Time-Lapse, in Situ Imaging of Ice Crystal Growth Using Confocal Microscopy

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ABSTRACT: Ice crystals nucleate and grow when a water solution is cooled below its freezing point. The growth velocities and morphologies of the ice crystals depend on many parameters, such as the temperature of ice growth, the melting temperature, and the interactions of solutes with the growing crystals. Three types of morphologies may appear: dendritic, cellular (or fingerlike), or the faceted equilibrium form. Understanding and controlling which type of morphology is formed is essential in several domains, from biology to geophysics and materials science. Obtaining, in situ, three dimensional observations without introducing artifacts due to the experimental technique is nevertheless challenging. Here we show how we can use laser scanning confocal microscopy to follow in real-time the growth of smoothed and faceted ice crystals in zirconium acetate solutions. Both qualitative and quantitative observations can be made. In particular, we can precisely measure the lateral growth velocity of the crystals, a measure otherwise difficult to obtain. Such observations should help us understand the influence of the parameters that control the growth of ice crystals in various systems.

INTRODUCTION

Understanding and controlling the growth of crystals, and in particular of ice crystals, is of interest in many domains, from geophysics to biology to food engineering. In materials science, the growth of ice crystals in colloidal suspensions is used in processing routes called “ice templating” or “freeze-casting,” where the unidirectional growth of the crystals and their successive removal by sublimation are used to template porosity in various materials. The microstructure, architecture, and properties of these materials are related, to some extent, to the morphology of the crystals grown during freezing. The dimensions and shape of ice crystals can be controlled, for example, by the cooling rate, the temperature gradient, the concentration and the composition of additives and particles, the pH condition, or the presence of externally applied magnetic or electric field. The ice crystals may exhibit three different morphologies: dendritic, cellular (fingerlike), or smoothed and faceted structures, as described by Gibbs–Curie–Wulff equilibrium. Two such examples are given by snowflakes or ice crystals in saline solutions.

Several additives have been proposed to control the growth morphologies of ice crystals, with varying degrees of success. These additives can be water-soluble solutes (sucrose, ethanol, and glycerol) or polymers (PVA and PEG). If these additives all affect, to some extent, the freezing point, growth kinetics, and morphologies of the ice crystals, the latter almost always adopt cellular or dendritic morphologies. A form of Gibbs–Curie–Wulff equilibrium could be achieved by using ice-binding proteins in solution (also called “anti-freeze proteins”, AFPs). Ice shaping is the result of their binding to ice. The first proteins to be discovered were the anti-freeze glycoproteins (AFGPs) in Antarctic fishes, followed by the report of Duman and DeVries of a helical peptide in the serum of winter flounder. Both AFPs and AFGPs can control the freezing temperature, the crystal growth, and the size of ice crystals during freeze–thaw cycles in the serum of fishes, insects, and plants that experience subzero temperatures.

Zirconium acetate (ZrAc) and zirconium hydroxyacetate (ZrHAc) are probably the only inorganic compounds that mimic the ice-faceting properties of AFMs. The growth of ice crystals in the presence of ZrAc probably occurs under the Gibbs–Curie–Wulff equilibrium. ZrAc can thus be used as a simple model system to grow faceted crystals and measure their growth kinetics. ZrAc in water is organized as tetrameric stacks, which might mimic the repetitive motif of certain AFPs.

