



OPEN Detection and characterization of colorectal cancer by autofluorescence lifetime imaging on surgical specimens

Alberto Ignacio Herrando^{1,2,3✉}, Laura M. Fernandez², José Azevedo², Pedro Vieira², Hugo Domingos², Antonio Galzerano⁴, Vladislav Shcheslavskiy^{5,6}, Richard J. Heald², Amjad Parvaiz², Pedro Garcia da Silva¹, Mireia Castillo-Martin⁴ & João L. Lagarto¹

Colorectal cancer (CRC) ranks among the most prevalent malignancies worldwide, driving a quest for comprehensive characterization methods. We report a characterization of the ex vivo autofluorescence lifetime fingerprint of colorectal tissues obtained from 73 patients that underwent surgical resection. We specifically target the autofluorescence characteristics of collagens, reduced nicotinic adenine (phosphate) dinucleotide (NAD(P)H), and flavins employing a fiber-based dual excitation (375 nm and 445 nm) optical imaging system. Autofluorescence-derived parameters obtained from normal tissues, adenomatous lesions, and adenocarcinomas were analyzed considering the underlying clinicopathological features. Our results indicate that differences between tissues are primarily driven by collagen and flavins autofluorescence parameters. We also report changes in the autofluorescence parameters associated with NAD(P)H that we tentatively attribute to intratumoral heterogeneity, potentially associated to the presence of distinct metabolic subpopulations. Changes in autofluorescence signatures of malignant tumors were also observed with lymphatic and venous invasion, differentiation grade, and microsatellite instability. Finally, we characterized the impact of radiative treatment in the autofluorescence fingerprints of rectal tissues and observed a generalized increase in the mean lifetime of radiated adenocarcinomas, which is suggestive of altered metabolism and structural remodeling. Overall, our preliminary findings indicate that multiparametric autofluorescence lifetime measurements have the potential to significantly enhance clinical decision-making in CRC, spanning from initial diagnosis to ongoing management. We believe that our results will provide a foundational framework for future investigations to further understand and combat CRC exploiting autofluorescence measurements.

Abbreviations

CRC	Colorectal cancer
NAD(P)H	Nicotinamide adenine (phosphate) dinucleotide
MRI	Magnetic resonance imaging
FDG-PET/CT	Fluorodeoxyglucose positron emission tomography combined with computed tomography
CT	Computed tomography
ECM	Extracellular matrix
PBS	Phosphate buffered saline
BMI	Body mass index
RT	Radiation therapy
ROI	Region of interest
IRF	Instrument response function
RR	Redox ratio

¹Biophotonics Platform, Champalimaud Foundation, Avenida Brasília, 1400-038 Lisbon, Portugal. ²Digestive Unit, Colorectal Surgery, Champalimaud Foundation, Avenida Brasília, 1400-038 Lisbon, Portugal. ³NOVA Medical School, Universidade Nova de Lisboa, Campo Mártires da Pátria 130, 1169-056 Lisbon, Portugal. ⁴Department of Pathology, Champalimaud Foundation, Avenida Brasília, 1400-038 Lisbon, Portugal. ⁵Becker & Hickl GmbH, Nunsdorfer Ring 7-9, 12277 Berlin, Germany. ⁶Privolzhsky Research Medical University, Minina and Pozharskogo Sq, 10/1, Nizhny Novgorod, Russia 603005. ✉email: ignacio.herrando@research.fchampalimaud.org

OMI	Optical metabolic imaging index
SD	Standard deviation
IQR	Interquartile range
CV	Coefficient of variation
ADC	Adenocarcinoma

Colorectal cancer (CRC) is largely spread worldwide. Only for 2020, it was reported 10% of new cases and 9.4% CRC related deaths among all other types of cancers were reported¹. In general, CRC diagnosis relies on endoscopic assessment of the colonic tract along with obtention of biopsies for histological characterization. In large lesions, biopsies are often randomly collected and, therefore, histological characterization is based on a minimal portion of tissue and then extrapolated to the entire lesion. For this reason, a biopsy that does not detect cancer cells does not always rule out the presence of cancer in patients. Also, pathology reports (including histological analysis of polyps) are time consuming and usually, unnecessary biopsies can overload the laboratory capacity, increasing the times to deliver diagnostic reports.

In addition, even being the gold standard in diagnosis, histology also relies on pathologists' expertise and their access to technical resources for optimal characterization. The diagnostic accuracy of conventional pathological assessment for colorectal samples is generally high. While the specificity of these biopsies in the diagnosis of colorectal malignancy approaches 100%, reported sensitivities vary widely between 50 and 100%^{2–4}, depending on technique, volume of tissue, and number of samples obtained. In addition, significant inter-operator variability exists^{5–8}, when assessing the reproducibility of histologic identification of dysplasia in colorectal biopsies. This variability underscores the need for improved diagnostic methods. Following endoscopic assessment and histological confirmation, malignant tumors are extensively studied with imaging that eventually guide clinical decision making^{9–12}. Traditional imaging modalities such as Magnetic Resonance Imaging (MRI), Fluorodeoxyglucose Positron Emission Tomography combined with Computed Tomography (FDG-PET/CT), or contrast-enhanced Computed Tomography (CT) provide valuable diagnostic information pre- and post-operatively that can aid and improve clinical assessment. However, besides the subjectivity introduced by radiologists' interpretation of the images, these methods offer low molecular and cellular specificity. Furthermore, they are usually not suitable for regular use during surgery or in combination with minimally invasive imaging for rapid characterization of tissue. Accordingly, without immediate objective feedback, clinicians usually encounter significant challenges when evaluating lesions detected during endoscopic procedures, e.g. neoplastic against non-neoplastic polyps or malignant lesions against benign fibrotic tissues.

The absence of tools for real-time quantitative and objective assessment of CRC has prompted the development of new optical technologies that could assist in clinical decision-making. Examples include hyperspectral imaging^{13–15}, reflectance¹⁶, autofluorescence¹⁷, or Raman spectroscopy¹⁸. Among these, autofluorescence spectroscopy stands out as a promising technology due to its inherent sensitivity to functional and structural changes in tissues. In particular, spectrally-resolved autofluorescence lifetime measurements capture the fluorescence signatures from key natural fluorophores within cells and tissues, offering multiparametric characterization of biochemical, molecular, and physicochemical processes^{19–21}. Notably, NAD(P)H, flavins, and collagens are extensively studied endogenous fluorophores in the context of cancer research^{22–32}. NAD(P)H and flavins are metabolic coenzymes involved in various redox reactions^{28,33,34}, while collagen is a major component of the extracellular matrix (ECM)^{35–37}. Autofluorescence lifetime measurements targeting these molecules can thus indicate metabolic or structural changes associated with malignant transformations^{38–43}.

Autofluorescence lifetime measurements have been extensively utilized to investigate cancer metabolism. This contributed significantly to the understanding of how cancer proliferates and invades tissues^{27,44,45}. Cancerous tumors were identified as highly metabolically heterogeneous, indicating that metabolic phenotypes may differ from one cell to another^{46–49}. In addition, although aerobic glycolysis is often found in malignant tumors, oxidative phosphorylation still contributes significantly to energy production in cancers and play an active role in cancer metabolism^{50,51}. On the top of all the metabolic modifications, cancer also induces a reconfiguration of the collagen scaffold promoting tumor growth, invasion, and metastatisation^{35,37,52}. These changes are known to affect collagen autofluorescence characteristics⁵³. In consideration of all these factors, fluorescence lifetime measurements have a valuable informative potential. As a consequence, many researchers attempted to translate them into clinical practice, by implementation of single point or imaging instruments^{54,55}. In the colorectal field, Mycek et al.⁵⁶ pioneered the application of fiber-based fluorescence lifetime spectroscopy in the identification of colorectal polyps *in vivo*. Similar work was carried out by Coda et al.⁵⁷ albeit in colorectal biopsies. Clearly, both studies demonstrated that autofluorescence lifetime can play a relevant role in the future of CRC diagnosis; yet, there have not been significant advancements in the field in recent years.

Here, with the aim of advancing the clinical implementation of autofluorescence lifetime measurements in colorectal applications, we sought to understand the autofluorescence signatures associated with CRC. Using a custom-built fiber-based imaging system⁵⁸, we conducted multidimensional autofluorescence lifetime measurements of freshly resected CRC surgical specimens obtained from 73 patients. We report the autofluorescence fingerprints considering the underlying clinical and histopathological data, malignant tumor variables, and treatment strategies. To the best of our knowledge, this is the largest study aiming to characterize the *ex vivo* autofluorescence signatures of colorectal tissues in light of a multitude of underlying clinicopathological features. We anticipate that the findings presented here offer a valuable framework for future *in vivo* investigations.

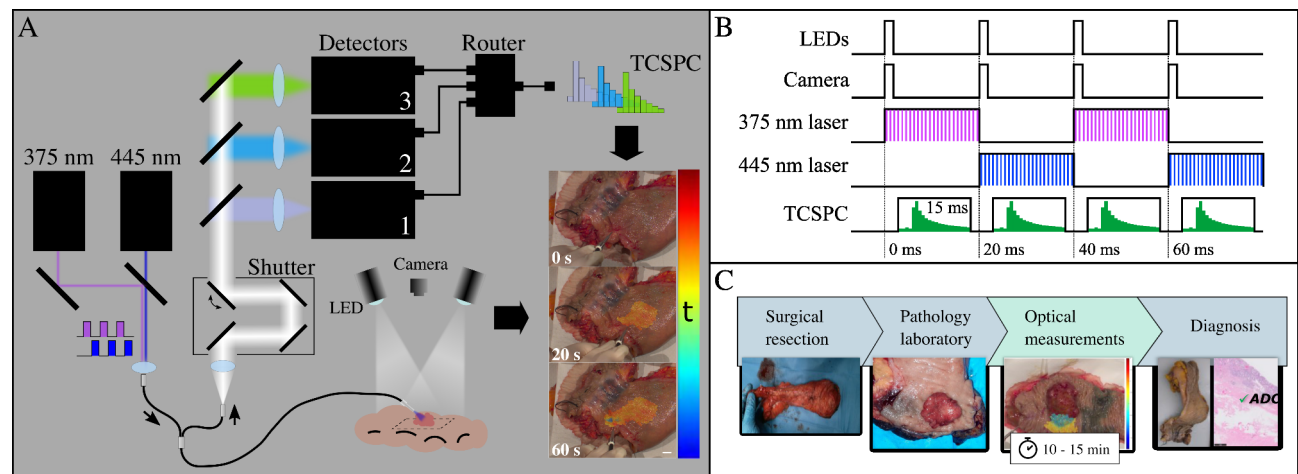


Fig. 1. (A) Schematic representation of the optical layout of the instrument. Black arrows indicate the signal direction. Bottom right corner: Live recording is augmented with false color maps representing time-resolved spectroscopic information obtained from the sample. (B) Timing diagram for the real-time acquisition. LEDs and cameras are triggered simultaneously at 50 Hz. Lasers are multiplexed at 50 Hz for sequential excitation at different wavelengths. TCSPC measurements are carried out at 50 Hz (25 Hz for each excitation wavelength) with an integration time of 15 ms. (C) Clinical workflow of the sample after surgical excision including estimative time for time-resolved autofluorescence measurements.

