Orange/Red Fluorescence of Active Caries by Retrospective Quantitative Light-Induced Fluorescence Image Analysis

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Key Words

Biofilm · Caries · Clinical study · Digital image analysis · Red fluorescence

Abstract

This retrospective clinical study determined the association of caries activity and orange/red fluorescence on quantitative light-induced fluorescence (QLF) images of surfaces that progressed to cavitation, as determined by clinical visual examination. A random sample of QLF images from 565 children (5–13 years) previously enrolled in a longitudinal study was selected. Buccal, lingual and occlusal surface images obtained after professional brushing at baseline and every 4 months over a 4-year period were analyzed for red fluorescence. Surfaces that progressed ($n = 224$) to cavitation according to the International Caries Detection and Assessment System (ICDAS 0/1/2/3/4 to 5/6 or filling), and surfaces that did not progress ($n = 486$) were included. QA2 image analysis software outputs the percentage increase of the red/green components as ΔR and area of ΔR (area ΔR) at different thresholds. Mixed-model ANOVA was used to compare progressive and nonprogressive surfaces to account for correlations of red fluorescence (ΔR and area ΔR) between surfaces within a subject. The first analysis used the first observation for each surface or the first available visit if the sur-

face was unerupted (baseline), while the second analysis used the last observation prior to cavitation for surfaces that progressed and the last observation for surfaces that did not progress (final). There was a significant ($p < 0.05$) association between red fluorescence and progression to cavitation at thresholds $\Delta R0$, $\Delta R10$, $\Delta R20$, $\Delta R60$, $\Delta R70$, $\Delta R80$, $\Delta R90$ and $\Delta Rmax$ at baseline and for $\Delta R0$ and $\Delta R10$ at the final observation. Quantification of orange/red fluorescence may help to identify lesions that progress to cavitation. Future studies identifying microbiological factors causing orange/red fluorescence and its caries activity are indicated.

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The optical phenomenon of tooth autofluorescing on illumination with ultraviolet light was first observed by Hans Stubel [Stubel, 1911; Buchalla, 2005]. This property has been applied in caries detection devices as a means of detecting incipient carious lesions [Benedict, 1928; Alfano and Yao, 1981; Bjelkhagen et al., 1982; Sundstrom et al., 1985; de Josselin de Jong et al., 1995; Zandona and Zero, 2006]. The concept held previously was that carious enamel lesions do not fluoresce [Benedict, 1928], and deep dentinal caries appeared dark brown, orange brown and pinkish orange when caries was debrided. It was shown later that carious lesions fluoresce in the red region

Table 1. Description of QLF parameter terminologies

Terminology	Definition
1 ΔR $\Delta R_{\text{pixel}} \cong \frac{(R/G)_{\text{lesion}} - (R/G)_{\text{sound}}}{(R/G)_{\text{sound}}} \times 100\%$	The change in the ratio of red and green pixels in the lesion area or area of interest compared to the sound area of the tooth; this is expressed in percentage; ΔR is based on various thresholds at $\Delta R0$, $\Delta R10$, $\Delta R20$, $\Delta R30$, $\Delta R40$, $\Delta R50$, $\Delta R60$, $\Delta R70$, $\Delta R80$, $\Delta R90$, $\Delta R100$, $\Delta R110$, $\Delta R120$ and total
2 Area of red fluorescence ($A\Delta R$)	The area of ΔR equivalent or higher to a specific threshold at $\Delta R30$, $\Delta R70$ and $\Delta R120$
3 Plaque wizard patch	A rectangular contour which can be adjusted to any size or shape which is placed around the lesion area, but should be in the sound area of the tooth
4 ΔF	The average loss of fluorescence in the carious surface (the depth of the lesion) compared to the fluorescence in the sound tooth area; ΔF is expressed as a percentage
5 Area (A)	The area of the carious lesion surface; it is expressed in mm ²
6 ΔQ	The percentage of green fluorescence loss (ΔF) times the area of the lesion (A); it is expressed as %px ² , resembling lesion volume

of the spectrum [König et al., 1999; Buchalla, 2005; Slimani et al., 2014]. Several studies have demonstrated heterogeneous emission spectral bands in the carious region compared to the sound surface of the tooth [Buchalla et al., 2004a, b; Buchalla, 2005; Zezell et al., 2007]. The long-standing question is whether the chromogenic autofluorescence originates from the tooth structure or from bacteria, or whether both contribute to this autofluorescence. It has been shown that macromolecular porphyrin might be responsible for the autofluorescence [Buchalla et al., 2008].

