

## Viability in *ex vivo* cultured cancellous bone

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**Background and Introduction:** Biocompatibility studies are carried out either in 2D monolayer culture or in animal studies. Three dimensional bone organ cultures are required to reduce the number of animal studies performed, while simultaneously ensuring a more natural environment than that provided by monolayer culture of isolated cells. Due to the highly impervious nature of the calcified bone matrix and fatty marrow it is relatively difficult to ensure adequate supply of soluble factors to the central regions of the explant. The reduced rate of mass transfer to the central parts of the explant also produces an extra complication when using standard viability techniques, with the central regions often remaining completely unstained. A number of viability assays are toxic and the longer incubation time required for maximal penetration can cause complications by killing the external layers of cells. Radioactive labelling of viable cells is routinely performed by the addition of 25  $\mu\text{Ci}/\text{ml}$   $^3\text{H}$ -glycine into the culture medium for 24 hours. This method of viability assessment however does have some major drawbacks.  $^3\text{H}$  is a weak  $\beta$ -emitter, resulting in long exposure times. Total assay time can be over five weeks, when including time for embedding the sample in resin and sectioning. Therefore we have developed improvements for various viability assays for their suitability in assessing the viability of 3D bone explants.

**Methods:** Cancellous bone cores 10 mm in diameter and 5 mm high were prepared from human femoral heads or ovine distal femurs. Assays were performed on either whole cores or 250  $\mu\text{m}$  sections cut from fresh unfixed cores.

**MTT-** Whole bone cores were incubated with 10 ml of Thiazolyl Blue Tetrazolium Bromide solution (MTT-5 mg/ml in DMEM + 10% FCS) at 37°C for 8 hours or at 4°C for 5 hours followed by 37°C for 3 hours. Alternatively 250 $\mu\text{m}$  sections were incubated with 500  $\mu\text{l}$  MTT solution for 4 hours in a humidified 37°C environment.

**Live/Dead-** Whole bone cores were incubated with 10 ml staining solution (25 mM cell tracker green (live), 1  $\mu\text{g}/\text{ml}$  ethidium homodimer 1 (dead) in DMEM) at 37°C for 6 hours. All incubations were carried out in the dark. After incubation, the cores

were washed in PBS. The cores were then processed, embedded in methylmethacrylate (MMA) and 6  $\mu\text{m}$  sections prepared.

**Radiolabelling of Bone Cores -** Whole cores were incubated with 2ml of DMEM containing 10% FCS and 25  $\mu\text{Ci}$  of  $^3\text{H}$ -glycine for 24 hours at 37°C. The cores were dehydrated and embedded in MMA. Sections were cut and incubated with emulsion at 4°C for 3 weeks. The slides were developed, fixed and counterstained.

**LDH-** Bone explants were sectioned to 250 $\mu\text{m}$ . The sections were incubated in 500 $\mu\text{l}$  reaction medium (5% Polysep, 2 mM Gly- Gly, 0.75% NaCl, 60mM lactic acid, 1.75 mg/ml NAD, 0.3mg/ml Nitroblue Tetrazolium (NBT) pH8) for 4 hours at 37°C. Sections were then washed with warm (50°C) water, rinsed with PBS and fixed with 4% formaldehyde at 4°C.

Samples were imaged with a Zeiss photomicroscope (Axioplan Imaging) and processed with AxioCam and Axiovision software.

**Results:** MTT staining was temperature dependant. In cores incubated entirely at 37 °C, a ring of viability was seen around the outside of the core, while the central region remained unstained. This showed that the outside cells were actively using all the MTT. Pre-cooling cores and incubating at 4°C to reduce cell activity, prior to transferring to 37 °C lead to a uniform staining throughout the core. MTT staining of 250  $\mu\text{m}$  sections demonstrated punctate staining within the osteocytes, often appearing as a ring (Fig 1).

The cells stained evenly throughout the sections, with central areas also staining as viable. Due to the thickness of the section, marrow integrity was well maintained and marrow cells produced a more diffuse type of staining (M). Fluorescent live dead staining was problematic both due to dye penetration problems and a low signal to noise ratio caused by the autofluorescence of the bone. It was also demonstrated that on whole cores neither dye penetrated more than 500  $\mu\text{m}$ , leaving the central parts of the core unstained. Radio labelling with  $^3\text{H}$  Glycine lead to stained osteocytes throughout the core. This method has the disadvantage of the inherent problems with radioactivity and the fact that total processing time is over 4 weeks.

LDH staining of 250  $\mu\text{m}$  sections led to excellent marrow structure preservation and uniform staining of marrow and osteocytes throughout the core (Fig. 2a). Exciting the bone with UV light caused autofluorescence which highlighted the stained live osteocytes as dark dots (Fig. 2 b). As no embedding in resin is required, and the LDH assay only requires 4 hours incubation, viability can be assessed within 8 hours of harvesting the sample.

