Video Article

Scalable Fluidic Injector Arrays for Viral Targeting of Intact 3-D Brain Circuits

Stephanie Chan[1], Jacob Bernstein[1], Edward Boyden[1]

[1]Biological Engineering, Brain and Cognitive Sciences, and McGovern Institute, Massachusetts Institute of Technology

Correspondence to: Edward Boyden at esb@media.mit.edu

Abstract

Our understanding of neural circuits--how they mediate the computations that subserve sensation, thought, emotion, and action, and how they are corrupted in neurological and psychiatric disorders--would be greatly facilitated by a technology for rapidly targeting genes to complex 3-dimensional neural circuits, enabling fast creation of "circuit-level transgenics." We have recently developed methods in which viruses encoding for light-sensitive proteins can sensitize specific cell types to millisecond-timescale activation and silencing in the intact brain. We here present the design and implementation of an injector array capable of delivering viruses (or other fluids) to dozens of defined points within the 3-dimensional structure of the brain (**Figure. 1A, 1B**). The injector array comprises one or more displacement pumps that each drive a set of syringes, each of which feeds into a polyimide/fused-silica capillary via a high-pressure-tolerant connector. The capillaries are sized, and then inserted into, desired locations specified by custom-milling a stereotactic positioning board, thus allowing viruses or other reagents to be delivered to the desired set of brain regions. To use the device, the surgeon first fills the fluidic subsystem entirely with oil, backfills the capillaries with the virus, inserts the device into the brain, and infuses reagents slowly (<0.1 microliters/min). The parallel nature of the injector array facilitates rapid, accurate, and robust labeling of entire neural circuits with viral payloads such as optical sensitizers to enable light-activation and silencing of defined brain circuits. Along with other technologies, such as optical fiber arrays for light delivery to desired sets of brain regions, we hope to create a toolbox that enables the systematic probing of causal neural functions in the intact brain. This technology may not only open up such systematic approaches to circuit-focused neuroscience in mammals, and facilitate labeling of brain regions in large animals such as non-human primates, but may also open up a clinical translational path for cell-specific optical control prosthetics, whose precision may enable improved treatment of intractable brain disorders. Finally, such devices as described here may facilitate precisely-timed fluidic delivery of other payloads, such as stem cells and pharmacological agents, to 3-dimensional structures, in an easily user-customizable fashion.

Video Link

The video component of this article can be found at http://www.jove.com/video/1489/

Protocol

1. Constructing Stereotaxic Clamp

1. The stereotaxic clamp is used for attaching the injector array to the stereotax, so that the injectors are parallel to the stereotaxic arm.
2. Each lab needs only one clamp, unless it is anticipated that more than one surgery may take place simultaneously. In this case, make the same number as the anticipated maximum number of simultaneous surgeries. It might also be advisable to make an extra, in case of damage to the first.
3. To make the stereotaxic clamp, firstly, a 1.5 mm outer diameter (OD) steel cannula is cut to 2 inches in length, and one of the ends is Dremeled down until flat.
4. Next, a small piece (approximately 0.5 x 0.5 cm) is cut out of PCB proto-board, to be used as a spacer.
5. Using a laser cutter to ensure that any cuts made are perpendicular to the surface of the material, two identical rectangles (1/2" x 3/8") are cut from 1/8" acrylic sheeting, each rectangle having two circular holes in opposite corners of the rectangle. The first hole has diameter 1.5mm, and is just large enough to hold the metal cannula tightly. The second hole has diameter 1/16" (**Figure 1C**).
6. The metal cannula is inserted into the 1.5 mm hole of one rectangle, then through the 1.5 mm hole of the second. The bottom end of the cannula is aligned with the bottom face of the bottom rectangle, forming the shape of a hockey stick.
7. While keeping the spacer tightly held between the two rectangles, this structure is cemented together by using hot glue around the cannula and 1.5mm holes, being careful to avoid gluing the spacer to the rectangles. Modeling clay may be helpful to hold things together during this process.
8. After the glue has dried, a 1-72 screw is inserted into the 1/16 hole from the top side of the rectangles. Then a 1-72 hex nut is screwed onto the end of the 1-72 screw, and tightened. The screw and nut serve to hold the clamp together.

