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Anterior Segment Organ Culture Platform for Tracking Open Globe Injuries and Therapeutic Performance

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TITLE:

Anterior Segment Organ Culture Platform for Tracking Open Globe Injuries and Therapeutic Performance

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SUMMARY:

Open globe eye injuries may go untreated for multiple days in rural or military-relevant scenarios, resulting in blindness. Therapeutics are needed to minimize loss of vision. Here, we detail an organ culture open globe injury model. With this model, potential therapeutics for stabilizing these injuries can be properly evaluated.

ABSTRACT:

Open globe injuries have poor visual outcomes, often resulting in permanent loss of vision. This is partly due to an extended delay between injury and medical intervention in rural environments and military medicine applications where ophthalmic care is not readily available. Untreated injuries are susceptible to infection after the eye has lost its watertight seal, as well as loss of tissue viability due to intraocular hypotension. Therapeutics to temporarily seal open globe injuries, if properly developed, may be able to restore IOP and prevent infection until proper ophthalmic care is possible. To facilitate product development, detailed here is the use of an anterior segment organ culture open globe injury platform for tracking therapeutic performance for at least 72 h post-injury. Porcine anterior segment tissue can be maintained in custom-designed organ culture dishes and held at physiological intraocular pressure. Puncture injuries can be created with a pneumatic-powered system capable of generating injury sizes up to 4.5 mm in diameter, similar to military-relevant injury sizes. Loss of intraocular pressure can be observed for 72 h post-injury confirming proper injury induction and loss of the eye's watertight seal. Therapeutic performance can be tracked by application to the eye after injury induction and then tracking intraocular pressure for multiple days. Further, the anterior segment injury model is applicable to widely used methods for functionally and biologically tracking anterior segment physiology, such as assessing transparency, ocular mechanics, corneal epithelium health, and tissue viability. Overall, the method described here is a necessary next step toward developing

biomaterial therapeutics for temporarily sealing open globe injuries when ophthalmic care is not readily available.

INTRODUCTION:

Open globe (OG) injuries can result in permanent loss of vision when not treated or at least stabilized following injury[1]. Delays, however, are prevalent in remote areas where access to ophthalmic intervention is not readily available, such as in rural areas or on the battlefield in military scenarios. When treatment is not readily available, the current standard of care is to protect the eye with a rigid shield until medical intervention is possible. In military medicine, this delay is currently up to 24 h, but it is anticipated to increase up to 72 h in future combat operations in urban environments where air evacuation is not possible[2–4]. These delays can be even longer in rural, remote civilian applications where access to ophthalmic intervention is limited[5,6]. An untreated OG injury is highly susceptible to infection and loss of intraocular pressure (IOP) due to the watertight seal of the eye being compromised[7,8]. Loss of IOP can impact tissue viability, making any medical intervention unlikely to restore vision if the delay between injury and therapeutic is too long[9].

To enable the development of easy-to-apply therapeutics for sealing OG injuries until an ophthalmic specialist can be reached, a benchtop OG injury model was previously developed[10,11]. With this model, high-speed injuries were created in whole porcine eyes while IOP was captured by pressure transducers. Therapeutics can then be applied to assess their ability to seal the OG injury site[12]. However, as this model uses whole porcine eyes, it can only assess immediate therapeutic performance with no way of tracking longer-term performance across the possible 72 h window in which the therapeutic must stabilize the injury site until the patient reaches specialty care. As a result, an anterior segment organ culture (ASOC) OG injury model was developed that detailed in this protocol as a platform for tracking long-term therapeutic performance[13].

ASOC is a widely used technique for maintaining avascular tissue of the anterior segment, such as the cornea, for multiple weeks post-enucleation[14–17]. The anterior segment is maintained under physiological IOP by perfusing fluid at physiological flow rates and preserving the trabecular meshwork outflow region, the tissue responsible for regulating IOP, during ASOC setup[18,19]. The ASOC platform can maintain tissue physiologically, induce an OG injury using a pneumatic-powered device, apply a therapeutic, and track injury stabilization for at least 72 h post-injury[13].

Here, the protocol provides a step-by-step methodology for using the ASOC platform. First it details how to set up and fabricate the ASOC platform. Next, the protocol details how to aseptically dissect the anterior segment and maintain the trabecular meshwork, followed by setting up anterior segment tissue in custom-built organ culture dishes. Then, it details how to create open globe injuries and apply therapeutic immediately following injury. Lastly, the protocol provides an overview on characterization parameters that are possible for use with this method that assesses functional, mechanical, and biological properties of the eye and how well the injury was stabilized. Overall, this model provides a much-needed platform to accelerate

product development for stabilizing and treating open globe injuries and improve the poor vision prognosis following injury.

PROTOCOL:

Before performing this protocol, be aware that there are legal and ethical requirements in place for the use of animals in research and training. If live animals are used for the source of ocular tissue, seek approval by the local ethical or legal authority (IACUC or Ethics committee, etc.) before beginning. If there is any question in obtaining approval for the use of animals, do not proceed. We previously determined and reported that fresh porcine eyes obtained and used within 24 h post-mortem compared closest to *in vivo* physiology and fared well for these studies (Animal Technologies, Tyler, TX, USA)[10,13]. No live animals were used throughout this protocol, using a tissue vendor to obtain tissue within 24 h.

NOTE: Prior to tissue arrival, fabricate the organ culture dishes (**Supplementary Protocol 1**), clamping rings (**Supplementary Protocol 1**), dish stands (**Supplementary Protocol 1**), pressure transducer data collection setup (**Supplementary Protocol 2**), and pneumatic puncture platform (**Supplementary Protocol 3**). Sterilize the dishes, tools, and supplies and prepare the work areas. It's useful to have a non-sterile area to perform gross dissection on the eyes, as they usually come with connective, extra orbital tissue attached. Execute these first steps on an open, clean work surface, and then transfer the eyes aseptically into a BSC II cabinet for micro-dissection (cabinet #1). Ensure the BSC II cabinet utilized for micro-dissection is optimally separated from the dish assembly BSC II cabinet (cabinet #2) to minimize airflow and maximize workspace. Set up the micro-dissection cabinet with a dissecting microscope and a way to visualize the work surface (camera or eyepieces protruding from the cabinet).

1. Sterilization steps, supplies (see Table of Materials for more details), and setup

1.1. Prepare the kit and gas-sterilize the following items (1 kit for each eye): ASOC dish, clamping ring, two fluidic connectors with O-rings, two 18 G needle hubs, four screws, two lengths of PE-100 tubing (length of distance should be long enough to extend from the dish inside the incubator to the syringe pump and pressure transducer data collection setup), two 18 G 90° bent needle hubs, two 3-way valves.

1.2. Prepare and autoclave the following kits.

1.2.1. Prepare and autoclave the micro-dissection instrument kit, containing one pair of fine forceps, one pair of Vannas scissors, one pair of medium toothed forceps, one pair of large scissors, cotton swabs, and a razor blade or scalpel.

1.2.2. Prepare and autoclave the assembly instrument kit, containing one pair of medium toothed forceps, one pair of surgical scissors, and one L-key.

1.2.3. Prepare and autoclave the daily kit (quantity: one per day of culture), containing one L-key to tighten clamping rings to dishes each day as needed.

1.2.4. Autoclave four 100 mL beakers for disinfecting and storing eyes and anterior segments.

1.2.5. Autoclave the puncture objects.

1.3. Gather the following sterile items: Petri dish (1 dish/eye), gauze (1–2/eye), dish stand, 20 mL syringes (1/eye), 10 mL syringes (1/eye), nylon syringe filters (1/eye).

1.4. Prepare sterile media: DMEM with 4% FBS, 1x Glutamax, 1x Gentamicin, 1x Antibiotic-Antimycotic (AA; approximately 30–40 mL complete media/eye).

1.5. Prepare AA-PBS: PBS with 1x AA (~500 mL).

1.6. Prepare the gross dissection tool pack: clean and dry large surgical scissors and forceps.

1.7. Set up the non-sterile dissection workspace: Gather supplies from gross dissection tool pack, enucleated porcine eyes submerged in PBS and on ice, surgical drape, 100 mL beaker with PBS. Lay out the surgical drape and items required for gross dissection.

1.8. Set up the sterile dissection workspace: Gather supplies from micro-dissection instrument kit, sterile gauze, dissecting microscope, betadine solution, sterile PBS, sterile media, four sterilized 100 mL beakers, sterile Petri dish. Aseptically transfer to BSC II cabinet #1. Set up the cabinet for visualizing eyes on the dissecting microscope.

1.9. Set up the ASOC assembly workspace: Gather gas-sterilized kits (dish kit and lid kit), assembly instrument kit, sterile media, dish stands, sterile Petri dishes, sterile syringes, and syringe filters. Aseptically transfer to BSC II cabinet #2. Set up the hood for dish assembly.

1.10. When eyes are stabilized and ready for puncture (72 h post-setup), aseptically transfer them to a BSC II cabinet. Set up the OG injury induction work pace: Pneumatic powered injury induction device (assembly detailed in **Supplemental 3**) and Lab Jack and cross-tracking vise to hold the ASOC dish.

2. Dissection of tissue

2.1. Prepare the porcine tissue using non-sterile dissection workspace.

2.1.1. Procure enucleated porcine eyes from a local abattoir, animal studies, or vendor. Maintain the eyes on ice submerged in PBS during delivery and use immediately upon receiving.

2.1.2. Cut away the extraorbital tissue and trim the conjunctiva leaving only the corneoscleral shell and optic nerve. Perform the dissection under non-sterile conditions with large surgical scissors and forceps in a gross dissection tool pack.

2.1.3. Place the eyes back into fresh PBS on ice until all eyes required for experimental setup have been preliminary/gross dissected.

2.1.4. Submerge the eyes in 10% betadine solution for 2 min in closed containers and transfer aseptically to BSC II cabinet #1. Perform all the subsequent works under sterile conditions to minimize contaminations during setup.

2.2. Sterilely dissect anterior segments.

2.2.1. After 2 min in betadine solution, transfer the eyes into three serial washes of sterile AA-PBS to remove excess betadine solution from the ocular surface while maintaining sterility of the ocular tissue. After three washes, maintain the tissue in AA-PBS until further use.

