

Dual-excitation multispectral fluorescence lifetime imaging handheld endoscope for label-free metabolic and biochemical imaging of the oral mucosa

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This work introduces a dual-excitation, multispectral-detection, frequency-domain fluorescence lifetime imaging (FLIM) handheld endoscopy system for comprehensive autofluorescence imaging of the oral mucosa. The excitation wavelengths and spectral detection channels were selected to preferentially image the fluorescence of endogenous fluorophores relevant for oral epithelial cancer detection. The endoscope dimensions and optical specifications enable direct imaging of oral lesions with minimal patient discomfort. Comprehensive imaging experiments using standard fluorophores demonstrate the capabilities of the FLIM endoscope to measure a wide range of fluorescence lifetimes (0.1–12 ns) with high precision (~100 ps). Clinical *in vivo* label-free metabolic and biochemical endoscopic FLIM imaging was demonstrated by imaging a malignant oral lesion and healthy mucosa in a patient undergoing a tissue biopsy procedure.

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Introduction. The incidence of oral cancer has been increasing over the last 20 years, with over 58,000 new cases expected annually, according to the American Cancer Society (ACS) [1]. When diagnosed at early stages, the 5-year survival rate is 87%. However, when diagnosed at intermediate or advanced stages, the 5-year survival rate drops to 69% and 39%, respectively. Unfortunately, benign oral lesions are often difficult to distinguish from dysplasia or early-stage cancer. As a result, only about 30% of patients are diagnosed at early stages despite the fact that the oral cavity is easily accessible for direct examination. Hence, there is a critical need for new clinical technologies that will facilitate reliable early diagnosis of oral cancer and dysplasia.

There are several morphological, biochemical, and functional biomarkers of oral dysplasia and cancer that modulate tissue autofluorescence [2]. Both epithelial thickening and collagen degradation occur in dysplasia and cancer, resulting in a decreased contribution of collagen to tissue autofluorescence. Increased metabolic activity, a hallmark of epithelial cell malignant transformation, induces subtle changes in oral tissue autofluorescence originating from mitochondrial flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and the reduced form of nicotinamide adenine dinucleotide and its phosphorylated forms (NAD(P)H), which are highly specific to oral dysplasia and cancer. Additionally, endogenous protoporphyrin IX (PpIX) has been observed in dysplastic and malignant oral lesions [3]. Previous investigations have demonstrated the capability of fluorescence lifetime imaging (FLIM) as a significant tool for cancer detection [4–9]. In this Letter, we describe a novel dual-excitation multispectral FLIM endoscopy system specifically designed for label-free metabolic and biochemical imaging of the oral mucosa (Fig. 1).

Imaging system design. The endoscopy system was developed based on a versatile, practical, and cost-effective frequency-domain (FD) FLIM design recently introduced by our group [10]. Simultaneous fluorescence excitation at 375 nm and 445 nm is achieved using frequency-multiplexed digital pulse modulation of continuous-wave (CW) diode lasers (Toptica iBeam-Smart-375 and iBeam-Smart-445). Time-resolved fluorescence detection across four emission spectral bands is implemented using fixed-gain avalanche photodiodes (APD) (Hamamatsu C12702-11). The FD-FLIM engine, implemented using a common field-programmable gate array (FPGA) available in a development board (Xilinx ZedBoard Zynq-7000), provided synchronized laser modulation and fluorescence signal digitization. Simultaneous multispectral time-resolved fluorescence signal digitalization is performed at 250 MHz sampling rate using a four-channel ADC FPGA mezzanine card (Abaco

