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Elucidation of soft tissue flap histologic margins within a canine vocal fold

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Summary. Background: Histologic identification of implanted soft tissues in experimental animal models can be challenging, as donor tissue often strongly resembles the recipient bed. We have encountered this dilemma following implantation of a Composite Thyroid Ala Perichondrium flap (CTAP) into a vocal fold. The CTAP procedure is the first to utilize a vascularized flap for vocal fold reconstruction, making data to confirm or refute its viability critical. The current study evaluated several tissue stains to define precisely the histologic margins of CTAPs at two weeks post-implantation in a canine model.

Methods: Initial testing exposed canine cadaveric tissues to four stains (tattoo ink, Congo red, 4'6-diamidino-2-phenylindole, and henna) across four time periods. Tattoo ink alone withstood histologic processing. An exposure of 1 minute adequately delineated CTAP boundaries. The study concluded with a canine *in vivo* evaluation of a CTAP exposed to tattoo ink for 1 minute. After a two-week recovery period, vocal folds were harvested and evaluated histologically.

Results: Tattoo ink proved to be a safe and effective histologic marker *in vivo*, where the histologic margins of the implanted CTAP were clearly demarcated by a thin band of tattoo ink, soft tissue reactions were minimal, and interference with standard, special, or immunohistochemical stain assessments did not occur.

Conclusions: Tattoo ink provides a reliable means of demarcating a CTAP within a vocal fold and demonstrated that CTAPs survive transplantation. Further, tattoo ink demarcation may serve as a useful histologic marker for those wishing to assess tissue implants in other *in vivo* models.

Key words: Vocal fold, Augmentation, Reinke's space, Tattoo ink, CTAP

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Introduction

Histologic differentiation of experimental soft tissue implants can be challenging, particularly when implanted tissue resembles the recipient bed. These challenges were encountered during an investigation of soft tissue flaps for use in vocal fold (VF) reconstruction. The study, "Local Vascularized Flaps for Augmentation of Reinke's Space", reported that histologic evaluation of Composite Thyroid Ala Perichondrium flaps (CTAPs) did not consistently demonstrate the presence of the flap 1-month after delivery into a canine VF (Dailey et al., 2011). The CTAP is a rotational flap of tissue composed of perichondrium and fat drawn from the anterior larynx. It is delivered through an anterior window directly into the vocal fold for reconstruction thereof in cases of soft tissue defects. Further examination of this potential treatment modality requires a means of tracking a soft tissue CTAP within the treated VF of a live model. This study aimed to evaluate previously described *in vivo* tissue markers in order to identify one that would precisely define CTAP histologic margins at 2 weeks

post-implantation in a canine model, not incite an inflammatory response or influence interaction of the tissues and not preclude additional tissue analysis, such as immunochemistry. Tattoo ink, henna paste, 4', 6-diamidino-2-phenylindole (DAPI) and Congo red were chosen for evaluation with a hypothesis that one or more of these markers would facilitate tracking of an implanted CTAP, demonstrate flap viability and afford pathways for other *in vivo* staining applications.

Materials and methods

The experiment was designed to be a tiered evaluation of successful staining across various exposures in order to conserve fresh (previously unfrozen) canine vocal fold and CTAP tissues.

Phase 1 involved evaluation of the following parameters for each stain: 1) the degree of absorption in CTAP-like tissues across several time periods, 2) whether it was useful in outlining the tissues, and 3) whether it survived standard processing, embedding, and hematoxylin and eosin (H&E) staining.

Previously frozen sternohyoid muscle and contiguous fat and fascia (tissues similar in composition to CTAPs) were utilized for Phase 1. Tissues were stored at -80°C and were then thawed to room temperature prior to sample harvest. Once thawed, samples of sternohyoid muscle with fat attached distally were harvested, sectioned to roughly 2.5 cm in length and 0.5 cm in height and depth, and placed in 0.9% Sodium Chloride solution (Baxter, Deerfield, IL, USA). Next, Congo red (Sigma-Aldrich, St. Louis, MO, USA), Shelly® henna paste (Kaveri Enterprises, Delhi, India), Star Brite Colors™ Tribal Black tattoo ink (Papillon Supply & Mfg., Enfield, CT, USA), and DAPI (Invitrogen, Carlsbad, CA, USA) were obtained. The four stains were evaluated across the following four time periods: a dip (samples were placed into each stain just long enough for the tissue to be completely covered by stain and were then removed without hesitation), 30 seconds, 1 minute, and 5 minutes. The DAPI, tattoo ink, and Congo red were undiluted. Henna was tested as a solution (diluted to approximately 2g/ml with distilled water) at each of the four time periods. Due to its viscous nature, undiluted henna paste was tested at the 5-minute interval only.