2.2.2. Hemisect the eye using a razor blade/scalpel and curved scissors. Place the eye on an AA-PBS-soaked gauze and create an incision with a sterile razor blade or scalpel near the equator of the eye (60/40 split with 40 on the anterior side). Using curved surgical scissors, hemisect the eye to isolate the anterior eye (corneal half).

NOTE: The cut around the anterior segment needs to be continuous to prevent jagged, rough edges in the sclera that will create fluid leaks after setup in the organ culture.

2.2.3. Use microscissors as a shovel to scoop vitreous humor from the anterior segment. Remove the lens from the anterior segment using microscissors. Leave the anterior segments in AA-PBS until further dissection steps.

NOTE: All the eyes that will be dissected can be held at this step and one by one taken through the remainder of the dissection process.

2.2.4. With a dissecting microscope, cut back iris to iris root gradually, radially until trabecular meshwork (TM) is visible. The TM is a pigmented tissue that comprises fibers circumferentially oriented around the corneoscleral shell. Careful cuts into the iris toward the iris root will expose the depth of the TM under the tissue.

2.2.5. Cut 360° around the iris at the same depth as the initial cut into the tissue to expose the entire TM region. Clean up any remaining residual iris covering TM as is necessary.

2.2.6. Trim the ciliary body remnants posterior to the TM, leaving only a thin band of tissue posterior to the TM region (approximately 1 mm).

2.2.7. Place the dissected anterior segment (AS) in media until further setup in ASOC in BSC II cabinet #2.

NOTE: All eyes can be held at this point prior to ASOC setup if a single user is performing dissection and organ culture dish assembly.

3. Setting up anterior segments in organ culture dishes

3.1. Place a single AS in a Petri dish with AS inverted (cup up). Using a cotton swab, wet in the media and gently dab in the center of the cornea to remove any pigment. Using forceps to hold the eye and the same swab, wipe the cotton swab around the sclera to remove extra pigment.

3.2. Invert the AS and place on top of bottom part of the dish over the elevated region, centering the cornea over the elevated region in the dish. Place the clamping ring on top of the newly placed AS.

3.3. Place four screws into the corresponding holes to hold the ring in place with the AS under the ring. Gently hand-tight the screws with the L-key.

NOTE: The tightening step will happen daily throughout the experiment, so the goal of the initial tightening here is to ensure the media does not leak while avoiding breaking the clamping ring.

3.4. With a sterile Petri dish set, place the top over the dish and invert the setup. Attach the dish stand. Attach the fluidic connectors with O-rings to threaded ports on the bottom of the dish.

3.5. To one fluidic connector, attach an 18 G 90° bent needle hub, a length of tubing, an 18 G needle hub, a nylon syringe filter, a three-way valve, and a 20 mL syringe filled with media.

3.6. To the second fluidic connector, attach an 18 G 90° degree bent needle hub, length of tubing, an 18 G needle hub, a three-way valve, and the barrel portion of a sterile 10 mL syringe (this will act as a reservoir to catch liquid and bubbles from the ASOC).

3.7. With three-way valves open appropriately to the syringes, gently push media through the system using the fluidics connector port identified in step 3.5 to inflate the AS, fill media in the tubing, and eventually the reservoir.

NOTE: If media leaks into the ASOC dish, the AS is not secured tightly enough with the clamping ring.

3.8. Remove bubbles by gently pushing media into the dish and inverting the dish to push out the bubbles and into the reservoir.

3.9. Place the dish and stand upright. Place the bottom portion of a Petri dish underneath the feet of the stand, careful not to ensnarl the tubing.

4. Starting anterior segment organ culture

4.1. ASOC is now ready for incubation. Place the ASOC dish into the cell culture incubator (37 °C, 5% CO_2). Ensure the height of the ASOC dish in the incubator above the pressure transducers is known and accounted for to calculate IOP accurately (**Supplementary Protocol 4**).

4.2. Direct the tubing lines out through the bottom of the 37 °C, 5% CO_2 incubator door so that they do not interfere with the opening and closing of the door. Attach the 20 mL syringe to the syringe pump set at 2.5 µL/min.

4.3. Position the tubing line with the reservoir at the pressure transducer instrument. Connect the side 3-way valve to the pressure transducer setup while flowing PBS through the line to avoid air bubbles entering the tubing lines.

NOTE: Empty the PBS from the reservoirs after the system is set up to reduce the likelihood of reservoir contamination with microbial growth for the duration of the organ culture.

4.4. Initiate IOP data collection by first ensuring a microSD card is present for saving data files. Then, turn on the pressure transducer setup to begin data collection.

NOTE: Details for setting up the pressure transducer data collection device are provided in **Supplementary Protocol 2**.

5. Daily maintenance of ASOC

5.1. After the ASOC has had 24 h to equilibrate, remove the dishes from the 37 °C, 5% CO_2 incubator and place them into tissue culture hood.

NOTE: On pressure data acquisition, these time periods look like spikes as the ASOCs are removed from the incubator (height change) and adjusted in the tissue culture hood.

5.2. Check for leaks under each dish on the Petri plate. If present, check for tight fluidic connections under the dish and re-tighten if necessary. Check for leaks in the dish top using a sterile L-key to tighten the screws in the clamping ring.

NOTE: The AS sclera tissue will compress and reduce thickness by 24 h, and the clamping ring will need to be tightened.

5.3. Aspirate the media from the dish well.

NOTE: The trabecular meshwork is filtering fluid from the media being pumped into the ASOC. Therefore, media will be present in the ASOC dish along the edges.

5.4. Repeat steps 3.7 and 3.8 to remove any trapped air bubbles.

5.5. Refill syringes on the syringe pumps, ensure the syringe pumps are operating, and confirm the alignment of valves for perfusion into the ASOC. Return the ASOC dish to the 37 °C, 5% CO_2 incubator.

NOTE: Optimally, these steps should be performed daily. However, the use of a 20 mL starting volume of media, the ASOC dish well volume, and a pump rate of 2.5 µL/min should be sufficient to let the system run for several days undisturbed.

6. OG injury induction with pneumatic-powered puncture device

NOTE: Construction of the pneumatic puncture device is detailed in **Supplementary Protocol 3**. OG injuries are induced after IOP has stabilized, which normally occurs after 3 days in culture. Acceptable IOP values are 5–20 mmHg based on physiological IOP, which can be determined by evaluating the IOP data files or setting LED indicators in the pressure measurement system as described in **Supplementary Protocol 2**.

6.1. Prepare the BSC II cabinet for OG injury induction as detailed in step 1.10. Connect the puncture platform to a compressed air line. Attach the sterile puncture object to the chuck.

NOTE: An air compressor can be used to power the device, but tank compressed air or built-in laboratory lines can suffice if pressure is greater than 50 psi.

6.2. Set the pressure regulator on the puncture platform to 50 psi for adequate puncture force on objects up to 4.5 mm in diameter. Position the cross-tracking vise on the lab jack in front of the puncture platform to hold the ASOC dish during injury induction.

6.3. Remove ASOC setup from 37 °C, 5% CO_2 incubator, and place in the cross-tracking vise perpendicular to the puncture platform (**Figure 2**) after removing the lid and setting it aside. Keep the anterior segment perfusing but close off the 3-way valve port to the pressure transducer to prevent over-pressurization damage to the transducer.

6.4. Extend the piston arm to its maximal distance and position the corneal apex within 1 mm of the puncture object. Retract the piston arm and bring the anterior segment 1 cm closer to the puncture object.

NOTE: This distance has been optimized for high-efficiency injury induction without hitting the ASOC dish.

6.5. Fire the puncture device by turning it on and opening the solenoid valve with the second switch on the device. To retract the device, press the second switch again to remove the puncture device from the eye. Verify proper injury induction by visual inspection and media leaking from the injury site.

6.6. Remove ASOC dish from the vise; place the lid back onto the dish assembly and open the fluidic line to the pressure transducer. Place the ASOC back into the 37 °C, 5% CO_2 incubator.

NOTE: At this point, therapeutic can be applied to the AS to assess its efficacy for sealing OG injuries.

7. Removing ASOC from culture

NOTE: Depending on endpoint analysis (see **Representative Results** for possible endpoint methods), the AS needs to remain in the ASOC dish inflated while other methods require AS tissue isolated from the culture chamber. The below methodology describes how to take AS out of the organ culture dishes and to remove the rest of the setup.

7.1. Remove the ASOC dishes from the 37 °C, 5% CO_2 incubator. Close the 3-way valve to the syringe and reservoir and disconnect the tubing from the system. Discard the syringe, reservoir, and filter. Place the 3-way valves, tubing, and needle hubs in a separate container for washing and sterilizing.

7.2. Disconnect the needle hubs from the fluidic connections on the bottom of the dish. Unthread the fluidic connectors and o-rings. Place all items in a container for washing and sterilizing.

7.3. Remove the four screws from the clamping ring using the L-key. Carefully remove the clamping ring.

7.4. Using forceps, remove the AS from the dish and, depending on endpoint analysis and image, place in fixative or appropriate biohazard waste.

8. IOP data analysis

8.1. Connect the microSD card to a computer to remove the **.txt** file containing data from the most recent experimental run.

NOTE: The file is named in the code controlling the microcontroller and should be updated for each experiment (see **Supplementary Protocol 2**).

8.2. Import the data into a spreadsheet.

8.3. Organize the data into 12 columns: Time (in min) and mV signal for each of 11 pressure transducer channels. The first ten channels correspond to ten ASOC experimental setups. The final transducer signal is for a sensor open to air as a control channel to confirm mV signal did not alter due to changes in the input signal. Plot control channel versus time to confirm mV signal was consistent throughout.

8.4. Convert the mV signals for ten channels into mmHg using slope-intercept equations generated from the initial calibration of each sensor (see **Supplementary Protocol 4**).

8.5. Plot time (convert to days to simplify data interpretation) versus each of the ten channels to determine how the IOP fluctuated across the experimental time course.

8.6. Determine average IOP values at key points in the data to more easily compare values between each and how they are altered before and after OG injury induction. Average 2–3 h of data for each 24 h interval to determine IOP at each day of ASOC.

NOTE: Representative IOP results are shown in **Figure 4**.

