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Author

Susie S. Cha1, Mark E. Bucklin1,2, Xue Han1

Author affiliation

1Boston University, Department of Biomedical Engineering, Boston, MA 02215

2Boston University, School of Medicine, Boston, MA 02118

Keywords

wide-field optical imaging, in vivo, silicone elastomer, cranial window …

# Abstract

Wide-field epifluorescence imaging of a living brain has substantially expanded our ability to perform high-throughput detection of neural activity at a spatial and temporal resolution sufficient to capture rich cellular dynamics of large interconnected networks of neurons. Yet optical signals are highly sensitive to light scattering, and the preservation of optical clarity through craniotomies is critically important for high-resolution imaging. Longitudinal imaging in cortex, however, is often limited due to granulation tissue growth between the brain and the imaging access window, and its inhomogeneous structure induces a significant level of optical scattering, thus severely compromising spatial resolution. To address this concern, we report the design of a polydimethylsiloxane (PDMS)-based cranial window system that can faithfully prevent the optically obstructive tissue from growing under the craniotomy. Additionally, the two-part system is constructed to allow for continued full tissue access after its initial installation. Utilizing the system, we demonstrate a year-long course of wide-field imaging recording the simultaneous activity in thousands of cortical neurons in awake mice.

1. Cranial window system that provides more functionalities to extend our capability using in vivo optical imaging to probe the complexity of the brain
   1. Long-term maintenance of optical quality
   2. Easy and repeatable tissue accessibility
2. Goal of the project was to design a system that
   1. Performs reliably
   2. Easily adaptable
   3. Easily adoptable
3. To achieve, used silicone elastomer (vs. convention – glass)
   1. Major advantage – can easily incorporate shapes and features to develop a system that can adapt to various experimental requirements
   2. Provide equivalent optical quality to glass
4. Achieved by developing a multi-stage system composed of a headplate with an integrated chamber and a cranial window
   1. Window was casted incorporating features to block tissue growth within image field
      1. Achieve image durations extending beyond a year to record calcium dynamics of hundreds of individual neurons using wide-field epifluorescence microscope
   2. Headplate provided the base for window installation which can be temporarily detached to perform acute manipulation on the underlying tissue
5. Easily adaptable, demonstrated through providing an example of two systems with similar design concepts but incorporated distinct design features
   1. Bilateral hemispheric windows
   2. Whole-brain window
6. Designs of the reported system are available in open source and can be easily adopted by developing the parts through rapid prototyping

# Introduction

In vivo optical imaging provides a means to visualize the structural features and functional dynamics of brain tissue, and to measure how these change over time1–3. Optical techniques for observing neural activity have advanced due to evolving digital imaging technology, and the development of increasingly effective functional indicators such as the genetically encoded fluorescent calcium sensor GCaMP6f4.

The ability to observe and record from the same brain region for extended periods is critical for longitudinal experiments tracking long term changes5–8. This ability relies heavily on maintaining a clear optical light path by forming a stable non-scattering interface with neural tissue overlying the targeted brain region. Using a wide-field fluorescence microscope with a scientific-CMOS camera, we can record activity in hundreds of distinct neurons in the hippocampus and other subcortical brain regions of awake behaving mice9. The method used to create an optical window in subsurface regions allows the quality of optical access to remain stable for several months. The approach is not without drawbacks, however; stable optical access is delayed by pooling and coagulation of blood on the window surface resulting from unavoidable vascular damage inherent to the implantation procedure5. The period of delay post implantation can range from 3-8 weeks before tissue repair and phagocytic debris removal processes subside to yield optical access to targeted brain tissue with stable imaging quality.

Attempts to image neocortical regions on the surface of mouse brain typically use a small glass disc fixed to the cranial surface to seal and protect the craniotomy, and to provide optical access to the brain10,11. This approach, however, is often challenged by progressive deterioration in image quality. Consequently, the reliability and average duration of optical access has been insufficient for long-term studies. The degradation is observed as a cloudy layer that gradually covers the imaging field, and is thought to arise from the natural inflammatory response that follows a craniotomy10–15. As granulation tissue grows, its inhomogeneous structure scatters light at the interface between brain tissue and optical window, which consequently degrades image quality and blurs fluorescence signals. Image quality in wide-field microscopy is more sensitive to scattering along the light path than scanning microscopes like two-photon or confocal, and is therefore particularly susceptible to this degradation1,16.

While glass is c­­hemically inert and comes in many shapes and sizes, cementing glass to the top of the skull leaves a fluid filled gap between the window and brain surface which is quickly filled by granulation tissue. This process, thought to be a mechanism for dura and bone regrowth, eventually disrupts the high optical clarity for chronic imaging12, and hence is a common target for efforts to extend image quality through a cranial window7,12.

