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Intravitreal Injection and Quantitation of Infection Parameters in a Mouse Model of Bacterial Endophthalmitis

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TITLE:

Intravitreal Injection and Quantitation of Infection Parameters in a Mouse Model of Bacterial Endophthalmitis

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SUMMARY:

We describe here a method of intravitreal injection and subsequent bacterial quantitation in mouse model of bacterial endophthalmitis. This protocol can be extended for measuring host immune responses and bacterial and host gene expression.

ABSTRACT:

Intraocular bacterial infections are a danger to the vision. Researchers use animal models to investigate the host and bacterial factors and immune response pathways associated with infection to identify viable therapeutic targets and to test drugs to prevent blindness. The intravitreal injection technique is used to inject organisms, drugs, or other substances directly into the vitreous cavity in the posterior segment of the eye. Here, we demonstrated this injection technique to initiate infection in the mouse eye and the technique of quantifying intraocular bacteria. *Bacillus cereus* was grown in brain heart infusion liquid media for 18 hours and

resuspended to a concentration 100 colony forming units (CFU)/0.5 µL. A C57BL/6J mouse was anesthetized using a combination of ketamine and xylazine. Using a picoliter microinjector and glass capillary needles, 0.5 µL of the *Bacillus* suspension was injected into the mid vitreous of the mouse eye. The contralateral control eye was either injected with sterile media (surgical control) or was not injected (absolute control). At 10 hours post infection, mice were euthanized, and eyes were harvested using sterile surgical tweezers and placed into a tube containing 400 µL sterile PBS and 1 mm sterile glass beads. For ELISAs or myeloperoxidase assays, proteinase inhibitor was added to the tubes. For RNA extraction, the appropriate lysis buffer was added. Eyes were homogenized in a tissue homogenizer for 1-2 minutes. Homogenates were serially diluted 10-fold in PBS and track diluted onto agar plates. The remainder of the homogenates were stored at -80 °C for additional assays. Plates were incubated for 24 hours and CFU per eye was quantified. These techniques result in reproducible infections in mouse eyes and facilitate quantitation of viable bacteria, the host immune response, and omics of host and bacterial gene expression.

INTRODUCTION:

Bacterial endophthalmitis is a devastating infection that causes inflammation, and, if not treated properly, can result in loss of vision or blindness. Endophthalmitis results from the entry of bacteria into the interior of the eye[1-5]. Once in the eye, bacteria replicate, produce toxins and other noxious factors, and can cause irreversible damage to delicate retinal cells and tissues. Ocular damage can also be caused by inflammation, due to the activation of inflammatory pathways leading to inflammatory cell influx into the interior of the eye[1,5,6]. Endophthalmitis can occur following intraocular surgery (post-operative), a penetrating injury to the eye (post-traumatic), or from metastatic spread of bacteria into the eye from a different anatomical site (endogenous)[7-10]. Treatments for bacterial endophthalmitis includes antibiotics, anti-inflammatory drugs, or surgical intervention[3,4,11]. Even with these treatments, vision or the eye itself may be lost. The visual prognosis after bacterial endophthalmitis generally varies depending upon the treatment effectiveness, the visual acuity at presentation, and the virulence of the infecting organism.

Bacillus cereus (*B. cereus*) is one of the major bacterial pathogens that causes post-traumatic endophthalmitis[7,12]. A majority of *B. cereus* endophthalmitis cases have a rapid course, which can result in blindness within a few days. The hallmarks of *B. cereus* endophthalmitis include quickly evolving intraocular inflammation, eye pain, rapid loss of visual acuity, and fever. *B. cereus* grows rapidly in the eye compared to other bacteria which commonly cause eye infections[2,4,12] and possesses many virulence factors. Therefore, the window for successful therapeutic intervention is relatively short[1-7,11-25]. Treatments for this infection are usually successful in treating endophthalmitis caused by other less virulent pathogens, but *B. cereus* endophthalmitis commonly results in greater than 70% of patients suffering from significant vision loss. About 50% of those patients undergo evisceration or enucleation of the infected eye[7,16,22,23]. The destructive and rapid nature of *B. cereus* endophthalmitis calls for immediate and proper treatment. Recent progress in discerning the underlying mechanisms of disease development have identified potential targets for intervention[19,26,27]. Experimental mouse models of *B. cereus*

endophthalmitis continue to be useful in discerning the mechanisms of infection and testing potential therapeutics that may prevent vision loss.

Experimental intraocular infection of mice with *B. cereus* has been an instrumental model for understanding bacterial and host factors, as well as their interactions, during endophthalmitis[28]. This model mimics a post-traumatic or post-operative event, in which bacteria are introduced into the eye during an injury. This model is highly reproducible and has been useful for testing experimental therapies and providing data for improvements in standard of care[1,6,19,29,30]. Like many other infection models, this model allows for independent control of many parameters of infection and enables efficient and reproducible examination of infection outcomes. Studies in a similar model in rabbits over the past few decades have examined the effects of *B. cereus* virulence factors in the eye[2,4,13,14,31]. By injecting *B. cereus* mutant strains lacking individual or multiple virulence factors, the contribution of these virulence factors to disease severity can be measured by outcomes such as the concentration of bacteria at different hours of postinfection or the loss of visual function[13,14,27,31,32]. In addition, host factors have been examined in this model by infecting knockout mouse strains lacking specific inflammatory host factors[26,29,33-35]. The model is also useful for testing potential treatments for this disease by injecting novel compounds into the eye after infection[30,36]. In this manuscript, we describe a detailed protocol which includes infecting a mouse eye with *B. cereus*, harvesting the eye after infection, quantifying intraocular bacterial load, and preserving specimens to assay additional parameters of disease severity.

PROTOCOL:

All procedures were performed following the recommendations in the Guide for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center (protocol numbers 15-103, 18-043, and 18-087).

1. Sterile glass needles

1.1. Turn **On** the needle pipette puller.

1.2. Adjust the **Heater** knob until the display shows 12.6.

1.3. Open the door and manually feed the 5 µL capillary tube through the upper clamp and heater filament until half of the tubing extends below the filament (**Figure 1A**).

1.4. Confirm that the capillary tube is in the "V" groove in the upper clamp. Tighten the upper clamp.

1.5. With the lower clamp open, manually adjust the vertical slide up until the lower clamp reaches its upper limit. Confirm that the tube is in the "V" groove in the lower clamp. Tighten the lower clamp.

1.6. Close the door and confirm that the 'Ready' light is on. Push the **Start** button to initiate the heater and pulling sequence (**Figure 1B**).

1.7. Ensure the heater turns off automatically after heat and gravity pulls the capillary tube apart (**Figure 1C**), and the slide moves downward.

1.8. Open the door. While holding the top of the capillary tube, untighten the top clamp. Remove the upper capillary tube and set it aside.

1.9. Hold the capillary tube in the bottom slide and untighten the lower clamp. Remove the lower capillary tube and set it aside.

1.10. Use a microelectrode beveler with a 0.05 µm alumina abrasive grinding plate to bevel the pulled capillary tube to create the injection needles.

