ORIGINAL ARTICLE

In vitro quantitative light-induced fluorescence to measure changes in enamel mineralization

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Abstract A sensitive, quantitative method for investigating changes in enamel mineralization of specimens subjected to in vitro or in situ experimentation is presented. The fluorescence-detecting instrument integrates a Xenon arc light source and an object positioning stage, which makes it particularly suitable for the nondestructive assessment of demineralized or remineralized enamel. We demonstrate the ability of in vitro quantitative light-induced fluorescence (QLF) to quantify changes in mineralization of bovine enamel discs that had been exposed in vitro to a demineralizing gel (n=36) or biofilm-mediated demineralization challenges (n=10), or were carried in situ by three volunteers during a 10-day experiment (n=12). Further experiments show the technique's value for monitoring the extent of remineralization in 36 specimens exposed in vitro to oral multispecies biofilms and document the repeatability of in vitro QLF measurements (n=10) under standardized assay conditions. The validity of the method is illustrated by comparison with transversal microradiography (TMR), the invasive current gold standard for assessing experimental changes in enamel mineralization. Ten discs with 22 measurement areas for comparison demonstrated a positive correlation between TMR and QLF (r=0.82). Filling a technological gap, this QLF system is a promising tool to assay in vitro nondestructively localized changes in mineralization of enamel specimens.

Keywords Dental enamel · Dental caries · Tooth demineralization/remineralization · Image processing · Computer-assisted

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Introduction

Dental caries is caused by metabolic end-products (in particular lactic acid) produced from carbohydrates in bacterial biofilms—dental plaque—colonizing smooth surfaces and fissures of teeth [6]. Main factors determining the pathogenicity of plaque are believed to be host diet, plaque composition, and plaque diffusion properties. However, the relative importance of these parameters is not entirely clear. In recent years, in vitro and in situ biofilm models have gained considerable attention to study the pathogenic mechanisms and to act as aids in the development of efficient antimicrobials or plaque-modifying agents for use as ingredients in caries-preventive oral care products [7, 8]. To measure demineralization and remineralization of enamel underneath such biofilms, transverse microradiography (TMR), which requires the controlled destruction of the enamel specimen, is currently the method of choice. Clearly, a reliable, quantitative, nondestructive, and site-specific detection system is highly needed.

Quantitative light-induced fluorescence (QLF), designed for clinical application [5], is a promising method meeting these requirements if it is adaptable to standardized in vitro application [1, 12]. In this paper, we describe a new commercially available in vitro QLF system allowing the quantification of changes in mineral content of experimental bovine enamel discs, usable for in vitro biofilm experiments [8] or in the course of in situ studies [7].

Materials and methods

In vitro QLF

The experimental setup of the instrument (Inspektor Research Systems BV, Amsterdam, The Netherlands) is shown in Fig. 1. It is a gradual modification of earlier systems described by Al-Khateeb et al. [1] and Pretty et al. [12]. White light is produced in a control box by a xenon arc lamp, filtered by a Hoya 370 nm bandpass filter and sent via a liquid light-guide to the holder, resulting in violet-blue excitation light on the specimen with peak wavelength at 405 nm and FWHM of 23 nm. The holder consists of a rotation and translation sample stage and a Sony CCD Camera (DXC-LS1P) equipped with a 12-mm focal lens and a >520 nm high-pass filter to record autofluorescence light emitted from enamel/dentin specimens. The camera, protected from the influx of ambient light by a black fabric hood, is mounted at a fixed focal distance of 33.35 mm from the specimen surface. Instrument calibration is done after a 15-min warm-up using a special stable fluorescent grid with known grayscale gradients (Inspektor). Images recorded by the video camera are grabbed real-time by a personal computer and instantly compared with a previously recorded reference image of the same specimen. To precisely position a disc on the stage, the live image is lined up with the red outline of the reference image using the "QLF-Patient" software (version 3.0.026; Inspektor). Optimum specimen orientation can also be controlled by the video repositioning module of the "QLF-Patient" software, but high correlation levels (e.g., 96–99%) are only obtained with control specimens which were not subjected to demineralization or remineralization. When optimum specimen positioning is achieved, a definitive image is stored in bitmap format. Pre- and postintervention images of specimens are compared by subtraction analysis using the software "Subtract 1.1.0.4" (Inspektor). Outcome measures are ΔF, defined as the percent change from the pre- to the postintervention image in fluorescence radiance in each image point averaged over the analysis area, and ΔQ, defined as the total volumetric fluorescence change (=ΔF multiplied by the area of analysis):

Fluorescence change in pixel i = $\Delta F(i)$

$$= \frac{F_{post}(i)}{F_{pre}(i)} \times 100 \, in \, \% \tag{1}$$

Average fluorescence change in postintervention image

$$= \overline{\Delta F_{post}} = \frac{\sum\limits_{image} \Delta F(i)}{A_{image}}, \tag{2}$$

where A_{image} corresponds to the analysis area.

