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# Decellularized Periodontal Ligament Cell Sheets with Recellularization Potential

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## APPENDIX

## MATERIALS & METHODS

### Confocal Imaging

The coverslips were fixed with a 4% paraformaldehyde solution at pH 7.4 (Sigma-Aldrich, Castle Hill, NSW, Australia) in phosphate-buffered saline (PBS) for 20 min and thereafter rinsed with PBS. The cells were then permeabilized for 5 min in Triton X-100 (0.2%) in PBS followed by 2 rinses in PBS. The samples were then incubated for 10 min in bovine serum albumin (BSA 1%, Sigma-Aldrich) in PBS. Solutions of primary IgG antibodies against human collagen I and fibronectin (Life Technologies, Invitrogen, Carlsbad, CA, USA) were diluted in BSA/PBS (1%); antibody solution was added to the designated coverslips and incubated for 45 min. Samples were rinsed with PBS and incubated for another 45 min protected from light in BSA (1%) in PBS containing fluorescently labeled secondary antibody Alexa 633 goat anti-mouse antibody (5 μg/mL, Alexa Fluor, Catalog #A-21126, Invitrogen), 4′,6-diamidino-2-phenylindole (DAPI, 5 μg/mL), and phalloidin–tetramethylrhodamine B isothiocyanate conjugate (Phalloidin-TRITC, 0.8 U/mL; Life Technologies, Invitrogen). We obtained controls for non-specific staining by omitting the primary antibodies. The coverslips were rinsed with PBS then mounted on a microscope slide with mounting medium (ProLong Gold, Invitrogen). Confocal imaging was done with a Leica SP5 microscope with excitation wavelength of 633 nm.

### DNA Content

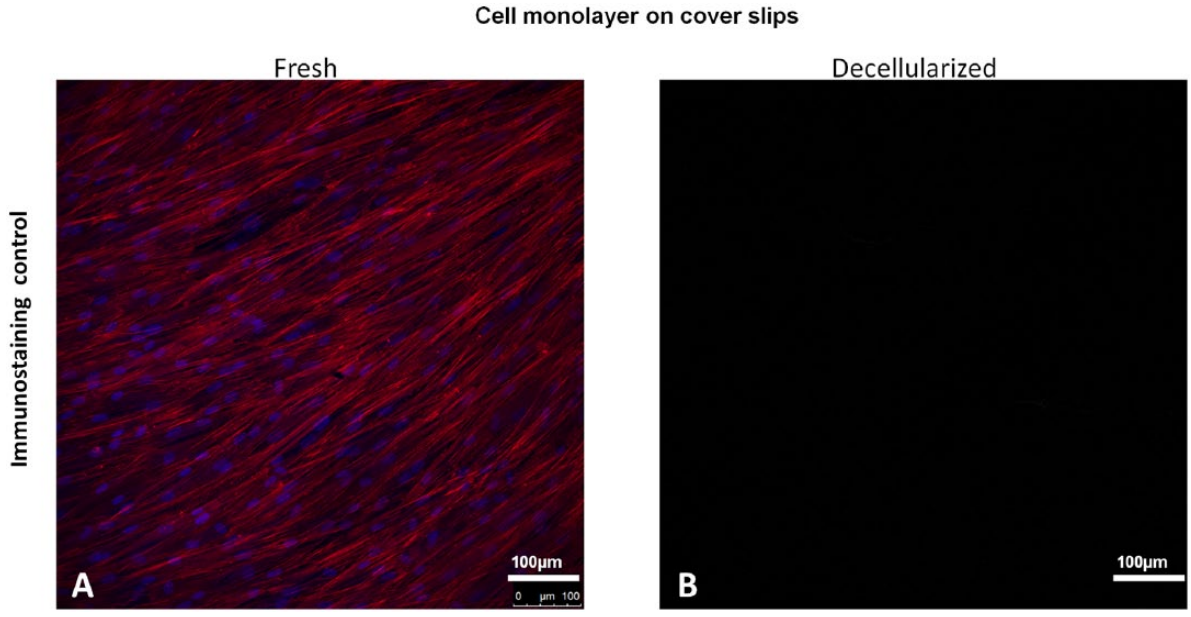
All samples were transferred to new sterile 24-well culture plates, sealed with parafilm, and placed at -80°C for 48 hr. The samples were thawed at room temperature, then transferred into Eppendorf tubes containing 300 μL of Proteinase K solution (Life Technologies, Invitrogen) diluted in PBE (1:50) for a final concentration of 0.5 mg/mL, then placed at 65ºC for 8 hr in a thermo-mixer (Eppendorf® Thermomixer®, BioExpress, Hamburg, Germany). Samples were diluted in phosphate-ethylene diamine tetraacetic acid buffer (PBE, 1:20). Standards were prepared with λ DNA standard (Quant–iT™ PicoGreen®, Life Technologies) by the dilution series method according to manufacturer's instructions, then plated in black 96-well plates (MicroWell™–NUNC™, Thermo Fisher Scientific). PicoGreen dye solution prepared according to the manufacturer's instructions (Quant–iT PicoGreen®), was added to each sample, protected from light, and incubated for 10 min. The fluorescence (excitation 485 nm, emission 520 nm) was measured by means of a fluorescence plate reader (BMG POLARstar®, Ottenberg, Germany). The standard curve of λ DNA was used to calculate the final DNA content of the samples.

