



Enabling high-resolution diagnostic oral confocal laser endomicroscopy in mice[☆]

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ARTICLE INFO

Keywords:

Oral cancer
Confocal Laser Endomicroscopy
Imaging
Parpi-FL
Acriflavine
Digital biopsy
Fluorescent methods

ABSTRACT

Therapeutic prevention of oral squamous cell carcinoma (OSCC) will avoid significant morbidity and mortality. To observe and measure the *in vivo* efficacy of therapeutic challenges, microscopic-level diagnosis without animal sacrifice is required. This study introduces a refined diagnostic methodology for non-invasive cellular-level imaging for diagnosis of micro-lesions by utilizing high-resolution scanning-fibre confocal laser endomicroscopy (ViewnVivo) with topical fluorescence imaging agents. We detail the development and standardization of imaging protocols using a fluorescent, cell-permeable cancer-targeting agent (PARPi-FL) as a cancer-targeting agent and a pan-cytoarchitectural (acriflavine) agent in a pre-clinical murine 4-NQO induced OSCC model. We provide comprehensive methodology for the *in vivo* identification of the progressive stages of oral carcinogenesis from microscopic lesions, supported by an annotated signature guide correlating with conventional histopathology. Our findings demonstrate that *in vivo* CLE imaging with both PARPi-FL and acriflavine clearly distinguishes between histologically normal and pathological oral tissue. Tissues with histologic dysplasia and carcinoma demonstrated PARPi-FL positivity and an aberrant nuclear staining pattern with acriflavine, compared to the regularly spaced nuclear staining of normal nuclei. Crucially, this methodology detects microscopic changes not visible to the naked eye, but histologically abnormal. Our observation model of progressive oral carcinogenesis has the potential to accelerate standardised interrogation of early molecular diagnostic applications and novel therapeutic efficacy, whilst reducing the need for animal sacrifice. This will result in faster validated translation to human applications, advancing effective early oral cancer detection and prevention.

1. Introduction

Head and neck squamous carcinoma (HNSCC) develops in the surface mucosa of the oral cavity, oropharynx, nasopharynx, larynx and hypopharynx and globally represents some of the most common cancers [1–5]. Over 90 % of head and neck cancers, excluding cutaneous malignancies are squamous cell carcinomas, with oral squamous cell carcinoma (OSCC) being the most common anatomical subtype [3,6]. Diagnosis of OSCC, particularly tongue cancer requires careful

management [7], and late-stage diagnosed OSCC is associated with a poor 5-year survival rate of ~ 40 %, due to limited advancements in targeted therapy in the last 30 years, with metastases and locoregional recurrences resistant to current therapies [3]. Oral potentially malignant disorders (OPMD) are at an elevated risk of progression to OSCC. Thus, developing an inexpensive, non-invasive test for early OSCC detection could significantly improve patient outcomes [8]. Pertinently, early detection of micro-lesions and treatment of HNSCC/OSCC dramatically improves the 5-year survival rate to 84 % and enhances the quality of

[☆] This article is part of a special issue entitled: 'Editors Collection - YMETH' published in Methods.

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<https://doi.org/10.1016/j.ymeth.2025.04.015>

Received 18 February 2025; Received in revised form 14 April 2025; Accepted 24 April 2025

Available online 24 April 2025

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life, often eliminating the need for invasive surgical procedures [9].

The oral cavity is accessible for diagnosis through direct observation, and since oral cancers develop from the epithelial surface, this allows for visual inspection and, in principle, diagnosis. Unfortunately, it is not possible to distinguish between OPMD and early-stage microscopic cancers by visual inspection alone. Therefore, OSCC diagnosis relies on an invasive, painful, time-intensive physical surgical scalpel biopsy and analysis by a pathologist. Even an expert pathological analysis cannot, however, always provide a conclusive diagnosis. A non-invasive, non-destructive and rapid method technology that would allow visualization of tumor versus non-tumor lesions could dramatically improve screening, detection and monitoring of small potentially malignant lesions, and therefore survival outcomes, for this cancer.

Previous attempts to image the oral cavity using fluorescence visualisation devices or to diagnose oral neoplastic lesions in humans and murine pre-clinical models have had limited success [10–15]. A recent study combined a micro-gradient-lens-based side-view endomicroscopy system with transgenic mice expressing fluorescently labelled cells that was able to provide non-invasive longitudinal cellular-level *in vivo* imaging [16]. While capable of single-cell imaging, this approach was limited to the buccal mucosa due to only side-view availability and therefore too specialised for monitoring more general OSCC outcomes in pre-clinical studies. All methods so far described, relied on fluorescence intensity imaging to distinguish between normal and cancerous tissues, which is difficult to quantify objectively and did not provide single-cell and micro-scale resolution. Thus, achieving reliable, non-invasive, cellular-level longitudinal imaging of the entire murine oral mucosa remains a challenge.

A recent systemic review and meta-analysis of the diagnostic accuracy of various confocal laser endomicroscopy (CLE) devices for non-invasive imaging of human OSCC highlighted the potential benefits of this approach, including high micro-sensitivity and specificity for diagnosing OSCC [17]. However, the review, which included three *in vivo* studies using fluorescein as the imaging agent, did not evaluate OPMD or epithelial dysplasia. While promising, the limited number of existing studies and small sample sizes suggest a potential risk of bias and restricts meaningful conclusions about the ability of CLE diagnostic imaging to distinguish between human OPMD, oral epithelial dysplasia and OSCC. Additional studies with a standardized methodological approach are therefore needed to provide more conclusive evidence that could facilitate changes in OSCC diagnostic clinical practice [17–19].

In addition to fluorescein, other imaging agents have more recently been trialled in conjunction with CLE imaging. PARP1 (polyadenosine diphosphate-ribose polymerase) plays a crucial role in the cellular DNA damage response and is elevated in the nucleus of many tumour types, including oral cancer [20]. PARPi-FL, a fluorescent non-mutagenic small molecule inhibitor of PARP1, derived from the therapeutic AZD2281 (olaparib) [21], can cross cellular and nuclear membranes and binds to PARP1/2 [22]. We have previously validated the uptake, 95 % sensitivity and specificity, and use of PARPi-FL as a cancer fluorescent imaging biomarker in *ex-vivo* studies conducted in mice, pigs and fresh human specimens of cancers in the oral cavity, pharynx and oesophagus, with PARPi-FL delivered topically and imaged using the ViewnVivo® FIVE2 [22–24]. Acridine (C₁₄H₁₄ClN₃), a topical antiseptic, also serves as an exogenous fluorophore when applied to the oral cavity, labelling all cell nuclei and permitting pan-cellular cytoarchitectural visualization [25,26].

We have conducted feasibility studies on human OSCC diagnostics using another CLE device, the InVivage® (OptiScan PTY Ltd, Australia), in conjunction with imaging agents such as PARPi-FL [23,25,27], acridine, and fluorescein [26,28]. These *in vivo* pre-clinical trials, supported by matched histopathology and documentation, demonstrated the painless and rapid capture of images at the microscopic level from multiple mucosal sites, improving diagnostic precision and accuracy [26]. These studies preferentially localized fluorescent contrast to OSCC with PARPi-FL [27] and utilized annotation software [25], without

requiring physical tissue biopsy. However, for further proof-of-principle of these combined technologies, an increased sample size and variety of lesion types are needed to address data reproducibility for quantitative predictive analysis.

To ensure a consistent methodological approach, in this study we evaluated the ViewnVivo® Five 2 CLE device (OptiScan PTY Ltd, Australia), designed specifically for pre-clinical and translational research using identical technology to the InVivage® also coupled with the imaging dyes; fluorescent PARP inhibitor (PARPi-FL) [23,24], and acridine, used in previous human studies [25–27]. We describe the use of topical fluorescent imaging methods to investigate the potential of live CLE imaging technology for oral pre-cancer/cancer diagnosis in the 4-NQO pre-clinical OSCC model [29–32].

Ulcerations and oral keratosis (leukoplakia) that cannot be scraped off are the most common OPMD [8,33–35]. These are clinical terms for lesions appearing as deep cracks in the surface epithelia or flat whitish plaques on the tongue and buccal mucosa of both humans [8,33] and mice [32]. Leukoplakia, particularly erythroleukoplakia has tumorigenic potential [8,33], and therefore requires monitoring over time for the possibility of transition to malignancy. We describe the methods, reagents, and models enabling live, instantaneous assessment of microscopic oral pathological changes to; (1) accelerate the translational potential of instantaneous early oral pre-cancer/cancer diagnostics for the detection of minute pathological changes before the onset of clinically detectable lesions, and (2) enable live, non-sacrificial serial monitoring of OSCC and oral disease therapy trials in pre-clinical models, eliminating the need for physical biopsy and potentially replacing it in clinical practice.