Quantitative light-induced fluorescence (QLF-CLIN; Inspektor Research Systems, Amsterdam, The Netherlands) employs the optical property of fluorescence for caries detection [Waller et al., 2012]. Inspektor™ QLF Pro employs an intraoral camera that illuminates the tooth with light in the violet-blue wavelength (290–450 nm) and captures fluorescence above 520 nm by a high-pass filter. Under these conditions, the sound areas of the tooth fluoresce green; however, an orange/red fluorescence can also be observed on these images. A new device, the QLF-D Biluminator™ 2, has been introduced using a single lens reflex camera with blue and white LED lights with a peak wavelength of 405 ± 20 nm (violet). White LEDs are used for standard white light images, while the blue LEDs provide excitation light for fluorescent imaging. The filter in this device allows the images of sound tooth surfaces to have a whitish appearance instead of green, while demineralized areas look darker, and bacterially infected areas show a bright red fluorescence. The

orange/red fluorescence has been assumed to originate from metabolic byproducts of oral bacteria within the dental biofilm called porphyrins [König et al., 1998; van der Veen et al., 2006; Volgenant et al., 2013]. In vitro oral biofilm studies have shown a correlation between the red fluorescence captured through the QLF-D Biluminator with caries risk severity [Lee et al., 2013; Kim et al., 2014]. A proprietary software (Inspektor Pro System) allows analysis of the images reporting three parameters: the average loss of fluorescence denoting lesion depth (ΔF , %), lesion area (A, mm²) and lesion volume (ΔQ , %px²). In the QLF-D Biluminator the ratio of red to green fluorescence in percentage (ΔR , %) and the area of red fluorescence ($A\Delta R$) are reported. Red or orange fluorescence (ΔR) is represented as the percentage ratio increase of the red to green components in comparison to sound surface. The red fluorescence area ($A\Delta R$, px²) is equal or higher than a specific threshold of ΔR (table 1). Red or orange fluorescence is believed to emanate from the excitation of fluorophores from bacterial byproducts on illumination through blue-violet light [van der Veen et al., 2003]. A preliminary in vitro study [Alammari et al., 2010] showed the relationship between the ΔR and ΔF to ICDAS and histology scores, requiring clinical validation.

The relationship between the QLF parameters (ΔF , $A\Delta R$ and ΔQ), the visual appearance of the lesions and its clinical behavior longitudinally has been demonstrated previously by our group [Ferreira Zandona et al., 2003, 2010]. During the capture and analysis of these clinical images the red fluorescence observed by others [Coulthwaite et

al., 2006; Bittar et al., 2014] was also seen on the images captured in these longitudinal studies. Thus, the objective of this study was to determine whether there is an association between the intrinsic orange/red fluorescence seen in QLF images of surfaces that progressed to cavitation over a period of time compared to surfaces with non-cavitated lesions that did not progress to cavitation, as determined by clinical visual examination using the criteria of the International Caries Detection and Assessment System (ICDAS).

Study Participants and Methods

This is a retrospective study based on the images captured during a longitudinal study previously published [Ferreira Zandoni et al., 2013]. Detailed information of participant selection, sample size calculation, examinations conducted and the analysis of fluorescence in QLF images have previously been published [Ferreira Zandoni et al., 2010; Fontana et al., 2011; Ferreira Zandoni et al., 2012, 2013]. In summary, in January 2007, children ($n = 565$) aged 5–13 years from kindergarten to 9th grade enrolled in public schools from the area of Aguas Buenas, Puerto Rico, were recruited into the study. Consent and assent if the child was over 7 years old was obtained. The study was approved by the Institutional Review Board (IRB) of Indiana University (IU-IRB 0608-15) and the University of Puerto Rico (UPR-IRB A1340107). The inclusion criteria included having at least 1 permanent molar with at least 1 unrestored surface, no medical problems and tolerance for the examinations performed.

Comprehensive Oral Examinations

The children underwent examinations at baseline and at 8, 12, 18, 24, 28, 32, 36, 40, 44 and 48 months. It is important to note that the examinations were conducted after a professional toothbrushing; thus most, if not all, the biofilm was removed from the surfaces of interest. A visual examination based on ICDAS, which ranges from 0 to 6 [Pitts, 2004], was performed at each time point as well as an examination of the teeth with QLF. Fluorescent images of occlusal and buccal surfaces of all permanent molars and lingual surfaces of upper molars were obtained at each examination. At the end of the 4-year study fluorescent images of surfaces that progressed to cavitation (ICDAS 0/1/2/3/4 to 5/6 or filling) and a random sample of images of surfaces that did not progress to cavitation were analyzed longitudinally using proprietary software (QLF 2.00 g).

