Electronic Supplementary Material (ESI) for Lab on a Chip.

The tooth on-a-chip: a microphysiologic model system mimicking the biologic interface of the tooth with biomaterials

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Supplementary information

Dentin preparation

Third molars extracted for orthodontic reasons were mounted in cement and placed in an Accutom-5 (Struers, Copenhagen, Denmark) and cut into (500 µm W x 1 mm H x 4.5 mm L cut perpendicular to the dentin tubules) (**Supplementary Figure 1**) and then polished with a series of 1200, 2400 and 4000-grit abrasive paper, with the addition of distilled water. Fragment thickness was verified with a digital caliper, the adequate fragments were sterilized with UV light, and stored in sterile 0.25% sodium azide, at 4 °C until further use.

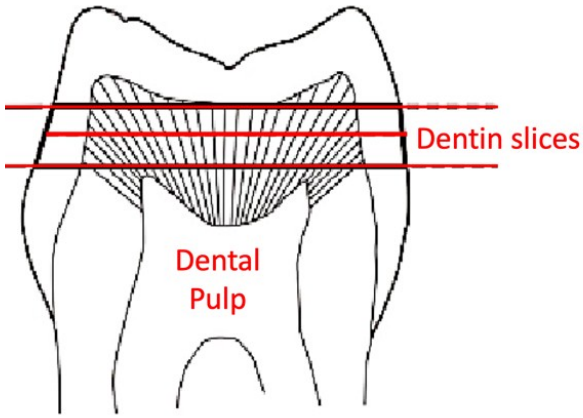

Figure S1: Schematic of dentin slice areas of choice.

Diffusion through the dentin tubules on-a-chip

The system was tested for leakage around the dentin fragments before use, and our experiments showed that even when the device was connected to a syringe pump and actively perfused, the coloured dye would penetrated through the dentin within seconds (**Supplementary Figure S2**). Also, in the absence of fluid flow, the dye could diffuse passively through dentin tubules in as little as 10 minutes (**Supplementary Figure S3**).

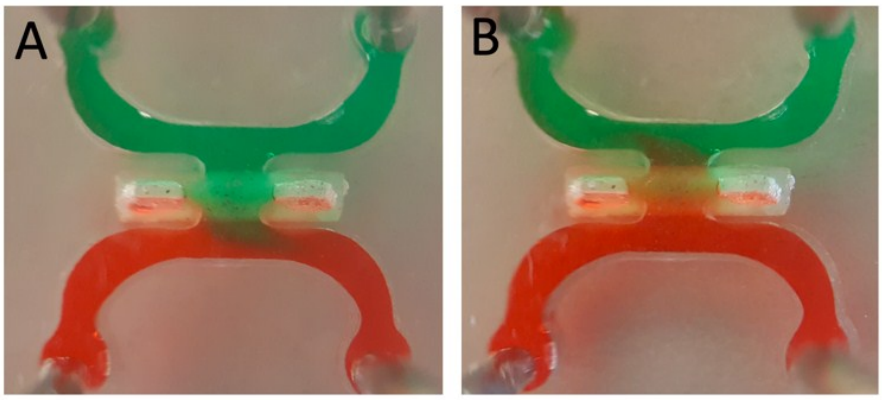

Figure S2: Forced diffusion of colored dye through dentin.

