Journal of Visualized Experiments

A rabbit model of aqueous-deficient dry eye disease induced by concanavalin A injection into the lacrimal glands: Application to drug efficacy studies

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59631R3
Full Title:	A rabbit model of aqueous-deficient dry eye disease induced by concanavalin A injection into the lacrimal glands: Application to drug efficacy studies
Keywords:	Dry eye, dry eye disease, dry eye syndrome, rabbit model, Concanavalin A, tear break up time, Schirmer's test, tear osmolarity, rose bengal staining, ocular drug development
Corresponding Author:	Robert Honkanen, MD Stony Brook Medicine Stony Brook, NY UNITED STATES
Corresponding Author's Institution:	Stony Brook Medicine
Corresponding Author E-Mail:	Robert.Honkanen@stonybrookmedicine.edu
Order of Authors:	Robert Honkanen, MD
	Liqun Huang
	Basil Rigas
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Stony Brook, New York, United States of America

TITLE:

A Rabbit Model of Aqueous-Deficient Dry Eye Disease Induced by Concanavalin A Injection into the Lacrimal Glands: Application to Drug Efficacy Studies

AUTHORS AND AFFILIATIONS:

Robert A. Honkanen[1], Liqun Huang[2], Basil Rigas[2]

[1]Department of Ophthalmology, Stony Brook University, Stony Brook, NY, USA

[2]Department of Preventive Medicine, Stony Brook University, Stony Brook, NY, USA

Corresponding Author:

Basil Rigas

basil.rigas@stonybrookmedicine.edu

Email Addresses of Co-authors:

Robert A. Honkanen Robert.Honkanen@StonyBrookMedicine.edu

Liqun Huang Liqun.Huang@StonyBrookMedicine.edu

KEYWORDS:

dry eye, dry eye disease, dry eye syndrome, rabbit model, Concanavalin A, tear break-up time, Schirmer's tear test, tear osmolarity, rose bengal staining, ocular drug development

SUMMARY:

This article describes the development of a method to induce acute or chronic dry eye disease in rabbits by injecting concanavalin A to all portions of the orbital lacrimal gland system. This method, superior to those already reported, generates a reproducible, stable model of dry eye suitable for the study of pharmacological agents.

ABSTRACT:

Dry eye disease (DED), a multifactorial inflammatory disease of the ocular surface, affects 1 in 6 humans worldwide with staggering implications for quality of life and health care costs. The lack of informative animal models that recapitulate its key features impedes the search for new therapeutic agents for DED. Available DED animal models have limited reproducibility and efficacy. A model is presented here in which DED is induced by injecting the mitogen concanavalin A (Con A) into the orbital lacrimal glands of rabbits. Innovative aspects of this model are the use of ultrasound (US) guidance to ensure optimal and reproducible injection of Con A into the inferior lacrimal gland; injection of Con A into all orbital lacrimal glands that limits compensatory production of tears; and use of periodic repeat injections of Con A that prolong the state of DED at will. DED and its response to test agents are monitored with a panel of parameters that assess tear production, the stability of the tear film, and the status of the corneal and conjunctival mucosa. They include tear osmolarity, tear break-up time, Schirmer's tear test, rose bengal staining, and tear lactoferrin levels. The induction of DED and the monitoring of its parameters are described in detail. This model is simple, robust, reproducible, and informative. This animal model is suitable for the study of tear physiology and of the pathophysiology of DED as well as for the assessment of the efficacy and safety of candidate agents for the treatment of DED.

INTRODUCTION:

Dry eye disease (DED) is a chronic condition with high prevalence and morbidity[1-4]. Inflammation plays a key role in its pathogenesis[5,6]. The pathophysiology of DED is conceptualized as deriving from either under-production or over-evaporation of tears; the former is also known as aqueous-deficient DED[7]. Sjögren's syndrome, an extensively studied prototypical cause of DED, affects primarily the lacrimal glands (LGs) and is a striking example of their importance in the pathogenesis of DED. DED is often treated with artificial tears which provide temporary relief, or with cyclosporine or lifitegrast, both of which suppress ocular inflammation. None of the available treatments for DED are optimal, necessitating the development of new agents[8,9].

The search for new therapeutic agents for DED is hampered by three major challenges: the lack of a recognized druggable molecular target, which may be elusive given the pathophysiological complexity of DED; the sparsity of promising agents; and the lack of animal models that recapitulate key features of DED.

As with most drug development efforts, informative animal models of DED are a crucial investigative tool, notwithstanding the axiomatic statement that no animal model completely recapitulates a human disease. Mouse, rat, and rabbit models of DED are the most commonly used while dogs and primates are used infrequently[10,11]. Most of the more than 12 rabbit DED models reported to date attempt to reduce tear production by either removing LGs or by impeding their function[12-16]. Such approaches include the surgical resection of the ILG; closure of its excretory duct; and impairing LG function by irradiation or injection of one of the following: activated lymphocytes, mitogens, botulinum toxin, atropine, or benzalklonium. Major limitations of these methods are their inconsistency and the frequent partial suppression of tear production.

Concanavalin A (Con A), a lectin of plant origin, is a potent stimulator T-cell subsets and has been used in experimental models of hepatitis[17] and DED[18]. The original Con A-based model was reported to offer significant advantages, including its relative simplicity; inflammatory cell influx in the LGs, mimicking diseases such as Sjogren's; stimulation of the proinflammatory cytokines IL-1β, IL-8, and TGF-β1; reduced tear function monitored by measuring tear fluorescein clearance and tear break-up time (TBUT); and drug responsiveness shown for an anti-inflammatory corticosteroid.

When this promising method was applied, in addition to its advantages, limitations were identified that necessitated its overall revision and drastic improvements. Three critical shortcomings of the method are documented. First, the model was an acute one; the induced DED subsided after about 1 week. Second, the response of the animals was inconsistent. As demonstrated, in "blind" transcutaneous injections to the Inferior LG (ILG), Con A was delivered only randomly to the targeted gland. Detailed study of the anatomy of the ILG revealed that its size could vary as much as 4-fold[19] making such injections "hit-or-miss" efforts. Finally, even when the ILG was injected, the superior LG (SLG) frequently compensated for the reduced tear flow, making the model problematic.

These key limitations were overcome by introducing three modifications to the method, generating a superior animal model of DED. First, the injection of Con A into the ILG was performed under ultrasound (US) guidance, ensuring that Con A entered the gland. The success of the injection was confirmed by obtaining a post-injection US image, as shown in **Figure 1**. Second, to remove the compensatory tear contribution of the SLG, both the palpebral and orbital portions of this gland were injected with Con A. Finally, this acute model of DED was converted to a chronic one by repeated injections of Con A every 7-10 days. DED of 2 months' duration is readily achieved in these rabbits. The success of this approach has been amply documented[19].

As already mentioned, an important application of animal models of DED is to determine the efficacy and safety of candidate therapeutic agents. The utility of this model was demonstrated by the study of phosphosulindac (OXT-328), a novel anti-inflammatory small molecule[20,21] administered as eye drops. Its efficacy was demonstrated based on a panel of parameters of DED[19]. The relative simplicity and informative nature of this model also allowed side-by-side comparison of phosphosulindac to the two FDA approved drugs for DED, cyclosporine and lifitegrast, demonstrating its strong preclinical superiority.

PROTOCOL:

All animal studies were approved by the Institutional Review Board of Stony Brook University and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

1. Animals and housing

1.1. Acquire New Zealand White (NZW) rabbits weighing 2-3 kg.

1.2. House the rabbits singly in cages with strict temperature (65 ± 5 ˚F) and humidity (45 ± 5%) control. Lighting should have a 12 h on/off cycle.

1.3. Provide unlimited access to water and standard rabbit chow. Eliminate dietary enrichments as they may contain vitamin A that affects the eye.

1.4. Acclimate the animals for at least 2 weeks prior to baseline measures or induction of dry eye.

2. Methods of anesthesia and euthanasia

NOTE: All procedures require mild sedation except for Con A injection that requires moderate sedation.

2.1. For mild sedation, inject acepromazine (1 mg/kg) subcutaneously over the shoulders using a 26-gauge needle. Endpoint for mild sedation: animals maintain a relaxed head position with ear lobes no longer fully upright.

NOTE: If the appropriate endpoint is not reached, an additional injection of acepromazine may

be given. Animals should always remain awake, responsive to touching of their whiskers, and never show slowed breathing.

2.2. For moderate sedation, first give the animals acepromazine as above. After the endpoint is reached (see the note above), give isoflurane using a gas mask with O_2 flow set at 1 L/min and isoflurane delivery set to 5% (**Figure 2**).

2.3. Administer isoflurane until the rabbit's body tone is completely relaxed and ears are completely floppy.

NOTE: No compensatory muscle movements should occur when the animal is turned on its side; breathing always remains spontaneous.

2.4. Spontaneous recovery occurs within 2-5 min: signs include spontaneous head movements and increased or normal muscular tone. After the experimental procedure is completed with moderate sedation, observe the rabbits for about 30 min or until their behavior returns to normal.

NOTE: Ophthalmic ointment is not required during either form of sedation. 1) In mild sedation, animals are still alert and maintain a blink reflex. In moderate sedation, the inhibition of the blink reflex is so short that the ocular surface is not at risk. 2) Placement of ophthalmic ointment on the ocular surface precludes visualization of structures assessed during testing.

2.5. Euthanasia: Use an overdose of intravenous pentobarbital (100 mg/kg).

3. Removal of nictitating membrane

3.1. Perform the removal during the acclimation period (usually the first week) to allow complete and accurate evaluation of the cornea.

3.2. Injection to the right nictitating membrane

NOTE: If the nictitating membrane of both eyes is to be removed, it is simplest to do this in one session. Start with one eye and proceed as described. For clarity of description, this method starts with the right eye.

3.2.1. Place the rabbit in an appropriately sized restraining bag.

3.2.2. Induce mild sedation as described in step 2.1.

3.2.3. Apply 25 µL of preservative-free lidocaine to the right eye using a micropipette.

3.2.4. Place a flexible wire lid speculum between the eyelids.

3.2.5. Using 0.3 forceps (or equivalent), grasp the nictitating membrane at its apex and extend it over the cornea.

3.2.6. Inject lidocaine 1% with 1:100,000 epinephrine subconjunctivally into the base of the nictitating membrane using a 26-gauge sharp needle (**Figure 3A**). A moderate bleb should form over the nictitating membrane.

3.2.7. Remove the speculum.

3.3. Perform an identical injection to the left nictitating membrane.

3.4. Cutting the nictitating membrane

3.4.1. After approximately 5 min, place the lid speculum back in the right eye. Grasp and retract the nictitating membrane at its apex using 0.3 forceps (or similar).

3.4.2. Cut the nictitating membrane off at its base using Westcott scissors or equivalent (**Figure 3B**).

NOTE: Bleeding is minimal and does not typically require cautery. Nevertheless, a high-temperature battery cautery is always kept nearby in case additional hemostasis is needed.

3.4.3. Remove the speculum.

3.4.4. Place topical antibiotic ointment on the eye (e.g., neomycin, polymyxin, bacitracin, and hydrocortisone).

3.5. Leave the Harderian gland intact. The Harderian gland is sometimes seen when the nictitating membrane is retracted.

NOTE: If a large white mass or tissue elevation is seen in the nasal or superior subconjunctival region after the nictitating membrane is removed, the membrane was resected too close to its base allowing the Harderian gland to spontaneously prolapse. To prevent this in subsequent procedures, leave more of the nictitating membrane at the base.