Although several methods have been proposed to follow the growth of ice crystals in situ, none of them can provide three-dimensional (3D) real-time observations without affecting the system. Optical microscopy is limited to two-dimensional (2D) surface observations. Computed X-ray tomography can provide time-lapse 3D observations, but the local absorption of synchrotron X-rays increases the temperature and affects the growth behavior and morphology of the ice crystals. Optical interferometry has been used to track the freezing front, but it cannot provide information about the morphology of ice.
crystals. Transmission electron microscopy can be used to image the growth of crystals and the behavior of particles repelled by the latter, but such experiments cannot yet be run routinely and the interaction of the electron beam with the sample is always questionable. Near-infrared imaging spectroscopy was used to investigate the morphology of ice crystals in biological materials, but freezing was not performed in situ in these studies. Crystal growth was followed by image analysis, which allowed us to carefully select the $x-z$ plane to measure: to get an overall insight into the ice crystal geometries and growth kinetics, we select a priori a plane where we expect that the sulforhodamine B fluorescence will dominate the transparency of the ice and that we will observe a few large crystals. Because of the supercooling effects when cooling from room temperature, we opted for a seeding protocol. The solution is quickly frozen to trigger the nucleation of ice, and the sample is then brought back to a temperature of $-10^\circ C$ and thermalized for 10 min. The ice recrystallizes at this temperature, leaving just a few crystals. We can then use these seeds to grow larger crystals in controlled conditions.

The critical point of the water/ice phase transition, the ice faces a density change of approximately $-8\%$. This change in density must be accommodated either by changing the volume (fixed internal pressure) or by changing the internal pressure (at fixed volume). We hypothesize that a change in internal pressure occurs, and the excess of pressure is accommodated by lifting the top glass slide, that is, by increasing the volume: an effect similar to frost weathering (ice can exert pressures on the order of thousands of bars). Due to this supercooled state and by observing small crystals after the freezing burst, we speculate that in a free-falling temperature experiment there are two stages for ice growth: the rapid dendritic/cellular growth followed by an apparent regular Gibbs–Curie–Wulff equilibrium growth when the crystal tips reach the position where $T = T_{\text{eq}}$.

**Imaging Ice Crystals.** An example of what we can image both in the $x-z$ and in the $x-y$ planes is shown in Figure 2.

The growth orientation on the $x-y$ plane can be tilted with respect to the edge of the image (Figure 2b). In the $x-z$ plane, the ice crystals may thus be probed at a certain angle, which partially explains the elongated morphology of the crystals along the $x$ direction (Figure 2a).

By collecting a series of similar images of the same $x-z$ section as a function of time (Figure 2), the growth of ice crystals can be visualized as 3D objects (Figure 3).

We can use such series of images to measure the transversal and longitudinal growth velocities of the ice crystals. Let us consider two cross sections at different times (Figure 4) of the sample shown in Figure 3. A straight line is drawn across the face of the crystal or the facet of the ice crystal moves perpendicular to this line. The series of frames is then resliced along the line without interpolation. The resulting image intensity is renormalized and binarized. A simple code written in Octave searches for the edge and displays the results in terms of the length of trajectory and growth velocities.

**RESULTS**

**Freezing Process.** We first verified that sulforhodamine B has no effect on the morphology of the ice crystals. A sample of 60 µL of a pristine 1 mM sulforhodamine B solution was diluted in 1 mL of deionized water. A sample of 12 µL was prepared and frozen. The results of the 3D topography of the frozen solution at the temperature of approximately $-22^\circ C$, cooled at $\Delta T = -1^\circ C/min$, is shown in Figure 1.

![Figure 1. Three-dimensional topography of brine channels (with sulforhodamine B) in ice. Visualization of the trace of sulforhodamine B cooled from room temperature to approximately $-22^\circ C$ at the rate of $\Delta T = -1^\circ C/min$. The fluorophore is expelled from the frozen water and concentrate in small rounded pockets. Similar effect is found in freezing of salty water and the formation of brine pockets. The pockets are aligned along the freezing direction $y$. The thickness is 28 µm.](image-url)

No regular structure is observable in the sulforhodamine B trace. As expected, water, while freezing, rejects brine, which also contains the fluorophore accumulated at the grain boundaries. With the proper fluorophore, we can thus quantitatively and thermodynamically follow the growth of ice crystals, with or without ZrAc in the solution.

The freezing of #2(TMA-OH) and #2(NaOH) solutions was observed as a function of time in the $x-y$ plane at full cooling velocity to refine the sample position, to check the extent of condensation, and to capture the longitudinal dendritic growth of the ice crystals.

