Radiation Treatment of Organotypic Cultures from Submandibular and Parotid Salivary Glands Models Key In Vivo Characteristics

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Abstract

Hyposalivation and xerostomia create chronic oral complications that decrease the quality of life in head and neck cancer patients who are treated with radiotherapy. Experimental approaches to understanding mechanisms of salivary gland dysfunction and restoration have focused on in vivo models, which are handicapped by an inability to systematically screen therapeutic candidates and efficiencies in transfection capability to manipulate specific genes. The purpose of this salivary gland organotypic culture protocol is to evaluate maximal time of culture viability and characterize cellular changes following ex vivo radiation treatment. We utilized immunofluorescent staining and confocal microscopy to determine when specific cell populations and markers are present during a 30-day culture period. In addition, cellular markers previously reported in in vivo radiation models are evaluated in cultures that are irradiated ex vivo. Moving forward, this method is an attractive platform for rapid ex vivo assessment of murine and human salivary gland tissue responses to therapeutic agents that improve salivary function.

Keywords

Medicine; Issue 147; submandibular salivary gland; vibratome culture; radiation; parotid salivary gland; organotypic culture; xerostomia

Introduction

Proper salivary gland function is essential to oral health and is altered following head and neck cancer treatment with radiotherapy¹. In 2017, nearly 50,000 new cases of head and neck cancer were reported in the United States². Due to the tissue-damaging and frequently irreversible effects of radiation therapy on surrounding normal tissues such as salivary glands, patients are often left with severe side effects and diminished quality of life^{2,3,4}.

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The video component of this article can be found at <https://www.jove.com/video/59484/>

Disclosures

The authors have nothing to disclose.

Common complications caused by radiation damage manifests in symptoms such as xerostomia (the subjective feeling of dry mouth), dental caries, impaired ability to chew and swallow, speech impairments, and compromised oral microbiomes^{2,3,4}. These symptoms collectively can lead to malnutrition and impaired survival in affected individuals⁵. While salivary gland dysfunction in this population has been well-documented, the underlying mechanisms of damage to acinar cells have been disputed, and there is little integration among different animal models^{6,7}.

The current methods of studying salivary gland function and radiation-induced damage include the use of in vivo models, immortalized cell lines, two-dimensional (2-D) primary cell cultures, and three-dimensional (3-D) salisphere cultures^{8,9,10,11,12}. Traditionally, cell culture models from immortalized cell lines and 2-D cultures involve single layered cells cultured on flat surfaces and are valuable for fast, easy, and cost-effective experimentation. However, artificial cell culture conditions can alter the differentiation status and physiological responses of cells exposed to various conditions, and the results often fail to translate to whole organism models^{14,15}. In addition, immortalized cell cultures require modulation of p53 activity, which is critical for the salivary gland response to DNA damage^{16,17}.

3-D salisphere cultures are enriched for stem and progenitor cells at early time points in culture and have been useful for understanding the radiosensitivity of this subset of salivary gland cells^{9,18}. A critical limitation of all these culture models is they are ineffective in visualizing the 3-D structure of the salivary gland, including the extracellular matrix (ECM) and cell-cell interactions over various layers, which are crucial in modulating salivary secretion¹⁵. The need for a method that encompasses the behavior of the tissue as a whole but can also be manipulated under laboratory conditions to study the effects of treatment is necessary to further discover the underlying mechanisms of radiation-induced salivary gland dysfunction.

Live tissue sectioning and culture has been documented previously^{19,20} and is often used to study brain-tissue interactions²¹. In previous studies, parotid (PAR) salivary gland tissue from mice was sectioned at approximately 50 μm and cultured for up to 48 h, and analysis of viability, cell death, and function was performed thereafter¹⁹. Su et al. (2016) expanded on this methodology by culturing human submandibular glands (SMGs) sectioned at 35 μm or 50 μm for 14 days²⁰. The proposed method is an advancement in that it includes both parotid and submandibular salivary glands sectioned at 50 μm and 90 μm and evaluation of the cultures for 30 days. The ability to cut a range of tissue thickness is important in evaluating cell-cell and cell-ECM interactions that are relevant for cellular processes including apical-basolateral polarity and innervation for secretion. Furthermore, the salivary gland slices were irradiated while in culture to determine the feasibility of this culture model to study radiation-induced salivary gland damage.