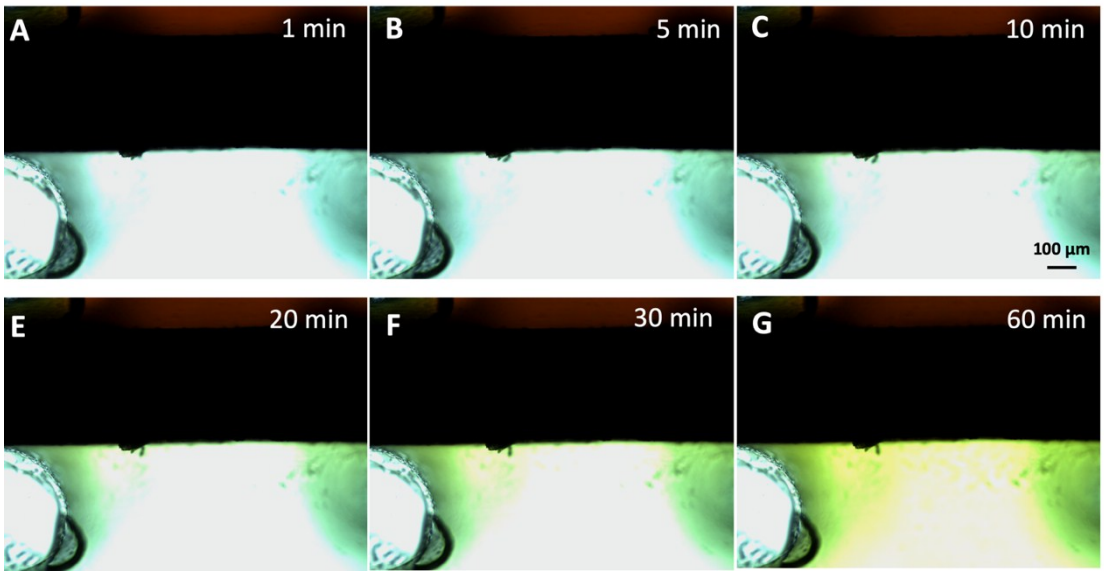

Figure S3: Passive diffusion of colored dye on-chip.

Cell culture

Stem cells from apical papilla (SCAPs) (Donation from Anibal Diogenes, University of Texas) were cultured in a Minimal Essential Medium Eagle, alpha modification (α MEM, Gibco, ThermoFisher Scientific, Waltham, USA) supplemented with L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich), and 10% embryonic stem cell fetal bovine serum (eFBS, ThermoFisher). Culture media was changed every 3 days. Cells were maintained in a humidified incubator (5% CO_2, 37°C). After reaching 80% of confluency, cells were treated with 0.05% trypsin and passaged to subsequent T75 culture plates. Only cells from passages 3 to 10 were used. Before use in experiments, cells were pre-differentiated for 7-10 days in

differentiation medium (10 nM dexamethasone, 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid) (all from Sigma–Aldrich) and 10% eFBS.

Each chip with dentin was treated with 17% ethylenediaminetetraacetic acid (EDTA) for 45 s to promote cell adhesion, and rinsed thoroughly. Next, 20 μL of a 10^5 SCAP ml^{-1} media suspension was seeded into the pulp chamber and incubated vertically for 1 h (37 °C, 100% humidity, 5% CO_2) for cell attachment onto the dentin walls. Next, the reservoirs were filled with differentiation medium.

During the optimization of the device we tried different cell concentrations to fabricate a consistent monolayer of cells adhered to the dentin in 24 h without cells attached to the glass. We tested different cell densities and methods to fabricate the monolayer, thus we learned that when chips are seeded with less than 10^5 cells/chip (5 x 10^6 cells/mL) few cells would attach to the glass forming a heterogeneous monolayer on dentin that would fully cover dentin only after 2-3 days. The minimum cell number to have a monolayer in 24 h with few cells covering partially the glass on the 'pulp chamber' was 10^5 cells/chip. At the beginning of our study, when we were including all cells in our calculations, the cell count results did not correspond to the cell loss we were seeing in the monolayer. So, we realized that the cells on the glass were interfering with the results. To overcome the influence of cells on glass, for the cell count analyzes we only considered cells located up to 60 μm away from the dentin as part of the monolayer. We chose this distance based on pilot studies on the monolayer formation where we could observe in the confocal microscope that cells attached to dentin had actin fibers oriented parallel to dentin while cells attached on the glass have actin fibers either perpendicular to dentin or in a random arrangement. To avoid an overestimated metabolic activity, we optimized our protocol to collect a 18 h baseline metabolic activity of the cells using Alamar Blue before the application of the dental materials. Afterward, we treated the dentin with dental products, 24 hours later rinsed with DPBS, and incubated cells with Alamar Blue every other day for one week. We normalized each chip according to the results of its own baseline, and this has minimized the variance that cells on the glass could potentially bring.

