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TITLE:

In Vivo Targeted Expression of Optogenetic Proteins Using Silk/AAV Films

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SUMMARY:

Here, we present a method for delivering viral expression vectors into the brain using silk fibroin films. This method allows targeted delivery of expression vectors using silk/AAV coated optical fibers, tapered optical fibers, and cranial windows.

ABSTRACT:

The quest to understand how neural circuits process information in order to drive behavioral output has been greatly aided by recently-developed optical methods for manipulating and monitoring the activity of neurons *in vivo*. These types of experiments rely on two main components: 1) implantable devices that provide optical access to the brain, and 2) light-sensitive proteins that change neuronal excitability or provide a readout of neuronal activity. There are a number of ways to express light-sensitive proteins, but stereotaxic injection of viral vectors is currently the most flexible approach because expression can be controlled with genetic, anatomical, and temporal precision. Despite the great utility of viral vectors, delivering the virus to the site of optical implants poses numerous challenges. Stereotaxic virus injections are demanding surgeries that increase surgical time, increase the cost of studies, and pose a risk to the animal's health. The surrounding tissue can be physically damaged by the injection syringe, and by immunogenic inflammation caused by the abrupt delivery of a bolus of high-titer virus. Aligning injections with optical implants is especially difficult when targeting small regions deep in the brain. To overcome these challenges, we describe a method for coating multiple types of optical implants with films composed of silk fibroin and Adeno-associated viral (AAV) vectors. Fibroin, a polymer derived from the cocoon of *Bombyx mori,* can encapsulate and protect

biomolecules and can be processed into forms ranging from soluble films to ceramics. When implanted into the brain, silk/AAV coatings release virus at the interface between optical elements and the surrounding brain, driving expression precisely where it is needed. This method is easily implemented and promises to greatly facilitate *in vivo* studies of neural circuit function.

INTRODUCTION:

The past decade has produced an explosion of engineered light-sensitive proteins for monitoring and manipulating neural activity[1]. Viruses offer unparalleled flexibility for expressing these optogenetic tools in the brain. Compared to transgenic animals, viruses are far easier to produce, transport, and store, allowing fast implementation of the newest optogenetic tools. Expression can be targeted genetically to distinct neuronal populations, and viruses designed for the retrograde transport can even be used to target expression based on neuronal connectivity[2].

Viruses are usually introduced with stereotaxic injections, which can be time-consuming and challenging. Precisely targeting small regions can be difficult, while driving expression over broad areas often requires many injections. Moreover, when an optical device is subsequently implanted into the brain to deliver light *in vivo*, the implant must be properly aligned with the viral injection. Here, we describe an easily-implemented method for delivering viral vectors to the tissue around an implanted device using silk fibroin films[3]. Silk fibroin is commercially available, well-tolerated by neural tissues, and can be used to produce materials with varied properties. Silk films can be applied to implants using common laboratory equipment like microinjection pipettes or hand pipettes. Silk/AAV films eliminate the requirement for two surgical procedures and ensure that virus-mediated expression is properly aligned to the optical implant. The resulting expression is constrained to the tip of fibers, and results in less unwanted expression along the fiber track than stereotaxic injections.

In addition to producing targeted expression at the tip of small fibers, silk/AAV films can be used to drive widespread (>3 mm diameter) cortical expression beneath cranial windows. *In vivo* 2-photon imaging of fluorescent activity sensors has become an indispensable tool for evaluating the role of neuronal activity in driving sensory and cognitive processing. However, to drive uniform expression over the broad cortical areas, experimenters often perform multiple injections. These injections can be extremely time-consuming and can lead to inconsistent expression across the field of view. In contrast, silk/AAV-coated cranial windows are extremely easy to manufacture, greatly reduce the time required for surgeries, and most remarkably drive expression hundreds of microns below the cortical surface.

PROTOCOL:

All experiments involving animals were performed in accordance with protocols approved by the Harvard Standing Committee on Animal Care following guidelines described in the US NIH *Guide for the Care and Use of Laboratory Animals*. Adult C57BL/6 mice of either sex (6-15 weeks of age) were used for all experiments.

1. Obtain Aqueous Silk Fibroin

1.1. Prepare or purchase aqueous silk fibroin (5-7.5% w/v).

2. Mix Aqueous Silk with AAV Expression Vectors

2.1. Choose an AAV expression vector to drive the optogenetic protein or fluorescent indicator of choice.

Note: To minimize the volume of silk/AAV that must be applied to implants while still driving robust expression, stock-titer AAV (stock titers typically obtained from vector cores are around ~10^{13} gc/mL) is recommended.

2.2. Immediately prior to coating implants, thaw an aliquot of AAV and combine with 5-7.5% aqueous silk fibroin (this mixture will be referred to as silk/AAV). In a 200 µL PCR tube, mix aqueous fibroin and AAV in a 1:1 ratio (for cranial windows use 1:4) immediately prior to the application. Gently pipette the solution in and out several times to thoroughly mix the fibroin and AAV.

2.3. Keep silk/AAV mixture on ice prior to use.

3. Prepare Equipment for Fabrication and Storage of Silk/AAV-Coated Devices

3.1. Procure equipment for coating optical fibers and Gradient-Index (GRIN) lenses (**Figures 1, 2**).

3.1.1. Construct a stable ferrule holder. To hold ceramic ferrules, drill 1.25 mm holes in a block of ¼" sheet acrylic. Tap holes to insert set screws from the side to hold ferrules in place.

Note: Any clamp can be used for this purpose.

3.1.2. Position a manipulator with sub-millimeter precision to move the optical fibers (stereotaxic apparatus or other precision micromanipulator).

3.1.3. Assemble a stable holder to position the microinjector.

3.1.4. Use a stereoscope to visualize optical fibers and silk droplet.

3.1.5. Position a light source to illuminate the optical fibers.

3.2. Prepare equipment for coating cranial windows (**Figure 3**).

3.2.1. Choose any P10 pipettor.

3.2.2. Obtain a container with lid.

Note: Any container with a silicone bottom is suggested—the soft bottom facilitates lifting up cranial windows.