The purpose of this salivary gland organotypic culture protocol is to evaluate maximal time of culture viability and characterize cellular changes following ex vivo radiation treatment. To determine the maximum time sections that are viable post-dissection, trypan blue staining, live cell staining, and immunohistochemical staining for cell death were performed.

Confocal microscopy and immunofluorescent staining were utilized to evaluate specific cell populations, morphological structures, and levels of proliferation. Tissue section cultures were also exposed to ionizing radiation to determine the effects of radiation on various markers in this 3-D model. Induction of cell death, cytoskeletal disruption, loss of differentiation markers, and compensatory proliferation in irradiated ex vivo cultures were compared to previous studies of in vivo models. This methodology provides a means to investigate the role of cell-cell interactions following radiation damage and provides an experimental model to efficiently evaluate the efficacy of therapeutic interventions (gene manipulations or pharmacological agents) that may be less suitable for in vivo models.

Protocol

1. Preparation of vibratome

1. Spray detachable components of the vibratome including the buffer tray, blade attachment, agarose block mold, and laboratory film with 70% ethanol, then UV-sterilize for at least 30 min.
2. Place and secure an additional sheet of laboratory film over the buffer tray to prevent ice from falling in.
3. Fill the ice chamber with crushed ice, remove the laboratory film from the buffer tray, and fill the buffer tray with 100 mL of ice-cold 1x phosphate buffered saline (PBS) solution supplemented with 1% penicillin-streptomycin-ampicillin (PSA).
4. Place a stainless steel razor blade into the blade holder. Use the screwdriver to tighten/loosen and change the angle of the blade.

2. Preparation of tissue sample in agarose block and sectioning using the vibratome

1. Autoclave all necessary dissection and sectioning tools including forceps, scissors, and paintbrushes.
2. Prepare 3% low melting point agarose in sterile 1x PBS and microwave until the agarose dissolves into solution. Ensure that the solution does not boil over and swirl the bottle periodically to mix.

NOTE: Prevent the low-melting agarose from solidifying by keeping it in a warm water bath. The low-melt agarose is fluid at 37 °C and sets at 25 °C. However, the water bath should be below 40 °C in order to pour the agarose around the tissue at physiological temperature.

3. Isolate salivary glands and place dissected tissue into 2 mL ice-cold 1x PBS supplemented with 1% PSA in a sterile, 30 mm culture dish.
4. Using autoclaved forceps, remove salivary glands from 1x PBS + 1% PSA solution and position at the bottom of an embedding mold. Fill the mold with liquid 3% low melting point agarose to cover the tissue.

5. Using the forceps, adjust the tissue to the middle of the block and position the salivary gland in the appropriate plane. The best cross sections for the submandibular and parotid glands are in the vertical plane.
6. Place agarose block on ice for 10 min to harden.
7. Carefully run a razor blade around the outer edge of the agarose to loosen and let the block slide out onto the UV-sterilized laboratory film from step 1.1.
8. Use a razor blade to cut out an agarose box containing the salivary gland. Ensure that the plane of section is straight and parallel to the opposite side of the block (the surface that will be glued down). Since the agarose does not infiltrate the tissue, do not trim off too much agarose around the tissue so the tissue will be well supported.
9. Use superglue to attach the block to the cutting surface.
10. Section at 50 μm or 90 μm thickness by vibratome at a speed of 0.075 mm/s and frequency of 100 Hz.

NOTE: Setting the vibratome at the highest frequency and lowest blade speed provides the most optimal slices.
11. Collect sections in a 24-well tissue culture dish containing ~1 mL ice-cold PBS per well using an autoclaved, natural hair paintbrush, placing the brush in 70% ethanol between slice collections to maintain sterility.

3. Culturing sections

1. Autoclave a micro-spatula and forceps.
2. Make stock vibratome culture media with or without fetal bovine serum (FBS): DMEM/F12 media supplemented with 1% PSA, 5 $\mu\text{g/mL}$ transferrin, 1.1 μM hydrocortisone, 0.1 μM retinoic acid, 5 $\mu\text{g/mL}$ insulin, 80 ng/mL epidermal growth factor, 5 mM L-glutamine, 50 $\mu\text{g/mL}$ gentamicin sulfate, and 10 $\mu\text{L/mL}$ trace element mixture. Add an appropriate amount of FBS to make 0%, 2.5%, 5.0%, or 10% solutions.
3. Prior to sectioning, add 300 μL of pre-warmed media and place a 12 mm diameter, 0.4 μm pore-size membrane insert into each well of a 24-well tissue culture plate. The media should reach the membrane bottom to create a liquid-air interface for culture.
4. Gently lay the salivary sections on top of the membrane insert using the micro spatula and culture at 37 $^{\circ}\text{C}$ in humidified 5% CO_2 and 95% air atmosphere incubator.
 1. Add approximately 300 μL primary culture media into the well and a few drops into the membrane inserts (approximately 40 μL) every other day or as needed. Proper culturing showed that the cells are able to survive and be maintained for up to 30 days ex vivo.

