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# Polarized light scanning cryomacroscopy, part I: Experimental apparatus and observations of vitrification, crystallization, and photoelasticity effects

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ABSTRACT

Cryomacroscopy is an effective means to observe physical events affecting cryopreservation success in large-size specimens. The current study aims at integrating polarized-light in the study of large-size cryopreservation, using the scanning cryomacroscope as a development platform. Results of this study demonstrate polarized light as a visualization enhancement means, including the following effects: contaminants in the CPA solution, crystallization, fracture formation, thermal contraction, and solute precipitation. In addition, photoelasticity effects are used to demonstrate the development of residual stresses and the potential for stress relaxation above the glass transition temperature. Furthermore, this study suggests that the ability to periodically switch between non-polarized light and polarized light is an essential feature of investigation. When using polarized light for example, a dark region may represent a free-of-stress and free-of-crystals material, or fully crystallized material, which may potentially experience mechanical stress; switching to a non-polarized light would help to distinguish between the different cases. The analysis of thermo-mechanical stress in cryopreservation is essentially based on four key elements: identification of physical events, knowledge of physical properties, thermal analysis of the specimen, and description of the mechanical behavior of the cryopreserved material (also known as the constitutive law). With the above knowledge, one can investigate the conditions to preserve structural integrity. While the current study aims at identification of physical events, critical knowledge on physical properties and mechanical behavior has already been developed in previous studies. The companion manuscript (Part II) aims at providing means for thermal analysis in the specimen, which will serve as the basis for a multi-scale analysis of thermo-mechanical stress in large-size specimens.



## 1. Introduction

Vitrification—the suppression of ice formation—is considered an important alternative approach to cryopreservation (*vitreous* in Latin means *glass*) [13,20,21,42]. Although controlling ice formation *per se* has long been recognized critical to cryopreservation success [24,27,44], additional systemic phenomena may affect cryopreservation success, such as the inherent toxicity of cryoprotective agents (CPAs) [14] and thermo-mechanical stresses (also referred to as *thermal stress*) [31,44]. In fact, reduction of toxicity and thermal stress represent competing needs as described here on. Since the CPAs are inherently toxic, it is necessary to use their lowest possible concentration in order to promote glass formation [7,26]. In turn, the critical cooling rate to promote vitrification is inversely related to the CPA concentration necessary to promote vitrification, where the increase in cooling rate is associated with increase in thermo-mechanical stress. When the thermal stress exceeds the strength of the material, structural damage follows with fracture formation as the most spectacular outcome [39]. However, the cooling rate is not the only driving force to thermal stress, where crystallization and constrained contraction by the specimen container may also lead to structural damage [31].

At the cellular level, visualization of cryoprotocols is commonly done with cryomicroscopy means [8,19,22,25,36,40,43]. When cryomicroscopy is used to analyze large-size cryopreservation, representative micro-slices may be exposed to conditions similar to

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those that would exist in a large specimen at strategic points, such that a complete picture of the process can be piecewise-constructed. Unfortunately, since the kinetics of crystallization and, independently, the formation of stress are often affected by the size and shape of the sample (i.e., surface effects), the correlation between microscopic effects and large specimen vitrification may be weak. To bridge this gap, the cryomacroscope has been invented, with the objective of *in situ* visualization of macro-scale physical events [30].

Over the past decade, the development of cryomacroscopy has resulted in four prototypes for various applications. The first-generation prototype (Cryomacroscope I) was developed to study vitrification in a 15 mL vial, using a dedicated passive cooling mechanism, magnetic recording means, and a monochrome CCD camera [30,37]. Earlier cryomacroscopy results demonstrated that micro-fractures in the glassy state may serve as ice nucleation sites during the rewarming phase of the cryoprotocol [37], following cursory reports of this phenomenon [45,46]. That early device has been demonstrated as a critical tool to investigate mechanical stresses induced by the container walls [37]. Follow on studies focused on the correlation between crystallization, fracture formation, and functional recovery of blood vessels [1,2].

A dedicated apparatus to study solid-mechanics effects in thin films was the design objective for Cryomacroscope II [32]. This apparatus was designed to measure fracture strain, repeatability of fracturing events, patterns of fracture formation, and effects of stress concentration induced by tissue specimens [32]. In contrast to Cryomacroscope I, which was designed to study commonly practiced cryopreservation protocols, Cryomacroscope II was designed specifically to investigate solid mechanics effects, where the thin-film configuration was chosen for simplicity in modeling.

