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Non-Invasive Intravital Imaging of Head and Neck Squamous Cell Carcinomas in Live Mice

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Abstract

Head and neck squamous cell carcinoma is one of the most common cancers with a 50% 5-year survival rate. Understanding the mechanisms that control development, progression, and spreading of the tumor to distal sites is of paramount importance to develop effective therapies. Here, we describe a minimally invasive procedure, which enables performing intravital microscopy of the mouse tongue in models for oral cancer and provides structural and dynamic information of the tumors at cellular and subcellular level.

1. Introduction

Each year 55,000 Americans are diagnosed with head and neck squamous cell carcinoma (HNSCC), which represent nearly 5% of all diagnosed cancers in the United States. With more than 600,000 new cases per year worldwide, oral cancer is considered the tenth most common cancer in the developed world [1]. Despite the recent advances in surgical procedures and treatment modalities, the 5-year survival rate for oral cancer patients is approximately 50%, and has remained largely unchanged for more than 30 years [1]. Even favorable prognosis in patients that respond to available treatments is often confounded by recurrences and secondary neoplastic lesions, which are generally less sensitive to therapy. Indeed, the features of oral malignant cells lead to tumor invasion and spread (metastases), which makes this cancer difficult to treat. Understanding the biology of the metastatic and invasive processes in the tumor microenvironment is critical for developing novel strategies for oral cancer prevention and treatment.

The aim of this protocol is to provide a powerful imaging tool to investigate the mechanisms regulating the development and the progression of head and neck tumors in live animals. Our approach is based on combining intravital two-photon microscopy, a technique that

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enables imaging various cellular and sub-cellular processes in live animals [2,3], with two complementary oral cancer models, which ensure the access to the tumor in a minimally invasive fashion, thus enabling daily imaging for several weeks. Specifically, we use: 1) a tongue tumor xenograft model based on immune-deficient mice injected with human HNSCC cells [4,5], which provides information on the molecular machinery regulating tumor growth and the invasive process, and 2) a carcinogen-induced tongue cancer model, which provides information on the early stages of the transformation from the normal oral tissue into a pre-malignant lesion, and the progression to malignancy [6,7].

We envision that this method will lead to 1) a deeper understanding of the mechanisms controlling tumorigenesis and cancer progression in HNSCC, 2) the development of more effective therapies, and 3) the development of better tools for early detection of pre-malignant lesions. Finally, this approach will serve as a paradigm to investigate several aspects of epithelial tumor biology in animal models.

2. Comparison with alternative techniques for tongue tumor imaging

There are various alternative methods available for tongue imaging, including MRI, CT, fluorescence-mediated tomography (FMT) and confocal microscopy. MRI and CT are whole body noninvasive imaging methods that offer a large amount of information. However, these techniques have limited imaging resolution and only recently have been adapted to small animals [8–11]. FMT is a quantitative method using trans-illumination to reconstruct 3D maps of fluorochromes that is based on reconstruction algorithms. FMT is often combined with CT and MRI for improved photon reconstruction and image visualization, thus enabling multimodal imaging. Cellular and subcellular resolution in living tissue can be achieved by confocal microscopes, which have become widespread, since they are user friendly and relatively inexpensive [9]. However, penetration depths are usually limited to 50–60 μm . This limitation has been overcome by multiphoton microscopy (MPM) that in the last two decades has achieved depth resolutions of 200–500 μm with the capability of generating tomographic z-stacks in multiple channels. Furthermore, MPM can be combined with other non-linear optical techniques, such as second and third harmonic generations (SHG and THG) [12,13] or fluorescence life-time imaging (FLIM) [14], thus providing the unique opportunity for simultaneous imaging of tumor cells and their microenvironment. Finally, MPM offers the opportunity to analyze structural and dynamic features of the tumor at different levels of resolution ranging from the overall lesion to specific area of the tumor (e.g. tumor margins), or to individual cells and subcellular structures.

