Original Article

Evaluation of the temporal relationship between formalin submersion time and routine tissue processing on resected head and neck specimen size

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Background: Studies show formalin fixation may shrink resected tissue, altering critical elements such as tumour size and surgical margins. To date, no literature exists examining time-dependent shrinkage of head and neck resection specimens. This paper aims to evaluate the temporal relationship between formalin submersion time and resected specimen size.

Methods: Twelve male merino sheep were utilised in this study. In each animal tissue was resected from four locations: tongue, labial mucosa, buccal mucosa and sternocleidomastoid (SCM) muscle. Dimensions of the tissue were recorded immediately post-resection and subsequently after 6, 12, 24, 48, 72 and 96 hours of submersion in formalin.

Results: After 96 hours of submersion, median shrinkage of specimen length in relation to initial size was 15.5% (11.56–19.5%; P<0.05) and shrinkage in width was 16.7% (13.2–20%, P<0.05). The rate of shrinkage for both length and width was maximal within the first 24 hours, after which shrinkage was markedly reduced. There was generally no difference in tissue shrinkage between individual regions.

Conclusions: These findings indicate formalin fixation time results in measurable and significant changes in specimen size of about 16% which evolves and then plateaus at the 24-hour mark. Based on this study all specimens should be submersed in formalin for 24 hours prior to sectioning, as any further tissue shrinkage is then negligible. Standardised fixation protocols should be considered to allow for accurate comparison of surgical specimens.

Keywords: Formalin; head and neck cancer; shrinkage; tissue processing; histology

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Introduction

Histopathological analysis of resected tissue remains a cornerstone of oncologic management, determining both operative success and directing adjuvant therapy. The head and neck region, in particular, may present significant challenges to ensuring operative tumour resection and thus rely more heavily on histological assessment to confirm primary site clearance (1). Contributing hurdles include a complex three-dimensional anatomy, unpredictability of tumour spread amongst the various tissue types, as well as

the endeavour to preserve critical organ functions such as swallowing and vocalisation. As a result, tumour size and margin status are routinely reassessed histologically to confirm operative findings (2,3).

Evidence suggests that preparation of resected specimens for analysis can have a significant impact on tissues, with multiple studies demonstrating a reduction in size of specimens after processing has been performed (1,4,5). This finding has been consistent, to varying degrees, across tissues including that of the head and neck (1,5-7). The observed shrinkage can have significant ramifications on the histological findings and in particular that of tumour size and margin clearance, two elements that play key roles in determining patients' consideration for adjuvant therapy. Currently adopted practices within head and neck oncology incorporates the discrepancies seen by recommending an intra-operative surgical margin of 1 cm be obtained to ensure a final "clear" histological margin of greater than 5 mm (8).

The majority of observed size reduction is thought to occur immediately post-resection due to contraction of intrinsic elastic tissue (1). Prior to final processing, mounting and staining, all specimens undergo a period of formalin submersion. This step allows formaldehyde, the primary fixative agent within formalin, to penetrate the specimen and fix the tissue such that cellular architectural integrity is preserved throughout histological processing (9). Whilst all resections are placed within formalin promptly after removal, the time of submersion can vary greatly. Adequate submersion time is paramount to ensure complete formaldehyde penetration of the tissue, which can be influenced by many factors such as size of tissue, concentration of formalin, fluid volume and tissue composition (9). In practice, the formalin fixation time is often also influenced by logistical and staffing factors such as day of the week and technician/pathologist availability that results in variability of submersion time between comparable resection specimens.

We aim to investigate the impact of formalin fixation time on the dimensions of resected tissue from distinct anatomical sub-regions of the head and neck. We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/ajo-20-46).

Methods

Animals

A total of twelve male merino sheep were used to conduct this study. Animals were of 2–4 tooth age. Use of animals was approved via scavenger ethics by the South Australian Health & Medical Research Institute in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Ed (Ethics Number: SAM-147). All sheep were utilised for an otolaryngology training course immediately prior to tissue resection. Oral cavity and neck tissue taken for the present study was not manipulated or handled during the preceding course.

