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TITLE:

The Chick Chorioallantoic Membrane *In Vivo* Model to Assess Perineural Invasion in Head and Neck Cancer

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SUMMARY:

Perineural invasion is an aggressive phenotype for head and neck squamous cell carcinomas and other tumors. The Chick chorioallantoic membrane model has been used for studying angiogenesis, cancer invasion, and metastasis. Here we demonstrate how this model can be utilized to assess perineural invasion *in vivo*.

ABSTRACT:

Perineural invasion is a cancer that surrounds or invades the nerves. It is associated with poor clinical outcome for head and neck squamous cell carcinoma and other cancers. Mechanistic studies have shown that the molecular crosstalk between nerves and tumor cells occurs prior to the physical interaction and invasion of nerves. There are only a few *in vivo* models to study perineural invasion, especially to investigate early progression, before physical nerve-tumor interactions occur. The chick chorioallantoic membrane model has been used to study cancer invasion because the basement membrane of the chorionic epithelium mimics that of human epithelial tissue. Here we repurposed the chick chorioallantoic membrane model to recapitulate perineural invasion, grafting rat dorsal root ganglia and human head and neck squamous cell carcinoma cells onto the chorionic epithelium. We have demonstrated how this model can be useful to evaluate the ability of cancer cells to invade neural tissue *in vivo*.

INTRODUCTION:

Perineural invasion (PNI) is an understudied phenotype in cancer, which is associated with high disease recurrence and poor survival in patients with head and neck squamous cell carcinoma (HNC)[1]. PNI is defined microscopically as tumor cells within or surrounding the nerves[2,3]. When PNI is detected, patients are likely to receive adjuvant therapies such as elective neck dissection and/or radiation therapy[4,5]. However, these therapies are aggressive, and not PNI-specific. In fact, there is no therapy to block PNI, primarily because the mechanisms underlying nerve-tumor interactions are still poorly understood.

Different molecular mechanisms have been implicated in nerve-tumor attraction; tumors and stromal cells release neuropeptides and growth factors to promote neuritogenesis[6,7]. When cultured together *in vitro*, HNC cells and dorsal root ganglia (DRG) both have a robust response; effects on tumor cell invasion and neuritogenesis can be seen after a few days in culture[6,8,9]. However, there is a lack of appropriate *in vivo* models to recapitulate tumor-nerve interactions prior to the invasion. Here we present an *in vivo* PNI model that we developed to study early interactions between HNC cells and nerves[6]. We adapted the chick chorioallantoic membrane (CAM) model to include a neural component, grafting a DRG in the CAM, followed by a graft of cancer cells to mimic an innervated tumor microenvironment.

The CAM model has been successfully used to assess the invasion of cells through the basement membrane, mimicking early invasive stages of carcinomas and melanoma[10-12]. The CAM is comprised of an upper chorionic epithelium, intervening mesenchyme, and lower allantoic epithelium. The chorionic epithelium is structurally similar to human epithelium[10,13] in that the collagen-IV-rich basement membrane simulates the basement membrane that separates the oral epithelium from the underlying connective tissue. Since the first tumor grafts were performed in the CAM in 1913[14], many adaptations of the method were developed to allow for the assessment of angiogenesis[15-17], tumor progression, and metastasis[18]. Importantly, the technique of grafting tumors onto the CAM has changed very little, but the applications are continuously evolving. Assays of increasing complexity have been published, including drug screening[19], bone tissue engineering[20], and nanoparticle-based anticancer drugs[21].

Our laboratory developed a CAM-DRG model in which a mammalian DRG is isolated and grafted onto the surface of the upper CAM. After the DRG becomes incorporated in the CAM, HNC cells are grafted near the DRG and allowed to interact with the DRG before the entire *in vivo* system is harvested and analyzed. Importantly, the system allows *ex-vivo* visual observation of both the DRG and the tumor by fluorescence labeling. This protocol comprises multiple steps with different levels of complexity performed within 17 days, from incubating eggs to harvesting the CAM (**Figure 1**). Cells expressing different proteins of interest can be tested in this model to elucidate the molecular pathways responsible for nerve invasion in cancer, and also for screening drugs to directly target neural invasion. Cells pre-treated with a candidate drug can be grafted on the CAM and the occurrence of PNI measured in comparison to untreated controls. In fact, the CAM model has been used for drug screening as an intermediate step between *in vitro* studies and pre-clinical *in vivo* trials in rodents[19].

The experimental design will vary with the hypothesis. For instance, if testing the role of a specific

protein on PNI, the experimental group would include DRG grafted with tumor cells overexpressing the protein, while the control group should include DRG with cells stably transfected with empty vector. If assessing the role of the nerves on metastasis, the control group should not include the nerve component. Several different experimental designs can be used to address specific questions.

PROTOCOL:

All experiments using rats in this protocol are done in accordance with IACUC (Institutional Animal Care and Use Committee) rules from our institution. Experiments with chicken embryos in this study do not require IACUC approval because the embryos are euthanized before hatching.

1. Egg Incubation (estimated timing: 5 min, day zero)

1.1 Get pathogen-free fertilized commercial Lohmann White Leghorn eggs, preferably on the first day post-fertilization. Incubate six eggs per experimental group in an egg humidifying incubator at 38 °C and 60% humidity for 8 days with hourly rotation. Use a regular rotation of the eggs to prevent the embryo from sticking to the egg membranes.

NOTE: Before incubation, keep the eggs in an 18 °C refrigerator to halt embryo development for a maximum of 1 week.

2. Harvesting and preparation of DRGs (estimated timing: 2 h 30 min, day 8)

NOTE: Experiments with mice and rats require approval from the Institutional Animal Care & Use Committee (IACUC). In some countries, the use of fertilized chicken eggs also requires approval.

2.1 Get six to seven week-old Sprague Dawley rats (~200 g in weight) to extract DRGs.

NOTE: One rat should yield ~40 cervical and thoracic DRGs. Mouse DRG also integrates in the CAM, however, the conditions for this species need to be optimized independently.

2.2. In a laminar flow cabinet, harvest DRG from cervical and thoracic regions following the protocol for mouse DRG extraction published elsewhere[22]. Follow **Figure 2** for orientation on how to harvest DRGs.

2.2.1. Euthanize the rat by exsanguination under anesthesia from Ketamine (40-90 mg/Kg)/ Xylazine (10 mg/Kg) intraperitoneally injected. Clean the rat skin with 70 % ethanol and remove the rat spine using a scissor. Do not perform cervical dislocation because this would damage the cervical DRGs.

2.2.2. Separate the cervical, thoracic and lumbar regions with the same scissors, following the schematic anatomic representation and gross images provided in **Figure 2A-D**. Place the spine sections in a 10 cm culture dish with 1x PBS to keep tissues wet.

2.2.3. With a delicate bone scissor, open the vertebral bones in the dorsal and ventral aspects, separating the spine in two lateral halves (**Figure 2E-F**). Place the tissue sections in a clean 10 cm dish with fresh 1x PBS.

2.2.4. Using forceps, gently detach the spinal cord from the vertebral bones to visualize the DRGs (**Figure 2G**).

2.2.5. With fine forceps held underneath each DRG, grasp it and pull it out from the bone cavity in which it is lodged. Do not hold the DRG directly because this will cause tissue damage. Do not trim the axon bundles from the DRG (**Figure 2H**).

NOTE: Avoid using lumbar DRGs since these have reduced integration in the CAM. For DRG region location, follow the schematic illustration and gross anatomic images on **Figure 2A-D**.

2.3. Immediately after harvesting, place each DRG into DMEM culture medium supplemented with 2% Pen/Strep and 10% FBS to help prevent bacterial contamination of the DRGs. Group all DRGs in the same 6 cm culture dish with 4 mL of culture medium. Incubate DRGs for 1 h in the cell culture incubator at 37 °C. During this time, prepare the eggs to receive the DRG as described below (step 3).

2.4. When 2% Pen/Strep prophylactic antibiotic treatment is complete, transfer DRGs to a new culture dish with DMEM culture medium supplemented with 1% Pen/Strep plus 10% FBS and containing 1.25 µg/mL of red fluorescent dye. Incubate for 1 h in the cell culture incubator.