Channel	Excitation wavelength	Emission range collection	Targeted molecules
1	375 nm	380–420 nm	Collagens
2		456–484 nm	NAD(P)H
3		500–550 nm	Elastin – NAD(P)H - FAD/FMN
4	445 nm	456–484 nm	FAD/FMN
5		500–550 nm	

Table 1. Excitation wavelengths and emission spectral bandwidths collected with our optical setup in correspondence with channel acquisition number and targeted molecule.

Materials and methods
Patient selection

We enrolled 73 patients aged 18 and above who were diagnosed with either colon or rectal cancer and did not have any other concurrent malignancies. Subsequently, we collected 73 colorectal samples. Among these samples, one subtotal colectomy specimen was included, from which we conducted two distinct measurements (for right and left-sided lesions), resulting in a total of 74 colorectal measurements. All patients were staged and surgically treated according to the standard of care in our institution, derived from the decision of the digestive oncology multidisciplinary team. An informed consent was signed prior to any procedures and retrieval of clinical data. The study was approved by the Champalimaud Foundation Ethics Committee (protocol number 2021020203) and was performed in accordance with the Declaration of Helsinki. All patient information was pseudonymized before processing and data crossover was exclusively handled by physicians.

Autofluorescence lifetime instrumentation

Autofluorescence lifetime measurements were carried out using a custom instrument that was previously described in detail elsewhere⁵⁸. A simplified optical layout of this instrument is shown in Fig. 1A. Briefly, two pulsed laser diodes operating at 20 MHz provided multiplexed excitation at 375 nm and 445 nm in 20 ms intervals, as illustrated schematically in Fig. 1B. Excitation light was delivered to the sample by means of a bifurcated fiber optic bundle (FiberTech Optica, Canada) consisting of a single 300 μm fiber at the excitation end, seven 200 μm fibers at the detection end, and a common branch at the sample end. The fiber bundle was handheld and moved over the specimen to obtain sequential single point measurements. The autofluorescence generated at the sample was collected and directed to the detection module that split the autofluorescence into three spectral bands, making up a total of five detection channels (CH1: 375 nm/ 400 ± 20 nm, CH2: 375 nm/472 ± 14 nm, CH3: 375 nm/525 ± 25 nm, CH4: 445 nm/472 ± 14 nm, CH5: 445 nm/525 ± 25 nm, excitation/emission, see Table 1). These five channels were specifically selected to separate and maximize the signal from key endogenous fluorophores, namely collagens, NAD(P)H and flavins. The autofluorescence signal was captured by three hybrid detectors (HPM-100-40-CMOUNT, Becker and Hickl GmbH). A router (HRT-41, Becker and Hickl GmbH)

and a single TCSPC module (SPC-130 EM, Becker and Hickl GmbH) were used to record the autofluorescence intensity decays from each spectral channel simultaneously.

Our setup also included two white LEDs (MNWHL4, Thorlabs, USA) that illuminated the sample uniformly and a USB color camera (FFY-U3-16S2C-C, FLIR, USA) that recorded the movement of the fiber over the specimen. Since our system operates as a single-point device, optical measurements through the fiber bundle do not carry spatial information. Instead, spatial information is provided by the USB camera that records measurements. The camera is synchronized with the LEDs, capturing each frame under bright illumination. This setup allows for tracking the position of the fiber as it moves over the tissue, enabling reconstruction of autofluorescence images from single point measurements. LEDs and camera were operated at 50 Hz and synchronized with laser excitation and TCSPC acquisition, which were also operated at 50 Hz (alternating between 375 nm and 445 nm) as illustrated in Fig. 1B. This electronic synchronization enabled sequential single-point measurements at two excitation wavelengths in dark conditions during the off-period of the LEDs, alternating with bright illumination periods during which white light images were captured. For every two consecutive frames, one frame is captured with 375 nm excitation (invisible to the camera) and the next with 445 nm excitation (visible to the camera). This alternating scheme enables precise localization of the excitation spot. The workflow begins with capturing synchronized frames under alternating excitations wavelengths. Since the 445 nm light is visible in the blue channel but not in the green channel of the RGB images, we can differentiate the excitation spot by examining the blue channel. By subtracting the green channel from the blue channels in consecutive frames, we effectively eliminate most of the sample background, including specular reflections from the white light. The resulting processed images highlight the excitation spot, allowing us to track and segment the region of interest accurately. This segmentation process ensures that the fluorescence lifetime measurements are correctly overlaid with the corresponding spatial information from the camera images. This image acquisition, processing and segmentation methodology was thoroughly detailed in our previous work⁵⁸.

Sample collection

After surgical resection, samples were promptly transported to the Pathology laboratory for processing according to standard procedures. Surgical specimens were opened carefully to avoid damaging the lesion harbored in the lumen of the organ, following the standard approach for macroscopic assessment. The specimens were then cleaned with running water, rinsed with ~300 cc of phosphate buffered saline (PBS) solution, and dried with absorbent paper. Optical measurements were carried out subsequently, typically within 15 min of reception. Figure 1C summarizes the workflow of the specimen from the operating room to the pathology laboratory.

Data collection

Optical measurements were specifically targeted towards normal tissues, adenocarcinoma, or adenomatous tissues. Only patients with a final diagnosis of normal, adenocarcinoma, or adenomatous lesion were included. For patients with other diagnoses, only normal tissue was taken into consideration for our analysis. For patients with pelvic recurrence but no malignant tumor within the mucosa, only normal tissues were measured. Information about smoking or alcohol consumption was recorded as a simple yes/no binary status, without considering consumption scales. Body Mass Index (BMI) was calculated from measurements obtained during preoperative assessment. Previous treatments were registered with particular interest in radiation therapy (RT, see supplementary Table S1). Tumor location was categorized using preoperative imaging studies and surgeon's perception. To avoid subjectivity regarding precise tumor location, measurements were grouped as follows: "right colon" when measurements were collected proximal to the splenic flexure; "left colon" when probing from the splenic flexure up to 15 cm from the anal verge; and the last segment was considered as "rectum". The vascular ischemia time was calculated from the last marginal vessel ligation to the start of our measurements. Histological information was retrieved from the final pathology report.

Representative regions of interest (ROI) from normal, adenocarcinoma, and adenomatous tissues were chosen based on expert pathologist criteria (MCM and AG). ROIs ranged between 624 and 9234 pixels depending on the extension of each lesion. In samples with adenocarcinoma or adenomatous lesions, care was taken to select ROIs that were as distant from each other as possible. Each ROI was labeled as "normal", "tumor" (for malignant lesions) or "adenoma" (for benign lesions) in concordance with the final pathologic diagnosis. To avoid any ambiguity and to maintain clarity throughout the manuscript, we will refer to malignant neoplasia as "Tumor" and benign neoplasia as "Adenoma" from this point forward.

Data analysis

Autofluorescence lifetime data were processed in MATLAB (R2022b, The MathWorks, Inc., USA) using a non-linear least square fitting function to minimize the goodness of fit chi-square value, χ^2 . ROIs were selected as described above and numerical data were extracted from them. Autofluorescence decay data obtained from CH2 – CH5 were analyzed using a biexponential decay function $I(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}$, from which the intensity weighted mean lifetime τ_{mean} was calculated as $\tau_{\text{mean}} = (a_1 \tau_1^2 + a_2 \tau_2^2) / (a_1 \tau_1 + a_2 \tau_2)$, where a_1 , a_2 and τ_1 , τ_2 refer to the pre-exponential factors and lifetime values of the short and long decay components, respectively. We opted to fit CH1 data to a single exponential function, considering the significantly lower number of photons collected in this channel (~100 photons per decay) relative to other detection channels⁵⁹. The values of χ^2 varied between 0.8 and 1.5 for the entire dataset. Fluorescence decays were analyzed considering constant background offset, time-varying background, and incomplete decay estimation. The instrument response function (IRF) was measured for all channels (by removing band-pass filters) using excitation light scattered off a reflective surface. Day-to-day calibrations were realized independently for both excitation wavelengths using reference slides with measured fluorescence lifetimes of 0.9 ns and 3.6 ns, at 375 nm and 445 nm excitation, respectively. Fluorescence lifetime measurements were further validated using POPOP ($\tau = 1.36$ ns in ethanol⁶⁰) and Coumarin 6 ($\tau = 2.72$

ns in ethanol⁶¹). Relative rates of glycolysis and oxidative phosphorylation were estimated using the optical redox ratio (RR), calculated as the normalized autofluorescence intensity of NAD(P)H divided by that of flavins, at 375 and 445 nm excitation respectively, which in our instrument is equivalent to the ratio of autofluorescence between Channels 2 and 5, i.e. $RR = I_{CH2} / (I_{CH2} + I_{CH5})$. Further metabolic characterization was obtained through determination of the optical metabolic imaging index (OMI)^{62,63}, calculated for each pixel as follows

$$OMI = \frac{RR}{\langle RR \rangle} + \frac{CH2 \tau_{mean}}{\langle CH2 \tau_{mean} \rangle} - \frac{CH5 \tau_{mean}}{\langle CH5 \tau_{mean} \rangle},$$

where the angle brackets refer to the average value within the ROI.

Histopathological assessment

After fixation in 10% buffered formalin, samples were processed according to the standard guidelines in our Pathology Service. Following gross sectioning of the specimen, the resulting sections were segmented (to fit into inclusion cassettes) and carefully selected by an experienced pathologist (MCM and AG) for paraffin embedding. Subsequently, 5–10 µm thick sections were produced using a microtome and stained with hematoxylin and eosin for diagnosis. Mismatch repair status by immunohistochemistry is routinely investigated at our institution and additional staining was done if requested by the treating clinician. Representative histology images for different conditions are presented in Supplementary Fig. S1.

Statistical analysis

Histopathological assessment served as reference for statistical analysis to evaluate differences between groups. Descriptive statistics were used to summarize the results, including measures of mean and median values, variance, standard deviation (SD), and interquartile range (IQR) to understand the spread or dispersion of the data. We quantified the spread of the data within each ROI through the coefficient of variation (CV), calculated as the ratio between standard deviation and mean. The CV measures the relative variability of a set of data points with respect to their mean, providing a normalized measure that facilitates comparison of the degree of dispersion across different datasets. A larger CV indicates greater relative variability within a set of data.

Differences and correlations in both independent and paired variables were computed based on normality tests. Parametric data underwent analysis using t-test or ANOVA, paired t-test or Pearson's correlation coefficient. Non-parametric data were assessed using the Mann-Whitney or Kruskal-Wallis' test, Wilcoxon's rank-test for paired variables and Spearman's correlation coefficients. To investigate potential differences in autofluorescence lifetime between various tissue types (normal, adenoma and tumor), we employed a linear mixed-effects model to quantify the average change between each type of tissue^{64,65}. A fixed effect coefficient and its standard error (SE) was calculated to determine variations in autofluorescence lifetime from one tissue type to another. A 95% confidence interval was applied, and the alpha error was set at 5%, ensuring a reliable assessment of relationships and differences in the study variables.

