Journal of Visualized Experiments

Revised surgical approach to induce endolymphatic hydrops in the guinea pig

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60597R1
Full Title:	Revised surgical approach to induce endolymphatic hydrops in the guinea pig
Section/Category:	JoVE Neuroscience
Keywords:	ear, hearing, cochlea, inner ear, Meniere's disease, endolymphatic hydrops, otology, audiology, otolaryngology, hearing loss
Corresponding Author:	Jeffery Lichtenhan Washington University in Saint Louis School of Medicine St Louis, MO UNITED STATES
Corresponding Author's Institution:	Washington University in Saint Louis School of Medicine
Corresponding Author E-Mail:	jlichtenhan@wustl.edu
Order of Authors:	Carla V. Valenzuela
	Choongheon Lee
	Craig A. Buchman
	Jeffery Lichtenhan
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	St Louis, MO USA

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript Click here to access/download;Manuscript;60597_R1.docx

TITLE:

A Revised Surgical Approach to Induce Endolymphatic Hydrops in the Guinea Pig

AUTHORS AND AFFILIATIONS:

Carla V. Valenzuela, Choongheon Lee, Craig A. Buchman, Jeffery T. Lichtenhan

Department of Otolaryngology – Head and Neck Surgery, Washington University School of Medicine, Saint Louis, MO, USA

Email addresses of co-authors:

Carla V. Valenzuela (valenzuelac@wustl.edu)
Choongheon Lee (c.lee@wustl.edu)
Craig A. Buchman (buchmanc@wustl.edu)
Jeffery T. Lichtenhan (jlichtenhan@wustl.edu)

Corresponding author:

Jeffery T. Lichtenhan (jlichtenhan@wustl.edu)

KEYWORDS:

Endolymphatic hydrops, endolymphatic sac obliteration, endolymphatic sac ablation, Auditory Nerve Overlapped Waveform, animal model of Meniere's disease, extradural approach

SUMMARY:

This article demonstrates an extradural approach to obliterate the guinea pig endolymphatic sac and injure the endolymphatic duct with a fine pick in order to induce experimental endolymphatic hydrops.

ABSTRACT:

Endolymphatic hydrops is an enlargement of scala media that is most often associated with Meniere's disase, though the pathophysiologic mechanism(s) remain unclear. In order to adequately study the attributes of endolymphatic hydrops, such as the origins of low-frequency hearing loss, a reliable model is needed. The guinea pig is an good model because it hears in the low-frequency regions that are putatively affected by endolymphatic hydrops. Previous research showed that endolymphatic hydrops can be induced surgically via intradural or extradural approaches that involve drilling on the endolymphatic duct and sac. However, whether it was possible to create an endolymphatic hydrops model using an extradural approach that avoided dangerous drilling on the endolymphatic duct and sac was unknown. The objective of this study was to demonstrate a revised extradural approach to induce experimental endolymphatic hydrops at 30 days post-operatively by obliterating the endolymphatic sac and injuring the endolymphatic duct with a fine pick. The sample size consisted of seven guinea pigs. Functional measurements of hearing were made and temporal bones were subsequently harvested for histologic analysis. The approach had a success rate of 86% in achieving endolymphatic hydrops. The risk of cerebrospinal fluid leak was minimal. No perioperative deaths or injuries to the posterior semicircular canal occurred in the sample. The

presented method demonstrates a safe and reliable way to induce endolymphatic hydrops at a relatively quick time point of 30 days. The clinical implications are that the presented method provide a reliable model to further explore the origins of low-frequency hearing loss that can be associated endolymphatic hydrops.

INTRODUCTION:

Endolymphatic hydrops is an enlargement of scala media. The presence of endolymphatic hydrops can be measured using the cross-sectional area of scala media. It is thought that clinical endolymphatic hydrops can be associated with low-frequency sensorineural hearing loss, such as seen in Meniere's disease, but the origin(s) of the hearing loss remain unclear. To adequately study the origins of low-frequency hearing loss associated with endolymphatic hydrops, a reliable model is needed.

In 1965, Kimura and Schuknecht described how to induce endolymphatic hydrops in the guinea pig using an intradural approach[1]. Their technique involved using a posterior cranial fossa approach to access the operculum and subarcuate fossa. The steps involved incising dura, retracting the cerebellum with a Ringer's solution soaked cotton pad, and drilling across the endolymphatic duct and the intermediate portion of the endolymphatic sac. Bone wax was then placed into the operculum to separate the endolymphatic duct from the distal endolymphatic sac. The craniotomy defect was closed by placing absorbable gelatin powder (e.g., Gelfoam) and reapproximating the overlying muscles. Histologic evidence of endolymphatic hydrops was consistently found at post-operative days 1, 3, 7, 14, 21, and 30, demonstrating that the intradural approach was a reliable protocol to induce histologically-confirmed endolymphatic hydrops. Using the same intradural approach as Kimura and Schuknecht, but with different time points, Salt and DeMott confirmed that scala media in the second turn of the cochlea was significantly enlarged at day 4 and beyond[2]. While the actual morbidity of inducing a cerebrospinal fluid (CSF) leak using Kimura and Schuknecht's intradural approach was not reported in the original study, the presence of a CSF leak could increase the risk of meningitis. It has been suggested that loss of CSF could lead to an outflow of perilymph, resulting in a simultaneous temporary expansion of the endolymphatic volume in the guinea pig[3]. An extradural approach to inducing endolymphatich hydrops would be a safer option.

