***Potential for direct application of blue light for photo-disinfection of dentine***

*Sherif A. Mohamad1* \**, Michael R. Milward1, Sarah A. Kuehne1,2, Mohammed A. Hadis1, William M. Palin1, Paul R. Cooper1,3*

1Institute of Clinical Sciences, School of Dentistry, University of Birmingham, 5 Mill Pool Way, Edgbaston, Birmingham, UK, B5 7EG

2Institute of Microbiology and Infection, University of Birmingham, 5 Mill Pool Way, Edgbaston, Birmingham, UK, B5 7EG

3Department of Oral Sciences, Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, PO Box 56, Dunedin 9054, New Zealand

\*Corresponding author: SMA857@bham.ac.uk

**Abstract:**

The direct application of light for photo-disinfection potentially provides a safe and novel modality to inhibit or eliminate cariogenic bacteria residing upon and within dentine. This study aimed to both; characterize the pattern of transmission of 405 nm light through molar dentine at different tooth locations, as well as, determining the irradiation parameters that are antibacterial for *Streptococcus mutans* under various growth conditions, including lawns, planktonic cultures, and biofilms. To determine the amount of light (405 nm) transmitted at different anatomical tooth locations; irradiance values were recorded after blue light (470 - 4054 mW/cm2) had traversed through occlusal, oblique, and buccal dentine sections; and three thicknesses - 1, 2 and 3 mm were investigated. To determine tubular density; scanning electron micrographs from 2 mm outer (dentine-enamel junction) and inner (pulp) dentine sections were analysed. For photo-disinfection studies; *S. mutans* was irradiated using the same 405 nm wavelength light at a range of doses (110 – 1254 J/cm2) in both biofilm and planktonic cultures. The inhibitory effect of the irradiation on bacterial lawns was compared by measuring zones of inhibition; and for planktonic cultures both spectrophotometric and colony forming unit (CFU) assays were performed. A live/dead staining assay was utilised to determine the effect of irradiation on bacterial viability in mature biofilms. Data indicated that increasing dentine thickness decreased light transmission significantly irrespective of its orientation. Occlusal and oblique samples exhibited higher transmission compared with buccal dentine. Oblique dentine 405 nm light transmission was comparable with that of occlusal dentine independent of section thickness. An increased tubule density directly positively correlated with light transmission. Irradiation at 405 nm inhibited *S. mutans* growth in both biofilm and planktonic cultures and a dose response relationship was observed. Irradiation at doses of 340 and 831 J/cm2 led to significant reductions in bacterial growth and viability; as determined by CFU counting and live/dead staining. Data suggests that phototherapy approaches utilising a 405 nm wavelength have therapeutic potential to limit cariogenic bacterial infections both at the surface and within dentine.

**Highlights:**

* Dentine microstructure and tubular density affect 405 nm light transmission.
* Higher light transmission was achieved in occlusal and oblique dentine in comparison with buccal dentine.
* 405 nm light inhibit *S. mutans* initial biofilm formation, and also kills bacteria in mature biofilms.
* The first study to utilise the same light source for dentine light penetration studies and photo-disinfection.
* These data have the potential to inform clinical translational approaches for caries prevention, management and treatment.

**Keywords:** 405 nm, blue light, light transmission, dentinal tubules, caries, *S. mutans*.

1. **Introduction**

Dental caries is a prevalent and debilitating disease which affects all ages and sectors of the population. Despite advancements in early detection and treatment, it remains one of the most common bacterially driven dental diseases globally and is a significant healthcare and economic burden. Advancement of the disease can cause significant pain and suffering for the patient and eventually can result in tooth loss **[1]**,**[2]**. The key causative factor is the initial infection by the Gram positive facultative anaerobic bacterium, *Streptococcus mutans* **[3]**.*S. mutans* rapidlygrows on the tooth surface and ferments a range of sugars which acidifies the local environment enabling further colonisation by acidogenic and acid producing bacteria. Consequently, the conditions generated by the bacterial biofilm result in demineralisation and breakdown of the tooth’s hard tissues, enabling the bacteria to infiltrate through the tooth’s enamel and dentine **[4-8]**. Notably, *S. mutans* can be isolated from both incipient and cavitated carious lesions **[9],[10]**. If incipient caries is untreated, deeper lesions develop, leading to pulp infection, tissue necrosis, peri-apical pathologies and ultimately tooth loss **[11]**.

Effective disease management therefore should aim to minimise *S. mutans* infection at an earlier a stage as possible alongside modification of the patient’s diet. The delivery of chemical antibacterial compounds (e.g. in toothpastes or mouth washes) to inhibit initial biofilm formation and growth is the current treatment of choice, however this approach is not always effective **[12-15]**. When a carious lesion is advanced, its clinical treatment involves the removal of the infected tissue which is then replaced with a restoration which aims to restore the tooth’s functionality. However, these restorations have a finite lifespan and frequently re-treatment procedures are required due to secondary caries, resulting in additional tooth tissue loss over time **[16]**.

The use of direct and indirect light therapy approaches has previously been explored for photo-disinfection of dental tissues. Photodynamic therapy (PDT) provides one such approach utilising a light-activated sensitizer to generate reactive oxygen species (ROS) which subsequently exert antibacterial action **[17]**. Studies have explored its potential use for the treatment of dental caries *in vitro* and *ex vivo,* using photosensitizers such as toluidine blue and curcumin activated by wavelengths of 450 nm and 633 nm, respectively. Variable results were obtained and diffusion of the photosensitizer within the biofilm was shown to be a potential limitation of this approach **[18],[19].**

Recent studies have now indicated that direct light irradiation could be used for bacterial killing. Compared with PDT, direct light potentially results in excitation of locally derived bacterial chromophores which subsequently release ROS, exerting an antibacterial affect **[20]**. Intracellular porphyrins are proposed as the main endogenous photosensitizers, which have an absorption peak within the violet/blue spectral range, i.e. 390 - 425 nm **[21-23]**.Indeed, a recent *in vitro* study has indicated that irradiation at 405 nm could be used directly to inhibit cariogenic bacteria residing within biofilms **[24]**.Other *ex vivo* studies have also demonstrated the potential efficacy of direct light application for dental tissue disinfection, however, frequently, neither details of the light parameters, nor the orientation of the dentinal tissues used were reported **[25-28].**

Importantly, for dental applications, photo-disinfection approaches should be able to kill bacteria on the tooth surface and located within the dentinal tubules **[8]**. Notably, the dentine’s tubular structure is complex and has an S-shaped curvature or linear orientation depending upon their location within the tooth. Furthermore, tubules are conical due to the deposition of intratubular dentine which has been deposited at increased levels at the outer dentinal margins as the tooth ages **[29],[30]**. Consequently, dentine is optically anisotropic, and light scattering, also due to dentine’s non-homogenous composition, is relatively high in the near ultraviolet - Near Infrared light spectrum. Notably, at the dentine’s outer-surface scattering is lower compared with locations closer to the pulp where there is a higher dentinal tubule density with wider diameters **[31]**.

Notably, the light dose/energy density (J/cm2) is directly related to reductions in bacterial viability and the delivered dose is dependent upon both; light irradiance/power density (W/cm2) and irradiation time (seconds). Therefore, decreased irradiation times for antibacterial action can potentially be obtained by using high energy irradiation **[32]**. To identify potential therapeutic irradiation parameters for use in a clinically relevant time-frame for dental tissue photo-disinfection, it is important to determine the optical characteristics of dentine at the target wavelength. Notably, differences in absorption and transmission at different anatomical tooth locations will influence how light could be delivered to disease areas. This study, for the first time, utilises the same light source, which is comprehensive photo-physically characterised, and aims to both; i) determine 405 nm light transmission through dentine, and ii) identify 405 nm light irradiation parameters that have direct antimicrobial efficacy for *S. mutans* which have the potential to be used both prophylactically and to treat different stages of carious infection*.*

**2. Materials and Methods**

**2.1. Dentine specimens**

Seventy-five non-carious human permanent molars (aged 20 – 40 of approximately equal gender) were used in this study (Ethical Approval Ref.: BCHCDent398.ToothBank / REC Ref.: 14/EM/1128 / IRAS Ref.: 161303). All teeth were stored at -80°C prior to use.