1.11. Pipette enough water onto the grinding plate to cover the surface (**Figure 1D**).

1.12. Turn **On** the beveller so that it begins to rotate at 60 rpm. Place the pulled capillary tube into the pipette clamp in the "V" groove. Tighten the clamp and adjust the angle of the pipette clamp to 30°.

1.13. Adjust the tip of the pointed edge of the capillary tube approximately two-thirds distance away from the center of rotation of the grinding plate.

1.14. Lower the clamped capillary tube by adjusting the coarse control knob at the top of the manipulator. Continue to lower the capillary tube until the tip of the capillary tube extends below the surface of the water and contacts the abrasive surface of the grinding plate.

1.15. Monitor the beveling progress using the microscope attached to the beveller. After 5 min, adjust the fine control to raise the glass needle from the grinding plate.

1.16. Remove the glass needle and place it under 10x magnification to check the tip of the capillary tube (**Figure 1E,F**).

1.17. To ensure that there are no blockages, insert the glass needle into a pipette holder on a syringe and push air into a 1 mL tube of 99% ethanol. If air bubbles form at the tip of the needle, the needle has no blockages and can be used for microinjection. This process also ensures the sterility of the glass needles.

1.18. One capillary tube can hold up to 5 µL fluid. Mark the capillary tube into 10 sections to calculate the distances on the needle to mark for 0.5 µL volumes. Mark a scale with that distance that holds 0.5 µL for future preparation. For a 5 µL needle, distances of 0.7 mm apart equate to 0.5 µL (**Figure 1G**).

2. *Bacillus cereus* culture

2.1. Perform all procedures in this section under Biosafety Level 2 conditions.

2.2. Streak freezer stock of *B. cereus* ATCC 14579 for single colony isolation on a 5% sheep blood agar plate and incubate overnight at 37 ˚C (**Figure 2A**).

2.3. Pipette 5 mL of sterile Brain Heart Infusion (BHI) broth into a 10 mL sterile snap-cap tube (**Figure 2B**). Using a sterile loop or needle, pick a single colony of *B. cereus* ATCC 14579 from the agar plate and inoculate the liquid BHI.

2.4. Vortex the sample briefly. Place the snap-cap tube into a rotating incubator. Set the temperature at 37 °C and speed at 200 rpm. After 18 h, remove the culture from the incubator (**Figure 2B**).

3. Bacterial dilution for intravitreal injection

3.1. Perform all procedures in this section under Biosafety Level 2 conditions.

3.2. Calculate the volume of the overnight *B. cereus* culture to add to 10 mL of fresh BHI to achieve 200 colony forming units per microliter (CFU/µL). For example, an overnight culture of *B. cereus* in BHI replicates to approximately 2 x 10^8 CFU/mL, which can then be diluted to 200 CFU/µL by pipetting 10 µL of overnight culture into 10 mL of fresh BHI.

3.3. Pipette 1 mL of the freshly diluted culture into a 1.5 mL microcentrifuge tube. Maintain this tube on ice until intravitreal injection. Perform the intravitreal injection within 60 min of diluting the bacterial culture.

4. Mouse intravitreal injection

4.1. Perform all procedures in this section under Biosafety Level 2 conditions.

4.2. Prepare the procedure site by placing medical underpads on the operating table.

4.3. Turn **On** the microinjector and open the gas valve on the compressed air tank attached to the microinjector. Adjust the tank regulator until the gas delivery is approximately 60 psi.

4.4. On the microinjector, press **Mode** until the screen shows **Balance**. Press **Balance** and place the computer mouse on the operating table. Connect the stainless-steel pipette holder to the tubing attached to 'Fill/Output' of the injector. This tube is always connected to the injector.

4.5. Insert the capillary needle to the other end of the pipette holder by screwing tight the pipette holder connector around the needle.

4.6. Turn on the ophthalmic microscope and turn on its light to an intensity of 50%. Adjust the microscope over the procedure site on the operating table and adjust the microscope to the desired focus.

4.7. Before the procedure begins, confirm that the mouse is adequately anesthetized by testing the pedal withdrawal reflex.

4.8. Place the anesthetized mouse on the medical underpads with its nose pointed to the right and leaning on its left side.

4.9. Look at the right eye of the mouse through the ophthalmic microscope and open the lids by opening the tongs of a reverse action forceps on either side of the eye to expose the injection site (**Figure 3C**).

4.10. Fill the capillary needle with the 200 CFU/µL dilution by left clicking the mouse pad connected to the microinjector (**Figure 3D**).

4.11. Secure the animal's head with the left hand and place the tip of needle at the limbus of the eye. With the needle in the bevel up position and at 45° angle, puncture the mouse eye, but ensure that only the sharp tip of the needle (~0.5 mm) is inserted when injecting.

4.12. Once the needle tip is inserted, move the left hand from the mouse head to the mouse pad and right click on the mouse pad to inject 0.5 µL of the *B. cereus* dilution. To prevent leakage, leave the needle tip inside the mouse eye for 2-3 seconds before removing (**Figure 3E**)[1,19,26,27,32,34,36-38].

4.13. Release the forceps and place the mouse into a cage that is sitting on a warming pad. Monitor these mice until they have recovered from anesthesia (**Figure 3F**).

4.14. Once mice are recovered from the anesthesia, return the cage to its proper rack. If the mice will be subjected to retinal function analysis by electroretinography, cages should be returned to a dark room for proper dark adaptation.

5. Harvesting tube preparation

5.1. Place 1 mm sterile glass beads into 1.5 mL screw cap tubes.

5.2. Sterilize these tubes in an autoclave on a dry setting. Let the tubes cool to room temperature before use.

5.3. Add 10 mL of 1x sterile phosphate-buffered saline (PBS) to a sterile 15 mL centrifuge tube.

5.4. Add 1 tablet of protease inhibitor cocktail tablet into the tube. Mix by vortexing[19,27,29,34,35].

5.5. Pipette 400 µL of 1x phosphate-buffered saline (PBS) containing protease inhibitor into each autoclaved harvest tube. Label the tubes and place on ice. (**Figure 4A**).

6. Harvesting the eyes

6.1. Perform all procedures in this section under Biosafety Level 2 conditions.

6.2. Euthanize the mouse by CO_2 inhalation. Use a secondary method to confirm euthanasia.

6.3. Hold the euthanized mouse head secure and open the fine tip forceps on either side of the infected eye. Push down towards the head to proptose the eye. Once the tongs are behind the globe of the eye, squeeze the tongs together. Pull forceps away from the head to detach the eyeball (**Figure 4B**).

6.4. Immediately place the eyeball into a labeled harvesting tube. Place tubes on ice for no more than 60 min (**Figure 4C**).

7. Intraocular bacterial count

7.1. Perform all procedures in this section under Biosafety Level 2 conditions.

7.2. Confirm that all harvest tubes are tightly closed and are balanced while in the tissue homogenizer (**Figure 5A**). Turn on the tissue homogenizer for 1 min to homogenize the samples. Wait for 30 s, then turn on for another minute. Place tubes on ice (**Figure 5B,C**).

