Journal of Visualized Experiments

# A minimally invasive lesion technique for muscles intrinsic to the odontophore of Aplysia californica.

--Manuscript Draft--

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|---|---|
| **Article Type:** | Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE60030R2 |
| **Full Title:** | A minimally invasive lesion technique for muscles intrinsic to the odontophore of Aplysia californica. |
| **Keywords:** | Aplysia, Biomechanics, Neurobiology, Minimally invasive surgery, Feeding, Lesions |
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Dear Editor,

We are very grateful for the helpful comments and feedback you provided on our revised manuscript. We have carefully made corrections to the text as well as answered a number of question that were raised and documented these changes in an additional response document. We believe that the revised version of the protocol should now be suitable for publication in the Journal of Visualized Experiments.

We look forward to hearing from you soon,
Catherine Kehl and Hillel Chiel

**TITLE:**

# A Minimally Invasive Lesion Technique for Muscles Intrinsic to the Odontophore of *Aplysia californica*

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**KEYWORDS:**

*Aplysia*, biomechanics, neurobiology, minimally invasive surgery, feeding, lesions

**SUMMARY:**

Here we present a protocol for minimally invasive surgical lesioning of muscles intrinsic to the feeding apparatus of the marine mollusk *Aplysia californica* to understand the roles of these muscles during feeding behavior.

**ABSTRACT:**

*Aplysia californica* is a model system for studying the neural control of learning and behavior. This animal has a semi-open circulatory system, making it possible to access many of its internal structures without causing any significant damage. Many manipulations can be easily performed both in vivo and in vitro, making it a highly tractable model for the analysis of behavior and neural circuitry. To better understand the functions of muscles within the feeding grasper, we have developed a technique for lesioning them without opening the main body cavity of the animal or damaging the outer layers of the feeding organ (i.e., the buccal mass). In this technique, the grasper is partially everted, allowing direct access to the musculature. This procedure allows animals to recover quickly and reliably. This has made it possible to lesion the I7 muscles and sub-radular fibers, allowing us to show that both muscles significantly contribute to the opening in vivo.

**INTRODUCTION:**

The feeding system of *Aplysia californica* has a long history of use as a model system for understanding learning and memory[1], motivated behaviors[2,3], and the interaction between behavior, biomechanics and neural control during feeding[4]. It has highly accessible neural circuitry, with a relatively small number of large, identifiable neurons. The animal has a semi-open circulatory system, making it possible to access many of its internal structures without causing significant damage. It is also robust to many manipulations both in vivo and in vitro, making it a highly tractable model for the analysis of behavior and neural circuitry.

To understand the neural patterns that give rise to feeding behaviors, it is important to describe the underlying mechanics of the soft structure that makes up the feeding organ, the buccal mass[4]. While there has been work done to characterize the outer muscles that make up the buccal mass[5,6], the inner muscles of the underlying structure within the buccal mass that controls the surface of the grasper, the odontophore, have been largely inaccessible to in vivo experimentation. Although there have been in vitro studies on the functions of some of these muscles[7,8], the lack of direct access to these muscles has made it difficult to study their role in intact, behaving animals.

Most techniques for electrode implantation or lesions in Aplysia or similar molluscan species require that the body wall be opened[9-12.] Opening the body wall causes epithelial injury, and the incision must be securely sealed to prevent hemolymph escape. Even more serious difficulties are posed when attempting to reach the inner muscles of the grasper of *Aplysia* (muscles underlying the radular surface or within the odontophore): having entered through the main body cavity, one must then go through some portion of the muscular wall of the buccal mass to gain access to the interior structures (**Figure 1A**). This accumulated injury and difficulty of access has made the approach through conventional means problematic because animals do not recover well from these surgeries (of animals with full eversions, only 17% regained any feeding ability, N=12. Around 85% of non-everted animals regained the ability to feed, N=84).

The I7 muscle, which has been characterized as a radular opener[8], is deep inside the odontophore itself, further complicating access. It stretches between the base of the radular stalk (**Figure 1C**) and the underside of the radular surface, through a lumen in the odontophore (**Figure 1C**). On three sides of the I7 muscles are walls of muscle, and the fourth wall consists of the radular stalk. For the purposes of a biomechanical study, major impairment to any of these structures would compromise the normal function of the feeding apparatus. We developed a novel approach of working the odontophore out through the jaws, and conducting the surgery through an incision to the thin, cartilaginous radular surface, that made it possible to lesion the I7 muscle, as well as newly-described fine muscle fibers that run just beneath the radular surface, which we refer to as the sub-radular fibers (**Figure 1C**).

[Insert **Figure 1**]

**PROTOCOL:**

*Aplysia* are invertebrates and thus not subjected to IAUC approval. To minimize discomfort to animals, ensure that they are fully anesthetized before applying the surgical techniques described below.

**1. Animal selection and anesthetization**

1.1. Select an active animal by offering it seaweed and confirming that bite intervals are no greater than between 3 and 5 s.

1.2.1. Anesthetize the animal with 0.333 molar magnesium chloride (see **Table 1**) by injecting near the head with an 18 G needle on a 60 mL syringe so that the highest concentration of anesthetic will be around the buccal mass.

1.2.2. Take care to penetrate both the outer epithelium and the inner tissue layer with the needle. Ensure that the injection is roughly under the rhinophore, halfway between the rhinophore and the foot, and the needle should enter obliquely, pointing in the direction of the jaws.

1.3. After 10 min, gently attempt to insert a pin into the gill and rhinophore, verifying that these do not retract, to ensure sufficient anesthetization.

1.4. Ensure that the lips and jaw of the slug are relaxed so that the odontophore can be exposed.

NOTE: The wrinkling on the lips of **Figure 2A** indicates that the animal's lips and jaw are not sufficiently relaxed for the surgical procedure to be performed without damage. The smooth, relaxed lips of **Figure 2B** indicate that the jaws are fully relaxed.

[Insert **Figure 2**]

1.5. If an animal's lips are not relaxed, inject an additional 30 mL of magnesium chloride and wait another 5 min. If this does not result in lip relaxation, return them to an isolated container with good water flow to allow them to recover (see step 4) and proceed with a different animal.

**2. Exposing the radular surface**

2.1. Position the slug so that the head hangs downward, allowing the buccal mass to settle against the jaws.

2.2. Apply pressure with the thumb and forefinger to push the buccal mass toward the jaws, holding the buccal mass in place.

2.3. Rotate the jaws so that they are visible. At the same time, maintain the pressure on the buccal mass so that the prow of the buccal mass is visible through the jaws. (**Figure 3**).

[Insert **Figure 3**]

2.4. Gently work the tips of the blunt forceps into the cleft of the odontophore and use them to lever the radular surface through the jaws. If the jaws are not sufficiently relaxed, use the forceps to gently grasp the edge of the cleft to assist this process.

CAUTION: This pressure does risk greater damage to the animal.

2.5. Once the surface is exposed, work the jaws clear of the anterior portion of the radular surface all the way around the perimeter. This makes the odontophore less likely to retract (**Figure 4**). Ensure that no more than half of the walls of the odontophore is exposed.