3.3. Prepare equipment to store finished implants (**Figure 4**).

3.3.1. Obtain a small (1-5 L) vacuum chamber.

3.3.2. Make sure that there is space to store implants in a 4 °C refrigerator.

4. Apply Silk/AAV Film to Devices

4.1. Coating optical fibers to drive focal expression at the fiber tip

4.1.1. Prepare chronic fiber implants as previously described[4].

4.1.2. Prior to use, rinse implants with ethanol, then with ultrapure water to ensure that the optical fibers are clean.

Note: Silk films adhere more reliably to clean glass surfaces.

4.1.3. Prepare a device to hold fiber ferrules. For typical 1.25 mm diameter ferrules, use a block ¼ inch clear acrylic, with ~1.3 mm holes, and tapped set screws entering from the side to hole implants firmly in place (**Figure 1A**).

4.1.4. Mount the ferrule holder into a stereotaxic apparatus (or any manipulation solution with submillimeter precision) equipped with a microinjector. Place the ferrule holder above the microinjector and apply the silk/AAV mixture from below.

Note: This is because applications of large volumes from above resulted in silk/AAV that was not restricted to the tip. However, the application of many small sequential volumes from above or below can produce AAV/silk deposits that are confined to the tip (although we prefer to apply from below).

4.1.5. Pull a standard intracranial injection pipette from borosilicate glass capillary.

4.1.5.1. To make it easier to deposit silk/AAV onto the optical fiber, ensure that the diameter of the injection pipettes is smaller than the fiber itself.

4.1.5.2. To produce an injection tip with a clean flat tip of the desired diameter, hold one pipette in each hand and use the thicker part of the taper on one pipette to score the other pipette at the desired break location.

4.1.5.3. Gently rub back and forth in a sawing motion (the glass-on-glass scoring method).

4.1.5.4. After scoring the pipette, apply gentle pressure to the tip of the scored pipette with the body of the other pipette to achieve a clean break.

4.1.6. Position a stereoscope to give a clear view of the optical fiber faces.

Note: Magnification should be sufficient to accurately position the injection pipette above the face of optical fibers.

4.1.7. Insert fiber implants into holder with the brain-side of the optical fiber facing downward.

4.1.8. Load the injection pipette with silk/AAV solution, as for any standard intracranial injection[5]. Load the amount required for the number of implants being made, plus ~30% extra to accommodate losses due to pipettes clogging. For example, if 10 implants are being made, then load with 100 nL deposits and withdraw ~1.3 µL.

Note: Silk/AAV may dry at the pipette tip in between ejections, which can clog the pipette. Large diameter pipettes (50-100 µm) are less likely to clog. Clogs can be dislodged by gentle brushing down the tip of the pipette with a wet paper wipe or alcohol swab.

4.1.9. Maneuver the injection pipette until it is touching or nearly touching the center of the optical fiber surface. Eject 10-20 nL of silk/AAV solution. Withdraw the pipette.

Note: The rate of delivery is not critical, but typical rates are 5-20 nL/s.

4.1.10. Observe the bolus of silk/AAV on the flat surface which appears as a liquid dome that dries to a flat film within ~1 min **(Figure 1B**).

4.1.11. Repeat steps 4.1.9-4.1.10 until the desired amount of silk/AAV is deposited (a total of 20-200 nL for most applications). When preparing multiple implants, apply silk/AAV to one implant and then move on to coat other implants before returning to the first.

4.1.12. Allow 1 h for drying before moving implants.

4.1.13. Vacuum desiccate overnight at ~125 Torr (-25 in. Hg), 4 °C. Do this by placing the entire ferrule holder into a vacuum chamber.

4.1.14. Evaluate the shape and position of the resulting silk film under a high-power microscope. Ensure that films are confined to the tip of the optical fiber surface, be relatively thin (>100 µm), and symmetrical (**Figure 1C**).

Note: Large or asymmetrical silk/AAV films may dislodge from the fiber during implantation (**Figure 1D**). The most common cause of problems arises from the application of single large volumes rather than the sequential application of many small volumes.

4.2. Coating tapered optical fibers to drive expression along the fiber axis

4.2.1. Obtain tapered optical fiber implants and perform steps 4.1.2-4.1.8, except that the tapered fiber is positioned laterally such that it is perpendicular to the injector (**Figure 2A**). Position the injector above the tapered fiber.

Note: Loading liquid droplets onto tapered fibers poses added challenges, because surface tension tends to cause droplets to jump back onto the injection pipette or migrate up the tapered fiber. Smaller injection pipettes (30-50 µm diameter) help to overcome this problem but increase the risk that the injection pipette will clog. Due to surface tension, droplets tend to adhere to the area of largest surface area, so the optimal injection pipette size is dependent on the size of the tapered fiber and one's tolerance for the occasional clog.

4.2.2. Position the silk/AAV injection pipette against the side of the optical fiber at the beginning of the taper. Make sure the injection pipette is touching the optical fiber.

4.2.3. Eject 20 nL of silk/AAV to start the coating process. Ensure that the droplet adheres to the optical fiber and remains at the interface of the fiber/pipette. Gently wick the droplet towards the end of the fiber tip as the silk/AAV dries (~45 s). Keep the injecting pipette in contact with the drying droplet to avoid clogging the pipette tip.

Note: Each deposit should coat approximately 400 µm of the tapered fiber (**Figure 2B**).

4.2.4. When the first bolus has dried almost completely, eject another 20 nL and continue wicking the droplet along the taper.

Note: The liquid silk will adhere to the dried silk, anchoring one end of the droplet as the pipette moves along the taper.

4.2.5. Repeat step 4.2.4 by ejecting small amounts of silk/AAV, and gradually drawing the solution up the side of the taper. 5-6 ejections are sufficient to traverse the surface of a 2.5 mm taper.

4.2.6. To drive more uniform expression around all sides of the fiber, rotate the fiber and repeat steps 4.2.2-4.2.5 until the desired amount of silk/AAV has been deposited.

4.2.7. If a hanging strand of dried silk/AAV extends beyond the fiber tip, carefully cut the strand with scissors, or use the ejection pipette to bend the strand back and adhere it to the taper of the fiber.

