Invited Paper

Imaging and etching, soft x-ray microscopy on whole wet cells

J. R. Gilbert[1], and J. Pine[2]

[1]EECS Department, 39-561, Massachusetts Institute of Technology, Cambridge, MA 02139
[2]Division of Physics, Mathematics and Astronomy, California Institute of Technology, Pasadena, CA 91125

ABSTRACT

We have produced images of whole wet tissue culture cells with the Stony Brook/BNL scanning transmission x-ray microscope (STXM). For fixed cells we have taken images at theoretical resolutions of ~50-75nm, and in practice have measured FWHM of features down to near 100nm, without any exotic image processing. For un-fixed (i.e., initially live) cells we have imaged with 100nm pixels and measured features down to 250nm. In order to do this we have developed, tested and used a wet cell for maintaining fixed or live cells on the STXM stage during imaging. Our design of the wet cell and the culture substrates that go with it make the STXM compatible with almost all standard systems for surface adherent tissue culture.

We will show some new images of whole wet fixed and unfixed cells, with visible sub-micron features. We will also report data that helps to characterize the tissue damage due to x-ray absorption during STXM imaging.

1. INTRODUCTION

We have been working towards the ability to produce images of whole wet cells, both fixed and unfixed, with the Stony Brook/NSLS scanning transmission x-ray microscope (STXM) that has been constructed over the last several years. A description of the STXM has been given elsewhere[1]. We will just touch on the incident flux calibration method that we used at the time this work was done. This paper is, for the most part, about the images taken with the STXM and their analysis.

In order to take good images of cells, and in order to estimate what the limitations of soft x-ray imaging technology may be it is essential to reach some understanding of radiation damage in STXM imaging. We take a very phenomenological approach to the subject, trying to observe, at near the pixel by pixel level, the damage in image series.

2. METHODS

We make thin, 100-200nm window membranes of low tension LPCVD silicon nitride[2], supported by a silicon frame. The membranes provide a 3mm square culture window centered in a 9.7mm square silicon chip[3]. The Caltech wet cell[4] is used to mount these culture chips on the STXM stage and maintain the cell cultures grown on them in a controlled environment during imaging.

0-8194-0914-6/92/$4.00

Primary chick Dorsal Root Ganglion (DRG) cultures were grown on the culture chips. The cells in culture were primarily DRG neurons and fibroblasts. The cells were grown for 1-2 days at 37°C in air before imaging (or fixation).

Estimation of the absolute number of photons incident on an image pixel, is done by using a dry wet cell (one containing no medium or cells), and a calculation of the losses between the front window of the wet cell and the photon counter of the STXM. We usually calibrate F_0, the number of incident photons per msec per 100mA of ring current. This calibration is dependent on λ and must be redone if there are changes or instabilities in the beamline or synchrotron ring. This calibration is more fully discussed elsewhere[5]. Generally using this method of estimating F_0 we expect an accuracy of no better than 50% from one series of images to another.

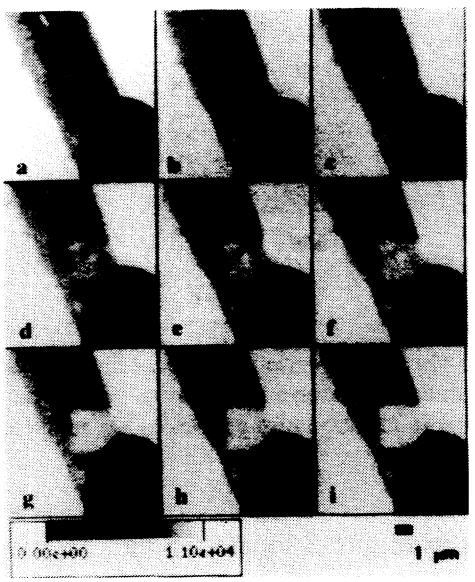

Figure 1 Radiation Series An array of the observation images in the Nov15 series. Each image has been slightly cropped relative to the image in Figure 2 , which is the first image in the array (a). All the images have been normalized together to give background transmission 100% (white), and full black is 0% transmission.