Immunofluorescence

On day 1 and 7, chips from each group (n=4) were rinsed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (v/v) for 1h, rinsed with PBS, permeabilized with 0.1% (w/v) Triton X-100 for 15 min under agitation. Unspecific biding sites were blocked with 1.5 % (w/v) bovine serum albumin (BSA) for 1 h. After washing with PBS, chips were incubated Actin Red 555 (cat. # R37112, Molecular Probes, ThermoFisher) for 1h, rinsed with PBS and incubated with NucBlue (cat. # R37606, Molecular Probes, ThermoFisher) for 30 min at 37 °C. Chips were imaged using a confocal microscope (Zeiss, LSM 880, Germany) with an objective of 20x (Zeiss, Plan-Apochromat 20x/0.8 M-27). The depth of imaging was 100-200

μm, split into at least 20 Z-stacks. Three-dimensional (XYZ) Z-stacks were converted into TIFF files using Zen or Imaris software (v9.1, Bitplane – Oxford Instruments, Zurich, Switzerland).

ISO cytotoxicity tests

To validate the chips as a microphysiological platform to test dental materials, the following samples were used: (a) 2-hydroxyethyl methacrylate (HEMA) (cat. # X9687044, Esstech, PA, USA) dissolved in cell culture medium (10 mM, 0.84% v/v), (b) 37% phosphoric acid gel (PA) (Ultradent Products Inc., South Jordan, UT, USA) dentin etching for 15 s and (c) 35% phosphoric acid dentin etching followed by Adper Single Bond 2 (SB) (cat. #51102, 3M/ESPE, St Paul, MN, USA) application according to the manufacturer recommendation. Briefly, dentin was acid-etched for 15s, rinsed 3 times with distilled water or until complete removal of the acid, dried with absorbent paper cone, then adhesive was applied and light-cured for 20s with a dental light (Valo Ultradent Products Inc, South Jordan, UT, USA). The materials were all introduced to the 'cavity side' of the dentin after, thus forming an interface akin to the dentin-pulp interface of a restored tooth (n=4). We then compared on-chip experiments against experiments performed using the International Organization for Standardization (ISO-10993-1) part 5 [18]. To that end, discs of Adper Single Bond 2 were prepared with 20 μL of the adhesive placed inside cylindrical molds of polydimethylsiloxane (PDMS) (4 mm diameter x 2 mm height) and light-cured for 10 seconds with a Valo Light (Valo Ultradent Products Inc, South Jordan, UT, USA) at a power density of 1650 mW/cm^2. To assure aseptic conditions, the discs were prepared inside a cell culture hood and measured with a digital caliper immediately after the cure were and then immersed in wells of a 24-well plate filled with 400 μL of SCAPS culture medium for 24 h to keep the same weight/volume proportion of adhesive and liquid as the chip and keeping the ISO recommended range of 0.5-6.0 cm^2/mL [18, 22]. After the 24 h, the elute was filtered with a 0.22 μm syringe (TPP, Darmstadt, Germany) and stored at 4 ^{o}C until use. For HEMA, cells were cultured for 24 h in a 10 mM solution dissolved in cell culture medium. To prepare the phosphoric acid group, 20 μL of the acid gel were dispensed onto a filter paper and immersed into 400 μL of SCAP cell culture medium for 15 s, next the elute was syringe-filtered and stored at 4 ^{o}C until use.

SCAPS were seeded in a 96-well plate (10^4 cells/well) in 200 μL of cell culture medium and after 24h, the culture medium was replaced with the Single Bond, HEMA and phosphoric acid extracts. Untreated cells cultured with SCAP medium served as controls. All groups had n = 6. Cells were incubated for 24h with the extracts and cell culture medium was replaced by regular SCAP medium, and cell cultures were followed for 7 days.

We measured the pH on the cell side before and after adding the phosphoric acid on the cavity side, and we found out that the average pH of the cell medium dropped from 6.5 to 4.5 in as

little as 30s (**Supplementary Figure 4**).