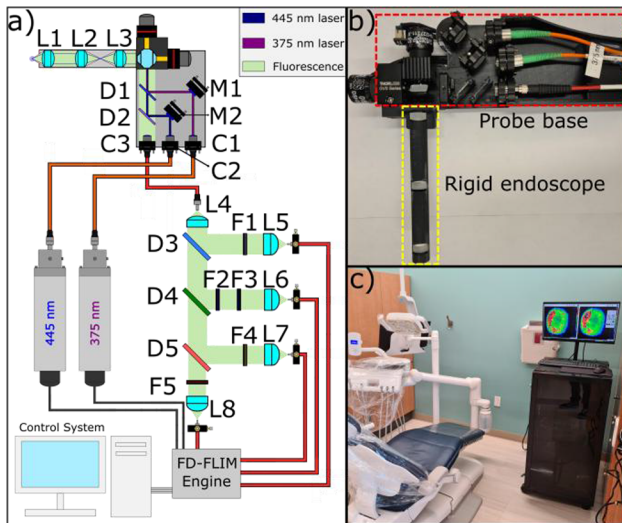


Fig. 1. (a) Schematic of the dual-excitation multispectral FD-FLIM endoscopy system. (b) Photograph of the handheld endoscopic probe. (c) Photograph of the FD-FLIM endoscopy system installed in a dental office at the Texas A&M College of Dentistry, Dallas, Texas.

FMC104). The use of the FPGA also permits real-time calculation of both the magnitude and phase frequency response of the fluorescence emission across multiple modulation frequencies and emission spectral channels. Dual-excitation multispectral FD-FLIM imaging and real-time frequency response calculation is performed at a pixel rate of 12.5 kHz.

To deliver the excitation light into the handheld endoscope probe (Fig. 1(a)), each laser output is coupled into 3 m multimode fibers (NA = 0.22, $d = 50 \mu\text{m}$, Thorlabs FG50UGA). In the handheld probe base, each laser excitation beam is collimated (C1–C2, Thorlabs F671FC-405) and combined into a common optical path by a series of reflective (M1–M2: Thorlabs BB05-E01) and dichroic (D1: Chroma T387lp; D2: Chroma ZT442dcrb-UF1) mirrors. The common optical path is aligned with the center of the lower two galvanometric mirrors (Thorlabs GVS202) used for simultaneous scanning of the two excitation beams onto the sample through a rigid endoscope built using three achromatic double lenses (L1–L3). The sample fluorescence emission is collected through the same lenses (L1–L3), de-scanned by the galvanometric mirror set, and coupled (C3, Thorlabs F220-SMA) into a 3 m multimode fiber (NA = 0.22, $d = 600 \mu\text{m}$, Thorlabs FG600AEA).

A photograph of the handheld endoscopic probe is shown in Fig. 1(b). The 3D-printed probe base (Fig. 1(b), red dashed box) is 71 mm in width and 210 mm in length and has holders for mounting the galvanometric mirrors, the rigid endoscope, and the alignment optics. The base was designed so that the height of the collimators and reflective/dichroic mirrors can be adjusted to match the optical center height of the lower galvanometric mirror. To facilitate the alignment of the optical paths for both the excitation beams and fluorescence emission, kinematic mounts (Thorlabs MK05 and MK11F) were used to position both the mirrors M1–M2 and the collimators C1–C3. The three optical fibers are fixed to the base to reduce the tension force on the collimators.

Oral epithelial lesions are typically smaller than 10 mm in length/width and can be found at any location inside the oral

cavity. To reach most oral lesions without inducing major discomfort to the patient, a rigid endoscope was designed with a field of view (FOV) of $\sim 78 \text{ mm}^2$ (circular FOV, $\sim 10 \text{ mm}$ in diameter), lateral resolution of $\sim 250 \mu\text{m}$, length $> 100 \text{ mm}$, and diameter $< 15 \text{ mm}$. Simulations were conducted in Zemax to determine components for a rigid endoscope with these specifications. A simple system with three achromatic lenses (L1–L3, $f = 25 \text{ mm}$, $d = 12.5 \text{ mm}$, Edmund Optics 65-971), where L1 works as an objective and L2–L3 serve as an image relay, was found to meet these requirements. The 3D-printed rigid endoscope consists of a lens tube (length, 123 mm; outer diameter, 13.5 mm) with slits for pressure holding the three lenses L1–L3 (Fig. 1(b), yellow dashed box). Lens L3 is placed at a distance to the midpoint between the two galvanometric mirrors equal to its focal length. Lens L2 is placed to form a telescopic system alongside lens L3, while Lens L1 is placed at the focal point of L2.

