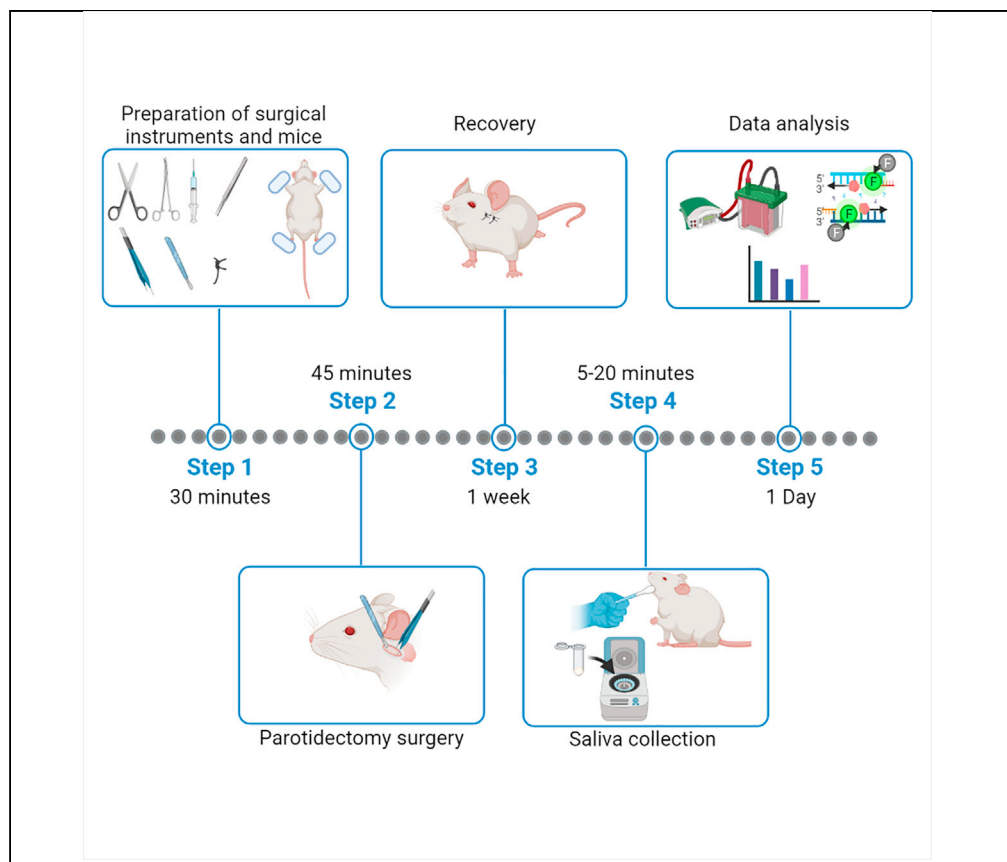


Protocol

Protocol for parotidectomy and saliva analysis in mice



The murine parotid salivary glands develop postnatally, shaping oral mucosal immunity in early and adult life. This protocol details the surgical removal of the parotid glands (parotidectomy) of mice. We also describe a protocol for saliva collection to enable manipulation and measurement of physiological and immunological salivary functions. Our saliva collection approach has been modified from published protocols to enable saliva collection from young mice, which can be challenging.

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Highlights

Protocol to collect
saliva from mice

Safe technique for
surgical removal of
the murine parotid
salivary gland

A detailed
description for
measuring the weight
and volume of
collected murine
saliva

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Protocol

Protocol for parotidectomy and saliva analysis in mice

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SUMMARY

The murine parotid salivary glands develop postnatally, shaping oral mucosal immunity in early and adult life. This protocol details the surgical removal of the parotid glands (parotidectomy) of mice. We also describe a protocol for saliva collection to enable manipulation and measurement of physiological and immunological salivary functions. Our saliva collection approach has been modified from published protocols to enable saliva collection from young mice, which can be challenging.

For complete details on the use and execution of this protocol, please refer to Koren et al. (2020).

