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1 **TITLE**

2 Intra-Operative Neural Monitoring of Thyroid Surgery in a Porcine Model

3

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55   **SHORT ABSTRACT**
56   This study aims to develop a standard protocol of intra-operative neural monitoring of thyroid
57   surgery in a porcine model. Here, we present a protocol to demonstrate general anesthesia, to
58   compare different types of electrodes, and to investigate the electrophysiological
59   characteristics of the normal and injured recurrent laryngeal nerves.
60

61   **LONG ABSTRACT**
62   Intraoperative injury to the recurrent laryngeal nerve (RLN) can cause vocal cord paralysis,
63   which interferes with speech and can potentially interfere with breathing. In recent years,
64   intraoperative neural monitoring (IONM) has been widely adapted as an adjunct technique to
65   localize the RLN, detect RLN injury, and predict vocal cord function during the operations. Many
66   studies have also used animal models to investigate new applications of IONM technology and
67   to develop reliable strategies for preventing intraoperative RLN injury. The aim of this article is
68   to introduce a standard protocol for using a porcine model in IONM research. The article
69   demonstrates the procedures for inducing general anesthesia, performing tracheal intubation,
70   and experimental design to investigate the electrophysiological characteristics of RLN injuries.
71   Applications of this protocol can improve overall efficacy in implementing the 3R principle
72   (replacement, reduction and refinement) in porcine IONM studies.
73

74   **INTRODUCTION**:
75   Although thyroidectomy is now a commonly performed procedure worldwide, postoperative
76   voice dysfunction is still common. Intraoperative injury to the recurrent laryngeal nerve (RLN)
77   can cause vocal cord paralysis, which interferes with speech and can potentially interfere with
78   breathing. Additionally, injury to the external branch of the superior laryngeal nerve can cause
79   a major voice change by affecting pitch and vocal projection.
80

81   Intraoperative neural monitoring (IONM) during thyroid operations has obtained wide
82   popularity as an adjunct technique for mapping and confirming the RLN, the vagus nerve (VN),
83   and the external branch of the superior laryngeal nerve (EBSLN). Because IONM is useful for
84   confirming and elucidating mechanisms of RLN injury and for detecting anatomic variations in
85   the RLN, it can be used to predict vocal cord function after thyroidectomy. Therefore, IONM
86   adds a new functional dynamic in thyroid surgery and empowers surgeons with information
87   that cannot be obtained by direct visualization alone[1-10].
88

89   Recently, many prospective studies have used porcine models to optimize the use of IONM
90   technology and to establish reliable strategies for preventing intraoperative RLN injury[11-20].
91   Porcine models have also been used to provide practitioners with essential education and
92   training in clinical applications of IONM.
93
94   Therefore, the combination of animal models and IONM technology is a valuable tool for
95   studying the pathophysiology of RLN injury[21]. The aim of this article was to demonstrate the use
96   of a porcine model in IONM research. Specifically, the article demonstrates how to induce
97   general anesthesia, perform tracheal intubation, and set up experiments for investigating the
98   electrophysiological characteristics of various RLN injury types.
99
100 **PROTOCOL**
101
102 The animal experiments were approved by the Institutional Animal Care and Use Committee
103 (IACUC) of Kaohsiung Medical University, Taiwan (protocol no: IACUC-102046, 104063,
104 105158).
105
106 **1.   Animal Preparation and Anesthesia**
107
108 1.1.   Porcine animal model
109
110 Note: This study applied the protocol described in the literature to establish a prospective
111 porcine model of IONM[11-19,22].
112
113 1.1.1. Use KHAPS Black or Duroc-Landrace pigs (3-4 months old; weighing 18-30 kg).
114
115 1.1.2. Ensure that the experimental protocol is consistent with national/international
116 regulations and guidelines for animal experiments, including the 3R principles (replacement,
117 reduction, and refinement). Obtain ethical approval of the experimental protocol from the
118 committee for care and use of experimental animals at the relevant institution.
119
120 1.2.   Anesthesia induction
121
122 1.2.1. Pre-anesthesia preparations
123
124 1.2.1.1.   Withhold food 8 hours before anesthesia and withhold water 2 hours before
125 anesthesia.
126
127 1.2.1.2.   Pre-medicate with intramuscular azaperone (4 mg/kg) at 2 hours before
128 anesthesia. Use a 500 mL saline bottle to fabricate a face mask for each piglet. Trim as needed
129 to ensure a secure fit to the snout.
130
131 1.2.1.3.   Use the weighing function on the operating table to measure the net weight of
132 each piglet **(Figure 1A)**.

133

134   1.2.1.4.   Maintain body temperature with a circulating water mattress set to 40 °C.

135

136   1.2.2. Induce general anesthesia (GA) with 2-4% sevoflurane at a fresh gas flow of 3 L/min *via*
137   the face mask with the piglet in a prone position. An adequate depth of anesthesia is usually
138   achieved in 3-5 minutes. Confirm the depth of anesthesia by no severe movement to pain due
139   to peripheral venous catheterization.

140

141   1.2.3. Identify a superficial vein on the outer side of one ear and sterilize the selected region
142   (about 6 x 6 cm^2) with 75% alcohol. For maximum safety, use a 24-gauge peripheral intravenous
143   catheter.

144

145   1.2.4. Administer intravenous anesthetic such as propofol (1-2 mg/kg) or thiamylal (5-10
146   mg/kg) to alleviate noxious stimulation by direct laryngoscopy.

147

148   Note: Use of neuromuscular blocking agent (NMBA) is not suggested. In subsequent
149   experiments, NMBA may complicate intubation by depressing spontaneous breathing and may
150   diminish electromyography (EMG) signals. Additionally, sevoflurane inhalation combined with a
151   bolus of propofol or short-acting barbiturates is reportedly sufficient for facilitating tracheal
152   intubation.

153

154   1.3.   Tracheal intubation **(Figure 1B)**

155

156   1.3.1. Prepare the equipment and materials required for EMG tube intubation: a size #6 EMG
157   endotracheal tube, a face mask for assisted ventilation, two slings to hold the mouth open, one
158   gauze strip to pull the tongue, a blunt tip suction catheter, a veterinary laryngoscope with 20cm
159   straight blades, an elastic bougie, a 20-mL syringe, a stethoscope, and adhesive tape.

160

161   1.3.2. Position the piglet in a prone position on the operating table. Align the head and body
162   to ensure clear visualization of the upper airway.

163

164   1.3.3. Direct the assistant to apply traction of the upper and lower jaw to maintain an
165   adequate mouth opening and to avoid rotation or overextension of the head. Cover the tongue
166   with gauze and pull the tongue out to optimize the visual field.

167

168   1.3.4. Hold the laryngoscope upside down and place it directly in the oral cavity to depress the
169   tongue.

170

171   1.3.5. Directly visualize the epiglottis and use the laryngoscope to press the epiglottis
172   downward toward the tongue base.

173

174   1.3.6. When the vocal cords are clearly identified, gently advance the elastic bougie into the
175   trachea. Slight rotation of the elastic bougie may be required to overcome resistance. Next,
176   advance the EMG tube at the mouth angle to a depth of 24 cm.

177

178 1.3.7. Inflate the EMG tube cuff to a volume no larger than 3 mL. If ventilation by manual
179 bagging reveals no obvious air leakage, *in situ* deflation of the EMG tube is feasible.

180

181 1.3.8. When the EMG tube is placed at the proper depth, confirm the free passage of fresh gas
182 by manual bagging. Further confirm the proper tracheal intubation by end-tidal carbon dioxide
183 ($etCO_2$) monitoring (capnography) and chest auscultation for early identification of inadvertent
184 esophageal or endobronchial intubation.

185

186 Note: Capnography showed both the $etCO_2$ waveform and the digital value in mmHg. When
187 esophageal intubation occurred, $etCO_2$ was absent or near zero after 6 breaths. When the EMG
188 tube was in the correct place, the typical $etCO_2$ waveform and adequate value (usually >30
189 mmHg) was noted. Furthermore, the breathing sound of a bilateral lung filled is clear and
190 symmetric as determined by chest auscultation.

191

192 1.3.9. Use medical tape to fix the EMG tube at the mouth angle. Since the tube usually
193 requires adjustment during IONM experiments, do not fasten the tube to the snout.

194

195 1.3.10. Connect the EMG tube to the ventilator. Continuous capnography is mandatory for
196 monitoring the $etCO_2$ value and curve throughout the experiment.

197

198 1.4.   Anesthesia maintenance **(Figure 1C)**

199

200 1.4.1. After the EMG tube is fixed, position the piglet on its back with the neck extended
201 (**Figure 1C**). Maintain general anesthesia with 1-3% sevoflurane in oxygen at 2 L/min.

202

203 1.4.2. Ventilate the lungs in volume-control mode at a tidal volume of 8-12 mL/kg, and set the
204 respiratory rate to 12-14 breaths/min.

205

206 1.4.3. Begin physiologic monitoring, including capnography, electrocardiography (ECG)and
207 monitoring of oxygenation (SaO_2).

208

209 **2.   Equipment Setting and Animal Operation** (Figure 1D)

210

211 2.1.   Equipment Setup

212

213 2.1.1. Connect the channel leads from the EMG tube to the monitoring system.

214

215 2.1.2. Set the monitoring system to run a 50 ms time window. Set pulsed stimuli to 100 µs and
216 4 Hz. Set the event capture threshold to 100 µV.

