Pulsed x-ray microscopy of biological specimens with laser plasma sources.

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ABSTRACT

The development of a compact, affordable, high-resolution x-ray microscope will have a strong impact on the biological and medical sciences. We discuss the potential that pulsed, laser-plasma x-ray sources have to this development. Several approaches to the high-resolution analysis of dried and *in-vitro* biological specimens with laser-plasma sources are described. We discuss the details of the laser and plasma conditions required for optimum x-ray generation, and the various x-ray optical and x-ray electro-optical imaging systems which could be incorporated into a compact, laser-plasma x-ray microscope.

I. Introduction.

At the present time detailed high resolution analysis of sub-cellular biological and life science structures is most commonly performed by electron microscopy. It is well-known that while this technique can provide Angstrom resolution, it suffers from a small depth of field and the need to significantly treat the samples (drying, dying, sectioning, coating etc.,) before analysis. X-ray microscopy in principle will avoid many of these limitations. While being limited to the resolution in the 100A range, it's ability to probe the internal structure of *in-vitro* assemblies provides biologists and life scientists the opportunity to observe complex features in their natural, even live state. Most x-ray microscope development has so far been made using large synchrotron sources. The latter limits x-ray microscopy to being primarily a limited research tool centered around a complex beam line located at a major synchrotron facility. The use of a laser-plasma x-ray source, however, makes plausible the development of a compact x-ray microscope having a size and cost comparable to a conventional electron microscope. The overall design of such a laser plasma-based x-ray microscope is illustrated in fig.1. One could envisage such a system being part of the standard analytical hardware available in major research and medical institutions. In this paper, we review the characteristics of laser-plasmas as they pertain to a microscope source, and discuss the various imaging technologies which can be incorporated into such a source. In the latter, we describe some new results obtained with both reflective optical microscopy and with high-resolution contact microscopy of dry and wet cellular structures. We also discuss the potential incorporation of high magnification electron-optical imaging systems into a compact x-ray microscope and the capability it will give for real-time imaging.

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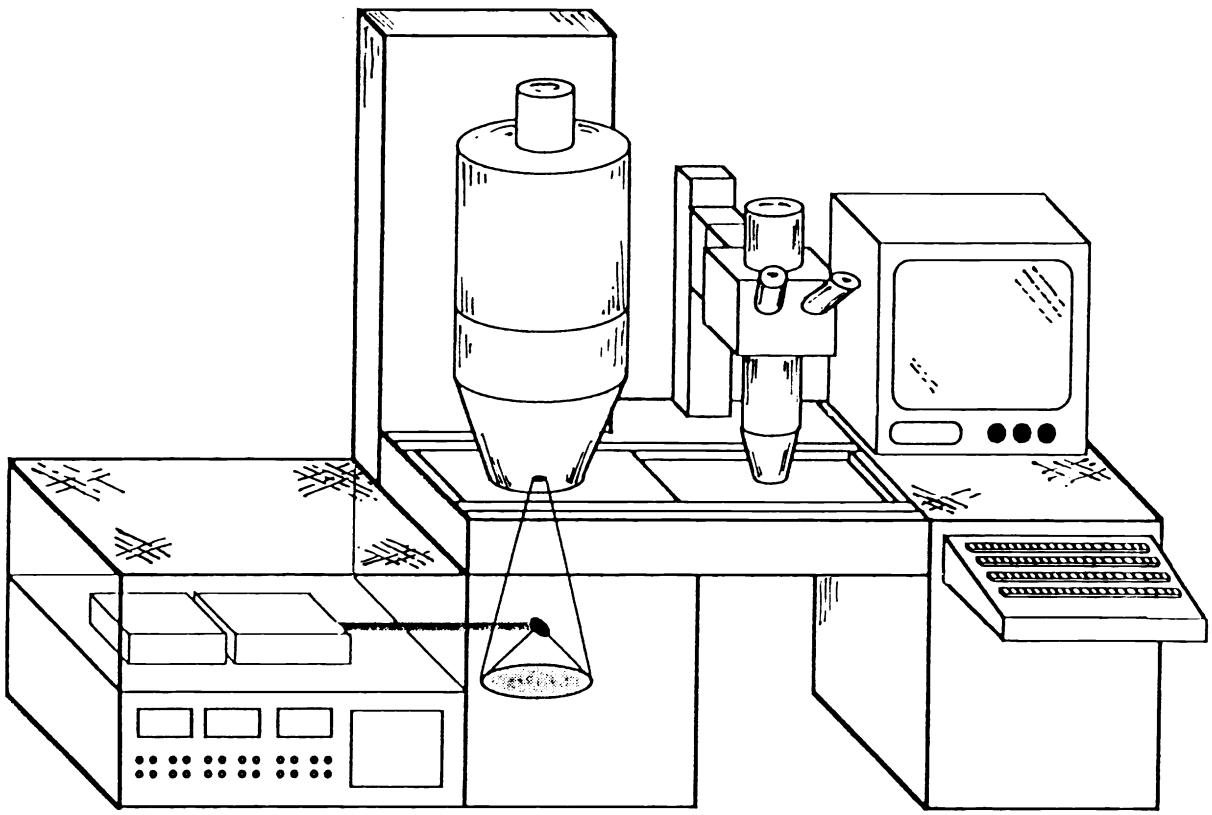