3.6. Allow the ocular surface to heal for at least 1 week before further manipulations are made or ocular surface assays are performed.

4. Measurement of dry eye parameters and collection of tear samples

NOTE: Measure DED parameters based on study protocol needs (e.g., at baseline and specified time points thereafter). Measurements for DED should be done in the following order, with rigorous effort to faithfully replicate them each time. Test all animals at approximately the same

time of day (± 1 h) to minimize circadian variations. These measurements usually require a team of two investigators.

4.1. Place the rabbit in a restraining bag. Induce mild sedation.

4.2. **Tear Osmolarity**[22]

4.2.1. Manually blink the eyelids 5-10 times to evenly distribute the tear layer on the ocular surface.

4.2.2. Gently retract the lower lid.

4.2.3. Sample tears with the TearLab Osmometer at the junction of the palpebral and bulbar conjunctiva along the lower fornix, just posterior to the base of the truncated nictitating membrane.

4.2.4. Measure osmolarity using the TearLab Osmolarity Test following the manufacturer's instructions.

4.3. **Tear break-up time** (TBUT)

4.3.1. Darken the room for this assay.

4.3.2. Place a wire lid speculum between the eyelids.

4.3.3. Apply a 50 µL drop of 0.2% fluorescein over the corneal surface using a micropipette. If even distribution of the fluorescein over the cornea is not obtained with the first drop, place a second drop.

4.3.4. Immediately start a timer.

4.3.5. Observe the pre-corneal tear film under a blue light. The TBUT is defined as the time taken to develop black dots, lines or obvious disruption of the fluorescein film (**Figure 4**). If needed, use surgical loupes that provide +1.50 magnification to better visualize early signs of break-up. Monitor for up to 1 min; if break-up as defined here occurs after 1 min, record TBUT for only 60 s.

4.4. **Schirmer's Tear Test** (STT)

4.4.1. Apply a 25 µL drop of preservative-free lidocaine to the ocular surface.

4.4.2. Place a Weck cell surgical spear into the inferior fornix to absorb residual lidocaine and any tear fluid. If needed, use the lower eyelid to cover the proximal end of the sponge to help keep it in place (**Figure 5A**).

4.4.3. After approximately 30 s, remove the Weck sponge.

4.4.4. Immediately insert a Schirmer's tear test strip into the space between the cornea and the palpebral conjunctiva at the mid-point of the lower lid.

4.4.5. Immediately start a timer (**Figure 5B**).

4.4.6. After 5 min, measure the length of the moistened portion of the strip; this is the STT value.

4.4.7. Perform measures in triplicate and report the average of the 3 readings as the STT value.

4.5. **Collection of tear samples**

4.5.1. To collect tear samples for assaying levels of various analytes in them such as lactoferrin, after the STT value is recorded at 5 min, leave the strip in place until wetting of at least 20 mm is obtained.

NOTE: If adequate wetting does not occur after DED has been induced, advance the strip deeper into the lower fornix to help reach this endpoint in reasonable time.

4.5.2. Cut the moistened strip and place immediately into 490 µL of chilled Tear Collection Buffer (4% BSA, 1M NaCl, 0.1% Tween-20 in PBS with proteinase inhibitor cocktail).

4.5.3. Keep the samples on ice until they can be stored at -80 °C, where they should remain until assayed.

4.6. **Rose Bengal Staining** (RBS)

4.6.1. Apply 50 µL of 1% preservative-free lidocaine to the cornea using a micro-pipette.

4.6.2. After 30 s, place 25 µL of 1% rose bengal on the ocular surface and manually blink the eyelid to distribute it evenly.

4.6.3. Immediately start a timer.

4.6.4. At 3.5 min, place a wire lid speculum between the lids.

4.6.5. At 4.0 min, photograph the superior conjunctival and corneal surface (**Figure 6**).

NOTE: Adjust to the type of camera being used. Typical settings: digital single-lens reflex camera, aperture priority mode (aperture 13 or greater), ISO 6000, 100 mm macro lens attached with two 12.5 mm extension tubes, manual focus mode, lens at maximum magnification, and illumination from macro/ring flash set to automatic with through-the-lens mode. The ring flash's focus lamp

is turned on to aid focus on the cornea.

4.6.6. Complete all photos for both eyes within 1 min.

4.7. Score ocular surface staining using the NEI method[23] modified as follows**.** Don not grade 6 separate conjunctival zones. Score the superior conjunctiva of each eye. This is the portion of the conjunctival surface easily photographed without manipulating the globe. Manipulation could artefactually change the staining of the ocular surface.

4.8. **Tear lactoferrin levels** are a surrogate measure of tear production from the lacrimal glands. Assay tear lactoferrin in tears collected as above by using an enzyme-linked immunosorbent assay[24] kit following the instructions of the manufacturer.

5. Induction and treatment of dry eye

NOTE: Three portions of the orbital lacrimal gland system are injected.

5.1. Sedate the rabbits with acepromazine 0.2 mg/kg subcutaneously.

5.2. Shear off the fur in the periorbital and scalp area and completely remove any residual fur using Nair. Leave the skin entirely smooth for better visualization of the anatomical hallmarks and US-guided injection of concanavalin A (**Figure 7**).

5.3. Induce moderate sedation as described above.

5.4. **Injection of the palpebral portion of the superior lacrimal gland** (PSLG)

NOTE: Perform injection of PSLG first.

5.4.1. Apply to the appropriate eye 25 µL of preservative-free lidocaine 1% with a micropipette.

5.4.2. Evert the upper eyelid and apply gentle medial pressure to the posterior orbital rim until the protuberance marking the palpebral portion of the gland is seen. The PSLG appears as a small bulbous elevation in the posterior (temporal) portion of upper lid.

NOTE: To view the gland tissue during the learning process, apply 5% fluorescein to the area (**Figure 8A**). Tears can be seen streaming from the bulbous PSLG. Application of fluorescein is not needed for the administration of Con A; it is done only for illustration purposes to show the gland tissue.

5.4.3. Using fine-toothed forceps and a 27-gauge needle on a tuberculin syringe, directly penetrate the gland using a transconjunctival approach. Advance the needle 2 mm into the tissue and inject 500 µg of Con A in a volume of 0.1 mL (**Figure 8B**).

NOTE: This injection can sometimes be painful. If necessary, keep the animals under isoflurane until this injection is completed.

5.5. **Injection of the orbital superior lacrimal gland** (OSLG)

NOTE: OSLG follows in rapid succession.

5.5.1. Apply medial pressure to the globe causing the OSLG to protrude from the posterior incisure (see ref[25] for anatomy, if needed). Apply medial pressure to the globe (**Figure 9**, *red arrow*) with the protuberance of the OSLG from the posterior incisure. The protuberance serves as gross localization to find the posterior incisure.

5.5.2. Use curved forceps with tips closed to indent the area until the bony opening in the skull is felt. This will be slit-like with an anterior/posterior direction under the protuberance.

5.5.3. Apply modest pressure with forceps to leave an indentation in the skin, which will serve as the landmark for needle placement (**Figure 10A**).

5.5.4. Insert a needle (tuberculin syringe with a 27-gauge, 5/8-inch needle) perpendicular to the skin over the indentation mark (**Figure 10B**) ~ ¼ inch into the incisure, then redirect the needle posteriorly and externally towards the lateral canthus aiming for the midpoint between the injection site and bony orbital rim.

NOTE: If the incisure is not precisely targeted with the needle, the skull blocks its advancement.

5.5.5. Once the hub of the needle is reached, slowly inject 1000 µg of Con A in a volume of 0.2 mL (**Figure 10C**).

5.6. Complete the injection of both the PSLG and OSLG within 2-3 min.

5.7. Remove the animal from isoflurane sedation (if not yet done). Injection of the inferior lacrimal gland (ILG) can usually be completed without further sedation.

5.8. **Injection of the inferior lacrimal gland**

5.8.1. View the animal from the side. The prominence of the ILG can be seen along the lower anterior portion of the orbit (**Figure 11A**).

5.8.2. Draw a vertical line using a surgical marking pen or suitable permanent marker on the skin where the superficial part of the ILG gland transitions from its superficial (more external) resting place on the zygomatic bone to its more medial location in the orbit. This is typically inferior to the anterior limbus (**Figure 11A**).

5.8.3. Identify the end of the zygomatic bone by sweeping the vertically-held US probe across this

line on the skin. The ILG transition occurs where the image of the gland changes from clearly circumscribed (hyperechoic line of the zygomatic bone is seen along the lower edge of the gland in the image) to one without a recognizable medial border (the zygomatic bone echo is no longer present, **Figure 1**).

5.8.4. Observe the relative position of the hand-piece to the line drawn on the skin when the US screen shows this change. This is the "injection site" where Con A should be given.

5.8.5. Control the depth of injection so as to place Con A into the gland at a point just medial to the zygomatic arch bone.

5.8.6. Determine the depth of injection as follows: Set the desired depth of injection as the depth of the zygomatic bone (hyperechoic signal) plus 1 mm. Subtract this value from the known length of the needle (15 mm in this example).

5.8.7. Insert the needle into the gland at the "injection site" ~12 mm, then slowly withdraw it until the length of the exposed needle outside the body (measured with surgical calipers) is equal to the difference calculated in 5.8.6 (**Figure 12**). Inject 1000 µg of Con A in 0.2 mL.

NOTE: To ensure that the capsule of the gland is *pierced* and not simply pushed by the needle, the needle should be inserted ~12 mm or almost to the hub before its withdrawal begins.

5.8.8. Repeat the US to confirm the success of the injection. The ILG should show a characteristic hypoechoic space (**Figure 1**).

NOTE: The ILG injection is the one best tolerated by the animals[26] and is, therefore, done last.

5.9. Complete the entire procedure to inject all glands of both eyes within 10 min. This will require achievement of competency in the procedure.

NOTE: A single set of injections into the 2 orbital lacrimal glands will induce acute DED lasting 1-2 weeks.

5.10. For DED of longer duration, inject Con A exactly as above every 7 days. Up to 6 such injections have been successfully performed.

6. Post-procedure care

6.1. Following injection of Con A, monitor the animals in their restraining bags for at least 10-20 min, or until the anesthetic effect has worn off.

6.2. Do not leave the animals unattended until they have regained sufficient consciousness to maintain sternal recumbency. Do not return them to their individual cages until they are fully

recovered.

6.3. Post-procedure pain is usually mild and lasts less than 48 h. Assess pain with the rabbit grimace scale. If needed, give a single dose of subcutaneous ketorolac (5 mg/kg). For more severe pain, give subcutaneous buprenorphine 0.1 mg/kg every 8 h.

REPRESENTATIVE RESULTS:

Con A injections induced a strong inflammatory response in the lacrimal glands characterized by a dense lymphocytic infiltrate (**Figure 13**), accompanied by decreased tear production. All tear parameters were markedly altered (**Table 1** and **Table 2**). In addition, tear lactoferrin levels were suppressed (control = 3.1±0.45 *vs.* Con A injected = 2.7±0.02 ng/mg protein (mean ± SEM); $p<0.03$). The end result was a compromised corneal and conjunctival epithelium, evidenced by increased rose bengal staining (**Figure 6**).