4. Irradiation of salivary gland sections

1. Treat sections with a single dose of radiation (5 Gy) using cobalt-60 (or equivalent) irradiator.
 1. Transport sections to irradiator facility using a covered styrofoam container to avoid fluctuations in temperature. In addition, care needs to be taken during transport back to the laboratory incubator to ensure that media does not splash onto culture lid and induce contamination.
 2. Place the 24-well plate containing salivary gland sections 80 cm from the radiation source, in the center of a 32" x 32" radiation field. Radiation dose calculations and corresponding time in irradiator will vary by instrument and cobalt-60 decay.
2. Continue monitoring and culturing these sections as described in section 3.

5. Viability staining

1. Aspirate old media and wash slices twice with sterile, pre-warmed 1x PBS.
2. Stain sections with trypan blue dye.
 1. Use a micro-spatula to move the slice from the culture to a glass slide.
 2. Add 0.4% trypan blue solution to the vibratome slice with enough volume to cover the tissue (10–20 μ L).
 3. Incubate slices at room temperature (RT) in the trypan blue for 1–2 min for the dye to penetrate the tissue. Nonviable cells will be stained blue, and viable cells will be unstained.
3. Stain sections with calcein, AM live-cell dye.
 1. Add enough volume of stain to cover the section in one well of a 24 well-plate (200–300 μ L).
 2. Incubate slices at RT for 15 min to allow dye penetration.
 3. Carefully remove the section from the well and place on a glass slide. Mount slices with 1 drop of mounting media.
 4. Image sections on a fluorescent microscope at excitation/emission wavelengths of 488 nm and 515 nm.

6. Antibody staining vibratome sections

NOTE: The following provides a general antibody staining protocol specific for Ki-67; however, this protocol can be used with any antibody. All washes are conducted at RT unless otherwise noted.

1. In the multi-well tissue culture dish, aspirate off media and wash at least 2x with sterile 1x PBS.
2. Fix sections using 4% paraformaldehyde (PFA) overnight at 4 °C.

3. Aspirate off 4% PFA and wash 3x with PBT [1x PBS, 1% bovine serum albumin (BSA), 0.1% Triton X-100].
NOTE: Sections can be stored in 1x PBS at 4 °C and stained at a later time. Maximum storage time tested in this manuscript was 3 weeks. Longer storage time will need to be optimized by the individual user if that is desired.
4. Permeabilize sections using 0.3% Triton X-100 in 1x PBS for 30 min.
NOTE: This step can be modified and optimized for specific antibody staining and permeabilization.
5. Pipet off permeabilization solution.
6. Wash slices 3x for 5 min with 1x PBS, 1% BSA, and 0.1% Triton X-100 (PBT).
7. Block the slices with blocking agent with 1% normal goat serum for 1 h.
8. Wash the slices 3x for 5 min with PBT.
9. Incubate the slices overnight at 4 °C with 500 µL anti-Ki67 rabbit monoclonal antibody diluted in 1% BSA in 1x PBS.
NOTE: For phalloidin staining, skip step 6.9 and proceed using the protocol for the specific phalloidin used. For DNA counterstain, skip to step 6.15.
10. Aspirate anti-Ki67 rabbit monoclonal antibody.
11. Wash the slices 6x for 5 min in 1x PBS.
12. Incubate the slices in 500 µL of fluorescently conjugated secondary antibody compatible with the anti-Ki67 antibody diluted in 1% BSA in 1x PBS at RT for 1.5 h covered from light.
NOTE: Based on secondary antibody used, incubation time with the secondary antibody can be further optimized by the individual user. In addition, the secondary antibody is light-sensitive. All subsequent washes must be performed in the dark.
13. Aspirate secondary antibody.
14. Wash slices for 3x for 5 min with 1x PBS.
15. Wash slices for 5 min in deionized water.
16. Counterstain slices with DAPI (1µg/mL) for 20 min at RT.
17. Wash slices (once) for 5 min in deionized water.
18. Mount slices with one drop (~40 µL) of mounting media. To avoid excess bubbles, slowly place the coverslip onto the mounting media on the slide, starting with a 45° angle.
 1. To prevent crushing the thick sections with the coverslip, mount each slice with a spacer. A spacer can be created by placing a rim of vacuum grease in a square around the tissue section.