The fluorescence emission is transmitted through the collection fiber to an optical cage system, where the emission is collimated (L4, $f = 40 \text{ mm}$, $d = 1 \text{ in.}$, Thorlabs LA1422-A) and separated into four spectral channels through a series of dichroic mirrors (D3, Chroma AT440dc; D4, Semrock FF506-Di03; D5, FF01-647/57-25) and optical filters (F1, Chroma ET405/40x; F2, Chroma AT465lp; F3, Semrock FF02-475/50-25; F4, Semrock FF01-550/88-25; F5, Semrock FF01-647/57-25). The resulting emission spectral channels were customized to preferentially image the oral mucosa autofluorescence originating from collagen ($405 \pm 20 \text{ nm}$), NAD(P)H ($482 \pm 18 \text{ nm}$), FAD and FMN ($550 \pm 44 \text{ nm}$), and PpIX ($647 \pm 28 \text{ nm}$). The output of each spectral channel is coupled (L5–L8, $f = 25 \text{ mm}$, $d = 1 \text{ in.}$, Thorlabs LA1951-A) into a multimode fiber (NA = 0.50, $d = 600 \mu\text{m}$, Thorlabs FP600ERT) to transmit the emission light onto the active area of an APD installed inside an RF-shielded enclosure.

Imaging system optical specifications. The optical resolution, scan range, and co-alignment of the two excitation beams were measured as follows. The galvanometric mirrors were centered at the middle of the sensor of a CMOS camera (Allied Vision Manta G-419) mounted at the focal plane of L1. The voltage of each scanning mirror was varied independently, until the maximum scan angle (12.5°) was reached. At each voltage value, the resolution ($1/e$ beam diameter) and the distance to the center of each laser beam were imaged and measured for each axis, as shown in Fig. 2.

For both excitation wavelengths, the resolution was fairly constant at the middle of the scan range ($< 200 \mu\text{m}$ for 375 nm; $< 150 \mu\text{m}$ for 445 nm), before starting to increase with the distance to the center due to spherical aberration introduced by the lenses. Overall, the resolution was $< 300 \mu\text{m}$ for the two excitation wavelengths in both axes. The improved resolution with 445 nm excitation stems from system alignment: the endoscope's focal plane was optimized for 445 nm, leaving 375 nm slightly out of focus. Although 375 nm offers higher theoretical resolution, focusing for it would further degrade 445 nm performance due to its longer wavelength. We prioritized 445 nm to balance resolution across both wavelengths. There is a notable difference in the scan range between the two axes, due to the difference in distance between each scanning mirror and the first lens (L3) of the relay system, with the y axis (closer mirror) scanning larger angles before clipping compared to the x axis (farther mirror). The resulting FOV has an elliptical shape (semi-minor axis, 4 mm; semi-major axis, 5 mm) with an area

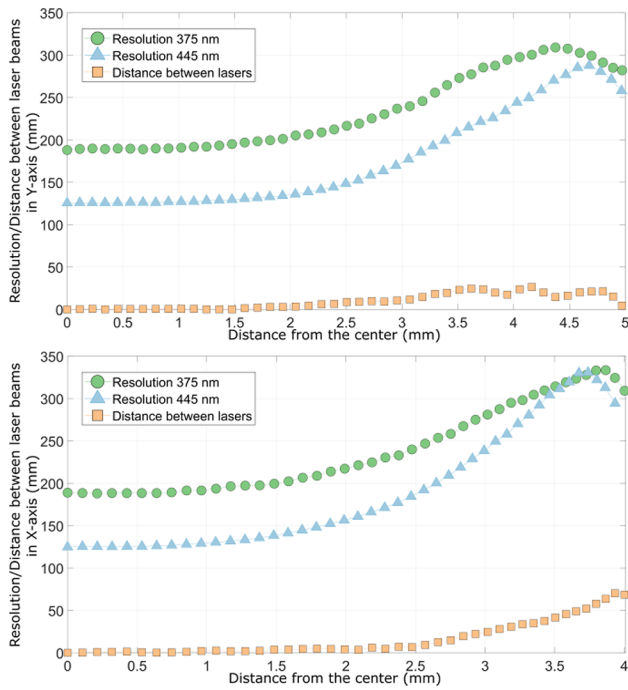


Fig. 2. Optical resolution, scan range, and co-alignment of the two excitation beams measured in both scanning axes. Resolution was measured as the $1/e$ beam diameter. Co-alignment was measured as the distance between the two excitation beams. The scan range in the y axis is larger than in the x axis.