7.3. Serially dilute each sample 10-fold by sequentially transferring 20 µL of the homogenate into 180 µL of sterile 1x PBS. Dilute until a factor of 10^{-7} is reached (**Figure 5D**).

7.4. Label each row of a warm, square BHI plate with the proper dilution factors. Transfer 10 µL of each dilution in a row to the top BHI plate that is tilted approximately 45°. Let the sample run until it almost reaches the bottom of the plate, then lay the plate flat (**Figure 5E**)[39].

7.5. When sample is absorbed into the BHI agar, transfer the plate to a 37 °C incubator. Colonies should begin to be visible 8 h after being placed in the incubator.

7.6. Remove the plate from the incubator before the growth of the *B. cereus* colonies interferes with identifying individual colonies (**Figure 5F**).

7.7. For an accurate representation of the concentration in the sample, count the row that has between 10-100 colonies. For example, a row with the dilution fraction of 10^{-4} that has 45 colonies will have a concentration of 4.5×10^5 CFU/mL.

7.8. To calculate the total number of bacteria per eye, multiply the concentration by 0.4, which represents the milliliter volume of 1x PBS used to homogenize the eye. For example, the 4.5 x

10^5 CFU/mL concentration would translate to 1.8 x 10^5 CFU *B. cereus* per eye.

8. Preservation of samples

8.1. Place homogenate samples in a labeled freezer box and place this box into a -80 °C freezer. These samples can be used later for inflammatory mediator analysis by ELISA.

REPRESENTATIVE RESULTS:

Generating a reproducible inoculum and accuracy of the intravitreal injection procedure are key steps in developing models of microbial endophthalmitis. Here, we demonstrated the intravitreal injection procedure using Gram-positive *Bacillus cereus*. We injected 100 CFU/0.5 µL of *B. cereus* into the mid-vitreous of five C57BL6 mice. After 10 h postinfection, we observed intraocular growth of *B. cereus* to approximately 1.8 x 10^5 CFU/eye. **Figure 1** demonstrates the construction of glass needles to deliver the bacteria into the midvitreous of the mouse eyes. *Bacillus cereus* growing on a blood agar plate and in culture tubes is shown in **Figure 2**. **Figure 3** shows the mouse intravitreal injection procedure using an air pressurized injection system. **Figure 4** demonstrates the process of harvesting the infected eyes after the desired time postinfection. **Figure 5** shows the technique for homogenizing the infected eyes. **Figure 6** demonstrates the intraocular bacterial counts from five different mouse eyes at 10 h postinfection. **Figure 7** depicts the overall procedure and a graphical representation of a mouse intravitreal injection.

FIGURE LEGEND:

Figure 1: Making beveled glass needles. Glass needles were made from disposable microcapillary pipettes using a needle/pipette puller and a micropipette beveler. (**A**) Clamped glass capillary tube in the needle/pipette puller. (**B,C**) Creation of glass needles using the desired voltage. (**D**) Beveling the glass micropipettes. (**E,F**) Glass micropipettes before and after beveling. (**E**) Scaling the glass needles. Space between two black points holds 0.5 µL.

Figure 2: *Bacillus cereus* ATCC 14579. (**A**) *Bacillus cereus* growing on a blood agar plate. Individual colonies of *B. cereus* typically display clear zones of hemolysis on a blood agar plate. (**B**) Turbid overnight cultures of *B. cereus*. (**C**) Gram-staining of *B. cereus*. *B. cereus* are Gram-positive rod-shaped bacteria. (**D**) Electron micrograph of *Bacillus cereus*. This electron micrograph shows rod shaped *Bacillus cereus* with hair like structures called flagella.

Figure 3: Mouse intravitreal injection. The injection procedure is performed using a pressurized air injector with viewing of the operating field using a commercial microscope (**A**) ketamine and xylazine drug to anesthetize the mouse. (**B**) Administration of ketamine and xylazine by intraperitoneal injection to anesthetize the mouse. (**C**) Clamping periocular skin back to proptose the eye. (**D**) Filling up the needles with bacteria using the air pressurized injector. (**E**) Intravitreal injection. (**F**) Monitoring infected mouse after anesthesia.

Figure 4: Harvesting mouse infected eye. Post-infection, after the desired time point, harvest infected mouse eyes using sterile tweezers. (**A**) PBS containing sterile glass beads. (**B**) Harvesting of the infected eye. (**C**) Harvest tube containing a mouse eye.

Figure 5: Processing the harvested eye for intraocular bacterial count. (**A**) Harvest tube with infected eye clamped tightly to a tissue homogenizer. (**B**) Infected eyes were homogenized twice for 1 min each. Track dilution (**D**) of the eye homogenates (**C**) and subsequent plating (**E**) for the bacterial quantitation. (**E**) Representative individual *Bacillus cereus* colony after track dilution.

Figure 6: Intraocular bacterial counts at 10 h postinfection. M, mouse number. CFU, colony forming units.

Figure 7: Mouse intravitreal injection. (**A**) Overall flowchart of the mouse intravitreal injection procedure. (**B**) Graphical presentation of the intravitreal injection. During the procedure, sterile, beveled glass needles containing a culture of *B. cereus* are inserted into the midvitreous of the mouse eye, and *B. cereus* are delivered using an air-pressurized microinjection system.

DISCUSSION:

Even with the availability of potent antibiotics, anti-inflammatory drugs, and vitrectomy surgery, bacterial endophthalmitis can blind a patient. Clinical studies have been useful in studying endophthalmitis; however, experimental models of endophthalmitis provide quick and reproducible results that can be translated to progress in standard of care, resulting in better visual outcome for patients.

The vitreous volume of the mouse eye is approximately 7 µL[40]. This small volume only allows for a limited amount of material to be injected. Volumes greater than 1.0 µL should not be injected in order to avoid ocular damage. The process requires specific equipment and practice of techniques to ensure reproducibility and accuracy. Endophthalmitis has been studied in primates, swine, rabbits, rats, guinea pigs and in the mouse[28,41-45]. Among the larger species, eyes are much closer in size to human eyes, and intravitreal injections into larger eyes can be performed without special equipment. Except for the mouse, the absence of knockout strains and reagents to study the host immune responses in other animal models constrains their usefulness in experimental endophthalmitis.

Endophthalmitis occurs most frequently as a complication of cataract surgery. This infection can also occur following penetrating ocular trauma or systemic infection. The visual outcome in this disease partly depends on the virulence of the infecting pathogen. In mice, visual outcome also depends on their immune status. Understanding the mechanisms of disease pathogenesis has been facilitated by studying the disease in experimental animal models[28]. The protocol for mouse intravitreal injection and the quantification of infection parameters can be adapted to study endophthalmitis initiated with almost any type of bacterial or fungal pathogen[28]. Furthermore, this protocol can also be applied and modified to study anatomical changes, inflammatory processes, and the gene expression profiles of both the bacteria and host during infection.

