An optical-axis freezing stage for laser-scanning microscopy of broad ice–water interfaces

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Key words. Confocal, cryopreservation, crystal growth, directional solidification, freeze drying, freezing, glycerol, image analysis, lake ice, laser-scanning microscope, saline.

Summary

This article presents a method to view a dynamic ice interface along the axis of ice growth using a laser-scanning microscope. A deep liquid volume is chilled from below so that ice growth is directed upward toward the microscope objective. The interface is made visible by rejection of fluorescent dye from the solid phase into the liquid. Images of the interface morphology in water with solutes of interest to cryobiology illustrate the imaging capability. These images are processed to quantify the lamellar structure of the ice interface. The optical-axis cryostage provides advantages over horizontal arrangements because (1) immersion objectives enhance, rather than disturb, the desired thermal gradient, and (2) features in the ice interface are not confined within a narrow capillary tube or microscope slide. This arrangement loses some of the thermal control found in planar freezing stages, and the dynamic, refractive interface presents challenges to confocal microscopy.

Introduction

The interface between growing ice and liquid water can take many shapes, from a smooth plane to a field of spines less than 20 µm wide (e.g. Hobbs, 1974; Körber, 1988). The microscopic interface morphology (flat, columnar, or branched) correlates with four local variables: the temperature gradient near the interface, the local melting point (determined by local chemical concentrations), the mobility of molecules near the interface and the crystal structure of the solid. The shape, temperature and chemical concentrations in the growing ice field can critically affect microscopic inclusions, such as biological cells, that are viable over a narrow range of physicochemical environments. Although microscale thermal and chemical conditions are difficult to measure directly, the shape and movement of the solid–liquid interface can be observed using microscopic techniques and are indirect indicators of the chemical conditions. In addition, the interface morphology directly influences the eventual mechanical properties of ice, such as its load-bearing capacity (Gold, 1963; Hobbs, 1974) and texture. As such, the microstructure of ice growth has implications for medicine, civil engineering and frozen food production (Desrosier & Tressler, 1977).

The net or average direction of growth of the phase front is dictated by internal thermal gradients, which are in turn the result of externally applied cooling and the extent of the material. Although ice fields can be quite large (e.g. lake freezing), most analysis can be reduced to the scale of a few centimetres. Of particular interest in medicine are situations in which bulk liquid (e.g. a cell suspension in a cryovial) or thick tissue (e.g. a visceral transplant) is frozen by external chilling. In addition, crystal growth itself can be altered by the proximity of solid walls (Billia et al., 1996). These conditions have generated interest in microscopic analysis of ‘large’ ice fields, which we shall define as situations where (a) the temperature difference between the surface and the interior cannot be neglected, or (b) the volume is large enough that the ice morphology is not altered by proximity to the walls of the container. Optical microscopy of thick specimens presents two primary challenges: (a) bringing the objective sufficiently close to the target, and (b) accommodating deviation of light as it traverses the intervening medium. To address these challenges, we have developed a microscope freezing stage to view the interface morphology as a cross-section by viewing through the liquid, along the direction of growth.

Background

The fundamental relationships between the interface morphology, temperature distribution and chemical conditions at the interface are common among many materials, including aqueous solutions, metal alloys and organic compounds.
(Fleming, 1974). At low solidification rates, the growth of ice is governed by heat and mass transfer and depends little on the crystal structure of the material. As the interface velocity increases, the orientation of crystal axes with respect to the net growth direction plays an increasing role in determining the interface morphology (e.g. Hardy & Coriell, 1969). In addition, chaotic aspects become more pronounced, and many unique but similar structures result from the same initial conditions. Studies of ice growth in isothermal, subcooled water have shown that ice is typically a hexagonal crystal with trifold symmetry; growth is fastest along each of three co-planar, evenly spaced axes (the a-axes), and is slowest along the perpendicular c-axis (review by Hobbs, 1974).

The solid–liquid interface morphology has been well studied for certain ice growth conditions. Steep temperature gradients and low interface velocity correspond to planar interface morphology, while shallow thermal gradients and high interface velocity lead to more highly branched (dendritic) growth. At moderate ratios of velocity to gradient, the ice forms a stable lamellar interface (Fig. 1), and with increased velocity more undulations develop along the ridges (Harrison & Tiller, 1963). Further increases in velocity lead to the formation of spikes or dendrites whose sharpness and branching increase with velocity (review by Körber, 1988). The components of a freezing solution affect the interface morphology significantly; for example, adding glycerol to solutions of physiological saline increases the tendency toward side branching (Ishiguro & Rubinsky, 1994, 1998).

**Biological consequences**

Because extreme cold inhibits both life-sustaining and life-degrading processes, freezing may be used both to destroy tumour cells (cryosurgery) and to store biological material (cryopreservation). Cells are at the greatest risk at temperatures that allow ice to form and recrystallize (0 to −60 °C). Ice that crystallizes out of dilute solutions is nearly pure H₂O, so it rejects dissolved salts, proteins and dyes into the adjacent liquid. The solutes become concentrated, drawing water out of the living cell to maintain osmotic equilibrium across the plasma membrane. The resulting contraction of the plasma membrane and concentrated intracellular solutions can damage internal components and cause irreversible injury. Rapid freezing minimizes the efflux of water, but risks intracellular ice formation. Successful freezing protocols often include a cryoprotective agent, such as glycerol, to avoid excessive contraction and intracellular ice. Cells are also subject to mechanical damage from lateral movements of the surrounding ice crystals, which have been shown to rupture cell membranes (Ishiguro & Rubinsky, 1998). The mechanical and chemical displacements are highly dependent on the shape of the ice–water interface.

