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Injection of hydrogel biomaterial scaffolds to the brain after stroke

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SHORT ABSTRACT:

Stroke is a global issue with minimal treatment options and no current clinical therapy for regenerating lost brain tissue. Here we describe methods for creating precise photothrombotic stroke in the motor cortex of rodents, as well as subsequent injection of hydrogel biomaterials to study their effects on tissue regeneration after stroke.

LONG ABSTRACT:

Stroke is the leading cause of disability and the fifth-leading cause of death in the United States. Approximately 87% of all strokes are ischemic strokes and are defined as the sudden blockage of a vessel supplying blood to the brain. Within minutes of the blockage, cells begin to die and result in irreparable tissue damage. Current therapeutic treatments focus on clot removal or lysis to allow reperfusion and prevent more severe brain damage. Although transient brain plasticity may salvage some of the damaged tissue over time, a significant fraction of patients are left with neurological deficits that will never resolve. There is a lack of therapeutic options to treat neurological deficits caused by stroke, emphasizing the need to develop new strategies to treat this growing patient population. Injectable biomaterials are currently being designed to enhance brain plasticity and improve endogenous repair through the delivery of active agents or stem cells. One method to test these approaches is to utilize a rodent stroke model, inject the biomaterial into the stroke core, and assess repair. Knowing the precise location of the stroke core is imperative for accurate treatment after stroke, therefore a stroke model that results in a predictable stroke location is preferable to avoid the need for imaging prior to injection. The following methods will cover how to induce a photothrombotic stroke, how to inject a hydrogel in a controlled and precise manner, and how to extract and cryosection the brain while keeping the biomaterial intact. In addition, we will highlight how these same hydrogel materials can be used for the co-delivery of stem cells. This protocol can be generalized to the use of other injectable biomaterials into the stroke core.

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DISCLOSURES:
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Keywords

Stroke; photothrombotic stroke; biomaterials; hydrogel; injectable hydrogels; hydrogel scaffolds; neurobiology; biomedical engineering

INTRODUCTION:

Stroke is the leading cause of disability and the fifth-leading cause of death in the United States[1]. Approximately 87% of all strokes are ischemic, while a majority of the remaining 13% are hemorrhagic[2]. An ischemic stroke is defined as the blockage of blood flow in an artery to the surrounding tissue. This occlusion results in oxygen deprivation and subsequent necrosis that often leads to permanent disability in surviving patients. While there has been a decrease in the mortality rate of stroke[3], its prevalence is expected to increase by 3.4 million people by 2030[4]. This increase in disabled survivors and consequent economic burden has led to a push for stroke research that focuses on mechanisms of neural repair. Following stroke there is an inflammatory period that leads to the formation of a scar that prevents the necrotic region from expanding. The region surrounding the necrotic core is termed “peri-infarct” and there is strong evidence that the plasticity in this region, which includes increased angiogenesis, neurogenesis, and axonal sprouting, is directly linked to the observed recovery in animal models and humans [5]. Since there are no *in vitro* models that can properly replicate the complex interactions following stroke, animal models are essential for stroke research.

There are several *in vivo* models that can be used to produce ischemic stroke. One of the most common models used in mice is middle cerebral artery occlusion, or MCAo, through distal or proximal (via intra-arterial filament) occlusion. The proximal model, also known as filament MCAo (fMCAo), typically result in large ischemic strokes that encompass anywhere from 5% to 50% of the cerebral hemisphere, dependent upon a number of factors[6]. In these models, a suture or filament is advanced from the internal carotid artery into the base of the middle cerebral artery (MCA) and kept in place for a specific period of time. This method for occlusion, which can be made temporary or permanent, produces an infarct that is centered in the striatum and may or may not involve overlying cortex[6]. The resulting stroke size is highly variable, and imaging techniques, such as laser doppler, are required to confirm the effectiveness of the procedure in each mouse. Intra-arterial or intra-luminal filament occlusion that lasts greater than 30 minutes produces strokes at the larger end of the size range. Some investigators have focused on shorter filament occlusion times, which require substantial experimental focus and lab validation[7]. Filament MCAo models in mice follow similar stages of cell death, ischemic progression, and formation of a peri-infarct region seen in human stroke cases; however, the larger strokes more closely resemble the disease state of malignant cerebral infarctions, which are less common, less treatable human strokes[6]. Meanwhile, distal MCA occlusion requires a more involved surgery and craniectomy. In this model, the distal part of the MCA that runs along the surface of the brain is directly occluded with a suture tie or cauterization. In some variations of the technique, the carotid arteries are unilaterally or transiently-bilaterally occluded. A benefit of the distal MCAo is that it produces a cortical-based stroke that is less variable in size

than the filament model. However, the distal model produces poorer behavioral output due to transection of the ECA, which is also a concern with fMCAo[6].

An alternative stroke model that is known for being less invasive is the photothrombotic (PT) model. The PT model results in a well-defined location of ischemia and is associated with a high survival rate[8]. The technique relies on a photosensitive dye injected intraperitoneally that allows for intravascular photo-oxidation simply by irradiating the desired tissue with a light or laser. Upon excitation, oxygen radicals are formed that cause endothelial damage, which activates platelet aggregation and clot formation in the irradiated area[8]. The tight control over stroke size and location, as well as high reproducibility of the PT model, makes it ideal for studying biomaterials. While precision is made possible through use of a laser and stereotaxic coordinates, there are some disadvantages that may make this model less ideal for particular studies. Unlike the fMCAo model, the PT stroke model is not able to be reperfused. Therefore, materials for investigating neuroprotective agents specific to damages following reperfusion or the mechanisms following reperfusion would not be useful here[8]. Additionally, due to the microvascular insult of the PT model, relatively little ischemic penumbra is seen. Instead, local vasogenic edema occurs, which is uncharacteristic for human stroke, making this model undesirable for preclinical drug studies focused on the peri-infarct area[6, 8].