Efforts to overcome this problem by adding purely mechanical features to the cranial window have involved attaching spacers made of agarose11,17, silicone18,19 and glass7 to the window’s brain-facing surface that compensate for the thickness of removed bone. These approaches report delaying tissue regrowth for up to a few months before optical quality deteriorates. These modest results indicate a valid basis underlying this approach and suggest that extending this strategy by starting with a design and material not limited by the fixed form of flat glass optical windows could yield some improvement. Additional elements of a chronic cranial imaging window intended to mitigate degradation by granulation tissue typically target the primary source stimulating the process, inflammation. These include the aseptic design of seals and features, selective use of biocompatible materials, and perioperative administration of anti-inflammatory and antibiotic drugs7,20,21.

While these designs have improved longevity, they remain limited in terms of long-term access to the cortical tissue. The ability to access and manipulate tissue during real-time imaging facilitates novel ability to characterize the dynamic processes in both physiological and pathological conditions22. Several strategies have been reported to gain access to regions below glass cranial windows by incorporating features such as an access port sealed with elastomer23, infusion cannula24, or the use of microfluidic channels17. Nonetheless, the approaches limit the tissue accessibility to a single designated site predetermined before an experiment begins and do not offer uniform access over the imaging area.

To address the relative restrictions using glass as cranial windows, a number of alternative efforts have highlighted the use of silicone elastomer for cranial windows25–29. For example, polydimethylsiloxane (PDMS) is optically clear, non-toxic and chemically inert and can be molded to take any shape or exhibit any desired feature, not necessarily sacrificing the imaging field of the window. These properties combine to offer a remarkably versatile material, particularly favorable for prototype development for projects with demanding specifications for biocompatibility and optical performance. A well-known and widely used example is the artificial dura for in vivo optical imaging in nonhuman primates25,26,30. This chronic implant device is placed in and covers a craniotomy and sits protected within a chronic cranial recording chamber. It mitigates tissue regrowth, and interfaces with a cylindrical insert – also made of PDMS – for optical imaging of neocortex. Additionally, the artificial dura is thin enough to enable access to underlying tissue for penetrating electrodes, which penetrate easily and leave a tight seal after withdrawal. Yet the efforts for translating this design windows for small research animals using silicone elastomer have thus far been limited. And a system with long-lasting high optical clarity and flexible tissue accessibility remains to be developed or explored for rodent models.

In this paper, we describe a design and demonstrate a cast silicone chronic cranial imaging window system, developed to facilitate longitudinal imaging experiments in mouse neocortex. The primary capability requirements for this design are:

1. Long-term stability of an optically clear light-path to cortical surface
2. Intermittent physical access to imaged region at any point in study

The system was designed considering biocompatibility and optical performance to facilitate integration in place of the removed bone flap enabling us to achieve sustained periods of optical clarity, extending beyond a year in some mice and allowing for both high spatial and temporal resolution using a wide-field microscope. Additionally, the two-part system consisting of a fixed headplate with integrated neural access chamber and optical insert, allowed flexible access to the underlying tissue. The utility of our design is demonstrated through chronic optical imaging of calcium dynamics in the cortex and acute interventions to the tissue upon detachment and replacement of the window from the headplate. Adaptation

# Results

Here we report the design for a head-fixation and cranial window device, and the procedures for surgical attachment. The sections below describe the features of each component, and also report the critical elements that contribute to the performance and capabilities of our optical imaging window system. The following sections provide a detailed report of the system performance observed during evaluation. Adaptation

## Cranial Window **System**

Many design features, and procedures for implantation were introduced and developed to mitigate tissue growth for the sustained optical quality of the window. Other features were included to enhance imaging performance in awake behaving animals, to facilitate repeatable localization of image fields across sessions and animal subjects.

The cranial implant system is composed of two parts: a headplate with an integrated chamber, and a silicone optical insert. The headplate is bonded to the dorsal surface of the animal’s bare skull. The optical insert – sometimes referred to as a “cranial window” – seals the chamber and establishes an optical interface with the animal’s brain through craniotomy sites in the chamber floor (Fig 1a).

### Headplate

The bottom surface of the headplate is curved to conform to the dorsal skull surface of a typical mouse. This feature aids alignment during installation, and a large surface area enables a strong adhesive bond to the skull surface. Adhesive cement is applied continuously along all points of contact to create a permanent bond along the entire perimeter. The cement applied along this joint effectively seals the bottom of the aseptic chamber and is critical for its long-term integrity.

The wide area of skull-to-headplate attachment provides a mechanically stable coupling between the animal’s skull and the headplate holder, which is fixed to the microscope table. The headplate is bonded to all skull plates, which stiffens the skull tremendously. Additional rigidity is provided by a central support structure that contacts the skull along the sagittal suture. All these features combine to provide a very rigid attachment to the mouse cranium, which drastically reduces its motion relative to the imaging system (Fig 1b). Remaining brain motion is then primarily movement relative to the skull, and may originate from physiological forces (i.e. cardiorespiratory) as much as behavioral forces from animal movement; suppressing this intracranial motion is addressed in the design of the silicone window insert described below.