Tissue resection

For each animal, tissue was resected using cold steel scalpel excision from four independent sites. Labial mucosa, tongue mucosa, buccal mucosa and sternocleidomastoid (SCM) muscle tissue were harvested by the same operator and of approximately equal size. Once collected, tissue specimen length and width were immediately measured using a digital Vernier calliper (Kincrome, Scoresby, Victoria, Australia), which reported to 0.001 mm accuracy. Length was defined by the specimen's maximal measurement, with width being defined as the maximal diameter in a plane perpendicular to length. The location of measurements was marked with ink to ensure accurate future corresponding measurements. Specimens were then submerged in 10% buffered formalin at a greater than 10:1 ratio (volume of formalin: volume of tissue).

Temporal analysis in formalin submersion

To assess for time-dependent effect of formalin on tissue size, resected specimens from the oral cavity and neck of six sheep were utilised.

After initial measurements and submersion, specimens remained in formalin for the duration of the study and removed only for the purpose of repeat measurement at six time points post-excision: 6, 12, 24, 48, 72, 96 hr. The ink markers were used to retake the same measurements with identical equipment and operator.

Analysis of processing effect

To assess for any potential shrinkage from the processing of resected specimens, a further six sheep were utilised.

After resection and initial measurement as described above, specimens were submersed in formalin for at least 24 hours. Specimens were then processed, sectioned, paraffin embedded, and slide mounted with hematoxylin and eosin (H&E) stain as per standard protocol by an independent pathology group (Clinpath Laboratories, Adelaide, Australia). Tissue processing was performed on a Leica Peloris processing machine utilising an 8-hour protocol with Xylene as the clearing agent. In brief, specimens were washed in 10% formalin prior to 6 washes in absolute ethanol to dehydrate the tissue. Specimens were then submerged in 3 washes of xylene and finally immersed in liquid paraffin wax prior to being embedded into paraffin blocks. Once processed and mounted, specimens were remeasured by the same independent pathology group to obtain final histological dimensions.

Statistical analysis

For the nonparametric data collected, Wilcoxon signed-rank tests were utilised to compare the dimensions of tissue between the various time points. Friedman analysis was utilised to compare results between regions. Where applicable, numerical data is given in the form of median (25^{th} percentile–75^{th} percentile). All statistical analysis was conducted using GraphPad Prism 6.0 software (San Diego, CA, USA).

Results

Tissue composition

Four pieces of tissue were collected from each animal, resulting in 48 samples in total. Tissue collected from the tongue, buccal mucosa and labial mucosa were all of composite nature, including the mucosa, submucosa, and muscle. SCM resections were of muscle only. Specimens taken from tongue, muscle and buccal mucosa all maintained their initial shape throughout the 4 days of formalin submersion. All specimens from labial mucosa, however, would inherently curl on itself in one plane after resection (*Figure 1*). *Figure 1* demonstrates representative images of collected specimens.

Temporal shrinkage effects of formalin submersion

Resected tissue specimens were serially measured at predetermined time-points after being submerged in formalin for up to 96 hours. After 96 hours of formalin submersion, median shrinkage of specimen length in relation to initial size was 15.5% (11.56–19.5%; P<0.05; *Figure 2A*). Similarly, the median reduction in relative width amongst resected tissue was 16.7% (13.2–20%, P<0.05; *Figure 2B*). When comparing between subsequent individual time-points, shrinkage was only statistically significant within the first 24 hours of submersion (*Figure 2A*,*2B*). Whilst shrinkage was ongoing after 24 hours, no statistically significant differences were observed between consecutive time-points.

Almost 90% of the pooled overall shrinkage occurred within the first 24 hours, with subsequent 24-hour time-periods contributing only smaller amounts of further shrinkage (*Figure 3*).

Across regions, the only statistically significant difference was identified between the shrinkage patterns between the SCM and labial mucosa (Friedman rank sum difference =14.00, P<0.05; *Figure 4*). In general, however, no differences in shrinkage patterns were seen amongst individual regions. Furthermore, no differences were observed when comparing oral tissue to the SCM tissue.

Shrinkage effects of histological processing

To examine the effect that histological processing may have on tissue specimen dimensions, we serially measured tissue length and width at different stages of processing (*Table 1*). When compared to initial size (24.44±8.6 mm), the average shrinkage after 24 hours of formalin submersion was 15.1% (20.54±7.6 mm; P<0.05) and the average shrinkage after histological processing was 34% (15.94±6.5 mm; P<0.005).

