

Video Article

Murine Salivary Functional Assessment via Pilocarpine Stimulation Following Fractionated Radiation

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Abstract

Hyposalivation is commonly observed in the autoimmune reaction of Sjögren's syndrome or following radiation injury to the major salivary glands. In these cases, questions remain regarding disease pathogenesis and effective interventions. An optimized technique that allows functional assessment of the salivary glands is invaluable for investigating exocrine gland biology, dysfunction, and therapeutics. Here, we present a step by step approach to performing pilocarpine stimulated saliva secretion, including tracheostomy and the dissection of the three major murine salivary glands. We also detail the appropriate murine head and neck anatomy accessed during these techniques. This approach is scalable, allowing for multiple mice to be processed simultaneously, thus improving the efficiency of the work flow. We aim to improve the reproducibility of these methods, each of which has further applications within the field. In addition to saliva collection, we discuss metrics for quantifying and normalizing functional capacity of these tissues. Representative data are included from submandibular glands with depressed salivary gland function 2 weeks following fractionated radiation (4 doses of 6.85 Gy).

Video Link

The video component of this article can be found at <https://www.jove.com/video/57522/>

Introduction

Salivary gland disorders include syndromes of dysregulated or impaired secretion leading to either overproduction (sialorrhea) or underproduction (xerostomia and hyposalivation) of saliva¹. In both cases, there is an interest in improving our understanding of salivary gland biology towards the end goal of therapeutic development².

The salivary glands are highly radiosensitive organs, and are often damaged during head and neck cancer radiotherapy, leading to permanent dry mouth (xerostomia)^{3,4}. Unlike other radiosensitive tissues, however, salivary gland turnover rate is relatively low, and the mechanism of secretory loss is poorly understood^{5,6}. In this unique injury setting, tissue regeneration and radioprotection strategies require salivary functional assessment. Experimentally, murine saliva collection is a particularly valuable tool in evaluating the gland response to both radiation and therapeutic agents.

Here, we present a method for performing and quantifying stimulated saliva secretion using pilocarpine, a potent muscarinic agonist⁷. Pilocarpine stimulates the autonomic nervous system to induce gland secretion^{8,9}. To complete this test appropriately, a tracheostomy is required to ensure that the mouse maintains a patent airway throughout the procedure, and to reduce the risks of choking and aspiration from pooled secretions in the oral cavity¹⁰.

This is a terminal procedure, culminating in the removal of the three major salivary glands: the parotid (PG), the submandibular (SMG), and the sublingual (SLG). For functional studies, gland weights are recorded and are often used to normalize saliva measurement^{11,12,13}. This data is particularly important in radiation studies, wherein gland atrophy is an expected outcome^{14,15}.

There is variability in the literature with regards to how stimulated saliva secretion is performed and reported¹⁶. For example, pilocarpine doses within the literature span at least three orders of magnitude^{17,18,19,20,21,22,23}. Here, we present an optimized high dose pilocarpine protocol with the intent of improved reproducibility in method execution, as well as providing a modular platform of techniques (tracheostomy, saliva collection, and gland dissection) that can be adapted as needed.

In addition to protocol demonstration, we include representative functional data of saliva flow at 2 weeks following fractionated radiation (4 doses of 6.85 Gy) to the SMG region.

Protocol

All *in vivo* procedures outlined below were approved by the University Committee on Animal Resources at the University of Rochester, Rochester, NY.

1. Preparation

- Using an analytical balance, weigh 20 mg of pilocarpine. Dissolve it in 2 mL of sterile saline in a microcentrifuge tube.
NOTE: Because pilocarpine is light sensitive and loses activity over time, this solution should be prepared the day of injection, and protected from light until administered.
- Using an analytical balance, weigh and identify all collection tubes and glass capillaries. The number of collection tubes and glass capillaries should match the number of mice.

