IMAGE ANALYSIS OF BONE CHIPS GENERATED FROM OSTEOGENIC HUMAN PERIOSTEAL CELLS AND BIODEGRADABLE MATRIX
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Introduction. Here we present confocal laser microscopy (CLM) analysis of a bioimplant (bone chip), consisting of osteogenic cells seeded onto biodegradable matrix, to be used for the treatment of residual bone defects, such as those occurring after the surgical removal of the radicular bone cyst of the upper jaw. Current treatment regimes for the reconstruction or improvement of function of damaged bone rely on autologous or allogenic tissue grafts. Tissue engineering is a new promising treatment, as it eliminates the problems of donor site morbidity of autologous grafts and the immunogenicity of allogenic grafts. This strategy involves seeding of autologous osteogenic cells in vitro onto biodegradable scaffolds. The cambium layer of the periosteum contains osteogenic and chondrogenic progenitor cells at different stages of differentiation. A small piece of periosteum biopsy contains a sufficient pool of cells, which were first proliferated in vitro, than seeded onto suitable biodegradable scaffolds to be finally transplanted back into the site of the defect. To promote the new tissue formation and to stimulate the bone healing response a sufficient number of osteogenic cells must be introduced at the site of bone defect and the cells should be adequately distributed throughout the grafted volume. The assessment of initial seeding cell density, cell viability and cell distribution is essential in the preparation of the in vitro engineered tissue. To meet this end, we here utilized confocal laser microscopy.

Methods. We have used a CLM based approach to evaluate the in vitro growth of cells derived from human periosteum for the development of tissue-engineered bone chips. A biopsy of local periosteal tissue (periosteal sheet dimensions were 5 mm x 10 mm) was taken with patients consent after treating a dentogenic radicular cyst of the upper jaw (approved by the Ethical Commission of the Republic of Slovenia). After enzymatic digestion, cells (a few hundred) were proliferated in three passages of monolayer cell conditions, yielding 20-30 million cells. Cell suspension was seeded into biodegradable matrix (0.6 x 4 x 4 mm). Fibrin sealant was used to encapsulate the cell-biodegradable matrix assembly (the bone chip), which was then cultured for a week in cell culture medium supplemented with osteogenic differentiation factors.

For CLM analysis bone chips were stained with discriminative vital fluorescent dyes Calcein AM and Ethidium homodimer. Cells with intact membranes display green fluorescence and nuclei of the dead cells are stained with red fluorescence.

CLM acquires a series of optical sections of the object, which allows three-dimensional analysis of the bone chips such as the estimation of the cell seeding density, viability within the bone chip and the estimation of cell proliferation. The fibres of the biodegradable matrix optically obstruct the emission of fluorescence, therefore we monitored only cells in the surrounding fibrin glue.
Results and Discussion. A custom software tool ParticleCO was developed to count particles in three-dimensional stacks of optical images. However, it can also be used in any other imaging application in which the counting of particles in a stack of images is of interest. ParticleCO is a menu driven program. It represents a powerful aid for rapid counting of particles in images and it provides tools to visualize results in 3D charts (see Figure). Optical sections of a bone chip were taken at successive optical planes with the Z-axis interval 5.5 μm. Live and dead cells in each optical slice were counted. Cell number and the position of each cell in a stack were stored. From the 3D imaging frame size and the number of cells in a stack, we determined the cell density in a certain volume of fibrin glue. The 3D display of cell coordinates shows a homogenous seeding density in stacks taken from different regions of fibrin glue surrounding the biodegradable matrix (Figure B). We have counted the number of cells seeded onto the biodegradable matrix (0.6 x 4 x 4 mm) to be 15 000-20 000. Cell viability on day 1 after seeding was 75%-90%. Following the incubation period in the differentiation factors supplemented culture medium the cells were positive for alkaline phosphatase staining, an early but not specific marker of osteogenic differentiation and after 2-3 weeks for osteocalcin (by immunostaining), a specific marker of the mature osteoblast phenotype. After 3 weeks cells stained positively for bone mineral deposition by von Kossa staining method. These results show that the bone chips represent a suitable tissue-engineered product to be used in surgical filling of the medium size radicular bone cysts.

![Figure](image-url)

**Figure. A:** Confocal laser scanning micrograph of the tissue-engineered bone chip. Optical sections were taken at successive optical planes on the day 1 post-seeding. Sections were then superimposed to get an extended focus view (left). The y-z confocal section is displayed on the right, which corresponds to the perpendicular section marked by the vertical line on the left image. Live cells appear bigger and are stained green, the nuclei of dead cells appear smaller and are stained red. Note that on post-seeding day 1 cells display a round shape. Scale bar: 100 μm.

**B:** A 3D scatter diagram showing cell distribution and viability on day 1 post-seeding of cells onto the biodegradable matrix (the view represents a small section of the whole bone chip). Larger light (green) circular marks represent live cells and smaller dark (red) marks represent dead cells. Stack size: x = 1303 μm, y = 1303 μm, z = 205.5 μm. Note that x-axis was stretched by a factor of 6 to allow a clear view of the cell distribution in 3D. In this 3D image 350 live cells were counted of all 15 000-20 000 cells in the whole bone chip.