2. Device, pre-clinical model, tools, anaesthesia and micro-imaging enhancement acquisition

2.1. Device

Scanning Single Fiber Endomicroscopy (FIVE) systems, such as the second generation Five2 ViewnVivo® (hereafter referred to as ViewnVivo®, Fig. 1A, Table 1), are miniaturized, non-destructive, point-scanning confocal microscopes. They offer micro-resolution (optical slice thickness/resolution; lateral (0.55 µm) and axial (5.1 µm)), and a field of view (475 x 475 µm), with user-controlled z-axis translation (up to 400 µm). This enables the isolation of individual cell layers (single frame, continuous capture, Z-stack or roll-back (60 frames)) and the acquisition of megapixel images [36].

The ViewnVivo® features a 488 nm illumination source and user selectable wavelength detection for imaging of PARPi-FL (Abs_{max} 507 nm/Em_{max} 525 nm) and acridine (Abs_{max} 416 nm/Em_{max} 514 nm). This portable imaging device, equipped with a 3.5 mm diameter x 66 mm long hand-held flexible probe for enhanced accessibility, holds significant potential for various clinical applications [37], including oral cancer diagnosis, as demonstrated by our previous studies [19,23–25].

2.2. Induction of OSCC using 4-NQO in C57BL/6 mice

In vitro models often fail to capture early-stage transformation, and human tumour xenografts cannot mimic the immune system's impact on tumorigenesis or facilitate the testing of novel combination therapies. To overcome these limitations, we conducted our studies using a murine model of OSCC induced by chronic oral administration of the tumorigenic compound 4-nitroquinoline-1-oxide (4-NQO) in drinking water (Fig. 1B) as previously described. [32] Wild type mice (C57BL/6 background, 10–14 weeks) or mice lacking genes impacting cell death pathways targeted by frequent genome alteration in human HNSCC [38]; TNF (Tumour Necrosis Factor; *Tnf*^{-/-}) [39] or MLKL (Mixed Lineage Domain Like pseudokinase; *Mkl*^{-/-}) [40] on a C57BL/6 background were treated with drinking water with or without (control) 100 µg/ml 4-NQO (Sigma) for 14 weeks and then given unadulterated drinking water ad

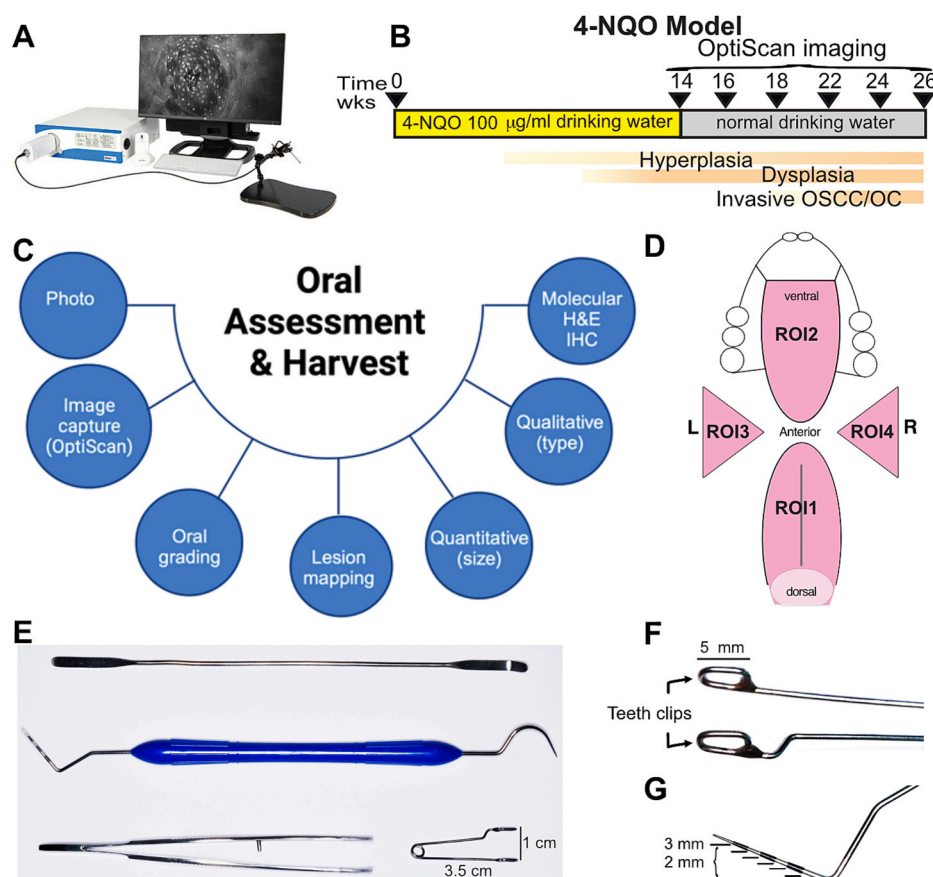


Fig. 1. Imaging device, model and oral examination tools. A) CLE imaging equipment used in this study. A) ViewnVivo® (OptiScan Imaging Ltd, Australia) with scanning probe attached. B) Schematic of murine OSCC tumour model induced with the 4-NQO carcinogen in the drinking water. C) Schematic of full analysis conducted at intervals or tissue harvest. D) Example of oral cavity clinical diagram used for lesion mapping on tongue dorsal and ventral surfaces and L, R buccal mucosa. E) Tools used for murine clinical examination; stainless-steel laboratory micro spatula (top), Perio Probe-Sickle clinical lesion measuring tool (middle), parafilm coated forceps (bottom, left), stainless steel Celentano Murine Mouth Opener (CMMO) (bottom, right). F) Detailed images of CMMO and G) Perio Probe-Sickle clinical lesion measuring tool.

libitum (Fig. 1B). This model induces mutations and DNA strand breaks, replicating damage caused by tobacco use and ultraviolet light, thereby reflecting the clinical setting. 4-NQO induced oral lesions follow the typical clinical trajectory from hyperplasia, epithelial dysplasia to carcinomas [29–31]. Lesions develop at the epithelial surface, permitting visual inspection, clinical grading, lesion mapping, photographic recording and image capture (Fig. 1C, D, Table 1). A previous study using this model demonstrated, through conventional H&E pathology, ex-vivo ViewnVivo® CLE imaging and immunohistochemistry, that PARP1 is overexpressed in tumour lesions compared to normal tissue and is absent from the surrounding tongue musculature [24], and laid the groundwork for this *in vivo* study.

2.3. Optimising murine anaesthesia for imaging timepoints

We found that the anaesthetic method chosen is crucially important to obtain high quality images with the ViewnVivo®. Inhaled anaesthetics, such as isoflurane (Iso), although quick to work, did not provide sufficient sedation time, typically 30–60 min per mouse, required for oral cavity scoring and imaging of multiple areas with multiple agents. Initially, ketamine-xylazine (KX) was administered intraperitoneally (Table 2), but this combination was also unsatisfactory due to insufficient depth of sedation, resulting in facial muscle twitching and compromised image quality and was of insufficient duration (< 30 min). Subsequently, we assessed medetomidine-midazolam-fentanyl (MMF, Table 2) [41]. MMF induced an extended and deep state of anaesthesia, allowing for clinical oral examination, standard/multiple oral lesion

mapping, and imaging with the ViewnVivo® coupled with imaging dyes. The ViewnVivo® output image quality of the oral cavity was optimal under MMF anaesthesia, since mice were sufficiently and reliably sedated and allowed rapid post-recovery with naloxone-flumazenil-atipamezole (NFA) antagonization with minimal adverse effects (Table 2) [41]. The MMF/NFA anaesthetic/reversal combination was therefore used in all subsequent studies (Fig. 1C) [42].

2.4. Step by step live *in vivo* oral imaging digital biopsy

4-NQO-induced OSCC tumour lesions develop on the epithelial surface of the tongue (dorsal/ventral), lips, buccal mucosa, floor of the mouth, oropharyngeal sites and buccal mucosa [32], mirroring human disease. This permits visual inspection, scoring (Table 3, adapted from Tang et al) and [29] lesion mapping performed by a qualified dentist with expertise in oral medicine/pathology (A.C), and progressive imaging (Fig. 1C). Specialized tools (Table 1, Fig. 1E–G) [32,43] were used to facilitate oral examination with photography documentation as standard. We developed a step-by-step workflow (Fig. 2) in a pilot study.

Step by step live imaging protocol

Step 1. – Anaesthesia

Sedate mice with MMF administered i.p. (Table 2), place mice on a heated platform and liberally apply sterile eye lubricant (Table 1). After righting reflex loss, insert CMMO (Table 1) into oral cavity.