Tissues were allowed to drip dry for approximately 3 seconds upon removal from saline and were then fully submerged into each stain for the duration of the time period. Following staining, tissues were dabbed dry with gauze and placed into individually labeled histology cassettes to prevent leaching of excess stain. Cassettes were placed into individual containers of 10% neutral buffered formalin (NBF) to prevent cross-contamination of samples during the 12 to 24 hour fixation period. The tissues underwent standard histologic processing, paraffin embedding, and were serially sectioned (5 micron thickness). The slides were cover-slipped unstained and were evaluated by a board certified pathologist (JT) to determine which stain(s) adequately outlined the tissues.

A standard H&E (Newcomer Supply, Middleton, WI, USA) histologic staining evaluation was performed on the tissues that were determined to be adequately outlined with experimental stain. The H&E slides were quantitatively analyzed by first scanning them at 6x magnification using PathScan Enabler IV's (Meyer Instruments, Houston, TX, USA) QuickScan software

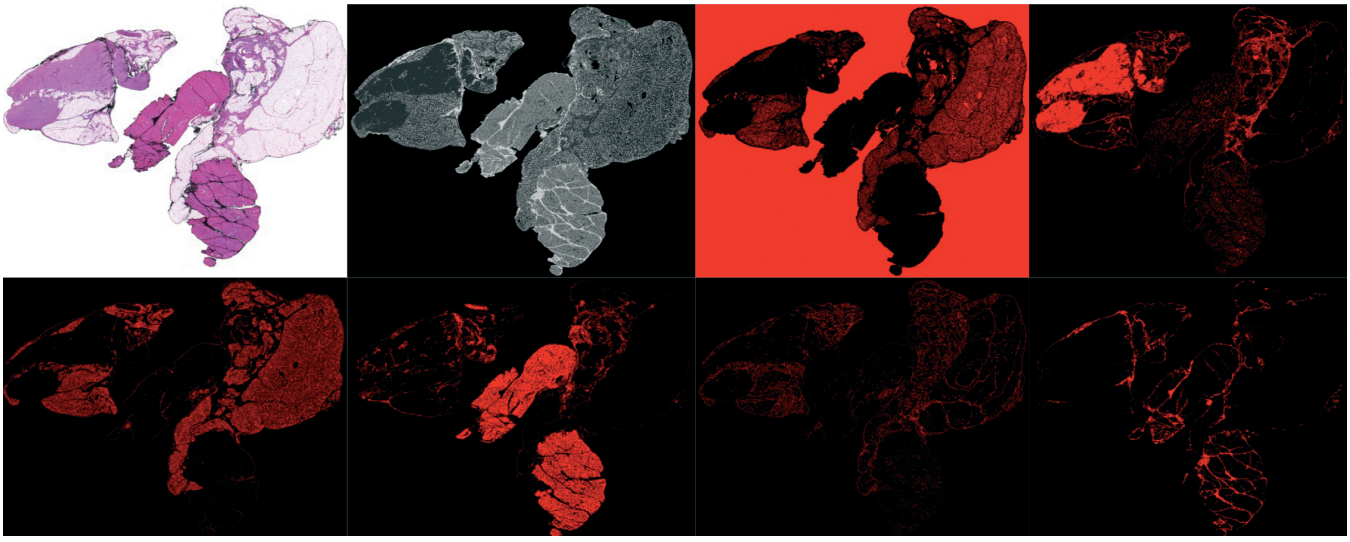

Fig. 1. Example of Image J K Means Clustering Results. Phase 1 sternohyoid muscle/adipose tissue stained with tattoo ink for 5 minutes, mounted and stained with Hematoxylin and Eosin, and scanned at 6x magnification. Top row (left to right): Image of original slide, image after K means clustering, background layer (red), dark hematoxylin (red). Bottom row (left to right): Light Hematoxylin (red), dark Eosin (red), light Eosin (red), tattoo ink (red).

and cropping the images to relatively uniform dimensions using Adobe Photoshop. Image J's 1.42q {NIH, Bethesda, MD, USA} K Means Clustering plug-in was then used to segment image layers (Fig. 1) and calculate the density of experimental stain in each sample. Experimental stain density was expressed as a percentage of the total hematoxylin, eosin, experimental stain, and background pixels on each scanned slide image. Qualitative analysis of the images was performed to evaluate any interference with H&E staining or assessment and the effectiveness of experimental stain(s) at adequately outlining the target tissues at various exposure times.

Phase 2 assessed tissue exposure times for the most effective stain from Phase 1 in order to determine an exposure time that would adequately delineate CTAP tissue while limiting time expenditure and excess dye exposure, which may lead to tissue inflammation, cumulative toxicity, or flap desiccation once implemented *in vivo*.

