Site-Specific Preparation of Intact Solid–Liquid Interfaces by Label-Free In Situ Localization and Cryo-Focused Ion Beam Lift-Out

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Abstract: Scanning transmission electron microscopy (STEM) allows atomic scale characterization of solid–solid interfaces, but has seen limited applications to solid–liquid interfaces due to the volatility of liquids in the microscope vacuum. Although cryo-electron microscopy is routinely used to characterize hydrated samples stabilized by rapid freezing, sample thinning is required to access the internal interfaces of thicker specimens. Here, we adapt cryo-focused ion beam (FIB) “lift-out,” a technique recently developed for biological specimens, to prepare intact internal solid–liquid interfaces for high-resolution structural and chemical analysis by cryo-STEM. To guide the milling process we introduce a label-free in situ method of localizing subsurface structures in suitable materials by energy dispersive X-ray spectroscopy (EDX). Monte Carlo simulations are performed to evaluate the depth-probing capability of the technique, and show good qualitative agreement with experiment. We also detail procedures to produce homogeneously thin lamellae, which enable nanoscale structural, elemental, and chemical analysis of intact solid–liquid interfaces by analytical cryo-STEM. This work demonstrates the potential of cryo-FIB lift-out and cryo-STEM for understanding physical and chemical processes at solid–liquid interfaces.

Key words: cryo-scanning transmission electron microscopy, cryo-focused ion beam lift-out, solid–liquid interfaces, spatially resolved electron energy loss spectroscopy

INTRODUCTION

Solid–liquid interfaces play a pivotal role in many fields, such as crystal growth in solution and hydrogels, which can guide crystals to unique morphologies and properties (Henisch, 1988; Asenath-Smith et al., 2012); biomineralization, where biological organisms form composite materials through organic–inorganic interfaces (Weiner & Addadi, 2011); and energy materials research, where electrode–electrolyte interfaces play a key role in the operation, performance, and safety of batteries (Tarascon & Armand, 2001; Goodenough & Kim, 2010). Transmission electron microscopy (TEM) and scanning transmission electron microscopy (STEM) provide atomic-resolution structural, elemental, and bonding information about solid–solid interfaces in hard materials (Muller, 2009; Botton, 2012; Mundy et al., 2014), but have thus far been limited in their applications to solid–liquid interfaces. This is due to the fact that in conventional electron microscopy these samples must be dried before entering the high vacuum of the microscope column, which can alter structure and chemistry of solid–liquid interfaces.

Cryo-TEM is a well-established technique in the biological sciences designed to address this issue and allows the near-native structure of thin frozen-hydrated specimens to be investigated at the nanoscale (Müller et al., 2008; Kourkoutis et al., 2012; Milne et al., 2012; Lučić et al., 2013; Cheng et al., 2015). Samples are cryo-immobilized by rapid freezing, which vitrifies the solution and avoids the formation of ice crystals that can damage the biological structure (McDowall et al., 1983; Dubochet et al., 1988). However, specimens that cannot be snap-frozen directly to an electron transparent thickness, such as whole cells, must be thinned before analysis in the cryo-TEM. Traditionally, cryo-ultramicrotomy served this function by using a diamond blade to slice thin sections from a larger sample (Al-Amoudi et al., 2004). However, cryo-ultramicrotomy lacks site specificity, and the physical cutting process induces unavoidable image artifacts (Al-Amoudi et al., 2005). As a result, cryo-focused ion beam (cryo-FIB) was developed in recent years as an alternative thinning method that addresses these issues.

Traditional FIB lift-out at room temperature is routinely used for site-specific preparation of high-quality TEM samples from bulk materials and devices (Giannuzzi & Stevie, 2005). In this method, a focused beam of ions (typically Ga+) is used to site-specifically remove material around a site of interest, forming a cross-sectional slice, or lamella, which is extracted from the bulk sample and transferred to a TEM grid using a nanomanipulator. Subsequently, the lamella is thinned to electron transparency...
for TEM work. The first attempt to modify this technique for preparation of frozen-hydrated biological samples was made by installing a liquid nitrogen cooled stage in a FIB, which enabled thinning of whole frozen cells and subsequent imaging of sub-cellular structures by cryo-TEM (Marko et al., 2006a, 2006b, 2007). In contrast to the traditional FIB lift-out preparation, these cryo-TEM samples were prepared directly on the TEM grid by milling entirely through the frozen specimen at a shallow angle, leaving a thin lamella nearly parallel to the supporting grid. Although variations of this “on-grid” cryo-FIB technique have been successfully applied to a range of cells (Marko et al., 2007; Hayles et al., 2010; Rigort et al., 2010; Rigort et al., 2012; Villa et al., 2013; Arnold et al., 2016), it is limited to samples on the order of microns thick. Thicker samples may be too thick to mill entirely through or require longer milling times with increased beam currents, which can induce sample damage. For preparation of bulk soft materials or large samples with internal solid–liquid interfaces, such as hydrogels with embedded crystals or electrochemical energy storage devices, a technique similar to the traditional FIB lift-out method was needed, with the added challenge of maintaining the sample temperature below the devitrification point during the entire process. In recent years, developments of cooled nanomanipulators in combination with existing cryo-stages have enabled proof of concept demonstrations of cryo-FIB lift-out of cryo-immobilized soft materials, such as frozen-hydrated Caenorhabditis elegans worms or hydrogels with embedded collagen fibrils (Rubino et al., 2012; Parmenter et al., 2014; Mahamid et al., 2015; Parmenter et al., 2016). In addition, cryo-FIB lift-out was recently also adapted for preparation of samples with buried solid–liquid interfaces for cryo-STEM analysis (Zachman et al., 2015).