Fig.1. Design concept of a compact, stand-alone, laser-plasma x-ray microscope.

II. Advantages of a laser plasma source for x-ray microscopy of biological specimens.

The primary advantages of a laser plasma x-ray source stem from it's compactness and flexibility. As has been previously mentioned, the spectral brightness of laser plasma x-ray sources can be comparable to the brightest available synchrotrons[1]. Laser plasma x-ray sources have the advantage of being compact, moveable and tolerable of modest vacuum requirements. The x-ray emission spectrum of laser plasmas is rich in bright broad (Planckian) continuum emission and in narrow atomic emission lines, and can easily be varied to suit specific microscopy needs. Typical characteristics of the x-ray emission of laser plasmas are illustrated in fig.2. This selectivity in x-ray wavelength has a potential high dividend for x-ray microscopy in facilitating elemental analysis of features within biological structures by difference imaging with emission at two different wavelengths. Laser plasmas are point sources which can be highly reproducible, a requirement for precision x-ray optical systems having extremely high alignment specifications. Lastly pulsed laser plasmas introduce the time domain element into x-ray microscopy. Whereas exposure times for microscopy with synchrotrons are measured in seconds, laser plasmas can provide x-ray emission in pulses ranging from several nanoseconds duration to less than one picosecond. This introduces the possibility of capturing kinetic, chemical, or morphological changes in biological structures in time frames of interest to understanding complex biological processes. No other type of microscopy can offer this capability.

Many measurements of the x-ray emission from laser plasmas have been made in the spectral regions of interest for biological microscopy[2–5], that is in the so-called 'water window' (2.3-4.4nm), and at other, element specific wavelengths. However these measurements have been made with laser and

target characteristics more germane to other applications of laser plasmas (such as laser fusion and x-ray laser generation). Moreover these studies have not had reason to consider the effects of plasma and particulate blowoff from the laser plasma, an important additional issue for microscopy, as it is for the use of laser-plasmas x-ray sources for lithography[6,7], where the integrity of expensive x-ray optics in close proximity to the target must be preserved. To the author's knowledge, no detailed laser-plasma design study has yet been made specifically to satisfy all the needs of pulsed x-ray microscopy.