Step 2. – Score, record, photograph

Assess mice clinically for development/extent of oral disease (ante-roventral and dorsal tongue, palate, floor of the mouth, lips, buccal

Table 1
Oral Examination and Imaging Equipment.

Purpose	Name	Brand name/ Manufacturer/Reference
CLE imaging	ViewnVivo®	OptiScan PTY, Vic, Australia
Murine mouth opener	Celentano Murine Mouth Opener (CMMO)	[32,43]
White light for clinical oral examination	580 Lumen HI powered zoom headlamp	Perfect Image, Vic, Australia
Oral examination	Stainless-steel micro spatula	Sigma-Aldrich
Lesion measuring tool	Perio probe-sickle	Medi Dent Disposable International, Australia
Clinical digital photography	Nikon D5600 105 mm lens	Nikon Corporation, Japan Nikon AF-S MICRO NIKKOR 1:2.8 G ED
Digital camera ring flash	MACRO Ring Flash (MF18) Meike MK-14EXT TTL for Nikon	Nissin Digital, Japan Meike Global
Removal of food debris/ biofilm	Mini-cotton swab	Daiso Industries Co. Ltd, Japan
Dye application	Disposable micro- applicator brush (1.5 mm)	OZavenue, eBay
Sterile eye lubrication	Poly Visc	Alcon Labs, Texas, USA
Nuclear fluorescence imaging agent	Acridiflavine (0.1 % in distilled water)	Pharmacy
Molecule-targeted fluorescence imaging agent	PARPi-FL (1000 nM in 30 % PEG300/sterile PBS)	[22,23,24,25,27,29]

Table 2
Anaesthesia dosage and antagonization for mouse oral imaging.

Purpose	Name	Brand name/Manufacturer
Anaesthesia –agonist	KX, intraperitoneal (i.p.)	
	Xylazine 10 mg/kg	Ilium Xylaxil-20, 20 mg/mL, Troy Laboratories Pty, Ltd, NSW Australia
	Ketamine 80–100 mg/kg	Ketamine, 200 mg/2mL Baxter Pharmaceuticals, Baxter India Private Ltd, Haryana, India
Anaesthesia –agonist	MMF, intraperitoneal (i. p.)	
	Midazolam 5 mg/ Kg	Dormicum® 5 mg/mL, Roche Pharma AG, Grenzach-Wyhlen, Germany
	Medetomidin 0.5 mg/Kg	DOMITOR®, 1 mg/mL, Orion Corporation, Espoo, Finland
	Fentanyl 0.05 mg/ Kg	Fentanyl®-Janssen 0.1 mg/2mL, JANSSEN-CILAG, Neuss, Germany
	NFA, intraperitoneal (i. p.)	
Anaesthesia –antagonist	Naloxone 1.2 mg/ kg	Junalox hydrochloride, 400 mg/1 mL, Juno Naloxone Juno, Naloxone hydrochloride, 400ug/1 mL, Juno Pharmaceuticals, Vic, Australia
	Flumazenil 0.5 mg/ kg	Flumazenil, 500ug/5 mL, Anexate (Australia) Pharmaco Ltd
	Atipamezole 2.5 mg/kg	Antisedan 5 mg/1 mL, Atipamezole Zoetis Australia Pty, Ltd, NSW Australia

mucosa as described [32,43]), using white light aided by mini-spatula/parafilm coated tweezers (Table 1). Use perio probe-sickle (Table 1) to quantitatively measure oral lesion size (mm). Use visual clinical gross lesion grading system (0 – 4, Table 3) to qualitatively map and score (Fig. 1D). Photograph standard anatomical Regions Of Interest (ROIs)/lesions with flash fitted digital camera (Table 1).

Step 3. Prepare cleansing swabs

Pre-soak (5 s) sterile (autoclaved) x2 double sided cotton swabs in

Table 3
Murine oral lesion graded scoring.

Grade	Description	Features
0	Normal	No visible lesions
1	Mild	Epithelial discoloration, erythema, erosions
2	Intermediate	Mild, hyperplastic lesions
3	Severe	Small, berry-like, elevated lesions
4	Most severe	Ill-defined, indurated, exophytic lesions, cauliflower-like surface

sterile PBS/saline.

Step 4. Cleanse oral cavity

Remove food debris/biofilm from the entire oral cavity prior to imaging by gently wiping with cotton swabs (tongue dorsal (5 s), ventral (5 s), buccal mucosa bilaterally L + R (5 + 5 = 10 s).

Step 5. Prepare PARPi-FL for application

Soak (5 s) x4 micro-applicator brushes in PARPi-FL (Table 1).

Step 6. Coat oral cavity with PARPi-FL (approximately 10–15 µL of solution/microbrush).

Gently paint oral cavity surfaces with micro-applicator brushes soaked with PARPi-FL (tongue dorsal (5 s), ventral (5 s), buccal mucosa bilaterally L + R (5 + 5 = 10 s) and incubate for 1 min.

Step 7. Repeat step 3

Step 8. Repeat step 4

Remove unbound excess PARPi-FL with clearing step to increase signal specificity and reduce background noise.

Step 9. Micro-image with ViewnVivo® probe

CLE image by placing probe in contact with tongue/oral mucosa of anesthetized mice following order of 4 ROIs 1; mid-tongue dorsal 2; mid-tongue ventral 3; mid-left buccal cheek mucosa and 4; mid-right buccal cheek mucosa plus additional areas of clinical abnormality/exposed lesions as recorded on oral map (Fig. 1D).

Step 10

Repeat steps 5–9 with 0.1 % acridiflavine (approximately 10–15 µL of solution/ microbrush).

Step 11. Reverse anaesthesia

Administer NFA (i.p.) to reverse MMF anaesthesia (Table 2).

The probe was directly applied to the predefined regions of interest (ROIs 1–4) within the oral cavity or mapped lesions (Fig. 1D, Fig. 2, step 9, arrow) for image acquisition. A Z-stack mechanism, capable of imaging up to 400 µm in depth, was utilized to obtain progressive images of cell layers. For all experiments, imaging was performed using a band-pass filter of 515–575 nm (LP515), with a scanning speed of 1 frame per second (1024 x 1024 pixels) and an aspect ratio 1:1. Auto brightness was set between 33–80 %, gain levels were adjusted to 2400 for acridiflavine and 2700 for PARPi-FL. Laser power output ranged from 200 to 1000 mW, depending on the site and the uptake of PARPi-FL and acridiflavine. Based on previous work PARPi-FL applied topically to oral tissues penetrates up to 100–200 µm, primarily localizing within the epithelial layer and superficial lamina propria [44]. This pattern reflects the high expression of PARP1 in epithelial and malignant cells. Similarly, acridiflavine has been reported to penetrate to a depth of approximately 200–300 µm, depending on tissue characteristics (e.g., keratinization) and exposure duration. CLE images were recorded (~70–100 mm). Imaging was conducted with a lens NA = 0.3, comparable to a 10 × objective, and grayscale images were visualized on a monitor for optical diagnosis (HPZ25n), as performed in previous *ex vivo* and human OSCC feasibility studies [23,25,26] but refined for this analysis. To prevent cross-contamination between imaging dyes, imaging of mice was performed sequentially, firstly with PARPi-FL and then after extensive washing of the probe and imaging tools with 70 % ethanol, followed by imaging with acridiflavine. When imaging cohorts of mice, post-acridiflavine imaging, the probe and all tools were decontaminated, and operator gloves and absorbent benchcoat replaced to avoid carryover of acridiflavine dye.