Cryo-FIB lift-out is inherently site-specific, as the user defines the area in which the TEM lamella is prepared. As a result, methods are needed to localize subsurface structures of interest before milling, and to guide the sample preparation process. For biological specimens this issue has been addressed recently using a correlative approach, where fluorescent labeling of molecules and cryo-fluorescence light microscopy (cryo-FLM) can be used to localize structures of interest before cryo-TEM or cryo-FIB preparation (Sartori et al., 2007; Schwartz et al., 2007; Rigort et al., 2010; Mahamid et al., 2015). The sample must then be transferred to the FIB and properly aligned by correlating in situ cryo-FIB images with the ex situ cryo-FLM images to identify the region of interest. In addition to the inherent limited resolution of the light microscope used, which is worsened by thick or cryogenic specimens (Sartori et al., 2007; Plitzko et al., 2009; Mahamid et al., 2015), this correlation process itself introduces additional uncertainty to the expected positions of the structures (Sartori et al., 2007; Schellenberger et al., 2014). The result is an uncertainty in the final colocalized position of a structure typically on the order of a micron (Sartori et al., 2007; Agronskaia et al., 2008; van Driel et al., 2009), though with added fiducial markers and a more involved correlation process this can now be reduced by an order of magnitude (Kukulski et al., 2011; Schellenberger et al., 2014; Schorb & Briggs, 2014; Arnold et al., 2016). In addition, the added handling and transfer steps introduced by such a two-instrument method increase the likelihood of compromising the sample integrity through contamination or structural damage, especially at cryogenic temperatures (Agronskaia et al., 2008; Faas et al., 2013). Although these techniques can be used to localize labeled biological specimens with sufficient accuracy for cryo-FIB lift-out, alternative techniques are needed to localize subsurface structures that lack fluorescent labels.

Here, we present an in situ localization technique to identify elementally distinct subsurface structures directly in the cryo-FIB. We use energy dispersive X-ray spectroscopy (EDX) mapping to identify structures embedded microns below the surface of a cryo-immobilized material. Using these maps as guides, site-specific cryo-FIB lift-outs of targeted structures can be performed. We demonstrate this capability on silica hydrogel samples with iron oxide particles grown in their interiors (Asenath-Smith et al., 2015; Asenath-Smith & Estroff, 2015). EDX mapping is used at various stages in the lift-out process to identify regions of interest, localize subsurface particles, and monitor site-specific milling.

A lamella containing the structure of interest can then be lifted-out and subsequently thinned uniformly to electron transparency. The high quality of the resulting lamellae enabled the structure of inorganic particles embedded in hydrogel to be examined at the nanoscale by cryo-STEM. Elemental distributions across the interfaces, as well as the local bonding environments of the hydrogel and particles, were obtained using EDX and electron energy loss spectroscopy (EELS) in the cryo-STEM. These results demonstrate for the first time that cryo-FIB lift-out, in combination with cryo-STEM, can provide access to the structure and chemistry of intact solid–liquid interfaces at the nanometer scale.