The region of analysis is defined by one or more freely moveable and sizable patches that overlay the images (not shown). Similarly, a reference patch can be defined to outline a control region, which, for example, is protected from demineralization/remineralization by a nail varnish cover. Use of a

Fig. 1 Experimental setup
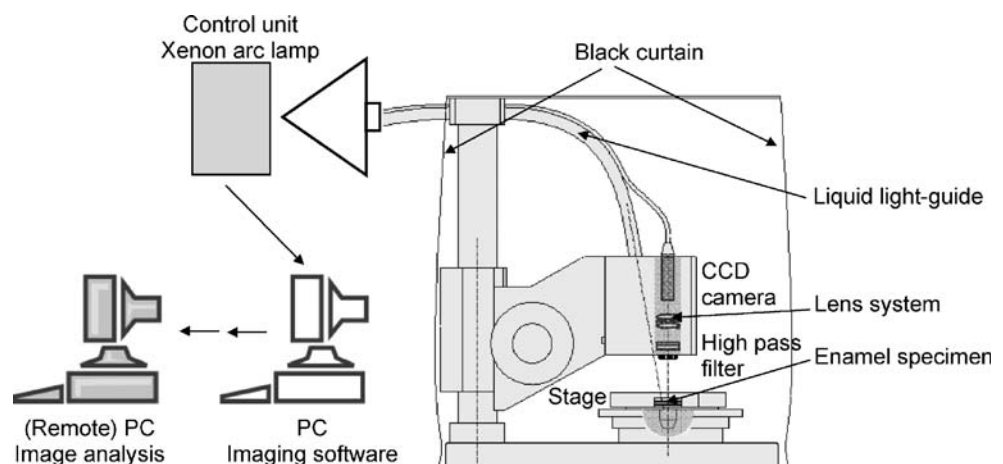
reference patch leads to a correction of the fluorescence radiance of each pixel, which is defined as follows:

$$Corrected\ fluorescence\ in\ pixel\ i = \frac{\sum\limits_{refpatch,\,pre} F(j)}{\sum\limits_{refpatch,\,post} F(j)}$$

$$\times F_{post}(i)$$

$$= \frac{\overline{F_{refpatch,\,pre}}}{\overline{F_{refpatch,\,post}}} \times F_{post}(i). \qquad (3)$$

Specimen areas affected by comparable degrees of demineralization or remineralization are definable by threshold levels for ΔF and may be visualized by a pseudo color illustration of the subtraction image.

Bovine enamel disc preparation, QLF analysis

Bovine enamel discs with a diameter of 6.8 mm were prepared from lower jaw incisors by first excising a cylinder perpendicular to the labial surface of the teeth with a diamond-coated corer under constant flow of cooling water and then grinding the cylinder from both sides using 500 sand paper to a final thickness of 1.5–1.6 mm with the enamel–dentin junction located approximately equidistant from both surfaces. Discs received a small notch at the edge (visible in Fig. 2) for disc alignment during QLF measurements and were stored in the dark in distilled water at 4°C until used. They were sterilized by exposure to ethylene oxide at room temperature before experimental use during in vitro or in situ studies.

For QLF measurements, discs were removed from storage fluid and gently cleared from any drops of fluid using a paper napkin. Then, after 20 min (±1 min) at room atmosphere on a paper napkin with the enamel side up, they were positioned one-by-one on the QLF stage to record the first disc image (base line image). When recording follow-up or postintervention images of the same disc, the preparatory steps were the same and then the disc was precisely positioned on the QLF instrument stage by the optimum alignment of the live image with the stored baseline image (using "QLF-Patient" as described above).

In situ demineralization experiment

The in situ experiment was performed with three volunteers who wore two acrylic appliances on the buccal side of the mandibular premolar/molar regions day and night [7] during a 10-day test period. Approval of the regional ethical committee (Bergen, Norway) was obtained. Each appliance had two standardized chambers in which bovine enamel discs were fixed using silicone (Dimension

Garant L, Espe, Seefeld, Germany). Test subjects performed habitual mechanical oral hygiene using fluoridated toothpaste in the morning and evening (Solidox Fluor, Lilleborg AS, Oslo, Norway; 1500 ppm F⁻ as NaF) and adhered to their regular diet with the appliances temporarily removed and stored in saline (0.9% NaCl) during meals and tooth cleanings. Appliances were dipped twice daily into either deionized water or 26.3 mM NaF (500 ppm F⁻; Riedel de Haën, Seelze-Hannover, Germany); treatment assignment to the left or right appliance was random. In addition, all appliances were immersed eight times daily (every second hour from 8 AM to 10 PM) for 10 min in sugar solution (3% glucose, 3% sucrose, 70 mM NaCl, 70 mM KCl, 2 mM MgCl₂; pH 5.5) to promote enamel demineralization. The dipping into water or NaF solution was done at least 30 min before the first (morning) or at least 30 min after the last exposure to saline or sugar solution (evening). At the end of the test period (day 11) appliances were removed from the mouth and plaque was carefully scraped off the disc surfaces with blunt plastic curettes (ZI 10; Deppeler, Rolle, Switzerland). Discs were then thoroughly washed in saline and stored at 4°C in saline until assessed by QLF. Spot tests showed no evidence for biofilm remnants.