BEFORE YOU BEGIN

In this manuscript, we describe a step-by-step protocol for surgical excision of the murine parotid salivary gland (parotidectomy). The protocol further describes an efficient approach for collecting saliva under physiological conditions or upon stimulation with pilocarpine (Kondo et al., 2015), enabling measuring and analyzing the murine saliva. The amount of naturally produced saliva is limited and might be too low for certain analyses. The use of pilocarpine overcomes this caveat by increasing the amount of saliva produced by the mice. Whereas pilocarpine increases the amount of saliva collected, it could influence the saliva quality such as the protein concentration, cellular content, and biochemical activities.

Before starting, please note the following:

1. Use sterile surgical tools only; it is recommended to perform the surgery in a biological hood to avoid wound contamination.
2. Get familiar with the anatomic area of the parotid gland to avoid unnecessary tissue damage during surgery (Maruyama et al., 2019). It is recommended to perform the first surgery on a cadaver.
3. Saliva collection protocol was performed on 2–12 weeks old C57BL/6J mice. The parotidectomy protocol was performed on two adult mouse strains (8–12 weeks old), C57BL/6J and BALB/c.
4. All the materials and tools should be prepared before starting the procedure as the schedule is tight, and there is no room for preparing material through the procedure.
5. Make sure you have the correct anesthesia and pain killers for post-surgery treatment.
6. For saliva volume/weight measuring make sure that the scale and pipettor you are using are calibrated.

Animal procedures

All animal protocols approved by the Hebrew University Institutional Animal Care and Use Committee (IACUC).



KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Chemicals, peptides, and recombinant proteins</i>		
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich	Cat#: D1408-500; Lot: RNBH8797
Fetal Bovine Serum	Biological Industries	Cat#: 04-127-1A; Lot: 1720444
Carprieve (carprofen)	Norbrook Laboratories	Cat#:143-52-92436-00
Sedaxylan (Xylazine)	Eurovet Animal Health B.V.	Cat#: 6031-2-02/03
Clorketam (Ketamine)	Vetoquinol	Cat#: ST01250
Sodium Chloride Intravenous Infusion BP 0.9% w/v (Saline)	Baxter Healthcare Ltd UK	Cat# IE1323
Pilocarpine hydrochloride	Sigma-Aldrich	Cat# P6503
Ethanol 70%	ROMICAL	BATCH#: V0H095140I
<i>Experimental models: organisms/strains</i>		
C57BL/6J	The Jackson Laboratory	JAX:000664
BALB/c	The Jackson Laboratory	JAX:000651
<i>Other</i>		
Mosquito Hemostat 12cm	ExLin	Cat# : ED-010-12
Sterile Collection Swab, 20mm break point	ZYMO	Cat#: C1052-50
Parafilm	Bemis	Cat#: PM-996
Warming lamp		100 Watt
2-mL microfuge tube	LIFEGENE	#Cat: LMCT2.0B
0.5-mL microfuge tube	SSlbio	#Cat: 1110-09
Centrifuge	Thermo Scientific	MICROCL 17
Toothed Curved forceps 16cm	Hu-Friedy	Cat#: 125-001
Oral swab – sponge	HALYARD	REF: 12243
Vicryl suture thread 5-0/6-0	ETHICON	Cat#: W9501
Gauze Pad	SION MEDICAL	LOT#: PC103048
Surgical blades 15	Swann-Morton	REF: 0105
Scalpel blades holder	Swann-Morton	Cat#: 7721551
Needles 16G x 1 1/2" (1.6 x 40mm)	BD Microlance™ 3	LOT#: 191015 REF#: 300637
Duct-tape 19MM 13M	Fisherbrand	Cat#: 15-910-5G
Scissors	AS	Cat#: 11-004-11

MATERIALS AND EQUIPMENT

Prepare the following working solutions:

Anesthesia solution

Dilute 1 mL of Ketamine and 0.5 mL Xylazine in 10 mL sterile saline. Use 15 μ L per gram body weight (BW) for a mouse. This amount of anesthesia will keep the mouse anesthetized for 30 min at least. The anesthesia solution can be kept at room temperature (25°C) for a few days.