Discussion

Complete surgical clearance of primary cancers within the head and neck continues to provide the best local tumour control (2). Operative excision provides the additional benefit of subsequent complete pathological and microscopic assessment of the tumour, allowing for important measures such as tumour size, grade, invasive front, perineural or lymphovascular spread and marginal

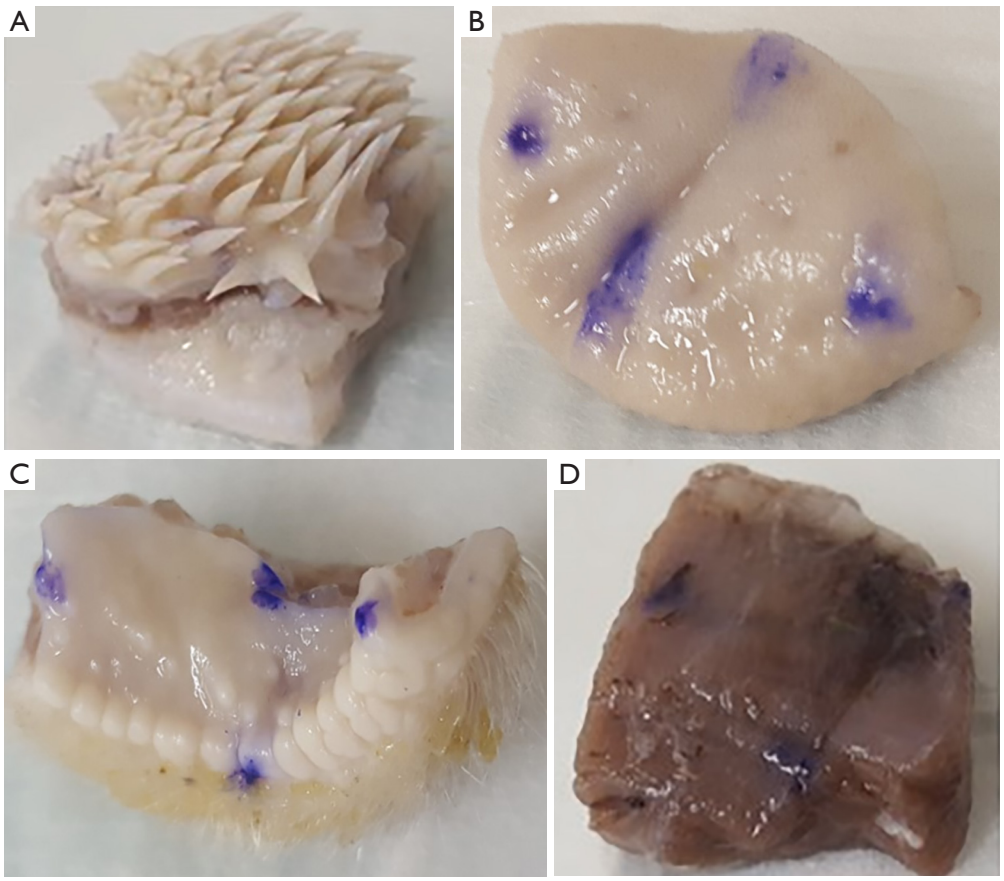

Figure 1 Representative images of resected head and neck specimens. Images taken of sheep specimen immediately post-resection, prior to formalin-submersion. Figures show buccal mucosa (A), tongue (B), labial mucosa (C) and SCM muscle (D). All specimens were marked with blue ink to maintain consistency of subsequent dimensional measurements. (C) Curling of the labial mucosa along one axis due to the intrinsic nature of the ovine lip. All other sites maintained initial shape throughout experimentation. SCM, sternocleidomastoid.

clearance to be reported accurately. These measures can have significant ramifications for patient in terms of both prognosis and determining subsequent management pathways (8). Inadequate marginal clearance, for example, increases risk of locoregional recurrence and detrimentally effects 5-year survival rates (2,8). As such, radiotherapy with or without concurrent chemotherapy is usually recommended if margins are close or involved. This additional treatment and its associated toxicity mean that it is imperative that final histologic specimens are produced in a repeatable, reliable manner and there is comparability between patients and centres to ensure the most consistent delivery of care is subsequently provided to patients. In modern practice, fixation of tissue using formalin is generally the most commonly used and accepted as best-practice (10). The ability for formalin to retain histologic cellular architecture as well as its capacity to allow for immunohistochemistry and molecular testing make it an appropriate choice for tumour histology. Formalin fixes tissue by inducing hydroxymethylene-based protein cross-links and coordinated calcium bonds in addition to tissue dehydration (11). Whether these mechanisms also contribute to the observed shrinkage is not well established.