**Directional solidification**

An ice front can be forced to grow in a specific direction and at a specific rate by imposing a controlled thermal gradient. Heat is typically removed by using a chilled stream of liquid or gas (e.g. chilled N₂), and finer temperature control may be achieved by adding a resistive heater (Diller & Cravalho, 1970; Reid, 1978) or thermoelectric cooler. For example, Harrison & Tiller (1963) arranged a series of strip heaters under a stationary, inclined water channel that was chilled from below. The thermal gradient was propagated by sequentially shutting off the heaters, and ice growth was observed with a dissecting microscope.

Microscope stages for directional solidification have been effective for observing phase front morphology and cell entrapment in growing ice (review by Körber, 1988). To view a fixed point on a microscope slide as an ice interface passes, a microscope slide may be supported on two chilled blocks that lie on either side of the field of view, with one end above and one below the melting temperature. The temperature at both ends is lowered to cause the ice front to propagate along the slide. Stationary temperature gradient stages have been used to solidify succinonitrile as a model of dendritic growth and interface instability in metals (e.g. Somboonsuk et al., 1984; Venugopalan & Kirkaldy, 1984) and to measure and correlate temperature and concentration fields and phase interface morphology in freezing aqueous solutions (Kourosh et al., 1990a,b).
The Bridgman solidification method – used to grow monocrystalline semiconductors – involves moving a sample continuously through a steady temperature gradient. Rubinsky & Ikeda (1985) adapted this method for microscopy of biological material, noting that a moving interface can be held in the field of view continuously with steady-state thermal conditions. Unlike stationary devices, a Bridgman or ‘pusher’ stage can maintain a constant cooling rate at the interface while varying the two rate factors (velocity and temperature gradient). Rubinsky’s original design placed specimens between a microscope slide and cover slip and has been adopted by other researchers (Cosman et al., 1989; Hubel et al., 1992; Ishiguro & Rubinsky, 1994; Namperumal & Coger, 1998), and modified to use flat capillary tubes (Lipp & Körber, 1993; Nunner, 1993; Tatsutani & Rubinsky, 1998).

Most of the previously mentioned studies used brightfield microscopy, but fluorescence may also be used to reveal the ice–liquid interface because the exclusion of solutes by crystalline ice segregates dye into the liquid and leaves the ice dark. Ice appeared corrugated when fluorescent protein solutions were frozen on a stationary directional solidification stage and viewed with a laser scanning microscope (Evans et al., 1996). Confocal laser scanning microscopy (Wilson, 1990) has provided a three-dimensional (3-D) view of ice growth around red blood cells suspended in an acridine orange solution (Ishiguro & Koike, 1998). The ice growth was horizontal (i.e. perpendicular to the viewing axis), and the dendrites could be seen clearly enough to determine the orientation of the c-axis. In a similar study of frozen muscle tissue, the muscle fibre bundles could be seen in cross-section down to a depth of 40 μm (Ishiguro & Horimizu, 1999).

Most biologically relevant ice crystallization studies have used thin water/ice layers that facilitate transmitted-light microscopy; measured thicknesses include 15 μm for sucrose solutions under a cover glass (Reid, 1984) and 100 μm for collagen solutions and suspensions in flat glass capillary tubes (Schoof et al., 2000). The primary disadvantage of using samples thin enough for transmission microscopy is that confining the sample potentially alters the interface morphology. Methods to observe broad interfaces that are not restricted by the container walls have included physical sectioning and turning the optical axis along the direction of growth. To obtain physical sections, salt solutions were directionally solidified in plastic tubes, then were sectioned and photographed at −40 °C to avoid melting or recrystallization (Rohatgi & Adams, 1967). Ice morphology has been observed indirectly in collagen scaffolds created for cell seeding (Schoof et al., 2001). In this process, directional solidification rejects suspended collagen from the growing ice phase, and subsequent lyophilization (sublimation at low pressure) leaves a protein matrix with pores where the ice had been. The matrix is not observed during solidification, but scanning electron micrographs of the finished scaffold indicate that the ice had a lamellar or plate-like structure.

Although the preceding studies have contributed extensively to our knowledge of ice growth and ice–cell interaction, they have not revealed a cross-sectional view of the broad, moving ice interface (which we define as ice at least 1 mm from any container wall). This goal may be achieved by placing the direction of solidification along the optical axis. This strategy has been used with oblique illumination to view very slow (0.1–1.0 μm s−1) planar ice growth (Ketcham & Hobbs, 1968), and for macroscopic observations of ice formation in a watertight box with a transparent top and a chilled base (Bianchi & Viskanta, 1994; McNulty et al., 1994). Because broad interfaces with lamellar interfaces are interesting on a microscopic scale, we have developed a fluorescence cryomicroscope stage that places the direction of solidification along the optical axis (Neilis & Diller, 1998). A temperature gradient is imposed across the bulk of the sample while the ice interface remains largely free from interaction with the container walls. This device reveals a cross-section of the arrangement of projections from a broad freezing front, perpendicular to the viewpoint in common horizontal cryomicroscope systems. The interface morphology in water, with and without solutes of interest to cryobiology, is used to illustrate the imaging capability as well as techniques to quantify the morphological structure of growing ice.

Materials and methods

Experimental system

The optical-axis directional solidification system consists of a sample cup, freezing stage, liquid nitrogen transfer system, confocal microscope, desktop computer for system control and necessary interfaces (Fig. 2). The cup rests on top of a nitrogen-cooled copper heat sink, which freezes the solution upward toward the microscope objective. The phase interface is viewed through the liquid layer over the ice, and the ice volume may extend far below the interface. Ice and liquid are differentiated by a fluorescent dye, which is excluded from the ice and concentrated in the adjacent liquid. A custom program run in LabView (National Instruments, Austin, TX, U.S.A.) provides a single user interface that transmits images and integrates the thermal and scanning controls.