Alternatives: Isoflurane can also be used to anesthetize the mice.

Pain killer

Dilute 100 μ L of Carprofen in 5 mL sterile saline.

Note: Always use a fresh solution.

Pilocarpine solution

Dissolve 3 mg of pilocarpine hydrochloride in 50 mL sterile saline. Inject mice with approximately 6 μ L per gram BW. (Final concentration 0.375 mg/kg BW).

Note: We usually use freshly made solutions.

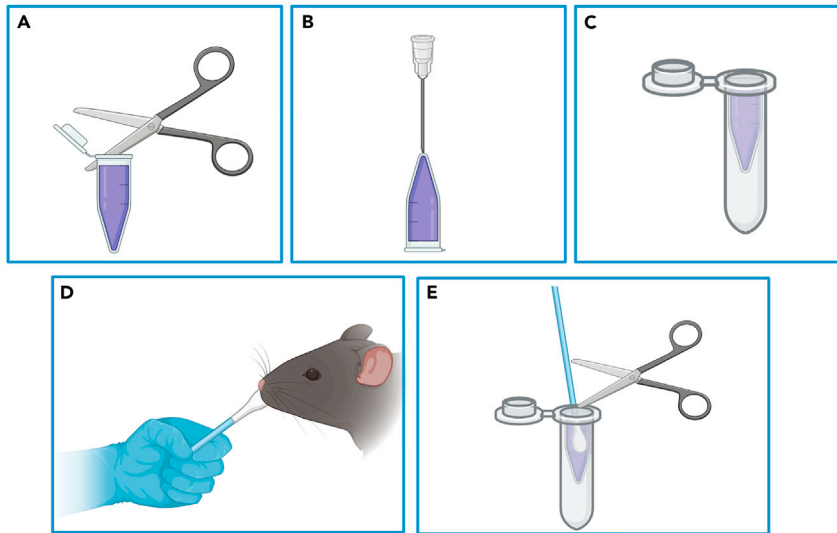


Figure 1. Saliva collection without pilocarpine injection

- (A) Remove the 0.5 mL tube lid.
 (B) Use the 16-gauge needle to make a hole in the bottom of the 0.5 mL tube.
 (C) Place the 0.5 mL tube in the 2 mL tube.
 (D) Use the collection swab to collect saliva.
 (E) Place the collection swab in the 0.5 mL tube and cut the holder.

⚠ **CRITICAL:** Pilocarpine hydrochloride can be fatal if swallowed or inhaled, prepare the solution in a chemical hood with all the safety instruments.

Alternatives: In theory, all reagents and resources listed in the ‘key resources table’ can be substituted with equivalent items from other suppliers. However, it should be noted that the protocol has been calibrated to the reagents listed in the above table and alternatives have not been tested on the protocol performance.

STEP-BY-STEP METHOD DETAILS

Collecting saliva without the injection of pilocarpine

⌚ **Timing:** ~ 20 min for 5 mice

Note: We performed this procedure without anesthetizing the mice, nevertheless, it can be performed on anesthetized mice if preferable. In case you want to use the saliva for bacterial quantification or RT-PCR analysis, make sure to use sterile equipment.

1. Take a 0.5 mL tube and remove the lid using scissors (Figure 1A).
2. Using a 16-gauge needle, perforate the bottom of the 0.5 mL tube and place it in a 2.0 mL centrifuge tube (Figures 1B and 1C).

Note: It is better to prepare the equipment and materials before performing the protocol.

⚠ **CRITICAL:** Be careful, if the gauge slips, it can cause a serious injury.

3. Hold the mouse with one hand, and with the other hand, use the collection swab to collect saliva from the oral cavity by moving the swab for 30 s, make sure to reach all areas - buccal and beneath the tongue (Figure 1D).

Note: for bacterial quantification, make sure the swab does not touch anything but the oral cavity.

4. Take the swab out, place it in the perforated 0.5 mL tube in the 2 mL tube (Figure 1E).
5. Seal the tube using parafilm and place it on ice while collecting other samples to prevent the saliva from drying.
6. After sampling the mice, remove the parafilm and centrifuge at 8000 g for 3 min while the 2 mL tube lid is open.
7. Take the 0.5 mL tube with the swab out, the saliva is in the bottom of the 2 mL tube.