The overall goal of biomaterial strategies in stroke is to either deliver bioactive agents or to act as a surrogate extracellular matrix for brain tissue growth. One strategy that we will explore using our methods is to deliver hydrogel directly into the stroke core, as opposed to the peri-infarct tissue where many current cell therapies are delivered[9]. The rationale for this approach is that delivery into the necrotic tissue found in the core will avoid disrupting the surrounding healthy or recovering tissue. We assume diffusion of any active agents included within the biomaterial will be able to reach the peri-infarct from the core, especially since we find that delivery of hydrogel biomaterials reduces the thickness of the glial scar[10]. This is important since the peri-infarct region has been shown to exhibit neuroplasticity after stroke, making it an attractive target. Furthermore, the delivery of a surrogate matrix to the stroke core can be loaded with angiogenic[11] or neurogenic[12] factors to guide the formation of new tissue, as well as cells for delivery[13]. Cell delivery is greatly enhanced by using a matrix because it protects cells from the harsh injection forces and local environment present during delivery, as well as encourages differentiation and engraftment[14].

These injectable therapeutic biomaterials have clinical relevance in stroke applications, as there are currently no medical therapies that stimulate neuronal recovery after stroke. The underlying neural circuits involved in recovery lie in brain tissue that is adjacent to the stroke core[15], while the stroke core itself is devoid of viable neural tissue. We anticipate that delivering a biomaterial into the necrotic stroke core has potential to stimulate the adjacent tissue toward regenerative processes through a number of mechanisms previously mentioned, including depot release of growth factors[12], stimulation of tissue in-growth and promotion of recovering brain tissue development[10, 11], alteration of immune responses[16], and delivery of stem cell-derived therapeutics[13, 17]. However, to effectively study the possibility of these applications, a consistent and reproducible method for inducing stroke and injecting biomaterials is needed. The PT stroke model uses techniques that offer precise

control over the orientation and location of the stroke. A laser attached to the stereotaxic device guides orientations, and pumps attached to the stereotaxic devices control the injection rate of the material without the need for additional forms of imaging. Therefore, we have chosen to describe the methods for performing a PT stroke in the motor cortexes of mice and for injecting biomaterials into the stroke core. Here, we use microporous annealed particle (MAP) hydrogels as the biomaterial for injection with no added cells or growth factors. Additionally, we explain how to successfully retrieve the brain with intact biomaterial, and we discuss immunochemistry assays used to analyze the stroke outcome with and without injection of biomaterials.

PROTOCOL:

Ethics statement

The experiments reported in this video were conducted in accordance with IUCAC at Duke University and University of California Los Angeles. 8 to 12-week-old male C57Bl/6J mice were used in this study. The animals were housed under controlled temperature (22 ± 2 °C), with a 12hr light-dark cycle period and access to pelleted food and water *ad libitum.* Analgesia and sedation protocols are described as approved by the IUCAC but might differ from protocols used in other laboratories.

1. Preparation of the Material and Instruments

1.1 Prepare photothrombotic dye prior to surgery. Weigh out 15 mg of Rose Bengal into a 2 mL Eppendorf tube. **CAUTION**: Rose Bengal can cause serious eye damage/irritation.

1.1.1 Dissolve the Rose Bengal in 1.5 mL of sterile filtered 1xPBS to make a working concentration of 10mg/mL.

1.1.2 Vortex the tube until no clumps are visible, indicating the dye has fully dissolved, then cover in foil until further use. **Note**: Amount of dye required will depend on the number of mice, where dye is injected IP at a concentration of 10 μL/g, e.g., 200 μL for a 20 g mouse.

1.2 Turn on heating pad in order to maintain the mouse body temperature during anesthesia (37°C).

1.3 Prepare autoclaved scissors, forceps, cotton, vet eye ointment, surgical glue or suture material, a blue or black surgical marker, and sterile alcohol and beta iodine wipes. Prepare bone drill with sterilized drill bit heads.

1.4 Turn on bead sterilization to 160°C to allow for sterilization of instruments between mice. Set aside a 50mL conical tube of sterile Saline or 1xPBS solution for later use in maintaining a hydrated operation area.

1.5 Attach the laser to the stereotaxic device. **CAUTION**: Use laser safety goggles whenever the laser is on following this point in the procedure. **Wearing laser safety goggles**, measure the power of the laser (mW).

1.5.1 Adjust the current on the laser console, following the laser manufacturing instructions, until the laser power output reads 10mW, and set the current as a pre-set on the home screen console. Then adjust the current again to establish another pre-set where the laser output power reads 40mW. Now turn the laser off until further use. **Note:** The 10 mW will be used to orient the laser prior to use.

1.6 Prepare a clean cage for mice after surgery. Then, place the mouse into the induction chamber with an isoflurane concentration of 4% to anesthetize it until spontaneous movement stops and its breathing comes to a slow, steady rate.

1.7 Once asleep, weigh the mouse to determine how much rose bengal dye to inject. Then, transfer and secure the mouse to the stereotaxic device with a maintenance isoflurane concentration of 1.5%. **Note:** Increasing isoflurane concentrations can dilate vessels and prevent sufficient occlusion or consistently-sized strokes between mice.

1.8 Apply vet eye ointment to both eyes. **Note:** watch breathing and temperature throughout surgical procedure and pinch toes occasionally to ensure the mouse cannot feel anything.

2. Photothrombotic Stroke Model

2.1 Shave the fur off of the mouse's head and aseptically prepare the area by using an alcohol wipe followed by a beta iodine wipe. Repeat three times. **Note:** If necessary, use an extra alcohol wipe at the end to clean off all beta iodine as it causes skin irritation which leads the mice to itch their skull after surgery.

2.2 Using small operation scissors, make a lateral incision between the ears to the eyes, about 1–2cm. **Note:** It is easiest to do this by pulling the skin upward with forceps to create a tent, cutting once downwards, then inserting the scissors into the opening and creating a continuous midline incision.

2.3 Separate the skin and press lightly on the parietal bones with the forceps to locate the bregma. Mark the bregma with a dot using the surgical marker. **Note:** Bone fragment movement highlights the frontal border of the parietal bones. The bregma is the center point at which the frontal and parietal bone fragments meet (Figure 1a).

2.4. **Wearing laser safety goggles**, move the laser over the skull of the mouse, fixing the stereotaxic device at a 90° angle. Select the 10 mW pre-set and turn on the laser.

