

Video Article

Preparing Adherent Cells for X-ray Fluorescence Imaging by Chemical Fixation

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Abstract

X-ray fluorescence imaging allows us to non-destructively measure the spatial distribution and concentration of multiple elements simultaneously over large or small sample areas. It has been applied in many areas of science, including materials science, geoscience, studying works of cultural heritage, and in chemical biology. In the case of chemical biology, for example, visualizing the metal distributions within cells allows us to study both naturally-occurring metal ions in the cells, as well as exogenously-introduced metals such as drugs and nanoparticles. Due to the fully hydrated nature of nearly all biological samples, cryo-fixation followed by imaging under cryogenic temperature represents the ideal imaging modality currently available. However, under the circumstances that such a combination is not easily accessible or practical, aldehyde based chemical fixation remains useful and sometimes inevitable. This article describes in as much detail as possible in the preparation of adherent mammalian cells by chemical fixation for X-ray fluorescent imaging.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52370/>

Introduction

X-ray fluorescence imaging allows for both the identity and quantity of elements present in a sample to be spatially resolved. Incident X-rays, of an energy selected to be greater than the electron binding energy of the heaviest element of interest, overcome the binding energy of inner-shell electrons to the nucleus¹. This creates a 'hole' in the electron shell. As higher-energy electrons fall down into these holes, fluorescent X-rays are emitted whose wavelength is dependent on the energy separation of those orbitals. Since the energy spacing of the orbitals is characteristic of a given element, the X-ray fluorescence emission also has characteristic wavelengths, dependent on the element. It is this emission at a characteristic wavelength that allows the identification of the elements present. Calibration of the fluorescence intensity allows the quantitation of the elements present.

X-ray fluorescence microscopy (XFM) has become increasingly utilized, partly due to the development of very brilliant X-ray synchrotron sources, such as those at Spring-8 in Japan, the European Radiation Synchrotron Facility (ESRF) in France, and the Advanced Photon Source (APS) in the US². These sources provide very high intensity X-ray beams. At the same time, improvements in X-ray optics, such as zone plate technology, allowed the focusing of these beams to sub-micron spots, albeit rather inefficiently³. With very high-intensity beams, even a relatively small amount of light that can be focused is sufficient to excite the endogenous metals in cells, producing signal that can be measured with currently available detector technology. Thus, studying the chemical biology of metals in the cell is one application in particular that makes use of many of the recent developments in this technique⁴⁻¹⁰.

There are many critical factors to be considered while applying XFM to investigate the elemental distribution and quantification of cultured mammalian cells or other biological samples. Firstly, the sample needs to be kept intact, both structurally and with respect to its elemental composition, in order for the measurement to be meaningful. Secondly, the sample must also be preserved in some way so that it is hardy to the radiation damage that can be caused by a focused X-ray beam. One way that a sample can meet both of these criteria at once is to be rapidly frozen into a vitreous, amorphous ice^{11,12}. Rapid freezing is often achieved through various cryopreservation techniques such as plunge freezing or high pressure freezing¹³⁻¹⁶. It is generally accepted that cryopreservation preserves overall cellular architecture and chemical compositions in biological samples as close to native state as possible. Chemical fixation, on the other hand, due to the slow and selective penetration of fixatives into cells and tissues as well as subsequent changes in membrane permeability, may allow various cellular ions especially the diffusible ions such as Cl, Ca and K to be leached, lost or relocated, thus rendering investigation of these elements suboptimal¹⁷⁻¹⁹. Despite the clear advantage of cryo-fixation over chemical fixation in general, for adherent mammalian cells in particular, cryopreservation has various limitations²⁰⁻²³. The most obvious one is that not every research lab has easy access to cryopreservation instruments. Most current high pressure freezers or even plunge freezers are costly and owned only by a subset of cryo facilities, which may be far from where cells are incubated. The benefit of cryopreservation might be traded for the disadvantage of travel stress placed on the cells. Thus, while cryopreservation is surely the most rigorous way to preserve samples for X-ray fluorescence analysis, it is certainly not the most accessible to all researchers under all

circumstances; nor is it always essential — if the metals of interest are tightly bound to fixable macromolecules, and the resolution at which the sample will be imaged is greater than the damage to the ultra-microstructure that might occur during drying. Mindful of the caveats²⁴, chemical fixation and drying may be a suitable choice.

