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Title: Construction and implantation of a microinfusion system for sustained delivery of neuroactive agents

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Abstract: Experimental protocols used for chronic infusion of neuroactive agents within regions of the brain often utilize a mini-osmotic pump system. Agents are commonly delivered via a stainless steel cannula with a diameter of 0.30 mm or greater. Systems utilizing a cannula of this caliber may impose trauma to the area of interest resulting in architectural damage, thereby compromising structural integrity and normal functioning. As neuroscience inquiry becomes more sophisticated, investigation of brain structures and circuitry requires improved levels of accuracy and higher resolution. We have developed a method for the preparation and implantation of a chronic infusion system within the brain utilizing a borosilicate microcannula with a tip diameter of 50 microns. This technique reduces damage to the local environment and diminishes reactive gliosis at the site of infusion. The configuration of the microinfusion system is also able to conform to the surface of the animal's skull, precluding the need for large cranial pedestals, and thus

facilitating closure of the scalp incision and reducing the risk of infection. We demonstrate reliable sustained delivery of a dye having a representative molecular weight using an in vitro model and in vivo studies in rats.

*** Revision Notes**

15 August 2007

Dear Dr. Gerhardt:

I would like to express my appreciation to you and the reviewers for offering further consideration for publication in the Journal of Neuroscience Methods of our manuscript, *Construction and Implantation of a Microinfusion System for Sustained Delivery of Neuroactive Agents*. The paper has been significantly re-worked based on the reviewers' thoughtful suggestions, and we have added a new experiment demonstrating reduced localized trauma using the proposed system. I am hopeful that in its revised form you will see it as being suitable for your journal. While I address each comment in turn below, I first would like to make a general statement about how the methods and data are presented:

The object of this paper is to describe a technique for chronic infusion of neuroactive agents with a system that reduces the delivery cannula some 5-10 fold. We do not claim *superiority* of this system over conventional methods, but demonstrate that the principles for reducing trauma and targeting small structures can be applied, and that reliable delivery is not compromised by doing so. It is to be decided by the scientist whether the method we present here is preferable for her or his experiments.

Thank you.

Miles

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Response to Reviewers

Reviewer #2 acknowledges the paper as being well written and commends the detailed and instructive description of the technique. This reviewer enumerates a number of valid points:

1. The authors state in the abstract and in the introduction that cannulas with diameters of 10-100 microns were used in the investigation. In the MoM section, it is stated that a 50 micron cannula was used for the experiments presented. Finally, in Fig 1, which depicts the trauma caused by cannula, the diameter of the cannula is said to be 70 microns.
This discrepancy must be explained, or corrected.

In these experiments, all microcannulas were made with a tip diameter of 50 μm. This has been corrected throughout the paper, and Fig. 1 has been replaced by Fig. 6.

2. If the authors had the possibility to manufacture cannula with a diameter of 10 microns, why were such a cannula not used for delivery of fast green?
Is it because such cannulas become rapidly obstructed? Were different cannula diameters investigated in vivo? This should be commented in the manuscript.

50 μm was chosen as suitable for illustrating the concepts of this methodology. Indeed, a smaller microcannula could most likely have been used without problems of obstruction – at least with Fast Green. Other, more viscous, infusates may obstruct smaller diameter microcannulas. We have added mention in the Discussion that 50 μm microcannulas were the only ones tested here, but smaller diameters may be utilized.

Abstract:
The abstract must be revised in order to better reflect the content of the manuscript.

1. The material (borosilicate) used to manufacture cannula should be stated.

Done.

2. The diameter of common used stainless steel cannula is said to be 0.31 mm. In the manuscript body, the diameter is said to be 0.25 mm - 0.5 mm. What is the diameter commonly used? From which reference is the value 0.31 mm obtained?

28 gauge is one of the most commonly used diameters of stainless steel delivery cannulas. This gauge can actually be found to range from 0.3 – 0.32 mm. Thus, for comparisons in this paper, we have used the Plastics One product, with a diameter of 0.3 mm. This has been corrected in the Abstract. And the most commonly used gauge is now given in the introduction.

3. The sentence "This technique allows increased precision and minimal trauma to the target region thereby reducing gliosis and other reactive cellular processes".
When reading this, one would assume that the process of gliosis has been investigated and presented in the manuscript. However, this was not done and the sentence should be rephrased.

We have rephrased the sentence and we have added an experiment which indeed demonstrates reduced reactive gliosis (Fig 6).

Materials and methods:

1. What was the brand of the steel cannula depicted in fig 1A, which was used for comparison with the 70 micron borosilicate cannula? This should be stated.

We now provide the product information (page 3).

2. The borosilicate cannula in fig 1A seems to have a conical shape. Is the tip of the cannula 70 microns, or is the entire part of the cannula that was implanted 70 microns in diameter? Please explain this.

This figure has been replaced by the more descriptive Figure 6.

3. Fig 5 shows in vitro testing of the osmotic pump fitted with a borosilicate cannula. This image and parts of the manuscript, it seems to me at least, reflects the function of the osmotic pumps rather than the effects of the cannula diameter. Would one expect that reducing the cannula diameter would decrease the flow-rate from the pump? Fig 5 should still be included in the manuscript, as it clearly depicts how in vitro testing of the MOP-cannula can be done.

Agreed. This figure does in fact demonstrate a suitable test for researchers needing to optimize their procedure. But the figure also illustrates the ability of the microinfusion system to function – that is, to allow unimpeded delivery of a solution. This is stated in the figure legend.

Results and discussion:
No comments or objections.

Finally, a general question, possibly of interest for the authors:
A smaller cannula might very well lead to less gliosis and tissue damage. However, the texture of the material implanted is also important for reducing cell activation and tissue response. Is this something that could be done to the borosilicate cannulas?

Interesting point – although perhaps out of the scope of this paper. It may be possible to coat the borosilicate cannula with an inert coating, e.g., silicone or a “de-activating” or anti-inflammatory agent.

Reviewer #3: General comments:

Authors describe here an alternative to the cannula of Alzet mini-osmotic pumps: a custom made microcannula. Even if the rationale for such an improvement is easy to conceive (i.e.: less damage to brain tissue), a clear demonstration of the claimed (in Abstract, Introduction and Discussion) advantages (small targeted structure, increased precision, minimal trauma, reduced gliosis or reactive processes, reduced risk of infection) of their construction, over the commercially available pump and cannula has not been shown. This demonstration is of particular importance because the fabrication of the microcannula needs special equipments and seems to be rather complicated to reproduce.

Reviewer #3 begins by stating that it is "easy to conceive" that if the delivery cannula is many-fold smaller, there will be decreased damage and diminished reactive gliosis. There is an enormous body of data supporting these principles. Moreover, by reducing invasiveness and allowing a surgical wound to be entirely closed (as opposed to only partially closed *around* a skull pedestal), one skilled in the art should understand why this reduces the risk of morbidity (e.g. infection and discomfort). However, this reviewer makes the point that, nevertheless, the authors do not support the claimed advantages with formal experiments.