**2.1.1. Dentine light transmission**

Teeth were fixed in acrylic blocks using impression compound sticks (Kerr®; USA) to enable sectioning. A water-cooled low speed saw (IsoMet™, BUEHLER®, USA) was used and a primary cut was made to remove the surface layer of enamel. Dentine discs (n=45); from randomly selected samples, were sliced and assigned to three main groups; cross-sectional occlusal, oblique and longitudinal buccal sections. Each group contained three sub-groups of thicknesses: 1 mm, 2 mm, and 3 mm (n=5 each). Polishing of surfaces using a carborundum stone (CARBORUNDUM®, France) was performed; after which discs were washed under running tap water and stored at room temperature in distilled water (E-POD®, Millitrack®, Germany) until light transmission measurements were undertaken. Compressed air was administrated to surface dry specimens prior to testing. A spectrometer; USB4000-VIS-NIR (Ocean Optics, USA); connected with 200 µm optical fibre sensor /detector and a glass cosine corrector (5 mm), were calibrated using deuterium/halogen light source DH2000 (Ocean Optics, USA). Transmission of light at 405nm (AURA light engine®, lumencor®, USA) in each sample was recorded with the dentine disc aligned between the light source (7 mm) above, and the detector below. Specimens were oriented with the pulpal side downward [see **Supp.** **figure 1**]. Increasing irradiation power settings were applied to investigate the potential of delivering light which was able to penetrate the dentine and may be used for antibacterial action in a clinically relevant time-frame. An irradiance reading (mW/cm2) was obtained for 10 increasing power outputs of the light source (470, 968, 1473, 1923, 2360, 2774, 3152, 3503, 3768, and 4054 mw/cm2). Each reading was recorded in triplicate and an average value was calculated. The percentage of light transmitted was calculated in reference to an initial characterization of the light source; measuring its absolute irradiance at distances of 1, 2, 3 mm between the light source and the sensor through atmospheric air. This percentage was calculated using an average of 10 measurements (i.e. at 10 gradual power settings) for each sample; after which, a mean from 5 measurements (per group) was calculated.

**2.1.2. Dentinal tubule characterisation**

Dentine discs (n=30) from non-carious molar teeth were randomly sectioned and assigned to six groups (2 mm thick) (n=5 per group). To assess the dentinal tubule density at different depths, three groups (occlusal, oblique, and buccal) were obtained with two sub-groups within each group; namely outer and inner. Outer sections were adjacent to the dentine-enamel junction (DEJ), while inner sections were located adjacent to the pulp chamber. All sectioning/slicing procedures were performed as described above. To ensure complete smear layer removal in preparation for imaging, samples were rinsed under running tap water, then each sub-group was treated in an ultrasonic bath (In-Ceram Vitasonic, VITA; Germany) for 10 minutes in a mixture of 5% sodium hypochlorite (Acros Organics, Fisher Scientific, UK) and 17% ethylene-diamine-tetraacetic acid (EDTA) (CanalPro EDTA 17%; COLTENE); relying on a previously published protocol **[33].** This was followed by a further 10 minute treatment in an ultrasonic bath of distilled water. Specimens were allowed to dry at 37°C (Hybaid Shake ‘n’ Stack, ThermoFisher Scientific; USA) for at least 72 hours before imaging. Specimens were gold sputter coated (Emitech K550X, Quorum Technologies; UK) under argon for 3 minutes at 20 mA at a distance of 45 mm to obtain a gold coating thickness of 21 nm. Specimens were imaged using a scanning electron microscope (SEM) (EVO MA10, Carl Zeiss; Germany). Micrographs were captured at 2000x magnification under high vacuum and electron high tension (EHT) voltage of 20 kV. Dentinal tubule numbers were quantified using ImageJ software (National Institutes of Health; USA). Subsequently, the number of tubules per unit area (mm2) were calculated using the following formula; *‘n X 106 / Z’*, where *‘n’* is the actual tubule count per image and *‘Z’* is the area of the image in μm2 **[34]**.

**2.2. Bacteria:**

*S. mutans* (ATCC 3209) was retrieved from frozen stocks (-80°C) and incubated (37 °C) aerobically (Heracell™ 150i, ThermoFisher Scientific, USA) for 24 hours on brain heart infusion (BHI) agar (Sigma-Aldrich®, USA). Subsequently, a representative colony was inoculated into 10 ml of BHI broth (Sigma-Aldrich®, USA), and incubated (37 °C) overnight (18-24 hours) in a shaking incubator at 100 rpm (NB-205, N-Biotek, Korea).

**2.2.1. Light irradiation characterisation in bacterial cultures**

Closed plate irradiation was used to avoid cross-contamination of bacterial cultures. Irradiation (AURA light engine®, Lumencor®, USA) from beneath the plates was performed to minimize the Rayleigh scattering of blue light in air **[35]**, and maximize its penetrationwithin bacterial layers **[36]**. Light characterisation was performed with the light source at a distance of 5 mm from the underside of the culture plate to better represent the use of light in a dental cavity where direct application to a cavity wall would not be clinically feasible. To determine irradiance values, the detector USB4000-VIS-NIR (Ocean Optics, USA) was placed in contact with the agar surface orientated towards the underside of the agar dish [see **Supp.** **figure 2(a)**]. Using the 405 nm wavelength the irradiance readings (mW/cm2) were obtained at 10 gradual power outputs (470 - 4054 mW/cm2). Readings were recorded three times and an average was obtained. Data were used to generate a calibration curve. A calibration curve; using a similar approach was also generated for black-walled 96 well plates (4titude®; UK), with the light source placed in contact with the flat transparent underside of the well (6mm); with the sensor in contact with the base of the well. As the diameter of the light source (7 mm) and the well were comparable, this experimental set-up ensured consistent saturation of the light beam within each black-walled well in the culture plate. No light leakage between wells was detected [see **Supp.** **figure 2(b)**]. Calibration curves were used to estimate irradiation doses applied using each experimental set-up; ‘*Dose (J/cm2) = Irradiance (W/cm2) X Time (seconds)’*.

**2.2.2. *S. mutans* viability analysis**

**Bacterial lawns:** Overnight bacterial cultures were diluted in fresh BHI broth to an optical density (OD) of 0.1 (≃ 108 CFU/ml) using a 7315 spectrophotometer (Jenway, UK) with an absorbance wavelength of 600 nm. Using a sterile cotton swab (Sterilin™, Thermofisher Scientific, USA); *S. mutans* was lawned onto the BHI agar. Subsequently, a non-irradiated plate served as the negative control; while experimental plates were exposed at six increasing doses of 110, 154, 273, 366, 456, and 573 J/cm2 – one dose per plate. Each dose was delivered using two different regimens; either with i) increased time/decreased power (IT/DP), or ii) increased power/decreased time (IP/DT)[see **Table 1**]. This protocol was adopted to identify optimal conditions which may have direct clinical application whilst minimising any potential heating effects. Following irradiation agar plates were incubated (37 °C) for up to 24 hours.Standardized images were obtained (SX620 HS, Canon, Japan) for each plate and diameter of zones of inhibition (ZOI) were measured using ImageJ software (National Institutes of Health; USA). Each ZOI was measured at three different orientations along horizontal, vertical and diagonal axes, and an average ZOI diameter was obtained. Studies were repeated four times.