In vitro biofilm demineralization experiments

For the comparison of TMR and QLF (see below), ten discs representing a broad range of demineralization were selected. They stemmed from several biofilm experiments carried out under different experimental conditions. Briefly, six-species in vitro biofilms composed of *Streptococcus oralis*, *Streptococcus sobrinus*, *Actinomyces naeslundii*, *Veillonella dispar*, *Fusobacterium nucleatum*, and *Candida albicans* were grown on bovine enamel discs in 24-well cell culture plates (static batch cultures with periodical medium changes) as described previously [8, 15]. Seven discs were derived from experiments using the so-called "feeding model". This implies that discs with biofilms were kept in saliva except for a series of three (three discs), four (two discs), or five (two discs) 45-min exposures to 65% medium/30% saliva/2.5% glucose/2.5% sucrose on days 2 and 3 of the 64.5-h experiment [17]. The other three discs were derived from experiments with the standard 70:30 model [8], whereby biofilms were kept throughout the incubation period in 70% saliva and 30% medium supplemented with Sörensen phosphate buffer and a balanced glucose/sucrose mixture. For these three discs/biofilms, the buffer conditions were adjusted to 20, 80, or 100% of the regularly used buffer strength, while the carbohydrate concentration was 2.5, 5, and 5%, respectively. All discs were harvested after 64.5 h, freed of any biofilm
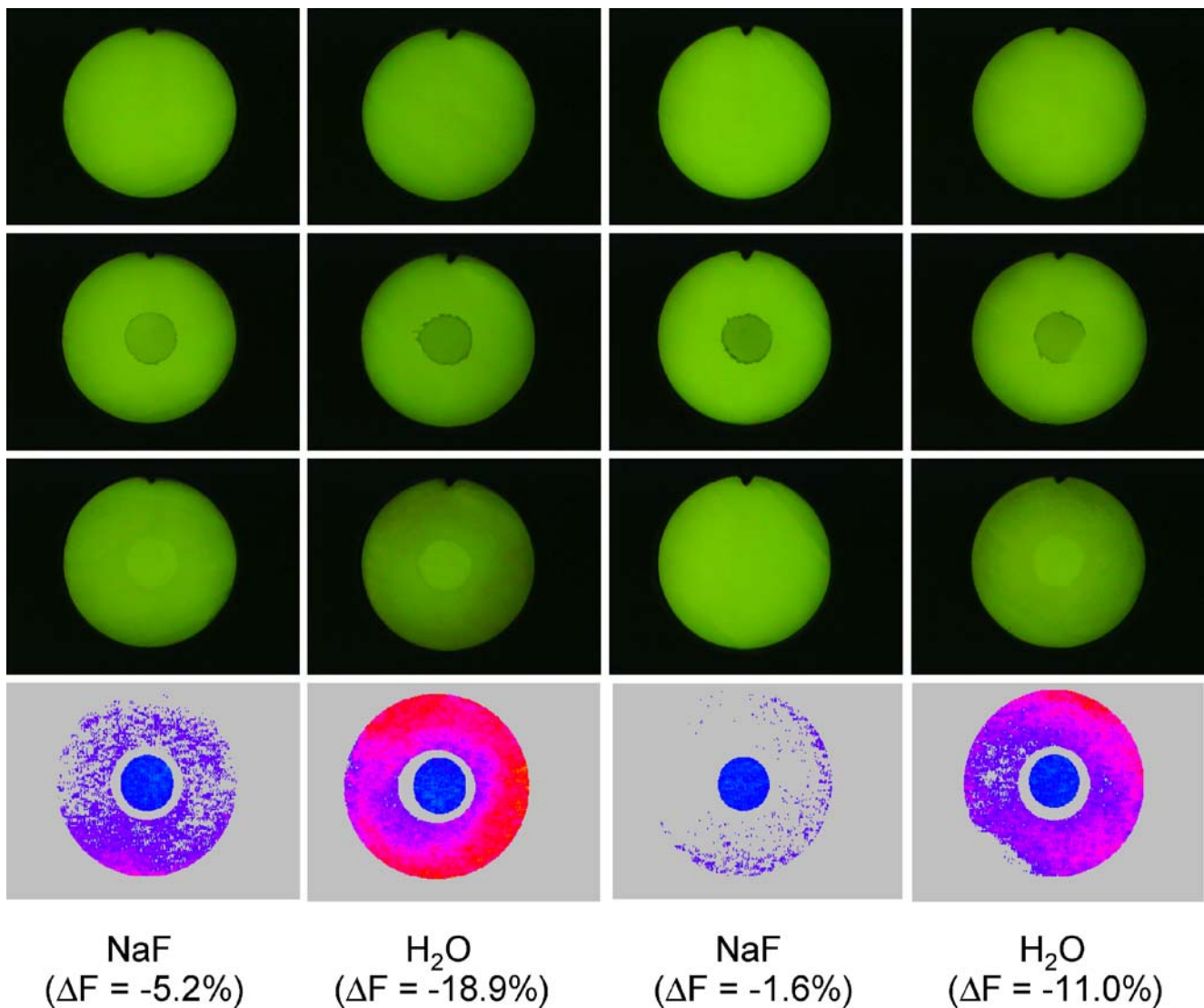
Fig. 2 Demineralization of bovine enamel discs in situ. The images in the *top two rows* show four different discs measured by QLF at baseline before (*first row*) and after (*second row*) the application of the central circular acid-resistant nail varnish cover. The *third row* shows the same discs after the in situ experiment. Pseudo color representations of the subtraction images are displayed in the *fourth row*. The *blue center* represents the reference; the *colored outer ring* represents the test area. The subtraction images were generated with a ΔF threshold of -5%, coloring only points with a loss in fluorescence intensity exceeding 5%. *Purple areas* display the least, while *bright red* areas the most demineralization