NOTE: The total 2 h interval between harvesting DRGs and completion of fluorescence labeling should be sufficient to prepare the eggs; avoid keeping DRGs for extended periods of time in the incubator.

3. Preparation of eggs for DRG grafting (estimated timing: 1 h for a dozen eggs, day 8)

3.1. In a laminar flow cabinet, dim the light and transilluminate the eggs to check for viability and embryonic phase. Exclude eggs with poor vasculature, non-fertilized eggs, or eggs not consistent with day 8 post-fertilization. Hold the egg gently with the naturally-occurring air sac toward the light source (**Figure 3A**).

3.2. With a pencil, mark the egg shell to receive the openings (**Figure 3A-B**).

3.2.1. First, identify the attachment of the developing embryo to the CAM as a dark moving vessel attached to the egg membrane and mark this area to avoid interventions in this region.

3.2.2. Second, choose the operating window area as a well vascularized area at least 2 cm from the embryo attachment and draw a 1.5 cm diameter circle. Approximately 1 cm from the operating window, draw a 0.5 cm square in a less vascularized area.

3.2.3. Third, draw the air sac region to exclude it from the operation area. Mark the middle of the air sac with a cross.

3.3. With a rotary tool and engraving drill, 3 mm in diameter, drill the egg shell in the marked square (**Figure 3C**). Use blunt forceps to remove the egg shell without removing the outer egg shell membrane (the white membrane right under the shell) (**Figure 3D**). Work carefully to avoid accidental perforation of this membrane.

3.4. Using the same drill as in step 3.3, make a pinpoint drill perforation in the marked cross in the air sac area to allow air flow into the egg (**Figure 3E**). Be careful not to apply too much pressure to the egg, to avoid breaking or damaging it.

3.5. Place 30 µL of HBSS in the square opening, over the intact outer egg shell membrane (**Figure 3E**). With a 30-G syringe needle, make a pinpoint perforation in the outer membrane in this square area (**Figure 3F**).

3.6. Place the egg in the light source to visualize the air sac. Apply pressure to an eyedropper rubber bulb and place it in the small perforation made in the air sac area (step 3.4). Release pressure in the bulb until you see the separation of the two membranes in the open window area (**Figure 3G**); repeat this step as many times as you need to achieve complete separation of the membranes in the operating window area.

3.7. Repeat steps 3.1-3.6 for all eggs.

3.8. Using the same drill as in step 3.3, drill the circular operating window being careful not to rupture the outer egg shell membrane (**Figure 3H**). Clean the egg surface by gently sticking an adhesive tape to remove all loose particles.

3.9. With blunt forceps, remove the egg shell from the drilled area (**Figure 3I-J**). Next, with the same forceps, remove the outer egg shell membrane (**Figure 3K**), being careful not to introduce small shell particles inside the egg to minimize contamination.

3.10. Identify the CAM approximately at 1 cm depth from the egg surface. Cover each opened egg temporarily with paraffin wax membrane to avoid contamination (**Figure 3L**).

3.11. Repeat steps 3.8-3.10 for all the eggs. Place the eggs back in the egg incubator without rotation until the DRGs are ready for grafting.

4. Grafting of DRG on the CAM (estimated timing: 40 min, day 8)

4.1. Prepare a 6 cm culture dish with HBSS medium, to wash the DRGs before implantation. Bring the prepared eggs to the cell culture laminar flow cabinet. Remove the paraffin membrane from the egg (**Figure 4A**).

4.2. With fine sterile forceps, gently grasp one DRG from inside the culture medium. Dip it into the HBSS medium to remove the excess medium that contains the fluorescent dye. Hold the DRG very gently; otherwise, it will stick to the forceps.

4.3. Place the DRG on the CAM, being careful not to puncture the membrane (**Figure 4B-C**). If necessary, use another pair of forceps to help detach the DRG from the tip of the forceps when placing it on the CAM.

NOTE: Keeping the DRG wet with HBSS medium will also facilitate detachment from the forceps.

4.4. Cover the egg with a sterile transparent film dressing. Cover all the windows and punctures made in the egg shell to avoid bacterial contamination (**Figure 4D**).

4.5. After grafting DRGs in all eggs, incubate the eggs in a humidifying incubator at 38 °C and 60% humidity for 2 days, without rotation.

5. Grafting of tumor cells on the CAM (estimated timing: 1 h 30 min, day 10)

5.1. At 48 h before grafting the cells, plate the cells needed in culture plates. Calculate 0.5 to 1 x 10^6 cells per egg for UM-SCC-1 cells in order to generate three dimensional tumors in the CAM. Be aware that cell number may vary by the cell line.

5.2. Aspirate medium and add DMEM culture medium supplemented with 1% Pen/Strep plus 10% FBS and 2.5 µg/mL of green fluorescent dye. Incubate for 1 h at 37 °C in the cell culture incubator. Then, check fluorescence intensity on the microscope, aspirate medium, wash once with 1x PBS, and add 0.25% trypsin for up to 10 min. Neutralize trypsin with DMEM supplemented medium.

5.3. Centrifuge at 250 x g for 4 min to form a cell pellet. Aspirate DMEM medium and re-suspend in HBSS to wash the excess fluorescent dye. Count cells using a hemocytometer.

5.4. Bring the eggs to the cell culture laminar flow cabinet. With scissors and forceps, open the transparent film dressing that covers the egg (**Figure 4E-F**).

5.5. Centrifuge the calculated number of cells again, aspirate the HBSS medium and re-suspend at a final concentration of 0.5 or 1x10^6 cells per 5 µL of same medium (**Figure 4G**). Prepare the amount needed for the total number of eggs (5 µL of cell suspension per egg).

5.6. Place 5 µL of cell solution onto the CAM, about 2 mm from the DRG (**Figure 4H**). Keep uniform distances between the DRG and cells. Be very careful not to disturb the egg to minimize spreading of the cells.

NOTE: To start cell implantation, choose eggs on which the CAM surface is visually dry. If the

surface is too wet, cells can spread and do not form regular tumors.

5.7. Cover the egg with a new film dressing as in step 4.5. Graft the cells in all eggs. Incubate the eggs in a humidifying incubator at 38 ^{o}C and 60 % humidity for 7 days, without rotation.

6. CAM harvesting (estimated timing: 1 h for dozen eggs, day 17)

6.1. Prepare 6-well plates with 4% PFA (paraformaldehyde) pH7.0, one well per egg, 2 mL per well.

6.2. Bring the eggs to a laboratory bench. Using a needle attached to a syringe to perforate the film dressing, drop around 300 µL of PFA over the CAM to slightly stiffen the CAM, thus facilitating the harvesting process. Repeat this for all eggs.

6.3. With a scissor, remove the upper half of the egg shell (where the operating window is located) with the CAM attached to it (**Figure 4I**). Reduce the size of this half to approximately 3 cm in diameter, keeping the portion of the CAM where DRG and tumor cells were grafted in the center (**Figure 4J**). Grasp the CAM with fine forceps and detach it from the egg shell while placing it into the PFA. Orient the DRG and cancer cells facing upwards (**Figure 4K-L**).

NOTE: The 3 cm area should include both the DRG and cells. The DRG is easily seen as a small lump attached to the CAM, however tumor cells are sometimes difficult to identify on the gross exam.

6.4. Alternatively, with a scissor, widen the operating window removing the egg shell from the top of the egg while keeping the CAM in place; remove approximately 1 cm beyond the operating window (**Figure 4M**) and identify the DRG and cells on the CAM (**Figure 4N-O**). With delicate fine forceps, hold the CAM at one of the edges and lift it gently. With sharp delicate scissors, gently cut the CAM to remove a circular area, approximately 3 cm in diameter (**Figure 4P**), and place the CAM in PFA with DRG and cancer cells facing upwards.

NOTE: Avoid stretching or holding the CAM with forceps in multiple places to minimize tissue damage that may generate microscopy artifacts.

6.5. Place each CAM membrane in one well (**Figure 4L**). Gently grasp the edges of the CAM to spread the tissue open in the PFA or gently shake the plate until the CAM is unfolded, to avoid fold artifacts.