217

218 2.2.   Surgical procedure

219

220 2.2.1. Wear sterile surgical gloves and use povidone iodine with cotton swabs to disinfect the
221 neck surgical site.
222
223 2.2.2. Make a transverse collar incision about 10-15 cm in length with a scalpel to expose the
224 neck and the larynx.
225
226 2.2.3. Raise the subplatysmal flap 1 cm cranially from the clavicle to the hyoid bone.
227
228 2.2.4. Remove the strap muscles and visualize the tracheal rings and nerves. Use monopolar
229 and bipolar electrocautery to assist the surgical dissection and hemostasis.
230
231 2.2.5. Localize, identify, and carefully expose the EBSLN, RLN, and VN with a handheld
232 stimulation probe.
233
234 2.2.6. Position an automated periodic stimulation (APS) electrode on one side of VN for
235 stimulating during continuous IONM (CIONM). Connect the APS electrode with the monitoring
236 system. Set pulsed stimuli to 1 Hz, 100 μs, and 1 mA.
237
238 2.3.   At end of experiments, euthanize all piglets by the veterinarian.
239
240 **3.   Electrical Stimulation**
241
242 Note: To apply the 3R principle in porcine IONM studies, always perform repeatable
243 electrophysiology studies that do not cause nerve injury before performing experiments that
244 may cause nerve injury. This can be used to study the intensity, safety, and cardiopulmonary
245 effects[11,17]. The IONM equipment can be classified as stimulation equipment or recording
246 equipment (**Figure 2A**).
247
248 3.1.   Evaluate the baseline EMG responses of the target nerves, including the EBSLN, RLN,
249 and VN **(Figures 2B, 2C)**.
250
251 3.1.1. Start with an initial stimulation current of 0.1-mA current and increase stimulation in
252 0.1-mA increments until an EMG response is detected and recorded.
253
254 3.1.2. Further increase the current until the maximal EMG response is obtained.
255
256 3.1.3. Record the baseline amplitude, latency, and waveform of the EMG response.
257
258 3.1.4. Define the minimal stimulus level as the lowest current (mA) that clearly evoked EMG
259 activity of >100 μV. Define the maximal stimulus level as the lowest current that evoked the
260 maximal EMG response.
261
262 3.2.   Evaluate the Safety of electrical stimulation[11,19]
263

264   3.2.1. Apply a continuous 1-minute stimulus at the fifth tracheal ring level of the VN or RLN.
265

266   3.2.2. Progressively increase the stimulus current from 1 mA to 30 mA.
267

268   3.2.3. During VN stimulation, evaluate hemodynamic stability by monitoring of heart rate, ECG,
269   and invasive arterial blood pressure.
270

271   3.2.4. Finally, evaluate nerve function integrity by comparing EMG responses proximal to the
272   nerve stimulation site before and after each level of stimulation is applied.
273

274   3.3.   Effect of anesthetics (muscle relaxants and their reversals)[12,20]
275

276   Note: Improper use of NMBAs is a potential cause of unsuccessful IONM. The proposed animal
277   model was used to compare recovery profiles among different depolarizing NMBAs (*e.g.,*
278   succinylcholine) and nondepolarizing NMBAs (*e.g.*, rocuronium) at varying doses and to identify
279   the optimal NMBA for use in IONM. The animal model can also be used to evaluate the
280   effectiveness of NMBA reversal drugs (*e.g.*, sugammadex) for rapidly restoring neuromuscular
281   function suppressed by rocuronium.
282

283   3.3.1. Firstly, apply C-IONM and use the automatically calibrated baseline latencies and
284   amplitudes of EMG as control data.
285

286   3.3.2. Administer a bolus injection of 0.3 mg/kg rocuronium in a volume of 10 mg/mL and
287   observe the real-time EMG changes.
288

289   3.3.3. Three minutes after injection, perform one injection of 2 mg/kg sugammadex in a volume
290   of 100 mg/mL as a rapid bolus. Record the recovery profile of laryngeal EMG for 20 minutes.
291

292   **3.4.   Stimulation electrodes** (Stimulation probes/dissectors) **(Figure 3)**[17]
293

294   Note: There are different types of stimulation electrodes that can be used for nerve stimulation
295   during IONM, *e.g.*, monopolar probes (**Figure 3A**), bipolar probes (**Figure 3B**), and stimulation
296   dissectors (**Figure 3C**).
297

298   3.4.1. To mimic direct stimulation of nerves during surgery, apply 1 mA stimulation to the
299   EBSLN, RLN, and VN without overlying fascia.
300

301   3.4.2. To mimic indirect mapping and localizing of the nerve position before visual
302   identification during surgery, apply 1 mA stimulation at a 1- and 2-mm distance away from the
303   nerves at overlying fascia.
304

305   3.4.3. Record and compare the EMG responses between different types of stimulation
306   electrodes.
307

308 **3.5.   Recording electrodes (EMG tubes/needle electrodes/pre-gelled skin electrodes) (Figure**
309 **4)**

310
311 **3.5.1.   Use the animal model to evaluate how rotation or upward/downward displacement of**
312 **the EMG tube electrode (Figure 4A) affects the stability of the EMG signal. Additionally, use the**
313 **animal model to compare the EMG responses between different electrode types (*e.g.*, needle**
314 **electrodes and adhesive pre-gelled electrodes, Figure 4B) and different recording approaches**
315 **(*e.g.*, transcutaneous/percutaneous and transcartilage approaches, Figures 4C and 4D) in terms**
316 **of feasibility, stability, and accuracy during IONM.**

317
318 3.5.2.   For a feasibility study, apply a 1 mA stimulus current to bilateral EBSLNs, VNs and RLNs.
319 Record and compare EMG responses evoked by each electrode tested (*i.e.,* EMG tube,
320 transcutaneous, percutaneous, and transcartilage electrodes).

321
322 3.5.3.   For a stability study, evaluate and compare EMG signal stability in C-IONM under
323 experimentally induced cricoid/tracheal cartilage displacement.

324
325 3.5.4.   For an accuracy study, evaluate and compare the accuracy of the tested electrodes in C-
326 IONM for identifying EMG signal degradation under RLN injury.

327
328 4.   **RLN injury study** (Figure 5)

329
330 4.1.   In accordance with the 3R principle, perform RLN injury experiments in the porcine
331 model after all repeatable electrophysiology studies are completed. Perform tests of nerve
332 segments from proximal nerve segments to distal nerve segments (*i.e.,* proceed from the
333 caudal part of the RLN to the cranial part of the RLN).

334
335 4.2.   Use C-IONM to confirm and compare patterns of real-time changes in evoked laryngeal
336 EMG signals during and after acute RLN injuries with different injury mechanisms (*e.g.*, traction,
337 clamping, transection, or thermal injuries) **(Figures 5A and 5B)**. Use C-IONM for continuous
338 real-time display and recordation of EMG changes and sequential recoveries throughout the
339 experiment (**Figure 5C**).

340
341 4.3.   Collect injured RLN segments for histopathological analysis of morphological alterations
342 caused by the nerve injury experiments.

343
344 4.4.   Traction compression/stretch injury

345
346 Note: Traction compression or stretch injuries are the most common intraoperative RLN injuries.
347 Experimentally induce traction stress and observe the resulting electrophysiological EMG
348 changes and histopathological changes.

349
350 4.4.1.   Traction compression injury[13]

351

4.4.1.1.   Wrap a thin plastic loop (*e.g.*, a vascular loop 1.3-mm wide) around the RLN and use a force gauge to apply retraction with 50 g of tension (**Figure 5A**). This scheme mimics an RLN trapped against a dense, fibrous band or a crossing artery at the region of Berry's ligament during medial traction of the thyroid lobe.

4.4.2. Traction stretch injury[16]

4.4.2.1.   Wrap the RLN with a wider elastic material (*e.g.*, a 10-mm wide silicone Penrose drain), and use a force gauge to retract the RLN with 50 g of tension) This scheme mimics an RLN adhered to or encased in the goiter capsule and stretched forward during medial traction.

4.5.   Clamping injury

Note: Intraoperative mechanical trauma to the RLN usually results from poor exposure or visual misidentification of the RLN. [13,16]

4.5.1. After the traction compression RLN injury experiment, pinch the distal segment of the RLN with hemostatic forceps for one second. This scheme mimics the nerve being inadvertently clamped owing to visual misidentification as a vessel during the operation. Record the accompanying EMG signal change for comparison with further histopathological findings of the nerve specimen.

4.6.   Thermal injury

Note: Most intraoperative RLN thermal injuries result from thermal spread when electrocautery devices and various energy-based devices (EBDs) are used to induce hemostasis near the RLN. Like traction injury, thermal injury is rarely visible to the naked eye. Therefore, perform animal IONM experiments to determine the best model for evaluating the pathophysiology of RLN thermal injury and to test the thermal tolerance[14] and the safety of EBDs[15,18].

4.6.1. Use C-IONM to register the EMG changes continuously throughout the experiment.

4.6.2. For the activation study, investigate how energy-based devices (EBD) can be safely applied for hemostasis and dissection near the RLN during surgery (**Figure 5B**).

4.6.2.1. Activate the EBD (electrothermal bipolar vessel sealing system, set power at level 2, and the energy discontinues automatically by 2 to 4 seconds) at a 5-mm distance away from the RLN.

4.6.2.2. If EMG signals remain stable after several tests, perform a further test at the narrower distance (*e.g.*, 2-mm, and followed by 1mm distance).

4.6.2.3. If any substantial EMG change occurs after any test the experiment is complete and followed by continuous real-time EMG recording for at least 20 minutes.