remnants by maximum vortexing, and stored in distilled water at 4°C until investigated by QLF.

TMR analysis and comparison with QLF

After the completion of the QLF image recordings, ten enamel discs with different levels of demineralization (from the above-described six-species in vitro biofilm experiments) were subjected to TMR [10, 19]. Briefly, specimens were cut by taking three parallel sections of approximately 500 μm thickness at 1.5 and 3.2 mm from the left and at 1.5 mm from the right side (Fig. 3). The sections were lapped parallel to a thickness of 110 μm with aluminum oxide abrasive powder, before five equidistant TMR scans were made for each section (Fig. 3). Integrated mineral loss (ΔZ, vol.% micrometer) and lesion depth (micrometer) were measured and averaged for every slice.

To match approximately the regions assessed by QLF and TMR, virtual rectangular patches were constructed with the QLF Subtract software and laid over images of the areas covered by the three TMR sections (Fig. 3). Three QLF patches were used because, for technical reasons, the locations of the 15 TMR sections per disc could not be matched exactly with 15 corresponding QLF patches. The
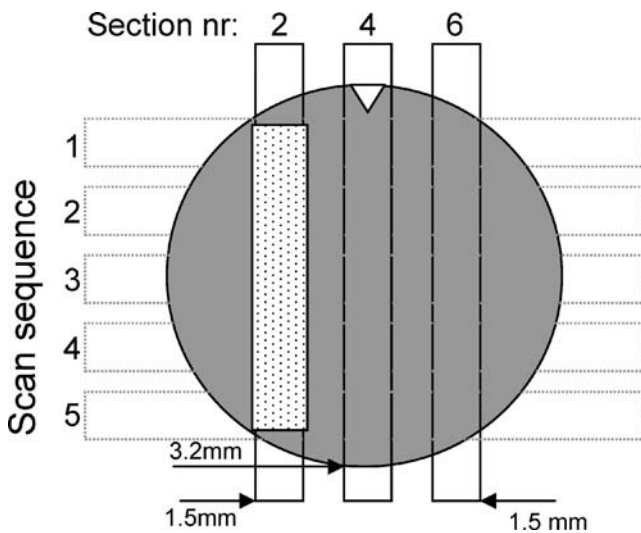
Fig. 3 Comparison of TMR and QLF. Of each disc section (labeled 2, 4, 6) prepared for TMR, five equidistant scans (labeled 1 through 5) were made. The *dashed overlay* over section 2 indicates the virtual patch used to limit the area considered for QLF analysis. Analogous QLF patches were laid and measured over sections 4 and 6

three patches include with certainty the entire area investigated by TMR, but they additionally cover immediately adjacent areas not assessed by TMR. Eight sections from eight discs were omitted from the TMR–QLF comparison due to technical problems with at least one of the five TMR scanned regions per section. This left 22 sections from ten discs for analysis.

Repeatability and dehydration experiments

To determine the repeatability of QLF measurements, ten bovine enamel discs were measured four times exactly 20 min after removal from water. Between measurements, discs were put back into water, and the QLF instrument was shut down for ≥ 2 h, thus mirroring the experimental QLF procedures used for longitudinal demineralization or remineralization studies.

To estimate a possible effect of the disc hydration status on QLF fluorescence radiance (F), ten bovine enamel discs were measured six times each at various time intervals after removal from storage fluid and the results compared to a previously registered baseline image, which was made 20 min after the removal from storage fluid. In contrast to the repeatability experiment, these data were recorded in a single session without the QLF instrument being shut down between measurements.

In vitro biofilm remineralization experiments

Remineralization was studied with bovine enamel discs that were predemineralized in vitro by exposure to acid gels before being used as biofilm carriers for 64.5 h and before

being exposed to either $CaCl_2$ or to water. In brief, 36 bovine enamel discs were measured by QLF and then covered at room temperature and 100% humidity with 150 mg of demineralizing gel [0.1 M acetate, 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 0.15 M KCl, 6% (w/v) hydroxyethyl cellulose; pH 4.8] [14]. The gel was replaced daily except on weekends for about 2 weeks. Thereafter, discs were washed thoroughly in distilled water and stored. Shortly before the start of the biofilm experiment, discs were subjected to a second QLF measurement followed by gas sterilization. Biofilms were grown using the standard 70:30 model [8] except that saliva was supplemented with 5 mM $CaCl_2$ (test group) or water (control group). Discs were harvested after 64.5 h, freed of any biofilm remnants by maximum vortexing, and stored in distilled water at 4°C until investigated by QLF. The experiment was repeated four times, twice with three discs and twice with six discs per group. In addition, in a similar fifth experiment, of which only three representative discs are shown, 10 and 15 mM $CaCl_2$ supplements were employed in addition to 5 mM $CaCl_2$.