Larynges previously frozen at -80°C were thawed to room temperature and samples of CTAP tissue were excised from the larynges, sectioned to approximately 2x0.5x0.5 cm, and placed in saline. Upon removal from saline, the tissues were stained with tattoo ink for the time periods previously described in Phase 1. The slides were stained with standard H&E. Qualitative and quantitative analyses were performed as described in Phase 1.

Phase 3a was performed to: 1) determine whether the most effective stain from Phase 1 would persist through cadaveric surgical implantation of the CTAP into Reinke's space in a previously frozen canine larynx, 2) determine the tested exposure time frame that most appropriately delineated the implanted tissue, 3) evaluate whether the experimental stain leached into surrounding tissues, and 4) compare data to Phases 1 and 2.

Phase 3a involved evaluation of CTAPs stained for 1 minute (left) or 5 minutes (right) prior to CTAP reparative procedure and subsequent fixation and whole vocal fold sectioning. Bilateral, inferiorly based CTAPs were created on canine cadaveric larynges that had been frozen and then thawed, as previously described. Next, a minithyrotomy was created along the inferior and medial aspects of each thyroid ala (Fig. 2). A myringotomy knife was inserted into the lamina propria of each VF, creating a pocket for each flap to be inserted into after experimental staining.

The left CTAP was immersed in tattoo ink for 1 minute prior to insertion into the left VF pocket and the right CTAP was immersed in the ink for 5 minutes before insertion into the right pocket. The CTAPs were left in place for histologic sectioning. The entire larynx was placed into a container of 10% NBF for 12-24 hours.

Following fixation, the vocal fold was isolated and sectioned coronally into left and right anterior, middle, and posterior sections. After standard processing and embedding, H&E staining was performed. Qualitative and quantitative analyses were performed as previously described.

Phase 3b involved evaluations to qualitatively assess: 1) staining of a freshly harvested CTAP inserted into the superficial vocal fold (Reinke's space), 2) any histologic staining differences between fresh and frozen tissues, and 3) the exposure time frame that most appropriately delineated the implanted tissue. Phase 3b was identical to Phase 3a, except freshly harvested (not frozen) canine larynges were utilized.

Phase 4 included assessment of: 1) any detrimental effects on the CTAP or surrounding VF tissue due to experimental stain exposure, 2) the presence and/or quantity of stain leaching into surrounding tissues, 3) the positioning of the margins of the flap and native VF, and 4) whether the experimental stain interfered with standard and special histological stain assessments.

Phase 4 evaluated a CTAP exposed to tattoo ink for 1 minute prior to insertion and fixation within the left VF lamina propria in a living canine model. As part of a UW-Madison Institutional Animal Care and Use Committee-approved protocol ("Histologic and Rheologic Evaluation of Canine Vocal Folds Implanted with Vascularized Soft Tissue", approved September 23, 2011), a left vocal fold CTAP reparative procedure was performed in a 15-month-old male beagle as previously described (Dailey et al., 2011), utilizing the Seldinger technique to dilate the vocal fold tunnel. The CTAP was exposed to tattoo ink for 1 minute prior to insertion and fixation within the pocket of the VF via a helical spring

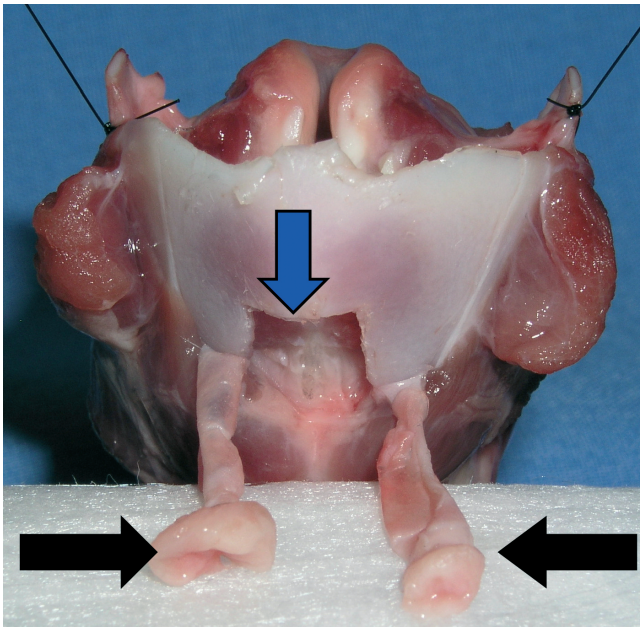

Fig. 2. Canine larynx mounted on a laryngeal dissection station. A bilateral minithyrotomy (approximately 14 mm tall x 10 mm wide) through the thyroid ala (blue arrow). Bilateral CTAPs prior to staining and insertion into the pocket in the lamina propria of the vocal fold (black arrows).

anchor (Fig. 3).

