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ORIGINAL ARTICLE

In vitro investigation of fluorescence of carious dentin observed with a Soprolife® camera

Ivan Panayotov · Elodie Terrer · Hamideh Salehi · Hervé Tassery · Jacques Yachouh · Frédéric J. G. Cuisinier · Bernard Levallois

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Abstract
Objectives Our aim was to determine the origin of the red fluorescence of carious dentine observed with the Soprolife® camera.
Methods We conducted in vitro studies to evaluate the origin of the red fluorescence using acids and matrix metalloproteinase (MMP) to mimic caries and methylglycoxal (MGO) to evaluate the effect of glycation reactions on the red fluorescence. In every step of these models, we detected the changes of dentin photonic response with Soprolife® in daylight mode and in treatment mode. A Raman spectroscopy analysis was performed to determine the variations of the dentin organic during the in vitro caries processes. Raman microscopy was performed to identify change in the collagen matrix of dentine.
Results The red fluorescence observed in carious dentine using a Soprolife® camera corresponds to the brownish color observed using daylight. Demineralization using nitric acid induces a loss of the green fluorescence of dentine. The red fluorescence of carious dentine is resistant to acid treatment. Immersion of demineralized dentine in MGO induces a change of color from white to orange-red. This indicates that the Maillard reaction contributes to lesion coloration. Immersion of demineralized dentine in an MMP-1 solution followed by MGO treatment results in a similar red fluorescence. Raman microspectroscopy analysis reveals accumulation of AGE's product in red-colored dentine.
Conclusions Our results provide important information on the origin of the fluorescence variation of dentine observed with the Soprolife® camera. We demonstrate that the red fluorescence of carious dentine is linked to the accumulation of Advanced Glycation End products (AGE).
Clinical relevance The study provides a new biological basis for the red fluorescence of carious dentine and reinforces the importance of the Soprolife® camera in caries diagnostics.

Keywords Dentine · Caries · Maillard reaction · Fluorescence · Raman spectroscopy

I. Panayotov · H. Salehi · H. Tassery · J. Yachouh · F. J. G. Cuisinier (✉) · B. Levallois
EA4203 Laboratory of Biology and Nano-science, Université Montpellier 1,
Montpellier, France
e-mail: frederic.cuisinier@univ-montp1.fr

E. Terrer · H. Tassery
Preventive and Restorative Department, Mediterranean University,
Marseille, France

Introduction

Proper treatment of dental caries demands detection of carious lesions at an early stage [1]. Because previous caries and caries risk assessments are the best predictors of future caries, the development of a technology to detect and quantify early carious lesions and carious activity may be the best method of identification of patients who require intensive preventive intervention [2]. Several new detection methods based on the fluorescence variation of dentine have been recently developed [3]. Three main imaging techniques based on the fluorescence response of the organic components of teeth have been developed for use in caries detection. The commercially available devices are as follows:

1. Fluorescence system. The DIAGNOdent® (KaVo Dental, Lake Zurich, Ill.), which uses a laser, reacting in part on extrinsic components like porphyrins operating at a fixed wavelength of 655 nm.
2. Combination of camera and fluorescence system: (a) the QLF® (QLF-clin, Inspektor Research Systems BV, Amsterdam, Netherlands), which uses an arc lamp with emission in the wavelength domain of 290–450 nm and

looks at the change in transmission from the green fluorescence occurring in the dentin body changed due to micro-porosities in enamel as a result of the acid attack, (b) the Canary® system (Quantum Dental Technologies, Toronto, Canada) is low powered and laser based, which uses a combination of heat and light (Frequency Domain Photothermal Radiometry and Modulated Luminescence (FD-PTR and LUM)) to examine directly the crystal structure of teeth and map the area of tooth decay, and (c) Soprolife® camera (Acteon, La Ciotat, France), which uses an LED light with an excitation wavelength of approximately 450 nm [4–6].

The Soprolife® selectively amplifies fluorescence signals to accentuate the specificity of the fluorescence images. As a result, any carious lesion or diseased tissue is detected based on the variation of its autofluorescence compared to the healthy area of the same tooth. The brown color of caries in daylight is perfectly superimposable over the dark red fluorescence detected with the Soprolife®. Sound dentine fluoresces green, and enamel has no fluorescence, although a bluish color is often observed from the diffusion of the green light emitted by dentine.