4.2.8. Allow 1 h for drying before moving implants.

4.2.9. Vacuum desiccate overnight in 4 °C. The entire ferrule holder can be placed into a vacuum chamber.

4.2.10. Evaluate the shape and position of the resulting silk film under a high-power microscope.

Note: Films need not be entirely uniform but should not have bumps that extend more than 100 µm beyond the surface of the fiber to minimize damage to surrounding tissue during implantation (**Figure 3C**). To minimize film size, it is critical that each droplet is completely dry before subsequent deposits are made.

4.3. Coating GRIN lens implants

4.3.1. Obtain GRIN lenses[6,7] and repeat steps 4.1.2-4.1.8. The injector can be mounted above.

4.3.2. Deposit silk/AAV in a single ejection (1 µL for a 1.0 mm diameter lens).

Note: This will yield a dome of liquid that adheres to the face of the lens, and dries to produce a uniform film (100-200 µm thick). However, in the event that a single large ejection dries unevenly and produces a film that is thicker near the edges of the GRIN lens, try depositing multiple smaller droplets (100-200 nL) in the center of the lens surface (allowing each droplet to dry before depositing the next) to ensure that the film will drive expression at the center of the field of view.

4.3.3. Allow 1 h for drying before moving implants.

4.3.4. Evaluate the shape and position of the resulting silk film under a high-power microscope to ensure that the film covers the surface of the lens.

4.4. Coating glass cranial windows

4.4.1. Prepare glass cranial windows by adhering two 3 mm diameter round coverslips (no. 1 thickness) to one 5 mm diameter window with optical adhesive (for details, see Goldey *et al.* 2014[8]).

4.4.2. Mix silk:virus in a ratio of 1:4 to reduce the total amount of silk in the film. Excessive quantities of silk do not dissolve beneath cranial windows after implantation. Titration experiments may be required to determine the ratio and volume that gives the desired expression profile.

4.4.3. Hand pipette a 5 µL droplet onto the surface of the 3 mm (brain facing) coverslip. The droplet should spread out to cover the entire glass surface (**Figure 3**).

4.4.4. Allow 2-3 h for drying before moving windows.

5. Storing silk/AAV-coated implants

5.1. Store silk/AAV-coated optical fibers in a cooled vacuum desiccator (~125 Torr, 4 °C) prior to use (**Figure 4A**).

5.2. Do not store cranial windows and GRIN lenses under vacuum, as large silk films stored under vacuum fail to fully dissolve after implantation. Implant cranial windows and GRIN lenses immediately after drying, or within a day of manufacture if stored at atmospheric pressure and 4 °C.

6. Implanting the Devices

6.1 Prepare animals for implant surgery as previously described[4].

6.1.1. Briefly, anesthetize mice with an intraperitoneal injection of ketamine/xylazine (100/10 mg/kg) and check the depth of anesthesia using a gentle toe-pinch. Shave the skull in the area of the implant and clean the scalp with iodine and alcohol.

6.1.2. Mount animals in a stereotaxic device and supplement anesthesia using a mixture of oxygen and isoflurane (1-2%). Make an incision in the scalp over the area of interest, and perform a craniotomy large enough to accommodate the implant.

6.2. Implant optical fibers[9] and microendoscope lenses[10] according to previously published procedures. Handle implants with care, as the silk/AAV deposit can be knocked off by an imperfect craniotomy, or by the implant catching on the edge of the skull. Lower the implant into the brain slowly (~2 mm/min).

6.3. Implant cranial windows as describe previously[8]. Do not touch the coated side of the window and avoid rinsing the window with fluid if performing gavage, as this may wash away the virus. To achieve maximal expression, perform a durotomy.

7. Evaluating the Expression and Troubleshooting

7.1 To evaluate expression of virally-expressed proteins, allow ~2-3 weeks for the virus to drive expression, then perform intracardial perfusion with 4% paraformaldehyde in phosphate buffered saline solution[11] and process brain tissue for fluorescent microscopy[12].

7.2. Evaluate the expression by using fluorescent microscopy to image the expression pattern of fluorophore-tagged optogenetic proteins.

7.3. If the level of expression is insufficient, increase the amount of virus in coatings by either increasing the total volume of the silk/AAV coating, or preferably by using a higher titer virus.

REPRESENTATIVE RESULTS:

To assess the success of silk/AAV films in driving expression, we perfused animals 2-3 weeks after implantation and prepared brain slices from the region of interest. Fluorescence images of fluorophore-tagged optogenetic proteins (ChR2-YFP) provided a measure of the extent of expression (**Figure 1D**). Typical optical fibers (230 µm diameter) can readily accommodate 200 nL

of silk/AAV. With practice, experimenters can achieve highly reliable expression around the tip of implanted fibers (**Figure 5**).

To assess expression driven by silk/AAV-coated cranial windows, begin imaging beginning 7-10 days after implantation. We have used two-photon imaging for visualization, but other methods such as fluorescence imaging with a CCD could also be used. Two possible issues with coated cranial windows are insufficient expression, and silk films that fail to dissolve and obscure the field of view. To increase expression, we suggest performing a durectomy prior to implanting the window, and/or increasing the amount of virus in the film. We achieved the best expression using a 1:4 mixture of silk and stock-titer AAV, respectively. While this represents a substantially larger number of viral particles than are usually used in stereotaxic injections, the decreased surgical time counters the marginal additional cost of virus. Meanwhile, if silk films fail to dissolve beneath the window, further reduce the amount of silk used to coat the window. The total amount of silk in coated windows is 10-100 times more than on fiber implants, and the film is less embedded in tissue and thus may not be exposed to the same levels of proteolytic activity than can dissolve silk films[13]. However, the presence of some silk is essential to achieving expression beneath windows[3], likely because a film made of virus alone is washed away by interstitial fluid during surgery.