6.6. Euthanize the embryo (day 17) by quick decapitation. Fix the harvested tissues in PFA for 4 h at room temperature. After fixation, replace PFA with 1x PBS and store tissues in PBS at 4 °C until embedding in paraffin for sectioning. Avoid over-fixation that will damage the delicate vasculature of the CAM.

REPRESENTATIVE RESULTS

When optimized, this method has near 100% embryo survival and DRG integration in the CAM. Representative results of DRG integration are shown in **Figure 5A-B**. The integration of DRG in the CAM is important since it provides viability to the DRG tissue during the experiment. Microscopically, the DRG is seen within the connective tissue of the CAM (H&E stain). Blood vessels are often seen inside the DRG tissue, suggesting that the CAM blood supply is nurturing the grafted tissue. Implanted tumors are also identified on the CAM by H&E; depending on how much invasion is present, tumors might present with none to numerous tumor islands invading the connective tissue (**Figure 5C-D**). The representative **Figure 5E-F** shows the harvested CAM on brightfield imaging and merged fluorescence. UM-SCC-1 cells overexpressing Galanin receptor 2 presented increased invasion of the DRG in comparison to control cells (**Figure 5G-H**). Cancer-DRG interaction is observed as cancer cells presenting directional invasion toward the DRG (**Figure 5H**).

Data analysis is performed in different ways. The directional invasion of cancer cells toward the DRG is observed as a dichotomous variable and the number of eggs presenting this pattern of invasion is counted in each group. Statistical differences between groups are calculated using a binomial test of proportions. The proximity between cancer cells and DRG, and tumor area are measured using ImageJ[6] and differences between groups is assessed using Student`s t-test. To assure accuracy with ImageJ analysis, all the images from the same experiment should be taken on equal light and exposure settings. After adjusting image threshold and brightness of all images using the same criteria, the analyze particles tool is used to measure tumor area and the linear measurement tool measures tumor-DRG distances. It is important to use the constant setup of the size of particles analyzed for all images across different groups. In some instances, tumors grow thicker and can be manually measured with a digital caliper, allowing for volume measurement.

Using sections of paraffin-embedded CAM tissue, H&E stain or immunohistochemistry for epithelial cells (Cytokeratin antibody reactive for human species) can be performed, allowing for assessment of invasion within the connective tissue. Invasion is quantified as the number of tumor islands in the connective tissue per egg. Immunofluorescence for collagen IV can be used to verify invasion of cancer cells through the basement membrane. Also, if using GFP-labeled cancer cells, identification of these cells in the tissue sections is facilitated without the need for performing a Cytokeratin stain. Metastasis and angiogenesis analysis in CAM experiments are discussed elsewhere[10,17].

FIGURE LEGENDS:

Figure 1: Experiment timeline including the major steps on days 0, 8, 10 and 17.

Figure 2: DRG extraction on day 8. A. Rat schematics illustrating the anatomical location of the spine. **B.** Diagram of the rat vertebra configuration showing different body regions; green for cervical, dark blue for thoracic, orange for lumbar and light blue for sacral vertebra. **C-D.** Ventral aspect of the rat spine after surgical excision; separation of the regions as illustrated in **B**. **E.**

dissection of the vertebra to open the spinal cord canal, separating the vertebral body into two lateral sections containing the DRG. The section should cut through the dorsal and ventral aspect of each vertebral bone at the midline. **F.** Gross aspect of the opened thoracic spine. **G.** After the spinal cord is displaced, DRG are easily visible in the vertebral canals (arrow heads pointing 3 DRGs). **H.** Stereomicroscopic image of one DRG (arrow) with the corresponding axon bundles (arrow head). Scale bars: **C**, **D**, **F**, and **G**, 1 cm; **H**, 1 mm.

Figure 3: Preparation of the eggs on day 8. A-B. Identification of egg vasculature and markings prior to the procedure. Arrows on A point to the naturally-occurring air sac. **C-D.** Drilling and opening of the egg shell on the square opening mark. The arrow on **D** points to the intact outer egg shell membrane after removing the shell with the help of blunt forceps. **E.** The marked cross on the air sac is perforated with the drill to allow the flow of air into the egg (arrow head). 30 µL of HBSS medium is placed onto the outer egg shell membrane on the square opening. **F.** With a fine syringe needle, the outer egg shell membrane is perforated where the water was previously placed. **G.** Pressure is applied to a rubber eyedropper bulb while attaching it to the perforation drilled on the air sac. When finger pressure is released, water is vacuumed, generating an artificial air sac (white arrows) that should extend to the operating window. **H.** The edges of the operating window are drilled in an almost parallel position to the egg shell, to avoid accidental perforation. **I-J.** Removal of the egg shell with blunt forceps. **K.** Remove the outer egg shell membrane with blunt forceps, being careful not to introduce particles on the CAM (observed at ~1 cm below the surface). **L.** Eggs are covered temporarily with a paraffin wax membrane and put back in the incubator.

Figure 4: Grafting of DRG, cells, and harvesting of CAM: On day 8: A. CAM easily observed after paraffin wax membrane removal. **B-C.** With fine forceps, DRG is placed onto the CAM. **D.** Egg is covered with film dressing and put in the incubator; arrows point to the openings that are covered. **On day 10: E-F.** Film dressing is removed and DRG is located (arrow head on **F**). **G-H.** 5 µL of cell solution is dropped onto the CAM at a ~2 mm distance from the DRG. **On day 17: I-L** and **M-P** demonstrate two different approaches used to harvest the CAM. **I.** Egg shell is opened with a fine scissor starting on the air sac drilled perforation until the upper half of the egg is removed. **J.** Egg shell with the CAM is reduced in size to approximately 3 cm. **K-L.** With fine forceps, CAM is detached from the egg shell and placed in PFA. **M-O.** Widening of the operating window is performed to visualize the DRG and cancer cells on the CAM. Arrowhead points to the tumor and arrow points to the DRG. **P.** The CAM is grasped with fine forceps, cut out with a sharp scissors, and placed in PFA as shown in **L**.

Figure 5: Representative results. A. H&E section showing the integration of the DRG in the CAM; arrowhead indicates DRG. **B.** Higher magnification of **A**; arrow shows CAM blood vessels in the DRG and arrow heads show a DRG axon bundle. **C**. UM-SCC-1 cells grafted onto the CAM and harvested on day 14, four days after grafting (H&E stain). **D.** Higher magnification of **C** showing invasive tumor islands in the CAM connective tissue (arrows). **E.** Gross stereomicroscopic image of the CAM grafted with UM-SCC-1-GALR2 cells and rat DRG, harvested on day 17. **F.** Merged fluorescence and brightfield images highlighting the DRG labeled in red and cancer cells labeled in green. **G-H.** Fluorescence stereomicroscopy of the CAM grafted with DRG and UM-SCC-1-

GALR2 versus control cells, illustrating directional invasion of UM-SCC-1-GALR2 cells to the DRG (**H**). Scale bars: **A-D,** 500 µm; **E-H**, 2 mm.

Table 1: Troubleshooting table

DISCUSSION:

The CAM-PNI *in vivo* model presented here addresses the deficits of previous models by demonstrating nerve-tumor interaction before the physical invasion of the nerve by tumor cells. Most *in vivo* studies of PNI focus on tumor spread and inhibition of motor function, and depend upon direct injection of tumor cells into sciatic nerves[23-25]. The sciatic nerve injection is an *in vivo* model of PNI where cancer cells are injected into a mouse or rat sciatic nerve where the tumor subsequently grows. Injection models are useful to show destructive tumor progression and pain resulting from tumor cells within nerves. The sciatic nerve model is also suitable for the study of factors that allow cancer cells to thrive in the nerve but lacks the ability to evaluate the early phase of PNI, because it introduces cells directly into the nerve, bypassing nerve sheaths. In a different approach, surgically implanted orthotopic tumor grafts were used to characterize the importance of adrenergic and cholinergic nerve fibers in promoting prostate cancer progression, thus suggesting a prominent role of nerves in tumor progression[26]. This model consisted of chemical ablation of murine sympathetic and parasympathetic nerves. The parasympathetic fibers infiltrated tumor tissues, a process related to PNI, but the model was not specifically used to assess physical interactions between the nerve and tumor, failing to replicate early steps of PNI. The CAM-DRG model allows investigation of interactions between the nerve and cancer during PNI. Furthermore, murine models are expensive and time-consuming when compared to the CAM model. We advocate using the CAM-DRG model for mechanistic studies of PNI.