Injection of the three orbital LG tissues produced a consistent and uniform DED state unlike the states achieved by previous methods[18,27]. Key contributors to this result were the US-guided injection of the ILG and the injection of the OSLG. **Table 1** summarizes the salient results of this method. All changes are consistent with severe DED.

A single set of Con A injections produces DED lasting about 1 week; all clinical parameters normalize by day 10 (**Table 2**). Sequential Con A injections about 1 week apart extend the duration of DED accordingly. For example, the second set of Con A injections on day 7 maintains DED for 2 weeks and so on. After approximately 5 sets of injections, the DED state often becomes permanent without the need for further injections.

When the rabbits with Con A-induced DED were treated with the novel agent phosphosulindac, it markedly suppressed the disease. For example, following one week's treatment with this agent TBUT increased markedly compared to vehicle-treated animals (43.6±4.0 vs. 12.2±2.8 s; $p<0.001$; mean ± SEM respectively, for these and following values) while tear osmolarity was normalized (294±4.6 vs. 311±2.0 mOsm/L, $p<0.002$). Mechanistically, phosphosulindac decreased the levels of two crucial interleukins, IL-1β (8.4±1.2 vs 21.2±6.6 pg/mg protein; $p<0.03$) and IL-8 (4.9±1.7 vs. 13.5±5.0 pg/mg protein; $p<0.05$)[19].

FIGURE AND TABLE LEGENDS:

Figure 1. Ultrasound image of the inferior lacrimal gland. *Upper Panel:* The ILG as it moves deeper in orbit to lie beneath the zygomatic arch. The dashed line represents the line on skin across which the US probe is swept. *Middle Panels:* As the hand-piece is swept across this line, the examiner looks for loss of the zygomatic bone echo that is present in the left image (*arrow*) and disappears in the right. *Lower Panels:* Images of the ILG taken before (*left*) and after (*right*) injection of Con A. Development of a large cystic space within the gland confirms proper delivery. Reprinted with permission[19].

Figure 2. Gas mask sedation. This photograph shows the gas mask providing brief moderate sedation with isoflurane.

Figure 3. Removal of the nictitating membrane. (**A)** Injection of lidocaine/epinephrine. **(B)** Truncation of the nictitating membrane at its base with Westcott scissors. **(C)** The ocular surface visualized more readily after removal of the nictitating membrane.

Figure 4. Tear break-up time measurement. (**A)** Uniform green tear film appearance of the corneal surface under blue light immediately following application of fluorescein drops. **(B)** Corneal surface that has already undergone marked break-up evidenced by multiple dark circles and linear streaks in the fluorescein. Break-up time is recorded as soon as the first dark spot or line develops. The two light blue circles are reflections of the light source off of the cornea.

Figure 5. Schirmer's tear test. **(A)** The proper placement of the Weck-Cel sponge in the lower fornix to remove any residual topical lidocaine solution and baseline tears. By placing the posterior edge of the triangular sponge under the lower lid margin, one can maintain a very uniform technique to dry the ocular surface prior to placement of the tear strips. **(B)** A tear strip appropriately placed at the mid-position of the lower lid between the globe and lower lid (palpebral conjunctiva).

Figure 6. Rose bengal staining. **Upper:** Photographs of the corneal surface. Left: No rose bengal staining is present before treatment with Con A. Right: Diffuse corneal and conjunctival staining is seen in the upper nasal quadrant post-injection (upper right). **Lower:** Conjunctival impression cytology from the superior bulbar conjunctiva. Left: Numerous goblet cells are present before treatment. Right: Epithelial cells are present but goblet cells are absent post-treatment.

Figure 7. Preparation of rabbit for concanavalin A injections. (**A**) Small shears are used to remove fur, allowing easier visualization of landmarks to identify the orbital superior lacrimal gland. (**B**) Nair is used to remove hair that remains after shearing.

Figure 8. Injection of the palpebral lacrimal gland. (**A**) The palpebral lacrimal gland, appearing as a bulbous elevation in the posterior temporal portion of the upper lid. Tears are seen streaming from the surface of this gland after applying a drop of 2% fluorescein. (**B**) The palpebral lacrimal gland is being injected while the rabbit is receiving moderate sedation. One investigator retracts the eyelid, optimizes exposure of the gland, and secures the mask while the second investigator injects the gland.

Figure 9. Localization of the orbital superior lacrimal gland. Changes in skin contours indicate the location of the OSLG as it protrudes through the posterior incisure. Alternating medial pressure on the globe (*large arrow*) causes the superior orbital gland to prolapse, which is seen as a small elevation in the skin. This elevation will increase in size each time the pressure is applied (*small arrows*). The location of this gland is usually in line with the posterior orbital rim.

Figure 10. Injection of the orbital superior lacrimal gland. (**A**) Application of gentle pressure to the skull with fine-toothed forceps in the area which prolapsed as in **Figure 9**. A thin slit-like opening in the skull can be palpated. Leaving a small indentation mark with the forceps greatly

aids placement of the needle during injection. (**B**) The needle is being inserted perpendicularly through the incisure. If placed incorrectly, its passage is stopped by the bony skull. (**C**) The needle is in final position angled towards the lateral canthus.

Figure 11. Localization of the inferior lacrimal gland. (**A**) The prominence of the superficial portion of the ILG seen through the lower lid. The curvilinear pen mark denotes the lower position of the gland. The vertical line, under the nasal limbus, denotes the approximate position where the ILG transitions to a deeper position within the orbit and serves as a visual reference for the US. **(B)** US hand-piece sweeping across the area of the vertical line; the US monitor will show where the zygomatic bone ends, where the ILG transitions and where the Con A injection should be given ("injection site").

Figure 12. Injection of the inferior lacrimal gland. Injection of the ILG is done at the location identified by US. The depth of injection is calculated as described in the text (step 5.8.6). Calipers (seen behind the needle) ensure that the needle is placed at the proper depth before injection.

Figure 13. Histology of the lacrimal glands. Tissue sections of a normal inferior lacrimal gland with typical tubulo-alveolar structure (**A**) and following injection of Con A (**B**), showing marked lymphocytic infiltrate with effacement of structure. Similar inflammatory infiltrates are seen in the superior lacrimal glands.

Table 1. Effect of injection technique and number of injection sites on dry eye severity.

Table 2. Effect of repeated Con A injections into ILG on duration of DED.

DISCUSSION:

Rabbits are highly attractive for the study of DED. Their cornea and conjunctiva have a surface area closer to that of humans compared to mice and rats; their complement of drug metabolizing enzymes such as esterases, and histology of their lacrimal glands are similar to those of humans, and their eyes are large enough for informative pharmacokinetic studies. Compared to pigs and monkeys, with which they share similar features, they cost less and their experimental manipulation is easier. If mechanistic studies are contemplated, a relative drawback of the rabbit, compared to mice, is that fewer reagents (e.g., monoclonal antibodies) are available. On the other hand, the rabbit is far superior to mice for pharmacokinetic and biodistribution studies because individual tissues are easily dissected and of sufficient size for analytical work, avoiding "sample pooling."

A critical general parameter is the acclimation period of the rabbits. The animals are shipped from the vendor under conditions that often do not ensure a transportation environment of the appropriate temperature or humidity. Some animals may have already developed dry eye upon arrival. A two-week period of acclimation is recommended. Equally important is scrupulous attention to the humidity and temperature of the space where the study rabbits are housed in the vivarium. Deviations in either condition can induce huge variations in their eye status. Have back-up humidifiers and dehumidifiers on hand. If the central system fails, act quickly to restore

ambient humidity using the back-up equipment. Bear in mind that such unfortunate developments are more common in the summer months. The three most critical steps, however, for successfully inducing DED in rabbits are: 1) the skillful use of US imaging to identify the ILG and to direct and confirm injection of Con A; 2) ensuring injection of both the ILG and the two parts of the SLG; and 3) reliably and reproducibly assaying the parameters of DED.

Developing the required experimental skill is not trivial but should not deter any serious investigator. Expect the learning curve to be completed within five iterations. An US imaging system of reasonable quality is essential. Recognition of anatomical hallmarks by US is important, therefore, the investigator should review the rabbit anatomy. The excellent description of rabbit anatomy by Davis[25], a classic, can be immensely helpful. Also keep in mind the variation in the size of the ILG. The corollary to this is that the success of Con A must always be confirmed with follow up imaging. Variations in the response to Con A in a group of rabbits is most often due to the injection technique (unsuccessful or partially successful injection) or to ignoring the capacity of residual lacrimal gland tissues to compensate with overproduction of tears. For those who wish to master the injection technique, injecting methylene blue followed by prompt anatomic dissection can be helpful; visualization is achieved if it reaches the lacrimal gland or spills onto neighboring tissues. To date, this injection method has been performed over 270 times by the authors without a single complication.

Assaying the five parameters of DED presented above can be as tricky as is their determination in clinical practice. Although circadian variations have not yet been formally documented in any of them, there is enough background evidence of such phenomena in the eye[28] that they should be assayed at the same time of day (± 1 h), especially when repeat assays are to be performed and compared to each other. Consistency in performing these assays is essential. A team of two is required. Four or more investigators in the same room participating in the assays can be disruptive, given that some steps require strict timing. Appropriate and high-quality photographic documentation, where indicated, is important.

This model is ideally suited for drug development studies. Mastery of the animal model and assay techniques ensured excellent reproducibility[19] of efficacy and safety studies.

This is a powerful experimental approach because it eliminates the confounding variability of prior models, has streamlined the animal model and essentially standardized assaying the five parameters of DED. The successful application of this model to the study of a candidate therapeutic agent has affirmed its practical utility as an informative animal model for a disease in desperate need of novel agents and of a deeper understanding of its pathogenesis.

ACKNOWLEDGMENTS:
All animal studies were completed in accordance and compliance with all relevant regulatory and institutional guidelines. All studies were approved by the Institutional Review Board of Stony Brook University and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

These studies were supported in part by a Targeted Research Opportunities grant from the Stony Brook University School of Medicine and a research grant from Medicon Pharmaceuticals, Inc., Setauket, NY. The authors thank Michele McTernan for editorial support.

DISCLOSURES:

The authors declare no competing interests except for BR who has an equity position in Medicon Pharmaceuticals, Inc. and Apis Therapeutics, LLC; and LH, an employee of Medicon Pharmaceuticals, Inc. with an equity position in Apis Therapeutics, LLC.

REFERENCES:

1 Paulsen, A. J. et al. Dry eye in the Beaver Dam Offspring Study: prevalence, risk factors, and health-related quality of life. *American Journal of Ophthalmology.* **157** (4), 799-806, doi:10.1016/j.ajo.2013.12.023, (2014).

2 Vehof, J., Kozareva, D., Hysi, P. G. & Hammond, C. J. Prevalence and risk factors of dry eye disease in a British female cohort. *British Journal of Ophthalmology.* **98** (12), 1712-1717, doi:10.1136/bjophthalmol-2014-305201, (2014).

3 Tan, L. L., Morgan, P., Cai, Z. Q. & Straughan, R. A. Prevalence of and risk factors for symptomatic dry eye disease in Singapore. *Clinical and Experimental Optometry.* **98** (1), 45-53, doi:10.1111/cxo.12210, (2015).

4 Craig, J. P. et al. TFOS DEWS II Report Executive Summary. *The Ocular Surface.* **15** (4), 802-812, doi:10.1016/j.jtos.2017.08.003, (2017).