9. To attach the hex nut to the bottom face of the bottom rectangle, small amounts of 5-minute epoxy are dropped around the edge of the hex nut, without allowing the epoxy to get into the threads of the hex nut or screw. Any modeling clay is removed, and it is confirmed that the spacer can be held firmly in place by tightening the screw.

2. Preparing the system for the customized injector array: Hamilton pump and stereotax

1. The injector array system can be customized to virtually any set of coordinates in the brain.
2. The user first locates coordinates of desired injection sites in a mouse (or other species) brain atlas, converting to the appropriate coordinates used by the stereotax (here, X-, Y-, and Z-coordinates). The number of injection sites (three in **Figures 1A and 1B**) will hereafter be called k.
3. k number of 10 µl Hamilton syringes are placed in an injection/withdrawal syringe pump such as this one from Harvard apparatus.
4. Next, 3-foot-long pieces of polyethylene tubing are securely attached to the needle of each Hamilton syringe.
5. For each piece of polyethylene tubing, an F-252 tubing sleeve from Upchurch Scientific is slid over the open end and attached to a P-627 tubing adapter using the included nut and ferrule, also from Upchurch Scientific.

3. Constructing the Customized Injector Array

1. Each injector array is customized to the set of coordinates that the user chooses. However, for sets of coordinates that differ only by a translation and/or rotation, the same injector array can be used.
2. Considering only the relative X and Y coordinates of the injection sites, k holes are drilled into a PCB proto-board of thickness 1/32", with the same relative spacing. These holes are drilled using a Modela mini mill and a .011" diameter drill bit from Mcmaster-Carr. Be sure to use a slow rate of drilling (Z-speed) to avoid breaking or wearing out the drill bit. Before removing the board from the mill, a rectangle is drilled around the small holes, using a larger drill bit of diameter 1/32", to avoid wearing out the smaller one. Code for the mini mill can be generated easily using MATLAB; sample code is provided in the following files.
   i. generate.m - MATLAB code for generating Modela code from desired coordinates
   ii. holes_ex.rml - Modela code for drilling ring pattern (eight holes) into Proto-board
   iii. outline_ex.rml - Modela code for drilling rectangle around holes

3. 245 µm OD/100 µm inner diameter (ID) fused silica capillary tubing, available from Polymicro Technologies, is cut into k number of 3 inch pieces. These comprise the individual injectors. A disposable piece of capillary tubing is poked through each of the holes, to clear away debris without clogging the actual injectors. The injectors are then inserted halfway into each hole, so that they are held tightly and parallel to one another. The injectors are epoxied to the board, forming the structure of the injector array. All that remains is to trim the injectors to the correct length.
4. The injector array is attached to the stereotaxic clamp by placing one corner of the proto-board between the acrylic rectangles, and then by tightening the screw of the stereotaxic clamp. Next, the metal cannula of the clamp is attached to the stereotax, using the attachment mechanism of the stereotax.
5. All of the following is done under a microscope. For one of the outer injectors (that is, one that can be approached from the side with a straight edge, without bumping into any of the other injectors), the tip extending beyond the bottom of the proto-board is cut with scissors. For cortical injections, it is appropriate to have the injector extending approximately 5mm beyond the surface of the proto-board. For deeper injections, this number can be increased by the corresponding increase in depth. The tip is flattened with a Dremel tool. Injector tips can also be ground at an angle as an extra precaution against clogging, if precision in depth is less important.
6. Next, a stable reference point is chosen within range of the stereotaxic arm, and the injector array is moved so that the flattened tip of the outer injector, is at this reference point.
7. A second injector is chosen, along with its corresponding coordinates. Considered is the relative difference in height (z-direction) between the first and second injector coordinates. The injector array is moved along the Z axis by this relative distance. The second injector is trimmed and Dremeled to the correct length, so that the tip of the second injector is now flat and at the height of the reference point. The injector array can be moved in the X and Y directions to facilitate comparison of the injector tip with the reference point.
8. This process of trimming is repeated for the remaining injectors.