Mouse intravitreal injection and subsequent analysis of infection parameters consist of several critical steps (**Figure 7**). The glass needle must be accurately created, marked, and sterilized. The sharp end of beveled glass needle and proper scaling determines the adequate delivery of the

bacteria and globe puncture. If the end of the needle is short and is not appropriately beveled, it could create a large hole on the globe which could cause leakage, contamination of the globe, and an inaccurate disease outcome. Therefore, it is recommended to observe the glass micropipettes while beveling under 10x optical zoom of a bright field microscope. To ensure the delivery of the correct quantity of bacteria, dilutions should be calculated beforehand, and the proper volume scale should be marked on the glass needle. Parameters for needle pulling and beveling using other types of equipment may vary.

The described intravitreal injection technique utilizes an air-pressurized microinjector system for the proper delivery of the bacteria into the mouse eye[1]. The appropriate use of this microinjector is crucial for the reproducible infection parameters. Since the air pressure determines the speed of delivery, excessive force might rapidly inject a larger volume into the eyes, causing excessive intraocular pressure and/or globe rupture. Therefore, the recommendation is to use a pressure of 10-13 psi to fill and inject during the procedure. Another potential issue is leakage of the bacterial solution from the glass needles after filling. Leakage could result in the injection of inaccurate volumes into the mouse eyes. Always check connections between the injection system, tubing, and glass needles before injections.

Injecting accurate quantities of bacteria is vital for the reproducibility of experimental bacterial endophthalmitis. Different experimental endophthalmitis models require inoculation of specific numbers of bacteria[12,28,46-49]. For *B. cereus* endophthalmitis, injecting 100 CFU is needed to initiate a reproducible infection[1]. Since bacterial growth depends on the growth medium and conditions, the growth environment, media, and dilutions must be repeated each time. Therefore, it is recommended to test the culture conditions before the experiment to reproduce the accurate inoculum in the appropriate volume for injection. As noted above, the upper limit for intravitreal injection into mouse eyes is 1.0 µL[28]. An excessive volume elevates the intraocular pressure which could result in glaucoma, detach the retina from the posterior segment, or rupture the globe. Intravitreal injection and the resulting infection in mice may not perfectly mimic *B. cereus* endophthalmitis in a human. Most animal models of human disease are limited in this way. Known quantities of *B. cereus* are injected into the eye after growth in nutrient-rich media. For clinical cases of *B. cereus* endophthalmitis, the infecting quantities are not known and the sources of contamination vary. However, the advantages of using characterized organisms and mouse strains and generating reproducible infection courses outweigh these limitations.

Proper harvesting of infected eyes is another critical step. Maintaining aseptic conditions is essential to avoid any cross-contamination which could interfere with interpretation of the data. Therefore, the recommendation is to disinfect the workbench and all instruments with 70% ethanol before harvest. Bacterial numbers increase rapidly inside the vitreous environment during infection, which may cause swelling of the eye. Therefore, care should be taken to gently remove the eye during harvesting prevent rupture of the globe. Furthermore, the cap of the harvest tube containing the infected eye must be adequately tightened before placing the tube in tissue homogenizer. A loose cap will result in leakage and contamination of the tissue homogenizer leading to inaccurate quantitation of bacteria in the leaking tubes. The eye homogenization procedure also elevates temperatures of the tubes. Therefore, it is

recommended to homogenize 1 minute at a time. Elevated temperatures could impact the quantitation of some infection parameters. While this method does not provide the number of bacteria within specific locations of the eye, when combined with histological methods, we can estimate where bacteria might be localized. Localization of *B. cereus* in the eye during endophthalmitis has been reported in rabbits, whose eyes are larger and more easily dissected into subcompartments.[2]

Intravitreal injection mimics the delivery of organisms to the posterior segment of the eye, which initiates infection. This initial step facilitates the qualitative and quantitative study of infection parameters in a highly reproducible mouse model of experimental endophthalmitis. These models are also used to estimate ocular inflammation by quantifying myeloperoxidase in infiltrating neutrophils, identifying specific cell types by flow cytometry, quantifying cytokines and chemokines by real-time PCR and/or ELISA, and observing ocular architecture by histopathology[1,6,19,20,26,27,34,35,38]. Infected mouse eyes are harvested with different diluents depending on the type of infection parameters to be measured. For gene expression analysis, a different lysis buffer is required[26]. For histopathological examination, harvested eyes are placed in a fixative solution. The multitude of genetic knockout mice also facilitates the study the role of various immune factors and cells. Intravitreal injection is therefore a mainstay technique for researchers in the field of intraocular infections and therapeutics.

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DISCLOSURES:

The authors have no financial conflicts to disclose.

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Figure 1

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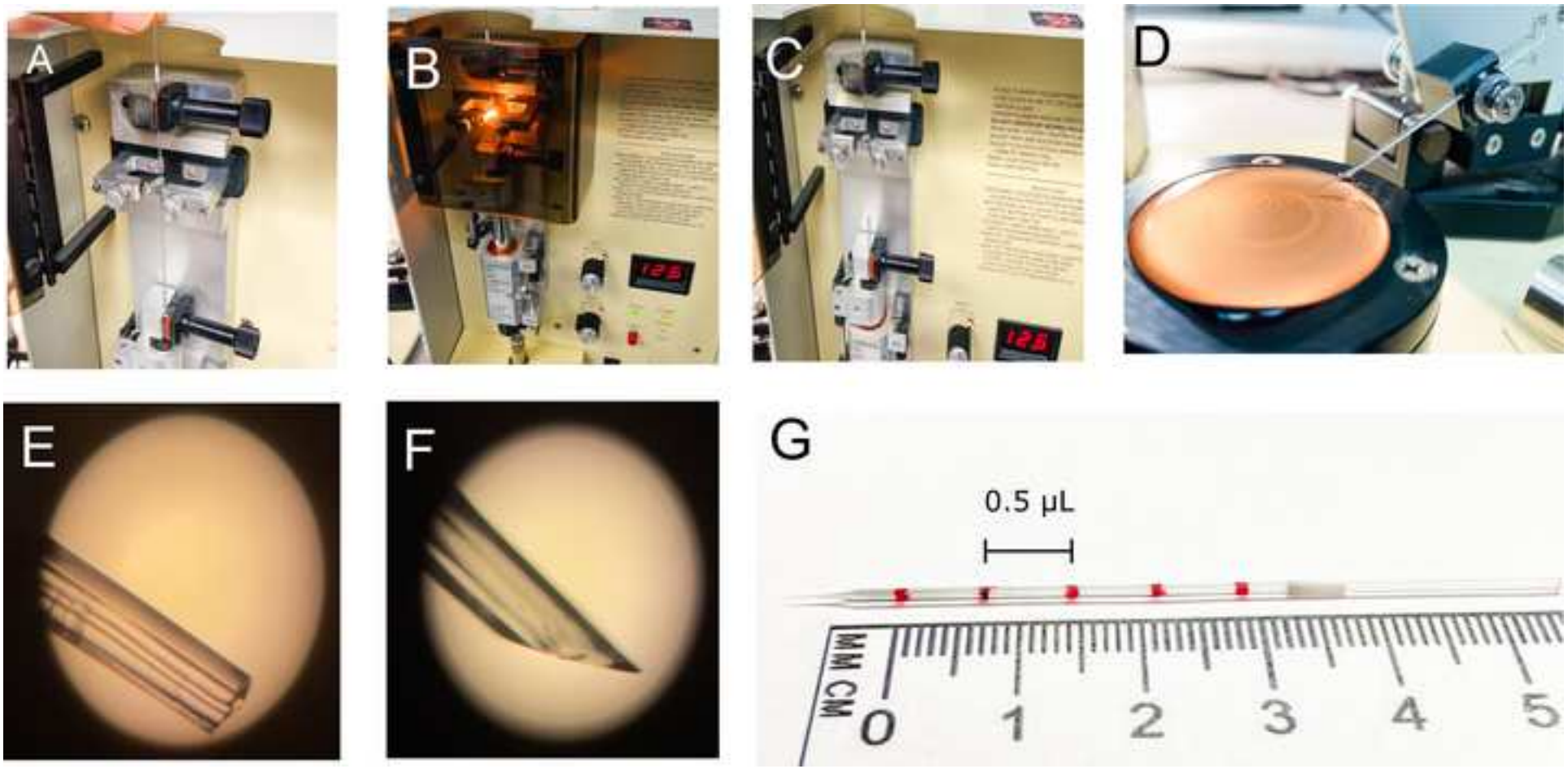