2.4.1 Adjust the laser's x- and y-axis until it is directly over the bregma. Reset the x and y on the digital display console, and then move the laser **1.8 mm** left of the bregma. Now bring the laser as close to the skull as possible without letting it touch the skull.

2.4.2 Move the laser to **2.2 mm** left of the bregma to ensure that it can move without any obstructions. Turn off the laser.

2.5 Inject the appropriate amount of rose bengal dye intraperitoneally (IP) using a disposable 29G needle. Immediately start a timer for **7 min** to allow dye to circulate systemically. Select the pre-set for 40mW without turning the laser on. **Note:** Once comfortable with

the procedure, the dye can be injected before aseptic preparation of the mouse and finished before the 7 min are over to save time.

2.6 Turn on the laser (40mW) when the 7 min timer goes off, while **wearing laser safety goggles,** and set a **10 min timer**.

2.6.1 After 10 min, move the laser to 2.2mm left of the bregma without turning it off, and set **another 10 min timer**.

2.6.2 Once the second 10 min timer has gone off, change the laser back to 10mW and move the laser to **2.0 mm** left of the bregma. Mark this spot with the surgical marker. Then turn off the laser.

2.6.3 Lift the laser and unlock it from the 90° angle so it can be moved away from the mouse. At this point, apply the sterile Saline or PBS with cotton if the surgical area is dry.

2.7 Drill in small bursts at the 2.0 mm mark perpendicular to the surface of the skull. **Note:** Be careful to drill slowly to avoid drilling too deeply and hitting the brain. There will be a slight drop/change in resistance once the drill has gone through the skull, and drilling may cause bleeding.

2.7.1 Use cotton to absorb any excess blood. **Note:** It is typical to see some blood from nicked arteries in the skull after drilling – excessive blood means the drill bit hit the brain. If this happens, record the mouse identifier and move on.

2.8 Place a small wipe next to the mouse. Using the forceps, pull the skin close together to prepare for gluing. **Note:** Suturing can be done here instead. Suturing typically requires only 3 sutures.

2.8.1 Using the forceps, grab the two sides of the skin and pull them together and upward. Dab off any large drops of surgical glue at the end of the tip on the wipe before applying to the mouse.

2.8.2 Apply the surgical glue sparingly and hold skin together with forceps for 10 seconds. Continue this method until there are no visible openings. **Note:** The glue comes out quickly – do not squeeze the bottle. Avoid gluing the skin to the skull or getting glue in the eye.

2.9 After gluing, or suturing, place the mouse in the new cage to recover from anesthesia. **Note:** It takes 5–10 min for the animal to recover, and it helps to rest half of the cage on a heating pad.

3. Sham Operation

Perform all procedures identically to the operation described above except inject 1xPBS or Saline instead of rose bengal dye.

4. Injection of hydrogel or other biomaterials

Injection of biomaterials are performed at a user-defined time. We have performed injections between 5- and 7-days post-stroke. 2 – 6 μL of hydrogel are injected (user-defined).

4.1 Turn on heating pad in order to maintain the mouse body temperature during anesthesia (37°C).

4.1.2 Prepare autoclaved scissors, forceps, cotton, vet eye ointment, surgical glue or suture material, and sterile alcohol and beta iodine wipes. Prepare the bone drill with sterile drill bit heads and the 25 μL glass Hamilton syringes with metal 30G needles.

4.1.3 Turn on bead sterilization to 160°C to allow for sterilization of instruments between mice.

4.1.4 Attach the injection pumps to the stereotaxic device. Set flow rate to 1 μL/min and set volume to 4 μL.

4.1.5 Prepare a clean cage for the mice after surgery.

4.2 Place the mouse into the induction chamber with an isoflurane concentration of 4% to anesthetize it until spontaneous movement stops and its breathing comes to a slow, steady rate.

4.2.1 Once asleep, transfer and secure the mouse to the stereotaxic device with a maintenance isoflurane concentration of 1.5%. Apply vet eye ointment to both eyes.

4.2.3 Shave any fur that may have regrown of the mouse's head, and aseptically prepare the area by using an alcohol wipe followed by a beta iodine wipe. Repeat three times. **Note:** If necessary, use an extra alcohol wipe at the end to clean off all the beta iodine as it causes skin irritation and leads the mice to itch their skull after surgery.

4.3 Using the small scissors and/or forceps, reopen the skin above the brain. Use cotton with sterile saline or PBS to clean any debris from the skull. **Note:** A white-yellow circle (the stroke, Figure 1b) and the burr hole from the drill should be visible. If the dead tissue is not visible, likely no stroke occurred. If the burr hole is not centered above the stroke, re-drill to center it.

4.4 Orient the injection pump over the mouse and lock at 90°. Clean the syringe with sterile saline or PBS solution before backloading material.

4.4.1 Once clean, disassemble the glass syringe so that there is no needle. Backload the syringe using a 25 μL positive displacement pipette with the 10 μL of hydrogel (or other biomaterial here with or without cells).

4.4.2 Using the syringe plunger, push the gel all the way to the front of the syringe. Then, reassemble the syringe and continue pushing until the gel visibly exudes from the needle.

4.5 Place the syringe onto the pump. **Note:** The back of the pump may need to be adjusted so the syringe fits. Do this by adjusting the flow rate to 30 uL/min, select WITHDRAWAL or INJECT depending on the direction it needs to move, and then press START. Hit STOP when the back of the pump is at the correct location.

4.5.1 Screw the back of the pump so the syringe is secure. If adjustments were previously made, make sure the flow rate is set back to 1 μL/min and is set to inject.

4.6. Move the pump in the x and y direction to orient it over the hole. Move the pump down in the z direction until the needle of the syringe touches the top of the hole.

4.7 Reset the z on the digital display console. Then, move the syringe down in the z direction **0.750 mm. Note:** If the syringe bends or there is resistance, the skull either healed or was not completely drilled through and needs to be re-drilled.