The animal was humanely euthanized for laryngeal harvest at two weeks post-reparative procedure and the vocal folds were bisected, sectioned, fixed, and processed as previously described. Five-micron serially sectioned slides were stained with the following: H&E, Gömöri trichrome (VWR, Radnor, PA, USA), Alcian Blue (Sigma-Aldrich, St. Louis, MO, USA), and Smooth Muscle Actin (SMA) (SMA mouse anticlonal - Sigma-Aldrich, St. Louis, MO, USA). Standard Immunofluorescence for Ki67 (rabbit polyclonal - Abcam, Cambridge, MA, USA) and SMA with DAPI counter stain was also performed.

Results

Phase 1: Congo red and DAPI were not visible upon histologic examination with a fluorescence microscope. Henna solution was only faintly visible upon standard histologic examination at all time frames, but staining did qualitatively appear to increase with exposure time. Henna paste was negligibly visible at the only tested exposure interval of 5 minutes (Fig. 4). These results confirmed our hypothesis that DAPI, Congo red, and Henna paste would not adequately demarcate the target tissues, survive histologic processing or persist long-term, and would therefore not delineate CTAP-like tissues.

Tattoo ink provided a very visible tissue outline for each exposure, showing clear delineation at even the dip and 30-second time periods. As with henna solution, tattoo ink staining intensity appeared to increase with exposure time. Tattoo ink survived fixation, processing, and embedding, and did not interfere with H&E staining. Quantitative analysis of H&E slides utilizing Image J confirmed the presence of tattoo ink staining in all four

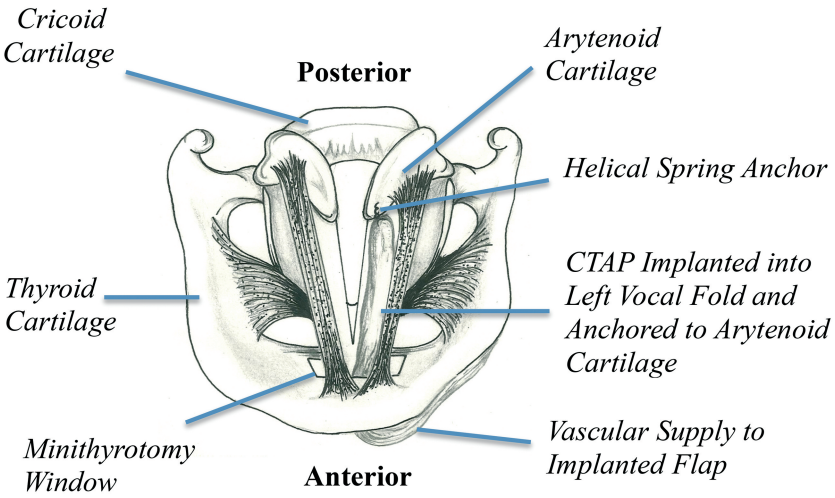

Fig. 3. Superior view schematic of a transverse section of a canine larynx demonstrating insertion of a left CTAP into the lamina propria of the vocal fold following immersion in tattoo ink for 1 minute.

Fig. 4. Phase 1 Sternohyoid muscle/adipose tissue unstained (left), stained with henna paste for 5 minutes (middle), and stained with tattoo ink for 5 minutes (right). The sample stained with tattoo ink for 5 minutes provides clear delineation of tissue boundaries.