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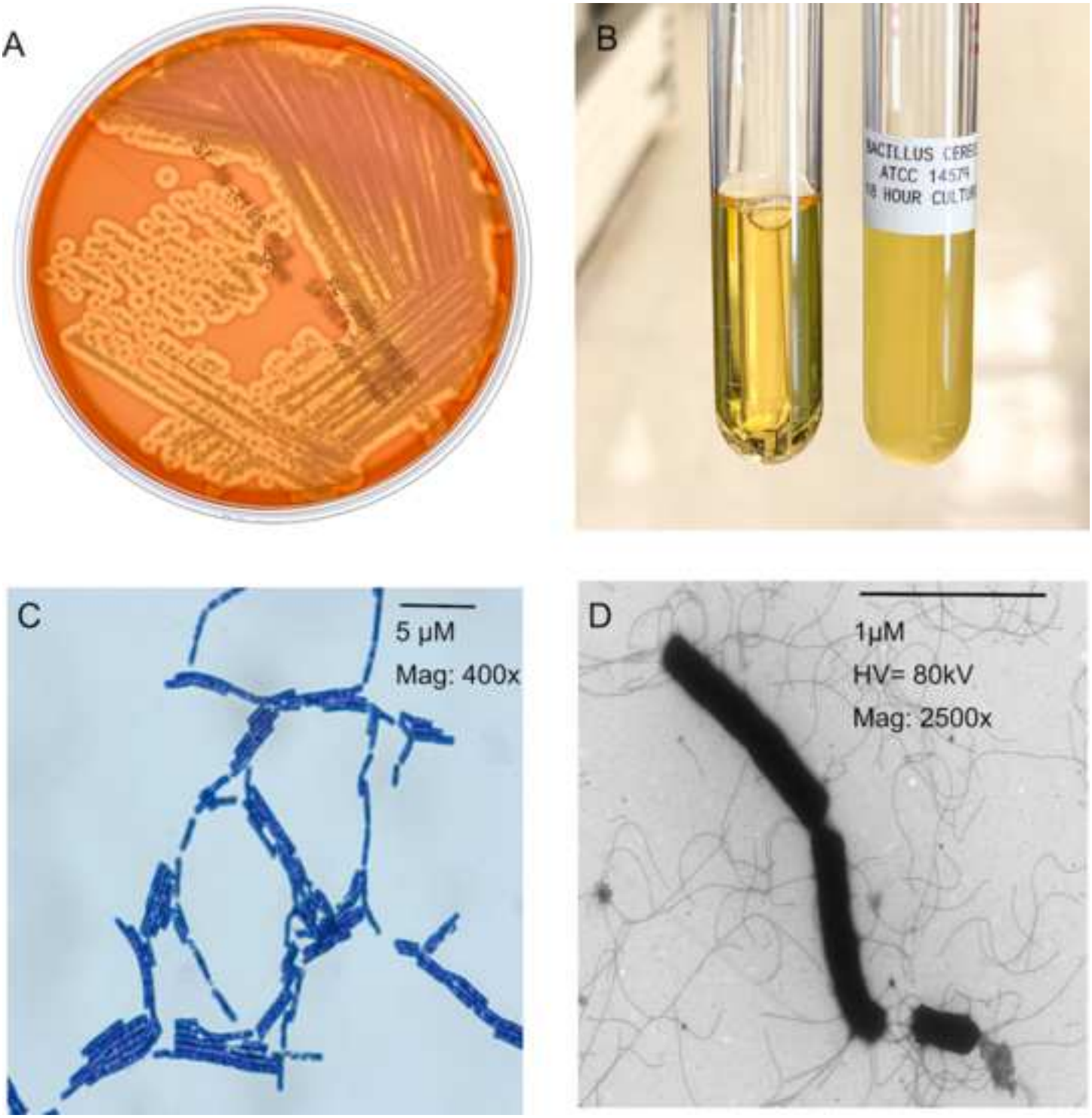