3. TISSUE DAMAGE IN IMAGING

Radiation damage is thought of as the relation between dose incident or absorbed and the observed "damage", usually lethality or biological or chemical activity. In our case we observe only the absorption in a pixel column. So we observe radiation damage as the relation between photons absorbed and the observed absorption in the pixel column.

In order to examine radiation damage more closely we observed the damage using a "radiation damage series" or radiation series. This is two interleaved sequences of STXM images. We take an "observation image" at low dwell time and dx=0.1μm, and follow it with a "damage image" over a small region of the observation image area. A damage image is taken at long dwell time and smaller dx. It has a much greater incident photon flux per square μm than the observation image. We then repeat this cycle of observation and damageuntil the region subjected to the damage images has been completely destroyed (i.e. gives transmission T=100% in the STXM image.) An example of a radiation series is shown in Figure 1.

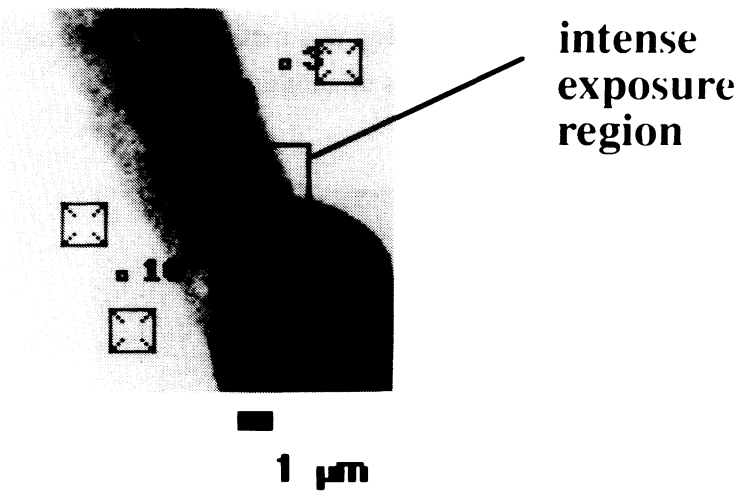

Figure 2 Master Image This is the first observational image of the Nov15 radiation series. The large box labeled "intense exposure region" outlines the damage image area. The three medium squares with ticks at their corners are the background regions used for normalization of the series[6]. The small numbered squares are described in the text. The image was taken at λ=36.4Å and dx=0.1μm.

The small numbered squares in Figure 3 locate the data regions of this series. Regions 3, 8, and 10 are in background areas, regions 1, 2 and 4 are control regions of the cell which receive only observational doses, regions 5, 6 are in the high exposure area of the cell, region 7 is in the high exposure area--but over the gold focus mark, and region 9 is a control region over the gold mark. Gold focus marks are always upstream of the sample and in this batch of culture chips the gold mark transmitted 57% of the normal flux, which gives a lower incident flux to regions 7 and 9. All these regions are 3x3 pixel areas whose means give us one data point per observational image, and the set of data points from one region give the data series that are graphed below in Figure 3.

In order to make more sense of this data we convert the pixel column transmission T of the images in Figures 1 and 2 into N_c, the number of carbon atoms over a $1\mu m^2$ column that would give that transmission. We also use the observed sample transmission and the estimated F_0 , in order to construct ΔE the cumulative absorbed energy in eV per $1\mu m^2$ column.