2. When laying the coverslip, the edges of the coverslip can be sealed with vacuum grease. Press down on the edges of the coverslip with your thumb to firmly adhere it onto the slide. Alternatively, seal the coverslip onto the microscope slide using clear nail polish.

7. Imaging vibratome sections

1. Image the stained slides within 5 days of staining.
2. Obtain optical sections of the stained vibratome slices using a confocal microscope. With a confocal microscope, obtain a z-stack at a defined step size or take individual images depending on the user's experimental design. Images can be examined on any computer screen after confocal collection.

NOTE: Due to the thickness of the vibratome slices, it is recommended that a confocal microscope or a scope with z-stack capability is used to visualize details within the samples. For the images used in this manuscript, a 63x oil objective was used; however, this can be tailored and further modified by the individual user and the specific scope used. Recommended pixel resolution for each objective and zoom factor with the assumed Nyquist sampling of 2.5 pixels in X and Y for the smallest optically resolvable structure was used. However, some commentators suggest 2.3 pixels, while others suggest 2.8 pixels. Please refer to the Handbook of Biological Confocal Microscopy²² for further details on how to make these calculations.

Representative Results

Primary 2-D cultures are grown in fetal bovine serum (FBS) supplemented media while primary 3-D salisphere culture are typically cultured in serum-free conditions^{10,11}. In addition, the two previous studies utilizing vibratome cultures from salivary glands cultured their sections in 0% or 10% FBS supplemented media^{19,20}. Mouse submandibular slices were sectioned at a thickness of 50 μm using a vibratome and optimal culture conditions were determined using a series of FBS concentrations (0%, 2.5%, 5.0%, and 10%). To determine survival characteristics, bright-field microscope images were taken at culture days 1, 4, 7, 14, and 30 post-sectioning (Figure 1). Additionally, gland sections were stained with 0.4% trypan blue dye and imaged with a bright-field microscope at 40x at the indicated time points (Figure 2A). Non-viable cells were stained blue and viable cells remained clear.

Similarly, to determine the survival characteristics of thicker slices sectioned at 90 μm , bright-field microscope images with and without trypan blue staining were taken at day 30 in cultures supplemented with 2.5% FBS (Figure 2B). Due to the slice thickness, 90 μm sections stained with trypan blue dye were imaged whole then cut in half in order to evaluate the center of the slice (Figure 2B). As a confirmation, a live-cell dye was utilized to evaluate cell survival in different FBS culturing conditions (Figure 2C). In bright-field images, high levels of translucent, surviving cells in tissues sectioned at both 50 μm and 90 μm were observed. Interestingly, sections became darker and overall tissue area condenses over time in culture, yet a significant portion of the section appears to survive the 30-day culture period (Figure 1, Figure 2). This condensation was most evident in the 0% and 10% FBS

culturing conditions. Trypan blue positive cells were observed on the perimeter of all sections regardless of culturing conditions, and there was an increase in trypan blue positive cell area in 0% FBS culture conditions when compared to the higher FBS culturing conditions (Figure 2A,2B).

Using the live cell stain, the sections cultured in 0% showed the lowest amount of staining, and the addition of FBS to the culture media improved the amount of live cells. Taken together, sections cultured in 0% FBS showed visible tissue condensation, elevated trypan blue stained areas, and the lowest levels of live cell staining. The addition of 2.5% FBS to the cultures improved the amount of translucent tissue, decreased the trypan blue positive area, and increased the levels of live cell staining. Additional increases in FBS concentration did not appear to improve survivability of the tissue; therefore, 2.5% FBS in the vibratome media was the optimal FBS concentration and utilized as the culture condition for all subsequent experiments.