396

397 <mark>4.6.3. For the cooling study, evaluate the cooling time to determine post-activation optimal EBD</mark>
398 <mark>cooling parameters.</mark>

399

400 <mark>4.6.3.1. Contact the activated EBD on the RLN directly after a 5 second cooling time.</mark>

401

402 <mark>4.6.3.2. If the EMG signals remain stable after three tests, test the shorter cooling time (*e.g.*, 2</mark>
403 <mark>seconds, and followed by 1 second).</mark>

404

405 4.6.2.4. If the EMG remains stable after repeated tests, confirm the safety of the EBD by
406 touching the RLN immediately after activation.

407

408 **REPRESENTATIVE RESULTS:**
409 Electrophysiology study
410 *Baseline EMG data, minimal/maximal stimulus level, and the stimulus-response curves*
411 Using a standard monopolar stimulating probe, the obtained minimal stimulation level for VN
412 and RLN stimulation ranges from 0.1 to 0.3 mA, respectively. In general, the stimulus current
413 correlated positively with the resulting EMG amplituderesponse[11,17]. The EMG amplitude
414 plateaued at the maximal stimulation levels of 0.7 mA for VN stimulation, and 0.5 mA for RLN
415 stimulation[11].

416

417 *Electrical stimulation (intensity, safety, and cardiopulmonary effect)*
418 In the safety study, there is no unwanted effects on EMG signal or hemodynamic stability
419 observed after continuous pulsatile VN and RLN stimulations in the setting of 1 mA to 30 mA. In
420 addition, baseline EMG amplitudes and latencies of the VN or RLN were relatively unchanged
421 after the nerves was stimulated by a high-current. Therefore, it was suggested that an
422 intermittent high stimulus current during IONM was not harmful to the VN or RLN[19].

423

424 *Effects of anesthetics (muscle relaxants and their reversals)*
425 Experimental comparisons of NMBAs of this animal model showed that different types and
426 doses of muscle relaxants have different natural recovery profile. For example, recovery times
427 for succinylcholine (1 mg/kg) and low-dose rocuronium (0.3 mg/kg) were significantly shorter
428 than that for standard dose rocuronium (0.6 mg/kg). The experiments for NMBA reversals
429 confirm that sugammadex (reversal of rocuronium) effectively and rapidly restores
430 neuromuscular function suppressed by rocuronium[20].

431

432 *Stimulating electrodes (stimulation probes and dissecting stimulators)*
433 Typically, IONM is performed with a commercially available ETT-based surface recording
434 electrode system (*i.e.,* a so-called EMG tube). However, a limitation of the clinical use of EMG
435 tubes is the need to maintain constant contact between the electrodes and vocal cords during
436 surgery to obtain a robust EMG signal. False IONM results can result from an EMG tube that is
437 mispositioned during intubation (*e.g.*, due to incorrect insertion depth, incorrect tube size, or
438 rotation of the electrode) or from an EMG tube that is displaced during surgical manipulation or
439 neck retraction (*e.g.*, causing rotation or upward/downward displacement of the electrode).

440
441   Experimental comparisons of stimulating electrodes showed that the stimulation
442   probes/dissectors evoked typical EMG waveforms from the EBSLN/RLN/VN with 1 mA current.
443   The stimulating current correlated positively with the resultant EMG amplitude. In monopolar
444   probes and stimulating dissectors, maximum EMG was elicited by <1 mA. In bipolar probes,
445   maximum EMG required a higher current. In all groups, evoked EMG amplitudes decreased as
446   the distance from the probe/dissector to the nerve increased. Evoked EMG amplitudes also
447   decreased in stimulated nerves that had overlying fascia. Therefore, the animal model
448   confirmed that both stimulation dissectors and conventional probes are effective to evoke
449   EBSLN, RLN, and VN waveforms to monitor real-time nerve function during surgery[17]. Various
450   stimulation probes/dissectors are now available in IONM system for specific stimulation
451   requirements, surgical monitoring application and the preference of the users.
452
453   *Recording electrodes (EMG tubes, needle electrodes, and pre-gelled skin electrodes)*
454   The feasibility study confirmed that the EMG tube electrodes on the vocalis, the
455   transcutaneous/percutaneous needle electrodes, and the transcutaneous/transcartilage pre-
456   gelled electrodes were effective for recording typical evoked laryngeal EMG waveforms from
457   the VN and RLN under 1 mA stimulation. **Figure 6** shows that transcutaneous/transcartilage
458   pre-gelled electrodes generally recorded lower EMG amplitudes compared to EMG tube and
459   needle electrodes.
460
461   In the stability study, real-time EMG tracings were compared before and after tracheal
462   displacement was experimentally induced. **Figure 7** shows that the change in contact between
463   EMG tube electrodes and vocal folds after tracheal displacement significantly changed the
464   recorded EMG signals. However, tracheal displacement had no apparent effect on electrode
465   contact quality or on EMG signal quality from the transcutaneous or transcartilage electrodes.
466   The accuracy study evaluated the accuracy of real-time signals in reflecting adverse EMG
467   degradation during RLN stress experimentally induced by continuous VN stimulation with the
468   APS electrode. When RLN traction stress was experimentally induced, the EMG tube electrodes
469   on the vocalis muscle and the transcartilage/percutaneous/transcutaneous electrodes recorded
470   similar patterns of progressive degradation in EMG amplitude **(Figure 8)**.
471
472   RLN injury study
473   *Traction injury*
474   Typical real-time EMG changes during RLN traction revealed a progressive amplitude decrease
475   combined with a latency increase (the so-called "combined event"). In addition, the EMG
476   signals gradually recovered after release of traction **(Figure 9A)**. The histopathology study
477   showed that morphological changes occurred mostly in outer nerve structures such as the epi-
478   and peri-neurium. Structures in the endoneurium remained relatively intact[13,16].
479
480   *Clamping injury*
481   All RLNs showed an immediate LOS (within less than 1 s) after acute mechanical injury was
482   experimentally induced. In addition, no gradually EMG recovery can be observed in a short
483   period of time after the injury **(Figure 9B)**. The histopathology study showed that distortion of

484 the epineurium and perineurium was greater in the clamping injury group compared to the
485 traction injury group[13,16].
486
487 *Thermal injury*
488 During the thermal injury study, the real-time EMG reveals a combined event, which then
489 rapidly degrades to LOS **(Figure 9C)**. The reaction time before LOS and the severity of
490 electrophysiologic injury may be related to the dose of thermal stress[14]. Studies of EBDs reveal
491 that the safe activation distance to the RLN and the cooling time vary by EBD type. For example,
492 the safe activation distances and cooling times are 5 mm and 1 second for monopolar
493 electrocautery (15 watts), 3 mm and 1 second for bipolar electrocautery (30 watts), 2 mm and 3
494 to 10 seconds for the Harmonic scalpel, and 2 mm and 2 to 5 seconds for the Ligasure system,
495 respectively. Notably, the Harmonic scalpel should be cooled for more than 10 seconds or
496 cooled by a quick (2 seconds) muscle touch maneuver before it touches the RLN. The Ligasure
497 system should be cooled for more than 2 seconds or cooled by a quick muscle touch maneuver
498 before it touches the RLN[15,18]. The histopathological examination of the thermal injured nerves
499 showed relatively severe damage to the inner endoneurium with less distortion of the outer
500 nerve structure[16].
501
502 **FIGURE AND TABLE LEGENDS**
503
504 **Figure 1. Preparation and anesthesia of KHAPS Black/Duroc-Landrace Pigs for IONM research.**
505 (**A**) Net weight of each piglet was measured before anesthesia. (**B**) An assistant maintained an
506 adequate mouth opening while traction was applied to the upper and lower jaw. A
507 laryngoscope was then used to press the epiglottis downward toward the base of the tongue.
508 When the vocal cords were clearly identified, the elastic bougie was gently advanced into the
509 trachea. The EMG tube was then inserted to a depth of 24 cm at the appropriate mouth angle.
510 (**C**) The piglet was placed on its back with the neck extended. The channel leads from the
511 recording electrodes were connected to the monitoring system. Physiologic monitoring was
512 performed during the study. (**D**) The neck and the larynx were exposed for experiments.
513
514 **Figure 2. The multifaceted electronic equipment and principle of the IONM system.** (**A**) The
515 basic equipment included the neural stimulating electrodes (stimulator) and the recording
516 electrodes (connected to the ETT). (**B**) The stimulating electrodes can be used to determine the
517 location and functional status of the EBSLN, RLN, and VN during IONM. (**C**) The evoked EMG
518 response is displayed on an LCD screen.
519
520 **Figure 3. The various stimulation electrodes available for use in IONM.** (**A**) Monopolar probes
521 (**B**) bipolar probes, and (**C**) stimulation probes/dissectors. The selection of stimulation
522 probes/dissectors used for IONM depends on the specific stimulation requirements, the specific
523 application desired and the preference of the surgeon.
524
525 **Figure 4**. **Various recording electrode types are available for use in IONM.** (**A**) The EMG ETT
526 electrodes include (**1a**) Trivantage (**1b**) Contact Reinforced (**1c**)–Standard Reinforced, and (**1d**)-
527 FLEX EMG Tubes); (**B**) (**2**)-adhesive pre-gelled electrodes and (**3**)-needle electrodes. (**C** and **D**)