For the violin plots here depicted, white dots represent median values and bars represent the interquartile range if not otherwise specified. In addition, dots contained within the clouds are individual measurements.

Results

Study population

From January to December 2022, a total of 74 colorectal samples from 73 patients were measured using our autofluorescence lifetime imaging system. Females represented 51% (37/73), the mean age was 62.2 years old (SD 13.6) and the average BMI was 25.54 (SD 4.1). Approximately 55% of patients reported occasional alcohol consumption (40/73), whereas only 18% reported smoking in the past year (14/73). From 74 samples, approximately 65% of our measurements (48/74) were obtained from colonic resections, from where 10 adenoma measurements were retrieved. One patient underwent surgery due to multiple polyps, with only two of them being measured (on the right and left colon). These were analyzed independently with corresponding adjacent normal mucosa as control. Left sided samples represented 40% of our cohort (30/74 measurements), harboring 6 of 10 adenomas. In contrast, right sided samples constituted the 24% of our measurements (18/74), carrying the remaining 4 adenomas from our study group. Finally, rectums represented 35.6% (26/74) of the samples, of which 11 received radiative treatment before surgery. We accounted the marginal vessel ligation as the last vascular element providing blood supply to the samples and the beginning of the ischemic period, which had a median of 50.5 min (IQR 41–72). We processed the fluorescent maps as described above and, following an expert pathologist's criteria, we retrieved 74 ROIs labeled as normal, whilst 56 were identified as tumoral, and 10 as adenomas. All the cancerous tissues were consistent with adenocarcinoma histology, being the "not otherwise specified" subtype the most common subtype (86%, 48/56). On the other hand, benign lesions were diagnosed as adenomas being the tubular type the most common among them (7/10). Additionally, they carried predominantly low-grade dysplasia, of which 3 had small foci of high-grade dysplasia (2 within tubulo-villous type and 1 within the tubular type). Demographic and clinicopathological data are summarized in Table 2.

Autofluorescence lifetime signatures of colorectal tissues

Collagen types I-IV, NAD(P)H and flavins are the dominant tissue fluorophores within the colorectal mucosa. In our system, their autofluorescence signals are best captured by CH1, CH2, and CH5, respectively. For this reason, results presented here focus on these channels. Autofluorescence lifetime parameters averaged across all specimens for each tissue type are presented in Supplementary Table S2. Figure 2A-D illustrates autofluorescence lifetime maps of channels 1, 2, and 5 in representative cases with and without tumor. To enhance interpretation of optical patterns, average autofluorescence lifetime parameters were computed for various tissue types (normal,

Age, mean (SD)	62.2 (13.6)
Gender, n (%)	
Male	36 (48.6)
Female	37 (51.3)
BMI, mean (SD)	25.54 (4.1)
Location, n (%)	
Right	18 (24.3)
Left	30 (40.5)
Rectum	26 (35.1)
Habits, n (%)	
Smoking	14 (18.9)
Alcohol intake	40 (57.1)
Radiation (only for rectum, N = 26)	11 (42.3)
Ischemia time, median (IQR)	50.5 (41–72)
Histology	
Normal, total	74
Adenocarcinoma, total	56
NOS	48
Mucinous	6
Undifferentiated	1
Signet ring cell	1
Adenoma, total	10
Tubular	7
Tubulo-villous	3
Low grade dysplasia	10
High grade dysplasia	3*

Table 2. Demographic information and tumor-related characteristics. BMI: Body Mass Index; SD: Standard deviation; NOS: not otherwise specified. *Small foci.

adenoma, and adenocarcinoma) across all non-irradiated specimens. These parameters are presented both as absolute values and relative to normal tissue (see Fig. 3 and Supplementary Fig. S2). From these data, there are three key observations.

First, there is significant interpatient variability across all optical parameters and tissue types, which is reflected in the large dispersion of the data around the absolute means. To mitigate the potential bias introduced by such variability in our analysis, relative values were calculated between transformed tissue (tumor and adenoma) and normal measurements (Fig. 3B–L). Analysis of relative values allowed us to visualize specific trends in the optical data.

This brings us to the second key result: tumor tissues yield significantly shorter fluorescence lifetimes than normal tissues in CH1 and CH5, but not in the other channels. This pattern emerged in most samples, as shown in Fig. 3C and L. Interestingly, while we did not observe a discernible trend in the mean autofluorescence lifetime of CH2 (Fig. 3E, F), analysis of its short lifetime component a_1 , often associated to free-NAD(P)H (see Fig. 3G, H, I), suggest that tumor metabolism primarily relies on glycolysis. In comparison to malignant tissue, adenomas and normal tissue showed significantly less proportion of a_1 component, suggesting less reliance on glycolysis. It is worth highlighting a shoulder observed in relative data indicative of bimodal distribution (see Fig. 3H, data in red), suggesting that different metabolic phenotypes may be harbored in this group.

Third, the autofluorescence lifetime signatures of adenoma tissues are in general more similar to normal tissues than to adenocarcinoma. This is particularly evident in CH1–CH4. Figure 2B shows an example of this similarity, where the lesion highlighted in Fig. 2B1 is undistinguishable from the surrounding normal tissue in the autofluorescence lifetime maps. In CH5, adenomas exhibit a significantly shorter fluorescence lifetime compared to normal tissue, while displaying similarities to the fluorescence lifetime observed in tumors (see Fig. 3J). Therefore, our results indicate that adenomas are comparable to normal tissue in CH1 (average decrease of -0.101 ns (SE 0.106), in adenoma with respect to normal tissue, p NS), yet exhibit greater resemblance to tumors tissue in CH5 (average increase of 0.076 ns (SE 0.14), in tumors with respect to adenomas, p NS). This observation could serve as a valuable tool in differentiating benign from malignant tissue. However, a larger sample size is essential to enhance the robustness and reliability of these findings. Interestingly, we observed significantly increased RR and OMI in adenomas relative to both normal and tumor tissues (see Supplementary Fig. S2G–L). This trend was observed in almost all specimens, contrasting with the high variability in tumor data across the cohort. These findings appear to suggest that tumors are metabolically more heterogeneous than adenomas between patients.

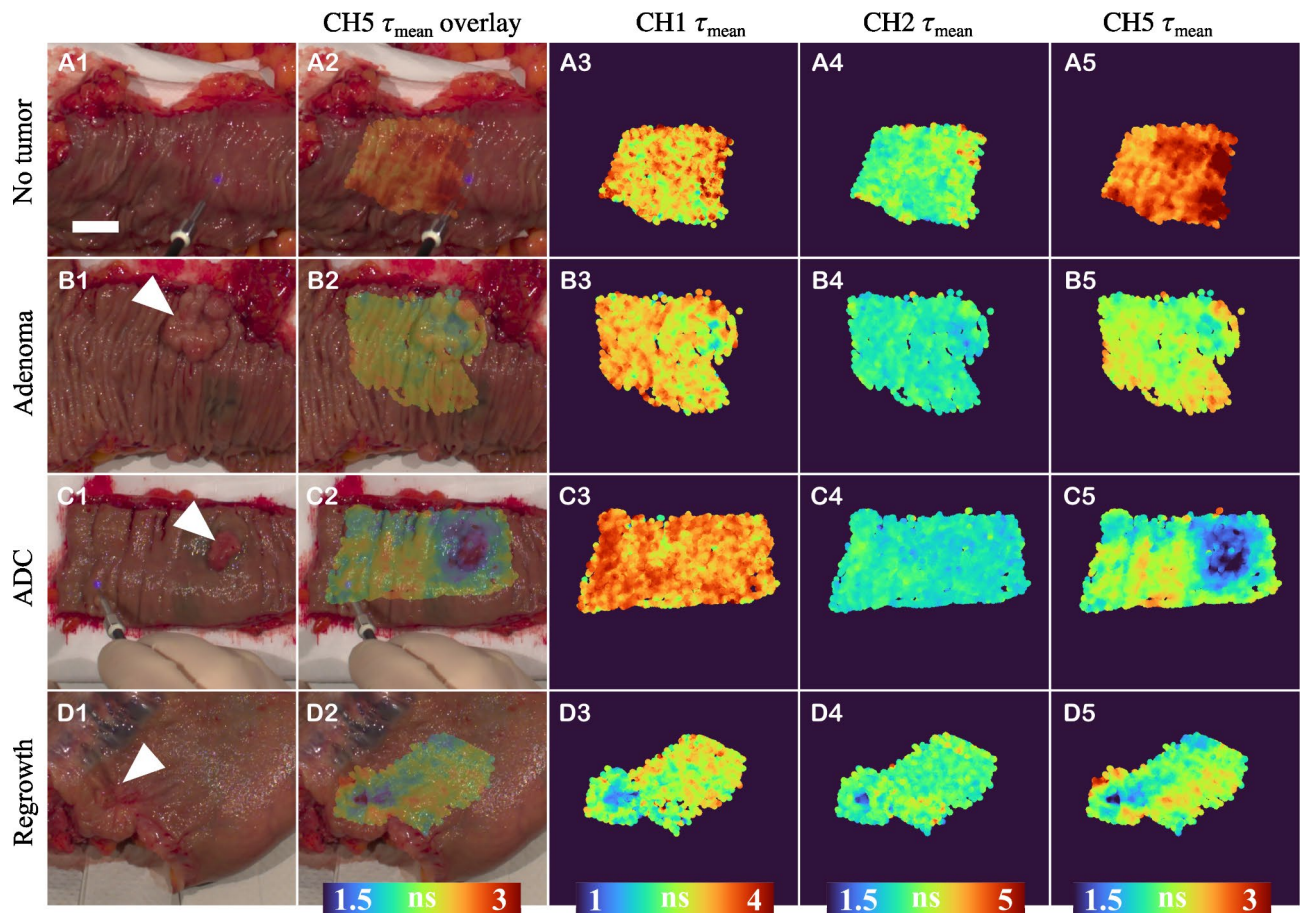


Fig. 2. (A)–(D) Representative examples (in rows) illustrating the differentiation potential of optical measurements (in columns) obtained with our instrumentation. From left to right: *Column 1.* White light image, targeted lesions are pointed with a white arrow. *Column 2.* White light image augmented with a false color map of the averaged lifetime collected from CH5. *Column 3, 4 and 5.* Fluorescence lifetime maps obtained from CH1, CH2 and CH5, respectively. Scale bar 2 cm. The color bars for the lifetime measurements are tailored to the specific lifetimes of the target molecules in each channel. This adaptation was made to enhance the visual contrast and accurately represent the differences in fluorescence lifetimes within each sample. ADC: adenocarcinoma.