Some advantages to the CAM-DRG approach include assessment of PNI and other phenotypes, such as tumor growth, metastasis, and angiogenesis. Identification of human DNA on the lower CAM and/or in the liver and lungs can be used for detection of metastasis of human cancer cell lines[10], a more sensitive experimental approach compared to tissue sectioning and staining, which may not reveal small metastases.

The CAM-DRG method has some limitations, including the short observation time frame. The immune system of the embryo is physiologically active by day 18[27], when rejection and an inflammatory process may take place, limiting the experimental time. It is also important to consider the distance when grafting tumor cells close to the DRG; larger DRG-cancer distances might impair the molecular interactions between tumor cells and nerve, or could delay the physical contact between both components of the model. Also, if the embryos are older than stipulated in this protocol, embryo movements might displace the tumor cells. Therefore, it is important to have multiple replicates and compare control and test groups.

Since the immune system is not fully developed before day 18[27], the tumor microenvironment in the CAM is similar to that of the immunosuppressed murine models often used for cancer studies. Therefore, this model is not useful to assess the role of immune cells in tumor progression.

Another limitation is the restricted availability of reagents for chicken species, such as antibodies, cytokines, and primers.

Accurately performing this protocol requires practice; however, it can be done by a laboratory member without the need for a specialized core facility. Drilling of the egg shell requires training. Practicing on grocery (non-fertilized) eggs is recommended before attempting this model for the first time. High embryonic survival and success of the model can be achieved if some critical steps to avoid infection are followed: appropriate antibiotic prophylaxis of DRGs in 2 % Pen/Strep, working in a laminar flow cabinet, and avoiding dispersion of egg shell particles onto the CAM. It is also crucial to keep stable humidity during the total egg incubation time. We recommend increasing the number of eggs per group until the technique is mastered. The most frequent problems for inexperienced laboratory personnel are egg contamination and inaccurate technique for cell implantation.

DRG extraction also requires training; practice harvesting DRGs for *in vitro* experiments[8] before attempting the *in vivo* model is recommended. The *in vitro* DRG culture is an opportunity to optimize conditions and improve technique to shorten the duration of DRG extraction. Special attention to extraction technique is required when grasping the DRG with forceps. The DRG should not be held directly; pressure should be applied underneath it. We recommend the use of the magnifying lens to better visualize the DRG during extraction. For some critical steps in this protocol, a troubleshooting table is provided (**Table 1**).

Importantly, when performing this model for the first time, all conditions should be optimized for the desired cell line. This model was optimized for rat DRG and the HNC cell lines UM-SCC-1, UM-SCC-22B, and UM-SCC-81B. The use of mouse DRG and other cancer cell types may require optimization. With a higher concentration of grafted cells, tumors tend to grow thicker and stiffer, which facilitates tumor measurements. Taking into consideration multiple eggs for each group and an appropriate concentration of cells for each egg, several million cells may be required for each experiment. To facilitate planning, knowledge of the doubling time of the cells should be taken into consideration.

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DISCLOSURES:
The authors declare no competing interests.