In 1989, Andrews and Bohmer described two extradural surgical approaches to reach the endolymphatic sac and duct, via either a middle cranial fossa approach or posterior cranial fossa approach, to obliterate the endolymphatic sac[4]. They described removing the operculum, and then either drilling off the intermediate portion of the endolymphatic sac or using a fine pick to disrupt the endolymphatic sac and duct. In 1993, Lee, Wright, and Meyerhoff described a similar approach, which included drilling through the endolymphatic sac and duct, but differed in that they also simultaneously obstructed the cochlear aqueduct[5]. They demonstrated the presence of endolymphatic hydrops, as assessed via histology, at four weeks after obliterating the endolymphatic sac and obstructing the cochlear aqueduct. Megerian et al. was the first to publish a video article demonstrating an extradural obliteration of the endolymphatic sac and duct that involved drilling directly on the medial portion of the operculum to enter into the endolymphatic sac and duct[6]. They demonstrated histologic

evidence of endolymphatic hydrops in a guinea pig sacrificed at 28 weeks after surgery, as well as hearing loss in the 16 kHz region[6]. Whether it was possible to induce histologically confirmed endolymphatic hydrops and low-frequency hearing loss at an early time point using extradural approaches was unknown.

The overall goal of this report is to demonstrate an extradural approach to induce experimental endolymphatic hydrops at 30 days post-operatively by obliterating the endolymphatic sac and injuring the endolymphatic duct with a fine pick. The rationale behind the use of this technique is the advantage of avoiding the need to drill on the petrous temporal bone, thereby removing the risk of accidentally injuring the dura and causing a CSF leak, mitigating the possibility of injuring the posterior semicircular canal, and reducing the risk of injury to the sigmoid sinus.

PROTOCOL:

All procedures listed immediately below in the Protocol section were conducted under protocols 20180133 and 20150091, both of which were approved by the Washington University Institutional Animal Care and Use Committee.

1. Anesthetic induction and monitoring of vital signs

NOTE: This study used pigmented NIH-strain guinea pigs obtained from an in-house breeding colony.

1.1. Use guinea pigs of either sex, weighing at least 350 grams.

1.2. Place the guinea pig in a neonatal warming isolette and give a ketamine/xylazine mixture intraperitoneally (50 mg/kg ketamine and 10 mg/kg xylazine) for induction anesthesia. Observe the guinea pig until it loses the toe-pinch reflex.

1.3. Once loss of toe-pinch reflexes occurs, shave the posterior neck and head of the guinea pig with a hair trimmer typically advertised for human use.

1.4. Inject a subcutaneous fluid bolus of 12 mL of lactated Ringer's solution into the back of the animal.

1.5. Place the guinea pig supine on a warming pad with legs raised and place 27.5 G butterfly needle intraperitoneally. Verify that the butterfly needle is in the correct position in the intraperitoneal space by ensuring only air is aspirated. If blood or fluid is aspirated, there is concern for delivery into the vascular or bowel system.

1.6. Flip the guinea pig over to the prone position and secure the head to a stereotactic holder.

1.7. Place a pulse oximeter and secure on a foot. If using pigmented guinea pigs, pigmented paws can prevent reading oxygen saturation. Therefore, place the pulse oximeter on any paw

that is not pigmented.

1.8. Insert a rectal temperature probe to monitor body temperature. The rectal probe is part of a warming blanket system that maintains body temperature at 38 °C. Do not turn on the warming blanket until the rectal probe is in place to avoid overheating the warming blanket. If having difficulty placing the rectal probe, it can be laid alongside the guinea pigs body.

1.9. Apply lubricant by hand to both eyes of the guinea pig to prevent corneal abrasions.

1.10. Administer supplemental oxygen via a rubber tubing positioned near the nose to maintain oxygen saturation levels above 90%.

1.11. Give enrofloxacin 0.5 mg/kg subcutaneously as an antibiotic prophylaxis.

1.12. Give 0.25 mg/kg bupivacaine/epinephrine subcutaneously at the anticipated incision site for local anesthesia and vasoconstrictive effects.

1.13. Provide maintenance anesthesia every 20 minutes for 4 cycles and then only as needed based on vital signs. Routinely monitor the depth of anesthesia by body temperature, respiration rate, and heart rate.

1.14. Monitor vital signs every 15 minutes (temperature, respiratory rate, heart rate, and oxygen saturation).

2. Surgical preparation

2.1. Once the head of the guinea pig is positioned securely in a stereotactic holder, place a piece of masking tape over the back to provide adequate tension along the skin overlying the occiput. Secure the ends of the tape to the stereotactic holder.

2.2. Liberally prep the skin overlying the occiput and posterior neck with iodine scrub and solution in a sterile fashion three times.

2.3. At this point, use sterile precautions and autoclaved instruments. Place sterile drapes over the guinea pig.

3. Surgical procedure

3.1. Using a 15 blade, make a small, midline incision along the posterior occiput extending down into the posterior neck. Once under the skin, use iris scissors to detach the right posterior cervical muscles from the occipital bone. If any bleeding occurs while cutting the muscles, control by applying pressure with a sterile cotton ball.