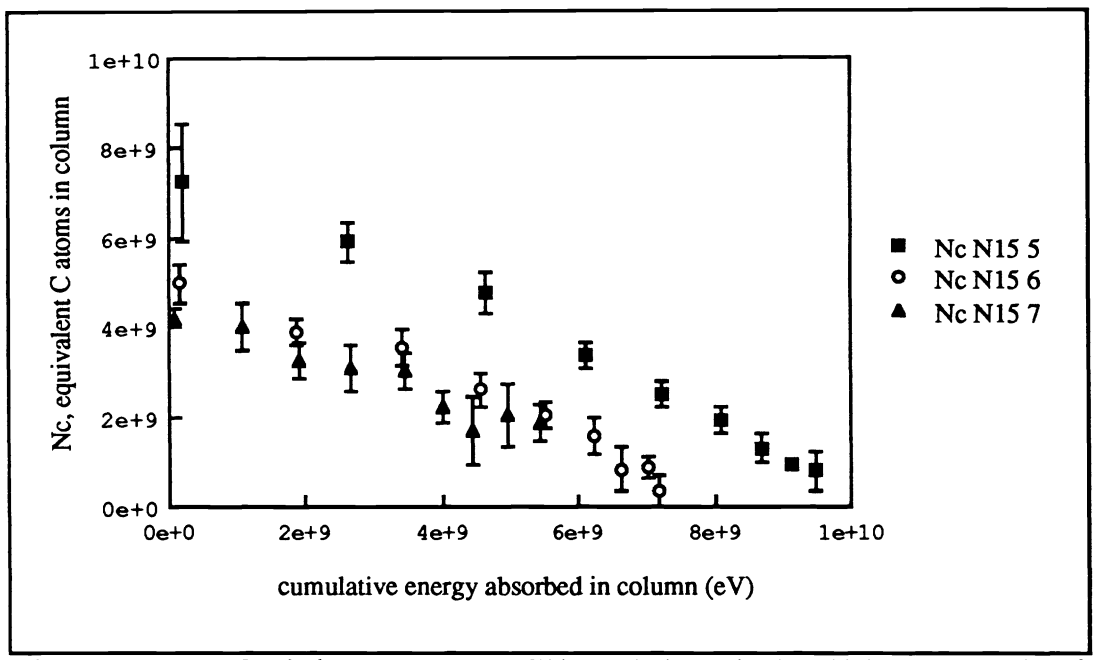

Figure 3 **Radiation Damage** This graph shows the three high exposure series of Figures 1 and 2, with the y-axis scaled to Nc, the equivalent number of carbon atoms in a column above a $1\mu m^2$ area. The x-axis is the cumulative energy that has been absorbed in the column during the radiation series. The error bars correspond to the standard deviations of the transmission in each 3x3 region.

To analyze the series in Figure 3 we use equation 1. Here we introduce the "hardness" of the sample α. The quantity $\alpha\Delta E$ gives the change in number of carbon atoms due to the absorption of energy ΔE. For the series graphed in Figure 3 a straight line fits quite well. And the observed slopes of the fit lines are close.

$$T=e^{\left(-\mu_{Nc}(\lambda)N_i-\alpha\Delta E\mu_{Nc}(\lambda)\right)} \qquad [1]$$

$\mu_{Nc}(\lambda)$ is the atomic absorption coefficient of carbon at wavelength λ.

N_i is the initial number of carbon atoms worth of absorption above background.

ΔE is the cumulative absorption above background in eV.

both N_i and ΔE are normalized to a $1\mu m^2$ column.

What kind of estimates do we get for the ratio of atoms lost to energy absorbed (the "hardness" α)? Our current best estimate is -0.78 ± 0.35 [C atoms change]/[eV absorbed], with the dominant error being our error in estimation of the absolute incident flux[7]. Using this estimate and equation 1 we can now estimate how much damage we cause in each STXM image.

4. IMAGES

Figure 4 shows a high resolution image of a region of a fixed fibroblast. The image was taken with pixel size dx=32nm and dwell time of 10msec. The image has a background noise of 1.35% (that is sd noise in the background areas of the image.) Using our hardness estimate of -0.78, we estimate damage at 69% mass loss in taking this image. Taking high resolution images can be very destructive.