FIGURE AND TABLE LEGENDS:

Figure 1: Applying silk/AAV films to optical fibers. (A) Chronic fiber implants are placed fiber side-down into a holder (inset) mounted on a XYZ translator. A fixed microinjector below the fibers dispenses silk/AAV onto the fiber tips. A stereoscope allows visualization of the process. **(B)** Apply silk/AAV to fiber tips in small volumes (10-20 nL). After ejecting a bolus, retract the pipette and allow ~60 s for the droplet to dry to a flat film. Repeat the process until the required volume has been applied to the fiber tip. **(C)** Inspect silk coatings. Optimal coatings should be centered on the fiber tip (left), while improper coatings extend outward from the fiber face making them more prone to dislodge from the fiber (right). **(D)** Representative fibers coated with 200 nL of silk/AAV, and the resulting AAV-driven ChR2-YFP expression 2 weeks after implantation. The compact silk/AAV coating on the left resulted in robust expression, while the coating on the right protruded past the face of the fiber and resulted almost no expression, likely because the silk/AAV did not adhere to the optical fiber during implantation. Scale bars 0.2 mm (fibers) and 1.0 mm (brain slices).

Figure 2: Setup for coating tapered fiber implants. (A) The microinjector is mounted above the tapered fiber holder, and tapered fibers are positioned orthogonally to the ejection syringe. **(B)** Begin at the widest point (inset) and eject small volumes while moving the ejection syringe towards the point of the taper. This results in a continuous coating along the length of the taper. **(C)** Representative tapered fiber coated with silk mixed with Fast Green to aid in visualization.

Figure 3: Coating cranial windows. Silk/AAV can be applied cranial windows using a hand pipette. A standard 3 mm diameter window can be coated with a 5 µL droplet, which will slowly dry to a flat film. Inset: GCaMP6f expression resulting from silk/AAV-coated cranial windows implanted

with and without durectomies. This figure has been adapted from Jackman *et al.* (2018)[3].

Figure 4: Storing silk/AAV coated implants. (**A**) To remove residual moisture and preserve viral efficacy, implants should be stored under vacuum at 4 °C until used. Implants stored this way remain viable for at least 7 days. (**B**) Expression resulting from 4 silk/AAV coated fibers implanted after 7 days of storage.

Figure 5: Silk/AAV-GFP coated optical fibers reliably drive expression. Fluorescent images of slices from 24 consecutive striatal implants. Each implant was coated with 100-400 nL of 1:1 silk/AAV-GFP. This cohort of implants indicates the ability of silk to restrict expression to the implant site (in this case the dorsal striatum). GFP fluorescence is indicated in green; DAPI staining is shown in blue.

DISCUSSION:

The use of silk/AAV to target the expression of optogentic proteins overcomes limitations of approaches that are currently in use. Although many studies successfully use AAV injections to express optogenetic proteins, it is challenging to align expression to the tip of optical fibers, to regions around the length of tapered fibers, and to the viewing region of a GRIN lens. Because of misalignment between optical components and optogenetic expression, stereotaxic injections can be unreliable, and many experiments fail. The silk/AAV labelling method we describe here solves this problem. It also simplifies the procedure by eliminating a second surgical step and in some cases eliminating the need for a second surgery. It can also be difficult to use viruses to obtain widespread expression beneath cranial windows, and experimenter typically perform long surgeries to inject virus in multiple locations. The ability to obtain widespread expression over large cortical regions by simply coating cranial windows with silk/AAV is a simplification that eliminates the need for the many invasive injections.

Another potential advantage of the silk/AAV method is that it might induce less inflammation in the neural tissues compared to viral injections. Injecting high-titer AAV into the brain can cause inflammatory responses such as reactive astrocytosis that have the potential to alter cellular and circuit properties[14,15] (although such potential complications are usually ignored). Silk films induce little immunogenic response on their own[13] and silk/AAV films are expected to release virus over the course of many hours or days[16], which may lower the viral load in the surrounding tissue and reduce immunogenic responses. With conventional approaches in which implantation of a device is preceded by an AAV injection, inflammatory responses can arise from both the implantation and the injection. In the future it will be desirable to systematically compare conventional approaches and the silk/AAV method to determine whether silk/AAV films reduces overall inflammatory responses.

Several steps are critical to the successful use of silk/AAV films. Most importantly, the coating of optical fibers must be done carefully as described in the Methods and the location of the dried films should be assessed carefully by visual inspection under a microscope to ensure that the films are compact, in the correct location, and adhere to the face of the optical fiber. Any silk/AAV on the sides of the optical fiber will lead to expression outside the region of interest, and

misshaped films that protrude beyond the face of the fiber may break off during implantation and lead to unreliable or no expression. The techniques we describe for applying silk/AAV to implantable devices can be adapted to use of any materials are that are readily available and allow the precise deposition of small volumes of silk/AAV.

A bit of practice is required to achieve accurate, reproducible results. If expression is observed along the track of the fiber, it is likely that the silk film dried on the side of the fiber rather than the fiber face. Repeat the manufacturing process and closely inspect dried implants for signs that films are drying on the side of the fiber. Because silk/AAV films are optically transparent, it may help to practice applying silk mixed with dye (Fast Green or a similar dye) to better visualize the shape of resulting films (**Figure 2C**). If there is no expression, it is probable that the silk film dislodged from the fiber tip during implantation. We suggest the use of stock-titer virus when making implants. For optical fibers, this reduces the total volume that must be applied to small diameter fibers. If the size of the coating is a concern consider waiting longer between each 10 nL application to allow complete drying of the deposited droplet. Silk/AAV droplets dry faster under a warm lamp. For cranial windows, high-titer virus may be necessary to supply adequate viral load across the pia or dura. Certain types of implants may dissolve silk and release AAV more readily than others. We have found that cranial windows implanted over the surface of the brain require a lower ratio of silk/virus to achieve reliable expression, perhaps owing to different cerebral spinal fluid dynamics or protease activity. If expression cannot be increased by increasing effective AAV concentrations, decreasing the volume of aqueous silk is a plausible alternative.

Finally, it is important to store the optical components properly and implant them quite soon after they are prepared. We have shown that coated fibers that are refrigerated under vacuum can be stored for many days prior to use. Vacuum storage removes residual moisture[17] which may reduce the solubility of silk films, and also help maintain viral efficacy. Ideally, optical fibers should be implanted within 24 hours of fabrication. However, we find that silk/AAV-coated fibers stored under vacuum drive similar levels of expression when implanted 7 days after fabrication (**Figure 4B**). In contrast, coated cranial windows and GRIN lenses drove the most reliable expression when they were dried at room temperature and used within hours of preparation. The reason for this disparity remains unclear. Further studies may be needed to refine preparation and storage conditions to further extend the storage time.