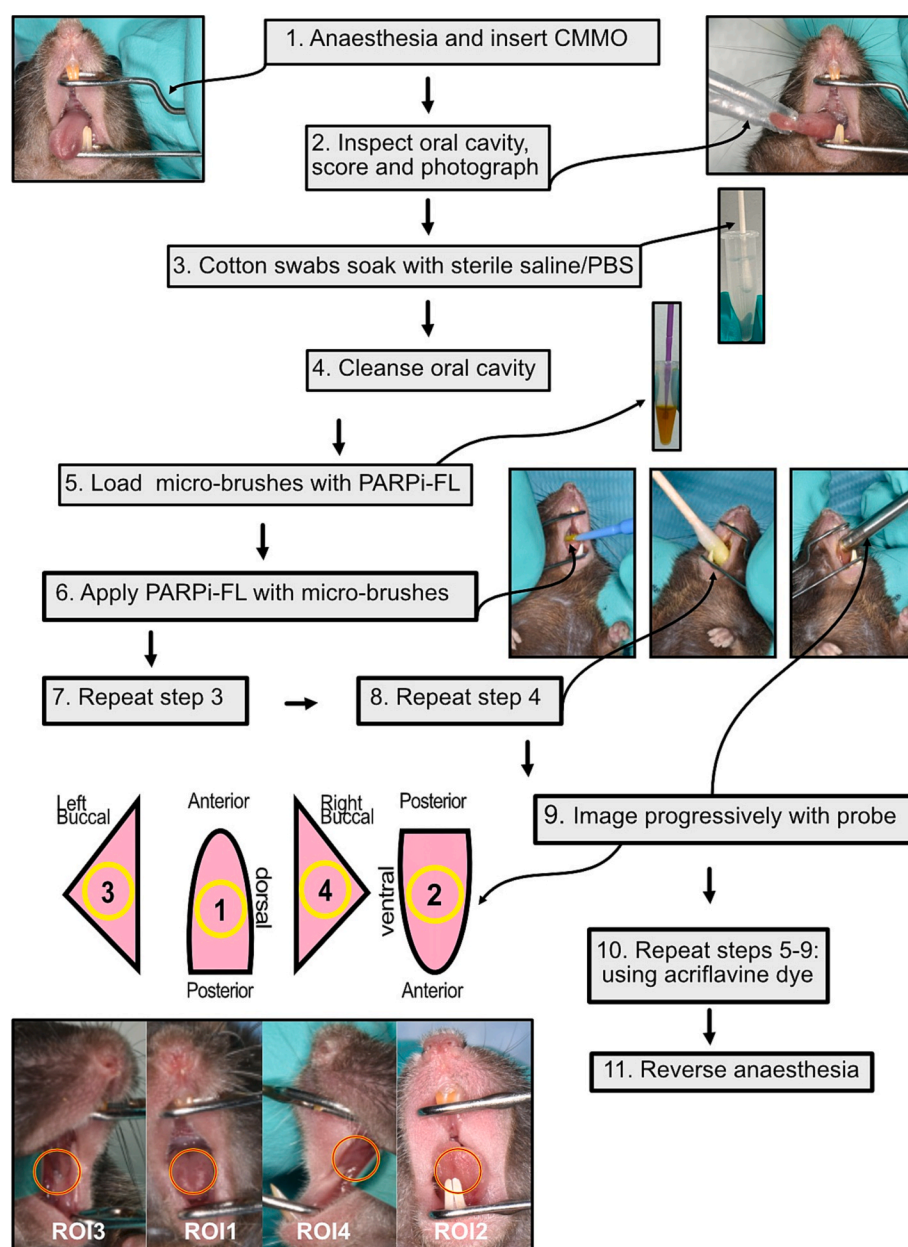


Fig. 2. Workflow diagram for oral scoring, topical dye application and oral imaging Numbers in brackets indicate the steps in [section 2.4](#) of this manuscript where the methods are explained in detail.

3. Results and discussion

3.1. Live CLE with imaging agents in the normal murine oral mucosa

We performed *in vivo* imaging of the oral cavity with the View-nVivo® in anesthetized wild-type C57BL/6 (wt) mice aged 10–15 weeks, the typical age for initiating 4-NQO treatment. A pilot study was conducted to ascertain baseline steady state imaging data from control wt mice, which were not part of the 4-NQO study. The oral cavity was clinically scored according to our scoring criteria ([Table 3](#)), which indicated that the entire oral cavity was clinically normal ([Fig. 3A](#)). Using our established protocol ([Fig. 2, steps 1 to 11](#)) four intra-oral regions of interest (ROIs): 1) mid-tongue dorsal, 2) mid-tongue ventral, 3) mid-left buccal cheek mucosa and 4) mid-right buccal cheek mucosa was assessed ([Fig. 2 step 9, Fig. 3B](#)). At the micro-level, no nuclear PARPi-FL positivity was observed in these areas at steady state ([Fig. 3C](#)). However, we noted non-specific natural light emission of

the non-nucleated dorsal papillae of the tongue with both PARPi-FL and acriflavine, presenting as a regularly spaced array. Low-level background staining of the dorsal tongue was also observed with both imaging agents ([Fig. 3C, D](#)). Positive nuclear acriflavine staining was observed in the squamous epithelial cells of the buccal mucosa (fluorescent staining regularly distributed and non-overlapping and with a regular ovoid shape) ([Fig. 3D](#)). These findings align with previously reported live CLE results of normal human oral mucosa stained with PARPi-FL [[25](#)] or acriflavine [[25,26](#)].

3.2. Live CLE with imaging agents in 4-NQO-treated mice detects oral abnormalities

Building on the pilot study, a cohort of wt control and 4-NQO-treated mice (16, 18, and 21 weeks post 4-NQO treatment initiation, [Fig. 4A](#), $n = 3$ –10/timepoint) were clinically assessed under MMF anaesthesia ([Fig. 4B](#)) and subsets also assessed for pathology ([Table 4](#)) at euthanasia

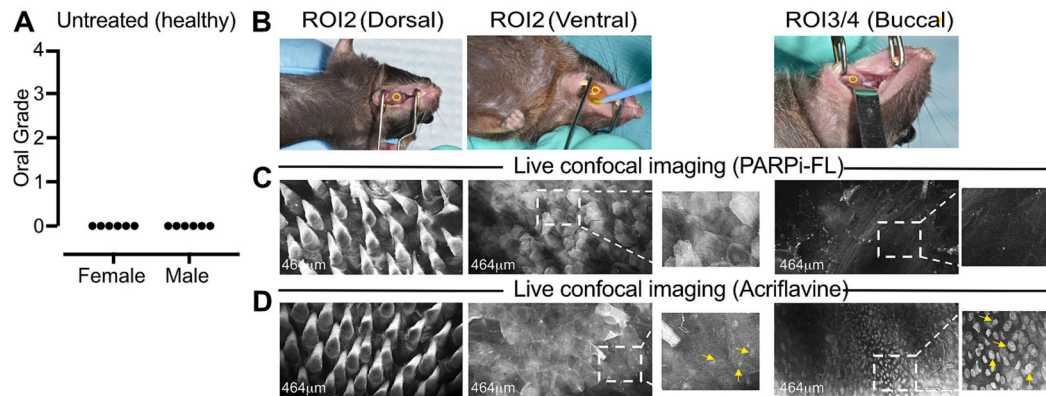


Fig. 3. Standard CLE analysis with imaging agents of normal murine oral cavity. A) Graphical representation of oral clinical scoring under anaesthesia of naïve wt (control, n = 6 male, n = 6 female) mice. B) Correlative representative white light photomicrographs of standard anatomical sites (Regions of Interest: ROIs) of the oral cavity used in this study; tongue dorsal (ROI1), ventral mucosa (ROI2) and buccal mucosa (ROI3/4) with C) correlating greyscale optical biopsy CLE images using the ViewnVivo® in conjunction with PARPi-FL or D) acriflavine topical staining. Yellow arrows indicate individual stained nuclei. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4B). Oral clinical scoring of control mice revealed no abnormalities (grade = 0, Fig. 4C). Control mice were also CLE imaged at the 4 ROIs with the ViewnVivo® using the topical contrast dyes according to the step-by-step live imaging protocol. Imaging of each ROI demonstrated an absence of nuclear staining across all timepoints, except regularly spaced, homogeneously acriflavine stained ovoid nuclei in the buccal epithelial mucosa (Supplemental Fig. 1A–C). This finding was consistent with normal mucosae pathology at the time of necropsy 0–5 weeks later (grade 1, Fig. 4D) and as described above (Section 3.1).

Next, we clinically assessed 4-NQO-treated wt mice (16, 18 or 21 wks, Fig. 4A). At these stages, the visual clinical grade ranged from 2 to 4 (Fig. 4B), increasing in severity with time. Whole tongue pathology was assessed at necropsy in a subset of mice at each timepoint, when diverse lesion types were observed (grades 2–6, maximum grade indicated, Fig. 4C). Prior to pathology assessment, mice were live CLE imaged with PARPi-FL and acriflavine at the 4 ROIs (Fig. 4D–F). In general, no clinical abnormalities were visually observed at the 4 ROI sites. However, in contrast, to ROI CLE imaging of control treated mice (Fig. S1A–D), the 4-NQO treated mice showed readily distinguishable micro-epithelial changes. Non-specific natural light emission of the non-nucleated dorsal papillae (ROI1) was again evident with both PARPi-FL and acriflavine imaging, but also appeared disordered with gaps, even though appearing clinically normal (Fig. 4D–F, Fig. S1E–G). This appearance correlated with architectural loss of specialised epithelium on later pathology analysis (Fig. 4G, Fig. S1H).