4. Assembling the entire system

1. The stereotaxic clamp and customized injector array, both already constructed, are required for this part.
2. The back end of each injector (the end that has not been trimmed) is inserted into a blue F-240 tubing sleeve and, using a P-235 nut and P-200 ferrule from Upchurch Scientific, is attached to the threaded adaptor already connected to the Hamilton pump by polyethylene tubing. Manufacturer's instructions can be consulted for details.
3. Using a 27-gauge needle, silicone oil is injected into the back of the Hamilton syringes so that the entire system is filled from the Hamilton syringe to the injector tip, with no air bubbles at all.
4. As the Hamilton syringes are re-placed in the Hamilton pump, the greatest possible volume of silicone oil is maintained in the syringes.
5. If the experiment requires more syringes than the pump is designed for (two in this case), the syringes can be spaced with small pieces of plastic (e.g., needle caps) to keep them parallel to one another, and to ensure that all pieces are pushed by the pump to the same extent. Or, a multi-rack upgrade kit can be purchased from Harvard Apparatus to hold up to 10 syringes at once.

5. Injections/surgery

1. The process of injecting with a parallel injector is very similar to that of injecting with a single pipette.
2. It is essential to use slow refill/infuse rates throughout this process, because fast pumping might put stress on the joint between large and small tubing. Recommended max rate: 2 µl/min for loading virus, and 0.1 µl/min for infusing virus.
3. An anesthetized mouse is placed in the stereotax, and remains anesthetized throughout the experiment.

4. Prepare the mouse as needed. For example, with a scalpel, a single cut is made down the midline of the skin, from between the eyes to between the ears. The skin is pulled back to expose the skull, and the fascia is cleaned off. A pulled glass pipette is attached to the stereotax. The positions of the ear bars are adjusted until bregma and lambda are aligned to the same height, and so that the line connecting them is parallel to the Y-axis of the stereotax. The axes of the stereotax are oriented according to the coordinate system in which the injection coordinates have been calculated. The stereotaxic is zeroed with the tip of the glass pipette at bregma, and then the tip is positioned slightly above the skull at the X- and Y-coordinates of one of the injection sites.

5. Using a dental drill, the skull below the tip is carefully pared away until there remains an extremely thin layer of bone. The glass pipette can be lowered and raised to check that the hole being drilled is centered at the correct position. Using a 30-gauge needle, a tiny piece of the layer is gently picked off, at the correct X- and Y- coordinates, so that the dura mater is exposed. This hole should be just large enough to fit one of the injectors (0.25mm wide). See **Figure 1D** for diagram. In this way, small, 0.25mm wide holes are made in the skull, at the X- and Y-coordinates corresponding to each injection site.

6. The glass pipette is discarded in a sharps container, and the customized injector array is attached to the stereotax using the stereotaxic clamp.

7. In order to correctly set the angle of the injector array, two outer injectors are chosen to be calibrated to a given reference point, as follows. After one of the injectors is matched with the reference point, consider the relative X- and Y- distances between the injection sites relative to this injector and one other. The axes of the stereotax are adjusted so that entire injector array is moved in the X- and Y-directions by these relative distances. If the second injector is not now aligned with the reference point, the metal cannula is loosened and rotated. This process is repeated iteratively until the injector array is angled correctly.

8. Before filling the injectors with virus, the injectors are filled with silicone oil until the syringes are at the 2 µl point (or greater). This provides a buffer zone, so that any air bubbles or clogging in the tip can be easily removed by pushing oil forward using the Hamilton pump, without having to refill the entire system with silicone oil from the back of the syringes, as describe previously.