**Materials and Methods**

**Sample Synthesis and Freezing**

A detailed description of the procedures used to synthesize the silica hydrogel and embedded iron crystals was described previously (Asenath-Smith et al., 2015; Asenath-Smith & Estroff, 2015). In brief, the silica hydrogel was produced by acidifying sodium metasilicate nonahydrate (0.5 M) with an equal volume of hydrochloric acid (1.0 M), and allowed to reach full gelation by setting for 24 h at 30°C. Subsequently, iron chloride hexahydrate solution (180 or 900 mM) was added to the surface of the silica hydrogel. The iron chloride solution was allowed to diffuse throughout the interior of the hydrogel by setting for 24 h at 30°C. To initiate the crystallization of iron (oxy-)–hydr(–) oxide nanocrystals within the hydrogels, the vessels were sealed and heated to 100°C. The particular crystallization protocol employed in this work provides a well-documented pathway to the formation of iron oxide (α-Fe₂O₃, hematite) after 4 weeks of reaction, which proceeds
through iron oxyhydroxide(s) [e.g., \(\beta\)-FeO(OH)] precursors that can be isolated after shorter reaction times, for example, 4 h (Blesa & Matijević, 1989; Asenath-Smith & Estroff, 2015). After cooling for 4 h at ambient conditions, specimens (~1 cm\(^3\)) were carefully extracted from the hydrogels, taking care to retain the structural integrity of the hydrogel. The specimens were rinsed by soaking in de-ionized water for 2 h; with water exchanges (6 ×) to remove all soluble salts. The specimens were stored under refrigeration in sealed vials until use.

To preserve the structures in their liquid environment, small pieces of the hydrogels (as seen in Fig. 6a) were removed with a razor blade and stabilized by rapid plunge freezing into slush nitrogen. Typically, plunge freezing of aqueous solutions can vitrify samples up to microns thick, whereas high-pressure freezing can vitrify samples up to hundreds of microns thick, but is slightly more involved to perform (Echlin, 1992; Studer et al., 1995). Formation of small ice crystallites in the hydrogel was not deemed prohibitive for the purposes of this study, so the simpler plunge freezing method was used.

**Instrumentation**

To maintain the sample at a temperature below the devitrification point throughout the lift-out procedure a Quorum PP3010T Cryo-FIB/SEM Preparation System (Quorum Technologies Ltd., East Grinstead, West Sussex, UK) was installed on an FEI Strata 400 FIB (FEI Company, Hillsboro, OR, USA). The system provides a cooled stage and anticontaminator in the interior of the FIB, as well as a separate cooled stage and anticontaminator in a preparation chamber attached to the FIB. The preparation chamber enables platinum sputter coating, controlled sublimation, and fracturing of frozen samples in a high vacuum before transfer into the FIB. To cool the system, gaseous nitrogen is flowed through a heat exchanger in a large liquid nitrogen dewar and subsequently through vacuum isolated Teflon lines to the FIB and preparation chamber. Figure 1a shows the cryo-FIB stage, thermally isolated from the rest of the FIB by a ceramic plate, and the Teflon cooling lines. During operation, the stages are maintained well below the devitrification temperature of the sample, typically ~165°C. The anticontaminators are operated at lower temperatures, typically ~192°C, to reduce ice contamination on the sample. To maintain the sample’s vitreous state during lift-out, an OmniProbe 200 Nanomanipulator (Oxford Instruments, Abingdon, Oxfordshire, UK) was fitted with a cooled needle assembly, as shown in Figure 1b. A ceramic rod thermally isolates the assembly from the uncooled shaft, and a copper braid attached to the anticontaminator in the FIB provides cooling. Temperature measurements using a Type T thermocouple soldered to the lift-out needle confirmed the minimum temperature is well below the devitrification temperature of water, as shown in Figure 2. The cooling rate of the needle nearly parallels the other system components, which confirms good thermal transport through the braid and connections.

Frozen samples are transferred into and out of the FIB on aluminum stubs placed in a shuttle that fits the cryo-stage. Sample stubs are loaded into the shuttle under liquid nitrogen in a workstation outside the FIB, and a vacuum transfer device is used to transport the shuttle from the workstation to the preparation chamber and back. The thermal mass of the shuttle ensures that no significant temperature change occurs during the <1 min transfer. The shuttle is further transferred into and out of the cryo-FIB chamber through a valve connecting it to the...
preparation chamber. Once the final lamella has been prepared and the shuttle is transferred back to the workstation, the lamella is removed from the shuttle under liquid nitrogen, placed in a cryo-TEM grid storage box, and stored in a liquid nitrogen dewar.

To attach a lamella to the cooled nanomanipulator needle in preparation for lift-out and subsequently to a TEM half-grid for thinning, an FEI Selective Carbon Mill (SCM) gas injection system (GIS) is used. The SCM introduces water vapor into the chamber, which has previously been shown to selectively enhance FIB milling of polymer-based materials at room temperature (Stark et al., 1995). The cryogenic sample temperature in cryo-FIB, however, results in the water vapor depositing nearly uniformly as amorphous ice, enabling sample attachment. A schematic of these cryo-FIB components is provided in Figure 3a. To ease handling and storage of the final lamella, as well as prevent the half-grid from falling through the cryo-TEM holder tip while loading under liquid nitrogen, we adhere the half-grid to a molybdenum slotted grid using M-Bond 610 epoxy, providing a rigid support. The half-grid is attached such that the curved side of the grid is in contact with the slotted grid, ensuring sufficient clearance for the nanomanipulator to reach the sample attachment location without contacting the slotted grid. These lift-out grids are transferred into and out of the cryo-FIB on a sample stub modified in-house to include a small lip and cam mechanism, which secures the grid to the stub and allows for simple grid removal under liquid nitrogen. Figure 1c shows a shuttle with a modified grid in the cam mechanism of the customized stub.