### Chamber

The chamber in the headplate center facilitates physical access to neural tissue by protecting the craniotomy sites between interventions. Once the headplate is bonded to the animal’s skull, the floor of the chamber is formed by the central support structure that traverses and fuses the sagittal suture, the skull surface surrounding each craniotomy, and a flat ledge that extends laterally. The joints between the skull surface edges of the central support, anterior and posterior walls, and the lateral ledge are sealed during the headplate attachment procedure. This bottom seal is crucial for maintaining an aseptic environment for the protection of the exposed brain tissue. When the dura mater is left intact during the craniotomy, the space within the chamber is continuous with the epidural space.

### Optical Insert

The insert has optically flat top and bottom rectangular surfaces. The bottom brain-facing surfaces are positioned to form a flat interface with the intact dura through each craniotomy. The body of the insert provides a clear light-path between top and bottom surfaces. The walls of the body are tapered to increase the angle of unimpeded light collection/delivery at the image field. This increases the numerical aperture for imaging through high power lenses, and also expands options for off-axis illumination. The tapered body is extended to the brain surface via vertical-walled columns that traverse each craniotomy. These columns fill the space made by removal of the bone flap during craniotomy, and their bottom surface gently flattens the brain tissue, positioning the cortex in a horizontal plane for convenient wide-field imaging. Both the top and bottom surfaces are made optically clear by integrating microscope slides in the mold when casting. The dimensions of the insert are depicted in figure 1-b.

Inserts are fabricated in batches using an optically transparent silicone elastomer. We vacuum cast the part in a PTFE and glass mold with an aluminum frame inclusion that gets embedded near the upper surface. This frame provides a site for attachment and sealing to the rim of the chamber, as well as structural reinforcement. This helps to establish and maintain a flat optical surface at the top of the insert, parallel to the headplate (Fig 1b). We constructed inserts with the bottom optical surface parallel to the top, which works well for imaging medial cortical regions. For imaging lateral cortical regions (e.g. visual or auditory cortex) the mold can be adapted to produce inserts that form a flat image plane with consistent controllable angle relative to the headplate. For any desired angle, this capability greatly simplifies recording from a consistent image plane across sessions and animals. The medial cortical region imaged in the demonstration provided here was square in shape (2 mm X 2 mm), at a horizontal angle of 0 degrees, and extended from 0.83 mm to 2.83 mm symmetrically off the midline (Fig 1a).

## Installation and Usage

The surgical installation procedures for this multi-stage implant device were adapted from a combination of procedures in common use for attachment of headplates and cranial windows in mice, and similar devices used for optical imaging in primates. The specific protocol evolved during 3 distinct trial runs, and the final protocol is summarized here and detailed in methods and materials below. The 18 mice reported here received the same version of headplate and optical insert. These mice were used for simultaneous development of other projects not described here. Minor changes were made to the surgical procedures from one batch to the next, each with discernable effect; see the discussion for details.

Because this is a multi-stage implant, the procedure for installation can be separated into multiple distinct surgeries depending on experimental requirements. The first stage includes headplate attachment to bare skull, centrally aligned along the AP axis with the bilateral sites over the cortical region of interest. Once the headplate is securely bonded, bilateral craniotomy can be made through the skull in the base of the chamber. If the second stage of installation is performed separately, the chamber is given a temporary silicone seal to protect the craniotomy. We delayed the second stage of installation for at least 2 to 3 days to allow for mouse recovery.

The second stage involves installation of the optical window, and may be directly preceded by injection of virus, pharmaceutical compounds, exogenous cells, or any other substance of interest. The optical window is installed in the chamber with the assistance of a stereotaxic holder, which enables fine height adjustment and holds the window’s position while being secured in place. The angle of the window’s top surface is held parallel with that of the headplate. The chamber is partially filled with sterile agarose to displace all air from the chamber when the optical insert is lowered into place. The height is adjusted to provide full contact between the insert’s bottom surface and the dura, which also places the insert’s frame in close proximity to the rim of the chamber. Dental cement is applied to form a joint between the headplate and the frame of the optical insert, fixing the insert in place and aseptically sealing the chamber.

The optical insert can be detached and reattached at any time to provide physical access to the neural tissue and/or for window replacement (i.e. for mid-experiment injections or window damage repair respectively). Detachment is relatively easy, accomplished by etching away the joint between headplate and optical insert. Window replacement uses the same procedure as the second-stage installation described above.

The replacement procedure was attempted 5 times, 4 of which were successful in preserving or restoring optical quality to “like-new” condition, without inflicting detectable tissue damage. Three windows were removed and replaced following damage to the top surface of the optical insert, inflicted by feisty cage-mates with sharp incisors (at 91, 83, and 172 days post-installation; 91 days case unsuccessful). The remaining two cranial windows were removed at 20 days post-installation to facilitate direct tissue access for a study requiring cellular graft to the imaged region. We found that the removal needs to be performed slowly, taking great care to avoid capillary rupture in the exposed brain and surrounding granulation tissue. During each of these procedures, we observed the pattern of granulation tissue growth into the peripheral area of the chambers. Photos of the typical growth (as observable with window removed) at day 172 is shown in figure 3-d, and described in more detail below.