Freezing stage

Sample cups (Fig. 3a) were made by gluing copper or aluminium foil (∼30 μm and 80 μm thick, respectively) between annular sections of pipe insulation made of closed-cell foam (inner diameter 18 mm). One foam annulus forms the cup (depth 8–11 mm), while another centres the cup on the pipe cap. The insulation is watertight at its inside and outside diameters, and its cut ends were melted smooth on a hot plate to seal the surface. A coating of flat black paint reduces reflection from the foil. To augment heat transfer into the upper boundary, cups intended for immersion objectives have an aluminium plate with a viewing port.

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The base temperature of the sample cup is imposed solely by the sample cup and the objective collar, so it permitted lateral or convection at the cover slip helps maintain the desired vertical temperature gradient. The temperature at the upper boundary of the sample cup can be recorded but is not controlled.

The thermal control routine in the LabView interface uses a proportional-plus-integral algorithm to regulate the base temperature in the sample cup, using feedback from the lower thermocouple. An integral term with an infinite impulse response is used to avoid a data queue and reduce calculation time. Equation (1) shows the complete output function in which \( I(t) \) is the control current, \( T \) is temperature in degrees Celsius, and \( t \) is time in seconds. The output current is updated once per second:

\[
I_{\text{out}}(t + 1) = (2 \text{ mA/°C})(T_{\text{base}} - T_{\text{target}}) + [I_{\text{out}}(t) + 14I_{\text{out}}(t - 1)]/15. \tag{1}
\]

The thermal measurement and control

Every sample cup includes a copper–constantan thermocouple glued to the inner bottom face of the cup. The thermocouple wires (Omega, Stamford, CT, U.S.A.; type TT-T-36, diameter 0.125 mm) were mounted horizontally to minimize heat conduction. During some trials, temperature readings were also collected at the upper plate and near the focal plane. Thermocouple voltages were converted to temperature data by signal conditioning modules (Analog Devices, Norwood, MA, U.S.A.; model 6B11) and transmitted to the desktop computer.

The base temperature of the sample cup is imposed solely by a controlled flow of cold nitrogen. The laboratory service vacuum draws liquid nitrogen from a one-litre dewar, through the freezing stage, then through a proportional solenoid-operated globe valve (ASCO, Florham Park, NJ, U.S.A.; model SD8202G3V) that regulates the gas flow. The computer interface adjusts output current to the solenoid valve controller (ASCO; model 8908A00), with options to hold or to chill at a constant rate. Heat conduction through an immersion objective or convection at the cover slip helps maintain the desired vertical temperature gradient. The temperature at the upper boundary of the sample cup can be recorded but is not controlled.

The thermal control routine in the LabView interface uses a proportional-plus-integral algorithm to regulate the base temperature in the sample cup, using feedback from the lower thermocouple. An integral term with an infinite impulse response is used to avoid a data queue and reduce calculation time. Equation (1) shows the complete output function in which \( I(t) \) is the control current, \( T \) is temperature in degrees Celsius, and \( t \) is time in seconds. The output current is updated once per second:

\[
I_{\text{out}}(t + 1) = (2 \text{ mA/°C})(T_{\text{base}} - T_{\text{target}}) + [I_{\text{out}}(t) + 14I_{\text{out}}(t - 1)]/15. \tag{1}
\]

The sample cup was observed to descend slightly during cooling due to thermal contraction of the copper cap. To provide a temperature sensor that remained fixed relative to the focal plane, a thermocouple was attached to the cone of the immersion objective and was suspended vertically so that its tip lay in the focal plane. The thermocouple length was made adjustable by running the insulated wires through a glass capillary that was bent to provide a tight fit. The correct wire length was ascertained by focusing on a glass slide and extending the junction to touch the glass. The vertical thermocouple passed perpendicular to isotherms in the liquid, so lateral heat conduction caused it to read several degrees high. A linear correction function for the mobile vertical thermocouple was developed using a fixed horizontal thermocouple. Because the vertical thermocouple became trapped in the ice, its use was restricted to experiments in which the objective stayed fixed with respect to the sample cup.

Imaging system

The cryostage was mounted on a Zeiss LSM 10 confocal laser-scanning microscope (CLSM) with a single photomultiplier tube (Carl Zeiss, Thornwood, NY, U.S.A.). Fluorescence images were captured digitally by laser scanning in confocal and non-confocal modes. Each 256 × 256-pixel image took 0.7 s to scan and 2 s to save to disk. Images presented here were captured using 488-nm illumination and a green band-pass emission filter.

Table 1 lists properties of the primary objectives, which included two immersion objectives and two dry objectives used with cover glass. A small plastic tent was installed around the dry objectives to prevent atmospheric vapour from condensing on the cover glass. The tent enclosed only the volume between the sample cup and the objective collar, so it permitted lateral...
access to the cryostage and allowed the cover slip to be dried effectively with low nitrogen flow.

The LabView program transmits scanning commands and receives images via a General Purpose Interface Bus (GPIB). Each image is scanned using one of two subroutines. The first routine scans a single horizontal plane every three seconds, capturing a time series of \(x-y\) images of the lamellar interface in cross-section as it passes. The second routine captures one vertical (\(x-z\)) scan every 10 s. Both routines scan and transfer 256 \(\times\) 256 images to the desktop computer, then place time values in the file names for correlation with the temperature history. The thermal control loop is dormant during scanning, and the scanning intervals were chosen to allow the temperature to stabilize between scans.

**Image capture**

Ice growth was observed in four aqueous solutions (Table 2). Every solution included 5 p.p.m. (13 \(\mu\)m) sodium fluorescein (Na-Fl) (Sigma, St. Louis, MO, U.S.A.; F-6377); absorbance of the 0.9% saline irrigation at 488 nm was 0.142 cm\(^{-1}\) as measured

---

Table 1. Primary objectives.