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Figure 1

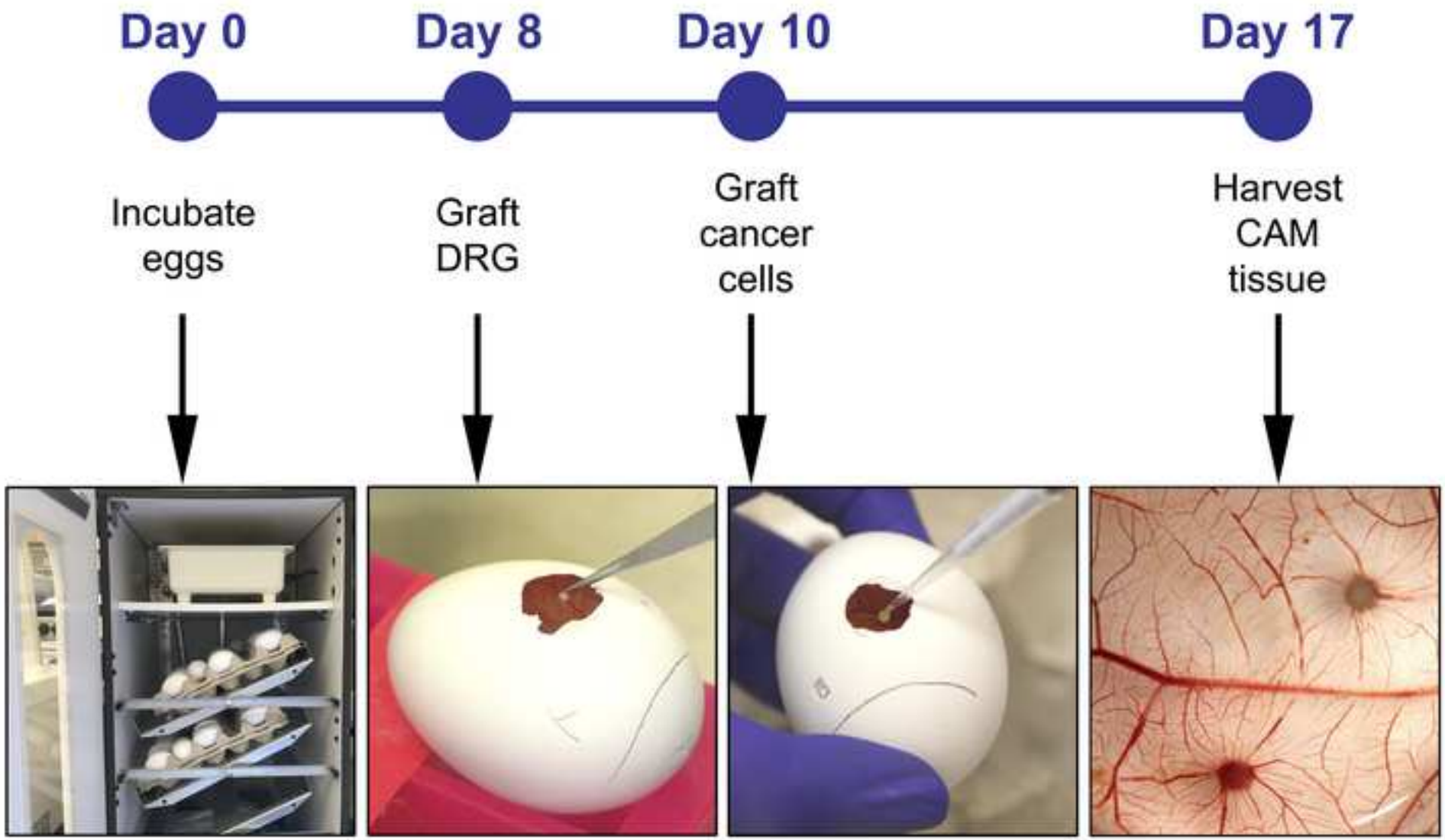

Figure 2

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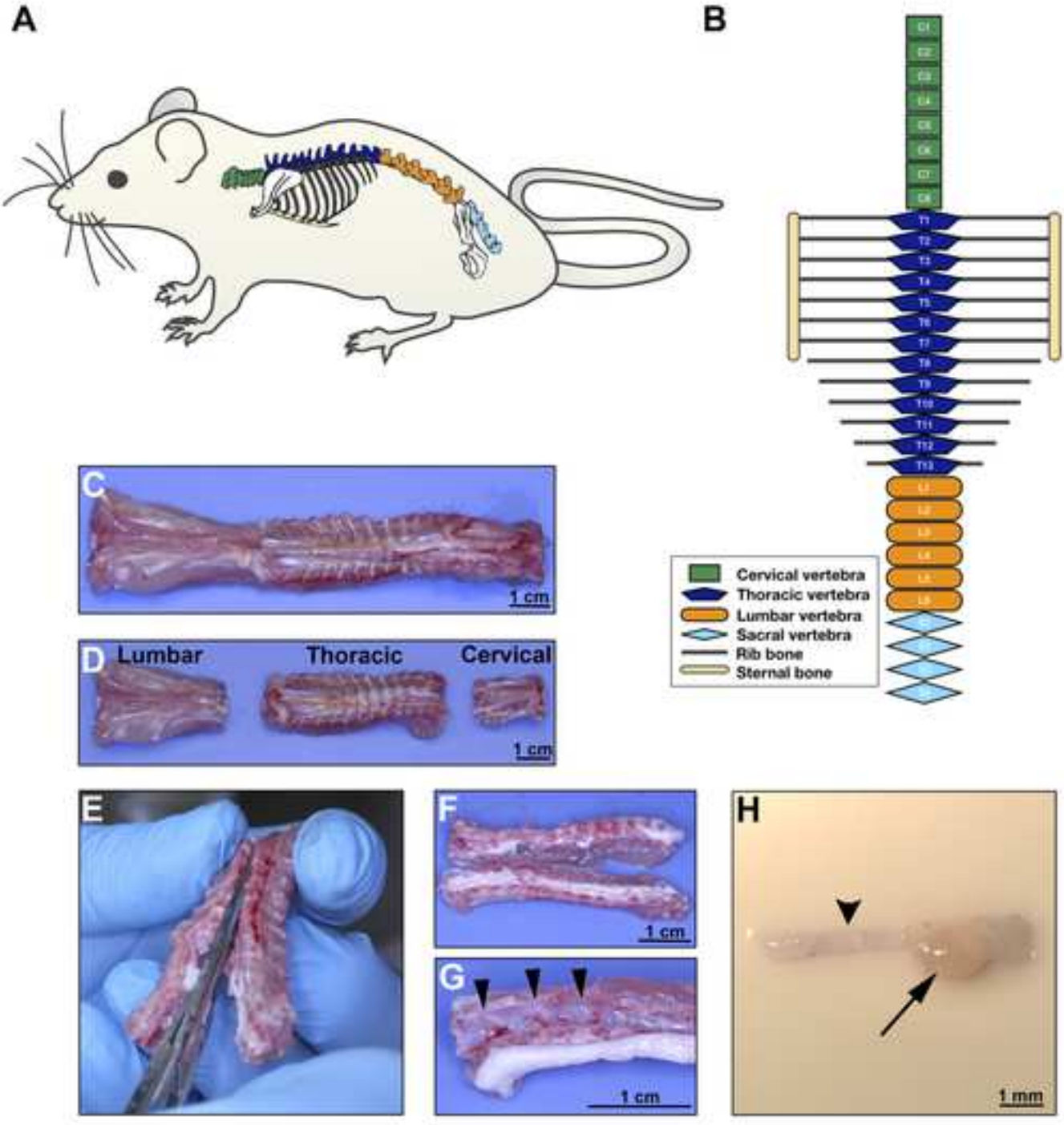