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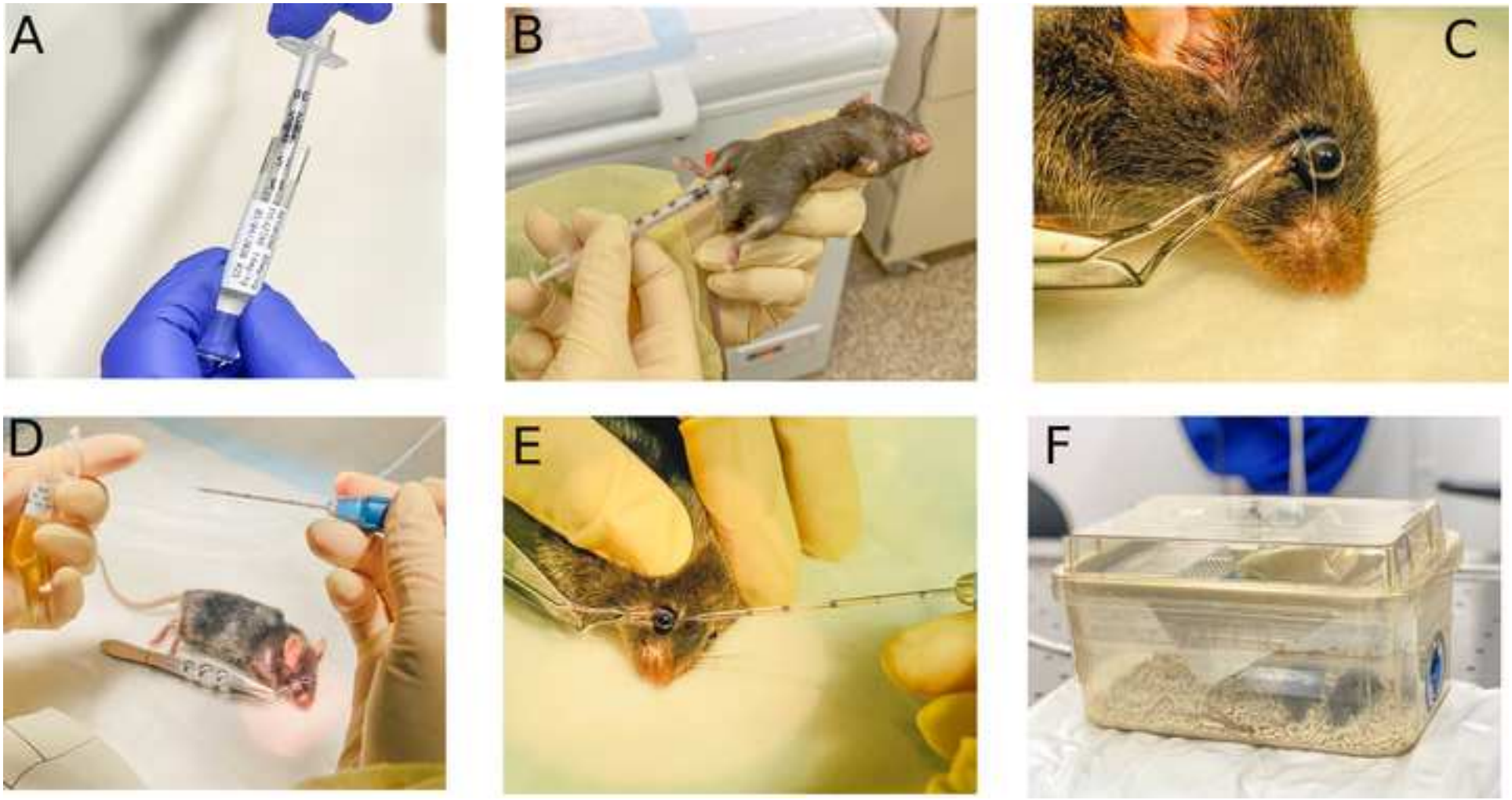

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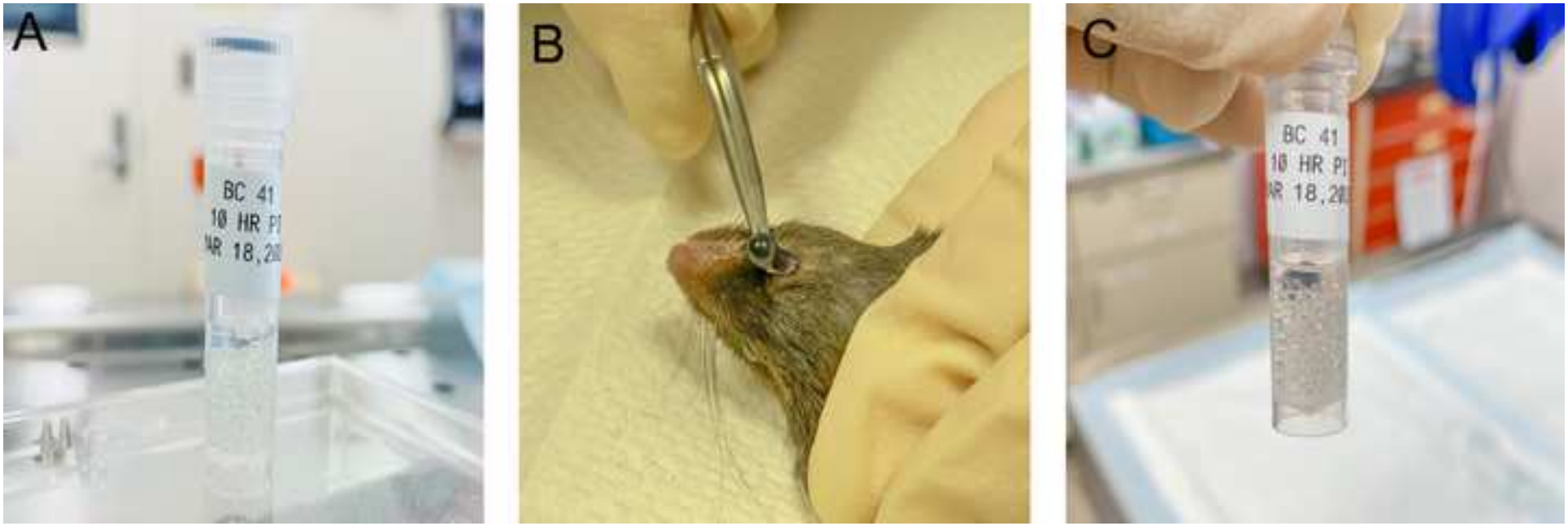

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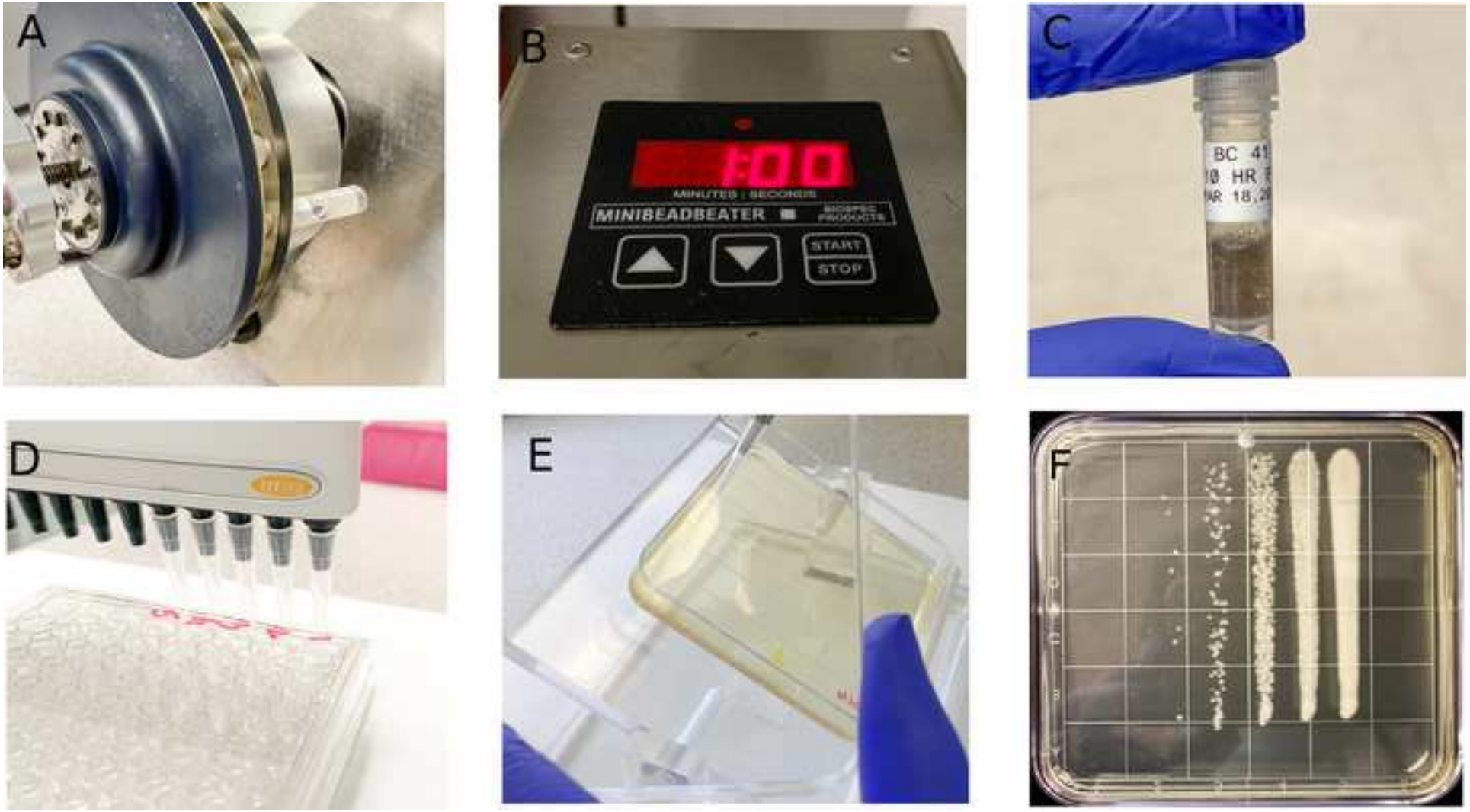