[Insert **Figure 4**]

NOTE: A full eversion of the odontophore will cause major muscle damage from which the animals are very slow to recover.

**3. Surgical incisions**

3.1. Once the radular surface is fully exposed, arrange the slug under a dissection scope for the surgery.

3.1.1. Alternatively, use a wide rubber band and a third hand to stabilize the jaws and radular surface for the surgery, especially while learning. This, however, adds time and increased tissue damage to the procedure, which makes it less ideal over the long term.

3.2. Place the radular surface so that the cleft side faces the investigator.

3.3. Gently grasp the radular surface, near the radular base, so that a horizontal fold is formed perpendicular to the anatomical crease. Use fine scissors to cut through this fold, making an incision along the anatomical crease (**Figure 5**).

[Insert **Figure 5**]

3.4. Extend this initial incision to 3-5 cm to allow access to the interior of the buccal mass.

3.5. Adjust light so that it points directly back through this incision.

3.6. Part the edges of the incision so that the back of the lumen of the odontophore and the thin vertical strands of the I7 muscle are visible. (**Figure 6**)

[Insert **Figure 6**]

3.7. Reach into through the incision, grasp both strands of I7, and pull them up through the incision, where as much as the muscle can be cut away as is practical (**Figure 7**).

[Insert **Figure 7**]

NOTE: With practice, it is usually more effective to locate I7 by feel than by sight.

**4. Post-operative care**

4.1. After lesions have been performed, grasp the anterior tentacles, and push down on the radular surface to return the slug to its original configuration.

4.2. Place post-surgical animals in a protected environment with good water flow. Increased oxygenation speeds the recovery. Ensure that the animals are alert and responsive on the day after surgery. If this is not the case, it can be assumed that they will not recover.

NOTE: Animals will usually begin to feed on the first or second day after surgery. Even animals that are having trouble biting should be offered seaweed, as it is our anecdotal observation that an animal's recovery is improved by its attempts to eat.

**5. For sub-radular fiber lesion**

5.1. Follow the steps from 1.1 through 3.5

5.2. Insert the tip of a small straight scalpel blade (#11 or similar) through the incision with the sharp edge angled upwards. Gently scrape the fine muscular fibers from the underside of the radular surface. (**Figure 8**).

[Insert **Figure 8**]

5.3. Return to step 4.1.

**REPRESENTATIVE RESULTS:**

Previous work had suggested that the I7 muscle contributed to the opening of the grasper[8]. Our own anatomical studies suggested that the sub-radular fibers might also contribute to grasper opening. To test these hypotheses, animals were induced to generate bites both before and after receiving a surgical procedure. Sham animals were subjected to all the surgical steps, including the incision in the radular surface, but no internal muscles were removed. Animals subjected to an I7 lesion had both I7 muscles removed. Animals subjected to a sub-radular fiber lesion had ~25% of the sub-radular fibers removed immediately beneath the incision. Sham lesions had no significant effect on the width of the opening at the peak of biting, whereas both I7 and sub-radular fibers lesions did significantly reduce bite width (**Figure 9**).

[Insert **Figure 9**]

**FIGURE AND TABLE LEGENDS:**

**Table 1: Magnesium Chloride dosage by bodyweight.**

**Figure 1**: **Anatomical Overview**. (**A**) Location of the buccal mass within *Aplysia.* (**B**) External anatomy of odontophore. The surface of radula and radular sac are yellow; muscles composing the odontophore are shown in red, based on their actual colors. (**C**) Sagittal section of odontophore, showing the location of sub-radular fibers (curved pink line) and I7 muscle (straight pink line). Cross section of the I6 muscle is shown in dark red.

**Figure 2: Tension and Relaxation in Anesthetized *Aplysia* Mouths.** (**A**) *Aplysia* showing a high degree of muscle tension around the lips. This correlates with jaw tension and contraindicates proceeding with the surgery. (**B**) *Aplysia* with relaxed lips, showing the inside of jaws (light grey). Colors again correspond to those observed in the animal.

**Figure 3: Supporting the Buccal Mass Against the Inside of the Jaws.** Fingers support the buccal mass that has been pushed up against the inside edge of the jaws until the tip of the prow can be seen.

**Figure 4: Partial Eversion of the Odontophore.** The radular surface is fully exposed, but the sides of the odontophore are not uncovered, making this only a partial eversion. Further eversion will likely result in damage to the animal.

**Figure 5: Location of Incision to the Radular Surface.** (**A**) Radular surface, with an incision. (**B**) Radular surface with circles showing where the strands of the bilateral I7 muscle attach; dotted lines show the location of the descending muscles underneath the radular surface.

**Figure 6: Location of I7 through the Radular Surface Incision.** Looking through the incision, both strands of I7 can be seen between the inner surfaces of I4.

**Figure 7: Pulling the I7 Muscle Strand Through the Incision.** The I7 muscle is highly elastic and can be pulled up through the incision for removal.

**Figure 8: Lesioning the Subradular Fibers.** The edge of the scalpel blade is angled upwards through the incision to the underside of the radular surface so that it can gently scrape away the sub-radular fibers.

**Figure 9: Results of Lesions on Opening Width During Peak Biting.** Data shown are the differences between the averaged normalized opening width of the radula before and after the surgical procedure for 5 animals in each of the 3 groups (sham, I7 lesion, or SRF lesion), with each animal serving as its own control. Averages were taken of 5 bites before, and 5 bites after the surgical procedure to determine the mean normalized difference. Opening width was the

distance from the center of radula to the radular edge at the peak protraction, normalized by the distance from the inner surface of the radular base to the cleft-side edges of the radular surface. The differences are shown as the means plus or minus the standard deviation. After establishing that the difference data were normally distributed, the probability that the lesion had no effect was determined (i.e., the null hypothesis was tested that the effects of the surgical procedures would be zero, on average) by applying a paired t-test to each independent group. The data demonstrates that the sham lesion had no significant effect, whereas a lesion of the I7 muscles or a lesion of the sub-radular fibers did have a significant effect on radular opening ($p < 0.031$ for the I7 lesion group, indicated with a single asterisk, or $p < 0.002$ for the SRF lesion group, indicated by a double asterisk).

**DISCUSSION:**

The most critical steps within the protocol are the need to ensure that the animal is fully anesthetized, and that the eversion of the buccal mass is just enough to access the underlying muscles. It may require some practice to perfect these steps, but once they are mastered, the yield from surgeries is likely to be greater than 85% of all experiments done. The most important way to properly modify and troubleshoot the protocol is to spend time doing dissections of the buccal mass so that the locations of the internal muscles are completely clear to the investigator. Because the suggested incision through the radular surface inevitably causes some damage to the underlying sub-radular fibers, it may be appropriate to modify the exact location of the incision to avoid specific regions of these fibers.