528   The EMG tube is designed to touch the vocal fold through intubation (**I**), and the adhesive pre-
529   gelled or needle electrodes can be used in transcutaneous (**II**), percutaneous (**III**), or
530   transcartilage (**IV**) approach for EMG recording during IONM.
531
532   **Figure 5**. **Continuous IONM was performed *via* APS of the VN (*) to investigate real-time EMG**
533   **changes in the RLN during (A) traction and (B) thermal injury.** (**C**). Throughout the experiment,
534   the C-IONM system displayed and continuously recorded the induced EMG changes and
535   sequential recoveries in real time.
536
537   **Figure 6**. **Comparison of evoked EMG responses between four different types of recording**
538   **electrodes.** The feasibility studies indicated that all electrode types (*i.e.,* EMG tube,
539   transcutaneous, percutaneous, and transcartilage electrodes) accurately recorded typical
540   evoked laryngeal EMG waveforms from the RLN under 1 mA stimulation.
541
542   **Figure 7**. **Comparison of real-time EMG tracings before and after experimental tracheal**
543   **displacement. For stability study, tracheal displacement was experimentally induced.** Changes
544   in contact between the EMG tube electrodes and vocal folds caused significant variation in
545   recorded EMG signals. (**A**) Electrodes in the normal position recorded strong EMG signals. (**B**)
546   Electrodes with slight upward displacement (1 cm) recorded relatively weaker EMG signals. (**C**)
547   Electrodes with moderate to severe upward displacement (2 cm) showed an EMG LOS.
548
549   **Figure 8**. **Comparison of real-time EMG tracings during experimental RLN experimental RLN**
550   **traction injuries between four different types of recording electrodes.** The accuracy studies
551   showed that, when RLN traction stress was experimentally induced, all electrode types (*i.e.,*
552   EMG tube, transcutaneous, percutaneous, and transcartilage electrodes) recorded similar
553   patterns of progressively degrading EMG amplitude.
554
555   **Figure 9**. **Comparison of real-time EMG changes and sequential recoveries after different RLN**
556   **injury types.** (**A**) In traction injury, the EMG signals gradually degraded under nerve stress and
557   gradually recovered after release of traction. (**B**) In clamping injury, the EMG signals showed an
558   immediate LOS and no recovery. (**C**) In thermal injury, the EMG signals revealed a combined
559   event and then rapidly gradually degraded to LOS with no recovery.
560
561   **DISCUSSION**
562   Injury to the RLN and EBSLN remains a significant source of morbidity caused by thyroid surgery.
563   Until recently, nerve injury could only be identified by direct visualization of trauma. The use of
564   IONM now enables further functional identification of the RLN by applying stimulation and
565   recording the contraction of the target muscles. Currently, however, both conventional
566   intermittent and continuous IONM systems have some technical limitations in false-positive
567   and false-negative interpretations. Hence, suitable animal models are necessary to these
568   clinical issues.
569
570   Recently, plenty of animal experimental studies have tried to overcome pitfalls of IONM and to
571   investigate new applications. Most of these studies have used medium-sized animals such as

572 canine/dog[23-25]and porcine/swine/mini-pig[11-19,22,26-29]. Canine models of the RLN and laryngeal
573 function are well-established and highly mimic human anatomy, size and physiology. The
574 porcine model is the oldest animal applied in RLN research[30-31]. The first experiments in live pigs
575 performed by Galen in the second century A.D. demonstrated functional alterations in a
576 transected RLN. Currently, the porcine model is most commonly used for IONM research
577 because its anatomy and physiology are very similar to those in humans. Experimental pigs
578 have a medium size that enables easy handling and are widely available at a relatively low
579 cost[21].
580
581 This article demonstrates standard protocols for using the porcine model in IONM research,
582 including protocols for general anesthesia and tracheal intubation. The 3R principle is
583 implemented in the design of experiments for investigating electrophysiological characteristics
584 of RLN injuries. Key issues in the use of the proposed porcine model include(1) EMG parameter
585 characteristics and safety considerations when applying electrical stimulation[11,17,19], (2) the use
586 of muscle relaxants and reversals[12,20,32], (3) stimulating and recording electrodes[17], and most
587 importantly (4) models of RLN injuries[13-16,18] that cannot be accurately quantified in humans.
588 The protocols were set up to induce different severity and types of RLN injuries. Recorded real-
589 time EMG data were correlated with postoperative vocal cord function and histopathology
590 examinations. Although some data from experimental studies are inapplicable to clinical
591 practice, our porcine model provides a valuable research platform not merely in understanding
592 technology of IONM, but also in guiding future experiments to improve surgical strategies for
593 lesser RLN injuries during thyroid surgery.
594