Generally no freezing was observed until the temperature of the Peltier stage was below $-15^\circ C$. Due to the supercooled state of the solution, the ice initially bursts into small dendritic crystals, which densely populate the slab, hindering any observation. These crystals will grow until the temperature reaches the equilibrium melting temperature $T_{\text{eq}}$. This obliges us to carefully select the $x-z$ plane to measure: to get an overall insight into the ice crystal geometries and growth kinetics, we select a priori a plane where we expect that the sulforhodamine B fluorescence will dominate the transparency of the ice and that we will observe a few large crystals. Because of the supercooling effects when cooling from room temperature, we opted for a seeding protocol. The solution is quickly frozen to trigger the nucleation of ice, and the sample is then brought back to a temperature of $-10^\circ C$ and thermalized for 10 min. The ice recrystallizes at this temperature, leaving just a few crystals. We can then use these seeds to grow larger crystals in controlled conditions.

The supercooled state can be explained by the lack of nucleation points, the colligative depression of the freezing point caused by the electrolyte, and the confined state of the solution (analogous to a similar effect in very small capillaries). We believe that due to the confinement, the nucleating water must override a change in internal pressure. At the critical point of the water/ice phase transition, the ice faces a density change of $-8\%$. This change in density must be accommodated either by changing the volume (fixed internal pressure) or by changing the internal pressure (at fixed volume). We hypothesize that a change in internal pressure occurs, and the excess of pressure is accommodated by lifting the top glass slide, that is, by increasing the volume: an effect similar to frost weathering (ice can exert pressures on the order of thousands of bars). Due to this supercooled state and by observing small crystals after the freezing burst, we speculate that in a free-falling temperature experiment there are two stages for ice growth: the rapid dendritic/cellular growth followed by an apparent regular Gibbs–Curie–Wulff equilibrium growth when the crystal tips reach the position where $T = T_{\text{eq}}$.

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From the analysis of several crystals grown in controlled freezing upon seeding, we can obtain a mean value of the transverse growth velocities $v_t$ (Table 1). For a cooling rate of $\Delta(T) = -1^\circ$C/min, the growth velocity in the TMA-OH solution is slightly lower than that in the NaOH solution. However, no differences are found for a cooling rate of $\Delta(T) = -2.5^\circ$C/min. The growth rate of the crystals in the NaOH solution is the same for the two cooling rates. We cannot at this point conclude whether the growth of the crystals is kinetic- or diffusion-limited. At this stage, the liquid water surrounding the crystals is heavily charged in solutes, which slow down the freezing. The diffusion of the solutes is very limited because of the low temperature and the already high solute concentration.

To better understand how the morphology of the ice crystal changes during its growth, we developed a Python code that searches for the liquid-crystal interface. Each frame is first filtered using a Gaussian filter that smoothens the image, which is then segmented. Isocontours are drawn, and the largest closed one is selected as the ice crystal area. The filter and the detection of contours are obtained from the Skimage and Trackpy35 packages. The code developed to track the contours and compute the growth velocities is available on Figshare for anyone to review, reuse, improve, or adapt.36 In Figure 5, the evolving contours of the central crystal from Figure 3 are shown together.

The growth of the crystal follows several steps by changing its apparent geometry from quasi-triangular to hexagonal. The apparent geometry depends on the viewing angle, as found previously. In Figure 6, we select five frames from Figure 5 with different geometries.
From these contours, we can measure the temporal evolution of the perimeter and area of the crystal as well as its growth velocity. The results for four representative crystals grown from the #2(TMA-OH) solution cooled at $\Delta(T) = -1 \, ^\circ\text{C}/\text{min}$ are shown in Figure 7. The four crystals show similar growth dynamics. The same conclusion can be drawn from the instantaneous velocity results (Figure 8). The weighted average velocity $\nu_A$ is $6.1 \pm 0.4 \, \mu\text{m}^2/\text{s}$ (computed for one of the crystals).