Other factors in a successful X-ray fluorescence imaging experiment include proper analysis. X-ray fluorescence imaging is fundamentally X-ray fluorescence emission spectroscopy combined with raster-scanning to provide spatial resolution. The X-ray fluorescence emission spectra collected contain a combination of overlapping emission peaks, background, and the elastic and inelastic scattering peaks of the incident beam. Software that enables the de-convolution of these contributions, and the fitting of the emission peaks, has been a critical development to this field²⁵. Also, the development and commercial distribution of thin-film standards of known composition, used to calibrate fluorescence intensity relative to material quantity, has also been very important.

This protocol provides a description of the preparation of adherent cells by chemical fixation and air drying. A vital step in this process is the growth of the cells on the silicon nitride windows, which often do not adhere well, making gentle rinsing in a particular fashion key to success.

Protocol

1. Preparation of Instruments, Substrates, Culture Media and Dishes

1. Handling of Silicon Nitride (Si_3N_4) windows.
 1. Open the capsule slightly by squeezing one end containing the window in such a way as to not squeeze the window itself, while rotating the other end of the capsule with the other hand.
 2. Check a pair of reverse, fine-tipped tweezers under stereomicroscope to make sure there are no adhesive, gaps or bends at the tip. Otherwise, the windows may be broken by the tweezers or fly off of them during the subsequent steps.
 3. Remove the window from the capsule by carefully grasping the frame of the window with tweezers, while gently squeezing the capsule near the open end applying pressure in a direction so as to make the capsule wider where the edges of the window need to come out.
2. Window pre-wash (optional).
 1. If desired, pre-wash windows by gently dipping them into distilled water for 5 sec, followed by 2 min wash in 70% ethanol and 2 min wash in 100% ethanol.
3. Affixing and sterilizing windows on the culture dish.
 1. To affix the windows, set the window with flat side up at the bottom of the culture dish, place a piece of tape on a clean glass slide. Cut about a quarter inch strip off down the long side of the tape. Take a slice of tape off the short side (at $\frac{1}{8}$ " by $\frac{3}{4}$ ") with a clean razor blade.
 2. Use tweezers to apply the slice of tape to the edge of the grid or window. Use enough to securely adhere it without covering the window opening itself. Manipulating the adhered tape with tweezers, set the window down onto the bottom of the culture dish. Use the tip of the tweezers to adhere tape to the bottom of the culture dish firmly.
 3. Apply another tape strip to the other edge and adhere this to the bottom firmly with the tip of the tweezers.
 4. Once all windows are taped, sterilize windows in culture dishes with ultraviolet (UV) radiation. Accomplish this by placing the dish under the UV lamp in a laminar flow hood for about 1 hr.
 5. (Optional) If windows are to be pre-coated with poly-lysine, do so following window sterilization. Coat the window with 10 μl of sterile 0.01% poly-L-lysine solution for 1 hr in a 37 °C incubator, and then rinse the remaining solution off with the culture media.
4. Prepare buffers for final wash before XFM.
 1. Prepare either a Tris-glucose or piperazine-*N,N'*-bis(ethanesulfonic acid) (PIPES) -sucrose buffer free of any trace metals with osmolality and pH equivalent to Dulbecco's phosphate buffer saline (D-PBS) without calcium and magnesium. Use one of these buffers to rinse cells after cells are chemically fixed.
 1. To make the Tris-glucose solution (10 mM Tris, 260 mM glucose, 9 mM acetic acid,) add 1.4 g of glucose, 36 mg of Tris base and 16 μl of acetic acid to 20 ml of ultrapure water. Then, adjust final volume to 30 ml with ultrapure water. No pH adjustment is needed. Store at RT up to one week.
 2. To make the PIPES-sucrose (20 mM PIPES, 200 mM sucrose) add 0.18 g of PIPES and 2.0 g of sucrose to 20 ml of ultrapure water and adjust the pH to 7.4 with small amounts of concentrated acetic acid. Then, adjust final volume to 30 ml with ultrapure water. Store at RT up to one week.