9. A sterile piece of Parafilm is placed on the skull, and the injectors are gently lowered onto the Parafilm. For coordinates with greatly varying depths, a custom-milled part (e.g. a staircase-shaped object) could facilitate loading.

10. The following parts assume that 1 µl of virus is to be loaded at each site (the following quantities should be scaled down if smaller quantities are desired). In order to guarantee that >1 µl of virus is injected at each site, 1.5 µl of virus is pipetted onto the Parafilm or staircase at the tip of each injector.

11. With the refill rate of the Hamilton pump set to 1 µl/min, 1.2 µl of the virus is refilled into each injector.

12. The tip of the longest injector is zeroed at bregma, and then the injector array is shifted to the X- and Y- coordinates of that injector. If clogging is an issue, say if many deep targets are involved: even before inserting the injector tips into the brain, it is sometimes advisable to start the pump infusing, until virus can be seen emerging from all the injector tips. This removes any air bubbles at the injector tips, and also ensures that there is no clogging. In the case of clogging, the Hamilton pump is set to infuse a brief pulse at a higher speed of 2 µl/min, to gently clear out clogging.

13. The injector array is then lowered, through the small holes made with the 30-gauge needles, to the correct Z-depth.

14. 1 µl of virus is injected through each injector, at 0.1 µl /min.

15. The injections are left alone for 30 minutes.

16. After the injector array is slowly extracted from the brain, the Hamilton pump is set to infuse at the same rate of 0.1 µl /min, in order to check for clogging in each injector.

17. Then the injectors are cleaned by refilling and infusing 1.5 µl of ethanol at 2 µl/min.

18. Finally, the injectors are refilled with silicone oil to maintain the 2 µl buffer zone in each Hamilton syringe.

19. All equipment should be sterilized before and after the procedure, according to your institution's biosafety and animal use protocols.

6. Representative Results

The parallel injector array speeds up a surgery roughly by a factor equal to the number of injectors, not counting setup and recovery time, although individual times will depend on the skill of the practitioner. For a 1 microliter injection, we typically saw lentivirus expression in a sphere of approximately diameter 1mm (**Fig. 1E**). The precision of the injection was such that the variability in tip positioning, from trial to trial, was about 45 microns (standard deviation of the distance from the tip position to the intended tip position).
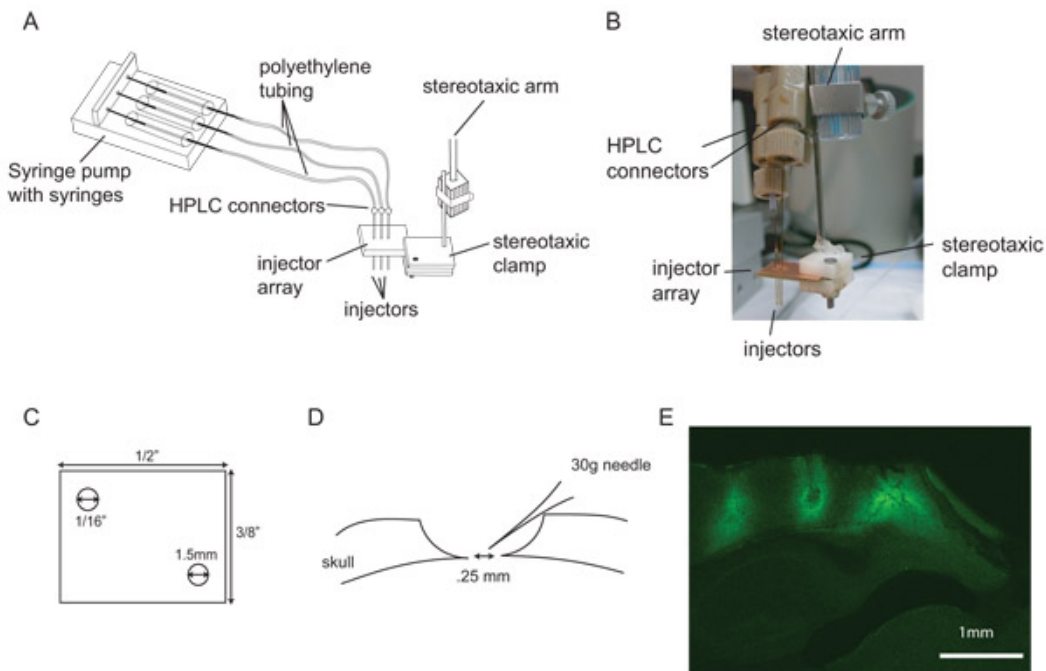
Figure 1. Design, implementation, and use of a parallel virus injector array. **A**, schematic of the parallel injector array system, showing a triple injector configuration, for three simultaneous injections. **B,** photograph of a triple parallel injector array as diagrammed in **A**. **C**, stereotaxic clamp, shown in outline from the top. **D,** illustration of technique for efficient, damage-minimizing, opening of holes in skull for injector insertion into brain: with a dental drill, thin the skull down to ~50 microns thickness, then use the tip of a sharp needle to open a small craniotomy. **E**, fluorescence image showing channelrhodopsin-2 (ChR2)-GFP-labeled cells in three mouse cortical regions, as targeted by the triple injector array shown in **B**.