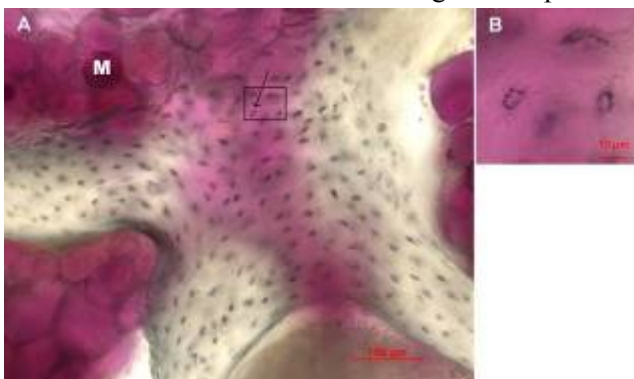


Fig. 1. Sections (250  $\mu\text{m}$  thick) were cut from unfixed, undecalcified ovine cancellous bone cores and incubated with MTT for 4h. Punctate staining could be seen within the osteocytes, often appearing as a ring (arrowhead- magnified in B). The cells stained evenly throughout the sections, with central areas also staining as viable. Due to the thickness of the section, marrow integrity was well maintained and marrow cells produced a more diffuse type of staining (M).

**Discussion and Conclusion:** Taken together these results of non-radioactive viability methods indicate that viability assessment of whole cancellous bone cores leads to artefacts which are caused by poor diffusion. All these assays are routinely used in 2D cell culture systems, yet each required modifications to be suitable for use with cancellous bone. These artefacts can be overcome by preparing 250 $\mu\text{m}$  thick fresh sections prior to application of the assays. Fluorescent live/dead staining had additional complications caused by the autofluorescence of the bone generating a high signal to noise ratio, making assessment of osteocyte viability impossible. MTT staining was difficult to interpret due to the punctate nature of the stain.

We found that lactate dehydrogenase staining of 250  $\mu\text{m}$  thick unfixed sections led to excellent viability determination of osteocytes within the mineralised matrix. It also maintained marrow organisation. Decreasing the viscosity of the LDH assay solution used in published methods led to a improved penetration into the calcified matrix. Quantification of thick sections is aided by using the autofluorescence of the bone to highlight the darkly stained osteocytes against the fluorescing

bone. The optimisation of viability methods is an extremely valuable tool in biomaterial assessment in explant cultures of bone and should also be of use for tissue engineering studies. As the LDH assay has few handling steps, data is obtained rapidly.

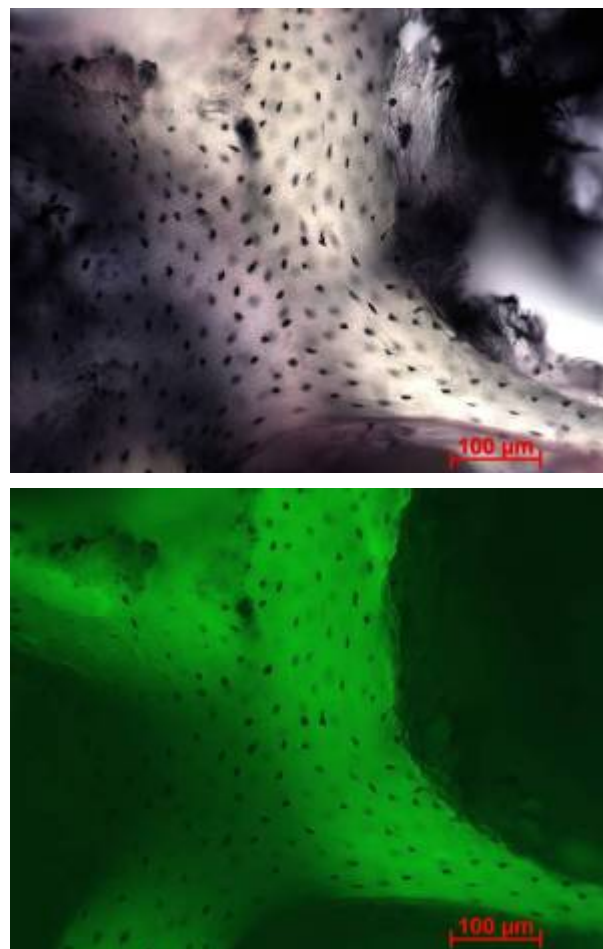


Fig.2. Sections of human cancellous bone (250  $\mu\text{m}$  thick) were stained for LDH activity for 4h. Sections were mounted using a water based mountant and viewed immediately. The brightfield image shows the presence of numerous, darkly staining osteocytes (A). The corresponding image using the autofluorescence of the bone (515-565nm emission filter) enhances the contrast, leading to clearer definition of viable cells (B).

**ACKNOWLEDGEMENTS:** This work has been funded by 3R #7801 and ESA MAP AO99-122. Thanks to Dr. Thomas Perren (Davos Hospital), Dr. Heinz Bereiter (Chur Hospital) for supplying human tissue.