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X-Ray Microimaging for the Life Sciences
Proceedings of the Workshop
May 24-26, 1989
at
Berkeley, California

* * *
David Attwood
Bob Barton
Editors

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Foreword

The workshop brought together the physical and biological science communities to explore the potential for imaging of macromolecular to sub-organelle structures, in their natural state, with spacial resolutions beyond those hitherto demonstrated. Emphasis was on direct imaging techniques applied to biological problems of structure and function, sequencing, and mapping. The requisite high brightness radiation sources, such as short wavelength undulators and x-ray lasers, were also addressed. In addition there were overviews of current and projected capabilities utilizing tunneling techniques, electron microscopy, and x-ray crystallographic techniques.

The Program Committee comprised the following fifteen workers from multiple disciplines with interests in the above applications to the life sciences.

David Attwood, LBL, Chairman
Ryszard Gajewski, DOE/BES
Robert Glaeser, UC Berkeley
Gerald Goldstein, DOE/OHER
Joe Gray, LLNL
Sol Gruner, Princeton
John Hearst, UC Berkeley
Janos Kirz, SUNY, Stoney Brook
Brian Newnam, LANL
Charles Rhodes, University of Illinois, Chicago
Stephen Rothman, UCSF
David Sayre, IBM
Szymon Suckewer, Princeton
Joseph Wall, BNL
Edwin Westbrook, ANL
DNA Structure:

The DNA in a single mammalian cell is a double helical structure consisting of nucleotides connected along the double helix by phosphodiester bonds and across the double helix by hydrogen bonding as illustrated in Figure 1 for two nucleotide pairs. The nucleotides pair through hydrogen bonding; A with T and G with C. The dimensions of the
AT and GC pairs are both ~11Å and the separation of the nucleotide pairs along the DNA double helix is ~ 3.4Å (Figure 2). Thus, X-ray resolution would have to be at the atomic level to reveal structural differences in the nucleotides or nucleotide pairs that could be used for DNA sequence analysis. The identification of specific nucleotides may be facilitated, however, by labeling them so that they can be detected more easily. For example, single stranded DNA molecules (i.e. half of the DNA double helix) can be synthesized in which the methyl groups (CH3; see Fig. 1) in the Ts are replaced by iodine. In this way, an iodine molecule can be placed at the location of each T in the DNA sequence. The problem then becomes one of determining the spacing between the iodine molecules and complementing this with information about the separation of the other bases obtained in the same way6. To be useful, x-ray imaging approaches to DNA sequence analysis must compete with commercial instruments that can analyze DNA sequences at the rate of ~10,000 nucleotides per day.

The structure of DNA molecules may also be determined by labeling short DNA sequences with gold using nucleic acid hybridization. In this procedure, single stranded DNA is produced and reacted with short (e.g. few nucleotide long), distinctly labeled sequences of single stranded DNA that are homologous to a portion of the DNA molecule under investigation. In this case, the labels might be gold microspheres a few nanometers in diameter7 which increase the scattering cross section and contrast. The problem then reduces to analysis of the locations of the distinctive labels. This scheme may be useful for detection of cloned DNA sequences that are partially homologous over part of their length. This capability would facilitate identification of contiguous cloned sequences as required for the physical mapping that is a prerequisite to DNA sequencing1.
Chromatin structure:

The \(~6\times10^9\) nucleotides that comprise the DNA in a mammalian cell, if stretched linearly, would form a molecule, roughly a meter in length and 20 Å in diameter. This material must be packaged into a mammalian cell nucleus \(\sim 5\ \text{µm}\) in diameter. This is accomplished in a hierarchical manner that is only partly understood by complexing DNA with organizing proteins to form chromatin (Figure 3). The first level of organization is the DNA double helix itself. At the next level, the DNA is coiled around a histone octamer (called a nucleosome) to form a \(~11\ \text{nm}\) diameter fiber. These fibers are then organized into \(~30\ \text{nm}\) diameter fibers. The existence and nature of these structures have been determined using a battery of physical and chemical techniques including neutron diffraction, electron microscopy and chemical analysis. Higher levels of organization have been difficult to infer, however; because of the requirement by most analysis techniques for substantial nuclear disassembly. For example, transmission electron microscopy requires comparatively thin samples to avoid complications derived from beam transmission through a complex thick section and scanning electron microscopy gives only surface information. Electron microscopy also requires sample fixation with the attendant distortion. Other procedures such as neutron diffraction and chemical analysis require more-or-less homogeneous samples and thus are poorly suited to analysis of complex structures such as are found in mammalian nuclei. Thus, the need remains for imaging tools capable of providing structural information about the organization of chromatin features larger than a few hundred Angstroms without disassembled or distorted by fixation. X-ray imaging will be useful in such studies if sufficient resolution can be achieved to distinguish between closely packed, low contrast structures in unfixed cells and if images can be obtained before the structures being studied are degraded by the imaging process. One approach here is to use high brightness sources such as X-ray lasers to generate images in a few hundred picoseconds while the structures are "inertially confined".

Figure 3
Chromatin dynamics:

An important aspect of chromatin structure is that it changes continuously during the process of DNA replication and gene expression. In DNA replication, for example, the hierarchical chromatin structure must disassemble or rearrange to make the DNA molecule available to the proteins and enzymes required for DNA replication (Figure 4). These may include enzymes to unwind double stranded DNA, proteins that stabilize single-stranded DNA, a DNA polymerase to catalyze DNA synthesis, RNA primase to initiate Okazaki fragment synthesis, DNA ligases, etc. Overall, the replication complex may "spread along" a region of DNA for several hundred Angstroms and it may move as along the DNA at rates up to 200 Å per sec. Interestingly, bacteria are able to replicate DNA almost an order of magnitude faster than mammalian cells; possibly a result of the fact that bacterial chromatin structure is less complex than that in mammalian cells and therefore more readily disassembled and reassembled. However, the exact nature of the replication complex (location, proteins involved, etc) is not well known. Similar dynamic changes are associated with DNA transcription.

X-ray imaging may contribute to the understanding of these processes if it can provide images of the replication or transcription complex in living
cells or, at a minimum, provide information about the locations and identities of the proteins that comprise these complexes. The latter may be facilitated by labeling the proteins and enzymes of interest with few nanometer gold spheres. To be useful, images should be obtained sufficiently rapidly (<msec) so that they are not blurred by the movement associated with cell function (e.g. movement of a replication complex along the DNA) or caused by Brownian motion.

Acknowledgement:

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RESEARCH DIRECTIONS IN DOE’S LIFE SCIENCES PROGRAMS
Gerald Goldstein, Department of Energy, Office of Health and Environmental Research, Washington, D.C. 20545

The Office of Health and Environmental Research (OHER) is the life sciences research arm of the Department of Energy. Historically, research in the life sciences originated in the Atomic Energy Commission as part of its mission to protect the health of the atomic energy workforce and the general public as well, and to explore the beneficial applications of atomic energy in biology and medicine. As a result of reorganizations within the Government, first establishing the Energy Research and Development Administration, then the Department of Energy, OHER is now responsible for studying the impacts of exposure to energy-related chemicals as well as radiation. Organizationally, OHER is one of the four outlay offices within the Office of Energy Research under the overall supervision of the Director of Energy Research, the others being the Office of Basic Energy Sciences, the Office of Fusion Energy, and the Office of High Energy and Nuclear Physics. OHER is internally organized and managed largely along disciplinary lines and consists of four Divisions. The Human Health and Assessment Division deals with medical research, the Health Effects Research Division with biology and biochemistry, the Ecological Research Division with environmental research, and the Physical and Technological Research Division with associated physical science. The total FY 1989 budget for these activities is $257 million.

The OHER program has two main objectives: (1) to develop the knowledge base necessary to identify, understand, and anticipate the long-term health and environmental consequences to energy use and development; and (2) to utilize the Department’s unique scientific and technological capabilities to solve major scientific problems in medicine and biology. Major program activities performed to achieve these objectives include:

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In addition to science oriented research aimed at improving our fundamental understanding in areas important to OHER objectives, several large scale multidisciplinary efforts have been mounted addressing key programmatic and societal issues that require a well organized and integrated approach involving a variety of scientific specialities. Some examples and their goals are:

- Human Genome Program - to provide the biological and computational resources and the instrumentation needed to completely characterize the human genome at the molecular level.

- Structural Biology - equip and staff capital intensive DOE facilities to develop and apply advanced techniques for the study of biological structure and function.

- Radon Research Program - to develop information to reduce the uncertainties and thereby enable an improved health risk estimate for people exposed to radon and its progeny.

- Acid Rain - to provide qualitative and quantitative understanding of the atmospheric chemistry processes associated with acid precipitation.

- Global Climate Change - to develop and improve the capabilities to estimate the range of regional and global climate change resulting from increasing carbon dioxide and other greenhouse gases.

- Subsurface Science Program - to define the physical-chemical-biological processes and interactions that control transport and stabilization of contaminants in the subsurface environment.

We anticipate that with the development of new high brightness, coherent synchrotron light sources, and x-ray lasers microimaging techniques will make an important contribution in structural biology research particularly in determining the three-dimensional structure of biological cells and subcellular components. Eventually a revolutionary advance in gene sequencing technology could emerge if it becomes possible to create an image of DNA molecules with sufficient spatial resolution to identify the individual bases and determine their order.
Chromosome Organization

The human genome contains $6 \times 10^9$ base pairs (bp) [diploid] or 204 cms of DNA packaged into 46 chromosomes. It is generally believed that chromosomes from all higher organisms each contain a single DNA molecule as has been shown for lower eukaryotes, such as yeasts. Yeast molecular genetics has identified three chromosomal elements essential for replication and equal segregation of chromosomes to daughter cells; i) the ends of the chromosomes or telomeres, ii) the centromere and iii) an origin of DNA replication. In Chinese hamster metaphase chromosome 2 (Figure 1) the centromere is the constriction at the center of the chromosomes located at the junction of sister chromatids and provides attachment points for the contractile mechanism to separate chromosomes into the daughter cells. Chromosome lengths, locations of centromeres and DNA dye banding patterns are used to identify chromosomes. The origins of the distinctive banding patterns are not well-understood but probably reflect a reproducible pattern of DNA folding induced by the interactions of sequence specific DNA regions with proteins [1]. Chromosomes contain enormous lengths of DNA, e.g., human chromosome 16 contains a 3·7 cm DNA molecule packaged into a metaphase chromosome of length 2·5 μm to give a linear DNA packing ratio of 15,000. To account for such high packing ratios earlier models for metaphase chromosomes were based on a series of linear coiled coils.
As we shall see a more realistic model to consider is the transverse packing of DNA loops into the thickness of metaphase chromatids.

Chromosomal Proteins and DNA Loops

Isolated chromosomal material consists of long DNA molecules associated with up to twice their weight of chromosomal proteins. Major proteins are the highly conserved group of 5 basic proteins, the histones that are equal in weight to that of DNA. Non-histone proteins are heterogeneous and contain proteins associated with the different chromosome functions, e.g., replication and gene expression as well as those associated with chromosome organization. Some of the proteins associated with long range order in chromosomes have been identified by treating chromosomes with high ionic strengths or with detergents to remove the histones and most other non-histone proteins leaving a small group of proteins tightly bound to the DNA. Electron micrographs of these depleted metaphase chromosomes show quite remarkable structures consisting of a residual protein "scaffold" of the metaphase chromosome surrounded by a halo of DNA. At higher resolution DNA loops can be observed to emerge from and return to the same point on the protein scaffold. (Figure 2) Two major scaffold proteins have been isolated, Sc1 and Sc2. Sc1 has been identified as topoisomerase II, which relaxes negatively supercoiled DNA through double stranded DNA cuts, and is essential at metaphase for the separation of sister chromatids to the daughter cells. Recent work from my laboratory shows also that histone kinases and topoisomerase II work in tandem in the process of chromosome condensation. [2] The size range of the DNA loops is 5 kbp to 120 kbp with an average size of about 50 kbp. Thus, the human genome of $3 \times 10^9$ bp DNA (haploid) corresponds to 60,000 loops of 50 kbp length that is in the range 50,000-150,000 genes thought to be required to specify a human being. This raises the possibility that each DNA loop defines a genetic unit of one or a small number of linked genes. In Drosophila Laemmli has shown that the group of 5 histone genes are located on a 5 kbp loop and that some developmentally regulated genes are single copy genes located on their own DNA loops. [3] The distribution of DNA loops has been shown not to change during development. Thus, questions related to developmental process of gene expression probably involve differential packaging of chromosome loops that determine the availability to factors of those genes required to specify and maintain a particular cell type.
Chromatin is a Repeating Subunit Structure

DNA and its associated chromosomal proteins, histones and non-histone proteins, is called chromatin. Digestion of chromatin in nuclei with micrococcal nuclease, which cuts double stranded DNA, gave a ladder of DNA lengths in multiples of a basic unit length of about 190-200 bp. This seminal observation showed that chromatin contained a repeating subunit. For most somatic tissues this subunit, the nucleosome, contains 195 ± 5 bp DNA the histone octamer [(H3_2 H4_2)(H2A,H2B)_2] and one histone H1. Prolonged, micrococcal nuclease digestion results in well-defined subnucleosome particles; i) the chromatosome with 168 bp DNA, the histone octamer and H1 and ii) the nucleosome core particle with 146 ± 1bp DNA and the histone octamer. [See 4] The core particle can be obtained in large quantities and has been subjected to extensive structural studies.

Neutron scatter techniques have particular application [5] to core particle structure determination because of the ability, by changing H_2 O : D_2 O mixtures, to “contrast match” either the DNA component (at 65% D_2 O) or the protein component (37% D_2 O). By neutron contrast matching core particles the radius of gyration of the DNA was shown to be 5.0 nm while that of the histone octamer was 3.3 nm. These measurements reversed the previously widely held view that histones were complexed on the outside of the DNA.

Analysis of the neutron basic scatter functions for the core particle gave the low resolution solution structure of the core particle (Figure 3). The regular structure of the core particle gives crystals that have been solved by x-ray diffraction to 7 Å resolution. [6] This structure is identical to that of the solution structure from neutron scatter but at the higher resolution, shows that α-helical segments of histones H3 and H4 interact with the DNA in its shallow groove whereas DNA regulatory proteins recognize and bind their specific sequences in the DNA deep groove. Another significant feature of the 7 Å structure is that the DNA does not bend uniformly around the core of the histone octamer but follows a path of straight segments with tight bends between these segments. This has relevance to the question of nucleosome positioning, i.e., whether some or all nucleosomes are located at precise positions on DNA sequences for functional reasons.

Figure 3. Core particle solution structure from neutron scatter. The hole is less than 1 nm. N-terminal regions of histones H2A and H2B are mobile in core particles (Braddock et al. 1981 [11]).
footprinting shows that some nucleosomes can be positioned to within a basic pair on a specific DNA sequence, e.g., Xenopus 5S rRNA gene DNA can be precisely located on a nucleosome. Analysis of 177 core particle DNA sequences shows that there is a code relating a DNA sequence to its "bend-ability." This has relevance not only to the locations of nucleosomes but also to the functions of sequence specific DNA factors controlling gene expression.

Chromatosomes and Nucleosomes

Extrapolating from the 1.7 turns of DNA of pitch 3.0 nm coiled around the core particle, Figure 3, the chromatosome's 168 bp of DNA is equivalent to 2 turns of DNA. These two turns require the binding of the fifth histone H1. Based on the structure of the core particle, and the conformation of histone H1 a model has been proposed for the chromatosome (Figure 4). A feature of the conformation of histone H1 is that the long flexible "arms" of the molecule have the potential to be involved in long range interactions in chromosome organization. The H1 "arms" undergo a pattern of phosphorylations through the cell-cycle, which would modulate these interactions. The fundamental structural units of chromosomes is the nucleosome. However, to describe the nucleosome beyond the model for the chromatosome requires a knowledge of the paths of the DNA, which links nucleosomes. Those paths are unknown at the present time. This lack of knowledge impedes considerably our understanding of higher order chromatin structures.

Higher Order Chromatin Structures

Chromatin in low ionic strength is in the form of a 10 nm diameter string of nucleosomes. This form is observed also when chromatin spills out of lysed nuclei. Neutron scatter studies of this 10 nm chromatin fibril give a mass per unit length equivalent to one nucleosome per 10 ± 2 nm, i.e., a DNA packing ratio of between 6 and 7 to 1. With increase of ionic strength the 10 nm fibril undergoes a transition to the "30 nm" fibrils. (Figure 5) Most of the DNA in interphase nuclei and metaphase chromosomes is contained in "30 nm" fibrils. Neutron scatter studies give a diameter for this fibril in solution of 34 nm and a mass/unit length, which is equivalent to 6 to 7 nucleosomes per 11 nm of fibril, i.e., a DNA packing ratio of 40 to 50:1. Because of the
paucity of hard structural data several models have been proposed for the 34 nm fibril including both one and two start helices. The simplest model that is consistent with available structural data is of a supercoil or solenoid of 6 to 7 radially arranged disc-shaped nucleosomes with a pitch of 11.0 nm and diameter 34 nm. Basic questions concerning the location of histone H1 and the linker DNA remain unanswered. In the metaphase chromosome the 34 nm filament appears to fold back on itself and coil into a 50-60 nm fibril.

Packaging of Chromosome Loops

The EM pictures of histone depleted metaphase chromosomes (Figure 2) shown transverse loops of DNA attached to the protein scaffold. The average size of these DNA loops is 50 kbp or 17 μm. With the measured DNA packing ratios, 17 μm of DNA could be packaged into 2.6 μm of the 10 nm fibril or into 0.4 μm of the 34 nm supercoil or solenoid of nucleosomes. Thus, the packaging of a DNA loop into the thickness of a sister chromatid (Figure 1) may require just one more order of chromatin folding. This additional order of folding is probably the 50-60 nm fibril observed in metaphase chromosome.

Architecture of the Cell Nucleus

In a diploid human cell the 2·04 m of DNA is packaged into a cell nucleus about 10⁻⁵ m in diameter. In general, the size of a cell nucleus is roughly proportional to the amount of DNA it contains. Most cell nuclei have minimum surface to volume ratios, i.e., are spherical. Departures from this shape may occur in specialized cells, e.g., nuclei that are genetically very active probably need to increase their surface area in response to the demand for increased RNA synthesis. Because of the enormous lengths of eukaryotic DNA it has long been assumed that the cell nucleus is highly organized. So far, however, we have had only glimpses of this order, e.g., telomere organization is maintained through interphase and metaphase chromosomes have the same arrangement in nuclei. There is a protein framework called a nuclear matrix that is thought to play a major role in the organization of the cell nucleus. The nuclear matrix has two major elements; a mesh of
protein fibers, called lamins, on the inside surface of the nucleus and an internal protein network. In the current model for DNA organization in the cell nucleus there are specific DNA attachments to both the inner periphery of the nuclear membrane and to the internal protein network. Chromatin takes different forms in different phases of the cell cycle. At metaphase the familiar condensed chromosomes organized by a chromosomal scaffold are observed. Following cell division the chromosomes redistribute throughout the nucleus into condensed chromatin regions (heterochromatins) associated with the inner surface of the nuclear membrane and dispersed chromatin (euchromatin) inside the nucleus. Much of our limited knowledge of the architecture of the cell nucleus comes from electron microscopic studies of cell sections. There is a great need for other microscopic methods that image the hydrated states of nuclei at different stages of the cell cycle and the states of nuclei from different specialized cells.

Conclusion

We are still a long way from understanding the structure/function relationships of eukaryotic chromosomes despite their central importance to biology. Relevant to this understanding will be the sequence information from the Human Genome Project. Although much interest focuses on the mapping and sequencing of genes, non-coding DNA regions clearly contain sequences involved in the organization and functions of chromosomes. The constancy of banding patterns of individual metaphase chromosomes (Figure 1) reflects a highly reproducible pattern of long-range DNA folding, most probably directed by specific DNA-protein interactions and possibly by unusual DNA structure, e.g., bent DNA segments. Superimposed on this long range order is the packaging of the DNA loops, which involves mainly the histones together with other poorly defined structural and regulatory proteins. A major biological question is how the different packagings of loops might determine the states of different gene families, i.e., permanently repressed, potentially active (e.g., heat shock genes) and active genes. DNA control regions of active genes must be accessible to gene regulatory proteins whereas regulatory regions of the permanently repressed genes of a particular cell type may be packaged so that they are inaccessible. Such packaging may also determine the availability of DNA regions to chemical damage. Thus, a knowledge of the organization of chromosomes is essential to an understanding of the central processes of differentiation and development as well as the processes of DNA damage in chromosomes. Such information is also essential to the ultimate understanding of chromosome organization in the cell nucleus. It is to be hoped that the x-ray imaging capabilities of the Advanced Light Source will provide images of hydrated chromosomes and nuclei at resolutions down to 10-0 nm.

Acknowledgements

Work in the neutron scatter determinations of the solution structure of nucleosomes, chromatin and DNA-protein complexes is supported by the Department of Energy and Los Alamos National Laboratory.
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INTRODUCTION TO X-RAY IMAGING METHODS

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X-rays are one of the three energy regions of photons or electrons in which mean free paths in condensed matter are larger than wavelength, permitting imaging of the internal structure of condensed matter (Fig. 1). The x-ray region is special among these regions in that its mean free path and wavelength give it the potential of imaging whole biological cells at nanometer resolution.

To realize this potential, high-resolution x-ray imaging techniques suitable for the imaging of general specimens are needed. At present, the only high-resolution x-ray techniques are those of x-ray crystallography and are limited to special types of specimens. This paper provides an introduction to the status and possible future of general high-resolution x-ray imaging methods.

Basically the situation is that focussing systems, which are the basis for high-resolution imaging with light and electrons, also exist for x-rays but due to the short wavelengths involved require extremely accurate fabrication. (This consideration is one of the reasons why most of the work to date has been done with x-rays of wavelength 20Å and above.) Resolutions at present are near 300Å, with 100Å regarded as possible in the next few years. To supplement these, methods of imaging which do not use focussing are also being studied. Ultimate resolutions to 30Å for focussing methods, and possibly to 10Å for non-focussing methods, can be envisaged. These numbers refer to purely optical limitations, however, and resolutions will inevitably be affected by radiation damage as well (see below).

Imaging methods: review.

In this paper we regard the process of imaging a specimen by radiation as equivalent to that of placing a large number of dots on a sheet of paper, the location of each dot corresponding to the location of a site in the specimen where a reaction has occurred between the incident radiation and the specimen. An image is thus a map of reactivity sites in a specimen.

Images may be formed in several different ways: (a) by focussing of product particles of the reactions to points which are focal images of their points of origin (conventional microscopy); (b) by focussing of the incident particles to confine reactions to single pixels of the specimen (scanning microscopy); (c) by high spatial-resolution examination of the transmitted beam at a position immediately behind the specimen (contact imaging); and (d) by recording the spatial distribution of a particular group of product particles, the coherently scattered particles (diffraction imaging and holography). The distribution of these latter particles is within a Fourier transformation of being a specimen image. In (c) and (d), the specimen is acting to image itself.
Broadly speaking, (a) and (b) are extremely good techniques when good focussing elements are available, and are the mainstay of visible-light and electron imaging; (c) avoids the need for focussing elements but encounters a degradation of resolution with thick specimens; (d) can provide the highest-quality imaging of any of the methods and imposes minimal equipment requirements, but has problems of its own, including the so-called phasing problem. Holography provides one solution to the phasing problem and is included here under (d). (d) is the method mentioned earlier as the basis of x-ray crystallography.

Resolution of imaging methods: review.

For methods (a), (b), and (d), resolution is poor if particles are gathered over a small collection angle and good if gathered over a large angle. Fig. 2 diagrams the resolution in units of the wavelength for different collection angles. For full collection the resolution can reach a value of half the wavelength, i.e. nanometers or fractions of nanometers in the x-ray region.

For method (c), resolutions cover a similar range, but the parameter which degrades resolution is feature-to-detector distance rather than collection angle. Since feature-to-detector distance cannot be entirely controlled in whole-cell imaging, (c) is ultimately less desirable* than the other methods in that application. For the present, however, (c) offers better resolution than (a), (b), or (d).

X-ray imaging methods

Fig. 3 specializes the discussion to the x-ray region and indicates the status and possible future of these methods in that region. Some explanatory notes follow.

Column 1. Methods are grouped roughly chronologically, with x-ray contact imaging dating from 1895-6. Conventional and scanning x-ray microscopies, and x-ray holography, were first attempted or proposed in or near the 1950s-60s. X-ray diffraction imaging was proposed for general specimens around 1980, having existed in crystallography since 1912-13.

Column 3. Resolution levels are approximate. In contact imaging, early estimates of resolution to 50Å (1) have been questioned and somewhat larger estimates suggested as more likely (2). In very recent work, structures have been seen in human chromosomes which have been tentatively identified with the 100Å chromatin fiber (3). In zone plate

*A technique has been proposed by the author in which several contact images are recorded simultaneously at different distances from the specimen and used to eliminate the resolution deterioration in (c), but the technique calls for very high accuracy in reading the images and is not currently practical at x-ray wavelengths.
Fig. 1. Summary of imaging regions. The slanted straight lines give wavelengths vs. energy (upper line photons, lower line electrons). The curved lines give mean free path vs. energy (upper curve photons in water, lower curve electrons in typical polymers). Imaging of internal structure can take place where mean free path is larger than wavelength. The vertical bars and shaded regions show the utilization of these regions; upper ends give an indication of the size of specimen which can be examined, and lower ends the size of the smallest features which can be seen. The two dense shaded regions denote electron and visible-light microscopy. The solid vertical line shows x-ray crystallography, and the dashed vertical bar shows the x-ray imaging which forms the subject of this article.
Except in contact case:

\[
\text{Resolution} = \frac{\lambda}{2 \sin \theta}
\]

\((\theta = \text{half-angle of collection})\)

In contact case:

\[
\text{Resolution} \approx \sqrt{\lambda d}
\]

\((d = \text{feature-to-detector distance})\)

Fig. 2. Foil for discussion of resolution. The resolutions shown on the diagram are in units of the wavelength. The diagram and the resolution formulae give resolutions which are slightly different (but close) to the Rayleigh definition of resolution.
<table>
<thead>
<tr>
<th>Method</th>
<th>Main current implementation</th>
<th>Present resolution level</th>
<th>Present minimum exposure</th>
<th>Turn-around</th>
<th>Present resolution goal</th>
<th>Feasible optical limit?</th>
<th>Sensitivity to radiation damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact imaging</td>
<td>X-ray resist EM/STM readout</td>
<td>100A</td>
<td>Nanosecs</td>
<td>Hours</td>
<td>Same</td>
<td>Same</td>
<td>Low</td>
</tr>
<tr>
<td>Conventional m'scopy</td>
<td>Zone plates</td>
<td>300A</td>
<td>&quot;</td>
<td>Seconds</td>
<td>100A</td>
<td>30A</td>
<td>&quot;</td>
</tr>
<tr>
<td>Scanning m'scopy</td>
<td>Zone plates</td>
<td>&quot;</td>
<td>Seconds</td>
<td>Seconds</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Medium</td>
</tr>
<tr>
<td>X-ray holography</td>
<td>Fresnel h'graphy</td>
<td>&quot;</td>
<td>Nanosecs</td>
<td>Day</td>
<td>100A</td>
<td>&quot;</td>
<td>Low</td>
</tr>
<tr>
<td>Diffraction</td>
<td>Low-noise collimator</td>
<td>70A</td>
<td>Hours</td>
<td>Months</td>
<td>Observation below 70A; try to image</td>
<td>10A</td>
<td>High</td>
</tr>
</tbody>
</table>

Fig. 3. Main table. See text for additional discussion.
work, the author is not aware of any images at resolutions below 500Å, but photographs of zone plates with geometries which should be capable of imaging at close to 200Å have been published (4). In holography, the best reported image reconstruction is at 400Å, but signal strength in holograms to better than 200Å has been reported (5). In diffraction studies, no imaging has been attempted as yet, but diffraction pattern to 70Å has been seen (6).

Column 4. Times are for single images except for diffraction imaging, where the estimate is for full 3-dimensional imaging. Full 3D imaging is likely to be standard in diffraction imaging because of needs imposed by the phasing problem. Times are based on present best sources. In addition, times reflect differences in the degree of parallelism in the methods (cf. scanning vs. conventional microscopy), and in the frequency of production of the particles observed (cf. diffraction imaging, which records a type of product particle for which the production cross-section is fairly low; holography uses the same particle and experiences some difficulty in obtaining sufficient signal in a current single x-ray laser pulse (7).

Column 5. The turnaround given for contact imaging is for the usual form involving examination of the exposed resist in an EM or STM. In forms using electronic detection (8) the image resolution is somewhat poorer but the imaging is real-time.

Column 6. Plans for holography also include plans to change from Fresnel to Fourier holography.

Column 7. The 30Å (10Å) entries assume that methods will exist which employ collection angles of almost a full half-sphere (full sphere) respectively. The column is labelled "optical limit" to emphasize that limitations from radiation damage are not considered in this column.

Column 8. Entries are given in relative terms, as no reports exist as yet of damage having been seen at present resolution levels. (Note: Damage effects at 70Å resolution (not yet confirmed) may have been seen in diffraction imaging experiments just prior to the time of writing this abstract. Diffraction imaging and holography expose the specimen to large amounts of incident radiation; see note on column 4. They also directly observe the Fourier transform of the image, and thus give a natural opportunity to look for damage effects at high resolutions.)

See also other papers, especially (9,10), in this conference.

References
(5) M. Howells, this conference.
(6) D. Seyre et al., recent work.
(7) J. Brase, this conference.
(9) S.P. Newberry, ibid.
(10) C. Rhodes, this conference.
Brightness, Coherence and Imaging at X-Ray Wavelengths

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Coherence, in our daily lives, refers to a systematic connection between events, actions, or policies. The word implies similar relationships among the field amplitudes used to describe electromagnetic radiation. Mathematically, one utilizes a mutual coherence function, $\Gamma$, as a measure of the degree to which electric field amplitudes at one point can be predicted, if known at some other point, as a function of space and time:

$$\Gamma(P_1, P_2; \tau) \equiv \langle E_1(t+\tau)E_2^*(0) \rangle$$

(1)

When fields are correlated over great spatial separations ($P_1$ far from $P_2$) and great time separations (large $\tau$), we tend to speak of coherent fields, i.e., a situation where there is a high expectation of being able to predict the field at one space-time point knowing it at another. At the other extreme are incoherent fields, where fields are relatively uncorrelated such that the expectation value $\langle \cdots \rangle$ quickly goes to zero for small spatial ($P_1, P_2$) or temporal ($\tau$) separations.

Fully coherent radiation, in which fields are perfectly correlated over all spatial dimensions and over all time, requires a single point source, oscillating with a pure frequency for all time. Real physical sources, of finite size, bandwidth and duration, do not meet these mathematically precise requirements, and are said to be "partially coherent". This brings us to notions of "regions of coherence" and "coherence time"; that is, spatial and temporal measures over which field amplitudes are well correlated. In some cases, in which energy flow is in a rather well-defined direction, it is useful to introduce the concepts of longitudinal and transverse coherence -- measures of the region of coherence along the direction of propagation and normal thereto. The longitudinal "coherence length," often referred to as the temporal coherence length, $l_{coh}$, is closely related to the spectral bandwidth, $\Delta \lambda$, viz.,

$$l_{coh} = \frac{\lambda^2}{\Delta \lambda}$$

(2)

where $\lambda$ is the wavelength.

Transverse coherence refers to field correlations transverse to the direction of energy propagation and is often referred to as spatial coherence. The subject of spatial coherence is important. Whether one is attempting to encode a complex wavefront -- as in holography -- or focus radiation to a smallest possible focal spot, it is necessary that the radiation field be coherent, or well behaved, over sufficiently large transverse dimensions. Often this requirement is overlooked because researchers using modern visible light sources -- lasers -- tend to deal with radiation in which transverse mode selection, internal to the laser itself, guarantees the desired spatial properties. Where this is not the case, one speaks of the radiation as being "so-many times diffraction limited," and external (to the
laser) techniques, such as a lens-pinhole spatial filter, are employed to pass only the spatially-coherent radiation (lowest order mode).

Working at x-ray wavelengths we must also be cognizant of the issues of spatial and temporal coherence. Longitudinal coherence lengths are generally several microns for both x-ray lasers and undulators, the two primary sources of partially coherent x-rays. Such dimensions are quite suitable as they are well matched to typical absorption lengths and can be extended as needed using spectral filtering methods -- albeit with concomitant losses of photon flux. Similarly, spatial filtering techniques are also required to achieve desired degrees of transverse coherence. This spatial filtering is required because the radiation phase space is overly large for both undulator radiation and x-ray laser radiation. Note that the phase space volume of undulator radiation is set primarily by the "emittance" of the relativistic electron beam, and lack of mode-control in the early expanding plasma laser experiments. Spatial coherent radiation is characterized by a space-angle product

\[
d \cdot \theta \equiv \frac{\lambda}{2\pi} \quad (3)
\]

where \(d\) is the source diameter and \(\theta\) is the far field radiation half angle, both measured as \(1/\sqrt{e}\) quantities. Note that in terms of a radial measure, rather than a diameter, as is the custom in the accelerator community, this would take the form \(r \cdot \theta \equiv \lambda/4\pi\), leading to an emittance criterion \(\sigma_r \sigma_\theta \equiv \lambda/4\pi\), where \(\sigma_r\) and \(\sigma_\theta\) are the rms radius and half angle measures of the electron bunch, and where \(\pi \sigma_r \sigma_\theta\) is the emittance.