Discussion

In recent years, a number of genetically-encoded optical sensitizers have enabled neurons to be activated and silenced *in vivo* in a temporally-precise fashion, in response to brief pulses of light (e.g., [1,4,5,6,7,8,11]). A key method with which neurons have been sensitized to light in the mammalian brain, is via viruses such as lentiviruses and adeno-associated viruses (AAV), which can deliver genes encoding for opsins to brains of animals ranging from mice to monkeys, in a safe and enduring fashion (e.g., [2,9,10]). Viruses allow faster turnaround time than do transgenics, especially for organisms that are not genetic model organisms such as rats and monkeys, and for opsins may enable high expression levels that may not be possible in transgenic scenarios. Here we demonstrate a parallel injector array capable of creating, in a rapid timescale, "circuit-level transgenics," enabling entire 3-dimensional brain structures to be virally targeted with a gene, in a single surgical step. The injector array comprises one or more displacement pumps that each drive a set of syringes, each of which feeds into a polyimide/fused-silica capillary via a high-pressure-tolerant connector. The capillaries are sized, and then inserted into, desired locations specified by custom-milling a stereotactic positioning board, thus allowing viruses or other reagents to be delivered to the desired set of brain regions. To use the device, the surgeon first fills the fluidic subsystem entirely with oil, backfills the capillaries with the virus, inserts the device into the brain, and infuses reagents slowly (<0.1 μL/min).

This technology will enable a wide variety of new kinds of experiments, such as millisecond-timescale shutdown of complexly-shaped structures (such as the hippocampus) at precise times during behavior, temporally-precise inactivation of bilateral structures that may act redundantly (such as the left and right amygdala), and the perturbation of multiple discrete brain regions (e.g., driving two connected regions out of phase to study how cross-region synchrony depends upon activity within each region, or stimulating inputs to a region while silencing a subset of the targets in order to understand which of the several targets are critical for mediating the effects of those inputs). For large brains like those in the primate, in which we have recently demonstrated optical cell-type specific neural activation[3], perturbing activity in a behaviorally-relevant area may require viral labeling of large, complex structures. We note that parallel injector arrays may be used to inject almost any payload – drugs, neuromodulators, neurotransmitters, or even cells – in complex 3-D patterns in the brain, in a temporally-precise manner. Finally, from a translational standpoint, it is possible that rapid, patient-customized gene therapy or drug delivery devices may be rapidly custom-designed and fabricated to match individual brain geometries, supporting new treatments for a variety of pathologies, potentially through the use of optical control molecules.