NOTE: The reason to avoid using PBS in the final rinse is that the concentrations of phosphate, chloride and potassium in PBS are so high that it can interfere with XFM if it is not completely removed before air drying.
5. Prepare buffers for cell plating.
 1. Prepare or take out all media, such as RPMI medium 1640, 1x trypsin-EDTA solution, D-PBS, and any other reagents as needed for the particular adherent cell line as would normally be done for passaging the adherent cells of interest, and warm them to 37 °C.

2. Plating of Cells

1. To the sterilized culture dish containing the windows, in the laminar flow hood, gently add media (10 ml for a 100 mm culture dish), along the side of the culture dish with it tilted at an angle. Avoid creating unnecessary surface tension or dropping media directly onto surface of silicon nitride windows. As media is added, slowly relieve the tilt angle to coat the grid and window with media.
2. Prepare the adherent cell line of interest appropriately, trypsinizing or scraping the cells off of the plates as usual for passaging the cell line. Perform any cell counting, or other preparation necessary before applying the cells to the fresh plate.

3. Add cells to the culture dish with windows at a cell density that is needed to typically reach 50%-70% confluence within 24-72 hr, and incubate at 37 °C in a humidified incubator with 5% carbon dioxide.
4. Observe the cells' growth occasionally, using an inverted light microscope, until the desired cell density (usually 50%-70% confluence) is reached.

3. Fixation of Cells

1. Prepare fresh 4% paraformaldehyde in D-PBS by diluting a purchased stock (such as 25% paraformaldehyde) and adjusting the pH to 7.4 with acetic acid (to avoid adding excess sodium to the sample).
2. When cells have been grown as desired, and any vital stains (such as Hoescht) have been applied and imaged, remove the media by gentle aspiration, tilting the dish at a 45° angle with one hand, and aspirating by hand pipette with the other.
NOTE: Instead of pipetting spent media, an alternative is to pick the window up, dip the window into microcentrifuge tubes with D-PBS twice and then place it into a new culture vessel with fixative solutions for 20 min followed by 2 times of PBS wash to remove the residual fixatives. If this is chosen, go directly to step 3.5.
3. Rinse the dish gently with D-PBS and replace the removed liquid immediately by gently adding the fresh 4% PFA/PBS while holding the dish at a 45° angle, pipetting towards the side of the dish, and slowly relaxing the tilt angle to allow the fixative solution to cover the windows. Keep the cells covered with the fixative for 20 min at RT.
4. Remove the liquid by gentle aspiration, again as above, tilting the dish slightly and aspirating by hand.
5. Replace the removed liquid by gently adding some of the PIPES/Sucrose solution, using the tilting and pipetting method described in Step 3.3.
6. Repeat Steps 3.4 and 3.5.
7. Prepare the materials for drying.
 1. Gather lint-free towels such as Kimwipes, and pull one out of the box so it is handy very quickly and easily.
 2. Prepare a clean, non-level place to set the window to dry. Set out a rubber grid mat of the kind used in electron microscopy, which has ridges so the window can be propped on one end while it dries. This will keep the window from getting stuck to the drying surface as it dries, and drain any remaining buffer to the edge of the sample.
8. Carefully tweeze the tape attached to window to hold the window up out of the liquid. Retrieve windows that come off of the tape by applying a small amount of PIPES/sucrose to get them to float and then picking them up with the reverse tweezers.
9. Carefully (without touching the window itself) and thoroughly dab any excess PIPES/sucrose from the edge of the window with a Kimwipe. Also daub the back indented area using a rounded fold of a Kimwipe.
NOTE: The goal is to have as little crystallization of salts or buffers on the surface of the window as possible.
10. Place the window onto the grid mat. Make sure that the window is not lying flat on the grid mat, but propped up on an edge (using the ridges of the grid mat) and touching as little of the grid mat as possible. Let the window dry.