Microscopy does not in general require radiation with good coherence properties; indeed, one should not employ more coherence than is required for a particular task.\(^3\) Visible light microscopy, common to all schools and research laboratories, uses highly refined optical instruments based on use of incoherent light. X-ray microscopy using incoherent synchrotron radiation has provided well-defined images of biological material,\(^4\) and more recently of electronic microcircuits and test patterns resolved\(^5\) to 500 Å, with indications of steady progress in the future.\(^6\)

Two situations in which coherence is required for high spatial resolution x-ray microimaging, involve scanning x-ray microscopy\(^7\) and x-ray holography.\(^8\) In scanning microscopy the beam is focused to the smallest possible spot size. Because high spatial resolution is achievable only with relatively inefficient (typically 5% to date) Fresnel zone plates, the scanning systems offer a significant advantage with regard to reduced radiation damage to biological material. Achieving this high spatial resolution requires not only a very precisely fabricated lens, but also a radiation source that is sufficiently close to being "point-like" in nature that its image in the focal plane -- the finite spot size that sets scanning microscope spatial resolution -- is determined only by the finite wavelength and lens F-number, e.g.,

\[
\Delta x \equiv 1.2 F \lambda \quad (4)
\]

A radiation source which can be focused to a spot size given by Eq. (4) is spatially coherent; it appears to radiate a smooth spherical wavefront containing little or no information about the source's finite spatial size, structure or intensity distribution. Achievement of the required spatial coherence has to date been accomplished by pinhole spatial filtering. Because Fresnel zone plates exhibit a strong chromatic aberration, they require use of narrow spectral bandwidth radiation, \(\Delta \nu \lambda \equiv 2N\), where \(N\) is the number of zones. As a consequence, the radiation must also have a degree of temporal (longitudinal) coherence length given by
\[ L_{\text{coh}} = \frac{\lambda^2}{\Delta \lambda} = \frac{N \lambda}{2} \]  

(5)

Thus we observe that use of a Fresnel zone plate lens in a scanning x-ray microscope requires radiation that is both temporally and spatially coherent at the desired x-ray wavelength.

Biological imaging utilizing scanning x-ray microscopy has recently\(^9\) achieved two significant milestones: (1) spatial resolution well beyond that of the visible light microscope, and (2) images of biologically viable samples in a wet and unmodified state (neither fixed, sectioned nor stained). The goal of this microscopy effort is to study a model biological process -- that of secretion -- with a space-time dynamic capability not previously possible. The trade-offs between resolution and damage, determinations of short-term functional viability, careful radiation budgeting and microscope engineering will determine the eventual success of this effort. Early results of zymogen granules in vivo, resolved to better than 1000 Å, have been obtained with protein content measured to sub-femtogram sensitivity.\(^9\) Measurements of larger populations, resolved to values approaching 500 Å, are in progress.\(^10\)

Spatially and temporally coherent radiation -- at least to the extent needed to permit formation of well modulated interference fringes -- is required for the encoding of complex scattered wavefronts in holographic microscopy of biological specimens. Using the same pinhole spatial filtering and monochromator spectral filtering as described above for scanning microscopy, Howells, Jacobsen, Kirz and colleagues have recorded and recently reconstructed x-ray holograms, achieving image plane spatial resolutions well below 1000 Å.\(^11\) Done in collaboration with S. Rothman, these reconstructions also involve microimaging of zymogen granules.

X-ray lasers, first reported only a few years ago,\(^12,13\) have natural coherence lengths of order 100 microns, high (single-pulse) peak power, and very short (sub-nanosecond) pulse duration. Such lasers, at least as developed so far, are multimode devices and therefore require spatial filtering or mode-selecting of some kind.\(^14,15\) J. Trebes, using what is essentially a far-field mode selection technique, has recorded and reconstructed the first x-ray laser hologram with sub-nanosecond duration, 206-Å wavelength radiation.\(^16\) Further experiments directed towards high spatial resolution, short time duration, holographic microimaging utilizing x-ray laser techniques and chromatin structure, are presently being considered.\(^17\) Chromatin, the protein structural material that determines the folding and packing of DNA, is particularly interesting as a subject because it is believed to be characterized by various spatial features covering the "mesoscopic" scales between those observable by visible light and those by electron microscopies. It thus provides both a valuable scientific opportunity to confirm these features, and a range of spatial scales to be examined as spatial resolutions improve.\(^18\) Careful analysis of the relationship between achievable spatial resolution and concomitant radiation damage, including a strategy for minimization of that damage in holographic microimaging, is a cornerstone of that effort.\(^19\)

With ever brighter, partially coherent radiation sources becoming available, both synchrotron-based and x-ray lasers, it is interesting to consider the relationship between brightness and coherence, and how that relationship depends explicitly and implicitly on wavelength \(\lambda\). One way to express brightness is photon-flux-emitted-per-unit-source area \(\Delta \Omega\), into solid angle \(\Delta \Omega\). Brightness is an important concept in radiation theory as it is a conserved quantity in lossless, aberration-free optical systems. Of further interest to us for the purpose of probing element-specific spectral features is spectral brightness (SB) -- the brightness per normalized spectral bandwidth, viz.,


\[ SB = \frac{\text{photons/sec}}{\Delta A \cdot \Delta \Omega \cdot (\Delta \lambda / \lambda)} \]  

(6)

where \( \Delta \lambda / \lambda \) is often, by convention in the synchrotron community, taken at a value of 0.1% bandwidth.

Total radiated power, \( P \), is the energy per photon multiplied by the photon flux:

\[ P = h \nu \cdot \text{photons/sec} \]  

(7)

With spectral brightness as given in Eq. (6), the relation to total radiated power \( P \) can be written as

\[ P = h c \cdot SB \cdot \Delta A \cdot \lambda \cdot \Delta \lambda \]  

\( \lambda^2 \)  

\[ \lambda^2 \]  

(8)

where the factor \( hc/\lambda \) is due to the units change from watts to photons/sec, and \( \lambda^2/\Delta \lambda \) is recognized as the longitudinal coherence length. Equation (8) is a relationship for the total power. If we wish to determine that fraction of the power which is spatially coherent, \( P_{\text{coh}} \), we must compare the product \( \Delta A \cdot \Delta \Omega \) to that of spatially coherent radiation. Noting that \( \Delta A = \pi d^2 \) and that \( \Delta \Omega = \pi \theta^2 \) for a narrow radiation cone of half angle \( \theta \), one observes that by spatial filtering one can choose to pass only that fraction of the radiation that satisfies the spatial coherence condition \( d \cdot \theta = \lambda / 2\pi \). Thus one observes that

\[ P \rightarrow P_{\text{coh}} \text{ as } \Delta A \cdot \Delta \Omega = \pi^2 (d \cdot \theta)^2 \rightarrow \left( \frac{\lambda}{2} \right)^2 \]  

(9)

Taking these limits in Eq. (8) above, one obtains

\[ P_{\text{coh}} = \frac{hc \left( \frac{\lambda}{2} \right)^2 \cdot SB}{L_{\text{coh}}} \]  

(10)

showing that for fixed coherence length, coherent power is proportional to spectral brightness times wavelength squared \( \lambda^2 SB \), further showing that coherent power declines rapidly at shorter wavelengths, even for fixed spectral brightness. Of course, spectral brightness itself often declines with wavelength. Indeed, bright radiation is more difficult to generate at shorter wavelength.

Let us just note before closing that had we chosen to express spectral brightness in terms of power (rather than photon flux) per unit area, unit solid angle and unit relative bandwidth, we would not have had to make the units change \( hc/\lambda \), and Eq. (10) would have taken the form \( \lambda^3 SB \), showing the cubic fall off when brightness is written in power units, as it sometimes is. Thus if one had a mechanism to generate radiation at rather constant power across a certain spectral range, either through saturated x-ray lasing or use of constant field undulators, one would still have coherent power dropping dramatically with
shorter wavelengths. The cubic dependence arises from the ever more demanding phase space limits in the two orthogonal transverse directions \((\lambda/2\pi)^2\), and the stated requirement of fixed longitudinal coherence length \((\lambda)\), assuming fixed bandwidth. Curves illustrating these general trends, which have been published elsewhere,\textsuperscript{14,20,21} are moving targets in these very exciting days of ever-improving x-ray source\textsuperscript{12,23} and optical\textsuperscript{22} development. The time is ripe for major advances, in both the life and physical sciences, using short wavelength coherent radiation.

Acknowledgement

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References

2. See, for example, A. Siegman, Lasers (University Science Books, Mill Valley, CA, 1986).
6. E. Anderson (LBL) and D. Kern (IBM/Yorktown Heights), private communication.
7. Scanning x-ray microscopy, designed to minimize radiation damage to biological specimens while achieving high spatial resolution and elemental sensitivity, was pioneered by J. Kirz, his students and colleagues, at SUNY Stony Brook and Brookhaven National Laboratory.
8. X-ray holography has been pioneered in Japan by K. Kikuta, S. Aoki, K. Kohra and their colleagues at the U. of Tokyo and the Photon Factory in Tsukuba; by M. Howells, C. Jacobsen, J. Kirz and their colleagues in the U.S. using synchrotron radiation at Brookhaven's NSLS; by J. Trebes, D. Matthews and colleagues at Lawrence Livermore National Laboratory using visible light pumped x-ray lasing in a neon-like selenium plasma; and by D. Joyeaux, F. Pollack and colleagues using the ACO synchrotron facility in France.
9. This effort is led by S. Rothman of UCSF and LBL's Center for X-ray Optics; see, for example, early results by S. Rothman et al., Biochim.Biophys.Acta 991, 485 (1989).


17. The multidisciplinary team planning these experiments involves J. Trebes, J. Gray, D. Matthews, R. London, M. Rosen and others at Lawrence Livermore National Laboratory. See papers in these proceedings.

18. J. Gray, these proceedings.

19. R. London, these proceedings.


22. N.M. Ceglio, "The Revolution in X-Ray Optics," J. X-Ray Science. and Techn. (Academic Press, to be published); also, these proceedings.

Some Terminology

- **Photon Flux**
  \[ \mathcal{J} = \text{photons/sec} \]

- **Brightness**
  \[ B = \frac{\text{photons/sec}}{\Delta A \cdot \Delta \Omega} \]

- **Spectral Brightness**
  \[ SB = \frac{\text{photons/sec}}{\Delta A \cdot \Delta \Omega \cdot \left( \frac{\Delta \lambda}{\lambda} \right)} \]

- **Power**
  \[ P_T = h\nu \cdot \text{photons/sec} = \frac{SB \cdot \Delta A \cdot \Delta \Omega \cdot \frac{\Delta \lambda}{\lambda} \cdot hc}{\pi^2(d\cdot\theta)^2} \]

- **Coherent Power**
  \[ P_{coh} = \left( \frac{\lambda}{2} \right)^2 \frac{SB \cdot hc}{\ell_{coh}} \text{ when } \Delta A \cdot \Delta \Omega \rightarrow \frac{\lambda^2}{4} \]

- **Coherent Power**
  \[ P_{coh} = P_T \cdot \frac{\lambda^2(2\pi)^2}{(\text{phase space})^2} \]

*d\cdot\theta = \frac{\lambda}{2\pi} \text{ for Gaussian rms measures} \]
Coherent Radiation

Electromagnetic radiation propagating in such a manner that the wave nature is simply described and interrelated over large distances of space and time, e.g., a spherical wave in which the phase at one point \( P_1 \) is well correlated with the phase elsewhere \( P_2 \).

**Mutual Coherence Function:**
\[ \Gamma(P_1, P_2; \tau) = \langle E_1(t) E_2^*(t) \rangle \]

**Partial Coherence**

Full coherence requires a point source oscillating with a single frequency for all time \((-\infty \leq t \leq \infty)\): Laboratory sources are finite in both spatial extent and duration and thus are partially coherent:

\[ \text{"d"} \theta = \frac{\lambda}{2\pi} \]

\[ l_{coh} = \frac{\lambda^2}{\Delta \lambda} \]
Partial Coherence
Following Wolf (M. Born and E. Wolf in *Principles of Optics*, Ch. 10. Also Hech, *Optics*, Ch. 12.

**Temporal coherence** relates to finite bandwidth, and for a reasonable degree of coherence is characterized by a "coherence length" in the direction of propagation $l_{coh} \equiv \frac{\lambda^2}{\Delta \lambda}$. If written as $N_c = \frac{\lambda}{\Delta \lambda}$ one sometimes speaks of the "number of waves" of coherence, viz.,

$N_c = \frac{\lambda}{\Delta \lambda}$.

**Spatial coherence** relates to the finite spatial (lateral) extent of the emitting source or radiation. Generally this concept is useful in the case of reasonably high degrees of coherence and refers to the degree of coherence along a surface normal to the propagation direction. For a radiation field in which phase is predictable across the entire lateral extent of vibrations, one often speaks of "full spatial coherence" or "defraction limited radiation."

**Coherence Length: destructive interference due to finite bandwidth**

- not dependent on cause of $\Delta \lambda$
- important in phase-sensitive experiments

Definition: $\ell_c = N\lambda_1 = (N - \frac{1}{2})\lambda_2$

$N = \frac{1}{2} \frac{\lambda^2}{\Delta \lambda}$

$\ell_c = N\lambda_1 \approx \frac{1}{2} \frac{\lambda^2}{\Delta \lambda}$
Partially Coherent Radiation Approaches
Uncertainty Principle Limits

\[ \Delta x \cdot \Delta p \geq \frac{1}{2} \hbar \]
\[ \Delta x \cdot \Delta k \geq \frac{1}{2} \]
\[ 2 \Delta x \cdot \Delta \theta \geq \frac{\lambda}{2\pi} \]

Spherical wavefronts occur in the limiting case
\[ d \cdot \theta = \frac{\lambda}{2\pi} \] (spatially coherent)

Propagation of a Spherical Gaussian Beam

(Following Siegman, Lasers, 1986)

\[ \frac{I}{I_0} = e^{-r^2/2r_0^2} \]

Where \( r(z) = r_0 \sqrt{1 + \left( \frac{\lambda z}{4\pi r_0^2} \right)^2} \)

In the far field, \( z >> \frac{4\pi r_0^2}{\lambda} \)
we define \( \theta \equiv \frac{r(z)}{z} = \frac{\lambda}{4\pi r_0} \)

If \( d \equiv 2r_0 \), then
\[ d \cdot \theta = \frac{\lambda}{2\pi} \] (Spatially coherent)
Fresnel Zone Plate Lens for Diffractive Focusing of X-Rays:

Applications of X-Ray Microscopy

Imaging Microscope

\[
\frac{1}{p} + \frac{1}{q} = \frac{1}{f}
\]

\[
M = \frac{q}{p}
\]

(Göttingen/BESSY)
Development of X-ray Microscopy as an Analytic Tool for the Study of Future Deep-submicron Devices and Circuits

Vladimirsky, Kern, Attwood, Meyer-Ilse (LBL, IBM, Göttingen, BESSY)

Pattern: two levels of an experimental 0.1 micron silicon MOSFET test circuit*

The Scanning X-ray Microscope*
Requires Spatially Coherent Radiation

- minimizes sample damage with inefficient lenses
- achieves high spatial resolution with spatially coherent radiation
- zone plate lens also requires temporal coherence

*Pioneered by J. Kirz & his colleagues
500 Å Fresnel zone plate
700 Å/300 Å bar/space ratio
1200 Å gold on 1000 Å silicon nitride
6000 Å thick apodized region
Outer diameter: 62 μm
1 mm focal length at 31 Å
First View of an Unaltered Subcellular Component
No fixation, no staining, no sectioning, aqueous environment

50 K cps

06 FEB 20 CNTM 10 PED MED2×2. INT5

λ = 32Å
Radiation dose ≥ 1 megarad

LBL, Stony Brook, NSLS, IBM, UCSF

Soft X-ray Microscopy of an Unaltered Zymogen Granule

First image

Second image

50 K cps

80 K cps

06 FEB 20 CNTM 10 PED MED2×2. INT5

λ = 32Å
Radiation dose ≥ 1 megarad per image

LBL, Stony Brook, NSLS, IBM, UCSF
Multiple View Imaging

Nano-CAT scan  Gabor holography  Off-axis holography

Encoding Complex Wavefronts - Holography

Incident wave (\(\lambda\))

Object and scattered wave

Mirror

Reference wave

"Film"

Interference pattern
The X-Ray Holography Experiment of Aoki et al. (1972)

Synchrotron x-rays, dispersed to
\[ \lambda = 60\,\text{Å} \]
\[ \Delta \lambda = 0.03\,\text{Å} \]

Object: three 3 μm slits, 9 μm apart

7 μm slit for spatial coherence
2.5 μm slit sets spatial resolution

Note: \( \ell_{\text{coh.}} \approx \frac{\lambda^2}{\Delta \lambda} \approx 12 \, \mu\text{m} \)

X-ray holographic microscopy at sub-1000 Å resolution

(Goal: towards 100 Å across, 1500 Å depth)

By: M.R. Howells & C. Jacobsen, Lawrence Berkeley Laboratory (CXRO); J. Kirz, State Univ. NY at Stony Brook; and K. McQuaid & S.S. Rothman, U.C. San Francisco

Hologram

Image

Enlarged image
(480 Å pixel size)

Electron micrograph of similar object

Source
- \( \lambda = 25 \) Å
- Undulator
- Brookhaven x-ray ring
- 79 minutes
- \( 8 \times 10^{11} \) photons

Monochromator

Pinhole

 Resist
- Copolymer
- IBM
- Gold shadowed
- TEM readout

Sample
- Zymogen granules
- Rat's pancreas

39
Evolution of Synchrotron Radiation

Today's Synchrotrons:
- Continuous electron trajectory
- "bending" radiation
- "X-ray light bulb"

Tomorrow's Synchrotrons:
- Many straight sections (periodic magnets)
- Tightly controlled electron beam
- "Undulator" and "wiggler" radiation
- "Laser-like"
- Tunable

Coherent X-Rays, Tuneable Across A Broad Spectral Region, are Generated.

Magnetic undulator (N periods)
- Flux concentrating steel (Fe) pole pieces

Relativistic electron beam, 
\[ E_e = \gamma m_0 c^2 \]

\[ \lambda_x = \frac{\lambda_u}{2\gamma^2} \]
\[ \theta \approx \frac{1}{\gamma \sqrt{N}} \]
In the past half decade or so there has been a technological revolution in our ability to generate, control, manipulate, focus, and detect x-rays. The emergence of x-ray lasers and synchrotron insertion devices has increased the brightness of laboratory x-ray sources eight to twelve orders of magnitude over what was available in the late 1960's. In addition, the past few years have been witness to significant advances in the development of normal incidence x-ray mirrors and beamsplitters, diffraction limited x-ray lenses, x-ray microscopy, x-ray holography, x-ray waveguides, and CCD x-ray detector arrays. Utilizing these new capabilities, workers in the field are taking the first steps toward the development of sophisticated soft x-ray optical systems, including soft x-ray interferometers, high intensity x-ray lasers and projection optics for x-ray lithography. Details of these developments are discussed in the references listed below. Highlights of the oral presentation are provided in the attached figures.

References:


2. N. M. Ceglio,"Revolution in X-ray Optics," LLNL report #UCRL-1010
Table 1:
A summary of soft x-ray lines for which amplified spontaneous emission (ASE) has been reported. All performance values are measured quantities. T.B.D. (to be determined) indicates that in these experiments output power was not explicitly measured.

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>Lasing medium</th>
<th>Measured gain</th>
<th>Gain length demonstrated</th>
<th>Output power demonstrated</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>279.3Å; 284.7Å</td>
<td>Ne-like Cu</td>
<td>1.7 cm(^{-1})</td>
<td>(\alpha L \approx 2.7)</td>
<td>T.B.D.*</td>
<td>[22]</td>
</tr>
<tr>
<td>232.2Å; 236.3Å</td>
<td>Ne-like Ge</td>
<td>4.1 cm(^{-1})</td>
<td>(\alpha L \approx 6.2)</td>
<td>T.B.D.*</td>
<td>[22]</td>
</tr>
<tr>
<td>206.3Å; 209.6Å</td>
<td>Ne-like Se</td>
<td>(4-6) cm(^{-1})</td>
<td>(\alpha L \approx 16)</td>
<td>(~ 10^6) watt</td>
<td>[3, 32]</td>
</tr>
<tr>
<td>166.5Å; 164.1Å</td>
<td>Ne-like Sr</td>
<td>4.0; 4.4 cm(^{-1})</td>
<td>(\alpha L \approx 8.1); 9.7</td>
<td>T.B.D.*</td>
<td>[18]</td>
</tr>
<tr>
<td>131Å; 132.7Å</td>
<td>Ne-like Mo</td>
<td>4 cm(^{-1})</td>
<td>(\alpha L \approx 7)</td>
<td>(10^3) watt</td>
<td>[19]</td>
</tr>
<tr>
<td>106.4Å</td>
<td>Ne-like Mo</td>
<td>2 cm(^{-1})</td>
<td>(\alpha L \approx 4)</td>
<td>(10^2) watt</td>
<td>[19]</td>
</tr>
<tr>
<td>182Å</td>
<td>H-like C</td>
<td>6.5 cm(^{-1})</td>
<td>(\alpha L \approx 6.5)</td>
<td>(10^5) watt</td>
<td>[4]</td>
</tr>
<tr>
<td>105.7Å</td>
<td>Li-like Al</td>
<td>1.0 cm(^{-1})</td>
<td>(\alpha L \approx 2.0)</td>
<td>T.B.D.*</td>
<td>[24]</td>
</tr>
<tr>
<td>81Å</td>
<td>H-like F</td>
<td>5.5 cm(^{-1})</td>
<td>(\alpha L \approx 2.8)</td>
<td>T.B.D.*</td>
<td>[23]</td>
</tr>
<tr>
<td>71Å</td>
<td>Ni-like Eu</td>
<td>1.1 cm(^{-1})</td>
<td>(\alpha L \approx 3.8)</td>
<td>(~ 10^2) watt</td>
<td>[14]</td>
</tr>
<tr>
<td>50.3Å</td>
<td>Ni-like Yb</td>
<td>1.2 cm(^{-1})</td>
<td>(\alpha L \approx 2.0)</td>
<td>(~ 10^2) watt</td>
<td>[14]</td>
</tr>
</tbody>
</table>

Future progress in x-ray optics: Predictions

Things to look for in the 1990's:

- X-ray lasers operating at \(\lambda < 44\)Å
- Efficient normal incidence x-ray mirrors and beamsplitters operating at \(\lambda < 44\)Å
- Many different XUV lasers operating at \(\lambda < 1000\)Å
- High intensity x-ray lasers operating at focussed intensity \(> 10^{12}\) watt / cm\(^2\)
- The 'new field' of nonlinear x-ray optics
- Demonstration of 'efficient', classical, soft x-ray waveguides, with \(t/\lambda \geq 10^3 \sim 10^5\)
- X-ray holography of live cells at \(\delta < 500\)Å
- X-ray microscopy of live cells at \(\delta \approx 100\)Å
Comparison of spectral brilliance values for a variety of x-ray sources ranging from electron impact and discharge sources (available in the 1960's) to synchrotron and laser produced plasma sources (available in the 1970's and 1980's) and ultimately soft x-ray laser sources (available in the mid-1980's). The reader should note that for x-ray laser and laser produced plasma sources the values quoted are peak values, whereas for the other sources average values are presented.
Transmission electron micrograph of Mo/Si multi-layer $d = 115\text{Å}$

$N = 25$

(a)

Mo/Si multilayer mirror data. (a) TEM micrograph of a mirror designed for x-ray laser cavity use at 206.3Å and 209.6Å. (b) Measured x-ray reflectivity versus wavelength for the mirror shown in (a). (c) Measured x-ray reflectivity of a mirror designed for x-ray laser cavity use at 131Å and 132.7Å.
Schematic illustration of multilayer beamsplitter behavior along with measured reflectivity and transmission data for a beamsplitter designed for use at 131Å.

Time resolved data for multipass amplification at 206.3Å in Neon-like Selenium (Experiment #17071705) are presented. Total cavity length was 8.5 cm; length of the gain medium was approximately 2 cm. The reduced amplification of the third and higher number passes was due to the short gain lifetime.
A possible Projection X-ray Lithography system utilizing a laser produced plasma as the x-ray source, a reflecting x-ray mask, and a series of normal incidence, multilayer coated spherical mirrors for the condenser and imaging optics.
DIRECT X-RAY MICROIMAGING

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X-ray microimaging may be classified into two basic approaches, direct and diffractive imaging. There are several contributions to this workshop concerning diffractive imaging, such as Gabor and Fourier transform holography, and soft X-ray diffraction. In this contribution we provide a brief review of the direct imaging methods, their relative merits, and speculate about future directions of the field.

Conceptually the simplest form of imaging is contact microscopy. In its earliest forms it goes back nearly to the discovery of X-rays. An X-ray sensitive sheet is placed next to the specimen, an exposure is made, the sheet is developed, and examined under a microscope. Traditionally the sheet is photographic film, and the microscope is optical. A considerable improvement in resolution was achieved with the replacement of film by X-ray resist, and the replacement of the optical by the electron microscope. This newer form of contact microscopy was developed largely between 1975 and 1980 at IBM by Feder, Spiller, and Sayre. More recently a careful analysis of the technique was performed by P.C. Cheng and D. Shinozaki.

Contact microscopy has the advantage that it does not place heavy demands on the X-ray source. It requires moderate collimation and wavelength selection, but has no real need for coherence. It is compatible with single flash exposures, and this form of imaging has been used at IBM, the Rutherford Lab, (Rosser et al.), at the Princeton X-ray laser, (DiCicco et al.) and elsewhere. A wider choice of wavelengths is available with synchrotron radiation sources, and longer duration contact exposures have been made at the Daresbury lab, (A. D. Stead et al.) at the Photon factory, (Shinozaki et al.), and at the NSLS, B. J. Panessa-Warren et al.). Laboratory sources have also been used.

A particularly interesting variant of the contact scheme has been under development by Polack et al. in France. As a detector they use a photoemissive surface. The electrons emitted are imaged using high resolution electron optics, so the image is made in real time.

The resolution depends on the detector. The best results have been obtained with PMMA, although some radiation induced roughening is observed at the highest resolution. Ultimately the effects of diffraction must be considered: The resolution will be limited to the square root of the product of the wavelength and the distance between the feature in the specimen, and the effective resist depth.

Schmahl's group in Gottingen has pioneered the use of Fresnel zone plates as magnifying elements in X-ray microscopy by direct magnification. The specimen is illuminated by monochromatic X-rays, and the magnified image is recorded on photographic film, or on a CCD detector. Up to now synchrotron radiation has been used for illumination (especially at BESSY), although the technique is compatible with flash exposure, given
the required monochromaticity. There is no need for spatial coherence.

The technique is fast and convenient. The resolution is determined by the zone plate. The Gottingen instrument was upgraded recently by an optical prefocusing system, that saves the specimen from being exposed to radiation before final positioning.

Some disadvantages of this microscope are the small field of view, the requirement that the specimen or wet cell be placed into a vacuum system, and that the specimen is exposed to extra radiation, due to the inefficiency of the post-specimen components. This extra dose is being reduced with the development of more efficient zone plates and detectors.

A new development, suggested by Schmahl and Rudolph, is a modification of the instrument to provide phase contrast. This extends the wavelength range of good contrast considerably. The phase contrast microscope does require, however, that the incident beam have at least moderate spatial coherence.

One can also use a zone plate or a mirror to focus the x-ray beam to form a microprobe. This is the basis of scanning microscopy, where the specimen is mechanically scanned across the microprobe. Such instruments are in operation or under development at Daresbury by Burge's group from King's College, at BESSY by the Gottingen group (B. Niemann et al.), and at the NSLS by the collaboration including the NSLS (H. Rarback), LBL (D. Attwood, E. Anderson, P. Batson), UCSF (S. Rothman, K. Goncz), IBM (D. Kern), and SUNY (H. Ade, C. Buckley, S. Hellman, J. Kirz, S. Lindaas, I. McNulty, M. Oversluizen). While these microscopes use zone plates and synchrotron radiation, Trail and Byer at Stanford are developing an instrument that uses a normal incidence mirror and a table-top laser/plasma source.

In the scanning microscope typically a proportional counter is used to detect the transmitted X-rays. The resolution is determined by the size of the microprobe, which in turn depends on the focusing element. For diffraction-limited performance the beam incident on the focusing element must be spatially coherent, and for zone plates it must also be monochromatic. The image is formed serially, a pixel at a time, therefore the method is not compatible with single flash imaging.

Scanning results naturally in a digital image that lends itself to quantitative interpretation in terms of specimen absorptivity maps. With images of the specimen at two or more wavelengths, this interpretation can be extended to elemental mapping. The specimen need not be placed in a vacuum chamber, and need not be exposed to excess radiation: X-rays that pass through the specimen fall directly onto an efficient detector.

Because of the need for coherent illumination, scanning microscopy benefits particularly from high brightness. Work at the NSLS makes use of the recently commissioned soft X-ray undulator, and Center for X-ray Optics / IBM zone plates with line widths as small as 40 nm. An interferometric prefocusing system has been implemented recently.
All these direct imaging methods have achieved a resolution of order 50 nm. All three have been used with wet biological specimens. What are the likely directions of future development? One can look forward to improved resolution. This requires better optical elements, and there are efforts at LBL's Center for X-ray Optics and elsewhere to this end. To exploit these, better microscopes will be needed, with improved reliability, user-friendliness, and flexibility in environmental control (temperature, humidity, etc.). With improved resolution come inevitably an increased radiation dose to the specimen. It will be important to study the effect of this dose, the temporal and spatial development of damage.

Additional information will become available with microscopes that use alternative signals. Photoelectron microscopes, now under development, will supply information on elemental and chemical state distribution at surfaces. High resolution fluorescence microscopes, using harder X-rays, will map the distribution of trace elements.

New sources have a profound effect on X-ray microscopy. Most imaging methods require coherent illumination, and the coherent power available is directly proportional to the brightness of the source. We can look forward to dramatic improvement with undulators planned for the Advanced Light Source, the Advanced Photon Source and PEP. The development of advanced X-ray lasers and FEL-s will open exciting new directions in flash microimaging.

Bibliography:

1/ X-Ray Microscopy II, edited by D. Sayre, M. Howells, J. Kirz, and H. Rarback (Springer, Berlin 1988) is the proceedings of the most recent international symposium on the subject.


Radiation Damage and its Influence on Source Requirements for High Resolution X-Ray Holography

Richard A. London

University of California Lawrence Livermore National Laboratory

Summary

This paper outlines the talk given at the conference on X-ray Microimaging for the Life Sciences held May 24-26, 1989 in Berkeley, California. Much of the material is described in greater detail in ref. 1.

Soft x-ray holography offers the possibility of obtaining high resolution, 3-D images of living cells and organelles therein. To achieve a specified resolution, a certain number of photons must be scattered by the smallest features of interest within the sample. This requires a certain irradiating fluence, the magnitude of which depends on the wavelength of the x rays and the scattering cross-sections of the features. Unfortunately, irradiation of the sample will be accompanied by the absorption of x rays. If the dose is large, the sample will be damaged, possibly compromising the quality of the image.

A theoretical study of the scattering and absorption of x rays during the creation of a hologram is described. Using a new prescription for scattering by condensed biological materials (e.g. protein and/or DNA) within the aqueous environment of a cell, we estimate the irradiating fluence required for a certain resolution and the associated sample dose. The relative merits of different x-ray wavelengths are discussed. A wavelength of about 44Å, just outside the "water window" (23.2 – 43.7Å), appears to be optimal in that the required fluence and dose are minimized, while reasonable x-ray penetrability is maintained. Estimates are given for the minimum source energy required and the maximum duration of an exposure to capture an image before blurring due heat induce motion. The use of colloidal gold tagging can enhance image contrast and reduce the required irradiating fluence and sample damage.

A multi-disciplinary group has been studying the feasibility of producing high resolution 3-D images of living cells using soft x-rays

- **Physics**: R. London, D. Matthews, M. Rosen, A. Szöke, and J. Trebes
- **Biology**: J. Gray, D. Peters, and D. Pinkel
- **Elec. Engineering**: J. Brase and T. Yorkey

Activities of holography study group (and talks at microimaging meeting)

- Define candidate biological objects (talk by Gray)
- study x-ray interactions, damage, and source requirements (this talk)
- design x-ray holography system
- develop simulation and reconstruction codes (talk by Brase)
- field analog holography experiments using visible light
- develop appropriate x-ray lasers (talk by Matthews)

The main part of this paper concerns x-ray interactions with the sample

- scattering is essential to make a hologram.
- absorption is detrimental, it leads to damage and limits sample thickness.
- previous work has been done by Solem et al\(^2\) and Howells and Jacobsen\(^3,4\).
we reconsider interaction properties of a wet biological sample and explore optimal x-ray wavelength considering three criteria:

1. minimize the required x-ray fluence
2. minimize the absorbed dose and subsequent damage
3. maximize the penetration length (i.e. sample thickness)

• we study limits on exposure time due to heat buildup, explosive motion, and natural motions of living samples.
• we discuss implications for x-ray source.

The following formulas are used to study the x-ray interactions

• define a resolution element as smallest resolvable feature
• assume a certain number of photons \( N_S \) must be scattered by each resolution element, as determined by signal/noise and detector efficiency
• required fluence: \( F = N_S \frac{h}{\sigma_S} \) [units: erg/cm\(^2\)], where \( \sigma_S \) = cross section of the resolution elements [units: cm\(^2\)]
• absorbed dose: \( D = F K_a \) [units: erg/g, Mrad = 10\(^6\) erg/g]
  where \( K_a \) = absorption opacity [units: cm\(^2\)/g]
• e-folding penetration depth: \( L = 1/(K_a \rho) \) [units: \( \mu m \)]

We assume that the smallest resolvable features can be approximated as spheres (Figure 1.)

