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Three-dimensional microscopic freezing and thawing behavior of biological tissues revealed by real-time imaging using confocal laser scanning microscopy

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ABSTRACT

Three-dimensional behavior of ice crystals and cells during freezing and thawing of biological tissues was investigated microscopically in real time by using a confocal laser scanning microscope (CLSM) and a fluorescent dye. Fresh white meat of chicken was stained in physiological saline, and then frozen and thawed under two different thermal protocols. The CLSM noninvasively produced tomograms of the tissues to clarify the pattern of freezing, the morphology of ice crystals in the tissues, and the interaction between ice crystals and cells. The results were compared and correlated with observed histological changes in the post-thaw tissues.

1. Introduction

Freezing and thawing of biological tissues are fundamental phenomena in cryopreservation and cryosurgery. Cryopreservation is the preservation of biological tissues *in vitro* by freezing, and cryosurgery is the destruction of biological malignant tissues *in vivo* by freezing [1,2]. Cryopreservation is commonly utilized in the medical field, agriculture, fishery, stock raising, and food engineering, and is useful in preserving tissue-engineered equivalents. These applications will benefit from fundamental research of bioheat transfer.

Survival of biological tissue after freezing and thawing depends on the type of tissue, the thermal history during cooling and warming, and the type and concentration of cryoprotectant used in the cryopreservation. Criteria that consumers use to determine the quality of food include texture, taste, smell, and nutritional value. However, detailed phenomena during freezing and thawing and the mechanisms responsible for injuries incurred during freezing and for the protection by cryoprotectants are not well understood [3–5]. The thermal history and chemical additives of biological tissue are macroscopic handling conditions that control the viability and quality of the tissue. These conditions directly determine the microscopic mechanical and chemical conditions surrounding the cells in the tissues. They also affect the biologically large molecules (e.g., proteins and lipids), water, and ice at the molecular level. Investigation of the microstructure of biological tissue during freezing and thawing under these various macroscopic handling conditions will help clarify the mechanisms responsible for the freezing injuries and for the protection by cryoprotectants.

The freezing and thawing of tissue generally proceed transiently and spatially in three-dimensions (3D). Therefore, 3D observation in real time is required to understand the details of the microstructure of tissues during freezing and thawing. For such observation, commonly used optical and electron microscopy have limitations. One limitation is that these techniques require fixation of the sample; consequently, the same sample cannot be monitored during freezing and thawing [6–8]. Another limitation is that they produce only 2D images. For example, real-time observation of a single layer of cells (e.g., epidermis of an onion) by using optical microscopy yields only a 2D image averaged in the direction of thickness of the sample [9].

By contrast, confocal laser scanning microscopy (CLSM) with a fluorescent dye is a noninvasive method that produces 3D optical tomograms of biological materials for high spatial-resolution without fixation or slicing of a sample. This method was applied by Ishiguro and Koike to visualize the 3D behavior of ice crystals and cells during the directional solidification of red blood cell suspensions [10,11] and to clarify the effect of a cryoprotectant and cooling rate on the microstructure.

In this study, this CLSM/dye method for real-time 3D observation was applied to clarify the 3D behavior of ice crystals and cells during the freezing and thawing of biological tissues. Fresh white meat (muscle tissue) of chicken was used as the material and

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Nomenclature

AO	acridine orange
CLSM	confocal laser scanning microscope
CT	connective tissues
H	cooling rate (°C/min)
IC(ex)	extracellular ice crystals
IC(in)	intracellular ice crystals
MF	muscle fibers (muscle cells)
T	temperature (°C)
t	time from start of cooling (min)
W	warming rate (°C/min)
x	distance in horizontal direction perpendicular to muscle fibers (μm)
y	distance in parallel direction to muscle fibers (μm)
z	distance upward in vertical direction from sample upper surface (μm)

acridine orange as the dye. The pattern of freezing, the morphology of the ice crystals in the tissue, and the interaction between ice crystals and cells were investigated for two different thermal protocols. The results were compared and correlated with observed histological changes in tissues due to freezing and thawing.