Collection of saliva after stimulation with pilocarpine

⌚ Timing: ~ 45 min for 5 mice

Note: Before you begin, prepare sponge pieces to collect saliva, cut a sponge into small pieces to a cylinder shape with a diameter of 0.5 cm and 1.5 cm height or boxes of the same size (Figure 2G).

8. Anesthetize the mouse by injecting the mice intraperitoneally (IP injection) with 200 mg/kg body weight (BW) – approximately 200–300 μ L of anesthesia solution per mouse, and wait 3–4 min until the mouse is fully anesthetized. Verify that the mouse is well anesthetized.
9. Inject the mouse with pilocarpine, IP injection of 0.375 mg/kg BW.

Note: It is important to follow the recommended concentrations/quantities of pilocarpine precisely as a slight overdose in addition to the anesthesia may be lethal. We found this protocol suitable for mice that are older than 2 weeks.

10. It takes 2–3 min for the pilocarpine to induce saliva secretion, in the meantime, lay the mouse on its side to avoid choking from excess saliva (Figures 2A and 2G).
11. Use a curved forceps to open the mouth of the mouse, and with another forceps, insert the sponge in a way that fills up the oral cavity. Make sure the sponge is stable and not inserted too deep (Figures 2B–2E and 2H–2J).

⚠ CRITICAL: If the sponge is inserted too deep it may cause suffocation. Do not use sharp forceps.

12. Set timer for 15 min, keep an eye on the mouse, move the mouse/sponge if it shows any signs of suffocating.
13. Using forceps, take the sponge out of the oral cavity and place it in the perforated 0.5 mL tube. Place the tube in a 2 mL tube, seal it with parafilm, and place it on ice so the saliva will not dry out (Figure 2F).
14. Do the same to the rest of the mice.
15. After taking all sponges out of the mice, remove the parafilm and centrifuge the tubes at 8000 g for 3 min while the 2 mL lid is open.
16. At the end of centrifugation, saliva should be at the bottom of the 2 mL tube. You can now measure the volume of the saliva.
17. Keep the mice warm using a warming light until they are fully awake from anesthesia.

Measuring volume/weight of saliva

⌚ Timing: ~ 5 min for each tube

For measuring the weight of the saliva, it is recommended to use a sterile 2 mL Eppendorf tube.