3. Experimental design

3.1 Animal models

3.1.1 Tongue tumor xenograft model—Tumor xenograft models in immunocompromised mice are widely used in the cancer biology field and for anticancer drug testing. Indeed, they have several advantages including 1) rapid tumor growth and metastasis when compared to other models, 2) the possibility to easily perform genetic manipulations in human-derived cancer cells, and 3) a well-defined tumor area close to the site of injection. On the other hand, the disadvantages of this model include lack of 1)

heterogeneity of tumor cells, 2) tumor-host response mechanisms, and 3) information on the initial stage of tumor development. The method described herein is based on the injection of human-derived HNSCC cells in the tongue of immuno-compromised mice (i.e. nude or SCID) (Fig. 1A). Most of the HNSCC cell lines developed so far form solid tumors within the first 2–3 weeks of the injection and only few metastatic cell lines disseminate to the cervical lymph-nodes and then to distal sites in a period between 5 and 15 weeks post-injection [4,5].

3.1.2 Carcinogen-induced tongue tumor model—Carcinogen-induced tumor mouse models are well-established and recapitulate the initial steps of malignant transformation of the basal oral epithelium into squamous cell carcinoma. The carcinogen initiates the genetic mutations in the exposed mucosal cells and promotes cell proliferation and malignant transformation concomitantly with microenvironment modifications. The use of the DNA adduct-forming agent, 4-nitroquinoline-1 oxide (4NQO), which serves as a surrogate for tobacco exposure, leads to a 100% incidence of heterogeneous oral malignant lesions, many of which evolve spontaneously into highly malignant invasive SCCs [6,7]. However, the main disadvantages of this model are 1) the long time required to achieve malignant transformations, and 2) the fact that is not possible to predict the location of the developing lesions and their outcome (i.e. benign vs. malignant) (Fig. 1B). Indeed, the carcinogen is provided in the drinking water for 16 weeks and the cancerous lesion become visible only around week 18–20.

3.2 Tumor labeling

3.2.1 Tongue tumor xenograft model

Lentivirus-mediated engineering of HNSCC cells: HN12, Cal27 and Hep2 are some of the most commonly used HNSCC cell lines, known to form solid tumors, and OSCC3, SCC2 and UMSCC17B are the HNSCC cell lines reported to show invasive and metastatic behavior when injected in immunocompromised mice [5]. Prior to the injection, these cells are genetically engineered by either introducing the gene of interest fused with a fluorescence protein or by ablating the target protein using short hairpin RNA interference (shRNA). The preferred method to introduce genetic material is in vitro transduction mediated by Avian leukosis virus (ALV) pseudo-typed lentiviral-based vectors [5,15]. One of the main advantages of this approach is the integration of the gene/shRNA into the host genome and a relatively high yield of transfected cells. This combined with the introduction of fluorescent reporters enables the selection of the transfected cells by FACS and the rapid generation of homogeneous cell lines that can be cultured for several passages.

3.2.2 Carcinogen-induced tongue tumor model

Transgenic mouse with fluorescent basal layer: 30–40% of the oral squamous cell carcinomas are primarily generated in the basal epithelial layer of the tongue. For this reason, we generated a mouse model in which this cell population is fluorescently labeled. The main strategy was based on the use of a mouse expressing the Cre-recombinase (Cre) reporter Rosa^{mT/mGFP} (mTomato) in which a plasma membrane-targeted peptide tagged with the tandem-tomato protein (mT) is replaced by a similar peptide tagged with GFP

(mGFP) upon Cre expression [16]. In this mouse, all the cellular membranes are fluorescently labeled and, specifically in the tongue, the papillae in the dorsal surface and the blood vessels in the ventral side can be easily visualized (Fig. 3A and 3B). This mouse was crossed with a strain which expresses the Cre-recombinase enzyme under the control of the Keratin 5 promoter, which is active in the basal layer (Fig. 3A) [17]. In the derived strain (K5-GFP/mTomato), the expression of the mGFP was primarily restricted to the basal layer (Fig. 3B, arrows), and to a smaller extent to few cells in the papillae (Fig. 3B, asterisks).

3.3 Microenvironment labeling

3.3.1 Vasculature and stromal labeling—One of the main landmarks of living tissue is the vasculature. In order to visualize the blood vessels in vivo, we routinely use fluorescent dye-conjugated dextrans. When administered by intravenous injections, dextrans rapidly appear in the circulation within a minute. Dextrans come in different sizes (molecular weight ranging from 3 KDa to 2,000 KDa) and they should be selected according to the experimental design. Large dextrans (MW > 500K), which are retained in the vasculature for several hours before being excreted through the urine, are the ideal choice to label blood vessels, measure blood flow, and probe for the leakiness of tumor blood vessels. On the other hand smaller dextrans (MW 40–70 KDa) diffuse through the fenestrated capillaries and are internalized primarily by stromal cells, thus providing indications on the microenvironment surrounding the tumors. Fluorescent dextrans can be produced in-house or easily purchased from commercially available sources.