Silk/AAV-coated cranial windows have considerable potential because they drastically shorten surgical times and are extremely simple to manufacture, but at present this method has limitations. Coated cranial windows uniformly label large areas of cortex and drive sufficient expression in layer 2/3 for GCaMP imaging, with somewhat less expression in deeper layers. However, stereotaxic injections drive more robust expression and provide more control over the layers targeted for expression. Reliable expression was only achieved when the dura was removed. Although the dura is often removed for many 2-photon imaging experiments to improve image quality[8], for many experiments it is desirable to obtain labelling in a less invasive manner. We have therefore explored our ability to use silk/AAV to label cortical regions without removing the dura. We obtained some labelling, but it is possible that this was a consequence of

damaging the dura in the process of preparing the craniotomy. Further study is needed for coated cranial windows to be used to reliably label the cortex without removing the dura.

The preparation of aqueous silk fibroin from cocoons of Bombyx Mori is described in detail in Rockwood *et al.* (2011)[18]. Aqueous silk fibroin is now commercially available (5% w/v). Although most of our experiments were performed using aqueous silk fibroin stocks prepared in our lab (5-7.5% w/v), we have obtained similar results using commercial aqueous fibroin. Aqueous fibroin is stable at 4 °C for up to 3 months, after which it spontaneously transitions from liquid to hydrogel[18]. We recommend that fibroin stocks be divided into ~1 mL aliquot and stored at -80 °C. A 1 mL working aliquot (sufficient for coating hundreds of implants) can be stored at 4 °C and used until it begins to gel. Be careful not shake, vortex, agitate or aggressively pipette aqueous fibroin, as shear forces can lead to gelation[19,20].

Silk/AAV films permit a wide range of expression patterns, from widespread cortical expression under cranial windows, to precise subcortical expression at the tip of a small-diameter optical fibers. These techniques were developed to take advantage of common AAV expression vectors but could likely be used to disperse other expression vectors like Lentiviruses or rabies viruses into the brain. Silk films could also be manufactured into three-dimensional shapes to improve viral release into tissue. For example, in order to drive strong expression beneath cortical windows without the use of a durotomy, cranial windows could be coated with arrays of silk microneedles that would pierce the dura and release virus in deeper cortical layers[21]. Further refinement will likely lead to improved properties of virus release, and new applications for silk/AAV films.

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DISCLOSURES:

The authors have nothing to disclose.