Dental caries is generally acknowledged as a process whereby bacterial acids destroy hard dental tissues. It is therefore not surprising that a great deal of caries research has been devoted to the de- and re-mineralization of enamel or dentine. Another notable but less investigated feature of the caries process is the Maillard reaction, which was previously described for dental caries coloration [7]. The Maillard reaction is a sugar–protein reaction of amino acid degradation in the presence of natural monosaccharides and encompasses a huge range of intermediates and products. It is also known as glycation or non-enzymatic browning. Numerous Maillard reaction products have been elucidated; many of which are formed in tissues during ageing and diabetes. Kleter et al. have demonstrated the presence of fluorescent amino acids such as hydroxylysylpyridinoline, lysylpyridinoline, pentosidine, and carboxymethyllysine in carious dentine [8].

Based on previous data, the first hypothesis tested in our work was that the fluorescence of carious dentine is caused by demineralization. The second hypothesis was that it is caused by the destruction of the demineralized dentine organic matrix by matrix metalloproteinase (MMP). The third hypothesis tested was that the Maillard reaction-produced advanced glycation end-products (AGEs) of the dentine organic matrix are responsible for the red fluorescence.

The objectives of this study were addressed with a series of experiments intended to investigate the origin of dental tissue fluorescence during caries using the following: (1) in vitro imaging of dental tissues treated with acid and matrix metalloproteinase (MMP) to mimic caries and methylglycoxal (MGO) to evaluate the Maillard reaction using the Soprolife® camera, (2) Raman microspectroscopy to determine the accumulation of AGE product in dentine appearing red with the Soprolife® camera in fluorescence mode.

Methods

Specimen preparation

Written consent was obtained prior to the extraction of eight healthy wisdom teeth at the University Hospital of Montpellier. In addition, two molars presenting occlusal caries (caries codes 5 and 6, ICDAS, International Caries Assessment and Detection System) were included in the study [9]. Under daylight observation, carious dentine presented a brown color, and a dark red color when observed with the Soprolife® camera in treatment mode.

Freshly extracted teeth were sectioned in the longitudinal axis by means of a diamond saw (Isomet 1000, Buehler, Lake Bluff, USA) with a thickness of up to 0.5 mm. Samples were ground to 0.25 mm and polished on carbide disks using diamond pastes (6, 1, and 0.25 μm) on an Escil polishing machine (Escil, Lyon, France). Finally, specimens were thoroughly cleaned in water with an ultrasonic bath for 5 min. Four samples were demineralized in 2.5 % aqueous nitric acid solution (pH=1) for 9 days. Four samples were demineralized using a four-in 4 % aqueous solution of lactic acid. Demineralization solutions were changed every 2 days.

The nitric acid demineralized samples were then processed as follows:

- Four non-carious samples and two carious samples were incubated for 2 days in a solution of 4 mL of MMP1 (Sigma-Aldrich Corporation, St. Louis, USA) at a concentration of 3 mg mL^{-1} in phosphate buffered saline (PBS) at room temperature.
- Two demineralized healthy samples were incubated in methylglycoxal (Sigma-Aldrich Corporation, St. Louis, USA) at a concentration of 10 mM in a PBS solution at 37 °C for 4 weeks (Fig. 1).
- Two demineralized healthy samples were incubated in methylglycoxal (Sigma-Aldrich Corporation, St. Louis, USA) at a concentration of 1 M in a PBS solution at 37 °C for 4 weeks (Fig. 1).

Soprolife® camera

The Soprolife® intra-oral camera utilizes two groups of LEDs that can illuminate tooth surfaces in either daylight mode (white light) or blue light mode (wavelength of 450 nm with a bandwidth of 20 nm, centered at ±10 nm around the excitation wavelength). The camera provides an anatomical image (daylight mode) superimposed on an

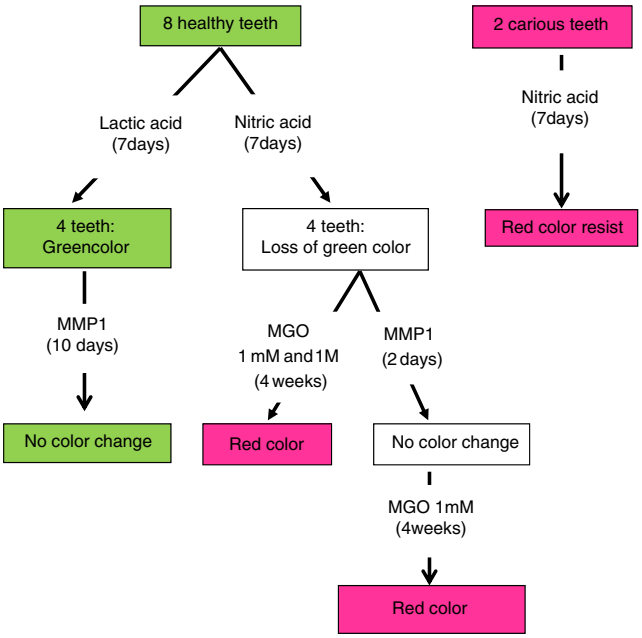

Fig. 1 Sample preparation workflow. Colors indicate the color of dentine observed with the Soprolife® camera in treatment mode after the chemical treatments symbolized by arrows