To determine the viability of the submandibular ex vivo tissue slices post-dissection, proliferative and apoptotic markers were evaluated at days 1, 3, 7, 14, and 30 in culture. The proliferative activity was assessed by Ki67 immunostaining in a subset of culture slices (Figure 3A). Ki67 positive cells were observed at all time points evaluated and continued to be present at day 30 in culture, with minimal differences between time points. Similarly, the degree of apoptosis was evaluated by cleaved caspase-3 immunostaining in a separate subset of sections (Figure 3B). A low level of cleaved caspase-3 positive cells was observed at all time points up to day 14 in culture, while day 30 conditions appeared to have a small increase in the number of cleaved caspase-3 positive cells in some areas. Evaluation of the tissue edges did not reveal higher levels of cleaved caspase-3, which does not recapitulate the trypan blue staining (Figure 2). Overall, these results suggest that cues for proliferation and viability remained present in the vibratome cultures during the 30-day evaluation period.

Thick section vibratome cultures allow the opportunity to evaluate interactions between cellular constituents of a particular tissue at a depth that includes more than one epithelial cell thickness in each direction. In addition, it is important to be able to culture both major salivary glands, as they differ in the composition of salivary proteins produced, histological architecture, radiosensitivity, and other critical features. To determine different cellular populations in submandibular gland cultures, submandibular sections were stained with E-cadherin (E-cad) to detect epithelial cells, smooth muscle actin (SMA) to detect myoepithelial cells, and actin filaments (phalloidin) to detect cytoskeletal structures during the 30 days in culture (Figure 4A).

E-cadherin staining was observed on the membranes of a majority of cells and detected throughout the 30-day culture period. SMA+ cells were detected throughout the culture period, with similar levels at each time point. Cytoskeletal organization of actin filaments also appeared to be maintained at each time point evaluated. In contrast, there were cellular markers that were not consistently maintained during the entire evaluation period and these included CD31 (vasculature), TUBB3 (neurons), and Aquaporin-5 (Aqp-5, acinar marker) (Figure 4B). Vascular structures through the confocal stacks were clearly observed at days 1 and 3 post-culture; however, these structures appeared fragmented at day 7. Similarly,

neuronal processes were intact during the first day of culture, appeared diminished at day 3, and were subsequently lost at day 7 in culture. Aqp5+ cells were observed at days 1, 3, 7, and 14 in culture; albeit, at day 14, the overall staining level appeared to be reduced, with a more granular resemblance in the remaining positive cells. These data suggest that submandibular cultures contain the diversity of tissue constituents with maintenance of the vascular and neuronal cell types for shorter culture periods, and epithelial and myoepithelial cell types for longer culture periods.

While vibratome-sectioned cultures have been previously reported for the parotid salivary gland, the cultures were maintained for 48 h, limiting the timeframe during which they could be studied. In order to determine whether parotid glands can be maintained for longer, murine parotid glands were sectioned and cultured for 1, 3, 7, or 14 days. Fewer sections were obtained from the parotid gland due to its smaller size in mice; therefore, a 30-day culture period was not attempted. Slices were evaluated for proliferation by immunofluorescent staining using antibody against Ki67. Similar to the submandibular cultures, Ki67 positive cells were observed at all time points, indicating that the cells are maintaining a degree of proliferation in culture (Figure 5A).

In addition, the maintenance of functional acinar markers (α -amylase and Aqp5), an epithelial marker (E-cad), and vascular (CD31) and neuronal (TUBB3) cell populations were evaluated. Amylase is one of the most abundant proteins produced by differentiated parotid epithelial cells and frequently lost during the 2-D culturing of primary parotid cells. In the vibratome cultures, amylase was observed in the acinar cells and excluded from the ductal cells throughout the 14-day culture period (Figure 5B). Similar to the submandibular cultures, Aqp5+ cells were present in the parotid cultures at each time point, with the day 14 cultures exhibiting reduced levels compared to earlier time points (Figure 5C). E-cadherin levels were also maintained on the membranes of a majority of cells during the 14-day culture period (Figure 5D). Neuronal structures appeared to be maintained during the 7 day culture period and were not assessed at later time points (Figure 5E). In contrast, vascular structures appeared intact during the first day in culture, and only smaller structures were present at days 3 and 7 in culture (Figure 5F). These data suggest that parotid cultures maintain their proliferative capabilities and most functional capabilities for 7-14 days in culture and potentially exhibit a more intact tissue structure for a longer time frame.