4. Sample Mounting and Imaging

1. Once the sample has dried, verify the presence of cells on the windows using a light microscope. Perform this verification, before preparing the samples for transport to a synchrotron.
2. Package the windows for transportation to the synchrotron beamline hosting your experiment. Gently affix the windows with tape on two sides to a glass slide, then affix the glass slide with tape to the bottom of a plastic Petri dish. Tape the Petri dish shut.
NOTE: Prior to this point, these steps may be carried out in any typical lab. The following steps would best be done at a synchrotron beamline.
3. Remove the tape from the window gently with tweezers. Use nail polish, in a thin even coating along the edge of the window, to secure the windows to an aluminum holder provided by the beamline collaborators at the synchrotron.
4. Insert the aluminum holder into a kinematic mount, and then place it into position in the X-ray microscope. Mount the sample on a bracket connecting it to motorized stages, inside the sample chamber at the beamline.
5. After exiting the sample X-ray microscope instrument area (a hutch made of lead-filled walls), setup the scan. Using beamline-specific software, choose the appropriate area of the sample to scan, while viewing the position of the X-ray beam on the sample using a camera equipped with a video cross-hair and pre-aligned with a downstream scintillator camera.
6. Choose the appropriate resolution for the sample, based on an estimate of the size of the smallest feature of interest. For imaging single mammalian cells (~20-40 µm in size), use a resolution of 0.25-0.5 µm. For imaging areas of tissues, and distinguishing cell layers from each other, use a resolution of 2-20 µm.
7. Choose the appropriate dwell time for the sample, based on an estimate of the quantity of the metal of interest present. For a quick overview scan, or if high quantities of metals are present, use fly scans with dwell times of 30-300 msec per pixel. If little material is present, or to measure metals of low relative abundance, use step scans with dwell times of 1-2 sec per pixel.
8. Program the scans into the beamline-specific software. Acquire a raster-scan image. Work together with the beamline collaborators to analyze the data with software that will identify the characteristic energy peaks for each element.

Representative Results

The ability of X-ray fluorescence imaging to provide information about biological samples is contingent upon these samples being prepared in such a way that they are robust to radiation damage on the time-scale of the experiment, and yet their chemical and structural features are well preserved. In viewing the result of a sample that has been prepared as described above and imaged, it is possible to see that there is variation in the elements present — indicating that fixation preserved these aspects of the cell (**Figure 1**).

Conversely, looking instead at a sample where this process did not go well, and the buffer remains present during drying, extensive crystal formation from the molecules present creates structural damage to the cells and also interferes with the collection of the X-ray fluorescence spectra (Figure 2).

These images are generated by per-pixel fitting of the X-ray fluorescence spectrum at each point in the image. This means that each spectrum, collected at each pixel, has been individually analyzed. When viewing the panels in these images generated from the fitted data, the value assigned to each point in the image is the integrated sum of a Gaussian for the characteristic emission of a given element (e.g., iron) as best fits the X-ray fluorescence spectrum collected at that point. For example, a single pixel in the image has a value (number) for iron, which is the sum of the area under a Gaussian curve at the characteristic emission energy for iron, that fits and models the data for the emission spectrum of that point on the sample. The data is displayed with threshold values above each image (max and min) that assign the high- and low-ends of the color-table (at the bottom of each image) to specific values within the image.

