

Detection of Hsp 70 expression in linkage with chondrocyte regeneration using confocal microscopy following Nd: YAG ($\lambda = 1.32\text{mm}$) laser irradiation of cartilage

Introduction

During embryonic development, chondrocytes differentiate from mesenchymal progenitor cells. In the mature organism, terminally differentiated chondrocytes do not undergo mitotic division and exhibit minimal metabolic functions consisting mainly of maintaining the extra-cellular matrix surrounding their lacunae. When cartilage is damaged, its inherently low mitotic activity and lack of perfusion limit any potential for adequate repair or regeneration. Because cartilage makes up the dynamic load bearing and smooth articulation functions of human joints, irreparable damage caused by trauma or degenerative arthritis and joint diseases lead to significant structural defects and medical complications. Hence, the development of methods that could potentially induce regeneration and proliferation in senescent chondrocytes would be of substantial value.

In previous studies, proliferating chondrocytes were identified on the edge of the laser-irradiated regions in cartilage tissue using flow cytometry and whole mount double antibody system (Nitro blue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate NBT/BCIP antibody staining) [1]. For these studies, tissue samples were placed in culture with media containing 5-Bromo-2'-deoxyuridine (BrdU), a brominated analog of thymidine that is selectively incorporated into DNA during S-phase of the cell cycle. Subsequently, fluorescent linked antibodies against BrdU were used to label proliferating chondrocytes for analysis using flow cytometry while NBT/BCIP staining was used to detect BrdU in the whole mount (Figure 1). However, limitations in these methods prevent observation of the proliferating chondrocytes in their native state as residents of the extra-cellular matrix (ECM) in cartilage. Flow cytometry necessitates the reduction of the cartilage tissue to its cellular components by digestion of the ECM in order to detect FITC (Fluorescein Isothiocyanate): fluorescein tagged BrdU incorporated cells. Consequently, this method omits information regarding the expression of the chondrocyte's phenotypic metabolism characterized by the proteins, or the products of chondrocyte growth, that form the ECM.

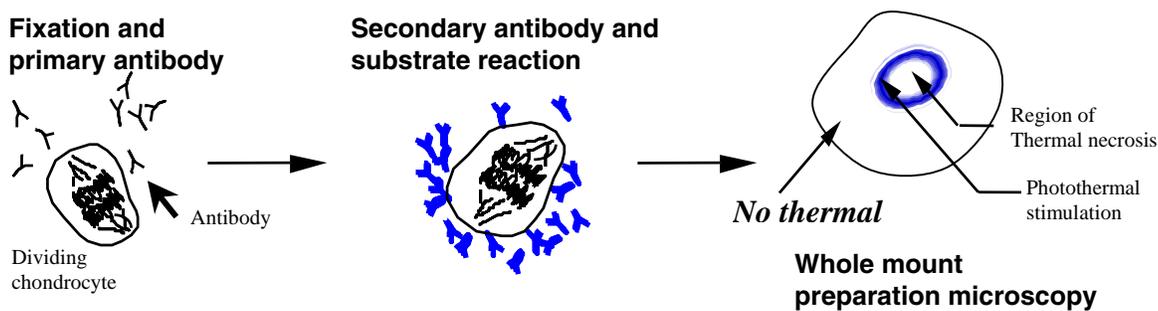


Figure 1: A double antibody system (NBT/BCIP) is used to label BrdU incorporated during cell division. An enzyme-linked dye is then used to stain proliferating cells, which show up as a blue ring on whole mount specimens.

Also, flow cytometry cannot provide any detail with respect to the spatial distribution of thermal damage/modification within the laser-irradiated specimen. Similarly, whole mount antibody staining provides only limited information with respect to the extent of regions where proliferating cells exist surrounding the irradiated spot (Figure 2). While BrdU antibody systems allow the detection of DNA synthesis in chondrocytes, they do not provide information with respect to the regulatory cascades triggered by laser-generated heat in cartilage that may be responsible to the observed regenerative process. The next steps required to study the photothermal activation of chondrocyte growth need to identify which specific cellular, matrix, or molecular mechanisms are triggered.