To minimize ice contamination during transfer to the cryo-TEM, samples are loaded into a Gatan 626 Cryo-Transfer Holder (Gatan Inc., Pleasanton, CA, USA) under liquid nitrogen. A shield on the holder tip is closed over the sample during the brief transfer to the microscope.

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**Figure 2.** Nanomanipulator needle cooling profile. The needle must be maintained below the devitrification temperature of the sample to prevent structural alterations and damage due to crystallization during lift-out. A T-type thermocouple was soldered to a cooled nanomanipulator needle (inset) and the temperature was measured as a function of cooling time. The approximate minimum devitrification temperature of water is shown as a dashed line, which the needle surpasses with under 15 min of cooling and stabilizes well below.

**Figure 3.** Schematic of the interior arrangement of the cryo-focused ion beam (FIB) and lift-out process. a: Cryo-FIB interior components consist of a cooled OmniProbe nanomanipulator, an FEI selective carbon mill gas injection system (which provides water vapor injection), a Quorum cold stage, and electron and gallium ion beams, separated by 52°. Steps to produce a lamella from a bulk sample include milling trenches on either side of the site of interest (b,d), lowering the nanomanipulator needle to the surface of the initial lamella, attaching the needle with water vapor, and cutting the lamella free and lifting it out (c,e). The lamella is then attached to a half-grid with water vapor, the needle is cut free, and the lamella is thinned to electron transparency.
For this work, a 200 kV monochromated FEI Tecnai F20 ST (S)TEM equipped with beryllium cryo-blades to reduce ice contamination on the sample in the vacuum of the microscope was used. A high-angle annular dark field (HAADF) detector at camera lengths of 100–300 mm was used for imaging, whereas a Gatan imaging filter 865-ER and an 80mm2 Oxford X-Max detector were used to acquire EELS and EDX spectroscopic data, respectively.

**In Situ Localization of Subsurface Structures**

Given the site-specific nature and limited throughput of cryo-FIB preparation, localization of subsurface structures of interest before lift-out is crucial for achieving an acceptable yield of these structures in the lamellae produced. Although this challenge was recently addressed in cryo-FIB preparation of fluorescently labeled biological specimens using correlative cryo-FLM and cryo-FIB (discussed above), alternative methods are needed for buried structures that lack fluorescent signatures.

Ideally, feature localization should be performed in situ, i.e. directly inside the FIB, as this would simplify the process compared with a correlative two-instrument approach. Depending on the experimental set-up, dual-beam FIB/scanning electron microscope (SEM) systems provide access to a range of signals, some of which can be used to detect subsurface structures. Backscattered electrons (BSE), for example, can escape from a moderate depth in the sample due to their high energy, revealing subsurface information. In addition, modern silicon drift X-ray detectors enable two-dimensional mapping of characteristic X-rays produced in the sample within reasonable timescales. EDX can therefore also be used to probe the interior of the sample. To evaluate the capabilities of these two signals, a model system of iron oxide particles grown in a silica hydrogel and protected by a 10 nm platinum capping layer was chosen. We performed Monte Carlo (MC) simulations of electron trajectories and sample interactions using MC X-Ray (Gauvin & Michaud, 2009) and CASINO (Hovington et al., 1997), with parameters chosen to mirror our experimental set-up (discussed below). An example of electron trajectories in a sample without an iron particle calculated with CASINO is shown in Figure 4a and demonstrates the range of 30 keV electrons in water.