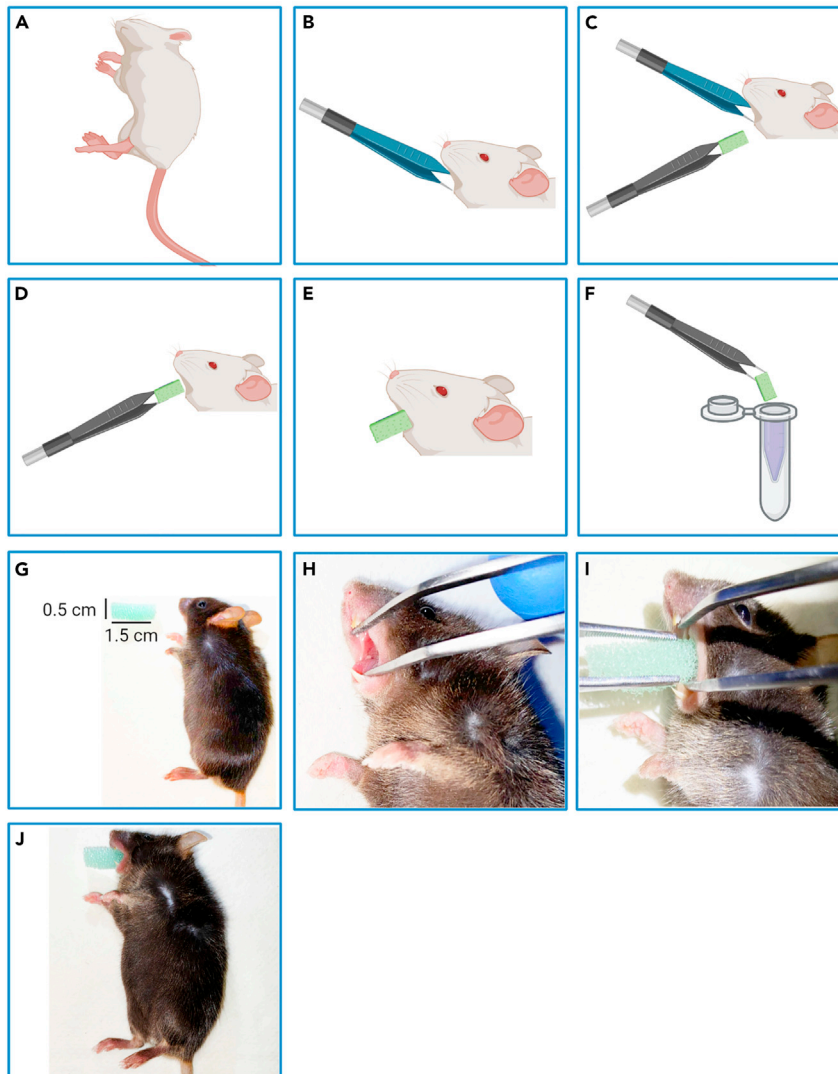


Figure 2. Saliva collection with pilocarpine stimulation

(A and G) Lay the mouse on its side after the injection of anesthesia and pilocarpine.
 (B and H) Open the mouth of the mouse with forceps.
 (C, D, and I) Hold the mouth open, use forceps to place the sponge in the oral cavity.
 (E and J) Wait 15 min for the sponge to absorb the saliva.
 (F) Remove the sponge and place it in the 0.5 mL tube for centrifugation.

18. Label each 2 mL tube and measure its weight while empty before saliva collection.
19. After collecting saliva, take the perforated 0.5 mL tube with the sponge out.
20. Close the 2 mL tube and weigh again with the saliva inside.
21. Calculate the net weight of the saliva.

For measuring the volume of the saliva

22. After centrifugation, remove the 0.5 mL tube with the sponge out.
23. If pilocarpine was used, use a pipettor, set it to a large volume of liquid (100–150 μ L for instance), pull out all liquid, and then set again the pipettor to a smaller volume until the liquid reaches the margin of the tip. This is the volume of the saliva.

24. If pilocarpine was not used, the amount of saliva might be too little and viscous for direct measurement. Therefore, dilute the saliva with a known amount of PBS (100 μ L for instance), collect the sample as described in the preceding step (step 23). Calculate the saliva volume by subtracting from the volume indicated by the pipettor the volume of the PBS.

Parotid gland extraction surgery (parotidectomy)

⌚ Timing: ~ 45 min per mouse

Note: While this study focuses on the parotid glands, it's worth mentioning that the submandibular glands produce the majority of saliva under physiological conditions. The submandibular glands were also reported to play an important immunological role.

Before performing the surgery for the first time, practice the procedure on a cadaver.

25. Anesthetize the mouse by IP injection with 200 mg/kg BW (approximately 200–300 μ L of anesthesia solution per mouse).
26. After verifying the mouse is well anesthetized, fix the mouse on a clean platform using duct tape placed on the limbs (Figures 3A and G).
27. Shave a 1 cm \times 0.5 cm area right under the mouse ear lobe using an electric pet shaver (Figure 3H). Disinfect the area with 70% medical ethanol.
28. Make a 4 mm horizontal incision on the skin placed 1–2 mm below the ear lobe to expose the glands underneath (Figures 3B, 3C, and 3I).

Note: There are two similar glands in this area: the posterior is the parotid gland, while the anterior is the extraorbital lacrimal gland. We recommend locating both glands to make sure the removal is performed on the parotid gland.

29. After identifying the parotid gland, use the curved forceps to pull the gland out, use a scalpel to separate the gland from the surrounding tissue (Figures 3D, 3E, and 3J–3K). Cut the gland out right beneath the lower margin of the gland (Figure 3L). Bleeding is unlikely unless a blood vessel was accidentally ruptured.