image produced by the autofluorescence (blue light) of the sample. The camera is equipped with an image sensor (a 0.25-in. CCD sensor) consisting of a mosaic of pixels covered with filters of complementary colors. The collected data, related to the energy received by each pixel, enable an image of the tooth to be retrieved and stored in a computer.

At each stage of analysis, we recorded pictures of the wet samples with the intra-oral fluorescent Soprolife® camera in daylight mode, diagnostic mode, and treatment mode. Daylight mode corresponds to the image registered using the four white light LEDs. Diagnostic and treatment images are produced under blue light from four blue LEDs. The difference between diagnostic and treatment modes involves difference in CCD settings. Treatment mode is designed to increase the red part of the electromagnetic spectrum.

We observed samples in hydrated state to avoid the modifications of teeth that have been previously described [10].

Micro-Raman spectroscopy

To investigate the chemical composition of different dentine samples, Raman spectra were recorded at 298 K with a LabRAM ARAMIS IR^2 confocal micro-Raman spectrometer (Jobin-Yvon S.A., Horiba, France) equipped with a BX41 Olympus microscope, a 1,800 grooves per mm grating, and a CCD detector cooled by a Peltier effect module. Samples were mounted on an XYZ motorized stage with 0.1-μm step resolution. The microscope lens was a long working distance ×50 objective. The spectral resolution was ±1 cm^{-1}. Raman spectra were obtained by excitation with 632.8 nm radiations from a He–Ne laser generating less than 12 mW on the samples. Due to fluorescence, samples were under the laser for photobleaching for 15 min before spectral recording. The acquisition time was 90 s with three accumulations.

Spectra obtained from Raman microscopy were normalized using Peakfit v4.12 (USA) software. The Gaussian amplitude in the second derivative method was chosen for fitting the peaks [11].

We also compared modes intensities ratio between and for amide I (1,670 cm^{-1}) and AGE products (1,550–1,600 cm^{-1}) [12, 13]. An increase of this ratio indicates altered collagen quality induced by Maillard reaction.

Results and discussion

Under observation with the Soprolife® intra-oral camera, teeth showing caries appeared acid green in treatment mode, and the carious tissues at the base of the three cavities produced a red color (Fig. 2). Sound dentine fluoresces green, and enamel has no fluorescence, although a bluish color is often observed from the diffusion of the green light emitted by dentine.

More precisely in active and arrested carious lesion, visual signals in fluorescent mode look different depending on the tissue structure [4]. Healthy dentin looks always acid green in fluorescence, as the infected one appears green black. In active caries, infected/affected dentin interface looks bright red fluorescence and yellow brown in daylight. Even caries color is not always a perfect reliable measure; this tissue is fairly easily eliminated with a manual excavator [14, 15]. Abnormal dentin at the end of excavation looks gray-green, sometimes with a very slight pink transparency.

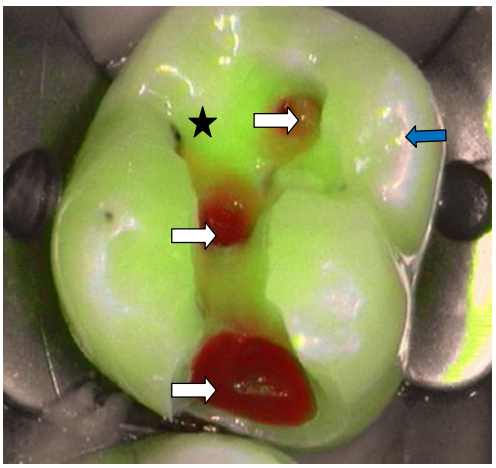

Fig. 2 Aspect of dental caries (ICDAS:5) with Soprolife® camera in treatment mode. *Black star* healthy dentine, *white arrows* dentine decay, *blue arrows* enamel surface

In arrested lesion, the infected/affected interface looks dark red and dark brown in daylight mode. This tissue is more difficult to eliminate with a manual excavator. Abnormal dentin at the end of the excavation looks light gray green with systematically persisting shady pink fluorescence at the bottom of the preparation, opposite the pulpal wall, which don't need to be more removed. It should not be at that time considered as false-positive signal.