## Evaluation of System Performance

Throughout development we implanted several prototypes to test the effect of various features and conditions. The cranial window design and surgical procedures described in this paper were attempted with 18 mice. Cranial window condition was evaluated by direct observation and evaluation of fluorescence dynamics in processed video recorded during periodic 5-minute imaging sessions. Direct (bright-field) observation with a stereoscopic microscope was useful for evaluating quality of the optical interface with brain tissue, as well as for tracking progression of granulation tissue growth in the surrounding space at the edges of the craniotomy. Analysis of cell dynamics measures from processed fluorescence imaging video indicated actual usability of the window for longitudinal studies requiring activity metrics at a cellular level.

### Experimental Batches

The first batch served as a short trial-run for the prototype and procedures whose performance in early tests suggested strong potential for long-term reliability. We ran the first batch for 4-6 weeks to get a better assessment of what we could expect for long-term viability. With this design and minor modifications to the surgical procedure, we felt comfortable using the window in studies with long-term requirements that would also allow for continuous assessment of the window’s performance in parallel. The first batch of windows was installed in April-May 2017 (N = 7) and was evaluated 2-3 times/week for just over 1 month. Several more were implanted for use in a long-term imaging study beginning with a second batch in June 2017 (N = 6), and a third in October 2017 (N = 5). The results of these runs are reported below, summarized in figure 3-b.

### Sustained Optical Quality Extended over a Year

In the first batch of 7 mice, image quality provided by the window was more than sufficient to record cell dynamics across both image regions beginning 4 days after the window-installation and fluorophore-transfection procedure and was sustained for several weeks. At 4-6 weeks post-implant this batch of mice was evaluated and 6 of the 7 mice were discontinued and the installation procedure was adjusted for the next batch. The decision to discontinue in each case was based on observed deterioration in either the health of the mouse (4 out of the 6) or the optical quality of the window (2 out of the 6). See the discussion section for the mechanisms we suspected to underlay and procedural adjustments made to address these issues.

We continued to observe and image the 7th mouse to this day. Progression of the optical quality and fluorophore expression characteristics in bilateral image regions is depicted in figure 4 for this mouse. Optical quality at the window-tissue interface has remained consistent for longer than 18 months at the time of this manuscript’s preparation. The structure of granulation tissue surrounding the window at 12 months is described in detail below and depicted for this mouse in figure 3-d.

Similar to the first batch, the second batch of 6 mice was observed and recorded for some time (3 – 5 months) before discontinuing all except one most exceptional mouse. This mouse received a window replacement at day 83, and was imaged periodically for 11 months before terminating due to a health concern unrelated to the surgical procedure.

The imaging period for the last batch was extended beyond what was required for the stem-cell study to more thoroughly test the longer-term limits of sustained optical quality. Of 5 mice, 1 mouse did not recover as promptly as expected following the craniotomy procedure and was immediately discontinued. We observed consistent performance on long-term optical sustainability, extending over 12 months on average among the 8 windows. Half of these windows remain to be imaged to this day, and the rest were discontinued either followed by capillary rupture within image field or the deterioration of the health of the aged animals.

Figure 3-a presents transfected cells surrounding the initial virus injection site that can be identified throughout the field-of view of 1.3 mm x 1.3 mm. In this data set, estimates of cell number ranged from XX to XX with a relatively small variation among few randomly selected days between XX and XX.

### Direct Observation of Cranial Chamber

We periodically examined the imaging chamber condition in all implanted mice using a stereoscopic microscope. Degradation of the optical interface was found frequently in prototypes/procedures that preceded the one mentioned here. This was observed as progressive extension of a cloudy white inhomogeneous layer across the brain-facing surface of the optical insert. Using the design and procedures reported in this paper, this type of degradation rarely occurred, limited to the cases reported above in Batch 1.

Remarkably, but not unexpectedly, tissue growth surrounding the insert was evident in all cases, regardless of window clarity. The tissue appeared highly vascularized, and grew from the craniotomy edge outward along the chamber floor (figure 3-c). This growth is a natural response to the tissue damage inflicted by any craniotomy procedure. The difference observed here is only that the growth does not extend under the window. Instead, it forms a non-adhesive interface with the vertical walls of the transcranial columns and diverges upward into the aseptic chamber, replacing the agarose filling between the optical insert and the adhesive cement covering the skull and chamber floor. To further investigate the structure of granulation tissue growth into the peripheral chamber areas we detached the optical insert for unobstructed observation in several mice. An especially gnarly example from a 6-month duration window is pictured in figure 3-d.

### In vivo Tracking of Transplanted Stem Cells over Long Term

The ability to separate the installation procedure into Stage 1 and 2 was critical to capture the integration process of transplanted stem cells from day 4 and day 563 (figure 4). In addition to window installation, the experiment entails dissection of progenitor cells from E13.5 mouse embryos prior to transplantation which sequences require to be carried out in a timely manner. Tailored to this specific application, we performed Stage 1 of the installation procedure two days in advance, allowing time for tissue dissection immediately before conducting Stage 2 on day 0 (figure 4-b).