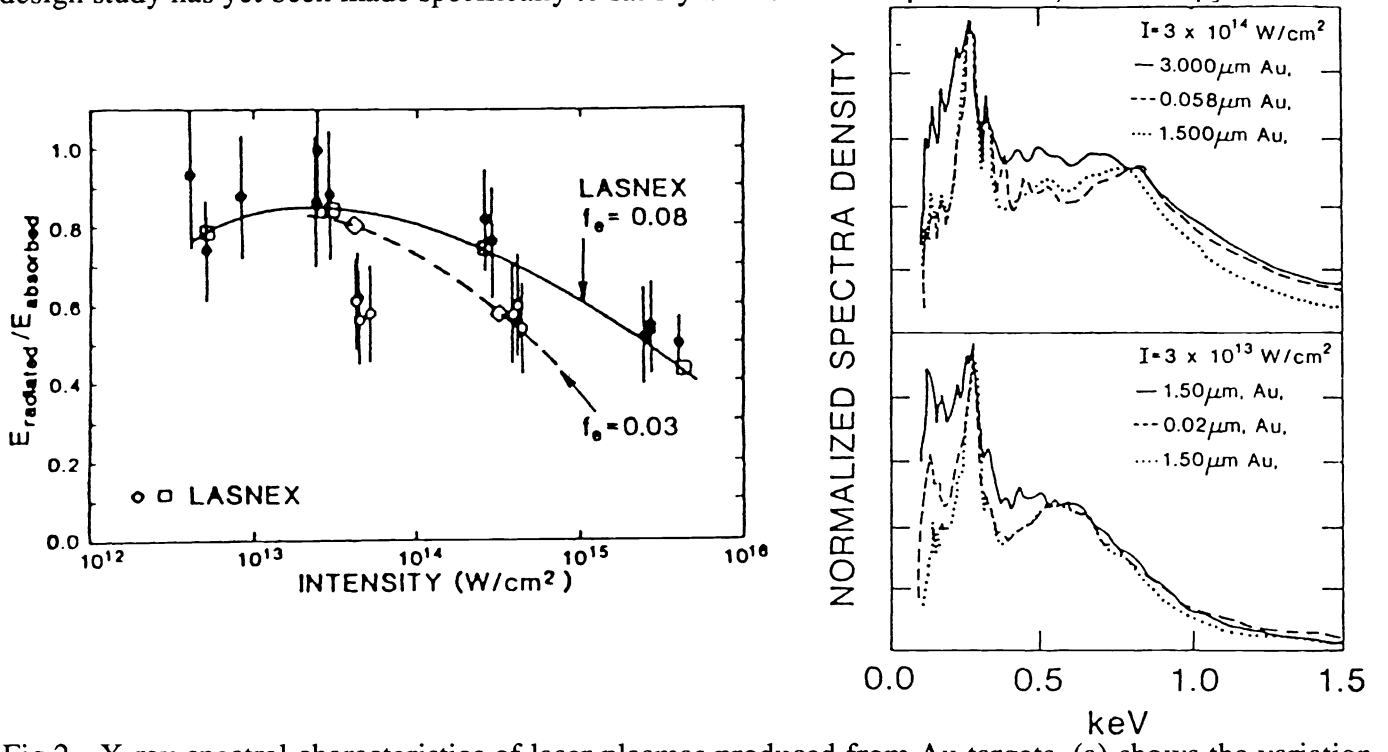


Fig.2. X-ray spectral characteristics of laser plasmas produced from Au targets. (a) shows the variation of x-ray conversion efficiency with laser intensity, and (b) indicates the variation of the x-ray spectrum with laser intensity. (from ref. 2)

III. X-ray optical requirements for a laser-plasma x-ray microscope.

In determining the specifications of x-ray optical components in a laser plasma-based x-ray microscope, we first establish the primary performance requirements of the overall system. We assume that the required resolution must be in the range of current imaging capabilities, ~50nm. This resolution, or better, has been demonstrated with Fresnel zone-plate imaging[8,9], contact imaging[10,11], and, at longer wavelengths, with normal incidence reflective optical imaging[12]. Moreover we assume that the system must be capable of real-time image acquisition. Current advanced image array detectors have pixel sizes in the 6μm range. Assuming a minimum image contrast ratio of ~10, this implies the need for an overall image magnification of ~$3x10^3$-10^4 This level of image magnification cannot easily be met with x-ray optics alone. Although most x-ray microscopes today rely primarily on optical elements having modest image magnification and long-time image processing of an image recorded on resist, film, or through image scanning, we believe the optimum microscope will require real-time image acquisition and thus will incorporate a combination of x-ray image magnification and electron-optical image magnification. Such a system is shown schematically in fig. 3. The specimen to be analyzed will be irradiated with monochromatic radiation from the laser plasma source. This could be facilitated by either a normal incidence multilayer mirror collector or a zone-plate condenser. An image of the specimen in the backlit radiation is then created with either a Schwarzschild microscope or Fresnel lens with a magnification (20-50) commensurate with the cathode resolution capability of an electro-optical image magnifier such as an x-ray-sensitive zoomtube[13] or an x-ray photoelectron microscope[14,15]. A zoomtube having an image

magnification of 40-200 and a cathode resolution of ~1μm has already been demonstrated[13]. Moreover, a photoelectron microscope having a resolution of 0.1μm and a magnification of 1000 should be developed in the near future[16]. At some loss in resolution, but with considerable gain in sensitivity and simplicity, the latter could be used in a simple contact or 'proximity' imaging mode.