Statistical analyses

Statistical analyses were done with StatView 5.01 (SAS institute, Cary, NC, USA). Differences in demineralization (ΔF) achieved in situ after intermittent exposure of discs to 26.3 mM NaF or water were tested by the paired t test. Similarly, differences in remineralization (ΔF) achieved in vitro underneath biofilms grown in the presence or absence of $CaCl_2$ were tested by the paired t test. Both QLF repeatability and the relationship between F and specimen hydration were evaluated by repeated measure ANOVA followed by Student–Newman–Keuls post hoc tests to isolate pairwise differences. Before using these parametric tests, all respective data sets were tested for approximate normality of data distribution using the Kolmogorov–Smirnov test (K–S test). TMR and QLF were compared correlating ΔZ with ΔF, and lesion depth with ΔQ.

Results

To demonstrate the ability of the QLF instrument to quantify sensitively plaque-mediated demineralization, we performed an in situ study with three volunteers who wore two acrylic appliances day and night, each holding two bovine enamel discs as test specimens. The test persons accomplished simultaneously two treatments by exposing one of the appliances twice daily briefly to NaF, the other to water (control). Figure 2 shows sequential images of four representative enamel discs, two from each treatment.

Posttreatment, areas of demineralization are readily identifiable by the darkened zones visible in the images of the third row. For comparison the top row displays images recorded after disc preparation. The dark central circular areas in the images of the second row are due to a nail varnish cover applied briefly before the start of the in situ experiment. The purpose of these covers is to generate a control region where the underlying enamel is protected from any plaque or further host-mediated effects. (Note that the images of the second row serve only to define the exact position of this control region. For ΔF calculations, the pair of images made before the application and after the removal of the nail varnish cover are compared.) In the fourth row, pseudo coloring indicates regions of different demineralization with pink-red representing maximum levels observed. Demineralization was consistently higher in the water treatment with mean ΔF levels of -10.5 (± 6.6 SD; $n=6$) for the water group and -3.1 (± 2.5 SD; $n=6$) for the NaF group. The difference was statistically significant (paired t test, $p=0.0155$; K–S test, $p>0.999$).

To compare the performance of this instrument with an established procedure for measuring enamel demineralization or remineralization, bovine enamel discs derived from multispecies biofilm demineralization experiments were first assessed by QLF and then by TMR as outlined in Fig. 3. The observed mean ($\pm$SD) ΔF values (QLF) for the 22 vertical patches ranged from -8.4 (± 1.7) to -47.8 (± 3.4), and the ΔZ values for the corresponding TMR sections ranged from 62.6 (± 49.4) to 1894.6 (± 188.1). Values of ΔZ and ΔF, and of lesion depth (TMR) and ΔQ were scatterplotted against each other (Fig. 4). The data revealed a positive correlation between corresponding TMR and QLF readings with correlation coefficients of 0.820 and 0.854, respectively.

Repeatability of QLF measurements is depicted in Fig. 5a. As expected, ten unused bovine enamel discs displayed significantly different F values ranging between
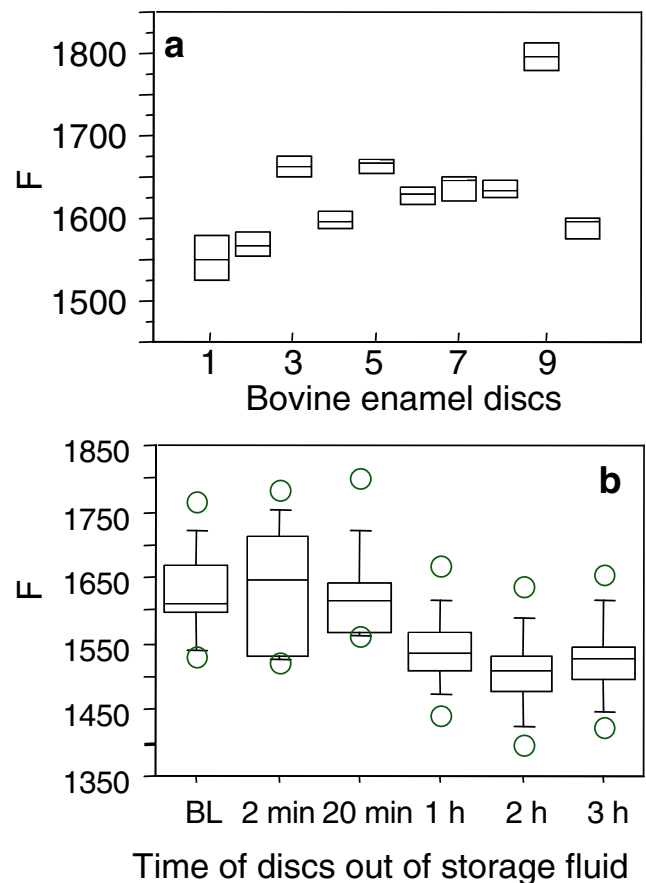
Fig. 5 **a** Box plot depicting fluorescence radiance (F) of ten bovine enamel discs measured 20 min after removal from storage buffer, four times each. F differences among discs were significant ($p=0.0001$, repeated measure ANOVA; $p>0.999$ in K–S test). Variability among repetitive measurements was insignificant, except for one outlier presumably due to a technical problem. **b**. Comparison of six consecutive QLF measurements of the same ten discs at different time points after removal from storage buffer indicating an effect of increasing dehydration on F. Measurements made after 1 h or later were significantly different from measurements made after 2 and 20 min ($p=0.0001$, repeated measure ANOVA; $p=0.676$ in K–S test)