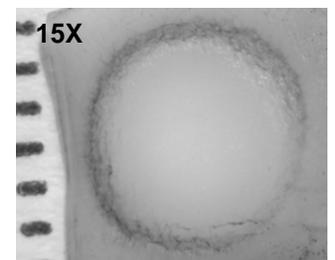


Figure 2: a ring of proliferating cells surrounding laser spot.

One class of proteins that merit investigation is the heat shock proteins (Hsp). Hsps are expressed constitutively at physiological temperatures in cells, and function as molecular chaperones. Likewise, they play a key role in protecting cells from a wide range of environmental stresses and facilitate cellular repair following injury [2, 3]. As a feature of the Hsp stress response, its induction reduces injury upon exposure to subsequent related stressors [2]. The inducible 72-kDa member of the heat shock proteins (Hsp70) is associated with protection against hyperthermia, prevention of apoptosis, and promotion of wound healing. In laser-irradiated skin, several studies have observed up regulated expression of Hsp70 coinciding with these ameliorative effects [4, 5]. In chondrocytes, it has been reported that the accumulation of Hsp 70 in chondrocytes limit induction of apoptosis [3]. While the expression of Hsp 70 in response to hyperthermia in chondrocytes is largely unknown, the induction of Hsp70 as a sequela of other stressors in chondrocytes suggests it likely plays a key role in the regulatory cascade triggered by thermal injury.

Objectives

This project will use confocal microscopy to investigate the cellular response to photothermal stimulation within the laser spot and surrounding thermally modified tissue where regeneration has been observed. The confocal microscope combines the flow cytometer's capability to detect fluorescent labels with the advantage of the whole mount staining in visualizing proliferation zones in relation to laser-irradiated sites. Additionally, confocal microscopy can visualize the distribution of both live and dead cells after irradiation using Live/Dead® cell viability assay (Figure3). Furthermore, the confocal microscope allows construction of

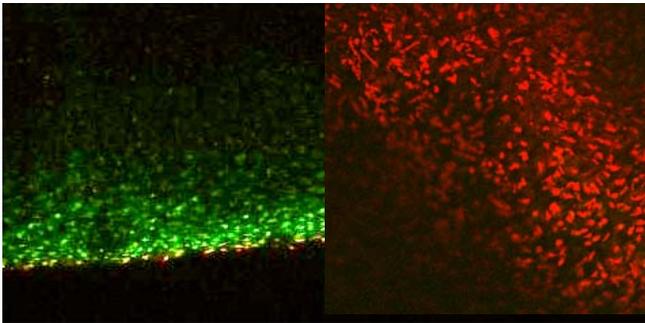


Figure 3: In whole mount confocal microscope images of cartilage samples, the Live/Dead® assay labels live cells with a green fluorescent agent (a) and dead cells with red fluorescent agent (b).

optical cross-sections in tissue, which facilitate 3-D volumetric renderings. This minimizes artifacts that may result from direct contact of the superficial layers of cartilage with the culture media. Finally, confocal microscopy allows the study of other fluorescently tagged molecules, antibodies or metabolic precursors—making it an ideal method to detect specific protein or cytokine expression that may be involved in regeneration.

In this study, we propose to observe the *in vivo* response of chondrocytes in Nd: YAG ($\lambda = 1.32\text{mm}$) irradiated rabbit nasal septal cartilage tissue maintained in culture. The specific aims of this investigation are:

1. Assess spatial extent of thermal injury immediately after Nd: YAG laser irradiation using confocal microscopy and Live/Dead® reduced biohazard cell viability assay.
2. Use confocal microscopy to detect chondrocyte proliferation in intact cartilage specimens with fluorescent linked antibodies to BrdU.
3. Determine whether Hsp 70 expression increases in the same spatial location where regeneration occurs