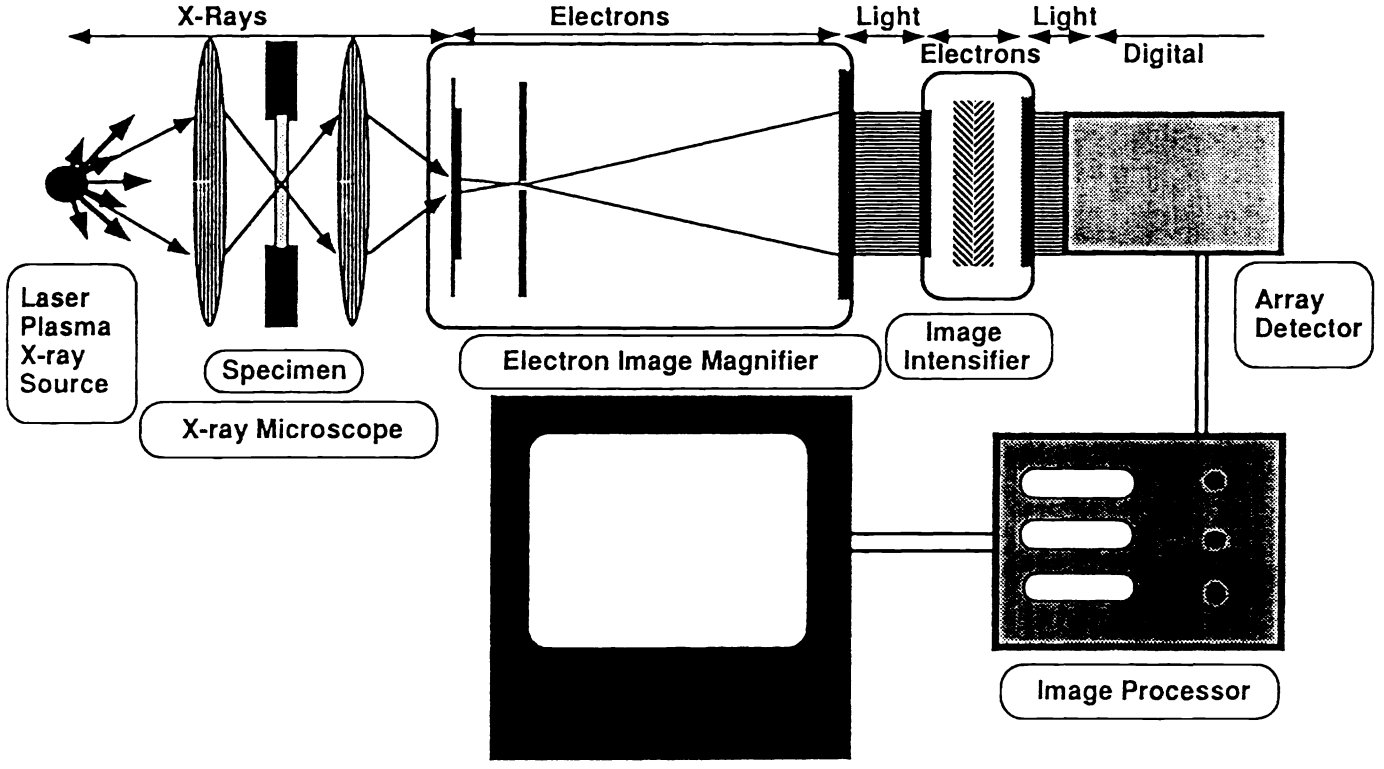


Fig.3 Possible structure of an x-ray - photoelectron-optical microscope.