Autofluorescence lifetime signatures with clinical and histopathological parameters

Next, we investigated the impact of different clinical and histological features in the autofluorescence signatures of normal and tumor tissues. Adenoma tissues were left out of this analysis given the relatively low number of cases ($n = 10$). Only paired measurements were considered for this analysis, i.e., specimens from which we collected from both normal and tumor regions. This is because the autofluorescence lifetime signatures of tumors were analyzed in light of the corresponding signatures from normal regions, due to the high interpatient variability, as shown in Fig. 3. Results of this analysis are presented in Fig. 4 and complemented in Supplementary Figs. S3 and S4. Overall, our data indicate that the autofluorescence lifetime signatures remain relatively consistent across location, i.e., differences of relative values between non-irradiated normal and tumor tissues are maintained irrespective of the specific location. Likewise, we did not find significant variations when data were grouped by gender or correlated to BMI. We also did not observe distinctive correlations between optical parameters and the time registered from marginal vessel ligation to optical measurements (see Supplementary Figs. S3 and S4), despite of the relatively ample interquartile range of registered ischemia times (41–72 min).

The autofluorescence signatures of tumors were also analyzed considering early (T stage 1 or 2) and advanced (T stage 3 or 4) pathological T staging (see Fig. 4D–G and Fig. S5–S8). Differences between early and advanced tumors were revealed in the short lifetime component (a_1 and τ_1) obtained from CH5, indicating that bound-flavins are more abundant in early-stage compared to advanced tumors. Interestingly, we have also observed that CH1 τ_{mean} is moderately correlated to tumor size ($r = -0.57$, $p < 0.01$) in early tumors but not in advanced tumors (see Fig. 4D, E), suggesting that CH1 autofluorescence may report initial changes in tissue architecture when the tumor starts growing. Despite the generally similar trends observed in CH1 and CH5, as depicted in Fig. 3, no correlations were observed between CH5 τ_{mean} , CH5 a_1 or CH5 τ_1 and tumor size (see Supplementary Fig. S5).

Tumors with deficient mismatch repair (dMMR, microsatellite instable) and proficient mismatch repair (pMMR, microsatellite stable) have significantly different CH2 short and long relative lifetime components

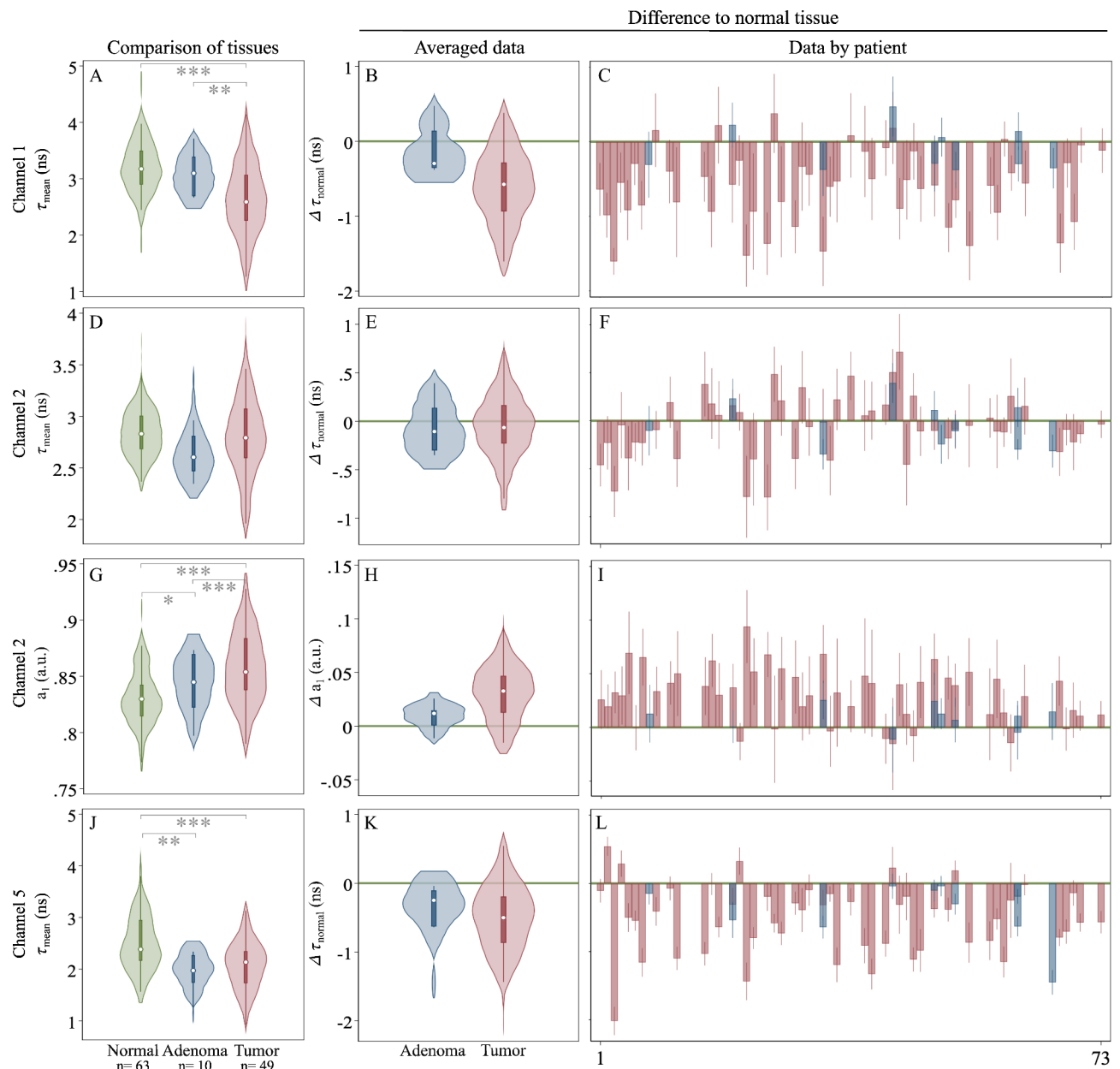


Fig. 3. Absolute and relative fluorescence lifetimes measured in CH1, CH2, and CH5 from normal (green), adenoma (blue) and tumor (red) tissues. (A), (D), (J) Average autofluorescence lifetime in CH1, CH2, and CH5, respectively, in non-radiated specimens. (B), (E), (K) Relative autofluorescence lifetimes of adenoma (blue) and tumor (red) tissues with respect to their corresponding normal (green line) tissue (i.e., $\Delta\tau = \tau_{Tumor|Adenoma} - \tau_{Normal}$), collected from CH1, CH2 and CH5, respectively, in non-radiated specimens. (C), (F), (L) Relative autofluorescence lifetimes in CH1, CH2, and CH5 for each patient across the cohort. (G), (H), (I) Absolute and relative values of CH2 a_1 in normal, adenoma, and tumor tissues. Linear mixed-effect model was used to calculate p values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

($\Delta\tau_{1,dMMR} = -0.12 \pm 0.13$ ns, $\Delta\tau_{1,pMMR} = -0.01 \pm 0.14$ ns, $p < 0.01$; $\Delta\tau_{2,dMMR} = -0.17 \pm 0.71$ ns, $\Delta\tau_{2,pMMR} = 0.39 \pm 0.64$ ns, $p < 0.01$; see Fig. 4L and M and Supplementary Fig. S6). This is despite of the general lack of contrast observed in CH2 data. Interestingly, dMMR and pMMR tumors yield similar relative CH5 τ_{mean} ($\Delta\tau_{mean,dMMR} = -0.18 \pm 0.18$ ns, $\Delta\tau_{mean,pMMR} = -0.59 \pm 0.23$ ns, $p > 0.05$) in contrast to the results presented for CH2 data. CH5 short and long components also remain similar between dMMR and pMMR tumors (Supplementary Fig. S7 C5 and D5).

Considering other histological features, population of free-NAD(P)H (CH2 a_1) was found to increase with high differentiation grade and lymphatic invasion (see Fig. 4K and N). Accordingly, relative CH2 a_1 was significantly larger in tumors with high grade of differentiation compared to low differentiation grade ($\Delta a_{1,high} = 0.04 \pm 0.03$, $\Delta a_{1,low} = 0.03 \pm 0.03$; $p = 0.02$). Similarly, tumors with lymphatic invasion had significantly larger

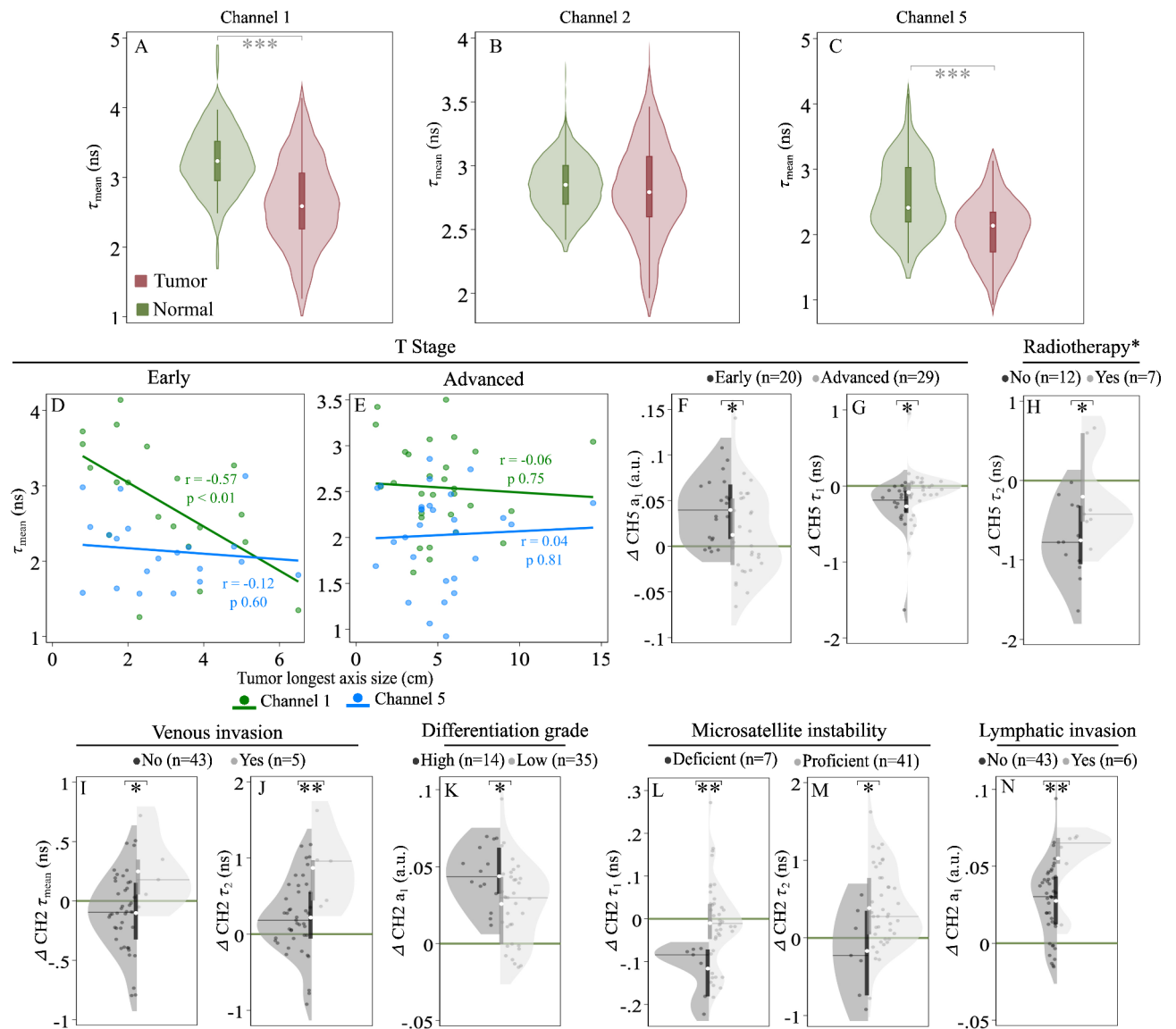


Fig. 4. (A)–(C) Averaged autofluorescence lifetimes of paired normal (green) and tumor (red) regions from non-irradiated specimens, in CH1, CH2, and CH5, respectively of $n = 49$ samples. (D), (E) Scatter plots correlating CH1 (dark green) and CH5 (light blue) mean lifetimes with tumor largest axis size for early and advanced tumors, respectively. (F)–(N) Relative values comparison between relevant histological features of tumors. Components of violin plots from (F)–(N) are constituted by: white dots representing the mean, darker horizontal lines that illustrate the median, and vertical bars the interquartile range (IQR). Paired tests were used to calculate p values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