Figure 7. Growth dynamics. Area of individual crystals vs time. #2(TMA-OH) at $\Delta(T) = -1 \, ^\circ\text{C}/\text{min}$. The area was computed for the four crystals in the inset.

Figure 8. Area velocity. #2(TMA-OH) at $\Delta(T) = -1 \, ^\circ\text{C}/\text{min}$ postseeded. Area velocities computed using the data in Figure 7.

Nevertheless, we should stress that because the cooling stage does not provide a good control of the temperature gradient, our measurements are somewhat position-dependent. The same procedure was used to measure the longitudinal growth velocities $\nu_l$ in both solutions. The values obtained for the two solutions and two cooling rates are given in Table 2.

The values of $\nu_l$ depend only on the cooling rates; neither NaOH/HCl nor TMA-OH contents seem to play any role.

Table 2. Average $\nu_l$: $\Delta(T)$ in $^\circ\text{C}/\text{min}$, $\nu_l$ in $\mu\text{m}/\text{s}$

| $\Delta(T)$ | #2(NaOH) | #2(TMA-OH) |
|-------------|-----------|------------|
| $-1$        | $0.9 \pm 0.4$ | $0.9 \pm 0.5$ |
| $-2.5$      | $2.4 \pm 0.5$ | $2.4 \pm 0.4$ |

A typical 3D topography of ice crystals grown from the #2(TMA-OH) solution (after final cooling at $T = -21 \, ^\circ\text{C}$) is shown in Figure 9. Similar morphologies were obtained for all the solutions and cooling rates.

Figure 9. Three-dimensional topography of ice crystals grown from the #2(TMA-OH) solution cooled at $\Delta(T) = -1 \, ^\circ\text{C}/\text{min}$. The tips of the ice crystals look like sharp chisels. Front view (top) and back view (bottom). Dimensions are $400 \times 200 \times 46 \, \mu\text{m}^3$.

A more careful observation of single $x$–$z$ images from the 3D topography shows that single crystals can result from the effective merging of smaller ones protruding from the cold side: the sequence in Figure 10 of six cross sections illustrates such merging. In the first cross section (namely “frame 1”), where $\Delta y = 0 \, \mu\text{m}$ represents the beginning of the sequence, we see three crystals, whose partial contours are highlighted by dotted lines. Two of them are already partially merged. At frames 18 and 47, we observe a cross section at $\Delta y = 27.2$ and $73.6 \, \mu\text{m}$ from the beginning, and the gap among the three crystals closes, so that at frame 72 ($\Delta y = 113.6 \, \mu\text{m}$) only one crystal is visible. Finally, at frame 95 ($\Delta y = 150.4 \, \mu\text{m}$), the geometry is perfectly hexagonal and the shape and size are retained till frame 116 ($\Delta y = 184 \, \mu\text{m}$).

DISCUSSION

The freezing point of the solution is of particular importance. Best results, in terms of growing hexagonal crystals, can be achieved if, after the rapid freezing due to supercooling, one is able to bring back the solution to a temperature close to the melting point and melt until a few small crystals remain. In this case, only the small crystals that remain will grow and the solute concentration in the liquid phase will closely approximate the concentration of the unfrozen solution. A similar method is generally used, for example, to distinguish single ice crystals grown in a solution with green fluorescent protein (GFP) tagged AFP37,38 and to identify their positions with respect to the ice faces. In the current experiments, we cannot tag the ZrAc with a fluorophore because the fluorophore has to be smaller than the tagged molecules to avoid or reduce the interference with the kinetic and dynamic processes of freezing. Moreover, small fluorophores (such as the pyrene-based ones37) fluoresce under UV light that is unavailable for us with the current equipment, and tagging ZrAc with a fluorophore could limit the ability of ZrAc to self-assemble.