We currently are investigating the practicality of two forms of x-ray imaging which could be incorporated into a laser plasma x-ray microscope of the type described above. In the first we investigate the limits of contact microscopy for analyzing *in-vitro* biological objects. In this investigation we study the effects of the x-radiation on the specimen, the degree to which the image contrast can be enhanced, and the resolution limits that can be achieved. In the second approach we examine the potential that Schwarzschild optics have in combination with laser-plasma x-ray sources for microscopy. We have made investigations with several Schwarzschild optics coated with x-ray multilayer coating with reflectivities centered at different wavelengths (4.42nm, 7nm and 17nm). We discuss the primary issues of resolution and contrast aswell questions of practicality, stability and ease of use in so far as envisaging such an optic in a compact x-ray microscope system.

IV. High resolution contact imaging of in-vitro biological specimens.

Many previous studies of contact microscopy of biological and life science objects have been reported. Several different sources have been used[17-22], including pulsed e-beam devices and synchrotrons, and a few recent investigations have been made with laser-plasma sources[21,22].

In the present studies small water specimen cells, 1-3μm thick, having a 100nm thick SiN window and backed with polymethylmethacrylate (PMMA) photoresist, fig.4(a), were used to contain stretched, live human chromosomes. They were irradiated with the x-rays generated by one pulse from a high power laser irradiating a planar Au target located 2cm away. The plasma was created on the target by 26J energy, 526nm wavelength, 1ns duration laser pulses with a focal spot intensity of ~ 10^{13}W/cm^2.

The specimen cell was positioned at an angle of 45° to the normal to the target and the laser beam axis. The stretched chromosomes were prepared by the surface-spreading technique and whole-mounted directly on the PMMA photoresist.

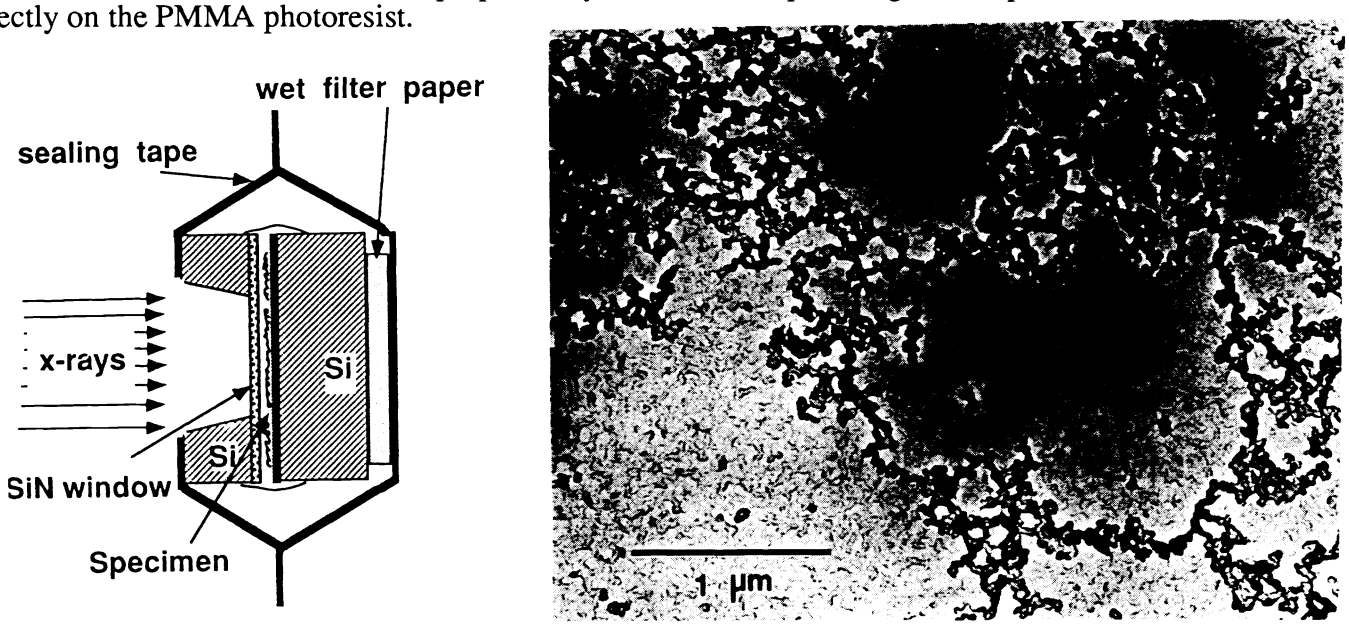
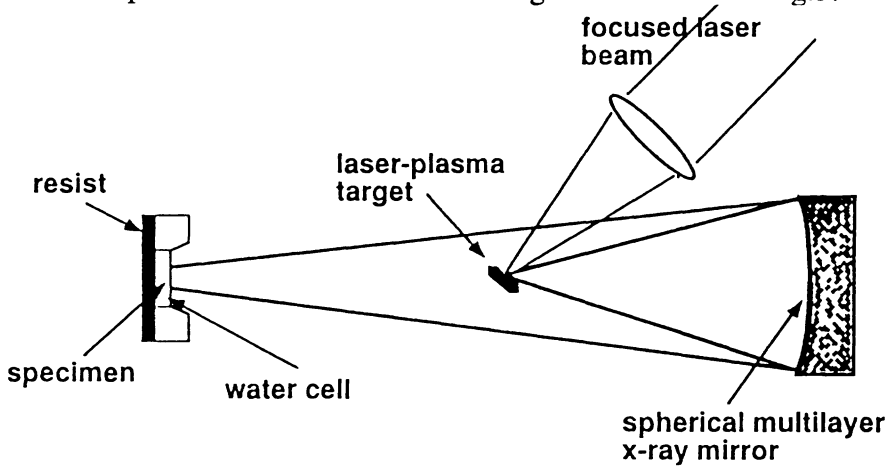