We take the scattering cross section from the Rayleigh-Gans formula\(^5\)

\[
\sigma_S = \frac{\pi^2 d^4}{2 \lambda^2} |n_p - n_w|^2
\]

where \( n_p, w \) are the complex indices of refraction of protein and water.
• valid for small phase shifts (equivalent to the Born approx.)

let \( n = 1 - \delta - i\beta \) \( \Rightarrow \sigma_S \propto (\delta_p - \delta_w)^2 + (\beta_p - \beta_w)^2 \)
• optical constants (\( \delta \) and \( \beta \)) are calculated from atomic scattering factors given in Henke\(^6\) tables.
• both the real and imaginary parts of the index are important
• the difference between protein and water is important.
standard parameters for examples

- protein composition = H$_{49}$ C$_{33}$ N$_9$ O$_9$ S$_1$; density = 1.35 g / cm$^3$
- diameter of spherical resolution element: d = 300 Å.
- minimum number of scattered photons from each resolution element: $N_s = 10^3$.

We illustrate the wavelength dependence of the scattering cross section, the fluence and the dose in Figures 2, 3, and 4.

Figure 2. Scattering cross section for a 300 Å protein sphere in vacuum and in water.

Figure 3. Fluence for a 300 Å protein sphere in water to scatter 1000 photons. The 43.7 Å C K-edge is indicated.
We compare the estimated dose with several characteristic doses

\[
\begin{array}{ll}
\text{process} & \text{dose (erg / g)} \\
\text{kill a hardy bacterium} & 10^8 \\
\text{chemical damage} & 10^9 - 10^{11} (??) \\
\text{boil water (\(\Delta T = 100K\))} & 4 \times 10^9 \\
\text{x-ray holography at 44\(\AA\)} & 2 \times 10^{11} (\text{d/300\(\AA\)})^{-4}
\end{array}
\]

Results for choice of wavelength, and resulting fluence and dose

- we suggest an optimal wavelength \(\approx 44 \text{\(\AA\)}\) for protein in water to minimize fluence and dose.
- the advantage of doing holography in the water window (\(\lambda = 23.3 \text{ to } 43.7\text{\(\AA\)}\)) appears to be a fallacy.
- for resolution = 300\(\AA\) at \(\lambda = 44\text{\(\AA\)}\), we estimate:
  - fluence \(= 3 \times 10^7 \text{ erg / cm}^2\)
  - dose \(= 2 \times 10^{11} \text{ erg / g}\)
  - penetration depths \(= 2\mu\text{m (in both water and protein)}\)
- fluence and dose scale as \(d^{-4}\).
- fluence and dose are higher than Howells\(^3\) and Jacobsen\(^4\); main difference is that they assume an ellipsoidal resolution element rather than a spherical one.

Gold tagging to enhance contrast in x-ray imaging

- colloidal gold tagging has been developed for 15 years for electron microscopy.
- marking of specific sites can be accomplished using antibodies attached to gold particles 50 \(\text{\(\AA\)}\) to 1500\(\text{\(\AA\)}\) in diameter.
- for x-ray holography, the required fluence and dose are reduced by a factor of 60 using 300\(\text{\(\AA\)}\) gold tags (see Fig. 5 for fluence).
• the optimal wavelength is still > 43.7 Å, with a broader range acceptable range.

Figure 5. Fluence to scatter 1000 photons by 300 Å spheres of protein and gold

We now consider the limitations placed on the duration of the x-ray exposure by studying the scaling of several characteristic timescales

<table>
<thead>
<tr>
<th>process</th>
<th>natural timescale (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>biological dynamics</td>
<td>(10^-3 ?)</td>
</tr>
<tr>
<td>brownian motion</td>
<td>2x10^-3 d^2 R_b</td>
</tr>
<tr>
<td>Exposure duration</td>
<td></td>
</tr>
<tr>
<td>conductive cooling</td>
<td>6x10^-2 D R_c^2</td>
</tr>
<tr>
<td>hydro - expansion</td>
<td>3x10^-11 D^-1/2 d</td>
</tr>
</tbody>
</table>

The symbols in the preceding tables are defined as: d is resolution, scaled to 300Å; D is dose, scaled to 10^{11} erg / g; R_b is the radius of the object undergoing brownian motion, scaled to 1 μm; and R_c is the size of the cooled boundary of the sample where the heat is removed, scaled to 10 μm.

Several conclusions are drawn based on the dose - timescale considerations

• gold tagged samples may be imaged at low dose (~ 3x10^9) for which hydro motion is probably not an issue.
• natural samples require high doses (~ 2x10^{11} erg / g) for which there are two possible modes of imaging:
  long pulse (t > 10^-2 s), in which conduction keeps the sample cool.
  ultra short pulse (t < 3x10^-11 s), in which image is captured before hydrodynamic blurring compromises the resolution, as suggested by Solem^2.
• natural processes at resolution scale may be take place in ~10^-3 s (as indicated by brownian motion), requiring short exposures.
Conclusions

- A study of the scattering properties of biological materials in water implies that the water window (23.3Å - 43.7Å) may not be optimal for holography.
- \( \lambda = 44 \text{ Å} \) minimizes the fluence and dose for a protein sample in water, while maintaining reasonable penetrability, and is therefore suggested as optimal.
- x-ray lasers with 300 µJ of coherent energy in a 50 psec pulse should be sufficient to produce holograms with 300Å resolution of natural samples.
- with gold tagging, a factor of 60 in source energy can be saved. The dose is also reduced, and the short-pulse length requirements are alleviated.
- **By virtue of their short pulse (~50 ps) x-ray lasers can avoid the damage problem and image fast biological processes with high (~300Å) resolution**

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References


NEW BIOLOGICAL SCIENCE WITH X-RAY MICROIMAGING

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There are two central motivations for developing new scientific methods. One is, of course, to accomplish what established methods cannot, or can only accomplish in a limited or less satisfactory fashion. A second is for comparison: to test the complementarity of established methods. That is, are results obtained by one method congruent with those obtained by another independent means of measurement? In regard to microscopic imaging in biology, this means that we seek to ground our view of microscopic structure on more than a single methodological standard, with whatever particular uncertainties that standard presents. These are the motivations that underlie the current impetus for the development of x-ray microimaging methods.

Our knowledge of the internal structures of cells has been shaped in great part by 40 years of study applying and developing the methods of electron microscopy. This has led to the evolution of a model of the biological cell that contains defined structures with established details and known spatial relationships1. Belief in the fidelity of this model to the natural cell rests in great part on the understanding that the preparative procedures commonly used in electron microscopy, procedures that greatly modify the natural object, do not alter or distort intracellular structure as to form, location or high resolution detail. Even though the cell as seen in the electron microscope most certainly resembles the natural object, because the procedures commonly applied greatly modify it, questions of the faithfulness of the image often remain.

Nonetheless, in the absence of satisfactory comparative methods, models based on electron microscopic images have been the only available — indeed, the gold — standard for cell ultrastructure. X-ray microimaging represents one potentially powerful comparative method that will both permit us to look at the structures of the cell in a new light, x-radiation, and without the major alterations in the sample required by common electron microscopic practice2. Unlike electron microscopy, x-ray imaging
is suited for viewing a wide range of biological structures in their natural state; that is, in the absence of fossilizing preparative procedures or other alterations, save extraction from the tissue or cell of origin.

Even though the electron microscope is capable of a resolution of better than 1 nm, its overarching importance in biology has come not from this remarkable resolving power, but as a result of the technique establishing structural features within cells at more moderate resolution, ranging in scale from 10 to several thousand nanometers. This is because structures on this size scale form the major components of the cell. It has been this "mesoscopic" resolution that has been central to the description of cellular ultrastructure and that has allowed for relatively detailed images, not only of the contents of the biological cell, but of the interior of the great majority of its substructures, from the nucleus to the mitochondrion, as well as permitting resolution of the smallest subcellular structures, such as ribosomes and microtubules. It is just for structures on this scale of dimensions that x-ray imaging is most promising. Even if we were limited to current capabilities and expected short term improvements in resolution, ranging say between 10 and 50 nm, x-ray microscopy should provide an excellent comparative tool.

Developing a method to accomplish what other methods cannot, or can only accomplish in a limited or less satisfactory way, applies to x-ray imaging as well. There are three central features. The first we have already touched upon; the ability to examine samples at high resolution without preparation alteration - natural imaging, if you will. The second is in major part a result of the first: x-ray methods allow for dynamic imaging; i.e., following events in real time, at high resolution. Finally, x-ray microimaging allows for the quantitative determination of various elements of interest within the object, as well as obtaining chemical information (both spectroscopic and inferred from elemental data) at the spatial resolution of the microscopy.

Whatever uncertainty there may be concerning the fidelity of electron microscopic images, there is no uncertainty in regard to dynamic measurements. Electron microscopy does not allow them. Requirements for sample preparation, such as dehydration and sectioning, and the vacuum environment of the machine, make such measurements beyond the technique's capabilities. Although there have been some ingenious attempts to overcome this limitation, notably quick freezing of samples before preparing them for viewing, biologists have been hard at work seeking other means to follow processes on the sub-optical size scale in real time. Much effort has been applied to actually achieve visualization at
the resolution limit of visible light (confocal microscopy), as well as examining unresolved structures beyond it [fluorescent and video (contrast) enhanced microscopy]4.

X-ray imaging allows the environmental conditions that are prerequisite for useful dynamic imaging of biological material. The sample can be kept in an aqueous environment at body temperature, whole, and without the addition of foreign substances, such as heavy metals or dyes, to provide imaging contrast (imaging contrast is provided naturally based on the native chemical content of the object)5. Where proper resolution is not the criterion of merit, one should be able to examine features beyond the resolution limit of x-ray microimaging in much the same way as has been done with visible light microscopy, offering the possibility of gaining information as to location and relative motion of very small objects.

The ability to determine quantitatively the presence of various elements of importance in biological cells and subcellular structures and to obtain chemical information about various organic substances within them at the spatial resolution of the microscopy, is made possible by the tunable nature of synchrotron radiation. L and K absorption edges for numerous elements of importance in biology are in the soft x-ray domain normally used for x-ray microimaging. By crossing these edges and making subtraction images, or otherwise establishing a predominant absorptive element (such as carbon in the "water window"), contrast can be provided quantitatively in terms of the elemental content of a sample5,6. As a result, elemental and in some cases chemical maps of structure can be obtained. It should be possible, for example, to measure the concentration and distribution of some important ions within cells at the resolution of the microscopy, or to measure variations in protein content both within an organelle and from object to object5.

Of course, x-ray imaging has its limitations. Most notably, as with electron microscopy, it is a radiation intensive method. Although magnitudinally less radiation intensive than the electron microscope for features in the resolution range I have been discussing, when very high resolution is the goal, radiation exposure increases severely, whether x-rays or electrons form the probing beam7. The radiation sensitivity of enzymes may also limit the potential for following enzyme-dependent events over time in single objects, although little structural damage would be expected at the doses required for mesoscopic resolution. Even so, one should still be able to follow time-dependent events in different objects in the same field by sequential viewing. How severe and general the constraints of radiation dose will be, and whether means can be found to overcome or ameliorate
them, is an important and complex issue that needs study and experimental evaluation. Whatever these limitations may be, it is clear nonetheless that using x-rays to illuminate samples microscopically has much to offer the life sciences. It is a method whose substantial potential remains to be tapped.

Acknowledgements:

This work has been supported by the Director, Office of Energy Research, Offices of Health and Environmental Research and Basic Energy Sciences, of the Department of Energy under Contract No. DE-AC03-76SF00098; and the U.S. Department of Defense, Air Force Office of Scientific Research, under University Research Initiative Contract No. F49620-87-K-0001; and a program development grant from Lawrence Berkeley Laboratory.

References


Prototypical cell as observed applying electron microscope technique (Acinar cell; mammalian pancreas)

RER -- rough-surfaced endoplasmic reticulum
CV -- condensing vacuoles
Z -- zymogen granules
L -- lumen
G -- Golgi membranes
N -- nucleus
Golgi apparatus
Rough-surfaced endoplasmic reticulum (Note ribosomes attached to membranes.)
The Biologist's Dream

- High-resolution imaging
- Unaltered cells and subcellular structure
- Concentration of elements in microenvironments
- Dynamics of cell processes

Preparative procedures commonly used in electron microscopy may raise questions concerning the fidelity of the image that is produced.
High-Resolution Imaging of Biological Material in its Natural State

Soft X-Rays Offer Unique Opportunities

- Soft x rays penetrate many cells and most cellular substructure; therefore, sectioning can be avoided.

- The sample can be viewed while suspended in water at atmospheric pressure and body temperature.

- By using radiation in the "water window", contrast can be based on natural cellular content, without adding foreign substances.

---

**X-ray Absorption by Protein in the Water Window**

![Graph showing x-ray absorption by protein in the water window.](image)

**Table: X-ray Absorption by Generic Protein**

<table>
<thead>
<tr>
<th>Element</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>50.0%</td>
</tr>
<tr>
<td>Oxygen</td>
<td>23.0%</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>18.0%</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>7.0%</td>
</tr>
<tr>
<td>Sulfur</td>
<td>2.0%</td>
</tr>
</tbody>
</table>

---

---
First View of an Unaltered Subcellular Component
No fixation, no staining, no sectioning, aqueous environment

Please see page 35, where this illustration is reproduced in color (as the upper figure).

The Editors

IBM

X-ray Lens for High Resolution X-ray Microscopy
(Y. Vladimirsky, LBL and D. Kern, IBM)

Gold Fresnel zone plate ZP 70-62
f = 1.3 mm at \( \lambda = 31 \, \text{Å} \), F/23
156 working zones
900 Å/500 Å bar/space ratio
1300 Å thick zones, 5000 Å thick central region
1000 Å silicon nitride substrate

66
400 Å Zone Plate Lens
for High Resolution X-ray Microscopy

X-ray Holograms of Highest-ever Resolution
Lawrence Berkeley Laboratory
National Synchrotron Light Source
SUNY Stony Brook
UC San Francisco

Source
- \( \lambda = 25 \) Å
- Undulator
- Brookhaven x-ray ring
- 79 minutes
- \( 8 \times 10^{11} \) photons

Monochromator
Pinhole

Resist
- IBM
- Copolymer
- Gold shadowed
- TEM readout

Sample
- Zymogen granules
- Approx. 1 \( \mu \)m diameter
- Rat’s pancreas
Reconstruction of zymogen granule hologram (with phase retrieval).

Natural imaging—a walk through a biological forest...

A selection of additional intercellular structures suitable for high resolution imaging with x rays, as observed using electron microscopic techniques, was presented at the workshop (from D.W. Fawcett, The Cell, and other sources).
X-ray holography: 
early experience in microimaging

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Beginning with Baez's initial consideration of the technique in 1952, there is a long history of theoretical and experimental efforts in x-ray holographic microscopy. While the promise of the technique has long been recognized, it is only recently that progress in x-ray sources (undulators at electron storage rings) and detectors (high resolution photoresists for recording Gabor holograms) has led to demonstrations of submicrometer resolution imaging. Joyeux et al. have recorded holograms of silica skeletons of diatoms using 100 Å x-rays; they have demonstrated 0.5-0.6 μm resolution with a visible light reconstruction, and are implementing a UV reconstruction scheme which should allow sub-1000 Å resolution images to be obtained with rapid turnaround time. We have recorded holograms of rat pancreatic zymogen granules using 25 Å x-rays, and have obtained sub-1000 Å resolution images by numerically reconstructing holograms that have been enlarged using an electron microscope and subsequently digitized. Images of standard electron microscope grid bars have indicated that our current technique gives a system resolution of 600 Å or better (Figure 1).

We have recently recorded holograms using 19 Å x-rays from the X-1A undulator beamline at the National Synchrotron Light Source. Coherent illumination is obtained through the use of a monochromator and pinhole arrangement, and we can now record holograms with an exposure time of about three minutes. Figure 2 is an image of 1.09 μm polystyrene spheres obtained by numerically reconstructing an x-ray hologram. As can be seen, the spheres appear to be structureless objects, and the image is only slightly corrupted by the twin-image noise inherent in the Gabor holographic geometry. The lack of internal structure in these spheres has been confirmed by viewing them in a 1.5 MeV high voltage transmission electron microscope (HVEM), as can be seen in Figure 3. These images suggest that we can indeed obtain essentially artifact-free images of organic materials.
We have begun to use this technique to study zymogen granule structure. Figure 4 is a HVEM image of granules which have been placed in a dilute sucrose solution to trigger enzyme release, and then centrifuged and rinsed before being air-dried on a carbon film. Granules prepared in this way show internal structure that we believe is caused by partial release of digestive enzymes. Figure 5 is a reconstructed image obtained from an X-ray hologram of similarly prepared granules. The X-ray holographic image shows much the same type of granule structure as has been observed in the HVEM, although image interpretation is complicated by the presence of twin-image noise. In order to address this problem, we have recently begun work on developing iterative algorithms for the reduction of twin-image noise in numerically reconstructed holograms.

The attractions of holography as a soft X-ray imaging technique include the simplicity of the X-ray optics (only a monochromator plus pinhole is required), the ability to make use of single-shot X-ray sources (such as X-ray lasers) when they become available at the required wavelength and brightness, the natural way in which phase contrast can be used in holographic imaging, the fact that the focussing of the image is accomplished in the reconstruction stage (without additional exposure of the specimen to X-rays), and the possibility of extension to diffraction tomography for achieving high-resolution, three-dimensional images. These theoretical advantages have long been recognized, but our experimental experience has given us greater confidence in the possibility of realizing the potential of X-ray holography for microimaging.

**Acknowledgements**

We acknowledge valuable help and advice from P. Batson, J. Boland, M. Caldarolo, K. Conkling, C. Dittmore, T. ErmacK, R. Feder, J. Grendell, D. Joel, T. Kondakjian, K. McQuaid, H. Rarback, and D. Sayre, as well as the generous assistance of the staff at the National Synchrotron Light Source at Brookhaven National Laboratory and the National Center for Electron Microscopy at Lawrence Berkeley Laboratory. This work was supported in part by the National Science Foundation under grant BBS-8618066 (J.K.) and the Director, Office of Energy Research, Office of Basic Energy Sciences, Materials Sciences Division, of the U.S. Department of Energy under contract DE-AC03-76SF00098.

**References**


**Figure 1.** Line scans across an x-ray hologram of a transmission electron microscope grid bar (bottom line), the reconstructed image of the grid bar (middle line), and the estimated x-ray transmission profile through the grid bar (top line). Examination of the copper grid bar edge with an optical microscope indicated that the bar had an average wall slope of $45 \pm 4^\circ$, while the $1/e$ absorption length of 25 Å x-rays in copper is 0.17 μm. The reconstructed image pixel size is 280 Å, and the grid bar edge is sharp to two pixels or less in the reconstructed image; from this, we estimate that the system resolution is 600 Å or better.
Figure 2. Image of 1.09 μm polystyrene spheres obtained by numerically reconstructing a 19 Å x-ray hologram. This hologram was recorded in 3.5 minutes using the NSLS X-1A undulator beamline. (Scalebar= 1 μm).

Figure 3. 1.5 MeV transmission electron micrograph of 1.09 μm polystyrene spheres from the same lot as those shown in Figure 2. This image is very similar to the x-ray holographic image of the polystyrene spheres.
Figure 4. 1.5 MeV transmission electron micrograph of rat pancreatic zymogen granules that have been prepared in a way which triggers digestive enzyme release (see text), and then air-dried on a carbon film. These unfixed, unsectioned, and unstained granules appear as low contrast, non-uniform objects of 0.5–0.7 μm diameter.

Figure 5. Image of zymogen granules (prepared in a manner similar to those shown in Figure 4) obtained by numerically reconstructing an x-ray hologram. While the image is complicated by twin-image noise, the granules appear similar to those shown in Figure 4.
Computational and Visible Light Experiments for X-ray Laser Holography*

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Due to the fact that most biological objects of interest are embedded in a complex three-dimensional environment, it is difficult to estimate the usefulness of x-ray images of complex objects using usual measures of resolution such as Rayleigh criteria. We are approaching this problem for the design and characterization of x-ray holography systems by developing computational tools for the simulation of x-ray hologram formation and for their computational reconstruction. These efforts are closely coupled with a scaled visible light experimental program which validates both the simulations and the reconstruction techniques. The use of simulations allows us not only to establish resolution limits for complex objects but also to vary the parameter space in a controlled manner to optimize imaging system designs.

Hologram simulation

The diffraction based simulation treats the scattering volume as a sequence of layers in the propagation direction z. In each layer the index of refraction, n, is specified as a function of transverse coordinates x and y. We assume that n(x,y) is constant through the layer thickness Δz. A wave of arbitrary form is propagated from layer to layer. The attenuation and phase shift of the propagating wave due to the material in a single layer is

\[ t(x,y) = e^{-k\beta(x,y)\Delta z} e^{i\alpha(x,y)\Delta z}. \]  

where

\[ n(x,y) = 1 + \alpha(x,y) + i\beta(x,y) \]  

and k is the wavenumber of the illumination. The incident field is multiplied by t(x,y) and then propagated through distance Δz by convolution with a quadratic phase function [1].

\[ U_{n+1}(x,y) = U_n(x,y) t_n(x,y) \ast \ast Q(x,y) \]  

\( U_n \) is the field at the start of the nth layer, \( \ast \ast \) indicates two-dimensional convolution, and

\[ Q(x,y) = \exp(i\kappa\Delta z) \exp\left[ \frac{i\kappa}{2\Delta z} (x^2 + y^2) \right]. \]  

This process is repeated in turn for each layer of the scattering volume. Then a final propagation step calculates the field at the detector. For Gabor holograms the reference

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wave is part of this propagated field: for a Fourier Transform hologram a separate spherical wave (or quadratic in the paraxial approximation) is added. The detected hologram intensity is then calculated.

Two other resolution-limiting effects must be included in the simulation: finite detector resolution and limited incident beam intensity. We already have limited detector resolution by the discretization imposed by the convolution implementation using discrete Fourier transforms. To study detector resolution effects we initially calculate the hologram intensity at a higher resolution and then low-pass filter the resulting image. In this way we can easily evaluate the effects by varying the detector resolution with a single hologram.

The finite illumination intensity can reduce achievable resolution due to the resulting Poisson distributed quantum noise. To generate a quantum-limited hologram we first calculate the detector intensity as described above. These relative intensities are then scaled to mean photon numbers using

\[ m(x,y) = a N_{\text{inc}} I(x,y) \]  

where \( a \) is the detector element area, \( N_{\text{inc}} \) is the incident photon flux density (photons/cm\(^2\)), and \( I(x,y) \) is the simulated hologram intensity. The final quantum-limited hologram is then formed by using \( m(x,y) \) as the mean for realizing a Poisson distributed random variable for each detector position.

**Hologram reconstruction**

Reconstruction from a single hologram is done using diffraction techniques similar to those used for simulation. The hologram intensity is illuminated by a plane wave for the Gabor geometry or a spherical wave for the Fourier transform geometry. The resulting field is then propagated to a detector using equation (3). The detector distance can be incremented such that the entire scattering volume is reconstructed.

When reconstructing a single hologram in three dimensions the longitudinal resolution is typically much worse than the transverse resolution. The widths of the point spread function are \( \sim \lambda / \text{NA} \) and \( \sim \lambda / (\text{NA})^2 \) for the transverse and longitudinal directions respectively where NA is the numerical aperture of the imaging system. For a 3D object, however, the achievable resolution may be reduced due to the interference of scattering from adjacent layers of the object. A possible solution is to illuminate the object from many angles— to do x-ray holographic tomography.

For a weakly scattering object a single hologram contains information corresponding to a spherical surface in the Fourier transform of the object (the Ewald sphere) [2]. To reconstruct the object with good 3D resolution we must fill in the Fourier space out to the limiting spatial frequency defined by the illumination wavelength. The basic reconstruction procedure consists of propagating single holograms through the object volume and summing the resulting low resolution images [3,4]. Fundamental questions remain on the number of views required for given resolutions and the extent to which a priori knowledge can be used in the imaging process.
Visible light experiments

Our development of computational tools for simulating and reconstructing x-ray holograms is closely linked to an experimental program in visible light holography. The experiments are scaled from x-ray wavelengths of 5 nm to visible light wavelengths of 500 nm. The resolution goal scales from 30 nm to 3 μm. We have demonstrated good agreement between experiment and simulation and a capability to computationally reconstruct both Gabor and Fourier transform holograms made with visible light.

Simulations of contrast enhancement techniques

As an initial test of our simulation and reconstruction techniques we have simulated holograms of a 300Å protein fiber in water. The object and calculated hologram intensity (not photon limited) are shown in Fig's 1a and 1b. For this experiment the object is confined to a 300Å layer; it is essentially two dimensional. There is no resolution reduction due to inter-layer interference. The reconstructed image is shown in Fig. 1c. The distortion is due mainly to the superposed virtual image.

We next imposed a source intensity of $10^{15}$ photons/cm$^2$. The hologram reconstruction is shown in Fig. 2a. If the source intensity is reduced by a factor of 10 to $10^{14}$ photons/cm$^2$ the reconstruction quality deteriorates as shown in Fig. 2b.

The addition of contrast enhancing elements can make the lower source intensity image useful. Figures 3a, 3b, and 3c show the 300 Å protein fiber labelled with 500 Å and 100 Å gold spheres, the hologram generated at the low source intensity, and the corresponding image reconstruction. The gold tags can clearly be located and provide useful structural information.

Summary

We have developed and validated techniques for simulation and computational reconstruction of both Gabor and Fourier transform holograms. We are just beginning to apply these tools to a systematic study of the achievable resolution in x-ray imaging of complex three-dimensional objects.

References


Figure 1a  A simulation of a 30 nm diameter protein fiber in water. The fiber is confined to a plane transverse to the x-ray propagation direction.

1b The hologram generated by illuminating the protein fiber with 5.0 nm x-rays. The detector resolution is 100 nm and its size is 2.5 μm on a side. The detector distance from the object is 100 μm which gives a numerical aperture of 0.0125 (F40).

1c The reconstruction of the hologram in Figure 1b.
Figure 2a Reconstruction of the hologram of Figure 1b where the source intensity has been set to $10^{15}$ photons/cm$^2$. The image is degraded from the ideal reconstruction of Figure 1c but still useful.

2b Source intensity reduced to $10^{14}$ photons/cm$^2$. No trace of the fiber is detectable.
Figure 3a The 30 nm protein fiber with alternating 50 and 10 nm gold tags attached.

3b The hologram generated from the tagged protein fiber. The imaging system is identical to that for Figure 2b.

3c Reconstruction of the gold-tagged hologram at a source intensity of $10^{14}$ photons/cm². There is now clearly useful structural information.
GENOME SEQUENCING BY DIRECT IMAGING X-RAY COLOR HOLOGRAPHY
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I. INTRODUCTION

The human genome contains \(-10^5\) genes encoded by approximately 3 to 5% of the total \(3 \times 10^9\) base pairs of DNA. These genes reside in 24 chromosomes which are unique to each individual, except, of course, identical offspring. Currently available techniques for sequencing DNA are inadequate for oligonucleotides of length greater than 600 nucleotides and are most effective in the 300 nucleotide range. The two commonly used methods for DNA sequencing are the Maxam–Gilbert\(^1\) and Sanger\(^2\) methods. Automated sequencers are now available which use, basically, the Sanger dideoxy termination method\(^3\). The sequencing rates of these conventional methods are extremely low and involve very labor intensive procedures, requiring on the order of two man-years of effort for the determination of the sequence of a single 100 kilobase segment\(^4\). Since the full human genetic complement is of vast size, representing \(3 \times 10^8\) bases, a fast and accurate method for DNA sequencing is needed. Therefore, we set as a goal a sequencing rate in the range of \(10^2\) bp/s with an accuracy of \(1\) error per \(10^6\) bases, a value exceeding that set by the fidelity of current enzymatic processes. These values would permit the accurate determination of the sequence of the full human genome in one year. An approach involving rapid direct imaging of large segments of DNA is desired. A properly constructed x-ray Fourier-transform holographic microscope appears to combine these features. A basic x-ray holographic instrument, as described in Ref. (5) has been designed. This concept, with appropriate modifications, and the use of presently available x-ray sources, appears applicable to the task of genome sequencing with a resulting rate of sequence determination in the range of \(10^2\) bp/s.

II. OVERALL CONCEPT

The overall approach can be understood with reference to the discussion given in Ref. (5). The concept involves the use of Fourier–transform holography in which a small metallic scattering sphere, used to produce the reference wave, is located near the sample of interest. The interference pattern produced by the combination of the reference wave and the signal scattered from the sample generates the holographic record which, upon reconstruction, forms the spatial image of the sample.

A. Basic Geometry

The holographic information is recorded directly on an x-ray sensitive charge-coupled device (CCD) located at an appropriate distance from the sample. For genetic analysis, the sample becomes a DNA fragment with a length of \(100\) kb mounted in a plane (x-y) perpendicular to the direction of propagation (z) of the incident x-ray beam, and parallel to the CCD recording surface. With this arrangement, the two-dimensional holographic exposure relates to a two-dimensional object.

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confined to the transverse plane. Since high spatial resolution is obtained more easily in the transverse (x-y) dimensions than in the longitudinal (z) direction\textsuperscript{6}, the reconstruction of high resolution images is more readily achieved in this geometry.

B. Reference Scatterer

The wavelength region that pertains to the imaging of the genome is nominally in the region of 5 Å so that sufficient spatial resolution is available for distinguishing the individual bases. Although Ni (Z = 28) and Os (Z = 76) were found to be the best materials for the generation of the reference wave by the microspheres in the spectral zone defined by the water window, as discussed in Ref. (5), analysis for the shorter wavelength region involved in this case indicates that tungsten (W) (Z = 74) is the optimum substance. A suitable tungsten sphere would then be used to generate the required reference wave. It is of paramount importance that the reference spheres be smooth to minimize speckle. It is also desirable that they be round to minimize distortions in graphic reconstruction of the holograms. Fortunately, both the surface tension forces and the damping rates of oscillations for a freezing liquid drop are extremely high and more than sufficient to provide the requisite roundness and smoothness.

C. X-Ray Source

Two types of sources can be considered for the production of the holographic exposures. They are (1) x-ray lasers, the technology appropriate in the discussion in Ref. (5), and (2) synchrotrons. The effects of radiation damage on living hydrated\textsuperscript{7} samples determines a minimum peak brightness for the construction of high resolution images that can only be met by a laser system. In contrast, the samples of DNA that would be used for assays of the base pair sequence can be prepared to be much harder against radiation damage. In addition, full structural information on the duplex structure is not needed. Specimens can be fixed to have a very high tolerance to radiation damage\textsuperscript{8,9}, a fact which permits the exposures to be made at a rate consistent with existing synchrotron facilities. Studies\textsuperscript{10} indicate that the undulator at the PEP facility is capable of producing coherent power $P_Y$ in the range $5 \times 10^{-7} \leq P_Y \leq 5 \times 10^{-6}$ W at $\hbar \omega \sim 2$ keV.

D. Tagging Bases

Relatively simple chemical procedures can be used to provide specific and unique heavy atom labels for the four bases (A, C, G, T). Among the atomic markers under consideration are F, Si, S, Cl, Br, I, and Hg. Since these tagged bases can involve very small structural alterations in comparison to the configuration of the original unaltered bases, they can also serve as substrates of available DNA polymerases. Consequently, faithfully labeled DNA duplex strands can be produced from DNA segments cut from the human genome by available procedures using the current library of restriction enzymes. Such labeled strands, formed by DNA synthesis with the altered bases on natural templates derived from the human genome, would serve as the samples for the holographic exposure.

The labeling atoms can, on account of the atomic number dependence of the absorption edge structure\textsuperscript{11}, have considerably different x-ray scattering powers. By taking separate holographic exposures at each of the four different wavelengths corresponding to four characteristic edges of the tagging atoms, data are selectively acquired on the positions of the individual bases. These four resonantly produced
holograms can be individually stored and, upon reconstruction, will form a composite image revealing the base pair sequence of the DNA fragment. We have given the name of 'color holography' to this method.

E. Enzymatic Processes

DNA synthesis in vivo proceeds with an extremely high fidelity. However, since the labeled duplex strands prepared for x-ray holographic exposure will be produced by DNA synthesis in vitro with the labeled bases, the intrinsic error rate associated with the polymerase under these conditions is important. We note, however, that one can design systematic procedures for DNA synthesis which will serve to isolate and eliminate the errors arising from degraded enzymatic action on the modified bases. An example of such a procedure is outlined in Ref. (12).

F. DNA Sample Preparation and Mounting

The preparation and mounting of the samples for holographic exposure will resemble in many respects the procedures commonly used for the preparation of specimens for electron microscopy. Assuming suitable DNA fragments are made available with the use of known cloning procedures, these fragments would be sandwiched between two thin foil layers (~ 100 Å thick) made of carbon, beryllium or Formvar. A possible embodiment of the complete target system, including both the reference sphere and the labeled DNA fragment, is shown in Fig. (1). Fossilization of the DNA fragment is expected to produce a very high tolerance to x-ray exposure and minimize the effects of radiation damage, since very little momentum transfer is produced by x-rays of this energy.

Fig. (1): A possible embodiment of the target system involving a tungsten sphere and a labeled DNA fragment placed on a grid-supported carbon foil. After placement, the DNA fragment is coated with a thin carbon layer.
G. Computational System for Holographic Image Reconstruction

A calculational system is currently being developed designed specifically for the general reconstruction of images from x-ray holographic data. These developments are discussed in Ref. (7). The DNA samples under consideration have several salient features which differentiate them from the wider general class of unknown structures. (1) The use of resonant scattering from the tagging atoms confers a high contrast to the object. (2) Since the sample is confined to the transverse plane, only two dimensions are represented in the object space. (3) The specimen, on account of its thin thread-like structure, only actually occupies a small fraction of the potential object space, namely, ~ 1%. (4) The system has a known structure, and in particular, a known base pair separation. An optimum algorithm for rapid image reconstruction will take account of these considerations. Although a high spatial resolution on the order of ~ 5 Å is required to resolve the bases, the criterion of spatial resolution alone does not appear to be the leading parameter for the understanding of an optimal system. This arises since the actual resolution achievable depends, not only on the wavelength and scattering angle, but also on other experimental factors such as signal-to-noise. Also, the coherent process gives a square law scattering intensity dependence for the number of tags occurring within λ/2. The real outcome desired is a minimum error rate on the assignment of bases in a given sequence. Therefore, we have begun a program of studies of the dependence of the error rate on various experimental parameters.