**Planktonic culture assay:** *S. mutans* was grown, sub-cultured, and diluted as described above. Subsequently, 200 µl of diluted cultures were transferred into a well of a black-walled 96-well plate. Two control wells were not exposed to light in each plate and a range of doses were applied including: 249, 340, 608, 831, 1014, and 1254 J/cm2. (IT/DP) and (IP/DT) modes were also investigated [see **Table 2**]. Doses were applied to individual wells in each plate. Subsequently, plates were incubated at 37°C for up to 24 hours. To determine bacterial cell viability and killing after irradiation, turbidity and colony forming units (CFU) assays were performed. For the turbidity assessment, the OD of wells was read at 600 nm using an ELx800™ Microplate Reader (BioTek™, USA). The survival rate was calculated based on the equation: *‘Treated wells OD / Control wells OD X 100’*. Experiments were performed in triplicate. Two doses were investigated; 340 J/cm2 (378 mW/cm2 for 15 minutes) and 831 J/cm2 (418 mW/cm2 for 33 minutes) to assess reductions in colony forming units.Colony counts for samples in irradiated and non-irradiated wells were performed using the Miles and Misra method **[37]**. The number of colonies per millilitre (CFU/ml) was calculated using the formula: ‘*Average number of colonies for a dilution x 50 x dilution factor’*. Finally, ‘Log10’ of each (CFU/ml) was calculated. This experiment was repeated three times; each in duplicate (two plates per experiment).

|  |  |  |
| --- | --- | --- |
| **Irradiance (mW/cm2)** | **Irradiation time** | **Dose (J/cm2)** |
| 122 | 15 minutes | 110 (IT/DP) |
| 251 | 7 minutes + 13 seconds | 110 (IP/DP) |
| 172 | 15 minutes | 154 (IT/DP) |
| 251 | 10 minutes + 12 seconds | 154 (IP/DT) |
| 152 | 30 minutes | 273 (IT/DP) |
| 251 | 18 minutes + 6 seconds | 273 (IP/DT) |
| 122 | 50 minutes | 366 (IT/DP) |
| 191 | 31 minutes + 54 seconds | 366 (IP/DT) |
| 152 | 50 minutes | 456 (IT/DP) |
| 191 | 39 minutes + 42 seconds | 456 (IP/DT) |
| 191 | 50 minutes | 573 (IT/DP) |
| 251 | 38 minutes | 573 (IP/DT) |

**Table 1:** Irradiation parameters applied when exposing *S. mutans* lawns to 405 nm light. The light source was placed at a 5 mm distance from the underside of the agar plate (see **Supp. Figure 2**).

|  |  |  |
| --- | --- | --- |
| **Irradiance (mW/cm2)** | **Irradiation time** | **Dose (J/cm2)** |
| 277 | 15 minutes | 249 (IT/DP) |
| 539 | 7 minutes + 40 seconds | 249 (IP/DT) |
| 378 | 15 minutes | 340 (IT/DP) |
| 539 | 10 minutes + 30 seconds | 340 (IP/DT) |
| 338 | 30 minutes | 608 (IT/DP) |
| 539 | 18 minutes + 48 seconds | 608 (IP/DT) |
| 281 | 50 minutes | 831 (IT/DP) |
| 418 | 33 minutes + 7 seconds | 831 (IP/DT) |
| 338 | 50 minutes | 1014 (IT/DP) |
| 418 | 40 minutes + 25 seconds | 1014 (IP/DT) |
| 418 | 50 minutes | 1254 (IT/DP) |
| 539 | 38 minutes + 46 seconds | 1254 (IP/DT) |

**Table 2:** Irradiation parameters applied when exposing planktonic cultures to 405 nm light. The light source was placed in contact with the base of a well in a 96 well plate (see **Supp. Figure 2**).

**Mature biofilm assay:** Overnight cultures were vortexed (IEC Centra CL2, Thermofisher Scientific, USA), then centrifuged at 5,000 RPM for 5 minutes. Afterwards, the supernatant was removed and replaced with 10 ml of sterile phosphate buffered saline (PBS), vortexed, then re-centrifuged. This step was repeated, before replacing the supernatant again with 10 ml of sterile PBS. Subsequently, the OD was adjusted to 0.5 Mcfarland standard (Pro-Lab Diagnostics, UK) (equivalent to 1.5 X 108 CFU / ml) using PBS. 40 µl of the diluted culture was pipetted into each well of a 96-well plate; supplemented with 160 µl of BHI broth - with 1% sucrose (Sigma-Aldrich®, USA). The cultures were incubated at 37°C, shaking at 80 rpm for four days. On the fourth day of culture, media was removed from wells; replaced with PBS, and the formed biofilms were irradiated. Two wells per 96-well plate were irradiated for each dose of 340 J/cm2 and 831 J/cm2. Two control wells were included containing one positive control (non-irradiated) and a negative control which included biofilm treatment with 70% ethyl alcohol for 20 minutes; applied immediately prior to imaging. Immediately after light delivery, the live/dead staining protocol (Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit, ThermoFisher Scientific, USA) was performed. To prepare a working solution, 3 µl of SYTO® 9 stain and 3 μL of propidium iodide were added to 1 ml sterile PBS. After PBS removal from wells, 200 µl of the working solution was added to each well and the plate was incubated in the dark at room temperature for 30 minutes. The stains were removed by washing with PBS, and biofilms were gently disrupted by aspiration; and 100 µl was pipetted into a µ-Dish 35 mm high Glass Bottom (ibidi ®, Germany) for imaging using a confocal microscope (LSM 700, Carl Zeiss; Germany) with excitation/emission at 480/500 nm and 490/635 nm. Image analysis for quantification of the live/dead bacterial cell percentages was performed using ImageJ software. To estimate the percentage of dead cells, the following formula was used: *‘(Dead Cells/Total Cell Number) X 100’*. The experiment was performed three times; in duplicates.

**2.2.3. Light absorption and temperature measurement in media** The light detector USB4000-VIS-NIR (Ocean Optics, USA) was positioned beneath the agar and absolute irradiance values were obtained. These values were deducted from reference values to calculate the amount of light absorbed by the agar. The percentage of light absorbed was based on an average of 6 measurements. The amount of blue light transmitted to the surface of the BHI agar was 67.86 % ± 2.03 when irradiating the plate at a 5 mm distance; indicating that the percentage of blue light absorbed before reaching the agar surface was 32.14 %. For BHI broth; absorbance was determined through 200 µl fresh BHI broth in well of a 96-well plate using a microplate reader (BioTek™, USA). The transmission percentage was calculated using the formula ‘*Absorbance (OD) = - log10 Transmittance’* **[38]**. Absorbance readings were obtained three times. The percentage of light transmitted through the BHI broth was calculated to be 29.36 % ± 0.24. Therefore, the amount of light absorbed in the broth was 70.53%.

K- Type thermocouples attached to a TC-08 data logger (Pico Technology; UK) were used for temperature measurements. Thermocouples were either attached to the surface of the agar or positioned within wells containing 200 µl broth following generation of access portals within the plastic ware. All temperature investigations were performed at room temperature in triplicate. Temperature elevation was always directly related to the power/ irradiance setting administered regardless of either; the duration of exposure or the eventual dose. Once the irradiation commenced, temperatures increased until a stationery plateau phase was reached. The highest temperature reached at the BHI agar surface was 31.18°C, and 37.99°C for the BHI broth [see **Supp. table 1**]. Both temperatures are within the range of normal incubation and growth for *S. mutans* **[39]**.

**2.3. Statistical analysis:**

Light transmission through dentine and tubule quantification data were analysed using one-way ANOVA; for comparisons of more than two groups. Tukey’s post hoc test was applied for pairwise comparisons. For antimicrobial data; the Kruskal-Wallis test was used to determine significant differences between groups. The *Bonferroni test* was applied for pair-wise comparisons. The significance value was set at *p ≤ 0.05*. Data were analyzed using SPSS 17 (IBM®, USA).

**3. Results**

**3.1. Dentine light transmission**

Data demonstrated that light transmission decreased as dentine thickness increased for all orientations. Percentage of light transmitted through 1 mm dentine; in all sections (occlusal, oblique, buccal), was statistically significant compared with both the 2 and 3 mm dentine specimens (P<0.001). The amount of light transmitted through either occlusal or oblique sections was consistently greater than the amount of light transmitted through buccal sections at all three thicknesses studied. No significant differences were found between occlusal and oblique dentine in terms of light transmission for the thicknesses examined.In the 1 and 3 mm dentine sections, light transmission in occlusal sections was significantly greater than in buccal dentine sections (P=0.033; P=0.040, respectively). It is notable that the percentage of light transmitted through each specimen was not significantly different regardless of the light source’s power output. Light therefore appeared to be transmitted along the tubules occlusally and obliquely; compared to degrading at sites of change of orientation in tubular direction buccally [see **Figure 1** & **Supp**. **table 2**]. Dentinal tubule quantification data revealed that occlusal and oblique dentine sections exhibited the highest tubular density in comparison with the buccal dentine. These data correlated directly with the light transmission results where the higher tubular density detected in the occlusal and oblique sections yielded higher percentages of light transmission. This was in contrast to the buccal sections which exhibited a lower dentinal tubular density. There were no significant differences between any outer and inner counts within the same section. Notably, both the inner occlusal (P=0.024) and inner oblique (P=0.004) counts were significantly higher than the inner buccal counts [see **Figure 2**].