5 Baudouin, C. et al. Clinical impact of inflammation in dry eye disease: proceedings of the ODISSEY group meeting. *Acta Ophthalmologica (Copenhagen).* **96** (2), 111-119, doi:10.1111/aos.13436, (2018).

6 Calonge, M. et al. Dry eye disease as an inflammatory disorder. *Ocular Immunology and Inflammation.* **18** (4), 244-253, doi:10.3109/09273941003721926, (2010).

7 Pflugfelder, S. C. & de Paiva, C. S. The Pathophysiology of Dry Eye Disease: What We Know and Future Directions for Research. *Ophthalmology.* **124** (11S), S4-S13, doi:10.1016/j.ophtha.2017.07.010, (2017).

8 Buckley, R. J. Assessment and management of dry eye disease. *Eye (London, England).* **32** (2), 200-203, doi:10.1038/eye.2017.289, (2018).

9 Clayton, J. A. Dry Eye. *New England Journal of Medicine.* **378** (23), 2212-2223, doi:10.1056/NEJMra1407936, (2018).

10 Barabino, S. Animal models of dry eye. *Archivos de la Sociedad Española de Oftalmología.* **80** (12), 693-694; 695-696 (2005).

11 Stern, M. E. & Pflugfelder, S. C. What We Have Learned From Animal Models of Dry Eye. *International Ophthalmology Clinics.* **57** (2), 109-118, doi:10.1097/IIO.0000000000000169, (2017).

12 Chen, Z. Y., Liang, Q. F. & Yu, G. Y. Establishment of a rabbit model for keratoconjunctivitis sicca. *Cornea.* **30** (9), 1024-1029, doi:10.1097/ICO.0b013e3181f1b0fc, (2011).

13 Gilbard, J. P., Rossi, S. R. & Gray, K. L. A new rabbit model for keratoconjunctivitis sicca. *Investigative Ophthalmology & Visual Science.* **28** (2), 225-228 (1987).

14 Guo, Z. et al. Autologous lacrimal-lymphoid mixed-cell reactions induce dacryoadenitis in rabbits. *Experimenal Eye Research.* **71** (1), 23-31, doi:10.1006/exer.2000.0855, (2000).

15 Burgalassi, S., Panichi, L., Chetoni, P., Saettone, M. F. & Boldrini, E. Development of a simple dry eye model in the albino rabbit and evaluation of some tear substitutes. *Ophthalmic Research.* **31** (3), 229-235, doi:55537, (1999).
16 Xiong, C. et al. A rabbit dry eye model induced by topical medication of a preservative benzalkonium chloride. *Investigative Ophthalmology & Visual Science.* **49** (5), 1850-1856, doi:10.1167/iovs.07-0720, (2008).
17 Heymann, F., Hamesch, K., Weiskirchen, R. & Tacke, F. The concanavalin A model of acute hepatitis in mice. *Lab Animal.* **49** (1 Suppl), 12-20, doi:10.1177/0023677215572841, (2015).
18 Nagelhout, T. J., Gamache, D. A., Roberts, L., Brady, M. T. & Yanni, J. M. Preservation of tear film integrity and inhibition of corneal injury by dexamethasone in a rabbit model of lacrimal gland inflammation-induced dry eye. *Journal of Ocular Pharmacology and Therapeutics.* **21** (2), 139-148, doi:10.1089/jop.2005.21.139, (2005).
19 Honkanen, R. A., Huang, L., Xie, G. & Rigas, B. Phosphosulindac is efficacious in an improved concanavalin A-based rabbit model of chronic dry eye disease. *Translational Research.* **198** 58-72, doi:10.1016/j.trsl.2018.04.002, (2018).
20 Huang, L. et al. The novel phospho-non-steroidal anti-inflammatory drugs, OXT-328, MDC-22 and MDC-917, inhibit adjuvant-induced arthritis in rats. *British Journal of Pharmacology.* **162** (7), 1521-1533, doi:10.1111/j.1476-5381.2010.01162.x, (2011).
21 Mattheolabakis, G. et al. Topically applied phospho-sulindac hydrogel is efficacious and safe in the treatment of experimental arthritis in rats. *Pharmaceutical Research.* **30** (6), 1471-1482, doi:10.1007/s11095-012-0953-8, (2013).
22 Osmalek, T., Froelich, A. & Tasarek, S. Application of gellan gum in pharmacy and medicine. *International Journal of Pharmaceutics.* **466** (1-2), 328-340, doi:10.1016/j.ijpharm.2014.03.038, (2014).
23 Lemp, M. A. Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. *Contact Lens Association of Ophthalmologists Journal.* **21** (4), 221-232 (1995).
24 Dal Piaz, F., Braca, A., Belisario, M. A. & De Tommasi, N. Thioredoxin system modulation by plant and fungal secondary metabolites. *Current Medicinal Chemistry.* **17** (5), 479-494, doi:CMC - AbsEpub/2010 - 012 [pii], (2010).
25 Davis, F. A. The Anatomy and Histology of the Eye and Orbit of the Rabbit. *Transactions of the American Ophthalmological Society.* **27** 400 402-441 (1929).
26 Lima, L., Lange, R. R., Turner-Giannico, A. & Montiani-Ferreira, F. Evaluation of standardized endodontic paper point tear test in New Zealand white rabbits and comparison between corneal sensitivity followed tear tests. *Veterinary Ophthalmology.* **18 Suppl 1** 119-124, doi:10.1111/vop.12178, (2015).
27 Zheng, W. et al. Therapeutic efficacy of fibroblast growth factor 10 in a rabbit model of dry eye. *Mol Med Report.* **12** (5), 7344-7350, doi:10.3892/mmr.2015.4368, (2015).
28 Wiechmann, A. F. & Summers, J. A. Circadian rhythms in the eye: the physiological significance of melatonin receptors in ocular tissues. *Progress in Retinal and Eye Research.* **27** (2), 137-160, doi:10.1016/j.preteyeres.2007.10.001, (2008).