2. Tracheostomy

- Weigh C57/BL6 mice using an analytical balance.
- Using a 0.5 mL syringe with 29G x ½" needle, anesthetize mice with an intraperitoneally injected sterile saline solution of 100 mg/kg ketamine and 10 mg/kg xylazine. Proceed to the following step when the mouse no longer responds to stimuli (*i.e.* absence of the pedal withdrawal reflex), which generally occurs within 5 to 10 min following injection.
- After dispensing lubricant on a cotton-tipped applicator, gently apply to eyes and place the mouse in a supine position on the stage. The anesthetized animal must be kept on warm surface.
NOTE: All steps are performed at room temperature.
- Secure the nose, limbs, and tail to the stage using surgical tape.
- Clean and wet the neck with an alcohol wipe.
NOTE: This will help prevent fur from entering subsequent incisions.
- Raising the ventral midline neck skin with forceps, make a small, superficial cut using dissecting scissors. Guide the scissors into the opening and slowly open the blades subcutaneously to separate tissue planes.
- Keeping the skin raised, carefully cut to 1 cm below the mouth.
- Make two lateral incisions at the inferior and superior aspects of the first cut. Using forceps, gently reflect away the skin to enable access to head and neck structures.
- Using the dissecting microscope at 8X magnification, visualize the submandibular glands (midline: **Figure 1**).
- Using fine forceps, gently lift the glands to expose the four infrahyoid (strap) muscles overlying the trachea. Avoid tearing or disrupting the surrounding vasculature or excretory ducts.
- If collecting saliva from more than one mouse, repeat steps 2.1 - 2.10 with each mouse before continuing.
NOTE: This procedure can be safely performed on up to 5 mice concurrently.
- With small dissecting scissors, remove the medial portion of strap muscles while staying as close to midline as possible. Cut only as much as necessary to visualize trachea and keep strap muscles out of the way during the procedure. Avoid nicking any nearby vessels because if there is volume depletion secondary to significant hemorrhage, pilocarpine will not be effective at inducing secretion.
- Reflect the strap muscles away from the trachea.
- Visualize the larynx, trachea, and thyroid gland. Ensure that the trachea is clear of overlying tissue.
- Make a horizontal incision in the trachea inferior/posterior to the thyroid using small dissecting scissors. Ensure that the airway is patent and clear of fluid.

3. Saliva Collection

- Remove the tape near mouth and angle dissection stage downward (45°, cranially) to assist with saliva flow.
- Using a 0.5 mL syringe with 29G x ½" needle, perform intraperitoneal injection of 10 µL/g body weight pilocarpine for a total dose of 100 mg/kg.
- Start a timer. If dosing multiple mice, reduce lead time by moving quickly and by having an assistant simultaneously inject the remaining mice.
- Using standard forceps, open the mouth for capillary tube access.
- Once a bead of saliva is observed in the mouth, place the proximal end of the capillary tube into the fluid, with the distal end placed into a collecting tube positioned below the dissecting stage (both tubes pre-weighed).
NOTE: In C57/BL6 female mice 6 - 10 weeks of age, gross salivation occurs within 2 min of pilocarpine injection.
- If saliva is building in the mouth, but not flowing through the tube, reattempt previous step. Ensure that the tube is not placed directly against a mucosal surface, which can obstruct the inlet.
- Collect saliva for a total of 12 min following pilocarpine stimulation. Ensure that the stoma remains clear.
NOTE: Increased tidal volume and secretions (salivary, urinary, fecal) are normal during this time.
- If mice have depressed saliva function (*e.g.*, due to injury or intervention), transfer the remaining fluid from the mouth to the collection tube using a P200 micropipette. If collecting from multiple mice, ensure that tips are changed.
- Record the weight of collected saliva plus tubes 12 min after pilocarpine injection.

4. Gland Dissection

- Euthanize mice by CO₂ euthanasia (as described in the AVMA Guidelines for Euthanasia of Animals: 2013 Edition) followed by thoracotomy, taking care to preserve the structures of the head and neck. For this reason, avoid cervical dislocation as a method of euthanasia.

2. Place the mouse in a supine position on the stage. Reposition glands in the original site over the trachea (**Figure 1**).
3. Using a dissecting microscope under 8X magnification, visualize the major glands: the PG, SMG, and SLG. To distinguish the glands during the dissection, identify that the PG is diffuse, lying lateral to the SMG and SLG.
NOTE: If dissecting the PG, this should be done first because it is the least defined and most likely to be torn.
4. Grasp the tail of the PG with forceps, pulling it away from underlying structures, and gently apply tension before cutting the head of the PG with dissecting scissors.
5. Breach the capsule surrounding the SMGs using forceps. Gently separate the left and right SMG.
6. Free the dorsal aspect of the SMG from adhered nonglandular tissue using forceps.
7. Remove the SMG from the neck by drawing Wharton's duct taut with forceps and cutting the duct with dissecting scissors.
8. Gently separate the SLG from the SMG using forceps.
NOTE: The SLG is in the superolateral aspect of the SMG.
9. Using an analytic balance, record SMG weights.