To better gauge and model the ice crystallization, the optical vertical resolution and the ability to isolate noninteracting crystals during freezing should be optimized. Both conditions are inherent to the statistical analysis of velocities and
The ice crystal section expands monotonically, going through the shape change previously described, with a preference for the x-direction. This can be explained by the finite size of the liquid slab in the z-axis, whereas the x- and y-directions seem infinite from the point of view of the ice crystal. It would thus be worthwhile to compute the surface free energy of the ice when in solution with and without ZrAc. Within our range of measurements and setup, we cannot identify whether the accretion of crystals is diffusion-limited or kinetic-limited.30

To perform an optimal experiment, it is strongly needed to control the sample temperature with great precision, on the order of less than 0.1 °C. We observed that the vertices of the ice crystals were the first to reshape when the temperatures became less stable. The tips, in particular, are the weakest features. This observation raises the following question: if we model the ice needles as a pyramid on the top of a hexagonal prism (which correspond to the morphologies reported in presence of ice-shaping proteins), what will be the maximum ratio between the basal plane and the pyramid height at the thermodynamic equilibrium? Thus, which are the equilibrium crystallographic planes of ice in the presence of such additives? The observations obtained using the procedure described here could be used to validate or invalidate the different models proposed for this problem. ZrAc is easier to obtain and use when compared with AFPs; it can thus be used as a model system to grow faceted ice crystals, which is difficult to achieve with other additives.

We hypothesize that, as proved,30 ZrAc is organized as stacks, whose length is dependent on the concentration. Such stacks, through an adsorption mechanism similar to that of antifreeze proteins, hinder the dendritic and cellular growth of ice crystals. The regular stacks intuitively resemble the repeating motif of several antifreeze proteins. The adsorption mechanism is mediated by the acetate anions. To follow where the ZrAc goes while water freezes, one could exchange one acetate with a small fluorophore, for example, pyrene-based fluorophores,39 whose size and weight are comparable with the ones of Zr tetramers (bearing in mind the limitations previously discussed on the length and weight of the fluorophore). In this way, we may be able to track the dynamics of ZrAc clusters in the proximity of the ice crystal and, by measuring the local fluorescence intensity, we may be able to link the local density with the velocity of the ice front.41 Similarly, we could identify how the ZrAc settles on the ice face and whether the mechanisms are indeed similar to those of AFPs.42,43 Finally, by using a pH-sensitive fluorophore in solution, we can quantitatively and thermodynamically follow the freezing process to gain further insights into how the pH distribution is linked to Zr tetramers and stacks.

### EXPERIMENTAL METHODS

#### Experimental Set-Up.

All measurements were carried out using a Leica TCS SP8 (Leica-Microsystems, Mannheim, Germany) confocal laser scanning microscope. Confocal laser scanning microscopy has been primarily developed to image cells, proteins, or other biological machines tagged with a fluorophore.44 The technique is also of interest for studies in materials science,45 albeit rarely used so far.

The microscope is equipped with two external continuous laser sources, at 488 (blue) and 552 nm (green), driven to the optical section by two independent optical fibers. The maximum power on the optical plane is 10 mW for both of them. At these wavelengths, the absorption coefficient for

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**Figure 10.** Merging ice crystals. Selected sections from a series of 3D topography of ice crystals grown in the #2(TMA-OH) solution cooled at $\Delta(T) = -2.5$ °C/min. Top to bottom: the three crystals, highlighted by dotted contours on the top subfigure, merged into one and through an intermediate step become a single crystal with an hexagonal geometry.
distilled water is approximately $0.45 \times 10^{-3}$ cm$^{-1}$ at 552 nm and approximately $0.25 \times 10^{-3}$ cm$^{-1}$ at 488 nm.\textsuperscript{46,47} The minimal absorption and the small intensities of both laser beams imply that no energy is deposited along the optical path through the sample of few tens of micrometers, as opposed to X-ray tomography where the intense and collimated beam may locally melt the ice or change the temperature gradient.\textsuperscript{24,48} To focus the laser light at the region of interest, the sample stage is equipped with 3-axis micrometric drive. Submicrometer resolution for the $z$-axis (or the vertical direction) is ensured by a galvanometric stage with a run of 200 $\mu$m.