We agree that all of the potential advantages of reducing the delivery cannula size by 5-10 fold were not experimentally tested and proven in this manuscript. Truly, this was not the goal of the paper; however, we acknowledge this reviewer's point and have therefore re-written a large part of the paper, being careful to use language that does not make unsupported claims. In its revised form, this manuscript now describes a method for building and surgically implanting a chronic microinfusion device that employs a delivery microcannula 5x smaller than conventionally used delivery cannulas. Further, we demonstrate that a) the system can reliably deliver a neuroactive agent over at least four days, and b) it indeed reduces physical trauma to the local environment and dramatically reduces reactive gliosis. It will be the decision of the experimenter whether the described method is superior or preferable for her or his applications.

Reviewer #3 further comments that the method requires special equipment and is difficult to construct. The only (possibly) specialize equipment is an electrode puller, a surgical drill (e.g., Dremmel) and a fiber optic lamp. These items are quite common in neuroscience laboratories and other research facilities. Regarding difficulty in construction, an inexperienced, naïve technician can learn this fabrication within a day. (I have taught this construction to 3 undergrads and a high school student, with no neuroscience experience, in a matter of hours.)

Major points:

1) The entire materials and methods section is not sufficiently well described to allow the reproduction of this study and needs to be revised to add precisions about the different techniques and equipments. Particularly, company names and locations from which equipments and tools were obtained is often not mentioned. Technical details must be added. How to lower the flow rate of the pump by paraffin coating (more precisely and exactly, how much time is it dunked into paraffin, what temperature is the paraffin)? How the final flow rate obtained is determined?

Reviewer #3's first comment is in direct contradiction to that of Reviewer #2, who stated that the paper is well written and provides detailed and instructive description of the technique. We originally attempted to compose the paper to provide succinct yet ample guidance for the experimenter and we feel that excessive detail, particularly regarding information that is known or easily acquired by a skilled scientist, would add unacceptable volume to the manuscript. Nevertheless, we have provided company names and locations, and while we did not include details for routine preparation of ALZET MOPs, we now point out that this information is provided by the manufacturer.

2) The description for the fabrication of the microcannula must be clarified and better described. How to cut the microcannula to 50µm? With what type of microforceps?

We describe preparation of the microcannula tip in section 2.2.1, line 5 (page 4). Microforceps are common to most labs, and they are provided by numerous companies. We specify the company on page 5.

3) How to determine the 'predetermined distance' to impose a right angle to the microcannula?

The predetermined distance is based on the depth of the target within the brain. Since one benefit of the present method is the ability to custom-build the system, this distance will be decided by the researcher based on her or his particular design. We presumed the experimenter would have the basic ingenuity to determine the specifications of his or her own customized system.

Nevertheless, the following information was added to section 2.2.1 (page 5):
"The predetermined length of the microcannula distal to the imposed right angle is decided based on the depth of the brain region to be targeted (e.g., 5 mm) and taking into account the skull thickness (~1mm) and working distance above the skull (e.g., 1-2 mm). Thus the predetermined distance from the tip of the microcannula for the present demonstrations was 7-8 mm."

4) Authors say that their microcannula tip range is from 10 to 100 µm. How do they measure the diameter of the fabricated microcannula? Is it possible to make a microcannula of a specific diameter? How?

Diameters of electrodes, cannulas, microforceps, etc. are easily measured with a dissecting scope or a standard microscope at low power using a stage micrometer. Again, this is working knowledge many scientist possess. However, we added the following sentence in section 2.2.1 (page 5/6):
"A microscope stage micrometer can be used to guide and confirm the desired diameter."

5) Also, describe exactly how the polyethylene tubing is attached to the stainless steel tubing.

The second sentence of section 2.2.2 was reworded thusly (page 5):
"A length of polyethylene tubing (Plastics One, Roanoke, VA , #C312 VT; OD, 1.22 mm; ID: 0.72 mm) is attached to the area of stainless steel tubing previously occupied by the flange (~4 mm) by inserting the stainless steel tubing into the lumen of the polyethylene tubing and securing with a suitable adhesive around the outer circumference of the connection."

6) Twelve rats were studied but there is no description of the different groups. How many rats for each time point, how many received the microcannula construct vs the original Alzet system? How much rat were sacrified because of microcannula breaking?

This is clarified in section 2.1 "animals and design" (page 3/4). There were no mishaps with microcannulas so no animals were sacrificed for that reason. Microcannulas virtually never break if the experimenter is reasonably skilled and cautious.

7) Figure 1 is incomplete. First, it doesn't describe for how much time the 2 cannulas have been implanted (in B and C). Secondly, this figure only shows the 'holes' leaved by the different cannulas and gives no ideas about neuronal cell death, microgliosis and astrogliosis around the canullas. Another figure or pictures in figure 1 must be added to show the difference between the 2 cannulas regarding those 3 aspects.

These points are acknowledged and a more formal experiment was conducted allowing the construction of a revised figure that includes a demonstration of reduced trauma and diminished reactive gliosis. This is now Figure 6.

8) Since the fabrication of the authors' pump and microcannula system implies the break down of the Alzet system to reuse the Alzet pump with another cannula, authors must demonstrate that their system function as well or better than the original system to worth the reconstruction of a system that is already ready to use. This was not demonstrated since the results shown are mainly only those of the authors' microcannula. No controls with the original Alzet system were done! Those controls must be added to the Results section and in figures. The figure 6 must be completed with results from those controls animals.

We are concerned that Reviewer #3 may have a misunderstanding. Neither the authors nor the manufacturer recommends reusing the mini-osmotic pumps. This method demonstrates preparation of the system using fresh, unused pumps, therefore the point about controls using the original ALZET is moot. Furthermore, Figure 6 does not describe a comparison, but rather it illustrates reliable sustained function of the device and method described.

9) Authors must explain why in figure 5 leakage of their system is visible in 2 points of their montage and how this affects the reliability of their system.

We presume the reviewer is referring to not only the obvious infusion of dye into the smaller Eppendorf tube, but the appearance of light blue-green coloration at the interface of the stainless steel/polyethylene tubing and the pump itself within the 15 ml conical tube. This is a good observation, but does not represent leakage. During the insertion of the stainless steel/polyethylene tubing into the pump, dye is forced into the tubing/pump interface and stains the cured adhesive. This is what has been mistaken as a "leak". Further careful inspection of this figure reveals that over time the saline in the 15 ml conical tube does not become colored, indicating that leakage is not occurring.

10) Also, the flow rate shown in figure 5 is clearly more than 0.125µl/hr since after 5 minutes (in A) more than 0.01 µL of the coloured solution is released. Please add another in vitro test that is comparable to the in vivo study.

The reviewer appears to be estimating the volume of dye that seems to be flowing downward in panel A of this figure. The dye exiting the tip of the microcannula is highly concentrated relative to saline, and its molecular weight is much greater. Upon contacting saline, the dye molecules begin to disperse, coloring the surrounding saline. And because the system is entirely still, the dye molecules tend to flow downward due to their increased mass. Nevertheless, it would seem that more than 0.01 µL would account for what is seen in panel A. Dye accumulation at the microcannula tip before placing in the Eppendorf tube must also be taken into consideration. An additional consideration (that is also provided by the pump manufacturer) is that pump rates, while accurate over long periods of time, at any given time may be increased or decreased. It is quite possible that the dye volume in panel A is greater than 0.01 µL. However, this *in vitro* preparation is designed to demonstrate unimpeded flow through the microcannula and not to test pump function or dye volume.