4.8 Press START on the pump console to inject 4 – 6 μL of the hydrogel at a rate of 1 μL/min.

4.9 Set a 5 min timer when the pump stops injecting to allow the gel to begin crosslinking.

4.10 After 5 min, slowly pull up the syringe by turning the z nob. Watch to see if any material is accidently pulled or leaking from the hole. Unlock the pump and move it away from the mouse when the needle is far enough away from the skull.

4.11. Using forceps, surgical glue, or sutures, close the skin above the head. Place the mouse in the clean cage and observe its recovery. It should take about 5 – 10 min for the mouse to recover from anesthesia.

5. Perfusion

5.1 Anesthetize the mouse using isoflurane.

5.2 Fix the animal in the supine position and open the abdominal cavity with a median cut. Optional: Clamp the descending abdominal aorta to use less fixative and PBS.

5.3 Cut open the diaphragm and move up the sides of the rib cage to open the chest cavity. Insert a perfusion cannula into the left ventricle and cut an opening in the right atrium. Start to slowly perfuse with 10–20 mL of PBS or until the liquid runs semi-clear.

5.4 Once clear, switch to 4% PFA for another 10 – 20 mL. Note: Unpinning the arms allows them to contract as the PFA infuses. Once they stop moving, wait another 30 seconds.

5.5 Decapitate the animal and pull back the skin to expose the skull. Using blunt, thin forceps, carefully remove the pieces of the skull to expose the brain. **Note:** Special care needs to be taken when removing the skull above the stroke site. Small surgical scissors can be used here to help cut away the skull. **The biggest challenge is gel getting stuck to the skull and coming out of the brain as the skull is removed.**

5.6 Once the brain is detached, place in 10 – 15 mL of 4% PFA overnight (no longer than 24 hrs).

5.7 Move the brains from PFA to 30% sucrose the following day. **Note:** The brain is ready for cryosection once it sinks to the bottom (about 2 – 3 days). It can also be left for storage at this point.

6. Cryosectioning and staining

6.1 Take the brain out of sucrose and place on a glass slide. Cut a small portion of the back of the brain off with a razor blade to create a flat portion to be mounted onto a chuck.

6.1.1 Place a dollop of Optimal Cutting Temperature Compound (OCT) onto the chuck, and then place the brain, flat side down, onto the chuck in the OCT. Place this specimen on dry ice until frozen or in the cryostat on the freezing block. **Note:** OCT can be added to fully encompass the brain to help prevent the material from separating from the brain during sectioning.

6.2 Cut the brains serially on a cryostat at −20°C at 30 μm thick sections across 10 gelatin coated slides. **Note:** Take care when sectioning so as not to lose the hydrogel. If this part is difficult, place some OCT on the top of the brain over the gel (Figure 2). Store at −80°C until further use.

6.3 Take slides out of the −80°C and place on a staining tray to allow them to dry. **Note:** Slides that have not be frozen but just sectioned can also be stained immediately.

6.4 Using a hydrophobic pen, draw a circle around the brain slices on the slide. Once dry, rehydrate the brain slices with 1x filtered PBS and place on a belly dancer at room temperature for 15 min. **Note:** This takes about 1 mL of buffer per slide with 9 – 12 brain sections.

6.5 Wash the slides with 1x filtered PBS for 5 min at room temperature, repeat three times.

6.6 Block slides for 30 min to an hour with 10% donkey serum in 0.01% PBS-Triton X at room temperature on the belly dancer. **Note:** This takes about 200 μL per slide.

6.7 Incubate the primary antibody in 10% donkey serum with 0.01% PBS-Triton X overnight at 4°C on a shaker or belly dancer. Test primaries first to determine correct concentrations. **Note:** Here we used GFAP (1:400), Iba1 (1:250), Glut1 (1:500) (Figure 3).

6.8 Wash slides with 1x filtered PBS the following day for 5 min at room temperature, repeat three times.

6.9 Incubate with secondary antibody in 10% donkey serum in 0.01% PBS-Triton X and DAPI for 2 hrs on the belly dancer at room temperature. **Note:** 1:1000 was used for all secondaries and DAPI.

6.10 Wash the slides for 5 min with 1x filtered PBS.

6.11 Allow sections to dry at room temperature in the dark. **Note:** This can take 20 min to 4 hrs; placing the slides in a desiccator can speed up the process.

6.12 Dehydrate slides in ascending concentrations of alcohol (1 min per solution) of 50%, 70%, 95%, 100%, and 100%.

6.13 De-fat in first bath of xylene for 1 min, then second bath of xylene for 5 min.

6.14 Quickly take the slides out one-by-one and add mounting medium while brain sections are wet with xylene. Quickly add a glass coverslip on top. **Note:** Use a cover slip that is correct length and thickness needed for the slide and microscope used for imaging, respectively.

6.15 Get rid of any bubbles under the coverslip by pressing gently with a pipette tip in an outward motion from the center of the slide. Allow DPX to dry for 4 hrs to overnight in the dark at room temperature.

6.16 Use a razor blade to scrape off any dried mounting medium that spilled onto the slides. Wipe down slides with lens cleaner. Slides can now be imaged with fluorescence microscopy. **Note:** It is best to store slide in the dark or at 4°C until imaging is finished.

DISCUSSION:

Here we demonstrate an easily reproducible, minimally invasive, permanent stroke model and describe how to inject a biomaterial into the infarct five days after stroke. The use of the photothrombotic dye rose bengal and a 520 nm collimated laser connected to the stereotaxic device gives us the ability to position the stroke at the motor cortex of the mouse with enhanced precision. Five days after stroke, the location of the infarct is visible by eye at the center of irradiation, 2.0 mm medio-lateral to the bregma. Hydrogel is then accurately injected into the stroke core using syringe pumps. Mortality during the surgery and after stroke in this model is virtually absent, with only a handful of mice dying after surgery due to anesthesiological complications. Despite the advantages of using PT stroke for biomaterial applications, there are several biological limitations and technical difficulties that should be addressed.