3.2. Using a combination of a #3 mm, #2 mm, and #1 mm diamond burr with a 5-0 suction

and sterile irrigation, perform a craniotomy that is bounded by the external occipital crest laterally, lamboidal ridge superiorly, the occipitomastoid suture line medially, and the dorsal margin of the foramen magnum inferiorly.

3.2.1. Gently place a small piece of saline-moistened cotton under the bone while separating the occipital bone from the dura.

3.3. Skeletonize the sigmoid sinus with a #0.5 mm diamond burr and carefully remove the bone overlying it.

3.4. Once the sigmoid sinus is exposed, gently retract the sigmoid sinus medially using a cotton ball and switch to using a 3-0 suction.

3.4.1. Carefully retract the emissary vein which is located ventrally.

3.5. Identify the operculum as a slit like structure that is located within the petrous temporal bone. The subarcuate fossa will be situated superior-posteriorly and the sigmoid sinus will be medial to it. The extra-osseous portion of the endolymphatic sac is then visualized as a clear sac entering the operculum and attached to the dura overlying the sigmoid sinus. The operculum is oval shaped, approximately 3 to 4 mm by 1.5 to 2 mm. However, as seen from the surgical view, the operculum appears as an approximate 1 mm slit. The visible portion of the sac from the surgical view is approximately the same size as the visible portion of the operculum, if not smaller.

3.6. Apply gentle retraction to the sigmoid sinus medially in order to clearly visualize the extra-osseous portion of the endolymphatic sac and increase the tension between the extraosseous and intraosseous portions of the endolymphatic sac.

3.6.1. Use a fine angled pick to gently expunge the intermediate portion of the endolymphatic sac. It is critical that the expungement process leave no visible connection between the dura and the operculum; then place a fine pick inside the operculum to broadly scrape along the inside of the bone to injure it.

3.6.2. Turn the fine pick in the direction of the endolymphatic duct and blindly disrupt the lining. At this point some bleeding may occur from a vessel within the operculum. It can be controlled with a small piece of cotton.

3.7. Dry the empty operculum with a small piece of cotton. Using the 3-0 suction as needed to keep the cotton dry.

3.8. Obtain bone dust by using a small curette to scrape along the squamosal portion of the temporal bone. Generously pack the operculum with bone dust. Use a cotton ball and suction to keep the area dry while packing it with bone dust.

3.9. Apply bone wax to the operculum to seal it. Ensure that there is no excess bone wax dislodged into the skull.

3.10. Use bone wax to cover the craniectomy defect.

3.11. Approximate the posterior cervical muscles with 4-0 braided, absorbable suture in an interrupted fashion.

3.12. Perform a subcuticular closure using a 4-0 braided, absorbable suture.

4. Post-procedure care

4.1. Remove the guinea pig from the custom stereotactic holder and transfer to a warming isolette.

4.2. Give 2 mg/kg Atipamezole and 24 mL of lactated Ringer's solution (subcutaneously away from the incision). Give lactated Ringer's solution due to the diuretic effects of xylazine.

4.3. Obtain vital signs every 15 minutes until the guinea pig fully emerges from anesthesia.

4.4. Give an additional 12 mL fluid bolus of lactated Ringer's solution about 2 hours from end of surgery during the recovery period.

4.5. Once the guinea pig is alert, ambulating, voiding, and having bowel movements, return the guinea pig to the animal facility. Approximately 4 hours are needed for the guinea pig to emerge completely from anesthesia.

4.6. Monitor guinea pigs twice daily for the first three post-operative days.

4.7. Give a 12 mL fluid bolus of lactated Ringer's solution subcutaneously twice a day for the first three days until the guinea pig reaches the pre-operative weight. If the guinea pig reaches its pre-operative weight prior to the third post-operative day, then stop fluid boluses. If the guinea pig continues to lose weight after the first three days, use a supplement nutrition shake typically advertised for human consumption mixed with crushed guinea pig food pellets.

4.8. Monitor guinea pigs weekly until their end point.

REPRESENTATIVE RESULTS:

The presented method used an extradural approach to obliterate the endolymphatic sac and injure the endolymphatic duct with a fine pick in seven guinea pigs consisting of two males and five females. The average duration of surgery was 2 h from incision to closure. The total drill time ranged from 5-10 min. An average of 4 h was needed for the guinea pig to fully emerge from anesthesia. There were no intra-operative or post-operative deaths in the sample. There were no injuries to the posterior semicircular canal or dura in any of the guinea pigs. Injury to

the sigmoid sinus occurred in the one guinea pig (excluded for the data analysis).