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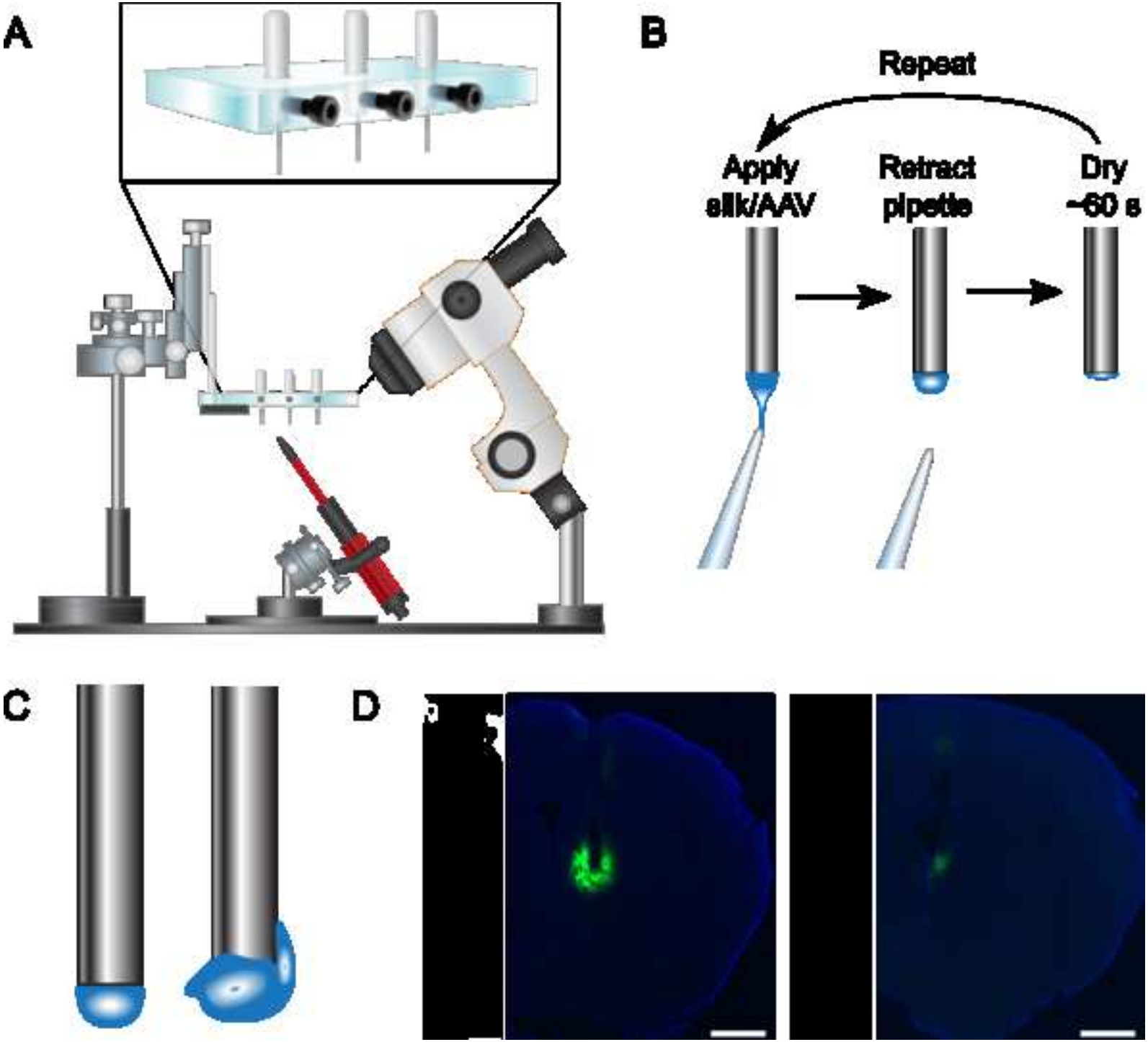
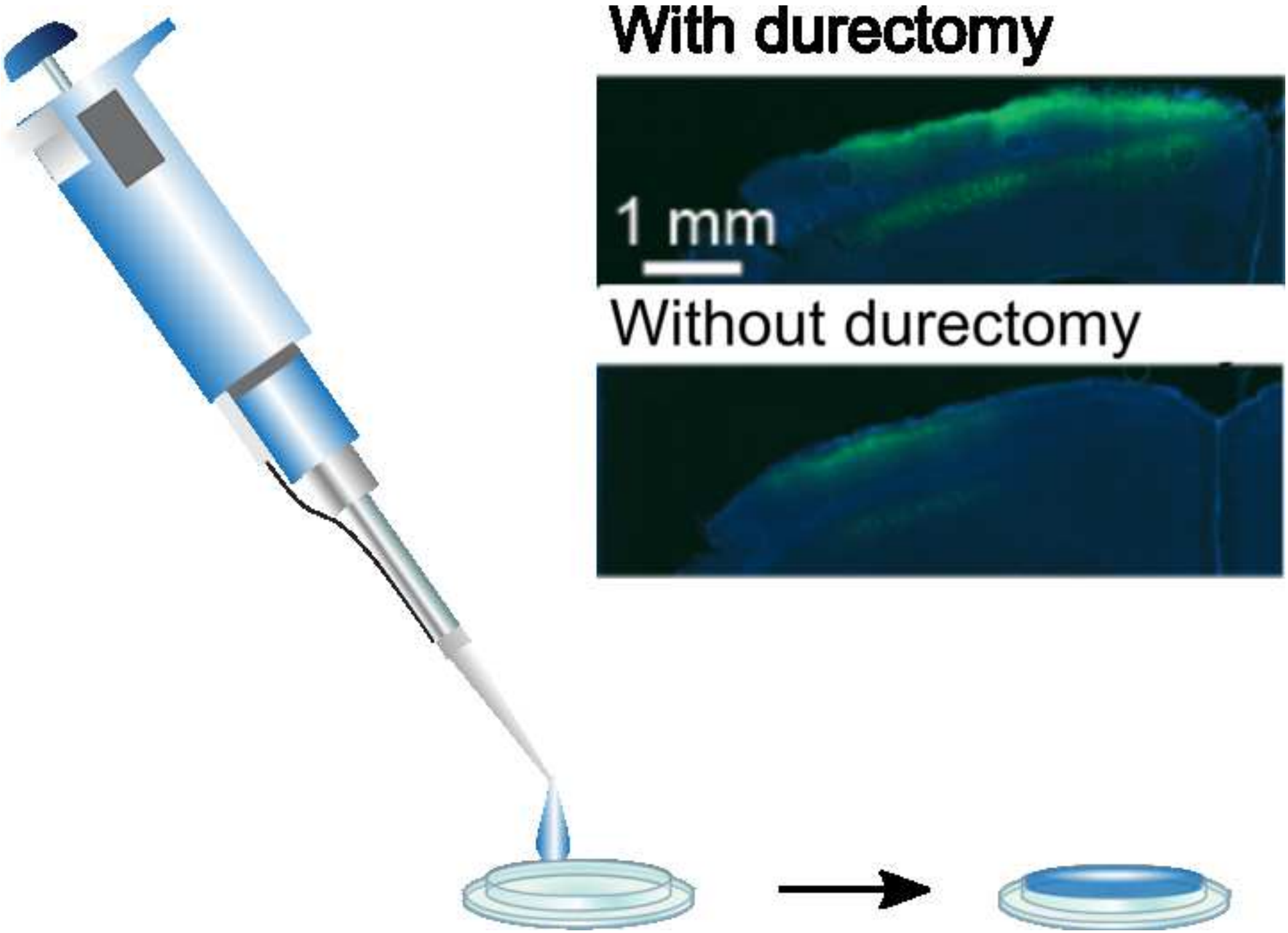


Figure 2



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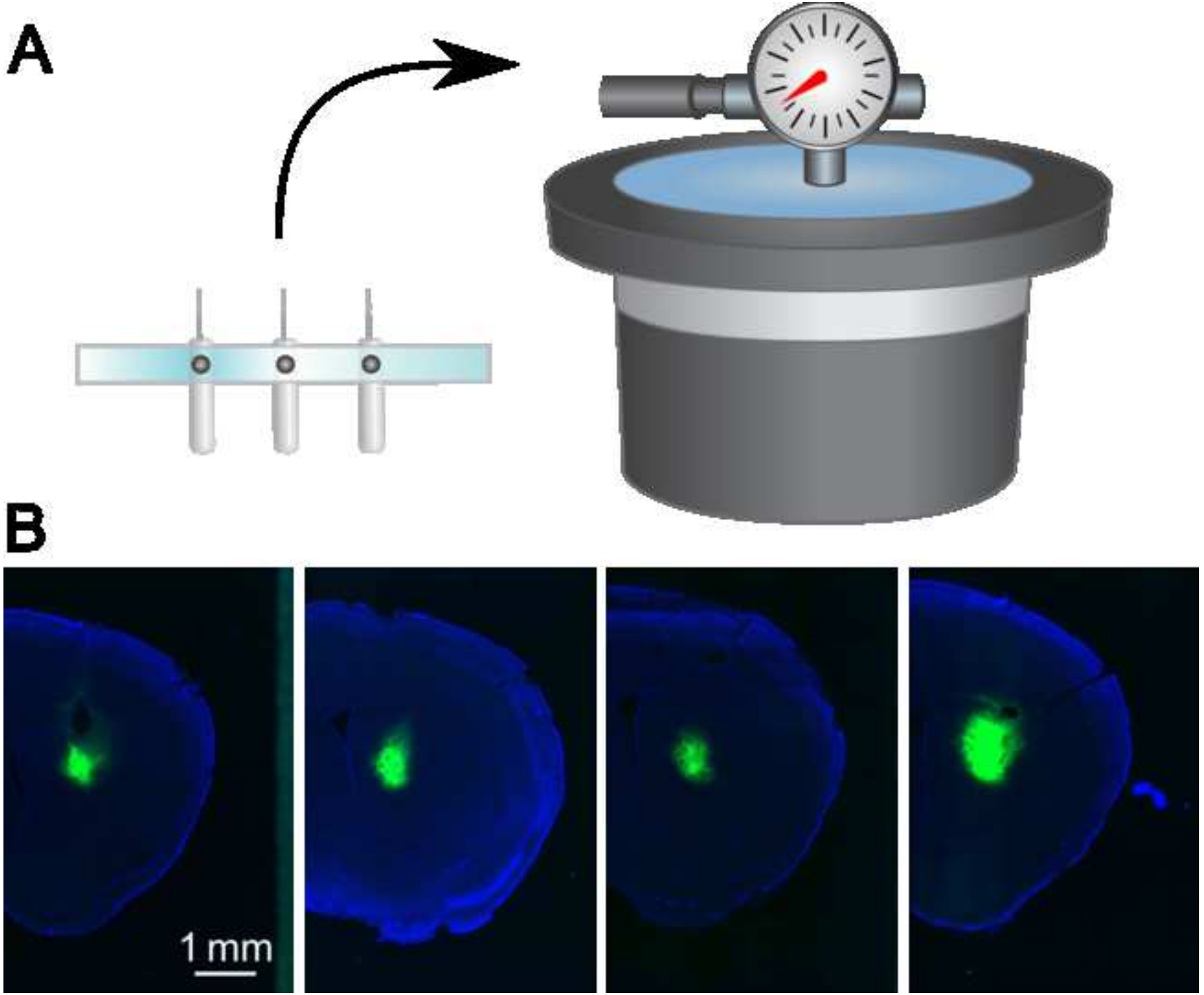