C:\Users\hp\Desktop\New folder\Manuscript 1_Final version\Pics Finals\Fig. 11000.tif

**Figure 1: (a)** Bar chart showing of light transmission percentages through occlusal, oblique, and buccal dentine; at 3 separate tissue thicknesses (n=5) (mean +/- S.D.). Significance level set at P ≤ 0.05. The different letters (A-F), within each of the different thickness groups indicate statistically significant differences between the differently oriented sample groups. **(b)** SEM images of buccal dentine sections at the locations where a change of orientation in tubular direction occurs. The tubules are not circular, but rather elongated; occluding the light beam instead of allowing transmission**.**

C:\Users\hp\Desktop\New folder\Manuscript 1_Final version\Pics Finals\Fig. 21000.tif

**Figure 2: (a)** Representative SEM images of outer (DEJ) and inner (pulp) dentine sections from each group; occlusal, oblique, and buccal. These Images were used to quantify the dentinal tubules. **(b)** Bar chart showing the tubular density through occlusal, oblique, and buccal dentine (n=5) (Mean +/- S.D.). Significance level set at P ≤ 0.05. Different letters are statistically significantly different.

**3.2. *S. mutans* viability following irradiation**

Results indicated that 405 nm light irradiation inhibited *S. mutans* growth in a dose dependent relationship based on zone of inhibition (ZOI) measurements. There were no significant differences in ZOI diameter between single doses when applied either in the IT/DP or IP/DT mode. Delivering a dose of 573 J/cm2 in both modes; IT/DT and IP/DT resulted in a significantly greater ZOI compared with the 110 J/cm2 (IP/DT) (P=0.012) (P=0.016). Delivery of 573 J/cm2 IP/DT resulted in a significantly wider ZOI compared with 110 J/cm2 IT/DP delivery (p=0.047) [see **Figure 3**]. All agar plates were re-incubated for an additional 48 hours; and ZOIs were still maintained indicating that the effect of 405 nm light was bactericidal rather than bacteriostatic. To address the hypothesis that 405 nm irradiation may exert an indirect affect due to i) activation of media components, or ii) degradation of nutrients, the agar was irradiated prior to the seeding of the bacterial lawn. Notably, bacteria grew over the area where the agar was illuminated and no ZOI was observed. Additionally, irradiation with the plate lid removed, directly from above, was performed and ZOIs were also observed. To address the hypothesis that blue light may be specifically degrading or sensitizing BHI agar; irradiation of bacterial lawns was conducted on Tryptone Soy Agar, similar inhibitory effects were observed.

To determine the effect of light on planktonic bacterial growth, turbidity and CFU analyses were performed following culture irradiation at 405 nm. Using turbidity measurements, a trend was detected indicating a dose response relationship using either the IT/DP or IP/DT mode of delivery. The dose of 1254 J/cm2 delivered using the IP/DT modality produced significantly lower survival percentages compared with 249 J/cm2 irradiation when using both modes; IT/DP (P=0.037) and IP/DT (P=0.021)**.** As was the case with irradiation of bacterial lawns on agar, there was no significant differences; within a single dose, between its IT/DP mode and IP/DT mode [see **Figure 4(a)**]. To better characterize the potential killing effects of 340 and 831 J/cm2 irradiation, planktonic cultures of *S. mutans* were irradiated which lead to 0.6 and 0.92 log10 reductions in growth. These values were statistically significant compared with the non-irradiated controls (P=0.038) (P=0.002) [see **Figure 4(b)**]. Image analysis of irradiated *S. mutans* biofilms revealed that delivery of 340 and 831 J/cm2 light resulted in 48 ± 1.38% and 54 ± 6.38% killing, respectively [see **Figure 5**]. Notably, these biofilms were irradiated solely in PBS, which therefore supports a direct antibacterial action of the light rather than this effect being due to activation of an intermediary compound as would occur due to PDT.

C:\Users\hp\Desktop\New folder\Manuscript 1_Final version\Pics Finals\Fig 31000.tif

**Figure 3:** **(a)** Representative images showing the difference between *S. mutans* i) control plate (not irradiated), ii) an agar plate exposed to 405 nm light at a dose of 110 J/cm2 , and iii) at a dose of 456 J/cm2 . As shown, the higher dose produced a greater area for the zone of Inhibition (7 mm diameter) compared with the lower dose (4 mm). **(b)** Bar chart showing the effect of 405 nm light on *S. mutans* lawns; when the light source was at 5 mm from agar plate bottom. Each dose was administrated in an (IT/DP) and (IP/DT) modes (n=4) (Mean +/- S.D.). The same symbols show statistically significant differences. Significance level set at p ≤ 0.05.

C:\Users\hp\Desktop\New folder\Manuscript 1_Final version\Pics Finals\Fig. 41000.tif

**Figure 4:** Bar charts showing the effect of different 405 nm light doses on *S. mutans* planktonic cultures (n=3). **(a)** Effect of blue light on bacterial survival percentages using OD readings at 600 nm. Each dose was administrated in either (IT/DP) or (IP/DT) mode. The same symbols show statistically significant differences. **(b)** Bar chart showing log10 reductions for *S. mutans* planktonic cultures; in response to 405 nm light doses of 340 and 831 J/cm2. Irradiation parameters for 340 J/cm2 were 378 mW/cm2 for 15 minutes; while for 831 J/cm2, were 418 mW/cm2 for 33 minutes. Superscripts (\*) represent a statistically significantly difference relative to the control. Significance level set at P ≤ 0.05. Mean +/- S.D. shown.

C:\Users\hp\Desktop\New folder\Manuscript 1_Final version\Pics Finals\Fig. 5p. 11000.tif**Figure 5: (a)** Representative confocal microscope images demonstrating *S. mutans* maturebiofilms i) Positive and negative controls, ii) biofilm exposed to 405 nm light at a dose of 340 J/cm2 , and iii) at a dose of 831 J/cm2. These Images were used to estimate the percentage of dead cells. **(b)** Bar chart showing the percentage of dead cells in response to both doses of 340 and 831 J/cm2 (n=3) (Mean +/- S.D.). There was no statistical significant difference between the effects of the two experimental doses used.

1. **Discussion**

Previously blue light has been reported to have the highest absorption and lowest penetration through dentine **[40-42]** whilst also having the potential to kill cariogenic bacteria **[24],[28]**. Consequently, the current studies were performed to better characterise how 405 nm light may be applied for the treatment and management of dental disease. Data indicated that the dentinal tubule orientation played a significant role in the transmission of 405 nm light; as the percentage of light transmitted through cross-sectional dissected occlusal and oblique dentine was always significantly higher than light transmitted through longitudinally dissected buccal dentine. Fried *et al*. reported that if light is delivered perpendicular and not along the path of the tubules, it will penetrate only ~100 microns and this is in part due to dentine being a bi-refractive tissue as it contains hydroxyapatite crystals as well as having a complex dentinal tubule structure **[43]**. Interestingly, Hariri *et al.* obtained similar results to those reported here confirming that the refractive index of cross-cut dentine is less than dentine cut either obliquely or longitudinally. In longitudinal and oblique dissections, hydroxyapatite crystal and collagen orientation were reported as being key properties which influenced light propagation **[44]**.

It is notable that considerable variation was detected in the light transmission data and this likely related to several factors. The extracted teeth used in this study were obtained from a relatively broad age range and therefore specimens would likely exhibit considerable heterogeneity in tissue structure potentially due to the presence of intratubular dentine and hence tubule width **[44]**, **[45]**. Furthermore, the presence of sclerotic dentine may also have been a factor influencing transmission as its presence could not be excluded **[46]**.

Our data, along with that from several other groups, support the use of direct light application for antibacterial action against *S. mutans*. Previously, in similar studies, a range of light sources have been used including a halogen lamp (400-500 nm), a plasma arc (450-480 nm), and an LED (450-480 nm). Notably, it has been shown that *S. mutans* and *Enterococcus faecalis* lawns required a 7-10 times higher dose for killing compared with *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Furthermore, *S. mutans* eradication was only demonstrated when using the plasma arc (450-480 nm); at a dose of 159 J/cm2 **[47]**. Results from the current study, however, have now shown that bacterial inhibition can be achieved using a LED light source at a wavelength of 405 nm, and lower doses than those previously reported can also be used.