Orange/Red Fluorescence Analysis

The same images previously analyzed were analyzed for orange/red fluorescence by red fluorescence analysis (QA2 software; Inspektor Research Systems). Surfaces ($n = 224$) that progressed to cavitation according to ICDAS (ICDAS 0/1/2/3/4 to 5/6 or filling) and surfaces that did not progress ($n = 486$) were included. [Ferreira Zandoni et al., 2013]. The plaque wizard patch of QA2 of the QLF-D Biluminator was used to analyze the images obtained from the Inspektor QLF Pro. For red fluorescence analysis the plaque wizard was applied on the same area of the carious lesion surface which had been analyzed previously to monitor the progression of

caries. It was applied on the last visit for sites that did not progress, or if there was a filling or a cavitation with ICDAS 5 or 6, it was applied on the visit prior to the filling/cavitation. A similar plaque wizard was applied at all other visits including the first observation, which was at baseline, or the first visit after eruption if the tooth was unerupted at baseline. QA2 outputs the percentage increase of red to green components as ΔR and $\text{area}\Delta R$ at different thresholds. For intraexaminer reliability 60 surfaces were chosen randomly, including surfaces that progressed to cavitation and those that did not progress, for repeat analysis by a single, experienced examiner.

Statistical Analysis

Statistical analysis was performed with SAS 9.3 (SAS Institute Inc., Cary, N.C., USA). Mixed-model ANOVA was used to compare surfaces that progressed to cavitation and those that did not progress to account for correlations of red fluorescence (ΔR and $\text{area}\Delta R$) between surfaces within a subject. The ranks of the data were used in the analysis because of nonnormal distributions. Two sets of analyses were performed. The first analysis used the first observation for each surface, which was at baseline or the first available visit if the surface was unerupted at baseline. The second analysis used the last observation before progression for surfaces that did progress to cavitation and the last observation for sites that did not progress. The area under the receiver operating curve (ROC) was calculated to determine the sensitivity and specificity of the threshold for the red fluorescence areas. Intraclass correlation coefficients were calculated to determine the level of intraexaminer repeatability.

Results

A statistically significant association ($p < 0.05$) was seen between red fluorescence and surfaces that progressed to cavitation. Analysis of the initial or first observation of the tooth surfaces showed that sites that progressed to cavitation had a significantly higher percentage of red fluorescence for specific thresholds of $\Delta R0$, $\Delta R10$, $\Delta R20$, $\Delta R60$, $\Delta R70$, $\Delta R100$, ΔR_{max} and total than sites that did not progress. The red fluorescence area (ΔR : the area at $\Delta 70$, px^2) correlated with the percentage of red fluorescence observed in the first observation ($p = 0.0191$). The simple plaque score (SPSTM) was borderline significant ($p = 0.0522$) at the initial observation (table 2).

At the first observation the mean value (and standard error, SE) of red fluorescence values was 4,955 (SE = 285) for surfaces that progressed to cavitation and 3,038 (SE = 166) for surfaces that did not progress. As shown in table 2 the sites that progressed to cavitation had significantly higher $\Delta R0$, $\Delta R10$, $\Delta R20$, $\Delta R60$, $\Delta R70$, $\Delta R100$, ΔR_{max} and total at the first observation than sites that did not progress. Even at the thresholds where there were no significant differences between the sites, there was a tendency for the sites that did not progress to have lower

Table 2. Red fluorescence analysis of baseline and final observations of progressive and nonprogressive surfaces