REPRESENTATIVE RESULTS:

Images captured via Optical Coherence Tomography (OCT) are shown for OG injured eyes to illustrate how a successful injury induction looks. **Figure 3** shows images for control and OG injured AS tissue immediately after injury and 72 h later. Two views are shown: cross-sectional images through the injury site and top-down maximum intensity projection (MIPs) to visualize the surface area of the image. Control eyes show no noticeable disruption in the cornea, while clear injuries can be located that cross the entire cornea after OG injury. From MIPs, it is evident that injuries are irregular in shape and size, but the injury size does decrease over 72 h. Previously, this effect has shown to be significant for a number of injury sizes tested[13].

The primary data output for the OG injury model described in this protocol is intraocular pressure over the course of the experimental setup. Data is recorded in units of millivolts as an output from each pressure transducer which can be converted into mmHg via calibration (**Supplementary Protocol 4**). Example IOP data vs the experimental time course is provided for eyes that are considered acceptable and others that would not be considered usable (**Figure 4A**). From the pressure trace data, eyes were attached to sensors after 24 h in culture, but IOP continues to fluctuate over the first 72 h in culture. Physiological IOP for AS tissue in organ culture is approximately 8–10 mmHg, so 2x and ½x range was decided upon as a gate for usable IOP values after values have stabilized (5–20 mmHg). Only eyes that were in that range would be allowable for use with the remainder of the protocol. From prior experiments, we had a 90% success rate that was achieved in ASOC setup for eyes stabilizing in the required range (**Figure 4B**).

The results for how IOP changes due to OG injury and therapeutic intervention are also provided (**Figure 4C,D**). After OG injury induction, pressure should significantly drop and remain that way until the tissue is removed from ASOC (**Figure 4C**). If an eye after injury induction does not

decrease in pressure, this indicates that a successful injury was not induced as IOP should be reduced if the watertight seal of the eye is present. However, smaller injury sizes may self-heal, which could result in IOP being restored. If therapeutic is applied to the eye after OG injury induction, restoration of IOP can be tracked during ASOC. This concept is demonstrated with data showing a Dermabond adhesive applied to 2.4 mm OG injuries (**Figure 4D**). Average results for five separate ASOC experiments with and without therapeutic are shown and it is evident the therapeutic is increasing IOP. This method can measure the efficacy of the therapeutic for restoring IOP and track whether that pressure is restored across the key 72 h post-OG injury.

Further, the ASOC protocol is adaptable for use with a wide range of characterization endpoints to meet the end user's experimental requirements. During culture, outflow media leaving the eye can be collected on a daily or even hourly basis which can be utilized for tracking protein level changes occurring during ASOC, after OG injury induction, or after therapeutic is applied. For instance, gelatin zymography has been previously performed to detect matrix metalloproteinase levels to track wound healing and tissue remodeling[20]. Further biological endpoints are possible after removing tissue from culture via traditional immunohistochemistry methods for assessing tissue viability[21,22], tracking pathophysiological changes to the cornea[23,24], or antibody-based staining for any protein of interest[25,26].

Functional corneal metrics can also be obtained from eyes maintained in ASOC. Corneal epithelium integrity can be assessed via a fluorescein eye stain and image acquisition using a blue light source[27,28]. After removal from culture, corneal tissue can be assessed for transparency through simple image acquisition[13]. Traditional ocular imaging can also be performed to assess tissue structure with or without therapeutic intervention. OCT images, as shown in **Figure 3**, can create cross sectional images through the cornea and can be captured non-invasively, potentially allowing image collection while maintaining tissue in culture. Other imaging modalities such as slit-lamp microscopy, ultrasound, or *in vivo* confocal microscopy can also be adapted for acquiring further anatomical information.

Lastly, assessment of mechanical properties of the anterior segment can be captured to understand the effect of the OG injury or subsequent therapeutic on the underlying tissue. While IOP data collection alone highlights how the integrity of the watertight seal of the eye has been compromised, we have previously shown that additional test metrics can be measured to tease out additional mechanical features[10,11]. Ocular compliance, a lumped mechanical property describing how intraocular pressure changes due to inflation (change in volume/change in pressure), can be measured with a syringe pump to inject sudden small volumes of fluid into the eye and recording the resulting pressure increase with a pressure transducer. Higher compliance indicates the tissue is less stiff and can be used to track how therapeutic material properties differ from the underlying corneal tissue. Leak rate from the eye or a traditional outflow facility can be measured and calculated to determine the precise fluidic flow rate leaving the eye per unit of pressure[20,29]. Lastly, with regards to therapeutic testing, burst pressure can be measured to determine the maximum pressure the eye can hold prior to the therapeutic failing. This can be used to compare performance to uninjured eyes or to track changes in performance with time[12,13].

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of the ASOC setup. Eyes are held in custom-built organ culture dishes and held in place with a clamping ring. ASOC media is infused via syringe pump through Valve A and connected to a pressure transducer, and subsequent data acquisition with Valve B. Open ports in each valve are highlighted in blue while yellow indicates closed channels.

Figure 2: Overview of the OG injury setup. (**A**) Pneumatic powered injury device setup. From left to right, compressed air is introduced to the device via a compressed air line, which passes through a regulator to set pressure at 50 psi as measured by the pressure gauge. Two solenoid valves are connected to a linear actuator to direct expansion/retraction of the drill chuck holding the puncture object. Vise is positioned in front of the puncture device to hold the eye at the appropriate x, y, z positioning. (**B**) Representative ASOC is placed in front of the injury induction device. Further details of the device and its construction are detailed in **Supplementary Protocol 3**.

Figure 3: Optical Coherence Tomography Images of ASOC OG injury experiments. Images are shown for uninjured, control eyes, and OG injured eyes immediately post-injury and 72 h post-injury. Views are shown as cross-sections through the cornea (**left side**) and top-down maximum intensity projection views of the corneal surface (**right side**). The figure has been adapted with permission from Snider et al.[13].

Figure 4: Representative IOP results for ASOC experiments. (**A**) Raw IOP data for the first 72 h of ASOC setup. Eyes are punctured at 72 h so the first 3 days of data are assessed to determine whether IOP stabilizes in the acceptable IOP range (5–20 mmHg). From the representative results, three of the five eyes fall within the acceptable IOP range, while one has IOP too high and one has IOP too low (falling outside of the highlighted yellow region on the plot). (**B**) Stabilized IOP for n = 50 ASOC setups from previous experiments to demonstrate the typical success rate with the ASOC method. (**C**) IOP for uninjured eyes compared to three different OG injury sizes after injury induction for 72 h. The loss of IOP is evident, with no signs of recovery. (**D**) Injured IOP results compared to injuries treated with a Dermabond adhesive. While the error rate is high due to some eyes being sealed and others not, the method can track changes to IOP over the 72 h period post-injury. The figure has been adapted with permission from Snider et al.[13].

DISCUSSION:

There are critical steps with the ASOC OG injury platform that should be highlighted to improve the likelihood of success when using the methodology. First, during the anterior segment dissection, preserving the trabecular meshwork is essential but challenging to do correctly. If the TM is disrupted, the eye will not maintain physiological pressure and will not meet eligibility criteria for experimental use. It is recommended to practice the dissection process under normal conditions first rather than introducing the additional aseptic technique challenges until proper dissections are obtained. Second, when setting the eyes in the ASOC dishes, it is imperative that they are tight enough to prevent fluid from leaking but loose enough to prevent damaging the ASOC dishes. If the eye is not secured tightly, fluid will leak out from the eye through non-

physiological means resulting in little or no IOP. However, the clamping ring holding the eye down is plastic and can be easily broken if overtightened. It is essential to clamp the eyes down over 2 days as the scleral tissue under the ring will compress and loosen the tissue during the first 24 h. It is recommended to tighten the rings just until resistance to tightening is felt on day 1 and follow this up by re-tightening to similar levels after 24 h in culture for best results.

Third, it is critical to fully understand where fluid flow is directed at all times when using this model. Each ASOC dish is connected to multiple three-way valves to direct fluid flow from the syringe pump or 10 mL syringe reservoir and connect to pressure transducers. Different instances of the setup process require valves to be positioned in such a way so as to flush air bubbles from the eye or to protect pressure transducers from over-pressurization. Care should still be taken to understand what is open/close at all times prior to critical protocol steps. Lastly, maintaining sterility throughout the ASOC OG injury protocol is critical but easy to lose across the multi-step, multi-day process. Perfusion media contains high levels of antibiotics and antimycotics to prevent this, and eyes are submerged in betadine prior to set up to prevent contaminations, but there are still critical steps where mistakes are most likely. During the initial setup in the dish, avoid contact with the eyes while tightening clamping rings in place and keep lids on the dishes at all times when not in use. A more likely exposure step is during the day-to-day ASOC maintenance. It is important to do these routine steps in a biosafety cabinet, even if it seems they can be quickly accomplished without removing the eyes from the incubator. Carefully following the protocol and maintaining good aseptic technique should minimize contamination risks across the 6-day ASOC experiments.

Overall, the ASOC OG injury platform is unique from other methodologies looking at open globe injuries due to two key criteria. First is the injury induction method. The high-speed pneumatic injury device utilized induces injuries with a high force amount. This allows for inducing injuries with objects that are not especially sharp nor with a small diameter. This more closely mimics injuries that are irregular in shape; high-speed shrapnel injures resulting from explosive devices[30,31]. The pneumatic device can easily be fitted with irregular-shaped shrapnel mimicking objects to create injuries more challenging to heal compared to previous methods using lasers, needles, or scalpel blades to create clean, precise injury geometries[32–34]. Second, the ASOC methodology allows for tracking injury progress and therapeutic performance beyond initial injury induction. Being able to track out to 72 h was not possible in the previously developed benchtop OG injury platform[10–12] and was the motivation behind developing this protocol. In fact, cell viability remained high in the corneal endothelium for at least 1 week in ASOC[13]. ASOC is the only means this long-term characterization can be accomplished without transitioning into costly *in vivo* experiments.

The main applications for the ASOC platform are two-fold. First, the model can be utilized for further characterizing open globe injuries, especially considering how they change with time. In the previous study, OG injuries were characterized in this manner and wound healing was observed over 72 h following injury[13]. Further tracking different injury sizes, shapes, locations for 72 h or even longer with regards to biological changes occurring will inform critical medical decisions that have to be made following OG injuries. Certain injury parameters may allow for

self-healing by the cornea, or other parameters may be more severe if the intervention is not applied within the first 24 h. This information will be invaluable for triaging patients when limited medical supplies or evacuation resources are available.