of $\sim 63 \text{ mm}^2$. The distance between the two excitation beams was $< 50 \mu\text{m}$ across the full Y -scan and most of the X -scan, i.e., less than $1/5$ of the optical resolution of either excitation at any beam position, demonstrating co-alignment throughout the FOV.

Imaging speed and excitation power. All FLIM images were acquired at 12.5 kHz with 160×160 pixels ($\sim 2 \text{ s}$ per image), using average powers of 11 mW (375 nm) and 17 mW (445 nm) at the sample. To ensure safe *in vivo* imaging, maximum permissible exposure (MPE) was calculated per ANSI laser safety guidelines [11] (see Supplement 1). Calculations confirm excitation levels remain at least an order of magnitude below ANSI limits.

Validation with fluorescence standards. To quantify the precision of the dual-excitation multispectral FLIM endoscopy system to measure fluorescence lifetimes, a set of 12 fluorescence standard solutions were imaged: POPOP, NADH, anthracene, DPA, coumarin-1, 9CA, rhodamine B, Rose Bengal, fluorescein, FAD, coumarin-6, and rubrene (the solvent and concentration for each fluorophore solution are provided in Supplement 1, Table S1). Each fluorophore solution was loaded into a quartz cuvette and imaged using the handheld FLIM endoscope. Fluorescence average lifetime ($\tau_{\text{AVG-FDFLIM}}$) was calculated from FD-FLIM data via nonlinear least squares curve fitting [12]. The average lifetime ($\tau_{\text{AVG-TCSPEC}}$) was also measured using a calibrated time-correlated single-photon counting (TCSPEC) spectrometer (DeltaPro-DD, Horiba, Japan) using the same excitation wavelengths and emission spectral channels as in the handheld endoscopy system. Table 1 summarizes the average lifetimes measured upon specific excitations (λ_{ex}) and emission channels (λ_{em}) for each fluorophore solution. Good agreement

Table 1. Measured Average Lifetimes of Fluorescence Standards

Fluorophore	λ_{ex} (nm)	λ_{em} (nm)	$\tau_{\text{avg-TCSPEC}}$ (ns)	$\tau_{\text{avg-FDFLIM}}$ (ns)
POPOP	375	405 ± 20	1.289	1.274 ± 0.009
		482 ± 18	1.289	1.282 ± 0.014
		550 ± 44	1.294	1.299 ± 0.031
NADH	375	482 ± 18	0.499	0.520 ± 0.011
		550 ± 44	0.494	0.482 ± 0.009
ANT	375	405 ± 20	4.137	4.102 ± 0.059
DPA	375	405 ± 20	6.123	6.180 ± 0.063
		405 ± 20	3.149	3.065 ± 0.068
Coumarin-1	375	582 ± 18	3.207	3.314 ± 0.022
		550 ± 44	3.217	3.252 ± 0.040
9CA	375	482 ± 18	12.014	12.542 ± 0.121
		550 ± 44	11.932	12.386 ± 0.148
Rhodamine B	375	550 ± 44	1.616	1.574 ± 0.032
			1.547	1.569 ± 0.063
Rose Bengal	375	647 ± 28	0.102	0.109 ± 0.072
			0.092	0.098 ± 0.007
Fluorescein	445	482 ± 18	3.857	3.956 ± 0.089
		550 ± 44	3.944	3.937 ± 0.047
FAD	445	550 ± 44	2.959	3.399 ± 0.093
		647 ± 28	3.560	3.564 ± 0.048
Coumarin-6	445	482 ± 18	2.442	2.468 ± 0.009
		550 ± 44	2.457	2.499 ± 0.019
Rubrene	445	550 ± 44	7.433	7.473 ± 0.049
		647 ± 28	7.446	7.512 ± 0.094

between the two instruments was confirmed using correlation and Bland–Altman plots (Supplement 1, Figs. S1 and S2).