ROI CLE imaging of 4-NQO treated mice with acriflavine at the 4 ROI sites (Fig. 4D–F) was predominantly negative but occasionally detected small clusters of positively staining nuclei even in areas appearing both clinically and visually normal (Fig. 4F, ROI2, Fig. S1G, ROI2). CLE imaging with acriflavine of the buccal (ROI3/4) of this cohort of wt 4-NQO treated mice in most instances and as expected demonstrated regularly spaced positive ovoid nuclei with homogeneous acriflavine staining (Fig. 1F, ROI3/4). Occasionally, the nuclear density per captured image also appeared to be elevated (Fig. S1G, ROI3/4). CLE imaging with PARPi-FL of ROI sites from 4-NQO treated mice, which appeared clinically normal was generally negative (Fig. 4E, Fig. S1F). Overall random ROI CLE imaging coupled with acriflavine was the most powerful combination to detect potential “abnormal” areas not visible to the naked eye and which on later pathology assessment were also graded normal (grade 1: Fig. 4G, Fig. S1H).

To assess the consistency of our findings, ROI CLE imaging with acriflavine of an additional cohort of *Mik1*^{-/-} treated 4-NQO mice (n = 3) and control treated (n = 2) was performed. This analysis confirmed that

ROI screening of clinically normal lesions in mice prone to developing oral cancer (4-NQO treated) may be detected at the micro-level before clinical visibility as minuscule abnormal areas of detected nuclear positivity (Fig. 4H–J), compared to control mice of the same genotype where the detection of acriflavine nuclear positivity was absent (Fig. 4K).

At times the ROI areas imaged (dataset from Fig. 4A–C) correlated with areas of dorsal/ventral tongue visible clinical abnormalities (Fig. 5A), which were also imaged (16 weeks (11 abnormalities from 7 mice), 18 weeks (10 abnormalities from 3 mice) and 21 weeks (12 abnormalities from 3 mice) in 4-NQO treated mice. In these instances, live imaging with PARPi-FL of clinically scored lesions (Fig. 5B, left, middle grade 2 and 6 respectively) detected clusters of positive staining nuclei, which on subsequent tissue harvest were confirmed as areas of non-normal pathology (Fig. 5C, left, middle). Changes in regular nuclear size/shape were also occasionally observed in the buccal mucosa on CLE imaging with acriflavine staining (Fig. 5B, right), correlating with underlying inflammation including multinucleate giant cells on pathology analysis (Fig. 5C, right).

In summary, where epithelial changes were located on the dorsal tongue this was associated with a loss of epithelial thickness (papillae disorganisation/loss), therefore microscopic nuclear positivity at the single cell level could be observed by CLE imaging. On the tongue ventral surface where papillae are ordinarily absent, areas of micro-abnormality were more readily observed by CLE imaging with acriflavine, even in the absence of an abnormality to the naked eye. Acriflavine nuclear positivity may be associated with multiple cell types; epithelial, connective tissue or inflammatory and small micro-areas of positivity may be detected in the absence of a clinical or histopathological lesion.

3.3. Correlative accuracy of imaging clinically defined oral lesions from oral keratosis to carcinogenesis with conventional histopathology reporting

3.3.1. Imaging pre-malignant oral lesions (epithelial ulcerations, erythema erosions and keratosis)

Having defined the ability of CLE with fluorescent agents to identify microscopic abnormal areas that cannot be seen with the naked eye through clinical scoring, we evaluated the qualitative diagnostic accuracy of CLE imaging for a range of lesions observed clinically, from early OPMD to dysplasia and invasive malignancy (Table 3), comparing them with traditional H&E pathology (Table 4). The desired lesion type for imaging was identified by clinical scoring, imaged with CLE and fluorescent agents (Table 1), followed by excision for pathology preparation and reporting.

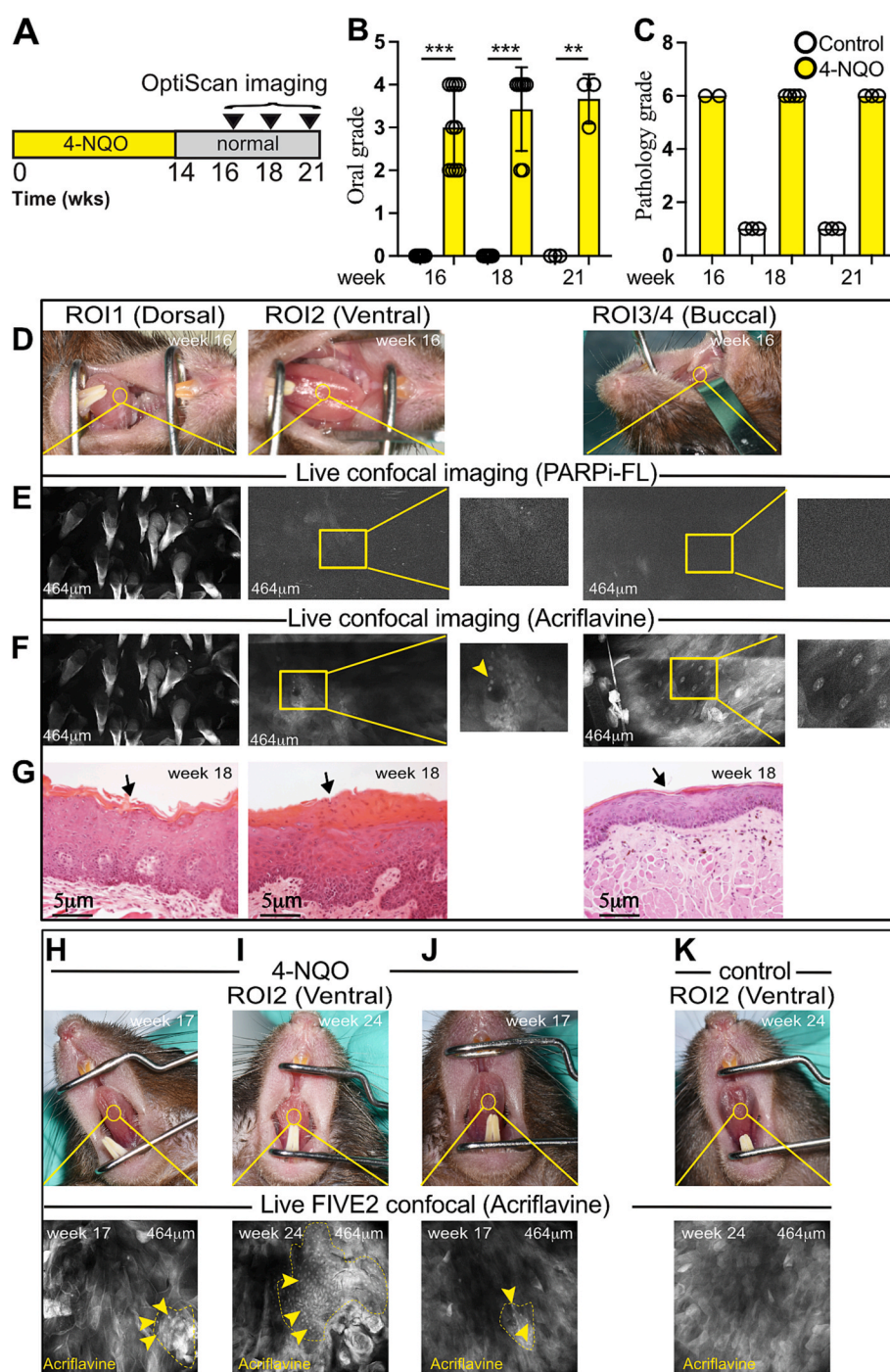


Fig. 4. ROI imaging of murine induced OSCC with PARPi-FL and acriflavine. A) Schematic of 4-NQO oral tumour model induced in wt mice via drinking water with CLE imaging timepoints indicated. B) Graphical representation of oral clinical scoring ($n = 3-10$ /timepoint) and C) correlative histopathological scoring at the time points indicated in A), each circle represents an individual mouse. D) Representative white light photomicrographs indicating imaged ROIs, with correlative greyscale optical biopsy live imaging using the ViewnVivo® in conjunction with E) PARPi-FL or F) acriflavine topical staining and G) histopathological photomicrographs of imaged ROIs. H-J) Representative white light photomicrographs of clinically normal ROI2 area from 4-NQO treated or K) control treated mice, with H-K) correlative greyscale optical biopsy live imaging using the ViewnVivo® in conjunction with acriflavine. Clinical scoring: 4-NQO mice $n = 10$, $n = 3-10$ /timepoint) and control mice $n = 6$, $n = 3-6$ /timepoint. Dotted yellow line indicates lesion area, solid yellow line and circle/square indicates area or lesion imaged or magnified area *in vivo* or *ex-vivo*. Black arrows in G) indicate surface imaged, yellow arrowheads F, H-J) representative positive staining nuclei. Statistical analysis; paired t test ** $P < 0.01$, *** $P < 0.001$. Error bars = SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

An additional data set of wt mice post-4-NQO administration ($n = 18$) with ulceration or keratosis was used to facilitate this analysis of mild grade lesion types (Table 3). Clinical keratotic type lesions (including leukoplakia) developing on both the dorsal and ventral

surfaces were identified as small flat whitish plaques (typically 0.2–3 mm) in 4-NQO treated mice. In general, while these lesion types were clearly visible using the CLE ViewnVivo® as gaps in the papillae (dorsal surface), specific PARPi-FL nuclear staining was either absent or at a low

Table 4
Oral pathology grading of H&E-stained sections.

