

Localization Of Proteins And Nucleic Acids Using Soft X-ray Microscopy

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INTRODUCTION

Imaging cells using a variety of microscopy techniques has provided information about the organization of cells and subcellular structures that is critical for our understanding of cellular function. The challenge has been to obtain the best resolution morphological information about cells that are examined in a state most closely resembling their natural environment. Soft x-ray microscopy is proving to be a very powerful method in that one can examine whole, hydrated cells, avoiding potential artifacts introduced by the dehydration, embedding and sectioning that is required for electron microscopy. By using a cryostage, we can examine cells that have been rapidly frozen and viewed in a state more closely approximating that seen in living cells. These cells are free of chemical fixation artifacts and, since they are viewed by utilizing the contrast provided by examining them in the water window, do not require chemical enhancement agents. Furthermore, immunolabeling techniques can be used to obtain information about the distribution of proteins and nucleic acids at five times better resolution than light microscopy. We describe the use of soft x-ray microscopy to visualize cellular structures in whole, hydrated and cryo-fixed cells as well as the power of immunocytochemistry to localize specific molecules.

IMAGING MAMMALIAN CELLS USING X-RAY MICROSCOPY

We used the soft x-ray microscope (XM-1) at the Advanced Light Source to examine whole, hydrated mammalian cells. Cells were grown on silicon nitride windows, fixed in 2% glutaraldehyde, then examined in the fully hydrated state. These cells reveal excellent ultrastructural details of the cell nucleus and cytoplasm. The nuclear membrane in the cell shown in Figure 1 is not present since this cell is in mitosis. Instead, we see two clusters of chromosomes in the center of the cytoplasm as they begin moving to opposite regions of the cytoplasm.

CRYO X-RAY MICROSCOPY OF MAMMALIAN CELLS

The ultimate goal of cell biologists/microscopists is to obtain information about the structure of cells at the best possible resolution with minimal perturbation of the cellular ultrastructure during processing. Rapid freezing techniques have long been recognized as an excellent way to achieve this goal, and several rapid freezing devices are commercially available and routinely utilized by a small number of laboratories. Unfortunately, techniques for examining these well-frozen cells in ways that do not require subsequent, potentially damaging processing have been more difficult to develop. Viewing rapidly frozen whole cells using high voltage electron microscopes, which provide superb resolution, has not been possible since these microscopes are restricted to imaging very thin ($< 1 \mu\text{m}$) objects. As a consequence, the well-frozen cells must then be processed using techniques such as freeze-substitution and low-temperature embedding followed by preparation of thin sections. The use of soft x-ray cryo-microscopy, however, provides an excellent solution to this problem since whole cells up to $10 \mu\text{m}$ thick can be examined following cryo-fixation. We used a cryo-fixation

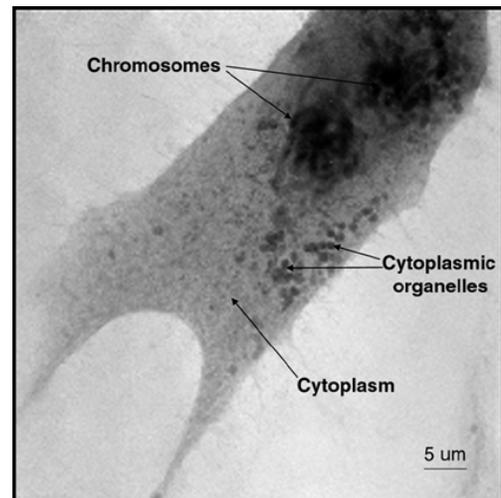


FIGURE 1. Mouse 3T3 fibroblast that was fixed in 2% glutaraldehyde and viewed in the hydrated state. The cell is undergoing division and the separating chromosomes are apparent as they begin moving to opposite poles. Numerous organelles are seen in the cytoplasm.