In vivo imaging of oral lesion. The dual-excitation multispectral FLIM endoscopy system was installed at the Texas A&M College of Dentistry (Fig. 1(c)). Following an IRB-approved protocol, a patient with an oral lesion in the left mandibular gingiva was imaged using the FLIM endoscope. An additional image was acquired from a healthy-appearing area on the contralateral side. After imaging, a biopsy was taken from the lesion center. Histopathology confirmed well to moderately differentiated squamous cell carcinoma (SCC).

The normalized multispectral fluorescence intensity maps indicate the fraction of the emission fluorescence detected at each emission spectral channel (λ_{em}) upon a specific excitation (λ_{ex}): $I_{\lambda_{\text{ex}}, \lambda_{\text{em}}}(x, y) = \frac{I_{\lambda_{\text{ex}}, \lambda_{\text{em}}}(x, y)}{\sum_{\lambda_{\text{em}}} I_{\lambda_{\text{ex}}, \lambda_{\text{em}}}(x, y)}$, where $I_{\lambda_{\text{ex}}, \lambda_{\text{em}}}(x, y)$ is the map of the absolute fluorescence intensity of emission spectral channel λ_{em} upon excitation λ_{ex} , and the sum is overall the emission spectral channels. The absolute multispectral fluorescence intensity maps were also used to calculate the redox ratio map reflecting the relative contribution of FAD ($I_{445, 550}(x, y)$) and NAD(P)H ($I_{375, 482}(x, y)$) to the tissue autofluorescence: $\text{REDOX}(x, y) = \frac{I_{445, 550}(x, y)}{I_{445, 550}(x, y) + I_{375, 482}(x, y)}$. In the FLIM endoscopy FOV of the healthy contralateral side, these maps were spatially homogeneous (Fig. 3, top). Stronger fluorescence was observed at the $482 \pm 18 \text{ nm}$ channel upon 375 nm excitation (associated with NAD(P)H contribution) and at the $550 \pm 44 \text{ nm}$ channel upon 445 nm excitation (associated with FAD and FMN contribution). The REDOX map was also spatially homogeneous with values ~ 0.5 . In the FLIM endoscopy FOV of the malignant gingiva oral lesion, all maps were spatially heterogeneous, as the FOV

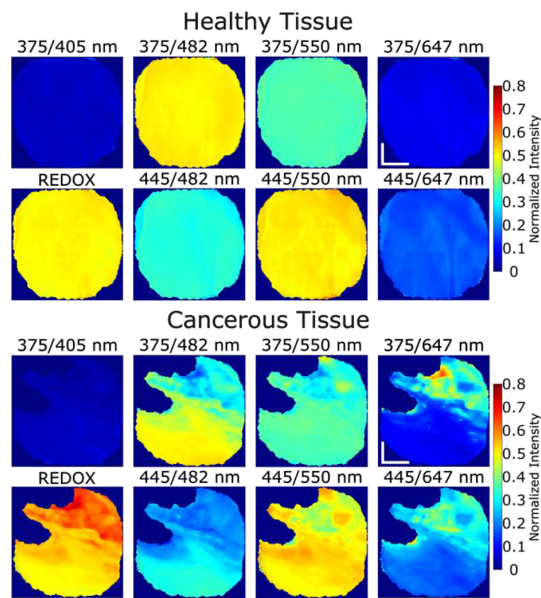


Fig. 3. Normalized multispectral fluorescence intensity maps for each combination of excitation and spectral emission channel ($\lambda_{ex}/\lambda_{em}$) and REDOX maps for healthy contralateral mucosa (top) and malignant gingiva oral lesion (bottom). Scale bar: 2 mm.