Note: Beware of cutting the external/common carotid artery.

30. Close the wound by a simple skin suture technique using a sterile vicryl surgical suture (three sutures should be enough) (Figures 3F and 3M).
31. Treat the mouse with painkillers, IP injection of 100 μ L Carprofen solution.
32. Release the mouse and put it back in the cage. Keep all the mice warm after surgery under a warming light. Wait for the mice to awake from the anesthesia and make sure they are moving just fine.
33. Watch the behavior of the mice and their ability to eat food and drink water on the following days.

EXPECTED OUTCOMES

It is expected that the extraction of the parotid salivary gland will result in a decreased secreted saliva to the oral cavity, as a result, an increase in the oral microbial load and change in the microbial diversity may occur. Weight loss is unlikely to happen, nevertheless, it is recommended to verify that the mice are eating and drinking properly.

Due to the parotidectomy-associated alteration in the load and diversity of the oral microbiota, periodontal diseases might develop.

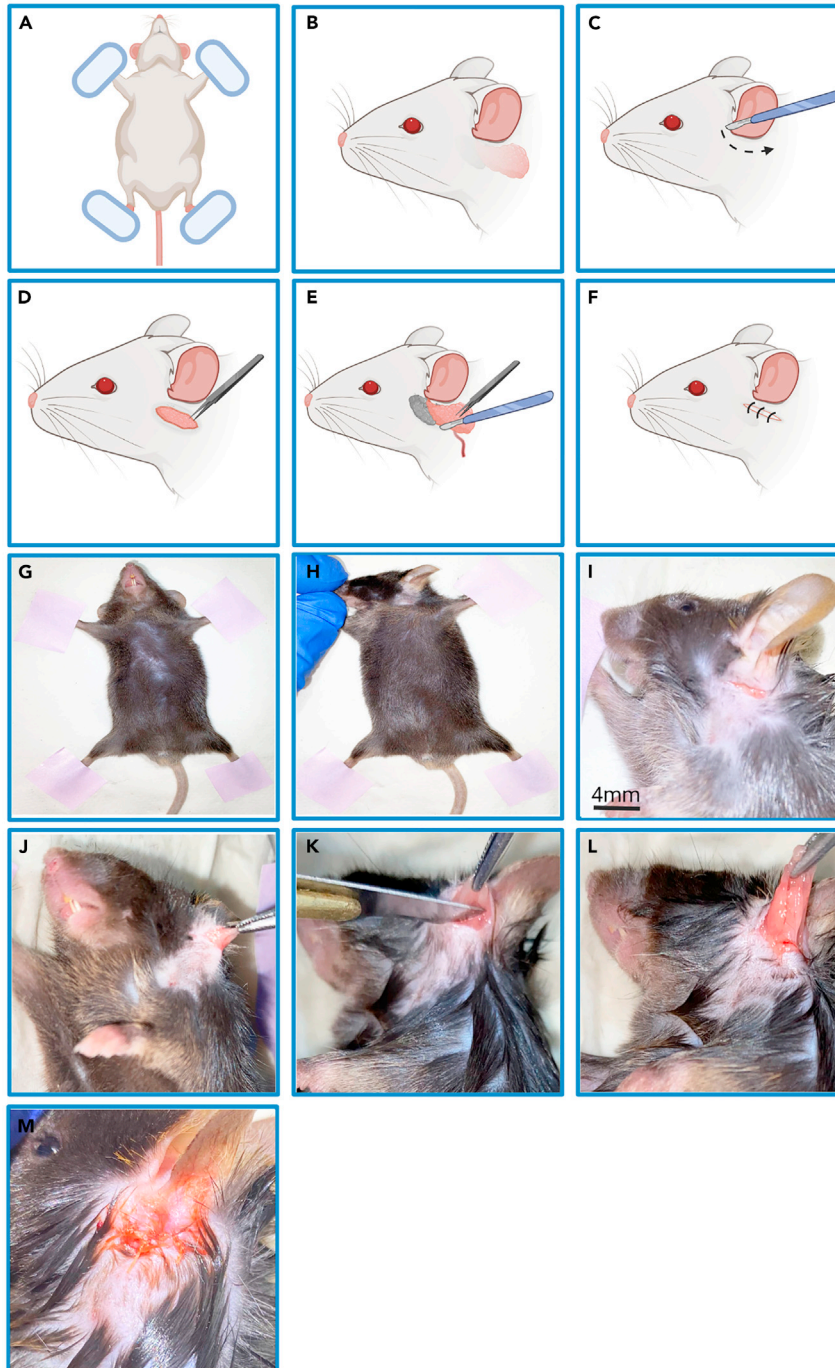


Figure 3. Parotid salivary gland extraction surgery

(A and G) Fix the limbs of the anesthetized mouse to perform with duct tape.
 (B and H) Find the appropriate position to reach the wanted area.
 (C and I) Make a horizontal incision under the ear lobe.
 (D and J) Pull the parotid gland out with forceps.
 (E and K) Separate the gland from the surrounding tissues with a scalpel.
 (L) Pull the gland and watch for the blood vessel.
 (F and M) Close the wound with surgical sutures.

LIMITATIONS

The parotid salivary glands are fully developed during weaning (around three weeks after birth), thus it is difficult to collect a measurable amount of saliva during the neonatal period. In addition, the neonates might not survive the pilocarpine injection.

TROUBLESHOOTING

Problem 1

When cutting parts of the gland, a major blood vessel (the carotid vessel) might be damaged. (step 29)

Potential solution

Locate the blood vessel while pulling the gland out and try avoiding it during the procedure. If the blood vessel was damaged by mistake, cover the wound with a sterile gauze pad, press gently to stop the bleeding, and suture the wound as fast as possible.