600 **DISCLOSURES**
601 The authors have nothing to disclose.
602

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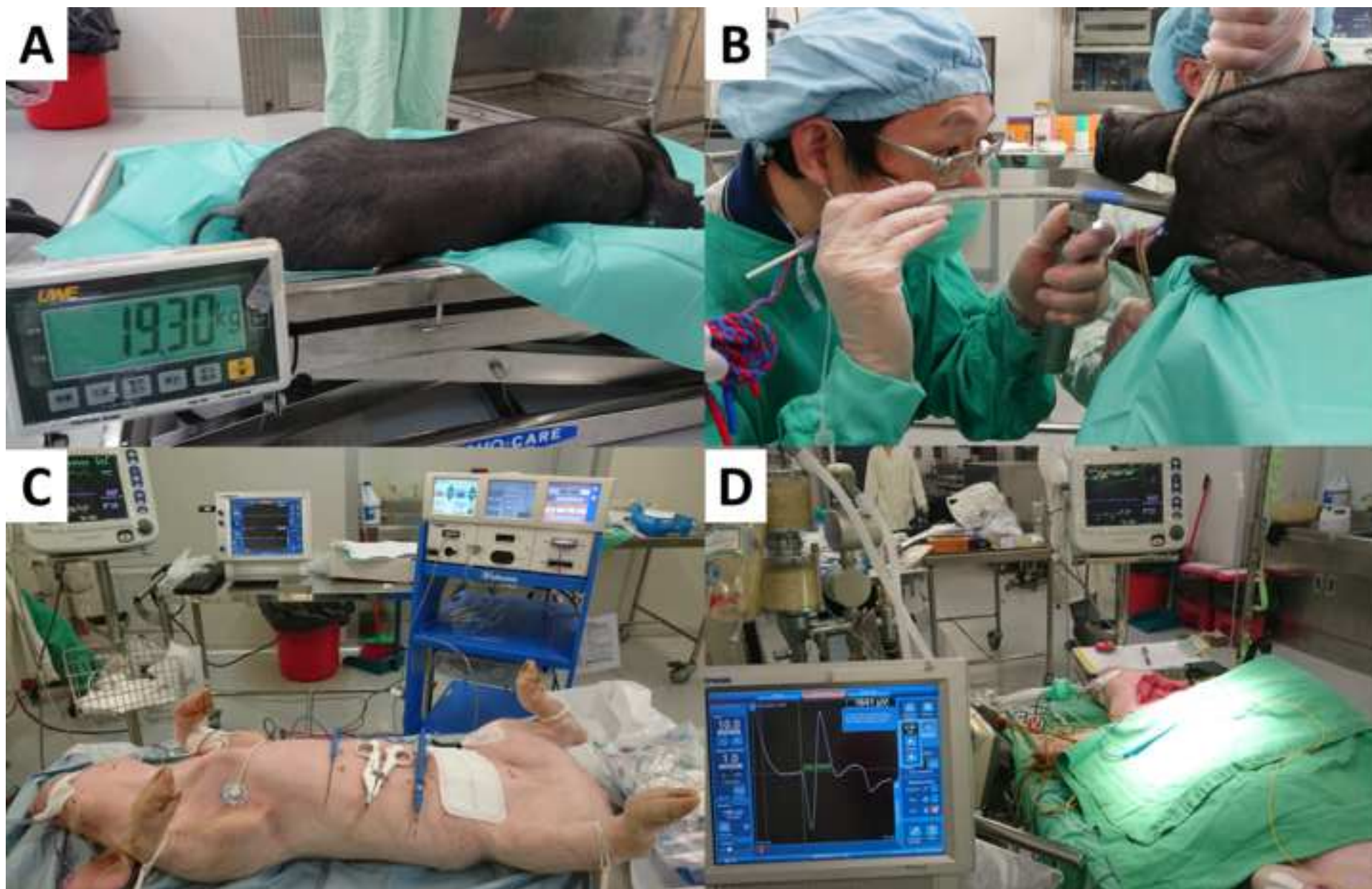648   characteristics in a porcine model. *Laryngoscope.* **127** (4), 998-1005 (2017).
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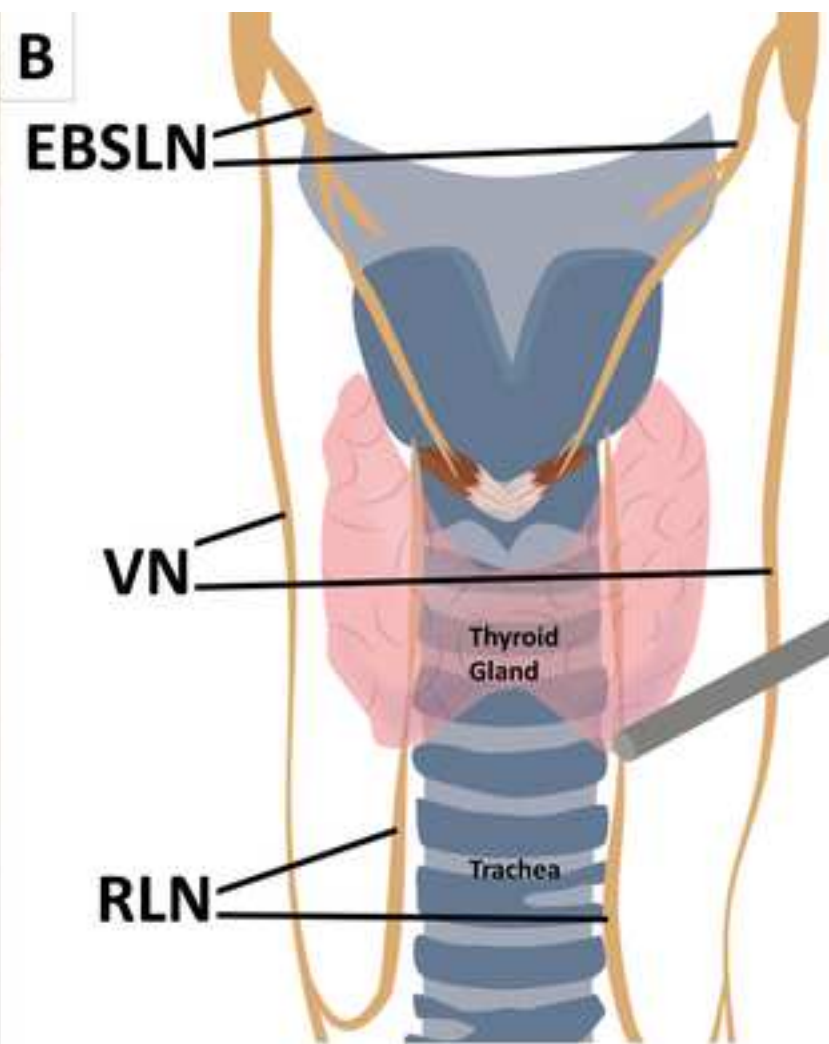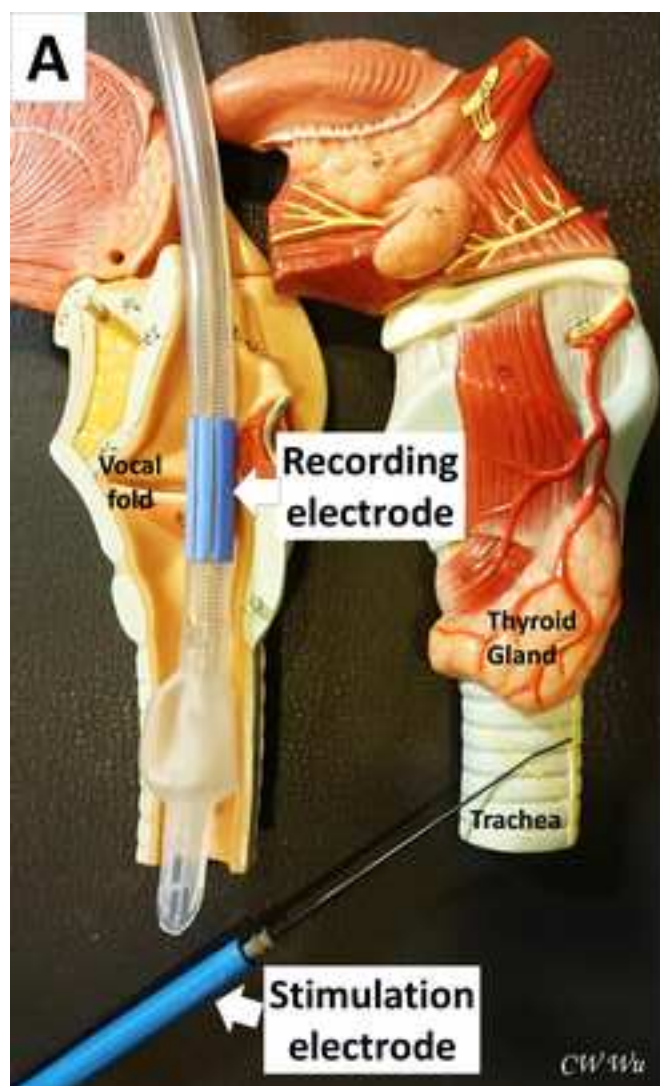651   19   Lu, I. C. *et al.* Safety of high-current stimulation for intermittent intraoperative neural