11) Please explain and demonstrate how the microcannula allows the targeting of smaller structures than the original pump. Maybe a smaller time course in needed along with the comparison with controls.

The rationale for this question is not clear. However, it may be now addressed with the added experiment and illustrations in Figure 6. Note that standard cannulas can result in a lesion in excess of 250 µm in diameter. If the desired target is 3 mm in diameter, one could argue that there occurs relatively less damage* than if the target is 0.5 mm in diameter (e.g., hilar region of the dentate gyrus), in which case half of the structure under study would be destroyed. Using a microcannula reduces this damage and most likely ensures more normal function of the area of interest.

(*However this argument is fallacious because one is still destroying a large amount of tissue that may comprise a substructure or contribute to the function of the brain region overall.).

12) Authors say (in Discussion) that their method requires 'skills in such techniques', please explain what and how much skills are needed, how much surgeries or attempts must been done until we 'rarely experience breakage'.

This is difficult to address, as levels of ability are quite variable. In our experience, researchers who are conscientious and careful ("exacting") acquire the necessary skill to use this method within a day. "Skilled in such techniques" refers to techniques in fabricating apparatus and performing stereotaxic surgery.

Minor points:

a) Please specify for how much time in vitro and in vivo the sustained delivery was in the Abstract (last sentence).

Corrected

b) Last sentence of the Materials and methods section 2.1. needs a dot (.).

Corrected

c) Please change the term 'subject(s)' for animal(s) or rat(s) in the Abstract and Discussion.

Corrected

d) First sentence of the Materials and methods section 2.2.2., add 'Alzet' (...stainless steel 'Alzet' MOP tubing...)

Corrected

e) Figure 4 C and D, a higher magnification or closer view for those pictures would be greatly appreciated.

Acknowledged, but higher power would compromise appreciation for the entire set-up of the system.

f) Add 'in vivo' in the Materials and methods section 3.2. title.

Corrected

g) Specify for how long the system can be implanted in the animals and if the pump can be refilled or not while it is implanted.

We demonstrate the ability for sustained delivery (for at least 4 days) of the system described. The length of time of an experiment is determined by the design and the model of the MOP. And as mentioned above, the re-use of pumps is not recommended. This information is provided by the pump manufacturer, and it has also been added to the discussion (page 12).

h) Is the microcannula suitable for mice? Do the authors have tested it in mice?

These studies do not include mice; however, it seems evident that a system that requires less skull surface area, reduces the risk of infection, and is less traumatic would be preferable over conventional methods for mice. This point has been added to the discussion (page 12).

i) Please precise the figure legends (time points, magnifications).

Corrected. Scale bars have been added where appropriate.

*** Title page-incl. type of article and authors' name and affiliation**

Construction and implantation of a microinfusion system for sustained delivery of neuroactive agents

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Running Head: **microinfusion system**

Abstract

Experimental protocols used for chronic infusion of neuroactive agents within regions of the brain often utilize a mini-osmotic pump system. Agents are commonly delivered via a stainless steel cannula with a diameter of 0.30 mm or greater. Systems utilizing a cannula of this caliber may impose trauma to the area of interest resulting in architectural damage, thereby compromising structural integrity and normal functioning. As neuroscience inquiry becomes more sophisticated, investigation of brain structures and circuitry requires improved levels of accuracy and higher resolution. We have developed a method for the preparation and implantation of a chronic infusion system within the brain utilizing a borosilicate microcannula with a tip diameter of 50 microns. This technique reduces damage to the local environment and diminishes reactive gliosis at the site of infusion. The configuration of the microinfusion system is also able to conform to the surface of the animal's skull, precluding the need for large cranial pedestals, and thus facilitating closure of the scalp incision and reducing the risk of infection. We demonstrate reliable sustained delivery of a dye having a representative molecular weight using an *in vitro* model and *in vivo* studies in rats.

Keywords: mini osmotic pump, chronic infusion, microcannula, fast green

1. Introduction

Direct infusion of neuroactive agents allows specific brain regions to be studied while bypassing the blood brain barrier. The applications of this approach in neuroscience are diverse and include altering the level of brain activity in discrete subregions (Berretta et al., 2004; Gliddon et al., 2005; Kim et al., 2000), investigating the actions of psychotropic agents (Clinton et al., 2006; Di Benedetto et al., 2004), providing controlled models of brain inflammation (Hauss-Wegrzyniak et al., 1998; Marchalant et al., 2007; Rosi et al., 2004), studying the mechanisms of addiction (Kim et al., 2005; Lockman et al., 2005; Zhang et al., 2006), and enabling long-term administration of trophic factors (Naert et al., 2006; Radecki et al., 2005; Takahashi et al., 2006).

Standard methods for chronic delivery of agents often utilize a mini-osmotic pump (e.g., ALZET Osmotic Pumps, Cupertino, CA) loaded with a neuroactive agent, which is delivered within the brain through a stainless steel cannula with a diameter that may range from 0.25 mm (Williams et al., 1987) to 0.5 mm, but most commonly is approximately 0.3 mm (Plastics One, Roanoke, VA ; see Fig 6A). While delivery cannulas of this size may be suitable for many applications in which high levels of precision are not required and trauma to the microenvironment is not a major concern, these cannulas are suboptimal for delivering agents to small, delicate sites in which the cannula is comparable in size to (or larger than) the targeted structure itself, or where the function must not be compromised by traumatic insult.

Presented here is a method for the preparation and implantation of a chronic infusion system for delivery of neuroactive agents within the brain utilizing a "microcannula" with a much reduced tip diameter. This technique allows small substructures to be targeted and reduces trauma to the of the area of interest. The present procedure is thus taught as an alternative to conventional methods for researchers who desire to minimize disruption to the brain region under investigation.

2. Materials and methods

2.1. *Animals and design*

Eighteen adult (400-450 gm) male Sprague-Dawley rats were used to demonstrate the procedure and test the function of the present method. Twelve animals were implanted with microinfusion systems and contributed to a time-course evaluation of sustained function over 4 days. Six animals were used to compare level of trauma and reactive gliosis 5 days after implantation of either a standard 28 ga delivery cannula

(N=3) or a microcannula (N=3) attached to a saline-filled mini-osmotic pump. Animals were housed in clear plastic cages, and maintained on a 12-hour light/dark schedule, with food and water provided ad libitum. All procedures were approved by the McLean Hospital Institutional Animal Care and Use Committee, in compliance with applicable federal and local guidelines for experimental use of animals.

2.2. *Microinfusion system assembly*

Figure 1 illustrates the components and assembly of the microinfusion system. We recommend constructing and implanting the system using sterile technique. In addition, in order to prevent introduction of air into the system, it is assembled while submerged within a saline bath. For the present tests, mini-osmotic pumps (MOPs) were employed (ALZET, model #1002, Cupertino, CA) with a 14 day duration, flow rate of 0.25 µL /hr, and a fill capacity of 98.6 µL. However, since excessive flow rates can deluge smaller areas of interest in the brain and cause structural damage, the flow rate for these studies was decreased to 0.125 µL /hr by coating one half of the MOP with paraffin as instructed by the manufacturer. For evaluation of local trauma, MOPs were filled with sterile saline. For evaluation of sustained function of microinfusion systems, Fast Green (1% in saline, Fisher Scientific, Park Lane Pittsburgh, PA) was chosen as a test solution because of its low toxicity, its ability to diffuse within viable brain tissue, and because it has a molecular weight (808.84) at the upper end of the range for "small molecules" (<1000) that are commonly of interest for chronic infusion (e.g. muscimol, picrotoxin, fluoxetine, etc.).

