for this comment. We have added that to the text.

It is not described whether the procedure can be performed multiple times per mouse. What would be a meaningful treatment interval in case of multiple injections (I assume this is highly dependent on the infused substance).

In principle the method can be performed multiple times, although one has to consider the target brain region, type of infusate. Moreover, for the decision regarding the intervals we would definitely take into account the *in situ* half-life of infusate, its distribution volume and expected biological reaction. We have elaborated on this topic in the discussion section of the updated manuscript.

Is there a need of antibiotic prophylaxis when applying the procedure.

Thank you for this remark. We have added this point in the revised version of the manuscript.

Is the system advantageous compared to continuous osmotic infusion e.g. by miniosmotic pumps?

The advantages of the CED protocol proposed in this manuscript depend on the scientific question being asked. As opposed to the miniosmotic pumps, CED enables perfusion of larger anatomic areas for intermittent drug delivery with minimal tissue damage. This is particularly important when a high dose of the infusate is necessary to obtain the biological effect and when an extended exposure leads to tolerance or systemic side effects. Intermittent delivery also resembles more closely the current clinical CED applications. We have elaborated on this topic in the discussion section of the updated manuscript.

Reviewer #3:

Major Concerns:
NONE

Minor Concerns:
TITLE line 3: I would question the authors choice of full title, local versus convection enhanced delivery. , exclude one of your deliveries

Yes, we agree that CED itself implies local therapy, so the word “local” was redundant in the title.

ABSTRACT, line 32: Given the small volumes it would be only a representation of a ramping regime of infusion to be convection. Is ramping of such a small volume possible

We fully agree that to benefit from the advantages of CED, it is required to use infusate volumes sufficient to form a pressure gradient at the catheter opening. We do not recommend infusing less than 5µl of volume with the ramping regimen described here. We are regularly performing CED with the volumes of 5 µl, in which the ramping-up injection itself is extended to 11 minutes 30 seconds. We think that this procedure gives a good balance between creating convection and avoiding damage to the target brain region. As mentioned in the introduction, infusing higher volumes is feasible.

Line 35: I would rephrase this sentence to remove, to name a few.

Sentence has been corrected.

INTRODUCTION, line 46: The original CED authors should be references here. Bobo, Chen ref 21R.H. Bobo, D.W. Laske, A. Akbasak, P.F. Morrison, R.L.Dedrick, E.H. OldfieldConvection-enhanced delivery of macromolecules in the brain

Thank you very much for pointing this out, the citation has been corrected.

PROTOCOL, line: 142: Diluted antibody, as opposed to antibody solution.

We have corrected the sentence.

Line 146 Ensure correct surgical setup, to include necessary tools and reagents to allow for a successful surgery to be completed

The sentence has been corrected.

DISCUSSION, line 273 confirmation of correct references to the 1990's these papers utilise the Bobo, Chen of the 1990's)

The citation has been corrected.

Minor comments :
Sucrose, as opposed to sucrose
US or UK theatre theater tumour tumor

We now adhere to US English throughout the manuscript.

CED script to be used