Problem 2

It might be possible that after the surgery the mice will have some difficulty eating or drinking properly (step 33).

Potential solution

Provide the mice soft food and water on the floor of the cage for at least 3 days.

Problem 3

A difficulty locating the parotid gland at the time of surgery (step 28)

Potential solution

Perform the surgery several times on mouse cadavers and learn the anatomic area of the surgery. If the parotid gland cannot be found at the time of surgery, a larger incision will ease locating it.

Problem 4

Mistakenly removing the external Lacrimal gland instead of the parotid gland due to their anatomic resemblance and proximate location (step 29).

Potential solution

During the surgery, make sure to identify each gland, separate them gently and pull the parotid gland out.

Problem 5

The collected saliva is not passing through the 0.5 mL tube to the 2 mL tube after centrifugation (step 7, 16).

Potential solution

When perforating the 0.5 mL tube, make sure to remove plastic chips that may clog the hole in the bottom of the tube.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Avi-Hai Hovav (avihaih@ekmd.huji.ac.il).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze data.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

K.Z., Y.S., O.B., and F.L.S. performed experiments; K.Z. conceptualized the study and calibrated the system; K.Z. and A.-H.H. wrote the paper; A.-H.H. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Kondo, Y., Nakamoto, T., Jaramillo, Y., Choi, S., Catalan, M.A., and Melvin, J.E. (2015). Functional differences in the acinar cells of the murine major salivary glands. *J. Dental Res.* 94, 715–721. <https://doi.org/10.1177/0022034515570943>.
- Koren, N., Zubeidat, K., Saba, Y., Horev, Y., Barel, O., Wilharm, A., Heyman, O., Wald, S., Eli-Berchoer, L., Shapiro, H., et al. (2020). Maturation of the neonatal oral mucosa involves unique epithelium-microbiota interactions. *Cell Host Microbe* 29, 197–209. <https://doi.org/10.1016/j.chom.2020.12.006>.
- Maruyama, C.L., Monroe, M.M., Hunt, J.P., Buchmann, L., and Baker, O.J. (2019). Comparing human and mouse salivary glands: a practice guide for salivary researchers. *Oral Dis.* 25, 403–415. <https://doi.org/10.1111/odi.12840>.