Figure 1

Figure 2

Click here to access/download;Figure;Fig 2-renumbered-Gas.mask.eps

Figure 3

Click here to access/download;Figure;Fig 3-renumbered-Nictating.eps

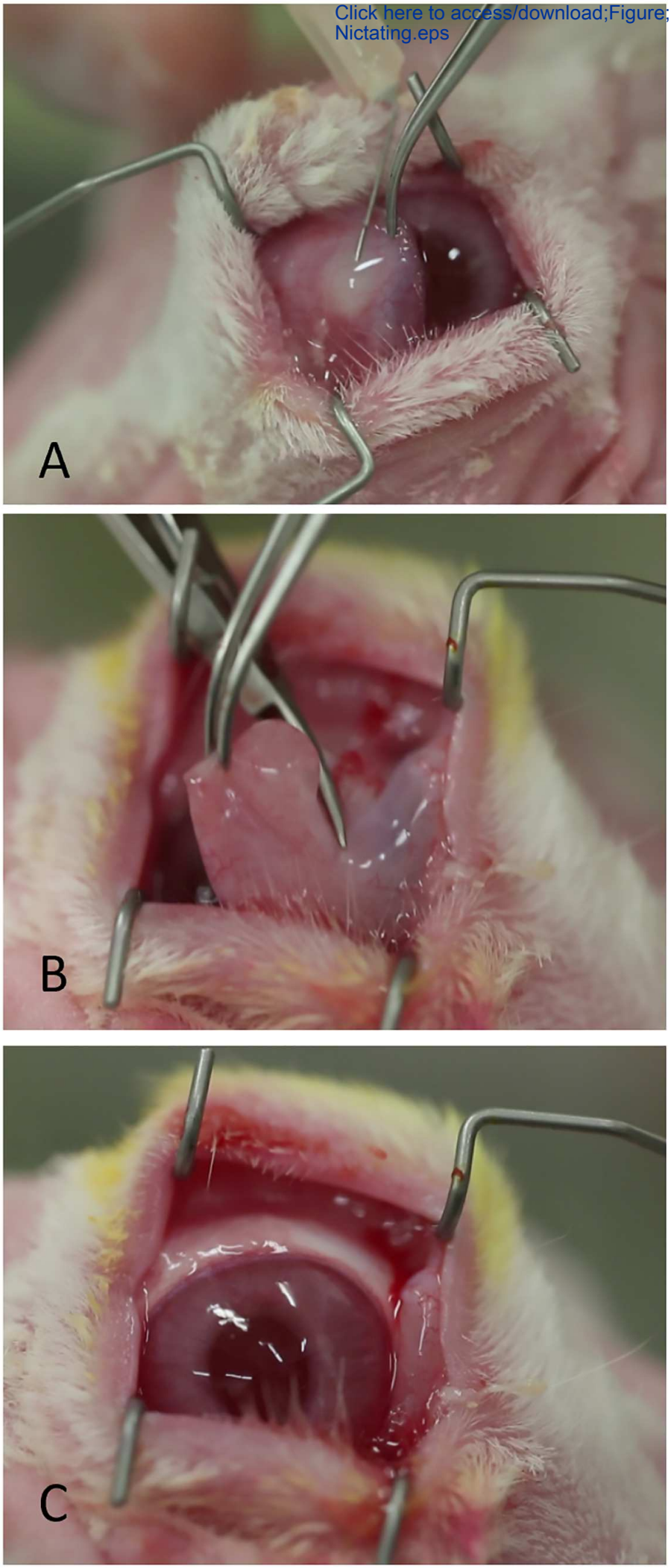


Figure 4

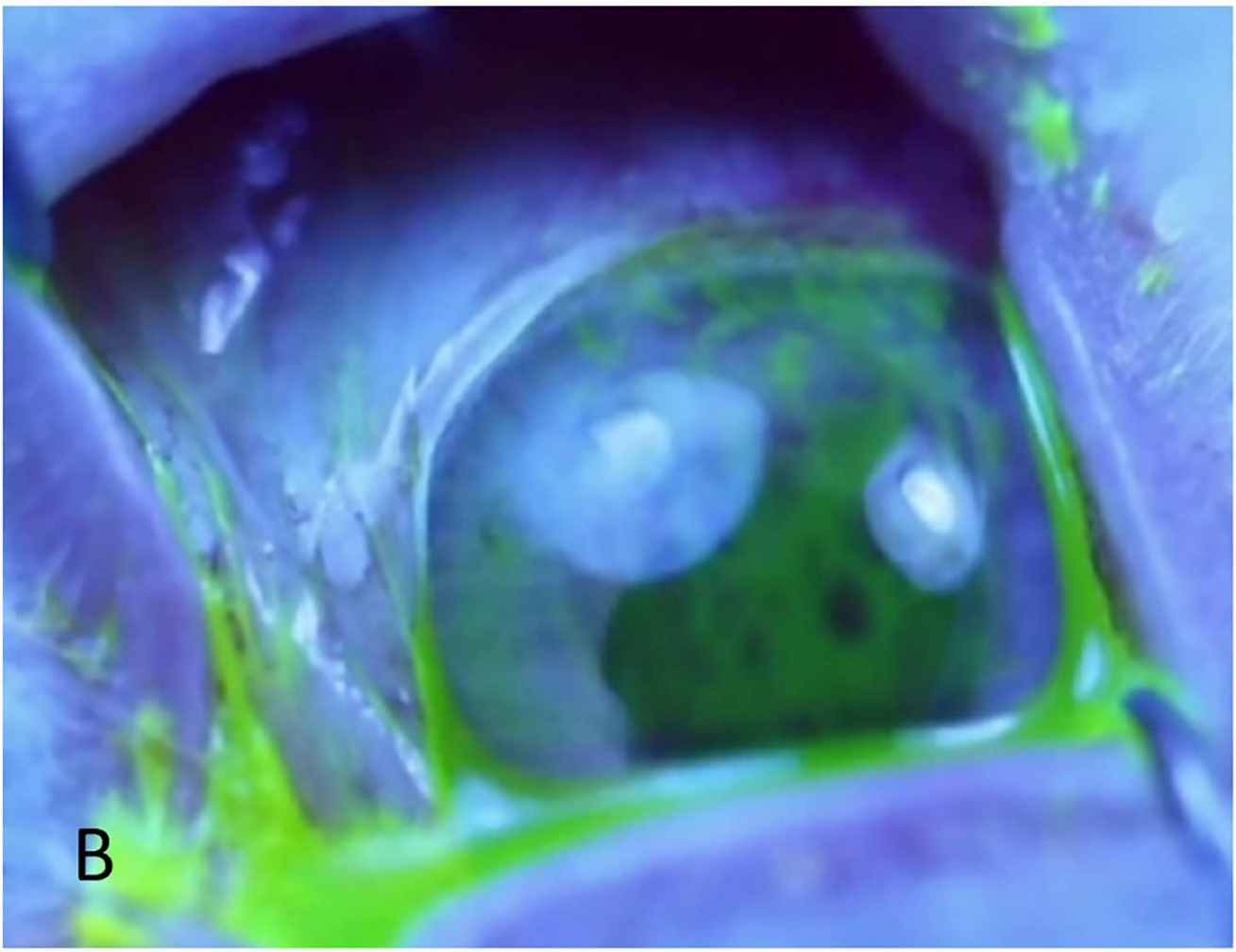


Figure 5