One limitation of the surgical technique is that it may have non-specific effects on feeding responses, such as the strength of protraction. One way to overcome this limitation is to have animals serve as their own controls. In addition, it is critical to have a sham lesion group which is subjected to the entire surgical protocol except for the removal of the specific muscle (i.e., I7 or the SRFs). By following these suggestions, an investigator will reduce the effects of variability between animals and have an intrinsic measure of the non-specific effects of surgery.

Previous work has used approaches through the body wall to lesion or record either from nerves[13,14], or muscles[15,16,17]. In our laboratory, we have anecdotally observed that body wall incisions are often accompanied by a significant loss of hemolymph and thus of body volume. Animals often require several days to recover from this, and if the body wall lesion is not carefully sutured, animals may not recover. In addition, post-mortem examination of the animals reveals considerable scarring around the incision and a strong immune response (anecdotal observations). In contrast, animals show no loss of hemolymph or change in body volume after recovery from the protocol described here (based on observations in 96 animals).

Future applications of the technique may extend it to other muscles within the feeding apparatus of *Aplysia,* and to other animals. We have focused on the removal of the I7 muscle and sub-radular fibers. These same general surgical techniques also allow access to most of the other muscles of the odontophore. Some of these, such as the internal portion of the I5 muscle, are best accessed through the radular surface. Others, like the inner leaflets of I4, may be better

reached through the exterior epithelium of the odontophore. We have made preliminary trials where an incision under the radular cleft of the partially everted odontophore allowed access for a sharpened hook to be inserted that could then be used to lesion another muscle within the odontophore, muscle I8[8]. Because the surgical protocol described here does not open the main body cavity, no suturing is required.

The protocol that we have described may be of general interest to other investigators working on soft tissue structures that would otherwise be difficult to manipulate, e.g., the feeding apparatus of other mollusks. More generally, this protocol could suggest other novel surgical approaches to the analysis of soft structures such as tongues, trunks or tentacles[18].

**ACKNOWLEDGMENTS:**

We would like to acknowledge the hard work that Sherry Niggel, Sisi Lu, and Joey Wu put into improving and validating these protocols.