Grade	Description
1	Normal
2	Hyperplasia
3	Mild dysplasia
4	Moderate dysplasia
5	Severe dysplasia
6	Invasive carcinoma

level (Fig. 5D–F). In contrast, acriflavine positive nuclei were detectable within areas defined as ulcers/keratotic lesions (Fig. 5 D–F, yellow dotted area/arrowheads), whereas no nuclear acriflavine positivity was observed on the normal tongue mucosal epithelium (Fig. 3). Post imaging pathology may show normal pathology (grade 1, Fig. 5D–F), however this is not too surprising since such microscale lesions can be (are often) lost during the histological process. Our observations underline the power of live imaging with acriflavine to examine keratotic lesions at a cellular level with the potential to identify lesions requiring further monitoring.

3.3.2. *Imaging advanced lesions: Mild hyperplastic lesions, squamous papilloma to OSCC*

Mice exposed to 4-NQO provide an opportunity to explore imaging signatures associated with a range of lesion types. We utilized the previous dataset to facilitate CLE imaging of intermediate to advanced lesions in our murine 4-NQO model (clinical grade 2–4, Table 3), including squamous papilloma (whitish raised plaque lesion, typically associated with a mild hyperplastic pathology n = 5), berry-like (small red elevated round lesion n = 7) and those lesions with a cauliflower-like surface (ill-defined, white indurated exophytic lesions n = 17) with some examples pooled from an additional dataset of *Tnf*^{−/−} mice (squamous papilloma n = 5, berry-like n = 2). These more advanced lesions are associated with moderate to high-grade dysplasia and invasive OSCC in both mice [29–32] and humans [34,45].

Following CLE imaging with PARPi-FL and acriflavine, lesions were excised, processed for histopathology and reported to provide a CLE image and physical biopsy comparative. Imaging of oral squamous papilloma areas was performed with PARPi-FL, which, in general, was associated with low-level background staining (Fig. 6A, B). Acriflavine staining and imaging of this lesion type was associated with clusters of positively staining nuclei (Fig. 6A, B), not found in the normal control oral cavity (Fig. 3, Fig. S1A–D). Excised imaged lesions were confirmed as hyperplasia/mild dysplasia histologically (Fig. 6A, B, grade 2–3). Advanced-type lesions, defined as berry/cauliflower-like in the 4-NQO model (Table 3) showed more numerous nuclei staining and therefore a more clearly defined differential elevation in nuclear contrast after PARPi-FL imaging (Fig. 6C) compared to benign lesions (Fig. 5, Fig. 6A, B). In addition, nuclear PARPi-FL and acriflavine imaging correlated well, with both imaging agents demonstrating distinct nuclear presence with increased crowding (Fig. 6C–E). Excised imaged lesions were confirmed as mild to severe dysplasia or invasive carcinoma (Fig. 6C–E, grades 3, 5, 6).

In conclusion, acriflavine positive nuclei were observable by CLE imaging in any lesion, whether mild or severe and the pattern of staining indicative of the severity of the lesion. On the other hand, PARPi-FL positive nuclei were only found in advanced lesions. We have demonstrated that either dye, PARPi-FL or acriflavine combined with CLE imaging has the ability to detect and stratify oral lesions without a physical biopsy. The ability to distinguish virtually between low- and high-grade dysplasia, requires a more rigorous quantitative image analysis and possibly facilitated by using both dyes.

3.4. *Longitudinal sequential imaging*

A significant advantage of non-invasive CLE imaging, both experimentally and clinically, is the ability to monitor individual lesions longitudinally without requiring multiple biopsies. As an example, we tracked the progression of an oral lesion in a wt 4-NQO treated mouse (Fig. 7 A, B). We observed the accumulation of acriflavine positive nuclei from week 17 to 25 post 4-NQO initiation, as the clinical lesion advanced from keratotic (grade 1) to squamous papilloma (grade 2), which coincided with the appearance of PARPi-FL positive nuclei. As another example, the accumulation of PARPi-FL positive nuclei was used to track lesion advancement from wart-like/mild to clinical berry-like stage in *Mkl1*^{−/−} mice treated with 4-NQO (Fig. 7 C). In each instance the accumulation of acriflavine and PARPi-FL nuclear staining at lesion sites over time was associated with clinical lesion progression.

3.5. *Limitations of murine oral cavity CLE imaging with fluorophore agents*

Certain limitations were encountered during CLE imaging, particularly in accessing posterior ventral surfaces and the floor of the mouth in mice due to probe size but this would not be a limitation in patients. Additionally, keratinization of cauliflower-like lesions hindered accurate detection, due to reduced imaging dye penetration, limiting the z-stack function. Such lesions, when observed in patients clinically, particularly when they exhibit rapid growth, irregular shape, or are located in high-risk areas, are already considered suspicious and often warrant surgical excision to rule out malignancy and prevent further complications [46].

4. **Conclusions**

We present the first live *in vivo* application of oral CLE imaging in a murine model, utilizing two complementary stains; PARPi-FL and acriflavine. This study establishes and validates a robust and comprehensive methodology for generating real-time digital biopsies at the micro and single-cell level within the murine oral cavity, achieving resolution comparable to conventional histopathology while employing non-toxic fluorescent agents. Key advancements include the detection of micro-areas of abnormality not visible to the naked eye, optimization of anaesthesia protocols, the standardization of topical staining procedures and the correlation of bio-imaging data with clinical observations and conventional biopsy pathology scoring. This approach holds significant translational potential for human OSCC detection, offering distinct advantages over traditional clinical sampling methods. Specifically, the murine model enables the imaging of multiple tumours at varying stages of OSCC progression, facilitating the study of specific genetic lesions and the evaluation of novel fluorophores. Importantly, this method facilitates the detection of oral abnormalities before they become clinically apparent, a capability of considerable clinical value.

Additionally, this approach enables longitudinal monitoring of tumour development at the microscopic level, thereby reducing the need for large animal cohorts in therapeutic studies. Despite some limitations, such as probe size and the challenge of imaging highly keratinized lesions, the use of identical dyes and CLE technology in both murine and human studies [25–27] ensures the translational relevance of our findings. Building on this work should provide proof-of-principle for clinical trials involving non-invasive virtual real-time biopsies for OSCC, particularly in evaluating precancerous lesions and screening. The transition from hyperplasia to dysplasia remains challenging to diagnose without biopsy, underscoring the need for reliable predictive methods. Our study suggests that CLE imaging, coupled with appropriate fluorescent agents, could bring us closer to predictive human OSCC diagnosis. However, future advancements will require the development of specialized AI tools for quantitative image analysis, coupled with optimal fluorophores, beyond the scope of this study.