## Adaptability

* we then explored the ability of the cranial implant system to be adapted to provide greater utility
* building upon the original configuration, we adapted the system to accommodate an enlarged window, proving both complete coverage of dorsal cortex and similar overall functionality of the original system
* while much of the functional performance of the original system were realized by its physical structures, we customized existing features to meet the new design requirements and constraints for proper functioning
* we also incorporated new features to enhance system performance
* below, detailed technical descriptions associated with the reconfiguration

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* 1. the headplate and the window frame were scaled to integrate a larger window
* 2. raised rims were added to the bottom surface of the headplate where it contacts squamosal suture of mouse cranium to supplement skull-to-headplate attachment
  + to accommodate for the restricted attachment area due to the removal of a larger fraction of parietal and frontal bone
* 3. a thin skirt was added to the perimeter of the bottom surface of the optical insert which gently wraps around the intact tissue and prevents tissue ingrowth
  + in replacement of the vertical column
  + to accommodate for endocranium curves
* 4. incorporated nuts and bolts to facilitate attachment/detachment between the modules
  + in replacement of using dental cement
  + allows fine height adjustment, positioning, and installation of the window without the use of stereotaxic apparatus
  + added silicone coating to the chamber’s inner walls that form a seal with the optical insert upon its attachment
* 5. incorporated an auxiliary module that protects the integrity of the window using a magnetically-coupled protective cap
  + in replacement of tape

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* wide-field imaging of vasculatures
  + the mechanical barrier effectively blocks tissue growth (figure)
  + figure
* 2-photon imaging of transplanted cancer cells

# Discussion

**Opening paragraph (key concepts to include)**

* we developed a cranial window system for in vivo optical imaging in mice
* specifically, we developed a system that can provide sustained optical quality and tissue accessibility demonstrated through stem cell imaging
* the performance was achieve by using silicone elastomer to incorporate the critical features
* silicone-based window can easily incorporate features to be functional and provides good optical interface to record cellular activities through wide-field imaging
* device design, two part component window designed to block tissue growth
* performance, duration, access by taking off
* additionally, the reported system can be adapted for different applications demonstrated through the two versions
* special focus on searching for the critical features to achieve the desired performance without compromising the optical quality
* also easier to systematically test features and optimize towards fulfilling desired outcomes

The goal of this project has been to facilitate long-term studies requiring sustained optical access and intermittent physical access to the neocortex of intact brains in small animal research models – such as rats and mice. Specifically, we require a bilateral cortical windows suitable for wide-field imaging, and access to the underlying tissue for virus-mediated gene delivery and injection of exogenous labeled cells. We needed this access to be available as soon as possible post-installation, and for the optical quality to be sustained for several months. Experiment duration is limited using current window designs by progressive degradation of the optical light-path at the brain-to-window interface caused by highly scattering tissue growth. The device/system described here successfully fulfills the requirements of this objective, giving a stable and reliable optical window with unrestricted access for longer than one year.

**Main finding: sustained optical quality (Fig 3)**

* the implant effectively blocks the granulation tissue from growing within the image field, yielding a prolonged image duration often extending beyond a year
* critical elements that facilitate the maintenance of the long-term optical quality
  + system design features
    1. mechanical barrier (Ref)
    2. adjustable height

🡪 provides a better fitting to replace the removed bone flap

🡪 incorporating both methods may have a synergetic effect on impeding the tissue growth

🡪 observed divergence of granulation tissue (Ref)

* other factors
  1. anti-inflammatory drugs (Ref)
     + cortical steroid
     + NSAID
  2. air-tight seal within the chamber
     + specific to the system design
* all four elements are equally critical to achieving the sustained optical quality
* implementing the reported method may eliminate the primary reason for pre-terminating the image session due to tissue ingrowth
* the system can facilitate studies that require long-term observation such as aging or disease progression

Refer to the methods section for the specifics of surgical procedures for headplate installation and insert attachment. These procedures were established after testing the variable formulations in protocol. Of particular note, we found that administration of both a corticosteroid and a nonsteroidal anti-inflammatory drug had a substantial impact on the viability of the optical interface. Additionally, the silicone insert must be attached at the correct height, which must be determined by observation of the contact between the dura and the bottom surface of the insert. The insert must be depressed very slightly until full contact is made across the entire window, but pressing beyond necessary will quickly exert an undesired increase in intracranial pressure, increasing inflammation and adverse outcomes. Lastly, sealing the chamber is absolutely vital to the viability of the optical interface, as well as health of the animal. This includes a permanent seal between the chamber and skull, and a reversible seal between the chamber rim and optical insert. One must also ensure the agarose fill displaces all air within the chamber prior to sealing. Any remaining air pockets will be susceptible to bacteria growth and may disrupt normal intracranial and intermembrane pressures.