Figure 6

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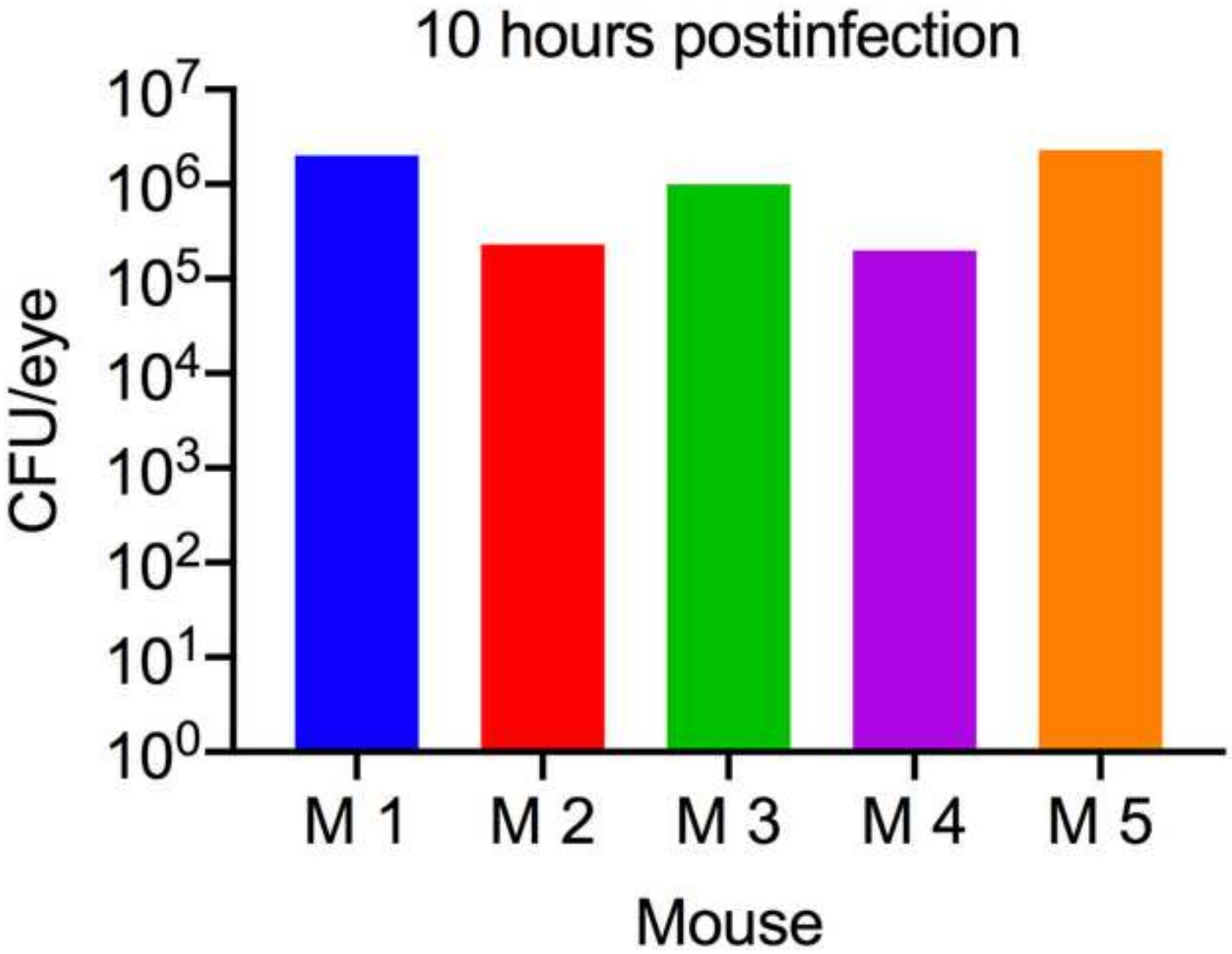