Representative Results

When performing high dose pilocarpine stimulated saliva collection, it is important to maintain the airway to prevent aspiration or choking from secretions in the oral cavity. A schematic of the tracheostomy is provided (**Figure 1**). Following tracheal incision, the stoma must remain clear of tissue and fluids.

To enhance capillary action during saliva collection, mice should be positioned with their heads downwards at a 45° angle. These steps can be performed on >1 mouse, though it is not recommended to exceed 5 mice at one time (**Figure 2**). Once collection is completed, it is recommended that tubes be weighed quickly to avoid evaporative losses.

Following saliva collection, mice are euthanized. Cervical dislocation should not be performed, as it may damage head and neck structures. Mice are returned to the dissecting stage, and the glands should be repositioned over the trachea, as originally located. Gland dissection (**Figure 3**) should begin with the PG, due to both its position and diffuse consistency. The SMG and SLG are firm, and can be removed easily.

Following 4 fractions of 6.85 Gy radiation to the murine SMG, saliva function significantly decreases by 2 weeks (**Figure 4**). Both total saliva secretion, and secretion normalized to gland wet weight were plotted. Using either metric, salivary functional capacity is reduced, as expected, at 2 weeks following irradiation²⁴.

Gland tissue can be fixed and sectioned for histological stains and/or immunohistochemistry. Hematoxylin and Eosin staining (H & E) shows similar gross SMG architecture both with or without pilocarpine stimulation (**Figure 5**). Staining for Nkcc1, a membrane sodium-potassium-chloride transporter, shows similar cell morphology irrespective of pilocarpine stimulation (**Figure 6**).

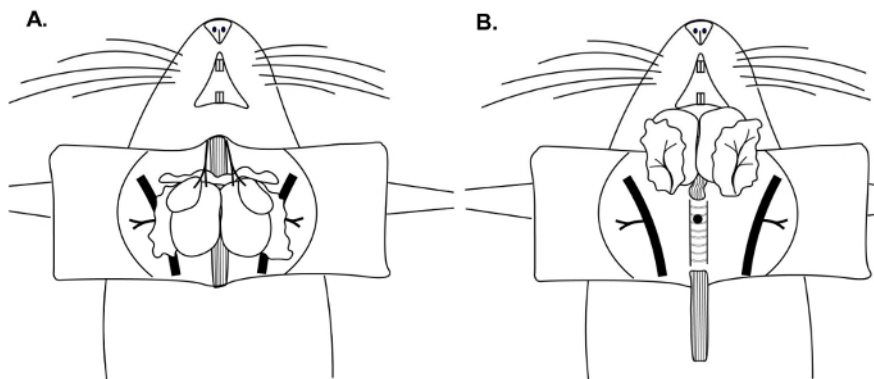


Figure 1. Tracheostomy schematic. (A) After opening the neck region, visualize the major structures. (B) To complete a tracheostomy, gently lift, but do not detach, the SMGs without disrupting neighboring structures, including blood supply, innervation, and excretory ducts. Cut the strap muscles to expose tracheal cartilage before making an incision into the trachea. Leave SMG in place and trachea exposed during entire saliva collection procedure. [Please click here to view a larger version of this figure.](#)



Figure 2. Saliva collection. Following pilocarpine stimulation, angle mice downwards and collect saliva through capillary tubes into pre-weighed collection tubes, which are placed below the dissection stage. [Please click here to view a larger version of this figure.](#)