Materials and Methods

Tissue Preparation

Nasal septal cartilage will be harvested from rabbits obtained from a local abattoir (B&B Rabbit Co., Fontana). Tissues will be stripped of perichondrium and cut into uniform slabs (3 cm x 1.5 cm), and will then be divided into three groups for each protocol. Cartilage samples will be irradiated using an Nd: YAG laser with varying time (4, 6, 8, and 12 seconds) and power (4, 6, and 8 watts). During irradiation, surface temperature of each specimen will be monitored with a thermopile. The specimens will then be washed with antibiotics (PBS 1X with amphotericin and gentamycin) three times. At this point, the first group will be stained using the Live/Dead® reduced biohazard cell viability assay, and imaged with the confocal microscope to determine the distribution of laser injury. A second group of specimens will be maintained in

tissue culture with medium containing BrdU for 7 days and will undergo confocal imaging with fluorescently labeled antibodies to BrdU. The final group will be used to examine the expression of Hsp70.

BrdU assay

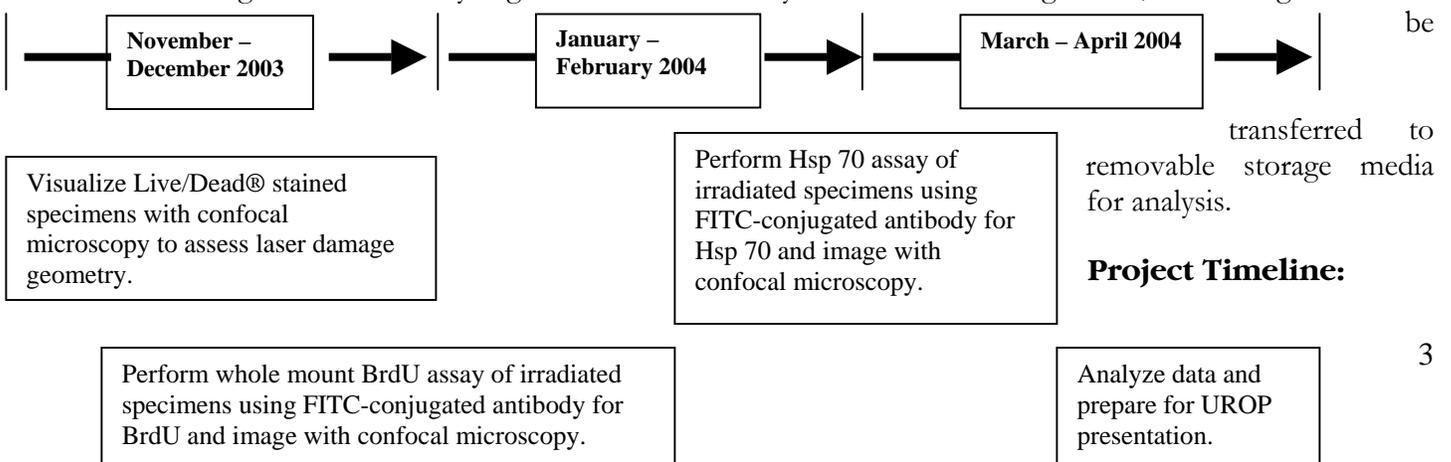
After culture with BrdU for seven days, specimens will be fixed in carnoys overnight and then washed with increments of 100%, 75%, 50%, 25%, and 10% ethanol, followed by rehydration in distilled water. They will then be stained with the FITC: fluorescein-linked antibody that will bind to all cells that have incorporated BrdU. The confocal microscope will then be used to take images of fluorescent cells in intact specimens. We anticipate the FITC signal at 525nm (green fluorescence signifying BrdU incorporated chondrocyte) will originate from a ring of proliferating cells surrounding the circular region of laser irradiation.