H. Exposure Requirements

Recently published work on partially coherent radiation sources at x-ray wavelengths provides an excellent basis for estimating the exposure time required for DNA sequencing by the color-holographic method. We estimate PEP UND could produce about 5 x 10^-7 W of spatially coherent x-rays at a quantum energy of ~ 2 keV with a temporal coherence length of 1 micron. The temporal coherence required for a signal-to-noise (s/(s+n)) of 1/2 is about 1 micron. This is 1.5 x 10^9 photons per second at 6.07 Å. Since a 1.5-micron square contains ~ 2 x 10^6 transverse pixels, the rate is about 800 photons per pixel per second. A pixel containing a tagged nucleotide will scatter a photon approximately every 9 seconds. The Rose criterion would then require ~ 200 seconds for exposure. Finally, taking into account both the fact that PEP can operate at the ESRF brightness, an increase of roughly one hundred-fold, and the decrease due to losses in the monochromator and focusing system, for which we assume a one to ten percent range for transmission, a final exposure time in the region of ~ 20 – 200 s results. If this is accomplished at four colors, the total exposure requirement is ~ 80 – 800 s. This gives ~ 125 – 1250 bp/s for this step in the sequencing process. At this rate, a complete exposure of the entire human genome could be recorded in one year. In order to account for uncertainties in these estimates, we will accept a rate of ~ 100 bp/s as a reasonable figure characterizing this approach. If the chemistry and the computational reconstruction can be handled in a sufficiently parallel fashion, so that the exposure rate is the limiting process, a dramatic advance in the speed of genome sequencing would be made.

III. ACKNOWLEDGEMENTS

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IV. REFERENCES


17. This range is based on the use of a bent crystal focusing monochromator system. A $\Delta \lambda / \lambda \sim 10^{-4}$, which is in the range of our needs for the exposures envisaged, can be achieved in this manner.
Possible Involvement of Radiation Damage in Soft X-ray Microscopy of Mammalian Cells

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When specimens are observed by soft x-ray microscopy, they always absorb many photons leading to the radiation damage of them. Table 1 shows the summary of reported results for estimated absorbed dose. In addition, Dr. London reported another estimated values at this Workshop. In most cases, estimated absorbed doses are not smaller than 10^4 Gy (1 Gy = 100 rad). It is, therefore, of interest to study the probable effects of radiation on mammalian cells when they absorbed 10^4 Gy.

The relationship between the radiation effect and the contrast mechanism is summarized in table 2. When the image is produced by absorption contrast, photons are always absorbed at the imaged site and hence the radiation damage (called "initial damage") should always be produced at the site where you observed. On the other hand, in the case of scattering or phase contrast, there should be no energy absorption from imaging photons at the imaged site but initial damage may be produced by the absorption of other photons in the related area. This initial damage may secondarily induce the structural change of the site where you observed. In any case, much initial damage should be produced. The possible phenomena following to the initial damage are summarized in table 3.

In the next step, the frequencies of radiation effects were reviewed and then estimated for the cells irradiated with 10^4 Gy of 1.5 keV x-rays (Al-K x-rays) in table 4. The energy of 1.5 keV was chosen simply because the experimental results were obtained for cell survival, chromosome aberrations and DNA strand breaks and also it is closer condition to x-ray microscopy than the energy of x-rays widely used for radiation biology. The estimated frequencies at 10^4 Gy were obtained by the linear extrapolation of those radiation effects except for cell survival which was estimated by using the equation shown in the legend for table 4. The results show that non-repairable DNA double strand breaks will be produced at the frequency of 1.18 x 10^3 per cell when the cells were irradiated at 10^4 Gy. Since the total DNA per cell is 6 x 10^9 base pairs, the results indicated that roughly one double strand break per every 3 x 10^6 base pairs should be produced in average. This means that there will be only 100-200 nucleosomes between the breaks. If the radiation dose is 10^7 Gy, the breaks will be in every nucleosome.

As shown above, the radiation damage is not preventable and is not small enough to be ignored. The time scale may be the only possible factor for finding way to prevent the problem of radiation damage. Unfortunately, there are not enough data for the time scale of radiation effect on structural changes. Table 5 shows the sequential process of radiation effects leading to the structural changes in macromolecules and the time scale obtained for the degradation of single-stranded DNA. The time scale for the structural change of DNA (Mw = 2.8 x 10^6) was an order of 10^3 sec. Another experimental data were reported for the interphase death of
mammalian cells irradiated with x-rays (Goldstein and Okada, Radiat. Res., 39, 361-373, 1969). When the cells were irradiated with 1.8 kGy of x-rays, most of the cells were stained with eosin Y indicating the changes in cellular membrane within one hour. The rate of the staining was dose dependent up to 1.8 kGy, hence higher dose may result in faster change in cell structure.

In conclusion, (1) radiation effects on the cells irradiated with $10^4$ Gy of 1.5 keV x-rays were estimated for the induction of structural changes in relation to the chromosome structure in mammalian cells; (2) time scale for the structural change may be slower than the achievable time for x-ray microscopy; (3) short pulse (less than msec) exposure is recommended for the observation of chromosome fiber in hydrated mammalian cells by x-ray microscopy (the time may depend on the size of the molecule to be observed); (4) x-ray microscopy will be the most accessible instrument to reveal the time sequence of the radiation effect on the structural change.

(Table 1)

"Estimated absorbed dose"

<table>
<thead>
<tr>
<th>Specimens (thickness, 2-10 μm)</th>
<th>absorbed dose (Gy)</th>
<th>Ref.</th>
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<td>Absorption</td>
<td></td>
<td></td>
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<tr>
<td>Sayre et al.</td>
<td>$&gt;10^7$</td>
<td>$&gt;10^4$</td>
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<tr>
<td>Solem</td>
<td>$4\times10^7$</td>
<td></td>
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<tr>
<td>Rudolph and Schmahl</td>
<td>$&gt;5\times10^4$</td>
<td></td>
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<tr>
<td>Morrison</td>
<td>$&gt;10^6$</td>
<td>$440-3500$</td>
</tr>
<tr>
<td>Phase contrast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rudolph and Schmahl</td>
<td>$&gt;5\times10^4$</td>
<td></td>
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<tr>
<td>Scattering</td>
<td></td>
<td></td>
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<tr>
<td>Solem</td>
<td>$5\times10^7$</td>
<td>$9\times10^5$</td>
</tr>
<tr>
<td>Howells</td>
<td>$5.8\times10^4$</td>
<td></td>
</tr>
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</table>

Specimens (thickness, 2-10 μm) were exposed to x-rays at the wavelength of 3 nm.; Expected signal-to-noise ratio (S/N) is 5 except **; *, expected resolution; **, S/N=3, resolution =50 nm; ***, resolution=20 nm.
(Table 2)

"Contrast mechanism and radiation effect"

<table>
<thead>
<tr>
<th>interaction</th>
<th>absorption</th>
<th>scattering or phase shift</th>
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<tbody>
<tr>
<td>energy transfer</td>
<td>photoelectric</td>
<td>elastic</td>
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<tr>
<td>effect</td>
<td>ionization of the element followed by Auger effect</td>
<td>little</td>
</tr>
<tr>
<td>effect</td>
<td>production of polycationic element and emittance of Auger electrons</td>
<td>none</td>
</tr>
<tr>
<td>probable consequence</td>
<td>breakdown of chemical bond (&quot;initial damage&quot;)</td>
<td>no change</td>
</tr>
</tbody>
</table>

(Returned to the original (intact) state)

The initial damage is repaired.

Changes in chemical structure

The change may/may not be bigger than the expected resolution.

Biological modifications of damage

The size of the initial damage is amplified biologically to the size enough to be observable.

[e.g., chromosome aberration]
"Summary of radiation effect"

<table>
<thead>
<tr>
<th>frequency/cell</th>
<th>observed/Gy</th>
<th>estimated/10^4 Gy</th>
</tr>
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<tbody>
<tr>
<td>cell survival*</td>
<td>D_{10} = 3 Gy</td>
<td>10^{-27}00000</td>
</tr>
<tr>
<td>(Chinese hamster V79)</td>
<td>0.9</td>
<td>0.9 x 10^4</td>
</tr>
<tr>
<td>chromosome aberrations. (human lymphocytes)</td>
<td>118</td>
<td>1.18 x 10^5</td>
</tr>
<tr>
<td>DNA single strand breaks</td>
<td>0.9</td>
<td>3 x 10^6</td>
</tr>
<tr>
<td>double strand breaks</td>
<td>118</td>
<td>1.18 x 10^5</td>
</tr>
<tr>
<td>(non-repairable)**</td>
<td>11.8</td>
<td>1.18 x 10^5</td>
</tr>
<tr>
<td>(Chinese hamster V79)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cells were irradiated with Al-K X-rays (1.5 keV).

*Estimated survival is calculated by the equation,

\[ SF = \exp(-0.57D-0.062D^2). \]

**Non-repairable fraction of DNA double strand breaks was estimated to be 10% of the initial breaks from Koval and Kazmar, 1988.

(Table 5)

Sequential process of radiation effect leading to the structural changes in macromolecules

```
\begin{itemize}
  \item Irradiation \rightarrow \text{production of free radicals such as } \cdot OH
  \item \text{damage in molecules}
  \item \text{changes in structure}
\end{itemize}
```

<table>
<thead>
<tr>
<th>process</th>
<th>time scale (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>physical (1)</td>
<td>10^{-18} - 10^{-12} *</td>
</tr>
<tr>
<td>chemical reaction (2)</td>
<td>10^{-12} - 10^{-7} *</td>
</tr>
<tr>
<td>structural change (3)</td>
<td>10^{-3} **</td>
</tr>
</tbody>
</table>


**for the degradation of single-stranded DNA (Mw=2.8x10^6) irradiated with electrons (98 Gy) from Washino and Schnabel, 1982.
LIMITATIONS TO SIGNIFICANT INFORMATION IN MICROSCOPY OF BIOLOGICAL MACROMOLECULES AS A CONSEQUENCE OF RADIATION DAMAGE.

Robert M. Glaeser
Cell and Molecular Biology Division, Lawrence Berkeley Laboratory, and Biophysics Department, University of California, Berkeley.

Physical Background

Should there be limitations as a consequence of radiation damage, or is there no physical limit to what can be done? All types of currently useful short-wavelength radiation (Breedlove and Trammel, 1970) represent a flux of ionizing radiation as well as a wave-field that is suitable for diffraction and/or imaging. Obviously, then, there must ultimately be some physical limitation associated with their use for microscopy. The question is to define what the limitations should be.

A self evident limitation will occur if the exposure which one uses to record an image is above a certain critical value ($N_{cr}$ quanta per unit area) at which the biochemical function or even the detailed structure of the macromolecule has been destroyed. Under these conditions one might still produce some kind if an image, but the information contained in the image will no longer be significant. The very thing that one had hoped to learn from the measurement would itself have been destroyed by the measurement.

A second limitation enters into the story if the exposure is kept below this critical exposure. For any finite exposure there will be a precisely determined amount of statistical variation in the number of quanta per picture element (shot noise, or "counting error"). This shot noise becomes more and more severe as the pixel size decreases, i.e. as the resolution increases. Thus, very small features and/or very low contrast features can no longer be reliably detected, if images are taken with exposures less than the "critical dose", and if that critical dose is too low.
Psychophysical experiments which were performed by Rose (1948) have established that there is a kind of "uncertainty principle" which says that one can detect arbitrarily small features (at high enough contrast) or one can detect features with arbitrarily weak contrast (at low enough resolution), for any value of the critical dose, $N_{cr}$. However, one cannot simultaneously have arbitrarily high resolution and arbitrarily low contrast. This "uncertainty principle" relationship, given in the panel below:

**ROSE'S "UNCERTAINTY PRINCIPLE" BETWEEN CONTRAST, RESOLUTION, AND CRITICAL DOSE**

\[ \frac{d \cdot C}{\sqrt{N_{cr}}} \geq \frac{K}{1} \]

- $K \approx 1$ for parallel lines.
- $K \approx 5$ for isolated disks.

$N_{cr}$ depends upon the specific specimen material, the biological function being studied, and the resolution (ultrastructure is more sensitive than gross morphology.)

can be used to estimate whether a particular type of high resolution imaging is in principle possible, or whether there is a fundamental physical limitation that stands in the way.
Imaging Biochemical or Cellular Processes.

The "Rose equation" has been used to evaluate the potential of high voltage electron microscopy for the study of living cells (Glaeser, 1975). The results are shown in Figure 1 as a comparison of the electron exposure (dose) that is needed to image substructures of a given size, on the one hand, and the biological effects, on the other hand, that are known to occur at such exposures. Although only a crude and overly optimistic estimate was made, assuming that all image features would have 10 per cent contrast (regardless of their size or composition), one could already conclude that high voltage EM is not able to produce images at a resolution better than can be achieved with the light microscope, without using exposures that are so great that they would destroy any relevant biochemical or dynamic function of the cell.

A similar evaluation can be made of the limitations that apply to soft X-ray imaging. Sayre et al. have published detailed calculations of the rad dose that must occur in obtaining statistically defined images, as a function of feature size and specimen thickness (Sayre et al., 1977). The model used for the calculation and the results are shown in Figure 2. Using these calculations one can discuss whether a particular type of soft X-ray imaging task is feasible or not. Once again, as for high voltage electron microscopy, the conclusion is that there are few, if any, questions about biochemical or cellular functions which can be answered by soft X-ray imaging at a resolution that is better than that of the light microscope. Some examples of important biochemical or cellular processes that one would like to "image", and the reasons why soft X-ray imaging of these processes is limited by radiation damage, are presented in the Table below. This Table does not rule out the possibility, however, that soft X-ray imaging might in some cases be superior to light microscopy, at the same resolution, because of new contrast mechanisms which would be unique to "tunable" X-ray imaging.
REPRESENTATIVE EXAMPLES OF INTERESTING STRUCTURAL AND FUNCTIONAL OBJECTIVES IN CELL AND MOLECULAR BIOLOGY

I. Sub-nanometer. Molecular substructure.
   A. Tertiary structure of proteins, i.e. size, position, orientation of helices and β-sheet domains. Experimental studies of protein folding.
   B. Linear sequence of extended-chain polymers
      (~ 3.5 Å resolution for polypeptides
      ~ 8 Å resolution for nucleic acids.)

   Required dose exceeds $10^{12}$ rad.
   $10^{12}$ rad ~ 60 eV / Å^3.
   Every chemical bond is broken more than ten times over.

   Desired objectives are impossible.

II. Below 10 nm. Supramolecular structure.
   A. Assembly and disassembly of microtubules or actin filaments (treadmilling).
   B. Hormone-induced binding of G-protein to trans-membrane receptor.
   C. On-rates and off-rates of promoters, repressors, and polymerases.
   D. Rotational step-size in the bacterial flagellar motor.

   Required dose exceeds $10^9$ rad.
   ATPase/GTPase etc. activities are inactivated well below $10^6$ rad.
   Polymer structures will be extensively nicked and/or crosslinked, precluding functional assembly reactions.

   Desired objectives are impossible.
III. Below 100 nm. Functional operation of complex assemblies.

A. Precise measurement of bending (i.e. local curvatures) of cilia and eukaryotic flagella.

B. Measurement of A-band and I-band length and width during contraction of skeletal muscle.

C. Chromosome-to-pole movement in isolated mitotic apparatus (a fishing expedition).

| Required dose exceeds 10^6 rad. ATPase activity and cytoplasmic motility are stopped during the first frame. |

Desired objectives are impossible.

IV. Below 1μm.

A. Requires justification that contrast mechanism is superior to any available mode in the light microscope.

B. Required dose down to 10^3 rad, so that tens or hundreds of successive frames are possible, depending upon which enzymes are needed for the observed cell-function.

V. Any imaging objective.

A. Calculate (or look up) the dose required to achieve that imaging task.

B. Ask whether the specimen remains intact and/or functional for that rad-dose.
Imaging Subcellular and Molecular Structure

The static structure of macromolecules and macromolecular assemblies is normally well preserved at much higher doses of ionizing radiation than those which destroy enzymatic or biochemical function. Thus, the critical dose for fading of the electron diffraction pattern of protein crystals at high resolution is well above $10^9$ rad at low temperature. (See for example the data of Glaeser and Taylor (1978) for catalase or the data of Hayward and Glaeser (1979) for bacteriorhodopsin). Relatively low resolution images, showing individual protein molecules, can even be obtained with doses up to $10^{10}$ rad (Figure 3 and Figure 4), but severe "bubbling" and morphological destruction occurs, on the size-scale of 1000 Å and larger, at doses of $3 \times 10^{10}$ rad (Glaeser and Taylor, 1978, Figure 5). It is not unreasonable to suppose that comparable images could be obtained with soft x-rays, at the same doses.

How to beat the 'Uncertainty Principle'

The limitations discussed above would obviously be pushed back if one could find some way to increase the critical dose, $N_{cr}$. At "very low" doses, below $10^6$ rad, some progress might be made with radioprotectant chemicals. These are quite useless at high doses, however, such as $10^9$ rad, because the radioprotectant itself is just as easily destroyed as is the material that one wishes to image. The only known mechanism to increase $N_{cr}$ beyond $10^{10}$ rad is to somehow "fossilize" the specimen. Standard electron microscopy achieves this by a variety of techniques, including negative staining, staining thin sections of plastic-embedded materials, or creating metal-shadowed replicas. Negative staining can actually preserve the initial structure at better than 10 Å resolution (Glaeser, 1971). Unfortunately the high resolution features of such fossilized specimens are still as sensitive
to radiation damage as they are in unstained specimens. In the range of 25 Å resolution, however, fossilization works very well. Experiments with soft x-ray imaging could therefore seek to extend what can already be done in electron microscopy by using "stains", "negative stains", or shadowing materials which once again exploit the tunability of synchrotron radiation as the basis for image contrast.

An alternative approach is to artificially increase $N_{cr}$ by summing the images of "n" identical objects, so that the statistical definition of the final image is determined by the value $nN_{cr}$, rather than $N_{cr}$. This approach has been the basis for the development of a whole new branch of crystallography, called "electron crystallography" (Glaeser, 1985). Recent progress in this field is exemplified by the images of bacteriorhodopsin at 2.8 Å (Baldwin et al., 1988) of OmpF porin at 3.5 Å (Zemlin et al, In Press) and of Light Harvesting Complex at 3.7 Å (Kühlbrandt and Downing, 1989). While it is certainly too early to look to x-ray imaging for such high resolution, it is not out of the question to think of important problems in cell and molecular biology where the same principle might be used at lower resolution to overcome a limitation of resolution/contrast that might otherwise stand in the way of getting an answer.
References


Zemlin et al., J. Mol. Biol., In Press
**IMAGING REQUIREMENTS AND RADIATION DAMAGE AT 1 MeV**

<table>
<thead>
<tr>
<th>Resolvable structures</th>
<th>Minimal electron exposure required for detectability (coulomb/cm²)</th>
<th>Rad dose/picture corresponding to electron exposure on the left</th>
<th>Biological effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells (10μ)</td>
<td>$10^{-10}$</td>
<td>100</td>
<td>Reproductive cell death (animal cells)</td>
</tr>
<tr>
<td>Cell nucleus (2μ)</td>
<td>$10^{-9}$</td>
<td>$10^{-3}$</td>
<td>Inactivation of T1 bacteriophage</td>
</tr>
<tr>
<td>Mitochondrion (5000 Å)</td>
<td>$10^{-7}$</td>
<td>$10^{-5}$</td>
<td>Reproductive cell death (E. coli)</td>
</tr>
<tr>
<td>Tumor virus (1000 Å)</td>
<td>$10^{-6}$</td>
<td>$10^{-1}$</td>
<td>Enzyme inactivation</td>
</tr>
<tr>
<td>Ribosomes, chromatin fibers (200 Å)</td>
<td>$10^{-4}$</td>
<td>$10^{-7}$</td>
<td>Stoppage of cell motility (protozoa)</td>
</tr>
<tr>
<td>Cell membranes (100 Å)</td>
<td>$10^{-3}$</td>
<td>$10^{8}$</td>
<td>Reproductive death (Micrococcus radiodurans)</td>
</tr>
<tr>
<td>Enzymes (50 Å)</td>
<td>$10^{-2}$</td>
<td>$10^{9}$</td>
<td>Disordering of crystalline valine</td>
</tr>
<tr>
<td>Nucleic acids (25 Å)</td>
<td>$10^{-1}$</td>
<td>$10^{11}$</td>
<td>Disordering of crystalline adenosine</td>
</tr>
<tr>
<td>10 Å resolution</td>
<td>$10^{-12}$</td>
<td>$10^{13}$</td>
<td>Change in characteristic UV spectrum of nucleotide bases</td>
</tr>
<tr>
<td>5 Å resolution</td>
<td>$10^{-13}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** A rough estimation of the electron dose that is required to image subcellular features of various sizes, and the corresponding (a) rad dose, and (b) biological and biochemical consequences of such rad dose exposures. (From Glaeser, 1975).
Overall view of the specimen model, showing division of the specimen into regions of types 1 and 2. (b) Close-up of regions, showing background material and features. As suggested in (b), the manner in which the feature material is distributed in the vertical dimension is immaterial in the theory given here.

Minimum radiation dose for photon microscopy in mode X1, specimen B = F1 = water, F2 = protein, \( t_F = d \). Photon wavelength chosen at each point in plane for lowest dosage to specimen.

Figure 2. The specimen model used by Sayre et al. (1977) and the estimated rad dose for soft x-ray imaging as a function of feature size \( t_F \) and total specimen thickness \( t \).
Figure 3. High resolution image of frozen hydrated catalase taken with nearly the maximum permissible electron exposure. (From the Ph.D. thesis of K. Taylor, UC Berkeley, 1975). There is an impression that individual unit cells are barely discernable, without using spatial averaging.
Figure 4. High resolution electron micrograph of a frozen hydrated specimen of bacterial outer membrane material, including a two-dimensional crystalline layer of "surface protein", which has a clearly visible lattice-constant of 145 Å. (From Taylor, 1977). The bilayer structure (profile) of the outer membrane can be seen clearly in small vesicles.
Figure 5. An example of the bubbling and gross morphological distortions of structure that happen in frozen hydrated catalase crystals at electron doses that correspond to approximately $3 \times 10^{10}$ rad or higher. (From the Ph.D. thesis of K. Taylor, UC Berkeley, 1975). The catalase crystals are surrounded by a thin "puddle" of frozen water, which exhibits crystalline "bend contours" due to strong electron diffraction.
Time-Resolved Macromolecular Crystallography
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Cornell University, Ithaca, New York 14853-2703

Much of our knowledge of the fundamentals of molecular biology comes from
analysis of the three-dimensional structure of macromolecules, obtained largely by x-ray
crystallographic investigations and more recently, also by two-dimensional nuclear
magnetic resonance techniques. Although this approach is achieving notable successes, it
has a major limitation. Conventional crystallographic techniques can probe only static
structures, and yield a space average over all molecules in the crystal, typically $10^{13}$ in
number, and a time average over the duration of the x-ray exposure. However, the heart of
biological processes such as catalysis, during binding, viral uncoating, antigen-antibody
recognition, and muscle contraction lies in dynamic changes in structure that extend over a
very wide time range (Fig. 1), rather than in static structure. How are these dynamic
changes in structure to be observed? Techniques are needed that can probe atomic
structures in real time, as the biological process is unfolding: time-resolved
crystallography.

A time-resolved crystallographic experiment (reviewed recently by Moffat (1989))
has three main components: reaction initiation, reaction monitoring and time-resolved x-ray
data acquisition, and data analysis.

Reactions Initiation

In reaction initiation, a structural reaction must be initiated rapidly, uniformly and in
a manner that does not damage the crystal, which must be of a size and quality suitable for
high resolution x-ray analysis. Initiation may involve change in a chemical parameter such
as reactant concentration, achieved via diffusion (which is unfortunately very slow and
hence not generally suitable), photoactivation of a stable, biochemically inert precursor into
an authentic reactant (for example, the conversion of caged ATP to ATP; reviewed by
McCray and Trentham, 1989), photoactivation of a naturally light-sensitive system such as
carboxymyoglobin (Parkhurst and Gibson, 1967) or photoactive yellow protein (Meyer,
1985; Meyer et al., 1987; McRee et al., 1989), or light-induced pH jump (McCray and
Trentham, 1989). Alternatively, initiation may involve change in a physical parameter such
as temperature or pressure.

If the initiation reaction or any of the subsequent structural reactions are
irreversible, then only a single-shot experiment is possible; but if all reactions are fully
 reversible, then multiple repetitions are possible to enhance the signal-to-noise or the time
resolution, via signal averaging or even stroboscopic experiments in cases where a
repetitive, pulsed x-ray source such as a storage ring is used.

Light activation is an attractive technique, since pulsed lasers of sufficient power,
tunability, repetition rate, and with suitably short pulses are readily available, and the
dimensions of the beam are well-matched to those of crystals, a few hundred $\mu$m.
However, temperature jumps and temperature gradients arising from laser-induced heating
must be minimized, through careful selection of the photoactivating wavelength and use of
reactions of high quantum yield for photoactivation. Artefacts may also arise if
photochemical intermediates or side products are chemically reactive, as is often the case
(McCray and Trentham, 1989).
Reaction Monitoring

A structural change within the crystal produces a simultaneous change in the x-ray diffraction intensities, and it is the variation in these intensities with time that constitutes the raw data of a time-resolved crystallographic experiment. The variation may arise from a change in the continuous molecular transform (the desired result), from changes in the sampling of that transform by change in the reciprocal cell, and by artefacts such as change in source intensity, crystal orientation, or radiation damage. For a variety of reasons, the polychromatic Laue x-ray diffraction technique is well-suited to time-resolved experiments. The time resolution that can be achieved is proportional to the inverse of the intensity per unit wavelength falling on the crystal (see equation 10 of Moffat, 1989). Present values for the time resolution at a relatively low brightness, dipole source such as CHESS lie in the 10 to 2200 ms range for unfocussed x-rays, depending on the crystal and the desired precision of measurement. These values will drop by a factor of 10 to perhaps 1000 at high brightness, dedicated, high current sources such as the ALS, APS and ESRF, particularly when they employ focussing Laue optics and an undulator source.

A time resolution of a few μs approaches the circulation time of a single bunch of electrons or positrons in a storage ring such as CESR (2.56 μs) and hence the true exposure time becomes the x-ray pulse length, namely 120 ps in CESR. This suggested to us (Moffat et al., 1987) that a single bunch exposure of 120 ps might be feasible when the Argonne/CHESS/Spectratech undulator (Bilderback et al., 1989) was installed at CHESS and CESR was run briefly in dedicated, high brightness, single bunch mode. To isolate the x-rays from a single bunch, an ultrafast x-ray shutter train was constructed (LeGrand et al., 1989). X-ray exposures of 120 ps were indeed successfully recorded under these conditions (Szebenyi et al., 1989) on single crystals of an indole alkaloid and of the protein, hen egg white lysozyme (Fig. 2, taken from LeGrand et al., 1989), using the low noise Kodak storage phosphor detector (Bilderback et al., 1988). Subsequent quantitative analysis of these single bunch images has shown that quite accurate structure factors can be extracted with merging R-factors in the 8-10% range against reference monochromatic data (manuscript in preparation by the MacCHESS group). However, the very weak incident source intensity means that only a relatively small fraction of the predicted data was actually recorded. It is clear that a further substantial gain in intensity per unit wavelength is needed before extensive protein data can be recorded with 120 ps exposures. This presents an attractive target for sources such as the ALS, APS and ESRF.

Data Analysis

Time-resolved data may be acquired in either a streak camera mode, or on a time point by time point basis (Moffat et al., 1986; Amemiya et al., 1987). In either case, it can be shown (Y. Chen and K. Moffat, unpublished; Moffat, 1989) that under certain plausible - but not yet established - assumptions, the time course of all x-ray intensities is given by a sum of exponentials, in which the exponents are identical for every reflection but whose coefficients vary from reflection to reflection. The number of exponentials is \( \frac{1}{2} N(N+1) \), where \( N \) is the number of distinct structural species involved, typically 2-4. Although the time course of each reflection is likely to be very noisy, the exponents may be extracted from the entire data set with relatively high precision, since they are substantially over-determined. With knowledge of the exponents and the kinetic mechanism, the coefficients may then be determined for each reflection. Finally, these coefficients enable individual, time-independent difference electron density maps to be calculated, each of which provides the difference in structure between a particular intermediate and the starting structure. That
is, the time-independent structures of the intermediates may be determined separately, rather than just their complicated, time-dependent superposition (Moffat, 1989).

**Results and Prospects**

The results to date are few in number and preliminary in nature, as summarized by Gruner (1987) and Moffat (1989). Those which perhaps best illustrate the potential for the future were obtained on the large, highly regulated enzyme phosphorylase b by Hajdu, Johnson and colleagues (Hajdu et al., 1987a, 1987b, 1988, 1989), using both monochromatic and Laue x-ray diffraction techniques at the Daresbury synchrotron. By judicious choice of solution conditions, the conversion of the substrate heptenitol to heptulose-2-phosphate could be slowed to the point where diffusion could be used as an easy means of reaction initiation in the crystal. A series of "snapshots", each representing a different stage of completion of the reaction, was obtained (Hajdu et al., 1987a), with a time resolutions of tens of minutes.

The problems today arise more in the area of reaction initiation -- enzymology -- than in the x-ray experiment itself. It is an interesting challenge to devise suitable means of reaction initiation for a particular system, to avoid damage to the crystal, and to satisfy the several assumptions that underlie a time-resolved experiment (Moffat, 1989). Light-initiated reactions offer promise, particularly on robust, well-ordered crystals with a high quantum yield such as phototoxic yellow protein (McRee et al., 1986, 1989). The advent of dedicated, high brightness synchrotron sources is essential if structural reactions in the important μ-ms domain (Fig. 1) are to be probed routinely, and certainly if extension to the ps domain is contemplated.

**Acknowledgements**

Supported by NIH grants GM 36452 and RR01646.

**References**


Fig. 1 Time scale of structural changes in macromolecules and of x-ray diffraction experiments using synchrotron and laboratory x-ray sources.

Fig. 2. X-ray Laue diffraction pattern from a single crystal of the enzyme hen egg white lysozyme. Pattern recorded on a novel Kodak storage phosphor x-ray detector with an exposure time of 100 picoseconds, using a single pulse of x-rays emitted by an undulator at CHESS, the Cornell High Energy Synchrotron Source. The undulator is a collaboration between CHESS and Argonne National Laboratory. (Taken from LeGrand et al., 1989.)
DNA Base Pair Sequencer:

Scanning Tunneling Microscope Plus Infrared Radiation

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Lawrence Berkeley Laboratory

May 31, 1989

I) Motivation

There is a large amount of effort going into the sequencing of the base pairs on the human genome. Presently it is estimated that the position of one base pair is determined every 3 minutes (throughout the whole world), but since there are approximately 6 billion base pairs on human DNA the job will take 36,000 years to complete at this pace. It has been proposed that there be a national project, on the scale of other large scale scientific projects (comparable to the SSC or the space station) with a price tag of around $4 billion, to determine this sequencing within a reasonable time frame.

II) Basis

Present methods of sequencing base pairs use chemical methods of isolating and identifying them. This is obviously a tedious and time consuming task. Any quicker less cumbersome method would obviously be of great value. It has also been proposed that the sequencing can be done by X-ray imaging. However, the necessary resolution requires X-rays of a few keV. Such energetic photons are efficient at photo ionizing K shell and other tightly bound electrons. This will produce highly energetic and reactive atoms in the molecule which almost certainly will cause severe damage. After looking up the ratio of elastic scattering to photo ionization we concluded that the X-ray flux required to make an image would completely destroy DNA. We propose that a combination of Scanning Tunneling Microscopy (STM) and Infrared Excitation (IRE) of the different base pairs may be able to do the sequencing. Such a method could be rapid, accurate, and nondestructive to the molecule. The scanning tunneling microscope has the required

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resolution while an infrared laser provides selective excitation of each of the four base pairs. The combination provides identification of a particular base. If this proves to be a feasible sequencing method it would be much cheaper, easier, and faster. Note that the STM moves at a rate of (about) 1000 Å/sec and hence one instrument can sequence at a rate of 30 base pairs per second.

Note, also, that the method proposed has application far beyond DNA sequencing and thus should prove useful, if established, in many different fields of science. For example, it could be used to study the structure of inert substances as well as that of a range of biological samples. The radiation need not be in the infrared; energy can be transferred from electronic excitation into vibrational modes or if electronic excitations remain localized sufficiently well they might produce image discrimination.

III) Concept

The Scanning Tunneling Microscopy has demonstrated resolution on the atomic scale. In fact images of DNA (Fig. 1) have been made although there are many questions about the interpretation of these images. Nevertheless it appears that STM will provide a powerful new tool in the investigation of DNA. It’s power would be greatly enhanced if a means to identify directly the atoms and molecular groups it was looking at were available.

The different base pairs have quite different vibrational structure as is shown in Fig. 2. It can be seen that thymine has a unique imide substructure, cytosine a characteristic exocyclic amine at C-6 (different from that of adenine and guanine), and guanine a unique amicile-like NH at Position 1. Adenine does not have unique functional groups but the exocyclic amine is in a somewhat different chemical environment than that of guanine and cytosine and, as a result, absorbs at a somewhat different infrared frequency. The infra-red absorption spectra are shown in Fig. 3, and it can be seen that preferential excitation of the different bases seems quite possible.