**Main finding: tissue accessibility (Fig 4)**

* the implant configuration consisting of two parts facilitates the installation procedure to be separable into distinctive stages carried out across days
* similarly, the reversed sequence can be performed for repeatable tissue access at later time points
* Ref: the process may be comparable to a previously reported method of removing the entire glass window to access the tissue
* here, however, we employed a faster and simpler method to reversibly detach and successively reattach the window without risking tissue damage compared to the laborious process required of glass windows
* the approach is advantageous over designating a fixated access port (Ref), providing full access without compromising the image field
* the practicality of separating the installation operations of a complete system has yet been explored
* the example of a comprehensive recording of the integration process of transplanted stem cells reported in this paper particularly well demonstrates the advantages of the ability
  + it may save time and resources – especially during the prototype stages – by allowing time to ensure each implanted animal fully recovers from the initial procedure
  + the delay allows normalization of the immune response or the heightened inflammation triggered by craniotomy before attempting a tissue intervention that is sensitive to these conditions (e.g. viral or cell injections)
  + the most remarkable advantage, however, is the ability to image the first tissue intervention from day 0

The ability to separate the operations necessary for installation of a complete system has a number of advantages, and is particularly well supported using the reported system. It may save time and resources – especially during the prototype stages – by allowing time to ensure each implanted animal fully recovers from the initial procedure. Additionally, the delay allows normalization of the immune response or the heightened inflammation triggered by craniotomy before attempting a tissue intervention that is sensitive to these conditions (e.g. viral or cell injections). The most remarkable advantage, however, is the ability to image the first tissue intervention from day 0.

**Main finding: adaptation (Fig 5)**

* the system framework is adaptable to accommodate various applications or to enhance its performance to reflect new technologies and demands
* here, we demonstrated the technical feasibility to future-proof the original system to adapt to continuously evolving image sensor technologies, allowing access to cellular interactions across multiple brain regions using wide-field imaging Ref
* as an inherent aspect of any design process, the adaptation of the original design evolved over the course of prototyping and testing to ensure consistent overall functional performance
* the iterative process, however, is much feasible with the major progresses of manufacturing and its increased versatility, providing better quality, customization, lower cost and shorter production time Ref
* in an effort to compare various manufacturing technologies, we explored manufacturing the finalized product design through a number of companies and advanced with 3D metal printing with overall satisfaction at i.materialise – we had also developed the parts through other rapid prototyping companies including Shapeways and Sculpteo
* additionally, various features and functions of the silicone insert were transformed and extended to conform to new design requirements, some requiring distinctively different design approaches
* we could benefit from the versatility of silicone elastomer to cover a spectrum of design strategies to optimize its configuration
* in fact, we found that a seemingly subtle physical difference can contribute to more than one function
* for example, the inclusion of a thin skirt extending below the optical insert, which was incorporated to provide protection against tissue growth within the image field, may also facilitate the brain to conform to the optical interface of the window over time resulting in a flat imaging plane, optimal for wide-field imaging, which was unachievable previously Ref
* overall, the design principles established from the initial development are robust and can be applied to new developments or refinements while preserving all qualities of the original implant
* the CAD designs of the reported systems are accessible in open source and can be modified and extended accordingly to the evolving demands and technologies
* We, the authors, are calling for replication, adaptation, evaluation (i.e. continued open / shared development).

**Further improvements**

* primarily explored the ability to mold precise and complex features using silicone elastomer to discover configurations to improve image performance
* encapsulate electrodes or optical guides Ref
* More significantly, the encapsulation of carbon, metal colloidal particles or quantum dots into polymer hydrogel networks will impart them with exclusive thermal, sonic, optical, electrical or magnetic properties
* the polymer interface may provide means to penetrate through directly Ref for electrophysiological recording or drug infusion allowing recording and/or manipulation during imaging session
* thickness of the window, chromatic aberration, wide-field and 2-photon imaging
* lenses
* As an innovative means to
* casting into practically any shape, size, or form
* embed/ Integrate
* new materials for fabrication

**Conclusion**

* here, we took an unique approach of using silicone elastomer to develop a cranial window system with the specific goals to build a system that performs reliable to provide long-term maintenance of optical quality and an easy and repeatable tissue access
* additionally, we prove the system can be easily adaptable for different applications
* the develop of such device will eliminate the physical barrier we have to probe the complexity of the brain – capability to facilitate longitudinal optical imaging experiment in mice

http://npi-med.com/news/top-5-reasons-consider-silicone-molding-medical-device-project/ - we used silicone elastomer to develop the window which provides versatility to add functions

# Materials and Methods

## Device development and fabrication

Components were designed using SolidWorks. Prototypes were fabricated using CamBam to generate toolpaths in G-code for machining on a CNC mill. The headplate and window frame were milled from aluminum plate. The mold for casting PDMS-based windows was designed in three parts. The middle component was milled from PTFE. The outer components were made using a laser-cutter and acrylic sheet (Supplementary). CAD files are shared on XXXXX(github?)XXXXX.