The guinea pigs underwent a second procedure on the day of sacrifice (post-operative day 30) to make auditory function measurements that included the Auditory Nerve Overlapped Waveform (ANOW) and cochlear compound action potentials (CAPs). ANOW and CAP measurements were made, and analyses performed, made using methods described previously[7-9]. The ANOW is a purely neural measurement that originates from neural excitation in the apical cochlear half[7-9]. Following the auditory function tests, the ears were immediately harvested and prepared for histologic analysis using methods previously describe[10]. Successful histological preparation was completed in six ears, but one ear showed tears in the Reisner's membrane. The ear with tears was eliminated from histological analysis but kept in physiological analysis. The cross-sectional area of scala media were measured using ImageJ[11]. Histologic analysis of the temporal bones revealed endolymphatic hydrops in six out of the seven guinea pigs throughout the right cochlea compared to the left cochlea (**Figure 1**). In **Figure 1**, the scala media cross sectional area on the operated, right ear (red) is enlarged compared to the contralateral, left ear (blue), demonstrating endolymphatic hydrops in the right ear. The cross-sectional area of scala media across each turn was also quantified and compared to control guinea pigs (**Figure 2**). Measures from one ear are not included in **Figure 2** because of a histological preparation problem that caused the Reisner's membrane to tear. Control guinea pigs had either undergone sham surgery (in which a craniotomy was made but the endolymphatic sac and duct were identified but not disturbed) or had not undergone any surgery other than that needed to make auditory function measures. As compared to the control, the cross sectional area was generally larger in ears surviving 30 days after obliteration of the endolymphatic sac (**Figure 2**). ANOW thresholds (≤ 1 kHz) were increased in six out of seven guinea pigs that demonstrated endolymphatic hydrops compared to control guinea pigs, demonstrating the presence of low-frequency hearing loss (**Figure 3**). Wave 1 of the auditory brainstem response, or the cochlear compound action potential (CAP), thresholds were within the normal range at frequencies above 8 kHz in six out of the seven guinea pigs (**Figure 3**).

Figure 1. Histologic images of a mid-modiolar cut of guinea pig cochlea. This guinea pig survived 30 days after obliteration of the endolymphatic sac using an extradural approach.

Figure 2. Cross-sectional area of scala media as a function of cochlear length. Measures from six of seven individual ears are in red. Gray dashed lines represent ±1 standard deviation of measures from the Control ears.

Figure 3. Auditory function measurements (ANOW and CAPs) measured on post-operative day 30. ANOW measures are ≤ 1 kHz and CAP measures were made > 1 kHz. Measures from individual ears are in red. Gray dashed lines represent ±1 standard deviation of thresholds for Control guinea pigs.

DISCUSSION:

The presented extradural method had a success rate of 86% in achieving histologically confirmed endolymphatic hydrops and low-frequency hearing loss. The method reliably

achieved histological evidence of endolymphatic hydrops by post-operative day 30, consistent with prior studies that used an intradural approach[2]. The significance of the method with respect to existing methods is that a CSF leak is not required, thus removing a potential confounding variable that has been suggested to result in a compensatory, temporary expansion of the endolymphatic volume[3]. Overall, the method demonstrates a quick, safe, and reliable way to induce experimental endolymphatic hydrops.

The presented method has several strengths compared with prior studies. First, the approach was extradural, minimizing the potential morbidity and confounding effects of a CSF leak. Second, by using a fine pick instead of a drill to expunge the endolymphatic sac and injure the endolymphatic duct, the method avoids any potential injury to the posterior semicircular canal. A critical step is ensuring no visible connection between the dura and the operculum. Third, using a fine pick in the temporal bone instead of a drill, the method minimized the potential for acoustic trauma caused by drilling on the petrous temporal bone. Finally, the method provides an optimized peri-operative animal protocol to ensure a rapid recovery and successful post-operative course of the guinea pigs. A limitation of the method is the use of ketamine/xylazine, which may be overcome by using a stereotaxic device that allows isoflurane delivery.

The scientific implications of the results are the development of a safe and reliable way to induce endolymphatic hydrops at a relatively quick time point of 30 days. The clinical implications are that the method provides a reliable model of endolymphatic hydrops in order to further explore the origins of the associated low-frequency hearing loss. Future applications of the method will be used to further study the origin(s) of low-frequency hearing loss associated with endolymphatic hydrops. In conclusion, the presented method is a modified occipital, extradural approach that involves obliterating the endolymphatic sac and injuring the endolymphatic duct with a fine pick to induce experimental endolymphatic hydrops at 30 days post-operatively in the guinea pig.

ACKNOWLEDGMENTS:
We thank Shannon M. Lefler for assistance with figures and the Table of Materials. Research reported in this publication was supported by the National Institute of Deafness and Other Communication Disorders within the National Institutes of Health, through the “Development of Clinician/Researchers in Academic ENT” training grant, award number T32DC000022 (C.V.V.) and by R01 DC014997 (J.T.L). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

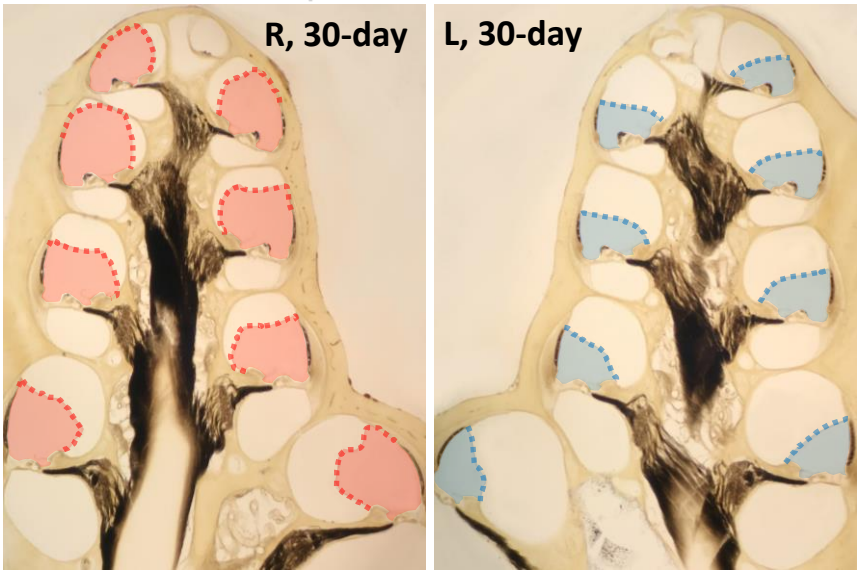
DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Kimura, R.S., Schuknecht, H.F. Membranous Hydrops in the Inner Ear of the Guinea Pig after Obliteration of the Endolymphatic Sac. *Pract oto-rhino-laryng.* **27**, 343-354 (1965).
2. Salt, A.N., DeMott, J. Time course of endolymph volume increase in experimental hydrops measured in vivo with an ionic volume marker. *Hearing Research.* **74** (1-2), 165-172