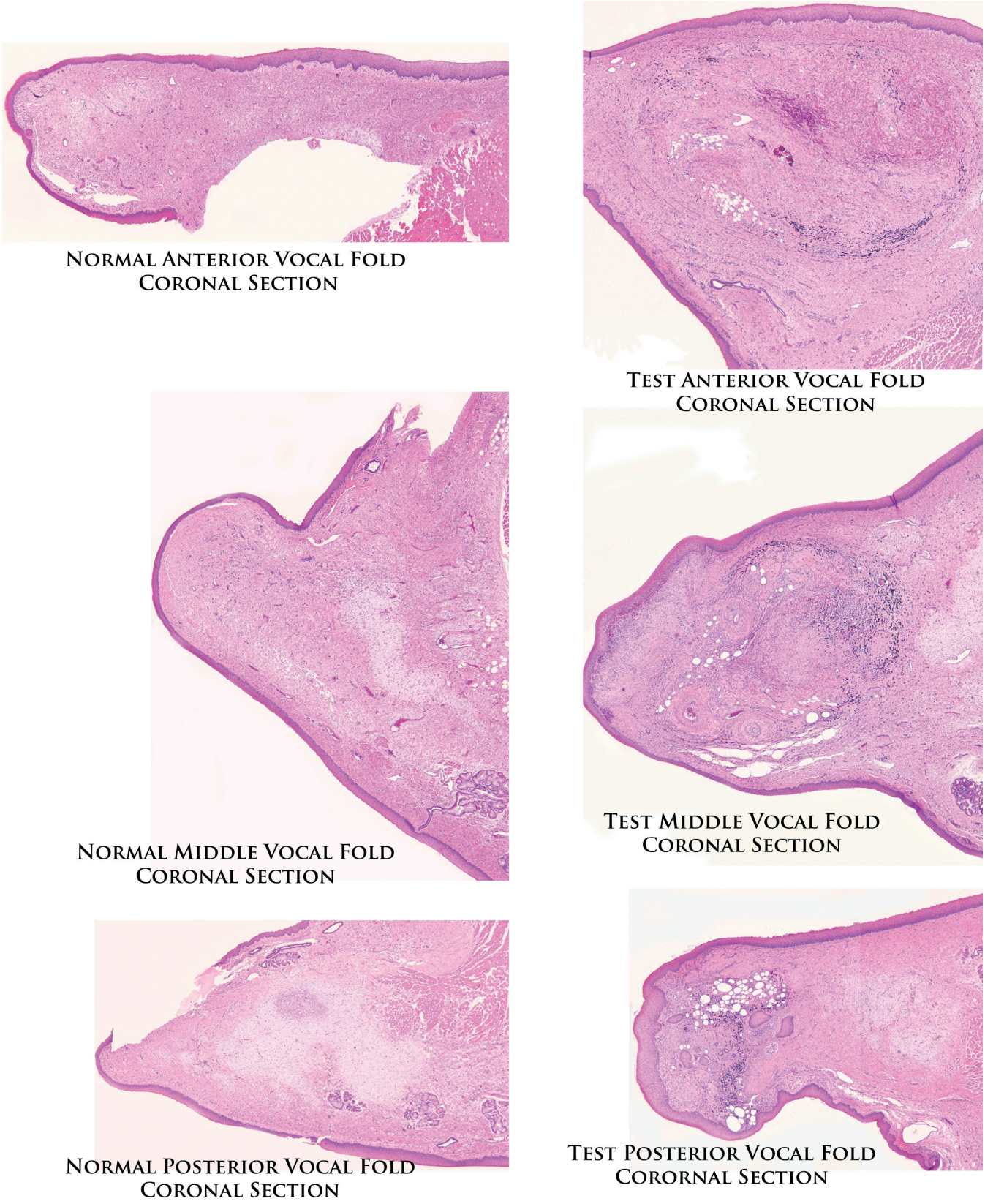

Fig. 5. Phase 4: Control right vocal fold coronal sections (left) and test left vocal fold coronal sections (right) showing implanted tattoo inked CTAP. The CTAP is in the correct near-epithelial location and there is no evidence of significant foreign body reaction.

samples (dip, 30 seconds, 1 minute and 5 minute ink exposure times). Qualitative H&E evaluation confirmed that tattoo ink provided an adequate outline of the tissue at all exposure intervals without interfering with H&E staining. Phase 1 experiments confirmed our hypothesis that tattoo ink was capable of adequately outlining the tissue, surviving histologic processing, and persisting long-term, without interfering with H&E staining, so tattoo ink alone progressed to further phases of the study.

Phase 2: Qualitative and quantitative evaluation revealed visible ink within all tested samples and an overall positive correlation between dip time and tattoo ink staining density. Qualitative review suggested that the dip and 30 second intervals may provide too fine of a delineation around the CTAP tissue to be of merit, especially when used *in vivo* for extended survival times. Therefore, it was determined that only the 1 and 5-minute ink exposure times were appropriate for further testing.

Phase 3a: Histologic evaluation revealed that tattoo ink persists within Reinke's space and adequately outlines an implanted CTAP, without leaching into surrounding tissues. The implanted CTAP was not successful at remaining fully within the VF pocket in all cases, likely due to retraction, which would necessitate affixing an implanted CTAP within Reinke's space as part of the procedure. While qualitative review revealed that 5-minute samples contained excessive ink, the 1-minute sections were adequately stained without excess. Based on these results, it was determined that the 1-minute soak timeframe would adequately outline the implanted tissue, allow for practical *in vivo* application, decrease surgical and anesthesia time, and minimize the potential for inflammatory response.

Phase 3b: Qualitatively, there were no significant differences between results of the frozen versus fresh (3a vs. 3b) trials.