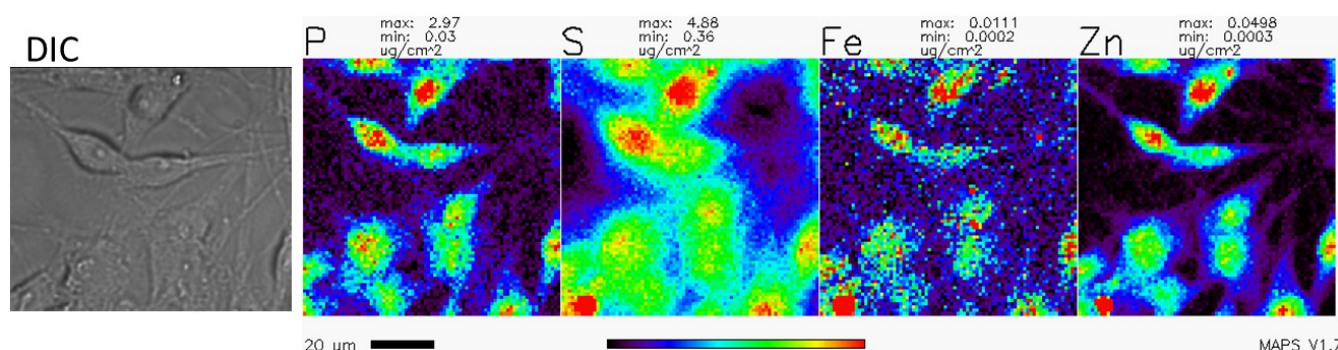


Figure 1. X-ray Fluorescence Image of a Human SH-SY5Y Cell. Each panel in this image displays different information about the same cells. The first panel, labeled DIC, is the optical differential interference contrast micrograph of the cells. The following panels, left to right, are the phosphorus (P), sulfur (S), iron (Fe), and zinc (Zn) images of the same cells, showing their distribution over the area of the cell. The scale bar shown is 20 μm . [Please click here to view a larger version of the figure.](#)

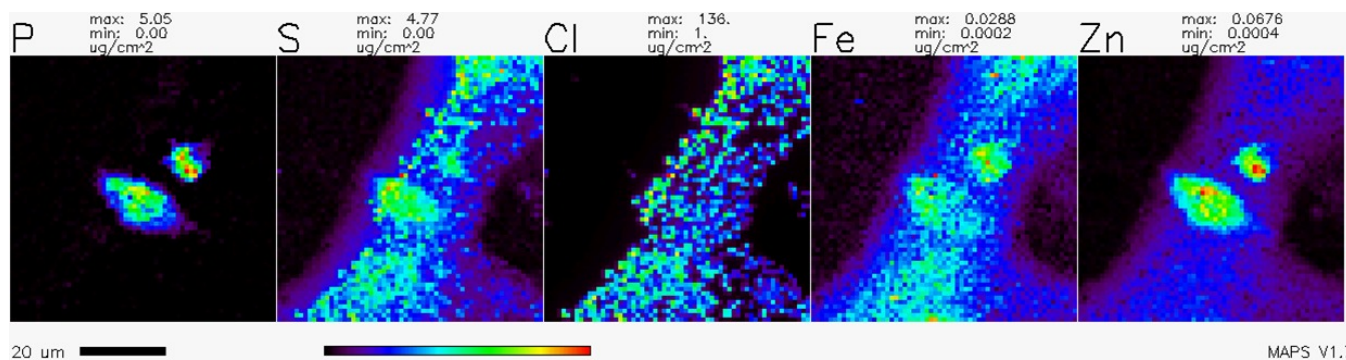


Figure 2. X-ray Fluorescence Image of a Rat B103 Cell, prepared with poor or insufficient removal of buffers. Two cells are visible in the phosphorus (P) panel, and are slightly visible in the iron (Fe) and zinc (Zn) panels. However, the presence of crystallized buffer, visible as a smear of particulates through the center of the image, obscures most of the information. The scale bar shown is 20 μm . [Please click here to view a larger version of the figure.](#)

Discussion

X-ray fluorescence imaging is useful in many fields, including geosciences, materials science, and chemical biology²⁶⁻³⁴. Advances in synchrotron X-rays, and their focusing, have produced very high-intensity beams. Focused X-ray beams sufficient to excite the endogenous metals in cells now exist, producing signal that can be measured with currently available silicon drift detector technology. And studying the chemical biology of metals in the cell is one application in particular that makes use of many of the recent developments in this area.