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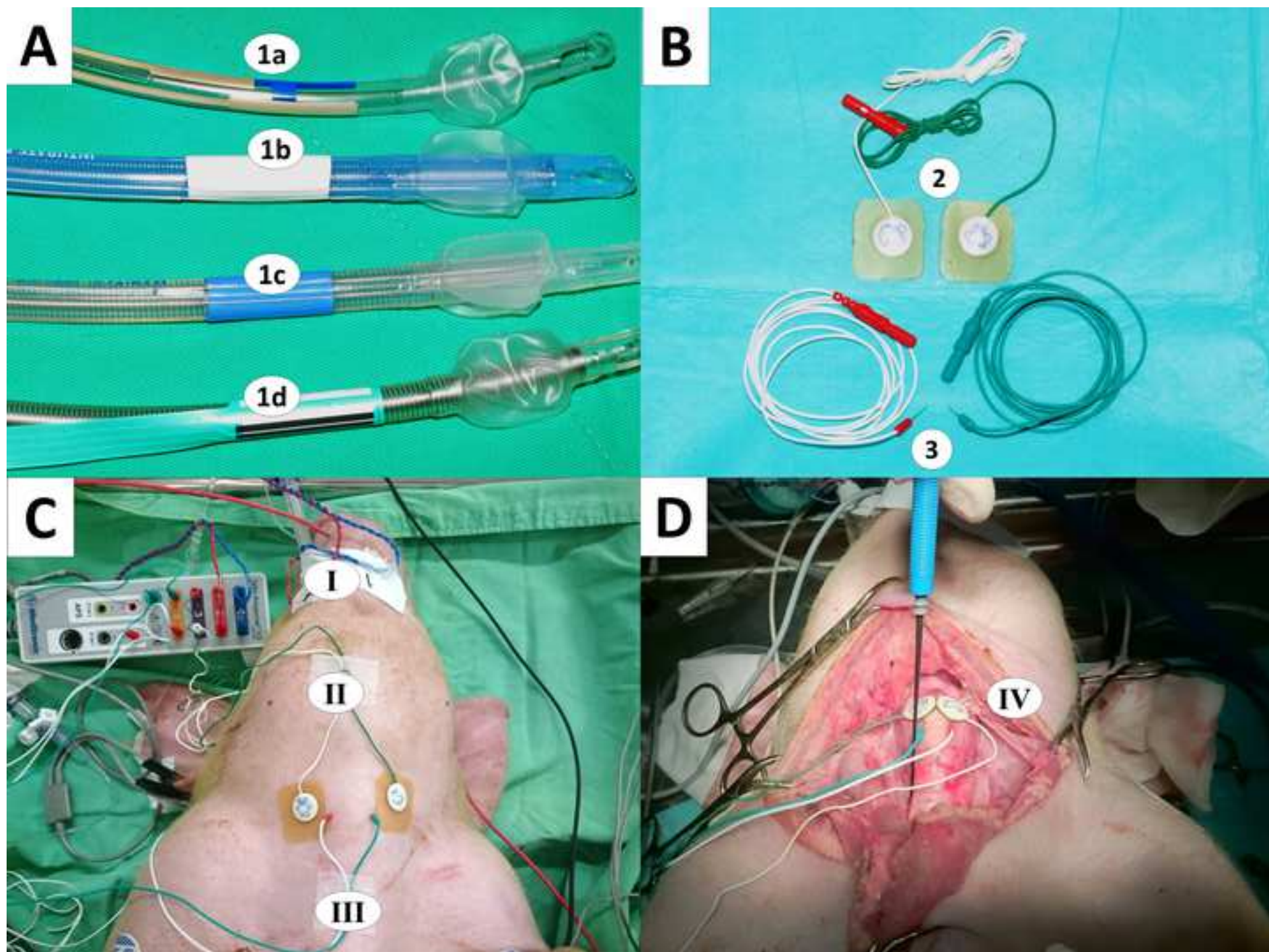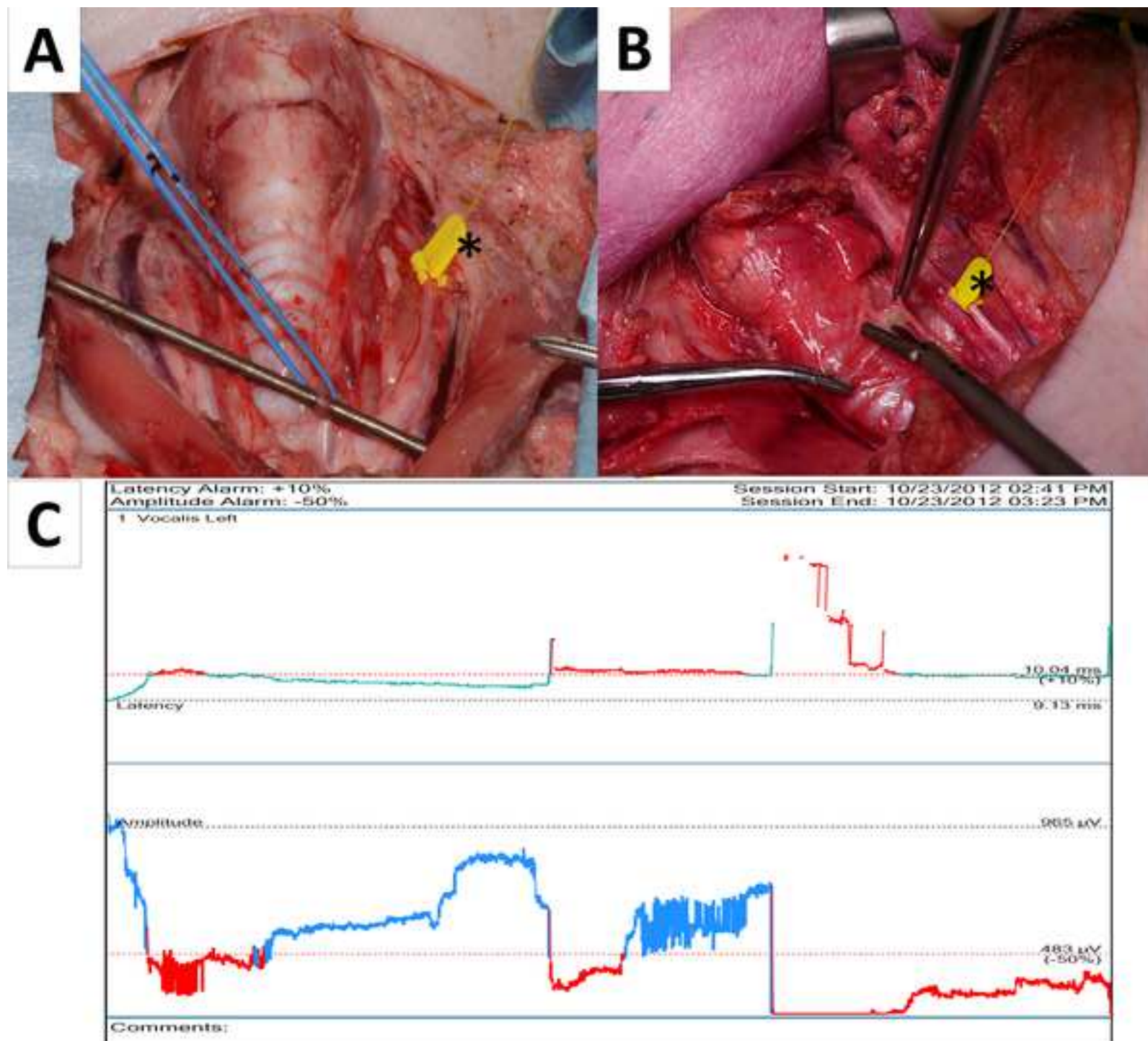655   *Laryngoscope.***126** (4), 1014-1019 (2016).
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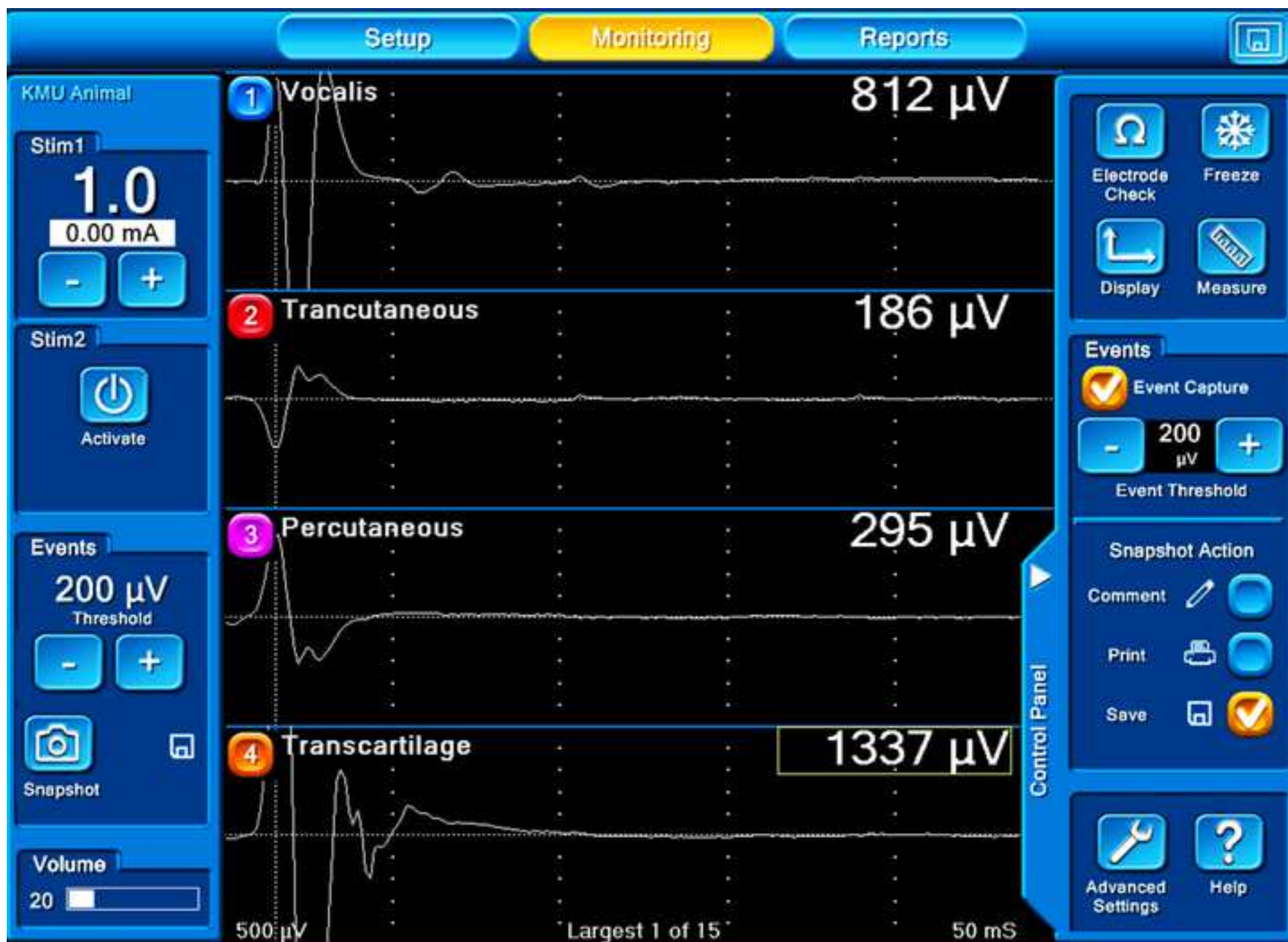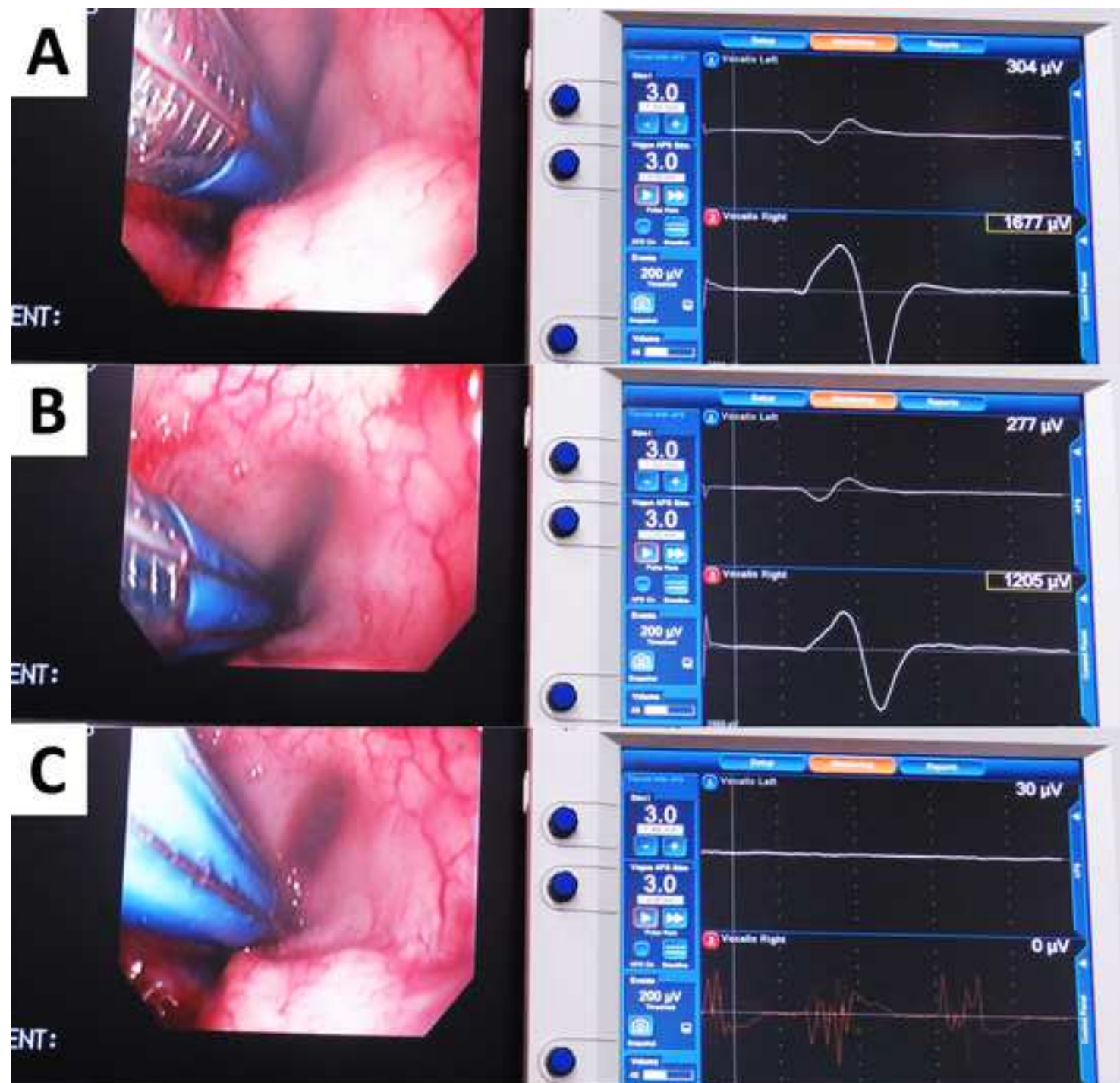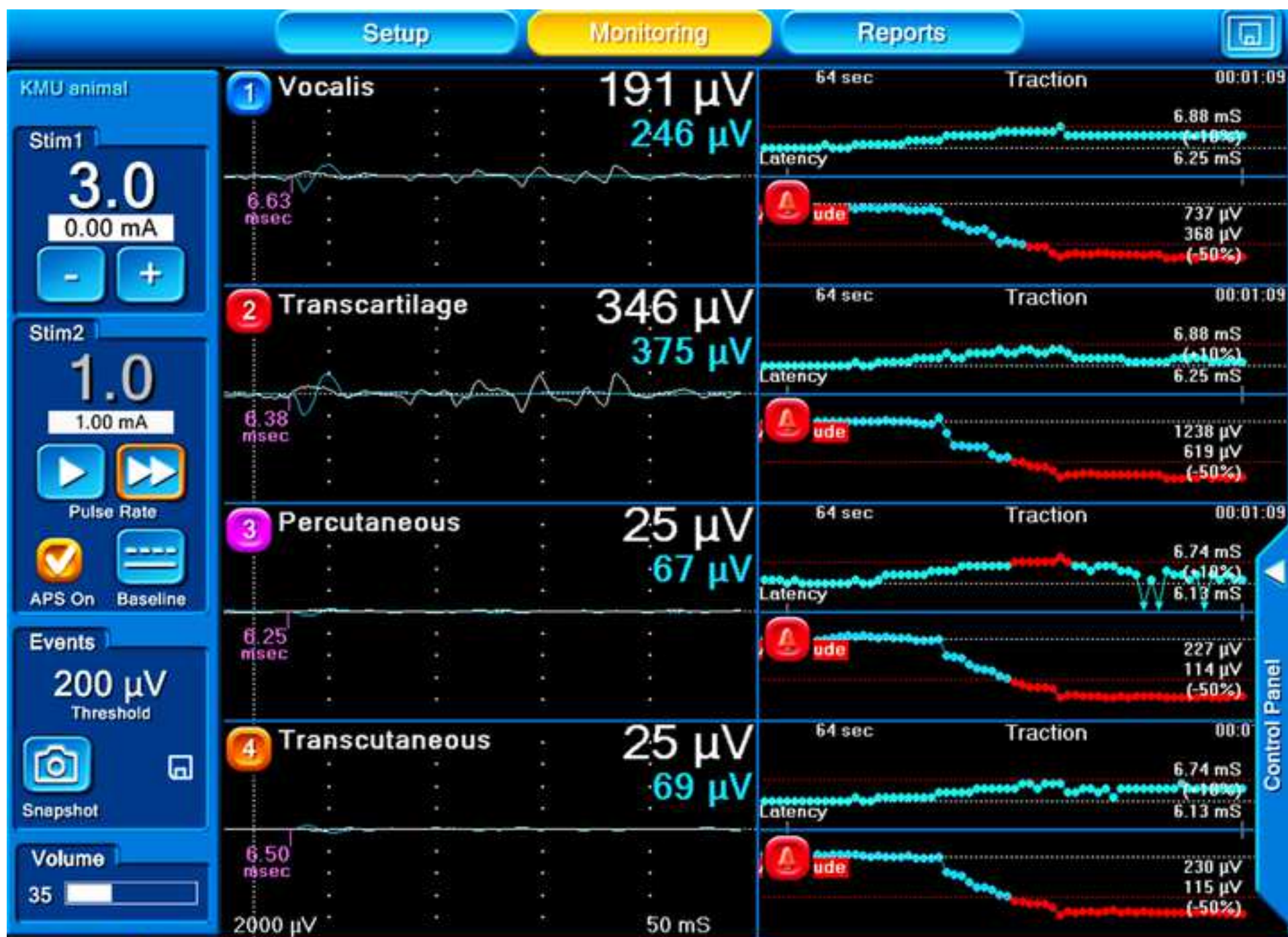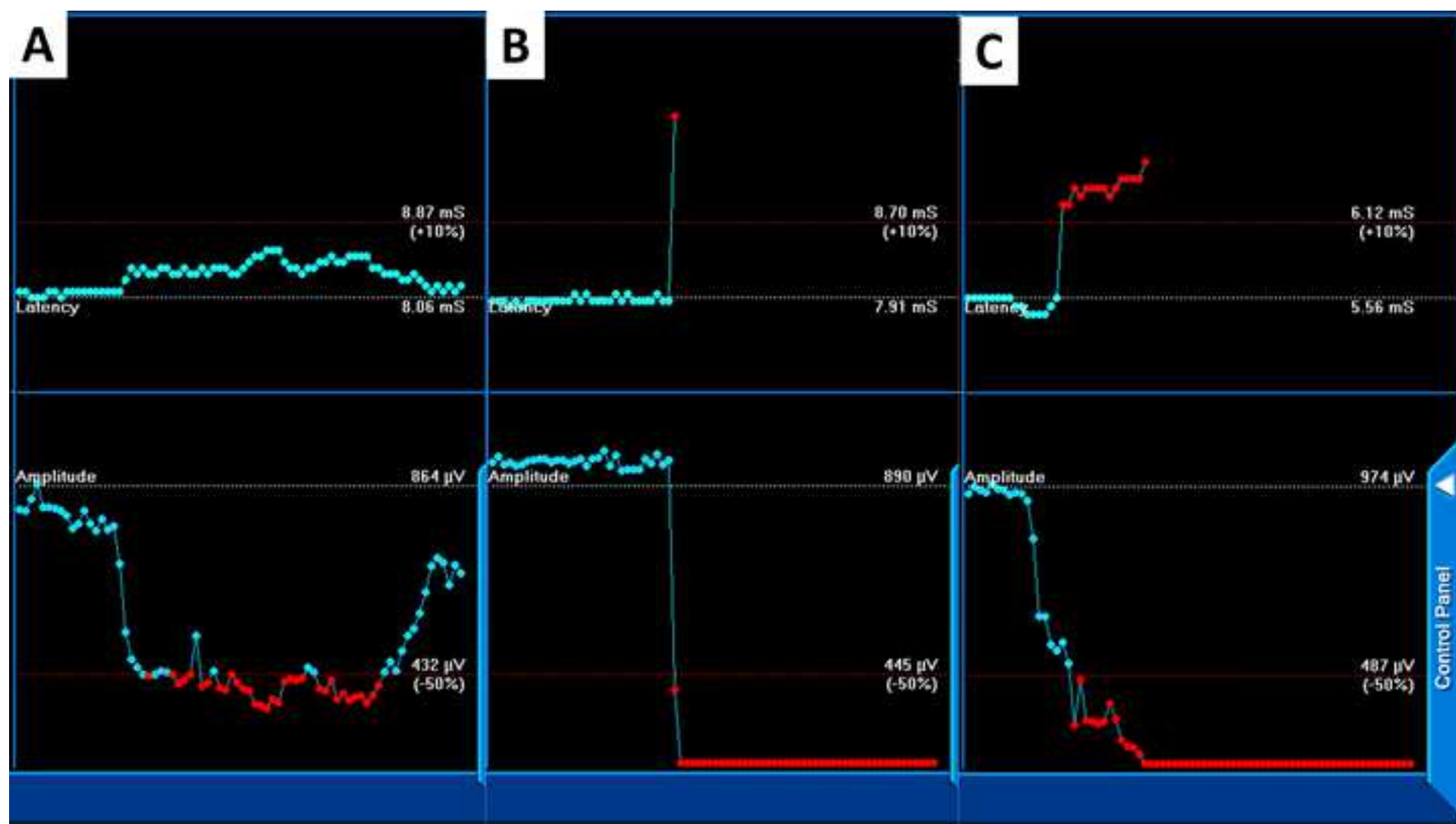688 *Laryngoscope.* **127** (1), E51-e52 (2017).
689