**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1



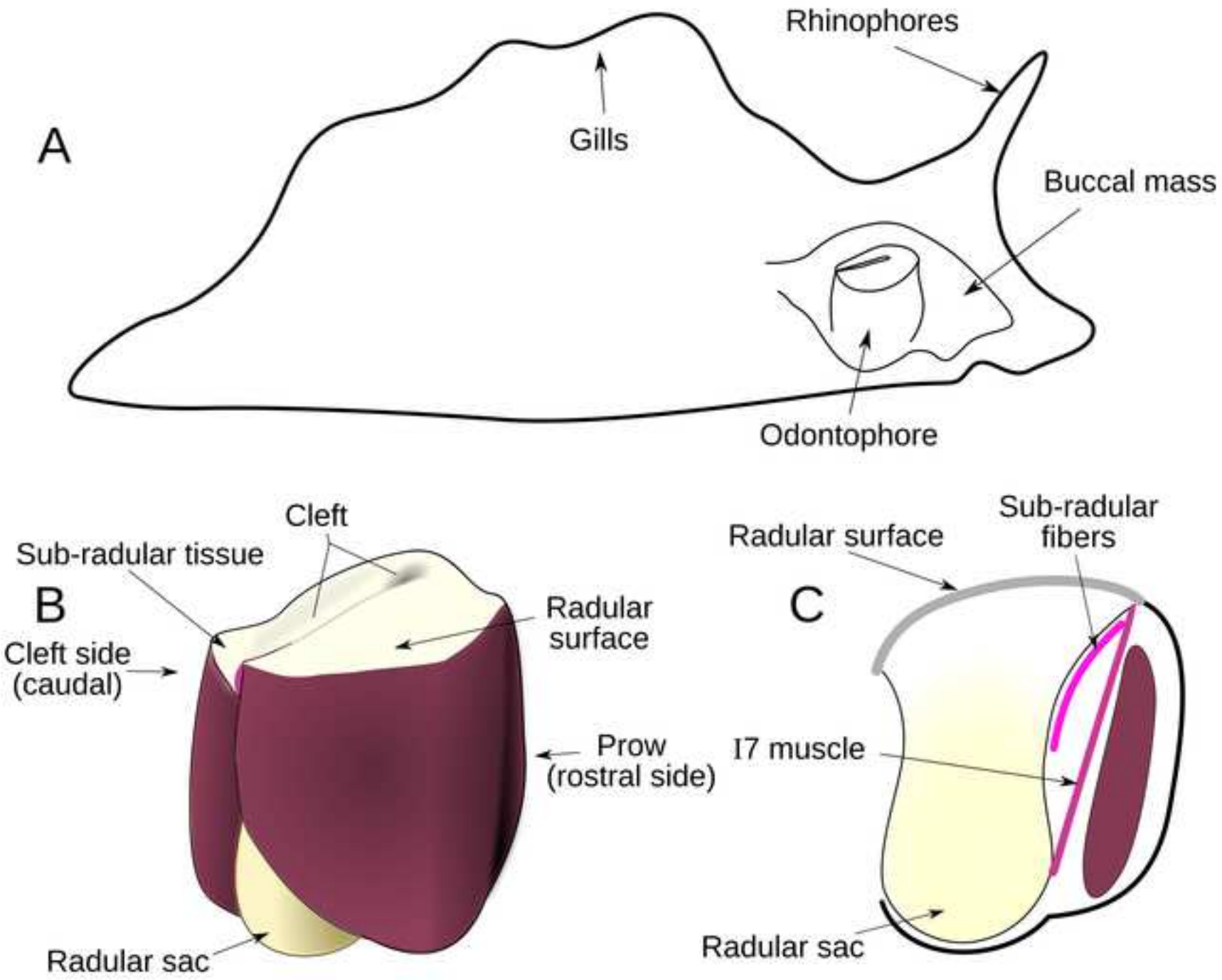

Figure 2



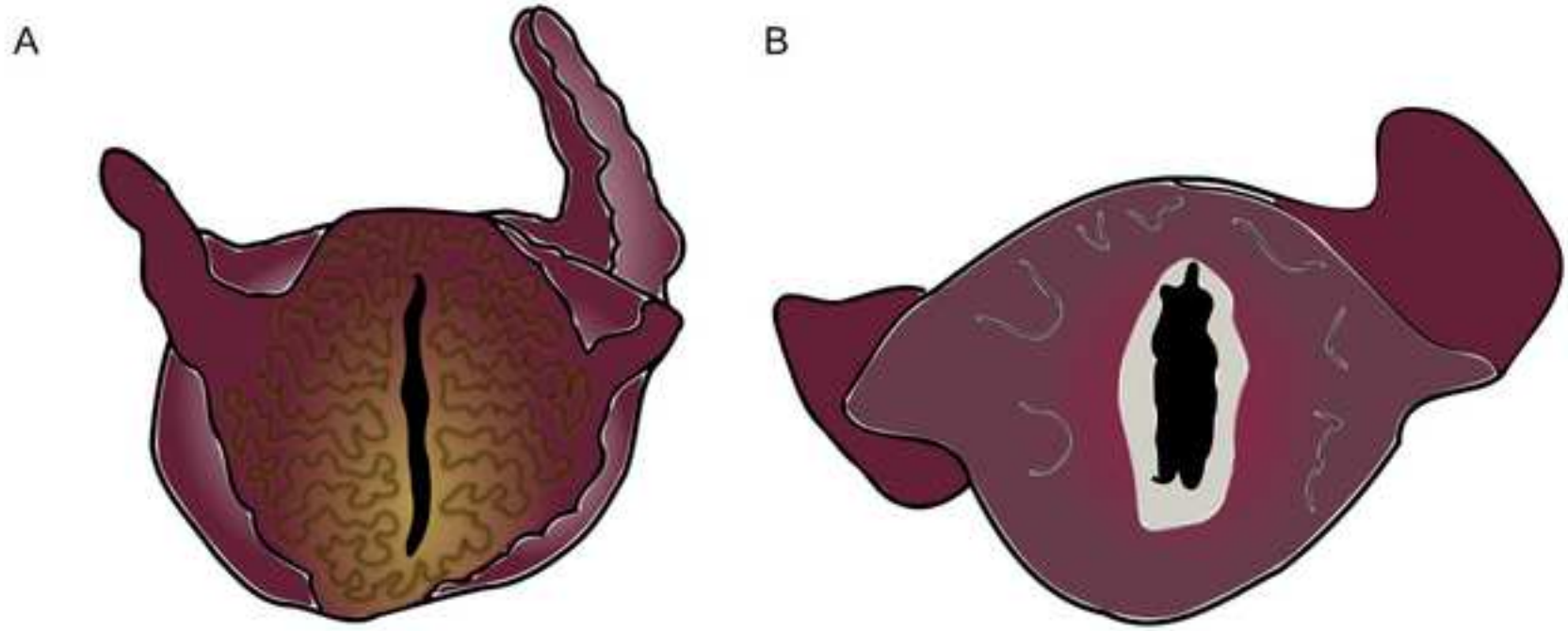

Figure 3

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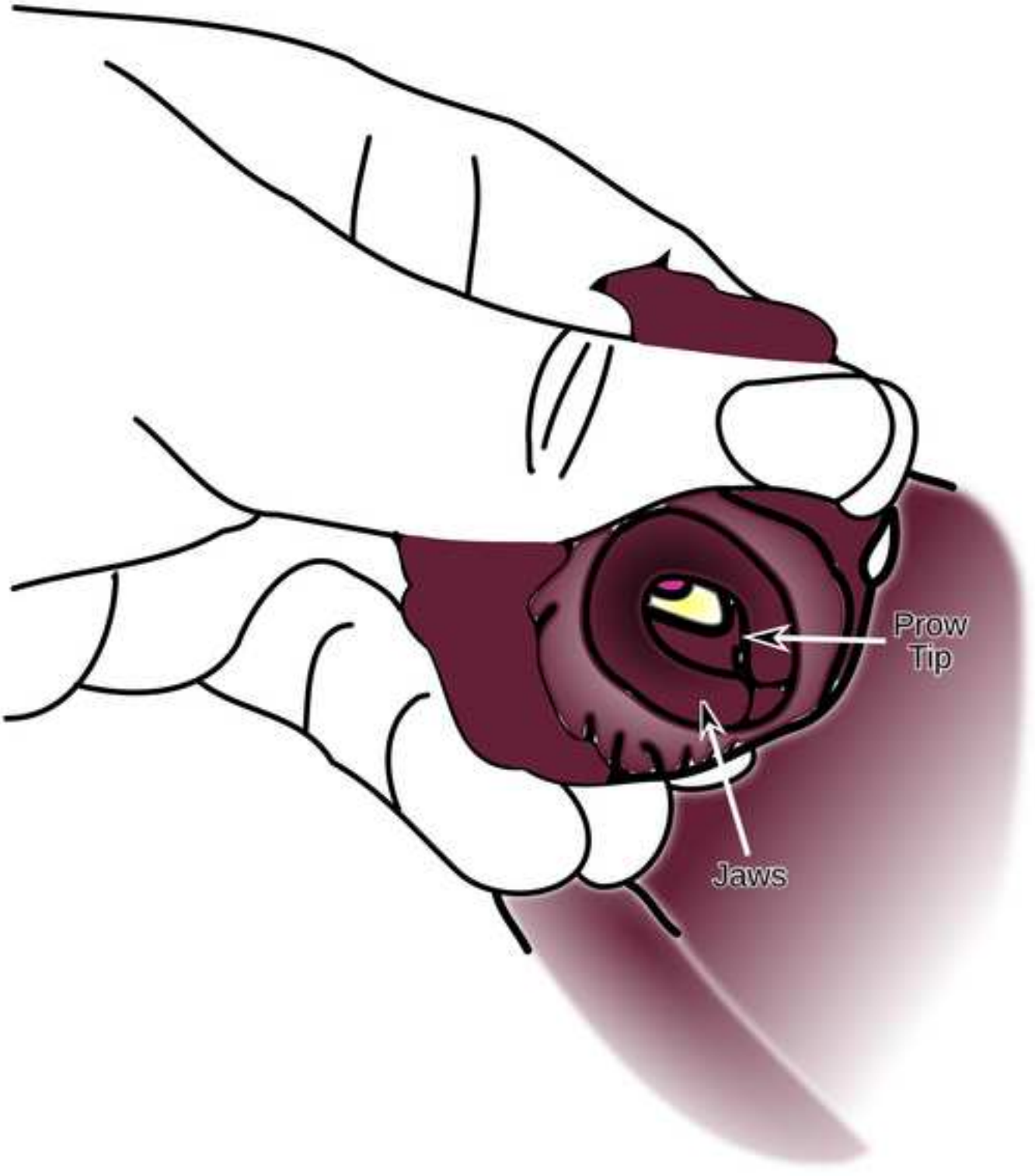