Red fluorescence parameter	First or baseline observation			Final observation or last before progression		
	progressed to cavitation (n = 224)	did not progress (n = 486)	p value	progressed to cavitation (n = 224)	did not progress (n = 486)	p value
SPS	0.045±0.021	0.019±0.011	0.0522	0.067±0.031	0.029±0.009	0.8458
Area ΔR30	0.069±0.035	0.039±0.019	0.3948	0.142±0.086	0.044±0.012	0.4164
Area ΔR70	0.016±0.010	0.009±0.006	0.0191*	0.022±0.017	0.006±0.002	0.4774
Area ΔR120	0.000±0.000	0.000±0.000		0.003±0.003	0.000±0.000	0.1905
ΔR0	1,341±87	752±48	<0.0001*	1,449±96	867±57	<0.0001*
ΔR10	43.00±5.06	26.87±3.14	0.0009*	51.17±6.27	32.00±3.49	<0.0001*
ΔR20	5.96±1.45	2.56±0.48	0.0029*	9.55±2.70	3.72±0.70	0.3863
ΔR30	2.37±0.83	0.84±0.28	0.3770	4.43±1.96	1.46±0.44	0.3603
ΔR40	1.38±0.58	0.49±0.25	0.2837	2.76±1.63	1.01±0.37	0.2869
ΔR50	0.95±0.44	0.34±0.20	0.0538	2.04±1.35	0.62±0.27	0.4398
ΔR60	0.64±0.29	0.23±0.13	0.0167*	1.27±0.83	0.37±0.18	0.2233
ΔR70	0.33±0.16	0.12±0.07	0.0191*	0.71±0.43	0.21±0.12	0.4749
ΔR80	0.17±0.09	0.04±0.03	0.0511	0.42±0.25	0.13±0.10	0.7095
ΔR90	0.07±0.05	0.02±0.01	0.1440	0.33±0.23	0.09±0.08	0.3285
ΔR100	0.02±0.01	0.00±0.00	0.0373*	0.28±0.20	0.07±0.07	0.4253
ΔR110	0.01±0.01	0.00±0.00	0.1416	0.22±0.17	0.06±0.06	0.1905
ΔR120	0.00±0.00	0.00±0.00		0.18±0.15	0.04±0.04	0.1905
ΔRmax	44.08±1.43	35.85±0.75	<0.0001*	47.71±2.14	40.51±1.05	0.0002*
Total	4,955±285	3,038±166	<0.0001*	5,113±287	3,204±169	<0.0001*

Values are means ± SE. * p < 0.05, statistically significant.

Fig. 1. Comparison of mean red fluorescence values at baseline or first visit and final visit of observation for surfaces that progressed to cavitation and those that did not progress at ΔRmax and ΔR10. * p < 0.05.

values than those that did progress to cavitation. The area at threshold ΔR70 (areaΔR70) was significant for lesions that progressed to cavitation compared to the area at ΔR30 and that at ΔR120. Analysis of the final observation showed that sites that progressed to cavitation had significantly higher ΔR0, ΔR10, ΔRmax and total than sites that did not progress (p < 0.0001). SPS scores were not significant at the last visit. The mean red fluorescence values at various thresholds during the final observation are included in table 2. The areas of thresholds at ΔR30, ΔR70 and ΔR120 were not significantly different.

Comparisons of red fluorescence values at ΔRmax and ΔR10 during the first and final observation are illustrated in figure 1. For both the first and final observations there was a significant difference for ΔRmax and ΔR10 for surfaces that progressed to cavitation compared to nonprogressive surfaces. Carious surfaces that progressed to cavitation showed greater red fluorescence values during the final observation at ΔR10. Surfaces that did not progress showed higher red fluorescence values at the first observation at ΔRmax. Therefore, the threshold kept at maximum level of cutoff was greater compared to the red fluo-

Fig. 2. Dot plot comparison of surfaces that progressed to cavitation and those that did not progress, with total values of red fluorescence during the first observation (a) and the final observation (b).

rescence value at $\Delta R10$. Red fluorescence is detected during the first observation with the maximum level of cutoff. Also, during the final observation of nonprogressive surfaces $\Delta Rmax$ showed higher red fluorescence values.

Dot plots are shown comparing surfaces that progressed to cavitation and nonprogressive surfaces based on total values of red fluorescence during the baseline or first observation (fig. 2a) and during the final observation (fig. 2b). The surfaces that progressed to cavitation and nonprogressive surfaces were significantly different in both cases ($p < 0.0001$).

The area under the ROC was calculated based on the continuous variables in the red fluorescence analysis data. Sensitivity and specificity were calculated based on the ROC. The ROC was plotted and was evaluated to determine the appropriate tradeoff between sensitivity and specificity. Figure 3 illustrates the ROC for the final observation (not progressed) or the last observation before progression to cavitation for the 'total' to predict progression. We can see from the curve that the best combination was a sensitivity of 65% with a specificity of 63%.