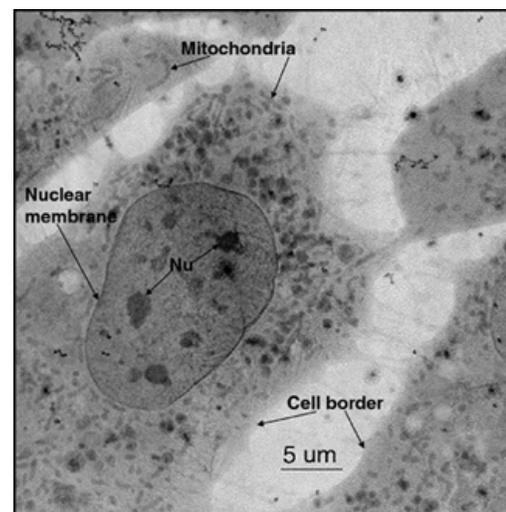


FIGURE 2. Mouse 3T3 fibroblast cells that were rapidly frozen and examined in the cryostage. The nucleus contains several nucleoli (Nu) and is surrounded by a distinct nuclear membrane. Numerous vesicles and organelles, such as mitochondria are seen in the cytoplasm.

apparatus and cryo-stage built by the Center for X-ray Optics (CXRO) and in operation at XM-1 at the ALS to examine initially live mammalian cells, such as the 3T3 fibroblast shown in Figure 2. The nuclear membrane surrounding the nucleus is well preserved, as are numerous cytoplasmic organelles such as the long, tubular mitochondria. These cells were remarkably stable during viewing and demonstrated no apparent radiation damage, even after repeated imaging with the x-ray microscope (data not shown). Imaging rapidly frozen whole cells using cryo-tomography will enable us to obtain unique three-dimensional information about cells and interactions of intracellular organelles.

IMMUNOLocalIZATION OF PROTEINS IN MAMMALIAN CELLS

A powerful method for identifying structure-function relationships of cells and proteins in cells is to determine the subcellular location of these proteins using immunocytochemistry. This requires chemical fixation of the cells followed by incubation in antibodies that recognize a specific protein followed by a tagged probe detectable by the microscope. We used a technique routinely used for electron microscopy and recently used for x-ray microscopy (1,2) that involves silver-enhancement of gold-tagged antibodies to examine the distribution of proteins in whole hydrated cells. To evaluate the ability of this technique to reveal well known nuclear structures, we undertook a series of immunolabeling experiments for proteins localized in defined nuclear subdomains. The nucleus of the cell is extremely thick and has been difficult to study using electron microscopy. Therefore, attempts to examine nuclear structure have relied on the use of extensive extraction procedures. We examined the distribution of nuclear pore complexes, the visualization of which is particularly challenging in conventional electron microscopy. Nuclear pore complex (NPC) is a large ($50\text{-}100 \times 10^6$ D) collection of proteins which organize the ~ 9 nm openings in nuclear membranes of eucaryotic cells. After mild permeabilization, tumor mammary epithelial cells were fixed in 2% paraformaldehyde, 0.1% glutaraldehyde, and 0.1% Triton X-100 in a cytoskeletal buffer. They were then treated with SuperBlock to prevent non-specific binding of antibodies to other proteins, incubated in anti-NPC primary antibodies, rinsed, then incubated in secondary antibodies tagged with 1.4 nm gold particles. After antibody labeling the cells were fixed in 2% glutaraldehyde to stabilize them, then incubated in silver to enhance the size of the gold particles for viewing in the x-ray microscope. Silver enhanced label revealed numerous and uniformly distributed nuclear dots in cells labeled with both primary and secondary antibodies, a pattern that is characteristic of NPC (Figure 3).