Results from the current study showed that 405 nm light was capable of inhibiting *S. mutans* growth inplanktonic cultures and these outcomes are in agreement with other similar studies. *S. mutans* planktonic cultures exposed to a xenon lamp (450-490 nm) at a dose of 686.4 J/cm2 showed more than 90% growth inhibition **[48]**. Chebath-Taub *et al.* reported delivery of a range of doses from 68 to 680 J/cm2 using a plasma arc lamp at wavelengths between 400-500 nm. All doses resulted in *S. mutans* killing and also interfered with biofilm re-formation **[49]**. In our *S. mutans* biofilm studies results demonstrated the ability of the 405 nm light to kill 48% (340 J/cm2) and 54% (831 J/cm2) of bacterial cells in mature (4 days old) biofilms. Notably, the overall dose administered by De Sousa *et al.* **[24]** falls within the same range as was used here although they irradiated *S. mutans* biofilms twice daily; for 5 days; each at 72 J/cm2. Their irradiation protocol resulted in a significant reduction in extracellular polysaccharides, as well as, a two-fold decrease in live biomass. Gomez *et al.* **[50]** also proposed that irradiating 12-16 hours old *S. mutans* biofilms, using a quantitative light-induced fluorescence system with a peak wavelength of 405 nm, led to a significant reduction in total biomass at doses as low as 9.26 J/cm2. However, this reduction was only observed in biofilms grown in sucrose free media**.**

Results showed differences between effective antibacterial doses observed on bacterial lawns or planktonic cultures. There were also variations through light exposure reciprocity; as a longer time delivery for a given dose led to wider ZOIs. These effects likely occurred due to the variations used for the experimental set-ups. Higher irradiation doses were required to kill bacteria in a broth medium, compared with the exposure required on the surface of solid agar. Data indicated that this difference was attributable to the scattering and absorption effects on the 405 nm light in the broth, thus affecting the penetration depth**.** Indeed, it is well recognised that irradiation conditions, as well as, the half-life of ROS; are major modulating factors with regards to achieving disinfection **[51-54]**. However, there was no notable differences between the effects of the two doses investigated; on both colony counts and mature biofilms. Furthermore, it has been reported that blue light can exert enhanced bacterial killing in biofilm cultures **[49],[55]**. Indeed, Steinberg *et al.* **[56]** demonstrated greater reductions in CFU counts following light irradiation for *S. mutans* biofilms compared with the levels of bacterial death detected in planktonic cultures irradiated under similar conditions**.**

It is therefore apparent that the direct delivery of 405 nm blue light has the potential for use to manage and treat carious infections. Based on the data presented here applying an exposure time of 7 minutes with an irradiance of 251 mW/cm2, a dose of 110 J/cm2 could be used to inhibit bacteria on the tooth surface. Using the same irradiation time but with higher irradiance parameters of 280 - 325 mW/cm2, has the potential to treat bacteria at up to a 1 mm depth within dentine tubules at different tooth locations. This light application regimen would therefore easily penetrate to sufficient dentine depths which have been reported for bacterially invaded tubules **[57]**. Delivery of the same dose at less exposure times can be achieved by either; applying a higher power setting, or reducing the distance between the light source and the target surface **[55]**. Furthermore, there is the potentially that greater bacterial killing at lower doses could be expected *in* *vivo*; as survival may not be as optimal as it is *in vitro* under the rich nutrient growth conditions used here **[47]**. Notably, the antimicrobial effects observed here are not based on the photo-thermal effects that are utilised by infra-red diode and carbon dioxide lasers which are potentially hazardous to healthy tissues **[58],[59]**.

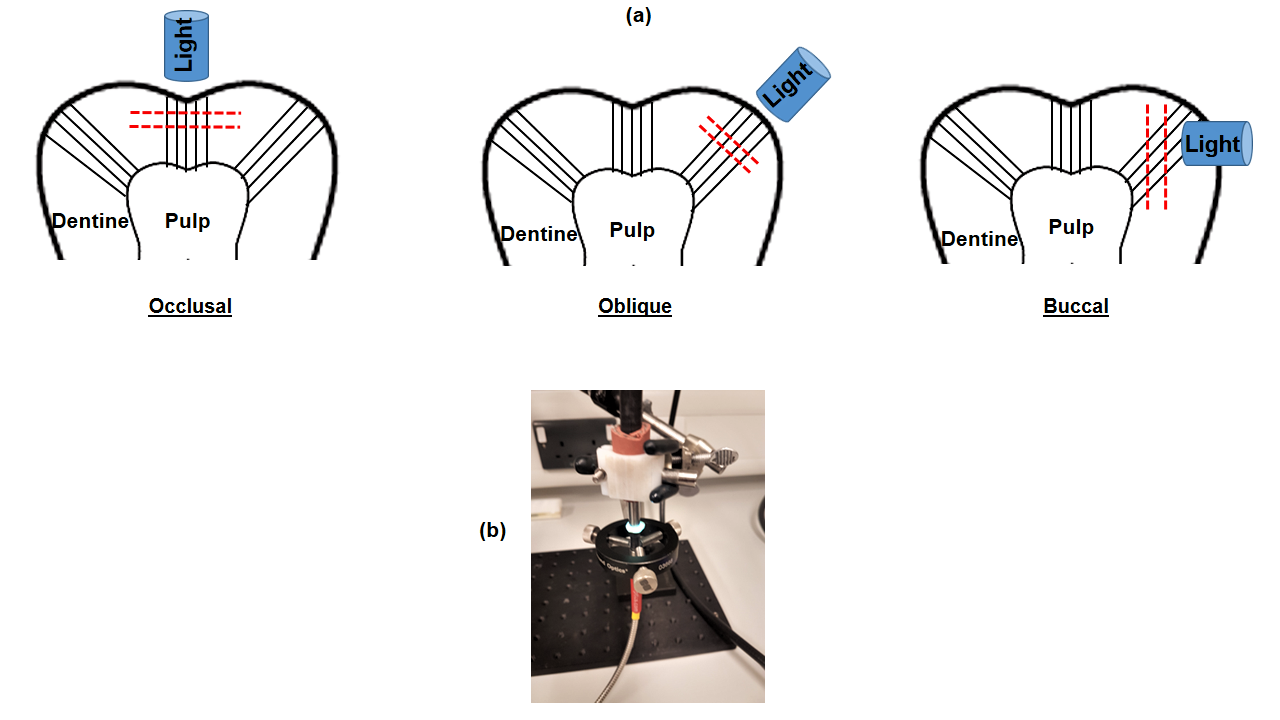
To our knowledge, this is the first study which has investigated both the transmission of blue light in coronal dentine; along with determining the antibacterial effects using the same light source. The data we have presented here indicates that direct blue light delivery has the potential to be used to treat and manage carious infections at different stages of disease. Our findings can therefore be used to underpin future studies which utilise well-established complex multispecies biofilm models to identify light delivery parameters for antibacterial killing which have the potential for use prophylactically and in a clinically relevant timeframe. Notably, we are also aware of the ability of 405 nm light to stimulate a positive response in host cells and tissues **[60-62]**, therefore there is the possibility to utilise PBM to stimulate innate repair responses within the tooth through the dentine. Future translational studies will therefore require the concomitant development of a suitable therapeutic device along with further *in vitro, ex vivo* and clinical trials to demonstrate the safety and efficacy of dental phototherapy.

