

1 **Supplementary Material**

2 **Materials and Methods**

3 *The analysis of densities of GFAP, S100B, vimentin and collagen type IV by Image*

4 *J*

5 The distribution pattern of GFAP, S100B, vimentin and collagen type IV in each
6 region were analysed using the Plot Profile analysis tool (ImageJ version 1.41o; National
7 Institutes of Health). Ten random regions of interest (ROI) from at least five samples of each
8 group were analysed. (S. Fig. 1). The plot displays column average: the x-axis representing
9 the horizontal distance and y-axis representing the vertical average pixel intensity.

10 The protein framework was also analysed by calculating fractal dimensions (D_F) using
11 the box dimension method (D_B) to provide indication of how the fractions fill the space
12 completely is calculated based on the formula below:

13
$$\text{Fractal dimensions } (D_F) = \log N_\epsilon / \log \epsilon$$

14 When N = the number of parts and ϵ = scale

15 The fractal dimensions which define the “self-similarity” between parts of network to
16 the whole image in the particular area was calculated and plotted to the graph using a box
17 counting dimension method (D_B) according to the formula below:

18
$$(D_B) = \lim_{\epsilon \rightarrow 0} [\log N_\epsilon / \log \epsilon]$$

19 When N = the number of parts and ϵ = scale where the limit is found as the slope of
20 regression line (m). The slope of the regression line (m) and the correlation (r^2) were
21 calculated according to the formula below:

22
$$m = (n\sum SC - \sum S \sum C) / (n\sum S^2 - (\sum S)^2)$$

23
$$r^2 = [(n\sum SC - \sum S \sum C) / \sqrt{[(n\sum S^2 - (\sum S)^2)(n\sum C^2 - (\sum C)^2)}]]^2$$

24 When S = log of scale or size, C = log of count, n = number of sizes, m = slope of the
25 regression line and r^2 = the correlation for the regression line [Plotnick et al., 1993].

26 ***Fluorescence in-situ hybridization (FISH)***

27 Sections (Supplementary Table1) were treated with 0.2 M HCl for 10 min, washed
28 with de-ionised water and treated with 1% Triton X-100 for 90s and washed again with de-
29 ionised water [Nadkarni et al., 2010]. Digestion was performed at 37°C with proteinase K,
30 lysozyme and mutanolysin as described previously [Nadkarni et al., 2010], to facilitate
31 penetration of the probe into the bacteria. Sections were incubated in 25% formamide
32 overnight at 46°C [Nadkarni et al., 2010] using a Hybaid OmniSlide system (Hybaid)
33 followed by washing at 48°C using the Hybaid wash module. Finally, each slide was rinsed
34 with 2 rinses of ice-cold water and dried with cool air. Slides were cover-slipped using
35 ProLong Gold antifade reagent (Molecular Probes) and stored in the dark at 4°C [Charadram
36 et al., 2012].

37

38 **References**

- 39 Plotnick R, Gardner R, O' Neill R: Lacunarity indices as measures of landscape texture.
40 Landscape Ecology 1993;8:201–11.
- 41 Nadkarni MA, Simonian MR, Harty DW, Zoellner H, Jacques NA, Hunter N. Lactobacilli are
42 prominent in the initial stages of polymicrobial infection of dental pulp. J Clin Microbiol.
43 2010; 48:1732-40.
- 44 Charadram N, Farahani RM, Harty D, Rathsam C, Swain MV, Hunter N. Regulation of
45 reactionary dentin formation by odontoblasts in response to polymicrobial invasion of
46 dentin matrix. Bone. 2012; 50:265-75.

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48 **Legends:**

49 **Supplementary Table 1. FISH method**

50 **S Figure 1. *The example showing the ten random region of interest (ROI) from at least five***
51 ***samples of each group were analysed.*** The pixels and distribution pattern of GFAP in green,
52 in each region were analysed using the Plot Profile analysis tool (ImageJ version 1.41o;
53 National Institutes of Health, see S Methods). (Odontoblast nuclei in red).

54 **S Figure 2. Showing 3-D reconstruction images acquired by confocal laser microscopy for**
 55 **an advanced carious lesion. (A) to (C):** different angle of 3-D reconstructions using 40 µm
 56 frozen sections and viewed by confocal acquisition software, 40-50 images of 0.8 µm step
 57 size were acquired as a Z-stack by confocal laser microscopy (collagen type IV in green;
 58 S100B in red, while odontoblasts are shown in blue. OD: odontoblast; PD: pre-dentin; DT:
 59 dentin).