The light-detection section includes a rotating head that can contain six objectives: two of the positions are occupied by a Leica HCX PL APO 40x dry objective and a Leica HCX PL APO CS 20x dry objective: working distances of each is 670 $\mu$m. This short length hampers the use of any encapsulating box filled with nitrogen to avoid the condensation of water on top of the glass slide, as described in ref 37. The collected light is detected by two photomultiplier detectors that are fed with photons whose wavelengths can be independently selected. The smallest optical window is 5 nm, and the two spectra cannot overlap. With this setup, one can independently capture two fluorophores. The digitalization of the integrated light is at 8-bits.

For the data collection, the 20x objective was used to image the $x$-$y$ plane and the $x$-$z$ section for all resolutions. For example, at a $x$-$y$ hardware resolution of 1024 $\times$ 1024 px$^2$ (pixels$^2$), the area sampled is $775 \times 775$ $\mu$m$^2$ at the minimum optical magnification of $M = 0.75x$ and a frame rate of 0.388 frames/s for the scanning frequency of 400 Hz, whereas for the $x$-$z$ section a hardware resolution of 1024 $\times$ 256 px$^2$ gives a scanned section of $400 \times 100$ $\mu$m$^2$ at the minimum optical magnification of $M = 1.45x$ and a frame rate of 0.357 frames/s at a scanning frequency of 100 Hz. For the measurements in the $x$-$z$ section, the optical plane is fixed and the sample is translated along the $z$-direction by the galvanometric stage. The microscope is controlled using the proprietary Leica LAS-AF software.

The sample stage was modified to accommodate a three-stage Peltier cooling device (Agilent Technologies), which is water-cooled using a chiller (Minichiller, Huber) connected through the sample of few tens of micrometers, as opposed to X-ray tomography where the intense and collimated beam may locally melt the ice or change the temperature gradient. To focus the laser light at the region of interest, the sample stage is equipped with 3-axis micrometric drive. Submicrometer resolution for the $z$-axis (or the vertical direction) is ensured by a galvanometric stage with a run of 200 $\mu$m.

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The sample stage was modified to accommodate a three-stage Peltier cooling device (Agilent Technologies), which is water-cooled using a chiller (Minichiller, Huber) connected from the backside. The temperature on the sample side is measured by a Pt100 sensor and controlled by a Lakeshore 336 temperature controller that drives the current amplifier of the Peltier stage. For this setup, the minimum temperature is $-25$ °C. However, we can confidently reach a temperature of $>-21$ °C under the working conditions. Below this value, the temperature cannot be stabilized for times longer than a few tens of minutes. The temperature measurement in the 0 to $-21$ °C range is accurate within 0.02 °C. The maximum linearly stabilized cooling rate is $\pm 5$ °C/min. Faster cooling rates are possible initially, but the linearity is lost around $-15$ °C.

**Sample Preparation.** A solution of zirconium acetate (in-house preparation of Saint-Gobain) at the initial concentration of 22.6 g/L (solution #1) of Zr (gravimetrically measured)\textsuperscript{17} and the original pH of 2.6 (measured using precalibrated Oregon Scientific pH-meter) was diluted in deionized water to obtain a solution with equivalent starting Zr concentrations of 13.3 g/L ($pH = 3.2$). Two identical batches were prepared. This concentration was chosen because it is the lowest concentration at which ice faceting has been observed.\textsuperscript{18} The pH values of one (#2(NaOH)) of the batches were then adjusted to $pH = 4.0 \pm 0.1$ by adding 25 mol/L NaOH solution and HCl solution at 37 wt % (Sigma Aldrich). The latter, #2(TMA-OH), was carefully diluted with TMA-OH (tetramethylammonium hydroxide, Sigma Aldrich) at the concentration of 10 wt % in water without overshooting pH $\approx 4$. The objective was to titrate with two different base solutions to assess whether the ionic strength of the bases and the inorganic/organic nature of the bases influence the growth morphologies and/or kinetics. TMA-OH is a weak base ($pK_a = 4.2$ vs 0.2 for NaOH). The final Zr concentrations for the TMA-OH solutions were estimated to be 11.2 g/L.