Phase 4: Histologic analysis revealed that the CTAP was in the correct near-epithelial location in all vocal fold sections and was demarcated by a thin band of tattoo ink (Fig. 5). The flap was determined to be viable with no necrosis or vascular obstruction. Small numbers of pigmented macrophages were noted in all sections. The ink appeared to stay around the periphery of the flap in all sections and was not noted to have leached into any surrounding tissues.

Gömöri trichrome histologic evaluation highlighted mature collagen of the flap surrounded by native loose connective tissue (Fig. 6). Alcian blue stain accentuated compositional differences between the implanted flap, comprised mainly of mature collagen and adipose, and the surrounding native myxoid tissue. Smooth muscle actin (SMA) staining highlighted blood vessels within the CTAP on the operated side in all sections, indicating the flap was receiving an adequate blood supply. Dense myofibroblast SMA staining under the epithelium suggests that the surgical site was healing within normal limits and supports prior observations of flap assimilation (Fig. 7a). The tattoo ink did not interfere with Gömöri trichrome, Alcian blue, or SMA assessments.

The left posterior vocal fold sections were processed for immunofluorescence with Ki67 and SMA (Fig. 7b). Red nuclei stained by Ki67 demonstrated the viability of the flap by illuminating cells undergoing nuclear proliferation within the flap. Bright green staining for SMA highlighted blood vessels in and around the flap showcased myofibroblasts and fibroblasts below the

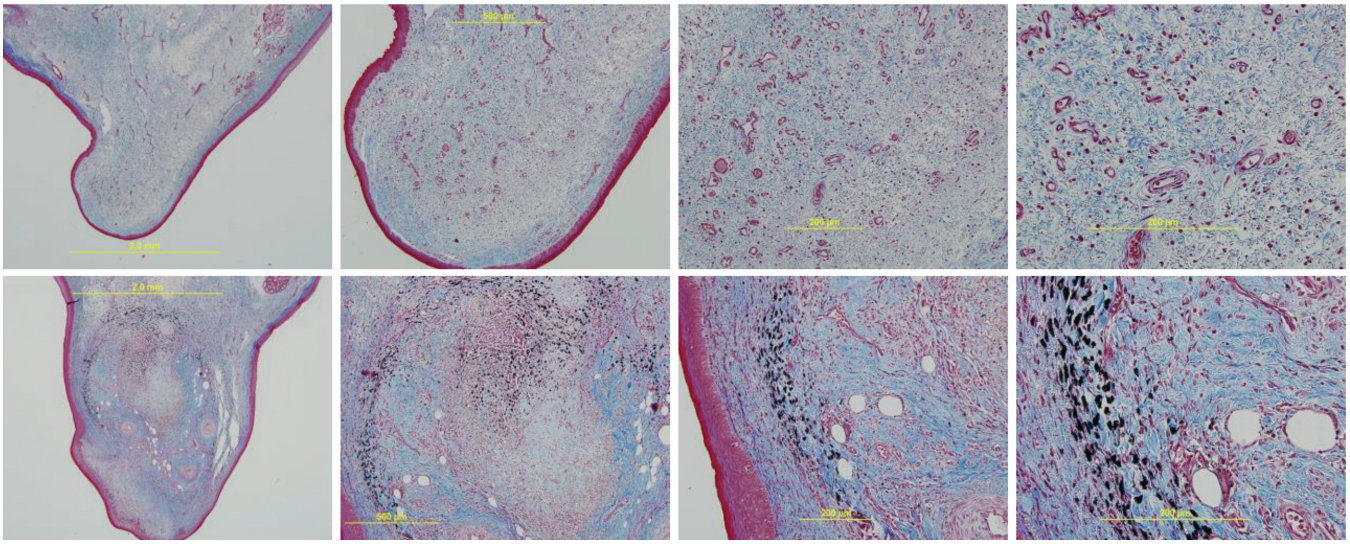

Fig. 6. Phase 4: Anterior coronal vocal fold sections stained with gömöri trichrome. Top row: Control (right VF) at 4x (left), 10x (left middle, 20x (right middle), and 40x (right). Bottom row: Test (left VF) at 4x (left), 10x (left middle), 20x (right middle) and 40x (right) demonstrating clear outline of tattoo ink surrounding the implanted CTAP. Darker blue staining in test sections highlights the mature collagen composition of the implanted flap. Lighter blue staining in the control sections demonstrates the native myxoid composition of the VF. Tattoo ink does not interfere with trichrome staining.

epithelium of the vocal fold. Adipose cells did not take up stain, appearing brownish-black. Remaining nuclei were counterstained with DAPI, and appeared blue in color. Tattoo ink was visible as small, dark black circles and did not interfere with the immunofluorescent assessment.