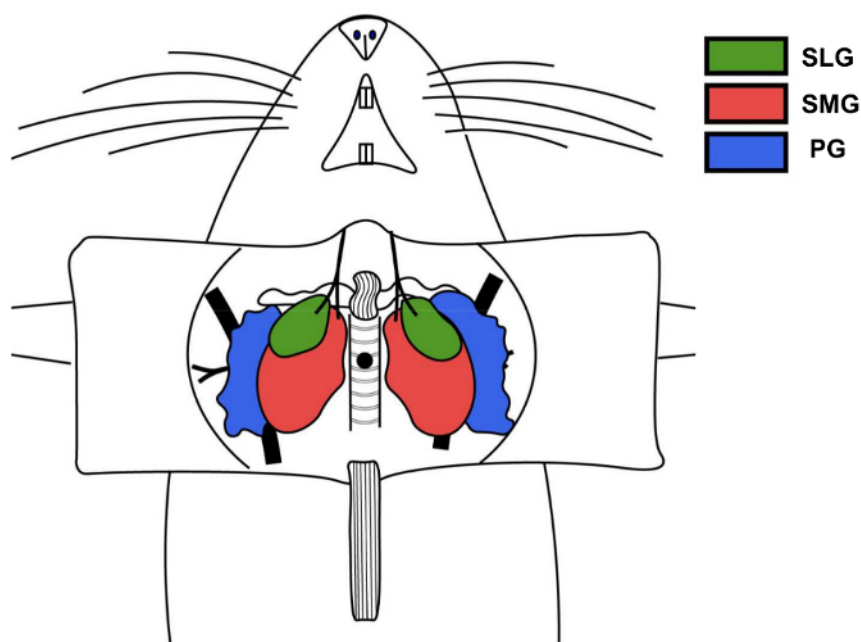


Figure 3. Major salivary gland dissection. Once mice have been euthanized, reposition and separate the left and right SMG. Carefully separate each gland from surrounding nonglandular tissues and blood vessels. The PG is a diffuse, lateral structure. The SMG and SLG are joined, but can be cleanly separated with forceps. [Please click here to view a larger version of this figure.](#)

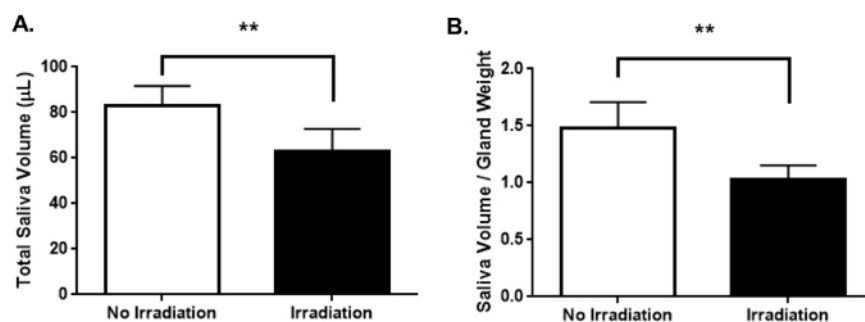


Figure 4. Representative secretion data. Two weeks following fractionated radiation (4 doses of 6.85 Gy) to the SMG, pilocarpine-stimulated saliva secretion was measured. Salivary function is reported as total saliva volume (A) and total saliva volume (B) normalized to total SMG wet weight. As expected, there is a significant decrease in the functional capacity of the SMG (mean $\pm$ SD $**p < 0.01$ using two-tailed unpaired t-test). [Please click here to view a larger version of this figure.](#)