Fig.4. (a) Contact irradiation scheme. (b) Contact image of stretched in-vitro human chromosomes obtained with a single nanosecond burst of laser plasma x-rays.

After irradiation, the resist was developed by dissolving the damaged material in a mixture of methyl isobutyl ketone and isopropyl alcohol. Visualization of the three-dimensional morphology of the developed resist was then made by transmission electron microscopy of a thin ethylene replica of the resist made by plasma polymerization[23]. This technique of analysis is described in more detail elsewhere[24]. A typical image is shown in fig. 4(b). This shows details of the stretched in-vitro chromosome structure. Clearly visible are the superbead structure of chromatin fibrils, as described by Watanabe [25], composed of supertwisted DNA molecules. In fact the supertwisted DNA fiber, which has a dimension of ~10nm can be seen connecting the superbeads. This image clearly demonstrates the capability of obtaining nanosecond-time-resolved x-ray images with a resolution down to the resolution limit of the resist, using a laser plasma. Although the TEM images of these chromosomes appears quite good, we believe the image contrast can be further improved. The x-rays used to irradiate the sample in these experiments possessed a broad spectral range, not optimized for maximum image contrast of the chromosomes. This will best be achieved with x-rays in the water window. To this end we are currently using 4.4nm wavelength x-rays from a laser plasma obtained as in the configuration shown in fig.5.

Fig.5.

Scheme for monochromatic, water-window contact x-ray microscopy.



In this configuration a spherical W/C multilayer x-ray mirror[26] is used to focus 4.4nm wavelength x-rays onto the specimen cell. This approach should result in higher contrast images and in less radiation damage to the specimen from highly absorbed radiation from the x-ray source.

The principal limitation and disadvantage of this form of contact microscope is the complexity and delay involved in processing the x-ray image. This can be removed if contact microscopy could be integrated with a special form of x-ray-sensitive photoelectron microscope. Considerable progress has been made in the last few years in developing low energy photo-emission microscopes[14,15]. The spatial resolution of these instruments can now reach below 100nm[14,15]. The development of highly resolving transmission x-ray photocathodes having a spatial resolution in this range, and their incorporation with a similar electron-optical system to that used in a photo-emission microscope[16], would provide this capability. Such a system could be used in combination with contact imaging to provide high magnification, real-time images of biological specimens.