Discussion

Inclusion criteria for markers in this study included that they be: non-toxic to living cells, quickly and easily applied, readily available, inexpensive, capable of persisting 2-4 weeks or more *in vivo* in a canine model, minimally inflammatory in nature and able to withstand histologic and immunohistochemical processing and embedding. Henna, Congo red, DAPI, and tattoo ink are readily available, inexpensive, and quickly and easily applied.

Lawsonia inermis, or Henna, has been proposed for use as a naturally occurring histological stain (Malakzadeh, 1968) that is temporary and consists of a plant-based dye (Ahmadian and Fakhree, 2009), presumed to elicit minimal immune response *in vivo*. In addition to its staining capabilities, Henna has shown promising properties as an antimicrobial (Malekzadeh, 1968), anti-inflammatory, analgesic, antipyretic, and anticancer substance (Endrini et al., 2007), but was unable to adequately outline tissues in Phase 1 of this study.

Congo red is one of the most widely used histologic stains and is perhaps best known for its use in detecting and diagnosing amyloidosis. Congo red has been utilized for visualization of brain amyloids both *in vivo* and antemortem (Wilcock et al., 2006). It has been demonstrated, however, that both azo groups and the sulfonic acid groups of Congo red have disadvantageous *in vivo* effects, as they may cause the dye to interfere with protein aggregation and folding (Frid et al., 2007). Further, Congo red was not visible on histologic examination in this experiment.

DAPI is a readily available, blue, fluorescent stain with specificity for nucleic acids. It has been useful for DNA studies in pollen (Coleman and Goff, 1985), yeast (Allan and Miller, 1980), tobacco (Moscone et al., 1996), bats (Santos and de Souza, 1998), mice (Zijlmans et al., 1997), and humans (Schnedl et al., 1977), but has also been classified as a mutagenic substance (Invitrogen, 2013) and was not visible on histologic examination in this study.

Carbon black dyes, such as tattoo or India ink, have been used *in vivo* to visualize the anatomic location or patency of lymphatic or vascular structures (Tata and Anderson, 2002; Nagy, 2010), demarcate histologic margins upon surgical resection of tissue (Janfaza,