<table>
<thead>
<tr>
<th>Mag.</th>
<th>NA</th>
<th>WL (mm)</th>
<th>Imm.</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3X</td>
<td>0.20</td>
<td>10</td>
<td>dry</td>
<td>Zeiss Neofluar 1600/-*</td>
</tr>
<tr>
<td>10X</td>
<td>0.30</td>
<td>5</td>
<td>dry</td>
<td>Zeiss Plan-Neofluar 0/0.17</td>
</tr>
<tr>
<td>20X</td>
<td>0.50</td>
<td>3.2</td>
<td>water</td>
<td>Olympus UMPlanFl</td>
</tr>
<tr>
<td>40X</td>
<td>0.75</td>
<td>1.0</td>
<td>water</td>
<td>Zeiss Achroplan Ph2 0/0</td>
</tr>
</tbody>
</table>

*With appropriate tube-length adapter.

Table 2. Aqueous solutions used for broad freezing interface.

<table>
<thead>
<tr>
<th>Solution*</th>
<th>Osmolality†</th>
<th>(T_{melt})‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtered water</td>
<td>0</td>
<td>0 °C</td>
</tr>
<tr>
<td>Saline irrigation (0.9 wt% NaCl)</td>
<td>291 mOsm</td>
<td>-0.54 °C</td>
</tr>
<tr>
<td>9.5 vol.% glycerol in water</td>
<td>1617 mOsm</td>
<td>-3.11 °C</td>
</tr>
<tr>
<td>10 vol.% glycerol in PBS</td>
<td>-1993 mOsm§</td>
<td>-4 °C</td>
</tr>
</tbody>
</table>

*All solutions contained 0.0005 wt% uranine (fluorescein disodium salt).
†Measurements on Osmette freezing point osmometer.
‡Calculated from measured osmolality.
§Out of measurement range; extrapolated from solutions 2 and 3.
on a Beckman DU 530 spectrophotometer. Ten to 20 mL of the test solution, of which about 5 mL was needed to fill the sample cup, was poured into a flask and used for multiple runs. Between runs, the cryostage was warmed above 10 °C and the solution was removed from the cup, mixed in the flask and returned to the cup to provide uniform initial conditions. Images were captured when the ice front was approximately 5 mm from the cup base.

Image processing and analysis

Image processing

Following capture, the mean grey level for each image was computed using NIH Image (U.S. National Institutes of Health, available at http://rsb.info.nih.gov/nih-image/). Images that had been captured with pixels in a 2×3 aspect ratio were scaled 67% vertically using linear interpolation. The ‘Reslice’ and ‘Project’ functions in NIH Image were used to produce 2-D and 3-D projections of image data.

Fourier analysis

Square regions measuring \(2^N \times 2^N\) pixels were selected from images that have consistent lamellar orientation and high contrast between ice and liquid. The regions were converted to the frequency domain using a 2-D fast Fourier transform (FFT), and the magnitude plot was searched for the pixel \((u_p, v_p)\) with the highest value [neglecting the 0th order peak, i.e. \((u_p, v_p) \neq (0,0)\)]. The coordinates \((u_p, v_p)\) represent the spatial frequency of the periodic lamellar ice: the ice lamellae lie at right angles to the line connecting \((-u_p, v_p)\) and \((u_p, v_p)\), and the dominant lamellar spacing \(\lambda\) is proportional to \((u_p^2 + v_p^2)^{-1/2}\). In our system, \(\lambda = (27.3 \, \mu\text{m}) N (u_p^2 + v_p^2)^{-1/2} + M\), where \(M\) is the primary objective magnification. Computations were done using MathCAD (MathSoft).

Euclidean distance map

An alternative measure of lamellar spacing is the average distance from each pixel to the nearest lamellar ridge. The Euclidean Distance Map (EDM) function in NIH Image takes as input any rectangular, binary image and replaces the value of each black pixel with the integer distance to the nearest white pixel. To measure the lamellar spacing, grayscale images were processed as follows:

- If necessary, add 1 to ensure no pixels are zero-valued.
- Manually trace the lamellar ridges using zero-valued pixels as markers.
- Threshold at zero to create a binary skeleton of lamellae.
- Apply EDM function.
- Calculate histogram of EDM and export to spreadsheet.
- Calculate average distance and convert pixels to millimetres.
- Multiply by four to obtain average interlamellar spacing.

Results

Optical performance

Figure 4 compares typical images captured with each of the four primary objectives. All four images show physiological saline with 5 p.p.m. Na–Fl, but the cooling rates and camera gain settings differ. The structure of individual lamellae is rounder and more brick-like when viewed through the 40× objective than through the other (longer working distance) objectives. Given equal camera settings, the contrast was higher for objectives with lower numerical aperture (NA) (6.3× and 10×) while the vertical resolution was better with the higher-NA objectives (20× and 40×).

Interface morphology

As seen in Fig. 5, the interface in filtered water was almost planar when the base of the cup was chilled at 5 °C min\(^{-1}\) (the chilling rate refers to the target temperature for the thermocouple in the base of the sample cup). In this time series, the brightness increased quickly and uniformly (Fig. 5b–c), then rapidly faded to black. The darker regions in Fig. 5(b) show the leading part of the ice; here the dye is segregated into a bright fissure and the ice remains uniformly black. The large bright region in Fig. 5(b) shows where the dye is pushed ahead of the interface; this region eventually captured the dye in pores, making these features visible behind the interface (bright dots in Fig. 5e,f). Gas bubbles formed (dark circles in Fig. 5d,e), then were trapped by the interface.