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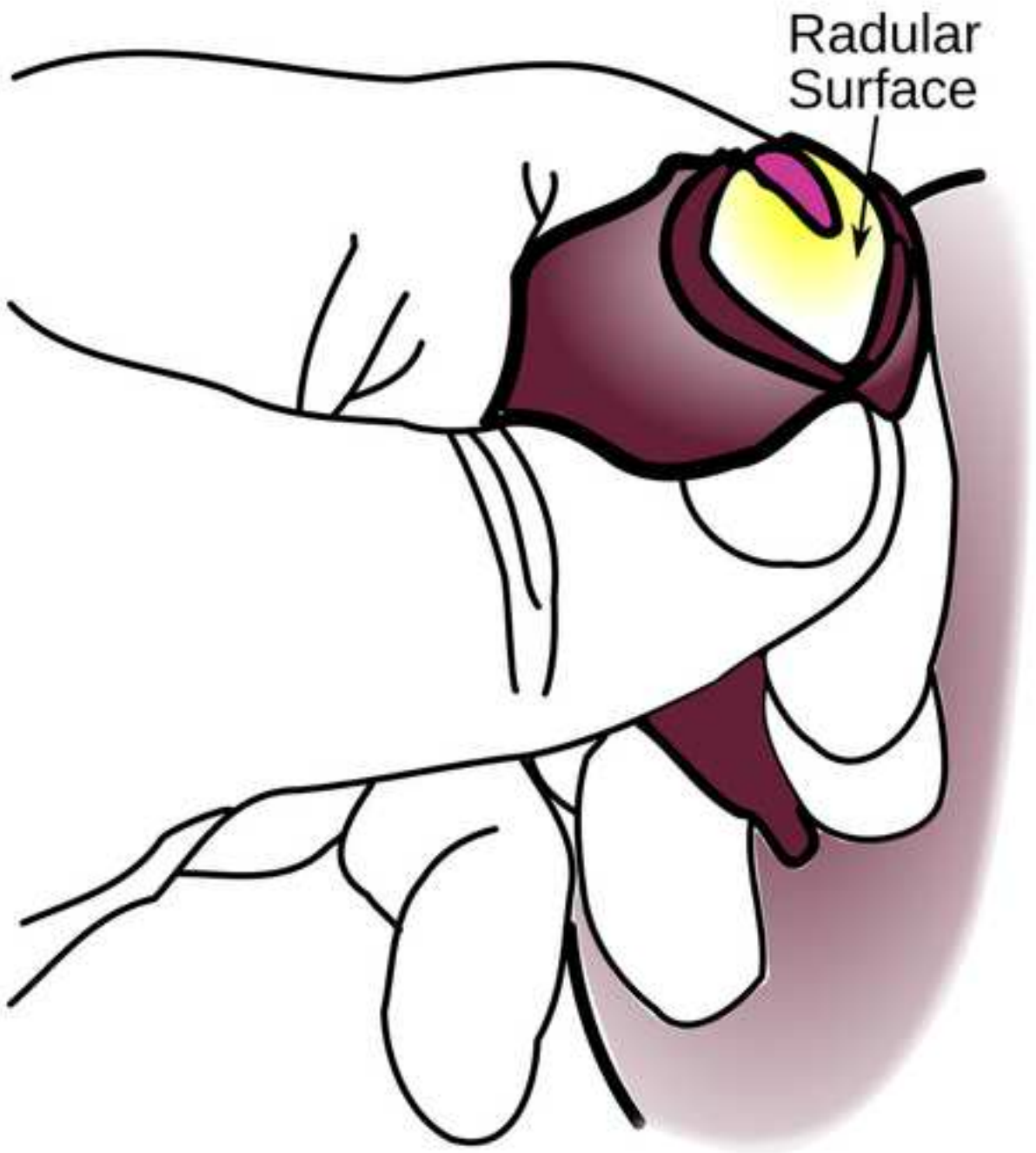