Figure 4

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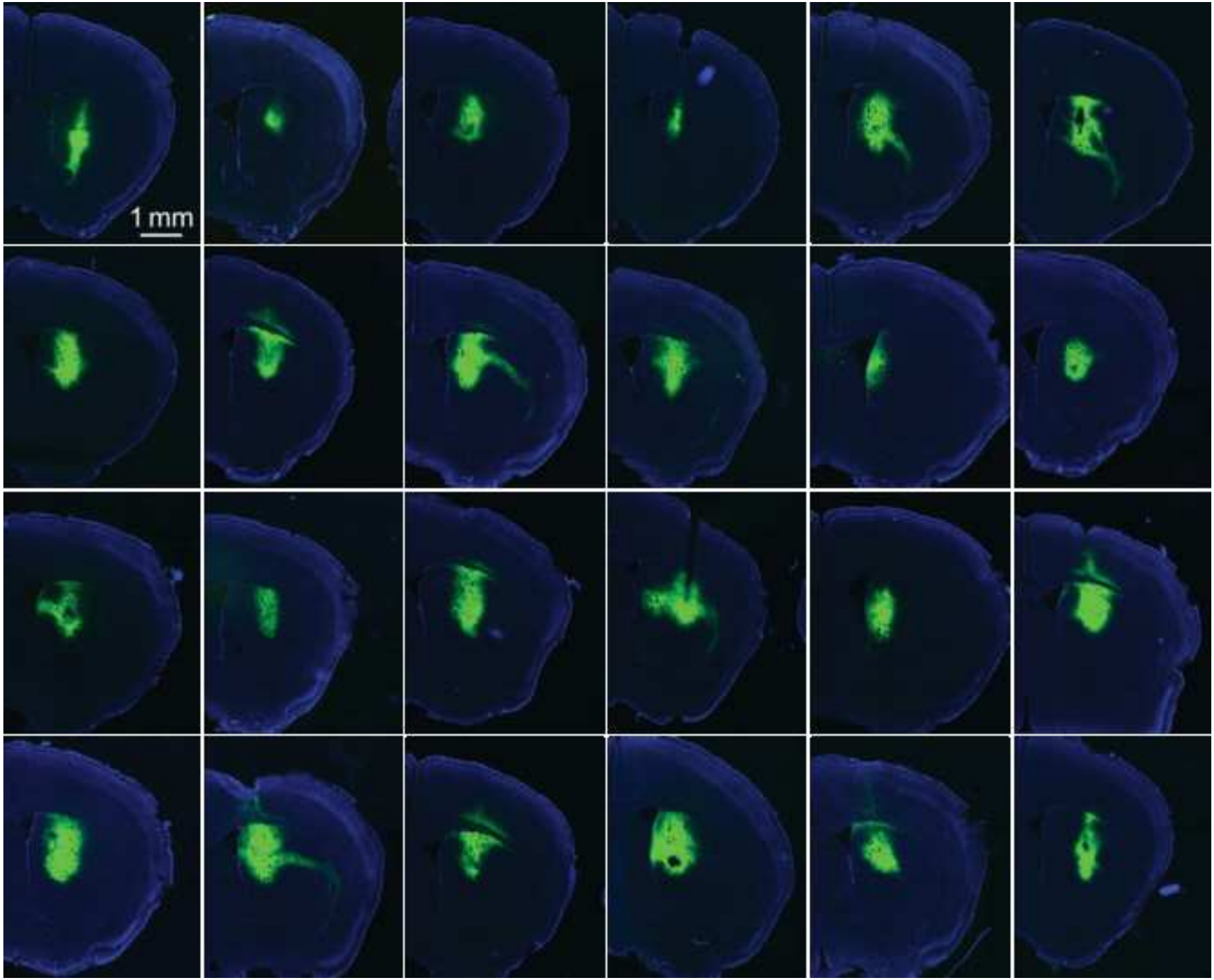