(1994).
3. Walsted, A., Garbarsch, C., Michaels, L. Effect of craniotomy and cerebrospinal fluid loss on the inner ear. An experimental study. *Acta Oto-Laryngologica.* **114** (6), 626-631 (1994).
4. Andrews, J.C., Bohmer, A. The surgical approach to the endolymphatic sac and the cochlear aqueduct in the guinea pig. *American Journal of Otolaryngology.* **10** (1), 61-66 (1989).
5. Lee, J.R., Wright, C.G., Meyerhoff, W.L. Modified occipital approach to the endolymphatic sac and cochlear aqueduct of the guinea pig. *American Journal of Otolaryngology.* **14** (2), 165-169 (1993).
6. Megerian, C.A. et al. Surgical induction of endolymphatic hydrops by obliteration of the endolymphatic duct. *Journal of Visualized Experiments.* (35) (2010).
7. Lichtenhan, J.T., Cooper, N.P., Guinan, J.J., Jr. A new auditory threshold estimation technique for low frequencies: proof of concept. *Ear and Hearing.* **34** (1), 42-51 (2013).
8. Lichtenhan, J.T., Hartsock, J., Dornhoffer, J.R., Donovan, K.M., Salt, A.N. Drug delivery into the cochlear apex: Improved control to sequentially affect finely spaced regions along the entire length of the cochlear spiral. *Journal of Neuroscience Methods.* **273**, 201-209 (2016).
9. Lichtenhan, J.T., Hartsock, J.J., Gill, R.M., Guinan, J.J., Jr., Salt, A.N. The auditory nerve overlapped waveform (ANOW) originates in the cochlear apex. *Journal of the Association for Research in Otolaryngology.* **15** (3), 395-411 (2014).
10. Lichtenhan, J.T., Hirose, K., Buchman, C.A., Duncan, R.K., Salt, A.N. Direct administration of 2-Hydroxypropyl-Beta-Cyclodextrin into guinea pig cochleae: Effects on physiological and histological measurements. *PloS One.* **12** (4), e0175236 (2017).
11. Schneider, C.A., Rasband, W.S., Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods.* **9** (7), 671-675 (2012).

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

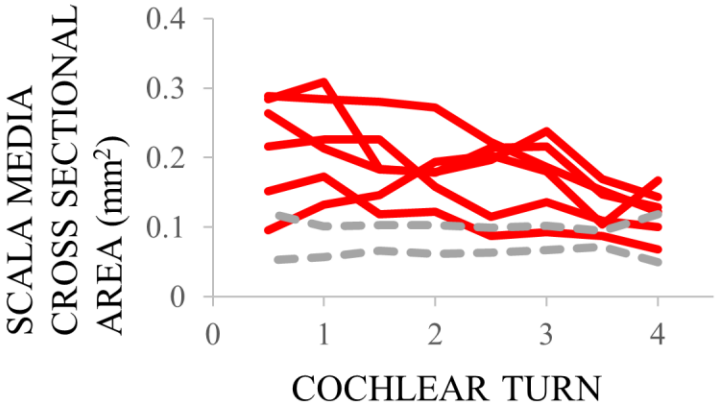
Click here to access/download;Figure;Valenzuela_Lichtehan_JOVE_2ndSub_