1. **Acknowledgment**

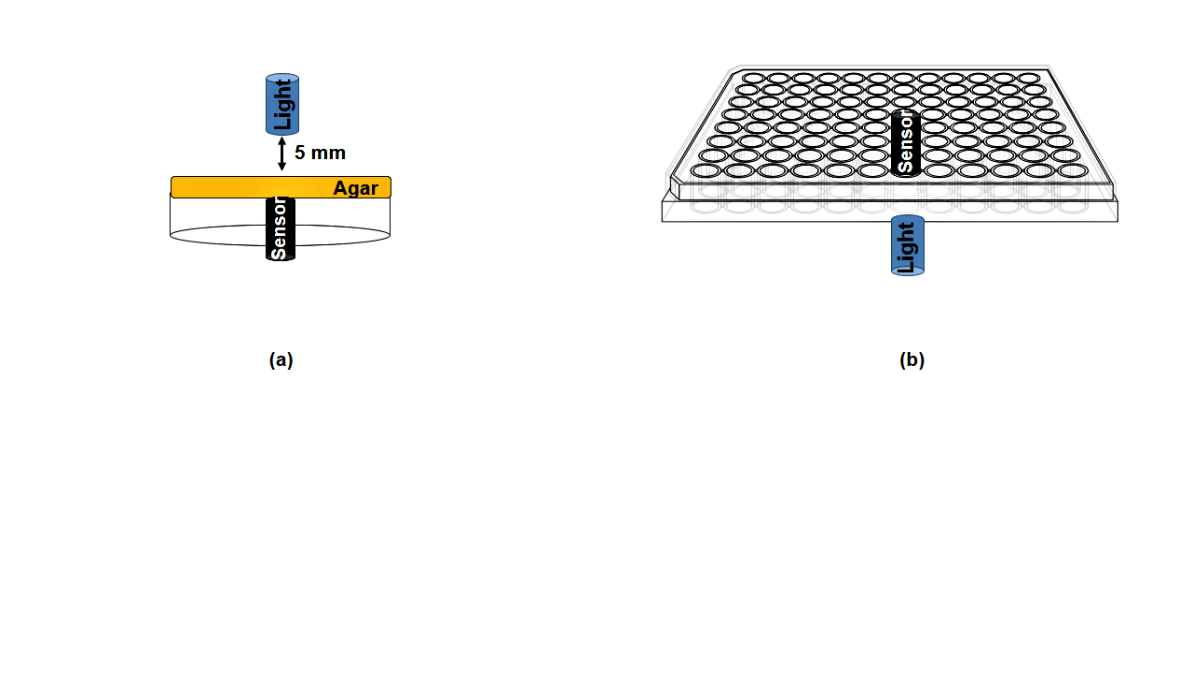
The author would like to thank Soher Jayash for her generous assistance with confocal microscopy. We also acknowledge the School of Dentistry, University of Birmingham for supporting this work.