Click here to access/download;Figure;Fig 5-renumbered-Schirner.eps

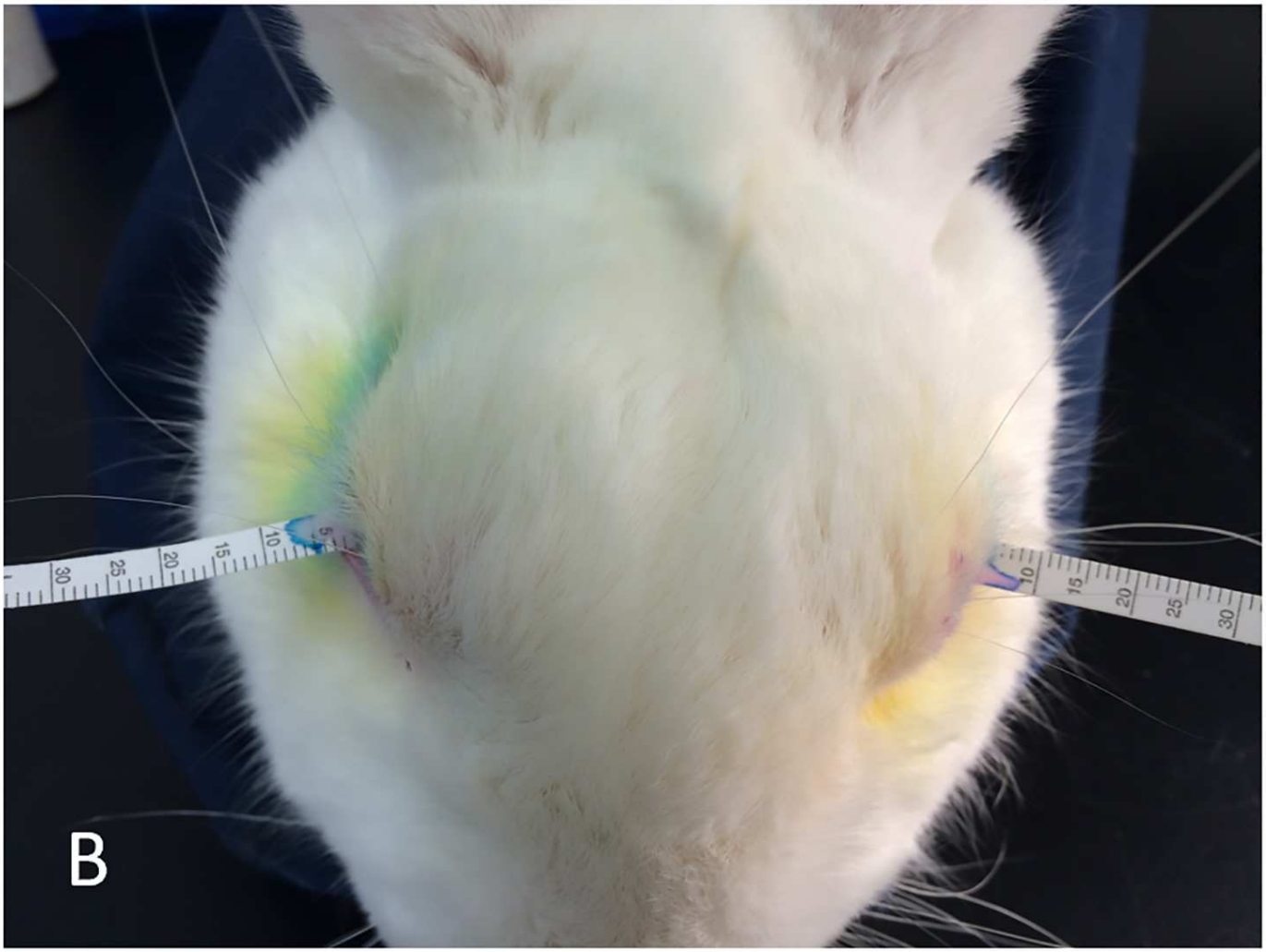


Figure 6

Click here to access/download;Figure;Fig.6-FINAL.TIFF

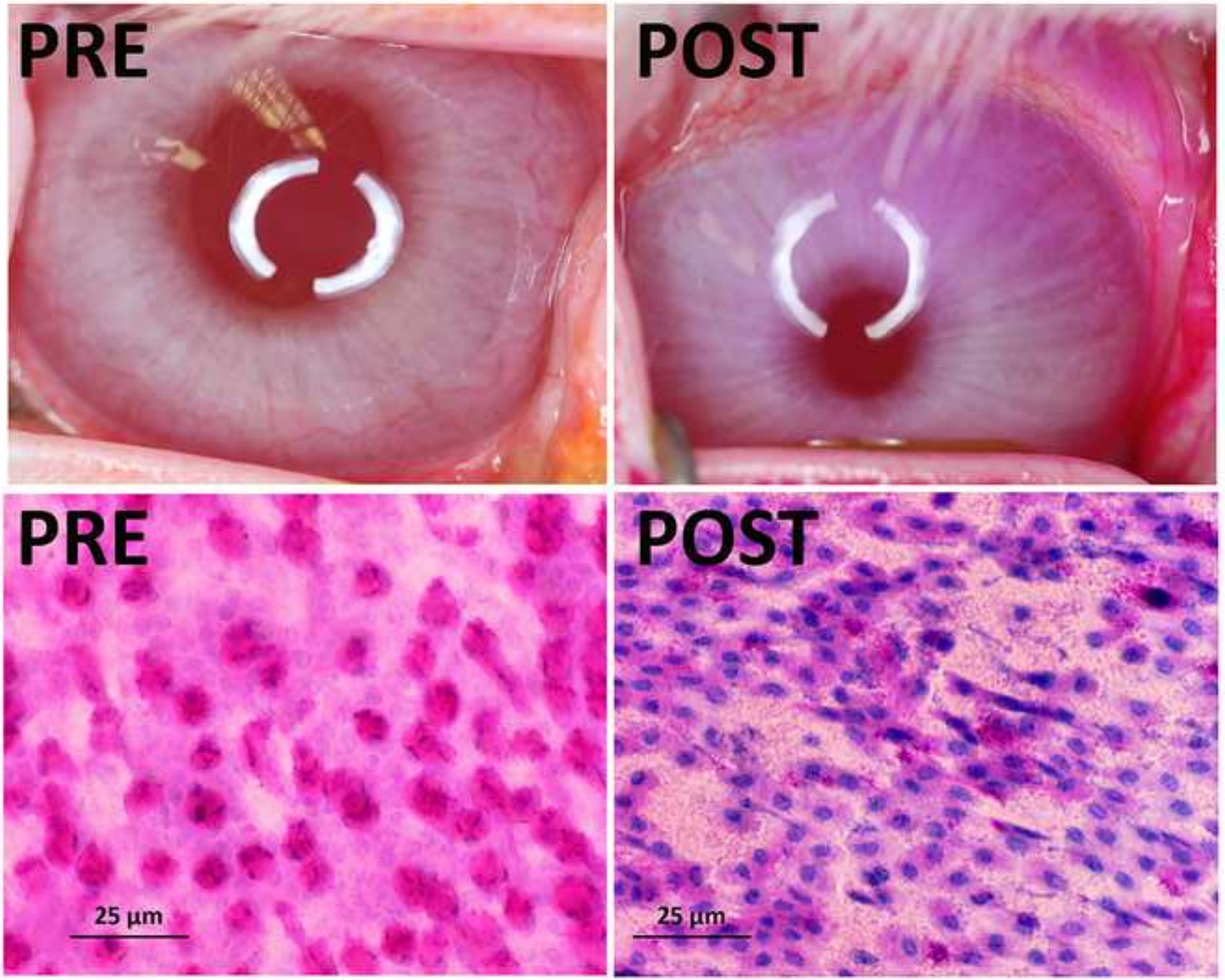


Figure 7

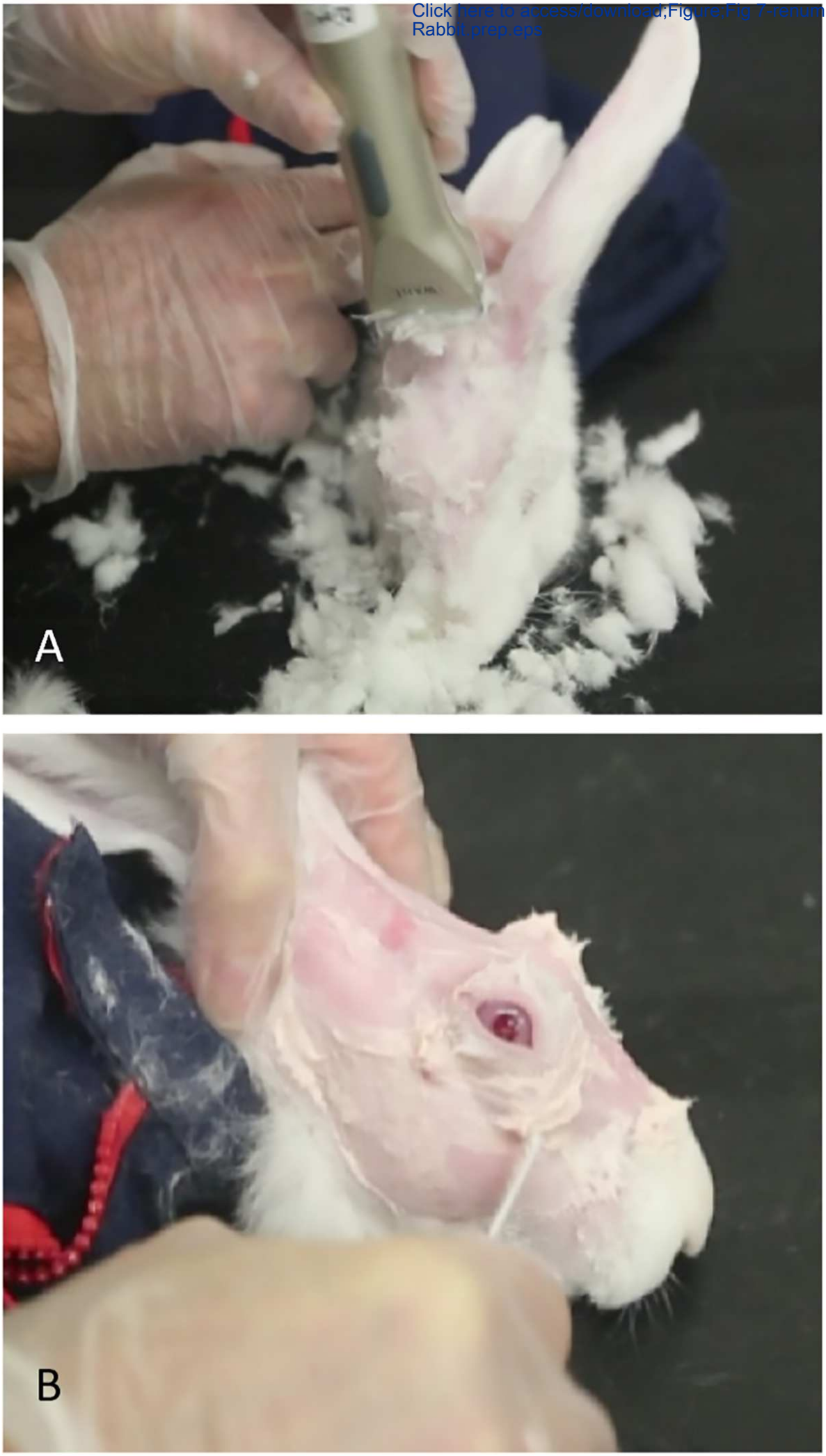
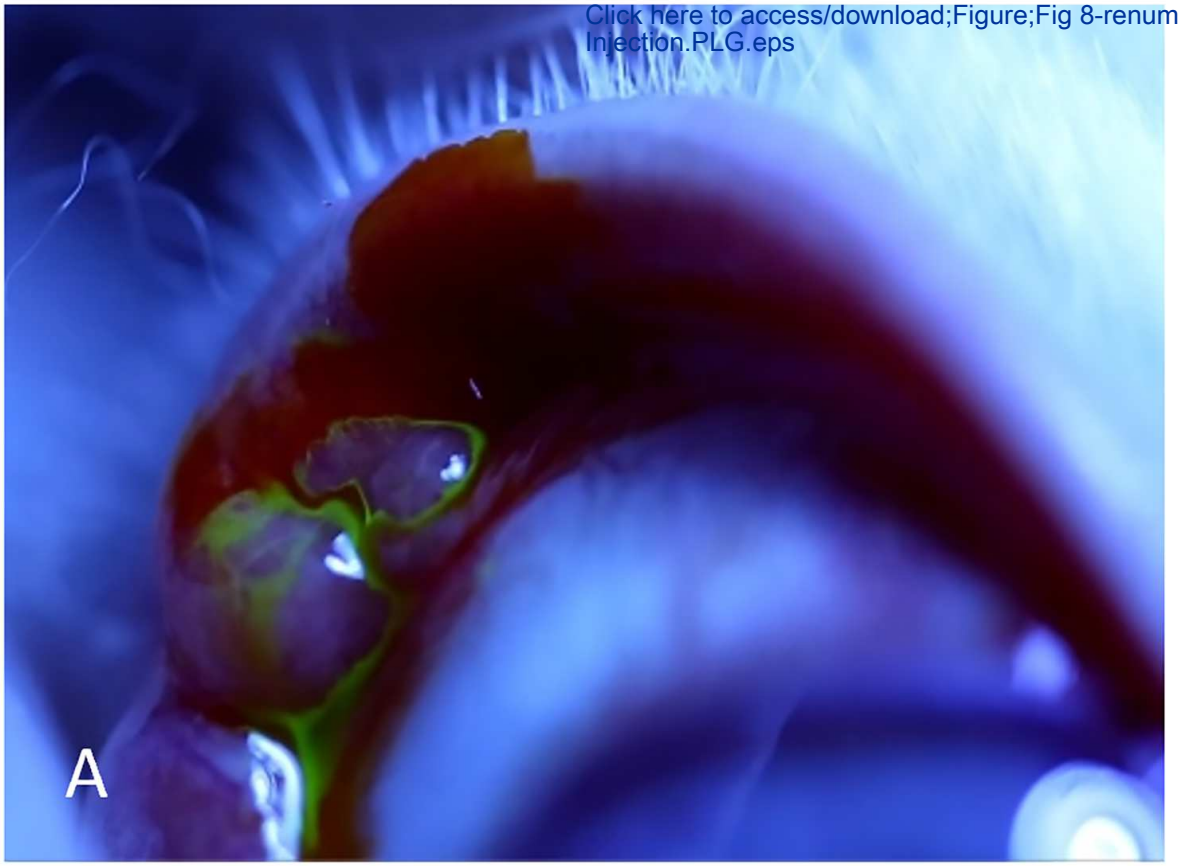