The functional utility of the vibratome culture model was addressed by treating parotid or submandibular cultures with a single dose of radiation (Figure 6, Figure 7, Figure 8). Previous work in irradiated mouse models has focused on the parotid gland and demonstrated the induction of apoptosis that peaks at 24 h, induction of compensatory proliferation starting at day 5, disruption of actin filaments starting at day 5, and loss of differentiation markers (e.g., amylase) by day 14^{23,24,26}. Irradiation of parotid vibratome cultures led to increases in apoptosis at day 1, increases in proliferation at day 7, disruption of actin filaments at day 7, and reductions in amylase at day 7 (Figure 6). Irradiation of submandibular vibratome cultures led to increases in apoptosis at days 1 and 3, increases in proliferation at day 7, and disruption of actin filaments at day 7 (Figure 7). E-cadherin levels appeared relatively intact in both parotid and submandibular cultures, which was similar to in vivo observations²⁶. The functional acinar cell markers Aqp-5 in submandibular gland

sections and amylase in parotid gland sections decreased at day 14, compared to corresponding untreated time points (Figure 8). These data suggest that radiation-induced tissue changes that were observed in vivo were also observed in irradiated vibratome cultures.

Discussion

Salivary gland research has utilized a number of culture models, including immortalized 2-D cultures, primary 2-D cultures, 3-D salsphere cultures, and 3-D organ cultures from embryonic explants to ascertain questions on underlying biology and physiology. These culture models have yielded insightful information across a diverse array of research questions and will continue to be important tools in salivary research. The limitations of these culture models include modulation of p53 activity during immortalization, transient viability of primary cultures, loss of differentiation and secretory proteins in culture, and inability to evaluate cell-cell, cell-ECM and polarity interactions in adult tissues. The first 3-D organotypic slice culture (vibratome sectioned cultures) method for salivary glands was published in 2008¹⁸; however, this technique has been largely underutilized in this field despite frequent use in other fields. The work cultured parotid sections for 48 h, which limits the ability to study these sections for chronic effects following radiation treatment or utilize transfection or transduction protocols for phenotypes following manipulation of specific genes. The method described here has been optimized to allow for longer time in culture, yield high resolution images through confocal microscopy, provide a method to study intra- and intercellular dynamics on a 3-D section, and evaluate radiation-induced changes during a culture period of at least 14 days.

While each step is required for implementation of the technique, some steps are crucial to successful sectioning and culture maintenance. These include cutting the salivary gland slices, maintaining the slices in culture, and staining and imaging the slices with confocal microscopy. The presented protocol poses some challenges that require patience and practice in order to obtain optimal slices for analysis. The following suggestions will assist in carrying out this protocol successfully. It is imperative to completely isolate the salivary gland from the surrounding connective tissue following dissection. Residual connective tissue causes the vibratome blade to drag the gland out from the agarose block and requires the tissue to be re-embedded in agarose. This can be a major limitation since multiple re-embedding can increase the chances of contamination and decrease the viability of the slices. This is especially crucial for culturing parotid glands, since the parotid glands are more lobular in structure and therefore more likely to have extraneous tissue.

The addition of 1 % penicillin-streptomycin-amphotericin B (PSA) to all liquids including the buffer tray, the slice collection dish, and the vibratome media minimizes contamination of the slices post-dissection. Variation of agarose concentration was used to optimize successful cutting. Due to the density of the salivary glands, 1.9% agarose was too soft, and the glands were easily dislodged from the block. Vibratome sectioning in other fields have used 5.0% agarose; however, this caused jagged cuts and slices were suboptimal. After testing several agarose percentage conditions, 3% agarose was the most optimal to support

the weight and firmness of the tissue. Notably, the agarose concentration utilized in Warner et al. and Su et al. was also 3%^{19,20}.

Additionally, the angle, frequency of vibration, and advancing speed of the blade can be modified based on the tissue to be sectioned. For submandibular and parotid salivary glands, a 15° angle, speed of 0.075 mm/s, and frequency of 100 Hz were appropriate for sectioning. Due to the softness of the salivary glands, the optimal cutting conditions required the blade to advance slowly through the tissue at a high vibration. For immunofluorescent staining, the permeabilization, duration of incubations, and wash steps are essential for optimal stains. If positive staining only appears in the outer layers of the tissue slice, a more stringent permeabilization with proteinase K may be needed, while uneven staining or high background staining may require a less stringent staining with 0.2% Triton X-100. The incubation times were optimized for high signal and low background, which may need to be tailored to specific primary antibodies. Longer wash steps are essential in reducing high background and this may be tailored to the specific antibodies used.