3.3.2 Imaging the extracellular matrix—In long-term experiments, where it is essential to image the same area over multiple days, it is of paramount importance to determine landmarks that are constant for several weeks. Due to tumor-associated angiogenesis, the vasculature can be used as a reference point only for a short period of time (4–5 days). A viable alternative is the use of photo-convertible or photo-switchable probes that can be engineered into either the tumor cells or the animals [18]. Alternatively, the extracellular matrix located at the surface of the tongue has a very slow turnover and can be used for this purpose. As mentioned before, multiphoton excitation enables capturing SHG signals produced by type I collagen when arranged in fibers [12] and as shown in Fig. 4, they provide simple and quite reproducible tissue landmarks. The only drawback is that due to small changes in the orientation of the tissue it is not always possible to achieve perfect alignment of the volumes generated by z-scans, without specifically designed imaging software.

3.4 Tongue imaging through the tongue holder

To minimize the motion artifacts due to the heartbeat and the respiration, we designed a device to hold the mouse tongue. The holder is composed of two flaps connected by a flexible hinge. The lower flap has a groove that can accommodate a thin square coverslip. The tongue is sandwiched in between the two flaps, which are kept in place by a screw. The holder is secured to a metallic platform by a metallic pin that is tightened by a screw (Fig. 2). Finally, the platform is placed on the microscope stage.

3.5 Length of imaging sessions

This non-invasive surgery-free approach allows for repeated long term imaging in the same animal. In a single session, it is possible to acquire time-lapse movies in the same animal for 4–6 hours. However, due to the effects of repeated exposure to anesthetics, we recommend at least 1–2 day intervals between imaging sessions. For daily imaging, in order for the animals to fully recover, we recommend lighter anesthesia and to not exceed 30–45 minute imaging time.

3.6 Main limitations of the approach

The tongue-imaging approach that we developed is a non-invasive procedure that requires minimal preparation that can be applied to investigate several physiological or pathological processes for a long period of time up to 20–25 weeks. However, there are also some limitations concerning the condition of the tongue at the time of imaging. For example, if the tumor is at a very advanced stage it could affect the movements of the tongue (e.g. it cannot be pulled out and cannot stably rest on the tongue holder). If possible, we prefer to image from the ventral side of the tongue, due to the thinner mucosal lining and the lesser papillae coverage which allows us to image deeper in the tissue. Due to the depth limitation of the technique, it is difficult to reach tumors that form or are injected deep in the middle of the tongue. For this reason, we recommend injecting tumor cells close to the surface and primarily in the anterior one third of the tongue.

3.7 Examples of the main applications of the tongue imaging

In this section we want to show some examples of the structural and dynamic information that can be acquired using this method. We want to emphasize that this approach is extremely flexible and can be adapted to answer a broad variety of questions.

3.7.1 Imaging tumor margins in tongue xenograft—One of the strengths of our method is to image details of the progression of the leading edge of migrating tumors and to acquire information on the dynamics of subcellular structures. In Fig. 3 we show the volume rendering of a section of a tumor acquired after day 17 and 19 from the injection of the cells. Specifically, we injected two populations of OSCC3 cells, which we have previously shown to form solid tumors in the tongue and to metastasize rapidly to the cervical lymph-nodes. These cells lack the small GTPase Rab25 and its re-expression blocks metastatic activity [4]. We engineered the OSCC3 cells to either express GFP-Rab25 (green) or m-Cherry (red) and co-injected in the tongue. After 17 and 19 days we acquired z-scans in the same area of the tumor (Fig. 4 upper panels). SHG signal was used to locate the same area and to orient the volumes. We could detect that the imaged area was depleted of cells lacking Rab25. A similar experiment was carried out imaging another area of the tumor for 4 consecutive days (D23–D26), showing that cell lacking Rab25 migrate away from the primary tumor, whereas cells expressing Rab25 are relatively static (Fig. 4, lower panels). Additional published data showed that cells lacking Rab25 invade the lymphatic vessels and that cells expressing Rab25 are confined in the primary tumor site (4). To investigate the characteristics of the tumor edges at a cellular level we imaged individual cells using a high magnification objective (60X). We performed time-lapse analysis in OSCC3 cells expressing GFP and