In terms of biological limitations, PT is a not a stroke model that offers the option for cerebral reperfusion, a process that can occur spontaneously in human strokes or in response to endovascular clot retrieval/lysis. Additionally, unlike the MCAo model that mimics the etiology of most human strokes by occluding a major cerebral vessel, PT impairs blood flow through many smaller vessels. A technical difficulty of injecting hydrogel biomaterials is their tendency to adhere to the syringe during surgery or to the skull during extractions. Designing a hydrogel that remains as a liquid during injection and then gels *in situ* can address the first issue; however, preventing the gel from sticking to the dura mater is often unavoidable since the injection site and stroke core reside at the surface of the brain. This can make it difficult to extract the brain tissue while keeping the biomaterial intact. As such, special care must be taken when removing pieces of skull from the brain to ensure that the dura mater does not lift with the skull and unintentionally uproot the gel.

Cryosectioning brains also requires technical skill because both the handling process and blade transection can easily detach the gel from its surrounding tissue, thereby limiting data collection regarding cell infiltration. Detachment is exacerbated when the material is not fully integrated with the tissue. Preparing the sample with OCT creates a reinforcement shell that helps to minimize material separation during cutting.

Before injecting biomaterials, we recommend optimizing the desired stroke size depending upon rodent strain and laboratory setup. Smaller or larger infarcts can be made by adjusting three main parameters: laser output intensity, beam magnification, and irradiation time. Other factors that have also been shown to affect stroke size include concentration of isoflurane[18] and amount of time the dye is allowed to circulate prior to irradiation. Regarding laser output intensity (power/area or mW/cm^2), we found that a laser power of 10 mW was sufficient to produce a small infarct with horizontal diameter slightly smaller to that of the laser beam. Higher laser power yielded marginally wider but substantially deeper infarcts (data not shown) due to the laser beam having a gaussian irradiation profile. By increasing the intensity, the center point of maximum irradiance can penetrate deeper past the skull, as well as cause the slight increase in the spot size penetration of the laser. Other stroke models have thinned the skull by sanding it prior to applying light or laser in order to increase light penetration and subsequent reproducibility of clotting while maintaining a lower laser intensity[19]; however, this can cause hemorrhaging in the skull and takes a fair amount of time during surgery and skill to master without accidently damaging the brain in the process. Similar to intensity, increasing the laser beam diameter also increased infarct size; however, laser intensity and beam diameter do not scale linearly with respect to one another but follow the equation, $power\ density = \frac{laser\ power\ (W)}{\pi r^2}$, where r is the radius of the beam. In terms of irradiation time, we found that a minimum of 10 min was needed to yield reproducible strokes. To increase the horizontal diameter of the infarct while keeping the vertical depth constant, we applied the laser to adjacent locations for 10 min each, sequentially. To address the influence of isoflurane concentration and circulation time of rose bengal, we found that 1.5% isoflurane concentration and 7 min of circulation after IP injection resulted in consistent formation of strokes. These five parameters were optimized for our particular use. We required a large enough stroke to cause behavioral changes yet not interfere with the subventricular zone, where we aimed to study the effect of our material on neural progenitor cells. In addition to stroke size, we also needed to determine the optimal gel volume to inject. At minimum, we needed enough gel to entirely fill the stroke cavity because we found that gaps between gel and surrounding tissue led to similar results as our control groups, while contact between gel and tissue resulted in fewer reactive astrocytes and increased cellular infiltration. We arrived at a lower limit of 4–6 μL of gel, which produced no adverse effects after injected (data not shown).

Regarding assessing stroke outcome, there are several options beyond IHC staining, such as magnetic resonance imaging, behavioral tests, histological analysis, and TTC (2,3,5-triphenyltetrazolium chloride) staining. It is highly recommended to use TTC for preliminary optimization of stroke due to its ease of use and immediate results. Previous behavioral testing in our lab has looked at the Cylinder test/spontaneous forelimb task, the grid-walking test, and the pasta test[11, 20]. It should be noted that rotarod testing did not show

significant decreases in behavioral outcomes after the PT stroke method was implemented (data not shown). Thus, behavioral tests should assess very complex tasks that require significant cortical input.

Here we demonstrated the technique of injecting hydrogels after stroke without the delivery of growth factors or cells. However, hydrogels have also been utilized for cell transplantation, as well as drug and growth factor delivery, and may prove to be a valuable resource for both studying biology after stroke and investigating new methods of therapy. In the context of stroke, our lab has injected non-porous hyaluronic acid hydrogels encapsulating induced pluripotent stem cell-derived neural progenitor cells[13, 17] at concentrations ranging from 25,000 cells/μL to 50,000 cells/μL of gel. The use of a hydrogel resulted in an increase in cell viability and differentiation. Other labs have also reported cell differentiation and increased tissue regeneration in response to hydrogels used to transplant stem cells after stroke [21–23]. Additionally, we have injected hydrogels with growth factors after stroke that lead to tissue regeneration and behavioral improvement after stroke[12, 13]. In terms of material design, injectability is a valuable feature that serves to minimize invasiveness; therefore, hydrogels should be formulated as either sol-gels (liquid-to-solid), shear thinning (G' > G" under static conditions; G" > G' during flow), or granular gels comprised of building blocks whose diameter is less than the inner diameter of a needle[11, 24, 25]. Optimization should then be performed to test diffusion of drugs or growth factors, cell viability under a range of cell concentrations, gel conditions, and injection parameters, such as speed, needle gauge, injection depth, and day of injection relative to when stroke was induced. For example, day of material injection has ranged from one day post-stroke to as long as three weeks post-stroke[26, 27]. Here, we report five days but have previously injected seven days after stroke when transplanting cells. These days were chosen based on the timeline of the inflammatory response as well as the peak activity of angiogenesis and neurogenesis seen one week after stroke[5, 28–30]. Volume characterization should also be performed at every injection time point, as the stroke volume will decrease over time due to atrophy. Given that these parameters are optimized prior to injection, the methods here are sufficient to guide users to inject biomaterials with the addition of cells and/or growth factors and drugs.