Second, the ASOC OG platform can be used for developing and testing product development. For this application, the organ culture platform can fill a number of roles. During initial product development, shorter time frames can be tested with a range of product formulations to determine what is most effective. The organ culture system can be configured for even greater high-throughput for this application with additional syringe pumps to move beyond the ten simultaneous experiments possible with the system detailed here. For more refined products, longer time points can be evaluated to assess performance for 72 h or potentially even longer. Lastly, wound healing evaluation may be possible when evaluating biologically active products that may permanently treat OG injuries rather than temporary stabilization.

However, there are limitations with the ASOC OG platform that should be taken into consideration. First, while the model allows for longer-term assessment of therapeutics, it is missing all tissue of the eye outside of the corneoscleral shell, such as the iris and lens. These additional tissues are likely to be influenced by the OG injury and may play a role in injury progression. Similarly, an isolated anterior segment is missing immune response elements that would be included when transitioning from the ASOC model to subsequent animal testing. Next, the model is only suitable for creating corneal OG injures and potentially limbal OG injuries. Scleral or posterior OG injuries cannot be induced with this method. However, many of these injury types result in damage to the retina, making any temporary stabilization therapeutic unlikely to prevent loss of vision[35,36]. Lastly, injuries with the model out to 72 h post-injury were only tracked. ASOC has been utilized in other applications out to 2 weeks, so the model can likely be utilized for these applications, but it has not been tested at this time[37–39].

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no competing interests. The views expressed in this article are those of the author(s) and do not reflect the official policy or position of the US Army Medical Department, Department of the Army, Department of Defense, or the US Government.

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Figure

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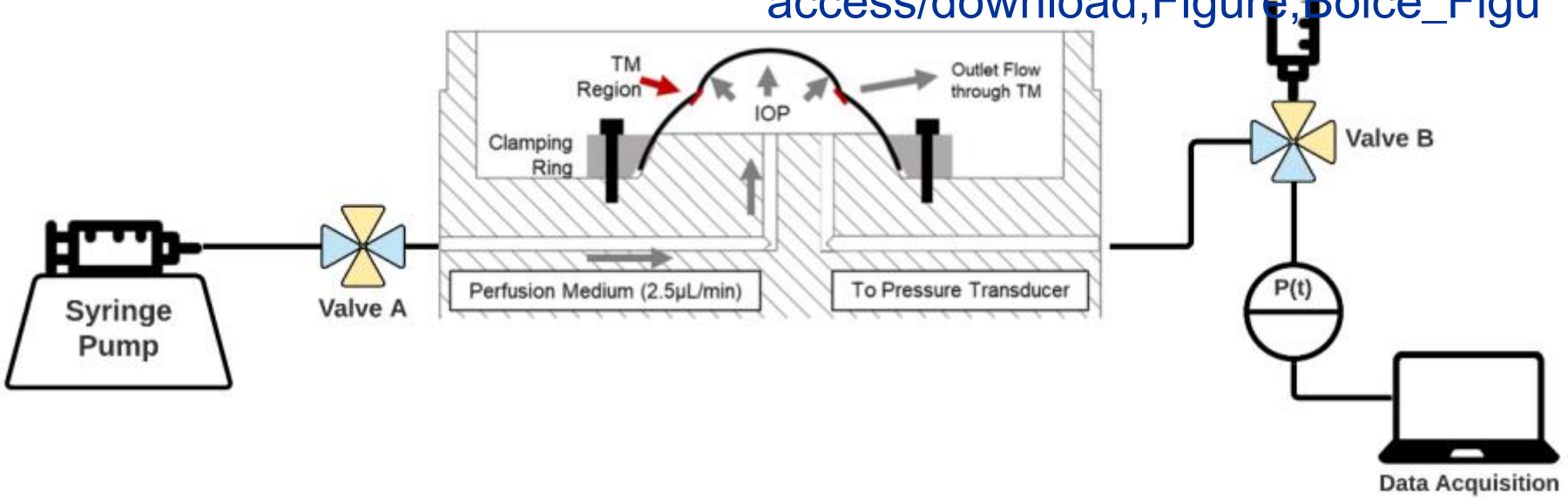

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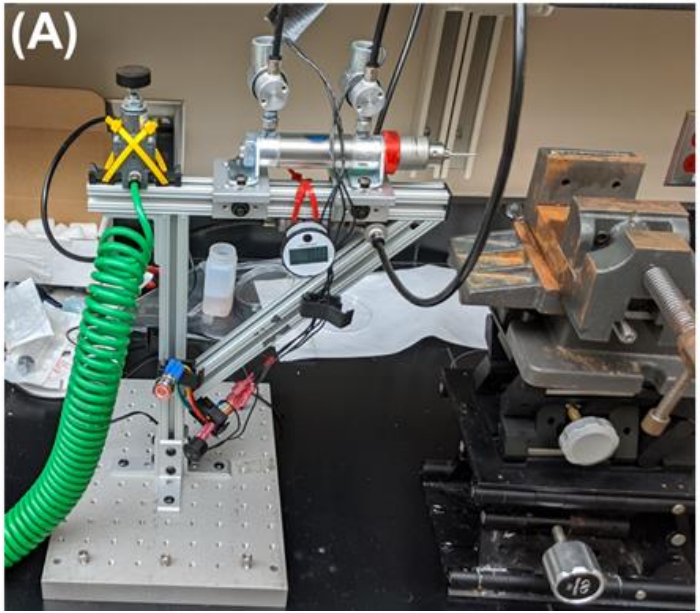

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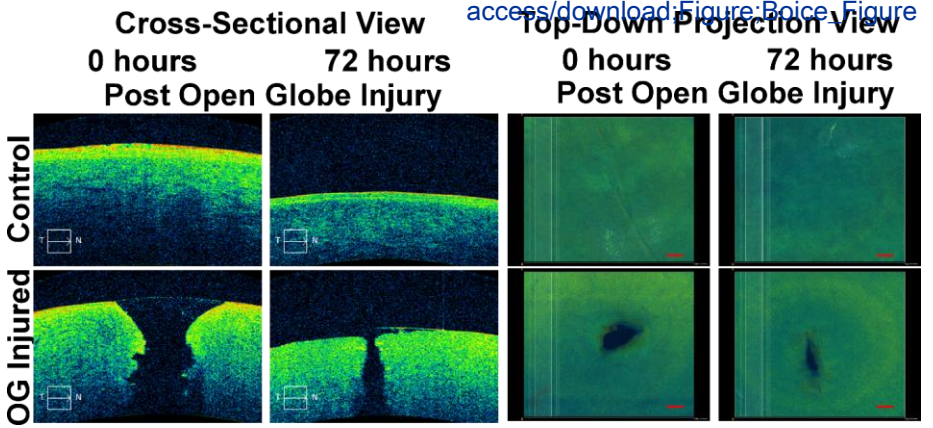

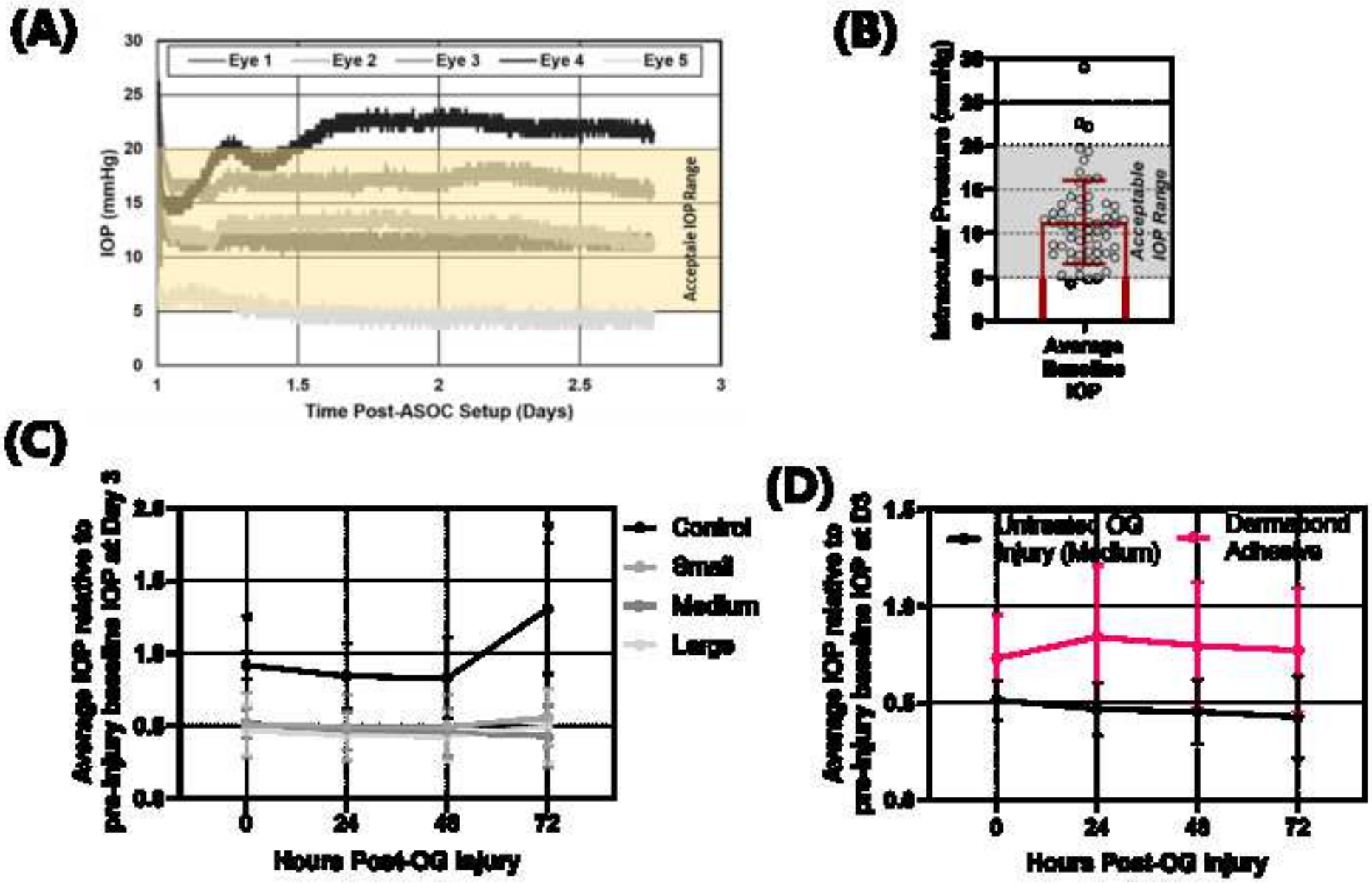

Dear Dr. Nguyen,

We would like to thank you and the Reviewers for the thorough review of our manuscript and for giving us the opportunity to respond to the comments. We have reproduced the Reviewer comments below in italics, followed by our responses. Changes to the manuscript are highlighted in red in the revised version for the Reviewers' convenience and are reproduced in our responses below when possible. We hope that our responses address all the Reviewers' concerns and that our study is now suitable for publication.