If we excite a base by shining infrared light on a wide area (a few 1000 square microns), but including the base under the tip, at one of it’s characteristic frequencies, the resonant base will be excited. The excited base dimensions will expand and contract with the
vibrations. As a result, their distance to the STM tip should vary periodically. Because the tunnelling current depends exponentially on the distance between the surface being observed and the STM tip (i.e. very non-linearly), the average current will depend on the degree of excitation. Roughly the tunnelling current increases by a factor of ten for every Angstrom decrease in sample tip separation. If the excitation reduced the minimum separation by only 0.04 Å the average current would increase by 10%. The STM should be able to pick out the excited base. The infrared laser colors, so to speak, one type of base pair in the area illuminated (perhaps 100 μm X 100 μm), and the STM images the excited pairs with the desired resolution. Thus the illuminated area can be "large", while the microscope tip gives the desired resolution. A schematic diagram of the proposed configuration is shown in Fig. 4.

We might comment that other configurations can be considered. For example, the laser radiation can be applied directly to the STM tip or a second tip in close proximity to it might be used. The radiation can be brought to the viewing region by using a coaxial waveguide configuration; such a geometry does not have a frequency cut off and so a very wide range of very high frequencies can be brought to the tip (possibly up to the near UV; however, at very high frequency the attenuation is so large that guiding over distances greater than say 100 microns may not be possible ). For small tips, the sample only sees the near field in the vicinity of the tip; i.e. over a region much smaller than the wave length of light being used. Such a configuration is much more complicated than the one we propose and we believe that the simpler scheme should be tried first.

IV) Potential Problems

There are a number of effects which undoubtedly play an important role in the concept.

First, the natural vibrations of the base will be modified by the presence of the substrata on which it is situated. We believe it will not be too large, but the effect must be investigated.

Second, the strong electric field at the STM tip will distort the bases and also alter their natural frequencies. A voltage of 1V across a distance of 3Å yields a field of 3x10^7 V/cm which is significant, but smaller than a typical atomic field.
Third, the excitation will probably be rapidly shared among all the modes of a base causing it to heat up (The vibrational relaxation time is in the picosecond range.); despite this energy sharing there should still be an observable effect; in fact thermal expansion of the heated base may contribute to the change in separation from the STM tip. It is, however, necessary that the energy spreads only slowly to different bases, which we believe to be the case.

Fourth, the heating of a base sufficient to see an effect may prove too severe and the base may come apart. This in itself might be used as a means of identification, provided the "coming apart" doesn't effect other bases in the chain. (A quick estimate, based on a typical frequency of 2000 cm\(^{-1}\) and an oscillating mass of 10 Daltons, gives a temperature rise of about 1500 K for an oscillation amplitude of 0.04 Å.). One should be able to use all kinds of box car integration techniques to pull small signals out of noise so that non-damaging intensities can be used. For example, an oscillation amplitude of 0.01 Å gives a 2% effect in STM current, while corresponding to a temperature rise of only (about) 100°. The sophistication of the experimental technique will be a strong factor here.

Fifth, the laser will heat the sample and tip and cause expansion which can cause unexpected changes in the image. These effects might be overcome by having a steady radiation on the observing area, whose level can be changed to compensate for the laser power, or switching continuously between the requisite four different laser frequencies, but maintaining a steady laser power on the sample. Clearly, the "off time" only has to be small compared to a thermal relaxation time, which should be easy to arrange.

Sixth, other unanticipated effects can be expected. These can only be found through experiment.

V) Acknowledgments

The authors have learned much chemistry from conversations with John Hearst, Richard Mathies, John Porter, Jonathan Sessler, Herb Strauss, and Ignacio Tinoco. They are also indebted to Charles Cantor, Bob Glaeser, John Holzrichter and Wigbert Siekhaus for valuable discussions and constructive criticism.
Fig. 1. An STM picture of DNA.

Fig. 2. The structure of the base pairs in DNA.
Fig. 3. The infra-red absorption spectra of the four monoribonucleotides in neutral D₂O solution. (Figure from G. J. Thomas, Biopolymers Z, 325 (1969).

Fig. 4. A schematic of the infrared source and STM.
The Requirements for Improvement in Biological Microscopy
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Introduction

It is sometimes overlooked that biological systems exhibit a structural micro-diversity many orders of magnitude more complex than that found in inorganic systems. The latter are considered complex if they consist of only a few separate structural phases while, in the former, each of the millions of macromolecules in a typical cell may be considered as a separate phase in that each molecule is spatially almost totally unrelated to any other molecule. The disparate scale of the orderliness characterizing biological and non-biological systems raises the question of whether it makes sense to even talk about biological structure at all.

Our persistence in doing so may be based on our common knowledge of the immense effective change that takes place in a mosquito when it is squashed: the atoms, molecules and even many of the cells making up the mosquito remain largely unaffected by this process but the mosquito no longer works. We ascribe its failure to operate to some level of structural organization that has been destroyed by the act of squashing. Biological structure, then, becomes those aspects of the spatial organization of the constituent parts that permits the mosquito, the cell or even the virus or enzyme to function.

To this rather fuzzy and negative definition of biological structure can perhaps be ascribed the relative weakness of the theoretical foundation upon which the organized study of biological structure rests. Compared with the quantitative aspects of physics, molecular biology or even biochemistry the statements emerging from those who study structure often seem vague and hard to generalize from.

Fortunately for those who study structure, these structural concepts, vague or not, also provide the context without which many of the more quantitative experimental approaches could not have been initiated. We might see an analogy in the way that "hard data" of archaeology are interpreted in the light of the context provided by the more diffuse constructs of history.

The study of biological micro-structure produces images which show the spatial relationships between the components of the cell. These images may be amazingly detailed but in general, living systems cannot be studied in the same detail as dead ones and so this detail is often purchased at a heavy price in terms of the possibility of either preparative artifact or sampling error. We cannot learn the structure of Chicago by extrapolating from the blueprints of a single building because the building might not be characteristic and the blueprint will in any case give us little information regarding its furnishings or landscaping. Blueprints from many different types of building would help reduce the sampling problem but much would still be missed without access to information on the city's transportation, communication, economic and social structure. These more general topics would provide a context to help us interpret the blueprints.

Given the task of using images to investigate the function of Chicago, what are the characteristics that we should look for in an imaging method?
Speed and Simplicity:

Speed is needed to permit many specimens to be viewed: an absolute requirement to reduce the chance of sampling error. Simplicity is of course an aspect of speed, but in addition it has the effect that experts on the structure of cities need not also be experts on the imaging method used.

Spatial Resolution.

Useful information regarding biological systems can be derived over a wide range of size scales from the molecule to the organism but inevitably efforts are made to reduce the size of the smallest discretely sampled volume of the specimen. However, for several reasons resolution per se is not an end in itself. The most distinctive feature of biological specimens is that they were at one time alive and therefore changing with time. Considerable advantage is attached to any system of microscopical study which permits observation of living, changing specimens without disrupting them. In practice this means that light microscopy is far more useful than its resolution limit might indicate, especially if its use is supported by subsequent study of the same cell by electron microscopy. In addition to the live/dead question, the quest for greater resolution is also tempered by the interactions of contrast mechanism and information content as they effect image interpretation.

Contrast and Information Content: Images are not made simply of signals from the specimen, they are made of changes in those signals or contrast. Yet even contrast by itself is not sufficient because it is also necessary that the feature causing the contrast be something in which the observer has an interests: something that contains information.

For instance, though it may be easy to image a colloidal gold particle on the surface of a specimen, this ability provides little information unless the position of the particle has been previously defined by a highly specific labelling protocol. When the gold becomes a marker for a specified biomacromolecule, the contrast becomes information. To become knowledge however, it must still be interpreted.

Ease of Interpretation We see images with our eyes but we interpret them with our mind. The connection between the two has been honed in each of us by long years of practical experience in evaluating images. In normal life, these are in general the three-dimensional images of surfaces. As the type of image derived from the specimen progresses from a 3-D surface image to a 2-D transparent section and finally to lists of numbers or spectra representing various quantitative aspects of the specimen, our ability to appreciate the structural implications of the data in an unambiguous way is considerably reduced.
The State of the Art in Biological Microscopy

Microscopical information regarding biological specimens overwhelmingly comes from light and electron microscopes.\textsuperscript{1} For biologists the distinction between these two types of instrument is far more fundamental than that between photons and electrons or even between low and high resolution. The important distinction is that between quanta which represent either less or more energy than that of common covalent bonds. In reality, it is the distinction between living microscopy and dead microscopy, a distinction meaningless in the microscopy of material science specimens but not so in biology.

Fortunately for the study of biological structure, there have recently been major advances in microscopical instrumentation on both sides of this dividing line. It is even more fortunate that these two developments have enhanced our ability to derive high-contrast images that are three dimensional, easy to interpret, and rich in information. These two instruments are the confocal light microscope (CLM) and the low voltage scanning electron microscope (LVSEM).

Confocal Light Microscopy

The quantitative evaluation of microscopic images has always been complicated by the effect of out-of-focus structures on the final image. These effects can be greatly reduced if the conventional light microscope is replaced by a scanning-confocal light microscope. In such an instrument two conditions are met: 1) only a single point of the sample is illuminated at any time and 2) this point on the sample is then imaged onto the pinhole at the entrance to the photodetector. Because little light from out-of-focus planes will pass through the pinhole, only in-focus data are recorded. Moreover, as only one point on the specimen is sampled, the specimen or the light beam must be scanned to produce an image.

Early confocal instruments required the sample to scan between two objective lenses, effectively preventing their use with living cells. However, two recently developed designs permit the sample to remain stationary. The Laser Scanning Confocal Microscope\textsuperscript{2} uses rapidly oscillating mirrors to cause the beam of a laser to scan over the sample producing either fluorescent or scattered light, some of which then enters the objective and is de-scanned by the same mirrors onto a stationary detector pinhole. The Tandem Scanning Reflected Light Microscope\textsuperscript{3}, (TSM), uses a symmetrical pattern of paired holes, precisely positioned on opposite sides of a spinning disk in the intermediate image plane. These holes serve first as the source of point illumination and then as the detector pinhole.

The laser scanning system provides more quantitative information since the photomultiplier tube detector (PMT) is more linear than the video-based system currently used to record the real images produced by the disk scanning microscopes. However, because the TSM utilizes a large number of scanning points in the field at one time, it provides an almost real-time image. This is essential if a living biological specimen is changing rapidly, especially
if many focus levels are to be imaged sequentially. The imbalance in measurement accuracy will diminish when the SIT video cameras presently used on the TSM’s are replaced by cooled, charge-coupled devices (cooled CCDs). These and other technical aspects of the technique are discussed at length in the Handbook of Biological Confocal Microscopy to be published in conjunction with the August 1989 meeting of the Electron Microscope Society of America in San Antonio.

The image in Fig. 1 has good contrast, adequate resolution and provides specific information of biological relevance namely the spatial distribution of actin-containing structures in a tapeworm larva. The 30 planes of the image required 10 minutes to acquire and an additional 2-3 minutes were required for the computer display and photographic recording of the data. Specimen was chemically fixed and is therefore dead but 100 x improvements in the photon efficiency of more modern confocal light microscopes may soon permit similar images to be made of living specimens.

Low Voltage Scanning Electron Microscope

The scanning electron microscope (SEM) was developed to study surface topography in the 1950’s. Though applied to biological investigation in subsequent decades, its low resolution (relative to the transmission electron microscope) and the requirement to cover the specimen surface with a relatively thick (20nm) layer of heavy metal resulted in few high resolution studies of intracellular structure. Recent improvements in the SEM, in particular the coupling of a high-brightness, field-emission electron source and a short focal-length, low-aberration objective lens have had the effect of substantially removing these limitations. The new instruments produce an acceptably small beam size (3-4nm) at low (1kV) beam voltages and low voltage SEM (LVSEM) can provide excellent image contrast without requiring the heavy metal specimen coating.

The LVSEM can provide a 3-D image of biological surfaces which cannot only be easily understood in terms of the micro-topography of structure down to the level of macromolecules, (Fig.2) it can also accurately localize specific biochemical molecules on this surface with the aid of Au-conjugated markers. Stereo images can be made rapidly by an observer requiring only a minimum of training. However, at present the optimal methods of sample preparation are still strongly specimen-dependent and require some skill to implement successfully. Progress is being made in this area, especially in cryo-preparative techniques. Fortunately their development is greatly assisted by the ease with which a variety of artifacts can be identified simply by analyzing the information-rich images produced by the LVSEM.

Discussion

In the 1940’s electron microscopy was rather primitive by today’s standards. It was under-utilized by biologists until reliable methods were
developed for specimen preparation in the 1950's. Thereafter, it became an indispensable component of cell-biological investigation.

More recently, the rapid development of novel imaging systems and spatially-resolved spectroscopies has given rise to the question of the utility of their application to the imaging of biological structure.

It is clear that to succeed any new method must not only work but work better than methods that are currently available. The improvement could be in finer spatial resolution, new contrast mechanisms or both.

Broadly speaking these new systems can be usefully divided into those that do or do not produce ionization damage in the specimen. Regarding those methods that do produce ionization damage and are therefore precluded from useful observations on living cells as that term is normally understood, this condition represents a much more severe test than that faced by electron microscopy in its early days. At that time there was no other source of structural information in the size range from 0.2 µ to 2.0nm. Now there are many. Not only are there many methods but they have been extensively used by thousands of researchers for over three decades. A considerable level of competence in the detection of artifacts and the interpretation of both images and spectral data has been gained and correlated with studies on the biochemical and molecular biological level.

It is true that by far the majority of the $10^7$ electron micrographs exposed per year in the US record images of cells which have been subjected to the effects of chemical fixation and staining. Though these procedures are recognized as harmful to fine biological structure, it would be wrong to conclude that many biologists see this as a serious limitation, at least down to 3-4nm level. Not only have the artifacts of fixation and dehydration been meticulously documented but the results have also been compared with a sizeable number of studies on hydrated or frozen-hydrated samples.¹

If there is a significant "hole" in our ability to image the structure of dead cells as has been provided by the electron microscope, it is probably in the range below 4nm (see Glaeser, this proceedings). Here radiation damage poses limits more severe than those associated with fixation and drying and the only escape is either electron crystallography or X-ray diffraction: two techniques that permit spatially averaging data from many subunits. A careful consideration of the damage and contrast mechanisms associated with either electrons or X-rays for direct imaging has shown that the chance that any imaging or diffraction technique will provide reliable information regarding, for instance, the base sequence of DNA is effectively zero. Even in this size range, there seems to be no theoretical or practical reason to image with particles or photons other than the electron. This preference applies not only to the primary interactions between radiation and the specimen but also to the quality of available lenses, their quantum efficiency and that of available detectors.

If alternative imaging methods seem unlikely to successfully challenge the various types of electron microscopy in terms of the contrast produced by concentrations of proteins or other organic molecules, what of other contrast
mechanisms? Atoms respond to X-rays differently than they do to photons. Could advantage be taken of this difference to map electrolytes, sulfur or phosphorus? Here the data is sparse but again the competition is stiff. Electron energy loss spectroscopy (EELS) in both the imaging and spot scanning mode is well advanced as is energy dispersive X-ray microanalysis. The problem is not so much instrumentation but specimen preparation and the competition at present comes not from X-ray microscopy but from the ion sensitive fluorophors such as FURA-II. This substance changes its fluorescent properties on binding micro-molar concentrations of Ca++. Using it, Ca++ concentrations have been determined in living cells at an effective resolution only slightly less than that of the EM techniques and with the immense advantage that the observations are carried out on functioning cells. Changes in response to external stimuli have been measured on a time-scale of hours to milli-seconds. It seems unlikely that X-ray-optical techniques will offer the best chances of improvement on this performance during the next few years.

**Conclusion**

Striking technical advances in X-ray sources and X-ray optics now make it possible to produce X-ray images under conditions thought impossible only a few years ago. However, major questions remain as to whether this new instrumentation can find useful application in elucidating biological structure. On the whole, given the sampling problem (implied by the immense cost of the sources needed for X-ray microscopy) the lack of any significantly new contrast mechanisms, the relative inefficiency of the lenses and detector system, and the strong competition from other micro-techniques, it is hard to identify a potentially useful role for X-ray microscopy in biology at this time.

**Acknowledgements.**

This work supported by NIH DRR 570-17 to the Integrated Microscopy Resource. Thanks are due to S. Paddock and H. Ris for figs. 1 and 2 respectively.
References


Fig. 1: Stereo confocal light microscope image of nerve cells. This image, which is described in the text, was made at the IMR by S. Paddock using a specimen prepared by Oaks.

Fig. 2: Xanthophore of goldfish
Xanthophore of goldfish spread on glass, fixed in 2.5% glutaraldehyde in 0.1 M Hepes buffer (pH 7.0) with 0.05% saponin and 0.2% tannic acid and critical point dried. The cell interior is exposed by dry-cleaving, i.e. by touching double-stick tape to the cell surface. The preparation was coated with a thin layer of platinum by argon ion-beam-sputtering and photographed with the Hitachi S-900 LVSEM at 1.5 KV. The image shows tubular regions of the endoplasmic reticulum connected to microtubules oriented in parallel. We see a fine network of microfilaments and a variety of granules of unknown nature. The complexity of cytoplasmic organization is visible even in this very small area close to the bottom membrane of the cell.
Electrons in circular motion at low velocity emit radiation in a non-directional pattern, as shown in Figure 1. At velocities approaching the velocity of light the radiated power increases dramatically and the pattern is folded forward into a cone with a full opening angle of approximately 
\[2\gamma^{-1} = 2mc^2/E,\]
where \(mc^2\) is the rest mass energy of the electron (0.51 MeV) and \(E\) is the total energy. The opening angle is only 1 milliradian for an electron energy of 1 GeV and correspondingly smaller at higher electron energy. Thus synchrotron radiation has intrinsically high brightness (flux per unit source area per unit solid angle). This geometrical property is exploited differently when the source is a bending magnet, a wiggler, or an undulator.

The total power radiated is given by
\[P(kW) = 1.267 \times 10^{-2} E^2(\text{GeV}) I(A) < B^2(T) > L(m),\]
where \(< B^2(T) >\) is the magnetic field in Tesla averaged over the length \(L\) in meters, allowing for constant fields (bending magnets) or alternating fields (wiggler and undulator magnets). For sinusoidal fields \(< B^2 >\) is replaced by \(B'_0/2\), where \(B'_0\) is the peak field.

**Bending Magnets**

Bending magnets produce a large horizontal fan of continuum radiation, much larger than \(\gamma^{-1}\) and much larger than the acceptance of one experiment. See Figure 2. The vertical opening angle remains small. Collecting optics can increase the flux delivered to an experiment by accepting larger horizontal angles, but the brightness is not increased. The smooth spectrum in Figure 3 is characterized by the critical energy given by \(\epsilon_c(\text{keV}) = 0.665 B(T) E^2(\text{GeV})\). This is the half-power point of the spectrum.

**Wiggler Magnets**

A wiggler magnet is a device with several poles, which, when inserted into a straight section of a storage ring, produces one or more oscillations of the electron beam but no net deflection or displacement of the beam. The deflection angle in each pole is large compared to \(\gamma^{-1}\). Figure 2. The spectrum produced by a wiggler, particularly a wiggler with many poles, does have some structure, especially at low photon energies. This structure is due to interference effects and is discussed more in the next section on undulators. At the higher photon energies for which the wiggler is most often used, the structure is usually very small and the spectrum approaches the smooth continuum characteristic of bending magnets.

If the amplitude of the electron beam oscillation is smaller than the electron beam dimension, the wiggler can usually be regarded as a linear succession of bending magnet sources producing a spectrum with a critical energy that is determined by the magnetic field and with an intensity enhanced by the number of poles as in Figure 3. If the amplitude of the electron beam oscillation

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is large compared with the beam dimension, the source points are no longer co-linear and the geometric pattern becomes more complicated.

Wiggler magnets can produce beams with a wide range of spectral and geometric properties. The simplest wiggler consists of one strong central pole, which is the main source of radiation, and two weaker, opposite polarity end poles. Together these end poles cancel the deflection of the central pole. In effect the device has two equivalent full poles which produce a single-oscillation of the electron beam. The central pole generally has a higher magnetic field than the ring bending magnets, resulting in a hardening of the spectrum. Such single oscillation wiggles, also called wavelength shifters, with superconducting fields up to about 5 Tesla are in operation at the Daresbury SRS and the Photon Factory. The fan of radiation from wavelength shifters is usually very large and the beam is easily shared by several experimental stations.

Most wiggles employ vertical magnetic fields making use of the smaller aperture requirement to achieve high magnetic field. The resultant radiation is highly polarized in the horizontal plane, as it is also with bending magnet beams. The Photon Factory superconducting wiggler\cite{1}, however, employs a horizontal magnetic field, causing the electron beam to execute a vertical oscillation and producing a unique vertically polarized synchrotron radiation beam.

Wiggles now in use produce total radiated power up to several kilowatts and power density on beam line components up to about $10 \text{ kW/cm}^2$. New designs for beam line components have been developed to handle these large thermal loads\cite{2}. More powerful wiggles on existing rings and on proposed new rings will present even more severe thermal problems. Clearly we are approaching fundamental thermal limits on wiggler beams, i.e., we can produce higher power and power density than can be handled on beam line components. Undulators reduce thermal problems because of their quasi-monochromatic spectrum; this is one reason for the increasing interest in undulator sources.

**Undulators**

An undulator magnet is a device with $N$ periods of alternating magnetic field (i.e. $2N$ effective poles with alternating polarity) in which each pole produces an angular deflection of the order of $\gamma^{-1}$, the natural emission angle of synchrotron radiation. Thus the intrinsic high brightness of the radiation is preserved and enhanced. Furthermore, interference effects in the radiation produced at many essentially co-linear source points result in a modified spectrum\cite{3,4,5,6}, see Figure 4, with tunable quasi-monochromatic peaks at wavelengths given by $\lambda_n = \lambda_u(1 + K^2/2 + \gamma^2\theta^2)/(2n\gamma^2)$ where $\lambda_u$ is the period length of the magnetic field, $n$ is the harmonic number ($n = 1$ is the fundamental), $K = 0.934 B(T) \lambda_u/(cm)$, and $\theta$ is the observation angle. The wavelength of the light can be most conveniently varied by changing the magnetic field and hence the value of $K$.

For $K \ll 1$, harmonics are very weak and the spectrum consists essentially of one peak, the fundamental. As $K$ increases towards 1, the fundamental moves to longer wavelength, the power in the fundamental increases (reaching a maximum at about $K = 1.2$), and more harmonics appear. For $K \gg 1$, many closely spaced harmonics appear. Since each harmonic has a finite width, the many closely spaced harmonics eventually blend into the continuum characteristic of bending magnets and wiggles. For $K \approx 4$ to 5, the device is generally considered a wiggler, although there is still structure in the spectrum, particularly around the very low energy fundamental peak. $K$ is also given by $\gamma \delta$, where $2\delta$ is the full angular deflection of the electron beam. Thus undulators (devices for which $K \approx 1$) produce a very narrow photon beam and wiggles (devices with $K \gg 1$) produce wider beams.

The angular divergence of the electron beam at the undulator source point in the storage ring must also be considered. If it is comparable to or larger than $\gamma^{-1}$, the interference effects are smeared out. If the electron beam angular divergence is of the order of $\gamma^{-1} N^{-1/2}$, the on-axis brightness produced by an undulator can increase as the square of the number of undulator periods. Thus, in new low emittance rings that satisfy this condition, it should be possible to achieve 3 to 4 orders of magnitude enhancement in brightness (compared with ring bending magnets) from undulators with about 100 periods. This is a main driving force behind the design of future rings.
From the above discussion, it may seem that the designer of an undulator generally seeks to achieve a short period (to reach short wavelengths and also to maximize the number of periods in a given straight section length) together with a peak magnetic field high enough to reach a K-value of about 1 (for high brightness in the fundamental and perhaps a few harmonics). High fields are most easily obtained by reducing the gap. The minimum gap of the magnet is determined by the aperture required by the electron beam. Although full vertical apertures of about 1 cm are adequate for a stored beam in most multi-GeV rings, larger apertures are often needed for injection and tune-up. Flexible vacuum chambers[7] offer an attractive solution to this need.

Most undulators are planar arrays with magnetic fields alternating in one direction. It is also possible to use helical fields, or a pair of planar arrays with different field directions, to produce elliptical, circular and other polarizations[8,9,10,11].

Permanent Magnet Technology

Permanent magnet technology is making it possible to achieve shorter period devices than can readily be achieved with conventional electromagnets and, in some cases, superconducting magnets. In conventional electromagnets the power density in the coils increases rapidly as the period goes down, eventually making it impossible to cool the coils. In the absence of coils, permanent magnets can be scaled down to very short periods. The peak field decreases exponentially as the ratio of the gap to the period. This sets a practical minimum period of about one to two times the gap. The minimum gap is determined by the aperture requirement for the electron beam.

The use of permanent magnets, particularly using rare-earth/cobalt material, for short period undulators was suggested independently around 1979 by Halbach at LBL and by Kulipanov and Vinokurov at Novosibirsk, leading to the construction of the first such devices in both laboratories at about the same time. The LBL device[12], designed and constructed in collaboration with SSRL, was a 2-meter-long, 30-period magnet that was tested in the SPEAR storage ring in late 1980 and used for the first experiment in 1981.

The first permanent magnet insertion devices were made with no iron, as shown in Figure 5. The peak, on-axis magnetic field in such a device is given approximately by

\[ B_o = 2B_r e^{-\pi g/\lambda_u} \frac{\sin(\pi/M)}{\pi/M} \left[ 1 - e^{-2\pi h/\lambda_u} \right] \]

where \( g \) is the full magnet gap, \( \lambda_u \) is the period length, \( M \) is the number of blocks per period, and \( h \) is the height of the blocks. It is most convenient to build such a device with four blocks per period \((M = 4)\) and with blocks of square cross section \((h = \lambda_u/4)\). In this case, for \( B_r = 0.9 T \) (which is readily available) the above equation reduces to \( B_o(T) = 1.28 e^{-\pi g/\lambda_u} \). Halbach[13,14,15] gives more general expressions for the field.

For devices containing iron, also called hybrid devices, the peak field on-axis is given by

\[ B_o = a e^{(-g/\lambda_u)(b-cg/\lambda_u)} \]

where \( B_r = 0.9 \)

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The first column is typical for samarium cobalt material and the second for neodymium-iron.
Figure 1: Radiation patterns of electrons in circular motion at low velocity (top) and at velocity approaching that of light (bottom).

Figure 2: Radiation patterns from electrons in bending magnets (top), wigglers (middle), and undulators (bottom).

Figure 3: Spectral distribution of radiation from the bending magnets of the SPEAR storage ring at 3 GeV and two wigglers now in operation in SPEAR.

Figure 4: Comparison of bending magnet, wiggler, and undulator spectra calculated for the 1.5-GeV Advanced Light Source at LBL. Courtesy Kwang-Je Kim, LBL.
Figure 5: Schematic drawing of a pure permanent magnet undulator based on concepts developed by K. Halbach, LBL.

References

Review of X-ray Lasers


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This extended abstract will describe progress to date in the field of x-ray lasers and the application of the soft x-ray lasers to microscopy. Soft x-ray lasers offer unique features which complement other light sources and microscopic techniques. We will describe the different techniques for generating a population inversion and producing a soft x-ray laser and review the progress to date and near term prospects in increasing the output power and progressing to shorter wavelengths. We want to stress that we don't see this as a competition between the different techniques. Getting detailed information about life processes inside cells is a challenging task and the more techniques available to obtain that information the better.

The first unambiguous demonstration of lasing action in the soft x-ray region of the spectrum came in 1984 by groups at the Lawrence Livermore National Laboratory\(^1\) and Princeton University\(^2\). To date x-ray laser action has been generated by one or two methods: (see Fig. 1)

a) recombination lasers based on hydrogen or lithium-like ions or
b) collisionally pumped lasers based on neon or nickel-like ions.

Both schemes rely on a high power pulsed laser to create the appropriate conditions in a plasma and in both schemes the population inversion necessary for stimulated emission and gain is brought about by fast radiative decay of the lower level. In the recombination scheme for hydrogen-like ions, a laser is used to create a plasma with a high fraction of totally stripped ions. After the laser pulse, the plasma is cooled rapidly and undergoes fast three body recombination. In some cases the plasma is cooled by adiabatic expansion. One unique feature of the Princeton laser is that the plasma is confined in a magnetic field and cooled by radiation losses. The magnetic field maintains a high electron density which is beneficial as the three body recombination rate scales as the electron density squared. It also helps shape the plasma into a long thin geometry suitable for a laser. Three body recombination puts a high population into upper excited levels which decay downward by collisional radiative cascade. Level 2 in hydrogenic ions decays rapidly by radiation and a population inversion is built up between levels 3 and 2. The atomic structure of lithium-like ions is similar to hydrogen-like ions and the same method works there also\(^3,4\). In this case the 3-2 transitions has a high radiative decay rate and gain can be generated on the 4-3 and 5-3 transitions. The lithium-like sequence has the advantage of a shorter wavelength lasing transition for ions of similar ionization potential i.e. a better "quantum efficiency".

The neon-like scheme was applied by Lawrence Livermore National Laboratory. Here a high density, high temperature plasma is generated by a large neodymium laser, Novette or Nova. In the neon-like plasma, a large population of ions is collisionally excited to the 3p level. The 3s level has a relatively low population since it has a fast radiative transition to ground and a population inversion is built up between the 3p and 3s levels. The same scheme also works in nickel-like ions and here as in the case of lithium-like ions there is an advantage in using the Ni-like sequence to access the shortest possible wavelengths. A recent review of work at Livermore is given in reference \(^5\) and current work is described in the article by D. Matthews in this proceedings. In summary, both approaches use a high power laser to create an appropriate plasma and rely...
on fast radiative decay to deplete the lower level in order to generate a population inversion. The major difference is that in one case the upper level is populated through recombination and the other by collisional excitation.

There are a number of laboratories around the world heavily engaged in x-ray laser research. We would like to mention the pioneering work of G.J. Pert, S.A. Ramsden et. al.\textsuperscript{6} at Hull in England on the recombination scheme for carbon fibers at 18.2 nm which was later taken up at the Rutherford Appleton Laboratory\textsuperscript{7}. This work is now part of an international effort involving at 7 different institutions in England; France (Orsay); Japan (I.L.E.) and the U.S.(N.R.L). Jaegle et. al.\textsuperscript{3} have exploited lithium-like ions to achieve a gain-length of 3 at 10.5 nm in a 6 cm long aluminium plasma. Gain measurements in Li-like aluminum have recently been reported by Moreno et al.\textsuperscript{8}. The recombination approach has also been used at Laboratory for Laser Energetics at Rochester\textsuperscript{9} to produce gain on the CVI 18.2 nm transition in a radiation cooled selenium/formvar plasma. The collisional excitation scheme has also been demonstrated by a group at NRL\textsuperscript{10} at wavelengths from 19.5 nm to 28.5 nm in neon-like germanium and copper. In Japan, Herman et. al.\textsuperscript{11} have demonstrated gain at various wavelengths including a measurement of gain of 2 cm\textsuperscript{-1} in the 8.1 nm transition in hydrogen-like fluorine. Kato et al.\textsuperscript{12}, in a controversial experiment, have reported gain-lengths of GL = 1 at 5.4 nm and GL = 0.9 at 4.5 nm.

A third potential method to achieve x-ray lasing is resonant photo-pumping in a two component plasma.\textsuperscript{13} Resonant photo-excitation has been recently demonstrated in the x-ray range by Monier et al.\textsuperscript{14} A hydrogen-like Al\textsuperscript{XIII} resonance line was used to pump a neon-like Sr\textsuperscript{XXIX} resonance line resulting in a factor of two increase in fluorescence from the upper state of the pumped transition. However the pumping efficiency was less than expected. Several difficulties need to be overcome in order to successfully apply this method to generating x-ray lasing.

Current research directions include extending the collisional excitation and recombination schemes to higher output energy and to shorter wavelengths. The current state of the art is represented in Fig. 2. The enhancement, E defined as the increase in output intensity as compared to spontaneous emission is given by:

\begin{equation}
E = \frac{I_{\text{out}}}{I_{\text{spont}}}
\end{equation}
only when the gain-length exceeds 4.6 that the output intensity exceeds spontaneous emission by more than an order of magnitude and one can talk about an x-ray laser beam.

Why is it so difficult to achieve high gain in the the wavelength range below 10 nm? The details vary according to the scheme but the simplest way to understand it is to look at the pump-rate needed to offset the spontaneous emission rate, $A$, in producing an inversion. The gain is proportional to the Einstein $B$ coefficient. However the ratio scales as:

$$A / B = 1 / \lambda^3$$

To generate gain at shorter wavelengths one needs a higher pump-rate to offset the higher spontaneous emission rate. This results in a very severe scaling on the required laser power. That is, to go from 20nm to 2nm needs a $1000x$ increase in laser power. Using the full power of Nova may be one way to do this. However the essential requirement on laser power may be met in a more modest sized laser with a picosecond or femtosecond pulse duration. There are a number of groups investigating the physics relevant to more efficient or shorter wavelength schemes in particular the

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Fig. 2. Plot of gain length achieved to date vs. wavelength (references 1-10). The dashed line marks $GL = 4.6$, which corresponds to an enhancement of 10.

Fig. 3. Plot of equation 1 showing the enhancement of stimulated emission over spontaneous emission as a function of gain-length. The dashed line marks $GL = 4.6$, which corresponds to an enhancement of 10.
work by Harris et al. 15,16 and Falcone et al. 17 on Auger ionization and of Rhodes et al. 18 on multiphoton processes. At Princeton we are investigating a two laser approach to lasing in the region near 1 nm in which a Nd or CO₂ laser creates a plasma column of an appropriate ionization stage which is subsequently excited by a terawatt - subpicosecond laser pulse 19. More details of the current research directions are contained in reference 20. For special journal issues devoted to x-ray lasers see references 21 and 22.