visualized dynamic membranous protrusions emanating from the plasma membrane of cells at the tumor edge (Fig. 5 center panels). Using the same imaging settings we imaged the distribution of GFP-Rab25, which in the tumor cells is localized in very dynamic intracellular vesicles that undergo fusion at the cell periphery (Fig. 5, lower panels and insets).

3.7.2 Imaging tumor progression in the carcinogen model—Tumors were induced by administration of the carcinogen 4NQO, as previously described (Fig. 6A). The tongues of K5-Cre/mTomato mice were imaged from the dorsal side before (Fig. 3A, right panel) and after the administration of 4NQO (Fig. 6B). Specifically, using low magnification objectives (10X or 25X) we scanned almost the entire surface of the tongue. For each individual field of view, a z-scan was acquired, and all the volume renderings were stitched together to generate the whole image. Under control conditions the basal layer, highlighted by the GFP, appears homogeneously distributed throughout the tongue. On the other hand, after 12 weeks the basal layer significantly expanded and clustered in few areas corresponding to the cancerous lesions (Figure 6B, insets 1 and 2). The lesions undergo small but detectable changes in the course of 5 weeks (Fig. 6B). Moreover, the basal layer compartment expanded into the fungiform papillae, as visualized by higher magnification volume rendering that reveal details of their cellular structure (Fig. 6C lower panels).

3.8 Materials

3.8.1 Animals—Female athymic (*nu/nu*) nude mice were from Harlan Sprague Dawley, Frederick, MD, female SCID mice were from (National Cancer Institute at Frederick, Frederick, MD), mTomato and K5-CreER^{T2} knock-in mice were from Jackson laboratory (cat# 007576 and 029155 respectively). 5–6 weeks old and 20–25 g, were used in this study.

3.8.2 Cell lines—HNSCC cell lines (HN12, CAL27, and OSCC3) were acquired and maintained as described in [4].

3.8.2 Reagents

- 0.9% Normal saline (Quality Biological, Inc., cat. No. 114-055-101)
- Xylaxine (Anased, 100 mg/ml Akorn, Decatur, IL)
- Ketamine (Ketaved, 100 mg/ml, Fort Dodge Animal Health, Fort Dodge, IA)
- Isoflurane (Forane, 100 ml, Baxter, Deerfield, IL)
- 70kDa Texas Red (cat # D-1864) and 2000 kDa Tetramethylrhodamine Dextran (cat # D-7139) (Molecular Probes, Carlsbad, CA).
- UltraPure Distilled water (Invitrogen, cat. no. 10977-015)
- Ophthalmic Ointment (Neomycin and Polymyxin B sulfates, Bacitracin Zinc and Hydrocortisone, Bausch & Lomb, NDC 24208-785-55)
- 4NQO (Sigma-Aldrich)