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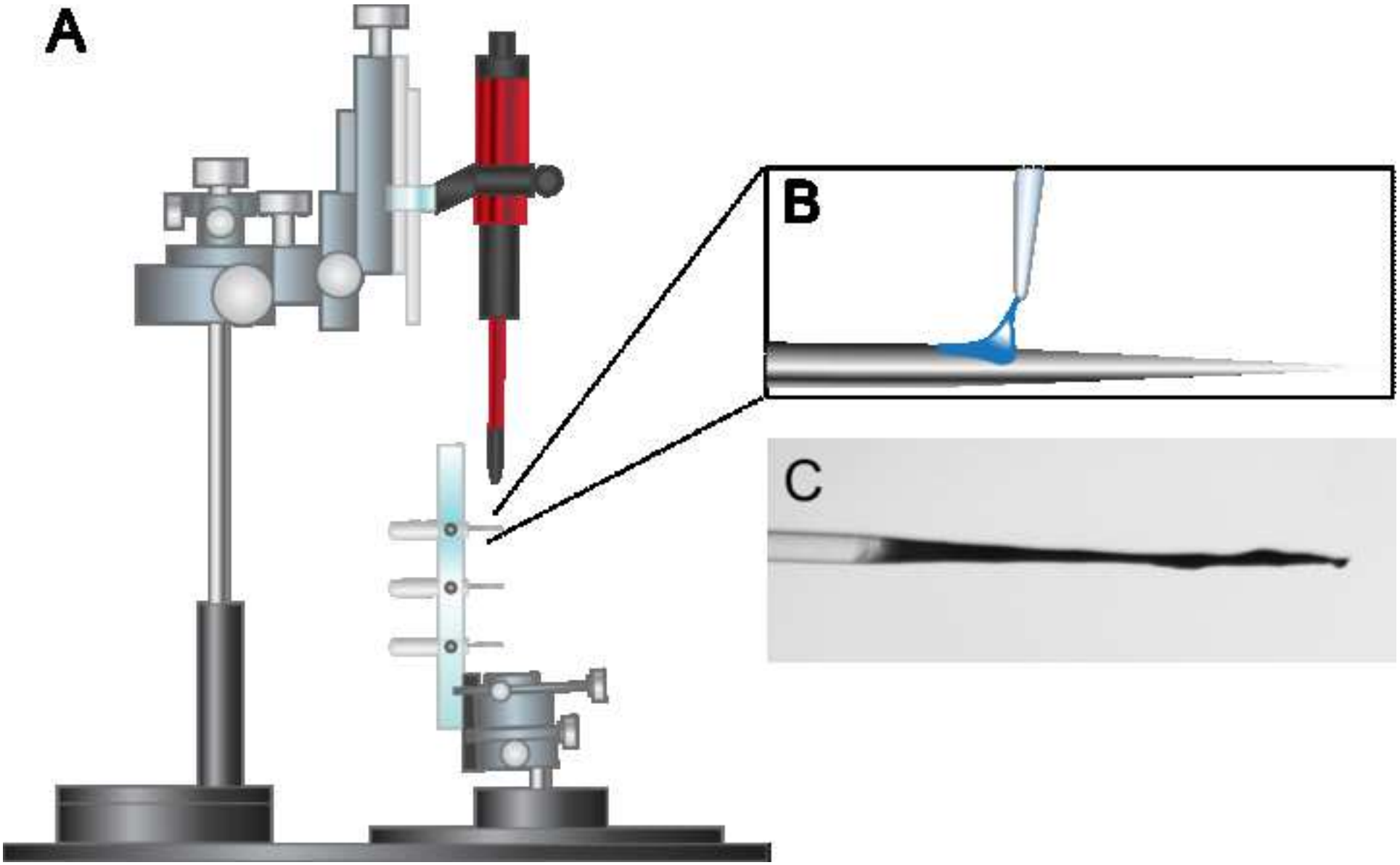

Name of Material/ Equipment	Company	Catalog Number
Aqueous silk fibroin	Sigma	5154-20ML
Microinjector to deposit silk/AAV	Drummond	3-000-207
Manipulator to hold implants	Narashige	MM-33
Stereoscope to visualize silk deposits	AmScope	SM-6TX-FRL
Vacuum chamber to store implants	Ablaze	N/A
Optional, implant holder for storage	N/A	N/A
Optical fiber	Thorlabs	FT200EMT
Ferrules	Kientec	FZI-LC-230
Various materials for manufacturing chronic fiber implants	Various	N/A
Tapered fiber implants	Optogenix	Lambda-B
GRIN lenses	GoFoton	CLH-100-WD002-002-SSI-GF3
Small glass cranial windows	Warner	64-0726 (CS-3R-0)
Large glass cranial windows	Warner	64-0731 (CS-5R-0)
Various materials for manufacturing cranial windows	Various	N/A

Comments/Description

Aqueous Silk Fibroin (5% w/v) for making films

Nanoject III nanoliter injector

Micromanipulator

3.5X-45X Trinocular articulating zoom microscope with ring light

3.5 Quart Vacuum Vac Degassing Chamber

To store premade optical fibers, drill a grid of ~4 mm-deep holes with a diameter just larger than the ferrule diameter into a plastic block.

Ø200 µm Core Multimode Optical Fiber for fiber implants

LC Zirconia Ferrule for fiber implants

For detailed procedure, see Ung K, Arenkiel BR. Fiber-optic implantation for chronic optogenetic stimulation of brain tissue. Journal of visualized experiments: JoVE. 2012(68).

Tapered fiber implants

GRIN lenses

Small round cover glass, #0 thickness

Small round cover glass, #0 thickness

For detailed procedure, see Goldey GJ et al. Removable cranial windows for long-term imaging in awake mice. Nature protocols. 2014 Nov;9(11):2515.

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Aug 17, 2018

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1. The editor has formatted the manuscript to match the journal's style. Please retain the same. Done.
2. Please address specific comments marked in the manuscript. Done.
3. Please refer to the figures in the order of the numbering. So figure 1 will be referred before 2, 3, 4 etc. Done.
6. Please use imperative tense throughout the protocol, as if directing someone how to perform the protocol. We now use the imperative tense.
7. Please ensure that the protocol is made up of discrete steps and consider moving the discussion about the steps to the discussion section. We now use only action steps in the protocol.
8. After all the formatting please ensure that the protocol is no more than 10 pages in length and the highlight is no more than 2.75 pages in length including headings and spacings. The protocol is 8 pages, with 4 more pages of representative results, figure legends, discussion and citations.

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