Hsp70 assay

The Hsp70 studies will be similar to the BrdU experiments described above. Tissue will be fixed after seven days in tissue culture and then stained with a FITC conjugated antibody for Hsp70. Since this antibody has not been studied in cartilage previously, one anticipated challenge might be the lack of adequate penetration of this agent to substantial depths within the specimen. Possible solutions include the partial digestion of the ECM to increase permeability to the antibody. However, Souil E. et al has demonstrated on numerous occasions the effectiveness of the Hsp70 antibody in penetrating skin, albeit the mechanical properties of skin differ substantially from those of cartilage. We anticipate the identification of FITC signal in an annular pattern surrounding the laser spot.

My specific responsibilities in this project will include:

1. Harvesting cartilage specimens: I will order rabbit heads, recover the nasal septal cartilage, remove perichondrial tissue, cut the specimens into uniform slabs using a microtome, and properly dispose of unused rabbit parts.
2. Laser irradiation, calibration, and temperature measurement: I will responsible for the use of the laser instrumentation, including positioning of the laser, calibrating the thermopile, setting the irradiation parameters, and turning on the laser.
3. Tissue culture and preparation, which consists of using sterile techniques that I have become proficient in during biochemistry laboratory training during the last summer.
4. Staining the samples with FITC-conjugated antibodies: During the summer, I focused my effort on learning the BrdU assay protocols involving NBT/BCIP staining and flow cytometry. This laboratory experience allowed me to work extensively with antibody staining techniques including use of FITC-conjugated antibodies. For this project, I believe I possess the necessary know-how to effectively label the samples with antibodies.
5. Imaging stained tissue with the confocal microscope: The confocal microscope is a precise instrument that can also be used to define multiple focal points within a single specimen to produce clear and highly contrasted optical sections. Over the past few months, I have become comfortable with taking images of stained cartilage specimen using this instrument (see Figure 3 for examples of my work with confocal microscopy) under minimal supervision. I am confident of my ability to use the confocal microscope to collect the necessary data for this project.
6. Recording results and analyzing data: data will mainly be collected in image form; these images will then



Itemized Budget:

<u>Item</u>	<u>Quantity</u>	<u>Source/Catalog #</u>	<u>Cost</u>
5-Bromo-2-deoxyuridine (BrdU)	2	Sigma/B 5002	\$10.98 X 2
Rabbit heads	5 shipments of 10 heads at \$4 per head	B&B Rabbit Co.	\$200.00
Sheep Serum	2	Sigma/S 2263	\$15.70 X 2
Live/Dead® Reduced Biohazard Viability/ Cytotoxicity Kit #1	2	Molecular Probes/ L-7013	\$200 X 2
Anti-Hsp70: FITC conj. 200ug	2	Stressgen/SPA-810FI	\$349 X 2
In Situ Cell Proliferation Kit, FLOUS	2	Roche/1810720	\$510 X 2
		TOTAL:	\$2371.36

References

1. Rasouli A, Sun CH, Basu R, Wong BJF (2003). "Quantitative Assessment of Chondrocyte Viability After Laser Mediated Reshaping: A Novel Application of Flow Cytometry." Lasers in Surgical Medicine **32**: 3-9.
2. Morris SD (2001). "Heat shock proteins and the skin." Clinical and Experimental Dermatology **27**: 220-224.
3. Terauchi R, Takahashi KA, Arai Y, Ikeda T, Ohashi S, Imanishi J, Mazda O, Kubo T (2003). "Hsp70 Prevents Nitric Oxide-Induced Apoptosis in Articular Chondrocytes." Arthritis & Rheumatism **48**(6): 1562-1568.
4. Souil E, Capon A., Mordon S, Dinh-Xuan AT, Polla BS, Bachelet M (2001). "Treatment with 815-nm diode laser induces long-lasting expression of 72-kDa heat shock protein in normal rat skin." British Journal of Dermatology **144**: 260-266.
5. Capon A, Souil E, Gaunthier D, Sumian C, Bachelet M, Buys B, Polla BS, Mordon S (2001). "Laser Assisted Skin Closure (LASC) by Using a 815-nm Diode-Laser System Accelerates and Improves Wound Healing." Lasers in Surgical Medicine **28**: 168-175.