The Princeton soft x-ray laser has been used for contact microscopy of biological specimens and a description of the microscope design and images of the biological specimens obtained are described in the article by R. J. Rosser et al. in this proceedings. Microlithography is an application closely related to the contact imaging of biological specimens and has become an area of intense national competition with large scale investment in compact synchrotrons. X-ray laser technology promises to offer significant economic advantages in this field. A diatom (silicified skeleton of planktonic algae) is a high contrast object and can be regarded as a lithography mask. Figure 4. shows a contact image of a diatom fragment recorded with one shot of the Princeton soft x-ray laser in PMMA co MMA resist.


The Development of Coherent X-ray Lasers for X-ray Holography*

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Abstract

We will describe several of our research paths to develop an x-ray laser capable of producing the flux required to obtain x-ray holograms of biological structures in live cells. These paths include optimization of our current Ni-like Yb laser which operates at 5.02 nm as well as the introduction of a new Ni-like Ta laser which operates at 4.48 nm, i.e., is the near optimum wavelength for holographic imaging of biological objects. Current predictions indicate that we will need to produce at least 20 μJ of spatially-coherent radiation in a pulselength of 50 psec and focussed at the sample to a beam of 10 μm diameter. We will provide a detailed description of how we hope to meet these requirements. In addition, some comments will be given on future trends in x-ray laser development, namely towards more efficient "table top" systems.

* Work performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract number W-7405-ENG-48.
We are pursuing the possibility of producing x-ray holographic images of living microorganisms using an x-ray laser as the source. A schematic of the technique is shown in Fig. 1. The concept is to create a spatially-coherent beam of x-rays of suitable wavelength, focus it onto a sample holder within which is contained a reference scatterer as well as an in vitro biological microstructure and then record the fourier transform hologram of the event. Reconstruction of the original object with a spatial resolution of ~300 Å would be accomplished by numerical techniques. The purpose of this talk is to list the source requirements and to describe how we plan to tailor the development of the x-ray laser to suite this purpose.

X-ray scattering cross section calculations of London et al. combined with numerical simulations of holography systems lead to the x-ray laser source requirements listed in Table 1. These results assume 3 simultaneous views are being formed of the object and that we can focus the x-ray laser beam to 10 μm dia. from an initial dimension of 100 μm dia. Both of these requirements appear to be readily feasible using a segmented Schwarzschild multilayer mirror optic to split initial x-ray beam into 3 beamlets. We separate the source requirements into two cases depending on whether or not we use gold tagging to enhance the x-ray scattering and reduce source strength. Basically, without gold tagging, we need a 44 Å laser, that is fully spatially coherent with a pulselength of 50 psec or less and with 1200 μJ of output energy. With the tagging there is no longer a short pulselength requirement and we can diminish the energy needed by 60 fold.

The development of an x-ray laser suitable for holography can thus be divided into 3 topics: wavelength, power and coherence. We will also discuss as a fourth topic some future x-ray laser designs which may allow true "table top" geometry instead of the large facilities such as Nova which are currently in use.

To date, we have been able to demonstrate x-ray lasers at wavelengths as short as 50.3 Å. Progress by many of our colleagues has also been excellent. To date, more than 30 x-ray laser transitions with wavelengths ranging between 300 and 50 Å have been published worldwide. Fig. 2 shows the wavelength scaling vs. atomic number of lasant ion of various types of x-ray lasers presently under development. Only minor extrapolations of many of
these schemes will be necessary to achieve the appropriate wavelengths (~44 Å) for holography. The development, therefore, of the x-ray laser to ever shorter wavelengths is forthcoming. Of more immediate concern, however, is the demonstration that sufficient coherent energy at short pulselengths can be extracted from these amplifiers.

For holography, the incident x-ray beam needs to be coherent over the size of the object to be imaged. The required longitudinal coherence is expected to be easily produced, based on the expected (Doppler) width of the lasing lines. Longitudinal coherence lengths are estimated to be 300 microns, which is more than adequate for x-ray holography experiments on biological structures which are only several microns thick. An actual measurement of the longitudinal coherence will be attempted before the end of 1989.

Transverse coherence is more difficult to achieve. Initial considerations have indicated that the x-ray laser may not be very coherent (of order $10^4$ transverse coherence modes in the beam), because of the large Fresnel number of the lasing plasma. Several techniques have been proposed to improve the coherence. The idea has been to use a two (or more) stage laser, in which the first stage (the "oscillator") provides a coherent, but small aperture (and therefore low power) signal which is then expanded and injected into a large aperture second stage "amplifier". A modification of this technique can produce a coherent beam by two-pass amplification, i.e., by combining a single x-ray laser amplifier and a small aperture between it and a multi-layer mirror. The first pass radiation would be spatially filtered by the mirror aperture, and then sent through the plasma for a second pass enabling most of the power to be extracted. An output aperture at the opposite end of the laser spatial filters both the first pass and the second pass radiation. Figure 3 shows the schematic arrangement for this double pass concept for the improvement of the spatial coherence.

Some very recent considerations suggest that by a combination of refraction and gain-guiding, the coherence of a simple exploding foil amplifier may be much greater than initially expected, making it easier to achieve the required degree of transverse coherence. In an x-ray laser, spontaneous emission generated at one end of the laser is amplified as it propagates along the laser axis. Refraction bends most of the radiation out of the amplifier, preventing its amplification. Only rays from a very limited transverse region at the end of the laser receive significant amplification. Because rays which are
bent out of the plasma experience less gain than those which stay within the plasma for the whole length, the transverse region which contributes significant stimulated emission is narrowed by the effect of refraction. Gain guiding adds to this effect. This narrowing of the emission region, improves the coherence. We are now engaged in modeling the coherence of the x-ray lasers with an electromagnetic field description of the radiation. Preliminary theoretical results indicate that the coherence may be quite high for standard LLNL x-ray lasers with the number of spatial modes expected to be 10 or less. Experimental tests of this concept are upcoming at our Nova laser facility.

The first x-ray laser scheme we are developing to actually reach the desired wavelength of ~ 45 Å produces relatively high output power but at a moderate pulse duration (~ 100 psec). The inversion scheme is the Ni-like 4d-4p laser first demonstrated by MacGowan et al.\textsuperscript{8,13} in Eu and Yb. The wavelength can be discretely tuned by changing the element being used as the laser. The most appropriate element for producing laser transitions for wet cell holography is Ta where the strongest laser line is calculated to appear at 44.8 Å. An optimized version of gain predictions\textsuperscript{14} using x-ray amplifiers similar to those used for Eu and Yb lasers indicate that the Nova 2 Beam laser facility may be able to produce ~11 gainlengths of amplification with this scheme. This corresponds to ~200 µJ of laser light in a pulse of ~100 psec (FWHM). Assuming the amplifier has no mode selection properties and has, according to a simple coherence model, 10\textsuperscript{4} modes, this gives ~0.02µJ /mode which is far too low to be useable. However, given the theoretical treatment by Strauss, London and Rosen\textsuperscript{12} the amplifier may well be an excellent mode selector owing to its inherent properties of beam refraction and gain guiding. This effect would mean that the Ta amplifier would support only 1 spatial mode, thus we could have more than adequate coherent energy. This amplifier will be tested soon by MacGowan and co-workers\textsuperscript{15} as will its coherence properties be diagnosed by Trebes et al.\textsuperscript{16}

There are two techniques for significantly enhancing the coherent output energy of the Ni-like Ta laser. The first method would be to significantly enhance the gain while maintaining the same pump power required to produce the x-ray laser. In fact, the relatively low pump laser energy requirements of this scheme may make it suitable as a "table top" x-ray laser. The laser scheme that has been recently suggested by M. Rosen and D. Matthews\textsuperscript{17} is a modification of the conventional nicke-like scheme and, for Ta, it is predicted to increase the
laser gain coefficient from 5.5 to 9 cm$^{-1}$. The method uses a low intensity (low energy) long pulse duration (typically, 1 nsec FWHM) heating laser pulse to create an x-ray laser medium with no gain. This same medium is then rapidly heated with a second short time-duration, high intensity pulse which produces inversion in the Ni-like 4d to 4p levels thus producing gain. If this concept works, an amplifier 2 cm in length will saturate with 18 gainlengths and provide 10 to 30 mJ of output energy at a pulselength of 20 to 50 psec, which is far more power than necessary. Indeed, with this amount of excess power the required degree of coherence could be achieved by simply placing a pinhole in the far field of the x-ray laser beam.

The second technique for increasing the coherent power is the use of a mirror plus aperture. In essence, we propose developing a double-pass$^{18}$ amplifier wherein a spherical multilayer mirror and aperture at one end reflects only a single spatial mode for reamplification. D. Stearns of LLNL has calculated that such a mirror which uses C/Cr layers optimized for 45Å would have a theoretical reflectivity of $\approx$ 50%. Using this mirror, as many as 16 gainlengths may be achievable with the conventional Ni-like Ta scheme. This is near saturation and should be more than sufficient coherent power for the holography application.

In addition, two lower risk paths exist for producing sources for preliminary x-ray laser holography experiments. These use the neon-like strontium laser at 165 Å and nickel-like Yb laser at 50.3 Å. Both these lasers have already been demonstrated$^{8,19}$. The strontium laser at 165 Å has the potential for achieving a multi-mode output of 1mJ. While this wavelength is too long for holography of live cells, there is sufficient x-ray penetration through protein at this wavelength to permit holography of gold labeled thin specimens. These specimens would also be thin enough to permit analysis by electron microscopy. This will allow direct comparison of x-ray holography with electron microscopy. It should be possible to use the existing Ni-like Yb x-ray laser at 50.3 Å to study wet gold labeled samples in preliminary experiments. By double pass amplification of the present (non-optimized) Yb laser we have determined that 10 µJ/mode could be obtained even under the very conservative assumption that the amplifier has no mode discrimination. Significantly more coherent output could be obtained from the strontium laser. In both these cases the lasers could be used in holography experiments with resolutions of 500-1000 Å,
We are also aggressively seeking to produce x-ray lasers that do not depend on pumping from large laser facilities such as Nova. These efforts are critical for the development of relatively low cost, accessible x-ray lasers for use by the general scientific community. For example, with the Mg H-like recombination laser\textsuperscript{20} it may be possible to produce a powerful, 45.5 Å x-ray laser by using high power, but not high energy, pump lasers. Pumping in this manner not only is less expensive, but also far more efficient and can provide high repetition rates. We plan to perform experiments this summer with 1 to 10 TW lasers which operate at 1 psec. Eventually, we\textsuperscript{21} hope to have a 100 to 1000 TW, 1 psec laser available. Moreover, as was recently pointed out by Corkum and Burnett\textsuperscript{22}, it may be possible to invert the K-shell of low Z atoms when using multiphoton ionization, i.e., produce x-ray lasers from n=2 to n=1 transitions. If this is realized we could achieve the desired x-ray holography wavelengths simply by fully ionizing carbon. This may be possible to achieve even with 10TW "table top" lasers. In addition to the possibility of utilizing these compact short pulse lasers at x-ray laser drivers, substantial work is in progress at LLNL to develop low cost high average power glass lasers\textsuperscript{23}. These would be comparable in performance to the Nova 2 beam facility except at a reduced cost and size and with a much higher repetition rate. Progress in these kinds of laser technologies indicate that the cost and availability of x-ray laser will improve significantly from the 4-5 shots a day currently available at major laser facilities to repetition rates of several per minute.
References

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6) J. Gray(LLNL), private communication.


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23) K. Manes (LLNL Laser Program), private communication.
Fig. 1. X-ray laser holography concept.

Fig. 2. X-ray laser wavelengths achievable with various inversion schemes.
Fig. 3. Double pass amplifier for producing a fully coherent x-ray laser at 44 Å.

Table 1. Characteristics of x-ray laser source necessary to produce x-ray holograms of living cells.

<table>
<thead>
<tr>
<th>Object</th>
<th>XRL spatially coherent energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>wet cell, single view</td>
<td>120 microjoules @ 50 psec</td>
</tr>
<tr>
<td>wet cell, 3 views</td>
<td>1,200 microjoules @ 50 psec</td>
</tr>
<tr>
<td>wet cell, gold labeled</td>
<td></td>
</tr>
<tr>
<td>single view</td>
<td>2 microjoules</td>
</tr>
<tr>
<td>wet cell, gold labeled</td>
<td></td>
</tr>
<tr>
<td>3 views</td>
<td>20 microjoules</td>
</tr>
</tbody>
</table>
FEASIBILITY/BIOLOGICAL APPLICATIONS OF NEUTRON MICROSCOPY

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Abstract

The neutron microscope is a new instrument permitting a better understanding of life processes at subcellular levels, e.g., in genetics, cell biology, physiology and pharmacology. New knowledge and understanding of biological functions at the organ and organism level are possible, e.g., real time, high spatial resolution distribution of nitrogen. The neutron microscopes using cold neutrons were built in W. Europe and the USSR, but their performances are severely limited by existing neutron detectors. To obtain submicron spatial resolution and to shorten the time necessary to obtain the image, we suggest the use of a new high spatial resolution imager: the superconducting granular detector (SGD). Using cryogenic techniques neutron detector with a few micron spatial resolution (2048 x 2048 pixels) can be developed and would permit the neutron microscopy with submicron resolution.

1.0 INTRODUCTION

The application of x-ray microscopy to structural biology has received much renewed attention due to recent advances in x-ray sources and optics. Compared to optical light, x-rays offer the possibility for finer resolution because of their shorter wavelength. Compared to electrons, x-rays have the advantage of higher penetrability through water, thereby enabling the structure of living cells or organisms to be studied, avoiding unknown changes which may occur in the process of sample preparation for electron microscopy. They also offer the possibility of capturing rapid dynamical biological processes, using short exposures.

The major problem of x-ray microscopy/holography seems to be a radiation damage. Radiation dose as high as 10 GRad was calculated, leading to motion artifacts; the biological structures within an aqueous environment are virtually boiled. This leads to a need of using ultra-short exposures in order to capture the image before the induced motion will blur the image. Thus, x-ray lasers were advocated. We should like to point out that a much less expensive alternative is the use of cold neutrons in which case the radiation damage is up to five orders of magnitude smaller. In the same time, using Triga reactors, the millisecond timing seems possible.

The scattering of neutrons has been the classical method for obtaining structural information about diverse biological materials and processes. For example, diffraction studies provide information on the detailed arrangement of atoms in molecules as well as about the long-range periodicity in the proteins. New intense sources of cold neutrons may permit real-time, in vivo neutron microscopy. Featuring spatial resolution comparable to optical microscopy and contrast paralleled only by NMR methods, neutron microscopy provides a new imaging technique of high importance for biology and medicine. In these applications, 2-dimensional neutron detectors with spatial resolution of a few microns are necessary and must have high Quantum Detection Efficiency (QDE).
2.0 BIOLOGICAL INTEREST IN NEUTRON MICROSCOPY

Neutron microscopy is a new instrument applicable to research in genetics, cell biology, physiology and pharmacology. New knowledge and understanding of biological function at sub-cellular levels are possible, e.g., real time, high spatial resolution distribution of nitrogen. The neutron microscopes using cold neutrons were built in Western Europe and the USSR, but their performances are severely limited by existing neutron detectors.

Micron spatial resolution will be required in neutron microscopy to obtain important biological information at the subcellular and cellular level. In the following, we show that spatial resolution of a few microns is possible using ultra cold neutrons. When "monochromatized neutrons" are used, the fundamental limitations to neutron microscopy are at 0.1 μm level.

Neutrons interact with matter quite differently than photons or electrons. Therefore, they "sense" a different contrast. Thermal-neutron scattering in combination with controlled proton-deuteron exchange is well established as an extremely powerful method for probing the hydrogen distribution in biological objects. The use of cold or ultra-cold neutrons permits extension to selective sensing of distribution of other nuclei, e.g., nitrogen. The distribution of some trace elements and/or heavy metal poisons can be studied. Furthermore, the contrast available with neutrons is orders of magnitude higher than in x-ray/electron microscopy.

The neutron microscope measures the number of absorbed or scattered neutrons. When only the number of forward going neutrons is measured, the neutron microscope senses the amount of hydrogen for which the incoherent scattering cross-section is very large \( \sigma(H) = 79.7 \text{ barn} \). This points to the first biological applications of neutron microscopy. We will obtain in vivo images of hydrogen distribution; spatial resolution of a few microns and quantitative imaging are possible. Thus, in this application the neutron microscopy will be a technique similar to NMR imaging, but with much better spatial resolution. One of the powerful techniques of in vitro studies is to partially replace hydrogen with deuterium. Neutron microscopy should permit images of deuterium distribution with a submicron spatial resolution.

We should note that the incoherent scattering of neutrons on hydrogen is due to hydrogen spin, and is independent of neutron velocity. The absorption cross-sections, of atomic nuclei, e.g. C, N, O, however, depends strongly on velocity, \( \sigma \propto 1/V_{\text{neutron}} \). Thus by imaging the same object with neutrons of different velocities, the incoherent scattering can be subtracted and the image of only the absorbing nuclei obtained. Furthermore, if the same biological object is studied at two different temperatures, and after being imaged with mirrors, the fraction of reflected scattered neutrons will change. This is so, because the scattering of ultracold neutrons on hydrogen leads to energy gain and with rising specimen temperature, there is an increase of the number of more energetic neutrons which don't reflect from mirrors. This permits us to differentiate between neutrons absorbed on nitrogen and scattered from hydrogen.

We would like to comment about the biological applications of neutron microscopy. For thin samples, the contrast is dominated by absorption. The biological samples consist essentially of hydrogen (H), carbon(C), nitrogen(N) and oxygen(O). The absorption cross-sections for thermal neutrons are \( \sigma = 0.33, 0.0032, 1.88 \text{ and } 2. \times 10^{-4} \text{ barn} \) for H, C, N and O, respectively. It can be seen
that the dominating contribution to the absorption is from nitrogen. Thus neutron microscopy gives the unique possibility of imaging in vivo the distribution of nitrogen. Thus it is complementary to NMR imaging of hydrogen and carbon but promises much better, submicron spatial resolution.

Many commercially available isotopes have very large neutron absorption cross-sections, e.g., 955 barn for $^7$B; 4010 barn for $^6$Li; 40 barn for $^{42}$Ca; 70 barn for $^{40}$K; 20,800 for $^{113}$Cd; 50,000 barn for $^{149}$Sm and 14,000 barn for $^{155}$Eu. This permits very good contrast for studies of compounds/pharmaceuticals which include these isotopes. Note that two of the above mentioned isotopes are isotopes of lithium and potassium, which permit important studies of neurophysiological effects of psychotropic compounds at a microscopic level. Furthermore, use of $^{42}$Ca will permit excellent contrast in bone studies. Use of $^{113}$Cd will give information about physiology of heavy metal concentration and poisoning. Finally, samarium and europium are trace elements.

Finally, we should like to point out that using the Triga type reactors the good timing can be achieved. Typical power of Triga reactors is up to MegaWatt, with time rise of a few milliseconds. Furthermore, a large flux is possible due to small dimension of critical assembly. Thus, biologically important gated studies with submillisecond time resolution are possible using 10 kHz neutron choppers and assuming fast neutron detectors.

### 3.0 Neutron Microscope

The availability of cold neutrons (CN) and especially ultracold neutrons (UCN)\(^{(3)}\) has opened the possibility of neutron microscope. This appears feasible since ultracold neutrons are totally reflected from suitable mirror materials even at large angles of incidence, and thus anastigmatic imaging systems can be developed. In essence, such neutron microscopes are quite similar to UV microscopes developed in the 1940s\(^{(4)}\). In the UV microscopes magnifications of about 4,000x to 25,000x are possible and under favorable conditions the spatial resolution is of the order of 0.3 microns.

Let us first consider the optics of neutron microscopes. One uses "neutron mirrors," i.e., the effect of total reflection of neutrons from appropriate metallic film. The critical angle of reflection is a strongly rising function of neutron wavelength, i.e., the refraction index is very large for ultracold neutrons. Technically, glass mirrors are coated with appropriate metal, e.g. $^{58}$Ni-Mo amalgamate. Thus, the know-how necessary to build the optical part of a neutron microscope already exists. Furthermore, in recent decades many ultracold neutron facilities have been developed. Thus, we believe that it is neutron detector development which is most necessary.

We pointed out above the biological importance of the neutron microscope as a sensor of absorption on nuclei. The technical implementation requires acquisition of images at two different wavelengths of neutron radiation. We note that due to the very low velocity of ultracold neutrons, the deflection due to the Earth's gravity field is a very efficient and simple method of velocity selection. However, as UCN beams are curved by gravity the wavelength dependence of curvature gives rise to chromatic aberrations of mirror systems. The properties of a concave mirror as a system for UCN imaging were first described by Frank in 1972\(^{(5)}\) and implemented by A. Steyerl et al./\(^{(6)}\) a few years ago. The first neutron imaging system was achieved by use of a "zone mirror" consisting of a totally reflecting, blazed zone grating deposited on a concave spherical mirror with vertical optical axis (see Fig. 1).
ultracold neutrons at the high-flux reactor in Grenoble, a sharp achromatic image of an object slit was obtained.

The next step in the development of neutron microscopy was done by the Moscow group which developed a complete neutron-microscope(7) which brings together:

- a mirror optics system; and
- a detector to visualize the neutron image.

The spatial resolution seems to be due to intrinsic spatial resolution of the neutron detector [ZnS scintillator coated with LiF]. Thus, development of neutron detectors with very good QDE and excellent spatial resolution, say a few microns, is necessary for further improvements in neutron microscopy. For example, with magnification of 50 (see Steyerl et al.) the detector spatial resolution of five microns would permit the spatial resolution of 0.1 microns. We believe, however, that it will be easier to diminish the magnification to facilitate the problem of chromatic aberration and yet, reach submicron resolution.

4.0 HIGH SPATIAL RESOLUTION DETECTORS FOR NEUTRON MICROSCOPY

The neutron microscopy requires detectors with(8)

- large surface and micron spatial resolution;
- good Quantum Detection Efficiency (QDE ≥ 50%);
- linear response as function of neutron energy;
- digital readout to permit image subtraction and enhancement.

The best spatial resolution among existing neutron detectors can be obtained with B/Li loaded photographic emulsion. However, the QDE is very low and good signal/background is difficult to achieve. Furthermore, nuclear emulsion is a detector with analog readout and highly nonlinear response function. Also, it can not be used in dynamical studies. The existing electronic neutron detectors have good QDE (say 50%) but millimetric spatial resolution (e.g. He-3 detectors).

The main characteristics of detectors which must be considered are spatial resolution, linearity, and dynamic range. Note that we talk here about "system spatial resolution," not the "intrinsic spatial resolution." For example, for thick detectors (gas detectors) the spatial resolution is often degraded by the parallax effect and curved detectors are required. Obviously, quantum detection efficiency (QDE) is very important. Furthermore, good photon rejection is necessary to obtain a good neutron-to-background ratio. No single detector, either now in operation or likely to be constructed in the near future, can satisfy all the demands simultaneously. All conventional techniques are mature and only minor improvements are expected. On the other hand, better and possibly new types of position sensitive detectors will become a must with the new cold neutron sources. We argue that a new neutron detector based on superconducting granular detector (SGD) may be used in neutron microscopy. The preliminary specifications of such a detector are:
- Surface: \(100 \text{ cm}^2\)
- Spatial resolution: intrinsic 4096 x 4096 pixels
- Efficiency (QDE): 90\%
- Photon background rejection: better than 10\(^{-3}\)
- Timing: better than 0.1 \(\mu\text{sec}\)

For cold neutrons, the excellent QDE is possible with a detector only a few microns thick. It can be shaped into a rotation paraboloid and "parallax limited" submicron spatial resolution is possible. The system autonomy is defined as a time between liquid helium refilling; if necessary it can be extended to about a week.

Table A shows a comparison of some existing 2-D position sensitive detectors:

<table>
<thead>
<tr>
<th>Diameter [cm]</th>
<th>Resolution</th>
<th>QDE: Thermal</th>
<th>Timing</th>
<th>Homogeneity</th>
<th>Curved-Detectors</th>
<th>Photon Rejection</th>
<th>SIND*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas(MWPC)</td>
<td>Scintillators</td>
<td>SIND*</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>20</td>
<td>100</td>
<td>4096x4096</td>
<td>90%</td>
<td>1 micro sec.</td>
<td>Difficult</td>
<td>10^3</td>
<td>1-10</td>
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<tr>
<td>512x512</td>
<td>256x256</td>
<td>90%</td>
<td>95%</td>
<td>5%</td>
<td>Yes</td>
<td>10^2</td>
<td></td>
</tr>
<tr>
<td>1 micro sec.</td>
<td>0.1 nsec.</td>
<td>1 micro sec.</td>
<td>1 micro sec.</td>
<td>0.1%</td>
<td>Yes</td>
<td>10^4</td>
<td></td>
</tr>
</tbody>
</table>

*SIND = Superconducting Imaging Neutron Detector.

It can be seen that this new detector has much better performance than existing detectors, especially for cold neutrons. The main advantages of the new detector is the possibility of a few microns spatial resolution. Furthermore, we note an improved QDE. For time-of-flight measurements timing much better than with scintillators may be possible. For imaging applications the excellent spatial resolution and nanosecond timing will allow neutron microscopy. Another application of the new neutron detector will be in protein crystallography.

The new class of neutron detectors is based on the measurement of temperature change in micron-size, superconducting structures. Each pixel is essentially a structure thermally independent of substrate: heating will occur whenever an alpha particle produced by stopped neutron interacts in the metal. The physics of this device was described in the other contribution in these Proceedings (also see references).

A possible implementation takes advantage of the metastability of type-I superconductors and the very small specific heat of these materials. Each pixel consists of a plurality of spherical grains dispersed into highly granulated boron or lithium hydride. Typically, 10\% of detector volume is in superconducting grains. Taking into account the relevant densities \(d(\text{Sn}) = 7.3 \text{ g/cc, } d(B) = 2.34 \text{ g/cc and } d(\text{LiH}) = 0.82 \text{ g/cc}\) the specific density of the detector is \(d_1 = 2.84 \text{ g/cc}\) and \(d_2 = 1.47 \text{ g/cc}\) for boron and lithium loaded detectors, respectively. Similarly, the fractional weights of the converter are \(X = 74.3\%\) and \(X = 44\%\).
We propose to use the reactions

\[ {^{10}}B + n \rightarrow {^7}\text{Li} + \alpha + 2.79 \text{MeV} \ (6.1\%) \]
\[ \rightarrow {^7}\text{Li} + \alpha + 2.31 \text{MeV} \ (93.9\%); \text{ and} \]
\[ {^6}\text{Li} + n \rightarrow {^3}\text{H} + \alpha + 4.786 \text{MeV}. \]

The range of alpha particles is much longer than the typical distance between grains, i.e., at least one superconducting grain is heated by the reaction products. Taking into account the detector composition, an average of 0.7 MeV and 2.4 MeV are deposited in the superconductor for reactions (a) and (b), respectively. However, at low temperature, the specific heat of metals is very small, e.g., at \( T = 1.5^\circ\text{K} \) the specific heat of a tin grain with \( R = 20 \text{ microns} \) is only 340 keV/\( ^\circ\text{K} \). Thus, a temperature increase of more than a degree is expected and will bring the temperature over the transition temperature. Change of state from superconducting to normal leads to a drastic change of magnetic properties; magnetic fields penetrate metals freely when superconducting currents disappear. The process of change of state is fast (a few nanoseconds) and with very sensitive electronics, based on Superconducting Quantum Interference Devices (SQUID’s), temperature changes as small as \( 10^{-5} ^\circ\text{K} \) are measurable.

This sensing principle can be generalized to an array of superconducting dots evaporated on both sides of very thin boron foil. With 5 micron-thick boron, practically all ultracold neutrons are absorbed; these produce MeV alpha particles which escape, to be stopped in a few microns of very high density superconductor, e.g. Ir (22.42 g/cc), Os (22.84 g/cc), Rh (20.53 g/cc) or Pb (11.35 g/cc). To increase the cross-section for neutron absorption a lithium film insulated from superconductors by oxide or LiF foil can be used. In such a configuration, QDE of 90% and spatial resolution of better than 5 microns is expected. It should be noted that a single superconducting pixel represents a magnetic dipole. The detected change of flux, due to the change of state, depends on the distance between the superconductor and the SQUID readout loop. With 3 SQUID loops one can establish the position of the irradiated pixel. Such a configuration is a superconducting position sensitive neutron detector.
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NEW HIGH SPATIAL RESOLUTION X-RAY DETECTOR

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Abstract:

We describe the development of a new class of solid state particle/radiation detectors. The detector consists of a large array, say \(10^5 \times 10^5\) of superconducting sensors and provides spatial resolution of a few microns with a reasonable number of readout channels. Subnanosecond time resolution is possible. Furthermore, order-of-magnitude improvements in maximum radiative dose are expected; a dose of over one Gigarad should not destroy the detector.

1. Introduction

One- and two-dimensional electronic position-sensitive detectors have become the preferred means of data collection in crystallography laboratories. A number of such devices have been constructed for X-ray applications, since they are capable of increasing the rates of data collection by one or two orders of magnitude compared to single counter diffractometers. Even more important is the reported\(^{(1)}\) improvement in the quality of collected data.

The use of 2-D detectors at synchrotron radiation (SR) facilities permits the technique of time-resolved X-ray diffractions which is a powerful tool for studying dynamic processes. It is possible\(^{(2)}\) to calculate the extent of changes in the structure as a function of time from the changes in the X-ray pattern. Studies which requires both the use of area detectors and synchrotron radiation are short-lived intermediates in, e.g. enzymatic reactions, and rapid time-resolved transient states\(^{(3)}\). Other examples are fiber diffraction and small angle scattering. The phenomena occurring during crystallization processes can be studied\(^{(4)}\).

The development of better detectors is essential for further progress. The main detector characteristics which have to be considered are spatial resolution, maximum count rate (overall and local), linearity, and dynamic range. Obviously, quantum detection efficiency (QDE) is very important but, at the O\(_1\) k-edge line (8 keV), all electronic detectors have reasonable QDE, typically 50-80\%. In UV and soft x-ray however, the QDE of conventional detectors is rather bad; the use of superconducting detectors will permit a factor five improvement. Furthermore, the detector stability is important. It is directly linked to that of the signal processing electronics.
In practice, we have three classes of detectors:

a) gas detectors with submillimeter resolution and large surface;
b) TV cameras (512 x 512 pixels with \( \phi \leq 5 \text{ cm} \));
c) solid-state imagers with spatial resolution as good as 25 x 25 \( \mu \text{m}^2 \) but very small surface.

It is not possible to specify the "best" detector for diverse biological studies; different experiments may demand conflicting qualities from the detector. Existing detectors cannot satisfy all the demands simultaneously; these techniques are mature and only minor improvements are expected. On the other hand, better and possibly new types of position sensitive detectors will become a must with the new UV and X-ray sources, e.g. new SR facilities. We argue that a new X-ray detector based on energy sensitive superconducting structures may become the best example of next generation focal 2-D detectors, e.g. it can feature the spatial resolution of 4096 x 4096 pixels with surface of 20 cm x 20 cm.

Next table shows a comparison of some existing 2-D position sensitive detectors with SGD proposed by ARC. For laboratory applications with conventional X-ray sources the main advantages of the new detector are improved spatial resolution. Furthermore the improved energy resolution may be important in some applications. Much higher count rates would permit obtaining the data for a single crystal within a few hours rather than a few days. For applications at synchrotron X-ray sources, the most important are orders of magnitude higher count rates and better spatial resolution.

**Comparison of diverse 2-D detectors of X-rays.**

<table>
<thead>
<tr>
<th></th>
<th>Gas(MWPC)</th>
<th>TV</th>
<th>CCD</th>
<th>SGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size: ( \phi[\text{cm}] )</td>
<td>30</td>
<td>8</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Resolution</td>
<td>512x512</td>
<td>512x512</td>
<td>1024x1024</td>
<td>4096x4096</td>
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<tr>
<td>QDE</td>
<td>85%</td>
<td>70%</td>
<td>550%</td>
<td>90%</td>
</tr>
<tr>
<td>Max. flux [ph/s]</td>
<td>( 5 \times 10^4 )</td>
<td>( 10^8 )</td>
<td>( 5 \times 10^5 )</td>
<td>( 10^9 )</td>
</tr>
<tr>
<td>Readout time</td>
<td>1-10\mu\text{sec}</td>
<td>20\text{msec}</td>
<td>10\text{msec}</td>
<td>10\text{nsec}</td>
</tr>
<tr>
<td>Homogeneity</td>
<td>1.5%</td>
<td>&gt;1%</td>
<td>&lt;1%</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

We and our collaborators believe that within a few years we can develop the following X-ray detectors.

1. **High Spatial Resolution X-ray Detector (HSRD).**
   - Energy range: 0.1-20keV
   - Energy resolution: \( \Delta E/E \approx 5\% \) at 5 keV
   - Area: 10 cm x 10 cm
   - Spatial resolution: 2048 x 2048
   - Temperature: 1.2K
   - Readout: Josephson junctions
   - Count rate: \( 10^7 \text{ photons/sec} \)
2. **Extreme Spatial Resolution X-ray Detector (ESRD).**

- Energy range: 0.1-20 keV
- Energy resolution: $dE/E \approx 5\%$ at 5 keV
- Area: 10 cm x 10 cm
- Spatial resolution: 4096 x 4096
- Temperature: 1.2 K
- Readout: Magneto-optic
- Count rate: $10^9$ photons/sec.