3.8.3 Equipment

- Insulin syringe (Easy Touch, 29 gauge, .5 cc, 1/2 inch)
- Kendall Monoject hypodermic needle polypropylene hub (25 gauge, 0.5mm, 5/8 A)
- Cotton Tipped Applicators (Puritan, 15cm, cat. no. 806-WCL)
- Toothless forceps (Braintree Scientific, Braintree, MA)
- Induction Isofluorane chamber and nose cones (Isofluorane V 1.9, Braintree Scientific, Braintree, MA)
- MicroTherma 2T Thermometer with RET-3 and IT-21 thermocouple probes (Braintree Scientific, Braintree, MA)
- Hydropac water (Lab products)
- Heat Pads (Kent Scientific, Torrington, CT)
- Microscope
 - Laser IX81 inverted confocal microscope, equipped with a Fluoview-1000 scanning unit (Olympus America, Center Valley, PA) modified for multiphoton microscopy as described in [19]. As a laser source, a tunable Ti:sapphire femtosecond laser (Chameleon Ultra II, Coherent Laser Group, Santa Clara, CA) was used, and the power was modulated using a combination of neutral density filters (Chroma Technology, Rockingham, VT). A beam expander (LSM Technologies, Stewartstown, PA) was used to modulate the size of the beam that was then directed into a scanning head (Fluoview 1000, Olympus America). The emitted signal was aimed into a custom-made set of three non-descanned detectors (LSM Technologies). Two dichroic mirrors and the barrier filters were purchased from Chroma Technology, and three cooled photo multipliers (PMTs, Model number, R6060-12) were purchased from Hamamatsu (Bridgewater, NJ). The first PMT (510-nm dichroic mirror, 400- to 480-nm barrier filter) detected the endogenous fluorescence and the second harmonic signal. GFP signal was detected with the second PMT (570-nm dichroic mirror, 505- to 560-nm barrier filter). mCherry, mRFP and td-tomato were detected on the third PMT (590- to 650-nm barrier filter).
- Objectives
 - All the objectives used in this method are from Olympus America
 - 10X UPLSAPO 10X N.A. 0.4 air objective Olympus
 - 25x XLPL25XWMP, N.A. 1.05 water immersion objective
 - 40X UPLSAPO 40XS N.A. 1.25 silicone oil objective
 - 60X UPLSAPO60x, N.A 1.2 water immersion objective

- Imaging platform and tongue holder device. The dimensions and details for the fabrication of the tongue device and the imaging platform are freely available upon request.
- XY Motorized Stage HLD117 (Prior, Rockland, MA) equipped with software to perform automated tiling and stitching.

3.9 Set up of the experiments

3.9.1 Anesthesia—Intraperitoneal (IP) administration of ketamine [100 mg/kg] and xylazine [10 mg/kg] mixed with normal saline. The prepared solution can be kept at room temperature (25°C) for up to 7 d.

3.9.2. Lentiviral infection of genes interested in HNSCC cell lines—cDNAs encoding for the gene of interest are subcloned into the intermediate vector pENTRsfII and transferred to the lentiviral expression vector pLESIP [15]. Lentiviral stocks are prepared and titrated with HEK-293T cells as packaging cells. SCC cells were infected with the virus for 16 hours. After that, cells were returned to normal growth medium. Infected cells are isolated with fluorescent activated cell sorting (FACS) and selected with puromycin (1 µg/ml).

3.9.3 Xenograft tumor generation—Athymic nude mice or SCID mice are anesthetized using isoflurane 2–5% in the induction chamber then transferred onto a downdraft table, where they are maintained under continuous anesthesia through nose cone. The tip of the tongue is gently pulled out of the mouth by using toothless tweezers. Cells (0.5 million to 5 million cells suspended in 0.05 ml media without serum) are loaded in an insulin syringe and injected in the submucosa of the lateral/anterior 1/3 of the tongue. To this end, the needle is inserted up to approximately 1–2 mm. Animals are fed with soft dough diet from the day of injection. One week after the injection, animals in each group are monitored and prepared for imaging.

3.9.4 Chemical carcinogenesis model—The carcinogen-induced tongue tumors develop by administering 50 mg/ml of 4NQO in the drinking water. We use the highest grade of 4NQO and formulate aseptically with sterile propylene glycol, as a vehicle in the chemical hood on low heat overnight. The carcinogenic water is changed once a week. 4NQO water treatment is terminated between 8 and 14 weeks and replaced with regular drinking water till the end of experiment. The carcinogen exposure, handling and waste disposal are conducted under the institute guidelines (NIH Department of Occupational Health & Safety; DOHS). Personal protective equipment (PPE; ie. double gloves, lab coat, goggles) are worn at all times whenever exposed to chemical or animals. Contaminated cage bedding and water bags are treated as hazardous waste.

4.0. Procedures

4.1 Pre-imaging preparation (10 min)

1. Anesthetize the mouse by intraperitoneal injection of ketamine-xylazine mixture (10 µl per g body weight). Place the mouse on a heating pad to maintain its body temperature at 37 °C throughout the imaging procedure.

CAUTION - All experiments dealing with live animals should be conducted in accordance with all relevant animal use and care guidelines and regulations. All experiments in this study were approved by the National Institute of Dental and Craniofacial Research (NIDCR, National Institute of Health, Bethesda, MD, USA) Animal Care and Use Committee.