Cryomacroscope III has been designed to investigate physical events associated with vitrification in the presence of synthetic ice modulators (SIMs) [33]. The main improvement in Cryomacroscope III over Cryomacroscope I is that it has been redesigned as an add-on to a commercially available top-loading controlled-rate cooler, thereby improving its potential dissemination for the benefit of the cryobiology community. Additional improvements include a high-speed color camera, fiber-optics illumination, and a dedicated computer code to create a post-processed movie of the experimental study. Results of this study indicated improved suppression of crystallization with the application of SIMs and unexpected phenomenon of solutes precipitation during rewarming [33].

While Cryomacroscopes I-III were designed to visualize physical events with a stationary camera in a somewhat similar arrangement to the cryomicroscope, Cryomacroscope IV was designed for vertical scanning of samples larger than the field of view of the camera, using a computer-controlled stepper motor and a carriage system. Cryomacroscope IV was designed as an add-on unit for commercially available, top-loading, controlled-rate cooling chambers. These scanning capabilities enabled cryopreservation investigations on a wide range of specimen configurations, containers, and common thermal protocols [15]. Cryomacroscope IV served as a development platform for the integration of polarized-light in the current study.

The history of light polarization began with the discovery of the double refraction effect by Erasmus Bartholinus in 1669 [3]. Visualization of mechanical stresses in transparent-amorphous materials using polarized light was first documented by David Brewster in 1815 [5,6], which is also known as photoelasticity [18]. The physical principles of light, optics, and their role in photoelasticity have been well documented in the literature of the past two centuries, and are addressed herein only briefly, for the completeness of experimental-setup presentation.

When two similar polarization filters are placed on a common axis, parallel to one another in an orthogonal polarization orientation, any light ray pointing in the same axial direction will be blocked by the filters couple. The filter closer to the light source is commonly referred to as the *polarizer* and the other filter is commonly referred to as the *analyzer* or *decoder*. Any object placed in between the two filters may refract the propagating light, potentially causing some of it to pass through the analyzer. It is this refraction effect that may enhance visualization, even from small objects that otherwise could not have been seen using the same available optics. In the context of microscopy and cryobiology for example, Luyet [23] used polarized light to better view intermixed frozen and glassy regions in cells, whereas Menz and Luyet [25] enhanced the appearance of fractured surfaces at the cellular level.

Photoelasticity is a whole-field, stress-analysis method based on a coupled optical-mechanical property called birefringence, which is characteristic of many transparent media [18]. When a photoelastic specimen is placed between the polarizer and analyzer, fringe patterns are displayed, related to the difference between the principal stresses. In that setup, any unstressed area will appear dark as all the light will be blocked by the orthogonally oriented filters (hence the term *dark-field photoelasticity*). Areas of relatively higher stress will brighten due to altered polarization of the refracted light allowing it to pass through the analyzer filter, with increasing intensity that may extend over the entire visible spectrum, and even create a spectrum-repetition pattern as the stress further increases [18]. An experimenter may estimate the magnitude of the stress by quantifying the light spectra, but this process requires some *a priori* knowledge about the field of stress for calibration purposes.

The current study presents the first device prototype for polarized-light cryomacroscopy, using the established scanning cryomacroscope as a development platform [15]. The current study aims at demonstrating polarized-light as a visualization-enhancement means of physical events, as well as effects of photoelasticity for the study of cryopreservation by vitrification.

## 2. Experimental apparatus

The scanning cryomacroscope has been presented previously [15] and is described here in brief only, for the completeness of presentation (Fig. 1). Emphasis in the current presentation is given to the newly added polarized-light means (Fig. 2). In general, due to the harsh environment surrounding the sample, all electronic components and mechanisms are placed externally to the cooling chamber. Light is delivered by various fiber-optics bundles, while the image of the specimen is delivered by means of a borescope (Hawkeye HH2992, Gradient Lens Corporation, Inc., NY, USA). The cryomacroscope comprises of the following key elements:

(i) A commercial cooling chamber and controller (Kryo 10-16 controlled by Kryo 10-20, Planer PLC, UK).
(ii) A cryogenic stage placed within the cooling chamber (Fig. 2). This unit is essentially the experimentation platform, which has been modified for the purpose of the current study.
(iii) A high-speed, light-sensitive, CCD camera (Grasshopper, Point Grey Research, Inc., BC, Canada), connected to the external end of the borescope.
(iv) A stepper motor and a controller (AMH-22 controlled with DCB-274, Advanced Micro Systems, Inc., VT, USA) to facilitate vertical scanning.
(v) A T-type thermocouples array (uncertainty of ±0.5 °C or 0.4% of full scale) for monitoring the thermal history via a computerized data acquisition system (OMB-DAQ-56, Omega Engineering, Inc., controlled by DaqView™).