In vitro caries model—influence of demineralization

After 9 days of incubation in a 2.5 % aqueous nitric acid solution, tooth sections completely lost their green color (Fig. 3). The loss of fluorescence indicates that dentine fluorescence is not due to collagen alone but to the interplay of calcium phosphate crystals and collagen. During demineralization with lactic acid of a sound tooth observed with the Soprolife® camera in treatment mode, the dentine green fluorescence persists to 7 days (Fig. 4). Enamel persists during these 7 days, showing that demineralization is incomplete. This demineralization is forming pits on dentine surface due to an inhomogeneous acid demineralization. This shows that a complete demineralization as obtained with nitric acid is needed to lose the green fluorescence of dentine. Fluorescence spectroscopy previously performed on sound root dentine also indicated that the strongest emitted fluorescence at a 450-nm excitation is an emission of approximately 500 nm (see Fig. 2 of reference [16]). This property was proposed in a previous fluorescence study using the same excitation light [17]. More recently, using an excitation light of 515 nm, the opposite phenomena were reported, and the authors concluded that the chromophores responsible for the green fluorescence of dentine must be organic in nature [18].

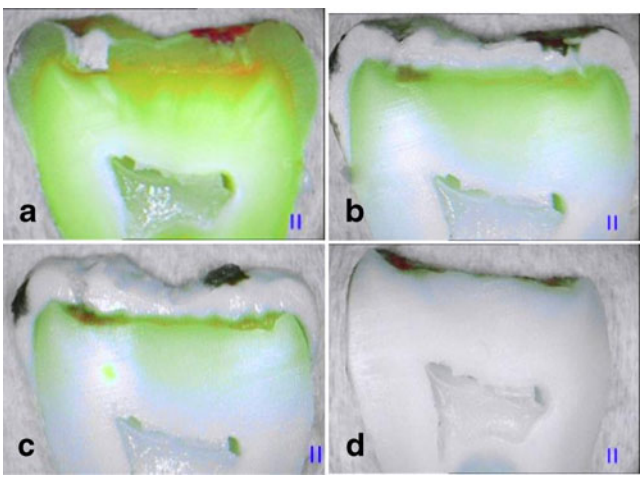

Fig. 3 Kinetics of demineralization with nitric acid of a sound tooth observed with the Soprolife® camera in treatment mode. **a** Day 0, **b** day 3, **c** day 6, **d** day 9. Enamel is absent, and dentine is white

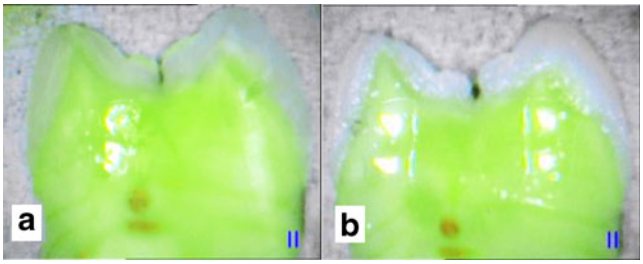

Fig. 4 Kinetics of demineralization with lactic acid of a sound tooth observed with the Soprolife® camera in treatment mode. Dentine green fluorescence persists to 7 days in lactic acid. Surface is irregular with trace of dissolution. **a** Day 0, **b** day 7

These results of sound dentin demineralization didn't explain the strong red fluorescence of caries. The red color of carious dentine observed in the bottom of cavities (Fig. 2) extends deeper into the dentine. We compared dentine fluorescence of sound and caries-affected dentine during nitric acid demineralization (Fig. 5). Comparison between images obtained in daylight mode (Fig. 5a) and treatment mode (Fig. 5b) shows that the red fluorescence corresponds to the brownish color of carious and infiltrated dentine. The green color of dentine is observed under the carious dentine, which has a red color. As for the sound teeth, demineralization in aqueous nitric acid solution induces a loss of dentine's green color when observed with Soprolife® camera in treatment mode. In contrast, the red carious dentine maintains its reddish color following demineralization (Fig. 5d). The loss of the mineral matrix of dentin induces losses of fluorescence of sound dentine and is not responsible of the strong red fluorescence of carious dentin observed with the Soprolife camera in treatment mode. The reason of this red fluorescence must be searched in structural changes of the organic matrix of dentine.