![Fig. 4. Comparison of primary objectives. All solutions are 5 p.p.m. fluorescein in PBSA; dye is white and ice is dark. The white bar in each image is 0.1 mm. (a) 6.3x objective, non-confocal, base of cup chilled at 8 °C min\(^{-1}\); (b) 10x objective, non-confocal, 8 °C min\(^{-1}\); (c) 20x objective, non-confocal, 5 °C min\(^{-1}\); (d) 40x objective, confocal mode, variable chilling rate.](http://rsb.info.nih.gov/nih-image/)

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Saline and glycerol solutions typically produced lamellar interfaces with one or more groups of parallel lamellae, as shown in Fig. 4 for saline solution and Fig. 6 for a 9.5% glycerol solution. In Fig. 6, the lamellar ridges are sharply defined, and the edge definition decreases with depth into the ice. Deeper images contain features that appear to be lateral branches but that could also be refractive artefacts.

The lamellar ice morphology revealed by the optical-axis cryostage is consistent with the groups of ‘platelets’ (flat unbranched dendrites) reported by Rohatgi & Adams (1967), who used polarization microscopy to show that each group of parallel platelets arose from one grain. They also found that the platelet spacing and thickness in some groups increased with distance from the chill plate, causing them to eclipse less favourably orientated platelet groups. The undulatory ridges seen in Fig. 4 are similar to the undulations reported by Harrison & Tiller (1963). The results for a given solution and cooling rate were consistent in form, but every trial produced a unique interface pattern. The broad patterns were more complex than those seen in thin specimens, and the morphologies observed in horizontal cryostages could be reproduced by inserting vertical glass surfaces in the vertical cryostage to restrict the length of the lamellar ridges (manuscript in preparation).

Figure 7 shows two vertical (x–z) scans of a lamellar interface. In both cases, a vertical stage travel of 2 mm encompassed three regions: upper, with dilute dye; middle, with ice and concentrated dye; and lower, with dye-free ice. Most of the interface detail was contained in a 0.5-mm vertical range, where interstitial dye is clearly visible as dark vertical streaks. With the 10× objective, these streaks were difficult to distinguish from the ice–liquid boundary, whereas with the 20× objective and a lower dye concentration, the ice appears very sharp and triangular in cross-section. The triangles are not laterally symmetric, but in no case is ice seen to overhang the interstitial liquid.

The time series in Fig. 6 has been reformatted to show the history of two individual scan lines, with temporal resolution...
of 3 s. The resulting $x$--$t$ and $y$--$t$ plots (intensity vs. distance and time) in Fig. 8 show the anisotropy of lamellar growth rates in graphical form. The lamellar ridge is nearly horizontal and the lamellae thicken at a rate that appears to be constant.

The time series of filtered water (Fig. 5) has been projected such that dye is opaque and ice is transparent (Fig. 9). The reconstructed surface suggests that as time progresses the dye is constricted into narrow channels and the fluorescence is extinguished as the temperature falls.

**Image analysis comparison**

The lamellar spacing in a $128 \times 128$ selection from Fig. 6(c) has been calculated using both the FFT and the EDM methods. Figure 10(a,b) show the original image selection and the FFT calculated from it. Figure 10(c,d) contain the manual markup.
of the lamellar ridges and the resulting distance map. The dominant spacing calculated from selections from several images is compared in Table 3 to the results of direct measurement with the length tool in NIH Image.

### Thermal performance
The ability of the cryostage to follow a linear cooling program is shown by the thermal histories in Fig. 11. These data were collected by chilling at 5 °C min⁻¹, then scanning a time series using the 20× objective. The cup base temperature did not deviate by more than 1 °C from the desired 5 °C min⁻¹ chilling rate except during nucleation and scanning. When the LabView routine took remote control of the CLSM, it suspended other operations. Therefore, the thermal control program looped every second when not scanning and every 3 s while running 256 × 256-pixel x-y scans, and the temperature increments used by the thermal control loop were adjusted accordingly.

Ice nucleated spontaneously, usually with $T_{\text{base}}$ between −3 and −5 °C, without the use of nucleating agents. The nucleation transient is visible in the plot of $T_{\text{base}}$. The valve control current was steadier before nucleation than after, but none of these transients appeared to affect the phase interface. Some larger transients were introduced, when the temperature control was paused for scanning. Temperature oscillations increased greatly when the base thermocouple became thermally decoupled from the pipe cap. For example, using 1-mm polystyrene from a plastic culture dish in lieu of the metal foil introduced so much thermal resistance that the control system had difficulty maintaining stable temperatures, even at moderate cooling rates (data not shown).

The traces in the right column of Fig. 11 show the average grey level of each image in the corresponding time series of x–y scans, normalized to the highest average in each series. The glycerol brightness trace spans 120 s.

### Table 3. Lamellar spacing and direction comparison.

<table>
<thead>
<tr>
<th>Image</th>
<th>Fourier transform</th>
<th>Manual (NIH Image)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spacing (µm)</td>
<td>Angle (°)</td>
</tr>
<tr>
<td>Fig. 4(a)</td>
<td>61</td>
<td>18°</td>
</tr>
<tr>
<td>Fig. 4(b) left</td>
<td>52</td>
<td>153°</td>
</tr>
<tr>
<td>Fig. 4(b) right</td>
<td>55</td>
<td>18°</td>
</tr>
<tr>
<td>Fig. 4(c) left</td>
<td>58</td>
<td>0°</td>
</tr>
<tr>
<td>Fig. 4(c) right</td>
<td>49</td>
<td>56°</td>
</tr>
<tr>
<td>Fig. 6(c)(10a)</td>
<td>26</td>
<td>116°</td>
</tr>
</tbody>
</table>