Figure 5



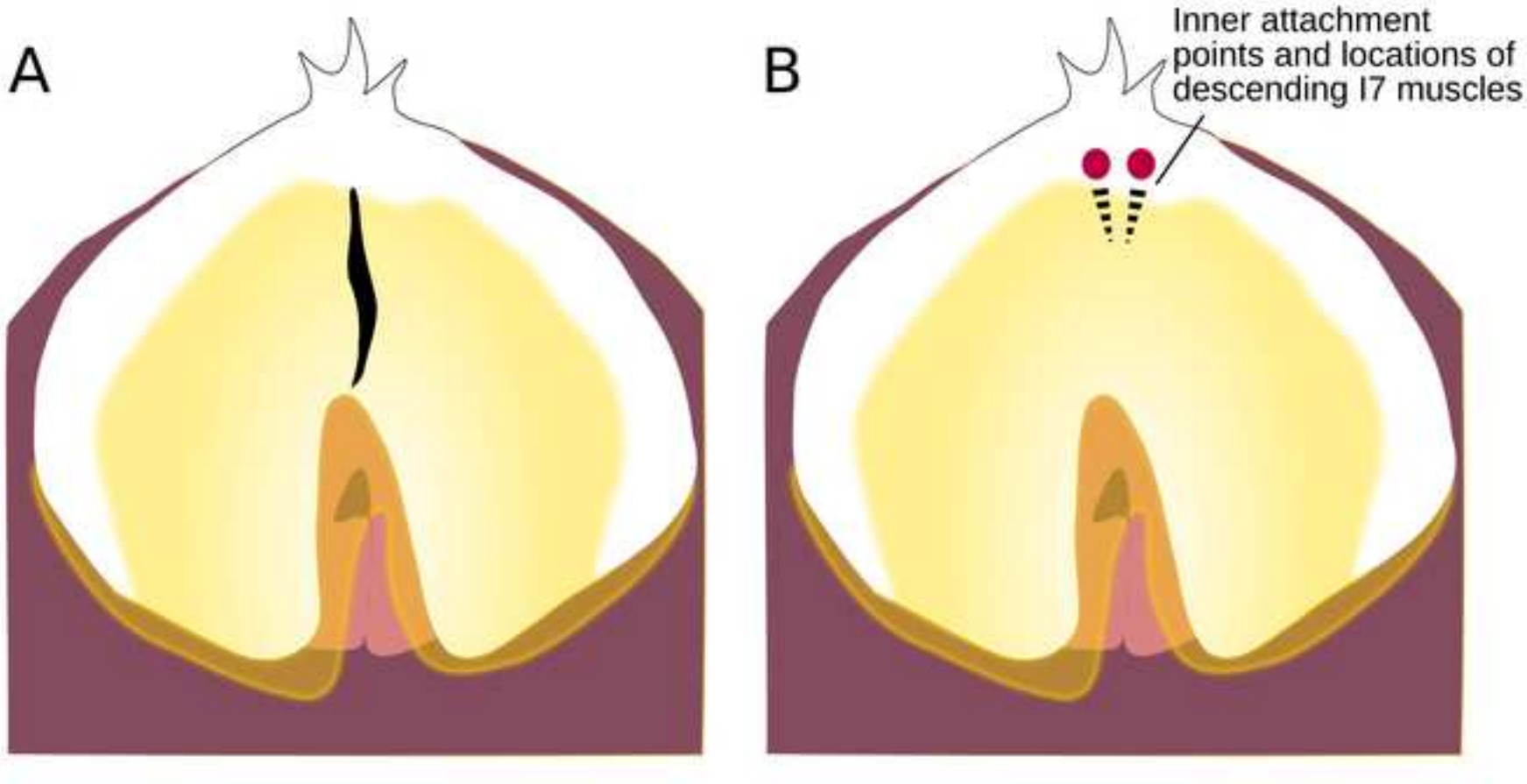

Figure 6

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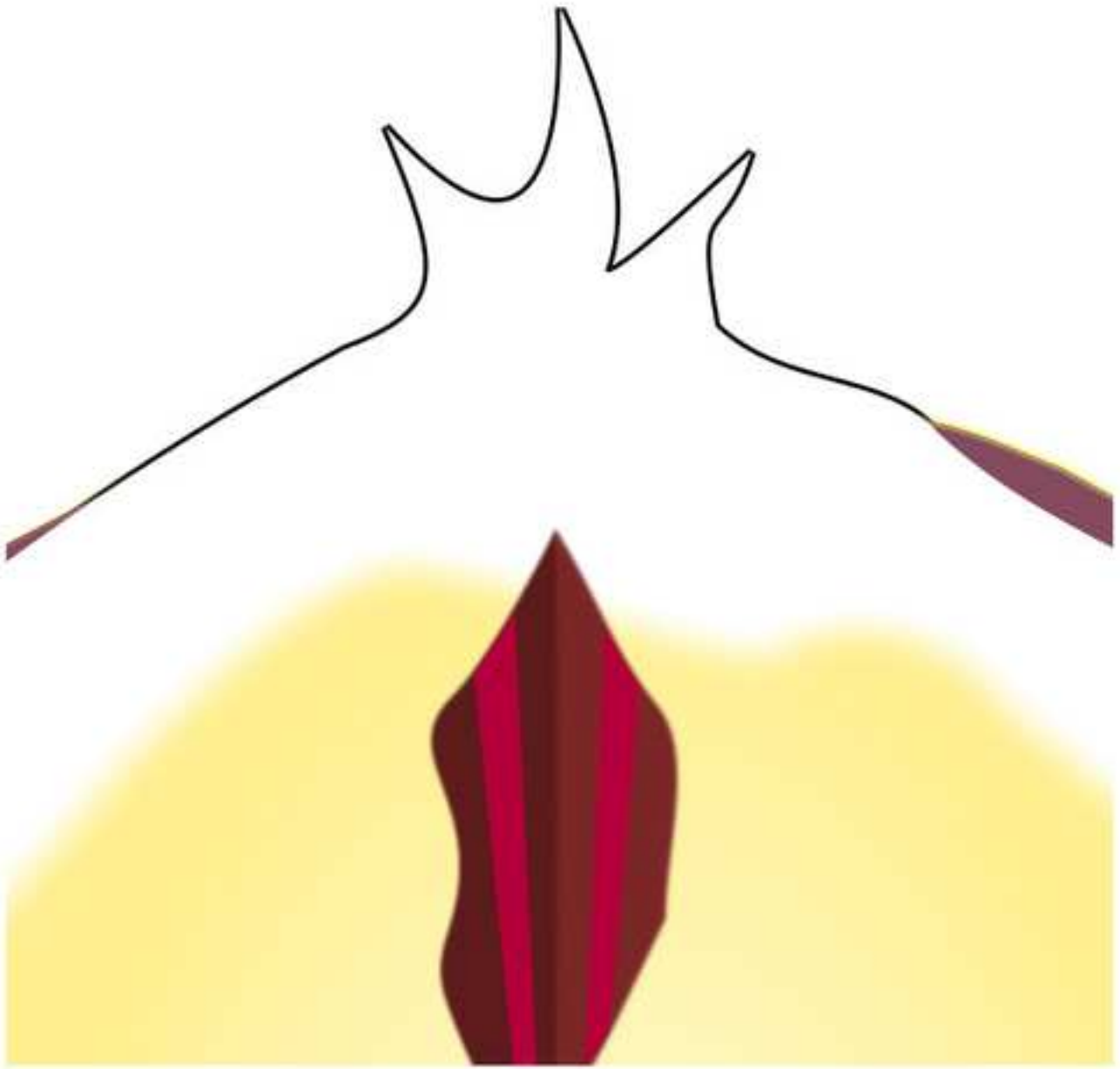

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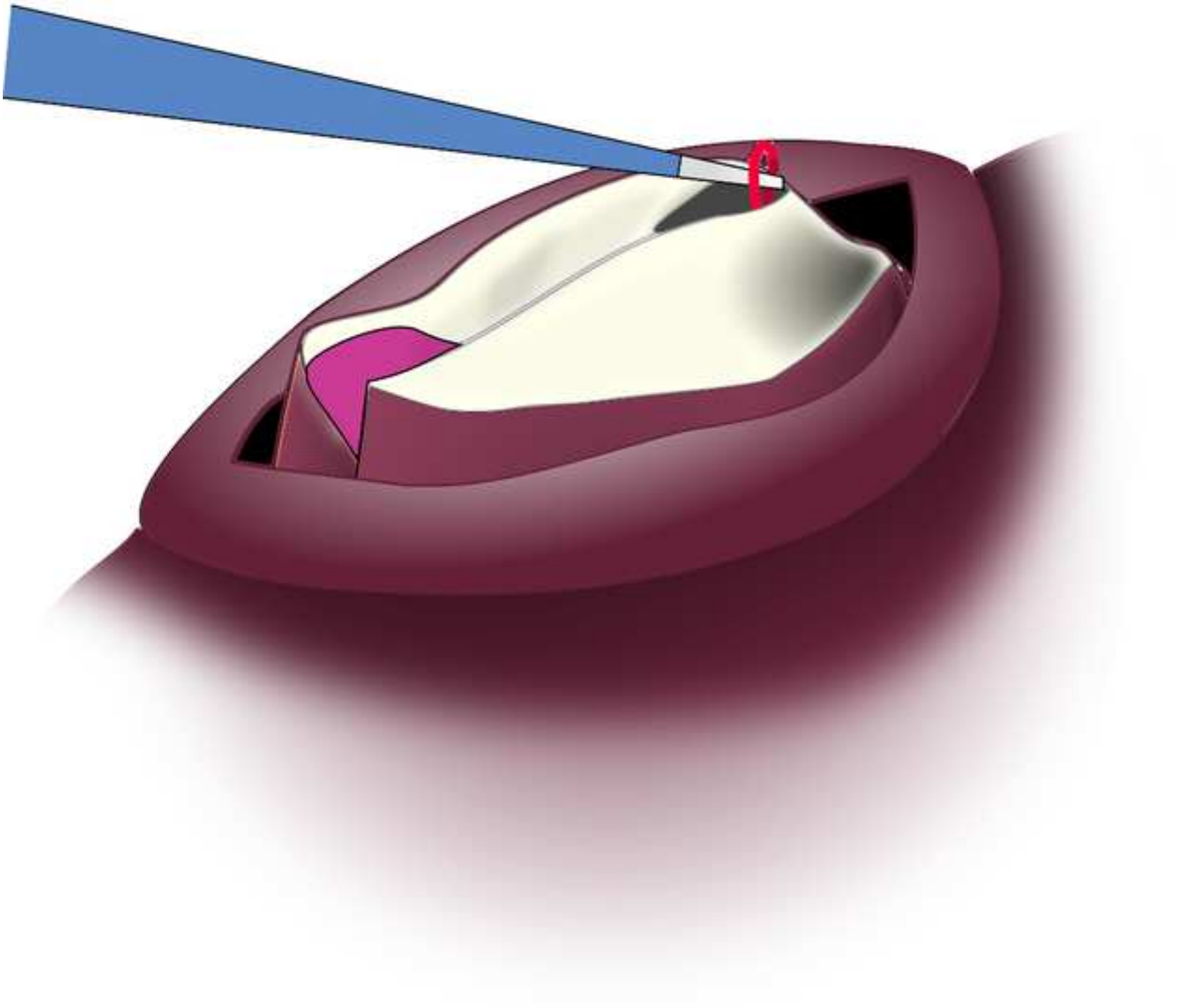