INSERT FIGURE 1 ABOUT HERE

2.2.1. *Microcannula preparation*

The microcannula can be fashioned using a standard horizontal or vertical electrode puller (Stoelting Co., Wood Dale, IL) with settings for producing a glass electrode with a long, gently tapering shank (Fig 2A). Borosilicate tubing is suitable for this purpose (part 1B100F-6, 1.0 mm, 6 in; World Precision Instruments, Inc., Sarasota, FL), as it has a relatively low melting point, allowing subsequent bending with focused heat. The distal-most portion of a straight microcannula can be cut with dissection scissors or a controlled break can be made with microforceps (World Precision Instruments, Inc., Sarasota, FL) to give the desired final tip diameter (50 µm for the

present demonstrations), and a microscope stage micrometer is useful to guide and confirm the desired diameter. The cut tip can be briefly heated, or “fire-polished”, to remove rough or sharp edges. The heating coil of the electrode puller or a bunsen burner is then used to impose a right angle onto the microcannula at a predetermined distance from the tip of the microcannula (Fig 2B) by placing the borosilicate tubing within the heating coil (or a bunsen flame) and applying gentle force at the distal portion with forceps as the tubing is heated. The predetermined length of the microcannula distal to the imposed right angle is decided based on the depth of the brain region to be targeted (e.g., 5 mm) and taking into account the skull thickness (~1 mm) and working distance above the skull (e.g., 1-2 mm). Thus the predetermined distance from the tip of the microcannula to the imposed right angle for the present demonstrations was 7-8 mm. A diamond pencil is then used to cut the borosilicate tubing approximately 8 mm proximal to the right angle.

INSERT FIGURE 2 ABOUT HERE

After the desired number of microcannulas has been made, we recommend sterilization with ethylene oxide or using an autoclave. The distal-most 1-2 mm of the microcannula can be colored with a sterile surgical marker (or permanent ink prior to sterilization) to allow the microcannula tip to be easily visualized during the surgical procedure.

2.2.2. *MOP preparation*

MOPs were prepared according to the manufacturer’s instructions. Briefly, the plastic flange from the stainless steel MOP tubing (“flow modulator”) is first removed using scissors or rongeurs. A length of polyethylene tubing (Plastics One, Roanoke, VA, #C312 VT; OD, 1.22 mm; ID: 0.72 mm) is attached to the area of stainless steel tubing previously occupied by the flange (~4 mm) by inserting the stainless steel tubing into the lumen of the polyethylene tubing and securing with a suitable adhesive around the outer circumference of the connection. LumaBond (myNeuroLab, Inc., St. Louis, MO) is particularly useful as it cures within seconds upon exposure to focused light (e.g., from a fiber optic lamp). The length of tubing should approximate the distance between the base of the animal’s skull and the rostral-most aspect of the scapulae (e.g., approximately 1.5-3.0 cm for laboratory rats). The pump is filled as instructed, and the

stainless steel ALZET tubing, with the length of polyethylene tubing attached to its end, is inserted into the filled pump. A small volume of solution from the pump will be forced around the outside of the pump-tubing interface (and should be dabbed away) and also into the proximal region of the polyethylene tubing. The filled pump with attached tubing is then incubated in sterile saline for 12 hours at 37°C. If the pump is functioning properly, after this incubation period, fluid will be seen to have traveled a few millimeters within the polyethylene tubing.

2.2.3. *Construction of microinfusion system*

The pump with its attached tubing is immersed in a 10 cm Petri dish containing sterile saline, and the remaining air within the polyethylene tubing is removed using a syringe filled with the same solution contained in the pump and which has been fitted with small-diameter tubing that can be inserted within the polyethylene tubing. Solution can thus be injected into the polyethylene tubing to replace the air. Similarly the microcannula is immersed into the saline bath and air is removed by injecting with solution. This is easily achieved with a second solution-filled syringe fitted with flexible (e.g., silicone) tubing that fits over the large (proximal) end of the microcannula.

The proximal end of borosilicate tubing is then inserted into the polyethylene tubing. Note that this may require dilation of the polyethylene tubing by firmly pressing the closed tips of microforceps into the lumen thereby flaring the opening for easy insertion of the borosilicate tubing. The air-free infusion system is then removed from the saline bath and placed on a sterile surface and gently dried with gauze or a cotton swab. The microcannula-polyethylene tubing connection is secured with a circumferential coat of adhesive (e.g., LumaBond).

If the system has been assembled properly, and if the MOP continues to function as it should, during the final step of this procedure a droplet of solution will usually appear at the tip of the microcannula as the osmotic mechanism within the pump continues during preparation. MOP flow rate can be decreased proportionately by coating the appropriate surface area with paraffin as instructed by the manufacturer. For the present demonstrations, 50% of each MOP was coated by briefly dunking it into molten paraffin and allowing it to cool, thus reducing the flow rate by half to 0.125 μL/hr. The completed microinfusion system is then submerged in sterile saline and stored at 37^{o} C until implantation.

2.3. Implantation of microinfusion system

The microinfusion system is implanted using standard stereotaxic methods as previously described (Cooley, 1990). After preparation of the surgical field, a burr hole is drilled for entry of the microcannula. If desired, skull screws can also be positioned to stabilize the bonding compound used to secure the microcannula. The compound used in this laboratory (Geribond, Den-Mat Inc., Santa Maria, CA) does not require additional anchoring; however, the skull surface must be prepared by cleaning with an acetone-dampened cotton swab. A 1-2 mm diameter "hook" is made at the distal end of the alignment wire (see Fig. 1), allowing it to encircle the bend in the borosilicate tubing. It is then secured (using LumaBond), in an orientation along the same axis as the distal portion of the microcannula (that is to be advanced into the brain, see Fig. 3B). The infusion system is then mounted on the stereotaxic carrier (Fig. 3), clamping the alignment wire onto the holder. Sterile suture is used to tether the infusion pump to the top of the stereotaxic manipulator, thus suspending it and preventing loss of orientation during the procedure due to the weight (and torque) of the pump (Fig. 3B). The microcannula can then be positioned in the desired orientation (e.g. precisely vertical) by using forceps to gently bend the alignment wire distal to the carrier clamp. The microcannula tip is positioned over a point of reference (e.g., bregma or lambda) and then maneuvered to the point of entry into the brain. The coordinates used for the animals in these experiments were: Bregma +0.2 mm, lateral 3.2 mm, and ventral 5.0 mm beneath dura with the animal's head in a skull-flat position.