Fig. 11. Nucleation transients and fluorescence transients. Rows (a), (b) and (c) show data for water, physiological saline and 9.5 vol.% glycerol solution, respectively; see Figs 5 and 6 for experimental details. The three plots in the left column show the temperature measured at the cup base as the base was chilled from 0 °C to −10 °C at 5 °C min⁻¹ (each plot is a 120-s segment from a longer trial, starting with the solution at 15–20 °C). Note that the addition of solutes postpones nucleation, and that the nucleation transient is smaller when glycerol is included. The three plots in the right column show the average grey level of each image in the corresponding time series of x–y scans, normalized to the highest average in each series. The glycerol brightness trace spans 120 s.
Discussion

The optical-axis cryostage presents a compromise between the sample size and thermal controls needed for volumetric ice growth and the conditions for optimal confocal microscopy. System and protocol design involves performance tradeoffs among numerous physical parameters for observing a broad, dynamic ice interface under a thick liquid layer: (a) selection of objectives, (b) scanning mode, (c) refraction at and above the phase interface, (d) specimen depth and (e) thermal arrangement. These are discussed in order below.

Selection of objectives

Microscope objectives used with the optical-axis cryostage should have a working distance long enough to avoid interfering with the temperature gradient that drives ice growth. Choosing objectives for long working distance at the expense of NA does sacrifice some vertical resolution, but a lower NA is preferable when viewing between the steep walls of vertical lamellae because the marginal rays are less likely to intersect the phase interface.

A dry objective with cover slip has little influence on the thermal gradient because an air space insulates the cover slip from the objective. The cover slip provides a stationary upper surface, creating a constant liquid volume that is amenable to numerical modelling. This stationary boundary provides a natural reference point for measuring the progress of the ice. Unfortunately, the cover slip tends to collect bubbles that form when the gas solubility drops during phase change. In addition, the focal plane is typically under several millimetres of liquid, meaning that dry optics must be capable of scanning several millimetres below the cover slip. Because dry objectives are typically designed for specimens at minimal depth below a cover slip, deep scanning causes uncorrected divergence of the marginal and paraxial rays. The interface depth may be corrected for refraction of the marginal rays according to Eq. (2), where $n_2$ is the refractive index of the solution:

$$d_{\text{CORRECTED}} = d_{\text{MEASURED}} \left( \frac{n_2^2 - NA_{\text{avg}}^2}{1 - NA_{\text{avg}}^2} \right)^{1/2}. $$ (2)

An average value of numerical aperture, $NA_{\text{avg}} = \sin(\alpha_{\text{avg}})$, was used to account for the range of angles along which light travels between the specimen and the objective. The angle $\alpha_{\text{avg}}$ is a weighted average in spherical coordinates, assuming a beam intensity that is constant over the illuminated solid angle. For example, with the 10× objective, $n_2 = 1.33$, $\alpha = NA = 0.30$, $NA_{\text{avg}} = \alpha_{\text{avg}} = 0.22$, and $d_{\text{CORRECTED}}/d_{\text{MEASURED}} = 1.34$.

Immersion objectives provide better optical performance because they focus through a constant depth of liquid. Unlike horizontal cryostages, in which a vertical temperature gradient is undesirable, the optical-axis cryostage takes advantage of heat transfer through immersion objectives to maintain the desired gradient. The interface is warmed by conduction heat transfer or by the influx of water that fills the volume vacated by the rising objective. However, objectives should not approach the interface so closely that they suppress ice growth or cause morphological changes during vertical scanning. The 40× objective did noticeably retard ice growth near the lens, producing a more rounded ice interface typical of steep thermal gradients. The 20× objective permitted sharper lamellar ridges to form. Both the 10× and the 20× objectives were useful for measuring the ice growth rate, and the 20× objective provided the best combination of working distance and depth resolution.

Scanning mode

Non-confocal laser-scanning microscopy produced good contrast compared with conventional fluorescence methods and provided useful information about ice structure. Although these images did not match the resolution reported for confocal systems, tightening the vertical resolution via the confocal pinhole was counterproductive for two reasons. First, the pinhole reduced the light that is collected, requiring higher gain settings that increased electronic noise. Normally this noise is reduced by averaging images, but the moving interface precluded averaging. Second, the refractive ice–water interface displaces light travelling to the objective such that the apparent and actual positions of the fluorescing voxel do not coincide. In confocal mode, this displacement causes the light to be blocked by the pinhole plate, potentially eliminating features from a confocal image.

Confocal laser-scanning microscopy returns its best results when scanning narrow, stationary specimens at moderate depth through a medium of uniform refractive index. Volumetric fluorescent emitters such as dye solutions are harder to localize than line or point sources. Excellent 3- and 4-D images have been reconstructed by deconvolving the point spread function (PSF) of the primary objective to remove out-of-focus information (e.g. Carrington et al., 1995). However, the PSF of an ice interface varies in both space and time, and it does not appear theoretically possible to deconvolve an image stack to determine consistent functions for the phase interface location and dye concentration field.

Refractive and processing artefacts

Although the arrangement of the optical-axis cryostage provides an unimpeded view of the leading edge of the lamellar ice interface, the refractive interference increases as the focal plane reaches the bottom of the lamellar troughs because a majority of the light experiences refraction or reflection. The refractive index of ice (1.31) is lower than that of water (1.33; Weast, 1987), so light striking ice from the solution reflects totally at incident angles greater than 80°, or arcsin(1.31/1.33). Ice surfaces must lie more than 23° from vertical to avoid total internal reflection with our 10×/0.3NA dry objective, i.e. **© 2004 The Royal Microscopical Society, Journal of Microscopy, 216, 249–262**
[\text{arcsin}(0.3/1.33) + 10^\circ]. Of course, the fraction of light that is internally reflected decreases gradually from unity as the incident angle decreases and the preponderance of light from the 10\times and 20\times objectives maintains a shallow angle with the vertical lamellar surfaces. Therefore, the lamellar interface reflects most of the light downward into the interstitial spaces and increases the brightness of the fluorescent dye.