### Scanning Electron Microscopy

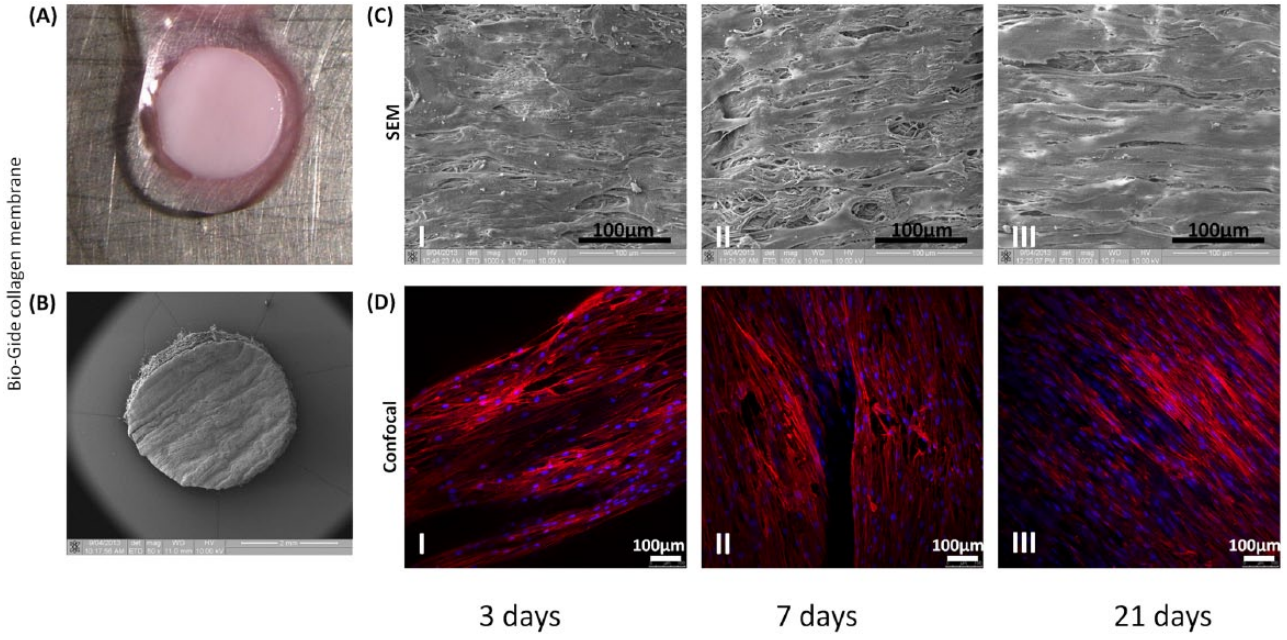
Fresh and decellularized cell sheets were fixed in glutaraldehyde (3%) for 1 day, then gradually dehydrated in gradient concentrations of ethanol, after which osmium tetroxide (Sigma-Aldrich) was utilized as a post-fixation agent for 60 min. The samples were dried overnight, subsequently mounted on adhesive stubs with cell sheets facing upward, and finally gold-coated under vacuum for 3 min. SEM imaging was performed utilizing a FEI Quanta 200 microscope operating at 10 kV with a working distance range of 11 to 15 mm.