2. Experimental materials

Fresh white meat (second pectoral muscle) of chicken was selected as the experimental material due to its transparency for fluorescence visualization and to its common use as food. Two pieces of white meat (each about 35 g in weight) was removed from a 40- to 45-day-old broiler (from Arbor Acres, Japan; about 2.0 kg after blood was removed by draining) immediately after slaughter. The meat was muscle tissue with homogeneous structure consisting of a bundle of muscle fibers (muscle cells) connected by connective tissues (Fig. 1), and each muscle fiber consisted of a bundle of myofibrils. The meat was stored at 4 °C for about 1 h, and then a sample (about $10 \times 10 \times 3$ mm) with original smooth surface (needed for visualization by CLSM) was removed carefully by using a microtome blade. The sample was then placed in physiological saline (0.154 M NaCl) containing acridine orange (AO) (0.4 mg/ml) at 4 °C and then left undisturbed for between 90 and 120 min to stain the sample.

3. Experimental apparatus and methods

Microscopic behavior of the ice crystals and cells in the tissues during the freezing and thawing was imaged in 3D in real time by using the CLSM/dye method and the temperature of the sample was controlled by using a cryostage.

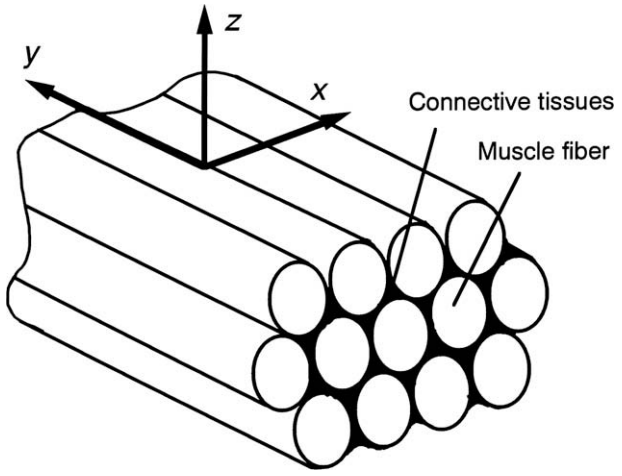

Fig. 1. Structure of tissues and coordinate system.

3.1. Cryostage

Fig. 2 shows a schematic of the cryostage attached to the CLSM and shows the coordinate system. The tissues were frozen and thawed on the cryostage at a uniform temperature according to the thermal protocol (see Section 3.2). The cryostage consisted of an aluminum (Al) block and a copper (Cu) plate (1.5 mm thick) that was lightweight (about 100 g in weight) for rapid vertical movement. The temperature at the cryostage surface was controlled by cooling with liquid nitrogen and by heating with a micro heating element. The sample was placed between a glass microslide (0.8–1.0 mm thick) and a glass coverslip (0.12–0.17 mm thick) so that the direction of muscle fibers was parallel to the x–y section and vertical to the x–z section with the original smooth surface of the sample upward. Good thermal contact between the microslide and the surface of the Cu plate was achieved by ethanol injected into a very thin gap between them.

3.2. Thermal protocols during freezing and thawing

The Cu-plate temperature was controlled between room temperature and a minimum temperature of −50 °C at a predetermined thermal protocol, which is defined as cooling-rate and warming-rate at the cryostage surface. The microstructure of the tissues during the freezing and thawing was imaged for two thermal protocols: (a) slow cooling (1 °C/min) and rapid warming (∼100 °C/min) and (b) rapid cooling (∼100 °C/min) and rapid warming (∼100 °C/min). The sample was maintained at the minimum temperature for 15 min. The rapid cooling rate and rapid warming rate are the attainable maximum rates for the cryostage used in this study and are defined as the time-averaged values between 10 °C and −50 °C. A new sample was prepared in every experiment and the experiments were repeated four or five times for each protocol.

3.3. Confocal laser scanning microscopy (CLSM)

A CLSM has two confocal pinholes and consequently has higher spatial-resolution, particularly a smaller depth of focus, compared with a typical optical microscope. The CLSM (Leica erect-type,

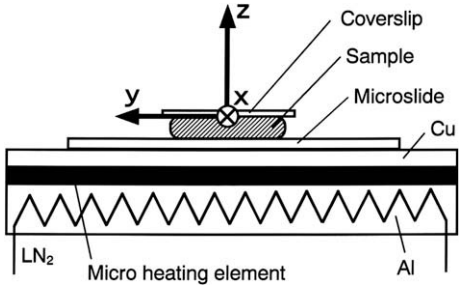

Fig. 2. Schematic of cryostage and coordinate system.