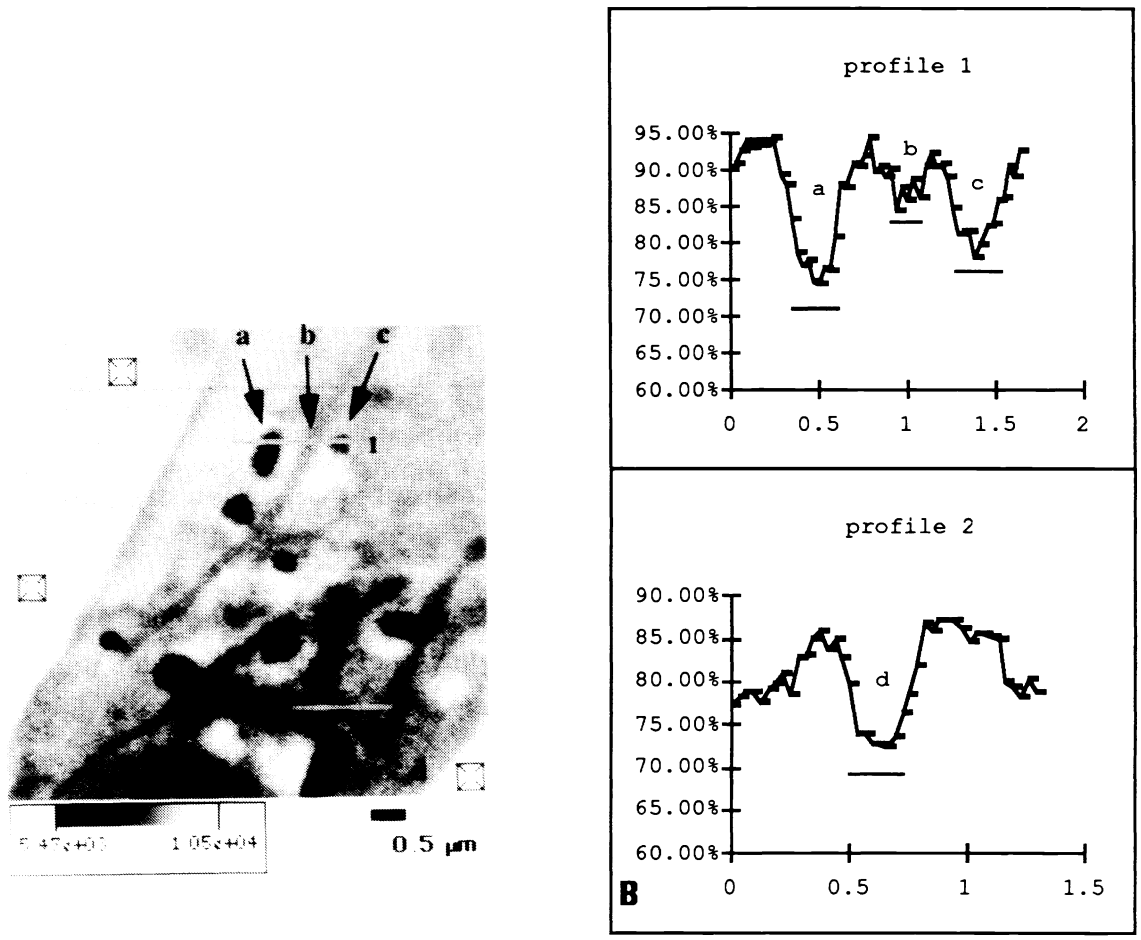

Figure 4 **Fixed Fibroblast** An image of a fixed fibroblast. **A** is taken at 32nm/pixel and 10msec/pixel, achieving 1.35% noise in the background areas. The labeled profiles 1 and 2 are graphed in **B** The labeled structures a, b, c and d that are profiled have estimated FWHM's of 0.30µm, 0.15µm, 0.30µm, and 0.28µm respectively. The fractional mass loss that we estimate using a hardness of -0.78 is 69%.

In Figure 5 we show an unfixed cell image taken with the STXM, and also two phase contrast images taken at 30 minutes and 3 hours after irradiation. After 30 minutes the cell looks relatively intact, but it is clearly showing gross damage after 3 hours. The cell is given about 1 MRad in absorbed dose (using a model of the cell as 10% carbon) and using our data on damage we would expect this image to cause 6% mass loss. However all our radiation series were taken on fixed cells and it is not obvious to us whether unfixed tissue should have a higher or lower "hardness", so that the 6% mass loss estimate is very tentative.