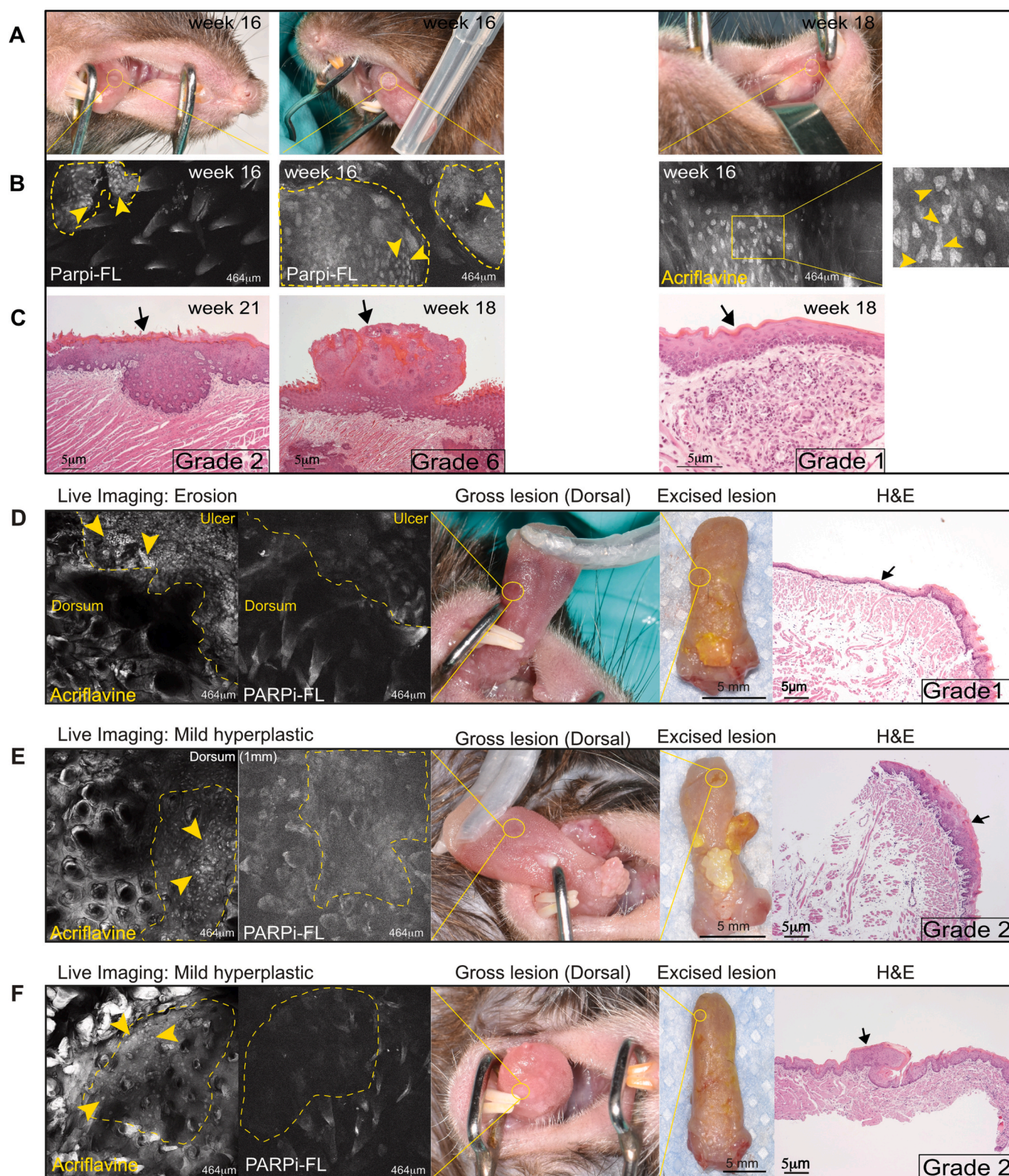


Fig. 5. CLE with imaging agents of murine oral lesions. A) Representative greyscale optical live biopsy CLE images using the ViewnVivo® in conjunction with PARPi-FL or acriflavine topical staining with correlative white light photomicrographs showing area scanned live *in vivo*, excised lesion and histopathological image from 4-NQO treated mice at the indicated timepoints, clinically scored as 1 mm nodular white lesion (left), 3 mm cauliflower-like lesions (middle) or buccal (right), with correlative greyscale optical biopsy with CLE in conjunction with PARPi-FL or B) acriflavine imaging agent and C) lesion histopathological photomicrographs at euthanasia. D) Histology, micrographs and CLE images with the induced stain of ulcer/erosive, grade 1 or E, F) keratotic (leukoplakia) grade 2, lesions from 4-NQO treated mice. Dotted yellow line indicates lesion area, solid yellow line and circle indicate lesion location *in vivo* or *ex-vivo*. Representative positive staining nuclei indicated by yellow arrow heads. Lesion imaged with ViewnVivo® in H&E section indicated by black arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

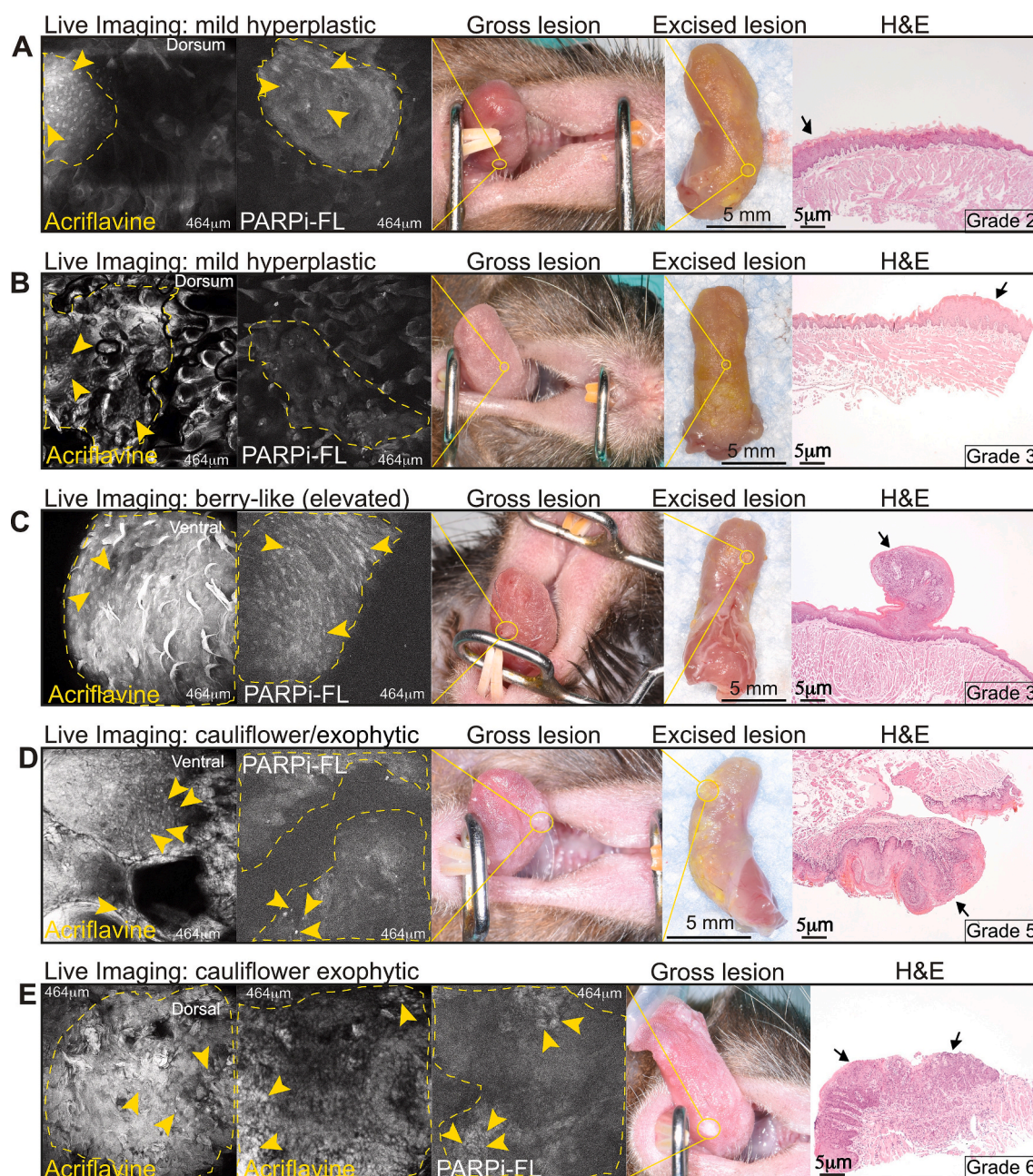


Fig. 6. CLE with imaging agents of transitional/advanced murine oral lesions. A, B) Representative greyscale optical biopsy CLE images using the ViewnVivo® in conjunction with PARPi-FL or acriflavine topical staining with correlative white light photomicrographs showing area scanned live *in vivo*, excised lesion and histopathological images from clinically observed papilloma, C-E) berry/cauliflower-like (leukoplakia) from 4-NQO treated mice. Dotted yellow line indicates lesion area imaged, solid yellow/black line and circle indicate lesion location *in vivo* or *ex-vivo*. Representative positive staining nuclei indicated by yellow arrow heads. Lesion imaged with ViewnVivo® in H&E section indicated by black arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5. Experimental section/methods

5.1. Ethics declaration

All experiments were approved by The Walter and Eliza Hall Institute Animal Ethics Committee (AEC 2018.042 approved 28/11/2018 and AEC 2023.014 approved 10/05/2023) in accordance with the Prevention of Cruelty to Animals Act (1986), the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes (1997), and with the ARRIVE guidelines [47]. Wild type mice or mice lacking TNF (*Tnf*^{-/-}) [39] or MLKL (*Mkl*^{-/-}) [40] all on a C57BL/6 background were used in this study. Mice were

housed under specific pathogen-free, temperature- and humidity-controlled conditions and subjected to a 12-hour light/dark cycle with *ad libitum* feeding.

5.2. The 4-NQO murine model of OSCC

OSCC was induced by chronic oral administration of 4-nitroquinoline-1-oxide (4-NQO at 100 µg/mL for 14 weeks in drinking water) followed by normal drinking water until oral cavity assessment, as previously described [32].