To gain insight into the depth-probing capabilities of different signals, electron scattering calculations allow for a comparison of the relative ranges of BSE and characteristic X-ray generation in a water sample. Using a simple atomic model and empirical data, Kanaya & Okayama (1972) estimated the range for electrons incident on a material, in microns, as follows:

\[
R_{KO} = \frac{0.0276 A E_o^2}{Z^2 \rho},
\]

where \(A\) is the atomic weight (g/mol), \(Z\) is the atomic number, and \(\rho\) is the density (g/cm³) of the target material, and \(E_o\) is the incident electron beam energy (keV). By using values appropriate for water, we can estimate the maximum range of 30 keV electrons in our hydrogel sample, \(R_{KO} \cong 24\) µm, which is well beyond the depth of a typical lamella (on the order of 10 µm). Assuming this energy dependence can be used to calculate the remaining beam energy at a particular depth [a simple continuous-slowing-down approximation (Reimer, 1998)], the maximum depth a primary electron could reach before returning to the surface is \(\sim R_{KO}/2\). Monte Carlo simulations previously performed on a variety of materials, however, show that this number typically remains below \(\sim R_{KO}/3\) (Goldstein et al., 2003). In comparison, the range where characteristic X-ray generation can occur, in microns, is given by Anderson & Hasler (1966):

\[
R_X = \frac{0.064}{\rho} (E_o^{1.68} - E_c^{1.68}),
\]

where \(E_c\) is the energy of the characteristic X-rays generated (keV). For Fe-Kα X-rays generated in water using 30 keV

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Results of electron scattering calculations and Monte Carlo simulations. a: Example of 30 keV electron trajectories in a water with 10 nm platinum capping layer, calculated with the CASINO program. Blue paths signify electrons that came to rest in the material, whereas red paths indicate backscattered electrons (BSE). b: Results from calculations of the Kanaya–Okayama primary electron range, characteristic X-ray generation range, and data for BSE range, showing the useful depth probed by the characteristic X-ray signal exceeds that of the BSE signal. Approximate excitation volumes of the signals are also shown, using the shape of total energy deposition contours from CASINO as a guide.
Electrons the range \( R_X \approx 0.73R_{KO} \), which exceeds the range for BSE by a factor of greater than two. The X-ray signal can therefore be used to probe deeper into the material. A schematic of the relevant depths for BSE and X-rays, as well as the approximate corresponding excitation volumes of these signals, is shown in Figure 4b.

To gain a more detailed understanding of these signals, as well as visualize the viability of BSE imaging and EDX mapping for localizing subsurface features of interest, we performed Monte Carlo simulations of electron scattering in our model system. We simulated a range of iron oxide particle sizes embedded in water at various depths and beneath a 10 nm platinum layer to assess detection limits. A 30 keV electron beam was scanned over a constant two-dimensional field of view centered on the particle, and the BSE and Fe-K\( \alpha \) X-ray signals were computed using appropriate detector configurations (MC X-Ray Microscope settings modified from the default: 30 keV beam, 0.5 cm EDX detector radius, and 45° detector take-off angle).

Figure 5a shows the BSE imaging and EDX mapping results for a 2.5 \( \mu \)m radius particle placed at various depths in the material. Here, the depths indicated mark the center of the particle, i.e., at a depth of 5 \( \mu \)m the highest point on the particle is 2.5 \( \mu \)m below the sample surface (for proper convergence of the simulation, one additional nanometer of material was placed over the particle in situations where its radius equaled its depth). These simulation results demonstrate that while the BSE signal may detect particles of a high atomic number material near the sample’s surface, the depth from which characteristic X-rays are detected, mapped by EDX, is much greater than that of the BSE signal. In fact, the X-ray range exceeds the depth of a typical lamella. An additional benefit to using the characteristic X-ray signal is the ability to differentiate clearly between elements of similar atomic numbers, which is more difficult with a BSE signal, especially for high atomic number materials (Heinrich et al., 1976).

The effect of particle size on the detected X-ray signal is shown in Figure 5b. Particles with radii from 10 nm to 2.5 \( \mu \)m were simulated up to 15 \( \mu \)m into the material (beyond a typical lamella depth). As expected, the depth at which particles can be detected by EDX decreases with decreasing particle size. Smaller particles, therefore, must sit closer to the surface of the sample to be localized. Experimentally, the maximum detection depth will also depend on factors such as background noise (Bremsstrahlung radiation and trace materials present) and acquisition time. Nevertheless, these simulations show with a high-quality sample and long enough acquisition times it may be possible to detect particles tens of nanometers in radius up to hundreds of nanometers deep into the material.

We also demonstrate this technique experimentally for the system described above, and show good qualitative agreement with the Monte Carlo simulations. In situ localization of embedded iron oxide particles by EDX during different stages of the cryo-FIB lift-out process is shown in Figure 6. At low magnifications, an overview of the distribution of elements present in the sample is obtained and used to identify regions of interest for milling (Fig. 6d). At higher magnification, individual particles are identified by EDX and the secondary electron signal is used to distinguish embedded particles from those at or near the surface of the sample (Figs. 6b, 6e). Once a suitable particle has been located, EDX mapping precisely guides the milling process (Fig. 6f), ensuring the final lamella contains the desired structure. As a result, this technique dramatically improves the yield of useful lamellae over milling “in the dark,” without EDX.