Fig. 4 Scatterplot of **a** shows ΔZ (TMR) vs ΔF (QLF), while scatterplot of **b** shows lesion depth (TMR) vs ΔQ (QLF)
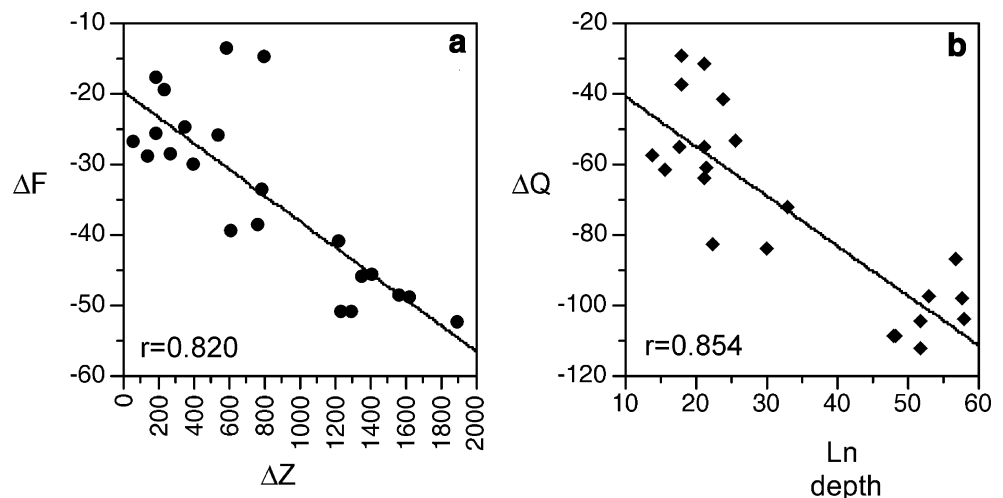
1520 and 1820 (repeated measure ANOVA, $p=0.0001$; K–S test, $p>0.999$). However, repeated measurements of these discs gave satisfactorily close results for each disc with values deviating, on average, from the originally measured value by 0.79% ($\pm1.68\%$ SD; range, -2.4 to 4.2%). Prolonged removal of discs from storage buffer will lead to a gradual decline in the hydration level of the discs that might affect fluorescence. Therefore, we measured the same ten discs at different time intervals after removing them from the storage fluid. Results revealed no significant differences among the measurements performed during the first 20 min, but after 60 min, F was significantly reduced in comparison to baseline values (Fig. 5b). F variability between discs was greatest after 2 min. For these reasons, all our QLF measurements are made 20 min after removal of the discs from the storage fluid.

The assessment by QLF of remineralization in vitro is summarized in Fig. 6. A succession of four experiments is described, in which 36 bovine enamel discs were first demineralized for 2 weeks, reaching ΔF values between -13.0 and -39.1, and then remineralized underneath an in vitro biofilm that was grown in 30% medium and 70% saliva with or without 5 mM $CaCl_2$ supplement. It is evident that all discs showed higher ΔF values after the biofilm experiment than before. In the presence of $CaCl_2$, the remission of fluorescence was significantly more pronounced (paired t test, $p=0.0002$; K–S test, $p>0.999$) than in the water control group. The remineralization effect is clearly visible (Fig. 7). The figure shows three represen-

tative discs before and after a 64.5-h biofilm experiment in the presence of different concentrations of $CaCl_2$. The association of pseudo color to ΔF scores facilitates the recognition of regional remineralization patterns.

Discussion

The instrument presented here offers a sensitive, standardized procedure to quantify in vitro demineralization and remineralization of experimental enamel specimens. As a particular advantage, it does not require destruction of the specimens and, thus, enables a longitudinal experimental design. It requires significantly less labor than TMR and shortens the analysis of large experiments with dozens of specimens to a few hours. A further distinct feature of the technique is excellent site specificity. Unlike alternative methods, in vitro QLF allows the precise local detection of demineralization or remineralization. Combined with techniques such as immunofluorescence or fluorescent in situ hybridization, it will now be possible to correlate the plaque microbiota with demineralization or remineralization patterns of an experimental enamel specimen.