## Window casting procedure

The PDMS-based windows were fabricated through vacuum casting procedure. Prior to casting, window frames and two glass coverslips (Corning, 2947-75x38), prepared in advance through plasma etching (30 sec, Power setting) and silanization using Trichlorosilane (Sigma-Aldrich, 448931-10G), were inserted into the mold. The mold was then placed between two custom-made acrylic plates with silicone gaskets in between and was assembled using bolts around the perimeter. The pressure control port (McMaster-Carr, 5454K61) was connected to the house vacuum line, and the fill port (McMaster-Carr, 2844K11) was connected to uncured PDMS polymer (Dow Corning Sylgard 184) (1:10 by weight), thoroughly mixed and degassed in advance. The liquid-state polymer was then drawn into the mold filling the volume in between the two coverslips using vacuum. Once polymer displaced all air, vacuum was released and positive pressure was applied through the pressure control port after plugging the fill port. While maintaining positive pressure, the polymer was cured at 75ºC for 12 hours. Finally, the windows were released from the mold and trimmed using scalpels. Windows were handled so as to protect the top and bottom surfaces from damage or debris. The completed window was sterilized in an autoclave before use.

## Surgical procedures

Animal care for surgical procedures is described below, and the details specific to each procedure are in the sections that follow. All procedures were approved by the Institutional Animal Care and Use at Boston University. Stereotaxic surgeries were performed on 6 to 8 weeks old female C57BL/6 mice (Charles River Laboratories). Pre-operative care for the initial headplate and craniotomy procedure included subcutaneous administration of meloxicam (NSAID, 2.5 ug/g) and buprenorphine (opioid analgesics, 0.3 ug/g), and intramuscular injection of dexamethasone (corticosteroid, 5 ug/g) one hour before surgery. Meloxicam and buprenorphine were continued postoperatively every 12 hours for 48 hours. Meloxicam was also given before and after procedures where brain tissue was exposed, i.e. those for intracerebral injection and window replacement. For all procedures described below, mice were placed under general anesthesia with isoflurane mixed with oxygen.

## Headplate installation and craniotomy

We shaved the top of the mouse’s head and sterilized the skin using 70% alcohol and 7.5% Betadine. We made a 1cm midline sagittal incision through the scalp using surgical scissors, and retracted laterally using a self-retaining retractor (WPI, 501968). To prepare the cranial surface, we applied 3% hydrogen peroxide to oxidize and facilitate removal of periosteal tissue with cotton tip swabs. The surface was then marked up before headplate installation followed by craniotomy. We targeted laterally symmetric craniotomies with edge length 2.2 mm centered at coordinates ±1.83 mm lateral to sagittal suture and 1.00 mm anterior to bregma. First, we used a surgical skin marker (FST, 18000-30) to roughly indicate the site of each craniotomy and enhance contrast of the edges to be etched. We etched the corners and edges using a dental drill with a FG 1/4 round carbide burr (Fig. 2 Step 1(L)). This way of marking the edges facilitates headplate placement and also aids recovery of the intended craniotomy position despite being covered by semi-transparent adhesive cement in the following steps.

We used a custom stereotaxic attachment (Supplementary) to position the headplate symmetrically aligned with the marked sites, and to hold it horizontal while bonding to skull. The headplate was anchored directly to the skull using either opaque or semi-clear quick adhesive cement (Parkell, S380). Subsequently, we began each craniotomy by drilling along the marked edges (Fig. 2 Step 2(L)). We frequently stopped to flush debris from the site using sterile saline and an aspirator. Once separated from the surrounding skull, the bone flap was carefully removed using a pair of sharp forceps (FST, 91150-20) and a 45º micro probe (FST, 10066-15) while keeping the dura intact (Fig. 2 Step 1(R)). At this point, we either attached the optical window or sealed the area with a layer of non-toxic silicone adhesive (WPI, KWIK-SIL).

## Optical insert attachment

The optical insert attachment can be performed immediately following the craniotomy or deferred to the day of injection as described below (Fig. 2 Step 4). First, we filled the headplate chamber with sterile 0.5% agarose solution, immersing the exposed brain. Enough agarose was added so as to overflow the walls of the chamber as the window is inserted, ensuring no air gaps remain in the space between the walls of the chamber and the window, below the joint to be sealed. Next, the window was placed in the chamber, directly over the craniotomy, in gentle contact with the exposed tissue. We used a ttaxic apparatus to adjust the window height and secure its position during attachment. This was aided by an attachment – similar to that used for headplate installation – which fixed the angle of the window’s top surface parallel with that of the headplate. The height adjustment required depressing the window until full contact was observed between the inner window surface and the dura. The window was tacked in place by applying an accelerated light-cured composite (Pentron Clinic, Flow-It ALC) in at least three points, bonding the window frame to the anterior and posterior walls of the headplate. At this point the guide was removed and the joint was prepped for sealing. Excess agarose (polymerized overflow from the window insertion step) was aspirated away to expose and clean the headplate surface surrounding the window. The chamber was sealed by filling the joint between headplate and optical insert with dental cement (Stoelting, 51458) using a P200 pipette. The window surface was protected by applying a double layer adhesive strip made of gaffers tape over a transparent adhesive film dressing (3M: Tegaderm, 70200749201).