One major application of this methodology is extended kinetic analysis following radiation exposure of salivary glands. Prior work has established both acute and chronic phase changes in irradiated salivary glands^{6,24,25,26}, and the vibratome culture system can be a powerful tool to dissect out the critical molecular events at specific time points after treatment. For example, radiation-induced cellular changes in the salivary gland that have been reported in the literature, including reductions in amylase, apoptosis of acinar cells, compensatory proliferation of acinar cells, loss of polarity and disruptions in cytoskeletal structure. Notably, vibratome cultures irradiated ex vivo exhibit similar alterations in these markers.

In addition, the acute phase response in salivary glands pivots around p53 activity; however, it is unclear what role p53 plays in later time points due to the ~5-day viability of primary cultures. This system would allow ex vivo, controlled disruption of p53 activity at later time points and uncover a role in chronic damage or regenerative responses. Furthermore, the compensatory proliferation response is initiated 5 days after radiation treatment, and it is difficult to delineate the molecular regulators of this response in transient primary cultures. The most widely used application of this methodology will likely involve cell-cell, cell-ECM, and polarity interactions in adult tissues. Impactful studies have been conducted in 3-D organ cultures from embryonic glands to uncover the intricate interaction between developing salivary glands and the neuronal or vascular network^{27,28,29,30,31}.

The method described here indicates that further optimization is needed for neuronal or vascular work in the submandibular cultures and possibly parotid cultures. Radiation damage also disrupts junctional regulators, induces collagen deposition, alters F-actin organization, and modulates secretory granules^{26,30,32}. Salivary gland regeneration studies are handicapped by the absence of an adult model to evaluate these interactions. This organotypic culture method can provide a system to apply advanced molecular techniques and further study the regulation of these mediators in a 3-D context and efficiently discover new therapies.

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Figure 1: Bright-field microscope images of 50 μ m submandibular sections.

Submandibular glands from female FVB mice (4-8 weeks old) were dissected, sectioned to 50 μ m thickness, and cultured on organotypic cell culture inserts for 1, 4, 7, 14, and 30 days post-dissection at 0%, 2.5%, 5.0%, and 10% fetal bovine serum (FBS) in media to determine culture characteristics and optimize culture conditions. Scale bars = 200 μ m.

Figure 2: Viability staining of submandibular sections.

(A) Bright-field microscope images of 50 µm submandibular dissected from 4- to 8-week old female FBV mice and stained with trypan blue (0.4%) at 1, 3, 7, 14, and 30 days in culture with media containing 2.5% fetal bovine serum (FBS). (B) Bright-field microscope images of 90 µm submandibular sections (left panel), stained with trypan blue (middle panel), cut in half and stained with trypan blue (right panel), then cultured to 30 days post-dissection in media containing 2.5% FBS. (C) Fluorescent images of 50 µm submandibular sections cultured in various FBS concentrations (0%, 2.5%, 5%, 10%) stained with calcein AM to indicate live cells (green) at culture day 7. Scale bars = 200 µm.

Figure 3: Evaluation of proliferative and apoptotic markers in submandibular organotypic tissue slices.

Submandibular glands from female FVB mice (4-8 weeks old) were dissected, sliced to 50 μm thickness, and cultured in media supplemented with 2.5% FBS on organotypic cell culture inserts for 1, 3, 7, 14, and 30 days post-dissection. At the indicated time points, slices were fixed and stained for proliferative (Ki67) and apoptotic (cleaved caspase-3) markers.

(A) Immunofluorescent staining of Ki67-positive cells (green) and the nucleus (blue). (B) Immunofluorescent staining of cleaved caspase-3-positive cells (red) and the nucleus (blue) from two viewpoints of a slice. The top row panel displays cleaved caspase-3-positive cells on the edge of a slice, and the bottom row panel displays cleaved caspase-3-positive cells from the middle of a slice. Representative confocal images were selected from multiple z-stacks per time point. Scale bars = 30 μm .