The QLF technique exploits natural fluorescence emitted by fluorophores from dentin and enamel. In demineralized enamel, the light absorption and scattering properties are altered, resulting ultimately in less fluorescence being remitted and caught by the camera lens from a demineralized surface than from a sound enamel surface [18]. How the
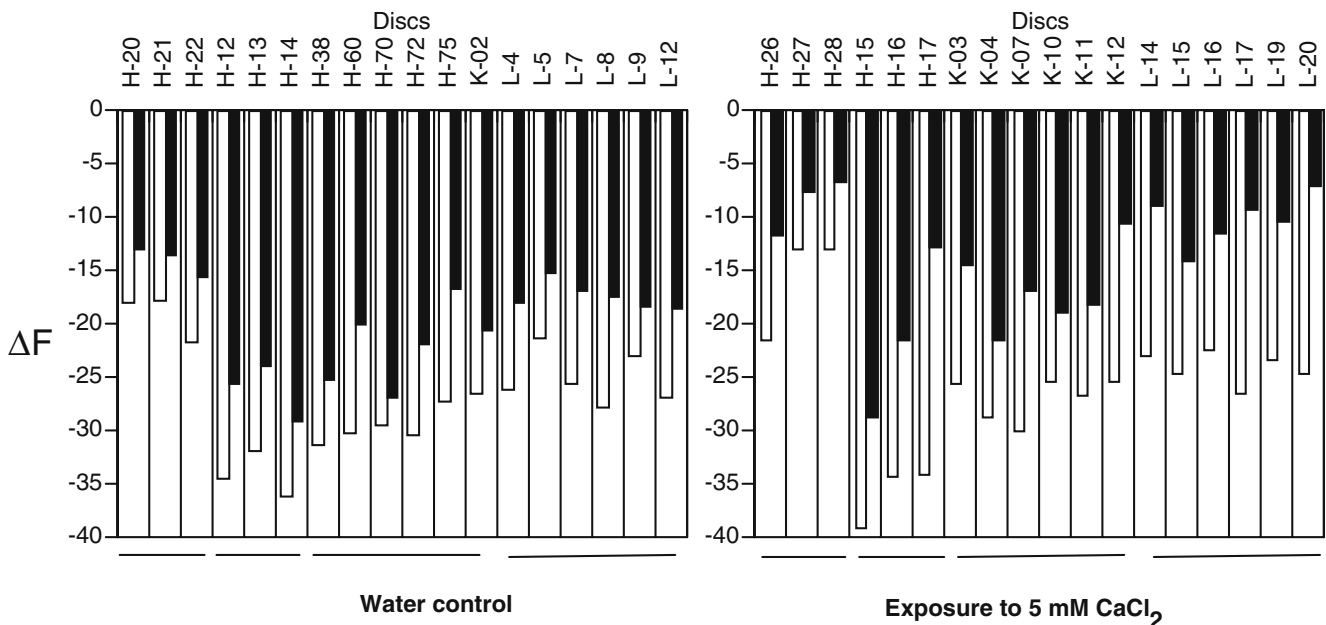
Fig. 6 Remineralization of 36 bovine enamel discs covered by in vitro biofilms. *Open bars* reflect the degree of demineralization (ΔF) achieved by a 2-week exposure to a demineralizing gel. *Black bars* indicate ΔF scores recorded at the end of a 64.5-h biofilm experiment, during which discs and biofilms were exposed to a 70%:30% mixture of saliva and culture medium supplemented with 5 mM $CaCl_2$ or water. Data are from four experiments as indicated along the x-axis. Differences among the two test groups were significant ($p=0.0002$, paired t test; $p>0.999$ in K–S test)