relative CH2 a_1 compared to tumors without it ($\Delta a_{1,LI} = 0.06 \pm 0.03$, $\Delta a_{1,no LI} = 0.03 \pm 0.03$, $p < 0.01$). Moreover, tumors with venous invasion yield longer mean autofluorescence lifetime in CH2, driven by the increase in the relative lifetime of bound NAD(P)H (see Fig. 4I and J). Finally, rectal tumors subjected to preoperative RT yield longer CH5 τ_2 compared to naïve tumors ($\Delta \tau_{2,RT} = 0.18 \pm 0.35$, $\Delta \tau_{2,no RT} = -0.75 \pm 0.83$, $p < 0.05$) (see Fig. 4H, and Supplementary Fig. S7). A similar pattern was observed in relative RR measurements, with radiated tumors presenting significantly lower RR compared to non-radiated tumors ($RR_{RT} = -0.08 \pm 0.03$, $RR_{no RT} = -0.01 \pm 0.04$, $p < 0.05$, see Supplementary Fig. S8–D7).

We have also investigated variations in the autofluorescence signatures of tumors harboring perineural invasion, budding, and extra tumoral nodes, but were unable to find significant correlations or trends in the data. A comprehensive overview of all autofluorescence lifetime parameters and histological features, are presented in Supplementary Figures S6 to S8.

Tumor heterogeneity can be quantified by NAD(P)H autofluorescence

Along with significant interpatient variability, our data are also suggestive of significant intra-sample variability. That is, autofluorescence data vary significantly within each sample. This is best captured by the large standard

deviation bars in sample-wise data (see Fig. 3 and Supplementary Fig. S2) but also clearly evidenced in the representative autofluorescence maps of Fig. 2A–D. We attempted to quantify this intra-sample variability of optical data using the CV within each ROI. The CV of the mean autofluorescence lifetimes of CH2 and CH5 are presented in Fig. 5. From these data resulted two key observations: (1) tumor tissues are more heterogeneous than normal tissues; (2) data do not vary equally across different optical parameters. Indeed, the variation of autofluorescence lifetimes in tumors is significantly higher than in normal tissues in CH2, with increases over 100% in most of patients (data not shown). Decreases in heterogeneity were observed in only 2 out of 49 samples (see Fig. 5B, top). These are despite the limited tissue discrimination provided by autofluorescence lifetime in CH2, as indicated in Figs. 2 and 3. Considering that CH2 predominantly probes NAD(P)H autofluorescence, these results could be indicative of metabolic heterogeneity within tumors. The increase in heterogeneity is less obvious in CH5 (Fig. 5, bottom row), with 19 out of 49 samples showing greater variation in normal tissues compared to tumor.

Autofluorescence diversity after radiation treatment

Radiotherapy is widely used for treating specific locally advanced rectal tumors due to its potent oxidizing effect on cancer cells, often resulting in cell death. As shown in Figs. 2D and 4H, radiation appears to alter the autofluorescence characteristics of tissues. To better comprehend these changes induced by RT, we conducted a subgroup analysis focusing on samples from rectal cancers, including both normal and tumor tissues. Samples were categorized based on whether patients underwent RT prior to surgery or not. The results presented in Table 3; Fig. 6, and Supplementary Fig. S9, demonstrate that RT promotes alterations in the autofluorescence signatures of both normal and tumor tissues, manifested by a general increase of the mean autofluorescence lifetime across all channels. On average, the autofluorescence lifetime increased 0.15 ns in normal tissues and 0.11 ns in tumor tissues, indicating that radiation affects both tissues equally. For this reason, differences observed between normal and tumor tissues appear to be maintained irrespective of previous radiative treatment (see Fig. 6C–F). We observed an increase in lifetimes in CH5 accompanied by a slight increment of heterogeneity

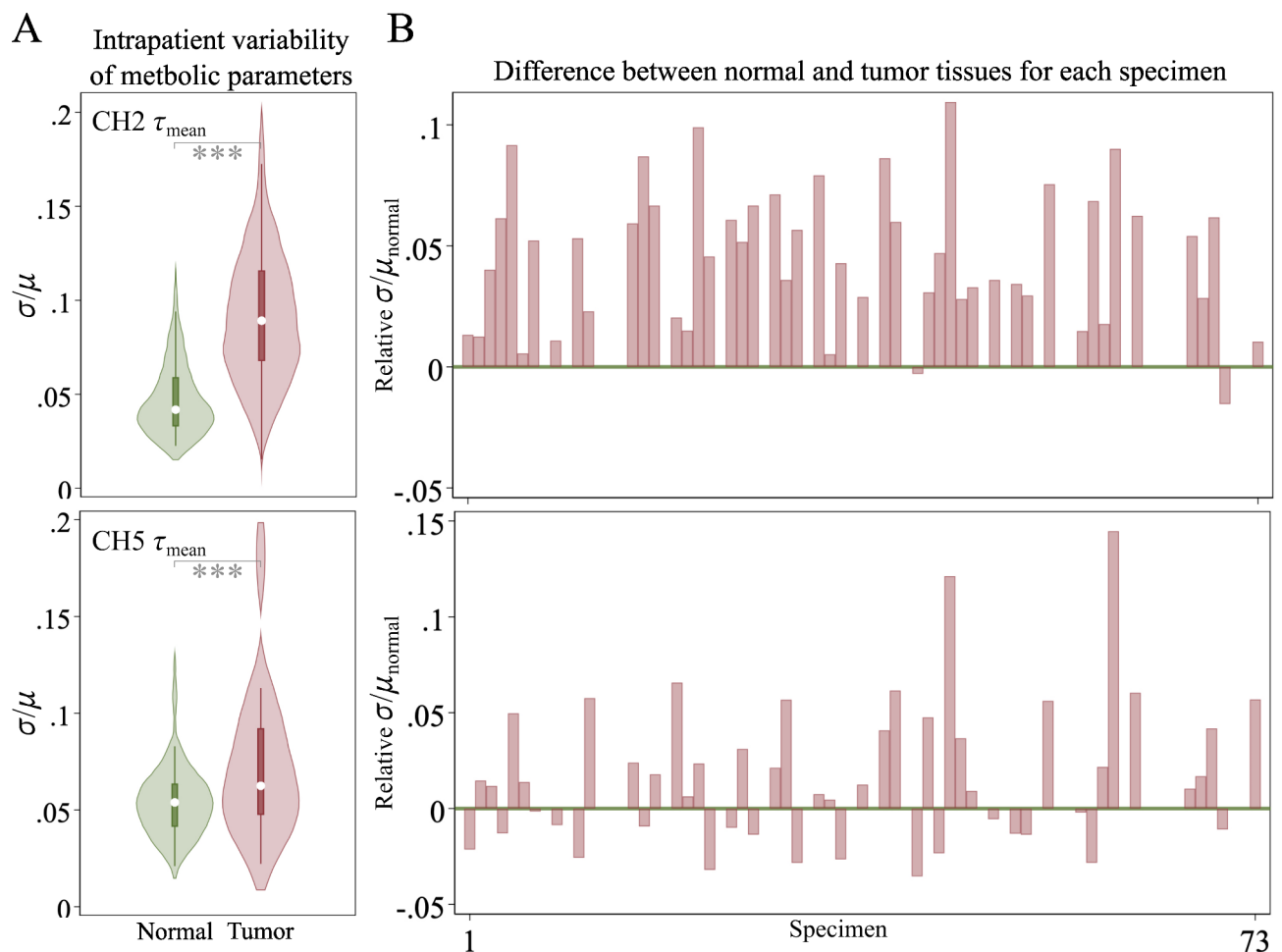


Fig. 5. (A) Tumor heterogeneity assessed by the coefficient of variation of mean autofluorescence lifetime data in $n = 49$ samples. White dots are the median and bars are the interquartile range. (B) Relative coefficients of variation of mean autofluorescence lifetime data across the cohort for CH2 and CH5. Paired tests were used to calculate p values (** $p < 0.001$).

Normal Tissue (<i>n</i> = 26)							Tumoral Tissue (<i>n</i> = 19)						
	n	CH1	CH2	CH3	CH4	CH5	n	CH1	CH2	CH3	CH4	CH5	
no RT	15	3.15 (0.67)	2.85 (0.22)	3.09 (0.34)	3.66 (0.52)	2.63 (0.58)	12	2.53 (1.01)	2.72 (0.55)	2.9 (0.5)	3.4 (0.51)	2.06 (0.61)	
RT	11	3.24 (0.34)	3.03 (0.31)	3.31 (0.46)	3.83 (0.46)	2.73 (0.60)	7	2.35 (0.46)	2.85 (0.49)	3.15 (0.43)	3.52 (0.57)	2.3 (0.51)	
<i>p</i> value		0.69	0.09	0.18	0.4	0.65		0.65	0.6	0.28	0.22	0.4	

Table 3. Average fluorescence lifetimes of normal and tumor tissues obtained from patients who previously underwent radiation treatment (RT).