Figure 4: Presence of cellular structures in submandibular organotypic tissue slices. Submandibular glands from female FVB mice (4-8 weeks old) were dissected, sliced to 50 μ m thickness, and cultured in media supplemented with 2.5% FBS on organotypic cell culture inserts for 1, 3, 7, 14, or 30 days post-dissection. At the indicated time points, slices were fixed and stained for their corresponding markers with the nucleus (blue). **(A)** Submandibular sections were evaluated at days 1, 7, 14, and 30 post-dissection for the levels of E-cadherin, smooth muscle actin (SMA), and F-actin (phalloidin). **(B)** Submandibular sections were evaluated at days 1, 3, and 7 for levels of CD31 (vasculature) and TUBB3

(neurons). Aquaporin-5 (Aqp5) was evaluated at days 1, 3, 7, and 14. Representative confocal images were selected from multiple z-stacks per time point. Scale bars = 30 μm .

Figure 5: Evaluation of proliferative and functional markers in PAR organotypic tissue slices. Parotid glands from female FVB mice (4-8 weeks old) were dissected, sliced to 50 μ m thickness, and cultured in media supplemented with 2.5% FBS on organotypic cell culture inserts for 1, 3, 7, or 14 days post-dissection. At the indicated time points, slices were fixed and stained for their corresponding markers with the nucleus (blue). **(A)** Immunofluorescent staining of Ki67-positive cells (green). **(B)** Immunofluorescent staining of amylase-positive cells (red). **(C)** Immunofluorescent staining of aquaporin-5 (Aqp5)-positive cells (green). **(D)** Immunofluorescent staining of E-cadherin (red) positive cells. **(E)** Immunofluorescent

staining of neurons indicated by TUBB3 (magenta) positive cells. **(F)** Immunofluorescent staining of the vasculature indicated by CD31 (red) positive cells. Representative confocal images were selected from multiple z-stacks per time point. Scale bars = 30 μm ; d = ductal cells.

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Figure 6: Cellular changes following irradiation of parotid organotypic tissue slices.

Parotid glands from female FVB mice (4-8 weeks old) were dissected, sliced to 50 μ m thickness, and cultured in supplemented with 2.5% FBS on organotypic cell culture inserts. On day 1 post-dissection, a subset of slices was exposed to 5 Gy radiation and maintained for 2, 4, or 8 days post-dissection (corresponds to days 1, 3, and 7 post-radiation). Immunofluorescent staining of untreated and irradiated parotid sections to determine levels of (A) Ki67 (green)-positive cells, (B) cleaved caspase 3 (red)-positive cells, (C) phalloidin (cyan)- positive cells, (D) amylase (red)-positive cells, and (E) E-cadherin (red) positive

cells. All nuclear staining utilized DAPI (blue). Representative confocal images were selected from multiple z-stack per time point. Scale bars = 30 μm .

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Figure 7: Cellular changes following irradiation of submandibular organotypic tissue slices. Submandibular glands from female FVB mice (4-8 weeks old) were dissected, sliced to 50 μ m thickness, and cultured in media supplemented with 2.5% FBS on organotypic cell culture inserts. On day 1 post-dissection, a subset of slices was irradiated with 5Gy and maintained for 2, 4, or 8 days post-dissection (corresponds to days 1, 3, and 7 post-radiation). Immunofluorescent staining of untreated and irradiated submandibular sections to determine levels of (A) Ki67 (green)-positive cells, (B) cleaved caspase 3 (red)-positive cells, (C) phalloidin (cyan)- positive cells, (D) aquaporin 5 (Aqp5) (green)-positive cells, (E) E-cadherin (red)-positive cells.

and (E) E-cadherin (red) positive cells. All nuclear staining utilized DAPI (blue). Representative confocal images were selected from multiple z-stack per time point. Scale bars = 30 μm .

Figure 8: Functional acinar markers in irradiated parotid and submandibular organotypic tissue slices.

Submandibular and parotid glands from female FVB mice (4-8 weeks old) were dissected, sliced to 50 μm thickness, and cultured in media supplemented with 2.5% FBS on organotypic cell culture inserts. On day 1 post-dissection, a subset of slices was irradiated with 5Gy and maintained for 14 days post-irradiation. Immunofluorescent staining of untreated (UT) and irradiated (IR) sections were used to determine levels of (A) aquaporin-5 (Aqp5) (green)-positive cells and (B) amylase (red)-positive cells. All nuclear staining utilized DAPI (blue). Representative confocal images were selected from multiple z-stack per time point. Scale bars = 30 μm .