V. Normal-incidence reflective x-ray optical microscopy.

An important component in the microscope design described above is the high-resolution x-ray objective. This can, in principle, be a reflective optic, using either grazing incidence optics or normal incidence, high-reflectivity, multilayer coatings, or a transmissive Fresnel lens. We have made considerable effort in the last few years to explore the potential of x-ray multilayer-coated Schwarzschild x-ray optical elements for high-resolution biological microscopy. It is well known that this approach to high-resolution microscopy presses the limits of x-ray mirror fabrication and efficient x-ray mirror design. A 120mm diameter, NA = 0.35, 15X Schwarzschild microscope coated with Ni/C multilayer mirrors for 7nm and having a measured resolution, limited by the recording film-grain size, of 0.5μm was developed by Kado et al[27,28], and separately Richardson et al [29] developed a smaller, 33mm diameter, NA = 0.28, 15X Schwarzschild microscope coated with a W/C multilayer mirror for 4.4nm, in the socalled 'water window' (2.5-4.4nm). In the present, we report a multilayer-coated Schwarzschild microscope for 17nm, of similar design to that in ref.28. In particular, we report it's application, for the first time with a laser plasma source to the imaging of biological specimens.

The biological specimens used in these studies were dried HeLa cells in their natural state. The cells were supported on a thin plastic membrane, and irradiated with x-rays from a laser plasma created from a thin Au target. These experiments were made with the setup shown in fig.6.

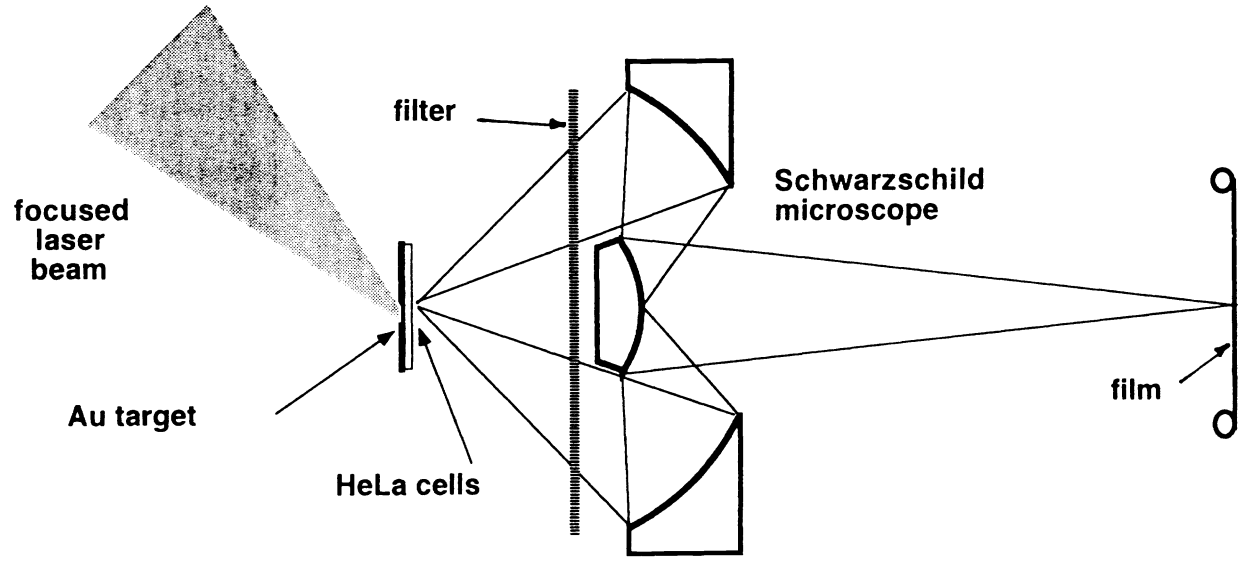


Fig.6. Experimental configuration for imaging x-ray microscopy of dried HeLa cells

The membrane supporting the HeLa cells was configured on a rectangular grid structure in direct contact with the 0.2μm thick Au laser plasma target, the whole assembly being positioned on a metallic washer on an accurate (1μm resolution) three-dimensional stepper motor-controlled target positioner in the center of an irradiation vacuum chamber at $5x10^{-4}$ Torr The grid structure was incorporated as an aid in aligning the specimen to be in the correct position relative to the focus position of the irradiating laser, and in the alignment of the Schwarzschild microscope to the specimen.