After the microcannula is advanced to the appropriate depth within the brain, the system is fixed into position with a pedestal compound (e.g., Geribond). The pedestal can be sculpted close to the surface of the skull to facilitate wound closure. After the pedestal compound has cured (~2 min), the alignment wire is cut flush with the pedestal using the drill's cutting wheel. A small amount of compound may be used to cover and smooth the remaining barb from the severed wire. The MOP is then inserted subcutaneously, positioned between the animal's scapulae. Finally, the wound is lavaged with sterile saline, and the incision closed using suture or wound clips (Fig. 3, panel D).

INSERT FIGURE 3 ABOUT HERE

2.4. Preparation and implantation of standard infusion system

The procedure for preparation of the standard infusion system is identical to that for the microinfusion system except the microcannula is replaced by an "osmotic pump connector cannula" with a diameter of 300 μm (28 ga; Plastics One, Roanoke, VA, #3300PM/SPC; see Fig 5a). Implantation of the standard system is also identical to that of the microinfusion system, including the attachment of an alignment wire to allow precise vertical positioning of the cannula of entry into the brain.

2.5. Histology and Analysis

To examine the microinfusion system's ability to provide sustained delivery of Fast Green, groups of three animals were sacrificed at 12 hr, 1 day, 2 days, and 4 days after surgery. Subjects were deeply anesthetized with sodium pentobarbital (120 mg/kg, ip) and transcardially perfused with 200 mL of 0.1M phosphate buffered saline (PBS) followed by 400 mL of 4% paraformaldehyde in 0.1M phospate buffer. Perfusion solutions were kept at 4^{o}C, with a pH of 7.4 and perfused at a rate of 50 mL per minute using a peristaltic pump. In order to remove the cannulas without damaging the post-mortem tissue, the perfused animal was fixed in a stereotaxic frame, the pedestal was secured with adhesive to a rigid wire attached to the manipulator arm clamp, and the skull was carefully removed from around the pedestal using a dental burr. The pedestal and the underlying cannula were thereby removed along the same trajectory in which the cannula was advanced for placement. Brains were then removed from the calvaria and immersed for 12 hours in the same fixative.

For evaluation of sustained infusion of Fast Green, sections with a thickness of 200 μm were cut using a Vibratome (myNeuroLab, St. Louis, MO) and wet mounted on glass slides. Representative sections were imaged using a Canon CanoScan LiDE 600F scanner. For evaluation of local trauma and reactive gliosis, 70 μm Vibratome sections were first wet-mounted and photographed unstained to prevent tissue distortion that may occur with histological processes, such as drying, staining, and dehydration. These same sections were then allowed to dry onto their gelatin-coated glass slides, and they were stained with hematoxylin and eosin (H&E), dehydrated with graded alcohols, and cover-slipped with Permount.

Adjacent 70 μm sections underwent a standard immunofluorescence procedure to detect glial fibrillary acidic protein (GFAP), which is expressed by astrocytes during the process of reactive gliosis in response to trauma (Ding et al., 2000; Ma et al., 1991).

Briefly, free-floating sections were rinsed with PBS and blocked with 10% normal donkey serum (NDS) and 3% bovine serum albumin in 0.1 M PBS with 0.3% Triton X-100 (PBS/Triton) for one hour and then incubated in rabbit anti-GFAP antibody (1:500; Sigma Chemical Co., St. Louis, MO) in PBS/Triton with 1% NDS for 12 hours at 4^{o}C. Sections were then rinsed with PBS and incubated with Alexa Fluor® 594 donkey-anti-rabbit secondary antibody (1:500; Molecular Probes, Inc., Eugene, OR) in PBS with 5% NDS for one hour at room temperature. The sections were thoroughly rinsed again with PBS and counterstained with NeuroTrace® green fluorescent Nissl stain (1:500 in PBS for 5 minutes; Molecular Probes, Inc., Eugene, OR). After a final rinse with PBS, sections were mounted on gelatin-coated slides and coverslipped with Slow-Fade (Molecular Probes). All photomicrographs were acquired using a Zeiss Axioskop microscope with AxioVision software (Carl Zeiss, Inc., Thornwood, NY).

3. Results

3.1 In vitro testing.

Prior to evaluation in animals, three microinfusion systems were prepared by placing their Fast Green-filled MOPs into in one compartment (15 ml conical tube) and placing their microcannulas in a second compartment (1 ml Eppendorf tube), both containing sterile saline at 37^{o}C. Each system demonstrated continuous flow of dye over 12 hours of observation. Figure 4 illustrates function of a typical system over 3 hours.

INSERT FIGURE 4 ABOUT HERE

3.2. Evaluation of in vivo *microinfusion system*

After perfusion, the removed microcannulas and their connections were carefully inspected, and all were found to be entirely intact. Thus, there occurred no breakage of the borosilicate glass within tissue, nor were there any defects in the connections of system components. A 10 μL saline-filled Hamilton syringe with a short length of silicon tubing attached to its needle was used to apply gentle flow to the cut end of the polyethylene tubing attached to the microcannula. All twelve of the microcannulas allowed continued flow and did not appear to be obstructed. Furthermore, after removing each MOP from the animals, the volume of the remaining solution was

measured and found to be appropriate for the MOP's flow rate (0.125 μL/hr) and the amount of time it was allowed to function.

3.3. In vivo delivery

Evaluation of coronal sections of tissue infused with Fast Green demonstrated little variability in distribution between animals within groups at any given time point (12 hrs, 1 day, 2 days, and 4 days). Figure 5 illustrates representative areas of Fast Green diffusion at these time points showing a progressive increase in dye infiltration over four days. Note that prior experiments using higher concentrations of Fast Green (e.g., 5-15%) resulted in rapid opacification of the parenchyma, thus obscuring accurate evaluation of the microinfusion system's function over time. While the full extent of 1% dye diffusion can be difficult to accurately determine on gross inspection, areas of infiltration were easily discernable at 2.5X magnification, and are indicated with dashed lines in Figure 5.

INSERT FIGURE 5 ABOUT HERE

3.4 Evaluation of trauma and reactive gliosis

Gross observation of freshly cut, unstained tissue sections showed that the microinfusion system resulted in a reduction in localized trauma at the site of delivery. The standard cannula was invariably seen to disrupt and displace a greater area of tissue (Fig. 6B & C), which is better appreciated at higher power with H & E (Fig. 6D & E). Moreover, blood was frequently seen accumulated at the standard infusion site (Fig. 6D), suggesting a greater degree of compromise to the vasculature and implying decreased integrity of the blood brain barrier.

The microinfusion system also resulted in greatly reduced reactive gliosis as demonstrated by decreased immunoreactivity for GFAP in the vicinity of infusion via a microcannula (Fig. 6G) when compared to a standard cannula (Fig. 6F). While microcannula-induced GFAP staining was elevated above normal levels (Fig. 6H), the striking escalation in reactive gliosis with standard cannula placement and infusion (6F) was not seen when the microinfusion system was employed.

INSERT FIGURE 6 ABOUT HERE

4. Discussion

Various studies have demonstrated that by decreasing the size of the delivery cannula for intracerebral infusions, tissue trauma and insult to the blood brain barrier are reduced (Perry et al., 1993), inflammation is decreased and the immune-response attenuated (Finsen et al., 1991), and reactive gliosis is diminished (Nikkhah et al., 1994). We present here a method for the chronic delivery of neuroactive agents within the brain via a microcannula with a diameter that is reduced 6-fold compared to that of conventionally-used delivery cannulas. We have demonstrated that this system reliably delivers a representative solution over time and the level of localized trauma and reactive gliosis is dramatically reduced. These studies employed a delivery cannula with a final tip diameter of 50 μm; however, smaller tip diameters may be utilized. The primary virtue of such microinfusion systems is their ability to deliver agents to very small, discrete targets, while incurring minimal trauma in the vicinity of the infusion.