This study aimed to investigate the temporal relationship between formalin submersion time and resected head and neck specimen size. Our results demonstrate that formalin submersion time independently effects tissue dimensions and highlights the need to standardise histology processing protocols. To our knowledge, this was the first occasion that an independent, temporal relationship has been identified between formalin submersion time and resected specimen size within this anatomical region. We have

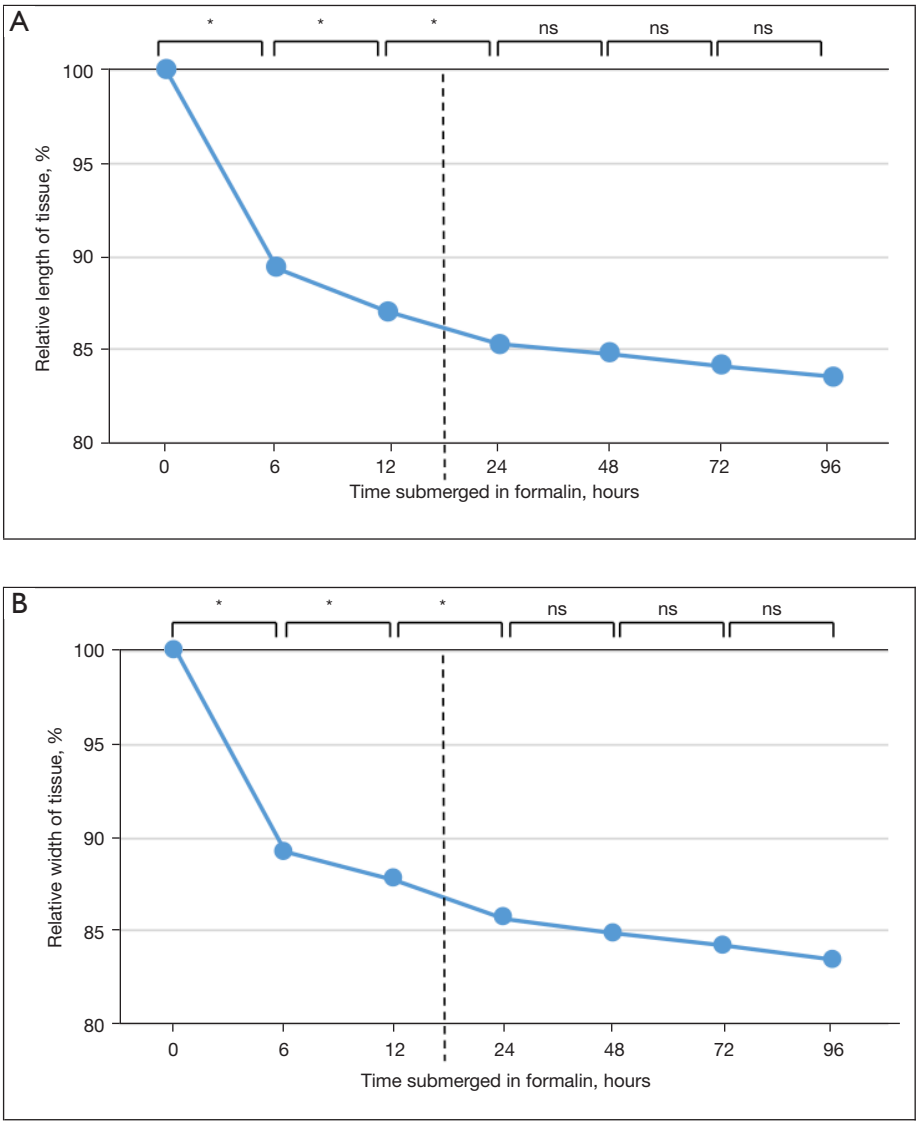

Figure 2 Overall relative shrinkage of tissue. Graphs demonstrating the relative shrinkage in both length (A) and width (B) of resected specimens after submersion in formalin. Y-axis represents relative shrinkage as a percentage of initial size with X-axis correlating to hours spent in formalin. Results from all specimens and all sites have been pooled together to demonstrate overall shrinkage. For both length (A) and width (B), there was statistically significant shrinkage between consecutive time points within the first 24 hours (P<0.05). Whilst shrinkage continued beyond 24 hours, the differences were non-significant. There was no difference between the shrinkage pattern between length and width. (Friedman analysis, P>0.05). X-axis is non-linear. Dotted line indicates point of transition. *, P<0.05; ns: statistical non-significance.

found that dimensions of resected tissue may shrink up to 16% when left in formalin for up to 4 days, the majority of the size reduction occurring within the first 24 hours. These results have substantial consequences, particularly when considering that both margin and tumour could be impacted by this shrinkage. Previous literature suggests that both normal and neoplastic tissue may be affected by tissue shrinkage (7,12,13), but it is yet unclear whether the rates of shrinkage are consistent between the two types of tissue and whether shrinkage typically results in overall upgrade (through loss of clearance) or downgrade (through tumour size reduction) in tumour staging. When histological