60
 61 **S Figure 3. FISH images of an advanced carious lesion.** Representative frozen section
 62 probed with universal bacterial FISH probe. (A) and (B): merged images showing no bacterial
 63 invasion of dental pulp. (C) and (D): merged images indicating positive reaction for bacteria
 64 (green color) only in dentinal tubules in dentin (DT). No bacteria were observed in dentinal
 65 tubules in dentin of healthy teeth (data not shown). Odontoblast (OD) nuclei stained with
 66 DAPI in blue, while differential interference contrast (DIC) images are shown in grey color.

67
 68
 69
 70 **Supplementary Table 1. FISH method**

71

Method	Healthy	Stage A	Stage B	Stage C ₂
FISH	2	4	6	5 ₇₃

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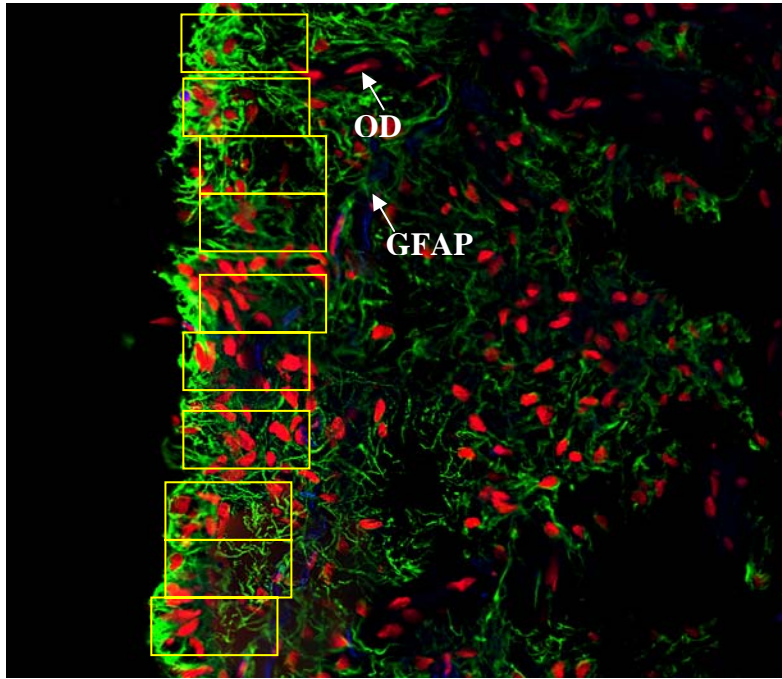
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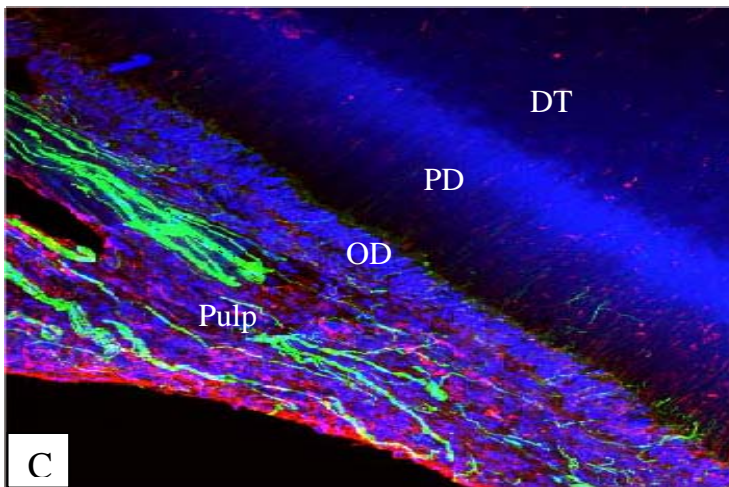
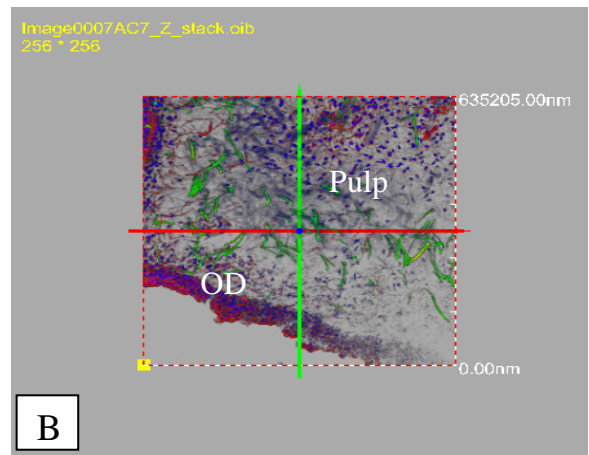
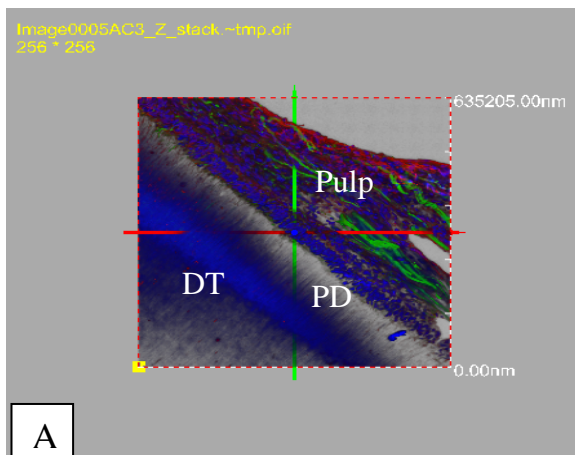
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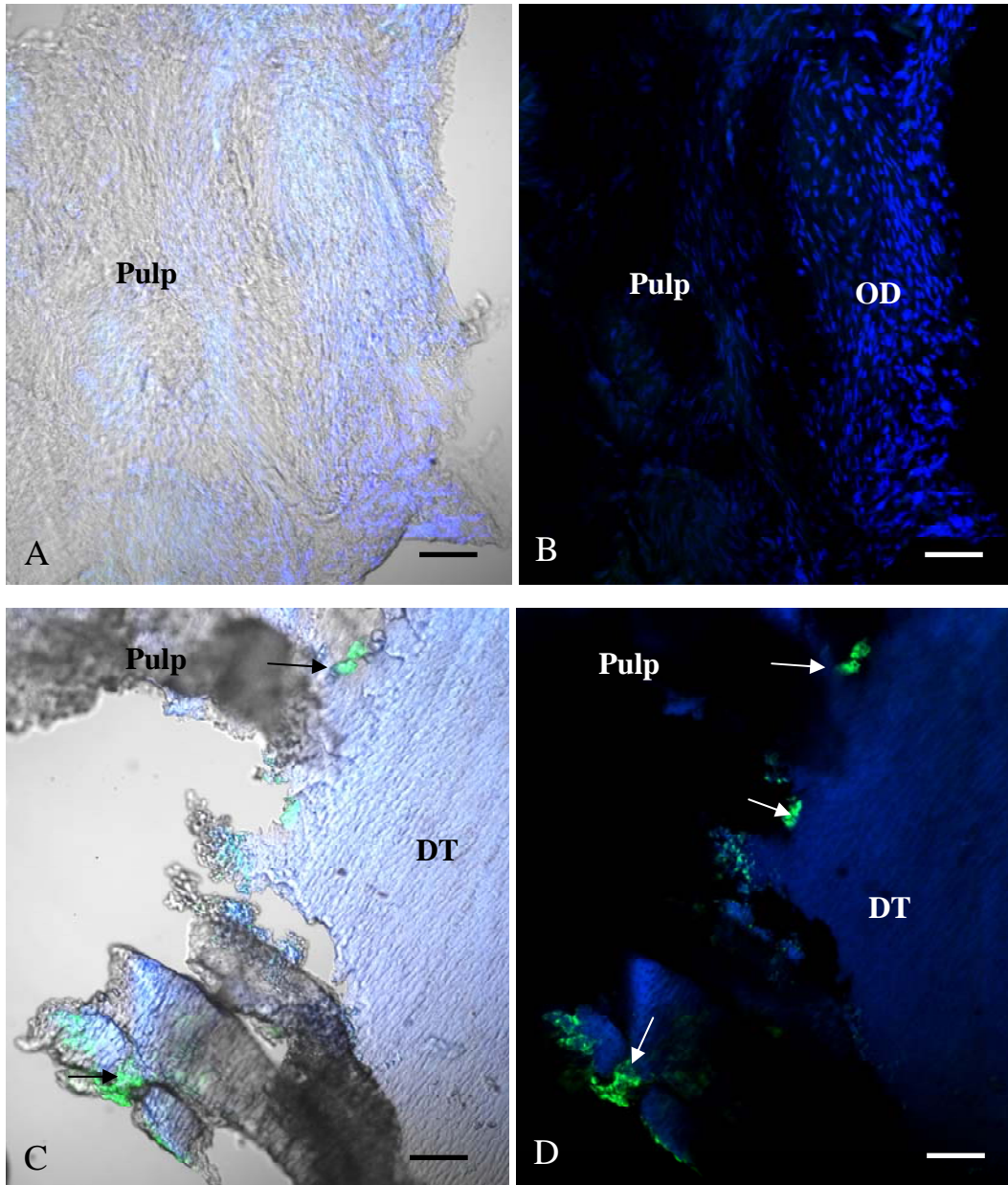


S Figure 1



S Figure 2

FISH



S Figure 3