The suggested detection scheme is based on the measurement of temperature change in micron-size, superconducting grains. To increase the device sensitivity, metals with high Debye temperatures are used. Further increases in sensitivity are obtained by using very thin films and small pixels, both made possible by using high atomic number and very high density materials. Each pixel is essentially a structure thermally independent of substrate: heating will occur whenever a photon interacts with the metal. Calculations show that at low temperatures for an energy deposition of a few keV, the temperature change of a single pixel is larger than 0.05°K, and easily measurable.

2.0 **Superconducting Granular Detector (SGD)**

Essentially, two classes of low temperature detectors have been developed, based on crystals (diamond and silicon) and superconductors. (6, 7) Due to their extremely small specific heats, the deposition of energy as small as a few hundreds eV leads to a measurable change of temperature in micron-size structures.

The above described low temperature devices are not very convenient as imaging devices; their limitations are due to the use of resistive readout, i.e. at least two wires have to be attached to each single sensor, which limits the number of distinct pixels to a few tens. In the following we discuss a class of devices in which the readout is by magnetic means; i.e. millions of physically separated sensors are readout in parallel by only a few channels of very sensitive electronics. These devices use the fact that even very small energy deposition can change the state of a superconductor, leading to a drastic change of its electromagnetic properties. To increase the detector sensitivity, a highly granulated superconducting medium is used.

The preferred implementation takes advantage of the properties of Type-I superconductors and the very small specific heat of these materials. Each pixel consists of a thick film of a superconductor (say 3 microns of Pb). X-ray energy brings the temperature over to the critical temperature; this leads to a large change of electromagnetic properties (a magnetic field penetrates metals freely when superconducting currents disappear). The process is fast (a few nanoseconds) and temperature changes as small as a few millikelvin are measurable. This structure is a superconducting bolometer based on the Meissner effect. This sensing principle can be generalized to an array of superconducting bolometers. It should be noted that a single superconducting grain represents a magnetic dipole. The detected change of flux, due to the change of state depends on the distance between the superconductor and the readout loop. **With 3 loops one can**
establish the position of the irradiated pixel. Such a configuration is a superconducting imaging bolometer.

The detector depends on the change of state from superconducting to normal in small grains of a few microns diameter (see ref. 8). The deposition of less than 1 keV of energy flips the state of the grains. This is easily detectable with ultrasensitive electronics based on either GaAs preamps or SQUID(9). This detector has been tested and shown to be sensitive to charged particles, photons and neutrons. The basic idea of the SGD is quite simple. One prepares a suspension of spherical grains of a Type I superconducting material, which will constitute the detecting medium. Since there are over 20 elements which are Type I superconductors, it is possible to obtain a wide range of material properties. Instead of grains, one can use other micron-size superconducting structures, e.g., arrays of evaporated squares or "dots."

To enable the detection of energy deposited in the grains, one first brings them into the superconducting state by immersion in a cryostat; if a large enough magnetic field \( H \) is applied to such grains, they will revert to the normal state if:

\[
H \geq H_T = \frac{2}{3}H_0(1 - T^2/T_C^2)
\]

where \( T \) is the temperature, \( T_C \) is the transition temperature (at zero field) at which the superconducting state is reached, and \( H_0 \) is the "critical field" at \( T=0 \). Equation (1) is a thermodynamic relation, analogous to the \( P(T) \) equation for the vapor pressure of a fluid. And, in a manner analogous to that for supersaturation of fluid, it is possible for the behavior of pure grains to show superheating (see Fig. 1). Depending on application, one can use either superheated superconducting grains or grains which return to the superconducting state after heat escapes.

To take advantage of the above principles in radiation detectors, one establishes an ambient magnetic field, \( H \), slightly smaller than \( H_{sh}(T) \), where \( T \) is the cryostat temperature and \( H_{sh}(T) \) is a superheating field. If radiation is absorbed by the grain, it will eventually be converted to heat, which in turn will increase the temperature of the grain by the amount \( dT = (3dE)/(4\pi R^3 C_V(T)) \) where \( dE \) is the deposited energy, \( R \) is the grain radius, and \( C_V(T) \) is the specific heat of the metal (typically about 1 keV/\( \mu m^3*K \) at 1.2°K). If \( H > H_{sh}(T + dT) \), the grain will revert to the normal state. It will remain in this state, even after cooling back to \( T \), unless the magnetic field is reduced to the supercooling point.

The change of state of the grain can be monitored when the grains are placed within a pickup coil connected to a very low noise voltage sensor. When the grain changes to the normal state (or "flips"), the magnetic field which had been excluded by the Meissner effect now penetrates freely. Effectively, a magnetic dipole has been removed from the interior of the colloid, and this changes the external field which threads the pickup coil. The change of magnetic flux leads to microvolt voltage pulse with nanosecond duration.

Above, we described an energy- and position-sensitive detector based on superconducting structures. Intrinsically, the detector is very fast;
the change of state requires less than 1 nanosecond. The appropriate electronics (Josephson junction flip-flops, used in supercomputers) are ultrafast and permit tens of picoseconds timing. Optionally, one can use very fast GaAs-Fet preamplifiers operating in liquid helium, which would give subnanosecond timing. While these super-fast readout electronics are under development, in most experiments somewhat slower and less-sensitive room temperature preamps based on silicon J-Fet's were used and timing of better than 10 nanoseconds has been achieved (D. Perret-Gallix, IAPP/CERN private communication (see Fig. 2). Much better performance is expected using GaAs Fet's operating at liquid helium. We recently developed two such GaAs preamps, one with rise time of 200 psec and the other a few nanoseconds (see Fig. 3). The preliminary tests show that the noise level when operated in liquid helium compares favorably with the noise of Si-Fet's. We are currently performing irradiation tests of SGD using the said preamps.

In one implementation, the detector structure is similar to a "solid-state" multiwire proportional chamber wherein two planes of readout loops permit two-dimensional localization. The signal sharing between closely placed loops permit the use of "center-of-gravity" methods to improve the spatial resolution; we expect that the spatial resolution will be ca. 0.1 x S where the spacing, S, of the readout wires can be as small as 100 μm. Such readout will however require 2N electronic channels. The localization method using superconducting delay lines will require a much smaller number of electronic readout channels. Actually, with four fast electronics channels, the 50 micron localization seems possible for a 10 cm x 10 cm detector. Two very fast preamps are sensing pulses induced/propagating in a superconducting pickup coil shaped into "meander delay line." The difference of time of pulse arrival is digitized by TDC's and stored in a computer or on-line processed by dedicated analog circuits to provide the localization in one direction. This method was used in the 1970's in many applications of gas detectors, especially the so-called drift chambers. The recent progress in TDC's 10 psec TDC's are being offered commercially; (G. Blanar, LeCroy, private information) permits the use of delay time method for count-rates of 10^9 ph/sec. On the other hand, the superconducting delay lines are dissipationless and permits the nanoseconds delays for subnanosecond rise-time pulses from SCD.

Due to the quantization and conservation of magnetic flux within a closed loop to a superconductor, a supercurrent is induced in the superconducting circuit. The supercurrent sets up a small magnetic field at the Superconducting Quantum Interference Device (SQUID) which is detected by well-known procedures. It should be pointed out that a SQUID can detect and localize a change of state of any single grain in the collection of trillions of grains. We mentioned above that the change of state of a grain is equivalent to the disappearance of a magnetic dipole. This leads to propagation of a spherical wave; the wave amplitude diminishes inversely proportional to the distance from the grain. Thus by using three identical pick up loops, each connected to an ultrasensitive magnetic sensor (DC-SQUID), one can localize the grain which flips. For redundancy, it's better to use four loops placed around the detector. The spatial resolution of such a system depends on the signal-to-noise ratio; with S/N ≥ 100 we expect a spatial resolution of ca. 100 μm for a 1 cm^2 detector. This S/N ratio has been achieved using RF-SQUID's (see Fig. 4).
Using the DC-SQUID's the S/N \( \geq 10^4 \) is expected. Furthermore, the possibility of recognizing single grain flips vs. multiple grain flips was confirmed experimentally (see Fig. 5). This property is important in some applications, e.g. in x-ray holography. DC-SQUID electronics featuring 11 SQUID's are commercially available, and systems featuring up to 100 DC-SQUID's are under development. Such DC-SQUID based systems are limited to \( 10^6 \) ph/second. However, much faster Josephson Junction flip-flops can be used. It is our opinion that the development of cryoelectronics appropriate for SGD readout is the important task of further SGD development, especially in applications requiring very high spatial resolution or recognition of many pixels (512x512 pixel arrays or larger).

The functioning of SGD as high QDE detector of X-rays is already proven. Thus the main challenge of the proposed program is to study the diverse localization methods. A small size detector will be tested in 1989 and the above described localization techniques will be implemented and critically compared.

3.0 The Advantages of SGD

Synchrotron Radiation Sources revolutionized many subfields of Material Sciences, e.g. biological applications. In section 1.3 we pointed out that the proposed detector compares favorably with other detectors when one considers: size, spatial and energy resolution. In this section we would like to point out two further arguments why SGD are particularly well suited for SR application.

The ideal focal plane detector for X-ray microimaging would have the following properties:

1) radiation-hard to a few hundreds of Mrad;
2) spatial resolution of a few microns;
3) sensitivity to selected wavelength;
4) fast, nanosecond readout using off-chip radiation-hard electronics.

The SGD satisfies all of these criteria.

Synchrotron Radiation is a predigious source of UV/X-ray photons; even after scattering the count rates of up to \( 10^9 \) photons/sec can be required. Unfortunately, classical X-ray detectors (gas chambers and CCD's) are rather slow, say a few \( \mu \)sec devices. The SGD's are subnanosecond devices; actually using very small grains and Josephson Junction flip-flops the 100 psec timing seems possible.

Other problems with existing detectors is the radiation damage. It has been investigated for three different types of imaging devices: silicon detectors including CCD's; gas detectors, and scintillators. A dose of 1 Mrad presents fatal problems for all three of these high spatial resolution detectors; SGD is radiation hard to over 1 Gigarad.

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One of the most important results of recent ARC efforts was experimental proof that superconducting detectors function properly after irradiation by a few GigaRad. Two series of experiments were performed:(7)

- in collaboration with the group of Prof. R. Huebener, Tubingen University, we analyzed the radiation-hardness of superconducting tunnel junctions (STJ);
- in collaboration with the group of Prof. B. Turrell, UBC we studied the radiation-hardness of a superconducting granular detector (SCD).

These results are highly significant because we used two different sources of radiation: medium energy electrons with $E_e=25$ keV, and high energy photons from a cobalt source ($E_y>1.5$ MeV).

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Fig. 1: Phase diagram for type-I superconductor; the * represents a typical grain in SGD. Note the presence of superheating and supercooling.

vertical scale = 2 mV
horizontal scale = 200 nsec

Fig. 2: Signal due to a change of state of a single grain in irradiated collection of billions of grains.
GaAs Cooled Preamp (4.2 K)

Fig. 3a: The schematics of 1 helium cooled GaAs preamps developed by ARC.

b: The performance of one of the GaAs preamps (gain = 4; rise time ≤5 nsec; noise ≤0.1 μV)

RADIATION INDUCED FLIP

Fig. 4: The signal/noise ratio for a single grain change in a collection of millions of grains placed in a pickup coil. For grains with $R = 8 \, \mu m$, cooled to $T = 3.1 \, ^{\circ}K$ the energy deposition of 10-20 keV gives QDE = 80%. (used superconducting electronics).

STEP SIZE DISTRIBUTION for $B = 200 \, GAUSS$

Fig. 5: Pulse height distribution in a focal plane detector (30x20 grain arrays) with spatial resolution of about 30 μm. Note the possibility of single vs. multiple grain flips.
A plasma focus operating in nitrogen is developed as an x-ray source for the laboratory type x-ray microscope. The radiation has to be emitted into the "water window" (2.4 nm - 4.4 nm) with a reciprocal relative bandwidth (RRB) of $\lambda/\Delta\lambda > 200$. The nearly coinciding Lyman-\(\alpha\) line of nitrogen VII $\lambda = 2.48$ nm and the second resonance line of nitrogen VI at $\lambda = 2.49$ nm are chosen. For the use in the microscope the end on diameter of the source has to be about 200 $\mu$m with a spatial jitter below 100 $\mu$m. A first version of the source including beamline and condenser stage has been tested. Spatial, temporal and spectral properties of the source have been investigated. With a pinhole-grating spectrograph spatial and spectral information are obtained simultaneously. First experiments with Fresnel condenser zone plates (CZP) as a tool for plasma soft X-ray emission diagnostics are presented. Experimental results show that the gas in the beamline can be used as a filter for undesired radiation. With improved efficiency of the zone plates and x-ray detectors the expected plasma emission of about 1 J into the full solid angle from a source with a diameter of 200 $\mu$m should be sufficient to achieve an exposure with one pulse. 

INTRODUCTION

An imaging x-ray microscope is designed in order to operate with a plasma focus source and matched zone plate optics. The source is demagnified by a CZP onto the object placed on a pinhole, which acts as a broadband filter due to the chromatic aberration of the CZP. The image is magnified by a micro zone plate (MZP) and recorded either by a CCD-camera or a photographic emulsion. As the samples are in wet surroundings, the wavelength region between the absorption K-edge of oxygen ($\lambda = 2.33$ nm) and the K-edge of carbon ($\lambda = 4.37$ nm), called water window, is best suited for contrast x-ray microscopy. In order to achieve the resolution of the MZP, narrow band radiation (RRB > 200) is needed. The linewidth of plasma emission lines can be estimated by calculating the values for Doppler effect and interatomic Stark effect, being the dominating broadening mechanisms. For achievable ion densities $n < 10^{20}$ cm$^{-3}$ and ion temperatures $kT < 2$ keV the RRB of a plasma emission line RRB > 1000 is high enough to fulfill the conditions for sufficient imaging properties, so that plasma focus sources can be used without additional narrowband filtering. As plasma sources are pulsed, nanosecond exposure time - which is advantageous to avoid blurring by particle
movement and radiation damage is possible.

In order to develop a laboratory x-ray microscope, four equally important subunits must be integrated: source, beamline, optical elements, and x-ray detector. A cooperating group at the Göttingen University is developing the zone plate optics and the x-ray detector. We are developing the source and the beamline of the microscope. The 1s-2p transition of N VII at $\lambda = 2.48$ nm and the closely neighboured 1s$^2$-1s3p of N VI at $\lambda = 2.49$ nm are chosen. Due to their small separation $\Delta \lambda = 0.0117$ nm ($\lambda/\Delta \lambda > 200$) these lines can be used simultaneously. Our aim is to maximize the mean spectral brightness (ISB) during an emission time as short as possible of these particular lines.

$$\text{ISB} = \int_{t_0}^{t_{\text{max}}} B_\lambda \, dt$$

$B_\lambda$ : spectral brightness (1)

With improved efficiency of the zone plates and detectors $0.1J$ x-rays per steradian emitted from a diameter of 200 $\mu$m are needed to achieve an exposure with a resolution of $\Delta \tau < 50$ nm with one pulse of $< 10$ ns duration.

THE X-RAY SOURCE

Figure 1: Plasma focus source NIXE for soft x-ray microscopy

Plasma focus devices have been mainly investigated in fusion research. The voltage of a low inductance capacitor bank is applied by means of a fast switch - usually a spark gap - to two coaxial electrodes located in several millibars of stationary nitrogen filling. A surface discharge creates a plasma
sheath along an insulator which is driven to the end of the electrodes as a shock wave due to the fast rising current. At the end of the electrodes the discharge current reaches its maximum and part of the plasma is accelerated towards the z-axis. An appreciable amount of the initially stored energy is transformed into kinetic and thermal energy of the plasma and magnetic energy in the surrounding of the plasma. Reaching the z-axis the kinetic energy is rapidly converted to thermal energy of the plasma. Thermalization and additional compression by the magnetic field leads to an increasing rate of inelastic collisions between electrons and ions and causes further ionization and generation of soft x-rays. The dynamics of the shock wave and the pinch are strongly influenced by the pressure of the neutral gas and the plasma current. Only a homogeneous and symmetrical plasma sheath leads to the necessary reproducibility of plasma focus discharges.

In the x-ray region recombination continua and line radiation are the dominating emission phenomena from high-z plasma sources, whereas Bremsstrahlung is negligible. To achieve maximum spectral brightness, the plasma emission has to be tailored for maximum line emission. To get a small source a matched pinch current has to be chosen. The optimum current for microscopy is in the range of a few hundred kA. Our NIXE (Nitrogen X-Ray Emitter) source (Fig. 1) for x-ray microscopy is designed to have a pinch current \(I = 100-300\) kA achieved with a capacitor bank of \(20\) \(\mu\)F, a discharge voltage \(U = 10-20\) kV and a total inductance of \(L < 30\) nH. A special design criterion is, that the x-ray source is located only \(80\) cm above floor level. The total height will be below \(3\) m at a magnification of 100 of the MZP.

The laboratory source has already been tested. First results have been obtained. Special attention has to be payed to the design of the beamline. The beamline is designed to act as spectral filter for interfering shortwave radiation and to protect the CZP from discharge debris. Only little \(N\) should be in the beamline as the absorption of \(N\) at \(2.48\) nm is high. With differential pumping and counterstreaming of oxygen or hydrogen the partial pressure is kept below \(5\times10^{-2}\) mbar as measured with a mass spectrometer. The total absorbing pressure path product is below \(6\) mbar\(\cdot\)cm having an absorption of below \(10\%\). It is planned to have oxygen as filter with about \(100 - 400\) mbar\(\cdot\)cm absorbing about 17\% - 50\% of the line but 92\% - 99.99\% below \(2.33\) nm wavelength.

**SOURCE DIAGNOSTICS**

To develop the source for x-ray microscopy the following parameters of the soft x-ray emission have to be measured:
- Emission duration
- Emission spectrum
- Total energy emitted into the lines
- Spatial dimensions of the source
- Spatial jitter of the source

Measurement of ISB requires simultaneous, temporal, spectral, and spatial resolution. This is partly possible with standard diagnostics:

- Microchannel plate detector
- Pinhole camera
- VUV spectrograph

Additional new methods for pinch plasma diagnostics are developed:

- Pinhole-grating spectrograph
- Monochromatic imaging of x-ray source by condenser zone plates⁹.

The spatial dimension and the jitter of the x-ray emitting region has been measured by means of a pinhole camera (diameter = 12.5 μm). As a 5 μm beryllium foil is used as a window, the system is much more sensitive to shorter wavelength continuum radiation than for the emission line at 2.48 nm. The emission region is a cylindrical column with a diameter of d = 400 μm and a length of up to 10 mm. The spatial jitter is below 1 mm.

The duration of the x-ray pulse has been measured with a microchannel plate detector (temporal resolution < 500 ps) behind 10 μm aluminum. The emission lasts less than 10 ns with a risetime below 1 nanosecond⁹.

Fig. 2: Pinhole-grating spectra of 2 mbar N₂ source

Fig. 3: Emitted energy vs. N₂ pressure.
Using a pinhole-grating spectrograph spectral and spatial resolved measurements of the emission has been done. A free-standing gold transmission grating incorporated into a pinhole gives spatial information in one direction and spectral information in the other direction. Maximum spectral resolution is below 0.05 nm for 2.48 nm wavelength. In our experiments reduced resolution of 0.2 nm has been chosen in order to increase the sensitivity. The spatial resolution is better than 100 μm. Fig. 2 shows typical results. To the left and the right of the zeroth order the spectral resolved images of the source is observed. Recombination continua are observed down to wavelengths below 1 nm. The relevant lines for microscopy at about 2.48 nm and the 1s2-1s2p line of N VI at 2.8 nm are resolved from the continuum.

Absolute values of the emitted energy have been estimated taking into account the grating efficiency (η = 0.08 ± 0.02 for wavelengths greater than 1 nm) and the geometry. Fig. 3 shows the total emitted energy into radial directions emitted from a region with a diameter of 200 μm and a length of 1 cm versus gas pressure into the relevant lines and into the total recombination continuum. The values are averaged over 5 discharges each. A maximum line intensity is observed with 2 mbar gas pressure. A sharp decline above 4 mbar is due to the inhomogeneities in the ignition phase of the discharge discussed above.

The emission has been recorded with higher spectral resolution of λ/Δλ = 500 using a 2 m grazing incidence VUV spectrograph. In Fig. 4 a typical result is shown. The response factor of the photoplate (Ilford Q2), the relative spectral efficiency of the grating, and the transmission of the gas have been taken into account. The background below the relevant lines at about 2.48 nm consists of a remarkable portion of continuum radiation diffracted into second order. The relevant lines are separated. The intensity ratio between the N VI and N VII lines is less than 0.4. The recombination continuum of N VI is located below 2.3 nm, the recombination continuum of N VII below 1.89 nm.

![Emission spectrum of the source](image1.png)
![Calculated spectrum at object](image2.png)
The undesired recombination continuum can easily be suppressed by filling the beamline of the microscope with oxygen (K-edge at $\lambda = 2.33$ nm). Fig. 4b shows the calculated influence of oxygen in the beamline on the spectrum. Nearly monochromatic radiation can be provided by the source. An additional advantage of an oxygen counterstream in the beamline is the protection of the CZP against eroded material from the electrodes.

The source has been imaged at 2.48 nm using a CZP (KZP 4; diameter 2.5 mm; focal length $f=1$ m; width of outermost zone $\delta r = 0.99$ $\mu$m). The CZP necessary for the laboratory microscope (KZP 7 $f=0.20$ cm $\lambda=2.48$ nm) is not yet available. Fig. 5 shows the optical arrangement. Imaging the source by means of CZP proved to be best suited for optimizing the plasma focus and the beamline due to the requirements of the microscope. The radiation emitted from the plasma is diffracted by the CZP with an efficiency $\varepsilon = 4\%$ into first order. After transmission through a foil (0.4 $\mu$m kapton; 0.2 $\mu$m vanadium) as filter for visible light, the image is recorded on the photo plate. The distance $d$ between object plane and image plane is $d = 4$ m. The achievable spatial resolution of 1.2 $\mu$m is given by the CZP.

Fig. 5: Optical set-up for zone plate imaging of plasma source

Due to the strong dependence of the focal length on $\lambda$, the image is sharply defined only for a particular wavelength. The diameter of the blur spot for the neighbouring N VII ls-3p line at 2.0910 nm is, e.g., 849 $\mu$m. After completion of the beamline now under construction this arrangement will be used for measuring absolute values of the ISB.
The spectral characteristic of the source is influenced by the initial pressure of the filling gas. Fig. 6a shows a picture taken at 3 mbar. Fig. 7 shows the profiles of the images, already corrected with respect to background. The half-width of the profile is reduced by changing the gas pressure from 1 mbar to 3 mbar and the peak value is raised by a factor of 1.26, so that the area within the margin of error is approximately the same.

The source diameter can be further reduced by a factor of 0.6 by adding 10% xenon to the nitrogen gas, not altering the ISB. The energy loss due to the ionization of the xenon and the accompanying impurity radiation might be advantageous. This experiment shows that the 1s-3p line of N VI makes only a small contribution to the image. The diameter of the blur spot of that line is expected to be 0.8 mm, whereas 0.3 mm is observed.

For x-ray microscopy a plasma focus is developed for the emission of line radiation of nitrogen VII at 2.48 nm wavelength. The time integrated spectral brightness is of special importance. The part of the microscope with the CZP up to the object plane is the best suited diagnostic technique to optimize the line emission. Spectral measurements with VUV and pinhole-grating spectrographs reveal the influence of the operating conditions upon the brightness. The continuum radiation from the plasma is suppressed by spectral filtering using...
oxygen in the beamline. The emitted energy in the relevant lines into 4\pi solid angle is about 200 mJ. The diameter of the source is below 0.3 mm.

ACKNOWLEDGEMENTS

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ABSTRACT

We describe the development of a new biologically non-invasive ultrasensitive light microscopy, based on combining the energy transfer "spectral ruler" method with the micro-movement technology employed in STM. We use near-field scanning optical microscopy, with micropipettes containing crystals of energy packaging donor molecules in the tips that can have apertures below 5 nm. The excitation of these tips extends near field microscopy well beyond the 50 nm limit. The theoretical resolution limit for this spectrally-sensitive light microscopy is well below 1 nm.

INTRODUCTION

The Near Field Scanning Optical Microscope (NSOM), developed by Lewis and co-workers, has enabled researchers to optically examine a variety of specimens without being limited in resolution to one half a wavelength of light [1-3]. All NSOM imaging is based on the fact that as an electromagnetic wave emerges from an aperture it is at first highly collimated to the aperture dimension. It is only after the wave has propagated a finite distance from the aperture that the diffraction which limits classical optical imaging takes effect. In the near field region a beam of light exists that is largely independent of the wavelength and determined solely by the size and shape of the aperture [4].

In a typical NSOM system a sub micron aperture is fabricated and brought to within several hundred angstroms of the surface to be imaged. The aperture is then illuminated and the spot of light that emerges is scanned across the surface generating a picture one point at a time. A feedback mechanism is incorporated to maintain a fairly constant distance between the aperture and sample, as the size of the near field spot is strongly dependent on the distance from the aperture. The system can be operated in a transmission or scattering mode, or alternatively, the sample can be illuminated and the near field collimation effect can be used to collect the light in the aperture obtaining the same resolution.

The most successful results to date have been obtained using a metal coated glass micropipette as an aperture [2]. Pipettes can readily be produced with inner diameters at the tip of several hundred angstroms [3]. Their tapered profile is almost ideal for probing recessed regions of non-planar surfaces. The metallic coating serves both to confine the light to the dimension of the pipette and as a conducting probe where tunneling feedback is employed. The details of pipette fabrication have been described elsewhere [3].

The current resolution limitation of the NSOM technique derives from the less than ideal characteristics of the aperture. There are no propagating electromagnetic modes in a subwavelength cylindrical metallic waveguide such as a
metal coated pipette. The least attenuated mode for a round aperture has been found to be the TE_{11} mode for which the energy decays at a rate of:

\[ E = E_0 \exp(-2 \times 1.81 \frac{L}{a}) \]

where \( L \) is the length of the aperture and \( a \) is the radius [4]. With a sufficiently rapid tapering of the pipette, however, this evanescent region can be kept short enough to obtain a fairly large throughput of light. What ultimately limits the resolution is the finite conductivity of the metallic coating around the pipette. The electromagnetic wave penetrates the coating and decays within it at a finite rate as well, given by:

\[ E = E_0 \exp\left(-\frac{d}{\delta}\right) \]

where \( d \) is the depth of the penetration and \( \delta \) is the extinction length of the metal. When the attenuation due to the waveguide effect exceeds the attenuation in the metal the contrast between the aperture and surrounding medium becomes insufficient for superresolution applications. The metal with the largest opacity in the visible region is aluminum for which \( \delta = 65 \) angstroms when the wavelength (\( \lambda \)) is 5000 Å. This yields a minimum usable aperture of about 500 angstroms. In practice, multilayered coatings are necessary to obtain good adhesion and a practical aperture should be expected to perform even worse.

The solution to this problem is to use the energy packaging capabilities of certain materials to circumvent the boundary problem of the edge of the aperture. According to fundamental understanding of energy propagation in materials, excitation can be confined to molecular and atomic dimensions under appropriate conditions [5]. By growing a suitable crystal within the submicron confines of a pipette, energy can be guided directly to the aperture at the tip instead of being allowed to propagate freely in the form of an electromagnetic wave. Such a material can be excited through an electrical or radiative process to produce an abundance of excitons that allows light to be effectively propagated through the bottleneck created by the subwavelength dimensions of the tip near the aperture. The excitons can be excited directly at the tip or they can be generated within the bulk of the material and allowed to diffuse to the tip via an excitonic (electric dipole, Förster) transfer [5,6]. In either case, in a suitable material these excitons will then undergo a radiative decay producing a tiny source of light at the very tip of the pipette.

The excitonic throughput is basically independent of the wavelength and is a linear function of the cross-section of the aperture.

**EXPERIMENTAL PROCEDURE**

To demonstrate the feasibility and usefulness of the method we chose to work with crystals of molecular anthracene. While this is not necessarily the best material available for this purpose, it is easy to work with and its electrical and radiative properties have been extensively characterized [5].

The crystal of anthracene was grown inside the very tip of a pipette from a benzene solution. The pipette was held vertically, pointing downwards, while a tiny drop of solution was injected into the top. The strong capillary action immediately pulled the liquid down into the tip. The benzene was then allowed to evaporate out
from the top, with the anthracene crystal precipitating inside the tip. By varying the concentration of the solution the size of the deposited crystal could be accurately controlled.

The source of excitation for our experiments was the 3638 line of an argon ion laser. Anthracene exhibits a very strong fluorescence in the blue with a quantum efficiency approaching unity when illuminated in the near U.V. The crystal can be illuminated either by directing light through the pipette as in the standard aperturing method, or alternatively by having an external beam incident on the crystal at the tip of the pipette. With the second method large amounts of energy can be brought to bear directly on the spot where the illumination is desired, with the upper limit being imposed only by the onset of photochemical bleaching. A fairly large (~1 micron) crystal was grown extending past the tip of the pipette and illuminated head on with several milliwatts at 3638. The spot of light produced was clearly visible to the naked eye under room lighting and is shown here magnified 100x.

To test the imaging capability of the light source a small crystal was grown inside a half micron pipette. In order to obtain high resolution images it is necessary for the anthracene to be confined to the inside of the pipette as much as possible so that the emerging beam remains collimated. Any excess material could readily be removed from the outer surface with a small drop of benzene. The test sample was a 2 micron aluminum grating. The imaging was done in the transmission mode with the excitatory beam being directed through the pipette. A filter was placed behind the grating to prevent any stray U.V. light from reaching the photomultiplier, confining the signal to the crystal fluorescence. The lifetime of the singlet state in anthracene, responsible for most of the luminescence, is sufficiently short (about ten nanoseconds) to allow the beam to be chopped and a lock-in detection scheme to be employed.

RESULTS AND DISCUSSION

The result of a single line scan 9 microns long and a complete two dimensional scan is shown in figure 4. The image is unprocessed and the resolution could be further improved by deconvoluting the image with the well defined shape of the pipette aperture.

For the smallest pipette experiment calibrated so far (ID 60 nm), the improvement in light transmission via the excitonic process is at least 150%. Extrapolating to smaller aperture dimensions, the improvement at 6 nm should be about a factor of $10^{13}$ and the throughput about $10^4$ photons/sec. Nanometer scanning optical microscopy has thus become a reality.

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Fig. 1. Micropipette exciton light sources excited with 5 mW of 363.8 nm of ultraviolet light form an argon ion laser. Tip only shown, magnification about 1000, ID = 150 nm.

Fig. 2. Same as Fig. 1, ID = 100 nm.

Fig. 3. Same as Fig. 1, ID = 60 nm.

Anthracene tip. Magnification 1000.
Fig 4. Scan of aluminum grating with dust particle (20-min scan, 20-Å steps, magnification about 10,000).
Microscopy with X-ray Lasers

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This extended abstract will describe the progress to date in applying the soft x-ray laser at PPPL to imaging biological specimens.

X-ray lasers offer the exciting prospect of enabling the study of dynamic processes in living cells. This arises naturally from the nanosecond duration of the soft x-ray laser pulse. One would sequentially take many flash images of living cells and later order them to form the sequence of action. The individual cells would not survive the exposure but radiation damage to structures inside the cell would not change their shape during the nanosecond exposure times. There are many processes occurring on millisecond time scales that would be ideal subjects for a flash image using a pulsed x-ray laser. Of course there is a high intensity limit beyond which the cell becomes a laser produced plasma and all structural information is lost, but this is only a significant problem at resolutions approaching the molecular level.

Work to date has consisted of contact microscopy using both the 18.2 nm laser directly and using a laser produced plasma source to obtain x-rays that fall within the water window 1.

In addition we are at an advanced stage of constructing an imaging microscope which we will start using in the fall.

The primary motivation for developing an x-ray microscope is to obtain images of hydrated specimens at an order of magnitude higher resolution than is possible with the conventional visible light microscope, without subjecting the specimen to artifact inducing preparation required by electron microscopy 2,3. This would be useful both in research, and in medical diagnosis. In research,

Figure 1. A schematic of the experimental setup of laser and contact x-ray microscope chamber (COXRALM) used to obtain the image in Fig.2.

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In clinical medicine the interest is in diagnosis of cancers. At present this is done by observing sections of tissue in a light microscope and comparing them with an histological atlas of known healthy and pathological cells. The shape of the cell, particularly the shape of the nucleus is important, not only in determining whether or not the section is malignant, but also the state of malignancy. Higher resolution imaging, especially of the nucleus of cells, will not only increase the accuracy of the diagnosis, but should also allow earlier detection of cancers.

![Image of HeLa cancer cell](image)

Fig. 2. An 18.2 nm contact x-ray image of a HeLa cancer cell. The gold coated resist image has been viewed with a scanning electron microscope.

X-ray lasers are not necessarily limited to large facilities. The one at Princeton, described in a paper by C.H. Skinner in these proceedings and in reference 5, fits inside a normal laboratory sized room and has a cost and scale similar to a CAT scan machine. Thus one could imagine in 10 years time or so a diagnostic facility based on x-ray laser microscopy at major hospitals.

A basis requirement for x-ray imaging is contrast between different elements. At 18.2 nm, the wavelength of the soft x-ray laser at Princeton, the ratio of the absorption cross section between carbon and oxygen is 3/1. Shorter wavelengths offer increased penetration and resolution. A second requirement is on the laser intensity. For high resolution contact microscopy one typically exposes a photo resist which is fairly insensitive requiring 100 mJ cm\(^{-2}\) for exposure. This flux level has been demonstrated x-ray lasers.