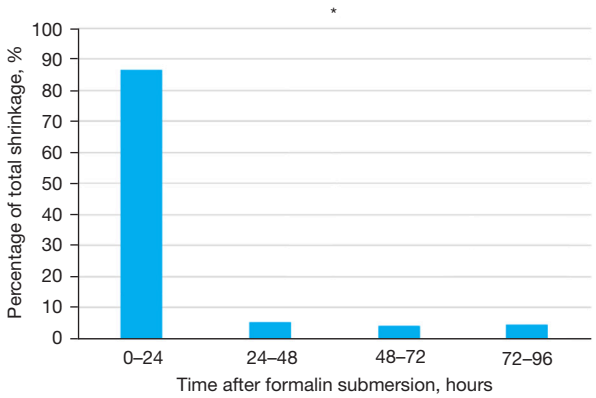

Figure 3 Contribution of total shrinkage by individual 24-hour periods. Comparison of the percentage of formalin-induced shrinkage occurring within 24-hour time periods. Over 85% of the total shrinkage occurs within the first 24 hours of formalin submersion. Subsequent days spent submersed in formalin contribute less than 5% (per day) towards total size reduction. *, P<0.05.

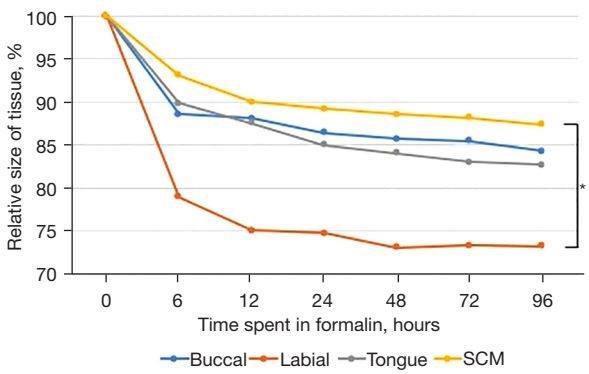

Figure 4 Comparison of shrinkage between sub-regions of the head and neck. Graphs demonstrating the relative shrinkage between individual anatomical sub-sites resected i.e., buccal and labial mucosa, tongue and SCM muscle. Y-axis represents relative shrinkage as a percentage of initial size with X-axis correlating to hours spent in formalin. Results from both length and width measurements were pooled. The only statistically significant difference was measured between the shrinkage patterns between SCM and labial mucosa (Friedman analysis, P>0.05). This was due to the initial curling of the ovine labial mucosa after resection and loss of tissue tension provided *in vivo*. There was no statistically significant difference between the shrinkage pattern between other regions (Friedman analysis, P>0.05). X-axis is non-linear. Dotted line indicates point of transition. *, P<0.05. SCM, sternocleidomastoid.

processing had been completed, there was a total relative size reduction in tissue of 34% amongst our data. These results were comparable to previous findings in which final histological size was found to shrink between 4% and 47% (1,14). In practice definitive tumour sizing is determined after complete histological processing and such a significant size reduction from initial measurements reinforces current practice that a more-than-adequate margin be obtained intra-operatively to ensure a final acceptable histological clearance (2).