Figure 3

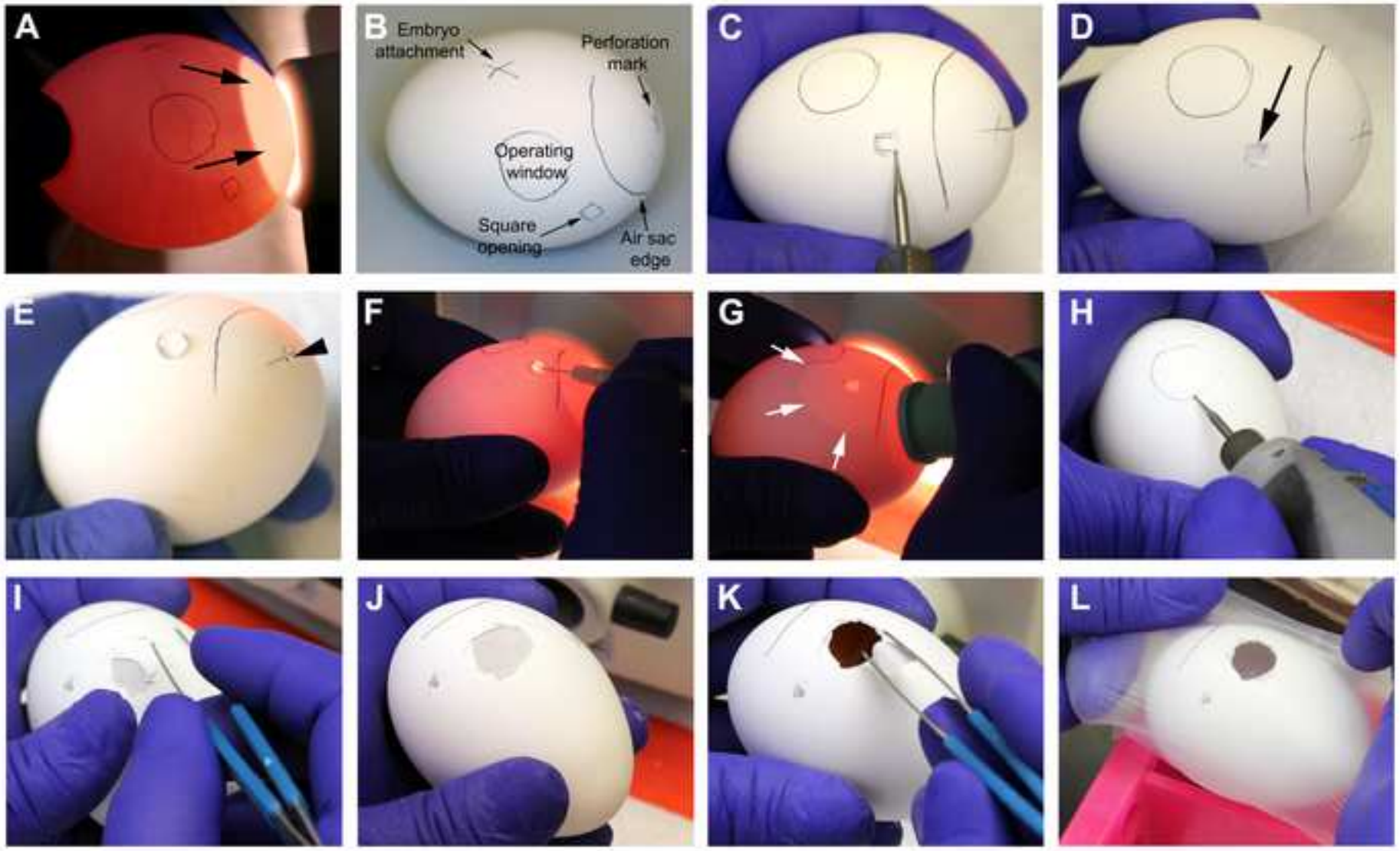

Figure 4

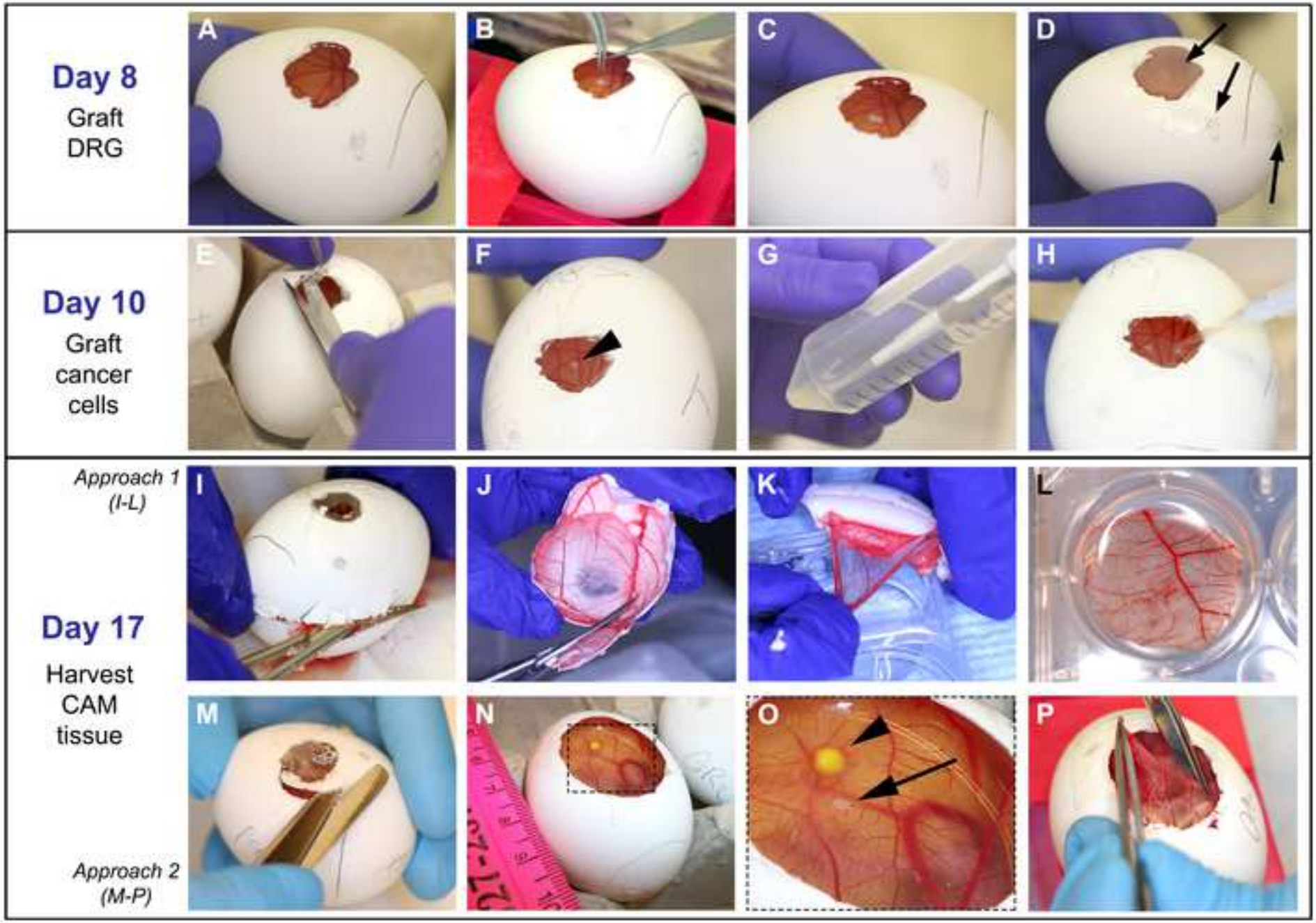

Figure 5

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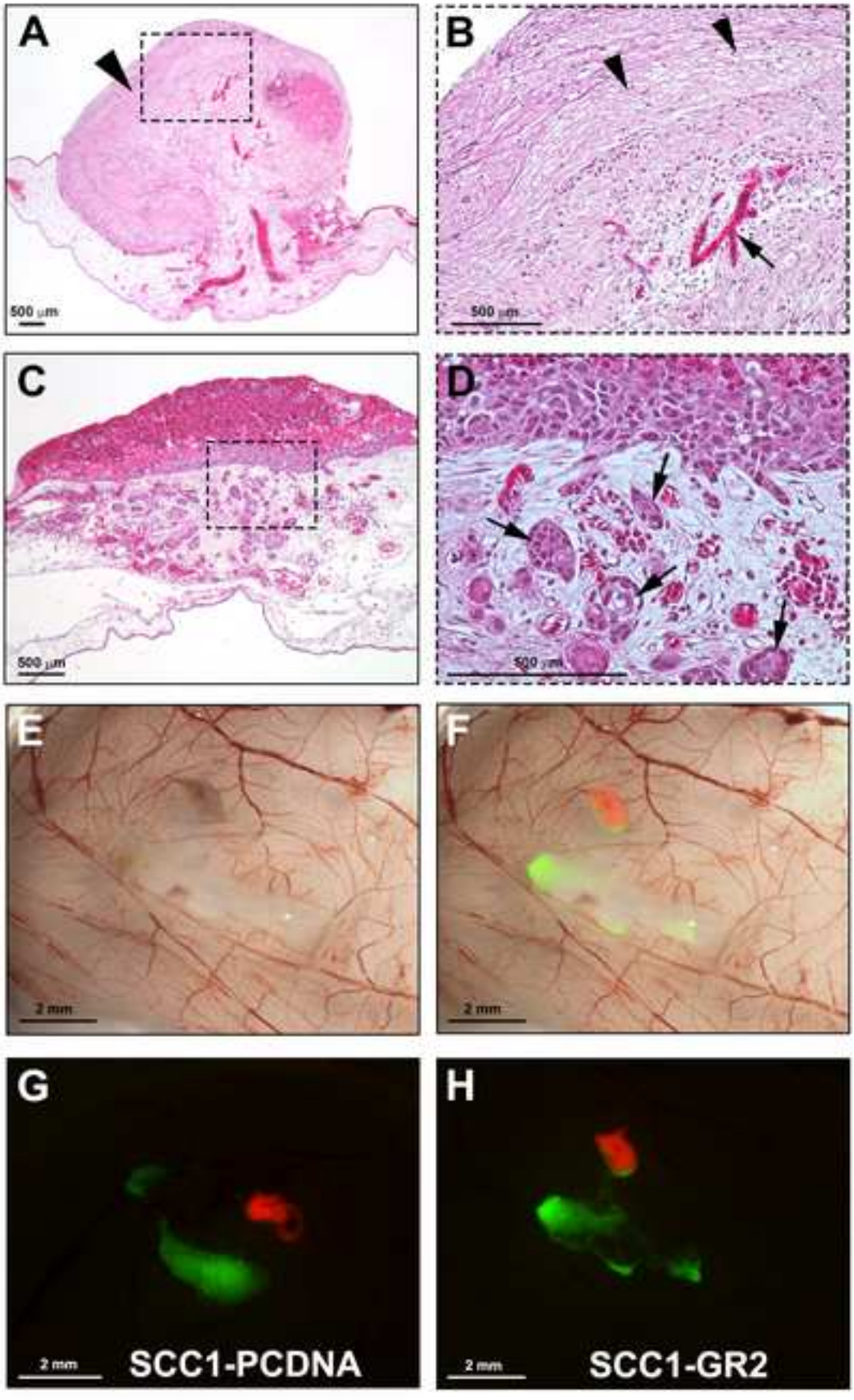

Troubleshooting table			
Step	**Problem**	**Reason**	**Solution**
3.2.1	Unable to identify the embryo attachment.	Attachment position is difficult to see while egg is still.	Rotate the egg quickly sideways to be able to see a long vessel attached to the egg membrane.
3.3 & 3.8	Perforation of the outer egg shell membrane while drilling.	Wrong positioning of the drill.	Position the drill almost parallel to the egg shell while drilling. If membrane is perforated in step 3.3 , there is no need to perform further perforation with needle as stated in step 3.5.
4.3	DRG sticks to the forceps.	DRG is dry.	Wet DRG again in HBSS and/or use a fine needle to help detach DRG from forceps.
5.2	Cells are not perfectly labeled with fluorescent dye.	Incubation time. Some cells require more time to label.	Keep cells for an additional hour in media with cell tracker.
5.6	Air bubble on the cell drop	Using all the fluid in the pipette tip.	Load 1µL more than the desired volume and do not use the final µL of the pipette when implanting cells. This will avoid air bubbles in the cells mix.
6.3	Unable to identify DRG or cells when harvesting the CAM	Small DRG, DRG got displaced, cancer cells spread.	If DRG is not seen, harvest a larger area of the CAM and place into a larger container for fixation. Check DRG and cell position under fluorescence in a stereo microscope, and then trim the CAM to a smaller size for paraffin embedding.