CRITICALSTEP - The body temperature of mice drops very quickly after anesthesia, especially in nude mice, if they are not warmed sufficiently during the preparation phase. It is fundamental to preserve the body temperature as close as possible to the physiological state (37°–38°C).

2. Apply eye ointment to the eyes of the mouse to prevent corneal damage.
3. Blood vessel labeling (optional - 10 min)

To label the blood vessels, if desired, inject your choice of fluorescent dye or other appropriate reagent intravenously. Otherwise, proceed to Step 4.

CRITICALSTEP - Intravenous injections can be administered either via the retro-orbital sinus or the tail vein. We recommend retro-orbital injection as it is usually faster and less technically challenging. However, care must be taken to ensure that the injection is administered correctly in order to avoid permanent mechanical damage to the mouse eye.

CRITICALSTEP - It is important to ensure that surgical anesthesia is attained before injection. Test for the absence of the hind foot reflex by gently pinching the mouse toes. The injection should be administered early in the procedure during the deepest stage of anesthesia to minimize discomfort to the animal. This also reduces the likelihood of sudden movements by the mouse (e.g., the mouse blink reflex) that may cause mistakes during injection.

4. Preparation and positioning of the tongue device (5 min)

Insert the rectangular glass coverslip (18×30 mm) into the tongue device (Fig 3). Secure the tongue device on the imaging platform with the screw and keep the clamp loosely open. Place the mouse in the prone position on the imaging platform. Use toothless forceps to gently pull out the mouse tongue and leave it at the corner of the mouth.

CRITICALSTEP – Handle the tongue very gently to avoid trauma and bleeding, which will interfere with the imaging. During the tongue positioning, do not to move the tongue beyond the lower incisors to avoid cuts and/or bleeding. Place the tongue device next to the tongue. Open the clamp using the clamp screw. Insert the toothless forceps through the opening of clamp and

gently grab the tongue. Pull the tongue through the clamp and tighten the screw to secure the tongue.

CRITICALSTEP - Sometimes the tongue muscles are not fully relaxed during the initial phase of the anesthesia, making it difficult to pull out the tongue. Wait for a few minutes to allow the tongue to relax. The clamp should be slightly tightened to prevent the tongue from slipping out. Avoid applying excessive pressure on the tongue since it will affect blood and lymph circulation.

CAUTION- The tongue should be pulled out enough to expose a large area for imaging. Avoid excessive pulling since it may cause suffocation. The tongue should be flattened and not rotated or twisted.

Once the tongue is in position, place the heat pad underneath the animal and insert the rectal probe to maintain the mouse body temperature.

4.2. Intravital imaging (30 min-2 h)

CRITICALSTEP- To avoid unnecessary waiting time, turn on lasers in advance of experiment setup so that the lasers will be ready for use once the tongue is prepared for imaging

1. Move the imaging platform onto the pre-heated stage on the microscope and maintain the body temperature at 37–38°C through the heated pad on the stage.

CAUTION- The oral cavity should be moistened with drops of water every 15 minutes to prevent oral dryness.

CRITICALSTEP- For long imaging sessions (>1 h), adequate depth of anesthesia should be monitored closely, and repeated half-doses of ketamine-xylazine solution should be administered periodically (approximately every 45–55 min) by subcutaneous injection. The injection should be performed carefully, to not cause any shifts of the imaging field.

4.2.1 Xenograft Imaging—For xenograft imaging we use a combination of different objectives: 10X, 25X, 40X, or 60X. Since the tumor cells are fluorescently labeled, the imaging area can be easily identified by epifluorescence microscopy. Using a 10X objective select the optimal field of view based on the location in the tumor and the brightness of the cells. Change the objective to a higher magnification and perform a pre-scan of the area to optimize the settings of the PMTs for each channel in order to visualize structural features with minimal background noise. Mice can be imaged for a few minutes to acquire single frames, z-stacks, short time sequences (Fig. 5) or for several hours (up to 4) to acquire either 3D or 4D time-lapse movies.