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

Click here to access/download;Figure;Valenzuela_Lichtehan_JOVE_2ndSub_

Standard Manuscript Template
Remove all gray text before submitting

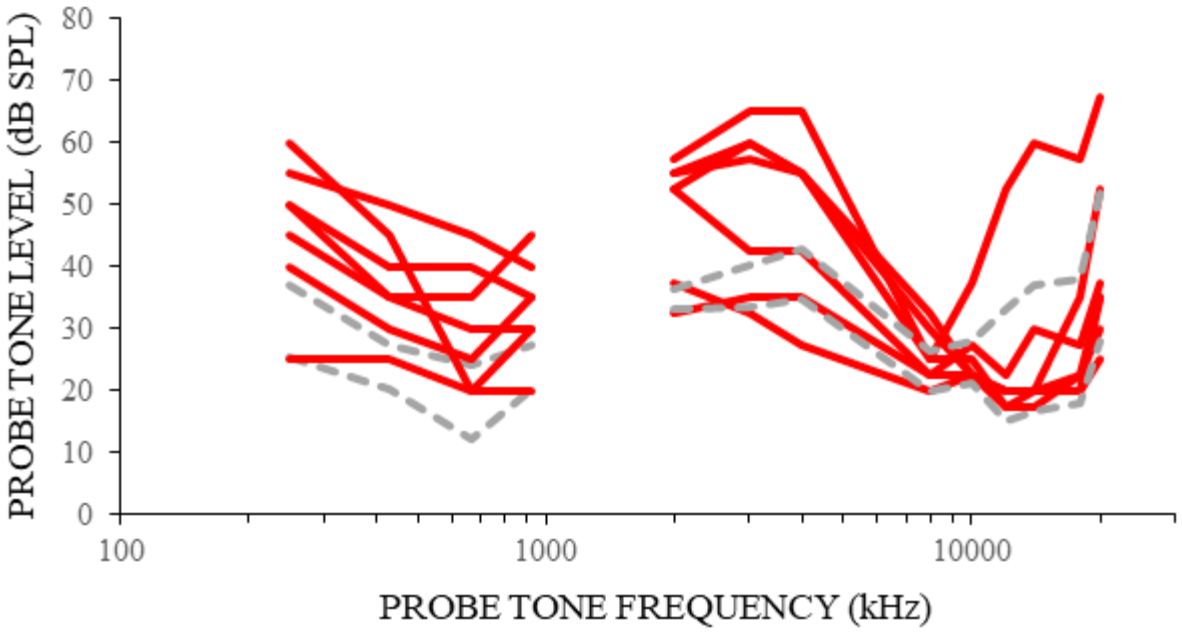


1

Click here to access/download;Figure;Valenzuela_Lichtehan_JOVE_2ndSub_

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

1



2

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_CVV_SML.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
12 mL syringe	Henke-Sass Wolf	5100-X00V0	
1 mL and 3 mL syringe	BD Precision(Ordered from Fischer Sci)	14-826-87 15859152	
27.5 butterfly gauge needle	Terumo Surflo Winged Infusion Set, Terumo Corporation, Japan) (Ordered from McKesson)	448407	
4-0 suture	McKesson	1034507	
4x4 gauze sponges	Dukal (Ordered form McKesson)	374454	
60mL syringe	Fisher Sci	22-031-375	
Anspach otologic drill	Anspach	SC2100	
atipamezole	Zoetis	107204-6	
autoclave	Fisher sci	15-103-0508	
autoclave bags	McKesson	524881	
bayonet separator	Olympus	AL 130564	
bupivicaine	auro Medics Pharma	555150-169-10	
clear sterile drape	3M	1020	
cotton balls	Fisherbrand (ordered from Fisher Sci)	22-456-885	
cotton swabs	McKesson	508716	
diamond burrs #3, #2, #1, and #0.5 mm	Anspach	QD8-3SD; QD8-2SD; QD8-1SD; QD8-05SD	
diaper pad	McKesson	945330	
disposable 15 blade	Swann-Morton	0305	

enrofloxacin	Hospira	0409-4888-01	
epinephrine	McKesson	63739-0456	
eye ointment	Dechra Vet Products	17033-211-38	
Freer elevator	Grace Medical	215100FX	
gelfoam	Pfizer (Ordered from McKesson)	82830	
hair trimmers	Oster Power Pro Cordless (ordered from Amazon)	078400-020-000	
iodine scrub	Purdue Pharma (ordered from mcKesson)	521243	
iris scissors	Olympus	CL-542114	
ketamine	Henry Schein Animal Health	55853	
lactated ringers	B. Braun Medical (ordered from McKesson)	186662	
lancet knife by Rosen	Grace Medical	151100FX	referred to as curette in the text
lubricant	Milex (ordered from Cooper Surgical)	MX5030	
masking tape	3M (ordered from fisher sci	19047259	
metal rectangle basin	Amazon	B07NQDBC6T	
needle holder	Olympus	CR 213015-ENT	
needles: 27 gage, 18 gauge	BD Precision(Ordered from Fischer Sci)	14-826-48 14-826-5D	

neonatal warming isollete	Air Borne Life Support Systems	731-1800	
operating microscope	Carl Zeiss	OPMI pico	
oxygen tank	AirGas	OX USP200	
pulse ox	CapnoTrue (Ordered from Medacx)	M-3090112001	
rectal probe with heating blanket	Harvard Apparatus	probe: PY2 50-7217 Heating Blanket: PY2 50-7214	
red body holder	Lichtenhan Lab	N/A	In-house product
right angle	Olympus	BV-230337	
rosen needle	Olympus	AM-130566	customized, it is the instrument I use to tear the sac
rubber tubing for O2 administration	Fisher Sci	14-171-104	
saran wrap	Fisher Sci	NC9617977	
stereotactic head holder	WUSTL Instrument Machine Shop	N/A	In-house product
sterile drapes	Cardinal Health	7553	
suction tube by Baron	Grace Medical	034903FX 034905FX	#3 and #5 Suction
tissue forceps adson brown	Grace Medical	325112FX	
Weitlander retractor	Olympus Grace Medical	BL200011 100313FX	
xylazine	Akorn	59399-110-20	

ALA

Click here to access/download;Author License Agreement (ALA);Author_License_Agreement JOVE Lichtenhan.pdf

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

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Revised surgical approach to induce endolymphatic hydrops in the guinea pig
Author(s):	Carla V. Valenzuela, Choongheon Lee, Craig A. Buchman, Jeffery T. Lichtenhan

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.