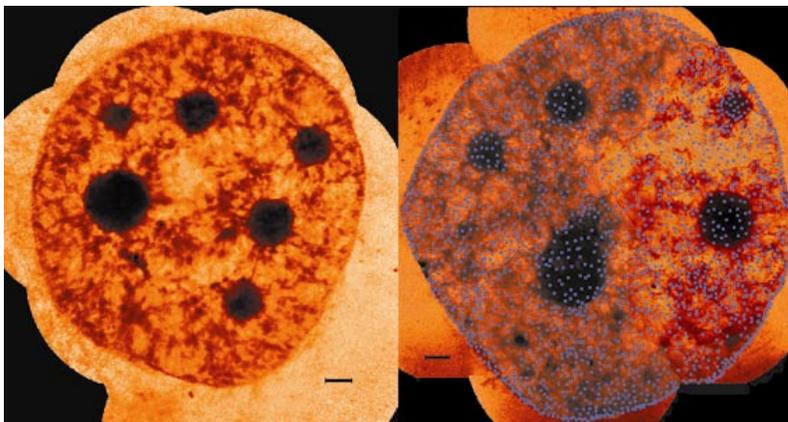


FIGURE 3. Distribution of nuclear pore complexes (NPC) in tumor mammary epithelial cells. The left image is the control, which was not exposed to primary anti-NPC antibodies but did receive secondary gold-tagged antibodies and silver enhancement, and is free of label. Blue dots in the right image are antibody labeled, silver enhanced NPC molecules. Scale bar = 1 μm .

IMMUNOLocalIZATION OF NUCLEIC ACIDS IN MAMMALIAN CELLS

It is also important to know the subcellular distribution of nucleic acids. RNAs, for example, are often positioned in distinct cellular locations where large numbers of proteins are needed upon short notice. We are using in situ hybridization to examine the distribution of RNAs in whole cells (Figure 4). Cells were fixed in 2% paraformaldehyde, 0.1% glutaraldehyde and 0.1% Triton X-100 in a cytoskeleton buffer, incubated in digoxigenin-labeled actin mRNA, then incubated in gold-labeled anti-digoxigenin antibodies that were subsequently enhanced with silver. The cells were then examined in the x-ray microscope and demonstrated a uniform distribution of actin mRNA throughout the cytoplasm and some mRNA particles in the nucleus. The control cell, which was not exposed to mRNA, contains no label. This technique will be of critical importance in the post-genomic era as we face the daunting task of determining the function of the vast number of genes and gene products identified as a result of modern molecular biology techniques.

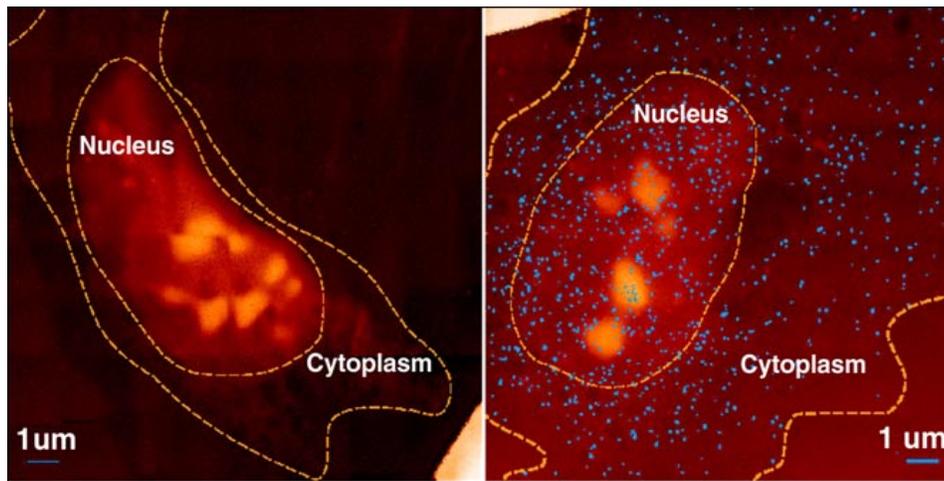


FIGURE 4. In situ hybridization for actin mRNA. Blue dots in the image on the right represent particles of digoxigenin-tagged actin mRNA that have been localized using antibodies against digoxigenin followed by silver-enhanced, gold labeled secondary antibodies. The image on the left is the control, which was not exposed to the digoxigenin-tagged actin mRNA, and is free of label.

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