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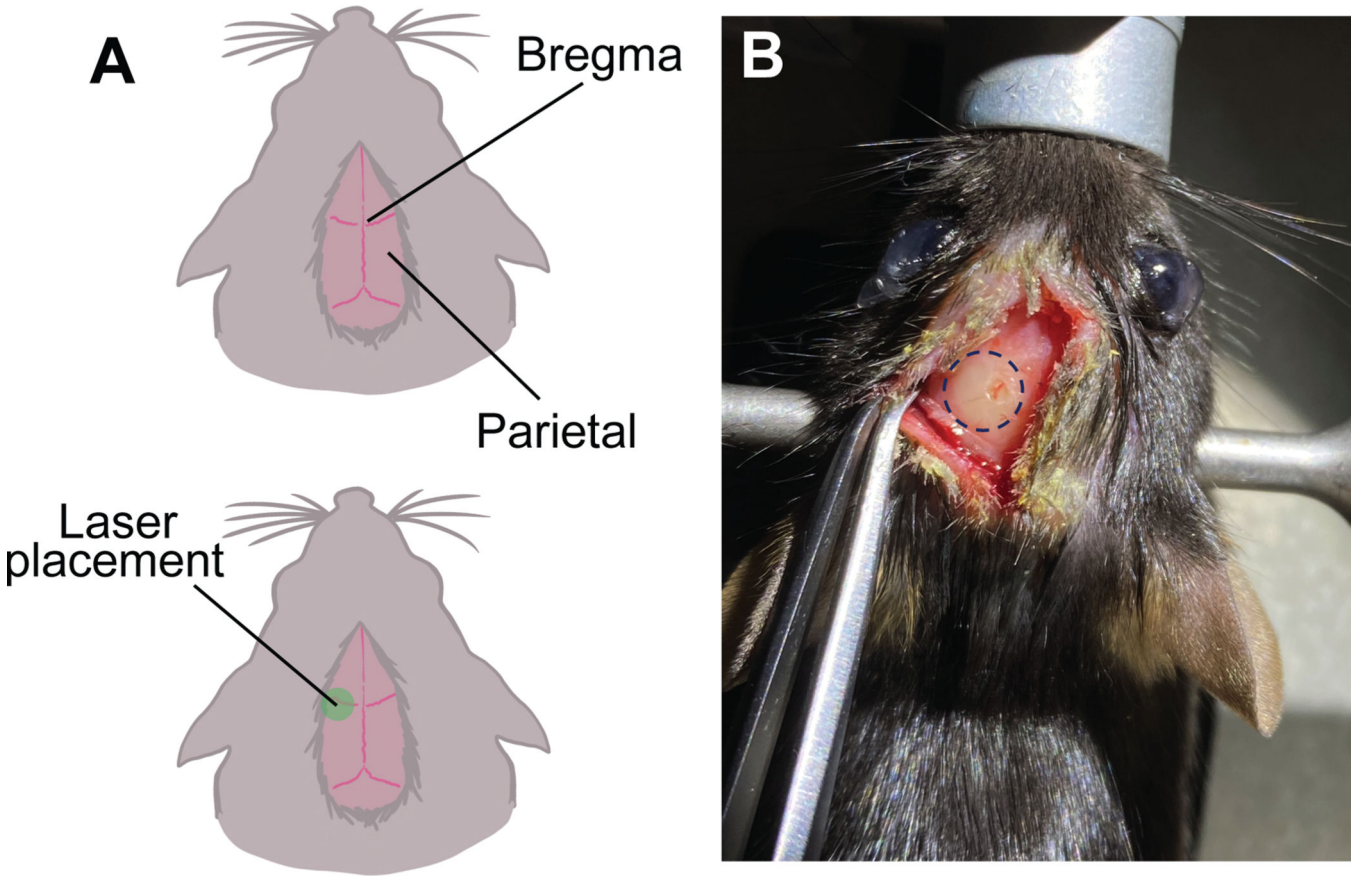

Figure 1. Schematic representation of bregma and localization of stroke.

a. The parietal lobes meet at the top to form the bregma. The laser placement is centered 2.0mm left of the bregma. b. Visual conformation of stroke and burr hole can be seen 5 days after stroke.