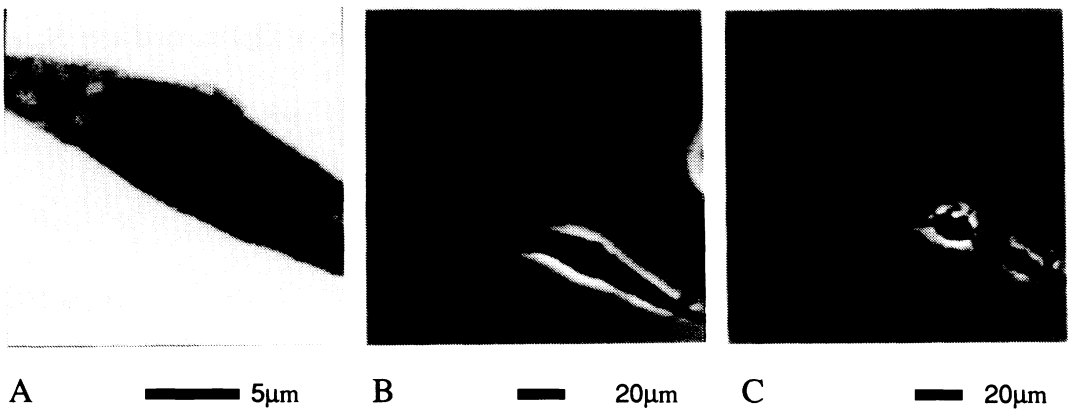

Figure 5 **Un-fixed Cell** **A**, is the STXM image of a cell, taken at 95nm/pixel, with a dwell time of 5msec/pixel. The cell received an absorbed dose of 10^7 Rads, and we estimate its mass loss to be 6% using a hardness of -0.78. **B** is a visible light phase contrast image of the cell about 30 minutes after STXM imaging, showing the cell still relatively intact. **C** is another phase contrast image taken 3 hours after irradiation, showing extensive damage.

5. CONCLUSION

We have shown several images of whole wet cells taken with the STXM. For fixed cells we have taken images at theoretical resolutions of ~50-75nm, and in practice have measured FWHM of features down to near 100nm, without any exotic image processing. For un-fixed (i.e., initially live) cells we have taken images with 100nm pixels and measured features down to 250nm. The observations of radiation damage that we've made, while preliminary, do suggest that the dose required to get low noise images at the highest resolution is pressing the limits imposed on STXM work by radiation damage. However, this is in the absence of any attempt to modify the environment of the cells to enhance radiation resistance. One suggestive feature of this work that may lead to some interesting experiments is the notion of "hardness" as a local feature of cells. We can in principal

extract the hardness, pixel by pixel, from two successive images. This would give us a hardness image of the cell at high resolution. Such a technique might illuminate new features or new interactions of cell tissue with preparation or fixative.

6. ACKNOWLEDGEMENTS

We would like to thank J. Kirz, C. Jacobsen , S. Williams and C.J. Buckley who all helped to take this data and explain the microscope to the authors. This work was supported by the NSF under grant DIR-8820376. Imaging took place at the NSLS, which is supported by the DOE Office of Basic Energy Sciences under Contract DE-AC02-76CH00016. The STXM is supported by the NSF under grant DIR-9005893. The zone plate used for these experiments was made by Dr. Erik Anderson, under a collaborative agreement between the LBL Center for X-Ray Optics and the Nanostructure Fabrication Group at IBM Research.

7. REFERENCES

1 C. Jacobsen, S. Williams, E. Anderson, M.T. Browne, C.J. Buckley,D. Kern, J. Kirz, M. Rivers, and X. Zhang "Diffraction-Limited Imaging in a Scanning Transmission X-ray Microscope," Optics Comm., **86,** 3/4 pp. 351 (1991).

2 C.H. Mastragelo, Y-C Tai, and R.S. Muller "Thermophysical Properties of Low-Residual Stress, Silicon-Rich, LPCVD Silicon Nitride Films," Sens. Actu. A, **23**, (1-3) pp.856-860 (1990).

3 J.R. Gilbert, Ph.D. dissertation, California Institiute of Technology, 1992, chapter 4.

4 J.R. Gilbert, Ph.D. dissertation, California Institiute of Technology, 1992, chapter 4.

5 J.R. Gilbert, Ph.D. dissertation, California Institiute of Technology, 1992, chapter 3.

6 J.R. Gilbert, Ph.D. dissertation, California Institiute of Technology, 1992, chapter 3.

7 J.R. Gilbert, Ph.D. dissertation, California Institiute of Technology, 1992, chapter 5.