1. **References**
2. Listl S, Galloway J, Mossey PA, Marcenes W (2015). Global economic impact of dental diseases. Journal of Dental Research 94(10):1-7. <https://doi.org/10.1177/0022034515602879>
3. Frencken JE, Sharma P, Stenhouse L, Green D, Laverty D, Dietrich T (2017). Global epidemiology of dental caries and severe periodontitis – a comprehensive review. Journal of Clinical Periodontology 44 Suppl 18:S94-S105. <https://doi.org/10.1111/jcpe.12677>
4. Loesche WJ (1996). Microbiology of Dental Decay and Periodontal Disease. In: Medical Microbiology - 4th edition.
5. Hamilton IR (2000). Ecological basis for dental caries. In: Oral Bacterial Ecology: The Molecular Basis.
6. Farges JC, Keller JF, Carrouel F, Durand SH, Romeas A, Bleicher F, Lebecque S, Staquet MJ (2009). Odontoblasts in the dental pulp immune response. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 312B(5):425-36. <http://doi.org/10.1002/jez.b.21259>
7. Shemesh M, Tam A, Steinberg D (2007). Expression of biofilm-associated genes of Streptococcus mutans in response to glucose and sucrose. Journal of Medical Microbiology 56(Pt 11):1528-35. <https://doi.org/10.1099/jmm.0.47146-0>
8. Love RM, Jenkinson HF (2002). Invasion of dentinal tubules by oral bacteria. Critical Reviews in Oral Biology and Medicine 13(2):171-83. <http://doi.org/10.1177/154411130201300207>
9. Cooper PR, McLachlan JL, Simon S, Graham LW, Smith AJ (2011). Mediators of inflammation and regeneration. Advances in Dental Research 23(3):290-5. <http://doi.org/10.1177/0022034511405389>
10. Zhou X, Li Y (2015). Supragingival Microbes. In: Atlas of Oral Microbiology - 1st edition.
11. Duggal MS, Curson, MEJ (2003). Dental disease / Etiology of Dental Caries. In: Encyclopedia of Food Sciences and Nutrition - 2nd edition.
12. Pitts NB (2001). Clinical diagnosis of dental caries: a European perspective. Journal of Dental Education 65(10):972-8. <http://doi.org/10.1177/154405910408301S03>
13. Featherstone JDB (2006). Delivery challenges for fluoride, chlorhexidine and xylitol. BMC Oral Health 6(Suppl 1): S8. <https://doi.org/10.1186/1472-6831-6-S1-S8>
14. Moshrefi A (2002) Chlorhexidine. The journal of the western society of periodontology / Periodontal abstracts 50(1):5-9.
15. Yates R, Jenkins S, Newcombe R, Wade W, Moran J, Addy M (1993). A 6‐month home usage trial of a 1% chlorhexidine toothpaste: (I). Effects on plaque, gingivitis, calculus and tooth staining. Journal of Clinical Periodontology 20(2):130-8. <http://doi.org/10.1111/j.1600-051X.1993.tb00327.x>
16. Gilbert R, Williams P (1987). The oral retention and antiplaque efficacy of triclosan in human volunteers. British Journal of Clinical Pharmacology 23(5):579-83. <http://doi.org/10.1111/j.1365-2125.1987.tb03094.x>
17. Lee Y (2013) Diagnosis and Prevention Strategies for Dental Caries. Journal of lifestyle medicine3(2):107-109.
18. Pattison DI, Davies MJ (2006) Actions of ultraviolet light on cellular structures. EXS (96):131-57. <https://doi.org/10.1007/3-7643-7378-4_6>
19. Williams JA, Pearson GJ, Colles MJ, Wilson M (2004). The photo-activated antibacterial action of toluidine blue O in a collagen matrix and in carious dentine. Caries Research 38(6):530-6. <http://doi.org/10.1159/000080582>
20. Araújo NC, Fontana CR, Bagnato VS, Gerbi MEM (2014). Photodynamic antimicrobial therapy of curcumin in biofilms and carious dentine. Lasers in Medical Science 29(2):629-35. <http://doi.org/10.1007/s10103-013-1369-3>
21. Lipovsky A, Nitzan Y, Gedanken A, Lubart R (2010). Visible light-induced killing of bacteria as a function of wavelength: Implication for wound healing. Lasers in Surgery and Medicine 42(6):467-72. <https://doi.org/10.1002/lsm.20948>
22. Hamblin MR, Viveiros J, Yang C, Ahmadi A, Ganz RA, Tolkoff MJ (2005). Helicobacter pylori accumulates photoactive porphyrins and is killed by visible light. Antimicrobial Agents and Chemotherapy 49(7):2822-7. <http://doi.org/10.1128/AAC.49.7.2822-2827.2005>
23. Lim CK, Rideout JM, Wright DJ (1983). High-performance liquid chromatography of naturally occurring 8-, 7-, 6-, 5- and 4-carboxylic porphyrin isomers. Journal of Chromatography 282:629-41. <https://doi.org/10.1016/S0021-9673(00)91640-6>
24. Bu W, Myers N, McCarty JD, O’Neill T, Hollar S, Stetson PL, Sved DW (2003). Simultaneous determination of six urinary porphyrins using liquid chromatography-tandem mass spectrometry. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences 783(2):411-23. <https://doi.org/10.1016/S1570-0232(02)00703-1>
25. De Sousa DL, Lima RA, Zanin IC, Klein MI, Janal MN, Duarte S (2015). Effect of twice-daily blue light treatment on matrix-rich biofilm development. PLoS ONE 10(7): e0131941. <http://doi.org/10.1371/journal.pone.0131941>
26. Ricatto LGO, Conrado LAL, Turssi CP, França FMG, Basting RT, Amaral FLB (2014). Comparative evaluation of photodynamic therapy using LASER or light emitting diode on cariogenic bacteria: An in vitro study. European Journal of Dentistry 8(4):509-14. <http://doi.org/10.4103/1305-7456.143634>
27. Lima JPM, Sampaio De Melo MA, Borges FMC, Teixeira AH, Steiner-Oliveira C, Nobre Dos Santos M, Rodrigues LKA, Zanin ICJ (2009). Evaluation of the antimicrobial effect of photodynamic antimicrobial therapy in an in situ model of dentine caries. European Journal of Oral Sciences 117(5):568-74. <https://doi.org/10.1111/j.1600-0722.2009.00662.x>
28. Burns T, Wilson M, Pearson GJ (1995). Effect of dentine and collagen on the lethal photosensitization of streptococcus mutans. Caries Research 29(3):192-7. <http://doi.org/10.1159/000262068>
29. Felix Gomez GG, Lippert F, Ando M, Zandona AF, Eckert GJ, Gregory RL (2019). Photoinhibition of Streptococcus mutans Biofilm-Induced Lesions in Human Dentin by Violet-Blue Light. Dentistry Journal 7(4):113. <https://doi.org/10.3390/dj7040113>
30. Berkovitz BK, Holland GR, Moxham BJ (1978) Color atlas oral anatomy. Wolfe Medical Publications. <https://doi.org/10.1016/0002-9416(79)90130-1>
31. Goldberg M, Kulkarni A, Young M, Boskey A (2011). Dentin: structure, composition and mineralization. Frontiers in Bioscience (Elite Edition) 3:711–35. <https://doi.org/10.2741/e281>
32. Zijp JR (2001). Optical properties of dentin. Groningen: s.n.
33. PJ Gwynne, MP Gallagher (2018). Light as a Broad-Spectrum Antimicrobial. Frontiers in microbiology 9:119. <https://doi.org/10.3389/fmicb.2018.00119>
34. Koçani F, Kamberi B, Dragusha E, Mrasori S, Haliti F (2012). The cleaning efficiency of the root canal after different instrumentation technique and irrigation protocol: A SEM analysis. Open Journal of Stomatology 2: 69-76. <http://dx.doi.org/10.4236/ojst.2012.22013>
35. Lo Giudice G, Cutroneo G, Centofanti A, Artemisia A, Bramanti E, Militi A, Rizzo G, Favaloro A, Irrera A., Lo Giudice R, Cicciù M (2015). Dentin morphology of root canal surface: A quantitative evaluation based on a scanning electronic microscopy study. BioMed Research International (4). <https://doi.org/10.1155/2015/164065>
36. Acton JC, Dawson PL (2004). Proteins in Food Processing. Woodhead Publishing Limited 603-4.
37. Bumah VV, Masson-Meyers DS, Cashin SE, Enwemeka CS (2013). Wavelength and bacterial density influence the bactericidal effect of blue light on methicillin-resistant Staphylococcus aureus (MRSA). Photomedecine and Laser Surgery 31(11):547-53. <https://doi.org/10.1089/pho.2012.3461>
38. Miles AA, Misra SS, Irwin JO (1938). The estimation of the bactericidal power of the blood. Journal of Hygiene 38(6): 732–49. <https://doi.org/10.1017/S002217240001158X>
39. Silva DFT, Mesquita-Ferrari RA, Fernandes KPS, Raele MP, Wetter NU, Deana AM (2012). Effective transmission of light for media culture, plates and tubes. Photochemistry and Photobiology 88(5):1211-6. <https://doi.org/10.1111/j.1751-1097.2012.01166.x>
40. Ranganathan V, Akhila C (2019). Streptococcus mutans: has it become prime perpetrator for oral manifestations?. Journal of Microbiology & Experimentation 7(4):207-213. <https://doi.org/10.15406/jmen.2019.07.00261>
41. Dogandzhiyska V, Angelov I, Dimitrov S, Uzunov T (2015). In vitro study of light radiation penetration through dentin, according to the wavelength. Acta Medica Bulgarica 42(2):16-22. <http://doi.org/10.1515/amb-2015-0013>
42. Ana Paula ST, Alonso JRL, Basso FG, Moriyama LT, Heling J, Bagnato VS, De Souza Costa CA (2013). LED light attenuation through human dentin: A first step toward pulp photobiomodulation after cavity preparation. American Journal of Dentistry 26(6):319-23.
43. Palin WM, Hadis MA, Milward MR, Carroll JD, Cooper PR (2015). Beam profile measurements for dental phototherapy: The effect of distance, wavelength and tissue thickness. Proc. SPIE 9309, Mechanisms for Low-Light Therapy X, 930905. <https://doi.org/10.1117/12.2077628>
44. Fried D, Glena RE, Featherstone JDB, Seka W (1995). Nature of light scattering in dental enamel and dentin at visible and near-infrared wavelengths. Applied Optics 34(7):1278-85. <http://doi.org/10.1364/AO.34.001278>
45. Hariri I, Sadr A, Shimada Y, Tagami J, Sumi Y (2012). Effects of structural orientation of enamel and dentine on light attenuation and local refractive index: An optical coherence tomography study. Journal of Dentistry 40(5):387-96. <http://doi.org/10.1016/j.jdent.2012.01.017>
46. Kienle A, Forster F K, Diebolder R, Hibst R (2003). Light propagation in dentin: Influence of microstructure on anisotropy. Physics in Medicine and Biology 48(2):N7-14. <http://doi.org/10.1088/0031-9155/48/2/401>
47. Zolotarev VM, Grisimov VN (2001). Architectonics and Optical Properties of Dentin and Dental Enamel. Optics and Spectroscopy 90(5):753-9. <http://doi.org/10.1134/1.1374665>
48. Feuerstein O, Persman N, Weiss EI (2004). Phototoxic Effect of Visible Light on Porphyromonas gingivalis and Fusobacterium nucleatum: An In Vitro Study. Photochemistry and Photobiology 80(3):412-5. [https://doi.org/10.1562/0031-8655(2004)080<0412:PEOVLO>2.0.CO;2](https://doi.org/10.1562/0031-8655(2004)080%3c0412:PEOVLO%3e2.0.CO;2)
49. Feuerstein O, Moreinos D, Steinberg D (2006). Synergic antibacterial effect between visible light and hydrogen peroxide on Streptococcus mutans. Journal of Antimicrobial Chemotherapy 57(5):872-6. <https://doi.org/10.1093/jac/dkl070>
50. Chebath-Taub D, Steinberg D, Featherstone JDB, Feuerstein O (2012). Influence of blue light on Streptococcus mutans re-organization in biofilm. Journal of Photochemistry and Photobiology 116:75-8. <https://doi.org/10.1016/j.jphotobiol.2012.08.004>
51. Gomez GF, Huang R, MacPherson M, Ferreira Zandona AG, Gregory RL (2016). Photo Inactivation of Streptococcus mutans Biofilm by Violet-Blue light. Current Microbiology 73(3):426-433. <https://doi.org/10.1007/s00284-016-1075-z>
52. Tomb, R. M., Maclean, M., Coia, J. E., MacGregor, S. J., & Anderson, J. G. (2017). Assessment of the potential for resistance to antimicrobial violet-blue light in Staphylococcus aureus. Antimicrobial Resistance and Infection Control 6:100. <http://doi.org/10.1186/s13756-017-0261-5>
53. Kochevar IE, Redmond RW (2000). Photosensitized production of singlet oxygen. Methods in Enzymology 319:20-8. <https://doi.org/10.1016/s0076-6879(00)19004-4>
54. Wu H, Song Q, Ran G, Lu X, Xu B (2011). Recent developments in the detection of singlet oxygen with molecular spectroscopic methods. Trends in Analytical Chemistry 30(1):133-41. <https://doi.org/10.1016/j.trac.2010.08.009>
55. Baier J, Fuß T, Pöllmann C, Wiesmann C, Pindl K, Engl R, Bäumler W (2007). Theoretical and experimental analysis of the luminescence signal of singlet oxygen for different photosensitizers. Journal of Photochemistry and Photobiology 87(3):163-73. <https://doi.org/10.1016/j.jphotobiol.2007.02.006>
56. Cohen-Berneron J, Steinberg D, Featherstone JDB, Feuerstein O (2016). Sustained effects of blue light on Streptococcus mutans in regrown biofilm. Lasers in Medical Science 31(3):445-52. <https://doi.org/10.1007/s10103-016-1873-3>
57. Steinberg D, Moreinos D, Featherstone J, Shemesh M, Feuerstein O (2008). Genetic and physiological effects of noncoherent visible light combined with hydrogen peroxide on Streptococcus mutans in biofilm. Antimicrobial Agents and Chemotherapy 52(7):2626-31. <https://doi.org/10.1128/AAC.01666-07>
58. Brittan JL, Sprague SV, Macdonald EL, Love RM, Jenkinson HF, West NX (2016). In vivo model for microbial invasion of tooth root dentinal tubules. Journal of applied oral science 24(2):126-135. <https://10.1590/1678-775720150448>
59. Dederich DN, Pickard MA, Vaughn AS, Tulip J, Zakariasen KL (1990). Comparative bactericidal exposures for selected oral bacteria using carbon dioxide laser radiation. Lasers in Surgery and Medicine 10(6):591-94. <https://doi.org/10.1002/lsm.1900100612>
60. Moritz A, Gutknecht N, Schoop U, Goharkhay K, Doertbudak O, Sperr W (1997). Irradiation of infected root canals with a diode laser in vivo: results of microbiological examinations. Lasers in Surgery and Medicine 21(3):221-6. [https://doi.org/10.1002/(sici)1096-9101(1997)21:3<221::aid-lsm1>3.0.co;2-s](https://doi.org/10.1002/(sici)1096-9101(1997)21:3%3c221::aid-lsm1%3e3.0.co;2-s)
61. Kushibiki T, Awazu K (2009). Blue laser irradiation enhances extracellular calcification of primary mesenchymal stem cells. Photomedicine and Laser Surgery 27(3):493-8. <https://doi.org/10.1089/pho.2008.2343>
62. Wang Y, Huang YY, Wang Y, Lyu P, Hamblin MR (2016). Photobiomodulation (blue and green light) encourages osteoblastic-differentiation of human adipose-derived stem cells: Role of intracellular calcium and light-gated ion channels. Scientific Reports 6:33719. <https://doi.org/10.1038/srep33719>
63. Zhu T, Wu Y, Zhou X, Yang Y, Wang Y (2019). Irradiation by blue light-emitting diode enhances osteogenic differentiation in gingival mesenchymal stem cells in vitro. Lasers in Medical Science 34(7):1473-81. <https://doi.org/10.1007/s10103-019-02750-3>