The microcannulas used in the construction of the infusion system we describe can be easily custom-built with predetermined lengths and tip diameters. The microinfusion system is designed to conform to the surface of the animal's skull allowing secure, low-profile fixation and precluding the need for a large cranial pedestal. The surgical wound can therefore be easily sutured over the streamlined microinfusion pedestal, thus reducing both the discomfort to the animal and the risk of infection. In addition, securing the system to the skull requires less skull surface area; therefore, additional or subsequent procedures may be performed without interference from a larger pedestal.

One should, however, take into account certain technical considerations when employing the methodology described here. A higher level of skill is required, and the production and implantation procedure is less time-efficient than it may be for standard infusion systems. On the other hand, this may be outweighed, or at least counter-balanced, by the time (and animals) spared by practicing high-precision technique. Another difficulty is that while the microcannula can be customized with virtually any diameter, a microcannula with a very small lumen can become obstructed, either by components of the infusate or by tissue encountered during implantation (e.g., blood, brain tissue). This risk is reduced by keeping the microcannula tip moistened (e.g., using a water- or saline-saturated cotton swab to prevent drying of infusate solutes) during the implantation procedure and by performing clean, "blood-free" surgery.

We recommend the researcher establish a microcannula tip diameter that is appropriate for the particular composition and/or viscosity of the solution that will be loaded into the MOP. This is easily achieved using an *in vitro* test system similar to the one described here (Fig. 4) or by simply immersing the assembled system in a 37^{o} C saline bath and monitoring the bath composition and/or measuring the volume of the MOP contents over time. Another disadvantage is that the microcannula is relatively fragile, particularly if its tip diameter is very small (e.g., ~10-50 microns). Accidentally breaking the microcannula during surgery usually requires that the entire system be replaced, as repairing or rebuilding that unit would extend surgery time considerably. Scientists skilled in such techniques and who practice exacting method rarely experience breakage, however.

At the conclusion of the experiment, the microinfusion system can be inspected to ensure that the microcannula has not been damaged and that it has remained patent. The volume of the mini-osmotic pump can also be measured to ensure that the appropriate amount of neuroactive agent has been delivered; we do not recommend recycling or reusing the pumps, however. While the present methods were demonstrated in adult rats, use of a system that reduces trauma and requires less skull surface area for fixation may be considered preferable over conventional methods for experiments with smaller animals, such as mice or young rats.

Although the present method requires a somewhat more advanced level of proficiency and skill by the experimenter, it offers to increase precision perhaps by an order of magnitude and to minimize experimental confounds associated with trauma to the region of interest. In our laboratory, we have found that this translates into more reliable and robust effects of the experimental intervention.

Figure Legends

Fig. 1. Assembly of the microinfusion system. (A) The components of the system placed in their relative positions for assembly. Note the flange from the flow modulater tubing has been broken away. We recommend the alignment wire be secured just before implantation (see text). (B) Assembled microinfusion system prior to attachment of alignment wire and implantation.

Fig.2. Preparation of microcannula. (A) An electrode puller (Stoelting Co. Wood Dale, IL) is used to produce a microcannula with a long, tapering shank. (B) The heating coil may be reoriented for ease of use, and the microcannula is placed within the coil, allowing a right-angle bend to be formed using forceps as the glass tubing is heated.

Fig. 3. Implantation of microinfusion system. (A) Positioning of microinfusion system onto stereotaxic instrument. (B) The MOP is tethered using sterile suture to prevent its weight from disrupting the orientation of the microcannula. The microcannula is positioned for precise vertical entry into the brain using the alignment wire. (C) A microinfusion system is positioned for placement and fixation within an animal. (D) The design of the microinfusion system allows easy closure of the incision over a low-profile pedestal, reducing the risk infection and minimizing discomfort to the animal.

Fig. 4. *In vitro* demonstration of microinfusion system function. (A) Flow of Fast Green dye is easily seen unimpeded from the microcannula at the beginning of an *in vitro* test period. (B) As flow continues, the reservoir solution becomes saturated (B), and ultimately opaque with coloration (C).

Fig. 5. *In vivo* demonstation of sustained microinfusion of Fast Green. (A) Twelve hours after intrastriatal placement of a Fast Green-loaded microinfusion system, dye is seen diffusing into the parenchyma surrounding the microcannula site. Observations at 1 day (B), 2 days (C), and 4 days (D) indicate continued delivery of Fast Green solution with increasing areas of dye infiltration. The outer limit of diffusion was observed under low-power microscopy and is indicated here with dashed lines. Scale bar, 2 mm.

Fig. 6. Localized trauma caused by a microcannula compared to a standard delivery cannula after 5 days of saline infusion. (A) A microcannula pulled from borosilicate

tubing with a tip diameter of 50 μm allows free, unrestricted flow of most neuroactive agents, yet is 6X smaller than a standard delivery cannula (28 ga, 0.30 mm). (B, C) Unstained, wet mounts of coronal sections (thickness, 70 μm) illustrating greater local tissue damage (arrows) caused by a standard cannula (B) when compared to a microcannula (C). (D, E) Higher power photomicrographs of H&E stained sections shown in B and C, respectively. The standard cannula caused more extensive traumatic injury as illustrated in D, having displaced a larger area of tissue and causing greater insult to the vasculature as demonstrated by the collection of blood within the lesion cavity (arrow). The lesion caused by the microcannula, however, is considerably smaller and less disruptive to tissue at the site of infusion. (F, G, H) Immunofluorescence against GFAP demonstrates dramatically increased numbers of GFAP+ astrocytes around the lesion of the standard cannula (F) when compared to that of the microcannula (G) and normal, unlesioned striatum (H). Scale bars: A & B, 1 mm; D - H, 250 μm.

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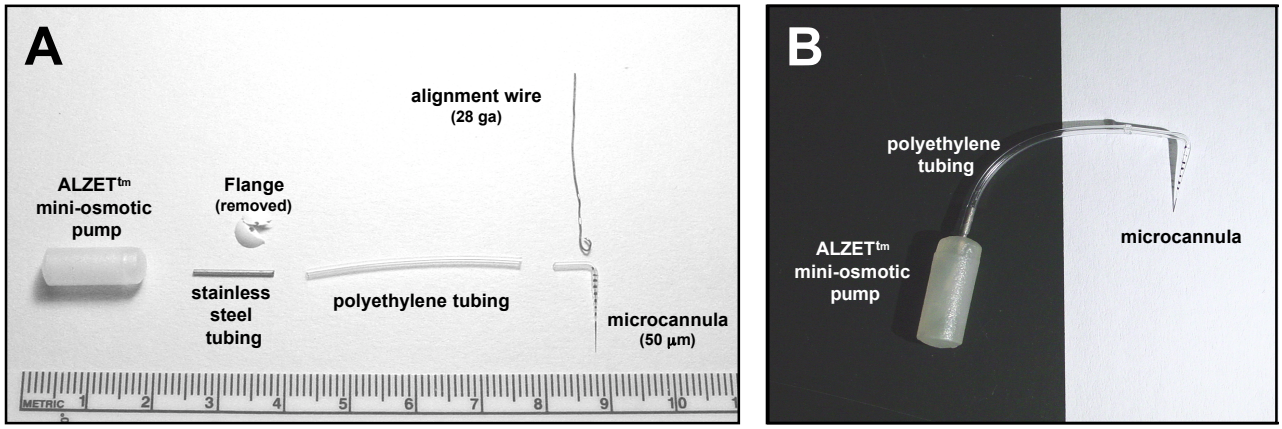
Figure