Sincerely,

Emily Boice

Editorial comments

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*
   Line 119: Bather?

Thank you for the observations. We have modified the manuscript to include those edits and provided those highlighted in red.

2. *Please present the numbered superscripted references numerically in order. Currently, it goes from reference 1 to reference 6-8. It should be 1, 2, 3, etc.*

We have renumbered the references to align with the flow of the manuscript.

3. *Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.*

We added in a sentence (Line 86-88) to further clarify that the tissue described was obtained from a vendor and no live animals were used for this work.

4. *Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol.*

We have corrected the text to reflect imperative tense and highlighted it in red throughout the manuscript.

5. *Please ensure that all incubation conditions are specified.*

We have added in the appropriate incubation conditions to the specific steps in section “Starting anterior segment organ culture”, “Daily maintenance of ASOC” and “Removing ASOC from culture”.

6. *Please use the SI abbreviation for hours: h instead of hrs*

We have corrected throughout the text and in Figure 4.

Reviewer 1

7. *It would be more convenient to do the bibliography in the order appropriate to the flow of the article.*

We have renumbered the references to agree with the flow of the manuscript.

Reviewer 2

8. *The scientific writing, especially in the protocol, lacks of standardization, please rewrite.*

Thank you for your observations. We have polished the manuscript to standardize the text and formatting and showed those modifications highlighted in red.

9. *How long this anterior segment organ culture can last? The authors said that they use this model to track therapeutic performance for at least 72 hours post injury. So please examine some important cells survival in the anterior segment organ culture. Does the corneal endothelial cells alive after culture for 3 days? We should try to ensure the corneal is alive, so we can investigate the therapeutic method for at least one week.*

The reviewer brings up an important point. In our previous study, we investigated cellular viability over a time course and saw that this organ culture system maintained high viability for up to one week. We have added a statement clarifying this on Line 472 and included the reference of our previous work. This should allow the manuscript to keep the focus on the methodology while pointing the reader to the results elsewhere.

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TITLE:

Supplementary Protocol 1 – Anterior Segment Organ Culture Part Fabrication

PROTOCOL:

For setting up in ASOC, first the culture dishes and supplementary components must be constructed. This includes the polycarbonate culture dish that houses the eye, clamping ring to hold the eye in place, and stand to hold each dish during culture in the 37°C, 5% CO_2 incubator. Design files for fabricating each of these are included with this protocol and all required supplies are detailed in the Supplementary Table of Materials.

1. A detailed list of parts is shown in the Table of Materials for this protocol.
2. ASOC Dish should be fabricated from polycarbonate through CNC milling methodologies. STL files for the dishes are attached to this protocol (Supplementary Figure 1A, "ASOC_Dish.stl").
3. Clamping rings are ideally CNC milled from polycarbonate as well but can also be laser cut from acrylic followed by boring out the center to fit the dish geometry via drill or lathe (Supplementary Figure 1B). STL files for the clamping rings are attached to this protocol ("ClampingRing.stl").
4. ASOC dish stands do not need to be sterilized before use as they are not in contact with ocular tissue and can thus be fabricated using traditional 3D printing methodologies (Supplementary Figure 1C). STL files for the dish stands are attached to this protocol ("DishStand.stl").
5. After fabrication, clamping rings need to be tested with 10-32 screws to confirm holes for securing the eye into the dish are bored to the proper diameter. Enlarge the holes via drill press if required.
6. ASOC dishes must be threaded to secure the 10-32 screws securing the eye with the clamping ring. This can be accomplished manually using a 10-32 tap. The fluidic ports for media flow and IOP measurement are too small to be CNC milled and must be drilled through the polycarbonate dish using a 1/16"diameter drill bit.
7. Next, the inflow and outflow ports on the bottom of the dish are 10-32 threaded polycarbonate pieces. Drill from the bottom of the dish with a 5/32" bit 6-7mm in depth followed by threading the port with a 10-32 tap.
8. Inflow/outflow ports are connected with a 3mm ID, 2mm width silicone O-ring between the fitting and the dish to ensure no fluid leak occurs through the connection site.
9. At this point, the ASOC dishes and clamping rings are prepared for sterilization following the main protocol and dish stands are ready for securing the eyes during incubation (Supplementary Figure 1D).

FIGURE AND TABLE LEGENDS:

Supplementary Figure 1. Images of the (**A**) ASOC dish, (**B**) Clamping Ring, (**C**) Dish Stand, and (**D**) Assembly of the 3 components.

TABLE OF MATERIALS:

Name	Company	Catalog Number	Comments/Description
1/16” Steel Drill Bit	McMaster-Carr	2901A111	
10-32 Tap Set	McMaster-Carr	2636A854	
10-32 Polycarbonate straight plug, male threaded pipe connector	McMaster-Carr	51525K431	
12’’ x 12’’ acrylic sheet, 1/8’’ thick	McMaster-Carr	85635K421	
18-8 Stainless Steel 5/8” length screws	McMaster-Carr	92196A271	
4mm ID x 2mm wide silicone O-rings	McMaster-Carr	5233T47	
5/32” Steel Drill Bit	McMaster-Carr	2901A117	
Clear gray polycarbonate disc, 1’’ long, 3’’ diameter	McMaster-Carr	8508K32	

SUPPLEMENTARY FILES:

- ASOC_Dish.STL
- ClampingRing.STL
- DishStand.STL

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TITLE:

Supplementary Protocol 2 – Intraocular Pressure Data Acquisition Device Construction

PROTOCOL:

Intraocular pressure is recorded using pressure transducers connected to each ASOC dish while in culture. Values are connected to each transducer and data is recorded by microcontroller for each IOP reading until eyes are removed from culture. Data is stored in real time to a micro-SD card connected to the microcontroller to remove the need for computer connection during data collection. Without a computer interface, it is tedious to access and assess the data while the experiments are ongoing which is essential to confirm IOP has stabilized in a physiological range. In response, LED indicators are included in the pressure readout and can indicate when values have stabilized in a healthy range without the need to stop and assess the data collected. All of this is housed in box enclosure and powered by DC power source. Here we detail the required parts for constructing the data acquisition setup and properly wiring the circuitry together.

1. A detailed list of parts is shown in the Table of Materials for this supporting protocol.
2. The box framework is shown in Supplementary Figure 2A. The overall dimensions of the rectangular enclosure were 11” x 6” x 3” (L x W x H). Enclosure parts were laser cut to shape and acrylic cemented together except for the lid which was loosely connected with tape.
   NOTE: The specifics of the box are not essential and can be of different shape and size and fabricated through different means to meet the end user’s capabilities.
3. Secure the Arduino microcontroller and micro-SD card reader to the floor of the box enclosure and connect LEDs with LED holders into the lid of the box assembly.
4. Solder pressure transducers as detailed in Supplementary Figure 3 to the Solder-able Breadboard. While only for use with 10 ASOC setups, 11 transducers are connected, with the extra sensor serving as an unconnected control recording baseline conditions.
   NOTE: The 11th transducer is not essential, but acts as a key check on IOP changes to confirm the signal from the transducer is biological and not due to a change in input electrical signal. Plotting values for sensor 11 vs. Time and confirming a stable signal indicates no issues occurred, but any deviation from this should be noted as it indicated non-experimental error being introduced.
5. Solder and attach the On-Off switch to the box enclosure and connect to the pressure transducer 5v power supply. Connect LEDs with wire and crimp connectors for easily swapping LEDs if a light burns out.
6. Connect the micro-SD card reader, LEDs and pressure transducers to the corresponding digital and analog terminals, respectively.
   NOTE: While this is the electrical setup used in this protocol, other microcontrollers and pressure transducer setups are compatible to fit the end user’s capabilities. IOP readings collected for 10 eyes simultaneously (~5 to 20mmHg value range) is the key aspect to ensure the setup can perform.
7. Attach silicone tubing to each of the pressure transducers and feed out through the box enclosure lid. Close the box with tape or other method.

NOTE: The control pressure transducer (sensor #11) does not need tubing connection and can be left unconnected in the box enclosure.

8. Connect tubing barb connector to luer fitting to each tubing piece, followed by 1 way valve, male-male luer adapter and 18G dispensing needle to easily connect to the PE 160 tubing used in ASOC dish setup (Supplementary Figure 2B).
9. To support the 10mL syringes connected to each ASOC dish, a t-slotted framing setup was constructed behind the box enclosure as shown in Supplementary Figure 2A.
   Custom syringe holder parts were fabricated using the "SyringeHolder.stl" file included with this protocol.
10. Prior to first use, pressure transducers and tubing connectors need to be flushed of air using DI water. Ensure the system is off during this process and sufficient time is given to allow electrical component to dry prior to use in the case of fluid leaking onto the circuitry.
11. Data acquisition is scripted using Arduino IDE open-source software, where data is recorded for each sensor every 11 seconds (1 sensor recorded per each second for 11 total sensors) and LEDs are gated by signal from each sensor to light up when within the 5 to 20mmHg acceptable IOP range. Data is stored as a table array along with time in a .txt file saved to the microSD card.
    NOTE: The gates for LED lights are determined via pressure transducer calibration as detailed in Supplementary Protocol 4.
12. Script is uploaded via USB cable and computer, but real time data collection only requires a 9V DC power supply and micro-SD card.