![Diagram of x-ray exposure process](image)

Figure 3. Contact microscopy. The image is formed by exposing an x-ray sensitive resist through a biological specimen. The specimen is then removed, and the latent image developed in the resist as a relief surface. This surface is then viewed at high magnification in an electron microscope.
Contact microscopy can be done using the 18.2 nm laser as a source, and the experimental arrangement for doing this is shown in figure 3.

An example of a contact micrograph produced using the x-ray laser is shown in figure 2. This is an electron micrograph of the relief pattern developed in the photoresist.

The specimen is a 100 nm thick section of a HeLa cell, in which the embedding medium (DGD) has been removed, and the specimen is supported on a 20 nm thick carbon film the x-ray during exposure. The resist is PBS which is developed in an MEK/IPA solution after exposure.

The cell nucleus, which measures about 10 microns across shows up clearly, and internal structure in the nucleus is visible down to about 100 nm, the limit being set by diffraction due to the specimen not being in intimate contact with the resist.

Better resolution should be possible using an imaging system. Figure 4 shows the schematic design of an imaging microscope intended for use with the 18.2 nm laser. The condenser is a multilayer coated Schwarzchild objective, chosen because this offers the maximum flux throughput 6. The imaging is down with a zone plate. The resolution of the system depends on the finest line width of the zone plate. Figure 4 shows the schematic design of an imaging microscope intended for use with the 18.2 nm laser. The condenser is a multilayer coated Schwarzchild objective, chosen because this offers the maximum flux throughput 6. The imaging is down with a zone plate. The resolution of the system depends on the finest line width of the zone plate. The specimen under study is placed at focus of toroid. Specimen under study is placed at focus of toroid.

Imaging zone plates have proven resolution of 50 nm and it has been possible to draw carbon zone plate patterns, which should work at the 18.2 nm wavelength with smallest line widths of 20 nm giving a theoretical resolution of 26 nm 7,8. A microscope based on this design is at an advanced stage of construction and will be used for experiments in the fall.
One current limitation of x-ray lasers is that the operating range falls short of the water window but intensive work is being done in institutions around the world to remove this limitation. In the mean time we are using a non-lasing (spontaneous emission) laser produced plasma source with a strong emission band at 4 nm in order to get some experience in this wavelength regime and compare the properties of this source to the soft x-ray laser.

The experimental set-up is shown in Fig. 5. A 3 nsec duration 20 - 30 J laser pulse was focussed onto a lead target creating a laser produced plasma with strong emission in the region around 4 nm.

![Fig. 6 Spectral emission of the laser produced plasma](image)

This source has been used for contact microscopy of biological specimens. The procedure used for contact microscopy is illustrated in figure 3.

The ultimate goal of the project is to obtain single shot stereo images of hydrated biological material in the water window region of the soft x-ray spectrum. This is some way off, and requires the development of shorter wavelength x-ray lasers. However, even at the 18.2 nm wavelength of the existing laser there is good contrast between protein and water and also between protein and DNA. The only drawback is that the total specimen thickness has to be very thin - less than 1 micron. A novel specimen chamber will allow us to do useful work at this wavelength and to develop the necessary technology. This technology will also be applicable to shorter wavelengths, so that when the water window soft x-ray lasers do become available we will be able to make immediate use of them.

CONCLUSIONS

Single shot soft x-ray contact microscopy has successfully been demonstrated using the 18.2 nm laser at PPPL. An imaging version is in the process of construction which will incorporate a multilayer reflecting condenser and an imaging micro zone plate. Such a system should reach resolutions of about 28 nm.

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b. Also at Mechanical and Aerospace Engineering Dept., Princeton University.

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Short Wavelength FELs as High Brilliance Sources

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We present here the concept of using a high gain FEL amplifiers with electron beams accelerated in high gradient structures powered by relativistic klystrons. Other authors\(^1\) have also considered x-ray FELs; the unique aspect of this paper is the use of high gradient acceleration.

The wavelength, power, and pulse width requirements stem from the objective of viewing biological structures in an aqueous environment with tomographic or holographic microscopy having resolution of several tens of nanometers over a field of view of about ten microns. London, et al.\(^2\) have shown that the greatest ratio of signal to dose occurs at a wavelength of about 44Å, at the carbon K-edge and that, at this wavelength, scattering from a protein sphere of 15nm diameter has a cross section of about \(10^{-14}\text{cm}^2\). For adequate signal strength, about 1000 photons should be scattered per resolution element. These numbers imply that the incident x-ray fluence should be about \(10^{18}\text{photons/cm}^2\), about 45J/cm\(^2\). The energy absorption resulting from this fluence is so great that significant blurring would occur if the exposure were any longer than about 30ps. With the FEL architecture under consideration here, the natural pulse length is 1-10ps, and so the required FEL peak power would be about 5-50MW.

One FEL architecture which can satisfy these requirements consists of a single pass, high gain wiggler amplifier with a high peak current electron beam accelerated in an RF high gradient structure which is powered by a high frequency relativistic klystron and which has a high emittance injector. The FEL wiggler which amplifies exponentially from spontaneous emission would saturate as the signal power nears several tenths of a percent of the electron beam peak power and so would not require tapering of the magnetic field near the end of the wiggler. This growth from noise is

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illustrated by the GINGER calculation shown in figure 1. The wiggle motion is confined to one plane, and the x-rays are consequently linearly polarized. The wiggler pole pieces are shaped to provide continuous focusing for the electron beam; this technique has been successfully demonstrated on the 25m PALADIN wiggler. For high gain, the electron beam energy is about 1-2GeV and the wiggler period is 2-4cm; the magnetic field is then great enough, 7-15kG, to equal or exceed the "Halbach limit" for hybrid wigglers with the gap between opposing pole pieces $\geq \lambda_w/4$, great enough to permit high pumping speed and to avoid the excitation of wakefield or resistive wall instabilities. These fields may imply the use of cryogenic, rare earth pole pieces or superconducting windings. For reasonable length wigglers, 10-25m, random field errors should be less than 0.1%,rms (the present state of art on the PALADIN wiggler is 0.14%, rms), and the beam should be steered to follow axis to within about 50um, which is about 1% of the beam tube radius (differential detectors can presently determine the position of electron beams within about 50um in beam tubes of about 2cm radius). These performance predictions for this FEL amplifier are based on numerical simulations conducted with FRED-3D, which tracks the evolution of the several radiation spatial modes. Even greater gain can be realized with the wiggler magnetic field tuned to be in resonance for 132Å x-rays and to amplify both the fundamental and the third harmonic, as indicated in the NUTMEG calculations depicted in figure 2.

The key features of the electron beam are its energy, 1-2GeV, its normalized emittance, 1-100mm-mrad, and its current, 3-10kA. The use of high frequency RF permits there to be a very great accelerating gradient, 100-200MeV, and, consequently, a relatively short RF accelerator, about 10m. Relativistic klystron driven by low energy induction accelerators are efficient (about 30%) sources of the RF power. A low emittance, high current injector has been developed by Sheffield, et al. The beam brightness is preserved through the accelerator by the suppression of the high order modes with a slotted accelerator structure patterned after that demonstrated by Palmer, et al. Compression of the pulse length from about 20ps to about 1ps prior to acceleration to full energy can boost the peak current from several hundred amperes to as much as 10kA. At the higher current, the gain is much increased, and, as illustrated in figure 3, the performance of the FEL is much less dependent on such factors as the energy spread of the electron beam.

This FEL would have high peak power, as much as $10^{10}$W, and very great spectral brilliance, $10^{31}$ photons/(sec mm$^2$ mrad$^2$).
band width). With a nominal wavelength of 44Å, it would be fully
tuneable and would have a natural bandwidth of 0.2% and, hence, a
longitudinal coherence length of 2.5μm. At the exit from the FEL,
the x-ray beam would have a spot size of about 100μm and a
divergence of about 20μrad. Each pulse of length 1-10ps would
contain as much energy as 1-10mJ (about 10^{13}-10^{14} photons per
pulse). The x-ray beam would have complete linear polarization and
would have about 85% of its power in the TEM_{00} mode. This design
can easily support a repetition rate of several Hertz.

In short, such an FEL could be exceedingly useful for biological
x-ray microscopy.

FOOTNOTES

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Growth from beam noise shows characteristic spectral evolution.

![Graph showing exponential growth of x-ray laser beam from spontaneous emission over the first 8 meters of wiggler.](image)

**Fig. 1**

1. This figure shows the results of a GINGER calculation of the exponential growth of the x-ray laser beam from spontaneous emission over the first 8 meters of wiggler. The effective noise level is about 100W. As indicated by the three inset spectra, there is little growth outside of a narrow bandwidth, which is given roughly by 1/2N where N is the number of wiggler periods. The beam and wiggler parameters for this calculation appear to the right of the graph.
2. These calculational results from NUTMEG numerical simulations illustrate the efficacy of tuning the wiggler field for a longer wavelength and then using a higher harmonic. Here the wiggler is tuned for 150Å, and both the fundamental and the third harmonic at 50Å are amplified. The signal gain at a wiggler length of 25 meters is plotted versus the wiggler period. For an electron beam of 1.5GeV and 4kA and for 1W of spontaneous noise, the exponential gain of the fundamental saturates at about $10^{10}$ W. At a wiggler period of 4 cm, the gain at 50Å is about 1000 greater using this technique than the gain obtained by tuning the wiggler magnetic field for resonance at 50Å. This technique lends additional flexibility in overcoming difficulties. A possible conclusion from these results would be to build a wiggler with a period of 4 cm rather than at 2-3 cm, which is a harder undertaking.
FEL is more tolerant of electron energy spread at higher current

3. The small signal gain at 25 meters from FRED-3D numerical simulations are plotted here versus the instantaneous energy spread in the electron beam. The beam and wiggler parameters used in the calculations appear on the right hand side. The three gain curves have three different peak currents. The assumed initial noise level is 1W, and thus the saturation at $10^{10}W$ would occur at the smaller signal gain of $10^8$ for the more realistic noise level of 100W. Clearly, the use of high current injector and/or temporal pulse compression to increase the peak current results in higher gain and in reduced sensitivity to flaws in the beam or the wiggler.
X-RAY MICROSCOPE WITH FREE-STANDING ZONE PLATES AT UVSOR(JAPAN)

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To develop X-ray microscope in VUV region, the use of a free-standing zone plate is inevitable. Thus, we have fabricated a free-standing zone plate, and tested its focal and magnifying features at UVSOR (a dedicated synchrotron facility belonging to the Institute for Molecular Sciences (Okazaki, Japan), 750 MeV, 120 mA). The test system was applied for the observation of mesh and dried biospecimens such as diatoms.

The present characteristics of the zone plates are: n=312, f=150 mm at 8 nm light, d=m=0.93 μm, and Au thickness = 2 μm with a central mask of 0.2 mm as a condenser zone plate. The same dimensional zone plate without masks was used as a micro zone plate.

We have tested zone plate imagings with two types of set-ups. One is with a plane-grating monochromator and a zone plate with a central mask at the beam line 6A2 of UVSOR, and the other is with two zone plates (with and without a mask) without a monochromator beam line 8A of UVSOR was used. Detectors (MCP or films) were located 2863.5 mm from MZP, which has a magnification ratio as 13.4, unless otherwise stated. MEM (Mitsubishi) or Minicopy (Fuji) films were used. Two types of MCP (Hamamatsu Photonics) were used; with a single layer or double layers. Obtained images were observed on fluorescent screen, which was usually monitored with SIT camera for accumulation.

The Cu #1000 mesh image and other biological samples were taken. Results were shown at Poster presentation.

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THE POTENTIAL OF PEP AS A HIGH-BRIGHTNESS SOFT X-RAY SOURCE FOR COHERENCE AND MICRO-IMAGING STUDIES

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Abstract

The Stanford Positron Electron Project (PEP) offers a number of technical advantages that, if properly exploited, can make it the brightest and most versatile soft x-ray source in the world for research in coherence and micro-imaging. Among these advantages are 1) the existence of long straight sections and high operating energies, which allows the development and installation of advanced weak-field undulators for versatile, polarized soft x-ray generation; 2) a possible emittance upgrade, utilizing a damping wiggler lattice, which would yield emittances of a fraction of 1 nm-rad, leading to the emergence of a new, coherent, mode of spontaneous synchrotron radiation (SR) emission; and 3) the possibility of applying longitudinal pulse compression techniques to the circulating electron bunch which could result in \( \approx 2 \) ps pulse lengths, greatly enhancing the temporal resolution of single-shot x-ray imaging. In this presentation, these and other features of PEP are graphically presented, and a brief enumeration and discussion of their application to advanced research in x-ray coherence and micro-imaging technology is given.

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I. Introduction

The Stanford Positron Electron Project (PEP) offers an unmatched opportunity for establishing the brightest and most advanced soft x-ray facility in the world for conducting research in coherence and micro-imaging. This observation is based in part on the following attributes of PEP [1]:

1) The high operating energies of PEP, ranging from 4 to 16 GeV, together with its long straight sections (exceeding 100 meters) permit the design and implementation of long-period, weak-field undulators with advanced features, allowing great flexibility in generating arbitrarily polarized photons, multi-color pulses, and spatial and angular modulation of the output radiation [2,3,4,5].

2) A future emittance upgrade, based on the installation of a wiggler lattice, is expected to lead to emittances of a fraction of 1 nm-rad [6]. This will not only make PEP the brightest soft x-ray source available, but is also expected to lead to a new mode of highly coherent spontaneous emission growth [7], which will make the radiation generated by a soft x-ray undulator in the lower part of its spectral range analogous in many respects to that emitted by an FEL.

3) The possibility of modulating the longitudinal dimension of the bunches in PEP is expected to be able to produce pulse lengths down to the order of a few picoseconds [8]. This will enhance the peak spontaneous brightness to levels unattainable anywhere else, with correspondingly high levels of peak coherent power. Since this pulse compression technique also directly modulates the beam density, the spontaneously coherent emission referred to above is likely to also be directly enhanced, making possible a further increase in both the peak and time-averaged coherent power to levels orders of magnitude higher than at any other storage ring facility. It is worthwhile to note that alternative, photon-pulse compression techniques optimally suited to PEP, have also been proposed [9]. The combination of the two attributes of enhanced coherent intensity and temporal compression will make PEP perhaps the only storage ring facility in the foreseeable future at which developmental research on single-shot imaging of picosecond-level resolution in high-resolution recording media could be viably addressed [10].
II. Soft X-ray Insertion Devices for PEP

The optimality of long-period, weak-field insertion devices for generating soft x-rays on PEP allows for their development with features greatly superior to conventional permanent-magnet (PM) insertion devices [11]. Among these are: 1) considerably lower cost; 2) absence of permeable materials; 3) electrical control of period length, for operation in the "constant-field, variable-period" mode [11]; and 4) generation of either electrostatic or magnetostatic fields. Several designs employing these features have already been developed at SSRL, and two of them are shown in Figs. 1 and 2. Each design generates a near-sinusoidal deflection field on axis by discretely sampling the voltage (Fig. 1) or current (Fig. 2) of a sinusoidal function with the desired period $\lambda_u$. In each case, the sampling period is $\lambda_{pv}$. For example, for the case of Fig. 1, the on-axis deflecting field is given approximately by

$$E_x(0,z) = \frac{-V_0 e^{-(\pi g_H'/M \lambda_{pv})}}{\lambda_{pv} \cosh^{-1}(g_H/d_w)} \left \{ \cos \left(2\pi z \frac{1}{\lambda_u} \right) + e^{-\pi g_H'/\lambda_{pv} \cos \left(2\pi z \left(\frac{1}{\lambda_{pv}} + \frac{1}{\lambda_u} \right) \right)} + e^{-\pi g_H'/\lambda_{pv} \cos \left(2\pi z \left(\frac{2}{\lambda_{pv}} + \frac{1}{\lambda_u} \right) \right)} + e^{-\pi g_H'/\lambda_{pv} \cos \left(2\pi z \left(\frac{2}{\lambda_{pv}} - \frac{1}{\lambda_u} \right) \right)} + \ldots \right \} \quad (1)$$

where, with proper design, the first cos term inside the brackets can be made dominant.

The output power curves for the undulator in Fig. 1 are shown in Fig. 3. The performance curves for the undulator in Fig. 2 would be identical. As is characteristic of fixed-field, variable-period insertion devices, it is seen that the tuning range is considerably greater than for a conventional PM undulator, and that, furthermore, the total radiated power remains constant, at an acceptable level with respect to engineering considerations.
Figure 1. Helical Electrostatic Undulator (HEU). By independent adjustment of voltages across each wire pair, a sinusoidal field with a continuously adjustable period and phase can be established along the z-axis for both the vertical and horizontal wire lattices.

Figure 2. Variable-period Transverse Magnetostatic Undulator (TMU). This structure is the magnetostatic analog of either of the electrostatic wire lattices shown in Fig. 1, and is described by an analogous set of equations.
Figure 3. Performance curves for the device shown in Fig. 1 on PEP. The curves are based on an assumed peak field strength of 1.5 MV/m between the wire pairs. It is noted that the device spans the soft x-ray range for all three running energies of PEP.
The following is a more detailed list of the depicted undulators' advantages:

For the undulator in Fig. 1:

1) Relatively low total output powers should allow use of simple, efficient, and relatively inexpensive monochromators reducing beam line instrumentation costs and enhancing both performance and throughput [10].

2) The problem of the potentially deleterious effects associated with large numbers of variable-gap devices on storage ring stability has recently started attracting significant attention. It is likely that an array of constant field (CF) devices, which should perturb the beam always by the same amount (insofar as total radiation losses are concerned), no matter how they are tuned, will ultimately prove to have the minimum and most easily controlled perturbative effects on storage ring stability. Electrostatic TEU’s have a further potential advantage in this regard in that it may be possible to apply real-time corrections and orbit compensations in times orders of magnitude faster than is possible with magnet coils.

3) The significantly lower amount of superfluous photon generation, in contrast to TMU’s, makes the variable-period CF transverse electrostatic undulator (TEU) a much more attractive device for FEL operation, both from the point of view of efficiency and enhanced control of the lasing process. In FEL operation, however, it should be pointed out that the constant-loss property of the TEU alluded to above can no longer be expected to apply.

4) Electronic control of field profiles simplifies the control aspects of the undulator/monochromator system, viz., the only moving subsystem needed would be the monochromator. This would result in more photons per unit time on experiments.

5) Continuous operation in the soft x-ray range, regardless of PEP's energy, at brightnesses superior (over broad energy ranges) to those of the ALS, ESRF, and APS [1,10].

6) Only one physical structure and control system are required.

7) Arbitrarily tunable polarization with high efficiency over the soft x-ray range, with usable polarized photons deliverable up to 120 KeV.

8) Arbitrary (band-limited) field profiles, including tapered-period profiles.

9) Sequential (TEU) and/or collateral (HEU) multi-color field profiles.
10) State switching, i.e., changing undulator field configurations between selected PEP pulses.

11) Operation of shorter sections of the undulator to optimize aperture matching, or coherence, requirements.

12) The field could be tailored to enforce near-sinusoidal transverse electron motion, minimizing the generation of odd harmonics in TEU operation.

13) Both the field and period could be varied over limited ranges for constant-K operation.

14) Field errors are an important consideration in TU's, especially for FEL operation. In the proposed HEU facility, automatic in situ correction of certain types of field error would be possible, making the device far less sensitive to certain mechanical tolerances than in corresponding conventional PM designs.

15) Due to the compact profile of the HEU, it should be possible to perform focussing functions on the beam with external magnetic fields. Hybrid insertion device configurations could also be implemented.

16) Unilateral bias offsets on the wire-pairs could be used to "raster-scan" the beam in the HEU. This could prove useful in generating uniform (time-averaged) radiation fields for x-ray lithography.

17) The same bias rastering, together with the ability of the HEU (or TEU) to rapidly change states, could also be used to feed more than one beamline with a maximally effective duty cycle.

18) The cost of the physical structure of the HEU, contrasted with that of a corresponding conventional PM undulator of the same length, is clearly negligible. For a restricted-period HEU, the cost of the entire system could also be made minimal in comparison to an equivalent magnetostatic facility.

For the undulator in Fig. 2:

19) In addition to possessing most of the desirable properties of TEU's and HEU's listed above, the constant-field variable-period magnetostatic undulator has an additional advantage in that its physical structure can be located outside of the vacuum enclosure.
III. Source Brightness and Possibilities for Soft X-Ray Imaging on PEP

In Fig. 4 two sequences of brightness curves are plotted, the lower sequence for the existing "low emittance" lattice on PEP, and the upper one for a projected "very low emittance" lattice.

In the first case, the indicated time-averaged brightnesses are comparable to those projected for the ALS and the APS storage rings, making the installation of a soft x-ray undulator on PEP immediately competitive in the short term with respect to all the imaging programs that are being planned for the other facilities in the future - but at a much lower cost.

Figure 4. Brightness curves for a 30 meter long soft x-ray polarizing undulator on PEP. Equal polarization components are assumed.

With regard to peak brightness, the PEP facility, in virtue of its 2200 meter diameter and the possibility of compressing the electron bunches, offers a 2 to 3 orders of magnitude increase over any other facility, now in existence or being planned. If one now adds to this the feasibility of inducing coherent gain in the PEP
device in the VUV range (following PEP's upgrade into its very low emittance mode), then in terms of both average and peak brightness the appropriate soft x-ray device on PEP remains unmatched by any other facility in the world for conducting imaging/coherence studies.

To give a brief quantitative indication of PEP's potential for coherent imaging (e.g., holography), we present a table below indicating the time intervals required to expose a 5mm × 5mm area in a high-resolution medium such as PMMA for a given amount of coherent power [10].

**TABLE 1**

Recording times for a 5mm×5mm area on PMMA @ a 1µ coherence length. Assumed energy density for full exposure is 1 J/cm². Lossy optical elements and a monochromator efficiency of 1% are assumed.

<table>
<thead>
<tr>
<th>Average Coherent Power [Watts]</th>
<th>Recording Time [sec]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0001</td>
<td>2000000</td>
</tr>
<tr>
<td>0.001</td>
<td>200000</td>
</tr>
<tr>
<td>0.01</td>
<td>20000</td>
</tr>
<tr>
<td>0.1 PEP</td>
<td>2000</td>
</tr>
<tr>
<td>1.0 (enhanced coherence)</td>
<td>200</td>
</tr>
<tr>
<td>10.0</td>
<td>20</td>
</tr>
<tr>
<td>100.0</td>
<td>2</td>
</tr>
</tbody>
</table>

In Fig. 5 we plot projected values of average coherent power for PEP from the device whose brightness curves are plotted in Fig. 4. It is clear that the potential of PEP for coherent imaging – over the range spanning absorption edges of interest for biology – is superior to that of the ALS. Perhaps of even greater interest is the attainable peak coherent power, which brings the per/pulse energy deliverable to a recording medium – in a span of ten or so ps – into the several-microjoule range. This clearly suggests the possibility of researching the development of ultra-fast, single-shot imaging techniques, some classes of which could hold
Figure 5. Coherent power curves for a 30 meter long soft x-ray undulator on PEP at 4 GeV.

promise as a means of circumventing the damage problem in conventional time-integrated coherent imaging.
IV. Summary

A brief examination of PEP's parameter range indicates that it is an ideal host machine for installing a high-brightness soft x-ray insertion device with a range of coherent power outputs and temporal modulations unmatched anywhere else. Both of these aspects strongly underlie the versatility and potential of PEP as a leading soft x-ray source for coherence and imaging studies. In addition, insertion devices of unprecedented performance and economy can be designed for this purpose, and, indeed, already have been [12]. Furthermore, PEP is already in operation, and a minimal lead time would exist between the funding of a soft x-ray undulator for PEP and the inception of the research outlined in the present workshop.

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SUPER-HIGH RESOLUTION HOLOGRAPHIC COMPOSITE
POLYMER GRAFTS FOR XUV APPLICATIONS

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Abstract

Advancement of holographic technology into the XUV brings with it many technical challenges. The resolution, optical quality and environmental stability of holographic materials become crucial issues at shorter wavelengths. Recent developments of a new class of XUV holographic materials based on graft concepts at Physical Optics Corporation (POC) point the way toward development of XUV holographic optical elements featuring high efficiency, high optical quality, conventional and unconventional functions, strong resistance to adverse environments and cost effectiveness.

1.0 INTRODUCTION

High efficiency XUV optical elements have various important applications ranging from the development of new and high intensity XUV sources, including X-ray lasers, X-ray free-electron lasers, synchrotron radiation and plasma sources, to X-ray microscopy and astronomy. Conventional XUV optics have been based on total-external-reflection (TER) mirrors\(^1\), multilayer reflectors\(^2\) and surface-relief gratings\(^3\). However, all these elements suffer from limited size, limited functionality, sophisticated fabrication process and high cost. Recently, XUV volume holographic optical elements (HOEs) based on a single step holographic recording have been proposed by Physical Optics Corporation as an entirely new approach to XUV optics\(^4,5\). The high efficiency XUV volume holographic structures can, in reflection-Lippman geometry, simulate deposited multilayers, while in more general geometries, they can perform various conventional or non-conventional that make them competitive to a majority of conventional XUV optical elements including uniform and nonuniform surface gratings, lenses, slanted (non-Snellian) mirrors, Fresnel zone-plates, concentrators/collimators, beam splitters, multilayer Fabry-Perot resonators, and binary optical elements. In addition, the XUV HOEs are easily scalable, can be obtained on various (glass, plastic; curved, flat) substrates, and have the potential for creating various optical elements (performing, for example, different operations for different wavelengths) in the same hologram volume.

As a major breakthrough in XUV holography, a new series of high resolution (20,000 l/mm) and high refractive index modulation (\(\Delta n=0.4\)) materials have been developed at POC's laboratory. These materials, called composite polymer grafts (CPGs), are produced by either reacting a photosensitive polymer with a photosensitizer in aqueous solution, within a cross-linking mechanism, or using a combination of polymerization and cross-linking. In addition to high resolution and high modulation, these materials exhibit high environmental stability, high laser damage threshold, low surface roughness, and cost effectiveness in mass production.
2. HIGH XUV DIFFRACTION EFFICIENCY OF HOLOGRAPHIC MULTILAYER STRUCTURES FROM COMPOSITE POLYMER GRAFTS

Due to the high refractive index modulation obtainable in POC's composite polymer grafts, high efficiency XUV volume holographic multilayers can be routinely fabricated at POC's optics laboratory. Consider a simple case where an XUV grazing incidence mirror is recorded in the visible region. The grating spacing is given by the well-known Bragg condition:

\[ \Lambda = \frac{\lambda_o}{2n_0 \cos \theta_0} \]  

(1)

where \( \lambda_o \) is the recording optical wavelength, \( \theta_0 \) is the angle of incidence and \( n_0 \) is the refractive index of the medium, related to the recording wavelength. The XUV diffraction efficiency of this type of reflection hologram is given by Kogelnik:

\[ \eta = \left| \frac{\text{sh}(v \text{cha})}{\text{sh}(a + v \text{cha})} \right|^2 \]  

(2)

where \( v \) is related to the coupling constant and \( a \) is related to the dephasing measure parameter, absorption constant and \( V \). Furthermore, at XUV wavelength \( \lambda \), the complex refractive index of the medium has the form:

\[ \hat{n} = n - j\beta = 1 - \frac{\pi e^2}{2\pi} N_a (f_1 + jf_2) \]  

(3)

where \( N_a \) is the number density of atoms, \( f_1 \) and \( f_2 \) are the atomic scattering factor components. The theoretical limit\(^4\) of diffraction efficiency \( \eta \) is obtained by assuming the thickness \( T \to \infty \):

\[ \eta_\infty = \left| \frac{\hat{F}}{1 + \sqrt{1 + \hat{F}^2}} \right|^2 \]  

(4)

where the complex holographic-strength parameter \( \hat{F} \) is given by

\[ \hat{F} = \frac{ab}{8} - j\frac{a}{4} \]  

(5)

where

\[ a = \Delta N_a/N_a \]  

(6)

\[ b = f_1/f_2 \]  

(7)

\( \Delta N_a \) is the peak-to-peak amplitude of modulation of \( N_a \).

In general, the a-coefficient cannot exceed two by definition, while according to Henke Tables\(^7\), the b-coefficient varies within the range 1 to 20. These two coefficients fully determine the asymptotic diffraction efficiency. Material density modulation \( \Delta N_a \) can be found via Eqs. 2 and 3 once the diffraction efficiency is measured. Then the refractive index modulation \( \Delta n \)
in the visible can be calculated by using the Lorentz-Lorenz formula $^8$, which can be reduced to the following form, assuming the average refractive index is 1.5 in the visible:

$$\frac{\Delta N_a}{N_a} = 2.5 \frac{\Delta n}{n}$$  \hspace{1cm} (8)

Figure 1. Experimental and Theoretical Diffraction Efficiency Curves of An XUV Holographic Multilayer Coating at $\lambda=11.4$ nm

Figure 2. Experimental and Theoretical Diffraction Efficiency Curves of An XUV Holographic Multilayer Coating at $\lambda=13.5$ nm

As an experimental verification, several CPG holographic mirrors were recorded using an Argon ion laser at $\lambda=488$nm in normal incidence Lippmann geometry. During the recording, the substrate sides of the samples faced the laser beam, and the reflection from the polymer coating-air interface provided the second beam to interfere with the incoming beam. These samples were measured at the University of Colorado at 14 XUV wavelengths (11nm-83nm) and within the entire range of incidence angles. Two of the experimental results
and their theoretical predictions are illustrated in Figures 1 and 2. Good agreement is observed between the experimental and theoretical results. The peak diffraction efficiency is about 28% at λ=11.4 nm which corresponds to an index modulation of Δn=0.4 in the visible. It is this high index modulation (or high material density modulation) that contributes to the high diffraction efficiency of POC's holographic multilayer structures.

3. MATERIAL STUDY

In this section, we will evaluate the laser damage threshold temperature stability and surface roughness of our composite polymer grafts, and compare these properties with some conventional materials.

Previous studies of dielectric multilayers in the UV have reported laser damage thresholds of 2-9 J/cm² for high reflectors⁹. A number of polymers have been studied for laser damage threshold in connection with the investigations into the possible use of these materials as laser optics. In the UV, FEP teflon has one of the highest reported damage thresholds at 3.2 J/cm² (when irradiated at 355 nm with 0.6 ns pulses).

Our test samples were prepared by spin coating plate glass substrates with a 15-20 micrometer thick CPG layer. Testing was performed with a Molectron MY-34 Nd:YAG laser frequency tripled to 354.7 nm and operating with a single axial mode etalon. The diameter of the irradiated area was calculated using the standard Gaussian beam relation d=2.44 f# and was found to be 83 micrometers. Pulse length ranged from 8-12 ns FWHM. Testing was performed by selecting a sub-threshold fluence and gradually increasing the fluence until damage occurred. The presence of damage was determined by observing the presence of a spark during exposure and by inspecting the irradiated sites after exposure with a 10x inspection loop.

The highest damage threshold we obtained is 21 J/cm². The reason for the high damage thresholds present in these elements is linked to their structure. Damage initiation may proceed by several mechanisms including: avalanche ionization, absorption and multiphoton absorption. Avalanche ionization is dependent on the electron ionization rate which is in turn dependent on the electric field strength. In dielectric multilayer coatings, the strongest electric fields are present at the boundaries between layers of different index. But in holographic coatings, with a sinusoidal index profile, there are no sharp index boundaries and as a result, electric field intensities are reduced.

Additionally, the difference between the highest and lowest index portions of holographic filters is on the order of 0.05-0.7. For a dielectric multilayer, by contrast, index difference may be 0.5-0.7. Thus, the sinusoidal index profile and small index difference present in holographic reflectors substantially reduce the probability of avalanche ionization as a mechanism of damage initiation. Absorption and damage initiated at impurities were clearly present in the films studied and are probably the cause of damage initiation in these reflectors.

High temperature stability tests were performed at POC in a closed oven under atmospheric pressure. The XUV holographic samples were first conditioned at 80°C for several hours before testing at a high temperature. Figure 3 shows temperature and optical characteristics after subjecting the filters to high temperatures.
From Figure 3, it can be easily observed that there is no significant effect on optical properties of holographic filters. The optical density and half-length full bandwidth remain virtually unaffected by an increase in temperature of 200°C. Thus, our XUV holographic filters based on POC's photopolymer survive temperature variations to at least 200°C. The refining of our holographic technique as well as further optimization of the recording material could further increase the temperature stability to above 200°C.

Figure 3. Optical Density (O.D.) versus wavelength as a function of temperature. O.D. spectra were recorded on a Varian 2300 Spectrophotometer after the sample was subjected to high temperature conditions.

In the discussion of surface reflection, the Fresnel equations are obtained by imposing the boundary conditions on the electromagnetic field at the interface between two materials. In the derivation of the Fresnel equations, it is assumed that the interface is perfectly smooth. In fact, the interface between two materials is never smooth. Even the best optical surfaces have rms surface roughness that are on the order of a few angstroms. The effect of surface roughness is to reduce the amount of light specularly reflected from an interface to a value that is lower than that given by the Fresnel equations. For work in the infrared, visible and near UV, this does not cause any great difficulties, yet in the XUV region the wavelength of light approaches the scale of surface roughness, and these effects can be significant.

Typical rms surface roughness values of the 23 most common materials used in deposited multilayers were determined by D.Windt. The range of these values is 1-3 nm. On the other hand, the rms values of our XUV holograms are measured at Los Alamos National Laboratory and TRW, and the results are around 2 nm (see Figure 4 for example) which is comparable to those of deposited multilayers. Moreover, this result is routinely obtained and can be further reduced under better coating and processing conditions.
Figure 4. Typical Surface Roughness Profile of XUV Mirrors

4. SUMMARY

We have demonstrated a new technology for producing high-efficiency XUV volume holographic diffraction elements using our high modulation, high resolution composite polymer grafts. It has been shown that our materials are advantageous in many respects over conventional materials used for XUV applications.

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6. ACKNOWLEDGEMENT

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