includes the oral lesion around the masked teeth and surrounding healthy mucosa at the bottom of the FOV (Fig. 3, bottom). The bottom region of the FOV showed similar normalized fluorescence intensity and REDOX values to the contralateral healthy side. In contrast, the gingival lesion area (see Fig. 4 for spatial context) exhibited weaker emission at 482 ± 18 nm and 550 ± 44 nm and stronger emission at 647 ± 28 nm for both excitations, the latter indicating increased PpIX fluorescence. The reduction in emission at the 482 ± 18 nm channel upon 375 nm excitation was more pronounced (lower NAD(P)H contribution), resulting in higher REDOX values in the malignant oral lesion, suggesting increased cellular metabolism via oxidative phosphorylation.

The corresponding fluorescence average lifetime maps $\tau_{avg, \lambda_{ex}, \lambda_{em}}(x, y)$ for each emission spectral channel upon a specific excitation are shown in Fig. 4 (notice wider lifetime range for the 647 ± 28 nm channel maps). In the FLIM endoscopy FOV of the healthy contralateral side, these maps were spatially homogeneous (Fig. 4, top), while for the malignant gingiva oral lesion, all maps were spatially heterogeneous (Fig. 4, bottom). The region at the bottom of the FOV showed similar fluorescence average lifetime values in all maps as in the healthy side. The region corresponding to the gingival lesion (for spatial context, see oral lesion photograph in Fig. 4) showed much longer average lifetime values (>8 ns) at the 647 ± 28 nm channel, another indication of increased PpIX fluorescence contribution. The gingiva lesion region also exhibited shorter average lifetimes at the 550 ± 44 nm channel upon 445 nm excitation, suggesting FAD and FMN quenching—another sign of increased cellular metabolism via oxidative phosphorylation.

Discussion and conclusion. A dual-excitation, multispectral FLIM handheld endoscope was developed for comprehensive autofluorescence *in vivo* imaging of the oral mucosa in a clinical setting. The capabilities of this novel tool for high-precision measurement of a wide range of fluorescence average lifetimes and label-free metabolic and biochemical imaging of the oral mucosa were rigorously demonstrated. While promising,

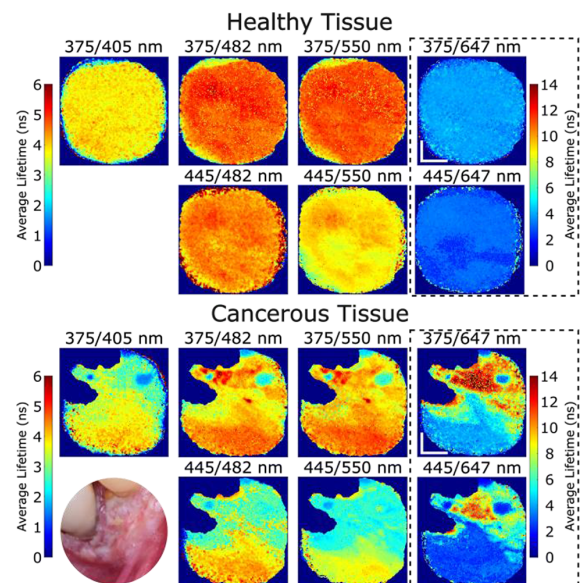


Fig. 4. Fluorescence average lifetime maps for each $\lambda_{ex}/\lambda_{em}$ for healthy contralateral mucosa (top) and malignant gingiva oral lesion (bottom). A photograph of the gingival malignant lesion is shown for anatomical reference. Scale bar: 2 mm.

the system has limitations that point to future improvements. Its spatial resolution does not support cellular-level imaging, and the lack of optical axial sectioning hinders clear separation of epithelial and submucosal signals. Ongoing research is evaluating the potential of dual-excitation, multispectral FLIM endoscopy for label-free early oral cancer detection in a multicenter clinical study.

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Disclosures. The authors declare no conflicts of interest.

Data availability. Data underlying the results presented in this Letter are not publicly available but may be obtained upon reasonable request.

Supplemental document. See Supplement 1 for supporting content.

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