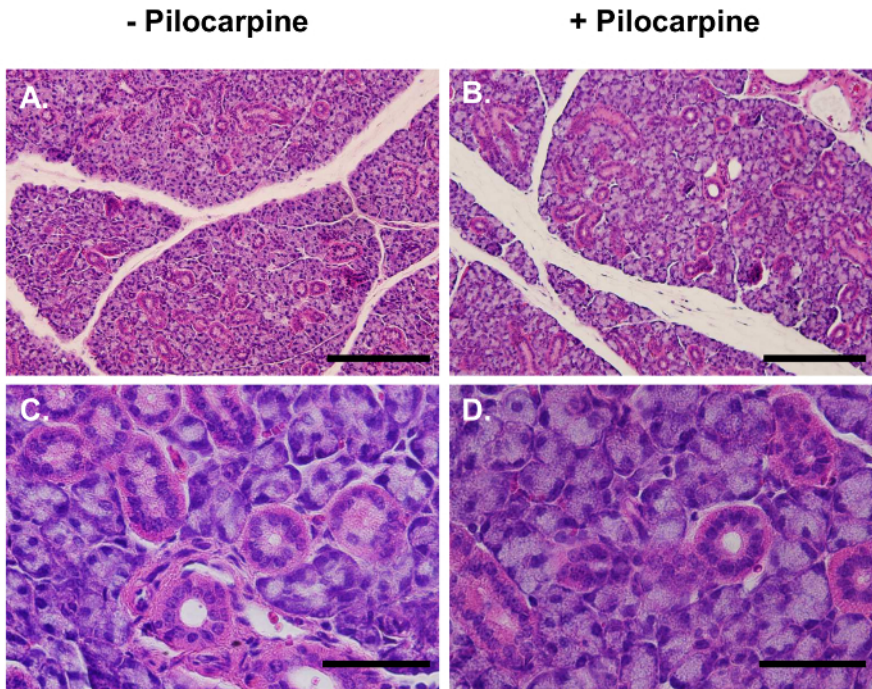


Figure 5. Hematoxylin and Eosin SMG staining. SMG tissue was harvested from a mouse after pilocarpine stimulation (100 mg/kg, Right) compared to control, untreated mouse SMG (Left). H & E staining shows gross gland structure at 10X (Scale bars: 200 μ m) (A, B) and 40X (Scale bars: 50 μ m) (C, D). [Please click here to view a larger version of this figure.](#)

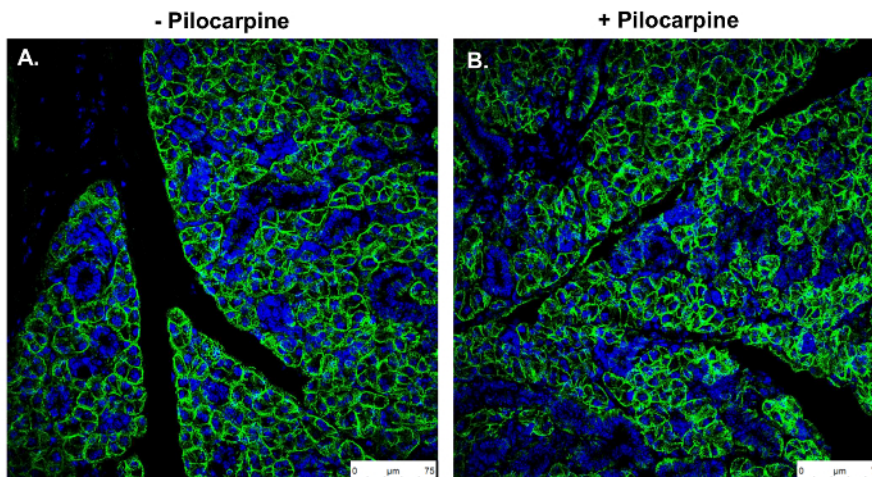


Figure 6. Nkcc1 SMG Immunohistochemistry. SMG tissue was harvested from a mouse after pilocarpine stimulation (100 mg/kg, Right) compared to control, untreated mouse SMG (Left). Nkcc1 is a membrane protein and can be used to evaluate cellular morphology. DAPI is used as a nuclear counterstain (Scale bars: 75 μ m). [Please click here to view a larger version of this figure.](#)

Discussion

We present a multistep method to assess salivary gland function, which can be applied to study gland injury and therapeutics. Our procedure involves tracheostomy, saliva collection, and gland dissection, each of which has experimental applications that can support an integrated study of salivary gland biology. For example, murine tracheostomy has been used for general airway management during procedures obstructing the oral cavity.

Proper dissection and tracheal incision are required for pilocarpine stimulated saliva secretion at 100 mg/kg. Alternatively, a reduced pilocarpine dose could avoid the need for tracheostomy and therefore be used for longitudinal assessment of saliva secretion¹⁶.

In addition to recording saliva secretion, this protocol also provides an accurate, consistent means to collect saliva for additional analyses. Such studies include rheology, proteomics, enzyme activity, saliva osmolarity^{25,26,27}, and biomarker discovery^{28,29}.

Finally, standardized dissection methods for the major salivary glands have utility beyond saliva collection. There is an interest in understanding gland development and response to injury. For effective investigations, it is important to perform consistent, clean dissections of the major salivary glands. Clean and efficient gland isolation is critical for cultures derived from primary cells that are mechanically and enzymatically dissociated from the major salivary glands^{30,31}, and for histologic and immunohistochemical preparations. Moreover, when staining for secreted proteins in gland tissue sections, it is critical to consider and control for the likely depletion of these products following stimulated saliva collection.

Quantifying salivary gland function in investigations of injury or intervention is a vital experimental technique. Gland histology is informative and may elicit key observations, yet conclusions are best supported by clear functional data.

Disclosures

The authors have nothing to disclose.

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