FIGURE AND TABLE LEGENDS:

Supplementary Figure 2. (**A**) Overview of the enclosure box setup and supporting frame for the 10mL syringes from the back port of the ASOC dish. (**B**) Magnified view of the fluidic connections attached to each pressure transducer.

Supplementary Figure 3. Detailed circuit diagram of the IOP data collection setup. Circuitry details connections to the Arduino microcontroller, SD card, ON-OFF switch, LEDs, and pressure transducers.

TABLE OF MATERIALS:

Name	Company	Catalog Number	Comments/Description
Electrical Components			
9v Power Supply	McMaster-Carr	70235K57	
Arduino IDE Open Source Software	Arduino	V 1.8.13	
Arduino Mega 2560 REV3 Microcontroller	Arduino	A000067	

Electrical Tape	3M Scotch	10457NA	
Female Crimp Connector	McMaster-Carr	7243K11	
Hook-Up Wire	Sparkfun	11367	Solid Core, 22AWG
LED, Basic Green	Sparkfun	COM-09855	
LED Holder, 5mm	Sparkfun	COM-11840	
Male Crimp Connector	McMaster-Carr	7243K12	
On-Off Switch	Sparkfun	11138	
Pressure sensor, 5 PSI Gauge	Digi-Key	480-3336-ND	Honeywell (SSCDANT005PGAA5)
SD Card Reader Module	HiLetGo	B07BJ2P6X6	
Solder-able Breadboard, Large	Sparkfun	12699	
Solder, Lead Free	Sparkfun	TOL-09325	
Box/Enclosure Components			
Acrylic Cement	McMaster-Carr	7517A1	
Cast Acrylic Sheet	McMaster-Carr	85635K421	12” x 12”, 1/8” thickness
Corner Bracket, 2” long, 1” rail	McMaster-Carr	47065T239	Quantity = 4
Syringe Holder	3D Printed		Attached STL File
T-Slotted Framing, 1” Rail, 12” Length	McMaster-Carr	47065T188	Quantity = 3
Fluidic Components			
BD Intramedic PE 160 Tubing	Fisher Scientific	14-170-12E	
Clear MasterKleer PVC Plastic tubing	McMaster-Carr	5233K51	50 ft, 1/16” ID, 1/8” OD
Male Luer to Male Luer Adapter	Cole Parmer	EW-45513-72	Pack of 25
One-way Stopcock	Cole-Parmer	EW-30600-01	Pack of 10
Stainless Steel 18G 90 degree angle dispensing needle	McMaster-Carr	75165A81	Pack of 10
Tube coupling – Barb to Luer	McMaster-Carr	51525K271	Pack of 10, 1/’16” ID tubing

SUPPLEMENTARY FILES:

- Syringeholder.STL

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Standard Manuscript Template

TITLE:

Supplementary Protocol 3 – Construction of the Pneumatic Powered OG Injury Device

PROTOCOL:

Open globe injuries are induced via a pneumatic powered linear actuator capable of producing high force injuries of various shapes and sizes. The system was constructed using readily available parts and supplies and includes the framework to hold the device static while inducing injuries. While an air compressor was utilized to generate the air pressure to drive the device, any compressed air source can be utilized as long as 50 psi can be generated. Below are instructions and diagrams to construct the device and a Table of Materials identifying each required component.

1. A list of required parts is detailed in the Table of Materials for this supplementary protocol.
2. Assemble the electrical components as detailed in Supplementary Figure 4B. Crimp connectors were used throughout for ease of connecting and disconnecting as was required.
3. Pressure components can be assembled as detailed in Supplementary Figure 4C. Ensure each connection is pressure tight by using Teflon tape for each threaded part.
4. Construct the t-slot framing setup to house the flow and electrical components as detailed in Supplementary Figure 4A.
5. Set the air pressure regulator at 50psi and ensure the relief valve in the system is set at 75psi. Power on the electronics and ensure the solenoid valves open and close with the momentary switch.
6. Connect compressed air to the system and ensure pressure in the system reached 50psi. Check for any leaks in the system by closing of the compressed air source and confirmed pressure holds in the system.
7. Turn on/off the solenoid valves to confirm they drive the linear actuator forward and back. The device is now ready for injury induction in ASOC setups as detailed in the protocol.

FIGURE AND TABLE LEGENDS:

Supplementary Figure 4: Overview of the open globe induction setup. (**A**) The injury device fully constructed. (**B**) Electrical diagram for controlling the solenoid valves for forwarding and retracting the linear actuator. (**C**) flow diagram for compressed air through the regulating valve and to the solenoid valves which direct air flow to the linear actuator and puncture object held in the drill chuck. Numbers indicate the required parts as identified in the Table of Materials for this supplementary protocol.

TABLE OF MATERIALS:

 # correspond to labels in the Supplementary Figure 4

#	Name	Company	Catalog Number	Comments/Description
	General Device Components			
9	304 Stainless Steel Cross Connector	McMaster-Carr	4464K311	1/8” NPT Female
7	304 Stainless Steel Straight Connector	McMaster-Carr	4464K711	1/8” NPT Male Quantity = 3
8	304 Stainless Steel Tee Connector	McMaster-Carr	4464K47	1/8” NPT Female
1	Air Cylinder Double Acting, 5/16” Bore, 1-½” Stroke	McMaster-Carr	6498K13	
14	Air Compressor	McMaster-Carr	9965K62	Any source of compressed above 50psi will suffice
6	Digital High Accuracy Pressure Gauge	McMaster-Carr	1287N1	1/8” NPT
2	Drill Chuck	Grainger	CSS-06 R-5/16 GE-DS	5/16” - 24
3	Electric Solenoid Control Valve	McMaster-Carr	61245K84	1/8” NPT, 12V DC Quantity = 2
11	Hard Nylon Plastic Tubing	McMaster-Carr	5548K84	¼” Tubing OD, 11/64” ID; 25ft Length
5	Precision Compressed Air Regulator (1 – 120psi)	McMaster-Carr	2227T2	1/8” NPT
4	Pressure Relief Valve	McMaster-Carr	5784T11	Set at 75psi, 1/8” NPT
10	Push-to Connect Rub Fitting	McMaster-Carr	5779K108	¼" Tubing OD, 1/8” NPT Male Quantity = 7
13	Thread Sealant Tape	McMaster-Carr	1726N12	3/8” wide, for Stainless Steel Threads
	Electronic Components			
15	DC Power Supply	Digi-Key	WSU120-1000	12V 1A DC
18	Female Crimp Connector	McMaster-Carr	7243K11	
17	Male Crimp Connector	McMaster-Carr	7243K12	
16	Push-Button Switch	API Electric	B07932GYNH	
20	On-Off Switch	SparkFun	11138	
19	Wire	McMaster-Carr	8054T15	

	Structural Framing			
27	1” T-Slotted Framing Bracket	McMaster-Carr	6498K992	Quantity = 2
23	4 Slot Rail , 1” L x W x H	McMaster-Carr	47065T101	Quantity = 2
22	Corner Bracket, 2” long, 1” rail	McMaster-Carr	47065T239	Quantity = 5
28	Foot Bracket for 5/16” Bore Size Air Cylinder	McMaster-Carr	6498K581	Quantity = 2
20	Optical Breadboard Plate	Newport	SA2-11	12”x12”, ¼” - 20
26	Tie Cable Holder	McMaster-Carr	47065T268	Quantity = 2
21	T-Slotted Framing, 1” Rail, 12” Length	McMaster-Carr	47065T188	Quantity = 1
29	T-Slotted Framing End Cap	McMaster-Carr	3136N2	Quantity = 1
24	T-Slotted Framing, End- Fed Single Nut	McMaster-Carr	47065T139	Quantity = 3
25	T Slotted Framing Snap Cap Cable Holder	McMaster-Carr	47065T389	Quantity = 2

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TITLE:

Supplementary Protocol 4 – Pressure Transducer Use and IOP Calibration

PROTOCOL:

Prior to first use and periodically after initial setup, the IOP recording system must be calibrated to confirm proper sensor performance and convert millivolt signal into units of pressure. This protocol walks through the methodology to collect calibration data and analyze the results.

1. Each transducer records pressure connected to its respective tubing inlet, which would require calibration 10 separate times for each transducer. Instead 2, 5-way manifolds can be connected to each of 10 ports to connect all transducers for a single calibration run for all sensors (Supplementary Figure 5A). Open all 1 way-valves so that each sensor is reading similar pressure. See Table of Materials for this supplementary protocol for supplies required.
2. Pressure will be precisely set using a 20mL syringe as a reservoir open to air (remove the plunger from the back after connection to the manifold setup). This allows pressure to be hydrostatically driven based on the cm-H2O the reservoir is held above the pressure transducer.
3. For simplicity with the calibration process, determine the height difference between the transducer box and ASOC dish placement. The height difference here will impact the signal read by the transducers. For properly accounting for this offset, the zero point on the calibration must be the height the dishes sit above the transducers during incubation.
   NOTE: Transducers should be lower than the ASOC but as close to the same height as possible. If the transducers are higher elevation, the pressure values may be too low for the sensors to detect. Example: If the ASOC dish is 8 cm above the transducer placement the zero point for pressure readings is 8 cm above sensors.
4. Starting at 0 cm, relevant to the ASOC dishes, record data for 10 seconds while each transducer is open to the reservoir and repeat every 10 cm-H2O for up to 100 cm-H2O in height.
   NOTE: To ensure adequate data is recorded, increase the data collection frequency to once every 0.1 seconds instead of 1 second so that multiple data points for each sensor are recorded across the 10 second interval.
5. Export signal from each transducer in units of millivolts and plot versus time to create a staircase-like plot (Supplementary Figure 5B), where each step is the 10 second hold on each pressure step. Average the values for each step to get eleven values for each sensor for 0 to 100 cm-H2O in 10 cm-H2O intervals.
6. Convert units of cm-H2O to mmHg by multiplying by 0.74. Plot pressure in mmHg versus signal in millivolts to create a calibration plot that should be linear for each sensor (Supplementary Figure 5C). Determine the slope, y-intercept, and R^2 value.
   NOTE: R^2 should be > 0.98 for a properly performing sensor; replace sensors if the linearity falls outside of this.
7. Determine the range of acceptable millivolt signal for each sensor by using the slope intercept equation to find the signal at 5mmHg and 20mmHg. These signal values can be

plugged into the IOP data collection script to ensure the LEDs indicate when eyes are at proper pressure.