It is not clear whether features that are visible when focusing into the lamellar troughs are real or are caused by refraction in the lamellar ridges. As expected in dendritic growth, secondary branches appear in the later images of Fig. 6. However, the branch locations coincide with clefts in the lamellar ridges, suggesting that the observed branches are spurious. The influence of intervening ice is also visible in x–z scans such as Fig. 7(a), where the lamellar surfaces appear to be straight lines extending from the spots of highest intensity. The regularity of these features suggests that they are due to the NA of the objectives rather than the interface morphology.

**Dye concentration**

The maximum usable dye concentration is limited by light absorption in the thick liquid layer. Because the entire cone of illumination passes through several millimetres of uniform dye solution, the dye in this solution must be barely detectable. A small volume of dilute dye at the interface would be undetectable, so imaging depends on multiplication of the dye concentration by exclusion from the growing ice. The amount of dye that is concentrated near the front depends on the interface morphology, the interface velocity and the mass diffusivity of dye in solution. The dye multiplication factor was highest at the planar interface (filtered water) and lowest when the front is concentrated near the front depends on the interface morphology, the interface velocity and the mass diffusivity of dye in solution. The dye multiplication factor was highest at the planar interface (filtered water) and lowest when the front comprised widely spaced lamellae (glycerol solutions).

The 5 p.p.m. Na-Fl was adequate for all ice morphology observations presented here and theoretically caused only 4\% attenuation in a 3-mm layer of saline solution (measured absorbance of 0.14 cm\(^{-1}\)). The progressively higher gain needed with dye concentrations below 1 p.p.m. introduces pronounced salt-and-pepper noise even in non-confocal images. In phosphate-buffered saline, the interface was barely visible with dye concentrations down to 0.05 p.p.m. (130 nm) in non-confocal mode, using the 20\times objective and chilling the base at 5 \textdegree C min\(^{-1}\).

Excessively concentrated dye suffuses the liquid with green emitted light, making the illumination diffuse rather than focused. When the dye concentration was near 50 p.p.m., the ice interface was visible as a reflecting surface rather than as a dye-exclusion volume. A similar effect is produced when the specimen is illuminated with a krypton UV bulb and viewed with epifluorescence microscopy. In this case, the objective illuminates a large volume, drastically reducing the contrast between ice and solution. Laser scanning illuminates only a small area, so higher contrast levels can be obtained. Therefore, although the confocal mode per se was not advantageous, laser scanning on the confocal microscopy system did enable the cross-sectional image capture that was desired from the optical-axis cryostage.

**Spacing analysis**

The analysis method best suited to measure lamellar spacing depends on the original image quality, the time available for manual manipulation and the information desired. Fourier analysis quantifies the two most striking characteristics of ice lamellae, namely their spacing and alignment in the x–y plane. The FFT is suitable for square images with a consistent lamellar field. Because the FFT does not require a binary skeleton, it can operate on images with fused/branching lamellae and broad intensity gradients, both of which complicate automated thresholding routines. Whereas the FFT method returns a single spacing vector (distance and direction), the EDM produces an unorientated distance population that can be averaged but that is more useful as a histogram. The EDM does not require images that are square or that have consistently orientated ice, although the 4\times scaling factor does assume parallel lamellae. Manual ridge marking for the EDM is time-consuming, but it is more reliable than automatic thresholding.

The histogram of the EDM provides some additional suggestions regarding how the interstitial space is distributed. For example, the largest spacing in the histogram equals the radius of the largest circle that can be inscribed among the lamellar ridges. The average lamellar spacing found using the EDM (Fig. 10c,d) is 33 \mu m, which exceeds the values reported in Table 3 by about 20\%. Whereas the FFT measures only the most dominant series of parallel lamellae, the EDM accounts for incomplete or half-length ridges, which leave additional open space and produce a larger average spacing.

**Thermal performance**

The optical-axis cryostage was able to establish base chilling rates of more than 30 \textdegree C min\(^{-1}\) (data not shown), enough to create a highly non-planar phase interface. The limiting factor with respect to interface velocity was the time needed to complete the raster scan: increasing the velocity increases the extent to which visible features will have changed between the beginning and end of the scan. Cooling rates within the sample cup are limited by the thermal properties of water, just as they are in bulk tissue, solution or suspension. The large thermal capacity of the pipe cap and the thermal resistance of water stabilize the feedback temperature against thermal oscillations and dissipate small temperature fluctuations at the base before they affect the interface. After nucleation the pipe cap becomes thermally coupled to ice, which has higher thermal conductivity and gives the base a higher effective thermal capacitance. The result is a longer response time that causes greater fluctuations in the nitrogen flow rate.

The thermal control algorithm was effective except during ice nucleation transients. The integral term in Eq. (1) is required.
because holding the temperature constant below ambient temperature requires a steady flow of nitrogen. The controller does not include an active heater, and instead the copper cap is heated passively by the surrounding stage base. This situation differs from pure proportional control systems, in which the equilibrium value ideally requires zero corrective input and the forcing function can take positive or negative values.

The solutions were separated from the heat sink by a foil liner in order to allow cups to be exchanged when the solution was frozen (the ice is difficult to separate from the lower boundary). This additional thermal resistance altered the boundary temperature, as shown by simultaneous measurements on the base of the freezing cup at the centre, and 4 mm and 8 mm from the centre. After nucleation when chilling at 5 °C min⁻¹, \( T_{\text{min}} = T_{\text{centre}} + 2.5 \, ^\circ \text{C} \), and \( T_{\text{min}} = T_{\text{centre}} + 7 \, ^\circ \text{C} \). When chilling at 10 °C min⁻¹, the differences were about 0.5 °C larger. The side of the pipe cap itself was colder than the \( T_{\text{centre}} \), indicating that \( T_{\text{min}} \) is high because the outer 2 mm of the pipe cap curves down away from the sample cup. The pipe cap itself experiences some radial temperature gradient because the liquid nitrogen impinges at its centre before spreading. Nonetheless, this temperature distribution did not cause any visible curvature in the area of the ice front that was viewable by the objectives.