The findings suggest that there is generally no significant difference in the shrinkage patterns between anatomical subsets of the head and neck. This is interesting given that the oral tissue is comprised of greater elastin content than the SCM and therefore expected to demonstrate greater shrinkage from intrinsic tissue contraction (1). This could suggest that the effects of tissue contraction occur instantly post-resection and that it had already taken place at the time of our initial post-resection measurement. Alternatively, the contribution of intrinsic tissue contraction to overall shrinkage may be less than previously estimated with formalin and tissue processing causing the majority of observed size reduction. The only observed difference between the SCM and labial mucosa was due to the inherent anatomical structure of the ovine lip and its tendency to curl on itself in one plane after resection. After this initial curling, however, the shrinkage pattern subsequently becomes comparable to other regions. Overall, the results suggest that specimens taken from the head and neck can be treated similarly in their histological preparation without needing to adjust for site variance. How tissue shrinkage may be influenced by age or in bony specimens that require prolonged decalcification is an area for further research.

Current guidelines for pathologists recommend that tissue spend no less than 6 and no more than 72 hours within formalin for optimal fixation conditions (15). This timeframe considers only that the tissue has been adequately penetrated by formalin and that it is not over-fixed, which can alter immunohistochemical suitability. Importantly, the effect of shrinkage has not been taken into consideration. At present, most pathology labs report a great variability in their formalin submersion time ranging from 8 to 72 hours. As our results demonstrate, however, specimens in formalin between 8 and 24 hours are still undergoing a phase of rapid size reduction. Retrieving specimens for processing during this early period does not give the tissue adequate time to

Table 1 Size of resected specimens at different stages of histology processing

Stage of processing	Measurement (± SD)	Shrinkage (%)	Significance (P value)
Post-resection	24.44 mm (±8.6)	–	N/A
Post-formalin fixation	20.54 mm (±7.6)	15.1	P<0.05
Final histology	15.94 mm (±6.5)	34	P<0.005

SD, standard deviation.

reach a relatively stable size, thus potentially influencing the final results. Whilst fixation for less than 24 hours may be appropriate for some head and neck specimens such as diagnostic biopsies, urgent samples and routine excisions, the ramifications for definitive tumour resections are much more significant. In practical terms, this results in two similar tumour resection specimens from similar patients being reported differently based solely on their formalin submersion time, which can ultimately alter the management and pathways of these patients despite similar underlying tumour and resection. This study suggests that pathology guidelines may need updating to take into consideration formalin-induced tissue shrinkage on head and neck tumour resection specimens. Based on these results, a standardised protocol with a minimum submersion time of 24 hours should be adopted for definitive tumour resection specimens to allow for dimensional changes to take place. This would increase the replicability of histology processing, reduce the variability in final histologic outcomes and provide increased consistency of care for head and neck cancer patients.

We do acknowledge the limitations of our study design, which may provide the impetus for further research in this area. Firstly, only healthy tissue has been assessed with no tumour component evaluated. There may be a differential effect of formalin on tumour compared to healthy tissue, which is not apparent in our results. Secondly, our study utilised a large animal model of head and neck resection and whilst we can expect the results to be reasonably translated to human tissue, further studies focussing on resected patient specimens is warranted.

Conclusions

Formalin submersion time and tissue processing has a significant impact on resected head and neck specimen size. From the time of resection to final histological assessment, specimens can shrink up to 34% when compared to their initial *ex vivo* size. The formalin-induced shrinkage of up to 16% evolves rapidly within the first 24 hours prior to plateauing. Tumour resection specimens should therefore be allowed to fixate in formalin for at least 24 hours prior to retrieval for processing. This standardised protocol can result in more comparable results across patients leading to improved and more consistent delivery of care.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://dx.doi.org/10.21037/ajo-20-46

Data Sharing Statement: Available at https://dx.doi.org/10.21037/ajo-20-46

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/ajo-20-46). SK reports grants from MedRobotics, outside the submitted work. JCH reports other from Tinnitus Treatment, outside the submitted work. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Use of animals was approved via scavenger ethics by the South Australian Health & Medical Research Institute in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Ed (Ethics Number: SAM-147).

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