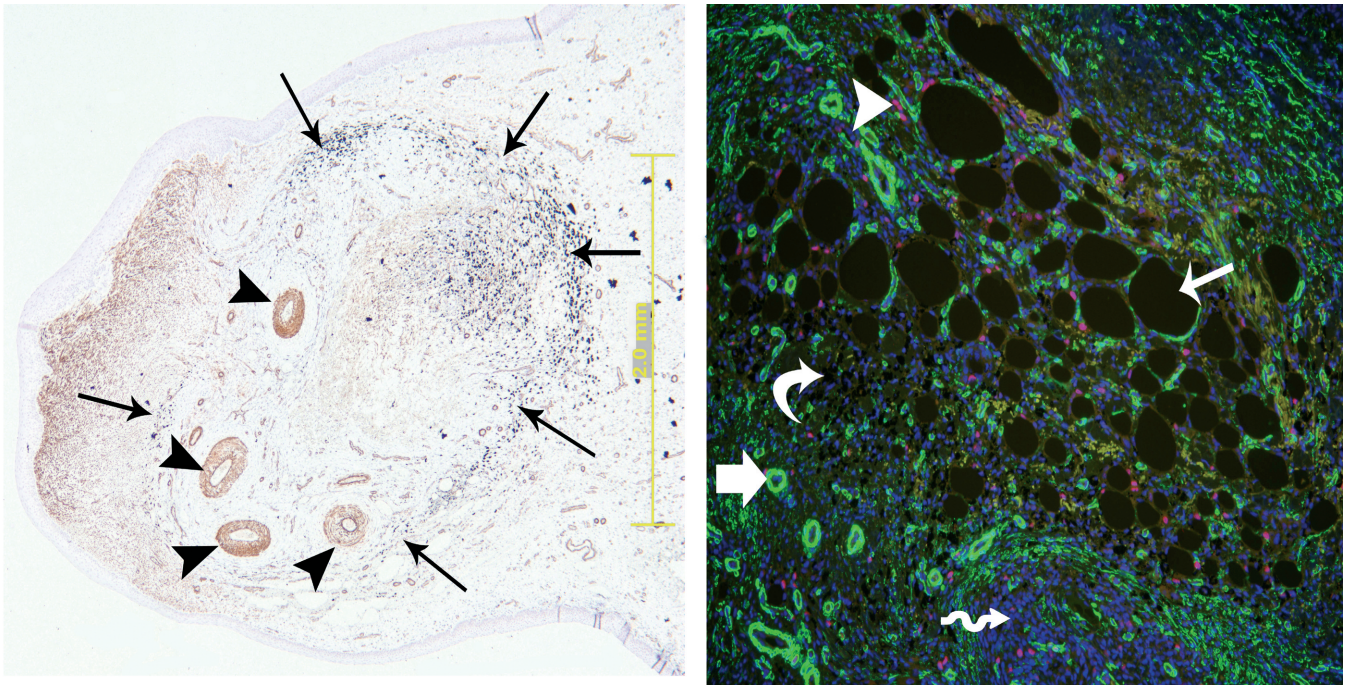

Fig. 7. A. (left) SMA staining of phase 4 *in vivo* left middle coronal section at 4x magnification. Small arrows point to tattoo ink outline of the implanted CTAP flap. Arrowheads point to dense SMA staining surrounding blood vessels within the flap. **B.** (right) Phase 4 *in vivo* left posterior coronal sections at 20x following combination of immunofluorescence results. Cells undergoing proliferation are highlighted by Ki67-positive red-stained nuclei (arrowhead). Other nuclei are stained blue by DAPI (squiggly arrow). Blood vessels in and around the flap are highlighted by bright green SMA staining (thick arrow) and tattoo ink is clearly visible as small, dark black dots (curved arrow). Fat globules did not uptake any stain and appeared brownish-black (thin arrow).

1980), and elucidate lesions, tumors or other defects upon colonoscopy (Nizam et al., 1996; Aboosy et al., 2005), thoracoscopy (Magistrelli et al., 2009), or endoscopy (Shaffer et al., 1998). In 1980, Dr. Parviz Janfaza reported positive results with minimal complications when utilizing India ink to demarcate tumor lesions in human patients prior to radiation or chemotherapy treatments (Janfaza, 1980). In 2012, Kim et al. utilized India ink for lesion identification during laparoscopic partial cystectomy procedures, reporting adequate visualization of lesion margins and no complications in the bladder (Kim et al., 2012). These cases utilized India ink to mark in situ tissues for surgical removal. Our study, however, demonstrates tattoo ink to also be an effective soft tissue marker for implantation and augmentation, allowing us to determine the ultimate success of the reparative procedure. Some studies have reported inflammation, fat necrosis, and abscess (Park et al., 1991; Coman et al., 1991) following *in vivo* demarcation with carbon black dyes, while many other studies (Nizam et al., 1996; Shaffer et al., 1998; McArthur et al., 1999; Aboosy et al., 2005; Magistrelli et al., 2009), including ours, report minimal or no histopathological complications.

We have demonstrated that, of the common previously described *in vivo* tissue marker methods, tattoo ink is superior in terms of its ability to survive histologic processing, persist long-term, and demarcate tissue without leaching or causing a significant inflammatory response. This, and prior research utilizing carbon black dyes *in vivo* in human patients (Janfaza, 1980; Nizam et al., 1996; Shaffer et al., 1998; McArthur et al., 1999; Aboosy et al., 2005; Tata and Anderson, 2002; Nagy, 2010; Magistrelli et al., 2009; Kim et al., 2012), suggests that tattoo ink can be utilized safely in a variety of species and on a variety of tissue types, making it a prime candidate for use in other long-term tissue implant tracking studies.

Conclusions must be tempered by a need for larger studies. Future potential studies to help clarify the efficacy and safety of using tattoo ink as an *in vivo* tissue elucidator should include a larger sample size and comparison to an idealized ink staining percentage. An increased sample size would assist with validation of our finding that there were no significant differences between tattoo ink staining densities in frozen versus fresh tissue. Additional studies could also assess the effectiveness of the dyes used only in Phase I of this study (congo red, DAPI, and henna paste) for staining CTAP tissue, rather than sternohyoid muscle and contiguous fat and fascia. These method modifications would allow for data generation beyond qualitative results. In Phase 3, implanted CTAPs were not consistently found within posterior-most sections of the VF pockets; therefore, further research aimed at identifying an effective method for CTAP affixation within a VF is in progress. Once an idealized CTAP affixation is established, pursuit of additional *in vivo* studies with longer post-surgical survival times to assess any long-term deleterious effects of tattoo ink exposure to the CTAP, vocal fold, or surrounding tissue would also be warranted.

Conclusion

The confirmed effectiveness of tattoo ink *in vivo* as a soft tissue implant marker is significant, allowing us to delineate the positioning and fate of our CTAPs within a vocal fold and proving definitively that, at two weeks post-repair, CTAPs remain viable. Additionally, as tattoo ink did not compromise histologic, immunohistochemical, or immunofluorescent assessments, is inexpensive, easy to apply, and non-inflammatory, it should prove useful for any researcher looking to define margins of implanted materials *in vivo*.

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Conflict of interest state, emt: This is original work that has been aprproved by all authors and has not been submitted elsewhere for publication. No party having a direct interest in the result of the research supporting this article has or will confer a benefit on the authors or on any organization or immediate family with which the authors are associated.

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