CRITICAL STEP - If the same area needs to be imaged for multiple days, acquire an initial image of the area at the lowest magnification (10X) and perform a z-stack to acquire the SHG signal. It is also crucial to orient the mouse in the same direction relative to the microscope. SHG signals are polarization dependent and the pattern will change if the mouse is placed on the scope in a different direction. Increase the magnification progressively by changing the objective, focus on the desired structures, and acquire

additional z-stacks that will be used as reference points (Fig. 4). In the next imaging session with the same animal it is fundamental to position the tongue in the holder with the same orientation, and to begin a continuous scan of the tongue with the 10X objective until the reference SHG pattern is found. At this point, the objective can be changed to the desired magnification and before acquiring the image make small corrections to the field of view to match the SHG reference patterns.

CRITICALSTEP- z-plane drift can occur occasionally during the time-lapse acquisition. The minor drift can be corrected by using the software processing in the image processing and analysis step. However, if the drifts cause the drastic changes from the starting imaging field, the scan field should be realigned before continuing.

CAUTION - While exploring the imaging field, z-plane advancement toward the tongue can break coverslip in the holder. If this occurs, the experiment should be temporary halted and the coverslip should be replaced.

4.2.3. Carcinogen model imaging—As previously mentioned it is not possible to predict where the cancerous lesions will form upon administration of 4NQO. For this reason, the best approach is to perform the scanning of the entire tongue using a 10X or a 25X objective. Set up the first field of view in the upper left corner of the tongue. Adjust the setting to acquire a z-stack and open the control of the motorized stage and set the scanning matrix to perform the tiling (4×5 for 10X objective 10×20 for the 25X objective). The software will automatically perform the 3D tiling and save the files as a concatenated tiff series and as a volume rendering (Fig. 6).

4.4 Termination of the experiment

1. Remove the animal with the imaging platform from the microscope stage.
2. Loosen the clamp screw and release the tongue from the tongue device holder. Gently press the tongue back into the mouth, avoiding the lower incisors.
3. If you intend to keep the mouse for further experiments, inject the animals subcutaneously with warm 0.9% normal saline at 40–80 ml/kg/24hr to replace the fluid loss. Place the mouse in the prone position on a heating pad and allow the mouse to recover. Monitor the mouse for anesthesia recovery and return it to its cage when it has recovered its ability to stand (righting reflexes are returning), which also indicates an ability to again regulate its body temperature.

CAUTION- Follow all relevant animal procedure guidelines for post anesthesia recovery

4. If the experiment is terminal, euthanize the mouse by carbon dioxide inhalation or other approved methods as determined by your institutions rules, regulations and guidelines.

4.5 Image analysis

Export the images in the appropriate format for subsequent analysis. We routinely use ImageJ FIJI or Metamorph to perform image corrections such as contrasting, changes in

brightness, application of median filters on the 12-bit images. 3D rendering and 4D movies are generated using Imaris (Bitplane).

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Highlights

- A non-invasive method to perform Intravital microscopy in tongue models for oral cancer
- This method allows to image cancerous lesions at the tissue, cellular, and subcellular level
- This method enables longitudinal studies up to 20 weeks

Figure 1. Schematics of the two main experimental models used to image tumor development in the mouse tongue

A. Xenograft model in immunocompromised mice. Human HNSCC cell lines are transfected with fluorescent probes and/or shRNA using lentiviral-based vectors and FACS sorting. Engineered cells are injected in the ventral anterior side of the tongue of immunocompromised mice (nude or SCID). Animals develop tumors within 2–3 weeks from the injection. Tumors are allowed to grow for a maximum period of 15 weeks. Metastatic cell lines invade the locoregional lymph-nodes in 5–10 weeks. **B.** Carcinogen model. 4NQO is administered to immunocompetent mice (e.g. C57B6 background) in the drinking water for 16 weeks. Cancerous lesions become visible on the surface of the tongue starting from 16–18 weeks. Tumors are allowed to grow for a maximum of 25–30 weeks.

Figure 2. Diagram of the tongue holder

A. The holder is composed of two plastic flaps connected by a flexible rubber hinge. The flaps have one hole each and the lower one has a groove that can accommodate a thin squared coverslip (18×30 mm). **B.** The tongue is pulled out of the mouth with toothless forceps and carefully sandwiched in between the two flaps, which are kept in place by a screw. **C.** The holder is secured to a pre-warmed metallic platform by a metallic pin, which is tightened by a screw. Finally, the platform is placed on the pre-warmed stage of an inverted microscope with the hole coinciding with the location of the objective. **D.** Overall view of the setup.