![Figure 5. Results of Monte Carlo (MC) simulations calculated with the MC X-ray program.](https://www.cambridge.org/core/...
Cryo-FIB Lift-Out Procedure

Cryo-FIB lift-out is conceptually similar to traditional FIB preparation of hard materials for TEM. In brief, once a structure of interest has been identified, cryo-FIB lift-out preparation proceeds as follows: material is removed with the ion beam from either side of the structure of interest in triangular-shaped trenches, leaving a vertical lamella between the two. The lamella is then nearly cut free from the bulk sample with the ion beam using a "J-cut" around the edges of the lamella, leaving a small section at the top to keep the lamella secure until it is attached to the lift-out needle. A cooled nanomanipulator needle is then brought in close proximity to the lamella, water vapor from the GIS is used to attach the two, and the lamella is fully cut free from the sample. It is then lifted out of the bulk sample, attached to a TEM half-grid, again using water vapor, and finally thinned to electron transparency. The frozen lamella is then removed from the cryo-FIB and stored under liquid nitrogen until it is transferred to the cryo-STEM. A basic schematic of this process is shown in Figures 3b and 3c. SEM images of the lift-out process are shown in Figure 7, including final lamellae produced by the methods below.

The first two steps in the lift-out process, trench milling and the "J-cut," mirror traditional FIB techniques. Here, we provide details on how to optimize preparation conditions depending on the sample. First, for electrically insulating samples, charge mitigation by a thin conductive layer on the sample surface greatly reduces deflections of the electron and ion beams, improving imaging and lamella quality. In this work, for example, ~10 nm of platinum was sputtered on the hydrogel samples in the preparation chamber before insertion into the cryo-FIB. Once in the FIB, the sample surface is tilted perpendicular to the ion beam and trenches are milled on either side of the structure of interest. The trenches are milled with either a "regular cross-section," where the milling region is repeatedly scanned with the beam, passing more frequently over the deep end of the trench, or a "cleaning cross-section," where a single slow pass of the beam is used, dwelling progressively longer toward the deep end of the trench. The cross-section used depends on the properties of the target material. For example, materials that redeposit a large amount while milling generally must be milled with a regular cross-section, preventing accumulation of redeposited material in the trench. This may result in the surface of the lamella becoming coated with redeposited material, however. Cleaning cross-sections may be used to ensure the lamella surface is free of redeposited material, however, this milling type can result in material redepositing in the trench behind the slowly progressing beam. With either cross-section type, the trenches are typically milled 5–10 μm wider than the

Figure 6. Energy dispersive X-ray (EDX) spectroscopy mapping for label-free in situ localization of subsurface structures for milling. a: At low magnifications, electron images reveal very little about subsurface structures, whereas (d) EDX maps provide elemental distribution information about structures up to microns beneath the surface. b,c: At moderate magnifications, secondary electron images can be compared with EDX maps, identifying and localizing subsurface structures with precision. f: EDX maps also provide the ability to guide milling of specific subsurface structures, significantly increasing the yield of desired structures lifted out over imaging with electron signals alone (c).
desired lamella width, and a minimum of a few microns deeper than the feature of interest, ensuring room for the J-cut to be performed. In addition, the trench opposite to the ion beam during the J-cut may be milled deeper, providing extra room for material sputtered from the J-cut. The deep ends of the trenches are positioned toward each other, as in Figure 3b, forming the cross-sectional lamella, typically 2–5 µm thick. Beam currents used to mill the trenches vary widely for different sample materials. For the soft hydrogel material, ~0.5–3 nA was used, taking typically upwards of 10 min to mill the trenches. Harder materials require increased current to achieve the same results. Once the trenches are complete, the sample surface is tilted back perpendicular to the electron beam, giving the ion beam access to the lamella face. A J-cut is then performed, milling through the lamella around the edges in an ~1–3 µm wide rectangular “J” shape, as shown in Figure 7a, nearly cutting it free from the bulk sample. A small bridge, a few microns thick, is left at the top of the lamella on the side opposite of where the needle approaches, supporting the lamella. Both the trench separation, which defines the initial lamella thickness, and the J-cut width depend on the sample material. As above, lamellae produced from materials that redeposit a large amount of material must be thin, and the J-cut must be narrow, to minimize redeposited material and avoid the lamella reattaching to the sample. Strongly charging samples, on the other hand, benefit from a wide J-cut to avoid lamella deflection and possible detachment during lift-out.