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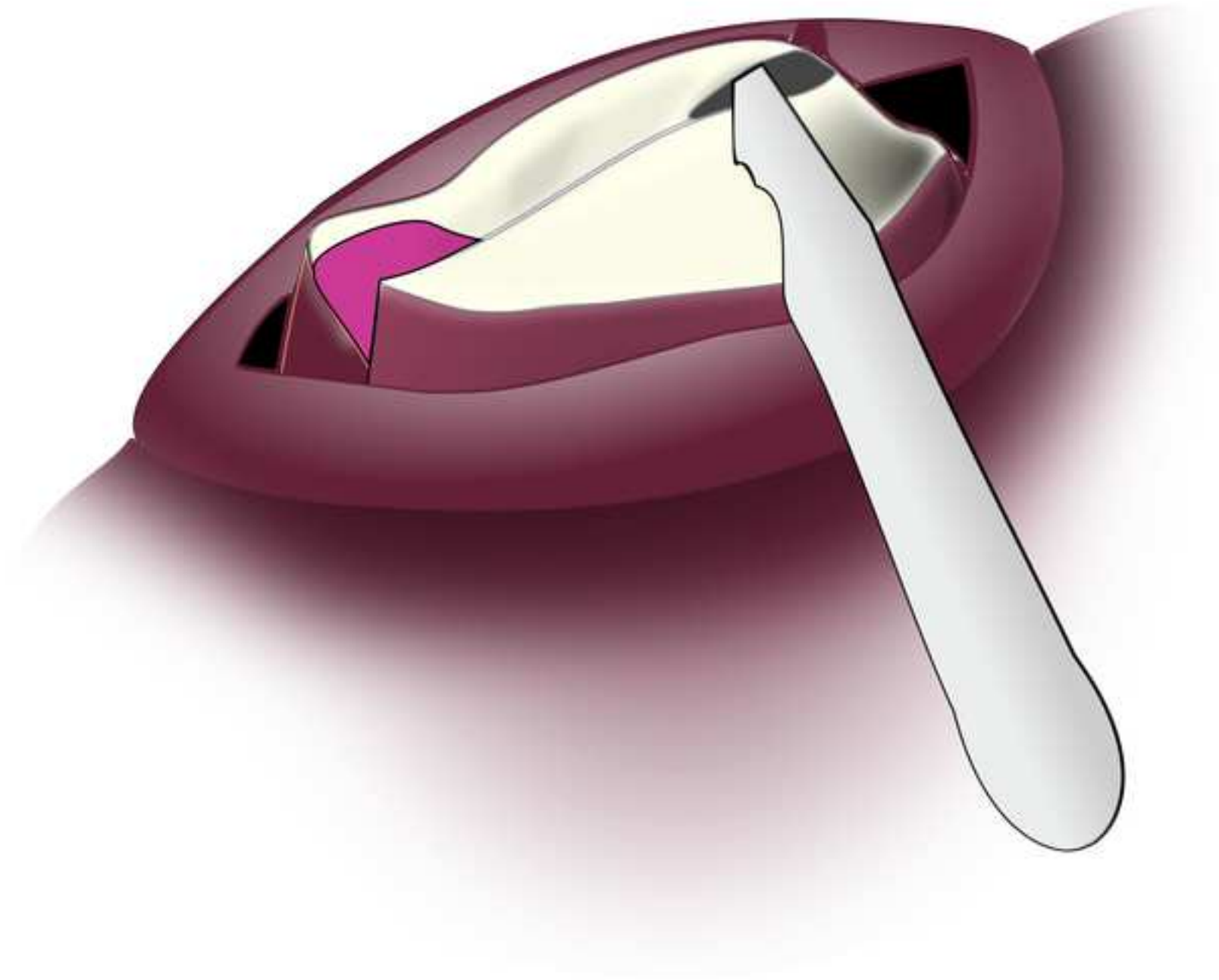

Figure 9

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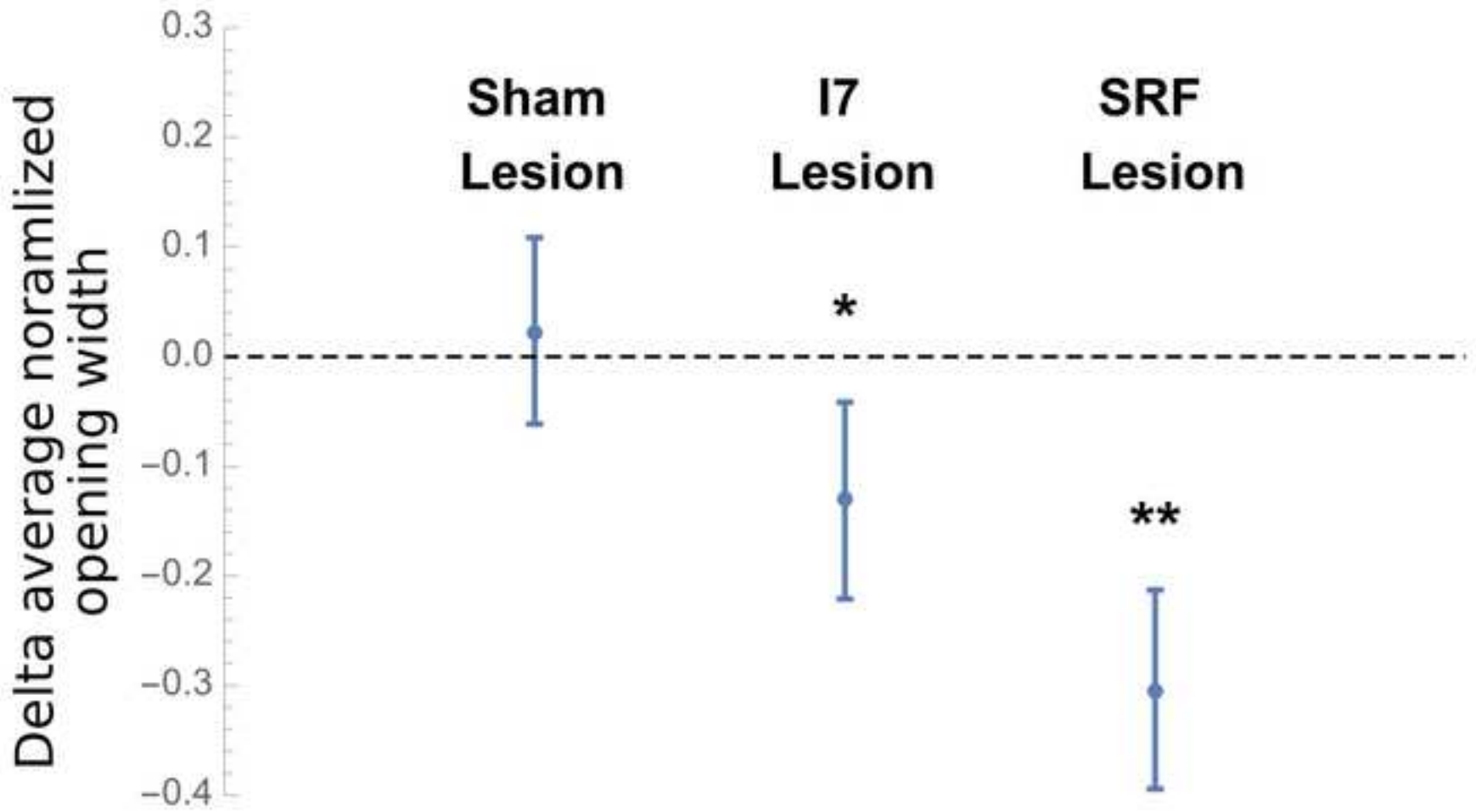