Figure 8

Click here to access/download;Figure;Fig 8-renumbered-Injection.PLG.eps



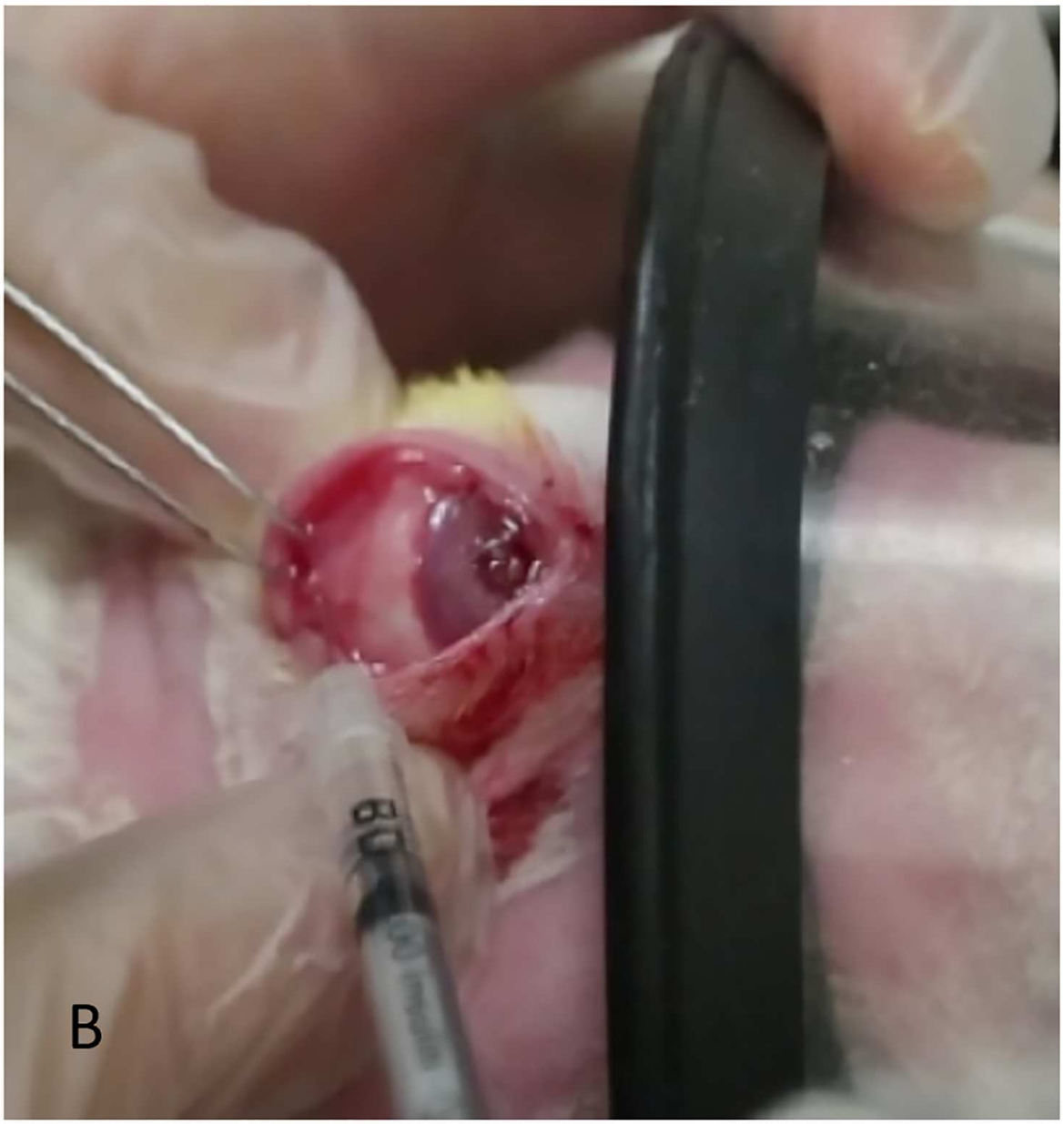


Figure 9

Click here to access/download;Figure;Fig 9-renumbered-Locate.OSLG.eps

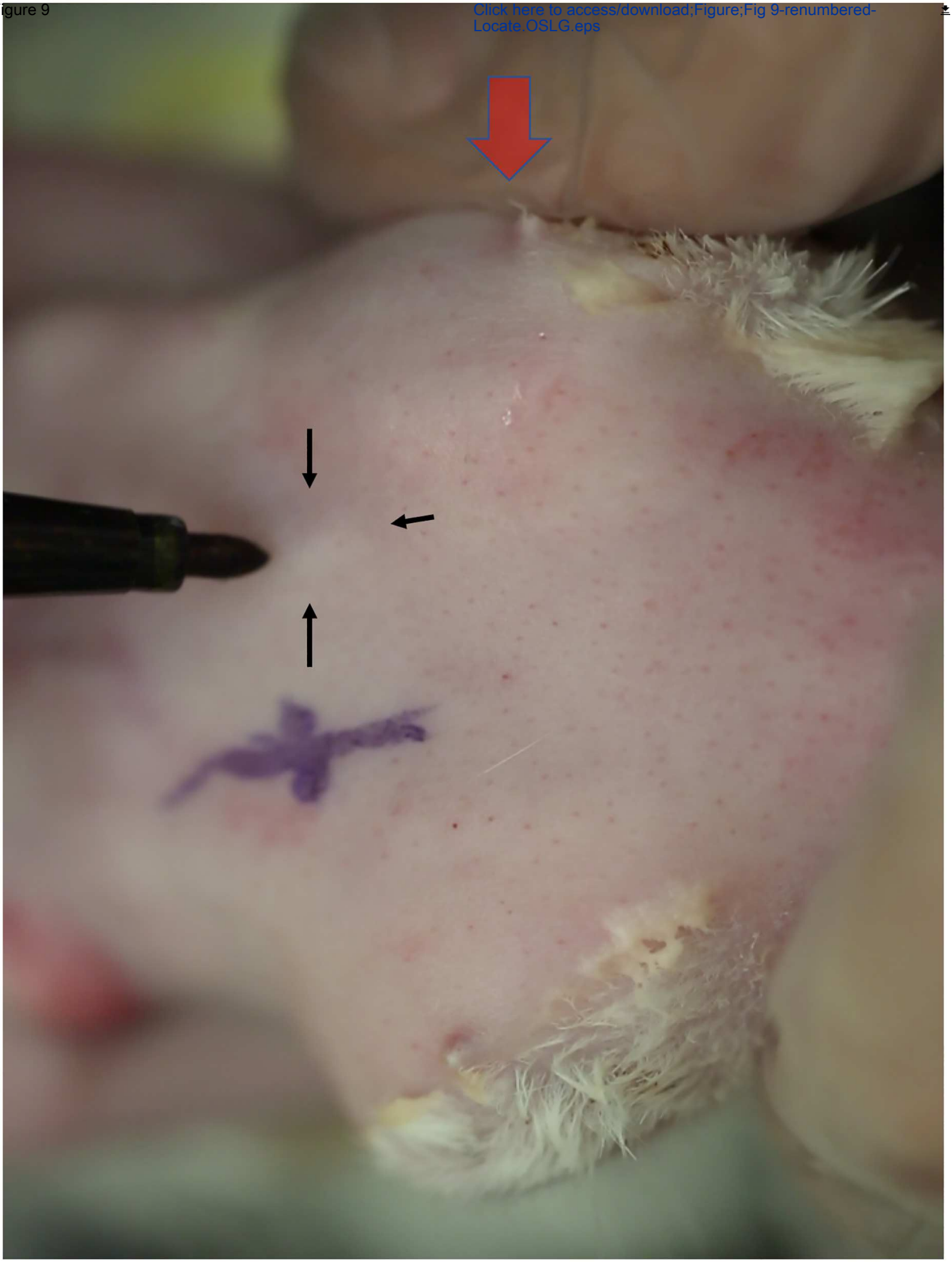


Figure 10

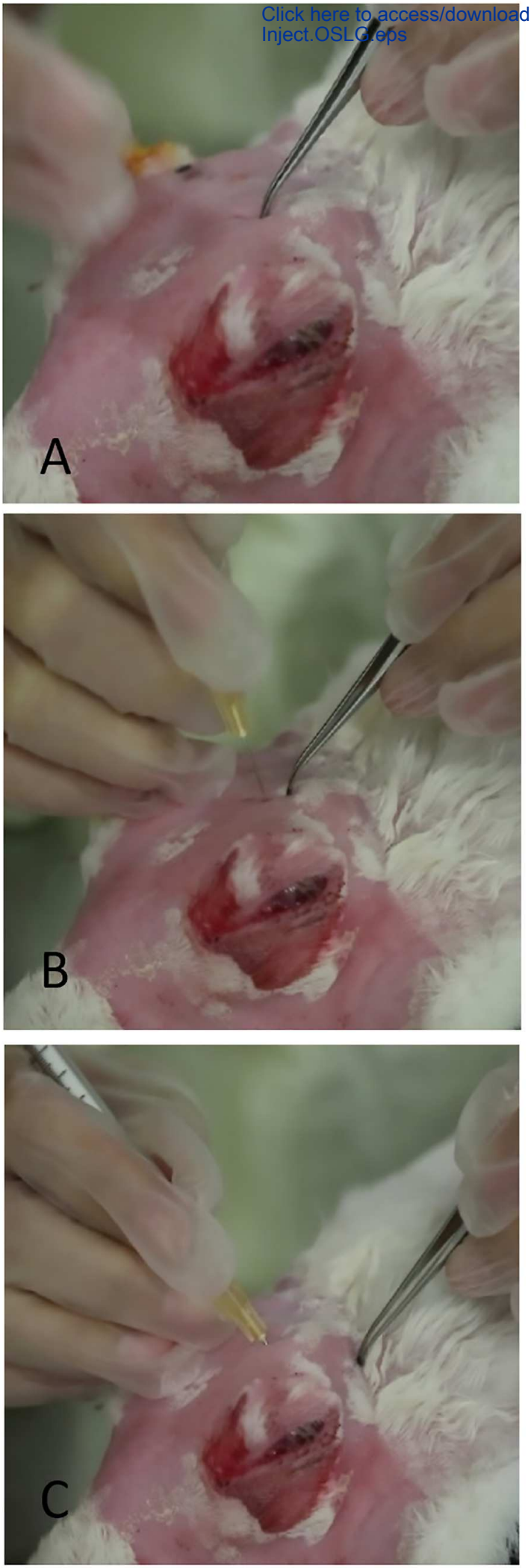


Figure 11

Click here to access/download;Figure;Fig 11-renumbered-Locate.ILG.eps

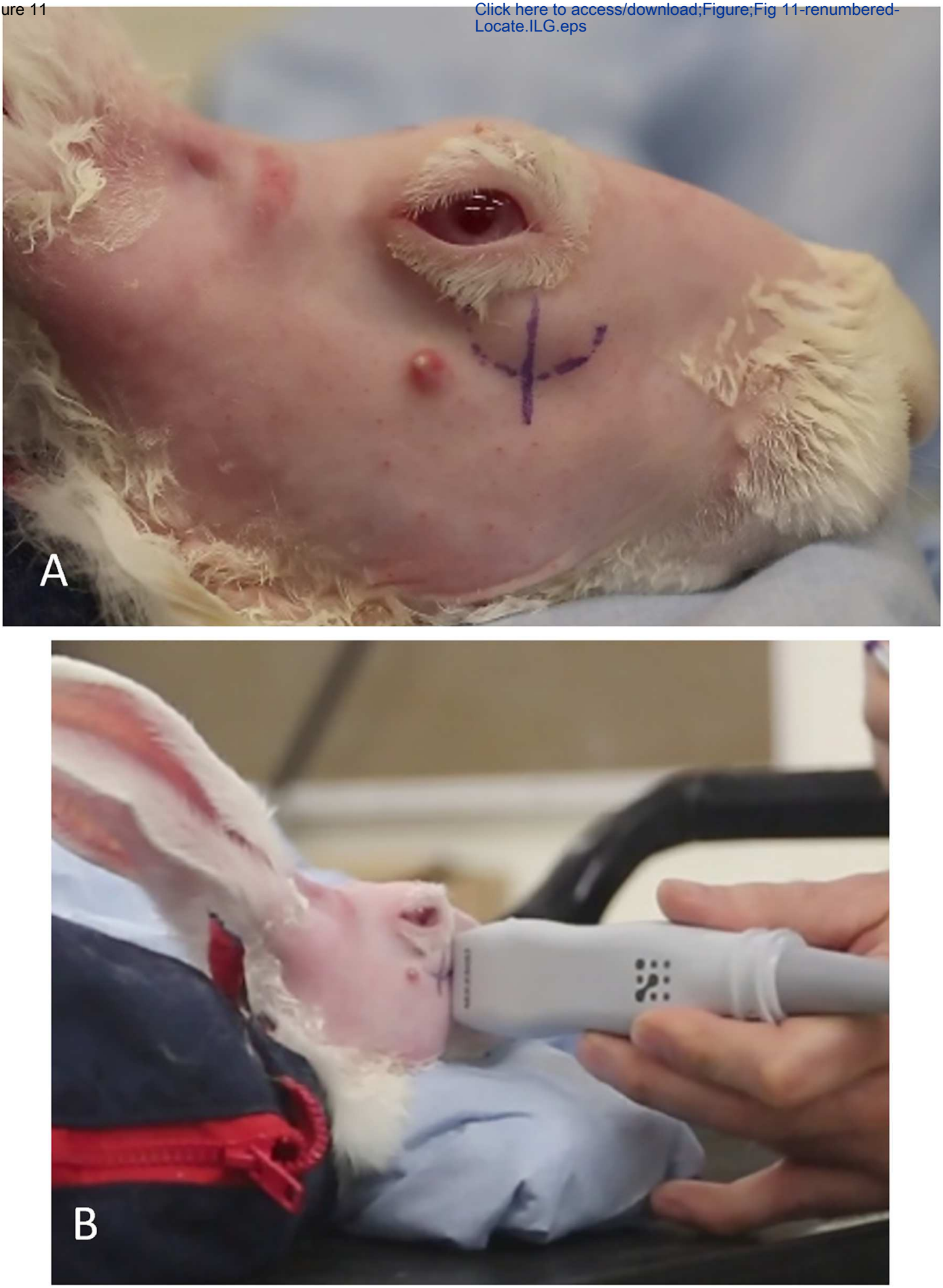


Figure 12

Click here to access/download;Figure;Fig 12-renumbered-Inject.ILG.eps

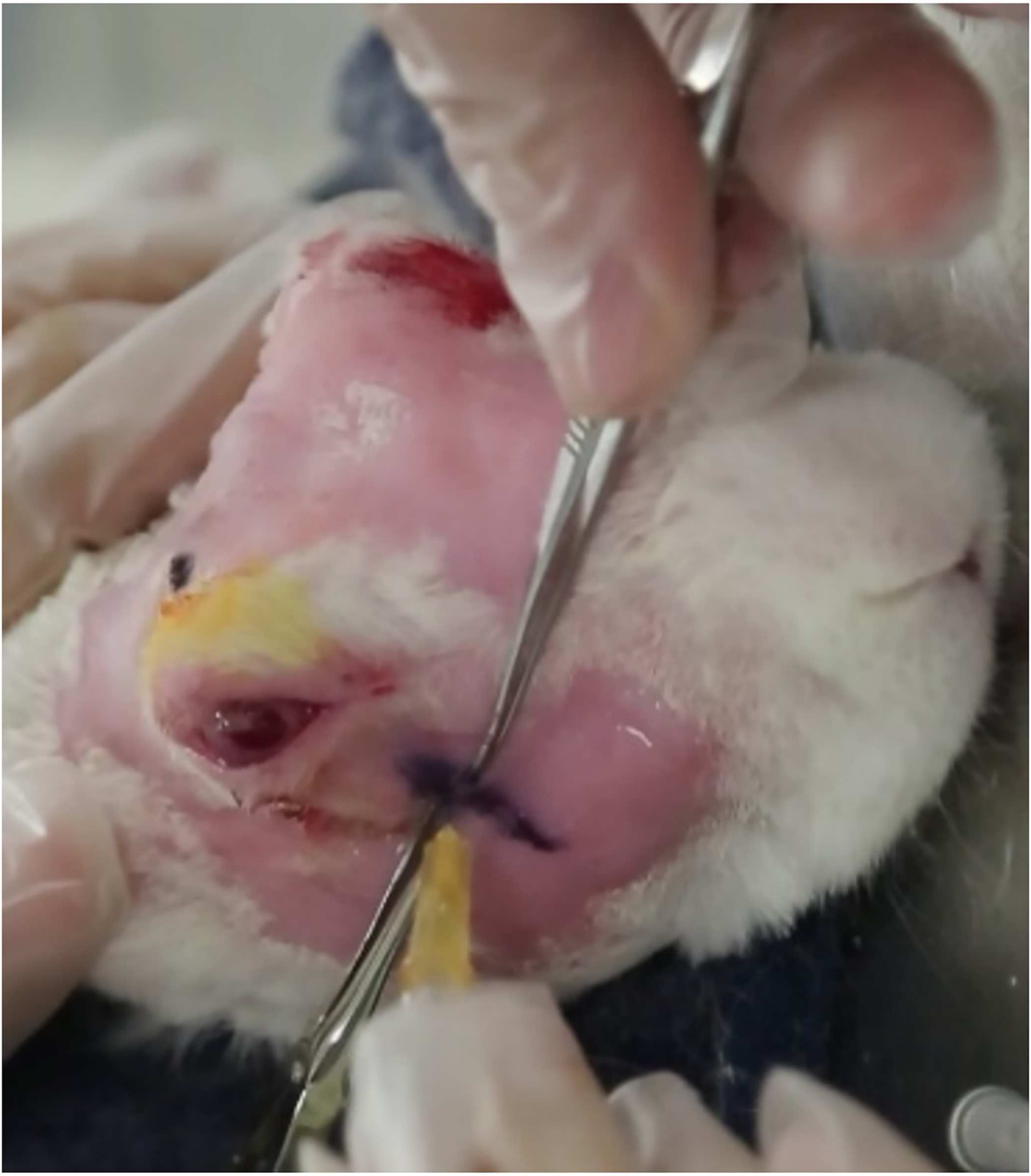


Figure 13

Click here to access/download;Figure;Fig 13-histology.tif

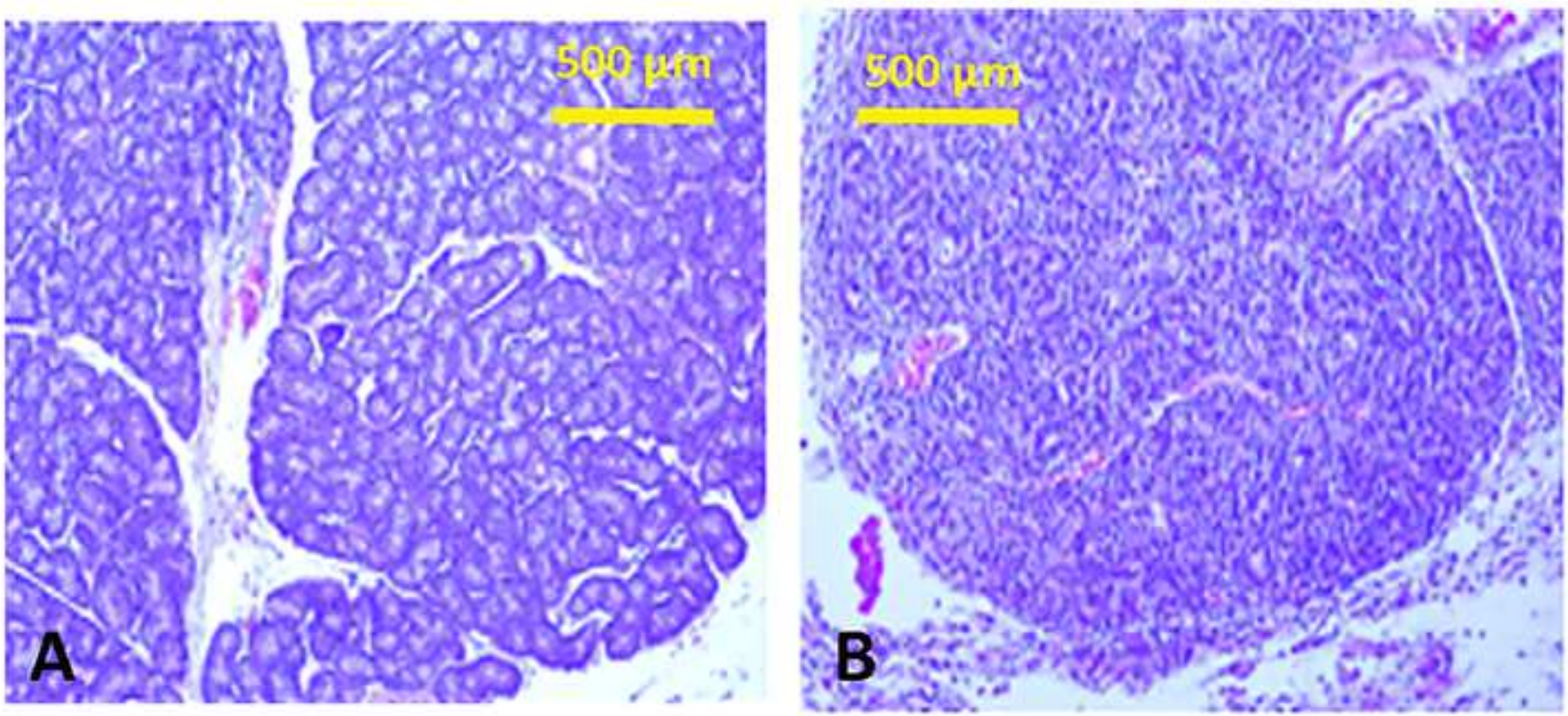


Table 1. Effect of injection technique and number of injection sites on dry eye severity

Injection Method	**TBUT,** sec		**Tear Osmolality,** Osm/L		**STT,** mm	
	Baseline	**Post-injection**	**Baseline**	**Post-injection**	**Baseline**	**Post-injection**
	mean ± SEM, *% change*					
ILG without US guidance *%change*	58.5 ± 1.5	44.5 ± 7.7 *-23%* p=0.05	297 ± 4.9	300 ± 3.8 *1%* p=0.36	15.2 ± 0.9	12.9 ± 2.2 *-15%* p = 0.21
US-guided ILG + PSLG *% change*	59.4 ± 0.6	11.4 ± 4.2 *-81%* p<0.0001	296 ± 4.7	326 ± 3.7 *10%* p<0.0001	15.1 ± 1.3	10.7 ± 1.8 *-29%* p<0.03
US-guided ILG + PSLG + OSLG *% change*	60 ± 0.0	6.2 ± 1.3 *-90%* p<0.0001	299 ± 2.9	309 ± 2.8 *3%* p<0.008	14.6 ± 0.9	9.9 ± 1.3 *-32%* p<0.002

All values were measured on day 6 following a single injection of Con A into the glands listed. All comparisons were made to baseline. *ILG, inferior lacrimal gland; PSLG, palpebral portion of superior lacrimal gland; OSLG, orbital portion of superior lacrimal gland; US, ultrasound.

Table 2. Effect of repeated Con A injections into ILG on duration of DED

	Baseline	Day 6 1 injection	Day 13 2 injections	Day 21 3 injections
TBUT, sec % change	58.5 ± 1.5	44.5 ± 7.7 *-24%* p=0.17	29.2 ± 7.8 *-50%* p=0.001	12.8 ± 3.9 -78% p<0.0001
TOsm, Osm/L % change	297 ± 4.9	300 ± 3.9 *1%* P=0.36	308 ± 4.9 *4%* p=0.04	313 ± 2.7 5% p=0.003
STT, mm % change	15.2 ± 0.9	9.3 ± 1.6 *-39%* p=0.17	12.9 ± 1.6 *-15%* p=0.13	7.4 ± 1.1 -51% p=0.008

Comparisons were made to baseline.

Table of Materials

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
100 mm macro lens	Canon EF 100mm f/2.8L IS USM	3554B002	
26 gauge needles (5/8)	Becton Dickinson and Company, Franklin Lakes, NJ	305115	Needles for injecting ConA into the lacrimal glands
27 gauge needles (5/8)	Becton Dickinson and Company, Franklin Lakes, NJ	305921	Needles for injecting ConA into the lacrimal glands
Aceproinj (acepromazine)	Henry Schein Animal Health,	NDC11695-0079-8	0.1ml/kg subcutaneously injection
Anesthesia vaporizer	VetEquip, Pleasanton, CA	Item # 911103	
Bishop Harmon Forceps	Bausch and Lomb (Storz), Bridgewater, NJ	E1500-C	Tissue forceps
Caliper	Bausch and Lomb (Storz), Bridgewater, NJ	E-2404	Caliper used to measure length of needle during ConA injection
Concanavalin A	Sigma, St. Louis, MO	C2010	Make 5mg/ml in PBS for injection into rabbit lacrimal glands
DSLR camera	Canon EOS 7D DSLR	3814B004	Digital single lens reflex camera