After the J-cut, the cooled needle is brought in close proximity to the lamella, as shown in Figure 7a, and attached with gas deposited from a GIS. In traditional FIB preparation a platinum-based organometallic precursor gas is injected into the chamber and “cracked” with the ion beam, depositing platinum metal locally and effectively “welding” the lamella to the needle. At cryogenic temperatures, however, many gases freeze on the cold surfaces, and as a result, a suitable gas must be chosen for cryo-FIB lift-out attachment. Although various options have been suggested for this purpose (Antoniou et al., 2012; Rubino et al., 2012; Mahamid et al., 2015), the following considerations led us to choose water vapor. First, the low atomic numbers of the elements in water minimize the effect of any remaining material on imaging in the cryo-STEM. In addition, the use of water avoids introducing artificial carbon, which is crucial for samples where carbon characterization is key. Finally, attachment by water deposition happens on a timescale that minimizes the effect of thermal drift of any component during attachment, while allowing the deposition to be monitored in real time through imaging with the electron beam, enabling the proper thickness to be selected. We typically deposit water vapor for ~10 s, which results in under a micron of amorphous ice on the cold surfaces and

Figure 7. SEM images of the lift-out process steps, performed on a bulk silica hydrogel with embedded iron oxide particles. a: Trenches are milled with the ion beam on either side of the desired structure, a “J-cut” is performed, and the needle is then cut free from the bulk sample, lifted out, and positioned near a transmission electron microscopic half-grid bar. c: Water vapor attaches the lamella to the grid, the needle is cut free, and the lamella is thinned to electron transparency. Final thinning reduces the lamella to a uniform thickness on the order of 100 nm. d: A top-down view shows the lamella thinness and homogeneity, here an early hydrogel lamella with a thickness of 150–180 nm. e: A top-down image of a lamella created from a solid–liquid interface with protective surface layer shows materials in this configuration can be thinned to <100 nm.
provides sufficient stability for attachment of the needle to the lamella. Once the needle is attached, the remaining small bridge is milled away, freeing the lamella from the bulk sample. If a long water deposition time or narrow J-cut are used, the ion beam direction and milling proceeds until all material within the rectangle is removed. This reduces the lamella thickness and removes “curtaining” from the surface. All milling steps are performed at 30 kV accelerating voltage, and for softer materials such as hydrogels, the initial thinning is usually performed at a beam current of ~50–100 pA. Depending on the initial lamella thickness, the first thinning steps typically remove ~1 μm or less from either side. As the lamella becomes thinner, the beam current and milling pattern size (perpendicular to the face of the lamella) are progressively reduced, increasing precision by minimizing probe tails and slowing the milling rate for increased control. The final thinning is typically performed with 10 pA or less, removing only nanometers of material. As the lamella approaches the final thickness, beam deflection, sample drift, and even bending of the lamella can occur. As a consequence, the beam shift knobs must be monitored vigilantly, as even a small beam drift or deflection of the lamella can rapidly destroy the sample. A protective layer on top of the lamella, such as platinum, can help this by decreasing the sensitivity of the lamella to probe tails and accidental beam exposure (Giannuzzi & Stevie, 2005).

The described cryo-FIB lift-out procedure provides sufficient control to produce homogeneously thin lamellae with smooth surfaces. For soft materials, such as hydrogels, lamellae down to ~100 nm thick can be produced. An early hydrogel lamella 150–180 nm thick is shown in Figures 7c and 7d. For materials more resilient to the beam, probe tails do less damage outside the intended milling area, allowing further thinning to be performed. An example of a <100 nm thick lamella from a solid–liquid interface protected by a hard metal capping layer is shown in Figure 7e. For soft materials that lack a protective surface layer but contain a resilient structure larger than the final lamella thickness, such as the iron oxide particle localized by EDX in Figure 6f, the resilient structure itself may be used to shield one interface with the soft material. In this case, the soft material above the structure is sacrificed, but the lower hard–soft interface is preserved. This approach also enables thinning of the lamella to <100 nm in thickness. An example of this is shown in Figure 8, where final thinning was performed on only the left half of the iron particle, shielding the soft hydrogel material below and preserving the interface between the two.

**RESULTS AND DISCUSSION**

**Cryo-STEM Imaging, EDX, and EELS**

The quality of lamellae produced by these techniques allows nanoscale structural, elemental, and bonding information to be obtained from the sample by cryo-STEM. A large range of STEM characterization techniques available for room temperature work are also available for cryo-STEM, including HAADF imaging and convergent beam electron diffraction (CBED) for structural determination, and EDX mapping and EELS to obtain elemental distributions and local bonding information. We demonstrate these techniques in Figures 8 and
9 for iron (oxy-, hydr-)oxide phase crystals grown in silica hydrogels and prepared by cryo-FIB lift-out. Figure 8a shows a HAADF STEM image of the hard–soft interface between a micron-sized iron oxide particle and the hydrogel in a lifted-out frozen lamella, where the material above the particle was sacrificed to preserve the soft hydrogel below the left portion of the particle, as discussed above. Additionally, HAADF STEM performed on precursor phase iron oxyhydroxide nanocrystals embedded in a silica hydrogel lamella (Fig. 9a) demonstrates nanometer-scale imaging resolution for characterization of hard–soft interfaces. The CBED pattern (Fig. 9a, inset) recorded on the particle confirms its crystallinity.