Fig. 7 In vitro biofilm-mediated remineralization of three representative bovine enamel discs in the presence of 15 (*top*), 10 (*middle*), or 5 mM CaCl₂ (*bottom row*). Images in the *third column* visualize the degree of site-specific remineralization using the pseudo *color scale* shown to the *right*. Δ*F* scores indicate the change in *F* with respect to the demineralized prebiofilm level
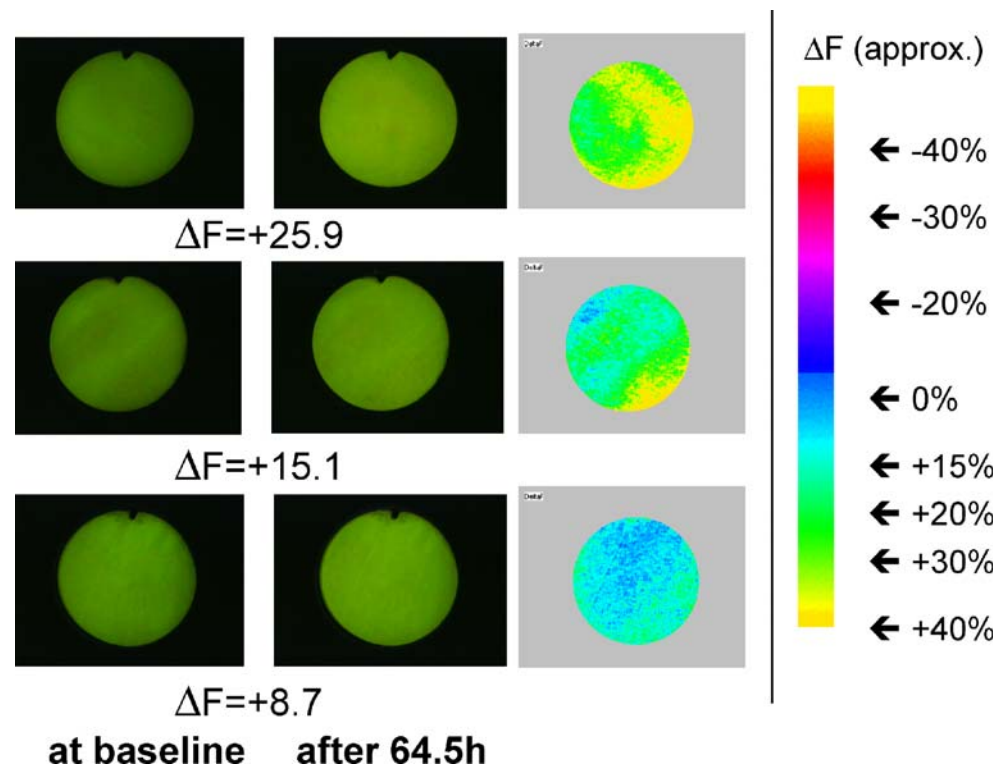
fluorescence of a lesion area is affected by external influencing factors such as bacterial metabolites is currently unknown. Nevertheless, fluorescence loss in both natural caries lesions and in vitro demineralizations was shown to correlate well with mineral loss quantified by TMR [3, 4, 16], the latter method being generally considered to represent the gold standard for mineralization analyses. In the present study, the comparison of ΔZ (TMR) with ΔF (QLF) and lesion depth (TMR) with ΔQ yielded correlation coefficients of 0.82 and 0.854, respectively, indicating a strong association between the QLF and TMR measurements. The value of 0.82 is lower than the coefficient of QLF to TMR correlation ($r=0.96$) reported by Ando et al. [3] for the assessment of chemically demineralized bovine enamel; however, these authors investigated very homogeneous lesions compared to the biofilm-formed lesions of the present study. It is similar to the coefficients described by Al-Khateeb et al. [1] and Hall et al. [9] for analyses of artificial carious lesions of either human ($r=0.84$) or bovine enamel ($r=0.74$ and 0.83). It also compares well with data from Ando et al. [4], who described coefficients of QLF to TMR correlation of $r=0.62$ and $r=0.88$ for early carious lesions in permanent and deciduous teeth, respectively. A still better correspondence between TMR and QLF data was likely prevented by the fact that it was technically impossible to match exactly the areas assessed by the two techniques. Our results indicate that in vitro QLF is suitable for the in vitro measurement of mineral density changes in experimental enamel specimens. Since dental caries—under current con-

ditions—is generally a slow disease, assessment of caries-preventive agents is often done in in situ or intraoral studies, whereby enamel or dentin specimens are subsequently analyzed with advanced laboratory techniques such as QLF and TMR. This way, effectiveness of agents can be evaluated long before any changes can be seen clinically. In vitro QLF is sensitive and particularly attractive for the detection of initial caries lesions. Its ability to score accurately very progressed lesions was not tested in this study because strongly demineralized bovine enamel discs tend to develop open cavities that, we thought, would most likely lead to doubtful results. It is interesting to note that Pretty et al. [13] reported recently a fine correlation between TMR and QLF ($r=0.91$) analyses of a broad bandwidth of acid erosion on human enamel. In their report, the authors point out that progressed erosions are characterized by a softened surface and crater formation, and they propose a new model including a "wall effect" to explain the accurate measurement of crater-like erosions by QLF.

With the instrument and software described, demineralization or remineralization is determined by comparing the change in fluorescence intensity of pre- and postintervention images pixel-by-pixel. Since such image pairs may be recorded months apart during in situ studies (with possible lamp changes and many instrument shut-offs in between), calibration of the instrument is critical and information on the repeatability of fluorescence intensity measurements is important. The repeatability tests performed in this study showed that calibration was reproducible and of sufficient precision

(Fig. 5a). Another variable that may influence F of a given specimen and requires standardization is enamel dehydration [2, 11]. Our data confirmed this relationship (Fig. 5b) and underlined the necessity to standardize the inevitable lag time between the removal of the test discs from the storage solution and the QLF measurement. We opted for a 20-min interval since data variation was smaller when discs were exposed for 20 min rather than 2 min to room atmosphere, and since this facilitated the coordinate handling and measurement of multiple discs.

In summary, the experiments of this study demonstrated that QLF performed in vitro with the described commercial instrument is applicable to the analysis of biofilm-mediated demineralization/remineralization of enamel specimens. The primary benefits of the assay system are sensitivity, site specificity, rapidity, noninvasiveness, and reproducibility. The system requires careful calibration and supervision, achieved best by an internal reference area that remains unaffected by any test treatment.

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