In vitro caries model—Maillard reaction and matrix metalloproteinase

Following demineralization, tooth sections were further treated with MGO and monitored Raman microscopy. After 14 days, the dentine color begins to change to an orange color, and two additional weeks in MGO turn the color of tooth sections homogenously red. Previous works reported that the Maillard reaction contributes to lesion discoloration [8]. A number of Maillard products, referred to as advanced glycation end-products (AGEs), are formed in tissues during ageing and diabetes [19]. In an in vitro model reaction, demineralized dentine reacted with glucose, acquiring a yellow stain [7]. AGE products are highly fluorescent [20], and the in vitro fluorescence increases as a function of time of incubation, as we observed. The time-dependent color increase reveals the existence of a biochemical reaction leading to accumulation of fluorescent products [21] (Fig. 6).

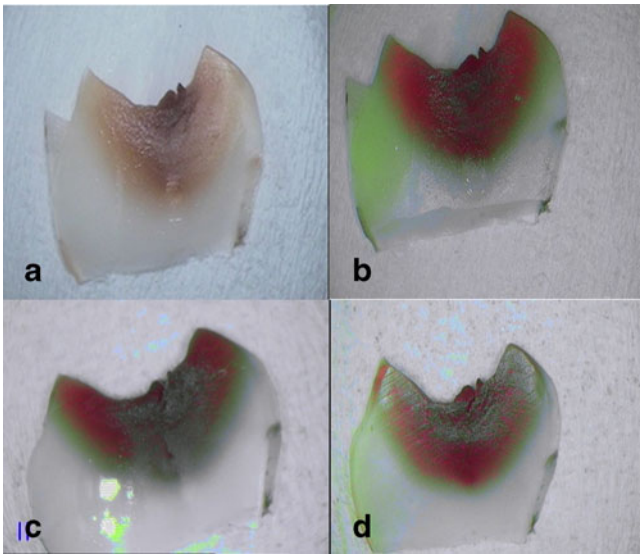

Fig. 5 Carious dentine demineralization in nitric acid observed with the Soprolife® camera. Molar code 6 ICDAS. **a** Day 0, white light mode: carious dentine has a brownish color; **b** day 0, treatment mode: carious dentine is dark red, and sound dentine is green; **c** day 15, treatment mode: carious dentine is dark, still red, and sound dentine is colorless; **d** day 27, treatment mode: carious dentine is still dark red, and sound dentine is colorless

A second experiment with MGO was performed by combining collagen alteration with MMP1 (Fig. 7). This experiment aimed to evaluate whether the orange red color observed during the MGO treatment of demineralized dentine would change. We expected that collagen degradation products would

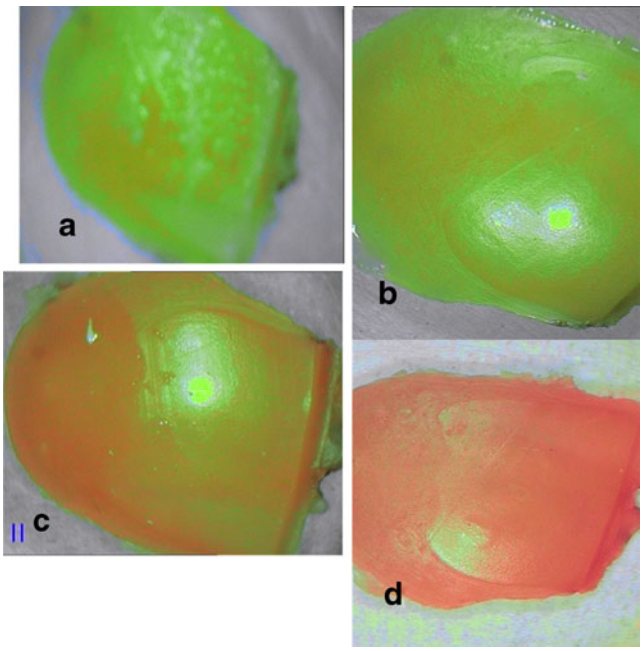

Fig. 6 Nitric acid-demineralized tooth treated with methylglycoxal monitored with the Soprolife® camera in treatment mode. **a** Day 0, **b** day 7, **c** day 14, **d** day 30. After 14 days, the dentine color starts to change to an orange color, which transitions to red by 30 days