Yet, the study of biological materials using X-ray fluorescence microscopy also presents special challenges, relative to other materials, because they are hydrated. To prevent radiation damage, ideally the samples would be frozen in vitreous ice during the measurement. However, chemical fixation and air drying are much more accessible to the novice, and may be appropriate if the metals of interest are inertly bound to fixable macromolecules, and the resolution at which the sample will be imaged is greater than the damage to the ultra-microstructure that might occur during drying.

In considering substrates for adherent cell XFM studies, an ideal substrate needs to meet the following basic requirements: 1) support robust cell growth without alternating normal proliferative and phenotypic cell growth, 2) must not have X-ray fluorescence that overlaps with those of elements of interest, 3) be optically transparent for cell growth assessment under light microscope before XFM, and 4) be able to withstand any physical and chemical manipulation during culturing and subsequent fixation. Historically, different substrates, ranging from thin polycarbonate foils/films³⁵⁻³⁷, formvar coated gold transmission electron microscopy (TEM) grids³⁸⁻⁴² and silicon nitride windows^{5,17,43-46}, have been used to grow mammalian cells and used for X-ray fluorescence imaging. In comparison among the existing and potential substrates for imaging adherent mammalian cells by XFM, silicon nitride membrane (Si_3N_4) windows are most suitable due to their low fluorescence background and relatively

simple elemental composition^{4-6,47,48}. In addition Si₃N₄ windows support robust cell attachment and proliferation. Compared to other commonly used substrates such as TEM grids, Si₃N₄ windows also have a much larger uninterrupted imaging area which is a particular advantage when these windows are used for X-ray tomography.

Si₃N₄ windows are commercially available from a limited number of vendors. Working with silicon nitride windows is an acquired skill. The windows are very fragile, and require great care to not create any twisting forces while handling them that might shatter them, or to drop them. Handling them by the edges using reverse tweezers is a popular approach. Most windows have thicknesses ranging from 30 nm to 500 nm and a width from 1 x 1 mm to 5 x 5 mm. In general, the thicker the membrane, the more robust they are to all kinds of handling stress. However, with increased thickness and less optical transparency, they will increase the background emission from the specimen. The 200 nm thick membranes are quite ideal. They have minimal impact on the measurement, yet are sturdy enough to be handled without much breakage.

Although many cell types tested can initially attach and robustly grow on the sterile Si₃N₄ windows, cells grown on silicon nitride windows are in general much more easily agitated than cells on traditional cell culture plates. They become easily detached, aggregated or rounded up even during routine media changes. We found pre-washed windows often became more hydrophilic; cells attach better and had less chance to aggregate together. The steps described in the protocol above delineate a gentle-washing approach to keeping the cells on the window. Some other steps that may be taken include coating the windows with poly-lysine, or laminin, just as one might coat a coverslip.

Another aspect of this method that can be the success or failure of a given experiment is agitation of the samples. In observing cells' growth on plastic culture vessels, the normal range of agitation resulting from pipetting and plate transportation doesn't seem to cause much harm. However, for cells grown on the silicon nitride windows, extra care during media change and plate transportation is needed. For some easily agitated cells such as mouse fibroblast cells NIH/3T3, the culture plates containing windows need to be handled carefully. They have to be carefully taken out from the incubator and gently set on the microscope stage or cabinet surface. Not necessarily every little physical shock will disturb the cells, but the risk of cell detachment increases without extra care. In addition, different batches of fetal bovine serum and silicon nitride windows may have certain effects on the attachment of cells on the window. Some sources of serum or batches of windows seem easier to work with, *i.e.*, without seeing too much disturbance by similar care. So, some trial and error for the cell line of interest may be advised.

With developments in cryogenic stages for X-ray fluorescence microprobes, as well as new research in the preparation of frozen hydrated biological samples, it is likely that sample preparation in the future will much more increasingly include frozen hydrated specimens. Many of the same challenges in working with the silicon nitride windows, and retaining cell adhesion exist there as well. Yet, mastering this technique remains a very good place to start in developing skills before attempting cryopreservation, and many times, is a perfectly suitable way to image samples.

Disclosures

The authors have nothing to disclose.

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