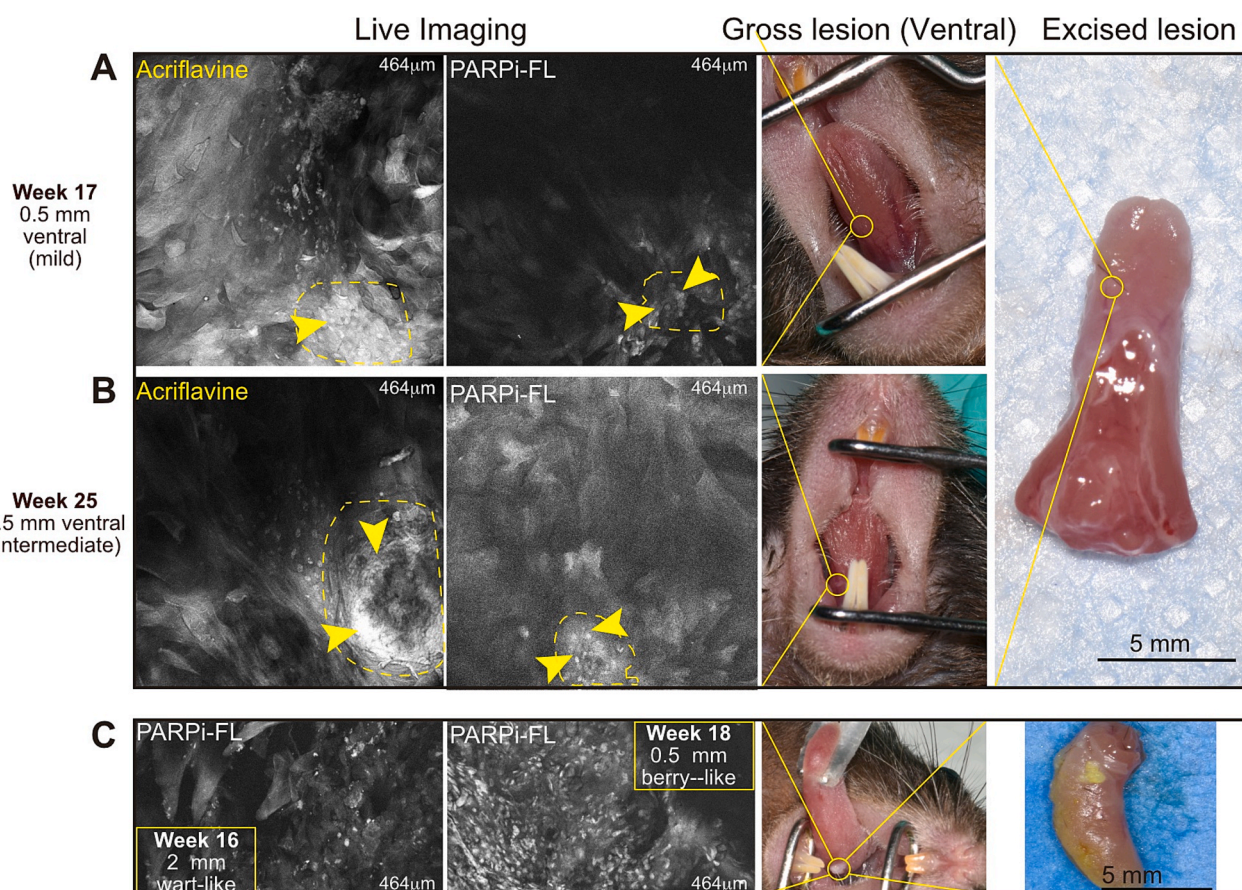


Fig. 7. Longitudinal CLE with imaging agents. A–C) Representative greyscale optical biopsy CLE images using the ViewnVivo® in conjunction with PARPi-FL or acriflavine topical staining at the indicated time points with correlative white light photomicrographs showing area scanned live *in vivo* at these timepoints and excised tongue at harvest. Dotted yellow line indicates lesion area imaged, solid yellow line and circle indicate lesion location *in vivo* or *ex-vivo*. Representative positive staining nuclei indicated by yellow arrow heads. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5.3. Preparation of traditional H&E histopathology biopsy

Subsequent to imaging, sample/lesion excision was performed. At necropsy, tongue specimens were collected and immersed in 10 % neutralized formalin overnight. The specimens were then paraffin-embedded (Tissue-Tek® TEC™, Sakura Finetek, USA), sectioned into 4 μm-thick slices, and automatically processed using a Tissue-Tek VIP® 6 AI Tissue Processor (Sakura Finetek, USA). Hematoxylin and Eosin (H&E) staining was performed using an automated stainer (Leica autostainer, XL, Leica Microsystems Pty Ltd, Vic Australia) followed by glass cover slipping (Leica CV50300, Leica Microsystems Pty Ltd, Vic Australia). Photomicrographs of H&E-stained sections were captured using an Axioplan 2 microscope (CarlZeiss, NSW, Australia) with objectives (×5/NA0.1, ×10/NA0.3, ×20/NA0.50).

5.4. Histopathological diagnosis

H&E-stained sections of the annotated tongue lesions were examined by a pathology registrar (J.R), who was blinded to the CLE data and 4-NQO exposure of the study groups. The findings were classified in line with the WHO Criteria for human oral epithelial dysplasia and squamous cell carcinoma [48]. The diagnosis was then further classified into 6 categories. (Table 4).

5.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism Data points

represent mean values, and error bars represent standard deviations. We used the paired *t* test to compare the differences between the groups with the level of significance for each result displayed as **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.

Ethics approval statement

All experiments were approved by The Walter and Eliza Hall Institute Animal Ethics Committee (AEC 2018.042 approved 28/11/2018 and AEC 2023.014 approved 10/05/2023).

Funding sources

This work was supported by The Garnett Passe and Rodney Williams Memorial Foundation (co-joint grant to LOR and JS ref: 2023_CG_Silke_Lim), IMPACT Philanthropy Application (Perpetual to LOR and JS ref: IPAP2023/0007), Technology Circle Donors (The Walter and Eliza Hall Institute of Medical Research to LOR and JS). LOR was also supported by a philanthropic donation to WEHI.

CRediT authorship contribution statement

Antonio Celentano: Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing. **James A. Rickard:** Methodology, Data curation, Writing – review & editing. **Jun Low:** Data curation. **Natasha Silke:** Data curation. **Ali Ibrahim Mohammed:** Data curation. **Elham Moslemi:** Data curation. **Rishi S. Ramani:** Writing –

review & editing. **Paula Demetrio De Souza Franca:** Writing – review & editing, Data curation. **Thomas Reiner:** Writing – review & editing, Data curation. **Michael J. McCullough:** Writing – review & editing. **Tami Yap:** Conceptualization, Writing – review & editing. **John Silke:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Lorraine A. O'Reilly:** Conceptualization, Investigation, Methodology, Project administration, Formal analysis, Funding acquisition, Supervision, Writing – original draft, Validation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: T. R. is a shareholder of Summit Biomedical Imaging and co-inventor on filed U.S. patent WO2016164771 that covers methods of use for PARPi-FL. T.R. is also a co-inventor on U.S. patent WO2012074840, covering the composition of matter for PARPi-FL. T.R. is a paid consultant for Theragnostics, Inc. This arrangement has been reviewed and approved by Memorial Sloan Kettering Cancer Center in accordance with its conflict-of-interest policies. The remaining authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to acknowledge Dr Lindsay Bussau (procurement, logistics & customer support manager at Optiscan (Australia)) for the initial support provided with the CLE technology. We also thank, S. Kelly, L. Spencer, D. Fayle, J. Leahy, J. Gilbert, G. Siciliano, M. Watters, J. Martin and N. Blasch for animal care, S. Holloway and J. House for veterinary care, C. Hall for genotyping and E. Tsui and team for histology preparation. We would also like to thank our consumers (D. Clements, A. Bradbury, A. Wilson, J. Byrne, N. Ross, L. Gilbert) for providing an insightful community perspective. Parts of Fig. 1 and some of the icons used to create the graphical abstract were created using BioRender.com under licence to The Walter and Eliza Hall Institute of medical Research, Australia in accordance with BioRender's Terms of Service and Academic Licence Terms ("License Terms").

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ymeth.2025.04.015>.

Data availability

Data will be made available on request.

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Glossary

- CLE:** confocal laser endomicroscopy
- OptiScan®:** second generation scanning single fiber endomicroscopy ViewnVivo® CLE device
- OPMD:** Oral potentially malignant disorders
- OSCC:** oral squamous cell carcinoma
- ROI:** region of interest
- PARP1:** polyadenosine diphosphate-ribose polymerase
- Parpi-FL:** fluorescent non-mutagenic small molecule inhibitor of PARP1