A precision method of alignment was vital to recording well-focused images of preselected regions of the HeLa cells distributed across the membrane. The position of the 200μm diameter laser focal spot was accurately registered on two CCD-recorded viewing systems attached to the target chamber, in separate laser irradiation experiments with thin foil targets, in which the laser plasma produced a small ~200μm diameter hole. This hole was then used to align the lateral position of the Schwarzschild microscope relative to the laser plasma x-ray source. The surrogate target was then replaced with the biological cell target, and aligned optically to the laser focus position. The axial position of the Schwarzschild microscope was then adjusted under vacuum by illuminating the grid and HeLa cell structure with white light and using a high-resolution CCD camera in the image plane of the microscope. precise lateral motion of the target structure them permitted selection of the desired cell structure for x-ray examination. The final x-ray image was recorded on Kodak 101 x-ray film, developed according to the calibration of Henke et al. A thin x-ray filter, (200nm Al on 300nm CH), was used to block visible light from the plasma from being detected. The duration of the x-ray emission is similar to the duration of the irradiating laser pulse (300ps). Thus the x-ray emission irradiate the sample before it is destroyed by the expanding laser plasma.

A typical single-shot x-ray image of dried HeLa cells recorded by the Schwarzschild microscope is shown in fig.7. The grid thickness and separation is 20μm and 100μm respectively. The individual, ~10μm diameter HeLa cells are clearly visible, with a resolution of ~500nm, limited by the grain size of x-ray film(5-7μm). Fig.8 shows the correlation between the optical image of a HeLa cell structure taken prior to irradiation with the resulting x-ray image of the same structure.

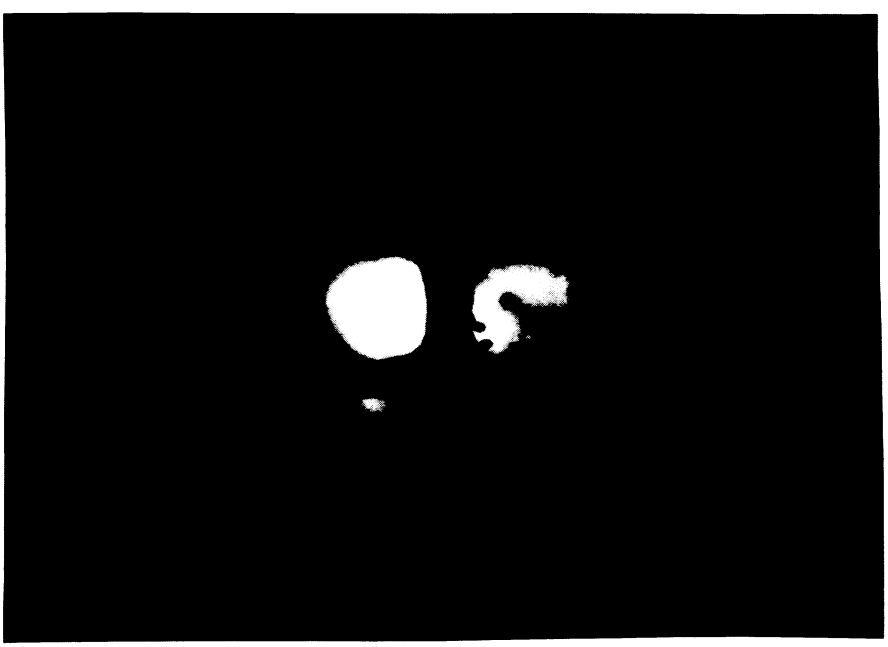


Fig. 7. X-ray image at 17nm of dried HeLa cells. Grid bar width 20μm, grid separation 100μm

a b

Fig.8. Corresponding optical (a) and x-ray (b) images of dried HeLa cells.

These images of these biological structures are limited in resolution by the film used for image recording. This limitation will be removed in future experiments with the use of an x-ray sensitive, electron-optical zoomtube image magnifier and direct, real-time, image acquisition[13]. The full potential of this approach will then be realized.

VI. Summary.

We have presented a concept of a practical x-ray microscope based on a compact laser plasma x-ray source. In addition, we have summarized our progress in both contact and imaging x-ray microscopy of biological samples in their natural state. In the future we plan to use these approaches towards two near term objectives. Firstly these techniques will be used to survey a wider range of biological samples to demonstrate the usefulness of x-ray microscopy to biological, medical and life sciences. Secondly, we will continue to develop these and the other technologies mentioned in this paper that are required for the successful fabrication of a practical stand-alone x-ray microscope.

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