### Cell-sheet Harvesting

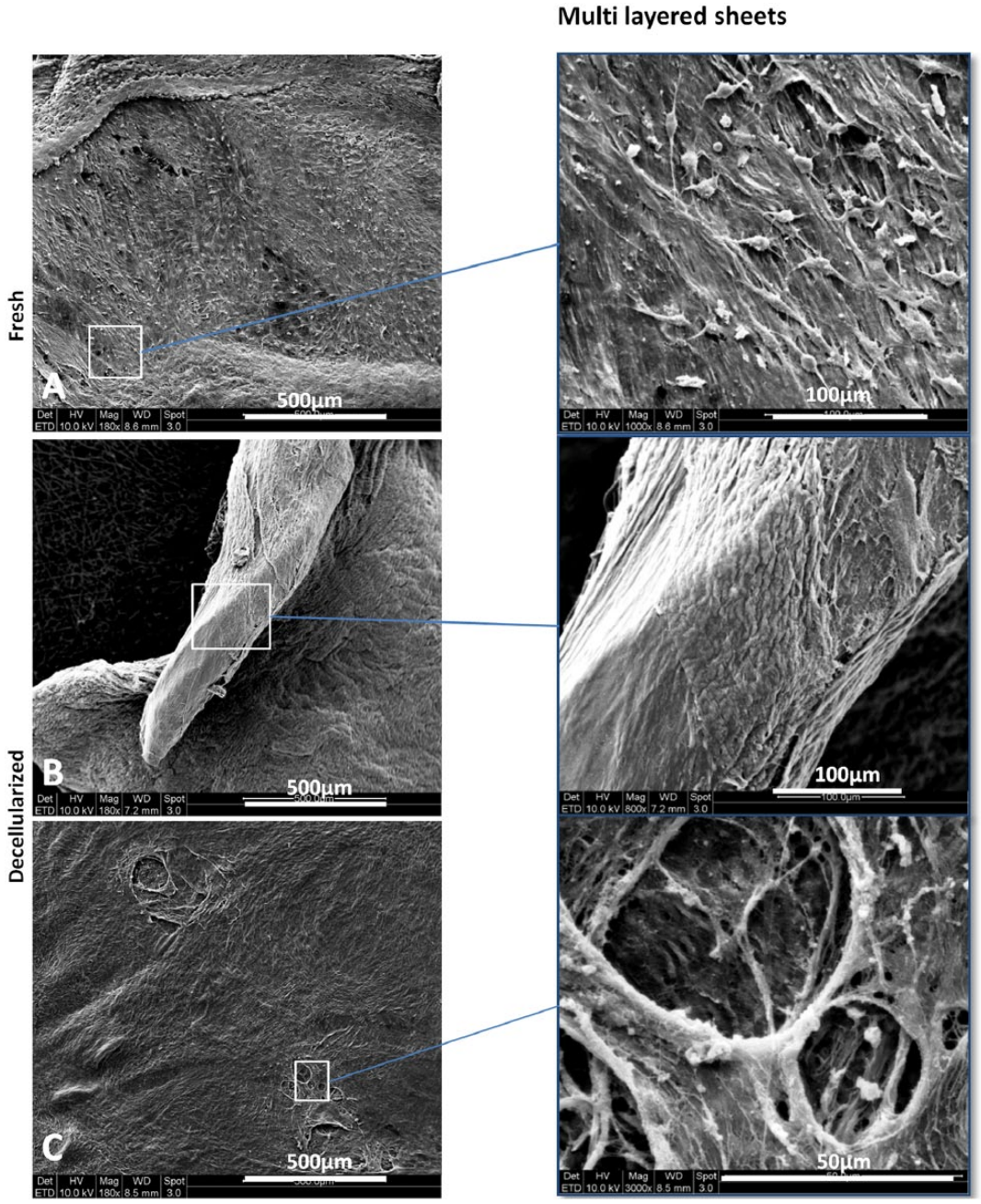
For cell-sheet harvesting, the PCL scaffold was positioned in the center of the 24-well plate, and the borders of the cell sheet were gently detached from the base of the well and pulled toward the edges of the scaffold by means of sterile fine-curved tweezers. This secured the cell sheet onto the PCL membrane, and the scaffold-cell sheet was placed into a 24-well plate with the cell sheets facing upward. To allow for cell adhesion of the cell sheet onto the membrane, the construct was further incubated for 4 hr. To prevent the drying of the cell sheet, a 25-μL quantity of medium was placed on the cell sheet. At the end of the adhesion period, 1 mL of medium was added to the well, and the cell sheets on the PCL scaffold were further incubated for 24 hr. For comparison with the PCL-single cell sheet construct, a multi-layered cell sheet consisting of 4 cell sheets was also prepared.




**Appendix Figure 1.** Secondary antibody control for immunostaining. **(A, B)** Nuclei (DAPI) in blue; actin filaments (phalloidin) in red.




**Appendix Figure 2.** Seeding of hPDL cells on Bio-Gide® collagen membrane over 3, 7, and 21 days. **(A)** Bio-Gide® membrane of 5 mm diameter. **(B)** SEM of membrane with no hPDL cells. **(C)** SEM showing hPDL cells on membrane at different time points. **(D)** Confocal imaging of hPDL cells showing nuclei (blue) and actin filaments (red).




**Appendix Figure 3.** SEM of multi-layered (4 layers) hPDL sheets. **(A-C)** hPDL multi-layered cell sheets before and after decellularization.

## Collagen Quantification

The samples were moved to a sterile 1.5-mL vial, and 100 µL of distilled water was added. The samples were sonicated for 30 sec at 3-second pulse and 1-second rest, with 50% amplitude. A 100-µL quantity of 10 N HCL was added *per* sample and incubated for 24 hr at 120ºC. The samples were left to cool and then were transferred to new microcentrifuge tubes and spun at 10,000 rpm for 3 min. Standards were prepared with serial dilutions. Samples were used undiluted. A 10-µL quantity of 10X Chloramine T solution and 90-µL of solution A were added to each sample in a 96-well plate, where samples and standards were plated in duplicate, then incubated for 20 min. A 50-µL quantity of 2X DMAB solution plus 50 µL of solution B was added to each well and further incubated for 30 min at 60ºC. Plates were read by means of a plate reader (BMG POLARstar®). Results were formulated according to equations provided in the manufacturer's protocol.