Sheet1

| Body Weight | Magnesium Chloride Dose |
|---|---|
| <200g | ½ bodyweight |
| 200–350g | 1/3 bodyweight |
| 350–450g | ¼ bodyweight |

**Table 1** Magnesium Chloride dosage by bodyweight.

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Blunt forceps | Fine Science Tools | 11210-10 | 2 pair |
| Scalpel blade (#11) | Fine Science Tools | 10011-00 | |
| Spring scissors | Fine Science Tools | 15024-10 | |
| Webcam | Logitech | c920 | for recording data |

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Please ensure that the title is crisp and avoid the use of the word novel.

We have changed the title to: "A minimally invasive lesion technique for muscles intrinsic to the odontophore of *Aplysia californica*."

Please also include what is being done in brief and what result and conclusion you derive from it in brief.

We have have reworded the abstract to be more concise and be clearer about the purpose of the experiments.

Old: "*Aplysia californica* is a model system for studying the neural control of learning and behavior. To better understand the feeding behavior, we have developed a novel protocol for lesioning muscles that lie deep within the feeding grasper. This new protocol does not require opening the main body cavity of the animal or damaging the outer layers of the feeding organ (the buccal mass). The ability to access muscles within the odontophore makes it possible to study their biomechanical functions as well as test hypotheses regarding the neural circuits that control them. In this novel technique, the grasper is partially everted, allowing direct access to the musculature. The new procedure allows animals to recover quickly and reliably. This has made it possible to lesion the I7 muscles and sub-radular fibers, allowing the quantification of their roles in radular opening."

New: "*Aplysia californica* is a model system for studying the neural control of learning and behavior. To better understand the functions of muscles within the feeding grasper, we have developed a technique for lesioning them without opening the main body cavity of the animal or damaging the outer layers of the feeding organ (the buccal mass). In this technique, the grasper is partially everted, allowing direct access to the musculature. This procedure allows animals to recover quickly and reliably. This has made it possible to lesion the I7 muscles and sub-radular fibers, allowing us to show that both muscles significantly contribute to opening *in vivo*."

Will you have the animal available during the filming of the procedure?

Our animal facility that is usually well stocked with *Aplysia*. My hope was to have multiple animals available during filming, both to show the procedure, and then, if there is interest, to show animals that have recently had the procedure performed and are performing our usual feeding assays.

Select from where? Do you rear these in the lab? How? Do you collect from the sea?

We maintain an animal facility (four 189 liter tanks at 16 ˚C with a 12/12 hour light dark cycle) with *Aplysia* that are live-caught off the Californian coast and shipped to us overnight. At any given time, there are many animals of an appropriate size available within the aquaria within our laboratory.

We cannot film the anesthesia process.

We understand, that is fine.

1.4, Note What is done in this case? Do you start fresh with another animal? Do you wait? Please provide all specific details with respect your experiment.

We have added an additional step to address this:

1.5 If an animal's lips are not relaxed, give them an additional 30 ml of magnesium chloride and wait another five minutes. If this does not result in lip relaxation, return them to an isolated container with good water flow to recover (see section 4) and proceed with a different animal.

We cannot have non numbered step in the protocol section. So, I have numbered this part as well.

Please expand all abbreviations during the first-time use.

Please write exactly how you would do this. You could refer to the previously stated step but include the step numbers. Please use complete sentences and bring out clarity in this part of the protocol.

We have reworked this section to address these concerns. It now reads:

5. For sub-radular fiber lesion:

5.1. Follow the steps from 1.1 through 3.5

<insert Figure 8>

5.1 Insert the tip of a small straight scalpel blade (#11 or similar) through the incision with the sharp edge angled upwards. Gently scrape the fine muscular fibers from the underside of the radular surface. (Figure 8).

5.2 Return to step 4.1.

Please describe the result with respect to your experiment, you performed an experiment, how did it helped you to conclude what you wanted to and how is it in line with the title. How do these results show the technique, suggestions

We have re-written this section to give better context with regard to the previous our research objectives. Our new text reads:

Previous work had suggested that the I7 muscle contributed to opening of the grasper[8]. Our own anatomical studies suggested that the sub-radular fibers might also contribute to grasper opening. To test these hypotheses, animals were induced to generate bites both before and after receiving a surgical procedure. Sham animals were subjected to all the surgical steps, including the incision in the radular surface, but no internal muscles were removed. Animals subjected to an I7 lesion had both I7 muscles removed. Animals subjected to a sub-radular fiber lesion had ~25% of the sub-radular fibers removed immediately beneath the incision. Sham lesions had no significant effect on the width of opening at the peak of biting, whereas both I7 and sub-radular fibers lesions did significantly reduce bite width (Figure 9).

Please include the color shadings details in the figure legend. What each color represent?

Thank you for this feedback; we have added explanatory notes in the listed figured legends, and the requested figured labels. In general, the colors reflect the colors observed in the animals.

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 the Figure Legends, but rather the Protocol.

The protocol focuses solely on the surgical technique, and therefore we are not including the behavioral measurements as part of the protocol. Because the effects of the surgery can be measured in many different ways, we do not think it is appropriate to make this a part of the protocol. If an investigator wished to understand the significance of the representative results, however, they would need the additional information that is provided in this legend.

[Figure 9] In the figure please include the x and y axis? What does it represent?

The labels along the x axis provide descriptive labels for the three independent groups; their position is otherwise unimportant. To the left of the y axis, we have now added a descriptive title: “Delta average normalized opening width”.

Data or results to support this claim?

We have added text in this section to address this issue: “Animals often require several days to recover from this, and if the body wall lesion is not carefully sutured, animals may not recover. In addition, post-mortem examination of the animals reveals considerable scarring around the incision and a strong immune response (anecdotal observations). In contrast, animals show no loss of hemolymph or change in body volume after recovery from the protocol described here (based on observations in 96 animals).”

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.

We have made these changes.