In addition to imaging the material’s structure, elemental and chemical bonding information across the hard–soft interfaces were obtained through cryo-STEM EDX and EELS, respectively. Figure 8b shows an EDX map of the micron-sized iron oxide particle and surrounding silica hydrogel material, revealing the distribution of silicon and iron across the interface. Cryo-STEM EELS data acquired from the hydrogel embedded nanocrystal shown in Figure 9a display a strong iron L-edge and oxygen K-edge prepeak (Fig. 9b, second column), consistent with published reference spectra for iron (oxy-, hydr-)oxide phase compounds (Chen et al., 2009). As expected, these signals were not present in the surrounding hydrogel, instead the O-K fine structure confirms the presence of water ice (Garvie, 2010).

A limiting factor for characterizing soft materials and solid–liquid interfaces by cryo-STEM is radiation damage. The critical dose where damage is induced is typically substantially lower in soft materials and liquids than in hard materials, which limits the obtainable signal-to-noise ratio (SNR) (Henderson, 1995; Egerton et al., 2004; Kourkoutis et al., 2012). This is evident from the difference in SNR of EELS spectra recorded on nanoscale and micron-sized crystals shown in Figure 9b. The nanocrystal was surrounded by hydrogel, which limited the acceptable dose for spectroscopy. However, in the case of the micron-sized particle, no hydrogel material remained above and below the particle, which resulted in a substantially larger acceptable dose and a greatly improved SNR (last column in Fig. 9b). However, in both cases we have shown that information about the bonding state of the sample can be extracted using cryo-STEM EELS.

CONCLUSION

Analytical cryo-STEM techniques combined with site-specific methods for preparing bulk frozen soft materials or solid–liquid interfaces will enable high spatial resolution characterization of biological, chemical, and physical processes that occur in these systems. Recent advances in cryo-FIB techniques have made progress in this direction, including proof of concept demonstrations of lift-out sample preparation methods. Here, we have reported developments of this technique that will enable more widespread utilization of cryo-FIB lift-out. Label-free in situ localization of subsurface structures of interest by EDX mapping, as demonstrated here, dramatically increases their yield in the lamellae produced. In addition, we discussed how to optimize the technique to create thin and homogeneous lamellae for cryo-STEM analysis of intact solid–liquid and hard–soft interfaces extracted from bulk samples. To demonstrate these capabilities, nanometer-resolution HAADF STEM imaging of iron oxyhydroxide nanocrystals embedded in a silica hydrogel lamella was performed. In addition, cryo-STEM EDX allowed elemental mapping across the solid–liquid interfaces extracted from bulk samples.

**Figure 9.** Nanoscale cryo-scanning transmission electron microscopic (STEM) high-angle annular dark field (HAADF) imaging and electron energy loss spectroscopy (EELS) of iron (oxy-,- hydr-)oxide crystals grown in silica hydrogel. a: Cryo-STEM HAADF imaging reveals the nanoscale structure of embedded nanoparticles, and (a, inset) convergent beam electron diffraction (CBED) confirms the particles’ crystallinity. Cryo-STEM EELS provides elemental and bonding information about the surrounding material (b, first column) and the embedded nanocrystal (b, second column). Lamellae containing larger crystals, such as the one shown in Fig. 8, lack hydrogel above and below the particle, which allows larger electron doses to be applied. This results in an EELS spectrum with improved signal-to-noise ratio (b, third column). The sample used in (b, third column) is taken after 4 weeks of reaction, whereas all others are taken after 4 h (900 mM iron chloride).
interface of an iron oxide crystal grown in a silica hydrogel and cryo-STEM EELS provided local bonding information. Our results establish that cryo-FIB lift-out provides a path to access internal solid–liquid interfaces with the liquids intact and stabilized by rapid freezing. In combination with analytical cryo-STEM, the technique can deliver nanometer scale structural, elemental, and chemical information about these interfaces. As a consequence, cryo-FIB lift-out and cryo-STEM will be able to facilitate advancements in fields where nanoscale information about solid–liquid interfaces is critical, such as crystal growth, biomineralization, and energy materials research.

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