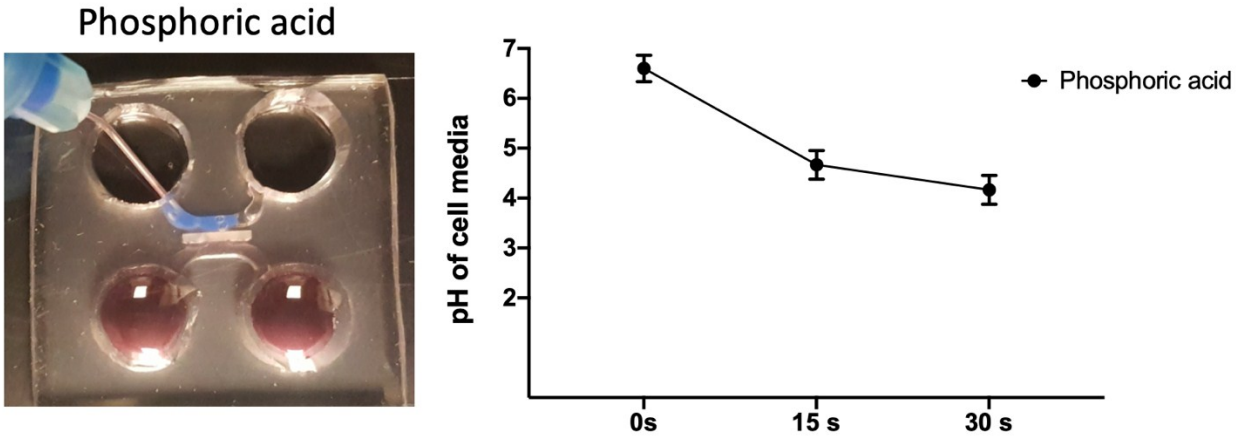

Figure S4: pH measurement of cell media on the pulp side before and after addition of phosphoric acid on the cavity side.

Inline metabolic activity assay with Alamar Blue

Briefly, 10% (v/v) Alamar Blue reagent (cat. # DAL1025, ThermoFisher) was added to the medium, and chips and 96-well plates were incubated for 18 h to allow viable cells to convert resazurin to resorufin. Subsequently, cell medium with Alamar Blue was collected and read using a microplate reader at 570 nm wavelength absorbance. For the Alamar Blue controls autoclaved Alamar Blue/SCAP medium solution was used as a 100% converted control (control A) and blank Alamar Blue/SCAP medium was used as a non-converted control (control B). To calculate the reduction of viability compared to the negative control, the following equation was used:

$$\%\, reduction\ of\ Alamar\ Blue = \frac{[(sample - control\ B) * 100\%]}{control\ A - control\ B}$$

Gelatinolytic activity of hybrid layer on-chip

To test the hypothesis that SCAPs may contribute to production of proteases and degradation of hybrid layer (HL) in the resin-dentin interface, we prepared the tooth on-a-chip with one group having a monolayer of SCAPs, (as described previously) and the other group with no cells. Dentin was etched for 15 s with 35% phosphoric acid gel, rinsed with continuous water irrigation, next, excess water inside the chamber was gently removed with paper cones in order to keep the cell culture medium on the opposite side of the dentin, where cells were seeded. Afterwards 7 µL of Adper Single Bond 2 adhesive labeled with 0.01% rhodamine-isothiocyanate was inserted on the etched dentin, then removed and reinserted in a back and forth movement simulating the two applications recommended in a clinical practice. After 20s

the chamber with cells was protected with a photomask and the adhesive was photocured for 20 s. DQ™ gelatin conjugated with fluorescein (EnzChek Gelatinase/Collagenase Assay Kit, cat # E-12055, ThermoFisher) was reconstituted in 1 mL of deionized water and the chip reservoirs were filled with 320 µL of cell culture medium and 80 µL of DQ™ gelatin. Chips were incubated at 37 ^{o}C, 5% CO2 and 100% humidity. Upon proteolytic digestion, fluorescein-labelled gelatin unquenches yielding highly fluorescent peptides that were live-imaged with a confocal microscope at 48 h using the same parameters describe above.

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