A
alignment wire
(28 ga)
ALZET™
mini-osmotic
pump
Flange
(removed)
stainless
steel
tubing
polyethylene tubing
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(50 µm)
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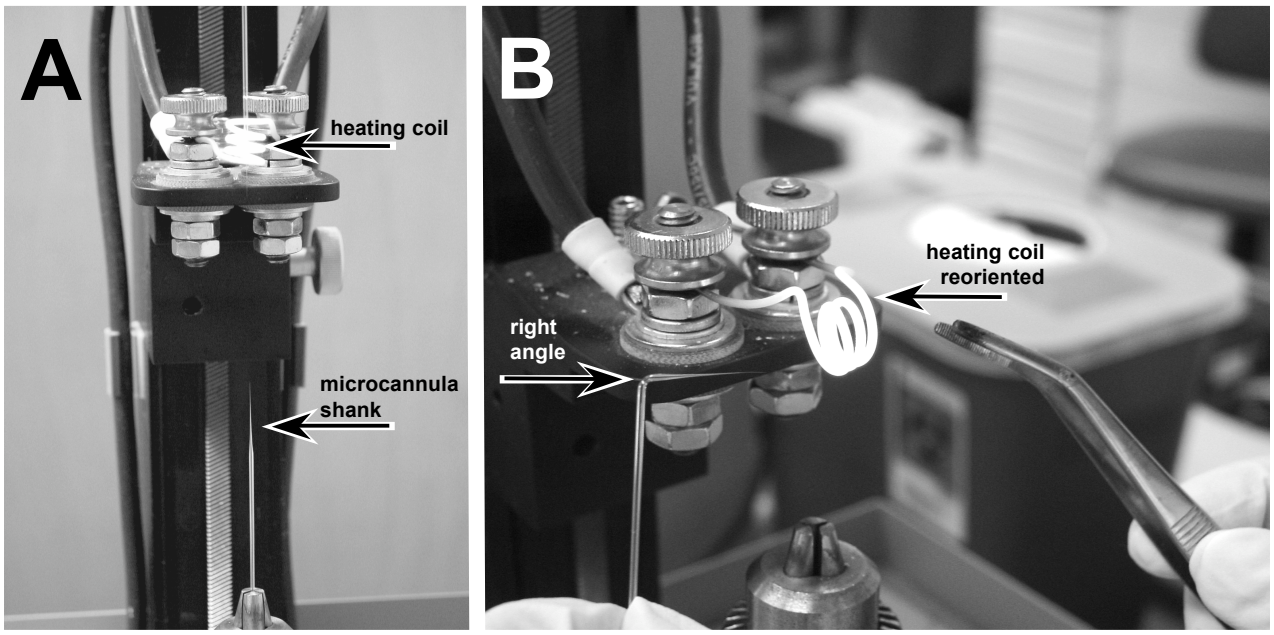
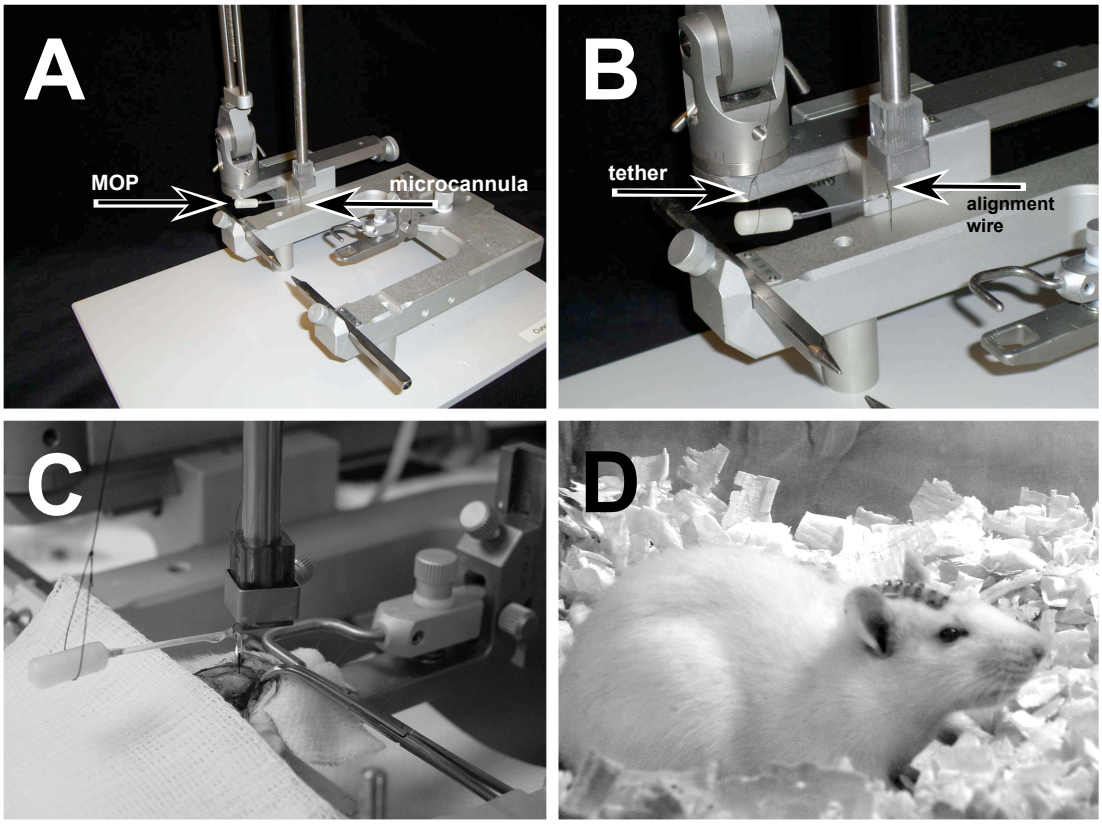
B
polyethylene
tubing
ALZET™
mini-osmotic
pump
microcannula