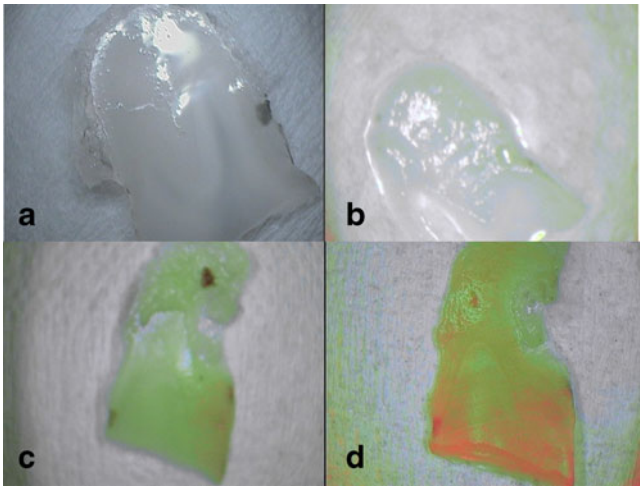

Fig. 7 Combined action of MMP1 and MGO as monitored by Soprolife® camera in treatment mode. **a** Colorless demineralized teeth. **b** After 2 days in MMP1, the specimen is colorless, but its shape was altered due to collagen destruction. **c** After 12 days in MGO, a green color appears. **d** After 19 days in MGO, a red color appears

be more sensitive to non-enzymatic glycation and a red shift would be observed. Following complete demineralization, a tooth section was immersed in an MMP1 aqueous solution for 2 days. Following this treatment, the sample started to lose its original shape, due to collagen degradation. Then, the section was immersed in an MGO solution. After 19 days in this solution, a red color began to appear, similar to the color obtained without collagen degradation.

These two experiments clearly indicate that the end-product of the Maillard reaction (AGE) induces the red color. Albeit, a careful comparison between the red color observed at the bottom of a dental cavity and the orange-red color obtained with the MGO bath indicates that they are different. This difference might be the result of differences in the thickness of the samples or the quantity of AGE products, which would depend on the extend of the Maillard reaction in the samples.

Micro-Raman spectroscopy of carious dentine

As we cannot prove the working mechanism of the camera with the camera as the only objective measure, four dentine samples were studied by Raman spectroscopy. There have been few studies of AGEs using Raman spectroscopy. Sebag et al. used a non-microscopy-based approach to show Raman spectral changes in the human vitreous that were attributed to AGEs [22]. Confocal Raman microscopy was used to quantify AGEs adducts in Bruch membrane [12, 13].

Table 1 gathers the Raman intensities of amide I and AGE's related product bands as well as their ratio. Ratios of this two organic bands increase in carious dentin and also in demineralized dentine treated with methylglycoxal. This

Table 1 AGE's band (1,550–1,600 cm^{-1}) intensity, Amide 1 band (1,670 cm^{-1}) intensity, and band intensities ratio of AGEs versus Amide 1

	Sound dentine	Carious dentine	MGO 10 mM	MGO 1 M
AGE's band (1,550–1,600 cm^{-1})	399	1,865	729	608
Amide 1 band (1,670 cm^{-1})	2,434	1,428	3,286	278
Ratio	0.16	1.31	0.22	2.19

gives a second indication that the red color of carious dentine observed with Soprolife® camera is certainly due to an accumulation of AGE product.

Conclusions

The Soprolife® camera selectively amplified the fluorescence signals of carious dentine. The brown signal of caries observed under daylight corresponds to the dark red fluorescence observed with the camera in treatment mode. We demonstrate that the green fluorescence of sound dentine is dependent on demineralization. However, a definitive proof would be to re-create the green fluorescence of a dentine collagen matrix during re-mineralization. As dentine mineralization is a complex process involving different proteins controlling the nature, size, and organization of calcium phosphate crystals [23], such a demonstration will be a challenging task.

The influence of the Maillard reaction on the appearance of a red coloration during caries is suggested. However, it is possible that this reaction is not the only one responsible, as the orange-red color is different from the deep red color observed at the bottom of dental cavities. The influence of bacterial metabolism (e.g., *S. mutans*) in association with Maillard reactions on the generation of the red color remains to be investigated.

Our experiment using MMP1 didn't generate any fluorescence variation. An experimental system closer to reality, using MMP produced by bacteria, might be a more informative approach.

In conclusion, our results provide important information on the origin of fluorescence variation of dental caries observed with the Soprolife® camera. These investigations should be continued and new parameters must be evaluated, as it is clear that destruction of dentine during caries is a complex process with many parameters to consider.

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Conflict of interest The authors declare that they have no conflict of interest.

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