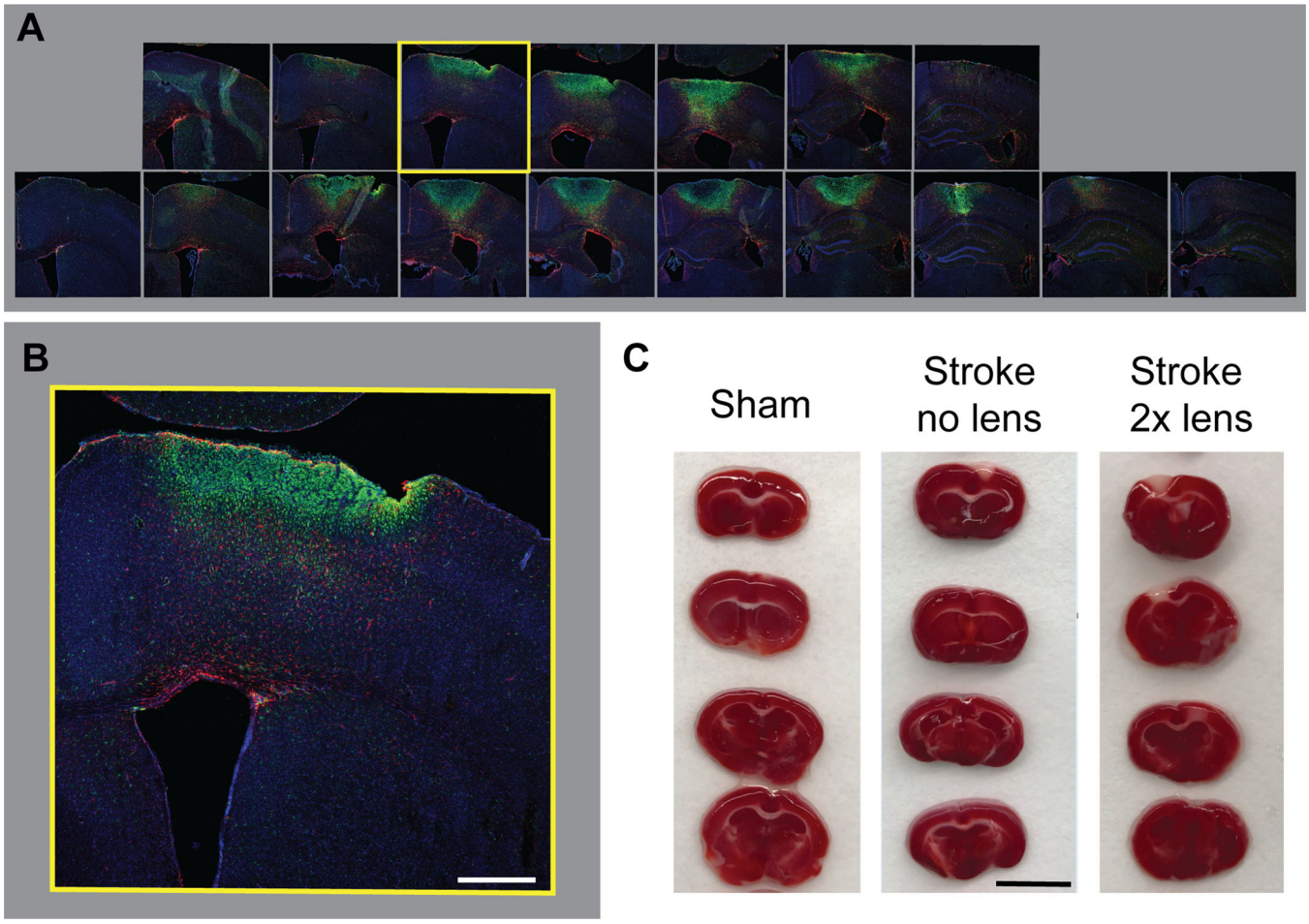

Figure 2. Injection and visualization of biomaterial.

a. Visual of gel injection during surgery. b. The gel can be visualized by eye when removing the skull. c. Once frozen and mounted, the gel will be visible in the brain while sectioning.

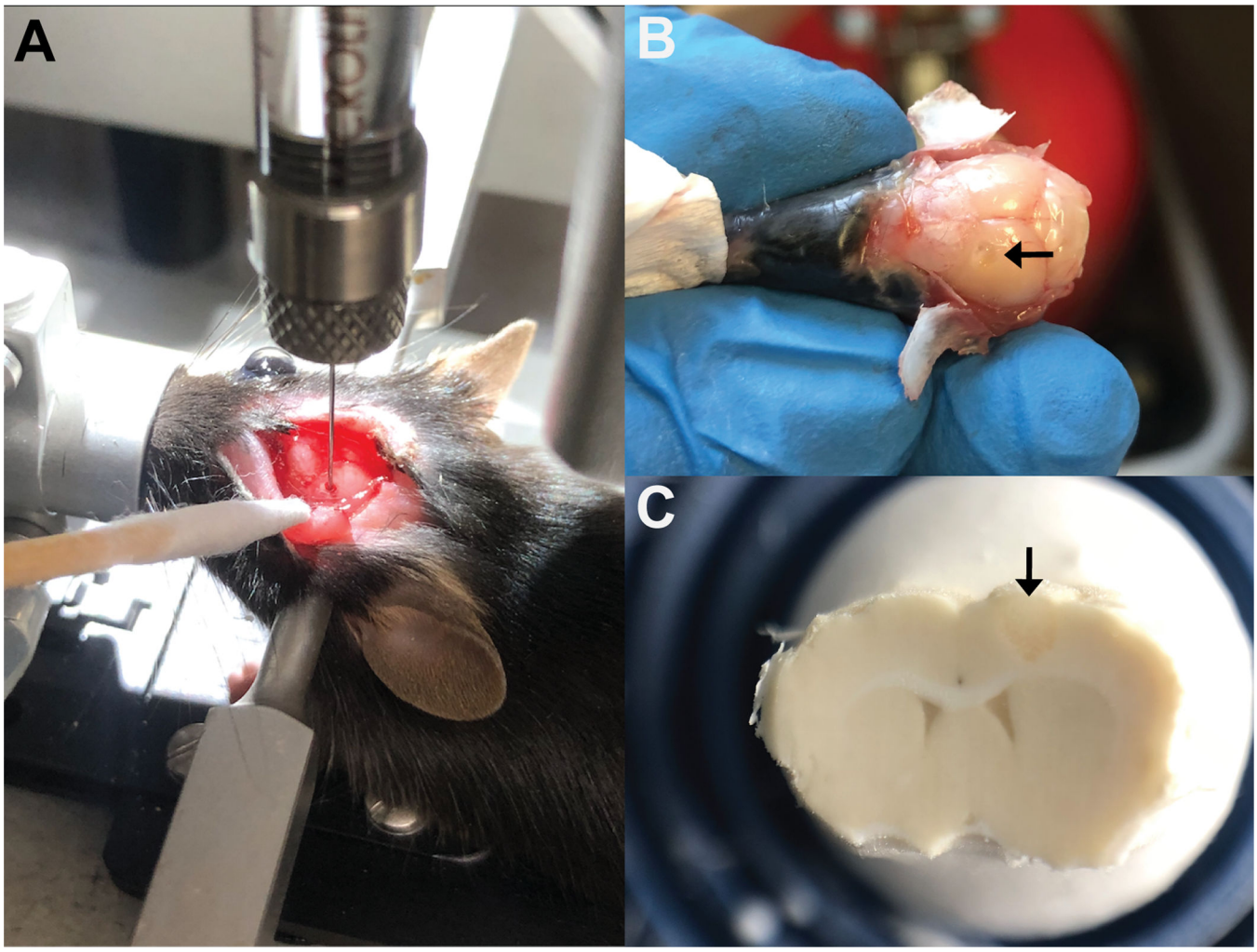

Figure 3. Immunohistochemistry stains.

a. Schematic of cryosectioning brains 30um thick. Several sections in the middle are chosen for image analysis. b. Stroke only, immunohistochemistry (IHC) stains 15 days after stroke. Astrocytes (GFAP+) cells are shown in red, microglia (Iba1+) are green, and vessels (GLUT1+) are white. Stroke + Hydrogel condition, IHC 10d post injection, 15 days after stroke. Astrocytes (GFAP+) cells are shown in red, microglia (Iba1+) are green, hydrogel material is white.