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:	Jeffery T. Lichtenhan
Department:	Otolaryngology
Institution:	Washington University School of Medicine in St Louis
Title:	Assistant Professor
Signature:	Jeffery Lichtenhan
Date:	7-23-19

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

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Authors: Done

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points

Authors: Done

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before 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.

For example: Ensure, Terumo Surflo Winged Infusion Set, Terumo Corporation, 116 Japan,

Authors: Done

4. Please ensure the Introduction includes all of the following including citations:

a) A clear statement of the overall goal of this method

Authors: We revised an existing sentence to read "The overall goal of this report is to demonstrate an extradural approach to induce experimental endolymphatic hydrops at 30 days post-operatively by obliterating the endolymphatic sac and injuring the endolymphatic duct with a fine pick."

b) The rationale behind the development and/or use of this technique

Authors: See response to 4c below.

c) The advantages over alternative techniques with applicable references to previous studies

Authors response to 4b and 4c: We revised an existing sentence to read "The rationale behind the use of this technique is the advantage of avoiding the need to drill on the petrous temporal bone, thereby removing the risk of accidentally injuring the dura and causing a CSF leak, mitigating the possibility of injuring the posterior semicircular canal, and reducing the risk of injury to the sigmoid sinus."

d) A description of the context of the technique in the wider body of literature

Authors: The second and third paragraphs of the Introduction provide this information.

e) Information to help readers to determine whether the method is appropriate for their application

Authors: Done.

5. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Authors: Done.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

7. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

Authors: Done.

8. The Protocol should contain only action items that direct the reader to do something.

Authors: Done.

9. Software steps must be more explicitly explained ('click', 'select', etc.).

Authors: Does not apply.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

Authors: Done.

11. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Authors: Done.

12. 1.4-1.5: How is this done?

Authors: Done.

13. How was the analysis performed? Please detail the protocol or provide citations to make this a stand-alone protocol.

Authors: Done. We now say "ANOW and CAP measurements were made, and analyses performed, made using methods described previously."

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please ensure that the live animal will be available for filming?

Authors: The filmable content is listed in the section "PROTOCOL" and is 2.75 pages. We ensure that the live animal will be available for filming.

15. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in Figure Legends, but rather the Protocol.

Authors: Done.

16. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

Authors: Done.

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e. "This figure has been modified from [citation]."

Authors: Does not apply.

18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

Authors: Done.

b) Any modifications and troubleshooting of the technique

Authors: Done.

c) Any limitations of the technique

Authors: Done.

d) The significance with respect to existing methods

Authors: Done.

e) Any future applications of the technique

Authors: Done.

19. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

Authors: Done.

20. Please include the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in a .xlsx file.

Authors: Done.

Reviewers' comments:

Reviewer #1:

Major Concerns:

In the results please note the degree of correlation between the histology and the physiology.

Authors: The current JOVE submission focuses on the Methods used for this research. We have, however, submitted a data-driven manuscript to a different journal where we included the correlation between the histology and the physiology and an interpretation on how this information has allowed us to learn something new about the ear.

Minor Concerns:

small typos line 48, line 180.

Authors: Thank you for catching this. Typos corrected.

In section ~ 3.2-3.5 in the written part it would be useful if very approximate sizes were given for the visible part of the operculum, the sac, etc.

Authors: We have now added the requested information to Protocol item 3.5: “The operculum is oval shaped, approximately 3 to 4 mm by 1.5 to 2 mm. However, as seen from the surgical view, the operculum appears as an approximate 1 mm slit. The visible portion of the sac from the surgical view is approximately the same size as the visible portion of the operculum, if not smaller.”

Section 4.5 notes 3-4 hours as recovery time but "Representative Results" notes an average of 4 hours. Please make it consistent.

Authors: Done.

Reviewer #2:

Major Concerns:

1. The extradural approach in this manuscript is not the first introduction in animal model. Egami et al introduced the method in the journal (Morphological and functional changes in a new animal model of Meniere's disease). After that, so many researchers followed the surgical method with more many populations' data. Moreover, there are new combined surgical methods with vasopressin for

endolymphatic hydrops.
Authors: We agree with the Reviewer. Our method is certainly not the first the first extradural approach. Indeed, most of the Introduction is a review of approaches used by others. Our motivation for publishing a revised method is that after studying all available content we still had to substantially improvise our surgical approach to create endolymphatic hydrops. The results of our improvisation is what we would like to document with video with JOVE. Our need to improvise demonstrates that a large amount of important information on how to complete this classic surgery is lacking in the available literature. Our goal is to build upon the terrific work done by others to help fill a void so future investigators can use this model to study endolymphatic hydrops.

The 86% hit rate of achieving endolymphatic hydrops in six out of seven guinea pigs suggests that vasopressin need not be added to our revised surgical method to achieve endolymphatic hydrops.

2. There were few figures to explain the surgical anatomy. According to the Megerian's paper(Jove 2013), this manuscript did not show the differences for the surgical procedures.
Authors: The video portion of this submission, which will follow the written submission that is currently be reviewed, will show the surgical anatomy and procedures.