8. Remove the manifold setup and close each 1-way valve until the IOP system is ready for use to minimize the chance of air bubbles getting trapped in the tubing lines.

FIGURE AND TABLE LEGENDS:

Supplementary Figure 5: (**A**) Diagram of setup for calibrating all 10 pressure transducers simultaneously with a hydrostatic reservoir. (**B**) Representative data collected during calibration showing transducer signal (mV) vs time (minutes). (**C**) Calibration plot for each of 10 transducers plotted as transducer signal (mV) vs pressure (mmHg).

TABLE OF MATERIALS:

Name	Company	Catalog Number	Comments/Description
5 port Manifold	Cole-Parmer	30600-43	Pack of 10 ports
20ml Syringe	BD	302830	
BD Intramedic PE 160 Tubing	Fisher Scientific	14-170-12E	
Stainless Steel 18G 90 degree angle dispensing needle	McMaster-Carr	75165A81	Pack of 10

Supplemental Figure 1

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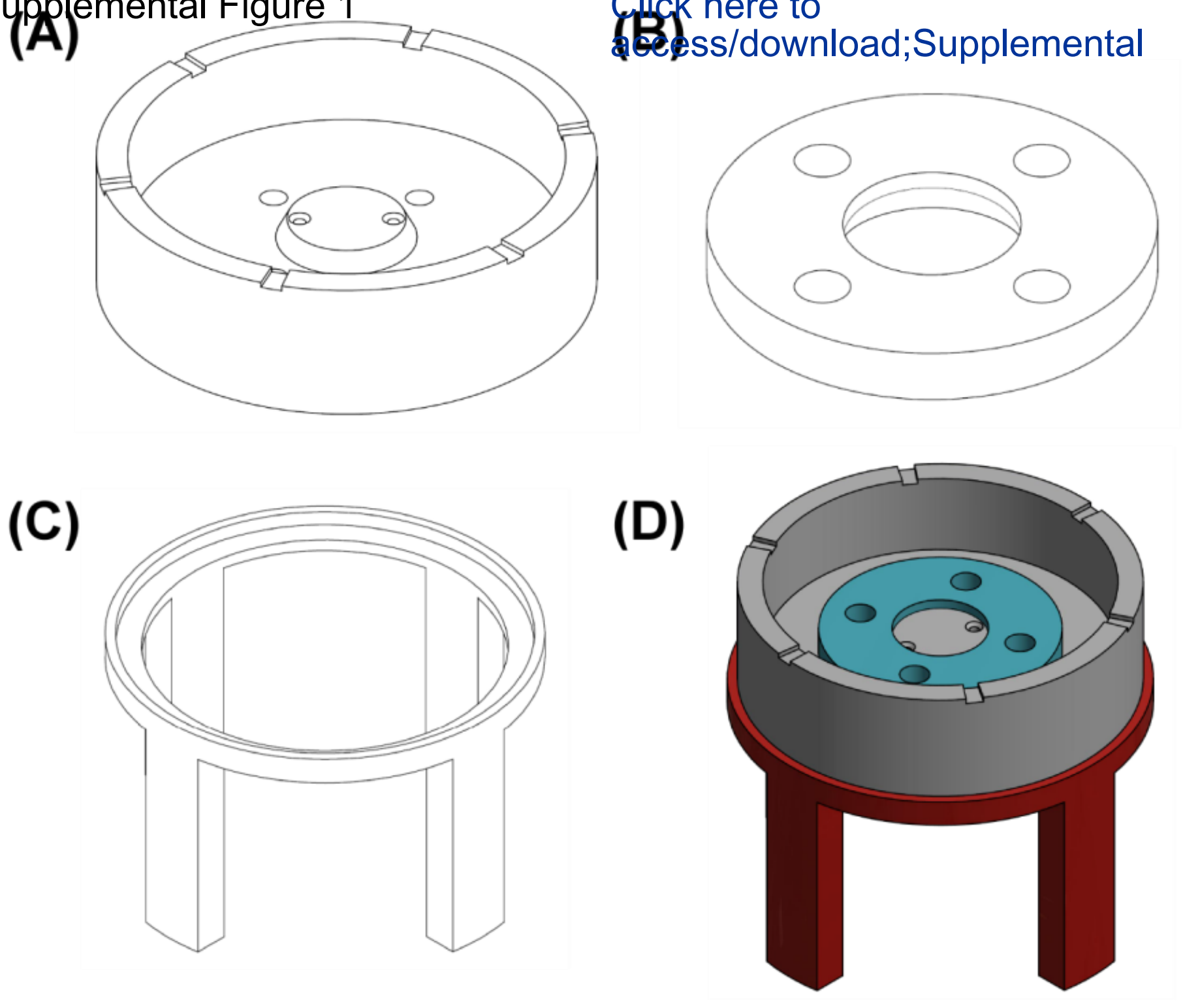

Supplemental Figure 2

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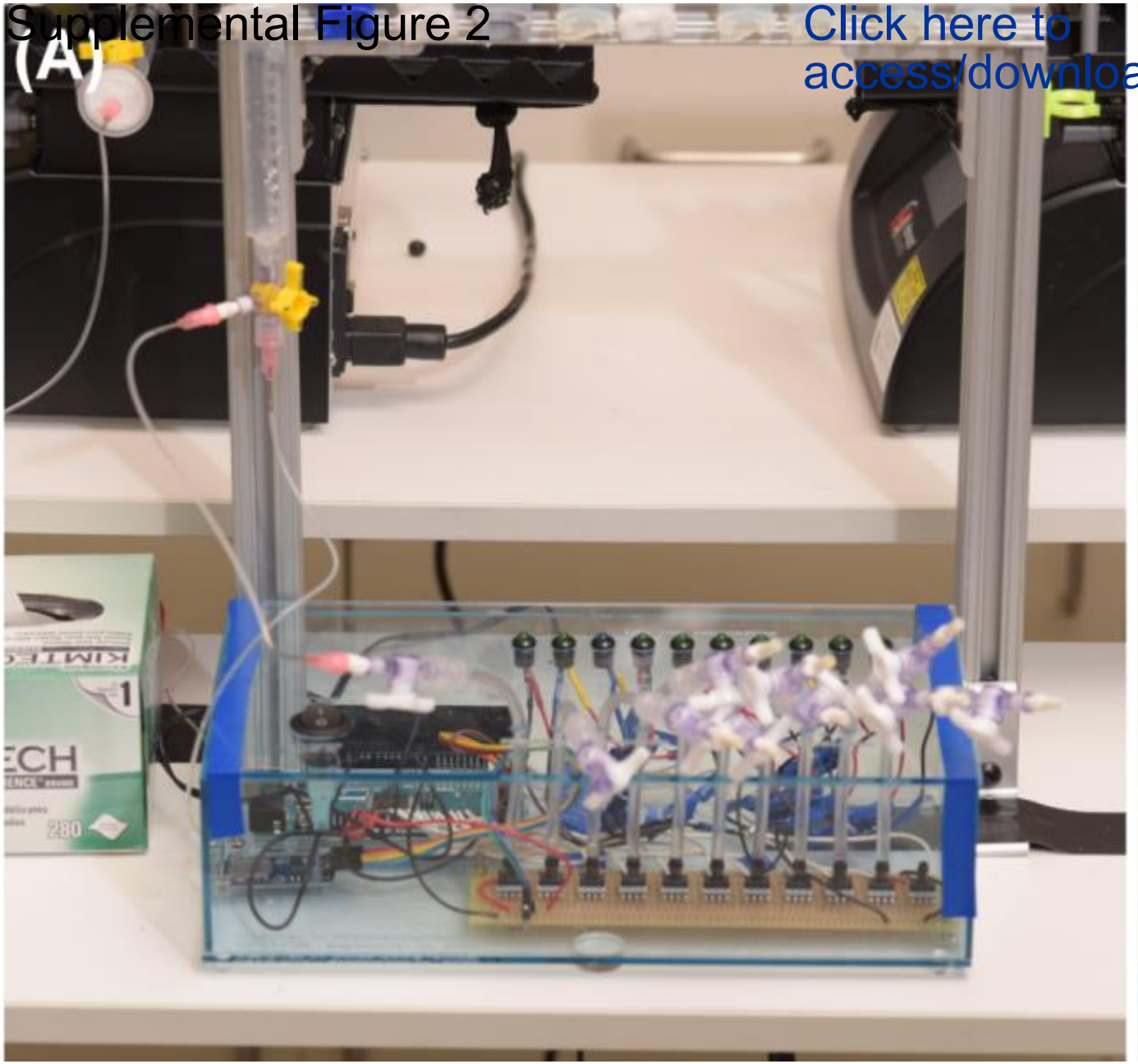