Name of Reagent/ Equipment	Company	Catalog Number
0.25% Trypsin-EDTA (1x)	Gibco	# 25200-056
ACE light source	SCHOTT North America, Inc.	
CellTracker Green CMFDA fluorescent dye	Life Technologies	# C7025
CellTracker Red CMTPX fluorescent dye	Life Technologies	# C34552
Cordless rotary tool	DREMEL	# 866
DMEM (1x)	Gibco	# 11965-092
DMSO	Fisher Bioreagents	# BP231-100
Dumont # 5 fine forceps	Fine Science Tools (FST)	# 11254-20
Egg incubator	GQF Digital Sportsman	# 1502
Engraving cutter	DREMEL	# 108
Extra fine Graefe forceps, curved	Fine Science Tools (FST)	# 11151-10
Extra fine Graefe forceps, straight	Fine Science Tools (FST)	# 11150-10
Fertilized Lohmann White Leghorn eggs		
Filter Forceps	EMD Millipore	# XX6200006P
Fine surgical straight sharp scissor	Fine Science Tools (FST)	#14060-09
HBSS (1x)	Gibco	# 14025-092
HI FBS	Gibco	# 10082-147
Paraffin wax membrane	Parafilm laboratory film	# PM-996
PBS (1x) pH 7.4	Gibco	# 10010-023
Pen/Strep	Gibco	# 15140-122
PFA (paraformaldehyde solution)	Sigma-Aldrich	# P6148-1KG
Sprague Dawley rats	Charles River laboratories	Strain code: 400
Tegaderm Transparent Film Dressing	3M	# 9505W

	Comments/Description
	Used to transilluminate the eggs
	Reconstitute 50µg in 20µL of DMSO and stock at -20^{o}C. Use 1µL of stock solution/mL of culture medium.
	Reconstitute 50µg in 40µL of DMSO and stock at -20^{o}C. Use 1µL of stock solution/mL of culture medium
	Used to drill the egg shell
	Dulbeecco`s Modified Eagle Medium
	Dimethyl Sulfoxide
	Used to harvest DRG
	Egg incubator equipped with automatic rotator, digital thermostat, temperature and humidity controls
	Used to drill the egg shell
	Used to graft DRG onto the CAM on day 8 and to harvest CAM tissue on day 17
	Used to graft DRG onto the CAM on day 8 and to harvest CAM tissue on day 17
	Fertilized eggs at early fertilization days, preferably on first day post-fertilization. Eggs used in this protocol are from Michigan State University Poultry Farm.
	Blunt forceps used to remove the egg shell
	Used to harvest the CAM tissue on day 17
	Hank`s Balanced Salt Solution
	Heat inactivated Fetal Bovine Serum
	Used to temporarily cover the egg openings until DRG grafting on day 8
	Phosphate Buffered Saline
	10,000 Units/mL Penicilin, 10,000 µg/mL Streptomycin
	Dilute in water to make a 4% PFA solution
	6-7 weeks old (190-210g in weight)
	Sterile, 6x7cm, used to cover the egg openings during incubation

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December 12, 2018

Dr. Vineeta Bajaj,
Associate Editor
JoVE

Dear Dr. Bajaj,

Attached please find a revised copy of our manuscript entitled " The Chick Chorioallantoic Membrane In Vivo Model to Assess Perineural Invasion in Head and Neck Cancer" (Manuscript #: JoVE59296), which as indicated in your correspondence of November 27, 2018 could be submitted as a revised manuscript after addressing the reviewers' comments.

The changes are detailed in the attachment and are summarized below. We thank the reviewers for the suggestions, which strengthened the manuscript. We look forward to hearing about the acceptability of the manuscript for publication in JoVE.

We appreciate the reviewer's positive feedback about the manuscript. Below, we have responded in detail to the Editor's and reviewers' comments, which are in italics to help in differentiating from our responses. All the changes implemented in the revised manuscript are highlighted in red.

Editorial comments: *Changes to be made by the author(s) regarding the manuscript:*

Comment #1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: Thank you. We have proofread the revised manuscript for spelling and grammar issues.

Comment #2: Please revise lines 71-75 to avoid previously published text.
Response: Thank you. These changes have been made in the revised manuscript.

Comment #3: Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.
Response: These changes have been made in the revised manuscript.

Comment #4: Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
Response: This change has been made in the revised manuscript.

Comment #5: Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
Response*:* These changes have been made in the revised manuscript.

Comment #6: 2.2: Please describe how to harvest DRG from cervical and thoracic regions. As this step is highlighted for filming, we need specific details.
Response: We added 6 sub-steps to section 2.2 to increase clarity on harvesting DRGs. However, it is still a summarized version of the entire published protocol from Sleigh *et al. BMC Res Notes (2016) 9:82* and cited in the current manuscript.

Comment #7: 2.4: Please specify incubation temperature.
Response: This change has been made in the revised manuscript.

Comment #8: 4.1: How large is the culture dish?
Response: This information has been added to the revised manuscript.

Comment #9: 5.3: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).
Response: This change has been made in the revised manuscript.

Comment #10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Response: This change has been made in the revised manuscript.

Comment #11. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response: This change has been made in the revised manuscript.

Comment #12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.
Response: This change has been made in the revised manuscript.

Comment #13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Response: This change has been made in the revised manuscript.

Comment #14. References: Please do not abbreviate journal titles.
Response: This change has been made in the revised manuscript.

Comment #15. Table of Materials: Please remove trademark (™) and registered (®) symbols and sort the items in alphabetical order according to the name of material/equipment.
Response: This change has been made in the revised table of materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The paper of Schmitd et al. (The chick chorioallantoic membrane in vivo model to assess perineural invasion in cancer) describes a co-culture model of CAM between rat dorsal root ganglia and human head and neck squamous cell carcinoma. The experiments are clearly presented and the co-culture model of interest.

Major Concerns:

Comment #1. The only problem is that the specificity of the dorsal root ganglia should be presented in a more convincing way, with a statistical approach, in order to demonstrate that any cells type (for example untransformed foreskin fibroblasts) are not able to invade the ganglia.

Response: We understand the concern about the specificity of the DRGs in attracting only cancer cells. However, it is unlikely that DRGs possess specificity for cancer cells and it is not our intent to imply this in the current manuscript. Nerves are implicated in many physiological processes, including regulation of stem cells in the skin[1] and are needed for regeneration and embryonic development of many tissues[2]. For these reasons, we do not expect that the DRG will specifically attract only cancer cells. However, the purpose of this model is to evaluate the impact on perineural invasion of specific proteins in tumor cells. Therefore, we are presenting a protocol for tumor cells. It is foreseeable that this model could be adapted to investigate the impact of the DRG on other cell types.

Minor Concerns:

Comment #2. In addition, why not to use GFP expressing cells in order to perform fluorescent labelling on histological sections?

Response: Thank you for this question. We used GFP-labeled cells in the CAM model with the same success as cells labeled with cell tracker (unpublished data) However, we have not used GFP-labeled cells for the CAM-DRG model, which is why we omitted this information in the protocol. It is very likely that GFP-labeling would work in the CAM-DRG model as well as it works on the CAM model and we have added this information in the representative results section.

Reviewer #2:

In this investigation Schmitd et al. developed a chorioallantoic membrane-dorsal root ganglia (CAM-DRG) model in which a mammalian DRG is isolated and grafted onto the surface of the upper CAM. After the DRG becomes incorporated into the CAM, head

and neck cancer cells are grafted near the DRG and allowed to interact with the DRG before the entire in vivo system is harvested and analyzed. This system allows ex-vivo visual observation of both the DRG and the tumor by fluorescent labelling, therefore, I believe that it is a very interesting model to evaluate the ability of cancer cells to invade the neural component in vivo. Therefore, I recommend the publication of this paper after minor revisions as follows:

Comment #1- Is there any possibility to quantify the invasion ability of cancer cells?
Response: Invasion of cancer cells through the basement membrane of the CAM is quantified by counting the number of invasive tumor islands in the connective tissue of the CAM. The quantification of tumor islands inside the DRG is not practical because of sampling issues on histologic sections. While tumor islands may be observed inside the DRG, this is infrequent, likely due to the location of sectioning. We believe that since the analysis is based on a few 5 μm sections of a 4-5 mm tissue, it is hard to capture the exact site at which PNI occurs. That is the reason why we rely on the fluorescence images to view PNI rather than histologic sections to quantify PNI. This quantification is performed by measuring how many samples had a directional migration of tumor cells toward the DRG and also by measuring distances between cancer cells and the DRG. We have increased clarity on how the analysis is done in the representative results section.