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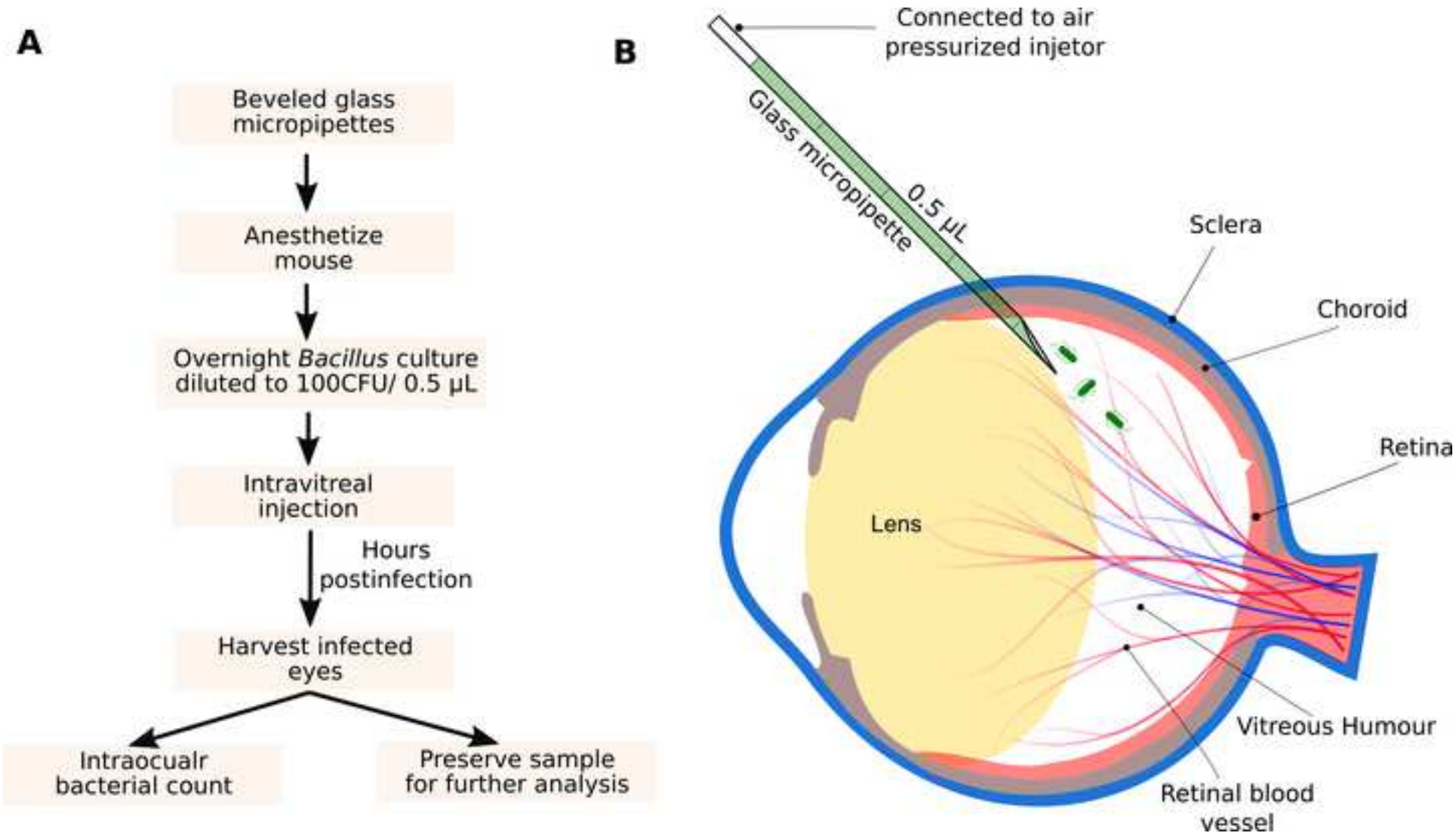

Click here to access/download;Table of Materials;JOVE 61749R1 Table of Materials.xlsx

Name of Material/Equipment	Vendor
2-20 µL pipette	RANIN
37°C Incubator	Fisher Scientific
Bacto Brain Heart Infusion	BD
Cell Microinjector	MicroData Instrument, Inc.
Fine tip forceps	Thermo Fisher Scientific
Glass beads 1.0 mm	BioSpec
Incubator Shaker	New Brunswick Scientific
Microcapillary Pipets 5 Microliters	Kimble
Micro-Pipette Beveler	Sutter Instrument Co.
Microscope Axiostar Plus	Zeiss
Microscope OPMI Lumera	Zeiss
Mini-Beadbeater-16	BioSpec
Multichannel pipette 30-300 µL	Biohit
Multichannel pipette 5-100 µL	Biohit
Needle/Pipette Puller	Kopf
PBS	GIBCO
Protease Inhibitor Cocktail	Roche
Reverse action forceps	Katena

Model/Catalogue Number	Comments/Description
L0696003G	NA
11-690-625D	NA
90003-032	NA
PM2000	NA
12-000-122	NA
11079110	NA
NB-I2400	NA
71900-5	NA
BV-10	NA
	NA
	NA
Model 607	NA
15626090	NA
9143724	NA
730	NA
1897315	Molecular grade
4693159001	Molecular grade
K5-8228	NA

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July 29, 2020

To the Editors and Reviewers of *Journal of Visualized Experiments*,

We are submitting our revised manuscript (JOVE61749R1) and thank the Reviewers for their efforts to improve the manuscript. We have addressed each comment to the best of our abilities in the paper and in a separate point-by-point response letter.

On behalf of all authors, I state again that there were no financial, personal, or professional interests that could be construed to have influenced the writing of this paper. All authors are aware and approve of this submission, its content, authorship, and the order of authorship.

We look forward to a thorough and positive review and appreciate the opportunity to submit our work to *Journal of Visualized Experiments*.

Best Regards,

Michelle C. Callegan, Ph.D.
OUHSC Ophthalmology

JoVE61749R1 (07/29/2020) RESPONSE TO REVIEWERS

We thank the Reviewers for their thorough and positive review of our JoVE submission. We address each comment below and denote where in the revised manuscript those changes can be found.

Editorial Comments:

• *…proofread the manuscript to ensure that there are no spelling or grammatical errors.*
Correct as suggested.

• *Avoid punctuating the title.*

Corrected as suggested, page 1.

• *Please list a minimum of 6 keywords/phrases.*
Keywords and phrases amended, page 1.

• ***Protocol Language:*** *Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique…1) Some examples NOT in the imperative: 1.11*
Addressed as suggested in 1.11 and throughout.

• ***Protocol Detail:*** *…* ***Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps. …***

Addressed as suggested throughout.

1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.
Ethics statement added, page 3.

• ***Protocol Highlight:*** *Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps…*
Relevant text has been highlighted in Sections 1-7.

• ***Discussion:*** *JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.*
The Discussion covers these areas.

• ***Figures:***
1) Fig 2: Add scale bars to all micrographs.
Added as suggested.

• ***References:***
1) Please spell out journal names.

Corrected as suggested.

• ***Commercial Language:*** *JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. 1) … replace all commercial sounding language in your manuscript with generic names that are not company-specific.*

Corrected as suggested throughout.

• ***Table of Materials:*** *Please sort in alphabetical order.*

Corrected as suggested.

Reviewer #1:

Minor Concerns:
For method 7.7, wouldn't the concentration be 4.5 x 10E5 CFU per 10 uL (rather than per mL) since 10 uL was plated?

For bacterial track dilution, the multiplication of a dilution factor with the respective colonies represent the CFU/mL. That is why 45 colonies at 4th dilution represents 45×10^4 CFU/mL or 4.5×10^5 CFU/mL.

Reviewer #2:

Major Concerns: *My only concern was that the authors only describe intravitreal injection of Bacillus or bacterial species and its quantification can only be applied for bacterial infections. The qualification of fungal spores/hyphae would vary and would not be applicable in this context. Hence the authors should change their title to specify only Mouse Model of BACTERIAL Endophthalmitis.*

We agree and have changed the title as suggested.

Minor Concerns:
It would also be useful for Point 3.2- that the authors specify the McFarland concentration used for qualification as initial load and overnight growth may vary among clinical strains.

We did not use a McFarland standard to quantify the concentration stated in the protocol. Point 3.2 (page 5) states that the growth of a *Bacillus* overnight achieves 2 x 10e8/10 uL. We included the dilution needed to achieve 200 CFU/uL of *Bacillus*. We discussed the variation in the growth of other species in the Discussion (full paragraph #1 on page 11).

Respectfully submitted,