Local gelation of the solutions was observed while adjusting using the bases: ZrAc eventually gelates at pH $> 4.4$. One night of stirring was enough for complete solubilization of the gelled part. Each solution was stained with 1 mM sulforhodamine B (Fluotechnik, France), 60 $\mu$L/mL. The absorption/emission spectra of sulforhodamine B are rather insensitive to pH in the range of 3–10,\textsuperscript{49} and its absorption band is excited by the laser light at $\lambda = 552$ nm (absorption peak at $\lambda_{max} = 566$ nm and emission peak at $\lambda_{max} = 584$ nm in water).\textsuperscript{50}

The fluorescent light is collected in the 575–625 nm range. To improve the image contrast at the liquid—solid interface, the reflected light of the blue laser ($\lambda = 488$ nm) is integrated in the 485–490 nm range. Following the method and setup proposed by Neils and Diller,\textsuperscript{50} we image the ice—water interface through the optical axis.

The measured sample is prepared as follows (Figure 11): a 22 $\times$ 22 mm$^2$ microscope cover slide (borosilicate D263M, VWR, thickness $\approx 170$ $\mu$m) is attached onto the copper plate using vacuum grease, which improves adhesion and thermal conductivity.

Half of the sample slide is in contact with the cold side of the stage (copper plate), whereas the other half is suspended in air because the copper plate is elevated above the surrounding metal plate, so there is no contact on this side. A temperature gradient parallel to the cover slip as well as perpendicular to its surface is thus created, and ice crystals grow from the cold side toward the hot side of the sample.
The images are post-processed and analyzed using Fiji. The only digital manipulations done before the analysis were contrast enhancement and normalization in the automatic mode. A cross section of the frozen sample #2(NaOH) is shown in two colors (Figure 13): magenta represents the fluorescent signal from sulforhodamine B diluted in water, whereas cyan is the reflected signal from different interfaces (top and bottom cover slides and water/ice interfaces). The dark hexagon-like regions are ice crystals.

A qualitative analysis was performed on the time and spatial x–z series, which we can use to reconstruct the topography of the ice crystals, using Fiji. The time series (x–z–t axes) can be used to image the growth of ice crystal, whereas the spatial series (x–y–z axes) show the 3D topography. Image segmentation was performed using the built-in tools available in Fiji.

**CONCLUSIONS**

We demonstrate here how laser scanning confocal microscopy can be used to investigate in situ the 3D growth of ice crystals, using a simple setup and a fluorophore. The approach was made using a model system, an aqueous zirconium acetate (ZrAc) solution, that results in faceted growth of ice crystals. Upon freezing, in analogy with ice-shaping proteins, ice crystals of predictable morphologies can develop in calibrated aqueous solutions. Because, like almost any solute, the fluorophore (sulforhodamine B) is expelled by the growing crystals, the ice crystals appear as black bodies immersed in a sea of fluorescence. We were able to follow qualitatively and quantitatively the kinetics of the growing crystals using solubilized ZrAc tetramers. This methodology can also be used to investigate, for example, the growth in the presence of simple polymers such as PVA, new promising ampholitic polymers, either tagged or untagged with fluorophores, such as it has been proved with ice-shaping proteins. As a minimal amount of energy is deposited along the optical path in the sample, confocal microscopy appears much more appropriate than X-ray computed tomography, which is known to induce artifacts in the growth morphology of ice crystals.

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### ADDITIONAL NOTES

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