Name	Company	Catalog Number	Comments
10% Normal Goat Serum	VWR	100504–028	For blocking buffer
2-ply alcohol pre pad, Sterile, Medium	Medline	MDS090735	
25uL Hamilton Syringe 702RN, no needle	Fishcer Scientific	14824663	Syringes used to inject biomaterials
25uL Positive displacement pipette	Gilson	M-25	
2x Beam Expander, 400–650nm	Thorlabs	GBE02-A	Laser beam expander
Adjustable Stage Platform	Kopf Instruments	901	
Anti-Glucose Transporter GLUT1 antibody, rabbit	Abcam	ab113435	
Anti-Iba1 Antibody, goat	abcam	ab5076	
BD Vacutainer Safety-Lok Blood Collection Sets. 25G, 12”	Medsupply	367294	For perfusions
BKF12- Matte Black Aluminum Foil	Thorlabs	BKF12	To cover anything that is reflective when using laser.
Bone Iris Mini Scissors - 3-1/2”	Sklar surgical instruments	64-2035	
C57BL/6 Mice	Jackson Laboratory	000664	8-12 weeks of age
Cage Assembly Rob	Thorlabs	ER3-P4	3” Long, diameter 6mm, 4 pack - for attaching laser to sterotax
Carbon Steel Burrs −0.5mm Diameter	Fine Science Tools	19007-05	For creating burr hole
Chromium(III) potassium sulfate dodecahydrate	VWR	EM1.01036.0250	
Compact Controller for pigtailed lasers	Thorlabs	CLD1010LP	
Cotton Swabs	VWR	89031-288	
CP 25 pipette tips	Gilson	F148012	
Donkey anti-goat IgG H&L (488)	abcam	ab150129	
Donkey Anti-rabbit IgG H&L (647)	abcam	ab150075	
Donkey Anti-rat IgG H&L (555)	abcam	ab150154	
EMS DPX Mountant	Elecron Microscopy Sciences	13512	Mounting solution for slides
EMS Gelatin Powder Type A 300 Bloom	Electron Microscopy Sciences	16564	For gelatin coating slides
EMS Paraformaldehyde, Granular	VWR	100504-162	For making 45 PFA
ESD Worstation kit	Elmstat	WSKK5324SB	Need for setting up the laser
Fiber Bench Wall Plate, unthreaded	Thorlabs	HCA3	Need for connecting laser to Kopf shaft
FiberPort	Thorlabs	PAF-X-5-A	FC/APC& APC, f=4.6mm, 350–700nm, diameter 0.75mm
Fine Scissors - straight/sharp-blunt/10cm	Fine Science Tools	14028-10	
GFAP Antibody, rat	Thermo Fisher Scientific	13-0300	
Heating Plate	Kopf Instruments	HP-4M	
ImmEdge Hydrophobic Barrier Pen	Vector Laboratories	H-4000	For staining slides
IMPAC 6-Integrated Multi Patient Anesthesia Center	VetEquip	901808	
Iodine Prep Pads	Medx Supple	MED MDS093917H	

Name	Company	Catalog Number	Comments
Jewlers Forceps #5	GFS chemicals	46085	
Laser Safety Glasses	Thorlabs	LG10B	Amber Lenses, 35% Visible light (googles versions available too)
M27-1084 Powerful LED Dual Goose-neck	United Scope	LED-11C	
Medical USP Grade Oxygen	Airgas	OX USP250	
Miltex Adson Dressing Forceps, Disecting-grade	Intefra Miltex	V96-118	
Mini Cord/Cordless Small Animal Trimmer	Harvard aparatus	72-6110	
Mini-pump variable flow	Thomas Scientific	70730-064	Pump for perfusions
Mouse Brain Matrices, Coronal Slices, 1mm	Kent Scientific	RBMA-200c	For TTC slices
Mouse Gas Anethesia Head Holder	Kopf Instruments	923-B	
Nanojet Control Box	Chemyx	10050	
Nanojet pump header	Chemxy	10051	Attach to stereotaxic device for injecting biomaterial
Needle RN 30G PT STY 3, 0.5 inch	Fishcer Scientific	NC9459562	
Non-rupture ear bars 60º	Kopf Instruments	922	
PBS buffer pH 7.4	VWR	97062 338	
Pigtaled laser 520 nm, 100mW, 5G Pin	Thorlabs	LP520-MF100	
Positive charge glass slides	Hareta	AHS90-WH	
Power engergy meter	Thorlabs	PM100D	Used to measure your mW laser output
Puralube Vet Ointment	Dr. Foster Smith	9N-76855	
Rectal Probe Mouse	kopf Instruments	Ret-3-ISO	
Rose Bengal Dye 95%	Sigma-Aldrich	330000-5G	
Shaft Modified 8–32 threaded hole 1/2” depth	Kopf Instruments	1770-02	For connecting laser to sterotaxic device
Slim photodiode power sensor	Thorlabs	S130VC	Used with power energy meter
SM1-Threaed 30 mm Cage Plate 0.35" thick 2 Retaining	Thorlabs	CP02	For connecting laser expander
Sol-M U-100 Insuline syringe with 1/2 unit markings 0.5 mL	VWR	10002-726	To inject rose bengal
StainTray Slide Staining System	Simport Scientific	M920-2	For staining slides
Sterotaxic device	Kopf Instruments	940	Small Animal Stereotaxic Instrument
Student Adson Forceps −1×2 teeth	Fine Science Tools	91127-12	
Student Fine Forceps - straight/broad Shanks	Fine Science Tools	91113-10	
Temperature Controller	Kopf Instruments	TCAT-2LV	
Tissue-Tek OCT compound	VWR	25608-930	
Triton X-100	VWR	97063-864	
Upper Bracket Clamp	Kopf Instruments	1770-c	For connecting laser to sterotaxic device
Vetbond Tissue Adhessive 3mL	Santa Cruz Biotechnology	sc-361931	
Vogue Professional My Manicurist	Bargin Source	6400	For Burrs

Name	Company	Catalog Number	Comments
VWR Bead Sterilizers	VWR	75999-328	
Tissue Tek OCT compound	Sakura	4583	For tissue embeding