One of the more-serious side effects of extended space flight is an accelerated bone loss [Bioastronautics Critical Path Roadmap, http://research.hq.nasa.gov/code_u/bcpr/index.cfm]. Rates of bone loss are highest in the weight-bearing bones of the hip and spine regions, and the average rate of bone loss as measured by bone mineral density measurements is around 1.2% per month for persons in a microgravity environment [T. Lang et al., JBMR 2004]. Figure 1 shows that an extrapolation of the microgravity-induced bone loss rates to longer time scales, such as a 2.5 year round-trip to Mars (6 months out at 0 g, 1.5 year stay on Mars at 0.38 g, 6 months back at 0 g), could severely compromise the skeletal system of such a person.

![Figure 1. Age-related bone loss in a 1g population of males (data from Atlas of Clinical Endocrinology: Osteoporosis, 2003) compared to a hypothetical person exposed to microgravity and partial gravity during a 2.5 year Mars trip. The model assumes a linear response of bone loss with g-level, and does not account for the possibility of new bone growth upon returning to 1 g, as no data yet exists for such an effect.](image)

It is well known that bone remodeling responds to mechanical forces. We are developing two-photon microscopy techniques to study bone tissue and bone cell cultures to better understand the fundamental response mechanism in bone remodeling. Osteoblast and osteoclast cell cultures are being studied, and the goal is to use molecular biology techniques in conjunction with Fluorescence Lifetime Imaging Microscopy (FLIM) to study the physiology of in-vitro cell cultures in response to various stimuli, such as fluid flow induced shear stress and mechanical stress. We have constructed a two-photon fluorescence microscope for these studies, and are currently incorporating FLIM detection. Current progress will be reviewed. This work is supported by the NASA John Glenn Biomedical Engineering Consortium.

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Biophotonics and Bone Biology

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Goal: Develop advanced fluorescence microscopy techniques to study bone cell physiology

Motivation:

- Cells cultured in microgravity exhibit different gene expression profiles.
- Cytoskeleton in space-based osteoblast cell cultures is less well-developed.
- T-cell lymphocyte (immune cells) activation is suppressed in microgravity

Microgravity has a harmful effect on human physiology

- Bone loss in hips and spine, 1% per month
- Immunodeficiency
- Loss of blood plasma, anemia
- Cardiac dysrhythmia

Like an accelerated osteoporosis

Ref. document: Bioastronautics Critical Path Roadmap
Normal trabecular (spongy bone) structures

Osteoporotic trabecular structures

Bone mineral density (BMD) loss: Effect of aging


Microgravity BMD loss: 1.2% per month (Lang et al., JBMR 2004)

Model a trip to Mars:

\[
\frac{d}{dt} BMD = \frac{d}{dt} BMD_{\text{aging}} + \frac{d}{dt} BMD_{\text{g-level}}
\]

\[
\frac{d}{dt} BMD_{\text{g-level}} = \frac{0.012 \cdot BMD}{\text{mo.}} (g^* - 1) \quad \text{Linear response model}
\]

\[
g^* = \frac{\text{local accel.}}{9.8 \text{ m/s}^2}
\]

Calculate

\[
BMD(t) = BMD(t_0) + \int_{t_0}^{t} \frac{d}{dt} BMD \, dt
\]
Bone loss
Hypothetical effects of aging and modeled trip to Mars

Mars trip: 6 mo. out (0g), 18 mo. stay (0.38g), 6 mo. return (0g)
Bone Remodeling: Balance between osteoclasts and osteoblasts

Use two-photon fluorescence microscopy to study macrophage, osteoclast and osteoblast cells
Background: Two-photon absorption

Single-photon absorption

488 nm

fluorescence

535 nm

Fluorescence intensity $\sim I_0$

820 nm

Two-photon absorption

820 nm

fluorescence

535 nm

Fluorescence intensity $\sim I_0^2$

Excitation rate (photons/s), $\phi$

1 - photon

$\phi_{1p} \approx 4P_0\eta_{1p}\sigma_{1p}\frac{(NA)^2}{hc\lambda}$

2 - photon, pulsed laser

$\phi_{2p} \approx 8\langle P_0 \rangle^2 \frac{\eta_{2p}\sigma_{2p}(NA)^4}{\tau_pf_p(hc\lambda)^2}$

$\left(\tau_pf_p\right)^{-1} \approx 10^5$

$\frac{\phi_{2p}}{\phi_{1p}} \approx 5 \cdot 10^{-4} / mW$
Advantages of two-photon excitation:

Fluorescence excitation is limited to the focal volume
  - confocal-like performance, but no need for pinhole in detection optics,
  - less photobleaching
  - improved contrast

Longer wavelength excitation
  - reduced Rayleigh scattering \((1/\lambda^4)\),
  - better depth penetration
  - less absorption/damage in tissue;
  - biological “optical window”
  - larger spectral gap in excitation/emission spectra

Disadvantages of two-photon:

- Large, expensive laser:
  - complete two-photon systems available commercially for $500k-$700k
- Slightly lower resolution due to longer excitation wavelength
Two-photon microscopy layout

M: mirror
S: shutter
f: filter
L: lens
D: dichroic
GM: galvonmeter mirrors (2)
PMT: photomultiplier tube
Fine tuning the optical alignment:

Scan a fluorescent lake sample (e.g., fluorescein in methanol), align scanhead, scope.

Point-spread-function measurement:
scan 0.093 µm diameter fluorescent microspheres in x,y,z

- Better alignment
- One-color representation

FWHM = 0.32 µm

FWHM = 0.72 µm
Add micro-incubator for 37 °C, 5% CO₂ control:

CHO cells expressing YFP;
Time lapse: 2 minutes/frame

Cells provided by Prof. Gabor Forgacs, U. Missouri and Dr. Rusty Lansford, CalTech

Mouse kidney section: z- scan
Application: Imaging bone tissue

Sample provided by: Melissa Knothe Tate, Cleveland Clinic Foundation

Human femur

Section from femoral head stained with basic fuschin and embedded in PMMA
Bone section, 12 µm depth, $\lambda_{\text{ex.}}: 810$ nm

435-485 nm
Autofluorescence
Mineralized matrix?

590-640 nm
Basic fuschin stain

640-700 nm
Autofluorescence
Collagen matrix
Confocal (top, 568 nm) versus Two-photon (bottom, 910 nm)

Cortical bone (femur)

20 micron depth
50 micron depth
70 micron depth
Producing osteoclasts in-vitro:

- Macrophage
- + RANKL
- Osteoclast precursor
- Macrophage
- Multinucleated osteoclast

TRAP+ (with RANKL treatment)

TRAP- (no RANKL treatment)
Fluorescence Lifetime Imaging Microscopy (FLIM)
- Presently adding FLIM capability to the microscope

Fura-2 dye (Calcium indicator), MC3T3 cells

Intensity is a function of many variables
(dye and Ca conc., excitation/collection efficiency)

- OK for qualitative imaging
- Quantitative data is possible but difficult

Example FLIM image: Elson et al., Optics and Photonics News, Nov ‘02

Fluorescence lifetime \( \tau = \frac{1}{\Gamma + k} \)
- \( \Gamma \), radiative decay rate
- \( k \), non-radiative decay rate

\( k = k(pH, Ca^{++}, \text{viscosity, membrane potential}) \)

- Provides quantitative data regarding cell physiology
Summary

- We are applying two-photon fluorescence microscopy techniques to the study of bone tissue and bone cell biology

- Ultimate goal is to understand bone loss in microgravity

- FLIM/FCS/protein expression will be used to study effects of fluid flow, acoustic vibrations, electro-mechanical forces on bone cells

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