Figure1

Figure2

Figure3

(A)

(B)

(C)

Figure4

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Figure5

Figure6

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Figure7

Figure8

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Figure9

Table of Materials

Name of Material/ Equipment	Company
Criticare systems	nGenuity
Intraoperative NIM nerve monitoring systems	Medtronic
NIM TriVantage EMG Tube	Medtronic
NIM Contact Reinforced EMG Endotracheal Tube	Medtronic
NIM Standard Reinforced EMG Endotracheal Tube	Medtronic
NIM Flex EMG Endotracheal Tube	Medtronic
Standard Prass Flush-Tip Monopolar Stimulator Probe	Medtronic
Ball-Tip Monopolar Stimulator Probe	Medtronic
Yingling Flex Tip Monopolar Stimulator Probe	Medtronic
Prass Bipolar Stimulator Probe	Medtronic
Concentric Bipolar Stimulator Probe	Medtronic
Side-by-Side Bipolar Stimulator Probe	Medtronic
APS (Automatic Periodic Stimulation) Electrode*	Medtronic
Neotrode ECG Electrodes	ConMed
LigaSure Small Jaw	Medtronic

Catalog Number

8100E

NIM-Response 3.0

8229706

8229506

8229306

8229960

8225101

8225275/ 8225276

8225251

8225451

8225351

8225401

 8228052 / 8228053

1741C-003

LF1212

Comments/Description

physiologic monitoring, including capnography, electrocardiography (ECG) and monitoring of oxygenation (SaO2)

monitor EMG activity from multiple muscles. If there is a change in nerve function, the NIM system may provide audible and visual warnings to help reduce the risk of nerve damage.

6 mm ID, 8.2 mm OD. The NIM TriVantage EMG Tube is a standard size, non-reinforced, DEHP-free PVC tube that features smooth, conductive silver ink electrodes and a cross-band to guide placement.