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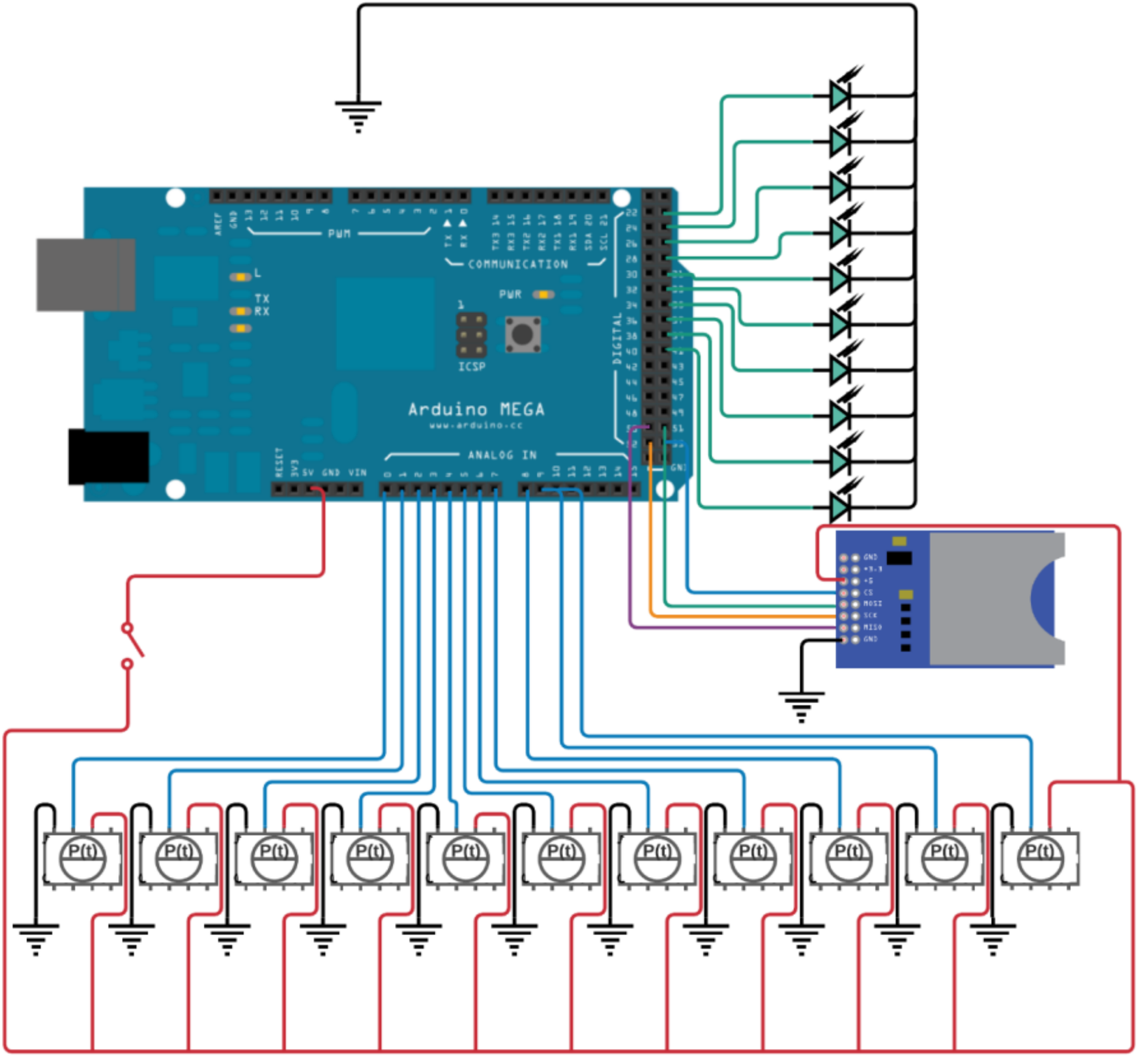

Supplemental Figure 4

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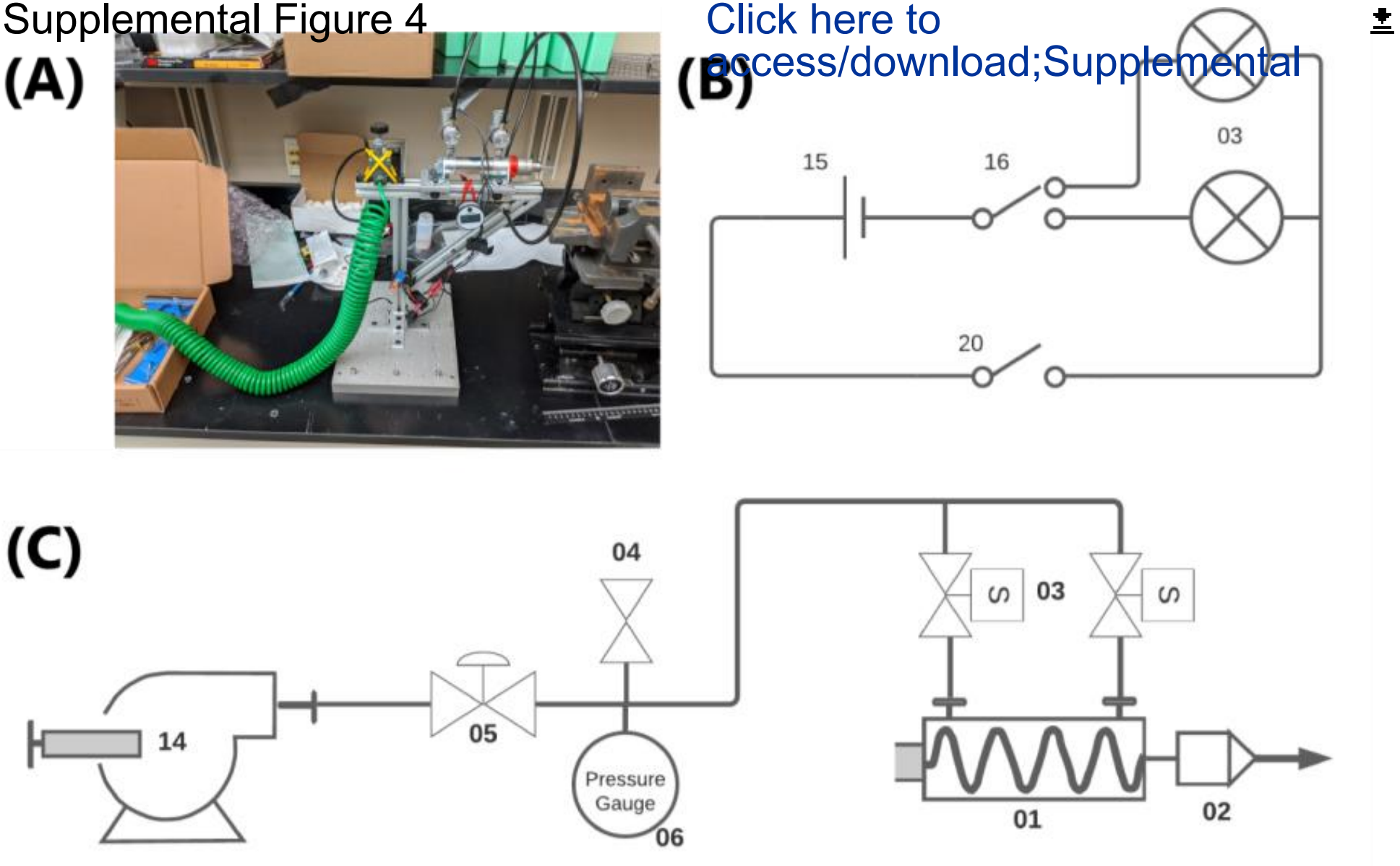

Supplemental Figure 5

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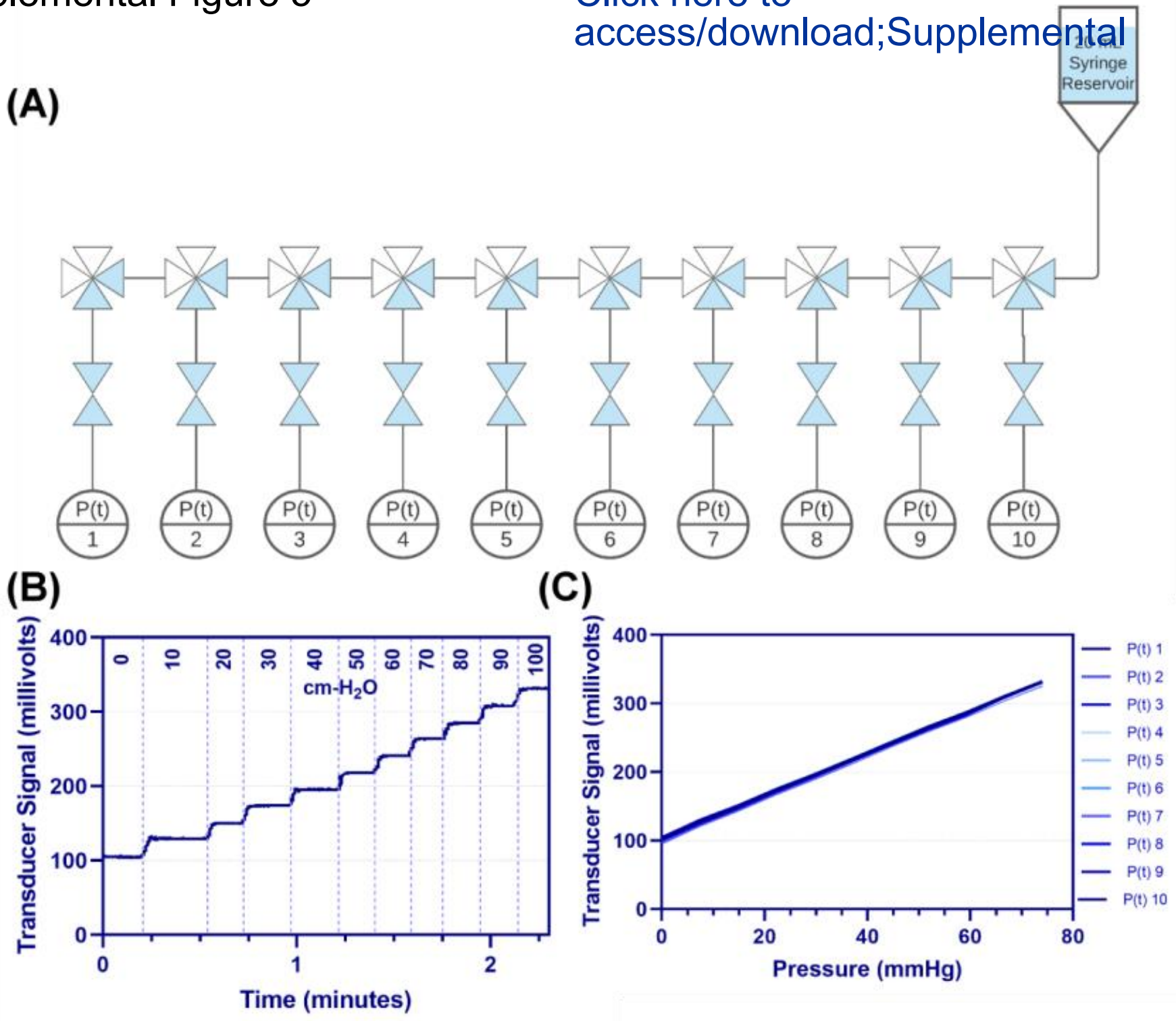

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Supplemental Coding Files

ASOCDish.stl