fluorescein	AKRON, Lake Forest, IL	NDC17478-253	Dilute to 0.2% with PBS to measure TBUT
Isoflurane	Henry Schein, Melville, NY	29405	
Lactoferrin ELISA kit	MyBiosource, San Diego, CA	MBS032049	Measure tear lactoferrin level
lidocaine	Sigma, St. Louis, MO	L5647	1% in PBS for anesthesia agent
macro/ring flash	Canon Macro Ring Lite MR-14EXII	9389B002AA	
Osmolarity tips	TearLab Corp., San Diego, CA	#100003 REV R	Measure tear osmolarity
PBS (phosphate buffered saline)	Mediatech, Inc. Manassas, VA	21-031-CV	

Rabbit, New Zealand White or Dutch Belted (as described in text)	Charles River Labs, Waltham, MA	2-3 kg	Research animals
Rose Bengal	Amcon Laboratories Inc., St. Louis, MO	NDC51801-004-40	1% in PBS, stain the ocular surface
Schirmer strips	Eaglevision, Katena products. Denville, NJ	AX13613	Measure tear production
Surgical Loupes +1.50	Designs for Vision, Bohemia, NY	Specialty item	Provide magnificantion of ocular surface while observing tear break up and performing Concanavalin A
TearLab Osmometer	TearLab Corp., San Diego, CA	Model#200000W REV A	Measure tear osmolarity
Ultrasound probe	VisualSonics Toronto, Ont	MX 550 S	Untrasonography-guide Con A injection for inferior lacrimal gland

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	A rabbit model of aqueous-deficient dry eye disease induced by concanavalin A injection into the lacrimal glands: Application to drug efficacy studies
Author(s):	Robert A. Honkanen, Liqun Huang, Basil Rigas

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☒ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Basil Rigas
Department:	Preventive Medicine
Institution:	Stony Brook Medicine
Title:	Professor
Signature:	
Date:	30 December 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Authors' Response to Editorial Comments
The updated manuscript, **59631_R2.docx**, was used to make our revisions.

Author responses are entered in blue font, below.

1. Line 304-309: JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

Text has been modified as follows. Commercial photography products have been added to Table of Materials and Reagents.

> NOTE: Camera settings; Adjust to the type of camera being used. Typical settings: digital single-lens reflex camera, aperture priority mode (aperture 13 or greater), ISO 6000, 100 mm macro lens attached with two 12.5 mm extension tubes, manual focus mode, lens at maximum magnification, and illumination from macro/ring flash set to automatic with through-the-lens mode. The ring flash's focus lamp is turned on to aid focus on the cornea.

2. Each table must be accompanied by a title in Figure and Table Legends after the Representative Results of the manuscript text.

Table titles have been added in Figure and Table Legends, as follows:

> Table 1. Effect of injection technique and number of injection sites on dry eye severity.
>
> Table 2. Effect of repeated Con A injections into ILG on duration of DED.

3. Figure 6: Please include a scale bar for all images taken with a microscope to provide context to the magnification used.

Scale bars have been added to figure 6, "Fig.6-Final.tiff".