6 mm ID, 9 mm OD. The NIM Contact EMG Tube continuously monitors electromyography (EMG) activity during surgery. An innovative design allows the tube to maintain contact,

6 mm ID, 8.8 mm OD. The NIM Standard EMG Tube continuously monitors electromyography (EMG) activity during surgery. Recording electrode leads are twisted pair. Packaged

6 mm. The NIM Flex EMG Tube monitors vocal cord and recurrent laryngeal nerve EMG activity during surgery. An updated, dual-channel design allows the tube to

Tips and Handles. For locating and mapping cranial nerves in the surgical field, the single-use Standard Prass Monopolar Stimulating Probe features a flush 0.5 mm tip

Tip and Handle, 1.0 mm/ 2.3mm. Featuring a flexible ball tip and flexible shaft, the single-use Ball-Tip Monopolar

Tips and Handles. The highly flexible single-use Yingling Monopolar Stimulating Probe allows stimulation in areas outside the surgeon's field of view. The platinum-iridium wire

The single-use Prass Bipolar Stimulating Probe features a slim, flexible tip that allows greater access to neural structures. The probe tip is 0.5 mm in distance

The single-use Concentric Bipolar Stimulating Probe features a 360° contact area. Insulation is complete to the active tip; cables and handles are

The single-use Side-by-Side Bipolar Stimulating Probe features probe tips that are 1.3 mm apart, allowing neural structures to be stimulated between the tips.

2 mm/ 3mm. The APS Electrode offers continuous, real-time monitoring. The electrode is placed on the nerve and can provide early warning of a change in nerve function.

The electrode is made of a clear tape material, which allows for continuous observation of the patient's skin during monitoring.

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electrothermal bipolar vessel sealing system for surgery

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Article Title: Porcine animal model for research of intra-operative neural monitoring in thyroid surgery

Signature: *Che-Wei Wu*

Date: 2018-01-28

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<u>**Cover letter**</u>

Editor, *The Journal of Visualized Experiments*

RE: Manuscript ID: JoVE57919R3 titled " Porcine Model in Intra-Operative Neural Monitoring in Thyroid Surgery "

Dear Editors,

   Thank you very much for reviewing our article and providing useful advice. We have tried our best to incorporate your comments into the revised manuscript and feel that the changes you suggested make our manuscript clearer and more informative for the readers. All the important changes have been highlighted in the revised manuscript. With these modifications, we look forward to your re-review for possible publication in *The Journal of Visualized Experiments*.

Sincerely,

I-Cheng Lu, MD, PhD (On behalf of all coauthors)
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June 12, 2018

The followings will respond to the points raised by the reviewer and the editorial

office:

Response to the Editoral

Comments:

1. The language in the manuscript is not publication grade. Please employ professional copy-editing services.

Response: Thank you very much for this important comment. The revised manuscript has been reviewed and edited by a BELS-certified technical editor and native English speaker. We will agree to pay additional charges for further copy-editing service by the journal for the accepted paper, if necessary.

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February 22, 2018

RE: Editing Certification

Dear Sir/Madam:

I am BELS-certified technical editor and native English speaker. This letter certifies that I edited the following manuscript, including language, grammar, punctuation, spelling, and style.
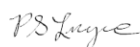
TITLE
Porcine model for research in intra-operative neural monitoring in thyroid surgery

AUTHORS & AFFILIATIONS
Che-Wei Wu[1], Tzu-Yen Huang[1], Hui-Chun Chen[2], Hsiu-Ya Chen[3], Tsung-Yi Tsai[1], Pi-Ying Chang[3], Yi-Chu Lin[1], Chiao-I Lin[1], Hsin-Yi Tseng[1], Pao-Chu Hun[4], Xiaoli Liu[5], Hui Sun[5], Gregory W. Randolph[5], Gianlorenzo Dionigi[6], Feng-Yu Chiang[1], and I-Cheng Lu[3]

The content and research findings were not changed in any way, and the authors reviewed the entire manuscript before its final submission. Please do not hesitate to contact me if you require further information.

Sincerely,

P S Lugue

Paul Steve Lugue
Technical Editor

2. Additional details are required in the protocol. Please see the comments in the attached manuscript.

Response:

Thank you very much. The protocol section has been revised accordingly.

3. The highlighting of the protocol is very discontinuous and does not tell a complete story. Please revise and ensure that only 2.75 page of protocol text is highlighted with the spaces and headers included.

Response:

Thank you very much. The highlighted protocol section has been revised as follows:

This study aims to develop a standard protocol of intra-operative neural monitoring of thyroid surgery in a porcine model. Here, we present a protocol to demonstrate general anesthesia, to compare different types of electrodes, and to investigate the electrophysiological characteristics of the normal and injured recurrent laryngeal nerves.

PROTOCOL-The animal experiments were approved by the IACUC of Kaohsiung Medical University, Taiwan.

1. Animal Preparation and Anesthesia

1.1. **Porcine animal model**

1.1.1. Use KHAPS Black or Duroc-Landrace pigs

1.2. **Anesthesia induction**

1.2.1. Pre-medicate with intramuscular azaperone (4 mg/kg) at 2 hours before anesthesia.

1.2.2. Induce general anesthesia with 2-4% sevoflurane at a fresh gas flow of 3 L/min *via* the face mask with the piglet in a prone position.

1.2.3. Identify a superficial vein on the outer side of one ear.

1.2.4. Administer intravenous anesthetic to alleviate noxious stimulation by direct laryngoscopy.

1.3. Tracheal intubation

1.3.1. Prepare the equipment required for EMG tube intubation: a face mask for assisted ventilation, two slings to hold the mouth open, one gauze strip to pull the tongue, a blunt tip suction catheter, a veterinary laryngoscope with straight blades, an elastic bougie, a syringe, a stethoscope, and adhesive tape.

1.3.2. Position the piglet in a prone position on the operating table.

1.3.3. apply traction of the upper and lower jaw to maintain an adequate mouth opening.

1.3.4. Hold the laryngoscope upside down and place it directly in the oral cavity to depress the tongue.

1.3.5. use the laryngoscope to press the epiglottis downward toward the tongue base.

1.3.6. When the vocal cords are clearly identified, gently advance the elastic bougie into the trachea. Next, advance the EMG tube at the mouth angle to a depth of 24 cm.

1.3.9 Use medical tape to fix the EMG tube at the mouth angle.

1.3.10. Connect the EMG tube to the ventilator.

1.4. Anesthesia maintenance

1.4.1. Maintain general anesthesia with 1-3% sevoflurane in oxygen at 2 L/min.

2. Equipment Setting and Animal Operation

2.1.1. Connect the channel leads from the EMG tube to the monitoring system.

2.1.2. Set the monitoring system to run 50 ms time window. Set pulsed stimuli to 100 µs and 4 Hz. Set the event capture threshold to 100 µV.

2.2.2. Use a transverse collar incision to expose the neck and the larynx.

2.2.3. Raise the subplatysmal flap.

2.2.4. Remove the strap muscles and visualize the tracheal rings and nerves.

2.2.5. Localize, identify, and carefully expose the EBSLN, RLN, and VN with a handheld stimulation probe.

2.2.6. Position an automated periodic stimulation electrode on one side of VN for continuous IONM.

2.3. At end of experiments, euthanize all piglets by the veterinarian.

3. Electrical Stimulation

3.1. Evaluate the baseline EMG responses of the target nerves.

3.2.3. During VN stimulation, evaluate hemodynamic stability by monitoring of heart rate, ECG, and invasive arterial blood pressure.

3.2.4. Evaluate nerve function integrity by comparing EMG responses proximal to the nerve stimulation site before and after each level of stimulation is applied.

3.3. Effect of anesthetics

3.3.1. Firstly, apply C-IONM and use the automatically calibrated baseline of EMG as control data.

3.3.2. Administer a bolus injection of rocuronium 0.3mg/kg and observe the real-time EMG changes.

3.3.3. Three minutes after injection, perform one injection of sugammadex 2mg/kg. Record the recovery profile of laryngeal EMG for 20 minutes.

3.4. Stimulation electrodes

3.4.1. To mimic direct stimulation of nerves during surgery, apply 1mA stimulation to the EBSLN, RLN, and VN without overlying fascia.

3.4.2. To mimic indirect mapping of the nerve position before visual identification during surgery, apply 1mA stimulation at a 1 and 2-mm distance away from the nerves at overlying fascia.

3.4.3. Record and compare the EMG responses between different types of stimulation electrodes.

3.5. Recording electrodes

3.5.1. evaluate how rotation or displacement of the EMG tube electrode affects the stability of the EMG signal. Additionally, compare the EMG responses between different electrode types and different recording approaches during IONM.

4. RLN injury study

4.2. Use C-IONM to confirm and compare patterns of real-time changes in evoked laryngeal EMG signals during and after acute RLN injuries with different injury mechanisms

4.4. Traction compression injury

Wrap a vascular loop around the RLN and apply retraction. This scheme mimics an RLN trapped at the region of Berry's ligament during medial traction.

4.5. Clamping injury

pinch the distal segment of the RLN with hemostatic forceps for one second. This scheme mimics the nerve being inadvertently clamped owing to visual misidentification as a vessel during the operation.

4.6. Thermal injury

4.6.2. Activation Study - To investigate how Energy-based devices (EBD) can be safely applied for hemostasis and dissection near the RLN during surgery.

4.6.2.1. Activate the EBD at 5-mm distance away from the RLN.

4.6.2.2. If EMG signals remain stable, perform a further test at the narrower distance.

4.6.3. Cooling Study- To evaluate the cooling time to determine postactivation optimal EBD cooling parameters.

4.6.3.1. Contact an activated EBD on the RLN directly after a 5 second cooling time.

4.6.3.2. If the EMG signals remain stable after three tests, test the shorter cooling time.