The uniformity of the base temperature could be improved by using a flat plate (in lieu of the pipe cap) and by allowing direct contact between the solution and the plate. Such an arrangement would eliminate the potentially uneven thermal coupling through the heat transfer paste, and the gap between the metal foil and the curved edges of the pipe cap. Because the liquid nitrogen is drawn through by applying a vacuum, the connection between the flat plate and the remainder of the freezing stage should be thermally insulating (to prevent heating of the rim of the plate), well sealed (to prevent inward leakage) and rigid (to prevent the vacuum from drawing the plate downward, as was observed in preliminary trials). Thermoelectric coolers were also tested, but rejected, as an alternative to direct application of liquid nitrogen. The amount of waste heat generated during solidification of several millilitres of solution is large and intermittent; given that a cooler imposes a temperature difference between its faces, such a system appears to require two control systems (one for the thermoelectric cooler and one for a nitrogen-cooled heat sink).

It is difficult to make accurate temperature measurements at locations above the cup base. There are no solid surfaces on which to affix thermocouples, and heat conduction through the copper thermocouple wires can overwhelm the convective heat transfer from water surrounding the small weld bead. Without accurate temperature data near the phase interface, the exact cooling rate and gradient were not known. In this regard, the optical-axis cryostage complements more thermally precise methods such as thin-specimen cryomicroscopy and differential scanning calorimetry in thermodynamic studies of ice crystallization.

Mounting a thermocouple vertically from the objective allows it to move with the focal plane, but also causes the readings to be high due to heat conduction through the wires. To improve our estimation of the interface temperature, we used a horizontal thermocouple to calibrate a vertical thermocouple and create a piecewise linear correction function. The temperatures reported in the captions to Figs 5 and 6 use this corrected data, which correlated well with the passage of the ice front.

**Conclusions**

The optical-axis freezing stage offers a way to observe interface morphology in a broad moving ice front, unconstrained by the container walls. Microscopic analysis shows that in saline and glycerol solutions ice grows as narrow, vertical lamellae that are amenable to 2-D phase-change modelling. The ice can be seen clearly in cross-section along the ridges and displays dimensions and complexity that have not been evident previously. As expected, the image quality suffers as the focal depth increases. The combination of optical arrangement and dynamic interface morphology is unique, so there is no basis for direct comparison, but the lamellar patterns are consistent with published results obtained by sectioning ice after freezing.

The optical-axis cryostage pushes the limits of deep scanning techniques and the LSM 10 microscope. Observing a dynamic phase interface under controlled thermal conditions requires compromises in some of the prerequisites for optimal confocal microscopy. Namely, the immersion objectives were chosen for long working distance at the expense of NA; the ice–water interface was reflective and refractive; the moving interface precluded image averaging; and volumetric fluorescent emitters such as dye solution were harder to localize than line or point sources. As a result, the images do not match the spatial resolution reported for confocal optical systems and desired for precise measurements of ice morphology and cell volume. Nonetheless, the cryostage provided unique morphological observations and invited exploration of the relevant optical challenges.

Performance of the optical-axis cryostage can be improved by further device engineering. Four potential modifications by which the optical and mechanical performance of the system may be enhanced are identified below.

**Ice tracking**

Adding a method to track or predict ice growth automatically would enable the control routine to keep the ice–water interface in focus. In addition to the quality of individual images, obtaining a time series that is consistently focused near the lamellar ridges permits the lateral velocity of slanted ridges, and hence their growth angle, to be calculated. Ideally, the ice height would be measured non-invasively, using a sensor that does not alter the ice height. This measurement may be done indirectly by comparing fluorescence spectra to determine

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local temperature (Aurich & Förster, 1984), a method that requires a microscope system with spectroscopic attachments.

Automated scan-plane rotation

Quantifying the lamellar morphology from vertical (x–z) scans is most easily done when the scans are perpendicular to the lamellae. The operator can orientate the scan plane by observing one x–y image of the lamellae, estimating a perpendicular line, and then rotating either the scan plane or the sample cup. This process could be automated by performing horizontal scans periodically until lamellae are first discernible. This image would then be transformed to the frequency domain using a 2-D FFT and analysed to determine the direction of maximum spectral energy. The line that passes through the first-order peaks is perpendicular to the dominant lamellae and therefore lies at the desired angle for x–z scanning.

Thermal design enhancement

The cryostage does not include a heater at the warm upper boundary, so the temperature gradient, cooling rate and interface velocity could not be set independently (setting one by controlling the base temperature alone would determine the other two). Ways to add this capability using an electrically resistive cover slip or a heated disc around the immersion objective were explored but were not implemented.

Image interpretation

Determining the physical location and true fluorescence intensity of each pixel or voxel is complicated by the deep fluorescent volume and refractive ice interface. Images of calibration specimens will help in the interpretation of subtleties of shading and the exact shape of the interface. Ideally, a calibration device would include submillimetre lamellar features made from a polymer that has the same optical properties and smoothness as ice. Such microscale features are achievable using photolithographic techniques, but the relevant materials (e.g. glass, silicon, epoxy, silicone) have refractive indices higher than that of water, so they do not mimic the refractive phenomena at the ice–water interface. To achieve the desired refractive effects, the water could be replaced with a liquid having a refractive index slightly higher than the test specimen.

Acknowledgement

This research was sponsored in part by the National Science Foundation Grant No. CTS-9632378.

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