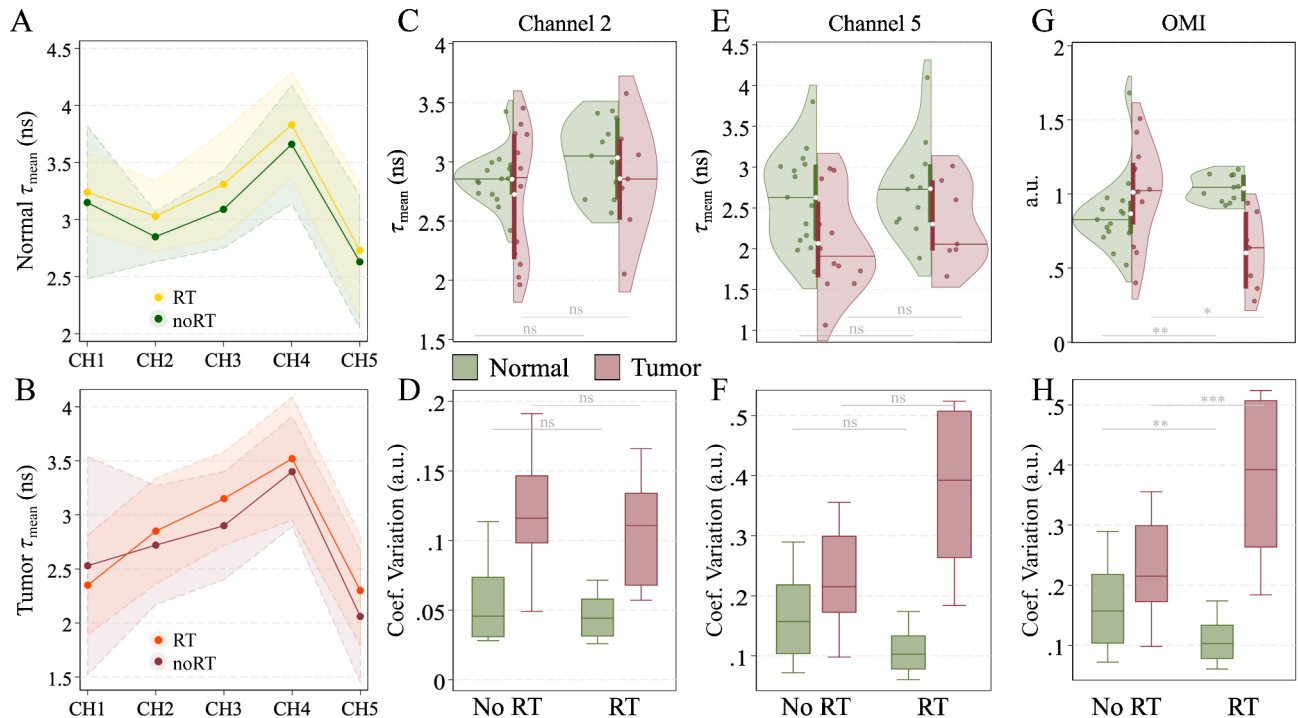


Fig. 6. Effect of radiotherapy in normal and tumor tissues. (A) and (B) Averaged lifetime values (dots) and SD (shaded area) of normal (green-yellow) and tumor (red-orange) tissues according to their previous radiation exposure across all channels. (C)–(H): Fluorescence lifetimes values obtained from CH2, CH5 and calculated OMI index (upper row) with their correspondent coefficients of variation in normal and tumor tissue of non-radiated (left) and radiated (right) specimens (lower row). Components of violin plots in (C), (E) and (G) are constituted by: white dots representing the mean, darker horizontal lines that illustrate the median, and vertical bars the interquartile range (IQR). p values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

following RT (Fig. 6E–F), perhaps suggestive of increased metabolic heterogeneity. Interestingly, this was not reflected in CH2 (Fig. 6C–D), where radiation did not significantly change lifetimes or heterogeneity. However, this observation is reinforced by OMI measurements (see Fig. 6G–H), indicating a higher OMI in non-radiated tumors and a lower OMI in radiated tumors, compared to normal tissues. This decrease in OMI in tumors following RT is accompanied by an increase in heterogeneity (Fig. 6H).

Discussion

This study describes the autofluorescence signatures obtained from CRC surgical specimens, exploiting multidimensional time-resolved autofluorescence fiber-based imaging. To the best of our knowledge, this is the largest study aiming to characterize the ex vivo autofluorescence signatures of colorectal tissues that also takes into consideration the underlying clinicopathological features. Our results confer solid evidence that label-free multiparametric autofluorescence lifetime measurements can provide additional functional and structural characterization of tumor and adjacent normal areas that could potentially aid clinical decision making. As outlined in previous work⁵⁸, isolating the autofluorescence signal of flavins from that of NAD(P)H by employing specific 445 nm excitation light was crucial to increase the specificity of the autofluorescence readout, permitting more accurate interpretation and comprehension of the results. This is a striking difference to alternative time-resolved autofluorescence systems that were previously employed in pre-clinical and clinical studies, exploiting excitation in the UV-range only^{56,66,67}. The specificity of these systems is limited by the inefficient excitation of flavins compared to collagens and NAD(P)H. In this regard, our optical setup is similar to that previously employed by Coda et al.⁵⁷, with the advantage of conferring spatial resolution to single-point measurements.

The results presented here show that flavins play a crucial role in distinguishing tissues through autofluorescence, while conversely, NAD(P)H contributes only slightly to this differentiation. Non-radiated tumors and adenoma tissues exhibited significantly shorter autofluorescence lifetimes in the emission band of flavins (CH5) compared to normal tissues. This is in line with the previous observations using excitation at 337 nm⁵⁶ and 435 nm⁵⁷. In further agreement with our results, Coda et al.⁵⁷ did not observe differences in the autofluorescence signature of adenomatous polyps against normal tissues, using 375 nm excitation and collection in the NAD(P)H emission range. Conversely, McGinty et al.⁶⁷ reported a significant increase in fluorescence lifetime excited at 355 nm from invasive colonic adenocarcinoma compared to adjacent normal areas, likely attributable to the summatory effect of various fluorophores present within the tissue, given the broad collection band used in this study. In comparison, our setup selectively collects fluorescence from NAD(P)H upon excitation with 375 nm (CH2 λ_{exc} : 375 nm; λ_{em} : 456–485 nm) and flavins when excited at 445 nm (CH5

λ_{exc} : 445 nm; λ_{em} : 500–550 nm). Therefore, we measured a different autofluorescence signature: (1) there was no difference between NAD(P)H autofluorescence lifetime of normal and tumor areas, and (2) flavins appear to decay faster in tumor tissue.

We demonstrate that signal emanated from non-radiated tumors and adenomas showed significantly shorter fluorescence lifetimes in the flavin band (CH5) compared to normal tissue. Results are also suggestive that CH1 autofluorescence lifetime could provide distinction between adenoma and cancer tissues. All together, these results highlight the potential of our optical setup to effectively distinguish between benign and malignant tissues, offering a meaningful contribution to current clinical decision-making process. Concerning CH1 output, we tentatively attribute the origin of the autofluorescence signal in this channel to collagen, although it is possible that NAD(P)H also contributes to the signal, considering its emission spectrum⁶⁸. The potential overlap between collagen and NAD(P)H in CH1 is important to consider, as they provide complementary information and can thus confound interpretation. Future work will aim to unravel the origins of this signal. Interestingly, normal tissues yield longer fluorescence lifetime in CH1 compared to CH2, suggesting a greater contribution of collagen to the former, owing to its characteristic long lifetime^{30,32}. In CH1, we observed significantly shorter fluorescence lifetimes in tumor compared to normal tissue. It is unclear whether this is a result of ECM structural remodeling to promote invasion^{69–71} or replacement of collagen fibers by cancer cells^{53,72}, thereby significantly increasing the contribution of NAD(P)H to CH1 autofluorescence. Similar observations were recently reported in lung squamous carcinoma⁷³. Notwithstanding, collagen signal from malignant tumors seemed dependent on tumor size and stage (Fig. 4D–E).

We have also investigated changes in the autofluorescence signatures of CRC considering tumor clinicopathological features using adjacent normal tissue as reference value, to mitigate the impact of interpatient variability in our dataset. This analysis enhanced visualization of specific trends and interpretation of the results. It is not clear at this point whether calibration with normal tissue would be required for every measurement of transformed tissue in a clinical context. This will be further explored in future work, as we continue to expand our database. Our data indicates that both short and long lifetimes of NAD(P)H decay faster in dMMR tumors (see Fig. 4L–M, and Fig. S6). Moreover, high-grade tumors were found to have more free-NAD(P)H compared to low-grade tumors (Fig. 4K). A comparable result was observed in tumors with lymphatic invasion (Fig. 4N). Additionally, bound-NAD(P)H and bound-flavins appear to be sensitive to venous invasion (Fig. 4I–J) and T staging (Fig. 4F–G), respectively. Taken together, while these results are still preliminary and therefore lack the necessary numbers for significant multivariate adjustment, they strongly indicate that tumor autofluorescence metabolic profile may be associated with relevant histological features that influence short and long-term oncological outcomes. The significance of these findings lies in their potential to improve tumor characterization, thereby assisting in clinical decision-making prior to treatment.

We investigated whether autofluorescence lifetime measurements could identify characteristic metabolic profiles in tissues. We consistently observed higher levels free-NAD(P)H in tumors compared to benign tissues (Fig. 3G–I), suggesting increased dependence on glycolysis. However, a considerable intra-tumoral variability was observed in our cohort, particularly in autofluorescence parameters associated to NAD(P)H. (Fig. 5). We hypothesize that this variability stems from the diverse metabolic phenotypes harbored within a tumor, thus laying the groundwork for further investigations and quantification of metabolic heterogeneity in tumors using a label-free, optical fiber-based approach. This is of significance, because cancer metabolic heterogeneity has recently been associated with local recurrence, distant metastasis, and overall survival^{74–80}. Consequently, the ability to quantify metabolic heterogeneity by label-free optical measurements would be of clinical interest.

Concerning the impact of RT in the autofluorescence fingerprint, we observed an overall increase in mean autofluorescence lifetime across all channels in radiated normal and tumor tissues (Table 3; Fig. 6 and Supplementary Fig. S9), albeit this increment was not statistically significant. Radiated tumors presented greater variability than naïve tumors, mainly in CH5 (Fig. 6F), suggesting increased heterogeneity. Intriguingly, the effect of radiative treatment was clearly observed through variation in OMI index. Radiated tumors exhibited a notable decrease in the OMI, accompanied by a significant increase in the variation of these values (Fig. 6G–H). RT effectively modifies tumor metabolism⁸¹ and, according to our results, these changes could be investigated by tissue autofluorescence. The exploitation of metabolic heterogeneity by multiparametric time-resolved fluorescence may unveil prospective biomarkers for evaluating pre-treatment radio resistance or treatment response in the management of rectal cancer, as suggested by van der Stel et al.⁸². Despite the promising results, further investigations involving a larger cohort are necessary to extrapolate these results to the clinical application.