## Window detachment and replacement

The dental cement connecting the window and headplate was etched away using a dental drill. Before removing the window, we thoroughly flushed debris from the surrounding surfaces with sterile saline. Replacement windows were installed using the same procedures described above for initial attachment. Localizing the replacement window to the same position was aided by the expansion of granulation tissue up to the walls of the prior window.

## Injection

The exposed brain was flushed with sterile saline before and after each injection. Injections were made using pulled glass micropipettes with inner tip diameter ranging from 40 and 80 um (WPI, 504949). The micropipette was initially back-filled with mineral oil, then secured onto a microprocessor controlled injector (WPI, NANOLITER2010). The micropipette was then front-loaded with virus or cells using a controller (WPI, Sys-Micro4). In general, an injection of 230 nL of cells labeled with AAV9.CAG.GCaMP6f (AV-9-PV3081, Penn Vector Core) at 106 cells/uL, or 230 nL of AAV9.Syn.GCaMP6f (AV-9-PV2822, Penn Vector Core). Injection was performed approximately 500 um deep into the cortex at the rate of 46 nL/min near the center of the imaging field, while avoiding blood vessels to maximize the observable cells around the injection site (Fig. 2 Step 3). The micropipette was left to sit for an additional 2 min at the injection site before slow withdrawal.

## Wide-field in vivo imaging and microscope setup

Wide-field epifluorescence imaging was accomplished using a custom microscope equipped with a sCMOS camera (Hamamatsu, ORCA Flash 4.0XX), 470 nm LED (Thorlabs, M470L3), excitation and emission filters of XX and XX, a dichroic mirror (XX), and a 10x objective lens (Mitutoyo, 378-803-3). Mice were positioned under the microscope for imaging using a custom headplate holder (Supplementary) and allowed to run on an air-supported spherical treadmill18 as pictured in Figure 2 Step 5. The camera recorded a field-of-view of approximately 1.3 mm x 1.3 mm using an image resolution of 2048 x 2048 or more commonly 1024 x 1024. Continuous image sequences were acquired at 40 to 60 frames-per-second for 5 to 7 minutes. We selected the field to image within each site by roughly centering around the injection site. To focus the microscope on labeled cells in the superficial layers of cortex, we focused on the surface vasculature to find a stable reference, then advanced the focal plane 50 to 150 um until multiple cells were distinguishable. A reference image of the selected image was recorded for each site and used later to reacquire the same field across image sessions. Alignment to this reference image relied primarily on using the major blood vessels as landmarks to guide microscope position in the XY plane. Image sequences were stored for subsequent processing and analysis.

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# Figures

## Figure 1 Design, assembly and installation of the polymeric optical window.

(a) Schematic of the window system assembled and installed to the animal. Cross section shows the placement of the window as the bottom plane contacts the brain as it extends below the surface of the skull. Side view shows the adjustable height between the window and the headplate to compensate the cavity from skull removal. (b) Top and side view of the polymer window and the headplate. The window frame and the headplate are made of aluminum, and the window is molded in PDMS. Exploded view shows how the two pieces can be assembled. Front side of the headplate includes a feature that avoids hitting the eyes of the animal. (c) Top and (d) side view of the parts. (f) Parts assembled and installed to the animal.

## Figure 2 Flexible experimental timeline for surgery, injection and imaging.

Two-part assembly allows flexible timeline to separate the procedure of headplate installation and craniotomy from injection which helps with optical clarity during the early period imaging. On Day -2 which can extend upto Day -7, headplate installation and craniotomy is performed. Step 1 shows the placement of the headplate above the intended craniotomy region (before) and the fixation to the skull using opaque Metabond (after). After at least 48 hours of recovery period, injection and window installation is performed on Day 0. Step 3 shows injection using a micropipette filled with Evans Blue dye for demonstration only. Step 4 shows window installed above headplate using dental cement. Any cavity between the two pieces are filled with sterile 0.5% agarose. Lastly, imaging session can start from Day 4. Step 5 shows a mouse freely running on a treadmill during a imaging session while its head movement is restrained. The microscope is installed above the animal. Step 6 shows after window removal (before) that allows full tissue access, and a new window installed (after) that allows continuation of imaging over the same region.

## Figure 3 Extended optical clarity by biocompatible design of the system.

(a) The space between the window and the skull filled with agarose is progressively replaced by granulation tissue over time. At 6 month, granulation tissue surrounds the polymer window and vasculature formation is visible resembling a wound healing process. By 1 year, the intermediate space is filled with tissue embedded with vasculatures. The figures are from different animal that represent the corresponding time-point from the initially window installation - the window was installed using opaque Metabond on the samples for Day 0 and 1 year and semi-clear Metabond on the sample for 6 month. (b) Snapshots with the field-of-view of 1.3 x 1.3 mm were taken over the same regions on both hemispheres using the major blood vessels as landmarks (circled same regions). The optical clarity maintains over time to detect cellular dynamics of individual cells. Some GCaMP6f labeled cells are visible. In the left hemisphere, vascular remodelings are notable during the early period.