**Supplementary material:**



**Supp. Figure 1:**  **(a)** Schematic diagram showing the direction of incident 405 nm light in relation to the orientation of dentinal tubules in occlusal, oblique, and buccal dentine sections. Black lines represent dentinal tubules, while red lines represent how each dentine disc was sectioned. **(b)** Image of the experimental set up showing a dentine disc sandwiched between the light source (AURA light engine®, lumencor®, USA) above and the detector below. The specimen was oriented with its occlusal side upward and pulpal side downward.

**Supp. Figure 2:** Diagrams showing 405 nm light characterization for the different experimental set-ups. **(a)** Irradiation from the underside of an agar plate at a 5 mm distance; with the sensor placed in contact with the surface of the agar. **(b)** Irradiation from the underside of the well in a 96-well plate; while the sensor was located inside the well and was in contact with its base.

|  |  |  |
| --- | --- | --- |
|  | **Irradiance (mW/cm2)** | **Temperature (°C)** |
|  | 122 | 24.98 ± 0.39 |
|  | 152 | 26.47 ± 0.56 |
| **Agar** | 172 | 26.92 ± 0.32 |
|  | 191 | 28.22 ± 0.13 |
|  | 254 | 31.18 ± 0.53 |
|  | 277 | 30.46 ± 0.37 |
|  | 338 | 31.47 ± 0.75 |
| **Broth** | 378 | 33.86 ± 1.24 |
|  | 418 | 35.27 ± 1.11 |
|  | 539 | 37.99 ± 0.40 |

**Supp. table 1:** Maximum temperatures reached corresponding to each irradiance applied on both the agar surface and in the broth media. Mean +/- S.D

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  | **Irradiance** | **(mW/cm2)** |  |  |  |  |
|  | **Atmospheric air** | 458 ± 0.8 | 945.2 ± 1.1 | 1440.5 ± 0.6 | 1884.5 ± 1.5 | 2299.8 ± 5.7 | 2723.8 ± 4.2 | 3059.3 ± 3.6 | 3383.6 ± 15 | 3680.3 ± 5.5 | 3938.6 ± 8.1 |
| **1 mm** | **Occlusal** | 105.4 ± 41.2 | 210 ± 80.7 | 317.6 ± 128.1 | 426.2 ± 181.7 | 520.1 ± 236.5 | 616 ± 283.2 | 703.8 ± 321.6 | 777.7 ± 347.8 | 855.8 ± 380.2 | 922.25± 417.5 |
|  | **Oblique** | 62.2 ± 18.3 | 129.2 ± 33.2 | 198.7 ± 51.3 | 269.9 ± 81.3 | 344.3± 109.9 | 421 ± 134.9 | 495.2 ± 172.3 | 579.4 ± 189.2 | 644 ± 213.8 | 710.8 ± 230.6 |
|  | **Buccal** | 49.8 ± 16 | 102.2 ± 32 | 150.2 ± 38.5 | 195.9 ± 44.6 | 241.7 ± 54.5 | 283.1 ± 70 | 330.9 ± 77.2 | 379.9 ± 86.7 | 427.3 ± 98.3 | 470.8 ± 103.8 |
|  | **Atmospheric air** | 453 ± 1.4 | 932.1 ± 1.6 | 1413.3 ± 3 | 1860.3 ± 5.9 | 2265.1 ± 5.6 | 2671.1 ± 8.2 | 3022.6 ± 3.3 | 3345.4 ± 4.7 | 3632.6 ± 12.3 | 3895.2 ± 5.4 |
| **2 mm** | **Occlusal** | 10.3 ± 5.2 | 21.2 ± 8.3 | 33.9 ± 12.3 | 50 ± 17.4 | 64.6 ± 21.1 | 80.4 ± 24.6 | 95.1 ± 28.9 | 113.1 ± 33.4 | 125.4 ± 38.6 | 140.2 ± 42.8 |
|  | **Oblique** | 12.7 ± 3.5 | 26.2 ± 7.6 | 40.6 ± 12.6 | 54.9 ± 19 | 69.5 ± 26.5 | 85.7 ± 35.5 | 102.3 ± 52.1 | 114.3 ± 57.9 | 122.7 ± 58.1 | 132.8 ± 52.7 |
|  | **Buccal** | 5 ± 3.3 | 10.6 ± 7.2 | 16.9 ± 11.5 | 23.8 ± 16.6 | 31.3 ± 22.2 | 39.3 ± 28.3 | 48.1 ± 33.7 | 56.3 ± 39.1 | 63.9 ± 43.4 | 71.1 ± 47 |
|  | **Atmospheric air** | 440.8 ± 0.3 | 905. ± 1.9 | 1384.6 ± 0.6 | 1805.8 ± 3.9 | 2229.4 ± 1.5 | 2605.3 ± 6.6 | 2949.7 ± 2.2 | 3291.2 ± 5.6 | 3571.6 ± 7 | 3815.5 ± 5.8 |
| **3 mm** | **Occlusal** | 3.1 ± 1 | 6.3 ± 2.2 | 9.3 ± 3.4 | 12 ± 4.8 | 15.9 ± 5.6 | 19.5 ± 6.8 | 22.8 ± 7.8 | 26.1 ± 8.4 | 29 ± 9.1 | 31.5 ± 9.9 |
|  | **Oblique** | 2.5 ± 1.7 | 5.2 ± 3.2 | 7.9 ± 4.6 | 10.3 ± 5.7 | 13 ± 6.7 | 19 ± 7.3 | 18.7 ± 8.2 | 22.4 ± 9.3 | 26.1 ± 11 | 30 ± 12.9 |
|  | **Buccal** | 1.1 ± 0.9 | 2.4 ± 1.9 | 3.8 ± 2.8 | 5.1 ± 3.7 | 6.4 ± 4.4 | 7.8 ± 4.9 | 9.1 ± 5.2 | 10.5 ± 5.5 | 11.7 ± 5.9 | 12.9 ± 6.2 |

**Supp. table 2:** Irradiance values recorded for 10 power outputs (left to right) for the light source; at 1, 2 & 3 mm distance between the light source and the sensor (n=3) (Mean +/- S.D.). Irradiances transmitted through occlusal, oblique, and buccal dentine; at 3 separate tissue thicknesses are shown.