Figure 3. Low and high magnification imaging of the tongue using transgenic mice

K5-GFP/mTomato mice were anesthetized and the tongue placed in the holder, as described in the legend to Fig. 2. The tongue was imaged by MPM (excitation wavelength 930 nm, emission filters 505–560-nm for the GFP and the 590–650 nm for the td-Tomato) with a 10X (A) or a 40X (B) objective. **A.** Low magnification imaging of the whole tongue using 3D stitching. Scanning was initiated from one of the anterior corners of the tongue. 200 nm z-stack were automatically acquired following the path described in the left diagram. Left images show the maximal projection of an individual field of view acquired on the dorsal (upper panel) or the ventral (lower panel) side of the tongue. In the dorsal side filiform (arrowheads) and fungiform (arrows) are shown. In the ventral side and area with blood vessels are shown (arrows). Bar 300 μ m. Right images show the overall reconstruction of the tongue highlighting the basal layer (green). Bar 1 mm. **B.** High magnification imaging of selected areas of the tongue. Left images show volume rendering of the dorsal (upper panel) or the ventral (lower panel) side of the tongue. Bar 100 μ m. Right panels show side view of the volume rendering of the filiform (upper) and fungiform (lower) papillae. The GFP expressing cells are primarily localized in the basal layer (BL) and to a lesser extent to the body of the papillae (asterisks). Bars 100 μ m.

Figure 4. Volume rendering of Xenograft in situ

A- OSCC3 cells were engineered to express GFP-Rab25 (green) or mCherry (red). Cells were mixed in a 1:1 ratio and injected in the tongue of a nude mouse. After 17 days (D17) the margin of the tumor was identified by epifluorescence and a z-scan (150 μm) was performed by using MPM (same parameters as legend to Fig. 2 and using a 25x water immersion objective). SHG signal was collected using a 400–480 nm emission filter. The two cell populations and the edge of the tumor are clearly distinguished in both the xy and xz view. After 2 days (D19), the tumor was imaged in the same location using the SHG signal as a reference point. As previously shown (4), a reduction in the number of cells

lacking Rab25 was observed, indicating that this cell population has a higher metastatic potential. Bars 150 μm . B- Maximal projections of z-stacks imaged over 4 consecutive days (D23–D26). Upper panels show the SHG signal and lower panels show a comparison of the behavior of cells lacking Rab25 (red) or cells expressing GFP-Rab25 (green). Cell lacking Rab25 migrate away from the primary tumor (arrows), whereas cell expressing GFP-Rab25 do not. Bar 100 μm (adapted from [3])

Figure 5. Time-lapse analysis of the tumor

OSCC3 cells were engineered to express either GFP-Rab25 or GFP and injected into the tongue of nude mice. After 5 weeks, the edge of the tumor was imaged by MPM (930 nm excitation wavelength) in time-lapse modality using a 60X water immersion objective. The upper panels show a close-up of a single cell expressing GFP (broken line) and a series of still images extracted from the time-lapse series showing the formation of membrane protrusions from the plasma membrane (insets). The lower panels show that Rab25 is localized in a series of dynamic vesicles that undergo fusion over time (insets). Bars 5 μ m. Time min:sec.

Figure 6. Long term imaging of the carcinogen-induced tumors

A. K5-GFP/mTomato mice were treated for 16 weeks with 4NQO as described in the Methods. The dorsal sides of the tongues were imaged by MPM once a week for 7 weeks (D12 to D19), as described in legend to Fig. 3, and volume rendering were generated. **B.** Cells expressing the GFP are organized in large clusters, which represent an expansion of the basal layer (compare to untreated animals in Fig. 3) Bar 1 mm. Inset 1 and 2 show the slow changes in the size of two main clusters. Bars 500 μm. **C.** An area with high GFP intensity was imaged by using a 25X objective. A z-scan was performed in the enlarged area showing a significant enrichment in GFP expressing cells in one fungiform papillae as a consequence of the expansion of the basal layer. The right panel shows the maximal projection of the Z-scan. Bar 150 μm.