Figure


A
heating coil
microcannula
shank
B
heating coil
reoriented
right
angle

Figure



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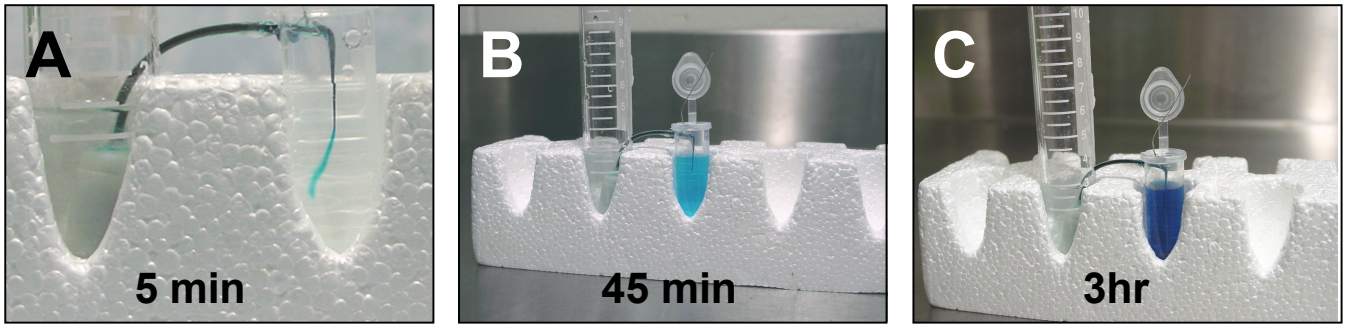

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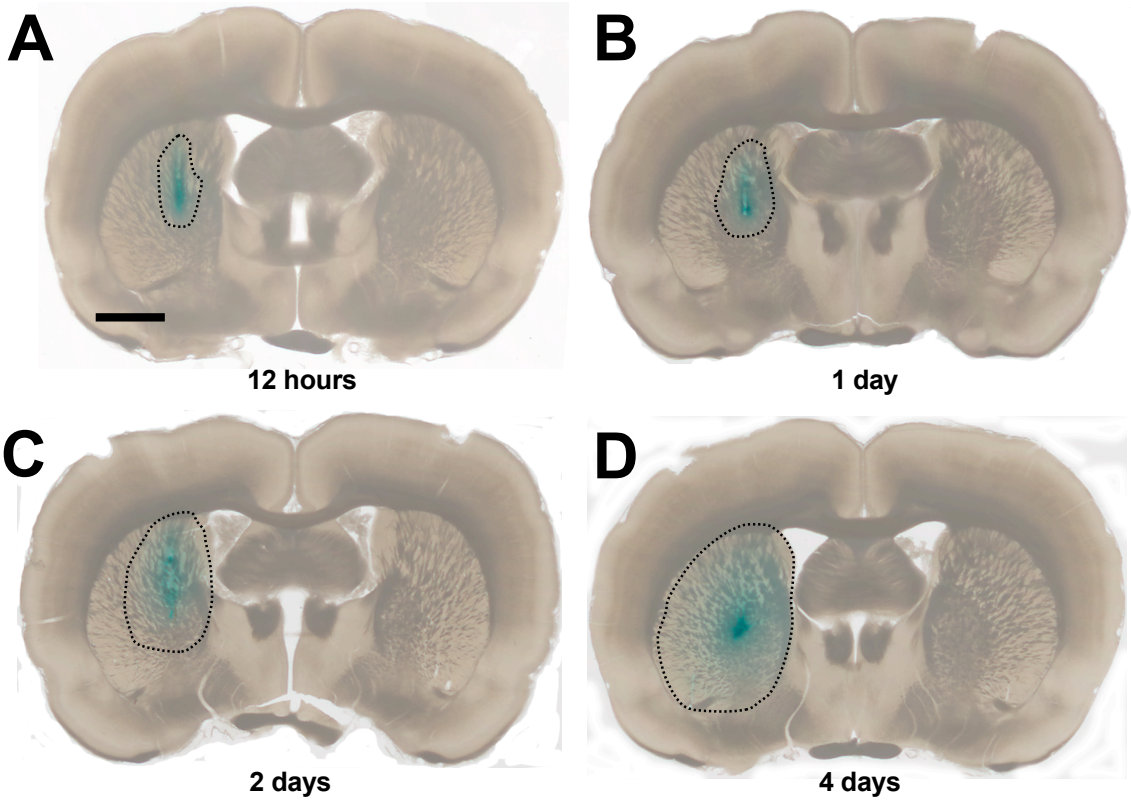

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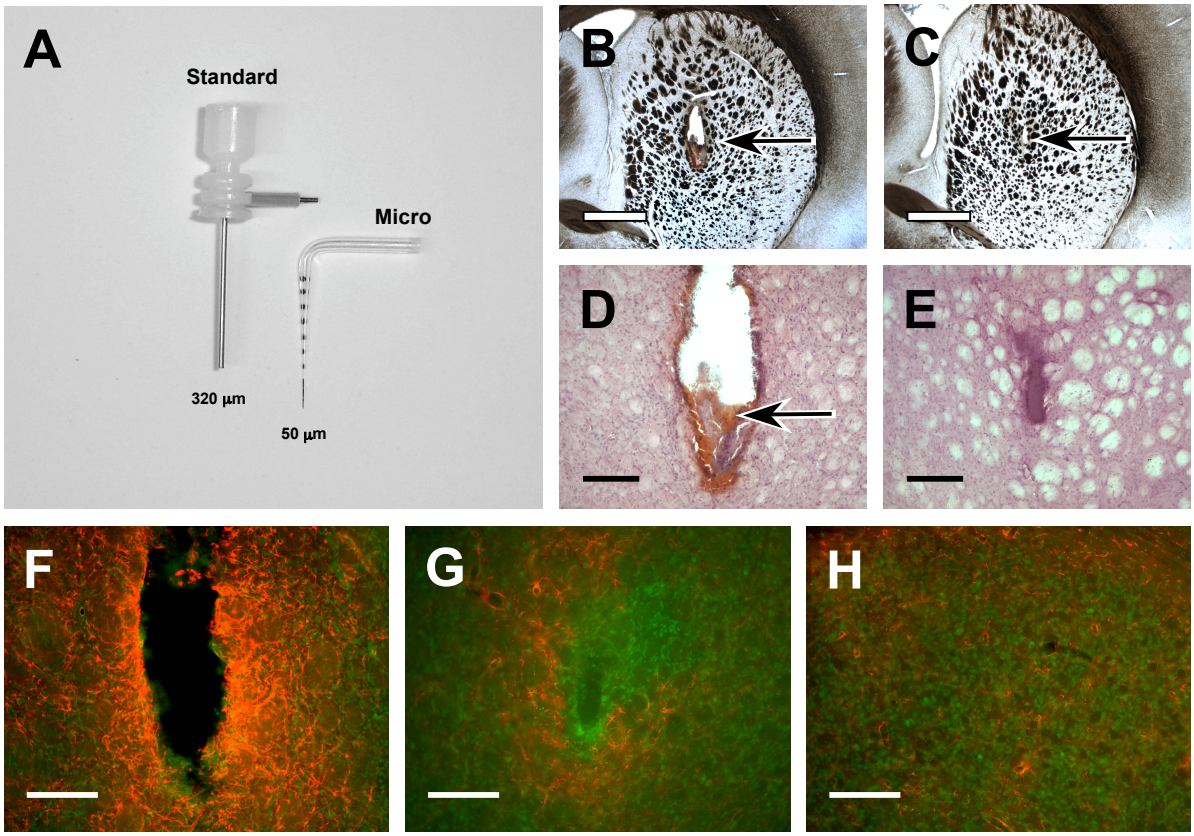

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