The intraclass correlation coefficients indicated good repeatability for all red fluorescence measurements: 0.99 for $\Delta Rmax$, 0.97 for red fluorescence total, 0.97 for $\Delta R0$, 0.87 for $\Delta R10$, 0.88 for $\Delta R20$, 0.89 for $\Delta R30$, 0.94 for $\Delta R40$ and $\Delta R50$, 0.92 for $\Delta R60$, 0.93 for $\Delta R70$, 0.96 for $\Delta R80$ and 0.78 for $\Delta R90$.

Discussion

Predicting caries activity is considered the 'holy grail' of cariology [Ismail et al., 2005]. To date, only subjective methods are available to determine whether a lesion will progress to cavitation or will arrest. It is known that not all early lesions progress to cavitation [Dirks, 1966]. Our study in this population also demonstrated that lesions with rapid changes in QLF parameters like the area, depth and volume of the lesion progressed to cavitation [Ferreira Zandona et al., 2013]. Identifying lesions that are likely to progress to cavitation can have a great impact on how dentistry is practiced and how the caries paradigm

Fig. 3. ROC predicting progression to cavitation from the final observation for red fluorescence total.

shift towards the nonsurgical management of dental caries can be implemented.

Most studies have focused on the red fluorescence of oral biofilm. The orange-to-red intrinsic fluorescence has been shown to be emanating from the oral biofilm (dental plaque) comprising all the oral bacterial species. It does not come from a single bacterial species [van der Veen et al., 2006]. A red excited fluorescence signal is likely to be derived from metabolic byproducts of oral bacteria in the dental biofilm [Koenig and Schneckenburger, 1994]. Red fluorescence emission seen on QLF images is proposed to be the result of excitation of endogenous porphyrins by the violet-blue light at a wavelength range of 380–500 nm. Fluorescing porphyrins in caries detected to some extent are protoporphyrin IX, coproporphyrin and uroporphyrin [Buchalla et al., 2008]. Red fluorescence from bacteria was shown to be an indicator of dentinal carious lesions [Lennon et al., 2006]. Higher levels of red-to-orange fluorescence can be an indicator of progression of lesions, as demonstrated in our study. Early detection of orange/red fluorescence may serve as a caries indicator to predict caries activity.

Our study focused on the tooth surfaces that were cleaned of visible biofilm before imaging. It showed that at specific thresholds or cutoff levels the orange/red fluorescence from lesions that progressed to cavitation was significantly higher compared to nonprogressive carious lesions. At low thresholds of $\Delta R0$, $\Delta R10$ and $\Delta R20$ at

baseline or the first available visit the orange/red fluorescence was significantly higher for the lesions that progressed to cavitation compared to lesions that did not progress. This indicates that the values of orange/red fluorescence have a potential to identify lesions that are likely to be active, that is, progress towards cavitation. This may be the first nonsubjective means to determine caries activity at a single time point. This can have a significant impact on dental care, ranging from caries risk to specific caries intervention.

Yet, there are several questions that remain to be answered. In our study, at every visit prior to capturing QLF images the teeth were brushed and flossed by study personnel. However, orange/red fluorescence was not only observable in the lesions, but also an apparent indicator of caries activity. What is the origin of this red fluorescence – are these metabolic products within the body of the lesion [Buchalla et al., 2008]? Could we just use the red fluorescence from the biofilm for caries prediction as some studies seem to indicate [Buchalla et al., 2010]?

Within the limitations of the study showing moderate sensitivity, quantification of orange/red fluorescence was able to distinguish carious lesions that progressed to cavitation from lesions that did not progress at a single observation. Though there are several factors involved in the increased contribution of orange/red fluorescence, future studies identifying the microbiological factors causing the orange/red fluorescence and its implication in caries

activity are indicated. Additionally, studies are needed to understand the role of porphyrins in active and arrested carious lesions.

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Author Contributions

Grace Felix Gomez, PhD student in Dental Sciences, Indiana University School of Dentistry, carried out the image analysis and wrote the paper, and also contributed to the conception of the article. Mr. George J. Eckert, Biostatistician supervisor, Indiana University School of Medicine, did the statistical analysis, tables and figures, and critically revised the manuscript for accuracy and approved it upon changes. Dr. Andrea Ferreira Zandona was the mentor for this project and the principal investigator of the parent study. She supervised the collection of images and the clinical assessment of the surfaces in the parent study. She facilitated the study design, the input of extensive ideas from conceptualization and data interpretation, as well as critically analyzing the paper and providing final approval upon changes.

Disclosure Statement

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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