Finally, our measurements did not reveal distinctive autofluorescence signatures in different regions of the colon and rectum (see Supplementary Fig. S2–S4). In contrast to our findings, García et al.⁶⁶ reported different lifetime patterns in various segments of murine colonic mucosa, including proximal/distal colon, middle colon, and rectum. This aligns with findings describing different mutational load patterns in various tumor locations⁸³, indicating differences beyond the typical right-left sidedness^{83,84}. However, García et al. measured different segments of the colon in the same specimen, eliminating the inter sample variability in their measurements. In our case, similarly to Coda et al.⁵⁷, specimens were obtained from a specific segment of the colon or rectum. Hence, comparisons between different segments can only be made from different patients. Naturally, it is possible that different regions of the colon yield different autofluorescence signatures, considering the diverse morphological and molecular characteristics of the tissue but, the magnitude of these differences would be comparable to those resulting from interpatient variability. In addition, different locations will be deprived of blood supply for different periods, depending on the complexity of the surgery. Vascular supply maintains nutritional support and oxygen delivery to tissue, consequently impairments of blood flow may suppose modification of energy metabolism^{85,86}. Nonetheless, despite of an ample variation of the ischemic period registered, our optical parameters did not correlate with ischemia time (Supplementary Fig. S3–S4). On this matter, Lukina et al.⁸⁷ showed that after

excision, if not supported, tissue suffers irreversible metabolic changes after 15 min. In comparison, the work of Lukina et al. was carried out in biopsies which are completely ischemic immediately after excision, while fresh samples are partially still alive when available for measurements. Nevertheless, ex vivo experiments demand caution when extrapolating results to what could be expected in vivo.

By employing a linear mixed-effects model, we demonstrated consistent variations in fluorescence lifetimes across different tissue types. However, the limited sample size restricts our ability to draw robust conclusions. Similarly, the relatively low number of certain histological characteristics (such as microsatellite instability), which reflects real-world scenarios, limits the robustness of our findings at this stage. While this study is preliminary, we believe that the assessment of 74 samples from 73 patients provides a reasonable foundation for constructing hypotheses and communicating our initial results. Currently, our group is actively collecting more samples to enhance the robustness of the comparisons described in this study. Another limitation stemmed from the lack of a one-to-one correlation between histopathology and optical features. Our pathology laboratory follows a standardized procedure for sample processing as described in the methods. Therefore, establishing a precise histological reference for our optical maps would be nearly impossible without significantly altering the current sample processing workflow. In addition, our optical maps are generated using information obtained tangentially from the sample's surface, and the selection of ROI is guided by an experienced pathologist. Unlike histological slides which evaluate tissue in a perpendicular fashion, this approach also introduces an element of subjectivity in the selection process.

In conclusion, we reported initial results of a large cohort of patients demonstrating that autofluorescence lifetime imaging can offer multidimensional characterization of colorectal tissues and be informative towards the clinical decision process. Specifically, we: (1) characterized the distinctive autofluorescence signatures of normal, adenoma, and tumor tissues considering the underlying clinicopathological features; (2) gathered evidence supporting the assessment of tumor metabolism and heterogeneity at the tissue level by means of autofluorescence lifetime measurements; (3) disclosed changes in the autofluorescence characteristics of normal and tumor tissues induced by radiotherapy. Altogether, our results indicate that autofluorescence measurements could aid clinical decision making in CRC, both for diagnosis and management. To that end, we emphasize that our fiber-based approach is adaptable to a minimally-invasive setting with relative ease. We are currently working on an endoscopic prototype to accelerate our research towards in vivo measurements. Enhanced characterization of tumors during endoscopic assessment can facilitate the establishment of a tailored treatment approach. Furthermore, monitoring variations in optical signatures may offer an effective and objective means to safely manage patients with rectal cancer who are candidates for organ preservation strategies.

Data availability

Raw data underlying this work is not publicly available at the time but may be obtained from the authors upon reasonable request to João L. Lagarto (Joao.lagarto@research.fchampilimaud.org).

Received: 14 May 2024; Accepted: 24 September 2024

Published online: 19 October 2024

References

- Sung, H. et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J. Clin.* **71**(3), 209–249 (2021).
- Johnson, G. G. R. J., Hershorn, O., Singh, H., Park, J. & Helewa, R. M. Sampling error in the diagnosis of colorectal cancer is associated with delay to surgery: A retrospective cohort study. *Surg. Endosc.* **36**(7), 4893–4902 (2022).
- Bökkerink, G. M. et al. Value of macrobiopsies and transanal endoscopic microsurgery in the histological work-up of rectal neoplasms: A retrospective study. *World J. Gastrointest. Oncol.* **9**(6), 251 (2017).
- Gondal, G. et al. Biopsy colorectal polyps is not adequate grading neoplasia. *Endoscopy* **37**(12), 1193–1197 (2005).
- Costantini, M. Interobserver agreement in the histologic diagnosis of colorectal polyps the experience of the multicenter adenoma colorectal study (SMAC). *J. Clin. Epidemiol.* **56**(3), 209–214 (2003).
- Jensen, P. et al. Observer variability in the assessment of type and dysplasia of colorectal adenomas, analyzed using kappa statistics. *Dis. Colon Rectum.* **38**(2), 195–198 (1995).
- Mollasharifi, T. et al. Interobserver agreement in assessing dysplasia in colorectal adenomatous polyps: A multicentric Iranian study. *Iran. J. Pathol.* **15**(3), 167–174 (2020).
- Smits, L. J. H. et al. Diagnostic variability in the histopathological assessment of advanced colorectal adenomas and early colorectal cancer in a screening population. *Histopathology* **80**(5), 790–798 (2022).
- Glynne-Jones, R. et al. Rectal cancer: ESMO Clinical Practice guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **28**, iv22–iv40 (2017).
- Argilés, G. et al. Localised colon cancer: ESMO Clinical Practice guidelines for diagnosis, treatment and follow-up†. *Ann. Oncol.* **31**(10), 1291–1305 (2020).
- Benson, A. B. et al. *NCCN Guidelines Version 3.2023 Rectal Cancer Continue NCCN Guidelines Panel Disclosures* (2023).
- Benson, A. B. et al. *NCCN Guidelines Version 2.2023 Colon Cancer Continue NCCN Guidelines Panel Disclosures* (2023).
- Deal, J. et al. Identifying molecular contributors to autofluorescence of neoplastic and normal colon sections using excitation-scanning hyperspectral imaging. *J. Biomed. Opt.* **24**(02), 1 (2018).
- Waterhouse, D. J. et al. First-in-human pilot study of snapshot multispectral endoscopy for early detection of Barrett's-related neoplasia. *J. Biomed. Opt.* **26**(10) (2021).
- Yoon, J. et al. First experience in clinical application of hyperspectral endoscopy for evaluation of colonic polyps. *J. Biophotonics* **14**(9) (2021).
- Gkouzonis, I. et al. Real-time tracking of a diffuse reflectance spectroscopy probe used to aid histological validation of margin assessment in upper gastrointestinal cancer resection surgery. *J. Biomed. Opt.* **27**(02) (2022).
- Lukina, M. et al. Interrogation of metabolic and oxygen states of tumors with fiber-based luminescence lifetime spectroscopy. *Opt. Lett.* **42**(4), 731 (2017).
- Wood, H. A. C. et al. Tri-mode optical biopsy probe with fluorescence endomicroscopy, Raman spectroscopy, and time-resolved fluorescence spectroscopy. *J. Biophotonics* **16**(2), (2023).