3. Using the pick could not be enough to keep the obliteration of endolymphatic hydrops. The clinicians know the injury of the endothelium of canal would be reversible. Why do you use the electro-cauterization? The endolymphatic sac is so small, the pick manipulation would be difficult. The blunt coagulation would be optional .
Authors: Our results show that a pick was enough to create endolymphatic hydrops. We agree with the Reviewer about reversibility, and have revised Protocol item 3.6 to more clearly communicate this. It now reads “It is critical that the expungement process leave no visible connection between the dura and the operculum, and then a fine pick be placed inside the operculum to broadly scrape along the inside of the bone to injure it.”

4. Figure 2 showed the cross-sectional area. But the portion of cross-section would be needed. The ratio from the apical to basal is so various. The comparison with absolute area is not informative. In the absolute area in control, is scala media similar? In Figure 1, you need to check whether the area of scala media were same in each turns.
Authors: The gray dashed lines of Fig. 2 are cross-sectional area measures of the control group and the red are area measures for the animals received the endolymphatic sac ablation surgery. The red lines are clearly different than the gray dashed lines with almost no overlap, demonstrating that we successfully created endolymphatic hydrops in all ears shown. Statistical results for the data driven version of this work submitted elsewhere show that this difference is significant. We thus disagree with the Reviewer that a proportion of the cross-section would be needed and that the comparison of the absolute area is not informative, as the data expressed in their current form clearly show the presence of endolymphatic hydrops in surgical ears.

We agree with the Reviewer that the ratio from the apex to base of hydropic ears would be variable (if our data were expressed that way). But, the variability is quite systematic with area of surgical ears (i.e., red lines) being less in the apex and greater in the base. That is to say, we think the reviewer is describing a “trend”, and not that a ratio would be “various”. The reason for the trend is because the cross sectional area of scala typmani and scala vestibuli in normal ears gradate gargantuanly by an approximate factor of 15 along the cochlear length, with area of scala typmani and scala vestibuli being

smallest in the apical half (e.g., Salt et al. 1995 Hearing Research, "Detection and quantification of endolymphatic hydrops in the guinea pig cochlea by magnetic resonance microscopy"). Thus, the scala media in a cochlear apex of an ear with endolymphatic hydropic does not have as much space to expand as compared to the base. For this reason, the red lines in Fig. 2 (i.e., scala media area of surgery ears) systematically vary from base to apex, the gray lines (i.e., scala media area of control ears) do not vary. Together these data show that our surgery robustly achieved endolymphatic hydrops.

The gray lines (i.e., control group measures) do not vary substantially, showing that are control data are consistent with previous reports on the guinea pig scala media of normal ears being essentially constant along the cochlear length for which we investigated (e.g., Salt et al. 1995 Hearing Research, "Detection and quantification of endolymphatic hydrops in the guinea pig cochlea by magnetic resonance microscopy"). The data in gray dashed lines and the additional data here address the Reviewer's direction to check whether the area of scala media were the same in each turns (of the control group): Fig. 1 left ear cross sectional area measures from apex to base in half turn increments: 0.077, 0.092, 0.107, 0.101, 0.115, 0.107, 0.112, 0.122. Our data show that cross sectional area of scala media of control ears does not vary substantially across cochlear turn, meaning that comparing to the area measures of surgical ears that do indeed vary across turns shows that we successfully induced endolymphatic hydrops.

5. In figure 3, you mean that the low tone freq. was under 800. In guinea pig, normal freq. is not same in human. Compared to control group, the thresholds across the whole freqs. were high in ANOW. It could not be evidences for the low tone loss in your animal model . The definition for low tone loss would be revised. The hearing tests in tone burst ABR would be added.

Authors: We define "low frequency" with two criteria: 1) the auditory brainstem response, and its wave 1 (the cochlear compound action potential), do not work adequately at 1 kHz and below (e.g., Fig. 7 of Lichtenhan et al. 2013 "A new auditory threshold estimation technique for low frequencies: proof of concept.", and many other reports as well as routine clinical usages of correction factors for quantifying threshold), and 2) the cochlear characteristic frequency place associated with the half-way demarcation point along the cochlear length, which is 1-2 kHz (Fig. 1 of Tsuji & Liberman 1997 The Journal of Comparative Neurology, "Intracellular labeling of auditory nerve fibers in guinea pig: central and peripheral projections"). We agree with the Reviewer that guinea pigs differ from humans but, with sincere respect, believe that our definition of "low frequency" is justified by functional data and anatomical landmarks and that our definition does not need to be revised.

The Reviewer is correct in that, compared to the control group, that all ANOW thresholds were higher than control. This is direct evidence that we successfully created low-frequency hearing loss in six out of seven animals that underwent endolymphatic sac ablation surgery.

The Reviewer requested that auditory brainstem response (ABR) measures be added. Those measurements were made and the measures of ABR wave 1 amplitude were presented in Fig. 3 of the original submission. We now clarify: "Wave 1 of the auditory brainstem response, or the cochlear compound action potential (CAP), thresholds were within the normal range at frequencies above 8 kHz in six out of the seven guinea pigs." CAPs are used instead of the entire ABR waveform, as endolymphatic hydrops is defined as a cochlear condition, and using a measure of cochlear origin (i.e., the CAP) is justified more than using measures from the brainstem.