The injector arrays are designed to be precise, both spatially and volumetrically. In the X- and Y-directions, this is accomplished by drilling very accurately placed holes using an inexpensive mini-mill, with the holes just large enough to fit the injectors, so that injectors are held parallel to one another, and in a precise location. In the Z-direction, the injectors are trimmed using a stereotaxic apparatus, allowing a level of precision equivalent to that of the stereotaxic surgery itself. The volumetric precision arises from the precision of the Hamilton pump, as well as the near-zero dead-volume connectors, adapted from the high-pressure liquid chromatography (HPLC) field. The injectors are made from fused silica capillary tubing, which is strong and rigid enough that it maintains precise shape and spacing under pressure, without the larger wall thickness of alternatives such as steel cannulas. Small modifications can easily be made to adapt the parallel injector array to a variety of experiments. For example, if a smaller volume of virus or finer spacing is required, smaller capillary tubing can be employed, along with a corresponding smaller

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drill bit. Future devices may utilize microfluidic channels and pumps, to increase the number of parallel injectors, to minimize the size (perhaps enabling such devices to be mounted on the heads of freely-moving animals).

Disclosures

All procedures were in accordance with the NIH Guide for Laboratory Animals and approved by the MIT Animal Care and Use and Biosafety Committees.

Acknowledgements

References

1. B. Chow, X. Han, X. Qian, and E. S. Boyden, *Frontiers in Systems Neuroscience. Conference Abstract: Computational and systems neuroscience.* doi: 10.3389/conf.neuro.10.2009.03.347 (2009).
2. Bi, A., Cui, J., Ma, Y.P., Olshevskaya, E., Pu, M., Dizhoor, A.M., Pan, Z.H. Ectopic expression of a microbial-type rhodopsin restores visual responses in mice with photoreceptor degeneration. Neuron 50, 23-33 (2006).
3. Han, X., Qian, X., Bernstein, J., Zhou, H., Franzesi, G., Stern, P., Bronson, R., Graybiel, A., Desimone, R., Boyden, E. Millisecond-Timescale Optical Control of Neural Dynamics in the Nonhuman Primate Brain. Neuron 62 (2), 191-198 (2009).
4. Zhang, F., Wang, L.P., Brauner, M., Liewald, J.F., Kay, K., Watzke, N., Wood, P.G., Bamberg, E., Nagel, G., Gottschalk, A., Deisseroth, K. Multimodal fast optical interrogation of neural circuitry. Nature 446 (7136), 633-639 (2007).
5. Boyden, ES., Zhang, F., Bamberg, E., Nagel, G., Deisseroth, K. Millisecond-timescale, genetically targeted optical control of neural activity. Nat Neurosci. 8 (9), 1263-1268 (2005).
6. Han, X., Boyden, E. S. Multiple-color optical activation, silencing, and desynchronization of neural activity, with single-spike temporal resolution. PLoS ONE 2, e299 (2007).
7. Szobota, S., Gorostiza, P., Del Bene, F., Wyart, C., Fortin, D.L., Kolstad, K.D., Tulyathan, O., Volgraf, M., Numano, R., Aaron, H.L., Scott, E.K., Kramer, R.H., Flannery, J., Baier, H., Trauner, D., Isacoff, E.Y. Remote control of neuronal activity with a light-gated glutamate receptor. Neuron 54 (4), 535-45 (2007).
8. Lima, S.Q., Miesenbock, G. Remote control of behavior through genetically targeted photostimulation of neurons. Cell 121 (1), 141-152 (2005).
9. Zhang, F., Wang, L.P., Boyden, E.S., Deisseroth, K. Channelrhodopsin-2 and optical control of excitable cells. Nat. Methods 3, 785 792 (2006).
10. Ishizuka, T., Kakuda, M., Araki, R., Yawo, H. Kinetic evaluation of photosensitivity in genetically engineered neurons expressing green algae light-gated channels. Neurosci Res. 54 (2), 85-94 (2006).
11. Luo, L., Callaway, E.M., Svoboda, K. Genetic dissection of neural circuits. Neuron 57 (5), 634-60 (2008).