19. Lakowicz, J. R., Szmacinski, H., Nowaczyk, K. & Johnson, M. L. *Fluorescence Lifetime Imaging Free Protein-Bound NADH* 89 (1992).
20. Marcu, L. Fluorescence lifetime techniques in medical applications. *Ann. Biomed. Eng.* **40**(2), 304–331 (2012).
21. Shcheslavskiy, V. I., Yuzhakova, D. V., Sachkova, D. A., Shirmanova, M. V. & Becker, W. Macroscopic temporally and spectrally resolved fluorescence imaging enhanced by laser-wavelength multiplexing. *Opt. Lett.* **48**(20), 5309 (2023).
22. Kolenc, O. I. & Quinn, K. P. Evaluating cell metabolism through autofluorescence imaging of NAD(P)H and FAD. *Antioxid. Redox Signal.* **30**(6), 875–889 (2019).
23. Georgakoudi, I. & Quinn, K. P. Optical imaging using endogenous contrast to assess metabolic state. *Annu. Rev. Biomed. Eng.* **14**, 351–367 (2012).
24. Lagarto, J. L. et al. Characterization of NAD(P)H and FAD autofluorescence signatures in a Langendorff isolated-perfused rat heart model. *Biomed. Opt. Express.* **9**(10), 4961 (2018).
25. Cao, R., Wallrabe, H., Siller, K. & Periasamy, A. Optimization of FLIM imaging, fitting and analysis for auto-fluorescent NAD(P)H and FAD in cells and tissues. *Methods Appl. Fluoresc.* **8**(2) (2020).
26. Cannon, T. M. et al. Characterization of NADH fluorescence properties under one-photon excitation with respect to temperature, pH, and binding to lactate dehydrogenase. *OSA Contin.* **4**(5), 1610 (2021).
27. Schaefer, P. M., Kalinina, S., Rueck, A. & von Arnim, C. A. F. Von Einem, NADH Autofluorescence—A marker on its way to Boost Bioenergetic Research. *Cytometry Part. A.* **95**(1), 34–46 (2019).
28. Heikal, A. A. Intracellular coenzymes as natural biomarkers for metabolic activities and mitochondrial anomalies. *Biomark. Med.* **4**(2), 241–263 (2010).
29. Marcu, L., Cohena, D., Maarek, J. M. I. & Grundfesta, W. S. *Characterization of Type I, II, III, IV, and V Collagens by Time-Resolved Laser-Induced Fluorescence Spectroscopy* (n.d.).
30. Lagarto, J. et al. Application of time-resolved autofluorescence to label-free in vivo optical mapping of changes in tissue matrix and metabolism associated with myocardial infarction and heart failure. *Biomed. Opt. Express.* **6**(2), 324 (2015).
31. Manning, H. B. et al. Detection of cartilage matrix degradation by autofluorescence lifetime. *Matrix Biol.* **32**(1), 32–38 (2013).
32. Shaik, T. A. et al. Monitoring changes in biochemical and Biomechanical Properties of Collagenous Tissues Using Label-Free and nondestructive optical imaging techniques. *Anal. Chem.* **93**(8), 3813–3821 (2021).
33. Zheng, J. Energy metabolism of cancer: glycolysis versus oxidative phosphorylation (review). *Oncol. Lett.* **4**(6), 1151–1157 (2012).
34. Vander Heiden, M. G. & DeBerardinis, R. J. *Underst. Intersections between Metabolism Cancer Biology Cell.* **168**(4), 657–669 (2017).
35. Mylonas, C. C. & Lazaris, A. C. Colorectal cancer and basement membranes: Clinicopathological correlations. *Gastroenterol. Res. Pract.* **2014** (2014).
36. Nebuloni, M. et al. Insight on colorectal carcinoma infiltration by studying perilesional extracellular matrix. *Sci. Rep.* **6** (2016).
37. Liang, Y. et al. Prognostic significance of abnormal matrix collagen remodeling in colorectal cancer based on histologic and bioinformatics analysis. *Oncol. Rep.* **44**(4), 1671–1685 (2020).
38. Pfefer, T. J., Paithankar, D. Y., Poneris, J. M., Schomacker, K. T. & Nishioka, N. S. Temporally and spectrally resolved fluorescence spectroscopy for the detection of high grade dysplasia in Barrett's esophagus. *Lasers Surg. Med.* **32**(1), 10–16 (2003).
39. Butte, P. V. et al. Diagnosis of meningioma by time-resolved fluorescence spectroscopy. *J. Biomed. Opt.* **10**(6), 064026 (2005).
40. Cicchi, R. et al. Non-linear imaging and characterization of atherosclerotic arterial tissue using combined SHG and FLIM microscopy. *J. Biophotonics.* **8**(4), 347–356 (2015).
41. Masters, B. R., So, P. T. C. & Gratton, E. Optical biopsy of in vivo human Skin: Multi-photon Excitation Microscopy. *Lasers Med. Sci.* **13**(3), 196–203 (1998).
42. Xiang, F. et al. Quantitative multiphoton imaging of cell metabolism, stromal fibers, and keratinization enables label-free discrimination of esophageal squamous cell carcinoma. *Biomed. Opt. Express.* **14**(8), 4137 (2023).
43. Rück, A., Hauser, C., Mosch, S. & Kalinina, S. Spectrally resolved fluorescence lifetime imaging to investigate cell metabolism in malignant and nonmalignant oral mucosa cells. *J. Biomed. Opt.* **19**(9), 096005 (2014).
44. Heaster, T. M., Landman, B. A. & Skala, M. C. Quantitative spatial analysis of metabolic heterogeneity across in vivo and in vitro Tumor models. *Front. Oncol.* **9** (2019).
45. Skala, M. C. et al. In vivo multiphoton microscopy of NADH and FAD redox states, fluorescence lifetimes, and cellular morphology in precancerous epithelia. *Proc. Natl. Acad. Sci. USA* **104**(49), 19494–19499 (2007).
46. Chekulayev, V. et al. Metabolic remodeling in human colorectal cancer and surrounding tissues: alterations in regulation of mitochondrial respiration and metabolic fluxes. *Biochem. Biophys. Res. Commun.* **4**, 111–125 (2015).
47. Reinsalu, L. et al. Energy Metabolic plasticity of Colorectal Cancer cells as a determinant of Tumor Growth and Metastasis. *Front. Oncol.* **11** (2021).
48. Robertson-Tessi, M., Gillies, R. J., Gatenby, R. A. & Anderson, A. R. A. Impact of metabolic heterogeneity on tumor growth, invasion, and treatment outcomes. *Cancer Res.* **75**(8), 1567–1579 (2015).
49. Kim, J. & DeBerardinis, R. J. Mechanisms and implications of metabolic heterogeneity in Cancer. *Cell. Metab.* **30**(3), 434–446 (2019).
50. Sengupta, D. & Pratz, G. Imaging metabolic heterogeneity in cancer. *Mol. Cancer* **15**(1) (2016).
51. J. ZHENG, Energy metabolism of cancer: glycolysis versus oxidative phosphorylation (review). *Oncol. Lett.* **4**(6), 1151–1157 (2012).
52. De Wever, O. & Mareel, M. Role of tissue stroma in cancer cell invasion. *J. Pathol.* **200**(4), 429–447 (2003).
53. Pouli, D. et al. Two-photon images reveal unique texture features for label-free identification of ovarian cancer peritoneal metastases. *Biomed. Opt. Express.* **10**(9), 4479 (2019).
54. Shcheslavskiy, V. I. et al. Fluorescence time-resolved macroimaging. *Opt. Lett.* **43**(13), 3152 (2018).
55. Lukina, M. et al. Label-free macroscopic fluorescence lifetime imaging brain tumors. *Front. Oncol.* **11** (2021).
56. Mycek, M. A., Schomacker, K. T. & Nishioka, N. S. Colonic polyp differentiation using time-resolved autofluorescence spectroscopy. *Gastrointest. Endosc.* **48**(4), 390–394 (1998).
57. Coda, S. et al. Fluorescence lifetime spectroscopy of tissue autofluorescence in normal and diseased colon measured ex vivo using a fiber-optic probe. *Biomed. Opt. Express.* **5**(2), 515 (2014).
58. Herrando, A. et al. Dual excitation spectral autofluorescence lifetime and reflectance imaging for fast macroscopic characterization of tissues. *Biomed. Opt. Express.* (2023).
59. Köllner, M. & Wolfrum, J. How many photons are necessary for fluorescence-lifetime measurements? *Chem. Phys. Lett.* **200**(1–2), 199–204 (1992).
60. Hinsdale, T. et al. Optically sectioned wide-field fluorescence lifetime imaging microscopy enabled by structured illumination. *Biomed. Opt. Express.* **8**(3), 1455 (2017).
61. Lagarto, J., Hares, J. D., Dunsby, C. & French, P. M. W. Development of low-cost instrumentation for single point autofluorescence lifetime measurements. *J. Fluoresc.* (2017).
62. Walsh, A. J. & Skala, M. C. Optical metabolic imaging quantifies heterogeneous cell populations. *Biomed. Opt. Express.* **6**(2), 559 (2015).
63. Walsh, A. J. et al. Optical metabolic imaging identifies glycolytic levels, subtypes, and early-treatment response in breast cancer. *Cancer Res.* **73**(20), 6164–6174 (2013).
64. Ruppert, D., Wand, M. P. & Carroll, R. J. *Semiparametric Regression* (Cambridge University Press, 2003).

65. Kenward, M. G. & Roger, J. H. Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* **53**(3), 983 (1997).
66. Alfonso-Garcia, A. et al. Assessment of murine colon inflammation using intraluminal fluorescence lifetime imaging. *Molecules* **27**(4), (2022).
67. McGinty, J. et al. Wide-field fluorescence lifetime imaging of cancer. *Biomed. Opt. Express* **1**(2), 627 (2010).
68. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy* (Springer, 2006).
69. Nebuloni, M. et al. Insight on colorectal carcinoma infiltration by studying perilesional extracellular matrix. *Sci. Rep.* **6**(1), 22522 (2016).
70. Liang, Y. et al. Prognostic significance of abnormal matrix collagen remodeling in colorectal cancer based on histologic and bioinformatics analysis. *Oncol. Rep.* (2020).
71. Provenzano, P. P., Eliceiri, K. W. & Keely, P. J. Multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) to monitor metastasis and the tumor microenvironment. *Clin. Exp. Metastasis*. **26** (4), 357–370 (2009).
72. Izuishi, K. et al. The histological basis of detection of adenoma and cancer in the colon by autofluorescence endoscopic imaging. *Endoscopy* **31**(7), 511–516 (1999).
73. Costa, S., Fang, Q., Farrell, T., Dao, E. & Farquharson, M. Time-resolved fluorescence and diffuse reflectance for lung squamous carcinoma margin detection. *Lasers Surg. Med.* **56**(3), 279–287 (2024).
74. Zhang, L. et al. Predictive value of intratumoral-metabolic heterogeneity derived from 18F-FDG PET/CT in distinguishing microsatellite instability status of colorectal carcinoma. *Front. Oncol.* **13** (2023).
75. Oh, B. Y. et al. Intratumor heterogeneity inferred from targeted deep sequencing as a prognostic indicator. *Sci. Rep.* **9**(1), 4542 (2019).
76. Liu, X. et al. Prognostic value of intratumor metabolic heterogeneity parameters on 18F-FDG PET/CT for patients with colorectal cancer. *Contrast Media Mol. Imaging* **2022**, 1–11 (2022).
77. Han, Y. H., Jeong, H. J., Sohn, M. H. & Lim, S. T. Clinical value of intratumoral metabolic heterogeneity in [18F]FDG PET/CT for prediction of recurrence in patients with locally advanced colorectal cancer. *Q. J. Nuclear Med. Mol. Imaging* **62**(4) (2018).
78. Zhang, M. et al. Metabolism-associated molecular classification of colorectal cancer. *Front. Oncol.* **10** (2020).
79. Rajput, A., Bocklage, T., Greenbaum, A., Lee, J. H. & Ness, S. A. Mutant-allele Tumor Heterogeneity scores Correlate with risk of metastases in Colon cancer. *Clin. Colorectal Cancer*. **16**(3), e165–e170 (2017).
80. Joung, J. G. et al. Tumor heterogeneity predicts metastatic potential in Colorectal Cancer. *Clin. Cancer Res.* **23**(23), 7209–7216 (2017).
81. Read, G. H., Bailleul, J., Vlashi, E. & Kesarwala, A. H. Metabolic response to radiation therapy in cancer. *Mol. Carcinog.* **61** (2), 200–224 (2022).
82. van der Stel, S. D. et al. Size and depth of residual tumor after neoadjuvant chemoradiotherapy in rectal cancer—Implications for the development of new imaging modalities for response assessment. *Front. Oncol.* **13** (2023).
83. Loree, J. M. et al. Classifying colorectal cancer by tumor location rather than sidedness highlights a continuum in mutation profiles and consensus molecular subtypes. *Clin. Cancer Res.* **24**(5), 1062–1072 (2018).
84. Guinney, J. et al. The consensus molecular subtypes of colorectal cancer. *Nat. Med.* **21**(11), 1350–1356 (2015).
85. Skala, M. C., Fontanella, A., Lan, L., Izatt, J. A. & Dewhirst, M. W. Longitudinal optical imaging of tumor metabolism and hemodynamics. *J. Biomed. Opt.* **15**(1), 011112 (2010).
86. Snyder, C. M. & Chandel, N. S. Mitochondrial regulation of cell survival and death during low-oxygen conditions. *Antioxid. Redox Signal.* **11**(11), 2673–2683 (2009).
87. Lukina, M. M. et al. Interrogation of tumor metabolism in tissue samples *ex vivo* using fluorescence lifetime imaging of NAD(P)H. *Methods Appl. Fluoresc.* **8**(1), 014002 (2019).

Acknowledgements

We would like to acknowledge the Hardware Platform of Champalimaud Foundation for the support in the development of the instrumentation. We also thank all personnel of Champalimaud Surgical Center involved in sample collection, and all technicians from the Pathology Service and the Champalimaud Foundation Biobank for their assistance with sample preparation. Alberto I. Herrando was supported by the European Union's Horizon 2020 research and innovation programme under Marie Skłodowska-Curie grant 857894 - CAST. Vladislav Shcheslavskiy was supported by RSF 23-15-00294.

Author contributions

A.I.H.: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing—original draft. L.M.F., J.A., P.V., H.D., R.J.H. and A.P.: Methodology; Resources; Writing—review & editing. A.G.: Methodology; Investigation; Validation. V.S.: Resources; Writing—review & editing. P.G.S.: Project administration; Supervision; Funding acquisition; Writing—review & editing. M.C.M.: Conceptualization; Methodology; Investigation; Validation; Resources; Supervision; Writing—review & editing. J.L.L.: Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing—original draft.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-74224-8>.

Correspondence and requests for materials should be addressed to A.I.H.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2024