Comment #2- The histology pictures A, B and C of figure 4 should be replaced by other pictures of better quality
Response: Thank you for the comment. We think the reviewer is referring to Figure 5. Figure 5A is a low magnification image of the entire DRG grafted onto the CAM. We replaced the previous Figure 5B with a higher magnification image to add more detail and show integration of the DRG in the CAM connective tissue and the DRG tissue. Figure 5C is a low magnification of tumor grafted onto the CAM. We replaced the previous Figure 5D with a higher magnification image to highlight the tumor islands invading the connective tissue.

Comment #3- In the discussion section the authors should compare their method with other methodologies used to assess the ability of cancer cells to invade the neural component (example using invasion xenograft mouse model).
Response: Thanks for the comment. The use of tumor xenografts is not a consistent method to assess PNI. In our experience and based on published literature, tumor xenografts generate very low rates of PNI, which may explain why the tumor xenograft model has not been reported in the literature as a method for investigating PNI.

Reviewer #3:

Manuscript Summary:

This manuscript presents a very exciting technique that involves leveraging the chick chorioallantoic membrane to model perineural invasion in head and neck squamous cell carcinoma. The authors have identified the paucity of models that can mimic the early events in the progression of perineural invasion and propose a model that can address this gap by serving as an excellent scaffold for rat dorsal root ganglia and human head and neck squamous cell carcinoma cells. They detail a series of steps that are required to employ this model in order to assess the ability of cancer cells to invade the neural component in vivo.

Major Concerns:

Comment #1. In figure 5H, the cancer cells seeded on the CAM appear closer to the DRG than in figure 5G, which makes it unclear whether the more extensive invasion occurred as a consequence of the proximity to the DRG or if it really was a phenotype from the genetic manipulation of the cell line. An H & E stained slide of the both control and GALR2 CAM tissue would also help to corroborate the invasion differences.

Response: We agree that in this specific picture the tumor-DRG distance is smaller than the representative control. Cells are grafted at equal distances in all groups. Since it is technically difficult to ensure completely equal distances across samples, we emphasize the need for several replicates in each group. It is also possible that the SCC1-GALR2 cells are closer because of the greater attractive potential they have towards the nerve. We agree that the image choice was not the most representative and have changed it.

Regarding differences in the invasive phenotype of the GALR2 cells, there are two types of invasion that can be assessed by the model: invasion through the basement membrane (shown in Fig. 5C-D) for which we use HE or IHC stain, and invasion toward the nerve component (Fig. 5G-H), for which we use the fluorescence images. We did not include HE images for the invasion of GALR2 cells because we would like to avoid confusion between these two distinct processes. However, you are right to assume that the GALR2 cells would present more invasion through the basement membrane. They are in fact more invasive, as shown in our previous publication[3]. Perineural invasion is difficult to quantify inside the DRG due to sampling issues on histologic sections. While tumor islands may be observed inside the DRG, this is infrequent, likely due to the location of sectioning. We believe that since the analysis is based on a few 5 μm sections of a 4-5 mm tissue, it is hard to capture the exact site at which PNI occurs. This is similar to the challenge of detecting PNI in human biopsy specimens of cancer. In the CAM-DRG model, we rely on fluorescence images rather than histologic sections to

quantify PNI. This quantification is performed by measuring how many samples had a directional migration of tumor cells toward the DRG and also by measuring distances between cancer cells and the DRG.

Comment #2. This manuscript only used head and neck cancer cell lines, therefore, the title should reflect this by changing the word "cancer" in the title to "head and neck cancer." Care should be exercised throughout the manuscript to avoid generalizing the utility of this PNI model in multiple cancers until a wider range of cancer cell lines have been tried on the CAM.
Response: Thank you for this comment. We have revised the title accordingly.

Minor Concerns:

Comment #1. Lines 117 and 129 include the timing, however, this can be omitted since every person performing the technique will likely require different time.
Response: This is a good comment about variations between individuals. The time was mentioned to provide an estimate for planning purposes for a person using this model for the first time. Therefore, we changed it to “estimated timing”.

Comment #2. Figure 2H has an arrow pointing at a DRG, but it is not entirely clear that this represents a reduced integration of the DRG in the CAM as the Note in line 147 suggests.
Response: We apologize for this confusion. The goal of figure 2H was only to show the DRG with the axon bundle attached to it and not to show integration. Figure 2H is a high magnification picture of an extracted DRG before it is grafted on the CAM. To avoid confusion, we changed the place where we mention figure 2H in the text. We also added another arrow to point the axon bundle to increase clarity.

Comment #3. The authors mention a decontamination period, but it is not specified what this incubation is decontaminating. Perhaps a sentence on this would help.
Response: We changed the wording of this part of the protocol. It is not a decontamination, but a prophylaxis with an increased dose of Pen/Strep (2% instead of 1%), to avoid potential bacterial contamination of the DRGs due to the harvesting process.

Comment #4. Section 3, line 165 should omit the timing portion.
Response: We changed it to “estimated timing” for planning purposes. Please see response to Comment #1 above.

Comment #5. In section 3.1, line 168 should contain exclusion of non-fertilized eggs.

Response: This information has been added to the revised manuscript.

Comment #6. An arrow that points to the air sac in figure 3A would help the reader see the naturally-occurring air sac.
Response: This change has been made in the revised manuscript.

Comment #7. In section 3.3, the authors should indicate what tool they will use to drill the egg shell in the marked square.
Response: This change has been made in the revised manuscript.

Comment #8. Section 3.4 needs to emphasize that this procedure should be performed carefully since applying too much pressure can damage or destroy the egg.
Response: This information has been added to the revised manuscript.

Comment #9. Section 3.5, line 194 needs to indicate that this step should be performed while holding the egg against the light source.
Response: It is not necessary to have the direct light source when perforating the membrane in the square opening; this step can be performed with room lighting.

Comment #10. Section 3.6 should mention that this step may require multiple attempts before succeeding.
Response: This information has been added to the revised manuscript.

Comment #11. Section 3.8 needs to specify what tool will be used to drill.
Response: This information has been added to the revised manuscript.

Comment #12. Section 4, line 222 should omit the timing portion.
Response: We changed it to “estimated timing” for planning purposes. Please see response to Comment #1 above.

Comment #13. Section 4.3, line 230 should indicate that sterile forceps should be used.
Response: This information has been added to the revised manuscript.

Comment #14. Figure 4D should have an arrow pointing towards all openings that should be covered.
Response: This change has been made in the revised figure.

Comment #15. Section 5, line 248 should omit timing portion.
Response: We changed it to “estimated timing” for planning purposes. Please see response to Comment #1 above.

Comment #16. Section 6, line 283, omit timing portion.
Response: We changed it to “estimated timing” for planning purposes. Please see response to Comment #1 above.

Comment #17. Section 6.3 needs better explanation and/or images because it is not entirely clear what the reader needs to be doing.
Response: We added more information to increase clarity to this step. We have also edited Figure 4I-K to better illustrate this step.

Comment #18. Paragraph three in the "representative results" section needs to be improved with a clearer explanation of the analysis. Perhaps some examples would help.
Response: This change has been made in the revised manuscript.

Comment #19. In figure legend for fig. 3, line 375 lists adding 30 ul of water, but in the manuscript is mentions using HBSS, please be consistent.
Response: Thank you for pointing out the discrepancy, which we have corrected in the figure legend. We use HBSS and not water.

REFERENCES:

1 Peterson, S. C. *et al.* Basal cell carcinoma preferentially arises from stem cells within hair follicle and mechanosensory niches. *Cell Stem Cell.* **16** (4), 400-412, doi:10.1016/j.stem.2015.02.006, (2015).

2 Boilly, B., Faulkner, S., Jobling, P. & Hondermarck, H. Nerve Dependence: From Regeneration to Cancer. *Cancer Cell.* **31** (3), 342-354, doi:10.1016/j.ccell.2017.02.005, (2017).

3 Scanlon, C. S. *et al.* Galanin modulates the neural niche to favour perineural invasion in head and neck cancer. *Nat Commun.* **6** 6885, doi:10.1038/ncomms7885, (2015).